THE FUNCTION OF γ-AMINOBUTYRIC ACID IN THE PITUITARY

by

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This Thesis is dedicated to my parents,

with much love.

I declare that the studies presented in this Thesis are the result of my own independent investigation with the exception of the analysis of homocarnosine by high performance liquid chromatography (Chapter 5) which was carried out by Mr. H. Dick.

This work has not and is not being concurrently submitted for candidature for any other degree.

Some of the results presented in this Thesis have been published as follows:

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AOAA	Amino oxyacetic acid
4AP	4-aminopyridine
APS	3-aminopropane sulphonic acid
BMI	Bicuculline methiodide
CRH	Corticotrophin releasing hormone
DA	Dopamine
DAGO	Tyr-D-Ala-Gly-NMePhe-Gly-ol
dbcAMP	N^6-2^o-O-dibutyryl adenosine 3':5'cyclic monophosphate
DIDS	4,4'-diiso-thiocyano-2,2'-disulphonic acid stilbene
DSLET	[D-Ser_2]Leu-enkephalin-Thr
EOS	Ethanolamine-O-sulphate
FSH	Follicle stimulating hormone
GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAG	γ-Acetylenic GABA
GH	Growth hormone
GHRH	Growth hormone releasing hormone
5-HT	5 hydroxytryptamine, serotonin
IAA	Imidazole acetic acid
IBMX	3-isobutyl-1-methyl xanthine
i.c.v.	Intracerebroventricular
i.v.	Intravenous
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LSC	Liquid scintillation counting
MBM	Medial basal hypothalamus
ME	Median eminence
αMSH	αMelanocyte stimulating hormone
NA	Noradrenaline
NI	Neurointermediate lobe of pituitary
PD	Pars distalis
PI	Pars intermedia
PIF	Prolactin release inhibiting factor
PN	Pars nervosa
PRF	Prolactin releasing factor
P4S	Piperidine-4-sulphonic acid

TEA	Tetraethyl ammonium bromide
THIP	4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol
TRH	Thyrotrophin releasing hormone
TSH	Thyroid stimulating hormone
VIP	Vasoactive intestinal peptide.

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ABSTRACT OF THESIS

Experiments were carried out using techniques of biochemical pharmacology to investigate the influence of γ-aminobutyric acid (GABA) on the pituitary gland. The properties and effects of GABA receptors in the gland, and the properties of GABA-releasing nerve terminals in the median eminence (ME) and neurointermediate lobe of the pituitary (NI) were studied.

The presence of $GABA_A$, $GABA_B$ and benzodiazepine binding sites in the pituitary was demonstrated by radioligand binding studies. $GABA_A$ sites were detectable in both pars distalis (PD) and NI, predominantly in PD, but in much lower concentration than in the CNS. $GABA_B$ sites were also detectable in PD, but not in NI. Both central and peripheral type benzodiazepine sites were present, but in contrast to $GABA_A$ sites, both types were more concentrated in NI. The central type sites were found to be linked to GABA receptors and to be predominantly of the BZ_1 subtype.

The effects of GABA and related compounds on prolactin secretion in vitro were examined in detail using a superfusion system. $GABA_A$ receptor agonists were found to have a biphasic effect on prolactin secretion, with a transient stimulation following by sustained inhibition. Evidence was provided, using both agonists and antagonists, that the two components of the response were mediated by different types or states of $GABA_A$ receptor complex. Benzodiazepines and a barbiturate were found to potentiate the stimulatory component of this response, but did not affect the inhibitory part. $GABA_B$ receptor effects were also observed on prolactin secretion, and their possible mediation by an effect on K^+ conductance was investigated.

GABA receptor-mediated effects were also found on the secretion of growth hormone, luteinizing hormone and adrenocorticotrophic hormone but not thyroid stimulating hormone. The GABA-containing dipeptide homocarnosine mimicked some but not all of these effects of GABA. This appeared to be attributable to homocarnosine itself as PD was found to contain undetectable homocarnosinase activity.

The properties of GABAergic terminals in ME and NI were studied using crude synaptosomal preparations. [^{3}H]GABA was accumulated by both regions with high affinity, showing the characteristics of neuronal uptake and was released by depolarising stimuli in a $Ca2^+$-dependent manner. Release from both regions appeared to be controlled by $GABA_A$ autoreceptors, and in addition $GABA_B$ autoreceptors were demonstrated in ME. Release from NI was inhibited by κ opioid receptor agonists, and experiments to investigate the mechanism of this effect indicated the involvement of chloride channels. A variety of other neurotransmitters and hormones had no effect on release from either area.

A $GABA_A$ receptor agonist was found to inhibit release of dopamine (a major prolactin inhibitory factor) from ME but not NI. This may be the means by which GABA agonists cause the stimulation of prolactin secretion reported in vivo.

Brattleboro rats were found to have much lower glutamic acid decarboxylase activity in NI than Long-Evans rats, but there was no alteration in the number of $GABA_A$ binding sites. This deficit was found to be confined to pars nervosa, and was interpreted as a lesion of the GABA innervation of that region.

These results provide new evidence indicating a diverse influence of GABA on pituitary function and clarify some of the mechanisms involved.

CONTENTS

CHAPTER 1

Introduction

1.1 GABA as a neurotransmitter

The neutral amino acid γ-aminobutyric acid, (GABA) was discovered to be present in brain in 1950; thereby identifying a previously unknown ninhydrin-positive substance present in much higher concentrations in brain than in peripheral tissues of several species (Awapara, Landura, Fuest and Seale, 1950; Roberts and Frankel, 1950; Udenfriend, 1950). The first suggestion that GABA might have an inhibitory function in the nervous system came from studies by Hayashi (1956) who found that topically applied GABA caused a marked depression of frog brain electical activity. Florey (1954) had previously described an extract of mammalian brain called Factor I, which had an inhibitory action on crayfish stretch receptor, and the inhibitory activity was subsequently identified as GABA (Bazemore, Elliot and Florey, 1957). McLennan (1959), however, found considerable inhibitory activity of a Factor I extract that was devoid of GABA.

The advent of microiontophoretic techniques permitted the study of the effect of GABA on individual neurones. GABA was found to inhibit all classes of spinal interneurones in the cat (Curtis, Phillis and Watkins, 1959) although those authors concluded at the time that GABA was unlikely to be the major inhibitory neurotransmitter in the spinal cord because its action was not antagonised by strychnine. Conversely, on the basis of a study of the effects of GABA on cortical neurones, Krnjevic and Phillis (1963) concluded that such properties were "eminently suitable" for a role for GABA as an inhibitory neurotransmitter.

Subsequent studies on the cerebral cortex provided evidence that GABA was indeed a major inhibitory neurotransmitter. GABA

hyperpolarised pyramidal and Purkinje cells by an increase in membrane conductance to chloride ions, in a manner similar to that produced by the inhibitory neurotransmitter released from synapses activated by electrical stimulation of the surface of the cerebral cortex (Krnjevic and Schwartz, 1966; Dreifuss, Kelly and Krnjevic, 1969). The discovery that the convulsant alkaloid bicuculline is an antagonist of the depressant effects of GABA (Curtis, Duggan, Felix and Johnston, 1970; 1971a) (although its specificity and potency have been criticised (Straughan, Neal, Simmonds, Collins and Hill, 1971)) provided a more precise means for studying the pharmacology of inhibitory pathways, and using this approach, Curtis and Felix (1971) concluded that GABA appeared to be the principal inhibitory neurotransmitter of the cerebellum and cerebral cortex.

1.2 Uptake and release of GABA

Slices of cerebral cortex but not peripheral tissues were originally shown to be able to greatly concentrate GABA by Elliot and van Gelder (1958), suggesting that uptake rather than metabolism is the mechanism of termination of the effect of GABA. There have since been carried out a very large number of studies on the uptake and release of GABA.

GABA is accumulated by neuronal tissue through an active high-affinity mechanism, which is dependent upon sodium (Iversen and Neal, 1968; Martin and Smith, 1972; Bennet, Logan and Snyder, 1972). An uptake carrier is also present in glial cells (Henn and Hamberger, 1971; Bowery and Brown, 1972; Schon and Kelly, 1974) with a similarly high affinity but a different pharmacological specificity: neuronal uptake can be preferentially inhibited by 2,4-diaminobutyric acid, and glial uptake by β-alanine (Iversen and Kelly, 1975).

Release of GABA from cat cerebral cortex has been detected *in vivo* (Jasper, Khan and Elliott, 1965) and could be altered by lesions or stimulation of the midbrain (Jasper and Koyama, 1969). Release could also be increased by electrical stimulation, in a Ca^{2+}-dependent manner (Iversen, Mitchell and Srinivasan, 1971). Similar properties have been observed *in vitro* but the degree of Ca^{2+}-dependency and the nature of Ca^{2+}-dependent release has been the subject of some uncertainty. K^{+}-induced release is generally not totally abolished in Ca^{2+}-free medium (Mulder and Snyder, 1974) and electrically-induced release has been reported to be abolished by some (Srinivasan, Neal and Mitchell, 1969) but not others (Potashner, 1978a). The release of GABA by K^{+} in a Ca^{2+}-independent manner is greater for exogenous than endogenous GABA (Haycock, Levy, Denner and Cotman, 1978) and may be a result of membrane transport rather than stimulus-secretion coupling as it is Na^{+}-dependent (Haycock et al, 1978; Szerb, 1979).

Glial cells can also release GABA in response to K^{+} (Minchin and Iversen, 1974), and this shows less Ca^{2+}-dependence than neuronal release (Sellstrom and Hamberger, 1977; Neal and Bowery, 1979). These points are discussed further in Chapter 8.

1.3 Metabolism of GABA

GABA is formed in the CNS of vertebrates from L-glutamic acid. This was first demonstrated by Roberts and Frankel (1950), and the enzyme responsible, glutamic acid decarboxylase (GAD) has since been purified (Wu, Matsuda and Roberts, 1973). Early studies suggested that GAD was confined to neurones (Salganicoff and de Robertis, 1965; Neal and Iversen, 1969), and this has been confirmed by immunohistochemical studies using antibodies against purified GAD

(McLaughlin, Wood, Saito, Barber, Vaughan, Roberts and Wu, 1974). Non-neuronal GAD has also been demonstrated (Haber, Kuriyama and Roberts, 1970) but this is a different enzyme which does not appear to cross-react with antibodies to authentic neuronal GAD (Wu, Chude, Wein, Roberts, Saito and Wong, 1978).

Although glutamate is the direct precursor of GABA, slices of rat brain form more GABA when loaded with glutamine than with glutamate (Benjamin and Quastel, 1972). This result led to the suggestion of a neuronal/glial cycle, whereby there is a flow of GABA and glutamate from nerve endings to astrocytes, and of glutamine from astrocytes to nerve endings (Hertz, 1979). Such a model depends upon high-affinity uptake of glutamine into neurones. This was demonstrated by Balcar and Johnston (1975), but others could only demonstrate low-affinity uptake (Baldessarini and Yorke, 1974).

GABA is degraded by undergoing a transamination reaction with α-ketoglutarate to produce succinic semialdehyde (Roberts and Bregoff, 1953) which can enter the tricarboxylic acid cycle. GABA transaminase has been localized immunohistochemically in the cerebellum (Barber and Saito, 1976), and was found to be present in neuronal cell bodies and dendrites and glial processes but not nerve terminals. This supported the results of subcellular distribution studies (Salganicoff and De Robertis, 1965). Therefore as a general principle, it seems possible that GABA released from a nerve terminal and reaccumulated by a terminal will be re-released, not metabolised, while if it is taken up by glial cells it will be metabolised. This anatomical arrangement would explain why drugs which inhibit GABA-transaminase greatly increase the amount of GABA

in brain without greatly affecting the actions of endogenous or exogenous GABA (Gottesfeld, Kelly and Renaud, 1972), again suggesting that the synaptic action of GABA is not predominantly terminated by metabolism. More recent immunohistochemical studies have localised GABA-transaminase in cerebellum in both neurones and glia (Chan-Palay, Wu and Palay, 1979) but using a histochemical approached applied to striatal efferent pathways, Vincent, Kimura and McGeer (1981) demonstrated that the enzyme was mainly present in GABAergic neurones, with little in glia.

GABA and its two associated enzymes are also involved in intermediary metabolism, in the "GABA shunt" whereby glutamine and glutamate can enter the tricarboxylic acid cycle (Roberts, 1956).

1.4 Anatomical considerations

The distribution of GABAergic markers in presumed strategic sites for neuroendocrine control will be discussed in this section. Four methods have been used to detect the presence of GABAergic neurones:

I) The measurement of endogenous GABA.

II) The measurement of GAD activity.

III) Autoradiography following uptake of [^{3}H]GABA.

IV) Immunohistochemical visualisation of GAD.

A fifth method, autoradiography of GABA binding sites, has not been applied to the pituitary or the hypothalamus, although membrane binding studies have been carried out. They have revealed the presence of GABA binding sites in both the hypothalamus (Williams and Risley, 1979) and pituitary (Grandison and Guidotti, 1979).

The measurement of endogenous GABA is complicated by rapid postmortem increases, which appear to be directly related to GAD

activity in the region (Tappaz, Brownstein and Kopin, 1977). Furthermore, both this method and the assay of GAD activity (even allowing for the validity of the assay method) are limited by the dissection, and the average values obtained for the dissected sample can be misleading. In a study of GAD activity and GABA content of hypothalamic nuclei, Tappaz et al (1977) found that the median eminence (ME) had the lowest content of both markers, and concluded that "GABAergic neurones might not be involved in neuroendocrine regulation at the median eminence level". Subsequently, immunohistochemical studies showed that GAD was mainly confined to the external layer of ME (Tappaz, Wassef, Oertel, Pant and Pujol, 1981). GABA was relatively evenly distributed compared to GAD, possibly reflecting the redistribution of released GABA into surrounding glial cells.

Nevertheless, the microchemical assay of GAD activity has provided evidence for a tuberoinfundibular GABA pathway. Deafferentation of the hypothalamus, or lesions separating medial from lateral hypothalamus did not affect GAD activity in ME, but partly reduced GAD activity in the arcuate and ventromedial nuclei (Tappaz and Brownstein, 1977). Administration of monosodium glutamate to neonate rats, a treatment which selectively lesions the arcuate nucleus, reduced GAD activity in ME (Walaas and Fonnum, 1978).

Infusion of [^{3}H]GABA into the third ventricle predominantly labelled neuropil adjacent to the site of infusion, but labelled and unlabelled cells could be differentiated (Makara, Rappay and Stark, 1975). Presynaptic terminals which had accumulated tritium could also be seen on electron microscopic examination, but the

accumulation by glial cells and the different pattern of labelling at different distances from the ventricle demonstrates the limited potential of this technique. Intracardiac injection of [^{3}H]GABA was successfully used to selectively label neuronal sites in the external but not the internal layer of ME (Tappaz, Aguera, Belin and Pujol, 1980), providing the first anatomical evidence pointing strongly towards GABAergic neuroendocrine control of the anterior pituitary.

These techniques were also used to investigate GABAergic markers in the pituitary*. GABA is synthesized in the pituitary (Anderson, Larsson and Pocchiari, 1961; Minchin and Beart, 1975) and endogenous GABA is found throughout the gland (Labella, Vivian and Queen, 1968; Beart, Schon and Kelly, 1974). The posterior pituitary was able to accumulate [^{3}H]GABA into glial cells and contained low but detectable GAD activity (Beart et al, 1979). The pars distalis (PD) was found to be devoid of GAD activity but contained higher GABA-transaminase activity than the posterior pituitary or the hypothalamus (Racagni, Apud, Locatelli, Cocchi, Nistico, diGiorgio and Muller, 1979). The precise neuroanatomical basis for this pattern of GABAergic markers being present in pituitary was unclear for some years.

*FOOTNOTE

The terminology used in this Thesis is as follows: the pituitary gland is divided into three lobes: pars distalis (PD), pars intermedia (PI) and pars nervosa (PN). The latter two, which are anatomically more closely related, are often considered together as the neurointermediate lobe (NI). The terms 'posterior pituitary' and 'neurohypophysis' will only be used in discussion of results where the original authors have used those terms.

The development of antisera to GAD however, allowed clarification of these results. GAD is generally considered to be a reliable marker for GABAergic neurones, and immunohistochemical studies have been carried out at both light- and electron-microscopic level. Studies of GAD-immunoreactivity (GAD-IR) in the hypothalamus and pituitary have been carried out by Vincent, Hokfelt and Wu (1982), Oertel, Mugnaini, Tappaz, Wise, Dahl, Schmechel and Kopin (1982) and Tappaz et al (1983).

PD was found to be devoid of GAD-IR, but there was a rich distribution of fibres in pars intermedia (PI) and scattered varicosities in PN. Terminals were described in apposition to glial cells and unlabelled axons and formed synapse-like contacts with secretory cells in PI, and apposed to terminals, pituicytes and fenestrated portal capillaries in PN. GAD activity was reduced by >95% after stalk sectioning, and no GAD-IR was observable, suggesting a central origin of this innervation. Clusters of magnocellular GAD-IR cell bodies were described in the posterior hypothalamus, and it was suggested that these might be the origin of the innervation of NI, but subsequent studies demonstrated a projection of these cells to the cortex, amygdala and striatum (Vincent, Hokfelt, Skirboll and Wu, 1983).

In ME, labelling was predominantly observed over the external layer, following outlines of capillary loops. Terminals were found in close contact with tanocytes and intermingled with bundles of unlabelled axons and neurosecretory endings, in some cases between the basement membrane and endothelial cells of the capillaries, but as in PN, no morphological synapses were found.

Some of the GABAergic projection to ME may arise from cell bodies in the arcuate nucleus, as discussed above, and GAD-IR cell bodies were observed there. Other candidate nuclei, eg the periventricular nucleus, may also project to ME (Wieg!and and Price, 1980) but there is no evidence to implicate GABA in this projection. GAD-IR terminals were found throughout the hypothalamus, and several areas of cell bodies were found but the weakness of the immune reaction, even after colchicine treatment, precluded definitive description. Groups of cell bodies were found adjacent to the magnocellular supraoptic and paraventricular nuclei, which may project with the peptidergic neurones to PN as well as locally. Overall, no obvious pattern of innervation was clear from these studies, although much is likely to be intrinsic to the hypothalamus (Tappaz and Brownstein, 1977).

Furthermore, these studies did not reveal any differential distribution of GAD-IR terminals in the external layer of ME, but a recent study has suggested that GAD-IR terminals are mainly observed in the lateral palisade zone, where there was a partial overlap with tyrosine hydroxylase-immunoreactive terminals (Everitt, Hokfelt, Wu and Goldstein, 1984). A conserable proportion of GAD-IR cell bodies in the arcuate nucleus also stained for tyrosine hydroxylase, suggesting coexistence of GABA and dopamine (DA) in tubero-infundibular neurones (Everitt et al, 1984).

Although GABA itself can now be detected immunohistochemically, (Storm-Mathison, Leknes, Bore Vaaland, Edminson, Haug and Ottersen, 1983) this approach has not yet been applied to the hypothalamus or pituitary.

Further to this important but essentially circumstantial

evidence pointing to a functional role for GABA in the hypothalamo-hypophysial system, a range of more direct physiological studies have been carried out. Evidence is available for GABA having regulatory effects on the secretion of a variety of pituitary hormones with examples from each zone of the gland.

1.5 Effects of GABA on secretion of Pars Distalis hormones

GABA may exert its various influences on PD hormone secretion either by a direct action on the endocrine cell or indirectly by acting on neurones which either themselves release transmitters into hypophysial portal blood or from synapses with other neurones that do. Theoretically, any number of intermediate neurones may be involved, and in vivo experiments measuring plasma hormone concentration provide no information on this, even when pharmacological or surgical attempts are made to elucidate the involvement of other systems. Thus although the results of Makara and Stark (1974) demonstrate a hypothalamic site of action of GABA in the control of adrenocorticotrophic hormone (ACTH) secretion, no information is obtained about the possibility of a direct effect on the pituitary, or whether the hypothalamic action is a direct effect on corticotrophin releasing hormone (CRH) secretion or not. Similarly, Vijayan and McCann (1979) attempted to use pimozide to distinguish DA-mediated from DA-independent effects of GABA on PD hormone secretion, though the confused state of the literature about the involvement of DA in, for example, the control of luteinizing hormone (LH) secretion (Weiner and Ganong, 1978) makes the interpretation of such multiple-variable experiments hazardous.

There is considerable evidence that GABA can affect the secretion of the PD hormones LH, ACTH, growth hormone (GH) and

thyroid stimulating hormone (TSH) by actions within the hypothalamus (Elias, Valenta, Szekeres and Grossmann, 1982; Racagni, Apud, Cocchi, Locatelli and Muller, 1982). Both stimulatory and inhibitory effects on each hormone have been reported, and in some cases in vitro experiments have provided evidence as to the mechanism of action: for example GABA inhibits the release of CRH (Burden, Hillhouse and Jones, 1974) and somatostatin (Gamse, Vaccaro, Gamse, DiPace, Fox and Leeman, 1980) which might account for the inhibitory effect of GABA on ACTH secretion (Makara and Stark, 1974) and the stimulatory effect on GH secretion (Vijayan and McCann, 1978b). Evidence for direct effects of GABA on cells of PD has been less forthcoming. In 1977, Schally, Redding, Arimura, Dupont and Linthicum isolated from hypothalamus a substance, distinct from catecholamines, which had inhibitory effects on prolactin secretion both in vivo and in vitro. This substance was identified as GABA. Subsequent studies have since confirmed the inhibitory effect of GABAergic agonists on prolactin secretion in vitro (eg Enjalbert, Ruberg, Arancibia, Fiore, Priam and Kordon, 1979a) but there have been no previous reports of effects on other PD hormones in vitro.

Although PD contains no GAD, it contains considerable amounts of GABA and GABA-transaminase (Racagni et al, 1979) and the concentration of GABA falls if the pituitary is ectopically transplanted. Inhibition of GABA-transaminase by central administration of ethanolamine-O-sulphate (EOS) increased PD GABA concentration and reduced plasma prolactin concentration, while peripheral administration of EOS did neither (Racagni et al, 1979), suggesting an 'over-flow' of GABA from the CNS to the pituitary.

The efficacy of EOS in apparently augmenting GABAergic function in this case contrasts with the generally slight effects of inhibition of GABA-transaminase on GABAergic function in the CNS (Gottesfeld et al, 1972). This may reflect the relative unimportance of metabolism of GABA in the CNS in the termination of its synaptic action, as discussed in Section 1.3 above and suggests that metabolism may be a more important, possibly limiting factor in the control of hypothalamo-hypophysial GABAergic function. Alternatively it could be argued from the results of Racagni et al (1979) that GABA-transaminase normally 'protects' PD from the effects of GABA. GABA has been detected in hypophysial portal blood, but in both dioestrous female and male rats the concentrations in peripheral and portal blood were not significantly different (Mulchahey and Neill, 1982; Mitchell, Grieve, Dow and Fink, 1983). The concentration of GABA in hypophysial portal but not peripheral blood was greatly increased by electrical stimulation of ME (Mitchell et al, 1983) or by EOS (Gudelsky, Apud, Masotto, Locatelli, Cocchi, Racagni and Muller, 1983). Intracerebroventricular (i.c.v.) injection of prolactin has also been reported to increase GABA secretion into hypophysial portal blood (Apud, Gudelsky, Masotto, Locatelli, Cocchi, Racagni and Muller, 1984a), providing evidence consistent with a negative feedback pathway.

In addition to GABA itself, higher concentrations of the GABA-containing dipeptide homocarnosine were found in hypophysial portal blood, than in peripheral blood, and its concentration was unaffected by electrical stimulation of ME (Mitchell et al, 1983). Schally et al (1977) had previously shown that homocarnosine could inhibit prolactin secretion <u>in vitro</u> and further experiments on this

substance have been carried out in this Thesis (Chapters 5, 7 and 12).

Evidence relevant to the control of prolactin secretion by GABA is discussed further in Chapter 5, and of other PD hormones in Chapter 7.

1.6 Effects of GABA on the Secretion of Neurointermediate lobe hormones

Again, effects of GABA on NI can be divided into direct and indirect, the latter particularly encompassing effects on the paraventricular and supraoptic nuclei, the origins of the peptidergic projections to PN.

GABA has been reported to inhibit the firing of cells in the supraoptic nucleus (Nicoll and Barker, 1971; Bioulac, Gaffori, Harris and Vincent, 1978), and to reduce the amplitude of the antidromic compound action potential in the neurohypophysial stalk (Zingg, Baertschi and Dreifuss, 1979) GABA has also been shown to depolarise these fibres (Loeffler, Desaulleus, Demeneix and Feltz, 1982). i.c.v. GABA and drugs that increase GABAergic transmission inhibited vasopressin secretion in vivo (Knepel, Nutto and Hertting, 1980) but GABA enhanced stimulated vasopressin secretion in vitro (Knepel, Nutto, Loeffler and Feltz, 1984). GABA has also been reported to have no effect on vasopressin secretion in vitro (Iversen, Iversen and Bloom, 1980). There is a brief report that GABA inhibited oxytoxin secretion in vitro (Dyball and Shaw, 1979) but this has not been confirmed.

The original demonstration that GABA inhibits α melanocyte stimulating hormone (αMSH) secretion in vitro (Hadley, Davis and Morgan, 1977) has recently been considerably refined. Taraskevich

and Douglas (1982) demonstrated that GABA depolarised PI cells in culture, causing a flurry of action potentials with subsequent quiescence. This effect showed the typical pharmacology of a $GABA_A$ receptor: bicuculline- sensitive, apparently mediated by opening chloride channels and potentiated by diazepam. Subsequent studies on the effects of GABA on αMSH secretion showed a biphasic effect (Tomiko, Taraskevich and Douglas, 1983), the stimulatory component of which has been shown to be $GABA_A$-receptor mediated, and the inhibitory component $GABA_B$-receptor mediated (Demeneix, Desaulles, Feltz and Loeffler, 1984; Taraskevich and Douglas, 1985).

Recent in vitro electrophysiological experiments on a mediobasal hypothalamus/NI preparation suggest that the dopaminergic innervation of PI is tonically active and causes substantial inhibition of the gland (Davis, Haas and Lichtensteiger, 1985). These results support conclusions from in vivo experiments (Passo, Thornborough and Ferris, 1981). Bicuculline had no effect on stimulus-induced inhibition of PI cell electrical activity, which was blocked by sulpiride, but no experiments on the effect of bicuculline on stimulus-induced PI cell action potentials were reported (Davis et al, 1985). The question as to whether GABA mediates this neuronal stimulatory input to PI, which would be compatible with the results of Taraskevich and Douglas (1982), is therefore unresolved. This type of preparation may provide considerable information as to the cellular function of the postsynaptic $GABA_A$ and $GABA_B$ receptors on melanotrophes.

The GABAergic innervation of NI may also contribute to the control of hormone secretion from PD. GAD-IR terminals were observed adjacent to fenestrated capillaries in PN (Oertel et al,

1982) and intrapituitary blood flow from PN to PD has been reported (Bergland and Page, 1979; Page, 1983). Removal of PN has been demonstrated to increase the concentration of prolactin in peripheral plasma (Oliver, Mical and Porter, 1977; Peters, Hoefer and Ben-Jonathan, 1981) and also to affect the secretion of LH (Fagin and Neill, 1982; Ben-Jonathan and Peters, 1982).

Although a variety of actions of GABA can be observed, these must be considered in the context of other influences. The control of prolactin secretion provides a useful example to consider.

1.7 The control of prolactin secretion

Prolactin secretion has long been recognised to be under the predominantly inhibitory control of the hypothalamus. This is illustrated by the increases in prolactin secretion following lesion of the pituitary stalk (Jacobsohn and Westman, 1945) or ectopic transplants in hypophysectomized rats (Everett, 1954). Following the demonstration of DA-containing nerve terminals in ME (Hokfelt 1967), DA was shown to inhibit prolactin secretion *in vitro* (Birge, Jacobs, Hammer and Daughaday, 1970). DA has been detected in hypophysial portal blood in sufficient quantity to inhibit prolactin secretion (Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977; Gibbs and Neill, 1978) and DA receptors have been demonstrated on lactotrophes (Goldsmith, Cronin and Weiner, 1979).

While there is general agreement about the importance of DA as a prolactin-inhibitory factor (PIF), there is considerably less evidence for the physiological involvement of other PIFs or prolactin-releasing factors (PRFs). As discussed above, GABA has been proposed to be a PIF, and is considered in detail in Chapter 5. The thyrotropin releasing hormone (TRH) metabolite

histidylproline-diketopiperazine has been demonstrated to inhibit prolactin secretion in vitro (Enjalbert, Ruberg, Arancibia, Priam, Bauer and Kordon, 1979b) as have catecholoestrogens (Linton, White, de Tineo and Jeffcoate, 1981) and somatostatin (Vale, Rivier, Brazeau and Guilleman, 1974) (although somatostatin has also been shown to stimulate prolactin secretion in vitro (Mitchell and Ogier, 1985). A peptidergic PIF has recently been described (Nikolics, Mason, Szonyi, Ramachandran and Seeburg, 1985).

The abrupt increase in prolactin secretion caused by the initiation of suckling has led to suggestions that withdrawal of tonic inhibition may not completely account for this. As DA is not the sole PIF, this cannot be very readily examined, but the involvement of stimulatory factors has been increasingly examined. A very large number of substances have been reported to have PRF activity (Leong, Frawley and Neill, 1983) including TRH (Tashjian, Barowsky and Jensen, 1971), opiates (Enjalbert, Ruberg, Arancibia, Priam and Kordon, 1979c) and vasoactive intestinal peptide (VIP) (Ruberg, Rotsztein, Arancibia, Besson and Enjalbert, 1978). VIP is found in brain and in hypophysial portal blood (Said and Porter, 1979) and antiserum to VIP has recently been shown to have pronounced effects on the stimulation of prolactin secretion by both suckling and ether (Abe, Engler, Molitch, Bollinger-Gruber and Reichlin, 1985). There is also evidence that oxytocin is a PRF: it is found in a projection to ME (Silverman, 1976) and in hypophysial portal blood (Horn, Robinson and Fink, 1985), and stimulates prolactin secretion in vitro (Lumpkin, Samson and McCann, 1983). The control of prolactin secretion may therefore have more in common with other PD hormones than has previously been recognised, in terms

of peptidergic stimulation (and inhibition) of secretion.

The apparent complexity of control at the level of the pituitary suggests that particular PIFs and PRFs may act only under certain conditions. This may be regulated by 'prolactin responsiveness factors' (Leong et al, 1983), which may or may not have direct effects on prolactin secretion. An interaction between DA and TRH has been demonstrated in vivo and in vitro, the presence or absence of DA reducing or increasing respectively the responsiveness to TRH in a more than additive manner (Fagin and Neill, 1981; Plotksy and Neill, 1982). These results are however complicated by a 'rebound' effect of withdrawal of DA seen in vitro, such that secretion is transiently stimulated above basal rates (Matsushita, Kato, Shimatsu, Katakami, Yanaihara and Imura, 1983).

Steroids may also modulate lactotrophe responsiveness: preincubation of PD cells in culture with oestradiol has been reported to prevent the inhibitory effect of DA but to enhance the stimulatory effect of TRH (Raymond, Beaulieu, Labrie and Boissier, 1978). Corresponding electrophysiological observations have also been made (Dufy, Vincent, Fleury, Du Pasquier, Gourdji and Tixier-Vidal, 1978; 1979). Such an interaction may participate in the proestrus prolactin surge.

A third method of control of lactotrophe responsiveness may be by a paracrine interaction with other cells, both with other lactotrophes (which may contribute to the pulsatile nature of prolactin secretion (Shin and Chi, 1979; Shin and Reifel, 1981) and other cell types, for example gonadotrophes. Luteinizing hormone releasing hormone (LHRH) was reported to have no effect on prolactin secretion from partially purified lactotrophes, but stimulated

prolactin secretion after addition of gonadotrophes (Denef and Andries, 1983). Recent in vivo experiments have provided support for this interaction (Debeljuk, Torres-Aleman and Schally, 1985).

The low concentration of GABA in hypophysial portal blood under basal conditions (Mitchell et al, 1983) suggests that it is not of major importance in the maintenance of tonic inhibitory control of prolactin secretion. The secretion of GABA from ME could be greatly increased by electrical stimulation (Mitchell et al, 1983) or by i.c.v. prolactin injection (Apud et al, 1984a) suggesting that GABA may provide a reserve of inhibitory control in feedback regulation. A further possibility is that GABA may modulate lactotrophe responsiveness to PRFs e.g. TRH and VIP, and experiments investigating this are described in Chapter 5.

Most studies investigating prolactin secretion in vivo or in vitro rely on radioimmunoassay measurements, but there is considerable evidence that prolactin is not a homogeneous substance. Suh and Frantz (1974) demonstrated two forms of prolactin in human plasma by gel filtration, with identical immunological properties. Variations in bioassay to radioimmunoassay potencies have been described for different forms of prolactin separated by gel electrophoresis (Asawaroengchai, Russell and Nicholl, 1978) and one of three bands separated by Sinha and Baxter (1979) had very high bioassay activity but no immunologic cross-reactivity with the major band. The presence of a cleaved form of rat prolactin has been demonstrated (Mittra, 1980a) which was present in different ratios to prolactin in different physiological states, eg pregnancy, and had potent mitogenic activity in mammary epithelial cells, a property not shared with

prolactin standards (Mittra, 1980b). The suggestion in these studies that different forms are differentially controlled and have different functions is supported by the demonstration that TRH and serotonin stimulated the secretion of different forms *in vivo* (Lawson, Gala, Chin and Haisenleder, 1980). Transformation of prolactin from one form to another may underly observations of depletion of pituitary prolactin during suckling (Grosvenor, Mena and Whitworth, 1980).

1.8 Aims of this Thesis

The primary aim of this Thesis was to provide further evidence for neuroendocrine actions and function of GABA, in both PD and NI. Three broad areas of approach were to be used:

I) Radioligand binding studies to investigate the GABA/benzodiazepine receptor complex in the pituitary.

II) *In vitro* superfusion of PD tissue to determine the effects of GABA on pituitary hormone secretion.

III) Investigation of the properties of the nerve terminals of the GABAergic innervation of ME and NI, and their interactions with other neuroendocrine transmitter systems, particularly with the dopaminergic innervation of both areas.

These various models were also to be used for the investigation of the principles and mechanisms of GABAergic transmission in general, particularly postsynaptic GABA receptor pharmacology of both $GABA_A$ and $GABA_B$ sites.

CHAPTER 2

General Methods

2.1. Animals

Animals used for the majority of experiments were male Wistar rats supplied by the University of Edinburgh Centre for Laboratory Animals. For the experiments in Chapter 12 homozygous Brattleboro rats were bred in the Department of Pharmacology, and Long Evans rats were purchased from Charles River, U.K. Ltd (Margate, Kent). The animals were maintained under controlled lighting (lights on 05.00-19.00h) and temperature (22°C) and had free access to tap water and Diet 41B (Oxoid, Basingstoke, Hants).

2.2. Dissection of tissues

For all dissections, rats were stunned, decapitated and their brains removed. The pituitary gland was then removed from the base of the skull and NI was then separated from the PD with fine forceps. For dissection of PI and PN, NI was put on a chilled glass plate under a binocular microscope. PI lobules of cells were visible, and could be carefully removed from PN using a pair of insect pincers. ME was dissected under a binocular microscope on a chilled glass plate according to Cuello, Horn, Mackay and Iversen (1973). The cut pituitary stalk was held with fine forceps and the borders of ME (defined by the presence of capillary loops) cut to a depth of 0.2-0.3mm with iridectomy scissors. The cerebellum was dissected from the pons, and after a coronal section at the caudal limit of the olfactory tubercles, the frontal cortex was dissected from the anterior block of brain. The hippocampus was dissected from the posterior block after making a midline sagittal section. When required, kidneys were also rapidly removed and were dissected free of the surrounding connective tissue before use.

2.3 Radioimmunoassays.

Numerous reviews on the techniques and principles of radio-immunoassay (RIA) have been published (Midgley, Niswender and Rebar, 1969; Kirkham and Hunter, 1971; Yalow and Berson, 1971; Yalow, 1980), and therefore only the methods used in this Thesis to measure hormones in in vitro perfusates of rat pituitary glands will be described here. Individual details of the methods of preparation and purification of radio-labelled hormones and the buffers used are given in the appendices.

The underlying principle of RIA is the competition between a fixed amount of radiolabelled antigen and the unknown amount of unlabelled antigen for binding sites on a limited number of primary antibody molecules. Binding of radiolabelled antigen is inversely dependent on the concentration of unlabelled antigen in the sample which can then be determined by reference to a set of standards of known concentration.

Hormones were labelled with ^{125}I (Na ^{125}I, 100mCi/ml, Amersham International) using modifications of the chloramine-T method (Greenwood, Hunter and Glover, 1963). In all assays, free hormone was separated from antibody-bound by the double antibody technique (Utiger, Parker and Daughaday, 1962). Bound hormone was retained for counting using a Berthold Mag310 Gamma counter (Scotlab Instrument Sales, Bellshill, Scotland).

Assay results were calculated by the inbuilt machine programme of the counter, by linear regression of the standard curves with logit B/Bo on the ordinate and log concentration on the abscissa. B/Bo represents the ratio of bound (standard or sample) to total

bound, and logit B/Bo = ln (B/Bo/(1-B/Bo)). Upper and lower limits of detection of assays were taken as the concentrations which caused 90% and 10% displacement respectively of labelled antigen from the primary antibody. The reproducibility and reliability of the assays were monitored by the inclusion of two known quality control pools in each assay, enabling calculation of intra- and inter-assay coefficients of variation (Rodbard, 1971). Additional quality control data was obtained by recording total counts, and blanks (no primary antibody) and total bound (no unlabelled antigen) as percentages of total counts, and the calculated equivalent concentrations of hormone corresponding to 20%, 50% and 80% B/Bo.

Standards in triplicate and samples in duplicate were aliquoted into disposable plastic tubes (LP3, Luckham Ltd) using automatic pipettes (Pipetman or Microman, Gilson) and reagents were dispersed using a Compupet automatic pipetting system (General Diagnostics).

2.3.1. Radioimmunoassay of prolactin

Pituitary superfusate concentrations of prolactin were measured using double antibody RIA kits supplied by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK, Baltimore, USA), as described by Pickering (1978). The assay is outlined in Appendix I. Standards (range 0.5-64ng/ml) were prepared from rat-PRL-RP-1 and rat-PRL-I-5 was used for iodination. The primary antiserum was rabbit anti-rat-PRL-S5, at a final dilution of 1:20,000. After incubation the antiserum-bound hormone was separated by precipitation with goat anti-rabbit gamma globulin (ARGG) (Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire) at a final dilution of 1:100.

A representative standard curve and quality control data are shown in Figures 2.1 and 2.2 respectively. The inter- and intra-assay coefficients of variation were 5.3% and 1.4% respectively, and the lower limit of detection was 12-16 ng/ml for 20µl samples of superfusate.

2.3.2. Radioimmunoassay for growth hormone.

Pituitary superfusate concentrations of GH were measured by double-antibody RIA using materials provided by NIADDK. The assay is outlined in Appendix I. Standards (range 0.5-32ng/ml) were prepared from rat-GH-RP-1 and rat-GH-I-4 was used for iodination. The primary antiserum was monkey anti-rat-GH-S4 used at a final dilution of 1:36,000. After incubation bound hormone was separated from free using anti-human gamma globulin (SAPU) at a final dilution of 1:150.

A representative standard curve is shown in Figure 2.3. The inter- and intra-assay coefficients of variation were 12% and 9% respectively. 40µl aliquots of a 1:20 dilution of superfusate were assayed and the lower limit of detection was 100-120 ng/ml.

2.3.3. Radioimmunoassay for luteinizing hormone

Pituitary superfusate concentrations of LH were measured using the ovine-ovine RIA developed by Niswender, Midgley, Monroe and Reichert (1968). The protocol is outlined in Appendix I. Standards (range 0.25-16ng/ml) were prepared from ovine LH (NIH-LH-S18) and ovine LH (LER-1056-C2) provided by Dr L. E. Reichert Jr, (NIADDK) was used for iodination. The primary antiserum (GDN-15) was provided by Dr. G.D. Niswender and was used at a final dilution of 1:240,000. After incubation of ^{125}I-LH with antiserum and standards/samples, bound was separated from free hormone using ARGG (SAPU) at a final dilution of 1:100.

A representative standard curve is shown in Figure 2.4 and quantity control data in Figure 2.5. The inter- and intra-assay coefficients of variation were 7% and 9% respectively. 100µl aliquots of supefusate were assayed in duplicate, and the lower limit of detection of the assay was 0.35 - 0.45 ng/ml.

2.3.4. Radioimmunoassay for ACTH

ACTH was measured in pituitary superfusate using double antibody RIA. The protocol used in this Thesis is given in Appendix II. Standards (range 3-200pg/ml) were prepared from synthetic human ACTH (1-39) provided by NIADDK, which was also used for iodination. The primary antiserum used was IgG-ACTH-1 (IgG Corporation, Nashville, USA) at a final dilution of 1:30,000. Bound was separated from free hormone using ARGG (SAPU) at a final dilution of 1:20.

A representative standard curve is shown in Figure 2.7. Only three ACTH assays were performed, and quality control data conformed to values previously established.

2.3.5. Radioimmunoassay for thyroid stimulating hormone

TSH was measured in pituitary superfusate by double antibody RIA using materials supplied by NIADDK. An outline of the protocol is given in Appendix I. Standards (range 0.3-50 ng/ml) were prepared from rat-TSH-RP-2 and rat-TSH-I-6 was used for iodination. The primary antiserum was anti-rat-TSH-S-2 at a final dilution of 1:10,000 and after incubation bound hormone was separated from free using ARGG (SAPU) at a final dilution of 1:100.

All results in this Thesis derive from one assay, the standard curve for which is shown in Figure 2.6. The intraassay coefficient of variation was 6.5%, and the limit of detection for 200µl sample was 0.14ng/ml.

2.4 Protein assay

All protein assays in this Thesis were carried out according to the method of Geiger and Bessman (1972). Aliquots of homogenate or bovine serum albumin standards (range 4–20μg) were made up to 200μl with distilled water in LP3 tubes and 500μl alkaline copper reagent was added. After 20 min 1ml of diluted Folin-Ciocalteau reagent was added, and absorbance of the solution was measured at 725nm using a Gilson model 250 spectrophotometer. Details of the method are given in Appendix III. Values for samples were read off from the standard curve, which showed linear absorbance against protein concentration over the range used.

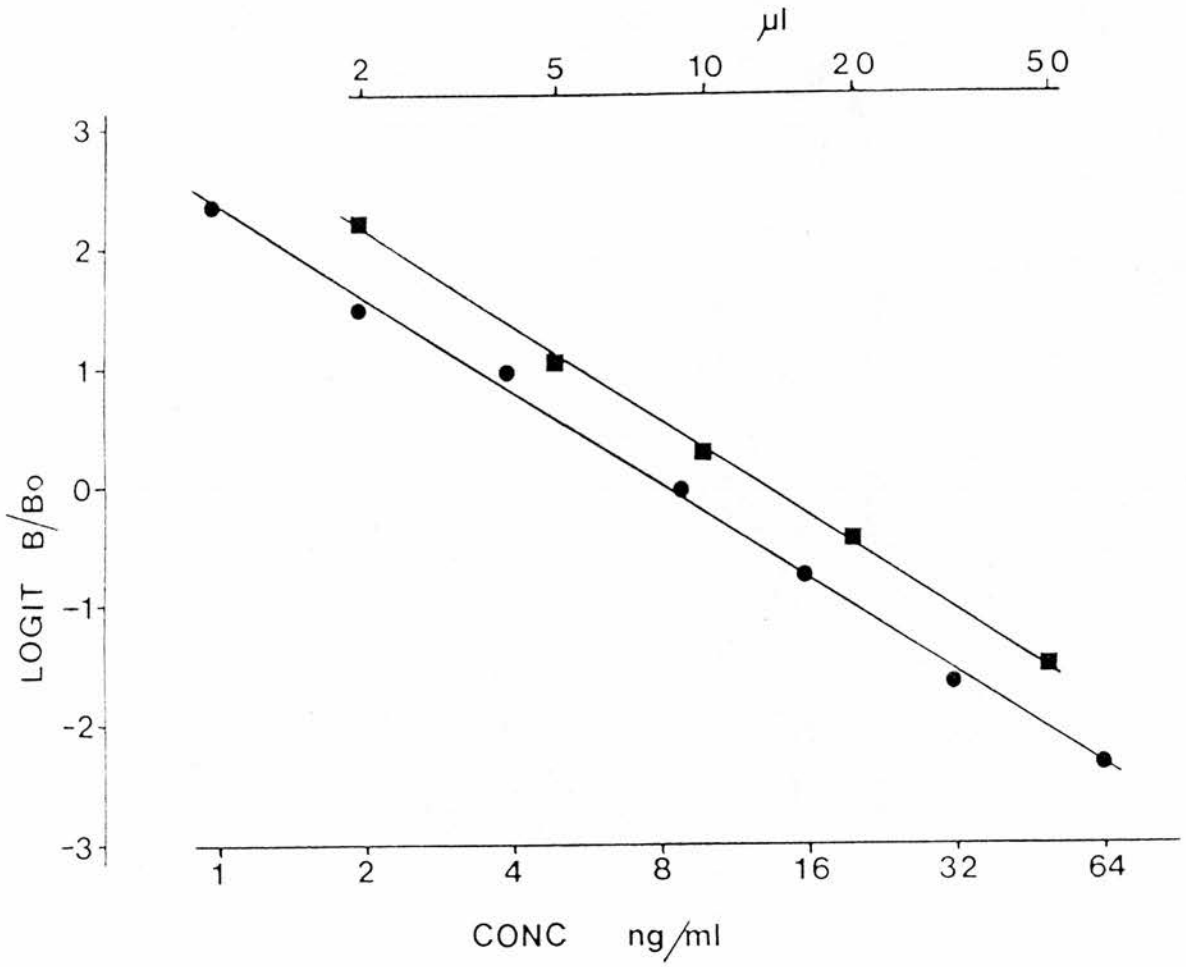


FIGURE 2.1

Prolactin: representative standard curve (●)
displacement by aliquots of PD superfusate ( ).

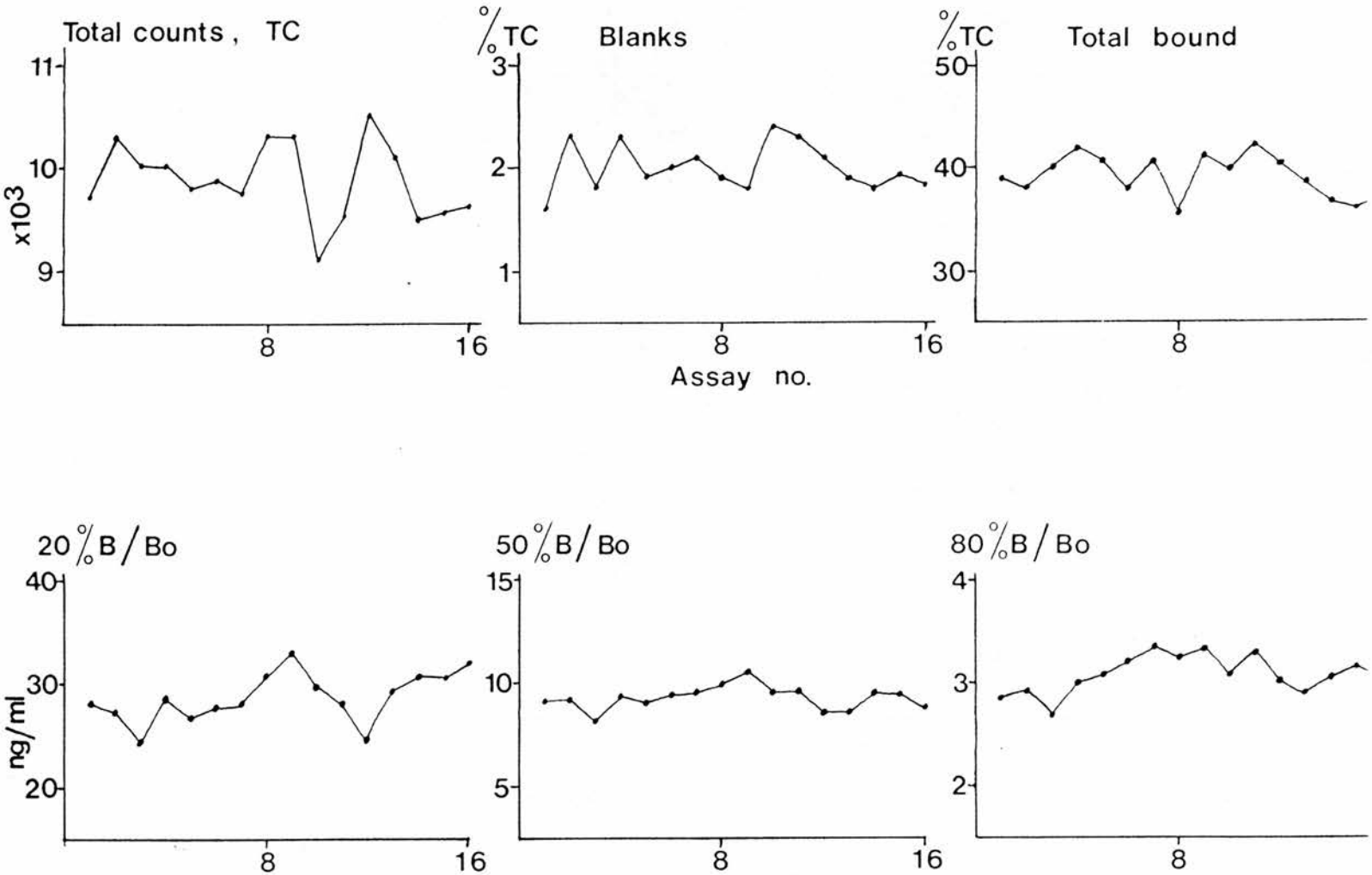


FIGURE 2.2

Prolactin: quality control data.
Total counts, Blanks and Total Bound as percentage of TC.
Prolactin (ng/ml) corresponding to 20%, 50% and 80% B/Bo.

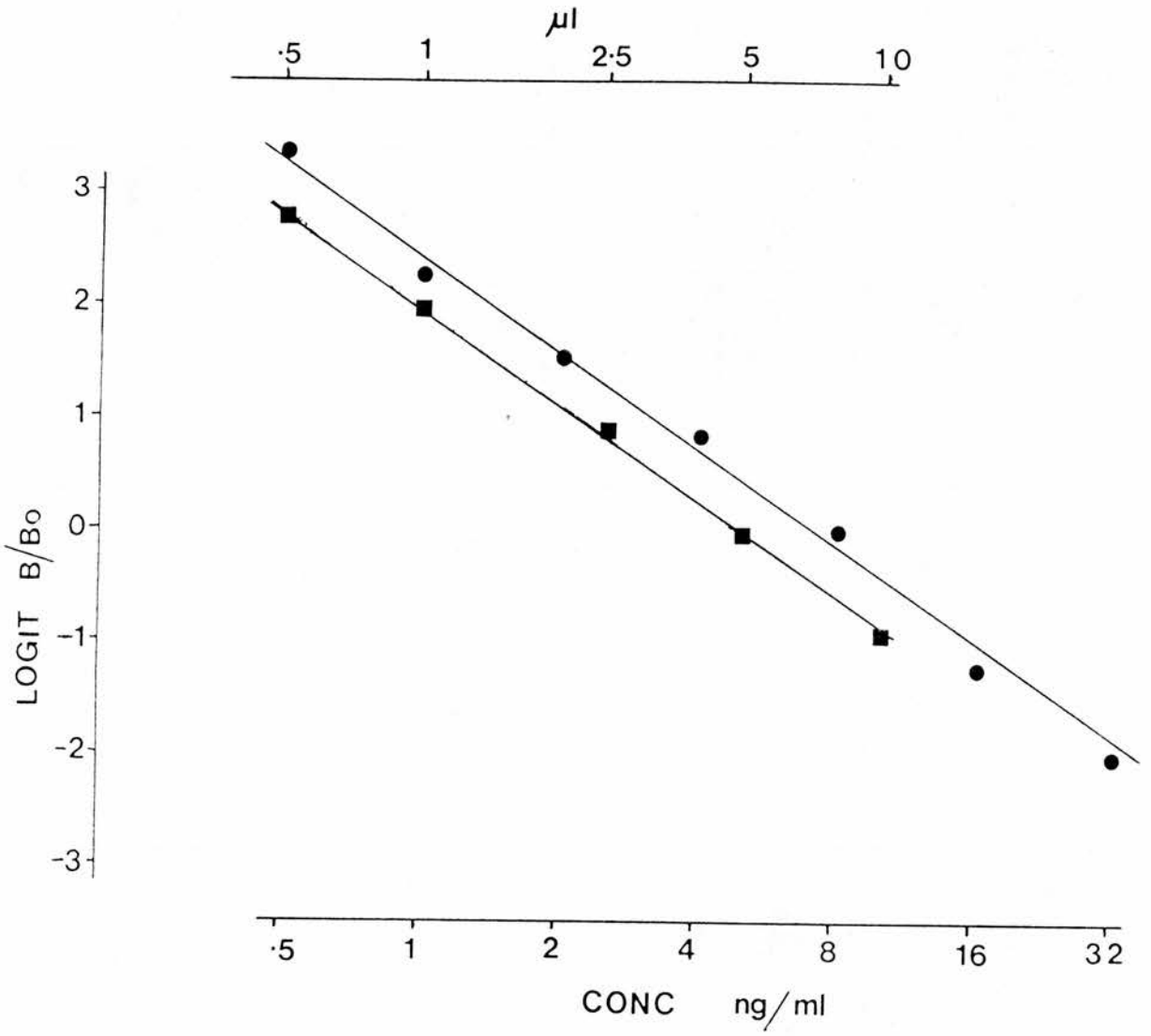


FIGURE 2.3

GH: representative standard curve ( )
displacement by aliquots of PD superfusate ( ).

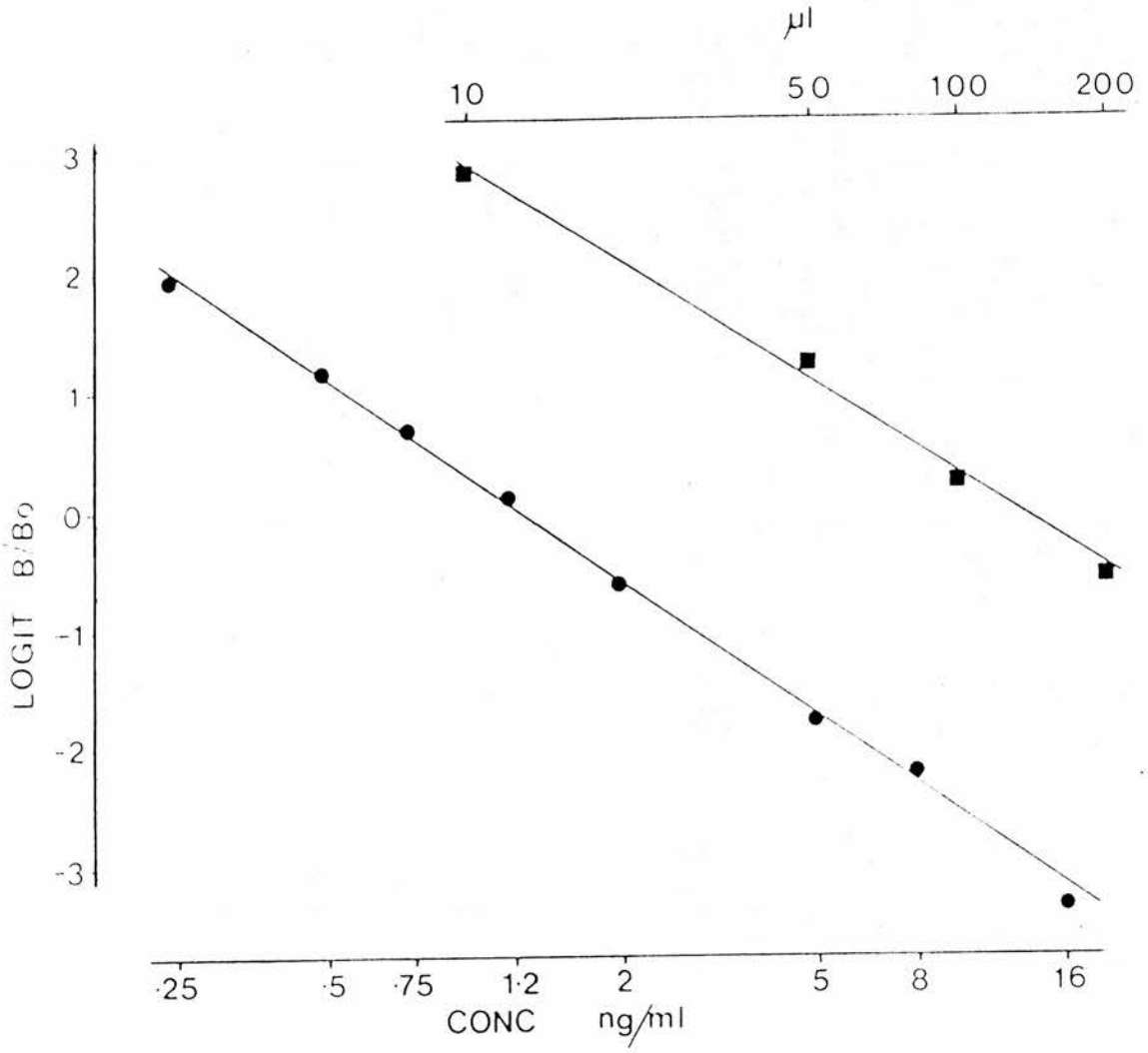


FIGURE 2.4

LH: representative standard curve (●)
displacement by aliquots of PD superfusate (■)

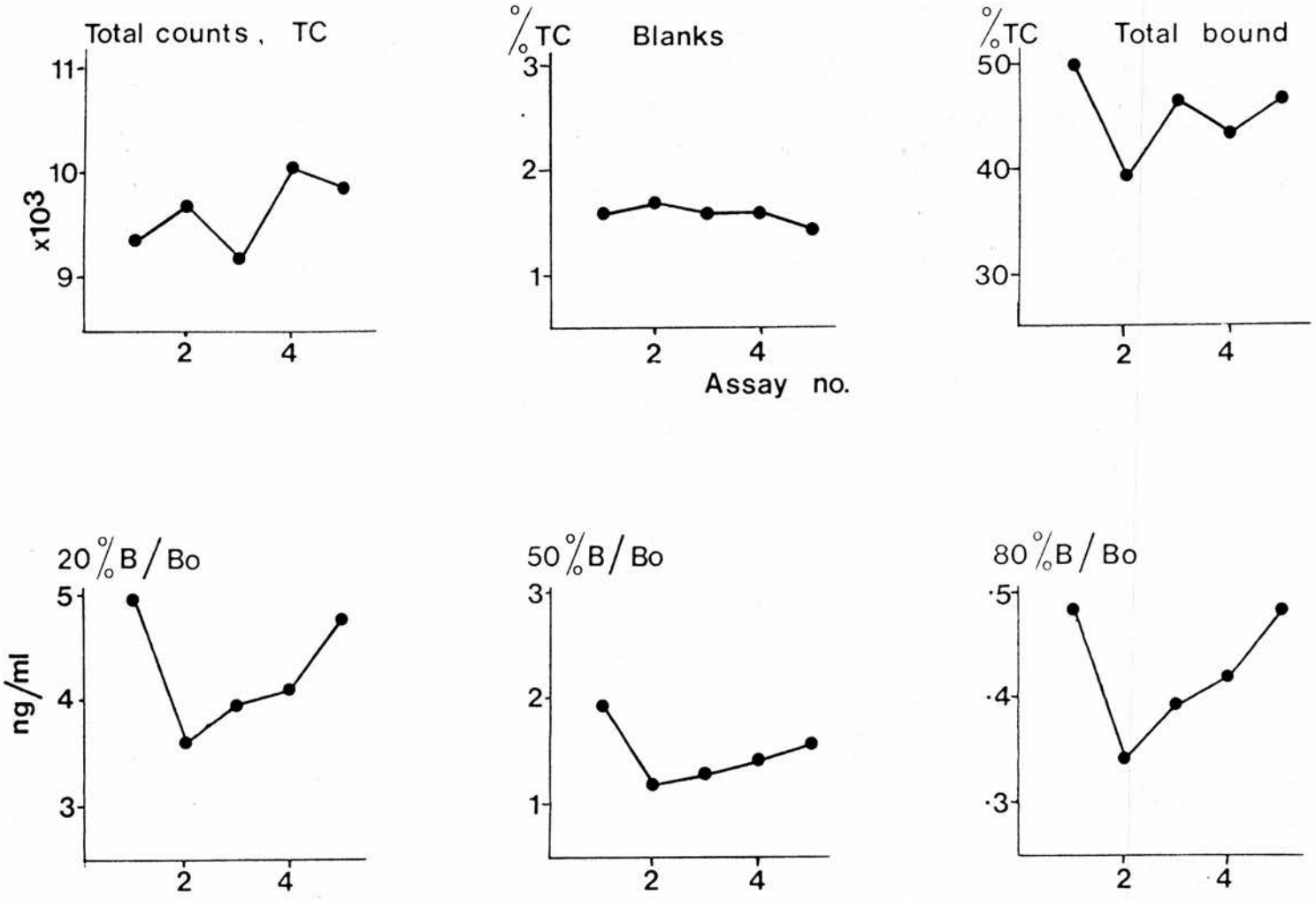


FIGURE 2.5
LH: quality control data

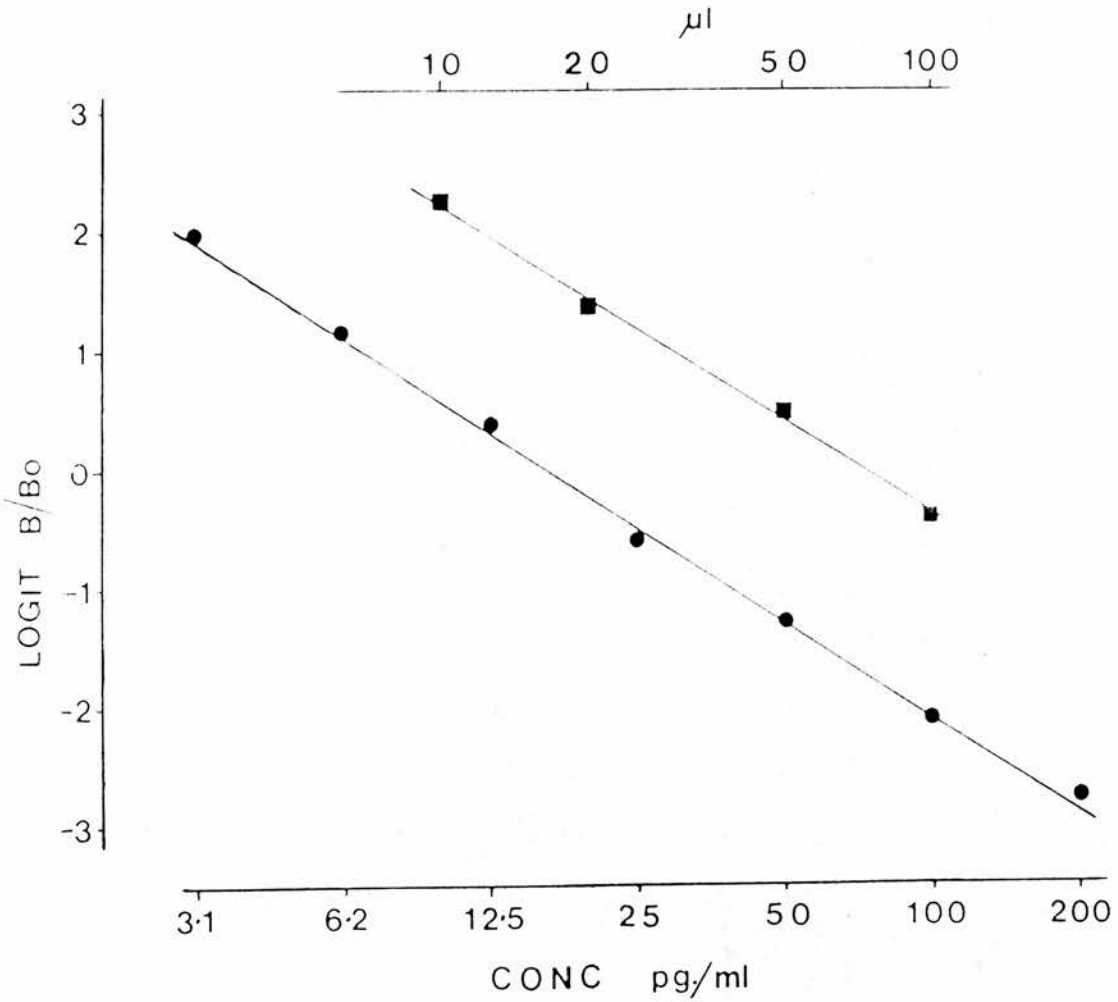


FIGURE 2.6

ACTH: representative standard curve (●)
displacement by aliquots of PD superfusate (■)

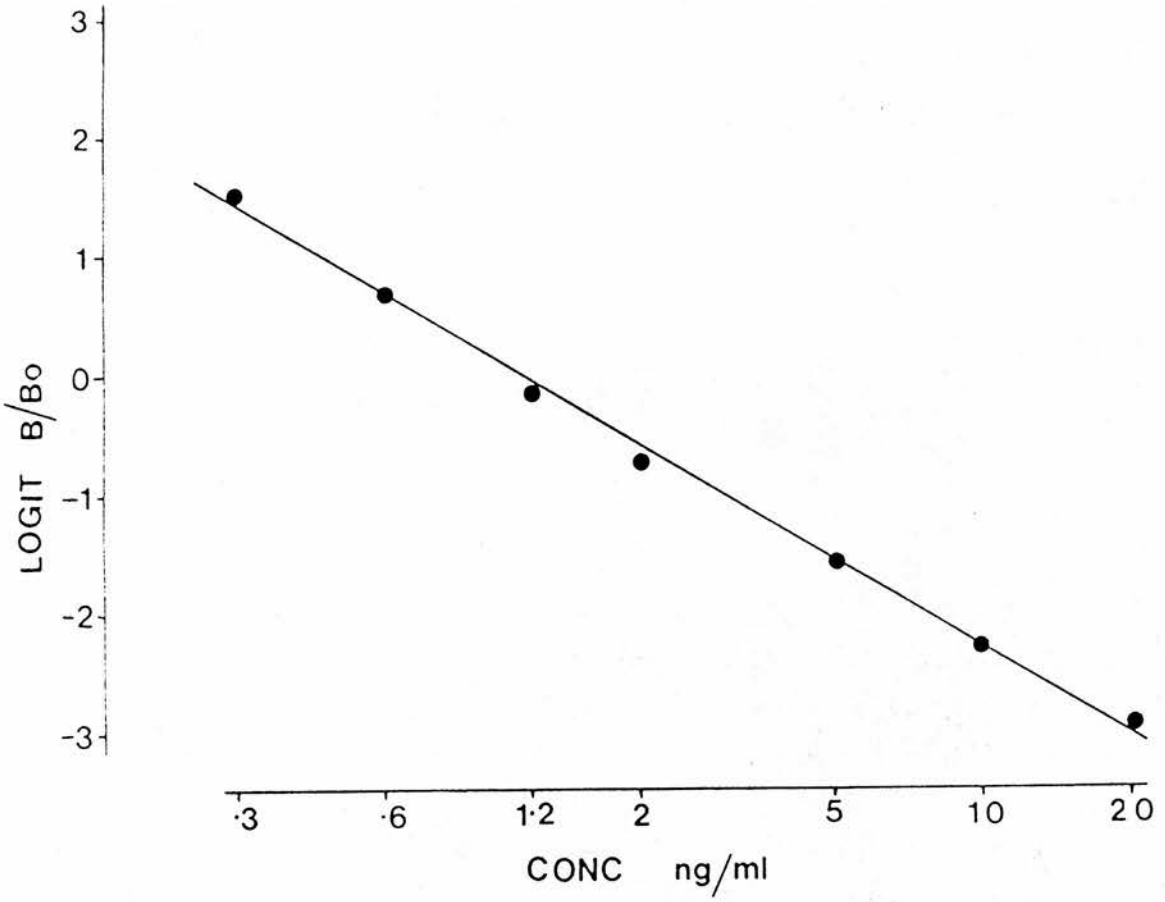


FIGURE 2.7

TSH: representative standard curve ( )

CHAPTER 3.

$GABA_A$ and $GABA_B$ binding sites in the pituitary gland.

3.1 Introduction

Synaptically-released GABA may interact with a variety of available recognition sites. These include postsynaptic or presynaptic receptors, or neuronal or glial high-affinity uptake sites. Early studies of binding sites for radiolabelled GABA showed their interaction with ligand to be Na^+- dependent (Sano and Roberts, 1963; Varon, Weinstein, Kakefuda and Roberts, 1968; DeFeudis, 1973), a characteristic of high-affinity uptake. Sodium-dependent binding of GABA also shows the same pharmacological profile as GABA uptake sites (De Feudis, 1973; Enna and Snyder, 1975). By contrast, the more recently detected Na^+-independent binding sites for [^{3}H]GABA show different characteristics (Enna and Snyder, 1975) and a pharmacological profile similar to that of postsynaptic GABA receptors, for both agonists and antagonists (Zukin, Young and Snyder, 1974; Wong and Horng, 1977; Greenlee, Van Ness and Olsen, 1978). GABA binding sites (Na^+-independent hereinafter) show apparent heterogeneity with two sites having different affinities but markedly similar pharmacological profile (Olsen, Bergman, Van Ness, Lummis, Watkins, Napias and Greenlee, 1981). These two sites have since been reported to show differences in that membrane treatment with either ammonium thiocyanate or diethylpyrocarbonate selectively abolished high-affinity [^{3}H]GABA binding, without altering low-affinity binding or GABAergic enhancement of [^{3}H]benzodiazepine binding (Browner, Ferkany and Ticku, 1981; Burch, Thyagarajan and Ticku, 1982). Conversely, sulfanilate treatment abolished low-affinity [^{3}H]GABA binding and GABAergic enhancement of [^{3}H]benzodiazepine binding (Burch et al 1982).

[^{3}H]Muscimol has also been used as a ligand for post-synaptic GABA receptors (Snodgrass, 1978; Beaumont, Chilton, Yamamura and Enna, 1978; Williams and Risley, 1979), and the sites thus labelled show many of the same characteristics as those labelled with [^{3}H]GABA. Pharmacological profile, regional and subcellular distributions of the sites labelled are similar, and, like [^{3}H]GABA binding, binding of [^{3}H]muscimol is enhanced by freezing and Triton X-100 treatment (Enna, Beaumont and Yamamura, 1978; De Feudis, 1980). [^{3}H]Muscimol also appears to bind to two sites in some reports (Beaumont et al, 1978; Williams and Risley, 1979; Jordan, Matus, Piotrowski and Wilkinson, 1982) but not others (Snodgrass, 1978).

In all the studies on Na^+-independent GABA binding sites cited here, the pharmacological profile has been of the $GABA_A$ receptor. A binding site which appears to correspond to the $GABA_B$ receptor has been described, which can be labelled by either [^{3}H]baclofen or [^{3}H]GABA (Bowery, Hill and Hudson, 1983). Interaction with this binding site shows an absolute requirement for divalent cations: Ca^{2+} is generally used and maximally enhances binding at physiological concentrations (Bowery et al, 1983). The pharmacological profile is clearly different from both postsynaptic $GABA_A$ receptors and GABA uptake sites, with only GABA, (±) and (-)baclofen being markedly active (Hill and Bowery, 1981). In the original studies, only one class of site was labelled using either [^{3}H](±)baclofen or [^{3}H]GABA (Hill and Bowery, 1981; Bowery et al, 1983) but subsequently, non-linear Scatchard plots have been obtained using [^{3}H](-)baclofen (which showed improved binding characteristics over the racemate) (Bowery, Hill and Hudson, 1985) and [^{3}H]GABA (Bowery et al, 1985; Karbon, Durman and Enna, 1983).

Radioligand binding studies using [^{3}H]GABA or [^{3}H]muscimol to label $GABA_A$ sites have been performed on PD from both female rats (Grandison and Guidotti, 1979; Fiszer de Plazas, Becu, Mitridate de Novara and Libertum, 1982; Racagni, Apud, Iuliano, Cocchi, Locatelli and Muller, 1983; Apud, Masotto, Cocchi, Locatelli, Muller and Racagni, 1984) and humans (Grandison, Cavagnini, Schmid, Invitti and Guidotti, 1982). Specific binding of [^{3}H]GABA was not detectable in male rat PD (Fiszer de Plazas, Seilicovich, Duvilanski, Gonzalez and Rettori, 1983).

In these studies on the rat, membranes were incubated in Triton X-100 before assay. This results in the conversion or loss of the low-affinity sites seen in untreated membranes to a single population of sites with high affinity (Beaumont et al, 1978), and in agreement with the results on CNS membranes, Scatchard plots of [^{3}H]GABA binding to Triton-treated PD membranes were linear (Grandison and Guidotti, 1979; Fiszer de Plazas et al, 1983). Scatchard plots of [^{3}H]muscimol binding, however, were curvilinear, with high and low affinity components (Racagni et al, 1983; Apud et al, 1984). Although some GABA-receptor mediated effects have been demonstrated in NI (Zingg et al, 1979; Taraskevich and Douglas, 1982; Demeneix et al, 1984) no binding studies have previously been performed in this region. These experiments set out to re-examine $GABA_A$ binding sites in PD, to investigate the presence of $GABA_A$ sites in NI, and $GABA_B$ sites in both areas, using both [^{3}H]GABA and [^{3}H]muscimol as ligands.

3.2. Materials and Methods

3.2.1. Materials

Aminobutyric acid, γ-[2,3-^{3}H]-([^{3}H]GABA, 82.6 Ci/mmol) and [methylene-^{3}H (N)]muscimol ([^{3}H]muscimol, 29.4 Ci/mmol) were

obtained from New England Nuclear, Dreiech, W. Germany. GABA, muscimol, and imidazoleacetic acid (IAA) were obtained from Sigma, Poole, isoguvacine was the gift of Dr. P. Krogsgaard-Larsen and (±)baclofen was the gift of Ciba Geigy, Basle, Switzerland. Other chemicals were Analar grade, BDH, Poole.

3.2.2.Membrane preparation

Male Wistar rats (150-250g) were stunned and decapitated, the pituitary gland removed, and dissected into PD and NI. Cerebellum and frontal cortex were also dissected and tissues were stored at -20°C. Membranes were prepared by homogenisation of tissue in 100 volumes of the appropriate ice-cold buffer (see below) using a high frequency homogeniser (Ystral, Dottingen, W.Germany) (setting 3 for 5 sec), and centrifugation at 48,000g for 10 minutes at 4°C. After discarding the supernatant, the membrane pellet was suspended in fresh buffer and stored at -20°C until use 1-3 days later. On the day of assay, the membranes were thawed and the washing procedure was repeated a further 4 times. Freeze/thaw treatment and repeated washing have been shown to be necessary to remove the very high levels of endogenous GABA present in brain tissue (Gardner, Klein and Grove, 1981).

3.2.3 Radioligand binding assays

Two radioligand binding assays were used:

(1)[^{3}H]GABA binding to $GABA_A$ and $GABA_B$ sites. Whole brain and pituitary region membranes were prepared in 50mM Tris HCl buffer, pH 7.4, containing 5mM $CaCl_2$. This concentration of Ca^{2+} ions has been shown to optimise binding to $GABA_B$ sites (Bowery, Hill and Hudson, 1983). [^{3}H]GABA was used as ligand at concentrations between 2 and 2,000nM for whole brain saturation analysis of

binding, and at 10nM for analysis of relative binding to PD and NI.

(2)[^{3}H]Muscimol binding to $GABA_A$ sites. CNS region and pituitary membranes were prepared in 50mM Tris HCl buffer, pH 7.1. [^{3}H]Muscimol was used as ligand at concentrations between 0.5 and 200nM for saturation analyses of binding to cerebellar and frontal cortex membranes, and up to 100nM for pituitary membranes. For estimation of the distribution of high and low affinity sites within the pituitary, concentrations of 2nM and 20nM were used.

In both cases, assays were carried out in duplicate on 200-600µg of membrane protein (in a final volume of 1 ml) in 1.5 ml polypropylene tubes (Sarstedt, W.Germany), and 100µM GABA was used to define non-specific binding. [^{3}H]GABA assays were incubated at 20°C for 15 min before being cooled on ice for 5 min, and [^{3}H]muscimol assays were incubated on ice for 60 min. Assays were terminated by centrifugation at 16,000g for 2 min at 4°C, the supernatant was aspirated and the tube and pellet washed superficially with 1.2ml of ice-cold buffer. 100µl of Protosol (New England Nuclear) was added to solubilise the pellet. After neutralisation of the Protosol with 100 µl of 1M HCl, the assay tube was placed in a scintillation counting vial and covered in 18 ml of liquid scintillation counting fluid NE265 (Nuclear Enterprises Ltd., Sighthill, Edinburgh). After thorough mixing radioactivity was counted at ≈47% efficiency in a Beckman counter. Protein determinations on aliquots of homogenates were performed as described in Section 2.4. In both cases binding was linear with protein concentration over the range used.

3.2.4. Calculation of results

Saturation analyses of binding of [^{3}H]GABA to whole brain membranes and [^{3}H]muscimol binding to brain regions and whole

pituitary membranes was analysed in two ways, both graphically after transformation of data according to Scatchard (1949), and by a computerised iterative least-squares method. Two computer programmes were used:

(i) a single-site programme to fit a straight line to data, used for analyses of [^{3}H]GABA binding to $GABA_B$ sites in whole brain,

(ii) a two-site programme that fitted a hyperbola to the data, used for [^{3}H]GABA binding to $GABA_A$ sites in whole brain and [^{3}H]muscimol binding in all regions. Both programmes generated K_D and B_{max} values for one or two binding sites as appropriate. For single-concentration analysis of binding of [^{3}H]GABA and [^{3}H]muscimol to pituitary regions, results are expressed as specific fmol bound/mg protein at that concentration.

The computer programmes were generously provided by Professor B. Ginsborg.

3.3. Results

3.3.1. [^{3}H]GABA assay characteristics

The [^{3}H]GABA binding protocol was based on that of Bowery, Hill and Hudson (1983) who developed it to optimise $GABA_B$ binding. In this study the assays were chilled on ice for 5 min before being centrifuged as this was found to increase the reproducibility of the assay without altering the amount of specific binding (Bowery et al, 1983). Both IAA and (±) baclofen caused concentration- dependent displacement of [^{3}H]GABA (10nM) binding from whole-brain membranes but neither drug caused complete displacement at 100μM (Figure 3.1.). The amount of IAA-displaceable binding at that concentration (69 ± 2%, n = 4) was equivalent to

that resistant to baclofen (66 ± 3%) and that sensitive to 40μM isoguvacine (66 ± 4%), the $GABA_A$ agonist originally used to discriminate [^{3}H]GABA binding under these conditions into $GABA_A$ and $GABA_B$ sites (Bowery et al, 1983). (Both isoguvacine and IAA show a considerable margin of selectivity for $GABA_A$ over $GABA_B$ sites (Hill and Bowery, 1981); IAA was used here because of restricted availability of isoguvacine). When high concentrations of either IAA or baclofen were included in the assay to suppress binding to one site, the other drug caused complete displacement (Figure 3.2.). Thus in the presence of 40μM IAA, (±)baclofen displaced the remaining binding with a Ki in the order of 200-300nM and caused 96 ± 1% displacement at 100μM (n = 4). Similarly, in the presence of 100μM (±)baclofen, IAA displaced the remaining binding with high potency. This demonstrates the ability of IAA and baclofen to selectively displace [^{3}H]GABA from $GABA_A$ and $GABA_B$ binding sites respectively in the protocol employed. Subsequently, binding of [^{3}H]GABA, displaceable by 100μM (±)baclofen, was defined as "$GABA_B$" and that resistant to displacement by 100μM (±)baclofen but sensitive to 100μM GABA as "$GABA_A$".

3.3.2 [^{3}H]GABA binding to brain and pituitary.

Saturation analysis of [^{3}H]GABA binding to whole brain membranes using this concentration of baclofen to discriminate binding gave the following results (Figure 3.3.): $GABA_A$ sites showed two components of differing affinity, as has been previously described for membranes prepared without Triton X-100 treatment (Olsen et al, 1981). The high affinity component had a K_D 6.9 ± 1.8nM, Bmax 169 ± 46fmol/mg protein, and the low affinity had a K_D 256 ± 35nM, Bmax 1790 ± 160fmol/mg protein (n = 4). $GABA_B$ sites

showed linear Scatchard plots, with K_D 28 ± 4nM, Bmax 419 ± 35fmol/mg protein (n = 4).

Specific binding of 10nM [^{3}H]GABA was detectable in both PD and NI, although less than in brain. In PD binding was 35% specific, (displaceable by 100μM GABA) accounting for ≃ 4,500dpm/assay, and was partly baclofen-sensitive and partly baclofen-insensitive. The concentration (as measured) of $GABA_A$ sites was 20.9 ± 1.7 fmol/mg protein, and of $GABA_B$ sites was 16.5 ± 1.8fmol/mg protein. In NI there were found to be fewer $GABA_A$ sites (5.5 ± 0.7 fmol/mg protein) and no detectable $GABA_B$ sites (baclofen-sensitive binding was 0.6 ± 1.4fmol/mg protein), n = 5 in all cases (Figure 3.4).

3.3.3 [^{3}H]Muscimol binding

The results of saturation analyses of [^{3}H]muscimol binding to frontal cortex and cerebellum membranes are shown in Figure 3.5. and Table 3.1. The affinity constants for the two components are similar to those reported by Williams and Risley (1979) who used a filtration assay. In our hands, however, the low affinity component was greatly reduced using a filtration assay (data not shown) and therefore a centrifugation method similar to that for the [^{3}H]GABA binding experiments described here was used.

The higher affinity and lower non-specific binding (approximately 50% using pituitary tissue) of [^{3}H]muscimol compared to [^{3}H]GABA enabled saturation analyses to be carried out on whole pituitary membranes. The results shown in Figure 3.6 and Table 3.1 show that the affinity constants of the two components are similar to those found in the CNS regions, but that the Bmax values are considerably lower.

The distribution of [^{3}H]muscimol binding sites within the pituitary gland was examined using ligand concentrations of 2nM and 20nM (Figure 3.7). At 2nM, approximately 70% of binding is to the high affinity site and at 20nM a similar fraction is to the low affinity site (Figure 3.6). Binding at 2nM comprised 9.5 ± 0.8 fmol/mg protein in PD and 3.0 ± 0.2fmol/mg protein in NI whilst at 20nM there was 21.3 ± 1.2fmol/mg protein in PD and 6.3 ± 0.4fmol/mg protein in NI (n = 5, PD; n = 4, NI). Thus the relative concentration of $GABA_A$ sites in PD found using [^{3}H]GABA was confirmed. The ratio of binding at 20nM to binding at 2nM was 2.2 in PD and 2.1 in NI. This suggests that the high and low affinity [^{3}H]muscimol binding sites are distributed in the same proportions between PD and NI.

3.4 Discussion

The presence of GABA binding sites in PD has been previously investigated using [^{3}H]GABA as ligand in both female rat (Grandison and Guidotti, 1979; Fiszer de Plazas et al, 1982) and human tissue (Grandison et al, 1982) although no binding sites were apparently detectable in male rats (Fiszer de Plazas et al, 1983). In all cases, linear Scatchard plots were reported, with K_D values of approximately 35nM, and Bmax values of 0.65 - 1.2pmol/mg protein. This contrasts with the results of Olsen et al (1981) who found that [^{3}H]GABA binding to frozen/thawed brain membranes without detergent treatment revealed two components with K_D values of ≃ 10nM and 300nM (similar to the values of 7nM and 250nM reported here and by others (Jordan et al, 1982). [^{3}H]Muscimol has also been shown to bind to two sites with K_D values differing by 30-fold in well-washed membranes (Beaumont et al, 1978) but after

Triton X-100 treatment, only one site, with high affinity, was found, although the Bmax was not altered. The results presented here using both [^{3}H]GABA and [^{3}H]muscimol confirm that two $GABA_A$ binding sites are present on CNS membranes, when one freeze/thaw cycle but not detergent is used during preparation and demonstrate very similar sites in the pituitary gland. Bmax values for [^{3}H]GABA and [^{3}H]muscimol binding to CNS membranes are similar to those reported in the literature, (small differences being attributable to the use of a whole tissue homogenate in these studies compared with semi-purified synaptosomal membranes by some others). However the Bmax values for [^{3}H]muscimol binding to pituitary membranes reported here are very much lower than the values reported elsewhere: the total Bmax for [^{3}H]muscimol here is 50fmol/mg protein, compared with values of 680fmol/mg protein (Fiszer de Plazas et al, 1982) and 1200fmol/mg protein (Grandison and Guidotti, 1979) using [^{3}H]GABA, although more comparable findings using [^{3}H]muscimol have also been reported (Racagni et al, 1983; Apud et al, 1984). These higher values are from experiments on PD tissue rather than whole pituitary as reported here, but the 'diluting' effect of NI tissue could not be sufficient to account for the greater than 10 fold difference. Saturation analyses of [^{3}H]GABA binding to pituitary membranes could not be performed, but the close parallels in ratios of sites for $GABA_A$ sites labelled by either [^{3}H]GABA or [^{3}H]muscimol (Figures 3.4 and 3.7) suggest that discrepancies caused by the different ligands cannot account for these large differences. This also suggests that any effect of Ca^{2+} on [^{3}H]GABA binding to $GABA_A$ sites (Corda and Guidotti, 1983) was of minor importance. A more likely

explanation for the large discrepancy is the use of Triton X-100 during membrane preparation. Although Triton X-100 has been reported to increase the K_D but not the Bmax values of [^{3}H]GABA and [^{3}H]muscimol binding to CNS tissue (possibly by removal of endogenous inhibitors such as GABA modulin (Toffano, Guidotti and Costa 1978)), it is possible that some experimental protocols may not allow detection of a significant proportion of the lower affinity sites until their affinity is enhanced by detergent treatment.

The results presented here demonstrate $GABA_A$ binding sites in NI and Figures 3.4 and 3.7 show that both [^{3}H]GABA and [^{3}H]muscimol bind specifically to NI membranes. Both [^{3}H]GABA and [^{3}H]muscimol label fewer sites in NI compared to PD (ratio PD:NI of 3.8 using [^{3}H]GABA, 3.2 and 3.4 using 2nM and 20nM [^{3}H]muscimol respectively). The close agreement in the ratios for the two ligands suggests that both are binding to the same sites.

A population of $GABA_B$ sites was detectable in PD, but not NI. Functional effects of these sites on PD hormone secretion are discussed in Chapters 5 and 7. In the present studies, [^{3}H]GABA binding to whole brain membranes (displaceable by 100μM baclofen) appeared to be a single component, with K_D28nM. No consistent curvature was observed even at the highest comcentrations used, in agreement with the observations by Bowery et al (1983), using both [^{3}H]GABA and [^{3}H](±)baclofen. Karbon et al (1983) have reported curvilinear Scatchard plots using [^{3}H]GABA to label $GABA_B$ sites in rat frontal cortex and five areas of cow brain, and suggested that the two populations were distinct, as only the low affinity sites were reduced after lesion of the dorsal noradrenergic bundle

in the rat. The sites measured in these studies may correspond to the high-affinity sites of Karbon et al (1983) which may explain the apparent deficit of $GABA_B$ binding sites in NI despite postsynaptic electrophysiological and biochemical effects of baclofen in that area (Demeneix et al, 1984; Taraskevich and Douglas 1985), if the two populations are anatomically and functionally distinct. Bowery et al (1985) have also reported curved Scatchard plots, using both [^{3}H](-)baclofen and [^{3}H]GABA and proposed that the inability to measure the low affinity component in earlier experiments (Hill and Bowery, 1981; Bowery et al, 1983) was caused by using the radioligand at too high a concentration. If the concentration of radioligand was kept low, and increasing concentrations of unlabelled ligand were used, the low affinity component could be detected. The technical difficulties of measuring a site of such low affinity ($K_D \simeq 1.2\mu M$) are very great and considerable further work is required to clarify this point, particularly in view of possible multiple mechanisms of action of baclofen.

[^{3}H]GABA binding in Na^+-free buffers (to eliminate binding to membrane uptake) meets many criteria of receptor identification (Zukin et al, 1974; Enna and Snyder, 1975; Greenlee et al, 1978). The studies here are in agreement with the view that both [^{3}H]GABA and [^{3}H]muscimol can be used to label GABA receptors (De Feudis, 1980; Jordan et al, 1982). Table 3.1 shows the results of saturation analysis of [^{3}H]muscimol binding to membranes from frontal cortex, cerebellum, and whole pituitary. The affinity constants for each of the two sites are similar in the three areas examined, and are similar to those reported by others (Beaumont et al, 1978; Williams and Risley, 1979). However in both the pituitary

and the frontal cortex it can be seen that the B_{max} of the high affinity components is much less than that of the low affinity component (a ratio of approximately 0.3) whereas in the cerebellum the B_{max} values are approximately equal. The preponderance of low affinity sites in frontal cortex has been previously reported using [^{3}H]GABA (Olsen et al, 1981; Burch et al, 1983) and it has been suggested that it is the low-affinity site which is linked to the benzodiazepine receptor (Browner et al, 1981; Burch et al, 1983). The significance of some regions containing a preponderance of low-affinity, possibly benzodiazepine-linked sites, if in fact the two populations are discrete (Olsen et al, 1981), is unknown. A significant point is that GABA binding sites are predominantly localised in PD, whereas the results in Chapter 4 show that central type benzodiazepine binding sites are approximately 4-fold more concentrated in NI than in PD. Thus it seems most unlikely that GABA and benzodiazepine binding sites are ubiquitously linked in a one-to-one ratio as has been suggested by some workers from receptor solubilisation experiments (Martini, Ricagni and Lucacchini, 1983).

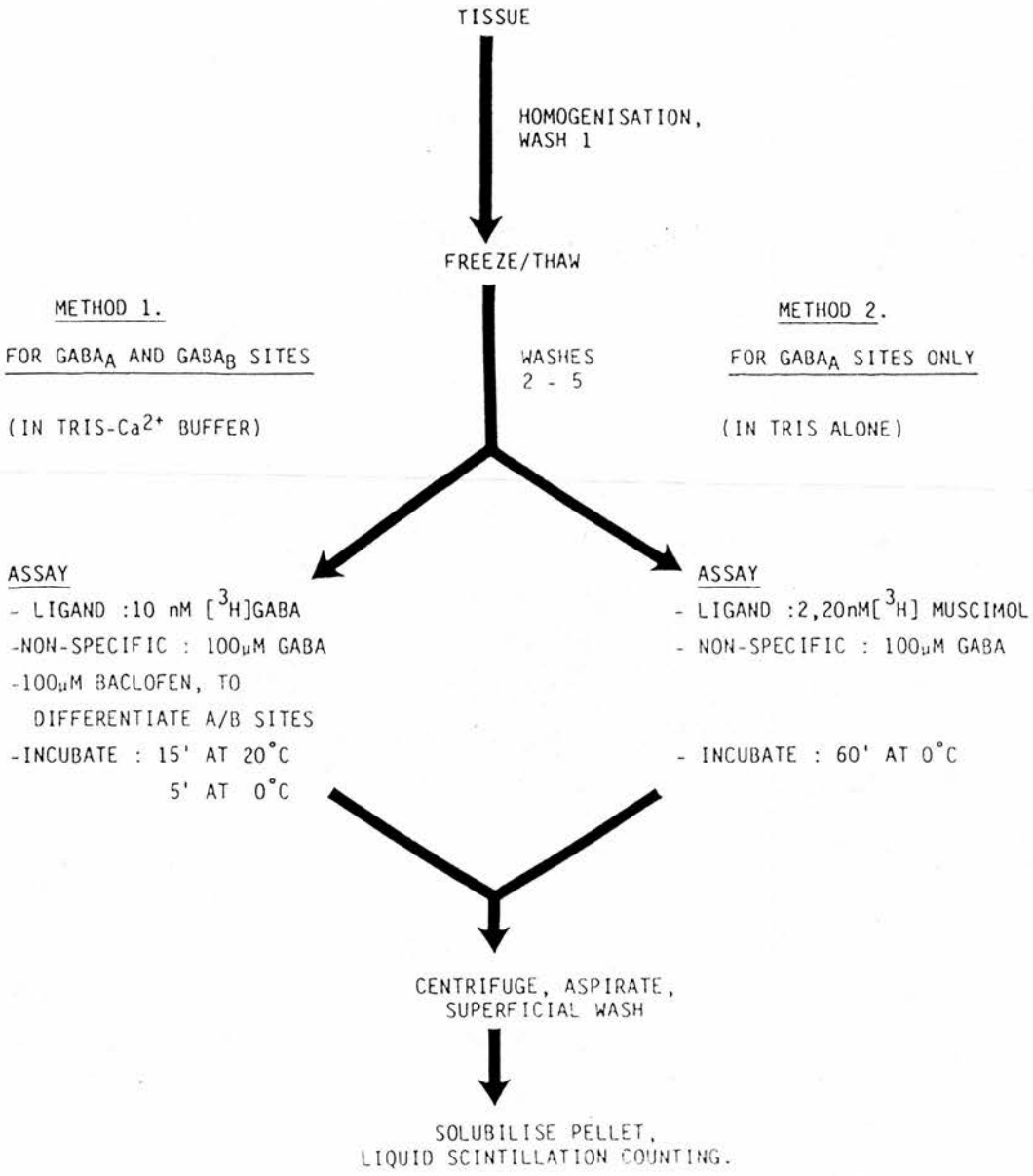


FIGURE 3.1

Outline of methods used to characterise and quantify GABA binding sites in the pituitary gland.

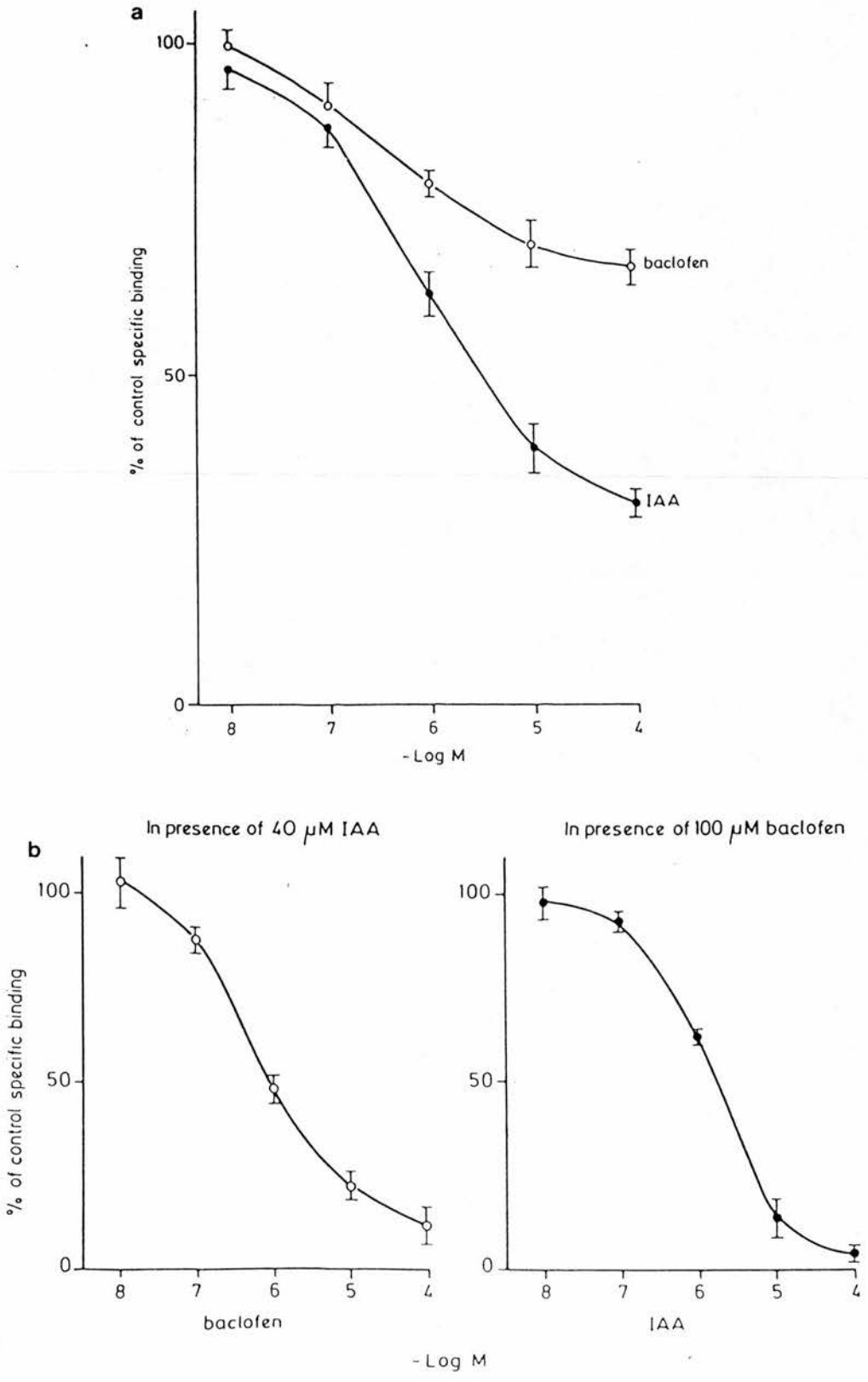


FIGURE 3.2

Displacement of [^{3}H]GABA binding to whole brain membranes in Ca^{2+}-containing buffer.
(a) Displacement by IAA (●) or baclofen (○) separately.
(b) Displacement by baclofen in the presence of 40μM IAA (○) and by IAA in the presence of 100μM baclofen (●).
Non-specific binding defined by 100μM GABA in all cases. Mean ± S.E.M., n = 4-5.

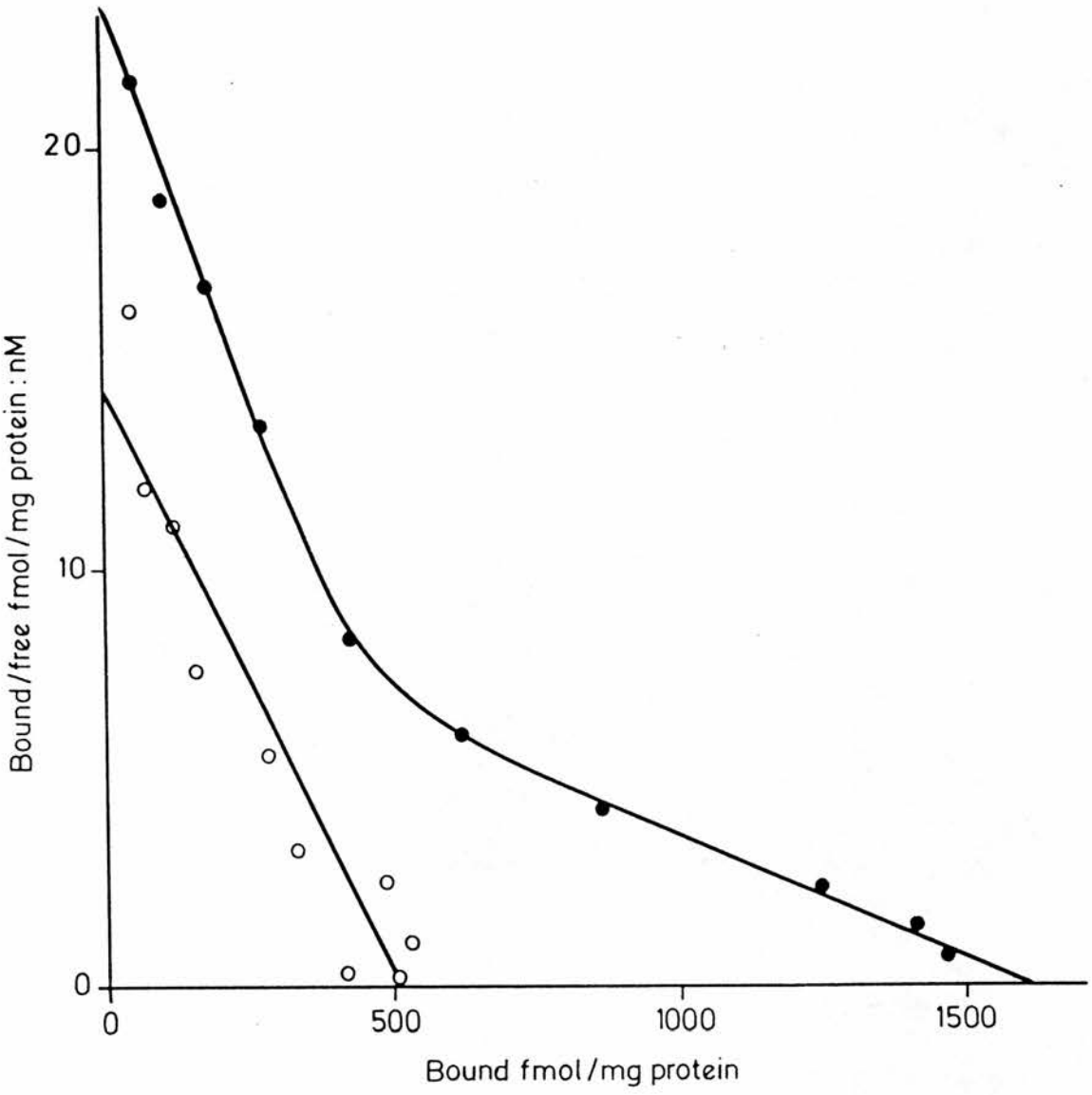


FIGURE 3.3

Scatchard plot of [^{3}H]GABA binding to $GABA_A$ and $GABA_B$ binding sites in rat whole brain
$GABA_A$ sites (●): binding not displaced by 100μM baclofen but displaced by 100μM GABA.
$GABA_B$ sites (○): binding displaced by 100μM baclofen.
Representative experiment, performed in duplicate.

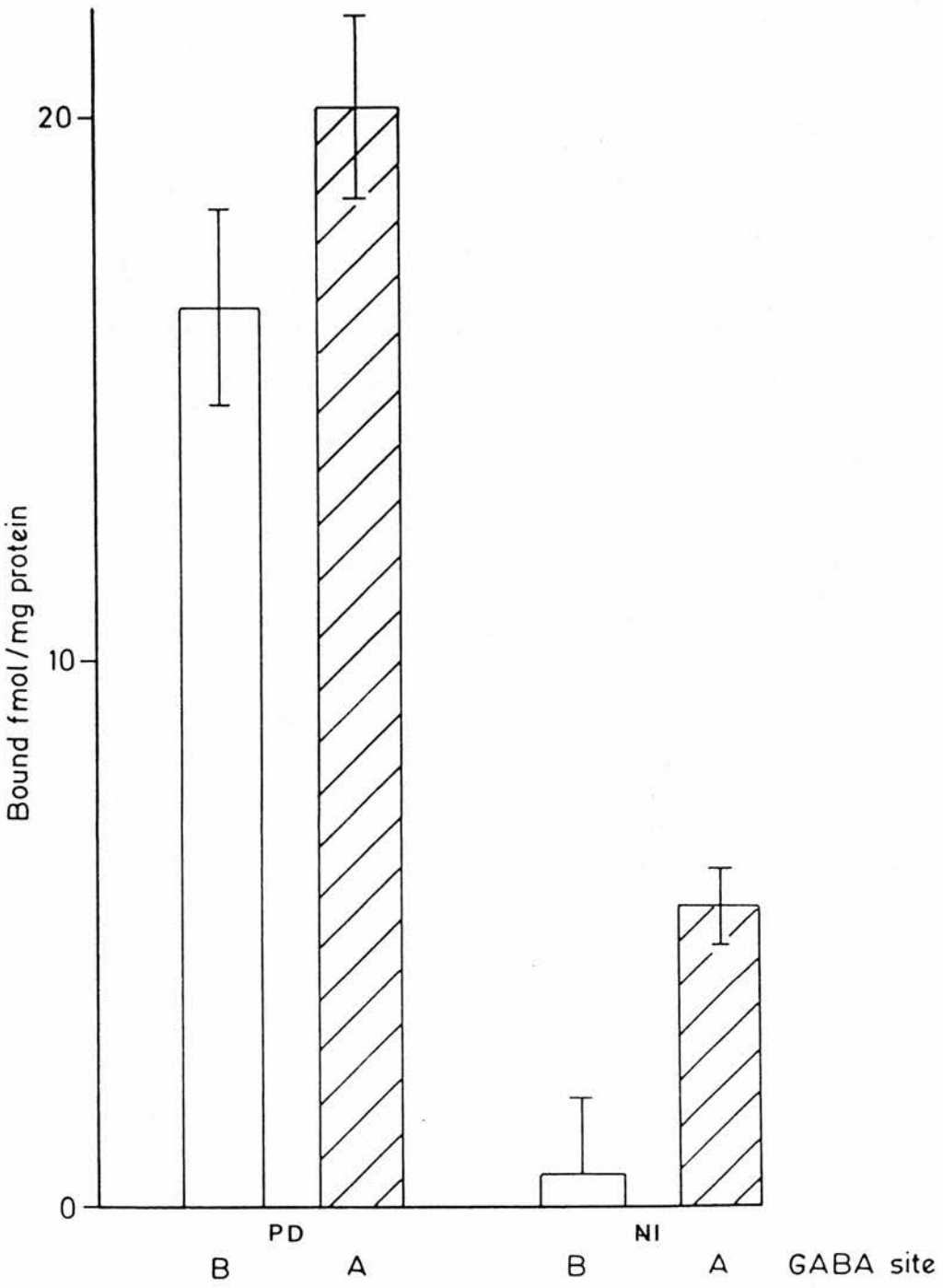
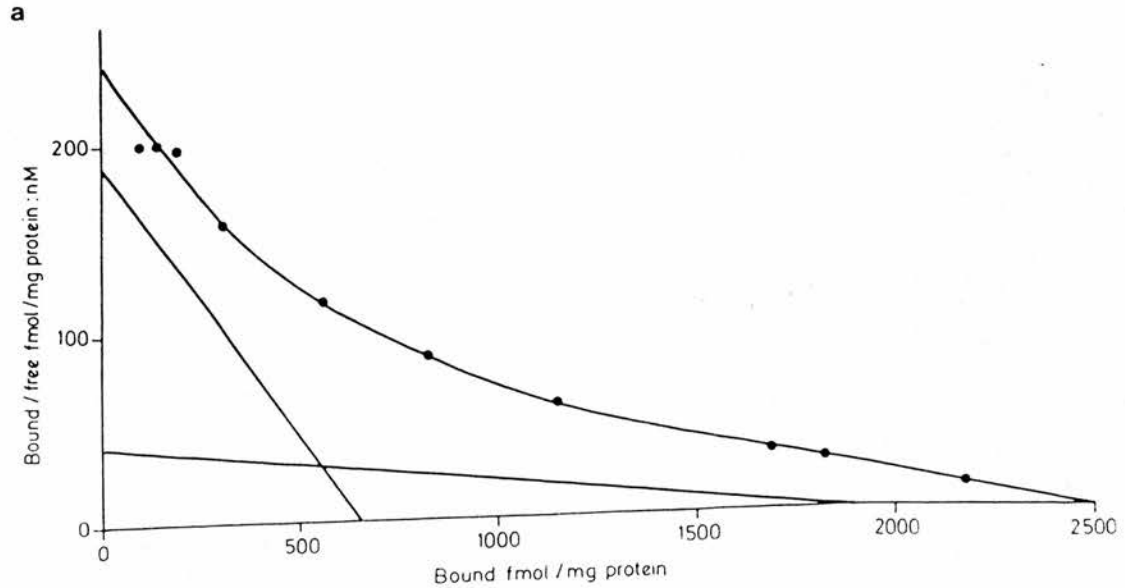


FIGURE 3.4

[^{3}H]GABA binding to $GABA_A$ and $GABA_B$ sites in PD and NI. [^{3}H]GABA concentration was 10nM. $GABA_A$ (hatched columns) and $GABA_B$ (open columns) binding sites were discriminated using 100μM baclofen. Mean ± S.E.M., n = 5.



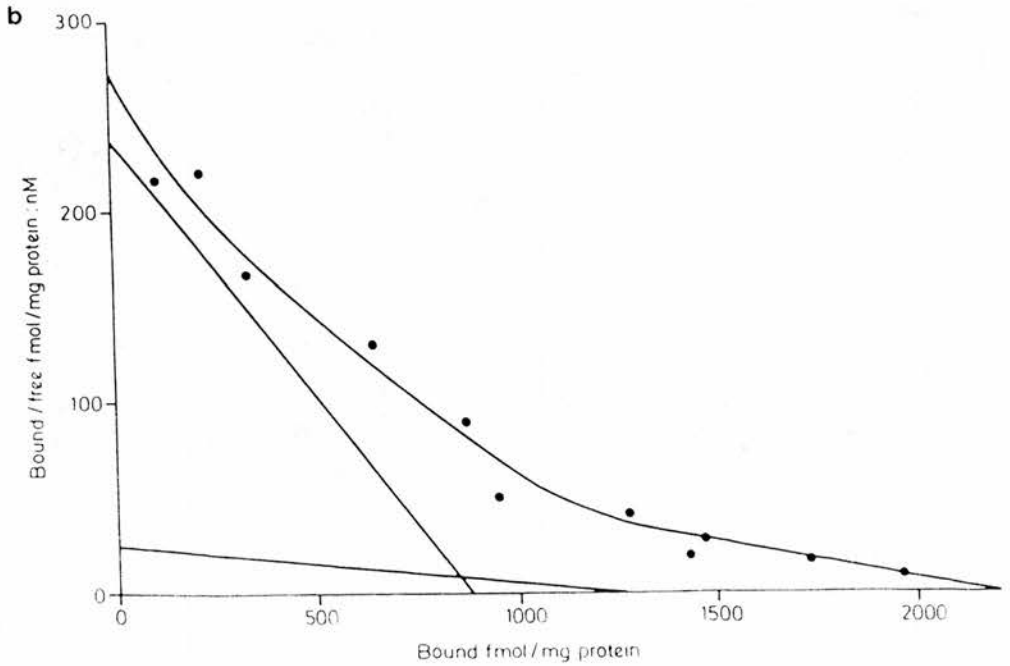


FIGURE 3.5

(a) Scatchard plot of [^{3}H]muscimol binding to Frontal Cortex membranes.

(b) Scatchard plot of [^{3}H]muscimol binding to Cerebellum membranes. Representative experiments. The straight lines represent the high- and low-affinity components of binding drawn independently. These data were derived from computerised least-squares fitting of the original saturation data.

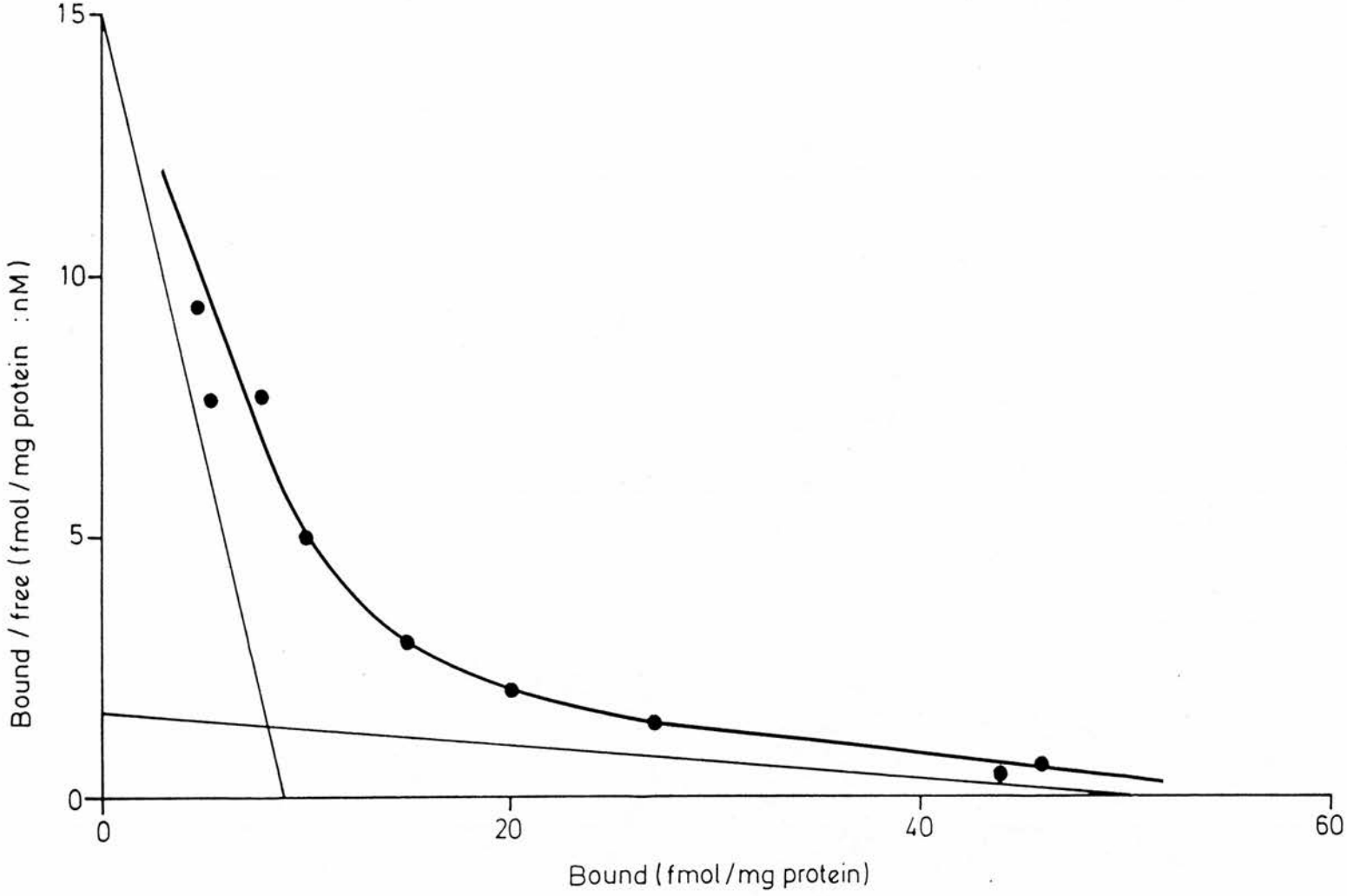


FIGURE 3.6

Scatchard plot of [^{3}H]muscimol binding to whole pituitary membranes. Individual components of binding derived as in Figure 3.5. Representative experiment.

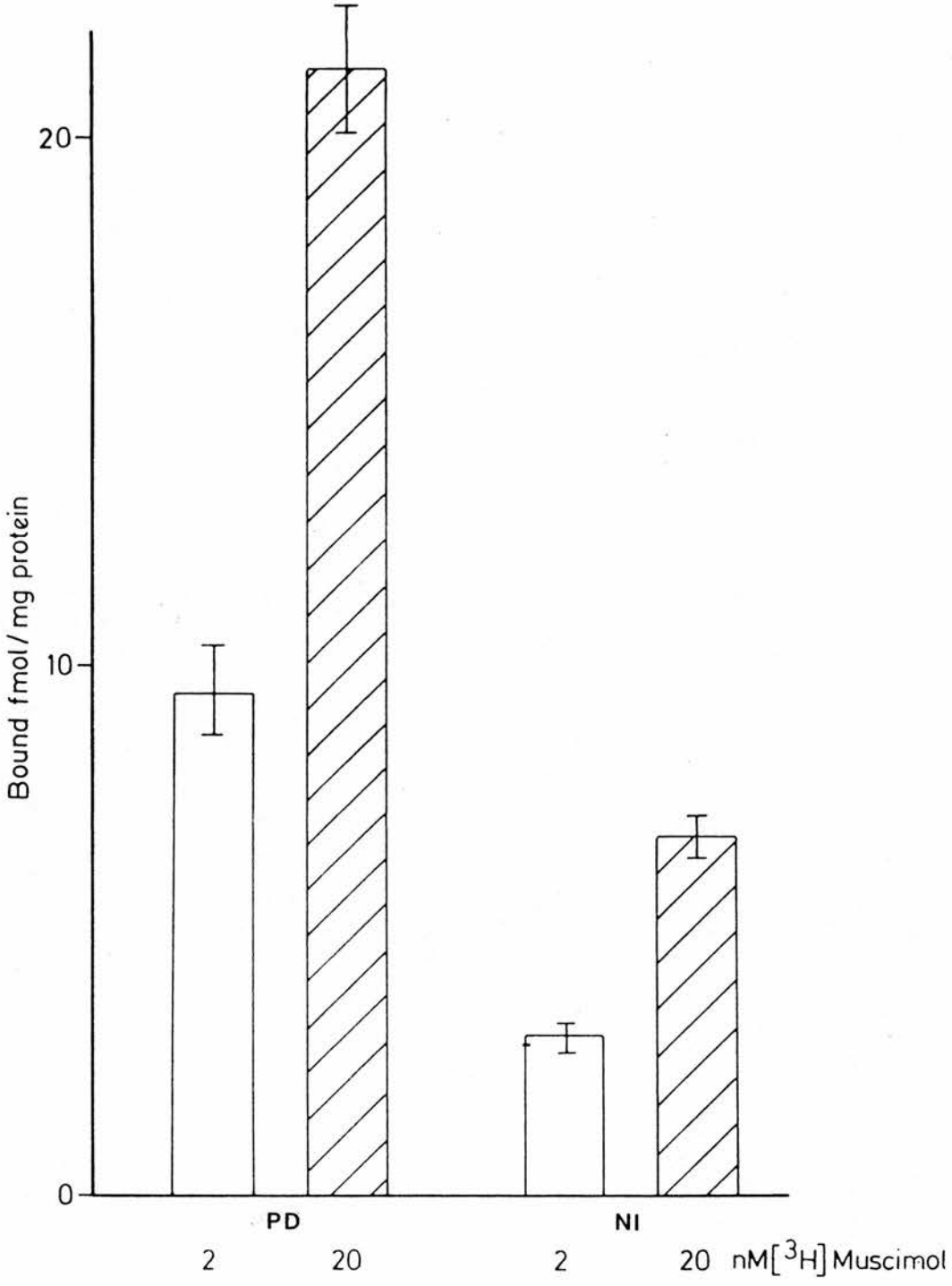


FIGURE 3.7

Binding of [^{3}H]muscimol to PD and NI membranes.
Open columns: binding at 2nM [^{3}H]muscimol.
Hatched columns: binding at 20nM [^{3}H]muscimol.
Mean ± S.E.M., n = 5, PD; n = 4, NI.

Table 3.1

	K_{D1} (nM)	B_{max1} (fmol/mg protein)	K_{D2}	B_{max2}
Pituitary	1.1 ± 0.1	9.4 ± 0.4	34.7 ± 3.0	46.8 ± 6.7
Frontal cortex	3.8 ± 0.2	608 ± 14	46.6 ± 4.2	2045 ± 69
Cerebellum	4.2 ± 0.2	1003 ± 45	44.7 ± 1.9	1051 ± 75

n = 3–4 Mean ± S.E.M.

Results of Scatchard analysis of [^{3}H]muscimol binding to membranes from whole pituitary, frontal cortex and cerebellum. K_D and B_{max} values were derived from computerised least-squares analysis of Saturation data.

CHAPTER 4

Analysis of benzodiazepine binding sites in the pituitary gland

4.1 Introduction

High-affinity binding sites for radiolabelled benzodiazepines were originally demonstrated by Squires and Braestrup (1977) and Mohler and Okada (1977a) on rat brain membranes, and the affinities of many benzodiazepines correlated well with their behavioural potency (Mohler and Okada, 1977a). Similar binding sites have been demonstrated in human brain (Braestrup, Albrechtsen and Squires, 1977; Young and Kuhar, 1979) and the affinities of many benzodiazepines at these sites correlate with human daily dosage. [^{3}H]diazepam and [^{3}H]flunitrazepam (FNM) also bind with high affinity to peripheral tissues, notably kidney (Braestrup and Squires, 1977; Regan, Yamamura, Yamada and Roeski, 1981), but there are great differences in pharmacological profile between these peripheral-type sites and the central-type sites, in particular the selectivity of clonazepam for central-type sites and the behaviourally-inactive benzodiazepine Ro5-4864 for peripheral-type sites (Gallagher, Mallorga, Oertel, Henneberry and Tallman, 1981; Regan et al, 1981). [^{3}H]Ro5-4864 has been used to label these peripheral-type sites in kidney and in brain (Marangos, Patel,Boulenger and Clark-Rosenburg, 1982; Schoemaker, Boles, Horst and Yamamura, 1983) and they can also be labelled by the non-benzodiazepine [^{3}H]PK11195 (Benavides, Quarteronet, Imbault, Malgouris, Uzan, Renault, Dubroeucq, Guerency and LeFur, 1983).

While evidence is accumulating for GABAergic control over the pituitary, it remains an open question whether GABA receptors there are modulated by benzodiazepine-recognising components. A close interaction between GABA receptors and sites recognising benzodiazepines is shown by allosteric effects on equilibrium

binding:GABA has been reported to enhance the binding of tritiated benzodiazepines to the central type site by an increase in affinity (Martin and Candy, 1978; Tallman, Thomas and Gallagher, 1978) but does not affect the peripheral type site (Marangos et al, 1982).

Further to the division of benzodiazepine binding sites into 'central-' and 'peripheral-' types, it has recently been suggested that the central type sites may not be homogenous, on the basis of their interactions with certain non-benzodiazepine compounds. These compounds, including triazolopyridazines such as CL 218872 (Klepner, Lippa, Benson, Sano and Beer, 1979) and esters of β carboline-3-carboxylate (Nielsen and Braestrup, 1980; Braestrup and Nielsen, 1981) show regional variations in affinity consistent with populations of two distinct subtypes of receptor, BZ_1 and BZ_2 (Braestrup and Nielsen, 1981) or type I and type II (Young, Neihoff, Kuhar, Beer and Lippa, 1981) being differentially distributed within the CNS. These differences can be detected by '<u>in vivo</u>' binding studies as well as <u>in vitro</u> (Minchin and Nutt, 1983).

Benzodiazepines however appear to have very similar affinities for the two putative subtypes. Kinetic analysis of the association and dissociation of [^{3}H]FNM have led to suggestions that benzodiazepine binding sites have different functional states with co-operative interactions rather than their being distinct molecular entities (Chiu, Dryden and Rosenburgh, 1982; Doble, Iversen and Martin, 1982; Quast and Mahlmann, 1982). Ethyl β-carboline-3-carboxylate (ECC)-induced dissociation of [^{3}H]FNM shows different kinetics to when dissociation is initiated by a benzodiazepine (Martin and Doble, 1983), and these data suggest that the binding mechanism for ECC (and other non-benzodiazepines) may be different

from that of benzodiazepines. This is supported by experiments involving photoaffinity labelling of benzodiazepine sites with [^{3}H]FNM (Hirsch, 1982; Brown and Martin, 1983). Nevertheless [^{3}H]FNM has been reported to bind to two proteins in hippocampus but only one in cerebellum (Sieghart and Karobath, 1980), and type I and type II sites appear to be differentially distributed within the substantia nigra and amygdala (Lo, Niehoff, Kuhar and Snyder, 1983; Niehoff and Kuhar, 1983) and change differentially after neurotoxin administration (Medina, Novas and De Robertis, 1983). It remains clear that benzodiazepine binding sites exhibit different properties in different regions and the possibility that these 'subtypes' may have distinctly different roles in vivo points to the importance of characterising the receptor subtypes present in the pituitary. Experiments were therefore carried out using [^{3}H]propyl-β-carboline as a selective ligand for BZ_1 type sites (Braestrup and Nielsen, 1981).

There is increasing evidence that the benzodiazepine class of drugs may exert neuroendocrine effects in addition to their well known profile of central actions as anxiolytics, hypnotics, anti-convulsants and muscle relaxants. For example, benzodiazepines have been reported to modify the plasma concentration of prolactin in the rat. A number of benzodiazepines have been shown to lower basal concentrations of circulating prolactin in male rats, and attenuate pharmacologically-induced increases (Grandison, 1980, 1981a; Lotz, 1982; Sibilia, Netti, Guidobono, Pagani and Pecile, 1985). The specific benzodiazepine antagonist, Ro15-1788 is reported to effectively block several of these effects (Lotz, 1982). Furthermore, diazepam markedly attenuated stress-induced prolactin release and the prolactin surge on the afternoon of

proestrus in female rats (Grandison, 1982). Diazepam also causes a prominent stimulation of GH secretion in man (Koulu, Lammintausta, Kangas and Dahlstrom, 1979).

The possibility that benzodiazepines might be acting directly on the anterior pituitary gland was raised by the demonstration of specific binding sites for [^{3}H]diazepam on rat pituitary cells in culture (Grandison, 1981b). However, [^{3}H]diazepam could not be displaced from these cells by clonazepam, but could be by Ro5-4864. Nevertheless, preincubation of these cells for 24 hours with diazepam, although at a very high concentration, potentiated the inhibition of prolactin release caused by receptor agonists for the putative prolactin release-inhibiting factors dopamine and GABA (Grandison, 1981b). Similar results have been reported by Schettini, Cronin, O'Dell and MacLeod (1984) and are discussed further in Chapter 6.

These studies therefore addressed the following questions.

i) Could any specific binding of [^{3}H]FNM to pituitary membranes be detected?

ii) Did this specific binding show a central or benzodiazepine peripheral type pharmacology profile, or was it a mixed population?

iii) What was the distribution of the two types of site within the pituitary?

iv) Was there any evidence for interaction between binding sites and GABA receptors?

v) Did the central-type sites appear to be of the BZ_1 or BZ_2 subtype, or a mixture?

4.2 Materials and Methods.

4.2.1. Materials

[Methyl-^{3}H]flunitrazepam ([^{3}H]FNM), specific activity 70-80 Ci/mmol and [propyl-2, 3-^{3}H]β-carboline-3-carboxylate ([^{3}H]PCC), specific activity 95 Ci/mmol were obtained from New England Nuclear. Laboratory chemicals (Analar) were obtained from BDH, and GABA from Sigma. Diazepam, clonazepam and Ro5-4864 were the gift of Hoffmann-La Roche, and PCC of Dr. I.L. Martin.

4.2.2. Membrane preparation

Male Wistar rats (150-250g) were stunned and decapitated, the pituitary gland removed, and dissected into PD and NI. Other CNS and peripheral tissues were also dissected, and all were stored at -20°C. Membranes were prepared by homogenisation of tissue in 100 volumes of ice-cold 25mM KH_2PO_4 buffer (pH 7.1) using an Ystral high frequency homogeniser (setting 3 for 5 seconds). Homogenates were centrifuged at 48,000g for 10 minutes at 4°C. After discarding the supernatant, the membrane pellet was resuspended in fresh buffer before centrifugation. The washing procedure was repeated a further three times to remove endogenous GABA (Gardner et al, 1981).

The final resuspension of membrane preparations gave concentrations of 200-600µg protein/ml for pituitary tissue (linearity over this range is shown in Figure 4.1) and 400-600µg protein/ml for CNS and kidney tissues. Aliquots of 500µl (≈ (area)) were used for radioligand binding assays. Protein determinations on aliquots of homogenate were performed as described in Section 2.4.

4.2.3. Radioligand binding assays.

The main series of experiments were carried out using [^{3}H]FNM as ligand at a concentration of 0.5nM or 1nM (see legends for

details) in a final assay volume of 2ml. For saturation analyses ligand concentrations between 0.1nM and 10nM were used for cerebellar and hippocampal membranes and up to 200nM for pituitary membranes. In experiments to analyse central benzodiazepine receptor subtypes [^{3}H]PCC was used as ligand at concentrations between 0.05nM and 5nM for saturation analyses.

Unlabelled benzodiazepines and PCC were dissolved in dimethylformamide which was present in the assay at concentrations between 0.0004% and 0.02% v/v and had no effect on specific binding in this range. Nonspecific binding was defined by 10μM diazepam or 200nM clonazepam in parallel incubations.

Assays in duplicate were incubated on ice for 90-180 minutes in the dark before being rapidly filtered through Whatman GF/B filters under vacuum and washed with 3 x 5ml ice-cold buffer via the incubation tube. Filters were transferred to scintillation vials and 9ml of scintillation fluid was added. Radioactivity was counted at ≃ 47% efficiency in a Beckman liquid scintillation counter.

To investigate the effects of GABA on [^{3}H]FNM binding to pituitary membranes, tissue samples were homogenised and washed in 50mM Tris Citrate buffer, pH 7.1, final resuspension being in phosphate buffer.

4.2.4. Radioligand purity and adsorption experiments

Radiochemical purity was tested by thin layer chromatography on silica gel plates (Whatman LK5D) using solvent systems of chloroform:acetone (9:1) for [^{3}H]FNM and ethyl acetate:methanol: ammonium hydroxide (5:1:1) for [^{3}H]PCC according to manufacturer's instructions. More than 97% of radioactivity in [^{3}H]FNM samples and more than 99% in [^{3}H]PCC samples co-migrated with the authentic compound (n = 5 in each case).

Account was also taken of adsorption phenomena reducing the free ligand/displacer concentration. Adsorption was assessed across the ligand concentration ranges used by experiments in which assay mixtures were carried through the standard preparation and incubation procedures before centrifugation for 3 minutes at 16,000g and 2°C. Tritium was counted in aliquots of supernatant. Under the conditions described (using 600µg protein/ml membrane preparation) whilst true free [^{3}H]FNM concentration represented 71-90% of added ligand through the range 0.1nM to 200nM [^{3}H]FNM added, the proportion of added [^{3}H]PCC remaining free varied from 82% at 50nM to only 30% at 0.05nM. Adsorption of both ligands was linear with protein concentration and appropriate corrections were made to the true free ligand concentrations in all calculations. The marked adsorption out of solution of some ligands such as PCC means that such phenomena must be taken into account to avoid artefactual distortion of kinetic binding data.

4.2.5. Calculation of results

Displacement data was also analysed as Hill and Hofstee plots. For Hill plots, data is plotted as

$$\log \frac{\%\ \text{displacement}}{100-\%\ \text{displacement}}$$

against log concentrations of displacing drug. Points were fitted by linear regression using a Texas programmable calculator. Hill coefficients were calculated as the inverse of the gradient of the line thus generated.

For Hofstee plots, data is plotted as:

$$\frac{\%\ \text{displacement}}{\text{concentration of displacing drug}}$$ against % displacement.

Linear Hofstee plots were fitted by linear regression and best lines for curved Hofstee plots were estimated by eye.

Saturation experiments were analysed after transformation of the data according to Scatchard (1949). Obviously linear Scatchard plots were fitted by linear regression, with the intercept on the abscissa giving the B_{max} value, and -1/gradient giving the K_D value. A curved Scatchard plot was obtained for diazepam-displaceable binding of [^{3}H]FNM to whole pituitary membranes (Figure 4.5). An estimate of the best line through the experimental points was fitted by eye. No numerical data were derived from this.

4.3 RESULTS

4.3.1. Discrimination of central and peripheral type benzodiazepine binding sites.

Experiments to establish suitable conditions for discrimination of [^{3}H]FNM binding to central- and peripheral-type sites were carried out using membranes from whole brain and kidney. Displacement of [^{3}H]FNM from both brain and kidney membranes by clonazepam and Ro5-4864 was concentration-dependent, showing sigmoid curves (Figure 4.2). Hill plots gave coefficients close to unity and Hofstee plots were linear, results entirely consistent with a bimolecular interaction at a single population of binding sites in both areas. In brain and kidney respectively, IC_{50}s of 0.86nM and 5,400nM for clonazepam and 80,000nM and 2.0nM for Ro5-4864 were obtained, demonstrating the specificity of clonazepam for the central-type site and of Ro5-4864 for the peripheral-type site. The curves showed that clonazepam at a concentration of 200nM caused approximately 100% displacement of [^{3}H]FNM from brain and negligible displacement from kidney membranes. This concentration was therefore used subsequently to define central-type binding.

4.3.2. Central- and peripheral-type benzodiazepine binding sites in the pituitary gland.

Specific binding of 1nM [^{3}H]FNM, defined by 10μM diazepam, was detectable in both areas of the pituitary : 28.3 ± 5.0fmol/mg protein in PD and 101 ± 14fmol/mg protein in NI (n = 5). Clonazepam and Ro5-4864 produced extended displacement curves with distinct plateau regions in both areas (Figures 4.3 and 4.4). Hofstee plots showed pronounced curvature and Hill coefficients were much less than unity (e.g. for clonazepam:0.29 in PD and 0.42 in NI). Figures 4.3 and 4.4 show that under the standard assay conditions used, central-type binding (i.e. that displaced by 200nM clonazepam) accounted for 27% of total specific binding in PD (7.6fmol/mg protein) and 34% in NI (34.2fmol/mg protein), a ratio PD:NI of 1:4.5. Peripheral-type (clonazepam insensitive) binding accounted for 20.7fmol/mg protein in PD and 66.4fmol/mg protein in NI, a ratio of 1:3.2. Under these conditions, specific binding to pituitary regions accounted for 800-2,000 dpm/assay representing approximately 60% of total binding, with a duplicate reproducibility in the order of 7%.

Scatchard plots of [^{3}H]FNM (0.1-200nM) binding to whole pituitary membranes was biphasic when 10μM diazepam was used to define non-specific binding (Figure 4.5), consistent with the presence of two sites. Using 200nM clonazepam to discriminate central and peripheral sites, binding was resolved into two linear components: a small population of high affinity clonazepam-sensitive sites and a larger population of lower affinity sites resistant to clonazepam. The high affinity site had a K_D of 1.9 ± 0.3nM, B_{max} 32.8 ± 2.6fmol/mg protein (n = 8), and the lower-affinity

site had a K_D of 34.7 ± 4.9nM, B_{max} 660 ± 16fmol/mg protein (n = 4).

The addition of 100μM GABA increased the specific binding of 1nM [^{3}H]FNM to whole pituitary membranes, washed in Tris citrate buffer, by 24% from 23.8 ± 1.1 to 29.6 ± 1.5fmol/mg protein (P <0.02, paired t test, Figure 4.6). Central-type binding (clonazepam-sensitive) was increased by 79% from 8.4 ± 1.1 to 15.0 ± 1.3fmol/mg protein (P < 0.05) but peripheral-type binding was not significantly altered (17.0 ± 1.3 to 14.7 ± 0.5fmol/mg protein, n = 4).

4.3.3. Subtypes of central benzodiazepine binding sites in pituitary and brain regions.

[^{3}H]PCC has been reported to be a high affinity specific ligand for the BZ_1 subtype of central benzodiazepine binding site and this ligand shows negligible binding to peripheral-type benzodiazepine sites (Mitchell and Wilson, 1984). [^{3}H]PCC bound to pituitary membranes in a specific, saturable manner throughout the concentration range 0.05-5nM. Scatchard plots of [^{3}H]PCC binding to whole pituitary membranes were linear (Figure 4.7) (using 200nM clonazepam to define non-specific binding), with a K_D of 0.33 ± 0.05nM, B_{max} 36.7 ± 4.8fmol/mg protein (n = 7). The B_{max} of [^{3}H]PCC binding sites was not significantly different from that of clonazepam-sensitive [^{3}H]FNM binding. The results of control experiments on hippocampal and cerebellar membranes are shown in Figure 4.8 and Table 4.1.

The displacement of bound [^{3}H]FNM (0.5nM) by unlabelled PCC also differed between cerebellum and hippocampus. In the cerebellum, PCC gave an IC_{50} of 1.3nM and clearly linear Hofstee

plots, but in the hippocampus, the apparent affinity of PCC was less, with an IC_{50} of 8.7nM and distinct curvature in Hofstee plots (Figure 4.9a, b). Hill coefficients of this displacement data were 0.96 and 0.71 in cerebellum and hippocampus respectively. When similar experiments were carried out using pituitary membranes, PCC showed a similarly high affinity to that in cerebellum (Figure 4.9a, IC_{50} of 1.1 ± 0.2nM, n = 4), giving linear Hofstee plots with no indication of curvature (Figure 4.9b) and Hill coefficient of 0.9 ± 0.1.

4.4. Discussion

The experiments on displacement of [^{3}H]FNM by clonazepam and Ro5-4864 in the brain and kidney (Figure 4.2) demonstrated the specificity of these two drugs and revealed the conditions necessary for the two binding sites to be differentiated in a mixed population. Similar experiments using pituitary membranes gave very different results from the single component Law of Mass Action displacement seen in the prototype areas. Extended displacement curves and non-linear Hofstee plots in both PD and NI suggested the presence of two distinct types of binding site (Figures 4.3 and 4.4). This was supported by the clearly non-linear Scatchard plot when diazepam is used to define non-specific binding (Figure 4.5). From Figure 4.2 it can be seen that 200nM clonazepam causes 100% displacement from brain and no significant displacement from kidney. The use of 200nM clonazepam to separate specific binding into clonazepam-sensitive (central) and -insensitive (peripheral) sites in the pituitary revealed populations of each type (Figure 4.5) with individually linear Scatchard plots. The clonazepam-sensitive sites showed higher affinity for [^{3}H]FNM

(1.9 ± 0.3nM) than the clonazepam- insensitive sites (34.7 ± 4.9nM). The affinity of [^{3}H]FNM for the clonazepam-sensitive site is similar to that found in CNS regions in this study (Table 4.1), consistent with this pituitary site representing a central benzodiazepine receptor. The affinity of [^{3}H]FNM for the clonazepam-insensitive sites is similar to that reported at the peripheral benzodiazepine receptor (Regan et al, 1981), being many fold lower than the affinity for central sites.

Further support for the idea of co-identity of clonazepam-sensitive sites here with central benzodiazepine receptors was provided by experiments to investigate the enhancement of tritiated benzodiazepine binding by GABA. Figure 4.6 shows that 100μM GABA significantly enhanced the binding of [^{3}H]FNM to well-washed pituitary membranes. Preparation in buffers containing divalent cation chetalors (e.g. citrate) has been reported to facilitate observation of GABA enhancement effects, probably by more efficient removal of endogenous GABA (Squires and Saederup, 1982). The use of 200nM clonazepam to define central-type binding sites showed that the increase in [^{3}H]FNM binding is limited to those sites. This is in accordance with previous reports that in contrast to CNS tissue GABA has no effect on [^{3}H]FNM binding to kidney membranes (Regan et al, 1981) nor on [^{3}H]Ro5-4864 binding (Marangos et al, 1982).

The pituitary population of central-type binding sites is clearly present in much lower concentrations than in CNS regions, with for example even NI having only 13% of the number of sites found in cerebellum but is similar to the number of GABA binding sites described in Chapter 3. Nevertheless, the similar

pharmacological properties of the sites and their interaction with GABA in these experiments strongly suggest that they may have a functional role in regulating local GABA receptors. The relatively small overall population might be explained in terms of a distribution restricted to certain cell types only, which individually could possess reasonably dense receptor populations. The experiments described in Chapters 6 and 7 demonstrate the presence of benzodiazepine receptors on lactotrophes and somatotrophes. These cell types make up approximately 60% of PD (Frawley, Bockford and Hoeffler, 1985), suggesting that these nonsynaptic receptors may be much less densely packed on these cell membranes than are the synaptic receptors e.g. in cerebellum.

Benzodiazepine binding sites in the CNS display different properties in different regions. In areas such as hippocampus and frontal cortex, but not cerebellum, the binding sites exhibit kinetics consistent with either a heterogeneity of sites or variable co-operative interactions between them (Squires, Benson, Braestrup et al. 1979; Braestrup and Nielsen, 1981; Ehlert, Roeske and Yamamura, 1981; Stapleton Prestwick and Horton, 1982; Chiu et al 1982.)

Furthermore, receptor solubilisation studies have shown multiplicity of [^{3}H]FNM photoaffinity-labelled proteins in some areas, including hippocampus and frontal cortex, but not cerebellum (Sieghart and Karobath, 1980; Sigel, Stephenson, Mamalaki and Barnard 1983; Asano, Yamada and Ogasawara, 1983). While evidence at present may not be sufficient to define unequivocally the existence of several distinct benzodiazepine receptor molecules (Martin, Brown and Doble, 1983) it appears that several sites can be defined in terms of their regionally different pharmacological properties (Squires et al, 1979; Braestrup and Nielsen, 1981). PCC binds with

high affinity to all benzodiazepine binding sites in cerebellum but in hippocampus shows a similar high affinity at only some sites with approximately 10-fold lower affinity at the remainder (Braestrup and Nielsen, 1981). The high affinity sites in cerebellum (and hippocampus) have been denoted BZ_1 and the lower affinity BZ_2 although differences between the cerebellar and hippocampal BZ_1 sites are apparent in their linkage to GABA receptors (Harris and Mitchell, 1984). It has been suggested that at low concentrations [^{3}H]PCC can be used as a selective ligand for the BZ_1 site (Braestrup and Nielsen, 1981).

By comparison of the relative B_{max} values of [^{3}H]FNM and [^{3}H]PCC binding it is possible to estimate the proportion of the two sites in an area. Thus the results of such Scatchard analyses in Figure 4.8 and Table 4.1 suggest that the cerebellum contains almost exclusively ($\simeq$ 92%) BZ_1 sites with similar B_{max} values for either ligand, whereas the B_{max} for [^{3}H]PCC in the hippocampus is only 50% of that for [^{3}H]FNM ($P < 0.001$), suggesting that BZ_1 and BZ_2 sites are present there in approximately equal numbers. These results on different CNS regions are in close accordance with those reported by other groups: Braestrup and Nielsen (1981) and Stapleton, Prestwich and Horton (1982) found values of 57% and 58% respectively for hippocampus, and both reported values of 91% for cerebellum.

This heterogeneity in the properties of the sites is confirmed by Hofstee plots of the displacement of bound [^{3}H]FNM by unlabelled PCC (Figure 4.9), with a single linear component in cerebellum but a clearly curvilinear plot in hippocampus. This together with Hill coefficients of 0.96 for cerebellum and 0.71 for

hippocampus, is consistent with the presence of only one site in the cerebellum but possibly two in the hippocampus.

Application of the same approaches to characterise the type(s) of central benzodiazepine binding site in pituitary revealed that [^{3}H]PCC binds there to a similar number of central sites to [^{3}H]FNM (Figure 4.7). This suggests that the benzodiazepine sites present are predominantly of the BZ_1 type. The results of PCC displacement curves of [^{3}H]FNM on pituitary membranes show an IC_{50} of 1.1 ± 0.2nM (very similar to that in the cerebellum (1.3nM), but rather lower than that in hippocampus (8.7nM)), linear Hofstee plots and a Hill coefficient close to unity (0.9). These results support those obtained by saturation analysis and strongly suggest that the central type benzodiazepine binding sites in the pituitary are predominantly of the BZ_1 type. It therefore appears that BZ_1 receptors must mediate any facilitation of GABA effects in the pituitary gland by benzodiazepines. The effectiveness of benzodiazepines in such systems will thus be characteristic of action at a specific BZ_1 type site and may be of use in the study of compounds with allegedly selective actions.

The presence of binding sites for [^{3}H]diazepam has been previously reported in both rat and human PD tissue (Grandison, 1981b; Grandison, Cavagnini, Schmid, Invitti and Guidotti, 1982; Voigt, Davis and Wyche, 1984) but clonazepam was reported not to cause any displacement in either tissue. In contrast, the results reported here have revealed a population of central type sites in both PD and NI, and suggest that the distribution of these sites with GABA receptors is not confined to the CNS.

Benzodiazepines have been reported to modify plasma prolactin concentrations, and the pharmacological profile reported (Grandison, 1982; Lotz, 1982) suggest that central-type rather than peripheral-type receptors are involved. The effects of benzodiazepines on prolactin secretion and interactions with GABA are discussed further in Chapter 6.

In addition to PD, benzodiazepine binding sites were found in NI, with both central and peripheral type binding sites being concentrated in NI rather than PD. The electrophysiological properties of PI cells have been reported to be affected by GABA and these effects were potentiated by diazepam (Taraskevich and Douglas, 1982). No other benzodiazepines were tested but as diazepam had no effect alone and only potentiated the effect of GABA, it seems unlikely that peripheral type sites were involved. Although GABA has been reported to have other effects on NI (Dyball and Shaw, 1979; Zingg et al, 1979; Demeneix et al, 1984), there are no reports on the involvement of benzodiazepines. There are clearly, therefore, likely to be a number of further actions of central type benzodiazepine sites in the pituitary not yet identified.

Since this work was carried out, an autoradiographic study has confirmed the presence of central-type benzodiazepine binding sites in the pituitary (Brown and Martin, 1984). These authors demonstrated (using [^{3}H]Ro15-1788) a minor population of sites in the PD but a dense population in PI. PN appeared devoid of sites.

[^{3}H]Ro5-4864 has been reported to bind to pituitary tissue with high affinity very similar to that in kidney and to that of a

minor population of peripheral type sites in the CNS (Schoemaker et al, 1983). The B_{max} of [^{3}H]Ro5-4864 in pituitary (436 ± 43 fmol/mg protein) is comparable to that reported here for clonazepam-insensitive [^{3}H]FNM binding (660 ± 16 fmol/mg protein). Schoemaker et al. (1983) reported that the affinity of FNM for [^{3}H]Ro5-4864 sites is approximately 20-fold lower than that of Ro5-4864 itself and in accordance with this the K_D value of 34.7 nM for clonazepam-insensitive [^{3}H]FNM binding here shows a similar ratio to the K_D values of 0.6 - 1.6 nM for [^{3}H]Ro5-4864 (Marangos et al, 1982; Schoemaker et al, 1983). The results reported here show that the peripheral type sites are predominantly localised in NI, in agreement with autoradiographic studies using [^{3}H]Ro5-4864 (Mohler and Richards, 1983; De Souza, Anholt, Murphy Snyder and Kuhar, 1985).

Ro5-4864 differs in structure from diazepam by only one substituent but lacks the classical benzodiazepine profile in behavioural tests in rodents (Zbinden and Randall, 1967). It has been reported to possess anticonvulsant activity against maximum electroshock in mice (deLorenzo, Burdette and Holderness, 1981) but others have found it to be inactive in that test (Pieri, Polc, Bonetti, Cumin, Scherschlicht and Haefely, 1983) or recently, proconvulsant in mice, guinea-pigs and rats (File and Mabutt, 1983; Pieri et al., 1983; Weisman, Cott, Paul and Skolnick, 1983). Benzodiazepines have been reported to exert various metabolic effects on transformed cells through peripheral type receptors (Strittmatter, Hirata, Axelrod, Mallorga, Tallman and Henneberry, 1979; Matthew, Laskin, Zimmerman, Weinstein, Hsu and Engelhardt, 1981; Wang, Morgan and Spector, 1982) and alterations in renal

benzodiazepine binding have been demonstrated in experimentally-induced and genetically linked states (Regan et al., 1981; Taniguchi, Wang and Spector, 1981; Del Zompo, Cherillard, Saavedra, Post and Tallman, 1982). One important feature of [^{3}H]Ro5-4864 binding sites, however, is that on subcellular fractionation they sediment in the nuclear rather than mitochondrial/plasma membrane fraction (Marangos et al., 1982) and are therefore unlikely to represent cell-surface receptors. [^{3}H]FNM has also been reported to bind to purified frontal cortical nuclear membranes with a K_D of 28nM (Bormann, Penney, Case and Averill, 1980), a value which is similar to that of clonazepam- insensitive binding in the pituitary reported here. Furthermore, when such binding sites have been described in the CNS, they appear to be relatively restricted to glial than neuronal elements (Gallagher et al., 1981; Marangos et al., 1982; Mohler and Richards, 1983). In contrast, recent electrophysiological studies have shown interaction of high (30μM) concentrations of Ro5-4864 with the $GABA_A$ receptor complex (Simmonds, 1984): Ro5-4864 antagonised both the effect of muscimol and the potentiation by phenobarbitone of the effect of muscimol but enhanced the potency of picrotoxin. Lower concentrations reduced the potentiation of the response to muscimol by flurazepam (Simmonds 1984) and it was suggested that Ro5-4864 was acting at sites not previously characterised in binding studies as a benzodiazepine antagonist (Pellow, File and Simmonds, 1984).

These studies have defined the distribution of benzodiazepine binding sites within the pituitary gland, revealing the presence of central-type (GABA-linked) sites in addition to the peripheral-type

sites which seem unlikely to relate to specific neurotransmitter function. The central sites present in pituitary have been further characterised as the BZ_1 subtype, which in other areas such as cerebellum shows prominent interaction with GABA receptors (Harris and Mitchell, 1984). Functional effects of the central-type sites in PD are described in Chapters 6 and 7.

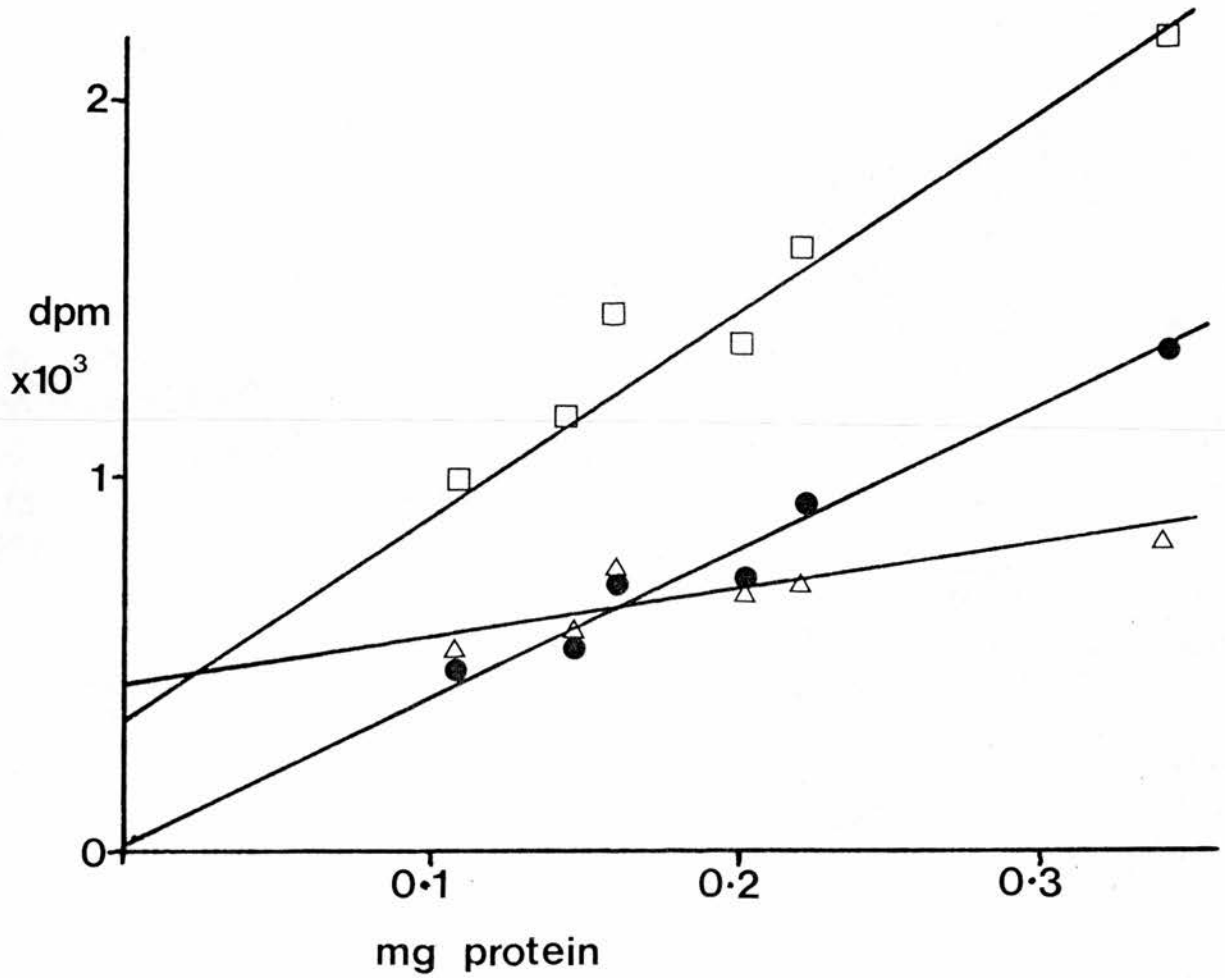


FIGURE 4.1

Linearity of binding of 1nM [^{3}H]FNM to whole pituitary membranes with protein concentration.

(□) Total binding.
(△) Non-specific binding (1μM diazepam)
(●) Specific binding

Lines were fitted by linear regression.

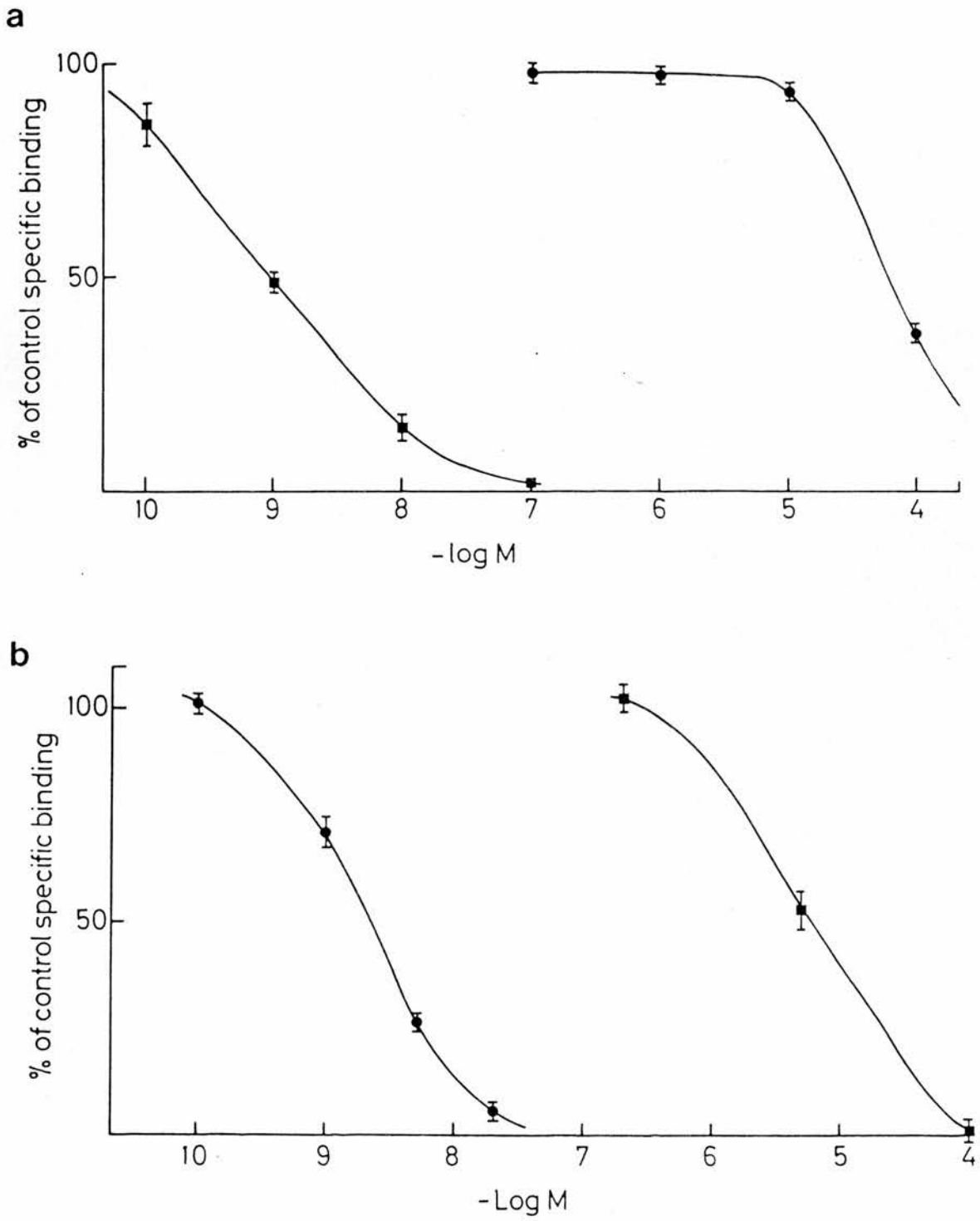


FIGURE 4.2

Displacement of 1nM [^{3}H]FNM by clonazepam (■) and Ro5-4864(●) from a) whole brain and b) kidney membranes. Assays were incubated at 0°C for 90 minutes. 10μM diazepam was used to define non-specific binding. Mean ± S.E.M., n = 4.

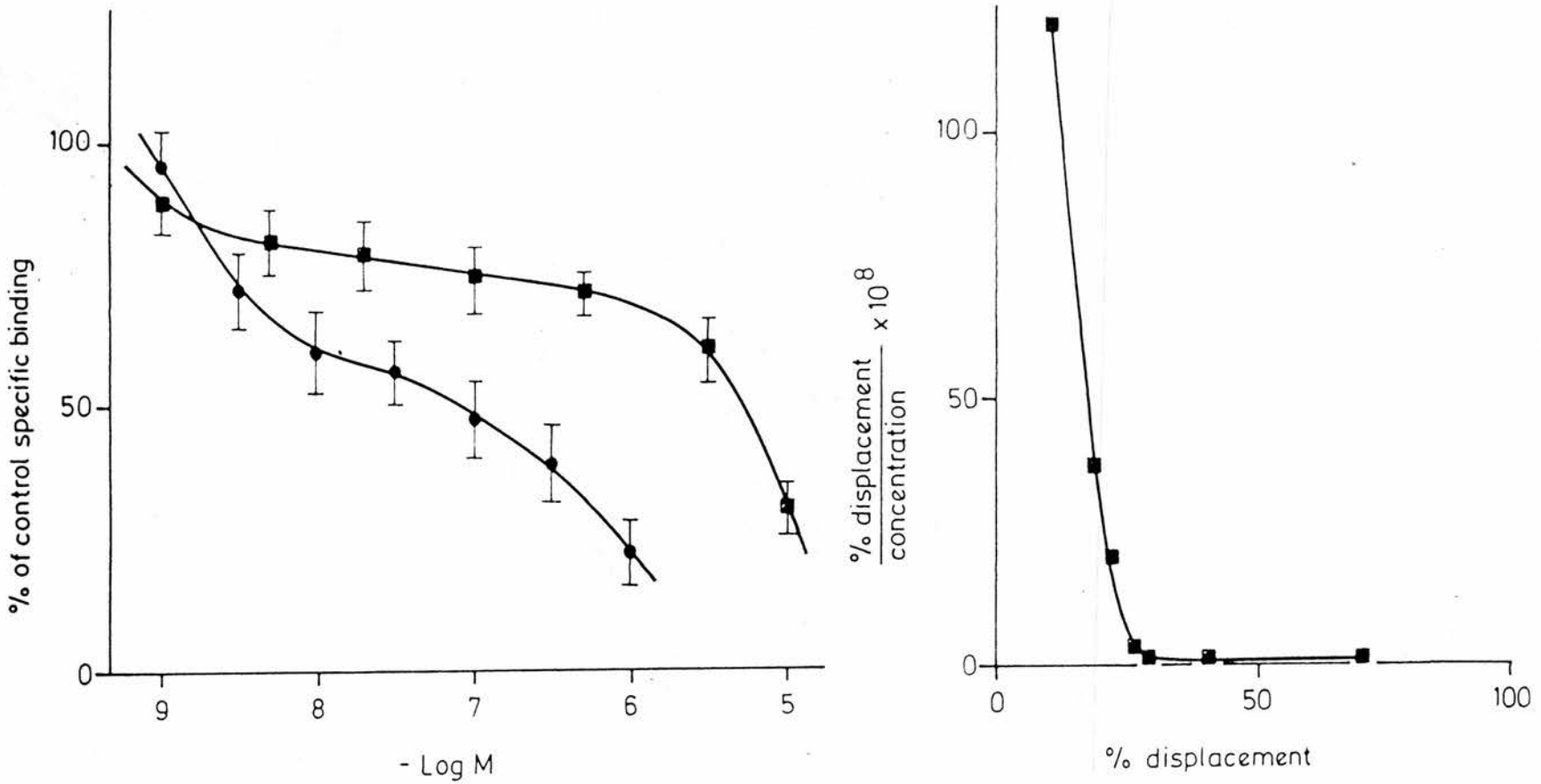


FIGURE 4.3

Displacement of 1μM [^{3}H]FNM from PD membranes by clonazepam (■) and Ro5-4864 (●).
(a) Displacement curves and (b) Hofstee plot of the data. Assays were incubated at 0°C for 90 minutes. 10μM diazepam was used to define non-specific binding. Mean ± S.E.M., n = 4.

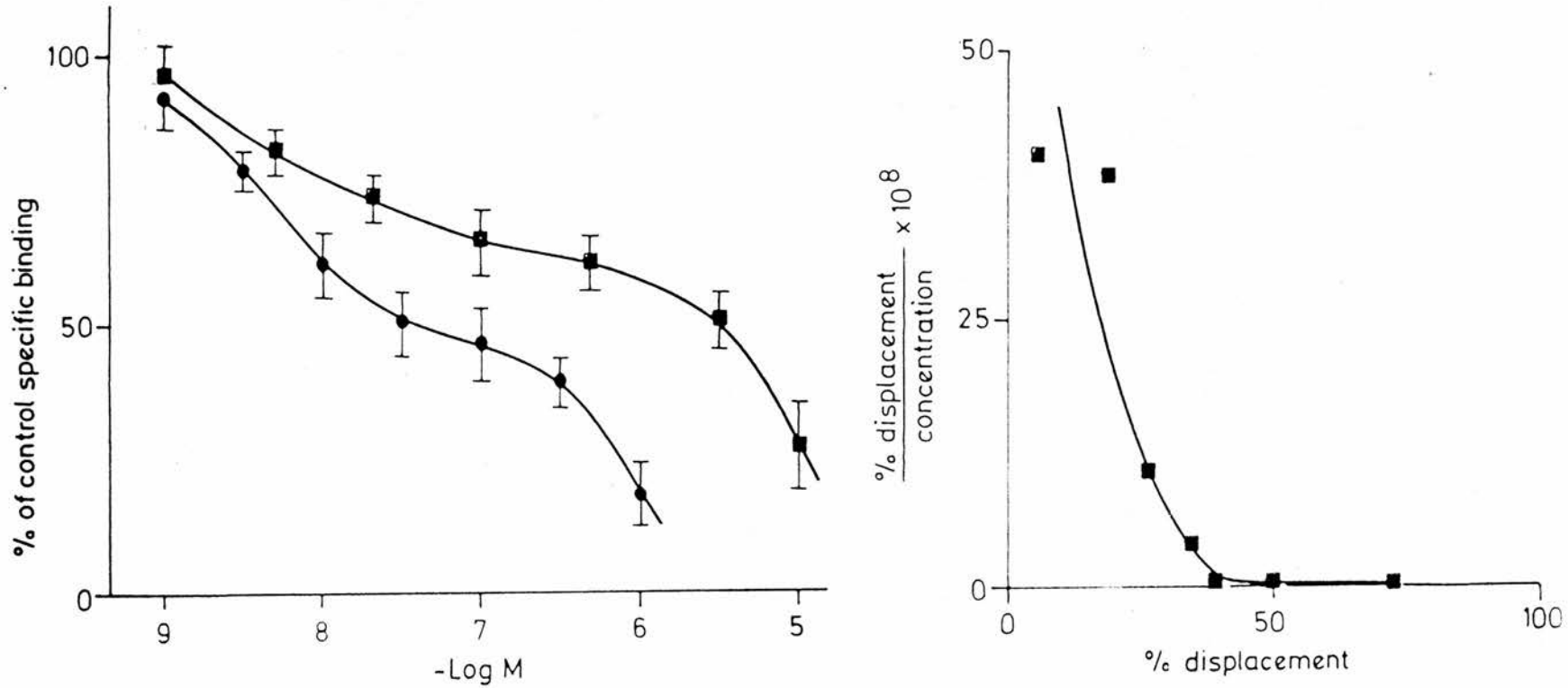


FIGURE 4.4

Displacement of 1nM [^{3}H]FNM from NI membranes by clonazepam (■) and Ro5-4864 (●).
(a) Displacement curves, and (b) Hofstee plot of the data. Assays were incubated at 0°C for 90 minutes. 10μM diazepam was used to define non-specific binding. Mean ± S.E.M., n = 4.

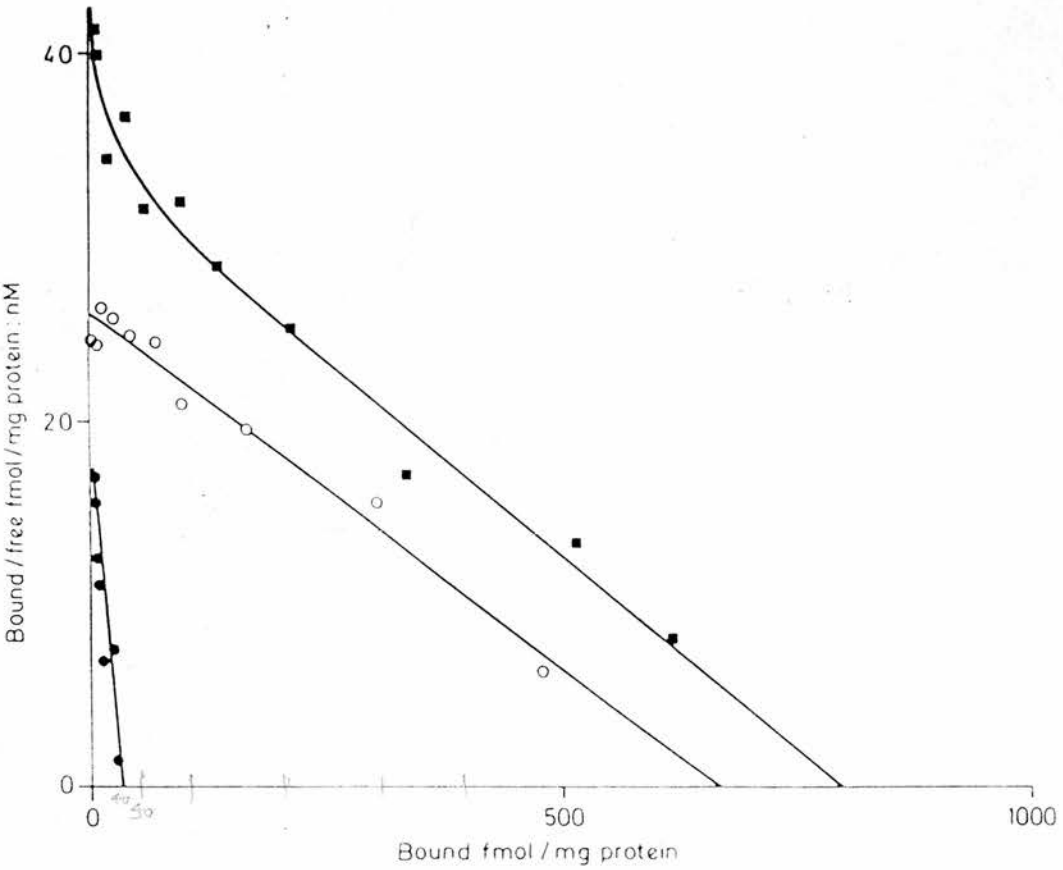


FIGURE 4.5

Representative Scatchard plot of [^{3}H]FNM binding to whole pituitary membranes.
(■) non-specific binding defined by 10μM diazepam.
(●) non-specific binding defined by 200nM clonazepam.
(○) binding in the presence of 200nM clonazepam, non-specific binding defined by 10μM diazepam.
Assays were incubated at 0°C for 120 minutes.

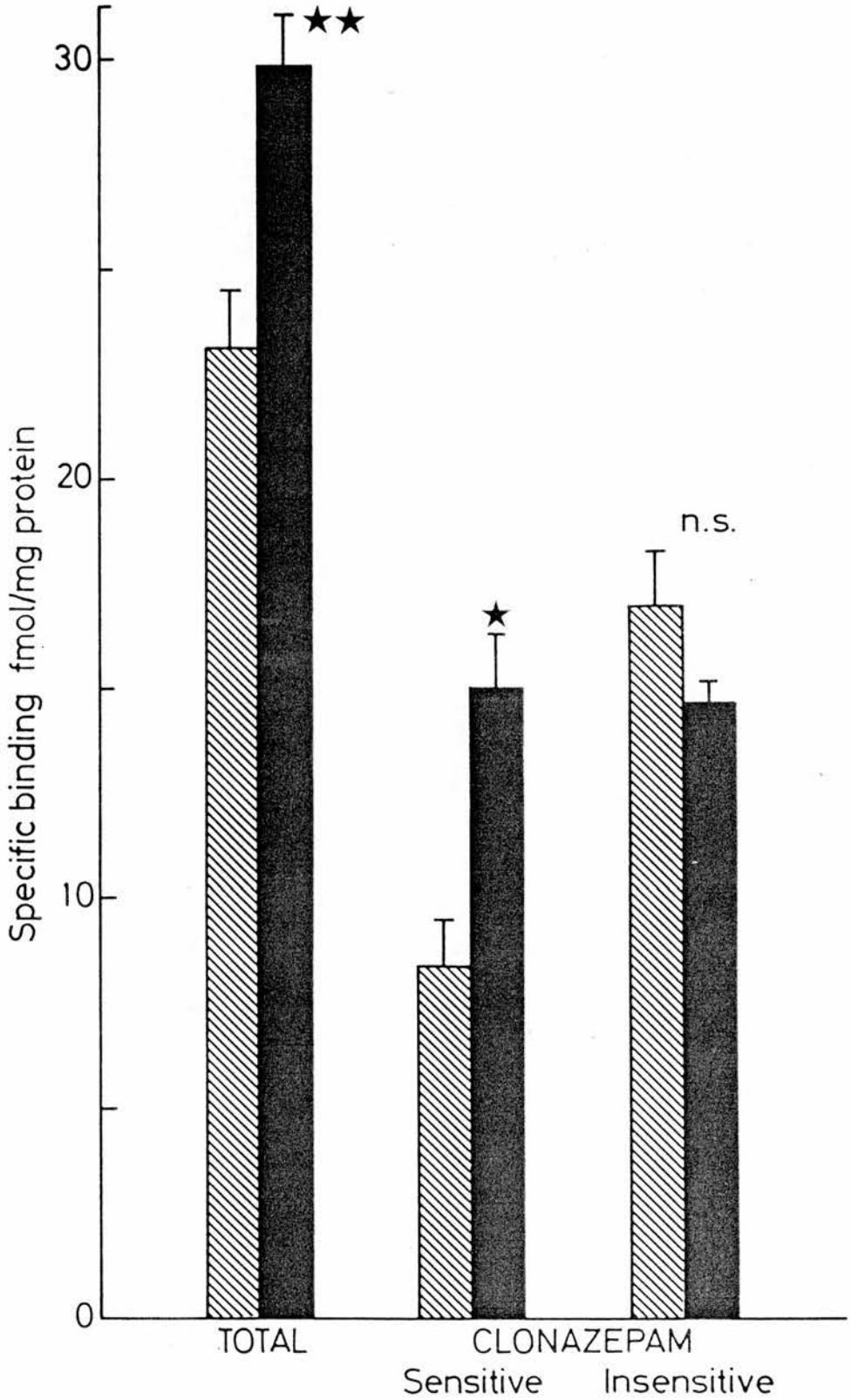


FIGURE 4.6

The effect of GABA on binding of 1nM [^{3}H]FNM to whole pituitary membranes. Columns represent specific binding of 1nM [^{3}H]FNM in absence (hatched columns) and presence (solid columns) of 100μM GABA.
TOTAL: non-specific binding defined by 10μM diazepam;
CLONAZEPAM SENSITIVE: non-specific binding defined by 200nM clonazepam;
CLONAZEPAM INSENSITIVE: binding in the presence of 200nM clonazepam, non-specific binding defined by 10μM diazepam.
Assays were incubated at 0°C for 90 minutes. Levels of significance determined by Students paired t test. *: $p < 0.05$, **: $p < 0.02$. Mean ± S.E.M. $n = 4$.

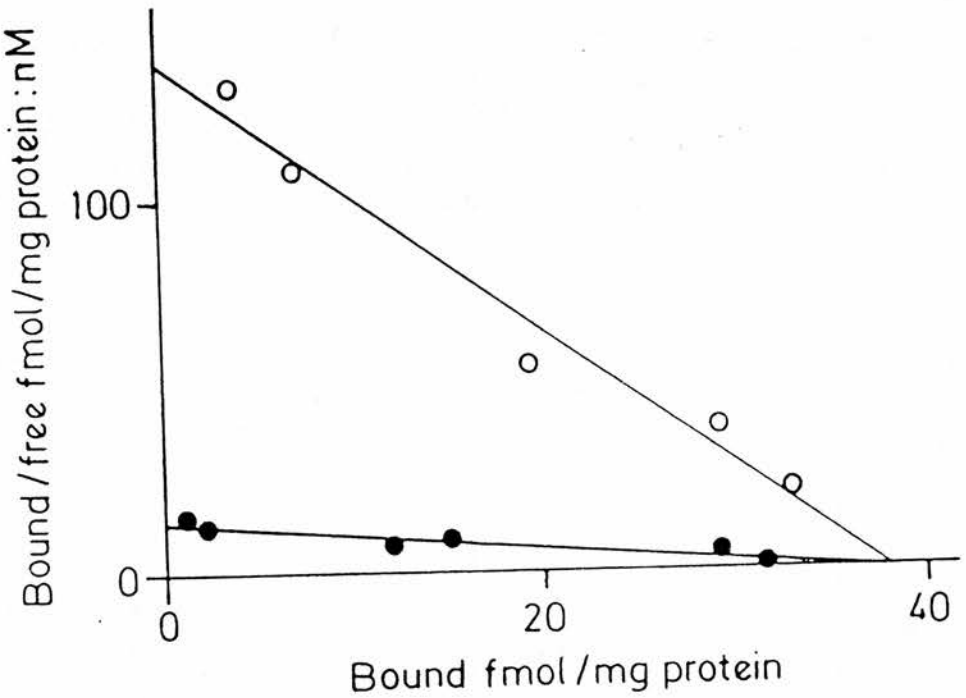


FIGURE 4.7

Representative Scatchard analysis of [^{3}H]FNM (●) and [^{3}H]PCC (○) binding to whole pituitary membranes. Assays were incubated at 0°C for 120 minutes. Non-specific binding was defined by 200nM clonazepam in both cases. Lines were fitted by linear regression.

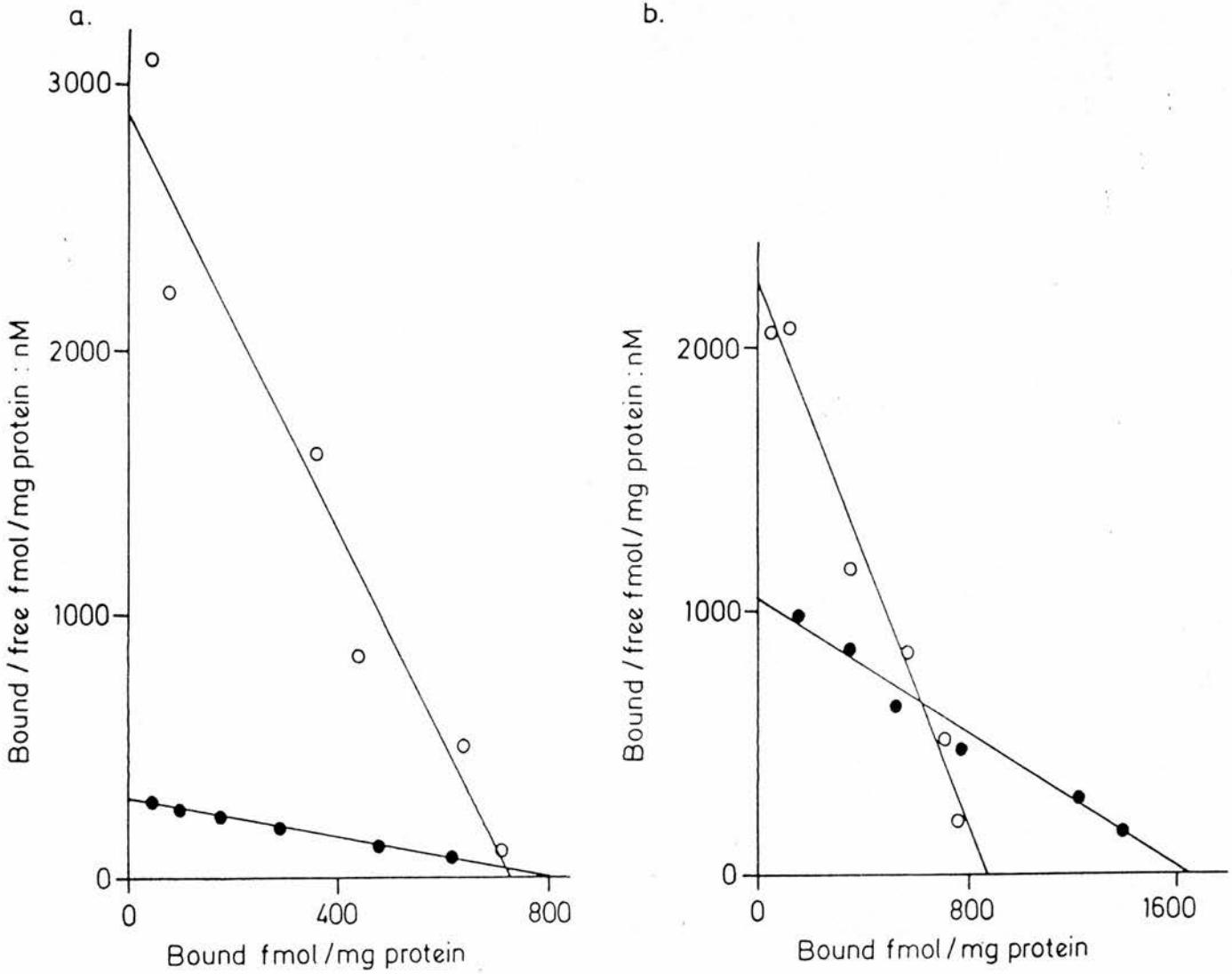


FIGURE 4.8

Representative Scatchard analysis of [^{3}H]FNM (●) and [^{3}H]PCC (○) binding to (a) cerebellum and (b) hippocampus membranes.
Assays were incubated at 0°C for 120 minutes. Non-specific binding was defined by 200nM clonazepam in all cases. [^{3}H]PCC was used in concentrations up to 10nM for cerebellum, 5nM for hippocampus. Lines fitted by linear regression.

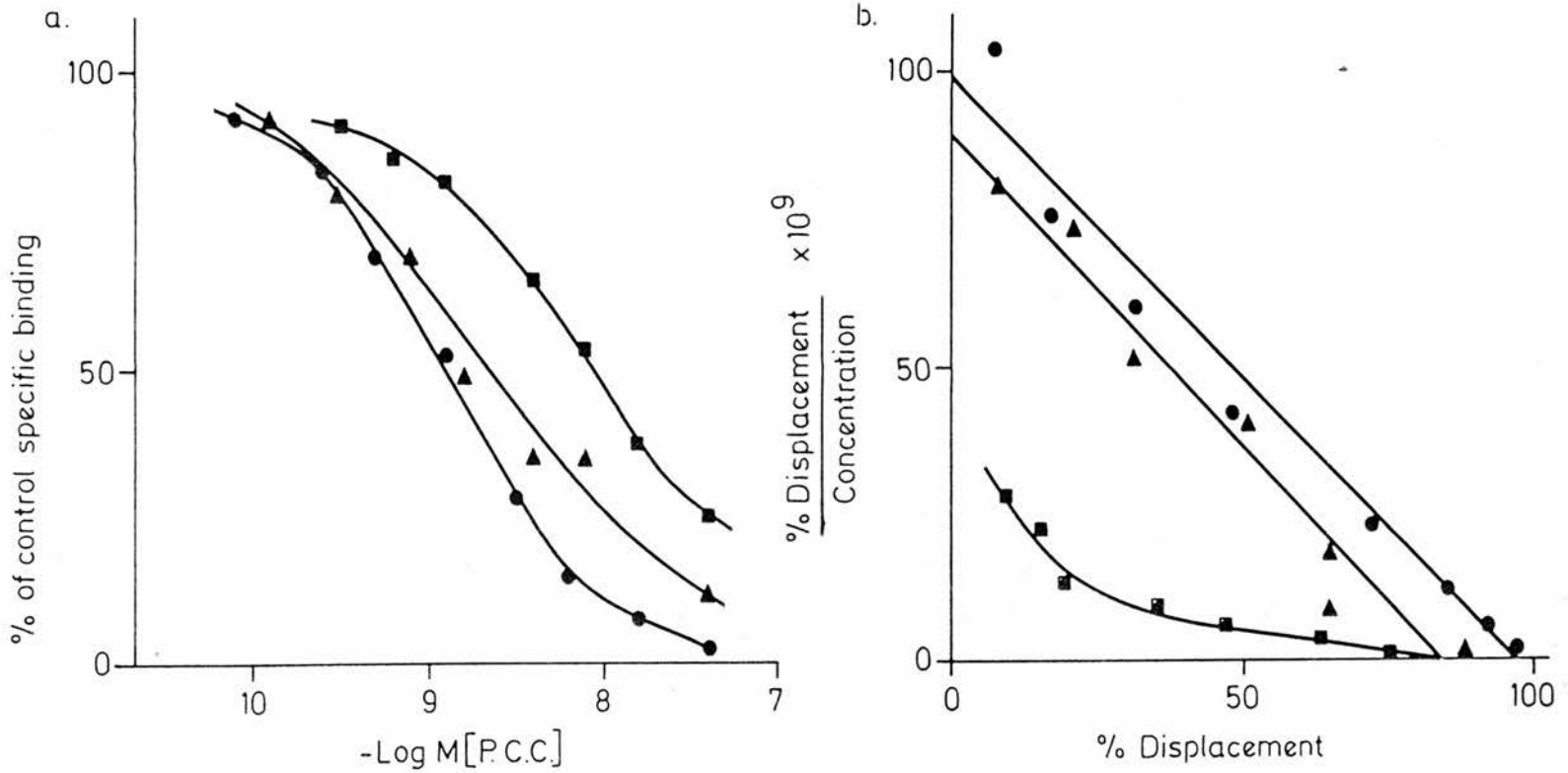


FIGURE 4.9.

a) Displacement curbes of bindings of 0.5nM [^{3}H]FNM by PCC from (●) cerebellum, (■) hippocampus and (▲) whole pituitary membranes.

b) Hofstee plots of the results in (a). Assays were incubated at 0°C for 180 minutes. 200nM clonazepam was used to define non-specific binding in all cases. Results are means of two experiments performed in duplicate for cerebellum and hippocampus, and representative of four performed in duplicate, for pituitary. Linear Hofstee plots were fitted by linear regression.

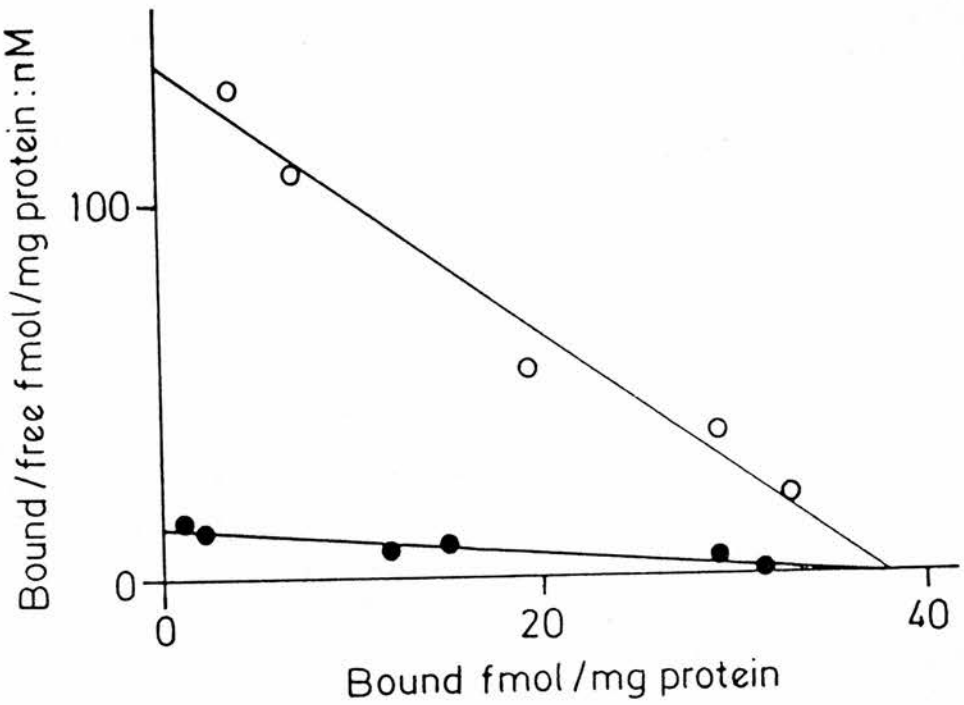


FIGURE 4.7

Representative Scatchard analysis of [^{3}H]FNM (●) and [^{3}H]PCC (○) binding to whole pituitary membranes. Assays were incubated at 0°C for 120 minutes. Non-specific binding was defined by 200nM clonazepam in both cases. Lines were fitted by linear regression.

Table 4.1.

		[^{3}H]FNM	[^{3}H]PCC
Pituitary	K_D(nM)	1.9 ± 0.3	0.33 ± 0.05
	B_{max} (fmol/mg protein)	32.8 ± 2.6	36.7 ± 4.8
Cerebellum	K_D	2.69 ± 0.02	0.28 ± 0.01
	B_{max}	865 ± 18	737 ± 34
Hippocampus	K_D	1.57 ± 0.01	0.42 ± 0.01
	B_{max}	1669 ± 47	836 ± 24

Results of Scatchard analysis of [^{3}H]FNM and [^{3}H]PCC binding to whole pituitary, cerebellum and hippocampal membranes. Mean ± S.E.M., n = 7–8 for pituitary, n = 4 for cerebellum and hippocampus.

CHAPTER 5

$GABA_A$ - and $GABA_B$ - receptor mediated effects on prolactin secretion *in vitro*

5.1 Introduction

Investigations of the involvement of GABA in the control of prolactin secretion do not date back as early as studies on other PD hormones (Makara and Stark, 1974; Ondo, 1974) but have since provided a wealth of data, at times conflicting, reflecting the variety of animal models, drugs and their routes of administration studied. GABA was originally reported to stimulate prolactin secretion after i.c.v. injection in both conscious female and anaesthetized and conscious male rats (Mioduszewski, Grandison and Meites, 1976; Ondo and Pass, 1976; Pass and Ondo, 1977) but intrapituitary injection was without effect (Ondo and Pass, 1976). This has been confirmed by others (Vijayan and McCann, 1979; Donoso and Zarate, 1981; Lamberts, Vijayan, Graf, Mansky and Wuttke, 1983). In contrast to these results, Schally et al isolated a PIF from porcine hypothalami which was distinct from catecholamines and which they identified as GABA (Schally, Redding, Arimura, Dupont and Linthicum, 1977). They found that GABA inhibited basal prolactin secretion *in vitro* and *in vivo* when administered intravenously (i.v.) after elevation of secretion by monoiodotyrosine or neuroleptics. These findings were supported by the demonstration that basal or elevated prolactin secretion could be inhibited *in vivo* by the GABA-transaminase inhibitors ethanolamine-o-sulphate (EOS), γ-acetylenic GABA (GAG) and amino-oxyacetic acid (AOAA) or muscimol (i.v.) (Locatelli, Cocchi, Racagni, Cattabeni, Maggi, Krogsgaard-Larsen and Muller, 1978; Racagni et al, 1979; Libertun, Arakelian, Larrea and Foglia, 1979; Locatelli, Cocchi, Frigerio, Betti, Krogsgaard-Larsen, Racagni and Muller, 1979; Grandison and Guidotti, 1979; Grandison, 1980; Debeljuk, Goijman, Seilicovich,

Diaz and Rettori, 1980; Apud, Racagni, Iuliano, Cocchi, Casanueva and Muller, 1981; Donoso and Zarate, 1981). An inhibitory action in vitro has also been confirmed (Lamberts and MacLeod, 1978; Enjalbert et al, 1979a; Grandison and Guidotti, 1979; Grossman, Delitala, Yeo and Besser, 1981; Matsushita, Kato, Shimatsu, Katakami, Yanaihara and Imura, 1983), and GABA was shown to inhibit spontaneous action potential firing in GH_3 cells (Israel, Dufy, Gourdji and Vincent, 1981), but it has been a consistent finding that GABA and muscimol do not inhibit prolactin secretion from ectopic PD transplants (Locatelli et al. 1978, 1979; Shin, Obonsaurin and Bates, 1984). This may be because of a long-term change in responsiveness of PD when removed from its normal environment and control mechanisms.

There is therefore considerable evidence that GABA can act directly on PD to inhibit prolactin secretion and specific binding sites for [^{3}H]GABA and [^{3}H]muscimol have been demonstrated in PD (Grandison and Guidotti, 1979; Chapter 3). A physiological role of GABA is supported by experiments which showed that bicuculline and allylglycine (an inhibitor of GAD) given i.v., potentiated the rise in plasma concentration of prolactin in male rats in response to injection of sulpiride (Debeljuk et al, 1980; Debeljuk, Seilicovich and Diaz, 1981). Other studies using bicuculline (i.v.) have yielded conflicting reports: it was found to have no effect in male rats after injection of pimozide (Shin et al, 1984) but in oestrogen-primed, ovariectomized rats, bicuculline methiodide reduced prolactin secretion (Locatelli et al, 1979; Casanueva, Apud, Locatelli, Martinez-Campos, Civati, Racagni, Cocchi and Muller, 1981).

Recently, bicuculline (i.v.) was reported to cause a transient stimulation of prolactin secretion followed by an inhibition in both male and ovariectomized female rats (McCann, Vijayan, Negro-Vilar, Mizunuma and Mangat, 1984) but no data was provided to support this.

In addition to reducing plasma prolactin concentrations, EOS (i.c.v.) increases the concentration of GABA in hypophysial portal blood (Gudelsky, Apud, Masotto, Locatelli, Cocchi, Racagni and Muller, 1983) and in PD (Racagni et al, 1979; Casanueva et al, 1981). The concentration of GABA in PD has also been used as an index of tuberoinfundibular GABAergic activity to investigate the possibility of a physiological role for GABA in suckling (Racagni and Apud, 1982). Pup separation caused a parallel fall in PD GABA concentration and plasma prolactin concentration, (i.e. an inverse correlation to that observed after EOS administration). Suckling caused an approximately 10-fold increase in plasma prolactin concentration within 30 min, at which time PD GABA was unchanged. After 2 hours, PD GABA concentrations were elevated, reaching their highest value during continuous suckling. From these results it was suggested (Racagni and Apud, 1982) that GABA may be involved in feedback mechanisms rather than in tonic regulation of prolactin secretion. AOAA has been reported to prevent suckling-induced prolactin secretion (Libertun et al, 1979) and sodium valproate administration to puerperal women prevented breast stimulation-induced prolactin secretion (Melis, Fruzzetti, Paoletti, Mais, Kemeney, Stringini, Boldrini and Fioretti, 1984) with little (Melis et al, 1984) or no (Libertun et al, 1979) effect on basal prolactin secretion. These studies, together with those discussed above, show the inhibitory potential of endogenous GABA, particularly when

prolactin secretion is elevated. GAD activity in MBH is decreased by apomorphine, and increased by neuroleptics (Prato, Clementi, Nicoletti, Canonico, Patti, Condorelli, DiGiorgio and Drazo, 1981; Nicoletti, Clementi, Prato, Canonico, Rampello, Patti, DiGiorgio and Scapagnini, 1983), treatments which decreased and increased plasma prolactin concentrations respectively. As the effect of sulpiride was prevented by hypophysectomy and to some extent mimicked by a pituitary graft (Nicoletti et al, 1983) it was proposed that the effects on GAD were mediated by prolactin, acting in a negative feedback manner. Similar experiments have been performed on DA turnover in MBH and ME (Moore, Demarest and Johnston, 1980). Prolactin itself has also been found to increase GAD activity in MBH, and the concentration of GABA in hypophysial portal blood after i.c.v. administration, but only after a delay of 16 hours (Apud et al, 1984).

While it is clear that GABA can stimulate prolactin secretion at a central site, possibly by inhibiting DA release into hypophysial portal blood (Vijayan and McCann, 1979; Casanueva et al, 1981; Racagni and Apud, 1982; see also Chapter 11) and can inhibit prolactin secretion by an action at the pituitary, a physiological role of GABA either in tonic inhibition or in feedback regulation remains unproven.

A transient stimulatory effect of GABA on electrical activity of cultured pars intermedia cells and on αMSH secretion (Taraskevich and Douglas, 1982; Tomiko et al, 1983; Demeneix et al, 1984) has been reported. The purely stimulatory $GABA_A$ receptor response on αMSH secretion and the inhibitory $GABA_B$ receptor response (Demeneix et al, 1984) contrasts with the inhibitory effect of GABA

and muscimol on prolactin secretion, even in superfusion systems when transient effects might be apparent (Grossman et al, 1981; Matusushita et al, 1983). The present studies were therefore undertaken to examine the effects of $GABA_A$ and $GABA_B$ receptor activation on prolactin secretion, using a superfusion system but avoiding the use of trypsin to disperse cells, as it abolishes spontaneous electrical activity in PD cells for several days (Israel, personal communication.).

5.2 Materials and Methods

5.2.1. Apparatus and materials

The superfusion chamber consisted of a Swinnex filter unit (Millipore, Harrow) containing a Millipore AP20 prefilter to support the tissue. The inlet at the top was attached to a length of 1mm bore silicone rubber tubing, the end of which was immersed in the superfusion medium. The outlet was attached by similar tubing to a peristaltic pump (model 510, Watson Marlow) and from this tubing passed to a fraction collector (model 2070 Ultrorac 11, LKB, Sweden).

All chemicals used in the superfusion medium and tetraethyl ammonium bromide (TEA) were obtained from BDH, Poole, except bacitracin and bovine serum albumin (BSA) which were obtained from Sigma. The following were also obtained from Sigma: GABA, muscimol, homocarnosine, IAA, picrotoxinin (PTX), strychnine, glycine, taurine, 3-isobutyl-1-methyl xanthine (IBMX), N^6-2^O-O-dibutyryl adenosine 3'5'cyclic monophosphate (dbcAMP), VIP, sodium propionate, propionic acid, 4-aminopyridine (4AP), collagenase, hyaluronidase, deoxyribonuclease and cholera toxin. Isoguvacine was obtained from Cambridge Research Biochemicals (Cambridge), 3-aminopropane sulphonic acid (APS) from Aldrich Chemical Co. (Milwaukee, WI, USA),

bicuculline methiodide (BMI) from Pierce Chemical Co., (Rockford, IL, USA), forskolin from Behring Diagnostics (La Jolla, CA, USA), 4,4'-diiso-thiocyano- 2,2'-disulphonic acid stilbene (DIDS) from Calbiochem Behring Corpn. (La Jolla, CA, USA). The following were generous gifts of the individuals or organisations named: (±)baclofen and its stereoisomers from Ciba-Geigy (Basel, Switzerland), piperidine-4-sulphonic acid (P4S) and 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol (THIP) from Dr. P. Krogsgaard- Larsen (Copenhagen).

5.2.2. Methods

Male Wistar rats (200-300g) were stunned, decapitated, and the pituitary gland removed (Section 2.3). NI was discarded, and PD was mechanically chopped (MacIlwain Tissue Chopper, Mickle Laboratory Engineering Co., Gomshall, Surrey) at 500μM intervals in two directions at 90°. Tissue was then transferred to a polystyrene tube (Luckham) containing ≈ 10ml of Krebs Bicarbonate medium (composition in mM: NaCl,127; KCl,3.83; $CaCl_2$,1.8; $MgCl_2$,1.18; KH_2PO_4,1.18; $NaHCO_3$,20; with 2g/l glucose, 30mg/l bacitracin and 0.1% BSA, pH 7.4) equilibrated with an atmosphere of 95%O_2/5%CO_2 at 37°C. Tissue was incubated for 1 hr under these conditions. Aliquots corresponding to tissue from 1-2 rats were then transferred into each superfusion chamber. The inlet line was reconnected, and superfusion with constantly- oxygenated medium at 37°C at a rate of 0.5ml/min was started. A diagram of this method is shown in Figure 5.1. After a period of 75 min, the collection of 2 min fractions was begun, and after a further 15 min the inlet lines were changed to medium containing drugs as appropriate. When antagonists were used, they were introduced 30 min before the

agonists. Fractions were collected into polypropylene scintillation vial inserts (Sterilin) and were stored at -20°C until assay (Section 2.3).

5.2.3 Preparation of dispersed cells

PD from male rats were roughly chopped using a razor blade and incubated in Earles Balanced Salt solution (Ca^{2+}- and Mg^{2+}- free with 1% BSA, Gibco) containing 0.1% collagenase, 0.1% hyaluronidase, and 0.02% deoxyribonuclease in a shaking waterbath at 37°C under an atmosphere of 95%O_2, 5% CO_2. Tissue was repeatedly triturated using fine, siliconised Pasteur pipettes to aid dispersal. After 45 min, the medium was centrifuged for 10min at 600g at 4°C, the supernatant discarded, and the cells resuspended in medium without the enzymes. The suspension was then filtered through a nylon mesh (80μM pore size) and mixed with Biogel P2 pre-equilibrated with perfusion medium. The resultant slurry was then introduced into the superfusion chambers, where it was supported on Whatman GF/B filters, and superfused as described above. Normal protocol was subsequently followed.

5.2.4 Calculation of results and statistical comparisons

Results were generally calculated as percentage changes from pre-drug basal secretion rate. Basal rate was obtained from the mean of 5 fractions immediately before addition of drugs, and peak values were used to express percentage stimulation. Inhibitory effects were quantified using a mean of 4-6 fractions when release appeared to have stabilised. In some cases, the prolactin concentration in each fraction is expressed as a percentage of mean pre-drug concentration. The results from experiments using

antagonists are expressed as percent antagonism of a control response carried out at the same time on a parallel superfusion.

Statistical comparisons were made using the Mann-Whitney U test.

5.3 Results

5.3.1. Effects of muscimol and baclofen on basal prolactin secretion.

The $GABA_A$ receptor agonist muscimol was found to have a biphasic effect on prolactin secretion, which consisted of an immediate, transient stimulation of secretion, followed by a sustained inhibition. An example of such a response to 10μM muscimol is shown in Figure 5.2. On washout of the muscimol, prolactin secretion recovered towards pre-drug baseline. A similar biphasic response to muscimol was also observed using collagenase-dispersed cells, in which 10μM muscimol caused 44 ± 8% stimulation and 45 ± 5% inhibition of prolactin secretion (n = 3).

To investigate the specificity of this effect, the established PIF DA was investigated in this system. 0.1μM DA caused a prompt inhibition of secretion (71 ± 3%, n = 3) without any transient stimulatory effect (Figure 5.3). The $GABA_B$ receptor agonist (±)baclofen had no effect on basal prolactin secretion at a concentration of 100μM (Figure 5.2). The results of further experiments investigating effects of baclofen on prolactin secretion are described in section 5.3.7.

5.3.2. Effects of other GABA agonists

GABA itself also had a biphasic effect on prolactin secretion, qualitatively the same as that of muscimol (Figure 5.4.). A quantitative analysis, using GABA and muscimol concentrations from 10nM to 30μM showed that muscimol was approximately 10-fold more potent than GABA at both effects (Figure 5.5). Figure 5.5 also shows

concentration-response results for isoguvacine and homocarnosine. Results for P4S, THIP and APS were similar to those for isoguvacine. From this figure it can be seen that both components of the response to muscimol and GABA are quantitatively similar at each concentration i.e. the stimulatory and inhibitory curves are nearly superimposed. By contrast, the curves for isoguvacine are well separated, only the inhibitory effect showing a clear concentration-dependence, the stimulatory effect being very small. The effects of homocarnosine are discussed further below. Therefore several analogues appeared to be less potent than GABA at causing stimulation of prolactin secretion, but were equipotent to GABA on the inhibitory response. This is illustrated by the responses to each of the agonists at a concentration of 10μM (Figure 5.6). At this concentration, GABA caused more than double the percent stimulation of prolactin secretion that the other agonists caused ($P < 0.05$ in each case) but there were no significant differences in the amount of inhibition.

5.3.3 Effect of homocarnosine and interaction with GABA.

Homocarnosine was not found to have a biphasic effect on prolactin secretion, but at 1mM, caused a prompt and sustained inhibition (28 ± 4%) (Figure 5.4). This effect was concentration-dependent (Figure 5.5), homocarnosine being approximately 100-fold less potent than GABA. The absence of any stimulatory effect, even at 2mM, despite a considerable inhibitory effect suggested that homocarnosine might not be acting specifically on GABA receptors. However, 10μM BMI was found to cause significant

antagonism of the effect of 1mM homocarnosine (28 ± 4% inhibition, 1mM homocarnosine alone, 17 ± 2% inhibition in the presence of 10μM BMI, n = 5, $P < 0.05$) (Fig. 5.7a) demonstrating that homocarnosine was indeed acting as a $GABA_A$ receptor agonist.

A further series of experiments was carried out to investigate the interaction between homocarnosine and GABA. Concentrations of GABA and homocarnosine were chosen that gave similar inhibitory effects. 3μM GABA caused 28 ± 2% inhibition, and 1mM homocarnosine caused 28 ± 4% inhibition. This concentration of GABA caused 14 ± 2% stimulation of secretion over pre-drug rate, but when recalculated to take into account the superimposed inhibitory effect (estimated as the mean response to 1mM homocarnosine at the fraction of peak stimulation), this value was 21 ± 3%. The combination of 3μM GABA and 1mM homocarnosine caused 32 ± 3% inhibition, and 2mM homocarnosine alone caused 31 ± 2% inhibition demonstrating compilation rather than addition of effects, consistent with a single site of action. The combination caused no stimulation of secretion above baseline, but secretion declined less rapidly. At the fraction previously observed to demonstrate peak stimulation, the combination caused 7 ± 3% stimulation over the effect of 2mM homocarnosine (mean response) (Figure 5.7b). This value was significantly less ($P < 0.05$) than that for GABA alone, suggesting that homocarnosine specifically antagonised the stimulatory effect of GABA, and that the response to the combination was not merely the arithmetical sum of the two responses.

To investigate whether significant contamination by GABA could in part account for sone of the inhibitory activity of homocarnosine, the sample of homocarnosine was subjected to analysis

by high pressure liquid chromatography with pre-injection derivatisation with O-phthalaldehyde and fluorescence detection. Using a method similar to that of Turnell and Cooper (1982), standards of GABA and homocarnosine were clearly separated. Homocarnosine was found to contain <0.03% free GABA. Furthermore, PD tissue was found to contain no detectable homocarnosinase activity (Chapter 12), suggesting that homocarnosine was not degraded by the tissue during the experiment. This was further checked by incubating chopped PD tissue at 37°C in oxygenated medium with 1mM homocarnosine. After 1hr, aliquots of medium were taken for determination of GABA by radioreceptor assay (Section 12.2.3). Under these conditions no GABA was detectable (< 10nM in tissue incubation medium). These results provide further evidence for the authenticity of the effect of homocarnosine.

5.3.4 Effects of glycine and taurine on prolactin secretion

Glycine (100μM) and taurine (1mM) were found to have no effect on basal prolactin secretion (Figure 5.8). Co-introduction of 100μM glycine increased the stimulatory response to 10μM muscimol from 65 ± 10% to 97 ± 4% ($P < 0.05$, $n = 5$) but did not alter the inhibitory effect: 51 ± 3% inhibition by muscimol alone, 50 ± 2% with glycine (Figure 5.8).

5.3.5 Antagonism of the effects of muscimol

BMI, PTX and strychnine were investigated as antagonists of the effects of muscimol. Two series of experiments were performed, using concentrations of muscimol of 10μM and 30μM. All three antagonists at 10μM antagonised the stimulatory effect of 10μM muscimol, with PTX and strychnine appearing especially potent (Figure 5.9). At this antagonist concentration, only BMI caused

significant inhibition of the inhibitory effect of muscimol, reducing it by ≈50% while PTX and strychnine were ineffective. At 100μM antagonist, all three virtually or completely abolished the stimulatory response, and all caused significant antagonism of the inhibitory response (Figure 5.9). In the second series of experiments, concentration-effect data was obtained for the three antagonists. 30μM muscimol alone caused 139 ± 23% stimulation and 57 ± 4% inhibition (n = 6). Figure 5.9 shows that PTX was approximately 3-fold more potent than BMI as an antagonist of the stimulatory response, with strychnine being of intermediate potency perhaps with a steeper concentration-response curve. The limits of accuracy of the results however prevent detailed interpretation of this. In agreement with the first series of experiments showing selective antagonism of the stimulatory response by PTX and strychnine, neither antagonist at any of the concentrations tested (i.e. up to 30μM for PTX and 100μM for strychnine) caused significant antagonism of the inhibitory response to 30μM muscimol. 30μM muscimol alone caused 57 ± 4% inhibition, 42 ± 4% in the presence of 30μM PTX and 52 ± 5% in the presence of 100μM strychnine (n = 5-6). BMI however, was an effective antagonist, reducing the response to 25 ± 5% at 100μM.

5.3.6. Effects of ionic substitution and ion channel blockade.

Two approaches were taken to investigate whether both components of the biphasic response to muscimol were mediated by increased chloride channel conductance. In the first, sodium propionate was substituted for NaCl in the medium, giving a residual $[Cl^-]_o$ of ≈6mM (4.5% of normal). In the second, the chloride channel blocker DIDS (Cabantchick, Kauf and Rothstein, 1978) was present in the

superfusate at a concentration of 100μM for 45 min before muscimol was introduced. Low-chloride medium caused a slight, ≃ 25% reduction in basal prolactin secretion which largely recovered in 30 min, before muscimol was introduced. In this medium, 10μM muscimol caused only 9 ± 2% stimulation and 24 ± 2% inhibition (n = 5, both $P < 0.05$ vs control, Figures 5.9 and 5.11). 100μM DIDS caused a delayed but sustained increase in prolactin secretion of 46 ± 5% (n = 5), and after this treatment, 10μM muscimol caused only 33 ± 1% stimulation and 26 ± 3% inhibition (n = 5, both $P < 0.05$ vs control, Figures 5.9 and 5.11). In neither case was the response to 30mM K^+ (using K_2SO_4) affected: 176 ± 12% control stimulation, 182 ± 10% in low-chloride medium, and 151 ± 13% in the presence of DIDS (n = 5 in each case).

Experiments were also carried out to investigate the effect of K^+-channel blockade by 4AP and TEA on the response to muscimol. Channel blockers were introduced 60 min before muscimol, and both caused a transient stimulation of secretion (Figure 5.15). In the presence of 1mM 4AP the stimulatory effect of 10μM muscimol increased to 110 ± 8% ($P < 0.05$ vs control, 65 ± 10%) while the inhibitory effect was unaltered (48 ± 5%, n = 5, control 51 ± 3%) (Figure 5.15). Both reponses to 10μM muscimol were unaffected in the presence of 10mM TEA; the agonist causing 60 ± 2% stimulation and 40 ± 2% inhibition (n = 5, figure 5.11).

5.3.7. Evidence for $GABA_B$ receptor effects on prolactin secretion.

As described in section 5.3.1., 100μM (±)baclofen had no effect on basal prolactin secretion (Figure 5.1.). This concentration of (±)baclofen also did not affect the stimulation of secretion caused

by 30mM K^+ or 3mM Ba^{2+} (Figure 5.12). 30mM K^+ caused 176 ± 12% stimulation alone, and 193 ± 7% with baclofen; 3mM Ba^{2+} caused 226 ± 34% stimulation alone, and 256 ± 35% with baclofen (n = 5 in each case). When tested on the stimulation of secretion caused by VIP however, an inhibitory effect was revealed. 10nM VIP caused a peak of 91 ± 4% stimulation of secretion, which was reduced to 62 ± 5% when 100μM (±)baclofen was cointroduced (n = 5, $P < 0.05$) (Figure 5.13). This effect was stereospecific: 100μM (+)baclofen had no effect on the response to 7.5nM VIP (92 ± 15% of control stimulation) but 100μM (−)baclofen caused a significant reduction to 59 ± 5% of control ($P < 0.05$, c.f. 68 ± 5% of control for (±)baclofen) (Figure 5.13).

VIP has been proposed to stimulate prolactin secretion by activation of adenylate cyclase (Dorflinger and Schonbrunn, 1983) and therefore the effect of baclofen was studied following superfusion with various substances that elevate intracellular cAMP concentrations. Forskolin, cholera toxin, IBMX and dbcAMP (the last two in combination as well as separately) were used. 10μM forskolin caused a slight, ≃ 30% stimulation of prolactin secretion which was transient, but neither 30ng/ml cholera toxin, 2mM dbcAMP nor 2mM IBMX had any apparent effect on prolactin secretion over the time-period studied. All were superfused for 40 min before the introduction of 100μM (±)baclofen, but under none of these conditions did baclofen have any effect (Figure 5.14).

Any possible interaction of baclofen with the transient stimulation of secretion caused by K^+ channel blockers was also investigated. 100μM (±)baclofen reduced the stimulation caused by 1mM 4AP from 99 ± 5% to 66 ± 6% ($p < 0.05$, n = 5, Figure 5.15)

without affecting the response to 10mM TEA (57 ± 10% alone, 67 ± 4% in the presence of baclofen). This effect of baclofen on 4AP-stimulated secretion was stereospecific: 100μM (+)baclofen had no effect (83 ± 10% stimulation) whereas 100μM (−)baclofen caused significant reduction of the stimulation (51 ± 6%, $n = 5$, $p < 0.05$, Figure 5.15).

5.4 Discussion

5.4.1 $GABA_A$ receptor effects

The transient stimulation of prolactin secretion, followed by a sustained inhibition (from which recovery could be demonstrated) caused by GABA and muscimol is different to any other $GABA_A$ receptor effect on pituitary hormone secretion. A purely stimulatory $GABA_A$ effect has been reported on αMSH secretion (Demeneix et al, 1984) and on LH, GH and ACTH secretion (Chapter 7). The inhibitory effect of GABA on αMSH secretion (Tomiko et al, 1983) has since been shown to be $GABA_B$ receptor mediated (Demeniex et al, 1984). Previous studies on the effects of GABA on prolactin secretion *in vitro* have used either static incubations (Lamberts and MacLeod, 1978; Enjalbert et al, 1979a; Grandison and Guidotti, 1979) under which conditions transient effects will not be apparent, or trypsin-dispersed cells which were subsequently superfused either immediately (Grossman et al, 1981) or the following day (Matsushita et al, 1983). Trypsin causes considerable damage to PD cells and abolishes spontaneous electrical activity for several days (Israel, personal communication), and this may be the reason why these groups were unable to detect the transient stimulatory effect. In this study, collagenase-dispersed cells responded very similarly to the chopped tissue preparation routinely used.

The electrophysiological responses of PI cells observed by Taraskevich and Douglas (1982) were suggested to correlate with the biphasic effect of GABA on αMSH secretion (Tomiko et al, 1983): thus GABA was considered to transiently depolarise the cells by an increase in chloride conductance, causing a transient burst of action potentials whose amplitude declined rapidly, with subsequent quiescence of the cells. The quiescence was attributed to the collapsed membrane resistance, rendering generation of spontaneous action potentials impossible (and, by implication, inhibiting hormone secretion). In these studies both components of the effect of muscimol were diminished in low Cl^- medium or in the presence of the anion channel blocker DIDS (Figure 5.9) suggesting that both components are the result of increased chloride channel opening, and therefore show close parallels with the results of Taraskevich and Douglas (1982). Spontaneous action potentials have been observed in normal PD cells (Ozawa and Sand, 1978; Taraskevich and Douglas 1977) as well as in transformed cells (Kidokoro, 1975; Dufy et al, 1979; Israel et al, 1981) which were stimulated by TRH and inhibited by DA, consistent with this hypothesis, and they have recently been directly demonstrated in lactotrophes (Ingram and Mason, 1985). There are, however, various results presented here which suggest that this relatively simple explanation for the biphasic effect of GABA and muscimol is inadequate, and that two different populations of $GABA_A$ receptors (or at least, two types or states of $GABA_A$ receptor complex), which cause a pure stimulation or inhibition of prolactin secretion are present.

The first indications of pharmacological discrepancies in the two effects was provided by the study of a range of GABA analogues:

THIP, P4S, isoguvacine and APS. These analogues have a rather more rigid structure than GABA or muscimol and are generally potent agonists (Krogsgaard-Larsen, Johnston, Lodge and Curtis, 1977; Krogsgaard-Larsen and Falch, 1981). They do show some differences in their effects, notably their reduced efficacy in vitro at stimulating benzodiazepine binding under certain conditions (Karobath and Lippitsch, 1979; Braestrup, Nielsen, Krogsgaard-Larsen and Falch, 1979; Wong and Iversen, 1985). These four $GABA_A$ agonists had a biphasic effect on prolactin secretion, but the amount of stimulation produced was low, relative to the amount of inhibition. Thus at 10μM these agonists produced less than half the amount of stimulation caused by equimolar GABA, but similar amounts of inhibition (Figure 5.5 and 5.6). This suggests a somewhat selective potency of these compounds for the inhibitory effect, in contrast with the non-selective action of GABA and muscimol, which retained a 10 fold difference in potency on both effects. This contrasts with the 'atypical' agonists potency both at displacing [^{3}H]GABA binding and at depressing cat dorsal horn interneurone firing (Krogsgaard-Larsen and Falch, 1981), in both of which models they are considerably more potent than GABA itself, but the percentage stimulation of secretion caused by these six agonists (at 10μM) correlates very well ($r = 0.96$) with channel lifetime recorded in fluctuation analysis experiments on cultured mouse spinal neurones (Barker and Mathers, 1981).

Dual $GABA_A$ effects of depolarisation at dendrites and hyperpolarisation at somata have been reported in studies on both hippocampal slices (Alger and Nicoll, 1979; 1982) and cultured mouse spinal neurones (Barker and Ransom, 1978a). Alger and Nicoll (1982)

found that THIP was surprisingly weak at causing depolarisation relative to its potency at causing hyperpolarisation, and concluded that two distinct GABA receptors may be involved. The demonstration here of two distinct types of $GABA_A$ response simultaneously on the same secretory cells obviates the possibility that in other models differences in the observed properties of $GABA_A$ responses may result from differences in agonist uptake or in ion gradients of the cells and their processes studied.

The GABA-containing dipeptide homocarnosine is present in hypophysial portal blood in high concentration (Mitchell et al, 1983) and has previously been briefly reported to inhibit prolactin secretion in vitro (Schally et al, 1977). In these experiments, homocarnosine differed from all the other $GABA_A$ agonists tested in that it caused no stimulation of secretion, even at a concentration of 2mM. Instead, a prompt and sustained inhibition of secretion was observed (Figures 5.4 and 5.5). This effect was both concentration-dependent and antagonised by BMI. Further evidence for the specificity of this effect despite the low potency is provided by the demonstration that neither glycine (100μM) or taurine (1mM) had any effect on prolactin secretion (Figure 5.8).

One interpretation of the action of homocarnosine is that there are two types or states of $GABA_A$ receptor complex on lactotrophes which mediate the two observed responses independently. In this analysis, homocarnosine is selective for the "receptor" mediating inhibition of secretion. The absence of the stimulatory effect could be explained in two ways, the first being that homocarnosine does not bind to the stimulatory $GABA_A$ site i.e. has no affinity for it, and the second being that homocarnosine binds but does not

'activate' it i.e. has affinity but no efficacy, and thus acts as an antagonist. As GABA and other agonists have efficacy at both sites, the response observed experimentally is the sum of two (independent) responses, while the response to homocarnosine is a 'pure' response to activation of one type. Therefore taking into account the inhibitory effect of homocarnosine allows reanalysis of the stimulatory effects of full or partial agonists. This was attempted using concentrations of GABA and homocarnosine which gave equal inhibitory responses. 3μM GABA caused 21% stimulation over the response to 1mM homocarnosine, but the combination caused only 7% stimulation over the effect of 2mM homocarnosine (Figure 5.7). If homocarnosine did not bind to the stimulatory site, then the combination would be expected to give a very similar result to GABA alone, as there is already an inhibitory effect equivalent to 1mM homocarnosine in the response to GABA. This was not the case, and these results suggest that homocarnosine specifically antagonised the stimulatory effect of GABA.

Agonists are not a powerful tool to demonstrate receptor multiplicity, and although homocarnosine appears able to act as an antagonist of one response and an agonist of the other, further pharmacological characterisation is required.

Bicuculline and PTX are both well-recognised $GABA_A$ antagonists (Curtis et al, 1971a; Gallindo, 1969; Hill, Simmonds and Straughan 1972) and strychnine is regarded as a glycine antagonist (Curtis et al, 1971b; Kelly and Renaud, 1973; Simmonds, 1983). In these experiments the quaternary N-methylated bicuculline methiodide was used as bicuculline itself is very unstable in solution (Olsen, Ban, Miller and Johnston, 1975). [^{3}H]BMI has been used to label GABA

binding sites (Mohler and Okada, 1977b), and in support of a direct interaction at the GABA receptor, bicuculline antagonises GABA responses competitively in the mammalian CNS (Simmonds, 1980b). The PTX binding site (using [^{3}H]dihydropicrotoxinin) (Ticku, Ban and Olsen, 1978) appears distinct, and PTX antagonises mammalian CNS GABA responses noncompetitively (Simmonds, 1980b) although 'mixed' effects are seen in lobster (Constanti, 1978). Strychnine has been reported to be approximately twenty times more selective an antagonist at glycine than GABA receptors (Simmonds, 1983), but appears to act competitively as a GABA antagonist, at the same site as bicuculline (Simmonds, 1982).

When tested against 10μM muscimol at an equimolar concentration, only BMI antagonised the inhibitory effect, and PTX and strychnine appeared especially potent at blocking the stimulatory effect (Figure 5.9). At higher concentrations of antagonist, all caused significant antagonism of the inhibitory effect. A more detailed comparison of the three as antagonists of the stimulatory effect of muscimol was carried out using a higher concentration of muscimol, 30μM, as the larger effect thus produced enables percent antagonism to be determined more accurately. These results confirmed the greater potency of PTX than BMI, and strychnine appeared intermediate (Figure 5.10). The selectivity of PTX and strychnine was also confirmed, as even at concentrations as high as 100μM, no antagonism of the inhibitory effect was observed. At this concentration of strychnine the stimulatory effect of muscimol was totally abolished, such that the response started to resemble that to homocarnosine i.e. an immediate inhibition of secretion. Lamberts and MacLeod (1978) also found that PTX was ineffective as

an antagonist of GABAergic inhibition of prolactin secretion in vitro, but others have found that it did antagonise the effect of GABA (Enjalbert et al, 1979a). These results, showing selective antagonism by strychnine (and to a lesser extent by PTX, as 30μM PTX caused a slight but not significant antagonism of the inhibitory response to 30μM muscimol) provide further evidence that the two components of the biphasic response to muscimol are mediated by independent receptor complexes with different pharmacological properties.

Glycine was found to have no effect on prolactin secretion alone at 100μM, but when co-introduced with 10μM muscimol significantly potentiated the stimulatory effect, without altering the inhibitory effect (Figure 5.8). Glycine and GABA act very similarly by opening anion channels (Barker and McBurney 1979a) but several lines of evidence suggest that independent receptor sites are involved: effects of GABA and glycine are selectively antagonised (Curtis et al, 1971a, b; Nicoll, Padjen and Barker, 1976), GABA is a very weak displacer of [^{3}H]strychnine binding (Muller and Snyder, 1978) and glycine is a very weak displacer of [^{3}H]GABA binding (Zukin et al, 1974), and cultured spinal cord neurones grown in the presence of one of the amino acids become unresponsive to that one, without responsiveness to the other being affected (Nelson, Ransom, Henkart and Bullock, 1977). Furthermore, if the amino acids shared one receptor, glycine, being inactive here, would be expected to antagonise, not potentiate, the response to muscimol. Barker and McBurney (1979a) found some cross-desensitisation, and suggested that the two independent receptors may share a common channel, which could still be activated by glycine when GABA receptors were largely

desensitised but which was resistant to activation by GABA when glycine receptors were only partially desensitised. If desensitisation contributes substantially to limiting the stimulatory response to muscimol, then a similar mechanism may contribute to the potentiation by glycine of the response observed here. These results suggest that glycine receptors can modulate GABAergic responses on lactotrophes, glycine itself potentiating the response and strychnine antagonising it. This modulation appears selective for the stimulatory response.

Strychnine is a potent antagonist of GABA-receptor stimulated benzodiazepine binding at some, but not all GABA/benzodiazepine receptor complexes (Braestrup and Nielsen, 1980), and benzodiazepines (and barbiturates) potentiate the stimulatory effect of muscimol on prolactin secretion without altering the inhibitory effect (Chapter 6). Furthermore, the agonists THIP, P4S, APS and isoguvacine are only weakly active as stimulators of both prolactin secretion (here) and benzodiazepine binding (Karobath et al, 1979; Braestrup, et al, 1979). The selective inhibitor of prolactin secretion, homocarnosine is inactive on benzodiazepine binding (Karobath et al, 1979).

It therefore appears that the $GABA_A$ receptor complex which upon activation causes a stimulation of prolactin secretion is associated with a variety of modulatory sites while the complex which causes inhibition of prolactin secretion is much simpler, having none of these. Despite these differences, both are chloride-dependent effects, and the GABA-binding part of the receptor complex may have similar properties in both cases, as BMI did not show any selectivity and muscimol and GABA showed similar potency ratios for both effects. Although both components of the

response to muscimol were found to be chloride-dependent, the possibility that other ion channels are involved exists. Potassium channels are of great importance in the regulation of membrane excitability, and a series of experiments were carried out to investigate the effect of K^+ channel block on the response to muscimol.

Potassium channels and their properties have been the subject of several recent reviews (Adams, Smith and Thompson, 1980; LaTorre and Miller, 1983; Petersen and Maruyama, 1984). 4AP blocks the transient K^+ current, I_A (Thompson, 1977) in molluscs and a similar current in hippocampus (Gustaffson, Galvan, Grafe and Wigstrom, 1982). This current is considered to have a major influence on excitability, as it is active at a more negative voltage than other K^+ currents, and slows the rate of depolarisation thus delaying generation of the action potential. Blockade of this channel by 4AP would therefore be expected to increase the frequency and duration of action potentials, and this has been observed in GH_3 cells (Ozawa and Kimura, 1979; Sand, Haug and Gautvik, 1980). 4AP was found to cause a stimulation of prolactin secretion (Figure 5.15) and subsequently, introduction of 10μM muscimol caused a considerably greater stimulation of secretion than in controls, but no change in the amount of inhibition (Figure 5.11). TEA blocks the voltage-activated late K^+ current, I_K (the delayed rectifier) (Hille, 1967) and in vertebrates, but not molluscs, the Ca^{2+}-activated K^+ current, I_C (Thompson, 1977; Adams, Constanti, Brown and Clark, 1982b). The main function of I_K appears to be to provide outward current to repolarize the membrane during a spike, and I_C is activated by increased

intracellular Ca^{2+} concentration, thus also contributing to repolarisation. TEA would therefore be expected to increase the duration of action potentials, which has been observed in GH cells (Taraskevich and Douglas, 1980). TEA caused a transient stimulation of prolactin secretion (Figure 5.15) but did not affect either the stimulatory or the inhibitory component of the response to 10μM muscimol (Figure 5.11). These results suggest that a current similar to I_A is of importance in limiting the stimulation of secretion caused by muscimol. This result is not unexpected, as I_A is a major influence on excitability in the mollusc (Adams et al, 1980) but demonstrates the ubiquity of both the channel and its effect. In similar experiments 4AP potentiated acetylcholine-induced GH release and ^{86}Rb efflux (a marker for K^+) (Schofield and Smith, 1981). The lack of effect of I_K block may reflect the more reluctant activation of this current in response to partial depolarisation (compared to I_A). Alternatively spike frequency rather than duration may be a more important factor in stumulus-secretion coupling (at least as measured here). I_C has been demonstrated in an ACTH-secreting cell line (Wong, Lecar and Adler, 1982) and may be of considerable importance in the control of secretion in both endocrine and exocrine glands (Petersen and Maruyama, 1984). The lack of effect of TEA on the response to muscimol is therefore perhaps surprising but indicates that in practice I_k and I_c are unlikely to be of importance in either modulating or even mediating any component of the $GABA_A$ responses. The lack of effect of TEA here contrasts with the potentiation of the stimulatory effect of TRH on GH secretion by TEA (Saith, Bicknell and Scholfield, 1982). TRH may act directly on

K^+ channels (Ozawa and Kimura 1979; Kaczorowski, Vandlen, Katz and Reuben, 1983; Barker, Dufy, Owen and Segal, 1983) which would explain this. Any further explanation of the present results is impossible without electrophysiological studies of the effect of muscimol and the properties of K^+ currents in lactotrophes. Similarly, the mechanism underlying the inhibitory effect of muscimol on prolactin secretion, and its apparent independence of K^+ currents, must await electrophysiological study.

In conclusion, the experiments using 4AP and TEA demonstrated that both are prolactin secretagogues. It was not possible to establish involvement of another channel type in the response to muscimol because of the importance of the currents blocked by 4AP and TEA in the regulation of membrane excitability and therefore basal hormone secretion, and similar problems would beset any investigation into the involvement of Ca^{2+} channels using a similar model.

5.4.2. $GABA_B$ receptor effects

100μM baclofen had no effect on basal prolactin secretion (Figure 5.2) or the stimulation of secretion caused by 30mM K^+ or 3mM Ba^{2+} (Figure 5.12). Ba^{2+} prolongs action potential duration in normal and transformed PD cells (Ozawa and Sand, 1978; Taraskevich and Douglas, 1980) possibly by blockade of I_M K^+ currents (Fatt and Ginsborg, 1958; Adams, Brown and Constanti, 1982a), and can also pass through Ca^{2+} channels more easily than Ca^{2+} itself (Hagiwara and Byerly, 1981). Both these effects have been observed in melanotrophes (Douglas and Taraskevich, 1980, 1982), where Ba^{2+} has been demonstrated to be a potent secretagogue (Douglas, Taraskevich and Tomiko, 1983). Ba^{2+} has

been used to demonstrate $GABA_B$ receptor-mediated inhibition of αMSH secretion (Demeneix et al, 1984). Baclofen was found to inhibit basal αMSH secretion by ≈25% and Ba^{2+}-induced secretion by up to 60%. These effects were interpreted primarily as a reduction in the Ca^{2+} component of each action potential, as observed electrophysiologically both in PI and on sensory neurones (Dunlap, 1981; Desarmenien, Santangelo, Occhipinti, Schlichter, Loeffler, Desaulles, Demeneix and Feltz, 1983). There was no such effect on prolactin secretion, implying that lactotrophes do not have $GABA_B$ receptors, or that if they do, they do not affect basal or Ba^{2+}-induced secretion through an apparent regulation of Ca^{2+} currents as in melanotrophes.

Early studies on $GABA_B$ receptors were concerned with their presynaptic mechanism of action (Bowery et al, 1980), in particular their effect on Ca^{2+} components of action potentials, as mentioned above (Dunlap, 1981; Desarmenien, Feltz, Occhipinti, Santangelo and Schlichter, 1984; Cherubini and North, 1984). More recently other mechanisms of action of baclofen have been investigated, particularly interactions with adenylate cyclase (Wojcik and Neff, 1984; Hill, 1985) and K^+ channels (Newberry and Nicoll, 1984a; Inoue, Matsuo and Ogata, 1985). These possible mechanisms of action are by no means mutually exclusive, as will be discussed below.

$GABA_B$ receptor binding is depressed by GTP (Hill, Bowery and Hudson, 1984), an essential cofactor in the activation or inhibition of adenylate cyclase (Rodbell, 1980). Investigations into the effects of baclofen on adenylate cyclase activity have been apparently conflicting: baclofen has been shown to potentiate the

stimulatory effect of catecholamines, histamine and VIP (Hill, 1985; Karbon and Enna, 1985). In contrast, baclofen inhibited the stimulatory effect of forskolin (Wojcik and Neff, 1984; Hill, 1985; Karbon and Enna, 1985). This inhibitory effect was shown to be dependent on GTP (Wojcik and Neff, 1984) and baclofen activated high-affinity guanosine 5'-triphosphatase (Wojcik, Cavella and Neff, 1985), suggesting involvement of N_i, the GTP binding protein that is inhibitory to adenylate cyclase. VIP is well recognised to stimulate prolactin secretion in vitro (Ruberg et al, 1978; Enjalbert, Arancibia, Ruberg, Priam, Bluet-Pajot, Rotsztejn and Kordon 1980). VIP activates PD adenylate cyclase (Robberecht, Deschodt-Lanckman, Camus, De Neef, Lambert and Christophe 1979; Giachetti, Borghi, Nicosia and Said, 1979) which has been proposed to be the mechanism of the biological effects of VIP (Dorflinger and Schonbrunn, 1983). Therefore if baclofen affects adenylate cyclase activity in lactotrophes, this might be reflected by an alteration in the prolactin-releasing effect of VIP: a potentiation if baclofen enhances the cAMP-producing effect of VIP and a reduction if the converse is true. When co-introduced, 100μM baclofen reduced the response to VIP by ≃ 35%, in a stereospecific manner (Figure 5.13). This suggested that baclofen might indeed be inhibitory to the activation of adenylate cyclase, but if that is so then baclofen might also cause an inhibition of prolactin secretion under other circumstances in which cAMP concentrations are elevated. In comparison, somatostatin has been shown to inhibit both receptor-mediated and non-receptor- mediated increases in cAMP and ACTH secretion (Heisler, Reisine, Hook and Axelrod, 1982). This was investigated using dbcAMP and the phosphodiesterase inhibitor IBMX,

and forskolin and cholera toxin. Forskolin is believed to act directly on the catalytic subunit of adenylate cyclase (Seamon and Daly, 1981) whereas cholera toxin acts on the N_s GTP binding protein (Bokoch, Katada, Northrup, Hewlett and Gilman, 1983). Both substances have been shown to cause very large and rapid increases in PD cAMP content, but inconsistent effects on the secretion of prolactin (Thorner, Hackett, Murad and MacLeod, 1980; Cronin, Myers, MacLeod and Hewlett, 1983). In these experiments, no marked effect of any of these treatments was observed, and 100μM baclofen, introduced 40 min later, was in no case observed to inhibit prolactin secretion (Figure 5.14). These results suggest two things:

1) the increase in cAMP caused by VIP may not be the major mechanism of the rapid stimulation of secretion observed in these cells.
2) consequently, any inhibitory effect of baclofen on cAMP accumulation is unlikely to be the cause of the observed inhibition of VIP-induced prolactin secretion.

These experiments in themselves do not provide any evidence for or against an effect of baclofen on adenylate cyclase activity in PD. TRH also increases cAMP accumulation in PD cells (Gautvik, Iversen and Sand, 1980) but its effect on prolactin secretion is likely to be a result of inhibition of I_A (Barker et al, 1983).

Baclofen has recently been reported to directly increase postsynaptic K^+ conductance in hippocampus (Newberry and Nicoll, 1984a; Inoue et al, 1985) and in the dorsolateral septal nucleus (Gallagher, Stevens and Shinnick-Gallagher, 1984). This effect (in hippocampus) could be antagonised by 4AP and was unaffected by TEA (Inoue et al, 1985), suggesting that it might be mediated by

facilitation of I_A-like currents. Both 4AP and TEA are prolactin secretagogues (see above) suggesting the involvement of currents equivalent or identical to I_A, I_K and I_C in the regulation of lactotrophe excitability. When cointroduced, 100μM baclofen reduced the prolactin-stimulating effect of 4AP in a stereospecific manner, without affecting the response to TEA (Figure 5.15). This suggests that baclofen can open K^+ (probably I_A-like) channels on lactotrophes, and parallels the results obtained using hippocampal slices. Although it is tempting to speculate as to the identity of the channel involved based on the pharmacological specificity of 4AP (Adams et al, 1980), discrepancies in the results obtained in the hippocampus between the baclofen-associated current and I_A (Inoue et al, 1985) prevent direct comparisons from being made.

These results provide a basis for a reexamination of the effects of VIP. Many other peptides have effects on K^+ currents e.g. TRH (Barker et al, 1983), opioids (Werz and Macdonald, 1983) and Substance P (Nowak and Macdonald 1982). This in turn may affect Ca^{2+} conductance (Werz and MacDonald, 1983; North and Williams, 1983). An alternative mechanism is suggested by the dependence of K^+ currents in Aplysia on transmitter-linked adenylate cyclase (Siegelbaum, Camardo and Kandel, 1982), and the ability of 8-bromo cAMP to inhibit I_C (Madison and Nicoll, 1982). In such a model the response to VIP may be produced by inactivation of a K^+ current mediated by an increase in cAMP. Baclofen might reduce either component but both the effect on Ca^{2+} conductance in sensory neurons and on K^+ currents in hippocampus appear to be independent of cyclic nucleotides (Dunlap and Fishbach, 1981; Newberry and Nicoll, 1984b; Dunlap, 1985). The inability of

non-receptor mediated increases in cAMP to cause a rapid prolactin response may reflect the importance of subcellular localization. In conclusion, these results provide evidence for $GABA_B$ receptors on lactotrophes which are capable of regulating the response to a physiological secretagogue. The inability of baclofen to inhibit basal or Ba^{2+} induced secretion of prolactin may reflect lack of association with Ca^{2+} conductances, and the reduction in response to 4AP (and VIP) suggests a direct involvement with a K^+ current (with or without an effect on adenylate cyclase).

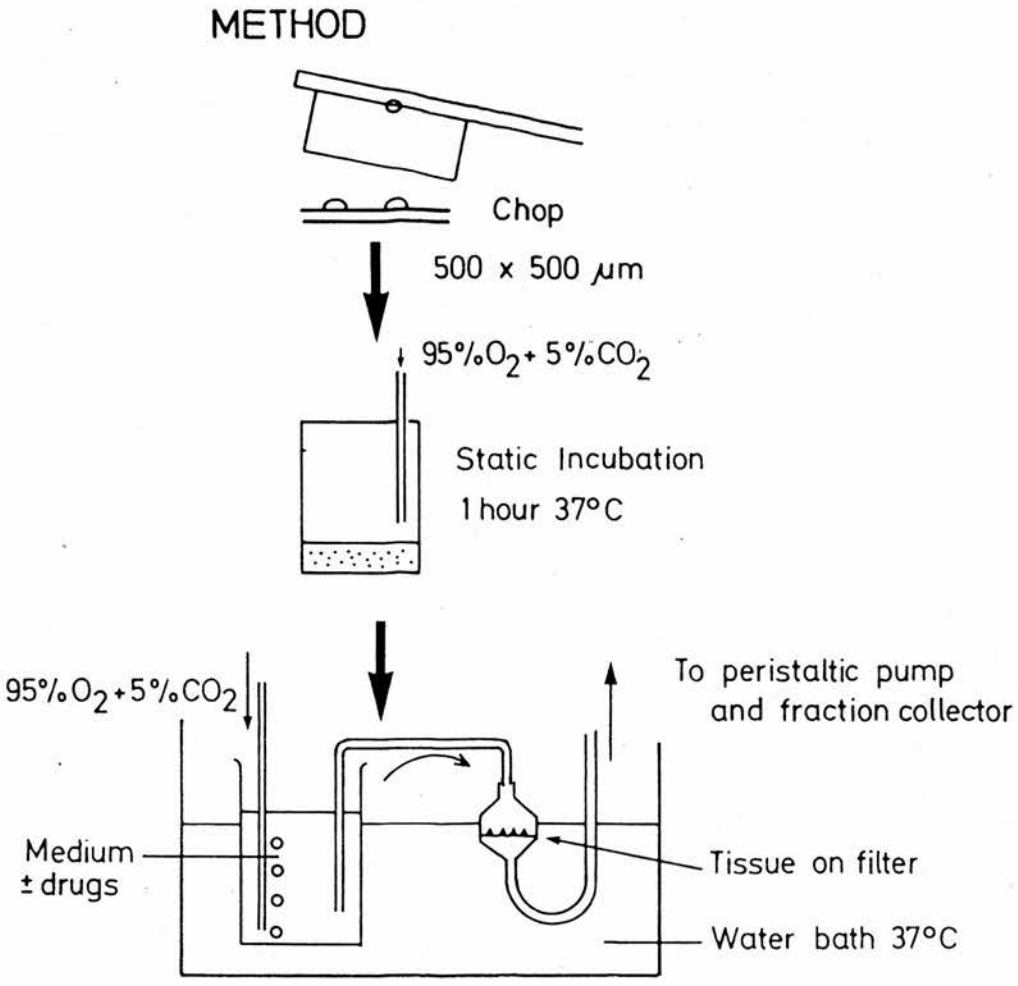


FIGURE 5.1

Method for preparation and superfusion of PD prisms.

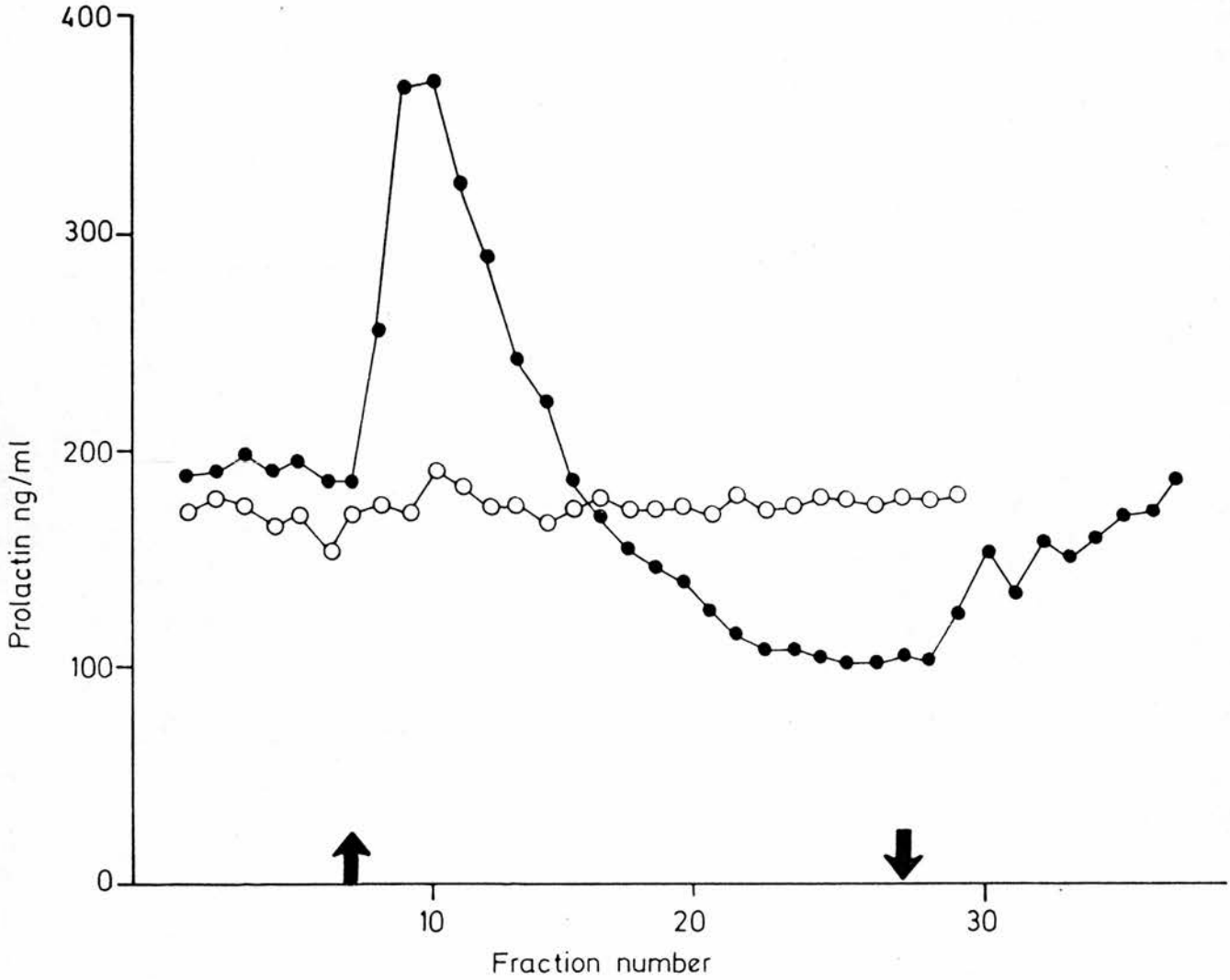


FIGURE 5.2

Effect of 10μM muscimol (●) and 100μM (±) baclofen (○) on prolactin secretion from PD prisms.
Drug introduced at ↑ and washed out at ↓
Representative experiments.

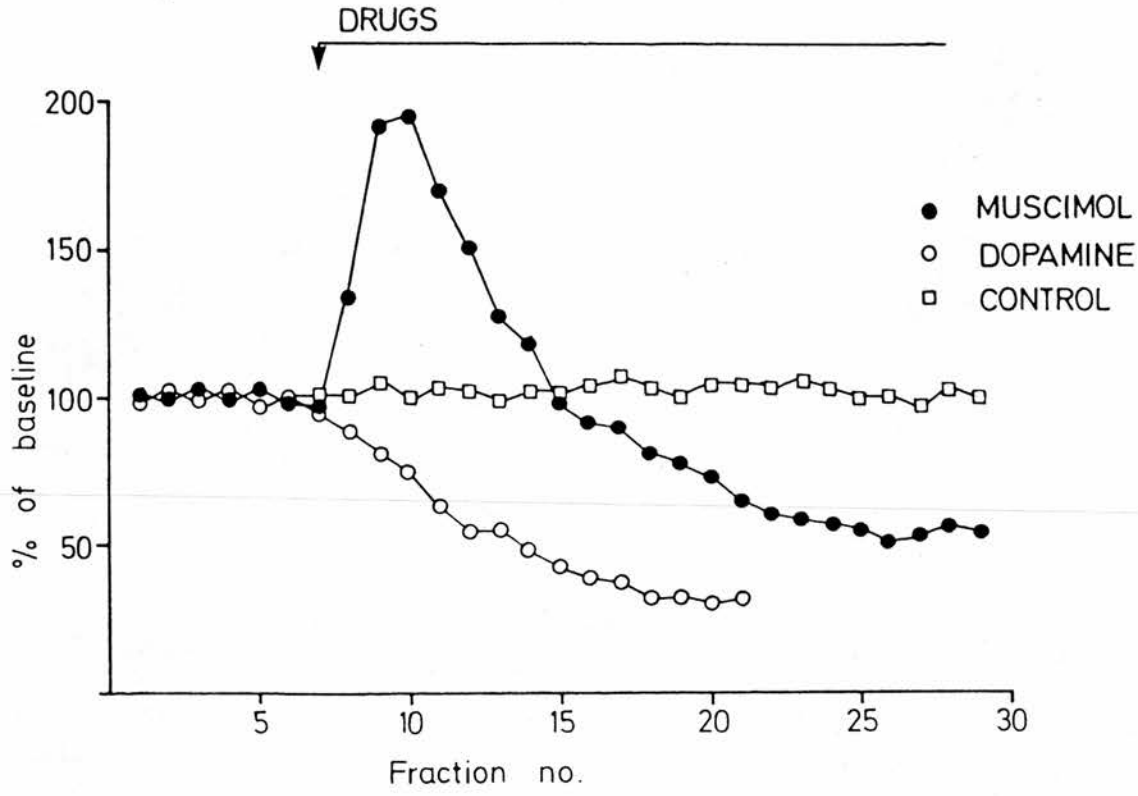


FIGURE 5.3

Comparison of the effects of 10μM muscimol (●) and 100nM DA (○) on prolactin secretion. Drugs were introduced at the arrow. Prolactin concentrations have been expressed as a percentage of pre-drug baseline concentration.

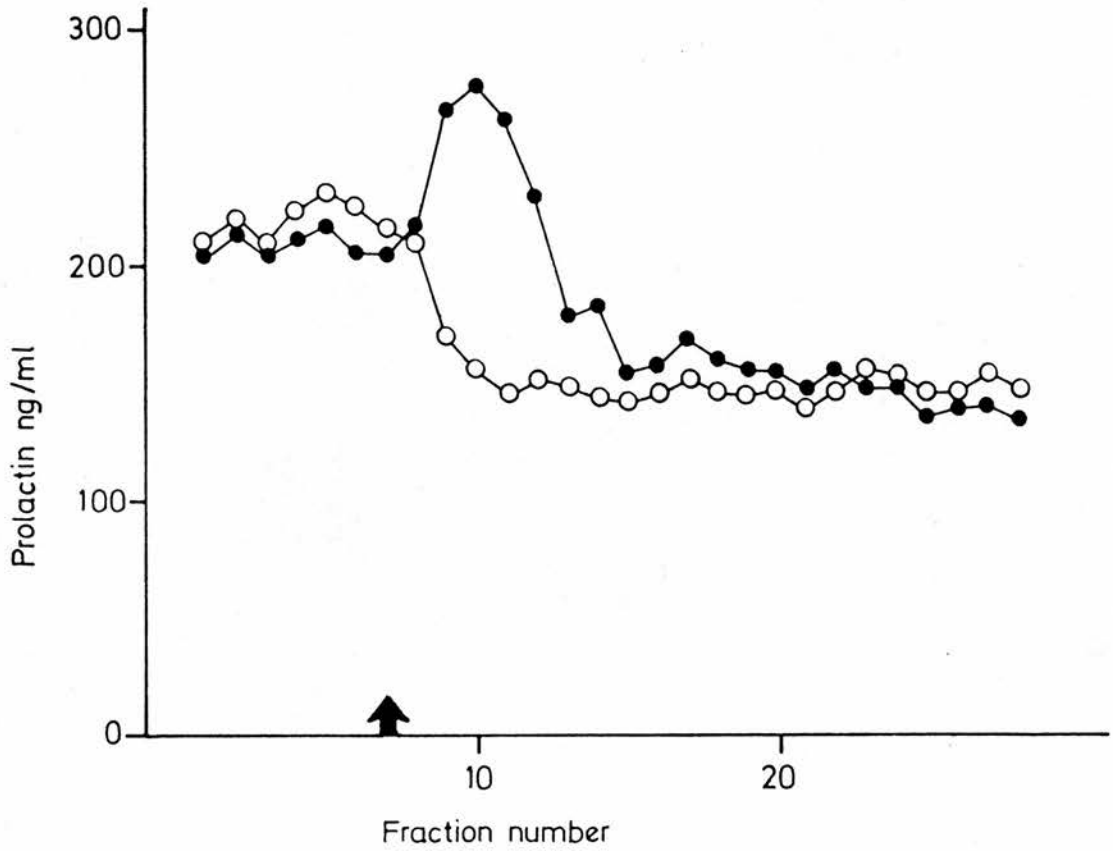


FIGURE 5.4

Effects of 10μM GABA (●) and 1mM homocarnosine (○) on prolactin secretion. Drugs were introduced at the arrow. Representative experiments.

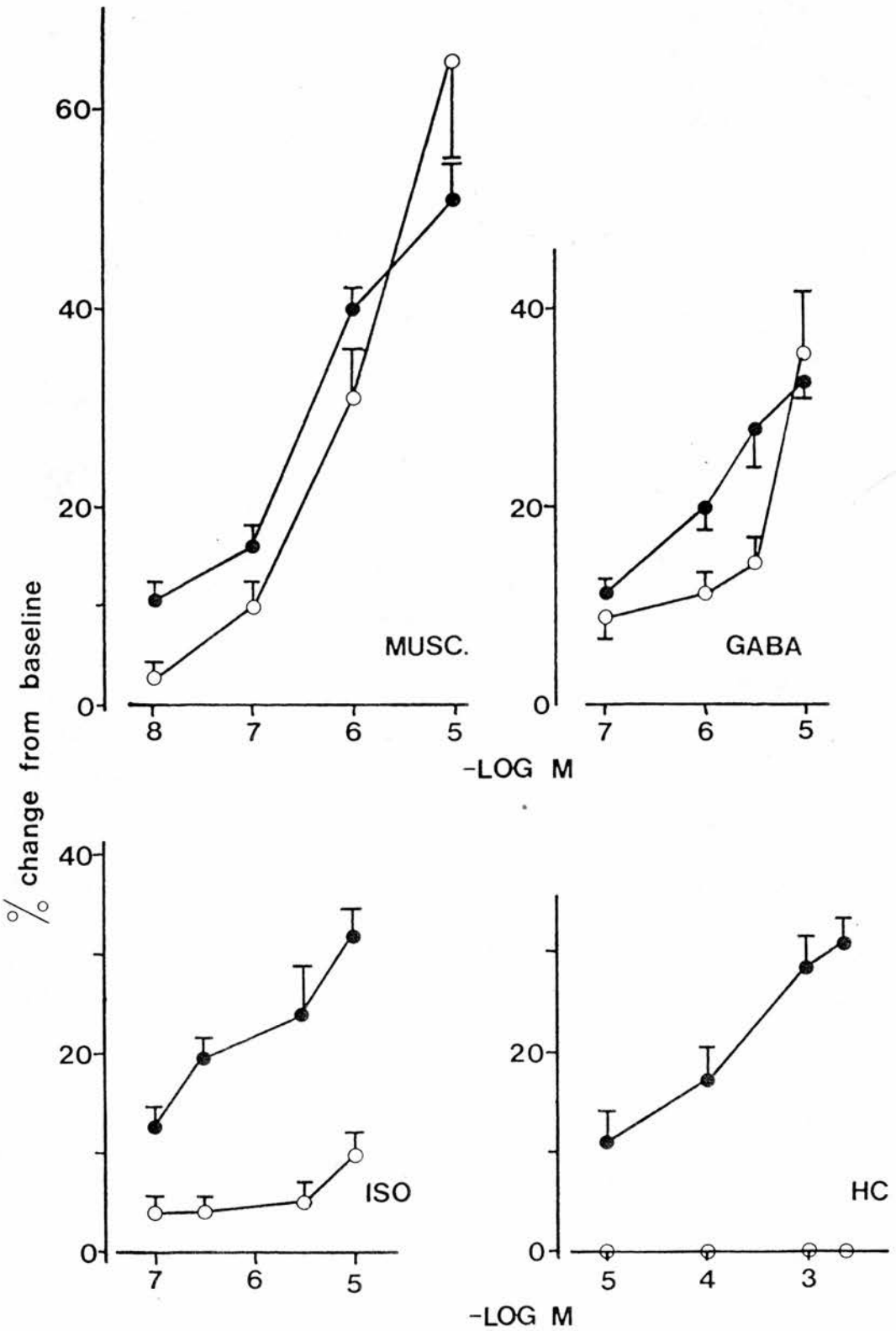


Figure 5.5

Concentration-response curves of the effects of some GABA agonists on prolactin secretion.
Results are expressed as percentage change from baseline.
(○) percentage stimulation
(●) percentage inhibition
Drugs: muscimol (MUSC); GABA; isogu vacine (ISO); homocarnosine (HC)
Mean ± S.E.M. n = 5-6 in each case.

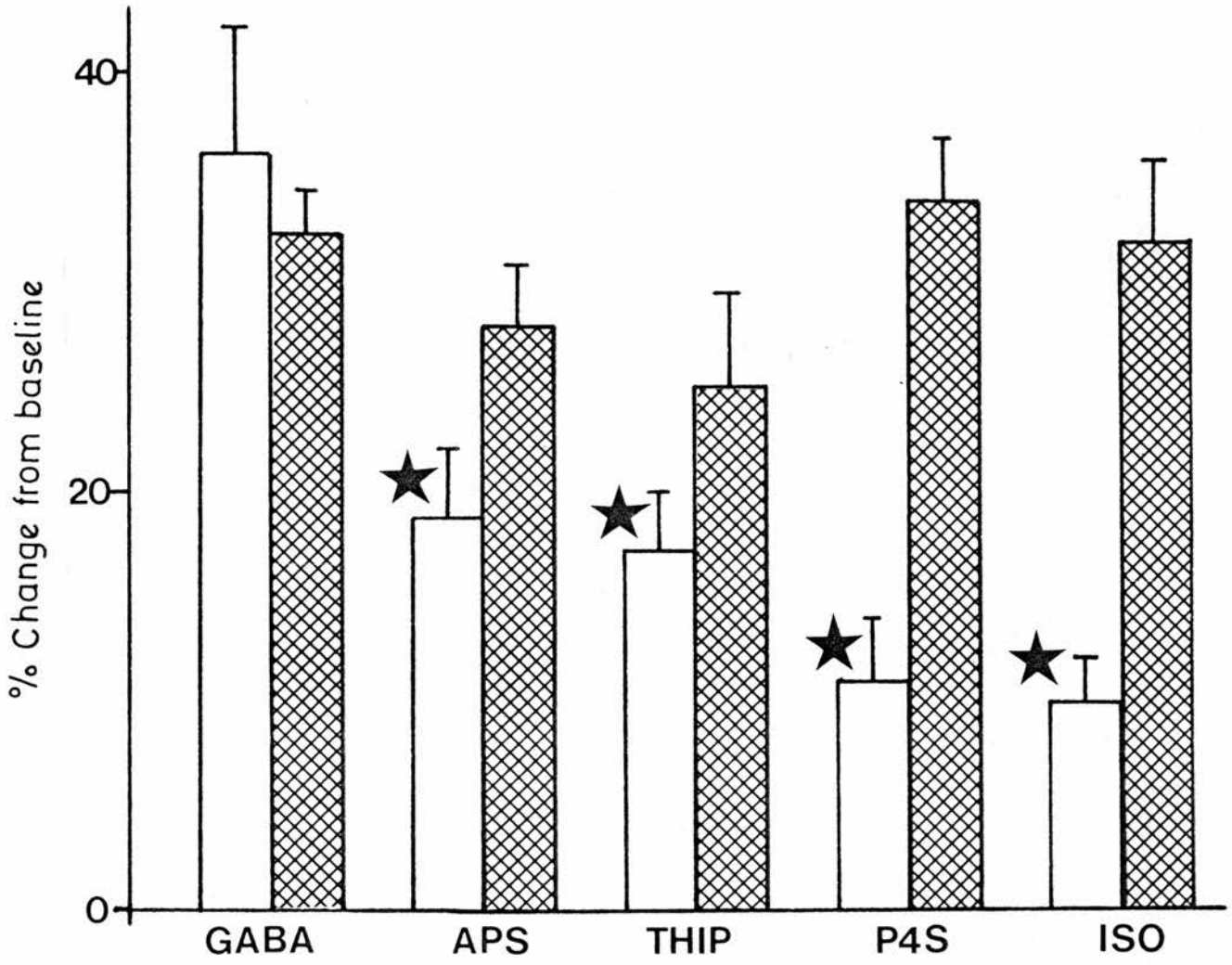
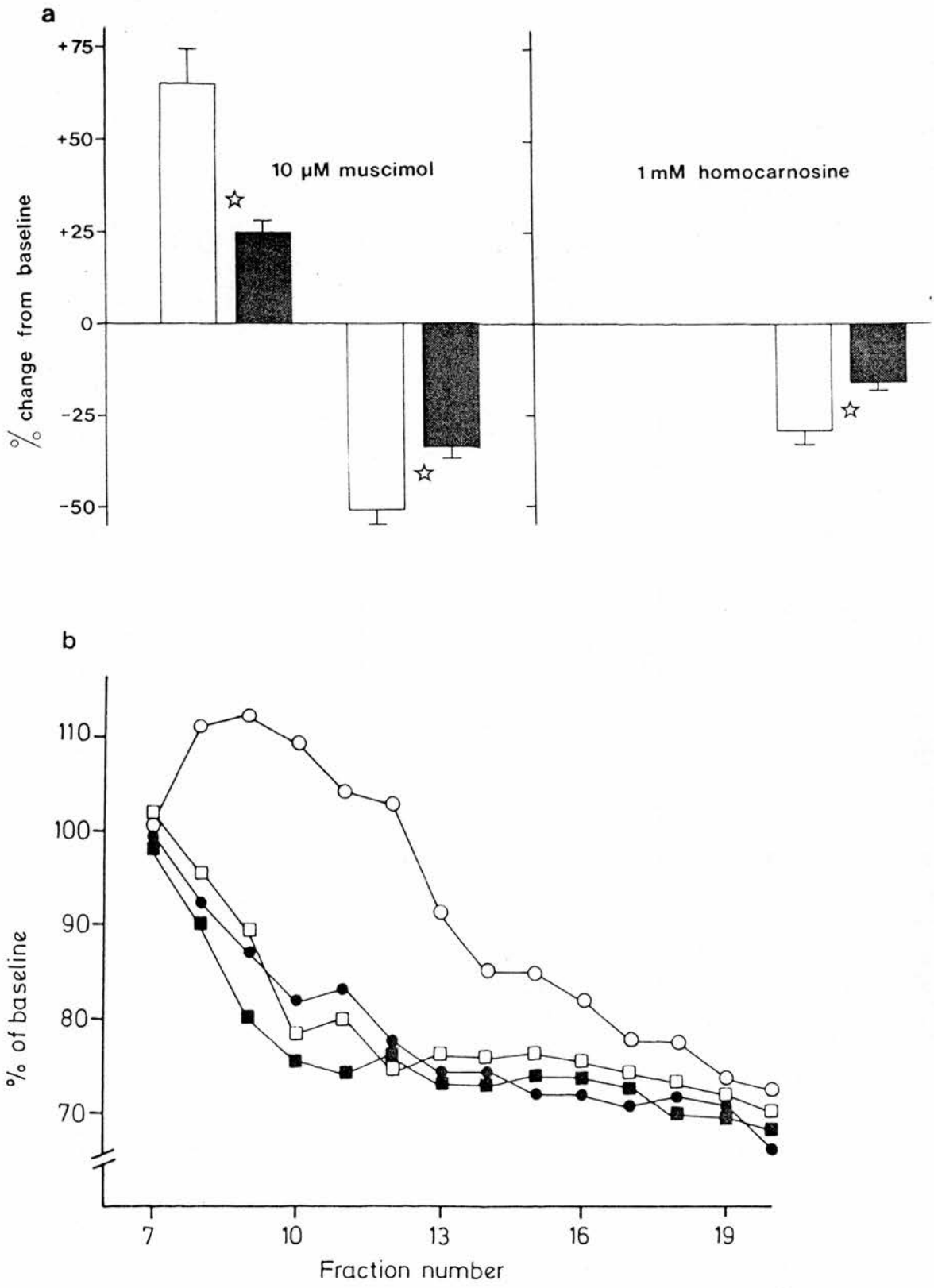


FIGURE 5.6

The effect of some GABAergic agonists on prolactin secretion. GABA, APS, THIP, P4S and Isoguvacine (ISO) were tested at 10μM.
Open bars: percentage stimulation of secretion
Hatched bars: percentage inhibition of secretion
*:$p<0.05$ vs response to GABA. Mean ± S.E.M., n = 5 in each case.


a
+75
+50
+25
0
-25
-50
% change from baseline
10 μM muscimol
1 mM homocarnosine
b
110
100
90
80
70
% of baseline
7
10
13
16
19
Fraction number

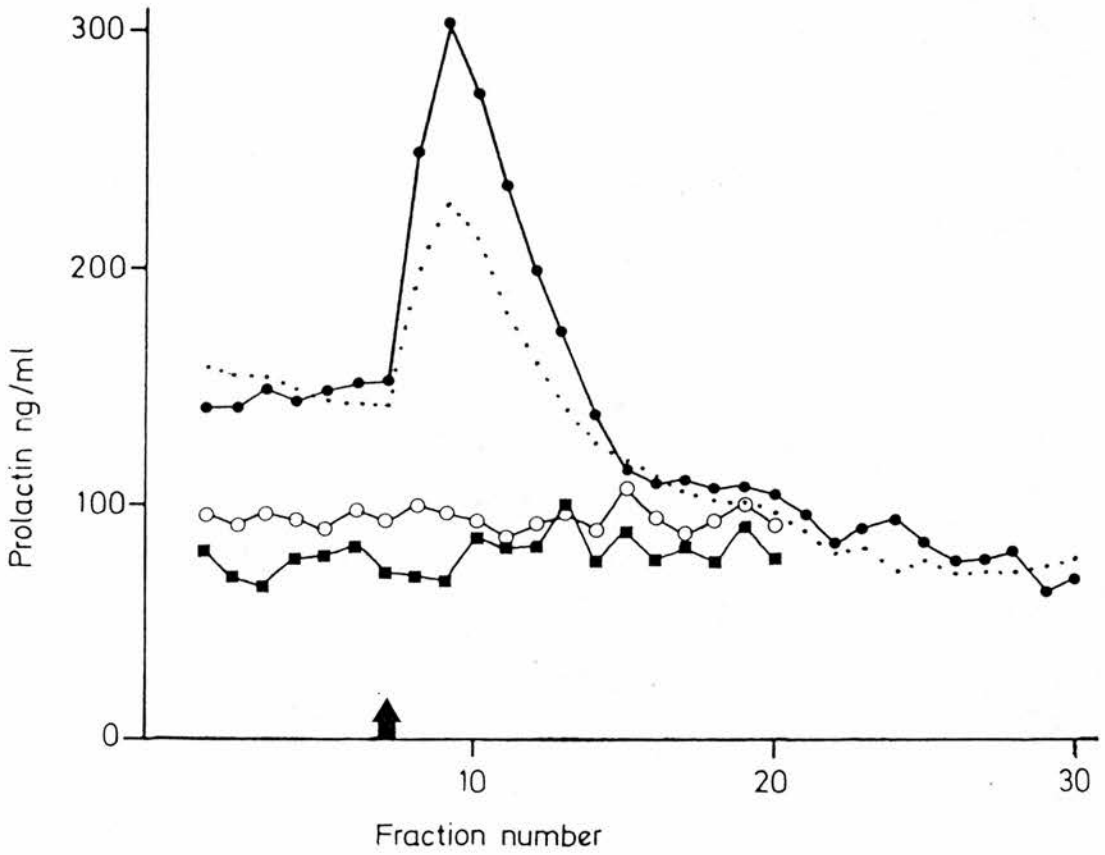
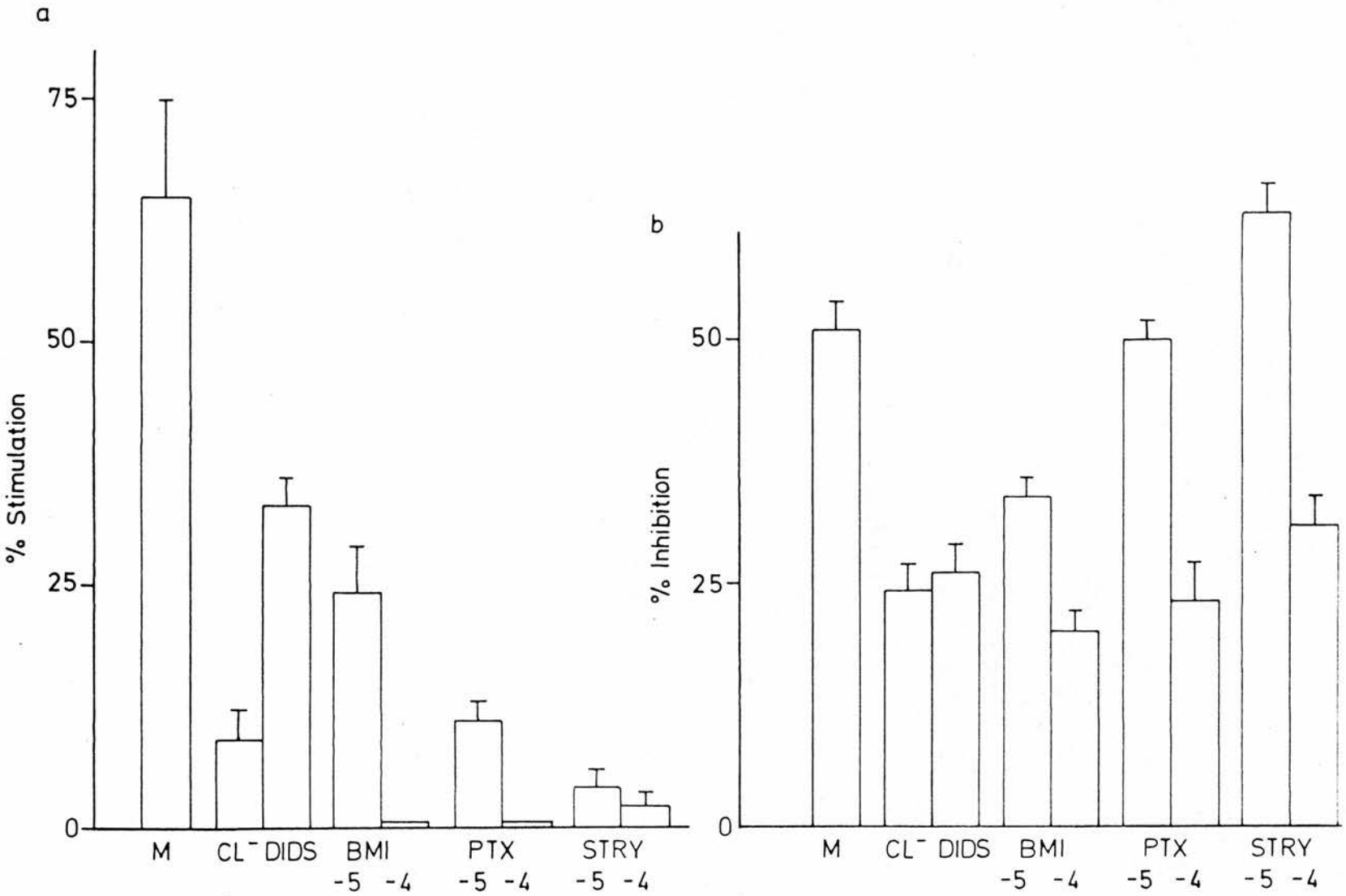


FIGURE 5.8

Effects of glycine and taurine on prolactin secretion and modulation of the effect of muscimol by glycine.

(○) 100μM glycine
(■) 1mM taurine
(●) 100μM glycine with 10μM muscimol

The dotted line shows a control response to 10μM muscimol.
Representative experiments, drugs were introduced at the arrow.


a
75
50
25
0
% Stimulation
M
CL⁻
DIDS
BMI
-5
-4
PTX
-5
-4
STRY
-5
-4
b
50
25
0
% Inhibition
M
CL⁻
DIDS
BMI
-5
-4
PTX
-5
-4
STRY
-5
-4

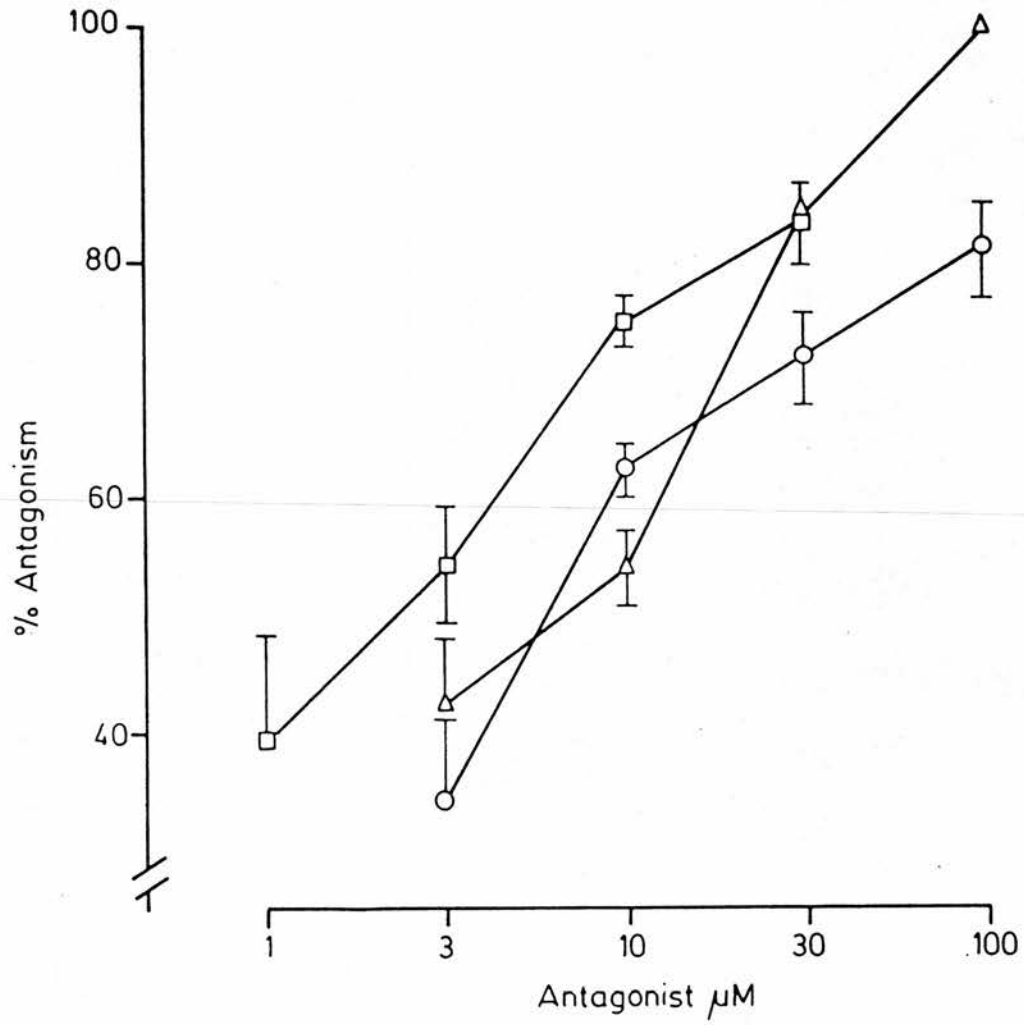


FIGURE 5.10

Antagonism of the stimulatory effect of 30μM muscimol on prolactin secretion. Antagonism by BMI (○)
PTX (□)
strychnine (△).
Results were calculated as percentage antagonism of control responses. Mean ± S.E.M., n = 5.

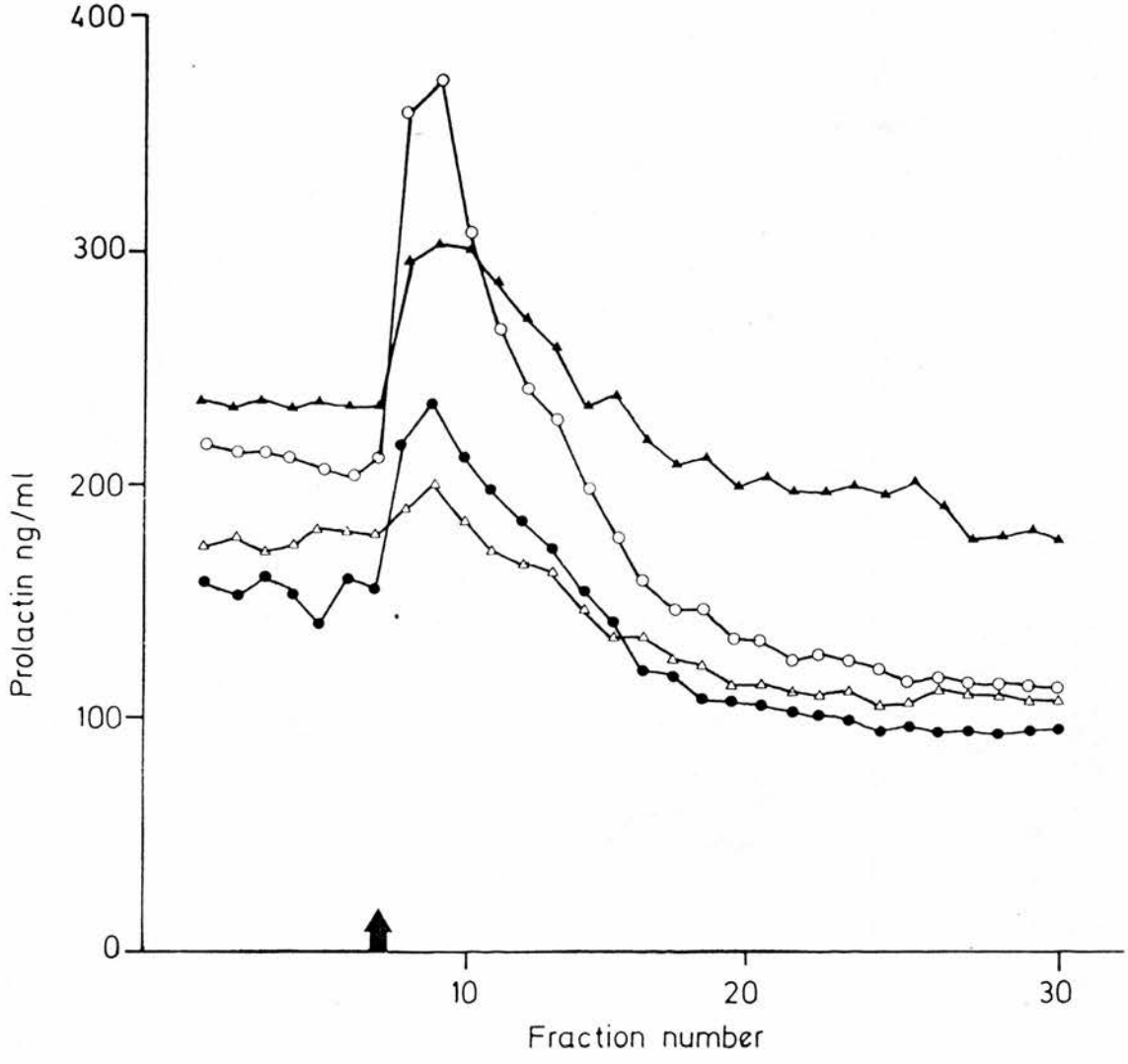


FIGURE 5.11

The effect of ionic manipulation on the effect of muscimol on prolactin secretion. Responses are shown to 10μM muscimol in the presence of

(△) low-chloride medium
(▲) 40μM DIDS
(○) 1mM 4AP
(●) 10mM TEA

Representative experiments. The response in the presence of TEA is effectively the same as control.

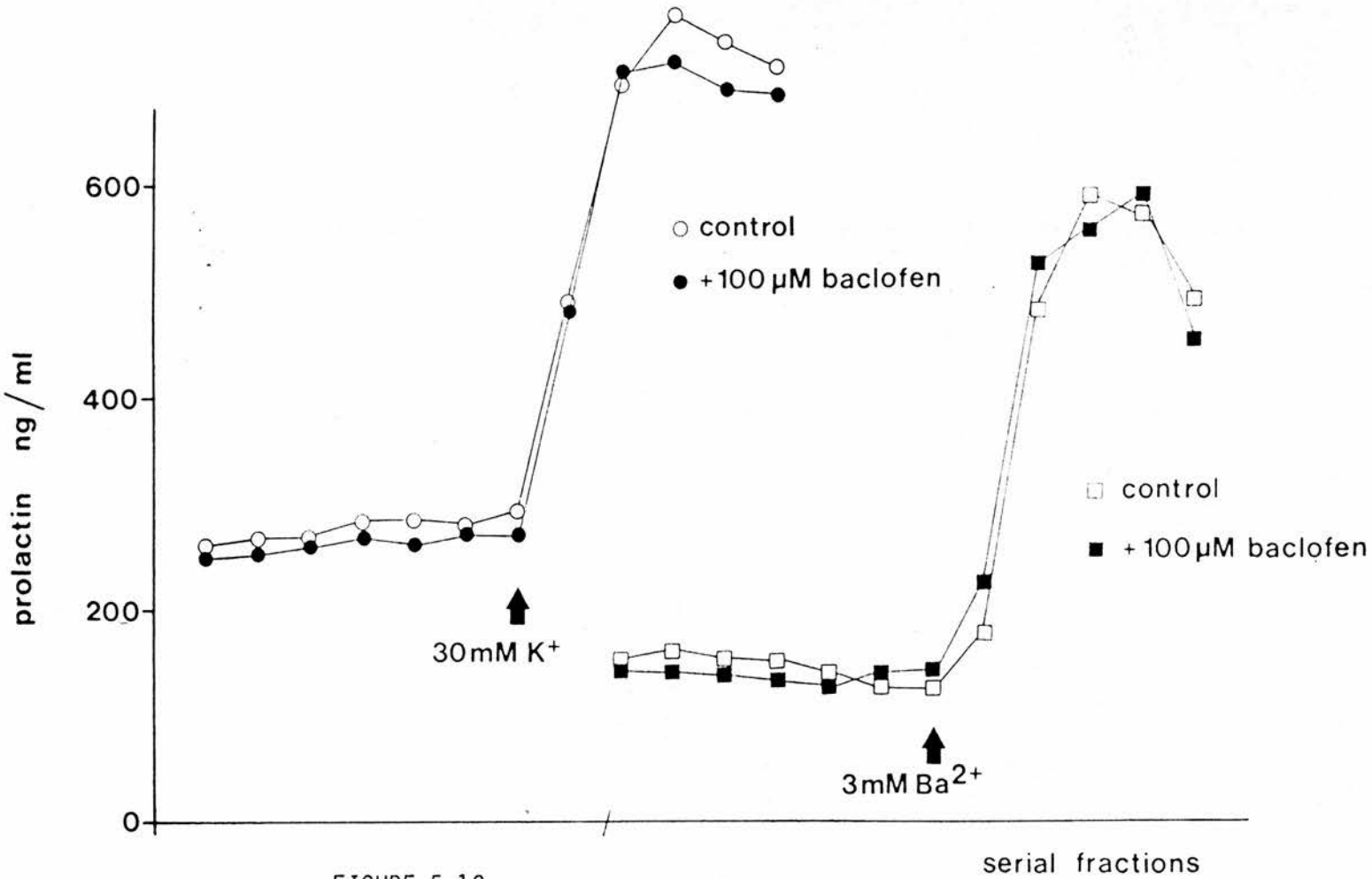


FIGURE 5.12

Effect of baclofen on K^+ and Ba^{2+} stimulated prolactin secretion.

(○) 30mM K^+ alone
(●) 30mM K^+ with 100µM (±)baclofen.
(□) 3mM Ba^{2+} alone
(■) 3mM Ba^{2+} with 100µM (±)baclofen

Representative experiments.

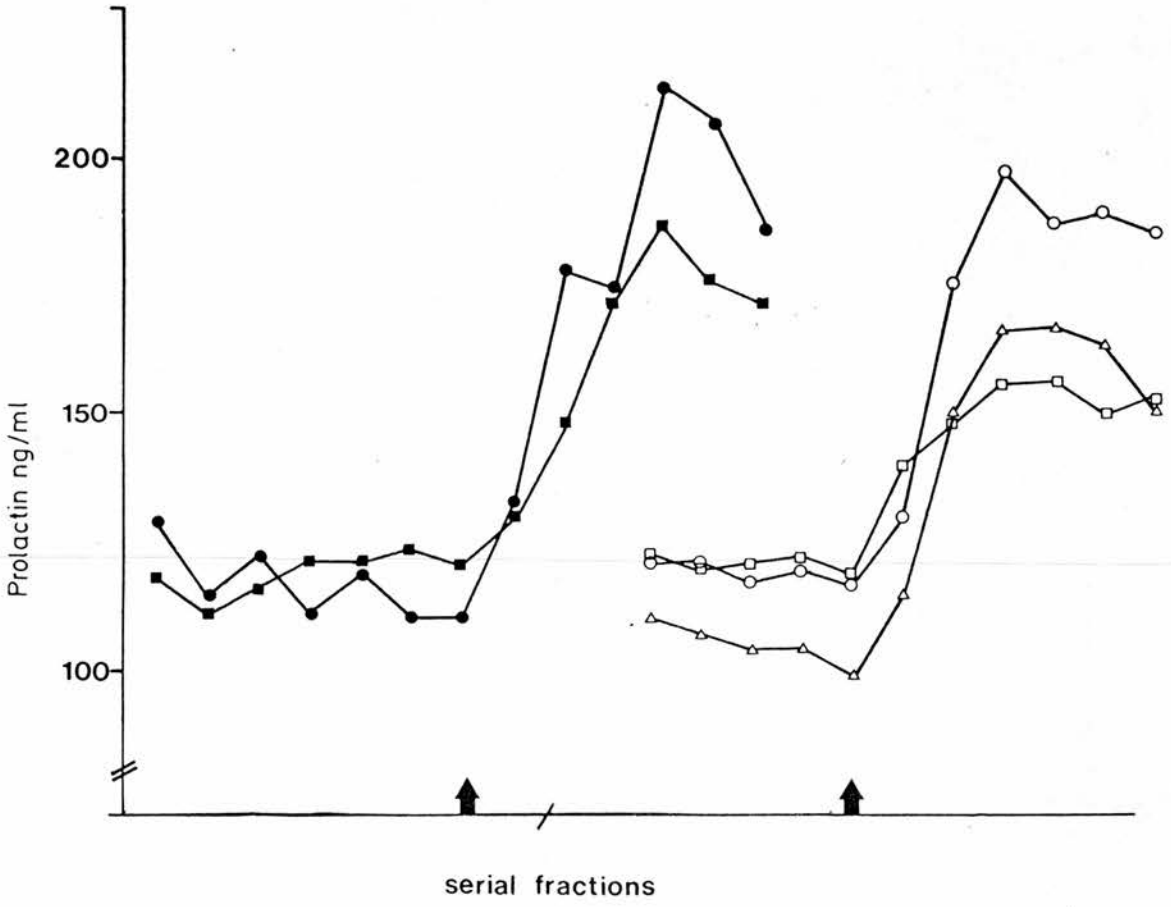


FIGURE 5.13

Inhibition of VIP stimulated prolactin secretion by baclofen.

(●) Effect of 10nM VIP
(■) Effect of 10nM VIP with 100μM (±)baclofen
(○) Effect of 7.5nM VIP
(□) Effect of 7.5nM VIP with 100μM (−)baclofen
(△) Effect of 7.5nM VIP with 100μM (+)baclofen

Representative experiments. Drugs were introduced at the arrows.

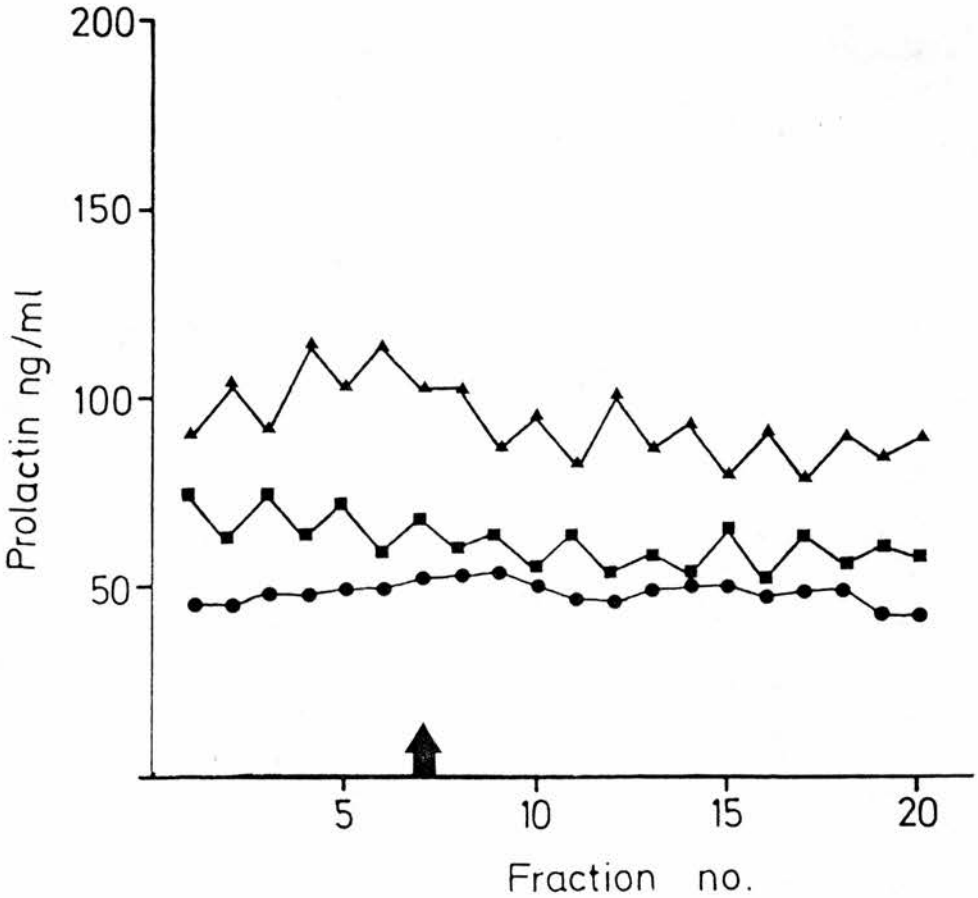


FIGURE 5.14

The effect of baclofen on prolactin secretion following treatment with drugs which alter intracellular cAMP concentrations. 100μM (±) baclofen was introduced at the arrow.

Treatments: (●) 10ng/ml cholera toxin
(■) 10μM forskolin
(▲) 2mM IBMX with 2mM dbcAMP
These drugs were introduced 40 min before baclofen.

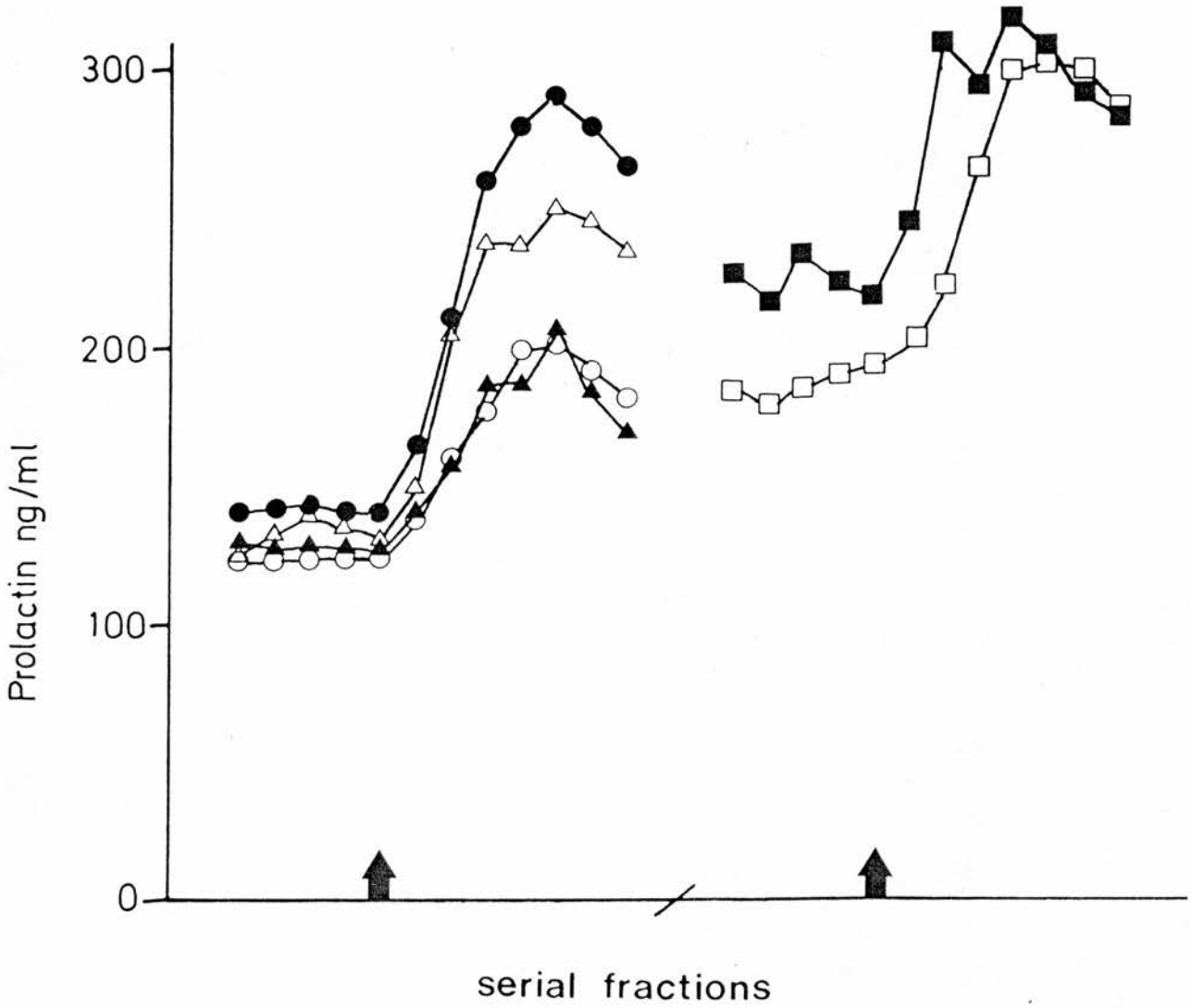


FIGURE 5.15

Effect of baclofen on 4AP- and TEA- stimulated prolactin secretion.

(●) 1mM 4AP
(○) 1mM 4AP with 100μM (±)baclofen
(▲) 1mM 4AP with 100μM (−)baclofen
(△) 1mM 4AP with 100μM (+)baclofen
(■) 10mM TEA
(□) 10mM TEA with 100μM (±)baclofen

Representative experiments. Drugs were introduced at the arrows.

CHAPTER 6.

Modulation of $GABA_A$ receptor effects on prolactin secretion by benzodiazepines and barbiturates.

6.1 Introduction

The hypothesis that the main mechanism of action of benzodiazepines is a facilitation of GABAergic effects is supported by a wealth of evidence. Schmidt, Vogel and Zimmermann (1967) provided the first report which, in retrospect, demonstrated a relevant synaptic mode of action of benzodiazepines. They found that diazepam facilitated primary afferent depolarisation in the spinal cord. Considerable evidence was subsequently provided that this presynaptic inhibition was mediated by GABA (Barker and Nicoll, 1972: Curtis and Johnston, 1974), enabling the work of Schmidt et al (1967) to be confirmed and enlarged upon (Polc, Mohler and Haefely, 1974). The effect of diazepam could be antagonised by bicuculline and by depletion of endogenous GABA by thiosemicarbazide (Polc et al, 1974), supporting the hypothesis that diazepam acted by facilitation of GABAergic transmission. Picrotoxin was also found to antagonise the action of diazepam in the spinal cord (Stratten and Barnes, 1971) and in the geniculate nucleus, another site of potentiation of presynaptic inhibition (Barnes and Moolenaar, 1971).

Benzodiazepines have also been reported to antagonise effects of GABA (Steiner and Felix, 1976; Gahwiler, 1976) or to be unable to potentiate responses to GABA (Dray and Straughan, 1976) although in this last study, the depressant effect of benzodiazepines alone was antagonised by bicuculline but not strychnine. Others have found that diazepam did not modify responses to exogenous GABA, but appeared to potentiate GABAergic transmission (Curtis, Lodge, Johnston and Brand, 1976). This is consistent with observations that inhibition of GAD by isoniazid or thiosemicarbazide prevented

the effects of benzodiazepines (Polc et al, 1974; Mao, Guidotti and Costa, 1974; Haefely, Kulcsar, Mohler, Pieri, Polc and Schaffner, 1975). It therefore appears that benzodiazepines are more easily able to potentiate the effect of endogenous, synaptically-released than exogenous GABA.

The facilitatory action of benzodiazepines on GABAergic synapses is now well documented, and current investigations are therefore aimed at determining the mechanism of this facilitation.

There are several possible sites of action within the GABAergic synapse: presynaptic effects on the release or reuptake of GABA, or on enzymes of synthesis and degradation, or post-synaptically on the GABA receptor and associated ion channels. Benzodiazepines have no effect on GABA uptake (Iversen and Johnston, 1971; Curtis et al, 1976), and although flurazepam appears to have some direct effect enhancing release of GABA (Nestoros and Nistri, 1978; Mitchell and Martin, 1978b) this was not observed with other benzodiazepines (Mitchell, 1979). Diazepam has been found to have no effect on GABA synthesis or degradation (Sawaya, Horton and Meldrum, 1975; Haefely et al, 1975), which argues against any major presynaptic effect of benzodiazepines. A direct postsynaptic action on GABA receptors is also unlikely as the effect of benzodiazepines can be prevented by depletion of endogenous GABA (see above) but more importantly benzodiazepines have been shown to be unable to displace [^{3}H]GABA from high- (or indeed, lower-) affinity binding sites (Snyder and Enna, 1975; Olsen, Ticku, Van Ness and Greenlee, 1978). Conversely, benzodiazepines have been shown to potentiate the effect of GABA in a synergistic manner (Kozhechkin and Ostrovskya, 1977), an effect

that was selective for GABA and not glycine (Choi, Farb and Fishbach, 1977). Similar results were obtained by Macdonald and Barker (1978) who proposed an allosteric mechanism of action equivalent to that frequently observed in enzyme biochemistry. These electrophysiological results are compatible with three potential mechanisms of action of benzodiazepines: to increase GABA receptor affinity, the number of available receptors, or to improve coupling between GABA receptor and chloride channel.

The demonstration of specific high-affinity binding sites for benzodiazepines (Mohler and Okada, 1977a; Squires and Braestrup, 1977) distinct from, but allosterically linked to GABA receptors (Tallman, Thomas and Gallagher, 1978), has provided a fresh model for the investigation of the mechanism of action of benzodiazepines (see Chapter 4). Although benzodiazepines were shown not to affect high-affinity [^{3}H]GABA binding, Guidotti, Toffano, Grandison and Costa (1978) showed that if fresh membranes were used (which show low-affinity [^{3}H]GABA binding), addition of benzodiazepines revealed a high-affinity component. Subsequently, these workers were able to purify an endogenous protein 'GABA modulin', which appeared to regulate high affinity GABA binding (Guidotti, Toffano and Costa, 1978; Toffano et al, 1978). More recently, benzodiazepines have been confirmed to increase low-affinity [^{3}H]GABA binding (Skerritt, Willow and Johnston, 1982; Skerritt and Johnston, 1983), although this result has not been readily reproduced by other groups.

These results therefore support a direct postsynaptic effect of benzodiazepines on the GABA-chloride channel complex, possibly by increasing receptor affinity.

Barbiturates also act to enhance synaptic GABAergic transmission and the effects of exogenous GABA but not glycine (Nicoll, 1975; Nicoll, Eccles, Oshima and Rubia, 1975; Ransom and Barker, 1976; Bowery and Dray, 1976; Barker and Ransom, 1978b), and their direct effects can be antagonised by picrotoxin (Nicoll, 1975). The application of fluctuation analysis has demonstrated that pentobarbital opens identical channels to those opened by GABA, but for much longer (Mathers and Barker, 1980), and that phenobarbitone can prolong the lifetime of the elementary conductance event activated by GABA and the time constant of decay of one population of spontaneous synaptic currents recorded in cultured spinal cord neurones (Barker and McBurney, 1979b). In this respect barbiturates differ from diazepam which is reported to have little effect on channel lifetime but increases the frequency of channel openings (a parameter which was somewhat decreased by pentobarbital)(Study and Barker, 1981). Furthermore, benzodiazepines and barbiturates bind to independent sites: barbiturates were found not to displace [^{3}H]diazepam binding (Squires and Braestrup, 1977) but potently displaced [^{3}H]dihydro-picrotoxinin ([^{3}H]DHP) and [^{35}S]t-butylphosphorothionate ([^{35}S]TBPS) binding, believed to be associated with the chloride channel (Ticku and Olsen, 1978; Squires, Cassida, Richardson and Saederup, 1983). In general, benzodiazepines do not displace [^{3}H]DHP binding with high affinity (Ticku, Ban and Olsen, 1978). Barbiturates interact allosterically with both [^{3}H]GABA and [^{3}H]bicuculline methochloride binding (Willow and Johnston, 1981; Olsen and Wong, 1984), the effect on [^{3}H]GABA binding being a slowing of dissociation from high affinity sites (Willow and Johnston, 1981).

The present studies were carried out to investigate whether any component of the $GABA_A$ receptor effect on prolactin secretion demonstrated in Chapter 5 could be modulated by benzodiazepines or barbiturates, which would provide functional evidence for the existence of the GABA receptor/effector complex with associated regulatory components (Olsen 1982) at a non-synaptic site outside the CNS. Benzodiazepines have been previously shown to inhibit prolactin secretion *in vivo* in a number of animal models (Grandison, 1980, 1982; Lotz, 1982) but the results of *in vitro* experiments were equivocal: benzodiazepines were found to have no GABA-potentiating effect in a dispersed cell superfusion system (Grossman et al, 1981) but potentiated the effect of GABA on prolactin secretion from statically incubated pituitary halves (Clemens and Shaar, 1981). More recently diazepam has been shown to potentiate the inhibitory effect of muscimol on prolactin secretion from cultured pituitary cells and to directly inhibit secretion at higher concentrations (Schettini, Cronin, O'Dell and Macleod, 1984).

The interaction between effects of benzodiazepines and GABA antagonists has been used to investigate their site of action within the GABA response mechanism (Simmonds, 1980a). As the effect of muscimol on prolactin secretion is reproducible and quantifiable, a similar series of experiments were carried out to examine whether the model proposed by Simmonds (1980a) could be supported.

6.2 Materials and Methods.

6.2.1 Materials

Muscimol, secobarbital and picrotoxinin (PTX) were obtained from Sigma, (Poole); bicuculline methiodide (BMI) from Pierce Chemical Co. (Rockford, IL, USA) and the benzodiazepines clonazepam,

flurazepam, Ro5-4864, Ro15-1788 and Ro11-6896 were the generous gift of Hoffmann La Roche (Basel, Switzerland).

6.2.2 Methods

The materials and methodology used in these experiments were as described in Chapter 5. Chopped PD tissue was superfused with oxygenated Krebs Bicarbonate medium at 37°C. Fractions were collected and assayed for prolactin by RIA (section 2.3). In most experiments 2 min fractions were collected, except in one series (results depicted in Figure 6.6) when 24sec fractions were collected.

After 90 min of superfusion, inlet lines were changed to medium containing muscimol, with benzodiazepines or secobarbital as appropriate. When antagonists were used, they were introduced 30 min before muscimol.

6.2.3 Calculation of results

The response to muscimol was calculated as percentage stimulation and inhibition, as in Chapter 5, using the mean of 5 pre-drug samples for baseline. When the rapid collection protocol was used, the prolactin concentration of each fraction after addition of drugs was also expressed as a percentage of pre-drug concentration, and the results in Figure 6.6 are plotted in this form. A line was fitted through the 5-6 fractions of the rising phase of the response in each experiment by linear regression, and the slope was taken as an index of the rate of increase in prolactin secretion. Correlation coefficients (for individual experiments) were >0.9 for muscimol alone and muscimol with secobarbital, and >0.75 for muscimol with Ro11-6896.

The results from experiments using antagonists are expressed as percent antagonism of a control response carried out at the same

time on a parallel superfusion line.

All data are expressed as mean ± S.E.M., from 5-6 experiments.

6.3 Results

Muscimol itself was found to have a biphasic effect on prolactin secretion (Chapter 5) which consisted of a transient stimulation of secretion followed by sustained inhibition. The effect of 1μM clonazepam was tested on a wide range of muscimol concentrations, from 10nM to 30μM. No effect was found on the inhibitory component of the response to muscimol at any concentration, but a potentiation of the stimulatory response was seen at low concentrations of muscimol. Thus the stimulatory response to 100nM muscimol was increased from 11 ± 1% to 27 ± 3% ($n = 5$, $P < 0.05$) (Figure 6.1) while the inhibitory response was 16 ± 2% alone and 13 ± 2% in the presence of clonazepam. The pharmacology of this potentiation was further investigated, and the results are shown in Figure 6.2. 10μM Ro15-1788 antagonised the effect of 1μM clonazepam, while having no effect on the response to muscimol itself. 1μM Ro15-1788 caused partial antagonism. 1μM Ro5-4864 did not affect either the stimulatory or inhibitory components of the response. These results suggest the involvement of the central- but not the peripheral-type benzodiazepine receptors, whose presence within the anterior pituitary gland was demonstrated by binding studies described in Chapter 4.

The effect of 1μM clonazepam was also investigated on the antagonism of the stimulatory response to 30μM muscimol by PTX and BMI. This concentration of muscimol gave a control response of 143 ± 15% ($n = 6$), which itself was unaltered by 1μM clonazepam, the combination causing 152 ± 18% stimulation. Concentration-response

curves for the antagonists are shown in Figure 6.3, results being expressed as % antagonism of a control response. Clonazepam shifted the curve for BMI to the right, such that BMI caused significantly less antagonism at 3μM and 10μM in the presence of 1μM clonazepam. The effect of PTX was clearly unaltered at 3, 10 or 30μM. Examples of individual experiments are shown in Figure 6.4. The discrepancy between these results and those of Simmonds (1980a) (using flurazepam) caused us to investigate the effect of flurazepam on this system. Flurazepam (1μM) reduced the antagonism caused by 3μM PTX from 54 ± 5% to 29 ± 6% (compared with 56 ± 4% in the presence of clonazepam) and that caused by 10μM BMI from 62 ± 2% to 39 ± 6% ($n = 5$, $P < 0.05$ in both cases)(compared with 47 ± 5% in the presence of clonazepam). Examples are shown in Figure 6.5.

The effects of the barbiturate secobarbital were investigated on the responses to muscimol using both the normal protocol and also in a series of experiments in which 24 sec (rather than the usual 2 min) fractions were collected. The effect of the benzodiazepine Ro11-6896 was also investigated in these experiments. Using the normal protocol, both 100μM secobarbital and 1μM Ro11-6896 potentiated the stimulatory response to 1μM muscimol from 20 ± 3% to 74 ± 4% (secobarbital) and 51 ± 6% (Ro11-6896) ($P < 0.05$, $n = 5$ in each case) while not affecting the inhibitory component: 43 ± 2% muscimol alone, 40 ± 4% with secobarbital and 43 ± 5% with Ro11-6896. Representative experiments are shown in Figure 6.6. Similar results were found using the rapid collection protocol: the peak stimulatory response of 23 ± 2% to muscimol was increased to 37 ± 2% by secobarbital and 54 ± 8% by Ro11-6896 ($P < 0.05$, $n = 5$ in each case, Figure 6.7). This protocol allowed the response to be

analysed in greater detail, as the rising phase of the response was spread over 5-6 fractions. The prolactin concentrations in these fractions were expressed as a percentage of baseline secretion (mean of 6 predrug fractions). The slope of the mean line constructed through these values gives a measure of the rate of increase in prolactin secretion, and was found to be
2.9 ± 0.6% per fraction with 1μM muscimol. This was increased to 6.8 ± 0.9 by 100μM secobarbital and 7.1 ± 1.4 by 1μM Ro11-6896 ($P < 0.05$, $n = 5$ in each case). Therefore both drugs were found not only to increase the peak response to muscimol but also the rate of increase in prolactin concentration, but as with clonazepam, no effect on the inhibitory response to muscimol was observed. None of the benzodiazepines or secobarbital were found to have any effect on prolactin secretion alone.

6.4 Discussion

The results presented here in Figures 6.1 and 6.6 demonstrate potentiation of a $GABA_A$ receptor effect by the benzodiazepines clonazepam and Roll-6896, which were without effect alone. This demonstrates that benzodiazepine receptors can be functional at non-neuronal, non-synaptic locations and supports the hypothesis of a widespread presence of this interaction (Olsen, 1982). PI cells are also responsive to diazepam (Taraskevich and Douglas, 1982) at what is probably a synaptic location (Oertel et al, 1982).

Two other observations were made:

a) that the benzodiazepine receptor involved showed central-type phamacology and

b) that only one part of the response to muscimol was potentiated.

The basis for conclusion (a) is that clonazepam and Ro5-4864

show great selectivity for the central-type and peripheral-type sites respectively (see Figure 4.2) and that the effect was antagonised by Ro15-1788. Ro15-1788 is a potent displacer of [^{3}H]diazepam binding, but in a variety of models showed no benzodiazepine-like action: it did, however, antagonise behavioural effects of benzodiazepines (Hunkeler, Mohler, Pieri, Polc, Bonetti, Cumin, Schaffner and Haefely, 1981). In situations in which GABAergic activity is compromised, eg after isoniazid treatment, Ro15-1788 has been reported to have anxiogenic activity and to cause convulsions (Corda, Guidotti and Costa, 1982), suggesting that it has some intrinsic β-carboline-like "inverse agonist" action (Braestrup, Nielsen and Honore, 1983). In electrophysiological studies it has been shown to have some partial agonist action (Macdonald, Skerritt and McLean 1984). Ro15-1788 did not affect the response to muscimol alone (Figure 6.2), and therefore appeared to have no detectable efficacy in this model. Peripheral-type benzodiazepine sites are present in PD in far greater abundance than the central sites (Chapter 4) but as yet no function can be ascribed to them. An association with Ca^{2+} channels has been suggested in the heart (Mestre, Carriot, Belin, Uzan, Dubroeucq, Gueremy, Doble and LeFur, 1985) and spinal cord (Skerritt, Werz, McLean and Macdonald, 1984). Ro5-4864 has recently been shown to reduce $^{45}Ca^{2+}$ uptake in GH_3 cells, an effect that was antagonised by PK 11195 but not mimicked by clonazepam, suggesting the involvement of a peripheral-type benzodiazepine receptor (Benavides, Burgevin, Doble, LeFur and Uzan, 1985). By this reasoning Ro5-4864 might be expected to influence stimulus-secretion coupling, but this was not demonstrated here at the concentrations required to exclude action

at central type sites. Ro5-4864-sensitive sites have also been associated (along with a putative GABA receptor) with activation of phospholipase A_2 (Majewska and Chuang, 1985): this involved potentiation of the effect of muscimol acting at a bicuculline-sensitive but picrotoxin-insensitive GABA receptor, and Ro5-4864 appeared to be an antagonist of the effects of other benzodiazepines e.g. diazepam. Ro5-4864 has also been reported to antagonise the effect of muscimol in the cuneate nucleus (Simmonds, 1984), although the mechanism for this is not entirely clear.

The second observation, the selectivity of the potentiation by both benzodiazepines and secobarbital of the stimulatory part of the response to muscimol is of interest in the light of the observations of the differential pharmacology of the two parts of the response described in Chapter 5. Strychnine was found to be a selective antagonist of the stimulatory part of the response, and has also been reported to be a potent antagonist of GABAergic stimulation of benzodiazepine binding in some regions (Braestrup and Nielsen, 1980). There are distinct differences between the mechanisms of action of benzodiazepines and barbiturates and the two classes of drug are recognised by distinctly different although interacting sites. It therefore appears that two different types of $GABA_A$ receptor/channel complex may be involved in producing the two effects on prolactin secretion:

(I) a benzodiazepine/barbiturate-linked complex, which also has associated sites for glycine and strychnine but at which homocarnosine apparently lacks efficacy,

(II) a complex that appears to be insensitive to these modulators, and glycine and strychnine, yet is activated by homocarnosine.

These results do not confirm the results others have previously obtained in vitro. The absence of an effect of benzodiazepines reported by Grossman et al (1981) may be attributed to the absence of a transient stimulation of prolactin secretion observed in their study. A potentiation of the inhibitory effect of GABA or muscimol was observed by Clemens and Shaar (1981) using hemipituitaries and by Schettini et al (1984) using cell cultures. The static incubation protocol used by these groups precludes the demonstration of transient effects, but nevertheless there is no ready explanation for the discrepancy. One possibility is that the effect of diazepam in those studies is related to its ability to depress Ca^{2+} action potentials (Skeritt et al, 1984; Cherubini and North, 1985) which occur in PD cells (Taraskevich and Douglas,1977) and therefore the potentiation of GABA effects is apparent rather than real. This would also explain the direct inhibitory effect of diazepam on basal and stimulated prolactin secretion (Schettini et al, 1984). Clarification must await further study, but the results presented here are consistent with the demonstration by Taraskevich and Douglas (1982) that diazepam potentiated the electrophysiological responses of PI cells to GABA, and $GABA_A$ receptor activation causes a stimulation of αMSH secretion (Tomiko et al, 1983; Demeneix et al, 1984). Ro11-6896 and secobarbital were also found to potentiate the stimulatory effect of muscimol on GH secretion (Chapter 7).

Despite the volume of research devoted to benzodiazepines, the mechanism by which they potentiate GABAergic responses is not fully understood. This problem was investigated by examining the effect of clonazepam on the ability of PTX and BMI to antagonise the

response to a fixed concentration of muscimol. Clonazepam was found to reduce the ability of BMI but not PTX to antagonise the response to muscimol (Figures 6.3 and 6.4) while not affecting the response to muscimol alone. This approach has been previously employed in electrophysiological experiments with brain slice preparations (Simmonds, 1980a). In that case however, (using flurazepam as the benzodiazepine) the potency of PTX but not BMI was reported to be selectively reduced. The lack of influence on the potency of BMI was suggested to reflect no change in the properties of the receptor itself while the reduced potency of PTX was suggested to reflect increased receptor-effector coupling or prolonged channel opening. Repeated series of experiments with that model have shown considerable variation in the interaction between muscimol, flurazepam and PTX (Simmonds, 1983b) and prolongation of channel open time has been shown to be a minor effect of diazepam (Study and Barker, 1981). Furthermore, results from binding studies have suggested instead, that alterations in binding site properties may be more crucial, in that a variety of benzodiazepines enhance the affinity of the low-affinity GABA binding site (Guidotti et al, 1978; Skerritt and Johnston, 1983). Recent patch-clamp experiments have supported this interpretation, suggesting that diazepam increases either the affinity of the GABA receptor or the number of available receptors, without affecting conductance or gating properties of GABA-activated channels (Bormann and Sakmann, 1984). The results presented here in Figure 6.3 are consistent with clonazepam enhancing the GABA receptor affinity, thus reducing the potency of BMI without altering the receptor-effector coupling. This is reflected in the lack of change of PTX potency, and the

absence of any potentiation of the effect of muscimol alone at this relatively high agonist concentration. It seems likely that a major contribution to this discrepancy, between the present results which support the ligand binding and patch clamp data and those of Simmonds (1980a), beside differences in the properties of the model used, is the use of flurazepam as opposed to clonazepam. Flurazepam is a relatively potent and apparently competitive displacer of [^{3}H]DHP binding (Leeb-Lundberg, Napias and Olsen, 1981) in comparison with its affinity for benzodiazepine receptors. Clonazepam (and Ro11-6896, also used here) are more selective compounds as they are less potent (and non-competitive) displacers of [^{3}H]DHP binding (Leeb-Lundberg et al, 1981) whilst they are more potent displacers of [^{3}H]benzodiazepine binding. Such an atypical action of flurazepam at [^{3}H]DHP binding sites could enhance its apparent effect on PTX potency. Indeed, such an effect was observed here as flurazepam considerably reduced the antagonism caused by both PTX and BMI. High concentrations of flurazepam have also been reported to antagonise GABA responses (Nistri and Constanti, 1978).

Another possible contributory factor is differences in the methodology used. In the cuneate slice, the lower part of the muscimol concentration-response curve was examined in the presence and absence of flurazepam and the antagonists (which is the part of the curve most sensitive to potentiation of the effect of muscimol (Simmonds, 1980a). In the studies presented here, a high concentration of muscimol was used to enable more accurate values for percent antagonism to be calculated, and the response to this concentration of muscimol was unaffected by clonazepam. The

experimental protocol used here does not allow more than one response to be measured on each set of superfused tissue, whereas many responses in the presence of different drugs can be measured in one cuneate slice. The use of part of the response curve sensitive to flurazepam may have contributed to the variation encountered (Simmonds, 1983b).

Lorazepam has also been found to reduce the potency of PTX in the cuneate slice (Simmonds, 1981). Lorazepam is not potent at displacing [^{3}H]DHP binding (Leeb-Lundberg et al, 1981) but is anomalously weak in antipunishment tests (Stephens, Kehr, Schneider and Braestrup, 1984). These discrepancies may reside with the model studied. One possibly important difference is the potency of strychnine to antagonise muscimol in these studies (Chapter 5) whereas it is much weaker than bicuculline in the cuneate slice (Simmonds, 1982).

Further experiments to confirm these findings could be carried out using β-carboline 'inverse agonists' which would be expected to reduce the effect of muscimol and enhance the potency of BMI in this model, paralleling their anxiogenic effects (Corda, Blaker, Mendeson, Guidotti and Costa, 1983).

In conclusion, these results demonstrate the potentiation of a $GABA_A$ receptor effect by benzodiazepines and a barbiturate. The interactions between clonazepam and the antagonists BMI and PTX are consistent with benzodiazepines enhancing GABA receptor affinity or the number of available receptors, without affecting the linkage between receptor and ion channel. These results also support the proposal that two separate types of $GABA_A$ receptor complex are involved in the biphasic effect of muscimol on prolactin secretion.

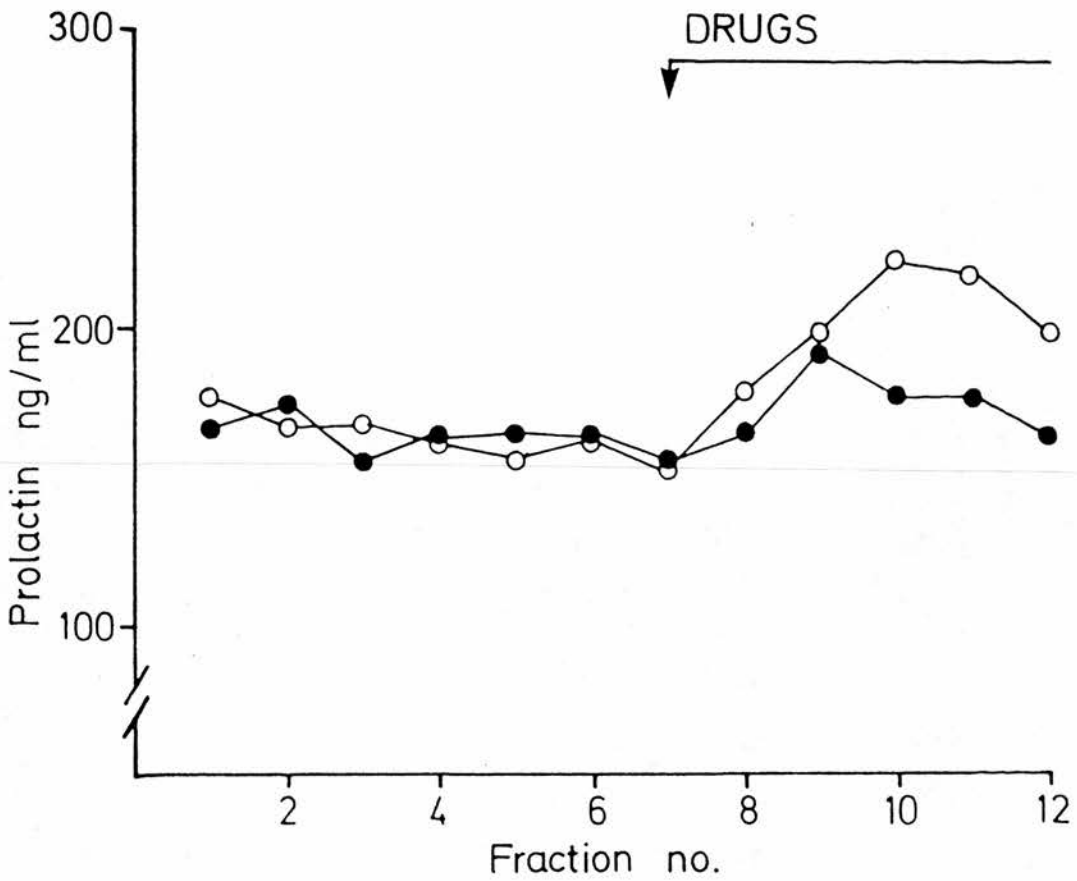
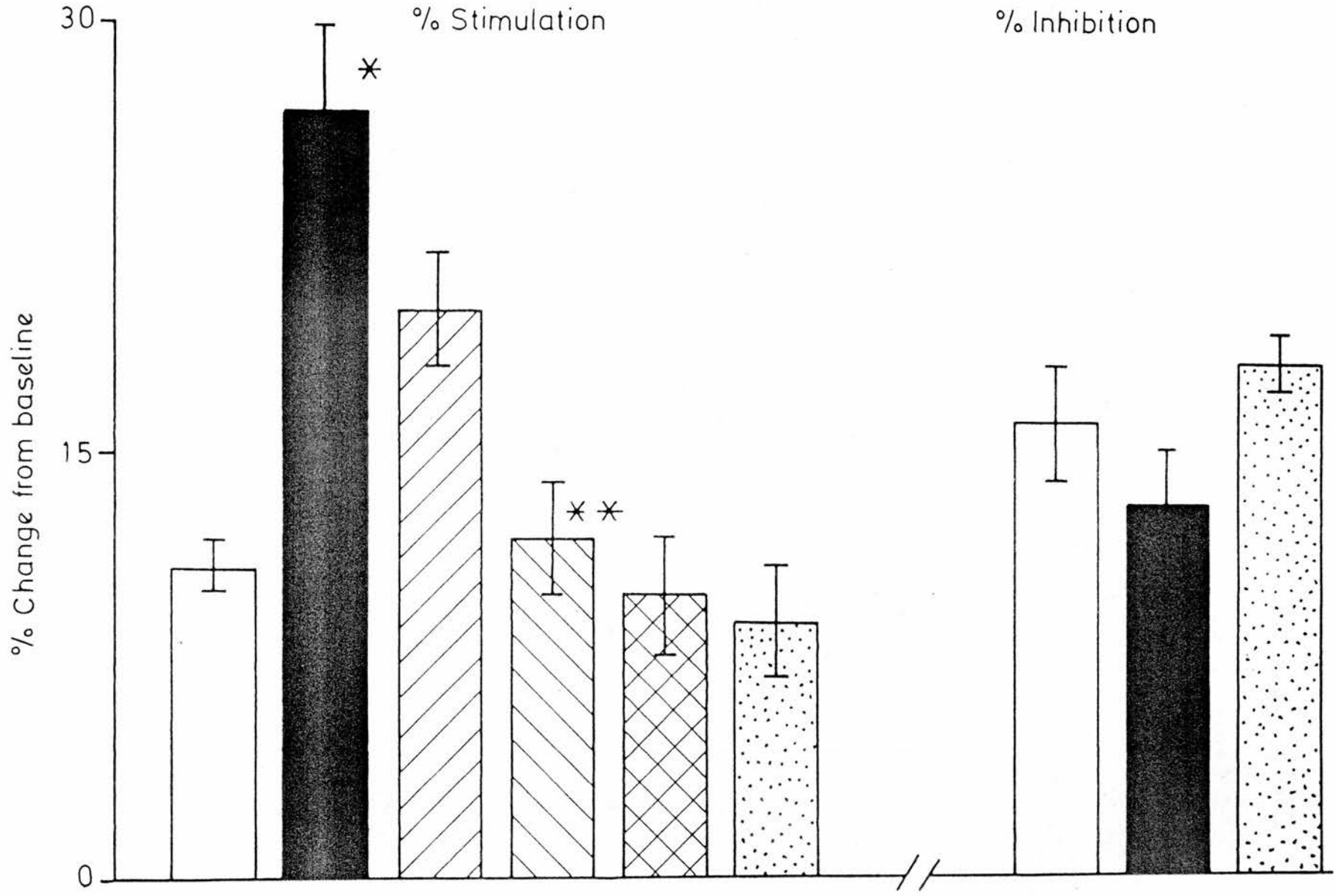


FIGURE 6.1

Potentiation of the stimulatory effect of muscimol on prolactin secretion by clonazepam. 100nM muscimol alone (●) or with 1μM clonazepam (○) was introduced at the arrow.


30
15
0
% Change from baseline
% Stimulation
% Inhibition
*
**

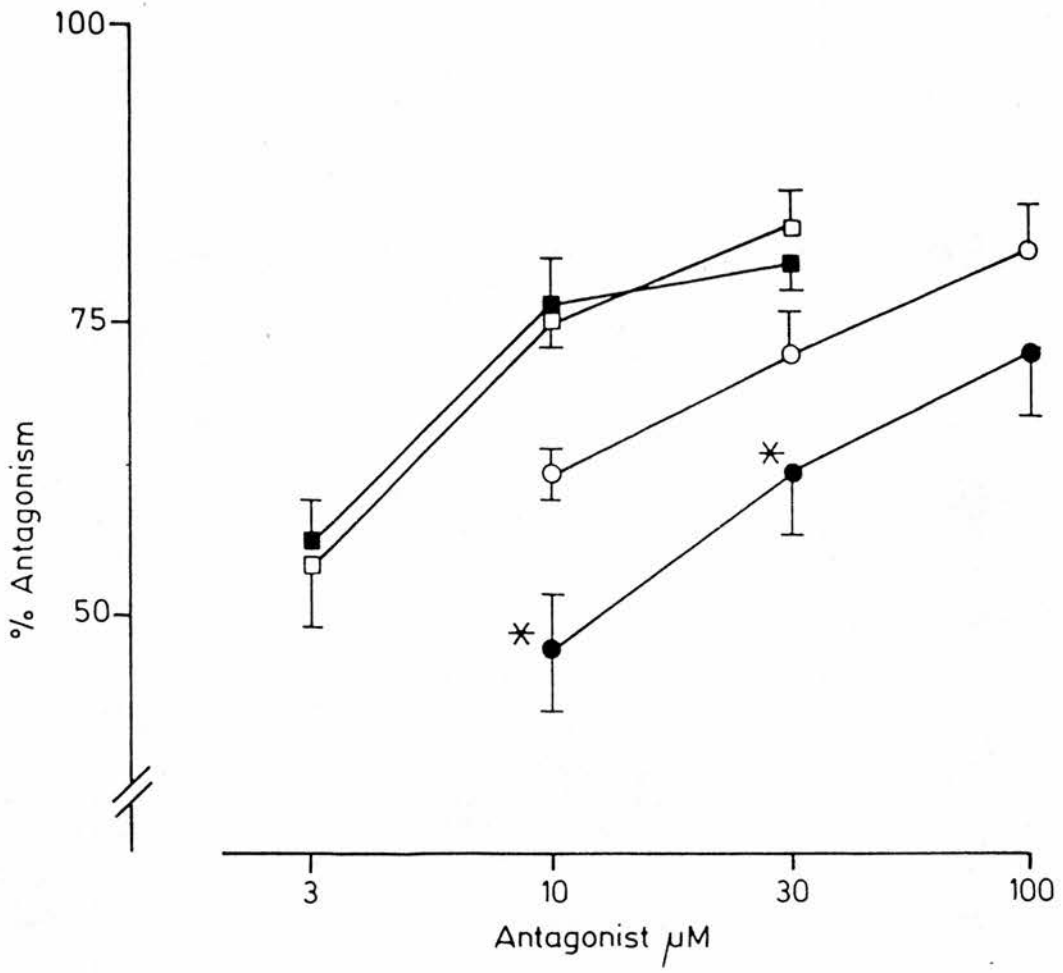


FIGURE 6.3

Interaction of clonazepam with antagonists.
Antagonism of the stimulatory effect of 30μM muscimol on prolactin secretion by (□)PTX and (○) BMI.
Antagonism in the presence of 1μM clonazepam: (■) PTX and (●) BMI.
Results expressed as percentage antagonism against antagonist concentration. Mean ± S.E.M., n = 5.
* $P < 0.05$ BMI + clonazepam vs BMI alone.

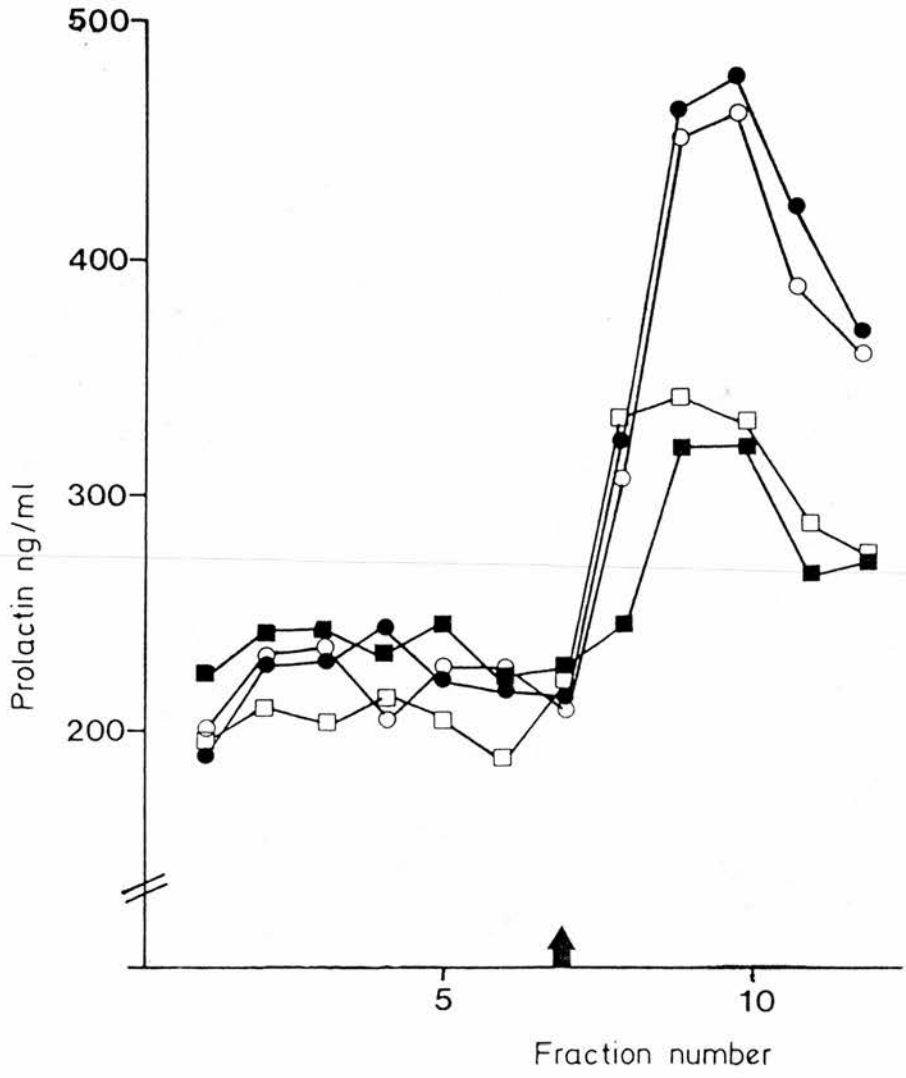


FIGURE 6.4

Example of the interaction of clonazepam with antagonism of the stimulatory effect of muscimol on prolactin secretion by BMI.

(●) 30μM muscimol
(○) 30μM muscimol + 1μM clonazepam
(■) 30μM muscimol + 10μM BMI
(□) 30 μM muscimol + 10μM BMI + 1μM clonazepam

Muscimol and clonazepam were introduced at the arrow, BMI was introduced 30 min previously. Note lower baseline of (□) than ( ).

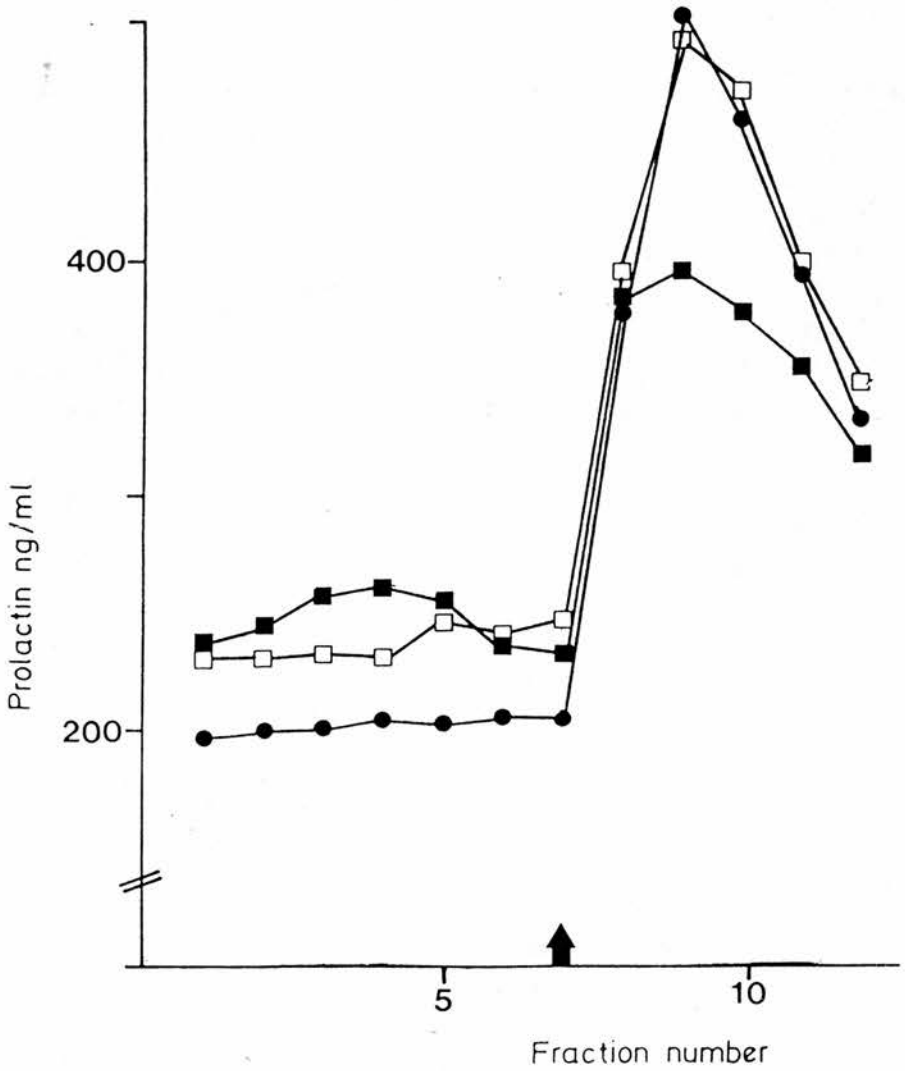


FIGURE 6.5

Example of interactions of flurazepam with antagonism of the stimulatory effect of muscimol on prolactin secretion by PTX.

(●) 30μM muscimol
(■) 30μM muscimol + 3μM PTX
(□) 30μM muscimol + 3μM PTX + 1μM flurazepam

Muscimol and flurazepam were introduced at the arrow, PTX was introduced 30 min previously.

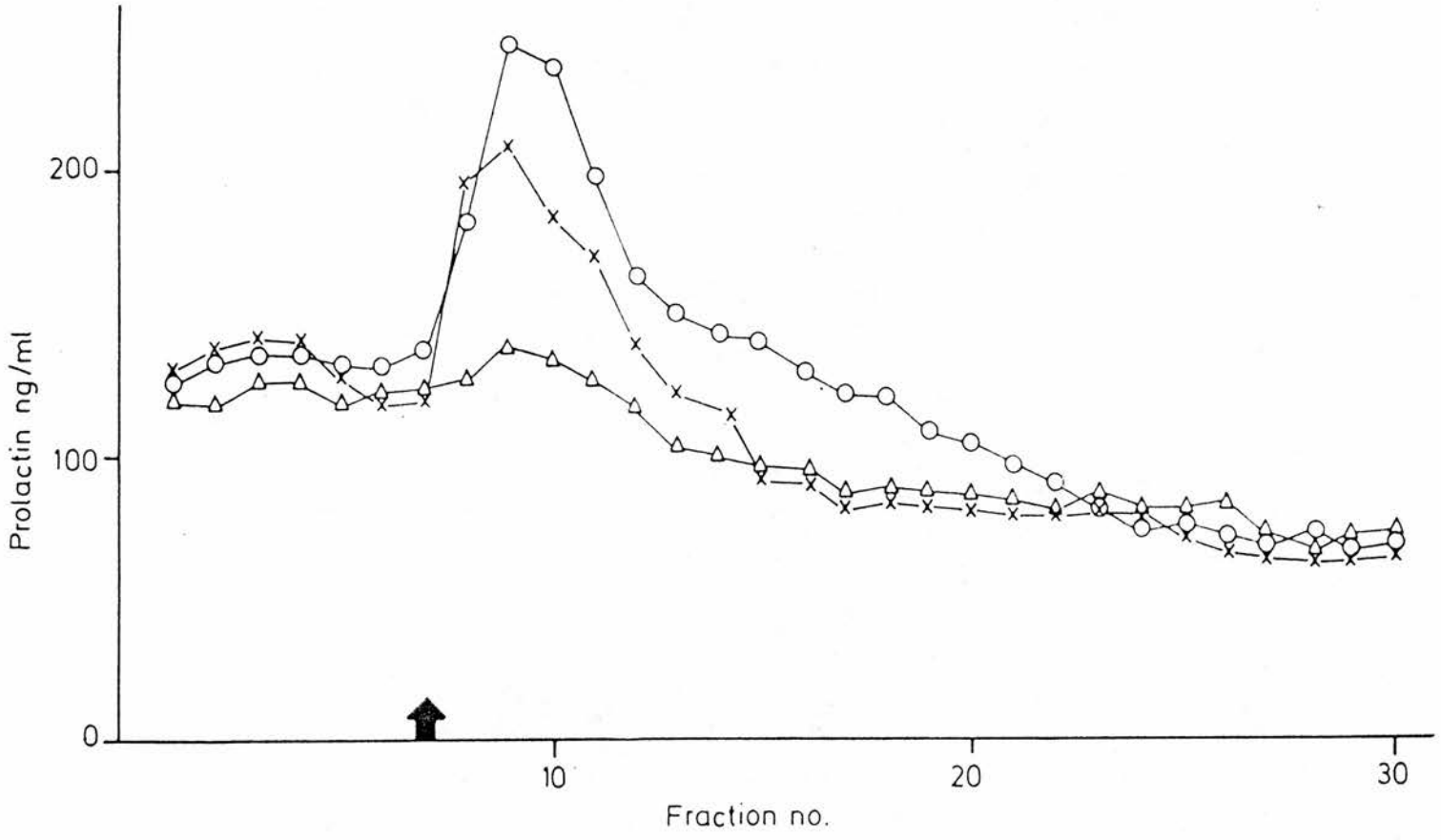


FIGURE 6.6

Effects of 1μM Roll-6896 and 100μM secobarbital on the response to 1μM muscimol.

(△) control, 1μM muscimol alone
(X) 1μM muscimol + 1μM Roll-6896
(○) 1μM muscimol + 100μM secobarbital

Drugs were introduced at the arrow. Representative experiments.

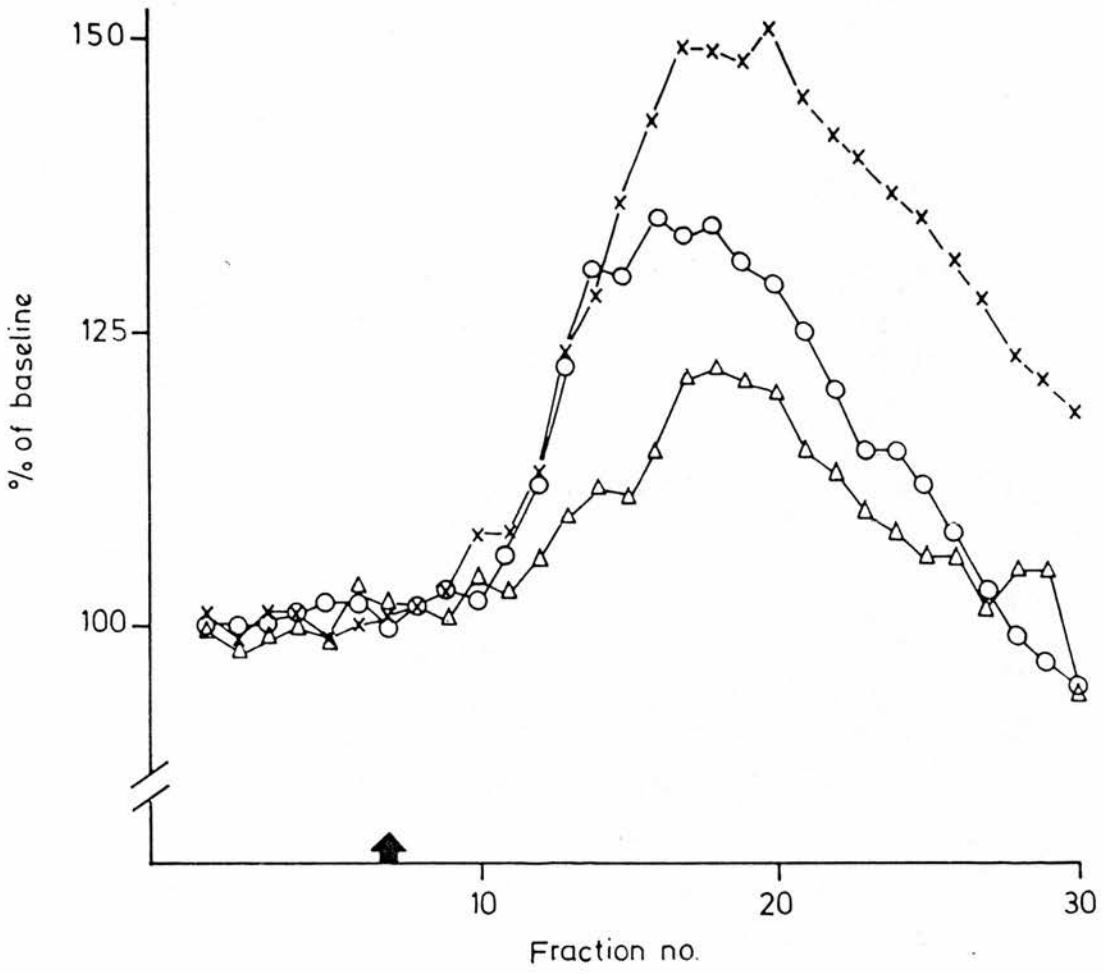


FIGURE 6.7

Effects of 1μM Ro11-6896 and 100μM secobarbital on the stimulatory effect of 1μM muscimol on prolactin secretion.
24 sec fractions were collected. Results are mean of prolactin concentration in each fraction as a percentage of pre-drug concentrations.

(△) 1μM muscimol alone
(X) 1μM muscimol + 1μM Ro11-6896
(○) 1μM muscimol + 100μM secobarbital

Drugs were introduced at the arrow. Standard errors (≤ 4%) are omitted for clarity. n = 5.

CHAPTER 7

Effects of GABA receptor agonists on the secretion of GH, LH, ACTH and TSH *in vitro*

7.1 Introduction

Although prolactin has been the PD hormone most subject to investigation in this field, there is considerable evidence that GABA is involved in some way in the control of the other PD hormones, GH, LH, ACTH and TSH. There are reports of no apparent effect of GABA on follicle-stimulating hormone (FSH) secretion (Ondo, 1974; Pass and Ondo, 1977; Vijayan and McCann, 1978a). As the results of experiments on these hormones are often conflicting, they will be discussed separately.

7.1.1. GABA and Growth Hormone Secretion

Vijayan and McCann (1978b) found that i.c.v. administration of GABA to conscious female rats caused a rapid increase in GH secretion, which was antagonised by bicuculline. This failed to confirm an earlier report that GABA i.c.v reduced plasma GH concentration in anaesthetized male rats (Bruni, Mioduszowski, Grandison, Simkins and Meites, 1977), but supported the results of Martin, Durand, Gurd, Faille, Audet and Brazeau (1978) who found that picrotoxin suppressed episodic GH secretion, providing evidence for a physiological stimulatory role for GABA. Bicuculline and picrotoxin have also been shown to prevent both the stimulation of GH secretion by a met-enkephalin analogue in conscious male rats (Katakami, Kato, Matsushita, Shimatsu and Imura 1981) and the ability of muscimol to stimulate GH secretion in man (Tamminga, Neophytides, Chase and Frohman, 1978). Administration of AOAA to elevate brain GABA concentrations also stimulated GH secretion (Turnbull and Slater,1970). This stimulatory GABAergic effect may be mediated, at least partly, by reduced release of somatostatin into hypophysial portal blood (Gamse et al, 1980; Takahara, Yunoki, Hosogi, Yakushiji, Kageyama and Ofuji, 1980).

Other results supporting those of Bruni et al (1977) of an inhibitory effect on GH secretion have also been obtained. Intraperitoneal injection of muscimol has been reported to inhibit GH secretion in female rats (Cocchi, Casanuva, Locatelli, Apud, Martinez-Campos, Civati, Racagni and Muller, 1981) but this was enhanced by bicuculline. Blockade of GABA synthesis or receptors stimulated GH secretion, and inhibition of GABA degredation inhibited GH secretion (Fiok, Acs and Stark, 1981a). These conflicting results may have been resolved by a recent study by Fiok, Acs, Makara and Erdo (1984). These authors demonstrated that systemic administration of muscimol inhibited anticipated GH surges in conscious male rats, an effect also produced by elevating brain GABA with GAG. Conversely, BMI triggered an early secretory peak. Muscimol also inhibited GH secretion in rats with anterolateral deafferentation of the medial basal hypothalamus, a procedure which elevates GH secretion by removing the influence of somatostatin (Brownstein, Arimura, Fernandez-Durango, Schally, Palkovits and Kizer, 1977; Karteszi, Fiok and Makara, 1982; Makara, Palkovits, Antoni and Kiss, 1983) and which therefore suggested that muscimol was acting by inhibition of GH-releasing hormone (GHRH) secretion. Fiok et al (1984) also suggested that the reason for the apparent discrepancy between these results obtained after peripheral administration of muscimol and BMI and those obtained after central administration is the anatomical localisation of somatostatin-containing and GHRH-containing cell bodies: the former, being in anterior periventricular regions (Hokfelt, Hellerstrom, Johnson, Luft and Arimura, 1975; Elde and Parsons, 1975; Dierickx and Vandersande, 1979) may be preferentially influenced by i.c.v. GABA (Makara et al, 1975) whereas GHRH-containing cells are found in

arcuate and ventromedial nuclei of the hypothalamus (Frohman, Bernardis and Kant, 1968; Martin, 1976; Block, Brazeau, Ling, Bohlen, Esch, Wehrenberg, Benoit, Bloom and Guillemin, 1983) and will therefore be more susceptible to the effects of peripherally-administered substances which only cross the blood-brain barrier poorly.

Taken in toto, these results suggest two centrally-mediated effects of GABA on GH secretion. The stimulation of GH secretion by BMI, and the inhibition by picrotoxin and bicuculline suggest that both may be tonically active. This explanation may underly the apparently paradoxical demonstration that diazepam-stimulated GH secretion in man is abolished by the GABA-transaminase inhibitor sodium valproate (Koulu, Lammintausta, Kanga and Dahlstrom, 1979).

Baclofen has been reported to inhibit stimulated GH secretion in man (Cavagnini, Invitti, DiLandro, Tenconi, Maraschini and Girotte, 1977) but others have reported rapid stimulatory effects in both man and rats (Koulu, Lammintausta and Dahlstrom, 1979, Fiok, Acs and Stark, 1981b).

Both GABA and muscimol have been reported to have no effect on GH secretion in vitro using hemipituitaries or dispersed cells (Tsushima, Irie and Sakuma, 1971; Vijayan and McCann, 1978b; Fiok et al, 1981a; 1984). Peripheral administration of GABA to neonate rats was observed to stimulate GH secretion (Acs, Makara and Stark, 1984), whereas in the adult muscimol inhibited GH secretion (Fiok et al, 1984). Subsequent study showed that this effect could be demonstrated in vitro using either GABA or muscimol, but only up to the 9th day post-partum (Acs et al, 1984).

7.1.2 GABA and Luteinizing Hormone secretion

Ondo (1974) demonstrated that i.c.v. GABA stimulated LH secretion in anaesthetized male rats. This was subsequently confirmed and enlarged upon (Pass and Ondo, 1977; Vijayan and McCann, 1978a, 1979), but more recently inhibitory effects of i.c.v. GABA and muscimol have been demonstrated (Lamberts, Vijayan, Graf, Mansky and Wuttke, 1983). This effect of muscimol was dependent on the steroid status of the animal, as it was present in ovariectomized rats, but not if they were treated restoratively with steroids (Lamberts et al, 1983). From this and results of measurements of catecholamine and GABA turnover after endocrine manipulations, it has been proposed that oestrogen-sensitive GABA neurones in the preoptic area/anterior hypothalamus modulate the stimulatory effect of noradrenergic neurones on LHRH neurones (Mansky, Mestres-Ventura and Wuttke, 1982; Fuchs, Mansky, Stock, Vijayan and Wuttke, 1984). A direct action of GABA on LHRH neurones was suggested to be unlikely as steroid-primed rats show great sensitivity to LHRH (Ramirez and McCann, 1963) and any direct effect would be reflected by changes in LH which was not observed (Lamberts et al, 1983). However, GAD-IR axons have recently been demonstrated to synapse directly onto LHRH-containing neurones (Leranth, MacLusky, Sakamoto, Shonabrough and Naftolin, 1985).

GABA has been reported to have no effect on LH secretion following direct injection into the pituitary (Ondo, 1974; Pass and Ondo, 1977).

7.1.3 GABA and Adrenocorticotrophic hormone secretion

Studies on ACTH secretion have been less conflicting, and have been of some therapeutic value. GABA i.c.v. inhibited

trauma-induced increases in plasma corticosterone, and picrotoxin and BMI elevated corticosterone concentrations when given i.c.v. to rats with hypothalamic deafferentation (Makara and Stark, 1974). GABA also inhibited the acetylcholine- or 5HT-stimulated release of bioassayable CRH from hypothalamic slices (Burden et al, 1974). An inhibitory involvement in the feedback of steroids or stress is suggested by several lines of evidence: bicuculline potentiated the stress-induced increase in plasma corticosterone, and prevented the dexamethasone-induced decrease in plasma corticosterone (Kameyama, Nabeshima, Banno and Kamata, 1983). Inhibition of GAD by mercaptopropionic acid reduced the effect of dexamethasone on basal and stressed corticosterone concentrations (Acs and Stark, 1978), and dexamethasone increased, whereas stress decreased, diencephalic GABA concentrations (Kameyama et al, 1983). A stimulatory effect of dexamethasone on GAD activity has been localised to the supraoptic nucleus (Acs, Palkovits and Stark, 1980) which may contain the cell bodies of part of the CRF innervation of the ME (Cummings, Elde, Ells and Lindall, 1983).

Sodium valproate given to rats for 3 months blunted stress-induced rises in plasma ACTH concentration, and this drug has proved to be of value in the treatment of Nelson's Syndrome, as has diazepam (Jones, Gillham, Attaher, Nicholson, Campbell, Watts and Thody, 1984). GABA and sodium valproate were without effect on ACTH secretion from human pituitary adenoma in vitro (Jones et al, 1984) suggesting that the effect of sodium valproate was to potentiate GABAergic inhibition of CRF secretion.

7.1.4 GABA and Thyroid Stimulating Hormone secretion

The existence of a centrally-mediated inhibitory effect of GABA on TSH secretion is suggested by the fall in plasma TSH concentration following i.c.v. administration of GABA (Vijayan and McCann, 1978b; Jordan, Poncet, Veisseire and Mornex, 1983). Picrotoxin and the GAD inhibitor semicarbazide were found to elevate the nocturnal low concentrations of TSH without affecting the diurnal peak, suggesting an involvement of GABA in the circadian rhythm (Jordan et al, 1983). AOAA also decreased plasma TSH concentrations (Matilla and Mannisto, 1980).

There is no evidence for a direct effect of GABA on TSH secretion. GABA was found to be without effect on TSH secretion from hemipituitaries (Vijayan and McCann, 1978b; Jordan et al, 1983) or on TRH-stimulated TSH secretion in vivo, although it was able to inhibit cold-induced TSH secretion (Matilla and Mannisto, 1980).

The results discussed above demonstrate pharmacological effects of GABAergic drugs on GH, LH, ACTH and TSH. Physiological involvement of GABAergic neurones is suggested for GH, ACTH and TSH by the effects of antagonists alone, and for LH by the steroid-dependency of the effect. Given the ubiquity of GABAergic neurones within the hypothalamus and the complexity of neurochemical control of these hormones, action at multiple sites (and hence conflicting experimental observations) appears near unavoidable. This is perhaps best illustrated by the evidence for GABAergic control of both somatostatin and GHRH secretion. Despite this wealth of possible function within the hypothalamus, the only report of a direct action on PD concerns GH secretion in neonate rats.

The aim of this study was to reassess the effect of GABA receptor agonists on GH, LH, ACTH and TSH secretion in vitro using the superfusion method demonstrated in Chapter 5 to have the resolution capable of revealing novel effects on prolactin secretion. The influence of both $GABA_A$ and $GABA_B$ receptors was investigated, as both are found in PD (Chapter 3) and may have opposing effects, as demonstrated on αMSH secretion from PI (Demeneix et al, 1984).

7.2 Materials and Methods

Materials were as described in Chapters 5 and 6; LHRH was obtained from Cambridge Research Biochemicals. Methods were also the same. Hormones were measured in aliquots of superfusate as described in Section 2.3.

Results were calculated as peak percentage changes from pre-drug baseline, and are expressed as mean ± S.E.M. Statistical comparisons were made by the Mann-Whitney U test.

7.3 Results

7.3.1 Growth Hormone

GABA and muscimol both produced a large, transient stimulation of GH secretion which peaked within 4 min and then rapidly declined, reaching approximately pre-drug concentrations in about 20 min (Figures 7.1, 7.3). At the end of the routine collection period, 30 min after drug addition, secretion was within 20% of pre-drug concentrations and no inhibitory effect was observed. The effect of muscimol was found to be concentration dependent from 0.1-10μM (Figure 7.2) and the effect of 10μM muscimol was reduced from 186 ± 21% to 20 ± 2% ($n = 5$, $P < 0.05$) by 10μM BMI, indicating mediation of the effect by a $GABA_A$ receptor (Figures 7.2, 7.3).

The selective $GABA_B$ receptor agonist baclofen (100μM) had no effect on basal GH secretion(Figure 7.1). 3mM Ba^{2+} was found to be a very potent secretagogue for GH, and caused 673 ± 53% stimulation of secretion alone. When 100μM (±)baclofen was co-introduced, the response was unaffected (659 ± 57% stimulation, n = 5 in each case).

The pharmacology of the $GABA_A$-receptor mediated stimulation of GH secretion was further investigated using drugs previously found of interest in the study of GABA receptor effects on prolactin secretion. The effect of 1μM muscimol was potentiated by both the benzodiazepine Roll-6896 and the barbiturate secobarbital: 1μM muscimol caused 117 ± 21% stimulation alone, 186 ± 9% in the presence of 1μM Roll-6896 and 260 ± 40% in the presence of 100μM secobarbital ($P<0.05$ in both cases, n=5-6, drugs introduced simultaneously, Figure 7.3). 10μM strychnine did not antagonise the response to 10μM muscimol, the combination causing 162 ± 35% stimulation(Figure 7.4a). Homocarnosine (1mM) had no effect alone on GH secretion, nor did it significantly affect the response to GABA. 10μM GABA caused 128 ± 12% stimulation alone, and 96 ± 19% in the presence of homocarnosine (Figure 7.4b).

7.3.2 Luteinizing Hormone

10μM muscimol caused a small transient stimulation of LH secretion (17 ± 2%, n = 5)(Figure 7.5) which rapidly returned to pre-drug concentrations. No subsequent inhibition was observed. Neither baclofen (100μM)(Figure 7.6) nor GABA itself (10μM) had any effect on basal secretion. This effect of muscimol was abolished by 10μM BMI (no stimulation of release could be observed), suggesting the involvement of a $GABA_A$ receptor, but the lack of effect of

GABA itself, probably because of its lower potency than muscimol, suggested the importance of studying stimulus-induced release. LHRH was used, being the physiological secretagogue, and caused a prompt, concentration-dependent stimulation of secretion that peaked in 4-6 minutes with a gradual subsequent decline. 10μM muscimol significantly potentiated the peak response to 300nM LHRH in a greater than additive manner when added simultaneously, from 117 ± 4% to 164 ± 6% (n=5, P<0.05), and this potentiation was antagonised by 10μM BMI (Table 7.1, Figure 7.5). 10μM GABA potentiated the response to 3nM LHRH but had no effect on the response to 300nM LHRH, although there was a trend towards an inhibition (Table 7.1). 10μM BMI antagonised the stimulatory effect of 10μM GABA on the response to 3nM LHRH, but in the presence of 10μM BMI, 10μM GABA caused a significant inhibition of the response to 300nM LHRH (Table 7.1). The effect of baclofen was investigated in parallel experiments. 100μM (±)baclofen had no effect on basal LH secretion, but reduced the response to 300nM LHRH from 117 ± 4% to 82 ± 8% (n = 5, P < 0.05, Figure 7.6, Table 7.1). These results suggest that an inhibitory $GABA_B$ response determines the effects of GABA at high LHRH concentrations, but a stimulatory $GABA_A$ receptor effect is predominant at lower LHRH concentrations. An alternative putative secretagogue, Ba^{2+} (3mM) was found to have no effect on secretion of LH in contrast to other hormones, although 50mM K^+ caused a similar response to 300nM LHRH (Figure 7.7). Subsequent studies have shown this to be a sex-dependent effect, as Ba^{2+} stimulates LH secretion from female PD tissue under identical conditions (R. Mitchell, unpublished observation).

7.3.3 ACTH

10μM Muscimol caused a transient stimulation of ACTH secretion rising to a peak of 39 ± 5% (n = 5)above baseline (Figure 7.8a). Secretion returned to approximately pre-drug rates within 20 min, and no subsequent inhibition was seen. 10μM BMI reduced this effect to 13 ± 3% ($P < 0.05$, n = 5). 100μM (±)baclofen had no consistent effect on basal ACTH secretion (although sometimes a transient inhibition was observed (Figure 7.8a), but reduced the peak stimulation caused by 3mM Ba^{2+} from 265 ± 23% to 162 ± 17% (n = 5, $P < 0.05$ Figure 7.8b). The response to K^+ (substituted for Na^+ in the medium) was also inhibited by baclofen: 50mM K^+ caused 170±10% stimulation alone and 104±7% in the presence of 100μM baclofen ($p<0.05$, n = 5), suggesting that on corticotrophes a $GABA_B$ receptor (as well as $GABA_A$) can be functionally active, but only in the stimulated condition.

7.3.4 TSH

Neither muscimol (10μM) nor baclofen (100μM) had any effect on basal TSH secretion (Figure 7.9). The effect of 3mM Ba^{2+} was also unaffected by baclofen, causing 88 ± 10% peak stimulation alone, and 91± 13% in the presence of 100μM baclofen (n = 5, Figure 7.9).

Discussion

These results demonstrate the apparent presence of GABA receptors on somatotrophes, gonadotrophes, and corticotrophes but not thyrotrophes. The possibility exists that the effects of muscimol demonstrated here are not direct in each case, but may be indirect, by a paracrine interaction between different cell types. Such an interaction between gonadotrophes and lactotrophes has been demonstrated (Denef and Andries, 1983), and could be investigated

using purified and cultured cell types. There are, however, differences in the responses of the various celltypes, with only LH and ACTH secretion following the same pattern (i.e. stimulatory $GABA_A$ response and inhibitory $GABA_B$ response) which perhaps suggests that such an interaction is not of major importance. An alternative possibility is that $GABA_A$ depolarisation-induced K^+ efflux (Deschenes and Feltz, 1976; Loeffler et al, 1982) for example, from somatotrophes, may account for the slightly increased release of LH (and ACTH).

$GABA_A$ receptor activation in these experiments (being a BMI-sensitive response to muscimol) was found to cause a transient stimulation of GH, LH and ACTH secretion. This is similar to that seen on αMSH secretion (Demeneix et al, 1984), but somewhat in contrast to prolactin secretion where a biphasic $GABA_A$ response is seen, with inhibition subsequent to the transient stimulation (Chapter 5). This purely stimulatory effect was clearest on GH secretion (Figure 7.1), suggesting that the inhibitory effect of $GABA_A$ agonists on prolactin secretion is not purely a consequence of the stimulation (Taraskevich & Douglas, 1982), but involves a quite distinct response mechanism that can be present or absent in different cell types.

Previous experiments have been unable to show any effect of GABA or, where investigated, muscimol on <u>in vitro</u> secretion of any of the hormones here examined (Fiok et al, 1981a, 1984; Jones et al, 1984; Vijayan & McCann, 1978b; Jordan et al, 1983), the only exception being that a stimulatory effect has been reported on GH secretion from neonate rat pituitaries, although the same authors were unable to find this effect using adult tissue (Acs et al, 1984). One

possible contributory factor, other than the inherent unsuitability of static incubations for the detection of transient events, is the high concentration of GABA-transaminase in PD (Racagni et al, 1979), which can prevent the inhibitory effect of GABA on prolactin secretion from being observed (Racagni et al, 1983). None of these reports used a superfusion system.

The mechanisms of action of $GABA_A$ and $GABA_B$ receptors on stimulus-secretion coupling are different. $GABA_A$ receptor activation results in an increased Cl^- conductance which (at least transiently) may depolarise the secretory cell membrane and cause a flurry of action potentials (Taraskevich & Douglas, 1982). This in turn will facilitate (again probably transiently) the release of hormone, and may be the mechanism underlying the effect of muscimol on the secretion of GH and ACTH. As gonadotrophes apparently show no spontaneous action potentials (Mason and Waring, 1984) but only an increased membrane electrical 'noise', even in response to LHRH (Mason and Waring, 1984) another mechanism may possibly be involved. Nevertheless, depolarisation with K^+ is an effective secretagogue in gonadotrophes, so partial depolarisation from $GABA_A$ receptor induced Cl^- movement may well promote the secretory mechanism. The transient nature of these responses may be a result of inactivation of voltage-dependent Ca^{2+} channels (Tomiko et al, 1981).

Evidence for endocrine responses to $GABA_B$ receptor activation is scanty, but stimulatory effects of baclofen on GH secretion in man and rat have been reported (Koulu et al., 1979; Fiok et al., 1981b). $GABA_B$ receptor activation results in inhibition of

neurotransmitter (Bowery et al, 1980) or hormone (Demeneix et al, 1984) release and blockade of calcium channels has been proposed as a mechanism of action (Dunlap, 1981; Dunlap and Fischbach, 1981). A hyperpolarising postsynaptic response to baclofen (as a result of increased K^+ conductance) (Newberry and Nicoll, 1984a) has also been reported in the hippocampus. Both of these type of effects would tend to inhibit secretion particularly in the stimulated situation. No effects of baclofen were found on basal secretion of any of the hormones here investigated, but baclofen was found to inhibit Ba^{2+}- and K^+-induced secretion of ACTH but not GH or TSH (or prolactin, Chapter 5) and LHRH-induced secretion of LH. Previous studies (Demeneix et al, 1984) have also shown greater effects of baclofen on stimulated than on basal secretion. Other possible mechanisms of action of $GABA_B$ receptors are discussed in Chapter 5, but no relevant experiments were carried out in this Chapter.

Barium is a potent secretagogue in PD, as shown here, and also in PI, where it has been used previously in the demonstration of a $GABA_B$ receptor effect. Barium is considered to act by facilitating sustained cell firing and the development of long-lasting action potentials by penetrating voltage dependent Ca^{2+} channels and blocking K^+ channels (Taraskevich and Douglas, 1980; Hagiwara and Byerly, 1981; Douglas et al, 1983; Adams et al, 1982a). Spontaneous action potentials have been detected in anterior pituitary tissue <u>in vitro</u> (Douglas and Taraskevich, 1978; Ozawa and Sand, 1978) and in cultures of purified somatotrophes provided that they have been cultured long enough to recover from the effects of trypsin used in preparation (Israel, personal

communication). LH secretion (from male tissue) was found to be unaffected by 3mM Ba^{2+}, which supports the above mechanism of action of Ba^{2+} in that ovine pars tuberalis gonadotrophes do not show action potentials, even in response to LHRH (Mason and Waring, 1984). This result does not provide evidence that LHRH acts ubiquitously by inhibition of the M current, an action which has been proposed to account entirely for its effect on bullfrog ganglia (Adams et al, 1982a) because Ba^{2+} also blocks this current.

The action of GABA in facilitating the LH response to a low (3nM) concentration of LHRH, but reducing the response to a high (300nM) concentration is potentially important in terms of a regulatory role. These effects appeared to be mediated by separate receptors, the potentiation of the response to LHRH being mediated by a $GABA_A$ receptor, but the inhibition of the response to LHRH being mediated by a $GABA_B$ receptor. If these results can be extrapolated to in vivo, the physiological role of GABA may be to sensitise gonadotrophes to low LHRH concentrations while suppressing the effects of high concentrations, and this perhaps only occurs under particular conditions in which the secretion of GABA into hypophysial portal blood is elevated. Similar effects of GABA acting at $GABA_A$ receptors to enhance neurotransmitter release at low secretagogue concentrations and at $GABA_B$ receptors to reduce release at high secretagogue concentrations, have been previously observed in the CNS (Bowery et al, 1980). The number of $GABA_A$ binding sites in the PD can be altered by steroid treatment (Racagni et al, 1983), which also increased GABA concentration in PD and GAD activity in ME (Nicoletti, Grandison and Meek, 1985) and this may support the idea of a variable GABAergic influence of gonadotrophe function.

Benzodiazepines and barbiturates commonly potentiate GABAergic responses (Ransom and Barker, 1976; Choi et al., 1977; Macdonald and Barker, 1978; 1979). Benzodiazepines potentiate the stimulatory effect of muscimol on prolactin secretion (Chapter 5), and there are central-type benzodiazepine binding sites in the PD (Chapter 4). Both the benzodiazepine Ro11-6896 and the barbiturate secobarbital were found to potentiate the stimulation of GH secretion caused by muscimol, demonstrating a further cellular location for $GABA_A$ receptor/modulator/chloride channel complexes (Olsen, 1982) outside the CNS. These complexes are therefore found on a large proportion of PD cells. The number of $GABA_A$ and central-type benzodiazepine binding sites in PD is much less than that found in CNS regions (Chapters 3 and 4) suggesting that their concentration on receptive cell membranes is much less at non-synaptic PD sites that at synaptic sites within the CNS. Benzodiazepines can modify plasma prolactin concentrations (Grandison, 1982; Lotz, 1982), and these results suggest that GH concentrations may also be affected.

In these experiments strychnine was found to be ineffective at antagonising the effect of muscimol on GH secretion. In contrast, muscimol-induced stimulation of prolactin secretion was very potently antagonised by strychnine, which was found to be slightly more potent than BMI (Chapter 5). The GABA-containing dipeptide homocarnosine, present in hypophysial portal blood in high concentrations (Mitchell et al, 1983) was without effect on GH secretion. Homocarnosine has modest affinity for $GABA_A$ receptors (Mitchell et al, 1983), but causes significant inhibition of

prolactin secretion in vitro (Schally et al, 1977; Chapter 5). Its lack of either agonist or antagonist action on GH secretion suggests that it has neither affinity nor efficacy at this particular $GABA_A$ receptor. The $GABA_A$ receptor mediating stimulation of GH secretion therefore shows both similarities and differences to the $GABA_A$ receptor mediating stimulation of prolactin secretion, being benzodiazepine- and barbiturate-linked but insensitive to strychnine and homocarnosine. This further emphasizes the novelty of the $GABA_A$ receptor effects on prolactin secretion, being the only pituitary hormone so far studied from the anterior or intermediate lobes which can be inhibited by $GABA_A$ agonists in addition to what must be now regarded as a near-ubiquitous stimulatory effect. If any of the responses demonstrated here are indirect, e.g. mediated by increased extracellular K^+, this might be a consequence of the ability of this tissue preparation to reveal such effects, as the structure of the tissue is largely preserved. If these responses were not observed using a dispersed cell preparation, this in turn might reflect the physiological importance of intercellular interactions of this kind.

These results provide further evidence for a neuroendocrine role of GABA in PD. Previously GABA has been associated only with the control of prolactin secretion at the level of the pituitary (Schally et al, 1977; Enjalbert et al, 1979) but these results demonstrate that both stimulatory and inhibitory GABAergic influences on other hormones require consideration in in vivo experiments.

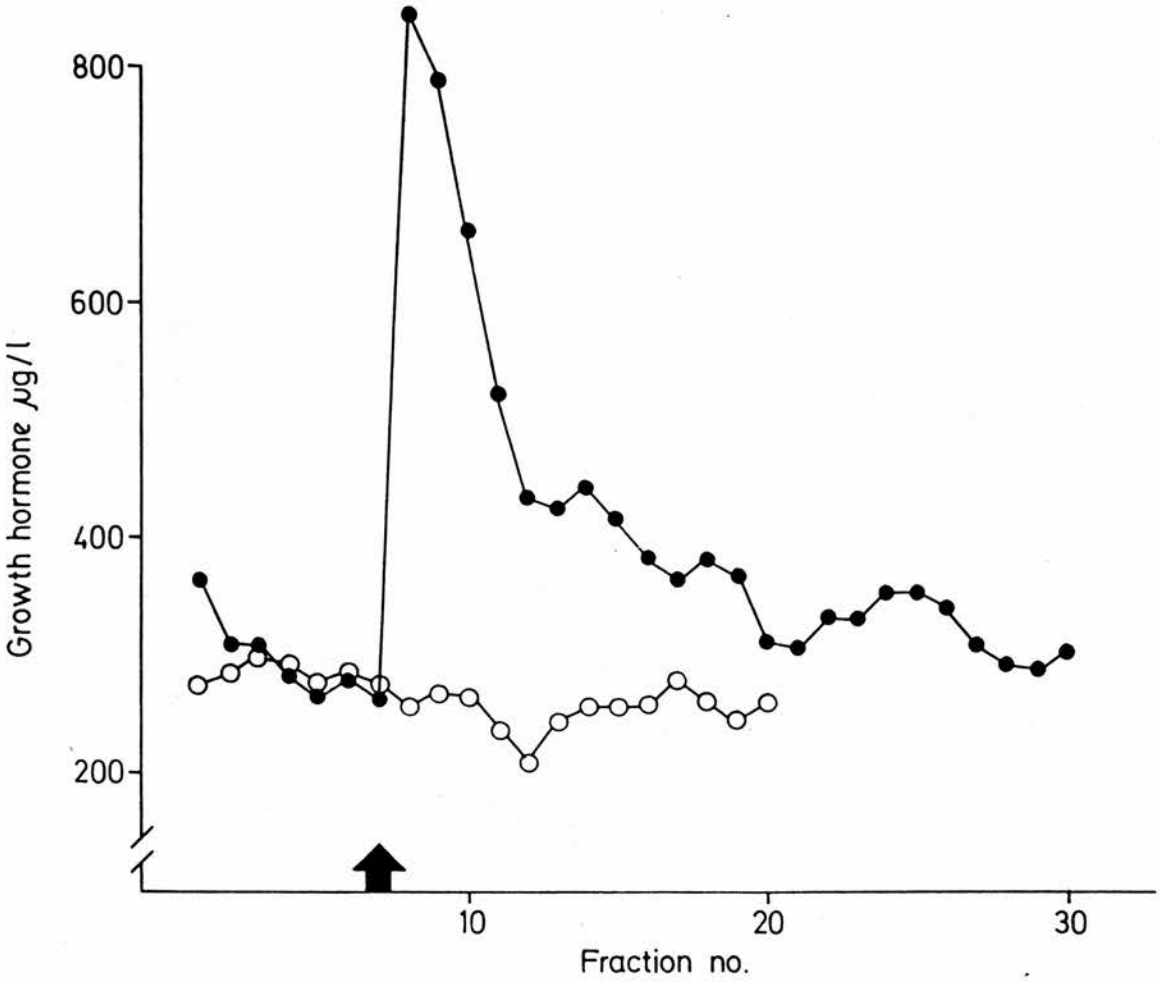


FIGURE 7.1

Effects of 10µM muscimol (●) and 100µM(±) baclofen (○) on basal GH secretion. Drugs were introduced at the arrow. Representative experiments.

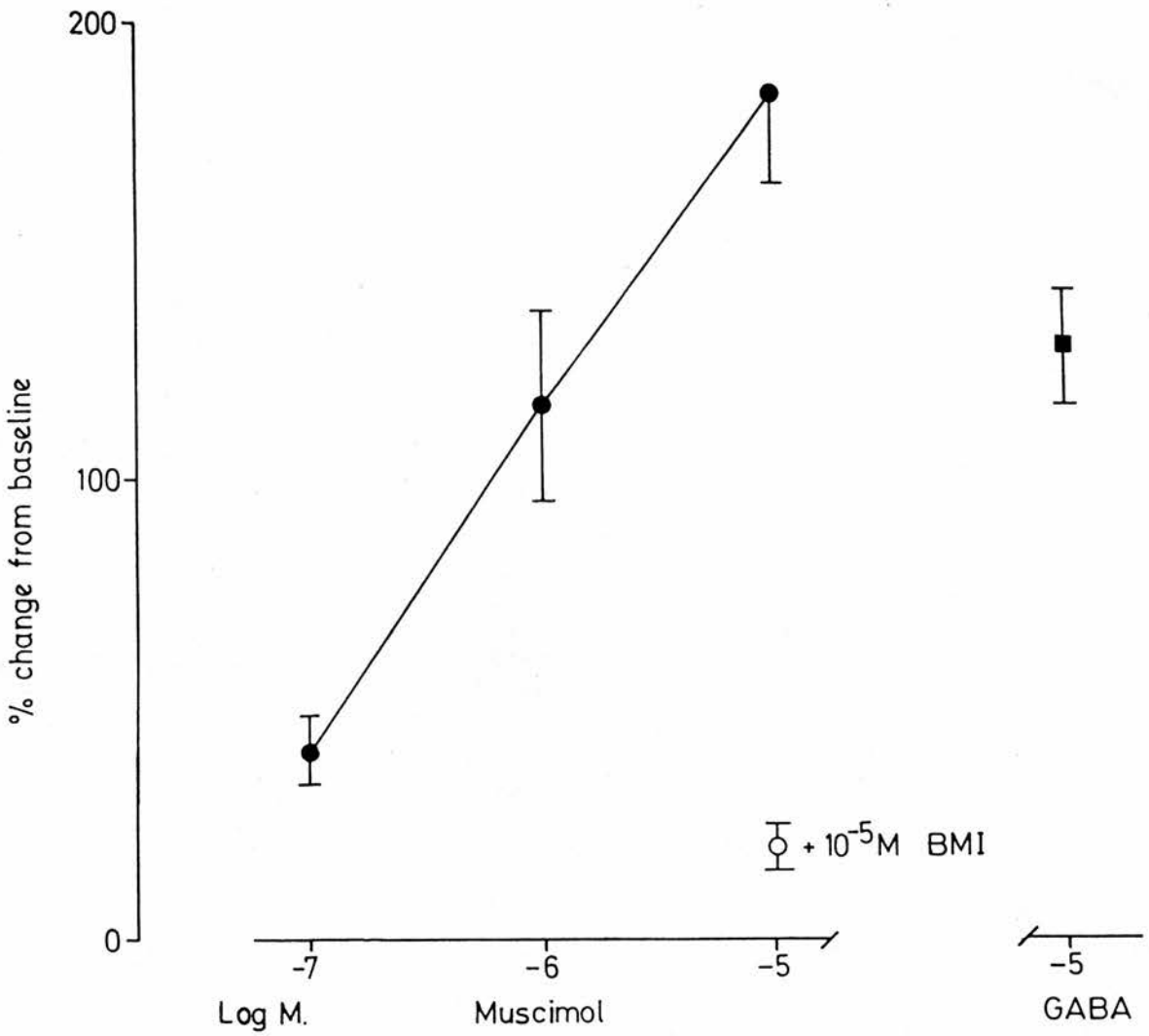


FIGURE 7.2

Stimulation of GH secretion by muscimol and GABA, and antagonism by BMI.

(●) Concentration-response curve for muscimol
(○) Effect of 10μM muscimol in the presence of 10μM BMI
(■) Effect of 10μM GABA.

Results are expressed as percentage increase in secretion, mean ± S.E.M., n = 5 in each case.

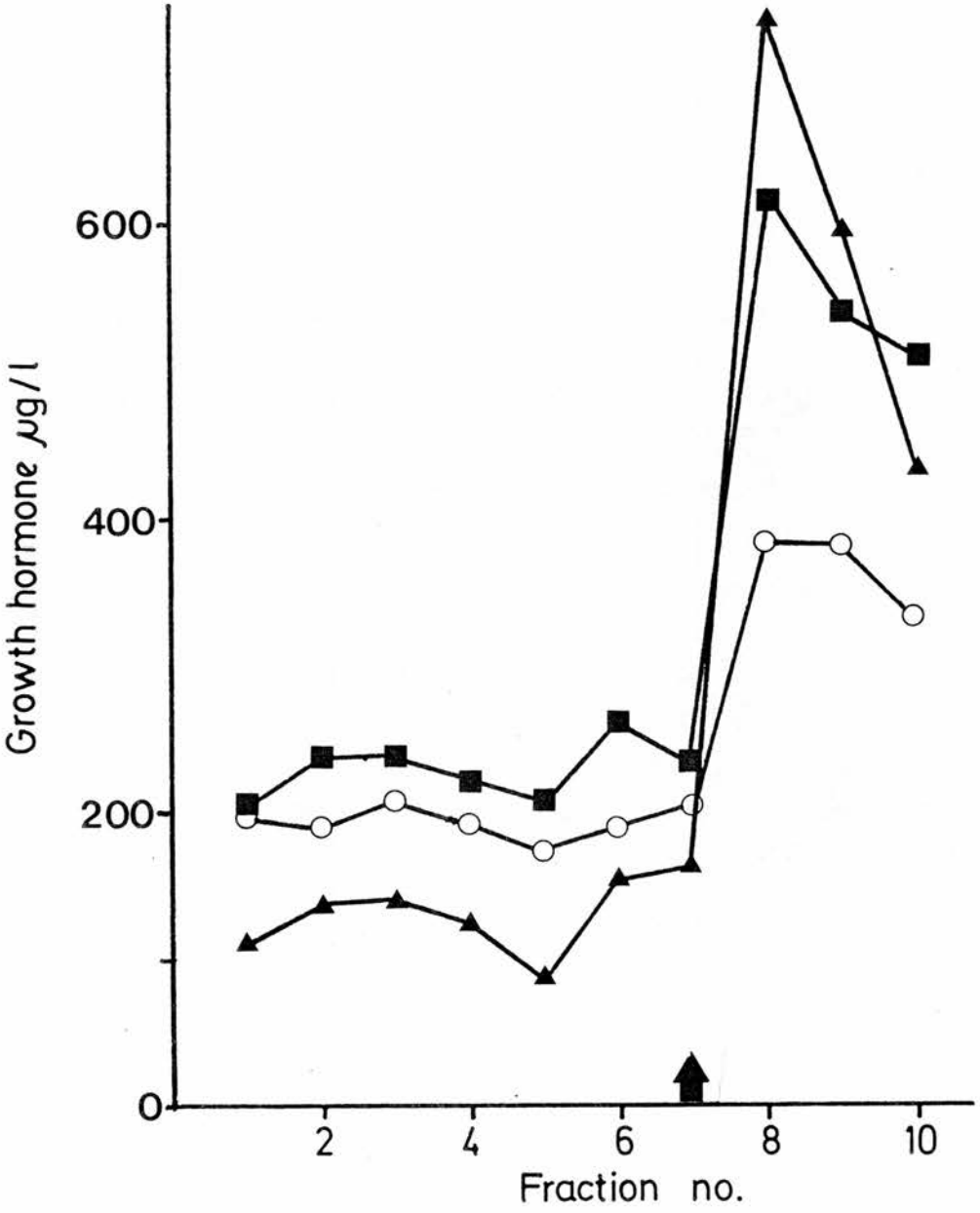


FIGURE 7.3

Modulation of the effect of muscimol on GH secretion by benzodiazepines and barbiturates.

(○) Effect of 1µM muscimol
(▲) Effect of 1µM muscimol + 1µM Roll-6896
(■) Effect of 1µM muscimol + 100µM secobarbital

Representative experiments. Drugs were introduced at the arrow.

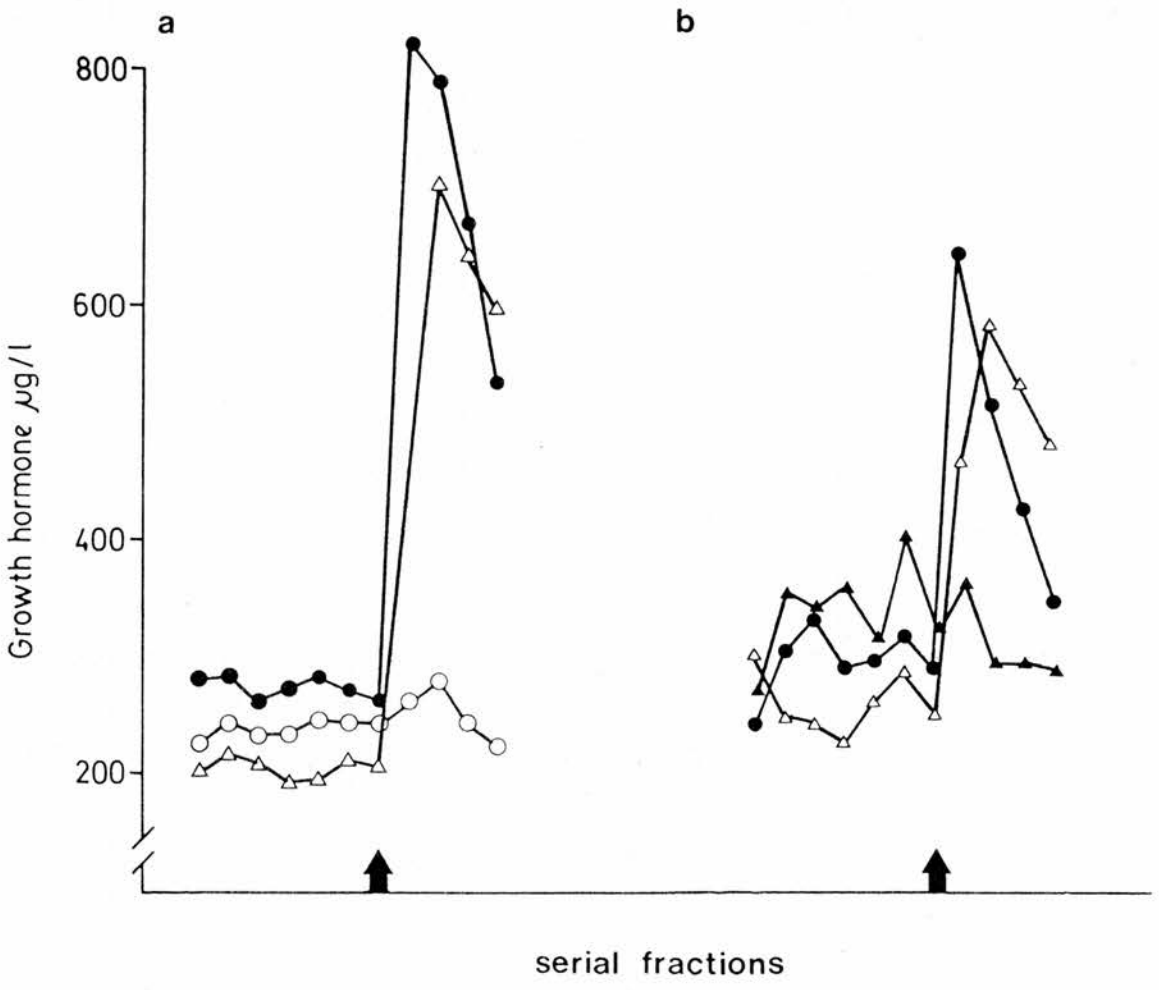


FIGURE 7.4

a) Selective antagonism of the effect of muscimol on GH secretion by BMI but not strychnine.

(●) Effect of 10µM muscimol
(○) Effect of 10µM muscimol in the presence of 10µM BMI
(△) Effect of 10µM muscimol in the presence of 10µM strychnine

Muscimol was introduced at the arrow. Antagonists were introduced 30 mins previously.

b) Effect of GABA and homocarnosine on GH secretion.

(●) Effect of 10µM GABA
(▲) Effect of 1mM homocarnosine
(△) Effect of 10µM GABA + 1mM homocarnosine

GABA and homocarnosine were introduced at the arrow.

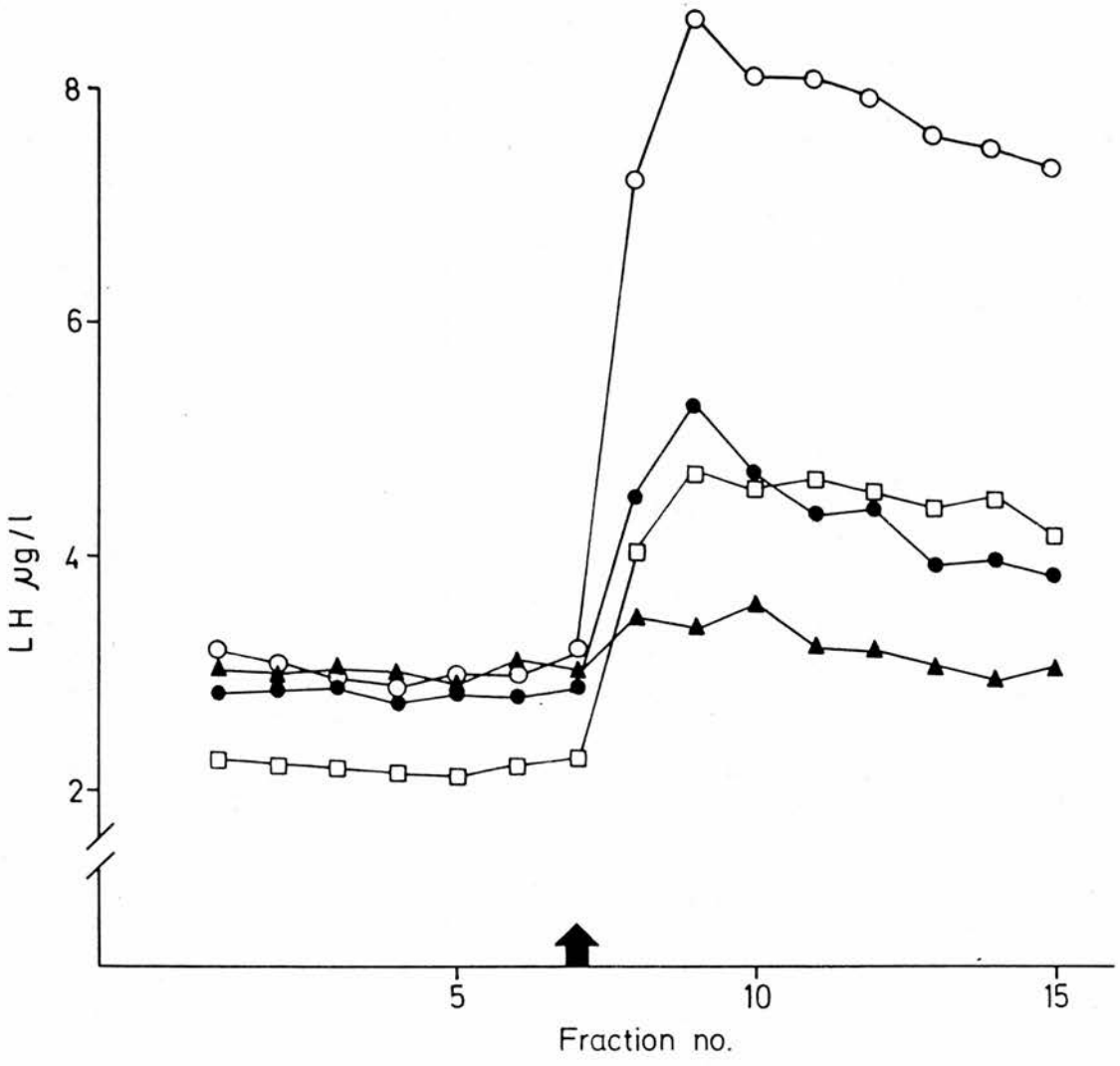


FIGURE 7.5

Interaction of $GABA_A$ receptors with LHRH-stimulated LH secretion.
(▲) Effect of 10μM muscimol
(□) Effect of 300nM LHRH
(○) Effect of 10μM muscimol + 300nM LHRH
(●) Effect of 10μM muscimol + 300nM LHRH in the presence of 10μM BMI.

Muscimol and LHRH were introduced at the arrow. BMI was introduced 30 mins previously. Representative experiments.

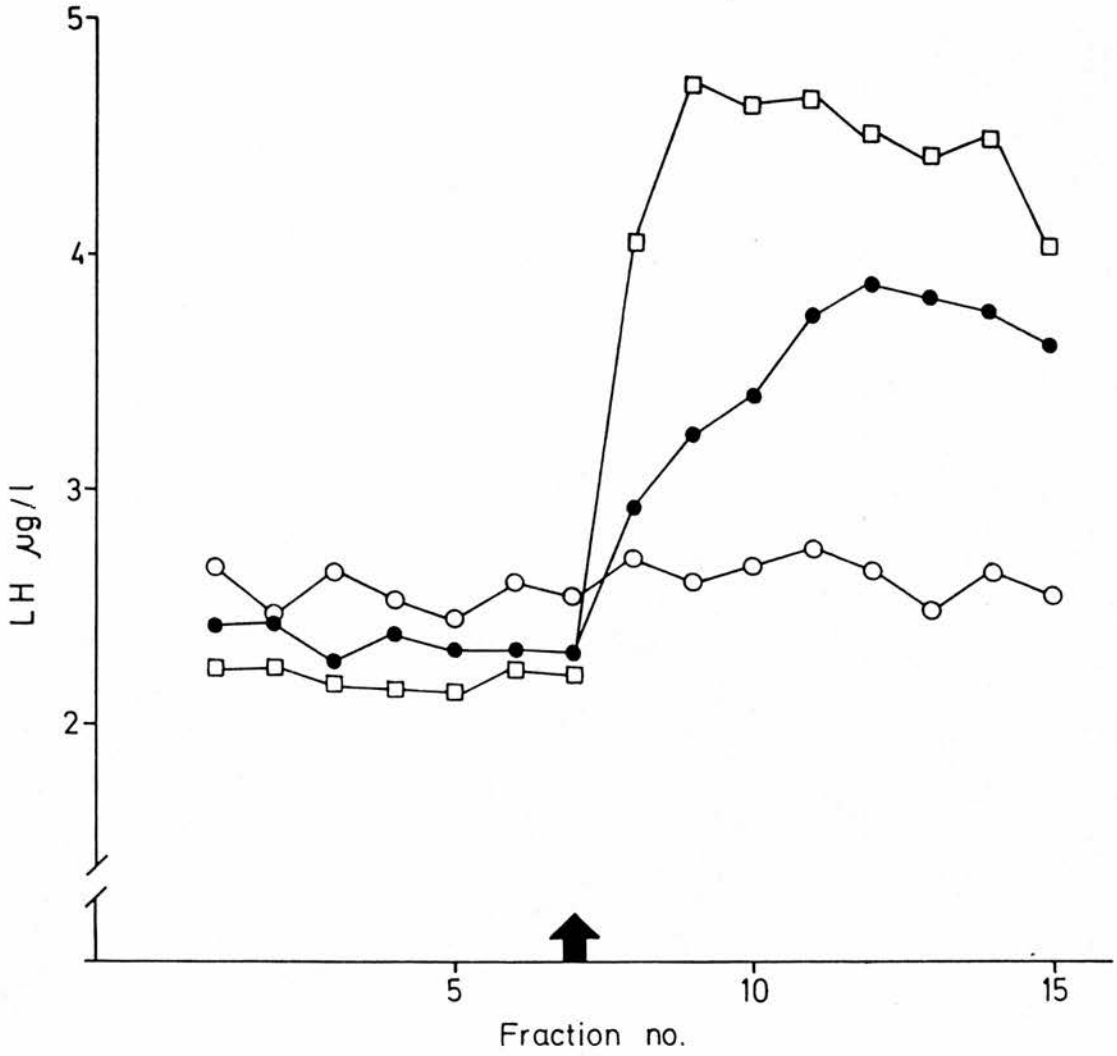


FIGURE 7.6

Interaction of $GABA_B$ receptors with LHRH - stimulated LH secretion.

(○) Effect of 100µM (±)baclofen
(□) Effect of 300nM LHRH
(●) Effect of 100µM (±)baclofen + 300nM LHRH

Baclofen and LHRH were introduced at the arrow. Representative experiments.

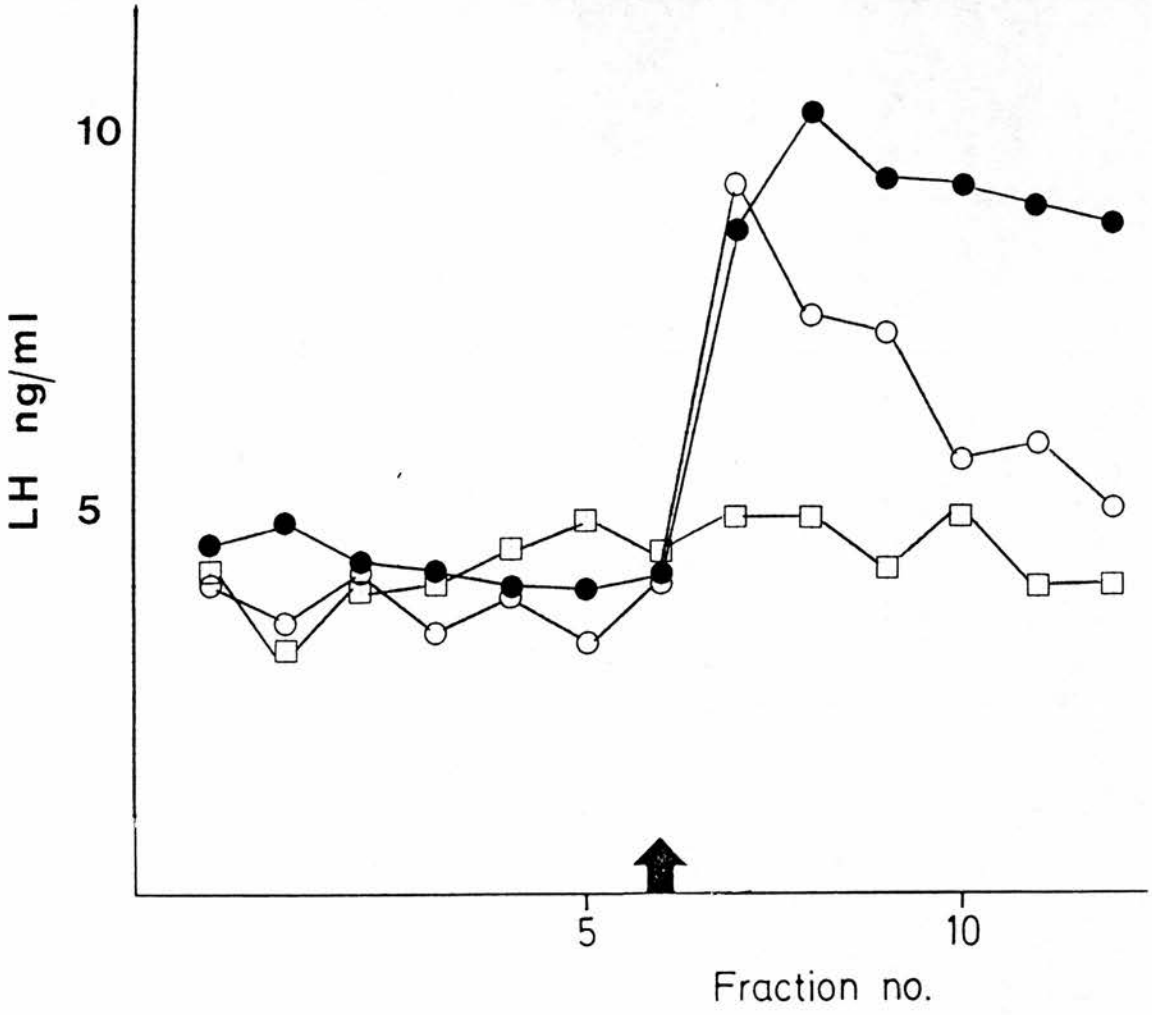


FIGURE 7.7

Secretagogue action of K^+ but not Ba^{2+} on LH secretion.

(○) Effect of 50mM K^+
(□) Effect of 3mM Ba^{2+}
(●) Effect of 300nM LHRH

Ionic-substituted medium or LHRH were introduced at the arrow.
Representative experiments.

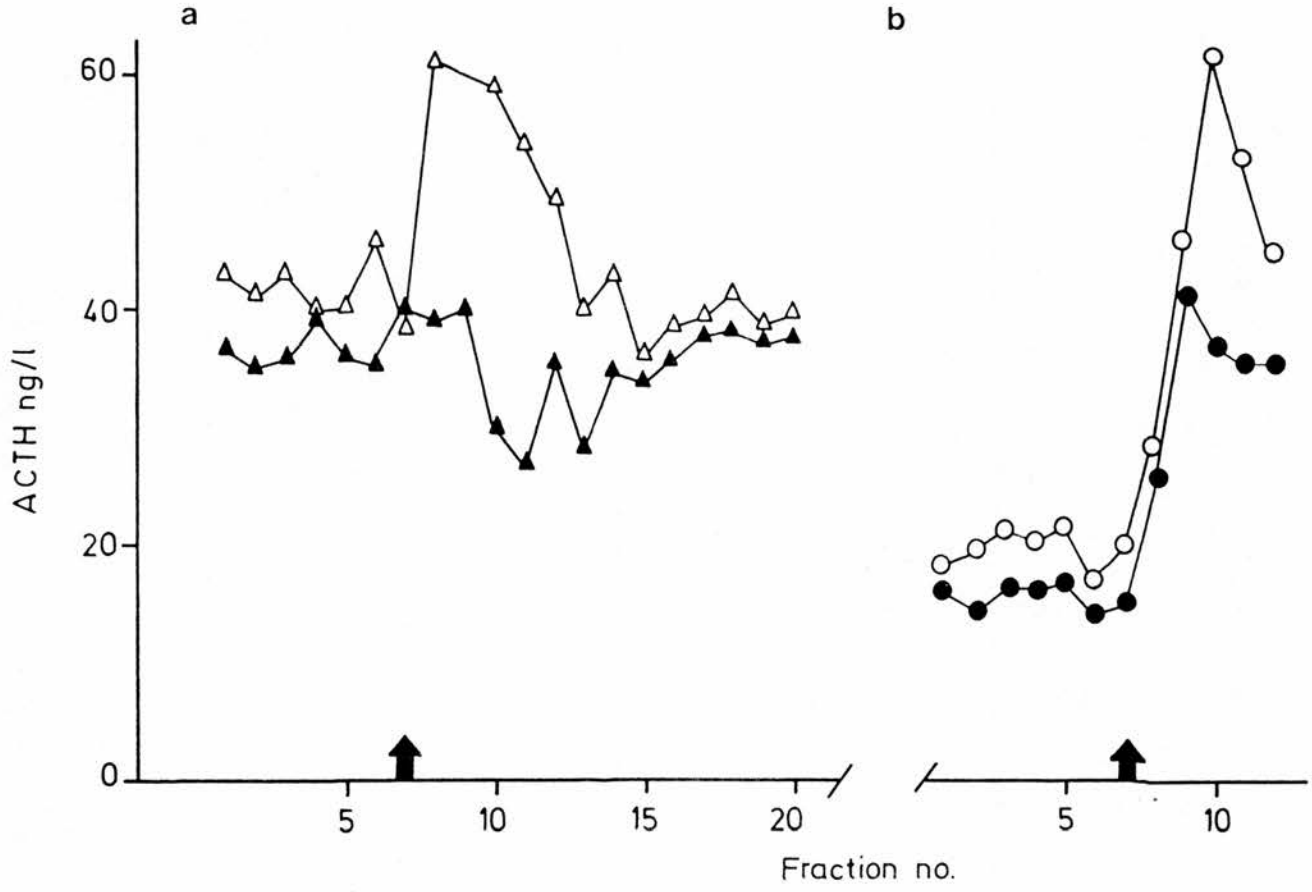


FIGURE 7.8

Effects of muscimol and baclofen on ACTH secretion.

a)(△) Effect of 10μM muscimol
(▲) Effect of 100μM (±) baclofen

b)(○) Effect of 3mM Ba^{2+}
(●) Effect of 3mM Ba^{2+} with 100μM (±) baclofen

Representative experiments. Muscimol, baclofen and Ba^{2+} were introduced at the arrows.

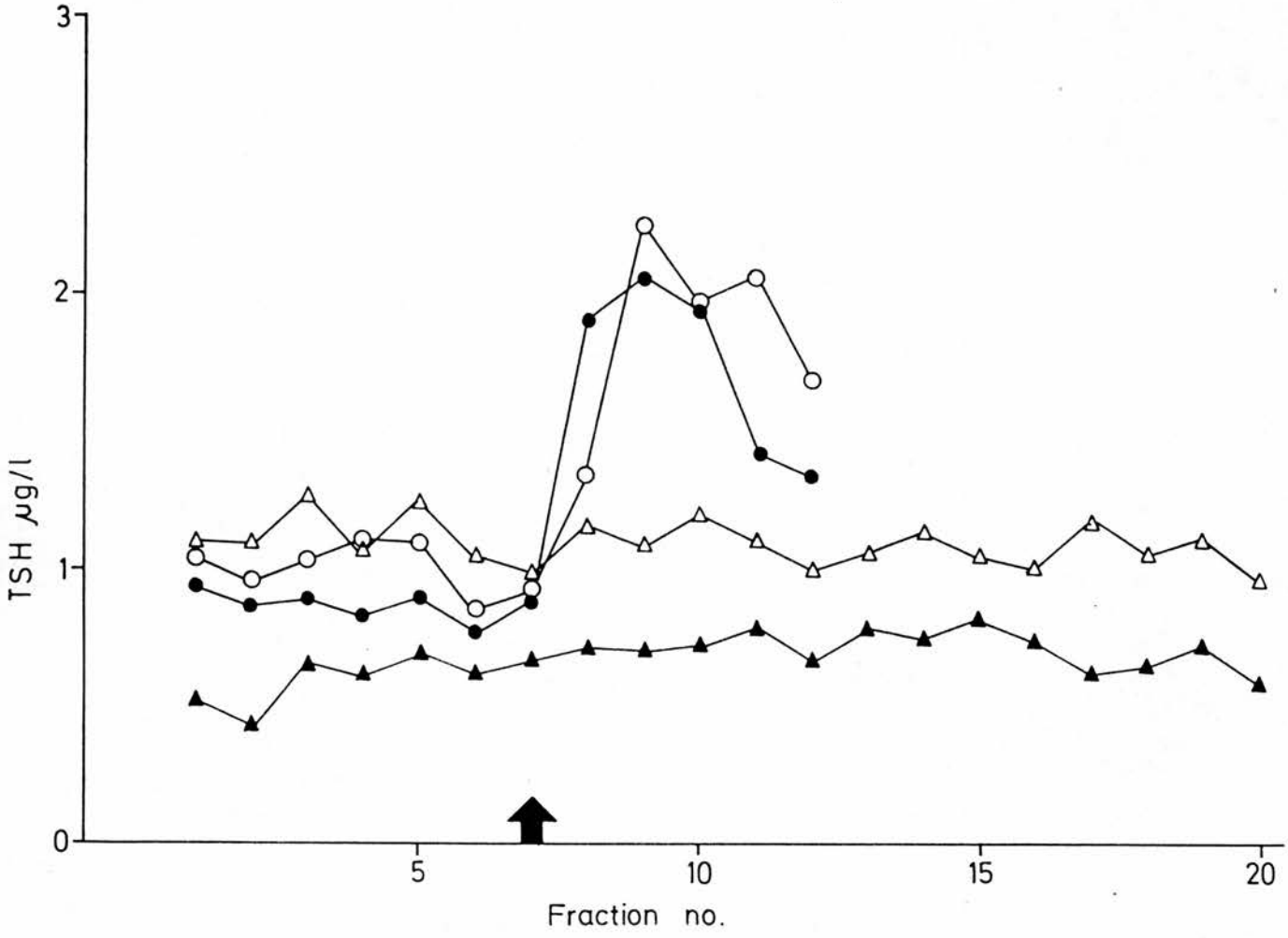


FIGURE 7.9

Effects of muscimol and baclofen on TSH secretion.

(△) 10μM muscimol
(▲) 100μM (±)baclofen
(○) 3mM Ba^{2+}
(●) 3mM Ba^{2+} + 100μM (±)baclofen

Representative experiments. Muscimol, baclofen and Ba^{2+} were introduced at the arrow.

TABLE 7.1

$GABA_A$ and $GABA_B$ receptor modulation of the effect of LHRH on LH secretion.

LHRH	3nM	300nM
Control	45 ± 6	117 ± 4
+ 10μM Muscimol	-	164 ± 6*
+ 10μM Muscimol + 10μM BMI	-	91 ± 9≠
+ 100μM Baclofen	-	82 ± 8*
+ 10μM GABA	75 ± 7*	78 ± 11
+ 10μM GABA + 10μM BMI	42 ± 5≠	52 ± 4*

Values are peak percentage stimulation of secretion.

* $P < 0.05$ vs control

≠ $P < 0.05$ vs Agonist alone

Mean ± S.E.M., n = 5 in each case.

CHAPTER 8

Characteristics of the uptake and release of [^{3}H]GABA by the median eminence and the neurointermediate lobe of the pituitary

8.1 Introduction

The median eminence (ME) contains very high metabolic activity corresponding to the GABA-synthesising enzyme GAD (Tappaz, Brownstein and Palkovits, 1976) and high concentrations of GABA itself (Van der Heyden, DeKloet, Koft and Versteeg, 1979). ME GAD activity is unaltered following deafferentiation of MBH (Tappaz and Brownstein, 1977) suggesting that it is present in neurones intrinsic to that area. Much lower GAD activity is present in the posterior pituitary and it was originally suggested to be confined to glial cells (Beart, Kelly and Schon, 1974). This was supported by autoradiographic localisation of [^{3}H]GABA accummulated in the posterior pituitary (Beart et al, 1974; Hamberger, Norstrom, Sandberg and Svanberg 1979). Release of [^{3}H]GABA from the posterior pituitary also showed glial characteristics (Minchin and Nordman, 1975). Similar experiments using ME tissue following either *in vitro* or *in vivo* accummulation of [^{3}H]GABA showed that it was taken up predominantly by neurones in the external layer of ME (Tappaz, Aguera, Belin and Pujol, 1980).

More recently, immunohistochemical studies using antisera to purified GAD as a marker for GABAergic neurones have been used to describe a dense plexus of GAD-like immunoreactivity in both the external layer of ME and the intermediate lobe of the pituitary, with a less dense innervation of the posterior lobe (Vincent et al, 1982; Tappaz et al, 1983). GAD activity and immunoreactivity in NI was virtually abolished by transection of the pituitary stalk (Oertel et al, 1982) suggesting that it is of central origin, but the precise location of the cell bodies remains unknown.

These studies were undertaken to examine the uptake and release of [^{3}H]GABA in ME and NI with the aim of establishing neurochemically the presence of GABA nerve terminals and revealing their characteristics.

One characteristic of GABA nerve terminals in several CNS regions is the presence of presynaptic autoreceptors (Mitchell and Martin, 1978a; Arbilla, Kamal and Langer, 1979). GABAergic nerve terminals in ME might be expected not to be subject to autoreceptor control if GABA is not released into a synaptic environment but into hypophysial portal blood (Mitchell et al, 1983). Similarly, DA terminals in ME have been shown to have a lower affinity uptake mechanism than in striatum (Demarest and Moore, 1979) and DA release was not influenced by autoreceptors (Annunziato, Cerrito and Raiteri, 1981). Others have reported the existence of DA autoreceptors, using the larger whole ME preparation (Sarkar, Gottshall, Meites, Horn, Dow, Fink and Cuello, 1983).

The role of DA as a major PIF is better established than that of GABA (Leong et al, 1983) and the apparent coexistence of tyrosine hydroxylase-immunoreactivity and GAD-IR has been described in some neurones projecting to ME (Everitt et al, 1984). Serotonin (5-HT) is present in the ME in both tanocytes (Sladek and Sladek, 1981) and nerve fibres (Bjorklund, Flack, Hromek, Owman and Wert, 1970; Palkovits, Saavedra, Jacobowitz, Kizer, Zaborsky and Brownstein, 1977) and may interact with GABA and DA in the control of prolactin secretion (Caligaris and Taleisnik, 1974; Wehrenburg, McNicol, Frantz and Ferin, 1980). Indeed the effect of 5-HT on plasma prolactin concentration can be antagonised by picrotoxin (Caligaris and Taleisnik, 1974). Similarly, there is both a dopaminergic and

serotonergic innervation of NI (Leranth, Palkovits and Kreiger, 1983) and both of these transmitters can affect hormone secretion from the PI in vitro (Bower, Hadley and Hruby, 1974). Therefore, in view of the morphological and functional connections between systems of these two transmitters and GABA, their effects on GABA release in both ME and NI were examined. Similar experiments have been carried out to determine whether prolactin, which may have a feedback effect on GABA synthesis in MBH (Nicoletti et al, 1983), also increases GABA release.

Vasopressin is found in ME as well as the posterior pituitary (Zimmerman, Stillman, Recht, Antunes and Carmel, 1977) in terminals of neurones of the paraventricular nucleus (Alonso and Assenmacher, 1981). This projection appears distinct from that to PN, both anatomically (Swanson and Sawchenko, 1980) and functionally, being involved in the control of ACTH secretion (Zimmerman et al, 1977; Gillies and Lowry, 1979; Rivier and Vale, 1983). An electrophysiological study, however, provided evidence that the ME projection consisted of axon collaterals of the neurohypophysial projection (Pittman, Blume and Renaud, 1978). The density of vasopressin binding sites in ME and PN (Brinton, Gee, Wamsley, Davis and Yamamura, 1984) suggests that vasopressin may be involved in the local control of release of other transmitters/releasing factors in both areas. αMSH is also found in nerve terminals in ME (O'Donohue, Miller and Jacobowitz, 1979), and may be involved in the control of prolactin (Khorram, Mizunuma and McCann, 1982) and LH secretion (Alde and Celis, 1980; Khorram, De Platis and McCann, 1984). The release of both vasopressin and αMSH from the pituitary can be affected by GABA, that of αMSH by a direct action (Tomiko et al,

1983) and that of vasopressin both centrally, possibly by an action within the supraoptic nucleus (Knepel et al, 1980; Arnauld, Cirino, Layton and Renaud, 1983) and directly (Knepel et al, 1984). An interaction within the neurohypophysis may be supported by the demonstration of increased [^{3}H]GABA uptake by that area following water deprivation (Hamberger et al, 1979). The possible influence of these hormones on [^{3}H]GABA release from ME and NI was therefore investigated.

8.2. Materials and methods

8.2.1. Materials

[^{3}H]GABA (aminobutyric acid, γ-[2,3-^{3}H]-), specific activity 80-83 Ci/mmol was obtained from New England Nuclear. The medium consisted of NaCl (133mM), KCl (5mM), $CaCl_2$(2.7mM), $MgSO_4$ (1.2mM), Tris (10mM) and glucose (10mM) pH 7.4, all Analar, BDH, and was equilibrated with 100% O_2.

β alanine, L-2,4 diaminobutyric acid, muscimol, dopamine, serotonin, αMSH, arginine vasopressin, ovine prolactin, and amino oxyacetic acid (AOAA) were obtained from Sigma. Bicuculline methiodide (BMI) was obtained from Pierce Chemical Co.

8.2.2. Tissue preparation

Male Wistar rats (200-250g) were decapitated and the brain rapidly removed. The pituitary gland was removed and NI separated from PD. The ME (including a small part of the pituitary stalk) was dissected from the brain on an ice-cold platform under a binocular microscope as described in section 2.2. Tissue was collected into cold oxygenated Krebs medium then transferred to a teflon/glass homogeniser. Pooled samples of ME or NI were homogenised in cold 0.32 M sucrose to produce a crude synaptosomal preparation of neurone terminals (Gray and Whittaker, 1960).

8.2.3. Uptake studies

NI and ME were homogenised in sucrose at a tissue concentration of one area/100µl (60-100µg protein for NI, 12-25µg protein for ME). 100µl aliquots were taken for individual determination of [^{3}H]GABA uptake and added to 8 volumes of cold Krebs medium. Samples were then preincubated at 25°C for 20 min before the addition of [^{3}H]GABA in 1 additional volume to give a final concentration of 1µM and sucrose dilution to 32 mM, in a final volume of 1 ml. After a further 5 min incubation, samples were filtered through Whatman GF/B filters under vacuum and washed with 3 x 5 ml medium at 25°C. Radioactivity incorporated into the filters was subsequently measured by LSC after overnight extraction into 10ml NE265 scintillation fluid. This standard protocol was adapted as follows to characterise the properties of [^{3}H]GABA uptake into the two regions:

a) Time-dependence: incubations were carried out for 1,2,5 or 10 min.

b) Temperature-dependence: control incubations were carried out on ice throughout, and filters were washed with cold medium.

c) Sodium dependence: choline chloride was substituted for NaCl in the medium.

d) Pharmacological properties: β alanine (1 mM) or L-2, 4 diaminobutyric acid (DABA) (500 µM) were present from the beginning of the preincubation period.

e) Kinetic analysis: a range of [^{3}H]GABA concentrations (1.0-20 µM) were used.

Appropriate blanks were subtracted in all cases derived either from detergent lysed preparations (1% Triton X-100) or tissue free incubation, which gave essentially identical results (approximately 30% of apparent uptake at 5 min in ME, 50% in NI). Aliquots of homogenate were taken for determination of protein content according to section 2.4.

8.2.4 Release experiments

Sucrose homogenates were diluted into medium to give a tissue concentration of 10 areas/4 ml and preincubated at 37°C for 15 min. 50nM [^{3}H]GABA was then added, with 10μM AOAA to inhibit catabolism of the accumulated transmitter through the longer time course of these experiments. After a further 15 min, aliquots corresponding to one dissected tissue area were transferred on to Whatman GF/B filters in parallel superfusion chambers and continuously perfused with oxygenated medium at 37°C at 0.5 ml/min. The superfusion apparatus was the same as that used for hormone secretion studies, and is described in section 5.2. After 30 min washout, 1 min fractions were collected. At the beginning of the 6th or 7th fraction, superfusion intake lines were transferred to medium containing depolarising concentrations of potassium ions and/or drugs as appropriate. After 12 fractions had been collected, radioactivity in each fraction and that remaining in the tissue was measured by LSC.

8.2.5. Chemical identity of released tritium

Aliquots of superfusate from both basal and K^+ pulse samples were passed through 3 x 0.6 cm columns of Dowex 50W (100-200) mesh in the H^+ form according to Iversen and Kravitz (1968). Acidic metabolites were eluted with 2 ml H_2O and were found to account

for only a few per cent of total radioactivity under these conditions. The remaining radioactivity (> 90%) was retained, to be eluted by 5ml of 1.5M NH_4OH indicating that its probable identity was unchanged authentic [^{3}H]GABA (n = 3-5 in each case).

8.2.6. Calculation of results and statistics

The results of uptake experiments were calculated as pmol GABA transported/mg protein/min. Kinetic data was derived by a computerised iterative least-squares program, as used in Chapter 3. Release data were calculated as fractional release coefficient (FRC) (i.e. radioactivity in a fraction as a percentage of tissue radioactivity at the beginning of that fraction). Values for percentage stimulation of release were obtained from the peak release coefficient and the mean of the two basal release fractions immediately preceding the stimulus. Significance of difference for uptake studies was by Students paired t-test, and for release studies was by Mann-Whitney U test, using values for peak percentage stimulation. In some cases percent stimulation of release was recalculated as a percentage of a control stimulation carried out at the same time on a parallel superfusion.

8.3 Results

8.3.1 Uptake of [^{3}H]GABA

Tritium was rapidly accumulated by both tissues when incubated with 1μM [^{3}H]GABA and uptake was approximately linear for 10 min (Figure 8.2) at these tissue concentrations, although ME showed a 20 fold greater capacity for uptake. Uptake was markedly reduced in sodium-free medium, by a mean of 89% in NI and 98% in ME, and also showed temperature-dependence, being reduced by a mean of 81% in NI and 90% in ME when incubations were performed on ice (Figure 8.3).

The pharmacological profiles of the uptake sites involved were investigated using β alanine and DABA as selective inhibitors of glial and neuronal [^{3}H]GABA uptake respectively (Iversen and Johnston, 1971; Schon and Kelly, 1974). Uptake was not significantly reduced in the presence of 1mM β alanine, but 500μM DABA inhibited uptake by 46% ($p < 0.001$) in NI and 56% ($p < 0.005$) in ME (Figure 8.3).

Kinetic analysis of [^{3}H]GABA uptake was carried out using a 5 min incubation period with [^{3}H]GABA concentrations between 1 and 20μM. For both areas, a double reciprocal plot gave a good fit to a single straight line component fitted by a computerised least squares method (Figure 8.4). Mean K_m values were 5.4μM and 5.0μM and Vmax values 17.2 pmoles/mg protein/min and 340 pmoles/mg/protein/min for NI and ME respectively, confirming the greater capacity of the GABA uptake carrier in ME.

8.3.2 Release of [^{3}H]GABA:

After an initial rapid washout phase, basal release of tritium was approximately constant within 30 min, with an FRC of approximately 0.5% in both regions. Depolarisation with 10-60mM K^+ replacing Na^+) rapidly caused a marked increase in the release of [^{3}H]GABA in a concentration-dependent manner (Figure 8.5). In Ca^{2+} free, Mg^{2+}- substituted medium (without any chelating agents) the response to 15mM K+ was greatly reduced (Figure 8.6) and basal release was slightly elevated. Thus the response to 15mM K+ was reduced from 211 ± 8% to 33 ± 5% increase in NI and from 129 ± 6% to 33 ± 3% in ME ($n = 5$ in each case, $P < 0.05$).

To investigate the presence of presynaptic GABA autoreceptors controlling transmitter release, the effect of the potent $GABA_A$

receptor agonist muscimol was investigated on stimulus-evoked release. 100nM muscimol was found to have no effect on basal release of [^{3}H]GABA (Figure 8.7) although higher concentrations caused some heteroexchange. A submaximal depolarising stimulus of 15mM K^+ was used, and muscimol and/or BMI were introduced at the same time as the K^+ stimulus. Muscimol (100nM) was found to reduce the peak response to 15mM K^+ by 24% in NI and by 26% in ME ($P < 0.05$, $n = 5$ in each case, Mann Whitney U test). This effect of muscimol was prevented by 10μM BMI (Figure 8.7, Table 8.1).

Effects of the neurotransmitters DA and 5-HT, and the peptide hormones prolactin, vasopressin and αMSH, were also investigated on stimulus-evoked release, and the results are shown in Table 8.2. None of these substances was found to affect [^{3}H]GABA release in either ME or NI.

8.4 Discussion

8.4.1 Uptake of [^{3}H]GABA

Previous studies have shown that when studying uptake of [^{3}H]GABA, it is necessary to use small (0.2mm x 0.1mm) slices or subcellular particles to reveal high-affinity uptake, as this is masked in larger slices by low-affinity uptake (Levi and Raiteri, 1973). The very small size of the tissue areas studied here precludes the use of slices, so a sucrose homogenate preparation was used. This preparation has been shown previously to accumulate [^{3}H]GABA with kinetics and inhibitor specificity similar to that of slices, into particles sensitive to osmotic lysis and with the density gradient characteristics of synaptosomes (Iversen and Johnston, 1971). The sub-physiological temperature of 25°C was used in these studies because it is the temperature at which [^{3}H]GABA

uptake is maximal in the absence of inhibitors of GABA catabolism (Iversen and Neal, 1968). AOAA was not used because there is evidence that it may modify kinetic measurements made on the uptake carrier (Snodgrass and Iversen, 1973). The results presented here show that both ME and NI tissue can accumulate [^{3}H]GABA in a temperature- and sodium-dependent manner, characteristic of [^{3}H]GABA uptake in other areas (Weinstein, Varon, Muhleman and Roberts, 1965; Iversen and Neal, 1968; Bennet et al, 1972; Martin and Smith, 1972; Jaffe and Cuello, 1980) and also of other neurotransmitter uptake systems (Bogdanski, Blaszkowski and Tissari, 1970; Baldessarini and Vogt, 1971; Sarkar et al, 1983). Uptake of [^{3}H]GABA was sensitive to inhibition by DABA but not by β alanine in both areas. The reported selectivity of these substances for neuronal and glial [^{3}H]GABA uptake (Iversen and Johnston, 1971; Schon and Kelly, 1974; Jaffe and Cuello, 1980), (with DABA about 20-fold selective for neuronal uptake and β alanine 200-fold selective for glial) predicts that the DABA concentration used here should inhibit neuronal uptake of 1μM [^{3}H]GABA by around 50%. It therefore appears that the uptake of [^{3}H]GABA described here shows neuronal specificity in both areas. These two substances do not have the lowest IC_{50} values reported (e.g. homo-β-proline has an IC_{50} value of 20μM for glial uptake and chlorpromazine has an IC_{50} value of 32μM for neuronal uptake (Iversen and Johnston, 1971; Larsson, Thorbek, Krogsgaard-Larsen and Schousboe, 1981) but they have the greatest selectivity ratios. Aminocyclohexane carboxylic acid is a rather more potent and selective inhibitor of neuronal GABA uptake than DABA (Bowery, Jones and Neal, 1976) but is not commercially available. These results are in agreement with

autoradiographic studies of in vivo uptake of [^{3}H]GABA into ME (Tappaz et al, 1980) which showed that label was accumulated over the external layer of ME with neuronal-uptake specificity. However autoradiography following in vitro uptake of [^{3}H]GABA into the posterior pituitary indicated that the label was localised almost exclusively over glial cell bodies and their processes (Beart et al, 1972; Hamberger et al, 1979). Subsequent immunohistochemical studies showed a plexus of GAD-IR nerve terminals in the external layer of ME and in PI with a less dense innervation of the PN (Vincent et al, 1982; Oertel et al 1982). The results presented here suggest that neurons in both ME and NI can accumulate [^{3}H]GABA, but the relative contributions of the intermediate and posterior lobes of the pituitary have not been investigated.

Kinetic analysis showed that the uptake mechanism had very similar Km values in the two areas, but the ME showed a considerably larger Vmax value. The Km values are in agreement with other reported values for both neuronal (Iversen and Neal, 1968; Jaffe and Cuello, 1980) and also glial uptake (Bowery and Brown, 1972; Schon and Kelly, 1974). The Vmax value for ME is approximately 1/5 that for frontal cortical tissue and rather greater than that of spinal cord (Iversen and Johnston, 1971). By contrast NI has a very low Vmax, in the same order as that of [^{3}H]GABA uptake in sensory ganglia (Schon and Kelly, 1974).

In these studies uptake of exogenous [^{3}H]GABA has been measured. There is evidence both for and against this high-affinity uptake system representing a homoexchange process. Iversen and Neal (1968) demonstrated a net uptake of GABA into cortical slices following incubation in medium containing [^{3}H]GABA, but that it

was smaller than the apparent uptake when only [^{3}H]GABA was measured. Others were unable to demonstrate a significant contribution to uptake from heteroexchange for GABA but could for glutamate (Levi, Bertollini, Chen and Raiteri, 1974). Net uptake could be greatly increased by prior depletion of GABA by cold-shock or high-molarity K^+ (Levi, Coletti, Poce and Raiteri, 1976; Regan and Roskoski, 1977), but preloaded [^{3}H]GABA could be released by unlabelled GABA with similar characteristics to those shown by high affinity uptake (Levi and Raiteri, 1974; Roberts, 1976). It is possible that the ability of tissue to retain GABA is disturbed in vitro, and interpretations of experiments using [^{3}H]GABA should therefore retain a degree of caution. More recent in vitro studies using nipecotic acid as an uptake inhibitor and measuring release of endogenous as well as tritiated GABA have provided evidence that reuptake is of major importance in removing GABA from extracellular spaces (Szerb, 1982). In vivo experiments collecting GABA released from the cerebral cortical surface also support neuronal uptake rather than metabolism as the physiological mechanism of removal of GABA (Moroni, Mulas, Moneti and Pepen, 1982, see also Chapter 1). If this is the case, then the use of exogenous GABA may be an equally valid technique to the measurement of released endogenous GABA because it will tend to label the same stores as released then reaccumulated transmitter. As demonstrated here, this type of experiment has revealed uptake kinetics in the areas of interest, closely similar to those of other CNS regions where the presence of GABA-releasing neurone terminals is well established.

8.4.2 Release of [^{3}H]GABA

The low and steady basal release of [^{3}H]GABA observed after an initial washout period was similar to that observed with other regions containing GABA neurone terminals. Both tissues here showed a prompt and sensitive response to K^+ (Figure 8.5), especially NI, and the effect of 15mM K^+ was largely abolished by perfusion with Ca^{2+}-free medium (Figure 8.6). This is in contrast to studies using whole neurohypophyses, in which K^+ was only able to stimulate release at very high concentrations (Minchin and Nordmann, 1974; Hamberger et al, 1979). Sellstrom and Hamberger (1977) demonstrated that such Ca^{2+} dependency was a characteristic of K^+ induced [^{3}H]GABA release from synaptosomes, but not from neuronal perikarya or glia, and Neal and Bowery (1979) found that glial [^{3}H]GABA release was insensitive to 25mM K^+. It thus appears that uptake and release of [^{3}H]GABA by the intact neurohypophysis is predominantly glial (Beart et al, 1974; Hamberger et al, 1979) whereas in the preparation of NI used here, uptake and release are mainly neuronal. This may be because most of the nerve terminals in the pituitary showing GAD-IR are in PI, and glial cells tightly-wrapped around neuronal terminals may also constitute a barrier to [^{3}H]GABA (Iversen and Kelly, 1975), which would be disrupted in the preparation used here.

The lack of complete dependency of K^+-evoked release on Ca^{2+} may be a result of residual Ca^{2+} in the tissue (Srinivasan, Neal and Mitchell, 1969) as cation chelators were not present in the superfusion medium. Ca^{2+}-dependency of evoked release has been the subject of considerable investigation, and has been consistently demonstrated for both endogenous and exogenous GABA (Mulder and

Snyder, 1974; Nadler, White, Vaca and Cotman, 1977; Potashner, 1978a). Basal release of [^{3}H]GABA from both areas was observed to be elevated in Ca^{2+}-free medium (Figure 8.6). This has also been observed for endogenous GABA (Potashner, 1978a) and may be the result of increased influx of Na^+ through a Na^+/Ca^{2+} exchanger (Szerb, 1979) causing release of Ca^{2+} from intracellular stores and thereby elevating secretion.

Presynaptic autoreceptors controlling the release of neurotransmitters have been demonstrated for a variety of neurotransmitters in both the central and peripheral nervous systems (Farnebo and Hamberger, 1971; Szerb and Somogyi, 1973; Starke and Montel, 1973; Starke, Reimann, Zumstein and Hertting, 1978; Langer, 1980). This interpretation is based on the inhibition by specific agonists and enhancement by antagonists of the release of previously accumulated radiolabelled transmitter in response to electrical or K^+ stimulation. Depending on the preparation used, a released transmitter may or may not (as here) accumulate around the terminals to concentrations sufficient to exert a tonic effect and therefore reveal an enhancement of control release by antagonists. Additionally, presynaptic autoreceptors may control the synthesis of neurotransmitter (Kehr, Carlsson, Linquist, Magnusson and Atack, 1972; Iversen, Rogawski and Miller, 1976). Similar studies using [^{3}H]GABA have led to the suggestion that GABAergic nerve terminals in frontal cortex and substantia nigra have presynaptic autoreceptors which, upon activation, inhibit release of the neurotransmitter (Mitchell and Martin, 1978a; Snodgrass, 1978; Arbilla et al, 1979; Brennan and Cantrill, 1979). These autoreceptors show similar pharmacology to postsynaptic $GABA_A$

receptors, for both agonists and antagonists (Mitchell and Martin, 1978a; Brennan, Cantrill, Oldfield and Krogsgaard-Larsen, 1981). This makes the demonstration of GABA autoreceptors in vivo very difficult, but action at such receptors by endogenous δ-amino-laevulinic acid has been suggested to account for some of the symptoms of porphyrias, which include hyperexcitability and convulsions (Brennan and Cantrill, 1979). It has also been proposed that GABA autoreceptors control synthesis of the amino acid (Carmona, Gomes and Trolin, 1981; de Mello, 1984). To demonstrate presynaptic receptor involvement in control of release, it is essential to use drugs that do not exchange with the transmitter i.e. are not substrates for the uptake system. The potent GABA receptor agonist muscimol is only a poor substrate for the GABA uptake system (Krogsgaard-Larsen and Johnston, 1975), and at the low concentration used here (100nM) effectively activated presynaptic receptors in both ME and NI without causing exchange (Figure 8.7). Higher concentrations induced significant elevation of basal [^{3}H]GABA release. Muscimol reduced the amount of stimulus-induced [^{3}H]GABA release in a bicuculline-sensitive manner, in both ME and NI (Figure 8.7, Table 8.1) by amounts similar to that in frontal cortex (Mitchell and Martin, 1978a). In contrast to the results of Brennan and Cantrill (1981), concentrations of muscimol activating the autoreceptor in ME and NI would never produce more than about 30% reduction of stimulated release before exchange distorted the results (even when repeating the 55mM K^+ stimulus used by these workers). The lack of effect of BMI on basal release, or on the response to 15mM K^+ suggests that the rapid superfusion system used here prevents released [^{3}H]GABA from re-accumulating

sufficiently to activate the presynaptic receptors. GABA receptor binding sites have been demonstrated in rat hypothalamus (Beaumont et al, 1978; Fiszer de Plazas et al, 1982) (although ME has not been specifically investigated) and also in NI (Chapter 3). These studies do not discriminate presynaptic and postsynaptic sites and it is not therefore possible to estimate the numerical contribution of the presynaptic receptors described here.

The results in Table 8.2 suggest that GABA release from terminals in both areas is independent of any of the several other transmitters investigated and also free from direct hormonal effects. The lack of effect of 5-HT on [^{3}H]GABA release from ME suggests that if its stimulatory effect on prolactin secretion is mediated by GABA (Caligaris and Taleisnik, 1974) then it may be acting indirectly or at the level of the cell body rather than the terminals in ME.

The PIF effects of both GABA and DA raise the possibility that they will be involved physiologically in the feedback regulation of prolactin secretion. Neuroleptic treatment causes a rapid increase in MBH GAD activity, and conversely apomorphine reduces MBH GAD activity (Prato et al, 1981). That the effects of these drugs might be mediated by changes in prolactin concentration was supported by the demonstration that hypophysectomy prevented the changes in GAD activity, and anterior pituitary grafts caused an elevation in GAD activity (Nicoletti et al, 1983). Direct injection of prolactin (i.c.v.) elevated MBH GAD activity and GABA secretion into hypophysial portal blood, but this was only observed after 16hr, and not after 4hr (Apud et al, 1984a). Similarly delayed effects on ME DA turnover have been reported (Moore, Demarest and Johnston,

1980). The present results show no direct effect of prolactin on [^{3}H]GABA release from ME and therefore do not support a rapid feedback mechanism at the level of the nerve terminals, although an effect on the cell bodies cannot be excluded. Prolactin has been shown to increase the release of both endogenous and tritiated DA from MBH slices (Perkins and Westfall, 1978; Foreman and Porter, 1981) but to have no effect on release of [^{3}H]DA from specifically ME tissue (Horn, 1984).

Immunohistochemical studies have shown presumed GABAergic neurones in close contact with other neurosecretory terminals in both ME and PN and with secretory cells in PI, as well as in the neurohaemal contact zone of ME (Oertel et al, 1982; Tappaz et al, 1983). There are therefore several sites at which GABA released from the nerve terminals in ME and NI investigated here might participate in neuroendocrine control and GABA has been demonstrated to affect the secretion of somatostatin (Gamse et al, 1980), CRH (Burden et al, 1974) and DA (Chapter 11) from MBH or ME. These neuroendocrine GABA systems (even that in ME, where GABA is not only secreted into a synaptic environment but also into hypophysial portal blood) appear to be similar to other CNS GABA neurones in that they are under autoregulatory control from presynaptic autoreceptors.

METHOD

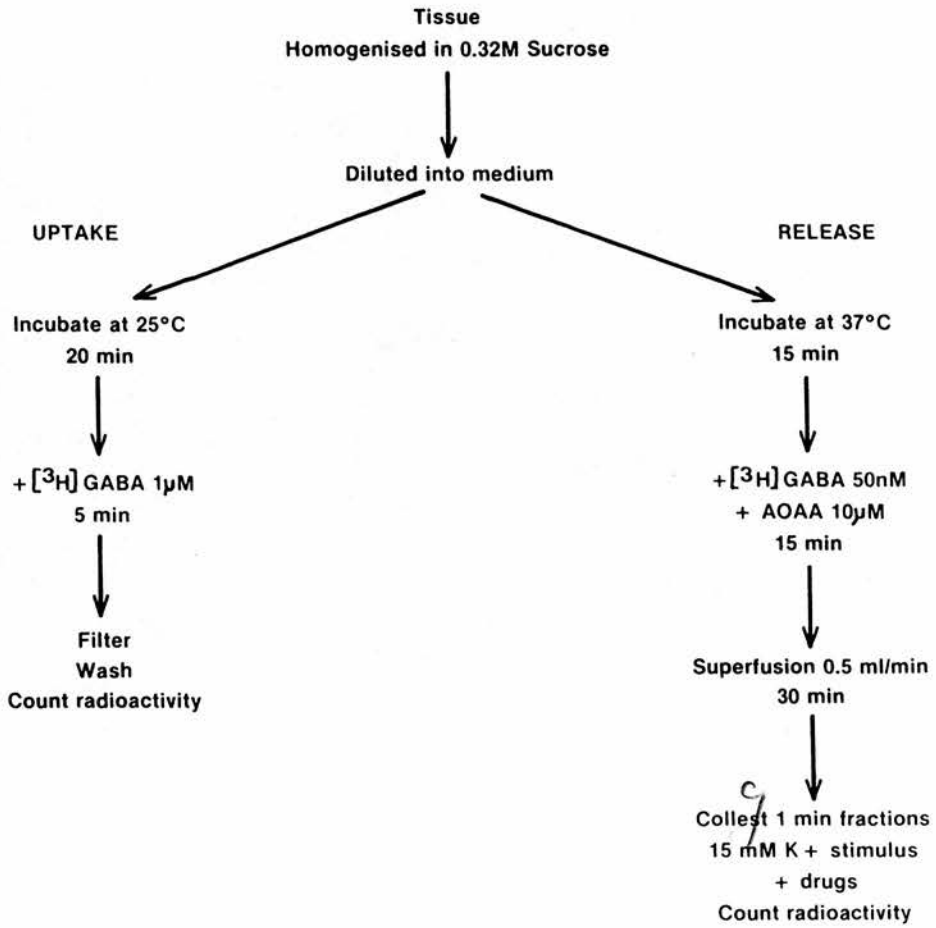


FIGURE 8.1

Outline of the methods used for the study of [^{3}H]GABA uptake and release by ME and NI crude synaptosomal preparation.

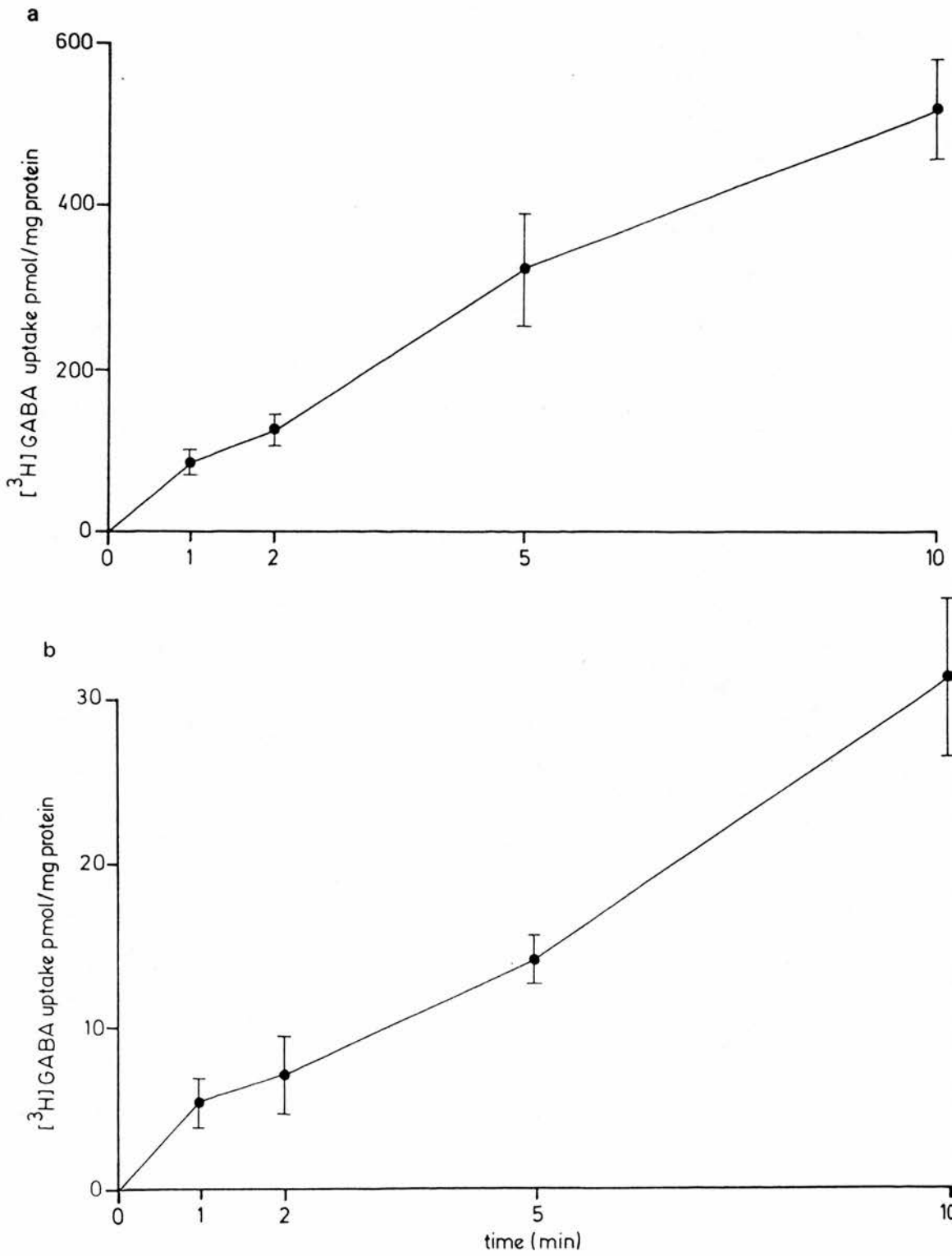


FIGURE 8.2

Accumulation of [^{3}H]GABA by a) ME and b) NI for different periods of time at 25°C. Values represent uptake of [^{3}H]GABA after subtracting tissue-free blanks. Blanks were ~ 20% of total uptake at 5min in ME and ~ 50% in NI. Mean ± S.E.M., n = 4.

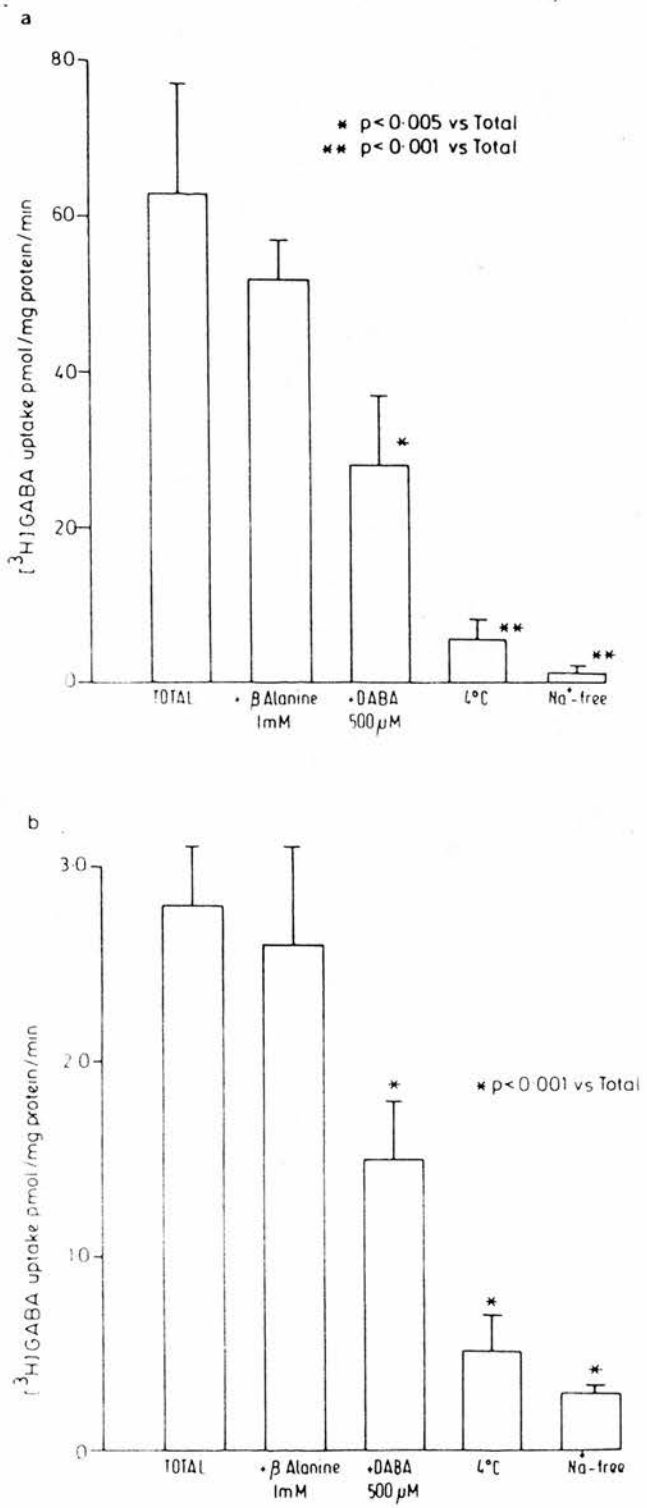


FIGURE 8.3

Accumulation of [^{3}H]GABA by a) ME and b) NI under various conditions. 5min incubation periods were used.
Total: total specific uptake over the 5min period.
1mM β alanine, 500μM DABA: incubations were carried out in the presence of these compounds.
4°C: incubations were carried out on ice throughout.
Na^+- free: choline was substituted for sodium in the medium.
Mean ± S.E.M., n = 4. Significance was assessed by paired Students 't' test.

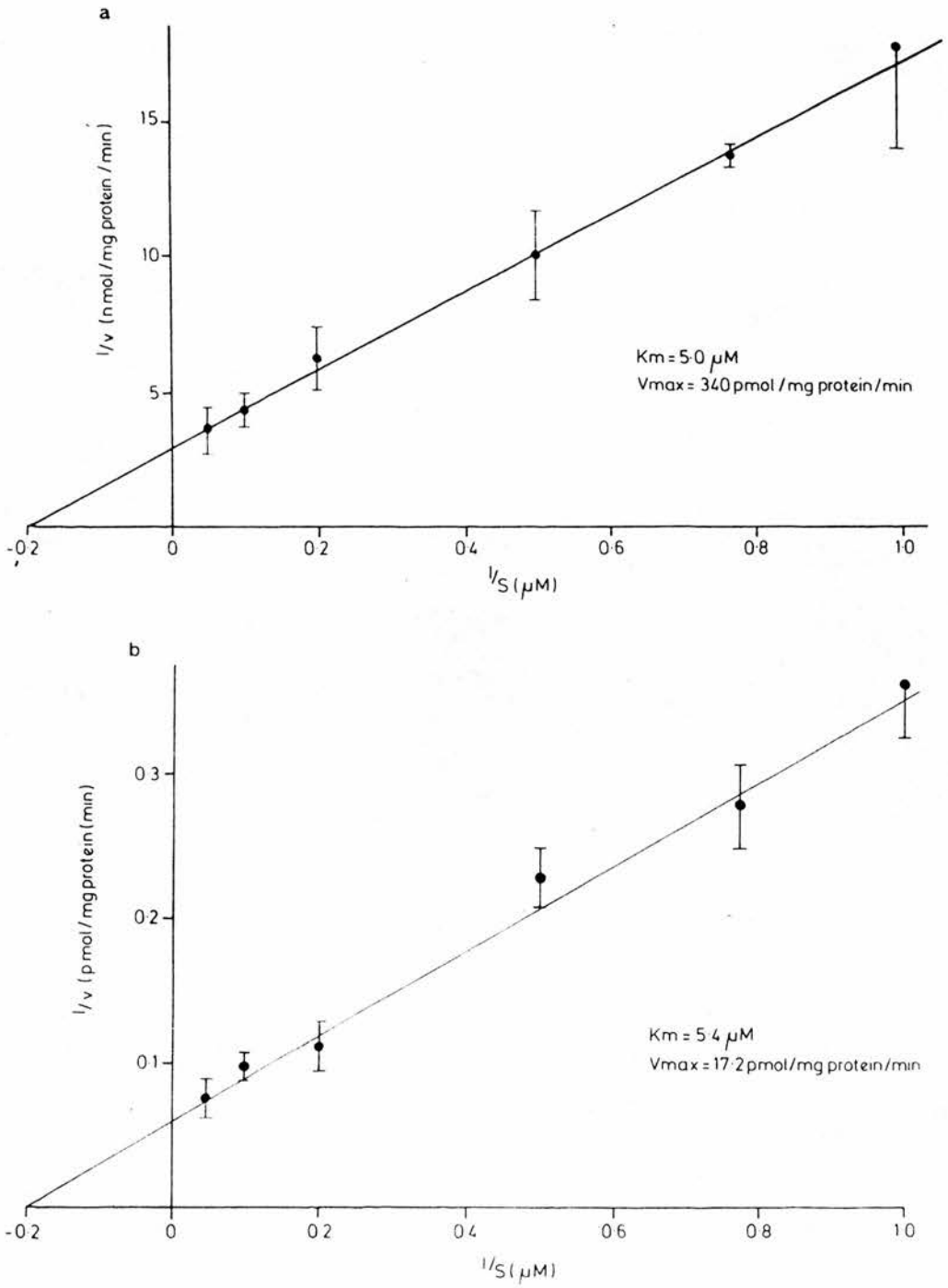


FIGURE 8.4

Kinetic analysis of [^{3}H]GABA uptake by a) ME and b) NI. Values are plotted in a reciprocal form.
S: [^{3}H]GABA concentration, from 1.0 to 20μM.
V: rate of specific accumulation of [^{3}H]GABA. Points were fitted by a computerised least squares method. Mean ± S.E.M., n = 4.

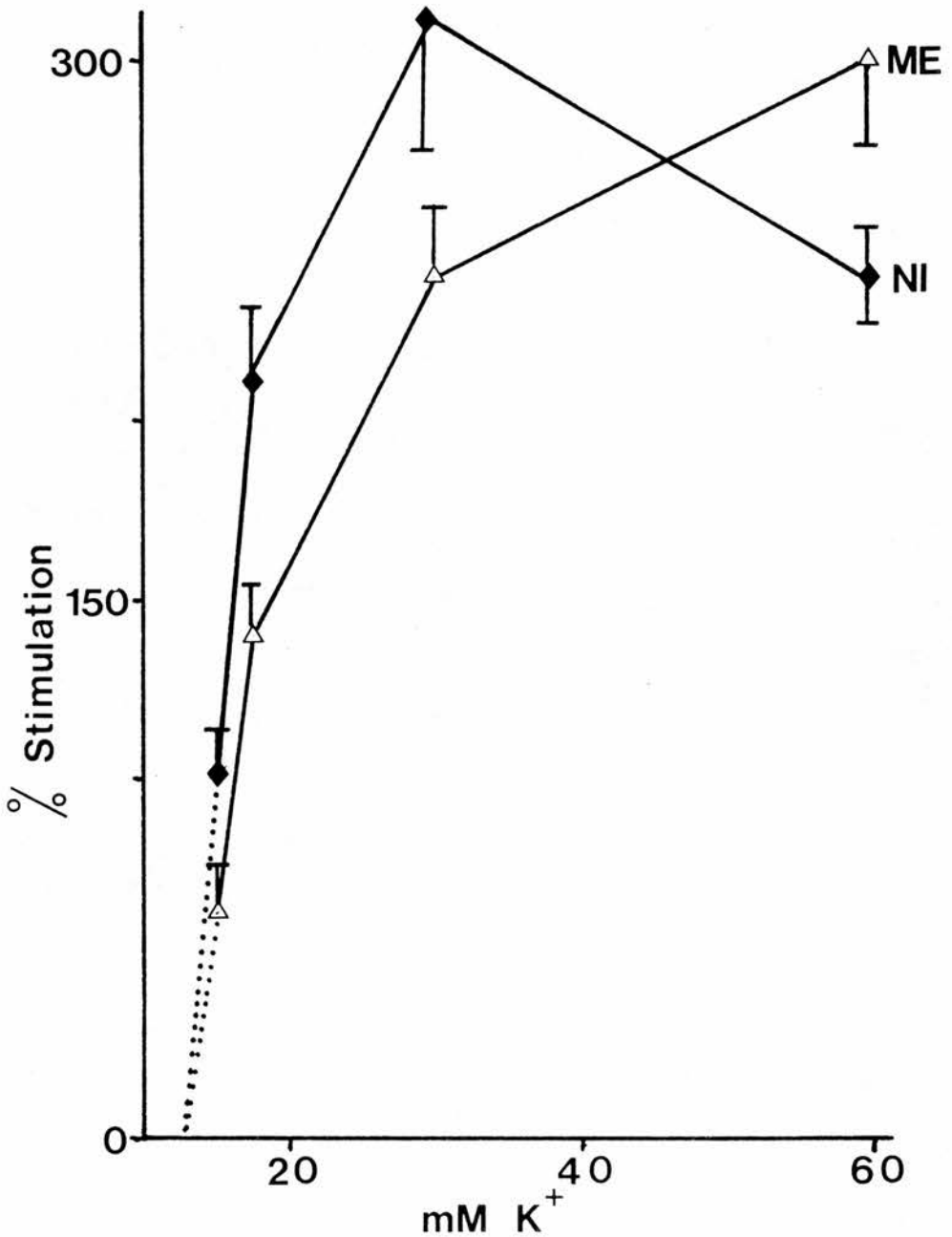


FIGURE 8.5

Concentration-dependence of the stimulation of [^{3}H]GABA release by K^+. Percentage stimulation of release from ME and NI by various K^+ concentrations (replacing equimolar Na^+). Mean ± S.E.M.,n = 5.

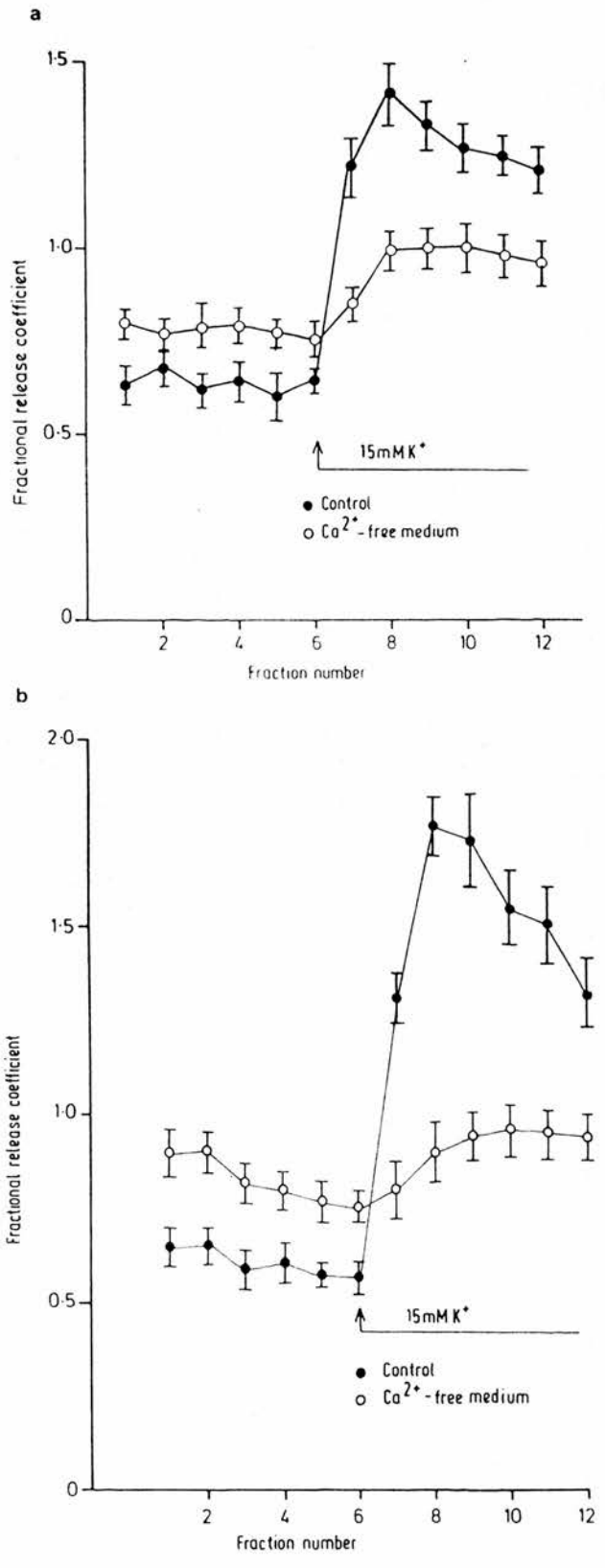


FIGURE 8.6

Release of [^{3}H]GABA by a) ME and b) NI. Filled circles: normal medium, open circles: Ca^{2+}-free medium. Superfusion medium was changed to one containing 15mM K^+ at the arrow. Release is expressed as FRC. Mean ± S.E.M., n = 5.

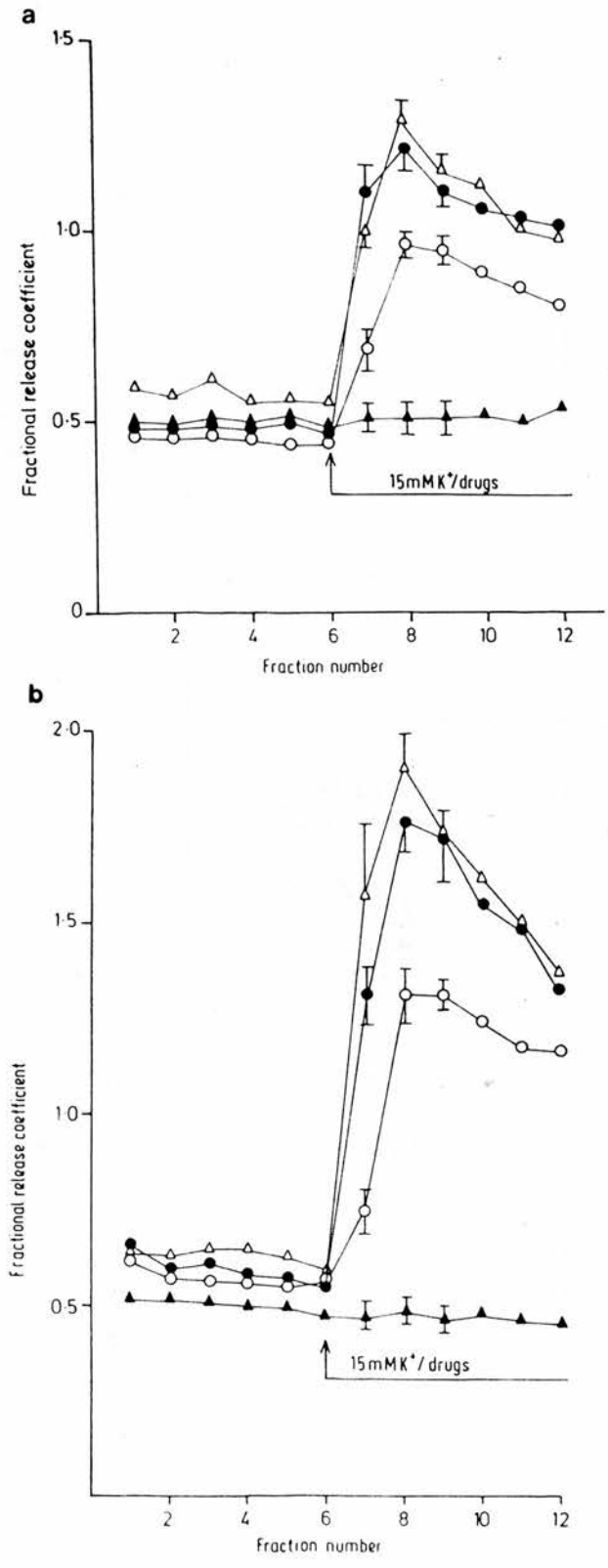


FIGURE 8.7

Autoreceptor-control of [^{3}H]GABA release from a) ME and b) NI. Medium was changed at the arrow from normal to:

(▲) normal + 100nM muscimol
(●) 15mM K^+
(○) 15mM K^+ + 100nM muscimol
(△) 15mM K^+ + 100nM muscimol + 10μM BMI

Mean ± S.E.M., n = 5.

Table 8.1

Evidence for $GABA_A$ autoreceptors in ME and NI.

a) % Stimulation of [^{3}H]GABA release from NI

15mM K^+	211 ± 8%
15mM K^+ + 100nM muscimol	161 ± 15%*
15mM K^+ + 100nM muscimol + 10μM bicuculline methiodide	229 ± 10%**
15mK^+ + 10μM bicuculline methiodide	210 ± 12%

* $P < 0.05$ vs K^+ alone
** $P < 0.05$ vs K^+ + muscimol

n = 5 in each case. Mean ± S.E.M.

b) % Stimulation of [^{3}H]GABA release from ME

15mM K^+	154 ± 10%
15mM K^+ + 100nM muscimol	114 ± 8%*
15mM K^+ + 100nM muscimol + 10μM bicuculline methiodide	135 ± 3%**

* $P < 0.05$ vs K^+ alone
** $P < 0.05$ vs K^+ + muscimol

n = 5 Mean ± S.E.M.

Values are peak percentage stimulation of release (expressed in terms of FRC) above basal. Drugs were introduced simultaneously with the K^+ pulse.

Table 8.2:

Effects of other neurotransmitters and neuropeptides on [^{3}H]GABA release

	ME	NI
prolactin (1μg/ml)	98 ± 5%	-
αMSH (1μM)	110 ± 7%	102 ± 7%
vasopressin (1μM)	90 ± 4%	108 ± 6%
dopamine (10μM)	96 ± 7%	98 ± 4 %
serotonin (10μM)	99 ± 5%	98 ± 6 %

Results are expressed as a percentage of the control K^+-induced stimulation of release of [^{3}H]GABA which represented some 129 ± 6% and 211 ± 8% increase over basal efflux, in ME and NI respectively. (mean ± SEM, n = 5 in all cases).

CHAPTER 9

Evidence for $GABA_B$ autoreceptors in the median eminence.

9.1 Introduction

The clinical use of the GABA analogue baclofen in the treatment of spasticity has prompted many studies on its actions on synaptic transmission, particularly in the spinal cord. Baclofen was found to cause a potent and long-lasting depression of cell firing of spinal cord, cerebral and cerebellar neurones, which was however bicuculline-insensitive (Curtis, Game, Johnston and McCulloch, 1974; Davies and Watkins, 1974), implying that the effect was not mediated by GABA receptors despite the structural similarities between the two compounds. On the basis of the depression of synaptic reflexes in the absence of effects on motorneurones, baclofen was proposed to act presynaptically to reduce the release of neurotransmitter (Davidoff and Sears, 1974), a suggestion which has received considerable support (Fox, Krnjevic, Morris, Puil and Werman, 1978; Curtis, Lodge, Bornstein and Peet, 1981). Neurochemical studies have also provided evidence that this may be a major effect of baclofen: Potashner (1978b) demonstrated that baclofen inhibited electrically- evoked release of endogenous aspartate and glutamate (but not GABA) from guinea-pig cortex, and similar results have been obtained in the rat olfactory cortex (Collins, Anson and Kelly, 1982) and cat spinal cord (Johnston, Hailstone and Freeman, 1980). The possibility that these effects of baclofen were mediated by a novel type of GABA receptor was proposed on the basis of experiments in which GABA and baclofen inhibited stimulated [^{3}H]noradrenaline release from rat atria in a bicuculline-insensitive manner, and subsequent studies identified specific recognition sites with similar properties within the CNS (Bowery and Hudson, 1979; Bowery et al, 1980). This apparently novel receptor site was designated

'$GABA_B$' in contrast to classical, chloride-channel-linked '$GABA_A$' receptors, and its major characteristics are stereospecific activity of baclofen, (-) baclofen being approximately equipotent to GABA, and insensitivity to muscimol and bicuculline (Hill and Bowery, 1981; Bowery et al, 1983).

$GABA_B$ receptors are found on sensory neurones (Dunlap, 1981) where electrophysiological studies have been carried out to clarify their mechanism of action. Baclofen reduced action potential duration in these cells, an effect common to several neurotransmitters which appeared to be a direct effect on Ca^{2+} conductance (Dunlap and Fischbach, 1981). The possibility that the observed effect on Ca^{2+} conductance might be secondary to increased K^+ conductance was suggested by the results of Desarmenien et al (1984), who showed that this effect of baclofen and GABA was prevented by loading cells with Cs^+. Similar results have been obtained using guinea-pig myenteric plexus (Cherubini and North, 1984) and such actions could provide a mechanistic explanation for $GABA_B$ receptor-mediated inhibition of transmitter release.

In the present experiments the presence of presynaptic $GABA_B$ receptors on GABAergic terminals in ME and NI was investigated: although presynaptic $GABA_B$ receptors are present on nerve terminals containing a variety of transmitters in different areas of the CNS, there have been no demonstrations of their presence on GABAergic terminals (Potashner, 1978b; Bowery et al, 1980; Collins et al, 1982). Few pharmacological tools other than the isomers of baclofen are available for the investigation of $GABA_B$ receptors; a major problem being the lack of a potent and specific antagonist.

Nevertheless δ-aminovaleric acid has been suggested to be a weak $GABA_B$ antagonist (Muhyaddin, Roberts and Woodruff, 1982) and its action in ME was also investigated.

9.2 Materials and methods

Materials and methods used in this chapter for the study of [^{3}H]GABA release from ME and NI were identical to those described in Chapter 8. (±)Baclofen and its stereoisomers were the gift of Ciba-Geigy, and δ-aminovaleric acid (δAVA) was obtained from Sigma.

Results were calculated as FRC, and hence values for percentage stimulation of release were obtained. Results were further analysed as a percentage of a control response carried out simultaneously in a parallel superfusion. Statistical comparisons were made using the Mann-Whitney U test, using values for peak percentage stimulation of release.

9.3 Results

The characteristics of basal and K^+-stimulated release in both ME and NI were described in Chapter 8. A submaximal (15mM K^+) stimulus was used throughout these experiments, and drugs were introduced simultaneously with this.

100μM (±)baclofen inhibited the stimulation of release by 15mM K^+ in ME but had no effect in NI. The peak stimulation was reduced to 64 ± 6% of control in ME (Figure 9.1, $P < 0.05$, $n = 5$) but remained at 101 ± 4% of control in NI. This concentration of (±)baclofen had no effect on basal release of [^{3}H]GABA from ME (Figure 9.1).

The effect of (±)baclofen was concentration-dependent (Table 9.1), and stereospecific. (-) Baclofen was approximately equipotent with the racemate, and (+)baclofen was without significant effect at concentrations up to 100μM (Table 9.1).

δAVA has been reported to have little $GABA_A$ agonist action, and to be a weak $GABA_B$ antagonist (Muhyaddin et al, 1982). Attempts to use it here were complicated by a degree of heteroexchange, both basal and K^+-stimulated release of [^{3}H]GABA from ME being enhanced. Thus 10μM δAVA potentiated the response to 15mM K^+ to 124 ± 5% of control ($P < 0.05$, $n = 5$), and was therefore included in control perfusions as well as those testing the effect of baclofen (Figure 9.2). 100μM (±)baclofen, in the presence of 10μM δAVA, slightly but still significantly inhibited release (83 ± 3% of control, K^+ + δAVA). This effect was, though, significantly less than that of 100μM (±)baclofen alone (64 ± 6% of control, K^+ alone).

Experiments attempting to use GABA itself as a $GABA_B$ agonist were also confounded by homoexchange, even at low concentrations (100nM). Fluspirilene has been used as a GABA uptake blocker in similar experiments, to allow the demonstration of GABA activity at autoreceptors (Mitchell and Martin, 1978a). However, in the present study, at a concentration necessary to prevent uptake (1μM, as in Mitchell and Martin, 1978a), fluspirilene was found to inhibit both basal and K^+-stimulated release of [^{3}H]GABA.

9.4 Discussion

These results demonstrate a selective inhibition of evoked release of [^{3}H]GABA from ME but not NI by baclofen. The effect was sterospecific, the (-) isomer being active and the (+) isomer being inactive at 100μM. This suggests that the effect is mediated by a $GABA_B$ receptor (Hill and Bowery, 1981). It was not possible to demonstrate an action of GABA itself, as considerable homoexchange was found at concentrations above 100nM, too low to

have any marked receptor-mediated effect without gross distortion of the results. The presence of presynaptic $GABA_A$ autoreceptors (Chapter 8) would also complicate interpretation.

δAVA is a weak $GABA_A$ receptor agonist in sympathetic ganglia (Bowery and Brown, 1974) but appears to be a $GABA_B$ antagonist (or very weak partial agonist) on noradrenergic terminals in rat anococcygeus (Muhyeddin et al, 1982) and in the guinea-pig ileum (Ong and Kerr, 1983). In that preparation, 500μM δAVA competitively antagonised the effect of both GABA and (±)baclofen, with a pA_2 of 4.1. Similar concentrations of δAVA in this preparation caused very considerable stimulation of basal [^{3}H]GABA release, probably by heteroexchange as δAVA shows considerable structural similarities to GABA. When 10μM δAVA was tested, heteroexchange was still prominent but as lower concentrations would not be expected to cause any antagonism, this concentration was used. The results obtained show that 100μM (±)baclofen caused slight inhibition of release in the presence of 10μM δAVA, but the effect appeared to have been partly antagonised. This result should be treated with great caution, as the entry of δAVA into the terminals and subsequent stimulation of release may have altered the receptor-mediated effect of baclofen, independent of any competitive antagonism. While these results are therefore consistent with δAVA being a $GABA_B$ antagonist, they do not provide any direct support as this is not a suitable model, and the absence of a potent and specific $GABA_B$ antagonist precludes further study.

Presynaptic $GABA_B$ receptors, which in all cases reported inhibit neurotransmitter release, have been demonstrated to affect the release of catecholamines, 5HT, acetylcholine and excitatory

amino acids in the CNS (Nistri, 1975; Potashner, 1978b; Bowery et al, 1980), and are found on both sympathetic and parasympathetic terminals (Bowery et al, 1981; Anwar and Mason, 1982; Muhyaddin et al, 1982; Kleinrok and Kilbinger, 1983). To this list should now be added CNS GABAergic terminals of the ME, although such regulation has not demonstrated elsewhere in the CNS. Previous experiments in which an effect of baclofen on release of GABA has been reported have not demonstrated the involvement of $GABA_B$ receptors. Kerwin and Pycock (1978) found that (+)baclofen (but not the (-) isomer) at 200μM stimulated basal release of [^{3}H]GABA from globus pallidus slices, and Roberts, Gupta and Shargill (1978) reported a slight enhancement of basal release of [^{3}H]GABA from whole brain synaptosomes by 50μM baclofen. These effects may represent an effect on the GABA uptake carrier, as baclofen causes weak but detectable inhibition of [^{3}H]GABA uptake (Kerwin and Pycock, 1978) although no uptake of [^{3}H]baclofen itself is detectable (Bowery et al, 1983). Curtis et al (1981) suggested that the inhibitory effect of baclofen on primary afferent depolarisation might be a result of a reduction in the release of GABA at axo-axonic synapses on 1a terminals, but any definitive evidence is lacking and autoradiographic study of $GABA_B$ receptors suggested that $GABA_B$ receptors in substantia gelatinosa were predominantly located on primary afferent terminals (Price, Wilkin, Turnbull and Bowery, 1984).

Electron-microscopic immunohistochemical studies have localised presumed GABAergic terminals in the external layer of ME among unlabelled axons and neurosecretory endings and in close vicinity to fenestrated capillary perivascular space (Tappaz et al, 1983). If

the $GABA_B$ receptors demonstrated here were present on GABAergic terminals in all these locations, then their effect would either be to inhibit GABA release into hypophysial portal blood or onto peptidergic or aminergic terminals. This in turn could effect the secretion of any of the anterior pituitary hormones. The effects of GABA on releasing hormone secretion have not been extensively investigated, but effects on somatostatin (Gamse et al, 1980) CRH (Burden et al, 1974) and DA (Chapter 11) have been reported. An effect on GHRH secretion has also been proposed (Fiok et al, 1984) and the stimulatory effect of baclofen on GH secretion (Fiok et al, 1981b) may therefore be in part mediated by disinhibition of GHRH secretion. The $GABA_B$ autoreceptors demonstrated here may therefore have a variety of functions in neuroendocrine regulation. The presence of $GABA_A$ autoreceptors in ME was demonstrated in Chapter 8, suggesting that GABAergic terminals in ME are subject to considerable autoregulation. The presence of both types of autoreceptor on one neurone would represent a certain functional redundancy and therefore seems perhaps unlikely, but the considerable inhibitory influence of the $GABA_B$ autoreceptors here (approximately 35% inhibition) suggests that they may well be present on a significant proportion of terminals. The maximal level of inhibition by $GABA_A$ autoreceptors (Chapter 8) and $GABA_B$ sites (here) are not possible to ascertain because of the exchange effects of most of the agonists at higher concentrations. In other brain areas, muscimol and other $GABA_A$ receptor agonists have been reported to cause up to 90% inhibition of evoked release of [^{3}H]GABA (Brennan et al, 1982) but such marked effects have never been observed (without exchange-efflux) in this laboratory.

Nevertheless, GABA and baclofen show similar potency at $GABA_B$ receptors (Hill and Bowery, 1981) and as little as 0.3μM GABA acts at $GABA_A$ autoreceptors in similar experiments (Mitchell and Martin, 1978a) to produce similar inhibition to that caused by 100μM baclofen here. It may be therefore, that $GABA_B$ autoreceptors are somewhat less sensitive than $GABA_A$ to accummulation of released GABA, and therefore act as a reserve of inhibitory control. The question of whether both types are present on one neurone remains to be established, and further studies are also required to establish the physiological significance of either of these sites.

TABLE 9.1

Inhibition of K^+-stimulated release of [^{3}H]GABA from ME by baclofen and its stereoisomers.

Baclofen (Concentration)	(±)	(−)	(+)
10μM	85±6*	83±2*	106±6
100μM	64±6*	63±4*	90±2
100μM + 10μM δAVA	83±3*≠	-	-

Mean ± S.E.M, n = 5–6
* $P<0.05$ vs control (Mann–Whitney U Test).
≠ $P < 0.05$ vs baclofen alone

Values represent stimulation of release as % of relevant control, K^+-evoked release. 15mMK^+ in the absence of drugs evoked some 150% increase in release above basal efflux. 100μM (±) baclofen had no effect on basal release under these conditions.

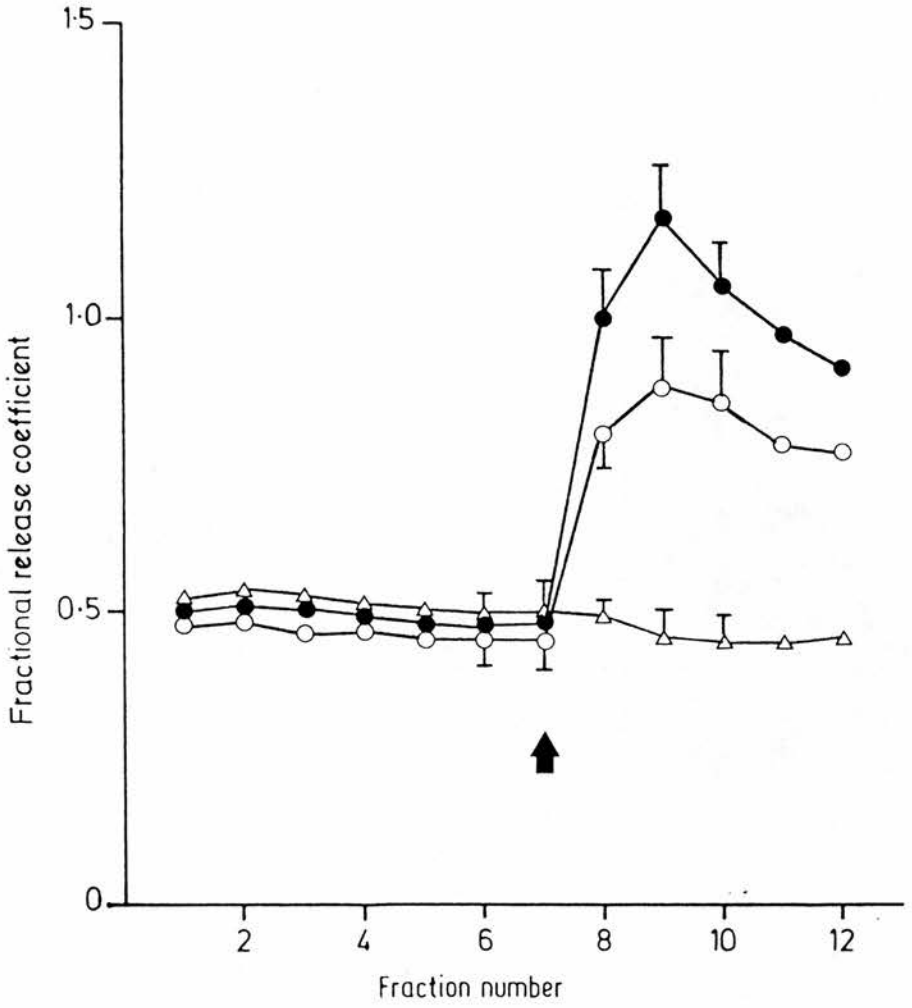


FIGURE 9.1

Inhibition of stimulated [^{3}H]GABA release from ME by baclofen. K^+/drugs were introduced at the arrow.

(●) 15mM K^+
(○) 15mM K^+ + 100μM (±)baclofen
(△) Effect of 100μM (±)baclofen on basal release.

Mean ± S.E.M., n = 5.

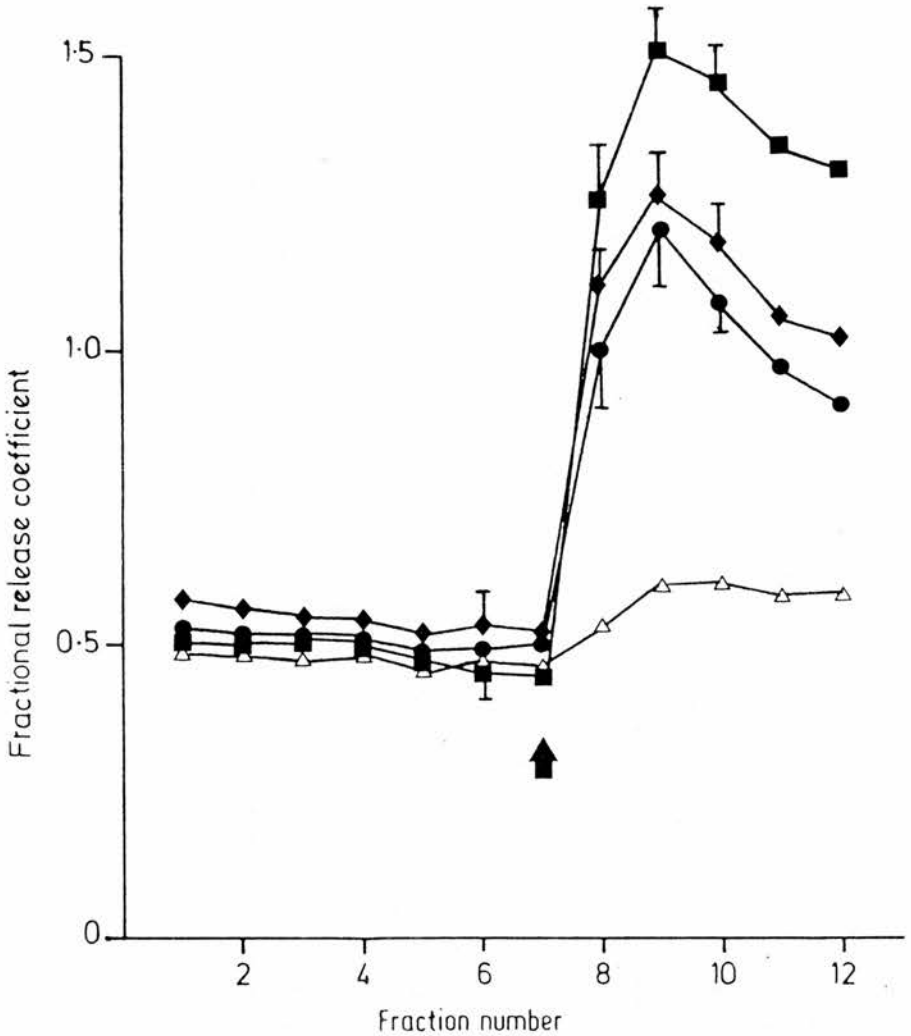


FIGURE 9.2

Antagonism of the effect of baclofen by δ-aminovaleric acid. K^+/drugs were introduced at the arrow.

(●) 15mM K^+
(■) 15mM K^+ + 10μM δAVA
(◆) 15mM K^+ + 10μM δAVA + 100μM (±)baclofen
(△) Effect of 10μM δAVA on basal release

Stimulated release: mean ± S.E.M., n = 5.
Basal release: mean of two experiments.

CHAPTER 10

Inhibition of [^{3}H]GABA release from the neurointermediate lobe by κ opioid receptors.

10.1 Introduction

All three opioid peptide families have been described in the pituitary: β-endorphin derived from pro-opioimelanocortin in corticotrophes and melanotrophes of PD and PI (Bloom, Battenberg, Rossier, Ling, Leppaluoto, Vargo and Guillemin, 1977), proenkephalin-derived leu- and met- enkephalin in nerve fibres in PN (Rossier, Battenberg, Pittman, Bayon, Koda, Miller, Guillemin and Bloom, 1979) and prodynorphin- (pro-neoendorphin-dynorphin-) derived peptides in PN and a few cells in PD (Watson, Akil, Ghazarossian and Goldstein, 1981) which may be gonadotrophes (Knepel and Schwanigner, 1985). These studies were all immunological and some of details of anatomical differentiation may no longer be tenable because of cross-reactivity, particularly between leu-enkephalin and dynorphin, as prodynorphin contains three copies of leu-enkephalin. Rossier et al (1979) first suggested that leu- enkephalin, apparently found in the supraoptic and paraventricular nuclei and in terminals in PN, might be involved in the control of vasopressin secretion and Martin and Voigt (1981) demonstrated coexistence of these two peptides. Subsequent studies using purified anti-prodynorphin antisera demonstrated that dynorphin but not leu-enkephalin coexisted with vasopressin, and that leu-enkephalin was present in the parvocellular area of the paraventricular nucleus (Watson, Akil, Fischli, Goldstein, Zimmerman and Nilaver, 1982). This has been supported by lesion studies (Millan, Millan and Herz, 1983).

Dynorphin was originally isolated from porcine pituitary, and shown to be extremely potent as a κ agonist in a number of assay systems (Goldstein, Tachibana, Lowney, Hunkapiller and Hood, 1979). The full 17 amino-acid sequence was subsequently determined

(Goldstein, Fischli, Lowney, Hunkapiller and Hood, 1982) and found to be widely distributed within the CNS (Watson et al, 1981). The concentration of dynorphin$_{1-17}$ is particularly high in PN, which also contains very large amounts of dynorphin$_{1-8}$ and α-neoendorphin (Seizinger, Hollt and Herz, 1981; Weber, Evans and Barchas, 1982; Suda, Tozawa, Tachibana, Demura and Shizume, 1982). Dynorphin$_{1-8}$ and α-neoendorphin were found to be regionally distributed in approximately equimolar concentrations, but this was not true of dynorphin$_{1-17}$, suggesting possible physiological differences in processing of the precursor (Weber et al, 1982). Dynorphin$_{1-8}$ is much more readily degraded by peptidases than dynorphin$_{1-17}$ and is less potent, which has been suggested to be more consistent with a transmitter than a hormonal role (Corbett, Paterson, McKnight, Magnan and Kosterlitz, 1982).

Functionally, opioids in PN are associated with vasopressin release. Morphine, β-endorphin and leu-enkephalin have been shown to inhibit stimulated vasopressin release in vitro (Iversen, Iversen and Bloom, 1980; AlZein, Lutz-Bucher and Koch, 1984) and in vivo, naloxone potentiated the stimulation of vasopressin release by some treatments such as angiotensin II infusion but not others (Knepel, Nutto, Anhut and Hertting, 1982). The concentration of β-endorphin in plasma was also raised by the treatments which were potentiated by naloxone (Knepel et al, 1982). Experiments with naloxone in vitro have been equivocal: naloxone was found to increase release of oxytocin but not vasopressin (Bicknell and Leng, 1982) but also to prevent the inhibitory effect of sulpiride on stimulated vasopressin release (Racke, Ritzel, Trapp and Muscholl, 1982). The enkephalins are somewhat selective δ agonists and β endorphin is equally active

at μ and δ receptors, while naloxone is most potent at μ receptors; δ and κ receptors requiring higher concentrations (Peterson, Robson and Kosterlitz, 1983). This suggests that vasopressin nerve terminals may have μ or δ receptors, but no κ-mediated effects in NI have been demonstrated.

GABAergic nerve terminals in NI are predominantly localised in PI rather than PN (Oertel et al, 1982; Vincent et al, 1982). In PI, GAD-IR terminals were observed in apposition to secretory cells and in both areas to unlabelled axons (Oertel et al, 1982). GABA may be involved in the control of αMSH and vasopressin secretion (Tomiko et al, 1983; Knepel et al, 1984) but the experiments in Chapter 8 demonstrated that neither hormone affected the release of [^{3}H]GABA. In this Chapter, the interaction of opioids selective for particular receptor types with the release of [^{3}H]GABA from NI was investigated, and subsequent experiments examined the ionic mechanism of action of the probable K receptor thus demonstrated.

10.2 Materials and Methods

10.2.1 Materials

Dynorphin$_{1-13}$, des-Tyr-dynorphin, Tyr-D-Ala-Gly-NMePhe-Gly-ol, (DAGO), [D-Ser$_2$] Leu-enkephalin-Thr, (DSLET) 4-aminopyridine (4AP) and quinine were obtained from Sigma. Naloxone was the gift of Erdo Laboratories.

Other materials were as described in Chapter 8.

10.2.2 Methods

The method for preparation of tissue, loading with [^{3}H]GABA and subsequent superfusion (and also treatment of data) was as described in Chapter 8. The channel blockers 4AP, TEA, quinine and DIDS were present in the medium from the start of superfusion when

appropriate i.e. 30 min before fractions were collected. When low-chloride medium was used, 114mM Na propionate was substituted for NaCl (≃ 83% substitution) and was present from the start of superfusion and during the K^+ pulse. Propionic acid was used to adjust the pH of the medium. In experiments to investigate the effect of dynorphin$_{1-13}$ on low-chloride-stimulated release, two methodologies were used: in the first the normal protocol was followed and normal medium was used to superfuse the tissue. At the 7th fraction, medium was changed to medium with 50% substitution of Na propionate for NaCl, with or without 1μM dynorphin$_{1-13}$. The second series of experiments were carried out using a slightly different protocol: the superfusion rate was increased to 1ml/min, and 6 sec fractions were collected. In this series, Na propionate was substituted for 33% of the NaCl in the test medium.

10.3 Results

10.3.1 Effect of opioid peptides on stimulated release of [^{3}H]GABA

Dynorphin$_{1-13}$ (1μM) reduced the stimulation of [^{3}H]GABA release evoked by 15mM K^+ but had no effect on basal release (Figure 10.1). The peak percentage stimulation of release was reduced to 42±5% of control by this concentration (n=6). This effect was concentration-dependent, with 10nM dynorphin$_{1-13}$ causing a slight but significant inhibition of secretion (Figure 10.2). The inhibition of release by 100nM dynorphin$_{1-13}$ was significantly antagonised by 1μM naloxone (introduced simultaneously) and the non-opioid derivative des-Tyr-dynorphin was without effect at 1μM (Figure 10.2), demonstrating that the effect of dynorphin$_{1-13}$ was mediated by an opioid receptor.

Dynorphin itself shows several fold greater affinity at κ rather than μ or δ receptors but cannot be considered a specific κ agonist. The pharmacological specificity of this effect was further examined using more selective agonists. DAGO is highly selective for μ sites (Handa, Lane, Lord, Morgan, Rance and Smith, 1981), DSLET shows several fold selectivity for δ over μ or κ sites (Garcel, Fournie-Zaluski and Roques, 1980) and U50488H is a newly developed compound apparently highly selective for κ sites (Lahti, Von Voigtlander and Barsuhni, 1982). DAGO and DSLET at 1μM were without effect on 15mM K^+-stimulated release, release being 104±11% of control in the presence of DAGO and 111±14% of control in the presence of DSLET (Figure 10.3). U50488H (1μM) caused a large reduction in stimulated release, to 43±6% of control (Figure 10.3), an effect similar in magnitude to that of dynorphin$_{1-13}$. The opioid receptor involved therefore shows every indication of κ-like pharmacology, and GABA nerve terminals in NI appear devoid of μ or δ receptors.

10.3.2 Ionic basis of κ receptor effect

Three approaches to investigate the ionic dependence of the effect of dynorphin$_{1-13}$ were made:

I) Blockade of ion channel and ionic substitution, with subsequent stimulation of release by 15mM K^+.

II) Stimulation of release by Ba^{2+}.

III) Stimulation of release by low-chloride medium.

10.3.2.1 K^+-stimulation after ion channel blockade and ionic substitution

4AP (1mM), TEA (10mM) and quinine (0.3mM) selectively block different subsets of K^+ channels (see Section 5.4.1), but were found to have no significant effect on the subsequent response

to 15mM K^+. None of these pretreatments affected the inhibitory effect of 1μM dynorphin$_{1-13}$ (Figure 10.4). 100μM DIDS, which selectively blocks anion channels (see Section 5.3.6), did not alter the response to 15mM K^+, but under these conditions the inhibitory effect of 1μM dynorphin$_{1-13}$ was significantly reduced (Figure 10.5). In low-chloride medium (≈83% substitution) the effect of 15mM K^+ was reduced to 75% of control, the inhibitory effect of dynorphin$_{1-13}$ was abolished (Figure 10.5). These data are summarised in Table 10.1.

10.3.2.2 Stimulation of release by Ba^{2+}

3mM Ba^{2+} ($MgCl_2$ substituted for $MgSO_4$ in the medium) caused 96±8% stimulation of release of [^{3}H]GABA (n=5). This was not significantly affected by 1μM dynorphin$_{1-13}$ (87±6% stimulation).

10.3.2.3 Stimulation of release by low-chloride medium

When the tissue was superfused with medium containing only 50% of normal chloride concentration release of [^{3}H]GABA was stimulated by 395±21%. Cointroduction of 1μM dynorphin$_{1-13}$ did not significantly affect this, the response being 465±26% stimulation, an increase of 18±6% over control (n=5). To enhance the resolution of this possible stimulatory effect of dynorphin$_{1-13}$, technical modifications were made: the rate of superfusion was increased to 1ml/min, and 6 sec fractions (compared with 1 min normally) were collected. The induced chloride gradient was also reduced, only 33% of chloride being substituted by propionate. Under these conditions, the low-chloride medium caused a peak of 159±13% stimulation of release, and this was increased to 287±21% by 1μM dynorphin$_{1-13}$ (181±7% of control, $p < 0.05$, n=5) (Fig 10.6).

10.4 Discussion

These results demonstrate that dynorphin$_{1-13}$ causes an inhibition of stimulated [^{3}H]GABA release from NI. This effect was concentration-dependent, antagonised by naloxone, and mimicked by U50488H, suggesting that it is mediated by a κ opioid receptor. Dynorphin$_{1-13}$ may also have some non-opioid actions (Przewlocki, Shearman and Herz, 1983), which are also caused by des-Tyr-dynorphin$_{1-13}$. This substance was tested on [^{3}H]GABA release, and found to be without effect at 1μM, providing further support for the presence of specific κ receptors. The lack of effect of DAGO and DSLET suggest that GABAergic nerve terminals in this area are free of μ and δ receptors, in contrast to dopaminergic and vasopressinergic terminals (Iversen et al, 1980; Vizi and Volbekas, 1980). κ receptors are also found on dopaminergic terminals in the striatum (Mulder, Wardeh, Hogenboom and Frankhuyzen, 1984), where, as found here, relatively high concentrations of naloxone are required for antagonism compared with δ-mediated effects.

There are several possible endogenous ligands for this effect. Dynorphin$_{1-17}$ is found in high concentrations in PN (Goldstein et al, 1982; Weber et al, 1982) but dynorphin$_{1-8}$ and dynorphin$_{1-13}$ have been suggested to be the predominant forms (Weber et al, 1982, Suda et al, 1982) with the co-derived peptide α-neoendorphin (Weber et al, 1982, Millan et al, 1983). The smaller peptides have been proposed to be neurotransmitters, as they have a short and rapid action (Corbett et al, 1982).

Dynorphin has been shown to coexist with vasopressin in the magnocellular projections to PN (Watson et al, 1982) and NI concentrations of dynorphin are altered concommitantly with vasopressin by endocrine manipulations and by lesions (Hollt,

Haarmann, Seizinger and Herz, 1980; Millan et al, 1982). GABA increased evoked vasopressin secretion in vitro (Knepel et al, 1984) and it is therefore possible that dynorphin co-released with vasopressin might indirectly regulate the secretion of vasopressin: vasopressin itself was without effect on [^{3}H]GABA release (Chapter 8). These results therefore may provide insight into the possible function of multiple neurotransmitters/hormones coexisting within one neurone, if one acts locally, perhaps in a negative feedback manner, and the other at a distant site. The local transmitter would therefore be an extra control, the magnitude of its effect being dependent on the activity of the GABAergic neurones in this case.

No evidence for the existence of μ or δ receptors was found. This could be taken to suggest that GABAergic terminals in NI are independent of regulation by β-endorphin and the enkephalins (Paterson et al, 1983), unlike dopaminergic terminals (Vizi and Volbekas, 1980) and vasopressin terminals (Iversen et al, 1980; AlZein et al, 1984) and therefore providing further evidence for functional differences between the three families of opioids. Most of the GABAergic terminals in NI are in PI (Vincent et al, 1982; Oertel et al, 1982), which suggests that most of the [^{3}H]GABA release measured here is from PI. Dynorphin$_{1-13}$ and U50488H inhibited the stimulated release of [^{3}H]GABA by ≈60%. The apparent absence of dynorphin from PI (Watson et al, 1982) may indicate that GABAergic terminals in PI have κ receptors which are not physiologically acted on by dynorphin. Alternatively, dynorphin$_{1-17}$ may act in PI at a distance from its site of release, as it is both extremely potent and resistant to degradation (Corbett et al, 1982).

Inhibition of neurotransmitter release is a common effect of opioids (Montel, Starke and Weber, 1974; Mudge, Leeman and Fischbach, 1979). κ receptor-mediated inhibition of neurotransmitter release has been reported (Mulder et al, 1984; Illes, Pfeiffer, von Kugelen and Starke, 1985), but stimulation may also occur (Ueda, Muramatsu and Fujwara, 1985). The mechanism of opioid receptor action has been studied electrophysiologically in locus coeruleus neurones, dorsal root ganglia and myenteric plexus. Mudge et al (1979) found that an enkephalin analogue decreased the duration of the action potential, an effect which could be mediated either by an increase in K^+ conductance or by a direct reduction in Ca^{2+} conductance. Enkephalin was found to open K^+ channels in locus coeruleus (Williams, Egan and North, 1982), which could be blocked by Ba^{2+}, and subsequently the reduction in the Ca^{2+} component of the action potential observed in these cells was shown to be a result of increased K^+ conductance (North and Williams, 1983). This effect may be common to both μ and δ receptors (Werz and Macdonald, 1982; 1983). While a direct increase in K^+ conductance appears to be established both centrally and peripherally (Morita and North, 1981) and can account for inhibition of neurotransmitter release, a direct effect on Ca^{2+} conductance remains to be confirmed. None of these studies, however, relates to receptors that are likely to be of the κ type. One recent report described the effect of dynorphin$_{1-17}$ on cultured dorsal root ganglion cells and implicated a reduction in Ca^{2+}-conductance (Werz and Macdonald, 1984). This apparent lack of mechanistic differentiation between quite different receptor subtypes is surprising, and it should be considered (as with all these studies) that dynorphin is not sufficiently κ-selective for unequivocal

identification of receptor type on the basis of that alone. Interestingly, in earlier reports enkephalins have also been considered to open chloride channels in cultured spinal cord neurones (Barker, Gruol, Huang, Macdonald and Smith, 1980). The receptor type was not then clearly identified, but may relate to the apparently Cl-channel-operating κ receptor here.

In the present experiments 4AP, TEA and quinine were used to block different subsets of K^+ channel (Adams et al, 1980; LaTorre and Miller, 1983; Cherubini, North and Suprenant, 1984). If dynorphin was acting by increasing K^+ conductance, one or more of these blockers would be expected to attenuate the inhibitory effect on release. This was not observed, suggesting that κ receptors may not influence K^+ conductance. The use of high-molarity K^+ as a depolarising stimulus may not be ideal for the investigation of K^+-conductance-mediated effects, but μ receptor activation was successfully shown to inhibit K^+ stimulated release of noradrenaline from frontal cortex (Montel et al, 1974). Experiments with K^+ channel blockers in that model would prove a useful test for the validity of this approach.

DIDS and low-chloride medium prevent the effect of substances acting by opening of chloride channels. This is illustrated by the antagonism of the effect of muscimol on prolactin secretion in Chapter 5. Both of these reduced the effect of dynorphin on K^+-stimulated release, and in low-chloride medium the effect of dynorphin was no longer significant. This contrasts with the lack of effect of changing chloride concentration on the effect of enkephalin in locus coeruleus (Williams et al, 1982), and suggests that opening of chloride channels may be at least part of the mechanism of action of κ receptors.

Ba^{2+} is a potent charge-carrier through voltage-dependent Ca^{2+} channels (Hagiwara and Byerly, 1981) which may account for its secretagogue action both on spontaneously active endocrine cells (Douglas et al, 1982; Chapters 5 and 7) and on neurotransmitter release (Douglas, Lywood and Straub, 1961; Boullin 1967). If dynorphin was acting by a direct effect on Ca^{2+}-conductance, then it might be expected to reduce the effect of Ba^{2+}. In analagous experiments, enkephalin and other transmitters reduced the duration of action potentials elongated by being recorded in Ba^{2+}-containing medium (Mudge et al, 1979; Dunlap, 1985). The lack of effect of dynorphin observed here suggests that a reduction in Ca^{2+} conductance is not involved, and that its site of action is prior to voltage-dependent Ca^{2+} channels.

Ba^{2+} also blocks some K^{+} channels (Fatt and Ginsborg, 1958; Brown et al, 1982), a property which has been used in investigation of μ-mediated effects on K^{+} conductance in locus coeruleus (Williams et al, 1982; North and Williams, 1983). This effect of Ba^{2+} is unlikely to be important here, as the secretagogue effect of Ba^{2+} on the synaptosomal preparation used is probably a result of penetration of Ca^{2+} channels and the K^{+} channel blockers were without effect on basal release.

Chloride-deficient medium elicited a marked release of [^{3}H]GABA from tissue previously superfused with normal medium, presumably caused by chloride movement in response to its artifically altered chemical gradient (Boakes, Turner and Virmani, 1984). As such chloride movement is likely to involve passage through membrane anion channels, receptors which open such channels should enhance ion movement, and thus may potentiate the effect on

release. Using the normal superfusion protocol and 50% substitution of chloride by the impermeant anion propionate, a slight but non-significant potentiation of release by dynorphin$_{1-13}$ was observed. A more rapid superfusion rate and fraction collection protocol revealed a prominent facilitation by 1μM dynorphin$_{1-13}$. This supports the results obtained using K^+-stimulated release in the presence of DIDS or low-chloride medium, and suggests that this κ receptor on GABAergic terminals in NI may act by enhancement of membrane anion conductance. This may not be a ubiquitous mechanism of action, as peripheral κ receptors appear to enhance acetylcholine release from parasympathetic terminals (Ueda et al, 1985) and to inhibit Ca^{2+} fluxes in erythrocytes (Yamasaki and Way, 1983), although such an effect on Ca^{2+} flux may be secondary to changes in anion conductance. Interestingly, in addition to reducing release, an increased intracellular chloride concentration caused by κ receptor activation may reduce GABAergic activity by inhibiting GAD (Roberts and Kuriyama, 1968).

In conclusion, these results demonstrate the presence of presynaptic κ but not μ or δ opioid receptors on GABAergic nerve terminals in NI, which may be acted on by an endogenous dynorphin to inhibit GABA release. The mechanism of action of this receptor may be to open chloride channels. [^{3}H]GABA release from NI is also controlled by $GABA_A$ autoreceptors (Chapter 8). $GABA_A$ receptors also act by opening chloride channels, and this suggests that there may be two populations of such receptor-controlled channels on GABAergic terminals in NI.

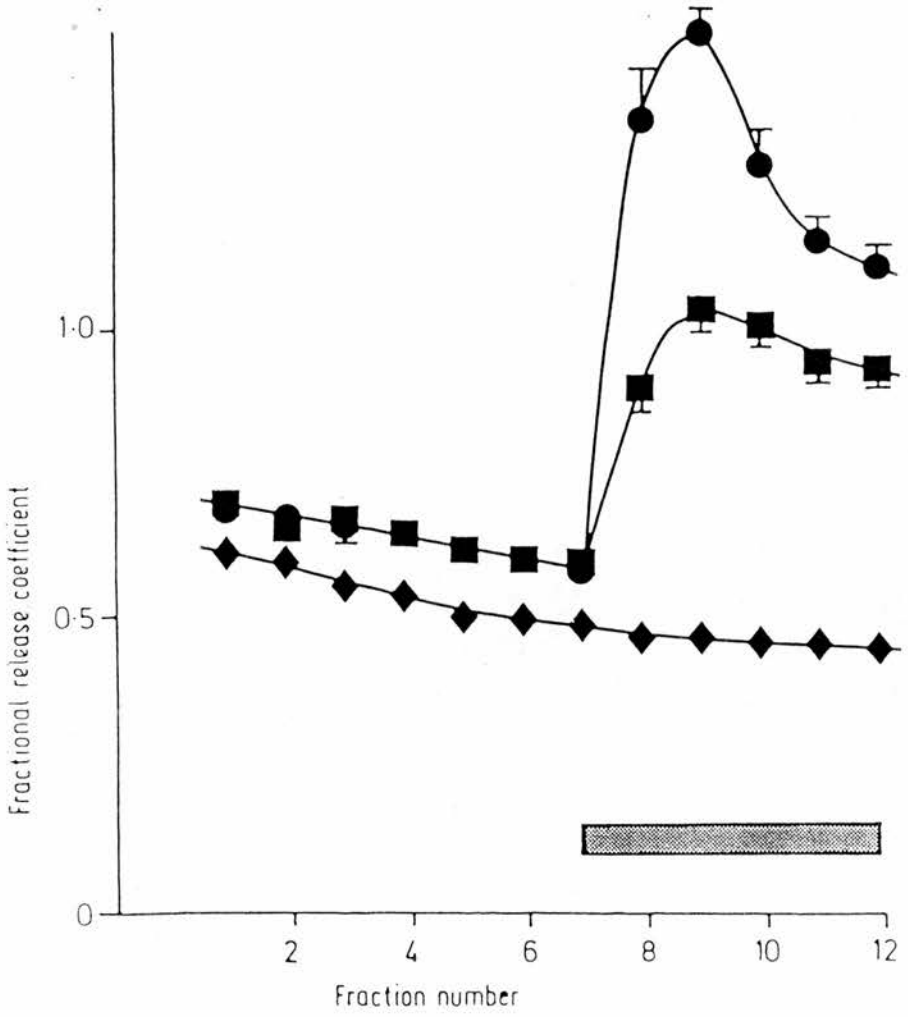


FIGURE 10.1

Inhibition of stimulated [^{3}H]GABA release from NI by dynorphin$_{1-13}$. K^+/dynorphin were introduced at the shaded bar.

(●) 15mM K^+
(■) 15mM K^+ + 1μM dynorphin$_{1-13}$
(◆) Effect of 1μM dynorphin$_{1-13}$ on basal release

Stimulated release: n = 5, mean ± S.E.M.
Basal release: mean of two experiments.

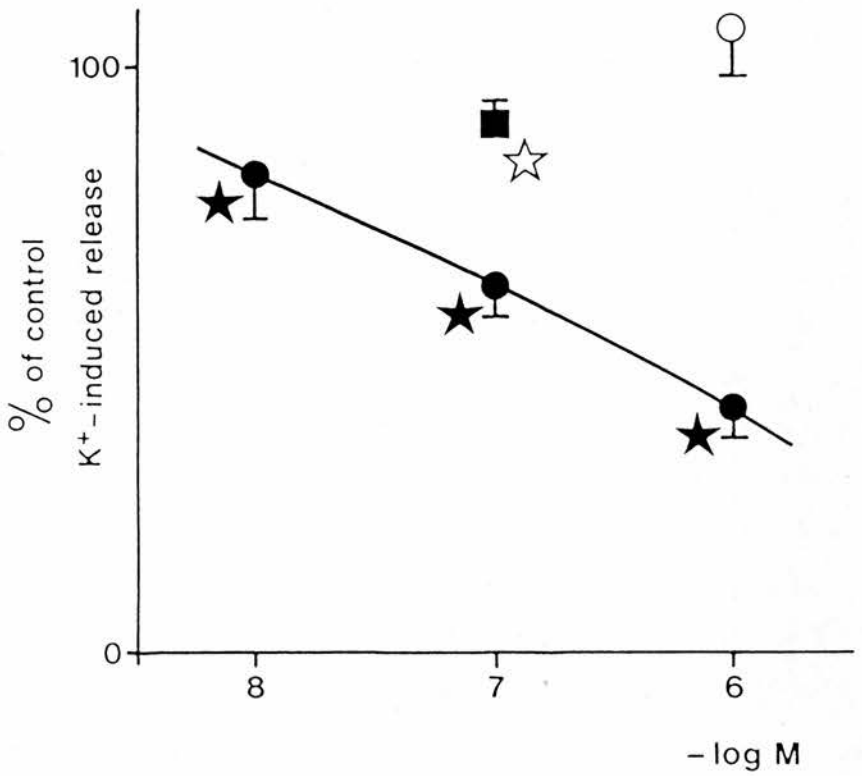


FIGURE 10.2

Concentration-dependence of the effect of dynorphin$_{1-13}$ on stimulated [^{3}H]GABA release from NI. Results are expressed as percentage of a parallel control superfusion.

(●) Dynorphin$_{1-13}$
(■) 100nM dynorphin$_{1-13}$ + 1μM naloxone
(○) 1μM des-tyr-dynorphin

★ $p<0.05$ vs Control
☆ $p<0.05$ vs 100nM dynorphin alone
Mean ± S.E.M., n = 5.

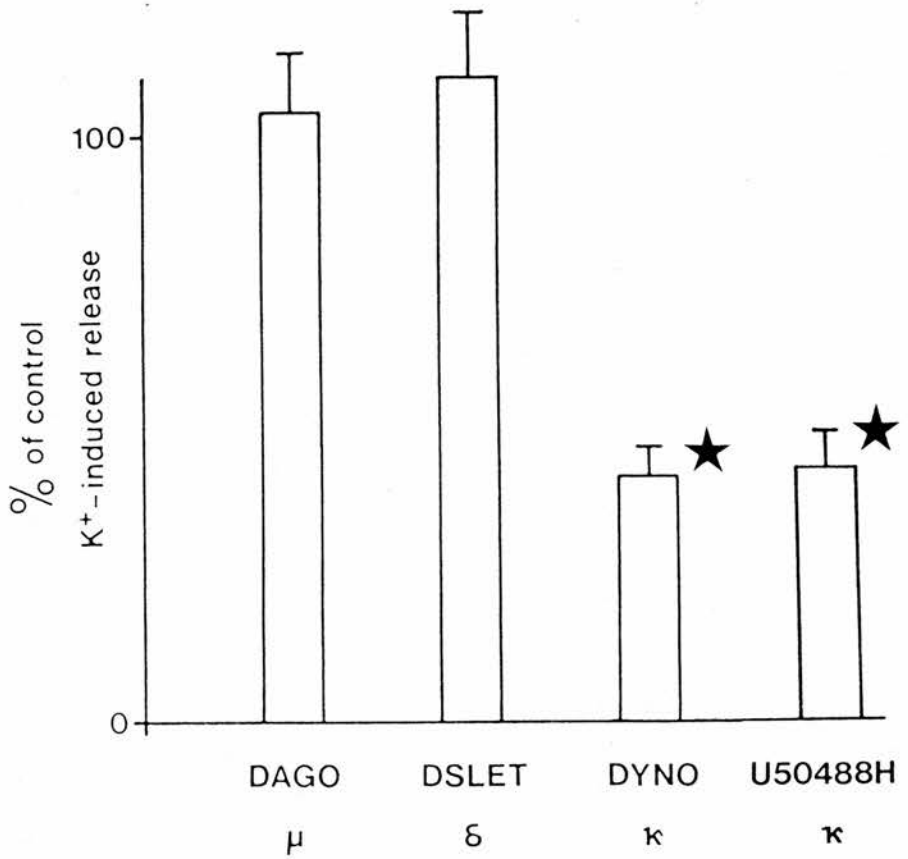


FIGURE 10.3

Receptor-specificity of the inhibition of [^{3}H]GABA release by opiate drugs. Results are expressed as a percentage of a control (K^+-only) stimulation of release carried out in parallel.

The peptides DAGO and DSLET had no significant effect on stimulated release, while Dynorphin$_{1-13}$ and U50488H (all drugs at 1μM) caused significant inhibition of release. Mean ± S.E.M., n = 5 in each case.

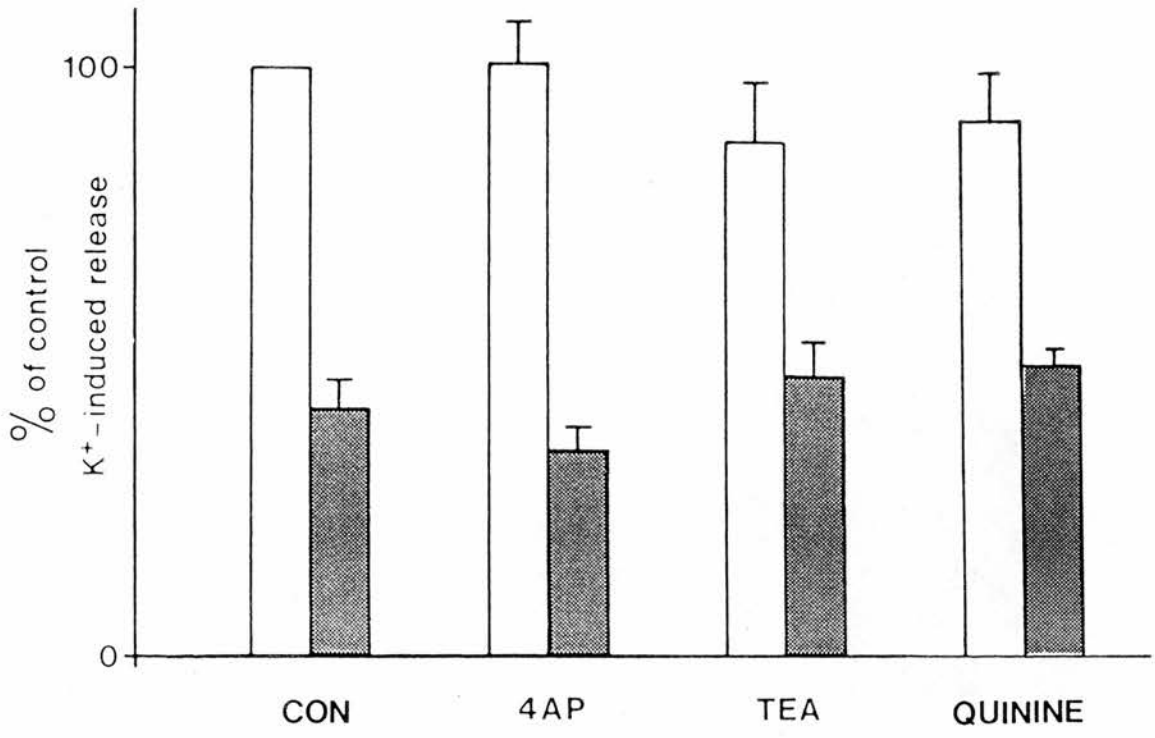


FIGURE 10.4

The effect of K^+-channel blockers on the inhibition of [^{3}H]GABA release by dynorphin$_{1-13}$.

CON control responses. Open column: normal medium, hatched column: normal medium + 1μM dynorphin$_{1-13}$.

Open columns: effect of 4AP (1mM), TEA (10mM) and quinine (0.3mM) on 15mM K^+-stimulated release. None of these treatments had any significant effect on release.

Hatched columns: effect of 1μM dynorphin$_{1-13}$ on K^+-stimulated release in the presence of the various channel blockers. Dynorphin$_{1-13}$ caused significant inhibition of release in each case, of similar magnitude to that in normal medium.
Mean ± S.E.M., n = 5.

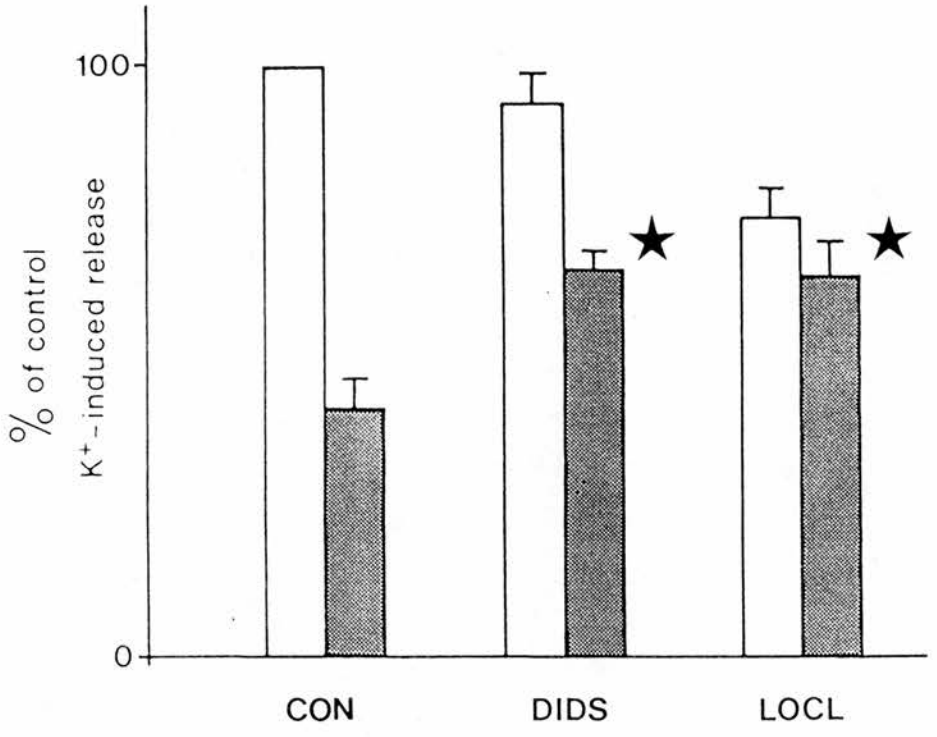


FIGURE 10.5

The effect of anion-channel blockade and low-chloride medium on the inhibition of [^{3}H]GABA release by dynorphin$_{1-13}$.

Open columns: effect of 15mM K^+ alone
Hatched columns: effect of 15mM K^+ + 1μM dynorphin$_{1-13}$
CON: normal medium, control responses
DIDS: medium containing 100μM DIDS
LOCL: medium containing Na propriate substituted for 83% of NaCl.

Mean ± .S.E.M., n = 5.
*: significant ($p<0.05$) attenuation of the effect of dynorphin$_{1-13}$

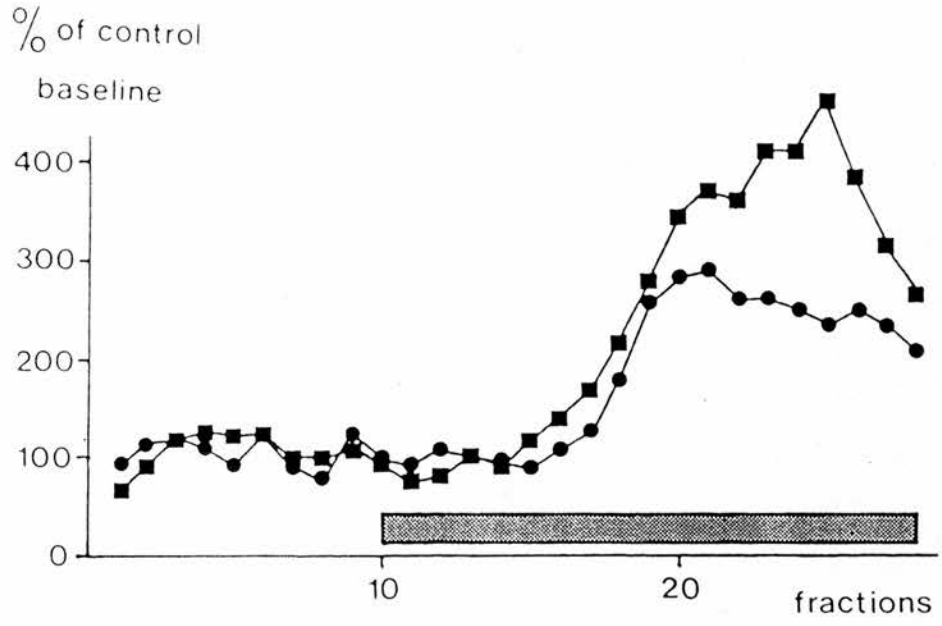


FIGURE 10.6

Effect of dynorphin on low-chloride stimulated release of [^{3}H]GABA from NI. Superfusion rate was increased to 1ml/min and 6 sec fractions were collected. Low chloride medium (33% substitution of Na propionate for NaCl) alone or with dynorphin$_{1-13}$ introduced at the hatched bar. The delay represents the dead-space of the apparatus.

(●) Low-chloride medium
(■) Low-chloride medium + 1μM dynorphin$_{1-13}$

Representative experiments. Results expressed as percentage of pre-stimulus baseline release.

TABLE 10.1

Effects of some ion channel-related drugs on the response to dynorphin$_{1-13}$

drug	concentration	% of control K^+-induced release	
		15mM K^+	15mM K^+ with 1μM dynorphin$_{1-13}$
-	-	100	42 ± 5*
4-aminopyridine	1	101 ± 7	33 ± 5*
tetraethylammonium	10	88 ± 10	48 ± 6*
quinine	0.3	92 ± 8	50 ± 3*
DIDS	0.1	94 ± 5	66 ± 3**,*
chloride deficient medium	114mM Na propionate replacing NaCl	75 ± 5*	65 ± 5**

Mean ± S.E.M., n = 5-7

* Significant inhibition compared to relevant controls
** Significant attenuation of inhibition
$p<0.05$, Mann-Whitney U test.

All ion channel drugs were present from the start of superfusion.

CHAPTER 11

The effects of GABA receptor agonists on [^{3}H]dopamine release from median eminence and pituitary neurointermediate lobe

11.1 Introduction

It is well established that there is a dense dopaminergic innervation of both ME and NI. The cell bodies of these tuberoinfundibularand tuberohypophysial neurones are found mainly in the arcuate nucleus with some in the periventricular nucleus (Bjorklund, Flack, Hromek, Owman and West, 1970; Jonsson, Fuxe and Hokfelt, 1972; Makara, Harris and Spyer, 1972) and there is also some evidence for a projection to ME from substantia nigra (Kizer, Palkovits and Brownstein, 1976). Dopaminergic terminals (demonstrated by fluorescence histochemistry and [^{3}H]DA uptake) in ME are found in close proximity to capillaries of the hypothalamo-pituitary portal vascular system and other nerve terminals and axons (Hokfelt, 1967; Cuello and Iversen, 1973) while those in NI are found in apposition to pituicyte processes, neurosecretory endings and secretory cells (Baumgarten, Bjorklund, Holstein and Nobin, 1972).

Dopaminergic terminals in both areas appear to show differences from mesencephalic dopaminergic terminals. The DA uptake carrier is of low affinity in both areas (Demarest and Moore, 1979a; Annunziato and Weiner, 1980) and tyrosine hydroxylase activity in ME does not appear to be subject to presynaptic control, although it is in NI (Demarest and Moore, 1979b). Evidence for presynapatic autoreceptors controlling DA release in ME is contradictory (Annunziato et al, 1981; Sarkar et al, 1983). Instead, the activity of tuberoinfundibular DA neurones may be subject to control by prolactin (Hokfelt and Fuxe, 1972; Annunziato and Moore, 1978) and tuberohypophysial DA turnover is increased by dehydration (Alper and Moore, 1982).

There are close parallels in both anatomy and function between these dopaminergic systems and the GABAergic innervation of ME and NI. For example, prolactin and dehydration also affect GABAergic function in ME and NI respectively (Apud et al, 1984a; Hamberger et al, 1979). GABA has been reported to stimulate DA release from several brain areas (Starr, 1978; Stoof, Den Breejen and Mulder, 1979; Bowery et al, 1980) including MBH but not NI (Sharman, Holzer and Holzbauer 1982). Negro-Vilar, Vijayan and McCann (1980) found that i.c.v. injection of GABA increased DA concentration in both ME and PD and decreased plasma prolactin concentrations, and inferred the mechanism to be a GABA-induced release of DA into hypophysial portal blood. Others, however, have reported that i.c.v. GABA stimulates prolactin secretion (Ondo and Pass, 1978; Grandison and Guidotti, 1979) and it has been proposed that GABA may act in that case by inhibition of DA release into portal blood (Casanueva et al, 1981). This controversy has been tested directly in the present experiments, by investigating the effect of $GABA_A$ and $GABA_B$ receptor agonists on the release of [^{3}H]DA from ME.

GABA and muscimol affect the amplitude of the antidromic compound action potential of hypothalamo-neurohypophysial axons (Zingg et al, 1979), implying the presence of presynaptic GABA receptors. Part of the effect may be on DA axons, in which case, GABAergic agents might affect DA release in NI. Both GABA and DA affect αMSH secretion from NI (Bower et al, 1974; Tomiko et al, 1983) and the possibility of interactions in NI was therefore also investigated.

11.2. Materials and Methods.

11.2.1. Materials

[^{3}H]Dopamine ([^{3}H]DA, 43.8 Ci/mmol) was obtained from Amersham International, ascorbic acid and pargyline from Sigma. (±)Baclofen, chlorimipramine and desimipramine were the generous gifts of Ciba-Geigy. Other chemicals and drugs were as described in Chapter 5.

11.2.2. Methods

The method and apparatus used was largely as described for [^{3}H]GABA release experiments described in Chapter 8. ME and NI were dissected as described in Section 2.2, and tissue pooled from 10 rats was homogenised in 400µl of cold 0.32M sucrose. The tissue homogenate was diluted into 3.2ml of oxygenated medium (composition as in Chapter 8, with 10µM pargyline and 130µM ascorbic acid), and incubated at 37°C for 15 min. [^{3}H]DA was then added to give a final concentration of 15nM, and sucrose concentration of 32mM. After a further 15 min, aliquots corresponding to two dissected areas were transferred to each superfusion chamber and superfused with oxygenated medium at 37°C at a rate of 0.5ml/min. After 30 min washout, basal release was approximately constant and 1 min fractions were collected. At the beginning of the 7th fraction, superfusion inlet lines were changed to medium containing 15mM KCl (replacing NaCl) with drugs as appropriate. Radioactivity in each fraction and that remaining in the tissue at the end of the experiment was measured by LSC, as described in Chapter 8.

11.2.3. Uptake specificity experiments

Experiments were carried out to assess the specificity of [^{3}H]DA uptake using the selective 5-HT and NA uptake inhibitors

chlorimipramine and desimipramine (Koe, 1976). 1μM chlorimipramine or 0.1μM desimipramine were present in the medium throughout an uptake period identical to that used for release studies i.e. 15 min preincubation and 15 min uptake in the presence of 15nM [^{3}H]DA, at 37°C. Samples were then filtered through Whatman GF/B filters under vacuum, washed with 3 x 5ml of medium at 37°C, and radioactivity in filters/tissue was measured as described in Chapter 8. Tissue-free (or detergent-lysed) blanks (~ 20% of total uptake for NI, 30% for ME) were subtracted to give values for specific uptake, and the percent inhibition of uptake by chlorimipramine and desimipramine was calculated for both ME and NI.

11.2.4. Calculation of results

Radioactivity in each fraction was expressed as a percentage of tissue radioactivity at the beginning of that fraction i.e. FRC. From this, values of peak percentage stimulation of release were calculated. In some cases these values were recalculated as a percentage of a control response carried out at the same time. Data are presented as mean ± S.E.M., and significance between two means (values as percent stimulation of release) were determined by Mann-Whitney U test.

11.3 Results

The possible contribution under these conditions of uptake of [^{3}H]DA into noradrenergic or serotonergic neurones was investigated using desimipramine and chlorimipramine at concentrations sufficient to cause prominent blockade of the NA and 5-HT, but not the DA uptake carrier (Koe, 1976). 0.1μM desimipramine caused only 11 ± 5% inhibition of uptake in ME, similar to that reported previously (Sarkar et al., 1983), and 15 ±

5% in NI, whereas 1μM chlorimipramine caused < 5% inhibition in ME and 20 ± 2% in NI (n = 5 in each case). These results demonstrate that under these conditions there is only a small contribution from noradrenergic or serotonergic neurones in either area. (Cuello, Horn, Mackay and Iversen, 1973; Bjorklund et al, 1970; Friedman et al, 1983).

After 30 min of washout, spontaneous release of [^{3}H]DA was steady with an FRC of ≃ 1% in both areas. Depolarisation with medium containing 15mM K^+ (replacing Na^+) rapidly caused a marked release of [^{3}H]DA from both areas (Figures 11.1 and 11.2), with a peak stimulation of 366 ± 10% from ME and 511 ± 54% from NI. This stimulation was reduced to 65 ± 3% (18% of control) in ME (Figure 11.1) and 79 ± 6% (15% of control) in NI (Figure 11.2) (n = 5, $P < 0.05$) by superfusion with Ca^{2+}-free (Mg^{2+}-substituted) medium (in the absence of cation chelators).

The possible influence of presynaptic GABA receptors on stimulus-evoked [^{3}H]DA release was investigated using muscimol as a selective $GABA_A$ receptor agonist and baclofen as a selective $GABA_B$ receptor agonist. 10μM muscimol reduced the response in ME to 79 ± 2% of control stimulus-induced release ($P < 0.05$ Figure 11.1) but had no effect on stimulated release from NI (106 ± 10% of control, Figure 11.2). 100μM (±)baclofen had no effect in either area (96 ± 3% in ME, 105 ± 14% in NI, n = 5 in all cases, Figures 11.1 and 11.2). The effect of muscimol was reversed (to 98±3% of control) ($P < 0.05$) by BMI (10μM) but was unaffected by strychnine (10μM, Table 11.1, antagonists introduced simultaneously with K^+/muscimol).

The pharmacology of this presynaptic $GABA_A$ receptor in ME was further investigated using homocarnosine. Homocarnosine was found to cause a concentration-dependent inhibition of stimulated [^{3}H]DA release from ME, reducing the peak response to 58 ± 3% of control at a concentration of 1mM ($P < 0.05$, $n = 5$). This effect appeared to be $GABA_A$ receptor-mediated as it was antagonised by 10μM BMI (Table 11.1). Neither muscimol nor homocarnosine had any effect on basal release of [^{3}H]DA at these concentrations.

11.4. Discussion

These results demonstrate that a crude synaptosomal preparation can be used for the study of DA release by ME and NI, as [^{3}H]DA is rapidly accumulated by the tissue and can be released by a K^+ stimulus in a reproducible and Ca^{2+}-dependent manner. This preparation allows the demonstration of direct presynaptic effects as interneuronal circuits will not be preserved, and in contrast to the whole ME preparation (Sarkar et al., 1983), released [^{3}H]DA is rapidly removed, thus preventing reuptake. A similar preparation has been used previously for the study of [^{3}H]DA release by ME (Annunziato et al, 1981) and for the study of [^{3}H]DA uptake by NI (Demarest and Moore, 1979a, Annunziato and Weiner, 1980) but this is the first demonstration of its use for the release of [^{3}H]DA by NI.

The effects of muscimol and homocarnosine on stimulated release in ME and the antagonism of these effects by BMI suggest that DA terminals in ME have presynaptic $GABA_A$ receptors and that activation of these results in reduced transmitter release. These results therefore provide a basis for the suggestion of Casanueva et al (1981) that the centrally-mediated stimulatory effect of GABA on prolactin secretion is mediated by reduced release of DA into

hypophysial portal blood and thus removal of a tonic inhibitory effect. Negro-Vilar et al (1980) suggested the opposite, that central administration of GABA increases DA release into portal blood, and this was supported by the demonstration of a stimulatory effect of GABA on DA release from MBH (Sharman et al., 1982). The tissue preparation in that case is very different to the one studied here, being > 10x larger, and containing several areas of cell bodies. It is therefore possible that GABA may have several sites of action on DA neurones in the hypothalamus.

The dense GABAergic innervation of ME (Vincent et al, 1982) with terminals adjacent to non-GABAergic axons and terminals as well as capillaries of the hypophysial portal system (Tappaz et al, 1983), provides an anatomical basis for this effect. The GABAergic innervation of ME appears to be most concentrated in the lateral palisade zone (Everitt et al, 1984), the region of ME which also receives the densest dopaminergic innervation (Ajika and Hokfelt, 1973). The functional significance of presynaptic regulation of dopaminergic function in ME is not clear, but both anatomical and physiological studies indicate some of the neuroendocrine systems which may be so influenced. The distribution of DA within ME parallels that of TRH and LHRH (Kizer, Palkovits, Tappaz, Kebabian and Brownstein, 1976), and tyrosine hydroxylase immunoreactivity in ME has been reported to show considerable overlap with that of LHRH and somatostatin (Agnati, Fuxe, Hokfelt, Goldstein and Jeffcoate, 1977). Axo-axonic contacts between dopaminergic and LHRH neurones in ME have also been demonstrated (Ajika, 1979). The dopaminergic innervation of ME may therefore have several functions other than as the source of DA for release into hypophysial portal blood. This is

supported by the demonstration that DA stimulates LHRH release from ME synaptosomes and MBH in vitro (Bennet, Edwardson, Holland, Jeffcoate and White, 1975; Rotsztejn, Drouva, Patou and Kordon, 1978) and there is also evidence for a stimulatory effect on somatostatin and TRH release (Negro-Vilar, Ojeda, Arimura and McCann, 1978; Maeda and Frohman, 1980). Increased DA turnover in ME is associated with the initiation of the proestrus LH surge (Rance, Wise, Selmanoff and Barraclough, 1981) but in vivo experiments on the effect of DA on LH secretion have given rise to confusion, and a steroid-dependent stimulatory effect of DA has been suggested (Vijayan and McCann, 1978c; Weiner and Ganong, 1978).

The multiplicity of endocrine effects of GABA makes the relevance of GABAergic regulation of ME DA release difficult to interpret, but it may contribute to some GABA-elicited responses e.g. inhibition of TSH secretion (Vijayan and McCann, 1979) by indirect inhibition of TRH secretion (Negro-Vilar et al, 1978). i.c.v. injection of muscimol has been shown to greatly reduce DA turnover in MBH (Fuchs, Mansky, Stock, Vijayan and Wuttke, 1984), and these experiments provide a possible site of action for this effect.

High concentrations of homocarnosine are present in hypophysial portal blood, although its origin is unknown as it does not appear to be released on electrical stimulation of ME (Mitchell et al., 1983). Homocarnosine appears to have weak partial agonist action at $GABA_A$ receptors as it can inhibit prolactin secretion in vitro (Chapter 5) and displace [^{3}H]muscimol binding at high concentrations (Mitchell et al., 1983). The present results suggest that homocarnosine may contribute to the GABAergic influence on DA terminals in ME.

The effect of muscimol in ME was antagonised by BMI but not by strychnine, as is most commonly observed for $GABA_A$ receptor responses, and this is consistent with the demonstration that recurrent inhibition in the tuberoinfundibular system is sensitive to the other classical $GABA_A$ antagonist, picrotoxin, but is resistant to strychnine (Yaki and Sawaki, 1975). This $GABA_A$ receptor also shows similarities with that causing inhibition of prolactin secretion in vitro, which is responsive to homocarnosine but is insensitive to antagonism by strychnine. Such a profile contrasts with that of the $GABA_A$ receptor complex mediating the transient stimulation of prolactin secretion in vitro which is not responsive to homocarnosine but is potently antagonised by strychnine (Chapter 5).

The recent demonstration of apparent coexistence of GABA and DA within ME (Everitt et al., 1984) raises the possibility that in addition to DA autoreceptors (Sarkar et al, 1983) and GABA heteroreceptors, GABA "co-transmitter autoreceptors" may be involved in the regulation of DA release from ME terminals. The presence of both $GABA_A$ and $GABA_B$ autoreceptors in ME was demonstrated in Chapters 8 and 9, and a schematic representation of the possible complexity of regulation of release of GABA and DA from a terminal in ME is shown in Figure 11.3. This hypothetical diagram shows all the GABAergic feedback pathways demonstrated in this Thesis and the presence of DA autoreceptors on one neurone, for which there is no direct evidence.

Muscimol was found to have no effect on [^{3}H]DA release from NI, and baclofen was without effect in both ME and NI. The dopaminergic projection to NI has been suggested to be the major

inhibitory innervation of that gland (Passo, et al, 1981; Davis et al, 1985) and may be involved in the control of peptide hormone synthesis and secretion in both PI and PN (Bower et al, 1974; Vizi and Volbekas, 1980; Lightman, Iversen and Forsling, 1982; Chen, Dionne and Roberts 1983). Recently the DA projections to PI and PN have been shown to have different properties (Lookingland, Farah, Lovell and Moore, 1985). Although any effect of muscimol or baclofen in only one of the lobes may therefore be less prominent here, since a mixed preparation was used, the results provide no evidence for any GABAergic influence on DA terminals in NI. Tuberohypophysial DA neurones therefore appear unusual in this respect, as DA release in a number of other areas can be affected by either $GABA_A$ or $GABA_B$ receptors (Starr, 1978; Stoof et al, 1979; Bowery et al, 1980).

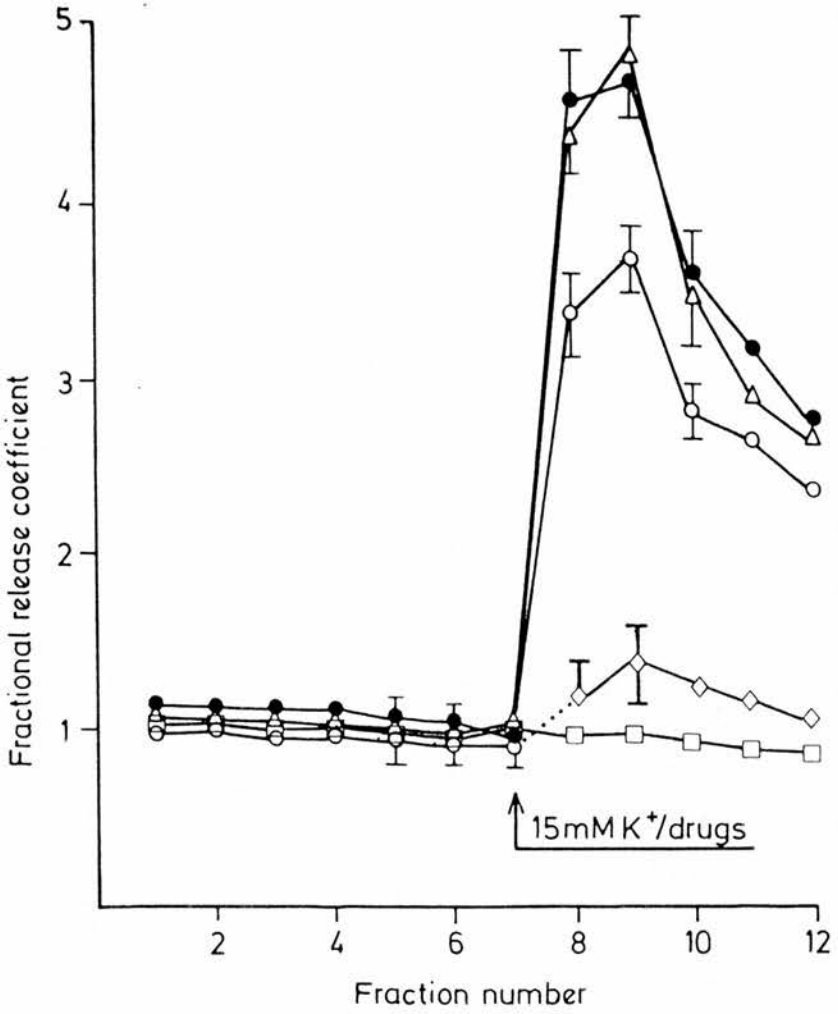


FIGURE 11.1

The effect of muscimol and baclofen on K^+-stimulated [^{3}H]DA release from ME. K^+/drugs were introduced at the arrow.

(●) Control response to 15mM K^+
(○) 15mM K^+ + 10μM muscimol
(△) 15mM K^+ + 100μM (±) baclofen
(◇) Response to 15mM K^+ in Ca^{2+}-free medium
(□) Effect of 10μM muscimol on basal release.

Mean ± S.E.M., n = 5 except muscimol on basal: n = 2.
Basal release in Ca^{2+}-free medium is not shown: effectively identical to (○).

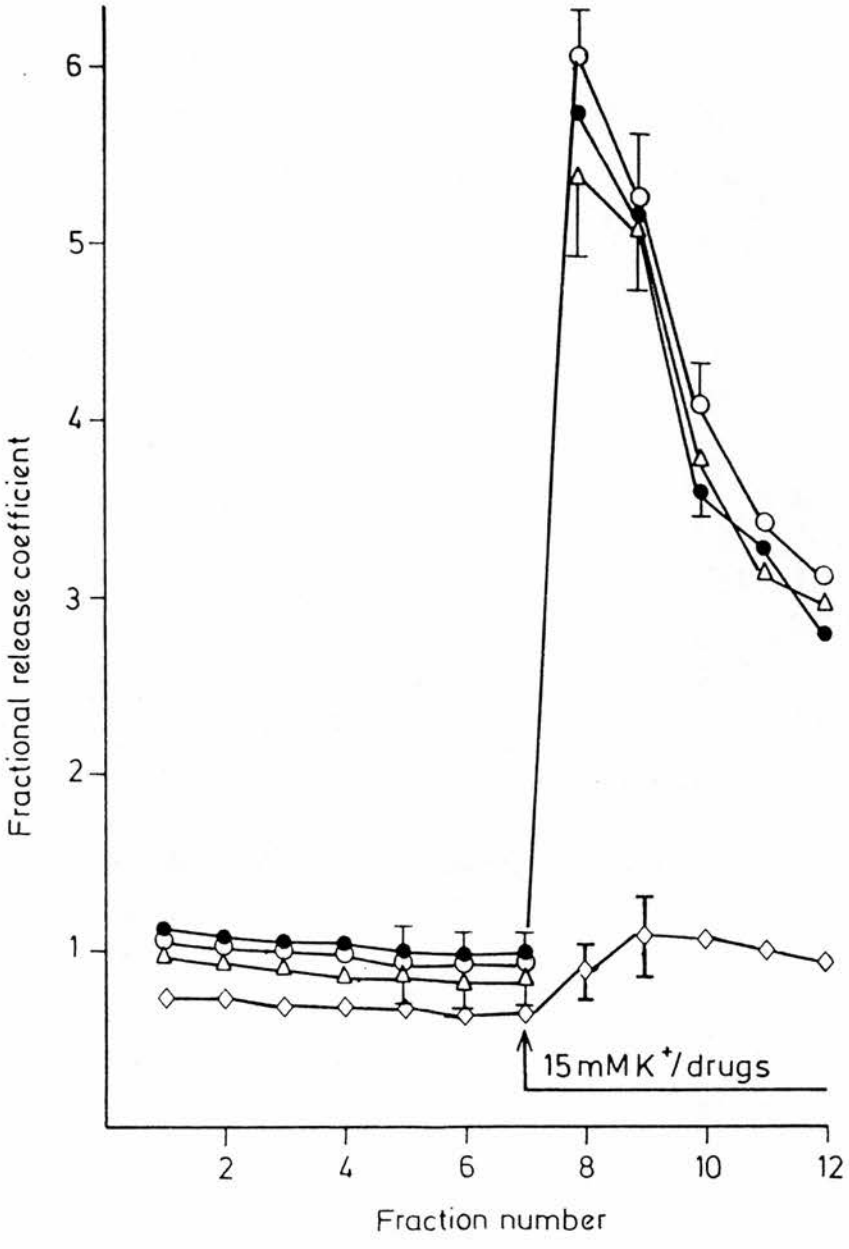


FIGURE 11.2

The effect of muscimol and baclofen on K^+-stimulated [^{3}H]DA release from NI. K^+/drugs were introduced at the arrow.

(●) Control response to 15mM K^+
(○) 15mM K^+ + 10μM muscimol
(△) 15mM K^+ + 100μM (±) baclofen
(◇) Response to 15mM K^+ in Ca^{2+}-free medium

Mean ± .S.E.M., n = 5.

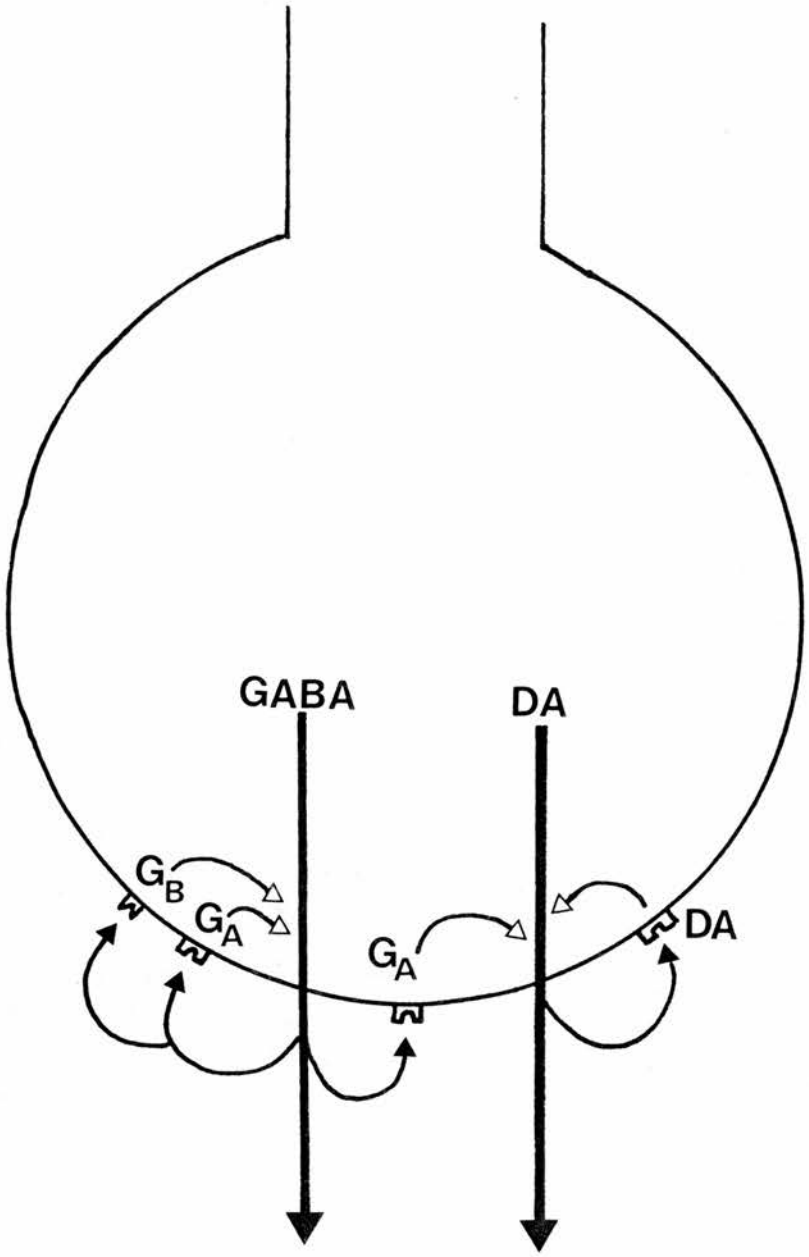


FIGURE 11.3

Schematic representation of inhibitory autofeedback mechanisms modulating GABA and DA release in ME. The diagram represents GABA and DA coexisting in a terminal in ME.

Large arrows: release of GABA and DA into perivascular space or synapse.
Small arrows, filled arrow heads: released neurotransmitter also binds to presynaptic DA, $GABA_A$ (G_A) or $GABA_B$ (G_B) receptors.
Small arrows, open arrowheads: intracellular inhibitory influence of receptor activation on release of neurotransmitter.

TABLE 11.1

Effects of GABA agonists on stimulus-induced release of [^{3}H]DA. Results are expressed as percentage of control response to 15mM K^+.

Drug	ME	NI
10μM muscimol	79 ± 2*	106 ± 10
10μM muscimol + 10μM bicuculline methiodide	98 ± 3**	-
10μM muscimol + 10μM strychnine	84 ± 2*	-
100μM homocarnosine	73 ± 4*	-
1mM homocarnosine	58 ± 3*	-
1mM homocarnosine + 10μM bicuculline methiodide	80 ± 4***	-
100μM (±)baclofen	96 ± 3	105 ± 14

Mean ± S.E.M., n = 5-6.

* $P < 0.05$ vs control

** $P < 0.05$ vs 10μM muscimol

*** $P < 0.05$ vs 1mM homocarnosine

Mann-Whitney U test on values for peak percentage stimulation.

CHAPTER 12

Investigation into the presence of homocarnosinase in the anterior pituitary gland.

12.1 Introduction

Homocarnosine (γ-aminobutyryl-L- histidine) was first identified in bovine brain by Pisano, Wilson, Cohen, Abraham and Udenfriend (1961). Subsequent studies demonstrated its presence in the brain of several animals with especially high concentrations in man (Abraham, Pisano and Udenfriend, 1967). It was not detectable in any peripheral tissue. Homocarnosine synthesis was demonstrated in frog brain *in vivo* and *in vitro* (Yockley and Marshall, 1969), and the enzyme involved, homocarnosine-carnosine synthetase, is mainly cytoplasmic and is regionally distributed in rat brain (Skaper, Das and Marshall, 1973; Ng and Marshall, 1978). Homocarnosine is degraded by a specific enzyme, homocarnosinase (Lenney, Kan, Sin and Sugiyama, 1977). High homocarnosinase activity is detectable in human brain (Kish, Perry and Hansen, 1979) but activity is low in rat brain (Ng and Marshall, 1978).

The distribution of homocarnosine does not simply parallel that of GABA (Young and Snyder, 1973) but the metabolism of the two substances is linked: myenteric plexus can synthesise both GABA and homocarnosine from glutamate (Jessen, Mirsky, Dennison and Burnstock, 1979) and the concentrations of both substances in brain and cerebrospinal fluid (CSF) are elevated by inhibitors of GABA-transaminase (Perry and Hansen, 1973; Palfreyman, Bohlen, Huot and Mellet, 1980). In Huntington's Chorea, the reduction in brain GABA content is accompanied by a reduction in homocarnosine content (Perry, Hansen and Kloster, 1973; Urquhart, Perry, Hansen and Kennedy, 1975). Genetic deficiency of homocarnosinase has been described, resulting in 'homocarnosinosis', which may cause paraplegia, mental deterioration and retinal pigmentation (Perry,

Kish, Sjaastad, Gjessing, Nesbakken, Schrader and Loken, 1979).

The related dipeptide carnosine has been proposed to be a neurotransmitter in the olfactory bulb (Margolis, 1974) but evidence for a functional role of homocarnosine in GABAergic transmission has been equivocal. In a number of models e.g. production of hyperexcitability and shortened barbiturate sleeping time, homocarnosine behaves like a GABA antagonist (Turnbull, Slater and Briggs, 1972) and endogenous homocarnosine has been suggested to act as a GABA antagonist, perhaps being responsible for dyskinetic movements after GABA-transaminase inhibition (Robin and Palfreyman, 1982). Iontophoretic application of homocarnosine onto cerebral and olfactory cortex neurones had little effect, although the neurones were depressed by GABA (MacLeod and Straughan, 1979). Homocarnosine has low but distinct affinity at GABA binding sites (Enna and Snyder, 1976; Mitchell et al, 1983), and Schally et al (1977) showed that concentrations as low as 6μM inhibited prolactin secretion in vitro. Very high concentrations of homocarnosine may be present in hypophysial portal blood (Mitchell et al, 1983) and further studies on the effects of homocarnosine on prolactin secretion in vitro were carried out in Chapter 5.

Human CSF contains homocarnosinase (Grove, Schechter, Tell, Rumbach, Marescaux, Warter and Koch-Weser, 1982) but rat plasma and CSF do not (Lenney et al, 1977; Grove et al, 1982). If the pituitary gland were to contain significant homocarnosinase activity, its action could elevate PD GABA concentrations above those measured in hypophysial portal blood, thus increasing GABAergic influences on PD function. The experiments described here were carried out to investigate this possibility.

12.2 Materials and Methods

The methodology used was based on that of Lenney et al, (1977) for the enzyme assay, with determination of GABA formed by the radioreceptor assay described by Mitchell et al, (1983).

12.2.1. Materials

N-ethylmorpholine, homocarnosine and GABA were obtained from Sigma. Tris and $CoSO_4$ were Analar grade, BDH, [^{3}H]muscimol (muscimol, [methylene - ^{3}H (N)]-, specific activity 20.6 Ci/mmol) was obtained from New England Nuclear. GABAase, NADP, and disodium α-ketoglutarate were obtained from Boehringer-Mannheim (Lewes).

12.2.2. Enzyme assay

Kidney and PD tissue from male Wistar rats was stored frozen at -20°C until use. Kidney tissue contains very high homocarnosinase activity (Lenney et al, 1977) and was therefore used to develop the assay. Tissue was thawed and homogenised at 20mg/ml for kidney or 50mg/ml for PD in cold 5mM N-ethyl morpholine buffer containing 1mM $CoSO_4$ (pH7.2) in a teflon-glass homogeniser. 250µl aliquots of homogenate were used in the assay, and were pipetted into 1.5ml polypropylene tubes on ice. 550µl of homogenisation buffer were then added, with 200µl of 2.5mM homocarnosine in buffer, giving a final concentration of 500µM. Tubes were then incubated at 30°C in a waterbath for the required time (generally 40 min), before the reaction was stopped by placing the assay tube in a boiling waterbath for 3 min. The tube was then centrifuged at 16,000 rpm for 10 min. The supernatant was assayed for GABA by radioreceptor assay. Blanks consisted of samples that were boiled immediately the homocarnosine was added.

12.2.3. GABA radioreceptor assay

In this assay, the unknown competes with [^{3}H]muscimol for high-affinity GABA binding sites on rat cerebellar membranes.

12.2.3.1. Membrane preparation

Cerebella from male Wistar rats were stored at -20°C until use. Tissue was homogenised in 50 vols of cold 50mM Tris HCl buffer, pH 7.1 using an Ystral high speed homogeniser and centrifuged at 48,000g at 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 50 vol Tris buffer containing 0.05% Triton X-100 detergent and incubated for 30 min at 37°C. This results in a preparation of near-uniform high-affinity binding sites for [^{3}H]muscimol, distinct from the mixed high- and low- affinity binding seen using non-detergent treated tissue (Wong and Horng, 1977, Beaumont et al, 1978; Chapter 3). The membranes were centrifuged as before, and washed a further seven times to remove the detergent, endogenous GABA, and endogenous inhibitors of GABA binding (Toffano et al, 1978, Gardner et al, 1981). After the final centrifugation, the pellet was suspended in 10 vol of buffer and stored frozen at -20°C. When required, membrane preparations were made up to 50 vol, centrifuged and homogenised again in 50 vol immediately before use.

12.2.3.2. Binding assay

Binding assays were carried out in 5ml polypropylene tubes and contained 500µl membrane suspension, 100µl [^{3}H]muscimol (final concentration 20nM), 10µl of a $^1/_{10}$ dilution of enzyme assay supernatant, 100µl of GABA (for non-specific binding and standard curve) and buffer to give a final volume of 1ml. 100µM GABA was used to define non-specific binding, and concentrations from 3-100nM

for standard curves. All determinations were made in duplicate. Assays were incubated on ice for 60 min, then filtered through Whatman GF/B filters under vacuum and washed three times with 5ml of ice cold butter via the incubation tube. Radioactivity bound to the filters was determined by LSC. Under these conditions, binding of [^{3}H]muscimol (approximately 18,000 dpm) was >95% specific.

Confirmation that the displacing activity of enzyme assays above that found in blanks was due to authentic GABA was carried out using the specific GABA-degrading enzyme mixture GABAase (Graham and Aprison, 1966). 5µl of the following mixture was added to the samples and buffer in the binding assay tubes: 20 units GABAase (from Pseudomonas fluoresencens), 1.6mg NADP, 3mg disodium α-ketoglutarate, 5µl 2-mercaptoethanol, 200µl Tris HCl buffer. Samples were then incubated at 37°C for 30 min before [^{3}H]muscimol binding was carried out.

Under these conditions, the final concentration of homocarnosine in the binding assay was 300nM. This concentration caused 3±2% displacement (n=4). The displacing activity of blanks (16±4% for kidney) was therefore not attributable to homocarnosine. As used GABAase was found to degrade >95% of authentic GABA (300nM), and the displacement caused by high concentrations of homocarnosine ($IC_{50}\simeq$ 50µM) was unaffected by GABAase.

12.2.4. Calculation of results

The apparent concentration of GABA in samples was calculated from the standard curve, derived at the same time from the same membrane preparation. Blank values were subtracted from this, to give the concentration of GABA formed. This was taken to be equivalent to the amount of homocarnosine degraded, thus giving the

activity of the sample after correction for the dilution involved. Data is presented as mean ± S.E.M.

The limit of sensitivity of the method (ie activity that would result in formation of GABA at a concentration double blank values) was calculated to be 0.4 μmol/hr/g tissue for PD.

12.3 Results

Formation of GABA by kidney homogenate used to characterise the method was found to be linear with time for 40 min (Figure 12.1) and with tissue concentration up to at least 10mg/assay (Figure 12.2). Activity in a preparation of supernatant (kidney homogenate centrifuged at 12,000g for 20 min) was found to be the same as homogenate. The amount of GABA formed by 250μl of supernatant in 20 min was 3.1 ± 0.6μmol/g, and by homogenate was 3.0±0.5μmol/g (n=4 in each case).

Similar experiments using PD homogenates showed minimal activity. After 40 min incubation, the apparent concentration of GABA in samples was estimated as 1.3±0.3nmol, giving an activity of 0.16μmol/hr/g, but this was well below the limit of sensitivity of the assay and cannot be considered reliable. Furthermore, the total 'GABA' present was not altered by treatment with GABAase. The apparent concentration of GABA in samples was 4.8±0.3μM before GABAase, 4.9±0.4μM after (n=4 in each case). PD therefore shows no detectable homocarnosinase activity. Immediate metabolism of any GABA formed in very unlikely as GABA-transaminase activity, although present in PD (Racagni et al, 1979), is dependent on the addition of α-ketoglutarate (Hall and Kravitz, 1967).

12.4 Discussion

These results do not provide any evidence for the hypothesis that PD tissue can hydrolyse homocarnosine to GABA, thus increasing the local concentration of GABA. Lenney et al (1977) also reported that homocarnosinase was not detectable in rat brain, but Ng and Marshall (1978) found some homocarnosine-degrading activity in rat brain homogenates. The absence of activity in high-speed supernatant suggests that activity measured in that study was not true homocarnosinase, which is equally active in homogenate and supernatant (Lenney et al, 1977; also present results). The rat therefore shows considerable differences from the human in homocarnosine metabolism. Human brain contains considerable amounts of homocarnosinase, although homocarnosine-carnosine synthetase is difficult to reliably measure because of post-mortem lability (Kish et al, 1979).

The concentration of homocarnosine in hypophysial portal blood is sufficient to cause considerable inhibition of prolactin secretion (Chapter 5). Homocarnosine also appeared to be able to antagonise the stimulatory effect of GABA on prolactin secretion, but was without either agonist or antagonist action at somatotrophe GABA receptors (Chapters 5 and 7). This variety of pharmacological activity is also seen in the CNS, as described in the introduction to this Chapter, and suggests that homocarnosine may be a potentially useful GABAergic compound as a basis for new drug development. The inhibitory $GABA_A$ receptors on lactotrophes and on DA terminals in ME (Chapter 10) appear to be the only models yet described where homocarnosine has considerable efficacy, but the affinity of homocarnosine for cerebellar GABA binding sites

(Mitchell et al, 1983) may suggest that negative results obtained elsewhere (eg MacLeod and Straughan, 1979) may be the result of too low a concentration being reached at synaptic loci.

Although homocarnosine can be formed from GABA by rat brain in vivo, this appears to be a slow process (Palfreyman et al, 1980). In contrast, the formation of GABA from homocarnosine is rapid in, for example human CSF (Grove et al, 1982). Basal GABAergic tone on PD in the human may therefore be more sensitive to alterations in homocarnosine concentration in brain and plasma. The concentration of homocarnosine in hypophysial portal blood was not altered by electrical stimulation of ME, although the secretion of GABA was greatly increased (Mitchell et al, 1983). The coexistence of GAD-IR with histidine-decarboxylase-IR has recently been demonstrated in magnocellular neurones in the rat hypothalamus (Takeda, Inagaki, Shiosaka, Taguchi, Oertel, Tohyama, Watanabe and Wada, 1984). The possibility therefore exists that these neurones may metabolise homocarnosine in some way. One possibility is that homocarnosine is a precursor and that histamine and GABA are co-transmitters. The is supported by the cytoplasmic location of carnosine-homocarnosine synthesis (Ng and Marshall, 1978). These neurones project to the neocortex (Vincent et al, 1983), but any projections within the hypothalamus are unknown. A metabolic/storage role for homocarnosine as a 'buffer' for GABA might explain why no increased secretion into hypophysial portal blood was observed. The origin of the homocarnosine is therefore unknown as sufficiently precise experiments to define the distribution of carnosine-homocarnosine synthetase have yet to be done. Considerable further study is needed to approach an

understanding of the function of this dipeptide, either in relation to GABA metabolism or GABAergic neurotransmission. Effects of homocarnosine not related to GABA should not be ignored, as the distribution of the two substances is not parallel (Young and Snyder, 1973). More detailed localisation of the enzymes involved in homocarnosine metabolism might shed considerable light on this problem. Immunohistochemical studies using antiserum against carnosinase have demonstrated the presence of that enzyme in vomeronasal nerves (Margolis, Grillo, Grannot-Reisfelt and Farbman, 1983) supporting the suggestion of Margolis (1974) that carnosine may be a neurotransmitter in the olfactory pathway. Carnosinase immunoreactivity was also found in other sensory neurones and analogous anatomical studies on homocarnosinase might provide important evidence for a role in the control of PD hormone secretion.

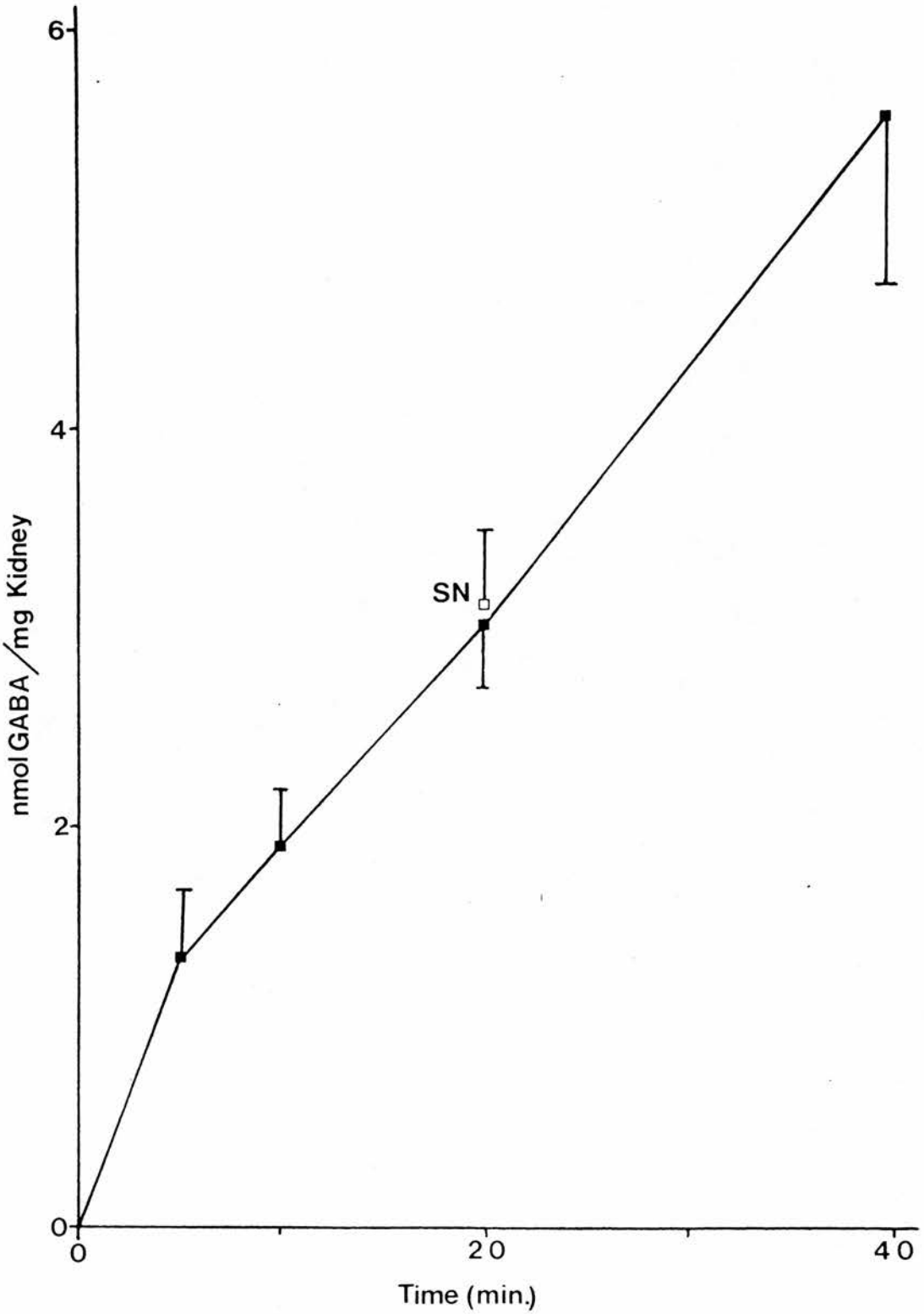


FIGURE 12.1

Time-dependence of formation of GABA from homocarnosine by kidney tissue. Assays (5mg tissue) were incubated at 30°C for the time stated.
SN: determination was carried out on high-speed supernatant equivalent to 5mg tissue, incubated for 20min. Mean ± S.E.M., n = 4.

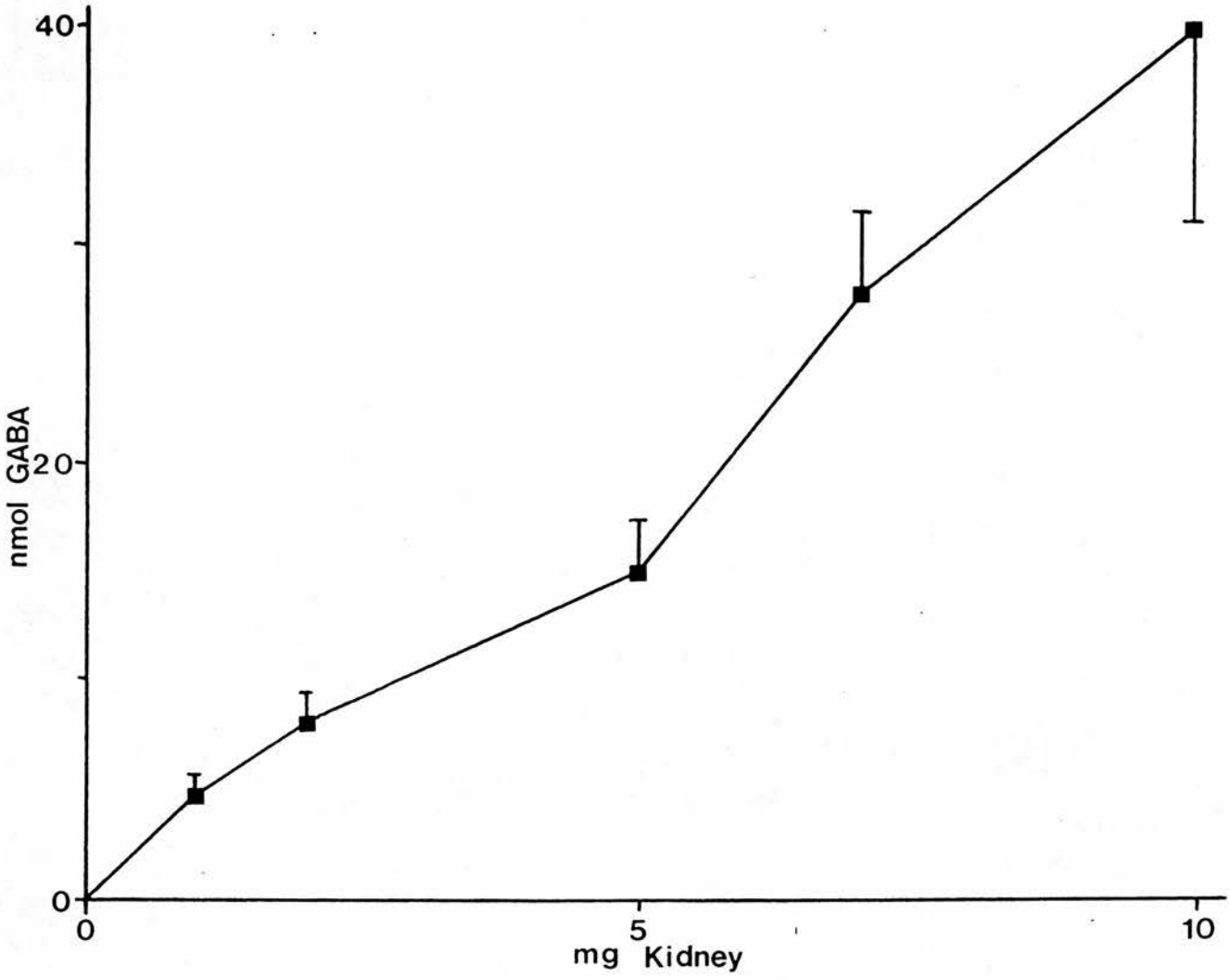


FIGURE 12.2

Tissue concentration-dependence of formation of GABA from homocarnosine by kidney tissue. Assays were incubated at 30°C for 20min. Mean ± S.E.M., n = 4.

CHAPTER 13

$GABA_A$ binding sites and Glutamic Acid Decarboxylase activity in the Neurointermediate lobe of Brattleboro rats

13.1 Introduction

Brattleboro rats suffer from hereditary diabetes insipidus, caused by inability to synthesize vasopressin and its associated neurophysin (Valtin, Sawyer and Sokol, 1965; Russel, Brownstein and Gainer, 1980; Schmale and Richter, 1984). The supraoptic and paraventricular nuclei show enlarged cells, and there is marked hypertrophy of PN (Sokol and Valtin, 1965) suggesting excessive activity, and homozygous Brattleboro rats are dehydrated even with free access to water (Valtin et al, 1965). Oxytocin synthesis appears to be unaffected (Sokol and Valtin, 1965; Russel et al, 1980) and the reduction in PN oxytocin content (Valtin et al, 1965) and increased plasma oxytocin concentration may be a result of dehydration (Dogterom, Van Wimermsma Greidanus and Swaab, 1977).

GABA may be involved in the control of vasopressin release. Feldberg and Rocha e Silva (1978) reported that bicuculline and picrotoxin, applied to the brain stem of cats, caused increased release of vasopressin and i.c.v. administration of GABA or AOAA reduced hypovolaemia-induced vasopressin release (Knepel et al, 1980). GABA also inhibited the firing of supraoptic neurones (Bioulac, Gaffori, Harris and Vincent, 1978; Arnauld, Cirino, Layton and Renaud, 1983), and the neurohypophysial antidromic compound action potential (Zingg et al, 1979). A direct effect of GABA on vasopressin release from PN *in vitro* has recently been demonstrated (Knepel et al, 1984).

Brattleboro rats also appear to have a GABAergic deficit of some description in PN. The concentration of GABA in the posterior pituitary of Brattleboro rats was greatly reduced compared to Sprague-Dawley rats, and showed little capacity for uptake of

[^{3}H]GABA (Hamberger et al, 1979). These results were interpreted in terms of GABA uptake by glial cells. The demonstration of a GABAergic innervation of NI from the CNS (Oertel et al, 1982; Vincent et al, 1982) suggested that these neurones may be involved. The present series of experiments were carried out to investigate this GABAergic deficit further. Two markers of GABA neuronal activity were measured: a postsynaptic marker, the number of $GABA_A$ binding sites, and a presynaptic marker, the activity of the GABA-synthesising enzyme glutamic acid decarboxylase. Brattleboro rats were compared to their parent strain, Long-Evans.

13.2 Materials and methods

13.2.1 Animals

Homozygous male Brattleboro rats (200-280g) were bred in the Department of Pharmacology. Urine excretion over a period of 24 hrs was measured using individual metabolic cages and was greater than 50% (v/w) in all rats used in this study. Male Long-Evans rats were obtained from Charles River (Margate) and all animals were housed as described in Section 2.1.

13.2.2 [^{3}H]Muscimol binding assay

$GABA_A$ binding sites were quantified using [^{3}H]muscimol. The method used was as described in Section 3.2 and will be only briefly outlined here. Pooled NI were homogenised in Tris HCl buffer, centrifuged and the membranes frozen. At the time of assay, membranes were thawed, and washed 4 times by repeated centrifugation. Aliquots in duplicate were then incubated with [^{3}H]muscimol (2nM and 20nM) on ice for 1 hr, and the assays were terminated by centrifugation, with superficial washing of the pellet. Non-specific binding was defined by the presence of 100μM

GABA. Radioactivity in the pellet was counted by LSC after being solubilised in Protosol. Protein content was determined as described in Section 2.5.

13.2.3 L-Glutamic acid decarboxylase assay

The method used in this thesis was based on that of Alberts and Brady (1959) and Tappaz, Brownstein and Kopin (1977) and depends on measurement of the amount of $[^{14}C]CO_2$ liberated from $[^{14}C]$ -L-glutamate.

13.2.3.1 Materials

L-[1-^{14}C] glutamic acid (59mCi/mmol) was obtained from Amersham International. All other reagents were obtained from Sigma (Poole) except KH_2PO_4, K_2HPO_4 and Triton X-100 (B.D.H., Poole).

13.2.3.3 Method

Fresh tissue (dissection as in section 2.3) was homogenised in 50µl of 0.1M K phosphate buffer containing 0.25% Triton X-100 and 0.1mM pyridoxal-5'-phosphate in an all-glass homogeniser. A 1M stock of K phosphate buffer was prepared by mixing 1M K_2HPO_4 and 1M KH_2PO_4 to give pH 6.5. Duplicate 10µl aliquots of homogenate were pipetted into 1.5ml polypropylene tubes (Sarstedt) and 10µl of incubation medium of the following composition was added (final concentrations in brackets): K phosphate buffer pH 6.5 (100mM), pyridoxal-5' phosphate (0.5mM), dithiothreitol (0.5mM), $[^{14}C]$glutamate (0.085mM), L-Na glutamate (10mM), Triton X-100 (0.25%), Na arsenite (1mM). Tubes were then put into plastic scintillation vials which contained 100µl methylbenzethonium hydroxide (hyamine, 1M in methanol). The vials were capped with a rubber seal, and incubated in a shaking waterbath at 37°C for 20

min. The reaction was stopped by injection of 200µl 6N H_2SO_4 into the tube using a 2ml syringe and needle, and the incubation was continued for a further 60min to ensure complete absorption of $[^{14}C]CO_2$ by the hyamine. At the end of that period, the rubber seals were removed and the outside of the reaction tubes washed with 2ml ethanol into the scintillation vials. 10ml NE265 scintillation fluid was added, and the vials were chilled overnight before radioactivity was counted. Blanks consisted of 10µl incubation medium carried through the whole procedure, and were determined in duplicate. Protein content of aliquots of homogenate were determined according to Section 2.4.

13.2.3.3 Calculation of results.

Specific $[^{14}C]CO_2$ evolution was found after subtraction of blank dpm from mean of sample dpm. Blanks were less than double background, and ME samples were approximately tenfold blank values. 0.202 µmoles $[^{14}C]$glutamate were added, containing 0.1µCi of radioactivity (equivalent to 220,000dpm). From this the activity of the sample was calculated as:

$$\text{specific dpm} \times \frac{0.202 \times 3}{220{,}000 \ \mu\text{g protein}} \quad \mu\text{mol/hr/}\mu\text{g protein}$$

Preliminary studies showed that the reaction was linear over the 20 min incubation used (Figure 13.1). Statistical comparisons were made using Students t test.

13.3 Results

13.3.1 $[^{3}H]$Muscimol binding assays

The experiments in Chapter 3 showed that $[^{3}H]$muscimol at 2nM and 20nM bound mainly (≈60%) to high-affinity and low-affinity sites respectively, and these concentrations were therefore used for the experiments in this Chapter. Binding of $[^{3}H]$muscimol was

considerably greater to NI from both Brattleboro and Long-Evans than from Wistar rats, but there was no difference between Brattleboro and Long-Evans rats. At 2nM, [^{3}H]muscimol binding comprised 23 ± 6 fmol/mg protein in Brattleboro NI, and 29 ± 4 fmol/mg protein in Long-Evans NI. At 20nM, the values were 157 ± 21 fmol/mg protein and 142 ± 18 fmol/mg protein respectively (n = 4 in each case). (Values obtained for Wistar NI in Chapter 3 were 3.0 fmol/mg protein at 2nM, and 6.8 fmol/mg protein at 20nM).

13.3.2 GAD assays

GAD activity in NI was much lower than in brain regions, but was readily detectable at 5-6 times blank dpm values in Long-Evans rats. The GAD activity of Long-Evans NI was 35.5 ± 1.9 pmol/hr/µg protein, which was considerably greater than the activity in Brattleboro rats: 20.1 ± 0.4 pmol/hr/µg protein (n = 5 in each case, $P < 0.001$). The hypertrophy of the Brattleboro PN may have accentuated this difference where expressed in terms of protein content, so GAD activity was also calculated per gland. Long-Evans NI had a GAD activity of 3358 ± 131 pmol/hr/NI and Brattleboro NI had an activity of 2680 ± 71 pmol/hr/NI. Therefore Brattleboro NI have 79% of the activity of Long-Evans NI, but the values were still significantly different ($P < 0.01$). The protein content of Long-Evans NI was 94.0 ± 5.5µg protein and of Brattleboro NI was 131.5 ± 6.5 µg protein.

Further experiments were carried out in an attempt to localise the depletion of GAD activity to PI or PN. It was not possible to directly determine GAD activity in PI due to the very small amounts of tissue involved, but determinations could be made on PN after removal of PI tissue. In these experiments, GAD activity in

Long-Evans PN was 23.5 ± 3.3 pmol/hr/µg protein (1182 ± 149 pmol/hr/gland) and 9.44 ± 0.76 pmol/hr/µg protein in Brattleboro PN (707 ± 46 pmol/hr/gland) ($n = 5$, $P < 0.01$ values per µg protein, $P < 0.02$, values per gland). This shows that Brattleboro GAD activity in NI was 57% of the activity of Long-Evans NI, and that PN activity is 40% of that in Long-Evans (values/µg protein). Recalculation of these results to express activity per gland rather than per µg protein, and subtraction of PN values from NI values to give an estimated value for PI, gives values of 2176 pmol/hr/PI for Long Evans (65% of total activity in NI) and 1973 pmol/hr/PI for Brattleboro (74% of total activity in NI). These results are summarised in Figure 13.2. Examination of the protein determinations shows that on average, protein content of PN was 54% of NI for Long Evans, and 57% for Brattleboro rats. No other analysis of the completeness of dissection of PI from PN was made.

GAD activity in ME from Brattleboro and Long Evans rats was also determined. Activity was 210 ± 43 pmol/hr/µg protein in Long Evans rats, and 203 ± 20 pmol/hr/µg protein in Brattleboro rats ($n = 4$ in each case).

13.4 Discussion

[^{3}H]Muscimol binding to NI membranes at both 2nM and 20nM was very similar in Long-Evans and Brattleboro rats. If these values can be taken to be indicative of $GABA_A$ receptors, then these results suggest that GABA receptor function in Brattleboro NI is unaltered. This will be discussed further below, in the light of the results on GAD activity.

The GAD assay used here is based on the stoichiometric liberation of [^{14}C]CO_2 and formation of GABA from

[^{14}C]glutamic acid. This method is widely used and extensively characterised, but several possible problems inherent in the method should be considered. In certain tissues, non-equivalence of CO_2 and GABA formation is observed (Kanzawa, Iversen and Kelly, 1976; Wu, Chude, Wein, Roberts, Saito and Wong, 1978), and the [^{14}C]CO_2 method gives over-estimates of GAD activity. Part of this discrepancy has been attributed to metabolism of [^{14}C]glutamic acid in the tricarboxylic acid cycle (Kanazawa et al, 1976), which was inhibited by the presence of arsenite (included in the incubation mixture here). GAD activity is detectable in many non-neuronal tissues, but shows different immunological properties (Wu et al, 1978). NI GAD activity, by the [^{14}C]CO_2 method appears to be neuronal, as suggested by immunohistochemical studies, because it is virtually abolished (giving lower values than PD or liver) following section of the hypophysial stalk (Oertel et al, 1982). The value for GAD activity in NI found here is very similar to that reported by Oertel et al (1982), as were values from trial experiments using PD tissue (two values of 3.3 and 4.8 pmol/hr/μg protein were obtained).

GAD activity in Brattleboro NI was significantly reduced compared to Long-Evans rats. This difference was particularly marked when expressed in terms of protein content, probably reflecting the hypertrophy of PN (Sokol and Valtin, 1965; PN from Brattleboro rats were found to contain ≃ 50% more protein than Long-Evans PN) but was still significant when expressed as activity per gland. GAD activity in PN (per μg protein) of Brattleboro rats was even more markedly reduced (to 40% of Long-Evans activity), but calculated, and therefore only approximate, values for PI GAD were

similar. The true neuronal GAD activity in Brattleboro PN may be even lower, as this value was approaching the sensitivity of the assay, and the value of non-neuronal GAD activity. These results therefore suggest that the depletion of GAD activity is predominantly localised to PN, and that the depletion is not merely a consequence of the tissue hypertrophy with 'dilution' of GABAergic terminals. GAD activity in PI may be normal in Brattleboro rats, but this remains to be determined directly.

GAD has been suggested to be the limiting factor in GABA synthesis (Tappaz et al, 1977), and therefore GABAergic transmission might be functionally reduced in Brattleboro PN. GABA binding sites have been reported to show denervation supersensitivity: high-affinity [^{3}H]GABA binding was increased by 81% (Bmax value) in substantia nigra following kainic acid lesioning of striatum (Waddington and Cross, 1978). In those experiments, nigral GABA concentration was reduced by less than 50%. In contrast, Hamberger et al (1979) found that GABA concentration in the posterior pituitary of Brattleboro rats was 30% of controls, but the results presented here show that there was no change in [^{3}H]muscimol binding. Taken together, these results suggest reduced GABAergic activity in Brattleboro PN, rather than depletion of GABA through increased activity (in which case GAD activity would be expected to be elevated, not reduced), although partial degeneration of GABAergic neurones is an alternative possibility, as GABA concentration and GAD are presynaptic markers. Postsynaptic supersensitivity (i.e. increased [^{3}H]muscimol binding), might be masked if the elements on which the binding sites occur have partly degenerated. Alternatively, if the [^{3}H]muscimol binding sites are

predominantly localised in PI rather than PN, where there appears to be no change in GAD activity, this would prevent observation of any change in PN.

There is a brief report that high concentrations of GABA inhibited oxytocin release in vitro (Dyball and Shaw, 1978) but this has not been confirmed. Oxytocin turnover in Brattleboro rats appears to be elevated (Valtin et al, 1965; Dogterom et al, 1977), and the low GAD activity observed here may reflect reduced GABAergic activity of oxytocin-associated GABAergic terminals. This interpretation could be investigated by measuring GAD activity under other conditions in which oxytocin secretion is altered and must remain hypothetical at present.

An association of GABA with vasopressin release has been more widely investigated, and recently GABA and other $GABA_A$ agonists have been reported to potentiate stimulus-induced vasopressin release in vitro (Knepel et al, 1984). Cells of the supraoptic nucleus of the Brattleboro rat are hypertrophied and show slightly greater spontaneous activity than those in normal rats (Dyball, 1974), but respond normally to osmotic stimulation (Dyball and Leng, 1985) despite an altered noradrenergic innervation (Schloer and Sladek, 1981). Dynorphin coexists with vasopressin in normal rats, and is present in vasopressin-deficient neurones in Brattleboro rats (Watson et al, 1982) although it may be in reduced concentration (Martin and Voigt, 1981). The GABAergic deficit demonstrated here may therefore be associated in some way with either the deficit of vasopressin, or the increased electrical activity of the still dynorphin-containing neurones. The experiments described in Chapter 10 demonstrated an inhibitory influence of dynorphin on GABA release

from NI. Increased release of dynorphin may therefore reduce GABAergic activity.

GAD activity in PI and ME appeared to be unaltered in Brattleboro rats, demonstrating a tissue-specific alteration in GABAergic activity. ME also receives a vasopressin innervation (Zimmerman et al, 1978), but there is no evidence to support an association with the GABAergic innervation of ME. The secretion of many PD hormones is altered in Brattleboro rats (Sokol and Zimmerman, 1982) and secretion of ACTH from NI in vitro is doubled (Baertschi and Beny, 1982). The absence of changes in GAD activity in either area suggests that GABA mechanisms are not of major importance in these hormonal alterations.

In conclusion, these results demonstrate a marked depletion of GAD activity in PN of Brattleboro rats, without concomitant alteration of GAD activity in PI or ME, or $GABA_A$ binding in NI. The relationship between this deficit, which may reflect reduced GABAergic transmission, and the neurohypophysial hormones (and opioids) is at present purely speculative, but provides circumstantial evidence for a physiological role in the regulation of the neurohypophysis.

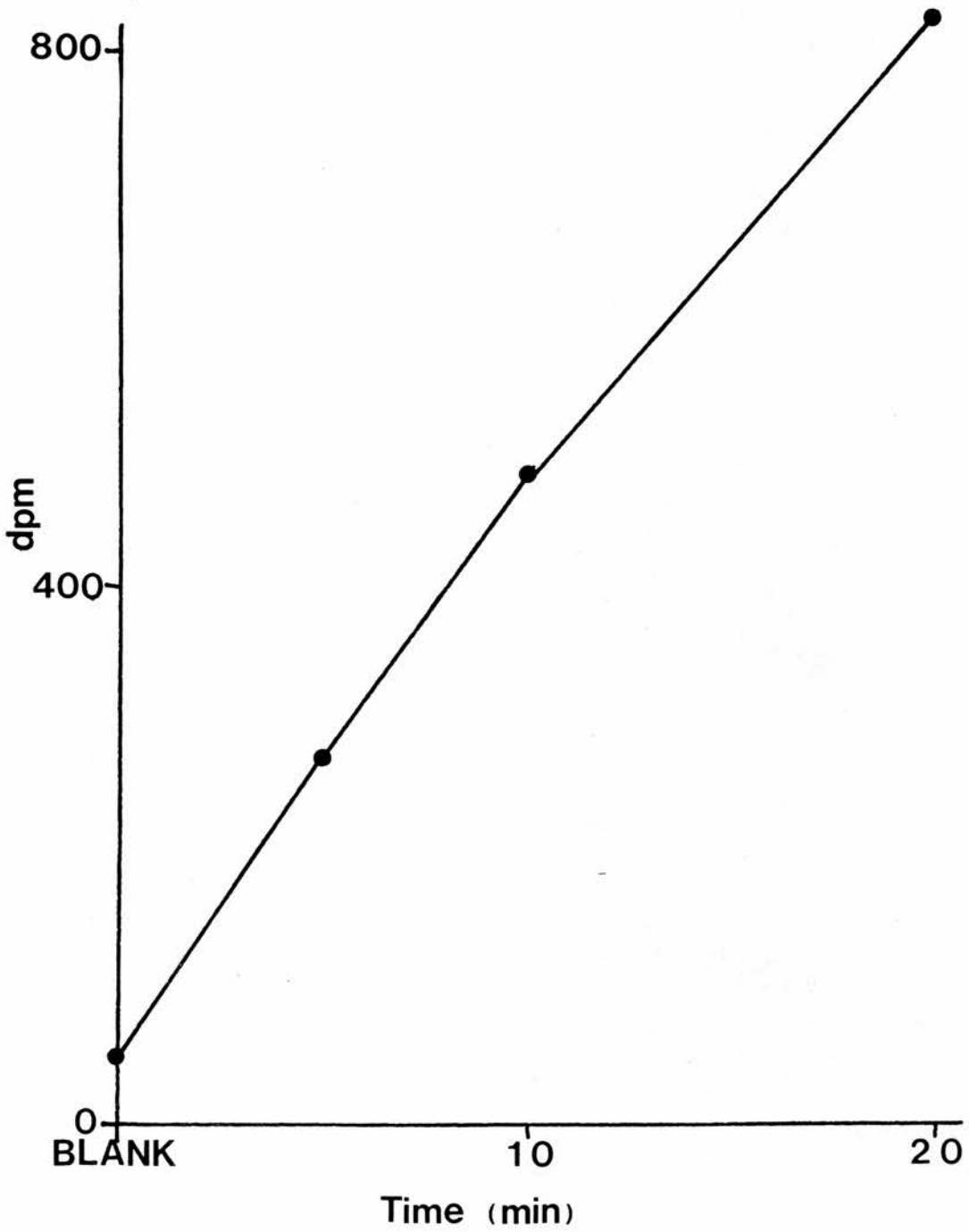
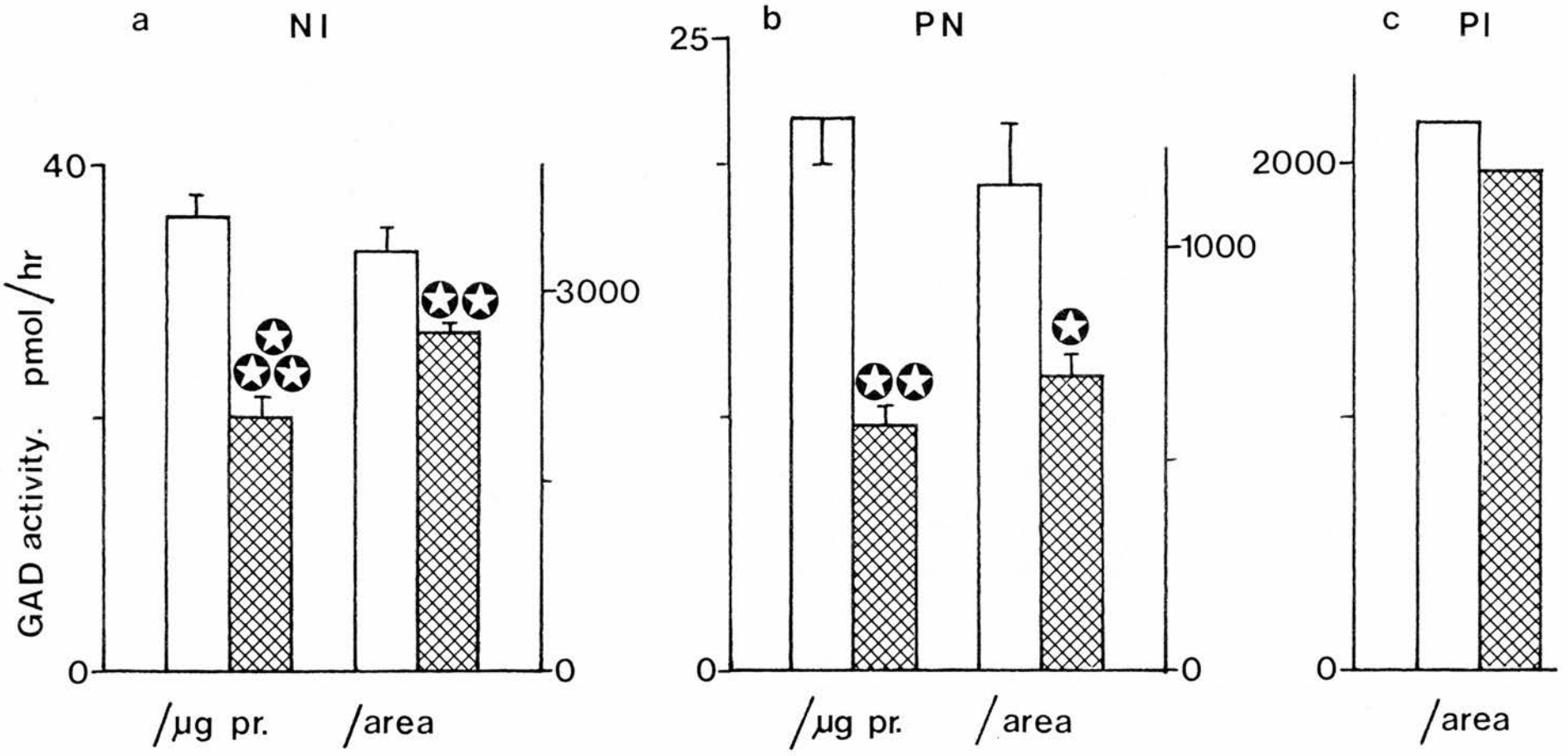


FIGURE 13.1

Linearity of [^{14}C]CO_2-evolution with time.
ME tissue (10µl of a 100µl homogenate of two ME) was incubated for the times indicated and the radioactivity evolved determined. Values are means of duplicate determinations (which differed by <5%). Tissue-free blank value is shown at zero time.


a
NI
40
0
3000
0
GAD activity. pmol/hr
/µg pr.
/area
b
PN
25
0
1000
0
/µg pr.
/area
c
PI
2000
0
/area

CHAPTER 14

Summary and Discussion

14.1 Summary

The control of hormone secretion from PD is predominantly under peptidergic control. The neurohormones GHRH, LHRH, CRH and TRH are well-established stimulators of GH, LH, ACTH and TSH respectively, and GH is also under the inhibitory control of somatostatin. The control of prolactin secretion may be slightly different (see Chapter 1): DA has long been recognised to be of considerable inhibitory importance but more recently strong evidence has been provided for the presence and importance of control by peptidergic PRFs (e.g. VIP) and PIFs. The importance of these substances is no longer in question, but their involvement in particular physiological states, and interaction with other substances in the control of PD hormone secretion, is largely unknown. It is in this context that the experiments described in this Thesis were carried out to examine whether the hypothesis of a role for GABA either as a neurohormone or as a neuromodulator in neuroendocrine control could be supported.

The evidence in the literature that GABA may be of importance in the control of PD function can be summarised as follows:

I) There is a dense GABAergic innervation of the external layer of ME, the site of secretion into hypophysial portal blood and final control thereof.

II) GABA, derived from the CNS, is found in PD.

III) GABA is found in hypophysial portal blood, and the concentration can be considerably elevated by stimulation of ME or i.c.v. injection of prolactin.

Most of the experiments in the literature have provided in vivo evidence suggesting that GABAergic systems may be of importance in the control of all the pituitary hormones and these experiments were

designed to investigate the mechanisms of such controls in a more detailed, localised way, using in vitro methodologies.

Radioligand binding studies were carried out to examine GABA binding sites in the pituitary (Chapter 3). [^{3}H]GABA was used to label $GABA_A$ and $GABA_B$ sites, and [^{3}H]muscimol to label $GABA_A$ sites. $GABA_A$ sites were found in both PD and NI, predominantly in the former. $GABA_B$ sites were found in PD, but were not detectable in NI. Scatchard analysis of saturation data suggested the presence of both high- and low-affinity $GABA_A$ sites, with low-affinity sites predominating, as found in the frontal cortex but not the cerebellum.

Central-type benzodiazepine binding sites (Chapter 4) were detectable in both PD and NI, but the distribution did not parallel that of $GABA_A$ sites, being found predominantly in NI (Section 4.3.2). A much larger population of peripheral-type benzodiazepine binding sites was also described, but, unlike the central-type sites, these were not linked to GABA receptors. Further studies using a β-carboline demonstrated that the pituitary central-type benzodiazepine sites were apparently predominantly of the BZ_1 sub-type, and showed properties similar to those in cerebellum but distinct from the mixed BZ_1/BZ_2 population in hippocampus.

These two sets of experiments have demonstrated that the pituitary contains binding sites of the GABA/benzodiazepine complex which are indistinguishable from those in the CNS, but the inverse distribution between PD and NI clearly shows that the two types of site are not ubiquitously linked in a constant ratio. It appears that PD contains a considerable population of benzodiazepine-independent GABA sites.

Preliminary experiments to investigate the interaction of benzodiazepines with GABA-mediated inhibition of prolactin secretion in vitro revealed a novel $GABA_A$ receptor-mediated effect: prolactin secretion was transiently stimulated, with subsequent sustained inhibition (Chapter 5). Both effects were produced by a number of $GABA_A$ agonists in a concentration-dependent manner with the exception of homocarnosine which at concentrations up to 2mM caused no stimulation of secretion. The rigid GABA analogues P4S, THIP, isoguvacine and APS were found to cause less stimulation than that caused by GABA, but very similar inhibition. These experiments provided evidence that the biphasic effect of $GABA_A$ receptor activation was more complex than originally thought, and that the two components might be independent. The model proposed by Taraskevich and Douglas (1982) was clearly not appropriate to a selective agonist such as homocarnosine, but extrapolation from melanotrophes to lactotrophes may be misleading and quite distinct GABA-evoked events may occur in this cell type. Experiments with antagonists provided further evidence that the two components of the biphasic response to muscimol were mediated by separate populations of $GABA_A$ receptors: BMI was found to antagonise both effects approximately equally, but PTX and strychnine selectively antagonised the stimulatory effect, only antagonising the inhibitory effect at very high concentrations. Conversely, glycine was found to selectively potentiate the stimulatory effect of muscimol. It therefore appears that homocarnosine and strychnine are selective drugs for the inhibitory and stimulatory effects respectively. With respect to the $GABA_A$ site mediating stimulation of prolactin secretion, the potency of strychnine as an antagonist and the

ability of glycine to potentiate the effect of muscimol in the absence of any direct effect, suggest that there may be a glycine receptor interacting in a modulatory fashion in some molecular complex with this GABA receptor and a chloride ion channel. Analogies may be drawn with the way in which benzodiazepines and barbiturates modulate GABA receptor function, though it is clear in this case, that the modulator acts with considerable basal tone. GABA and glycine receptors are generally independently linked to chloride channels (Hamill, Bormann and Sakmann, 1983) and similar modulatory effects have not been previously reported. The molecular nature of these interacting GABA and glycine recognition sites here is not apparent but is clearly of interest. If the two $GABA_A$ responses described here are indeed independent, then they may have separate physiological function. GABA, being active at both sties, will cause both effects, but this may be modulated by homocarnosine under certain conditions. If GABA was released into hypophysial portal blood in short bursts of high concentration, then the effect would be to stimulate prolactin secretion. More prolonged release would tend to inhibit secretion, and the continued presence of GABA (with consequent effects on membrane resistance) might also affect lactotrophe responsiveness. Responses to GABA are often limited by desensitisation, but the inhibitory effect measured here showed no tendency to decline over the time-course examined.

Baclofen had no effect on basal secretion of prolactin, or on K^+ or Ba^{2+}-stimulated secretion. It did, however, inhibit VIP-stimulated secretion in a stereospecific manner. Experiments to investigate whether this was a result of inhibition of adenylate cyclase proved inconclusive, as no effect of baclofen could be

demonstrated on tissue treated with drugs which affect cAMP concentrations. An interaction with K^+ channels, as demonstrasted in the hippocampus, was also investigated using 4AP and TEA. Both these drugs caused a stimulation of prolactin secretion, and the stimulation evoked by 4AP but not TEA was reduced by baclofen (stereospecifically). Based on the selective properties of 4AP and TEA, an interaction between $GABA_B$ receptors and transient K^+ channels, with properties possibly similar to I_A was proposed. The considerable importance of I_A in regulating membrane excitability suggests that these $GABA_B$ receptors may be of importance in the control of prolactin secretion particularly by affecting responsiveness to other PIFs and PRFs. It was tentatively suggested that this may be part of the mechanism of the interaction observed between VIP and baclofen. Substance P has PRF activity and affects K^+ currents, as does TRH. It is therefore possible that $GABA_B$ receptor effects on K^+ currents may be an important postsynaptic mechansim of regulation of peptide responses and indeed may not be necessarily confined to the pituitary.

The effects and mechanism of GABA-linked benzodiazepine receptors demonstrated in PD by radioligand binding studies were investigated using prolactin secretion as a model in Chapter 6. Clonazepam and Ro11-6896, but not Ro5-4864, potentiated the stimulatory effect of muscimol on prolactin secretion. No effect on the inhibitory part of the response was observed, and the effect of clonazepam was antagonised by Ro15-1788. Secobarbital also selectively potentiated the stimulatory response. These results demonstrate the presence of $GABA_A$ receptors linked to central-type benzodiazepine- and barbiturate-recognising components at a

non-synaptic location, not confined to the CNS. These results are of interest with relation to the binding studies carried out in Chapters 3 and 4, in which the existence of benzodiazepine-linked and -independent GABA receptors was suggested on the basis of their differential distribution. The results on prolactin secretion suggest that both types are present on lactotrophes, but have different effects, and provides further pharmacological characteristics of the two sites/effects. A peptide termed 'diazepam-binding inhibitor' (DBI) has been isolated from rat brain and characterised as having proconflict action, and DBI-immunoreactivity was found to be especially dense in MBH (Costa, Ferrero, Alko and Guidotti, 1985). If DBI, or a fragment, is an endogenous ligand for benzodiazepine receptors, then this system may be of particular value in the investigation of endogenous modulation of GABAergic transmission as it is easily accessible in vivo and in vivo, as well as being of physiological importance. Experiments addressing the molecular mechanism of benzodiazepine modulation of $GABA_A$ receptors were also carried out using this model. Clonazepam was found to reduce the potency of BMI as an antagonist of the effect of muscimol on prolactin secretion, but had no effect on the potency of PTX. This was interpreted as indicating an effect of clonazepam on the $GABA_A$ receptor itself, enhancing its affinity or the number of available receptors but not affecting the molecular linkage between receptor and ion channel.

GABAergic agonists were also found to effect the secretion of GH, LH and ACTH but not TSH in vitro (Chapter 7). Muscimol stimulated the secretion of GH, LH and ACTH: this effect was transient in each case, and its magnitude varied greatly between the

different hormones, being greatest for GH but only slight for LH. The possibility exists that the slight effect on LH secretion was indirect, perhaps mediated by elevated extracellular K^+. This would require cell purification for testing, and was not attempted. If the effect was indirect, this would not obviate any physiological importance but might reflect aspects of the organisation of PD i.e. relative distribution of the cell types within the gland, and electrical coupling between them. In no case was the secretion of the hormones inhibited by muscimol, secretion rates rapidly returned to pre-drug in the continued presence of muscimol, and the responses therefore differed from the biphasic prolactin response. GABA may contribute to the pulsatile nature of GH secretion, and the existence of GH-releasing factors other than GHRH has been recently proposed (Thomas, Groot and Arimura, 1985). In that study a transient increase in GH secretion in response to antisomatostatin antiserum was observed even after previous injection of antiGHRH antiserum. Part of this may be caused by GABA. In the continued presence of GABA, the responsiveness of the various cell types to other releasing/inhibitory factors might be altered despite secretion having returned to basal, but this was not investigated although it may be of considerable physiological importance. The effect of muscimol on GH secretion was demonstrated to be concentration-dependent, and antagonised by BMI but not by strychnine. GABA mimicked the effect of muscimol, but homocarnosine had no effect alone and did not affect the response to GABA. The effect of muscimol was potentiated by both Ro11-6896 and secobarbital.

The stimulatory $GABA_A$ receptor effects on prolactin and GH therefore show both similarities and differences in their pharmacology: both are linked to benzodiazepine/barbiturate sites, and both are unaffected by homocarnosine (although experiments described in Chapter 5 suggested some antagonistic action of homocarnosine on the stimulation of prolactin secretion). The major difference is that the prolactin response is antagonised by strychnine (and potentiated by glycine) whilst the GH response is not. The basic properties of the $GABA_A$ receptors on the two cell types are unlikely to be different, despite this, although the various agonists investigated on prolactin secretion would need to be tested on GH secretion for confirmation. The presence of different modulatory components may conformationally modify them in such a way as to alter the affinity for some agonists. If the interpretation of the glycine/strychnine effects on prolactin secretion discussed above is correct, then such modulatory sites would appear to be absent from somatotrophes. The effect of glycine on GH secretion was not investigated. Considerable numbers of PD cells appear to secrete both prolactin and GH (Frawley et al, 1985) and the selective inhibitory effect on prolactin secretion may therefore involve a separate (non GH-secreting) cell type or otherwise differential intracellular controls could operate.

Baclofen had no effect on basal secretion of any hormone, and did not affect Ba^{2+}-stimulated secretion of GH or TSH. It did, however, reduce K^+ and Ba^{2+}-induced secretion of ACTH and both $GABA_A$ and $GABA_B$ effects could be demonstrated on LHRH-stimulated LH secretion: $GABA_A$ receptor activation enhanced secretion, and $GABA_B$ receptor activation reduced secretion. GABA itself appeared

to act on both types of receptor: the $GABA_B$ effect predominated at higher LHRH concentrations, but at lower LHRH concentrations a $GABA_A$ response was observed, and these results suggest that GABA may regulate gonadotrophe sensitivity in vivo. As discussed above, the $GABA_A$ effect may be indirect, or may be the result of a paracrine interaction. Dynorphin appears to coexist in gonadotrophes (Knepel and Schwaniger, 1985) but no effects in PD have been described. Other, undiscovered, peptides may also exist in PD cells, which may regulate the effects of the large number of substances reported to affect PD hormone secretion. It therefore appears that lactotrophes, gonadotrophes and corticotrophes have both $GABA_A$ and $GABA_B$ receptors, somatotrophes have only $GABA_A$ receptors and thyrotrophes are devoid of GABA receptors.

The properties of GABA-releasing neurone terminals in ME and NI were studied using a crude synaptosomal preparation (Chapter 8). [^{3}H]GABA was rapidly accumulated by tissue in a time-, temperature- and Na^+-dependent manner. In both areas, the uptake carrier showed predominantly neuronal (rather than glial) specificity. Kinetic analysis demonstrated that the carrier in both areas had a K_m value of $\simeq$5μM, but the V_{max} value in ME was 20x greater than that in NI. Experiments in which muscimol reduced the effect of 15mM K^+, in a BMI-sensitive manner, suggested the existence of $GABA_A$ autoreceptors in both areas. A variety of neurotransmitters and hormones (DA, 5HT, αMSH, prolactin, vasopressin) had no effect on basal or stimulated release suggesting a lack of direct feedback control. These terminals in ME and NI therefore appear to have kinetic and autoregulatory characteristics identical to those of other GABAergic terminals, despite their

relatively specialised roles, in contrast to dopaminergic terminals in ME which have a lower-affinity uptake system than dopaminergic systems elsewhere and may not be subject to autoregulation. This may suggest that perhaps the majority of the GABAergic terminals in ME investigated here are involved in local, synaptically-mediated control rather than secretion into hypophysial portal blood, but this is not supported by the morphological evidence (Tappaz et al, 1983) It therefore appears that even neurosecretory GABAergic terminals do not have specialised properties.

Stimulated release of [^{3}H]GABA from ME but not NI was inhibited by baclofen, in a concentration-dependent and sterospecific manner (Chapter 9). Release was reduced to ≈65% of control by 100μM (±) or (-) baclofen, and 100μM (+) baclofen had no effect. Attempts to use δAVA as a $GABA_B$ antagonist were equivocal as this drug caused some heteroexchange, but it appeared to antagonise the effect of baclofen. These results suggest that GABAergic terminals in ME have inhibitory $GABA_B$ autoreceptors in addition to $GABA_A$ autoreceptors. This is the first demonstration of a $GABA_B$ autoreceptor, although presynaptic $GABA_B$ receptors have been demonstrated on the terminals of a variety of other neurotransmitters. The precise location and function of these receptors is unknown. Their distribution within ME could be determined by autoradiographic binding studies, perhaps in conjuction with lesions of the acuate nucleus (the proposed origin of the GABAergic innervation of ME) in an attempt to discriminate presynaptic from postsynaptic locations. A comparison with the distribution of $GABA_A$ receptors (both autoreceptors and on terminals releasing other transmitters) would also be of

considerable interest, but it might be difficult to get unequivocal data from such studies. One fundamental question is whether $GABA_A$ and $GABA_B$ autoreceptors coexist on one terminal. $GABA_A$ autoreceptors are likely to be of greater importance (as deduced from the magnitude of inhibition caused by various agonists), but this may merely reflect amore widespread distribution. The two types of autoreceptors may be associated with different population of GABAergic neurone, for example one type being associated with GABAergic control of some transmitters/neurohormones and the other with control of neurohaemal secretory GABAergic terminals. The presence of $GABA_B$ autoreceptors may reflect specialisation of a population of neurones, but their function is likely to remain elusive without the discovery of a potent and specific antagonist.

The effect of opioids on release of [^{3}H]GABA from NI was investigated in Chapter 10, to examine the possible function of dynorphins in that region. $Dynorphin_{1-13}$ caused a marked inhibition of stimulated release, an affect which was concentration-dependent and antagonised by naloxone. This effect was mimicked by the κ-selective agonist U50488H, but δ- and μ-selective peptides had no effect: GABAergic terminals in NI therefore appear to have only κ receptors, unlike dopaminergic and peptidergic terminals which have been reported to be influenced by δ/μ opioids. The effect of $dynorphin_{1-13}$ was prevented by superfusion with low-chloride medium, or medium containing DIDS but was unaffected by the K^+ channel blockers 4AP, TEA and quinine. $Dynorphin_{1-13}$ had no effect on Ba^{2+}-induced release, but potentiated the secretagogue effect of low-chloride medium. These results suggest that κ opioid receptors act by opening anion channels, unlike δ and μ receptors

which open K^+ channels and provides a function for the very high concentrations of dynorphin peptides in PN. The coexistence of dynorphin with vasopressin in PN suggests that this effect may be of importance in regulating the interaction between GABA and vasopressin, as a local feedback control. There are two conclusions suggested by the ionic dependence of the effect of dynorphin:K receptors may be ubiquitously linked to chloride channels, but this seems unlikely as there is evidence for an interaction with Ca^{2+} channels (Werz and Macdonald, 1984), or alternatively this may be a tissue-specific or sub-type-specific property. If this is the case, it might be related to the fact that the location is GABAergic terminals, and the increase in intracellular chloride concentration might inhibit GAD activity as well as GABA release thus increasing the effectiveness and duration of inhibition in vivo.

The interaction between GABAergic and dopaminergic tuberoinfundibular and tuberohypophysial systems was further investigated in experiments on the effects of GABA agonists [^{3}H]DA release from ME and NI (Chapter 11). Muscimol inhibited stimulated release from ME but not NI, and this was antagonised by BMI but not strychnine. Homocarnosine also inhibited release from ME, again in a BMI-sensitive manner, but baclofen had no effect in either area. The GABA receptor involved therefore appears to show similar pharmacological characteristics to the receptor associated with inhibition, but not stimulation, of prolactin secretion. Strychnine and homocarnosine therefore appear to be of use in investigating $GABA_A$ receptor pharmacology and may provide the basis for a putative subclassification of $GABA_A$ receptors. As DA in ME is involved in the control of the release of releasing factors into

hypophysial portal blood, in addition to its own release into portal blood, this may be the mechanism of action of some of the effects of GABA on hormone secretion observed in vivo, particularly the frequently reported stimulation of prolactin secretion following i.c.v. injection of GABA.

In several Chapters in this Thesis, the pharmacological properties of homocarnosine with respect to GABA receptor mediated effects have been demonstrated. In addition to being a direct, though weak $GABA_A$ agonist, homocarnosine may also be a source of GABA, if PD contains considerable amounts of the enzyme homocarnosinase. Experiments were carried out to investigate the presence of this enzyme in PD (Chapter 12), although previous reports had been unable to detect any activity in rat brain. No homocarnosinase was detectable, suggesting that any endocrine function of this substance at the PD is directly attributable to it and not to its conversion to GABA. The origin and fate of the high concentration of homocarnosine reported in hypophysial portal blood remain unknown, but the demonstration of GAD-IR and histidine-decarboxylase-IR in magnocellular neurones in the hypothalamus provides an intriguing possibility that these may be a major source of the molecule.

There is evidence that GABA is involved in the control of vasopressin secretion, and that Brattleboro rats, which are genetically unable to synthesise vasopressin, have some deficit of the GABAergic innervation of NI. This was investigated by measuring $GABA_A$ binding sites and GAD activity in NI (Chapter 13). No change in $GABA_A$ binding sites in Brattleboro rats compared to Long-Evans controls was detectable, but GAD activity was

substantially reduced. This deficit appeared to be confined to PN, with PI having normal GAD activity. GAD activity in ME was unaltered from controls. As GAD is the rate limiting enzyme in the synthesis of GABA, these results suggest that GABAergic transmission in PN is compromised. The relationship between this deficit and the absence of vasopressin in these animals is unknown but an association between the two is possible. The GAD deficit may be a consequence of atrophy through inactivity, excessive and prolonged inhibition by dynorphin, and/or changes in the water balance and hormonal alterations in the Brattleboro rats. These results also suggest that the Brattleboro rat is not merely an animal model of vasopressin deficiency but represents a more complex alteration of PN function.

14.2 Conclusion

The present results provide further evidence that GABA may be a neuroendocrine transmitter or neurohormone, acting directly on pituitary cells, particularly in PD. The importance of GABA in the CNS as the major inhibitory neurotransmitter may be reflected in the pituitary but our knowledge is at present insufficient to identify the precise nature of any tonic or phasic physiological roles. The absence of detailed knowledge about the functional organisation of PD is perhaps one of the more obvious stumbling blocks, as well as the difficulty of investigation of the function of ME without resort to traumatic manipulations of the physiological state of the animal. The present experiments have also demonstrated the widespread occurrence of the GABA/benzodiazepine/barbiturate 'receptor' association, here at non-synaptic sites, and provide convenient *in vitro* models for the detailed study of their interaction.

The importance of GABA in neuroendocrine control is suggested by the dense innervation of ME and NI, and the effect of GABA on the secretion of virtually every pituitary hormone. These effects should be seen in the context of peptidergic and aminergic control, but on the basis of experiments carried out in this Thesis and by other groups it is now clear that neuroendocrine control by GABA itself can indeed be complex with not only multiple sites of action but also multiple receptor types being involved. Thus for example GABA may act both on dopaminergic terminals in ME and on lactotrophes, both sites contributing to the control of prolactin secretion. The widespread effects on PD hormone secretion imply that GABA does not have just a specific function in controlling the secretion of one hormone, but may be of broader importance in regulating the sensitivity of PD as a whole to other releasing factors. If this was the case, then elucidation of the role played physiologically by such an action would be rather difficult, requiring intervention with several transmitter/hormone systems at once to establish their functional interaction.

Nevertheless, the following comments can be made:

i)The hypothalamo-hypophysial system may therefore be, at least in PD, an example of GABAergic transmission that does not involve point-to-point regulation in well-defined anatomical circuits but a more generalised influence. The same does not necessarily apply to NI (or indeed to effects within ME) where a 'classical' function seems more likely.

ii) GABA prevented the generation of action potentials in melanotrophes for as long as exposure was maintained (Taraskevich and Douglas, 1982). The same may well be true for somatotrophes and

corticotrophes, in which case the transient effect on secretion may be of little physiological importance, but an ensuing low membrane resistance would limit the secretagogue effects of substances which acted by alterations of cellular electrical activity. This effect of GABA on the electrical properties of the cell may be independent of the actions of other substances that act by way of chemical second messengers such as cyclic AMP, $Ca2^{+}$ or inositol phosphates.

iii) The inhibitory effect of GABA on prolactin secretion suggests a different physiological function to its effects on the electrical properties of other cell types, although the same comments may apply to the stimulatory effect. This inhibitory effect may itself be of direct physiological importance, as an adjunct to dopaminergic inhibition.

iv) The interaction of GABA with the response to LHRH demonstrates a possible physiological role of GABA, having both stimulatory and inhibitory effects, at different concentrations. The responsiveness of the anterior pituitary gland to LHRH varies considerably (as much as 150 fold) in different physiological states, and conceivably these differences can be brough about, at least in part, by the modulatory action of GABA. Indeed, steroid treatment has been demonstrated to alter GABA binding sites in PD. GABA might therefore contribute to the initiation yet limit the magnitude of the prooestrous LH surge.

In conclusion, these results provide a systematic neurochemical basis for hypotheses on the control or modulation of pituitary function by GABA and establish how broad ranging and multifacetted this action is. The new evidence provided should permit the design of further experiments to establish under which conditions GABA is of physiological importance in the control of the pituitary, and what its precise functions are in those circumstances.

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Appendix I Radioimmunoassays for prolactin GH, LH and TSH.

I.1 Stock solutions

0.01M phosphate buffered saline (PBS)

8.17g NaCl
0.1g Na merthiolate
0.25g $NaH_2PO_4 . 2H_2O$
1.193g Na_2HPO_4 anhydrous
per litre distilled water, pH 7.6

Antiserum buffer

500ml 0.01M PBS
9.306g ethylenediamine tetra-acetic acid disodium salt
1.7ml normal serum: rabbit for prolactin, LH and TSH
human for GH
pH 7.0

Assay buffer (0.01M PBS/1% BSA)

1l 0.01M PBS
10g bovine serum albumin

0.01M Borate buffer (for prolactin iodination)

0.618g boric acid
0.1g Na merthiolate
11.8ml 0.1M NaOH
per litre distilled water, pH 8.6

0.05M phosphate buffer

1.015g NaH_2PO_4 $2H_2O$
6.175g Na_2HPO_4 anhydrous
per litre distilled water, pH 7.6

Rinse for prolactin iodination

0.18g KI
0.8g sucrose
10ml distilled water

I.2 Iodination

Columns: Prolactin. 25 x 1cm Sephadex G50 coated with 200µl human albumin (4.5% in PBS). Eluted with 0.01M borate buffer.
GH. 20 x 1cm Sephadex G50 coated with 200µl human albumin (4.5% in PBS). Eluted with 0.05M Na barbitone buffer (pH 8.6).
LH. 12 x 1cm column Biogel G60 coated with 1.5ml egg white (5% in PBS). Eluted with 0.01M PBS.
TSH. 30 x 1cm column Sephadex G-75 coated with 4ml PBS/2% BSA. Eluted with PBS.

Hormones: Prolactin. Rat-PRL-I-S, 20µl aliquots of 250µg/ml in 0.01M $NaHCO_3$.
GH. Rat-GH-I-4, 50µl aliquots of 100µg/ml in 0.01M $NaHCO_3$
LH. LER-1056-C2, 10µl aliquots of 500µg/ml in 0.01M PBS.
TSH. Rat-TSH-I-6 20µl aliquots of 250µg/ml in 0.25M phosphate buffer.

$Na^{125}I$: 1mCi in 10µl for each iodination

Chloramine T: Prolactin: 1mg/ml in 0.05M phosphate buffer. 15µl used.
GH: 0.5mg/ml in H_2O. 10µl used.
LH: 5mg/ml in 0.01M PBS. 10µl used.
TSH: 1.5mg/ml in 0.05M phosphate buffer. 20µl used.

Sodium metabisulphate: Prolactin: 2.4mg/ml in 0.05M phosphate buffer. 50µl used.
GH: 1.2mg/ml in H_2O. 10µl used.
LH: 5mg/ml in 0.01M PBS. 25µl used.
TSH: 2.4mg/ml in 0.05M phosphate buffer 50µl used.

Protocol
I) Add 25µl 0.5M phosphate (pH 7.5) to hormone
II) Add 1mCi $Na^{125}I$
III) Add Chloramine T, mix gently.
IV) Reaction times: prolactin 20sec
GH 25sec.
LH 2 min
TSH 40sec
V) Add $Na_2S_2O_5$, mix gently
VI) Transfer to column
Prolactin: rinse vial with 2 x 100µl KI/sucrose solution.
VII) Elute with buffer.
Prolactin: borate buffer
GH, LH and TSH: 0.01M PBS
VIII) Collect fractions: 0.5ml into 0.5ml PBS/1% BSA for prolactin, GH and TSH. 0.5ml into 0.5ml 0.01M PBS/5% egg white for LH.
Estimate radioactivity per fraction. Retain peaks.

In all cases, iodinated hormone eluted before free iodine.
Prolactin and TSH were only used on the day of iodination, GH and LH could be used up to 20 days later following rechromatography.

I.3 Standards

Prolactin: rat-PRL-RP-1. 0.5, 1, 2, 4, 8, 16, 32, 64ng/ml in 0.01M PBS/1% BSA. Stored as 200µl aliquots.
GH: rat-GH-RP-1. 0.5, 1, 2, 4, 8, 16 32ng/ml in 0.01M PBS/1% BSA.
LH: NIH-LH-S18. 0.25, 0.5, 0.75, 1.2, 2, 5, 8, 16ng/ml in 0.01M PBS/1% BSA.
TSH: rat-TSH-RP-2. 0.3, 0.8, 1.2, 2, 5, 10, 20, 50ng/ml in 0.01M PBS/1%BSA.

I.4 Assay timetable

All incubations were carried out at 4°C

Day 1.	All assays:	200µl samples/standards. 200µl 0.01M PBS/1% BSA 200µl antiserum in buffer
Day 2.	All assays:	200µl ^{125}I-hormone in PBS/1% BSA ≃ 10,000 cpm for prolactin, LH and TSH ≃ 5,000 cpm for GH
Day 3.	GH only:	200µl AHGG in 0.01M PBS
Day 4	GH only:	Centrifuge tubes (30 min at 2000g, 4°C) aspirate supernatant and count pellet.
	Prolactin and LH:	200µl ARGG in 0.01M PBS
Day 5.	Prolactin and LH:	Centrifuge, aspirate, count.
Day 6.	TSH:	200µl ARGG in 0.01M PBS
Day 7	TSH:	Centrifuge, aspirate, count

Appendix II Radioimmunoassay for ACTH

II.1 Assay buffer

63mM Na_2HPO_4
13mM Na_2 EDTA
0.02% NaN_3
0.1% Triton X-100
250KIU/ml Trasylol (Bayer)
3.5% silicic-acid-cleaned BSA: 100mg SIL-A-200, 60-200mesh screen (Sigma) per ml, mixed for 30 min at 20°C, centrifuged at 2400rpm for 10 min. Collect supernatant.

Antiserum buffer

As assay buffer.

II.1 Iodination

The method used was based on Harmar and Rosie (1983). A HPLC apparatus consisting of a two channel pump in series with a three way valve, a sample injection valve and a disposable cartridge of bonded phase cyanopropyl (CN) silica was used.

Solvent A: 0.2% Trifluoric acid (TFA) in H_2O
Solvent B: 0.2% TFA in methanol

A solvent gradient from 100% solvent A to 100% solvent B was generated by changing the proportions of the two solvents delivered to the column

Hormone: Human-ACTH (NIADDK), 10µl of 200µg/ml in 0.01M HCl
Chloramine T: 1mg/ml 0.05M phosphate buffer.
Sodium metabisulphite: 25mg/ml 0.05M phosphate buffer.

Protocol:
I) Add 10µl 0.25M phosphate buffer to hormone
II) Add 1mCi $Na^{125}I$.
III) Add 10µl Chloramine T. Mix.
IV) Reaction time: 20 sec
V) Add 10µl sodium metabisulphite
VI) Add 180µl 0.25M phosphate buffer
VII) Add 620µl 10mg/ml KI/0.2% TFA
VIII) Inject into HPLC
IX) Elute at 1ml/min.
X) Label could be stored at -80°C for 3 weeks.

Collect 6 1min fractions for each gradient step: 0%, 50%, 60%, 100% methanol. ^{125}I-ACTH was found to elute with 60% methanol.

II.3 Standards

Human-ACTH (NIADDK). 3.1, 6.25, 12.5, 25.0, 50, 100 and 200pg/ml in assay buffer. Stored at -40°C in 100µl aliquots.

II.4 Primary antibody

IgG-ACTH-1 (IgG Corporation, Nashville, TN) diluted 1:10,000 in assay buffer + 1% normal rabbit serum.

II.5 Assay timetable

Day 1. 100µl samples/standards
100µl antiserum in buffer
Incubate at room temperature

Day 2. 100µl ^{125}I-ACTH in buffer
≃ 10,000 cpm
Incubate at room temperature for 8 hr.
100µl ARGG in 0.01M PBS
Incubate at 4°C

Day 3. Add 1.6ml of PBS/3% BSA
Centrifuge, aspirate and count

Appendix III Protein assay

All protein assays in this Thesis were carried out according to this method (Geiger and Bessman, 1972).

Reagents

0.1g $CuSO_4$ $5H_2O$
0.54g NaK-D-tartrate
made up to 20ml, distilled water

4g NaOH
20g $NaHCO_3$
made up to 200ml, distilled water

Alkaline copper reagent is prepared by mixing the above two solutions together.

Protocol: Standards (0–20µg) of bovine serum albumin and samples (5–20µl) are made up to 200µl with distilled water in LP3 tubes.
500µl alkaline copper reagent is added and mixed well.
Tubes are left for 20 min
1ml of diluted Folin-Ciocalteau reagent is added (1:10 in distilled water) and tubes are rapidly mixed.
After a further 20 min, absorbance is read at 725nm in a spectrophotometer and a standard curve is constructed.
Samples are read off the standard curve.

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Multiple receptors for γ-aminobutyric acid (GABA) in rat pituitary

By R. A. Anderson and R. Mitchell (introduced by G. Fink). *M.R.C. Brain Metabolism Unit, 1 George Square, Edinburgh*

Gamma-aminobutyric acid (GABA) may be involved in the regulation of hormone release from the pituitary gland (Racagni, Apud, Cocchi, Locatelli & Muller, 1982; Vincent, Hokfelt & Wu, 1982). Specific binding sites for GABA have been described in anterior pituitary (Grandison & Guidotti, 1979) but the contribution of different subtypes and their distribution within the gland has not been described.

Pituitary glands were removed from freshly killed adult male Wistar rats, dissected into anterior (AP) and neurointermediate (NI) lobes and stored at −20 °C. Tissue was homogenized and centrifuged in 100 vol. of cold buffer at 48000 g for 10 min, resuspended in fresh buffer and frozen. Before assay the membranes were washed a further four times. [^{3}H]GABA was used as a radioligand for both $GABA_A$ and $GABA_B$ receptors in Tris HCl buffer (50 mM, pH 7·1) containing 2·5 mM-$CaCl_2$ (Bowery, Hill & Hudson, 1983). [^{3}H]muscimol was used to label selectively $GABA_A$ sites in Ca^{2+}-free Tris buffer. Assays were terminated by centrifugation and pellets washed superficially before measurement of bound ligand. Specific binding of 10 nM [^{3}H]GABA to pituitary membranes showed a differential distribution of both A and B type sites, discriminated in the presence of 100 μM (±) baclofen ($GABA_A$: AP, 20·9 ± 1·7; NI, 5·5 ± 0·7; and $GABA_B$: AP, 16·5 ± 1·8; NI, 0·6 ± 1·4 fmol/mg protein; $n = 5$). Experiments using [^{3}H]muscimol at concentrations of 2 and 20 nM to label predominantly high- and low-affinity components of $GABA_A$ sites confirmed a relative concentration of $GABA_A$ sites in AP, with 9·5 ± 0·8 and 21·3 ± 1·2 fmol/mg protein ($n = 5$), respectively, but only 3·0 ± 0·2 and 6·3 ± 0·4 fmol/mg protein ($n = 4$) in NI. Scatchard analysis of [^{3}H]muscimol binding to pituitary membranes revealed two components of similar affinity to c.n.s. regions; K_{D1}, 1·1 ± 0·1 nM, B_{m1} 9·4 ± 0·4 fmol/mg protein and K_{D2} 34·7 ± 3·0 nM, B_{m2} 46·8 ± 6·7 fmol/ml protein ($n = 3$). This relatively large contribution of low affinity sites was found to be similar to that in frontal cortex but not cerebellum.

These results show the presence of moderate concentrations of both $GABA_A$ and $GABA_B$ receptors in AP but only a minor population of $GABA_A$ and no detectable $GABA_B$ receptors in NI.

R.A.A. is a Houldsworth scholar.

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$GABA_A$ receptors have a biphasic effect on prolactin release *in vitro* from anterior pituitary of the rat

By R. A. Anderson and R. Mitchell. *MRC Brain Metabolism Unit, 1 George Square, Edinburgh*

Gamma-aminobutyric acid (GABA) has been reported to inhibit the release of prolactin by a direct action on the pituitary both *in vitro* and *in vivo* (Schally, Redding, Arimura, Dupont & Linthicum, 1977). We have re-examined control by GABA of prolactin release from the anterior pituitary using a sensitive rapid superfusion technique which allows the measurement of transient effects.

Anterior pituitary (AP) glands from male Wistar rats (200–300 g) were chopped into 500 μm prisms and incubated for 1 h at 37 °C in oxygenated Krebs bicarbonate medium, pH 7·4, containing 0·1 % BSA, 2 g/l glucose and 30 μg/l bacitracin. Tissue from two pituitary glands was then superfused with medium at 0·5 ml/min. Fractions (2 min) were collected serially and prolactin was measured by radioimmunoassay. After 90 min the potent $GABA_A$ receptor agonist muscimol was added to the medium, and produced two effects which were both concentration-dependent from 10 nM to 100 μM; a rapid prominent stimulation of release, with a peak at 6–8 min, followed by a decline to a steady reduced rate of release within 30 min. The inhibitory effect was maximal (-51 ± 3 %; $n = 5$; mean $\pm$ S.E. of the mean) at 10 μM. The stimulatory component increased further with a peak increment of 117 ± 13 % at 100 μM ($n = 5$), and was inhibited by (+)bicuculline methiodide (BMI) and picrotoxinin in a concentration-dependent manner throughout the range 1–100 μM. The stimulatory effect of 10 μM muscimol was reduced from $+65 \pm 10$ % to $+24 \pm 5$ % and the inhibitory effect from -51 ± 3 % to -34 ± 2 % by 10 μM-BMI ($n = 5$, $P < 0{\cdot}05$ in each case). The antagonists alone had no effect on prolactin release. By contrast the established prolactin release inhibitory factor dopamine produced only inhibition of secretion (-71 ± 5 % at 0·1 μM, $n = 3$).

These results demonstrate a biphasic influence of $GABA_A$ receptors on prolactin release similar to the effects described on αMSH release (Tomiko, Taraskevitch & Douglas, 1983). The electrophysiological events accompanying these phenomena in melanotrophes (Taraskevitch & Douglas, 1982), which are likely to underly the changes in hormone release, may well be paralleled in lactotrophes. It is possible that a transient elevation of the output of several hormones may be a physiologically important aspect of pituitary response to GABA.

R.A.A. is a Houldsworth Scholar of the University of Edinburgh.

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Homocarnosine may selectively activate one of two $GABA_A$ receptor subtypes on lactotrophes in the rat

By R. A. Anderson and R. Mitchell. *M.R.C. Brain Metabolism Unit, 1 George Square, Edinburgh*

The GABA-containing dipeptide homocarnosine (HC) is found in high concentrations in hypophysial portal blood and has been shown to have moderate affinity for $GABA_A$ receptors (Mitchell, Grieve, Dow & Fink, 1983). We have investigated the effects of HC on prolactin secretion from an adenohypophysial slice preparation, which has previously been used to demonstrate a biphasic influence of $GABA_A$ receptors on lactotrophes (Anderson & Mitchell, 1984).

Anterior pituitary glands from male Wistar rats were prepared and superfused as previously described (Anderson & Mitchell, 1984). GABA produced a transient stimulation followed by prolonged inhibition of prolactin secretion both of which occurred throughout a similar concentration range. The $GABA_B$ receptor agonist (±) baclofen (100 μM) (Hill & Bowery, 1981) had no effect on basal secretion or on that induced by K^+ or Ba^{2+}. Muscimol was approximately tenfold more potent than GABA in both effects, but other $GABA_A$ receptor agonists which were equipotent with GABA at inhibiting prolactin secretion were markedly less potent at stimulation. The most selective amongst these was HC, which appeared to be totally specific for the site mediating inhibition, up to the highest concentration tested (2 mM-HC, 31 ± 2% inhibition, no significant stimulation).

This effect of HC was mediated by a $GABA_A$ receptor since, like both effects of GABA, it was sensitive to bicuculline methiodide (1 mM-HC, 28 ± 4% inhibition; 1 mM-HC with 10 μM bicuculline methiodide, 17 ± 2% inhibition; $P < 0{\cdot}05$, $n = 5$). It seems unlikely that the effect of HC may be partly due to its degradation to GABA by the anterior pituitary, since we were unable to detect any homocarnosinase activity (Lenney, Kan, Siu & Sugiyama, 1977) in the gland, in contrast to the kidney which showed a high activity of 9 μmol g^{-1} h^{-1}.

Although HC is approximately 100-fold less potent than GABA, its presence in high concentrations and its selectivity for one of two $GABA_A$ receptor-mediated effects suggest that it may be an important factor in the GABA-mediated control of anterior pituitary function.

R.A.A. is a Houldsworth Scholar of the University of Edinburgh.

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Presynaptic autoreceptors inhibit [^{3}H]GABA release from neurone terminals in the median eminence of the rat

By R. A. Anderson and R. Mitchell. *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ*

A role for γ-aminobutyric acid (GABA) in the control of anterior pituitary hormone secretion is supported by both autoradiographic and immunohistochemical studies, indicating a plexus of GABA neurone terminals in the hypothalamic median eminence (ME) (Tappaz, Aguera, Belin & Pujol, 1980; Vincent, Hokfelt & Wu, 1982). The present studies describe the characteristics of the uptake and release of [^{3}H]GABA by ME *in vitro*.

Tissue from male Wistar rats was homogenized in 0·32 M-sucrose before dilution into physiological medium (pH 7·4) (Mitchell & Martin, 1978). Uptake experiments were performed at 25 °C. For release experiments (at 37 °C), tissue was incubated with 50 μM-[^{3}H]GABA (including 10 μM-amino-oxyacetic acid) for 15 min and then superfused at 0·5 ml/min while collecting 1 min fractions.

[^{3}H]GABA was accumulated with an affinity (K_m) of 5·0 μM. β-alanine (1 mM) inhibited uptake into ME by only 18%, while L-2,4-diaminobutyric acid (500 μM) inhibited uptake by 56%, indicating that uptake in ME is mainly neuronal. Basal efflux of [^{3}H]GABA was around 0·5% of tissue content/min and was stimulated by elevated K^+ concentrations in a Ca^{2+}-dependent manner. The peak response to 15 mM-K^+ (154 ± 10% increase), was inhibited by 100 nM-muscimol (114 ± 8%, $P < 0·025$) and this was antagonized by 10 μM-bicuculline methiodide (135 ± 3%, $P < 0·02$ compared to muscimol), (mean ± S.E.M., $n = 4$ or 5, in each case).

These results indicate that neurosecretory GABA neurone terminals in ME have characteristics like those elsewhere in the CNS, including the presence of GABA receptors (Mitchell & Martin, 1978). These sites may regulate the secretion of GABA into hypophysial portal blood (which can be elicited *in vivo* by electrical stimulation of ME; Mitchell, Grieve, Dow & Fink, 1983), and thereby contribute to physiological control of anterior pituitary function.

R.A.A. is a Houldsworth Scholar of the University of Edinburgh.

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GABA-related anion channels, labelled by [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]TBPS), in rat pituitary

By R. A. Anderson, R. H. McAllister-Williams and R. Mitchell. *MRC Brain Metabolism Unit, 1 George Square, Edinburgh EH8 9JZ*

Multiple receptors for γ-aminobutyric acid (GABA) are present in both anterior and neurointermediate pituitary gland (Anderson & Mitchell, 1983*a*), where they may be involved in controlling the secretion of hormones such as prolactin (Anderson & Mitchell, 1984) and α-MSH (Tomiko, Tareskevich & Douglas, 1983). Recognition sites for benzodiazepines that represent a component of some $GABA_A$-type receptor complexes are also present (Anderson & Mitchell, 1983*b*). The experiments described here use a specific ligand ([^{35}S]TBPS) to label the picrotoxinin/barbiturate-sensitive site of the anion channel component in $GABA_A$ receptor complexes (Squires, Casida, Richardson & Saederup, 1983).

Pituitary glands were removed from adult male Wistar rats and dissected into anterior (AP) and neurointermediate (NI) lobes. Tissue was homogenized and centrifuged in 100 volumes of 5 mM-Tris HCl buffer, containing 1 mM-EDTA, pH 7·5 and washed three further times in fresh buffer. Binding of [^{35}S]TBPS was studied by the method of Squires *et al.* (1983), as described by McAllister-Williams & Mitchell (1984).

Specific binding of 2 nM-[^{35}S]TBPS (defined by 100 μM-picrotoxinin) was detected in both AP and NI at 278 ± 26 and 133 ± 13 fmol/g tissue respectively ($n = 5$). This relative concentration of sites in AP matches that of $GABA_A$ receptors (Anderson & Mitchell, 1983*a*) but contrasts with the distribution of benzodiazepine recognition sites (Anderson & Mitchell, 1983*b*). Displacement of [^{35}S]TBPS binding to AP membranes by muscimol (2–2000 nM) revealed two components, similar to observations in certain CNS areas such as cerebellum, where only some of the complexes are regulated by benzodiazepines (McAllister-Williams & Mitchell, 1984). These results demonstrate the presence of an anion channel component linked to $GABA_A$ receptors in the pituitary gland, as in the CNS, and are consistent with the existence of both benzodiazepine-regulated and benzodiazepine-independent $GABA_A$ receptor complexes in AP.

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Evidence for GABA receptors on rat somatotrophes and gonadotrophes

By R. A. Anderson and R. Mitchell. *M.R.C. Brain Metabolism Unit, 1 George Square, Edinburgh*

Gamma aminobutyric acid (GABA) may be involved in the control of secretion of many anterior pituitary (AP) hormones, although prolactin is the only hormone reported to be influenced directly at the pituitary (Racagni, Apud, Cocchi, Locatelli & Muller, 1982). We have here investigated GABAergic control of other AP hormones.

Anterior pituitary glands from male Wistar rats were chopped into 500 μM prisms and superfused as previously described (Anderson & Mitchell, 1984). Growth hormone (GH) and luteinizing hormone (LH) were measured by radioimmunoassay. The $GABA_A$ receptor agonist muscimol (10 μM) caused a stimulation of secretion of both hormones: $186 \pm 21\%$ for GH and $17 \pm 1\%$ for LH ($n = 5$). This effect on GH was concentration-dependent and the response to 10 μM muscimol was reduced to $20 \pm 2\%$ by 10 μM-bicuculline methiodide (BMI) which abolished the effect on LH. In both cases the response was transient; secretion returned to near pre-drug concentrations within 30 min. The $GABA_B$ receptor agonist baclofen (100 μM) was not found to have any effect on basal secretion of either hormone. We have also investigated the effects of muscimol and baclofen on LH release stimulated by LH-releasing hormone (LHRH). When introduced simultaneously, 10 μM-muscimol potentiated the effect of 300 nM-LHRH, increasing the peak stimulation from $117 \pm 4\%$ to $164 \pm 6\%$ ($P < 0{\cdot}05$). This was prevented by BMI ($91 \pm 9\%$ stimulation, $P < 0{\cdot}05$ *vs.* LHRH + muscimol, $n = 5$ in each case). By contrast, baclofen reduced the effect of LHRH (300 nM-LHRH + 100 μM baclofen: $82 \pm 8\%$ stimulation, $P < 0{\cdot}05$).

These results demonstrate direct effects of GABA agonists on secretion of AP hormones other than prolactin. The effect of muscimol on both GH and LH is in marked contrast to its effects on prolactin (Anderson & Mitchell, 1984), in that no inhibitory effect subsequent to the stimulation was seen here. Furthermore, these results demonstrate the presence of a $GABA_B$ receptor in AP, supporting the results of radioligand binding studies (Anderson & Mitchell, 1983).

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PRELIMINARY NOTES

CENTRAL-TYPE BENZODIAZEPINE BINDING SITES IN RAT PITUITARY GLAND ARE OF THE BZ_1 SUBTYPE

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SUMMARY Experiments were performed to investigate the subtype of central-type benzodiazepine binding site present in rat pituitary gland. B_{max} values for [^{3}H] n-propyl β-carboline-3-carboxylate and [^{3}H] flunitrazepam (for central-type binding only) were similar. Displacement of [^{3}H] flunitrazepam by n-propyl β-carboline-3-carboxylate gave a linear Hofstee plot and a Hill coefficient close to unity. These results suggest that the central benzodiazepine binding sites in the pituitary are predominantly of the BZ_1 subtype, as found in the cerebellum.

Benzodiazepines have been reported to affect the secretion of various hormones from the pituitary gland. Secretion of growth hormone is stimulated (Koulu, Lammintausta, Kangas and Dahlstrom, 1979) while prolactin secretion is reduced (Lotz, 1982). The possibility that some of these effects might be the result of a direct action on the pituitary was supported by the demonstration of both central- and peripheral- type benzodiazepine binding sites in pituitary of the rat (Anderson and Mitchell, 1983). As in the CNS, the central type sites are allosterically influenced by γ-aminobutyric acid (GABA), indicating their association with GABA-mediated control of the gland. Indeed, secretion of prolactin is modified by GABA (Schally, Redding, Arimura, Dupont and Linthicum, 1977) and we have recently demonstrated that clonazepam can potentiate the effect of muscimol on secretion of prolactin *in vitro* (Anderson and Mitchell, 1984).

Further to the division of benzodiazepine binding sites into 'central' and 'peripheral' types, it has recently been suggested that the central type sites may not be homogeneous, on the basis of their interactions with some non-benzodiazepine compounds. These compounds, including triazolopyridazines (Squires, Benson, Braestrup, Coupet, Klepner, Myers and Beer, 1979) and esters of β carboline-3-carboxylic acid (Braestrup and Nielsen, 1981), show regional variations in affinity consistent with populations of two distinct subtypes of receptor, BZ_1 and BZ_2, being differentially distributed within the CNS. Benzodiazepines, however, appear to have very similar affinities for the two putative subtypes. Kinetic analyses of the ligand interactions with these sites have led to suggestions that they represent different functional states with co-operative interactions rather than distinct molecular entities (see Martin, Brown and Doble, (1983) for review). Nevertheless it is clear that benzodiazepine binding sites exhibit regionally different properties and the possibility that subtypes may exert different roles *in vivo* points to the importance of characterising the receptor present in the pituitary.

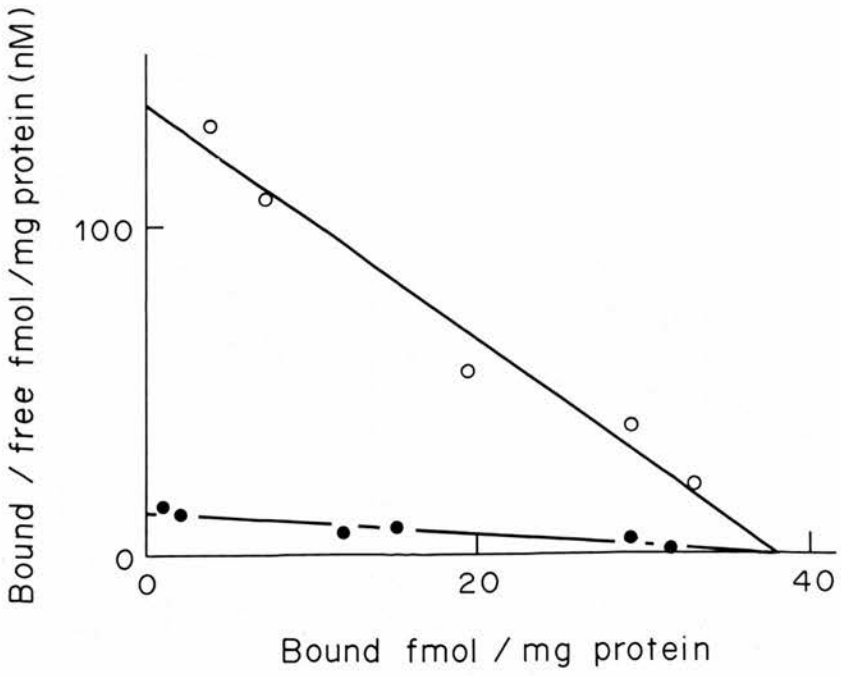
METHODS

Male Wistar rats (150–250g) were stunned and decapitated, the pituitary gland and brain regions removed and stored at -20°C. Membranes were prepared by homogenisation of tissue in 100 volumes of ice-cold 25mM KH_2PO_4 buffer (pH 7.1) using an Ystral high frequency homogeniser (setting 3 for 5 seconds). Homogenates were centrifuged at 48,000g for 10 minutes at 4°C. After discarding the supernatant, the membrane pellet was resuspended in fresh buffer before centrifugation. The washing procedure was repeated a further 3 times. The final resuspension of membrane preparations gave concentrations of 250–500μg protein/ml for pituitary tissue and 400–600μg protein/ml for CNS tissues. Aliquots of 500μl were used for radioligand binding assays, in a final assay volume of 2ml.

[^{3}H] Flunitrazepam, ([^{3}H] FNM, [methyl- ^{3}H] flunitrazepam, 70–80Ci/mmol, NEN) was used at concentrations between 0.1nM and 10nM for Scatchard analyses and at 0.5nM for displacement studies. [^{3}H] Propyl-β-carboline-3-carboxylate ([^{3}H]PCC, [propyl-2, 3-^{3}H] β-carboline-3-carboxylate, 95Ci/mmol, NEN) was used at concentrations between 0.05nM and 5nM for Scatchard analyses. Non-specific binding was defined by 200nM clonazepam. Adsorption phenomena were corrected for as previously described (Mitchell and Wilson, 1984). Assays

in duplicate (or triplicate for [^{3}H] PCC Scatchard analyses) were incubated on ice for 120 minutes in the dark, then filtered through GF/B filters under vacuum and washed with 3 x 5mls of ice-cold buffer via the incubation tube.

FIGURE 1.



Scatchard analysis of [^{3}H] FNM (●) and [^{3}H] PCC (○) binding to pituitary membranes. In [^{3}H] PCC experiments the proportion of specific binding was less than for [^{3}H] FNM (i.e. ~ 250dpm representing approximately one third of total binding at 0.1nM) and assays were therefore carried out in triplicate. Typical of 7-8 experiments.

RESULTS

Both ligands bound to pituitary membranes in a specific, saturable manner. When 200nM clonazepam is used to define non-specific binding, the binding of [^{3}H] FNM to the population of peripheral type sites present in the pituitary is discounted (Anderson and Mitchell, 1983). [^{3}H] PCC was found not to bind to peripheral type sites at concentrations up to 20nM. Scatchard analyses of the binding of both ligands were linear (Fig. 1), and the B_{max} values were similar: 32.8 ± 2.6fmol/mg protein (mean ± S.E.M., n = 8) for [^{3}H] FNM and 36.7 ± 4.8fmol/mg protein (n = 7) for [^{3}H] PCC. As in CNS regions, the affinity of [^{3}H] PCC was rather higher than that of [^{3}H] FNM: 0.33 ± 0.05nM vs 1.9 ± 0.3nM (see Table). The results from control experiments on hippocampal and cerebellar membranes are also included in the table. As reported by Braestrup and Nielsen (1981), [^{3}H] PCC binds to a similar number of sites to [^{3}H] FNM in the cerebellum, but significantly fewer (≃ 50% of [^{3}H] FNM B_{max}) in the hippocampus.

The displacement of bound [^{3}H] FNM (0.5nM) by unlabelled PCC also differed between cerebellum and hippocampus in accordance with the results of Braestrup and Nielsen (1981). In the cerebellum, PCC gave an IC_{50} of 1.3nM and clearly linear Hofstee plots but in the hippocampus, the apparent affinity of PCC was less with an IC_{50} of 8.7nM and distinct curvature (consistent with two components) in Hofstee plots (Fig. 2a, b). Hill coefficients of this displacement data were 0.96 and 0.71 in cerebellum and hippocampus respectively. When similar experiments were carried out using pituitary membranes, PCC showed a similarly high affinity to that in cerebellum (Fig. 2a, IC_{50} of 1.1 ± 0.2nM, n = 4), giving linear Hofstee plots with no indication of curvature (Fig. 2b) and Hill coefficient of 0.9 ± 0.1.

TABLE

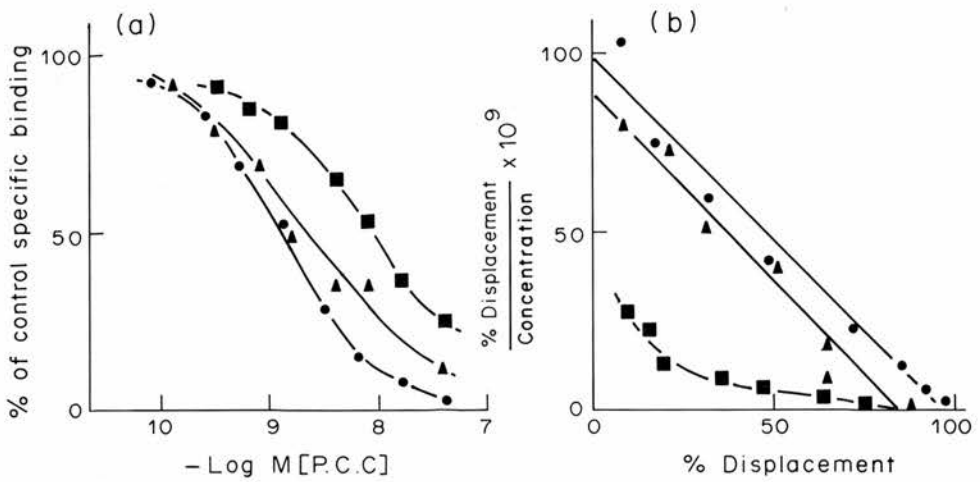
	[^{3}H] FNM	[^{3}H]PCC
Pituitary K_D (nM)	1.9 ± 0.3	0.33 ± 0.05
B_{max} (fmol/mg protein)	32.8 ± 2.6	36.7 ± 4.8
Cerebellum K_D	2.69 ± 0.02	0.28 ± 0.01
B_{max}	865 ± 18	737 ± 34
Hippocampus K_D	1.57 ± 0.01	0.42 ± 0.01
B_{max}	1669 ± 47	836 ± 24

Results of Scatchard analysis of [^{3}H] FNM and [^{3}H] PCC binding to pituitary, cerebellar and hippocampal membranes. Mean ± S.E.M. n = 7–8 for pituitary, and n = 4 for cerebellum and hippocampus.

DISCUSSION

PCC binds with high affinity to virtually all benzodiazepine binding sites in cerebellum but in hippocampus shows a similar high affinity at only some sites with approximately 10-fold lower affinity at the remainder. The high affinity sites in cerebellum and hippocampus (that can be selectively labelled by low concentrations of [^{3}H] PCC) have been denoted BZ_1 and the lower affinity, BZ_2 (Braestrup and Nielsen, 1981). By comparison of the relative B_{max} values of [^{3}H] FNM and [^{3}H] PCC binding it is possible to estimate the proportion of the two sites in an area. The results presented in the table confirm that under the present conditions, the cerebellum appears to contain almost exclusively (≃ 92%) BZ_1 sites with similar B_{max} values for either ligand, whereas the B_{max} for [^{3}H] PCC in the hippocampus is only 50% of that for [^{3}H] FNM ($p < 0.001$), suggesting that BZ_1 and BZ_2 sites are present there in approximately equal numbers. This heterogeneity in the properties of the sites is further demonstrated by Hofstee plots of the displacement of bound [^{3}H] FNM by unlabelled PCC (Fig. 2), showing curvilinearity (and a low Hill coefficient) in hippocampus but not cerebellum.

FIGURE 2.



a) Displacement curves of 0.5nM [^{3}H] FNM by PCC from (●) cerebellum, (■) hippocampus and (▲) pituitary membranes.
b) Hofstee plots of the results in (a). Results are representative of 4 experiments performed in duplicate.

Application of the same approach to the pituitary gland revealed that [^{3}H] PCC binds there to a similar number of central sites to [^{3}H] FNM (Fig. 1), suggesting that the sites present are predominantly of the BZ_1 type. This conclusion is fully supported by the results on displacement of [^{3}H] FNM by PCC showing a single component of similar affinity to that in cerebellum.

It therefore appears that BZ_1 receptors must mediate any facilitation of GABA events in the pituitary gland elicited by benzodiazepines. The effectiveness of benzodiazepines in such systems will thus be characteristic of action at a specified, BZ_1 type site. The presence of these sites in both anterior and neurointermediate lobes of the gland (Anderson and Mitchell, 1983) and reports of BZ-potentiated GABA responses in both areas (Anderson and Mitchell, 1984; Taraskevitch and Douglas, 1983) suggests that drugs active at BZ_1 receptors may have marked effects on a number of endocrine systems.

R.A.A. is a Houldsworth Scholar of the University of Edinburgh.

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