



**GABA AND GABA-RECEPTORS IN  
THE ENTERIC NERVOUS SYSTEM**

*A thesis submitted for the degree of  
Doctor of Philosophy*

by

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## DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any University.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying or loan.

Jennifer Ong

1985.

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### Corrigenda

p.2, line 7 - should read 'was likely to be an inhibitory ....'

p.19, line 20 - should read '(Beart et al., 1972, Bowery et al., ....)'

p.24, line 3 - should read '(GAD, EC.4.1.1.15), and ....'

p.24, line 5 - should read '(GABA-T, EC.2.6.1.19), as ....'

p.30, line 13 - should read '.. et al., 1975). Muscimol also has a low  
affinity for Na<sup>+</sup>-dependent GABA uptake sites (Krogsgaard-  
Larsen, 1981) ....'

p.32, line 3 - delete 'environmental factors', insert 'experimental conditions ....'

p.58, line 19 - should read '.... GABA, nor a direct effect ....'

p.74, line 7 - should read '.... gastro-intestinal tract ....'

p.110, line 12 - should read '.... as 2, 4 diaminobutyric ....'

## SUMMARY

In this thesis, properties of gamma-aminobutyric acid (GABA) and its receptors (GABA<sub>A</sub> and GABA<sub>B</sub>) have been characterised in the enteric nervous system of the guinea-pig.

Chapter one outlines the history of GABA as a neurotransmitter, where aspects of GABA pharmacology at GABA receptor sites are reviewed together with GABA metabolism, GABA binding studies, GABA release, uptake and inactivation. Finally, the functional organisation of the enteric nervous system together with the many putative transmitter substances is discussed, with particular emphasis on GABA in the myenteric plexus.

The actions of ethylenediamine (EDA) in isolated intestinal segments of the guinea-pig are investigated in chapter three. EDA releases both endogenous and tritiated GABA from the myenteric plexus, and responses to EDA mimic responses elicited by GABA, antagonised by GABA antagonists. Moreover, metabolic inhibitors of GABA synthesising and degrading enzymes alter responses to EDA. Ethylenediamine monocarbamate is the active compound releasing GABA in the intestine.

In chapter four, high-affinity saturable GABA uptake is established in isolated ileal preparations using selective neuronal and glial uptake inhibitors.

Chapter five demonstrates a barbiturate potentiation of GABA<sub>A</sub>-receptor-mediated ileal responses; with anaesthetic, sedative-hypnotic barbiturates being more potent than

anticonvulsants. Barbiturates reverse antagonism at the GABA<sub>A</sub>-receptor complex by picrotoxinin (PIC) and by a convulsant caprolactam, showing that GABA<sub>A</sub>-receptors in the myenteric plexus have an associated barbiturate-PIC site in relation to the chloride ionophore.

Chapter six analyses the interactions between GABA and 5-hydroxytryptamine (5-HT) and shows that prior application of GABA inhibits ileal contractile responses to 5-HT through two mechanisms: an early inhibition of the smooth muscle, engendered by a GABA<sub>A</sub>-receptor-mediated activation of its non-adrenergic non-cholinergic inhibitory innervation, and a second, more delayed and longer lasting, due to a GABA<sub>B</sub>-receptor-mediated depression of the cholinergic transmission resulting from 5-HT stimulation of the myenteric motoneurons.

In chapter seven, GABA antagonists most often reduce spontaneous activity occurring in the intestinal segments, as does 3-mercaptopropionic acid (3-MPA). Conversely, Avermectin Bla induces regular rhythmic contractions and relaxations of the longitudinal muscle in the ileum, blocked by bicuculline, PIC and 3-MPA. Finally, faecal pellet expulsion in isolated distal colon is slowed to a considerable extent by application of GABA antagonists.

In the final chapter, evidence is drawn together to establish GABA as a neurotransmitter in the the guinea-pig intestine.



## PUBLICATIONS

During the course of my study, the following papers have been published or submitted for publication.

Ong, J. and D.I.B. Kerr. (1983). GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated modification of intestinal motility. *Eur. J. Pharmac.* 86:9-17.

Ong, J. and D.I.B. Kerr. (1983). Interaction between GABA and 5-hydroxytryptamine in the guinea-pig ileum. *Eur. J. Pharmac.* 94:305-312.

Ong, J. and D.I.B. Kerr. (1984). Evidence for a physiological role of GABA in the control of guinea-pig intestinal motility. *Neuroscience Lett.* 50:339-343.

Ong, J. and D.I.B. Kerr. (1984). Potentiation of GABA<sub>A</sub>-receptor-mediated responses by barbiturates in the guinea-pig ileum. *Eur. J. Pharmac.* 103:327-332.

Ong, J. and D.I.B. Kerr. (1984). Evidence that 5-hydroxytryptamine does not mediate GABA-induced contractile responses in the guinea-pig proximal ileum. *Eur. J. Pharmac.* 106:665-668.

Kerr, D.I.B. and J. Ong. (1984). Evidence that ethylenediamine acts in the isolated ileum of the guinea-pig by releasing endogenous GABA. *Br. J. Pharmac.* 83:169-177.

Kerr, D.I.B. and J. Ong. (1984). GABA and GABA-receptors in the enteric nervous system. *Neuropharmacology.* 23:835-836.

Kerr, D.I.B. and J. Ong. (1985). GABAergic mechanisms in the gut: their role in the regulation of intestinal motility, in "GABAergic mechanisms in the mammalian periphery". S.L. Erdo and N.G. Bowery (eds), Raven Press, New York (in press).

Kerr, D.I.B., J. Ong, R.H. Prager and A.D. Ward. Caprolactam-barbiturate interaction at the GABA<sub>A</sub>-receptor-chloride ionophore complex in the guinea-pig intestine. *Eur. J. Pharmac.* (submitted).

Kerr, D.I.B. and J. Ong. GABA-dependent motility induced by Avermectin in the isolated intestine of the guinea-pig. *Neuroscience Lett.* (submitted).

Ong, J. Uptake blockers potentiate GABA-induced responses in the isolated ileum of the guinea-pig. *Br. J. Pharmac.* (submitted).

Ong, J. and D.I.B. Kerr. GABA-induced responses in various segments of the guinea-pig intestine. *Eur. J. Pharmac.*

(submitted).

Kerr, D.I.B. and J. Ong. Bicarbonate dependence of responses to ethylenediamine in the guinea-pig isolated ileum. Eur. J. Pharmac. (submitted).

#### ABSTRACTS

The following abstracts have been presented at meetings of learned societies:

Ong, J. (1981). Baclofen: Some effects in guinea-pig small and large intestine. Proc. Aust. Physiol. Pharmac. Soc. 12(2):114P.

Kerr, D.I.B. and J. Ong. (1981). GABA actions in guinea-pig ileum through release of endogenous GABA by ethylenediamine. Proc. Aust. Clin. Exp. Pharmac. Soc. 3P.

Ong, J. and D.I.B. Kerr. (1982). Baclofen inhibits non-cholinergic excitatory nerves in the guinea-pig small intestine. Proc. Aust. Physiol. Pharmac. Soc. 13(2):203P.

Kerr, D.I.B., A. Krantis, and J. Ong. (1983). GABA is a transmitter in the enteric nervous system. Proc. International Union Physiol. Sci. (29th Congress). 15:453P.

Kerr, D.I.B. and J. Ong. (1984). GABA depresses neurally-mediated responses to 5-HT in the guinea-pig ileum. Proc. Aust. Clin. Exp. Pharmac. Soc. 8:112P.

Ong, J. and D.I.B. Kerr. (1984). Barbiturate modulation of GABA-induced responses in the guinea-pig ileum. Proc. Aust. Physiol. Pharmac. Soc. 15(1):93P.

Kerr, D.I.B. and J. Ong. (1984). GABA released by ethylenediamine causes differential effects in various region of the guinea-pig intestinal tract. International Union Pharmac. (9th Congress, London). 163P.

Ong, J. and D.I.B. Kerr. (1984). Inhibitors of GABA metabolism alter responses to ethylenediamine in the guinea-pig intestine. International Union Pharmac. (9th Congress, London). 164P.

Kerr, D.I.B. and J. Ong. (1985). GABA-dependent motility induced by Avermectin in the guinea-pig isolated intestine. Proc. Aust. Physiol. Pharmac. Soc. 16(2):131P.

CHAPTER ONE. GENERAL INTRODUCTION.



## HISTORICAL REVIEW

$\gamma$ -Aminobutyric acid (GABA) was first synthesised in 1883 (Schotten, 1883) and then found present among the products of vital activity of micro-organisms (Ackermann, 1910). The presence of GABA in mammalian brain was later established independently in two separate laboratories at the same time (Awapara et al., 1950; Roberts and Frankel, 1950), and the first indications that GABA might play an important role in some physiological inhibitory function in the nervous systems, came from studies with convulsant hydrazides to prevent GABA synthesis (Killam, 1957; Killam and Bain, 1957), and the discovery of its presence in the peripheral nervous systems of various invertebrates in relatively high concentrations (Kravitz et al., 1963 b; Otsuka et al., 1967).

Early on, the discovery of an inhibitory agent, Factor I in the brain by Florey (1954) led to the isolation of GABA in 1957 (Bazemore et al., 1957), followed by a comparison of the effects of Factor I and GABA on isolated smooth muscle preparations (Florey and McLennan, 1959), Florey (1953;1954) already having shown an anti-acetylcholine (ACh) effect of Factor I using isolated ileal preparations from guinea-pigs and rabbits. These results later gave rise to the identification of GABA as the main active component of brain that could account for the Factor I activity, although it was originally thought that Factor I was some constituent similar to GABA. However, it was soon realised that some preparations of Factor I did not contain GABA and the

two substances did not appear to be completely identical (McLennan, 1959; 1960); nevertheless, upon purification of the brain extract, it was shown that GABA could account for the inhibitory activity of Factor I (Bazemore et al., 1957), hence disputing the hypothesis that Factor I might be the central nervous system (CNS) inhibitory transmitter. From then on, GABA became accepted as the inhibitory transmitter in the CNS.

Although GABA was first reported to be present in the vertebrate CNS in 1950 (Roberts and Frankel, 1950), and the subject of GABA metabolism already reviewed by Roberts (1956), with the chief metabolic pathways of GABA outlined (Fig. 1), its physiological function remained obscure at that time. The uniquely high concentrations of GABA localised within nervous tissues in the CNS suggested that it might have some connection with conduction of nerve impulses in the CNS, but the first definitive evidence for an inhibitory transmitter function of GABA at synapses come from studies of the effects of crude brain extracts on the crayfish stretch receptor system (Florey, 1960). Although apart from its inhibitory role in synaptic transmission, there is also evidence that GABA may function as an excitatory transmitter in at least some vertebrate and invertebrate preparations (Obata, 1976; Florey et al., 1975). However, the crustacean nervous system offered an attractive model for the study of inhibitory synapses at that time, as these occur peripherally in such organisms, and the possible role of GABA in inhibitory transmission was suggested by Bazemore et al. (1957) who showed that GABA was the major inhibitory substance in mammalian brain extracts that

inhibited discharges in the crustacean stretch receptor. Widespread interest in GABA was stimulated by the discovery that it occurred in high concentrations throughout the mammalian CNS (Baxter and Roberts, 1960). Subsequently, Kravitz et al. (1963a) showed the localization of GABA in crustacean presynaptic axons, based on preliminary findings that the concentration of GABA was highest in a nerve that contained only one motor and one inhibitory axon, where GABA was found in inhibitory neurones but not in motor fibers of crustacea, suggesting that GABA has a specific physiological role confined to inhibitory neurones. Following this, Kravitz et al. (1965) showed the existence of the metabolic machinery for GABA production and degradation in the lobster presynaptic inhibitory axons, where they demonstrated the presence of GABA degrading enzyme, GABA-transaminase (GABA-T) and GABA synthesising enzyme, glutamic acid decarboxylase (GAD). Otsuka et al. (1966) demonstrated a calcium-dependent release of GABA at neuromuscular junctions of the lobster, from inhibitory nerves but not from the excitatory axons in response to nerve stimulation; the amount of GABA released was proportional to the number of stimuli applied to the inhibitory nerve, depending on frequency and/or duration of stimulation. Exogenously applied GABA was found to have potent inhibitory effects on crustacean muscles and on the crayfish neuromuscular junction (Kuffler, 1960; Takeuchi and Takeuchi, 1965), and there have been extensive pharmacological analyses of GABA actions on crustacean stretch receptors and neuromuscular junctions (Edwards and Kuffler, 1959; Grundfest et al., 1959; McGeer et al., 1961; Dudel, 1965 a,b).

In crustacean muscle, GABA actions were found to be chloride-dependent, causing a selective increase in the permeability of the muscle membrane to chloride ions, thus tending to stabilize the membrane potential at or near to the chloride equilibrium potential (Boistel and Fatt, 1958; Grundfest et al., 1959; Kuffler, 1960; Takeuchi and Takeuchi, 1965). The postsynaptic inhibitory action of GABA on crustacean muscle was blocked by picrotoxin (Robbins and van der Kloot, 1958), and the actions of GABA at presynaptic inhibitory synapses on motorneurons were similarly sensitive to picrotoxin (Dudel and Kuffler, 1961; Dudel, 1965 a,b), which is effective in blocking GABA-induced chloride conductance in crayfish muscle (Takeuchi and Takeuchi, 1965). Such actions of GABA mimic those of the naturally occurring inhibitory transmitter, thus reinforcing the notion that the natural inhibitory transmitter might be identical to GABA itself. A convulsant, picrotoxin (the 1:1 mixture of the more potent picrotoxinin and the less potent picrotin) is known to antagonise some synaptic inhibition, including the effects of GABA upon crustacean muscle and stretch receptors (Curtis and Watkins, 1965), and it appears to act at the membrane region responsible for controlling the GABA-induced chloride flux, or on the link between the postsynaptic GABA receptor and the associated chloride ionophore complex (Ticku and Olsen, 1977). The alkaloid, bicuculline, has been found to antagonise not only the actions of GABA in the mammalian brain (Curtis et al., 1970 a), but also the actions of the naturally occurring inhibitory transmitter, and applied GABA, in the spinal cord, cerebral cortex, cerebellum and hippocampus (Curtis et al., 1970 b; 1971

a,b). Bicuculline has since become a very useful pharmacological tool to distinguish the actions of GABA in various brain regions.

The original findings that GABA has extremely potent inhibitory effects on invertebrate stretch receptor neurones, and muscles, prompted considerable interest that GABA might also be involved in inhibitory synaptic transmission in the mammalian CNS, and persuasive evidence has become available to support this view. Microiontophoretic application of GABA induces powerful inhibitory effects in spinal neurones (Curtis and Watkins, 1960, 1965), and causes hyperpolarisation of Deiter's neurones (Obata et al., 1967) as well as in cerebral cortical neurones (Krnjevic and Schwartz, 1966). GABA appears to be a very potent agonist on the spinal cord, although the sulphonic acid analogue of GABA, 3-amino-1-propanesulphonic acid (3-APS) may be slightly more potent than GABA on GABA receptors (Curtis et al., 1968 a). Muscimol is also a potent agonist at GABA postsynaptic receptor sites in a variety of systems (Curtis et al., 1971 b; Krosggaard-Larsen et al., 1977; Krosggaard-Larsen, 1978; Krosggaard-Larsen and Johnston, 1978; Krosggaard-Larsen and Falch, 1981). All these GABA actions, in the vertebrate CNS, are sensitive to both picrotoxin and bicuculline, and are commonly mediated by an increase in the chloride ion permeability of the postsynaptic membrane (see review by Iversen, 1972). This increase in chloride ion permeability is thus important for both the postsynaptic hyperpolarization of neurones by GABA at synapses on soma and dendrites, and the depolarization of primary afferent terminations at axo-axonic synapses, leading to a reduction in excitatory transmitter



output known as presynaptic inhibition (see review by Curtis and Johnston, 1974; Curtis, 1978).

Investigations on the action of GABA in several parts of the CNS have shown a striking similarity to the effects of synaptic inhibition, with a lowering of the neuronal membrane resistance and a more negative transmembrane potential, which is a reversal potential that can be altered by injection of chloride ions inside the cell, all of which observations are consistent with GABA being an inhibitory transmitter that acts by increasing chloride conductance (Krnjevic, 1974). GABA can induce both a hyperpolarizing and depolarizing response, as a result of alteration in chloride conductance. The GABA-induced hyperpolarization is caused by a net influx of chloride ions into the postsynaptic membrane and moves the membrane potential towards the chloride equilibrium potential, leading to a generation of an inhibitory postsynaptic potential (IPSP). That GABA may also be the transmitter for presynaptic inhibition was first proposed by Eccles et al. (1963), where a depolarizing response to GABA represents a depolarization of presynaptic terminals, leading to a reduction in the amount of transmitter released (presynaptic inhibition or primary afferent depolarization). Here, the increase in chloride conductance results in a net efflux of chloride ions, due to a high internal chloride concentration being maintained by an inwardly directed chloride pump.

Evidence that GABA is an inhibitory neurotransmitter, both in the vertebrate and invertebrate nervous system, is well docu-

mented (Sytinsky et al., 1978; Roberts and Hammerschlag, 1972; Otsuka, 1973; Iversen, 1972; Curtis and Johnston, 1974; Johnston, 1976; 1978; Nistri and Constanti, 1979; Krnjevic, 1974). GABA appears to fulfil all the criteria formulated for the identification of a classical neurotransmitter in the CNS (Werman, 1966; Steiner, 1971; Orrego, 1979; Fagg and Foster, 1983; Oja et al., 1977; Iversen, 1979), including the demonstration of the synthesis and storage of GABA within nerve cells, its release from terminals, its interaction with postsynaptic receptor sites and the consequent transient alterations in ionic mechanisms underlying the synaptic events sensitive to certain substances that antagonise these latter effects, as well as the processes of removal and inactivation of the transmitter from the synaptic environment. Understanding of the neurochemistry, neuroanatomy, neurophysiology and neuropharmacology of GABA in the CNS has thus been greatly advanced since its original isolation (see reviews by Curtis and Johnston, 1974; Oja et al., 1977; Snodgrass, 1983).

The extent to which GABA is ubiquitous within the CNS is reflected in the recognition that impaired transmission at synapses that use GABA as the transmitter appears to be implicated in a variety of neurological and psychiatric disorders (Enna, 1981; Krogsgaard-Larsen, 1981; Meldrum, 1982), making agents with direct or indirect GABA-mimetic properties potential therapeutic agents in a number of clinical disorders.

**GABA ANTAGONISTS : BICUCULLINE AND PICROTOXIN**

Consistent with pharmacological and electrophysiological observations that the convulsants bicuculline and picrotoxin selectively block GABA-mediated hyperpolarisation in the CNS (Curtis et al., 1971 a,b; Nistri and Constanti, 1979), bicuculline binds competitively to GABA receptor sites, whilst picrotoxin acts as a non-competitive antagonist by allosterically modifying the receptors or blocking GABA-activated ionophores (Olsen et al., 1978 b). Bicuculline, although shown to be a specific antagonist of GABA-mediated actions (Curtis et al., 1971 a), nevertheless has been questioned as a GABA antagonist (Godfraind et al., 1970; Straughan et al., 1971), since it has a variety of other unspecific effects such as inhibition of acetylcholinesterase activity (Svenneby and Roberts, 1974) and potentiation of acetylcholine (ACh) action (Miller and McLennan, 1974) when employed in relatively high doses. It has also been reported to be chemically unstable under physiological conditions, as its conversion to bicucine is quite rapid at pH 7 in aqueous solution, therefore caution should be exercised in experiments using bicuculline in solutions at physiological pH, and such solutions should not be kept for any prolonged period of time to avoid misleading negative results (Olsen et al., 1975). Alternatively, the quaternary salts bicuculline methiodide (BMI) (Pong and Graham, 1972) and methochloride (BMC) (Johnston et al., 1972) do not show rapid hydrolysis, as monitored by stability of their ultraviolet spectra and by retention of biological activity (Olsen et al., 1975), and hence, have preferentially been used in

most experiments performed in the present studies since they are the most convenient, specific antagonists at the GABA-receptor sites (Curtis and Johnston, 1974; Johnston, 1976; 1978).

Ligand binding studies show that BMI and dihydropicrotoxin (DHP), an analogue of picrotoxin, bind to different sites on the GABA-receptor  $\text{Cl}^-$ -ionophore complex (Olsen, 1981 a,b). A comparison of the relative potencies of bicuculline, and  $\text{Cl}^-$ -blockers such as picrotoxin, furosemide (Nicoll, 1978 a) and other antagonists of GABA-induced responses in rat cuneate nucleus slices (Simmonds, 1982) differentiates between the actions of bicuculline and picrotoxin, where the former is a competitive antagonist and the latter a non-competitive antagonist of GABA-mediated events in most vertebrate preparations; although picrotoxin appears to have both a competitive and a 'mixed' type antagonism in some invertebrate preparations (Nistri and Constanti, 1979).

Piretanide, an active  $\text{Cl}^-$ -transport blocker, selectively blocks primary afferent responses in the frog spinal cord (Wojtowicz and Nicoll, 1982), whilst furosemide, similarly a  $\text{Cl}^-$ -transport inhibitor, also acts by blocking the  $\text{Cl}^-$ -conductance increase elicited by GABA in the frog spinal cord (Nicoll, 1978 a).

The first direct claim that picrotoxin blocks a GABA-mediated inhibition in the vertebrate CNS was in the cuneate nucleus of the cat (Galindo, 1969), although it was already known

to block presynaptic inhibition in the spinal cord (Eccles et al., 1963). Since then, it has been shown that picrotoxin antagonises the action of GABA in various parts of the CNS (see review by Krnjevic, 1974) mainly through inhibiting the  $\text{Cl}^-$ -ionophore associated with the GABA receptor sites (Olsen, 1981 a,b). Picrotoxin has direct effects on crustacean muscle membrane conductance (Grundfest et al., 1959), and inhibition of GABA synapses by picrotoxin via the  $\text{Cl}^-$ -ionophore is non-competitive with GABA (Takeuchi and Takeuchi, 1969), suggesting that picrotoxin acts on the  $\text{Cl}^-$ -ionophore which is regulated by the GABA receptor (Olsen, 1981a, b; 1982). Due to lack of charge on the picrotoxinin molecule, such detailed pharmacological studies have proved almost impossible in the CNS, but binding studies with DHP emphasise that picrotoxin is a non-competitive inhibitor acting at a site remote from the GABA-receptor (Olsen et al., 1978 b; Ticku et al., 1978 a,b).

Bicuculline specifically antagonises GABA-mediated inhibition (Curtis et al., 1971 a,b; Curtis and Felix, 1971; Johnston et al., 1972), binds to GABA receptors (Mohler and Okada, 1977 a), and displaces GABA from the GABA receptors (Enna and Snyder, 1975; Olsen et al., 1978 a; Zukin et al., 1974). Bicuculline has been shown to antagonise GABA-induced responses in the dorsal root ganglion (Feltz and Rasminsky, 1974; Nishi et al., 1974), spinal cord (Curtis et al., 1971 b) and also on invertebrate neurones (Pigott et al., 1977). In most preparations, bicuculline antagonises only GABA-induced responses without affecting responses to other amino acids, suggesting that

bicuculline interacts specifically with GABA receptors (Curtis et al., 1971 a,b; Curtis and Felix, 1971).

Bicuculline has recently been shown to reversibly antagonise the membrane hyperpolarization responses mediated by GABA in mammalian spinal cord and cortical neurones studied in cell culture, in a competitive manner, and bicuculline also competitively inhibits [3H] GABA binding to both high and low affinity GABA sites, while [3H] muscimol binding to the high affinity site is also displaced by bicuculline, [3H] muscimol being a useful ligand for GABA receptor studies due to its specificity and potency (Snodgrass, 1978). This suggests that the high affinity site may be the physiologically relevant postsynaptic GABA receptor whilst the low affinity GABA receptors may be presynaptic (Nowak et al., 1982; Frere et al., 1982). However, in other preparations, bicuculline may demonstrate a non-competitive or 'mixed' type of non-competitive antagonism against GABA responses (Schechter and Tranier, 1977; Shank et al., 1974; Takeuchi and Onodera, 1972). In biochemical studies, [3H] BMC binds preferentially to low affinity GABA receptor sites (Olsen and Snowman, 1983), this being supported by the demonstration of autoradiographic localization of low affinity GABA receptors in various regions of the CNS (Olsen et al., 1984), which overlap with those for benzodiazepine receptors (Unnerstall et al., 1981).

## PERIPHERAL GABA RECEPTORS

It is well established that extrasynaptic GABA receptors exist in the peripheral nervous system, for example in the mammalian sympathetic and sensory ganglia, and on unmyelinated autonomic axons, none of which is thought to receive any form of GABAergic innervation (Adams and Brown, 1975; Bowery and Brown, 1972; DeGroat, 1972; Obata, 1974; Deschenes et al., 1976; Feltz and Rasminsky, 1974, Nishi et al., 1974; Brown and Marsh, 1978). As an example, GABA is not released from incoming fibres, but still increases chloride permeability of ganglion cells by depolarising the cell membrane if applied artificially (Adams and Brown, 1975). This effect is due to activation of the GABA receptors, and is blocked by bicuculline and picrotoxin (Bowery and Brown, 1974; DeGroat, 1970). Such depolarising effects, described on sensory neurones (Gallagher et al., 1978), and on unmyelinated axons in peripheral nerve trunks (Brown and Marsh, 1978) may be consistent with a depression of transmitter output related to presynaptic inhibition in the relevant pathways (Brown et al., 1981; Brown, 1979).

GABA also has a chloride-dependent depolarising action by acting on extrasynaptic receptors present on myelinated axons of both mammalian and amphibian peripheral nerves, such actions being activated by a range of GABA receptor agonists with potencies as follows: muscimol > 3-APS > GABA > 5-aminovaleric acid (DAVA), and these effects could be reversibly antagonised by

bicuculline and picrotoxin (Curtis and Johnston, 1974; Morris et al., 1983). It is again not entirely clear, however, if there is any functional role for such GABA receptors on peripheral nerve trunks. Sympathetic ganglia also take up GABA by a high affinity carrier system (Bowery and Brown, 1972), but the ganglionic carrier is located on neuroglial cells, surrounding the neurones and the nerve fibres, rather than on the neurones (Gallagher et al., 1978). Evidently a sodium-dependent GABA carrier complex exists on, and GABA is metabolised by GABA-T present in the glial cells (Brown, 1979). Given the presence of extrasynaptic receptors, such a neighbouring neuroglial uptake process assumes the role of a protective device against adverse effects of excess GABA on neuronal excitability, as there is a close anatomical correlation between the distribution of receptors and carriers (Brown, 1979), although there is no evidence that GABA ever reaches physiologically significant levels in these tissues. The significance of the role of extrasynaptic GABA receptors remains unresolved in such systems, but the peripheral GABAergic system may provide a useful model for studying GABA actions and transport, e.g. the mechanism of GABA release from glial cells is quite different from that of transmitter release from nerve terminals, there being no evidence for a vesicular or quantal release from glia, and unlike release from neurones, the rate of release from sympathetic glial cells is calcium-independent, and relatively insensitive to small changes in external potassium concentration (Brown, 1979).



## INTERACTIONS OF GABA WITH OTHER TRANSMITTER SYSTEMS

The presence of GABA receptor binding sites on serotonergic afferent terminals in the substantia nigra and on nigral efferent projections to the caudal mesencephalon (Gale, 1982), together with known interactions of GABA with other neurotransmitter systems such as dopamine (Waszczak and Walters, 1983), motilin, somatostatin, opiates and taurine (Chan-Palay et al., 1982), suggests that more than one transmitter system may be required to coordinate some physiological functioning, as such systems are intricate and complex.

GABA is known to directly affect the electrophysiological properties of pituitary pars intermedia cells, with the ionic and pharmacological characteristics of this action of GABA resembling those occurring at GABAergic synapses in the CNS, and suggesting a GABA-receptor-Cl<sup>-</sup>-ionophore complex influencing hormone output (Taraskevich and Douglas, 1982). High levels of GABA and its metabolic enzymes are also present in the hypothalamus (Okada et al., 1971; Fahn, 1976; van der Heyden et al., 1979). Therefore, it seems that GABA not only interacts with other neurotransmitter systems but also has a modulatory effect on hormonal function, GABAergic systems may thereby control hormonal secretion by modulating the release of several hypothalamic-hypophyseal hormones involved in behavioural regulation, either through their endocrine actions or their direct actions on the brain. Very likely the development of GABAergic drugs will be useful for treating

some types of hormonal or neuropsychiatric disorders (DeFeudis, 1984).

In addition, appreciable levels of GABA have been found in rat pancreatic islets (Okada et al., 1976; Taniguchi et al., 1977; Okada et al., 1982), and rat thyroid (Taniguchi et al., 1977). Interestingly, GABA is also present in other peripheral tissues such as the rat ovary and oviduct, with the GABA concentration in the rat oviduct being far greater than that in rat whole brain (Martin del Rio and Caballero, 1980; Martin del Rio, 1981; Schaeffer and Hsueh, 1982; Erdo et al., 1982; 1983 b; 1984). It is still unresolved as to what extent the importance of such findings might be, other than the postulate that GABA may be associated with the hormonal status in these tissues, and possibly related to the local control of blood flow (Erdo et al., 1985).

#### PHYSIOLOGICAL PROPERTIES OF GABA

Whilst it is generally accepted that GABA is a transmitter for postsynaptic (Curtis and Johnston, 1974), as well as presynaptic inhibition in the CNS (Anderson, 1978), but still, much remains to be learned about all aspects of the function of the GABA system in the CNS. GABA neurones are envisioned to play a key role at all levels of information processing, where the major neural system exerting tonic inhibition on pacemaker neurones might be an inhibitory system using GABA as a transmitter; as well, GABA can set the gain on the sensitivity of sensory

receptors to coordinate the function of the systems involved in perceptual integration, and can be concerned in neural regulation with regard to which neural circuits should be released for use at a particular time (Roberts, 1974). The anatomical substrates for all these have been seen, where immunocytochemical procedures at the light and electron microscopic levels indicate that GABAergic neurones form axo-dendritic, axo-somatic, axo-axonic and dendro-dendritic synapses in various regions of the CNS, including the spinal cord, cerebellum, cortex, hippocampus, olfactory bulb, retina, substantia nigra and striatum (Roberts, 1979 a). Correlations with biochemical, morphological and physiological studies of these regions have indicated that GABA neurones participate in information processing within and between neural subsystems by exerting tonic and phasic pre- and postsynaptic inhibition which results in the inhibition or in the discriminatory release of various neural functions (Roberts, 1979 a). Recent experimental results suggest that GABA-containing neurones may play a key role in controlling neural mechanisms in hypothalamic centers, thus changes in responses with altered GABA function might be observed in emotional reactivity, the sensation of pain, cardiac and respiratory functions, blood pressure, food and water intake (appetite control), sweating, galvanic skin response, insulin secretion, liberation of gastric acid, motility of the colon and many others (Roberts, 1979 a; DeFeudis, 1981 a,b; 1984; Enna, 1981; Kendall et al., 1982;), and, moreover, decreases in the efficacy of the GABA system could result in convulsive seizures (Roberts et al., 1960). It would be of great importance to be able to

specifically either decrease or increase the effectiveness of GABA neurones and influence the activity of the GABA system by influencing any of these factors: (a) activity of the GABA neurones themselves (b) GABA release (c) synthesis (d) postsynaptic actions (e) removal or synaptic inactivation by carrier-mediated transport or (f) enzymic degradation by metabolic destruction (Fig. 2). Enhancing the first four of the above processes, in combination with decreases in carrier-mediated transport and catabolic processes, leads to enhanced GABAergic functions, while the converse would result in decreased effectiveness of the GABA system.

## GABA RELEASE, UPTAKE AND INACTIVATION

Since the first demonstration that mammalian brain slices actively accumulate exogenous GABA (Elliott and van Gelder, 1958), it is now widely established that GABA released from neurones is subsequently taken up into both glial cell and neuronal compartments of the nervous tissue, and subsequently inactivated by the GABA-degrading enzyme, GABA-T (Fig. 1) in the tissues (Iversen, 1972), such uptake being sodium-dependent but with differing kinetics in each compartment (Schousboe, 1978). Thus GABA, largely neurally released, is inactivated by energy requiring transport processes (Curtis and Johnston, 1974) which may occur in either neurones or glial cells (Iversen and Kelly, 1975; Schousboe et al., 1977; Schousboe, 1978,a,b). Henn and Hamberger (1971) were the first to demonstrate that glial cells had a high affinity uptake system for GABA, and such a glial transport system has since been demonstrated in peripheral ganglia (Bowery and Brown, 1972; Young et al., 1973; Schon and Kelly, 1974; 1975; Roberts, 1976a,b), rat retina (Neal and Iversen, 1972), spinal cord explant cultures (Hosli et al., 1972) and primary culture of glial cells from cerebellum (Lasher, 1975) and cerebrum (Schousboe et al., 1977). From such studies, it is concluded that astrocytes may be involved in the inactivation of GABA, and the existence of a net inward transport as demonstrated by Hertz et al. (1978) indicates that the GABA uptake into astrocytes indeed represents a net uptake, in contrast to that found for peripheral ganglia (Roberts, 1976 b) and bulk-prepared glial

cells (Sellstrom and Hamberger, 1976). Amino-oxyacetic acid (AOAA), an extremely powerful inhibitor of GABA-T (Schousboe et al., 1974), was shown to be a relatively weak inhibitor of high affinity transport of GABA in brain cortex slices (Snodgrass and Iversen, 1973; Johnston and Balcar, 1974), although it was found to have no effect on GABA uptake into astrocytes (Schousboe, 1978). It is thus concluded that glial cells may well be involved in the inactivation and subsequent metabolism of GABA, this being supported by the relatively high activity of the GABA-T in these cells (Schousboe et al., 1977).

Based on structure-activity relations (SAR) studies using conformationally restrained GABA analogues, it is proposed that the GABA receptors, GABA transport systems, and GABA-T have basically similar yet distinctly different structural specificities (Krogsgaard-Larsen, 1978; Johnston et al., 1979 b; Krogsgaard-Larsen, 1981), suggesting that GABA adopts different conformations during interactions with the various bio-molecules concerned with each. So far as transport is concerned, 3-amino-cyclohexanecarboxylic acid (ACHC) has been shown to interfere specifically with neuronal GABA transport (Bowery et al., 1976 a; Neal and Bowery, 1977), and 2,4-diaminobutyric acid (DABA) has been previously classified as a specific inhibitor of the neuronal GABA carrier system (Schon and Kelly, 1974; Iversen and Kelly, 1975). By contrast, b-alanine (b-ala) is believed to be more inhibitory for glial GABA transport than for neuronal GABA transport (Schon and Kelly, 1975; Iversen and Kelly, 1975; Bowery et al., 1976 b), whilst (-) nipecotic acid (nip) is much more

potent than the (+) isomer in inhibiting the high affinity GABA uptake into astrocytes (Schousboe, 1978), although the latter may be a more specific blocker of neuronal GABA transport (Schousboe, 1978). Racemic mixtures of (+-) nip are thus said to be potent inhibitors of neuronal and glial GABA uptake, being a substrate for the transport carriers concerned (Krogsgaard-Larsen, 1980). However, although it has been shown that some GABA uptake inhibitors may induce epileptogenic and anti-convulsant effects, the myoclonus and seizures seen in audiogenic mice after intracerebrao-ventricular administration of uptake blockers such as (+) DABA or (+) cis ACHC (Meldrum et al., 1980), are unlikely to be a consequence of blockade of GABA uptake, as these may instead interfere with GABA-mediated inhibition by displacing GABA from intraneuronal stores and acting as 'false transmitters' or by other actions on GABA receptors or metabolism (Horton et al., 1979; Meldrum et al., 1980).

Studies with uptake blockers demonstrate a pronounced substrate specificity of the glial and, in particular, the neuronal GABA transport system, and it is evident that the GABA molecule is transported in a different conformation from that of its interaction with receptor sites. An example of this is muscimol, a structural analogue of GABA (Brehm et al., 1972), isolated by Muller and Eugster (1965) from *Amanita muscaria* and by Takemoto et al. (1964) from *Amanita muscaria*, is a potent GABA agonist on bicuculline-sensitive and strychnine-insensitive postsynaptic GABA receptors (Enna and Snyder, 1975; Enna et al., 1977 a; Krogsgaard-Larsen, 1978; Hori et al., 1978; Curtis et al.,

1971 a,b), but muscimol has no affinity for the glial uptake system (Schousboe, 1978), although it does have a weak effect on GABA uptake in the neuronal system (Schousboe et al., 1978), where it is also a weak substrate for the neuronal GABA transport carrier. Thus limitation of the agonist concentration of muscimol at the receptors by neural uptake is minimal (Johnston et al., 1978), as also with the glial uptake system (Schousboe et al., 1979; Scholfield, 1982). Conformational studies of a variety of compounds that have stereo-selective actions on GABA uptake, such as nip, DABA or ACHC indicate that GABA transport carriers are capable of interacting with a somewhat wider range of GABA conformations than are receptors. Neuronal transport carriers appear to bind GABA in more extended conformations than do glial carriers (Johnston et al., 1979 b), since (+) cis ACHC is a selective substrate for the former and b-ala for the latter (Bowery et al., 1976 a,b). Where the same GABA conformations may interact with some receptors and transport carriers, steric hindrance on opposite aspects of this same conformation may be capable of interfering with binding to receptors and transport carriers respectively, and would hence be consistent with one stereoisomer of a GABA analogue showing selectivity for receptors and the other stereoisomer for transport carriers (Johnston et al., 1979 b). Such findings are important for the development of drugs to manipulate pharmacologically functional GABA-mediated synapses, especially for use in certain pathological and neurological disorders.



## GABA RELEASE

GABA, released predominantly from neurones may be re-accumulated by nerve endings, but can also be metabolised by nonsynaptic regions of neurones or by astrocytes. Generally, glutamate, the source of GABA, is released in larger amounts than GABA and accumulated almost exclusively into astrocytes, whence it is converted to glutamine and returned to the neurones which utilize this glutamine for GABA synthesis. Although such interactions occur between neurones and astrocytes in the turnover of GABA, and its precursor glutamate, the full extent to which glutamine is returned from astrocytes to neurones, to compensate for the release of GABA and glutamate is not known (Hertz and Schousboe, 1980).

Synaptic vesicles enriched in GABA have been found (Storm-Mathisen et al., 1983), and the electrically-induced release of endogenous GABA from rat brain cortical slices is a TTX-sensitive, calcium-dependent process, which indicates a neural origin of the released GABA (Valdes and Orrego, 1978). Indeed, newly synthesised [3H] GABA from [3H] glutamic acid may be preferentially released from GABAergic nerve terminals in rat striatal slices, in response to the depolarization of neurones (Yoneda et al., 1984). It has also been shown that ornithine (Yoneda and Roberts, 1982; Yoneda et al., 1982), in addition to glutamine (Hamberger et al., 1979), can be transformed to GABA via glutamic acid in the mammalian CNS, but a positive correlation of glutamine synthetase (glutamine-synthesising enzyme) with

GAD in the rat CNS (Patel et al., 1985) suggests that GABA levels may largely be dependent on glutamine levels in neural tissue.

## GABA METABOLISM

In neural tissue, GABA is principally synthesised by the decarboxylation of glutamic acid through the action of a specific enzyme, glutamic acid decarboxylase (GAD), and degraded by GABA:2-oxoglutarate aminotransferase or GABA:2-keto-glutaric transaminase (GABA-T), as outlined in Fig. 1. The succinic semialdehyde resulting from this degradative step is incorporated into the Krebs cycle from whence the 2-ketoglutarate for glutamate resynthesis is provided through the GABA shunt; the latter steps occur in glial cells that provide glutamine which is transferred from glia to neurones, so supplying the substrate for neuronal GABA synthesis (see Chapter 3). It was originally proposed that at least two distinct types of GABA synthesising enzyme, glutamic acid decarboxylase (GAD), may exist in mammalian tissues (Haber et al., 1970 a,b; Drummond and Phillips, 1974), with one of them (GAD I) thought to be present in cerebral neurones and the other type (GAD II) in non-neural tissues such as the kidney and the heart. Although these two types of GAD showed different biochemical and immunochemical properties (Wu, 1976; 1977; 1980; 1982; Wu et al., 1978), a recent study, however, on comparative characterization of GAD in crude homogenates of oviduct, ovary and the hypothalamus suggests that the oviductal and the hypothalamic GAD may be identical (Erdo et al., 1984 a). Thus only one form of GAD may exist, most often associated with GABAergic innervation of the particular tissue.

GAD, the primary GABA synthesising enzyme present

predominantly in neurones, is restricted to certain neuronal populations, and has been purified to homogeneity from mouse brain (Wu et al., 1973). Since then, antibodies have been produced towards the enzyme, and used to visualize GAD location in the CNS (McLaughlin et al., 1974). Such pioneering efforts then led to the production of the rabbit antiserum to GAD to enable light and electron microscopic localization of GABAergic neurones in the CNS of several animal species (Roberts, 1979 b). Indeed, Wu et al. (1982) have reported their approaches to purify GAD to homogeneity, to characterize its properties and to determine its site of synthesis, storage and transport, together with the raising of antibody using purified enzyme preparations, in order to visualize its cellular and subcellular localizations by immuno-cytochemical techniques. A similar production and characterization of a specific GAD antiserum, following the isolation GAD and the purification via isolation of a GAD-anti-GAD-complex, has been described by Oertel et al. (1981 a,b,c). The immuno-cytochemical demonstration of GABAergic neurones in the mammalian CNS using GAD antiserum raised in sheep against rat brain GAD appears to be a specific probe for the localization of GAD-immunoreactivity (Oertel et al., 1982). Immuno-cytochemical methods have also been developed to visualize glutamate and GABA in the CNS neurones, by the use of antibodies raised against the amino acids coupled to bovine serum albumin with glutaraldehyde (Storm-Mathisen et al., 1983). The tissue localizations of glutamate-like and GABA-like immunoreactivities matched those of specific uptake sites for glutamate and GABA, and, in the case of GABA-like immuno-reactivities, also that for GAD.

In the dorsal column nuclei of the cat brain, GAD-immunoreactive boutons are localized on possibly presynaptic fibres, indicating that GABA is the transmitter responsible for the presynaptic inhibition (Westman et al., 1984). There is compelling immuno-cytochemical evidence for the coexistence of GABA and motilin in the cerebellum (Chan-Palay, 1982), as well as inter-relations between enkephalin and GABA in avian retina (Watt et al., 1984), and co-localization of GAD- and enkephalin-like immuno-reactivity in the rat neostriatum (Aronin et al., 1984). Also, GABAergic synapses present in the granule cell layer of the rat dentate gyrus have been examined using light and electron microscopes with GAD immuno-cytochemistry, (Kosaka et al., 1984), as have GABAergic synapses in the basal hypothalamus (Tappaz et al., 1983), whilst the presence of GAD and 5-HT in one nerve cell in the raphe nuclei has also been reported (Belin et al., 1983). Since the regional activity of GAD in the CNS correlates highly with the GABA concentration, it is likely that GAD is a good marker for GABAergic neurones (Fahn, 1976; Fonnum and Walberg, 1973; Kuriyama et al., 1966 a; Ribak et al., 1981). Nevertheless, immuno-histochemistry of GABA itself (Storm-Mathisen and Ottersen, 1983) will undoubtedly replace this in such studies.

Recently, the presence of GAD activity and high GABA levels have been detected in the rat oviduct and ovary (Martin del Rio and Caballero, 1980; Martin del Rio, 1981; Erdo et al., 1982; 1984a; Schaeffer and Hsueh, 1982), indicating a possible GABAergic innervation of the Fallopian tube (Erdo et al., 1984

a), although such high GABA levels have not been confirmed in other studies (Okada et al., 1982). Specific GABA receptor binding sites have been identified in both rat and human reproductive organs (Schaeffer and Hsueh, 1982; Erdo and Lapis, 1982 a,b; Erdo et al., 1983a; Erdo and Laszlo, 1984), and high affinity uptake systems for GABA have been demonstrated in tissue slices of the rat ovary (Erdo, 1983a) and fallopian tube (Erdo, 1983b).

A method for demonstrating the degradative enzyme GABA-T, developed by Van Gelder (1965), has since been used to demonstrate GABA-T activity in other CNS tissues (Vincent et al., 1980; 1981; 1982; Nagai et al., 1983; 1985). A pharmacohistochemical method for GABA-T, combined with retrograde tracing by horseradish peroxidase, has also been used to stain preferentially GABAergic neurones, and hence may be used to localize GABAergic pathways (Araki et al., 1984).

Compounds known to inhibit the activities of GAD and GABA-T can reduce the levels of GABA and hence provide valuable research tools, where abnormalities of motor function and behaviour can be induced by the local application of such compounds, indicating some functional roles of GABA pathways (Meldrum, 1975). GABA synthesis may be impaired by compounds that block the synthesis or coenzymic function of pyridoxal phosphate (PLP), including the hydrazides, by competitive inhibitors of GAD such as 3-mercaptopropionic acid (3-MPA), and by allylglycine or its metabolite, 2-keto-4-pentenoic acid (2-KPA), an irreversible inhibitor of GAD

(Meldrum, 1979; Horton et al., 1978). Conversely, drugs that inhibit GABA-T can increase the brain GABA content (Meldrum, 1979), such as  $\gamma$ -acetylenic GABA,  $\gamma$ -vinyl GABA which are more specific than AOAA or valproate (Gale and Iadarola, 1980; Iadarola and Gale, 1979; Seiler and Sarhan, 1980; Metcalf, 1979; Palfreyman et al., 1981), and potent anti-convulsant effects of the irreversible inhibitors of GABA-T, ethanolamine-o-sulphate (EOS) and gabaculine (GBL),  $\gamma$ -acetylenic acid and  $\gamma$ -vinyl GABA have been demonstrated in rodents and in primate models of epilepsy (Anlezark et al., 1976; Horton et al., 1977; Meldrum and Horton, 1978; Schechter et al., 1977).

A variety of alternative metabolic pathways have been postulated for GABA metabolism in mammalian brain. These include the formation of  $\gamma$ -guanidinobutyric acid by transamination with arginine; formation of GABA-containing peptides such as homocarnosine (GABA-histidine) and homoanserine (GABA-l-methyl histidine); formation of  $\beta$ -hydroxy-GABA,  $\gamma$ -butyrobetaine,  $\gamma$ -aminobutyrylcholine, or of homopantothenic acid (see review by Baxter, 1970). An interesting possibility is that succinic semi-aldehyde (SSA) can be converted to  $\gamma$ -hydroxybutyric acid by lactic dehydrogenase (Iversen, 1972; Fishbein and Bessman, 1964), which is present in the brain (Roth and Giarman, 1970 a,b), and the formation of small amounts of this substance from labelled GABA has also been seen (Roth, 1970). Nevertheless, the metabolism of GABA by GABA-T and SSA-dehydrogenase constitutes the major pathway for GABA metabolism in mammalian brain (Balazs et al., 1970).

## GABA BINDING STUDIES

GABA receptors have been identified in the CNS using in vitro receptor binding methodology (Zukin et al., 1974; Enna and Snyder, 1975; 1977 a,b; Enna et al., 1975; 1977 a; Olsen, 1976; Mohler and Okada, 1977 a; 1978 a). Such studies have characterized many of the properties of GABA receptor binding in nervous tissues, including bicuculline-sensitive, as well as picrotoxin-sensitive GABA-receptor binding (Mohler and Okada, 1978 b; Olsen et al., 1978; Olsen, 1976; 1981a,b; 1982; 1983). In particular, GABA binding to synaptic receptor or to uptake sites can be differentiated as  $\text{Na}^+$ -independent or  $\text{Na}^+$ -dependent respectively (Zukin et al., 1974; Enna and Snyder, 1975; see review by DeFeudis, 1983 a), where sodium can mask the presence of the high affinity class of GABA receptor related to synaptic receptor sites (Kurioka et al., 1981 a).  $\text{Na}^+$ -independent GABA binding occurs by an energy-independent, non-enzymatic, physicochemical process (Elliott and van Gelder, 1960; Sano and Roberts, 1963), whereas  $\text{Na}^+$ -dependent GABA binding, involved primarily in GABA uptake, is related to energy-dependent, enzyme-dependent transport (Varon et al., 1965; Weinstein et al., 1965; Kuriyama et al., 1968 a), some of which may be physically in close relation to synaptic or GABA receptors (DeFeudis, 1983 a; DeFeudis and Somoza, 1977; DeFeudis et al., 1979a; Kurioka et al., 1981 a,b). Such binding has proved to be a useful tool to correlate neuro-anatomical studies with localization of GABA receptors, where autoradiography has revealed the presence of extensive GABA-receptor binding sites in the CNS, using  $[^3\text{H}]$  GABA and  $[^3\text{H}]$



muscimol which are known to label GABA-receptor sites specifically (Enna and Snyder, 1977 a; Enna et al., 1978). In addition, Na<sup>+</sup>-dependent GABA binding to uptake sites occurs in the CNS (Sano and Roberts, 1963; Elliott et al., 1965; DeFeudis, 1973 a,b), very likely related to a 'carrier-mediated' process involved in the inactivation of synaptically-released GABA (Kuriyama et al., 1968a,b), but there has been little work on localization of these sites as such.

Muscimol, a structural analogue of GABA (Muller and Eugster, 1965; Brehm et al., 1972), exerts a potent, bicuculline-sensitive, depressant action by interacting at postsynaptic GABA receptor sites (Johnston et al., 1968; Curtis et al., 1971 a,b; Curtis and Johnston, 1974; Krosggaard-Larsen et al., 1975). Due to the high specificity of muscimol for the GABA receptor sites, there has been preferential use of muscimol as a GABA neuronal marker in in vitro binding studies aimed at characterizing and isolating GABA receptors, but such studies have been critically reviewed and analysed by DeFeudis (1980) who has suggested that results from such binding studies must be interpreted with extreme caution, in view of the observation that not all populations of the GABA receptors can be labelled with [3H] muscimol. Indeed, in considering certain electrophysiological data (Constanti and Nistri, 1981), the requirements for the binding sites for GABA and muscimol are not identical (DeFeudis et al., 1979 b,c; Johnston et al., 1979 a,b), thus either [3H] muscimol is capable of interacting with other population(s) of binding sites or [3H] muscimol is bound to some sites not labelled by

[3H] GABA (Olsen, 1982), thereby questioning the validity of using muscimol for detecting GABA receptors (DeFeudis, 1980; Jordan et al., 1982). Nevertheless, [3H] muscimol has proved to be a useful probe in autoradiographic studies, in particular, to localise the distribution of GABA receptor sites in various areas of the CNS. Furthermore, cellular localization of binding sites for tritiated GABA, muscimol, bicuculline methiodide and flunitrazepam, a benzodiazepine compound, in cultures of rat cerebellum and spinal cord using light microscopic autoradiography demonstrate that such binding sites are concentrated on neurones but not on glial cells (Hosli et al., 1980), although uptake of [3H] GABA was observed in almost all cultured glial cells (Hosli and Hosli, 1978). The lack of such binding sites on cultured glial cells is consistent with the lack of [3H] muscimol and [3H] GABA binding sites on glia shown in autoradiographic studies on CNS preparations (Chan-Palay, 1978; Kuhar et al., 1979).

Kinetic analyses of ligand binding data, and certain electrophysiological and behavioral methods, have revealed the possible existence of a multiplicity of GABA receptors in preparations of CNS tissues, but is not yet certain that these sites exist in vivo (DeFeudis, 1983 b). At least two classes with different kinetic constants for [3H] GABA or [3H] muscimol have been detected in various subcellular CNS preparations in both the presence and absence of sodium (DeFeudis, 1983 b), where the antagonism of the [3H] GABA binding by bicuculline appears to be competitive at the higher affinity site and non-competitive at the lower affinity

site, indicating the existence of multiple GABA receptors (DeFeudis, 1983 b).

Manipulation of environmental factors necessary for GABA binding studies has been investigated, with reports of the presence of endogenous inhibitors of GABA binding in mammalian brain (Greenlee et al., 1978 a) as well as the inhibition of GABA binding by endogenous GABA itself (Napias et al., 1980), it having been proposed that endogenous GABA in part determines the characteristics of [3H] GABA binding (Gardner et al., 1981). However, pretreatment with GABA, and modulatory ligands, enhances GABA receptor binding by the facilitated removal of endogenous substances (Maksay and Ticku, 1984) that are evidently endogenous modulators of the GABA receptor (Lagos et al., 1983). Thus, different populations of GABA binding sites have been reported, depending on the conditions of the binding assays, as e.g. treatment with the detergent Triton X-100, to reveal either Na<sup>+</sup>-dependent or independent binding sites (Enna and Snyder, 1975; Enna et al., 1977 a; Enna and Snyder, 1977 a). Indeed, a class of Na<sup>+</sup>-independent GABA postsynaptic binding sites in mammalian brain has been detected, where the binding capacity of the tissue is maximal and stable with time if the tissue is thoroughly washed to remove any endogenous inhibitor of binding (Greenlee et al., 1978 a).

There have been several reports of such endogenous inhibitors of binding of radioactive ligands to GABA receptors (Guidotti et al., 1978; Johnston and Kennedy, 1978; 1979; Toffano

et al., 1978). The latter have described an endogenous modulator of the GABA receptor, an acidic, heat stable protein, termed "GABA-modulin", as being distinct from, and having a higher molecular weight than GABA itself, although this was not detected by Napias et al. (1980); such discrepancies in their respective findings may be attributed to differences in experimental procedures, and conditions, in membrane treatment. Yoneda and Kuriyama (1980) have reported the presence of a non-GABA endogenous ligand called the GABA receptor binding inhibitory factor (GRIF), and more recently, Johnston and Skerritt (1984) have suggested that endogenous modulators of GABA binding (GABARINS) may be involved in benzodiazepine enhancement of low affinity GABA binding. However, some later study suggest that the endogenous inhibitor of GABA is indeed GABA itself, based on biochemical characteristics of such material (Napias et al., 1980; Lagos et al., 1983). Consequently GABA binding to specific GABA receptor sites is best measured using sodium-free assay conditions to minimize association of GABA with nonspecific sites such as the  $\text{Na}^+$ -dependent transport sites (Zukin et al., 1974; Enna and Snyder, 1975; Greenlee et al., 1978 b), and detergent treatment and the rigorous process of continuous freezing and thawing of the membranes (Enna and Snyder, 1975; Enna and Snyder, 1977 a) may improve the binding affinities of the ligands, but such harsh treatments can be considered potentially unphysiological, even though binding is enhanced.

In the periphery, specific GABA receptor binding sites have been detected in the fallopian tube, oviduct and ovary (Erdo et al., 1982; 1983 b; Erdo and Laszlo, 1984; Schaeffer and Hsueh,

1982), where the specific binding of [3H] GABA is displaced by muscimol, cold GABA, or bicuculline (Erdo and Laszlo, 1984). Moreover, a high affinity, Na<sup>+</sup>-dependent uptake system for GABA has been demonstrated in rat ovary slices (Erdo, 1983 a).

Studies with ligand binding techniques, although subject to certain limitations, especially with regard to their relevance to the in vivo situation (DeFeudis, 1980; Olsen, 1982), have been useful in understanding the relationships that exist between GABA receptors in the CNS and behaviour, where the neuronal pathways involved seem to be related to convulsions, extrapyramidal functions, analgesic mechanisms, effects of anxiety and environmental stresses, epilepsy, Parkinson's disease, Huntington's chorea, and certain hormonal influence, as well as behavioural disorders such as Alzheimer's dementia. Indeed, binding studies on GABA receptors in material from patients suffering from epilepsy (Lloyd et al., 1980), Parkinson's disease (Lloyd et al., 1977 b; 1980), or Huntington's chorea (Enna et al., 1976; Lloyd et al., 1977 a) may yet provide clues to the altered GABAergic functions in these disorders, for such alterations in cerebral GABA receptors are possibly involved in many such neurologic-psychiatric conditions, so that hopefully such further characterization of GABA receptor binding may be useful in providing clues to the treatment of assorted behavioral disorders.

In conclusion, using isolated preparations to obtain knowledge about drugs and drug-receptor interactions based primarily on binding studies and uptake studies, and to gain more

information about the pharmacological classification of drugs and their receptors in isolated tissues , would be valuable in defining drug action, and in the future design of more effective therapeutic agents (Kenakin, 1984), particularly in relation to GABAergic mechanisms.

**GABA<sub>A</sub>-AND GABA<sub>B</sub>-RECEPTOR PHARMACOLOGY**

GABA receptors in the mammalian brain, as well as on peripheral autonomic neurones, are not homogeneous. A clear distinction exists between receptors that are bicuculline- and picrotoxin-sensitive, and an entirely separate population of receptors that are insensitive to bicuculline or picrotoxin (Bowery et al., 1981 a,b). The bicuculline-sensitive GABA receptors have been designated GABA<sub>A</sub>-receptors (Hill and Bowery, 1981), with the rank order of the potencies of the agonists interacting at such receptor sites being muscimol > 3-APS > or equal to GABA > isoguvacine, whilst the bicuculline-insensitive GABA<sub>B</sub>-receptor sites are not sensitive to GABA mimetics such as muscimol, 3-APS or isoguvacine, but the b-(p-chlorophenyl)-derivative of GABA, baclofen, is stereospecifically active at such sites whereas it is devoid of activity at the GABA<sub>A</sub>-receptor site (Bowery et al., 1979; Hill and Bowery, 1981; Bowery et al., 1981 a,b; Bowery, 1982; Bowery et al., 1982 a). The [3H] (-) isomer of baclofen is an improved ligand for GABA<sub>B</sub>-receptor sites, exhibiting significant affinity for GABA<sub>B</sub> sites ( Bowery et al., 1985; Drew et al., 1984), whilst the labelled racemic form has a lower specific activity due to the presence of the inactive (+) isomer (Wilkin et al., 1981). Neuronal actions of GABA and its analogues were first reported in the CNS by Eccles et al. (1963), who showed that GABA and 3-APS produced changes in dorsal root potentials, consistent with a central depolarising action when applied to the exposed surface of the cat spinal cord. Picrotoxin, known to be anti-GABA (Robbins and van der Kloot, 1958), reduced the prolonged inhibi-

tion of spinal monosynaptic reflexes as well as primary afferent depolarization (Eccles et al, 1963), hence suggesting that such mechanisms have a GABAergic involvement, perhaps through GABA receptor sites. Eventually, bicuculline was introduced as a potent GABA antagonist in such experiments (Curtis et al., 1970 a; 1971 a,b).

Baclofen was first shown to cause muscle relaxation and relieve various forms of spasticity in man (Birkmayer et al., 1967). Early studies, however, showed that baclofen failed to mimic GABA when applied microiontophoretically to cortical neurones (Davies and Watkins, 1974), but would induce a bicuculline-insensitive depressant action on CNS neuronal firing in vivo (Curtis et al., 1974; Davies and Watkins, 1974). Since then, a number of effects induced by baclofen have been described. Baclofen has a presynaptic action at the crayfish neuromuscular junction, by depressing the amplitude of excitatory junction potentials (ejps) without affecting muscle input resistance, and reducing the frequency of spontaneous miniature ejps without affecting their size, hence depressing transmitter release from the excitatory nerve (Barry, 1984). Other actions of baclofen include inhibition of dopamine release from rabbit caudate nucleus presynaptically (Reimann, 1983), a bicuculline-insensitive depression of neuronal firing in rat hippocampal slices (Ault and Nadler, 1983 a), and anticonvulsant-like actions in similar preparations (Ault and Nadler, 1983 b). In the olfactory cortex, Cain and Simmonds (1982) showed that inhibitory postsynaptic activity is depressed by baclofen, and Scholfied (1983) reported



that baclofen blocks postsynaptic inhibition by reducing the ability of excitatory nerve terminals to activate the inhibitory cell, also in the olfactory cortex. Indeed, the reduction of excitatory transmitter effects as with substance P and excitatory amino acids, is well documented (Pierau and Zimmerman, 1973; Polc and Haefely, 1976; Potashner, 1978; 1979; Fox et al., 1978; Saito et al., 1975; Johnston et al., 1980; Kato et al., 1982), although it was originally unclear if such actions of baclofen might be in any way associated with GABAergic function.

GABA itself reduces the evoked but not the basal release of [3H] noradrenaline from sympathetic nerve endings in isolated atria and vas deferens of the rat; this action is insensitive to bicuculline or picrotoxin, and is not mimicked by any of the GABA<sub>A</sub>-receptor agonists muscimol, 3-APS or isoguvacine (Bowery and Hudson, 1979), but mimicked by baclofen. Both GABA and baclofen also reduce electrically induced noradrenergic twitch responses of the vas deferens, and cholinergic twitch responses in the guinea-pig ileum; these effects are also insensitive to both bicuculline and picrotoxin, being a chloride-independent event and are not mimicked by any of the GABA<sub>A</sub>-receptor agonists (Bowery et al., 1981 a,b). Moreover, Muhyaddin et al. (1982 a,b) have shown that GABA<sub>B</sub>-receptor sites are present on rat anococcygeus muscle, where baclofen reduces noradrenaline output by interacting with GABA<sub>B</sub>-receptor sites, this being antagonised by 5-aminovaleric acid (DAVA). The general consensus is that a major site of action of baclofen is on presynaptic nerve terminals where it depresses the release neurotransmitters by an

action at a novel GABA receptor site, GABA<sub>B</sub>-receptor, the (-) isomer of baclofen being > 100 fold more active than the (+) isomer (Bowery et al., 1980 a,b; 1981 a,b; 1982 a). Baclofen has become the drug of choice in the treatment of spasticity of spinal origin due to its antispastic efficacy at doses which do not produce sedation, its low frequency of serious side effects and its lack of organ toxicity (Sachais and Logue, 1977), since it can cross the blood-brain barrier (Keberle and Faigle, 1972), and does not interfere with GABA transport recognition sites (Tardy et al., 1978; Bowery et al., 1983 a). Furthermore, baclofen is resistant to metabolic degradation (Tardy et al., 1978). Earlier, it has been proposed to be a potential analgesic agent (Cutting and Jordan, 1980), in view of early reports that its action may be associated with inhibition of sensory input to the spinal cord, possibly through a process mimicking presynaptic inhibition (Pierau and Zimmerman, 1973; Pierau et al., 1975). In keeping with this, neurotransmission in isolated hemisectioned spinal cord preparations from immature rats is depressed by baclofen, resulting from a depression of the presynaptic release of excitant amino acid transmitters (Ault and Evans, 1981), and baclofen also inhibits activation of sympathetic preganglionic neurones by stimulation of dorsal roots in isolated spinal cord preparations of the rat (McKenna and Schramm, 1984). Presynaptic GABA<sub>B</sub> receptors have also been found on sympathetic (Starke and Weitzell, 1980; Bowery et al., 1981 a; Stone, 1981; Anwar and Mason, 1982; Hughes et al., 1982; Muhyaddin et al., 1982 a,b; 1983) and parasympathetic nerve terminals (Bowery et al., 1981 a).

Both GABA<sub>A</sub> and GABA<sub>B</sub> receptors are present on Ad and C primary afferents; the bicuculline- and picrotoxin-sensitive GABA<sub>A</sub>-receptor mediates depolarizations, and the responses are mimicked by muscimol, isoguvacine, 4,5,6,7-tetrahydroisoxa-zolo[5,4,-c]pyridin-3-ol (THIP) and 3-APS, but the baclofen-sensitive, bicuculline- and picrotoxin-insensitive GABA<sub>B</sub> receptor, on ganglion cell bodies of C and Ad fibres, instead shortens the calcium component of their action potentials (Desarmenien et al., 1982; 1984; Dunlap, 1981; McBurney, 1984). In cultured chick dorsal root ganglion neurones, GABA<sub>B</sub> receptor activation can be monitored as a shortening of the calcium component of action potentials (Dunlap, 1981), and also leads to a decrease of the voltage-sensitive calcium channel conductance in embryonic chick sensory neurones (Dunlap and Fischbach, 1978; 1981).

Autoradiographic localization of GABA<sub>B</sub> receptors in rat cerebellum shows that GABA<sub>B</sub> sites, unlike GABA<sub>A</sub> sites, are confined almost exclusively to the molecular layer of the cerebellum (Wilkin et al., 1981), and a comparison by Bowery et al. (1984 a,b) of the distribution and cellular locations of the two different receptor types in the brain show that their locations in various brain regions differ. Bowery et al. (1984 a,b) have also reviewed GABA<sub>B</sub>-receptor distribution, and presented a detailed discussion of the separation between GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor subtypes in various central and peripheral tissues. Both high and low affinity GABA<sub>B</sub>-receptor binding sites have been identified and appear to be anatomically distinct, low affinity sites

being associated with cerebro-cortical noradrenergic nerve terminals suggesting that GABA may regulate noradrenergic activity in the brain (Karbon et al., 1983), whilst autoradiographic and lesion studies in the spinal cord indicate that a large proportion of GABA<sub>B</sub>-receptors are present on primary afferent terminals (Price et al., 1984).

GABA receptor subtypes have been described on the basis of many characteristics (Nistri and Constanti, 1979; Allan et al., 1980; Costa, 1981; Alger and Nicoll, 1982). The clearest subdivision to emerge has derived from structure-activity studies performed in many systems (Bowery, 1982). Undoubtedly, a multiplicity of GABA<sub>A</sub>-receptor subtypes exist and these may include autoreceptors, pre- and post-synaptic, synaptic and extrasynaptic receptor types. Both neurochemical and electrophysiological studies show the existence of GABA<sub>B</sub> sites in addition to GABA<sub>A</sub> receptor sites (Bowery et al., 1980 a,b; Ault and Nadler, 1982 a,b; Cain and Simmonds, 1982). The characteristics of GABA<sub>B</sub>-receptor sites (Bowery et al., 1983 a; 1984 b), in contrast to GABA<sub>A</sub>-receptor sites, include the following :

- (1) Not activated by isoguvacine, 3-APS, piperidine-4-sulphonic acid or THIP, or other recognised GABA-mimetics of GABA<sub>A</sub>-receptors
- (2) activated by (-) baclofen in a stereospecific manner
- (3) not influenced by barbiturates or benzodiazepines (Bowery et al., 1984 a)
- (4) sensitive to the presence of GTP (Bowery et al., 1982 b;

Hill et al., 1984)

(5) not associated with chloride-ionophores

(6) dependent on physiological concentrations of divalent cations  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , and possibly associated with a  $\text{Ca}^{2+}$  channel (Dunlap and Fishbach, 1978; 1981; Dunlap, 1981; Desarmenian et al., 1982; McBurney, 1984), and acting by decreasing the inward flux of calcium giving rise to the reduction in evoked transmitter release at presynaptic terminals (Bowery and Hudson, 1979; Bowery et al., 1980 a,b; 1981 a,b; Kaplita et al., 1982; Hughes et al., 1982; Muhyaddin et al., 1982 a,b; Fillenz and Fung, 1983; Giotti et al., 1983 a; Kilpatrick et al., 1983; Ong and Kerr, 1983 a,b), or a reduction in calcium-dependent action potentials (Dunlap, 1981; Dunlap and Fishbach, 1981). A recent finding suggests that GABA decreases calcium currents in chick dorsal root ganglion cells by interfering with at least two types of calcium channels associated with two types of GABA<sub>B</sub>-receptor complexes, but the physiological consequences of this internal modulation of the GABA action for phenomena such as presynaptic inhibition are difficult to predict (Deisz and Lux, 1985).

Johnston and Allan (1984) reported a multiplicity of bicuculline-insensitive GABA-receptor sites that are not activated by (-) baclofen in the CNS, consistent with recent evidence for two possible pharmacologically distinct, bicuculline-insensitive actions of GABA in the rat hippocampal slices, only one of which is shared by baclofen (Ault and Nadler, 1983 a,b). Such a suggestion, that there may be two different classes of bicuculline-insensitive receptor sites in the CNS, is

supported by recent evidence that GABA interacts with GABA<sub>B</sub> binding sites in extended conformations, and that folded analogues of GABA may interact with a class of binding site insensitive to (-) baclofen and bicuculline (Drew et al., 1984), which in turn supports the evidence for two pharmacologically distinct bicuculline-insensitive actions of GABA in rat hippocampal slices (Ault and Nadler, 1983 a,b). There appears, in addition, to be a further class of bicuculline-insensitive receptor (suggested to be named GABA<sub>C</sub>) for which *cis*-4-aminocrotonic acid and *cis*-2-(aminomethyl)cyclopropanecarboxylic acid are specific agonists (Drew et al., 1984). Binding properties of these GABA analogues have been described, but there is as yet little pharmacological evidence on any specific role that they might play in GABAergic neural function.

It is as yet uncertain whether calcium channels are associated with GABA<sub>B</sub> sites situated on postsynaptic elements such as the Purkinje cell dendrites in the cerebellum (Bowery et al., 1983 b), but calcium involvement in GABA<sub>B</sub>-receptor mediated actions has been reported in the myenteric plexus of the guinea-pig (Cherubini and North, 1984 a,b), and dendrites of the hippocampus (Newberry and Nicoll, 1984; Nicoll and Newberry, 1984). Although, in addition, functional inhibitory postsynaptic GABA<sub>B</sub>-receptors are thought to exist, since late bicuculline-insensitive hyperpolarizing potentials are observed following orthodromic stimulation of hippocampal CA1 neurones (Newberry and Nicoll, 1984), mimicked by baclofen when applied to the dendrites of CA1 pyramidal cells (Newberry and Nicoll, 1984; 1985), both of which

may result from an increase in potassium conductance (Newberry and Nicoll, 1984; 1985; Nicoll and Newberry, 1984). Also in the invertebrate, presynaptic inhibition in *Aplysia* involves a decrease in the calcium current of the presynaptic neurone, possibly mediated through GABA<sub>B</sub>-receptor sites (Shapiro et al., 1980). Physiological concentrations of Ca<sup>2+</sup> or Mg<sup>2+</sup> are required to promote binding to the GABA<sub>B</sub>-receptor site but not to GABA<sub>A</sub>-receptor sites; and Triton X-100 treatment of synaptic membranes enhances ligand binding to GABA<sub>A</sub> receptors while reducing that to the GABA<sub>B</sub>-receptor sites (Enna and Snyder, 1977 a; Hill and Bowery, 1981; Bowery et al., 1982 a; 1983 a). The affinity of the GABA<sub>B</sub>-receptor site for its ligands is regulated by divalent cations, through common sites of action, in the following order of potency for increased binding: Mn<sup>2+</sup> = Ni<sup>2+</sup> > Mg<sup>2+</sup> > Ca<sup>2+</sup> > Sr<sup>2+</sup> > Ba<sup>2+</sup> (Kato et al., 1983), but Hg<sup>2+</sup> > Pb<sup>2+</sup> > Cd<sup>2+</sup> > Zn<sup>2+</sup> for inhibition (Drew et al., 1984). Thus, although calcium stimulates the binding of [3H] GABA to GABA<sub>B</sub>-receptor sites, it also modulates the interaction between GABA<sub>A</sub>- and benzodiazepine-receptor sites, whilst decreasing [3H] GABA binding to GABA<sub>A</sub>-receptor sites (Corda and Guidotti, 1983; Majewska and Chuang, 1984).

In the mammalian periphery, GABA<sub>B</sub> sites are present in the intestine, vas deferens and atria (Bowery et al., 1981 a; Muhyaddin et al., 1982 a,b; Giotti et al., 1983 a,b; Kleinrok and Kilbinger, 1983; Ong and Kerr, 1983 a,b; Kaplita et al., 1982), vascular muscle (Starke and Weitzell, 1981), anococcygeus muscle (Hughes et al., 1982; Muhyaddin et al., 1982 a,b), basilar artery

(Anwar and Mason, 1982), and also in the reproductive tract (Erdo et al., 1984 b).

No potent specific antagonist for the baclofen-sensitive, GABA<sub>B</sub>-receptors has yet been reported, except for DAVA, which appears to antagonise GABA<sub>B</sub>-receptor-mediated actions in the anococcygeus muscle (Muhyaddin et al., 1982 a,b; 1983). However, DAVA would be ineffective as a GABA<sub>B</sub>-receptor antagonist against GABA<sub>B</sub>-receptor-mediated events in the CNS, since it is also active as a GABA<sub>A</sub>-receptor agonist (Bowery et al., 1983 a). In addition, homotaurine has also been found to be a weak antagonist at GABA<sub>B</sub>-receptor sites in the intestine (Giotti et al., 1983 b), but it is a potent agonist at GABA<sub>A</sub>-receptor sites (Bowery et al., 1981 a; Ong and Kerr, 1983 a). Studies of GABAergic function would thus be greatly advanced with the availability of a specific and potent GABA<sub>B</sub>-receptor antagonist.

From time to time, more controversial actions of GABA have been described e.g. exogenous GABA inhibits serotonin release in the rat brain via GABA<sub>B</sub>-receptor sites which may be assumed to be located presynaptically on the serotonergic nerve terminals (Schlicker et al., 1984), but controversial results from previous studies have been reported where GABA either increases (Starr, 1979) or inhibits evoked [3H] serotonin release (Balfour, 1980; Bowery et al., 1980 a; Bowery, 1982). In other studies, GABA and 3-APS cause a release of catecholamine from isolated perfused dog adrenals in a dose-dependent manner, such release being antagonised by bicuculline and picrotoxin which suggests that



GABA may interact with a receptor located on chromaffin cells and/ or on splanchnic nerve terminals to evoke a calcium-dependent, but sodium and chloride-independent, release of catecholamines from adrenal medulla (Kitayama et al., 1984). Also Arbilla and Langer (1979) demonstrated that GABA facilitates the potassium-evoked release of norepinephrine from the rat occipital cortex, and neither picrotoxin nor bicuculline block this action of GABA. It is probable that in these tissues, a different type of GABA receptor exists. Also, Karbon et al. (1983) have described the presence of two GABA<sub>b</sub>-receptor binding sites for [3H] GABA in membranes from cerebral cortex of the rat, probably associated with noradrenergic terminals. An additional separation of GABA receptor sites (GABA<sub>1</sub> and GABA<sub>2</sub>) has been proposed on the basis of different binding affinities for [3H] GABA in rat brain synaptic membranes (Guidotti et al., 1979), but has not achieved general acceptance.

Both GTP and GDP decrease the saturable binding of [3H] baclofen and [3H] GABA to GABA<sub>b</sub> but not GABA<sub>a</sub> receptors sites, whereas GMP has negligible activity. This effect is specific to guanyl nucleotides and is not mimicked by high concentrations of ATP. Evidently, GTP reduces the affinity of the GABA<sub>b</sub>-receptor for GABA rather than producing any change in the apparent number of receptors (Hill et al., 1984), and it has been postulated that GABA<sub>b</sub>-receptor sites activation may be linked to adenylate cyclase activity, although the detailed operative mechanisms are not known (Dolphin, 1984; Hill et al., 1984; Hill and Dolphin, 1984; Karbon and Enna, 1983; 1985; Enna and Karbon, 1984; Karbon

et al., 1984; Wojcik and Neff, 1984).

## NUCLEOTIDE INVOLVEMENT IN GABA ACTIONS

Dolphin (1984) questions the functional relevance of the modulation of intracellular cyclic AMP levels by GABA<sub>B</sub> receptors, suggesting that GABA<sub>B</sub>-receptor-mediated inhibition of adenylate cyclase activity may imply that a change in the phosphorylation state of specific proteins due to a reduction in presynaptic cyclic AMP levels would reduce transmitter release either at the calcium influx or at the exocytosis step. In the cerebellum, some of the GABA<sub>B</sub> receptors that are negatively coupled to adenylate cyclase are situated presynaptically on parallel fibre terminals (Wilkin et al., 1981; Wojcik and Neff, 1984), there being a marked reduction of GABA<sub>B</sub>-receptor-mediated inhibition of adenylate cyclase in weaver mice which have a selective loss of granule cells, but not in nervous mice which are without Purkinje cells, suggesting that GABA<sub>B</sub>-receptors are mainly localized in the granule cells (Wojcik and Neff, 1984). On the other hand, Wilkin et al. (1981) showed that GABA<sub>B</sub>-receptors are in the molecular layer of the cerebellar cortex. The inhibition of basal adenylate cyclase activity by GABA or (-) baclofen at the GABA<sub>B</sub>-receptor sites is GTP-dependent (Wojcik and Neff, 1984). However, Hill et al. (1984) did raise the possibility that GABA<sub>B</sub>-receptor activation may either stimulate or inhibit adenylate cyclase activity, whereas Wojcik and Neff (1984) tend to suggest that GABA<sub>B</sub>-receptor activation would inhibit adenylate cyclase activity.

GABA<sub>B</sub> agonists such as GABA and baclofen potentiate the norepinephrine-induced cyclic AMP response in rat brain slices (Karbon and Enna, 1983; 1985; Karbon et al., 1984; Enna and Karbon, 1984; Hill and Dolphin, 1984; Hill, 1985 a), and also the cyclic AMP response that occurs during exposure to isoproterenol, adenosine, vasointestinal polypeptide (VIP) and histamine (Karbon and Enna, 1985), but depress the forskolin-induced rise in cyclic AMP levels (Hill, 1985 a). It is believed that such influence of GABA<sub>B</sub>-receptor activation on cellular cyclic AMP accumulation depends on the initial stimulus to adenylate cyclase, such that when adenylate cyclase is stimulated via an adrenoceptor, GABA<sub>B</sub>-receptor activation results in a potentiation of the response, whereas in the presence of direct stimulation of the enzyme, GABA and baclofen inhibit the rise in cyclic AMP. This inhibitory action of baclofen and GABA is dependent upon the presence of GTP (Wojcik and Neff, 1984), the inhibitory GTP binding protein is likely to be involved in the coupling between GABA<sub>B</sub>-receptors and adenylate cyclase enzyme and is hence responsible for mediating the GTP modulation of GABA<sub>B</sub>-receptors in vitro (Hill, 1985 a). GABA<sub>B</sub> agonists may play a role in modulating the responsiveness of neurotransmitter-coupled cyclic nucleotide systems in brain via a calcium-dependent process, as GABA<sub>B</sub>-receptor binding requires calcium (Hill and Bowery, 1981). GABA<sub>B</sub>-receptor activation influences neurotransmitter stimulated cAMP production by modifying some component of the cyclic nucleotide generating system, without affecting basal cAMP production itself, suggesting that GABA<sub>B</sub>-receptors are linked to adenylate cyclase, as

modulation of this enzyme may be responsible for some of the effects of GABA<sub>B</sub>-receptor activation (Enna and Karbon, 1984; Hill and Dolphin, 1984). Radioligand studies have shown that GABA<sub>B</sub>, but not GABA<sub>A</sub> receptors, are sensitive to inhibition by guanyl nucleotides (Bowery et al., 1982 b; Hill et al., 1984); such a reduction in receptor affinity induced by GTP is thought to be the result of a functional coupling between receptor and adenylate cyclase enzyme, which in turn, induces a state of low receptor affinity (Rodbell, 1980). Although DAVA has been reported to be a GABA<sub>B</sub>-receptor antagonist in peripheral tissues (Muhyaddin et al., 1982 a,b), various concentrations of DAVA employed (300 uM and 1 mM) did not prevent the inhibition of adenylate cyclase by baclofen (Wojcik and Neff, 1984).

**BENZODIAZEPINE-BARBITURATE-GABA-RECEPTOR-IONOPHORE COMPLEX**

Impairment of GABA function leads to seizures (Meldrum, 1979; Olsen, 1981 a), resulting from a defect in GABA biosynthesis, GABA synaptic release, GABA postsynaptic actions or the associated  $\text{Cl}^-$ -ionophore, whilst conversely an enhancement of GABA-mediated function can be effective in protecting against some forms of seizures (Iadarola and Gale, 1981; Krosgaard-Larsen, 1981; Meldrum, 1979; Olsen, 1981 b; Roberts, 1980). The latter could be manipulated by blocking GABA uptake into nerve terminals, blocking GABA catabolism by the enzyme GABA-T, using GABA-mimicking drugs, such as GABA agonists at the receptor sites or prodrugs, or more commonly, by the use of indirect potentiators of GABA inhibitory synaptic transmission acting at components of the receptor complex. Thus, the anticonvulsant action of benzodiazepine depends on enhancement of GABAergic inhibition through interaction at the GABA-benzodiazepine receptor complex (see review by Meldrum and Braestrup, 1984). Some clinically used anticonvulsants appear to fit in this category, by potentiating GABAergic function, including not only the benzodiazepines which have a receptor site on the GABA-receptor  $\text{Cl}^-$ -ionophore complex of the postsynaptic membrane (Braestrup et al., 1982; Costa and Guidotti, 1979; Olsen, 1981 b; Olsen and Leeb-Lundberg, 1981 a,b; Squires et al., 1980; Tallman et al., 1978), but also barbiturates and related depressants which have a separate receptor site on the GABA-receptor ionophore complex (Leeb-Lundberg et al., 1980; 1981; Olsen, 1981 a,b; 1982; Ticku and Olsen, 1978; Olsen et al., 1984).

Numerous studies show that both benzodiazepines and barbiturates have modulatory drug receptor sites on the GABA-receptor ionophore complex, with a close coupling of GABA and benzodiazepine receptor sites (Olsen, 1982), whilst other CNS depressants, including the barbiturates and pyrazolopyridine anxiolytics (e.g. etazolate) appear to modulate the binding of ligands to GABA and benzodiazepine receptor sites allosterically. The benzodiazepine-GABA-receptor ionophore complex, first proposed as being composed of an oligomeric structure consisting of at least three interacting components, namely the (a) benzodiazepine binding site (b) GABA receptor binding site and the (c) picrotoxinin (and related compounds) binding site (Fig. 3), exhibits allosteric interactions of a variety of centrally acting modulatory drugs which appear to facilitate GABAergic transmission, including barbiturates and benzodiazepines, that show stereoselectivity, anion dependence and brain regional selectivity (Ticku, 1983). The picrotoxinin binding site seems to have a pronounced three-dimensional structure, and properly positioned and oriented alkyl substituents at C-5 of barbiturates may lead to convulsant or depressant drug actions, e.g. where interactions at the hypothetical hydrophobic allosteric regulatory site of the  $\text{Cl}^-$  channel makes the compound convulsant by blocking the  $\text{Cl}^-$  channel (Ticku and Maksay, 1983). With the exception of bicuculline, other convulsants such as the bicyclophosphate esters inhibit GABA transmission and inhibit the binding of dihydropicrotoxinin (DHP) or t-butylbicyclophosphorothionate (TBPS) to the picrotoxinin site, preventing the allosteric enhancing effect of depressant

drugs on GABA and benzodiazepine binding (Ticku and Maksay, 1983). The barbiturate site can be assayed with tritium-labelled ligands such as [3H]-DHP, an analogue of picrotoxin (Olsen, 1981 a,b; Olsen, 1982; Ticku and Olsen, 1978), or related convulsants, such as sulfur 35-labelled [35S]-TBPS, (Squires et al., 1983). Barbiturate enhancement of GABA and benzodiazepine receptor binding is totally dependent on the presence of physiological amounts of chloride or other anions involved in inhibitory currents regulated by the GABA receptors (Olsen, 1981a, 1982).

A variety of evidence suggests that barbiturates act via the same sites as convulsants such as picrotoxinin, since the enhancement of GABAergic synaptic transmission (Nicoll, 1975 a,b) and of benzodiazepine receptor binding (Leeb-Lundberg et al., 1981) by barbiturates is blocked by picrotoxinin. Moreover, barbiturates have been shown to compete for binding of [3H]-DHP or [35S] TBPS (Ticku and Olsen, 1978; Squires et al, 1983; Ticku and Maksay, 1983; Ramanjaneyulu and Ticku, 1984 a,b), although the latest evidence suggests that barbiturates modulate [35S] TBPS binding allosterically by binding to a site distinct from the picrotoxinin recognition site, indicating that the barbiturate recognition site on the GABA-benzodiazepine receptor complex is distinct from the picrotoxinin/TBPS recognition site (Trifiletti et al., 1984).

A number of agents, including barbiturates, affect benzodiazepine-receptor affinity without interacting directly at the benzodiazepine-receptor site. As an example, pentobarbitone



(PB) enhances [3H] diazepam binding to brain benzodiazepine receptors in chloride-containing buffers (Leeb-Lundberg et al., 1980; Olsen and Leeb-Lundberg, 1981 a,b; Johnston and Willow, 1981), but PB does not act directly on GABA receptor recognition sites (Olsen and Leeb-Lundberg, 1981 a,b; Ticku, 1981). The barbiturate enhancement of benzodiazepine binding is reversed allosterically by bicuculline and competitively by picrotoxinin and related convulsants that block the GABA-receptor operated Cl<sup>-</sup>-channels, and which inhibit [3H] DHP binding (Olsen, 1981 a,b; Ticku, 1981). Barbiturate interactions with the GABA receptor complex show a chemical specificity and stereospecificity (Olsen and Leeb-Lundberg, 1981) that correlates well with the ability of these agents both to reverse GABA antagonist action (Bowery and Dray, 1978; Simmonds, 1981) and to enhance GABA-agonist postsynaptic receptor-mediated responses (Bowery and Dray, 1978; Evans, 1979; Haefely, 1980; MacDonald and Barker, 1978a; 1979 a,b; Nicoll and Wojtowicz, 1980; Schulz and MacDonald, 1981); there is also a reasonably good correlation with anaesthetic and hypnotic potency (Willow and Johnston, 1981; Ticku, 1981). Thus, based on the interactions between barbiturates and benzodiazepines at the GABA-receptor ionophore complex, there is considerable circumstantial evidence for a role of the GABA-receptor ionophore complex in seizure disorders, in the light of modulatory receptor sites on the GABA receptor complex for anticonvulsant drugs, including the benzodiazepines and barbiturates (Olsen, 1981 a,b; 1982; Olsen and Leeb-Lundberg, 1981; Olsen et al., 1984), suggesting that an impairment of GABA-mediated inhibitory transmission could contribute to the genesis of certain kinds of

seizure disorders.

Regulation of GABA receptors by barbiturates and related sedative-hypnotic and anticonvulsant drugs, as well as electrophysiological and neurochemical studies of the barbiturates and their possible mechanisms of actions have more recently been extensively reviewed (Johnston, 1983; Willow and Johnston, 1983), as have the effects of barbiturates on synaptic transmission (Nicoll, 1978 b; Richter and Holtman, 1982), with the demonstration that barbiturates enhance and prolong GABA-mediated synaptic inhibitions in the CNS, due to an alteration in the affinity of the GABA receptor for its ligand following an interaction of the barbiturate with a binding site closely associated with the picrotoxinin binding site. The mechanisms of actions of barbiturates and benzodiazepines, together with other known anticonvulsant and convulsant drug actions on vertebrate neurones in primary dissociated cell culture have also been reviewed (MacDonald, 1984).

Apart from the known anaesthetic and anticonvulsant actions of barbiturates, they have been found to possess other unspecific actions (Willow and Johnston, 1983), among which is the ability of anaesthetic barbiturates to depress, whereas convulsant barbiturates promote, release of excitatory amino acids, and their ability to alter activity of the plasma membrane  $Ca^{2+}$  pump (Willow and Johnston, 1979; 1983; Skerritt et al., 1983a). PB was also found to augment the GABA-evoked release of [3H] noradrenaline in hippocampal synaptosomes at a concentration of 1-10

uM, but 100 uM PB depressed this effect (Fung and Fillenz, 1984), the latter also being due to an effect on calcium movement in the nerve terminals.

A steroid anaesthetic, alphaxalone and PB share a common mode of action on the GABA system, potentiating depolarizing responses to superfused GABA and muscimol in rat cuneate nucleus slices (Harrison and Simmonds, 1984). Whilst the various components of the GABA receptor complex are characterized on the basis of the presence of (a) GABA receptors (b) chloride channels (c) regulatory sites where drugs act to influence any GABA-mediated transmission, it is still unclear if any such sites of drug action are involved in the normal physiological regulation of GABA-mediated inhibition. In particular, the identity of any endogenous ligands, and the degree of functional coupling at these sites, remains to be resolved (Simmonds, 1983).

### **BENZODIAZEPINES**

Anxiety may involve many brain neurotransmitters, one of which is probably GABA, and the benzodiazepine group of anxiolytics exert their effects via the GABA-benzodiazepine receptor  $\text{Cl}^-$  channel complex, (Braestrup and Nielsen, 1982). Earlier evidence supporting a GABAergic involvement for the mode of action of benzodiazepines includes (a) the specific ability of the benzodiazepines to relieve convulsions associated with an impairment of GABAergic function (b) the enhancement of GABA-mediated presynaptic inhibition and (c) the potentiation of post-

synaptic GABAergic mechanisms by benzodiazepines (Costa and Guidotti, 1979; Costa et al., 1975; Guidotti et al., 1978). Other actions of benzodiazepines may be to (d) block presynaptic  $\text{Ca}^{2+}$  uptake (Leslie et al., 1980) (e) reduce voltage-dependent  $\text{Na}^+$  and  $\text{K}^+$  ion conductances (Wang and James, 1979) (f) to increase chloride conductance (Vyskocil, 1977) and to (g) reduce repetitive firing of action potentials (Vyskocil, 1977; Wang and James, 1979). The use of benzodiazepines as a pharmacological tool has helped to elucidate details of the supramolecular nature of the GABA receptor regulatory unit and also has helped to characterize the specificity and efficacy of this group of drugs in the mediation of GABAergic function, since benzodiazepines have no direct GABA-mimetic properties (Costa and Guidotti, 1979).

The discovery of benzodiazepine receptors in 1977 (Squires and Braestrup, 1977) prompted an intensive search for endogenous ligands with benzodiazepine-like activity, or the opposite, with a number of possible ligands proposed (Braestrup and Nielsen, 1980; Mohler, 1982), although there is as yet no compelling evidence for the existence of any such an endogenous substance in the brain. Multiple benzodiazepine receptors exist (Martin et al., 1983; Squires, 1983), and the regional, cellular and subcellular localization of benzodiazepine binding sites has been described (Richards and Mohler, 1984). A greater understanding of the synaptic actions of the benzodiazepines (Haefely, 1983), and of the behavioural and neuropharmacological aspects of benzodiazepines in relation to specific GABAergic pathways, together with recent developments in benzodiazepine receptor research (Haefely,

1982; Yamamura et al., 1982; Squires et al., 1982), all lend further support for the presence of multiple, independent but interacting sites for benzodiazepines, influenced by various anions, at the GABA-receptor complex (Squires et al., 1982), as well as interacting recognition sites for picrotoxin (and related substances) and cations (Squires and Saederup, 1982). The finding that THIP, a GABA receptor agonist (unlike muscimol or GABA) does not enhance [3H] benzodiazepine binding to CNS tissue particulates (Braestrup et al., 1979) indicates that THIP might interact with a class of GABA receptors not associated with benzodiazepine receptors, although a piperidine derivative related to THIP, piperidine 4-sulphonic acid (4-PS), does alter benzodiazepine binding affinities at higher temperatures (Wong and Iversen, 1985).

Electrophysiological experiments have shown that benzodiazepines enhance both presynaptic and postsynaptic GABAergic inhibition at various sites throughout the CNS (Haefely et al., 1979; Costa et al., 1975); effects that do not involve the synthesis, release or inactivation of GABA, nor is there a direct effect of benzodiazepines as GABA agonists on the receptor recognition site (Braestrup and Nielsen, 1982). However, stimulation of GABA receptors by GABA enhances the affinity of benzodiazepine receptors for benzodiazepines in an anion dependent manner, suggesting the possibility of a close coupling of the GABA and benzodiazepine receptors to the  $\text{Cl}^-$  channel at the GABA-benzodiazepine-receptor  $\text{Cl}^-$ -ionophore complex on postsynaptic neuronal membranes (Tallman et al., 1980; Olsen, 1982; Karobath et al.,

1981). Although the benzodiazepines themselves do not open  $\text{Cl}^-$  channels when they occupy the benzodiazepine receptor sites, they increase the frequency of  $\text{Cl}^-$  channel opening (Study and Barker, 1981; 1982), thus GABA has greater success in opening  $\text{Cl}^-$  channels when the benzodiazepine receptor is stimulated by the benzodiazepines such as the diazepam, in contrast to barbiturates which slow GABA-dissociation at its receptor (Willow and Johnston, 1981b) and prolong the  $\text{Cl}^-$  channel opening time, hence enhancing GABA-mediated inhibition (Study and Barker, 1981; 1982). It has been proposed that a facilitation of GABA inhibition would explain the many central actions of the benzodiazepines (Polc et al., 1974; Costa et al., 1975; 1976; Mao et al., 1975; Haefely, 1978), and such drugs bind with high affinity to recognition sites on synaptic membranes (Mohler and Okada, 1977 b; Squires and Braestrup, 1977), which correlates with their muscle relaxant and anxiolytic action (Mohler and Okada, 1978 b).

An allosteric model for benzodiazepine receptor function has been proposed, since numerous binding studies show that benzodiazepine receptors can exist in different conformational states upon receptor activation, with a range of ligands at benzodiazepine receptor sites displaying heterogeneous binding characteristics (Ehlert et al., 1983 a,b). The reciprocal interaction between the benzodiazepines and GABA receptors is thought to be modulated by membrane lipids (Kuriyama and Ueno, 1983), and/or by an endogenous regulatory protein (GABA-modulin) which allosterically regulates the number of GABA and benzodiazepine receptors, and which competitively interacts with the benzodiazepines for

the regulation of high affinity recognition sites for GABA (Guidotti, 1980), but there appears to be some question as to whether the GABA-modulin molecule is part of the supramolecular organisation constituting the GABA-benzodiazepine-ionophore complex, or whether the GABA-modulin is a consequence of an homogenization artefact in the binding assay (Johnston and Skerritt, 1984).

Benzodiazepine receptor ligands with positive and negative efficacy have been described, and pharmacological experiments indicate that agonists, antagonists and inverse agonists constitute a whole continuum of agents with a graduated variety of efficacy at the receptor complex, and this, with the support of both biochemical and electrophysiological data, suggests that the benzodiazepine receptor allosterically up- or down-regulates the gain in the GABAergic system depending on the nature of the respective ligands (Braestrup et al., 1984).

Benzodiazepines potentiate the actions of both synaptically released and exogenously administered GABA on mammalian neuronal preparations (Polc and Haefely, 1976; Choi et al., 1977; MacDonald and Barker, 1978 b), e.g. Flurazepam, a benzodiazepine compound, potentiates GABA-mediated responses and attenuates preferentially the action of picrotoxin rather than bicuculline (Simmonds, 1980), whilst Gallagher (1978) showed that benzodiazepines selectively potentiate GABA-mediated inhibition in dorsal raphe neurones, and proposed a model for separate GABA and benzodiazepine entities coupled to a common ionophore (Gallagher et

al., 1980).

Distinct pharmacologically relevant receptor sites for GABA/bicuculline (Olsen, 1982; Enna and Snyder, 1977 a; Olsen et al., 1981; Olsen and Snowman, 1983), benzodiazepines (Tallman et al., 1980; Braestrup and Nielsen, 1981; Squires, 1981; Mohler and Richards, 1981), and barbiturate/picrotoxinin-like drugs (Olsen, 1982; Leeb-Lundberg et al., 1981; Leeb-Lundberg and Olsen, 1982) have all been well characterized. Reciprocal in vitro chloride sensitive interactions between these various receptors suggest their close association in the post-synaptic membrane, representing physiologically relevant processes within a functional GABA-receptor-ionophore complex. In addition, heterogeneity in benzodiazepine receptor and GABA receptor function has been proposed, with the suggestion of a speculative model involving multiple coupling states of a single type of GABA and benzodiazepine receptor in which they may be found associated with each other and with barbiturate/picrotoxinin receptors associated with the  $Cl^-$  channels (Leeb-Lundberg and Olsen, 1983). It appears that one class of benzodiazepine receptor might be associated with either a high or low affinity GABA receptor, and a further class might not be linked to GABA receptors, providing further evidence for a multiplicity of GABA and benzodiazepine receptors (Tallman et al., 1978; Karobath and Sperk, 1979; Squires et al., 1980), as two or more types of benzodiazepine receptors appear to exist in CNS subcellular preparations, only a portion of which may be functionally coupled to the GABA-receptor-ionophore complex (Squires et al., 1980; Olsen, 1982; Richards et al., 1982). In



addition, it has been found that there is an interaction between barbiturate and benzodiazepine receptor sites, as PB stimulates benzodiazepine binding, such action is mediated via TBPS sites, and the fact that PB inhibition of [35S] TBPS binding is not competitive suggests that PB does not act at exactly the same site as TBPS to modulate constituents of the GABA-receptor complex (Richter and Yamamura, 1985).

Studies in vitro have also revealed the existence of presynaptic GABA autoreceptors (Snodgrass, 1978; Mitchell and Martin, 1978; Brennan and Cantrill, 1979 a,b; Brennan et al., 1981), where GABA and its receptor agonists inhibit the release of [3H] GABA from synaptosomes with d-aminolaevulinic acid as a selective agonist for the GABA autoreceptors (Brennan and Cantrill, 1979 b; Brennan et al., 1981). The physiological release of GABA is subjected to negative feedback control through presynaptic GABA autoreceptors sensitive to bicuculline (Brennan and Cantrill, 1979 a), but these autoreceptors are not functionally coupled to benzodiazepine receptors, at least in the rat cerebral cortex (Brennan, 1982). As the depolarization-induced release of preloaded [3H] glutamate was augmented by GABA and by certain GABA agonists (except baclofen), a further population of presynaptic GABA receptors might also exist on glutamate-containing nerve terminals (Mitchell, 1980). Little is known of the precise agonist requirements for such presynaptic actions, other than for primary afferent depolarization, despite considerable advances in the design of GABA agonists and antagonists, together with increased knowledge of their therapeutic effects through their

respective actions on the receptors in GABA-mediated synapses (Krosgaard-Larsen, 1981; Krosgaard-Larsen and Christensen, 1980).

Avermectin Bla, a novel macrolide anthelmintic (Kass et al., 1980) and insecticidal (Ostlind et al., 1979) agent, has been reported to enhance the muscle relaxant activity of benzodiazepines (Williams and Yarbrough, 1979), and to cause a marked and sustained increase of GABA release from rat brain synaptosomes in a  $\text{Ca}^{2+}$ -independent manner, suggesting that such ability of the drug to release GABA may be the basis of its antiparasitic action (Pong et al., 1980). On the other hand, Fritz et al. (1979) showed that avermectin has a GABA-agonist like action by opening  $\text{Cl}^-$  channels in the lobster muscle membrane, this action being reversed by picrotoxinin. Avermectin enhances the binding of [3H] diazepam to brain benzodiazepine receptors (Williams and Yarbrough, 1979; Paul et al., 1980; Supavilai and Karobath, 1981; Pong et al., 1982; Drexler and Sieghart, 1984) and has been shown to possess binding affinity for a unique separate modulatory receptor site on the benzodiazepine-GABA-receptor  $\text{Cl}^-$ -ionophore complex (Supavilai and Karobath, 1981; Olsen and Snowman, 1985); this binding site being in intimate contact with the GABA-receptor  $\text{Cl}^-$ -ionophore complex, as Avermectin also enhances the binding of [3H] GABA (Pong and Wang, 1982).

In summary, the current state of knowledge suggests that there are at least five major, pharmacologically distinguishable binding sites on the GABA-benzodiazepine-barbiturate receptor

complex (Fig. 3), namely:

(a) GABA (b) benzodiazepine (c) avermectin (d) TBPS/picrotoxinin site (barbiturate site) and (e) an anion site (Drexler and Sieghart, 1984; Olsen and Snowman, 1985). The structure-activity requirements of the GABA receptor have been extensively reviewed (Krogsgaard-Larsen et al., 1983 a,b; 1984).

## THE ENTERIC NERVOUS SYSTEM

The autonomic nervous system has three main divisions based on the classification suggested by Langley (1921) and can be divided into the following:

- (a) Sympathetic, arising from the thoracolumbar spinal cord,
- (b) Parasympathetic, arising from cranial nerves or from sacral cord, and (c) enteric, comprising the neuronal plexuses of the gut (see Gershon et al., 1979). The sympathetic and parasympathetic divisions are seen to have distinct and often antagonistic actions on their target organs, whilst the enteric nervous system functions autonomously but under the combined influence of the other two systems.

The general outline of the innervation of the gastrointestinal tract has been extensively reviewed (Schofield, 1968), and is known to consist of intrinsic neural plexuses; the myenteric (Auerbach's), the submucosal (Meissner's), and deep muscular plexuses. The myenteric plexus is situated between the outer longitudinal and the inner circular smooth muscle layers of the muscularis externa, whilst the submucosal plexus lies in the submucosal layer, supplying the mucous membrane, glands and villi of the innermost mucosa. There are also plexuses of nerve fibres in the muscularis mucosae, around the intestinal glands, and in the villi forming a 'sub-epithelial' plexus. Perikarya of the myenteric neurones are, for the main part, found in the ganglia and primary meshwork of the plexus, the latter giving rise to a finer secondary meshwork that further branches to form the ter-

tiary meshwork innervating the intestinal smooth muscle (Fig.4). Each ganglion contains a variety of neurones with differing morphology and, a few cell bodies occur in the secondary meshwork, whilst 'Interstitial Cells of Cajal' are found at intersections of the tertiary meshwork (Thuneberg et al., 1982). The enteric ganglia consist of sensory elements, interneurons and motorneurons with an internuncial integrative circuitry, constituting an organised, complex network which processes afferent information and generates coordinated excitatory and inhibitory activity, ultimately at the level of the smooth muscle. All this is dependent on the intact integrative circuitry within the enteric ganglia, and can function independently of input from the CNS (Wood, 1975; 1981 a,b; 1983; 1984).

Unlike other peripheral nerves or ganglia, neurones in the myenteric plexus are not supported by endoneurial, perineural, or epineural connective tissue sheaths, but are supported by surrounding glial elements (Gabella, 1971; 1972; Cook and Burnstock, 1976), thus the enteric ganglia have a compact organization of neural and glial elements, with an absence of collagen and paucity of extracellular space (Gabella, 1972). Jessen and Mirsky (1980) have identified a glial fibrillary protein present in the glial cells of the rat myenteric plexus, similar to that in the CNS. In general, glial cells appear to play a complex supportive role in nervous tissue (Hertz, 1979), and are believed to be important in controlling the extracellular milieu surrounding nerve cells by buffering potassium (Orkand et al., 1966), by uptake of neurotransmitters (Hertz, 1979) and transport

of nutrients from the blood (Golgi, 1903), and finally by providing mechanical support for the neuronal matrix. They can also serve as a major restraining influence on the spontaneous activity of neurones by decreasing intrinsic excitatory levels of neuronal membranes below their spontaneous firing levels, where such restraint could be exerted by removing substances from the extraneuronal environment in the region of synapses (e.g. potassium or protons), by adding substances to it, or by regulating the diffusion of substances in such a way as to shunt the depolarizing ionic currents. Glial cells are likely involved in the 'blood-brain' barrier, but the existence of a similar barrier in the enteric nervous system is controversial although such a blood-myenteric plexus barrier may exist in the gut, similar to the blood-brain barrier of the CNS, interposed between the blood vasculature and the synaptic circuits of the ganglia. Tight junctions between the endothelial cells of capillaries that supply neuronal elements in the plexus likely constitute this barrier, the plexus itself being thought to be devoid of capillaries, as blood vessels do not enter enteric ganglia (Gershon and Bursztajn, 1978).

Glial cells are readily identified in the intramural ganglia on the basis of their shape, position and ultrastructural features (Gabella, 1972), being smaller and more numerous than the ganglionic neurones, which they surround, in the myenteric plexus of the guinea-pig ileum; however, it is not clear whether there are different types of glial cell within the enteric ganglia. An important role suggested for glial cells of the enteric

ganglia is related to the mechanical activity of the muscularis externa, including the myenteric and submucosal ganglia, where it is thought to be involved in structural re-arrangement of the ganglia, which allows changes in shape and sliding of structures past each other, yet at the same time holding them together during the marked changes in shape that occur during intestinal motility (Gabella, 1981).

#### **ENTERIC NEURONAL CLASSIFICATION**

Based on intracellular electrical recording from the cell body in the guinea-pig small intestine, four categories of enteric neurones with different electrophysiological properties and membrane properties have been classified namely: S/type 1, AH/type 2, type 3, or type 4 (see Wood, 1983). From the viewpoint of electrophysiology, enteric ganglion cells can be subdivided into two major classes: S/type 1 neurones, and AH/type 2 neurones (Wood, 1983; 1984). Of these, S/type 1 neurones have a low resting membrane potential with high input resistance, exhibit TTX-sensitive, sodium-dependent repetitive spike discharges, and commonly show fast excitatory postsynaptic potentials (EPSP's) as well as inhibitory postsynaptic potentials (IPSP's), whereas AH/type 2 neurones are distinguished by a higher resting membrane potential with lower input resistance, exhibit TTX-resistant calcium-dependent spikes of very limited train length, and show prolonged post-spike hyperpolarizing potentials (the AH property); however, processes of AH/type 2 cells do show typical-

ly TTX-sensitive spikes. Some low amplitude fast EPSP's are found in AH/type 2 cells, apparently arising at some distance from the soma, but the predominant synaptic potential of this cell type is a slow EPSP with which is associated a reduction or abolition of the AH property, so that the cell will fire repetitively, but AH/type 2 cells do also exhibit IPSP's. Although Dogiel (1899) described three morphologically different types of enteric ganglion cells, namely Type I cells (short club-shaped processes) and Type II cells (many long smooth processes) and Type III cells (multipolar with processes of intermediate length), it has been found impossible to correlate their electrophysiological characteristics with morphological structure. Neither interneurons, sensory, nor motor neurons have been unequivocally identified by electrophysiological techniques in the enteric nervous system, as they have been in the CNS.

#### MOTILITY

Contractile activity in the intestine is influenced by at least four factors : (a) intrinsic smooth muscle properties (b) intrinsic nerves (c) extrinsic nerves and (d) circulating or locally released hormone or chemicals. The movements of the muscle layers depend on the intrinsic properties of the muscle itself, and the influence of circulating hormones<sup>e</sup> and nerves. The muscle has the ability to undergo varying rhythmic changes in excitability along the length of the intestinal tract, and it is the contraction of the layers of the external musculature which propels the intestinal contents from the stomach to the rectum.



A basic feature of intestinal smooth muscle is the generation of cyclic changes in excitability of the smooth muscle cells, related to membrane potentials and commonly known as 'slow waves' that have been recorded from small and large intestines of all species studied in vivo (Daniel, 1968; 1975; Prosser and Bortoff, 1968; Prosser, 1974; Bortoff, 1976). Such slow wave activity (basal electrical rhythm) varies along the length of the gastrointestinal tract, the frequency decreasing along the length of the small intestine in a non-linear pattern (Alvarez and Mahoney, 1922), and can be recorded from either circular or longitudinal muscle layer of the intestine. Although the cellular origin of intestinal slow wave is uncertain, some studies have found that slow waves are generated by cells of the longitudinal layer and spread electronically into the circular muscle layer (Bortoff and Sachs, 1970), and it has been suggested that the 'Interstitial Cells of Cajal' function as intestinal pacemaker cells, responsible for regulating the slow wave activity (Thuneberg, 1982).

#### **THE MYOELECTRIC MIGRATING COMPLEX**

The origin and propagation of migrating myoelectric motor complexes, which are cyclical changes in the pattern of intestinal motility in fasted animals and humans, has been reviewed (Costa and Furness, 1982), with the recognition of the ability of the isolated intestine to transport its contents aborally (Cannon, 1911; Bayliss and Starling, 1899; Langley and Magnus, 1905; Crema et al., 1970, Trendelenburg, 1917; Kosterlitz and Watt, 1975; Costa and Furness, 1976). The classic work of

Bayliss and Starling (1899; 1900; 1901) better characterised the reflexes involved in the mechanism of peristalsis, but their major conclusion that "excitation at any point of the gut excites contraction above and inhibition below", which forms the "Law of the Intestine", was originally refuted by several authors because of an inability to detect the relaxation of the circular muscle in front of the advancing bolus (Alvarez and Zimmerman, 1927; Baur, 1928; Henderson, 1928; Alvarez, 1940). However, in essence the 'Law' holds, and a variety of stimuli evoke this polarised sequential activation of the enteric reflexes resulting in the propulsion of intraluminal contents (Costa and Furness, 1982). In the ascending reflex, there is a final excitatory cholinergic neurone, but in the descending inhibitory reflex, the final inhibitory neurones release some unidentified transmitter substance. These enteric reflexes project in all areas of intestine, and the neurones involved give a coordinated response to increased volume of the luminal contents, facilitating the passage of the contents in the aboral direction. Sympathetic noradrenergic nerves, which are of extrinsic origin, inhibit food propulsion along the digestive tract, whereas para-sympathetic cholinergic nerves augment the excitatory component of the peristaltic reflex.

Within the enteric nervous system, there exists a multiplicity of transmitter substances such as ACh (Kosterlitz and Lees, 1964), NA (Furness and Costa, 1974), 5-HT (Gershon, 1981), adenosine triphosphate (Burnstock, 1975), prostaglandins (Dajani et al., 1975), somatostatin (Hokfelt et al., 1975), substance P

(Hokfelt et al., 1974), VIP (Bryant et al., 1976; Fuxe et al., 1977), enkephalins (Elde et al., 1976; Schultzberg et al., 1978), GABA (Jessen et al., 1979) and an array of peptides (Furness and Costa, 1982), all of which have been proposed as enteric neurotransmitters or neuromodulators. About ten or more distinct types of enteric neurones have been distinguished, based on electrical, pharmacological, functional, histochemical, biochemical and ultrastructural characteristics (see review by Furness and Costa, 1982), but it is not known if there is any correlation between their structures and the transmitters involved, and furthermore, electrophysiological studies alone do not provide sufficient evidence for a transmitter function for most of the substances, in particular the enteric peptides. Nevertheless, the full range of synaptic potential types, both fast and slow excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs), can be evoked experimentally by electrical stimulation of presynaptic fibres in the myenteric plexus, or they may occur spontaneously, with presynaptic inhibitory mechanisms operative at both the fast and slow synaptic junctions; an example of which is a presynaptic action of 5-HT, where application of 5-HT to myenteric ganglia reduces the amplitude of stimulus-evoked fast EPSPs, but does not affect the depolarising response to iontophoretically applied ACh (North et al., 1980).

Ileal longitudinal muscles of the guinea-pig have, in addition to a cholinergic motor innervation, both an excitatory and inhibitory non-cholinergic, non-adrenergic (NANC) innervation, the NANC excitatory fibres are more densely distributed in the

terminal rather than in the proximal region, while in the case of NANC inhibitory <sup>6</sup>fires, the distribution is reversed (Bauer and Kuriyama, 1982). Circular muscle cells are, however, homogenously innervated by NANC inhibitory nerves.

Langley and Anderson (1895) were the first to report that contractions of the urinary bladder in response to pelvic nerve stimulation were "atropine-resistant", which finally led to the suggestion that there was a separate NANC innervation of the bladder (Ambache and Zar, 1970; Burnstock et al., 1972). The existing evidence for both NANC inhibitory and excitatory nerves in the vertebrate autonomic nervous system has been reviewed (Burnstock, 1979), with a greater emphasis of the NANC excitatory nerves discussed by Ambache and Freeman (1968). The presence of a NANC inhibitory neuronal system in the mammalian gastro-intestinal tract was first proposed in 1966 (Bennett et al., 1966), raising the possibility of its physiological and functional significance in the control of intestinal motility. Classically, the non-sphincteric smooth muscle of the gut has been considered to be controlled by parasympathetic cholinergic excitatory nerve and sympathetic adrenergic inhibitory nerves (Langley, 1921), but TTX-sensitive inhibitory junction potentials are recorded from intestinal smooth muscle cells during stimulation of intramural nerves in the presence of atropine and guanethidine (Burnstock et al., 1964), hence establishing the presence of NANC inhibitory nerves in the smooth muscle of the gut (Burnstock, 1972).

Evidence that ATP might be the transmitter liberated from

the endings of NANC inhibitory nerves in the gut was presented by Burnstock (1972; 1975), although this proposal is not universally accepted but, rather, is highly controversial, and constantly the subject of intense debate. This whole subject is further complicated by the considerable amount of controversial findings on the possibility that ATP may be the NANC transmitter in different regions along the gastro-intestinal tract, between and within a wide range of animal species (Daniel, 1979). These inconsistent results are highlighted by the problem that there is, as yet, no specific antagonist of 'ATP-receptors' available. However, it is thought by Burnstock (1975) that purinergic neurones are involved in inhibitory reflexes which facilitate the passage of intraluminal material through the gastro-intestinal tract, including participation in the "descending inhibition" of the peristaltic reflex.

A review of NANC autonomic nerves has been presented, as well as a review of the putative transmitters that might be present (ATP, polypeptides, dopamine, 5-HT, GABA), and the possibility raised of coexistence of some neurotransmitter substances (Burnstock, 1979). Apart from ATP being proposed as the NANC inhibitory transmitter substance, VIP has also been proposed as a possible candidate (Furness and Costa, 1982) but so far, firm evidence for this is lacking. Among all the proposed transmitter substances, there is growing evidence that 5-HT may play a role as a neurotransmitter in the gastro-intestinal tract (Gershon, 1981), to which the present study would add GABA as an inhibitory transmitter in the myenteric plexus.

## GABA IN THE ENTERIC NERVOUS SYSTEM

Using explant cultures of myenteric plexus containing enteric neurones from guinea-pig, grown for periods of up to 3 weeks, it was found that adrenergic neurones, VIP-containing neurones and putative GABAergic neurones are all represented in plexus explants. Employing autoradiography following uptake of [3H] GABA to label putative GABAergic neurones, no neuronal death was detected between days 1 and 5, while the number of neurones decreased between days 5 and 15 (Jessen et al., 1983 a,b). A small subpopulation of neurones, in explants of the myenteric plexus from the taenia coli, shows heavy autoradiographic labelling of [3H] GABA in the presence of  $\beta$ -alanine. This confirms previous studies that [3H] GABA uptake sites are present in tissue sections of the myenteric plexus in situ and in explant cultures (Jessen et al., 1979; Jessen, 1981; Krantis and Kerr, 1981 a; Saffrey et al., 1983), based on the evidence that high affinity neuronal GABA uptake sites are an exclusive property of neurones that utilize GABA as a neurotransmitter (Iversen and Kelly, 1975; Jessen et al., 1979, 1983 a). Enteric neurones in the myenteric plexus become selectively and heavily labelled with [3H] GABA, such uptake being unaffected by  $\beta$ -ala which preferentially blocks glial GABA uptake sites, but it is abolished by cis-ACHC, an inhibitor of GABA neuronal uptake, hence confirming the presence of high affinity GABA uptake sites in the myenteric neurones (Jessen et al., 1979; 1983 a; Krantis and Kerr, 1981 a; Saffrey et al., 1983).

Such autoradiography is thus a convenient way of revealing high affinity GABA uptake sites, where the cells that possess these sites are selectively labelled and can be detected by the resultant accumulated radioactive GABA using autoradiography. Culture preparations of the myenteric plexus from the guinea-pig taenia coli provide an excellent model for studies on the cell and tissue interactions involved in both histogenesis and maintenance, in enteric nervous tissues (Jessen et al., 1983 a;b).

Both endogenous GABA and GAD activity have been shown to be present in the myenteric plexus of the guinea-pig taenia coli, with the ability of the neurones to synthesise and accumulate [3H] GABA from [3H] glutamic acid, the immediate precursor to GABA in GABA synthesis (Jessen et al., 1979; Jessen, 1981). The demonstration of substance P and electrically-evoked release of endogenous and labelled GABA from guinea-pig ileal tissues, the release being calcium-dependent and neuronal in origin, confirms that GABAergic neurones are indeed present in the intestine (Jessen and Mirsky, 1982; Kerr and Krantis, 1983; Jessen et al., 1983 c; Taniyama et al., 1982 a; 1983 a; 1985; Tanaka and Taniyama, 1985), where GABA release appears to be under the control of bicuculline-sensitive autoreceptors (Taniyama et al., 1985). Hence, biochemical studies, together with autoradiographic evidence, support the presence of a population of GABAergic neurones in the intestine. Further confirmation of this is provided by the demonstration, in several species, of GABA, GAD, and GABA-T localized in the myenteric plexus from various levels of the intestine (Taniyama et al., 1982 b,c; Miki et al., 1983;

Taniguchi et al., 1982), and of high affinity [<sup>3</sup>H] GABA uptake (Krantis and Kerr, 1981 a; Jessen et al., 1979; 1983 a; Jessen, 1981; Jessen and Mirsky, 1982; Saffrey et al., 1983) with a subsequent calcium-dependent, TTX-sensitive evoked release of [<sup>3</sup>H] GABA (Kerr and Krantis, 1983; Taniyama et al., 1982 a,b; Jessen et al., 1983c; Jessen and Mirsky, 1982).

From earlier work, although GABA has been found to have diverse pharmacological actions in isolated intestinal preparations from different species of animals studied (Hobbiger, 1958 a,b; Inouye et al., 1960; Takahashi et al., 1961 a,b; Tsuchiya, 1960; Lewis et al., 1972), the true mechanisms, and significance, of the different modes of GABA actions in these preparations have not been clear. It is now conclusive that, at least in the guinea-pig isolated ileum, stimulation of GABA<sub>A</sub>-receptor sites leads to a bicuculline- and picrotoxin-sensitive cholinergic contractile response, sensitive to TTX (Krantis et al., 1980; Krantis and Kerr, 1981 b; Bowery et al., 1981 a; Giotti et al., 1983 a,b; Kaplita et al., 1982; Ong and Kerr, 1983 a,b), followed by a prolonged, delayed 'after-relaxation' due to a depression of ACh output, through activation of GABA<sub>B</sub>-receptor sites, insensitive to bicuculline and picrotoxin but antagonised by DAVA (Bowery et al., 1981 a; Giotti et al., 1983 a; Kaplita et al., 1982; Ong and Kerr, 1983 a,b). Fig. 5 of this thesis shows a typical representative response to GABA in the isolated ileum of the guinea-pig. In the rat duodenum, however, GABA predominantly induces a bicuculline- and picrotoxin-sensitive relaxation, by activating intramural non-adrenergic, non-cholinergic (NANC)



neurones, and this GABA<sub>A</sub>-receptor-mediated effect is mimicked by 3-APS but not by baclofen (Maggi et al., 1984). A similar small NANC-mediated relaxation occurs in the guinea-pig distal colon treated with hyoscine to block cholinergic activity (Krantis et al., 1980). In keeping with these actions, the GABA-evoked [3H] ACh release from postganglionic cholinergic neurones, through the bicuculline-sensitive GABA receptor is a calcium-dependent and TTX-sensitive mechanism in both the guinea-pig small intestine (Taniyama et al., 1983 b), and guinea-pig ileal synaptosomal preparations (Yau and Verdun, 1983). The bicuculline-sensitive GABA receptor mediating facilitation of ACh release in the intestine appears to exist on cell bodies (Krantis et al., 1980; Kleinrok and Kilbinger, 1983; Taniyama et al., 1983 b), whilst the bicuculline-insensitive GABA-receptor sites involved in the inhibition of ACh release are on nerve terminals (Bowery et al., 1981 a; Kleinrok and Kilbinger, 1983). Substance P has been shown to evoke both [3H] GABA and endogenous GABA release from the isolated small intestine of the guinea-pig in a calcium-dependent and TTX-sensitive manner, and bicuculline antagonises the release of [3H] ACh induced by substance P, suggesting that this latter release is partly mediated through the endogenous GABA released by substance P (Tanaka and Taniyama, 1985). Electrophysiological studies have also shown the localization of bicuculline-sensitive GABA receptors on cell bodies of myenteric neurones of the guinea-pig (Mayer et al., 1982; Grafe et al., 1979) and GABA causes a membrane depolarization in AH neurones associated with an increase in membrane conductance, effects due to a bicuculline-sensitive, rapidly desensitizing chloride

conductance activation. In addition to which, there is a bicuculline-insensitive, non-desensitizing depolarisation, and both GABA and baclofen inhibit the release of ACh as well as the transmitter mediating the slow epsps, all of which latter result from inhibition of an inward calcium current (Cherubini and North, 1984 a,b).

#### **GABA IN OTHER PERIPHERAL TISSUES**

GABA and baclofen stimulate the spontaneous motility of both longitudinal and circular muscle preparations from virgin rabbit oviducts, and this action was is not antagonised by bicuculline, atropine or TTX, indicating the presence of GABA<sub>B</sub> receptors in the oviductal musculature (Erdo et al., 1984 b), although GABA also increases ACh-induced responses in the isolated rat oviduct in a bicuculline-sensitive manner (Fernandez et al., 1984). It therefore appears that GABA<sub>B</sub>-receptor activation has many manifestations of responses, depending on animal species. Bicuculline-sensitive GABA<sub>A</sub> receptors have been identified in the Fallopian tube of the rat and the human (Erdo and Lapis, 1982 a,b; Erdo et al., 1983 b), GABA and GAD have been demonstrated in the Fallopian tube of the rat (Erdo et al., 1982), and the rat ovary (Martin del Rio and Caballero, 1980), together with a high affinity GABA uptake system in the rat ovary (Erdo, 1983 a), whilst an extrinsic GABAergic innervation exists in the rat oviduct (Fernandez et al., 1985) and in the Fallopian tube, but not in the ovary (Apud et al., 1984). Thus, in the reproductive tract, local GABAergic mechanisms may be involved in the regulation of

ovarian blood flow and hormone secretion in anaesthetized, pseudopregnant rats, where GABA applied topically on the surface of the ovary, significantly reduces the blood pressure in the femoral artery, increases ovarian blood flow, enhances the rate of estradiol-17- $\beta$  release and markedly decreases progesterone secretion (Erdo et al., 1985). GABA receptors are also found on the autonomic innervation of various organs, e.g. [ $^3$ H] ACh is released from preloaded postganglionic cholinergic neurones in the guinea-pig gallbladder, through a bicuculline-sensitive, calcium-dependent, TTX-sensitive mechanism (Saito et al., 1984) whilst Taniyama et al. (1983 c) have described a bicuculline-sensitive inhibitory effect of GABA on cholinergic neurotransmission in field stimulated guinea-pig urinary bladder, and Santicioli et al. (1984) a GABA $_B$ -receptor-mediated inhibition of field stimulated rabbit bladder smooth muscle, antagonised by DAVA (Muhyaddin et al., 1982 a,b), as well as homotaurine (Giotti et al., 1983 b), the latter being less effective as it desensitizes GABA $_A$ -receptor sites. In the rat anococcygeus muscle, a GABA $_B$ -receptor-mediated presynaptic inhibitory action on the excitatory adrenergic innervation, mimicked by baclofen, has also been demonstrated (Hughes et al., 1982). The current knowledge of GABAergic mechanisms and their functional relevance in a variety of peripheral tissues has been reviewed (Erdo, 1985).

The present thesis is directed towards elucidating GABAergic mechanisms in isolated intestinal segments of the guinea-pig, using ethylenediamine (EDA) as the principal substance to evoke GABA release, and to elicit both GABA $_A$ - and GABA $_B$ -receptor-

mediated responses, analogous to those of GABA in the intestine. The endogenous GABA content in the myenteric plexus can be manipulated by inhibitors of GABA synthesising (GAD) and degrading (GABA-T) enzymes which subsequently alter responses to EDA, such manipulation thereby provides further insight into biochemical aspects of GABAergic neurotransmission in the enteric nervous system. Pharmacological responses to GABA and its analogues, in the presence of selective neuronal and glial uptake inhibitors for GABA, are evaluated and analysed to demonstrate and characterise GABA uptake in the intestine. Not only interactions between GABA and other excitatory agents such as 5-HT, ACh or nicotine are studied here, but the potentiating actions of barbiturates on GABA-induced responses are also demonstrable in the intestine, indicating an allosteric coupling between barbiturate and GABA-receptor sites at a chloride ionophore, hence confirming the presence of a barbiturate-benzodiazepine-GABA<sub>A</sub>-receptor chloride-ionophore complex on the postsynaptic element of the cholinergic neurone in the myenteric plexus. Finally, the functional involvement of GABA in the control of myenteric neuronal function is further explored, suggesting a possible role of GABA in mediating intestinal motility.

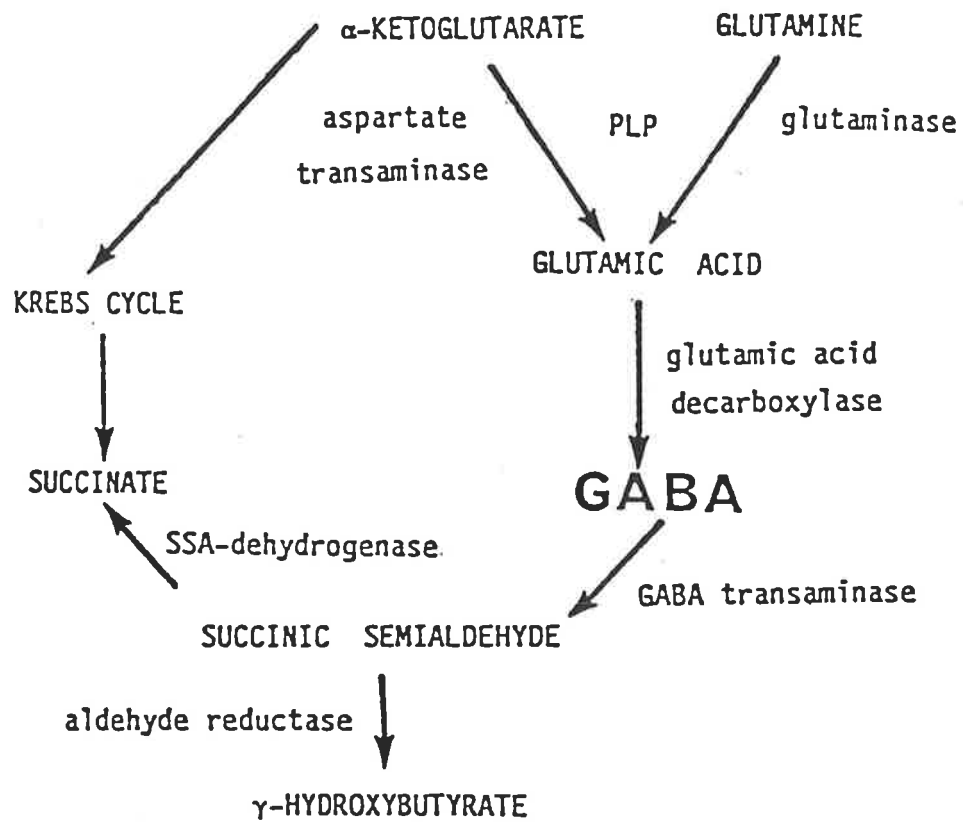


Fig. 1. Schematic representation of the metabolic pathways for GABA. PLP represents pyridoxal-phosphate dependent enzymes.

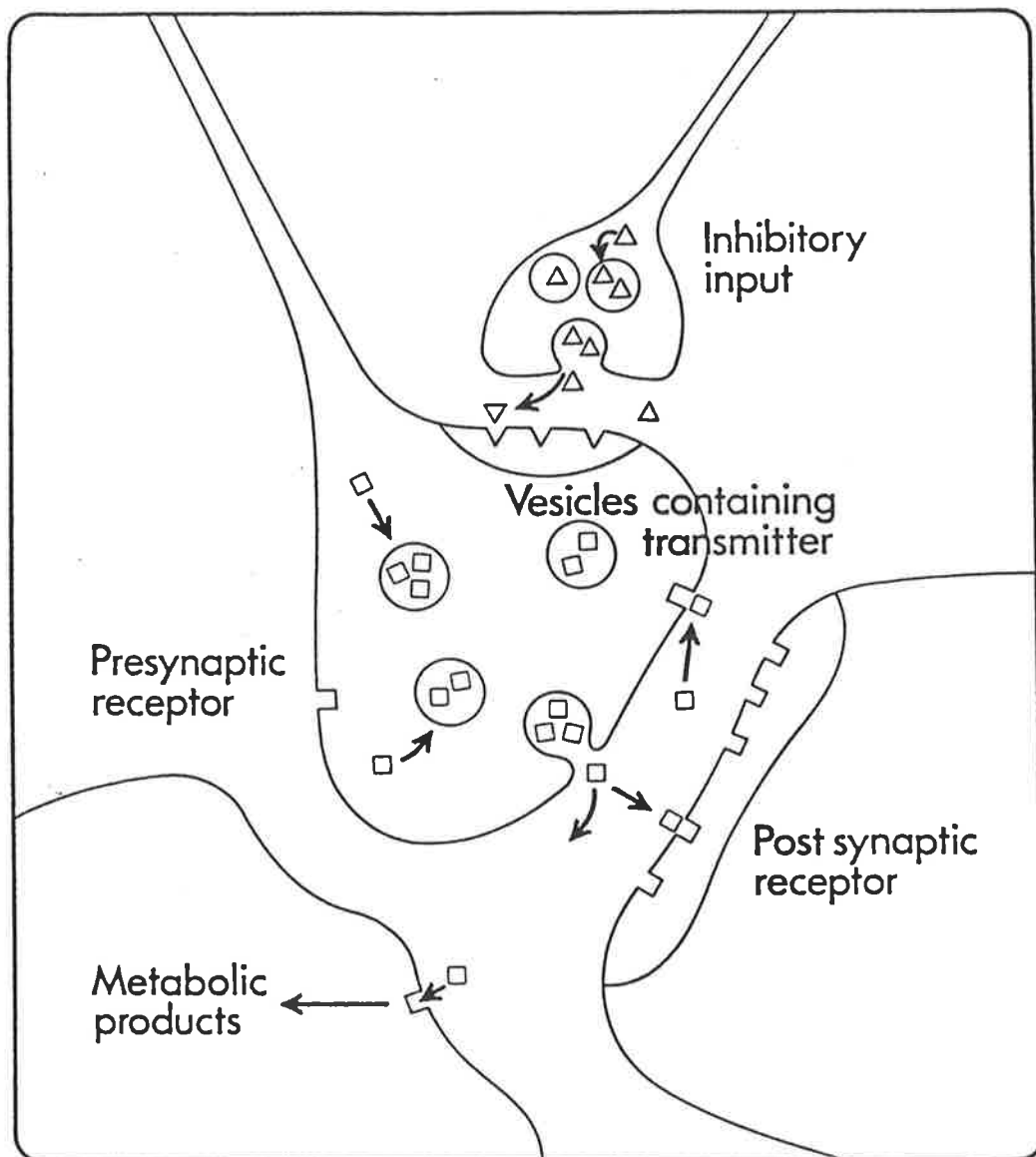


Fig. 2. Diagrammatic representation of release of neurotransmitters (e.g. GABA) at synaptic junctions.

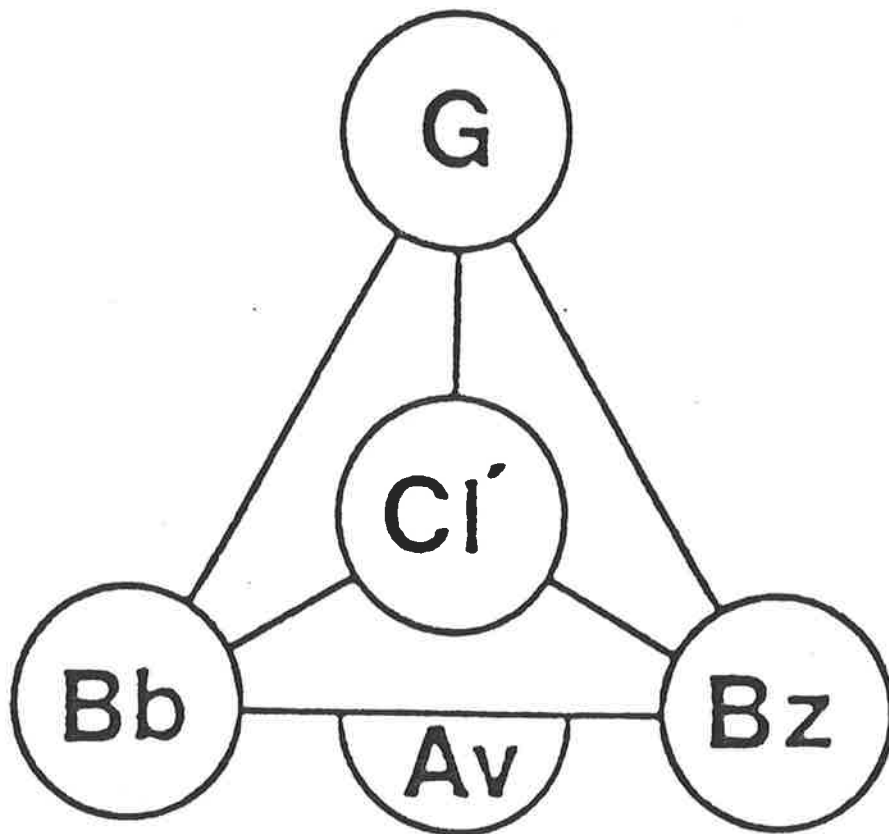


Fig. 3. Diagram of the GABA-benzodiazepine-barbiturate chloride-ionophore receptor complex, showing a separate active site for Avermectin (AV).

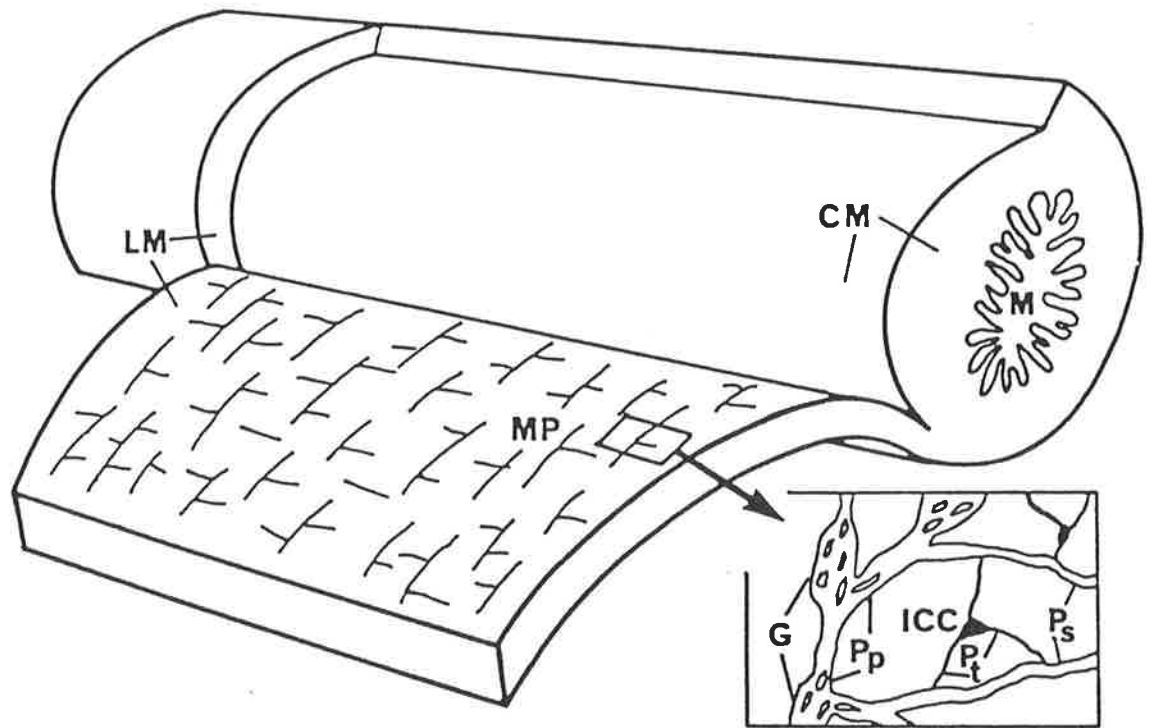


Fig. 4. Diagrammatic representation of a gut segment (not to scale), showing Auerbach's myenteric plexus (MP) on the inner surface of the reflected longitudinal muscle layer (LM), where it is found lying between the LM and circular muscle (CM). The CM is bounded internally by Meissner's plexus (not shown) which lies between the CM and mucosa (M) which forms the innermost layer lining the lumen of the gut. The insert illustrates ganglia (G) containing irregular shaped neurones, the primary plexus (Pp) which interconnects the ganglia, the secondary plexus (Ps) derived from the Pp, and the tertiary plexus (Pt) arising from branches of the Ps. Interstitial Cells of Cajal (ICC) are represented at intersections of the tertiary branches. The deep muscular plexus, derived from the MP, is found within the CM layer.



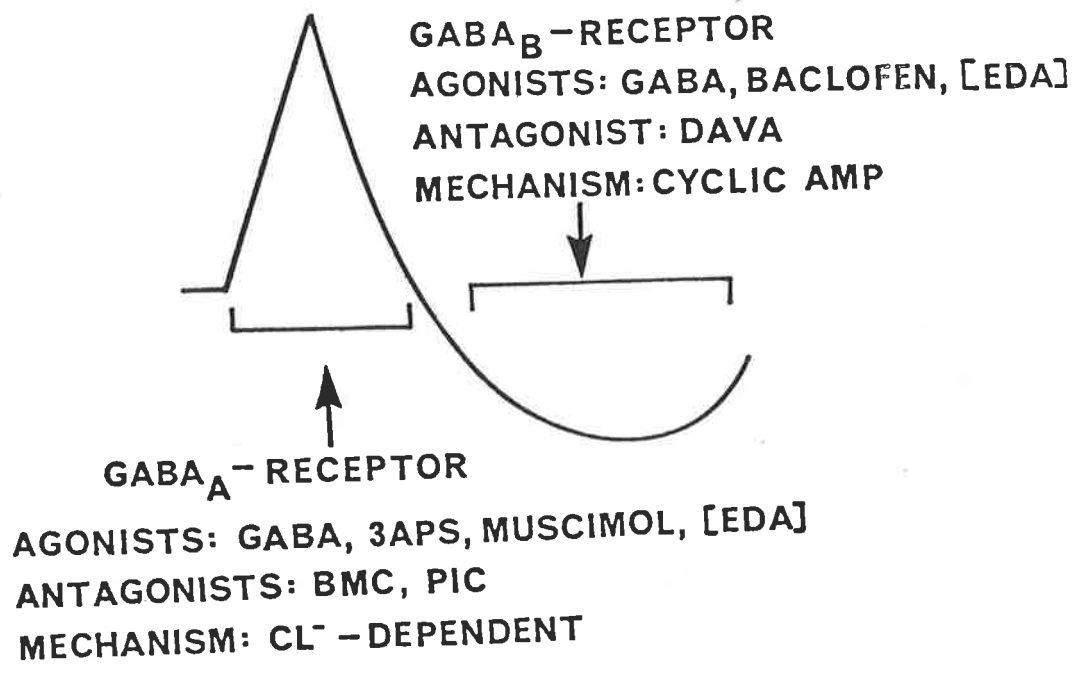


Fig. 5. A typical representative response to exogenously applied GABA in the isolated ileum of the guinea-pig.

CHAPTER TWO. METHODS.

## 2.1. METHODS

### 2.1.1. Organ bath studies

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of the intestine, 3-4 cm in length, were quickly removed, emptied of their contents and placed in modified Krebs solution of the following composition (mM):  $\text{Na}^+$  151.0;  $\text{K}^+$  4.6;  $\text{Mg}^{2+}$  0.6;  $\text{Ca}^{2+}$  2.8;  $\text{Cl}^-$  134.9;  $\text{HCO}_3^-$  24.9;  $\text{H}_2\text{PO}_4^-$  1.3;  $\text{SO}_4^{2-}$  0.6; glucose 7.7 (pH 7.4 at 37°C). The Krebs solution was continuously aerated with a gas mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Isolated segments of the tissues were then mounted vertically in a 10 ml organ bath containing the Krebs solution, and were left to equilibrate for 60 min before any exogenous drug application or electrical stimulation. Effects of drug treatments were examined on the resting tissue or on electrically evoked contractions of the tissues (twitch contractions), elicited through a pair of parallel platinum electrodes positioned around the segments of the intestines, using pulses (duration 0.1 ms, single pulses or a repetitive frequency of 0.1 or 0.2 Hz, supramaximal voltage 60 V) delivered from a Grass S48 stimulator to give transmural stimulation of cholinergic intrinsic neurones.

Mechanical activity of the longitudinal muscle was recorded isometrically at a resting tension of 1 g using a Grass Model FT03 force transducer. Changes in tissue tension were displayed on a Grass polygraph recorder. Drugs were applied within 15-20

min intervals, depending on the recovery of the tissue responses to baseline. Antagonists were added at least 10 min before agonists activities were tested, and in some experiments, the drugs were left in contact with the tissues in the bath for 30-60 min, depending on the experiments. Drug volumes used were never more than 1% of the bath volume. Student's t-test for paired and unpaired samples was used to assess the significance ( $P < 0.05$ ) of differences between mean values of the dose-response effects.

### 2.1.2. Efflux Studies

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of the intestinal tissues, 3-4 cm in length, were quickly removed and emptied of their contents. The tissues were incubated for 10-20 min in aerated Krebs solution (95%  $O_2$  and 5%  $CO_2$  pH 7.4,  $37^{\circ}C$ ) of the following composition (mM):  $Na^+$  151.0;  $K^+$  4.6;  $Mg^{2+}$  0.6;  $Cl^-$  134.9;  $HCO_3^-$  24.9;  $H_2PO_4^-$  1.3;  $SO_4^{2-}$  0.6;  $Ca^{2+}$  2.8; glucose 7.7. The tissues were then transferred to 5 ml Krebs solution, containing [3H] GABA (10 nM) (66 Ci mmol), amino-oxyacetic acid (AOAA) (0.1 mM), and b-alanine (b-ala) (1 mM) for 20 min at  $37^{\circ}C$ . AOAA and b-ala were subsequently present in the Krebs medium throughout each experiment to minimize metabolism of [3H] GABA and to prevent GABA uptake into glial cells. The tissues were then removed, blotted to remove excess incubating medium and suspended in glass perfusion chambers. After washing the tissues repeatedly over an equilibration period of 60 min to establish a basal efflux of [3H] GABA, 2 ml fractions of the superfusate were

collected over 2 min periods. Drugs such as ethylenediamine (EDA) were added at varying doses to the medium and 2 ml samples again collected. In experiments where 3-mercaptopropionic acid (3-MPA) was used, it was added to the medium at least 5 min before a dose of EDA was added.

When electrical stimulation was used to evoke release of [3H] GABA from the tissues, the tissues were suspended between platinum stimulating electrodes in the glass perfusion chambers containing 2 ml of the superfusion medium. Electrical stimulation from a Grass S48 stimulator was applied to the tissues, for 2 min periods, using 60 V, 0.1 ms duration at 10 Hz, and 2 ml fractions were then collected.

To test the influence of  $\text{Ca}^{2+}$  on the EDA-induced efflux of tritium, the Krebs solution was substituted by a low  $\text{Ca}^{2+}$  (0.6 mM) superfusion medium containing a high  $\text{Mg}^{2+}$  (10 mM), the remaining constituents being as in the Krebs solution, which also contained AOAA and b-ala, and 2 ml samples of the superfusate were collected over 2 min, with and without EDA. The tissues were returned to the normal Krebs solution, and EDA was again added to the medium to evoke [3H] GABA release.

The superfusate fractions were then each added to 5 ml of a Triton/toluene scintillator employing a Beckman LS 2800 counter, and the radioactivity measured by liquid scintillation spectrometry, and expressed as d.p.m after correction for quenching by the appropriate drugs. All experimental procedures were run in du-

plicate and were repeated at least twice, with at least 6 tissues being used from a minimum of 3 animals for each set of experiments. Statistical analysis using Student's t-test for paired and unpaired samples was done to assess the significance of differences between the means of samples.

CHAPTER THREE. ACTIONS OF ETHYLENEDIAMINE  
IN THE INTESTINE.

### 3.1. GENERAL INTRODUCTION

Aminophylline, a soluble complex of two molecules of theophylline to one of the simple diamine ethylenediamine (EDA) was originally reported to induce a release of [<sup>14</sup>C] GABA from superfused slices of rat striatum, such GABA-releasing properties being attributed to the EDA component of the molecule (Lloyd et al., 1982 a). It was also realised that the EDA-induced release of radiolabelled GABA from brain slices is calcium-independent (Lloyd et al., 1982 a), although EDA itself has relatively little effect on [<sup>14</sup>C] GABA uptake (Forster et al., 1981). From such studies, it was then suggested that EDA might well be a useful tool to investigate GABAergic mechanisms.

Other studies have shown that iontophoretic application of EDA to single cells, in the cerebral cortex or globus pallidus of rats, depresses neuronal firing of the cells with a potency comparable to that of GABA (Forster et al., 1981), inhibits the firing of rat neurones in vivo, and depolarises neurones of the superior cervical ganglion in vitro, all of which are mimicked closely by GABA. Bicuculline, a GABA<sub>A</sub>-receptor antagonist (Curtis et al., 1971 a,b), in such studies, appears to be twice as effective as an antagonist of EDA-induced actions than of GABA (Perkins et al., 1981). In ligand binding studies, EDA can displace specifically bound [<sup>3</sup>H] GABA, muscimol and baclofen in rat brain synaptosomal preparations (Bowery et al., 1982 c) by acting as a GABA agonist for both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites, albeit with low potency, and has also been shown to



enhance [3H] diazepam binding to rat brain membranes in a bicuculline-sensitive manner (Morgan and Stone, 1982 a; Davies et al., 1982).

EDA has, in addition, been shown to inhibit [3H] b-alanine (b-ala) uptake into rat cerebral cortex slices (Davies et al., 1982), the latter perhaps suggesting that EDA can counter-exchange with b-ala or rides on the b-ala transport system; thus, EDA may show some selectivity for the glial rather than the neuronal GABA uptake system. Recent studies show that [14C] EDA itself can be taken up into rat brain slices by a temperature-dependent and sodium-dependent process, and can subsequently be released through a calcium-dependent mechanism (Lloyd et al., 1982 b; Davies et al., 1983 a). On the other hand, EDA stimulates the release of [3H] GABA and [3H] b-ala from preloaded slices, whereas b-ala does not stimulate [14C] EDA release. This suggests that extracellular EDA can counter-exchange with intracellular GABA or b-ala, the accumulated EDA may then be bound or move to pools not directly accessible to GABA or b-ala exchange, and hence may not be acting simply as a substrate for the GABA transport sites (Davies et al., 1983 a). Although this evidence from uptake, release and receptor binding studies points to an action of EDA on GABA recognition sites, there is still controversy with respect to the exact site of EDA action, for example, at the GABA-receptor site itself or at a separate site mediated via allosteric mechanisms.

EDA interacts with neuronal postsynaptic GABA receptors on

Limulus and Helix central neurones, rat cerebellar and sympathetic ganglion neurones (Bokisch et al., 1982; 1984), and is said to possess anti-convulsant properties (Morgan and Stone, 1982 b). By virtue of a modulatory action at benzodiazepine receptors on rat brain synaptosomal preparations, EDA also potentiates [3H] diazepam binding, by increasing the number of receptors (Morgan and Stone, 1982 a), this being blocked by bicuculline methylbromide. The potency and efficacy of EDA differs from GABA, the EDA maximum being only some 40% that of the GABA maximum, with additivity studies showing that EDA does not act as a partial agonist at GABA-receptor sites. It is concluded that EDA and GABA do not act on the same receptor sites in potentiating [3H] diazepam binding, although EDA can act on the GABA-benzodiazepine-receptor  $\text{Cl}^-$ -ionophore complex (Morgan and Stone, 1982 a; 1983). Whilst, in relation to the GABA-benzodiazepine receptor complex, EDA and GABA do not appear to be completely interchangeable, since, although EDA and GABA are equipotent on rat cerebellar Purkinje cells, flurazepam potentiates GABA-induced responses more often than it does EDA-induced responses (Bokisch et al., 1984). Nevertheless, in rat hippocampal pyramidal cells, there is a close similarity in the directions (depolarising and hyperpolarising), reversal potentials, and  $\text{Cl}^-$ -dependence of responses to EDA and GABA, with similar potencies in this system (Blaxter and Cottrell, 1982). GABA and EDA produced a bicuculline- and picrotoxin-sensitive,  $\text{Cl}^-$ -dependent depolarization, and a bicuculline-resistant  $\text{K}^+$ -dependent hyperpolarization of the dendrites on slices of the rat hippocampal CA1 pyramidal neurones, with a bicuculline-sensitive  $\text{Cl}^-$ -dependent hyperpolarizing

response on the cell body (Blaxter and Cottrell, 1985). Benzodiazepines potentiate the dendritic depolarising responses and the hyperpolarising responses of the cell body to GABA and EDA without affecting the hyperpolarising response of the dendrites, indicating that GABA<sub>A</sub>-receptors mediate both the dendritic depolarising and hyperpolarising responses on the cell body (Blaxter and Cottrell, 1985). In the same study, bicuculline reduced the depolarising response to EDA and to GABA equally effectively, in contrast to that reported in the rat cervical ganglion, where bicuculline is more effective in blocking the depolarising response to EDA than to GABA (Perkins et al., 1981). It is also thought that in rat hippocampal pyramidal neurones, EDA acts by releasing endogenous GABA rather than as a GABA-mimetic (Blaxter and Cottrell, 1985).

Since GABA<sub>B</sub>-receptors are involved in the hyperpolarising response in the hippocampal pyramidal cells (Newberry and Nicoll, 1984), it is possible that the hyperpolarising response of the dendrites is mediated through GABA<sub>B</sub>-receptor sites (Blaxter and Cottrell, 1985). EDA and its analogues, when tested on cortical and pallidal neurones, show that they may be interacting with a novel, bicuculline-sensitive receptor to produce inhibition of cell firing, with the suggestion that such interaction requires two amine groups at a critical distance apart, as in EDA (Perkins and Stone, 1982). However, this concept is complicated by the observation that EDA also acts at GABA<sub>B</sub>-receptor sites, where the structural requirements for agonist activity are quite different (Bowery et al., 1982 c), e.g. in the rat anococcygeus muscle, a

tissue that possess dense adrenergic innervation, EDA was less potent than GABA in exerting a presynaptic inhibitory action on the excitatory adrenergic innervation of the muscle through bicuculline-insensitive GABA<sub>B</sub>-receptor sites (Hughes et al., 1982), and the notion of a critical charge separation for GABA-mimetic actions is less compelling.

Some controversy over the mechanism of EDA actions has been raised, Stone and Perkins (1984) maintaining that EDA interacts directly at the GABA<sub>A</sub>-receptor complex by virtue of its diamine property, whereas Curtis and Malik (1984) contend that EDA must first be converted to the monocarbamate which bears a closer structural analogy to GABA. Certainly, bicarbonate ions are essential for EDA to act as a GABA-mimetic (Hill, 1985 b) or to interact at uptake recognition sites (Davies et al., 1983 a). In radioligand binding assays, bicarbonate ions are found to increase the GABA-mimetic potency of EDA in rat brain synaptic membranes, and enhance the ability of EDA to potentiate [3H] diazepam binding to membrane preparations, by raising both the potency of EDA and its maximum effect, leading to the conclusion that the GABA-mimetic activity of EDA is dependent upon physiological concentrations of bicarbonate ions (Hill, 1985 b). Similarly, in isolated superior cervical ganglion preparations of the rat, EDA only induces a depolarising response in the presence of Krebs-bicarbonate medium but not in Krebs-phosphate solution, whereas responses to GABA and muscimol are unaffected in either medium (Hill, 1985 b). This could mean that bicarbonate ions, as such, are essential for these actions of EDA to be manifested, as

Hill (1985 b) suggests, or that EDA must first be converted to a structural analogue of GABA. In addition to which, EDA may instead act by releasing GABA, as earlier reported by Lloyd et al. (1982 a), and suggested by Blaxter and Cottrell (1985). However, much of this controversy remains unresolved.

Since GABA actions in the intestine have been well characterized, it seemed appropriate to investigate EDA actions in the enteric nervous system of intestinal preparations from the guinea-pig, known to possess GABAergic mechanisms (Jessen et al., 1979; Krantis et al., 1980; Krantis and Kerr, 1981 a,b,c; Krantis, 1982; Taniyama et al., 1982 a,b,c; Kerr and Krantis, 1983) and contain endogenous GABA (Taniyama et al., 1983 a). GABA stimulates both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites in the guinea-pig ileum (Bowery et al., 1981 a; Kaplita et al., 1982; Giotti et al., 1983; Ong and Kerr, 1983 a,b), the GABA<sub>A</sub>-receptor-mediated event is manifested as a cholinergic contractile response, sensitive to both bicuculline and picrotoxin, whereas the GABA<sub>B</sub>-receptor-mediated event is an 'after-relaxation' response, insensitive to both bicuculline and picrotoxin but antagonised by 5-aminovaleric acid (Muhyaddin et al., 1982 a,b).

In the present study, the guinea-pig isolated intestine has been used as a model to investigate the actions of EDA and to compare its pharmacological responses with those of GABA. It has also been aimed to test whether EDA is capable of releasing GABA in the guinea-pig intestine, rather than acting directly as a GABA-mimetic at GABA-receptor sites in the myenteric plexus of

the guinea-pig.

## GABA METABOLISM

Roberts and Frankel (1950) first reported the presence of GABA in the vertebrate CNS, and since then, GABA metabolism in the various neuronal compartments of the CNS has been well described (Roberts, 1976), following the elucidation of the major pathway of GABA metabolism (Roberts, 1956); likewise the understanding of the biochemistry and neurophysiology of the GABA system has been greatly advanced (Baxter, 1970; Iversen, 1972; Roberts, 1974; Meldrum, 1985). It is now accepted that the predominant synthetic pathway of GABA is through the decarboxylation of glutamate to GABA, catalyzed by the enzyme glutamic acid decarboxylase (GAD) and that the degradative pathway of GABA metabolism is through transamination with 2-oxoglutarate to succinic semialdehyde, the amine group being transferred to the 2-ketoglutarate of the Krebs cycle, catalysed by the enzyme GABA:2-ketoglutarate aminotransferase (GABA-T), both enzymes being pyridoxyl phosphate (PLP)-dependent. In this reaction, 2-ketoglutarate combines with the ammonia to form glutamic acid, thereby providing the GABA precursor. The succinic semialdehyde is either reduced to 3-hydroxybutyrate by succinic semialdehyde reductase, or undergoes oxidation through the action of succinic semialdehyde dehydrogenase (SSA-DH), with the di-nucleotide NAD as acceptor, to yield succinic acid which in turn joins the Krebs cycle (Baxter, 1976). This pathway, whereby the Krebs cycle is bypassed through the synthesis of glutamate, is called the 'GABA-shunt'. However, kinetic studies have shown that there are alternate pathways for GABA synthesis and degradation, as well as

alternate substrates for GABA-T, and differential compartmentalization of GABA pools in nervous tissue (Baxter, 1976; Roberts, 1976).

Purification and characterization of the GABA synthesising enzyme, GAD and the GABA degrading enzyme, GABA-T (Wu, 1976), followed by immuno-cytochemical and histochemical localization of the metabolic enzymes, which prove to be valuable tools as markers in probing GABA-containing neuronal elements in central nervous tissues, have been documented (Fahn, 1976; Fonnum and Walberg, 1973; Kuriyama et al., 1966 a; Ribak et al., 1981; Ohara et al., 1983; Oertel et al., 1981 a,b,c; Storm-Mathisen et al., 1983; Vincent et al., 1981; 1982; Nagai et al., 1983; 1985; Van Gelder, 1965; Robinson and Wells. 1973).

Both light and electron microscopic visualization of GAD and GABA-T in immuno-cytochemical preparations of rodent CNS (Barber and Saito, 1976; Wood et al., 1976; Storm-Mathisen, 1976) have made it possible to localize GABAergic neurones in the brain. In particular, the use of GAD as a marker has methodologic advantages, as it is not subjected to rapid postmortem changes in activity or distribution, and can be easily measured in small tissue samples. GABA-T, on the other hand, is not so suitable as a marker since it has a different subcellular and topographical distribution from GAD. Studies of the regional distribution of GABA in the brain have shown this to parallel with the distribution of GAD, whereas there is no such association between GABA and GABA-T or SSA-DH (Fahn, 1976). In many inhibitory nerves,



however, GAD is somewhat more highly concentrated in the presynaptic endings of the neurones and is not associated with mitochondria, whereas GABA-T is contained in mitochondria of all neuronal regions, and seems to be richer in the mitochondria of postsynaptic neuronal sites onto which GABA might be released (Roberts, 1974).

GABA levels of neuronal tissue can be manipulated by inhibiting the activities of GAD or GABA-T (Roberts, 1974; Meldrum, 1982; 1985). Among a range of drugs affecting GAD function are direct competitive inhibitors of GAD, such as 3-mercaptopropionic acid (3-MPA) (Lamar, 1970; Horton and Meldrum, 1973), or 2-keto-4-pentenoic acid (2-KPA), the active metabolite of allylglycine (Meldrum, 1975; Horton and Meldrum, 1977; Horton et al., 1978), as well as indirectly acting inhibitors that depress the coenzymic function of PLP, such as thiosemicarbazide (TSC) (Wood and Abrahams, 1971; Collins, 1973), all of which significantly reduce GABA content in nervous tissue resulting in an impairment of intrinsic on-going GABA-mediated inhibitory function that leads to convulsions (Meldrum, 1975). Conversely, GABA neurotransmission can be potentiated by elevating GABA levels within the nerve endings (Meldrum, 1982; Baxter and Roberts, 1961; Kuriyama et al., 1966 b; Gale and Iadarola, 1980), through the use of compounds that inhibit GABA-T activity and thus possess anticonvulsant properties (Loscher and Frey, 1978; Loscher, 1980 a,b). This elevation of GABA content can be achieved by inhibiting GABA-T activity with amino-oxyacetic acid (AOAA) (Wood, et al., 1978; Geddes and Wood, 1984), a potent carbonyl trapping agent

and unspecific inhibitor of the enzyme, which also inactivates a number of aminotransferase and decarboxylase enzyme systems as it complexes with the cofactor, PLP (Roberts and Simonsen, 1963; Da Vanzo et al., 1961). On the other hand, gabaculine (Rando and Bangerter, 1977), or ethanolamine-o-sulphate (EOS) (Anlezark et al., 1976; Fowler and John, 1972; Horton et al., 1977; Seiler and Sarhan, 1984), are all irreversible and specific inhibitors of GABA-T which prevent the transamination of GABA to its metabolic product, succinic semialdehyde (Roberts, 1974), so raising neuronal GABA levels.

A number of studies have sought to correlate the convulsant effects of various hydrazides with an inhibition of GABA synthesis caused by these compounds (Killam and Bain, 1957; Baxter and Roberts, 1959; Balzer et al., 1960; Tapia and Awapara, 1967; 1969), which also cause a reduction in GABA concentration in the brain after administration (Balzer et al., 1960; Maynert and Kaji, 1962). These compounds inhibit GAD mainly by inactivating PLP coenzymic function, since GAD activity is highly dependent on PLP (Meldrum, 1975). Convulsions induced by TSC, a carbonyl trapping agent, can be relieved by pyridoxal administration, despite a decline in brain GABA concentration (Baxter, 1969). Other carbonyl trapping reagents such as AOAA (Wallach, 1961; van Gelder, 1966) that are potent inhibitors of GABA-T both in vitro and in vivo, are also inhibitors of GAD in vitro, although they appear to inhibit GABA-T preferentially in vivo, with less effect on GAD activity. Thus, in practice, administration of AOAA leads to steady rises in GABA concentration in

the brain by some 500% (Wallach, 1961; van Gelder, 1966). A possible explanation for this may be that the pyridoxal-inhibitor complexes may be bound with higher affinity by GABA-T than by GAD, and so making reactivation of the former enzyme by PLP more difficult (Baxter, 1970).

#### **MODIFICATION OF GABA LEVELS IN THE CNS**

With improved understanding of GABA metabolism, the possible manipulation of GABAergic function has become feasible. Inhibitors of GAD such as 3-mercaptopropionic acid (3-MPA), and the hydrazides, and irreversible inhibitors of GAD such as 2-keto-4-pentenoic acid (2-KPA) (Meldrum, 1975; Horton et al., 1978), by lowering GABA levels produce generalized convulsions if given systemically, or focal convulsions if applied topically to the cortex (Meldrum, 1982); whilst pharmacological procedures used to enhance GABAergic inhibition, for example, have been used in therapeutic trials for epilepsy, movement disorders, schizophrenia or mania, all of which are thought to have an association with GABA (Meldrum, 1975; 1982).

Several of the compounds that inhibit GABA-T can increase the brain GABA level in experimental animals (Meldrum, 1979). Sodium valproate and  $\gamma$ -vinyl-GABA are said to induce an increase in GABA concentration selectively in synaptic terminals (Gale and Iadarola, 1980; Iadarola and Gale, 1979; Seiler and Sarhan, 1980), and their actions may be related to an enhanced release of GABA. Other GABA-T inhibitors used for these purposes are gabaculine (GBL), AOAA, ethanolamine-o-sulphate (EOS) and  $\gamma$ -acetyl-

nic GABA, although sodium valproate inhibits both SSA-DH and aldehyde reductase (Meldrum, 1982).

The pharmacology of GABA-T inhibitors has been reviewed (Palfreyman et al., 1981), and the effects of two GABA-T inhibitors, AOAA and hydroxylamine, on primary afferent depolarization (PAD) and presynaptic inhibition reported for the frog isolated spinal cord, where both AOAA and hydroxylamine consistently augment PAD and facilitate presynaptic inhibition (Davidoff et al., 1973) with a concomitant increase in GABA concentration. Although AOAA and hydroxylamine preferentially inhibit GABA-T in intact neural tissue and increase GABA concentration (Wallach, 1961; Baxter and Roberts, 1961), these reagents are carbonyl-trapping agents and can inhibit various enzymes requiring PLP, and as a consequence of this, the specificity of such GABA-T inhibitors remains to be further explored.

Other studies have shown that AOAA,  $\gamma$ -acetylenic GABA and, more particularly, sodium dipropylacetate (valproate) increase GABA levels in rat models (Eli and Cattabeni, 1983). Sodium valproate blocks both GABA-T and SSA-DH (Harvey et al., 1975), and it is well established that it is capable of elevating cerebral GABA concentrations both in rodents (Godin et al., 1969) and in man (Loscher and Schmidt, 1981). However, some reports suggest that sodium valproate can increase GABA levels by activating GAD (Loscher, 1981 a), e.g. Iadarola et al. (1979) showed that sodium valproate caused an accumulation of GABA in neuronal compartments where GAD is mainly localised, suggesting that

enhancement of GAD in the neuronal compartment may be a reasonable explanation for the localised accumulation of GABA, and Philips and Fowler (1982) have also suggested that the anti-convulsant activity of sodium valproate may be mediated at least in part by elevation of cerebral GABA levels through potentiation of GAD activity. Whilst earlier studies indicate that sodium valproate inhibits GABA-T (Simler et al., 1973) or SSA-DH (Van Der Laan et al., 1979) in vitro, recent electrophysiological studies have suggested a possible action of sodium valproate at the postsynaptic membrane of GABAergic synapses (Schmutz et al., 1979; Kerwin et al., 1980; Gent and Phillips, 1980), and alternative explanations for the anti-convulsant action of valproate may involve a modulation of the synaptic effect of GABA without any overall increase in the level of GABA in brain. In particular, augmentation of GABA synaptic actions has been reported (MacDonald and Bergey, 1979; Hackman et al., 1982; Harrison and Simmonds, 1982), one possible explanation for which comes from a very recent study showing that valproate at concentrations  $\geq$  or equal to 2.4 mM decreases the transport of GABA through the plasma membrane of Deiter's neurones, which reflects the physiological event of postsynaptic uptake of GABA by its uptake carrier. Such an effect reduces the efficiency of GABA postsynaptic inactivation processes and well might be a mechanism for the valproate-induced potentiation of GABA synaptic action (Hyden et al., 1984).

Although it is suggested that valproate preferentially increases the GABA concentration in nerve terminals, whilst AOAA

exerts its predominant effect on GABA levels in glial cells and neuronal perikarya (Iadarola and Gale 1981), when Loscher (1980 b) studied the catalytic inhibitors of GABA-T, as well as AOAA and valproate, for effects on neurochemical assays for GABA synthesis, receptor binding, uptake and metabolism in mouse and rat brain preparations, he found the following results: Gabaculine does not affect GAD activity, is a weak inhibitor of GABA receptor binding sites but a moderately potent inhibitor of GABA uptake, and a very potent inhibitor of GABA-T;  $\gamma$ -acetylenic GABA is similar to  $\gamma$ -vinyl GABA in that it is a weak inhibitor of GAD and GABA binding, but is virtually equipotent in inhibiting uptake and metabolism of GABA, except that  $\gamma$ -vinyl GABA does not decrease GAD activity. EOS was found to show virtually no inhibition of GAD and GABA uptake, but was a fairly potent inhibitor of GABA binding and, in this respect, 500 times more potent than as an inhibitor of GABA-T. AOAA is a powerful inhibitor of both GAD and GABA-T, but has very little affinity for receptor or uptake sites for GABA; and finally, valproate showed no effects on GABA neurochemical assays which could be related to anti-convulsant action, inhibiting GABA-T activity only in very high concentrations (Loscher, 1980 b). The binding of [3H] GABA to synaptic membranes is not affected by concentrations of valproate up to 10 mM, and at this concentration, valproate inhibited GABA uptake by only 20%. Gabaculine, isolated from natural sources and shown to possess anti-convulsant properties in several animal test systems of epilepsy (Metcalf, 1979), was found to be the most potent and specific inhibitor of GABA-T, more so than EOS. These results suggest that the anti-convulsant properties of

catalytic inhibitors of GABA-T tested may be at least in part mediated through a direct influence on GABA receptors and uptake sites. A number of the inhibitors of GABA catabolism described above have been tested and compared with other GABAergic drugs, with the finding that all GABA-T inhibitors elevate seizure thresholds and increase GABA levels in central nerve terminals (Loscher, 1981 b). Among the latest development of GABA-T inhibitors,  $\gamma$ -allyl GABA was found to be 2-3 times more potent than  $\gamma$ -vinyl GABA in inhibiting GABA-T, and has the great advantage in that it does not inhibit GAD in vitro (Jung et al., 1984). Hence such compounds may have potential use in investigating GABA metabolism.

Although GABA is formed from glutamic acid, there have been reports that GABA can be formed from putrescine in the brain and liver (Seiler et al., 1971; 1973). Konishi et al. (1977) confirmed the latter synthetic pathway for GABA, showing the GABA metabolic pool was different from that derived from glutamic acid in the brain. Indeed, GABA is widely distributed in various organs of mammals, its formation from putrescine also occurring in peripheral organs such as the kidney, liver, and the intestine. The enzyme involved in this formation was purified from small intestine and identified as a diamine oxidase, histaminase, the highest activity being detected in the small intestine (Tsuji and Nakajima, 1978), although the physiological significance of the formation of GABA from putrescine is not immediately clear. The finding that this pathway is active in chick embryonic brain at an early stage of ontogenesis indicates that GABA derived from

putrescine may play some important role in cell proliferation, maturation or differentiation, perhaps regulating protein synthesis for cell maturation or differentiation (Sobue and Nakajima, 1977; 1978). Thus, the role of putrescine involvement in GABA synthesis in the rat small intestine may be related to protein metabolism.

In the enteric nervous system of the guinea-pig, cat and human, the regional distribution of GABA, GAD and GABA-T in the intestine has been determined, and such findings strongly suggest the presence of GABA innervation in the myenteric plexus of mammals, including humans. There are species differences in the population of GABAergic neurones and GABA concentrations within the enteric neurones (Miki et al., 1983), but in the myenteric plexus of the guinea-pig, the formation of GABA from glutamic acid via GAD activity has been demonstrated (Jessen et al., 1979). Both GABA and GAD activity are also detected in organs such as the uterus, liver, kidney, adrenals, pituitary, skin, spleen, thyroid and pineal body (Tallan et al., 1954; Zachmann et al., 1966; Haber et al., 1970 a; Lancaster et al., 1973; Beart et al., 1974; Drummond and Philips, 1974) and in the ovary (Martin Del Rio and Caballero, 1980; Erdo, 1984; Erdo et al., 1982), and the fallopian tube (Erdo, 1984; Erdo et al., 1982; Martin Del Rio, 1981). GAD activity is also detected in the ovary and oviduct (Martin Del Rio and Caballero, 1980; Schaeffer and Hsueh, 1982; Martin Del Rio, 1981). Evidently, GABA synthesis and degradation occurs not only in the CNS but also in the periphery.



In the present study, EDA has been used to elicit GABAergic responses in various segments of the guinea-pig intestinal tract in vitro, and the effects of various GABA metabolic inhibitors of GABA synthesis or degradation have been tested against the intestinal responses elicited by EDA, which is thought to release endogenous GABA from the myenteric plexus of the intestine.

### 3.2 MATERIALS AND METHODS

Guinea-pigs of either sex, weighing between 200-400 g, were killed by a blow on the head and bled. Segments of the ileum, jejunum, duodenum and distal colon, each 3-4 cm in length, were quickly removed and emptied of their contents. The tissues were then mounted vertically in a 10 ml organ bath containing oxygenated normal Krebs-bicarbonate (Krebs BC) solution (pH 7.4, 37°C), the composition of the Krebs solution was described in Chapter 2.1. Where the bathing medium was switched to oxygenated Krebs-phosphate-buffered (Krebs PP/O<sub>2</sub>) solution or Krebs-HEPES-buffered solution (pH 7.4, 37°C), in the phosphate-buffered solution, the bicarbonate was omitted, being replaced with equimolar phosphate buffer (pH 7.4) and the Ca<sup>2+</sup> concentration reduced to 1.2 mM to prevent precipitation in the high phosphate solution, whilst in the HEPES-buffered solution, the bicarbonate was replaced by 5 mM HEPES buffer (pH 7.4), and the NaCl content increased to give the normal Na<sup>+</sup> concentration at 151 mM. Mechanical activity of the longitudinal muscle was recorded isometrically (see Chapter 2.1), and the effects of drug treatments were examined on the resting tissue or on electrically evoked contractions of the tissue stimulated by a pair of parallel platinum electrodes positioned around the tissue segment in the bath. The parameters for electrical stimulation of cholinergic intrinsic neurones were described in Chapter 2.1.

The guinea-pig vas deferens and the rat anococcygeus muscle were isolated and set up vertically in a 10 ml organ bath

containing magnesium-free Krebs-bicarbonate buffer solution at 32°C, the mechanical activity of the contractions was recorded isometrically, according to the method described in Chapter 2.1. Effects of drug treatments were examined on the resting tissue or on electrically evoked contractions of the tissue stimulated by a pair of parallel platinum electrodes positioned around the tissue segment in the bath. The parameters for electrical stimulation in the guinea-pig vas deferens were as follows: 3 Hz, 1 ms duration, 60 V, whilst the parameters for the rat anococcygeus muscle were 10 Hz, 1 ms duration, 50 V.

The equilibration period for the tissues in the organ bath was 60 min before any drug application or electrical stimulation. Drugs were applied within 15-20 min intervals, depending on the recovery of the tissue responses to baseline, and the contact time depends on the individual experiments. Some drugs were allowed to be left in contact with the tissues for at least 30 min. All antagonists were added at least 5-10 min before the appropriate agonists were tested, and drug volumes used were never more than 1% of the total bath volume. Student's t-test for paired and unpaired samples was used to assess the significance ( $P < 0.05$ ) of differences between mean values of the dose-response effects.

L-Allylglycine was injected intraperitoneally (0.4 g/kg) in the guinea-pig for at least 6 h until the animal started to convulse, before segments of the ileum were quickly removed and mounted in a 10 ml organ bath, and pharmacological studies were

performed as described above on the tissues. For the control experiments, saline solution was injected intraperitoneally in the guinea-pig for at least 6 h.

### **Synthesis of 2-keto-4-pentenoic acid**

2-keto-4-pentenoic acid was prepared from L-ally<sup>l</sup>glycine by oxidative deamination with L-amino acid oxidase according to the method of Reingold and Orłowski (1979), based on the original enzymatic synthesis by Collinsworth et al. (1973). Ether extraction yielded a clear, volatile oil of characteristic odor, and this was used for metabolic studies without further purification.

### **Synthesis of ethylenediamine monocarbamate**

The monocarbamate of ethylenediamine (EDAC) was prepared by Dr. D.I.B. Kerr, using the method of Katchalski et al. (1951), by passing CO<sub>2</sub> gas into a methanolic solution of EDA (free base) cooled to -10°C. The white powdery product was triturated, repeatedly washed with ether, dried and stored at -20°C. The identity of EDAC was established by NMR, confirmed by Dr. N.G. Bowery (Merck, Sharpe and Dohme, U.K.).

### **[3H] GABA efflux studies:**

The efflux studies were performed according to the method described in Chapter 2.1. Where uptake blockers for GABA were

used in such studies, the tissues were incubated at 37<sup>0</sup> C with amino-oxyacetic acid (AOAA) (0.1 mM) and the uptake inhibitors at known concentrations for at least 20 min before [3H] GABA (10 nM) (66 Ci mmol<sup>-1</sup>) was added to the incubating vessels containing oxygenated normal Krebs-bicarbonate solution. The tissues were then left to incubate in the presence of [3H] GABA for another 20 min at 37<sup>0</sup> C before transferring to glass perfusion chambers for efflux studies. Both AOAA (0.1 mM) and b-alanine (b-ala) (1 mM) were subsequently present throughout the experiment. In experiments where the incubating medium contained Krebs-phosphate-buffered/O<sub>2</sub> (Krebs PP/O<sub>2</sub>) solution, the composition used was as described previously (Table 1).

#### CHEMICALS:

The following chemicals were obtained from Sigma except for those indicated in parenthesis.

2,3-[3H]-GABA (Radiochemical Centre, Amersham)

GABA

3-amino-1-propanesulphonic acid

ethylenediamine dihydrochloride

amino-oxyacetic acid

b-alanine

atropine sulphate

tetrodotoxin

picrotoxinin (dissolved in 1:9 absolute alcohol and distilled water)

d-aminovaleric acid

muscimol  
baclofen (Ciba-Geigy)  
bicuculline methochloride (Pierce)  
3-mercaptopropionic acid (Koch-Light)  
thiosemicarbazide  
L-allyglycine  
2-keto-4-pentenoic acid (synthesised in this laboratory)  
nipecotic acid  
cis-aminocyclohexane carboxylic acid (Dr. D.I.B. Kerr)  
L-2,4-diamino-n-butyric acid dihydrochloride  
D,L-gabaculine hydrochloride (Calbiochem)  
sodium valproate (a gift from Dr. B. G. Priestly)  
ethanolamine-o-sulphate (Calbiochem)  
d-aminolaevulinic acid  
5-hydroxytryptamine creatinine sulphate  
acetylcholine chloride  
nicotine  
1,1-dimethyl-4-phenyl piperazinium iodide (Koch-Light)  
histamine  
4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridin-3-ol (THIP) (a gift  
from Dr. Krogsgaard-Larsen)

### 3.3. RESULTS: GABA PHARMACOLOGY AND [3H] GABA EFFLUX STUDIES

#### 3.3.1. Differential release of [3H] GABA from preloaded intestinal segments, using EDA as the releasing agent.

Within one hour of loading the different, individual segments of the guinea-pig intestinal tissues, i.e. duodenum, jejunum, ileum and distal colon, with [3H] GABA (10 nM) (66 Ci mmol<sup>-1</sup>) in separate vessels containing normal oxygenated Krebs-bicarbonate solution in the presence of AOAA (0.1 mM) and b-ala (1 mM), followed by subsequent 2 min-interval washes in normal Krebs solution with AOAA (0.1 mM) and b-ala (1 mM) present, the basal or resting efflux of tritium from the tissues had declined to a low, steady level during the equilibration period of 60 min. As seen in Fig. 1., addition of EDA to the superfusing medium induced a dose-dependent release of [3H] GABA in the ileum, this [3H] GABA release being prevented in a dose-related manner when 3-MPA was added to the superfusate for 5 min before the addition of EDA. Subsequent upon washing out the 3-MPA with Krebs solution for 10 min, the release of [3H] GABA by EDA was again observed. A similar pattern of [3H] GABA release was also observed in similarly loaded segments of duodenum, jejunum and distal colon, the release of [3H] GABA by EDA in these tissues also being blocked by 3-MPA (Table 1).

Exogenous application of EDA (1 mM) for 5 min to the superfusion medium of the different intestinal segments induced a differential release of [3H] GABA, the release was greatest in

the distal colon = ileum > jejunum > duodenum (Table 1). Such release was  $\text{Ca}^{2+}$ -dependent, as it was prevented by high  $\text{Mg}^{2+}$  (10 mM) and low  $\text{Ca}^{2+}$  (0.6 mM) in the Krebs-bicarbonate superfusate medium, the release by EDA being again observed upon the addition of normal  $\text{Ca}^{2+}$  into the Krebs solution (Fig. 2). The release was tetrodotoxin (TTX) (0.1  $\mu\text{M}$ )-insensitive.

### 3.3.2. EDA-induced release of [3H] GABA from preloaded ileum in the presence of uptake blockers.

In order to explore the compartment(s) of GABA stores from which GABA could be released, segments of ileum were preincubated in normal Krebs solution containing AOAA (0.1 mM) together with the various GABA uptake blockers such as d-aminobutyric acid (DABA) (0.1 mM), cis-aminocyclohexanecarboxylic acid (ACHC) (10  $\mu\text{M}$ ), nipecotic acid (nip) (1 mM), or b-ala (1 mM) for at least 20 min, in separate vessels, and then incubated for a further 20 min after the addition of [3H] GABA (10 nM) to the same solution containing the uptake blocker. The segments were then repeatedly washed with Krebs containing AOAA together with the appropriate uptake blocker, until the basal efflux reached a steady level, after which EDA (1 mM) was added to the superfusate still containing uptake blocker, and the resultant efflux of tritium measured.

Upon application of EDA (1 mM) to ileal segments loaded as described above, in the presence of the various uptake blockers, the ensuing release of [3H] GABA varied depending on the uptake



blocker being used (Fig. 3). There was a maximal efflux of label from control tissues loaded only in the presence of AOAA, with no added uptake blocker, (but the Krebs solution used for washing and during the EDA-induced efflux did contain AOAA and b-ala, to minimise uptake and metabolism of the label), by comparison with which, EDA induced a significantly ( $P < 0.05$ ) reduced efflux of label from tissues loaded in the presence of AOAA and DABA or ACHC, although somewhat less than that from tissues loaded in the presence of b-ala alone. Racemic nip is said to inhibit high affinity GABA uptake into both glia and neurones (Schousboe et al., 1978); with tissues loaded in the presence of nip (1 mM) alone, EDA induced a significantly ( $P < 0.05$ ) reduced release of [3H] GABA by comparison with that in the presence of b-ala alone. However, there was a more marked reduction of release from tissues loaded in the presence of combined inhibition of glial and neuronal uptake, as by loading in Krebs containing AOAA with both nip (1 mM) and b-ala (1 mM), with DABA (0.1 mM) and b-ala (1 mM), or with both b-ala (1 mM) and ACHC (10  $\mu$ M). There was no significant difference in the release observed under either of the latter combinations of uptake blockade during loading, but the release was significantly less than that seen when loading was carried out during inhibition of either neuronal or glial uptake alone (Fig. 3).

### 3.3.3. Effects of 3-MPA on electrically stimulated release of [3H] GABA in the ileum.

Electrical stimulation (10 Hz, 0.5 msec, 60V) induced a

release of [3H] GABA from the preloaded ileum in the presence of AOAA (0.1 mM) and b-ala (1 mM). Such release was prevented by 3-MPA (1 mM) (Table 2), and upon washing out the 3-MPA, there was a recovery of the electrically induced release of [3H] GABA.

#### 3.3.4. Effects of EDA on isolated intestinal preparations.

##### Ileum:

In all subsequent experiments, GABA was not applied to the bath before the addition of EDA. In the ileum, a transient dose-dependent contractile response was induced by EDA (1 mM), followed by an 'after-relaxation' (Fig. 4). These contractions were cholinergic and neurogenic in origin, being abolished by atropine (0.1  $\mu$ M) and TTX (0.1  $\mu$ M). The contractile response was also sensitive to bicuculline methochloride (BMC) (10  $\mu$ M) (Fig. 4), or picrotoxinin (PIC) (10  $\mu$ M), but the 'after-relaxation' was unaffected by either of these antagonists. However, the GABA<sub>B</sub>-receptor antagonist, 5-aminovaleric acid (DAVA) (500  $\mu$ M) did prevent the 'after-relaxation' phase of the ileal response to EDA, without affecting the initial contractile component or the response to transmural stimulation of cholinergic neurones (Fig. 4). Neither BMC, PIC nor piritanide affected ileal responses to ACh, transmural stimulation, or pharmacological activation of cholinergic motor neurones (c.f. Krantis and Kerr, 1981 b). The dose-response curve for the contraction elicited by EDA was displaced to the right in a parallel fashion by BMC (5, 10 and 50  $\mu$ M), in a competitive manner (Fig. 5), with an estimated pA<sub>2</sub> value of 5.8, similar to that for GABA (c.f. Krantis and Kerr,

1981 b). On the other hand, PIC (6 and 10  $\mu\text{M}$ ) gave a non-parallel shift of the dose-response curve for EDA to the right, with a depression of the maximum response in a non-competitive manner (Fig. 6).

When repetitive cholinergic twitch contractions of the ileum were challenged with EDA at varying concentrations, both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated effects were observed: a GABA<sub>A</sub>-receptor-mediated contraction superimposed on the twitch contractions, followed by a delayed depression of the twitch responses (GABA<sub>B</sub>-receptor-mediated effects). The superimposed contraction was antagonised selectively by BMC (10  $\mu\text{M}$ ) (Fig. 7), PIC (10  $\mu\text{M}$ ) (Fig. 8) or piretanide (10  $\mu\text{M}$ ), whilst the delayed depressant action was unaffected by these antagonists (Fig. 7,8). Conversely, the dose-dependent depressive effect of EDA on twitch contractions was antagonised by DAVA (500  $\mu\text{M}$ ), but the superimposed contraction was not affected (Fig. 9). DAVA (500  $\mu\text{M}$ ) alone, however, elicited a weak BMC- and PIC-sensitive contractile response, but gave no cross desensitization of the GABA<sub>A</sub>-receptors so that EDA and GABA evoked unchanged contractile responses. Antagonism of the EDA-induced responses by DAVA was competitive, the dose-response curve for the EDA-induced depression of electrically elicited twitch contractions being displaced to the right in a parallel manner by varying concentrations of DAVA (0.5, 0.8 and 1 mM) (Fig. 10), with an estimated pA<sub>2</sub> value of 3.8.

GABA alone elicited both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated

actions, whilst 3-amino-1-propanesulphonic acid (3-APS), muscimol and THIP, elicited only GABA<sub>A</sub>-receptor-mediated contractile responses, the potency being muscimol > 3-APS > GABA (see Chapter 4 of this thesis). THIP has so far proved to be the least potent agonist at ileal myenteric GABA<sub>A</sub>-receptor sites, and its action was also blocked by BMC, PIC and piretanide (Fig. not shown). Baclofen (50  $\mu$ M) induced a depression of cholinergic twitch contractions and a delayed 'after-relaxation' in the unstimulated ileal preparations, such actions were antagonised by DAVA (500  $\mu$ M) (Fig. 11) but not by BMC or PIC .

#### **Duodenum, jejunum and distal colon:**

Contrary to Krantis et al. (1980), the following experiments, demonstrating the pharmacological and physiological effects of EDA, as well as that of GABA and its analogues such as baclofen, 3-APS or muscimol, were all conducted on intestinal segments in the absence of atropine or guanethidine in the Krebs medium, in order to maintain inherent cholinergic activity. Under these conditions, both EDA (1 mM), and GABA (50  $\mu$ M) elicited pronounced relaxation responses in the duodenum, distal colon (Fig. 12) and also in the jejunum. Such responses were mimicked by baclofen (0.1 mM), whereas in the isolated ileum, EDA and GABA each induced a transient contraction, followed by an 'after-relaxation', but baclofen induced only relaxation responses. This particular ileal distal region was also highly sensitive to 3-APS and muscimol, both of which elicited solely contractile effects. However, in contrast, 3-APS and muscimol were

ineffective in the jejunum, duodenum and the distal colon, and only exerted weak contractile effects in these tissues at very high concentrations (5 mM). Other agents such as acetylcholine (ACh), histamine, 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP), 5-hydroxytryptamine (5-HT), or nicotine, however, all induced contractile responses in each of the tissues taken from all levels of the intestine.

It was difficult to construct a dose-response curve to the relaxations induced by GABA, EDA or baclofen in the jejunum, duodenum, or the distal colon, since dose-dependent relaxations only occurred over the following concentration range for GABA (50-500  $\mu$ M), EDA (1-10 mM), and baclofen (50-500  $\mu$ M), the upper ranges giving maximal relaxations. At doses higher or lower than the range employed, the tissues were either totally unresponsive at a lower dose, or simply relaxed to the same maximal extent as in the ranges used above, if the doses were higher. These relaxation responses induced by GABA (0.1 mM), EDA (1 mM) (Fig. 13) and baclofen (0.1 mM) were all ~~antagonised~~ antagonised by DAVA (500  $\mu$ M); although there was a slight antagonism with BMC (10  $\mu$ M) or PIC (10  $\mu$ M) for responses induced by GABA and EDA (Fig. 13), but not baclofen. Antagonism of the ileal responses to EDA, GABA, or any of its analogues, by BMC, PIC or DAVA are reported in section 3.3.4 of this chapter.

In the duodenum, using electrically stimulated repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60V), baclofen, GABA and EDA induced a depression of the twitch contractions at

the concentrations shown in Fig. 14 and 15, antagonised by DAVA (500  $\mu\text{M}$ ) but unaffected by BMC (10  $\mu\text{M}$ ) (Fig. 14), PIC (10  $\mu\text{M}$ ) (Fig. 15) or chloride ionophore blockers. Such results were also reproducible in the jejunum (Fig. not shown).

Also, in the rat anococcygeus muscle (10 Hz, 1 ms duration, 50 V) (Fig. 16) and guinea-pig vas deferens (3 Hz, 1ms duration, 20 V) (Fig. 17), baclofen, GABA and EDA all depressed repetitive twitch contractions, the depression being antagonised by DAVA (Fig. 16, 17) but not by BMC or PIC.

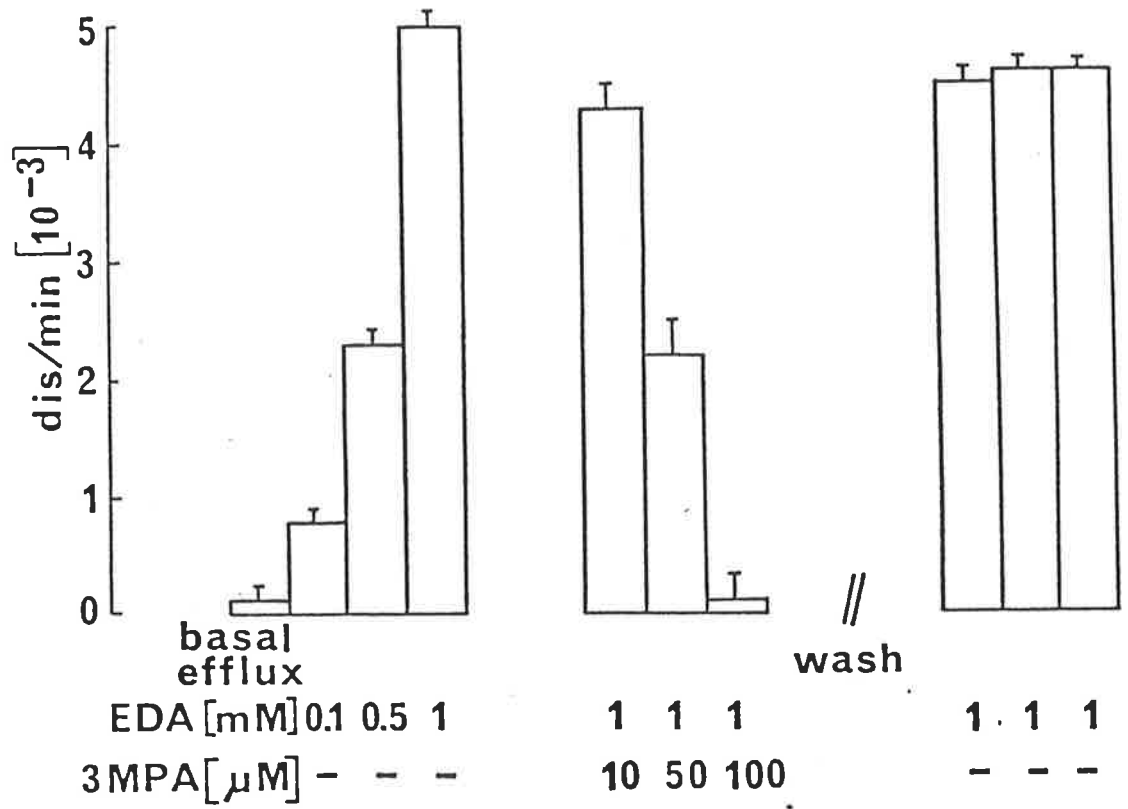


Fig. 1. Dose-dependent release of [3H] GABA by EDA in the preloaded isolated ileum of the guinea-pig. 3-MPA prevented the release of [3H] GABA by EDA, also in a dose-dependent manner; after washing out the 3-MPA from the tissues, [3H] GABA was released by EDA as before. Each column represents the mean of at least 10 values, with the vertical line showing s.e. mean. Results are expressed as d.p.m.

EDA-INDUCED [<sup>3</sup>H] GABA RELEASE (d.p.m. 10<sup>-3</sup> ± s.e.m.)

	EDA (1 mM)	EDA (1 mM) + 3MPA (50 μM)
DUODENUM	1.8 ± 0.05	0.1 ± 0.01
JEJUNUM	1.5 ± 0.1	0.2 ± 0.02
ILEUM	5.0 ± 0.03	2.2 ± 0.2
COLON	4.4 ± 0.04	1.2 ± 0.1

Table 1. EDA-induced release of [<sup>3</sup>H] GABA in preloaded isolated intestinal segments, the [<sup>3</sup>H] GABA release was prevented by 3-MPA. Results are expressed as d.p.m., and s.e. mean of each value is indicated. The number of experiments performed in each case was at least n=6.



TABLE 2

[<sup>3</sup>H] GABA RELEASE IN THE ILEUM (DPM x 10<sup>-3</sup>)

ELECTRICAL STIMULATION	3MPA (1 mM)	ELECTRICAL STIMULATION
4.8 ± 0.2	1.2 ± 0.02	4.2 ± 0.1 (6)

Table 2. Release of [<sup>3</sup>H] GABA in the isolated ileum induced by electrical stimulation (10 Hz, 0.5 ms, 60 V), and 3-MPA (1 mM) prevented the electrically-induced release, with s.e. mean of each value indicated. The number of experiments performed was n=6, indicated in parentheses.

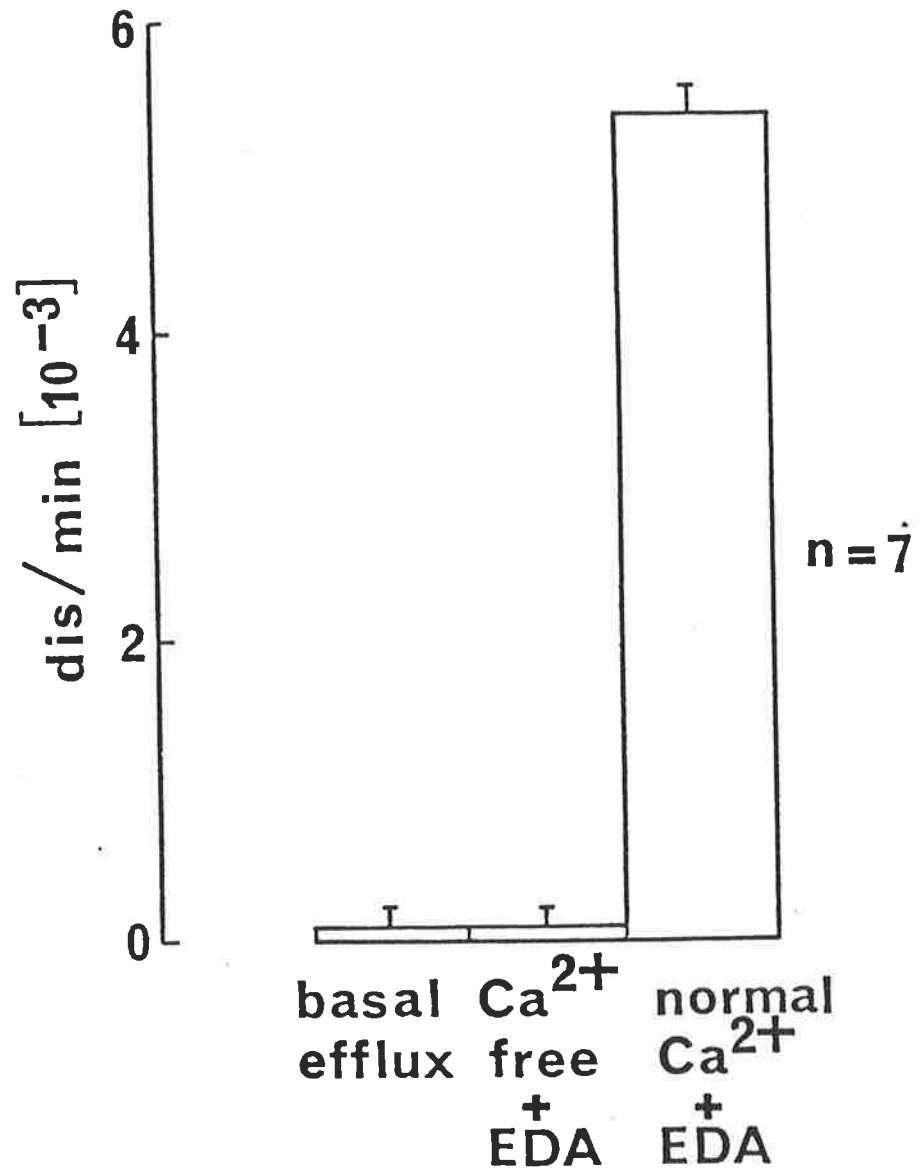


Fig. 2.  $\text{Ca}^{2+}$ -dependent release of  $[3\text{H}]$  GABA induced by EDA (1 mM) in the isolated ileum, where  $[3\text{H}]$  GABA release was prevented by low  $\text{Ca}^{2+}$  (0.6 mM), high  $\text{Mg}^{2+}$  (10 mM), and the release was induced by EDA in the presence of normal  $\text{Ca}^{2+}$  (2.8 mM).  $n$  indicates the number of experiments performed. Results are expressed as dis/min, and the vertical bar on each column indicates the s.e. mean of each value.

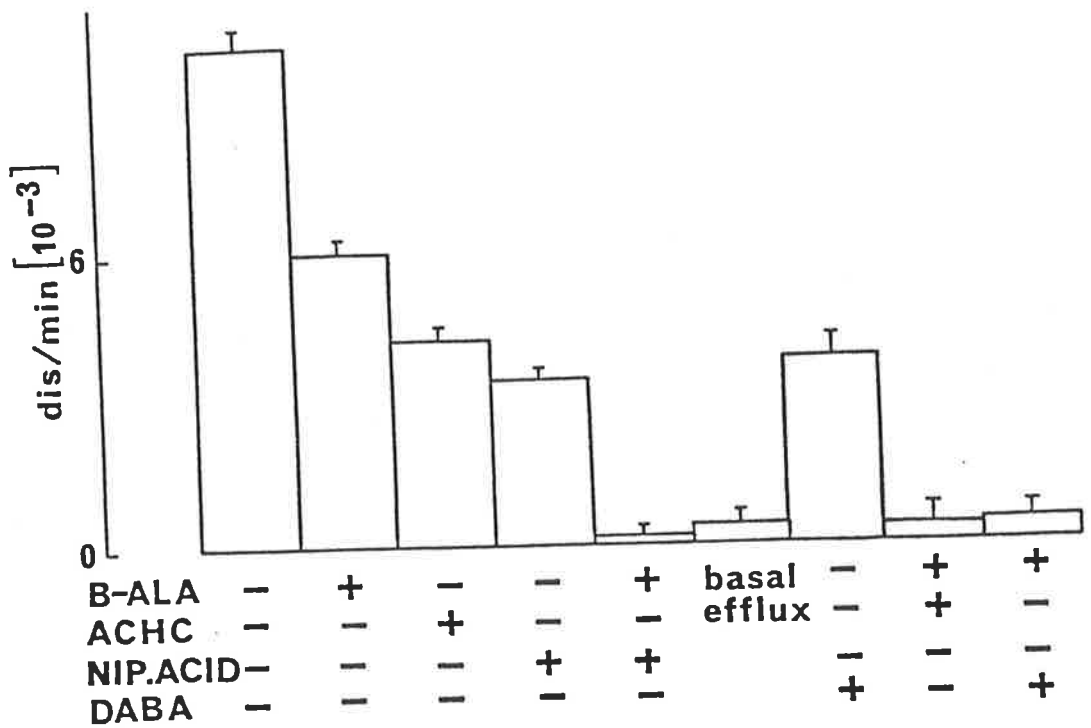


Fig. 3. Effects of b-ALA (1 mM), ACHC (10  $\mu$ M), DABA (100  $\mu$ M), NIP (1 mM) on EDA (1 mM)-induced release of [3H] GABA in the isolated ileum. Each column represents the mean of at least 6 value, with the vertical line showing s.e. mean. Results are expressed as d.p.m.

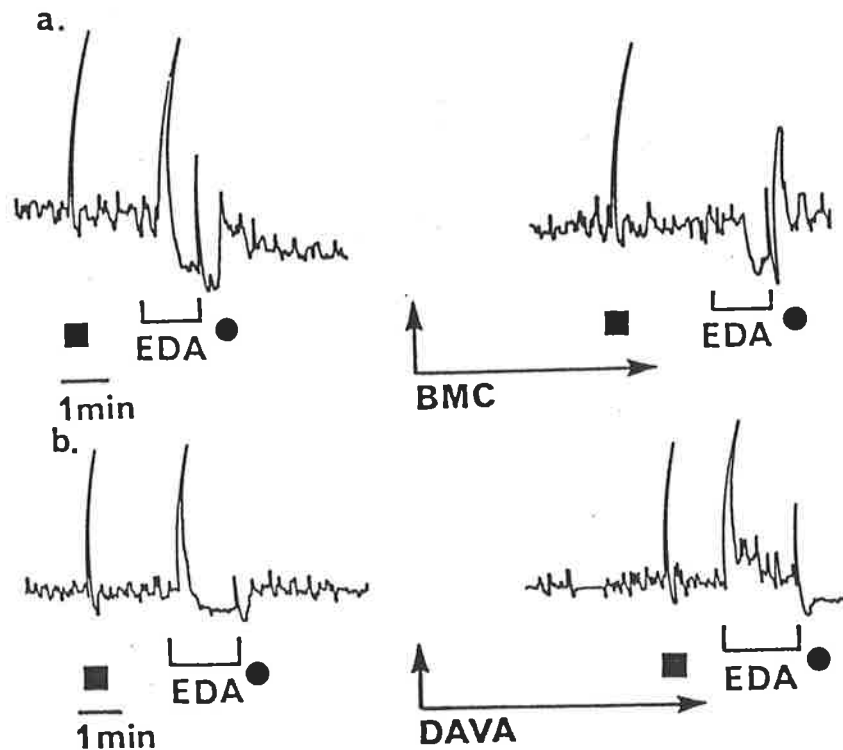


Fig. 4. Responses to exogenously applied EDA (1 mM) in the isolated ileum; antagonism of the contractile response to EDA by (a) BMC (10  $\mu$ M) and antagonism of the 'after-relaxation' by (b) DAVA (500  $\mu$ M). Both BMC and DAVA did not affect responses to transmural stimulation (0.1 ms duration, supramaximal voltage) as indicated by ■. (●) indicates tissue washout after each drug application. The number of experiments performed in each case was at least 6.

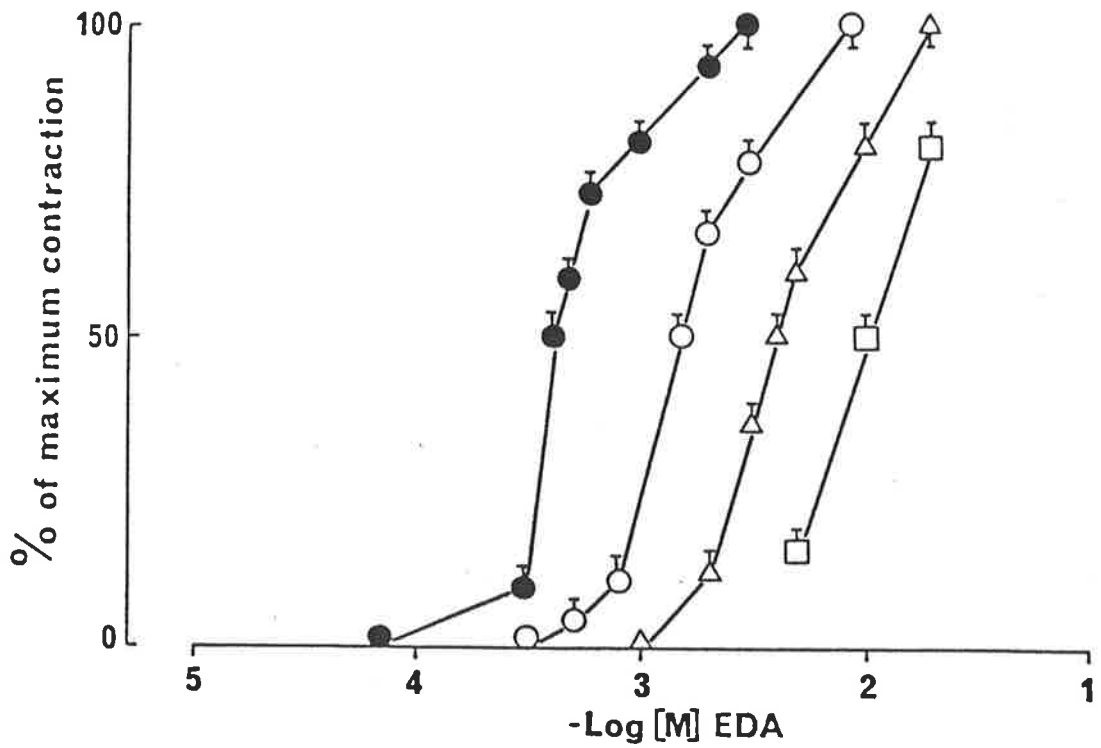


Fig. 5. Dose-response curves for contractile responses to ● EDA, in the presence of ○ BMC (5  $\mu$ M), △ BMC (10  $\mu$ M) and □ BMC (50  $\mu$ M). Results are expressed on the coordinate as a mean of the percentage of the maximal contraction induced by EDA, with each point representing the mean and vertical lines s.e. mean (n=8).

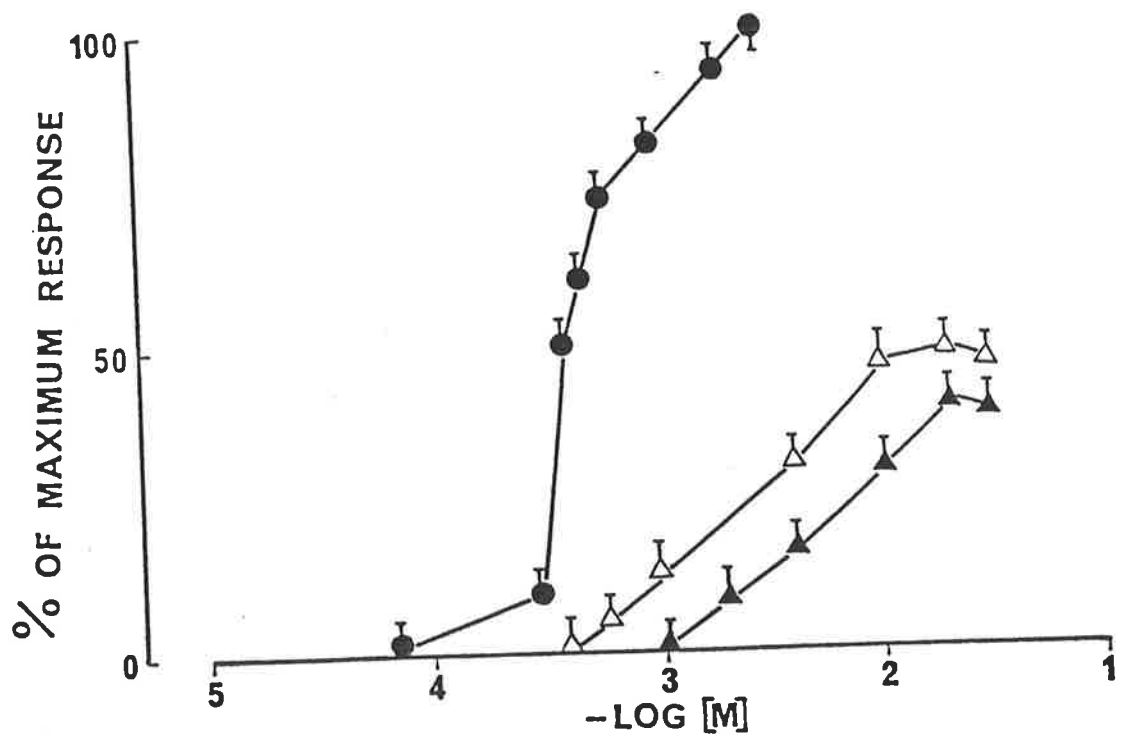


Fig. 6. Dose-response curves for contractile responses to ● EDA, in the presence of △ PIC (6 uM) and ▲ PIC (10 uM). Results are expressed on the ordinate as a mean of the percentage of the maximal contraction induced by EDA, with each point representing the mean and standard error of the mean (n=7).

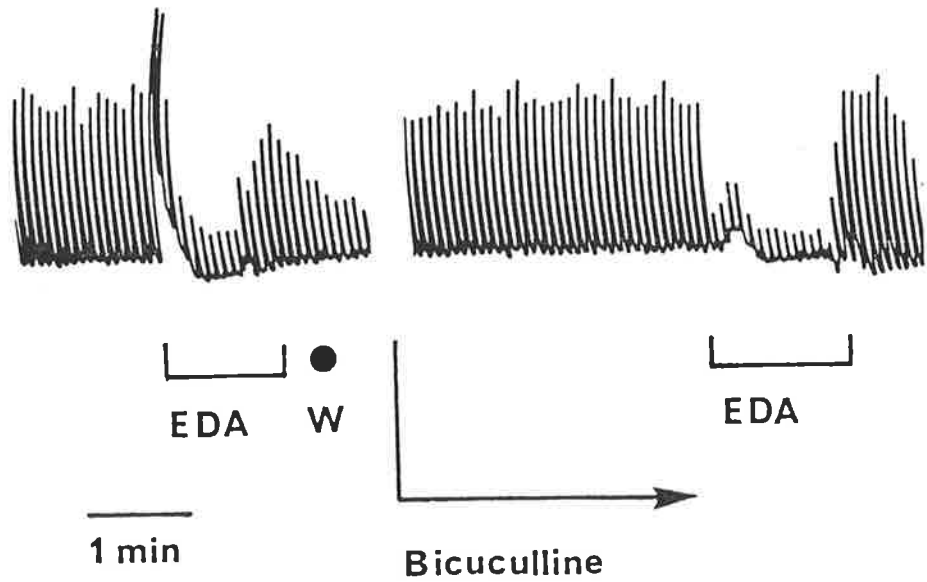


Fig. 7. Effect of BMC (10  $\mu$ M) on responses to EDA (1 mM) in electrically-stimulated isolated ileal preparations (0.1 Hz, 0.5 ms, supramaximal voltage). ● tissue washout.

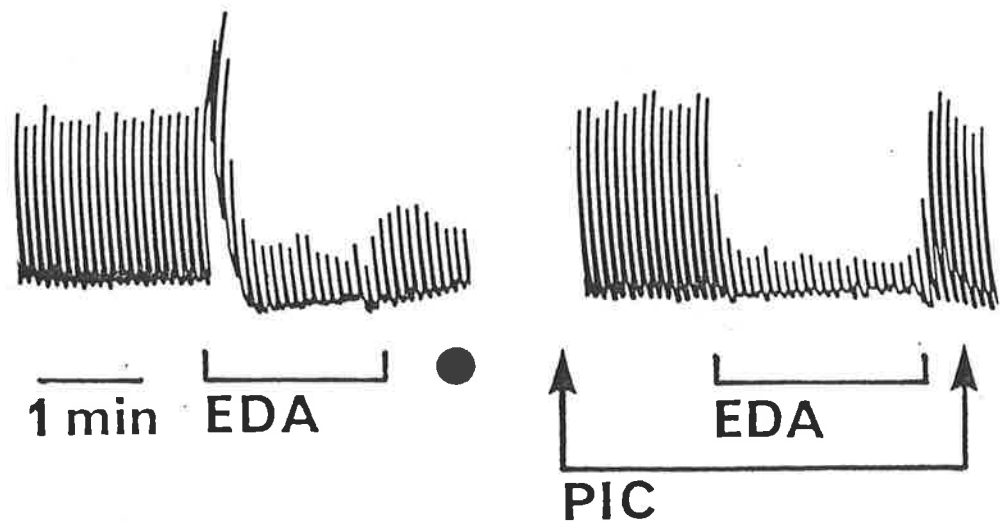


Fig. 8. Effect of PIC (10  $\mu$ M) on responses to EDA (1 mM) in electrically-stimulated ileal preparations (0.1 Hz, 0.5 ms, supramaximal voltage). ● tissue washout.

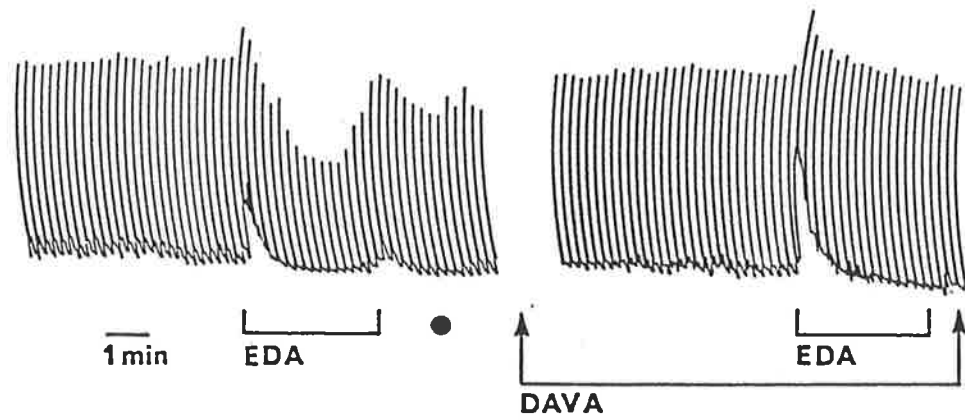


Fig. 9. Effect of DAVA (500  $\mu$ M) on EDA (1 mM)-induced responses on repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, supramaximal voltage). ● tissue washout.



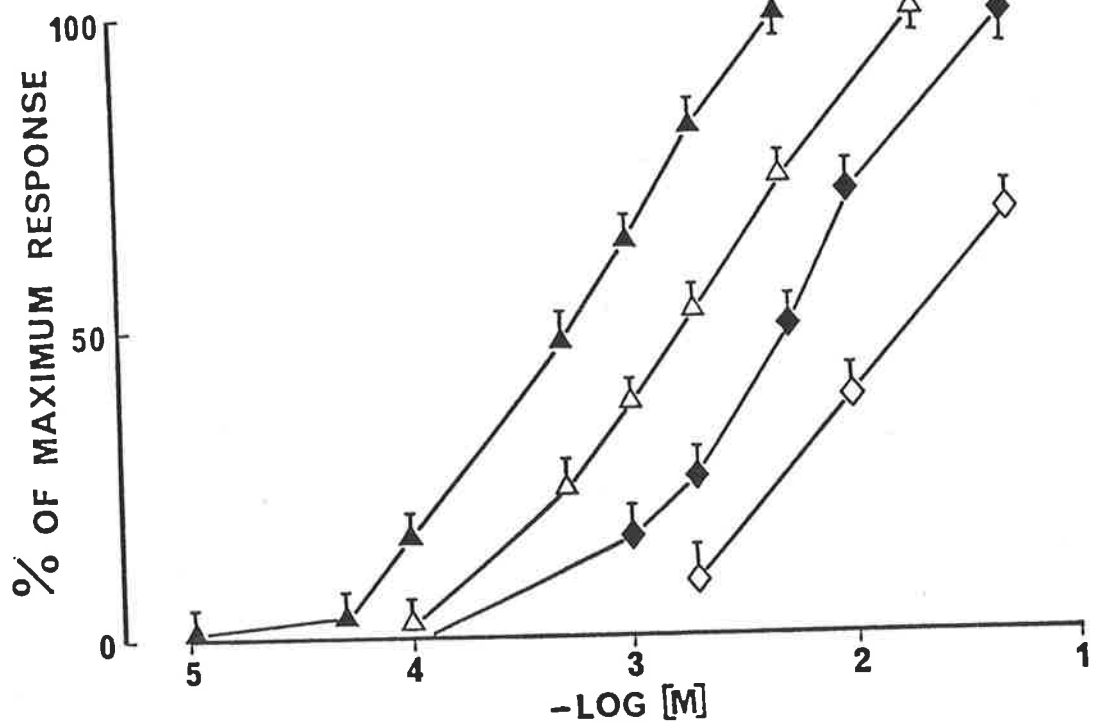


Fig. 10. Dose-response curves for the depression of the repetitive cholinergic twitch contractions induced by  $\blacktriangle$  EDA, in the presence of  $\triangle$  DAVA (500  $\mu\text{M}$ )  $\blacklozenge$  DAVA (800  $\mu\text{M}$ )  $\diamond$  DAVA (1 mM). Results are expressed as a percentage of the maximum inhibition of the twitch contractions induced by EDA. Each point is the mean and vertical lines show s.e. mean of at least 6 values obtained from 6 tissues using a minimum of 3 animals.

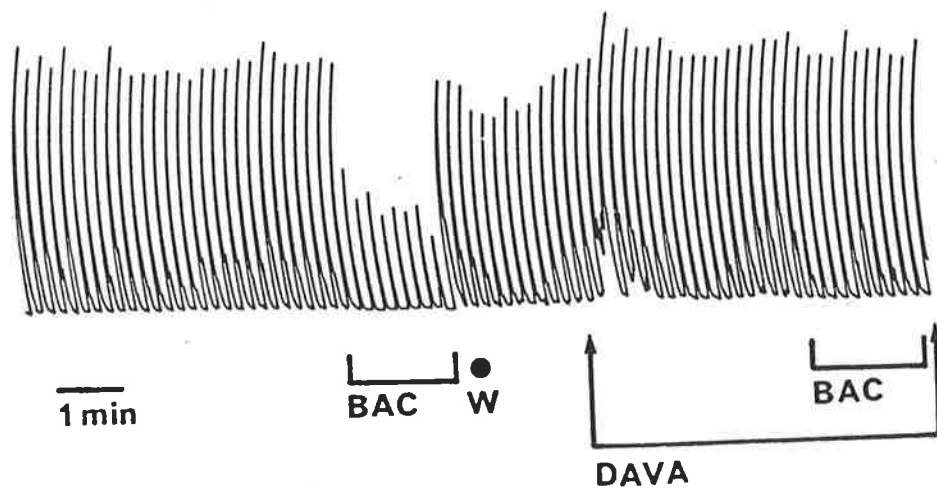
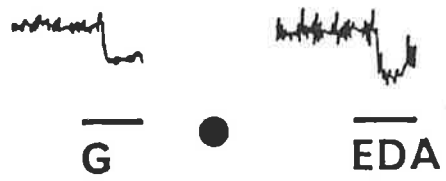


Fig. 11. Antagonism of the baclofen (50 uM)-induced depression of repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60 V) by DAVA (500 uM). ● tissue washout.

DUODENUM



DISTAL COLON

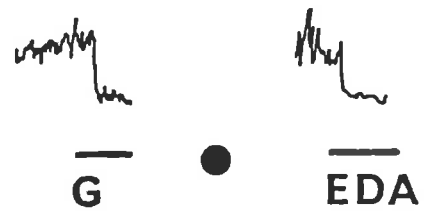


Fig. 12. Responses to EDA (1 mM) and GABA (50 uM) in the isolated duodenum and distal colon. ● tissue washout.

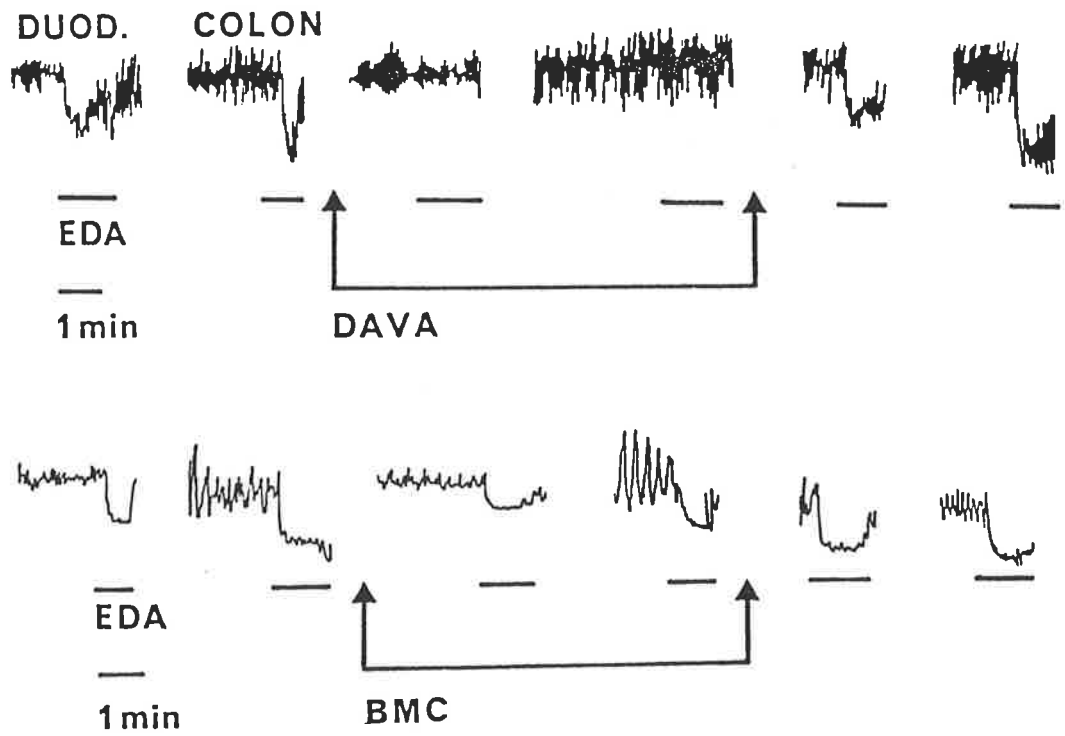


Fig. 13. Effects of DAVA (500 uM) and BMC (10 uM) on relaxation responses induced by EDA (1 mM) in the duodenum and the distal colon.

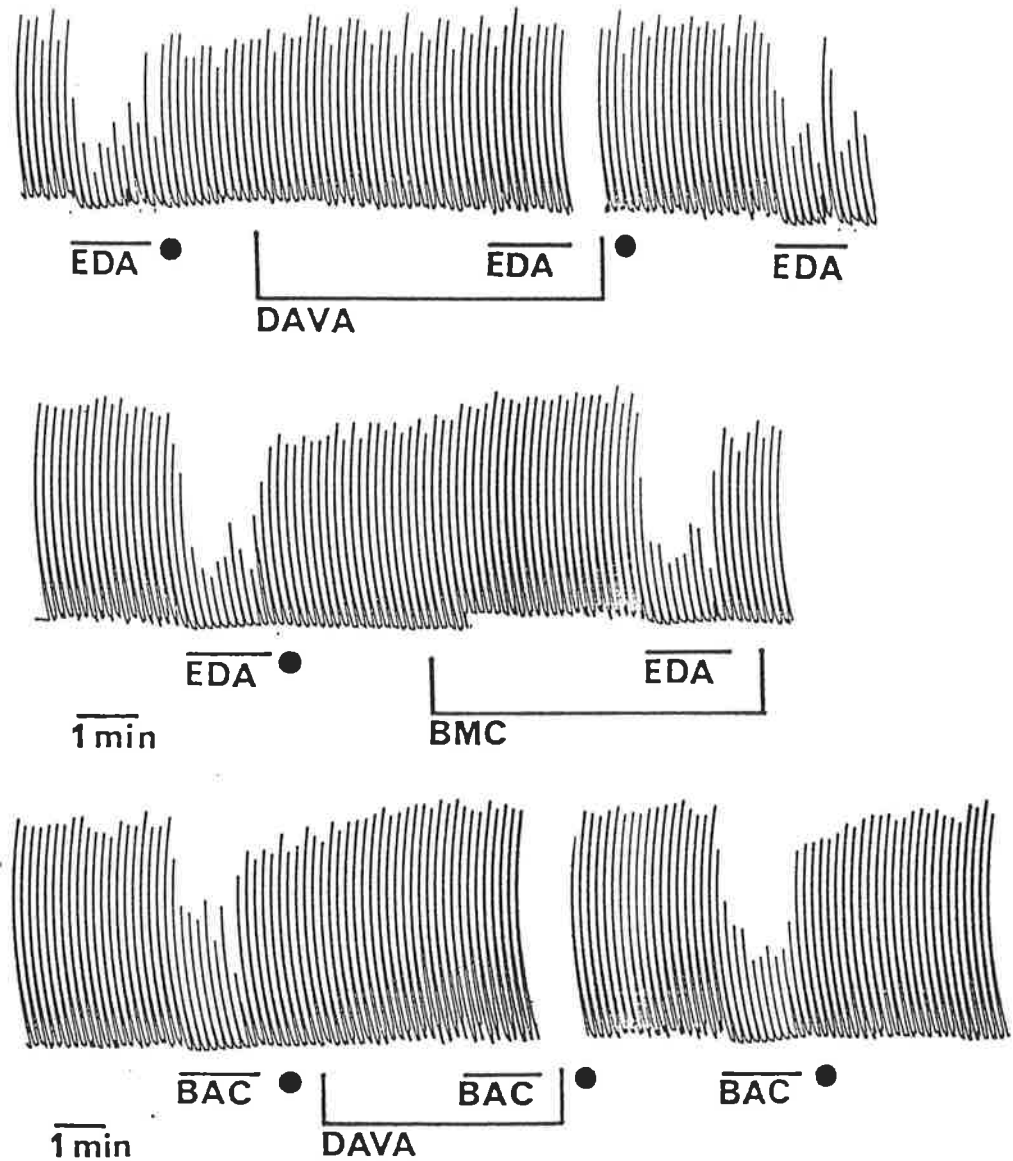


Fig. 14. Effects of DAVA (500  $\mu$ M), but lack of effect of BMC (10  $\mu$ M) on EDA (1 mM)-induced depression of repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60V) in the isolated duodenum. Antagonism by DAVA (500  $\mu$ M) on the baclofen (50  $\mu$ M)-induced depression of twitch contractions. ● tissue washout.

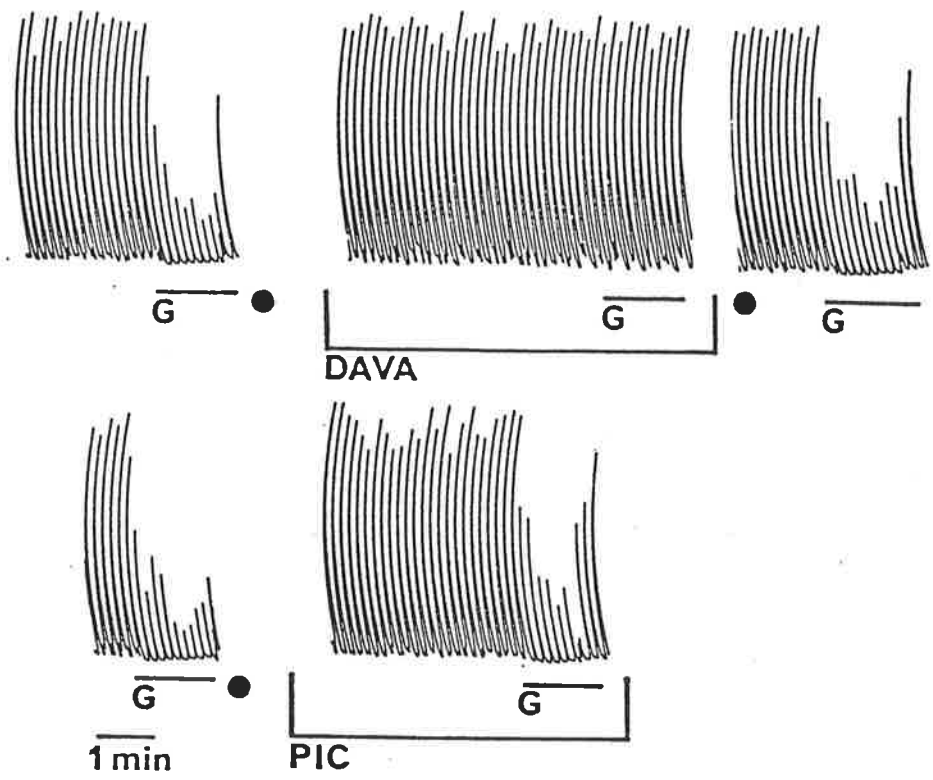


Fig. 15. Effects of DAVA (500  $\mu$ M) and PIC (10  $\mu$ M) on GABA (50  $\mu$ M)-induced depression of cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60 V) in the isolated duodenum. ● tissue washout.

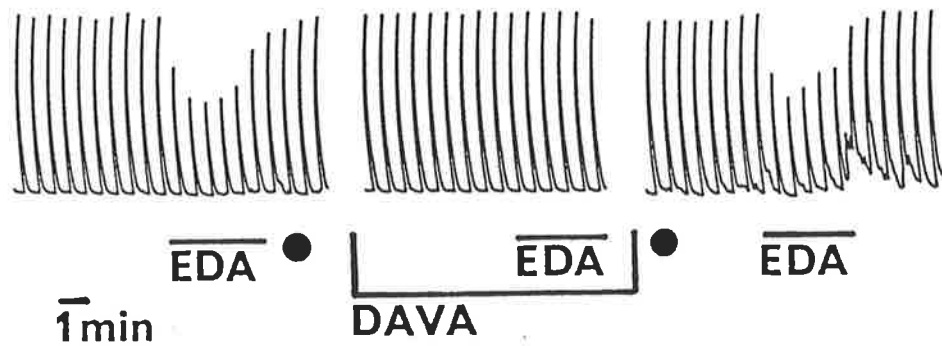


Fig. 16. Effect of DAVA (500  $\mu$ M) on EDA (1 mM)-induced depression of repetitive twitch contractions (10 Hz, 1 ms duration, 50V) in the rat anococcygeus muscle. ● tissue washout.

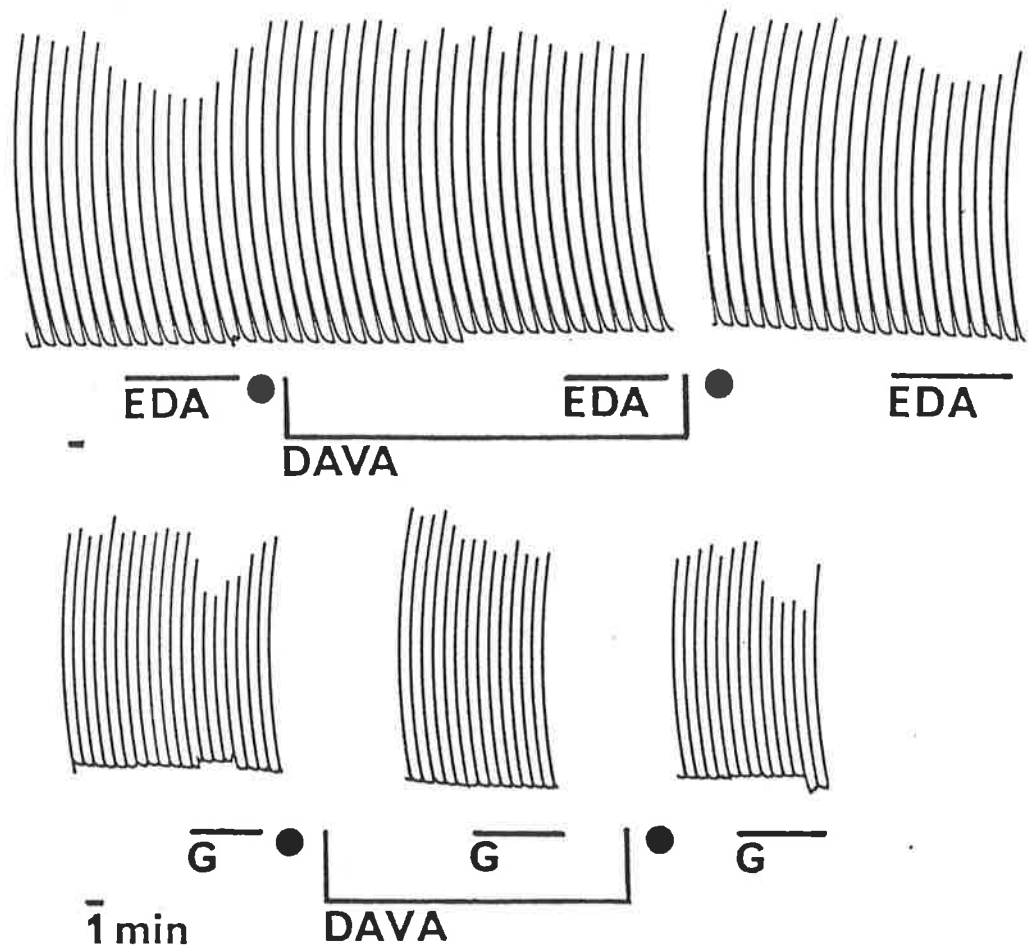


Fig. 17. Effects of DAVA (500  $\mu$ M) on EDA (1 mM)- and GABA (100  $\mu$ M)-induced depression of repetitive twitch contractions (3 Hz, 1 ms duration, 20V) in the vas deferens of the guinea-pig. ● tissue washout.



### 3.3.5. RESULTS: EDA MONOCARBAMATE

#### (a) Bicarbonate dependence of EDA-induced GABA-mimetic responses in the guinea-pig ileum.

There was a typical response to GABA (10  $\mu$ M), but no response to applied EDA (1 mM) in the guinea-pig isolated ileum maintained either in phosphate-buffered Krebs solution (Krebs PP/O<sub>2</sub>), or HEPES (25 mM)-buffered Krebs solution (Krebs HE/O<sub>2</sub>), both being gassed with 100% O<sub>2</sub> and containing no bicarbonate (Fig. 1). Yet a prompt response occurred if HCO<sub>3</sub><sup>-</sup> (5 mM) was then added to the bath already containing the EDA (Fig. 1). The critical concentration of added HCO<sub>3</sub><sup>-</sup> necessary for EDA to elicit ileal GABA mimetic responses (in Krebs PP/O<sub>2</sub> or Krebs HE/O<sub>2</sub>) was 2 mM, the content of the bath being altered by the addition of varying proportions of normal Krebs solution (Krebs BC) buffered with NaH CO<sub>3</sub>, and gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub> (5% Carbogen); generally, 5 mM HCO<sub>3</sub><sup>-</sup> has been used since this yielded the most consistent results. While this suggests that a minimum of some 2-5 mM HCO<sub>3</sub><sup>-</sup> is necessary for EDA to exert its action in releasing endogenous GABA from the myenteric plexus, it could also be that, instead, EDA must first combine with CO<sub>2</sub> (in equilibrium with HCO<sub>3</sub><sup>-</sup> in the bathing solution) to form ethylenediamine monocarbamate (EDAC), this being more nearly a GABA analogue than is the diamine itself. That the latter is more likely follows from the use of stock EDA (1 M) solution treated by gassing with 5% carbogen in 25 mM NaH CO<sub>3</sub> solution, bringing the pH to 7.4. Using either Krebs HE/O<sub>2</sub> or Krebs PP/O<sub>2</sub>, this CO<sub>2</sub> treated EDA (1 mM) induced typical GABA mimetic ileal responses, yet only 25  $\mu$ M

$\text{HCO}_3^-$  would then be present in the bath. Furthermore, EDA (1 M) treated by gassing with 5% carbogen with no  $\text{NaHCO}_3$  present, also yielded such responses, as Curtis and Malik (1984) showed in the CNS. Since this indicates that the monocarbamate, rather than the parent diamine, is indeed responsible for the ileal responses to EDA, authentic EDAC was tested in the presence and absence of  $\text{HCO}_3^-$ .

**(b) [3H] GABA efflux induced by EDA monocarbamate in the guinea-pig intestine.**

In normal Krebs-bicarbonate solution gassed with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  (Krebs BC) and containing AOAA (0.1 mM) and b-ala (1 mM), both EDA and EDAC (1 mM) induced a release of [3H] GABA from the guinea-pig, preloaded, isolated ileum (Table 1), this release being subsequently blocked by 3-MPA (1 mM); with a recovery of [3H] GABA release after washing out the 3-MPA. EDAC also induced a 3-MPA-sensitive release of [3H] GABA in phosphate-buffered Krebs gassed with 100%  $\text{O}_2$  (Krebs PP/ $\text{O}_2$ ), whereas EDA itself did not induce any such release in this solution (Table 2). There was no significant difference in the [3H] GABA release induced by EDAC in normal Krebs (Krebs BC) as compared to the release in Krebs PP/ $\text{O}_2$ .

**(c) EDA monocarbamate-induced responses in the isolated intestine.**

In the ileum maintained in either normal Krebs BC or Krebs

PP/O<sub>2</sub> solution, EDAC (1 mM) induced a neurogenic, cholinergic response followed by an 'after-relaxation'. Just as with GABA, such contractile responses were dose-dependent and could be antagonised by either BMC (10 µM) or PIC (10 µM) (Fig. 2), whilst the 'after-relaxation' was only antagonised by DAVA (500 µM). Such GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated responses induced by EDAC (1 mM) in either normal Krebs/CO<sub>2</sub> or phosphate-buffered/O<sub>2</sub> solution were prevented by 3-MPA (50 µM) (Fig. 3) and by pretreatment for 30 min with thiosemicarbazide (TSC) (100 µM) (Fig. 4), as with EDA described in section 3.3.6. of this chapter.

Using repetitive twitch contractions, EDAC induced a contractile response superimposed on the cholinergic twitch contractions, followed by a depression of the twitch height, in normal Krebs solution as well as in phosphate-buffered/O<sub>2</sub> Krebs solution (Fig. 4). Only the contractile response was antagonised by BMC (10 µM), or PIC (10 µM), whereas the depression of the twitch contractions was antagonised by DAVA (500 µM). Such EDAC-induced depressive actions were also blocked by 3-MPA or by pretreatment with TSC (100 µM) (Fig. 4).

In the distal colon of the guinea-pig, both EDAC (1 mM) and GABA (0.1 mM) induced only relaxation responses, in either normal Krebs BC (Fig. 5), or in phosphate-buffered Krebs PP/O<sub>2</sub> solution; such responses were mainly sensitive to DAVA (0.5 mM), and slightly blocked by BMC (10 µM) or PIC (10 µM), as shown in Fig. 5. Also, 3-MPA (50 µM) (Fig. 5) and TSC (100 µM) prevented these relaxation responses induced by EDAC in the two Krebs solutions

without affecting responses to GABA or baclofen, which still induced a relaxation response in Krebs PP/O<sub>2</sub> solution, blocked by DAVA (500 uM).

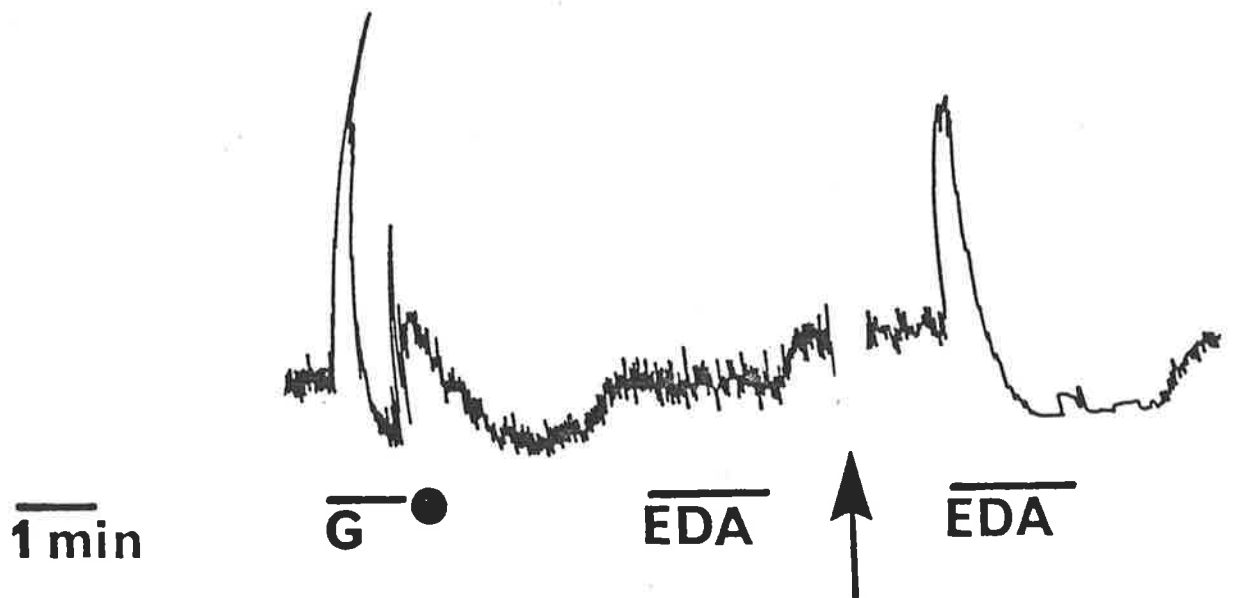


Fig. 1. Responses to GABA (10  $\mu$ M) and EDA (1 mM) in the isolated ileum maintained in Krebs-phosphate buffered solution gassed with 100%  $O_2$  (Krebs PP/ $O_2$ ) and containing no bicarbonate.  $\uparrow HCO_3^-$  (5 mM) added to the bath elicited a response to exogenously applied EDA (1 mM).  $\bullet$  tissue washout. The number of experiments performed was  $n=8$ .

EDA (dpm x 10 <sup>-3</sup> )	3MPA (dpm x 10 <sup>-3</sup> )	EDA (dpm x 10 <sup>-3</sup> )
(1 mM)	(50 uM)	(1 mM)
4.8 ± 0.3	2.1 ± 0.5	4.3 ± 0.2 (n=6)
EDAC	3MPA	EDAC
(1 mM)	(50 uM)	(1 mM)
4.9 ± 0.1	2.5 ± 0.3	4.1 ± 0.2 (n=6)

Table 1. Release of [3H] GABA induced by EDA (1 mM) and EDA carbamate (EDAC) (1 mM) in the isolated ileum, the release was reduced in a significant manner (P < 0.05) by 3-MPA (50 uM). Results are expressed as d.p.m., with ± s.e. mean of each value indicated. The number of experiments performed is shown in parentheses.

EDA (dpm x 10 <sup>-3</sup> ) (1 mM)	EDAC (1 mM)	3MPA (50 uM)	EDAC (1 mM)
0.5 ± 0.1	5.0 ± 0.2	2.3 ± 0.1	4.2 ± 0.4
(n=6)	(n=6)	(n=6)	(n=6)

Table 2. Lack of [3H] GABA release by EDA (1 mM) in Krebs-phosphate buffered medium, gassed with 100% O<sub>2</sub>, but EDAC (1 mM) induced a release of [3H] GABA which was reduced in a significant manner (P < 0.05) by 3-MPA (50 uM). Results are expressed as d.p.m., with ± s.e. mean of each value shown. The number of experiments performed is indicated in parentheses.

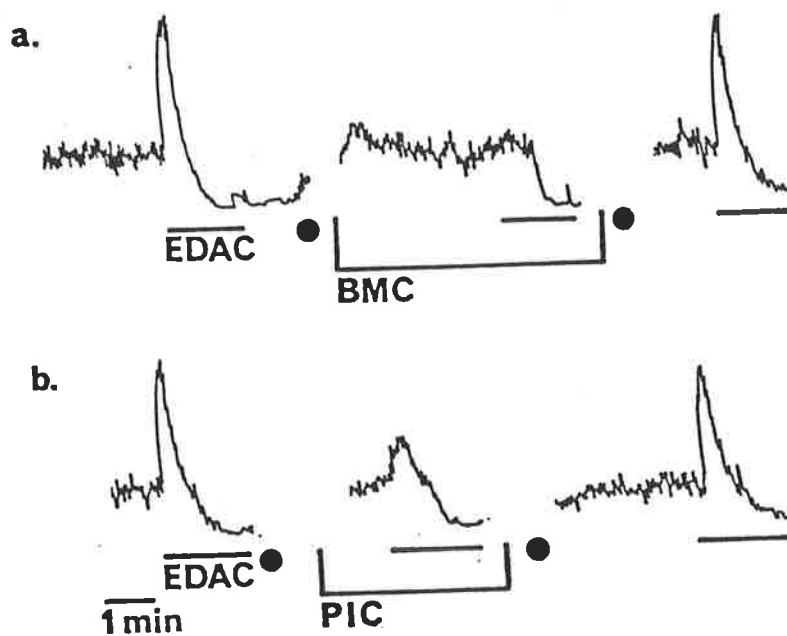


Fig. 2. Antagonism of the EDAC (1 mM)-induced contractile responses in the isolated ileum by BMC (10  $\mu$ M) or PIC (10  $\mu$ M) in Krebs-phosphate buffered medium. ● tissue washout. The number of experiments performed was n=8.



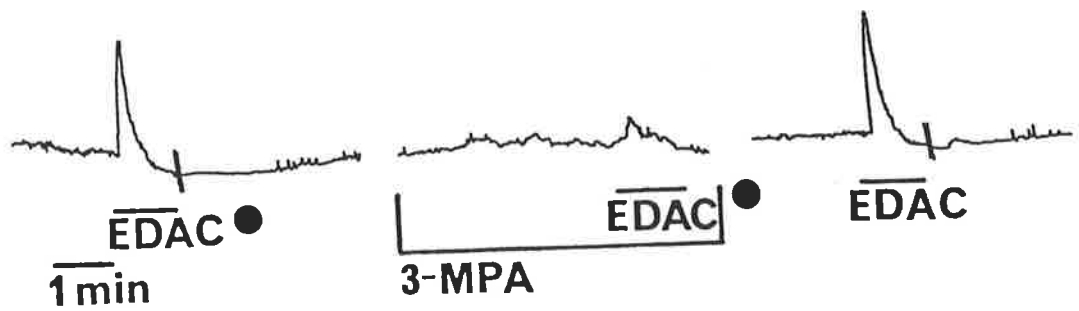


Fig. 3. Prevention of the EDAC (1 mM)-induced responses by 3-MPA (50  $\mu$ M) in the isolated ileum, maintained in normal Krebs-bicarbonate medium, with a recovery of the EDAC-induced responses upon tissue washout indicated by  $\bullet$ . The number of experiments performed was  $n=6$ .

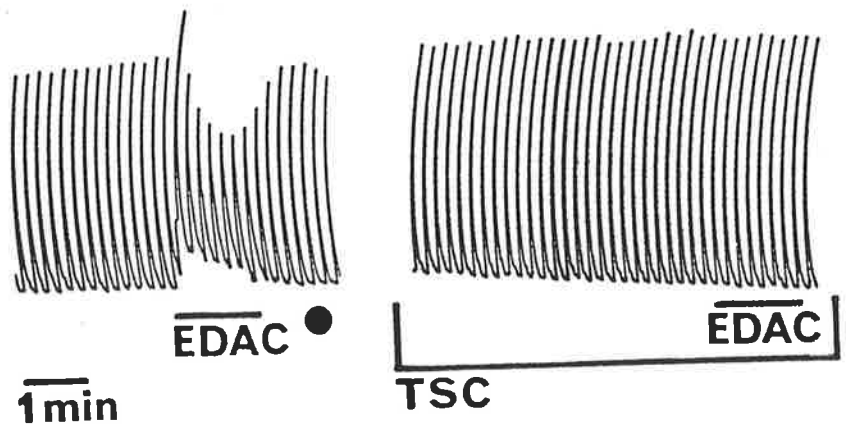


Fig. 4. Abolition of the EDAC (1 mM)-induced response on repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60 V) in the ileum maintained in normal Krebs-bicarbonate medium by TSC (100  $\mu$ M).  $\bullet$  tissue washout. The number of experiments performed was  $n=6$ .

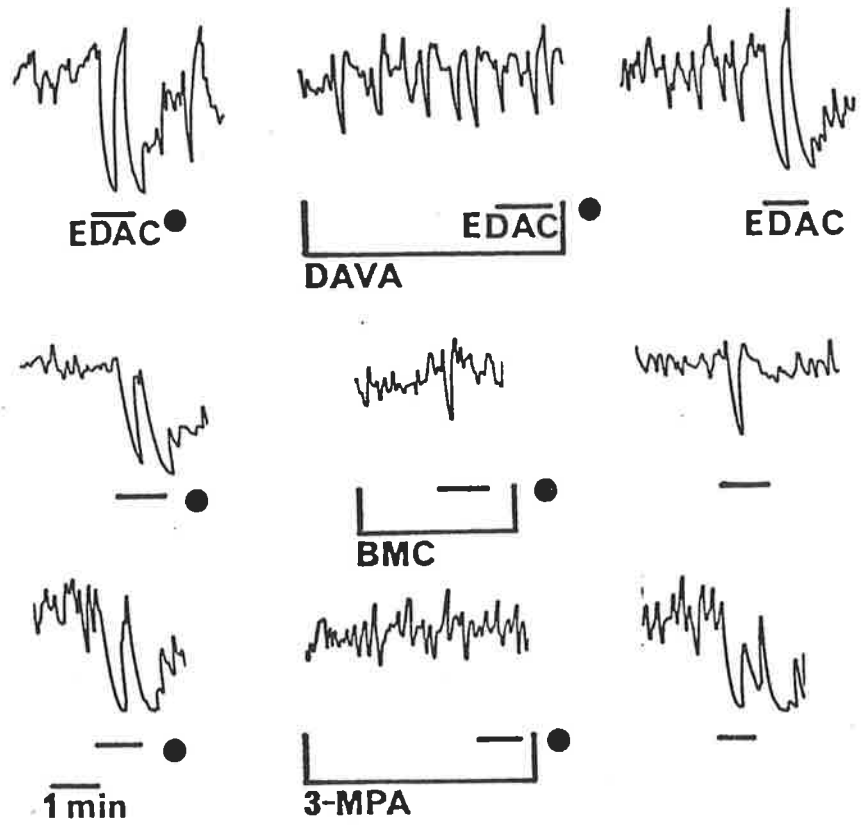


Fig. 5. Effects of DAVA (0.5 mM), BMC (10  $\mu$ M) and 3-MPA (50  $\mu$ M) on EDAC (1 mM)-induced relaxation responses in the isolated distal colon of the guinea-pig maintained in normal Krebs-bicarbonate medium. ● tissue washout and the bars indicate the presence of EDAC in each experiment. The number of experiments performed was at least n=6.

### 3.3.6. EDA and GABA METABOLISM

#### (a) Effects of 3-MPA on EDA-induced responses in guinea-pig intestinal segments.

Both the GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated actions induced by EDA in the ileum were prevented by 3-MPA (Fig. 1). 3-MPA was added in divided doses to a total of 1 mM over 3-5 min, then left in the bath for a further 5 min before responses to EDA, GABA, 3-APS, muscimol or baclofen were elicited. Only the responses to EDA were specifically prevented by 3-MPA (Fig. 1a), its action being dose-dependent with a threshold concentration around 50  $\mu$ M. It was necessary to increase the doses of 3-MPA gradually to a final maximal concentration of 1 mM, since a single application at 1 mM caused non-specific depressive effects on single twitch response to transmural stimulation of the ileum. The inhibitory effects of 3-MPA on EDA-induced responses declined after it had been in the bath for 15-20 min, but could be reinstated by additional doses, which suggests that 3-MPA was in some way rendered inactive in the bath rather than the <sup>s</sup>tissue becoming desensitized to 3-MPA. Both the GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated actions of EDA on the ileum returned after washing 3-MPA from the bath.

Using repetitive twitch responses to transmural electrical stimulation in the ileum, 3-MPA (50-500  $\mu$ M) itself, applied as above, had no effect on the twitch contractions, but prevented, in a dose-dependent manner, the depressive responses to EDA. Although 3-MPA blocked the GABA<sub>B</sub>-receptor-mediated depression of

the twitch contractions induced by EDA (1 mM) (Fig. 1b), it was without affect on the GABA<sub>B</sub>-receptor-mediated inhibition induced by baclofen (Fig. 1c). Similarly, 3-MPA did not affect responses to exogenously applied GABA (Fig. 1), 3-APS or muscimol, or other ileal stimulant substances such <sup>as</sup> 5-HT, ACh or histamine.

In the other intestinal segments such as the jejunum, duodenum and distal colon, 3-MPA (50-500  $\mu$ M) specifically prevented the relaxation responses induced by EDA (1 mM) (Fig. 2), without affecting responses to GABA (0.1 mM) or baclofen (0.1 mM), with a recovery of the responses to EDA after washing out the 3-MPA (0.5 mM). However, in the rat anococcygeus muscle, or the guinea-pig vas deferens, the EDA (1 mM)-induced depression of the twitch contractions was unaffected by 3-MPA (0.5 mM) (Fig. 3).

**(b) Effects of GAD inhibitors on responses induced by EDA in the isolated ileum.**

As shown above, EDA induced a dose-dependent transient contractile response, followed by a prolonged, delayed 'after-relaxation', and this contractile response was cholinergic and neuronal in origin. Their blockade with 3-MPA indicates that these components of the EDA-induced responses resulted from the release of endogenous GABA, such actions being identical to that induced by exogenously applied GABA. These responses to EDA (0.1-5 mM) were unaffected by allylglycine (ALG) when this was directly added to the bath at 50-500  $\mu$ M, but ALG did prevent responses to

EDA (Fig. 4), yet not responses to transmural stimulation of cholinergic neurones or to exogenously applied GABA (50  $\mu$ M), in preparations of ileum taken from guinea-pigs following the onset of convulsions, generally within 6 h of intraperitoneal injection of the ALG (0.4 g/kg). However, ALG, if injected intraperitoneally for 12-24 h before the animal was sacrificed, did not prevent EDA-induced responses, whilst for animals similarly injected intraperitoneally with saline, the EDA-induced responses were always unaffected.

Untreated preparations that responded to EDA were rendered unresponsive by bath application of 2-keto-4-pentenoic acid (2-KPA) (10  $\mu$ M), which is the active metabolite of ALG, the 2-KPA being added at least 30 min prior to testing by the addition of EDA (Fig. 5). There was no recovery of the EDA-induced responses, under 4 h, after washing out the 2-KPA. In the same way, ileal responses to EDA were also abolished when TSC (100  $\mu$ M) was added to the bath for at least 30 min before testing with EDA; Fig. 6 illustrates the abolition of the EDA (0.5 mM)-induced depression of repetitive cholinergic twitch contractions of the ileum as a result of treating the preparation with TSC (100  $\mu$ M) in the bath for 30 min before EDA was again added.

In preparations rendered unresponsive to EDA by application of TSC or 2-KPA, the responses were restored if GABA (50  $\mu$ M) was added to the bath and then washed out before EDA was again added (Fig. 4). This restoration of responses to EDA was also seen when GABA was added to those previously unresponsive preparations

taken from guinea-pigs treated with ALG intraperitoneally (Fig. 4). However, there was no such restoration of responses to EDA, in preparations treated with TSC, 2-KPA, or ALG, if blockers of neuronal and glial GABA-uptake (nif, 100  $\mu$ M; b-ala, 100  $\mu$ M) were added to the bath for 20 min prior to the addition of GABA (50  $\mu$ M) and subsequent testing with EDA. Yet these uptake blockers by themselves potentiated responses to EDA in control preparations not treated with TSC, 2-KPA or ALG (Fig. 7). In preparations where the EDA-induced responses did not consistently recover, even 3-4 h after being pretreated with TSC or 2-KPA, L-glutamine (100  $\mu$ M) was injected into the bath and left for at least 30 min, after which upon washing out, the EDA-induced responses returned (Fig. 8). L-glutamine itself at this concentration elicited a contractile response, sensitive to TTX and atropine, and such responses declined to the baseline within 2-3 min. Application of L-glutamine into the bath did not affect responses to GABA (50  $\mu$ M) (Fig. 8).

None of these agents used as GAD inhibitors (TSC, ALG, or 2-KPA) had any effect on responses to exogenously applied GABA, or on contractile responses to muscimol or 3-APS, neither did they affect the relaxant action of baclofen (50  $\mu$ M) (Fig. 5) or the baclofen-induced depression of twitch responses to transmural electrical stimulation (Fig. 4,5). Likewise, none of these GAD inhibitors had any influence on ileal responses to transmural stimulation, or to 5-HT, ACh or histamine.

Duodenum, jejunum and distal colon:

Preincubation of other intestinal tissues (jejunum, duodenum and distal colon) with TSC (100  $\mu$ M) for up to 60 min, or 2-KPA (10  $\mu$ M) within 30 min, specifically prevented the relaxation responses induced by EDA in these tissues without affecting responses to GABA or baclofen. There was no recovery of the EDA-induced relaxation in the tissues within 3 h of washing out the TSC or 2-KPA, these being essentially irreversible in preventing the GABA-induced activity by EDA. Similarly, although in vitro incubation of ALG for 1-3 h did not influence EDA-induced responses, following intraperitoneal injection of ALG in the animal for up to 6 h, in these tissues taken after convulsions had occurred, there was an abolition of EDA-induced responses, without affecting responses to GABA, baclofen, 3-APS or muscimol. But EDA-induced responses in the isolated intestinal tissues were not affected if the animal was sacrificed 12-24 h after intraperitoneal administration of ALG. In treated preparations where the EDA-induced responses did not recover even after some 4 h, re-loading the tissues with exogenously applied GABA (50  $\mu$ M), or the use of L-glutamine (0.1 mM), rendered the preparations again sensitive to EDA.

**(c) Effects of GABA-T inhibitors on EDA-induced responses in the ileum.**

AOAA (100  $\mu$ M) (Fig.9), EOS (100  $\mu$ M) and gabaculine (GBL) (100  $\mu$ M) (Fig. 10) all potentiated EDA (1 mM)-induced responses without affecting responses to GABA (50  $\mu$ M), baclofen (50  $\mu$ M) or to transmural electrical stimulation (0.1 ms duration), within at

least 30 min. This potentiation persisted despite washing out the irreversible inhibitors of GABA-T such as GBL and AOAA (Fig. 9,10).

**(d) Effect of uptake blockers on EDA-induced responses.**

Neuronal blockers such as ACHC, DABA, and nip, as well as glial uptake blockers such as b-ala, applied for 10-20 min, also potentiated EDA-induced responses in the isolated ileum, in a manner similar to that described for GABA in chapter 4. d-aminolaevulinic acid (d-ALVA) (500  $\mu$ M) potentiated EDA- and GABA-induced responses in the ileum without affecting responses to 3-APS, muscimol or baclofen (Fig. 11), but at a higher concentration, d-ALVA (1 mM) itself induced a contractile response blocked by BMC (10  $\mu$ M).

**(e) Barbiturate-EDA interactions in the ileum.**

Barbiturates such as pentobarbitone, barbitone and thiopentone potentiated the contractile responses induced by EDA without affecting the 'after-relaxation', and these barbiturates also reversed the EDA antagonism by PIC (Fig.12), but not that by BMC, similar to that as described for GABA in chapter 5.



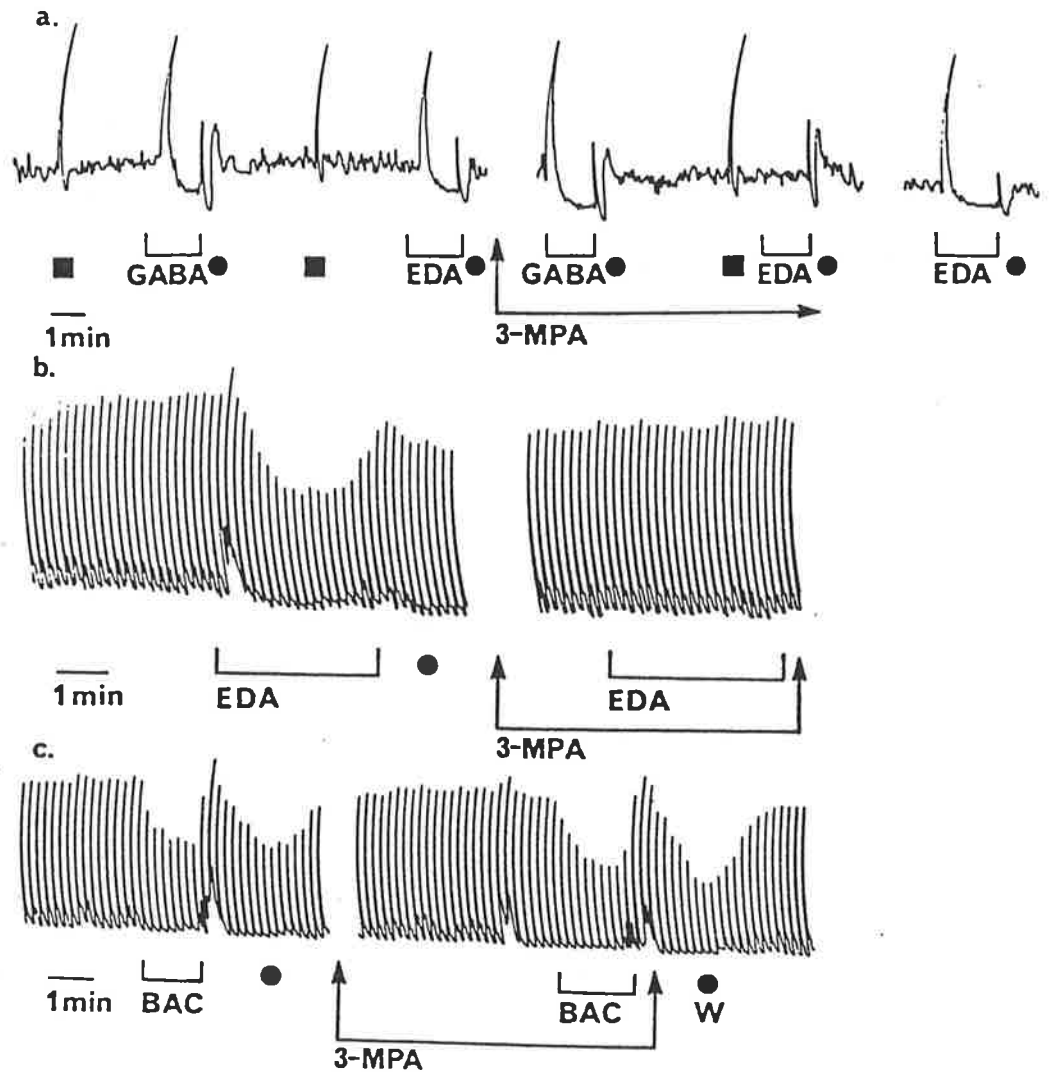


Fig. 1. Effects of 3-mercaptopropionic acid (3-MPA) on responses induced by GABA, baclofen (BAC) and EDA in the isolated ileum. (a) 3-MPA (1 mM) prevented the EDA (1 mM)-induced response without affecting responses to GABA (50 μM) or to transmural electrical stimulation of cholinergic neurones (0.1 ms duration). The number of experiments performed was n=6. (b) Using repetitive twitch contractions (0.1 Hz), 3-MPA (1 mM) prevented the EDA (1 mM)-induced responses, n=5. (c) 3-MPA (1 mM) did not affect the baclofen (50 μM)-induced depression of twitch contractions, n=6. ● tissue washout.

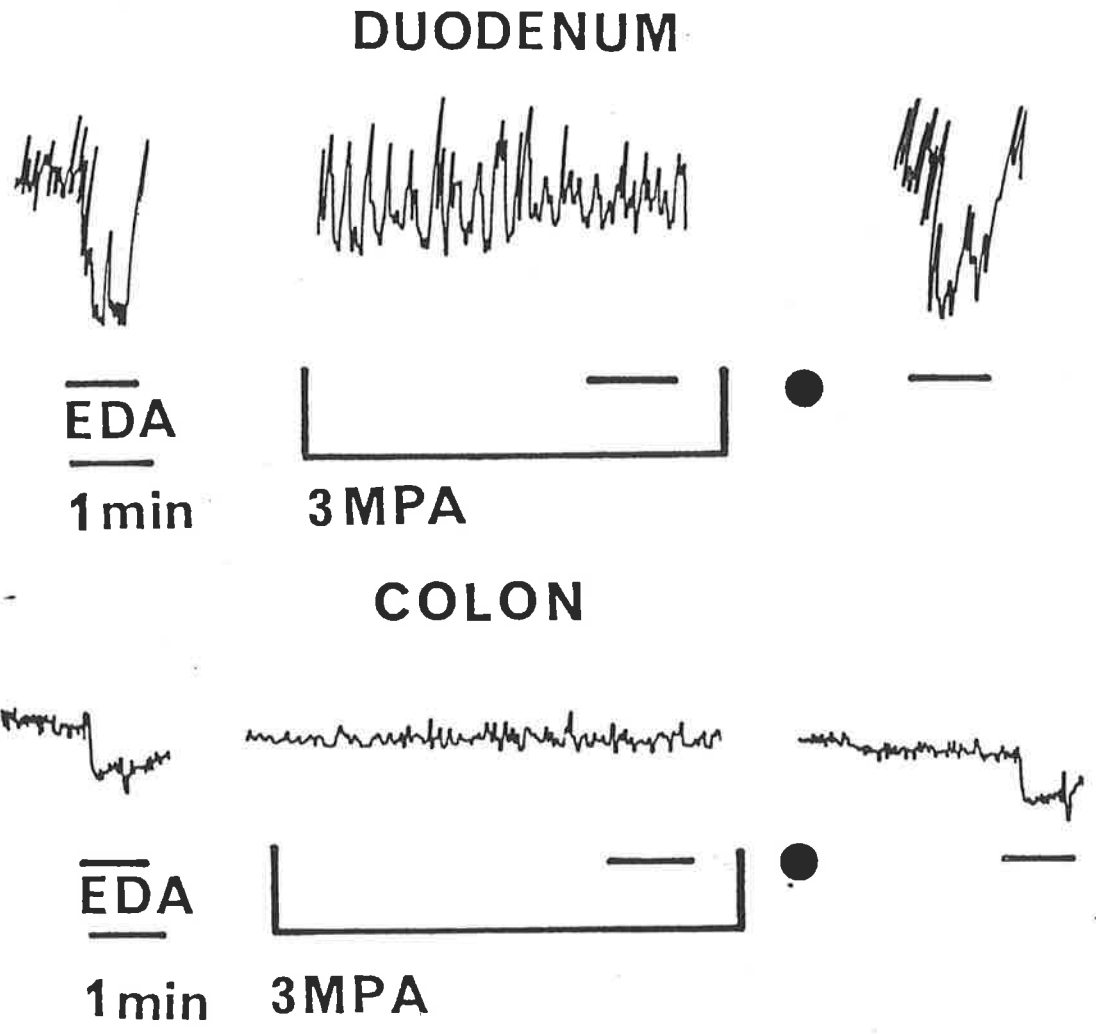


Fig. 2. Prevention of the EDA (1 mM)-induced relaxation in the duodenum and distal colon by 3-MPA (0.5 mM). ● tissue washout. The number of experiments performed was n=6.

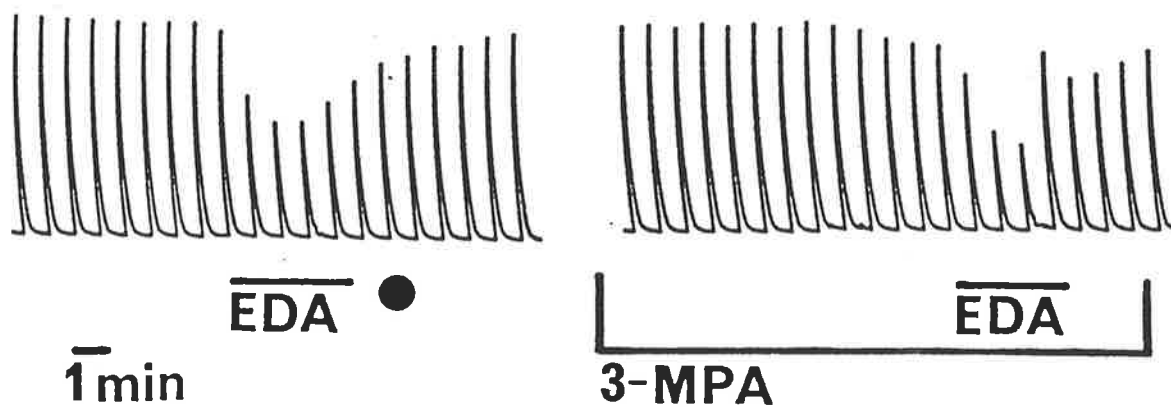


Fig. 3. Lack of effect of 3-MPA (0.5 mM) on EDA (1 mM)-induced depression of repetitive twitch contractions (10 Hz, 1 ms duration, 50 V) in the rat anococcygeus muscle.

## ALLYLGLYCINE (0.4 g/kg i.p.)

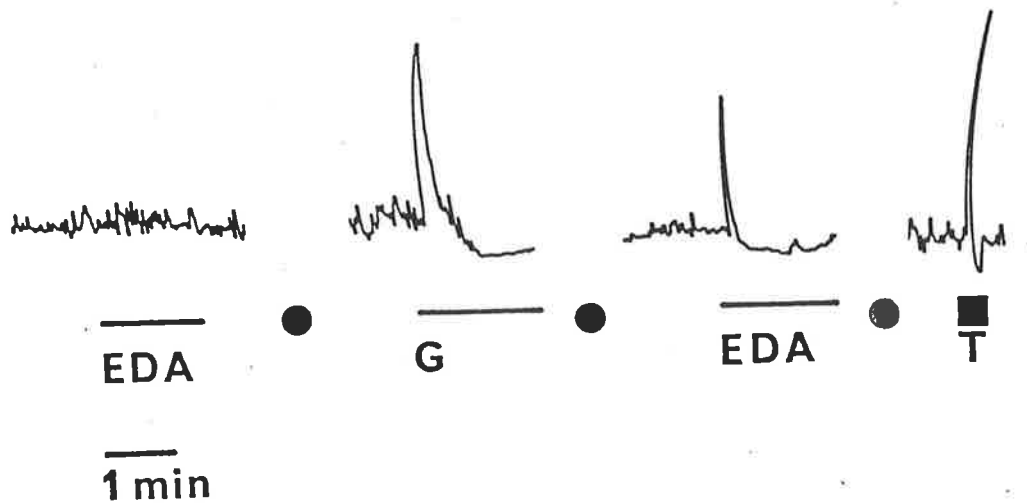
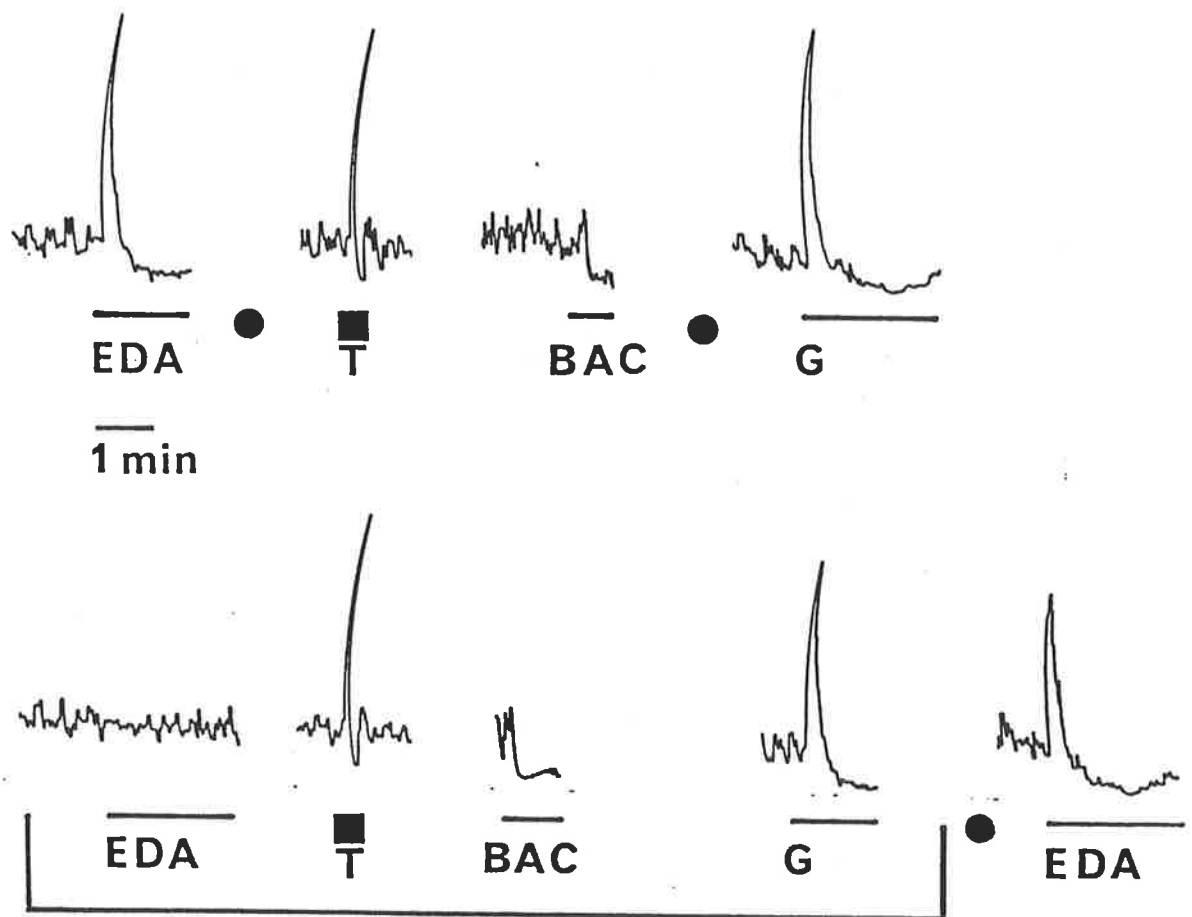


Fig. 4. When guinea-pigs were injected intraperitoneally with allylglycine (0.4 g/kg), the isolated ileum did not respond to EDA (1 mM) but responded to GABA (50 μM). After having injected GABA into the bath, there was a response to EDA (1 mM). Transmural electrical stimulation (■) was unaffected by pretreatment with allylglycine. The number of experiments performed was n=6. ● tissue washout.



### 2-KETO-4-PENT. ACID

Fig. 5. Abolition of the EDA (1 mM)-induced responses by 2-KPA (10 μM), whilst responses to baclofen (50 μM), GABA (G) (50 μM) or to transmural electrical stimulation (T: 0.1 ms duration, 60 V) were not affected by 2-KPA. The number of experiments performed was n=8. ● tissue washout.

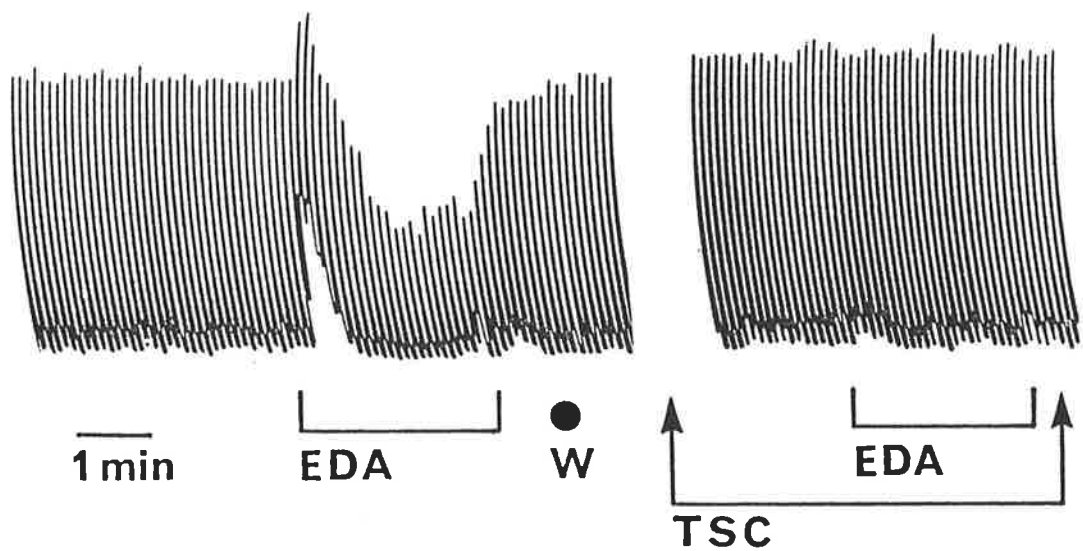


Fig. 6. Abolition of the EDA (0.5 mM)-induced depression of repetitive cholinergic twitch contractions (0.1 Hz, 0.5 ms, 60 V) of the ileum by pretreatment with TSC (100  $\mu$ M). The number of experiments performed was n=6. ● tissue washout.

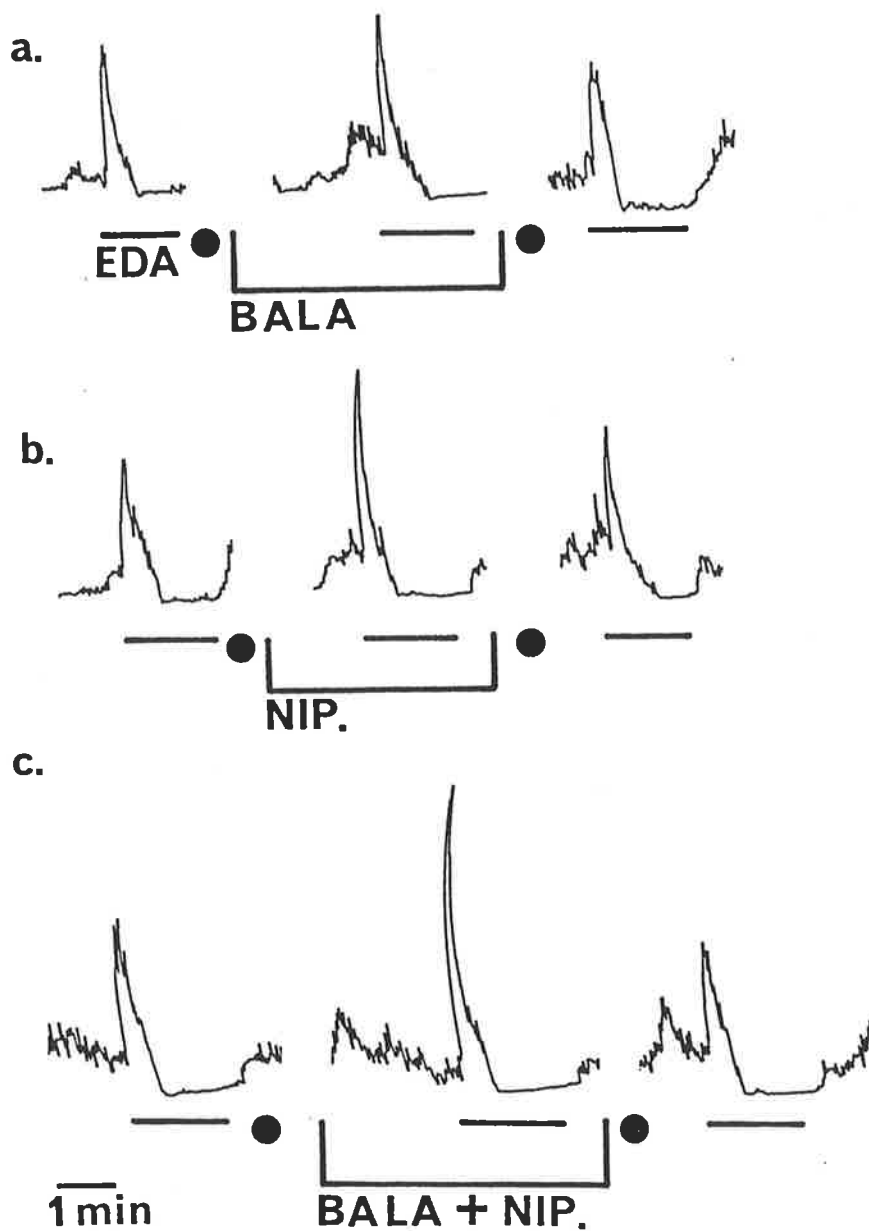


Fig. 7. Potentiation of the EDA (1 mM)-induced responses by b-ALA (100  $\mu$ M), NIP (100  $\mu$ M) and a combination of b-ALA and NIP. The number of experiments performed was  $n=6$ . ● tissue washout.

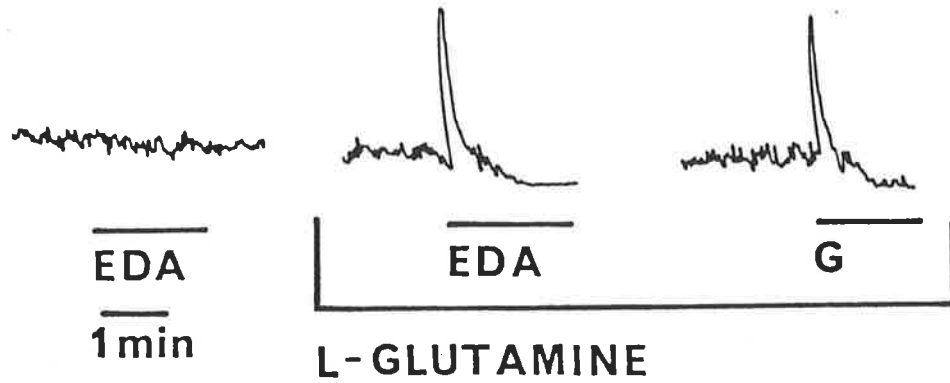


Fig. 8. In ileal preparations which did not respond to EDA (1 mM), upon injection of L-glutamine (100 uM) into the bath for at least 30 min, the tissues responded to EDA (1 mM). L-glutamine did not affect GABA (50 uM)-induced responses. The number of experiments performed was n=6.

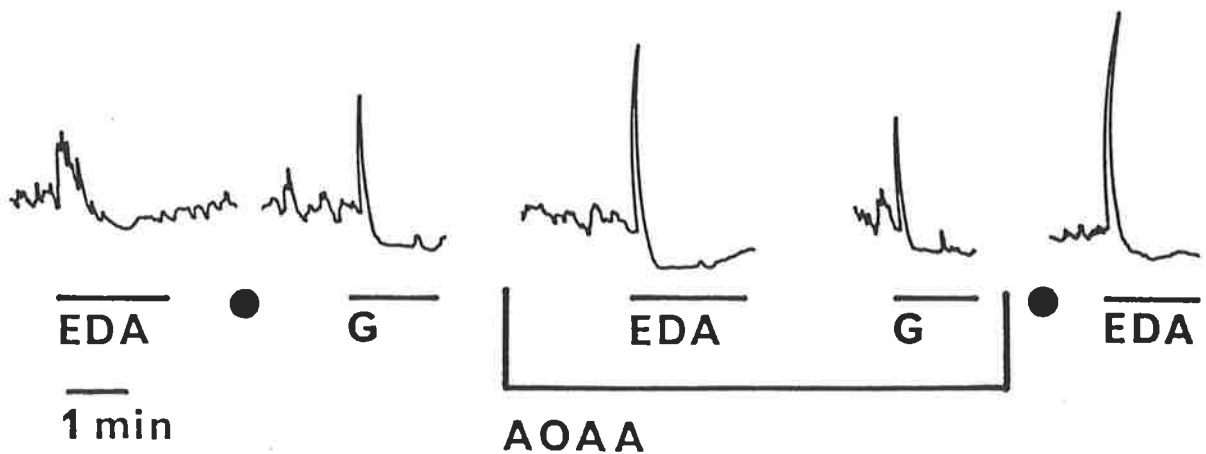


Fig. 9. Potentiation of responses to EDA (1 mM) by AOAA (100 uM). AOAA did not affect responses to GABA (50 uM). The number of experiments performed was n=6. ● tissue washout.



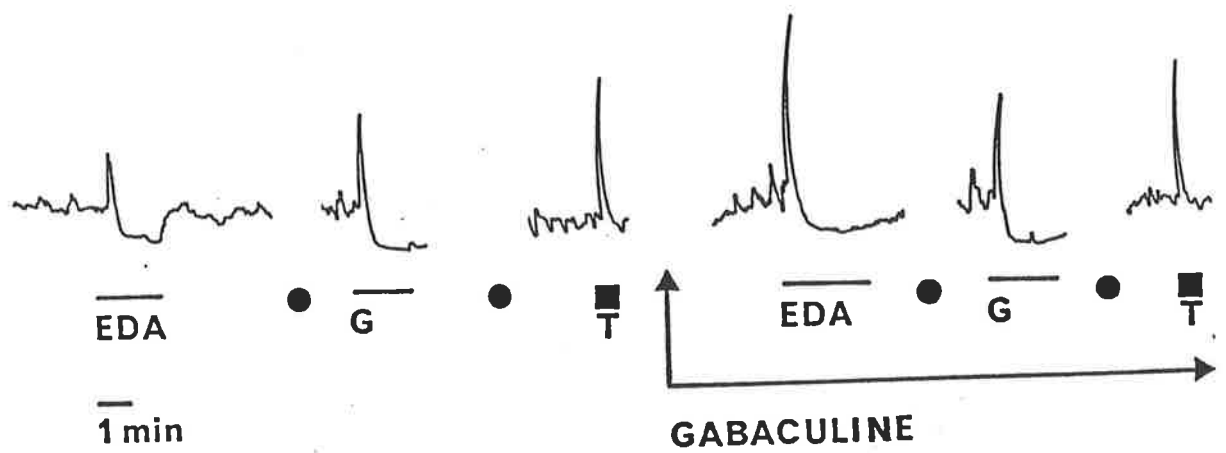


Fig. 10. Gabaculine (100  $\mu$ M) potentiated EDA (1 mM)-induced responses without affecting responses to GABA (G) (50  $\mu$ M) or to transmural electrical stimulation of cholinergic neurones (T: 0.1 ms duration). The number of experiments performed was  $n=6$ . ● tissue washout.

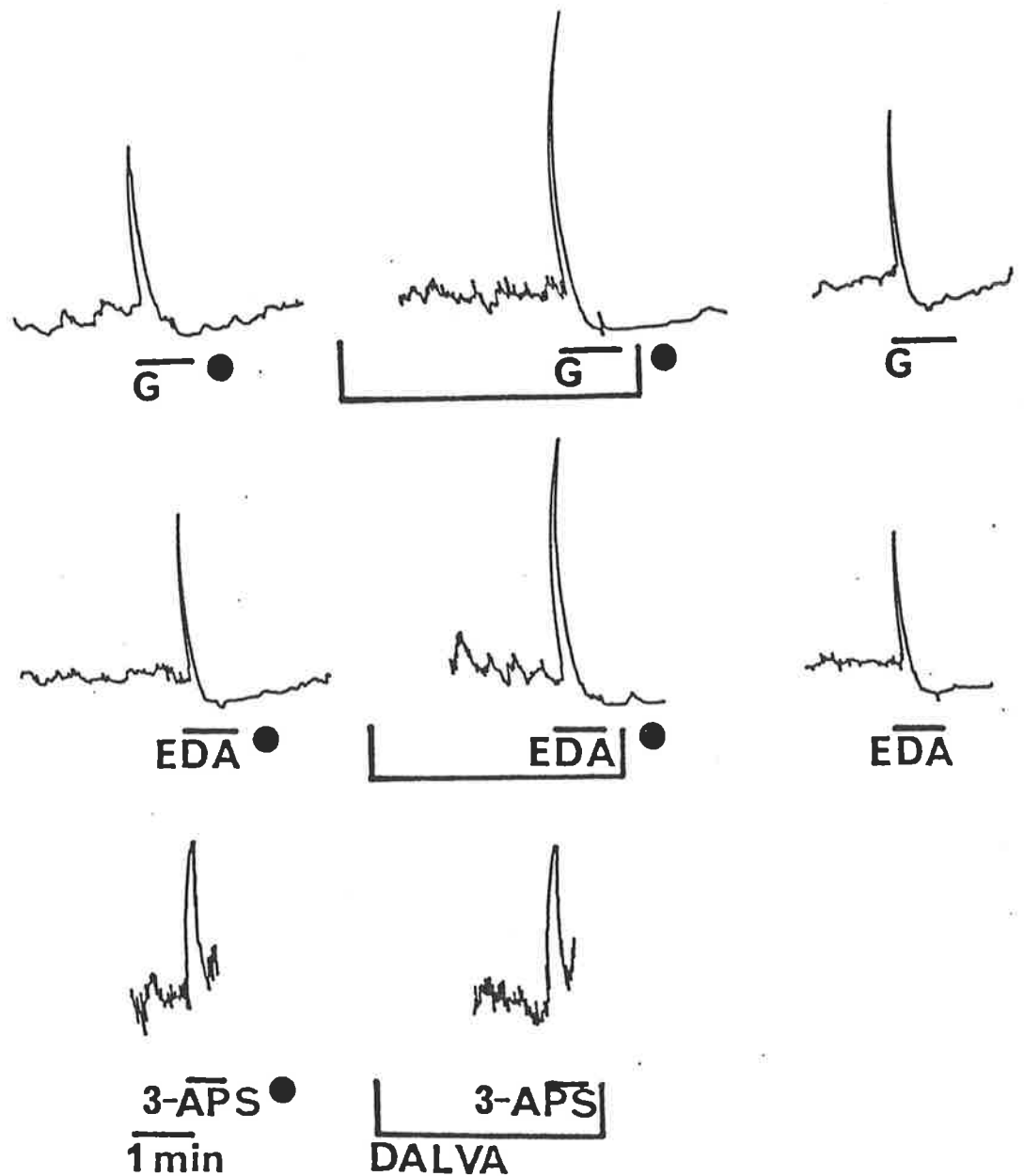


Fig. 11. Potentiation of GABA (G) (50  $\mu$ M)- and EDA (1 mM)-induced responses by d-ALA (0.5 mM) in the isolated ileum. d-ALA (0.5 mM) did not affect responses to 3-APS (10  $\mu$ M). ● tissue washout. The number of experiments performed was at least n=6.

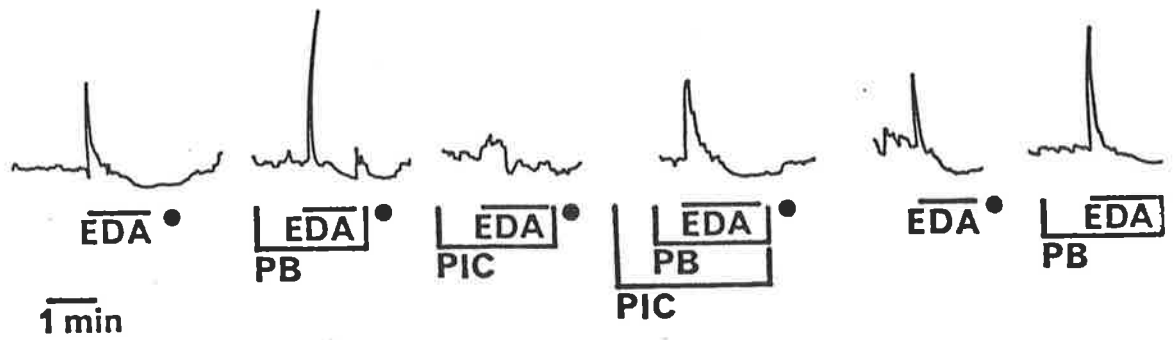


Fig. 12. Potentiation of the EDA (0.5 mM)-induced contractile responses by pentobarbitone (PB) (0.5 mM), and a reversal of the PIC (10  $\mu$ M)-induced antagonism of EDA (0.5 mM)-induced responses by PB (0.5 mM). After tissue washout as indicated by ●, PB again potentiated the EDA-induced contractile responses.

### 3.4. DISCUSSION

From the present results, it can be seen that EDA has the ability to release [3H] GABA at all levels of the guinea-pig intestine, with the observation that the proportion of [3H] GABA release differs along the intestinal tract, a greater release occurring from the distal colon and ileum than from the jejunum or duodenum. This [3H] GABA release was prevented by 3-MPA which was first reported to be a competitive inhibitor of GAD (Lamar, 1970; Karlsson et al., 1974) but later shown to prevent GABA release from brain cortical slices through a mechanism that is rapid in onset (Fan et al., 1981) and is known to possess convulsant properties (Loscher, 1979). In the ileum, the [3H] GABA-release induced by EDA was dose-related and calcium-dependent, in contrast to the calcium-independent, bicuculline-insensitive release observed in striatal slices (Lloyd et al., 1982 a). It is also inferred here that the [3H] GABA release induced by EDA in the intestine would be bicuculline-insensitive, since bicuculline did not affect the total ileal mechanical response to EDA, i.e. bicuculline only antagonised the contractile response without affecting the 'after-relaxation' mediated through the GABA<sub>B</sub>-receptor site.

That differing amounts of [3H] GABA were released by EDA, when loaded in the presence of various specific neuronal uptake blockers such as ACHC, DABA or nip, and of glial uptake blockers such as b-ala (Krogsgaard-Larsen, 1980), suggests that there is a specific uptake of radiolabelled GABA into the respective neuro-

nal and glial compartments, of the myenteric plexus, from which EDA can release GABA, with a greater degree of uptake into the neuronal (75%) than into the glial pool. Such results parallel the pharmacology of GABA in the presence of various GABA uptake blockers described in Chapter 4 of this thesis, showing potentiation of GABA-induced responses.

High affinity [3H] GABA uptake, followed by a TTX-sensitive and calcium-dependent release of [3H] GABA induced by electrical stimulation has been earlier demonstrated from the isolated myenteric plexus of the guinea-pig, where GABA is released mainly from terminals of neurones in the myenteric plexus (Jessen et al., 1983 c; Taniyama et al., 1982 a; 1983 a; Kerr and Krantis, 1983), with no such release of [3H] GABA being detected when nip and b-ala were present during preloading of labelled GABA into the tissues (Kerr and Krantis, 1983). The present results reinforce these previous observations that GABA can be taken up into myenteric neurones of the intestine and can then be subsequently released. The fact that 3-MPA inhibited electrically-induced [3H] GABA in the intestine shown in the present study suggests that the [3H] GABA is indeed released from intrinsic GABAergic neurones.

There are interesting implications in the differing characteristics and pharmacology of the GABA-mimetic responses to GABA, baclofen and other analogues such as 3-APS and muscimol, as well as the responses to the endogenous GABA released by EDA, in the jejunum, duodenum and distal colon, as compared to the ileum.

GABA, baclofen and EDA elicited only prolonged 'relaxation' responses in the former tissues, and the jejunum, duodenum and the distal colon were found to be relatively insensitive to 3-APS and muscimol, whilst in the ileum, GABA, EDA, 3-APS and muscimol all elicited neuronal transient contractile responses of cholinergic origin, sensitive to the GABA<sub>A</sub>-receptor antagonist BMC and the Cl<sup>-</sup>-ionophore blocker PIC, as a result of stimulating GABA<sub>A</sub>-receptor sites (Bowery et al., 1981 a; Kaplita et al., 1982; Giotti et al., 1983a; Ong and Kerr, 1983a,b). When using GABA and EDA, the ileal contractions were followed by a GABA<sub>B</sub>-receptor-mediated 'after-relaxation' evidently due to a depression in transmitter output, this effect being insensitive to both BMC and PIC, but antagonised by DAVA (Muhyaddin et al., 1982 a,b). In the ileum, BMC and DAVA are competitive receptor antagonists at GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites respectively, reflected by the shift of the EDA dose-response curves to the right in a parallel manner. Using EDA as the agonist, the estimated pA<sub>2</sub> values for BMC and DAVA are approximately 5.8 and 3.8 respectively, the latter being estimated from the EDA-induced depression of transmurally elicited ileal cholinergic twitch contractions. This pA<sub>2</sub> value for BMC is similar to that for BMC-antagonism of GABAergic ileal responses earlier reported by Krantis and Kerr (1981 b), and in the rat hippocampus, bicuculline reduces the depolarising responses to EDA and to GABA in the dendrites equally effectively (Blaxter and Cottrell, 1985), whereas Perkins et al. (1981) maintain that responses to EDA are more readily antagonised with bicuculline than are responses to GABA. PIC, on the other hand, is a non-competitive antagonist of EDA-induced ileal contractile

responses, mediated through the GABA<sub>A</sub>-receptor Cl<sup>-</sup>-ionophore complex as with GABA (Olsen, 1981 a). By contrast, the GABA-, baclofen- and EDA-induced relaxation responses in jejunum, duodenum and distal colon of the guinea-pig were predominantly blocked by DAVA and to a smaller extent, by BMC or PIC, suggesting that the relaxations in these tissues were mainly GABA<sub>B</sub>-receptor-mediated effects. However, in the rat duodenum, GABA predominantly induced a non-cholinergic, non-adrenergic relaxation which is BMC- and PIC- sensitive, as a result of stimulating GABA<sub>A</sub>-receptor sites, and 3-APS but not baclofen mimics this effect (Maggi et al., 1984).

In all segments of the intestine, so far, EDA-induced responses have evidently been due to the release of endogenous GABA, rather than any direct GABA-mimetic activation of the GABA receptor sites, as induced by GABA or baclofen, since pre-incubation with metabolic inhibitors of GABA synthesis, such as TSC (Abrahams and Wood, 1970; Collins, 1973) or 2-KPA (Meldrum, 1975), abolished responses to EDA in these tissues without affecting responses to GABA or baclofen. Both TSC and 2-KPA are inhibitors of GAD, the GABA synthesising enzyme, and presumably deplete the GABA content of these tissues, although direct measurement of the altered GABA contents have not yet been made. AOAA (Wood et al., 1978), EOS (Fowler and John, 1972) and GBL (Rando and Bangerter, 1977), on the other hand, specifically potentiated EDA-induced responses by inhibiting GABA-T activity, presumably by elevating the GABA level in the myenteric plexus, in which case more GABA was then released by EDA, resulting in the evident

potentiation of the responses. Also, the abolition of the EDA-induced responses by 3-MPA, within a short period (10 min) in the different segments, suggests that 3-MPA prevented EDA from releasing endogenous GABA in the intestine rather acting than as a competitive GAD inhibitor as proposed by Lamar (1970). In keeping with this, 3-MPA prevented the GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated responses induced by EDA, without affecting responses to GABA, 3-APS, muscimol, baclofen or transmural stimulation of cholinergic neurones. This supports previous studies that GABA-mediated responses in the spinal cord are not selectively depressed by 3-MPA (Davies et al., 1983 b). All of this suggests that EDA acts in the intestine, by releasing endogenous GABA rather than by acting as a GABA-mimetic, as has been reported by Perkin et al. (1981), and is in contrast to results reported by Bokisch et al. (1982) who found that neither 3-MPA nor any of the metabolic inhibitors of GABA affect responses to EDA in the superior cervical ganglion. An explanation of this contradiction could be that the latter system may not contain any significant GABAergic innervation so that the EDA actions reported there might instead be the result of EDA interacting with GABA-receptor sites rather than through a release of endogenous GABA. However, in hippocampal slices, EDA appears to act by releasing endogenous GABA rather than as a GABA-mimetic (Blaxter and Cottrell, 1985).

Although EDA is described as being almost equipotent with GABA in depressing neuronal firing, and is said to be more sensitive than GABA to antagonism by bicuculline in brain cortical slices (Perkins et al., 1981), in contrast, it was less



potent (approximately 100 fold) than GABA in the guinea-pig ileum, and was antagonised with a  $pA_2$  value identical to that found for exogenous GABA, the latter identity also argues against a direct agonist effect of EDA in the ileum. The GABA released by EDA must have been of endogenous origin since particular care was taken not to pre-expose the intestine to any exogenous GABA before testing the effects of EDA on the isolated intestinal preparations.

The apparent lack of contractile response to EDA, GABA, 3-APS, or muscimol, in the jejunum, duodenum and distal colon, where the GABA-receptor-mediated responses were primarily GABA<sub>B</sub>-receptor-mediated relaxations (antagonised by DAVA rather than BMC and mimicked by baclofen), however, indicates that such relaxation responses could possibly be due to a prejunctional action of GABA in depressing transmitter output from neurones that directly innervate the muscle and are responsible for its resting tone; whereas the small chloride-dependent, BMC-sensitive component of the relaxation response that persisted in the presence of atropine could be the result of a stimulatory action of GABA on the non-cholinergic, non-adrenergic (NANC) neurones, which then releases some unknown inhibitory transmitter to relax the muscle (Krantis et al., 1980).

These results shown in the present study highlight two important findings: firstly, the presence of GABA at all levels in the guinea-pig intestinal tract is substantiated by the evidence that EDA released endogenous GABA and hence induced

typical GABAergic responses; and secondly, the GABAergic-relaxation responses in the jejunum, duodenum and distal colon as compared to that in the ileum (contraction followed by an 'after-relaxation') may reflect a different organisation of the GABAergic innervation in these tissues, leading to the speculation that perhaps, the functional role of GABA in these segments may be more intricate and complex than at first seems, beyond the simple proposition that GABA has a modulatory role in intestinal motility (Ong and Kerr, 1983a; 1984a; Kerr and Ong, 1985; 1985 (in press)).

This investigation has also provided further evidence that GABA is present in the enteric nervous system, by testing the effects of various inhibitors of GABA metabolism on EDA-induced responses, where EDA has been shown to release endogenous GABA from the guinea-pig intestine. Since none of these inhibitors affect any intestinal responses to GABA or its analogues, nor those to a variety of other stimuli, it can be concluded that the observed alterations in EDA-induced responses were due to changed GABA levels in the intestine resulting from the actions of the various inhibitors of the GABA synthesising (GAD) and degrading enzymes (GABA-T), analogous to their actions on GABA metabolism and GABA levels in the CNS (Roberts, 1974; Roberts et al., 1976; Meldrum, 1975; 1982; 1985). But biochemical assays measuring existing GABA levels in the intestinal tissues after pre-treatment with the metabolic inhibitors have yet to be performed. ALG was only effective in making the tissues completely insensitive to exogenously applied EDA when ALG was given in vivo, within 6 h

but not after 12-24 h, and then watching for convulsions to develop before taking the ileum and testing for EDA-induced responses. But its metabolite 2-KPA, which is an active inhibitor of GAD, did inhibit EDA-induced ileal responses within 20 min when added to the bath. The results with both these agents are consistent with the notion that they eventually lower myenteric GABA levels sufficiently to prevent any EDA-induced responses in the ileum due to the release of endogenous GABA. Moreover, when GABA was applied to such depleted, unresponsive preparations, they would once more respond to EDA, results again consistent with the ability of EDA to release GABA from the myenteric plexus since it is likely that such exogenously applied GABA would be taken up into the depleted stores from whence it could be released by EDA.

There is still some controversy over the GABA-releasing action of EDA, with the latest view that it acts as a GABA-mimetic rather than as a GABA-releasing agent (Stone and Perkins, 1984), based on the observation that iontophoretic application of EDA is far more effective than GABA in depressing pallidal neuronal firing, and the apparent greater effectiveness of bicuculline in blocking EDA-induced responses as against GABA-induced responses (Perkins and Stone, 1982); all of which suggest that EDA is acting directly on the postsynaptic GABA receptor sites. Also, in the rat isolated superior cervical ganglion, a preparation which does not appear to contain GABA, the responses to both GABA and EDA are not changed by prolonged incubation with GAD inhibitors such as 3-MPA and TSC (Bokisch et al., 1982). However, to

the contrary, Blaxter and Cottrell (1985) suggest that EDA acts in the rat hippocamp<sup>U</sup>as by releasing GABA, rather than as a direct agonist at GABA receptor sites. Moreover, the present study shows that in isolated intestinal preparations, EDA releases both tritiated and endogenous GABA, and that such actions of EDA are inhibited by 3-MPA, known to act by preventing GABA release (Fan et al., 1981) rather than by inactivating GAD function as previously reported (Lamar, 1970; Karlsson et al., 1974; Rodriguez de Lores Arnaiz et al., 1972; 1973; Lingren, 1983; Roberts et al., 1978), since 3-MPA has a rapid action in the present study. Lloyd et al. (1982 a) showed that EDA could release endogenous GABA from brain slices, and hence could possess indirect neuronal depressant effects acting through such release, which prompted the present investigation.

In the isolated ileum of the guinea-pig, EDA induced a transient cholinergic contraction, sensitive to TTX, followed by a prolonged delayed 'after-relaxation', characteristics shared by exogenously applied GABA. The contraction is a chloride-dependent mechanism, sensitive to BMC and PIC and due to activation of GABA<sub>A</sub>-receptor sites, whilst the 'after-relaxation' is chloride-independent and sensitive to DAVA. All these effects of EDA are also prevented by 3-MPA without influencing the actions of GABA or its analogues in the ileum. Thus it is most likely that EDA releases endogenous GABA, as well as preloaded [3H] GABA, from the myenteric plexus, the released GABA then acting at GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites in the intestine (Bowery et al., 1981 a; Ong and Kerr, 1983a,b; Kerr and Ong, 1984a). The present results

reinforce this conclusion since inhibitors of GAD, known to deplete GABA levels in neural tissue, also eliminated ileal responses to EDA without affecting those to GABA or its analogues.

Further evidence that EDA acts in the intestine by releasing GABA is provided by the modification of EDA-induced responses following incubation of the ileal tissues with inhibitors of GABA-T that are known to raise GABA levels in neuronal tissues. Here, GBL (Rando and Bangerter, 1977; Rando, 1979; Mutsu and Deguchi, 1977; Wood et al., 1981; 1982), EOS (Anlezark et al., 1976) and AOAA (Kuriyama et al., 1966 b; Loscher and Frey, 1978; Wallach, 1961; van Gelder, 1966; Walters et al., 1978), all significantly potentiated EDA-induced responses without affecting responses to GABA or any of its analogues, or to transmural stimulation. Such potentiation was essentially irreversible since the EDA-induced responses remained enhanced throughout the experiment, even after a number of tissue washouts, which suggests that inhibition of GABA-T potentiated the EDA-induced, GABA-mediated ileal contraction and 'after-relaxation' by virtue of increasing the myenteric plexus GABA content, with the consequence that EDA was able to release more GABA. Moreover, GABA released in the myenteric plexus has to be retaken up into neurones or into glial cells before further metabolism, so that inhibition of the uptake process should also potentiate the actions of GABA released by EDA. Indeed, short-term prevention of GABA uptake, using neuronal and glial uptake blockers such as nip or b-ala (Krogsgaard-Larsen, 1980; Krogsgaard-Larsen et al., 1981 a) potentiated EDA-induced responses in the intestine (see Chapter 4 of this thesis), presumably as a result of prolonging the actions of endogenous GABA released by EDA. As a corollary of this, following the abolition of responses to EDA through inhibition of GAD, exogenously applied GABA no longer restored

responses to EDA if the tissues were incubated with these blockers of neuronal and glial GABA-uptake, prior to this application of GABA. This was in marked contrast to the restoration of EDA-induced responses by the simple application of GABA in depleted tissues, alluded to the above, and indicates that these uptake blockers prevented GABA from being reloaded into depleted GABAergic neurones of the myenteric plexus.

Estimation of the GABA content of the myenteric plexus following depletion, enrichment, and reloading of GABA in the myenteric plexus, as described here, would greatly strengthen the conclusion that the present results are to be explained in terms of altered GABA release under the various conditions employed. Nevertheless, the present results are entirely consistent with the notion that GABA is present in the intestine of the guinea-pig, as has been demonstrated by previous authors (Jessen et al., 1979; Kerr and Krantis, 1983; Miki et al., 1983; Ottersen, personal communication; Taniyama et al., 1982 a,b,c; 1983 a; Jessen et al., 1983 c; Tanaka and Taniyama, 1985).

In the substantia nigra and the pallido-entopenduncular nuclei in the cat, [3H] GABA synthesised from [3H] glutamine, has been shown to be released from GABA nerve terminals but not glial cells in vivo (Besson et al., 1981), as the precursor [3H] glutamine preferentially labelled the small glutamate precursor pool for GABA in nerve terminals (Shank and Aprison, 1977). In the ileum, the observation that some previously unresponsive preparations could respond to EDA upon incubating the tissues with L-

glutamine, suggests that L-glutamine, through the action of glutaminase, could boost glutamate and thus GABA pools in the enteric neurones; this newly synthesised GABA thereafter could be released by exogenous EDA, just as reloading with GABA in the bath enabled the EDA to release more endogenous GABA, after GABA itself has been taken up into the neurones in the myenteric plexus depleted of GABA by GAD inhibitors.

High concentrations of d-aminolevulinic acid (d-ALVA; at least 100  $\mu$ M) inhibited GABA and glutamate uptake and stimulated basal efflux of the amino acids in purified nerve endings, and at low concentrations (1  $\mu$ M), it inhibited the stimulated release of GABA from nerve endings probably by acting as an agonist at GABA autoreceptors (Brennan and Cantrill, 1979a,b; 1981). In another study, d-ALVA (1 mM) caused a slight inhibition of [U-14C]-GABA uptake into neurones and glia (Percy et al., 1981). D-ALVA was also shown to mimic GABA actions by increasing chloride conductance in the crayfish stretch receptor neurone (Dichter et al., 1977), hyperpolarising motoneurones and depolarising primary afferents in a manner similar to that of GABA (Nicoll, 1976). In the guinea-pig ileum, d-ALVA is a weak GABA<sub>A</sub>-receptor agonist, as it induced a contractile response at very high concentrations (> 0.5 mM), an effect which was blocked by BMC and PIC. However, it appears that d-ALVA inhibits GABA uptake, rather than releasing GABA, in the guinea-pig intestine, since it potentiated GABAergic responses induced by either EDA or GABA but not 3-APS, muscimol or baclofen at 0.5 mM, and this agrees with an earlier report that d-ALVA inhibits [3H] GABA uptake in a linearly competitive



fashion with maximal inhibitions at 0.5 mM (Brennan and Cantrill, 1979 b). d-ALVA never induced [3H] GABA release in the ileum, even at high concentrations (1-5 mM) which significantly stimulated [3H] GABA release in rat brain synaptosomes (Brennan and Cantrill, 1979 b).

Barbiturate potentiation of GABA<sub>A</sub>-receptor-mediated, EDA-induced contractile responses in the ileum was similar to the barbiturate enhancement of GABAergic responses elicited by GABA and its analogues described in Chapter 5 of this thesis. In keeping with this, PIC-induced antagonism of the EDA-induced responses was reversed by barbiturates, suggesting that this action of EDA is mediated through the release of endogenous GABA by EDA, the GABA then interacts with the GABA<sub>A</sub>-receptor at the chloride-ionophore complex (Olsen, 1981 a).

Among the earliest observations on EDA actions in the CNS was the observation that EDA releases labelled GABA from pre-loaded brain slices (Lloyd et al., 1982 a; Davies et al., 1983 a), yet almost all subsequent studies on EDA neuronal actions have stressed the notion that EDA acts directly on GABA receptors, with the implication that there is some critical spacing of the amine groups in EDA that bridges a pair of GABA<sub>A</sub>-receptor sites to activate two adjoining receptor complexes (Bokisch et al., 1984). To an extent, this problem still remains unresolved since the rat superior cervical ganglion is the only preparation, other than the intestine, where the effects of GABA metabolic inhibitors have been tested on EDA-induced responses (Bokisch et

al., 1982), but, unfortunately, these ganglia are apparently free of GABAergic innervation so that such metabolic inhibitors could not be expected to influence any EDA-induced responses in this tissue. In fact, EDA is markedly less active in the ganglion than in the CNS where EDA and GABA are near equipotent (Curtis and Malik, 1984), but obviously EDA does possess some GABA-mimetic properties in its own right. Nevertheless, in the intestine, EDA only induces GABA-mimetic responses by virtue of releasing GABA, as can be seen from the modification of EDA-induced responses by altering GABA metabolism through inhibition of its synthesizing and degrading enzymes (Kerr and Ong, 1984 c; Ong and Kerr, 1984b). There is no explanation, so far, for this difference in the sensitivity of these preparations to EDA, or in the mechanism whereby it acts. In addition, it is also apparent that EDA very likely is converted to the monocarbamate to exert its actions; indeed, (Hill, 1985 b) found EDA to be inactive in several preparations if bicarbonate ions are absent from the solution. In practice, EDA readily forms its monocarbamate (EDAC) in the presence of bicarbonate ions or more probably carbon dioxide which would be in equilibrium with bicarbonate in the bathing solution.

In the present experiments, EDAC was also shown to be the form in which EDA acts to release GABA, or [3H] GABA, from the myenteric plexus, in that synthetic EDAC still released GABA and evoked GABA-mimetic responses in the total absence of added bicarbonate or carbon dioxide. Alternatively, 2-5 mM of  $\text{HCO}_3^-/\text{CO}_2$  buffer could be added to the perfusion bath in the presence of

EDA, when such responses were again induced, or EDA could be treated, as by Curtis and Malik (1984), with  $\text{CO}_2$  in concentrated stock solution, where subsequent bath application of this treated EDA would contain < 1% of the physiological concentration of bicarbonate. All of this further indicates that EDA is converted to EDAC in order to exert GABA-mimetic actions in the intestine, rather than these originating from EDA itself, but does not require the presence of physiological concentrations of bicarbonate ions as Hill (1985 b) found elsewhere. Nevertheless, it is to be stressed that so far, apart from the report by Curtis and Malik (1984), this dependence on conversion to EDAC, for EDA to act, applies only in the intestine, and is related to GABA release by EDA rather than to any direct actions of EDA at GABA receptor sites.

In conclusion, EDA appears to act by releasing GABA through a yet unknown mechanism in the myenteric plexus of the guinea-pig intestine, rather than by acting as a GABA-mimetic as proposed by Stone and Perkins (1984). Furthermore, the active releasing agent appears to be the monocarbamate of ethylenediamine (EDAC) rather than EDA itself.

CHAPTER FOUR. EFFECTS OF UPTAKE BLOCKERS  
ON GABAERGIC ACTIONS.

#### 4.1. INTRODUCTION

Following neural release of a transmitter, the postsynaptic action of the transmitter is terminated by three possible processes, namely, (a) diffusion from the synaptic region, and subsequently transported by blood or taken up into extraneuronal tissues such as glia (b) exo-enzymatic destruction within the synaptic region or inactivation within the nerve terminal in which the transmitter was originally stored (c) uptake into pre- and postsynaptic elements, where in the former, the transmitter may be re-incorporated into the releasable pool, or may be catabolised as in the latter. Since a number of known transmitters often induce only transient effects on the postsynaptic receptor sites, efficient means of removal are required not only to ensure that the concentration of the synaptically-released transmitters in the synaptic cleft and the extracellular space remains relatively low, but also to recover the transmitters for re-use.

In general, it is commonly accepted that GABA is disposed of by specialised carrier-mediated active uptake into both neurones and glial cells, through high and low affinity transport mechanisms (Henn and Hamberger, 1971; Martin, 1976; Krosggaard-Larsen, 1980; Iversen and Kelly, 1975). GABA is then inactivated by the degrading enzyme, GABA-transaminase (Iversen and Kelly, 1975). It has been known for some time that mammalian brain slices, and homogenates containing synaptosomes, actively accumulate exogenous GABA from the extracellular space or external medium (Elliott and Van Gelder, 1958; Sano and Roberts, 1963;

Iversen and Bloom, 1972; Martin, 1973; Beart and Johnston, 1973; Iversen and Neal, 1968). Rat cerebral cortical slices rapidly accumulate [3H] GABA from a low concentration of the labelled amino-acid, giving rise to tissue-medium ratios as high as 100 to 1 (see Fig. 4-4 Iversen, 1972), in a temperature-dependent manner which requires the presence of sodium ions in the external medium. The rates of GABA uptake in various rat brain regions, as described by saturation kinetics, have an apparent  $K_m$  value of approximately 20  $\mu M$  (Bond, 1973; Iversen and Johnston, 1971; Iversen and Neal, 1968), and  $V_{max}$  0.116  $\mu moles/g/min$  (Iversen and Neal, 1968). The accumulation of GABA by the high affinity transport systems in brain preparations is said to reflect a net uptake, rather than a homo-exchange process (Iversen and Kelly, 1975). Although there has been some debate as to whether GABA uptake represents only net uptake or whether it includes exchange with endogenous GABA, high intracellular sodium levels appear to encourage a high GABA efflux rate and hence GABA exchange in synaptosomal preparations (Lake and Voaden, 1976), whilst net uptake of GABA appears to be mediated by high affinity transport in normal physiological conditions (Ryan and Roskoski, 1977).

From studies of labelled GABA uptake in different regions of the brain, it is possible to localize GABAergic (GABA containing and GABA releasing) neurones in the CNS, through various biochemical analyses demonstrating the accumulation of high amounts of radioactive GABA in the brain, particularly in nerve terminals and preterminal axons which are the primary storage sites for GABA (see review by Oja et al., 1977).

Autoradiographic studies have revealed specific GABA uptake sites in the CNS, both in vitro and in vivo (Hokfelt and Ljungdahl, 1972; Hosli and Hosli, 1976 a; Schon and Iversen, 1972; Iversen and Kelly, 1975), more particularly so in cerebellar and cortical cell cultures (Hosli and Hosli, 1976 a; Yu and Hertz, 1982), showing a number of neurones and glial cells that accumulate GABA, with a great proportion of these neurones being selectively GABAergic in nature (Storm-Mathisen, 1976; Yu and Hertz, 1982). Tissue culture is considered a valuable technique for identifying and localizing specific uptake sites on a cellular and ultrastructural level, the reasons being that the diffusion of the isotopes in the cultures is relatively rapid, therefore allowing short incubation times so as to avoid degradation of labelled substances during the uptake process (Balcar and Johnston, 1973; Schon and Kelly, 1974; Hosli and Hosli, 1976 b), and allowing a better preservation of the various cell types in the culture medium (Hokfelt and Ljungdahl, 1972; Hosli et al., 1972). Recent studies have shown that purified rat Schwann cells maintained in culture retain their ability to take up GABA by a high affinity uptake mechanism (Gavrilovic et al., 1984). There is a dense distribution of silver grains over the nucleus and cytoplasm in developing Purkinje cells in the rat embryo after being labelled with [3H] GABA in vivo, examined by means of light and electron microscopic autoradiography (Atoji, 1983). It is now well established that GABA uptake is not restricted to neurones, but also occurs in glial cells of the nervous system. This is supported by localization of radioactive GABA, administered in vivo, in glia (Schon and Kelly, 1974), as well as by the

demonstration of a high affinity GABA uptake system in many glial cell preparations (Henn and Hamberger, 1971; Henn, 1976; Hertz and Schousboe, 1975).

Although both neurones and glial cells have the ability to accumulate GABA, there are a number of differences which may distinguish GABA uptake of glia from that into nerve terminals, based on kinetic studies. For instance, the rate of GABA uptake into glial cells is much slower than that reported for nerve terminals (see review by Kelly et al., 1976), and in addition, a wide array of GABA analogues, and other substrates, has been designed and tested for inhibition of labelled GABA uptake, since the uptake processes also differ in their chemical specificity for the GABA analogues (Schousboe, 1981). L-2,4-diaminobutyric acid (DABA) appears to be specifically taken up into nerve terminals but not into glial cells (Simon and Martin, 1973), whereas b-alanine (b-ala) is a more selective substrate of the glial uptake system (Iversen and Kelly, 1975; Schon and Kelly, 1974). It is also realised that DABA is one of the most potent blockers of GABA uptake into nerve terminals (Simon and Martin, 1973; Iversen and Johnston, 1971), and has almost no effect on glial cell uptake in tissues such as the rat sensory (Schon and Kelly, 1974) and sympathetic ganglia (Bowery and Brown, 1972), which rapidly accumulates b-ala. In contrast to b-ala, which has very weak activity on the nerve terminal uptake system, DABA is accumulated very rapidly into cortical slices representative of neuronal elements (Dick and Kelly, 1975).



Studies of the effects of b-ala and DABA on GABA uptake and release in brain slices, synaptosomes, and glial cells (Iversen and Johnston, 1971; Simon and Martin, 1973; Simon et al., 1974; Minchin, 1975; Schon and Kelly, 1974; 1975; Bowery et al., 1976 a,b; Brennan and Cantrill, 1978; Iversen and Kelly, 1975; Weitsch-Dick et al., 1978) have also led to the conclusion that b-ala preferentially inhibits glial GABA uptake, whereas DABA interferes primarily with neuronal or presynaptic transport processes; Schousboe and coworkers have since confirmed these results in primary cultures (Schousboe et al., 1978; 1979; Schousboe, 1979 a,b). Like DABA, nipecotic acid (nip) is a substrate-competitive inhibitor of neuronal GABA uptake (Johnston et al., 1976 a,b), and by interacting with the neuronal transport system in a similar way to that for GABA, it may replace GABA and may itself be transported into neurones with a higher efficiency than GABA (Johnston et al., 1976 a,b). On the other hand, some studies have also shown that nip and its derivatives are also capable of inhibiting GABA uptake into glial cells (Schousboe, 1981). Another conformationally restricted analogue of GABA, cis-3-amino-cyclohexanecarboxylic acid (ACHC), is known to be a specific competitive inhibitor of GABA uptake into neurones (Beart et al., 1972), and appears to be more selective for neuronal GABA uptake, compared to glial, than are either DABA or nip alone (Johnston and Stephanson, 1976; Bowery et al., 1976 a; Neal and Bowery, 1977). A report on the interactions between conformationally restricted GABA analogues and the astroglial GABA carrier, and a review of the different GABA analogues on GABA uptake in both cultured astrocytes and brain cortical slices

has been presented by Schousboe et al. (1980; 1981 a,b).

Substances that specifically block the binding of GABA to transport sites and the inactivating system, yet that do not possess any GABA-mimetic or antagonistic properties at GABA-receptor sites, might be effective enhancers of GABA actions at synapses where GABA is normally liberated. Among the known compounds, (-) nip and DABA selectively enhance or prolong the effects of GABA on neurones in vivo, without influencing the time course of synaptic action at GABAergic synapses for which GABA is thought most likely to be the transmitter (Curtis et al., 1976). Both nip and DABA potentiate the actions of electrophoretically administered GABA on cat spinal cord, cerebellar and cerebral neurones (Curtis et al., 1976). More recent studies have shown that the inhibitory action of synaptically released GABA is also enhanced in the presence of various GABA uptake blockers (Matthews et al., 1982; Korn and Dingledine, 1983; Krnjevic, 1984), and these induce a marked increase in the effectiveness and duration of action of exogenously applied GABA (Johnston, 1976; Krosgaard-Larsen, 1980; Krnjevic, 1984). By contrast, a recent finding by Desarmenien et al. (1980) indicates that inhibition of glial GABA uptake by b-ala in the dorsal root ganglion does not affect the amplitude or time course of responses to GABA when the neurones under study are close to the source of GABA, presumably due to the insensitivity of the glial uptake mechanisms in this particular model.

In the enteric nervous system, high affinity [3H] GABA

uptake sites have been demonstrated in the myenteric plexus of the guinea-pig intestine, where [3H] GABA is taken up into both neurones and glial cells by high affinity transport processes (Jessen et al., 1979; 1983 a,b; Jessen, 1981; Krantis and Kerr, 1981 a; Saffrey et al., 1983), and can then be subsequently released by electrical stimulation (Jessen et al., 1983 c; Kerr and Krantis, 1983; Taniyama et al., 1983 a). Such high affinity uptake was selectively inhibited by specific neuronal and glial uptake blockers for GABA (Jessen et al., 1979; Krantis and Kerr, 1981 a; Kerr and Krantis, 1983). Thus, a high affinity GABA transport process appears to exist in the peripheral nervous system, so that GABA may be inactivated there through a mechanism analogous to that for GABA in the CNS (Martin, 1976). Such termination of GABA-mediated transmission in the CNS is thought to minimize over-activity of GABAergic neuronal function and to reduce the accumulation of extracellular GABA (Roberts, 1978), and it is thus assumed that the same reasoning applies to GABA uptake in the enteric nervous system.

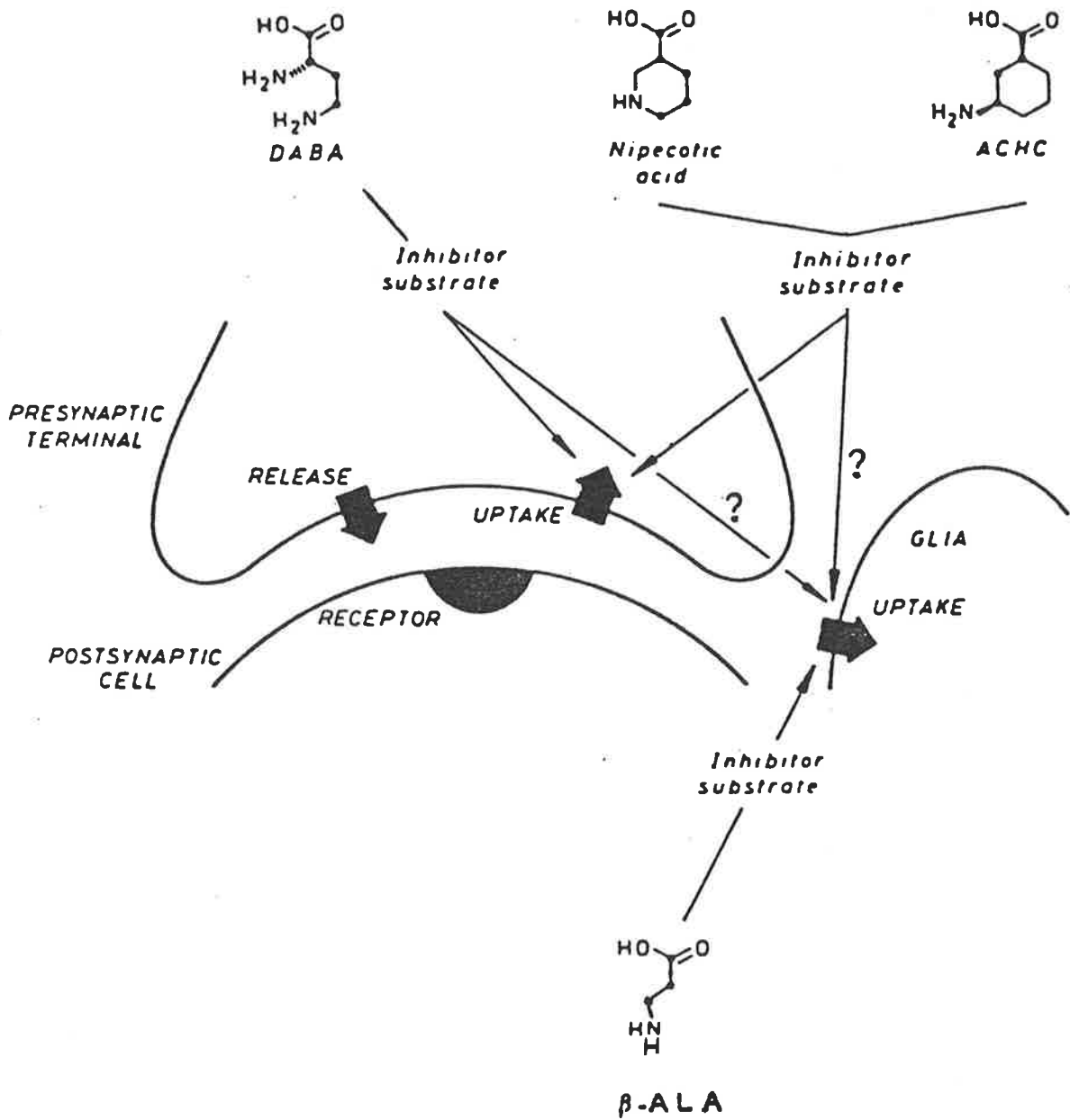
Exogenously applied GABA induces a transient, BMC- and PIC-sensitive cholinergic contractile response, which is followed by a delayed prolonged 'after-relaxation' in the guinea-pig isolated ileum (Kaplita et al., 1982; Giotti et al., 1983 a; Ong and Kerr, 1983 a,b). The neurally-mediated contractile response is the result of the release of ACh from cholinergic neurones, this conclusion being supported by efflux studies where GABA induces a tetrodotoxin-sensitive release of [3H] ACh in the myenteric plexus of the intestine, mediated through GABA<sub>A</sub>-receptor sites asso-

ciated with the chloride-ionophore (Taniyama et al., 1983 b; Kleinrok and Kilbinger, 1983). Such GABA-induced ACh release is also sensitive to BMC and furosemide, a chloride ion channel blocker, which inhibits chloride-dependent, GABA-mediated responses in the frog spinal cord (Nicoll, 1978 a), and blocks the chloride channels opened by GABA at the crayfish stretch receptor via blocking the chloride pump (Deisz and Lux, 1976). The 'after-relaxation' induced by GABA, on the other hand, is due to a depression of cholinergic output as a result of activating GABA<sub>B</sub>-receptor sites (Bowery et al., 1981 a), for which baclofen is a specific agonist (Hill and Bowery, 1981).

Although a previous study has shown that inhibitors of neuronal GABA uptake, but not glial cell uptake, potentiate the inhibitory effect of GABA on field stimulated vas deferens preparations, suggesting the presence of a predominantly neuronal GABA uptake system in this particular preparation (Bowery et al., 1981 c), the pharmacological effects of GABA uptake blockers on GABAergic responses have not been widely investigated in peripheral tissues. It is for this reason that the present study examines in detail, both quantitatively and qualitatively, the effects of various neuronal and glial uptake blockers on responses elicited by GABA and its analogues in the isolated intestine, to determine if GABA uptake similarly limits GABAergic neurotransmission in the enteric nervous system of the guinea-pig. In addition to which, the results from such studies should provide further insight into the characteristics of the GABAergic system in the intestine, and help to elucidate the uptake mechanisms

present.

The structures and proposed sites of actions of the respective neuronal and glial uptake blockers for GABA, used in the present study, are presented in a simplified schematic diagram shown on the following page, together with the possible mechanisms of uptake, release and postsynaptic actions of the transmitter. Specificity of neuronal versus glial uptake for DABA and nipecotic acid remains unresolved. However, it is undoubtedly clear that selective uptake blockers for GABA have been used extensively to investigate GABA uptake mechanisms in a variety of tissues, and in this case, the enteric nervous system has been used as a working model for studying such mechanisms.



#### 4.2. MATERIALS AND METHOD

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of isolated ileum, 3-4 cm in length, taken 2-3 cm from the ileo-caecal valve, were mounted vertically in a 10 ml organ bath containing Krebs-bicarbonate solution gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (composition as described in Chapter 2.1). Mechanical activity of the longitudinal muscle of the tissue was recorded as described (Chapter 2.1), and the tissue was allowed to equilibrate for 60 min in the organ bath before any drug application. Effects of drug treatments were then examined on the resting tissue. Some drugs were left in the bath to incubate with the tissue for at least 15 min before agonists were tested, whilst antagonists were added at least 5-10 min before testing the effects of the appropriate agonists. Tissue responses were allowed to recover to baseline level before any further drug application, and drug volumes were never more than 1% of the total bath volume. Student's t-test for paired and unpaired samples was used to assess the significance of differences between mean values of the dose-response effects. The experiments were performed in duplicate, and data was collected from a minimum of 6 tissues from at least 3 different animals.

#### CHEMICALS:

The following chemicals were obtained from Sigma except for those indicated in parenthesis.

GABA

atropine sulphate

tetrodotoxin

b-alanine

nipecotic acid

cis-3-aminocyclohexanecarboxylic acid (Dr. D.I.B. Kerr)

2,4-diaminobutyric acid

acetylcholine chloride

histamine dihydrochloride

5-hydroxytryptamine creatinine sulphate

baclofen (Ciba-Geigy)

bicuculline methochloride (Pierce)

picrotoxinin (dissolved in 1:9 absolute alcohol and distilled water)

muscimol

3-amino-1-propanesulphonic acid



### 4.3. RESULTS

#### 4.3.1. Effects of uptake blockers on ileal responses induced by GABA.

Exogenously applied GABA induced a transient, neuronal, cholinergic response, followed by a delayed, prolonged 'after-relaxation' (Ong and Kerr, 1983 b), this contractile effect being sensitive to atropine (0.7  $\mu$ M) and TTX (0.7  $\mu$ M). In the presence of either  $\beta$ -alanine ( $\beta$ -ala) (0.1 mM), (Fig. 1), nipecotic acid (nip) (0.1 mM), cis-3-aminocyclohexanecarboxylic acid (ACHC) (10  $\mu$ M) (Fig. 1), or 2,4-diaminobutyric acid (DABA) (0.1 mM), both GABA-induced contractile response and the 'after-relaxation' was potentiated, but these uptake inhibitors themselves did not alter the baseline of the isolated ileal preparations when applied at the concentrations used in the present experiments, nor did any of these inhibitors affect contractile responses to exogenously applied acetylcholine (ACh) (5 nM) (Fig. 1), histamine or 5-hydroxytryptamine (5-HT). The dose-response curves for the contractile responses induced by GABA were shifted significantly to the left in the presence of  $\beta$ -ala (0.1 mM), nip (0.1 mM), ACHC (10  $\mu$ M), or DABA (0.1 mM) at their effective concentrations (Fig. 2,3), this shift being more prominent over the lower dose-range of GABA (5-50  $\mu$ M). The potentiation of the contractile responses induced by GABA in the presence of DABA, ACHC or nip was significantly greater than that observed with  $\beta$ -ala, as seen from Fig. 2,3; whilst simultaneous addition of either DABA, nip or ACHC together with  $\beta$ -ala (Fig. 4,5,) caused a greater shift to the left of the GABA dose-response curves than did any of the uptake

blockers when applied alone (Fig. 2,3,), e.g. a combination of b-ala (0.1 mM) and DABA (0.1 mM) gave a significantly greater ( $P < 0.05$ ) potentiation of the GABA-induced responses than in the presence of DABA (0.1 mM) alone (Fig. 6).

In addition, the GABA-induced 'after-relaxation', but not the baclofen-induced relaxation, was potentiated by the uptake blockers. As shown in Fig. 7, the baclofen (10  $\mu$ M)-induced relaxation was unaffected by either b-ala (0.1 mM) or nip (0.1 mM). High concentrations of b-ala (0.5 mM), nip (> 5 mM) or DABA (1 mM) themselves elicited weak contractile ileal responses, sensitive to bicuculline methochloride (BMC) (10  $\mu$ M) and to picrotoxinin (PIC) (30  $\mu$ M). Such high concentrations were avoided since they generally had a depressant action on the GABA-induced contractile responses when left in the bath for 15 min or longer. As can be seen from the results presented in Fig. 2-5, ACHC is by far a more potent uptake inhibitor than either DABA or nip in the intestine.

#### **4.3.2. Effects of uptake blockers on GABA-induced responses in the presence of BMC or PIC.**

- BMC (5  $\mu$ M) caused a parallel rightwards shift of the dose-response curve for GABA-induced ileal contractile responses without affecting the maximal response, and this effect of BMC (5  $\mu$ M) persisted in the presence of b-ala (0.1 mM) (Fig. 8), nip (0.1 mM) (Fig. 9), DABA (0.1 mM) (Fig. 10), or ACHC (10  $\mu$ M), but

was less than that seen for BMC in the absence of GABA uptake inhibition (c.f. Kenakin, 1982). BMC (5  $\mu$ M) also shifted the dose-response curve for GABA in the presence of nip (0.1 mM) and b-ala (0.1 mM) significantly to the right (Fig. 11). PIC (30  $\mu$ M), on the other hand, not only shifted the dose-response curve for GABA to the right in a non-parallel manner, but also depressed the maximum response, indicating a non-competitive antagonism induced by PIC (Fig. 12). Upon incubating the tissues with b-ala, the dose-response curves for GABA in the presence of both PIC (30  $\mu$ M) and b-ala (0.1 mM) (Fig. 12) were again shifted to the right, but to a lesser degree than that seen with PIC alone (Fig. 12). Using PIC (30  $\mu$ M), there was also a rightwards shift of the dose-response curve for the GABA-induced contractile responses in the presence of DABA (0.1 mM), nip (0.1 mM), or a combination of either of these uptake inhibitors with b-ala (0.1 mM) (Fig. 13,14).

#### **4.3.3. Effects of uptake blockers on ileal responses induced by 3-APS or muscimol.**

Muscimol and 3-amino-1-propanesulphonic acid (3-APS) only elicited transient, dose-dependent, contractile responses in the guinea-pig ileal preparations. These responses to muscimol (5  $\mu$ M) or 3-APS (5  $\mu$ M) were unaffected by any of the GABA uptake inhibitors such as b-ala (0.1 mM), ACHC (10  $\mu$ M), nip (0.1 mM) or DABA (0.1 mM) (Fig. 15,16,17). As seen from these dose-response curves for muscimol and 3-APS, when compared to GABA (c.f. Fig. 2,3), the potencies of muscimol and 3-APS in inducing ileal

contractile responses were approximately equal, but some 4 -fold greater than that of GABA. These contractile effects were also atropine (0.1  $\mu$ M)- and TTX (0.1  $\mu$ M)-sensitive. None of the GABA uptake inhibitors caused any significant shift of the dose-response curves for ileal contractions induced by muscimol or 3-APS; neither did they influence the rightwards shift of these dose-response curves in the presence of BMC (10  $\mu$ M) or PIC (10  $\mu$ M) (Fig. 18-21), unlike that seen with GABA as the agonist.

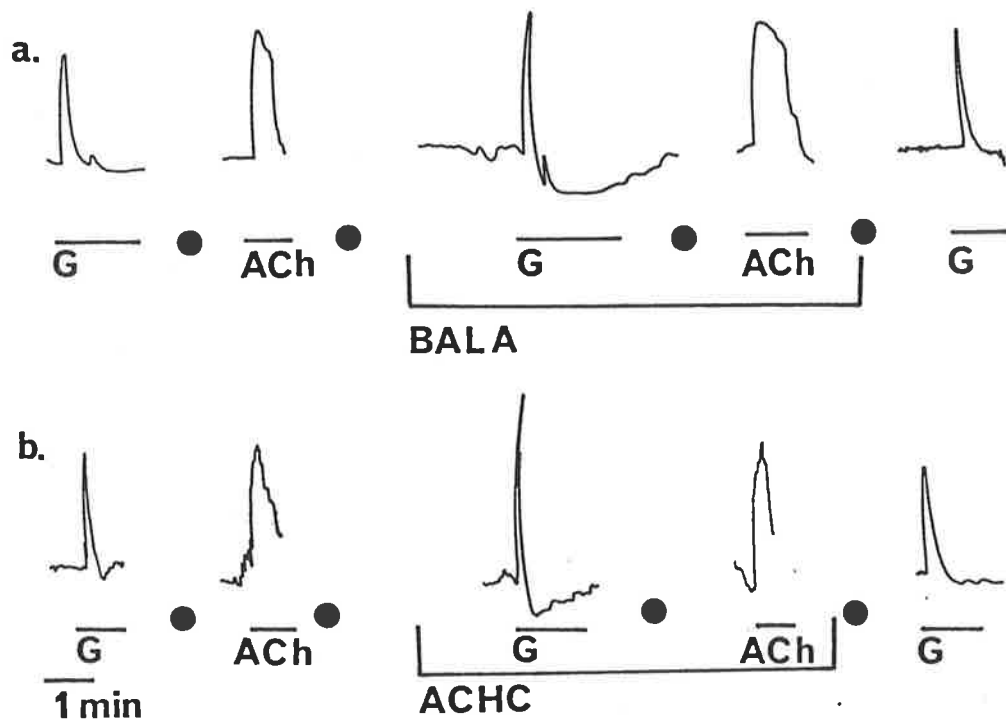


Fig. 1. Effects of BALA and ACHC on responses induced by GABA and ACh in the isolated ileum: (a) Significant potentiation ( $P < 0.05$ ) of GABA (10  $\mu\text{M}$ )-induced responses by BALA (0.1 mM), and the lack of effect of BALA on ACh (5 nM)-induced contraction. (b) Significant potentiation ( $P < 0.05$ ) of GABA (10  $\mu\text{M}$ )-induced responses by ACHC (10  $\mu\text{M}$ ), and the lack of effect of ACHC on ACh (5 nM)-induced contraction. After tissue washout, the GABA-induced contractile response recovered.

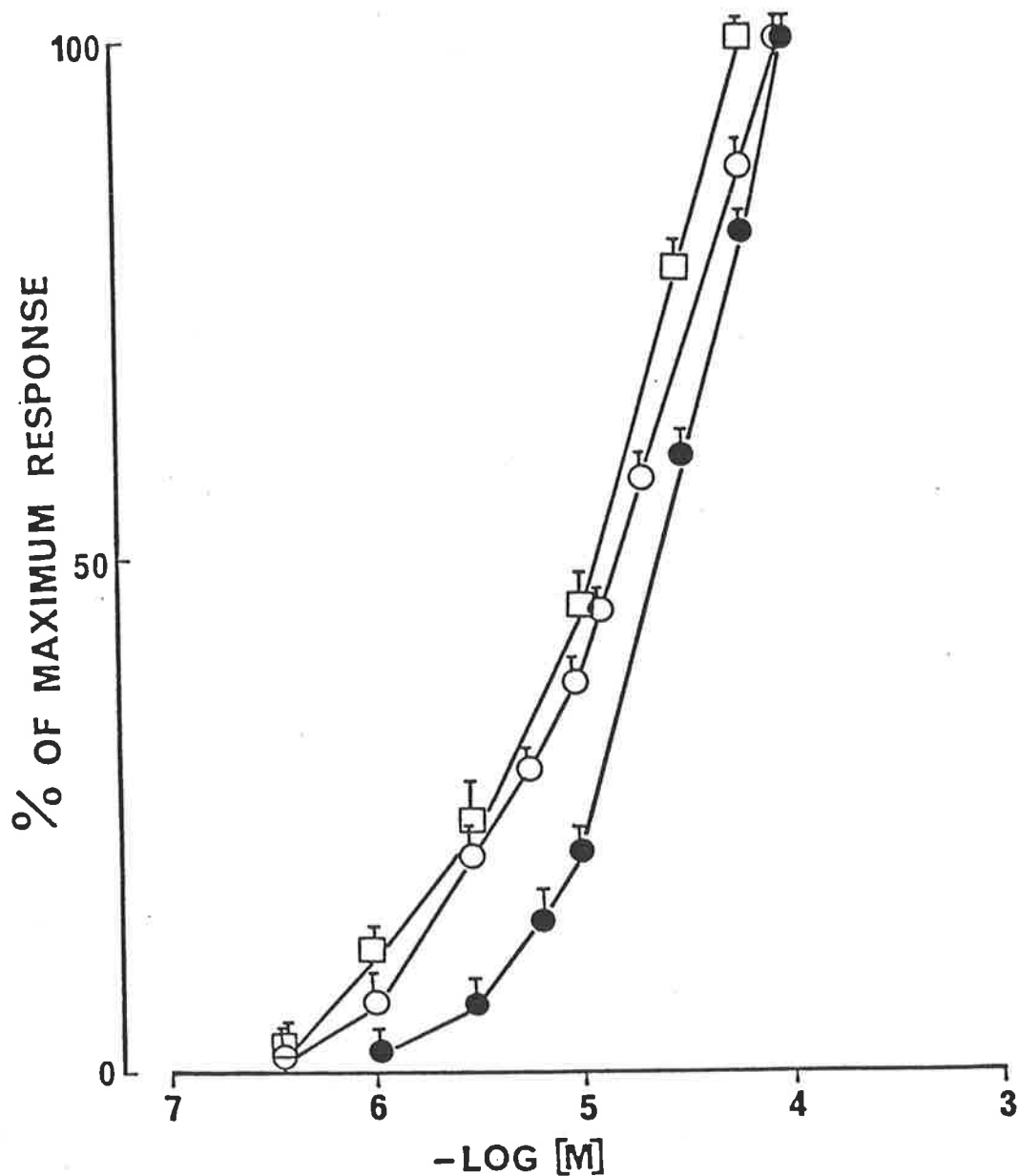


Fig. 2. Dose-response curves for contractile responses to ● GABA, and significant potentiating effects ( $P < 0.05$ ) of ○ BALA (0.1 mM) and □ NIP (0.1 mM) on the GABA-induced contractile responses over the lower dose-range of GABA (5-50  $\mu$ M). There was no significant difference in the dose-response curves for GABA in the presence of BALA or NIP. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean based on the number of experiments performed ( $n=6$ ).

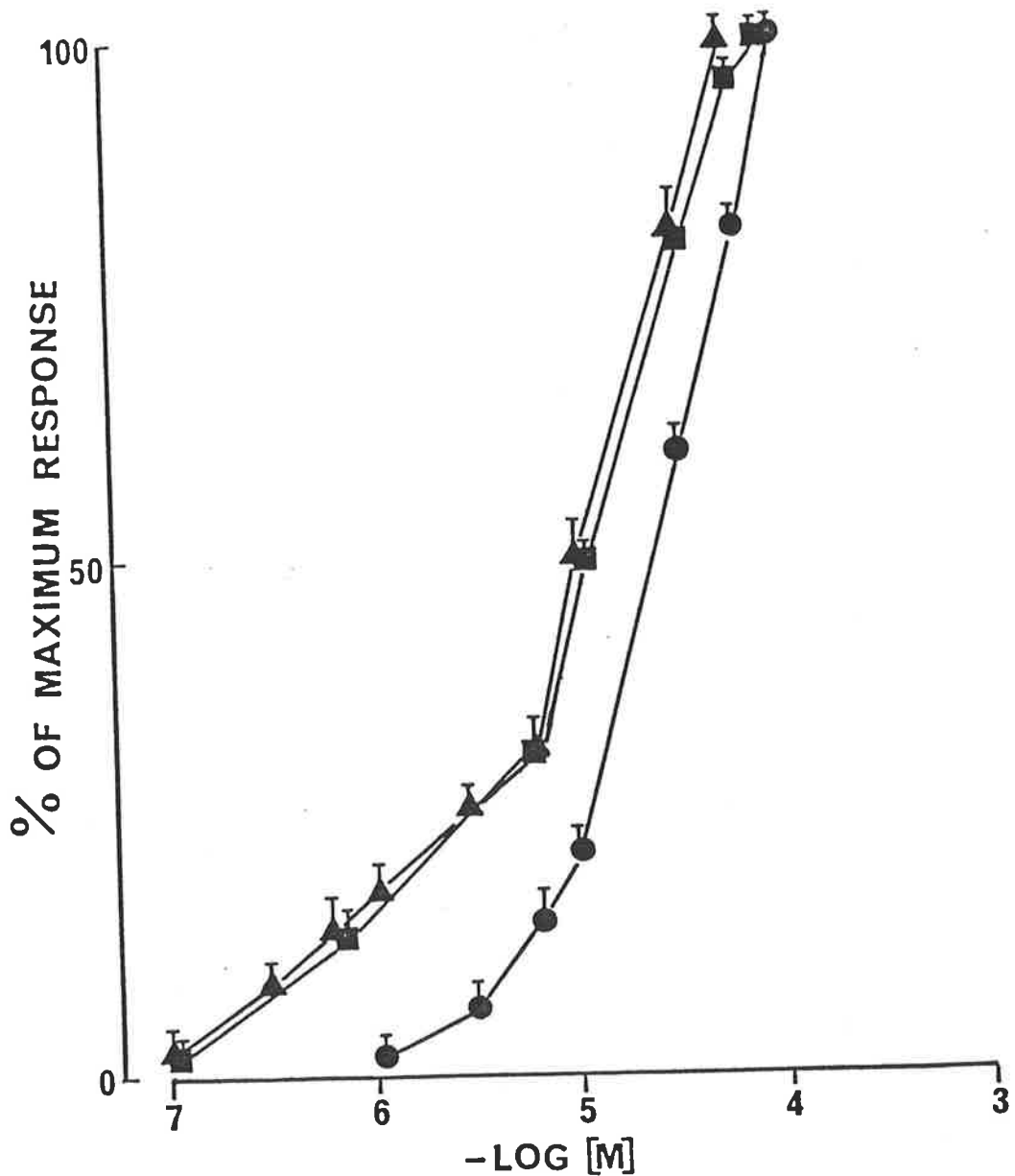


Fig. 3. Dose-response curves for contractile responses to ● GABA, and significant potentiating effects ( $P < 0.05$ ) of ■ ACHC (10  $\mu$ M) and ▲ DABA (0.1 mM) on the GABA-induced contractile responses over the lower dose-range of GABA (5-50  $\mu$ M). There was no significant difference in the dose-response curves for GABA in the presence of ACHC or DABA. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean based on the number of experiments performed ( $n=6$ ).

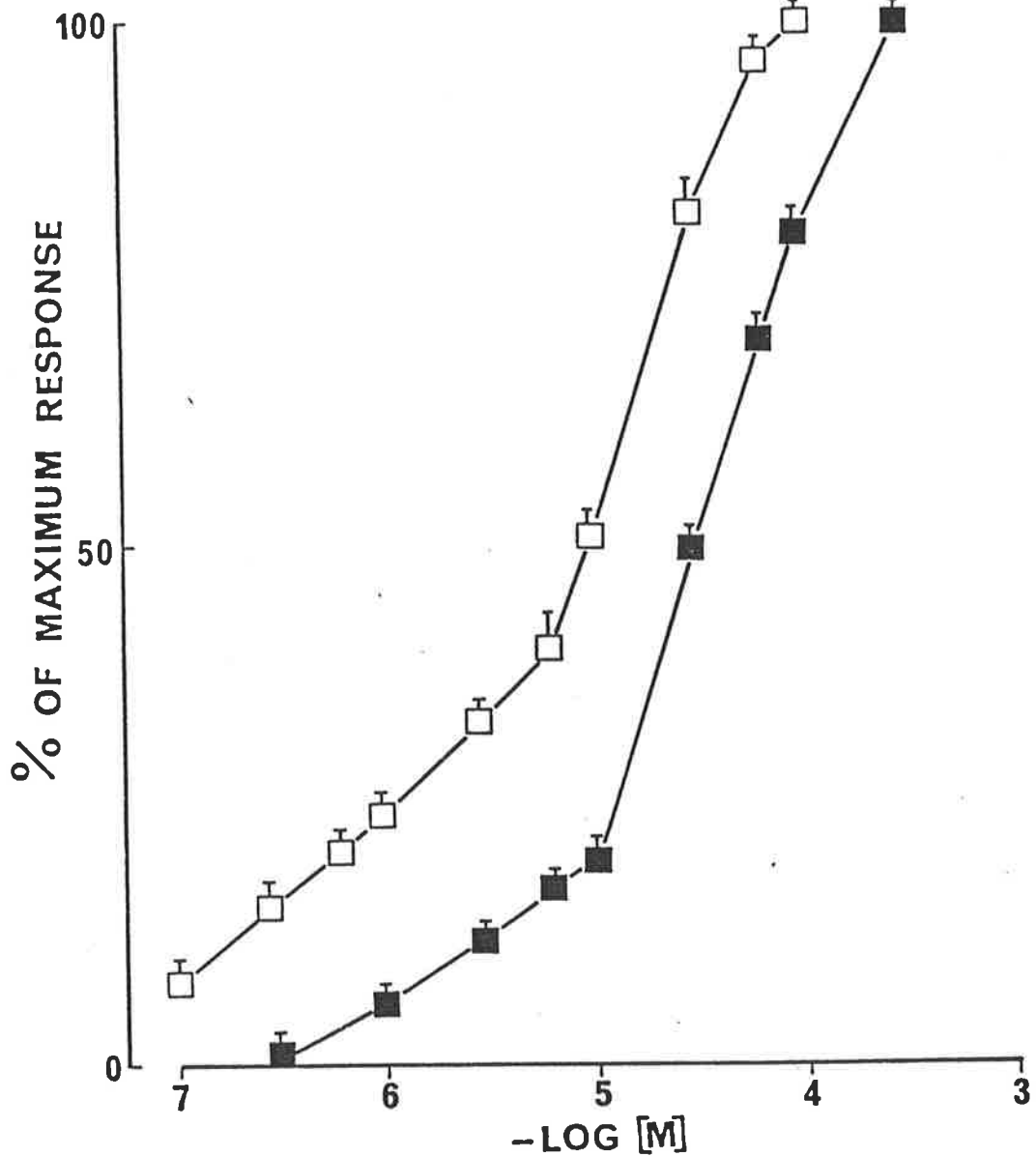


Fig. 4. Dose-response curves for GABA-induced contractile responses in the presence of a combination of  $\square$  BALA (0.1 mM) and DABA (0.1 mM), and a significant rightwards shift of the curve in the presence of  $\blacksquare$  BMC (5 uM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean (n=6).



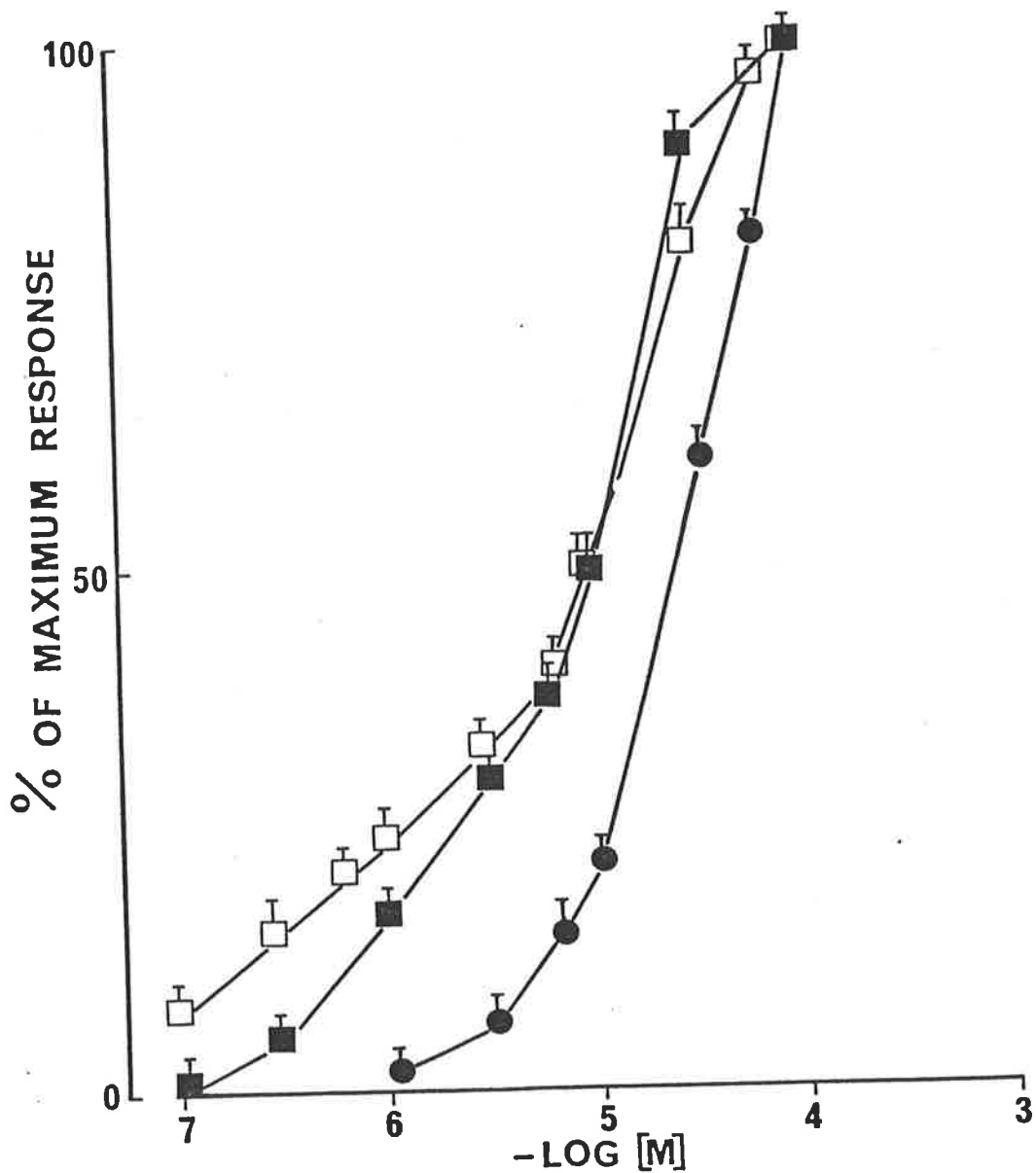


Fig. 5. Dose-response curves for contractile responses induced by ● GABA, and significant potentiating effects of the GABA-induced contractile responses by a combination of ■ NIP (0.1 mM) and BALA (0.1 mM) and □ DABA (0.1 mM) and BALA (0.1 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean based on the number of experiments performed (n=6).

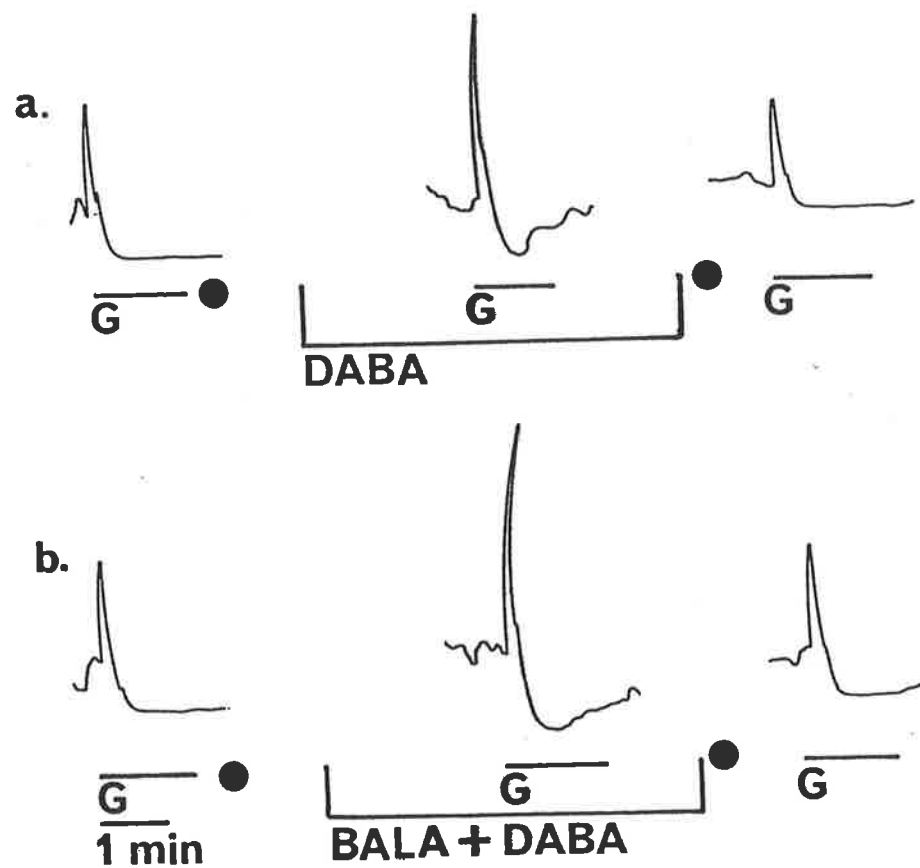


Fig. 6. Potentiating effects of DABA (0.1 mM) and a combination of DABA (0.1 mM) and BALA (0.1 mM) on the GABA-induced responses.

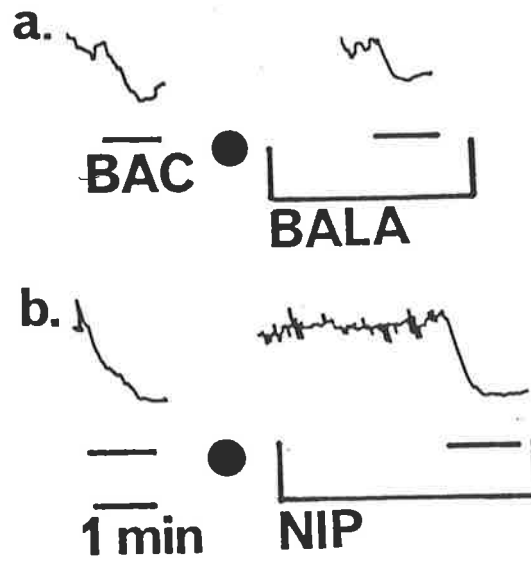


Fig. 7. Lack of effect of BALA (0.1 mM) and NIP (0.1 mM) on the baclofen (10 uM)-induced relaxation in the ileum.

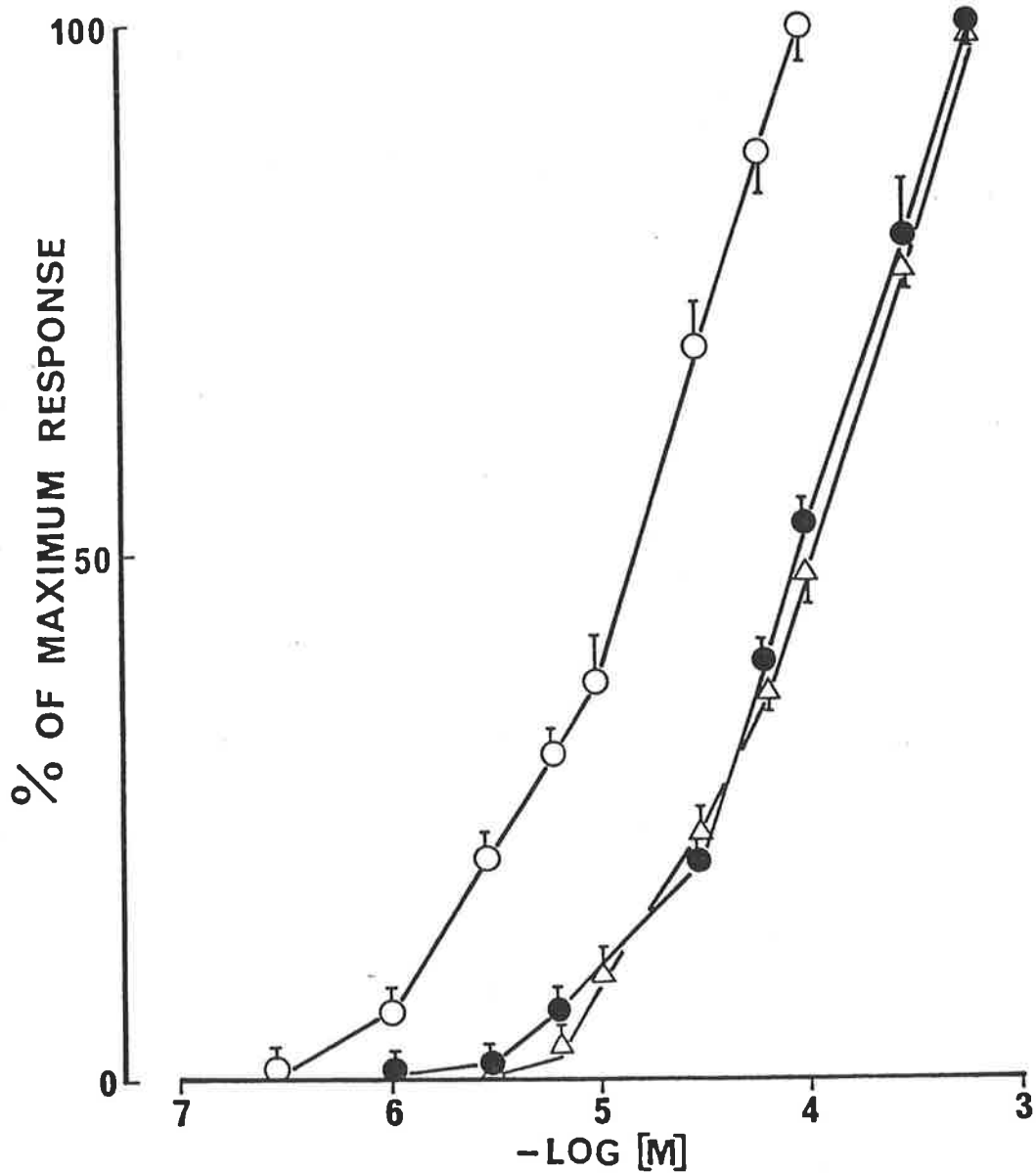


Fig. 8. Dose-response curves for contractile responses induced by GABA in the presence of  $\circ$  BALA (0.1 mM), and there was a significant ( $P < 0.05$ ) rightwards shift of the GABA dose-response curve by  $\Delta$  BMC (5  $\mu$ M) and by  $\bullet$  BMC (5  $\mu$ M) in the presence of BALA (0.1 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from 6 experiments.

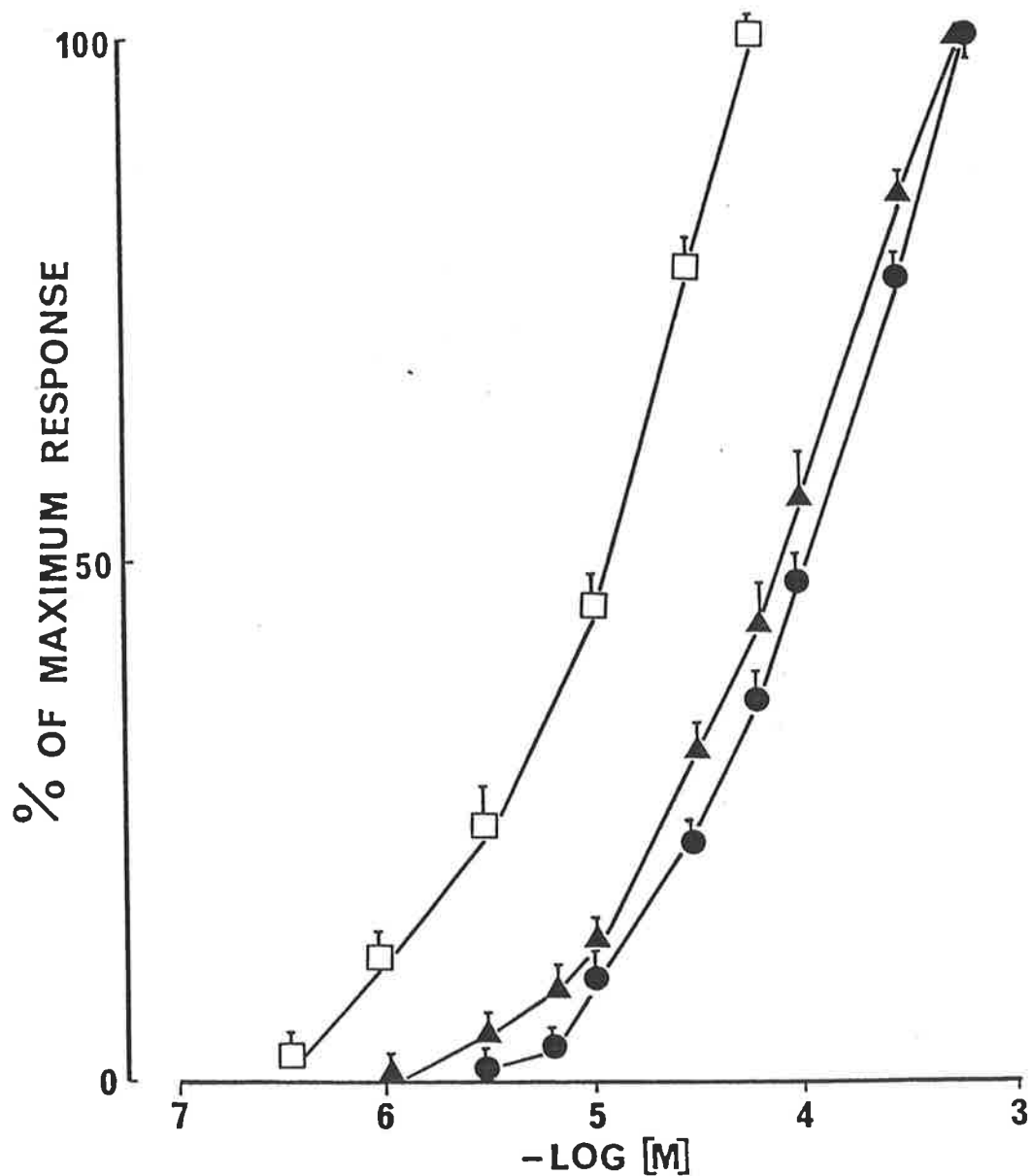


Fig. 9. Dose-response curves for GABA-induced contractile responses in the presence of  $\square$  NIP (0.1 mM),  $\bullet$  BMC (5  $\mu$ M) and there was a rightwards shift of the dose-response curve for GABA and NIP (0.1 mM) by  $\blacktriangle$  BMC (5  $\mu$ M). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from 6 experiments.

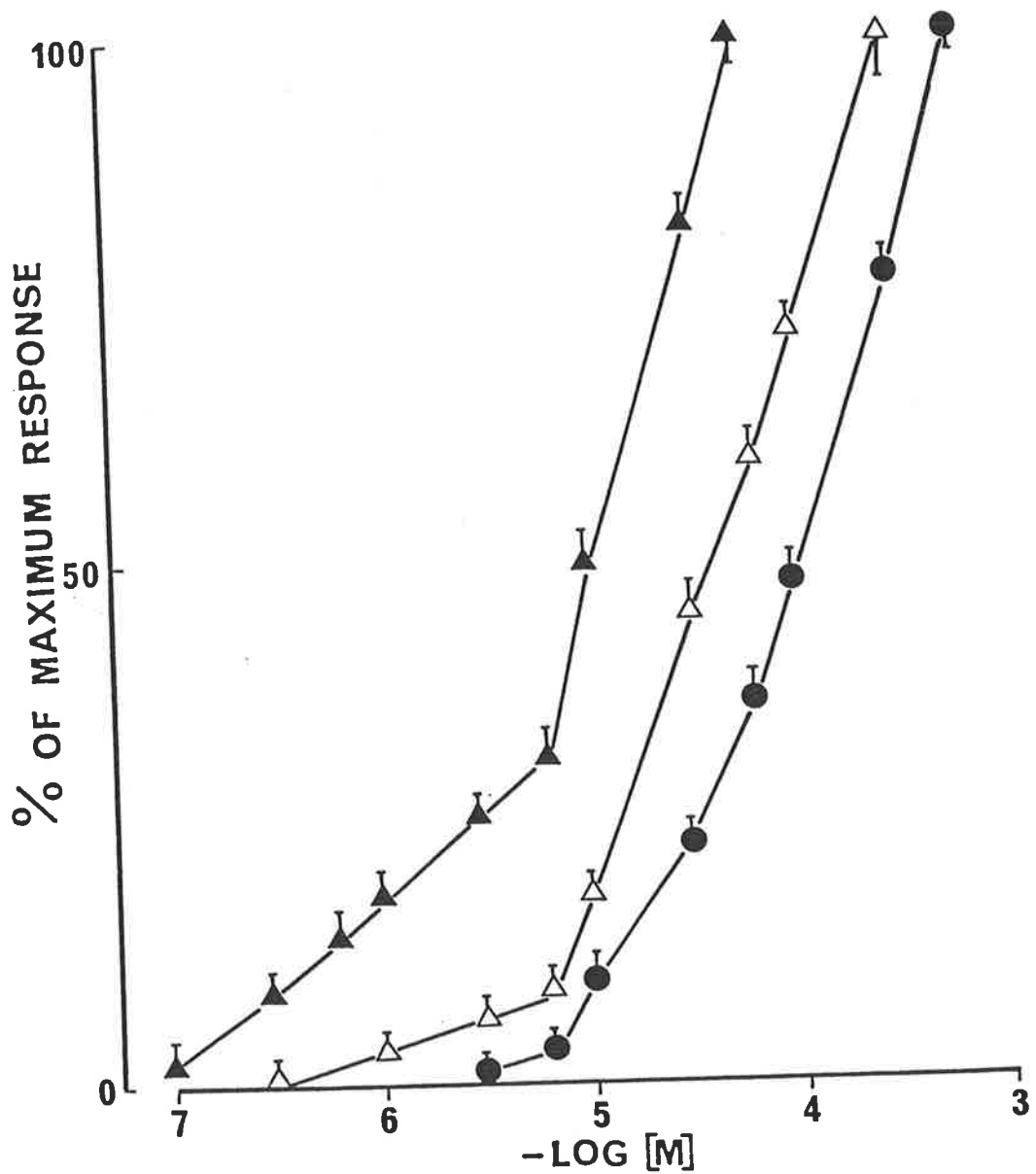


Fig. 10. Dose-response curves for GABA-induced contractile responses in the presence of ▲ DABA (0.1 mM), and a parallel rightwards shift of the dose-response curve for GABA alone in the presence of ● BMC (5 μM), and for GABA and DABA (0.1 mM) in the presence of △ BMC (5 μM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from 6 experiments.

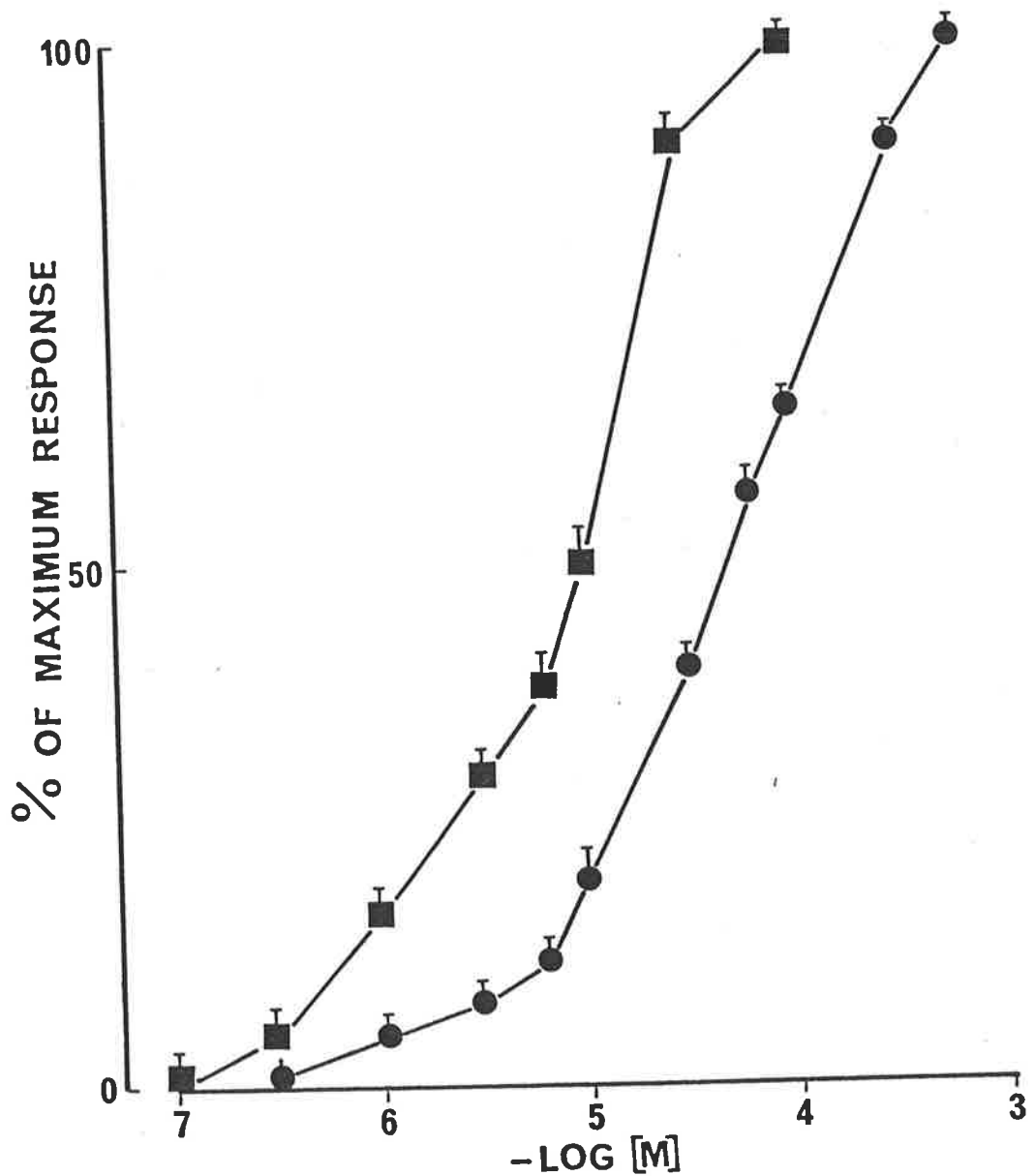


Fig. 11. Dose-response curve for the potentiation of the GABA-induced contractile responses by ■ NIP (0.1 mM) and BALA (0.1 mM), and a parallel rightwards shift of the dose-response curve by ● BMC (5  $\mu$ M). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from 6 experiments.

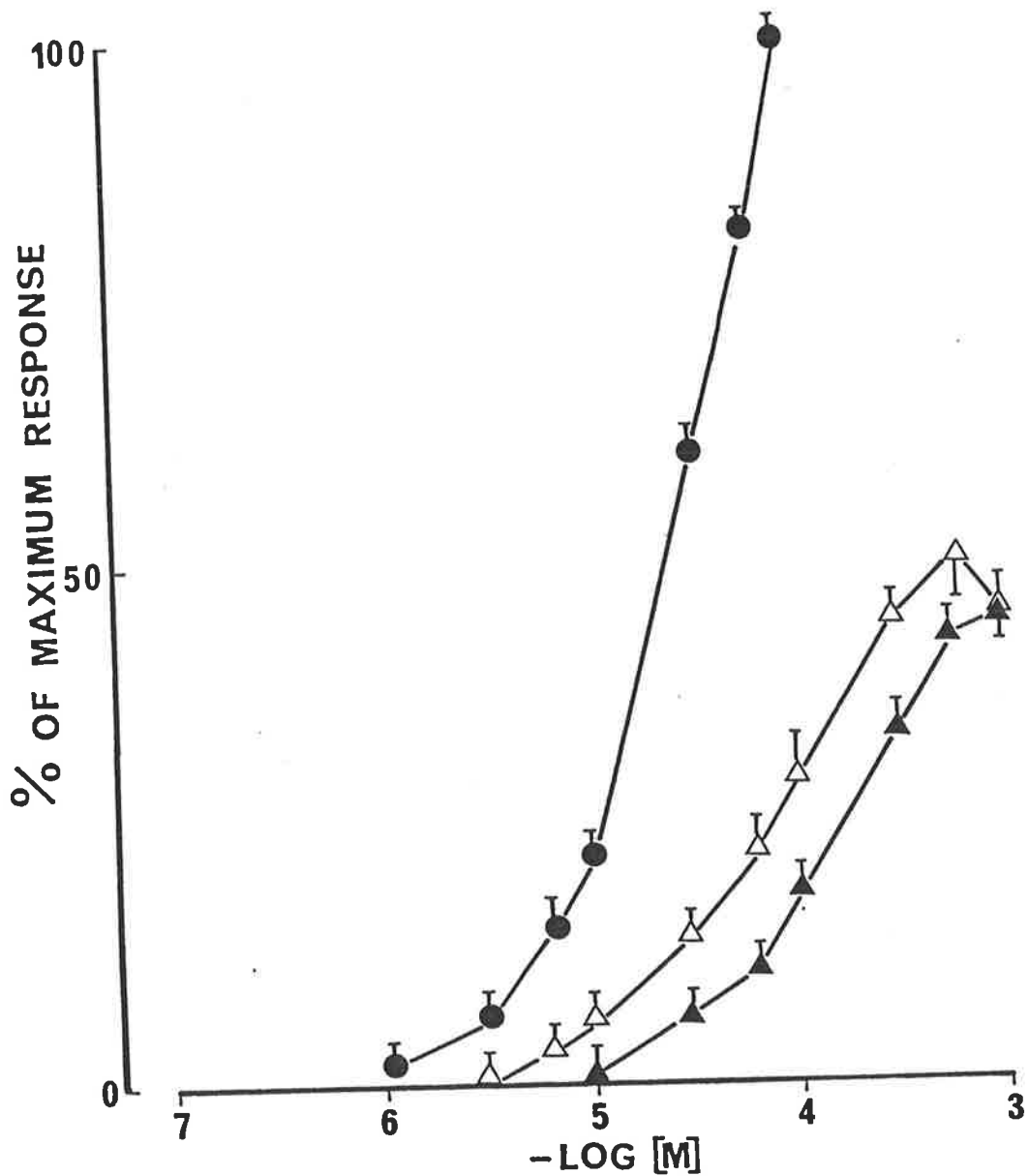


Fig. 12. Dose-response curves for the contractile responses induced by ● GABA, and in the presence of △ BALA (0.1 mM) and PIC (30  $\mu$ M), compared to that in the presence of ▲ PIC (30  $\mu$ M) alone. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from at least 6 experiments.



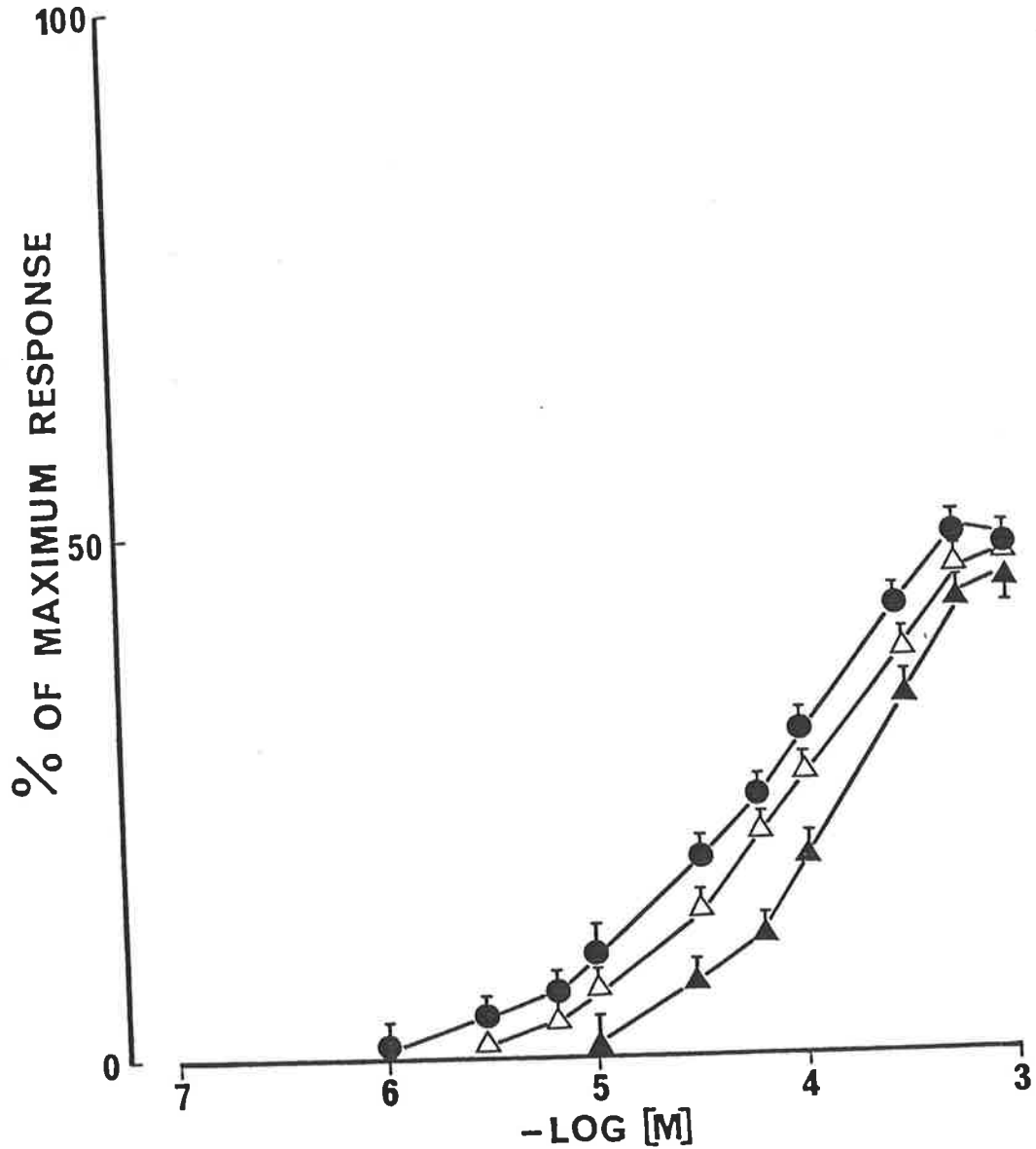


Fig. 13. Dose-response curves for the PIC (30  $\mu$ M)-induced antagonism of the contraction elicited by  $\blacktriangle$  GABA, and in the presence of  $\triangle$  DABA (0.1 mM) alone, and both  $\bullet$  DABA (0.1 mM) and BALA (0.1 mM). There was no significant differences between these curves. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from 6 experiments.

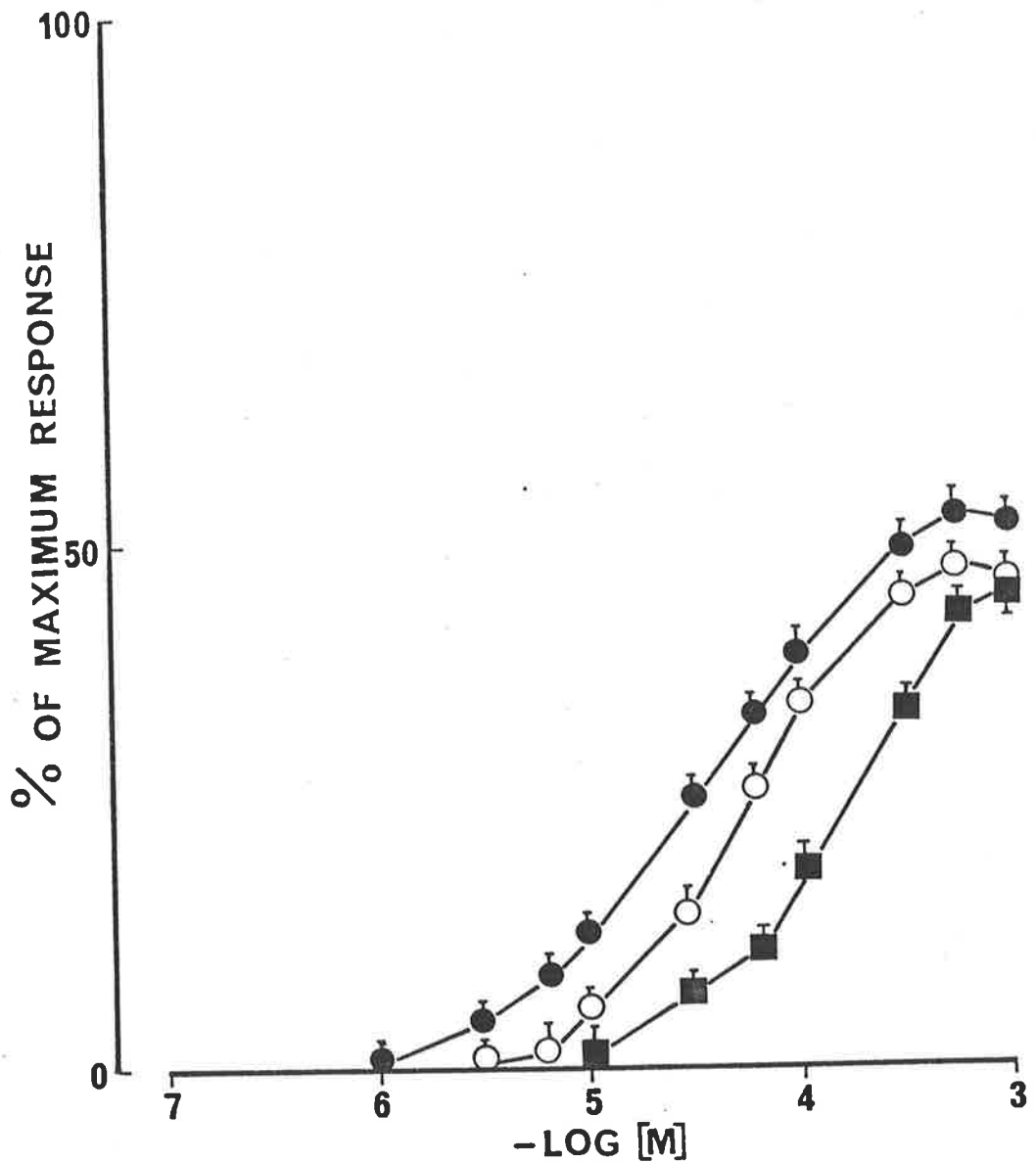


Fig. 14. Dose-response curves for the PIC (30  $\mu$ M)-induced antagonism of contractile responses elicited by  $\blacksquare$  GABA, in the presence of  $\circ$  NIP (0.1 mM) and  $\bullet$  NIP (0.1 mM) and BALA (0.1 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean from a minimum of 6 experiments.

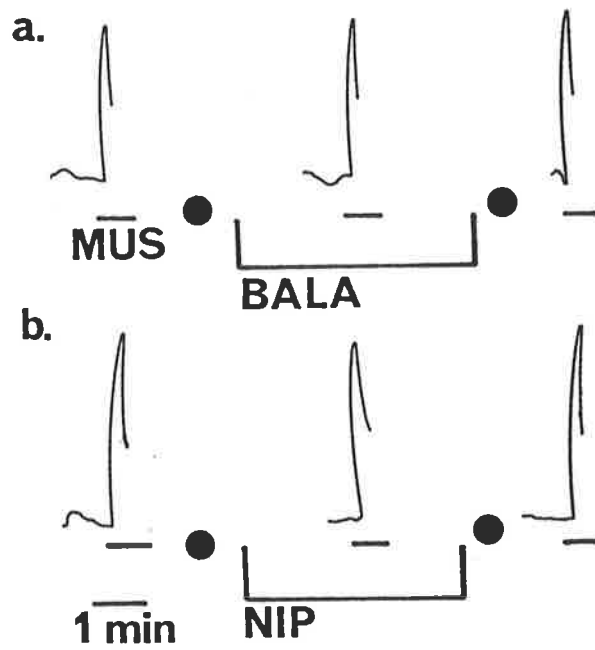


Fig. 15. Lack of effect of BALA (0.1 mM) and NIP (0.1 mM) on muscimol (5  $\mu$ M)-induced contractile responses.

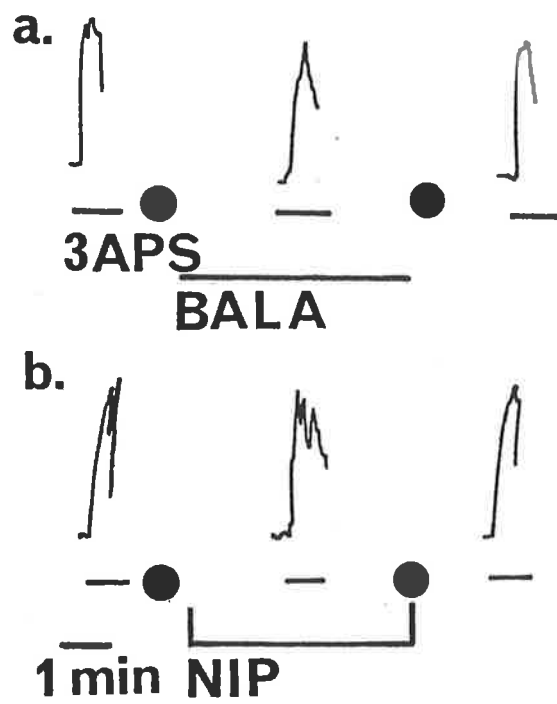


Fig . 16. Lack of effect of BALA (0.1 mM) and NIP (0.1 mM) on 3APS (5  $\mu$ M)-induced contractile responses.

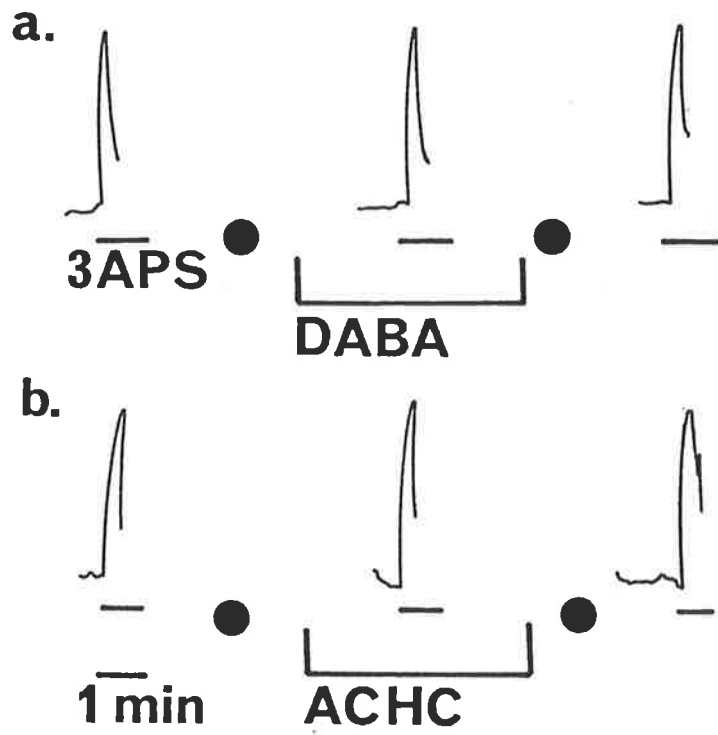


Fig. 17. Lack of effect of DABA (0.1 mM) ACHC (10  $\mu$ M) on 3APS (5  $\mu$ M)-induced responses.

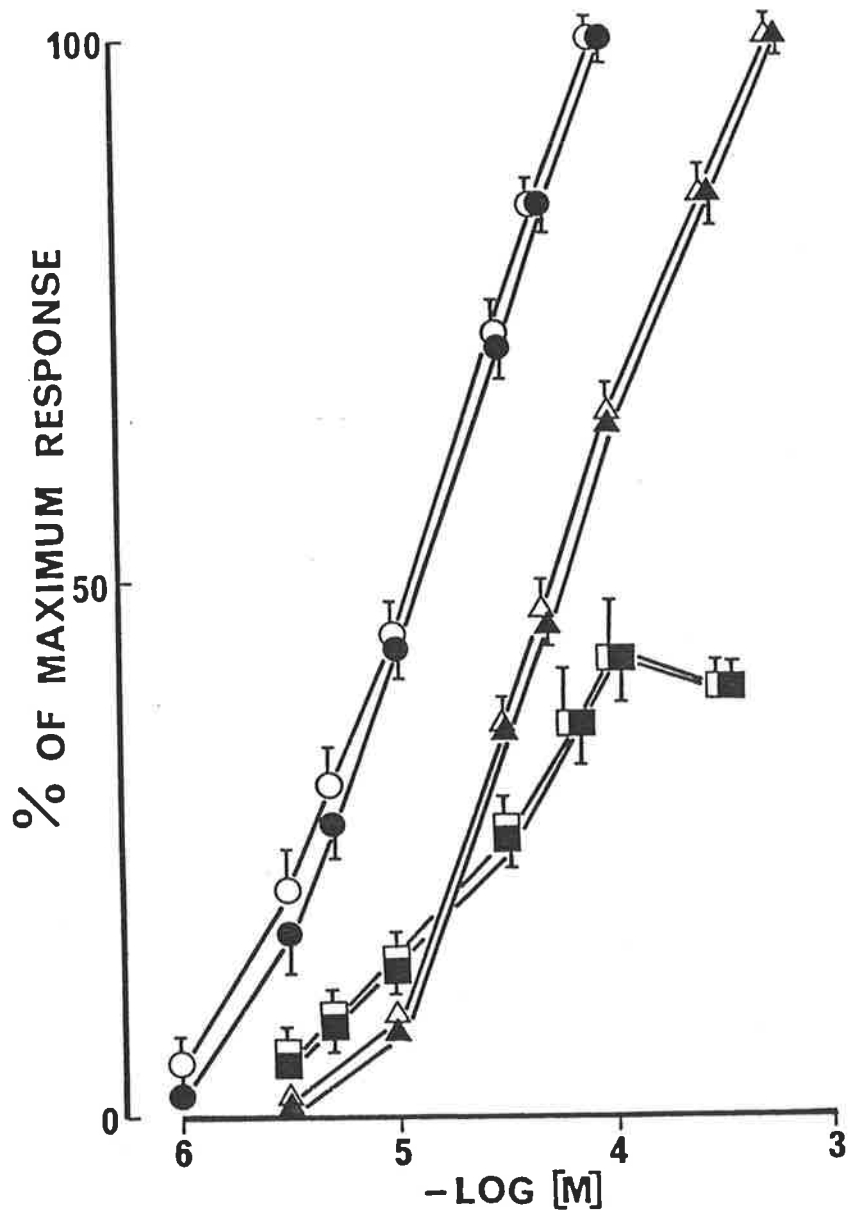


Fig. 18. Dose-response curves for contractile responses induced by ● muscimol, in the presence of ■ PIC (10  $\mu$ M), ▲ BMC (10  $\mu$ M) and lack of effect of ○ BALA (0.1 mM) on dose-response curves for muscimol in the presence of △ BMC (10  $\mu$ M) and □ PIC (10  $\mu$ M). Results are expressed on the ordinate as a percentage of the maximum contraction. Each point represents the mean and standard error of the mean from 6 experiments.

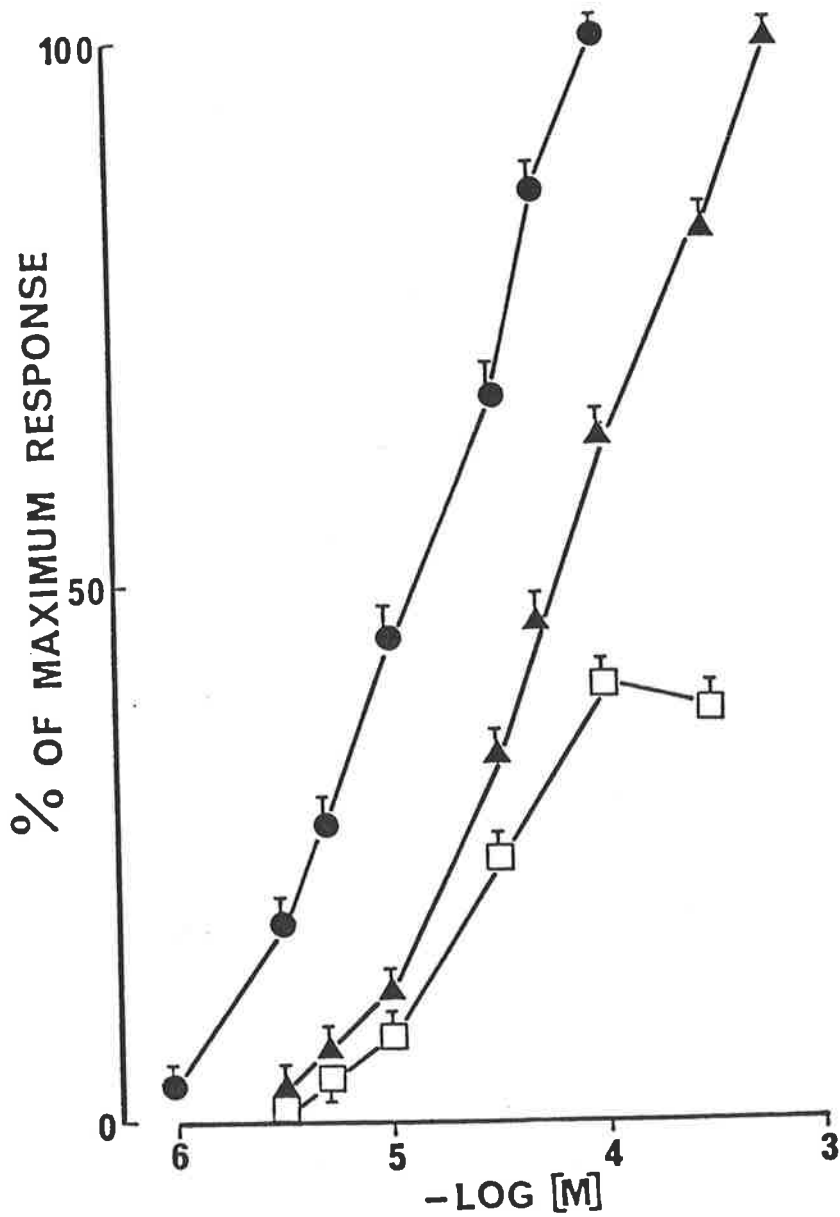


Fig. 19. Dose-response curves for contractile responses induced by muscimol in the presence of ● DABA (0.1 mM), ▲ DABA (0.1 mM) and BMC (10 uM) and □ DABA (0.1 mM) and PIC (10 uM). Results are expressed on the ordinate as a percentage of the maximum contraction. Each point is the mean and standard error of the mean from a number of experiments (n=6).

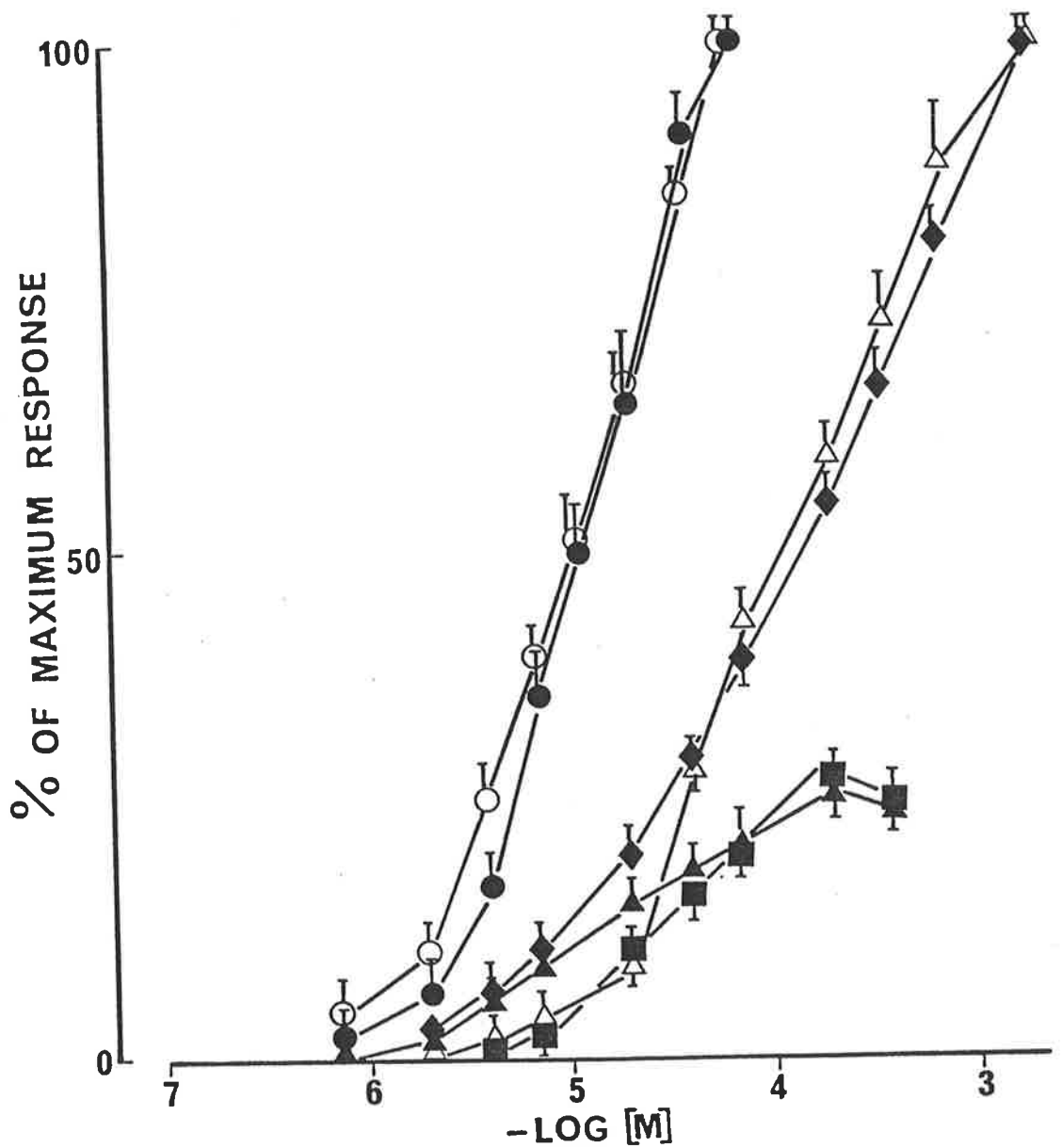


Fig. 20. Dose-response curves for contractile responses induced by ● 3APS, in the presence of △ BMC (10 uM), ▲ PIC (10 uM), and the lack of effect of ○ BALA (0.1 mM) on the dose-response curve. BALA (0.1 mM) did not affect the dose-response curves for 3APS in the presence of ◆ BMC (10 uM) and ■ PIC (10 uM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by 3APS. Each point represents the mean and standard error of the mean based on 6 experiments.



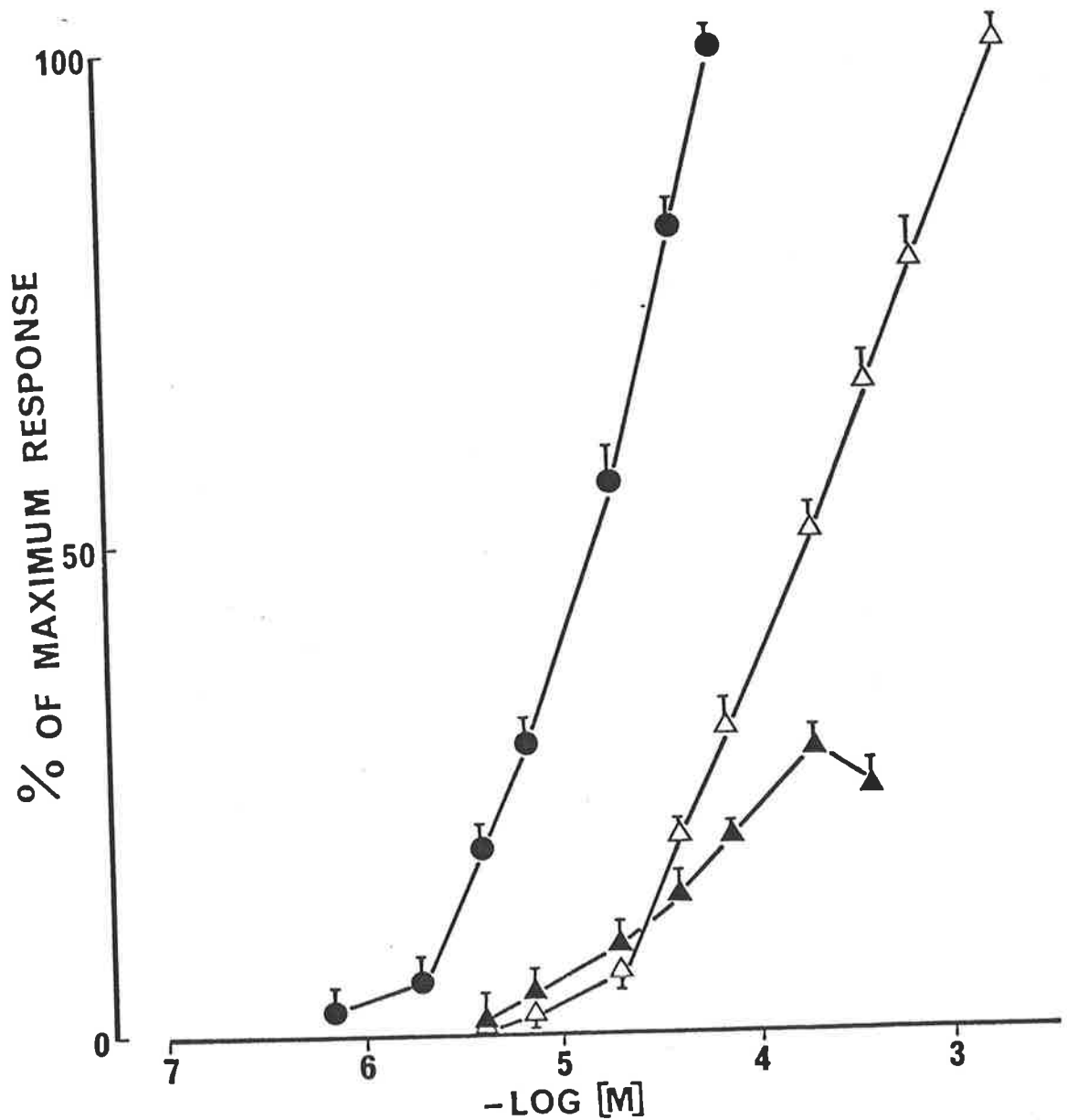


Fig. 21. Lack of effect of DABA (0.1 mM) on dose-response curves for ● 3APS, in the presence of △ BMC (10 uM) and ▲ PIC (10 uM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by 3APS. Each point represents the mean and standard error of the mean based on 6 experiments.

#### 4.4. DISCUSSION

There has been marked progress in the study of GABA transport in the CNS, where the limitation of synaptic function of GABA depends on the transport of GABA across membranes into the nervous tissue elements. Tissue localization of the transport systems, together with the physiologic factors which may influence GABA transport, as well as the kinetic aspects of the transport such as the dependence of the uptake mechanism on its environment (Martin, 1976; Storm-Mathisen et al., 1976; Storm-Mathisen, 1976), and also the inhibitory effects of structurally rigid analogues of high chemical specificity on such transport mechanism, have been intensively studied to explore the role of GABA uptake in synaptic function (Johnston, 1976; Krogsgaard-Larsen, 1978; 1980; 1981; Brehm et al., 1979; Schousboe, 1979 a; 1981; Larsson et al., 1981; 1983 a,b).

Nip, known originally to be a substrate-competitive inhibitor of the neuronal uptake for GABA (Krogsgaard-Larsen and Johnston, 1975; Johnston et al., 1976 a), proved to be a very potent inhibitor of GABA uptake into rat brain slices with little or no affinity for GABA receptor sites (Krogsgaard-Larsen and Johnston, 1978); thus, nip when used for studying the physiological role of GABA uptake, based on electrophysiological recording and microelectroretic application of nip on single neurones, enhances the depressant effect of simultaneously ejected GABA (Curtis et al., 1976). The GABA analogues DABA (Martin, 1976; Iversen and Kelly, 1975), and ACHC (Beart et al., 1972), are

inhibitors of the neuronal GABA uptake system, and are substrates for the GABA transport carrier (Neal and Bowery, 1977). ACHC appears to be more selective for neuronal as compared to glial uptake of GABA than are either DABA or nip (Bowery et al., 1976 a). Such inhibitors have little direct action on neuronal firing, and can be used in microelectrophoretic experiments to examine GABA inactivation in the synaptic environment, where they potentiate the depressant action of GABA (Curtis et al., 1976). By contrast, b-ala, however, is taken up into glial cells via a sodium-dependent, high affinity, structurally specific uptake process (Schon and Kelly, 1975), and such uptake of b-ala can be inhibited by various compounds (Johnston and Stephanson, 1976). b-ala has substantial use as a glial uptake inhibitor for GABA, and it also enhances GABAergic actions in the CNS (Curtis et al., 1976).

Whilst more sophisticated techniques have been developed to study GABA uptake and inactivation in the CNS, such studies on GABA transport have not been extended to the enteric nervous system in any specific detail, although the presence of a selective high affinity uptake system for GABA in the myenteric plexus of the guinea-pig taenia coli was first implicated by Jessen et al. (1979). Following this, active GABA uptake has since been confirmed to be present in various segments of the guinea-pig intestine (Taniyama et al., 1983 a; Krantis and Kerr, 1981 a; Kerr and Krantis, 1983; Kerr et al., 1983). However, the knowledge of the physiological significance of myenteric GABA transport and its mechanism at the molecular level is currently

scarce, but from the present investigation, it is suggested that GABA-mediated synaptic transmission in the intestine is terminated by the removal of GABA from the synaptic cleft into the surrounding cells by specific transport systems, where GABA is taken up into both neuronal and glial cell compartments, analogous to that for GABA in the CNS (Iversen and Kelly, 1975; Roberts et al., 1979). Such GABA uptake systems have been studied here in the guinea-pig isolated ileum, where the effects of the various neuronal and glial cell uptake blockers for GABA have been tested against responses induced by GABA and by its analogues, 3-APS, muscimol and baclofen, that are not significantly transported. The responses have been quantified accordingly in a dose-dependent manner.

The potentiation of the GABA-induced contractile responses in the guinea-pig isolated ileum by inhibitors of neuronal GABA uptake, such as ACHC (Neal and Bowery, 1977), nip (Krogsgaard-Larsen, 1980; Johnston et al., 1976 a), or DABA (Iversen and Johnston, 1971), and of glial uptake by b-ala (Schon and Kelly, 1974), suggests that exogenously applied GABA is removed from its site of action at the cholinergic neurones in the myenteric plexus by active transport into both neurones and glial cells. However, the more marked potentiation of such contractile responses elicited by GABA in the presence of the neuronal inhibitors, when compared to the responses in the presence of b-ala, indicates that a larger proportion of GABA is taken up into the neuronal compartment than into glial cells. The nature of the shift in the dose-response curves for GABA-induced contractile

responses produced by uptake inhibition, where there was a more significant shift of the curves to the left over the lower dose-range of the applied GABA, further suggests the presence of a saturable uptake mechanism in the intestine, with saturation occurring at the higher to maximal doses of GABA, similar to that for the catecholamines in the peripheral tissues (Langer and Trendelenburg, 1969), and GABA in the crayfish stretch receptor (Deisz et al., 1984).

In similar studies in the invertebrate, Roberts et al. (1979) showed that nip at 1 mM, gave a leftwards shift of the GABA dose-conductance curve of the crayfish stretch receptor, with the relative enhancement by nip being greatest at the lower GABA concentrations (c.f. Fig. 5 in Roberts et al., 1979); the addition of 1 mM nip by itself, did not affect membrane conductance at this concentration but enhanced the conductance increase produced by a submaximally effective concentration of GABA (40  $\mu$ M) (c.f. Fig. 2 in this chapter). The curve for muscimol was not affected by nip (c.f. Fig. 5 in Roberts et al., 1979) as muscimol has very low affinity for the GABA transport recognition site. In the absence of nip, lower concentrations of muscimol appeared to be far more potent than GABA in producing conductance changes, while at higher concentrations GABA appeared to be more effective, but in the presence of nip, GABA was more potent than muscimol over the entire dose-range. This suggests that, when removal by the transport system is eliminated in this preparations, GABA may actually have a greater affinity for the receptor site than muscimol. Also, in an independent study by Deisz

and Dose (1983), the slow development of the postsynaptic conductance change observed with lower concentrations of GABA is primarily due to the presence of a saturable uptake system of limited capacity, which limits the access of bath applied GABA, the response to low GABA concentration being greatly accelerated by nifedipine, whereas muscimol, being a poor substrate for the high affinity transport carrier, caused instantaneous dose-dependent conductance increases which were unaffected by nifedipine.

In the present study on the effect of these uptake blockers of GABA on the 'after-relaxation' induced by GABA in the guinea-pig isolated ileum, there was clearly an enhancement in the presence of the uptake inhibitors, but these did not affect the baclofen-induced 'after-relaxation', baclofen evidently not being taken up into either neurones or glia, confirming that baclofen has no affinity for the GABA transport system (Tardy et al., 1978). The potentiation of the GABA-induced 'after-relaxation' confirms that GABA uptake sites are associated with the GABA<sub>B</sub>-receptors of the tertiary plexus in the myenteric plexus, as already indicated by autoradiographic studies using [<sup>3</sup>H] GABA in the dissected longitudinal muscle-myenteric plexus preparation of the guinea-pig intestine (Krantis and Kerr, 1981 a).

DABA (Harris et al., 1973) and, more particularly, ACHC are said to be more relatively selective inhibitors of neuronal GABA uptake, with minimal GABA-mimetic activities (Beart et al., 1972; Bowery et al., 1976 a; Larsson et al., 1983 a), thus the potentiation, by DABA or ACHC of GABA-induced ileal responses over the

lower dose-range for GABA, can be ascribed to inhibition of a neuronal uptake in the myenteric plexus that limits the concentration of GABA at the GABA<sub>A</sub>-receptors under such circumstances. The additional potentiation with simultaneously added b-ala is then to be expected, since uptake into glia cells would, though evidently to a lesser degree, similarly limit GABA actions in the plexus. It follows that the combined inhibition of both glia and neuronal GABA uptake will allow a greater GABA concentration at the receptor sites over the lower dose-range, before substrate saturation of the uptake process limits the potentiating effect of uptake inhibition at higher GABA doses.

Nip appears to be less potent than either DABA or ACHC in potentiating the ileal contractile responses to GABA, and moreover, is said to be less specific in its action as a neuronal uptake blocker for GABA (Schousboe et al., 1979; 1981 b), since it is also an inhibitor of glia uptake (Krogsgaard-Larsen, 1980). Nevertheless, it has proved to be an effective agent in potentiating GABA actions in the ileum, with an additional potentiation when both nip and b-ala were used together. This indicates that, despite the contention that nip can inhibit glial uptake of GABA (Schousboe et al., 1979), there was a glial uptake component for GABA that could be inhibited with b-ala even in the presence of nip, leading to an increased potentiation of the GABA actions in the ileum in the combined presence of these uptake inhibitors. Unfortunately, all the samples of the uptake blockers used in the present study, such as ACHC, DABA and nip were racemates, consequently, it is not possible to assign true

relative potencies of the uptake blockers in the present experiments.

Contractile responses to muscimol and 3-APS, both of which are GABA-mimetics in the ileum (Kaplita et al., 1982; Giotti et al., 1983 a; Ong and Kerr, 1983 a,b; Kerr and Ong, 1984 a,b), were not affected by any of the GABA uptake inhibitors used. Thus, these GABA analogues were evidently not significantly taken up into myenteric neurones or glia by the GABA transport system. However, this appears to be in contrast to results reported by Brown and Scholfield (1984), who showed that nip potentiates the responses of the olfactory neurones to exogenous GABA and its analogues, 3-APS and muscimol, suggesting that there is a carrier-mediated transport system for 3-APS and muscimol in that tissue.

Muscimol has minimal affinity for GABA uptake system (Johnston, 1971) as it appears to be only a weak substrate for the high affinity GABA uptake system in cerebral cortical slices, this being inhibited by ACHC (Johnston et al., 1978), whilst the actions of iontophoretically applied muscimol in the CNS may be limited by an uptake process (Lodge et al., 1978). Desarmanien et al. (1980) have shown that muscimol is not a substrate for the GABA transporter in glial cells. However, there is a separate uptake mechanism for taurine in neurones and glia, insensitive to ACHC or DABA but sensitive to b-ala (Balcar and Chronwall, 1981), and it is possible that 3-APS, which is homotaurine, the higher analogue, could also be transported through this uptake mecha-



nism, yet the actions of 3-APS were unaffected by ACHC, DABA, or b-ala in the present study, and it is thus unlikely that significant uptake of 3-APS occurs through a taurine transport mechanism in the myenteric plexus. Previous studies have shown that 3-APS has, indeed, low affinity for the GABA carrier (Bowery et al., 1976 b), which prevents it from being taken up into either neurones or glial (Krogsgaard-Larsen et al., 1983 a; 1984), yet, nevertheless, it is a potent GABA-like depressant with little effect on GABA uptake (Beart and Johnston, 1973). Here, responses to 3-APS or to muscimol did not appear to be limited by uptake, unlike the responses to GABA where such limitation evidently did occur in the absence of uptake inhibition. Indeed, when both neuronal and glial uptake of GABA was inhibited then, over much of its dose-response curve, GABA appeared here to be more potent than muscimol in eliciting ileal contractions, contrary to earlier observations made in the absence of uptake blockers (Kaplita et al., 1982; Giotti et al., 1983a; Ong and Kerr, 1983 a,b).

Dose-response curves for GABA-induced ileal contractions were displaced to the right by BMC, a GABA<sub>A</sub>-receptor antagonist and by PIC, which acts at the chloride-ionophore complex. This rightwards shift of the GABA dose-response curves, due to BMC or PIC, persisted in the presence of uptake blockers, but the rightwards shift was less than that seen in their absence. This is evidently an example of the interaction between antagonism and potentiation by uptake inhibition as discussed by Kenakin (1982, c.f. Fig. 3), where simultaneous antagonism and potentiation, by

uptake inhibition, cause the dose-response curve to be shifted rightwards less than would occur with antagonism in the absence of concomitant uptake inhibition. These results, and the parallel observations of an apparently increased potency of GABA brought about by inhibition of GABA uptake, stress that observations on relative potencies of GABA agonists should take into account such limitation of agonist action by uptake processes.

A weak GABA-mimetic action of b-ala, nip or DABA cannot be excluded in the ileal preparations here, where they elicited BMC- and PIC-sensitive contractile responses at high concentrations, although other studies have shown that these uptake inhibitors do not interact with the binding sites for GABA at postsynaptic receptors in vitro (Krogsgaard-Larsen and Johnston, 1978). Moreover, the use of radiolabelled uptake blockers to specifically label the uptake substrate recognition sites (Breckenridge et al., 1981; Lloyd and Vargas, 1982) in autoradiographic studies in tissue preparations (Hosli and Hosli, 1978; Neal et al., 1979; Cunningham et al., 1981; Agardh and Ehinger, 1982; Schon and Kelly, 1975), shows that these uptake blockers can themselves be transported in to the neural or glial tissues (Schon and Kelly, 1975; Larsson et al., 1983 a,b), and released in a manner similar to that with GABA (Johnston et al., 1976 b; Minchin, 1979). Thus, although the uptake blockers primarily interact with GABA uptake sites and not with GABA-receptor sites (Krogsgaard-Larsen, 1978), the possibility that the uptake blockers may have a direct action at postsynaptic GABA receptor sites or its ionophore cannot be totally discounted (Lodge et al., 1977), e.g. b-ala is a potent

neuronal depressant (Curtis and Watkins, 1960), as well as an inhibitor of GABA receptor binding in vitro (Enna and Snyder, 1975).

There may be some coupling between the neuronal GABA uptake process and the release system by a yet unknown mechanism, as there is evidence that ACHC and related amino acids, but not nip, induce GABA release from synaptosomes (Early et al., 1981), whilst b-ala has been shown to depolarize neurones indirectly through the release of GABA from glia by an exchange mechanism (Bowery et al., 1976 b). There is evidence for indirect GABA-mimetic activities of DABA and ACHC in isolated cortical tissues (Galvan and Brown, unpublished), similar to results obtained in the present investigation, where both DABA and ACHC induced transient contractile responses of the ileum at very high concentrations. B-ala also exerts weak contractile responses of the ileum, and has been reported to have a direct depressant action on spinal neurones, possibly as a result of its GABA-mimetic effects (Curtis and Watkins, 1960). Alternatively, such GABA-mimetic actions of uptake blockers may reflect the consequent accumulation of endogenous GABA in the synaptic region following uptake inhibition, as suggested for nip by Krosgaard-Larsen et al. (1975). Indeed, the development of selective, rigid, conformational structures of GABA as specific neuronal or glial uptake inhibitors (Krosgaard-Larsen, 1980) may be a useful approach to raise GABA levels at the synapse and prolong the course of actions of synaptically released GABA, as well as to modulate the effects of GABA in both central and peripheral nervous systems,

where on-going intrinsic GABAergic transmission results in the release of endogenous GABA.

In the guinea-pig isolated vas deferens preparations, both DABA and ACHC enhance the GABA-induced inhibitory effect on electrically evoked twitch contractions, b-ala having no effect on the sensitivity of the tissue to GABA (Bowery et al., 1981 c). The presence of a neuronal uptake system is thus demonstrable in this particular model, and inhibiting this neuronal uptake system enhances the maximum inhibitory effect induced by GABA. However, the significance of the absence of a glial uptake system in this tissue is unclear, when compared to the guinea-pig ileum that does appear to have both neuronal and glial uptake.

In a broader context, since the uptake mechanisms in nerve terminals and glial cells are important means for terminating the actions of GABA, selective inhibition of these consequently may be relevant with respect to facilitation of GABAergic neurotransmission in diseases with impaired function of the GABA system, where GABA is both directly and indirectly implicated in the pathogenesis of Huntington's Chorea (McGeer and McGeer, 1976 a,b; Enna et al., 1977 b; Lloyd et al., 1975; 1977 a), Parkinson's disease (Hornykiewicz et al., 1976; Lloyd et al., 1975; 1977 b; McGeer and McGeer, 1976 b), and possibly of schizophrenia (Roberts, 1974) and epilepsy (Meldrum, 1975).

It is important to keep the extracellular GABA concentration

low, not only to prevent an undue continuous inhibitory action, but also to prevent desensitization of GABA receptors (Desarmenien et al., 1980). It is still debatable as to what extent the uptake of GABA into presynaptic terminals may be responsible for the termination or inactivation of GABA, since the major part of the high affinity GABA uptake into synaptosomes is due to a 1:1 homoexchange process (Levi and Raiteri, 1974; Raiteri et al., 1975), that can be of no physiological importance for the removal of GABA from synaptic clefts. Glial or postsynaptic uptake is therefore likely to be more important. Henn and Hamberger (1971) were the first to demonstrate that glial cells have a high affinity uptake system for GABA, and since then, such a glial transport system has been demonstrated in peripheral ganglia (Bowery and Brown, 1972; Young et al., 1973; Schon and Kelly, 1974; Roberts, P.J., 1976 a,b), rat retina (Neal and Iversen, 1972), and primary cultures of glial cells from cerebellum (Lasher, 1975; Bury and Lasher, 1975; Hosli and Hosli, 1976 a), and also in the cerebrum (Schousboe et al., 1977; Hertz et al., 1978). A difference between the synaptosomal and glial uptake systems, for GABA seem to be in their sodium dependence, as the synaptosomal high affinity transport for GABA requires 2-3 sodium ions per GABA molecule (Martin and Smith, 1972; Martin, 1973), whereas the corresponding uptake into cultured astrocytes requires only 1 sodium ion per GABA molecule (Hertz et al., 1978).

Muscimol, a potent BMC-sensitive GABA receptor agonist

at postsynaptic GABA-receptor sites (Curtis et al., 1971 b; Enna and Snyder, 1975; Enna et al., 1977 a; Krosggaard-Larsen et al., 1975; 1979 a,b; Krosggaard-Larsen, 1978) has no effect on GABA uptake systems, or only slight effect on GABA uptake into mini-slices which primarily represents neuronal uptake (Schousboe et al., 1978), although other reports suggest that muscimol does interact with GABA uptake processes (Johnston et al., 1978; Schousboe et al., 1979). In the present study, however, muscimol did not appear to have any affinity for either the neuronal or glial uptake systems in the guinea-pig isolated ileal preparations.

Glial accumulation of [3H] GABA is inhibited by b-ala but not by DABA (Schon and Kelly, 1974), and b-ala is a potent stimulator of [3H] GABA release from the glial cells of rat dorsal root ganglia, whilst DABA is less effective in inducing GABA release (Minchin, 1975). DABA is capable of exchanging freely with intrasynaptosomal GABA, primarily with neuronal pools of GABA, and b-ala exchanges with the glial pool (Brennan and Cantrill, 1978). It has been demonstrated that nip may enhance the spontaneous release of GABA in rat cortical slices, involving an intracellular heteroexchange mechanism (Szerb, 1982). Racemic mixtures of nip, employed at a concentration of 1 mM, inhibit GABA uptake into brain slices by 99% (Krosggaard-Larsen and Johnston, 1975), being a potent inhibitor of both neuronal and glial GABA transport (Krosggaard-Larsen, 1980; Krosggaard-Larsen and Johnston, 1975; Schousboe et al., 1978). (-) Nip is much more potent than the (+) isomer, and the IC50 for (+) nip is,

however, much higher for the glial transport than for the neuronal transport (Johnston et al., 1976 a), and so the (+) isomer may be a more specific blocker of neuronal GABA transport.

A  $\text{Ca}^{2+}$ -dependent, potassium-induced release of GABA has been observed from peripheral ganglia (Minchin and Iversen, 1974; Minchin, 1975; Roberts, P.J., 1976 a), where GABA is exclusively located in glial cells, and high affinity GABA uptake has been demonstrated into glial cells in peripheral ganglia (Bowery and Brown, 1972; Young et al., 1973; Schon and Kelly, 1974). A cellular high affinity GABA uptake can be of importance for termination of transmitter activity, provided it occurs with a reasonably high  $V_{\text{max}}$  and represents a net accumulation rather than a 1:1 homoexchange process. It has been concluded that the uptake of GABA into satellite cells of sensory ganglia also represents a homoexchange mechanism (Roberts, P.J., 1976 b), but there are no synapses on sensory ganglion cells. The glial cells in the sympathetic ganglia, which has high affinity carriers for GABA (Bowery and Brown, 1972), release GABA by a different mechanism from that in the neurones, as there is no evidence for vesicular or quantal release, whilst the rate of release is relatively insensitive to changes in external  $\text{K}^+$  concentration and is a  $\text{Ca}^{2+}$ -dependent process (Brown, 1979). The kinetic properties of the glial transport process (relatively low velocity, but high capacity), render it more suitable for effective buffering of extracellular GABA levels in the long term, and this is supported by experiments which show that inhibiting the glial carrier does not affect the duration of synaptic inhibition.

GABA release (Neal and Bowery, 1979), synthesis and degradation may also occur in glial cells (Haber et al., 1970 b). Not all glial cells in the nervous system are located in the vicinity of GABA-releasing nerve terminals. Glial cells in the peripheral ganglia, pineal or pituitary glands would not be exposed to GABA, since these structures do not contain GABA-inhibitory neurons, not do they receive any form of GABAergic innervation. Therefore, it seems likely that GABA plays some part in glial function unrelated to its inhibitory transmitter role. As exogenous [3H] GABA can be released from glial cells in sympathetic or sensory ganglia (Minchin and Iversen, 1974; Bowery et al., 1976 b), and GABA receptors exist on the surface of ganglionic neurons (DeGroat et al. 1972), it is possible to speculate that GABA could function as an inhibitory modulator released from glial cells, therefore controlling excitability of nearby neurons (Iversen and Kelly, 1975), but the absence of a GABAergic innervation in these tissues makes the significance of this somewhat obscure.

3-APS is a potent agonist with low affinity for the GABA carrier (Bowery et al., 1976 b), since 3-APS-induced depolarization of the sympathetic ganglion cells is not augmented by b-ala, although b-ala releases both endogenous and [3H] GABA from glial cells in these tissues, whereas muscimol appears to be taken up into nerve terminals by the high affinity GABA uptake system which is inhibited by the neuronal uptake inhibitor ACHC, but not



by the glial uptake inhibitor b-ala, and can then be subsequently released in a  $\text{Ca}^{2+}$ -dependent manner by high  $\text{K}^+$  stimulation (Johnston et al., 1978). It is thus suggested that GABA and muscimol can be counter-transported using the same mobile high affinity carrier, although the affinity of muscimol for the GABA high affinity carriers was at least 50 fold less than that for GABA. Nevertheless, the inhibitory action of muscimol is enhanced to a similar extent to that of GABA by (-) nip and (+) DABA (Lodge et al., 1978) when administered electrophoretically near spinal neurones of cats. From the present study in the ileum, both 3-APS and muscimol did not have an apparent affinity for either the neuronal or glial uptake processes.

More detailed stereostructure-activity studies on the conformation of GABA analogues may provide some indirect information about the molecular interaction of GABA with the transport carriers and receptor sites, where the GABA receptors and the transport carriers exhibit a different stereoselectivity. Drugs which have no affinity for the transport carriers may be useful as therapeutic agents in inhibiting the uptake of the transmitter. Although the uptake inhibitors (e.g. nip) has been shown to enhance the inhibitory effects of microelectrophoretic application of GABA on neuronal firing in the CNS (Curtis et al., 1976), there is less evidence that uptake limits the action of endogenous GABA, as other workers have shown that uptake inhibitors have no effect on inhibitory synaptic actions (Curtis et al., 1976; Krogsgaard-Larsen, 1980; Lodge et al., 1977), but, in the hippocampus, both in situ and in slices, blockers of GABA

uptake did produce a clear enhancement of synaptically-mediated inhibition (Matthews et al., 1982; Korn and Dingledine, 1983). Such discrepancies in experimental results may be due to differences in electrophoretic techniques employed.

Recent investigations of the EDA-induced physiological GABAergic responses, due to the EDA-induced release of endogenous GABA (Kerr and Ong, 1984a) show potentiation of GABAergic responses by uptake inhibitors, as well as a reduction of the EDA-induced release of [3H] GABA from the myenteric plexus of the intestine upon preloading with [3H] GABA in the presence of the uptake blockers (see Chapter 3 of this thesis). In the present work on uptake blockers and GABA-induced responses in isolated ileal preparations, it is concluded from such use of GABA uptake inhibitors that GABA actions in the myenteric plexus are terminated predominantly by an active, saturable uptake process into neurones, and also to a smaller extent, into the glial cells. Evidently, the selectivity and potency of such uptake inhibitors for GABA could be readily examined using the GABA-induced contractile responses of the ileum described here. Hence, this model may be useful for future evaluation and screening of potential GABA analogues designed for use as uptake inhibitors, which may bear some therapeutic and clinical implications, as in the CNS, where GABA uptake inhibitors are considered potential drugs in epilepsy research (Krogsgaard-Larsen et al., 1981 b). In particular, drugs blocking GABA reuptake into glial cells may have potential anti-convulsant properties as well as anti-epileptic clinical values. A selective blockade of glial uptake may

lead to an increase in the amount of GABA taken up by the neuronal carrier with subsequent increase of GABA in the GABA pool within the nerve terminals, hence increasing the GABA available for neurotransmission (Krogsgaard-Larsen et al., 1981 a).

In conclusion, GABA neurotransmission in isolated intestinal preparations may be facilitated in a flexible manner through pharmacological manipulation of the GABA transport processes associated with termination of GABAergic activity in the intestine.

CHAPTER FIVE. BARBITURATE-INDUCED POTENTIATION  
OF GABA ACTIONS.

## 5.1. INTRODUCTION

Anaesthetic and anticonvulsant barbiturates have been shown to prolong and enhance GABA-mediated synaptic inhibition in many areas of the central nervous system (CNS) (Lodge and Curtis, 1978; MacDonald and Barker, 1978 a; 1979 a,b; MacDonald, 1983; 1984; Nicoll, 1978 b,c,d; Ransom and Barker, 1976; Schultz and MacDonald, 1981), by augmenting presynaptic and postsynaptic inhibition at a variety of sites where GABA is believed to be a transmitter, therefore leading to more pronounced effects of inhibitory inputs on neuronal activity. Presynaptic inhibition in the spinal cord and cuneate nucleus is known to be potentiated by barbiturates (Eccles et al., 1963; Miyahara et al., 1966; Polc and Haefely, 1976), and similarly these drugs also potentiate postsynaptic inhibition in the cuneate, olfactory bulb and hippocampus (Nicoll, 1972; Nicoll et al., 1975). The increase in GABA-mediated hyperpolarization of frog motoneurons (Nicoll, 1975 a,b) and the enhancement of the depolarizing action of endogenous GABA on primary afferents at presynaptic inhibitory synapses (see review by Nicoll, 1978 b) by barbiturates point to an interaction between barbiturates and GABA, occurring at the level of the GABA-receptor  $\text{Cl}^-$ -ionophore complex (Olsen, 1981 a,b), where barbiturates such as pentobarbital (PB) appears to be capable of activating  $\text{Cl}^-$  channel mechanisms through engagement of receptor-like sites on the cellular membranes of cultured mouse spinal neurons (Barker and Mathers, 1981), producing direct increases in membrane  $\text{Cl}^-$  conductance (MacDonald and Barker, 1979 a; Schultz and MacDonald, 1981).

This enhancement of synaptic inhibition described above may reflect an important therapeutic role for barbiturates, and may underlie some of the pharmacological properties of these barbiturates, among which are direct GABA-mimetic activities, antagonised by the GABA antagonists bicuculline and picrotoxin (Nicoll, 1975 b; Bowery and Dray, 1978). Many electrophysiological, biochemical and neurochemical studies have shown that barbiturates exert a number of general effects on synaptic transmission, neuronal membrane properties and axonal conduction, uptake and release of other transmitters, ion transport ( $\text{Ca}^{2+}$  in particular), binding of neurotransmitters to receptor-ionophore complexes, and mitochondrial function, as well as having pre- and postsynaptic actions on a variety of synapses (see recent reviews by Nicoll, 1978 b; Johnston, 1983; Johnston and Willow, 1982 a,b; Richter and Holtman, 1982; Straughan, 1979; Olsen, 1981 a,b; Willow and Johnston, 1983). It has been proposed that the anti-convulsant actions of barbiturates are due to augmentation of GABAergic transmission, whereas the sedative/anaesthetic actions are due to a reduction of presynaptic calcium influx and resultant blockade of transmitter release (see review by MacDonald, 1984).

Whether or not the barbiturates produce anti-convulsant, sedative, hypnotic or anaesthetic effects depends to a large degree on the effective concentrations used. Phenobarbital (PhB) enhances GABA-induced responses but also antagonises glutamate

responses at anti-convulsant, therapeutic serum concentrations (25-100  $\mu\text{M}$ ), and does not activate membrane  $\text{Cl}^-$  ion conductance or reduce calcium-dependent action potentials below 100  $\mu\text{M}$ , hence it is likely that barbiturates produce anticonvulsant actions at least in part, by enhancing postsynaptic GABA responses. At high concentrations (0.2-5 mM), which are in the high anaesthetic and toxic drug ranges, PhB increases membrane  $\text{Cl}^-$  ion conductance, and this may contribute to deep anaesthesia and drug overdose (MacDonald, 1984). PB, on the other hand, augments GABA responses, antagonises glutamate responses, reduces calcium-dependent action potential duration, blocks calcium uptake by synaptosomes, and reduces neurotransmitter release from presynaptic nerve terminals at concentrations which are anaesthetic (50-150  $\mu\text{M}$ ). It is thus likely that the addition of presynaptic reduction of calcium entry, and thus neurotransmitter release, to an increased postsynaptic modulation of neurotransmitter action may underlie sedative/hypnotic and anaesthetic barbiturate actions (see current review by MacDonald, 1984).

Responses mediated through the GABA<sub>A</sub>-receptor  $\text{Cl}^-$ -ionophore complex are modulated by both barbiturates and benzodiazepines, a conclusion supported by ligand binding studies which demonstrate that the GABA receptor complex is part of a larger macromolecular protein assembly comprised of specific functional receptor subunits for GABA, benzodiazepines, barbiturates, picrotoxin and the associated  $\text{Cl}^-$ -ionophore. Reciprocal  $\text{Cl}^-$ -sensitive interactions have been shown to occur at this complex, as a result of allosteric binding of various drugs to the modulatory sites on the GABA-

receptor complex, so modifying GABA activation of the GABA-receptor site (Olsen, 1981 a,b; 1982). Barbiturates are known to augment GABA-induced responses and increase GABA binding at its receptor, whilst picrotoxinin reduces the GABA-induced responses by binding to a site independent of the GABA-receptor (recognition) site (MacDonald and Young, 1981; Ticku and Olsen, 1978). Such barbiturate modification at the ionophore complex has been shown to result in both a potentiation of GABA-induced responses, and a barbiturate reversal of their antagonism by PIC, yet not by bicuculline, in frog motoneurons (Nicoll and Wojtowicz, 1980). Such modulatory effects by barbiturates are here described in the intestine, where the actions of GABA and related drugs at GABA<sub>A</sub>-receptor sites are also mediated through a Cl<sup>-</sup>-ionophore complex (Bowery et al., 1981 a; Krantis and Kerr, 1981 b; Kaplita et al., 1982; Ong and Kerr, 1983 a,b).

In several other different preparations, such as isolated frog motoneurons, and immature rat dorsal root fibres, barbiturates potentiate GABA<sub>A</sub>-receptor-mediated actions, and are able to reverse the non-competitive antagonism of GABA by PIC, restoring the slope of the dose-response curve for GABA and the maximum response, but do not alter the dose-ratio for BMC-induced antagonism of the GABAergic responses, the latter acting at the GABA-receptor site itself and not at the Cl<sup>-</sup>-ionophore complex as does PIC (Evans, 1979; Nicoll and Wojtowicz, 1980; Simmonds, 1981). Thus, by examining the nature of the interaction between barbiturate potentiation and GABA antagonism, it is possible to distinguish the site of action of a particular antagonist at the GABA-



receptor  $\text{Cl}^-$ -ionophore complex.

Several relatively simple compounds with a lactam or lactone moiety are convulsants in the CNS (Elison et al., 1971; Klunk et al., 1982). In particular, caprolactams with alkyl substituents at C4 and C6 are considerably more potent than the parent lactam (hexahydro-2H-azepin-2-one), whilst caprolactams with bulky substituents at C7 of the ring are sedatives (Duong et al., 1976). These convulsant caprolactam derivatives are evidently antagonists at the GABA-receptor-ionophore complex (Kerr et al., 1976), which accounts for their convulsant properties, but little is known of the detailed mechanism of this antagonism beyond the demonstration of a PIC-like  $\text{Cl}^-$ -dependent partial inhibition of GABA<sub>A</sub>-receptor binding on synaptosomal preparations, (Skerritt et al., 1985). This has prompted a re-examination of the caprolactam-induced antagonism at the GABA-receptor-ionophore complex, using the isolated ileum of the guinea-pig in which the properties of the GABA<sub>A</sub>-receptor have been well characterised (Bowery et al., 1981 a; Krantis and Kerr, 1981 b; Ong and Kerr, 1983a,b). In the enteric nervous system of the guinea-pig, cholinergic neurones in the myenteric plexus of the ileum are stimulated by GABA which releases endogenous acetylcholine (ACh) from the neurones, resulting in transient ileal contractile responses, most often followed by a delayed and prolonged 'after-relaxation' (Bowery et al., 1981 a; Kaplita et al., 1982; Giotti et al., 1983 a; Ong and Kerr, 1983 a,b). The contractile response is  $\text{Cl}^-$ -dependent and bicuculline-sensitive, and is due to the stimulation of GABA<sub>A</sub>-receptor sites, whereas the 'after-relaxation' is a

GABA<sub>B</sub>-receptor-mediated effect. The latter is insensitive to bicuculline, and Cl<sup>-</sup>-independent, and results from a depressive action of GABA on the cholinergic motor neurones, so reducing ACh output (Bowery et al., 1981 a; Ong and Kerr, 1983a,b). This GABA<sub>B</sub>-receptor-mediated inhibition of ACh output is however, antagonised by a weak GABA<sub>B</sub>-receptor antagonist, 5-aminovaleric acid (DAVA) (Muhyaddin et al., 1982 a,b ; Ong and Kerr, 1983 b).

The present study investigates if the GABA<sub>A</sub>-receptor complex in the enteric nervous system of the guinea-pig isolated ileal preparation is at all similar to that found in the CNS, and to see if barbiturates such as PB, PhB, thiopentone (ThP) or barbital (Bb), might interact with the receptor-ionophore complex on neurones of the myenteric plexus in the intestine to potentiate the contractile responses induced by GABA, muscimol and 3-amino-1-propanesulphonic acid (3-APS), at the GABA<sub>A</sub>-receptor sites. In addition, it examines whether barbiturates can reverse the antagonism of such GABA-induced ileal contractions by PIC, and by a class of convulsant caprolactam derivatives (Kerr et al., 1976), since the latter has recently been shown to induce a PIC-like Cl<sup>-</sup>-dependent partial inhibition of GABA receptor binding (Skerritt et al., 1985) and are thought to be antagonists of GABA-induced responses at the PIC/barbiturate binding site. In particular it examines if there is a barbiturate-dependent reversal of the non-competitive antagonism of GABA-induced ileal contractile responses by the convulsant caprolactam TMC (4,6,6-tri-Me-caprolactam). In this way, it should be possible to discern if the convulsant caprolactams antagonise GABA<sub>A</sub>-receptor-mediated actions by acting

at the  $\text{Cl}^-$ -ionophore complex rather than at the GABA<sub>A</sub>-receptor site itself. . . . The latter can be arrived by comparing barbiturate actions on the effects of TMC and bicuculline-induced antagonism of the GABA<sub>A</sub>-receptor-mediated contractile responses in the ileum.

## 5.2. MATERIALS AND METHOD

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of the ileum, 3-4 cm in length, taken 2-3 cm from the ileo-caecal valve, and segments of the distal colon, also 3-4 cm in length, were quickly removed and mounted in a 10 ml organ bath containing oxygenated Krebs-bicarbonate solution (composition as described in Chapter 2.1). Mechanical activity of the longitudinal muscle was recorded according to the method described (Chapter 2.1), and the equilibration period for the tissues in the organ bath was 60 min. Electrical stimulation of intrinsic cholinergic neurones was elicited, using a pair of parallel platinum electrodes positioned around the tissues, and employing the parameters described in Chapter 2.1. Drugs were applied within 15-20 min intervals, and antagonists were added at least 5-10 min before agonists were tested, depending on the experiment. Student's t-test for paired and unpaired samples was used to assess the significance ( $P < 0.05$ ) of differences between mean values of the dose-response effects. All experiments were run in duplicate, and were repeated at least  $n=6$  times on tissues from at least 4 different animals.

### CHEMICALS:

All the chemicals used were from Sigma, unless otherwise indicated in parenthesis.

GABA

bicuculline methochloride (Pierce)

picrotoxinin (dissolved in 1:9 absolute alcohol and distilled water)

pentobarbitone

thiopentone (May and Parker)

phenobarbitone (British Drug Houses)

barbitone

atropine sulphate

tetrodotoxin

baclofen

muscimol

3-amino-1-propanesulphonic acid

acetylcholine chloride

histamine dihydrochloride

5-hydroxytryptamine creatinine sulphate

substance P

nicotine

4,6,6,-tri-Me-caprolactam (Dr. D.I.B. Kerr)

### 5.3. RESULTS

#### 5.3.1. Potentiation of GABA-induced responses by barbiturates in the guinea-pig ileum.

Exogenously applied pentobarbitone (PB) (0.2 mM) did not itself elicit any response in the isolated ileum, but brought about a transient contractile response to exogenously applied GABA (0.1  $\mu$ M), which was sensitive to TTX (0.1  $\mu$ M) and atropine (0.1  $\mu$ M), when applied prior to GABA. This subthreshold dose of GABA (0.1  $\mu$ M) in itself had no effect on the tissue in the absence of PB (Fig. 1). There was no 'after-relaxation' when both PB and GABA were present (Fig. 1). However, as shown in Fig. 1, a higher dose of GABA (5  $\mu$ M) elicited a contractile response followed by an 'after-relaxation', typical of a GABAergic response in the ileum, and PB (0.2 mM) enhanced the contractile response significantly without altering this GABA-induced 'after-relaxation'. Upon tissue wash-out, the control response to GABA (5  $\mu$ M) was re-established. The threshold concentration for PB to enhance GABA-induced responses was found to be 0.2 mM, there being a dose-dependent enhancement up to a maximum with PB (0.5 mM), after which the potentiation decreased when using PB at a concentration of 0.7-1 mM (n=6). The enhancing action of PB on the GABA-induced contractile responses was seen only over the lower dose range of exogenously applied GABA, where its dose-response curve was shifted to the left by PB (0.2-0.5 mM) in a dose-dependent manner (Fig. 2), such that the threshold concentration for GABA-induced contractions was lowered and larger

responses were seen with doses of GABA less than 50  $\mu$ M (Fig. 2). Similar potentiating actions by both thiopentone (ThP) and barbitone (Bb) on the GABA-induced responses were also seen (Fig. 2), such potentiation also being effective over this lower dose range of applied GABA, with a threshold concentration for the enhancement of GABA-mediated contractile responses by ThP at 80  $\mu$ M, and that for Bb at 0.5 mM.

Dose-response curves for GABA<sub>A</sub>-receptor-mediated ileal contractile responses were constructed in the presence of increasing concentrations of all the barbiturates used (ThP, PB, Bb and phenobarbitone (PhB)), up to concentrations that began to depress rather than potentiate the GABA-induced responses. There was no significant difference in the dose-response curves for the potentiation of the GABA<sub>A</sub>-receptor-mediated contractile effect of GABA in the presence of equipotent concentrations of ThP (100  $\mu$ M), PB (500  $\mu$ M) and Bb (5 mM), all points falling with no significant departure from the PB (0.5 mM) curve of Fig. 2. Using equal concentrations of ThP, PB and Bb (0.5 mM), their potentiating effects on the dose-response curves for GABA are compared in Fig. 2, the potentiating potencies being ThP > PB > Bb. PhB was the least effective potentiator in comparison with these 3 barbiturates (Fig. not shown), with a threshold concentration of 1 mM (n=4). As could be observed in a number of ileal preparations (n=32), these barbiturates never affected the GABA-induced 'after-relaxation' (c.f. PB effect on GABA response in Fig. 1), nor the baclofen-induced 'after-relaxation' or the baclofen(50  $\mu$ M)-induced depression of cholinergic repetitive twitch

contractions (n=6) (Fig. 3).

### 5.3.2. Barbiturate potentiation of responses induced by GABA analogues.

Muscimol and 3-amino-1-propanesulphonic acid (3-APS) also elicited dose-dependent contractile responses in the isolated ileum, with their relative potencies being muscimol approximately 8 fold more potent than GABA, and 3-APS 5 fold more potent than GABA, measured from the ED<sub>50</sub>s. PB(0.5 mM) significantly potentiated the contractile responses induced by muscimol (1  $\mu$ M) (Fig. 4) and 3-APS (5  $\mu$ M), both of which elicited only contractile effects without any 'after-relaxation', with a recovery of the control contractile responses after washing out PB. The dose-response curves for the potentiating effects of PB (0.5 mM) on responses to muscimol and 3-APS showed a significant shift to the left, akin to that with GABA, and similarly there was a greater shift over the lower dose range of the applied muscimol (0.1-50  $\mu$ M) (Fig. 5) or 3-APS (0.1-50  $\mu$ M) (Fig. 6). The potentiation of these ileal contractile responses to muscimol and 3-APS by PB was also dose-dependent, with a threshold concentration of PB at 0.2 mM and the maximal effect at 0.5 mM. The other barbiturates, ThP, Bb and PhB exhibited potentiating potencies on muscimol and 3-APS similar to those with GABA (Fig. not shown).



### 5.3.3. GABA-mimetic actions of the barbiturates.

High doses of ThP (0.5 mM), PB (>0.5 mM), Bb (>5 mM) and PhB (10 mM) often elicited GABA-like contractile responses, sensitive to bicuculline methochloride (BMC) (5-50  $\mu$ M) or picrotoxinin (PIC) (5-50  $\mu$ M); the antagonism of the contractile responses to ThP, PB and Bb by BMC and PIC (Fig. not shown). These contractile responses to PhB were only small relative to those elicited by ThP, PB or Bb. The enhancement of GABA-induced contractile effects by these barbiturates could not be due to these GABA-mimetic actions, since GABA-induced responses were still potentiated when GABA, muscimol or 3-APS were applied some 60s after the responses induced by the barbiturates had subsided. Higher concentrations of these barbiturates (ThP (1 mM), PB (1 mM), Bb (10 mM), PhB (>10 mM)) depressed the GABA-induced contractile responses, rather than augmenting these responses.

None of the barbiturates, ThP, PB, Bb and PhB at their effective concentrations, potentiated baclofen- or GABA-induced relaxations in the distal colon, and these barbiturates at the concentrations used to potentiate GABA-induced responses, did not affect cholinergic responses to electrical stimulation (c.f. Fig. 4), or any other muscle contracting agents such as ACh, histamine, 5-HT, or substance P.

#### 5.3.4. Barbiturate interaction with GABA antagonism by BMC or PIC.

GABA (10  $\mu$ M)-induced ileal contractions, but not the delayed prolonged 'after-relaxations', were completely antagonised by BMC (10  $\mu$ M), and the GABA-induced contraction potentiated by PB (0.5 mM) was also reduced in the presence of BMC (Fig. 7a). Upon tissue wash-out, the contractile response to GABA (10  $\mu$ M) recovered to the same amplitude as the control shown in the same figure. BMC, used at increasing doses (5-10  $\mu$ M), gave parallel rightwards displacements of dose-response curves for the GABA-induced contractile response in the guinea-pig ileum with no apparent depression of the maximal response (Fig. 7b), and a  $pA_2$  value of 5.8 for this competitive antagonism was estimated (c.f. Krantis and Kerr, 1981a). PB, used at 0.5 mM, shifted the dose-response curve for the GABA-induced contractile effect to the left over the lower dose range of GABA, in potentiating the contractile responses (Fig. 2 and 7b). In the presence of BMC at 5 or 10  $\mu$ M respectively, PB (0.5 mM) again shifted the dose-response curves to the left without altering the slope of the dose-response curve or the dose-ratio (Fig. 7b).

By contrast, PIC (10-50  $\mu$ M), not only displaced the dose-response curve for GABA to the right in a dose-dependent manner but also lowered the slope of the curve and reduced the maximal response (Fig. 8), suggesting a non-competitive antagonism by PIC. Moreover, when using PIC (10, 50  $\mu$ M) at essentially equipotent doses to that of BMC above (5-50  $\mu$ M), PB (0.5 mM) reversed these

effects by not only shifting the dose-response curve for GABA to the left in the presence of the different concentrations of PIC, but restoring the slope and the maximal response toward that of the control dose-response curve for GABA (Fig. 8). As shown in Fig. 9, only the GABA (5  $\mu$ M)-induced contractile response and not the 'after-relaxation' was antagonised by PIC (10  $\mu$ M), and PB (0.5 mM) partially restored the GABA-induced response in the presence of PIC, although this could also be described as a PIC reversal of the the potentiating effect of PB on the GABA-induced contraction. The response to GABA (5  $\mu$ M) recovered after washing out both the PB and the PIC. The GABA-induced 'after-relaxation' was unaffected by BMC or PIC at all concentrations used (c.f. Ong and Kerr, 1983b), and even in the presence of PB (0.2-0.5 mM), with or without these antagonists, the GABA-induced 'after-relaxation' remained unaltered from that induced by GABA alone (Fig. 7,9).

The dose-response curves for muscimol (Fig. 5) and 3-APS (Fig. 6) were similarly shifted to the right in a parallel manner by BMC (10  $\mu$ M), analogous to that for GABA, and the dose-response curve for muscimol was displaced to the left by PB (0.5 mM) (Fig. 5). In the presence of BMC (10  $\mu$ M), PB again shifted the dose-response curve for muscimol to left without altering the slope of the curve or the dose-ratio (Fig. 5). Likewise, PB also shifted the dose-reponse curve for 3-APS to left in the presence of BMC (10  $\mu$ M), in a manner identical with that described for muscimol (Fig. 6). On the other hand, as with GABA, PIC (10  $\mu$ M) displaced the dose-response curves for these agonists to the right in a

non-competitive manner, with a depression of the maximal responses for both muscimol (Fig. 10) and 3-APS (Fig. 11), and PB (0.5 mM) again reversed the depression of the responses by PIC (10  $\mu$ M), shifting the dose-response curves for muscimol (Fig. 10) and 3-APS (Fig. 11) to the left and restoring the slopes and maximal responses toward that for the control dose-response curves for muscimol and 3-APS respectively (Fig. 10, 11).

#### 5.3.5. Antagonism of GABAergic responses by TMC.

The GABA (8  $\mu$ M)-induced contractile response, but not the delayed 'after-relaxation', was antagonised in a dose-dependent manner by TMC (500  $\mu$ M) (Fig. 12), which shifted the dose-response curve for GABA to the right in a non-competitive manner, depressing the maximum contractile response induced by GABA, as shown with TMC (500  $\mu$ M) in Fig. 13. Although this antagonism was comparable to the antagonism of the GABAergic response by PIC (c.f. Krantis and Kerr, 1981), the potency of TMC was some 10 fold less than that of PIC, with a threshold concentration of 100  $\mu$ M. Contractile responses to exogenously applied 3-APS (5-50  $\mu$ M) or muscimol (5-10  $\mu$ M) were similarly antagonised by TMC in a non-competitive manner, but TMC did not affect the 'after-relaxation'; the baclofen (50  $\mu$ M) or GABA (50  $\mu$ M)-induced depression of cholinergic twitch contractions (Fig. 14) at any of the concentrations employed. All these GABAergic contractile responses were antagonised within 5-10 min by TMC (100-500  $\mu$ M), with complete recovery after 10 min upon washing from the bath. TMC was specific in its action against these GABAergic contractions, as ileal respon-

ses to ACh, 5-HT, nicotine or transmural electrical stimulation of cholinergic neurones were unaffected (see Fig. 14).

#### 5.3.6. Barbiturate interaction with GABA antagonism by TMC.

PB (0.5 mM) reversed the non-competitive antagonism of the GABA(8  $\mu$ M)-induced contractile response by TMC (500  $\mu$ M) (Fig. 12), shifting the dose-response curve for GABA leftwards, with a restoration of the slope and the maximal response toward that for the control GABA dose-response curve (Fig. 13). The antagonism of 3-APS- or muscimol -induced contractile responses by TMC was similarly reversed by PB (Fig. not shown).

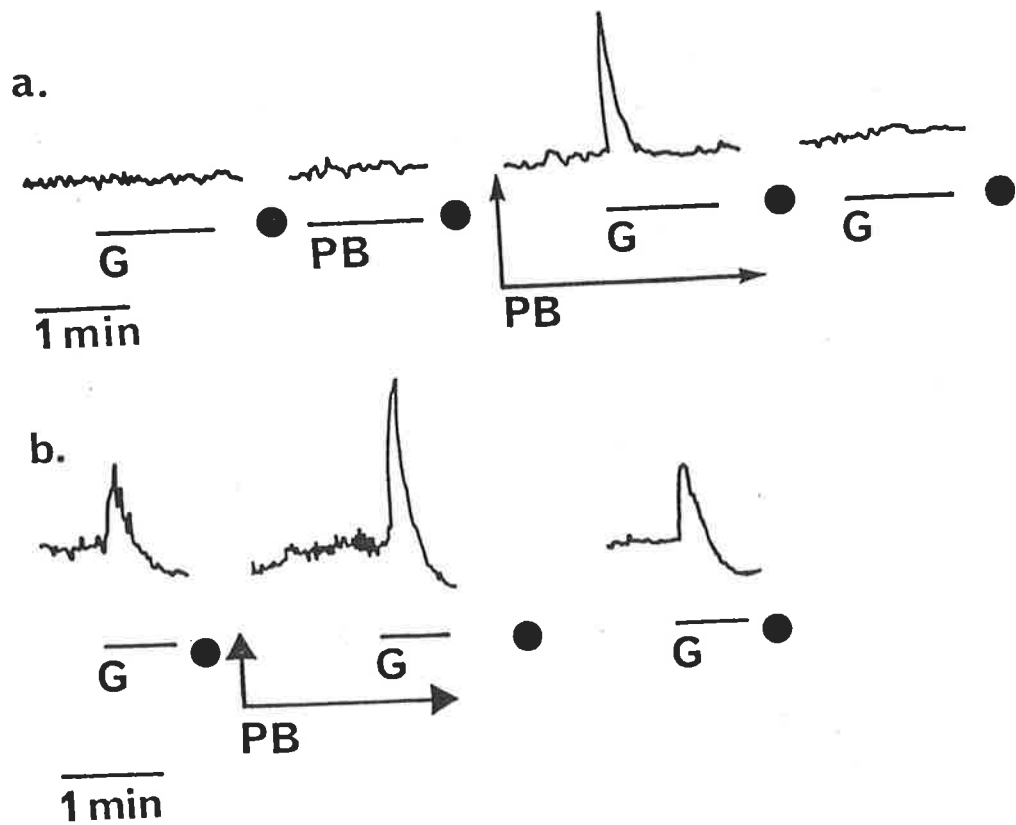


Fig. 1. Potentiation of the GABA-induced responses by PB in the isolated ileal preparations. (a) There was no response to exogenously applied GABA (0.1  $\mu$ M) or PB (0.2 mM), yet in the presence of PB (0.2 mM), a GABA-induced contractile response, but not the GABA-induced 'after-relaxation' was observed. Upon tissue washout, indicated by ●, no response to GABA (0.1  $\mu$ M) was observed. The number of experiments performed was at least  $n=10$  from a minimum of 4 tissues. (b) Potentiation of GABA (5  $\mu$ M)-induced contractile response, but not the 'after-relaxation' by PB (0.2 mM), the PB was applied 1 min before GABA. Control contractile responses to GABA prior to PB application and after PB washout are shown;  $n=32$ .

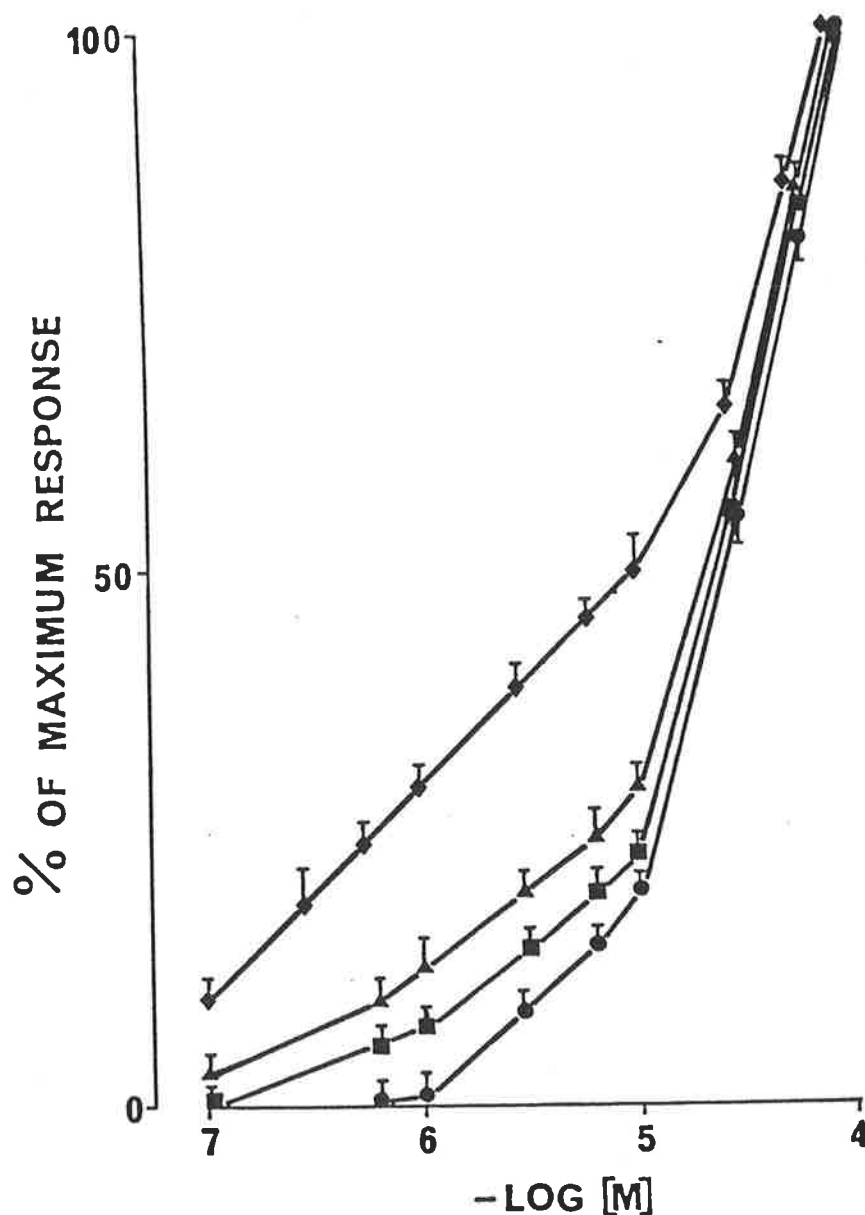


Fig. 2. Potentiating effects of barbiturates on the GABA-induced contractile responses in the isolated ileum.  $\blacklozenge$  thiopentone (ThP),  $\blacktriangle$  pentobarbitone (PB) and  $\blacksquare$  barbitone (Bb) (all at 0.5 mM). There was only a significant potentiation of the GABA-induced contractile responses by these barbiturates over the lower range ( $< 50 \mu\text{M}$ ) of the GABA dose-response curve. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean, and the number of experiments performed was  $n=8$ .

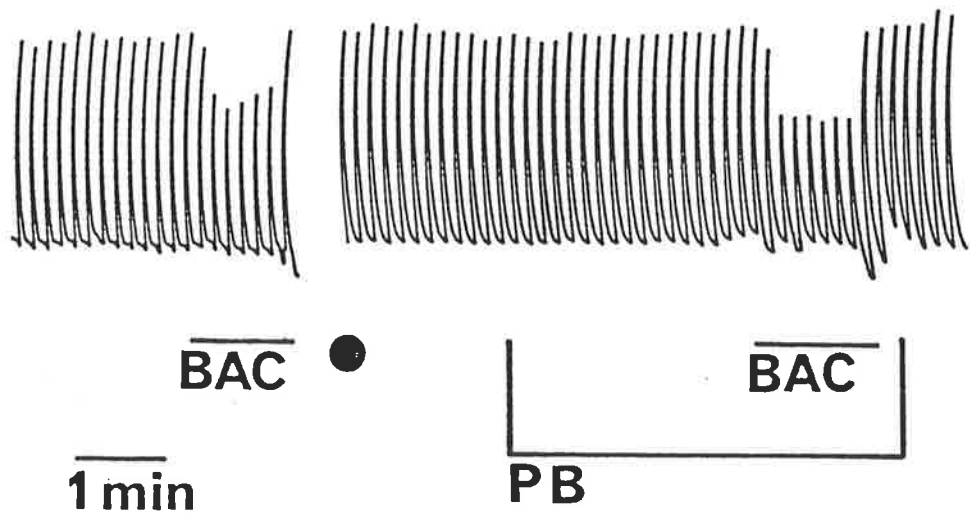


Fig. 3. Lack of effect of PB (0.2 mM) on electrically-induced cholinergic twitch contractions (0.1 Hz, 0.1 ms, supramaximal voltage) and the baclofen (50  $\mu$ M)-induced depression of repetitive twitch contractions, n=6.

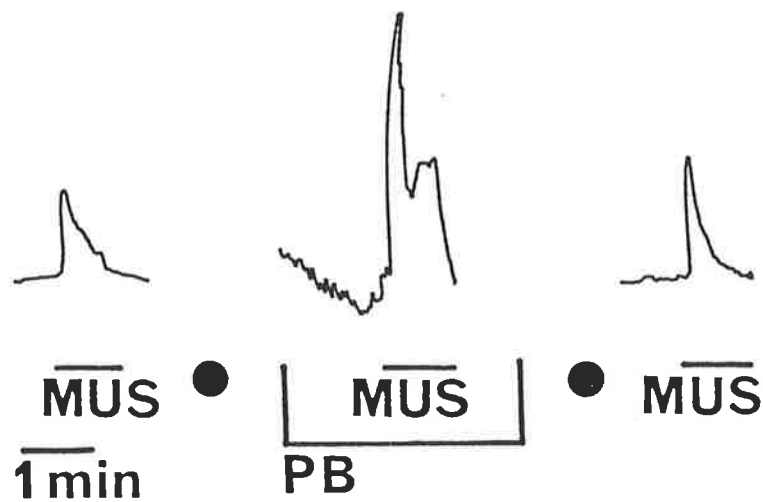


Fig. 4. Potentiation of muscimol (1  $\mu$ M)-induced contractile response by PB (0.5 mM), with a recovery of the muscimol-induced contraction after tissue washout, n=6.



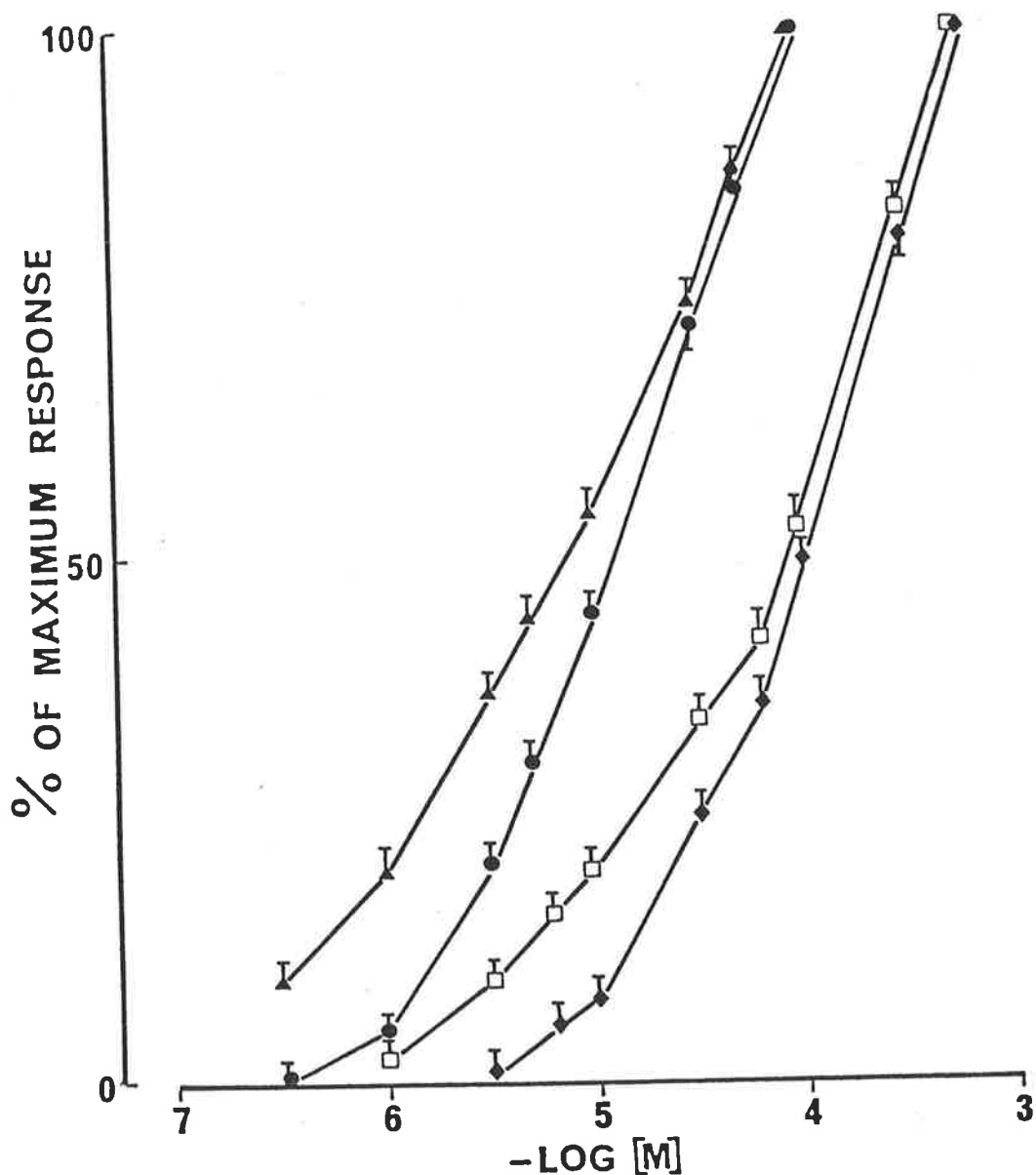


Fig. 5. Potentiating effects of ▲ PB (0.5 mM) on ● muscimol dose-response curve, and there was a significant potentiation of the muscimol-induced contractile responses by PB over the lower dose-range (< 50 uM) of the muscimol dose-response curve. There was a parallel rightwards shift of the muscimol dose-response curves in the presence of ◆ BMC (10 uM) and □ PB (0.5 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by muscimol. Each point represents the mean and standard error of the mean, and the number of experiments performed was n=8.

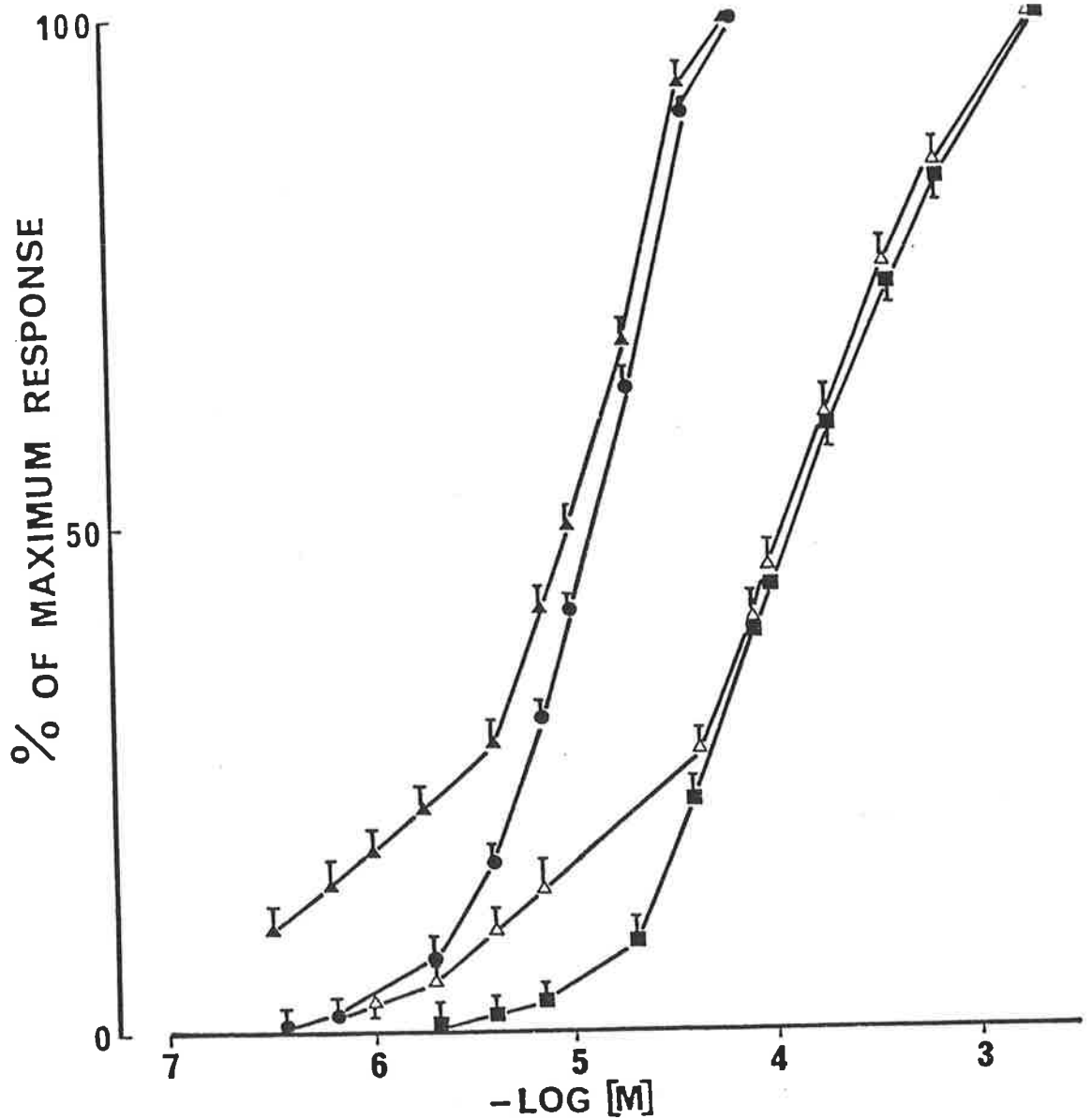


Fig. 6. Potentiating effects of ▲ PB (0.5 mM) on ● 3APS dose-response curve, with a parallel rightwards shift of the dose-response curves in the presence of ■ BMC (10 uM) and △ PB (0.5 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by muscimol. Each point represents the mean and standard error of the mean, and the number of experiments performed was n=8.

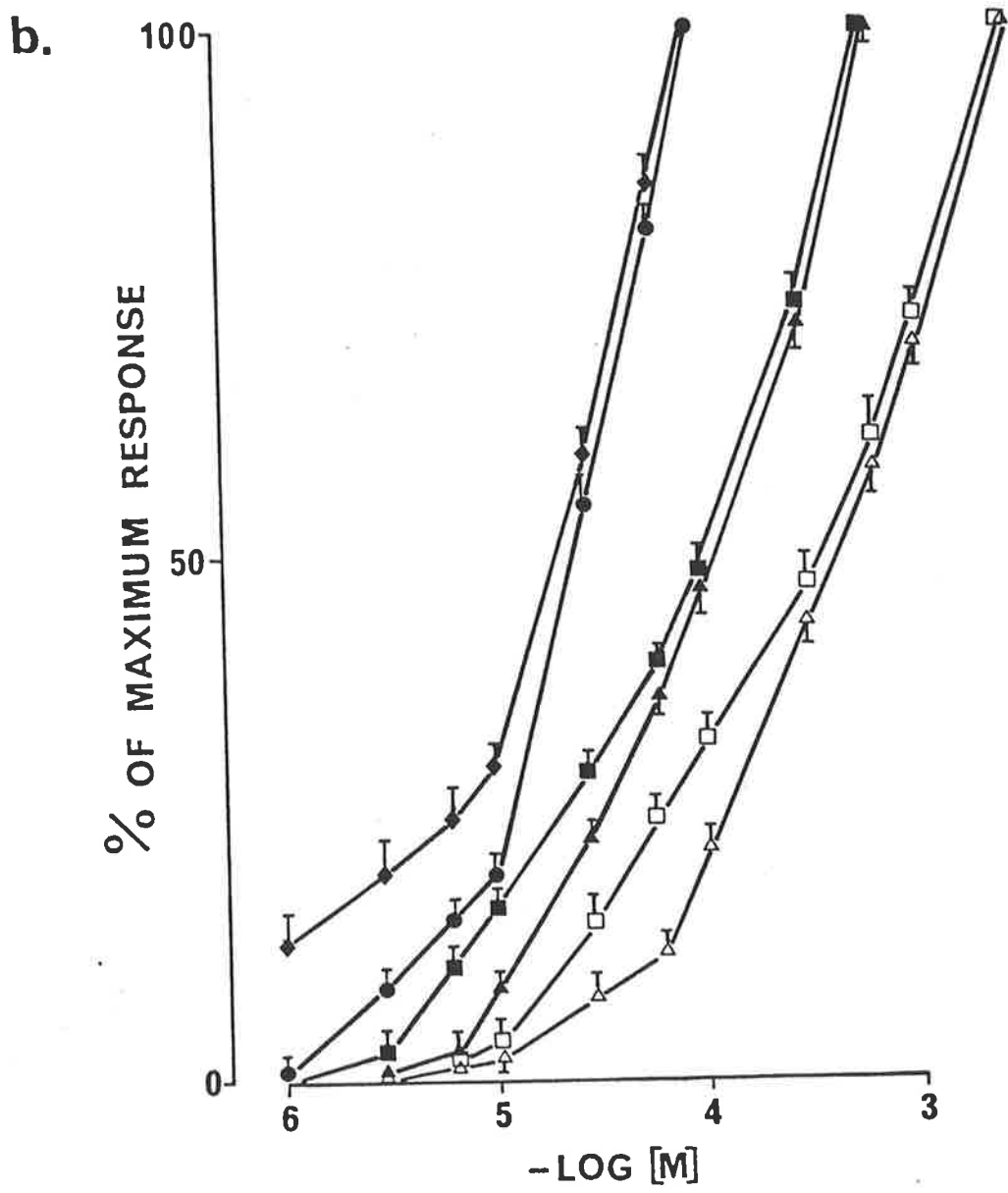
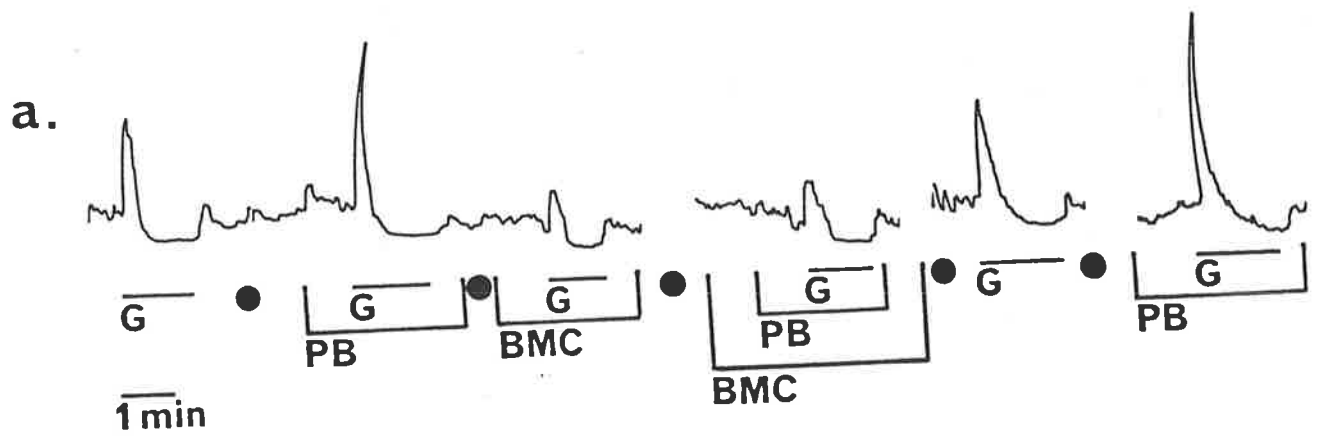


Fig. 7. (a) Antagonism of the GABA (10  $\mu$ M)-induced contraction but not the 'after-relaxation' by BMC (10  $\mu$ M), and potentiation of the GABA-induced contractile response by PB (0.5 mM), with a reduction of this potentiating effect by PB in the presence of BMC. Upon tissue washout, PB again potentiated the GABA-induced response.

(b) Dose-response curve for the ● GABA-induced contractile responses in the presence of ◆ PB (0.5 mM), ▲ BMC (5  $\mu$ M), △ BMC (10  $\mu$ M). PB (0.5 mM) shifted the GABA dose-response curves to the left in the presence of ■ BMC (5  $\mu$ M) and □ BMC (10  $\mu$ M). Results are expressed on the ordinate as a percentage of the maximum GABA-induced contractile response. Each point represents the mean and standard error of the mean based on n=10 experiments.

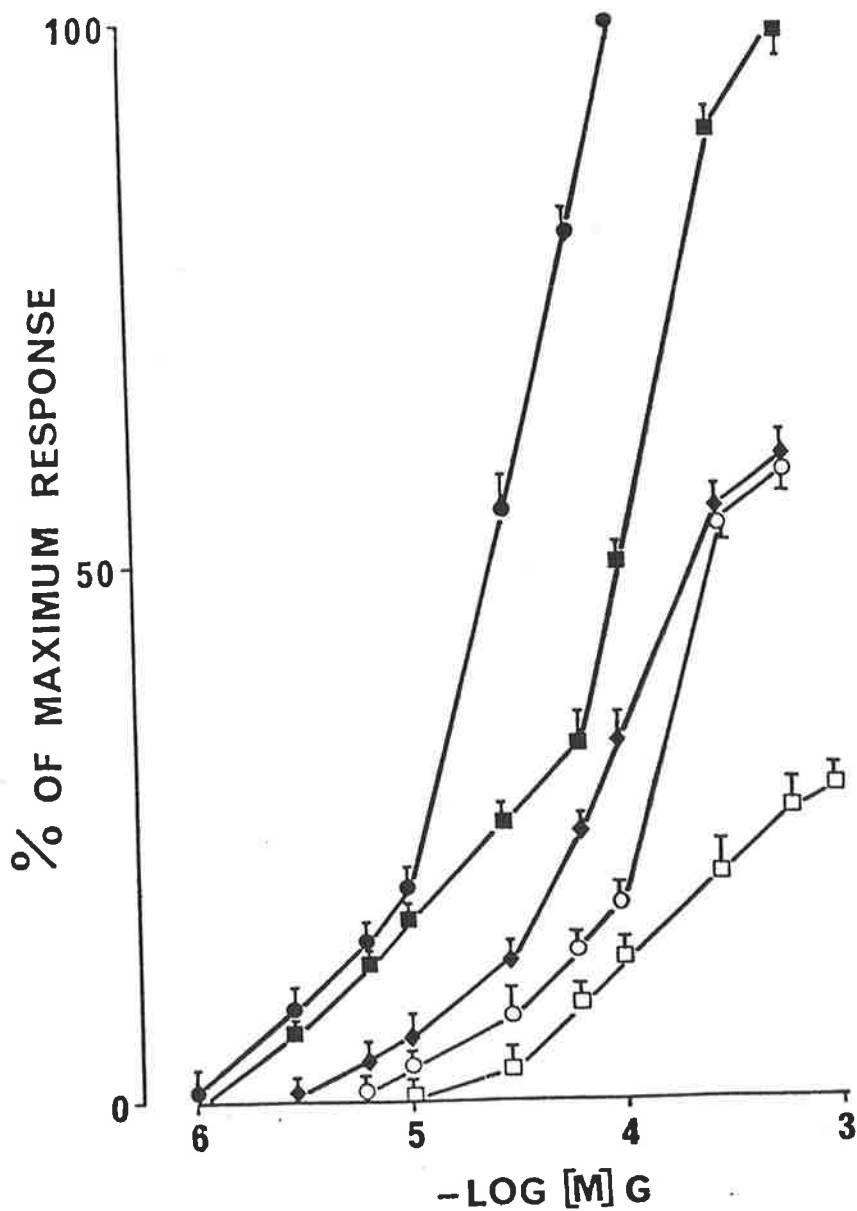


Fig. 8. Dose-response curves for the ● GABA-induced contractile responses in the presence of ◆ PIC (10 uM), □ PIC (50 uM). PB (0.5 mM) shifted the dose-response curves for GABA to the left in the presence of ■ PIC (10 uM) and ○ PIC (50 uM), restoring the slope and the maximum response toward that of the control GABA dose-response curve. Results are expressed on the ordinate as a percentage of the maximum GABA-induced contractile response. Each point represents the mean and standard error of the mean based on n=10 experiments.

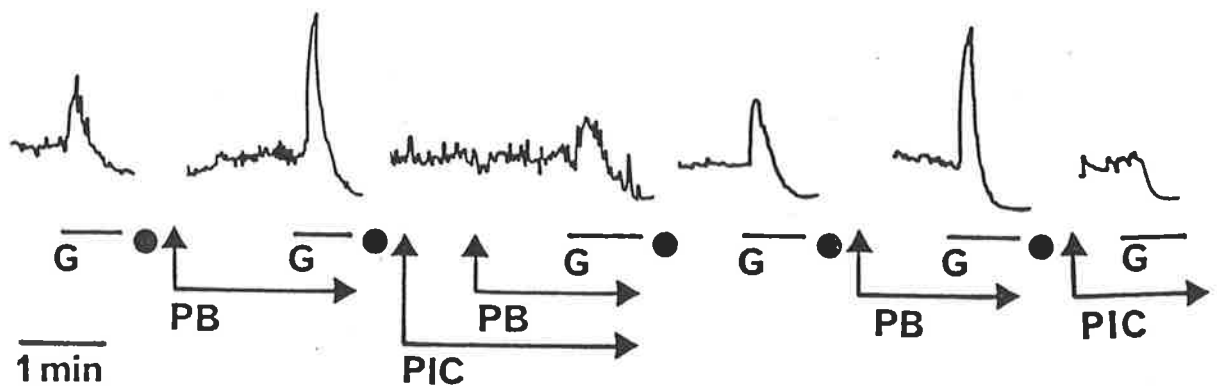


Fig. 9. PIC (10  $\mu$ M)-induced antagonism of the GABA (5  $\mu$ M)-induced contraction, but not the 'after-relaxation', and potentiation of the GABA-induced contraction by PB (0.5 mM), with partial restoration of the GABA-induced response in the presence of PIC (10  $\mu$ M) by PB (0.5 mM) or a PIC-induced reversal of the potentiating effect of PB on the GABA-induced contraction.

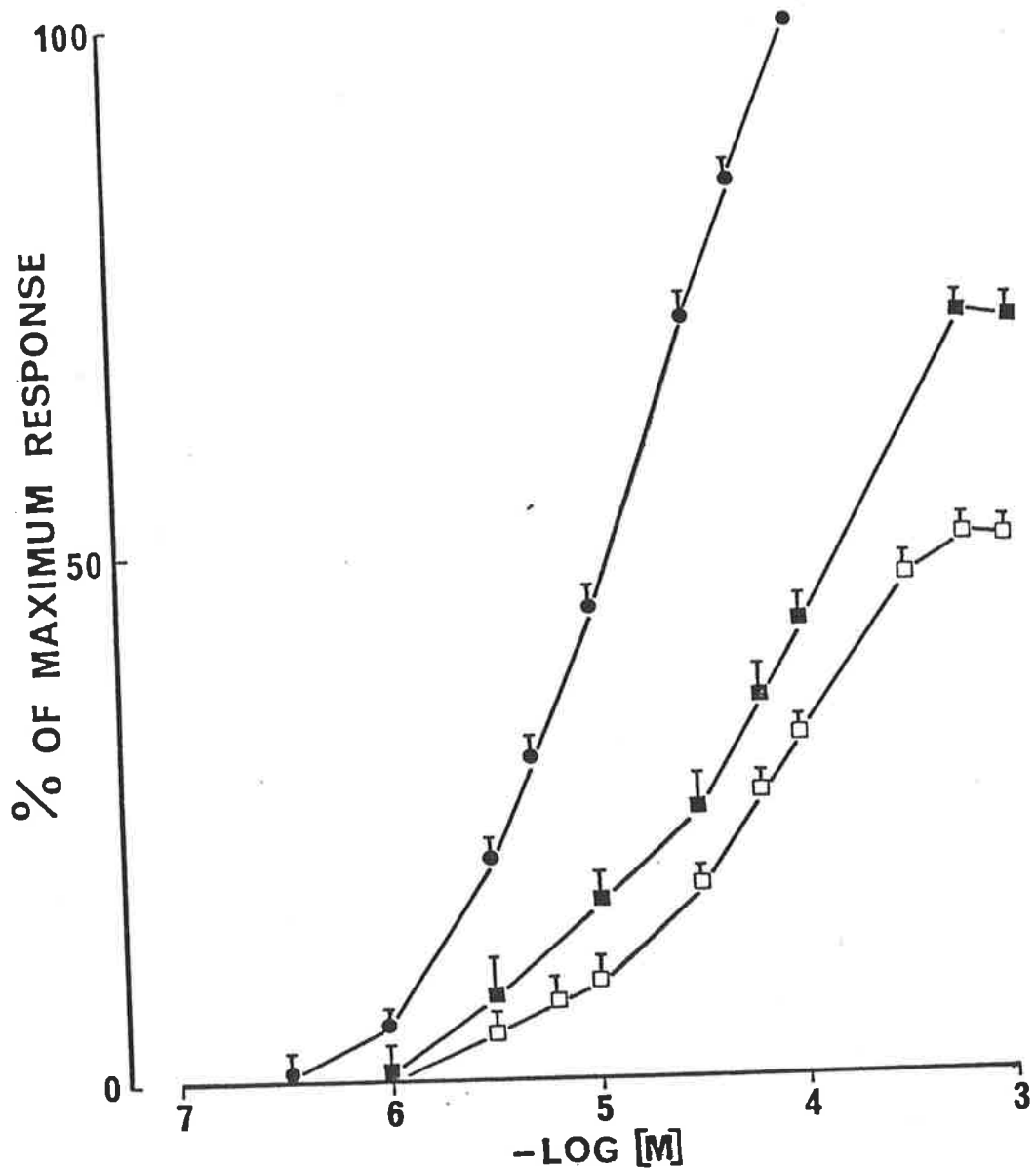


Fig. 10. Dose-response curves for the ● muscimol-induced contractile responses, in the presence of □ PIC (10  $\mu\text{M}$ ) and the leftward shift of the muscimol dose-response curve in the presence of PIC by ■ PB (0.5 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by muscimol. Each point represents the mean and standard error of the mean (n=8).

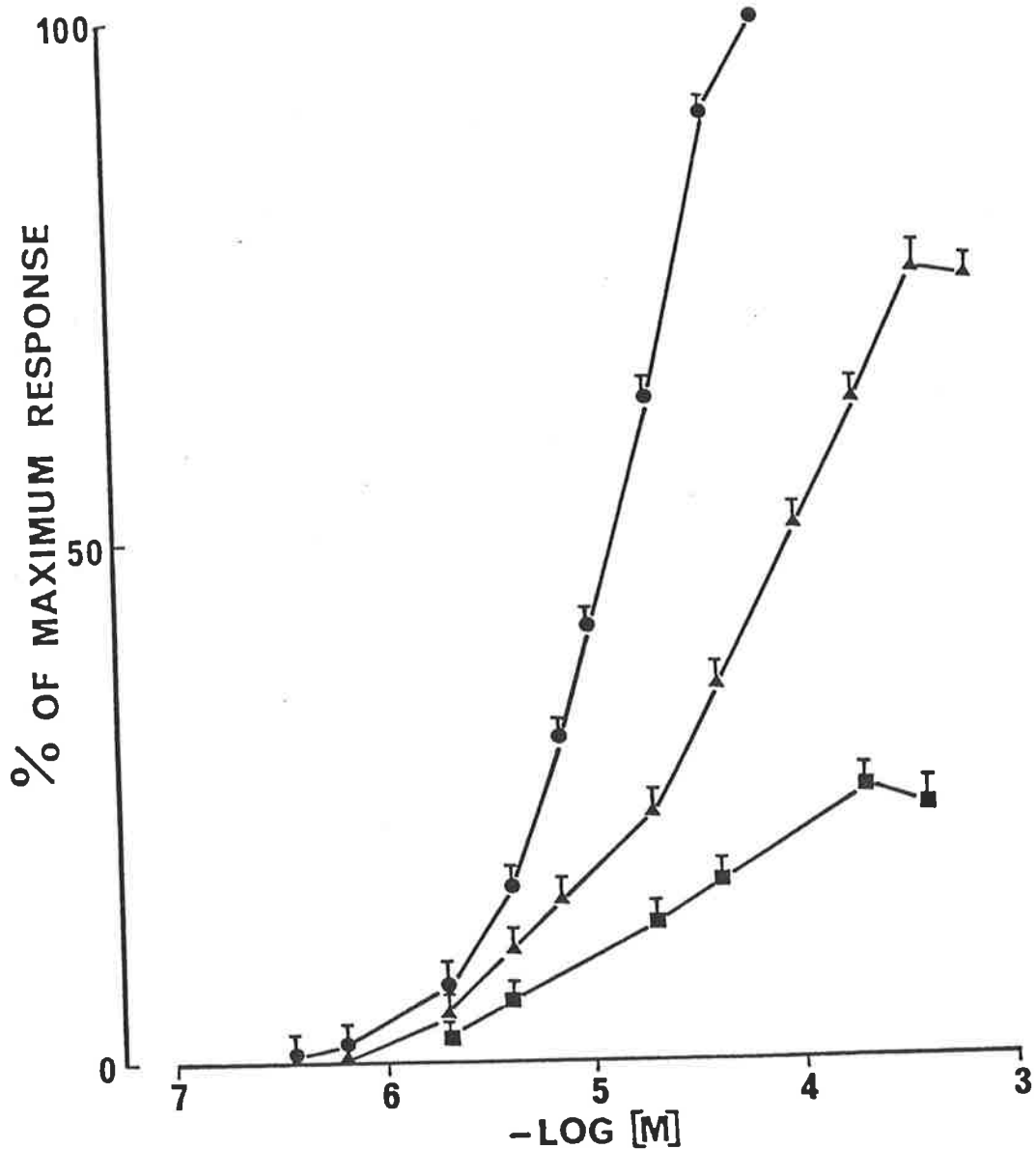


Fig. 11. Dose-response curves for the ● 3APS-induced contractile responses, in the presence of ■ PIC (50  $\mu$ M) and the leftward shift of the 3APS dose-response curve in the presence of PIC by ▲ PB (0.5 mM). Results are expressed on the ordinate as a percentage of the maximum contraction induced by 3APS. Each point represents the mean and standard error of the mean (n=8).



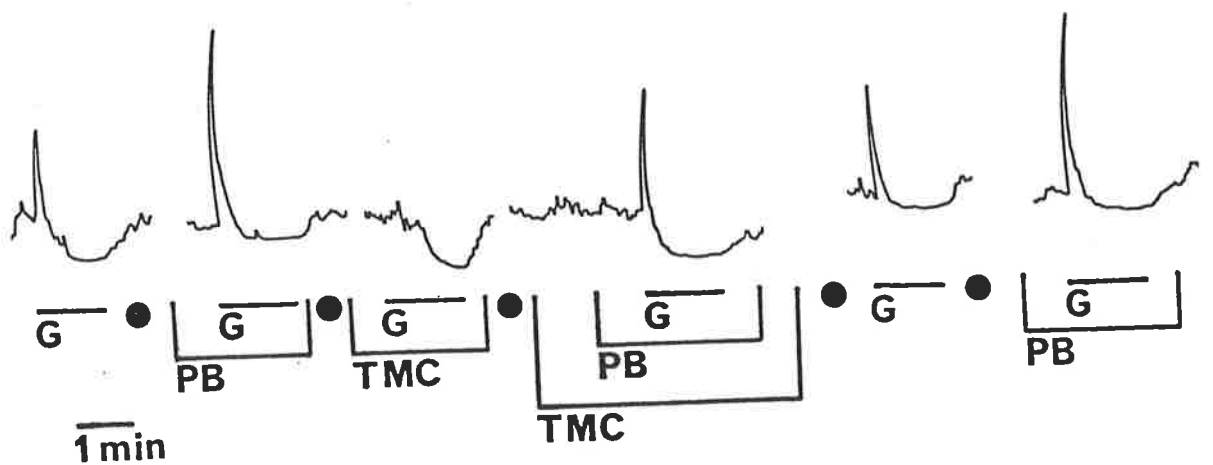


Fig. 12. Potentiating effect of PB (0.5 mM) on the GABA (8  $\mu$ M)-induced contractile response, and TMC (500  $\mu$ M) antagonised the GABA-induced contraction without affecting the 'after-relaxation'. PB (0.5 mM) reversed the antagonism of the GABA-induced contractile response by TMC (500  $\mu$ M).

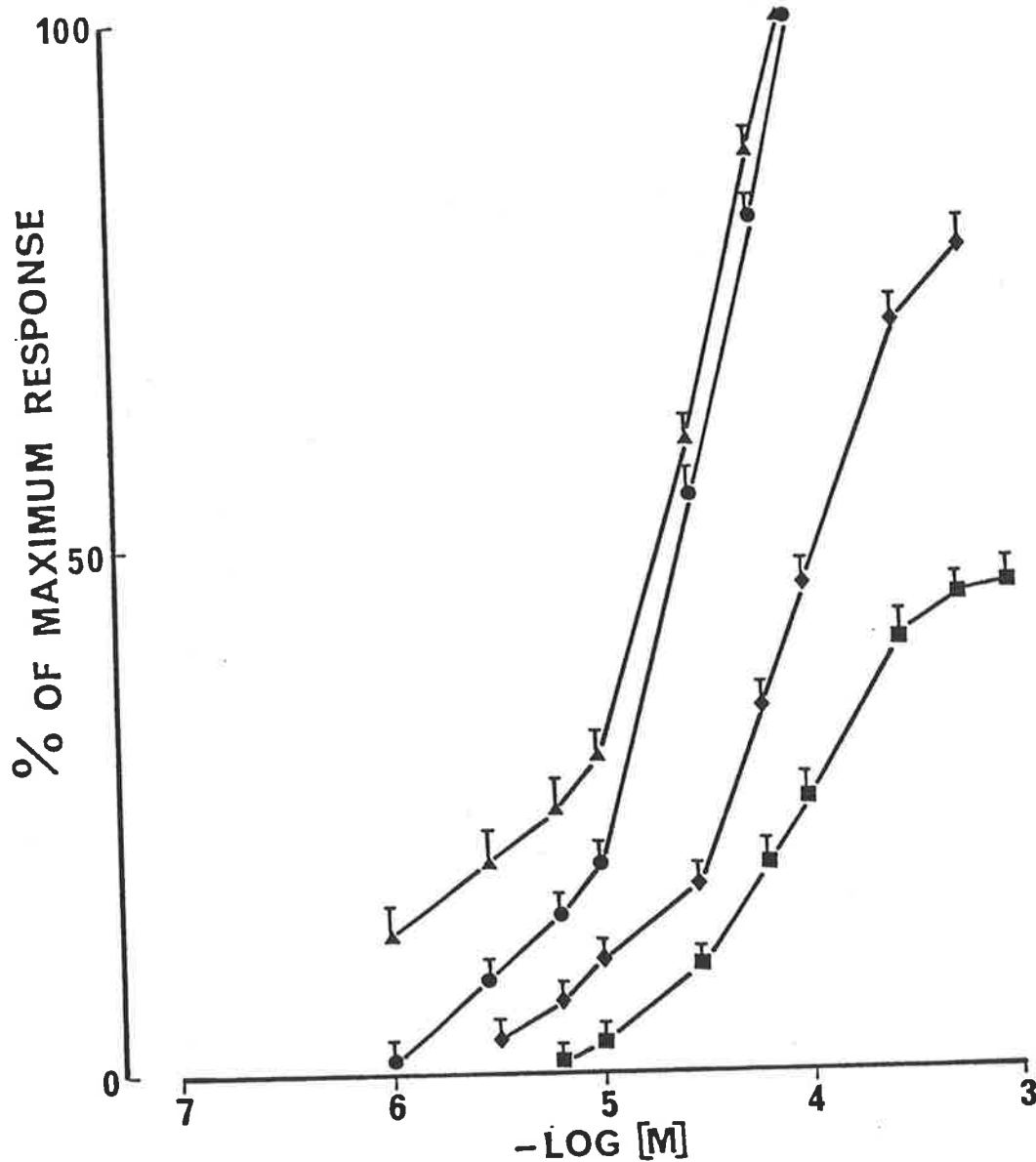


Fig. 13. Dose-response curves for ● GABA-induced contractile responses, in the presence of ▲ PB (0.5 mM), and ■ TMC (500  $\mu$ M) shifted the dose-response curve for GABA to the right in a non-parallel manner. ◆ PB (0.5 mM) shifted the dose-response curve for GABA in the presence of TMC to the left. Results are expressed on the ordinate as a percentage of the maximum contraction induced by GABA. Each point represents the mean and standard error of the mean based on 8 experiments.

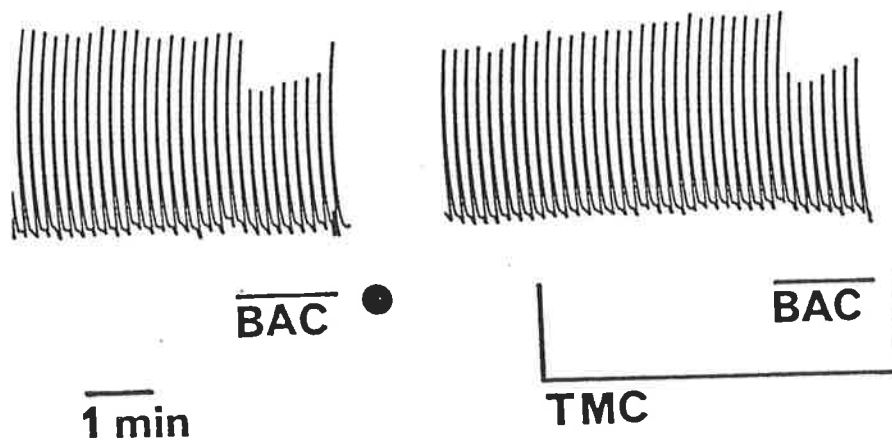


Fig. 14. Lack of effect of TMC (500  $\mu$ M) on repetitive cholinergic twitch contractions and on the baclofen (50  $\mu$ M)-induced depression of twitch contractions.

#### 5.4. DISCUSSION

The discovery of the ubiquitous distribution of GABA receptors throughout the CNS, studied *in vivo* and *in vitro* in a great variety of preparations where there is both direct and indirect GABAergic involvement in neuronal function, provides an obvious area of interest for the investigation of the actions of many different types of drugs potentially active at these sites. Among such are the barbiturates, which are claimed to have several actions, mainly sedative/hypnotic/anaesthetic effects, anti-convulsant effects, muscle relaxant, and even anti-anxiety actions, whilst some barbituric acid derivatives have excitant/convulsant effects and may also stimulate respiratory function, although most barbiturates depress cardiovascular and respiratory activity (see review by Richter and Holtman, 1982). Increasing evidence over the years supports a role for the major inhibitory neurotransmitter GABA in the action of many CNS depressant and excitatory drugs, and recent biochemical studies have demonstrated direct interactions between distinct GABA-receptor binding sites and separate receptor sites for two classes of drugs that are found to modulate GABAergic synaptic transmission, namely the benzodiazepines and barbiturates, as well a variety of depressant and convulsant or excitatory agents such as PIC (Olsen, 1981 a,b; 1982; Ticku and Olsen, 1978; Johnston, 1983; Willow and Johnston, 1983). All these receptors form the so called 'benzodiazepine-barbiturate-GABA-receptor-Cl<sup>-</sup> ionophore complex' (Olsen, 1982), and such drugs can bind to one or other of the components of this oligomeric complex, and, in

turn, can modulate the binding of ligands to other sites within this complex. Thus, reciprocal allosteric  $\text{Cl}^-$ -dependent interactions between receptor sites for GABA, barbiturates and the benzodiazepines occur at GABAergic synapses, and modulate the postsynaptic actions of GABA in the nervous system, facilitating GABAergic neurotransmission (Olsen, 1981 a,b; 1982; Haefely et al., 1979; Tallman et al., 1980). Such biochemical ligand binding studies have been supported by neurochemical and neurophysiological studies which suggest e.g. that depressant barbiturates such as PB produce their therapeutic effects by enhancing GABAergic transmission (Nicoll et al., 1975; Simmonds, 1981).

Radioactive GABA has been widely used to identify GABA receptor sites (Zukin et al., 1974; Enna and Snyder, 1975; Olsen et al., 1978 c), and to show that barbiturates modify GABA binding at these sites (Willow and Johnston, 1980; 1983). Chloride ions appear to have major effects on the interactions of all three receptors in such binding studies (Olsen, 1981 a; Johnston, 1983; Madtes, 1984; Olsen and Snowman, 1982), which is perhaps to be expected since the GABA<sub>A</sub>-receptor-mediated events are  $\text{Cl}^-$ -dependent (Nicoll, 1978 d; Krantis and Kerr, 1981 b), and PIC has been found to inhibit the postsynaptic increase in  $\text{Cl}^-$  permeability by interacting at a site separate from the GABA receptor, resulting in a reduction of the GABAergic response via a non-competitive block of GABA inhibitory synaptic transmission (Ticku and Olsen, 1977).

Radioactive (-) dihydropicrotoxinin (DHP), a biologically

active analogue of picrotoxin, binds specifically to synaptic membrane fragments from crayfish muscle (Olsen et al., 1978 b) and from mammalian brain containing GABA synapses sensitive to PIC (Nistri and Constanti, 1979). DHP binding is inhibited only by biologically active chemical analogues of PIC and not other inactive analogues (Olsen and Leeb-Lundberg, 1981 a,b; Olsen et al., 1979), indicating that DHP has some specificity as a probe for the picrotoxin binding site associated with the  $\text{Cl}^-$ -ionophore on the GABA-receptor complex (Ticku and Olsen, 1978). This occurs without altering GABA binding to its receptor (recognition) site (Olsen et al., 1978 a) since DHP binding sites appear to be distinct from GABA receptor sites (Ticku et al., 1978 a,b). DHP binding is also inhibited by depressant barbiturates such as PB ( $\text{IC}_{50} = 50 \mu\text{M}$ ), which is more potent in this respect than the anti-convulsant drug PhB ( $\text{IC}_{50} = 400 \mu\text{M}$ ) (Olsen et al., 1979). This suggests that the DHP binding site might well be involved in at least some of the actions of depressant barbiturates, but anti-convulsant activity is more difficult to correlate with anti-DHP binding efficiency at the DHP receptor site.

More recently, labelled  $[35\text{S}]$ -*t*-butylbicyclophosphorothionate ( $[35\text{S}]$  TBPS) has been introduced as a superior ligand for the PIC/barbiturate site at the ionophore complex (Ramanjaneyulu and Ticku, 1984b; Maksay and Ticku, 1985) where a range of barbiturates inhibit TBPS binding, although GABA agonists inhibit TBPS but not DHP binding (Squires et al., 1983; Ticku and Ramanjaneyulu, 1984). Since PIC does not influence the binding of GABA to its receptors, it is thought that barbiturates do not

act directly on GABA receptor sites to enhance binding, instead they modulate GABA actions on the  $\text{Cl}^-$ -ionophore (Olsen, 1981 a). The opening of the  $\text{Cl}^-$  channels is thus regulated by GABA receptor agonists binding to GABA<sub>A</sub>-receptor sites, modulated by depressant drugs which may potentiate the  $\text{Cl}^-$  permeability, leading to an enhancement or inhibition, or by excitatory drugs which may block the  $\text{Cl}^-$  channels and so reduce inhibition. Hence, it seems reasonable to postulate that endogenous ligands for these various sites could well be present in the nervous system, possibly involved in controlling excitability, sleep, and anxiety, all of which are associated with GABA actions in the CNS (Olsen and Leeb-Lundberg, 1981 b). The PIC binding site is the physiologically relevant PIC receptor and GABA receptor complex consists of different functional units interacting in a complex fashion to regulate the efficacy of GABAergic synaptic transmissions (White et al., 1985).

Eccles and Malcolm (1946) made the first observation related to the interaction between barbiturates and GABA receptors, when they found that PB prolongs the decay of the dorsal root potential in the isolated frog spinal cord. Ever since, many studies have shown that both anaesthetic and anti-convulsant barbiturates enhance GABA actions in a variety of in vivo and in vitro preparations (Nicoll, 1975 a,b; Nicoll, 1978 d; Bowery and Dray, 1976). One of the techniques, now extensively employed to study barbiturate modulation of GABA receptor function, is fluctuation analyses of the membrane current responses induced by GABA in spinal neurones grown in cell culture, to estimate the properties

of single channels activated by GABA (Barker and McBurney, 1979 a,b; McBurney and Barker, 1978; Barker et al., 1981). Iontophoretic application of PhB to cultured spinal neurones of the mouse prolongs unitary current fluctuations and conductance responses to GABA, the principle action of which is to increase the average opening time of the GABA-activated  $\text{Cl}^-$  channels, and <sup>to</sup> prolong the duration of miniature synaptic currents with a time constant of decay similar to the mean open-time of GABA-activated channels. This suggests that the enhancement of these GABA-mediated synaptic events by PhB is due to the postsynaptic action of the drug (Barker and McBurney, 1979 b), and is in keeping with the demonstration by Willow and Johnston (1981 a,b) that barbiturates enhance binding and slow GABA dissociation at its receptor. PB, the anaesthetic barbiturate, exerts its effects by 1) directly depressing membrane excitability through activation of GABA receptors, coupled to  $\text{Cl}^-$  conductance, 2) by modulating GABA-mediated events to enhance and prolong GABA-induced conductance change and 3) to depress glutamate excitation in a non-competitive manner (Barker and Ransom, 1978). In general, GABAergic activity appears to be facilitated by both PB (Ransom and Barker, 1975; 1976) and PhB (MacDonald and Barker, 1977). Recently, it was concluded that PB binds to the 'barbiturate receptors' located close to the GABA-receptor-chloride-ionophore complex, and directly affects the GABA-GABA-receptor interactions rather than the ionic channels in frog sensory neurones (Akaike et al., 1985).

Qualitative and quantitative differences between anaesthetic



and anti-convulsant barbiturates exist, according to studies by MacDonald and Barker (1978 a; 1979 b), who find that barbiturate anaesthetics abolish the spontaneous activity of cultured spinal cord neurones, directly increase membrane conductance and are more potent than anti-convulsants in augmenting GABA and depressing glutamate responses. Anti-convulsant barbiturates, however, have minimal GABA-mimetic inhibitory actions at high doses, apart from possessing the capability of abolishing PIC-induced convulsive activity. These differences between anaesthetic and anti-convulsant barbiturates are reflected in their clinical pharmacological properties. PhB is an effective anti-convulsant at clinical doses that produce minimal sedation, whilst deep sedation is produced by toxic doses, whereas conversely, PB is commonly used as an anaesthetic because of its dominant sedative action, but it produces unacceptable levels of sedation at anti-convulsant doses (Sharpless, 1970). Therefore, disparate pharmacological actions of these agents may underlie the different mechanisms of action for these barbiturates, possibly accounting for their clinical pharmacological properties and manifestations. Evidently, increased GABAergic inhibition and decreased glutamatergic excitation contributes to the anti-convulsant mode of action, whilst anaesthetic effects are ascribed to these actions but with the addition of a directly produced GABA-mimetic inhibition through increases in  $\text{Cl}^-$  conductance that further depress neuronal excitability (Schultz and MacDonald, 1981).

The isolated ileum of the guinea-pig has been used to demonstrate the pharmacology of GABA actions, where exogenously

applied GABA elicits a GABA<sub>A</sub>-receptor-mediated contractile event of cholinergic and neural origin followed by a GABA<sub>B</sub>-receptor-mediated depression of cholinergic output, which is a delayed prolonged 'after-relaxation', the latter being mimicked effectively by baclofen (Bowery et al., 1981 a; Ong and Kerr, 1983 a,b). This GABA<sub>A</sub>-receptor-mediated contraction is mimicked by muscimol and 3-amino-1-propanesulphonic acid (3-APS), which are both potent agonists at GABA<sub>A</sub>-receptor sites (Curtis et al., 1959; Krosgaard-Larsen et al., 1977; 1979 b). In the present experiments, the GABA<sub>A</sub>-receptor-mediated event has been further investigated to see if this Cl<sup>-</sup>-dependent, bicuculline-sensitive effect is at all analogous to that seen at the typical GABA<sub>A</sub>-receptor-Cl<sup>-</sup>-ionophore complex associated with a barbiturate/PIC binding site which occurs in the CNS (Olsen, 1981 a; 1982).

The potentiation of GABA-induced contractile responses by barbiturates in the isolated ileal preparations of the guinea-pig is evidently due to modulatory actions at GABA<sub>A</sub>-receptor sites, such receptor sites having a close interaction with the Cl<sup>-</sup>-ionophore and barbiturate binding site, since the contractile response elicited by GABA was both potentiated by barbiturates, PIC-sensitive, where PIC is known to be an antagonist of the Cl<sup>-</sup>-ionophore-mediated event acting at GABA-activated Cl<sup>-</sup>-ionophores (Takeuchi and Takeuchi, 1969; Ticku et al., 1978 b). The 'after-relaxation' induced by GABA or baclofen was unaffected by the barbiturates, hence, there is no evidence for interactions of these barbiturates with the GABA<sub>B</sub>-receptors responsible for the latter depressive action of GABA. Barbiturates also potentiated

ileal GABAergic responses elicited by muscimol, as was found in the CNS (Nicoll, 1975 a; Ransom and Barker, 1976; Brown and Constanti, 1978; Barker and Ransom, 1978; Simmonds, 1981), and by 3-APS, but had no influence on baclofen-induced relaxations. This provides further supportive evidence that the potentiating action of the barbiturates is directed primarily to the GABA<sub>A</sub>-receptor complex of the myenteric plexus, where the potentiating action of ThP was most potent, some 5 fold more active than PB which in turn was some 10 fold more active than Bb, whereas PhB was the least active barbiturate used.

In an attempt to correlate the potencies of the potentiating effects by these barbiturates with binding studies, Willow and Johnston (1980) demonstrated for the first time, contrary to Enna and Snyder (1976), an enhancement by PB of the binding of GABA to rat brain synaptosomal membranes. This enhancement by PB is concentration-dependent and appears to be due to an increase in the affinity of the high-affinity GABA recognition site for GABA, and is also inhibited by PIC (Willow and Johnston, 1980; 1981 a; Johnston and Willow, 1981; Willow, 1981; Olsen and Leeb-Lundberg, 1981 a; Asano and Ogasawara, 1982); such receptor binding by barbiturates is Cl<sup>-</sup>-dependent (Skerritt et al., 1983 b; Olsen and Snowman, 1982). In addition to PB, other barbiturates also enhance GABA binding to synaptosomal membranes (Willow and Johnston, 1981 a), and barbiturates such as PhB and Bb are less potent than PB in their ability to enhance GABA binding (Willow et al., 1981), whereas the therapeutically inactive parent barbituric acid itself is without effect, even at concentrations as

high as 5 mM. The rank order of the potentiating action of the barbiturates (ThP, PB, Bb and PhB) tested in the isolated ileum follows the ability of the individual barbiturates to potentiate GABA binding to membrane preparations in the CNS as shown by Willow (1981). However, such binding studies have not yet been described in the myenteric plexus, but it may well be inferred from the present pharmacological analyses that barbiturates also modify binding of GABA to membranes of neurones in the myenteric plexus of the guinea-pig where responses to lower doses of GABA were preferentially potentiated by these barbiturates.

Here, ThP, Bb and PB, at high doses also elicited PIC- and BMC-sensitive GABAergic contractions of the ileum, whilst PhB did not elicit such contractile responses, except on very rare occasions when it induced weak contractions at extremely high doses. Elsewhere, it has been reported that certain barbiturates can mimic GABA actions in vitro (Evans, 1979; Ransom and Barker, 1976; Nicoll, 1975 a; Nicoll and Wojtowicz, 1980; MacDonald and Barker, 1978 a; Barker and Mathers, 1981; Schultz and MacDonald, 1981; Barker et al., 1984), e.g. PB acts as a GABA agonist on postsynaptic receptor sites when applied directly onto spinal cord neurones, and increases neuronal  $\text{Cl}^-$ -conductance (MacDonald and Barker, 1978 a; Barker and Ransom, 1978). Higher concentrations of PB (40  $\mu\text{M}$ ) depolarizes primary afferent fibres, an action blocked by the GABA antagonists BMC and PIC (Nicoll, 1975 b), and directly hyperpolarizes frog motoneurones (Nicoll, 1975 a). Similarly in the isolated spinal cord of immature rats, PB produced a BMC-sensitive depolarization of dorsal root fibers

(Evans, 1979). In general, high concentrations of PB have a GABA-mimetic effect even in the absence of GABA (Higashi and Nishi, 1982; Mathers and Barker, 1980), PB is said to be 10 times more active than PhB in GABA-mimetic properties (MacDonald and Barker, 1978 a), comparable to the results observed in the present study.

The potentiating and the GABA-mimetic actions of barbiturates have been ascribed to their ability to alter the conductance characteristics of the  $\text{Cl}^-$ -ionophore coupled to GABA<sub>A</sub>-receptors, where barbiturates delay the dissociation of GABA from the receptors (Willow and Johnston, 1981 b) and prolong the life-time of opened  $\text{Cl}^-$ -channels (Barker and Mathers, 1981). Such modulatory actions may well be responsible for the effects of barbiturates on GABA<sub>A</sub>-receptors of the receptor-ionophore complex in the myenteric plexus, since the  $\text{Cl}^-$ -channels of the plexus are sensitive to PIC and other  $\text{Cl}^-$ -channel transport blockers such as furosemide and piretanide (Krantis and Kerr, 1981 b). Again in ligand binding studies, anaesthetic barbiturates displace radiolabelled DHP from brain membranes (Ticku and Olsen, 1978), reinforcing the notion that barbiturates may enhance GABA responses by binding to the  $\text{Cl}^-$ -ionophore site in close proximity to the GABA receptor site. By contrast, BMC interacts with the GABA-receptor (recognition) site to antagonise GABA responses without altering the enhancement of GABA binding by PB (Willow, 1981), consistent with other electrophysiological observations (Curtis and Lodge, 1977; Evans, 1979; Nicoll and Wojtowicz, 1980; Bowery and Dray, 1976;

1978 ), and the present results on barbiturate-BMC interaction in the myenteric plexus.

Further substantiating the similarity of the GABA<sub>A</sub>-receptor-ionophore complex in the myenteric plexus and in the CNS, results from this study on interactions between barbiturates and the non-competitive GABA antagonist PIC showed that barbiturates reversed the antagonism of the GABA<sub>A</sub>-receptor-mediated contractile responses by PIC but not the competitive antagonism with BMC. The dose-ratios of the dose-response curves for GABA in the presence of BMC were unaltered by PB, whereas the slope of the curve for the non-competitive antagonism with PIC was made more steep by the barbiturates, and the maximal response increased toward that of the control GABA-induced response in the absence of PIC. These results support similar findings by Nicoll and Wojtowicz (1980) in showing that barbiturates and PIC interact at a common, or very closely coupled site, which forms part of the ionophore complex, whereas BMC acts at a quite different site associated with the GABA<sub>A</sub>-receptor site itself. Nicoll and Wojtowicz (1980), using the frog isolated spinal cord, established that PB reverses the non-competitive antagonism of GABA responses by PIC, without altering the BMC antagonism of GABA, whilst the PB-induced direct hyperpolarization on the frog motoneurons is similar to that with GABA, being abolished by BMC and PIC. In the frog spinal cord, the threshold concentration for the PB-induced hyperpolarisation is about 10 fold more than the dose required to enhance GABA-mediated responses, in accordance with the present results, in that the threshold concentration of PB (>

1 mM) exerting a GABA-mimetic ileal contraction, here, was approximately 10 times more than the threshold dose required to enhance the GABA<sub>A</sub>-receptor-mediated contraction.

Simmonds (1981) found that PhB is 10 times less potent than Pb in having an agonist action on afferent fibres to the rat cuneate nucleus in vitro, and both barbiturates enhance the depolarising action of the GABA agonist muscimol, reducing the potency of BMC and PIC as an antagonist of muscimol. In his preparations, PhB causes only a small potentiation of muscimol, and reduces the potency of PIC in doses which do not affect the potency of BMC, whereas PB does not show such selectivity but shows a substantial potentiation of muscimol and reduction in potency of BMC. Contrasting results have been reported where both PhB and PB reduce the potency of PIC as a GABA antagonist on frog spinal cord motoneurons without affecting that of BMC (Evans, 1979; Nicoll and Wojtowicz, 1980), whilst in the study by Bowerly and Dray (1978) on the rat cervical ganglion, a wide variety of depressant drugs such as PB and other sedative/hypnotics were shown to reduce the potency of BMC with little potentiation of GABA-induced responses; but such observations were disputed by Brown and Constanti (1978) and Evans (1979). From a recent review by Johnston and Willow (1982 b), there appears to exist a multiplicity of GABA receptors, some of which may not be linked to barbiturate or benzodiazepine receptors, whilst others are linked to either one, and there are yet others that are apparently linked to both. Hence, it is conceivable that some GABA-evoked responses in particular tissues might not be poten-

tiated by the barbiturates.

In the isolated ileum, PB was far more potent than PhB in reversing the antagonism of PIC against GABA-induced contractile responses, and this correlates well with the order of potency in displacing (3H)-DHP binding and in reducing the potency of PIC, where the potency of PB > PhB (Ticku et al., 1978 b; Ticku and Olsen, 1978; Olsen et al., 1979; Simmonds, 1981). The conclusion from the present findings in the ileum, that barbiturates interact with PIC at a site separate from the GABA receptor is further reinforced in those binding studies where the enhancement of GABA binding by barbiturates is depressed by PIC but not by BMC (Willow, 1981). Such a mechanism is evidently responsible for the long known mutual, specific interaction between barbiturates and PIC in whole animals where each conteracts the other (Koppanyi et al., 1936; Marshall et al., 1937), although the basis for this interaction may not be as simple as this scheme suggests, as shown by Harrison and Simmonds (1983), who found a separation of GABA-potentiating and anti-PIC actions of barbiturates and allied compounds.

From the present investigation, the lactam TMC proved to be a non-competitive antagonist of GABA<sub>A</sub>-receptor-mediated contractions in the ileum, since it displaced the GABA-induced dose-response curve to the right in a non-parallel manner, causing a decline in the slope and a depression of the maximum GABA-induced response, in a manner analogous to that seen with PIC. PB reversed this non-competitive antagonism with TMC, shifting the



dose-response curve leftwards and restoring the slope and maximum response closer to that of the control dose-response curve, effects that parallel the interactions of GABA, PIC and barbiturates in the ileum and CNS. Ileal contractile responses to GABA analogues such as 3-APS and muscimol were also antagonised in a non-competitive manner by TMC, but neither the baclofen nor the GABA-induced 'after-relaxation', or depression of ileal cholinergic twitch contractions, were affected. Also, TMC was without effect on responses to exogenously applied ACh, 5-HT or nicotine, and did not influence responses to transmural electrical stimulation of cholinergic neurones in the intestine. From this, it can be concluded that the GABA antagonism exerted by these convulsant lactams is rather specifically directed against the 'PIC-barbiturate' site (Olsen, 1982) of the GABA<sub>A</sub>-receptor ionophore complex, there being no antagonism at the GABA<sub>B</sub>-receptor site (Hill and Bowery, 1981) in the guinea-pig ileum. These results confirm the original conclusion (Kerr et al., 1976) that the caprolactams antagonise GABA-induced responses in a PIC-like fashion.

Recently, Skerritt et al (1985) have demonstrated the specificity of C4, and C6 substituted caprolactams in GABA binding studies, these compounds exhibiting a PIC-like Cl<sup>-</sup>-dependent partial inhibition of GABA binding, most probably at the GABA-receptor coupled ionophore. This is consistent with the present results showing a non-competitive, barbiturate-reversible antagonism of GABA-mediated actions in the ileum. By contrast, caprolactams substituted at C5 lack convulsant activity, whilst some with bulky C7 substituents are depressants (Duong et al., 1976).

Thus, alkyl substitution at C4 and C6 on the caprolactam ring in particular imparts convulsant properties to the molecule, and it is most likely that this, as shown here for TMC, is due to a specific interaction of these agents at the ionophore complex associated with the neuronal GABA-receptor site. There is a more than superficial resemblance of the 4,6 alkyl substituted caprolactams to the convulsant <sup>a</sup> b-substituted butyrolactams where the b-alkyl groups have been proposed to be the 'effector' moiety responsible for occluding the Cl<sup>-</sup> ionophore channel (Klunk et al., 1982). In the same way, the depressant C7 substituted lactams recall the  $\gamma$ -substituted butyrolactones, and it is proposed that the latter occupy an adjacent site, but without occluding the Cl<sup>-</sup>-channel (Klunk et al., 1982). Thus these lactams with convulsant and depressant actions, may provide further insight into the nature and modulation of the Cl<sup>-</sup>-ionophore complex associated with neuronal GABA<sub>A</sub>-receptors.

Recent studies (Radulovic and Kazic, 1983; Mayer et al. 1981) on barbiturate actions on the isolated ileum of the guinea-pig have shown that high concentrations of PB and Bb inhibit cholinergic contractile responses produced by electrical stimulation, and contractions elicited by ACh and noradrenaline, indicating a postjunctional depressive action. It is also concluded by Radulovic and Kazic (1983) that PB when used in anaesthetic concentrations might depress smooth muscle contractility via an indirect action on the functioning of the voltage-dependent calcium channels, in reducing calcium influx, where the antagonism between PB and calcium appears to be non-competitive. PB has

also been shown to inhibit transmitter release by suppressing the calcium-dependent depolarization mechanism (Quastel et al., 1972), and to affect uptake and distribution of calcium in the heart (Nayler and Szeto, 1972). A further important presynaptic action of barbiturates is to block calcium uptake in presynaptic nerve terminals, therefore reducing the release of many transmitters (Blaustein and Ector, 1975; Ondrusek et al., 1979).

PhB, together with other anti-convulsants, has been shown to selectively inhibit potassium-evoked release of <sup>the</sup> excitant amino acid D-aspartate (Willow et al., 1980; Skerritt and Johnston, 1983). Furthermore, an enhancement of the evoked calcium-dependent release of GABA, but at the same time, also a marked decrease in the evoked release of aspartate, have been seen using rat olfactory cortical slices (see review by Straughan, 1979), where PB does not affect the resting release of endogenous GABA or aspartate. However, contradictory results from other studies on brain slices and synaptosomes show that PB inhibits the spontaneous and the potassium-evoked release of (3H) GABA, but potentiates the electrically-induced release (see review by Straughan, 1979). The present investigation did not detect any such depressive effect induced by any of the barbiturates (ThP, PB, Bb and PhB) when used at their respective concentrations that potentiated GABA-induced responses, there being no effect on cholinergic responses to electrical stimulation, or on responses to any other muscle contracting agents such as ACh, histamine, 5-HT or substance P. The potentiating concentrations of these barbiturates employed here were evidently below those sufficient to cause a postjunctional depression, but non-specific depressions were seen at concentrations above those eliciting GABA-mimetic responses.

The potentiation of the GABAergic responses by barbiturates observed in the ileum could not be due to alteration in GABA uptake, since it has been demonstrated that PB generally does not alter GABA uptake in brain synaptosomes (Olsen et al., 1977).

Moreover, barbiturates also potentiated responses to muscimol and 3-APS in the present study, which further excludes the possibility that the enhancement of the GABA<sub>A</sub>-receptor-mediated contraction is due to an inhibition of GABA uptake by the barbiturates since 3-APS has not, and muscimol only to a small extent, any substrate affinity for the GABA uptake sites (Beart and Johnston, 1973; Johnston, 1971; Johnston et al., 1978). However, it has been shown that PB and some other barbiturates do inhibit neuronal and astrocytic GABA uptake (Larsson et al., 1982), and it is therefore possible that some of the pharmacological actions of the barbiturates in the CNS, *in vivo*, might be due to inhibition of GABA uptake into astrocytes. PB is a much more potent inhibitor of GABA uptake into cultured astrocytes than into cultured neurones, where more than 1 mM PB appears to be the effective concentration (Hertz et al., 1980), and this inhibition of uptake might in turn enhance the effects of endogenously released GABA (Wood et al., 1980; Schousboe et al., 1981 a). The above observations again show that in the isolated ileum, PB would be unlikely to potentiate GABA actions by inhibiting GABA uptake, as the effective concentration of the drug used in the ileum was always less than 1 mM, as compared to the 1 mM PB seen by Hertz et al. (1980) to inhibit GABA uptake.

Apart from the barbiturates, the benzodiazepines (BZD) are also capable of potentiating GABA<sub>A</sub>-receptor-mediated ileal contractile responses, and this enhancing effect is antagonised by Ro 15-1788, an antagonist at the BZD receptor site on the receptor complex (Luzzi et al., 1984; Kerr and Ong, preliminary

studies, 1984). From the latter study, BZD's appear to potentiate only the GABAergic contractile responses in the guinea-pig isolated ileum, without affecting GABA<sub>B</sub>-receptor-mediated actions such as the 'after-relaxation', and this supports electrophysiological evidence that BZD's enhanced the Cl<sup>-</sup>-dependent, BMC-sensitive GABA-induced depolarizing action on the AH cells in the myenteric plexus of the guinea-pig small intestine (Cherubini and North, 1985), mediated through the GABA<sub>A</sub>-receptor site. One further possibility, in the actions of barbiturates on GABA-induced ileal contractile responses, is that the barbiturates in some way interact with BZD receptors at the GABA<sub>A</sub>-receptor complex (Leeb-Lundberg et al., 1981; Leeb-Lundberg and Olsen, 1982), but this has not so far been explored in the intestine.

In summary, in the guinea-pig ileum, the effects of barbiturates on GABA-mediated neurotransmission may be explained by their modulatory action on the GABA<sub>A</sub>-receptor site and not the GABA<sub>B</sub>-receptor site, leading to a significant augmentation of the GABA-induced contractile responses. The antagonism of the Cl<sup>-</sup>-dependent GABA-mediated contraction by PIC is reversed by the barbiturates, whereas the BMC-induced antagonism of such responses at the GABA<sub>A</sub>-receptor site is not altered, suggesting allosteric interactions of barbiturates with the PIC-receptor site, consistent with neurophysiological evidence that the depressant or convulsant actions of barbiturates involve modulation of GABAergic synaptic transmission at the postsynaptic GABA-receptor-ionophore level (Ransom and Barker, 1976). Results from

this study further reinforce binding studies in the CNS, which show that barbiturates can displace labelled DHP from the PIC binding site, yet both barbiturates and DHP have no direct effects on the GABA-receptor site (Olsen et al., 1979). Therefore, barbiturate/PIC and GABA receptor binding sites are physically distinct, with PIC having a close relation with the  $\text{Cl}^-$ -ionophore, although appropriate binding studies have yet to be performed on the myenteric plexus in the intestine to reveal the possible coupling of the GABA-receptor site with the barbiturate/PIC binding sites. On the basis of qualitative and quantitative results observed here, it is hypothesized that the depressant barbiturates potentiate GABA<sub>A</sub>-receptor-mediated ileal contractile responses via an allosteric interaction with the PIC-sensitive site which appears to be involved in the regulation of the  $\text{Cl}^-$ -ionophore. It is still premature to postulate if PIC and barbiturates could alter the life-time or the closing of the activated  $\text{Cl}^-$ -channel in the myenteric plexus, although it is believed that PIC and convulsant barbiturates may act by closing the ionophore, whilst the depressant and anti-convulsant barbiturates could prolong GABA-mediated inhibitory mechanism by increasing the life-time of the activated  $\text{Cl}^-$  ion channel (see review by MacDonald, 1984).

In conclusion, the isolated ileum is a simple in vitro preparation for studying barbiturate/benzodiazepine/GABA<sub>A</sub>-receptor interactions at the  $\text{Cl}^-$ -ionophore complex, and may prove useful in elucidating the mechanisms by which such interactions occur.

CHAPTER SIX. GABA AND 5-HYDROXYTRYPTAMINE

INTERACTIONS IN THE INTESTINE.



## 6.1. INTRODUCTION

Both GABA and 5-HT elicit neurally-mediated contractile responses of cholinergic origin in the isolated ileum of the guinea-pig (Gaddum and Picarelli, 1957; Brownlee and Johnson, 1963; Costa and Furness, 1979; Krantis et al., 1980). This contractile effect of GABA is rather transient, being followed by a brief period of inhibition at the smooth muscle of the intestine which is then succeeded by a delayed, prolonged 'after-relaxation' due to a diminution in transmitter release from the cholinergic motoneurons responsible for the contraction (Ong and Kerr, 1983 a,b). By contrast, the ileal contractile response to 5-HT, although somewhat resembling that of GABA, is not followed by either of these inhibitory phases (Costa and Furness, 1979). Ganglionic blocking agents do not alter the ileal contractile responses to 5-HT (Costa and Furness, 1979), nor to GABA (Krantis et al., 1980). This suggests that GABA might stimulate some interneurone causing it to release 5-HT which would then stimulate ileal cholinergic motoneurons mediating the contractions, rather than GABA acting through a direct stimulation of the motoneurons (Tonini et al., 1983); it being generally accepted that the 5-HT receptors eliciting the cholinergic responses are on the motoneurons of the myenteric plexus. The site of action of GABA in the myenteric plexus is, however, less clear although the contractile responses to GABA are mediated through GABA<sub>A</sub>-receptors coupled to a chloride-ionophore (Bowery et al., 1981 a; Krantis and Kerr, 1981 b; Ong and Kerr, 1983 a,b). Activation of these receptors could lead indirectly to ileal contractions

through the mediation of some non-cholinergic transmitter released by GABA, or, as is favoured, the ileal contractions could result from the release of ACh due to a direct GABA<sub>A</sub>-receptor-induced depolarization of the cholinergic neurones innervating the ileal smooth muscle, since such receptors commonly occur on autonomic neurones (Brown and Marsh, 1978).

Early in the investigations of GABA actions in the guinea-pig intestine, it was shown that contractile responses of the ileum induced by 5-HT or nicotine were reduced by prior application of GABA (Hobbiger, 1958 a,b; Inouye et al., 1960). There are several possible explanations for this 'antagonism' which has not been investigated since. In the first place, GABA and 5-HT might interact at the level of the 5-HT receptor on the ganglionic cell bodies of the cholinergic neurones responsible for the contraction of the ileum, and such interaction could result in either non-competitive or competitive antagonism of 5-HT, as implied by Inouye et al (1960). Alternatively, this depressant GABA action could be exerted at some region remote from the 5-HT receptors, as Hobbiger (1958b) suggested, possibly by a GABA-dependent presynaptic depression of transmitter output from cholinergic neurones, or else through some postjunctional inhibitory action indirectly induced by GABA on the ileal smooth muscle itself. Such a postjunctional anti-cholinergic action was indeed described by Hobbiger (1958 a,b) and by Inouye et al (1960), using exogenously applied ACh to stimulate the ileum, but this is not the entire explanation of the 'antagonism' since, as the present study shows, ileal responses to either 5-HT, nicotine or

DMPP, are also depressed by GABA acting through the GABA<sub>B</sub>-receptor sites on myenteric neurones.

As GABA receptors in the enteric nervous system can be differentiated into two types, GABA<sub>A</sub> and GABA<sub>B</sub>, based on their differing agonist and antagonist susceptibilities (Bowery et al., 1981 a; Kaplita et al., 1982; Giotti et al., 1983a; Ong and Kerr, 1983 a,b; 1984 c,d; Kerr and Ong, 1984 a,b; in press), the contributions of such GABA<sub>A</sub>-and GABA<sub>B</sub>-receptor-mediated actions of GABA to the depression induced by 5-HT in the guinea-pig intestine, are here described. Manipulation of varying experimental conditions (e.g. temperature, etc.), and the timing of drug application, have also been employed in the present study to investigate the depressive effects of various GABAergic agents on responses to different agonists stimulating the ileum.

Recently it has been suggested by Tonini et al (1983) that the GABA-induced ileal contractions are in fact due to an activation of the cholinergic motoneurones through an interneuronal release of 5-HT that is induced by GABA. Since the neuronal actions of GABA in the intestine are not affected by ganglionic blocking agents (Krantis et al., 1980), its actions could be mediated by direct stimulation of GABA receptors in the cell body of the effector neurone, or alternatively, GABA could act by interneuronal release of a neurotransmitter substance, such as 5-HT. Tonini et al. (1983), based on their observations that 5-HT desensitization results in decreased responsiveness to GABA in ileal preparations, suggest that the GABA<sub>A</sub>-receptor-

mediated ileal contractile response is largely mediated by 5-HT release which then, in turn, activates the motoneurons. Such a possibility, that GABA might cause its excitatory actions in the intestine by releasing a non-cholinergic transmitter, such as 5-HT, onto the motoneurons, had already been suggested (Kerr, personal communication); indeed, much of the present work was completed before the report by Tonini et al. (1983) appeared. However, the contrary conclusion, that 5-HT does not mediate the contractile response to GABA in the guinea-pig ileum, is more supported by the present evidence showing that contractile responses to GABA in the guinea-pig ileum can persist even after a 'desensitizing' dose of 5-HT which, as described by Huidobro-Toro and Foree (1980), depresses or abolishes the ileal response to an additional dose of 5-HT. Furthermore, if 5-HT does indeed mediate these responses to GABA, then they should not only always be depressed by desensitization to 5-HT, as Tonini et al (1983) report, but should also be abolished by antagonists of the neurally-mediated contractile response to 5-HT, such as quipazine (Lansdown et al., 1980). This study shows that such antagonism by quipazine, so far found to be the most specific available antagonist of the neurally-mediated 5-HT response, does not abolish contractile responses to GABA in the guinea-pig isolated ileum, contrary to the notion that they are mediated by 5-HT.

## 6.2. MATERIALS AND METHOD

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of the distal and proximal ileum, 4 cm in length, the distal ileum taken 2-3 cm from the ileo-caecal valve, and segments of the distal colon, also 4 cm in length, were quickly removed and mounted vertically in a 10 ml organ bath containing normal Krebs-bicarbonate solution, gassed in a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, the composition of the Krebs solution was as described in Chapter 2.1. Effects of drug treatments were examined on the resting tissue, the mechanical activity of the longitudinal muscle of the tissue was recorded as described in Chapter 2.1. In some experiments, the tissues were electrically stimulated with a pair of platinum electrodes (see Chapter 2.1). The equilibration period for the tissues prior to any drug application of electrical stimulation was approximately 60 min. Antagonists were added at least 10 min before agonists were added. ED<sub>20</sub>, ED<sub>50</sub> and ED<sub>70</sub> are defined as the effective doses of the drug required to produce 20%, 50% and 70% of the maximum control responses. Desensitization to any drug (e.g. 5-hydroxytryptamine (5-HT)) was induced by leaving a high dose of the drug, (5-HT, 5-50  $\mu$ M) in the bath for at least 3-5 min, a second, repeat, test dose of the drug being applied after the primary response had returned to the baseline. Drug volumes used never exceeded 1% of the total bath volume. All experiments were repeated in duplicate on at least 8 tissues from 4 different animals. Student's t-test for paired and unpaired samples was used to assess the

significance ( $P < 0.05$ ) of differences between mean values of the dose-response effects.

**CHEMICALS:**

The following chemicals were obtained from Sigma except for those indicated in parenthesis.

5-hydroxytryptamine hydrochloride or creatinine sulphate

tetrodotoxin

atropine

GABA

bicuculline methochloride (Pierce)

picrotoxinin (dissolved in 1:9 absolute alcohol and distilled water)

piretanide (Dr. A.H. Bretag)

furosemide (Dr. A.H. Bretag)

d-aminovaleric acid hydrochloride

acetylcholine chloride

3-amino-1-propanesulphonic acid

muscimol

nicotine

1,1-dimethyl-4-phenyl-piperazinium iodide

baclofen (Ciba-Geigy)

quipazine (Miles Laboratories)

histamine

ethylenediamine dihydrochloride

## 6.3. RESULTS

### 6.3.1. Interactions of GABA and 5-HT in the guinea-pig ileum.

In the distal ileum, dose-dependent contractions were induced by 5-HT (Fig. 1). At lower doses, up to 0.5  $\mu\text{M}$ , these contractions were essentially of neural and cholinergic origin, being abolished by TTX (0.3  $\mu\text{M}$ ) and atropine (0.7  $\mu\text{M}$ ), whilst at higher doses, the contractile responses were increasingly due to a direct action of 5-HT on the muscle, and only partially antagonised by atropine. For this reason, all the experiments conducted in present study used doses of 5-HT not exceeding 0.2  $\mu\text{M}$ , when investigating the mechanism of interaction between GABA and 5-HT on ileal preparations. Responses of the unstimulated ileum to GABA consisted of dose-dependent contractions, also of neural and cholinergic origin, which rapidly declined to the baseline, and were then followed by prolonged relaxations which, in preparations with high intrinsic basal tone, extended to a level below the baseline (Fig. 2), i.e. an 'after-relaxation' effect. The dose-dependent contractions were sensitive to BMC (5-50  $\mu\text{M}$ ) (Fig. 2), PIC (5-50  $\mu\text{M}$ ), and other chloride blockers such as piretanide and furosemide (c.f. Krantis and Kerr, 1981 b). The delayed prolonged 'after-relaxation', however, was only sensitive to DAVA (0.1-1 mM) (Fig. 2), but not to BMC or PIC. Interactions between GABA and 5-HT were examined after the addition of GABA into the bath. The nature of the resultant depression of the responses to 5-HT was critically dependent on the timing of this addition, with two different mechanisms involved

that corresponded in turn with the decline of the GABA-induced contractile response to the baseline, and with the prolonged 'after-relaxation' elicited by GABA.

There was no evidence of 5-HT-receptor sensitivity to antagonists of GABA<sub>A</sub>-receptor-mediated events, such as PIC (5-50  $\mu$ M, n=12) (Fig.5), piretanide (5-50  $\mu$ M, n=10) or BMC (5-50  $\mu$ M, n=12) (Fig. 2,4) as shown by the lack of displacement of the dose-response curves for 5-HT in the presence of either BMC or PIC (Fig. 1). Furthermore, none of these antagonists altered cholinergic responses to applied ACh, or to transmural electrical stimulation, although they did antagonise contractile responses to GABA, 3-APS or muscimol (vide Chapter 3).

### 6.3.2. GABA<sub>A</sub>-receptor-mediated depression of responses to 5-HT.

There was a BMC (5  $\mu$ M)-sensitive depression of the response to exogenously applied 5-HT (50 nM), provided that the response to 5-HT occurred during the period corresponding with the falling limb of the GABA (10  $\mu$ M)-induced contractile response (Fig. 2). This depressive effect of GABA on responses to 5-HT was dose-dependent (GABA 5-100  $\mu$ M; Fig. not shown). In general, 5-HT had to be applied within < 20 s of GABA for this effect to be seen, but not so early that the two contractile responses added. Following treatment with BMC, the contractile response to GABA was eliminated and GABA no longer depressed the response to 5-HT when added with the same timing as that used before BMC treatment (i.e. < 20 s after GABA); here the contractile response to 5-HT



occurred before the onset of the delayed, GABA-induced 'after-relaxation' in the presence of BMC (Fig. 2, n=12). This early GABA-induced depression of responses to 5-HT could also be prevented by pretreatment with PIC (5-50  $\mu$ M, n=6) or piretanide (5-50  $\mu$ M, n=6). In addition, the same type of depression was seen when using 3-APS (10-50  $\mu$ M) or muscimol (10-50  $\mu$ M) in place of GABA, and was also sensitive to BMC, PIC and piretanide, in at least 6 preparations. However, the early, transient period of depression was unaffected by treatment with DAVA (0.5 mM) (Fig. 2, n=16), the ileum still responded to 5-HT (50 nM) or to GABA (10  $\mu$ M) with a contraction in the presence of DAVA. Only the delayed GABA-induced 'after-relaxation' was eliminated by DAVA (Fig. 2), and GABA still depressed responses to 5-HT that occurred during the phase of the falling limb on the GABA-induced response (Fig. 2).

DAVA itself induced a brief BMC-sensitive contraction, and then within 1-2 min prevented the depression of electrically elicited twitch responses in the ileum by GABA or baclofen (see Chapter 3) without affecting contractile responses to GABA, 3-APS or muscimol.

In at least 6 preparations, GABA (10  $\mu$ M) also caused the same, early BMC-sensitive depression of responses to nicotine (NIC) (0.5  $\mu$ M), ACh (10 nM) and DMPP (0.5  $\mu$ M), when these were applied similarly within < 20 s of GABA, the same timing as with 5-HT, i.e. the responses occurred during the falling limb of the response to GABA. This depression of responses to NIC (Fig. 3),

ACh or DMPP by GABA was also sensitive to PIC (10  $\mu\text{M}$ ) and pirenitide (10  $\mu\text{M}$ ), but not to DAVA (0.5 mM) (Fig. 3). However, in our experience when working at 37°C, this early GABA-induced depression of all these cholinergic responses was sufficiently difficult to quantitate that this aspect was not pursued further.

### 6.3.3. GABA<sub>B</sub>-receptor-mediated depression of responses to 5-HT.

Responses to 5-HT were also depressed, or even abolished, by prior application of GABA (20  $\mu\text{M}$ ) when the response to 5-HT (2.5 nM) occurred during the period of the delayed 'after-relaxation' that followed the GABA-induced contractile response (Fig. 4), generally 30 s-1 min after the addition of GABA. Neither BMC (5  $\mu\text{M}$ ) (Fig. 4), PIC (5  $\mu\text{M}$ ) (Fig. 5) nor pirenitide (5-10  $\mu\text{M}$ ) altered this delayed depression which still occurred despite the abolition of any GABA-induced contraction in the presence of these agents. But this delayed depression was sensitive to DAVA (0.1 mM), which both abolished the 'after-relaxation' to GABA (20  $\mu\text{M}$ ) and prevented the delayed depression of responses to 5-HT (Fig. 4, n=6). DAVA (0.1 mM), alone, had no influence on responses to 5-HT (2.5 mM).

Responses to NIC (0.5  $\mu\text{M}$ ) or DMPP (0.5  $\mu\text{M}$ ) were similarly depressed when NIC or DMPP was applied 30 s -1 min after GABA (20  $\mu\text{M}$ ) (n=6) or baclofen (50  $\mu\text{M}$ ; n=8), but responses to ACh (10 nM) were unaffected if applied with this delay (Fig. 6), although the responses to ACh (1 nM) were depressed considerably if ACh was applied < 20 s after GABA (Fig. 6) (n=6). For ACh applied 30

s-1 min after either GABA (20  $\mu$ M) (Fig. 7) or baclofen (20  $\mu$ M) at their respective ED50s, there was no shift in the dose-response curve for ACh (Fig. 7) (n=6). DAVA (0.1 mM) also prevented the delayed GABA-induced depression of responses to NIC or DMPP. By contrast, if 3-APS (10  $\mu$ M) (Fig. 8) or muscimol (5  $\mu$ M) was used in place of GABA, there was no such depression of responses to 5-HT (2 nM) (Fig. 8) or NIC applied 30 s-1 min later, and there was no 'after-relaxation'. However, the ACh (10 nM)-induced contractile response was depressed if ACh was applied < 30 s after 3-APS (10  $\mu$ M) (Fig. 8). These results were all repeated on 6 different tissues in duplicate throughout each series of experiments.

The dose dependency of this delayed, GABA-induced depression of responses to 5-HT was assessed in two ways:

(a) Dose-response curves for 5-HT were constructed in the presence of GABA applied 30 s-1 min before the 5-HT. Three doses of GABA were used, corresponding to the ED20 (5  $\mu$ M), ED50 (20  $\mu$ M), and ED70 (50  $\mu$ M) for the GABA-induced depression of electrically elicited twitch contractions in the ileum (c.f. Ong and Kerr, 1983a). The dose-response curves were shifted to the right in a non-competitive manner, with an increasing depression of the maximum response to 5-HT as the dose of GABA was raised; but in the presence of DAVA (0.5 mM), there was no such displacement of the dose-response curve for 5-HT (Fig. 9a).

(b) Responses to 5-HT (0.2  $\mu$ M) were challenged by prior addition of increasing doses of GABA or baclofen; this concentration of 5-

HT was near the maximum usable in such experiments since direct actions of 5-HT on the ileal muscle began to interfere at higher doses. GABA or baclofen, applied 30 s-1 min previously, depressed the responses of 5-HT (0.2  $\mu$ M) in a dose-dependent manner with virtual abolition of the response at doses of 100  $\mu$ M GABA and above, whilst baclofen, added with a similar timing, produced an equipotent depression of responses to 5-HT (Fig. 9b). These effects were seen in the presence and absence of BMC (5-10  $\mu$ M), but there was no such GABA- or baclofen-induced depression of responses to 5-HT in the presence of DAVA (0.1 mM).

The dose-response curves for ACh applied at increasing doses 30 s-1 min after applying GABA, at ED20, ED50 and ED70, were not significantly different from the control dose-response curve for ACh alone (e.g. Fig. 7 for GABA ED50=20  $\mu$ M), nor were histamine (10 nM)-induced responses affected if histamine was applied such that the response occurred during the delayed, prolonged 'after-relaxation' phase induced by GABA, i.e. > 30 s after GABA. The histamine-induced contractile response was also unaffected by baclofen, but was depressed if the response occurred on the descending limb of the GABA-induced contractile response.

#### 6.3.4. Effect of temperature, and of cold storage on the ileum.

##### (a) Responses to GABA, and GABA and 5-HT interactions.

Hobbiger (1958 a,b) used a working temperature of 37°C, similar to the main part of the present study, whilst Inouye et

al (1960) maintained their preparations at 25 °C. In addition, both subjected some ileal preparations to prolonged cold storage. On repeating these various conditions, in eight fresh ileal preparations, at 25°C, there was a reduction in spontaneous activity whilst responses to GABA (10 µM) and 5-HT (10 nM) were slower, more prolonged, and some three fold less sensitive than those seen at 37°C (Fig. 10). In particular, the decline of the GABA (10 µM)-induced contraction back to the baseline was markedly slowed (Fig. 10), and parallel with this, there was a prolongation of the early GABA-induced depression of responses to 5-HT (10 nM) (Fig. 10), NIC (1 µM), DMPP (1 µM) or ACh (10 nM), which was insensitive to DAVA (100 µM), but was sensitive to BMC (10 µM), and PIC (10 µM). The DAVA (100 µM)-sensitive, GABA-induced depression of responses to 5-HT (10 nM) or NIC (1 µM) was also further delayed, and again coincided with the 'after-relaxation' as described in the previous sections. Similar but even more slowed and delayed effects were seen, with GABA and 5-HT interactions, using ileal preparations at 20°C, the lowest temperature found to give consistent, reproducible results (Fig. not shown).

When ileal preparations were subsequently used at 37°C, after having been kept in Krebs solution at room temperature for some 10 h, followed by a further 12-14 h at 6°C overnight, the responses to 5-HT were little altered, but there was a considerable reduction, or even abolition of the contraction elicited by GABA (10 µM), or EDA (0.5 mM) (Fig. 11) (n=6) and the early GABA- or EDA-induced depression of responses to 5-HT was correspondingly diminished or sometimes absent (Fig. not shown).

Contrasted with this, there was a prominent increase in the 'after-relaxation' induced by GABA (10  $\mu$ M) and EDA (0.5 mM), compared to the responses which were elicited in fresh ileal preparations maintained at 37<sup>0</sup>C (Fig. 11), which showed, as seen above, more prominent contractile responses to GABA (10  $\mu$ M) and EDA (0.5 mM), but a lesser 'after-relaxation'. Corresponding with the increased 'after-relaxation' in cold-stored preparation, an enhancement of the associated delayed DAVA (0.1 mM)-sensitive depression of responses to 5-HT was observed, e.g. in fresh preparations at 37<sup>0</sup>C, GABA (10  $\mu$ M) reduced the response to 5-HT (10 nM) by  $50 \pm 0.5$  % , but depressed it by  $80 \pm 0.5$  % after 'ageing' and cold storage (n=8).

**(b) Interactions of GABA and ACh in the isolated ileum.**

Both Hobbiger (1958a) and Inouye et al (1960) also showed that GABA depresses contractile responses of the ileum to ACh which acts directly on the smooth muscle. This depression was confirmed here, and was found to be BMC- and PIC-sensitive. This anti-ACh effect was best seen when using fresh ileal tissues at lower bath temperatures (e.g. 25<sup>0</sup>C) since it corresponded to the early, transient depression already described, and occurred only during the (slowed) decline of the GABA-induced contraction towards the baseline. Although sensitive to BMC (10  $\mu$ M) and PIC (10  $\mu$ M), this 'anti-ACh' action was not influenced by DAVA (500  $\mu$ M), and did not occur if the ACh (10 nM) was added during the delayed DAVA-sensitive 'after-relaxation' induced by GABA. Both 3-APS (10  $\mu$ M) and muscimol (5  $\mu$ M) caused a similar

depression of ACh (10 nM)-induced responses at 25°C when ACh was applied < 30 s after 3-APS or muscimol. Although repeated on more than 6 ileal preparations, this particular depressive action of GABA acting on ACh-induced responses at 25°C was not quantitated here, as was done by Inouye et al (1960).

#### **6.3.5. Interactions between EDA and 5-HT in the guinea-pig ileum.**

Interactions, very similar to those described above, were found when using EDA to replace GABA. Thus, responses to ACh (10 nM), NIC (2 µM), DMPP (5 µM), histamine (10 nM), or 5-HT (1 nM), were depressed if timed to occur during the declining phase of the EDA (0.5 mM)-induced contractile response, and this depression was prevented by BMC (10 µM) or PIC (10 µM), but not by DAVA (0.5 mM) (Fig. 12), whilst responses to NIC, DMPP, or 5-HT, but not ACh or histamine, were depressed if timed to occur during the delayed 'after-relaxation' of the EDA-induced response. The latter depression was sensitive to DAVA but not to BMC or PIC. These interactions between EDA and 5-HT were also found to be modified by altered (low) temperature and cold storage of the tissues as described in section 6.3.4 above (Fig. not shown).

#### **6.3.6 Interactions between GABA and 5-HT in the distal colon.**

In the distal colon, exogenously applied GABA (100 µM), EDA (1 mM) and baclofen (100 µM) induced relaxation responses, whilst DMPP (5 µM) and 5-HT (2 nM) induced contractile responses. The

5-HT (2 nM)-induced contraction, timed to occur during this relaxation, was depressed by GABA (100  $\mu$ M) (Fig. 13), EDA (1 mM) and baclofen (100  $\mu$ M), such depression being antagonised by DAVA (500  $\mu$ M) (Fig. 13), rather than by BMC (10  $\mu$ M), although a BMC-sensitive component was at times seen when using GABA or EDA. The depression of the DMPP (5  $\mu$ M)-induced contraction by GABA (0.1 mM) or EDA (1 mM) was also antagonised by DAVA (0.5 mM) (Fig. 13). Contractile responses to ACh (80 nM) were unaffected during this DAVA (0.5 mM)-sensitive relaxation phase. These results were repeated on at least 6 tissues from 3 different animals.

#### **6.3.7. Interaction of 5-HT desensitization and GABA in the proximal ileum.**

Responses to exogenously applied ACh (80 nM) or NIC (2  $\mu$ M) were never depressed when applied such that the responses occurred after the peak of the 5-HT (10 nM)-induced response, the two responses showing additivity. Desensitization to 5-HT, as demonstrated by the reduction of the response to a second, repeat test dose of 5-HT in the presence of the initial dose (10 nM), only ever partially blocked responses induced by NIC (2  $\mu$ M), and never those to ACh (80 nM), whilst in some preparations, 5-HT desensitization did not block the response to NIC (2  $\mu$ M).

As shown in Fig. 14, a high dose of 5-HT (10 nM) elicited a contractile response that subsequently declined, with virtual abolition of any response to a repeat, test dose of 5-HT (10 nM), an effect ascribed by Huidobro-Toro and Foree (1980) to desensi-



tization to 5-HT. Exogenously applied GABA (10  $\mu$ M) resulted in a transient ileal contraction followed by an 'after-relaxation', as described earlier in this section. In a number of preparations of the ileum (n=26), this response to GABA was unaltered in the presence of such desensitization to 5-HT (20 nM) (Fig. 14), but in some other ileal preparations (n=10), desensitization to 5-HT (20 nM), did abolish the contractile response induced by GABA (10  $\mu$ M), without affecting the 'after-relaxation'. The same variability of the depression of GABA-induced contractions by desensitizing doses of 5-HT was observed using a range of doses with both GABA (5-100  $\mu$ M) and 5-HT (5-50 nM). When it occurred, this depressant effect of 5-HT was readily washed-out, as shown by the recovery or the control response to GABA (Fig. 14).

#### 6.3.8. Quipazine antagonism

Quipazine (0.1-0.5  $\mu$ M) was found to antagonise only neurally-mediated 5-HT (0.5-50 nM)-induced responses, without affecting contractile responses to ACh (10 nM), NIC (5  $\mu$ M), GABA (5-50  $\mu$ M), or cholinergic responses to transmural stimulation. At high concentrations (> 0.5  $\mu$ M), quipazine itself elicited a contraction in the ileal preparations, and there are indications that quipazine may be a partial agonist at 5-HT-receptor sites on some peripheral tissues (Lansdown et al., 1980). In Fig. 14, quipazine (0.1  $\mu$ M) selectively antagonised the contractile response to 5-HT (5 nM), whilst the equipotent response to GABA (10  $\mu$ M), and to transmural stimulation, persisted in the presence of quipazine (Fig. 14) (n=24). Quipazine was readily washed-out

in all preparations, with a recovery of the response to 5-HT. Again, this selective depression of 5-HT-induced responses, but not the responses to GABA, was observed over a range of doses with both GABA (5-100  $\mu$ M) and 5-HT (5-50 nM).

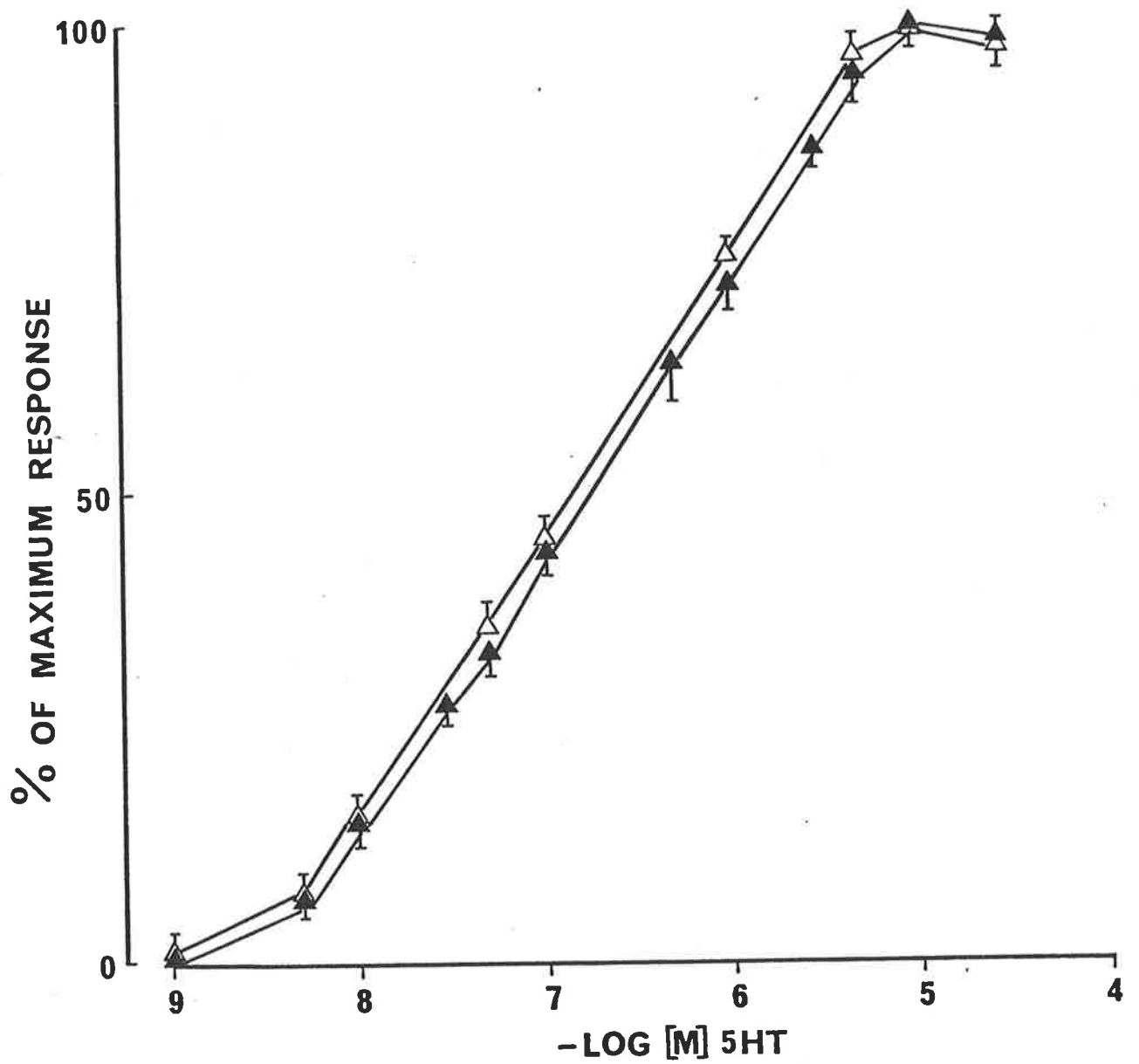


Fig. 1. Dose-response curves for contractions of the guinea-pig ileum induced by ▲ 5-HT (n=8), and in the presence of △ PIC (10  $\mu$ M) (n=12). Results are expressed on the ordinate as a percentage of the maximum contraction induced by 5-HT. Each point represents the mean and standard error of the mean.

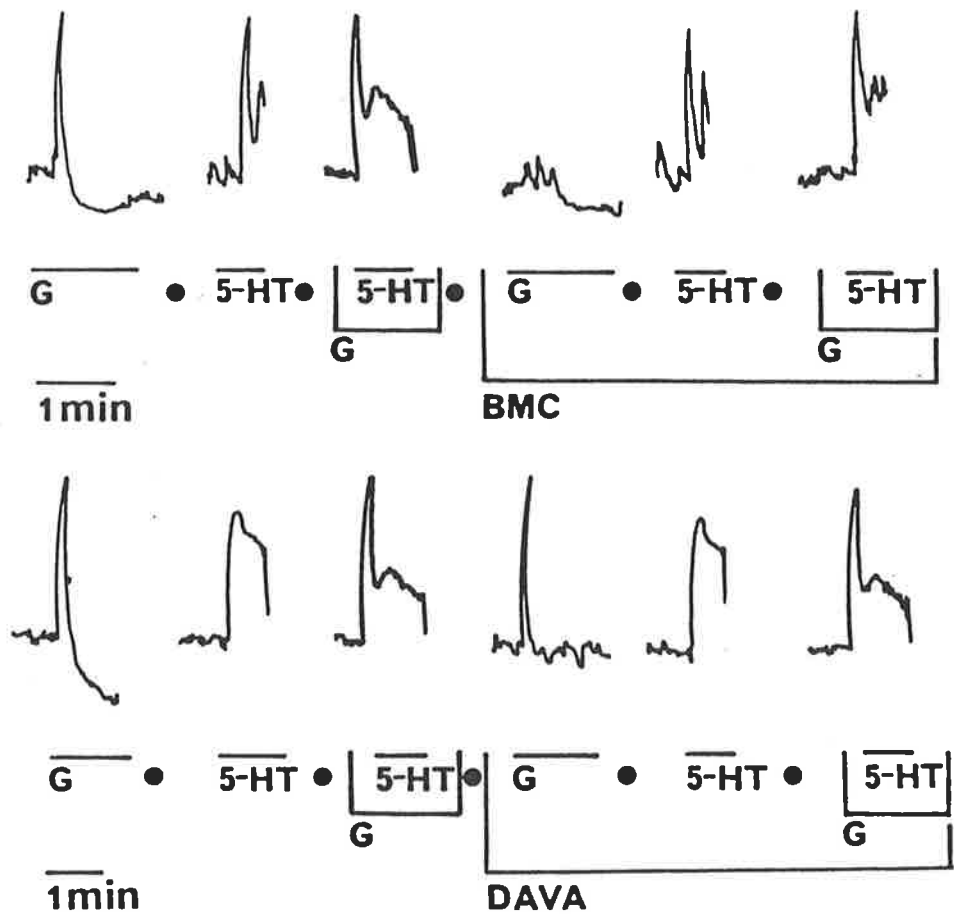


Fig. 2. Depression by GABA (G) of contractile responses to 5-HT applied < 20 s after GABA in the ileum, in the absence and presence of BMC and DAVA. (a) BMC (5  $\mu$ M) prevented the depression of the contractile response to 5-HT (50 nM) applied < 20 s after GABA (10  $\mu$ M). (b) In the presence of DAVA (0.5 mM), the response to 5-HT (50 nM) was still depressed by prior application of GABA (10  $\mu$ M) with the same timing (< 20 s) as in (a). BMC antagonised the GABA-induced contraction, whilst DAVA antagonised the GABA-induced 'after-relaxation'. • indicates tissue washout.

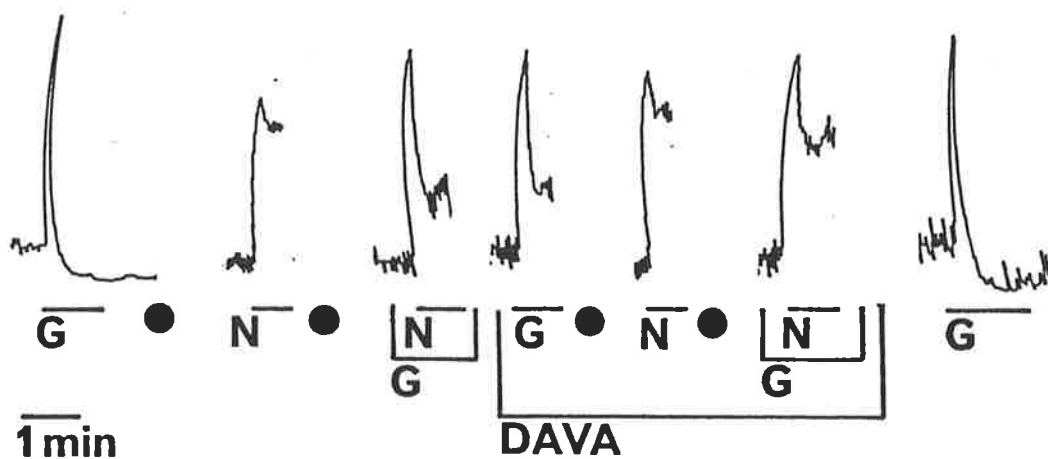


Fig. 3. Depression by GABA (G) of contractile responses to nicotine (N) applied < 20 s after GABA, in the absence and presence of DAVA. DAVA (0.5 mM) did not prevent the depression of the nicotine (0.5  $\mu$ M)-induced contractile response by GABA (10  $\mu$ M).

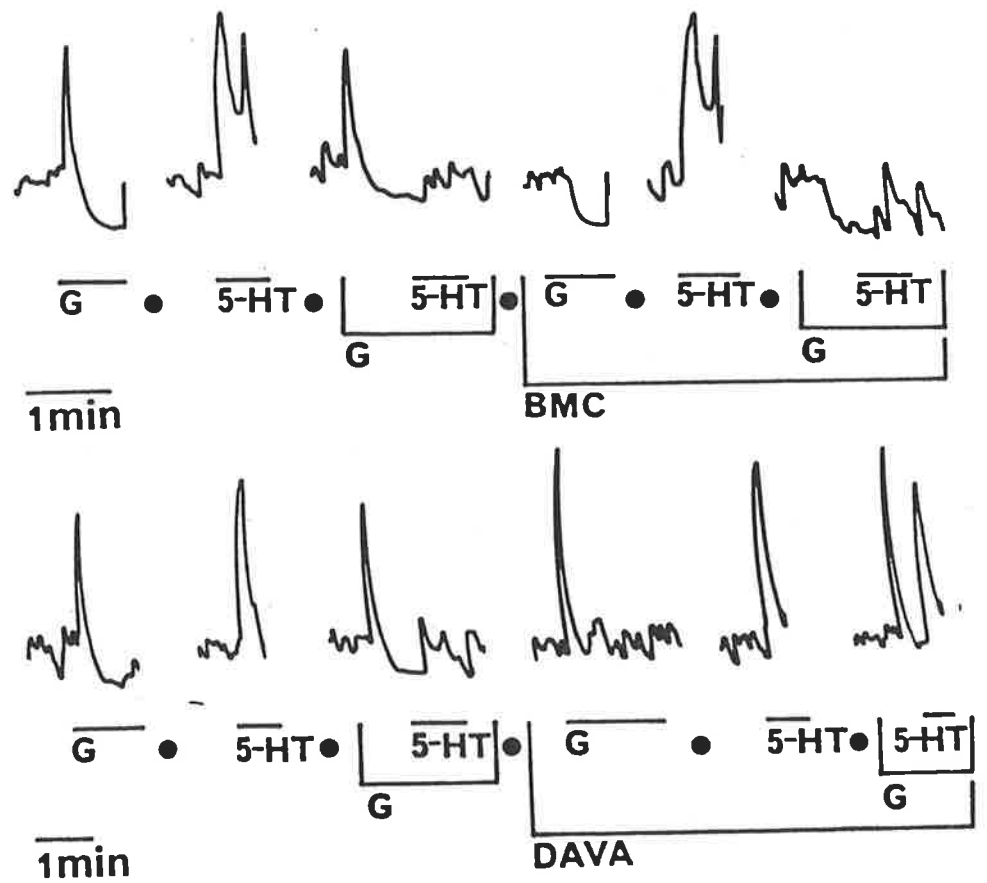


Fig. 4. Depression by GABA (G) of contractile responses to 5-HT, when 5-HT was applied > 30 s after GABA, in the absence and presence of BMC and DAVA. (a) BMC (5  $\mu$ M) did not affect the depression of the 5-HT (2.5 nM)-induced contractile response by GABA (20  $\mu$ M). (b) DAVA (0.1 mM) antagonised the GABA (20  $\mu$ M)-induced depression of the contractile response to 5-HT (2.5 nM).

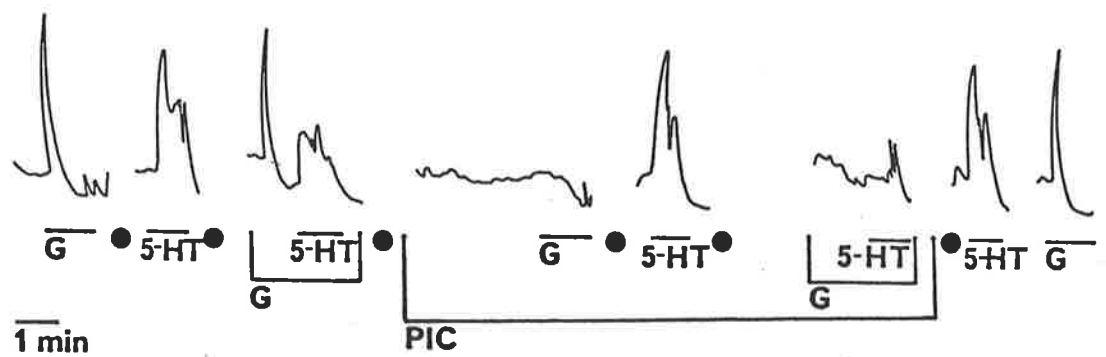


Fig. 5. Lack of effect of PIC (5  $\mu$ M) on the depression of the contractile response to 5-HT (2.5 nM) induced by GABA (20  $\mu$ M) when 5-HT was applied > 30 s after GABA. A recovery of the contractile responses to both 5-HT (2.5 nM) and GABA (20  $\mu$ M) was shown.

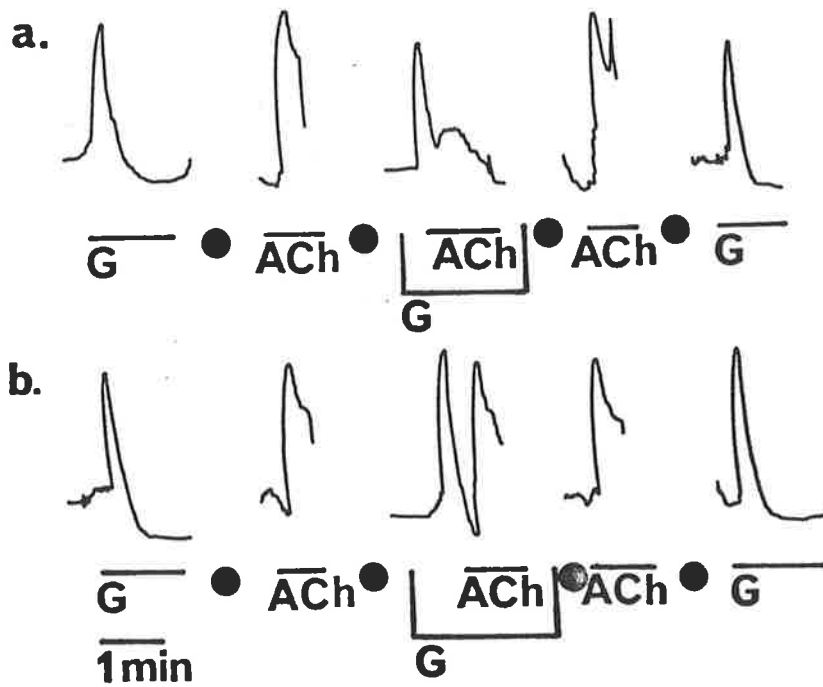


Fig. 6. (a) The contractile response to ACh (10 nM) was depressed by GABA (20  $\mu$ M) when ACh was applied < 20 s after GABA. (b) The contractile response to ACh (1 nM) was unaffected by GABA (20  $\mu$ M) when ACh was applied > 30 s after GABA. A recovery of control responses to ACh and GABA was shown.



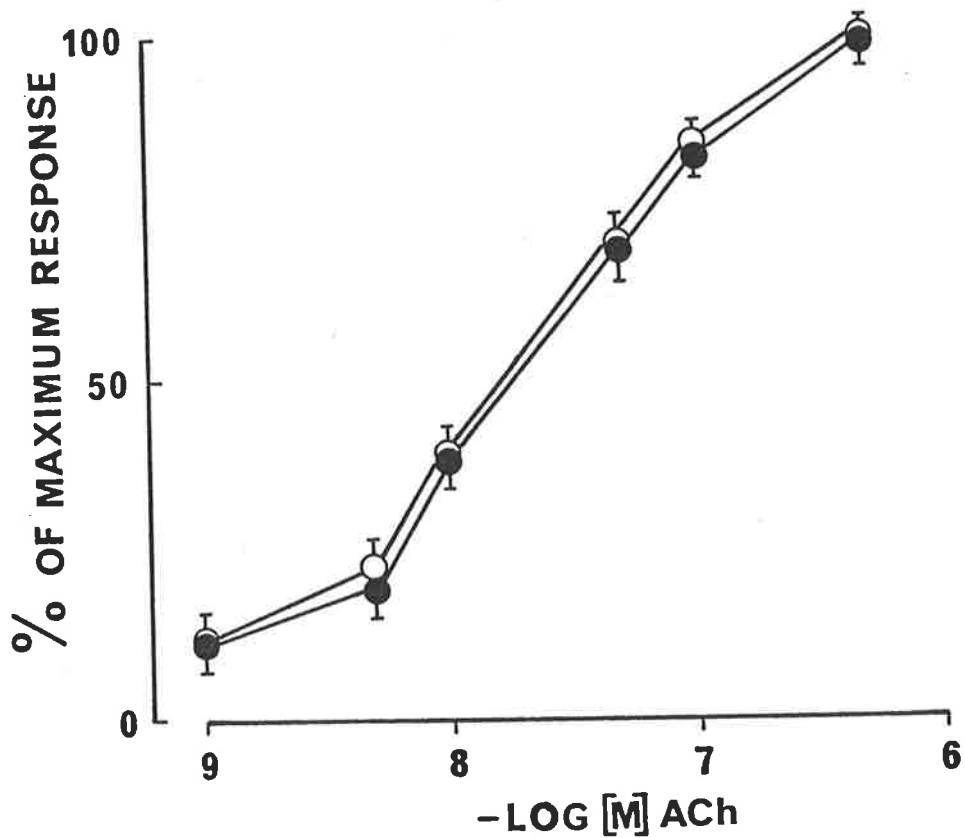


Fig. 7. Dose-response curves to contraction induced by ● ACh, with no significant shift in the dose-response curve for ○ ACh, when ACh was applied > 30 s after GABA at a ED50 value of 20  $\mu$ M. Results are expressed on the ordinate as a percentage of the maximum contractile response induced by ACh. Each point represents the mean and standard error of the mean based on the number of experiments performed n=6.

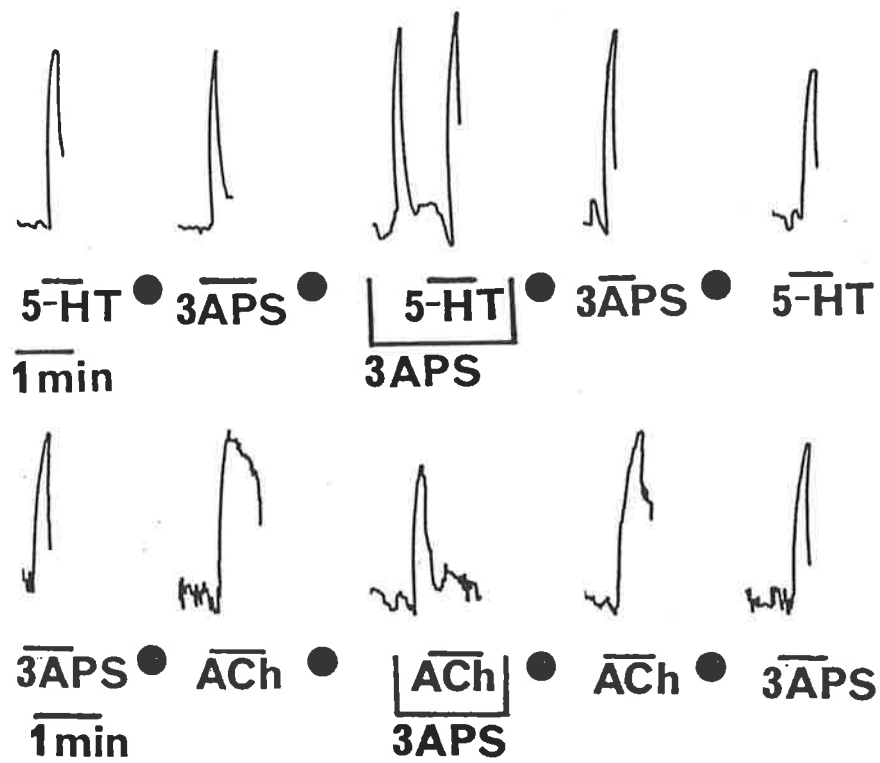


Fig. 8. Lack of depression of the 5-HT (2 nM)-induced contractile response by 3APS (10  $\mu\text{M}$ ) when 5-HT was applied > 30 s after 3APS, but there was a depression of the ACh (10 nM)-induced contractile response when ACh was applied < 30 s after 3APS (10  $\mu\text{M}$ ).

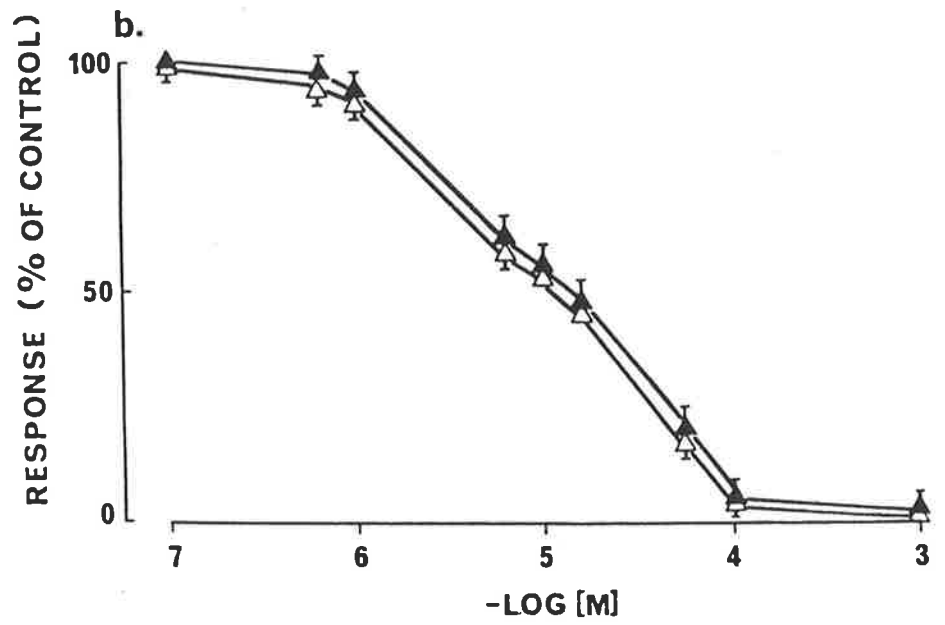
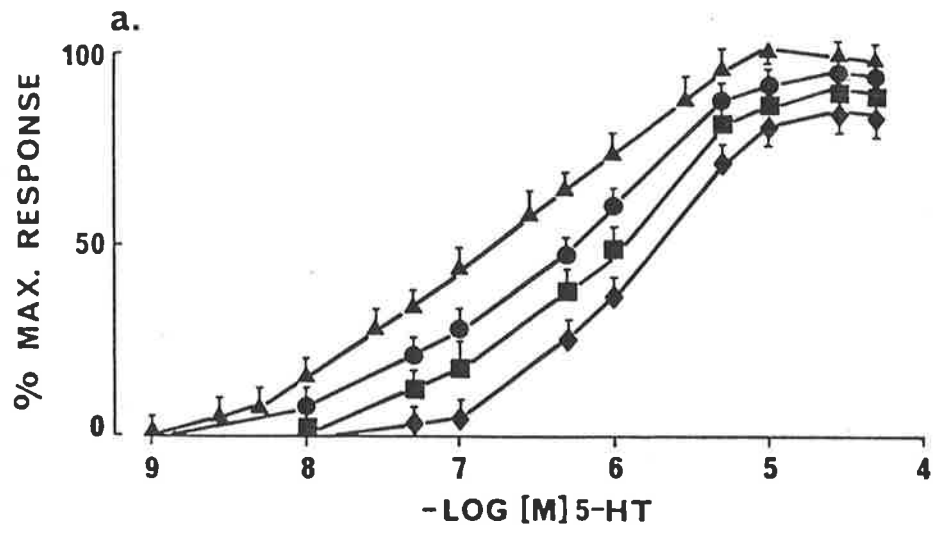


Fig. 9. (a) Dose-response curves for contractions induced by ▲ 5-HT applied 30 s-1 min after GABA, n=8; ● ED20 of GABA (5 uM), n=6; ■ ED50 of GABA (20 uM), n=6; and ◆ ED70 of GABA (50 uM), n=6; the results are expressed as a percentage of maximum contractile response (100%) induced by 5-HT. The depression of the dose-response curves for 5-HT by varying concentrations of GABA was significantly different from the control 5-HT dose-response curve ( $P < 0.05$ ). (b) Comparable dose-response curve for ▲ GABA- and Δ baclofen-induced depression of the contractile response elicited by ED50 of 5-HT (0.2 uM) applied 30 s-1 min after GABA, n=6. Ordinate: percentage of the control (ED50) response to 5-HT, ED50 of 5-HT is expressed as the dose required to produce 50% of the maximum contractile response induced by 5-HT. Each point represents the mean and standard error of the mean; n indicates the number of experiments performed.

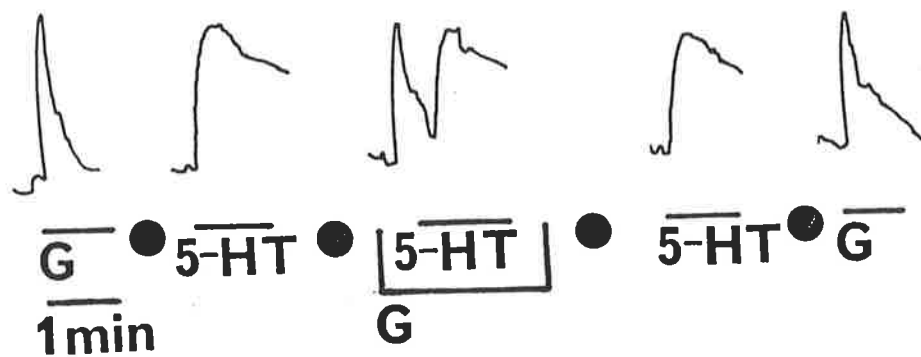


Fig. 10. Slowing and prolongation of the contractile responses to GABA (10  $\mu\text{M}$ ) and 5-HT (10 nM) in the guinea-pig ileum maintained at 25°C. There was a prolongation of the early GABA (10  $\mu\text{M}$ )-induced depression of contractile response to 5-HT (10 nM).

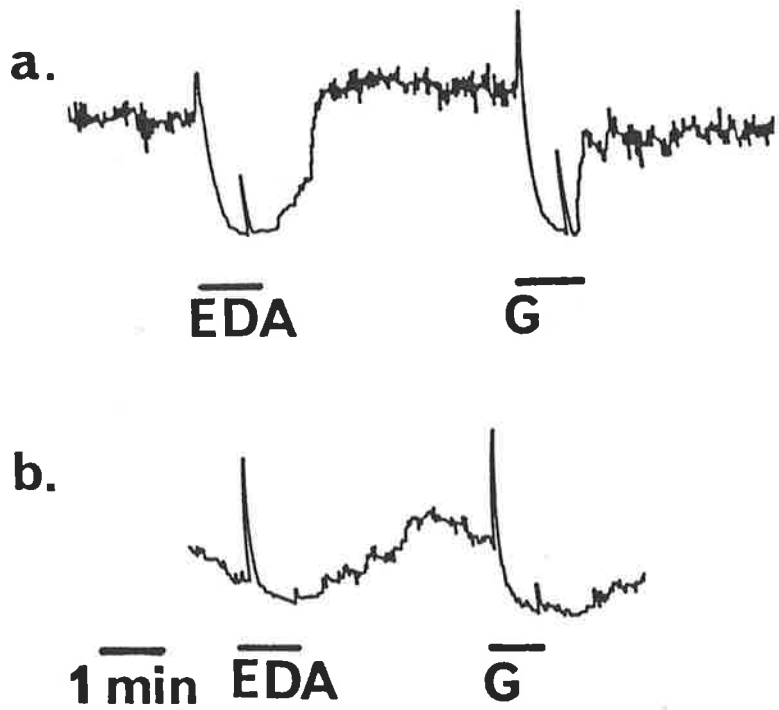


Fig. 11. At 37°C after overnight storage of the ileum at 6°C, both GABA (G) (10  $\mu\text{M}$ ) and EDA (0.5 mM) induced marked 'after-relaxation' responses, but a considerable reduction in the contractile responses. (b) Responses to GABA (10  $\mu\text{M}$ ) and EDA (0.5 mM) in fresh ileum maintained at 37°C.

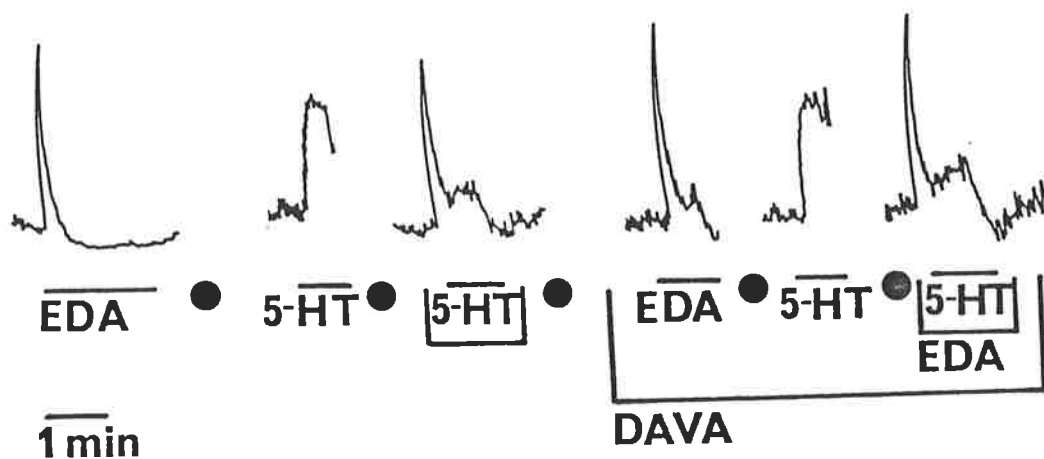


Fig. 12. Early depression of the contractile response to 5-HT (1 nM) by EDA (0.5 mM) when 5-HT was applied < 30 s after EDA, and DAVA (0.5 mM) did not prevent the early depression of the 5-HT contraction induced by EDA.

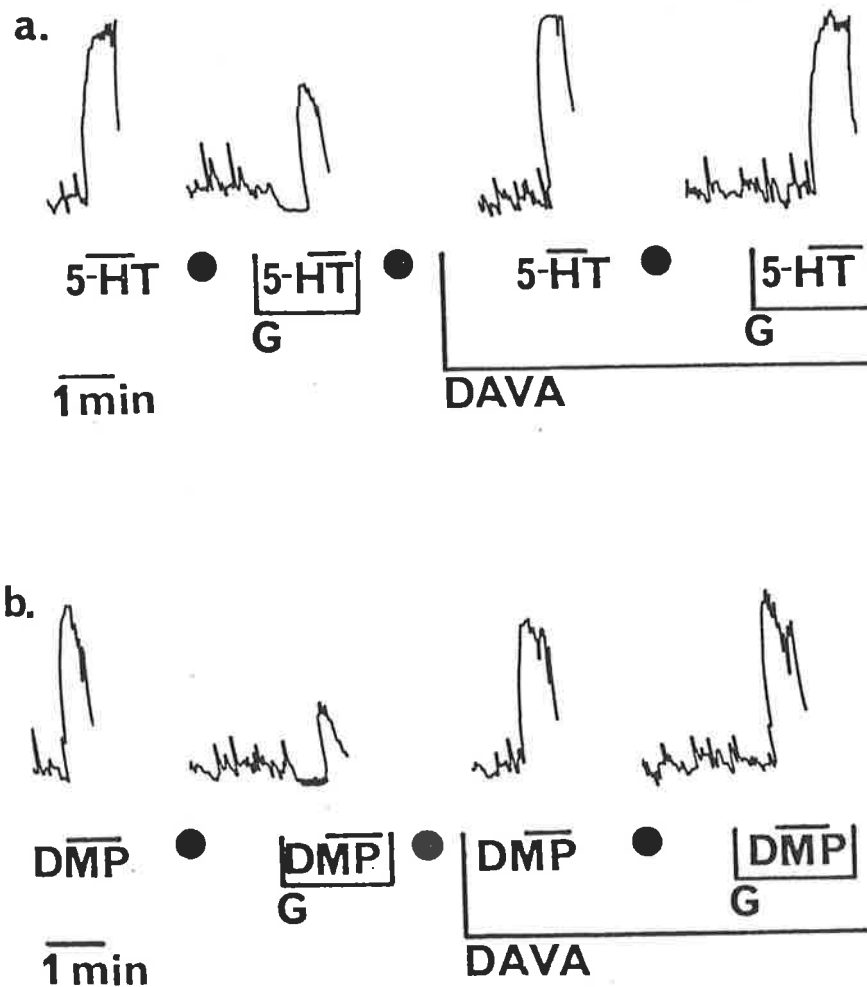


Fig. 13. (a) Depression of the contractile response to 5-HT (2 nM) by GABA (0.1 mM) when 5-HT was applied during the relaxation phase of the GABA (0.1 mM)-induced response in the guinea-pig distal colon. DAVA (0.5 mM) antagonised the depression of the 5-HT-induced contractile response by GABA. (b) Depression of the contractile response to DMPP (DMP) (5 μM) by GABA (0.1 mM) when DMPP was applied during the relaxation phase of the GABA (0.1 mM)-induced response in the distal colon. DAVA (0.5 mM) prevented the depression of the DMPP-induced contractile response by GABA.



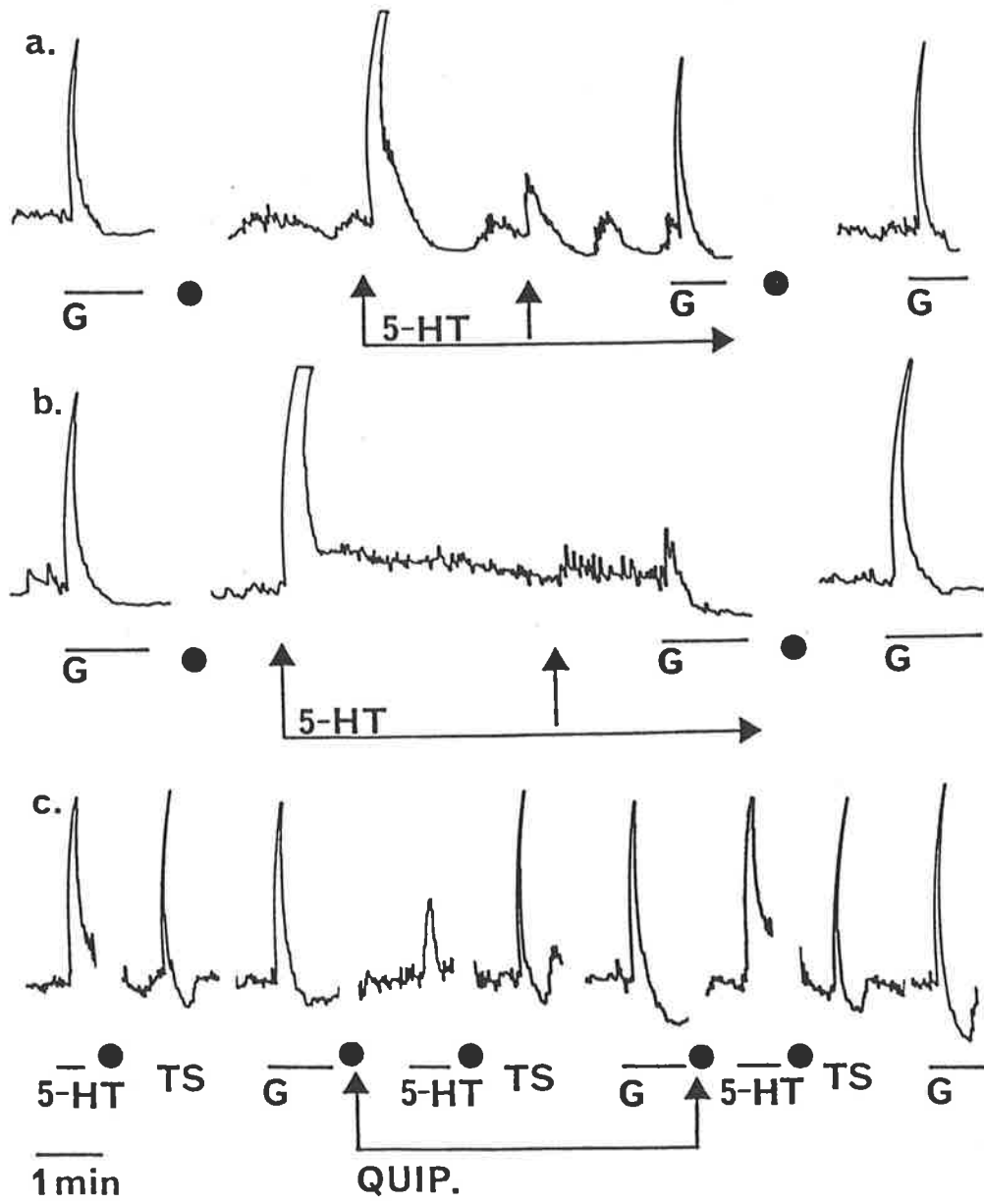


Fig. 14. (a) Desensitization to 5-HT in the guinea-pig proximal ileum was induced by applying an initial dose of 5-HT (10 nM) into the bath, followed by a repeat test dose of 5-HT (10 nM) without washout. Control contractile responses to GABA (G) (10  $\mu$ M) prior to 5-HT desensitization, during 5-HT desensitization and after washing out the desensitizing dose of 5-HT are shown (n=26). (b) Desensitization to 5-HT (20 nM total) abolished the contractile response to GABA (G) (10  $\mu$ M) in a different ileal preparation from a different animal (n=10). 5-HT desensitization did not affect the GABA-induced 'after-relaxation' in both (a) and (b). (c) Effect of quipazine (QUIP) (0.1  $\mu$ M) on contractile responses to equipotent doses of 5-HT (5 nM) and GABA (G) (10  $\mu$ M) and to transmural stimulation (TS) of cholinergic neurones (0.5 ms, supramaximal voltage). Control contractile response to 5-HT, GABA and TS prior to and after quipazine application are shown. All experiments were repeated in duplicate on at least 8 tissues from 4 different animals.

#### 6.4. DISCUSSION

This study has attempted to re-evaluate the initial explanation for the GABA-induced depression of contractile responses to 5-HT, ACh and nicotinic stimulants in the guinea-pig ileum (Hobbiger, 1958 a,b; Inouye et al., 1960), and has revealed two distinctly separate depressive actions of GABA in the intestine.

There was an early, short-lived depressive action of GABA against ileal contractions induced by 5-HT, NIC or DMPP, and postsynaptic excitatory agents such as ACh and histamine, only when these agonists were applied < 20 s after GABA, such that their responses were elicited during the period of the falling limb of the GABA-induced contractile response. Such timing appears to be critically important, because if the contractile responses to 5-HT or to any of the agonists occurred too early, they were additive with the GABA-induced contractile response, and if too late, only the responses to 5-HT, NIC or DMPP, but not to exogenously applied ACh or histamine were affected, evidently to be explained by a separate mechanism during the latter period. This early short-lived depressive effect was originally described by Hobbiger (1958a), and is to be seen in Fig 3. of that account. It is this particular depressive action of GABA that Inouye et al. (1960) described and found to be PIC-sensitive. In the present study, the early depressive action of GABA on responses to 5-HT, NIC, DMPP, ACh or histamine, was sensitive not only to PIC but also to BMC and chloride blockers such as piretanide, and this was also seen using EDA which releases GABA from the myente-

ric plexus (Kerr and Ong, 1984 a). In addition, the same early depression was elicited by 3-APS and muscimol, which are GABA<sub>A</sub>-but not GABA<sub>B</sub>-receptor agonists, and could be prevented by BMC, PIC or piretanide, all antagonists of GABA<sub>A</sub>-receptor-mediated events. It is thus unlikely that this particular depression could be due to a direct interaction of GABA and 5-HT at a common receptor site, as implied by Inouye et al. (1960), for neurally induced responses to NIC or DMPP were similarly depressed, as were responses to exogenous ACh or histamine which act directly on the ileal smooth muscle. Since GABA itself has no direct action on ileal smooth muscle (Krantis and Kerr, 1981 b), it must engender some neurally mediated inhibitory action at the postjunctional level on the smooth muscle to bring about this depression, presumably by activation of GABA<sub>A</sub>-receptors on myenteric neurones that bring about non-adrenergic, non-cholinergic (NANC) inhibition on the muscle itself.

There was, in addition, a longer-lasting depressive action of GABA on responses induced by 5-HT, NIC or DMPP, but not by ACh or histamine, when these agonists were applied within 30 s-1 min after GABA, i.e. during the 'after-relaxation' phase which corresponds with the delayed GABA<sub>B</sub>-receptor-mediated depression of cholinergic twitch contractions in the guinea-pig ileum, and which is only sensitive to DAVA but not to BMC, PIC or piretanide. Since ACh, or histamine, act directly on the smooth muscle of the intestine, and GABA did not influence responses to ACh applied with this timing, this delayed depressive effect of GABA must occur at the neuronal level in the myenteric plexus,

preventing neurally elicited cholinergic contractions. The same, delayed, depression of responses to 5-HT, NIC, or DMPP was also seen with baclofen, a specific GABA<sub>B</sub>-receptor agonist, and could be prevented by DAVA but not by BMC, PIC or piretanide, as also occurred when using EDA to release endogenous GABA from the myenteric plexus (Kerr and Ong, 1984 a). Furthermore, no such delayed depression was seen with 3-APS or muscimol, which both lack significant GABA<sub>B</sub>-receptor affinity (Bowery et al., 1981 b). It therefore follows that this depressive effect of GABA is mediated through GABA<sub>B</sub>-receptor sites on cholinergic neurones of the myenteric plexus, leading to a depression of ACh output as shown by Kleinrok and Kilbinger (1983).

Dose-response curves for 5-HT were altered in a non-competitive manner when constructed with appropriate timing in the presence of GABA or baclofen. The anomalous shape of these dose-response curves in the presence of high concentrations of GABA is to be explained by the dual action of 5-HT on the ileum, where it induces cholinergic contractions at doses less than 0.5  $\mu$ M, but increasingly causes a direct stimulatory action on the ileal smooth muscle at higher doses (Costa and Furness, 1979; Chahl, 1983). Only the cholinergic contractions induced by 5-HT could be affected through the GABA<sub>B</sub>-receptor-mediated depression of cholinergic transmission, consequently with doses of 5-HT in excess of 0.5-1  $\mu$ M one obtained here, in the presence of GABA (50  $\mu$ M), a dose-response curve for 5-HT acting directly on the muscle, as did Chahl (1983) using atropine to eliminate the cholinergic component of the responses to 5-HT. Furthermore, the

dose-response curves for ACh itself were unaffected by GABA or baclofen, showing that ACh responses are not depressed by GABA<sub>B</sub>-receptor-mediated actions in the intestine.

In the guinea-pig ileum, the 5-HT-induced contractile responses were insensitive to BMC or PIC, although, on the contrary, other studies have shown that some 5-HT-induced effects in the CNS can be antagonised by both BMC and PIC (Mayer and Straughan, 1981). PIC is reported to be an effective antagonist of 5-HT at several sites in feline autonomic and sensory ganglia (DeGroat and Lalley, 1973), and also blocks excitation by 5-HT of primary afferent neurones in the nodose ganglia and afferent axons in the carotid sinus nerve (Simonds and DeGroat, 1980), and rat hippocampal neurones (Segal, 1976). The mechanism of this antagonistic action of PIC against 5-HT is unclear since PIC is known to be a specific blocker of chloride channels (Takeuchi and Takeuchi, 1969) associated with the GABA-receptor chloride-ionophore complex (Olsen, 1981 a). One possible explanation could be that the action of 5-HT in these preparations is chloride-dependent, similar to that for GABA, hence explaining the PIC-induced antagonism of the responses elicited by 5-HT. However, in the guinea-pig enteric nervous system, neither PIC nor BMC affected any responses to 5-HT, as shown by the lack of displacement of the 5-HT dose-response curves in the presence of such antagonists. Also, in other preparations such as the rabbit nodose ganglion cells, PIC did not reduce responses to 5-HT (Wallis et al., 1982).

Using 25°C as a working temperature, Inouye et al. (1960) found a slowing of responses to GABA, 5-HT or ACh; it was thus possible for them to quantitate the early, short-lived depressant action of GABA, which was prolonged when working at 25°C so that the timing of drug additions was less critical. The essential basis of their findings was confirmed in the present study, but such a quantitation was not attempted. Hobbiger (1958b) noticed that "ageing" of ileal preparations, particularly with cold storage, would unmask or accentuate the 'delayed' depressant action of GABA, as was confirmed here; such cold storage often eliminated the initial contractile response to GABA or EDA, but always enhanced the GABA<sub>B</sub>-receptor-mediated effect, making more prominent the 'after-relaxation' and the delayed depression of responses to 5-HT and NIC or DMPP. The marked effects seen with GABA or 5-HT at such sub-physiological temperatures presumably may in part be due to a deficiency in the uptake system, which would be partially inactivated by low temperatures.

Although Krantis et al. (1980) described only a BMC-sensitive relaxation induced by GABA, in the guinea-pig distal colon maintained in Krebs solution containing hyoscine to suppress spontaneous contractile cholinergic activity, here, there was a marked BMC-insensitive relaxation of the colon induced by both GABA and baclofen, which was sensitive to DAVA. Contractile responses of the colon to 5-HT or DMPP, but not ACh, were depressed if timed to occur during this relaxation, and such depression was most often DAVA-sensitive, although at times a BMC-sensitive component was seen. However, the latter was more

erratic in its occurrence and this aspect was not further pursued. Evidently the DAVA-sensitive relaxation, and depression of neurally-mediated contractions, induced by GABA, EDA and baclofen in the colon, is analogous to the DAVA-sensitive 'after-relaxation' these agents induce in the ileum. It is likely also, although this requires more work, that the small BMC-sensitive component, in the GABA- and EDA-induced relaxation of the colon (Krantis et al., 1980), is in the same way analogous to the brief inhibitory phase seen during the declining limb of GABA- and EDA-induced ileal contractions.

The present investigation further shows that GABA can markedly modify activity evoked in the intestinal smooth muscle by cholinergic neurones of the myenteric plexus, and was aimed to clarify earlier studies showing that GABA has antagonistic actions on 5-HT, NIC or ACh responses. It is concluded that two depressive mechanisms are activated by GABA in the guinea-pig intestine:

- (a) an early, short-lived depression of contractile responses to agents directly, or indirectly, stimulating the intestinal smooth muscle, and mediated through GABA<sub>A</sub>-receptor sites on myenteric neurones leading to postjunctional inhibition on the muscle, and
- (b) a delayed, longer-lasting depression that is directed only against neurally-mediated stimulation of the muscle, and mediated through GABA<sub>B</sub>-receptor sites on the cholinergic innervation of the intestinal smooth muscle.



In agreement with Tonini et al (1983), desensitization to 5-HT can depress ileal responses to GABA; but in a significant number of preparations, even when responses to 5-HT (5-50 nM) had been virtually abolished by prior administration of 5-HT, contractile responses to GABA often persisted essentially at the control level. The 'desensitizing' dose of 5-HT (20 nM total) employed in experiments illustrated here should, from the results of Tonini et al (1983), markedly depress contractile responses to GABA (10  $\mu$ M), there being almost a 100 fold rightward shift in their dose-response curve for GABA constructed in the presence of 5-HT at only 5  $\mu$ M. There is no explanation for these discrepancies, but a range of doses of both GABA and 5-HT produced the same inconsistent results, leading to the conclusion that 'desensitization' to 5-HT is an unreliable tool in such studies. Since desensitization to 5-HT did not always abolish responses to GABA, some other explanation has to be sought for those examples where a depressive interaction between 'desensitization' to 5-HT and the contractile response to GABA could occur.

It cannot be that GABA and 5-HT interact at the receptor level in the myenteric plexus, for the converse depressive action of GABA on responses to 5-HT (Hobbiger, 1958 b) is non-specific and has a dual origin, a GABA<sub>A</sub>-receptor-mediated transient inhibition at the level the smooth muscle, responsible for the rapid return of the GABA-induced response to the baseline, and a GABA<sub>B</sub>-receptor-mediated prolonged depression of cholinergic transmission (Ong and Kerr, 1983 a,b). However, one possible explanation for the depressant action of desensitization to 5-HT upon ileal

responses to GABA would be that 'desensitizing' dose of 5-HT depress cholinergic transmitter output through a presynaptic mechanism, as has already been described by North et al (1980) in the myenteric plexus where they found that 5-HT acts prejunctionally to depress ACh output from cholinergic neurones. Such presynaptic inhibition by 5-HT in the enteric nervous system is evidently less prone to desensitization (Wallis, 1981) and could well persist in the presence of high doses of 5-HT. In fact, potent depolarizing influences of 5-HT on C-fibers have been observed (Pike, personal communication), and these do not desensitize but markedly altered depolarizing responses to GABA in an 'all or none manner', effects that could explain the depression of GABA-induced ACh release seen here with 5-HT.

Alternatively, 5-HT could also release some other substance that depresses the myenteric plexus and so prevent the ileal reponse to GABA. Some such effect is apparent in Fig. 3 of Tonini et al. (1983) where there was a persistent loss of tone in the ileum so long as the 5-HT was present, and the same phenomenon was occasionally observed in the present study. At any event, GABA is known to release ACh from myenteric synaptosomal preparations, where the responsible GABA receptor must be on the immediate presynaptic membrane. Thus GABA can directly release ACh from plexus neurones without the mediation of any other transmitter substance (Yau and Verdun, 1983), but whether or not 5-HT interacts with this release is not known. In the guinea-pig colon, however, 5-HT desensitization did not modify the GABA-induced non-adrenergic inhibitory responses, suggesting that the

action of GABA in this preparation is not mediated by 5-HT (Onori et al., 1984).

Quipazine appears to be a weak partial agonist with potent antagonist properties at 5-HT receptor sites on some peripheral tissues, particularly neuronal receptors (Lansdown et al., 1980), and it also behaves as an antagonist at 5-HT autoreceptors in the CNS (Martin and Sanders-Bush, 1982). Although quipazine is said to be a potent agonist on smooth muscle, including the intestine (Hong and Pardo, 1966), as well as in the CNS (Green et al., 1976; Jacoby et al., 1976), in the present work it has proven to be a reversible antagonist of neurally mediated contractile responses to 5-HT, and partial agonist. Here, quipazine (0.1  $\mu\text{M}$ ) antagonised responses to 5-HT without affecting responses to GABA or to transmural electrical stimulation of the cholinergic neurones in the ileum. Better and more selective antagonists of neural 5-HT receptors in the gut may yet be found, but so far quipazine has proven to be effective, and capable of discriminating between responses to 5-HT and to GABA. For the present purpose, the latter property is most important, since the selective abolition of 5-HT responses, but not equipotent responses to GABA, is not compatible with the notion that 5-HT mediates the contractile responses to GABA. These results, and the evident difficulties in obtaining consistent depression of contractile responses to GABA following desensitization to 5-HT, thus raise severe doubts that 5-HT mediates GABA-induced contractile responses in the guinea-pig ileum, but the lack of availability of a potent, specific antagonist for neurally-mediated 5-HT responses

in the intestine has so far prevented a definitive resolution of the problem.

CHAPTER SEVEN. MODIFICATION OF INTESTINAL MOTILITY

BY GABA ANTAGONISM.

## 7.1. INTRODUCTION

Bayliss and Starling (1899) were the first to show that local stimulation of the isolated intestine of the dog initiates a band of constriction on the proximal side and relaxation on the distal side of the stimulus point, creating a peristaltic wave consisting of 2 phases, ascending excitation and descending inhibition, which travels down the bowel with uniform velocity in an aboral direction, sweeping the intestinal contents before it. They thus demonstrated that extrinsic innervation of the gut is not essential for the coordinated reflex responses responsible for initiating intestinal movements, and concluded that intrinsic nerve plexuses form an integrative network in the enteric nervous system independent of input from the CNS. This supports Langley's (1921) classic concept that the sympathetic, parasympathetic and enteric nervous systems comprise three major divisions of the autonomic nervous system, with the enteric nervous system of the gastrointestinal tract functioning independently, in effect behaving as a "mini-brain".

Bayliss and Starling (1900) also recorded intrinsic rhythmical contractions, devoid of CNS control, occurring 10 to 12 times a minute in the intestinal wall of the dog, involving both circular and longitudinal muscular coats; these contractions were increased in amplitude, within limits, by increased tension, and in rate by increasing the temperature of the incubating vessel. Such studies were further pursued by Trendelenburg (1917), who devised a method using isolated intestinal preparations to ex-

mine reflex responses to intraluminal distension, and showed that these responses consist of circular and longitudinal muscle contractions starting at the oral end of the preparation, and travelling down to the aboral end. Contraction of the longitudinal muscle and inhibition of the circular muscle occur ahead of the advancing bolus, whilst circular muscle contraction and longitudinal muscle relaxation occur within the gut segment immediately behind the bolus. Thus the isolated segment of the intestine proves to be a useful experimental model to study the role of intrinsic nerves in determining motility patterns (Kosterlitz and Lees, 1964; Kosterlitz, 1968; Frigo et al., 1972; Costa and Furness, 1976).

Enteric ganglion cells are spontaneously active in isolated preparations without influence from the CNS, reflected by ongoing release of neurotransmitters (Paton et al., 1971), by changes in smooth muscle contraction when the neural activity is blocked by drugs (Wood, 1972), and by ongoing spike discharges, consisting of excitatory and inhibitory synaptic potentials detected in electrophysiological recording (Wood, 1975; 1981 a; Wood and Mayer, 1978). However, TTX, which blocks neural activity, does not completely abolish spontaneous contractions in isolated intestinal strips from some but not all species studied, and may change the amplitude or the frequency of the spontaneous contractions (Kao, 1966; Kuriyama, H et al., 1966), suggesting a myogenic origin for such activity subject to neural modulation.

Indeed, one of the fundamental properties of the intestinal musculature is the presence of myogenic mechanisms of excitation initiated by intrinsic pacemaker mechanism (electrical slow waves (Prosser and Bartoff, 1968). But these are normally under neural influences where nervous control of the longitudinal muscle layer of the intestine apparently differs from that of the circular muscle, since the former shows rhythmic contractions at the frequency of the electrical slow waves when the enteric neurones are active, whereas the circular muscle layer is quiescent (Wood, 1970). Yokoyama and Ozaki (1980) investigated the effects of gut distension on the myenteric plexus in the intestine and concluded that such stimulation causes an excitatory effect on specific mechanosensitive neurones in the plexus, which regulate intestinal muscle contractility leading to peristaltic movements, whilst localized spontaneous neuronal discharges are occasionally recorded from ganglia of the myenteric plexus (Yokoyama and Ozaki, 1978). It is now generally accepted that the intestinal musculature behaves physiologically as an extensive functional syncytium with myogenic pacemaker mechanisms, although the 'Interstitial Cells of Cajal' have been implicated in their control (Thuneberg, 1982).

In earlier studies, Hobbiger (1958 a,b) found that GABA would temporarily block guinea-pig ileal peristaltic activity resulting from raised intraluminal pressure, such effects of GABA were later confirmed and extended by Tsuchiya (1960) and Inouye et al. (1960), who showed a GABA-induced depression of spontaneous motility and a blockade of peristaltic reflexes in the



isolated ileum. Takahashi et al. (1961 a,b) have also reported pharmacological actions of GABA in the isolated ileum of the guinea-pig, but whilst they clearly recognised two different modes of GABA action in the intestine, unfortunately their methodology was unsuited to the elucidation of the actual mechanisms involved in these actions. Although initial studies by Hobbiger (1958 a,b) and Inouye et al. (1960) showed that applied GABA immediately depresses peristalsis in the isolated ileum, this effect does not in itself directly imply a GABAergic involvement in the control of peristaltic activity. However, more recently, it has been demonstrated that GABAergic mechanisms may very well be directly involved in the regulation of gastrointestinal motility, based on extensive studies of the pharmacological actions of GABA in isolated preparations of the guinea-pig intestine (Krantis et al., 1980; Krantis and Kerr, 1981 b; Ong and Kerr, 1983a). GABA antagonism by BMC, or GABA desensitization (tachyphylaxis), substantially reduces the speed of faecal pellet propulsion, and lowers the amplitude of the orad reflex contraction of the circular muscle associated with the pellet propulsion along isolated segments of the guinea-pig distal colon, there being little change in the relaxation preceding the passage of the pellet (Krantis and Kerr, 1981 c). It is not clear why Krantis et al. (1980) saw no influence of bicuculline on the peristaltic reflex examined in a model involving localized mechanical stretch of the gut wall (Costa and Furness, 1976), but the radial stretch due to a faecal pellet possibly represents a more physiological stimulus for peristalsis (Frigo and Lecchini, 1970) than does a focal stretch as used by Costa and Furness (1976) and

by Krantis et al. (1980).

Recently, much evidence has accumulated showing that GABA stimulates both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites in the guinea-pig intestine (Bowery et al., 1981 a; Kaplita et al., 1982; Giotti et al., 1983 a; Ong and Kerr, 1983 a,b; 1984 a,c) manifested through both excitatory and inhibitory actions due to activating and inhibiting cholinergic neuronal activity, and GABA may also activate some non-cholinergic inhibitory effector neurones (Krantis et al., 1980). Combined antagonism of GABA<sub>A</sub>-receptors and desensitization to baclofen, a specific GABA<sub>B</sub>-receptor agonist (Bowery et al., 1981 a) slows pellet expulsion in the distal colon to the same extent as does GABA desensitization alone, indicating that both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites are involved in this modification of peristalsis by GABA desensitization (Ong and Kerr, 1983 a). Also, in the same study, baclofen and GABA caused a BMC- and PIC-insensitive depression of spontaneous cholinergic contractions, mediated by the circular muscle, in the distal colon. This modification of intestinal motility through manipulation of GABA receptors, leading to a loss in co-ordination of peristaltic activity which contributes to the subsequent observed slowing or cessation of faecal propulsion, thus strongly suggests a GABAergic involvement in the co-ordination of peristalsis, and represents the first demonstration of inherent GABA actions outside the CNS.

Studies on other peripheral tissues show that GABA not only inhibits cholinergic neurotransmission in the guinea-pig urinary

bladder through BMC-sensitive GABA<sub>A</sub>-receptor sites associated with chloride channels (Taniyama et al., 1983 c), but also causes a BMC- and PIC-insensitive dose-related inhibition of electrically evoked twitch contractions in the rat anococcygeous muscle, by acting on GABA<sub>B</sub>-receptor sites on sympathetic nerve terminals in this preparation (Muhyaddin et al., 1982 b) resulting in a presynaptic inhibitory action on the excitatory adrenergic but not the inhibitory innervation of the anococcygeus muscle (Hughes et al., 1982). In the rabbit urinary bladder, however, it has been shown that prejunctional GABA<sub>B</sub> receptors do reduce ACh release, playing a BMC-insensitive modulatory role on parasympathetic excitatory postganglionic neurotransmission (Santicioli et al., 1983), contrary to that reported by Taniyama et al. (1983 c) in the guinea-pig urinary bladder. By contrast, GABA will induce a bicuculline-sensitive increase in ACh contractions in the rat oviduct, and it has been suggested that this enhancement of ACh-induced contractile responses may be due to some postsynaptic modulatory effect mediated through GABA<sub>A</sub>-receptor sites (Fernandez et al., 1984). However, these findings give little insight into any physiological mechanism whereby GABA may influence motility in the peripheral tissues, whereas there is already evidence for this in the intestine.

The present study examines the effects of GABA antagonism using BMC, PIC, and GABA desensitization, as well as drugs that modify the synthesis and release of GABA, such as 3-MPA (Lamar, 1970; Fan et al., 1981), on spontaneous rhythmic relaxations developed in the isolated ileum of the guinea-pig and rat, in

order to explore if GABA plays a physiological role in the generation of such spontaneous relaxations. A novel macrocyclic lactone antiparasitic agent, Avermectin Bla (AVM) causes a sustained increase in the rate of release of GABA from rat brain synaptosomes (Pong et al., 1980), and interacts with the picrotoxin/barbiturate and benzodiazepine modulatory sites of the GABA<sub>A</sub>-receptor chloride-ionophore complex (Olsen and Snowman, 1985). AVM has therefore also been tested against basal activity of intestinal preparations, to see if it induces any changes in spontaneous activity in these tissues.

## 7.2. MATERIALS AND METHODS

### ORGAN BATH STUDIES

Guinea-pigs of either sex, weighing between 200-400 g, were stunned by a blow on the head and bled. Segments of the ileum, 2-3 cm in length and taken 5-10 cm from the ileo-caecal valve, and also the taenia coli, were quickly removed and mounted vertically in a 10 ml organ bath containing modified Krebs-bicarbonate solution (see Chapter 2.1 for composition) bubbled continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and the tissues maintained at 37<sup>0</sup> C, pH 7.4. Mechanical activity of the longitudinal muscle was recorded isometrically at a resting tension of 1 g using a Grass model FT03 force transducer coupled to a Grass polygraph recorder. The tissue was allowed to equilibrate for 60 min in the organ bath before drug treatments were examined on the resting tissue. Drugs were applied to the bath within 15-20 min intervals and were allowed to remain in contact with the tissue for at least 5-10 min. Desensitization to GABA occurred when exogenously applied GABA remained in the bath for at least more than 5 min before a repeat test dose of GABA was again applied without washing out the initial dose. All experiments were repeated in duplicate over 20 tissues from a minimum of 10 animals.

Adult rats (Sprague-Dawley) of either sex, were killed by stunning and dislocation of the neck. Segments of proximal ileum, 3-4 cm in length, were quickly removed and mounted vertically in a 10 ml organ bath containing Krebs-bicarbonate medium

as described above, the mechanical activity of the longitudinal muscle was recorded isometrically.

#### FAECAL PELLET EXPULSION STUDIES

In a further series of experiments, motility, as measured by the rate of pellet expulsion, was investigated using freshly excised segments of guinea-pig distal colon maintained in Krebs solution at 37<sup>0</sup> C (c.f. Ong and Kerr, 1983a). The subsequent number of pellets expelled over 2 min intervals was measured in the absence and presence of pharmacological agents in the incubating vessel, and the rate of pellet expulsion has been expressed as the percentage of the sum of pellets expelled, as against the control number of pellets within 20 min. For statistical analysis, Student's t-test for paired and unpaired samples was used to assess the significance ( $P < 0.05$ ) of differences between mean values of the effects.

#### CHEMICALS:

The following chemicals were obtained from Sigma except for those indicated in parenthesis.

tetrodotoxin (Calbiochem)

atropine sulphate

bicuculline methochloride or bicuculline methiodide (Pierce)

picrotoxinin (dissolved in 1:9 absolute alcohol and distilled water)

GABA

5-aminovaleric acid

Avermectin Bla (dissolved in 0.1% DMSO; Merck, Sharp and Dohme)

3-mercaptopropionic acid

### 7.3. RESULTS

#### 7.3.1. Spontaneous relaxations in the guinea-pig isolated ileum.

Some ileal segments of the guinea-pig showed spontaneous relaxations that occurred with a rather regular periodicity throughout each experiment. Such neurally mediated relaxations, of consistent pattern and frequency, were unique to certain ileal preparations with particularly high resting tone, and were partially abolished, or sometimes totally abolished by TTX (0.1  $\mu$ M) which lowered the tone (Fig. 1). These relaxations, which lasted for up to 30 s could also be completely abolished by atropine (0.1  $\mu$ M) (Fig. 1), the relaxations resembling a mirror image of the more commonly observed spontaneous cholinergic contractions described in the next section. BMC (10  $\mu$ M), PIC (10  $\mu$ M), applied within 5 min, or desensitization to GABA (50  $\mu$ M total), as performed by applying a second, test dose of GABA into the bath without washing out the initial dose at 25  $\mu$ M, all consistently reduced or abolished these rhythmic spontaneous relaxations, or else in some preparations, altered their set pattern and frequency. As shown in Fig. 2, such rhythmic spontaneous relaxations were mostly reduced, or sometimes abolished for the duration of the various drug treatments, with rapid recovery of the relaxations upon washing out the drug. These results had been observed over at least 20 tissues from a minimum of 10 animals.

In some ileal preparations from the rat, bicuculline methiodide (BMI) or bicuculline methochloride (BMC) (10  $\mu$ M) completely abolished the relaxations (Fig. 3), and replaced them with a



contraction; the relaxations were similarly depressed by PIC (10  $\mu\text{M}$ ) or GABA desensitization (50  $\mu\text{M}$  total) (Fig. 3,  $n=6$ ). These results were consistently observed and could be repeated over some hours in all preparations exhibiting such spontaneous relaxations. Although atropine and TTX abolished these spontaneous relaxations by lowering the resting tone of the tissues, the latter effect did not occur in the presence of BMI or PIC. Indeed, in most of the ileal segments with a high resting tone, BMI and PIC often further raised the tone, or the high tone persisted, especially as the spontaneous relaxations ceased. Likewise, in the taenia coli of the guinea-pig, spontaneous relaxations were also blocked by BMC (10  $\mu\text{M}$ ) which then subsequently raised the tissue tone (Fig. 4).

### 7.3.2. Cholinergic spontaneous contractions of the isolated ileum.

In contrast to the above, the ileal segments sometimes exhibited rhythmic spontaneous contractions rather than relaxations. The amplitude of these was reduced by both TTX (0.1  $\mu\text{M}$ ) and atropine (0.1  $\mu\text{M}$ ) (Fig. 5), and they were considerably modified by BMC or BMI (10  $\mu\text{M}$ ), PIC (10  $\mu\text{M}$ ), or GABA desensitization (50  $\mu\text{M}$  total) (Fig. 6,7). Such spontaneous contractions were also blocked by 3-mercaptopropionic acid (3-MPA) (0.1 mM), that prevents GABA release (Fig. 7). These results were observed over at least 8 preparations from 4 animals.

In some essentially quiescent ileal preparations ( $n=10$ ),

application of the macrolide antibiotic Avermectin Bla (AVM, 1  $\mu\text{M}$ ), within 2-5 min, induced regular rhythmic activity consisting of contractions, with a small relaxation component relative to the level of intrinsic activity. The vehicle for AVM, DMSO, did not affect ileal basal activity. Often this AVM-induced rhythmic contractile activity was of a relatively higher amplitude than seen in other spontaneous rhythms, but was clearly of neural origin since it could be blocked by TTX (0.1  $\mu\text{M}$ ) or atropine (0.1  $\mu\text{M}$ ) (Fig. not shown). The AVM-induced activity was reduced or abolished by BMI or BMC (10  $\mu\text{M}$ ) (Fig. 8) and by PIC (10  $\mu\text{M}$ ) (Fig. not shown), as well as by treatment with 3-MPA (0.1 mM). The rhythm generally resumed upon washouts of these agents, but occasionally required re-application of AVM (1  $\mu\text{M}$ ), and often persisted for a time ( $> 20$  min) in the absence of GABA antagonism.

### **7.3.3. Non-cholinergic spontaneous contractions of the isolated ileum.**

Ileal preparations which were subjected to the presence of atropine (0.1  $\mu\text{M}$ ) throughout the experiment, often showed non-cholinergic spontaneous contractions. These atropine-insensitive contractions were reduced by BMC (10  $\mu\text{M}$ ), GABA desensitization (50  $\mu\text{M}$  total), and 3-MPA (0.1 mM).

### **7.3.4. Faecal pellet expulsion in the isolated distal colon.**

A measure of motility more directly related to peristalsis

is the rate of pellet expulsion from freshly excised segments of the guinea-pig distal colon, which contained on average 12 pellets. The isolated segments were maintained in Krebs solution, and showed (Fig. 9) a reduction in motility, as judged by the number of pellets expelled over a 20 min period during treatment with PIC, DAVA and/or desensitization to GABA (300  $\mu$ M) or baclofen (140  $\mu$ M). Desensitization was induced by leaving the indicated doses of GABA and baclofen in contact with the preparations throughout the 20 min period. DAVA (0.5 mM) significantly ( $P < 0.05$ ) slowed pellet expulsion to approximately the same extent as desensitization to baclofen (140  $\mu$ M). The reduction in motility during treatment with PIC (10  $\mu$ M), GABA desensitization (300  $\mu$ M total), or a combination of baclofen (140  $\mu$ M) and PIC (10  $\mu$ M) has been described in detail elsewhere (c.f. Ong and Kerr, 1983a, Fig. 6). The control response in Fig. 9 in the present study indicates the total number of pellets expelled, generally completely emptying the control colon within 20 min, and is represented as 100%. The number of experiments performed in each case was at least  $n=6$ .

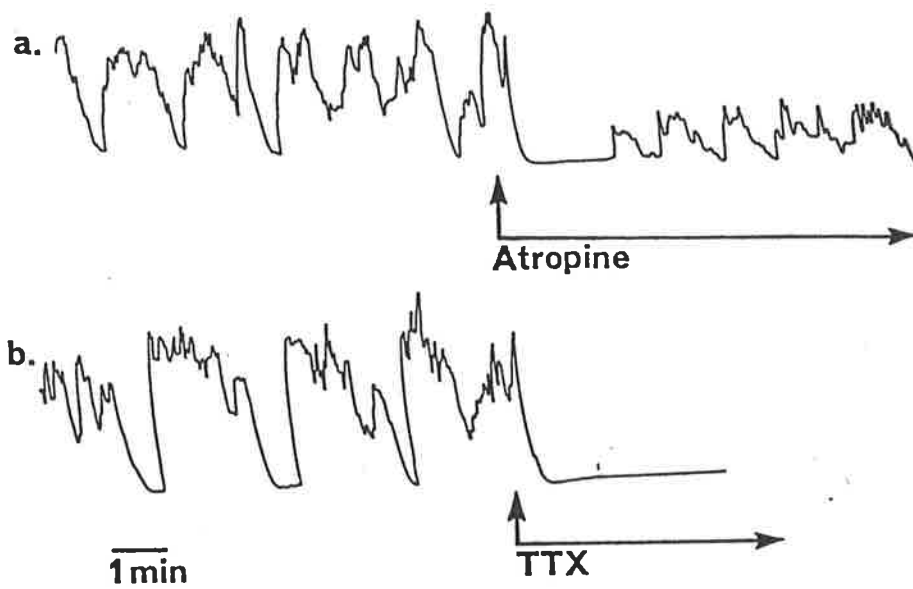


Fig. 1. Abolition of the spontaneous relaxations in the isolated ileum of the guinea-pig by atropine (0.1  $\mu$ M) and tetrodotoxin (TTX) (0.1  $\mu$ M), with a partial recovery of the rhythm in the presence of atropine.

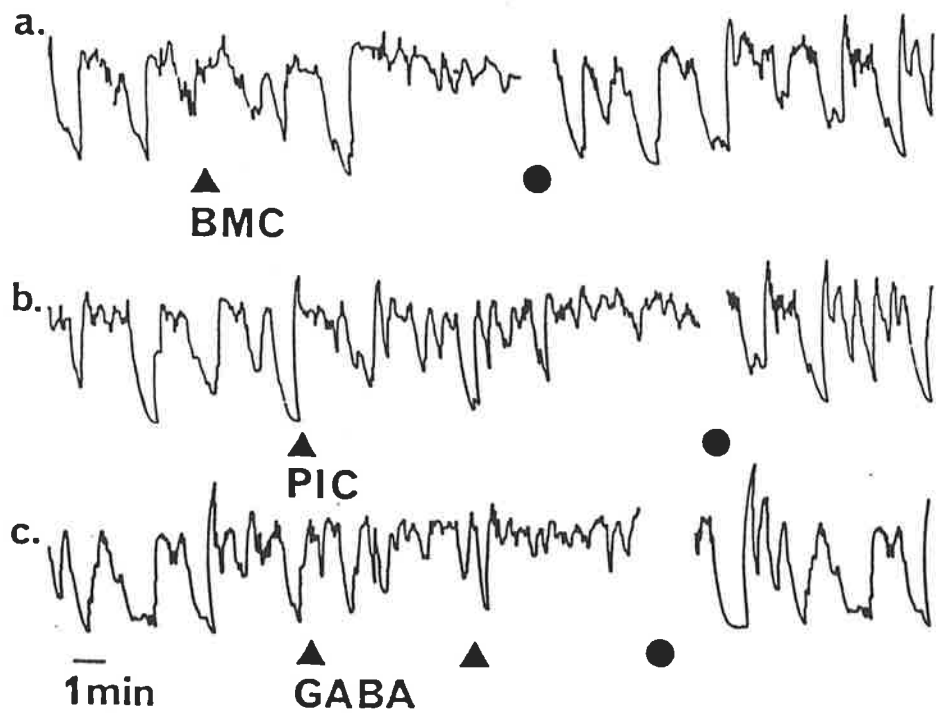


Fig. 2. (a) Abolition of the spontaneous relaxations in the guinea-pig ileum by BMC (10  $\mu$ M) and by (b) PIC (10  $\mu$ M) when the drugs were left in contact with the tissue for at least 5-10 min. (c) Significant reduction of the spontaneous relaxations in the presence of a desensitizing dose of GABA (50  $\mu$ M total). After tissue washout indicated by ●, the spontaneous relaxations returned. ▲ indicates the time when the drugs were injected into the organ bath.

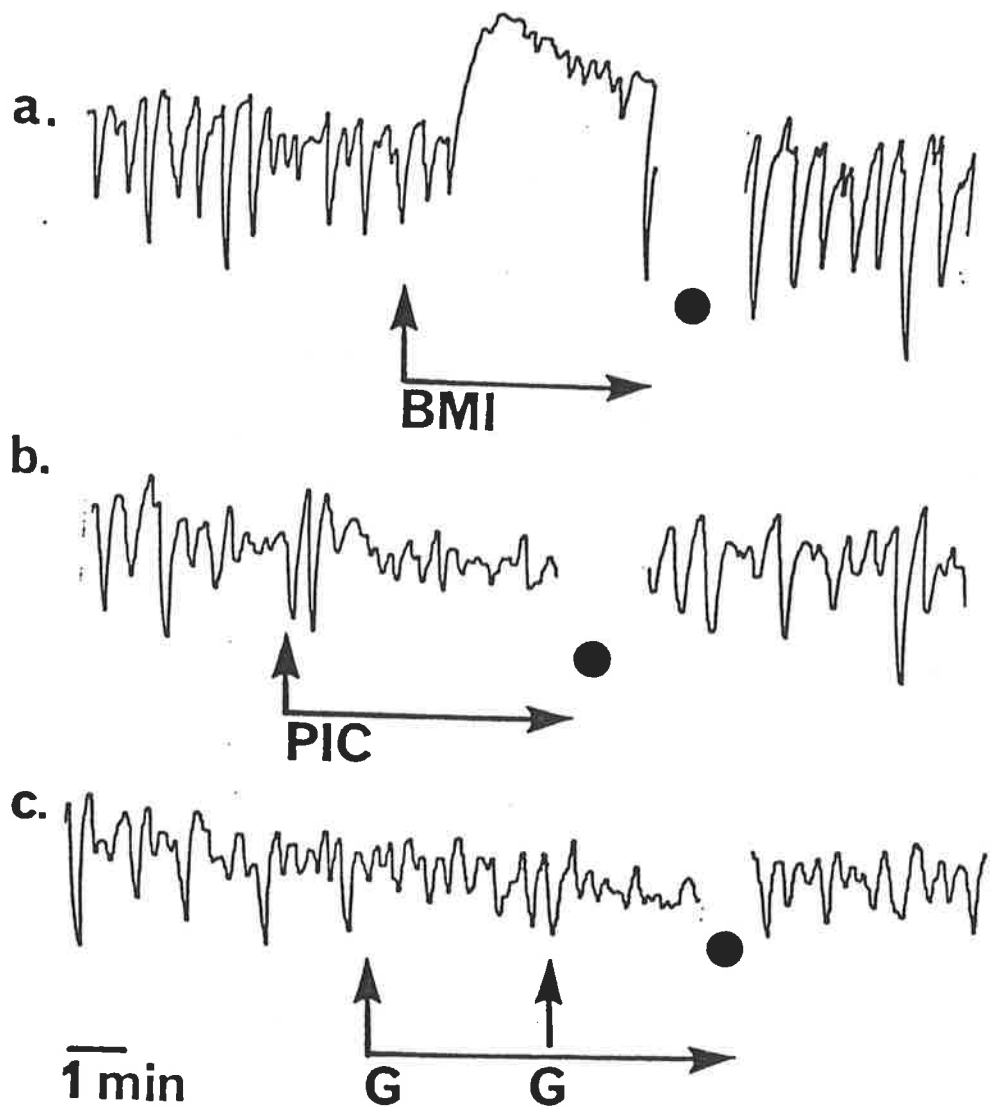


Fig. 3. In the rat isolated ileum, (a) BMI (10  $\mu$ M) completely abolished the spontaneous relaxations and contracted the tissue. (b) PIC (10  $\mu$ M) and (c) a desensitizing dose of GABA (50  $\mu$ M total) reduced the spontaneous relaxations.

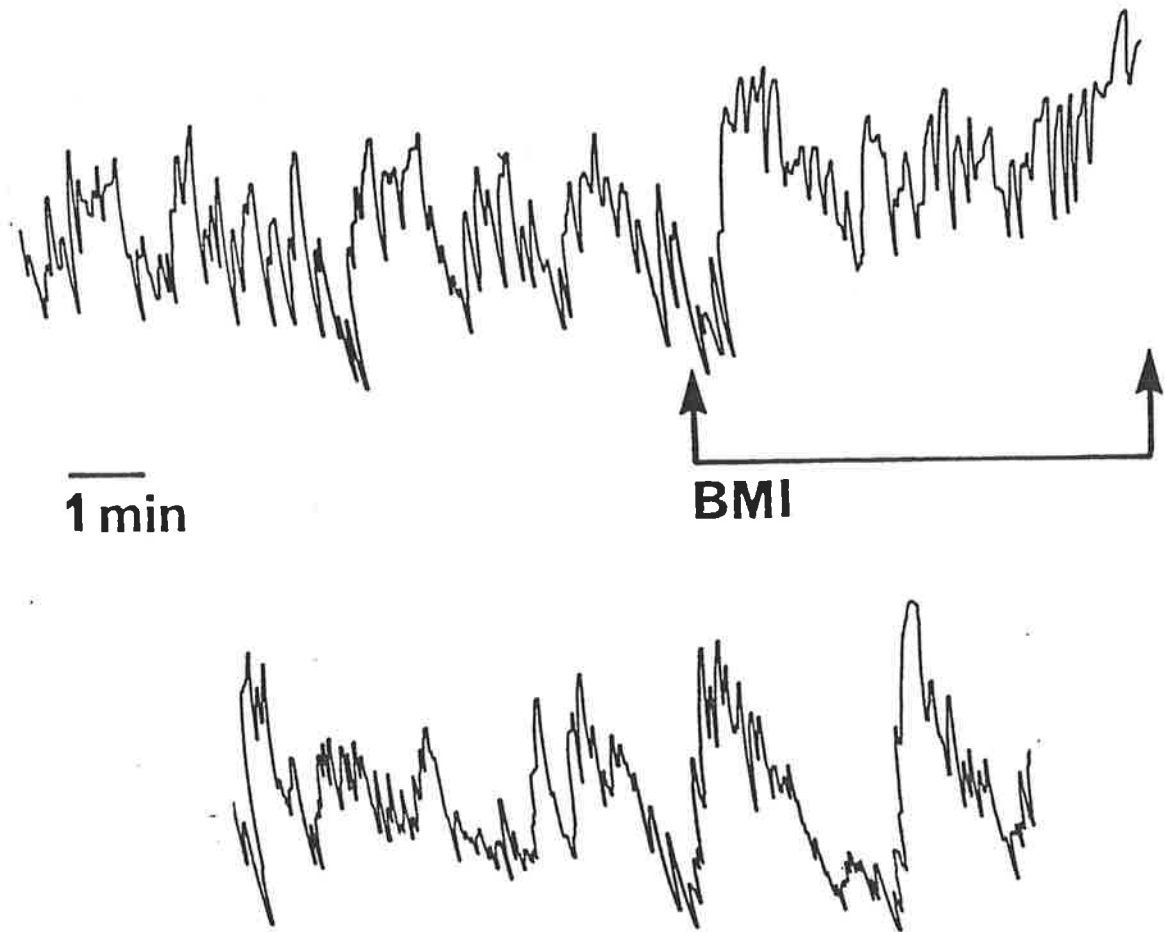


Fig. 4. In the guinea-pig taenia coli, BMI (10  $\mu$ M) raised the tissue tone and reduced the spontaneous relaxations. Bottom trace shows a recovery of the activity after washing out BMI.

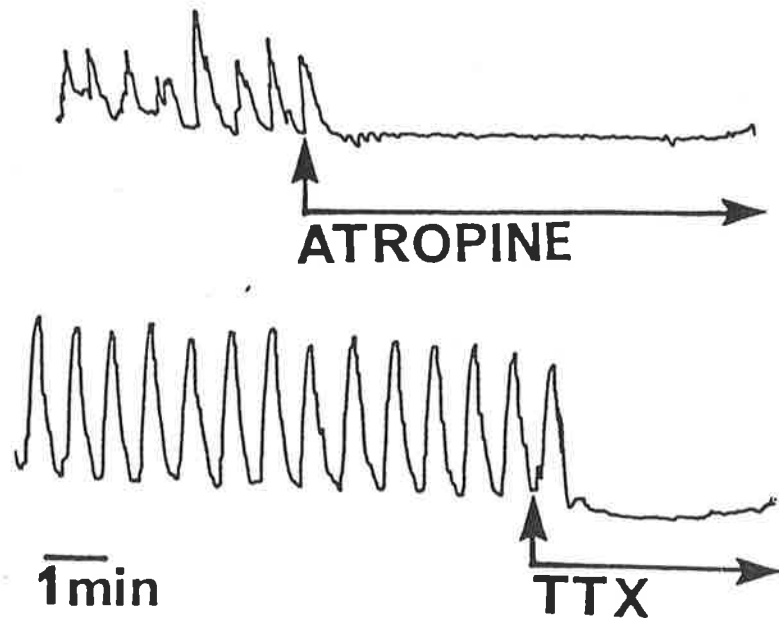


Fig. 5. Sensitivity of the spontaneous contractions to atropine (0.1  $\mu$ M) and TTX (0.1  $\mu$ M) in the guinea-pig ileum.



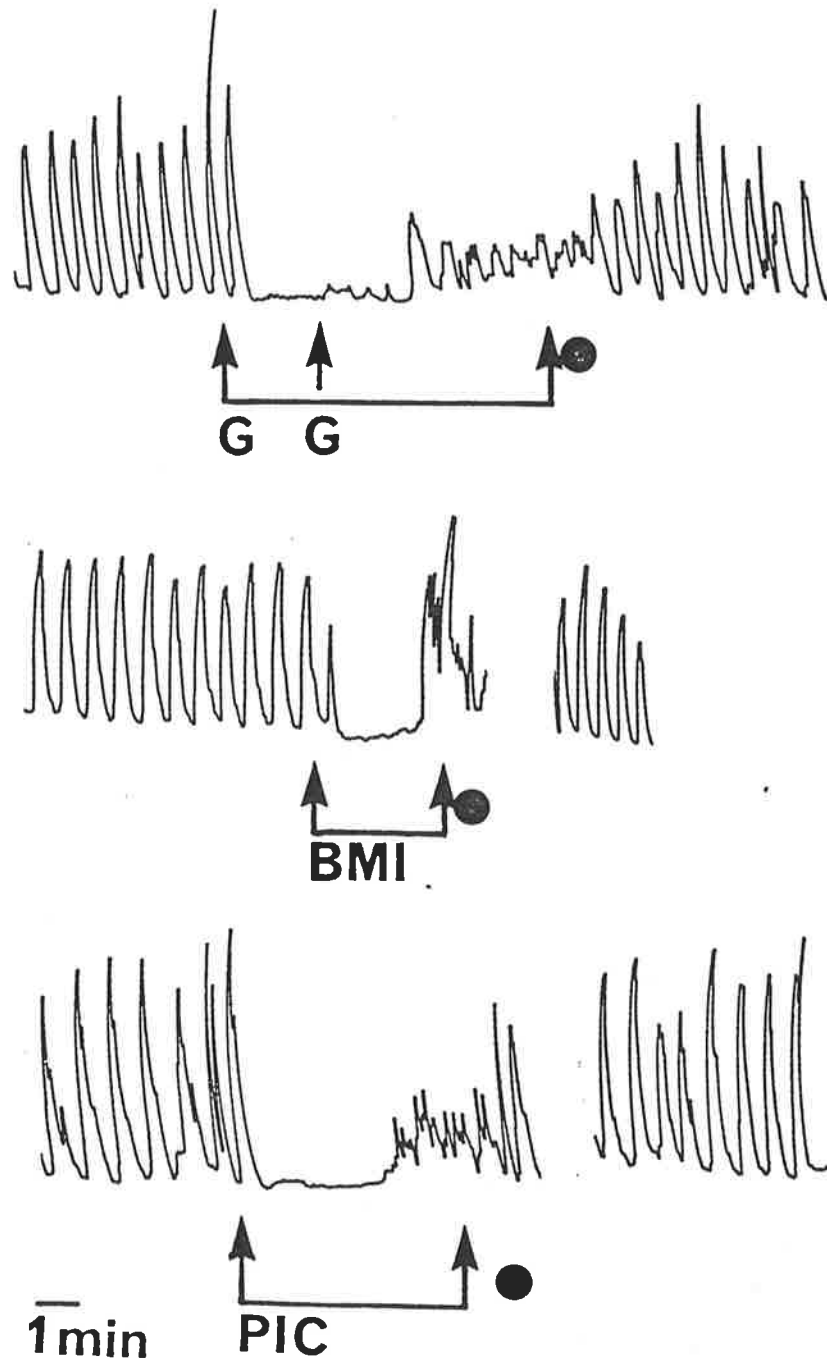


Fig. 6. Abolition of the spontaneous contractions in the presence of GABA desensitization (50  $\mu$ M total), BMI (10  $\mu$ M) and PIC (10  $\mu$ M), with a recovery of tissue activity upon washout.

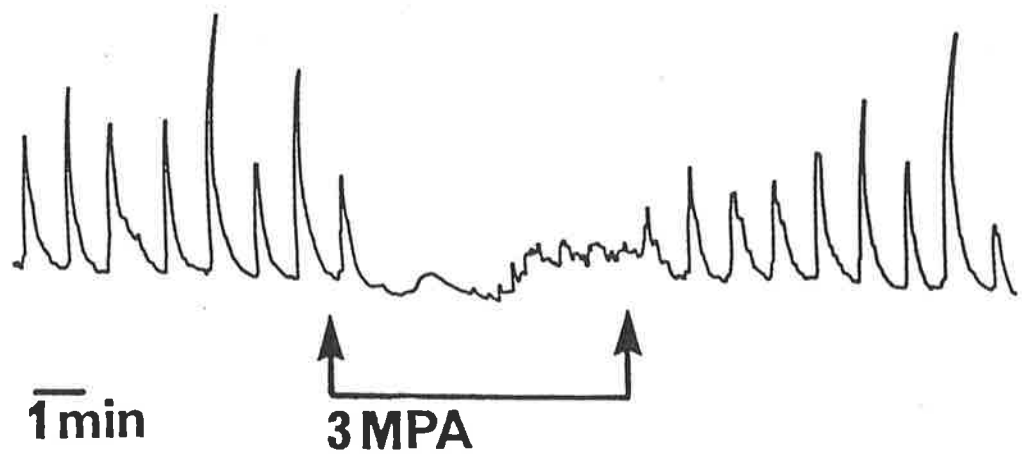


Fig. 7. Abolition of the spontaneous contractions in the guinea-pig ileum by 3-MPA (0.1 mM).

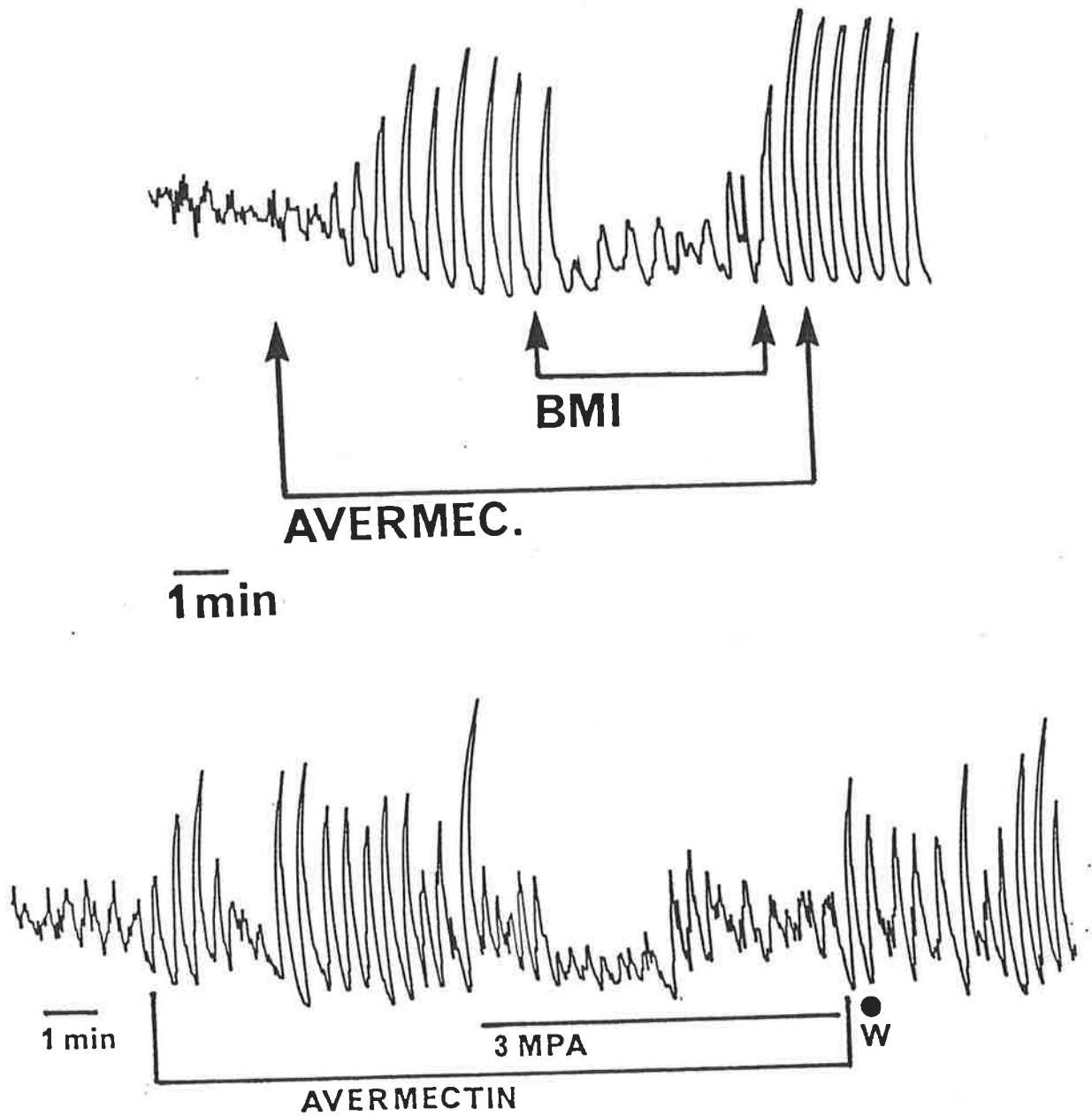


Fig. 8. Induction of rhythmic spontaneous contractions in the guinea-pig ileum by Avermectin (10  $\mu$ M), which were then reduced by BMI (10  $\mu$ M) and 3-MPA (0.1 mM).

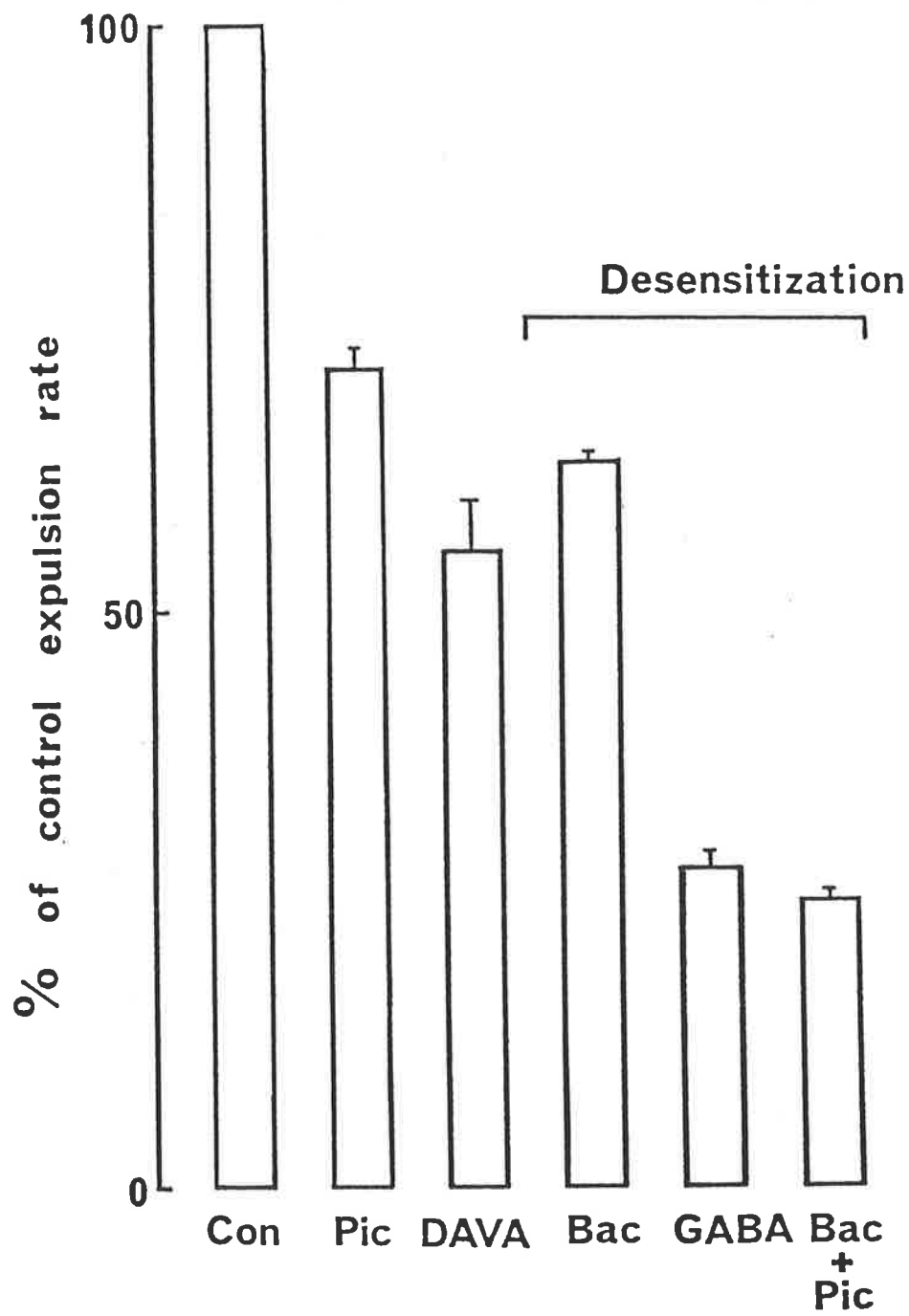


Fig. 9. Reduction in motility of freshly excised segments of guinea-pig distal colon, as judged by the number of faecal pellets expelled over a 20 min period during treatment with PIC, DAVA, and/or desensitization to baclofen (Bac) and GABA, and a combination of both bac and PIC. Each column represents the mean and standard error of the mean of 6 or more experiments and is expressed as the number of pellets expelled taken as a percentage of the control rate (generally 12 pellets in 20 min, which emptied the colon). Motility was significantly reduced by all treatments ( $P < 0.05$ ). Control (con),  $n=7$ ; PIC (10  $\mu\text{M}$ ),  $n=6$ ; DAVA (0.5 mM),  $n=6$ ; baclofen desensitization (Bac) (140  $\mu\text{M}$ ),  $n=6$ ; GABA desensitization (GABA) (300  $\mu\text{M}$ ),  $n=6$ ; Bac (140  $\mu\text{M}$ ) and PIC (10  $\mu\text{M}$ ),  $n=8$ . The number of experiments performed is indicated by  $n$ .

#### 7.4. DISCUSSION

Coordinated movements of the intestine are controlled by a local nervous mechanism present within the intestinal wall, even when isolated from the CNS (Mall, 1896; Bayliss and Starling, 1899; Langley and Magnus, 1905; 1906; Cannon, 1911, 1912; Thomas and Kuntz, 1926; Alvarez, 1940; Gustavsson, 1978). In addition to which, some inherent myogenic activity is also implicated in the generation of intestinal movements. With the improvement of Magnus's (1904) pioneering experiments of isolating plexus-free intestinal preparations of the circular muscle from the intestinal wall, Gunn and Underhill (1914) found it possible to make plexus-free isolated preparations that developed spontaneous rhythmic activity, and this was later confirmed by Alvarez and Mahoney (1922). In addition, Gasser (1926) studied the inherent rhythm of isolated circular muscle strips of the intestine, finding that such strips develop spontaneous rhythms, entirely myogenic in origin, representing an early demonstration of movements generated independently of Auerbach's plexus or other nervous elements. Nevertheless, it is clear that the intrinsic innervation of the intestine to a large extent normally controls spontaneous and reflexly initiated activity of the intestinal smooth muscle, particularly in the guinea-pig.

Myogenic activity in most regions of the gastro-intestinal tract consists of periodic membrane oscillations known commonly as slow waves (basal electrical rhythm) or pacemaker activity, and superimposed on them are spikes or action potentials, accom-

panied by contractile activity. Such slow waves are electrically coupled to give synchronous oscillations in both circular and longitudinal muscle layers, and represent cyclical changes in the excitability of smooth muscle cells. These slow waves, recorded in vivo from many parts of the small and large intestine in a number of species (Daniel, 1968; 1975; Prosser and Bortoff, 1968; Prosser, 1974; Bortoff, 1976) determine the patterns of intestinal movement, where synchronous contraction of the longitudinal and circular muscle layers is coordinated by slow waves propagated between the layers, and depends on the two layers becoming sufficiently excited to initiate a contraction. Activity of the slow waves is in turn determined by neural influences within the enteric nervous system, with cholinergic nerves being thought to be most important in initiating contractile activities (see review by Costa and Furness, 1982).

Propulsive movement of intraluminal contents along the intestinal tract depends on activation of ascending and descending enteric reflexes, manifested through the waves of contractions that sweep analwards, due to migration of neuronal excitability within the enteric plexuses (see review by Costa and Furness, 1982), but the contribution of the slow waves and migrating motor complex (MMC) to the propulsive activity of the intestine has yet to be explored further. Costa and Furness (1976) reported that the ascending excitatory neuronal pathways in the isolated intestine are partially cholinergic and partly involve a 5-HT-like transmitter substance acting on the smooth muscle, whilst the descending inhibitory reflex acts on the muscle

through non-cholinergic, non-adrenergic enteric inhibitory neurones in the guinea-pig intestine, for which the transmitter is still unknown. Faecal pellet propulsion in the distal colon is thus mediated by both cholinergic and 5-HT-containing neuronal pathways. These authors postulate that ascending excitatory reflexes and descending inhibitory reflexes, as well as descending waves of contraction that are cholinergic and neuronal in origin, all contribute to propulsion in such preparations.

Frigo and Lecchini (1970) showed that localized distention of the isolated distal colon of the guinea-pig and cat causes propulsive movements of the colon, and concluded that propulsion of a solid bolus in the intestine is the result of differential and coordinated contractions and relaxations of the circular muscle, but that the stoppage of such propulsion can be the result of an uncoordinated contraction of the circular muscle rather than an inhibition of the contractile mechanism. In a further study, Crema et al. (1970) found, in the guinea-pig distal colon, that both cholinergic and non-cholinergic excitatory neurones in the longitudinal and circular muscle layers are essential for propulsion, whilst the descending inhibitory reflexes and excitatory responses may involve nicotinic receptors. It has also been postulated that other pathways subserving the oral reflex contraction of the circular muscle may be independent of muscarinic receptor activation. These are known as 'hyoscine-resistant pathways', and may, however, involve 5-HT or a 5-HT-like substance (Tonini et al., 1981).



All in all, then, the control of intestinal motility by this intricate network of neurones consisting of sensory receptors, primary afferent neurones, integrative, and motor neurones, involves the ultimate participation of cholinergic excitatory, and non-cholinergic non-adrenergic inhibitory efferent neurones at the level of the smooth muscle, although the actual transmitter(s) involved in the latter remain undefined. It may also well be affected by locally released substances or circulating hormones such as prostaglandins, an array of peptides and other endogenous substances, all of which can influence gut motility (Furness and Costa, 1982).

Many electrophysiological studies using single cell recording have focussed on the electrical properties of the enteric neurones themselves, since a knowledge of single neurone properties may help elucidate the ways in which the enteric nervous system functions, and how such functions are related to gut motility (Yokoyama, 1966; Wood, 1970; 1973; North and Williams, 1977; Ohkawa and Prosser, 1972 a,b; Nishi and North, 1973; Hirst et al., 1974; Hirst and McKirdy, 1974; Hirst et al., 1975). Following the first report of electrical activity recording of single neurones in the intestinal wall (Yokoyama, 1966), several studies have reported the existence of different types of neurones, present in Auerbach's and Meissner's plexuses, and named accordingly to their known electrical properties. Based on their differential electrical properties and synaptic activities (Hirst, 1979; Wood, 1975; 1979), these are designated as "S" or "AH" cells defined in the introduction (Chapter 1, Enteric

Nervous System), but the functional significance of this subdivision remains essentially obscure. However, the properties of excitatory conduction through Auerbach's plexus, and those of efferent pathways in the reflex arc in the plexus, have been reported (Yokoyama et al., 1977; Yokoyama and Ozaki, 1978). In the latter study, it was shown that repetitive electrical stimulation of a ganglion of Auerbach's plexus produces an excitatory effect on the adjacent longitudinal muscle, but an inhibitory effect below the stimulated region, hence supporting the concept of the law of the intestine proposed by Bayliss and Starling (1899). It was also concluded that gut distention causes an excitatory effect on specific mechanosensitive neurones in the myenteric plexus involved in producing peristaltic movements (Yokoyama and Ozaki, 1980).

Nervous control of activity in the circular and the longitudinal muscle layers appears to differ, for the longitudinal muscle layer can show rhythmic contractions at the frequency of the electrical slow waves, whilst the circular muscle layer remains quiescent (Wood, 1970). Here, ACh, exclusively released from enteric neurones in the myenteric plexus itself, may be responsible for initiating the contractile responses of the longitudinal muscle (Paton and Zar, 1968), since the cholinergic motor innervation does not penetrate the longitudinal muscle layer, and there is a tonic output of ACh from the myenteric plexus. Furthermore, there is little tonic inhibitory control on the longitudinal muscle, as against the circular layer, and, this combined with tonic ACh release, may account for the

spontaneously active longitudinal muscle (North, 1982). Pacemaker cells, or reverberating circuits (Wood, 1970), probably constitute the input to these intrinsic inhibitory neurones on the circular muscle. Activation of the circular muscle could then be due to disinhibition at the smooth muscle, following neural inhibition of the continuously active inhibitory effector neurones. However, more work is required in this area before a complete, comprehensive understanding of the interplay between excitatory and inhibitory neuronal events and the functional output is achieved.

The present study has examined the pharmacological effects of GABA-related drugs on spontaneous, rhythmic activity in the longitudinal muscle of the isolated ileum of the guinea-pig, manifested as spontaneous relaxations in some tissues, and spontaneous contractions in others. The nature and characteristics of these spontaneous activities and how they may correlate with the known electrophysiological and neurophysiological properties of the enteric neurones is at present ill defined. However, the contractions are certainly cholinergic and neural in origin, being sensitive to both atropine and TTX, but the transmitter for the relaxations is unknown. Both generally occur with a rather regular rhythm, characteristic of each preparation. It is unclear which types of cell, based on the electrophysiological classification of "S" or "AH" cells, may be involved in regulating these spontaneous rhythms, or whether they are associated in any way with the slow waves or basal electrical rhythm described previously. It is also uncertain what initiates the rhythm.

However, the present evidence clearly points to GABA playing a physiological role in controlling these spontaneous activities, since antagonism of GABA<sub>A</sub>-receptor sites using bicuculline (BMC), or of GABA<sub>A</sub>-receptor-mediated events using picrotoxinin (PIC), most often reduced or sometimes even completely abolished spontaneous activity directly related to neurally-mediated inhibition or excitation in isolated ileal segments of the guinea-pig. The periodicity of the rhythm of such spontaneous relaxations or contractions was also affected upon application of these drugs, and furthermore, in some preparations, BMC raised the tone at a concentration that would not have anticholinesterase action (Breuker and Johnston, 1975), suggesting that there may be a GABAergic component in one of the inhibitory pathways responsible for the generation of the spontaneous activity. Spontaneous relaxations in the guinea-pig isolated taenia coli and the rat ileum were also affected by antagonism of GABA-receptor sites, thus implying a GABAergic influence in both the guinea-pig and the rat intestine. It would be interesting to see if such GABA-dependent spontaneous activities occur in the intact animal, although they are independent of any extrinsic innervation, since they are manifested in the isolated preparations.

Further strong indication of a GABA involvement in the control of intestinal motility comes from the present use of AVM, earlier shown to reduce lobster muscle membrane resistance through a PIC-sensitive increase in chloride conductance (Fritz et al., 1979), by acting either directly as a GABA agonist, or indirectly by causing a tonic release of GABA from nerve terminals of the inhibitory nerves. Of these, the latter appears to be more likely, since AVM causes a significant sustained increase in the rate of release of GABA from synaptosomes (Pong et al., 1980). Also, there is a specific and saturable binding of radiolabelled AVM to brain synaptosomes (Pong and Wang, 1979), and this is related to altered benzodiazepine binding (Supavilai and Karobath, 1981; Williams and Yarbrough, 1979; Tallman et al., 1978) probably at a separate specific site for AVM at the GABA-receptor complex (Supavilai and Karobath, 1981; Olsen and Snowman, 1985). Indeed, the interactions of AVM with the components of the complex is quite unique (Olsen and Snowman, 1985).

In some initially quiescent intestinal preparations from the guinea-pig, applied AVM induced high amplitude activity within a few minutes. This induced motility was atropine-sensitive and could be antagonised by either BMC or PIC. One possible explanation for this may be that AVM is capable of releasing endogenous GABA from myenteric neurones through a mechanism similar to that demonstrated in the CNS (Pong et al., 1980), GABA release from myenteric synaptosomes having already been seen (Yau and Verdun, 1983). Alternatively, AVM may be acting through modulation of the

GABA-receptor complex on myenteric neurones, where these sites are closely coupled to the benzodiazepine-barbiturate-GABA<sub>A</sub>-receptor chloride-ionophore complex (Olsen and Snowman, 1985). Or possibly, AVM induces intestinal motility by a combination of these mechanisms; certainly GABA release is involved because 3-MPA, known to block GABA release (Fan et al., 1981), will prevent this AVM-induced activity.

On the other hand, the spontaneous relaxations which developed in some intestinal preparations were also sensitive to GABA antagonists such as BMC, PIC, or GABA desensitization, and thus also involve GABAergic mechanisms. Since GABA has no action on ileal smooth muscle (Krantis et al., 1980; Krantis and Kerr, 1981 b), these GABA receptors must be located on neurones of the myenteric plexus where GABA may either stimulate the non-cholinergic non-adrenergic inhibitory neurones that innervate the smooth muscle of the intestine resulting in relaxations (Krantis et al., 1980), or may cause inhibition by an action at postsynaptic GABA-receptor sites on excitatory neurones of the myenteric plexus, although there is no evidence so far for the latter action. However, this does not exclude the further possibility that GABA may instead act in the plexus by inducing presynaptic inhibition through GABA<sub>A</sub>-receptor sites on excitatory neurones of the plexus, for GABA depolarises neurones of the ileum by a chloride-dependent mechanism (Krantis and Kerr, 1981 b) very reminiscent of that for presynaptic inhibition in the CNS (Levy, 1977).

Electrophysiological studies have so far failed to demonstrate BMC antagonism of any evoked synaptic potentials in myenteric neurones, indicating that myenteric GABA-containing neurones may innervate nerve terminals rather than cell bodies in the plexus, or else GABA acts at receptors on some part of the neuropil not so far accessible to electrophysiological recording. Iontophoretic application of GABA causes membrane depolarization of "AH" myenteric neurones in the guinea-pig small intestine but not the "S" neurones, where two effects are involved, namely (a) a bicuculline-sensitive, rapidly desensitizing chloride conductance activation and (b) a bicuculline-insensitive, non-desensitizing depolarization. But as yet there has been no report of bicuculline-sensitive spontaneous or induced synaptic potentials due to an increase in  $\text{Cl}^-$  conductance in either type of the myenteric neurones (Cherubini and North, 1984a,b). On the other hand, to the contrary, GABA has been shown to depolarize the membrane potential and decrease the input resistance of both "S" and "AH" myenteric neurones, blocked by bicuculline and picrotoxin (Mayer et al., 1982), suggesting that specific receptors for GABA are present on both classes of myenteric neurones. The "AH" cells are thought to include both afferent neurones in the peristaltic reflex arc, as well as ACh-releasing motoneurones (North, 1982; Szerb, 1982).

Further recent evidence on GABA in the intestine comes from its effect on synaptic transmission in the myenteric plexus, where applied GABA reduces the amplitude of the fast excitatory postsynaptic potentials (epsp's) without changing the amplitude

of the nicotinic response to iontophoretic application of ACh. Such an effect, related to presynaptic inhibition, may result from an inward calcium current, and is mimicked by baclofen, but is insensitive to bicuculline (Cherubini and North, 1984 a,b). Both cholinergic and non-cholinergic slow epsp's are also reduced in amplitude by GABA, and are insensitive to bicuculline, all of which suggests that GABA may inhibit release of an unknown transmitter, without influencing responses to applied ACh (Cherubini and North, 1984 a,b). The probable mechanism for this is comparable to the effect in embryonic sensory chick neurones, where GABA reduces the duration of calcium-dependent action potential (Dunlap and Fishbach, 1981). Thus, although there are no reports of synaptic potentials in myenteric neurones, that are mimicked by iontophoretic application of GABA or are antagonised by the GABA antagonists, this does not preclude the possibility that one could evoke GABAergic synaptic potentials in the isolated intestine.

Spontaneous inhibitory junction potentials that result from ongoing discharge of inhibitory neurones can sometimes be recorded from the smooth muscle of the guinea-pig taenia coli, where such inhibitory activity is manifested in the muscle as a steady hyperpolarization and decreased input resistance, both of which reduce the probability of pacemaker currents depolarizing the muscle to spike threshold (Wood, 1981 b). This continuous neuronal inhibition of the muscle, producing continuous inhibition of the autogenous activity of the circular muscle, can be converted to sharp phasic contractions of the muscle when the neuronal



discharge is blocked with TTX (Wood, 1981 b). The general function of intrinsic inhibitory neurones appears to be continuous suppression of activity of the inherently excitable intestinal musculature, consequently myogenic contractions occur when ongoing discharge of the inhibitory neurones is suppressed by inhibitory synaptic input from internuncial enteric neurones (Wood, 1981 b).

GABA releases ACh from crude myenteric synaptosomal preparations (Yau and Verdun, 1983), where GABA receptors must be located on the 'presynaptic' membrane of the synaptosomes, while the excitatory action of GABA on ileal myenteric neurones is potentiated by barbiturates (see Chapter 5 of this thesis), which is also characteristic of presynaptic inhibition (Nicoll, 1975 b). The periodic generation of such presynaptic inhibition at synapses on appropriate myenteric neurones would result in the observed rhythmic, BMC- and PIC-sensitive spontaneous relaxations. In the same way, GABA may be acting at GABA-receptor sites on cholinergic neurones inducing an intrinsic release of ACh responsible for generating the contractile rhythm, but a more complex explanation may be required to rationalise GABAergic initiation or inhibition of non-cholinergic spontaneous contractions, as there is as yet no conclusive evidence that GABA stimulates non-cholinergic excitatory pathways for which the transmitter is still unknown.

Since both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptors are present in the

myenteric plexus, the relative contribution of these receptor types in peristalsis was explored by using antagonists to the GABA<sub>A</sub>-receptor complex, and by desensitization to baclofen (Ong and Kerr, 1983 a). The rate of faecal pellet propulsion was significantly slowed by PIC, and by desensitization to 3-APS, yet they were not as effective as desensitization to GABA itself. The latter suggests that GABA<sub>B</sub>-receptors are indeed involved in peristaltic activity, and this conclusion has been confirmed here by desensitization to baclofen and by the use of DAVA, a GABA<sub>B</sub>-receptor antagonist, both of which slowed faecal pellet expulsion in the guinea-pig isolated distal colon. Furthermore, a combination of baclofen desensitization and PIC was equi-effective with simple GABA desensitization in reducing the rate of emptying of the colon (Ong and Kerr, 1983 a); each of these treatments would inactivate both of the GABA-receptor types, the dual inactivation being more effective than either alone.

Previous findings, where antagonism of GABA<sub>A</sub>-receptor sites slows intestinal motility (Krantis and Kerr, 1981 c; Ong and Kerr, 1983 a), as well as the change in the rhythm and a reduction in the amplitude of the spontaneous contractions of the circular muscle by baclofen, a specific GABA<sub>B</sub>-receptor agonist in the distal colon (Ong and Kerr, 1983 a), together with the present results, all suggest that GABA plays some key role in the control of peristalsis. Thus, activation of both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites may serve a modulatory role on neural mechanisms mediating gut motility, and this notion is reinforced in particular by the present observations, that some spontaneous activity of the

longitudinal muscle in the ileum is due to the action of endogenous GABA, as shown with AVM and GABA antagonism. This study provides for the first time evidence for a physiological role of GABA in regulating spontaneous and peristaltic motility.

**CHAPTER EIGHT. GENERAL DISCUSSION.**

## 8.1. GENERAL DISCUSSION

The majority of work in this thesis has employed isolated intestinal segments from the guinea-pig which provide a suitable convenient model for investigating GABAergic mechanisms. There is yet a great deal to be done to establish and extend the present knowledge of GABAergic transmission, and function, in the mammalian enteric nervous system, where the major areas for advance include: detailed quantitative and qualitative studies of GABA release, in particular, storage and inactivation of GABA from both intact and isolated intestinal preparations, between and across species, together with histochemical localization of the distribution of GABA-containing neurones and of the GABAergic innervation, as well as the localization of GABA metabolic enzymes, using GAD and GABA-T as markers. But, most importantly, the physiological implications of GABA in the control of enteric nervous functions, and the correlation of results from in vitro and in vivo preparations, warrant further investigation. Such knowledge of GABAergic mechanisms in the intestine may underlie the possible development of therapeutic agents in relation to gastro-intestinal diseases, as in the CNS, where GABA is commonly accepted as an inhibitory transmitter, and has been implied<sup>cat</sup> in several pathological disorders such as Huntington's Chorea, Parkinsonism, epilepsy, schizophrenia, spasticity and drug-induced dyskinesia (Roberts, 1979 a). Indeed, the design of drugs for clinical use in such conditions has advanced considerably over recent years, depending largely on the knowledge of GABAergic functions in the CNS, and the same may be expected in the enteric

nervous system.

EDA has proved to be a pharmacologically useful tool in inducing the release of both tritiated and endogenous GABA from myenteric neurones in the guinea-pig intestine, despite recent controversies regarding its mode of action as a GABA-mimetic rather than as a specific GABA releasing agent (Stone and Perkins, 1984). However, the actions of EDA may vary among preparations, and from species to species, depending on the extent of GABAergic innervation in the particular preparations used, and it is thus possible that in some preparations which appear not to have any GABA-containing neurones, e.g. in the superior cervical ganglion, EDA may preferentially act as a GABA-mimetic rather than by releasing endogenous GABA. But, in other preparations such as hippocampal and cortical slices, EDA acts by releasing endogenous GABA from GABAergic neurones (Lloyd et al., 1982 a; Blaxter and Cottrell, 1985). Therefore, it is necessary to exercise full caution in defining the actual mechanisms of EDA actions, but, from the present studies, EDA releases endogenous GABA from the myenteric plexus of the guinea-pig intestine, and the active compound is the EDA monocarbamate rather than EDA itself, since such action of EDA requires the presence of bicarbonate ions in the medium, supporting recent studies by Hill (1985 b). Further CNS studies should therefore concentrate on using the monocarbamate of EDA to evoke GABA release from neurones.

Knowledge of GABA metabolism in the guinea-pig enteric

nervous system is greatly advanced by the use of specific and selective inhibitors of the GABA metabolic enzymes, GAD and GABA-T. The marked potentiation of the EDA-induced responses by GABA-T inhibitors no doubt results from an increase in GABA content within the GABAergic neurones present in the myenteric plexus, whilst the abolition of such responses by GAD inhibitors is due to a depletion in the GABA level in the tissues. These findings suggest that endogenous GABA levels in the enteric neurones can hence be manipulated by metabolic inhibitors of GABA, although biochemical assays and direct measurements of the GABA content in the neurones, both before and after treatment with the metabolic inhibitors, should be performed, as this could provide useful information on the GABA pool per se in the neurones. Reloading the GABA depleted tissues with L-glutamine, a precursor of GABA synthesis demonstrated in Chapter 3, shows that GABA can be resynthesised from its usual precursor, and future studies using biochemical analyses, could measure the turnover rate of GABA present in the neuronal pool. Also, the use of glutaminase inhibitors such as methionine sulfoximine, a convulsant (Lamar and Sellinger, 1965), which inhibits the synthesis of glutamine, may be useful in this approach and further reinforces the presence of the GABAergic metabolic machinery in the enteric neurones. Recently, storage and release of endogenous and labelled GABA formed from [3H] glutamine in hippocampal slices have been studied (Szerb, 1984).

The use of the neuronal and glial cell uptake blockers for GABA in investigating the pharmacology of the GABAergic responses

in the guinea-pig isolated ileum (Chapter 4), suggests that the isolated intestinal preparation is a simple, effective model for studying transmitter uptake, analogous to that with the catecholamines (Langer and Trendelenburg, 1969). The significant potentiation of ileal responses induced by GABA and EDA, but not by 3-APS, muscimol or baclofen, using the various neuronal and glial uptake blockers, was shown by the leftwards shift of the dose-response curves for GABA over the lower dose-range of GABA in the presence of the uptake blockers. <sup>This</sup> indicates that saturation of uptake occurs at the higher dose-range for GABA, reminiscent of that for the catecholamines (Langer and Trendelenburg, 1969). Also, in the efflux studies for GABA, there was a differential release of tritiated GABA induced by EDA, following the use of neuronal and glial uptake blockers during preloading of the ileal tissues with labelled GABA, which implies that GABA is taken up to a greater extent into the neuronal compartments than into glial cells. Selective inhibition of the uptake of a transmitter by close structural analogues has to be achieved in a specific fashion because such analogues may themselves be transported into the neurones, inhibiting the accumulation of the transmitter and possibly exerting postsynaptic actions similar to that of the transmitter. The uptake inhibitors themselves should not have any affinity for the transport carriers, and should be devoid of any unspecific actions. It is now established that high affinity GABA uptake into the enteric neurones and glial cells exists, characteristic of GABAergic neurones demonstrated in the CNS (Iversen and Kelly, 1975; Johnston, 1978), and autoradiographic studies have shown [3H] GABA uptake into cell processes, axons



and dendrites, within all three meshworks of the myenteric plexus (Jessen et al., 1979; 1983 c; Krantis and Kerr, 1981 a). Thus, high affinity GABA uptake may limit GABAergic activity in the enteric nervous system, and the ileal preparation presents a convenient test system for examining the ability of various compounds to inhibit neural or glial uptake, which is of potential use in examining possibly clinically useful uptake inhibitors.

Both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor-mediated actions in the guinea-pig intestine have been described throughout this thesis, in support of previous studies (Bowery et al., 1981 a; Giotti et al., 1983 a; Kaplita et al., 1982; Ong and Kerr, 1983 a,b; 1984 c,d; Muhyaddin et al., 1982 a,b), and the use of baclofen (Hill and Bowery, 1981) to differentiate between the two classes of receptor subtypes, since baclofen is a specific GABA<sub>B</sub>-receptor agonist, is a useful approach to investigate GABAergic mechanisms of action in the enteric nervous system. Although DAVA is so far the only GABA<sub>B</sub>-receptor antagonist available, despite its low potency as an antagonist and also some GABA<sub>A</sub>-receptor agonist affinity, it can be used in studies on the intestine, but it is not suitable for use as a GABA<sub>B</sub>-receptor antagonist in the CNS on account of marked GABA<sub>A</sub>-receptor-mediated CNS actions. A more specific and potent GABA<sub>B</sub>-receptor antagonist has yet to be discovered, particularly, for elucidating GABAergic mechanisms involved in the control of intestinal motility.

Differential GABAergic actions have been found in the

different regions of the guinea-pig gastro-intestinal tract (Kerr and Ong, 1984 c; in press), suggesting that GABA plays diverse roles in the respective segments, with predominantly GABA<sub>B</sub>-receptor-mediated relaxations in the jejunum, duodenum, and the distal colon, but a GABA<sub>A</sub>-receptor-mediated excitatory response, followed subsequently by a GABA<sub>B</sub>-receptor-mediated inhibitory 'after-relaxation', in the terminal ileum. It is not clear why the ileum appears to be the only segment significantly contracted by GABA, but the possibility that prejunctional GABA<sub>A</sub>-receptor sites may occur on the cholinergic innervation of the ileal smooth muscle cannot be excluded, hence, GABA<sub>A</sub>-mediated presynaptic inhibition may occur at the level of the cholinergic innervation on the smooth muscle. In keeping with this, GABA releases ACh from ileal synaptosomal preparations (Yau and Verdun, 1983), and it is likely that this mechanism of action is functionally concerned with GABA<sub>A</sub>-receptor-mediated presynaptic inhibition in this segment of the gut, since direct GABA-induced stimulation of ACh release would be a unique action (Taniyama et al., 1983 b; Kleinrok and Kilbinger, 1983).

Part of the relaxation responses to GABA in other segments of the gastro-intestinal tract, in particular of the distal colon, is associated with non-adrenergic, non-cholinergic (NANC) inhibition. Thus, it is not surprising that GABAergic mechanisms and innervation in these various segments may well differ; in fact, Maggi et al. (1984) have found that the responses to GABA in the rat duodenum are predominantly 'NANC' relaxation responses

mediated through the GABA<sub>A</sub>-receptor sites, such responses being mimicked by 3-APS but not baclofen. It has been found, based on autoradiographic studies, that there is an abundance of GABA-containing nerve terminals projecting to the circular muscle in the ileum and colon of the guinea-pig (Kerr, personal communication), and furthermore, GABA-containing neurones have been directly demonstrated in the myenteric plexus by immuno-fluorescence with antibodies directed specifically to GABA (Ottersen, personal communication). Presynaptic GABAergic neurones innervating the circular muscle of the intestine thus seems plausible, although this has to be substantiated with histochemical localization of such neurones in future studies.

The abolition of the spontaneous, rhythmic relaxations developed in isolated intestinal preparations, from both the guinea-pig and the rat in vitro, by BMC, PIC, and GABA desensitization, as well as by 3-MPA, implies that such spontaneous relaxations are under a tonic GABAergic influence (Ong and Kerr, 1984 a), since the removal of such influence by GABA antagonism abolishes the relaxations, and sometimes, converts them to a contractile response, in particular with BMC or PIC. In view of the recent proposal that the 'Interstitial Cells of Cajal' (ICC) may be pacemaker cells, responsible for generating slow waves in the intestinal circular muscle, manifested as rhythmic oscillations (Thuneberg et al., 1982), and since the GABA-accumulating properties of many ICC's have been reported in autoradiographic studies (Krantis and Kerr, 1981 a), there is a possibility that the spontaneous relaxations observed in the present study may be,

in some way, related to the ICC. This strongly suggests that there is GABAergic involvement in these relaxations. It would be useful to extend neurophysiological studies in such investigations, to see if any electrophysiological responses induced by ICC (Daniel and Posey-Daniel, 1984) can be altered in any way by GABA antagonism. Nevertheless, the spontaneous, rhythmic relaxations of the isolated intestine warrant further investigation. Moreover, there is conclusive evidence that GABA is involved in the regulation of intestinal motility, as GABA antagonism slows faecal pellet expulsion and propulsion in the distal colon (Krantis and Kerr, 1981 c; Ong and Kerr, 1983 a), and the participation of GABA<sub>B</sub>-receptors in this has been confirmed here by the use of DAVA.

Preliminary observations on the initiation of rhythmic, spontaneous activity, manifested as contractions, in the longitudinal muscle of the ileum by Avermectin B1a, and the abolition of such activity by BMC and 3-MPA, indicates that Avermectin may influence intestinal motility through a GABAergic mechanism (Kerr and Ong, 1985) based on earlier reports of the interaction of Avermectin with the GABA-benzodiazepine-chloride-ionophore receptor complex (Supavilai and Karobath, 1981; Pong et al., 1982; Drexler and Sieghart, 1984; Olsen and Snowman, 1985), and of the releasing action of GABA from synaptosomal preparations by Avermectin (Pong et al., 1980). Lacking better understanding of the mode of action of Avermectin, the explanation of this rhythmic activity remains obscure, particularly since very little is known of the neural mechanisms underlying

the generation of spontaneous activity in the intestine, but GABAergic mechanisms are clearly involved.

Barbiturate-induced enhancement of the GABAergic responses, and the reversal of the PIC- and lactam-induced antagonism of the GABAergic responses by the barbiturates, suggests that barbiturates potentiate the GABA<sub>A</sub>-receptor-mediated contraction, but not the GABA<sub>B</sub>-receptor-mediated relaxation, in the guinea-pig intestine, by an allosteric interaction via the GABA-barbiturate-chloride-ionophore-receptor supramolecular complex (Olsen, 1981 a,b; 1982). It would be necessary to correlate such pharmacological studies with electrophysiological and ligand binding studies, to investigate further the mutual interactions of the receptor sites on the GABA-receptor complex. Furthermore, the effects of alcohol, and other related compounds may be investigated to see if their actions are mediated through the GABA-receptor complex, and it may therefore be feasible to design molecular antagonists of the pharmacological actions of barbiturates and ethanol, based on their interactions at the receptor-ionophore complex, recently reported by Mendelson et al. (1985) who showed that it is possible to reverse barbiturate and ethanol toxicity by using ligands which bind at the chloride-ionophore on the receptor complex. Such in vitro models as the isolated guinea-pig intestine provide a good system to analyse the actions of a number of compounds including the convulsant, depressant barbiturates, as well as the benzodiazepines, since it has been shown the benzodiazepines also enhance GABA<sub>A</sub>-receptor-mediated contractile responses in the guinea-pig intestine (Luzzi et al.,

1984; Kerr and Ong, unpublished observations).

Electrophysiological evidence indicates that GABA depolarizes the membrane potential, and decreases the input resistance of both S/type 1 and AH/type 2 myenteric neurones of the guinea-pig small intestine, these actions of GABA being blocked by bicuculline and PIC, and due to an increase in chloride conductance (Mayer et al., 1982). Cherubini and North (1984a), on the other hand, report that GABA causes membrane depolarization of "AH" neurones but not "S" neurones, and has two effects on the membrane of the myenteric neurones: firstly, a bicuculline-sensitive, rapidly desensitizing chloride activation, potentiated by the benzodiazepines (Cherubini and North, 1985), and secondly, a bicuculline-insensitive, non-desensitizing depolarization, mimicked by baclofen, that may result from inhibition of an inward calcium current, and this effect also leads to a reduction in transmitter release (Cherubini and North, 1984 b). GABA and baclofen reduce both the slow and fast EPSPs, the former action could be due to a presynaptic inhibition of ACh release, whilst the latter action could be due to a presynaptic inhibition of an unknown non-cholinergic transmitter-like substance (probably substance P). An advancement in this area would be to selectively stimulate GABAergic neurones, and see if any synaptic potentials are affected by GABA antagonists.

Recent advances of our understanding of the pharmacology of GABA actions in the enteric nervous system should encourage further investigations into the biochemistry, physiology and

morphology of GABAergic neurones, and may help to elucidate a physiological role of GABA in the intestine.

In summary, it is now generally agreed that the identification of a transmitter involves neurochemical evidence for its presence, synthesis, release and inactivation, together with pharmacological evidence that the putative substance and synaptically released transmitter have identical actions at the appropriate synaptic region, these actions being abolished by specific antagonists (Iversen, 1979; Orrego, 1979; Fagg and Foster, 1983). In practice, these criteria are rather rigorous and have seldom been met in their entirety, even for the best recognised transmitters. So far as GABA is concerned, these criteria have now been essentially met, albeit partly indirectly, in the myenteric plexus of the intestine: GABA and its synthesising enzymes are present in the plexus, together with degradative pathways and high affinity GABA-uptake (Jessen et al., 1979; Jessen, 1981; Miki et al., 1983); receptor sites are present and can be isolated in synaptosomal preparations that exhibit typical binding characteristics with GABA agonists and antagonists (Ong and Kerr, unpublished); calcium-dependent neural release of endogenous GABA, or [3H] GABA, that occurs upon electrical or chemical stimulation (Jessen et al., 1983 c; Kerr and Krantis, 1983; Taniyama et al., 1982 a; 1983 a), this released GABA eliciting pharmacological responses identical with those to exogenously applied GABA, as described in this thesis; antagonists exhibiting identical actions against endogenous and exogenous GABA, at both GABA<sub>A</sub>- and GABA<sub>B</sub>-receptor sites (Bowery et al., 1981 a; Kaplita

et al., 1982; Giotti et al., 1983 a; Ong and Kerr, 1983 a,b; Kerr and Ong, 1984 a,b), whilst spontaneous GABAergic activity is similarly antagonised by these agents (Ong and Kerr, 1983 a; 1984 a, Kerr and Ong, 1985). All of this is not only consistent with, but best explained by, GABA being an inhibitory transmitter in the myenteric plexus where it is involved in the coordination of intestinal motility.



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