

**"THE MODULATION OF CALCIUM TRANSPORT BY
NEUROACTIVE ~~AGENTS~~". SUBSTANCES .**

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ABSTRACT

This study investigated the effects of various neuroactive agents on basal and stimulated Ca^{2+} fluxes in rat brain and parallel effects on endogenous neurotransmitter amino acid release. The stimulation was chemically induced by veratrine or high potassium. The criterion for detecting a receptor-mediated effect was the inhibition of the response by the appropriate antagonist.

The effects of opioid-receptor agonists on veratrine-induced neurotransmitter release were studied. All the opioid compounds tested, in a dose-dependent manner, inhibited both parameters. Morphine inhibited, specifically, the veratrine-stimulated $^{32}\text{P}_i$ incorporation into proteins in preference to the $^{32}\text{P}_i$ incorporation into lipids. The inhibitory action was enhanced by GTP which suggested the involvement of a second messenger system.

The actions of exogenous noradrenaline, dopamine and 5-hydroxytryptamine were studied on slices of striatum or cortex. The neurotransmitters, at low concentrations, inhibited the veratrine-stimulated Ca^{2+} influx and amino acid release, but did not affect basal levels of the two parameters. Dopamine did not affect the stimulatory actions of veratrine on cortex slices, whilst noradrenaline was ineffective on stimulated striatal slices. Evidence is presented for the involvement of second messengers. The results are discussed in respect to a possible mechanism for the presynaptic regulation of neurotransmitter release.

The actions of various anticonvulsant drugs were compared. Diphenylhydantoin, carbamazepine and phenobarbitone inhibited veratrine-stimulated Ca^{2+} influx at concentrations within the therapeutic range. Ethosuximide and sodium valproate did not decrease veratrine-stimulated Ca^{2+} influx but did affect the release of amino acids. The ω -phosphono- α -amino carboxylic acids (AP4, AP5 and AP7) inhibited both the veratrine and the K^+ stimulated actions on hippocampal slices with increasing orders of potency. AP7 also inhibited Ca^{2+} influx induced by glutamate, aspartate and N-methyl-D-aspartate (NMDA). The inhibition of NMDA induced Ca^{2+} influx was dependent on external Mg^{2+} concentration. The actions of the NMDA antagonists to inhibit Ca^{2+} influx is discussed in respect to their anticonvulsant activity.

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CHAPTER 1

INTRODUCTION

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1.1 General introduction

The cellular components of the central nervous system (CNS) fall into two major categories, neurones and glia. The electrical impulses that transmit information throughout the CNS are carried by the neurones, whilst the glia, although unable to produce action potentials, act to maintain and support the neuronal cells.

The neurones are highly differentiated cells with an intact plasma membrane. The electrical currents carried by these cells are transmitted by the redistribution of ions across the membrane. Specialised junctions occur at the terminals of these cells, called synapses, and they aid the transmission of signals between neurones. Generally, neurones do not make contact but are separated by a gap of approximately 20nm in width. Chemicals released from the terminal can diffuse rapidly across this gap to interact with specific receptors on the postsynaptic membrane. The release of these chemicals, or neurotransmitters, is caused by depolarization of the presynaptic terminal, and for release to occur there must be an influx of Ca^{2+} ions into the presynaptic terminal.

The purpose of this study was to investigate further the coupling between Ca^{2+} entry and neurotransmitter release. In order to study this, the accumulation of Ca^{2+} ions in slices from rat brain was studied under a variety of conditions. Depolarization was simulated by a variety of chemicals, which were able to cause either a redistribution of ions across the membrane or alter the mechanism controlling Ca^{2+} homeostasis within the cell. The release of endogenous amino acids was also measured. As a result of these studies it was possible to discover more about the site of actions of certain drugs and how they affect the CNS. It was also possible to investigate mechanisms which exist to regulate the release of endogenous neurotransmitters.

From the knowledge of such mechanisms of control and drug action it may be possible to design neuropharmacological agents to treat neurological diseases which result from impaired Ca^{2+} transport and neurotransmitter release.

1.2 Brain slices: Their use in *in vitro* neurochemical investigations

The *in vitro* preparations of rat cerebrocortical, striatal, and hippocampal slices have been used in these investigations. Slices were prepared both manually using a McIlwain recessed guide and blade (McIlwain, 1961) or by a mechanical tissue chopper (McIlwain and Bubble, 1953). In either case the cell structure is thought to be maintained in a relatively intact state so that the metabolic characteristics of the tissue are minimally disturbed (McIlwain, 1951;

McIlwain and Bubble, 1953). Brain tissue does suffer some damage on slicing (Libet and Garard, 1939), and has a tendency to swell, but nevertheless such sections are histologically relatively intact and retain many biochemical characteristics of the organised structure. The optimal thickness of the slice is 300-400 μ m (Li and McIlwain, 1957; Bradford, 1972). Under these conditions the slices are able to take up, actively, glucose and to maintain an ionic gradient across their membranes. Slices greater than 400 μ m in thickness are unable to respire normally due to the problems of oxygen, glucose, and ions diffusing into the central regions. The innermost cells will suffer hypoxia or ischaemia and be permanently damaged. Slices were prepared so as to sever as few neurones as possible, inevitably the interneurones connecting the laminar layers of neurones would necessarily be damaged.

The resting membrane potentials measured in cortical slices are found to be in the range -40 to -60 mV and slices can be depolarized by both electrical and chemical stimuli, which is an indication of the viability of the slices to maintain an ionic gradient across their membranes.

Brain slices provide a useful tissue preparation in which to study Ca^{2+} uptake and neurotransmitter release. The synaptic terminals are maintained in an environment more comparable to the *in vivo* condition with respect to the presence of the glial cells which provide important modulatory influences.

1.3 Involvement of glial cells

The performance of the CNS is usually discussed in terms of neurones only, although neural tissue consists largely (75%) of glial cells outnumbering neurones 1:10. Although glial cells are so numerous little is known about their functions (Kuffler and Nicholls, 1976). This is due to the inability to separate neurones from glia as together they form interweaving networks. However, with the development of cell tissue culture it has been possible to study in more detail the structure and function of the glial cells. Oligodendroglia form myelin sheaths around the axons of the larger neurones, the presence of the sheaths increases the rate of conduction, and also provides insulation between conducting neurones. Astroglia have a much wider range of function in comparison to the oligodendroglia; the former are thought to be involved in the functioning of the blood-brain barrier (Fenstermacher, 1975). Astroglia play a role in guiding the growth and regeneration of nerve fibres during development and following injury respectively (Eccles, 1976). Additionally, glial cells have uptake and metabolic systems for some neurotransmitters to aid in the termination of transmitter actions at the terminals, eg γ -amino butyric acid (GABA) (Bowery and Brown, 1972).

As mentioned previously, glial cells do not produce action potentials (Kuffler and Nicholls, 1976). The glial membrane potential is higher than that of neurones and depends primarily on the distribution of K^+ ions. Glial cells can undergo depolarization by a rise in extracellular K^+ concentration produced when neurones are depolarized. Radiochemically-labelled GABA and glutamate can be released in a Ca^{2+} -dependent process from glial cells in the dorsal root ganglion when the neurones are depolarized by an increase in the K^+ content of the bathing medium (Minchin and Iverson, 1974). However Neal and Bowery (1979) found that in the rat retina, sympathetic, and spinal ganglia, K^+ did not greatly increase GABA release and this release was Ca^{2+} independent. They concluded that the release of endogenous GABA from glial pools is unlikely to be of any physiological importance.

Veratrine was used as a depolarizing agent in many of these studies. Several workers have reported that veratrine does not stimulate the release of endogenous neurotransmitters from glial cells (Minchin, 1980). Amino acid neurotransmitter release from glia of rat spinal cord slices (Minchin, 1977) was unaffected by veratrine as was the release of GABA from glia in rat retina or sympathetic or spinal ganglia (Neal and Bowery, 1979). Ca^{2+} uptake into neurones occurs at a higher rate than into astroglia and neuronal perikarya (Lazarewicz et al, 1977; Hertz, 1982). Primary neuronal cultures take up Ca^{2+} five times faster than do corresponding cultures of astrocytes.

1.4 Involvement of Ca^{2+} in neuronal function

It is generally agreed that calcium ions are involved in the maintenance of neuronal cell viability and function, eg stabilization of excitable membranes (Frankenhaeuser and Hodgkin, 1957), protein phosphorylation (Delorenzo, 1976; Greengard, 1979a), and excitation-secretion coupling at nerve terminals (Blaustein, 1975). The actions of neurotransmitters on the post synaptic membrane may also involve Ca^{2+} in the sequence of events leading to the response of the post-synaptic cell (Greengard, 1979b).

The electrophysiological experiments of Katz and Miledi (1969) who used the giant synapse of the squid stellate ganglion, have shown that depolarization of the presynaptic nerve terminal induces an increase in calcium ion conductance ($g_{Ca^{2+}}$) across the synaptic membrane. They also showed (Katz and Miledi, 1967 a and 1967b) that Ca^{2+} entry into nerve endings was involved in the release of acetylcholine from frog motor nerve terminals. They termed this the "calcium hypothesis". Llinas and Nicholson (1975) observed that the injection of aequorin, a calcium sensitive photoluminescent protein, into the presynaptic terminal of the squid giant

synapse produced an increase in aequorin luminescence on depolarization. Ashley et al (1984) have been able to demonstrate that a depolarization-induced increase in intracellular Ca^{2+} was involved in the release of acetylcholine in synaptosomes (pinched-off nerve terminals) using Quin-2, a fluorescent Ca^{2+} indicator. It therefore follows that neurotransmitter release is initiated by a transient increase in the intracellular Ca^{2+} concentration within the nerve terminal usually caused by the net influx of Ca^{2+} ion down a steep electrochemical gradient through voltage-dependent Ca^{2+} channels.

1.5 Regulation of Ca^{2+} within the nerve terminal.

1.5a Ca^{2+} influx.

Ca^{2+} influx is a passive process dependent upon the electrochemical gradient of Ca^{2+} across the synaptic plasma membrane. Calcium ions pass through channel-forming proteins embedded in the synaptic plasma membrane lipid-bilayer. The channels are gated in a voltage-dependent manner and may be modulated by neurotransmitters, hormones, and drugs. Ca^{2+} currents are "slow currents" since the rise in Ca^{2+} conductance with time after depolarization is slow as compared to Na^+ currents in the same membrane (Kostyuk, 1980). This is because the activation of Ca^{2+} conductance occurs at a higher positive membrane potential than the activation of Na^+ conductance. The mechanism of inactivation is as yet unknown.

In electrophysiological studies, the Ca^{2+} inward current (through voltage-dependent Ca^{2+} channels) is separated from the Na^+ inward current and a non-specific influx of Ca^{2+} through the Na^+ channels by the use of a specific Na^+ channel blocker, tetrodotoxin (TTX) (Kostyuk, 1980). The subsequent outward K^+ currents are prevented either by the addition of tetraethylammonium ions (TEA) or by the replacement of K^+ by internal perfusion of the cells being studied. Ca^{2+} channels are insensitive to TTX and TEA. Recently the use of patch-clamp techniques has enabled the properties of single Ca^{2+} channels to be measured within the membrane. The Ca^{2+} channel in the somatic membrane of molluscan neurones, has the following ion selectivity $\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$. The selectivity of the channel appears to depend on the size of the ion, thus Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in transmitter release experiments as they can permeate through the Ca^{2+} channel. A binding site for these ions has been proposed to be situated at the external opening of the Ca^{2+} channel (Kostyuk, 1980). Ions, eg Mg^{2+} , Co^{2+} , Mn^{2+} which have binding affinities greater than Ca^{2+} for this site are able to act as calcium channel blockers. A second Ca^{2+} binding site has been proposed

to exist on the inner side of the channel, this may well be involved in a negative feedback system, where a high internal Ca^{2+} concentration causes the Ca^{2+} channel to close (Kostyuk, 1980).

A second route for Ca^{2+} entry across the plasma membrane is via the $\text{Na}^+/\text{Ca}^{2+}$ antiport system. One Ca^{2+} ion may be exchanged for three or more Na^+ ions by counter transport (Blaustein and Oborn, 1975; Blaustein and Ector, 1976; Akerman and Nicholls, 1981), thus involving the movement of nett charge. The $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism is dependent upon the magnitude of the membrane potential (Akerman and Nicholls, 1981), thus Ca^{2+} could enter the cell by this mechanism in a comparable manner to the entry of Ca^{2+} by voltage-dependent Ca^{2+} channels. However, it is possible that there is a differentiation between the two systems, since on halving the external Na^+ concentration the Na^+ dependent Ca^{2+} influx is enhanced, whereas Sr^{2+} and Ba^{2+} uptake through the voltage-sensitive Ca^{2+} channel is not enhanced by this treatment (McGraw et al, 1982).

A number of experiments indicate that the mechanism for Na^+ -dependent Ca^{2+} influx is distinct from the mechanisms for the fast and slow phases of K^+ stimulated Ca^{2+} influx, since the concentration of Ca^{2+} required to half maximally (K_m) saturate the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism is much higher than the K_m for K^+ stimulated Ca^{2+} influx.

Thus there is evidence for two voltage-dependent Ca^{2+} influx mechanisms. Turner and Goldin (1985) found that depolarization at high K^+ levels had no effect on the rate of synaptosomal Na^+ efflux, which also suggests that $\text{Na}^+/\text{Ca}^{2+}$ exchange does not mediate depolarization-stimulated Ca^{2+} uptake.

A second type of calcium channel, the receptor-operated calcium channel, is an ion channel controlled or operated by a receptor for a stimulant substance and not by voltage changes across the membrane (Bolton, 1979). These receptor-operated channels appear not to be blocked as readily by general Ca^{2+} channel antagonists as do the voltage-dependent calcium channels (Bolton, 1979). However, Ichinda et al (1984), found that the receptor-operated channels were dose-dependently inhibited by the antagonists eg verapamil, Co^{2+} , diltiazem. There are however many difficulties involved in studying these channels, for example their ion-specification and much is still to be learned about their functioning.

1.5b Intra-terminal Ca^{2+} buffering.

The physiological intracellular Ca^{2+} concentration (Ca^{2+})_i in the cytoplasm of presynaptic terminals is in the order of 10^{-7} M at rest (Dipolo et al, 1976; Schweitzer and Blaustein, 1980) whilst the extracellular concentration is in the millimolar range. This concentration gradient,

together with a large negative resting potential (-60mV to -90mV) sets up a very steep electrochemical gradient for Ca^{2+} across the plasma membrane.

As a result of an action potential and the resultant increase in the calcium conductance of the membrane (Katz and Miledi, 1969) sufficient Ca^{2+} enters the terminal to raise $(\text{Ca}^{2+})_i$ to 10^{-6}M or 10^{-5}M , this increase triggers neurotransmitter release (Miledi, 1973). Only a very small fraction of cytoplasmic calcium is ionisable and freely diffusible. Baker (1972) provided evidence that of the $400\mu\text{M}$ calcium present in the squid axoplasm, only approximately $10\mu\text{M}$ was diffusible but unionizable and only $0.3\mu\text{M}$ was ionised as measured using aequorin with EGTA (ethylene glycol-bis [α -amino ethyl ether]N,N'-tetraacetic acid) buffer. The bulk of the unionised calcium was sequestered by energy-dependent processes into intracellular organelles. Quin-2 measurements have revealed that the intrasynaptosomal ionised Ca^{2+} level is $0.1\text{--}0.2\mu\text{M}$ (Ashley et al, 1984; Nachshen, 1984b) whereas the average total concentration of Ca^{2+} in the nerve terminal is $1\text{--}2\mu\text{M}$ (Nachshen, 1985a).

When ionized Ca^{2+} is taken up by neurones it is rapidly sequestered by intracellular organelles (Baker, 1972). For example, Ca^{2+} is buffered by mitochondria, smooth endoplasmic reticulum (SER), synaptic vesicles. The SER has a high affinity for Ca^{2+} ($K_m = 0.35\mu\text{M}$), but a low capacity ie $2\text{--}3\mu\text{mol/mg}$ synaptic protein. The mitochondria have a lower affinity for Ca^{2+} ($10\text{--}100\mu\text{M}$) but they have a greater storage capacity ($30\mu\text{mol/mg}$ synaptic protein) (Blaustein et al, 1978b,c, and 1980). ATP is required in the sequestration of Ca^{2+} by the SER and also by the mitochondria. It is unlikely that the mitochondria are involved in sequestering Ca^{2+} under normal physiological conditions since they are unable to sequester Ca^{2+} when the free ionisable Ca^{2+} concentration in the medium falls below $0.4\text{--}0.5\mu\text{M}$ (Blaustein et al 1978a). The SER appears to be more important in buffering the internal free Ca^{2+} at physiological Ca^{2+} concentrations (McGraw et al 1982).

Further evidence against mitochondrial involvement in regulating the resting level of Ca^{2+} in nerve terminals has been provided by the recent studies of Nachshen (1985). Evidence was obtained that the mitochondrial inhibitors valinomycin and FCCP (fluoro-carbonyl cyanide phenyl hydrozone) increased the intracellular-free Ca^{2+} levels (measured using Quin 2) by an indirect action on energy metabolism, rather than releasing Ca^{2+} sequestered within the mitochondria. It was also shown that rotenone and oligomycin abolished the mitochondrial membrane potential without causing a rapid depletion in intra-terminal ATP, whilst doing so these agents did not increase the Ca^{2+} concentration. Within the terminal there are also Ca^{2+} binding proteins which are able to sequester Ca^{2+} , for example, calmodulin (CaM), calcineurin, calsequestrin and S100 (Cheung, 1980; Bromstrom and Wolff, 1981; Baudier et al, 1982). It has

been found that CaM is the most widely occurring binding protein.

1.5c Ca^{2+} efflux

In order for Ca^{2+} to act as a secondary messenger in the nerve terminal, the resting $(\text{Ca}^{2+})_i$ must be maintained at very low levels. As the intra-terminal Ca^{2+} concentration rises after depolarization, organelles sequester the Ca^{2+} , but the excess must eventually be extruded across the plasma membrane. The primary method for Ca^{2+} extrusion is the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism (Blaustein and Ector, 1976). The Na^+ electrochemical gradient, directed into the cell in the resting condition, provides energy for the Ca^{2+} efflux. A second mechanism for Ca^{2+} extrusion may involve Ca^{2+} dependent ATPases which pump Ca^{2+} to the exterior, however the presence of these in the plasma membrane have not been shown unequivocally. A third extrusion process could involve a Ca^{2+} - Ca^{2+} exchange (Blaustein and Ector, 1976). Ca^{2+} efflux from synaptosomes is usually consistent with a Ca^{2+} carrier mechanism which extrudes Ca^{2+} in exchange for Na^+ or Ca^{2+} (Blaustein and Oborn, 1975).

1.6 Biochemical Basis of the Action Ca^{2+}

The biochemical events linking depolarization-dependent Ca^{2+} influx to the process of neurotransmitter release have yet to be elucidated. The source of the released transmitter is still an area of much controversy. Evidence for vesicular release comes from electrophysiological studies and electronmicroscopy (Pumplin et al, 1981). Several types of vesicle are present in the neurone which are used for packaging different neurotransmitters such as peptides (Hokfelt et al, 1980), acetylcholine, (Navone et al, 1984) and glutamate (Naito and Ueda, 1983).

Greengard and co-workers isolated a protein synapsin I (De Camilli and Greengard et al, 1983a and b). This protein has been found to be virtually neurone specific and appears to be associated with small synaptic vesicles containing acetylcholine and glutamate in preference to the larger dense-cored vesicles. Synapsin I appears to form a 'cage' around the small vesicles. The protein itself strongly resembles a protein found in erythrocytes (protein 4.1) (Baines and Bennett, 1985), which is able to link the skeletal protein fodrin to an integral membrane protein. Thus if synapsin I and the protein (4.1) are similar, the former could act as a bridge between the synaptic vesicle and important cytoskeletal elements. Synapsin I is known to be a substrate for phosphorylation by various kinases (Nairn et al, 1985). Cyclic AMP-dependent protein kinase and Ca^{2+} /calmodulin-dependent kinase I phosphorylates the protein at a site termed site I. A second

Ca^{2+} -dependent kinase, Ca^{2+} /CaM-dependent kinase II phosphorylates the synapsin I at site II. When the phosphorylation occurs the vesicle is released from its restraining cage and makes a "bee line" for the membrane. In a separate study Delorenzo et al, (1976) also showed that phosphorylation of a neurone-specific protein occurred on Ca^{2+} -dependent depolarization. They isolated this protein which is thought to be tubulin, a protein involved in the cytoskeletal system. Tubulin is phosphorylated by Ca^{2+} /CaM-dependent kinase.

Thus when Ca^{2+} enters the cell it binds to a calcium chelating protein, for example calmodulin (Cheung et al, 1980) and can then cause neurotransmitter release. The time required for these phosphorylation steps falls within the time limits given for neurotransmitter release.

Calmodulin is an ubiquitous protein which has four binding sites that are linked by a positive co-operative interaction. On binding Ca^{2+} the three dimensional structure of calmodulin changes and it can then cause phosphorylation of proteins.

Evidence is still accumulating for the non-vesicular release of neurotransmitters, however this necessitates a gating mechanism to allow for quantal release. One possible mechanism for such release involves the Na^+/K^+ ATPase. Changes in Na^+/K^+ ATPase activity appear to be inversely related to the efflux of neurotransmitter. Since Ca^{2+} inhibits this enzyme, it has been suggested that the physiological release process is triggered by this inhibition (Meyer and Cooper, 1981). However this enzymatic process may be too slow, and the concentration of Ca^{2+} too high, for this mechanism to be directly involved in neurotransmitter release.

The quantity of neurotransmitter released following a nerve impulse can be influenced by a variety of factors. In some cases neurotransmitters, released from a synapse, may feedback to "gate" the voltage-dependent Ca^{2+} channels (Forscher and Cooper, 1985).

In other cases, neurotransmitters may affect the production of secondary messengers following interactions with specific receptors. Two major second messenger systems are thought to be involved. The first utilises cyclic nucleotides eg, cAMP, cGMP. The second system is related to the metabolism of membrane polyphosphoinositides and the generation of inositide triphosphate (IP3) and diacylglycerol (DAG). The action of IP3 can lead to the mobilization of Ca^{2+} from intracellular stores and DAG is a key regulator of protein kinase C which is also a Ca^{2+} -requiring enzyme. Both these two systems appear to act by stimulating kinases (eg. cAMP- or cGMP-dependent protein kinases, Ca^{2+} /calmodulin- dependent kinases 1,2 and 3). It is quite clear that in many instances both cAMP and DAG modify release processes by acting at an intracellular site rather than altering Ca^{2+} influx directly. Cruetz et al (1985) reported that phospholipid C (the major enzyme involved in the formation of IP3 and DAG) is associated with chromaffin cell granules. DAG released by such an enzyme could act as a local regulator for

vesicular-contained neurotransmitters. In other cases, neurotransmitters and their secondary messengers may regulate Ca^{2+} influx. The cAMP-dependent phosphorylation of voltage sensitive Na^+ channels, K^+ channels, and Ca^{2+} channels has been demonstrated (Browning et al, 1985; Nairn et al, 1985). Phosphorylation may modify the properties of the channels and lead either directly or indirectly to changes in Ca^{2+} influx. The voltage-dependent Ca^{2+} channel is a substrate for cAMP-dependent protein phosphorylation (Curtis and Catterall, 1985) and phosphorylation leads to an increase in the probability of the channel opening and also an increase in the number of active channels.

1.7 Amino acids as neurotransmitters.

Much evidence has accumulated over the years to suggest that glutamate, aspartate, and GABA (γ -amino butyric acid) should be classed as putative neurotransmitters. Both glutamate and aspartate occur in millimolar concentrations in the CNS, since they are both extensively involved in intermediary metabolism. GABA is also present in concentrations 200-1000 fold higher than the classical neurotransmitters such as noradrenaline or acetylcholine.

1.7a Glutamate and Aspartate.

Prior to the work of Wofsey et al, no chemical evidence existed for the neurotransmitter role of these two major excitatory amino acids due to the inability to differentiate between the transmitter pool and the larger metabolic pool. The major breakthrough came when Wofsey et al (1971) demonstrated a Na^+ -dependent high affinity uptake of glutamate and aspartate into a unique population of synaptosomes isolated from rat brain and spinal tissue. Even more convincing evidence has recently appeared relating this high affinity uptake to specific neurones. The decrease in this high affinity uptake after lesions of specific pathways suggests the existence of major "glutaminergic" or "aspartatergic" tracts in the mammalian brain (Storm-Mathisen, 1977).

Many research groups have demonstrated a Ca^{2+} -dependent release of endogenous aspartate and glutamate on chemically or electrically stimulated slices or synaptosomes (Curtis and Johnston, 1974; de Belleruche and Bradford, 1972). Neuronal release can be distinguished from glial release due to the Ca^{2+} -dependency of the former as compared to the latter (Blaustein, 1975; Sellstrom and Hamberger, 1977).

Due to the different roles assigned to glutamate and aspartate, i.e. metabolic precursors or

neurotransmitters, it is not surprising that synthesis and metabolism are compartmentalized in a very complex manner. Several studies have shown that glutamine is an excellent precursor for the releasable pool of glutamate and GABA (Hamberger et al, 1979a and b; Young and Bradford, 1986). Aspartate is apparently produced by transamination with glutamate acting as an indirect precursor.

On release, glutamate and aspartate combine with excitatory amino acid receptors. Pharmacological evidence indicates that there are three subtypes of receptor (Watkins, 1981). The receptors are activated preferentially by N-methyl-D-aspartate (NMDA), quisqualate, and kainate. The relative specificity of glutamate and aspartate for these receptors is not fully known. The NMDA receptor sub-type may be characterised by the potent and specific antagonist 2-amino-5-phosphonovaleric acid (AP5; Perkins et al, 1982), and by its apparent sensitivity to the extracellular concentration of Mg^{2+} ions (Collingridge, 1985).

The two other receptor subtypes are relatively insensitive to AP5, however, specific antagonists do exist for these sites, for example, both α -D-glutamylsulphonic acid and L-glutamic acid diethyl ester have greater specificity to the kainate and quisqualate sites than to the NMDA sites (McLennan and Lodge, 1979).

Discrete localizations of the receptor subtypes have been found by autoradiographic techniques suggesting that they are associated with specific synaptic pathways (Monaghan et al, 1984; Monaghan and Cotman, 1985).

There is evidence to suggest that all three receptor subtypes are involved in neuronal transmission. NMDA receptor antagonists can act as anticonvulsants (Croucher et al, 1982), and they can also prevent the initiation of long term potentiation (Collingridge, 1985). Quisqualate and kainate receptors may well be involved in "normal" synaptic transmission. There is the possibility that modulation exists between the three receptor subtypes (Monaghan and Cotman, 1985).

1.7b GABA.

There is strong evidence to suggest that GABA is a major inhibitory neurotransmitter. Uptake studies using labelled GABA show that 30% of the cortical neurones utilise GABA (Bloom and Iverson, 1971). There are problems in interpreting these data since [^3H] GABA uptake may not be specific to GABAergic terminals, and part of the uptake may be into glial cells. Non-specific uptake into glial cells can be reduced by the use of uptake blockers.

Experiments using radiochemical-labelling techniques have revealed that glutamic acid is the

immediate precursor of GABA (Bradford and Ward, 1976). Central to the production of GABA is the GABA-shunt system; three enzymes are involved in this system. Glutamic acid decarboxylase (GAD) which converts glutamic acid to GABA and is found free in the intraneuronal space. GABA-oxoglutarate transaminase (GABA-T) converts GABA to succinic semialdehyde and is found in both neurones and glia. The succinic semialdehyde is then returned to the Krebs cycle by succinic semialdehyde dehydrogenase (SSADH). GABA is thus metabolised both pre- and post-synaptically in neurones and also in glial cells. However GABA is produced only in the presynaptic terminals due to the location of GAD from where it is released by a Ca^{2+} -dependent mechanism (Bradford, 1975; Neal and Bowery, 1979). GABA is removed from the synaptic cleft by a Na^+ co-porter (Blaustein and King, 1976). While it is presumed that GABA is stored in presynaptic vesicles in the same fashion as the catecholamines, direct evidence has been hard to accumulate. However data obtained by de Belleruche and Bradford (1973) suggest a cytoplasmic origin for the GABA released from synaptosomes.

Microiontophoretic application of physiological concentrations of GABA onto cortical interneurons mimics the hyperpolarizing actions of endogenous GABA (Krnjević and Scharf, 1966). In general, pharmacological agents that diminish GABA activity cause convulsions (for example bicuculline and picrotoxin), whilst those that enhance GABA activity (for example agonists, imidazolacetic acid and muscimol) are anticonvulsants.

CHAPTER 2

MATERIALS AND METHODS.

- 2.1 Preparation of brain tissue
 - 2.1a Slices prepared by McIlwain tissue chopper
 - 2.1b Slices prepared by recessed guide and blade
- 2.2 Calcium uptake into neuronal tissue
 - 2.2a Incubation of slices prepared using McIlwain tissue chopper
 - 2.2b Incubation of slices prepared by recessed guide and blade
- 2.3 Scintillation counting
 - 2.3a Preparation of samples
 - 2.3b Quantification of the calcium accumulation
- 2.4 Determination of protein levels
- 2.5 Measurement of tissue potassium content
- 2.6 Amino acid analysis
 - 2.6a Sample preparation
 - 2.6b Analytical system
 - 2.6c Quantitation of amino acid concentration
- 2.7 Incorporation of $^{32}\text{P}_i$ into cerebrocortical tissue
 - 2.7a Incubation of slices
 - 2.7b Determination of total $^{32}\text{P}_i$ incorporation
 - 2.7c Determination of the ratio of incorporation between lipid and protein fractions
- 2.8 Drugs

2.1 Preparation of brain tissue

Throughout this work female Sprague-Dawley rats (200g) were used. The rats were reared under controlled light-dark cycles and had continuous access to food and water. The rats were rapidly stunned, decapitated and their whole brain was removed onto a warmed (37°C) dissecting block.

2.1a Slices prepared by McIlwain Tissue Chopper.

Rat brain was removed as described above. The hippocampi were removed bilaterally and slices 300 μ m thick were prepared by a mechanical tissue chopper (McIlwain and Bubble, 1953). Cortices were also removed and sliced as for the hippocampi. The slices were placed in a petri dish containing oxygenated Krebs-Tris medium (mM: NaCl,138.0; KCl,5.0; MgSO₄,1.0; Tris,20.0; Glucose,10.0; CaCl₂,1.2; pH 7.4) at 4°C.

2.1b Slices prepared by recessed guide and blade.

Brain slices (approximately 10x5x0.3mm) were prepared from rat cerebral cortex using a recessed guide (300 μ m recession) and blade. Both blade and guide were kept moist during the slicing. The forebrain was bisected and one slice was prepared from each hemisphere. These slices were then halved, to give half slices weighing approximately 15-24mg fresh wet weight. Once cut, the slices were rapidly transferred to the appropriate volume of incubation medium, usually the Krebs-Tris medium. Striatal and hippocampal slices were prepared in an identical manner to the cortical slices, one slice being taken from each striatum or hippocampus. The slicing was directed so as to disrupt as few neurones as possible. This was achieved for the hippocampus by slicing along the longitudinal axis, normal to the ventricular surface. The lamellar structure of the hippocampus allows slices to be taken without destroying the neurones and their dendrites and axons.

2.2 Calcium uptake into neuronal tissue.

2.2a Incubation of slices prepared using McIlwain Tissue Chopper.

Hippocampal or cortical slices prepared by the method described in 2.1a were evenly

distributed between 12x1ml incubations in eppendorf tubes. This was achieved with the aid of a 5ml Finn Tip pipette, with the plastic tip cut down to give a diameter large enough for the easy passage of slices. The slices were pre-incubated for 30 minutes at 37°C, and during this time each eppendorf tube was regularly (every 5 minutes) aerated with pure oxygen and inverted. After 30 minutes a 50µl spike of $^{45}\text{CaCl}_2$ (0.14Ci/ mole final specific activity, Amersham International) was added followed, 1 minute later, by 50µl of various agonists dissolved in the buffered incubation medium. After a total incubation time of 12 minutes with the $^{45}\text{Ca}^{2+}$, the tubes were centrifuged (10,000g for 2 minutes in an "eppendorf" bench centrifuge). The supernatant liquid was retained and an aliquot taken for amino acid analysis. The pellet was washed once with 1ml of EGTA stop solution (mM: NaCl,120.0; KCl,5.0; MgSO_4 ,1.2; NaH_2PO_4 ,1.2; Tris,20.0; EGTA,30.0; pH 7.4), at room temperature, and twice with 1ml aliquots of K^+ -free- Na^+ medium (mM: NaCl,137.0; MgCl_2 ,1.2; NaH_2PO_4 ,1.2; Tris,20.0; Glucose,10.0; pH 7.4) by resuspending and resedimenting. The washed pellet was then dissolved in 1ml of 1N NaOH at 50°C and aliquots were then taken for the measurement of Ca^{2+} uptake, protein assay, and for determination of tissue K^+ content.

2.2b Incubation of slices prepared by recessed guide and blade.

Slices prepared from the three brain regions, cortex, striatum, and hippocampus were treated in an identical manner. The slices were individually incubated in 5ml beakers containing 4.5ml (final volume) of oxygenated Krebs-Tris medium. Pure oxygen was provided to each vessel by a gassing manifold. Twenty-five minutes into the incubation period antagonists were added followed, 5 minutes later by the addition of $^{45}\text{Ca}^{2+}$ (0.14 Ci/mole, final specific activity, Amersham International, 7.04×10^{-12} moles Ca^{2+}). The relevant concentration of depolarizing agent or agonist, dissolved in 200µl of the incubation medium, was added 1 minute after the addition of the radioactive Ca^{2+} . After a total of 12 minutes incubation with the Ca^{2+} , each slice was removed and immersed for 5 seconds in 2ml of the 30mM EGTA stopping solution (composition as above). Each slice was then transferred in quick succession through 3x2ml washes of K^+ free Na^+ washing medium. They were then solubilised in 1ml of 1N NaOH. Aliquots were taken for measurement of Ca^{2+} uptake, protein determination, and K^+ content of the slice.

2.3 Scintillation counting

2.3a Preparation of samples

Aliquots (500 μ l) were taken from the final NaOH digest, neutralised with a few drops of 2N HCl and then added to 10ml of a liquid scintillation cocktail (Packard, 199). The tissue content of $^{45}\text{Ca}^{2+}$ was counted in a Packard 300 CD Tricarb Liquid Scintillation Counter which had been programmed with a $^{45}\text{Ca}^{2+}$ quench curve.

2.3b Quantification of the calcium accumulation.

Calculations were performed to convert the DPM to nmoles total Ca^{2+} uptake/100mg protein/12 min. Due to the relatively short half life of $^{45}\text{Ca}^{2+}$ (164 days), the volume of $^{45}\text{Ca}^{2+}$ taken from the stock had to be corrected for decay to ensure that 0.4 μ Ci of $^{45}\text{Ca}^{2+}$ were always added to each incubation. The absolute amount of Ca^{2+} added in the form of $^{45}\text{Ca}^{2+}$ was of the order of 7×10^{-12} moles and this was not considered significant. The concentration of Ca^{2+} in the incubation medium was 1.2mM (5.4×10^{-3} moles/4.5ml.), so at the beginning of the incubation the ratio of hot $^{45}\text{Ca}^{2+}$: cold Ca^2 was:

$$\frac{\text{Hot calcium}}{\text{Cold calcium}} = \frac{0.4}{5.4 \times 10^{-3}} \quad \mu\text{Ci/nmole}$$

The hot calcium taken up into each slice was determined from the DPM values obtained from the scintillation counter:

$$\frac{Z}{2.2 \times 10^6} \quad \mu\text{Ci were taken up into the slice,}$$

where Z is the DPM value for that sample.

Since the ratio of hot calcium to cold calcium is considered to be constant throughout the incubation the total Ca^{2+} accumulated is given as follows:

$$\frac{5.4 \times 10^3 \times Z}{0.4 \times 2.2 \times 10^6} \text{ nmoles} \Rightarrow \frac{1.2 \times Z}{0.4 \times 10^3 \times 2.2} \text{ nmoles}$$

2.4 Determination of protein levels.

The protein content of slices following solubilisation in 1N NaOH was determined using a Technicon autoanalyser by the method of Lowry et al (1951). Bovine serum albumin standards were employed over the range 10-200 $\mu\text{g}/\text{ml}$.

2.5 Measurement of tissue potassium content.

Determinations of tissue potassium ion concentration were carried out on diluted aliquots of 1N NaOH tissue digests. Potassium was measured using a CB 900 Atomic Absorption Spectrophotometer (GBC Scientific Equipment, Victoria, Australia) reading at 764.4nm, with a lamp current of 0.045 nAmps. Standard solutions of potassium dissolved in 1N NaOH were used to quantitate values. The range was from 0.1-10 ppm, where 10 ppm is equivalent to 255 nequivalents/ml.

2.6 Amino Acid Analysis.

2.6a Sample Preparation

A 500 μl aliquot of the supernatant liquid was taken from 2.2a and was added to 250 μl of 0.025 N HCl containing norleucine so that the final norleucine concentration was 50nmole/ml. The samples were then stored at -18°C. A similar aliquot, 500 μl , was taken from the incubation medium from 2.2b. This was centrifuged (10,000g for 3 minutes, Eppendorf bench top centrifuge) to remove any tissue particles. The supernatant liquid was removed and added to 250 μl of 0.025N

HCl containing norleucine so that the final norleucine concentration was 50nmole/ml. Samples were again stored at -18°C.

Before analysis the samples were thawed and evaporated to dryness using a dessicator. The dessicator was attached to a vacuum pump via a "cold finger" attachment cooled by solid CO₂ and propan-2-ol. The amino acids were then extracted into methanol (1ml), centrifuged to remove insoluble salts and evaporated to dryness. This process removed all lipids which would interfere with later analysis of amino acid concentration. The amino acids were finally dissolved in 500µl of 0.025 M HCl for analysis.

2.6b Analytical system

Amino acid concentrations were measured by autoanalysis (Norris et al, 1980) using a chromospek 180 with fluorimeter J143 (Rank Hilger, Margate, Kent, UK). Programmed gradient elution (pH2 to pH12) was used with an acid buffer (0.05M citric acid, 0.15M LiCl, "super pure" water to 1000ml) and titrated against a basic buffer (0.05M boric acid and 0.2M LiOH) run time approximately 2 hours. The fluorogenic agent (Benson and Hare, 1975) was orthaphthaldehyde (OPA) with mercaptoethanol (800mg OPA, 3ml mercapto ethanol, "super pure" water to 1000 ml). This was performed in a Na(CO₃)₂ buffer adjusted to pH10 with NaOH and containing 2ml/litre sodium hypochlorite to maintain a quiet baseline. "Super pure" water was used throughout the preparation of the samples and buffers, and was prepared by deionising distilled water through a Millipore Super Q deioniser. To prevent bacterial growth 0.1% phenol was added to the buffers as a bactericide, this did not affect the detection of the amino acids of interest.

2.6c Quantitation of amino acid concentration

Since all amino acids do not react to the same extent with OPA, colour factors (F) were calculated for each amino acid. This was achieved by dividing the norleucine peak height by the other amino acid peak heights obtained from a standard mixture of amino acids (amino acid standards: British Drug House, Poole, Dorset). The standard stock was diluted to 25nmole/ml as this was within the sensitivity range of the machine. GABA and norleucine were added to the diluted standard.

Amounts of each amino acid in the sample were calculated using the formula:

$$\text{nmole amino acid} = \frac{A}{N} \times F \times C,$$

where A = height of amino acid peak under analysis

N = height of norleucine peak in sample under analysis

F = colour factor

C = concentration of norleucine (internal standard) present in sample under analysis

The final result is then corrected for dilutions during preparation and is then expressed as nmoles amino acid released/100mg protein.

2.7 Incorporation of $^{32}\text{P}_i$ into cerebrocortical slices.

2.7a Incubation of slices

Cerebrocortical slices were prepared as in 2.1b except that they were weighed immediately after preparation. The protein concentration as determined by the method of Lowry et al (1951, 2.4) could not be performed due to the highly radioactive state of the tissue following the experiment. Once weighed, each slice was suspended in 4.5ml (final volume) oxygenated Krebs-Tris medium for 30 minutes at 37°C. Subsequently $^{32}\text{P}_i$ was added (Amersham International, 0.2 mCi/ incubation) to each slice and the incubation allowed to continue for one hour. The depolarizing agent, dissolved in 200 μ l of the incubation medium was then added, and the experiment was terminated 1 minute later. Each slice was rapidly removed and washed in 15ml of ice cold K^+ -free Na^+ medium for 15 seconds. The slice was then either added to 1ml of 1N NaOH to determine total $^{32}\text{P}_i$ incorporation, or was added to 1ml of 10% trichloroacetic acid for further assay to determine the ratio of $^{32}\text{P}_i$ incorporation into protein and lipid.

2.7b Determination of total $^{32}\text{P}_i$ incorporation

After washing, each slice was placed in 1ml of 1N NaOH at 50°C to solubilise the tissue. A 100 μ l aliquot of the digest was taken, neutralised with a few drops of 2N HCl and added to 5ml of the liquid scintillation cocktail and counted as before (2.3). Final values are given as DPM/mg tissue wet weight.

2.7c Determination of the ratio of incorporation between lipid and protein fractions

Each slice was placed in an eppendorf tube containing 1ml of 10% trichloroacetic acid (50°C) to precipitate the protein. Each eppendorf tube was rapidly agitated during the 50 minutes using a "vortex whirly mixer". The suspension was finally centrifuged using an eppendorf bench top centrifuge to pellet the protein. The supernatant liquid containing the soluble lipid was removed and retained to determine the content of radioactivity. To extract any remaining lipid the precipitate was resuspended in 1ml of an ethanol:methanol mixture (1:1) containing acidified diethylether at 0°C. A 5 minute extraction period was made then the preparation was recentrifuged as above and the supernatant liquid retained for determination of $^{32}\text{P}_i$ content. This extraction procedure was repeated twice more, and the final precipitate was dissolved in 1ml of 1N NaOH at 50°C. A 100 μl aliquot of this digest was then taken for determination of radioactive content as described (2.7b).

The DPM values obtained from the three methanol/ethanol diethylether extractions were then pooled with the DPM value obtained from the initial (TCA) supernatant liquid. On correcting each value for volume, the final value gives an estimation as to the total $^{32}\text{P}_i$ incorporation into the lipid fraction. The DPM value obtained from the NaOH solubilised pellet gives an estimation of $^{32}\text{P}_i$ incorporation into proteins. Final values are expressed as DPM $^{32}\text{P}_i$ incorporation/mg tissue wet weight.

2.8 Drugs

Drugs were dissolved in Krebs-Tris medium unless otherwise stated. The opioids used in chapter 4 were dissolved in the incubation medium which was buffered to pH 7.4. Etorphine, ethylketocyclazocine, and the WIN 4444-1 compounds were dissolved in deionised water. Tifluadom was dissolved in ethanol and then diluted with the incubation medium until the final concentration of ethanol in the incubation vessel containing the brain slice was less than 0.3%.

For experiments involving noradrenaline or dopamine (chapter 5) the drugs were dissolved in medium containing nialamide (10^{-5}M) and ascorbate (10^{-4}M). These drugs were present as monoamine oxidase inhibitor and antioxidant respectively, and were found to have no effect on basal or stimulated levels of Ca^{2+} uptake, amino acid release, or the K^{+} content of the slices. Chlorimiprimine (10^{-6}M) was used in experiments with 5-hydroxytryptamine, and acted as an uptake blocker, again no change in either basal or stimulated levels were noted.

The anticonvulsants (chapter 6) were either dissolved in ethanol or the incubation medium. Again the final ethanol concentration in the presence of the brain slice was below 0.3%. The ω -phosphonic- α -amino carboxylic acids and their isomers were dissolved in 1N NaOH and then buffered to pH 7.4 with HCl.

CHAPTER 3

BRAIN SLICES: THEIR USE IN *IN VITRO* STUDIES

- 3.1 Introduction

- 3.2 Results
 - 3.2a Temperature of the cutting block
 - 3.2b Evaporational loss
 - 3.2c Washing procedure
 - 3.2d Variations between slicing techniques
 - 3.2e Time course of spontaneous and evoked Ca^{2+} uptake
 - 3.2f Effect of varying external Ca^{2+} on the uptake of Ca^{2+}
 - 3.2g Depolarizing action of veratrine
 - 3.2h Effect of varying external K^{+} concentration on Ca^{2+} uptake and amino acid release
 - 3.2i Depolarizing action of veratrine and high K^{+} on striatal slices
 - 3.2j The influence Ca^{2+} on spontaneous and stimulated amino acid release
 - 3.2k Effect of extracellular Na^{+} on veratrine-induced amino acid release

- 3.3 Discussion

3.1 Introduction

Many workers have used brain slices and have shown, by detailed experiments, that the slices are able to perform satisfactorily many of the metabolic functions of the *in vitro* brain (McIlwain et al, 1951; Toggenburger et al, 1983; Whittingham et al, 1984). Electrophysiological experiments on the rat brain show that the neurones are able to maintain a negative membrane potential and respond to depolarizing stimuli. The viability of the slice depends greatly upon the speed of preparation, the thickness of the slice and also the amount of damage caused on slicing. These factors will be analysed.

To ensure that the uptake of Ca^{2+} determined experimentally is a true indication of the specific uptake and not due to enhanced binding of Ca^{2+} to the external membranes, the washing procedure was studied and this is also discussed.

In the majority of the experiments two agents, either veratrine or K^+ at high concentrations, were used to stimulate Ca^{2+} uptake and to release endogenous neurotransmitters. Veratrine is a complex mixture of steriod veratrum alkaloids isolated from the roots of liliaceous plants, particularly of the veratrum genera. The alkaloids vary greatly in their activity with veratridine the most potent, and cevadine being less active. The other three alkaloids making up veratrine are much less potent in respect to veratridine. The relative proportion of the five alkaloids varies between batches and the molecular weight is calculated as the average of the five components.

Veratrine is known to depolarize certain excitable cells by blocking the inactivation of voltage-dependent Na^+ channels (Ulbrich, 1969; Honerjager, 1983). Veratridine has no action on the Na^+/K^+ ATPase.

High external K^+ concentrations cause depolarization by reducing the electrochemical gradient across the synaptic membrane (Goldring and Blaustein, 1973). As a result of the depolarization by either veratrine or high K^+ there should be a concomitant influx of Ca^{2+} ions into the brain slice in accordance with the Ca^{2+} hypothesis of Katz and Melidi (1967 a and b). This influx may be studied directly by following the uptake of radioactive Ca^{2+} . Uptake of Ca^{2+} across rat brain slice membranes has been shown by a number of workers (Leslie et al, 1983; Retz and Coyle, 1984) to be enhanced by high K^+ , veratrine, excitatory amino acids and their analogues, for example, N-methyl-D-aspartic acid or kainic acid. In the following section, evidence is given that rat brain slices taken from various regions can take up Ca^{2+} and release neurotransmitters on depolarization.

3.2 Results

3.2a Temperature of the cutting block

Basal and veratrine-stimulated Ca^{2+} uptake was enhanced and more consistent when cerebrocortical slices were prepared on a dissecting block warmed to 37°C as compared to slices prepared on a block held at 0°C or at 20°C (Table 3.1).

3.2b Evaporation losses

Reduction in the loss of volume by evaporation was assessed by measuring the loss in weight of the incubations at the end of the experiment. The loss in volume was greater for oxygenated incubations as compared to non-gassed incubations. The same was true for covered-gassed incubations and non-covered-gassed incubations where the latter lost more incubation medium (Table 3.2).

3.2c Washing procedure

Rapid serial dips in the wash media at room temperature removed $^{45}\text{Ca}^{2+}$ which was not closely associated to the brain slices. Rapid dipping in EGTA (30mM) chelated external $^{45}\text{Ca}^{2+}$ without affecting the internal stores providing that the wash was completed in 5 seconds. The wash size for ease of dipping and prevention of excess leaching was found to be 2ml. Three x 2ml washes in K^{+} free- Na^{+} medium of 5 seconds in length were found to remove, adequately, external Ca^{2+} chelated by EGTA without affecting the internal Ca^{2+} levels (Table 3.3).

3.2d Variations in slicing technique.

The viability of slices prepared by two slicing techniques were compared. Both hippocampal and cortical slices prepared manually showed a greater retention of K^{+} compared to McIlwain slices [$\mu\text{equiv}/100\text{mg protein} \pm \text{SEM}$, $n=6$; hippocampal (70.45 ± 8.4 to 41.42 ± 4.35); cortex (70.36 ± 1.72 to 19.99 ± 4.36) respectively]. Basal release of the three neuroreactive amino acids was lower from slices prepared manually and the percentage released on K^{+} - or veratrine- stimulation was higher than released from slices

prepared mechanically. Basal release of threonine from McIlwain slices was greater than the release from the manually prepared slices. However, on stimulating slices prepared by the two methods with either veratrine or K^+ there was no significant change in threonine release as compared to basal level (Table 3.4). The Ca^{2+} data show that there was a lower basal influx of Ca^{2+} into manually prepared slices as compared to the McIlwain slices (nmoles Ca^{2+} /100mg protein $\pm$ SEM, n=6: hippocampus: 2313 ± 39 , 4449 ± 158 ; cortex: 1424 ± 106 , 3901 ± 71 , respectively) (Table 3.4). Stimulation by both veratrine and K^+ caused smaller percentage increases for the McIlwain slices than for the manually prepared slices. Due to the lower basal levels of Ca^{2+} influx, endogenous amino acid release, and the higher level of K^+ content compared to the mechanically chopped slices manually chopped slices were used in preference throughout the rest of the work.

3.2e Time course of spontaneous and evoked Ca^{2+} uptake.

An initial early component of the Ca^{2+} influx into non-depolarized brain slices was completed within the first 5 second period, and this was followed by a slower rate of Ca^{2+} uptake. After 7 min there was no net change in the rate of Ca^{2+} entry. Veratrine-depolarized slices did not appear to have this very rapid early stage, however the slower component was enhanced by veratrine and the overall accumulation of Ca^{2+} by stimulated brain slices was greater than for resting brain slices (Fig 3.1).

3.2f Effect of varying external Ca^{2+} on the uptake of $^{45}Ca^{2+}$

The rate of basal Ca^{2+} uptake into cortical brain slices became saturable with increasing concentrations of Ca^{2+} in the incubation medium (Fig 3.2a). The plateau occurred at about 1.2mM $[Ca^{2+}]_o$. Values for K_m and V_{max} were obtained from an Eadie Hofstee plot (Fig 3.2b) and were 1.14mM and 2040 nmoles Ca^{2+} /100mg protein /12 minutes respectively. A Ca^{2+} concentration of 1.2mM was then utilized throughout all further experiments except where otherwise indicated.

3.2g The depolarizing action of veratrine

Increasing the concentration of veratrine induced a dose-dependent decrease in the level of K^+ retained within the slice (Fig 3.3). The extent of depolarization caused by veratrine, as

indicated by K^+ loss, became maximal at $50\mu M$. The accumulation of Ca^{2+} was similarly affected in a dose-dependent manner, being maximal at $50\mu M$ (184% of control). The release of aspartate, GABA and glutamate was stimulated by veratrine, again the maximal response occurred with $50\mu M$ veratrine (% of control; aspartate, 248; GABA, 256; glutamate, 168). The release of threonine, a non-neuroactive metabolic amino acid was not affected by any concentration of veratrine used. The stimulated release of other amino acids was enhanced by veratrine but to a lesser extent than glutamate, aspartate or GABA. The stimulatory effect of veratrine on Ca^{2+} uptake, K^+ content, and amino acid release was totally inhibited by $1\mu M$ tetrodotoxin (TTX) (Table 3.5)

3.2h Effect of varying external K^+ concentration on Ca^{2+} uptake and release of endogenous amino acid neurotransmitters.

Increasing concentrations of KCl were added to the incubation medium, and there was a dose-dependent increase in both the Ca^{2+} influx and in the endogenous amino acid neurotransmitter release (Fig 3.4). There was no significant stimulation of threonine release at any K^+ concentration used. Maximal stimulation of both Ca^{2+} uptake and amino acid release occurred at approximately 60mM, and 25mM K^+ was the lowest concentration which produced a significant response. On running parallel experiments with increasing Na^+ levels, instead of K^+ , no stimulation of Ca^{2+} uptake or neurotransmitter release was observed (Fig 3.4). The effects of high K^+ were resistant to $1\mu M$ TTX (Table 3.5).

3.2i Depolarizing action of veratrine and of high K^+ on striatal slices.

Depolarizing concentrations of both veratrine ($75\mu M$) and K^+ (56mM) significantly enhanced the influx of Ca^{2+} into striatal slices (178% and 196% of control respectively). The release of glutamate was similarly enhanced by both depolarizing agents (Table 3.6). The addition of $1\mu M$ TTX completely inhibited both the veratrine-induced Ca^{2+} uptake and amino acid release, whilst TTX at this concentration did not effect the high K^+ -induced effects.

4.2j Influence of Ca^{2+} on spontaneous and stimulated amino acid release.

Basal release of amino acids was reduced in the absence of external Ca^{2+} . Veratrine-

enhanced release of glutamate, aspartate, and GABA was not significantly affected by the removal of external Ca^{2+} . However the K^{+} -evoked release of these amino acids was greatly reduced and no longer significant when external Ca^{2+} was absent (Fig 3.5). The release of amino acids evoked by veratrine in the absence of Ca^{2+} was still abolished by $1\mu\text{M}$ TTX. The release of threonine was not significantly effected under these conditions.

3.2k Effect of extracellular Na^{+} on veratrine-induced amino acid release

Increasing the Na^{+} concentration of the incubation medium did not significantly affect the veratrine-induced depolarization or the amino acid release in Ca^{2+} -free medium. Control incubations contained $100\mu\text{M}$ sucrose. There was in general a slight, but not significant, increase in amino acid release in hyperosmotic conditions as compared to amino acid release in osmolar Ca^{2+} -free incubation media,

Table 3.1 **Effect of the temperature of the dissecting block on slice viability.**

nmole Ca ²⁺ /100mg protein			
Temp °C	0	20	37
Control	976 ± 367	1174 ± 306	1424 ± 106
Vt	1551 ± 436 (158)	2101 ± 384 (179)*	2819 ± 236 (198)***

Rat cerebral corteces were prepared as in 2.1b and dissected out onto the block. The block was either at 0°C, 20°C or 37°C. Slices were then cut and incubated as in 2.2b and stimulated with 75µM veratrine. The data are mean ± SEM, n=6. Values in parentheses are % of control. Significance of differences between control and stimulated conditions was calculated by Student's two-tailed t-test for unpaired samples (*P<0.05;*** P<0.005).

Table 3.2 Evaporational losses.

Treatment	% loss in weight (mean $\pm$ SEM)	(n)
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NOT GASED:

Open to air	0.76 $\pm$ 0.05	6
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Covered by parafilm	0.03 $\pm$ 0.01	6
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GASED:

Open to air	0.84 $\pm$ 0.09	6
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Covered by parafilm	0.06 $\pm$ 0.02	6
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Slices were prepared as in 2.1b and incubated in pre-oxygenated Krebs-Tris medium for 30 min at 37°C in a water bath. The volume of the incubation fluid was 4.5ml and no additions were made. Incubations that were gassed with pure oxygen carried a gassing line suspended just below the surface of the incubation medium. The rate of oxygen flow was regulated to a minimum using a fine adjustment attachment to the cylinder head. The rate of oxygen flow was approximately 5 bubbles per second. The vials containing the incubations were weighed before and after the incubation period and the percentage loss by weight calculated.

Table 3.3 **Effect of the washing procedure on basal Ca^{2+} uptake into cortex slices**

Time (sec)	Volume of washes (ml)	$\mu\text{molCa}^{2+}/100\text{mg protein} \pm \text{SEM}$			
		1	2	3	4
1	2.0	5.7 ± 0.4	5.1 ± 0.3	4.9 ± 0.1	4.7 ± 0.3
1	4.5	3.7 ± 0.3	4.2 ± 0.4	4.1 ± 0.2	3.9 ± 0.3
1	9.0	3.5 ± 0.4	3.3 ± 0.6	3.1 ± 0.3	2.0 ± 0.4
5	2.0	3.6 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.0 ± 0.2
5	4.5	2.1 ± 0.2	1.9 ± 0.4	1.8 ± 0.2	1.4 ± 0.3
5	9.0	1.8 ± 0.3	1.9 ± 0.1	1.0 ± 0.4	0.9 ± 0.3
15	2	1.4 ± 0.4	1.1 ± 0.4	0.9 ± 0.7	0.6 ± 1.6
15	4.5	1.4 ± 0.4	0.9 ± 0.5	0.6 ± 0.3	0.6 ± 0.8
15	9.0	0.9 ± 0.3	0.8 ± 0.4	0.7 ± 0.3	0.6 ± 0.4
30	2	0.6 ± 0.4	0.6 ± 0.4	0.5 ± 0.6	0.4 ± 0.1
30	4.5	0.4 ± 0.1	0.5 ± 0.3	0.5 ± 1.1	0.5 ± 0.4
9.0	0.45	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.3	0.5 ± 0.2

Cortex slices were prepared and incubated as described in 2.1b and 2.2b. The $^{45}\text{Ca}^{2+}$ was added after a 30 minute preincubation. The experiment was terminated by removing each slice in turn and transferring through EGTA and K^+ -free Na^+ washes of varying volume and number. The length of time of the wash was also varied. Data are expressed as mean $\pm$ SEM.

Table 3.4a and b.

Effect of different slicing techniques on the responses of cortical and hippocampal slices.

Rat brain cortex or hippocampus were manually (2.1b) or mechanically sliced with a McIlwain tissue chopper (2.1a). These slices were incubated in Krebs-Tris medium as described (2.2a or 2.2b). The uptake of Ca^{2+} , release of amino acid transmitters, and K^{+} -content were measured under basal, veratrine ($75\mu\text{M}$), or K^{+} (56mM) conditions. Data are mean $\pm$ SEM, $n=6$. Significance of difference between control and stimulated samples were calculated by Student's two-tailed t-test for unpaired samples (* $P<0.05$; ** $P<0.01$; *** $P<0.005$). Values in parentheses are % of control. The K^{+} -content of the slices stimulated by high K^{+} could not be measured.

Table 3.4a Variations between slicing techniques: Hippocampus

	MANUAL	McILWAIN
Ca ²⁺ uptake (nmol/100mg protein)		
Basal	2313 ± 39	4449 ± 158
Veratrine	4579 ± 164 (191 ± 14)***	5639 ± 642 (127 ± 26)
K ⁺	3673 ± 141 (159 ± 11)***	4732 ± 288 (106 ± 13)
K ⁺ -Content (μequiv/100mg protein)		
Basal	70.45 ± 8.4	41.42 ± 4.35
Veratrine	34.91 ± 1.86 (49 ± 3)***	29.36 ± 5.47 (71 ± 13)**
K ⁺	ND	ND
Amino acid neurotransmitter release(nmol/100mg protein)		
Glutamate:		
Basal	4437 ± 94	9835 ± 1068
Veratrine	12461 ± 246 (281 ± 14)***	17571 ± 178(178 ± 12)**
K ⁺	11473 ± 186 (258 ± 21)***	12762 ± 1041 (129 ± 31)
Threonine		
Basal	1463 ± 5	2635 ± 174
Veratrine	1283 ± 12 (87 ± 12)	2850 ± 30 (108 ± 11)
K ⁺	1599 ± 72 (109 ± 11)	2546 ± 163 (97 ± 20)
Aspartate		
Basal	2755 ± 205	3464 ± 245
Veratrine	4828 ± 72 (175 ± 2)***	5274 ± 499 (152 ± 12)**
K ⁺	4536 ± 174 (1649 ± 5)***	4703 ± 164 (136 ± 17)**
GABA		
Basal	664 ± 43	1668 ± 141
Veratrine	1894 ± 148 (285 ± 12)***	2771 ± 330 (166 ± 7)**
K ⁺	1774 ± 89 (267 ± 17)***	2434 ± 330 (145 ± 11)*

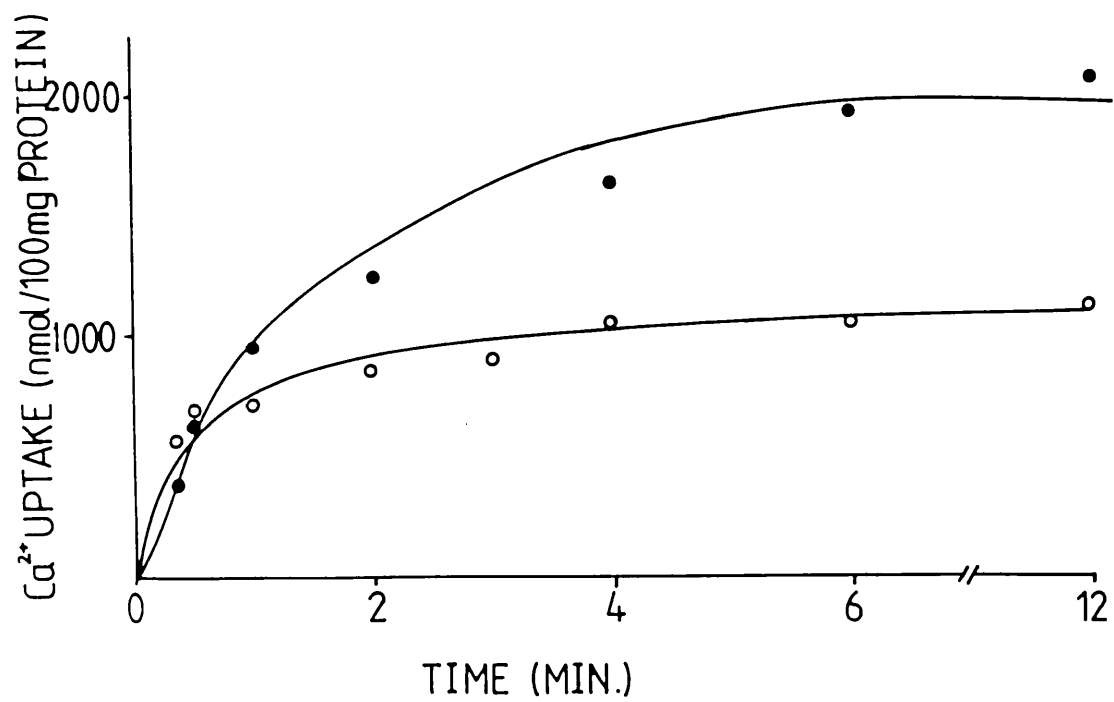
Table 3.4b Variations between slicing techniques: Cortical slices.

	MANUAL	McILWAIN
Ca²⁺ uptake (nmol/100mg protein)		
Basal	1424 ± 106	3901 ± 71
Veratrine	2804 ± 147 (197 ± 4)***	4704 ± 376 (121 ± 7)*
K ⁺	2763 ± 127 (194 ± 6)***	4503 ± 176 (115 ± 4)*
K⁺ Content (μequiv/100mg protein)		
Control	70.36 ± 1.72	19.99 ± 4.36
Veratrine	31.34 ± 2.76 (46 ± 2)***	16.31 ± 3.90 (82 ± 3)
K ⁺	ND	ND
Amino acid neurotransmitter release (nmol/100mg protein)		
Glutamate		
Control	2041 ± 159	2713 ± 1408
Veratrine	3432 ± 156 (168 ± 4)***	3314 ± 174 (122 ± 4)*
K ⁺	3418 ± 22 (167 ± 6)***	3294 ± 274 (121 ± 3)*
Threonine		
Control	393 ± 124	848 ± 86
Veratrine	632 ± 128 (161 ± 7)	1039 ± 628 (122 ± 7)
K ⁺	589 ± 187 (149 ± 14)	974 ± 114 (114 ± 11)
Aspartate		
Control	1150 ± 72	1842 ± 157
Veratrine	2852 ± 54 (248 ± 14)***	2382 ± 106 (126 ± 14)
K ⁺	3769 ± 38 (327 ± 38)***	2434 ± 102 (132 ± 6)*
GABA		
Control	260 ± 94	535 ± 136
Veratrine	667 ± 59 (256 ± 6)***	923 ± 103 (172 ± 8)*
K ⁺	740 ± 49 (285 ± 9)***	823 ± 154 (155 ± 6)

Fig 3.1 Time course for the spontaneous and evoked Ca^{2+} uptake.

Cortical brain slices were incubated in Krebs-Tris medium for 30min before the addition of $^{45}\text{Ca}^{2+}$. Veratrine ($75\mu\text{M}$) was added (where appropriate), one minute later. The total period of incubation in the Ca^{2+} was varied over the range 5 seconds to 15 minutes, after which the slices were removed and washed (2.2b). Data are mean $\pm$ SEM, $n=6$.

Basal uptake	1474 ± 112
Veratrine-stimulated uptake	2908 ± 198

Fig 3:1 TIME COURSE OF Ca^{2+} UPTAKE

Figs 3.2a and 3.2b.

Effect of varying external Ca^{2+} on the uptake of the Ca^{2+}

Rat brain cortical slices were incubated in Krebs-Tris medium containing Ca^{2+} ranging in concentrations from 0.2mM to 1.4mM. After 30 minutes preincubation $^{45}\text{Ca}^{2+}$ was added to each incubation. The $^{45}\text{Ca}^{2+}$ was of a sufficiently high specific activity so that it did not significantly alter the total Ca^{2+} concentration of any of the incubations. Slices were removed after the 12 minute incubation, washed (2.2b) and treated as before (2.3). Data are mean $\pm$ SEM, n=6.

Fig3.2 EFFECT OF VARYING EXTERNAL $[Ca^{2+}]$
UNSTIMULATED Ca^{2+} UPTAKE

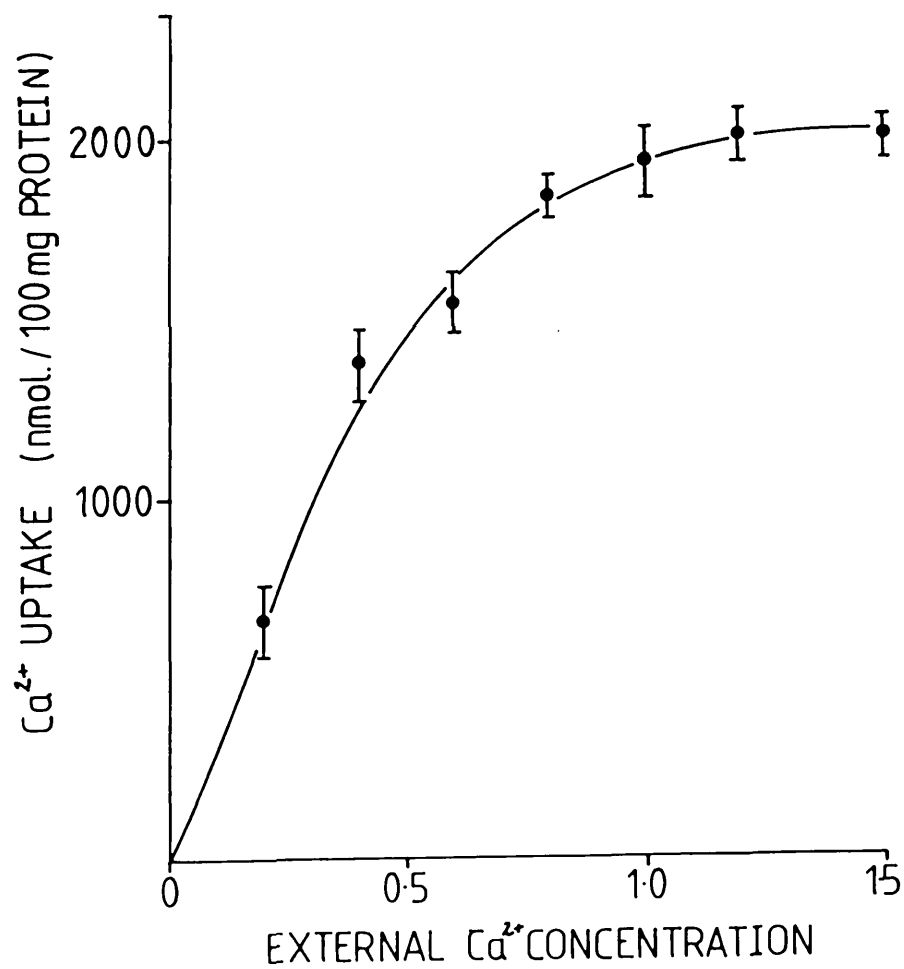


Fig 3.2 b EADIE-HOFTSEE PLOT FOR THE
EFFECT OF EXTERNAL Ca^{2+}

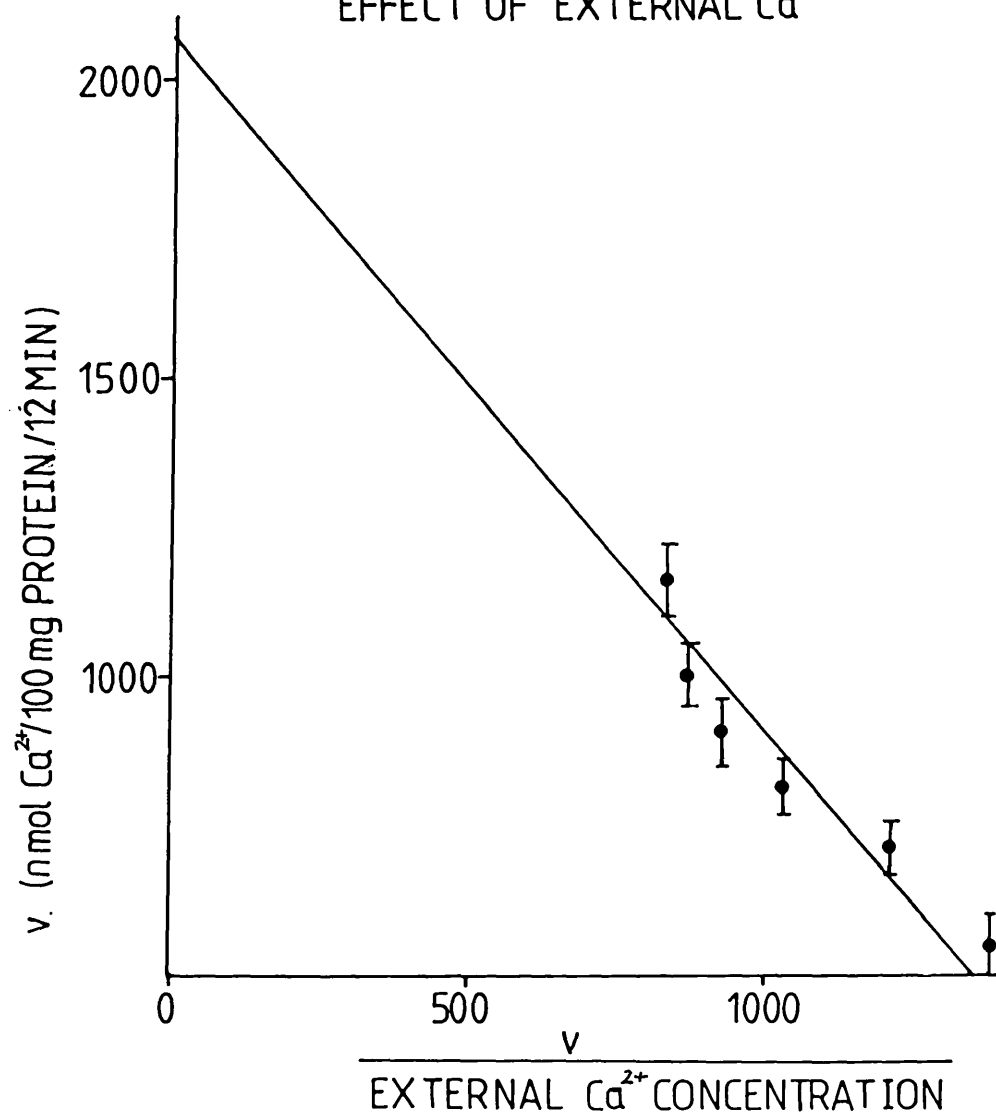


Fig 3.3 **Stimulatory action of veratrine on the uptake of Ca^{2+} , K^{+} -content, and amino acid release.**

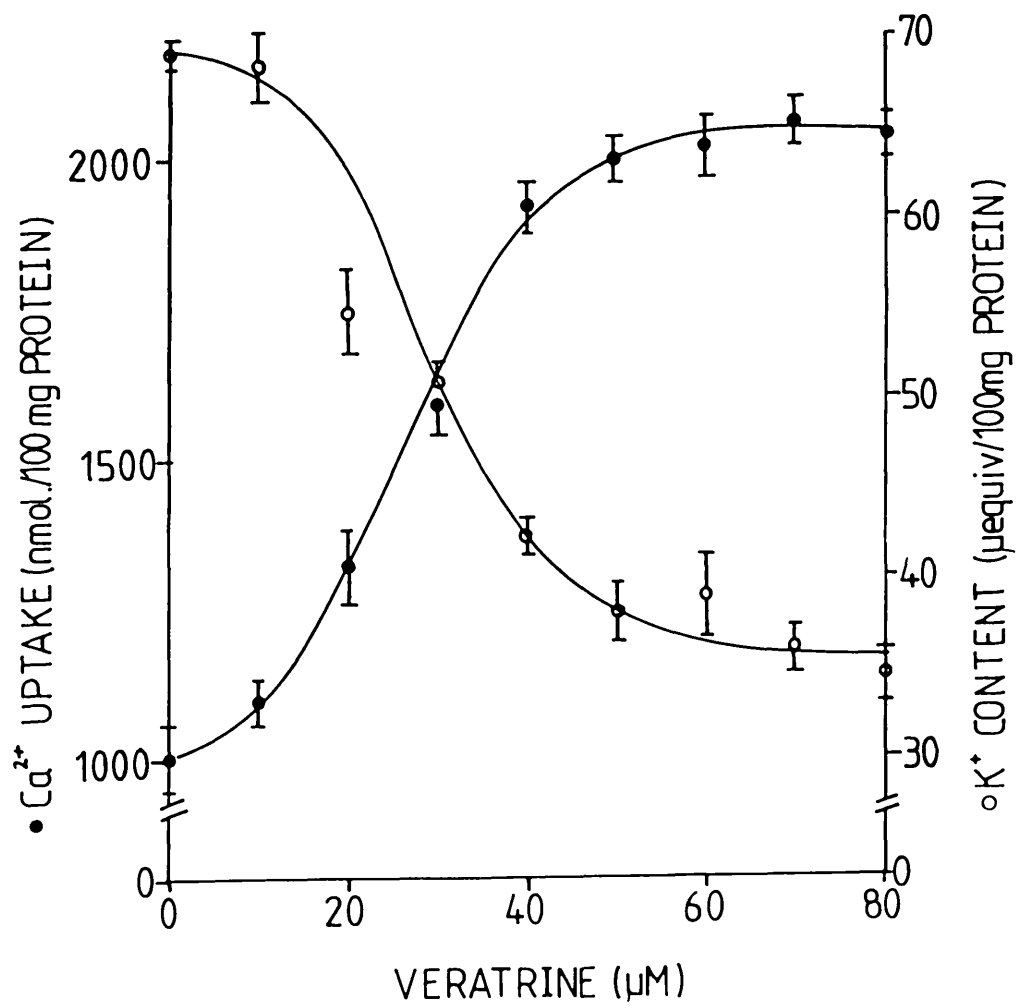
Cerebro-cortical slices were prepared and incubated as before (2.1b and 2.2b). The appropriate concentration of veratrine was added to each incubation 1 minute after the addition of $^{45}\text{Ca}^{2+}$. The experiments were terminated 11 minutes later.

Values are % of control $\pm$ SEM, $n=6$. Significance of difference between control and stimulated slices was calculated by Student's two-tailed t-test for unpaired samples, (* $P<0.05$; ** $P<0.01$; *** $P<0.005$).

Basal levels were:

Ca^{2+} uptake	1246 ± 127 nmol/100 protein
K^{+} -content	71 ± 6.4 μ equiv/100mg protein
glutamate release	2064 ± 159 nmol/100mg protein
aspartate release	2311 ± 137 nmol/100mg protein
GABA release	374 ± 26 nmol/100mg protein.

Fig 3.3a VERATRINE DOSE RESPONSE CURVES:
 Ca^{2+} UPTAKE AND K^+ CONTENT.



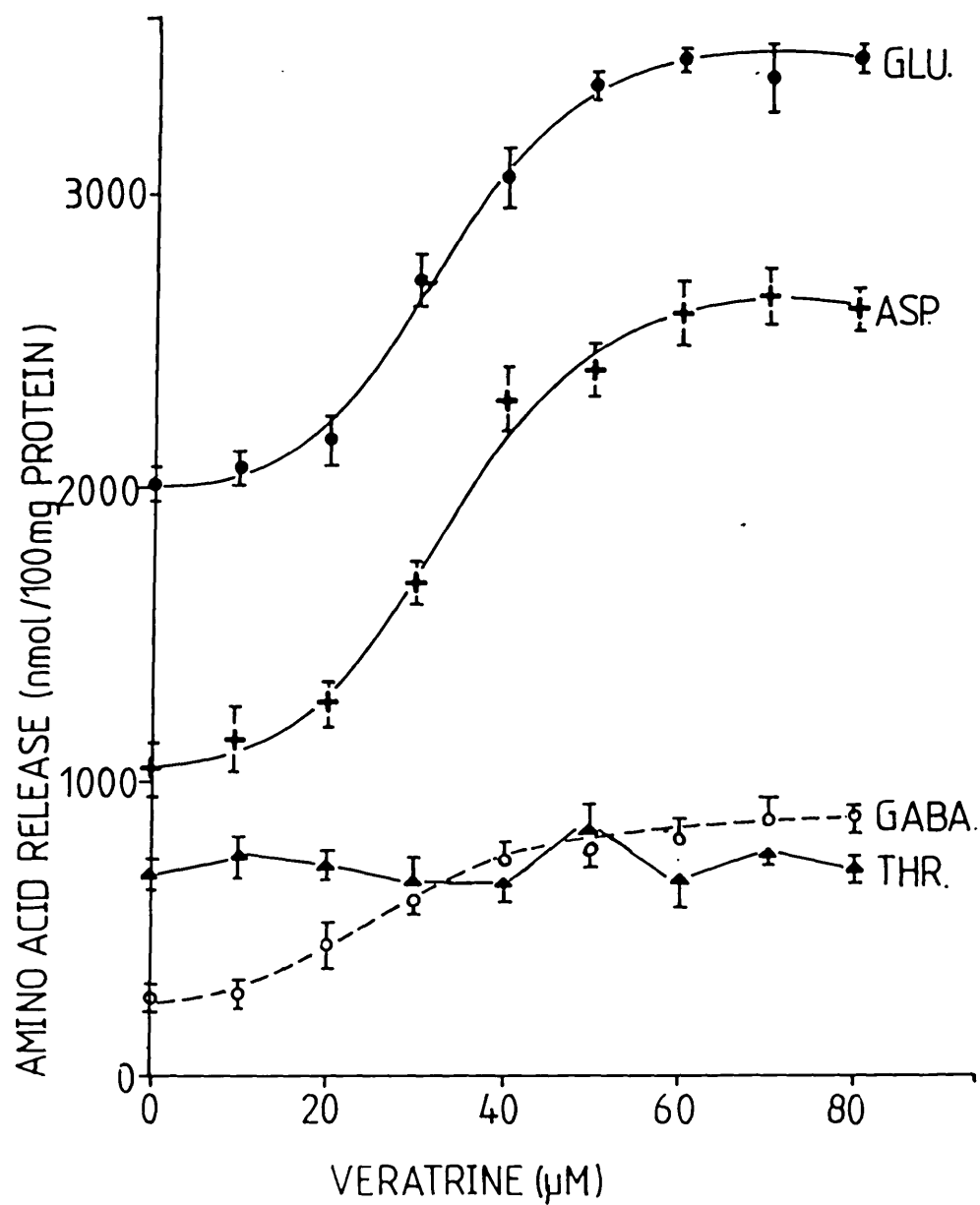


Fig3.3b VERATRINE DOSE RESPONSE CURVES :
AMINO ACID RELEASE

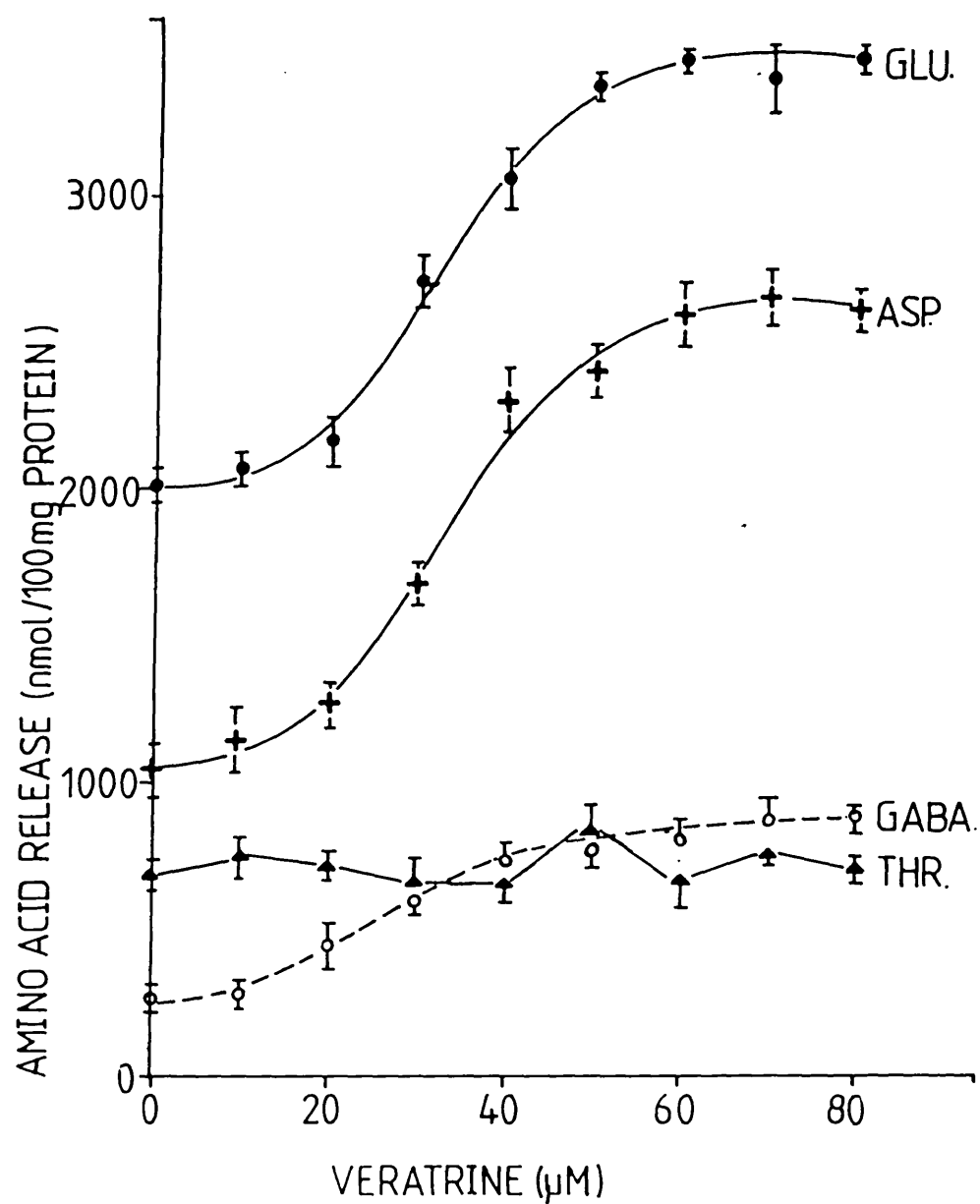


Fig3.3b VERATRINE DOSE RESPONSE CURVES :
AMINO ACID RELEASE

Fig 3.4 Effect of varying external K^+ concentration on Ca^{2+} uptake and release of endogenous amino acid neurotransmitters

Cerebro-cortical slices were prepared and incubated as in 2.1b and 2.2b. After the addition of $^{45}Ca^{2+}$, varying concentrations of K^+ were added and the experiment terminated 11 minutes later.

Values are % of control $\pm$ SEM, n=6. The significance of difference between control and K^+ -stimulated was calculated by Student's two-tailed t-test for unpaired samples (* P <0.05; ** P <0.01; *** P <0.005).

Basal levels were:

Ca^{2+} uptake	1364 ± 174 nmol/100mg protein
glutamate release	2641 ± 147 nmol/100mg protein
aspartate release	1886 ± 187 nmol/100mg protein
GABA release	224 ± 27 nmol/100mg protein.

Fig34a K^+ DOSE RESPONSE CURVE:
 Ca^{2+} UPTAKE

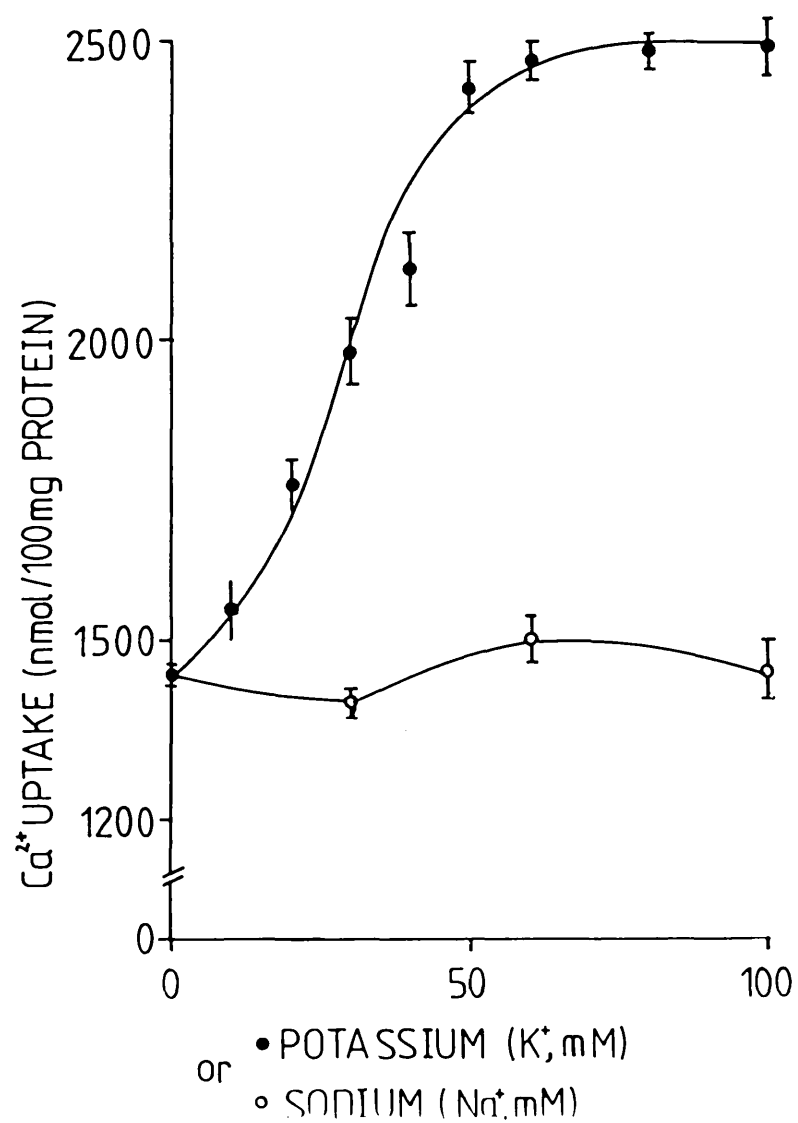


Fig 3.4b K^+ DOSE RESPONSE CURVES:
AMINO ACID RELEASE

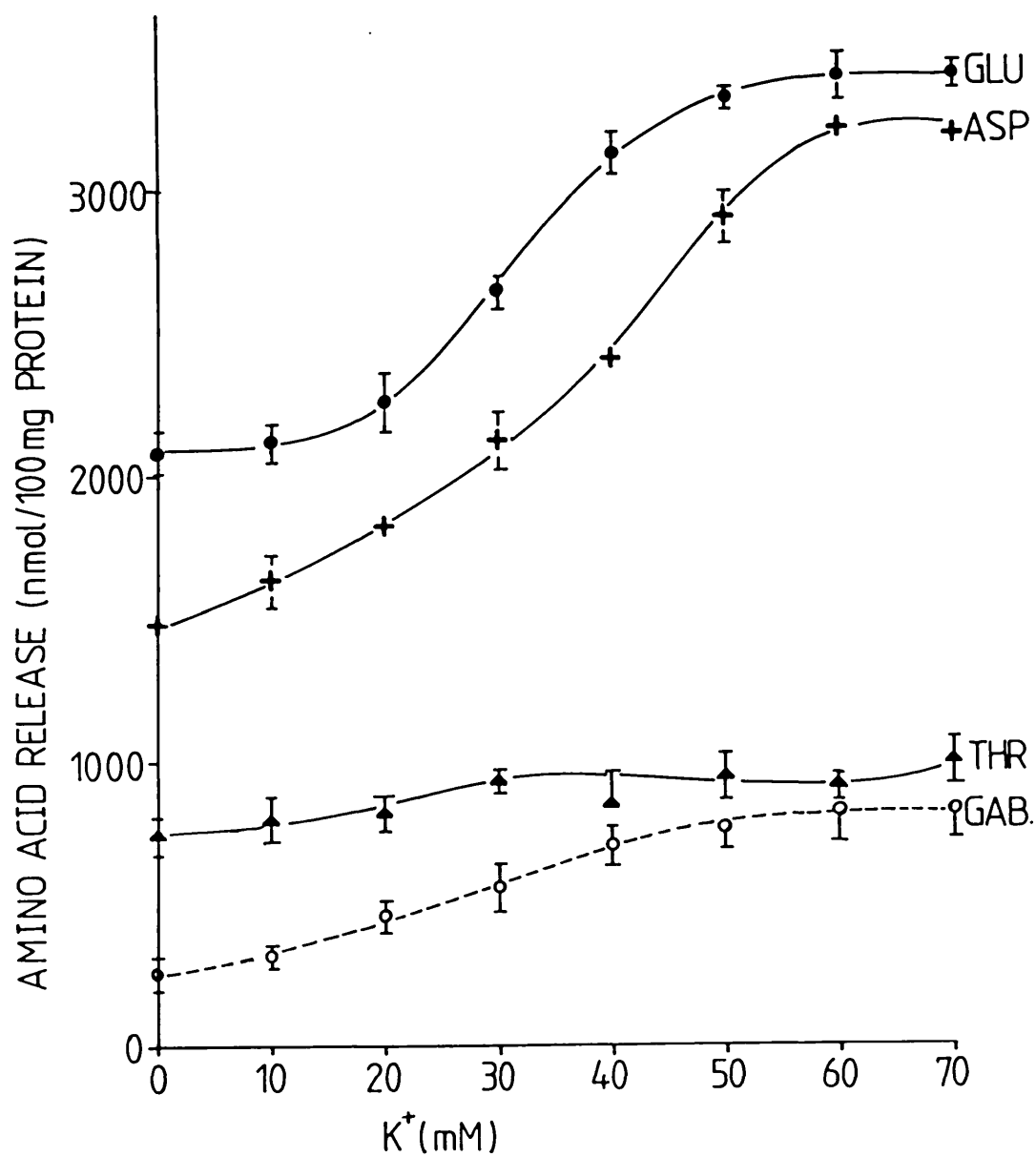


Table 3.5 The action of TTX on either veratrine or K⁺ stimulated slices.

Treatment	Ca ²⁺ uptake	K ⁺ content
	nmol/100mg protein	μequiv/100mg protein
Control	1364 ± 157	71.36 ± 1.98
TTX 1μM	1258 ± 134	72.98 ± 2.54
Veratrine 75μM	2987 ± 218	35.76 ± 3.23
Veratrine 75μM + TTX 1μM	1345 ± 245	69.38 ± 3.99
K ⁺ 56mM	2678 ± 263	ND
K ⁺ 56mM + TTX 1μM	2599 ± 2347	ND

Amino acid release (nmol/100mg protein)

	Asp	Glu	GABA	Thr
Control	1167 ± 91	2112 ± 231	234 ± 77	334 ± 12
TTX 1μM	1121 ± 123	1998 ± 123	245 ± 23	339 ± 23
Veratrine 75μM	2789 ± 334	3290 ± 234	567 ± 121	298 ± 12
Veratrine 75μM + TTX 1μM	1232 ± 132	1998 ± 215	224 ± 43	329 ± 32
K ⁺ 56mM	3121 ± 234	3145 ± 342	601 ± 103	345 ± 34
K ⁺ 56mM + TTX 1μM	3212 ± 239	3087 ± 267	597 ± 97	315 ± 32

Cortical slices were prepared and incubated as described (2.1b and 2.2b). TTX (1μM) was added to the appropriate incubation before the addition of the slice. The ⁴⁵Ca²⁺ was added 30 min into the pre-incubation period and the depolarizing agents were added 1 min later. The experiment was terminated 11 min later. The uptake of Ca²⁺ and the K⁺ content of each slice was determined as described (2.3 and 2.5). The level of amino acid released into the

incubation medium was also measured.

Table 3.6 Depolarizing action of veratrine and high K⁺ on striatal slices.

Treatment	Ca ²⁺ Uptake (nmol/100mg prot)	K ⁺ -Content (μ equiv/100mg prot)	Glutamate release (nmol/100mg prot)
Control	1047 $\pm$ 85	55.8 $\pm$ 4.9	1041 $\pm$ 165
Veratrine	1864 $\pm$ 136***	30.36 $\pm$ 0.26***	2036 $\pm$ 149***
Veratrine + TTX	1156 $\pm$ 98	54.4 $\pm$ 7.1	1163 $\pm$ 147
K ⁺	1763 $\pm$ 144	ND	1969 $\pm$ 214
K ⁺ + TTX	1807 $\pm$ 106	ND	1993 $\pm$ 174

Striatal slices were prepared and incubated as before (2.1b and 2.2b). TTX (1 μ M) was added to the incubation medium before the addition of the slice. Veratrine (75 μ M) or K⁺ (56mM) were used as the depolarizing agents. After the experiment was completed the slices were solubilised in 1N NaOH and samples of this digest were taken for determination of radioactive content and of K⁺-content. Aliquots of the incubation media were taken for amino acid analysis. The K⁺-content of the slices stimulated by K⁺ (56mM) could not be determined (ND).

Values are mean $\pm$ SEM, n=6. Significance of difference between control and stimulated samples was calculated by Student's two-tailed t-test for unpaired samples (*** P<0.005).

Fig 3.5 Influence of Ca^{2+} on spontaneous and stimulated amino acid release

Cortical brain slices were incubated in Krebs-Tris medium $\pm$ 1.2mM Ca^{2+} . The $^{45}\text{Ca}^{2+}$ was not added in this experiment and K^+ (56mM) or veratrine (75 μM) were added 31 minutes into the incubation, which was terminated 11 minutes later. Values shown are % of control $\pm$ SEM, n=6. Significance of difference between control and stimulated release was calculated by Student's two tailed t-test for unpaired samples, (* $P<0.05$; ** $P<0.01$; *** $P<0.005$).

Basal levels of release (nmol/100mg protein $\pm$ SEM, n=6)

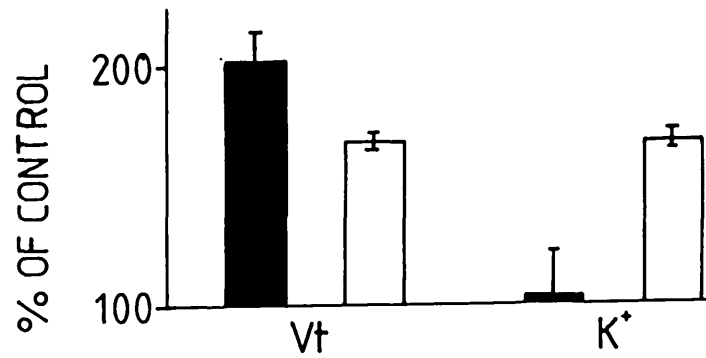
	Ca^{2+} free	Ca^{2+} 1.2mM
Glutamate	2477 $\pm$ 279	2041 $\pm$ 75
Aspartate	1519 $\pm$ 94	1150 $\pm$ 137
GABA	520 $\pm$ 94	374 $\pm$ 26
Threonine	914 $\pm$ 90	848 $\pm$ 51

□ + Ca^{2+}

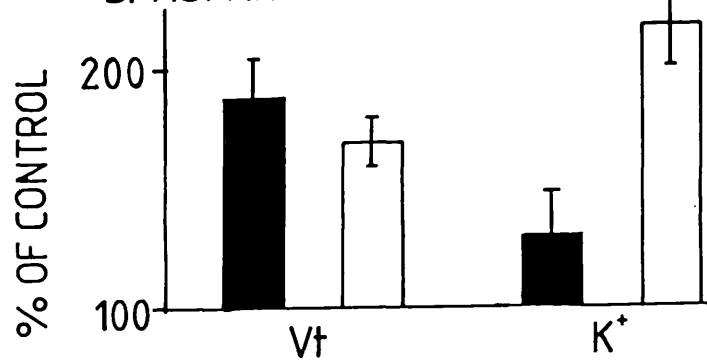
■ - Ca^{2+}

Fig 3.5 THE INFLUENCE OF Ca^{2+} ON SPONTANEOUS AND STIMULATED AMINO ACID RELEASE.

A. GLUTAMATE



B. ASPARTATE



C. GABA

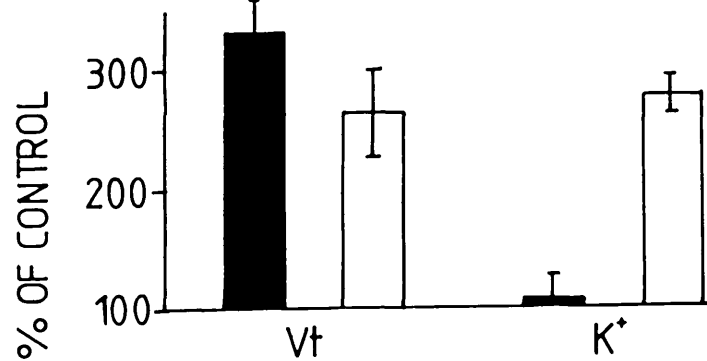


Table 3.7 **The effect of extracellular Na⁺ on veratrine-induced amino acid release.**

Treatment	% Depolarization	% Amino acid release		
		ASP	GLU	GABA
Na ⁺ (50mM)	42	216	207	366
Veratrine	44	209	194	394
None	-	187	187	356

Cortical brain slices were incubated in Ca²⁺ free Krebs-Tris medium containing either 50mM NaCl or 100mM sucrose. Incubations were carried out as for 2.2b, except that Ca²⁺ was not added. Veratrine (75μM) was used as depolarizing agent. At the end of the experiment, amino acid levels were measured in the incubation medium and the K⁺-content of each slice was determined.

Basal K⁺-content when 50mM Na⁺ was present was 56.37 ± 0.28 μequiv/100mg protein, and was 53.62 ± 1.64 μequiv/100mg protein in the presence of 100mM sucrose. Basal amino acid release was (nmol/100mg protein), 2064 ± 147; 1789 ± 146; 561 ± 72, for glutamate, aspartate, and GABA respectively. The values are expressed as mean ± SEM, n=6.

McIlwain, and co-workers in many of their studies (1951) have shown that the size of the brain slice is very important, in that slices upto 400 μ m in thickness are able to respire normally, are able to take up glucose actively, and to maintain an ionic gradient across their membrane. Slices above 400 μ m in thickness are unable to respire normally due to problems of diffusion of glucose and oxygen into the central areas and also the inability of waste products to diffuse out rapidly from these areas. Rates at which cortical brain slices metabolise glucose are very similar to rates for the *in vivo* brain (Schmidt et al, 1945) and for synaptosomes. Cortical synaptosomes respire in the region of 60 μ mole O₂/100mg protein/hour on incubation with 5mM glucose (Bradford and Thomas, 1969). To provide such a quantity of oxygen to the slice a continuous passage of oxygen into the medium is required to maintain the correct partial pressure. Excitation of the nerve, for example by depolarization with high K⁺, leads to a marked increase in the rates of oxygen uptake, aerobic glycolysis, carbon dioxide production and the amount of K⁺ released (Shanes, 1958; McIlwain, 1951).

This increased need for oxygen was supplied by a gassing line. If the oxygen concentration in the medium were to fall too low or the slices were prepared in excess of 400 μ m in thickness, then anoxia would develop. This results in an irreversable loss in ATP content and in phosphocreatine levels, which are the two main energy stores of the neurone. Prolonged anoxia depresses evoked responses in slices (Kass and Lipton, 1982), and there also appears to be gross disruption of tissue structure (Bak et al, 1980; Cohen, 1962).

To maintain a steady oxygen supply it was necessary to aerate the samples and this caused an increase in evaporation from the incubation medium (Table 3.2). The use of parafilm to reduce the evaporational loss, increased the difficulty of the experimental procedure for a variety of reasons. Firstly the parafilm had to be removed or punctured to allow the drugs to be added and secondly, condensation on the underside of the parafilm hampered the pipetting of drugs. It was therefore decided that the incubation vials should be left uncovered since there was only 0.59% by weight, loss of volume during a 30 minute incubation (Table 3.2), and this was considered to have negligible effects on the final results.

A study was made on whether a warmed (37°C) block, a 20°C block, or an ice-cold block should be used for dissection purposes. The results show (Table 3.1) that slices prepared from cortices maintained at 37°C were able to take up Ca²⁺ more consistantly than those prepared at 0°C or 20°C. Although slicing tissue at 4°C or 20°C rather than 37°C might be expected to cause less tissue damage, metabolic and electrical responses measured by Whittingham et al

(1984) do not support this hypothesis. Chilling the brain during slicing did not prevent, but only delayed, the post-decapitation loss of ATP. The fall in phosphocreatine levels was not prevented in the chilled slice as most of this loss occurred during the first 30 seconds following decapitation (Lowry et al, 1964) and prior to the time the slices were placed on the chilled block. Since the slicing technique used here was very rapid ie. under 2 minutes, unlike the 3-4 minutes required to prepare McIlwain chopped tissue, maintaining the slice at a temperature close to the physiological temperature may increase the rate by which the slices reach equilibrium.

Two types of slice preparation were used, McIlwain tissue chopped slices and manually prepared slices cut with a recessed guide and blade. The results (Table 3.4a and b) show that the basal uptake of Ca^{2+} is increased into chopped slices and the percentage stimulation with veratrine or K^{+} was reduced in comparison to manually prepared slices. The release of amino acids followed very similar patterns with an enhanced basal release and decreased stimulated release in comparison to the manually prepared slices. Such variations can be explained by the increased surface area damaged on mechanically slicing the tissue. Manually prepared slices had one cut surface, whereas chopped tissue had a minimum of two cut surfaces. Electronmicrographs by Garthwaite et al, (1979) show that sectioning by hand gives better morphology than mechanically chopped tissue. Whittingham et al (1984), provide several reasons for suspecting that trauma caused by slicing leads to changes in ATP levels and decreases in evoked responses. Histological studies (Garthwaite et al, 1979) have shown that a zone 50-100 μm deep on the cut surface of the slice is composed of dead or severely disrupted tissue. Thus in a slice 400 μm thick with one cut side, 25% of the volume would be composed of disrupted cells. ATP levels near *in vivo* values have been reported for slices with only one cut side (Yamamoto and Kurokawa, 1970; Ibata et al, 1971). Thus in chopped tissue with 3 or more cut edges upto 75% of the slice may be dead. The greater proportion of dead tissue in chopped slices may easily account for the variations in both Ca^{2+} uptake and stimulated responses. Increased basal levels of Ca^{2+} uptake may be the result of larger areas available for non-specific binding. The enhanced basal release of amino acids could be caused by excessive leakage of intracellular contents from the damaged cells. The reduced percentage stimulated levels are the result of the higher basal levels and the reduction in the number of viable neurones. Another problem with chopping tissue is that many interconnecting axons are severed on slicing. However, for example, when the hippocampus was sliced the cut was made longitudinally along the structure thus maintaining the dendritic and axonic connections. Thus this slice will provide a more valuable link between the *in vitro* and *in vivo* systems.

For Ca^{2+} uptake studies it was necessary to ensure that all the non-specific surface bound $^{45}\text{Ca}^{2+}$ was removed before counting. The presence of this surface-bound $^{45}\text{Ca}^{2+}$ would increase the background levels thus reducing the overall increase in Ca^{2+} measured on stimulation. The Ca^{2+} uptake was stopped by the removal of each slice and a rapid (5 second) wash in 30mM EGTA. EGTA is a very powerful Ca^{2+} chelator and is able to extract Ca^{2+} from internal stores, thus yielding apparently lower levels of Ca^{2+} uptake (Jansson et al, 1977; Wonacott et al, 1978; Scott et al, 1980). By exposing the slice to an EGTA solution for only 5 seconds it was found that there was minimal loss of Ca^{2+} from internal stores. However, if the slices were left in EGTA for longer than 5 seconds there was a large decrease in the basal Ca^{2+} uptake (Table 3.3).

Three serial washes were then performed to remove the Ca^{2+} -EGTA complex and any additional surface-bound Ca^{2+} . It was found that 3 x 2ml washes in the K^{+} -free Na^{+} medium provided the best conditions for removing the "extra" Ca^{2+} without affecting the internal stores. Basal levels of Ca^{2+} uptake were reduced when either the volume of wash medium or the number of washes were increased, probably by increasing the rate of Ca^{2+} diffusion from the internal stores.

Thus, manually prepared slices dissected at 37°C were used throughout the work reported here. They were incubated in oxygenated medium which was left uncovered throughout the experiment. The experiment was terminated by washing each slice in 1 x 2ml EGTA followed by 3 x 2ml washes in K^{+} -free Na^{+} medium, each wash was 5 seconds in duration.

A major shortcoming of these procedures is their poor time resolution, the shortest time achieved was 10 seconds which far exceeds the extremely fast Ca^{2+} influx that occurs during the first milliseconds of stimulation. Basal Ca^{2+} uptake consists of a fast phase of rapid accumulation in less than 5 seconds followed by a slow phase (Fig 3.1). A two phase uptake of Ca^{2+} into striatal slices has been recorded (Retz and Coyle, 1984). It is unlikely that the rapid phase is due to superficial binding of Ca^{2+} to the membrane. Nashchen and Blaustein (1980) suggested that the two phases represent Ca^{2+} influx via two separate populations of Ca^{2+} channel, since each phase had different properties in respect to inorganic Ca^{2+} channel blockers. It has been proposed that the role of the fast phase of entry is to modulate the phasic release of neurotransmitters following an action potential. The slow Ca^{2+} entry modulates the neurotransmitter release following a longer-lasting depolarization such as occurs on repetitive stimulation of nerve terminals (McGraw et al, 1982). Veratrine appears to enhance the slow phase of Ca^{2+} entry. Elevated concentrations of both K^{+} and of veratrine caused depolarization of neurones within slices. This depolarization would cause the opening of

voltage-dependent Ca^{2+} channels thus producing the increase in Ca^{2+} uptake (Figs 3.3,3.4 and Table 3.6). The strength of depolarization, induced by veratrine is indicated by the decrease in the K^+ content of the slice (Fig 3.3). The opening of the Ca^{2+} channels will allow Ca^{2+} to enter the terminal down its electrochemical gradient. The intracellular concentration will rise and trigger neurotransmitter release as hypothesised by Katz and Miledi (1967a)(Fig 3.3).

The cationic fluxes elicited by veratrine resemble more the fluxes occurring during physiological depolarization, since media containing high K^+ concentrations are either hypotonic (Bradford et al ,1973) or partially Na^+ deficient (Blaustein, 1975). High K^+ is known to affect glial cells, thus the results obtained from experiments in which brain slices are depolarized by high K^+ may be complicated to explain. However, prolonged depolarization by veratrine is very different from that of a brief nerve impulse. Veratrine is proposed to act preferentially on neuronal tissue and as such may be the better depolarizing agent. In most experiments both agents have been used to compare results. Veratrine-stimulated Ca^{2+} uptake into cortical slices is inhibited by TTX a selective blocker of the Na^+ channel (Table 3.5) (Narahashi et al, 1964). When added to brain slice preparations, $1\mu\text{M}$ TTX totally inhibited the veratrine-induced Ca^{2+} uptake, amino acid release, and K^+ loss, whilst TTX had no effect on either the Ca^{2+} influx or amino acid release induced by K^+ (Fig 3.5), suggesting that Na^+ channels are not involved in high K^+ evoked responses. In this study, maximal levels of Ca^{2+} uptake and amino acid release occurred with 60mM K^+ (Fig 3.4), and $50\mu\text{M}$ veratrine (Fig 3.3). These results are comparable to the results obtained by Blaustein et al (1972). Thus it appears that brain slices prepared as described above are viable and show stimulated responses in the presense of veratrine and K^+ . The blockade of veratrine-stimulated amino acid release by TTX in the absence of extracellular Ca^{2+} indicates that the action of veratrine under these observations is also mediated by Na^+ channels.

The tendency for the K^+ -stimulated amino acid release from cortical tissue be depressed in the absence of Ca^{2+} has been widely reported (de Belleruche and Bradford, 1972; Cunningham and Neal, 1981). A similar Ca^{2+} dependency is seen for noradrenaline and acetylcholine released from the rat cortex (Vargos et al, 1977; Piazzarro et al, 1986). The majority of workers report a lack of dependency on extracellular Ca^{2+} for veratrine-induced amino acid release from the mammalian cortex (Szerb, 1979; Minchin, 1980), but veratrine-stimulated release of noradrenaline and acetylcholine whether endogenous or exogenous was Ca^{2+} dependent (Levi et al, 1980; Piazzarro et al, 1986). However, Schoffelmeer and Mulder (1983) showed that the $[^3\text{H}]$ -noradrenaline release evoked by veratrine was Ca^{2+} -

independent, and that the Ca^{2+} dependency noted by the other workers had only resulted because of the short incubation time with veratrine. Sandoval (1980a and b) demonstrated that Ca^{2+} release from the mitochondria in the absence of external Ca^{2+} triggered GABA release. He used mitochondrial protonophores that destroy the mitochondrial potential and allowed mitochondrial Ca^{2+} efflux. Veratrine stimulates an increase in intracellular Na^+ concentration which may then allow Ca^{2+} efflux to occur from intracellular stores by reversing the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism present in the inner membrane of the brain mitochondria (Crompton et al, 1978; Nicholls, 1978). However, if this is the the situation then it is hard to explain why this Ca^{2+} cannot cause the release of noradrenaline or acetylcholine. It is possible that the efflux of Ca^{2+} from the intracellular stores caused by the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ antiport system is not sufficient to release neurotransmitters stored in vesicles (for example, noradrenaline) but may release extra-vesicular amino acids (for example, glutamate). A recent report by Piazzarro et al (1986) supports this view that veratrine may be able to differentiate between two pools of neurotransmitters, ie. vesicular and non-vesicular. Vesicular release is dependent on the presence of Ca^{2+} due to the exocytotic processes involved whilst non-vesicular release could occur due to depolarization possibly by making the terminals more leaky (Levi et al, 1980).

Nicholls and his co-workers (Sihra et al, 1984) proposed that veratrine caused GABA release by modulation of the plasma membrane potential rather than elevating cytosolic Ca^{2+} . They suggest that the Na^+ influx that occurred during veratrine-induced depolarization caused the efflux of a cytosolic pool of GABA by reversal of the Na^+ -dependent GABA uptake mechanism. The main evidence they presented to support this theory was that veratrine could release GABA from synaptosomes that had been incubated for one hour in a solution containing a high concentration of EGTA. Thus they concluded that there could be no internal Ca^{2+} left since all endogenous Ca^{2+} had been removed. However they did not check the endogenous levels which may have been large enough to support release.

When the Na^+ in the medium was replaced with Li^+ the synaptosomes were still depolarized. Lithium was unable to support the GABA uptake system, thus veratrine should not cause GABA release. However Cunningham and Neal (1981) found that synaptosomes still responded to veratrine by releasing GABA. When the extracellular Na^+ concentration was raised to reverse the the Na^+ gradient across the plasma membrane (Table 3.7), veratrine-induced amino acid release was not inhibited. If stimulated release were due to the reversal of Na^+ -dependent amino acid uptake, it was likely that the release would have been antagonised. Elevated sucrose concentrations were used as hypertonic controls. It is apparent that the

veratrine-stimulated release of amino acids in Ca^{2+} -free conditions was enhanced by the raising of the medium osmolarity. There was no change in the K^{+} -content of the slices in these varying conditions which suggests that veratrine ($75\mu\text{M}$) is still depolarizing the neurones.

Thus the evidence from the results presented here support the hypothesis that veratrine-induced amino acid release in Ca^{2+} -free media is regulated by the release of intracellular Ca^{2+} stores. K^{+} -stimulated amino acid release is, however, dependent on the influx of Ca^{2+} . Glial release of amino acids is thought to be Ca^{2+} -independent. Thus the almost complete inhibition of the K^{+} -evoked amino acid release in the absence of Ca^{2+} suggests that a large majority of this release is from the depolarized neurone.

CHAPTER 4

***IN VITRO* ACTION OF SOME OPIOID COMPOUNDS ON CALCIUM FLUXES, NEUROTRANSMITTER RELEASE, AND PHOSPHORYLATION.**

- 4.1** **Introduction**

- 4.2** **Results**
 - 4.2a Action of opioid compounds on Ca^{2+} fluxes, amino acid release, and K^{+} content
 - 4.2b Antagonistic effects of WIN 44441-3 and naloxone
 - 4.2c Stereospecificity of the actions of WIN 44441 isomers
 - 4.2d Effects of nucleotides on opioid action
 - 4.4e $^{32}\text{P}_i$ incorporation into cortical slices and the effects of morphine

- 4.3** **Discussion**

4.1 Introduction

It has been well established that narcotics stereospecifically activate opioid receptors in the CNS to elicit analgesia (Pert and Synder, 1973; Terenius, 1973). There is strong evidence for the existence of several different subtypes of opioid receptor (Paterson et al, 1983; Goldstein and James, 1984; Zukin and Zukin, 1984).

The concept of multiple opioid receptors originates from the pioneering work of Martin et al (1976), who studied the action of various opioid compounds on the chronic spinal dog preparation. Benzomorphan opioids had analgesic properties without being able to substitute for morphine by reducing morphine withdrawal symptoms. It was proposed that the benzomorphans act at the κ -receptor which is distinct from the μ -receptors which have a higher specificity for morphine. Many of the benzomorphans have cross reactivity with both μ - and κ -receptors, ethylketocyclazocine, the κ -prototype agonist, exhibits some μ -antagonistic activity, whilst U40-488H (trans-(+)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny)cyclohexyl] benzeneacetamide methane sulphonate) appears to be more specific for the κ -receptor (Vonvoigtlander et al, 1983; Goldstein and James, 1984). Certain benzomorphans also elicit hallucinogenic effects and it has been postulated that these effects are due to an interaction with a third receptor the σ -receptor, for which the prototype is N-allylnormetazocine (SKF 10,047). However, Wood (1982) has suggested that the σ -receptor may not be a true opioid receptor since SKF 10,047 is resistant to naloxone and is able to displace bound [^{3} H]-phencyclidine. These properties are in contrast to those of both μ - and κ - receptor agonists which are both sensitive to naloxone and are unable to displace [^{3} H]-phencyclidine binding.

A fourth receptor subtype, the δ -site, has the same characteristics as for μ - and κ -sites (see above) and has been proposed to be specific for the enkephalins (Lord et al, 1977). These receptor sites have been found to be highly concentrated in the mouse vas deferens.

Wood (1982) postulated the existence of a fifth subtype, the ϵ -receptor, which is specific for the β -endorphins. The ϵ -receptors are poorly defined and if they do exist they probably only compose a very small proportion of opioid receptors.

The μ - and δ -receptors have been localised in the rat cerebral cortex using autoradiographic techniques (Chang et al, 1979). Two different agonists were used to visualise these receptors, [^{3} H]-DAGO (Tyr-D-ala-gly-(Me)phe-gly-ol), a μ -selective ligand, and [^{3} H]-DADLE (D-ala²-D-leu⁵-enkephalinamide) a δ -selective ligand. The κ -receptors have been identified in the rat cerebral cortex (Goodman and Snyder, 1982; Burkard, 1984).

There has been much controversy as to whether these receptor subtypes exist as individual

receptors or whether they are interconvertible forms of the same receptor (Bowen et al, 1981). However, the results of selective protection experiments (Goldstein and James, 1984) are difficult to reconcile with an interconverting model for these sites. The criteria for differentiating between opioid receptors has been very unsatisfactory due to lack of specificity for both agonists and antagonists. Care must be taken in interpreting data from binding studies where selectivity of a particular ligand for a receptor does not necessarily mean that the receptor is involved in the physiological effects. Antagonists are of particular use in identifying opioid receptors since they tend to have parallel effects in both binding and pharmacological assays (Goldstein and James, 1984).

Many of the physiological effects of opioids may be due to an inhibition of neurotransmitter release. There are a number of reports of the naloxone-sensitive actions of opioid compounds in reducing the release of various neurotransmitters *in vitro* and *in vivo* (Montel et al, 1974; Arbilla and Langer, 1978; Coutinho-Netto et al, 1980 and 1982; Dhaliwal and Bradford, 1982; Jones and Marchbanks, 1982; Mulder et al, 1984). Neurotransmitter release may be inhibited by an effect of the opioid compound on the availability of Ca^{2+} in the nerve terminal. A number of studies have implicated Ca^{2+} to be involved in the pharmacological actions of narcotic analgesics (Gudelsky and Porter, 1979). Intracerebroventricular (ICV) injections of Ca^{2+} (Harris et al, 1975) antagonise the analgesic effects of morphine whilst EGTA potentiates such effects. Schmidt and Way (1980) have shown that Ca^{2+} enhances the hyperanalgesic response following withdrawal of morphine from both morphine dependent and control mice. From these observations it was concluded that both morphine and EGTA may produce analgesia by depleting a small Ca^{2+} pool in the nerve cell.

Harris et al (1976) also observed that acute treatment of morphine, 30 minutes before sacrifice, resulted in a significant depletion (30%) of the Ca^{2+} content in unlysed synaptosomes, the Ca^{2+} content in other subcellular fractions was not affected. When $^{45}\text{Ca}^{2+}$ was injected into the ICV areas in order to label brain Ca^{2+} pools, significant decreases in the synaptic vesicle content of Ca^{2+} were noted following morphine injections. Naloxone, a morphine antagonist, when used alone did not alter the Ca^{2+} distribution but injections of naloxone prior to morphine injections effectively prevented the depletion of Ca^{2+} (Cardenas and Ross, 1976).

Chronic morphine treatment appeared to act differently (Yamamoto et al, 1978; Cardenas and Ross, 1976). Morphine pellets planted *in vivo* increased synaptic Ca^{2+} concentration specifically in synaptic regions. The increase in Ca^{2+} content proceeded, in parallel, to a rise in cAMP levels. It has been suggested that these highly selective changes in both Ca^{2+} levels

and Ca^{2+} binding may represent mechanisms whereby morphine treatment interferes with synaptic transmission and by which chronic administration leads to the states of tolerance and dependence (Sharma, 1975).

Mule (1969), demonstrated that narcotic analgesics inhibited Ca^{2+} binding to phospholipids *in vitro*. The analgesic compound levorphanol has been shown to inhibit high affinity $^{45}\text{Ca}^{2+}$ binding to synaptic membranes in a dose-dependent manner (Ross and Cardenas, 1977). The inhibition being non-competitive and blocked by the narcotic antagonist naloxone. The endogenous opiate β -endorphin also has been shown to be a potent inhibitor of Ca^{2+} binding (Guerrero-Monez et al, 1979). Kakunaga (1966) reported that morphine blocked the K^{+} -stimulated entry of Ca^{2+} into rat brain slices. Guerrero-Monez et al (1979) showed that acute morphine treatment administered subcutaneously resulted in a significant reduction in the ATP-dependent $^{45}\text{Ca}^{2+}$ uptake into both lysed and intact synaptosomes. Chronic morphine caused the reverse effect, ie, the increase in the ATP-dependent $^{45}\text{Ca}^{2+}$ uptake.

In vitro work has produced conflicting results. Leslie et al (1982) were unable to detect morphine inhibition of Ca^{2+} fluxes, whereas End et al (1981) and Dhaliwal and Bradford (1982) were both able to show a decrease in $^{45}\text{Ca}^{2+}$ fluxes when high K^{+} or veratrine were used as stimulant. The differences in results may be due, in part, to the strength of stimulation. Dhaliwal and Bradford (1982) found that there was a requirement for submaximal stimulation by veratrine for the effects of morphine to be apparent on Ca^{2+} uptake and glutamate release from synaptosomes. Electrically stimulated noradrenaline release from cortical slices was inhibited by morphine especially when the frequency of stimulation was low (Montel et al, 1974).

Such decreases in Ca^{2+} fluxes would then have inhibitory effects on neurotransmitter release. Mudge et al (1979) showed that enkephalins not only inhibited substance P release from dorsal root ganglia in cell culture, but also reduced the duration of the Ca^{2+} action potential.

Apart from the direct effect on transmitter release, opiates are also found to modulate enzymes which regulate neurotransmitter production. Tyrosine hydroxylase is one such enzyme whose activity is depressed in the presence of morphine, thus reducing the levels of monoamines. The rate of activity of tyrosine hydroxylase is dependent upon cAMP produced by a Ca^{2+} -dependent adenylate cyclase.

As morphine and endogenous opiates affect cAMP levels it is possible that the underlying mechanism of analgesia may involve modulation of the phosphorylation processes (Klee and

Nirenberg, 1974; Sharma et al, 1975). Nehmad et al (1982) found that acute morphine treatment had an opposite effect to that of chronic morphine on the flux between bound and cytosolic calmodulin (CaM). As mentioned in Chapter 1, CaM bound to the synaptic membrane plays an important role in the mechanism of neurotransmitter release. Thus, increasing the amount of cytosolic CaM would decrease the rate of neurotransmitter release. A possible model for the action of the opioids in producing analgesia, is by binding stereospecifically to receptors, they can affect CaM distribution, adenylate cyclase activity and hence affect the level of cellular phosphorylation. It is well known that guanine nucleotides can regulate the interactions of opioid agonists with their receptors (Blume, 1978; Childers and Synder, 1978; Koski et al, 1981). It has been demonstrated that, under certain conditions, opioid receptors are coupled to adenylate cyclase and that the activity of this enzyme is modulated by guanine nucleotides (Cooper et al, 1982; Scheideler et al, 1983).

In this chapter a number of opioid agonists and antagonists have been studied to determine whether occupation of any one subtype of opioid receptor might affect Ca^{2+} influx and amino acid release from slices of rat cerebral cortex, either at rest or during periods of stimulation by veratrine. The concentration of veratrine used produced submaximal stimulation of these parameters. The effect of nucleotides on the actions of opioid agonists was also studied. The effect of morphine on the incorporation of $^{32}\text{P}_i$ into lipids and protein fractions of brain slices was compared.

4.2 Results

4.2a Action of opioid compounds on Ca^{2+} fluxes, amino acid release, and K^+ loss

All the opioid agonists used in these experiments inhibited veratrine-stimulated Ca^{2+} uptake, though with varying potency (Fig 4.1). The IC_{50} values (Table 4.1) are grouped according to the selectivity of the ligands for the opioid receptor subgroups. The selectivity is based on the results obtained from binding studies (James and Goldstein, 1984). Sufentanil (proposed μ -agonist), tifluadom (proposed κ -agonist) and bremazocine (proposed κ -agonist/ μ -agonist) were amongst the most potent in blocking Ca^{2+} uptake. Neither basal Ca^{2+} uptake or basal K^+ content of the cortical slices were affected by any of the opioid compounds used.

At higher concentrations (e.g. $100\mu\text{M}$) fentanyl, tifluadom, U50-488H, butorphanol and bremazocine greatly reduced the veratrine-induced loss of K^+ from the slices as well as inhibiting stimulated Ca^{2+} uptake (Table 4.2a and b). None of these compounds affected the K^+ content of the slices at concentrations causing 50% inhibition of stimulated Ca^{2+} uptake.

The relative order of potency for the μ -agonists in inhibiting the veratrine-evoked Ca^{2+} influx was sufentanil > etorphine > fentanyl > morphine. The order of potency for the κ -agonists/ μ -agonists and κ -agonists was, tifluadom > bremazocine > EKC > U50-488H > butorphanol > pentazocine (Table 4.1).

DAME (D-al²-met⁵-enkephalinamide) was only effective in inhibiting Ca^{2+} uptake at high concentrations ($100\mu\text{M}$). The proposed σ -agonist SKF 10,047 ($100\mu\text{M}$) totally inhibited Ca^{2+} uptake, and was 50% effective at inhibiting Ca^{2+} at $10\mu\text{M}$. None of the opioid compounds affected the basal release of the endogenous amino acids.

Veratrine ($25\mu\text{M}$) stimulated the release of glutamate, aspartate and GABA whilst the release of threonine was unaffected. The stimulated release of amino acids was reduced, by varying degrees, with all the opioid agonists (Table 4.3). In most cases the % inhibition of neuroactive amino acid release was similar to the % inhibition of Ca^{2+} uptake produced by these drugs. Morphine and sufentanil (Table 4.3) reduced the evoked release of the amino acids below resting levels.

4.2b Antagonistic effects of WIN 44441-3 and naloxone

Naloxone and WIN 44441-3 both had no effect on either basal or veratrine-stimulated Ca^{2+} uptake, K^+ content, or amino acid release from the slices. The inhibitory effects of the

μ -agonists used in this study were effectively antagonised by 1 μ M naloxone except for fentanyl (Table 4.4). Fentanyl at 1 μ M was only partially inhibited by 10 μ M naloxone, however, like the other μ -agonists, fentanyl was not effectively antagonised by 100 μ M WIN 44441-3 (Table 4.5).

Tifluadon and U50-488H were both preferentially antagonised by WIN 44441-3 than naloxone as were EKC, pentazocine, butorphanol and bremazocine.

The σ and δ agonists, SKF 10,047 and DAME respectively, were not significantly antagonised by either naloxone (1 μ M) or WIN 44441-3 (10 μ M). The action of opioid antagonists to inhibit stimulated amino acid release is reported in terms of their influence on stimulated glutamate release (Table 4.4 and 4.5). Similar inhibitory effects were found for aspartate and GABA release. Both WIN 44441-3 and naloxone had no effect on the release of the non-neuroactive amino acid threonine.

The effects of the μ -agonists on amino acid release were inhibited by 1 μ M naloxone (Table 4.4), again fentanyl was the exception. WIN 44441-3 (100 μ M) was ineffective as an antagonist against any of the actions of the μ -agonists, except against very low concentrations of etorphine. The inhibitory actions of the κ -agonists and κ -agonist/ μ -agonists on stimulated amino acid release were blocked by WIN 44441-3 (1 μ M) in preference to naloxone (10 μ M). Both naloxone (1 μ M) and WIN 44441-3 (10 μ M) were unable to affect the actions of either SKF 10,047 or DAME (Table 4.4 and 4.5).

4.2c Stereospecificity of the action of WIN 44441

WIN 44441-2 is the pharmacologically inactive (+) stereoisomer of WIN 44441-3. Table 4.6 shows the lack of effect the (+) isomer had on the inhibition of stimulated Ca^{2+} uptake and neurotransmitter release by 4 opioid agonists. WIN 44441-2 had no action on veratrine-stimulated depolarisation or on basal levels of Ca^{2+} uptake, K^{+} content, or amino acid release.

4.2d Effects of nucleotides on opioid action

Tifluadom, DAME, pentazocine, and morphine were each used at concentrations that produced half-maximal inhibition of veratrine-evoked Ca^{2+} uptake and amino acid efflux from slices. The addition of 10 μ M GTP had a small but significant, enhancing effect on the inhibitory actions of each of these opioid compounds (Table 4.7, Fig 4.2). GTP alone had no effect on either basal or stimulated Ca^{2+} uptake, K^{+} content, or amino acid release. When

GTP was inactivated, a sample was heated for 1 hour at 37°C, it had no significant effect on the inhibitory action of tiffanydom.

ITP, CTP and GMP were all unable to affect the inhibitory actions of morphine on either Ca^{2+} uptake or amino acid release (Table 4.8a and b). Similarly, ITP had no enhancing effect on the inhibitory actions of tiffanydom, DAME, or pentazocine on either of the above two parameters.

Both basal and stimulated K^+ content of the slices were not significantly affected by any of the nucleotides used, in the presence or absence of the opioid compounds (Table 4.7).

In order to confirm the specificity of action of naloxone and GTP a submaximal inhibitory concentration of the general Ca^{2+} channel antagonist D600 was employed. D600 (10 μM) inhibited both veratrine-induced Ca^{2+} uptake (43%; Table 4.9a) and amino acid release (eg, aspartate release was inhibited by 83%; Table 4.9). Neither naloxone nor GTP significantly altered the degree of inhibition produced by D600 on these parameters.

4.2e $^{32}\text{P}_i$ Incorporation into cortical slices and the effects of morphine

When cerebrocortical slices were stimulated by veratrine (75 μM or 25 μM) there was a significant increase in the incorporation of $^{32}\text{P}_i$. Veratrine (75 μM) induced a 105% increase in $^{32}\text{P}_i$ incorporation in respect to control (Table 4.10), and this was inhibited by TTX (1 μM). Morphine (100 μM) was without effect on the stimulated phosphorylation evoked by 75 μM veratrine. However, morphine (100 μM) was able to totally inhibit the increased phosphorylation evoked by 25 μM veratrine (Table 4.10). Naloxone (1 μM) abolished the inhibitory actions of 100 μM morphine. The addition of 10 μM GTP to the incubation enhanced the inhibitory action of morphine by 37% on veratrine-stimulated Ca^{2+} uptake, whilst 10 μM ITP was without effect.

The enhanced incorporation of $^{32}\text{P}_i$ into the cortical slices was further analysed in terms of the proportions of the responses which were detectable in either the protein or lipid fraction of the tissue (Table 4.11). On stimulation with 25 μM veratrine, 78% of the increased $^{32}\text{P}_i$ incorporation was recovered in the protein fraction with 32% being incorporated into the lipid fraction. In the presence of morphine (15 μM), the total $^{32}\text{P}_i$ content was inhibited by 19%, the majority of this decrease being due to a reduction in the $^{32}\text{P}_i$ incorporation into the protein fraction, with a smaller change in the incorporation into the lipid fraction. Addition of GTP (10 μM) in the presence of 15 μM morphine, the total $^{32}\text{P}_i$ incorporation was inhibited by a further 15%, with incorporation into both fractions being decreased by similar amounts.

Fig 4.1 Effects of opioid compounds on veratrine-stimulated Ca^{2+} uptake.

Slices were prepared as described in 2.1b and incubated as in 2.2b. The appropriate opioid compound was added after a 25 minute preincubation. Tifluadom and EKC were dissolved in deionised water, etorphine was dissolved in ethanol so that the final concentration of ethanol was 0.3%. The other opioid compounds were soluble in the incubation medium. Ca^{2+} uptake was measured as in 2.3 and the results are plotted as the percentage inhibition of $25\mu\text{M}$ veratrine-stimulated Ca^{2+} uptake against the log opioid concentration.

Results are mean $\pm$ SEM, $n=6$. Although not recorded all SEM values were with 10% of the mean. Basal Ca^{2+} uptake: 1424 ± 106 nmol/100mg protein; Veratrine ($25\mu\text{M}$) Ca^{2+} uptake: 2278 ± 202 nmol/100mg protein.

Fig4.1 EFFECT OF OPIOID COMPOUNDS ON
VERATRINE-STIMULATED Ca^{2+} INFLUX

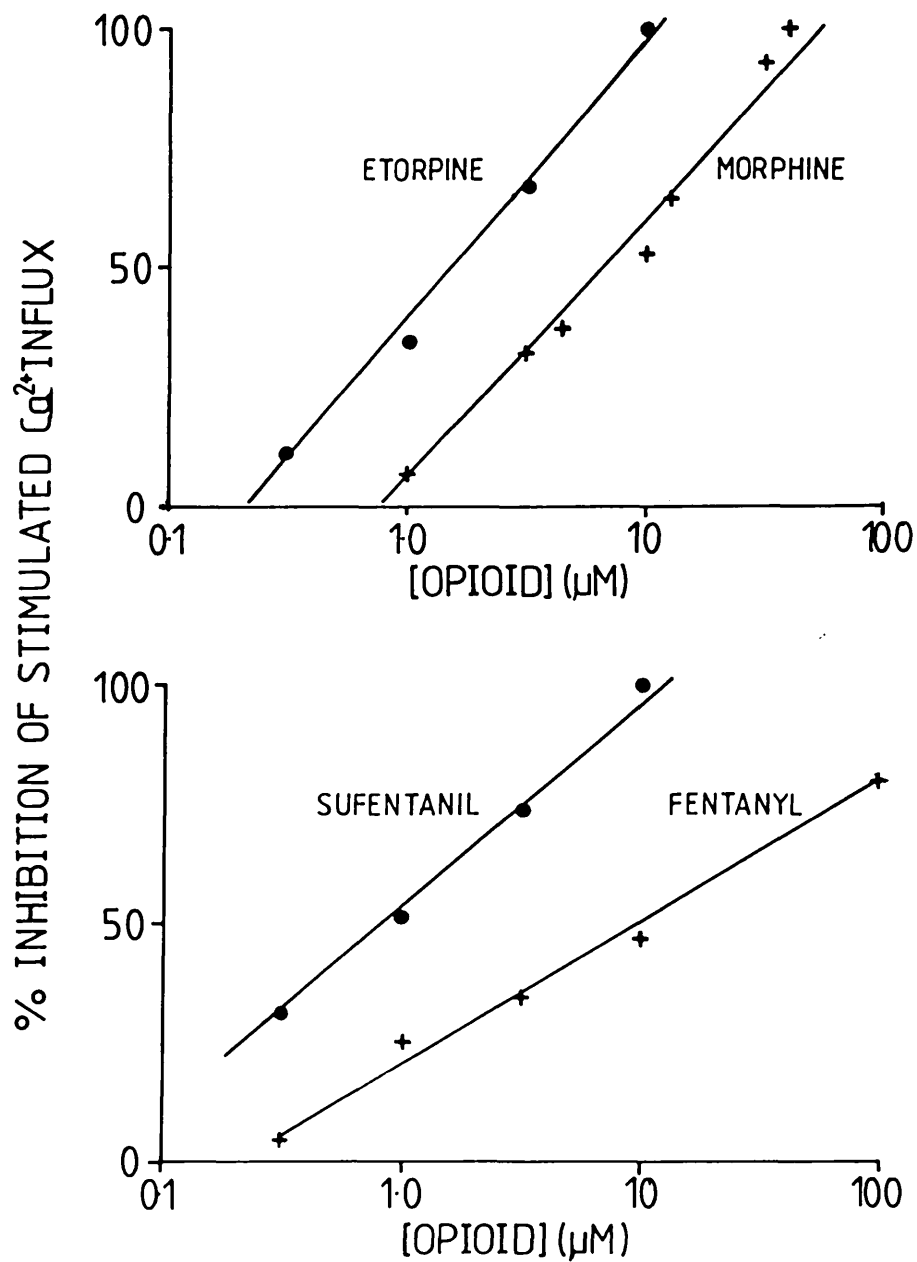


Fig 4.1 EFFECT OF OPIOID COMPOUNDS ON
VERATRINE-STIMULATED Ca^{2+} INFLUX

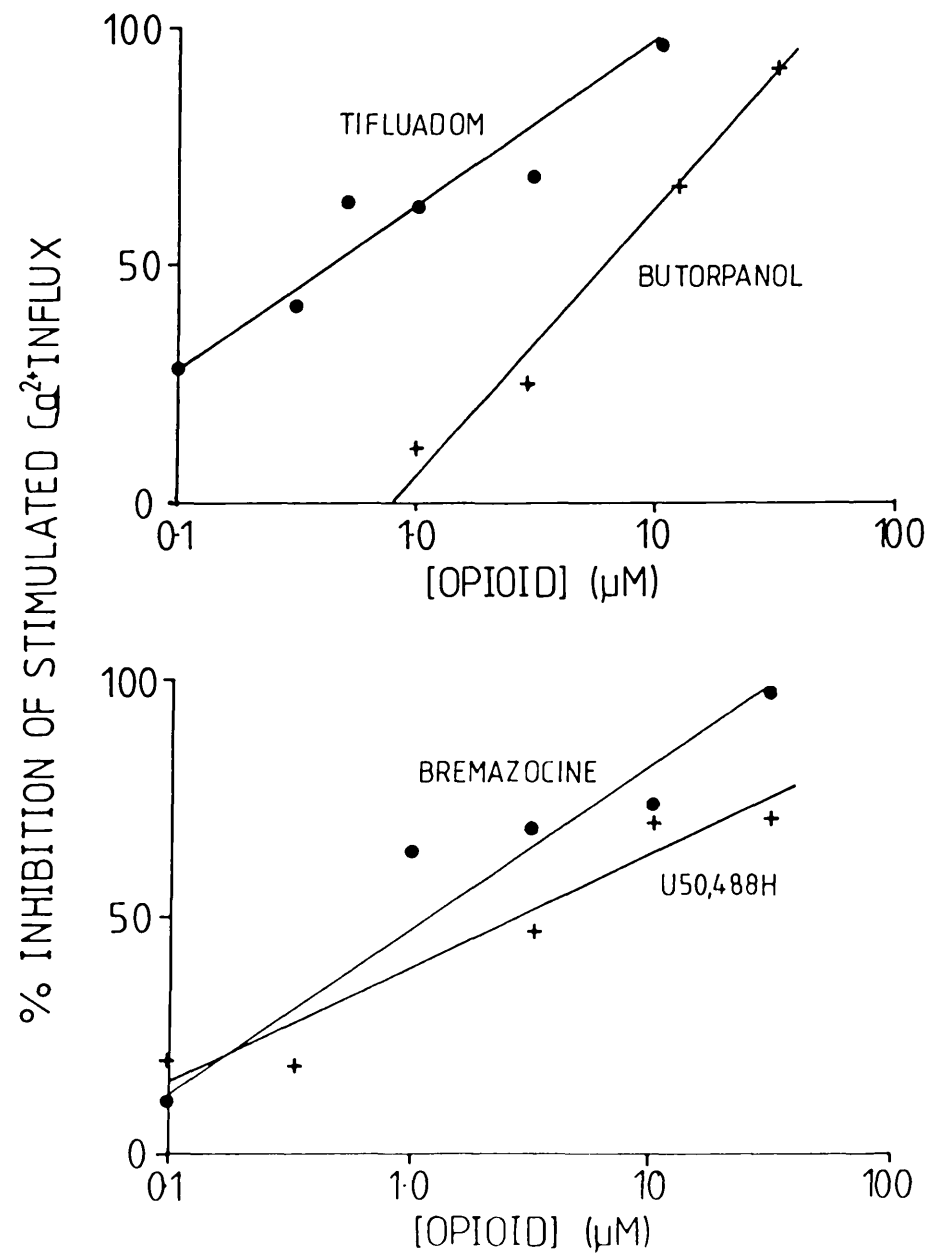


Fig4.1 EFFECT OF OPIOID COMPOUNDS ON
VERATRINE-STIMULATED Ca^{2+} INFLUX

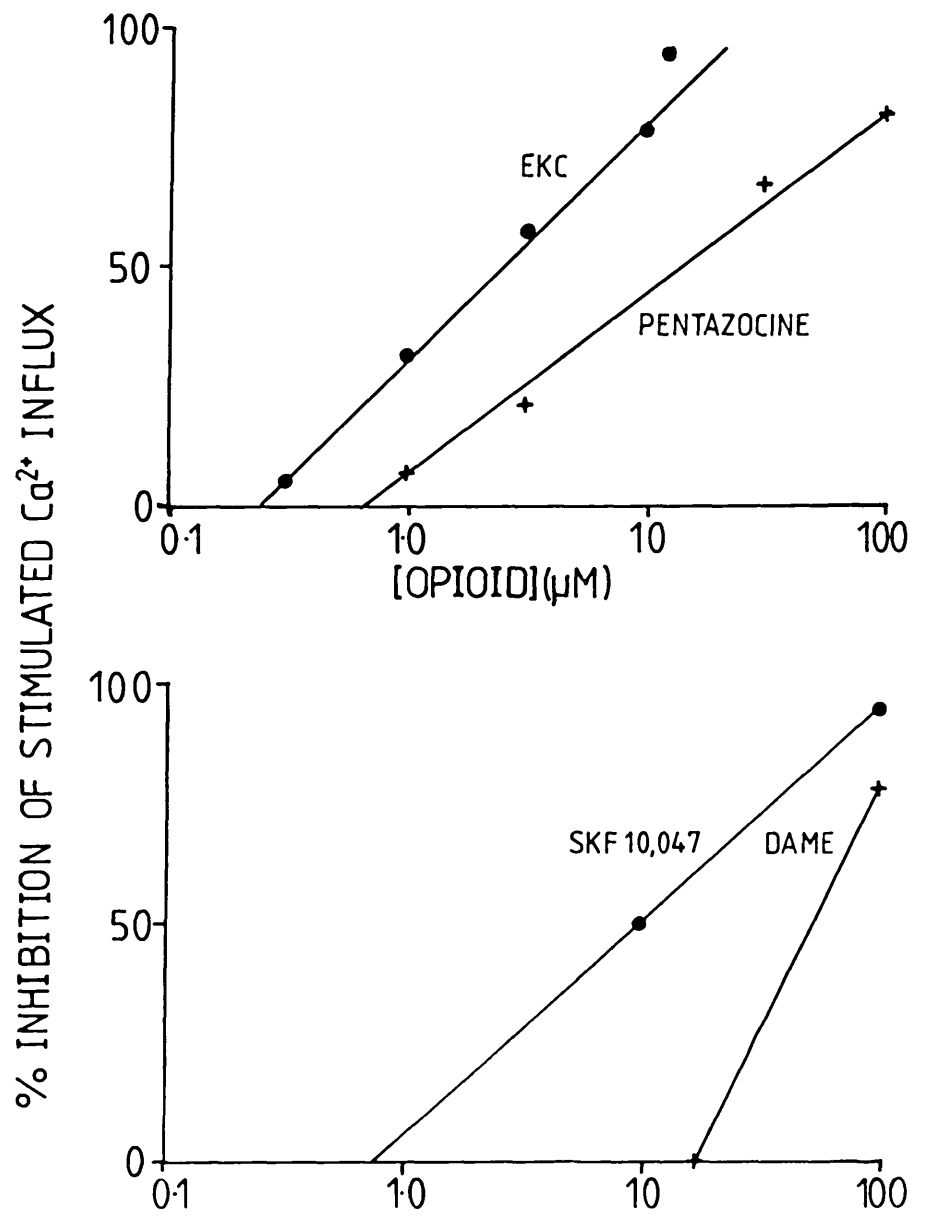


Table 4.1 **IC₅₀ values for the opioid compounds on Ca²⁺ uptake.**

Compounds	IC ₅₀ (μM)
Proposed μ-agonists	
Etorphine	1.7
Fentanyl	7.4
Morphine	8.1
Sufentanil	0.7
Proposed κ-agonists	
Tifluodom	0.5
U50-488H	4.3
Proposed κ-agonist/u-antagonist	
Bremazocine	1.4
Butorphanol	7.7
EKC	2.4
Pentazocine 11.2	
Proposed δ-agonist	
DAME	54.7
Proposed σ-agonist	
SKF 10,147	9.2

The IC₅₀ is the concentration causing 50% inhibition of the veratrine-stimulated Ca²⁺ uptake.

Slices were prepared as described (2.1b and 2.2b). Opioid compounds were dissolved as before (Fig 4.1). The IC₅₀ values were calculated from dose response curves (Fig 4.1) fitted by linear regression, correlation coefficients (r) were all between 0.96 and 1.00.

Table 4.2a **Effect of opioid compounds on veratrine-enhanced K⁺-efflux.**

Opioid compound	(μ M)	% Inhibition of K ⁺ -loss
Etorphine	100	0
	1	0
Fentanyl	100	113
	10	50
	1	0
Morphine	100	0
	1	0
Sufentanil	100	0
	1	0
DAME	100	0
	20	6
SKF 10,047	100	0
	10	32

Incubations were carried out as described (2.1b and 2.2b). The appropriate opioid compound was added after a 25 preincubation. The drugs were dissolved as before (Fig 4.1). The K⁺ -content of each slice was measured as in 2.5, and the % inhibition was determined.

Results are mean $\pm$ SEM, n=6. SEM were all within 10% of the mean. Basal K⁺-level, 89.7 $\pm$ 0.3 μ equiv/100mg protein; veratrine (25 μ M) level, 40.1 $\pm$ 0.7 μ equiv/100mg protein.

Table 4.2b **Effect of opioid compounds on veratrine-enhanced K⁺-efflux.**

Opioid compound	(μ M)	% Inhibition of K ⁺ -loss
Tifluadom	100	77
	10	16
	1	10
	0.1	2
U50-488H	100	89
	10	8
Bremazocine	100	64
	10	0
	1	0
Butorphanol	100	15
	41	13
	12	0
EKC	100	0
	1	0
Pentazocine	100	0
	1	0

Incubations were carried out as described (2.1b and 2.2b). The appropriate opioid compound was added after a 25 preincubation. The drugs were dissolved as before (Fig 4.1). The K⁺ -content of each slice was measured as in 2.5, and the % inhibition was determined.

Results are mean $\pm$ SEM, n=6. SEM were all within 10% of the mean. Basal K⁺-level, 89.7 $\pm$ 0.3 μ equiv/100mg protein; veratrine (25 μ M) level, 40.1 $\pm$ 0.7 μ equiv/100mg protein.

Table 4.3a **Effect of opioid compounds on veratrine-induced amino acid release.**

		% Inhibition of release		
Opioid agonist	(μ M)	GLU	ASP	GABA
Etorphine	100	82	84	71
	10	62	82	59
	1	28	32	41
Fentanyl	1	108	108	-
Morphine	100	150	93	105
	10	116	74	100
	1	36	19	28
Sufentanil	1	157	83	95
DAME	100	45	45	59
SKF 10,047	100	108	113	111
	10	102	117	103

Incubations were carried out as described (2.1b and 2.2b). The appropriate opioid compound was added after a 25 preincubation. The drugs were dissolved as before (Fig 4.1). Release of the endogenous amino acids into the incubation medium was measured (as in 2.5) and the % inhibition of the stimulated response calculated.

Results are mean $\pm$ SEM, n=6. SEM values were within 10% of the mean.

	Basal release	Veratrine (25 μ M) release
	(nmol/100mg protein)	
Glutamate (GLU)	1806 $\pm$ 185	2972 $\pm$ 165
Aspartate (ASP)	911 $\pm$ 157	1997 $\pm$ 139
GABA	205 $\pm$ 36	466 $\pm$ 54

Table 4.3b **Effect of opioid compounds on veratrine-stimulated amino acid release.**

		% Inhibition of release		
Opioid agonist	μM	ASP	GLU	GABA
Tifluadom	100	99	98	89
	10	69	86	4
	1	82	54	78
	0.1	66	41	78
U50-488H	100	108	98	81
	10	83	70	71
Bremazocine	100	93	101	93
	10	84	48	73
	1	33	30	19
Butorphanol	100	106	103	103
	41	100	109	93
	12	96	74	100
	4	-	86	69
EKC	100	100	85	100
	10	70	71	69
	1	20	43	46
Pentazocine	100	72	98	82
	10	50	66	58
	1	21	30	30

Slices were prepared and incubated as described in 2.1b and 2.2b. The appropriate opioid compound was added after a 25 minute preincubation. Tifluadom and EKC were dissolved in deionised water, all other opioids were dissolved in the incubation medium. The release of

endogenous amino acids into the incubation medium was measured (2.6), and the % inhibition of stimulated response calculated.

Results are the mean $\pm$ SEM, n=6. For basal and stimulated levels see legend to Table 4.3a.

Tables 4.4 a and b

The antagonistic effects of naloxone on veratrine-induced Ca^{2+} uptake and glutamate release

Slices were prepared and incubated as described in 2.1b and 2.2b. The appropriate opioid compound was added after a 25 minute preincubation. Etorphine was dissolved in ethanol and diluted so that the final concentration of ethanol in the incubation medium was less than 0.3%, tifluadom and EKC were dissolved in deionised water, all other opioids were dissolved in the incubation medium. Naloxone was added to the incubation medium before the addition of the slice.

Aliquots of the NaOH digest were taken to determine Ca^{2+} content. The release of endogenous amino acids into the incubation medium was measured (2.6). The results are expressed as % antagonism of opioid effect on veratrine ($25\mu\text{M}$)-stimulated Ca^{2+} uptake or glutamate release. The results are mean $\pm$ SEM, $n=6$. SEM values were less than 10% of the mean.

Table 4.4a The antagonistic effects of naloxone on veratrine-induced Ca²⁺ uptake and glutamate release.

		% Opioid inhibition				
		Ca ²⁺ uptake			Glutamate release	
Opioid compound (μM)		Naloxone(μM)			Naloxone(μM)	
		100	10	1	10	1
Etorphine	100	-	-	77	-	72
	10	-	-	98	-	96
	1	-	-	106	-	152
Fentanyl	100	-	-	1	-	-
	10	-	-	0	-	-
	1	-	41	12	66	0
Morphine	100	-	-	94	-	100
	10	-	-	100	-	100
	1	-	-	100	-	100
Sufentanil	100	-	-	28	-	-
	1	-	102	93	84	138
DAME	100	-	7	0	-	-
	20	-	-	0	-	-
SKF 10,047	100	-	7	0	-	-
	10	-	-	0	-	19

Table 4.4b The antagonistic effects of naloxone on veratrine-induced Ca^{2+} uptake and glutamate release.

% Opioid antagonism						
Opioid		Ca^{2+} uptake			Glutamate release	
compound	(μM)	Naloxone(μM)			Naloxone(μM)	
		100	10	1	100	10
Tifluadom	100	-	0	-	-	0
	10	-	0	-	-	0
	1	0	0	-	2	0
	0.1	0	-	-	2	0
U50-488H	100	-	22	-	-	0
	10	-	3	-	-	0
Bremazocine	10	-	20	-	-	66
	1	96	-	-	73	-
Butorphanol	41	-	65	-	-	48
	12	-	53	-	-	67
EKC	100	-	8	-	-	4
	10	-	5	-	-	85
	1	-	144	-	-	99
Pentazocine	100	8	-	-	0	-
	10	11	-	-	26	-
	1	57	-	-	53	-

Tables 4.5a and b.

Antagonistic effects of WIN 44441-3 on veratrine-induced Ca^{2+} uptake and glutamate release.

Slices were prepared and incubated as described in 2.1b and 2.2b. The appropriate opioid compound was added after a 25 minute preincubation. Etorphine was dissolved in ethanol and diluted so that the final concentration of ethanol in the incubation medium was less than 0.3%, EKC and tifluadom were dissolved in deionised water, all other opioids were dissolved in the incubation medium. WIN 44441-3 was dissolved in deionised water and added, at the right concentration, to the incubation medium before the addition of the slice.

Aliquots of the NaOH digest were taken to determine Ca^{2+} content. The release of endogenous amino acids into the incubation medium was measured (2.6).

The results are expressed as % antagonism of opioid effect on veratrine ($25\mu\text{M}$)-stimulated Ca^{2+} uptake or glutamate release. The results are mean $\pm$ SEM, $n=6$. SEM values are less than 10% of the mean.

Table 4.5a. Antagonistic effects of WIN 44441-3 on veratrine-induced Ca²⁺ uptake and glutamate release.

Opioid compound		% Opioid antagonism				
		Ca ²⁺ Uptake		Glutamate release		
		(μ M)	WIN 44441-3 (μ M)	WIN 44441-3 (μ M)		
			100 10	100 10 1		
Etorphine	100	38	-	0	-	-
	10	47	-	96	-	-
	1	30	-	83	-	-
Fentanyl	100	10	-	-	-	-
	10	-	1	-	-	-
	1	0	0	1	0	-
Morphine	100	49	-	22	-	-
	10	22	-	89	-	-
	1	0	-	23	-	-
Sufentanil	100	-	0	39	0	-
	1	25	3	39	0	-
DAME	100	-	2	-	-	-
SKF 10,147	100	-	2	-	-	-

Table 4.5b. Antagonistic effects of WIN 44441-3 on veratrine-induced Ca²⁺ uptake and glutamate release.

Opioid compound	(μM)	% Opioid antagonism				
		Ca ²⁺ Uptake		Glutamate release		
		WIN 44441-3 (μM)		WIN 44441-3 (μM)		
		10	1	10	1	100
Tifluadom	10	18	-	-	22	-
	1	77	-	-	96	-
	0.1	82	-	-	117	-
U50-488H	100	-	91	-	-	77
	10	-	91	-	-	131
Bremazocine	100	87	-	-	93	-
	10	91	50	-	113	97
	1	-	83	-	-	116
Butorphanol	41	-	97	-	-	100
	12	-	129	-	-	122
	1	47	-	-	-	125
EKC	10	105	34	-	99	-
	1	97	123	-	100	100
Pentazocine	100	40	-	-	32	-
	10	78	-	-	67	-
	1	100	-	-	100	-

Table 4.6 Comparison of the effect of WIN 44441 isomers on the actions of four opioids.**a, Action on Ca²⁺ uptake**

Opioid Compound	% Inhibition of Ca ²⁺ uptake	% Antagonism	
		WIN 44441-2	WIN44441-3
Etorphine	114	0	38
Morphine	112	0	49
EKC	61	2*	31
Pentazocine	61	2*	40*

b, Action on glutamate release

Opioid compound	% Inhibition of Glu release	% Antagonism	
		WIN 44441-2	WIN44441-3
Etorphine	82	0	0
Morphine	151	0	22
EKC	85	0	25
Pentazocine	98	0*	32*

Incubations of cortical brain slices were carried out as in Table 4.5. WIN 44441-2 and

WIN 44441-3 were added to the appropriate incubations at 100 μ M (or 10 μ M “*”), before the addition of the slice. Results are mean $\pm$ SEM, n=6.

Table 4.7a **The effect of GTP on the actions of tifuadom on amino acid release.**

Amino acid released/100mg protein				
Treatment	Asp	Thr	Glu	GABA
Control	919 ± 46	424 ± 53	2756 ± 204	330 ± 78
TiF.	967 ± 28	384 ± 53	2769 ± 376	342 ± 127
GTP	903 ± 219	453 ± 160	2749 ± 744	256 ± 64
GTP + Tif.	753 ± 99	424 ± 104	2728 ± 466	272 ± 54
Vt	1049 ± 52	419 ± 63	4558 ± 630	521 ± 95
Vt + Tif.	1005 ± 52	395 ± 60	3260 ± 680	443 ± 74
Vt + GTP	1042 ± 289	491 ± 60	3941 ± 392	509 ± 202
Vt + GTP + Tif.	997 ± 431	475 ± 120	2901 ± 341	429 ± 155

Cortex slices were prepared as described (2.1b and 2.2b). GTP (10 μ M) was added 1 minute before the end of the preincubation. Tifluadom (Tif; 0.5 μ M), was added 25 minutes into the preincubation. Veratrine (25 μ M) was used as the depolarizing agent. The level of amino acids released into the incubation medium was measured.

Results are mean $\pm$ SEM, n=6.

Table 4.7b **The effect of ITP on the actions of tiftuadom on amino acid release.**

Amino acid released/100mg protein				
Treatment	Asp	Thr	Glu	GABA
Control	535 ± 134	409 ± 79	2067 ± 209	125 ± 3
ITP	524 ± 26	519 ± 97	1627 ± 583	130 ± 9
ITP + Tif.	441 ± 31	595 ± 206	1933 ± 150	124 ± 12
Vt	732 ± 88	431 ± 44	3414 ± 34	297 ± 12
Vt + Tif.	696 ± 52	564 ± 101	3000 ± 194	280 ± 23
Vt + ITP	607 ± 124	479 ± 152	2159 ± 474	174 ± 41
Vt + ITP + Tif.	600 ± 23	464 ± 87	2232 ± 210	188 ± 45

Cortex slices were prepared as described (2.1b and 2.2b). ITP (10 μ M) was added 1 minute before the end of the preincubation. Tiftuadom (Tif; 0.5 μ M), was added 25 minutes into the preincubation. Veratrine (25 μ M) was used as the depolarizing agent. The level of amino acids released into the incubation medium was measured. Results are mean $\pm$ SEM, n=6.

Table 4.7c The effect of ITP and GTP on the actions of pentazocine on amino acid release.

Amino acid released/100mg protein				
Treatment	Asp	Thr	Glu	GABA
Control	1094 ± 214	566 ± 40	2216 ± 48	192 ± 160
GTP	1186 ± 180	486 ± 44	2202 ± 348	185 ± 63
Vt	2130 ± 160	476 ± 120	3264 ± 138	472 ± 50
Vt + Pent.	1730 ± 118	462 ± 80	2814 ± 29	506 ± 19
Vt + GTP + Pent.	1330 ± 174	534 ± 18	2588 ± 78	214 ± 71
Vt + ITP	2028 ± 152	680 ± 48	3202 ± 296	499 ± 72
Vt + GTP + Pent.	1834 ± 128	566 ± 84	2942 ± 142	394 ± 14

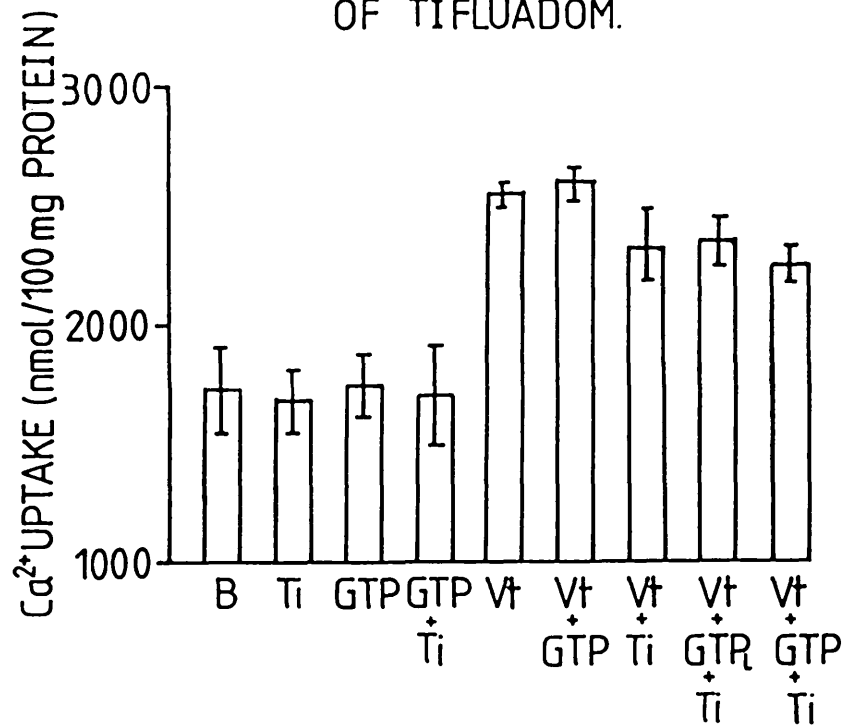
Cortex slices were prepared as described (2.1b and 2.2b). GTP (10 μ M) or ITP (10 μ M) were added 1 minute before the end of the preincubation. Pentazocine (Pent; 10 μ M), was added 25 minutes into the preincubation. Veratrine (25 μ M) was used as the depolarizing agent. The level of amino acids released into the incubation medium was measured. Results are mean $\pm$ SEM, n=6.

Fig 4.2 **The effects of GTP and ITP on the actions of tifluadom, pentazocine and DAME.**

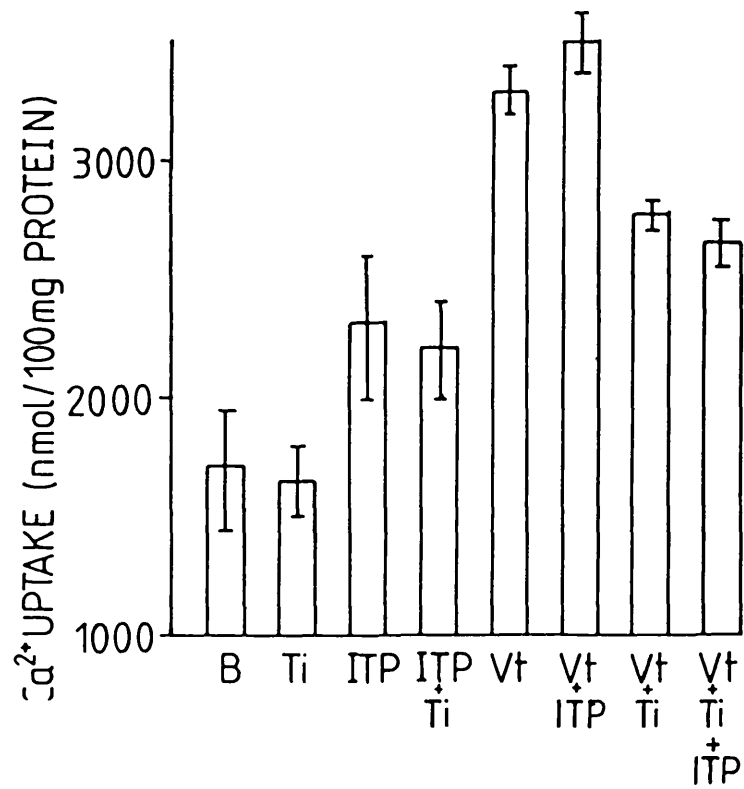
Cortex slices were prepared as described (2.1b and 2.2b). The nucleotides (10 μ M) were added 1 minute before the end of the preincubation. Tifluadom (Ti; 0.5 μ M), pentazocine (P; 10 μ M) or DAME (D; 50 μ M) were added 25 minutes into the preincubation. Veratrine (Vt; 25 μ M) was used as the depolarizing agent. Results are mean $\pm$ SEM, n=6. GTP (GTPi) was inactivated by heating at 37°C for 1 hour before addition to the relevant condition.

Fig4.2

THE EFFECT OF GTP ON THE ACTIONS
OF TIFLUADOM.



THE EFFECT OF ITP ON THE
ACTIONS OF TIFLUADOM



THE EFFECTS OF GTP AND ITP
ON THE ACTIONS OF PENTAZOCINE

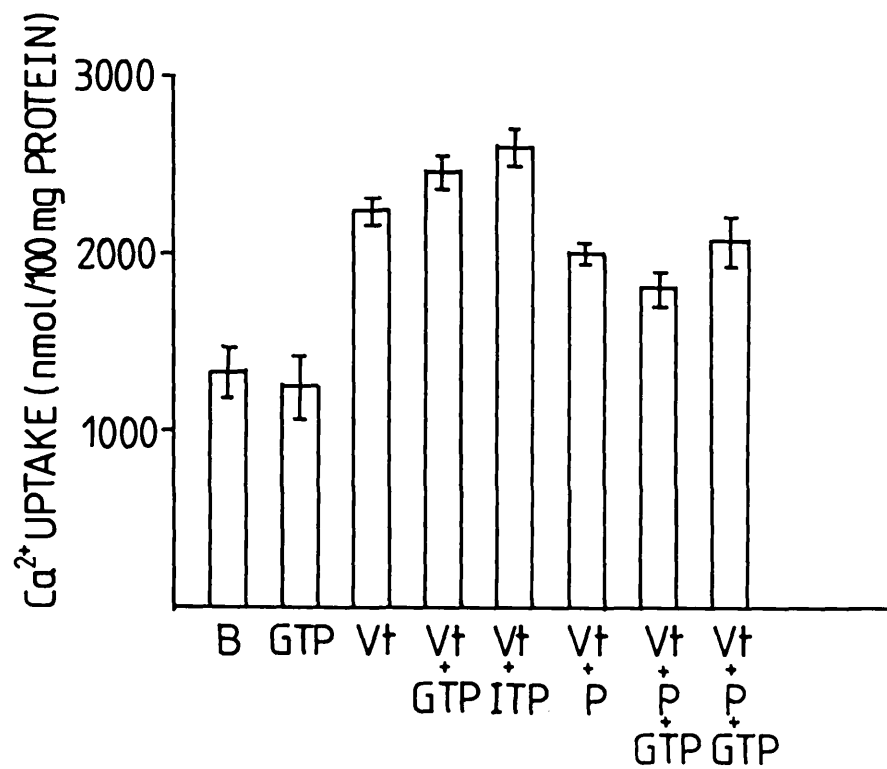


Fig 4.2
THE EFFECTS GTP AND ITP ON
THE ACTIONS OF DAME

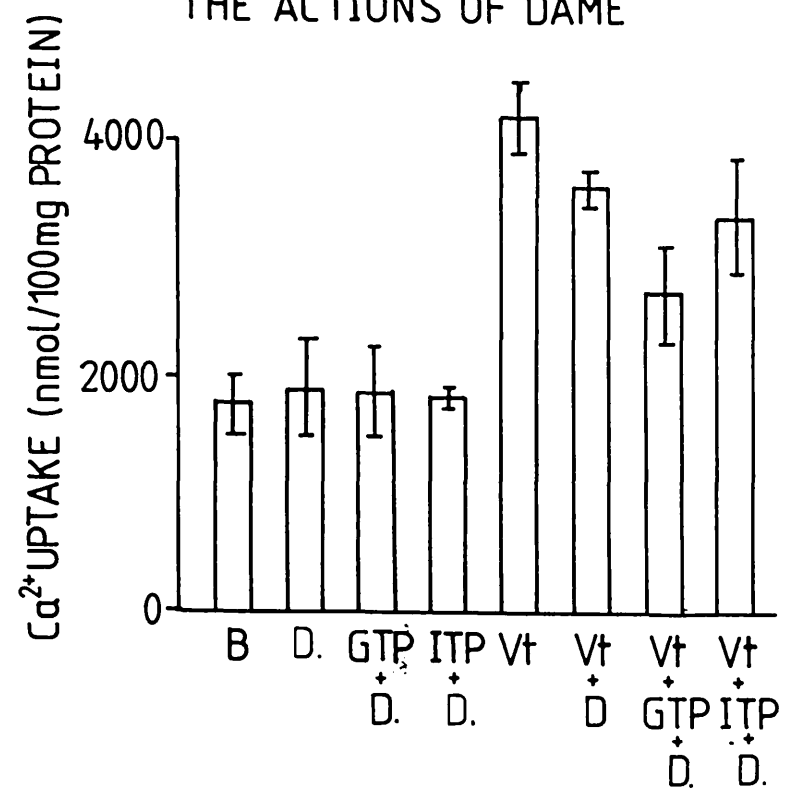


Table 4.8a **The effect of nucleotides on the action of morphine on Ca^{2+} uptake and the K^+ content of cortex slices.**

Treatment	Ca^{2+} Uptake nmol/100mg protein	K^+ Content $\mu\text{equiv}/100\text{mg protein}$
Control	1121 ± 96	67.4 ± 0.8
Vt	1506 ± 127	49.8 ± 0.4
Vt + Morphine	1321 ± 96	46.3 ± 1.3
Vt + GTP	1574 ± 72	47.3 ± 0.7
Vt + GTP + Morphine	1207 ± 72	46.3 ± 1.1
Vt + GMP + Morphine	1341 ± 94	49.7 ± 0.8
Vt + CTP + Morphine	1299 ± 104	46.4 ± 1.0
Vt + ITP + Morphine	1362 ± 78	47.1 ± 1.0

Cortex slices were prepared as described (2.1b and 2.2b). Nucleotides ($10\mu\text{M}$) were added 1 minute before the end of the preincubation. Morphine ($15\mu\text{M}$), was added 25 minutes into the preincubation. Veratrine ($25\mu\text{M}$) was used as the depolarizing agent. The Ca^{2+} uptake and K^+ content of the slices were measured as in 2.3 and 2.5 respectively. Results are mean $\pm$ SEM, $n=6$.

Table 4.8b **The effects of nucleotides on the action of morphine on amino acid release.**

Amino acid released/100mg protein				
Treatment	Asp	Thr	Glu	GABA
Control	838 ± 56	520 ± 48	1722 ± 184	210 ± 10
Vt	1828 ± 70	454 ± 32	2588 ± 58	440 ± 15
Vt + Mp.	1234 ± 52	600 ± 70	1862 ± 24	364 ± 16
Vt + GTP	1784 ± 14	420 ± 108	2548 ± 278	471 ± 19
Vt + GTP + Mp.	880 ± 86	454 ± 54	178 ± 76	240 ± 27
Vt + GMP + Mp.	1218 ± 56	482 ± 28	1982 ± 222	381 ± 21
Vt + CTP + Mp.	1182 ± 94	528 ± 38	1814 ± 134	365 ± 18
Vt + ITP + Mp.	1254 ± 70	452 ± 28	1892 ± 54	379 ± 42

Cortex slices were prepared as described (2.1b and 2.2b). Nucleotides (10 μ M) were added 1 minute before the end of the preincubation. Morphine (15 μ M), was added 25 minutes into the preincubation. Veratrine (25 μ M) was used as the depolarizing agent. The amino acid release into the incubation medium was measured (2:6). Results are mean $\pm$ SEM, n=6.

Table 4.9a **The effects of naloxone and GTP on the actions of D600 on Ca²⁺ uptake and the K⁺ content of the cortex slices.**

	Ca ²⁺ Uptake nmol/100mg protein	K ⁺ Content μequiv K ⁺ /100mg protein
Basal	1029 ± 165	54.9 ± 7.7
D600	1053 ± 259	48.8 ± 59
Vt	2028 ± 16	20.2 ± 2.4
Vt + D600	1601 ± 492	32.0 ± 1.7
D600 + Naloxone	964 ± 330	54.1 ± 5.0
D600 + Naloxone + Vt	1540 ± 106	26.3 ± 2.8
D600 + GTP	929 ± 214	53.5 ± 4.5
D600 + GTP + Vt	1647 ± 259	29.6 ± 2.3

Cerebrocortical slices were incubated as described (2.1b and 2.2b). Naloxone (1μM) was added to the relevant incubations prior to the addition of the slices. GTP (10μM) was added 1 minute before the end of the preincubation where required. After a 25 minute preincubation, 10μM D600 was added. Veratrine (Vt; 25μM) was used as the depolarizing agent. Results are expressed as mean ± SEM, n=6.

Table 4.9b **Effects of naloxone and GTP on the actions of D600 on amino acid release.**

Treatment	Amino acid release (nmol/100mg protein)		
	Asp	Thr	GABA
Basal	1048 ± 100	464 ± 58	205 ± 15
D600	1086 ± 112	345 ± 26	469 ± 51
Vt	1479 ± 112	350 ± 37	281 ± 19
Vt + D600	1117 ± 76	350 ± 37	281 ± 19
D600 + Naloxone	1049 ± 82	382 ± 37	278 ± 50
D600 + Naloxone + Vt	1125 ± 89	359 ± 60	315 ± 25
D600 + GTP	989 ± 106	349 ± 26	179 ± 23
D600 + GTP + Vt	1170 ± 67	444 ± 10	363 ± 26

Cerebrocortical slices were incubated as described (2.1b and 2.2b). Naloxone (1 μ M) was added to the relevant incubations prior to the addition of the slices. GTP (10 μ M) was added 1 minute before the end of the preincubation where required. After a 25 minute preincubation, 10 μ M D600 was added. Veratrine (Vt; 25 μ M) was used as the depolarizing agent. Aliquots of the incubation medium were taken for amino acid analyses. Results are expressed as mean $\pm$ SEM, n=6

Table 4.10 **Total $^{32}\text{P}_i$ incorporation into cerebrocortical slices.**

Cortex slices were prepared as before (2.1b and 2.7). Morphine was added after a 60 minute preincubation of the slice in the presence of $^{32}\text{P}_i$, the nucleotides (where required) were added 1 minute earlier. TTX ($1\mu\text{M}$), was present throughout the incubation with $^{32}\text{P}_i$. Veratrine (Vt) was used as the depolarizing agent, and was added 15 minutes after morphine (Mp). The experiment was stopped 1 minute after the addition of veratrine. The slices were treated as in 2.7b and total $^{32}\text{P}_i$ incorporation was determined. Data are mean $\pm$ SEM, $n=6$. Values in parentheses are the % inhibition of stimulated response, calculated as below:

$$\begin{array}{l} \text{\% inhibition} = 1 - \frac{\text{basal} - \text{test}}{\text{basal} - \text{stimulated}} \\ \text{of response} \end{array}$$

test = veratrine + antagonist

stimulated = veratrine alone

Table 4.10 Total $^{32}\text{P}_i$ incorporation into cerebrocortical slices.

Treatment	$^{32}\text{P}_i$ incorporation dpm/mg tissue wet weight	% of Control
Control	2968 $\pm$ 210	100
GTP 10 μM	2623 $\pm$ 134	97 $\pm$ 5
Morphine 10 μM	2590 $\pm$ 81	96 $\pm$ 3
Vt 75 μM	5530 $\pm$ 485	205 $\pm$ 18
Vt 75 μM + TTX	3076 $\pm$ 147	103 $\pm$ 64
Vt 75 μM + Mp	6259 $\pm$ 432	232 $\pm$ 16 (0%)
Vt 25 μM	3777 $\pm$ 129	140 $\pm$ 12
Vt 25 μM + Mp 15 μM	3480 $\pm$ 269	129 $\pm$ 24 (36%)
Vt 25 μM + Mp 100 μM	2806 $\pm$ 101	104 $\pm$ 9 (80%)
Vt 25 μM + Nx	3657 $\pm$ 214	136 $\pm$ 4 (15%)
Vt 25 μM + GTP 10 μM	3804 $\pm$ 123	141 $\pm$ 11 (0%)
Vt 25 μM + ITP 10 μM	3846 $\pm$ 361	142 $\pm$ 7 (0%)
Vt 25 μM + Mp 100 μM	4020 $\pm$ 78	146 $\pm$ 7 (0%)
+ Nx 1 μM		
Vt 25 μM + Mp 15 μM	3184 $\pm$ 157	118 $\pm$ 4 (73%)
+ GTP 10 μM		
Vt 25 μM + Mp 15 μM	3517 $\pm$ 191	130 $\pm$ 17 (33%)
+ ITP 10 μM		

Table 4.11 Proportional incorporation of $^{32}\text{P}_i$ into protein and lipid fractions of cortex slices, and the effects of morphine.

a) $^{32}\text{P}_i$ incorporation dpm/1mg tissue wet weight

Treatment	Lipid	Protein	Total
Control	1022 $\pm$ 121	1280 $\pm$ 131	2302 $\pm$ 222
Vt	1448 $\pm$ 80	2186 $\pm$ 176	3635 $\pm$ 239
Vt + Mp	1344 $\pm$ 134	1658 $\pm$ 28	2952 $\pm$ 99
Vt + Mp + Nx	1185 $\pm$ 133	1427 $\pm$ 205	2611 $\pm$ 103
Vt + Mp + GTP	1502 $\pm$ 91	2159 $\pm$ 89	3661 $\pm$ 202

b) % Change from control levels of incorporation

Treatment	Lipid	Protein	Total
Control	41 (32)	71 (78)	57
Vt + Mp	31 (42)	29 (29)	28
Vt + Mp + Nx	16 (53)	11 (47)	13
Vt + Mp + GTP	47 (36)	69 (64)	59

Rat cerebrocortical slices were prepared and incubated as in 2.1b and 2.7. Nucleotides (10 μM) or Nx (Nx; 1 μM) were added 1 minute before the end of the 60 minute incubation with $^{32}\text{P}_i$. Morphine (Mp; 15 μM) was added 1 minute later, followed by veratrine (Vt; 25 μM) 1 minute later. The incorporation of $^{32}\text{P}_i$ into the two fractions was determined as described (2.7c).

Data are mean $\pm$ SEM, n=6. Values in parentheses represent the proportion of the total response in the whole slice recovered in each fraction.

As mentioned in the introduction opioid receptor-subtypes have been identified by autoradiography in rat brain cortex, using selective ligands (Chang et al, 1979; Goodman and Snyder, 1982). In this study, the presence of pharmacologically active μ and κ -receptor sites in cortical slices has been shown and it is possible that active σ and δ -receptors are also present. All the different receptor agonists used appeared to affect veratrine-stimulated Ca^{2+} uptake and neuroactive amino acid release from the cortex slices. There were certain drugs which were more potent in affecting this Ca^{2+} influx than others but it was not possible to determine whether one receptor type was more effective than the others in mediating this effect because the variation in selectivity and potency of the agonists used in each group were greater than the variation between the receptor categories.

Amongst the μ -selective agonists, sufentanil was the most effective antagonist of both stimulated Ca^{2+} entry and amino acid release from the cortical slices. The relative order of potency of the μ -selective agonists based on these parameters was sufentanil > etorphine > fentanyl > morphine (Table 4.1). The κ -selective agonists, tifluadom and U50-488H were approximately equipotent, whereas amongst the κ -selective agonists/ μ -antagonists the relative order of potency at $10\mu\text{M}$ was bremazocine > butorphanol > EKC > nalbuphine > pentazocine. A similar order of potency was found for these compounds in binding studies using guinea pig brain membranes and in producing analgesia using mouse phenylquinone-induced writhing test (Schmidt, 1984). The concentrations required to inhibit Ca^{2+} uptake into cerebrocortical slices were of the same order as those that produced analgesia in the mouse, except for etorphine, sufentanil, and fentanyl, which required significantly higher concentrations to inhibit Ca^{2+} uptake than to elicit analgesia. However, it is very difficult to directly compare *in vivo* concentrations to those required *in vitro*. *In vivo*, drugs may not diffuse evenly and it is possible that there may be discrete regions of high drug density, so that the effective drug concentration may be several times greater than the overall tissue level.

Mulder et al, (1984) found that μ -, κ -, and σ -opioid receptor agonists differentially inhibited dopamine and acetylcholine release from rat striatal slices. The κ -agonists inhibited stimulated dopamine release but not acetylcholine release whereas the σ -agonists produced the reverse effect and μ -agonists had no detectable effect. Jones and Marchbanks (1982) observed that [D-al²,met⁵] enkephalin inhibited stimulated noradrenaline release from cerebrocortical slices, but had no effect on acetylcholine release. However, no differential effects on the stimulated release of glutamate, aspartate or GABA by any opioid were obtained in the

present study. The results support the view that presynaptic inhibitory opioid receptors are present on aspartatergic, glutamatergic and GABAergic nerve endings of the rat cerebral cortex. There is some evidence from studies on myenteric neurones to suggest that opioid receptors subtypes could exist on the same neurone (Cherubini and North, 1985).

The inhibitory action of DAME is of particular low potency and may be due to significant degradation of the peptide during the experiment, a very low concentration of σ -receptors in the cortex, or that the enkephalins have a different mode of action as compared to the μ - and κ -agonists. Jones and Marchbanks (1982) showed that DAME did not affect the stimulated uptake of Ca^{2+} into rat cerebrocortical synaptosomes, thus the enkephalin may have a post synaptic effect as has already been proposed (Jones and Marchbanks, 1982).

Basal Ca^{2+} uptake and neurotransmitter release were both unaffected by any of the opioid compounds added and this is in agreement with work by End et al (1981) and Dhaliwal and Bradford (1983).

At high concentrations, fentanyl, tifluadom, U50-488H, butorphanol and bremazocine all reduced the veratrine-induced decrease in K^+ content from slices (Table 4.2). This may be due to the opioids hyperpolarizing the neuronal cells, perhaps by acting on the Ca^{2+} activated K^+ channels (North and Williams, 1983) thus the plasma membrane would become less sensitive to veratrine. Several recent reports suggest that opioids, especially of the μ - and σ -subtype, exert their inhibitory effects by affecting membrane K^+ conductance (North, 1986). However, any hyperpolarizing effects of the opioids on the cerebrocortical slices may not be detected as changes in K^+ content since they may be very small and localised in comparison to total changes in K^+ content of the slice.

The analgesic effect of opioid compounds may be due to an inhibition of neurotransmitter release, which could be achieved by reducing the availability of free Ca^{2+} in the nerve terminal. Acute morphine treatment reduces both the Ca^{2+} content and Ca^{2+} binding capacity of the synaptosomal fraction of brain tissue (Ross, 1977; Ross and Cardenas, 1977; Yamamoto et al, 1978). This decrease in both Ca^{2+} content and binding was receptor-mediated since it was dose-dependent, naloxone reversible, and stereospecific. Dunlap and Fischbach (1978 and 1981) produced electrophysiological evidence that enkephalins act directly on the voltage-dependent Ca^{2+} channels of dorsal root ganglion neurones grown in cell culture. They suggest that this action of enkephalin may have a role in the presynaptic inhibition of neurotransmitter release. In these experiments, the opioids appeared to be acting by a receptor-mediated mechanism to inhibit Ca^{2+} entry into the cell. This effect was not a result of the opioids inhibiting the depolarizing action of veratrine, as shown by the lack of

effect on the veratrine-induced K^+ loss.

All the opioids tested, inhibited the stimulated release of endogenous amino acids with varying potencies. The observed effects of opioid agonists on veratrine-induced amino acid release were of particular interest since veratrine has been shown to stimulate release in a manner independent of Ca^{2+} influx (3.2j). This suggests that the opioid compounds are exerting an action at two sites, firstly at the voltage-dependent Ca^{2+} channel to inhibit Ca^{2+} influx and, secondly at a site distal to Ca^{2+} entry involved in the release of neurotransmitters. This proposal is further supported by the observation that some opioid agonists showed greater potency in inhibiting the amino acid release compared to inhibiting the Ca^{2+} influx (eg. morphine, sufentanil, fentanyl, butorphanol and SKF 10,047). Similarly, Dhaliwal and Bradford (1982) reported that morphine had a greater inhibitory effect on amino acid release as compared to its effects on Ca^{2+} uptake in cortical synaptosomes. It may be that these opioid compounds actually cross the plasma membrane and enter the nerve terminals to act at the proposed second site. An alternative hypothesis could be that the opioids, when they bind to their receptors affect the production of intracellular messengers e.g. cAMP, cGMP, and this would result in changes in the phosphorylation state of the neurone.

White and Bradford (1986) have shown that there is significant accumulation of [^3H]etorphine, [^3H]EKC, and [^3H]morphine in cerebrocortical slices. They found that 4.1, 7.7 and 7.2 nmol/100mg final tissue wet weight tissue of each drug respectively were taken up, which suggests that sufficient drug would be able to gain access to an internal site after a 15 minute incubation. Roth and Coscia (1984) have identified microsomal opioid receptors in rat forebrain preparations, which were distinct from those associated with the synaptic membranes in that they lack sensitivity to guanine nucleotides and proteases. These receptors may represent internalised and/or incompletely processed opioid receptors. There are reports of ligand mediated internalisation of receptors accompanied by loss of guanine nucleotide regulation for erythrocyte β -adrenergic receptors (Chuang et al, 1980). It is not known whether this process of internalisation occurs in the CNS, though Van Loon and George (1983) have reported that met-enkephalin was taken up and internalised by rat brain synaptosomes. They suggested that the internalisation of the receptor may be a method by which receptors are recycled. However, they also suggested that met-enkephalin may have been internalised with the opioid receptor for an action at an intracellular site. The uptake of [^3H]-opioids may therefore be due to internalisation and thus the opioids could induce analgesia by acting at an internal site.

The alternative method by which opioids could inhibit veratrine-induced amino acid

release could be by affecting the phosphorylation state of the neurone.

The opioid receptor has been reported to be coupled to adenylate cyclase by the guanine nucleotide-sensitive protein (Ni), (Sharma et al, 1975; Cooper et al, 1982). The relative affinities of opioids for their receptors compare favourably with their effectiveness as inhibitors of adenylate cyclase (Sharma et al, 1975). GTP (10 μ M) enhanced the inhibitory action of morphine, tifluadom, pentazocine, and DAME on both Ca²⁺ uptake and amino acid uptake. This effect was specific to GTP with no comparable enhancement by ITP, CTP, or GMP. It seems therefore that μ - and κ -receptors may be associated with the Ni protein.

The actions of naloxone and GTP were specific to opioid compounds, since the antagonists did not affect the inhibitory actions of D600 on either Ca²⁺ uptake or amino acid release. This is further evidence that the actions of opioids in inhibiting both amino acid release and Ca²⁺ uptake are receptor-mediated actions. Giradot et al,(1983) observed that the presence of EGTA abolished the ability of guanine nucleotides to enhance opioid inhibition. The enhancement was restored by the addition of the Ca²⁺/calmodulin complex. Thus the inability of GTP to enhance opioid inhibition in the absence of Ca²⁺ could be due to the requirement of Ca²⁺ for the binding and/or activity of GTP and Ni.

The involvement of cAMP in the action of narcotics has been proposed by many workers (Collier and Roy, 1974; Palmer, 1983). Brandt et al, (1976) found that morphine prevented the increase in cAMP levels induced by prostaglandin E in NB108-15 cells. Collier and Roy (1974) found a similar effect in rat brain homogenates.

Decreased levels of cAMP would lead to a reduction in the activity of cAMP-dependent protein kinases. One possible substrate of this enzyme is the voltage-dependent Ca²⁺ channel (Fedulova et al, 1981; Doreshenko et al, 1972). Thus, a reduction in cAMP levels would regulate Ca²⁺ influx through the channel. A reduction in cAMP could also effect the intracellular mobilisation of Ca²⁺ (Greengard, 1979a) and thereby effect neurotransmitter release.

Inhibition, by morphine, of the depolarization-induced phosphorylation has been observed to occur in cortical brain slices (Table 4.10). On further analysis, morphine appeared to affect the incorporation of ³²P_i into proteins rather than into the lipid fraction (Table 4.11).

Brain cortex slices were incubated for 60 minutes in the presence of high-specific activity ³²P-inorganic phosphate. This long incubation time allowed for diffusion and uptake of ³²P_i into the intracellular spaces and for the equilibrium of ³²P_i with the phosphorylated energy-storage products, for example ATP and phosphocreatine. Radiochemically-labelled ATP could not be used due to the inability of this substance to move across cell membranes. The

depolarizing agent, veratrine, was present for 1 minute before the incubation was stopped. Robinson and Dunkley (1983) have shown that maximal incorporation of $^{32}\text{P}_i$, stimulated by agonists, occurs in the first minute of exposure to that agonist after which there is a steady decrease in incorporation. This rapid decrease is probably due to the rapid fluxing between phosphorylated and dephosphorylated states of the proteins.

The inhibition of veratrine-stimulated phosphorylation was sensitive to the opioid antagonist naloxone indicating a receptor-mediated effect. The inhibition was enhanced by GTP. It is interesting that $15\mu\text{M}$ morphine caused a similar decrease of both veratrine-stimulated Ca^{2+} uptake and $^{32}\text{P}_i$ -incorporation, whilst GTP enhanced the inhibitory effects on these two parameters by different extents. GTP had a much greater effect on enhancing the inhibition of the $^{32}\text{P}_i$ incorporation rather than enhancing the inhibition of Ca^{2+} influx (75% to 33% respectively). This may be the result of the morphine- μ -receptor complex having a more potent effect on the Ca^{2+} channel without the intervention of GTP-sensitive secondary messengers. In contrast, the inhibition in the incorporation of ^{32}P -inorganic phosphate is possibly directly mediated by morphine, through a guanine-nucleotide system.

The ability of morphine to decrease the phosphorylation of proteins rather than lipids suggests that morphine acts to inhibit protein kinases linked to protein phosphorylation in preference to those kinases involved in lipid phosphorylation. O'Callagan et al, (1979) reported that the *in vitro* addition of morphine to rat striatal membranes, produced a decrease in the Ca^{2+} -regulated phosphorylation of several proteins, and that opioid compounds inhibited the cAMP-dependent phosphorylation of some membrane bound proteins. Williams and Clouet (1982) also observed an opioid-induced inhibition of Ca^{2+} /calmodulin-dependent phosphorylation of several proteins. Apart from the Ca^{2+} channel, whose activity is thought to be regulated by phosphorylation, several other proteins have been identified which are also regulated by Ca^{2+} /calmodulin protein kinases. These include the α and β subunits of neurotubulin (O'Callagan et al, 1982; Burke and Delorenzo, 1981). Thus, the inhibition of phosphorylation within the synaptic terminal may directly modulate amino acid neurotransmitter release by interfering with the cellular processes controlling the cytoskeleton (Vallano et al, 1985). Overall, the regulation of both Ca^{2+} and cAMP levels by opioid compounds will effect different protein kinases and hence produce a high degree of fine modulation of cellular processes.

The intracellular actions of Ca^{2+} are thought to be modulated by calmodulin, thus calmodulin may also be a site of opioid activity. Acute administration of morphine *in vitro* and *in vivo* increased the levels of calmodulin in the synaptosomal cytoplasm (Hanbauer et al,

1979; Hoss and Formaniak, 1983). Gnegy et al, (1977, 1982) demonstrated that calmodulin was equally distributed between the membranes and the cytosol of striatal neurones. Membrane-bound calmodulin is involved in the stimulation of adenylate cyclase, whilst cytosolic calmodulin is involved in the stimulation of Ca^{2+} -dependent phosphorylation. Thus, if morphine is able to displace membrane-bound calmodulin, this would lead to a reduction in adenylate cyclase activation and an increased rate of dephosphorylation, possibly leading to the reduction in phosphorylation noted in the results presented here (Tables 4.10 and 4.11).

Hippocampal adenylate cyclase can be inhibited by opioid compounds in a GTP-dependent manner. Calmodulin appeared to play a role in this system since its inactivation resulted in the loss of inhibition by the opiates. Addition of calmodulin restored this effect (Giradot et al, 1983). The above observations illustrate that complex interrelationships between cAMP and Ca^{2+} metabolism within the terminal. It is therefore likely that opioid compounds, when they combine with their receptors, can affect both the above mentioned systems resulting in changes in neurotransmitter release.

The opioids agonists are classified in Table 4.1 according to the receptor for which they appear to have greatest selectivity in binding studies. However, considerable cross-reactivity between the receptor types occurs. Therefore etorphine, for example, may cross react with σ - and κ -receptors to some extent (Rosenbaum et al, 1984). Opioid antagonists were employed to specify further the opioid receptor types which are involved in mediating these effects on Ca^{2+} influx.

Naloxone is primarily a μ -antagonist, but it is also capable of antagonising actions through κ - and σ -receptors. The order of potency being $\mu > \kappa > \sigma$ - (Takemori and Portuguese, 1984). WIN 44441-3 [(2,6,11S*)-(-)-1 cyclopentyl -5-(1,2,3,4,5,6-hexahydro-8-hydroxy-3,6,11-trimethyl-2, 6-methano-3-benzazocin-11-yl)-3-pentanone methane sulphonate] is a pure opioid antagonist which is thought to act with greater selectivity for the κ -receptor (Romer et al, 1982) but also acts on the μ - and σ -receptors (Ward et al, 1983).

WIN 44441-3 proved to be stereospecific in its actions in antagonising opioid inhibition of veratrine-stimulated Ca^{2+} uptake and amino acid release in these experiments (Table 4.6). The (+) isomer WIN44441-2 was totally ineffective even at $100\mu\text{M}$.

At low concentrations ($1\mu\text{M}$ - $10\mu\text{M}$) WIN 44441-3 was more effective than naloxone at antagonising actions through the κ -receptor. Naloxone ($1\mu\text{M}$) selectively antagonised effects that were mediated by the μ -receptor and appeared to be more effective than WIN 44441-3 on the μ -receptor. The actions of morphine, etorphine and sufentanil were effectively antagonised

by 1 μ M naloxone but required higher concentrations of WIN 44441-3 to be antagonised. Therefore the effects of these three opioid agonists on stimulated Ca^{2+} uptake and neurotransmitter release are thought to be primarily through the μ -receptor. However fentanyl (1 μ M) was only partially inhibited by 10 μ M naloxone and was not effectively antagonised by 100 μ M WIN44441-3. This result appears to be an anomaly. The actions of fentanyl may also be through the μ -receptor, but are unlikely to be via k-receptors.

Tifluadom and U50-488H were both preferentially antagonised by WIN 44441-3 over naloxone, indicating that they act primarily at the k-receptor in this system. Both these compounds are reported to be very selective k-agonists in other systems (Romer et al, 1982; Vonvoigtlander et al, 1983).

DAME and SKF 10,047 were not significantly antagonised by naloxone (1 μ M) or WIN 44441-3 (10 μ M). Since δ and σ selective antagonists were not used, the action of these drugs through δ - and σ - receptors respectively could not be confirmed. However, the lack of antagonism by naloxone and WIN 44441-3 implies that these agonists are not acting via μ - or k-sites.

Sufentanil is thought to be very selective for μ -receptors over k- and δ -receptors (Goldstein and James, 1984), but WIN 44441-3 partially antagonised the inhibition of Ca^{2+} influx by sufentanil. This suggests that WIN 44443-1 may not be absolutely specific for k-receptors, but certainly has a greater antagonistic effect at k- over μ -receptors in these experimental conditions.

In binding studies WIN 44441-3 has been shown to potently antagonise k- and μ - binding with much less effect on the δ -receptors, WIN 44441-3 is more effective than naloxone at μ -, k- and δ -receptors in guinea pig ileum and mouse van deferens (Michne et al, 1978; Ward et al; 1983). However in these experiments it appears that WIN 44441-3 is more selective to k- than μ -receptors.

CHAPTER 5

INHIBITORY EFFECTS OF NORADRENALINE, DOPAMINE, AND 5-HYDROXYTRYPTAMINE ON BRAIN SLICES.

- 5.1 Introduction
- 5.2 Results
 - 5.2a Effects of noradrenaline on Ca^{2+} fluxes, K^{+} content, and amino acid release in slices of cortex and striatum
 - 5.2b Action of noradrenergic antagonists
 - 5.2c Effect of dopamine on Ca^{2+} fluxes, K^{+} content, and amino acid release in slices of rat cortex and striatum
 - 5.2d Effect of dopaminergic antagonists and agonists
 - 5.2e Effect of 5-hydroxytryptamine on Ca^{2+} fluxes, K^{+} content, and amino acid release in slices of cortex
 - 5.2f The effect of metergoline, a 5-hydroxytryptamine antagonist
- 5.3 Discussion

5.1 Introduction

In 1969, Katz and Miledi demonstrated that the release of a neurotransmitter could be elicited by local depolarization of the nerve terminal even when the action potential was blocked. Therefore, it is conceivable that the amount of neurotransmitter released does not depend upon the nerve activity alone, but could also be modulated by various factors influencing the membrane potential of the nerve endings. This hypothesis was originally proposed to explain pharmacological data obtained from the peripheral nervous system and the first candidate for this effect was the neurotransmitter released by the neurone itself. For example, noradrenaline reduces or increases its own release by acting on noradrenergic receptors located on noradrenergic nerve endings (Starke, 1977; Westfall, 1977; Langer, 1980). These receptors are called "presynaptic autoreceptors" (Langer, 1974). Presynaptic autoreceptors are thought to exist for dopamine (Nowicky and Roth, 1977), 5-hydroxytryptamine (5-HT) (Farnebo and Hamberger, 1974) and acetylcholine (Kilbinger, 1977). In addition to the transmitter itself, other neurotransmitters can modulate, presynaptically, the transmitter outflow. Cholinergic control of noradrenaline release from noradrenergic nerve terminals has been well documented (Sakurai et al, 1982; Westfall, 1977). Similarly, noradrenaline controls acetylcholine release from peripheral cholinergic nerve terminals (Vizi et al, 1977).

Because of the complexity of the central nervous system, the existence of presynaptic control of transmitter release is difficult to demonstrate in the brain. However, Starke et al (1972) have shown, by the use of radiochemically-labelled noradrenaline ($[^3H]$ -NA) in superfused rat brain cortical slices, that both autoreceptors and presynaptic mechanisms involving other neurotransmitters may play a role in central neurotransmission. In this chapter the mechanisms by which three major neurotransmitters (noradrenaline, dopamine, and 5-HT) control the release of neuroactive amino acids were studied using slices of rat brain.

Dale, in 1906, first subclassified noradrenergic receptors on the basis of their function. However, Ahlquist (1948) suggested that the subtypes of adrenergic receptors should be differentiated by pharmacology and introduced the α and β terminology. Both these two subtypes have now been subclassified, the initial subclassification of the α -receptors was based on anatomical or topographical localisation; α_1 were postsynaptic, α_2 were presynaptic (Langer, 1974). This anatomical division was not particularly useful because considerable evidence suggests that receptors having similar pharmacological properties to the autoreceptors were not located presynaptically.

The present classification is now based, as originally, for α_1 and α_2 receptors on pharmacological differentiation. Thus phenylephrine is α_1 -selective, while clonidine is α_2 -selective, both these drugs are agonists. For the antagonists, prazosin is α_1 -selective, while yohimbine is α_2 -selective (Bylund and U'Pritchard, 1983).

There is considerable evidence that α_2 -agonists decrease and α_2 -antagonists increase the field stimulated release of preloaded [^3H]-noradrenaline from brain slices (Farnebo and Hamberger, 1971; Starke and Montel, 1973). Presynaptic autoreceptors controlling noradrenaline release have also been shown to be present in the rat hypothalamus (Gothert et al, 1979) and hippocampus (Frankhuyzer and Mulder, 1980) both structures contain noradrenergic terminals originating from the locus coeruleus or neighbouring nuclei, but no intrinsic noradrenergic neurones.

The effects of antagonists and agonists on stimulated noradrenaline release are resistant to TTX (Dismukes et al, 1977) and are observed in synaptosomes (De Langan and Mulder, 1980). This suggests that the receptors involved are located on the noradrenergic terminals. However, when the noradrenergic cortical innervation of the rat was destroyed by the administration of 6-hydroxydopamine there was an increase rather than the expected decrease in the number of binding sites for the preferential α_2 -ligand clonidine (U'Pritchard et al, 1979). Since α_2 receptors probably have both a pre- and a post-synaptic location, this may reflect a supersensitivity of postsynaptic receptors masking a decrease in the number of presynaptic sites. In fact, in the amygdala and septum, two areas with a noradrenergic innervation, the number of α_2 -binding sites decreased after lesion of the noradrenergic afferents (U'Pritchard et al, 1979).

The presence of dopamine presynaptic receptors has also been proposed. Farnebo and Hamberger (1974) initially showed that dopamine antagonists (neuroleptics) increased dopamine outflow whilst agonists (eg, apomorphine) decreased the evoked outflow of dopamine from rat striatal slices. These results were confirmed by several groups and showed that dopamine could control its own release (Arbilla and Langer, 1981).

There is much controversy over dopamine presynaptic receptor classification. For example, the existence of multiple receptors for dopamine has been proposed (Costall and Naylor, 1981), that there are four distinct receptor subclasses (D1, D2, D3, and D4; Seeman, 1980; Seeman and List, 1982), and yet again that only one receptor type exists (Laduron, 1982). However more recent evidence points to two receptor sub-types, the D-1 and D-2, which can be differentiated by their pharmacology (Cooper et al, 1986). The D-1 receptor sub-type appears to stimulate adenylate cyclase (Seeman, 1980; Kebabian and Calne, 1979)

and is activated by micromolar concentrations of dopamine and antagonised by micromolar concentrations of most neuroleptics (Seeman, 1977). The current definition of the D-2 sub-type is a receptor which is blocked by nanomolar concentrations of neuroleptics, stimulated by micromolar concentrations of dopamine, and is linked to adenylate cyclase in an inhibitory manner. However, there is evidence for a third receptor sub-type (D-3) which is not linked to adenylate cyclase and is sensitive to nanomolar concentrations of dopamine but is antagonised by micromolar concentrations of neuroleptics (List et al, 1980; Titeler et al, 1979). It has been suggested that the inhibitory actions of dopamine receptors on adenylate cyclase is often hard to detect due to the "blanking effect" of the D-1 receptor (Cooper et al, 1986). Thus the D-2 and D-3 receptors may well be a single receptor which exists in two states. Sibley et al (1982) have shown that dopamine agonists recognise two states of the D-2 receptor, but George et al (1985) reported that the two states could not be discriminated by antagonists.

Thus, although much controversy still exists over the number of receptor sub-types, results of functional studies indicate that the D-2 receptor is involved in presynaptic control (Langer, 1980) and is antagonised specifically by sulpiride and, less potently, by chlorpromazine.

The localisation of the presynaptic dopamine receptor is as yet uncertain since presynaptic actions of antagonists and agonists cannot be detected in synaptosomes (Raiteri et al, 1978). This may be because interneurons are involved in the reduction of dopamine release by an indirect method. The participation of interneurons is unlikely since the effects of neuroleptics and apomorphine on dopamine release are still observed when nerve activity is blocked by TTX, (Jackisch et al, 1980) but TTX-resistant interneurons may exist in the striatum (Fukuda and Kameyama et al, 1980). Several groups have recorded a decrease in the number of D-2/D-3 binding sites after lesions of dopaminergic terminals in the rat striatum (Nagy et al, 1978; Fujita et al, 1980).

The classification of 5-HT receptor subtypes is also unclear. Data could be interpreted in such a way as to indicate the presence of three anatomically and pharmacologically distinct 5-HT receptors (Bradley et al, 1986). Two receptor sub-types can be differentially labelled by [³H]-5-HT (5-HT₁) and [³H]-spiperone (5-HT₂). Martin and Sanders-Bush (1982), using preloaded ([³H] 5-HT) synaptosomes stimulated by K⁺, showed that the 5-HT autoreceptor and 5-HT₁ binding site have similar pharmacological characteristics and that they could be one and the same site. These two 5-HT receptors can also be differentiated by the use of stereoisomers of methiothepin (Hibert and Middlemiss, 1986) where the (-) methiothepin is a much more potent antagonist than (+) methiothepin at the 5-HT₁ site and the autoreceptor

whilst the two isomers are equipotent at the 5-HT₂ site.

Engel et al (1983) showed by binding assays using seven different antagonists that 5-HT₁ sites could be subdivided, viz, 5-HT_{1A} and 5-HT_{1B}. Blurton and Wood (1986) provide evidence for three independent binding sites, 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C}, for which spiperone has low, medium and high binding affinities respectively.

Binding of ³H [5-HT] to the 5-HT₁ receptor sub-type was affected by GTP, whilst GTP had no effect on the binding of [3H]-spiroperidol to the 5-HT₂ sub-type (Peroutka et al, 1979). Little is as yet known about the third receptor-subtype either in its pharmacology or its functions (Bradley et al, 1986).

It would appear from the foregoing that it has proved difficult to elucidate any conclusive evidence for the biochemical mechanism by which neurotransmitters can exhibit presynaptic control. However, there are several factors which are common to all presynaptic control systems.

Presynaptic control of release is detected only when release of transmitter is elicited by Ca²⁺ dependent mechanisms, for example, *in vitro* either by electrical field stimulation, or high K⁺ concentrations. The Ca²⁺ independent release of noradrenaline elicited by tyramine could not be modulated either by agonists or antagonists specific to the α₂-adrenergic receptors. Tyramine elicits release of noradrenaline by displacing the transmitter from vesicular binding sites and is therefore Ca²⁺ independent. Thus it is possible that the presynaptic feedback mechanism modifies the availability of calcium ions for the release process. Similarly, neuroleptics modify only the Ca²⁺ dependent release of dopamine (Kamal et al, 1981).

It has been reported that the inhibition of transmitter release by exposure to exogenous noradrenaline was more pronounced when the Ca²⁺ concentration in the medium was reduced from 2.6mM to 0.25 mM (Langer et al, 1975). The potentiation of the inhibitory effects of α₂-receptor agonists on neurotransmission by a reduction in the external calcium concentration indicated that the activation of presynaptic α₂-receptors may reduce the availability of calcium for the excitation- secretion coupling. Similarly, if the calcium concentration were maintained at its original level, but the frequency of the stimulation decreased, the inhibition of neurotransmitter release in the presence of agonists was enhanced (Starke and Montel, 1973; Dismukes et al, 1977). The evidence from the above experiments suggests that the presynaptic control of release is by a mechanism regulating depolarization-induced Ca²⁺ entry.

Several groups have suggested that noradrenaline controls its own release by an inhibition

of voltage-dependent calcium channels (De Langan and Mulder, 1980; Schoffelmeer et al, 1981). A decrease in cAMP formation may also be involved (Wemer et al, 1982). However, the role for cAMP is unclear since the evoked release of noradrenaline is increased *in vitro* by cAMP analogues (Wemer et al, 1982) but decreased by phosphodiesterase inhibitors which increase intracellular levels of cAMP (Dismukes and Mulder, 1976). The interpretation of these results is further complicated by the fact that phosphodiesterase inhibitors may interact with adenosine receptors, and the activation of these receptors may also lead to a reduction in transmitter release (Fredholm 1981).

In a manner similar to noradrenaline antagonists, it has been shown that the effects of haloperidol (a dopamine antagonist) on dopamine release varied with the frequency and duration of the stimulation (Hoffmann and Cubeddu, 1982); the maximum effect was observed when small trains of rapidly-delivered depolarizing pulses were applied. As well as controlling release, dopamine by stimulation of its autoreceptors, appears to control its own synthesis (Zigmond, 1985; El Mestikawy et al, 1986). Such control involves the first enzymatic step in the dopamine-biosynthetic pathway, ie. the formation, by tyrosine hydroxylase, of 3,4-dihydroxyphenylalanine (DOPA) (Roth, 1984). The receptors involved are thought to be located presynaptically. Haubrich and Pflueger (1982), reported that exposure of striatal synaptosomes to some dopamine agonists reduced their capacity to synthesise [³H] DOPA from [³H] tyrosine and that this effect could be reversed by dopamine antagonists. The biochemical mechanism for such control is thought to involve an inhibition of adenylate cyclase, which reduces the amount of phosphorylated tyrosine hydroxylase thus slowing down the production of DOPA.

Experiments have been performed to investigate the effects of three neurotransmitters namely, noradrenaline, dopamine and 5-HT, on both the influx of Ca²⁺ and on the release of endogenous amino acid neurotransmitters, evoked by veratrine or high K⁺. Specific antagonists of each neurotransmitter were used to indicate a receptor-mediated action.

5.2 Results

Experiments with noradrenaline and dopamine were performed using media containing nialamide (10^{-5}M , monoamine oxidase inhibitor) and ascorbate (10^{-4}M , antioxidant).

Basal and stimulated levels of Ca^{2+} uptake, K^{+} content, and release of endogenous amino acids were unaffected by these additions. Similarly chlorimpridine (10^{-6}M , indolamine uptake inhibitor) was present throughout the experiments involving 5-HT and did not affect basal or stimulated levels of the parameters mentioned above.

5.2a Action of noradrenaline on Ca^{2+} fluxes, K^{+} content, and amino acid release in slices of cortex and striatum.

With the knowledge that the presynaptic action of neurotransmitters is dependent on the frequency of stimulation, the ability of noradrenaline to affect Ca^{2+} influx into rat cortical slices evoked by varying veratrine and K^{+} concentrations was studied. Veratrine and K^{+} , both in a dose dependent manner, stimulated Ca^{2+} influx, however, noradrenaline ($100\mu\text{M}$) only inhibited the Ca^{2+} influx evoked by veratrine ($25\mu\text{M}$) or K^{+} (30mM) (Fig 5.1) whilst it had no significant inhibitory action against the Ca^{2+} influx evoked by veratrine ($75\mu\text{M}$) or K^{+} (56mM).

The endogenous amino acid release evoked by $25\mu\text{M}$ veratrine or 30mM K^{+} was also inhibited by $100\mu\text{M}$ noradrenaline, whereas the increased release evoked by maximal concentrations of the agonists was unaffected (Fig 5.1).

Throughout the remaining experiments, veratrine ($25\mu\text{M}$) or K^{+} (30mM) were used as depolarizing agents, to enable the responses of noradrenaline to be determined.

Noradrenaline ($100\mu\text{M}$) when added to unstimulated cortical slices did not affect Ca^{2+} uptake, endogenous amino acid release, or K^{+} content (Tables 5.1 and 5.2). Similar results were also obtained using striatal slices (data not given).

Noradrenaline ($1\mu\text{M}$ - $100\mu\text{M}$) significantly inhibited the veratrine-induced Ca^{2+} uptake into cortical slices (Fig 5.2). The IC_{50} was calculated to be $0.14\mu\text{M}$ (Fig 5.2). The Ca^{2+} influx into cortical slices evoked by $30\mu\text{M}$ K^{+} was also inhibited in a dose-dependent manner by noradrenaline (IC_{50} , $0.08\mu\text{M}$). TTX ($1\mu\text{M}$) did not reverse this inhibitory action of noradrenaline (Table 5.2).

Noradrenaline inhibited the veratrine-induced amino acid release from cortical slices in a dose-dependent manner (Fig 5.2a). Noradrenaline inhibited, to the same extent, the

stimulated release of glutamate, aspartate, and GABA whilst the release of threonine, an example of a non-neuroactive amino acid, was not affected by the addition of noradrenaline at any concentration.

Only at the highest concentration tested (100 μ M) did noradrenaline affect the K⁺ loss induced by veratrine. At lower concentrations, the monoamine had no effect, indicating that it was not altering the veratrine-induced depolarization (Table 5.2).

At all concentrations tested (0.01 μ M-10 μ M) noradrenaline was ineffective in inhibiting the Ca²⁺ influx evoked by veratrine into striatal slices.

5.2b Effect of noradrenergic antagonists

Three antagonists with varying specificities for the α_1 -, α_2 -, and β -adrenergic receptors were used to define the receptor which induced the inhibition of veratrine-stimulated Ca²⁺ uptake and amino acid release.

Propranolol (a β -adrenergic receptor antagonist (0.1 μ M-10 μ M) did not significantly reverse the inhibition induced by noradrenaline (100 μ M) of either the Ca²⁺ influx or the amino acid release (Tables 5.3 and 5.4). However, the presence of yohimbine an α_2 -adrenergic receptor preferring antagonist, (0.1 μ M), did reverse the inhibition induced by 100 μ M noradrenaline. Corynanthine (1 μ M; α_1 -preferring antagonist) partially reversed this inhibition, but even at 10 μ M the reversal was only 72% of stimulated level.

The antagonists had comparable effects in reversing the noradrenaline inhibition of veratrine stimulated amino acid release. Propranolol (10 μ M) had no effect, yohimbine potently reversed the inhibition (1.0 μ M $P < 0.01$), whilst corynanthine (10 μ M) as before had an intermediate effect (10 μ M causing a 50% reversal of the 100 μ M noradrenaline inhibition, Table 5.4).

5.2c Effect of dopamine on Ca²⁺ fluxes, K⁺ content, and amino acid release in cortical and striatal slices.

Dopamine (1 μ M-100 μ M) had no effect on basal amino acid release, K⁺ content or endogenous amino acid release when added to both cortical and striatal slices (data for striatal slices only is shown; Table 5.5a).

Veratrine (25 μ M) stimulated the influx of Ca²⁺ into both cortical and striatal slices but dopamine reduced the stimulated influx of Ca²⁺ into striatal slices only (Table 5.5a); the

monoamine was ineffective in reducing the veratrine induced Ca^{2+} influx into cortical slices (data not shown). The inhibition by dopamine of both the veratrine and K^{+} induced Ca^{2+} influx was dose dependent with IC_{50} values of $0.08\mu\text{M}$ and $0.41\mu\text{M}$ respectively (Fig 5.2b; Table 5.5b; Table 5.8).

Dopamine, in a dose dependent manner, inhibited the stimulated release of endogenous amino acids with an IC_{50} of $0.16\mu\text{M}$ for the inhibition of veratrine-stimulated glutamate release. The veratrine-induced release of aspartate and of GABA were both inhibited to an extent similar to the dopamine-reduced glutamate.

Dopamine (0.01 - $100\mu\text{M}$) did not affect the veratrine-induced K^{+} loss from striatal slices (Table 5.5a).

5.2d Effect of dopaminergic agonists and antagonists

Apomorphine acted in a comparable fashion as dopamine, in that it inhibited the veratrine-induced Ca^{2+} influx into striatal slices in a dose-dependent manner (Fig 5.2c; IC_{50} $0.02\mu\text{M}$).

Stimulated amino acid release was also inhibited by apomorphine, the veratrine-evoked releases of glutamate, aspartate, and GABA were inhibited to similar extents (Fig 5.2c; IC_{50} for the inhibition of glutamate release was $0.06\mu\text{M}$).

The potency by which two dopamine antagonists, chlorpromazine and sulpiride, reversed the inhibitory action of dopamine were compared. Both drugs were tested against the dopamine inhibition of both the veratrine-stimulated Ca^{2+} uptake and the amino acid release. It can be seen from Table 5.6 that sulpiride ($0.1\mu\text{M}$) and chlorpromazine ($0.1\mu\text{M}$) both significantly ($P < 0.05$) reversed the dopamine inhibition of veratrine-evoked Ca^{2+} uptake. The inhibition of stimulated glutamate release was reversed significantly ($P < 0.05$) by $0.01\mu\text{M}$ sulpiride, and also by $1.0\mu\text{M}$ chlorpromazine ($P < 0.05$) (Table 5.6).

5.2e Effect of 5-HT on Ca^{2+} fluxes, K^{+} content, and amino release in slices of cortex

5-HT ($0.01\mu\text{M}$ - $100\mu\text{M}$) had no effect on basal Ca^{2+} influx, K^{+} content, or endogenous amino acid release in cortical slices. However, there was a dose-dependent inhibition both of veratrine-induced Ca^{2+} influx (IC_{50} $0.02\mu\text{M}$) and of amino acid release (IC_{50} for inhibition of glutamate release, $0.1\mu\text{M}$; Fig 4.2.b, Table 4.8). There was no effect on the veratrine

induced K^+ loss from the cortical slices by any concentration of 5-HT.

5.2f The effect of metergoline, a 5-HT antagonist

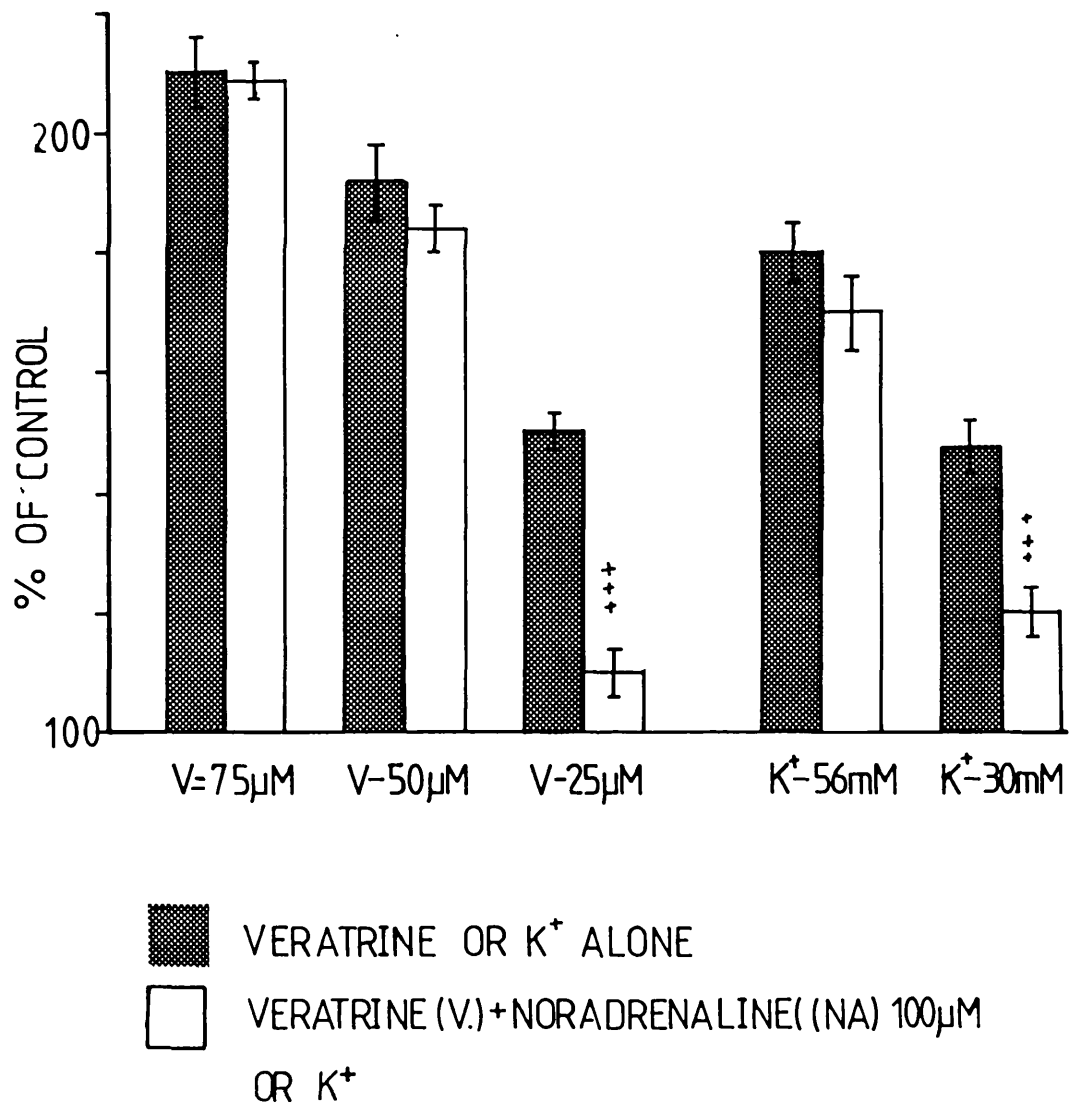
Metergoline, ($1\mu\text{M}$) caused a 91% reversal of the 5-HT induced inhibition of veratrine-stimulated Ca^{2+} influx. The inhibition of glutamate was also reversed (95%) by $1\mu\text{M}$ metergoline (Table 5.7).

Fig 5.1 Frequency dependency of the actions of noradrenaline on cortex brain slices

Rat cerebrocortical slices were prepared and incubated as described (2.1b and 2.2b). The incubation medium contained 10^{-5} nialamide and 10^{-4} M ascorbate. Noradrenaline was added 25 minutes after the beginning of the incubation to give a final concentration of $100\mu\text{M}$. Five minutes later $^{45}\text{Ca}^{2+}$ was added followed 1 minute later by the depolarizing agent. The experiment proceeded for 11 minutes, and was terminated as described (2.2b). Samples of the NaOH digest were taken for radioactive counting and K^{+} content. Aliquots of the incubation medium were analysed for amino acid content. Data are mean $\pm$ SEM (n=6).

Significance of difference between agonist (veratrine or K^{+}) and agonist plus noradrenaline were calculated by Student's two tailed t-test for unpaired samples (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$).

Fig 5.1
INHIBITORY ACTION OF NORADRENALINE: DEPENDENCE
ON THE LEVEL OF STIMULATION.



DATA ARE MEAN ± SEM n=9. † p<0.005 FROM CONTROL

Figs 5.2a, b, c, and d.

Dose response curves for the action of noradrenaline, dopamine, apomorphine, and 5-HT on both the veratrine-induced Ca^{2+} influx and glutamate release.

Rat cerebrocortical slices were prepared and incubated as described (2.1b 2.2b). The incubation medium contained either nialamide (10^{-5}M) and ascorbate (10^{-4}) (for experiments with noradrenaline, dopamine, and apomorphine) or chlorimiprimine (10^{-6}M) (for experiments with 5-HT). The neurotransmitters were added (at the appropriate concentration) 25 minutes after the beginning of the incubation. Five minutes later $^{45}\text{Ca}^{2+}$ was added followed 1 minute later by the depolarizing agent. The experiment proceeded for 11 minutes, and was terminated as described (2.2b). Samples of the NaOH digest were taken for determination of Ca^{2+} uptake. Aliquots of the incubation medium were analysed for amino acid content.

The curves were fitted by linear regression. The correlation coefficients (r) were found to fall between 0.96 and 0.99.

Fig 5.2a DOSE RESPONSE CURVES:
NORADRENALINE

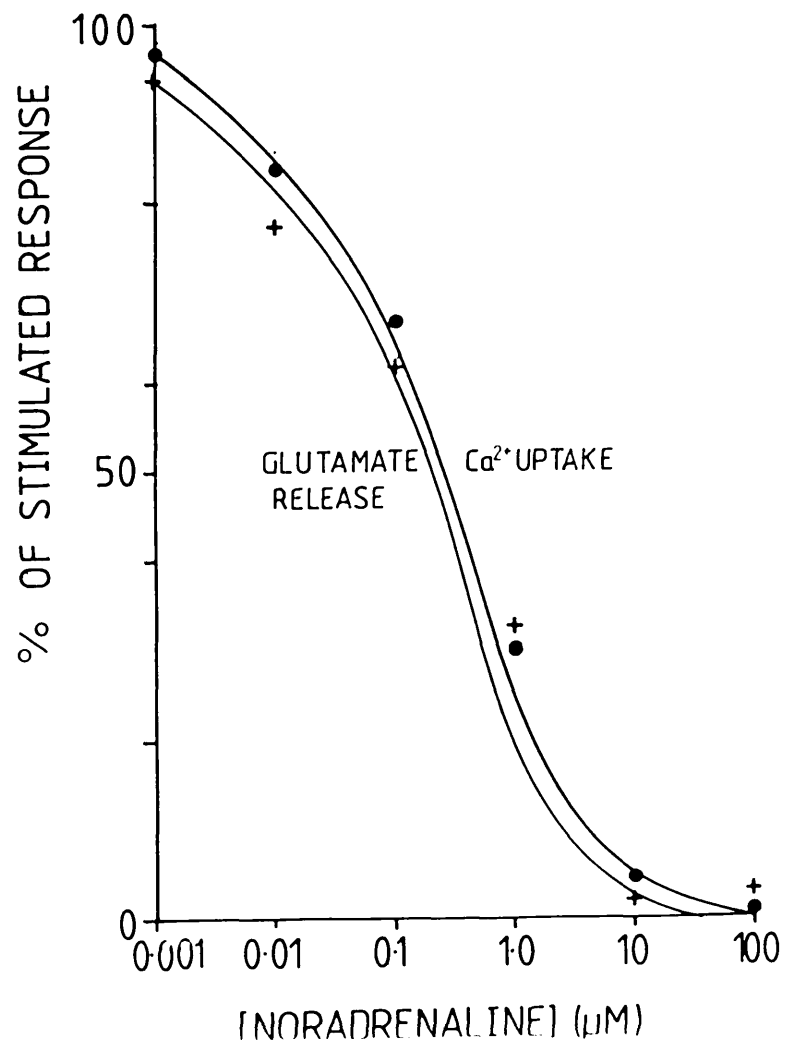


Fig 5.4b DOSE RESPONSE CURVES :
DOPAMINE

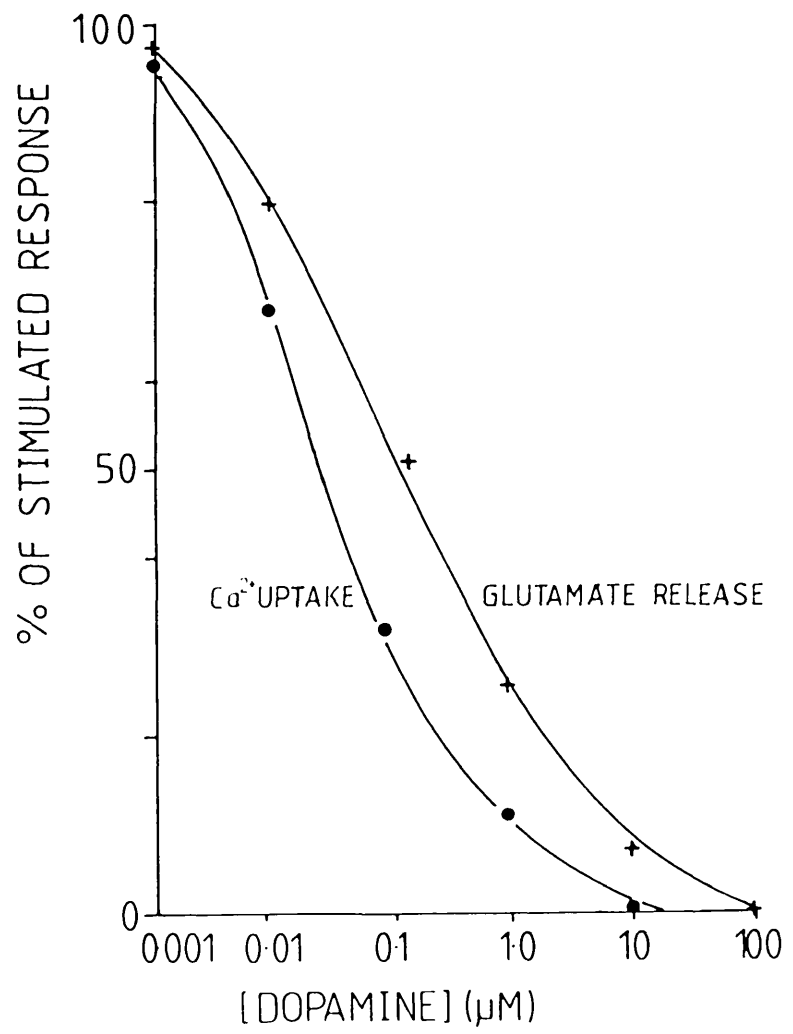


Fig 5.3c DOSE RESPONSE CURVE FOR THE ACTION
OF APOMORPHINE

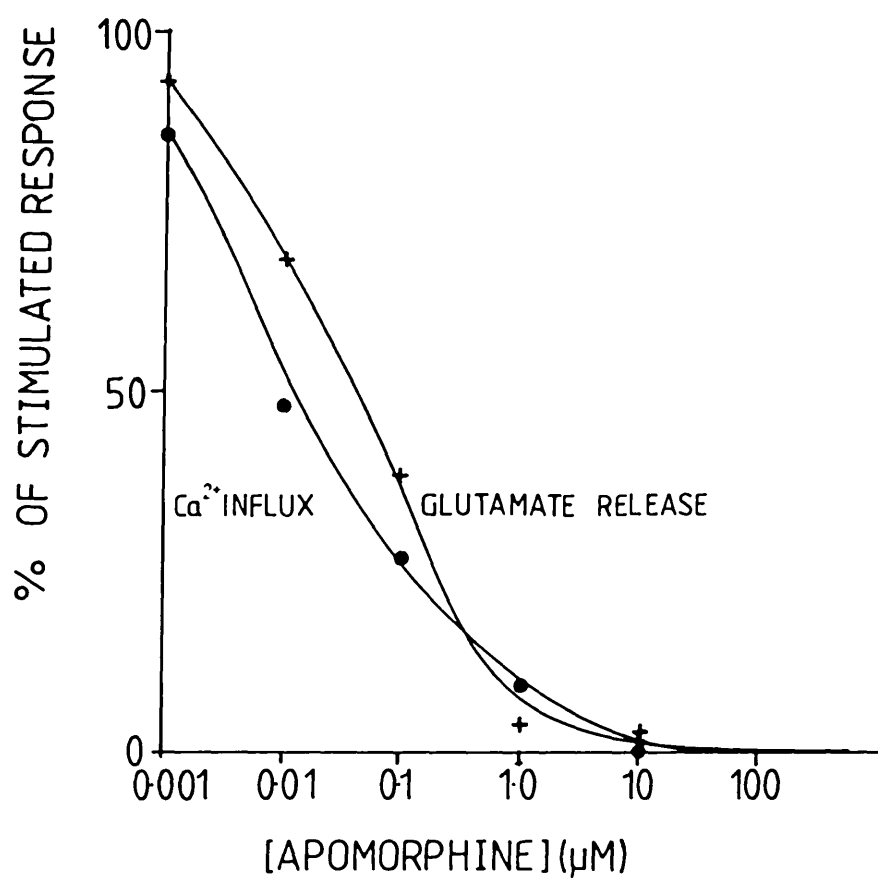


Fig 5.3d DOSE RESPONSE CURVES :
5- HYDROXY TRYPTAMINE

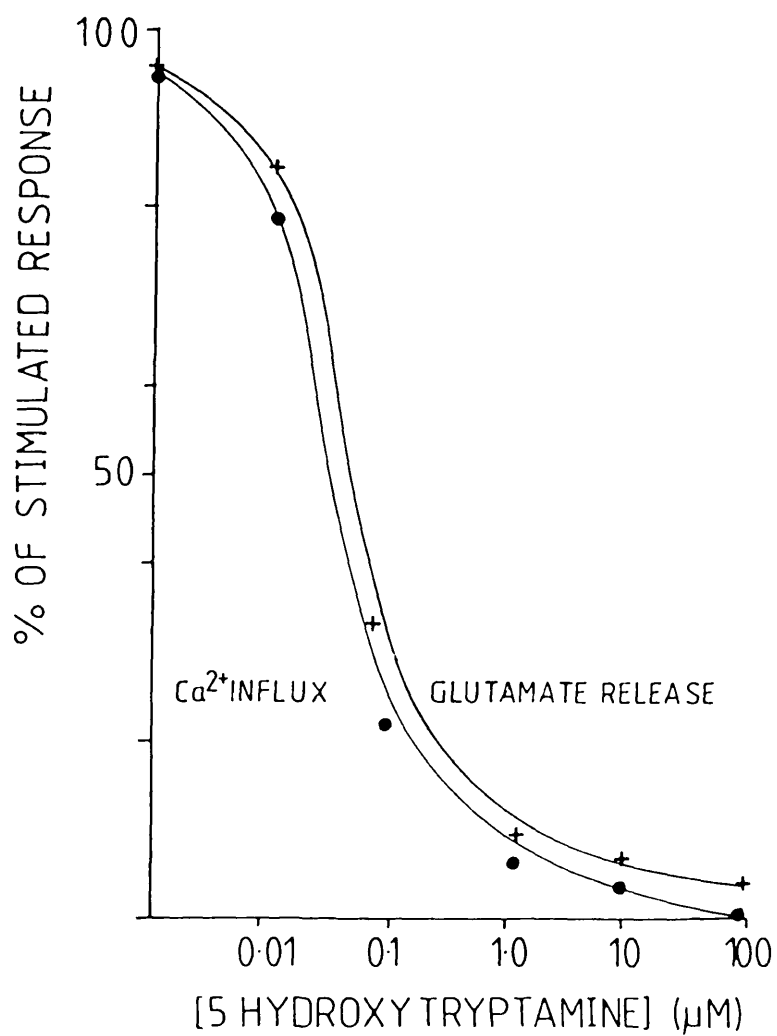


Table 5.1 **Effect of noradrenaline on veratrine-stimulated Ca^{2+} uptake, K^+ content and endogenous amino acid release from cortex slices.**

	Ca^{2+} uptake nmol/100mg	K^+ loss $\mu\text{equiv}/100\text{mg}$	Glu release nmol/100mg
Control	1113 ± 51	80.44 ± 2.47	1647 ± 113
NA $100\mu\text{M}$	1162 ± 62	76.19 ± 2.47	1564 ± 37
Vt $25\mu\text{M}$	1526 ± 69	48.68 ± 1.22	2486 ± 171
Vt + NA $0.001\mu\text{M}$	1507 ± 131	47.91 ± 1.64	2428 ± 131
Vt + NA $0.01\mu\text{M}$	1522 ± 167	52.67 ± 1.61	2311 ± 57
Vt + NA $0.10\mu\text{M}$	1423 ± 115	44.77 ± 2.36	2159 ± 94
Vt + NA $1.00\mu\text{M}$	$1223 \pm 79^*$	46.00 ± 1.56	$1924 \pm 107^*$
Vt + NA $10.0\mu\text{M}$	$1167 \pm 107^*$	45.97 ± 3.23	$1689 \pm 156^*$
Vt + NA $100\mu\text{M}$	$1115 \pm 91^*$	53.08 ± 3.58	$1632 \pm 134^{**}$

Cortex slices were cut and incubated as in 2.1b and 2.2b. Nialamide (10^{-5}M) and ascorbate (10^{-4}M) were present in the incubation medium. Noradrenaline (NA) was added, at the correct concentration, 25 min into the pre-incubation, Veratrine (Vt; $25\mu\text{M}$) was used as depolarizing agent. Samples of the NaOH digest were taken for determination of Ca^{2+} content, and K^+ content. Aliquots of the incubation media were taken for amino acid analysis. Values are mean $\pm$ SEM, $n=6$.

Student's two-tailed t-test was used to evaluate the significance of difference between depolarizing agent alone and depolarizing agent plus noradrenaline was (* $P<0.5$; ** $P<0.01$).

Table 5.2 **Effect of noradrenaline on K^+ -stimulated Ca^{2+} uptake in cortex slices.**

Treatment	Ca^{2+} uptake nmol/100mg protein	
Control	941 $\pm$ 114	
NA 100 μ M	1016 $\pm$ 132	(108)
K^+ 30mM	1392 $\pm$ 74 **	(149)
K^+ 30mM plus:		
NA 0.001 μ M	1394 $\pm$ 71 **	(148)
NA 0.01 μ M	1206 $\pm$ 106*	(128)
NA 0.1 μ M	1141 $\pm$ 123	(121)
NA 1.0 μ M	1036 $\pm$ 94	(110)
NA 10 μ M	914 $\pm$ 106	(97)
K^+ 30mM + NA 0.1 μ M	1126 $\pm$ 34	(119)
+ TTX 1 μ M		

Cortex slices were cut and incubated as in 2.1b and 2.2b. Nialamide (10^{-5} M) and ascorbate (10^{-4} M) were present in the incubation medium. TTX (1 μ M), where required, was added to the incubation vessel before the addition of the slice. Noradrenaline (NA) was added, at the correct concentration, 25 min into the pre-incubation. K^+ (K^+ ; 30mM) was the depolarizing agent. The K^+ content of the slices could not be determined due to the background level of K^+ . Values are mean $\pm$ SEM, n=6. Values in parentheses are % of control.

Student's two-tailed t-test was used to evaluate the significance of difference from control, (*P<0.05; **P<0.01).

Table 5.3 Action of adrenergic antagonists on the inhibition of veratrine-stimulated Ca^{2+} uptake by noradrenaline in cortex slices.

Treatment	Ca^{2+} uptake (% of Control)		
	Yohimbine	Corynanthine	Propanolol
Control	100	100	100
Antag $10\mu\text{M}$	98 ± 3	105 ± 5	111 ± 7
NA	105 ± 6	103 ± 9	97 ± 4
Vt	$147 \pm 12^{**}$	$137 \pm 6^{**}$	$139 \pm 7^{**}$
Vt + Antag $10\mu\text{M}$	$146 \pm 7^{**}$	$133 \pm 5^{**}$	$136 \pm 4^{**}$
Vt + NA	99 ± 7	96 ± 5	104 ± 3
Vt + NA + Antag $0.1\mu\text{M}$	$131 \pm 7^{**}$	103 ± 4	101 ± 7
Vt + NA + Antag $1.0\mu\text{M}$	$136 \pm 6^{**}$	$115 \pm 7^*$	107 ± 4
Vt + NA + Antag $10\mu\text{M}$	$151 \pm 6^{**}$	$127 \pm 3^*$	109 ± 10

Cortex slices were cut and incubated as described in 2.1b and 2.2b. Antagonists were dissolved and diluted in the Krebs-Tris medium, except for corynanthine which was dissolved in ethanol and then diluted so that the final concentration in the incubation containing the brain slice was less than 0.3% ethanol. The antagonists were added five minutes before the addition of noradrenaline (NA; $100\mu\text{M}$). The Ca^{2+} uptake was measured as described (2.3). Veratrine (Vt; $25\mu\text{M}$) was added as the depolarizing agent.

Values are mean $\pm$ SEM, $n=9$. Student's two-tailed t-test was used to evaluate the significance of difference from control (* $P<0.05$; ** $P<0.01$). Control Ca^{2+} uptake = 1119 ± 136 nmol/100mg protein.

Table 5.4 Action of adrenergic antagonists on the inhibition of veratrine-stimulated glutamate release by noradrenaline in cortex slices.

Treatment	Glutamate release (% of Control)		
	Yohimbine	Corynanthine	Propanolol
Control	100	100	100
Antag 10 μ M	106	106	97
NA	107	96	101
Vt	154**	142**	145**
Vt + Antag 10 μ M	151**	136**	138**
Vt + NA	86	104	99
Vt + NA + Antag 0.1 μ M	121	110	101
Vt + NA + Antag 1.0 μ M	164**	118	104
Vt + NA + Antag 10 μ M	169**	122*	106

Cortex slices were cut and incubated as described in 2.2b. Antagonists (Antag) were dissolved and diluted in the Krebs-Tris medium, except for corynanthine which was dissolved in ethanol and then diluted so that the final concentration of ethanol in the incubation was 0.3%. The antagonists were added five minutes before the addition of noradrenaline (NA; 100 μ M). Veratrine (Vt; 25 μ M) was added as the depolarizing agent. The amino acid content of the incubation medium was measured as described (2.6).

Values are mean $\pm$ SEM, n=9. Student's two-tailed t-test was used to evaluate the significance of difference from control. (*P<0.05; **P<0.01). Basal glutamate release 1456 $\pm$ 86 nmol/100mg protein.

Table 5.5a **Effect of dopamine on veratrine stimulated Ca^{2+} uptake, K^+ content, and endogenous amino acid release from striatal slices.**

Treatment	Ca^{2+} uptake nmol/100mg	K^+ content $\mu\text{equiv K}^+$	Glutamate nmol/100mg
Control	1047 ± 85	55.8 ± 4.9	1041 ± 165
DA 100 μM	1036 ± 104	54.13 ± 3.4	1103 ± 104
Vt 25 μM	$1769 \pm 76^{***}$	$35.15 \pm 1.9^{***}$	$1686 \pm 44^{***}$
Vt + DA 0.001 μM	$1732 \pm 73^{***}$	$34.59 \pm 2.9^{***}$	$1655 \pm 131^{***}$
Vt + DA 0.010 μM	$1545 \pm 104^{**}$	$35.71 \pm 2.4^{***}$	$1563 \pm 114^{***}$
Vt + DA 0.10 μM	1408 ± 53	$35.71 \pm 2.9^{***}$	$1389 \pm 96^{**}$
Vt + DA 1.00 μM	1227 ± 74	27.34 ± 7.8	1202 ± 94
Vt + DA 10.00 μM	1106 ± 141	$35.71 \pm 5.8^{***}$	1073 ± 151
Vt + DA 100.0 μM	1016 ± 174	$34.59 \pm 3.4^{***}$	968 ± 106

Striatal slices were cut and incubated as in 2.1b and 2.2b. Nialamide (10^{-5}M) and ascorbate (10^{-4}M) were present in the incubation medium. Dopamine (DA) was added, at the correct concentration, 25 min into the pre-incubation, Veratrine (Vt; 30mM) was used as depolarizing agent. Samples of the NaOH digest were taken for determination of Ca^{2+} content and K^+ content. Aliquots of the incubation media were taken for amino acid analysis. Values are mean $\pm$ SEM, n=6. are % of control. Student's two-tailed t-test was used to evaluate the significance of difference from control (**P<0.01; ***P<0.005)

Table 5.5b **Effect of dopamine on K⁺ stimulated Ca²⁺ uptake.**

Treatment	Ca ²⁺ uptake nmol/100mg protein
Control	1174 ± 136
K ⁺ 30mM	1560 ± 198 (132)**
K ⁺ + DA 0.001μM	1466 ± 73 (124)*
K ⁺ + DA 0.010μM	1424 ± 136 (121)*
K ⁺ + DA 0.10μM	1372 ± 106 (117)
K ⁺ + DA 1.00μM	1288 ± 146 (110)
K ⁺ + DA 10.00μM	1201 ± 177 (102)
K ⁺ + DA 0.1μM + TTX 1μM	1309 ± 62 (111)

Striatal slices were cut and incubated as in 2.1b and 2.2b. Nialamide (10⁻⁵M) and ascorbate (10⁻⁴M) were present in the incubation medium. TTX (1μM), where required, was added to the incubation vessel before the addition of the slice. Dopamine (DA) was added, at the correct concentration, 25 min into the pre-incubation. K⁺ (30mM) was used as depolarizing agent. Samples of the NaOH digest were taken for determination of Ca²⁺ content. The K⁺ content of the slices could not be determined due to the background level of K⁺. Values are mean ± SEM, n=6. Values in parentheses are % of control.

Student's two-tailed t-test was used to evaluate the significance of difference from control, (*P<0.05; **P<0.01).

Tables 5.6 a and b

Action of dopaminergic antagonists on the inhibition of veratrine-stimulated Ca^{2+} uptake and glutamate release by dopamine.

Striatal slices were prepared and incubated as described (2.1b and 2.2b). Antagonists (Antag) were dissolved in Ca^{2+} Krebs-Tris medium and added five minutes before dopamine (DA; $1\mu\text{M}$). Veratrine (Vt; $25\mu\text{M}$) was used as the stimulating agent and added 1 minute after the $^{45}\text{Ca}^{2+}$. The uptake of Ca^{2+} was measured as described (2.3). Samples of the incubation medium were taken for amino acid analysis.

Values are mean $\pm$ SEM, $n=6$. Values in parentheses are % of control.

Significance of difference between veratrine and veratrine plus dopamine plus antagonist were calculated by Student's two tailed t-test for unpaired samples (* $P<0.05$; ** $P<0.01$).

Table 5.6a Action of dopaminergic antagonists on the inhibition of veratrine-stimulated Ca^{2+} uptake by dopamine in striatal slices.

Treament	Ca^{2+} uptake nmol/100mg protein	
	Sulpiride	Chlorpromazine
Control	1313 $\pm$ 118	1314 $\pm$ 72
Antag 1.0 μM	1264 $\pm$ 106 (96)	1374 $\pm$ 136 (104)
DA	1364 $\pm$ 74 (104)	1360 $\pm$ 84 (103)
Vt	2394 $\pm$ 212 (182)	2108 $\pm$ 161 (160)
Vt + Antag 10 μM	2269 $\pm$ 147 (173)	2008 $\pm$ 148 (153)
Vt + DA	1464 $\pm$ 143 (112)	1448 $\pm$ 127 (110)
Vt + DA + Antag 0.01 μM	1522 $\pm$ 117 (116)	1647 $\pm$ 203 (124)
Vt + DA + Antag 0.1 μM	2092 $\pm$ 133 (159)*	1859 $\pm$ 169 (141)*
Vt + DA + Antag 1.0 μM	2414 $\pm$ 142 (183)**	2194 $\pm$ 103 (166)**

Table 5.6b Action of dopaminergic antagonists on the inhibition of veratrine-stimulated glutamate release by dopamine in striatal slices.

Glutamate release nmol/100mg protein		
Treatment	Sulpiride	Chlorpromazine
Control	1049 ± 119	1246 ± 174
Antag 1.0μM	1164 ± 107 (111)	1236 ± 26 (99)
DA	979 ± 136 (107)	1301 ± 126 (104)
Vt	1829 ± 185 (174)	1732 ± 146 (139)
Vt + Antag 1.0μM	1849 ± 194 (176)	1864 ± 198 (150)
Vt + DA	1076 ± 121 (103)	1353 ± 176 (109)
Vt + DA + Antag 0.01μM	1664 ± 184 (157)*	1307 ± 143 (106)
Vt + DA + Antag 0.1μM	1894 ± 116 (118)**	1541 ± 119 (124)

Table 5.7 Action of metergoline

Treatment	Ca ²⁺ uptake nmol/100mg protein	Glutamate release nmol/100mg protein
Control	1217 ± 156	1897 ± 210
Ant 1.0μM	1198 ± 210 (94)	1789 ± 102 (94)
5-HT	1250 ± 102 (102)	1982 ± 198 (104)
Vt	1919 ± 132 (151)**	2826 ± 153 (150)**
Vt + 5-HT	1341 ± 214 (110)	1998 ± 132 (105)
Vt + Ant 1.0μM	1879 ± 154 (156)**	2765 ± 231 (146)**
Vt + 5-HT + Ant 0.01μM	1435 ± 210 (118)	2145 ± 176 (113)
Vt + 5-HT + Ant 0.1μM	1629 ± 198 (134)	2678 ± 245 (141)
Vt + 5-HT + Ant 1.0μM	1867 ± 192 (153)*	2787 ± 148 (147)*

Cortex slices were cut and incubated as described (2.1b and 2.2b). Metergoline (Ant) was dissolved in ethanol and then diluted so that the final ethanol concentration in the incubation medium in the presence of the slice was less than 0.3%. The antagonist was added five min before the addition of 5-HT (1μM). Veratrine (VT; 25μM) was used as the depolarizing agent. The uptake of Ca²⁺ was measured as described (2.3). Samples of the incubation medium were taken for amino acid analysis.

Values are mean ± SEM, n=9. Values in parentheses are % of control. Student's two-tailed t-test was used to evaluate the significance of difference from control, (*P<0.05; **P<0.01).

Table 5.8 Comparison of the IC₅₀ values for the inhibition of veratrine-stimulated Ca²⁺ uptake and glutamate release by the three neurotransmitters.

	IC ₅₀ (μM)	
	Ca ²⁺ uptake nmol/100mg protein	Glutamate release nmol/100mg protein
Dopamine	0.08 (0.06-0.11)	0.16 (0.12-0.21)
5-HT	0.02 (0.01-0.04)	0.1 (0.07-0.14)
Noradrenaline	0.14 (0.01-0.24)	0.13 (0.05-1.7)

Values and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (1949). The results presented for dopamine were obtained using rat striatal slices, whilst cortex slices were used for the experiments involving noradrenaline and 5-HT. Veratrine was used as the depolarizing agent.

It has been established in recent years that in the peripheral nervous system as well as in the CNS the release of noradrenaline from noradrenergic neurones is inhibited by the activation of presynaptic α_2 -adrenergic receptors (Langer, 1977; Starke, 1977). This has also been shown to be true for dopamine and for 5-HT acting through their own specific presynaptic receptors (Farnebo and Hamberger, 1974; Middlemiss, 1981).

In the absence of depolarizing agents, noradrenaline (100 μ M) did not affect Ca^{2+} uptake or neurotransmitter release (Table 5.1). The results presented show, that noradrenaline at concentrations of 1 μ M and above reduced the Ca^{2+} influx induced by 25 μ M veratrine or 30mM K^+ , but did not modulate the influx induced by 75 μ M veratrine or 56mM K^+ (Fig 5.1).

Conceivably, when the neuronal membrane was exposed to a strong depolarizing stimulus (eg, veratrine 75 μ M or K^+ 56mM) at a normal physiological external Ca^{2+} concentration the resulting influx of Ca^{2+} ions was so great that the modulating system was overridden. It has been shown (Dismukes et al, 1977; Gothert, 1979) that at lower external Ca^{2+} concentrations even a strong depolarizing stimulus will provoke a moderate Ca^{2+} influx, which is susceptible to α_2 -receptor mediated modulation.

Although it has been suggested that the α_2 - receptors which modulate noradrenaline release are located presynaptically at or near nerve endings, there is no direct evidence as to their precise location. The inhibitory effect of noradrenaline on the K^+ -induced Ca^{2+} influx and the K^+ -induced amino acid release was still apparent even in the presence of TTX (1 μ M) (Table 5.2). This provides evidence against the involvement of an interneuronal loop in the presynaptic mechanism of the action of noradrenaline. Evidence from experiments utilising synaptosomes indicate that the receptors regulating the feedback mechanisms are located presynaptically (De Langan et al, 1979).

The results reported here show that only at high concentrations (100 μ M) did noradrenaline affect the K^+ loss due to veratrine. Changes in K^+ content usually indicate that the added substance is affecting the ability of veratrine to induce depolarization. Such action could occur by the blockage of the Na^+ channels held open by veratrine or by the drug acting upon K^+ channels leading to a rapid repolarization and possible hyperpolarization of the neurone. Noradrenaline (100 μ M) may well be acting to hyperpolarize the membrane. Dismukes et al (1977) suggested that the neuronal membrane may be hyperpolarized by an α_2 -adrenergic receptor -mediated lowering of the ratio of

Na^+/K^+ permeability. The hyperpolarizing action of noradrenaline has also been reported to occur in peripheral systems (Brown et al, 1979).

At lower noradrenaline concentrations ($<10\mu\text{M}$) no significant change in K^+ loss was detected. This may not mean that these lower concentrations did not hyperpolarize the cell, but rather that such small local changes in K^+ content could not be detected against the general high level of K^+ in the slice.

The data presented here show that Ca^{2+} influx into cortical slices induced by depolarization (veratrine $25\mu\text{M}$) is decreased by noradrenaline in a dose-dependent manner, the IC_{50} value being $0.14\mu\text{M}$ (Fig 5.2a). This IC_{50} value of $0.14\mu\text{M}$ correlates with data presented by Reichenbacher et al, (1982) who reported that the stimulated release of [^3H] noradrenaline could be inhibited by approximately 50% in the presence of noradrenaline concentrations of $0.01\mu\text{M}$ - $0.1\mu\text{M}$. As both the [^3H] noradrenaline release and the Ca^{2+} influx were inhibited by similar concentrations of noradrenaline, it suggests that inhibition of the voltage-dependent Ca^{2+} influx may be the method of presynaptic regulation.

However, noradrenaline did not inhibit stimulated Ca^{2+} influx into striatal slices even at a concentration of $100\mu\text{M}$. This correlates well with the low density of noradrenergic neurones and terminals in the striatum.

The inhibitory action of noradrenaline on stimulated neuroactive amino acid release was found to be dose dependent with an IC_{50} value of $0.029\mu\text{M}$ (0.05 - $0.17\mu\text{M}$ for the inhibition of the veratrine-stimulated glutamate release). This IC_{50} value falls within the 95% confidence limits of the IC_{50} value for inhibition of Ca^{2+} influx (Table 5.8), the significance of which will be discussed later. It is of interest that the stimulated release of each of the three amino acid neurotransmitters measured in these experiments was inhibited by noradrenaline in a dose-dependent manner, which suggests that presynaptic adrenergic receptors are present on terminals releasing glutamate, aspartate, and GABA.

Dolphin et al (1982) have shown that in the cerebellum, added noradrenaline decreased glutamate release. However, the presence of noradrenergic receptors on glutamatergic or aspartatergic terminals in the cortex has not been studied in detail (reviewed in Chesselet, 1984). The enhancing action of GABA on the cortical release of noradrenaline has been demonstrated (Taube et al, 1977; Arbilla and Langer, 1979), but there is little evidence in the literature to suggest that presynaptic noradrenergic receptors exist on GABAergic terminals. The addition of yohimbine, a preferential α_2 -antagonist has been shown to enhance the release of [^3H] GABA from mouse brain stem and rat cerebral cortex slices (Maurin et al, 1985). However, these latter results are thought to be due to the high ($>10\mu\text{M}$) concentration

of yohimbine being used which may have nonspecific effects on transmitter release which are not mediated by α_2 -adrenergic receptors. Yohimbine has been shown to have anticonvulsant activity in a convulsive mutant of the mouse (Chermat et al, 1979, 1981), but proconvulsant activity has also been detected (Worms and Lloyd, 1982). These pro- and anti-convulsant effects may be due to the actions of yohimbine on the GABAergic or glutaminergic systems, both systems being implicated in the origin of convulsions. This possibility is discussed in greater depth in Chapter 6.

Three antagonists were used in this study which have been defined as being specific to certain adrenergic receptor subtypes. This classification has been shown to hold true in electrophysiological and biochemical tests on many different animal systems (for example; Reichenbacher et al, 1982). Yohimbine, as has already been mentioned, is a preferential α_2 -antagonist; corynanthine, an α_1 -antagonist; and propranolol, a selective β -adrenergic antagonist. All three antagonists at the concentrations used in the test had no effect on either basal or veratrine stimulated Ca^{2+} uptake, K^+ content, or amino acid release. The lack of action of yohimbine on amino acid release is in contrast to the findings of Maurin et al (1985), as explained above.

The relative potencies of the three antagonists in reversing the noradrenaline induced inhibition of both veratrine stimulated Ca^{2+} influx and amino acid influx in this study was yohimbine > corynanthine > propranolol. This order of potency has been found to be characteristic of α_2 -adrenergic receptors (Reichenbacher et al, 1982). It may therefore be concluded that the receptors mediating the effects of noradrenaline are predominantly of the α_2 type. Corynanthine (α_1 -antagonist) only partially reversed the effect of noradrenaline which may reflect a cross-reactivity of this antagonist with the α_2 -receptor and/or that the α_1 -receptors may have been involved in some part of the mechanism. The total inability of the β -adrenergic antagonist to exhibit any reversal indicates the lack of β -adrenergic receptor involvement in this physiological system.

Yohimbine and corynanthine at concentrations which affected the inhibitory actions of noradrenaline did not reduce the veratrine-induced K^+ loss. Thus the actions of the antagonists do not appear to involve changes in the amount of neuronal depolarization.

Thus, noradrenaline decreases stimulated Ca^{2+} influx and amino acid release by an α_2 -receptor mediated action. The effect is dependent on the strength of stimulation and is not affected by the presence of TTX.

Dopamine ($0.001\mu\text{M}$ - $1.00\mu\text{M}$), in parallel to the actions of noradrenaline, caused no detectable stimulation of either basal Ca^{2+} influx or neurotransmitter release in slices taken

from either the cortex or striatum. The K^+ content of the slices was similarly unaffected by dopamine at any concentration. Dopamine in a dose-dependent manner inhibited both the veratrine- and K^+ -stimulated Ca^{2+} influx into striatal slice (IC_{50} of $0.08\mu M$ and $0.07\mu M$ respectively).

Apomorphine, a dopamine agonist, also had a dose dependent inhibitory action on veratrine stimulated striatal slices (IC_{50} $0.03\mu M$, Fig5.2c).

Dopamine was without effect on veratrine-stimulated cortex slices. These results compare favourably with Bannan et al (1981) who noted that cortical neurones lacked dopaminergic presynaptic terminals. Fadda et al (1984) showed that two dopamine agonists, apomorphine and N-n-propyl norapomorphine (NPA), were ineffective in decreasing the production of dopamine in the dorso-lateral frontal cortex, but the agonists were active in reducing dopamine production in the medial prefrontal cortex. It is possible that contrasting results concerning the existence of dopamine presynaptic receptors in the cortex depend upon which area is taken for analysis.

The dopamine inhibition of the K^+ -evoked Ca^{2+} uptake could not be reversed by $1\mu M$ TTX, indicating that interneurones are not involved in the mechanism of presynaptic inhibition. However, Fukuda and Kameyama (1980) reported evidence for the existence of TTX-insensitive neurones in the striatum, so that the inability of TTX to cause reversal of the inhibition may be due to the insensitive nature of the neurones rather than the lack of interneuronal involvement.

The veratrine-induced amino acid release was inhibited by both dopamine and apomorphine in a dose-dependent manner (IC_{50} 's of $0.169\mu M$ and $0.085\mu M$ respectively, for the inhibition of glutamate release; Figs 5.2 a and b). A reciprocal regulation appears to exist between dopamine and glutamate/aspartate in the striatum; glutamate increased dopamine release (Giorgiueff et al ,1977), whilst dopaminergic agonists decreased the release of glutamate in striatal slices (Rowlands and Roberts, 1980; Mitchell and Dogett, 1980). This latter effect appeared to be modulated through the action of dopaminergic presynaptic D-2 receptors located on the cortico-striate terminals (Schwartz et al, 1978). However, large concentrations of dopamine agonists were required to inhibit the release glutamate (Helmreich et al, 1982), and the pharmacology of the receptor involved did not appear to correlate directly with the D-2 receptor. This may be the result of the differential binding of agonists to the D-2/D-3 receptors mentioned in the introduction. Together with the decrease in the release of the two excitatory amino acids, dopamine also decreased the veratrine-stimulated release of GABA. Much evidence has accumulated for the effect of GABA on

dopamine release but little appears to be known about the reverse situation. Korf et al (1981) have shown that *in vivo* dopamine and dopamine agonists decrease GABA release in the striatum, but they found little evidence to suggest that this was a presynaptic effect. Many authors have pointed out that studies on GABA-dopamine inter-relationships depend greatly upon the area of the striatum studied (reviewed in Stoof and Kebabian, 1981), and whether monoamine oxidase inhibitors were present.

The classification of the receptor involved in mediating the effects of dopamine reported in this study was made by comparing the potency by which two dopaminergic antagonists reversed the inhibitory actions of dopamine; the antagonists used were chlorpromazine and sulpiride. Sulpiride is a substituted benzamide and is used clinically as a neuroleptic drug. It is also a potent D-2 receptor antagonist either in electrophysiological or in behavioural tests (Woodruff et al, 1983), but it is ineffective as an antagonist against the D-1 receptor. Chlorpromazine similarly is an antagonist whose activity is directed towards the D-2 receptor in preference to the D-1 receptor.

Both these antagonists reversed the inhibition of veratrine-induced Ca^{2+} uptake and of amino acid release in a dose-dependent manner. The IC_{50} for the reversal of Ca^{2+} influx being approximately $0.05\mu\text{M}$ (sulpiride) and $0.1\mu\text{M}$ (chlorpromazine), thus sulpiride was the more potent. The reversal of dopamine inhibition indicates a receptor-mediated effect, the receptor being of the D-2 subtype. El Mestikawy et al, (1986) showed that the rate of dopamine synthesis was greatly reduced by sulpiride ($1\mu\text{M}$ - $10\mu\text{M}$) since its action on binding to the D-2 receptor on the striatal dopaminergic terminals decreased tyrosine hydroxylase activity. The concentrations of sulpiride used in those experiments were in the same range as those found to be effective in this study.

The 5-HT innervation of the forebrain originates from the raphe nucleus (Kuhar et al, 1972). Thus, the presynaptic regulation of 5-HT can be studied in cortex slices, as the 5-HT-containing terminals are uniformly distributed in the superficial layers of the entire cerebral cortex in rats (Aghajanian et al, 1975).

In the results presented here exogenous 5-HT, in a dose-dependent manner, decreased the veratrine-stimulated Ca^{2+} uptake in the cortex slices, in the presence of chlorimipramine, a 5-HT uptake blocker. The IC_{50} for 5-HT for the inhibition of veratrine-stimulated Ca^{2+} uptake was $0.02\mu\text{M}$, which is comparable to the concentration of 5-HT which was required to decrease, by 50%, the efflux of [^3H] 5-HT from rat brain cortex slices (Gothert and Weinheimer, 1979).

The inhibition of K^{+} -induced Ca^{2+} influx by 5-HT was observed in the presence of

TTX, suggesting that interneurons do not play a role in this inhibitory effect, but rather that 5-HT presynaptic receptors are located on the presynaptic membrane. The inhibitory effects of 5-HT on the [^3H] 5-HT release was observed in synaptosomes (Cerrito and Raiteri, 1979) which supports a presynaptic localisation for these receptors.

5-HT did not influence the veratrine-induced K^+ -loss from the slice thus indicating that 5-HT was not acting to prevent Ca^{2+} influx or neurotransmitter release by affecting the level of depolarization.

Metergoline has been shown to have antagonistic properties at the 5-HT autoreceptor site (Cox and Ennis, 1982). Engel et al (1983) in experiments using rat brain cortex proposed that the 5-HT receptor may consist of two subtypes, 5-HT 1A and 5-HT 1B , and that the 5-HT presynaptic receptor has pharmacological similarities to the 5-HT 1B site. Thus, since the 5-HT effects reported here are inhibited by low concentrations of metergoline, the action is probably mediated by the 5-HT 1 presynaptic receptor. Addition of 5-HT to cortex slices caused a decrease in the stimulated release of glutamate, aspartate, and GABA. The inhibition was reversed by metergoline, which indicates that the effect may be mediated by the same receptor through which 5-HT induced the decrease in Ca^{2+} influx.

In summary, therefore, dopamine, 5-HT, and noradrenaline can reduce the veratrine-stimulated Ca^{2+} uptake (Table 5.8) and the evoked release of glutamate, aspartate, and GABA. The responses are receptor-mediated as they are sensitive to antagonists known through previous research to interact specifically with their respective presynaptic receptors.

It was found in the results presented here that modulation of Ca^{2+} uptake by the transmitters only occurred when the slices were stimulated by submaximal concentrations of veratrine or K^+ . The results compare favourably with those obtained by Gothert (1980) and Langer (1977), who found that when the Ca^{2+} concentration of the external medium was lowered, or the strength of stimulation decreased, the inhibitory action of the neurotransmitters on their release was enhanced. Thus, these results support the hypothesis that presynaptic regulation of transmitter release is by modulating Ca^{2+} influx.

Gothert (1980) compared the effects of 5-HT and methiothepin on the release of [^3H] 5-HT at different external Ca^{2+} concentrations. By constructing a double reciprocal plot, he found that V_{max} was not altered but that the K_{m} was changed. A change in V_{max} would be a result of activation of previously inactive varicosities, the alteration in K_{m} is compatible with the idea that these compounds exert their actions by modifying the affinity of the voltage-sensitive Ca^{2+} channel. An alternative explanation for the altered K_{m} could be that the compounds change the affinity of the intracellular secretory mechanism for external

Ca²⁺ ions. However, this possibility appears to be less probable, because it is likely that the presynaptic receptors themselves are located in the cell membrane and, in addition, because the voltage-sensitive Ca²⁺ channel has been shown to be the site of action of presynaptic modulators in noradrenergic neurones in which the stimulus-release coupling is very similar to that in 5-HTergic neurones (Gothert et al, 1979).

It is not known how receptor activation mediates a decrease in Ca²⁺ influx. There could be binding to a receptor which is somehow linked directly to the Ca²⁺ channel and that activation of the receptor would affect the kinetics of the opening and closing of the Ca²⁺ channel. Alternatively, receptor activation could lead to an alteration in the production of a secondary messenger eg cAMP and that could lead to modulations in neurotransmitter release and in Ca²⁺ influx.

The results shown in Table 5.8 indicate that noradrenaline inhibits both veratrine-stimulated Ca²⁺ uptake and amino acid release with similar IC₅₀ values. Dopamine and 5-HT both inhibit the veratrine-stimulated Ca²⁺ influx and amino acid release, however the IC₅₀ values for the inhibition of both these parameters are significantly different, although still very potent. This may indicate that the mechanism of presynaptic modulation by noradrenaline is different to the mechanism by which dopamine and 5-HT elicit their presynaptic responses.

In the majority of these experiments veratrine was used as the depolarizing agent. As shown previously, (Chapters 3 and 4) veratrine stimulates neurotransmitter release even in the absence of external Ca²⁺, and this has been attributed to an increase in intracellular Na⁺ concentration (Cunningham and Neal, 1981). This high intracellular Na⁺ concentration may cause a substantial release of Ca²⁺ from bound pools such as contained in mitochondria (Crompton et al, 1978). Schoffelmeer and Mulder (1983) showed that in Ca²⁺ free medium there was a substantial release of [³H] noradrenaline which could not be blocked by 50mM Cd²⁺ which is a potent Ca²⁺ channel blocker, however, release was blocked by 0.3 mM TTX, a Na⁺ channel blocker. The transmitter release facilitated by Ca²⁺ derived internally was subject to a small time delay as compared with the release induced in the presence of external Ca²⁺.

Thus if noradrenaline, dopamine, and 5-HT are to inhibit the veratrine-stimulated amino acid release at concentrations comparable to the blockade of Ca²⁺ uptake, then the involvement of a secondary messenger system is necessary.

The results presented here indicate that a secondary messenger mediates the actions of noradrenaline, and this hypothesis is supported by results obtained from other experiments. A

recent paper by Ullrich and Wollheim (1985) have shown that an insulin-secreting cell line possessed both α_1 - and α_2 -receptors. They showed that α_2 -adrenergic receptor action is exerted at a step later and distant from the point of Ca^{2+} entry, a hypothesis supported by results obtained by Mulder and Schoffelmeer (1985) for the action of noradrenaline on α_2 -receptors. Dismukes and Mulder (1976) proposed that a presynaptic adenylate cyclase system may link receptor activation and membrane alterations. Evidence for the α_2 -adenylate cyclase link in the CNS is not as well proven (see introduction to Chapter 5). Wemer et al (1982) and Schoffelmeer and Mulder (1983) both give more evidence to support the involvement of adenylate cyclase in the action of noradrenaline. Limberger and Starke (1984) showed that there was a delay between the addition of noradrenaline and the onset of inhibition of about 4 seconds. A delay of some seconds is not unexpected for effects mediated by adenylate cyclase linked receptors.

Dopamine is also able to inhibit Ca^{2+} influx and amino acid release, however the IC_{50} values for the inhibition of the parameters are significantly different (Table 5.8). Arbilla and Langer (1981) and many other workers proposed that the presynaptic D-2 receptor is not linked to the adenylate cyclase system, however, in a recent review Stoof and Kebabian (1985) present evidence that the D-2 receptor may be coupled to the adenylate cyclase by an inhibitory linkage. Schoffelmeer and Mulder (1985) proposed that dopamine inhibits further release of neurotransmitters by acting directly at the Ca^{2+} channel to prevent subsequent Ca^{2+} influx, but this does not explain why a reduction of veratrine-stimulated glutamate, aspartate, and GABA release is observed in the presence dopamine. However the results presented here give further support to the hypothesis that the presynaptic modulation of dopamine release involves a second messenger system.

El Mestikawy et al (1986) suggested that dopamine controls its own synthesis by modulating the main regulatory enzyme in the process ie tyrosine hydroxylase. This modulation is via presynaptic receptors that have the pharmacological characteristics of the D-2 receptor, ie sensitivity to (-) sulpiride etc. The intrinsic properties of the enzyme can be altered by two different phosphorylation processes, one involving cAMP-dependent protein kinases (Morgenvoth et al, 1975), and the other, Ca^{2+} dependent protein kinases (El Mestikawy et al, 1985).

Investigations by Roth and co-workers (Billingsley and Roth, 1982; Roth, 1984; Wolf and Roth, 1985) have led to the proposal that control of tyrosine hydroxylase activity by presynaptic dopamine autoreceptors may involve alterations in Ca^{2+} -dependent phosphorylation, brought about by the carboxymethylation of calmodulin. Preliminary data

support the suggestion that the reduction in tyrosine hydroxylase activity in dopaminergic terminals exposed to some dopamine agonists is the result of a lower proportion of the active form of the tyrosine hydroxylase enzyme, the active form of the enzyme is produced by a cAMP-dependent protein kinase (El Mestikawy and Hamon, 1985). Cooper et al (1986) have shown that by blocking D-1 receptors with the antagonist SCH 23390 that the D-2 agonists can inhibit adenylate cyclase activity. This process of inhibition occurs with the same requirements for GTP and Na^+ as has been found for other receptors which are known to inhibit the activity of adenylate cyclase by the involvement of the inhibitory GTP regulatory protein (Rodbell, 1980).

Since the release of amino acid transmitters is inhibited potently by 5-HT a second messenger system may be involved in its presynaptic mechanism of action. The effects of 5-HT were reversed by metergoline, an specific 5-HT₁ antagonist, which indicates that the actions of 5-HT are receptor-mediated.

Evidence is accumulating that the 5-HT₁ receptor subtype is coupled to adenylate cyclase. Peroutka et al (1979) showed that GTP can differentiate between 5-HT sub-sites. GTP lowers the binding affinity of the agonist [³H] 5-HT to the 5-HT₁ subsite, whilst the binding of the agonists to the 5-HT₂ site was not affected. In general the affinity of agonists but not antagonists for receptor binding is decreased by the guanine nucleotides. The evidence that 5-HT reduces cAMP levels on interacting with the 5-HT₁ site is still very controversial. Gothert (1980) suggested that the inhibitory action of 5-HT could be due to an inhibitory interaction with the phosphoinositide system.

Thus, noradrenaline, dopamine, and 5-HT may be acting at presynaptic receptors to modulate further neurotransmitter release. However, experimental evidence obtained by Kalsner (1985) does not support this hypothesis for negative feedback control. Although the results presented here support the concept of presynaptic modulation by a decrease in Ca^{2+} influx, it remains to be determined whether such a system functions *in vivo*.

CHAPTER 6

THE INHIBITORY ACTIONS OF ANTICONVULSANT DRUGS ON BASAL AND STIMULATED BRAIN SLICE ACTIVITY.

- 6.1 Introduction
- 6.2 Results
 - 6.2a Classical anticonvulsant drug action
 - 6.2b ω -Phosphonic- α -aminocarboxylic acids.
- 6.3 Discussion

Epilepsy is not a single, simple disorder of the central nervous system but rather refers to many different types of seizure activity produced by excessive neuronal discharge in certain regions of the brain (for example, Glazer, 1975). Seizures assume a wide variety of forms, usually involving loss of consciousness and sometimes convulsive movements. Status epilepticus (Wilder et al, 1977) represents an extreme situation where seizures occur so frequently that there is little time, if any, between seizures. Other types of epilepsy include partial seizure, tonic-clonic seizure or psychomotor seizure (Meldrum, 1982). However, there is a single common factor in epilepsy in that all attacks are associated with excessively, synchronous or sustained discharge in a group of neurones in the brain.

Several animal models have been developed to enable research into both the origin of epilepsy and the mechanisms of anticonvulsant drug action. Certain strains of animal are genetically prone to seizures. For example, DBA/2 mice are an inbred strain in which, within a critical age range, a fixed sequence of seizure responses can be induced by a loud noise (Croucher et al, 1982; Chapman et al, 1984). Baboons, *Papio papio*, from Senegal, show a genetically determined syndrome of photosensitive epilepsy (Meldrum, 1972). Other models rely on chemical or electrical stimulation of certain regions of the central nervous system to induce the state of epilepsy, eg cobalt implants (Dodd and Bradford, 1976), homocysteine (Freed, 1985), or kindling by electrical stimulation (Goddard, 1983; Racine, 1978). The responses of brain slices or synaptosomes prepared from epileptic and non-epileptic animals can be compared to give an *in vitro* test system (Leach et al; 1985). With the introduction of cell tissue culture it may be possible to produce bulk epileptogenic-type cells and thus direct electrophysiological and biochemical studies could be performed.

It is interesting to note that seizures induced in animals by any of the above methods are differentially sensitive to anticonvulsant drugs (Ferendelli and Daniels-McQueen, 1982) indicating, perhaps, the underlying nature and possible origin of the different seizure types mentioned. Although there is such a diversity of seizure types it is generally held that there is one common factor namely, an imbalance between the inhibitory (probably GABA; Meldrum, 1975) and excitatory (probably glutamate and/or aspartate; Storm-Mathisen, 1977; Fonnum, 1984) systems of the central nervous system. Evidence for the involvement of these two major transmitter systems in epilepsy results mainly from experiments using agonists or antagonists of GABA, glutamate or aspartate. GABA antagonists have a tendency to be proconvulsants, whilst glutamate antagonists are anticonvulsants in animal models of epilepsy.

Autoradiographic studies using labelled GABA suggest that GABA is released by approximately one third of all synaptic endings in the brain (Iversen and Kelly, 1975). Impairment of GABA-mediated inhibition, either by preventing GABA synthesis or by blocking its post-synaptic activity, leads to generalised seizures (Meldrum, 1975). Conversely, drugs which prevent the metabolism of GABA, for example γ -vinyl GABA, ethanolamine-o-sulphate, when administered systemically or intracerebrally to rats or mice, which prevent the metabolism of GABA, are associated with a massive rise in cerebral GABA levels and are potent anticonvulsants (Schechter et al, 1977; Meldrum and Horton, 1978).

Benzodiazepines and barbiturates, however, act to enhance the post-synaptic activity of released GABA (Blaustein and Ector, 1975; Olsen, 1981). The GABA receptor is thought to be a complex of at least three recognition sites: the GABA, the benzodiazepine and the barbiturate recognition sites. Associated with, or part of, these recognition sites is the chloride channel, the opening of which is regulated by GABA agonists binding to the GABA receptor. Barbiturates enhance the binding of GABA (Olsen et al, 1981), and there is evidence for positive co-operativity between the benzodiazepine and GABA sites (Tallman et al, 1975). The increased post-synaptic binding of GABA acts to decrease the severity of epileptic seizures.

The majority of recent work has focused on the involvement of glutamate and aspartate in the origin of epilepsy. Exogenous glutamate when administered systemically (Bhagara et al, 1971; Bradford and Dodd, 1975), or intracerebrally (Hayashi, 1954) induces convulsions. It has been reported that in kindled rats there is a decrease in cortical glutamine concentrations, this decrease correlates directly with the increase in the duration of the epileptic after-discharges (Leach et al, 1983). The reduction in glutamine may reflect an increase in activity of glutamate-containing neurones. This is supported by Peterson et al (1983) who reported that there was an increased level of glutamate released into the ventricles during a kindled epileptic seizure. Leach et al, (1985) showed that when cortical slices, prepared from either control or kindled rats, were exposed to veratrine, more endogenous glutamate and aspartate were released from the kindled tissue than from the control.

The released amino acids interact post-synaptically with receptors, which are classified according to their activation by N-methyl-D-aspartate (NMDA), quisqualate and kainate (KA) (Watkins and Evans, 1981). Various amino acid antagonists have been developed. Compounds which antagonise the excitatory action of NMDA, for example 2-amino-5-phosphonopentanoic acid (AP5) or 2-amino-7-phosphono- heptanoic acid (AP7), give protection against a wide range of experimental seizures including audiogenic and chemically-

induced convulsions (Croucher et al, 1982; Czuczwar et al, 1984), kindled seizures (Peterson et al, 1983), and photic induced seizures in baboon (Meldrum et al, 1983). Conversely, NMDA is a potent convulsant when injected centrally or systemically (Czuczwar and Meldrum, 1982; Meldrum et al, 1983).

Electrophysiological studies have shown that NMDA induces paroxysmal shifts in membrane potential with a characteristic burst discharge reminiscent of that recorded from an epileptic focus (Herrling et al, 1983). Thus the activation of NMDA receptors by glutamate and/or aspartate may be important in the initiation and/or spread of seizure activity. Unlike quisqualate or KA receptors, NMDA receptors are regulated by a voltage-dependent Mg^{2+} gate (Ault et al, 1980; Nowak et al, 1984; Mayer and Westbrook, 1985). In some preparations external Mg^{2+} concentrations as low as $10^2 \mu M$ began to inhibit the activity of NMDA receptors (McCulloch et al, 1974). Thus the presence of the Mg^{2+} block may restrict the involvement of NMDA receptors in "normal" neuronal activity (Collingridge, 1985). Specific antagonists of KA or of quisqualate have not yet been developed, however γ -D-glutamylglycine (DGG; Watkins 1981) and γ -aminomethylsulphonic acid (GAMS; Jones et al, 1984) are more specific to these two sites than to the NMDA receptor subtype.

When injected into the central nervous system, glutamate, aspartate, KA, and NMDA induce seizures. However, the excitatory amino acids and their analogues can cause degeneration of cell tissues (Garthwaite and Garthwaite, 1983; McBean and Roberts, 1985). Ben-Ari et al (1980) injected KA into the amygdaloid complex of the rat and electrophysiological activity typical of epilepsy was detected soon after the injection and the motor signs were apparent after one hour. Postmortem examination of the brain showed the expected neuronal loss and gliosis around the injection site. In addition, neuronal loss and degenerative changes were present at other sites where lesions are found after Status Epilepticus. These sites included various regions in the hippocampus, the contralateral amygdala and some cortical regions. In the same series of experiments, a correlation was found between the severity of the epileptiform activity and the severity of tissue damage, and Ben-Ari et al concluded that the tissue damage was due to the epileptogenic effect of the drug. The mechanism of KA action is unknown, however the drug has been reported to act on presynaptic receptors and cause an influx of Ca^{2+} into the presynaptic terminal (Pastuszko et al, 1984); this may well act to enhance glutamate release (Ferkany and Coyle, 1982). Kainic acid may be acting as a glutamate uptake blocker and hence increase the synaptic concentration of the excitatory amino acid (Johnston et al, 1979; McBean and Roberts, 1985).

Glutamate, aspartate, KA, and NMDA have been reported to increase Ca^{2+} influx into

the cellular (non-inulin) space of rat brain cortex slices incubated *in vitro* (Berdichevsky et al, 1983). KA also induces Ca^{2+} uptake into synaptosomes prepared from cerebral cortex and hippocampus (Pastuszko et al, 1984; Lazarwicz et al, 1986). In contrast, Retz and Coyle (1984) showed no effect of KA on $^{45}\text{Ca}^{2+}$ uptake into mouse striatal slices and Lazarwicz et al (1986) noted that $^{45}\text{Ca}^{2+}$ accumulation in bulk isolated astroglial cells was unaffected by KA.

Recent studies have provided evidence that glutamate may act to increase the intracellular free Ca^{2+} concentration by stimulating the metabolism of inositol phosphate. Sladeczek et al (1985) noted that glutamate, NMDA, and KA all enhanced a three-to-four fold increase in inositol triphosphate (IP3) in primary culture striatal neurones. Other groups who have found that whilst glutamate and aspartate increase inositol triphosphate turnover in slices of rat brain, NMDA and KA were inactive (Nicoletti et al 1986). They did however find that the stimulation of IP3 was inhibited potently by AP4, and they suggest that a class of excitatory amino acid receptor sites are coupled to phospholipase C, the enzyme that catalyses the hydrolysis of membrane inositol phospholipids. The physiological significance of such sites has yet to be determined.

Recently a close correlation has been observed between Ca^{2+} influx and toxic cell damage in a number of different test systems (Schanne et al, 1979; Farber, 1981). Intracellular Ca^{2+} levels are normally maintained at very low concentrations ($<10^{-7}\text{M}$) as compared to the extracellular Ca^{2+} concentration (10^{-3}M , Blaustein et al, 1980, Dipolo, 1976). It is reasonable to assume that an elevated internal Ca^{2+} content would disturb the tightly regulated metabolic reactions upon which normal cell activity depends. Increased intracellular concentration of Ca^{2+} is likely to affect the integrity of the mitochondrial membrane, and it is possible that excess Ca^{2+} ions would activate proteinases and phospholipases which would ultimately lead to cell lysis (Farber, 1981). Thus it is possible that alterations in the normal Ca^{2+} flux across neuronal membranes may be involved in epileptogenesis. Improved liquid ion-exchange electrodes have made possible the observation of large, rapid decreases in the concentration of extracellular Ca^{2+} ions during epileptic hyperactivity (spiking) (Pumain et al, 1983). Moreover, in defined cortical layers the decrease normally started shortly before the onset of each spike. It was also shown that glial cells were not responsible for such rapid changes and that these decreases may reflect the entry of Ca^{2+} into the neuronal cells.

The respective contribution of pre- and of post-synaptic Ca^{2+} entry to the decreases in extracellular Ca^{2+} concentrations is unknown, although with experiments using TTX and

Mg^{2+} , a large amount of Ca^{2+} uptake was into the post-synaptic site (Heinemann and Pumain, 1980). In penicillin foci the paroxysmal field potential is associated with intracellularly recorded paroxysmal depolarization shifts (PDE's; Matsumoto and Ajmone-marson, 1964). At the post- synaptic level an inward voltage- dependent Ca^{2+} current probably contributes to the envelope of depolarization of the PDEs. Such conductances have been shown to be present in neocortical neurones (Heinemann and Pumain, 1980) and orthodromically evoked dendritic bursts in pyrimidal neurones of penicillin-treated hippocampal slices, and are generated by such Ca^{2+} currents (Wong and Prince, 1979). The majority of the Ca^{2+} decreases are probably due to the entry of Ca^{2+} into postsynaptic membranes following PDE's. In addition, the lowering of the extracellular Ca^{2+} concentration, which results in reduced transmitter release, could limit the duration of synchronized activity and so the maximum Ca^{2+} decreases may coincide with the cessation of PDEs. Thus, the changes in membrane permeability to Ca^{2+} , and hence Ca^{2+} fluxes, may play an important role in epileptogenesis. The mechanism of action of some anticonvulsant drugs may involve an effect on Ca^{2+} fluxes.

The results of this chapter can be divided into two broad regions. Firstly, the abilities of some classical anticonvulsants to inhibit both the veratrine and high K^{+} influx of Ca^{2+} and release of endogenous amino acids were compared. These experiments were conducted on slices of rat cortex, in an attempt to elucidate the primary site of action of the anticonvulsant agents. The levels of released amino acids were measured to determine whether there were any differential effects of the drugs on the excitatory as compared to inhibitory amino-acid-containing neurones.

The second section investigates the effects of depolarizing agents and excitatory amino acids on Ca^{2+} fluxes in hippocampal slices. Measurements were made of the extent by which both the stimulated Ca^{2+} influx and the amino acid release were affected by excitatory amino acid antagonists. The antagonists were DL-2-amino-4-phosphono-butyric acid (AP4); DL-2-amino-5-phosphonopentanoic acid (AP5), and DL-2-amino-7-phosphonoheptanoic acid which are selective NMDA antagonists and GAMS (a selective KA/quisqualate antagonist).

6.1 Results

6.2a Classical anticonvulsants

None of the anticonvulsants tested, when added alone to unstimulated cortical brain slices, had any significant action on the basal level of Ca^{2+} uptake, K^{+} content, or amino acid neurotransmitter release.

Whilst diphenylhydantoin, carbamazepine and phenobarbitone showed significant effects on the $75\mu\text{M}$ -veratrine-induced Ca^{2+} influx (74%, 52% and 48% respectively at $100\mu\text{M}$; Figs 6.1 a, b and c). In contrast, neither valproate ($0.1\mu\text{M}$ - 10mM) nor ethosuximide ($0.1\mu\text{M}$ - 10mM) had any significant action on this parameter (Figs 6.2 a, b). Diphenylhydantoin was the most potent drug in inhibiting the stimulated Ca^{2+} influx (IC_{50} approximately $21\mu\text{M}$), whilst carbamazepine and phenobarbitone were less potent. (IC_{50} 's of $96\mu\text{M}$ and $176\mu\text{M}$ respectively; Table 6.2). The potencies of these and other drugs studied are given in Table 6.2 where the IC_{50} values are compared to clinically effective doses (Ferrendelli and Daniels-McQueen, 1982).

The tissue K^{+} content was monitored as a measure of the levels of membrane depolarization. Veratrine at $75\mu\text{M}$ caused a 61% loss of K^{+} from cerebrocortical slices (see legend to Fig 6.1). Diphenylhydantoin, at relatively low concentrations, ($10\mu\text{M}$ - 1mM ; Figure 6.1a) significantly ($P<0.05$ - $P<0.01$) reduced the extent of K^{+} loss from the cortex brain slices induced by the addition of veratrine (25%; $100\mu\text{M}$; Fig.6.1a). Carbamazepine ($10\mu\text{M}$ - 1mM) and phenobarbitone ($10\mu\text{M}$ - 1mM) had equivalent and significant ($P<0.05$) effects on K^{+} levels (Figs. 6.1 b and c). The IC_{50} values for the action in reducing stimulated K^{+} loss were in the low range ($5\mu\text{M}$, $11\mu\text{M}$ and $12\mu\text{M}$ respectively) and this inhibitory action seemed to be more closely associated with blockade of Ca^{2+} uptake than with inhibition of amino acid release. In contrast, whilst valproate and ethosuximide also reduced K^{+} loss evoked by veratrine, these effects only became significant at 10mM ($P<0.05$) where non-specific or toxic actions may be displayed (Figs. 6.2a and b).

Diphenylhydantoin ($10\mu\text{M}$ - 1mM) also reduced the veratrine-evoked release of glutamate, aspartate, and GABA (Fig 6.1 a) in parallel with its actions on Ca^{2+} uptake and K^{+} loss. The basal level of threonine (a non-neuroactive amino acid) released into the incubation medium remained unchanged throughout. Glutamate release was significantly reduced (27%; $P<0.05$) by $100\mu\text{M}$ diphenylhydantoin, whilst the release of aspartate and GABA was inhibited to approximately twice the extent of glutamate. At 1mM , diphenylhydantoin

inhibited the release of all three compounds to approximately the same extent (75%, Fig 6.1a). The stimulated release of amino acid neurotransmitters was likewise inhibited significantly ($P < 0.05$) by phenobarbitone (1mM) and carbamazepine (100 μ M) (Fig 6.1b and c). Ethosuximide at the concentrations tested (10 μ M - 10 mM; Fig. 6.2 b) showed no significant effect on the release of glutamate or aspartate, however GABA release was slightly enhanced (10% with 100 μ M, Fig. 6.2b). Valproate at 1.0mM reduced the release of aspartate by 91% ($P < 0.01$) from stimulated levels (Fig 6.2a) and reduced GABA release by 61%. This value for valproate is 2-3 higher than the clinically effective dose range (Table 6.2) but the effect on aspartate were detectable (59%; $P < 0.05$) at 100 μ M which is well below this range.

6.2b ω -Phosphonic- α -aminocarboxylic acids

Both veratrine (75 μ M) and K^+ (56mM) enhanced the basal influx of Ca^{2+} in hippocampal slices (to approximately 160% - 190% of control values; Table 6.3, Fig. 6.3). This was accompanied by marked increases in the efflux of glutamate (220%- 319% of control; Table 6.3), aspartate ($151 \pm 7\%$, $n=24$) and GABA ($330 \pm 36\%$, $n=24$). The efflux of the non-neuroactive amino acid threonine was unchanged (data not shown).

The amino acid receptor antagonists AP4, AP5, and AP7 at concentrations up to 1mM had no significant effect on Ca^{2+} uptake, amino acid neurotransmitter release, or K^+ content when added to hippocampal slices. (see Table 6.3). However, they all significantly inhibited both Ca^{2+} influx and amino acid release evoked by either veratrine (75 μ M) or K^+ (56mM) (Tables 6.3a and b, Figs.6.3 a,b,c). The estimated IC_{50} values for the inhibition of the two parameters (Table 6.4) show that AP4 was the least active whilst AP5 and AP7 showed greater and approximately equipotent activity. The maximum inhibition of Ca^{2+} influx observed in the presence of the antagonists was 61%- 79% of the stimulated response. This compared with complete inhibition of the veratrine- or K^+ induced Ca^{2+} influx in the presence of the general Ca^{2+} channel antagonist (-) D888 (10 μ M) (Figure 6.4). This concentration of (-)D888 was employed as it has been found to be the highest concentration which totally blocks Ca^{2+} entry without secondary effects on Na^+ channels (White and Bradford, 1986).

The action of AP7 was found to be stereospecific with D(-) AP7 showing much greater inhibitory potency than L(+) AP7 against both veratrine- and K^+ - induced Ca^{2+} influx (Table 6.4)

Veratrine- and high K^+ - evoked amino acid neurotransmitter release were also inhibited

by the antagonists in a similar order of potency as that for inhibition of Ca^{2+} influx (Table 6.4). The stimulated efflux of glutamate and of aspartate were more readily inhibited than the efflux of GABA (Figs 6.3 a, b, c). Indeed, after either AP4 or AP5 addition no significant inhibition of GABA efflux was observed at concentrations of $1\mu\text{M}$ - 1mM (Figs 6.3 a, b).

At concentrations greater than 1mM , AP4 significantly ($P < 0.05$) inhibited the fall in K^+ content of the slices induced by veratrine. AP5 and AP7 also partially inhibited the fall in K^+ content, this effect reaching significance at $300\mu\text{M}$ and $500\mu\text{M}$ respectively.

The homologous long chain fatty acids, butyric, valeric, and heptanoic acids ($10\mu\text{M}$ - 10mM) did not influence either basal or stimulated levels of Ca^{2+} influx, amino acid neurotransmitter efflux, or K^+ content (Table 6.5).

It seemed likely from the above results that the antagonists were active through blockage of the receptor-mediated action of excitatory amino acids (particularly glutamate) released by either high K^+ or veratrine. To test this hypothesis, the ability of AP7 to inhibit Ca^{2+} influx evoked by L-glutamate, L-aspartate, NMDA or KA was examined. Addition of the amino acid alone to the incubation medium resulted in depolarization of the hippocampal slices (as judged by the fall in K^+ content) and a dose-dependent stimulation of Ca^{2+} influx (Tables 6.6 and 6.7). Approximate ED_{50} values and maximum evoked increases in Ca^{2+} influx for the amino acids were ($n=6$), for glutamate ($100\mu\text{M}$; $158 \pm 10\%$ of control), for aspartate (1mM ; $161 \pm 12\%$), for NMDA ($50\mu\text{M}$; $153 \pm 7\%$), and for KA ($50\mu\text{M}$; $163 \pm 3\%$).

The relative inhibitory potencies of AP7 against the Ca^{2+} influx evoked by the different agonists were compared. AP7 ($500\mu\text{M}$), completely inhibited Ca^{2+} influx evoked by NMDA ($50\mu\text{M}$) (Tables 6.6 and 6.7). At the same concentration, AP7 only partially inhibited Ca^{2+} uptake induced either by L-glutamate ($44 \pm 6\%$, $n=6$), or by aspartate ($64 \pm 3\%$, $n=6$) and was inactive against Ca^{2+} influx induced by KA ($50\mu\text{M}$) (Tables 6.6 and 6.7). In contrast, the more effective 'KA receptor' antagonist GAMS (1mM) (Jones et al, 1984; Davies and Watkins, 1985) completely inhibited the Ca^{2+} influx evoked by KA ($50\mu\text{M}$). The fall in K^+ content of the slices induced by the agonists was also similarly reduced by AP7, the changes paralleling those of the Ca^{2+} influx (Tables 6.6 and 6.7).

Neurotransmitter release was evoked by the excitatory amino acid (Table 6.8). Glutamate release was stimulated by 143%, 139% and 154% of control by NMDA ($50\mu\text{M}$), by aspartate (1mM) and by KA ($50\mu\text{M}$) respectively. The release of aspartate was similarly stimulated, 143% by glutamate ($100\mu\text{M}$) and 150% by KA ($50\mu\text{M}$). GABA release was enhanced by all four excitatory amino acids (Table 6.8). The stimulated release of the neurotransmitter amino acids was inhibited by AP7 and GAMS, in proportion to the decreases in Ca^{2+} influx

(compare tables 6.6 and 6.7). AP7 (500 μ M) completely inhibited the increased glutamate release evoked by NMDA (50 μ M) whilst it only partially (56%) inhibited the glutamate release induced by aspartate (1mM) and was inactive against the glutamate release induced by KA (50 μ M). As before, GAMS (1mM) completely inhibited the latter response. AP7 (500 μ M) and GAMS (1mM) together totally inhibited the neurotransmitter release induced by glutamate (Table 6.8).

It has been proposed on the basis of electrophysiological observations that the NMDA receptor is gated by Mg^{2+} in a voltage-dependent fashion (Nowak et al, 1984; Mayer and Westbrook, 1985). The inhibitory action of Mg^{2+} appears to be ineffective at low membrane potentials. To test this hypothesis biochemically, the ability of NMDA (50 μ M) or KA (50 μ M) to induce both Ca^{2+} influx and amino acid neurotransmitter release in the presence of varying external Mg^{2+} concentrations was compared (Figure 6.5; Table 6.9). In incubation medium containing no added Mg^{2+} the basal Ca^{2+} influx was raised by 44% and basal glutamate release was also increased compared to 1.0mM Mg^{2+} . Under these conditions the small additional Ca^{2+} influx and amino acid release evoked by KA or by NMDA was statistically insignificant. When 0.1mM Mg^{2+} was added, the basal Ca^{2+} influx again increased (by 36%) but the response due to added NMDA were significantly enhanced (by 21%; $P<0.005$) over those seen in the presence of 1.0mM Mg^{2+} . In contrast, the response due to KA was not significantly enhanced by these low Mg^{2+} concentrations. In incubation medium containing 10mM Mg^{2+} the Ca^{2+} influx induced by NMDA was significantly reduced (from 41% to 9%; $P<0.05$) compared to the increase evoked in the presence of 1.0mM Mg^{2+} . Conversely the Ca^{2+} influx induced by KA was not significantly reduced in medium containing high Mg^{2+} (Figure 6.5).

To ensure that the ability of 10mM Mg^{2+} to inhibit the NMDA-induced Ca^{2+} influx was not due to calcium channel blockade by Mg^{2+} , the effects of 10mM Mg^{2+} on Ca^{2+} influx, K^+ , content and amino acid neurotransmitter release evoked by veratrine and high K^+ was studied. Mg^{2+} (10mM) did not suppress any of the above parameters evoked by either of these agonists (Table 6.10).

Figure 6.1 The effect of diphenylhydantoin, carbamazepine, and phenobarbitone on veratrine-induced Ca^{2+} influx, amino acid neurotransmitter release, and K^{+} content.

Brain cortex slices were prepared and incubated as described (2.1b and 2.2b). Diphenylhydantoin was dissolved in absolute ethanol and diluted with the Krebs-Tris medium so that the final concentration of ethanol in the incubation medium was less than 0.3%. The other two anticonvulsants were dissolved and diluted in the Krebs-Tris medium. The drugs were added 5 minutes before $^{45}\text{Ca}^{2+}$, and the experiment was terminated as usual and the tissue was solubilised in 1N NaOH. Aliquots were taken to measure both Ca^{2+} and K^{+} content. Samples of the incubation medium were taken for amino acid analysis.

Histobars represent stimulated Ca^{2+} influx or amino acid release as indicated on the ordinate.

■ glutamate; [] aspartate, ▣ GABA.

The plotted data [-●-●-] represent the extent of loss of K^{+} content of the tissue due to added veratrine. The first histobar or data point in each sequence represent the response level with veratrine alone in the absence of drug. Veratrine was added in all cases. Data are mean $\pm$ SEM, $n=6$. Significance of difference between veratrine alone and veratrine in the presence of the anticonvulsants were calculated by Student's two-tailed t-test, Ca^{2+} and amino acids, * $P>0.05$; ** $P>0.01$; *** $P>0.005$; K^{+} -content data, + $P>0.05$; + $P>0.01$.

	Basal levels	Veratrine (75 μM)
K^{+} ($\mu\text{equiv}/100\text{mg protein}$)	76.0 ± 1.72	21.64 ± 2.16
Ca^{2+} (nmol/100mg protein)	1963 ± 124	3243 ± 122
Glutamate (nmol/100mg protein)	2166 ± 152	3449 ± 273
Aspartate (nmol/100mg protein)	1776 ± 195	3690 ± 444
GABA (nmol/100mg protein)	205 ± 56	644 ± 45

a = DPH; b = carbamazepine; c = phenobarbitone

Fig 6.1a DIPHENYLHYDANTOIN

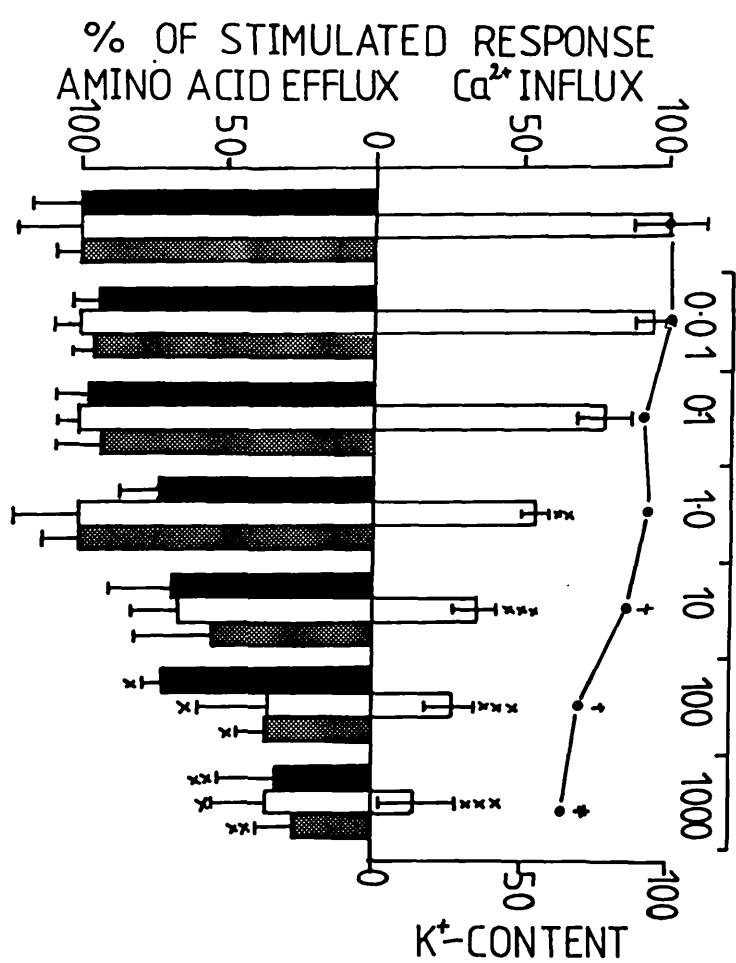


Fig 61b CARBAMAZEPINE

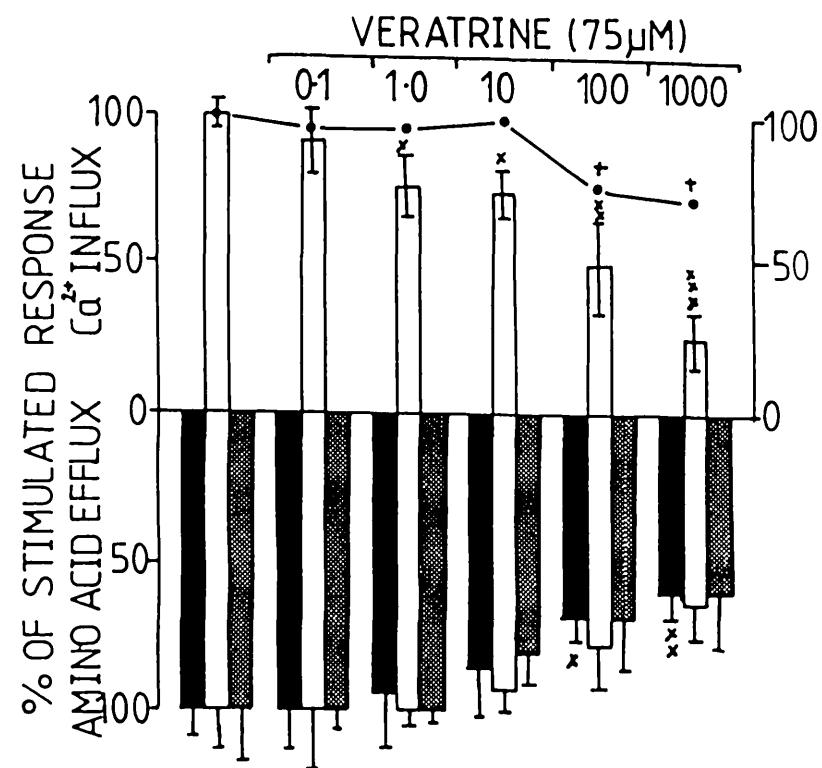


Fig 6.1c PHENOBARBITONE

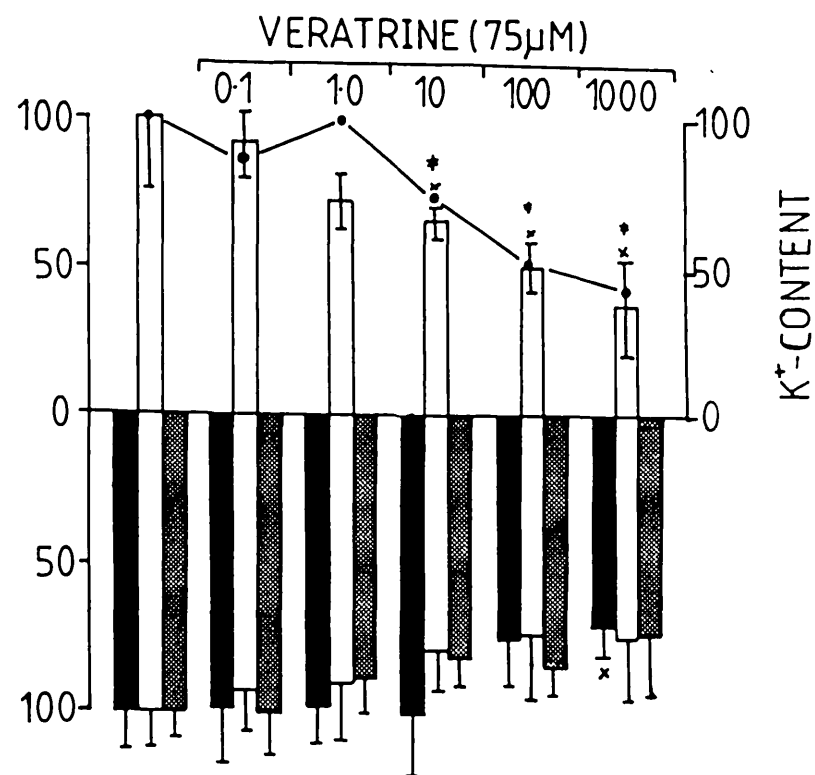


Figure 6.2 **The effect of sodium valproate and ethosuximide on veratrine-induced Ca^{2+} influx, amino acid release, and K^{+} content.**

The data for Ca^{2+} uptake, amino acid release and change in slice K^{+} content are represented as described in the legend for figure 6.1. Data represent the percentage changes from stimulated control for each parameter and are mean $\pm$ SEM (n=6).

Significance of differences between veratrine and veratrine in the presence of the anticonvulsants were calculated by Student's two-tailed t-test (Ca^{2+} and amino acids, * $P>0.05$; ** $P>0.01$; *** $P>0.005$; K^{+} -content data; + $P<0.05$; + $P<0.01$).

The K^{+} data was very variable in these experiments with SEM values of 10-20% from the mean. Significant differences from control were only seen with 10mM drug concentrations in either case.

For basal and stimulated levels see legend to Figure 6.1.

a = Sodium valproate; b = Ethosuximide

Fig 6.2a ETHOSUXIMIDE

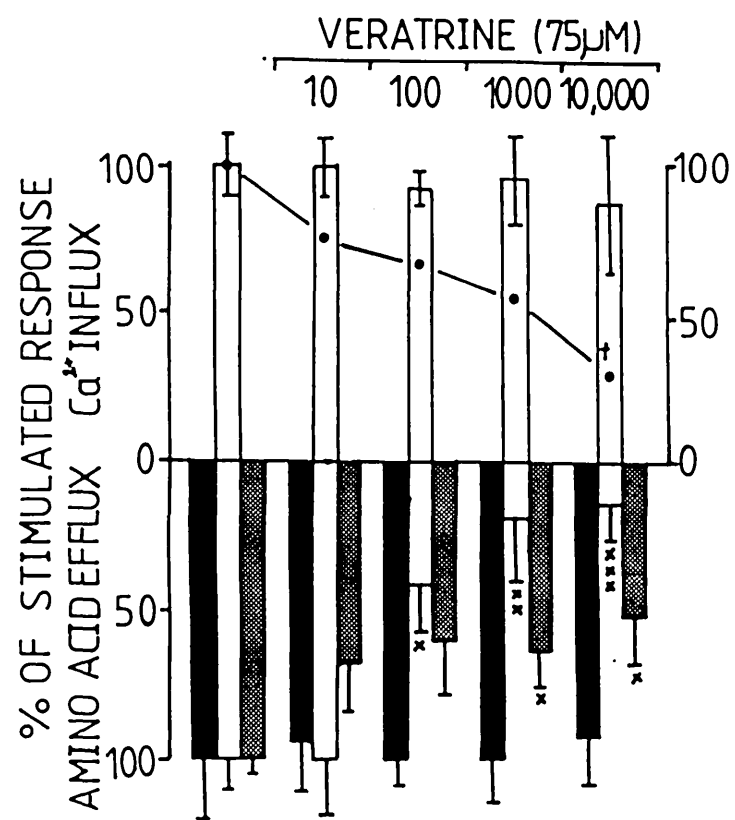


Fig 6.2b SODIUM VALPROATE

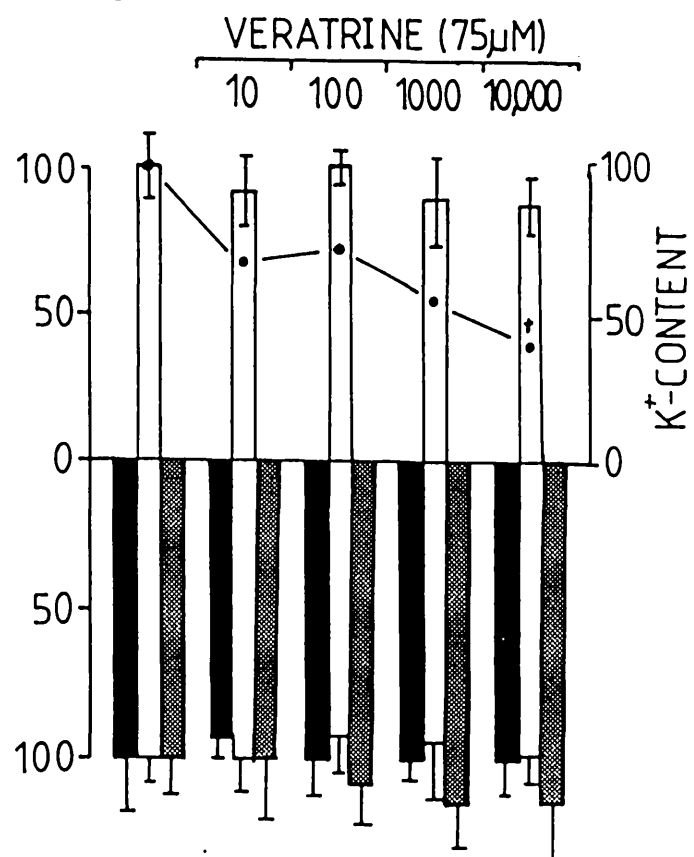


TABLE 6.1 Action of anticonvulsants on basal Ca^{2+} influx, K^+ loss, and glutamate release on cortex slices.

Treatment	Ca^{2+} influx nmol/100mg pr.	K^+ level $\mu\text{equiv}/100\text{mg pr.}$	Glu release nmol/100mg pr.
Carbamazepine			
Control	1528 ± 200	56.14 ± 3.55	2209 ± 197
1mM	1439 ± 76 (94)	61.39 ± 5.98 (109)	2224 ± 106 (101)
Diphenylhydantoin			
Control	1963 ± 124	55.76 ± 1.72	2166 ± 153
1mM	1804 ± 79 (92)	57.17 ± 1.43 (103)	2236 ± 103 (103)
Ethosuximide			
Control	1724 ± 186	59.53 ± 2.58	2191 ± 216
10mM	1907 ± 276 (111)	62.17 ± 3.64 (104)	1836 ± 209 (83)
Phenobarbitone			
Control	1682 ± 104	66.70 ± 4.94	2198 ± 173
1mM	1714 ± 113 (102)	75.08 ± 3.14 (113)	1983 ± 104 (90)
Valproate			
Control	1724 ± 186	59.53 ± 2.58	2191 ± 216
10mM	1810 ± 56 (105)	71.43 ± 9.52 (120)	1994 ± 350 (91)

Brain cortex slices were prepared and incubated as described (2.1b, 2.2b). Diphenylhydantoin was dissolved in absolute ethanol and diluted with the Krebs-Tris medium so that the final concentration of ethanol in the incubation medium was less than 0.3%. The other four anticonvulsants were dissolved and diluted in the Krebs-Tris medium. The drugs were added 5 minutes before $^{45}\text{Ca}^{2+}$, and the experiment was terminated as usual with the tissue being solubilised in 1N NaOH. Aliquots were taken to measure both Ca^{2+} and K^+ content. Samples of the incubation medium were taken for amino acid analysis. Data are mean $\pm$ SEM (n=6). Values in parentheses are % of control.

Table 6.2 **IC₅₀ values for anticonvulsant drugs on veratrine stimulated Ca²⁺ uptake and amino acid release**

	Ca ²⁺ uptake	glutamate release	aspartate release	effective antiepileptic level (μM) +
DPH	21	106	42	40-80
Carbamazepine	96	1016	1247	20-50
Phenobarbitone	176	1364	1574	60-160
Ethosuximide	>10,000	>10,000	>10,000	350-700
Valproate	>10,000	>10,000	264	350-650

IC₅₀ values were obtained graphically from log dose response curves. +, Taken from Ferrendelli and Daniels-McQueen, 1982.

Figure 6.3 Effect of ω -phosphonic α -amino carboxylic acids on Ca^{2+} influx and amino acid neurotransmitter efflux evoked by veratrine ($75\mu\text{M}$) and K^+ (56mM) in hippocampal slices.

Hippocampal slices were prepared and incubated as described previously (2.1b and 2.2b). The phosphono compounds were dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. The drugs were added 25 min into the incubation followed, 5 min later by $^{45}\text{Ca}^{2+}$. The depolarizing agent was added 1 min later and the experiment was terminated after a 12 min incubation with $^{45}\text{Ca}^{2+}$. Samples of the NaOH digest were taken for determination of Ca^{2+} . Aliquots of the incubation medium were taken for amino acid analysis.

Significance of differences between agonist responses alone and agonist in the presence of antagonist were estimated using Student's two-tailed t-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Representative values for both basal and stimulated Ca^{2+} influx and basal and stimulated glutamate efflux are shown in Table 3. (Values for aspartate, nmol/100mg protein: basal, 2269 ± 170 ; veratrine stimulated, 3980 ± 148 ; K^+ stimulated, 3671 ± 189 . Values for GABA, nmol/100mg protein: basal, 750 ± 63 ; veratrine stimulated, 3595 ± 113 ; K^+ stimulated, 2795 ± 255). Data are mean $\pm$ SEM, $n=6$.

■ glutamate [] aspartate ▣ GABA

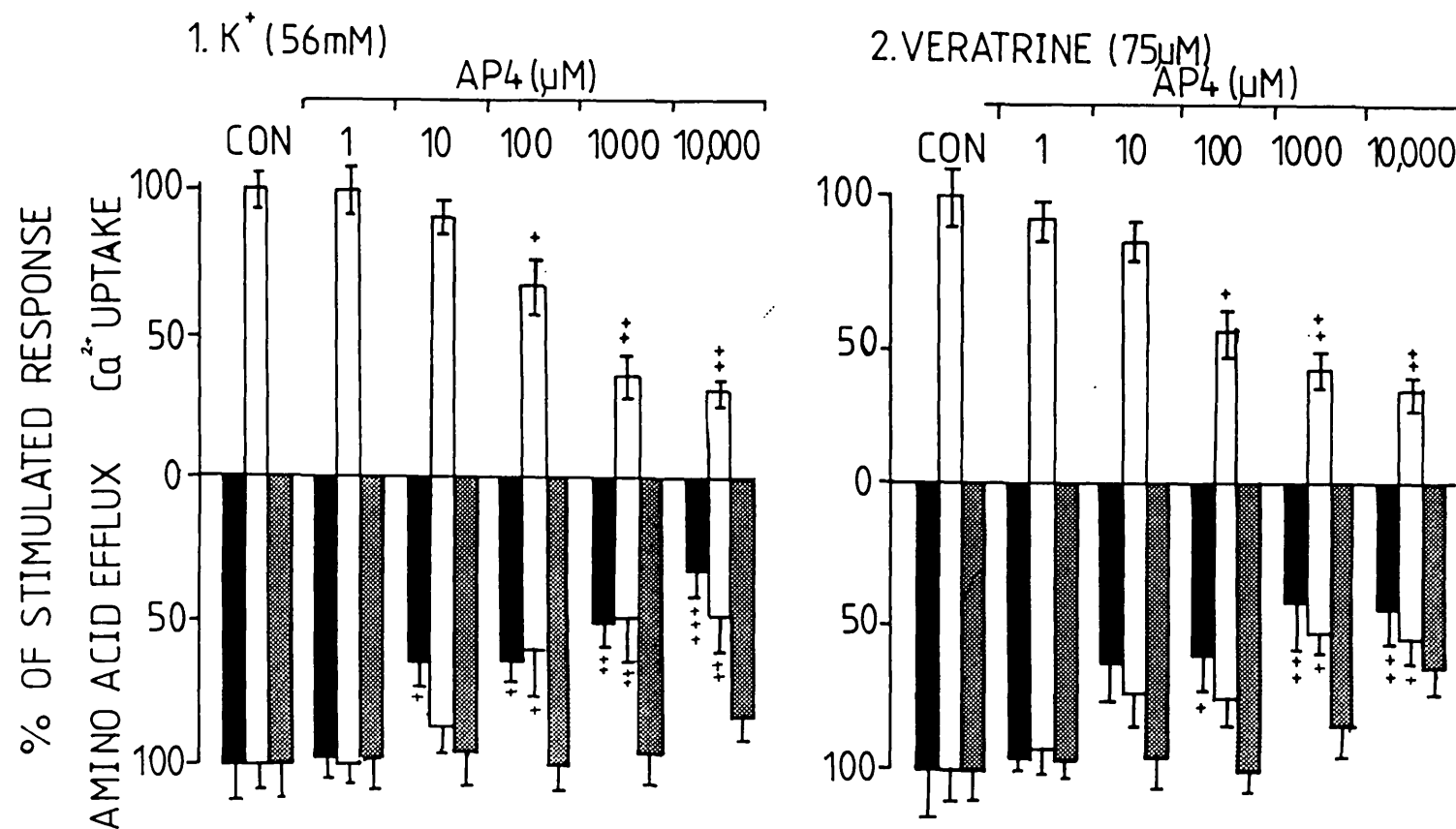
Fig 6.3a AP4 ON STIMULATED Ca^{2+} UPTAKE AND AMINO ACID RELEASE

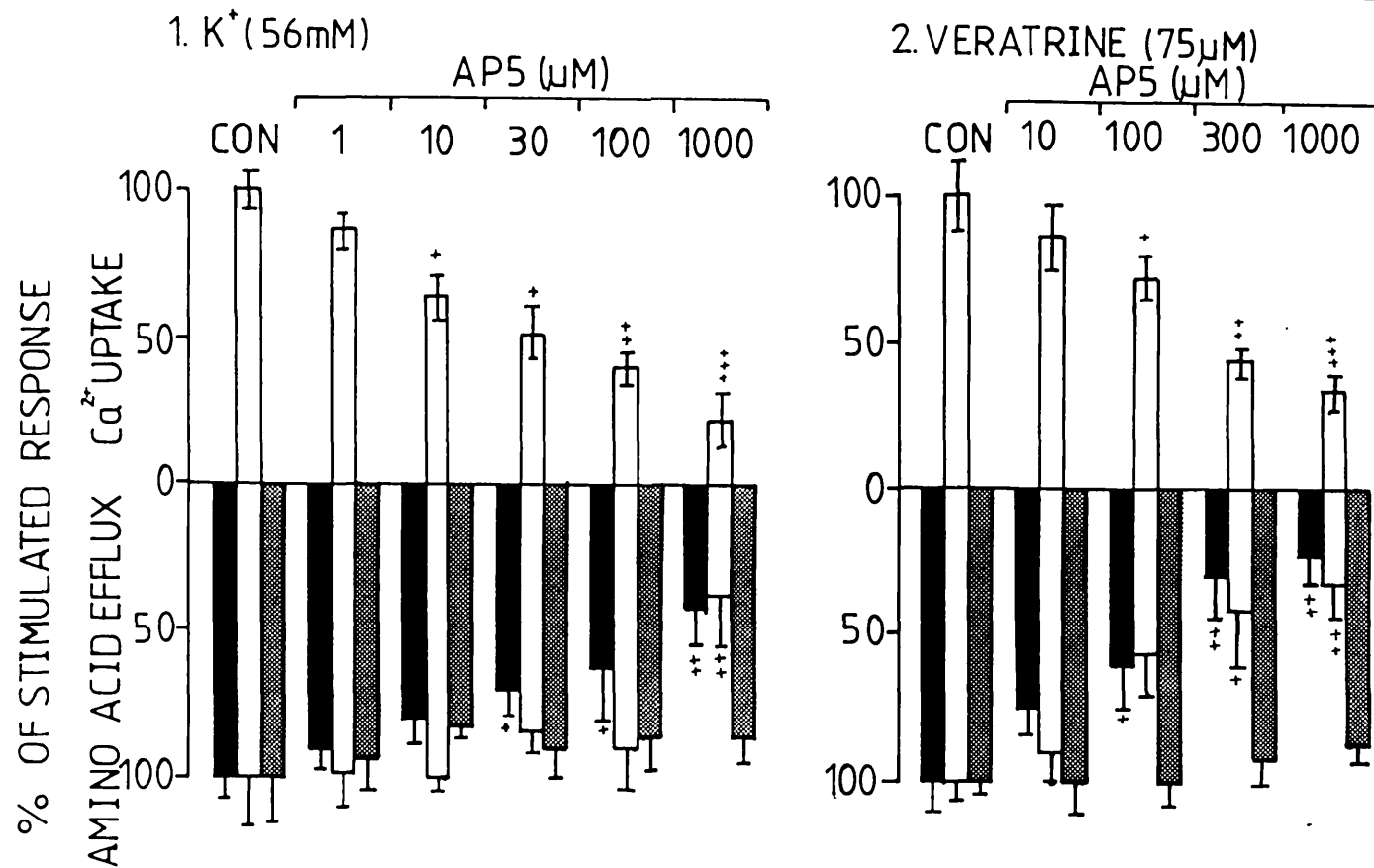
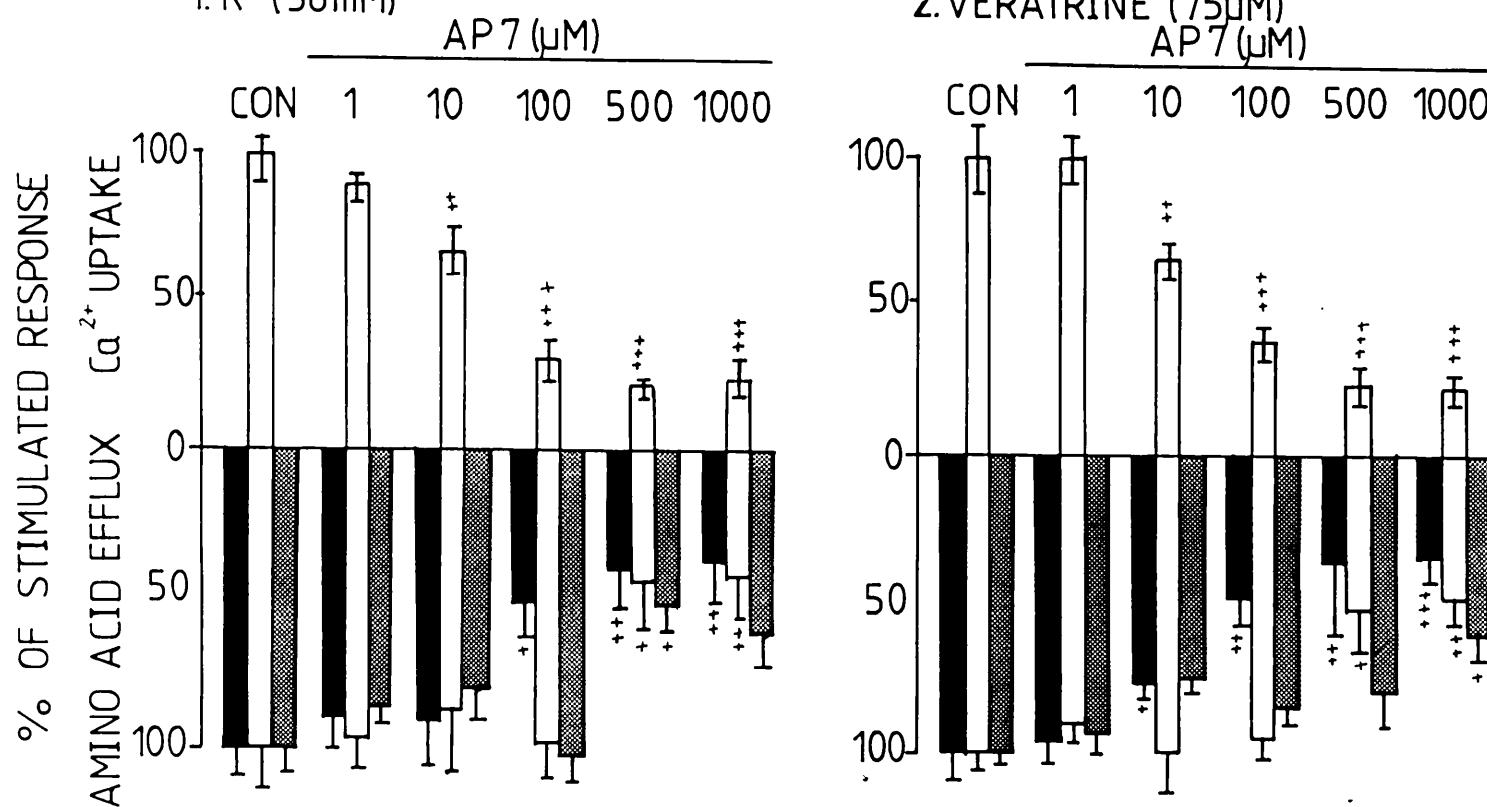
Fig 6.3b. AP5 ON STIMULATED Ca^{2+} UPTAKE AND AMINO ACID RELEASE

Fig 6.3c. AP7 ON STIMULATED Ca^{2+} UPTAKE AND AMINO ACID RELEASE1. K^+ (56mM)2. VERATRINE (75 μM)

Tables 6.3a and b.

Action of AP7 against Ca^{2+} influx, K^{+} content, and amino acid release evoked by veratrine and K^{+} in hippocampal slices.

Hippocampal slices were prepared and incubated as described previously (2.1b and 2.2b). AP7 was dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. The drug was added 25 min into the incubation, followed 5 min later by $^{45}\text{Ca}^{2+}$. The depolarizing agent was added 1 min later and the experiment was terminated after a 12 min incubation with $^{45}\text{Ca}^{2+}$. Samples of the NaOH digest were taken for determination of both Ca^{2+} and K^{+} content. Aliquots of the incubation medium were taken for amino acid analysis. The K^{+} content of slices incubated in the presence of K^{+} could not be determined.

Significance of differences were calculated using Student's two-tailed t-test.

Between control and antagonist alone, ns = not significant.

Between agonist and agonist in the presence of antagonist, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Data are mean $\pm$ SEM, $n = 6$.

Table 6.3a Action of AP7 against Ca^{2+} influx and K^{+} content evoked by veratrine ($75\mu\text{M}$) and K^{+} (56mM) in hippocampal slices.

Treatment	Ca^{2+} uptake nmol/100mg protein	% of control	K^{+} content μ equiv/100mg protein	% of control
Control	1678 ± 193	100	53.73 ± 0.88	100
AP7 $100\mu\text{M}$	1867 ± 103	111 ns	46.95 ± 2.81	87 ns
AP7 $500\mu\text{M}$	1764 ± 114	105 ns	46.19 ± 2.64	86 ns
AP7 1mM	1856 ± 106	111 ns	46.17 ± 2.74	86 ns
Vt ($75\mu\text{M}$)	3225 ± 182	192	21.61 ± 1.48	40
+ $1\mu\text{M}$ AP7	3326 ± 186	198	22.36 ± 0.74	42
+ $10\mu\text{M}$ AP7	2702 ± 137	161 **	24.02 ± 1.69	45
+ $100\mu\text{M}$ AP7	2235 ± 131	133 ***	26.91 ± 2.41	50
+ $500\mu\text{M}$ AP7	2164 ± 79	129 ***	28.36 ± 1.69	53 *
+ 1mM AP7	2157 ± 209	128 ***	28.43 ± 0.91	52 *
K^{+} (56mM)	3159 ± 188	188		
+ $1\mu\text{M}$ AP7	3037 ± 166	195		
+ $10\mu\text{M}$ AP7	2668 ± 67	159 **		
+ $100\mu\text{M}$ AP7	2113 ± 193	126 ***		
+ $500\mu\text{M}$ AP7	2049 ± 141	122 ***		
+ 1mM AP7	2033 ± 140	121 ***		

Table 6.3b Action of AP7 against amino acid neurotransmitter release induced by veratrine (75 μ M) and K⁺ (56mM) in hippocampal slices.

Treatment	Glutamate release	
	nmol/100mg protein	% of Control control
Control	7548 $\pm$ 373	100
AP7 100 μ M	6825 $\pm$ 666	90 ns
AP7 500 μ M	7036 $\pm$ 274	93 ns
AP7 1mM	8610 $\pm$ 171	114 ns
Veratrine (75 μ M)	24086 $\pm$ 1131	319
+ 1 μ M AP7	23647 $\pm$ 947	313
+ 10 μ M AP7	19827 $\pm$ 256	263 *
+ 100 μ M AP7	15440 $\pm$ 1193	205 **
+ 500 μ M AP7	13998 $\pm$ 374	185 **
+ 1mM AP7	13696 $\pm$ 145	181 ***
K ⁺ (56mM)	16613 $\pm$ 1397	220
+ 1 μ M AP7	16279 $\pm$ 1698	215
+ 10 μ M AP7	15283 $\pm$ 791	202
+ 100 μ M AP7	12168 $\pm$ 653	161 *
+ 500 μ M AP7	11808 $\pm$ 177	156 **
+ 1mM AP7	11729 $\pm$ 834	155 **

Table 6.4 **The IC₅₀ values for the inhibition of Ca²⁺ uptake and amino acid release by ω -phosphonic- α -amino carboxylic amino acids.**

	Ca ²⁺ uptake IC ₅₀ (μ M)		Glutamate release IC ₅₀ (μ M)	
	K ⁺ (56mM)	Vt (75 μ M)	K ⁺ (56mM)	Vt (75 μ M)
$\pm$ AP4	395.0 (62.5-2504.0)	620.0 (57.3-6708.0)	1055.0 (38.0-30722.0)	470.0 (32.1-6885.5)
$\pm$ AP5	40.0 (5.3-302.5)	305.0 (43.3-2146.0)	320.0 (58.4-1753.0)	98.0 (13.2-728.0)
$\pm$ AP7	53.0 (8.9-317.5)	45.5 (7.9-260.1)	NT	NT
D(-)AP7	12.5 (4.66-35.5)	20.0 (6.4-62.5)	NT	NT
L(+)AP7	1005.0 (144.0-6976.0)	310.0 (24.8-3871.0)	NT	NT

The IC₅₀ values and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (1949). NT = not tested.

Tables 6.5a and b.

Effect of the long chain fatty acids on either veratrine or K⁺ stimulated hippocampal slices

Hippocampal slices were prepared and incubated as described (2.1b; 2.2b). The fatty acids were diluted in Krebs Tris-medium and were buffered with 1N NaOH to pH 7.4 before additions were made. The fatty acids were added five minutes prior to ⁴⁵Ca²⁺. The depolarizing agent, veratrine (Vt; 75μM) or K⁺ (56mM) was added one minute after ⁴⁵Ca²⁺. Incubations were terminated 11 minutes later. Aliquots were taken from the solubilised slice digest to determine both Ca²⁺ and K⁺ levels. Samples from the incubation medium were analysed for amino acids.

K⁺ levels were not determined in the presence of 56mM K⁺.

Significance of differences were calculated by Student's two-tailed t-test for unpaired samples.

There was no significant difference between basal and fatty acids alone, or between agonist and agonist + antagonist. Data are mean ± SEM, n=6, and expressed as % of control.

Basal levels:

Ca ²⁺ ,	2527 ± 251 nmol/100mg protein;
K ⁺ ,	74.79 ± 0.74 μequiv/100mg protein
Glutamate,	6255 ± 169 nmol/100mg protein.

Table 6.5a **Effect of the long chain fatty acids on either veratrine or K⁺ stimulated hippocampal slices.**

	% of Control		
	Ca ²⁺ uptake	K ⁺ Content	glutamate release
Butyric acid (mM)	100	100	100
Butyric acid 0.001	89 ± 3	95 ± 4	97 ± 2
Butyric acid 0.1	89 ± 6	104 ± 4	99 ± 6
Butyric acid 10	96 ± 2	91 ± 3	104 ± 1
Veratrine (75μM)	159 ± 4	50 ± 3	171 ± 3
+ Butyric acid 0.001	152 ± 2	52 ± 6	169 ± 0.1
+ Butyric acid 0.1	171 ± 4	46 ± 2	172 ± 1.1
+ Butyric acid 10	168 ± 3	53 ± 1	168 ± 3
K ⁺ (56mM)	152 ± 6	-	161 ± 4
+ Butyric acid 0.001	151 ± 2	-	169 ± 2
+ Butyric acid 0.1	155 ± 1	-	163 ± 3
+ Butyric acid 10	162 ± 0.5	-	158 ± 6
Valeric acid (mM)	100	100	100
Valeric acid 0.001	97 ± 2	96 ± 1	106 ± 2
Valeric acid 0.1	91 ± 7	105 ± 0.1	103 ± 0.5
Valeric acid 10	108 ± 3	94 ± 3	98 ± 2
Veratrine (75μM)	168 ± 6	40 ± 4	165 ± 6
+ Valeric acid 0.001	159 ± 4	34 ± 1	166 ± 3
+ Valeric acid 0.1	168 ± 1	38 ± 0.3	166 ± 1
+ Valeric acid 10	161 ± 2	25 ± 0.2	140 ± 0.4

Table 6.5b Effect of the long chain fatty acids on either veratrine or K⁺ stimulated hippocampal slices.

	% of control		
	Ca ²⁺ uptake	K ⁺ Content	glutamate release
K ⁺ (56mM)	160 ± 1	-	168 ± 2
+ Valeric acid 0.001	152 ± 2	-	173 ± 3
+ Valeric acid 0.1	161 ± 0.4	-	184 ± 0.1
+ Valeric acid 10	118 ± 1	-	140 ± 0.2
Heptanoic acid (mM)	100	100	100
Heptanoic acid 0.001	116 ± 4	103 ± 3	98 ± 4
Heptanoic acid 0.1	96 ± 1	106 ± 2	104 ± 2
Heptanoic acid 10	103 ± 6	106 ± 4	113 ± 4
Veratrine (75μM)	168 ± 0.3	52 ± 6	172 ± 3
+ Heptanoic acid 0.001	183 ± 1	55 ± 1	168 ± 6
+ Heptanoic acid 0.1	193 ± 3	50 ± 5	169 ± 0.2
+ Heptanoic acid 10	164 ± 2	45 ± 0.3	163 ± 2
K ⁺ (56mM)	158 ± 0.4	-	160 ± 7
+ Heptanoic acid 0.001	156 ± 0.6	-	159 ± 0.4
+ Heptanoic acid 0.1	162 ± 2	-	158 ± 0.1
+ Heptanoic acid 10	145 ± 5	-	154 ± 2

Figure 6.4 **Relative inhibitory effects of AP7 and (-) D888 against Ca^{2+} influx evoked by veratrine ($75\mu\text{M}$) or K^+ (56mM) in hippocampal slices.**

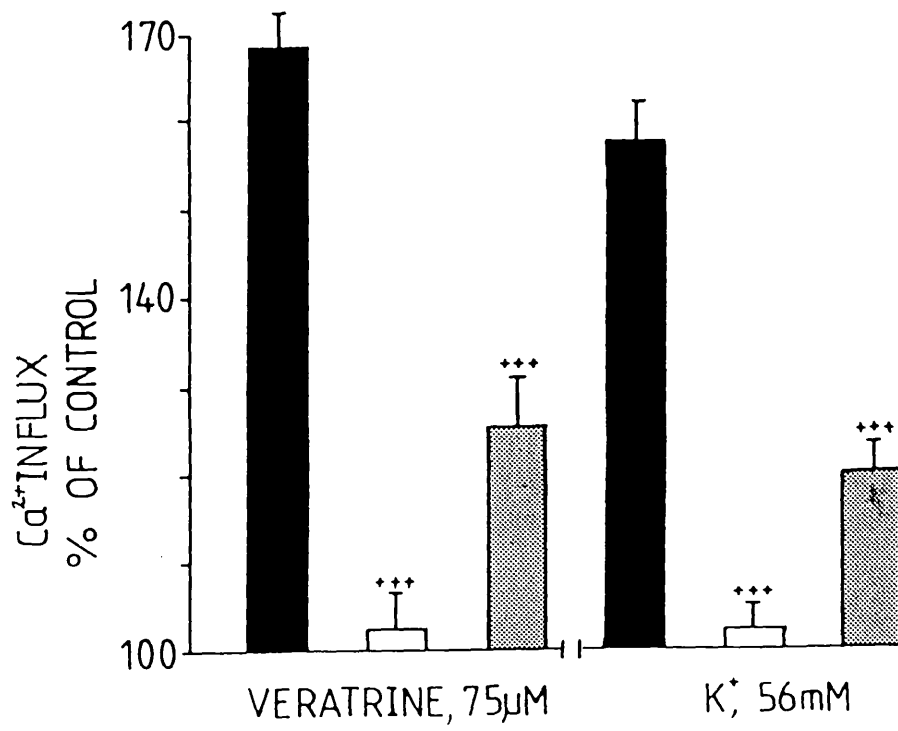
Hippocampal slices were prepared and incubated as described previously (2.1b and 2.2b). AP7 was dissolved in 1N NaOH and then buffered to pH7.4 in the incubation medium and was added 25 min into the incubation followed, 5 min later by $^{45}\text{Ca}^{2+}$. The depolarizing agent was added 1 min later and the experiment was terminated after a 12 min incubation with $^{45}\text{Ca}^{2+}$. Samples of the NaOH digest were taken for determination of Ca^{2+} . Values are mean $\pm$ SEM, n=6.

Basal Ca^{2+} influx; 1791 ± 44 nmol/100mg protein; veratrine stimulated Ca^{2+} influx; 3093 ± 189 nmol/100mg protein; K^+ stimulated Ca^{2+} influx; 2917 ± 134 nmol/100mg protein.

■ agonist alone; [] agonist + (-)D888, $10\mu\text{M}$;

▨ agonist + AP7, $500\mu\text{M}$.

Fig 6.4
(-)D888 AND AP7 VERSUS VERATRINE OR K^+
INDUCED Ca^{2+} INFLUX INTO HIPPOCAMPAL SLICES.



Tables 6.6 and 6.7

Inhibitory actions of AP7 and GAMS on the Ca^{2+} influx and the K^+ loss due to treatment of hippocampal slices with excitatory amino acid analogues.

Hippocampal slices were prepared and incubated as described (2.1b; 2.2b). The excitatory amino acid analogues, AP7, and GAMS were dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. AP7 ($500\mu\text{M}$) was added five minutes prior to the addition of $^{45}\text{Ca}^{2+}$ and GAMS (1mM) was added 1 min before the AP7. The excitatory amino acids, L-glutamate (Glu), L-aspartate, (Asp), NMDA or KA were added one minute after $^{45}\text{Ca}^{2+}$. Incubations were terminated 11 minutes later. Aliquots were taken from solubilised slice digest to determine both Ca^{2+} and K^+ levels. Samples from the incubation medium were analysed for amino acids.

Significance of differences were calculated by Student's two-tailed t-test.

Between control and agonist alone, a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.005$; ns = not significant.

Between agonist(s) and agonist(s) plus antagonist(s), * $P < 0.05$; ** $P < 0.01$; *** $p < 0.005$. Data are mean $\pm$ SEM, n=6.

Table 6.6a Inhibitory actions of AP7 and GAMS on the Ca²⁺ influx due to treatment of hippocampal slices with L-glutamate and L-aspartate

Treatment	Ca ²⁺ uptake nmol/100mg protein	% of control
Control	2518 ± 121	100
Asp 200μM	3367 ± 253	130 ± 8a
Asp 1mM	3772 ± 184	149 ± 18b
Asp 5mM	3826 ± 181	161 ± 12c
Asp 1mM + AP7 500μM	2965 ± 123	119 ± 11*
Glu 50μM	2836 ± 106	112 ± 8 ns
Glu 100μM	3122 ± 145	127 ± 14a
Glu 200μM	3394 ± 209	145 ± 12a
Glu 1mM	3481 ± 193	152 ± 19b
Glu 5mM	3952 ± 430	158 ± 10b
Glu 100μM + AP7 500μM	2781 ± 91	117 ± 1*
Glu 100μM + GAMS 1mM	2924 ± 134	116 ± 10ns
Glu 100μM + AP7 500μM + GAMS 1mM	2561 ± 127	104 ± 1*
Glu 100μM + Asp 1mM	4048 ± 53	161 ± 2c
Glu 100μM + Asp 1mM + GAMS 1mM	3061 ± 139	121 ± 4***
Glu 100μM + Asp 1mM + AP7 500μM + GAMS 1mM	2644 ± 203	106 ± 12***

Table 6.6b Inhibitory actions of AP7 and GAMS on the loss of tissue slice K⁺ content due to treatment of hippocampal slices with L-glutamate and L-aspartate

Treatment	K ⁺ content μ equiv/100mg protein	% of control
	86.58 $\pm$ 1.35	100
Asp 200 μ M	75.28 $\pm$ 1.98	89 $\pm$ 2a
Asp 1mM	74.93 $\pm$ 2.83	86 $\pm$ 2b
Asp 5mM	69.87 $\pm$ 3.75	81 $\pm$ 5a
Asp 1mM + AP7 500 μ M	79.35 $\pm$ 0.37	92 $\pm$ 1ns
Glu 50 μ M	81.36 $\pm$ 1.27	96 $\pm$ 7ns
Glu 100 μ M	79.64 $\pm$ 0.41	91 $\pm$ 3ns
Glu 200 μ M	77.69 $\pm$ 3.14	89 $\pm$ 2ns
Glu 1mM	75.14 $\pm$ 2.31	84 $\pm$ 3a
Glu 5mM	74.24 $\pm$ 3.13	77 $\pm$ 2a
Glu 100 μ M + AP7 500 μ M	81.09 $\pm$ 3.03	95 $\pm$ 3ns
Glu 100 μ M + GAMS 1mM	80.57 $\pm$ 5.2	95 $\pm$ 5ns
Glu 100 μ M + AP7 500 μ M + GAMS 1mM	85.84 $\pm$ 2.61	98 $\pm$ 3*
Glu 100 μ M + Asp 1mM	74.36 $\pm$ 2.61	86 $\pm$ 4a
Glu 100 μ M + Asp 1mM + GAMS 1mM	80.64 $\pm$ 1.72	95 $\pm$ 3ns
Glu 100 μ M + Asp 1mM + AP7 500 μ M + GAMS 1mM	83.92 $\pm$ 2.39	98 $\pm$ 7*

Table 6.7a. Inhibitory actions of AP7 and GAMS on the Ca^{2+} influx due to treatment of hippocampal slices with NMDA and KA.

Treatment	Ca^{2+} uptake nmol/100mg protein	% of control
Control	2518 $\pm$ 121	100
KA 5 μ M	2656 $\pm$ 86	105 $\pm$ 2ns
KA 25 μ M	2794 $\pm$ 167	111 $\pm$ 3ns
KA 50 μ M	3248 $\pm$ 142	129 $\pm$ 4a
KA 500 μ M	3651 $\pm$ 228	145 $\pm$ 8b
KA 5000 μ M	4094 $\pm$ 178	163 $\pm$ 3c
KA 50 μ M + AP7 500 μ M	3752 $\pm$ 235	149 $\pm$ 3
KA 50 μ M + GAMS 1mM	2555 $\pm$ 136	102 $\pm$ 3***
NMDA 5 μ M	2617 $\pm$ 137	104 $\pm$ 2ns
NMDA 25 μ M	2935 $\pm$ 107	117 $\pm$ 7a
NMDA 50 μ M	3298 $\pm$ 216	131 $\pm$ 4a
NMDA 500 μ M	3852 $\pm$ 134	153 $\pm$ 7c
NMDA 5000 μ M	3511 $\pm$ 414	139 $\pm$ 10ns
NMDA 50 μ M + AP7 500 μ M	2543 $\pm$ 109	101 $\pm$ 3***

Table 6.7b. Inhibitory actions of AP7 and GAMS on the K⁺ loss due to treatment of hippocampal slices with NMDA and KA.

Treatment	K ⁺ content $\mu\text{equiv}/100\text{mg protein}$	% of control
Control	84.58 ± 2.58	100
KA $5\mu\text{M}$	81.19 ± 1.76	$96 \pm 7\text{ns}$
KA $25\mu\text{M}$	78.65 ± 2.53	$93 \pm 3\text{ns}$
KA $50\mu\text{M}$	74.43 ± 2.40	$88 \pm 8\text{a}$
KA $500\mu\text{M}$	70.20 ± 2.57	$83 \pm 4\text{a}$
KA $5000\mu\text{M}$	39.67 ± 5.86	$53 \pm 9\text{c}$
KA $50\mu\text{M}$ + AP7 $500\mu\text{M}$	60.89 ± 3.04	$72 \pm 3^*$
KA $50\mu\text{M}$ + GAMS 1mM	85.42 ± 3.13	$101 \pm 4^*$
NMDA $5\mu\text{M}$	78.65 ± 1.78	$93 \pm 4\text{ns}$
NMDA $25\mu\text{M}$	76.67 ± 4.21	$91 - 3\text{a}$
NMDA $50\mu\text{M}$	73.58 ± 2.13	$87 \pm 7\text{a}$
NMDA $500\mu\text{M}$	75.27 ± 4.18	$89 \pm 4\text{a}$
NMDA $5000\mu\text{M}$	65.12 ± 6.42	$77 \pm 10\text{a}$
NMDA $50\mu\text{M}$ + AP7 $500\mu\text{M}$	86.27 ± 1.89	$102 \pm 9^*$

Table 6.8 Amino acid neurotransmitter release induced by NMDA, KA, aspartate or glutamate. The inhibitory actions of AP7 (500 μ M) or GAMS (1mM)

Hippocampal slices were prepared and incubated as described (2.1b; 2.2b). The excitatory amino acid analogues, AP7, and GAMS were dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. AP7 (500 μ M) was added 25 min into the incubation. $^{45}\text{Ca}^{2+}$ was not added. GAMS (1mM) was added 1 min before the addition of AP7. The excitatory amino acids, L-glutamate (Glu), L-aspartate, (Asp), NMDA, or KA were added 31 mins into the incubation period and the experiment was terminated 11 min later. Samples from the incubation medium were analysed for amino acids. Values in parentheses are percentage of stimulated control.

Significance of differences were calculated by Student's two-tailed t-test. Between agonist (s) and agonist(s) plus antagonist (s), *P<0.05; **P<0.01; ***P<0.005

Table 6.8 Amino acid neurotransmitter release induced by NMDA, KA, aspartate or glutamate. The inhibitory actions of AP7 (500 μ M) or GAMS (1mM).

	nmoles released/100mg protein	
	Glutamate	Aspartate
Control	6020 $\pm$ 178	2531 $\pm$ 172
Asp 200 μ M	7126 $\pm$ 210	ND
Asp 1mM	8368 $\pm$ 176	ND
Asp 5mM	8906 $\pm$ 231	ND
Asp 1mM + AP7 500 μ M	7043 $\pm$ 141 (45)**	ND
Glu 50 μ M	ND	2784 $\pm$ 298
Glu 100 μ M	ND	3619 $\pm$ 206
Glu 100 μ M + AP7 500 μ M	ND	3114 $\pm$ 167 (62)
Glu 100 μ M + GAMS 1mM	ND	3198 $\pm$ 122 (61)
Glu 100 μ M + AP7 500 μ M + GAMS 1mM	ND	2550 $\pm$ 165 (0)**
KA 5 μ M	6171 $\pm$ 143	2806 $\pm$ 192
50 μ M	9199 $\pm$ 102	3794 $\pm$ 116
500 μ M	9660 $\pm$ 535	4334 $\pm$ 83
KA 50 μ M + AP7 500 μ M	10016 $\pm$ 274 (127)	3492 $\pm$ 236 (76)
KA 50 μ M + GAMS 1mM	6149 $\pm$ 491 (4)**	2897 $\pm$ 264 (30)*
NMDA 5 μ M	6220 $\pm$ 164	ND
50 μ M	8582 $\pm$ 117	ND
500 μ M	8076 $\pm$ 327	ND
NMDA 50 μ M + AP7 500 μ M	6168 $\pm$ 197 (6)***	ND

Table 6.9 Amino acid release induced by NMDA and kainate: Effect of Mg^{2+} concentration

		nmol released/100mg protein	
		glutamate	aspartate
0mM Mg^{2+}			
	Control	8759 $\pm$ 565	4234 $\pm$ 532
	NMDA	15453 $\pm$ 462 (176)ns	ND
	KA	12216 $\pm$ 328 (139)ns	6945 $\pm$ 463 (164)ns
0.1mM Mg^{2+}			
	Control	7093 $\pm$ 364	2645 $\pm$ 223
	NMDA	12002 $\pm$ 254 (168)**	ND
	KA	10678 $\pm$ 559 (152)**	3982 $\pm$ 265 (151)*
1.0mM Mg^{2+}			
	Control	5784 $\pm$ 282	2143 $\pm$ 189
	NMDA	8254 $\pm$ 267 (143)***	ND
	KA	8673 $\pm$ 393 (150)***	3527 $\pm$ 284 (165)**
10mM Mg^{2+}			
	Control	5382 $\pm$ 250	1897 $\pm$ 239
	NMDA	6135 $\pm$ 274 (114)ns	ND
	KA	8126 $\pm$ 282 (151)**	2958 $\pm$ 232 (156)*

Hippocampal slices were prepared as described (2.1b, 2.2b). The slices were incubated in medium containing 0mM Mg^{2+} , 0.1mM Mg^{2+} , 1.0mM Mg^{2+} or 10mM Mg^{2+} medium. The excitatory amino acid analogues were dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. NMDA or KA were added 31 minutes into the incubation period. $^{45}Ca^{2+}$ was not added. Incubations were terminated 11 minutes later. Samples from the incubation medium were analysed for amino acids.

Values are mean $\pm$ SEM (n=6). Values in parenthesis are % of control, (control being the basal level for each individual Mg^{2+} concentration).

Significance of differences between control and agonist in each Mg^{2+} concentration were calculated by Student's two-tailed t-test. *P>0.05; ** P>0.01; *** P>0.005; ns, not significant. ND = not determined.

Figure 6.5 The effect of Mg^{2+} on the stimulation of Ca^{2+} influx by NMDA and KA in hippocampal slices.

Hippocampal slices were prepared as described (2.1b, 2.2b). The slices were incubated in medium containing 0mM Mg^{2+} , 0.1mM Mg^{2+} , 1.0mM Mg^{2+} or 10mM Mg^{2+} medium. The excitatory amino acid analogues were dissolved in 1N NaOH and then buffered to pH 7.4 in the incubation medium. NMDA or KA were added 1 min after the $^{45}Ca^{2+}$. Incubations were terminated 11 minutes later. Significance of differences between agonist responses and their relevant controls were calculated by Student's two-tailed t-test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Values are mean $\pm$ SEM; n=6.

■ Basal level; [] 50 μ M KA; ▨ 50 μ M NMDA

Fig 6.5
 Mg^{2+} ON KAINATE AND NMDA INDUCED Ca^{2+}
 INFLUX INTO HIPPOCAMPAL SLICES

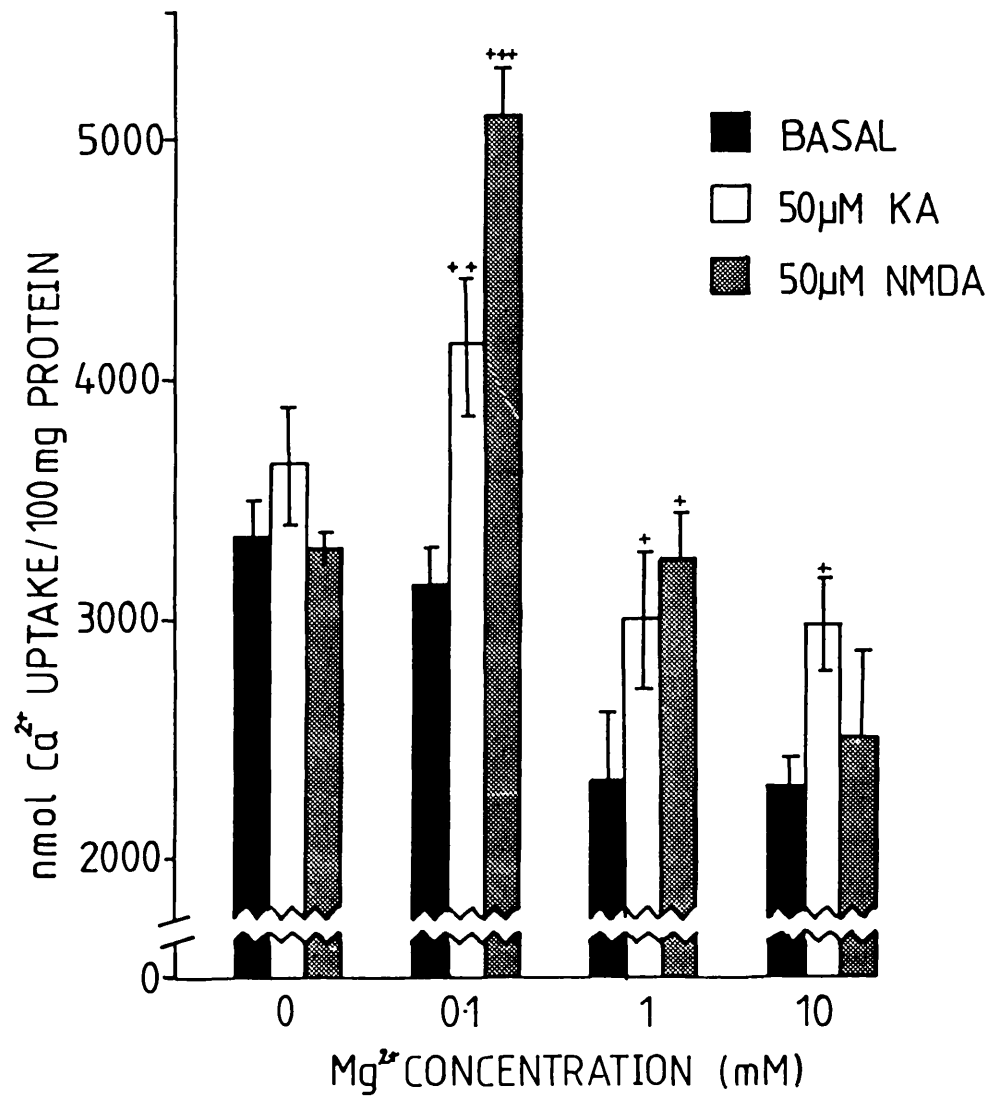


Table 6.10 **Effect of raising the external Mg^{2+} concentration to 10mM on Ca^{2+} influx, K^+ content, and glutamate release in hippocampal slices.**

Treatment	Ca^{2+} uptake nmol/100mg prot	K^+ content μ equiv K^+ /100mg	Glu release nmol/100mg prot
Control:			
1mM Mg^{2+}	1750 $\pm$ 127	74.36 $\pm$ 1.74	4168 $\pm$ 271
10mM Mg^{2+}	1713 $\pm$ 108 (102)	76.17 $\pm$ 0.63	4798 $\pm$ 362
Vt 75 μ M			
1mM Mg^{2+}	3582 $\pm$ 356	45.37 $\pm$ 1.96	7941 $\pm$ 136
10mM Mg^{2+}	3356 $\pm$ 263 (107)	50.74 $\pm$ 2.69	8364 $\pm$ 276
K^+ 56mM			
1mM Mg^{2+}	3411 $\pm$ 171	ND	8361 $\pm$ 314
10mM Mg^{2+}	3394 $\pm$ 123 (101)	ND	7693 $\pm$ 261

Hippocampal slices were prepared as described (2.1b, 2.2b). They were either incubated in normal Krebs Tris medium (1.0mM Mg^{2+}) or in high (10mM) Mg^{2+} Krebs medium. Veratrine (Vt; 75 μ M) or K^+ (56mM) were used as depolarizing agents. incubation with $^{45}Ca^{2+}$. Samples of the NaOH digest were taken for determination of both Ca^{2+} and K^+ content. Aliquots of the incubation medium were taken for amino acid analysis. Values are mean $\pm$ SEM (n=6). Values in parentheses are % of control. ND = not determined.

Although the action of anticonvulsant drugs is unknown, numerous physiological and biochemical correlations of such action have been reported (Baldino and Geller, 1981; De Boer et al, 1982; Greenberg et al, 1984). In the first part of this study three of the five anticonvulsant drugs ie. diphenylhydantion (DPH), carbamazepine (CBZ) and phenobarbitone (PB), used in the clinical treatment of epilepsy, have been shown to reduce both stimulated Ca^{2+} influx and amino acid release at relatively low concentrations in a dose-dependent manner. The IC_{50} values for the inhibition of stimulated Ca^{2+} influx for each of the three drugs were found to be close to the clinically effective dose (Ferrendelli and Daniels-McQueen, 1982) suggesting that the blockade of the Ca^{2+} influx may be an important factor in the mode of action of these drugs (Table 6.2).

The inhibitory action of DPH, CBZ and of PB on the stimulated Ca^{2+} influx has also been demonstrated in synaptosomes (Sohn and Ferrendelli, 1976; Pincus and Hsiao, 1981; Ferrendelli and Daniels-McQueen, 1982), which suggests that the anticonvulsant action of these drugs may involve an inhibition of presynaptic Ca^{2+} channels. DPH has also been found to inhibit neuronal Ca^{2+} currents (Selzer, 1978; McLean and Macdonald, 1983) and Ca^{2+} fluxes in neuroblastoma cells (Tuttle and Richelson, 1979). In addition, DPH inhibits intracellular uptake of Ca^{2+} by subcellular fractions (Yaari et al, 1978), although the mechanism of this action is unknown. However, the inhibition of Ca^{2+} across cellular and intracellular membranes might be due to a direct blockade of the Ca^{2+} channel or to an indirect effect via a decrease of Na^{+} influx and consequent reduction of the voltage-dependent Ca^{2+} influx.

Measurement of the K^{+} content of the slice provides a useful indication of the level of membrane depolarization. As the action of veratrine is directed against neurones, K^{+} loss from the slices is likely to be largely from neurones. Veratrine ($75\mu\text{M}$) caused a 61% loss of total K^{+} from cortical slices (Fig 6.1a) which DPH and CBZ were able to partially reverse ($10\mu\text{M}$ - $1\mu\text{M}$) with IC_{50} values of $5\mu\text{M}$ and $11\mu\text{M}$ which associates it more with the block of Ca^{2+} uptake than inhibition of amino acid neurotransmitter release. This reversal of the veratrine-induced depolarization indicates that these anticonvulsants may have an action on the voltage-dependent Na^{+} channels.

Ferrendelli and Daniels-McQueen (1982) reported that DPH and CBZ both inhibited Ca^{2+} influx into synaptosomes induced by 56mM K^{+} but at higher concentrations than that required to block the veratrine-stimulated uptake. They suggested that these two

anticonvulsants were able to inhibit both Ca^{2+} and Na^{+} conductances by separate and independent mechanisms. As the blockade of the Na^{+} channels occurs at far lower doses than Ca^{2+} channel blockade, this may be the more clinically relevant action.

A direct action of DPH on the Ca^{2+} channel has been reported by Greenberg et al, (1984) and more recently by Harris et al, (1985) and by Messing et al, (1985). DPH at clinically relevant concentrations inhibited the binding of [^3H]-nitredipine to the dihydropyridine site of the voltage-dependent Ca^{2+} channel. Greenberg et al, (1984) also reported that PB, CBZ, and valproic acid produced only minimal reductions in the [^3H]-nitredipine binding. Blockade of the Ca^{2+} channels by DPH may also explain the hypotension and hypoglycaemia experienced as side effects of DPH treatment (Bolger et al, 1983).

DPH and CBZ are known to reduce $^{22}\text{Na}^{+}$ uptake into clonal neuroblastoma cells and synaptosomes (Willow et al 1984), and they also depress neuronal Na^{+} currents in the same preparations (Perry et al, 1978). DPH and CBZ competitively inhibited the batrachotoxin-stimulated Na^{+} fluxes in neuroblastoma cells (Willow et al, 1984) and also batrachotoxin binding (Willow and Catterall, 1984) and may modify Na^{+} currents by interacting with the toxin binding site on the Na^{+} channel.

The inhibition of Na^{+} channels by DPH and CBZ is both voltage- and frequency-dependent (Perry et al, 1978). The voltage-dependency of the anticonvulsant action has been ascribed to the preferential binding of DPH and of CBZ to the Na^{+} channel in its inactive state. Thus the abnormal changes which occur during paroxysmal depolarizing shifts in experimental epileptic foci would be expected to increase the fractional block of Na^{+} channels. Similarly, the higher frequency firing of neurones in an epileptic focus would increase the probability of binding of the anticonvulsants to the Na^{+} channel in its inactive state (Willow et al, 1985).

Structurally, it is not surprising that DPH and CBZ act in a similar manner to block the voltage-sensitive Na^{+} channels since their three-dimensional forms are superimposable (Julien and Hollister, 1975; Willow, 1986). However, variations in their two-dimensional structures may explain their differing abilities to inhibit the binding of [^3H]-nitredipine.

Ethosuximide (ESX) and sodium valproate (VP), at all concentrations tested, failed to affect basal or stimulated Ca^{2+} influx (Table 6.2). These results are comparable to data obtained by Sohn and Ferrendelli (1976) and Greenberg et al, (1984). Greenberg et al, (1984) also found that the binding of [^3H]-nitredipine was not inhibited by either of the two drugs.

The three anticonvulsants found to inhibit Ca^{2+} influx were also able to reduce

veratrine-evoked amino acid neurotransmitter release, though less potently than the Ca^{2+} influx and mostly at concentrations considerably higher than the clinically effective doses (Table 6.2). Such discrepancies in concentration required to block the two parameters may be due to the action of veratrine in releasing intracellular Ca^{2+} stores (Bradford et al, 1986). Thus to block amino acid neurotransmitter release stimulated by veratrine ($75\mu\text{M}$) an intracellular site of action for these anticonvulsant drugs may be involved. Such a mechanism is supported by results from other groups.

De Boer (1982) showed that DPH and PB at concentrations above the clinically-effective doses inhibited the K^{+} -stimulated release of glutamate, GABA, and acetylcholine. However, DPH reduced the K^{+} -evoked [^3H]-noradrenaline release from rat brain synaptosomes at clinically effective concentrations (Pincus and Weinfield, 1985). These differences may be the result of the anticonvulsants differentially modulating the synthesis and/or release of neurotransmitters. However, the release of neurotransmitters other than amino acids may be more sensitive to reductions in Ca^{2+} influx, and may therefore be more important in seizure formation (eg, noradrenaline or acetylcholine; Sherwin et al, 1984).

Delorenzo (1980) showed that DPH and CBZ at therapeutic concentrations blocked the Ca^{2+} -dependent phosphorylation of two important synaptic proteins (DPH-L and DPH-M). PB had a slight inhibitory effect on protein phosphorylation whilst ESX had no inhibitory action. These effects on phosphorylation may be due to DPH and CBZ competitively inhibiting the action of the Ca^{2+} -calmodulin complex (Delorenzo, 1983). DPH-L and DPH-M are important phosphoproteins involved in vesicular release of transmitters, thus by blocking their activation by inhibiting Ca^{2+} -calmodulin - dependent protein kinases, a marked reduction in transmitter release would be expected.

The work of Delorenzo (1980) suggests that drugs lacking effects on the post-tetanic potentiation (PTP) for example, PB, ESX, and VP, also fail to affect Ca^{2+} and calmodulin protein phosphorylation, whilst those that do modify PTP (DPH and CBZ) also inhibit phosphorylation. Thus the ability of DPH and of CBZ to inhibit PTP may be related to their ability to block Ca^{2+} and calmodulin-stimulated protein phosphorylation.

DPH may also have effects on phospholipids. DPH has been shown to bind with varying abilities to some phospholipids and affect their interaction with Ca^{2+} . DPH enhanced the binding of $^{45}\text{Ca}^{2+}$ to phosphotidic acid and phosphotidyl serine (Chwen and Leslie, 1981). These authors suggest that the enhanced binding of Ca^{2+} to acidic lipids may be involved in alteration of Na^{+} or K^{+} transport. DPH may affect phosphotidylinositol turnover (Berridge, 1984), and thus provide a different mechanism whereby Ca^{2+} influx and amino acid release

are inhibited.

Other mechanisms for reducing the release of neurotransmitters may involve alterations in transmitter synthesis. ESX, at therapeutic concentrations has been shown to inhibit both GABA-transaminase and succinic semialdehyde dehydrogenase (two enzymes of GABA catabolism) in rat whole brain (Sawaya et al, 1975; Chapman et al, 1982). The result of the inhibition of these two enzymes would be an increase in the concentration of GABA and an enhanced action of GABA at the post-synaptic membrane. The results shown (Fig 6.2b) support this theory, as aspartate and glutamate levels are little effected, whilst stimulated-GABA levels are raised by clinically-effective concentrations of ESX.

VP did not inhibit Ca^{2+} uptake. However in the present study it appeared to differentially affect the stimulated release of amino acids, inhibiting aspartate whilst having no effect on glutamate or GABA release at relatively low levels ($100\mu\text{M}$). There is evidence from electrophysiological and biochemical studies that VP interacts with the GABAergic system (Godin et al, 1969; Anlezark et al, 1976). The acute administration of valproate increased brain-GABA content, presumably due to either increased rate of synthesis or to decreased rate of breakdown. Thus the anticonvulsant activity of VP may be rationalised in terms of enhanced synaptosomal- GABA content and consequent enhanced GABA release and post-synaptic inhibitory influences.

However, this hypothesis is not compatible with studies of GABA release from synaptosomes or from superfused cortex, in which there is no detectable rise in GABA release consequent upon VP administration (Abdul-Ghani et al, 1981a and b), although this may be due to re-uptake of the released amino acids by either high and low affinity uptake systems. The results given here show that at clinically effective doses (approximately $300\mu\text{M}$), VP did not significantly reduce GABA levels released by the stimulated rat cortex slices. However, there was a significant reduction in aspartate levels (IC_{50} , $264\mu\text{M}$; Table 6.2).

Acute administration of VP has been shown to substantially and specifically decrease the whole brain content of aspartate in rats (Schechter et al, 1978; Chapman et al, 1982). There appeared to be good correlation between the anticonvulsant potency of VP and its ability to lower aspartate levels (Chapman et al, 1984). Thus the action of VP in reducing aspartate levels correlate well with the data presented here.

Thus to summerise, DPH and CBZ inhibit both Ca^{2+} and Na^{+} fluxes. These two anticonvulsant drugs also affect intracellular phosphorylation by interacting with calmodulin. The likely result of these actions would be to inhibit stimulated Ca^{2+} influx, decrease veratrine-induced depolarization, and reduce stimulated amino acid release as reported here.

In the present study PB has also been shown to decrease Ca^{2+} influx, veratrine depolarization and amino acid release. However, the mechanism of PB action is not fully understood. ESX and VP acted differentially on amino acid release, and their respective modes of anticonvulsant action may be related to the reported increase in GABA or decrease in aspartate release shown.

In the second section of this chapter the mechanism of anticonvulsant action of the ω -phosphono- α -aminocarboxylic acids (AP4, AP5, and AP7) is discussed.

Hippocampal slices were used in these experiments as opposed to the cortex slices in the first section. Hippocampal slices are metabolically stable (Whittingham et al, 1984), and if prepared with the plane of the slice lying normal to the ventricular surface and the longitudinal axis, the lamellar structure is maintained. The hippocampal formation has long been recognised as a site of epileptogenesis (Kandel and Spencer, 1961), and the activities of the phosphonic amino acids have been studied *in vivo* in this structure (White et al, 1977 and 1979). When the glutamate content of tissue undergoing an epileptic seizure was recorded, it was found that there was an increase in glutamate levels as compared to non-epileptic tissue; this increase in release may have been the result of excessive Ca^{2+} influx into the epileptic neurones. Thus by decreasing the stimulated Ca^{2+} influx there would be a reduction in the severity of the epileptic seizure.

The effect of the phosphonic compounds on both the veratrine- and the K^+ -stimulated Ca^{2+} influx and amino acid release was measured. Both veratrine ($75\mu\text{M}$) and K^+ (56mM) significantly enhanced the basal influx of Ca^{2+} in the hippocampal slices (to 160%-190% of control levels; Table 6.3). AP5 and AP7 at $100\mu\text{M}$ caused a maximal block of 60%-70% of the Ca^{2+} influx into the hippocampal slices evoked by veratrine or high K^+ , whilst the general alkylamine Ca^{2+} channel blocker (-)D888 ($10\mu\text{M}$) caused total inhibition of this uptake.

This suggests that a large proportion of the stimulated Ca^{2+} uptake induced by these depolarizing agents was due to an action of the excitatory amino acids, presumably mainly glutamate and/or aspartate, released by the depolarizing treatments. The observations that the addition to the hippocampal slices of relatively low concentrations of glutamate ($100\mu\text{M}$), aspartate (1mM), KA ($50\mu\text{M}$), or NMDA ($50\mu\text{M}$), induced considerable Ca^{2+} uptake, which could be largely inhibited by AP7 or GAMS, supports this conclusion. Although $100\mu\text{M}$ glutamate caused only 29% of the maximal effect of veratrine (Tables 6.4a and b, 6.5a and b), the concentration of glutamate or aspartate reached in the intracellular spaces of the slice on addition of depolarizing agents could well be in excess of $100\mu\text{M}$ and the dose-

response data reported here show that glutamate or aspartate in excess of 200 μ M can induce much greater Ca^{2+} influxes (Table 6.4a and b). Thus, released glutamate and aspartate could together account for 60%-70% of the Ca^{2+} influx induced by veratrine which is predicted by the inhibitory action of AP7. Other workers have reported similar degrees of enhancement of Ca^{2+} influx into brain slices and synaptosomes (Berdichevsky et al, 1983; Pastuszko et al, 1984; Lazarewicz et al, 1986).

Relatively high concentrations (500 μ M) of AP7 were used in these experiments to guarantee the production of maximal inhibitory actions. However, the dose response histograms (Fig 6.3c) show that both 10 μ M and 100 μ M AP7 also have significant inhibitory effects on Ca^{2+} influx.

Both AP4 and AP5 displayed the ability to suppress Ca^{2+} uptake with AP5 exhibiting similar potency to AP7, and AP4 being some 10-fold less effective. However, AP4 was still moderately effective, causing 33%-46% inhibition of K^{+} - or veratrine-stimulated Ca^{2+} uptake, respectively, at 100 μ M (Fig 6.3a). The rank order of activity of the antagonists is consistent with the activities of the compounds in electrophysiological and behavioural studies (Croucher et al, 1982; Coutinho-Netto et al, 1980).

The stereoselectivity of the action of AP7 was observed in the much greater potency of D(-)AP7 compared to L(+)AP7, also in accordance with both electrophysiological and behavioural studies (Perkins et al, 1982; Croucher et al, 1982). This clearly indicates that receptor interaction is involved in the action of the antagonists. Receptor mediation was also indicated by the pattern of inhibition of Ca^{2+} uptake stimulated by NMDA or KA. Thus whilst AP7 (a specific "NMDA" receptor antagonist) abolished Ca^{2+} influx, and also neurotransmitter release both evoked by NMDA it had no effect on the KA-induced Ca^{2+} uptake or amino acid release. Conversely, the latter response were fully inhibited by GAMS, a quisqualate and "KA-receptor"- directed antagonist (Jones et al, 1984; Davies and Watkins, 1985).

The homologous long chain fatty acids, butyric, valeric, and pentanoic acids failed to influence either basal or stimulated levels of Ca^{2+} influx or amino acid neurotransmitter release (Tables 6.4a and b). This indicated that the actions of the antagonists are critically dependent on the presence of the phosphonic group and are not the result of non-selective membrane interactions mediated by the hydrophobic fatty acid side chains.

Inhibition by the NMDA antagonists of Ca^{2+} uptake induced by the depolarizing agents was paralleled by inhibition of amino acid neurotransmitter release, with a similar order of potency. Glutamate and aspartate efflux were most potently inhibited with GABA showing

some suppression by AP7.

The loss of K^+ from the slice induced by veratrine was significantly decreased (20%-22%; $P < 0.05$) by AP7 at 500 μM or greater, probably reflecting blockade of depolarization induced by the release of amino acids acting at NMDA receptors. This rationale is supported by the observation that AP7 completely blocked the K^+ loss due to NMDA and substantially prevented K^+ loss following the addition of glutamate or aspartate (Table 6.6). The latter would include the action of glutamate at the non-NMDA classes of glutamate receptors including "KA receptors". Indeed the K^+ loss induced by KA was blocked by GAMS which acts at these receptors, and the K^+ loss due to added glutamate was completely prevented by AP7 and GAMS added together, indicating that NMDA, quisqualate, and KA receptors were being activated by glutamate. This correlation between K^+ loss and Ca^{2+} uptake suggests that the Ca^{2+} uptake occurred, at least partially, through voltage-dependent Ca^{2+} channels, but does not exclude the presence of Ca^{2+} channels directly operated by NMDA receptors as suggested by Collingridge (1985) and others, as they would be inhibited in parallel. Indeed the differential action of AP7 on the recovery of K^+ content and on Ca^{2+} uptake induced by veratrine shown in Table 6.3, suggests that voltage-independent Ca^{2+} channels linked to NMDA receptors are present.

Autoradiographic studies using [^3H]-glutamate and [^3H]-aspartate on *in vitro* and *in vivo* hippocampal preparations have shown a zonal distribution of glutamate and aspartate binding sites, and that a high proportion of the glutamate receptors are of the NMDA subtype (Monaghan and Cotman, 1985; Monaghan et al, 1984; Storm-Mathisen and Otherson, 1984). The results obtained in this study show that approximately 44% of the Ca^{2+} influx stimulated by glutamate is inhibited by AP7 which indicates a high density of NMDA receptors in this structure in hippocampal slices.

The action of low concentrations of Mg^{2+} in specifically enhancing the effects of NMDA provides supporting evidence for the proposal that extracellular Mg^{2+} inhibits these receptors during normal synaptic transmission (Collingridge, 1985; Mayer and Westbrook, 1985). The unusual voltage-dependency of the NMDA receptor was recorded early in 1960 (Curtis and Watkins, 1960). More recently, Collingridge and co-workers (1983) have shown that hippocampal slices bathed in Ca^{2+} -free physiological saline fire in bursts similar to those caused by GABA receptor antagonists. They proposed that intracellular Mg^{2+} normally inhibits NMDA receptor activity. During excessive stimulation, for example, just prior to an epileptic seizure, when the neurones are becoming more depolarized the Mg^{2+} block would be removed and the NMDA receptors would function. This may explain why the

NMDA antagonists are inactive under normal conditions.

NMDA receptors may also be involved in the initiation of long-term potentiation (LPT). LPT is a phenomenon in which tetanic stimulation of a set of input fibres potentiates synaptic transmission in a particular pathway period varying in length from hours to weeks. LPT is blocked by AP5 (Collingridge et al, 1983) and high levels of Mg^{2+} , indicating that NMDA receptors are functional in situations of high stimulation in low- Mg^{2+} medium. The above results may also bear on the protective actions of the amino acid antagonists in animal models of epilepsy and cerebral ischaemia.

In relation to epilepsy, blockade of Ca^{2+} uptake and excitatory amino acid release would clearly have anticonvulsant effects, especially as GABA release is much less effected than the release of glutamate and aspartate. Indeed, the ω -phosphonic- α -aminocarboxylic acids show anticonvulsant actions in a wide range of animal models of epilepsy (Coutinho-Netto et al, 1981; Croucher et al, 1982; Meldrum et al, 1983; Peterson et al, 1984). AP5 and AP7 are the most universally active anticonvulsants in this respect. This is consistent with the relative potencies of these anticonvulsants as inhibitors of Ca^{2+} uptake and amino acid efflux reported here.

AP4 shows a quantitatively very similar action although much weaker, than the other antagonists. This is surprising in view of the complexity of action of AP4 at the synaptic level as suggested by the differing activities of its separate stereoisomers (Koerner and Cotman, 1981).

Moderate, brief increases in intracellular Ca^{2+} mediate neurotransmitter release, whilst chronic high intracellular Ca^{2+} can lead to excitotoxic cell damage (Schanne et al, 1979; Farber et al, 1981). Rothman (1984) has recently shown that anoxic neuronal death of cultured rat hippocampal neurones can be prevented by the excitatory amino acid antagonist γ -D-glutamylglycine. The antagonist similarly protected the cultured neurones from the toxicity of exogenous glutamate and aspartate, suggesting that the synaptic release of an excitatory transmitter may mediate anoxic neuronal death *in vivo*.

The present report and earlier reports (Berdichevsky et al, 1983; Pastuszko et al, 1984; Lazarewicz et al, 1986) of the stimulation of Ca^{2+} influx into brain slices and synaptosomes by excitatory amino acid receptor agonists suggest that this may therefore be a key element in the mechanism of anoxic/ischaemic death. Furthermore, blockade of excitatory amino acid-mediated Ca^{2+} uptake by amino acid antagonists, as reported here, may be an important factor in the prevention of anoxic neuronal death.

CHAPTER 7

CONCLUDING REMARKS

Brain slices have been used throughout this study and have provided a valuable model upon which to examine the effects of neuroactive agents.

Slices taken from three different regions of the brain, cortex, striatum and hippocampus, were shown to be viable and to contain active synaptic terminals. This was demonstrated by the ability of depolarizing agents to induce Ca^{2+} influx, amino acid release and K^{+} loss. The values obtained for these parameters were comparable to those results obtained by other workers.

Characteristics of the Ca^{2+} influx and amino acid release indicated that the two responses were related. Veratrine was found to stimulate amino acid release even in the absence of extracellular Ca^{2+} , the response was sensitive to the Na^{+} -channel blocker, TTX. Amino acid release induced by high concentrations of K^{+} was abolished in the absence of external Ca^{2+} and was unaffected by TTX.

The different properties of the two depolarizing agents were used in investigations to identify the sites of action of neuroactive agents.

The *in vitro* action of some opioid compounds was studied in chapter 4. Previous research showed that morphine inhibited the depolarization-dependent Ca^{2+} influx by a μ -receptor-mediated action. It was interesting to discover whether other opioid receptor subclasses could act in a similar manner.

It was found that all the other opioid compounds used inhibited the stimulated Ca^{2+} influx and amino acid release. The differing ability of naloxone and WIN 44441-3 to inhibit the action of the μ - and κ -agonists indicated the presence of functional μ - and κ -receptors in

the cortex. The action of the opioids on veratrine-induced amino acid release had the properties of an effect mediated by a cell-surface opioid receptor.

Morphine, at concentrations comparable to those which were found to block Ca^{2+} influx also inhibited the incorporation of $^{32}\text{P}_i$ into the protein fraction of the cortex slices. The enhancing effect of GTP on the inhibitory action of morphine on both Ca^{2+} and the incorporation of $^{32}\text{P}_i$ indicated the involvement of a second messenger system which is linked to the Ni regulatory subunit. The action of tifluadom and EKC were also enhanced by GTP which may indicate a similarity in the mechanism of μ - and κ -agonists.

In chapter 5, the possible presynaptic action of three neurotransmitters was investigated. Noradrenaline, dopamine, 5-HT and apomorphine (a dopaminergic agonist) all inhibited the depolarization-evoked uptake of Ca^{2+} and release of amino acid neurotransmitters. The concentrations required to induce this inhibition were comparable to those concentrations found to be active in other preparations.

The activities of the agonists were blocked by their specific antagonists indicating a receptor-mediated action. The action of noradrenaline appeared to be mediated by α_2 -receptors, whilst dopamine was acting via D-2 receptors.

The characteristics of blockade of veratrine-stimulated amino acid release indicated the involvement of a second messenger system in the presynaptic mechanism of action of these neurotransmitters and this was discussed.

One effect of anticonvulsant drugs is to inhibit the release of excitatory amino acids thus, the aim in chapter 6 was to investigate whether this decrease was due to a blockade of stimulated Ca^{2+} influx.

DPH, carbamazepine and phenobarbitone all inhibited Ca^{2+} influx and amino acid release at clinically relevant concentrations. The involvement of a second messenger in these effects was discussed. Sodium valproate, although it did not block Ca^{2+} influx, specifically and potently, inhibited the release of aspartate. Ethosuximide had no significant effect either Ca^{2+} influx or amino acid efflux. However, the efflux of GABA was enhanced. The action of these latter two anticonvulsants on amino acid neurotransmitter metabolism was discussed.

Hippocampal slices were used in investigations on the action of AP4, AP5 and AP7. The uptake of Ca^{2+} and the efflux of glutamate or aspartate induced by veratrine or K^+ was blocked by all three anticonvulsants. The maximum blockade was approximately 60%-70% compared to the 100% blockade with (-)-D600. Uptake of Ca^{2+} induced by glutamate, aspartate and NMDA was also inhibited by AP7, whilst GAMS blocked the action of KA and partially blocked that of glutamate.

The action of NMDA in stimulating Ca^{2+} uptake was Mg^{2+} -sensitive, low Mg^{2+} levels in the incubation medium selectively enhanced the response. It was concluded that Ca^{2+} uptake evoked by excitatory amino acids is receptor mediated, and that the released amino acids are responsible for a large part of the action of veratrine and high K^{+} in stimulating Ca^{2+} . It was also concluded that AP5 and AP7 were acting as anticonvulsants by preventing the postsynaptic activity of the released excitatory amino acids.

This thesis has looked at the methods by which different agents may act to affect the movement of Ca^{2+} and subsequent neurotransmitter release. There are many areas of research which would lead to a better understanding of the role of Ca^{2+} influx and neurotransmitter release and how it may be modulated by neuroactive drugs.

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MORPHINE INHIBITION OF CALCIUM FLUXES, NEUROTRANSMITTER RELEASE AND PROTEIN AND LIPID PHOSPHORYLATION IN BRAIN SLICES AND SYNAPTOSOMES

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Abstract—Morphine (1–100 μ M) was found to inhibit several concomitant events in brain slices and synaptosomes which are augmented by depolarizing agents. Thus, $^{45}\text{Ca}^{2+}$ uptake, amino acid neurotransmitter release, increases in 3',5' cyclic AMP levels and $^{32}\text{P}_i$ incorporation to proteins and lipids induced by veratrine (25 μ M) and by potassium (56 mM), were each inhibited in a dose related manner. These inhibitory actions of morphine were all prevented by naloxone (1 μ M).

Evidence is presented that morphine binding to a receptor on the synaptic membrane affects intracellular mechanisms involved in neurotransmitter release possibly via a second messenger system. An enhancing action of GTP on the inhibitory influences of morphine suggests that its actions are mediated at least in part, via a coupling of the receptor to adenyl cyclase in the outer membrane. This is supported by its inhibitory action on the capacity of depolarizing agents to increase cyclic AMP levels.

There have been many reports of the action of morphine in reducing the release and turnover of acetylcholine and biogenic amines both *in vivo* and *in vitro* [1–5]. Similarly, enkephalins have been shown to inhibit the release of these neurotransmitters and all of the inhibitory actions of morphine are prevented by naloxone indicating that they are specific and receptor mediated [6–10]. In addition, naloxone-sensitive suppression by morphine of amino acid neurotransmitter release evoked *in vivo* by depolarizing agents and by sensory stimulation has been reported [11, 12]. Parallel effects of morphine on Ca^{2+} uptake and amino acid neurotransmitter release from synaptosomes incubated *in vitro* have been demonstrated [13]. The present paper describes further features of this inhibition of Ca^{2+} fluxes and neurotransmitter release by morphine, and examines parallel effects of the drug on related systems including adenylate cyclase and protein and lipid phosphorylation, with a view to further defining the mode of action of morphine.

MATERIALS AND METHODS

Preparation of synaptosomes. Synaptosomes were prepared from cerebral cortex of female Sprague-Dawley rats, by the method of Gray and Whittaker [14] as modified by Bradford *et al.* [15].

$^{45}\text{Ca}^{2+}$ uptake by synaptosomes. The synaptosomal pellet was suspended in 1 ml of Tris buffered Krebs-Ringer medium of the following composition (mM); NaCl, 120.0; KCl, 5.0; MgSO_4 , 1.0; glucose, 10.0;

Tris-HCl, 20.0; pH 7.4. Incubation was performed in 5 ml conical flask at 37° for 30 min in a shaking water bath. A gas phase of oxygen was provided. Morphine sulphate (final concentration 1–100 μ M) was present for the last 15 min of incubation. The depolarizing agent and $^{45}\text{CaCl}_2$ were present for the last 20 min. $^{45}\text{Ca}^{2+}$ was added to give a final concentration of 1.2 mM, with specific activity 0.8 mCi/mmol.

Ice-cold EGTA solution of composition (mM); NaCl, 120.0; KCl, 5.0; EGTA, 30.0; MgCl_2 , 1.2; Tris-HCl, 20.0; NaH_2PO_4 , 1.2; pH 7.4, was added to terminate the reaction, and the synaptosomal suspensions were sedimented in a bench ultracentrifuge [16]. The supernatants were retained for amino acid analysis, and the pellets were washed three times in 1 ml of K^+ -free Na^+ solution, composition (mM); NaCl, 137.0; MgCl_2 , 1.2; Na_2HPO_4 , 1.2; Tris, 20.0; glucose, 10.0; pH 7.4. The resulting pellets were digested at 50° in an appropriate volume of 1 M NaOH for 20 min. Aliquots were taken for radioactivity and protein determination.

$^{32}\text{P}_i$ incorporation into synaptosomes. Synaptosomal pellets were suspended in Krebs-Ringer medium and 0.5 ml aliquots (4 mg/ml) were incubated at 37° for 5 min in the presence of morphine before the addition of drugs and $^{32}\text{P}_i$. The $^{32}\text{P}_i$ (Amersham International) was added to give a specific activity of 200 $\mu\text{Ci}/\text{ml}$ Ci. The synaptosomal suspension was precipitated by the addition of 1.5 ml of 15% TCA. The mixture was left on ice for about 15 min with frequent "vortex mixing" and the suspension was sedimented in a bench-top ultracentrifuge. The resulting protein was then taken for radioactivity counting.

Preparation and incubation of brain slices. Female Sprague-Dawley rats (200 g) were stunned and deca-

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‡ Abbreviations used: P_i , inorganic phosphate; TTX, tetrodotoxin.

pitated. Brains were rapidly removed and placed on a warmed glass plate [17] and cortex slices (40–80 mg) were rapidly cut (2 min in total) from the cerebral hemispheres using a razor blade and recessed guide [18]. Only one slice was taken from each. This was halved and then incubated in Krebs–Tris medium which had the composition (mM), NaCl, 138.0; KCl, 5.0; MgSO₄, 1.0; Tris–HCl, 20.0; glucose, 10.0; CaCl₂, 1.2; pH 7.4.

⁴⁵Ca²⁺ uptake by cortex slices. Brain slices (approximately 30 mg wet wt) were incubated at 37° in 4.5 ml Ca²⁺–Krebs–Tris medium (1.2 mM Ca²⁺) in 5 ml beakers in a shaking water bath for 30 min. Pure oxygen was bubbled continuously into the medium throughout the incubation. Weight measurements showed a <5% loss of volume due to evaporation.

Test compounds, dissolved in the Krebs–Tris buffer, were added 5 min prior to addition of ⁴⁵Ca²⁺. The ⁴⁵Ca²⁺ was added after 30 min of incubation (Amersham International), to give a final specific activity of 0.14 Ci/mmol. One minute later the depolarizing agent was added and after a further 10 min incubation with ⁴⁵Ca²⁺ the slices were transferred to 25 ml beakers for 15 sec, each containing 2 ml of EGTA stop-solution (described above). The slices were then washed in three separate 2 ml aliquots of K⁺-free Na⁺ medium, of the composition described above, at room temperature. Slices were then digested in 1 ml of 1 M NaOH at 50° for 30 min. The suspension was aliquoted and samples taken for analysis of K⁺ content by atomic absorptiometry and ⁴⁵Ca radioactivity was measured by scintillation counting as described elsewhere [19].

Incorporation of ³²P_i into brain slices. Cerebral slices were each suspended in 4.5 ml of Ca²⁺–Krebs–Tris medium for 30 min. Subsequently ³²P_i was added (Amersham International) to give a final specific activity of 200 µCi/per incubation.

The incubation was allowed to proceed for an hour and then the depolarizing agent was added. The incubation was terminated 1 min later. For total ³²P_i incorporation into the slice, the tissue was rapidly washed in 1 ml of ice-cold K⁺ free Na⁺ medium, digested in 1 ml of 1 M NaOH and samples were taken for counting of radioactivity. When the incorporation of ³²P_i into total protein and lipid fractions were estimated, the procedure described by Abdul Ghani *et al.* [20] was followed. The lipid fraction was removed by extraction with ethanol/methanol mixtures and acidified diethyl ether [20]. All supernatants were counted as was the final NaOH digest.

Amino acid analysis. Samples were analysed for amino acid content as described by Norris *et al.* [21] using a Rank Hilger Chromaspek J180 autoanalyser with fluorimeter J143.

3',5' Cyclic AMP assay. Cerebrocortical synaptosomes were suspended in 0.32 M sucrose containing 4 mM Tris–HCl pH 7.4 [22]. The preparation was stored at –20° until use.

The standard incubation mixture (150 µl) consisted of 0.3 mM ATP, 30 mM Tris–HCl, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 mM MgCl₂. The reaction was initiated by the addition of synaptosomal fractions (25 µg protein). Incubation was continued for 10 min at 37°, the relevant drugs being

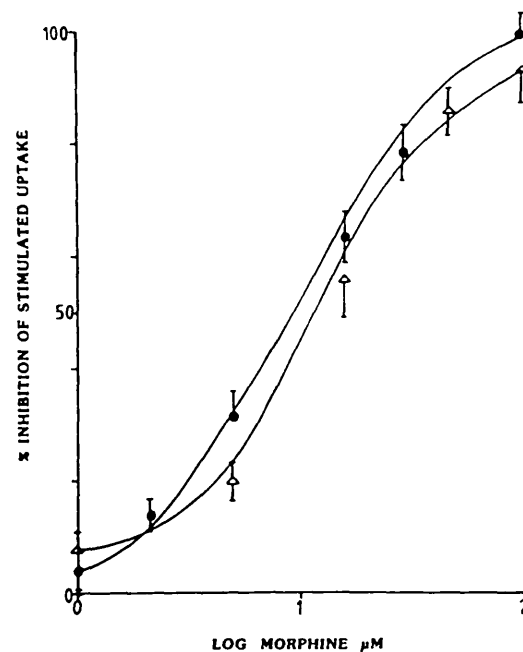


Fig. 1. Dose response curves for the inhibitory action of morphine on veratrine (25 µM) stimulated Ca²⁺ influx into both cortical brain slices and synaptosomes. Values are means ± S.E.M. and the lines were fitted by linear regression: ●, Brain slices (N = 18); △, synaptosomes (N = 12).

present for the last 5 min. The synaptosomal suspension was then centrifuged in a bench ultracentrifuge and the supernatant was retained for analysis.

3',5' Cyclic AMP levels in the supernatant were determined using an assay kit supplied by Amersham International.

Protein determination. Both synaptosomal and brain slice protein content were determined by the method of Lowry *et al.* [23] using bovine serum albumin as the standard.

RESULTS

The effect of morphine on resting and stimulated uptake of ⁴⁵Ca²⁺

Morphine added alone in the concentration range 1–100 µM had no effect on spontaneous ⁴⁵Ca²⁺ uptake or on amino acid release from either brain slices or synaptosomes. The dose–response curve for morphine inhibition of Ca fluxes is given in Fig. 1.

Using 25 µM veratrine as the depolarizing agent, ⁴⁵Ca²⁺ uptake increased by 29% ± 3% in brain slices and 54% ± 6% in synaptosomes. These responses were significantly (P < 0.001) inhibited by morphine (100 µM), the levels of ⁴⁵Ca²⁺ uptake approaching those of untreated controls. The IC₅₀ for morphine inhibition of 25 µM veratrine-evoked ⁴⁵Ca²⁺ uptake was 9.4 µM (5.3–16.7) in brain slices and 11.4 µM (8.7–14.9) in synaptosomes (Table 1).

High K⁺ (56 mM) also stimulated Ca²⁺ uptake and this response was found to be sensitive to morphine,

Table 1. IC_{50} values for the inhibition by morphine of both stimulated Ca^{2+} uptake and endogenous glutamate release, in cerebrocortical brain slices and synaptosomes

Stimulating agent	Brain slices N = 18		Synaptosomes N = 12	
	Ca^{2+} influx	Evoked glutamate release	Ca^{2+} influx	Evoked glutamate release
Veratrine 25 μ M	9.4 μ M (5.3–16.7)	18.4 μ M (16.3–19.8)	11.4 μ M (8.7–14.9)	26.0 μ M (16.1–41.9)
Potassium 56 mM	4.8 μ M (2.3–10.1)	3.9 μ M (2.0–7.6)	2.4 μ M (0.3–19.2)	0.5 μ M (0.02–11.6)

Tabulated data are for μ M morphine added, and were estimated from dose response curves over a concentration range 1–100 μ M morphine (see Fig. 1). In brain slices Ca^{2+} influx and evoked glutamate release were inhibited by 100% by 100 μ M morphine, and by 72–79% by 50 μ M morphine. In synaptosomes Ca^{2+} influx and evoked glutamate release were inhibited by 91% and 79% respectively by 50 μ M morphine, and by 86% and 55% at 15 μ M morphine. Evoked glutamate release was calculated as the difference between control and stimulated levels of glutamate in the incubation medium. Stimulation increased glutamate release by 9-fold in synaptosomes and by 2-fold in brain slices.

Data were collated from 6 experiments (brain slices) or 4 experiments (synaptosomes), each experiment involving triplicate samples for each condition. The 95% confidence limits (in parentheses) were calculated by the method of Litchfield and Wilcoxon [43].

with estimated IC_{50} values of 4.8 μ M (2.28–10.1) in brain slices and 2.4 (0.3–19.2) in synaptosomes (Table 1). All inhibitory actions of morphine on veratrine or K^+ -induced Ca^{2+} uptake were abolished by 1 μ M naloxone (Table 2).

Influence of GTP on morphine

The addition of GTP (1 μ M–10 μ M) significantly ($P < 0.005$) increased the inhibitory effect of 15 μ M morphine on the Ca^{2+} uptake induced by 25 μ M veratrine (Table 2).

Morphine (15 μ M) decreased stimulated Ca^{2+} uptake into brain slices by 48%, and in the presence of 10 μ M GTP this inhibitory action increased by 78%. In synaptosomes there was a 16% enhance-

ment of the inhibition induced by 15 μ M morphine on the addition of 10 μ M GTP.

GTP, when added alone had no influence on basal or stimulated Ca^{2+} uptake into either brain slices or synaptosomes. Other related nucleotides tested, GMP, CTP or ITP (all at 10 μ M) showed no enhancement of morphine inhibition (Table 2).

Action of morphine on neurotransmitter amino acid release

Morphine at low concentrations (1–15 μ M) inhibited the evoked release of endogenous neurotransmitter amino acids from both brain slices and synaptosomes when 25 μ M veratrine and 56 mM K^+ were employed as stimulation agents. All of these

Table 2. Effect of GTP and naloxone on morphine-induced inhibition of stimulated Ca^{2+} uptake

Condition	Brain slices N = 9	Synaptosomes N = 9
Control	11.21 $\pm$ 0.22	3.70 $\pm$ 0.14
GTP (10 μ M)	11.28 $\pm$ 5.25	3.75 $\pm$ 0.13
Veratrine	15.06 $\pm$ 4.22	8.16 $\pm$ 0.23
Veratrine plus morphine	13.21 $\pm$ 0.32 (48%)	6.33 $\pm$ 0.15 (41%)
Veratrine plus GTP (10 μ M)	15.14 $\pm$ 0.24	—
Veratrine plus morphine: plus GTP (10 μ M)	12.46 $\pm$ 0.21* (67%)	5.70 $\pm$ 0.09* (55%)
GTP (1 μ M)	12.07 $\pm$ 0.24* (78%)	5.61 $\pm$ 0.11* (57%)
GMP (10 μ M)	13.41 $\pm$ 0.33 (43%)	6.19 $\pm$ 0.22 (44%)
GDP (10 μ M)	—	6.37 $\pm$ 0.19 (40%)
CTP (10 μ M)	12.99 $\pm$ 0.63 (53%)	6.44 $\pm$ 0.23 (39%)
ITP (10 μ M)	13.62 $\pm$ 0.47 (37%)	6.33 $\pm$ 0.18 (41%)
Naloxone (1 μ M)	15.01 $\pm$ 2.47	8.14 $\pm$ 0.64

Veratrine was present at 25 μ M final concentration, and morphine at 15 μ M. Tabulated data are nmoles Ca^{2+} uptake/mg protein $\pm$ S.E.M. Values in parentheses are % inhibition by morphine or morphine plus GTP on the veratrine stimulated Ca^{2+} uptake. Data were collated from 3 experiments for both brain slices and synaptosomes, each experiment involving triplicate samples for each condition.

* $P < 0.005$ compared to veratrine plus morphine alone employing Student's two-tailed *t*-test.

Table 3. 3',5' Cyclic AMP levels in synaptosomes: effects of depolarizing agents and morphine

		pmol/mg Protein	Increase in levels above control (%)	Inhibition of cyclic AMP response by morphine (%)
Control		111 ± 12	—	—
Morphine	15 µM	110 ± 15	0	—
Morphine	100 µM	119 ± 17	8	—
Naloxone	1 µM	114 ± 11	2	—
K ⁺		170 ± 16	53 ± 5	—
K ⁺ plus:				
Morphine	1 µM	156 ± 13	41	23
Morphine	100 µM	125 ± 14	13	76
Morphine and Naloxone	100 µM } 1 µM }	166 ± 17	50	7
Veratrine 25 µM		183 ± 16	65 ± 6	—
Veratrine (25 µM) plus:				
Morphine	15 µM	142 ± 12	30	57
Morphine	100 µM	128 ± 15	15	76
Morphine and Naloxone	100 µM } 1 µM }	178 ± 17	60	7
Veratrine 75 µM		200 ± 19	80	—
Veratrine (75 µM) plus:				
Morphine	15 µM	186 ± 14	68	15
Morphine	100 µM	180 ± 15	22	22
Morphine and Naloxone	100 µM } 1 µM }	204 ± 18	85	0

Data was obtained from twelve samples from six experiments and represent the mean ± S.E.M.

Table 4. Total ³²P_i incorporation into cerebral tissue

		% Incorporation	
		Brain slices N = 9	Synaptosomes N = 15
Control		100	100
GTP	10 µM	98 ± 7	—
Morphine	100 µM	96 ± 14	109 ± 11
Veratrine	75 µM	205 ± 27	270 ± 21
Veratrine (75 µM) plus:			
Morphine	15 µM	—	241 ± 14 (17%)
Morphine	100 µM	232 ± 39 (0%)	245 ± 10 (15%)
Veratrine	25 µM	140 ± 21	217 ± 17
Veratrine (25 µM) plus:			
Morphine	15 µM	129 ± 7* (27%)	166 ± 13** (44%)
Morphine	100 µM	104 ± 12** (90%)	160 ± 9** (49%)
Morphine and Naloxone	100 µM	146 ± 16 (0%)	223 ± 11 (0%)
GTP	1 µM	—	—
Morphine and GTP	10 µM	141 ± 9 (0%)	—
Morphine and GTP	15 µM	118 ± 10† (55%)	—
Morphine and ITP	10 µM	130 ± 6 (28%)	—
K ⁺ 56 mM			200 ± 11
K ⁺ plus:			
Morphine	1 µM	—	161 ± 15 (39%)
Morphine	100 µM	—	153 ± 11 (47%)

Data represent % increases in ³²P_i incorporation, and are expressed as means ± S.E.M.

Values in parentheses are % changes from veratrine-stimulated level. Data were collated from 3 experiments for brain slices and 5 experiments for synaptosomes each experiment involving triplicate samples for each condition.

The control level of ³²P_i incorporation were:

brain slices = 29987 ± 210 dpm/mg tissue wet weight;

synaptosomes = 2681 ± 50 dpm/mg protein.

* P < 0.005; **P < 0.001, in comparison to veratrine (25 µM) alone; † = P < 0.001 in comparison to veratrine (25 µM) plus morphine (15 µM) employing Student's two-tailed t-test.

Table 5. Incorporation of $^{32}\text{P}_i$ into lipid and protein fractions of brain slices

	% Change from control levels of incorporation		Response in whole slice
	Lipid	Protein	
Veratrine	41% (32)	71% (78)	57%
Veratrine plus Morphine	31% (42)	29%* (29)	28%*
Veratrine plus Morphine + GTP	16%* (53)	11%* (47)	13%*
Veratrine plus Morphine + Naloxone	47% (36)	69% (64)	59%

Values in parentheses represent the proportion of the total response in the whole slice recovered in each fraction. The data was from different experiments as reported in Table 6. Data were collated from 3 experiments, each experiment involving triplicate samples for each condition. Veratrine was added at 25 μM , Morphine at 15 μM , GTP at 10 μM and Naloxone at 1 μM .

* $P < 0.005$ compared to veratrine alone employing Student's two-tailed t -test.

inhibitory actions of morphine were reversed by 1 μM naloxone (Table 2).

Depolarizing agents and 3',5' cyclic AMP levels in synaptosomes

An increase in cyclic AMP levels was observed to occur during incubation of synaptosomes with depolarizing agents. Thus, 15 μM veratrine caused an increase of $65\% \pm 6$ and 56 mM K^+ caused a $53\% \pm 5$ increase (Table 3). Morphine, had no significant effect on the increase (80%) in cyclic AMP levels induced by veratrine 75 μM , although the enhancement due to 25 μM veratrine was reduced by morphine (100 μM). Thus, cyclic AMP levels were increased by only 15% of control values by veratrine at 15 μM when morphine was present at 100 μM (i.e. 76% inhibition of response), and by only 50% when 15 μM morphine was used (Table 3). Its potency against K^+ -induced cyclic AMP increases was similar (Table 3). All of these inhibitory actions of morphine were prevented by naloxone (1 μM ; Table 3).

Action of morphine on the incorporation of $^{32}\text{P}_i$ inorganic phosphate into protein and lipid fractions

Incorporation into synaptosomes. Both K^+ (56 mM) and veratrine (25 μM and 75 μM) enhanced the incorporation of $^{32}\text{P}_i$ into synaptosomal proteins two- or threefold (Table 4).

When added alone, morphine (100 μM) was without significant influence on the rate of $^{32}\text{P}_i$ incorporation; however, it caused a substantial inhibition of the depolarization-evoked responses in this incorporation. Thus at 15 μM morphine the enhancement due to 75 μM veratrine was reduced by 17% whilst that caused by 25 μM veratrine was inhibited to a much greater extent (44%; $P < 0.001$; Table 4). Morphine was more potent in its action against K^+ -induced increases in $^{32}\text{P}_i$ incorporation (e.g. 1 μM morphine reduced this increase by 39%).

Incorporation into brain slices. As for synaptosomes, depolarization by veratrine or K^+ increased the incorporation of $^{32}\text{P}_i$ into brain slices; 75 μM veratrine causing a 110% increase in incorporation over and above that of basal levels; this increase

could be blocked by TTX. Morphine at (1 μM or 100 μM) caused no change in the phosphorylation stimulated by 75 μM veratrine. However, with veratrine at 25 μM , morphine (100 μM) caused a total inhibition of stimulated phosphorylation. Naloxone, 1 μM , was able to abolish this inhibitory effect of morphine (Table 4).

The action of D-al² met⁵ enkephalinamide. D-Ala²-met⁵ enkephalinamide at concentrations up to 100 μM did not show any action on veratrine-stimulated Ca^{2+} uptake by synaptosomes. In brain slices the enkephalin analogue at 100 μM decreased Ca^{2+} uptake by 100% (Table 6).

DISCUSSION

Inhibition of Ca^{2+} fluxes

Morphine was found to have no effect on either basal Ca^{2+} uptake or basal amino acid release. The drug selectively inhibited only the depolarization-induced influx of Ca^{2+} and it acted in a dose-dependent manner (Fig. 1). Some investigators have found no such effects with morphine [24] whilst others have shown inhibition by morphine of K^+ -evoked uptake *in vitro* when presented at concentrations of 0.1 μM [25] or 90 μM [26]. The negative results of some groups may be attributed to the high level of stimulation they employ. In this study and previous ones [13] morphine had no effect on inhibiting the Ca^{2+} influx induced by 75 μM veratrine but could block the influx due to 25 μM veratrine. This implies that the Ca^{2+} influx induced by the higher veratrine concentration was too great to be blocked by morphine even at 100 μM .

All the effects of morphine on Ca^{2+} influx could be blocked by naloxone showing that the actions of the drug are receptor mediated. Dextrophan which is the (+) isomer of levorphanol had no effect on Ca^{2+} influx (results not shown) indicating that the stereo specificity of the binding site is an important feature in the morphine interaction.

Inhibition of neuroactive amino acid release

Morphine was found to have an inhibitory effect

Table 6. Effect of D-alal² met⁵ enkephalinamide (DAME) on stimulated Ca²⁺ influx into cerebral tissue

Condition	Brain slices N = 9	Synaptosomes N = 12
Control	16.92 ± 1.07	3.80 ± 0.13
DAME (100 µM)	17.06 ± 6.53	3.71 ± 0.13
K ⁺	—	6.44 ± 0.15
K ⁺ plus DAME (100 µM)	—	6.39 ± 0.14
Veratrine	22.17 ± 11.41	8.21 ± 0.20
Veratrine plus DAME (100 µM)	15.97* ± 1.64 (100%)	—
DAME (15 µM)	21.98 ± 11.32 (0%)	8.36 ± 0.13 (0%)

Tabulated results are in nmoles Ca/mg protein ± S.E.M. Values in parentheses represent the % inhibition of stimulation.

Veratrine was used at 25 µM and K⁺ at 56 mM. Data were collated from 3 experiments for brain slices and 4 experiments for synaptosomes, each experiment involving triplicate samples for each condition.

* P < 0.005 in comparison to the stimulated level (either veratrine or K⁺) employing Student's *t*-test.

on neuroactive amino acid release as well as on Ca²⁺ influx; actions of morphine on acetylcholine release and biogenic amines have been previously reported [1–5].

The mechanism of the inhibition by morphine of neuroactive amino acid release induced by high K⁺ could involve regulation of presynaptic Ca²⁺ transport since both are inhibited in parallel. However, in the case of veratrine, neurotransmitter release is not wholly dependent on external Ca²⁺ which suggests that morphine must also have a more direct effect on the neurotransmitter release mechanism. This may occur at an internal site by preventing the action of Ca²⁺ released from internal stores [27]. There is evidence that morphine enters neural cells [42].

Action on adenylate cyclase

Other studies have indicated that noradrenaline may inhibit amino acid neurotransmitter release through interactions at a surface receptor which may be linked to adenylate cyclase [28] and subsequently affects intracellular phosphorylation.

Since morphine also inhibited the parallel rise in cyclic AMP levels, and GTP enhanced its actions, the drug may exert some of its effects via adenylate cyclase, including a longer term effect on neurotransmitter release. The relative affinities of narcotics for their receptors and their pharmacological potencies have been reported in accordance with their effectiveness as inhibitors of adenylate cyclase [29]. It is known that there is a selective nucleotide and ion regulation of agonist interactions with opiate receptors, and Blume *et al.* [30] reported that GTP was required for receptor-directed inhibition of adenylate cyclase in mouse neuroblastoma-glioma hybrid cells. The ability of GTP and Na⁺ to affect adenylate cyclase activity is mediated by the guanine nucleotide binding protein [31].

Acute administration of morphine both *in vivo* and *in vitro* apart from affecting cAMP levels also influences the level of soluble calmodulin. Delorenzo *et al.* [34, 35] have implicated calmodulin in mediating many events at the neuronal membrane. Acute

morphine increases the concentration of soluble calmodulin and evidence indicates that this is due to its removal from synaptic terminal membranes by the opiate. The membrane bound enzyme, adenylate cyclase, is found as two isoenzymes in the cerebral cortex, one of which is Ca²⁺ and calmodulin sensitive [36]. Removal of calmodulin from the membrane may well decrease cyclic AMP levels by acting on this isoenzyme of adenylate cyclase. Stimulation of cyclic AMP formation by prostaglandin E, in rat brain has been shown to be inhibited by morphine [37], and the observed inhibition correlates well with both antinociceptive potency and opiate receptor binding affinity.

Inhibition of ³²P_i incorporation

Inhibition of depolarization-induced phosphorylation was observed to occur in the presence of morphine in both brain slices and synaptosomes. Since the cyclic AMP dependent protein kinase system [38, 39] is involved in the phosphorylation of synaptic proteins, a decreased level of cyclic AMP could be expected to correlate with a reduction in phosphorylation of synaptic terminal proteins. One such protein is Synapsin I [40] a major component of the synaptic vesicle cage. The protein is phosphorylated by both cyclic AMP dependent protein kinases and calcium/calmodulin-dependent protein kinases [41]. The phosphorylation of Synapsin I by the Ca²⁺ calmodulin dependent protein kinase leads to a release of the vesicle from its cage and hence making it available for exocytosis. The effect of phosphorylating Synapsin I by cyclic AMP kinases is unknown. Thus if morphine acts to decrease the activities of adenylate cyclase and calmodulin combining with Ca²⁺ to phosphorylate protein then this may be a major mechanism by which synaptic transmission is reduced by morphine.

Relative potencies of the effects of morphine

The potency of morphine was similar whether inhibiting responses induced by veratrine (25 µM) or by K⁺ (56 mM). For instance, all the responses induced by both depolarizing agents were inhibited

between 24 and 25% by morphine at 1 μM in synaptosomes and by 5 μM in brain slices. The enhancement of both Ca^{2+} uptake and glutamate release by these two depolarizing agents could be inhibited by 87–94% with 100 μM morphine. However, augmentation of cyclic AMP levels and of $^{32}\text{P}_i$ incorporation in proteins and lipids showed lesser degrees of inhibition by morphine at 100 μM . Both Ca^{2+} and phosphorylation ($^{32}\text{P}_i$ incorporation) were decreased by morphine to a similar degree and both showed increased inhibition by GTP. No other nucleotide caused this enhancement indicating a possible involvement of the G-regulatory protein of adenylate cyclase.

In summary, the data presented here show that morphine influences a range of cellular processes via naloxone-sensitive receptors including Ca^{2+} entry through voltage-sensitive channels, phosphorylation processes and neurotransmitter release. Its inhibitory action on neurotransmitter release could be due to inhibition of Ca^{2+} entry during depolarization, but may also be due to interaction with surface receptors which inhibit Ca^{2+} entry during depolarization. It may also produce its inhibitory action by influencing phosphorylation of sites concerned with neurotransmitter release, either directly at an internal site or via interaction with adenylate cyclase at surface receptors, thereby inhibiting cAMP-dependent protein phosphokinases.

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Inhibitory actions of opioid compounds on calcium fluxes and neurotransmitter release from mammalian cerebral cortical slices

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- 1 The effects of opioid agonists on veratrine-stimulated Ca^{2+} influx and amino acid neurotransmitter release in rat cerebrocortical brain slices were studied. Inhibitory effects were seen on both of these parameters with all of the opioid agonists used.
- 2 None of the drugs used affected basal $^{45}\text{Ca}^{2+}$ uptake, basal K^+ content or basal amino acid release from the slices.
- 3 At high concentrations (100 μM) fentanyl, tifluadom, U50,488H, butorphanol and bremazocine greatly inhibited the depolarization of the slices by veratrine as determined by the reduced release of K^+ .
- 4 The opioid receptor subtypes at which the drugs were acting were characterized by the antagonistic effects of naloxone and WIN44441-3.
- 5 The opioid-induced inhibition of stimulated Ca^{2+} uptake and amino acid release were not antagonized by WIN44441-2, the inactive enantiomer of WIN44441-3.
- 6 It is concluded that opioid agonists acting through μ - and κ -receptors and probably through δ - and σ -receptors, have an inhibitory effect on Ca^{2+} uptake into cerebrocortical brain slices and the subsequent release of aspartate, glutamate and γ -aminobutyric acid (GABA).

Introduction

Previously, in our laboratory, morphine has been shown to inhibit depolarization-induced calcium influx and neurotransmitter release from rat cortical synaptosomes (Dhaliwal & Bradford, 1982). This effect was stereospecific and reversed by relatively low concentrations of naloxone, therefore morphine is likely to be acting through the μ -type opioid receptor. The inhibitory action of morphine on calcium influx and neurotransmitter release was postulated to be directed at the calcium channel.

As an extension of this work, a number of opioid agonists and antagonists have been studied to determine whether occupation of any other opioid receptor subtypes might effect calcium influx and amino acid neurotransmitter release from slices of cerebral cortex, either at rest or during periods of stimulation with veratrine.

Methods

Preparation and incubation of brain slices

Brain slices (approx. $1.0 \times 0.5 \text{ cm}$ and 0.3 mm thick) were prepared from rat cerebral cortex using a McIlwain recessed guide and a razor blade (McIlwain, 1961). These were incubated at 37°C for 30 min in 5 ml glass beakers each carrying a gassing line which delivered pure O_2 . The relevant opioid compound was added to the incubation medium (composition, mM: NaCl 138, KCl 5, MgSO_4 1.0, CaCl_2 1.2, NaH_2PO_4 1.2, Tris 20, glucose 10, pH 7.4), dissolved in 200 μl of this medium. When the effect of an opioid antagonist was studied, it was included in the incubation medium at the required concentration before the addition of the slice.

Uptake of $^{45}\text{Ca}^{2+}$

Five minutes after the opioid compound was added,

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$^{45}\text{CaCl}_2$ (Amersham International) was added (200 μl) to each beaker to give a final specific activity of 0.14 Ci mol^{-1} . Veratrine HCl (200 μl) was added 30 s later to give a final concentration of $25 \mu\text{M}$. The final volume of the incubation medium was 4.5 ml. After a total period of incubation with $^{45}\text{Ca}^{2+}$ of 12 min, the slices were removed and each was immersed in 2 ml EGTA stopping solution (composition, mM: NaCl 120, KCl 5, MgCl_2 1.2, NaH_2PO_4 1.2, Tris 20, EGTA 30, pH 7.4) at room temperature. The slices were each transferred in quick succession through $3 \times 2 \text{ ml K}^+$ free washing medium (composition, mM: NaCl 137, MgCl_2 1.2, NaH_2PO_4 1.2, Tris 20, glucose 10, pH 7.4). They were then made soluble in 1 ml of 1.0 M NaOH for 30 min at 50°C .

Measurement of $^{45}\text{Ca}^{2+}$

An aliquot of dissolved tissue was added to a liquid scintillation mixture (Packard 199) and counted in a Packard 300CD Tricarb liquid scintillation counter to determine the tissue content of radioactive calcium.

Measurement of K^+

K^+ was measured in the dissolved slices by atomic absorption spectrophotometry, using a SB-900 Atomic Absorption Spectrophotometer (G.B.C. Scientific Equipment, Victoria, Australia). Standard solutions of K^+ in 1 M NaOH were used to quantitate the K^+ levels.

Measurement of amino acids

At the end of the incubation, aliquots of the incubation medium were acidified with 0.025 M HCl containing the appropriate concentration of norleucine as internal standard. Amino acids were measured with a chromaspek J180 with fluorimeter J143 (Rank-Hilger, Margate, Kent). An acid buffer (0.05 M citric acid, 0.15 M LiCl) was titrated against a basic buffer (0.05 M boric acid, 0.2 M LiOH) using electronic programmed gradient elution from pH 2 to pH 12. The run time was 2 h. Orthophthaldehyde with mercaptoethanol (800 mg and 2 ml respectively in 1 litre distilled water) was used as the fluorogenic agent for analysis of the amino acids. This was carried out in 0.3 M NaHCO_3 buffer, pH 10. The column was 15 cm $\times$ 3 mm packed with $4 \mu\text{m}$ of 8% cross-linked polystyrene sulphonic acid resin.

Measurement of proteins

Protein levels were measured using the method of Lowry *et al.* (1951). Bovine serum albumin was used for the standards.

Opioid drugs

We should like to thank the following companies for their kind gifts: etorphine HCl (C-Vet Ltd); butorphanol tartrate (Bristol-Myers Co.); fentanyl citrate, sufentanil citrate (Janssen Pharmaceutica); U-50,488H (Upjohn Co.); bremazocine HCl, tifluadom HCl (Sandoz Ltd); ethylketocyclazocine methane sulphonate, pentazocine HCl, WIN44441-3, WIN44441-2 (Sterling-Winthrop); SKF 10,047 (NIDA); nalbuphine HCl, naloxone HCl (Du Pont).

Results

The effects of opioid agonists on Ca^{2+} uptake and depolarization

All of the drugs employed inhibited veratrine-stimulated $^{45}\text{Ca}^{2+}$ uptake, though with varying potencies (Table 1). (Neither basal $^{45}\text{Ca}^{2+}$ uptake nor basal K^+ content of brain slices were altered by any of these drugs).

At higher concentrations (e.g. $100 \mu\text{M}$) fentanyl, tifluadom, U50,488H (trans-($\pm$)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl] benzeneacetamide methane sulphonate), butorphanol and bremazocine greatly reduced the veratrine-induced release of K^+ from the slices as well as inhibiting $^{45}\text{Ca}^{2+}$ uptake, and could therefore be exerting at least part of their actions at these higher concentrations by reducing the depolarizing action of veratrine (Table 1).

The opioid agonists are classified in Table 1 according to the receptor for which they appear to have greatest selectivity in binding studies (James & Goldstein, 1984). However, considerable cross-reactivity between the receptor types occurs. Therefore etorphine, for example, may cross-react with δ - and κ -receptors to some extent (Rosenbaum *et al.*, 1984). Opioid antagonists were employed to specify further the opioid receptor types which are involved in mediating these effects on calcium influx.

Naloxone and WIN44441-3 antagonism of the effects on $^{45}\text{Ca}^{2+}$

Naloxone is primarily a μ -antagonist, but it is also capable of antagonizing κ - and δ -agonist activity, the order of potency being $\mu > \kappa > \delta$ (Takemori & Portuguese, 1984). WIN44441-3 [(2d, 6d, 115*)-($-$)-1 cyclopentyl-5-(1, 2, 3, 4, 5, 6-hexahydro-8-hydroxy-3, 6, 11-trimethyl-2, 6-methano-3-benzazocin-11-yl)-3 pentanone methane sulphonate] is a pure opioid antagonist which is thought to act with a greater selectivity for the κ -receptor (Romer *et al.*, 1980) but also acts on μ - and δ -receptors (Ward *et al.*, 1983).

All the μ -agonists in this study were effectively

Table 1 The effects of opioid drugs on calcium uptake into cerebrocortical slices and depolarization stimulated by veratrine

Opioid agonists (μM)		Inhibition of Ca^{2+} uptake* (%)	Inhibition of K^+ loss† (%)	% opioid antagonism‡					
				Naloxone (μM)			WIN44441-3 (μM)		
				1	10	100	1	10	100
μ-agonists									
Morphine	100	122	0	94					49
	10	85	0	114					22
	1	14	0	100					0
Etorphine	100	114	0	77					38
	41	93	17	98					47
	10	75	0	106					30
	1	32							
	0.1	0.2							
Fentanyl	100	102	113	1					10
	10	115	50	0				1	
	1	57	0	12	41			0	0
Sufentanil	100	111	0	28				0	
	1	96	0	93	102			3	25
κ-agonists									
TiFluadom	100	96	77		0			0	
	10	94	16		0			18	
	1	78	10		0	0		77	
	0.1	36	2			0		82	
U50,488H	100	87	89		22		76		
	10	68	8		3		91		
δ-agonist									
[D-Ala ² , Met ⁵] enkephalinamide	100	80	0	0				0	
	20	0	6	0				0	
σ-agonist									
SKF 10,047	100	106	0	0	7			2	
	10	52	32	0					
κ-agonist/μ-antagonists									
Ethylketo-cyclazocine	100	100	0		8				3
	41	103							
	12	93							
	10	78	0		25		34	105	
	3	56					100		
	1	31	0		144		123	97	
	0.3	4							
Pentazocine	100	61	0			8		40	
	10	56	0			11		78	
	1	30	0			57		100	
Nalbuphine	100	95	3			0		0	
	10	76	0			0		27	
Bremazocine	100	100	64					87	
	10	84	0		20		50	91	
	1	69	0			96	83		
Butorphanol	100	103	55						
	41	116	13		65		97		
	12	82	0		53		129		
	4	25							
	1	10						47	

Results are mean of 6 incubations from 2 experiments; s.d. values are all within 10% for Ca^{2+} content.

*Basal Ca^{2+} uptake $1424 \pm 106 \text{ n mol } 100 \text{ mg}^{-1} \text{ protein } 12 \text{ min}^{-1}$; $25 \mu\text{M}$ veratrine stimulated Ca^{2+} uptake $2278 \pm 202 \text{ n mol } 100 \text{ mg}^{-1} \text{ protein } 12 \text{ min}^{-1}$.

†Basal K^+ content $89.7 \pm 0.3 \mu\text{ equivalent } 100 \text{ mg}^{-1} \text{ protein}$; $25 \mu\text{M}$ veratrine stimulated K^+ content $40.1 \pm 0.7 \mu\text{ equivalent } 100 \text{ mg}^{-1} \text{ protein}$.

‡% opioid antagonism of effects on stimulated Ca^{2+} uptake.

antagonized by 1 μM naloxone except fentanyl (Table 1). However fentanyl, like the other μ -agonists was not effectively antagonized by 100 μM WIN44441-3. These results suggest that the actions of morphine, etorphine and sufentanil are primarily at the μ -receptor rather than the κ -receptor. The actions of fentanyl may also be through the μ -receptor, but are unlikely to be

through the κ -receptor.

Tifluadom and U50,488H were both preferentially antagonized by WIN44441-3 rather than naloxone, indicating that they act primarily at the κ -receptor in our assay system. Both these compounds have been found to be very selective κ -agonists in other systems (Vonvoigtlander *et al.*, 1982; Romer *et al.*, 1982).

Table 2 The effects of opioid drugs on glutamate release from cerebrocortical slices stimulated by veratrine

Opioid agonists (μM)		% inhibition of stimulated glutamate release*	% opioid antagonism†					
			Naloxone (μM)			WIN44441-3 (μM)		
			1	10	100	1	10	100
<i>μ-agonists</i>								
Morphine	100	150	117					22
	10	116	118					89
	1	36	100					23
Etorphine	100	82	72					0
	10	62	96					90
	1	28	152					83
Fentanyl	100	ND	ND					ND
	10	ND	ND				ND	
	1	108	0	66			0	1
Sufentanil	100	ND	ND					
	1	157	138	84			0	39
<i>κ-agonists</i>								
Tifluadom	100	98		0			0	
	10	86		0			22	
	1	54		0	2		96	
	0.1	41			0		117	
U50,488H	100	98		0		77		
	10	70		0		131		
<i>δ-agonist</i>								
[D-Ala ² ,Met ⁵] enkephalinamide	100	45	6				0	
	20	ND	ND				ND	
<i>σ-agonist</i>								
SKF 10,047	100	108	ND	ND			ND	
	10	102	19					
<i>κ-agonist/μ-antagonists</i>								
Ethylketo-cyclazocine	100	85		4				25
	10	71		85			99	
	1	43		99		100	100	
Pentazocine	100	98			0		32	
	10	66			26		67	
	1	30			53		100	
Nalbuphine	100	100			8		42	
	10	92			36		87	
	1	101					93	
Bremazocine	100	48		66		97	113	
	10	30			73	116		
	1	103						
Butorphanol	100	109		48		100		
	41	100		67		122		
	12	86				125		
	4							

Results are mean of 6 incubations from 2 experiments; s.d. values are all within 10% of the mean for glutamate release.

*Basal glutamate release 1806 ± 184 n mol 100 mg⁻¹ protein; 25 μM veratrine-stimulated glutamate release 2972 ± 165 n mol 100 mg⁻¹ protein.

†% opioid antagonism of effects on stimulated glutamate release.

ND not determined.

Table 3 Comparison of the effect of WIN44441 isomers on the actions of 4 opioid agonists

Opioid agonist	% inhibition of veratrine stimulated Ca^{2+} uptake	% opioid antagonism		% inhibition of veratrine-stimulated glutamate release	% opioid antagonism	
		WIN44441-2 (+)	WIN44441-3 (-)		WIN44441-2 (+)	WIN44441-3 (-)
Morphine	112	0	49	150	0	22
Etorphine	114	0	38	82	0	0
Ethylketocyclazocine	100	4	31	85	0	25
Pentazocine	61	2*	40*	98	0*	32*

*To obtain these values $10\text{ }\mu\text{M}$ WIN44441-2 and WIN44441-3 was used; $100\text{ }\mu\text{M}$ of these isomers was used for the other values. The stimulating agent was $25\text{ }\mu\text{M}$ veratrine.

[D-Ala², Met³] enkephalinamide and SKF 10,047 were not significantly antagonized by naloxone ($1\text{ }\mu\text{M}$) or WIN44441-3 ($10\text{ }\mu\text{M}$). This suggests that these agonists may be acting at opioid receptor types other than μ and κ . Since δ - and σ -selective antagonists (Cotton *et al.*, 1984; Tam, 1985) were not employed, the actions of these drugs through δ - and σ -receptors respectively could not be confirmed.

Sufentanil is thought to be very selective for μ -receptors over κ - and δ -receptors (Goldstein & James, 1984), but $100\text{ }\mu\text{M}$ WIN44441-3 partially antagonized the inhibition of Ca^{2+} influx by $1\text{ }\mu\text{M}$ sufentanil. This suggests that WIN44441-3 may not be absolutely specific for the κ -receptor, but certainly has a greater antagonistic activity at κ - over μ -receptors in these experimental conditions.

The effects of opioid agonists and antagonists on amino acid release

As previously found, veratrine caused differential tetrodotoxin-sensitive release of glutamate, aspartate and GABA from nerve terminals (Wedge *et al.*, 1977). There was no effect on amino acids such as threonine, which does not have the properties of a neurotransmitter. Basal neuroactive amino acid release was unaffected by the drugs used. However, opioid agonists did inhibit release stimulated by veratrine ($25\text{ }\mu\text{M}$) to various degrees. These actions are expressed in terms of their influence on glutamate release (Table 2), though very similar suppression of veratrine evoked-aspartate and γ -aminobutyric acid (GABA) release also occurred.

Actions of isomers of WIN44441

Table 3 shows the lack of effect that WIN44441-2 (the (+) optical isomer of WIN44441-3) had on the inhibition of stimulated Ca^{2+} uptake and neurotransmitter release by 4 opioid agonists. WIN44441-2 had no action on veratrine-stimulated depolarization or on the basal levels of Ca^{2+} uptake, K^+ content or amino acid release.

Discussion

Opioid receptor subtypes have been identified by autoradiography in rat brain cortex, using selective ligands (Chang *et al.*, 1979; Quirion *et al.*, 1981; Goodman & Snyder, 1982).

In our study all the different receptor agonists examined appeared to affect stimulated Ca^{2+} influx and neuroactive amino acid release from the slices. There were certain drugs which were more potent in affecting this $^{45}\text{Ca}^{2+}$ influx than others, but it was not possible to determine whether one receptor type was more effective than the others in mediating this effect because the variation in selectivity and potency of the agonists used in each group was greater than the variation between the receptor categories. However, amongst the μ -selective agonists, sufentanil was the most effective antagonist of Ca^{2+} entry and amino acid release from the cortical slices. At $1\text{ }\mu\text{M}$ the relative order of potency of the μ -selective agonists based on these parameters was sufentanil > fentanyl > etorphine > morphine (Table 1). The κ -selective agonists, tifluadom and U50,488H were approximately equipotent, whereas amongst the κ -selective agonists/ μ -antagonists the relative order of potency at $10\text{ }\mu\text{M}$ was bremazocine > butorphenol > ethylketocyclazocine > nalbuphine > pentazocine.

These results are of particular interest when compared to those of Mulder *et al.* (1984) who found that μ -, κ - and σ -opioid receptor agonists differentially inhibited dopamine and acetylcholine release from rat striatal slices. Jones & Marchbanks (1982) observed that [D-Ala², Met³]enkephalin inhibited stimulated noradrenaline release, but had no effect on acetylcholine release from cerebrocortical brain slices. We have found that none of the opioid receptor agonists used had differential effects on aspartate, glutamate or GABA release from cerebrocortical brain slices.

WIN44441-3 has proved to be stereospecific in its action in antagonizing opioid inhibition of veratrine-stimulated Ca^{2+} uptake and amino acid release in our experiments.

At high concentrations of fentanyl, tifluadom,

U50,488H, butorphanol and bremazocine there was a reduction in veratrine-induced release of K^+ from the slices. The opioid compounds may cause hyperpolarization of the membrane, so that it is less readily depolarized by veratrine (North & Williams, 1983). Any hyperpolarizing effect of the opioid compounds on the cerebrocortical slices would not necessarily be seen as a change of K^+ content, as the alteration of K^+ conductance may be too small to be detected, or may occur in a localized region which could be obscured by measurement of total K^+ content in the slices.

Many of the physiological effects of opioid compounds are likely to be due to an inhibition of neurotransmitter release. A reduction in transmitter release may be achieved by reducing the availability of free Ca^{2+} ions in the nerve terminal. Acute morphine treatment produces a selective decrease in both the Ca^{2+} content and the Ca^{2+} binding capacity of the synaptosomal fraction of brain tissue (Ross, 1977; Yamamoto *et al.*, 1978). Dunlap & Fischbach (1978) produced electrophysiological evidence that enkephalin acts directly on the voltage-dependent Ca^{2+} channels of dorsal root ganglion neurones grown in cell cultures. In our experiments opioid compounds appear to be acting like Ca^{2+} channel antagonists; inhibiting Ca^{2+} influx into cerebrocortical slices by a receptor-mediated mechanism. The effect of 100 μM

morphine and 1 μM sufentanil on glutamate release appears to be much greater than 100%, i.e. the stimulated release of glutamate is completely blocked, and there is additionally an inhibition of spontaneous release. When added alone, 100 μM morphine and sufentanil had no effect on basal amino acid release. However, 100 μM morphine produced only 93% inhibition of stimulated aspartate release, and 105% inhibition of stimulated GABA release, therefore this result seems to be anomalous. Similarly, 1 μM sufentanil produced 83% inhibition of stimulated aspartate release and 95% inhibition of stimulated GABA release. For other opioid compounds, where inhibition appears to be greater than 100%, it can be taken as 100% inhibition within experimental variation.

In conclusion, it appears from this set of experiments that the μ - and κ -opioid receptors, and possibly the δ - and σ -opioid receptors, have a role to play in mediating an effect on calcium influx into cerebrocortical slices in a fashion which allows regulation of amino acid neurotransmitter release.

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