REGULATION OF GLUTAMATE EXOCYTOSIS FROM ISOLATED NERVE TERMINALS (SYNAPTOSOMES): ROLE OF PRESYNAPTIC RECEPTORS

BY

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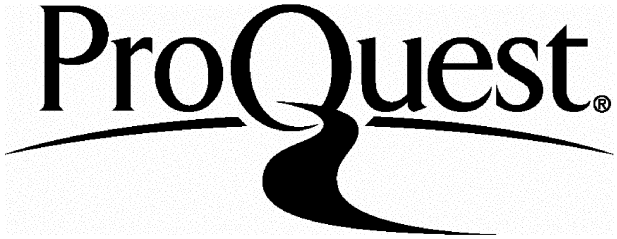

Abstract

Isolated nerve terminals (synaptosomes) can be used as a model to study glutamate exocytosis in the mammalian CNS. ω-CTx GVIA reduced Ca^{2+} entry into cerebrocortical synaptosomes and inhibited glutamate exocytosis. Furthermore, ω-CTx MVIIC completely inhibited glutamate exocytosis and Ca^{2+} influx into nerve terminals. From these toxin studies, it is proposed that glutamate transmission in the cerebral cortex may be controlled by class B N-type VSCC and class A P/Q type VSCC. In contrast, dihydropyridines did not alter glutamate release evoked by KCl, supporting the hypothesis that class C/D L-type VSCC are not coupled to release of fast acting transmitters. The role that presynaptic receptors play in the modulation of glutamate release was examined. The non-NMDA receptor agonists AMPA, kainate and domoate potentiated 4AP-evoked glutamate exocytosis and pharmacological analysis indicated the presence of a high-affinity kainate receptor (autoreceptor) on a population of cerebrocortical nerve terminals that is positively linked to glutamate exocytosis. (-)Baclofen, a $GABA_B$ receptor agonist, inhibited glutamate release from synaptosomes with a pharmacology that correlates closely with previous data indicating the presence of inhibitory $GABA_B$ receptors on glutamatergic nerve terminals (heteroreceptors). A direct reduction of Ca^{2+} entry into synaptosomes was observed with (-)baclofen and inhibition of glutamate release was insensitive to pertussis toxin (PTX), suggesting that $GABA_B$ receptors are negatively linked to VSCC coupled to glutamate exocytosis via a PTX-insensitive G-protein. Finally, the metabotropic glutamate receptor (mGluR) agonists 1S,3R-ACPD and L-AP4 strongly inhibited glutamate exocytosis from adult rat cerebrocortical synaptosomes, indicating that glutamate transmission in the adult mammalian CNS may be subject to modulation by presynaptic inhibitory group II and/or group III mGluRs (autoreceptors).

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List of Abbreviations

AA	arachidonic acid
Ach	acetylcholine
1S,3R-ACPD	(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid
AMPA	α-amino-5-methyl-4 isoxazole-propionic acid
4AP	4-aminopyridine
AP5	D-2-amino-5-phosphono-valerate
ATP	adenosine triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]_c$	cytosolic free Ca^{2+} concentration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	adenosine 3',5'-cyclic phosphate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
Con A	concanavalin A
ω-CTx GVIA	ω-Conotoxin GVIA
ω-CTx MVIIC	ω-Conotoxin MVIIC
DAG	diacylglycerol
DHPG	(S)-3,5-dihydroxy-phenylglycine
DHPs	1,4-dihydropyridines
$DiSC_2(5)$	3,3'-diethyl-thiadicarbo-cyanine iodide
DRG	dorsal root ganglion neurons
EGTA	ethylene glycol-bis (β-amino-ethyl ether) N,N,N',tetra acetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
Fura-2 AM	fura-2 acetoxymethyl ester
GABA	γ-aminobutyric acid
GAMS	α-D-glutamylamino-methylsulphonic acid
GDH	glutamate dehydrogenase
HBM	hepes buffered medium
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
HVA	high-voltage activated Ca^{2+} channels
IP_3	inositol 1,4,5 trisphosphate
KA	kainic acid
K_d	dissociation constant
L-AP4	L(+)-2-amino-4-phosphonobutyric acid
L-CCG-I	(2S,1'S,2'S)-2-(Carboxy-cyclopropyl)glycine
LDCVs	large dense-core synaptic vesicles
LTD	long-term depression
LTP	long-term potentiation
LVA	low voltage-activated Ca^{2+} channels
MARCKS	myristoylated alanine-rich C-kinase substrate
MCE	mercaptoethanol
mGluR	metabotropic glutamate receptor
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NBQX	6-nitro-7-sulphamoylbenzo-[f]quinoxaline-2,3-dione
NMDA	*N*-methyl-*d*-aspartate
NPY	neuropeptide Y
NSF	**N**-ethylmaleimide-**s**ensitive **f**usion protein
nval	norvaline
OPA	*o*-Phthaldialdehyde
PCP	phencyclidine
PCR	Polymerase Chain Reaction
PDBu	phorbol 12,13-dibutyrate

PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+}/phospholipid-dependent protein kinase
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PPF	paired pulse facilitation
PSP	postsynaptic potentials
PTKs	protein-tyrosine kinases
PTPs	protein tyrosine-phosphatases
PTX	pertussis toxin
RF	response factor
SBFI	Na^+-binding benzofuran isophthalate
SDS	sodium dodecyl sulphate
SNAP-25	synaptosome-associated protein 25
SNAPs	**s**oluble **N**SF **a**ttachment **p**roteins
SNARE	**SNA**P **re**ceptor
SSA	sulphosalicylic acid
SSVs	small clear synaptic vesicles
TPA	12-*O*-tetradecanoylphorbol13-acetate
t-SNARE	**t**arget-associated SNARE
TTX	tetrodotoxin
VAMP	vesicle-associated membrane protein
V_m	resting membrane potential
VSCC	voltage-sensitive Ca^{2+} channel(s)
v-SNARE	**v**esicle-associated SNARE

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CHAPTER ONE

1 Introduction

The brain can be divided into distinct levels based essentially on structure; systems, maps, layers, networks, neurons, synapses and molecules. With such a complex architecture, it was proposed by the Nobel prize winning neurobiologist David Hubel in the late 1970's, that the human brain may never be completely understood. Twenty years later, it is currently the 'decade of the brain' and neuroscientists are studying the brain at every level, from a whole systems approach down to the synapses and molecules that make up that system. As a result, a greater understanding of the brain is beginning to evolve and we may yet see a more positive conclusion than that invoked by Hubel.

1.1 Neurons, synapses and molecules

Brief History

As early as three centuries ago, Rene Descartes described the mind as an extracorporeal entity which expressed itself through the pineal gland. This is now known to be incorrect but he laid the foundations for subsequent brain researchers to build upon. A first appreciation of the cells of the nervous system, i.e. neurons, came from Claude Bernard at around 1850. Although Bernard realised that contacts formed by nerve cells on their targets were specialised, it was not until 1897 that these *synapses* were so named by Charles Sherrington.

In the late nineteenth/early twentieth century, Santiago Ramon y Cajal provided definitive morphological evidence for the existence of neurons and synapses. Cajal demonstrated that all synapses have two conserved elements; a presynaptic terminal and a postsynaptic target site. He also proposed the presence of a third element, the synaptic cleft, which is the space between the presynaptic and postsynaptic elements of the synapse. His principle of dynamic polarisation predicts that chemical synapses convey information in a unidirectional manner; information flows from the presynaptic to the postsynaptic cell (anterograde signalling). This theory largely holds but recent research has revealed that specific signals can be conveyed retrogradely from the postsynaptic cell to the presynaptic terminal (retrograde signalling).

1.2 Chemical synaptic neurotransmission

1.2.1 Introductory principles

The human brain has about one hundred billion neurons of which there are several hundred types. However, even with this vast diversity of neuronal type, neurons do have certain common features. There is a cell body/soma which contains the nucleus and is a site for protein synthesis. Cell bodies range from 5 - 60 μm in diameter. The surface area of the soma is greatly increased by the presence of dendrites which extend out from the cell body. Some neurons have dendrites with projecting dendritic spines, which further increases surface area. An axon extends from the soma through the axon hillock, branching and making up to one thousand synaptic contacts with other neurons, via presynaptic terminals or en passant varicosities. Nerve terminals, most of which are about 1 μm in diameter, and en passant varicosities release neurotransmitters in response to Ca^{2+} entry, which is triggered by the arrival of an action potential. Neurotransmitter is released into a synaptic cleft, which is the junction between the presynaptic nerve terminal and the apposed postsynaptic cell dendrite or soma. Once in the synaptic cleft neurotransmitter diffuses and binds to postsynaptic receptors, which evoke an all or nothing response. However, receptors are also located presynaptically on nerve terminals, activation of which can modulate the probability of neurotransmitter release. Released neurotransmitter is cleared from the synaptic cleft by enzymatic degradation or re-uptake into the nerve terminal or surrounding glial cells.

1.2.2 Ionic hypothesis of chemical synaptic transmission

Experiments performed by the electrophysiologists, Hodgkin and Huxley culminated in their description of how neurons transmit signals (Hodgkin and Huxley, 1952). Resting neurons have a membrane potential of ~ -70 mV. This membrane potential results from an unequal distribution of Na^+, K^+, and Cl^- across the neuronal membrane. Maintenance of this uneven distribution occurs through an ATP-dependent Na^+-K^+ pump that transports Na^+ out of and K^+ into

the neuron, keeping [K^+] inside the neuron 20 times higher and [Na^+] inside the neuron 10 times lower than [K^+] and [Na^+] outside the neuron. As a result, K^+ tends to diffuse out of the neuron through leakage K^+ channels, generating a negative potential relative to the outside. When no more K^+ can diffuse outwards, the resting membrane potential is reached (about -70 mV).

The Nernst equation can be used to calculate the theoretical resting membrane potential (V_m) of a neuron assuming that K^+ is the only ion that can permeate the neuronal membrane;

$$\text{Nernst equation: } V_m = \frac{-2.3RT}{nF} \log_{10} \frac{[K^+ \text{ out}]}{[K^+ \text{ in}]}$$

where R = gas constant (8.314 $J.K^{-1}.mol^{-1}$), T = absolute temperature in Kelvins (K), F = Faraday constant (0.0965 $kJ.mol^{-1}.mV^{-1}$) and n = number of electrons transferred from an oxidised state to a reduced state. When physiological [K^+ out] and [K^+ in] values are used, i.e. 5.5 mM and 150 mM respectively, V_m is ~ -88 mV (at 37°C). This theoretical resting membrane potential of a neuron is slightly different to experimentally derived values. This is because in real neurons, K^+ is not the only permeable ion; leakage of Na^+ and Cl^- across the neuronal membrane occurs. When all three ions are taken into account, using the Goldman-Hodgkin-Katz equation (Nicholls, 1994) V_m is ~ -69 mV (at 37°C), assuming the following values for a mammalian neuron:

Table 1. Typical ion concentrations across the mammalian neuronal plasma membrane

Ion Species	[Ion inside] (mM)	[Ion outside] (mM)
K^+	150	5.5
Na^+	15	150
Cl^-	9	125

If the resting membrane potential of a neuron is reduced by a critical amount, in the order of 15 mV, an action potential of some 100mV amplitude results which is generated as a result of the sequential activation of two classes of voltage-gated ion channels. Hodgkin and Huxley showed that upon injection of a depolarising stimulus into a length of nerve axon, Na^+ and K^+ channels open: the Na^+ channels open rapidly, the K^+ channels open with a delay. Na^+ influx into

the neuron through open Na^+ channels results in depolarisation of the neuronal membrane potential; the mechanism is regenerative in that further depolarisation will lead to more Na^+ channels opening, more Na^+ influx and more depolarisation. This process abolishes and eventually reverses the resting membrane potential of ~ -70 mV, ultimately driving the membrane potential towards the predicted equilibrium potential for Na^+ (+55 mV). The latter is not achieved because Na^+ channel inactivation along with opening of delayed rectifier K^+ channels terminates the action potential, restoring the resting membrane potential. The action potential is propagated without gain loss from the cell body region down the neuronal axon to the nerve terminal. Propagation occurs almost instantaneously between nodes separating myelinated axons[1].

At the nerve terminal, the action potential opens Na^+ channels, leading to nerve terminal depolarisation which eventually activates and opens Ca^{2+} channels. Ca^{2+} influx into the terminal triggers release of chemical transmitter. In this manner the ionic signal is converted to a chemical signal. Transmitter binds to ligand-gated ion channel receptors located on the postsynaptic membrane[2]. If the transmitter leads to opening of ion channels permeable to Na^+ , depolarisation of the postsynaptic membrane results causing neuronal excitation. However, if K^+ or Cl^- channels are opened by a ligand binding to the receptor, hyperpolarisation of the membrane potential results and synaptic inhibition can occur. It must be noted that along with fast synaptic ligand-gated signalling, slower synaptic responses can be achieved by activation of postsynaptic metabotropic receptors.

1.2.3 Neurotransmitters

There are three types of neurotransmitter (see Nicholls, 1994a):

Type I amino acids glutamate, GABA , glycine and possibly aspartate play a role in fast chemical neurotransmission; glutamate and GABA can also evoke slower metabotropic responses. They are stored at the nerve terminal in small

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[1] Shorter axons of interneurons do not require action potential propagation to be speeded up and therefore are unmyelinated.

[2] Transmitter can also feedback and bind to nerve terminal autoreceptors/heteroreceptors, activation of which can alter the probability of transmitter release.

clear synaptic vesicles (SSVs) of about 50 nm in diameter. The major portion of released amino acids are taken back up into the nerve terminal via neuronal amino acid transporters. However, some of the released amino acid is transported into nearby glial elements. Type I neurotransmitters are present in rat brain at μmoles/gram wet weight.

Type II neurotransmitters comprise acetylcholine (ACh), the catecholamines noradrenaline and dopamine, the indoleamine 5-hydroxytryptamine (5-HT) and the purines adenosine triphosphate (ATP) and adenosine. They mediate slow synaptic transmission and play a modulatory role in the CNS[3]. ACh is stored at the nerve terminal in small electron-lucid synaptic vesicles. Released ACh is rapidly degraded in the synaptic cleft by acetylcholinesterase. Catecholamines and 5-HT are stored at the nerve terminal in both SSVs and large dense-core synaptic vesicles (LDCVs) which are greater that 75 nm in diameter. Type II transmitters are present in the rat brain at nanomoles/gram wet weight.

Type III neurotransmitters contain the neuropeptides [met]-enkephalin and [leu]-enkephalin, substance P, somatostatin, neuropeptide Y and cholecystokinin. They act as neuromodulators. Neuropeptides are stored in LDCVs and are released ectopically, i.e. not directly into the synaptic cleft. After being released, neuropeptides must be newly synthesised by proteolytic cleavage of precursor peptide molecules made in the cell soma. New neuropeptide is then transported in LDCVs to release sites. Type III transmitters are present in rat brain at nanomoles to picomoles/gram wet weight.

1.2.4 Neurotransmitter receptors

Neurotransmitter ligands can bind to two types of receptor:

1) Ionotropic receptors are oligomers of distinct subunits containing an integral cation or anion conducting pore region. Ion conductance through the ligand-gated ion channel causes a rapid membrane potential change; these receptors mediate

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[3] There are some exceptions such as the fast acting ionotropic cholinergic nicotinic receptor responses or the ionotropic $5HT_3$ receptor responses.

* (Mintz et al., 1995)

most of the fast synaptic transmission in the CNS.

2) Metabotropic receptors are seven-transmembrane G-protein coupled receptors comprising a subunit mass which is packed almost entirely within the neuronal plasma membrane. Ligand binding stimulates the production of intracellular second messengers such as adenosine 3',5'-cyclic phosphate (cAMP), diacylglycerol (DAG), inositol phosphates and arachidonic acid (AA), which mediate slow, metabotropic neuronal responses.

1.3 The mechanism of neurotransmitter release (exocytosis)

No form of exocytosis is more exquisitely controlled or intensively studied than the regulated release of neurotransmitters from the presynaptic nerve terminal, the basis of synaptic transmission (Kelly, 1993). The protein chemistry of exocytosis of amino acid transmitters and ACh stored in SSVs, is beginning to be pieced together. Exocytosis of transmitters from LDCVs is not so well characterised. Discussion of LDCV exocytosis is not within the remit of this thesis.

The quantal hypothesis of exocytosis predicts that chemical transmitters such as ACh, are released as multimolecular packets called quanta. Each quantum of transmitter is packaged in a single SSV and is exocytosed by a Ca^{2+} trigger at specialised release sites within the nerve terminal called active zones. The influx of Ca^{2+} into the nerve terminal is thought to occur through Ca^{2+} channels sited close to active zones and may produce local intraterminal free [Ca^{2+}] of up to 100μM, which is thought to be required to trigger exocytosis of SSVs (Heidelberger et al., 1994). Ca^{2+} triggered SSV exocytosis requires less than 0.3 ms (Bruns and Jahn, 1995). The probability of exocytosis exhibits a non-linear dependence on the Ca^{2+} concentration with an apparent cooperativity of four (Dodge, Jr. and Rahamimoff, 1967). The theory relating to Ca^{2+} coupling to transmitter release has been termed the 'Calcium Hypothesis' of transmitter release (Zucker and Lando, 1986; Zucker and Fogelson, 1986).

The exocytotic-endocytotic cycle for SSVs in the nerve terminal requires the sequential filling of vesicles with transmitter, their specific translocation to the active zone of the presynaptic plasma membrane, docking of the SSVs with the plasma membrane ready for exocytosis, exocytosis itself (involves fusion of SSVs with the presynaptic plasma membrane) and membrane retrieval and recycling

(endocytosis). A complete picture of this cycle eludes us but significant advances have been made in the characterisation of numerous synaptic proteins thought to be involved in the regulated exocytosis of SSVs at mammalian synapses.

The interaction of vesicle-associated membrane protein (VAMP) also known as synaptobrevin (Link et al., 1992; Schiavo et al., 1992; Sudhof et al., 1993; Yamasaki et al., 1994) with the nerve terminal plasma membrane proteins syntaxin (Sudhof et al., 1993) and synaptosome-associated protein 25 (SNAP-25) (Blasi et al., 1993; Sudhof et al., 1993) is thought to mediate docking of SSVs with the presynaptic plasma membrane. Another protein munc18 (Hata et al., 1993) associates with syntaxin during docking. It has been proposed that during regulated exocytosis at mammalian synapses, SSVs are specifically transported to a fusion target on the nerve terminal plasma membrane containing syntaxin and SNAP-25. The 'SNARE' hypothesis (Söllner and Rothman, 1994) proposes that docking of SSVs at specific plasma membrane sites is achieved when a synaptic **v**esicle-associated SNARE (**v**-SNARE), probably VAMP/synaptobrevin, pairs with a cognate **t**arget-associated SNARE (**t**-SNARE) which would be one of the plasma membrane proteins syntaxin or SNAP-25. For plasma membrane-SSV fusion to occur it is thought that these SNAREs associate together with **N**-ethylmaleimide-**s**ensitive **f**usion protein (**NSF**) (Schiavo et al., 1995) and **s**oluble **N**SF **a**ttachment **p**roteins (**SNAP**s) (Clary et al., 1990) (SNARE ≡ **SNA**P **re**ceptor) in the presence of ATP (*in vitro*, a 20 Svedberg fusion particle composed of the aforementioned proteins is formed in the presence of non-hydrolysable ATP). The final stages of fusion are thought to involve an interaction between synaptotagmin, an SSV protein that binds Ca^{2+} (Petrenko et al., 1991; Brose et al., 1992; Li et al., 1995a), and the t-SNARE syntaxin. Syntaxin has been shown to associate with voltage-sensitive Ca^{2+} channels (VSCC) (Bezprozvanny et al., 1995) that are coupled to SSV exocytosis in the mammalian CNS (Dunlap et al., 1995; Luebke et al., 1993). It is thought that syntaxin may regulate Ca^{2+} entry and that the associated protein synaptotagmin senses this Ca^{2+} and drives SSV exocytosis by a currently unknown mechanism. Finally, a group of ubiquitous nerve terminal plasma membrane proteins termed neurexins (Ushkaryov et al., 1992) are thought be involved in SSV exocytosis; they appear to interact with the syntaxin-synaptotagmin-VSCC complex by a mechanism that is poorly understood.

Immediately after fusion and release of transmitter into the synaptic cleft, it is thought that synaptotagmin acts as a nucleus for clathrin coat assembly by serving as an AP2 receptor. AP2 is a protein complex that binds to a specific receptor on SSVs and plasma membranes to mediate assembly of clathrin for endocytosis (De Camilli and Takei, 1996). Rapid endocytosis of SSVs occurs in 1 to 5 seconds (von Gersdorff and Matthews, 1994) and has been proposed to occur by two possible mechanisms: endocytosis via clathrin-coated pits akin to receptor-mediated endocytosis (Heuser, 1989), or coupled exo- and endocytosis ('kiss and run') (Ceccarelli and Hurlbut, 1980). Another important protein involved in endocytosis of SSVs, dynamin, is a GTPase that can be phosphorylated by protein kinase C (PKC), dephosphorylated by calcineurin upon membrane depolarisation (Sihra et al., 1992) and binds to AP2 (Robinson et al., 1993; Robinson et al., 1994; De Camilli and Takei, 1996). At the nerve terminal plasma membrane, dynamin is thought to mediate the final constriction of fused synaptic vesicle membranes, leading to reinternalisation of SSVs after exocytosis (Takel et al., 1995).

A group of phosphoproteins unique to mammalian nerve terminals called the synapsins bind to SSVs and F-actin. Synapsin I is thought to restrain SSV mobilisation by causing SSVs to associate with the actin cytoskeleton. When phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II, synapsin I dissociates from SSVs and actin, freeing SSVs for docking, fusion and exocytosis. Thus, the synapsins regulate SSV availability and are believed to be centrally involved in the regulation of amino acid release from mammalian nerve terminals (Greengard, 1987; Sudhof et al., 1989; Nichols et al., 1990; Llinas et al., 1991; Nichols et al., 1992; Li et al., 1995b; Rosahl et al., 1995). Finally, the synapsins may also play a role in synaptic development in the mammalian CNS (Han et al., 1991) and organisation of the nerve terminal active zone (Jovanovic et al., 1996).

1.4 Neuromodulation

Synaptic transmission is a plastic process, being constantly modulated through changes both at the presynaptic digit and the postsynaptic element. Released transmitter can bind to G-protein-coupled receptors or ionotropic receptors located on dendrites and cell soma. This can lead to neuronal excitation

or inhibition depending on the type of receptor and signal transduction cascade activated. Alternatively, activation of presynaptic receptors can potentiate or depress transmitter release evoked by propagation of action potentials through the nerve terminal.

1.4.1 G-protein linked receptors

Modulation of synaptic transmission occurs via activation of metabotropic G-protein linked receptors. G-proteins linked to activation of metabotropic receptors are composed of 3 subunits, α, β and γ[4]. When the receptor has no ligand bound, the G-protein α, β and γ subunits are closely associated, with GDP bound to the α subunit. When transmittter binds to the receptor, a conformational change occurs, allowing the G-protein to dock with intracellular domains of the receptor. This causes a conformational change in the G-protein allowing GDP bound to the α subunit to be displaced by cytosolic GTP, relaxing the ligand-bound conformation of the receptor, facilitating dissociation of transmitter from its binding domain. Next, the GTP-bound α subunit dissociates from the β and γ subunits which remain associated as a $\beta\gamma$ dimer (β–γ subunits may define which receptor type G-proteins can associate with). The G-α subunit is highly mobile along the inner neuronal membrane surface and can shuttle between receptor and effector systems where it can activate/regulate important effector enzymes[5], which themselves control intracellular second messenger concentrations. These second messengers then mediate modulation of neuronal membrane ion channels and other downstream cellular effects.

G_s α subunits are stimulatory and activate adenylyl cyclase which generates the second messenger cyclic AMP (cAMP). cAMP activates cAMP-dependent protein kinase (PKA) which through phosphorylation effects can facilitate the opening of Ca^{2+} channels (Dolphin, 1995), regulate ionotropic receptors (Wang et al., 1991; Raymond et al., 1993; Greengard et al., 1991) and

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[4] cDNAs for at least 21 diffferent α subunits (grouped into G_s, G_i, G_q and G_o) have been discovered and at least 4 β and 6 γ subunits exist.

[5] It is now clear that along with the α subunit, $\beta\gamma$-subunits also interact with a number of effector enzymes.

affect proteins (e.g. the synapsins) involved in the exocytotic-endocytotic cycle for nerve terminal SSVs (Sihra et al., 1989). G_i is inhibitory and can inhibit adenylyl cyclase possibly through the G_i $\beta\gamma$-subunit which may compete with the cyclase for liberated G_s α subunits reducing the concentration of G_s α available for the cyclase. In this way Ca^{2+} channels can be inhibited (for review see Clapham and Neer, 1993).

G_q α subunits and $\beta\gamma$-subunits activate phosphoinositide-specific phospholipase C (PLC) which hydrolyses membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) with consequent generation of two second messengers inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1984). IP_3 binds to specific intracellular receptors to trigger Ca^{2+} mobilisation (Berridge, 1993), while DAG, together with membrane phospholipids and Ca^{2+} mediates activation of a large family of Ca^{2+}/phospholipid-dependent protein kinases (PKC). PKC facilitates activation of neuronal Ca^{2+} channels by direct phosphorylation (Shearman et al., 1989; Farago and Nishizuka, 1990) or more indirectly by destabilising the membrane potential by inhibiting K^+ channels (Barrie et al., 1991). PKC also has regulatory effects on ionotropic receptors (Dildy-Mayfield and Harris, 1994; Kelso et al., 1992) and metabotropic receptors (Haga et al., 1990), and at the nerve terminal has been shown to interact with the actin cytoskeleton (Terrian and Ways, 1995) and thus may be involved in regulation of SSV traffic.

Protein-tyrosine kinases (PTKs) and protein tyrosine-phosphatases (PTPs) are key enzymes in signal-transduction pathways for a wide range of cellular processes and are highly expressed in the nervous system (Wagner et al., 1991), which is consistent with the importance of tyrosine phosphorylation in neuronal function (O'Dell et al., 1991). Recent evidence suggests that neurotransmitter receptors may be regulated by tyrosine phosphorylation (Wang and Salter, 1994).

Another important neuromodulatory mechanism occurs through a direct membrane-delimited interaction between the activated G-α subunit and the neuronal ion channel itself, usually resulting in an increase in the activity of K^+ channels (causing hyperpolarisation) and/or a decrease in Ca^{2+} channel activity (Brown, 1990; Dolphin, 1990; Brown and Birnbaumer, 1990; Dolphin, 1995). These effects involve activation of the G_i and G_o subfamilies of G-proteins.

1.4.2 Neuromodulation through ionotropic receptors

Finally, it is notable that certain types of ionotropic receptors can mediate neuromodulatory effects, for example the NMDA glutamate receptor, along with some subtypes of non-NMDA glutamate receptors directly gates Ca^{2+} into neurons. Raising intracellular Ca^{2+} (this can also occur via IP_3 receptor-mediated Ca^{2+} mobilisation from internal Ca^{2+} stores) can lead to the activation of calmodulin and calmodulin-dependent enzymes such as Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) which have been implicated in modulation of ion channel activity, neurite extension and the induction of the persistent changes in synaptic transmission which occur in learning and can be recalled as memory (Hanson and Schulman, 1992; Lynch and Bliss, 1986; Kennedy, 1989).

1.5 Glutamatergic synaptic transmission

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. The 1980's saw an explosion of interest in glutamate as a transmitter due to the development of conformationally restricted analogues of glutamate that exhibited marked potency and specificity in depolarising central neurons.These agents included quisqualic acid (isolated from seeds), ibotenic acid (isolated from mushrooms) and kainic acid (isolated from seaweed). The first glutamate receptor classification proposed the existence of at least three distinct excitatory amino acid receptor subtypes based on agonist effects at central neurons, namely Kainate, Quisqualate and *N*-methyl-*d*-aspartate (NMDA) receptors (Watkins and Evans, 1981). This classification has been completely superceded with the discovery of a new family of metabotropic glutamate receptors (see below).

1.5.1 Ionotropic glutamate receptors

NMDA receptors

Postsynaptic NMDA receptors are selectively activated by NMDA whilst glutamate and possibly aspartate are endogenous ligands.
D-2-amino-5-phosphonovalerate (AP5) is a competitive antagonist at the NMDA receptor, whilst MK801 and phencyclidine (PCP) are open channel blockers.

which is antagonised by 7-chlorokynurenic acid. There is an allosteric glycine binding site on the NMDA receptor. Activation of the NMDA receptor requires glycine binding. The NMDA receptor also has a polyamine regulatory site which may be obligatory for exposing the glutamate binding site and thus for activation of the receptor.

The NMDA receptor effector mechanism is a cation channel which is permeable to Ca^{2+}, Na^{+} and K^{+}. Ca^{2+} entry into neurons through activation of NMDA receptors is thought to be associated with neuron modification and learning via short term memory processes (Collingridge and Bliss, 1995; Collingridge and Singer, 1990; Collingridge and Bliss, 1987). More specifically, NMDA receptors play a vital role in the induction (but not maintenance) of *long-term potentiation* (LTP) (Bliss and Collingridge, 1993). LTP is the most intensely investigated model underlying the biochemistry of learning and memory.

The NMDA receptor has been described as a coincidence detector as receptor activation and Ca^{2+} conductance requires simultaneous presynaptic release of glutamate and postsynaptic membrane depolarisation (probably via activation of postsynaptic non-NMDA AMPA-type glutamate receptors). Indeed, NMDA-induced LTP has been shown to be linked to an enhancement of presynaptic glutamate release (Canevari et al., 1994; Lynch et al., 1994). Under polarised conditions, NMDA receptor-mediated Ca^{2+} entry is prevented by Mg^{2+} which blocks the ion channel. Depolarisation relieves Mg^{2+} block.

NMDA receptors are abundant in the hippocampus. Excitatory postsynaptic currents (EPSCs) in the CA1 region of the hippocampus have two components: a fast voltage-insensitive component and a slower voltage-dependent component The voltage-dependent component is abolished by the NMDA receptor antagonist AP5. The fast component is abolished by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Hestrin et al., 1990). From this data, it has been postulated that CA1 hippocampal EPSCs comprise a fast non-NMDA α-amino-5-methyl-4-isoxazole-propionic acid (AMPA) receptor-activated component which decays rapidly leaving a slower NMDA receptor-mediated component, which arises due to AMPA-receptor depolarisation-induced relief of NMDA receptor Mg^{2+} block (Hestrin et al., 1990).

The first NMDA receptor was cloned in 1991 by the Nakanishi group and

was termed NMDAR1 (Moriyoshi et al., 1991). Four more subunits have been cloned, and named NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D (Monyer et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993). NMDAR1 and NMDAR2A-D subunits can be expressed together as hetero-oligomers yielding functional receptors. Co-expression may confer subtle but important biophysical differences between subtypes of NMDA receptors. NMDAR1 is expressed in almost all neuronal cells, NMDAR2A and NMDAR2B are prominently expressed in cerebral cortex and hippocampus, NMDAR2C predominates in the cerebellum and NMDAR2D mRNA is expressed in the diencephalon (Moriyoshi et al., 1991; Masu et al., 1993).

Non-NMDA receptors

These receptors can essentially be split into AMPA-type and kainate-type based on electrophysiological, pharmacological and molecular cloning data.

AMPA receptors

AMPA receptors mediate fast glutamatergic synaptic transmission. AMPA is the most selective agonist at AMPA receptors; kainic acid (KA) is also an agonist and glutamate is the endogenous ligand. Antagonists include CNQX and 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX), the latter being more selective. The AMPA receptor effector mechanism is a conductance of Na^+/ K^+ (low divalent ion permeability) through an integral ion pore, which elicits membrane depolarisation. In the hippocampus, AMPA receptor-mediated postsynaptic currents are voltage-insensitive, rapid in onset and decay with a time course of 4-8 ms (Hestrin et al., 1990).

In electrophysiological experiments AMPA evokes rapidly desensitising currents, whereas KA-evoked AMPA receptor currents are non-desensitising. For this reason, KA (whilst being less selective than AMPA) is the preferred agonist for studying the biophysical properties of AMPA receptors. Cyclothiazide is a benzothiadiazide diuretic that potently inhibits the rapid desensitisation of AMPA-preferring receptors (Partin et al., 1993; Rammes et al., 1994; Wong and Mayer, 1993; Yamada and Tang, 1993). Potentiation of AMPA responses by pretreatment

with cyclothiazide is not lost upon the administration of a competitive AMPA receptor antagonist (LY293558) (Rammes et al., 1994) or non-competitive antagonist (GYKI 52466) (Rammes et al., 1994; Johansen et al., 1995; Kessler et al., 1996). This suggests that cyclothiazide and GYKI 52466/LY293558 act at distinct sites on the AMPA receptor complex (Johansen et al., 1995; Kessler et al., 1996). Cyclothiazide shows selectivity towards AMPA receptors composed of 'flip' subunits (see below) (Johansen et al., 1995). Aniracetam, a nootropic drug, also reduces AMPA receptor desensitisation (Johansen et al., 1995), slowing the rate of decay of fast excitatory currents in the hippocampus (Isaacson and Nicoll, 1991). Postsynaptic AMPA receptors are involved in the maintenance of LTP (see Bliss and Collingridge, 1993; Collingridge and Singer,1990). It has been argued that in order for LTP to be sustained/maintained AMPA receptors are selectively modified perhaps by phosphorylation.

Molecular cloning has identified four closely related members of a family of AMPA-selective ionotropic glutamate receptors termed GluR1, GluR2, GluR3 and GluR4 (Boulter et al., 1990; Nakanishi et al., 1990) or GluR A, B, C and D (Keinanen et al., 1990). They show 30-40% homology to NMDA receptor clones. When expressed *in vitro* these receptors display a high affinity for AMPA, a binding site for CNQX and a low affinity binding site for KA, which can be antagonised by AMPA. A pentameric subunit structure is thought to form functional homomeric or heteromeric receptors. AMPA receptor subunits are highly abundant in the hippocampus, cerebral cortex and cerebellum (Hollmann and Heinemann, 1994). Each receptor subunit can exist in two forms, generated by alternative splicing of primary RNA transcripts, termed 'flip' and 'flop' (Sommer and Seeburg, 1992; Sommer et al., 1990). Prenatal brain expresses mostly 'flip' forms, which persists throughout adulthood. The 'flop' forms appear only from early postnatal stages onwards and are co-expressed with 'flip' forms in many cells. This developmental change in AMPA receptor structure has functional consequences. Prenatal receptors containing only 'flip' modules have slower desensitisation kinetics than adult receptors composed of both 'flip' and 'flop' modules. These differing desensitisation kinetics affect the ratio of peak: steady-state components of glutamate-activated currents in AMPA receptor channels. The steady-state component is virtually absent in channels assembled from subunits

in their 'flop' configuration (Seeburg, 1993).

AMPA receptor channels formed *in vitro* from GluR1, GluR3 and GluR4 (GluRA, GluRC and GluRD) subunits show high Ca^{2+} permeability (Hollmann et al., 1991). However, a special feature is observed in the 'flip' and 'flop' forms of GluR2 (GluRB) subunits, which may account for the particular properties seen in AMPA receptors of native membranes. Post-translational editing of the mRNA removes an amino group from a specific adenine base altering the codon for glutamine (*Q*) to arginine (*R*) at residue 586 in the putative second transmembrane segment of the polypeptide subunit (Burnashev et al., 1992). The unedited *Q* GluR2(GluRB) subunit forms a homomeric receptor with high Ca^{2+} permeability and does not conduct ions when strongly depolarised (beyond -50mV). This form decreases developmentally and is almost totally replaced by the edited *R* GluR2(GluRB) in mature neurons. AMPA receptors containing the *R* edited GluR2(GluRB) subunit have low Ca^{2+} permeability. GluR2(GluRB) subunits determine the ionic conductance properties of heteromeric AMPA receptors. AMPA receptors containing unedited GluR2(GluRB) subunits show greater Ca^{2+} permeability over those containing *R*-edited GluR2(GluRB) subunits.

Cloned AMPA receptor subunits when homomerically or heteromerically expressed show the following agonist sensitivity profile: quisqualate>AMPA> glutamate>KA (Hollmann and Heinemann, 1994). As with native AMPA receptors, AMPA-evoked currents are rapidly desensitising, whereas KA-evoked currents are not. Localisation of cloned AMPA receptor subunits, using immunostaining techniques, shows little evidence of presynaptic loci. Most immunostaining occurs at postsynaptic densities (Petralia and Wenthold, 1992; Hampson et al., 1992).

Kainate receptors

Kainate receptors are concentrated in a few specific areas of the CNS such as the stratum lucidum region of the hippocampus, where they co-exist along with NMDA receptors and AMPA receptors. This makes them difficult to characterise due to KA action at AMPA receptors, although domoate is a neurotoxin with potent and relatively specific agonist action at kainate receptors in the CNS (Debonnel

et al., 1989b; Debonnel et al., 1989a). KA does have a specific[6], markedly desensitising depolarising action at dorsal root C fibres (Pook et al., 1993) which can be blocked by α-D-glutamylaminomethylsulphonic acid (GAMS) (Davies and Watkins, 1985). However, most brain pathways contain kainate receptors and AMPA receptors in close association, and a lack of specific pharmacological antagonists to dissect out AMPA from kainate receptors has hampered the functional study of kainate receptors; CNQX and NBQX antagonise both receptor types. Saying this, novel AMPA receptor antagonists have enabled isolation of a class of rapidly desensitising glutamate receptors that are activated by kainate, but not by AMPA, in hippocampal neurons (Lerma et al., 1993, Lerma et al., 1997). Further study of hippocampal kainate receptors indicates that they do not participate in conventional fast synaptic transmission which is mediated by AMPA and NMDA receptors (Lerma et al., 1997). Instead, roles for kainate receptors in the regulation of transmitter release (Chittajallu et al., 1996) and neuronal development (Lerma et al., 1997) have been proposed. The kainate receptor effector mechanism is believed to be a cation conducting pore permeable to Na^+, K^+ and Ca^{2+} which elicits membrane depolarisation. Distinct high-affinity and low-affinity [^{3}H]kainate binding sites have been described on rat brain membranes (Young and Fagg, 1990) which may correlate to the two cloned kainate receptor subunit groups, low-affinity kainate receptors and high-affinity kainate receptors.

Low-affinity kainate receptor subunits consist of cloned polypeptide subunits GluR5, GluR6 and GluR7 (Bettler et al., 1992; Egebjerg et al., 1991; Sommer et al., 1992; Lomeli et al., 1992) and show 40% homology with GluR1-4 subunits (AMPA type). As is the case with the GluR2 AMPA subunit, RNA editing can occur at a proposed *Q/R* site in GluR5 and GluR6 subunits but not GluR7. *R* editing does not completely occlude Ca^{2+} permeability, as is the case with AMPA receptors containing GluR2 *R* edits.

Agonist profiles for currents obtained with expressed GluR5 and GluR6 receptor subunits are; domoate > KA > glutamate. AMPA elicits no current (Seeburg, 1993) whilst domoate and KA responses are rapidly desensitising

[6] There are no AMPA type receptors on dorsal root C fibres.

(Seeburg, 1993)[7]. Concanavalin A prevents kainate receptor desensitisation in dorsal root ganglion neurons (DRG) (Wong and Mayer, 1993). Expressed GluR7 subunits produce no currents (Egebjerg et al., 1991; Sommer et al., 1992).

Homomerically expressed GluR6 subunits can be selectively antagonised by the novel compound NS-102 (Verdoorn et al., 1994). Furthermore, NS-102 shows selective displacement of low-affinity [^{3}H]kainate binding sites on rat brain membranes (Johansen et al., 1993). The binding affinities for kainate obtained from [^{3}H]kainate competition binding studies using cloned GluR5 and GluR6 subunits correlate closely to low-affinity [^{3}H]kainate binding sites on rat brain membranes; both K_d's are in the region of 50-70 nM (Hollmann and Heinemann, 1994). Low-affinity kainate receptor subunit clones may make up the low-affinity kainate receptor described on native rat brain membranes.

Two high-affinity kainate receptor subunits termed KA1 and KA2 have been cloned using Polymerase Chain Reaction (PCR)-mediated DNA amplification (Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992). They show 35 - 45% sequence homology with GluR 1-4 subunits and GluR 5-7 subunits. KA1 and KA2 subunits are not subject to any form of post translational mRNA editing. When KA1 or KA2 subunits are expressed as homomers or KA1/KA2 heteromers, no currents are detectable, due possibly to rapid desensitisation, small ionic conductances or low open channel probability. It seems likely that kainate receptors comprising only KA1/KA2 subunits do not exist in native membranes. However, when KA2 subunits are co-expressed with GluR5 or GluR6 kainate receptor subunits, functional channels with novel properties are seen; kainate receptors comprising only GluR6 subunits show no sensitivity to AMPA but GluR6/KA2 channels can be gated by AMPA in a non-desensitising manner (Seeburg, 1993), as well as by KA and glutamate which elicit markedly desensitising responses.

High-affinity KA1 and KA2 receptor clones bind kainate with close correlation to the high-affinity [^{3}H]kainate binding site on native rat brain membranes (K_d 2-15 nM). AMPA binding in both cases is undetectable. High-affinity kainate receptor subunit clones may represent the high-affinity [^{3}H]kainate

[7] KA is non-desensitising at AMPA receptors.

binding site on rat brain membranes. However, in light of the fact that KA1/KA2 subunits when homomerically expressed produce no currents , it is conceivable that the high-affinity [^{3}H]kainate binding sites in rat brain do not represent functional kainate receptor channels[8].

KA2 subunits are highly expressed throughout the brain (Herb et al., 1992) indicating that high-affinity kainate receptors in one way or another may be involved in all central glutamatergic neuronal circuits of the brain. KA1 subunits are less abundant. Highest levels of KA1 expression occurs in the hippocampus, where high levels of KA1 subunit mRNA in granule cells has been interpreted as evidence for a possible presynaptic location of KA1 subunits (Werner et al., 1991).

1.5.2 Metabotropic glutamate receptors (mGluRs)

Glutamate and quisqualate stimulate inositol phosphate formation in a number of neuronal cell types (Sladeczek et al., 1985; Nicoletti et al., 1986) which led to the proposal of a new family of glutamate receptors linked to G-proteins. Definitive evidence arose in 1987, when it was shown that a glutamate receptor coupled to phospholipase C (PLC) could be expressed in oocytes following injection of rat brain mRNA (Sugiyama et al., 1987). Metabotropic glutamate receptors(mGluRs) are seven-transmembrane G-protein linked receptors coupled to the stimulation of intracellular second messengers. The molecular biology and pharmacology of mGluRs has taken off in explosive fashion since the first metabotropic glutamate receptor, mGluR1, was cloned (Houamed et al., 1991; Masu et al., 1991). mGluR1 is coupled to PLC, which stimulates inositol phosphate formation.

Since the cloning of mGluR1, seven other mGluRs have been cloned (named mGluR2 through mGluR8) using low stringency hybridisation screening with mGluR1 probes (Tanabe et al., 1992; Abe et al., 1992; Nakajima et al., 1993; Okamoto et al., 1994; Nakanishi, 1992; Duvoisin et al., 1995). Furthermore, 3 splice variants of mGluR1, α , β and *c* have been found (Pin et al., 1992). mGluRs show little sequence homology to other G-protein linked receptors and are

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[8] This *in situ* binding site may represent high-affinity kainate receptor subunits waiting to be co-assembled with low-affinity kainate receptor subunits (GluR 5-7).

- Immunocytochemical studies in the rat cerebral cortex and hippocampus indicate that some mGluR5 immunoreactivity is located in presynaptic axon terminals, suggesting that mGluR5 may function as a presynaptic receptor (Romano et al., 1995).

considerably larger. They have been assigned a place in one of three different groups on the basis of sequence similarity.

Group I contains mGluR1 and mGluR5 whose activation has been linked to phosphoinositide-specific PLC stimulation via G_q or G_o, and the subsequent hydrolysis of PIP_2 to IP_3 ,which effects Ca^{2+} mobilisation from intracellular Ca^{2+} stores (Abe et al., 1992). Agonists include quisqualate, (1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) (Irving et al., 1990) and (S)-3,5-Dihydroxyphenylglycine (DHPG) (Schoepp et al., 1994). mGluR1 is widespread in the brain and is particularly abundant in hippocampus and cerebellar Purkinje neurons (Masu et al., 1991). mGluR5 is restricted to a small population of cerebellar Golgi cells but is also localised to striatum, cerebral cortex, hippocampus and olfactory bulb (Abe et al., 1992).

Group II contains mGluR2 and mGluR3, whose activation causes a decreased activity of neuronal membrane bound adenylate cyclase via G_i or G_o (mGluR2 and mGluR3 responses are pertussis toxin (PTX)-sensitive) leading to a reduction in intracellular cAMP levels (Schoepp et al., 1992; Tanabe et al., 1993). 1S,3R-ACPD also activates group II mGluRs, however, (2S,1'S,2'S)-2-(Carboxycyclopropyl)glycine (L-CCG-I) shows selectivity for group II mGluRs over group I mGluRs (Hayashi et al., 1992). mGluR2 is abundant in the brain being present in the olfactory bulb (Kaba et al., 1994) cerebral cortex, striatum, cerebellar Golgi cells and dentate gyrus granule cells. mGluR3 is localised to glial cells, dentate gyrus granule cells and neurons of the cerebral cortex (Tanabe et al., 1993).

Group III contains mGluR4, mGluR6, mGluR7 and mGluR8 activation of which leads to a reduction in cAMP levels (Okamoto et al., 1994) as occurs with group II mGluRs. Agonists have been used with some success in order to distinguish between group II and group III mGluRs in central neurons; group III mGluRs are insensitive to 1S,3R-ACPD, whilst being uniquely sensitive to the synaptic depressant compound L(+)-2-Amino-4-phosphonobutyric acid (L-AP4) (Okamoto et al., 1994; Tanabe et al., 1993; Nakajima et al., 1993). mGluR4 and mGluR7 correspond well to the putative L-AP4 receptor which plays an important role in modulation of glutamate transmission in the CNS (Nakanishi, 1992). mGluR4 is expressed in low amounts in the granule cells of the cerebellum and

in the olfactory bulb (Tanabe et al., 1993). Expression of mGluR6 is restricted to the inner nuclear layer of the retina (Nakajima et al., 1993), corresponding to the retinal L-AP4 sensitive mGluR which regulates synaptic transmission between photoreceptor cells and on-bipolar cells of the visual system (Nawy and Jahr, 1990; Shiells and Falk, 1990). mGluR 7 is expressed in the cerebral cortex, olfactory bulb, striatum and hippocampus (Okamoto et al., 1994). It may have a presynaptic location (Shigemoto et al., 1996) and could be an inhibitory metabotropic glutamate autoreceptor.

Apart from PLC, stimulation of mGluRs can also lead to the activation of two other phospholipases, phospholipase A_2 (PLA_2)[9] and phospholipase D (PLD) (Schoepp and Conn, 1993; Schoepp, 1994). As well as reduction in cAMP levels by group II and group III mGluRs, increases in intracellular cAMP resultant from mGluR stimulation have been observed (Schoepp, 1994). Activation of mGluRs is also linked with modulation of ion channel currents (Chavis et al., 1995), modulation of ligand-gated ionotropic receptors (Kelso et al., 1992) and modulation of neurotransmitter release (Burke and Hablitz, 1994; East et al., 1995; Vazquez et al., 1995b; Herrero et al., 1992).

It has recently been reported that mGluRs may be involved in modulation of synaptic plasticity (Bashir et al., 1993b; Bashir et al., 1993a; Bortolotto and Collingridge, 1993). Under certain circumstances, LTP of AMPA receptor-mediated synaptic transmission can be induced by activation of mGluRs with 1S,3R-ACPD (Bortolotto and Collingridge, 1993). Putative antagonists of group I mGluRs block the induction of LTP, further confirming the role of mGluRs in LTP (Bashir et al., 1993a). However, mGluR antagonists will only block the induction of LTP in *naive* synapses, i.e. synapses that have never previously experienced LTP; synapses which have experienced LTP cannot be prevented from further LTP induction by use of mGluR antagonists (Bortolotto et al., 1994). From this data it has been proposed that mGluRs induce a conditioned state in *naive* synapses, activation of NMDA receptors takes the conditioned synapse to the potentiated state and maintenance of a potentiated synapse occurs through AMPA receptors. A conditioned synapse never reverts back to a *naive* synapse

[9] Ca^{2+}-activated PLA_2 generates arachidonic acid (AA), which may act as a neuronal retrograde messenger molecule involved in LTP.

and mGluRs are no longer needed in order to induce AMPA receptor-mediated LTP.

1.6 Ca^{2+} channels involved in glutamatergic synaptic transmission

First described in 1953, voltage-sensitive Ca^{2+} channels have been accepted as a ubiquitous component of all excitable cells. First reports of multiple Ca^{2+} channel subtypes came from Hagiwara and co-workers (Hagiwara et al., 1975). Electrophysiologically, Ca^{2+} channels can be divided up into two groups: low voltage-activated Ca^{2+} channels (LVA) and high-voltage activated Ca^{2+} channels (HVA).

Low voltage-activated Ca^{2+} channels

LVA Ca^{2+} channels have one member, the T-type Ca^{2+} channel which is found in cardiac muscle and neurons and is active at relatively negative membrane potentials (Tsien et al., 1988). These channels have tiny unitary conductances and rapidly inactivate. They play a role in generation of rhythmic pacemaker activity of cardiac myocytes and neurons.

High voltage-activated Ca^{2+} channels

The HVA Ca^{2+} channels group has multiple members which were originally distinguished by electrophysiological and pharmacological criteria (Nowycky et al., 1985), a division that still remains. Members of the HVA Ca^{2+} channel group are sub-classed according to sensitivity to the 1,4-dihydropyridines (DHPs). The DHP-sensitive class contains one member, L-type Ca^{2+} channels, which have very slow inactivation kinetics and large unitary conductances. The L- type Ca^{2+} channel is extremely heterogeneous at the molecular level and there are potentially many different isoforms of the L-type Ca^{2+} channel (Dolphin, 1995).
The following DHP-insensitive Ca^{2+} channels have been described and can be distinguished from one another using highly selective Ca^{2+} channels toxins (Olivera et al., 1994):
A) N-type Ca^{2+} channels have intermediate inactivation kinetics (Fox et al., 1987) and are exclusive to neurons. ω-Conotoxin GVIA, isolated from the fish eating

marine mollusc Conus geographus produces irreversible block of N-type Ca^{2+} channels (Regan et al., 1991). All ω-conotoxins isolated to date inhibit N-type Ca^{2+} channels, with varying binding affinities. Isolated from Conus magus, ω-conotoxin MVIIA (Bleakman et al., 1995), MVIIC (SNX-230) (Adams et al., 1993; Hillyard et al., 1992; Grantham et al., 1994) and MVIID (Monje et al., 1993) reversibly block N-type channels but MVIIC and MVIID show higher affinity for and preferential block of other HVA Ca^{2+} channels (see below).

B) P-type Ca^{2+} channels[10] have inactivation kinetics slower than the intermediary kinetics of N-type Ca^{2+} channels (Llinas et al., 1992). P-type Ca^{2+} channels are potently (low nM amounts) blocked by a peptide toxin, ω-Aga IVA, isolated from the venom of the funnel web spider Agelenopsis aperta (Mintz et al., 1991; Mintz et al., 1992) but are insensitive to ω-conotoxin GVIA. ω-Conotoxin MVIIC (ω-CTx MVIIC) also blocks P-type Ca^{2+} channels but with much lower affinity than ω-Aga IVA (Olivera et al., 1994).

C) Q-type Ca^{2+} channels first described in granule cells are blocked by ω-Aga IVA and ω-conotoxin MVIIC and can be distinguished from P-type Ca^{2+} channels based on channel inactivation kinetics and sensitivity to the above named toxins (Randall and Tsien, 1995), i.e. when cRNA encoding class A Ca^{2+} channel α_1 subunits, which are thought to represent native P-type Ca^{2+} channels, is injected into oocytes, resulting Ca^{2+} currents are rapidly activating/inactivating and potently blocked by ω-CTx MVIIC (SNX-230). In comparison to P-type purkinje Ca^{2+} channels, these α_{1A} channels are 100-fold less sensitive to ω-Aga IVA and 10-fold more sensitive to ω-CTx MVIIC (Sather et al., 1993). It is proposed that α_{1A} cRNA is capable of generating a Ca^{2+} channel phenotype different from P-type Ca^{2+} channels and may represent the Q-type Ca^{2+} channels described in the CNS. Q-type channels also support hippocampal synaptic transmission (Wheeler et al., 1994).

D) Recently, a putative O-type Ca^{2+} channel has been described in the brain, based on binding studies with ω-CTx MVIIC, showing brain binding sites with an affinity for ω-CTx MVIIC in the pM range (Adams et al., 1993; Kristipati et al., 1994). This is far higher than the binding affinity of ω-CTx MVIIC for N-type Ca^{2+}

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[10] First described in cerebellar Purkinje neurons.

channel sites or the potency of ω-CTx MVIIC at P-type and Q-type Ca^{2+} channels. This novel O-type Ca^{2+} channel binding site may have a presynaptic location (Kristipati et al., 1994) and may also be the Q-type Ca^{2+} channel defined electrophysiologically.

Some of the electrophysiologically and pharmacologically classified somatic VSCC types described above (A to D) are thought to be represented in nerve terminal membranes where they are coupled to the release of transmitters. It has been shown that glutamate release from rat cerebrocortical and striatal synaptosomes is insensitive to the N-type Ca^{2+} channel toxin, ω-conotoxin GVIA but partially blocked by the P-type Ca^{2+} channel toxin ω-Aga IVA (Turner et al., 1992; Turner et al., 1993). ω-Aga IVA blocks KCl-stimulated $^{45}Ca^{2+}$ uptake by rat brain synaptosomes (Mintz et al., 1992) and it has been proposed that multiple Ca^{2+} channel subtypes co-exist to regulate glutamate release: an ω-Aga IVA-sensitive P-type Ca^{2+} channel and an ω-Aga IVA-insensitive, ω-conotoxin-insensitive Ca^{2+} channel. However, it has been found that ω-conotoxin GVIA partially blocks (18%) the plateau phase of KCl-evoked elevations in rat cortical cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, and that ω-Aga IVA produces a 65% block (Bowman et al., 1993), suggesting the existence of presynaptic N-type Ca^{2+} channels as well as P-type Ca^{2+} channels.

Pocock and co-workers demonstrated that a toxin purified from the venom of the funnel web spider Agelenopsis aperta, named ω-Aga-GI, completely inhibits synaptosomal glutamate release evoked by 4AP or KCl (30mM) and causes a 50% reduction in KCl-evoked and 4AP-evoked elevation in $[Ca^{2+}]_c$ (Pocock and Nicholls, 1992; Coffey et al., 1994b). However, it is not known which VSCC ω-Aga-GI specifically targets.

Contrasting to cerebral cortex and striatal synaptosomes, glutamate release from hippocampal synaptosomes is partially inhibited (16%) by ω-conotoxin GVIA and potently by ω-Aga IVA (Luebke et al., 1993). It has been reported that ω-conotoxin GVIA blocks excitatory and inhibitory synaptic transmission in the CA1 region of the hippocampus (Dutar et al., 1989; Luebke et al., 1993) and cerebellum (Takahashi and Momiyama, 1993) but that it preferentially blocks inhibitory synaptic transmission (Horne and Kemp, 1991; Takahashi and

Momiyama, 1993). This indicates that the Ca^{2+} entry that triggers GABA and glutamate release at central synapses may be mediated by pharmacologically distinct Ca^{2+} channels. P-type Ca^{2+} channels appear to play a prominent role in excitatory amino acid release, although N-type Ca^{2+} channels are also clearly involved (Luebke et al., 1993; Tareilus and Breer, 1995). Similar results as to the Ca^{2+} channel types mediating hippocampal synaptic transmission have been observed in hippocampal slices using HPLC analysis of released amino acids (Burke et al., 1993) or by measuring presynaptic Ca^{2+} transients (Wu and Saggau, 1994). Some excitatory synaptic circuits in the hippocampus do not appear to use P-type Ca^{2+} channels for synaptic transmission but instead a combination of N-type and Q-type Ca^{2+} channels (Nooney and Lodge, 1996; Wheeler et al., 1994).

L-type Ca^{2+} channels do not appear to mediate hippocampal excitatory synaptic transmission (Horne and Kemp, 1991) or inhibitory transmission in the cerebellum and spinal cord (Takahashi and Momiyama, 1993). DHPs do not block glutamate release from cerebral cortex synaptosomes (Pocock and Nicholls, 1992) or KCl-evoked $^{45}Ca^{2+}$ uptake by rat brain synaptosomes (Reynolds et al., 1986). However, glutamate release in cultured cerebellar granule cells is blocked by DHPs, indicating a role of L-type Ca^{2+} channels in regulating glutamate release in cerebellar glutamatergic circuitry (Huston et al., 1995; Huston et al., 1990). However, DHPs do not inhibit glutamate release from cerebellar *synaptosomes* (Pocock et al., 1995) and it is argued that L-type Ca^{2+} channels modulating release in cultured cerebellar granule cells are located at somatic regions rather than at neurites.

The broad spectrum Ca^{2+} channel toxin ω-conotoxin MVIIC (ω-CTx MVIIC) inhibits a major portion of [^{3}H]glutamate release from synaptosomes (Turner et al., 1995). Furthermore, ω-CTx MVIIC inhibits 75% of KCl-evoked $^{45}Ca^{2+}$ uptake by crude rat brain synaptosome preparations (Hillyard et al., 1992) and inhibits KCl-evoked Ca^{2+} influx into hippocampal synaptosomes (Malva et al., 1995a). It is argued that ω-CTx MVIIC and ω-Aga IVA both inhibit a presynaptic Ca^{2+} channel containing the class A Ca^{2+} channel α_1 subunit (Malva et al., 1995a) but that, because of its broader specificity, ω-CTx MVIIC is less useful for probing Ca^{2+} channels linked to glutamate release than ω-conotoxin GVIA and ω-Aga IVA

(Dunlap et al., 1995). These highly specific arachnid and molluscan Ca^{2+} channel toxins validate the 'Calcium Hypothesis' of transmitter release which suggests a tight coupling between VSCC and exocytosis (Zucker and Lando, 1986).

1.7 Characterisation of glutamate exocytosis using synaptosomes

Nicholls and Sihra showed that guinea-pig brain synaptosomes release glutamate in a Ca^{2+}-dependent manner when depolarised with 30mM KCl (Nicholls et al., 1987; Nicholls and Sihra, 1986). Similar observations have been made with rat brain synaptosomes (Coffey et al., 1993). Elevated external KCl collapses the K^+ concentration gradient across the synaptosomal plasma membrane and causes a 'clamped' membrane depolarisation.

Depolarisation of the synaptosomal membrane potential cannot be measured electrophysiologically. Biochemical techniques are available which allow a qualitative assessment of the synaptosomal membrane potential and any changes thereof due to depolarisation. These include positively charged membrane potential-sensitive cyanine dyes which can be incorporated into the membrane leaflet of synaptosomes (Enkvist et al., 1988; Coffey et al., 1993) and $^{86}Rb^+$ which can be used to estimate the synaptosomal membrane potential by determining the K^+-equilibrium potential (Tibbs et al., 1996).

Depolarisation of synaptosomes with 30mM KCl causes a large, fura-2 detectable elevation in cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, characterised by a transient 'spike' of $[Ca^{2+}]_c$ which recovers to a sustained 'plateau' level (McMahon and Nicholls, 1991). This is believed to be the result of Ca^{2+} influx through VSCC linked to glutamate release (Nicholls, 1993).

Sanchez-Prieto and co-workers showed that the Ca^{2+} ionophore, ionomycin, elicits glutamate release (Sanchez-Prieto et al., 1987). However, ionomycin produces a modest release of glutamate in comparison to the Ca^{2+}-dependent glutamate release evoked by KCl (30mM) (Sihra et al., 1992). Ionomycin produces Ca^{2+} ionophores randomly on the synaptosomal membrane and it is argued that a lack of Ca^{2+}-influx through VSCC linked to glutamate release is why ionomycin is not very competent at releasing glutamate (Sihra et al., 1992), thus lending support to the 'Calcium Hypothesis' of transmitter release

(Zucker and Lando, 1986; Zucker and Fogelson, 1986).

Ca^{2+}-dependent glutamate release from synaptosomes can be evoked by the Na^+ channel activator, veratridine, implicating Na^+ channels in glutamate release (Sanchez-Prieto et al., 1987). Cytosolic free Na^+ levels can be measured using the fluorescent Na^+ probe, Na^+-binding benzofuran isophthalate (SBFI) (Deri and Adam-Vizi, 1993). Veratridine evokes a sharp rise in cytosolic free Na^+ levels which is thought to be a consequence of extracellular Na^+ influx through veratridine-sensitive voltage-dependent Na^+ channels.

A more physiological way of depolarising synaptosomes is to use a K^+ channel blocker, 4-aminopyridine (4AP), which evokes Ca^{2+}-dependent glutamate release (Tibbs et al., 1989b; Tibbs et al., 1989a) by depolarising synaptosomes (Coffey et al., 1993) and eliciting a large elevation in cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, (Tibbs et al., 1989a; Tibbs et al., 1996). It has been proposed that by blocking K^+ channels, 4AP destabilises the synaptosomal plasma membrane potential and thus increases the probability of repetitive activation and opening of voltage-dependent Na^+ channels, this leading to Na^+ influx and depolarisation. Depolarisation activates and opens VSCC, allowing Ca^{2+} influx into the nerve terminal, the trigger for exocytosis (Nicholls and Coffey, 1994; Tibbs et al., 1989a). This hypothesis is confirmed by the fact that the Na^+ channel blocker tetrodotoxin (TTX) inhibits a major portion of 4AP-induced Ca^{2+} influx into synaptosomes and completely attenuates 4AP-evoked Ca^{2+}-dependent glutamate release (Tibbs et al., 1989a). In contrast, glutamate release evoked by 30 mM KCl does not involve repetitive firing of Na^+ channels since TTX does not block Ca^{2+} influx or Ca^{2+}-dependent glutamate release evoked by 30 mM KCl (Tibbs et al., 1989a). This is because of the '*clamped*' depolarisation elicited by KCl (30mM).

By a mechanism similar to 4AP, dendrotoxin isolated from the African green mamba snake Dendroaspis angusticeps (Tibbs et al., 1989b) and Ba^{2+} (McMahon and Nicholls, 1993; Sihra et al., 1993) also evoke Ca^{2+}-dependent glutamate release both in a TTX-sensitive manner. At the concentrations used to evoke glutamate release, 4AP, dendrotoxin and Ba^{2+} block electrophysiologically defined delayed rectifier K^+ channels and transiently activated *A* type K^+ channels (Cook, 1988; Choquet and Korn, 1992). Currently,

it is not possible to define which K^+ channel 4AP, dendrotoxin and Ba^{2+} target. Delayed rectifiers and *A* type K^+ channels are both thought to be represented in presynaptic nerve terminals.

1.8 Modulation of glutamate exocytosis by presynaptic receptors

Activation of presynaptic receptors by a transmitter modulates the probability of neurotransmitter release from nerve terminals. Modulation of release of one transmitter by another occurs through activation of heteroreceptors, whereas autoreceptor activation causes self-regulation of transmitter release. Presynaptic receptor activation commonly attenuates transmitter release but can under certain circumstances enhance transmitter release.

The mechanism by which a presynaptic receptor transduces an extracellular chemical signal into a change in release of transmitter depends on the type of presynaptic receptor involved; activation of ligand-gated ion channel receptors may increase or decrease an integral Na^+, K^+, or Ca^{2+} conductance thereby altering the nerve terminal membrane potential. Alternatively, activation of presynaptic G-protein linked metabotropic receptors could lead to stimulation of specific effector enzymes and subsequent modulation of nerve terminal intracellular second messengers which activate protein kinases. Thus, protein kinase-mediated phosphorylation of nerve terminal K^+ or Ca^{2+} channels may regulate transmitter release. Metabotropic receptors can have a more *membrane-delimited* action whereby G-protein α subunits can directly interact with neuronal ion channels by a mechanism where no intracellular second messenger is involved.

1.8.1 Modulation by type II and type III transmitters

Presynaptic noradrenergic α_2-adrenoceptors inhibit glutamate release from spinal cord, thalamic, hypothalamic and cerebrocortical synaptosomes (Kamisaki et al., 1993; Kamisaki et al., 1992; Kamisaki et al., 1991) but not from striatal or cerebellar synaptosomes. It has also been shown that α-adrenoceptors inhibit excitatory transmission in the hippocampus (Scanziani et al., 1993). 5HT inhibits

KCl-evoked glutamate release from cerebellar synaptosomes (Maura et al., 1986) via activation of presynaptic $5HT_2$ (Maura et al., 1991) and $5HT_{1D}$ (Maura and Raiteri, 1996) heteroreceptors.

Adenosine inhibits glutamate release from cerebrocortical synaptosomes (Barrie and Nicholls, 1993; Budd and Nicholls, 1995) and hippocampal slices (Dolphin and Archer, 1983) through the activation of presynaptic A_1 receptors. Adenosine causes a reduction in the KCl-evoked elevation in cytosolic free Ca^{2+} concentration and ω-Aga IVA occludes these inhibitory effects of adenosine (Vazquez et al., 1995a; Budd and Nicholls, 1995) suggesting that the Ca^{2+} channel inhibited by adenosine may be a P-type Ca^{2+} channel. A_1 receptors are G-protein linked receptors; treatment of cells with PTX reverses adenosine inhibition of glutamate release and it has been proposed that activation of A_1 G-protein linked receptors inhibits the VSCC linked to glutamate release via a membrane-delimited effect (Dolphin and Prestwich, 1985).

Relatively little information has been obtained with respect to modulation of glutamate release by type III transmitters. It has been shown, however, that neuropeptide Y (NPY) potently inhibits glutamate release from hippocampal nerve terminals (Dunwiddie et al., 1990; Greber et al., 1994).

1.8.2 Modulation by type I transmitters

Heteroregulation by GABA

Presynaptic phaclofen-sensitive $GABA_B$ autoreceptors limit the release of GABA from cerebral cortex nerve terminals (Bonanno and Raiteri, 1993; Bonanno and Raiteri, 1992; Bonanno et al., 1989). $GABA_B$ autoreceptors in the spinal cord are phaclofen-insensitive, instead being antagonised by CGP 35348 (Bonanno and Raiteri, 1993). Differing phaclofen sensitivity suggests heterogeneity among $GABA_B$ autoreceptors. Presynaptic $GABA_B$ autoreceptors may be distinct from postsynaptic $GABA_B$ receptors, activation of which increases a K^+ conductance (Gahwiler and Brown, 1985) or inhibits Ca^{2+} currents in a PTX-sensitive manner (Scholz and Miller, 1991; Potier and Dutar., 1993). Presynaptic $GABA_B$ autoreceptors on inhibitory terminals do not appear to alter K^+ conductances (Lambert et al., 1991) indicating that the mechanism by which presynaptic $GABA_B$

autoreceptors limit GABA release may be due to an inhibition of VSCC. In support of this proposal, the $GABA_B$ receptor agonist (-)baclofen inhibits KCl-evoked rises in $[Ca^{2+}]_c$ (Stirling et al., 1989) and inhibits Ca^{2+} influx into hippocampal synaptosomes via a putative G-protein membrane-delimited mechanism (Tareilus et al., 1994).

As well as presynaptic $GABA_B$ autoreceptors, there is an accumulating library of evidence showing that $GABA_B$ heteroreceptors may be located on glutamate releasing nerve terminals. Activation of these receptors with (-) baclofen, a $GABA_B$ receptor agonist, inhibits glutamate release from cerebral cortex nerve terminals in a phaclofen-insensitive, CGP 35348-sensitive manner (Pende et al., 1993; Raiteri et al., 1992; Bonanno and Raiteri, 1992). In contrast to the cerebral cortex, (-)baclofen inhibits release of glutamate from neuronal cerebellar granule cell cultures in a phaclofen-sensitive manner (Huston et al., 1990) through activation of a PTX-sensitive G-protein linked receptor (Huston et al., 1993) that may inhibit N-type and P-type VSCC linked to glutamate release (Huston et al., 1995). (-)Baclofen-mediated inhibition of excitatory synaptic transmission in the hippocampus is believed to be due to activation of presynaptic $GABA_B$ heteroreceptors which are only partially PTX-sensitive (Potier and Dutar, 1993). This implies that there may be a heterogeneity amongst presynaptic $GABA_B$ heteroreceptors.

Self-regulation

Ionotropic glutamate autoreceptors

There have been numerous reports of a stimulatory action of the non-NMDA receptor agonist kainic acid (KA) on basal and depolarisation-evoked Ca^{2+}-dependent glutamate release from various neuronal preparations (Ferkany et al., 1982; Ferkany and Coyle, 1983; Pastuszko et al., 1984; Poli et al., 1985; Terrian et al., 1991; Gannon and Terrian, 1991). Other reports do not see any Ca^{2+}-dependent effects (Pocock et al., 1988; McMahon et al., 1989), instead observing Ca^{2+}-independent effects of KA via a putative KA-mediated inhibition of the nerve terminal plasma membrane acidic amino acid carrier. Two other non-NMDA receptor agonists domoate and AMPA stimulate KCl-evoked Ca^{2+}-

dependent glutamate release from synaptosomes (Terrian et al., 1991; Sherman et al., 1992). However, some reports using hippocampal synaptosomes fail to show stimulation of glutamate release with AMPA (Zhou et al., 1995). This may be due to rapid desensitisation of an AMPA-type receptor. Cyclothiazide, an AMPA receptor desensitisation inhibitor, unmasks AMPA stimulation of [^{3}H]glutamate release from rat hippocampal synaptosomes (Barnes et al., 1994) where the AMPA effect is antagonised by the non-competitive AMPA receptor antagonist GYKI 52466.

Curiously, as well as stimulation of glutamate release by non-NMDA receptor agonists, it has been reported that KA inhibits Ca^{2+}-dependent synaptosomal glutamate release (Solyakov et al., 1992; Chittajallu et al., 1996; Barnes and Henley, 1994) and that KA and domoate (but not AMPA) both inhibit hippocampal NMDA receptor-mediated excitatory postsynaptic currents by a presynaptic mechanism; an effect which is blocked by the GluR6 kainate receptor antagonist NS-102 (Chittajallu et al., 1996). From this it has been proposed that glutamate release, at least in the hippocampus, can be differentially modulated by presynaptic AMPA-type receptors and kainate receptors containing the GluR6 subunit (Chittajallu et al., 1996), adding further complexity to hippocampal glutamatergic synaptic transmission.

A mechanism for presynaptic non-NMDA receptor modulation of glutamate release remains elusive. Early reports showed that as well as stimulation of Ca^{2+}-dependent glutamate release, KA depolarises the synaptosomal plasma membrane potential (Pastuszko et al., 1984) and elicits a rapid increase in $^{45}Ca^{2+}$ uptake by crude synaptosomal fractions, whilst having no measurable efffect on cytosolic free Na^+ levels (Pastuszko and Wilson, 1985; Pastuszko et al., 1984). It was proposed that KA depolarises the nerve terminal membrane potential via activation of a ligand-gated Ca^{2+} conducting ion channel receptor. In hippocampal synaptosomes it has been shown that AMPA and KA both elicit rises in $[Ca^{2+}]_c$ (Malva et al., 1995b), with the conclusion that the CA3 sub-region of the hippocampus is enriched in a presynaptic high-affinity kainate receptor which modulates nerve terminal Ca^{2+} influx.

Metabotropic glutamate autoreceptors

It has been reported that stimulation of a Group I mGluR with 1S,3R-ACPD facilitates 4AP-evoked Ca^{2+}-dependent glutamate release from cerebral cortex synaptosomes and this effect is thought to be due to inhibition of a nerve terminal K^+ conductance (Herrero et al., 1992). Characterisation of the group I metabotropic glutamate autoreceptor shows that mGluR activation involves a rapid and transient increase in diacylglycerol (DAG) (Herrero et al., 1994), indicating that the mGluR is linked to phosphoinositide hydrolysis and activation of protein kinase C (PKC); use of phorbol esters to chronically stimulate synaptosomal PKC causes a large facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release via inhibition of a K^+ channel (Coffey et al., 1993; Barrie et al., 1991). Group I mGluRs may be physiological activators of nerve terminal PKC. However, the transient increase in PKC activity resulting from mGluR-mediated stimulation of DAG does not lead to a facilitation of glutamate release unless a concurrent rise in arachidonic acid (AA) levels occurs in the nerve terminal (Herrero et al., 1992; Lombardi et al., 1996; Collins et al., 1995; McGahon and Lynch, 1994). AA activates PKC in the absence of Ca^{2+} and phospholipids (Murakami et al., 1986; Murakami and Routtenberg, 1985) and together with the finding that AA does not alter mGluR-mediated phosphoinositide metabolism in synaptosomes (Vazquez et al., 1994), it has been proposed that a dual synergistic stimulation of PKC by mGluRs and AA is required for sustained facilitation of glutamate release (McGahon and Lynch, 1994; Vazquez et al., 1994). Complementary to this proposal, it has been shown that AA and DAG analogues synergise to facilitate glutamate release from synaptosomes (Zhang and Dorman, 1993) and persistently enhance synaptic transmission in the hippocampus (Bramham et al., 1994).

It is not known whether the source of AA that mediates facilitation of glutamate release is presynaptic or postsynaptic. It has been proposed that during synaptic strengthening, AA could be released postsynaptically in response to a Ca^{2+}-activated PLA_2 (activated by Ca^{2+} influx through NMDA receptors) and then diffuse from the postsynaptic membrane to the presynaptic nerve terminal where it could mediate its synergistic action on terminal PKC with mGluR-mediated DAG (Lynch et al., 1991; Nicholls, 1992; Fazeli, 1992). This *retrograde messenger* action of AA has been proposed to explain results showing a link between AA,

enhanced glutamate release and synaptic potentiation (LTP) (Bliss et al., 1989; Williams et al., 1989; Lynch and Voss, 1990).

As well as these positive-feedback group I mGluRs, negative-feedback mGlu-autoreceptors which limit glutamate release have been described. Early reports of L-AP4-mediated inhibition of hippocampal synaptic transmission (Koerner and Cotman, 1981) led neuroscientists to propose the existence of a novel presynaptic *AP4* glutamate receptor. For many years it was not known whether this *AP4* receptor should be classified as ionotropic or metabotropic. However, recent cloning of four L-AP4-sensitive mGluRs, the group III mGluRs, indicates that the *AP4* glutamate receptor described in the hippocampus in the 1980's is probably a group III mGluR, perhaps mGluR7, which is thought to have a presynaptic location in the hippocampus (Shigemoto et al., 1996). Group II and group III mGluRs can be distinguished by the unique sensitivity of group III mGluRs to L-AP4 whilst Group II mGluRs can be activated by 1S,3R-ACPD. Electrophysiological data indicate the presence of mGluRs in the brain, activation of which can depress glutamatergic synaptic transmission in the rat neocortex (Burke and Hablitz, 1994), primary somatosensory cortex (Cahusac, 1994), hippocampus (Baskys and Malenka, 1991), striatum (Lovinger, 1991; Lovinger et al., 1993) and basolateral amygdala (Rainnie and Shinnick-Gallagher, 1992). In the hippocampus mGluR inhibition of excitatory transmission is developmentally regulated (Baskys and Malenka, 1991), the effect being absent in adult rat brain.

Pharmacological analysis shows that mGluR inhibition of hippocampal excitatory transmission is mediated by group II and group III mGluRs (Vignes et al., 1995). However, in the striatum it is not clear whether mGluR inhibition of excitatory transmission is mediated by only group II mGluRs (Lovinger, 1991) or by both group II and group III mGluRs (Calabresi et al., 1996). Glutamate release from striatal synaptosomes is inhibited by the group III-selective mGluR agonist L-AP4 (East et al., 1995) which suggests the presence of group III mGluRs on striatal nerve terminals. Contrasting to the hippocampus, mGluR-mediated inhibition of striatal excitatory synaptic transmission is not developmentally regulated (Lovinger and Lambert, 1993).

However, similar to inhibition of glutamate release in the hippocampus, group II and group III mGluR inhibition of glutamate release from rat cerebral

cortex synaptosomes appears to be developmentally regulated. Inhibition is present in young rats where it is proposed that L-AP4 and 1S,3R-ACPD inhibit glutamate release via activation of a PTX-sensitive G-protein linked mGluR which may inhibit a P-type or Q-type VSCC linked to glutamate release by a *membrane-delimited* mechanism (cAMP is not involved) (Vazquez et al., 1995a; Herrero et al., 1996). However, L-AP4/1S,3R-ACPD-mediated inhibition of glutamate release appears to be absent in synaptosomes prepared from adult rat cerebral cortex (Herrero et al., 1992; Vazquez et al., 1995b).

1.9 Preparations used to study glutamate release

Brain slices

The brain slice can be used for electrophysiological studies as well as neurochemical studies and must be thin enough to allow adequate oxygenation of all the brain slice tissue but at the same time be thick enough (300 - 400 microns) to minimise the proportion of the slice rendered non-functional due to mechanical damage in the preparation process. In brain slices the integral neuronal circuitry is intact enabling it to be electrically stimulated. In this way patterns of electrical stimulation may be applied to mimic physiological inputs into the brain region of study.

When using the brain slice to look at regulation of transmitter release from nerve terminals it is not possible to study regulation of release by presynaptic receptors in absolute isolation, due to the intact neuronal circuitry whereby other influences impinge on the nerve terminal. Thus, glia, postsynaptic receptors on cell bodies and an inherent heterogeneity of transmitters influencing presynaptic nerve terminal receptors could all affect nerve terminal function.

Neuronal cell cultures

Primary neuronal cultures when studying transmitter release have the distinct advantage over the brain slice in that preparation of homogenous populations of neurons is possible. This provides for a system where there will be little transmitter heterogeneity so that the release of a single transmitter can be studied without influences from other transmitters. The cerebellar granule cell

primary culture preparation is glutamatergic and can be used to study glutamate release (Van-Vliet et al., 1989; Huston et al., 1993). Primary neuronal cultures share the same problem as brain slices in having nerve terminals (neurites) still attached to a cell body, so when studying presynaptic receptor regulation of transmitter release, postsynaptic receptor influences must be considered.

Isolated nerve terminals (synaptosomes)

When a crude mitochondrial fraction from the brain, prepared by differential centrifugation through sucrose, is examined using electron microscopy it is found to contain a huge abundance of nerve terminals that have been pinched off their axons during initial brain homogenisation. Isolated nerve terminals are called synaptosomes and have a diameter of 0.5 - 1 μm. They have an intact membrane, inside of which are one or two mitochondria and a large population of SSVs. These vesicles have neurotransmitter transporters and accumulate transmitters (Maycox and Jahn, 1990).

Brain synaptosomes are best made from regions which have a clearly defined layered organisation such as the cerebral cortex and hippocampus. A 'P_2' synaptosomal pellet is contaminated with free mitochondria, myelin and broken postsynaptic membranes. One way of removing these contaminations is to purify the synaptosomes through Percoll gradients (Dunkley et al., 1986; Dunkley et al., 1988). Once prepared, synaptosomes remain viable for 6 - 8 hours, reflecting the fact that *in situ*, the nerve terminal largely operates autonomously from the cell soma[11]. The synaptosomal preparation is the simplest system in which to study the uptake, storage and release of transmitters. It has the advantage over brain slices and neuronal cell cultures in that there are no functional postsynaptic receptors on cell bodies to confuse study of presynaptic receptor regulation of transmitter release. Although amino acids can be released from synaptosomes upon electrical stimulation (Bradford, 1970a; Bradford, 1970b, Bradford et al., 1973; DeBelleroche and Bradford, 1977), the release is largely Ca^{2+}-independent in nature. Alternatively, biochemical depolarising agents (such as elevated external KCl or 4AP) have been successfully employed to study the modulation of Ca^{2+}-dependent amino acid release (exocytosis) from isolated nerve terminals (see Nicholls and Coffey, 1994).

[11] Except for a slow replacement of proteins and membrane components, which would occur physiologically by axonal transport.

1.10 Quantitation of glutamate release

Isotopic detection

Synaptosomes, brain slices or neuronal cultures can be incubated with exogenous [^{3}H]glutamate (Barnes and Henley, 1994): [^{3}H] glutamate is taken up into the nerve terminal via the membrane glutamate transporter and can be accumulated into glutamatergic synaptic vesicles. Upon depolarisation vesicles exocytose their transmitter contents and [^{3}H]glutamate is assayed by liquid scintillation spectroscopy. This method suffers from the problem that *in situ*, although [^{3}H]glutamate is taken up into the nerve terminal cytosol, synaptic vesicles incorporate [^{3}H]glutamate slowly from the cytosol with the result that vesicular release of glutamate is underestimated while any release of glutamate from the cytosol is exaggerated[12] (Nicholls and Sihra, 1986).

Exogenous *D*-[^{3}H]aspartate has been used as an indicator for glutamate release (Potashner and Gerard, 1983). Ca^{2+}-dependency is a key characteristic indicating exocytosis of a transmitter. As it has been observed that *D*-aspartate can be released in a Ca^{2+}-dependent manner on stimulation of pathways that use glutamate as a transmitter, it has been proposed that *D*-aspartate is taken up into glutamatergic synaptic vesicles. However, Nicholls and Attwell have shown that although *D*-aspartate is transported into the nerve terminal on the plasma membrane acidic amino acid carrier, it does not compete with glutamate for uptake into glutamate containing synaptic vesicles, nor is it accumulated itself (Nicholls and Attwell, 1990), therefore indicating that *D*-aspartate cannot be reliably used as a marker for glutamate release.

Endogenous amino acid detection

To avoid the problems that may be encountered when using isotopic labels, endogenous glutamate release can be measured in two ways:

Firstly, chromatographic techniques such as high performance liquid chromatography (HPLC) can be used to detect released amino acids from

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[12] Cytosolic glutamate release occurs *in vitro* due to 'reversal' of the nerve terminal plasma membrane glutamate transporter, which normally transports glutamate into the nerve terminal. This is a result of prolonged biochemical depolarisation.

synaptosomes (Pende et al., 1993), brain slices or neuronal cell cultures (Patrizio et al., 1989). This method has the advantage that absolute concentrations of glutamate can be detected. However, it is not always the method of choice due to the fact that from experiment to end calculation of glutamate release is a discontinuous process which can be very labour intensive.

Alternatively, glutamate release from synaptosomes can be quantitated using an enzyme-linked assay (Sanchez-Prieto et al., 1987; Nicholls et al., 1987; Nicholls and Sihra, 1986), whereby released glutamate is oxidised by glutamate dehydrogenase (GDH), this being coupled to nicotinamide adenine dinucleotide phosphate ($NADP^+$) reduction to NADPH. When NADPH is excited at 340 nm in a fluorimeter it emits a maximum fluorescence signal at 460 nm. Following the increase in NADPH fluorescence provides a correlate to endogenously released glutamate. This assay has the advantage over HPLC detection of released glutamate in that it is an on-line continuous process. However, with this assay, absolute amounts of released glutamate cannot be measured and calibration using a standard amount of glutamate added at the end of each experiment is required.

1.11 PhD aims

- To characterise glutamate release from rat cerebral cortex synaptosomes using established protocols.
- To address which Ca^{2+} channel subtypes mediate glutamate release in the cerebral cortex using Ca^{2+} channel toxins.
- To study the regulation of Ca^{2+}-dependent glutamate release from cerebral cortex synaptosomes by ionotropic and metabotropic glutamate auto-receptors and presynaptic $GABA_B$ heteroreceptors. Receptor mechanisms underlying regulation of release will be investigated.

CHAPTER TWO

2 Methods and Materials

2.1 Preparation of rat brain synaptosomes

Reagents and equipment

- Sucrose - 320 mM.
- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- 23%, 10% and 3% Percoll gradient solutions containing sucrose (320 mM), EDTA (1 mM) and dithiothreitol (0.25 mM)-pH 7.4.
- Motor driven (Potter-Elvejham) Teflon pestle and glass tube.
- Hand-operated Dounce Pyrex glass pestle and glass tube.

P_2 method (carried out at 4 °C)

Adapted from McMahon and Nicholls (1991).

- A rat was sacrificed (200-250g ; Sprague-Dawley) and the brain was rapidly removed and placed into sucrose (320mM). The cerebral cortices were dissected and homogenised (7-8 up/down strokes) in 10 ml sucrose (320 mM) using a Potter-Elvehjam teflon pestle, motor driven at 900 rpm.

Male

- The homogenate was centrifuged at 3,000 x g for 2 min in 40 ml sucrose (320 mM). The pellet (P_1) was discarded and the supernatant (S_1) was recentrifuged at 14,500 x g for 10 min.The S_2 supernatant was discarded and the P_2 pellet was kept.
- 4 - 5 ml of sucrose (320 mM) was added to the P_2 pellet and a gentle swirling motion used to dislodge the white loosely compacted upper layer of the pellet which contains the synaptosomes; the darker mitochondrial layer beneath was left undisturbed. The synaptosomal suspension produced was removed and the protein concentration determined using a Bradford assay (Bradford, 1976). P_2 protein concentration was typically 8-10 mg per rat. 0.5 mg P_2 synaptosomal pellets were prepared by centrifugation through 4 volumes HBM (pH 7.4) at 10,000 x g for 1 min in a benchtop centrifuge.
- Pellets were used within 6 hours of preparation.

Percoll method

Adapted from Dunkley et al., (1986).

This method is the same as the P_2 but with the following additional steps and modifications:

- Percoll gradients (3-step) were prepared into 4 x 12 ml tubes; 2.5 ml of 23% Percoll was pipetted into the bottom, followed by layering of 2.5 ml of 10% Percoll and finally 2.5 ml of 3% Percoll.
- The entire P_2 pellet was resuspended in 8 ml of sucrose (320 mM). 2 ml of P_2 resuspension was layered on top of a 3-step Percoll gradient (x4). The four Percoll gradients were centrifuged at 34,000 x g for 5 min (from speed).
- The synaptosomes layered as a single band at the 23%/10% Percoll interface. The layer above this contained myelin and light membranes, and the layer at the bottom of the Percoll gradient tube contained mitochondria. For each of the four Percoll gradients, the myelin and light membrane layer was aspirated and discarded. The synaptosomal layers were harvested, combined and centrifuged at 27,000 x g for 10 min with 40 ml HBM (pH 7.4) in order to wash the Percoll away.
- The resulting pellet was gently resuspended in HBM (pH 7.4) using a hand-operated Dounce-type (glass pestle) glass homogeniser. Protein concentration was determined using the Bradford Assay. The protein yield was typically 2.5 - 3.5 mg per rat. 0.25 mg Percoll-purified synaptosomal pellets were prepared by centrifugation through approx. 30 volumes HBM (pH 7.4) at 3,000 x g for 10 min .
- Pellets were used within 6 hours of preparation.

2.2 Measurement of glutamate release

A. Enzyme-linked assay

Reagents

- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- BSA (16 μM), $CaCl_2$ (1 mM), EGTA (200 μM), $NADP^+$ (2 mM), GDH (50

units/ml), KCl, 4AP and glutamate (5 nmol).

Method

(Used with 0.5 mg P_2 and 0.25 mg Percoll-purified synaptosomes).

- Synaptosomes were resuspended in HBM (pH 7.4) containing 16 μM BSA and stirred in a thermostatted water bath at 37 °C. After 40 min incubation, synaptosomes were centrifuged for 1 min at 10,000 x g in a benchtop centrifuge. Synaptosomal pellets were resuspended with fresh HBM (minus BSA) (pH 7.4) and incubated in a stirred and thermostatted cuvette at 37 °C in a Perkin-Elmer LS-5 spectro-fluorimeter.

  Glutamate release was assayed by on-line fluorimetry (Nicholls and Sihra, 1986; Nicholls et al., 1987) as follows;

- $CaCl_2$ (1mM), $NADP^+$ (2 mM) and GDH (50 units/ ml) were added after 3 min (addition times of any *drugs* used are detailed in the results figure legends). After a further 7 min of incubation, KCl or 4AP was added to depolarise the synaptosomes. Fluorescence was monitored at excitation and emission wavelengths of 340 nm and 460 nm respectively and data accumulated at 2 s intervals. A standard (Std) of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence response monitored. The value of the fluorescence change produced by the standard was used to calculate the released glutamate as nmol glutamate/mg synaptosomal protein (nmol/mg). Cumulative data was analysed using Lotus 1-2-3.

- Cumulative glutamate (glu) release calculation:

$$\text{Glu release}_{\text{nmol/mg}} = \frac{\text{Sample fluorescence}}{\text{Glu Std fluorescence}} \times 5 \text{ nmol} \times \frac{1}{\text{synaptosomal protein (mg/ml)}}$$

Determination of Ca^{2+}-dependent glutamate release

- Synaptosomes contain a pool of cytosolic glutamate some of which is released upon depolarisation of synaptosomes with KCl or 4AP. This Ca^{2+}-independent release is measured by incubating synaptosomes in the absence of $CaCl_2$ (1mM) and by adding EGTA (200 μM) 1 min before depolarisation with KCl or 4AP (Total (Ca^{2+}-dependent & Ca^{2+}-independent)

glutamate release was in the presence of 1 mM $CaCl_2$). Once quantitated, cumulative Ca^{2+}-independent release data was subtracted from cumulative total release data using Lotus 1-2-3. This evaluates net Ca^{2+}-dependent release data resultant from exocytosis of SSVs containing glutamate.

B. Endogenous amino acid release analysis by HPLC

Reagents & Buffers

- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- BSA (16 μM), $CaCl_2$ (1 mM), KCl or 4AP.
- Sodium phosphate (12.5 mM; pH 7.2), acetonitrile, sulphosalicylic acid (SSA) (25%), *o*-Phthaldialdehyde (OPA)/mercaptoethanol (MCE) mix (5 mg/ml OPA with 5 μl/ml MCE), neutralising buffer mix (NB mix) (0.2 M Na-borate/5 M NaOH titrated to neutralise 4.1% SSA), internal standard stock containing norvaline (56μM), initial calibration mix containing reference amino acids; aspartate (5.3 μM), glutamate (5.6 μM), alanine (5.5 μM), GABA (5.5 μM) and internal standard norvaline (5.6 μM).

Method

Adapted from Graser et al., (1985).

- Synaptosomes were incubated as described for the enzyme-linked glutamate release protocol. However, $NADP^+$, GDH and a glutamate standard were not added and instead of being incubated in a fluorimeter, synaptosomes were incubated in a stirred and thermostatted cuvette at 37 °C in a water bath.
- After depolarisation, synaptosomes were centrifuged for 1 min at 10,000 x g and the supernatant (500μl) was rapidly aspirated and added to tubes containing 100 μl 25% SSA with 71.1 μl 56 μM internal standard stock (120 μl 560 μM norvaline, 100 μl 10X HBM, 780 μl H_20 and 200 μl 25% SSA). Acidified samples were immediately vortexed and frozen in liquid nitrogen to facilitate protein precipitation.
- Frozen samples were thawed at 4 °C and centrifuged at 10,000 x g for 5 min. Aliquots (600 μl) of deproteinated supernatant were removed and neutralised with 36 μl of NB mix (0.2 M Na-Borate/5 M NaOH titrated to

neutralise 4.1% SSA). Samples were vortexed and 100 µl aliquots loaded on a Gilson 231XL autosampler for automatic derivatisation with 100 µl *o*-Phthaldialdehyde (OPA)/mercaptoethanol (MCE) mix (5 mg/ml OPA with 5 µl/ml MCE). 20 µl of derivatised sample was injected via an automatic loop injector followed by reversed phase chromatography on a Beckman Ultrasphere C18 column (4.6 mm x 15 cm). Amino acid-OPA adducts were resolved by gradient elution with Buffer A (12.5 mM sodium phosphate buffer; pH 7.2) and Buffer B (acetonitrile) using a pair of Beckman System Gold 125 pumps. Amino acid-OPA adducts were detected post-column using a Shimadzu RF-535 fluorometric detector. Fluorescence data was accumulated and analysed using System Gold software.

Quantitation of sample amino acids

- System Gold software computes the retention time and peak area of detected sample amino acid-OPA adducts and relates them to those of reference amino acid (ref a-a) standards set up in an initial calibration experiment. In the initial calibration, the peak area associated with the fixed amount of internal standard norvaline (nval) is used to calculate the relative peak area ratio, termed the response factor (RF), for each reference amino acid peak using the formula;

$$RF = \frac{\text{ref a-a (nmol)}}{\text{nval (nmol)}} \times \frac{\text{peak area nval}}{\text{peak area ref a-a}}$$

The following formula is then used to calculate released amino acid glutamate (Glu) as nmol glutamate/mg synaptosomal protein (nmol/mg);

$$Glu = \frac{\text{peak area glu in sample}}{\text{peak area nval in sample}} \times \text{nval in sample (nmol)} \times RF$$

Glutamate release data from both the enzyme-linked assay and HPLC analysis is expressed in results chapters as nmol/mg protein/x min, where /x min is not a rate but describes the amount of glutamate released after x min.

2.3 Cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, measurements

Reagents

- Fura-2 AM (5 μM).
- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- BSA (16 μM), $CaCl_2$ (0.1 mM and 1 mM), KCl or 4AP.
- Sodium dodecyl sulphate (SDS) (0.4%) and Na-EGTA/1M tris (5 mM).

Introduction

- A popular method for determining intracellular free Ca^{2+} concentration uses fura-2 or a related fluorescent Ca^{2+} chelator. Ca^{2+} binding is a function of the free Ca^{2+} concentration and the dissociation constant (K_d) for the chelator; in the case of fura-2 half maximal binding occurs at 224 nM at 37 °C. When fura-2 binds Ca^{2+} the excitation spectrum of fura-2 changes (an increase in fluorescence at 340 nm and a decrease at 380 nm is observed). This enables the proportion of free fura-2 and Ca^{2+}-bound fura-2 (and hence the Ca^{2+} concentration) to be calculated from the ratio of emission intensity following excitation at 340 nm and 380 nm. An important feature of this ratiometric determination is that the signal is independent of dye loading or variations in the size of the cell.

  As the free acid, fura-2 possesses five carboxyl groups, making the molecule hydrophilic and therefore membrane impermeant. If each of these carboxyl groups are esterified to form acetoxymethyl (AM) esters, the resultant fura-2 AM becomes sufficiently hydrophobic to cross the plasma membrane. Once in the cell cytoplasm non-specific esterases cleave off the AM groups sequentially, regenerating fura-2 which is too hydrophilic to escape from the cell. After typically a 30 min incubation period much of the original fura-2 AM has accumulated in the cytoplasm as free fura-2. Any remaining fura-2 AM can be washed away.

Method

Adapted from Komulainen and Bondy (1987).

- Synaptosomes were preincubated in HBM/BSA(16μM) containing 5 μM fura-2 AM and 0.1 mM $CaCl_2$ for 35 min at 37 °C in a stirred cuvette. After fura-2 loading, synaptosomes were centrifuged in a microcentrifuge for 1

min at 10,000 x g. The pellet was resuspended in HBM (minus BSA) at 37 °C and the synaptosomal suspension stirred in a thermostatted cuvette in a Perkin-Elmer LS-5 spectrofluorimeter.

- $CaCl_2$ (1 mM) was added after 3 min (addition times of any *drugs* used are detailed in the results figure legends). After a further 7 min incubation, synaptosomes were depolarised with KCl or 4AP. Fluorescence data was accumulated at excitation wavelengths of 340 nm and 380 nm (emission wavelength 505 nm) at 3.5 s intervals. At the end of each experiment, a calibration was performed with 0.4% SDS and 5 mM Na-EGTA/1M tris* to give a maximum and a minimum fluorescence ratio signal respectively. Cumulative data was analysed using Lotus 1-2-3.

  Free $[Ca^{2+}]_c$ (nM) was calculated from the following equation previously described (Grynkiewicz et al., 1985);

$$[Ca^{2+}]_c = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where K_d = 224 nM at 37°C, R = experimental fluorescence ratio value (340/380), R_{min} = minimum fluorescence ratio value, R_{max} = maximum fluorescence ratio value, S_{f2} = minimum fluorescence value at 380 nm and S_{b2} = maximum fluorescence value at 380 nm.

*At pH known to neutralise the increase in $[H^+]$ consequent from EGTA binding of 1 mM $CaCl_2$.

2.4 Cytosolic free Na^+ measurements

Reagents

- SBFI AM (5 μM).
- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- BSA (16 μM), $CaCl_2$ (0.1 mM and 1 mM), KCl or 4AP.

Method

- SBFI AM is a Na^+ sensitive fluorescent dye. Experiments followed the protocol for free $[Ca^{2+}]_c$ measurements with fura-2 AM. Because calibration procedures for the quantitation of free $[Na^+]_c$ are not reliable, a calibration was not performed and thus free cytosolic Na^+ concentration was not

calculated. Instead, results are expressed as experimental fluorescence ratio values (340/380 ratio). Cumulative data was analysed using Lotus 1-2-3.

2.5 Membrane potential measurements using $DiSC_2(5)$

Reagents

- $DiSC_2(5)$ (4 μM).
- Hepes buffered medium (HBM) - (composition in mM: NaCl, 140; KCl, 5; $NaHCO_3$, 5; $MgCl_2.6H_20$, 1; Na_2HPO_4, 1.2; Glucose, 10; Hepes, 20)-pH 7.4.
- BSA (16 μM), $CaCl_2$ (1 mM), KCl or 4AP.

Method

- Synaptosomes were resuspended in HBM (pH 7.4) containing 16 μM BSA and stirred in a thermostatted water bath at 37 °C. After 40 min incubation, synaptosomes were centrifuged for 1 min at 10,000 x g in a benchtop centrifuge. Synaptosomal pellets were resuspended with fresh HBM (minus BSA) (pH 7.4) and incubated in a stirred and thermostatted cuvette at 37 °C in a Perkin-Elmer LS-5 spectro-fluorimeter.
- After 1.5 min incubation, 4 μM $DiSC_2(5)$ was added to the synaptosomes and allowed to equilibrate before the addition of $CaCl_2$ (1mM) at 3 min (addition times of any *drugs* used are detailed in the results figure legends). KCl or 4AP was added to depolarise the synaptosomes at 7.5 min and $DiSC_2(5)$ fluorescence was monitored at excitation and emission wavelengths of 646 nm and 674 nm respectively and data accumulated at 2 s intervals. Cumulative data was analysed using Lotus 1-2-3 and results are expressed in fluorescence units.

2.6 Reagents list

Name	Abbreviation	Source
Acetonitrile		BDH
γ-aminobutyric acid	GABA	Sigma
(1S,3R)-1-Amino cyclopentane-1,3-dicarboxylic acid	(1S,3R)-ACPD	Tocris Cookson
(S)-α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid	AMPA	Tocris Cookson
L(+)-2-Amino-4-phosphono-butyric acid	L-AP4	Tocris Cookson
4-aminopyridine	4AP	Sigma/RBI
Arachidonic acid	AA	Sigma/RBI
R(+)-Baclofen HCl	(-)Baclofen	RBI
S(-)-Baclofen HCl	(+)Baclofen	RBI
Bovine Serum Albumin (essentially fatty acid free)	BSA	Sigma
Bradfords Reagent		Biorad
8-Bromo-cAMP sodium	8-Br-cAMP	Sigma
$CaCl_2$		BDH
CGP 35348		Gift from Ciba Geigy Ltd.
ω-Conotoxin GVIA (Conus geographus)	ω-CTx GVIA	alomone labs/ TCS Biologicals Ltd.
ω-Conotoxin MVIIC (Conus magus)	ω-CTx MVIIC	Bachem (UK)
6-Cyano-7-nitroquinoxaline-2,3-dione	CNQX	Tocris Cookson
Cyclothiazide		Tocris Cookson
3,3'-diethyl-thiadicarbo-cyanine iodide	$DiSC_2(5)$	Molecular Probes, Inc.
Domoic acid	domoate	Tocris Cookson
Ethylene glycol-bis (β-amino-ethyl ether) N,N,N',tetra acetic acid	EGTA	Sigma
Fura-2 AM (cell permeable)	Fura-2	Molecular Probes, Inc.
D+Glucose		BDH
L-Glutamate Dehydrogenase (type II from bovine liver)	GDH	Sigma
L-glutamic acid	Glu	Sigma
GYKI 52466-HCl		RBI
Ionomycin, *Streptomyces Conglobatus*	Ionomycin	Calbiochem Corporation
Kainic acid	KA	Tocris Cookson
KCl		BDH
β-Nicotinamide adenine dinucle-otide phosphate	$NADP^+$	Sigma
Nimodipine		RBI
Norvaline	NVal	Sigma
NS-102		RBI
o-Phthaldi-aldehyde	OPA	Fluka/ Sigma
Orthoboric acid		BDH
Percoll		Pharmacia
Pertussis Toxin (Islet-activating protein)	PTX	Gift from Dr Karen Page RFHSM, UK.
Phaclofen		Tocris Cookson
Phorbol 12,13-Dibutyrate	PDBu	RBI
Rp-cAMPS triethylamine	Rp-cAMPS	RBI
SBFI AM (cell permeable)	SBFI	Molecular Probes, Inc.
Sucrose		BDH
Tetrodotoxin	TTX	Sigma

CHAPTER THREE

3 Characterisation of glutamate release from cerebral cortex nerve terminals (synaptosomes)

Introduction

Glutamate release from synaptosomes can be evoked by biochemical depolarisation of the synaptosomal plasma membrane potential with KCl (30mM) (Nicholls and Sihra, 1986) or the K^+ channel blocker 4-aminopyridine (4AP) (Tibbs et al., 1989b). Depolarisation leads to the opening of voltage-sensitive Ca^{2+} channels (VSCC) and Ca^{2+} influx into the nerve terminal (Tibbs et al., 1989a; McMahon and Nicholls, 1991). The 'Calcium Hypothesis' of SSV transmitter release (Zucker and Fogelson, 1986) predicts that high local cytosolic Ca^{2+} levels, $[Ca^{2+}]_c$, close to active zones where small synaptic vesicles (SSVs) containing transmitter cluster, trigger exocytosis of SSVs. The high local $[Ca^{2+}]_c$ hypothesis of SSV transmitter release is substantiated by neurochemical analysis of the Ca^{2+}-dependency of glutamate release from synaptosomes (Verhage et al., 1991).

The presynaptic Ca^{2+} channels that mediate glutamate release from cerebral cortex nerve terminals have been studied using Ca^{2+} channel toxins. In cerebral cortex synaptosomes, [^{3}H]glutamate release is inhibited by the P-type and Q-type VSCC toxins ω-Aga IVA and ω-conotoxin MVIIC (ω-CTx MVIIC) (Turner et al., 1992; Turner et al., 1993; Turner et al., 1995). ω-Aga IVA partially (50%) inhibits glutamate release and when ω-CTx MVIIC is applied to ω-Aga IVA-treated synaptosomes, further inhibition is seen (30-40%) (Turner et al., 1995). ω-Aga IVA potently blocks P-type VSCC but is 100 fold less effective at blocking Q-type VSCC. ω-CTx MVIIC blocks Q-type VSCC with 10 fold more sensitivity than its blocking action at P-type VSCC (Sather et al., 1993). Synaptosomal studies with ω-Aga IVA and ω-CTx MVIIC seem to indicate that P-type and Q-type VSCC control glutamate release from cerebral cortex nerve terminals. It has been proposed that ω-Aga IVA and ω-CTx MVIIC both inhibit a presynaptic Ca^{2+} channel containing the class A Ca^{2+} channel α_1 subunit (Malva et al., 1995a).

Studies to date have not shown any significant inhibition of glutamate release from cerebrocortical synaptosomes by the N-type VSCC toxin, ω-conotoxin GVIA (ω-CTx GVIA) (Turner et al., 1992) or by dihydropyridines (DHPs) (Pocock and Nicholls, 1992), which block L-type VSCC. This indicates that N-type and L-

type VSCC are not involved in controlling glutamate release in the cerebral cortex.

Glutamate release from synaptosomes can be measured using an enzyme-linked assay (Nicholls and Sihra, 1986; Nicholls et al., 1987). Synaptosomes contain a cytosolic pool of glutamate as well as vesicular glutamate. In glutamate release experiments some of this cytosolic glutamate is released upon depolarisation with KCl or 4AP. This cytosolic release is Ca^{2+}-independent and not believed to be physiologically important. It is measured by performing experiments in the absence of Ca^{2+} and presence of the Ca^{2+} chelator, EGTA (see Methods & Materials section 2.2.A). Once determined, the Ca^{2+}-independent release is subtracted from release in the presence of Ca^{2+} to provide a calculated net Ca^{2+}-dependent release. Ca^{2+}-dependent release is considered to be a result of exocytosis of SSVs containing glutamate.

The VSCC types that are coupled to glutamate transmission in the cerebral cortex have not been fully characterised, i.e. despite the results of Turner et al (1992) that show no inhibition of glutamate exocytosis from cerebrocortical synaptosomes by ω-CTx GVIA, [^{125}I]ω-CTx GVIA receptors are abundant in the cerebral cortex, which are believed to correspond to N-type VSCC (Gohil et al., 1994). A significant proportion of these receptors may have a presynaptic location. Thus, participation of N-type VSCC in glutamate exocytosis cannot be ruled out. Furthermore, there is a large population of [^{125}I]ω-CTx MVIIC receptor sites in the cerebral cortex that are not displaced by ω-CTx GVIA (Filoux et al.,1994). ω-CTx MVIIC blocks P-type, Q-type and N-type VSCC indicating that all three of these VSCC may participate in excitation-secretion coupling in the cerebral cortex. However, there are few data to show what effects this toxin has on transmitter release.

Results in this chapter show that KCl and 4AP both evoked similar levels of Ca^{2+}-dependent glutamate release but by two different mechanisms. KCl-evoked release was insensitive to the Na^+ channel blocker tetrodotoxin (TTX), whilst 4AP-evoked release was potently blocked by TTX; KCl elicits release via a 'clamped' depolarisation of the synaptosomal plasma membrane potential which rapidly inactivates Na^+ channels whereas 4AP causes asynchronous depolarisation due to repetitive firing of TTX-sensitive Na^+ channels (Nicholls and Coffey, 1994). The DHP, nimodipine did not inhibit KCl-evoked glutamate release

✳ (concentration known to chelate extrasynaptosomal Ca^{2+} (Nicholls and Sihra, 1986))

confirming previous reports that L-type VSCC do not control glutamate release in the cerebral cortex (Pocock and Nicholls, 1992). However, contrary to previous findings, ω-CTx GVIA, a selective N-type VSCC blocker, partially inhibited KCl-evoked glutamate release and inhibited KCl-evoked Ca^{2+} influx into synaptosomes. Studies also showed that ω-CTx MVIIC potently and reversibly inhibited KCl-evoked and 4AP-evoked glutamate release in a dose-dependent manner. Inhibition of Ca^{2+}-dependent glutamate release was 100% at 2 - 4 μM ω-CTx MVIIC. Complementary to the release data, ω-CTx MVIIC dose-dependently inhibited KCl-evoked and 4AP-evoked Ca^{2+} influx, whilst not affecting ionomycin-induced rises in $[Ca^{2+}]_c$. The data indicat that glutamate release from cerebral cortex nerve terminals is controlled by N-type and P/Q-type VSCC but not by L-type VSCC.

Results and discussion

KCl (30mM) evoked the release of 9.0 ± 0.5 nmol glutamate/mg protein/5 min in the presence of added $CaCl_2$ (1 mM) (Fig.1.i) and 3.0 ± 0.1 nmol glutamate/mg protein/5 min in the absence of external Ca^{2+} but presence of EGTA (200μM) (Fig.1.ii). Net KCl-evoked Ca^{2+}-dependent glutamate release was 5.8 ± 0.6 nmol/mg protein/5 min (Fig.1.iii). The glutamate release evoked by addition of 4AP (3 mM) in the presence of 1 mM Ca^{2+} was 8.9 ± 0.5 nmol/mg protein/5 min (Fig.2.i), similar to that evoked by KCl (30mM). However, 4AP-evoked glutamate release in the absence of external $CaCl_2$ (1 mM) but presence of EGTA was less than that obtained with KCl (1.5 ± 0.2 nmol/mg protein/5 min) (Fig.2.ii). Ca^{2+}-dependent glutamate release with 4AP (3 mM) was thus 7.45 ± 0.3 nmol/mg protein/5 min (Fig.2.iii), higher than the Ca^{2+}-dependent release evoked by KCl. This greater Ca^{2+}-dependent glutamate release with 4AP was not always apparent in experiments (data not shown) and thus may not be significant.

High KCl (30mM) and 4AP (3 mM) depolarise synaptosomes by two different mechanisms which can be distinguished using the Na^+ channel blocker, tetrodotoxin (TTX). In one set of experiments, KCl (30mM)-evoked glutamate release in the presence of 1 mM Ca^{2+} was 11.8 ± 0.4 nmol/mg protein/5 min (Fig.3.A.i). Preincubation of synaptosomes with TTX (2 μM) before KCl addition did not significantly alter glutamate release (11.3 ± 0.75 nmol /mg protein/5 min)

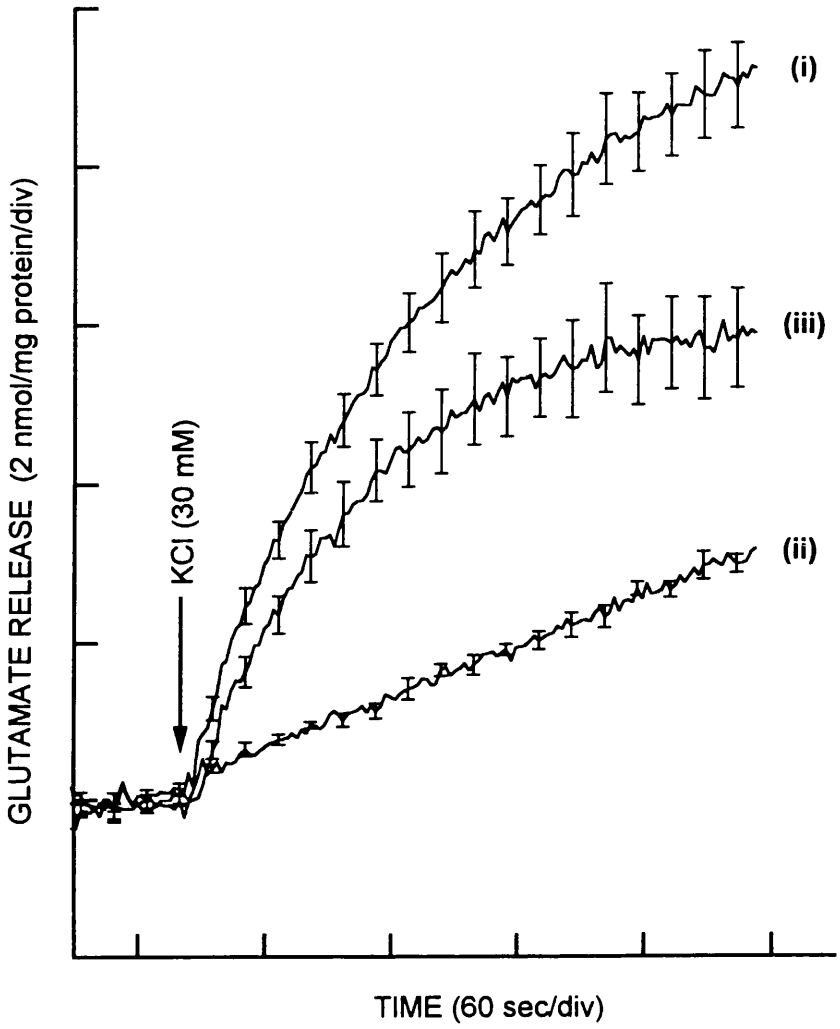

Fig.1. **KCl-evoked glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of KCl (30mM) in the presence of $CaCl_2$ (1mM) (i) or absence of 1mM Ca^{2+} (presence of 200 μM EGTA) (ii); (iii) Net Ca^{2+}-dependent glutamate release calculated by subtracting (ii) from (i). Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

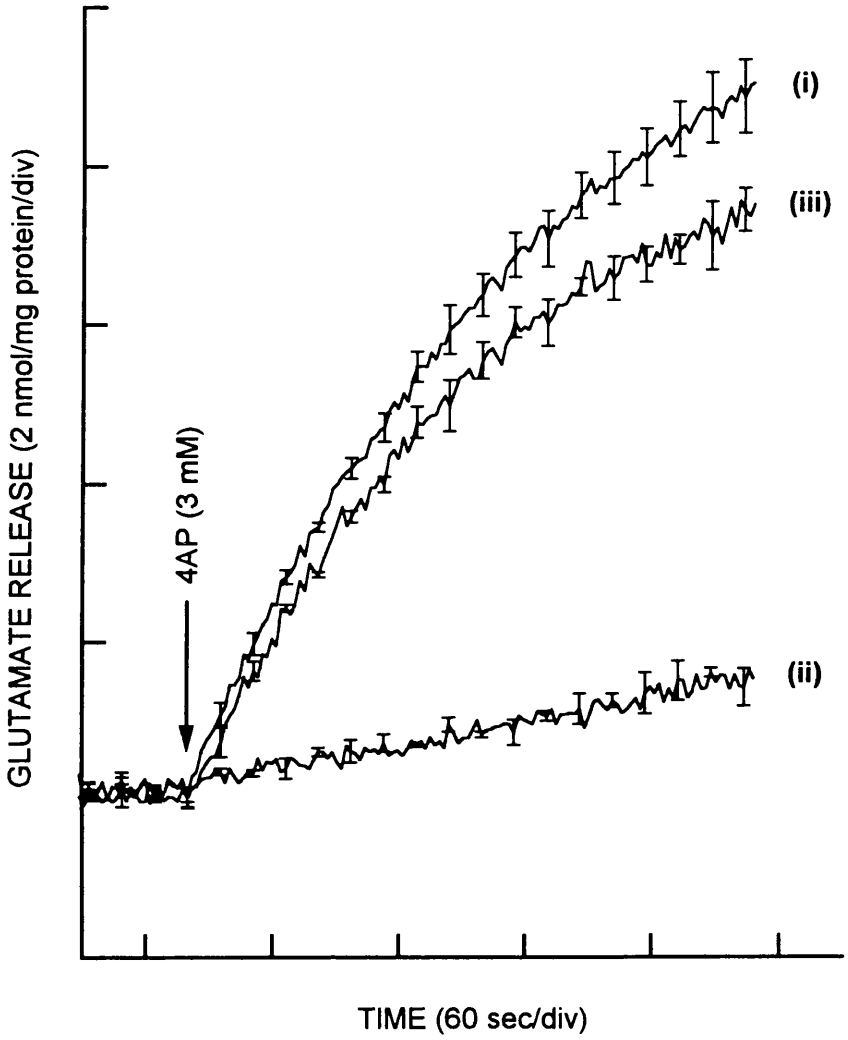

Fig.2. **4AP-evoked glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (3mM) in the presence of $CaCl_2$ (1mM) (i) or absence of 1mM Ca^{2+} (presence of 200 μM EGTA) (ii); (iii) Net Ca^{2+}-dependent glutamate release calculated by subtracting (ii) from (i). Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

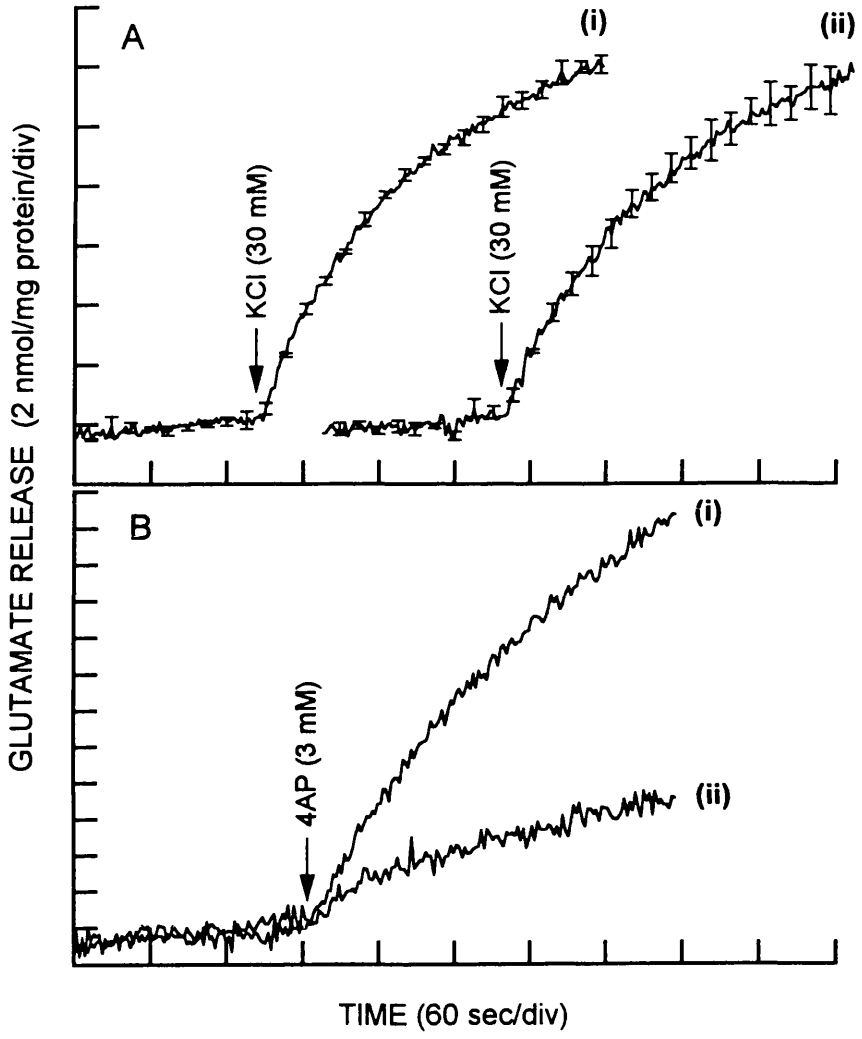

Fig.3. **TTX inhibits 4AP-evoked glutamate release but does not affect KCl-evoked glutamate release.** P_2 synaptosomes were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of KCl (30 mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with TTX (2 μM) for 3 min before KCl addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity. (B) (i) Glutamate release evoked by addition of 4AP (3 mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with TTX (2 μM) for 3 min prior to 4AP addition. Traces are single representative experiments.

* (although a $[Ca^{2+}]_c$ 'spike' with 4AP depolarisation can often be detected (Sihra et al; 1995). The absence or presence of a 4AP-evoked $[Ca^{2+}]_c$ 'spike' may depend on the fluorimetric instrumentation in which $[Ca^{2+}]_c$ measurements are made.)

(Fig.3.A.ii). In contrast, in a second set of experiments control 4AP (3 mM)-evoked glutamate release (22 nmol/mg protein/5 min) (Fig.3.B.i) was potently inhibited by preincubation with 2 μM TTX (7 nmol/mg protein/5 min) (Fig.3.B.ii).

KCl (30mM)-evoked release is not sensitive to TTX because it causes a 'clamped' membrane depolarisation for the duration of its application; TTX-sensitive Na^+ channels rapidly inactivate and do not contribute to KCl-evoked depolarisation. In contrast, 4AP-evoked glutamate release is potently inhibited by TTX supporting the belief that 4AP-mediated inhibition of nerve terminal K^+ channels destabilises the synaptosomal membrane potential to an extent that TTX-sensitive Na^+ channels fire repetitively to cause depolarisation (Tibbs et al., 1989a; Nicholls and Coffey, 1994).

Glutamate exocytosis (Ca^{2+}-dependent release) occurs when depolarisation causes voltage-dependent activation of Ca^{2+} channels that are linked to release; extracellular Ca^{2+} flows into the nerve terminal and triggers exocytosis of SSVs containing glutamate (Verhage et al., 1991). Depolarisation-induced rises in nerve terminal free cytosolic Ca^{2+} levels, $[Ca^{2+}]_c$, resultant from extracellular Ca^{2+} influx through voltage-senstive Ca^{2+} channels (VSCC) can be assessed in synaptosomes with fura-2 (Komulainen and Bondy, 1987). Elevation in $[Ca^{2+}]_c$ occurred after addition of KCl (30mM) and then followed a complex time-course (Fig.4.i). An initial $[Ca^{2+}]_c$ 'spike' reached 403 ± 5 nM and then declined over the course of 1 -2 min to a steady-state 'plateau' $[Ca^{2+}]_c$ of 350 ± 3 nM/5 min. 4AP (3 mM) evoked an increase in $[Ca^{2+}]_c$ lacking an initial $[Ca^{2+}]_c$ 'spike' phase (Fig.4.ii). The 4AP-evoked 'plateau' $[Ca^{2+}]_c$ was 368 ± 7 nM/5 min, similar to that elicited by KCl.

It is thought that Ca^{2+} influx through transiently activated VSCC is not a dominant factor in the release of glutamate, i.e. the transient Ca^{2+} 'spike' seen with KCl does not support release. In favour of this argument, it has been shown that while predepolarisation of synaptosomes prior to addition of Ca^{2+} removes the KCl-evoked $[Ca^{2+}]_c$ 'spike' leaving the 'plateau' phase of Ca^{2+} entry, the latter fully supports glutamate release (McMahon and Nicholls, 1991). A second line of evidence indicating that the transient Ca^{2+} 'spike' does not support glutamate release comes from the observation that 4AP, which produced little or no $[Ca^{2+}]_c$ 'spike' (Fig.4.ii) causes similar (if not greater) Ca^{2+}-dependent glutamate release

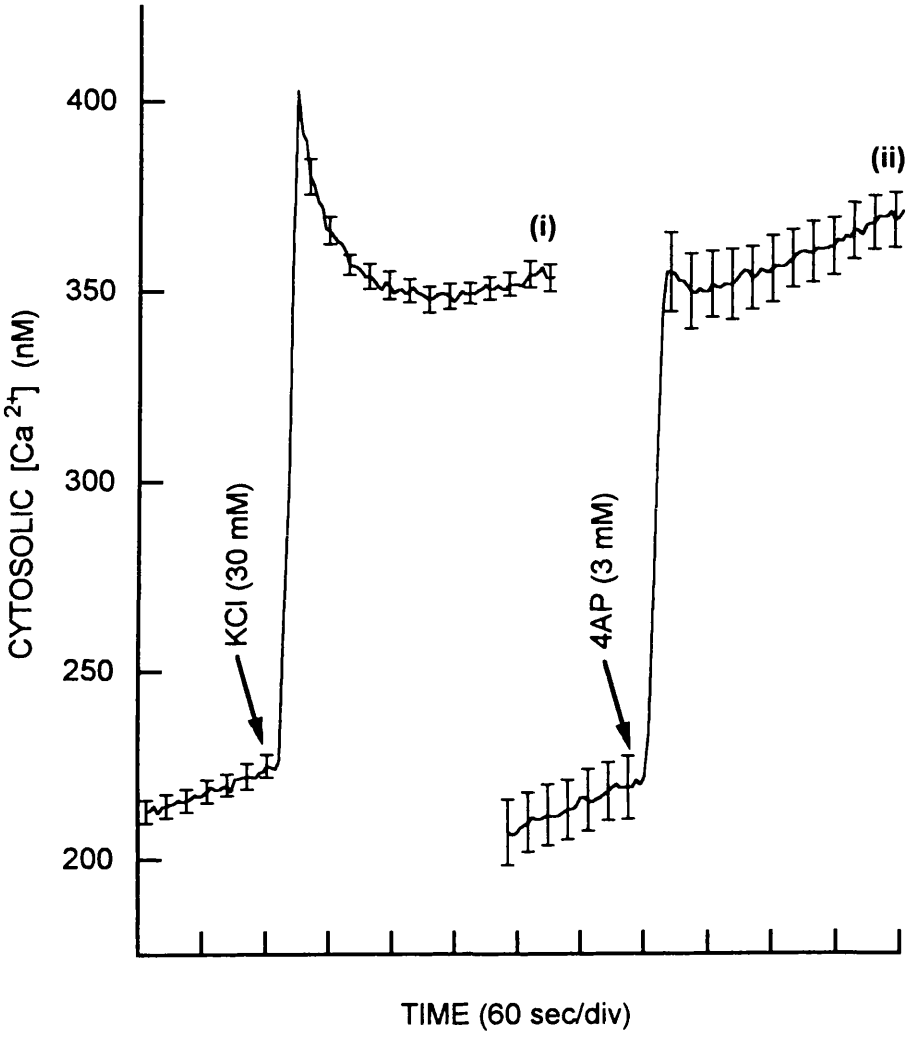

Fig.4. **Increased $[Ca^{2+}]_c$ following depolarisation with KCl and 4AP.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. Elevation in $[Ca^{2+}]_c$ occurred after addition of KCl (30mM) (i) or 4AP (3mM) (ii). Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 20 s for clarity.

to that elicited by KCl. Glutamate release evoked by both 4AP and KCl shows a rapid component, followed by an extensive slow phase (McMahon and Nicholls, 1991). Because 4AP produces no Ca^{2+} 'spike', but has a similar profile of release to that of KCl, it is likely that both release phases occur as a result of Ca^{2+} influx through non-inactivating Ca^{2+} channels (seen experimentally as a steady-state $[Ca^{2+}]_c$ 'plateau'). Indeed Ca^{2+}-dependent glutamate release evoked by pre-depolarisation with 30 mM KCl (to remove the Ca^{2+} 'spike') shows both the rapid phase and slow phase, further emphasising that transient 'spike' Ca^{2+} entry does not contribute to any phase of release (McMahon and Nicholls, 1991). In the present studies, predepolarisation release protocols remove transient Ca^{2+} influx and glutamate release evoked in the absence of a KCl-mediated transient Ca^{2+} 'spike' (10.2 ± 0.8 nmol/mg protein/5 min) (Fig.5. KCl Ca^{2+} -) was similar to release evoked in its presence (10.7 ± 1 nmol/mg protein/5 min) (Fig.5.Ca^{2+} KCl -). This result substantiates the previous findings that transient Ca^{2+} influx does not support glutamate release and that it is the influx of Ca^{2+} through non-inactivating Ca^{2+} channels which controls release.

Attempts have been made to characterise the presynaptic non-inactivating VSCC that are linked to exocytosis of glutamate. The L-type VSCC is a possible candidate as it has very slow inactivation kinetics and large unitary conductances. L-type VSCC are uniquely blocked by dihydropyridines (DHPs). Thus, to assess the contribution of the L-type VSCC in the control of glutamate release, the ability of DHPs to block glutamate release can be studied. The results of one such experiment are shown in Fig.5. KCl (30mM) evoked the release of 10.7 ± 1 nmol glutamate/mg protein/5 min in the presence of 1 mM Ca^{2+} (Fig.5. Ca^{2+} KCl -). Preincubation with the DHP, nimodipine (3 μM), before KCl addition did not alter glutamate release (11 nmol/mg protein/5 min) (Fig.5. Ca^{2+} KCl nim). However, it has previously been demonstrated that DHPs inhibit glutamate release most effectively under predepolarisation conditions (Huston et al., 1990), i.e. DHPs bind to open Ca^{2+} channels. Predepolarisation protocols can be used to activate and open VSCC, with subsequent release of glutamate upon addition of 1 mM Ca^{2+}. Glutamate release evoked using a predepolarisation protocol, where Ca^{2+} is added to synaptosomes 1 min after KCl (30mM) (Fig.5. KCl Ca^{2+} -), amounted to 10.2 ± 0.8 nmol/mg protein/5 min. Under these conditions, Ca^{2+}-evoked release

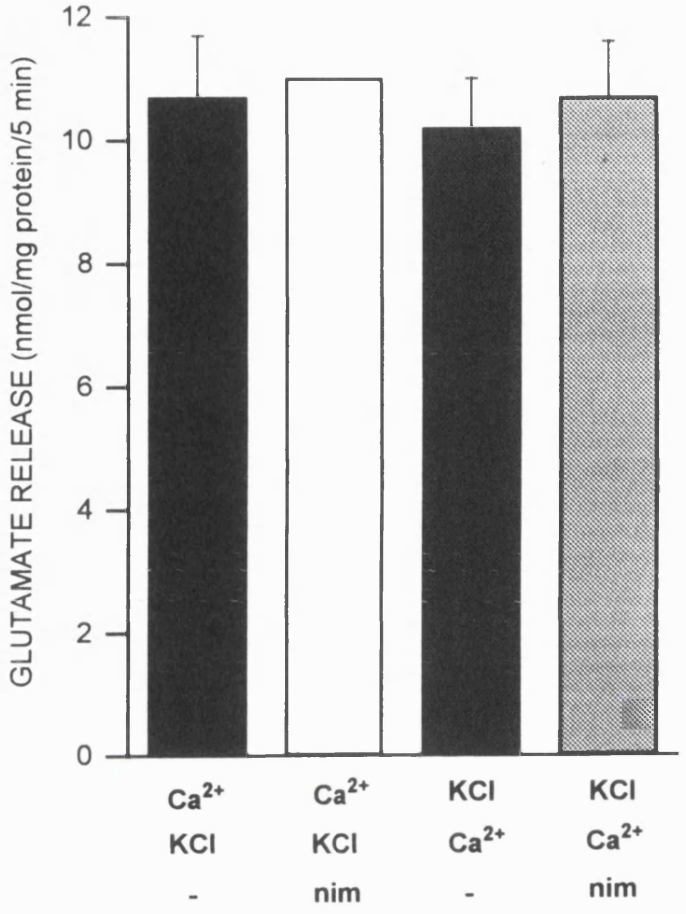

Fig.5. **Dihydropyridines do not inhibit glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (Ca^{2+} KCl -) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of KCl (30 mM) in the presence of $CaCl_2$ (1mM); (Ca^{2+} KCl nim) Preincubation with 3 μM nimodipine (nim) for 7 min before KCl addition. (KCl Ca^{2+} -) Glutamate release evoked by addition of $CaCl_2$ (1mM) in the presence of KCl (30 mM); (KCl Ca^{2+} nim) Preincubation with 3 μM nimodipine for 7 min before addition of $CaCl_2$ (1mM). Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals, except (Ca^{2+} KCl nim) which is a single representative experiment.

presynaptic VSCC that control hippocampal excitatory amino acid release may be conserved in the cerebrocortical circuitry,

in the presence of nimodipine was 10.7 ± 0.9 nmol glutamate/mg protein/5 min. (Fig.5. KCl Ca^{2+} nim). Thus, when maximal binding is facilitated, nimodipine still has no blocking action on glutamate release. These findings indicate that L-type VSCC do not control glutamate release from nerve terminals in the rat cerebral cortex and corroborate previous studies using guinea-pig synaptosomes (Pocock and Nicholls, 1992).

N-type VSCC have intermediate inactivation kinetics (Fox et al., 1987) and are insensitive to block by DHPs but are selectively blocked by ω-conotoxin GVIA (ω-CTx GVIA), isolated from the fish eating marine mollusc Conus geographus (Regan et al., 1991). It has previously been shown that [^{3}H]glutamate release from cerebral cortex synaptosomes is not inhibited by ω-CTx GVIA (Turner et al., 1992; Turner et al., 1993), indicating that N-type VSCC may not be coupled to the release of glutamate in the cerebral cortex. The major role of putative presynaptic N-type VSCC in this brain region (Gohil et al., 1994) appears to be regulation of the release of type II transmitters such as catecholamines (Oliver et al., 1994). However, in the hippocampus, glutamate release (Dutar et al., 1989; Luebke et al., 1993) and GABA release (Horne and Kemp, 1991; Takahashi and Momiyama, 1993) are partially inhibited by ω-CTx GVIA. This suggests that N-type VSCC can participate in exocytosis of type I transmitters in some synaptic circuits. As the

it seemed necessary to assess the contribution of N-type VSCC in controlling glutamate release in the cerebral cortex. KCl (30mM) evoked the release of 10.5 ± 0.7 nmol glutamate/mg protein/5 min in the presence of $CaCl_2$ (1 mM) (Fig.6.i) and 2.75 nmol glutamate/mg protein/5 min in the absence of external Ca^{2+} (presence of EGTA) (Fig.6.iii). Net KCl-evoked Ca^{2+}-dependent glutamate release was calculated to be 7.75 ± 0.7 nmol/mg protein/5 min. Preincubation with ω-CTx GVIA (2 μM) before KCl addition in the presence of 1 mM Ca^{2+} inhibited glutamate release to 7.7 ± 0.4 nmol/mg protein/5 min (Fig.6.ii). Ca^{2+}-independent release remained unaltered by ω-CTx GVIA (Fig.6.iv). Net KCl-evoked Ca^{2+}-dependent glutamate release in the presence of ω-CTx GVIA was calculated to be 4.95 ± 0.4 nmol/mg protein/5 min, thus, ω-CTx GVIA inhibited KCl-evoked Ca^{2+}-dependent glutamate release by 36%. This indicates that in the cerebral cortex, a substantial portion of KCl-evoked Ca^{2+}-dependent glutamate release is triggered by the influx of Ca^{2+}

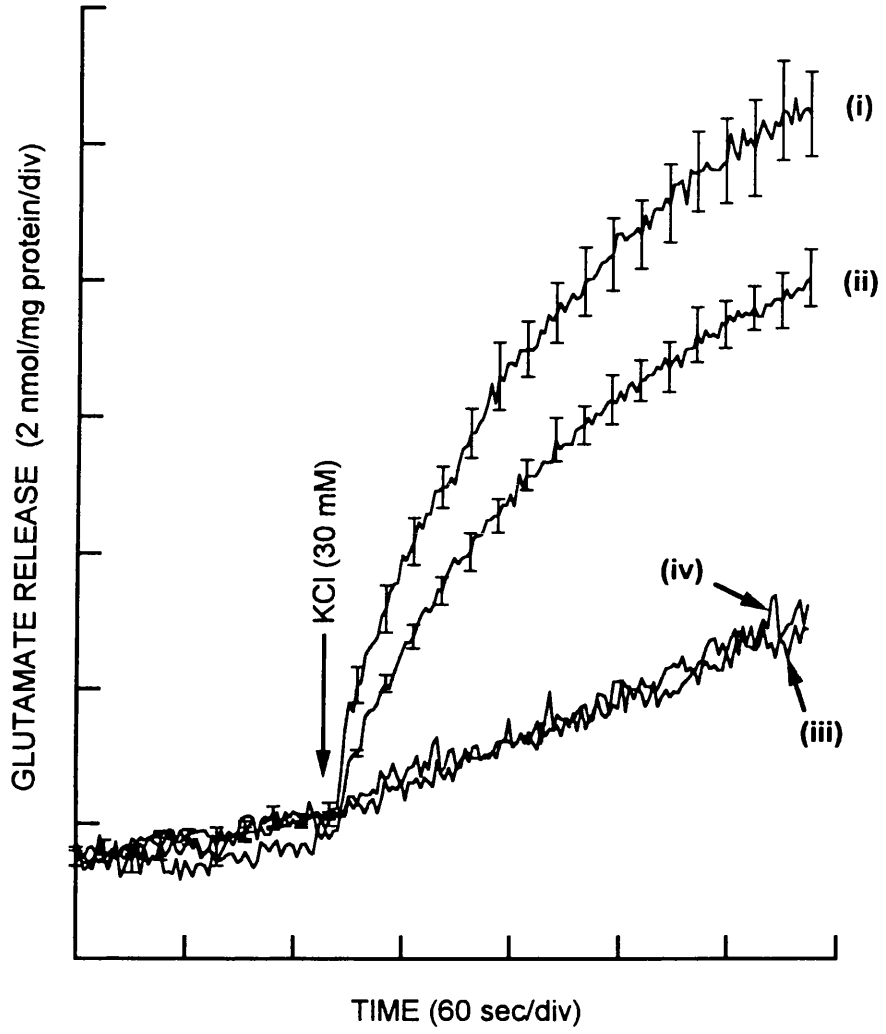

Fig.6. **ω-Conotoxin GVIA inhibits KCl-evoked glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of KCl (30 mM) in the presence of $CaCl_2$ (1mM) (i) or absence of 1mM Ca^{2+} (presence of 200μM EGTA) (iii); (ii) Preincubation with ω-CTx GVIA (2 μM) for 7 min before KCl addition in the presence of $CaCl_2$ (1mM); (iv) Preincubation with ω-CTx GVIA (2 μM) for 7 min before KCl addition in the absence of 1mM Ca^{2+} (presence of 200μM EGTA). Traces (i) and (ii) are the means ± SEM of independent experiments using synaptosomal preparations from four to six animals. Error bars are shown every 15 s for clarity. Traces (iii) and (iv) are single representative experiments.

through N-type VSCC. To confirm that ω-CTx GVIA inhibits glutamate release by blocking Ca^{2+} influx through N-type VSCC, experiments were performed to assess what proportion of the KCl-evoked rise in $[Ca^{2+}]_c$ is sensitive to 2 μM ω-CTx GVIA. A rise in $[Ca^{2+}]_c$ occurred after addition of KCl (30mM) having a characteristic transient 'spike' which declined to leave a $[Ca^{2+}]_c$ plateau of 286 ± 3 nM/5 min (Fig.7.i). Preincubation of synaptosomes with 2 μM ω-CTx GVIA reduced the KCl-evoked increase in $[Ca^{2+}]_c$ (Fig.7.ii). The transient $[Ca^{2+}]_c$ 'spike' was not inhibited by ω-CTx GVIA. This is not surprising based on electrophysiological data showing that N-type VSCC are not transiently activated (Fox et al., 1987). However, the $[Ca^{2+}]_c$ 'plateau' in the presence of 2 μM ω-CTx GVIA was decreased to 260 ± 1.5 nM/5 min. This amounted to a 21% inhibition of the non-inactivating phase of Ca^{2+} influx into synaptosomes by the toxin. This finding strongly suggests that inhibition of glutamate release by ω-CTx GVIA results from inhibition of the KCl-evoked non-inactivating phase of Ca^{2+} entry into cerebrocortical synaptosomes by ω-CTx GVIA. Based on the pharmacology of ω-CTx GVIA at electrophysiologically-defined somatic VSCC, it seems fair to propose that ω-CTx GVIA inhibits KCl-evoked Ca^{2+} influx by blocking presynaptic N-type VSCC. These results are in stark contrast to previous reports using rat cerebral cortex synaptosomes which fail to find any effect of ω-CTx GVIA on glutamate release (Turner et al., 1992) or Ca^{2+} infux . However, complementary to the experimental data shown in Fig.7, one report using cerebral cortex synaptosomes shows that ω-CTx GVIA when added to the plateau phase of $[Ca^{2+}]_c$ causes a 18% reduction in $[Ca^{2+}]_c$ (Bowman et al., 1993), indicating that indeed N-type VSCC contribute to the non-inactivating phase of Ca^{2+} influx that occurs upon depolarisation.

The results with ω-CTx GVIA showing a partial inhibition of glutamate release and Ca^{2+} entry indicate that additional VSCC types are coupled to glutamate exocytosis in the cerebral cortex. Much attention has been focused on the role that P-type VSCC might play in the release of the fast-acting transmitters glutamate and GABA. This attention has been necessary because L-type VSCC do not appear to participate in release of these transmitters and N-type VSCC seem to play a more prominent role in the release of type II transmitters. P-type VSCC have non-inactivating kinetics (Llinas et al., 1992), are potently blocked by

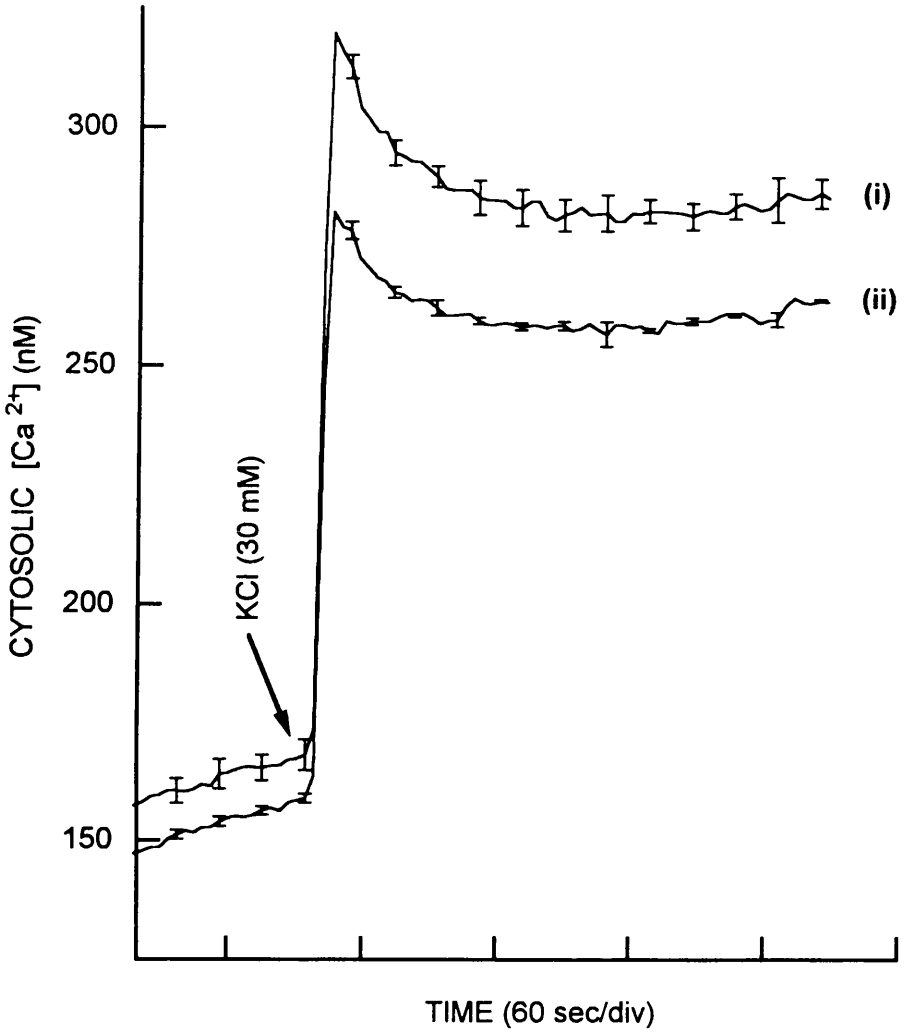

Fig.7. **ω-Conotoxin GVIA reduces the KCl-evoked increase in $[Ca^{2+}]_c$.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. (i) Elevation in $[Ca^{2+}]_c$ after addition of KCl (30mM); (ii) Preincubation with ω-CTx GVIA (2 μM) for 7 min before KCl addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 20 s for clarity.

low nanomolar amounts of a peptide toxin, ω-Aga IVA, isolated from the funnel web spider Agelenopsis aperta (Mintz et al., 1991) and are insensitive to ω-CTx GVIA. ω-Aga IVA also blocks electrophysiologically-defined Q-type VSCC which at certain synapses are found in close association with P-type VSCC (Wheeler et al., 1994). Q-type VSCC have faster inactivation kinetics than P-type VSCC and ω-Aga IVA blocks the Q-type VSCC with 100 fold less sensitivity than its block of P-type VSCC. Glutamate release from cerebral cortex synaptosomes is attenuated by low nanomolar amounts of ω-Aga IVA but the maximum inhibition that can be achieved is 50% (Turner et al., 1992; Turner et al., 1993). ω-Aga IVA also blocks 60% of the 'plateau' phase of KCl -evoked elevations in $[Ca^{2+}]_c$ (Bowman et al., 1993), indicating that a major portion of KCl-evoked non-inactivating Ca^{2+} influx into synaptosomes is carried through P-type VSCC. It is well accepted that P-type VSCC contribute to the control of glutamate release and synaptic excitatory transmission (Luebke et al., 1993; Burke et al., 1993; Wu and Saggau, 1994). However, some synaptic excitatory circuits in the hippocampus do not appear to use P-type VSCC but instead a combination of N-type and Q-type VSCC for transmission (Nooney and Lodge, 1996). Q-type VSCC are potently blocked by a toxin isolated from Conus magus called ω-conotoxin MVIIC (ω-CTx MVIIC) (Sather et al., 1993). ω-CTx MVIIC also blocks P-type VSCC but with 10 fold lower affinity as well as N-type VSCC with low affinity. Little data relating to the action of ω-CTx MVIIC at glutamatergic nerve terminals exists, although the toxin is known to inhibit 75% of KCl-evoked $^{45}Ca^{2+}$ uptake by 'crude' rat brain synaptosomes (Hillyard et al., 1992) and blocks Ca^{2+} influx into hippocampal synaptosomes (Malva et al., 1995a).

A series of experiments were performed to assess the effects of ω-CTx MVIIC on glutamate release and depolarisation-evoked rises in $[Ca^{2+}]_c$. Preincubation of synaptosomes with a range of doses of ω-CTx MVIIC (10nM - 4μM) inhibited Ca^{2+}-dependent glutamate release evoked by KCl (30mM) (Fig.8.A) and 4AP(3 mM) (Fig.9.A). Complete block of Ca^{2+}-dependent release occurred at 2 - 4 μM ω-CTx MVIIC. Ca^{2+}-independent glutamate release evoked by KCl or 4AP was not affected by the toxin (data not shown). The IC_{50} value for ω-CTx MVIIC inhibition of KCl-evoked Ca^{2+}-dependent release, derived from a log.dose-

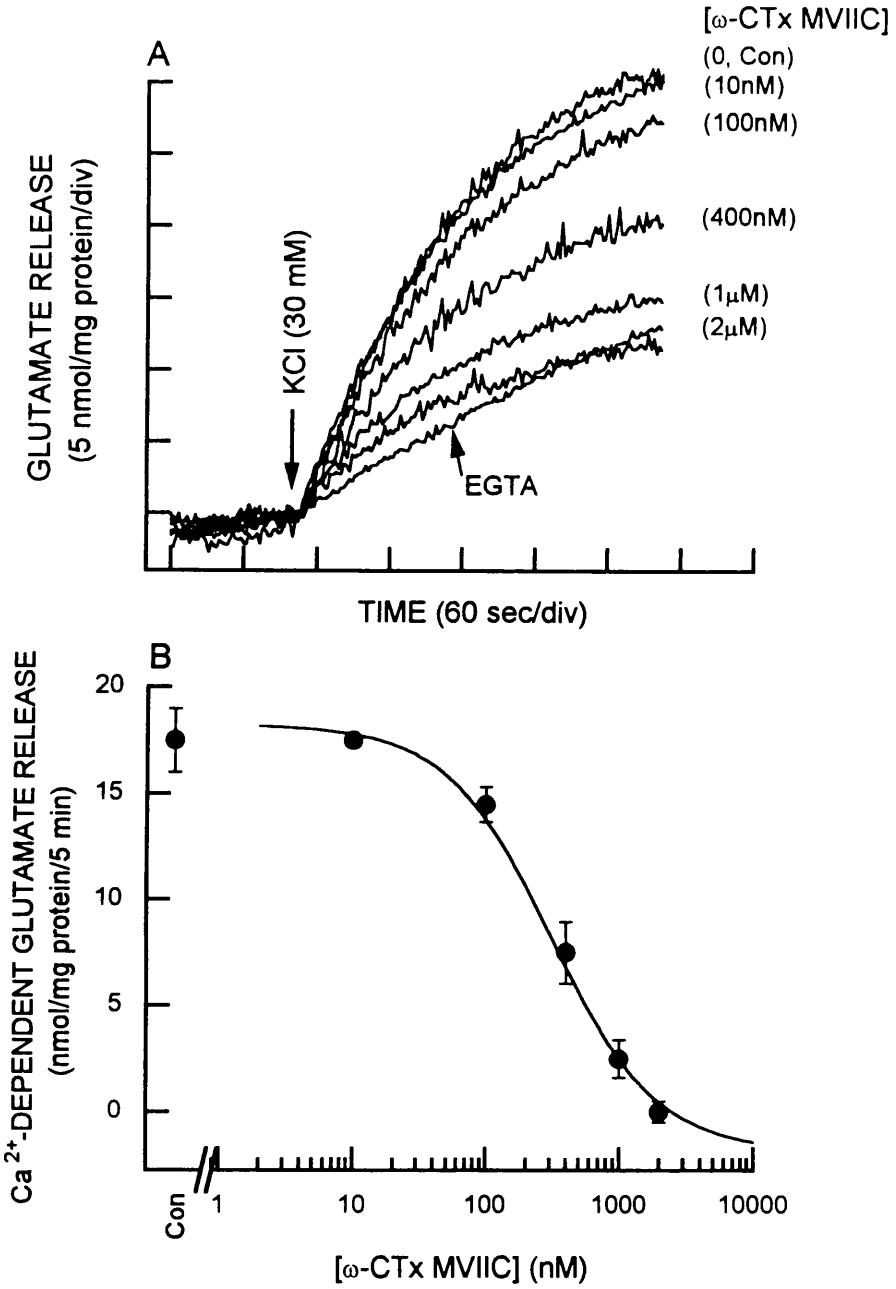

Fig.8. ω-CTx MVIIC dose-dependently inhibits KCl-evoked glutamate release. Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of KCl (30 mM) in the presence of $CaCl_2$ (1mM) (Con) or absence of 1mM Ca^{2+} (presence of 200 μM EGTA). Glutamate release was also evoked after preincubation with a range of doses of ω-CTx MVIIC (10 nM - 2 μM) for 2 min before KCl addition in the presence of $CaCl_2$ (1mM). Each trace is the mean of independent experiments using synaptosomal preparations from three animals. (B) Log. dose-response curve for ω-CTx MVIIC inhibition of KCl-evoked Ca^{2+}-dependent glutamate release, fitted using a logistic function. Data are the means ± SEM of three independent experiments.

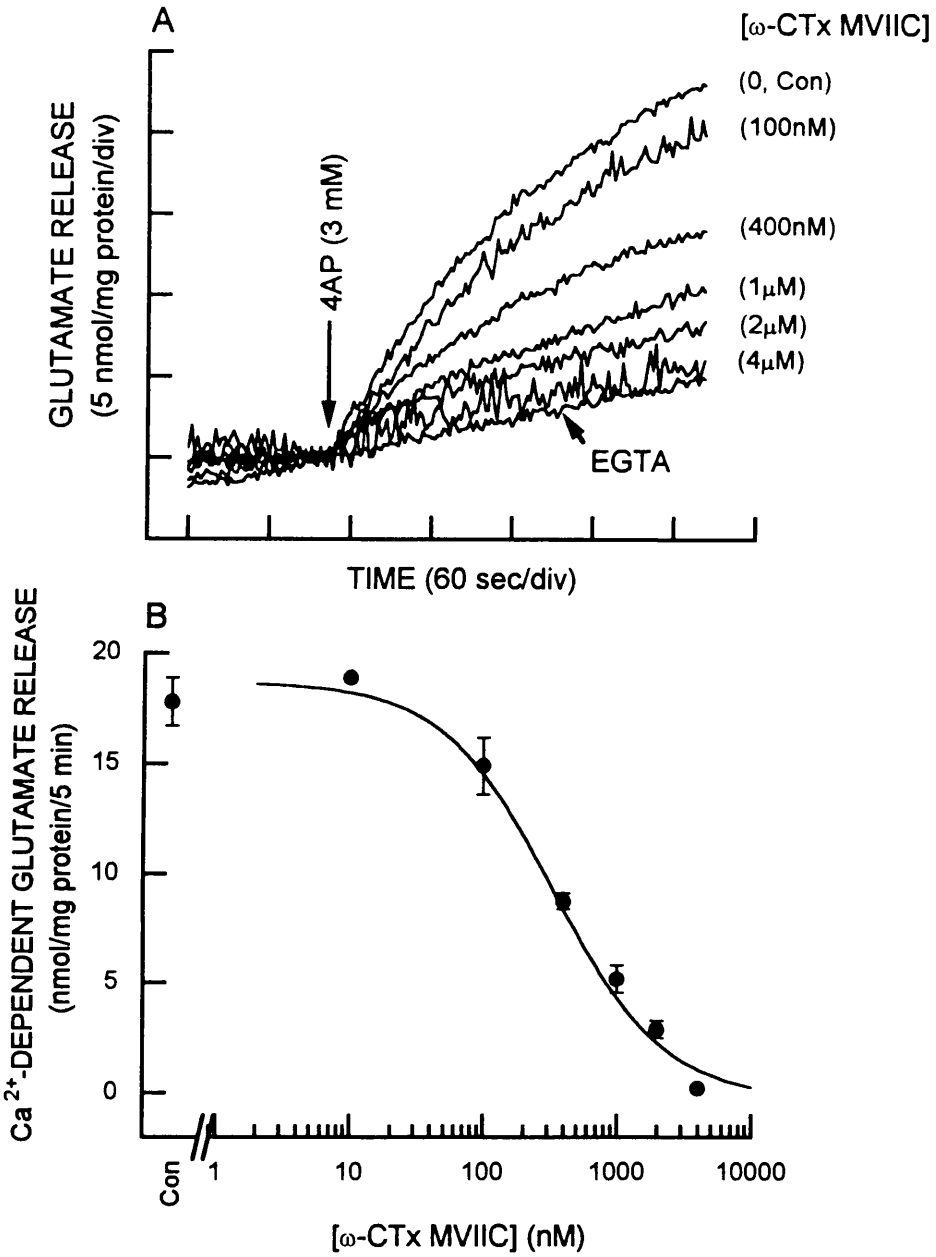

Fig.9. **ω-CTx MVIIC dose-dependently inhibits 4AP-evoked glutamate release.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (3mM) in the presence of $CaCl_2$ (1mM) (Con) or absence of 1mM Ca^{2+} (presence of 200 μM EGTA). Glutamate release was also evoked after preincubation with a range of doses of ω-CTx MVIIC (100 nM - 4 μM) for 2 min before 4AP addition in the presence of $CaCl_2$ (1mM). Each trace is the mean of independent experiments using synaptosomal preparations from three animals. (B) Log. dose-response curve for ω-CTx MVIIC inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release, fitted using a logistic function. Data are the means ± SEM of three independent experiments, except where the SEM is not shown n=2.

response curve (Fig.8.B) was 310 nM. A similar IC_{50} value of 330 nM was obtained from the log.dose response curve for ω-CTx MVIIC inhibition of 4AP-evoked Ca^{2+}-dependent release (Fig.9.B). The similarity of the IC_{50} values indicates two important points. Firstly, it strongly suggests that the depolarisation-evoked non-inactivating Ca^{2+} influx that mediates KCl-evoked glutamate release is common to that mediating 4AP-evoked glutamate release. However, it is possible that ω-CTx MVIIC could inhibit 4AP-evoked glutamate release by blocking Ca^{2+} channels and/or Na^{+} channels, based on the finding that 4AP evokes glutamate release via the repetitive firing of TTX-sensitive Na^{+} channels leading to depolarisation and activation of VSCC coupled to exocytosis. In contrast, KCl (30mM)-evoked glutamate release is TTX-insensitive and does not involve repetitive firing of Na^{+} channels. For this reason it can be argued that, in general, an inhibition of KCl-evoked glutamate release results only from block of VSCC linked to release. If the IC_{50} values for KCl-evoked and 4AP-evoked release had been significantly different this could have been indicating that ω-CTx MVIIC inhibits 4AP-evoked glutamate release by blocking an ion channel linked to release other than a Ca^{2+} channel, i.e. the toxin could be blocking the 4AP-evoked, repetitively firing Na^{+} channels. This does not seem likely because the IC_{50} values for KCl and 4AP are similar, suggesting that like KCl-evoked release, 4AP-evoked release is inhibited by a blocking action of ω-CTx MVIIC exclusively at the VSCC coupled to release. Furthermore, it validates the use of 4AP to study the effects of ω-toxins on excitation-secretion coupling because these ω-toxin ligands do not appear to have non-specific blocking effects, instead being highly selective VSCC ligands. This may be important because when studying transmitter release from neurochemical preparations, 4AP is argued to be a more physiological and thus preferable depolarising stimulus than elevated KCl.

It has been reported that ω-CTx MVIIC is a reversible blocker of VSCC (Grantham et al., 1994). The reversibility of ω-CTx MVIIC inhibition of glutamate release was tested. 4AP (3 mM) evoked the release of 20.6 ± 1.4 nmol glutamate/mg protein/5 min in the presence of added Ca^{2+} (Fig.10.i). Preincubation with ω-CTx MVIIC (1 μM) before addition of 4AP inhibited glutamate release to 9.4 ± 0.7 nmol/mg protein/5 min. (Fig.10.ii). When these ω-CTx MVIIC-treated synaptosomes were washed with fresh incubation medium (HBM), a second

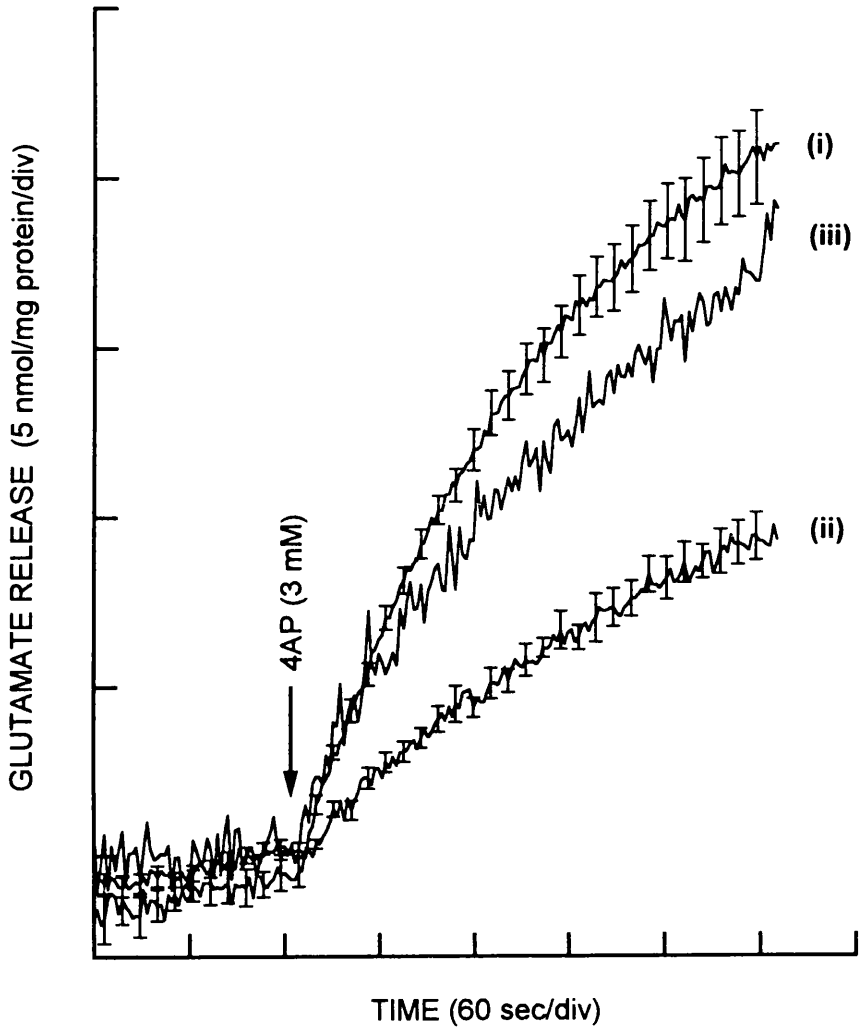

Fig.10. **ω-CTx MVIIC reversibly inhibits 4AP-evoked glutamate release.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (3mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with ω-CTx MVIIC (1 μM) for 2 min before 4AP addition; (iii) Restimulation with 4AP (1mM) after washing ω-CTx MVIIC-treated synaptosomes with fresh HBM. Traces (i) and (ii) are the means ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity. Trace (iii) is the mean of independent experiments using synaptosomal preparations from two animals.

depolarisation with 3 mM 4AP evoked the release of 17.8 nmol glutamate/mg protein/5 min. (Fig.10.iii (*n*=2)). The data indicates that ω-CTx MVIIC binds reversibly to its target Ca^{2+} channels(s) and the toxin can be washed off to give near control release levels upon restimulation. This makes ω-CTx MVIIC a useful ligand when probing the Ca^{2+}-dependency of glutamate release from repetitive stimulations. An irreversible ligand would not allow for multiple cycles of release.

$[Ca^{2+}]_c$ studies were performed to probe what effect ω-CTx MVIIC has on Ca^{2+} entry into synaptosomes. The elevation in $[Ca^{2+}]_c$ that occurred after addition of KCl (30mM) was inhibited by preincubation of synaptosomes with a range of doses of ω-CTx MVIIC (5nM - 4μM) (Fig.11.A). It can be seen in Fig.11.A that, as was the case with ω-CTx GVIA, ω-CTx MVIIC does not attenuate the KCl-evoked $[Ca^{2+}]_c$ 'spike' but caused near complete suppression of the 'plateau' phase of Ca^{2+} influx at a concentration of 2 μM ω-CTx MVIIC (IC_{50} - 400nM (Fig.11.B)). A lack of effect of ω-CTx MVIIC on the KCl-evoked $[Ca^{2+}]_c$ 'spike' indicates that the toxin blocks non-inactivating Ca^{2+} channels. Because ω-CTx MVIIC completely blocked glutamate release while only blocking Ca^{2+} entry through non-inactivating VSCC, this is strong evidence that the transient Ca^{2+} 'spike' is not coupled to the release of glutamate. Similarly, ω-CTx MVIIC dose-dependently inhibited the 4AP-mediated increase in $[Ca^{2+}]_c$ (Fig.12.A). In these experiments, the small 4AP-evoked $[Ca^{2+}]_c$ 'spike' observed was again not blocked by any concentration of ω-CTx MVIIC. At 2 μM, ω-CTx MVIIC-mediated inhibition of the 4AP-evoked $[Ca^{2+}]_c$ 'plateau' was not as complete as that observed with KCl. However, the two IC_{50} values for inhibition of the KCl-evoked and the 4AP-evoked $[Ca^{2+}]_c$ 'plateau' by ω-CTx MVIIC are very similar (KCl - 400 nM ; 4AP - 430 nM).

It seems reasonable to assume that the rises in $[Ca^{2+}]_c$ evoked by KCl and 4AP, and assessed with fura-2, occur due to Ca^{2+} influx through VSCC, because ω-CTx MVIIC, which is a VSCC blocker, causes up to 95% block of the KCl-evoked increase in $[Ca^{2+}]_c$. However, it was necessarry to confirm that this is the case; a rise in $[Ca^{2+}]_c$ can be elicited by the Ca^{2+} ionophore, ionomycin, but the resultant glutamate release is inefficient (Sihra et al., 1992). Ionomycin must produce a seven-fold greater increase in $[Ca^{2+}]_c$ compared to KCl or 4AP to trigger half the glutamate release observed with KCl (30mM) or 4AP (3 mM) (McMahon

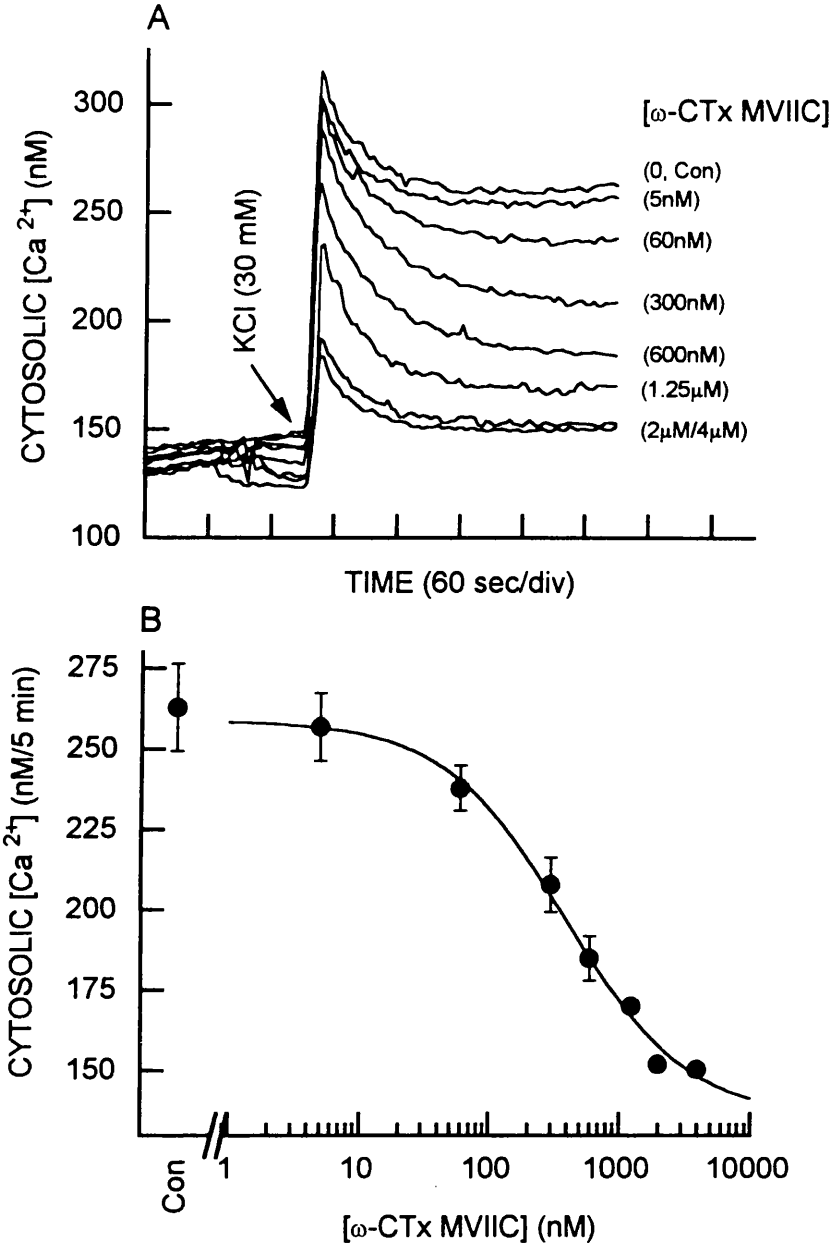

Fig.11. **ω-CTx MVIIC dose-dependently reduces the KCl-evoked increase in $[Ca^{2+}]_c$.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods and Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. (A) Elevation in $[Ca^{2+}]_c$ occurred after addition of KCl (30 mM) (Con), and after preincubation with a range of doses of ω-CTx MVIIC (5 nM - 4 μM) for 1.5 min before KCl addition. Each trace is the mean of independent experiments using synaptosomal preparations from two to three animals. (B) Log. dose-response curve for ω-CTx MVIIC inhibition of the KCl-evoked increase in $[Ca^{2+}]_c$, fitted using a logistic function. Data are the means ± SEM of three independent experiments, except where the SEM is not shown n=2.

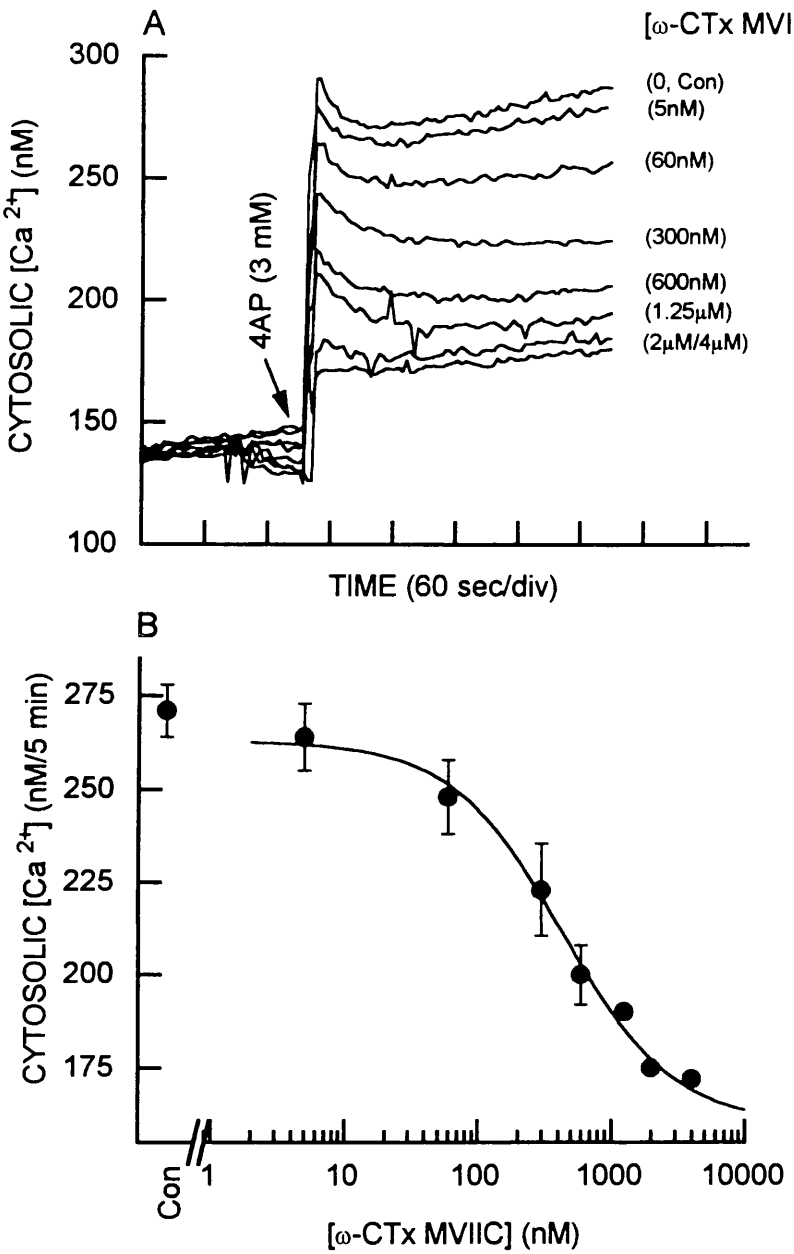

Fig.12. **ω-CTx MVIIC dose-dependently reduces the 4AP-evoked increase in $[Ca^{2+}]_c$.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods and Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. (A) Elevation in $[Ca^{2+}]_c$ occurred after addition of 4AP (3mM) (Con) and after preincubation with a range of doses of ω-CTx MVIIC (5 nM - 4 μM) for 1.5 min before 4AP addition. Each trace is the mean of independent experiments using synaptosomal preparations from two to three animals. (B) Log. dose-response curve for ω-CTx MVIIC inhibition of the 4AP-evoked increase in $[Ca^{2+}]_c$, fitted using a logistic function. Data are the means ± SEM of three independent experiments, except where the SEM is not shown n=2.

and Nicholls, 1991). It is argued that because ionomycin causes delocalised entry of Ca^{2+} at nonspecific points around the terminal plasma membrane it is far less effective at evoking glutamate release contained in SSVs, which require localised Ca^{2+} entry through active zone Ca^{2+} channels. Ionomycin (5 μM) evoked an increase in $[Ca^{2+}]_c$ similar to 30 mM KCl-evoked and 3 mM 4AP-evoked rises in $[Ca^{2+}]_c$ (291 ± 22 nM/5 min, n=3). Preincubation with 1 μM ω-CTx MVIIC did not reduce the ionomycin-induced elevation in $[Ca^{2+}]_c$ (288 ± 25 nM/5 min, n=3), indicating that ω-CTx MVIIC inhibits KCl-evoked and 4AP-evoked rises in $[Ca^{2+}]_c$ and subsequent glutamate release by specific blockade of Ca^{2+} channels linked to glutamate release.

ω-CTx MVIIC at low μM concentrations will block N-type VSCC (Hillyard et al., 1992), P-type and Q-type VSCC (Randall and Tsien, 1995). Results in this chapter show that N-type Ca^{2+} channels partially control glutamate release from cerebral cortex nerve terminals. The remaining release of glutamate can be inhibited by ω-CTx MVIIC . It has been previously shown that ω-Aga IVA inhibits glutamate release from cerebrocortical synaptosomes by only 50% (Turner et al., 1992), and thus it is tempting to propose that the results obtained in this chapter showing 100% inhibition of glutamate release by ω-CTx MVIIC, are due to an action of ω-CTx MVIIC at N-type, P-type and Q-type Ca^{2+} channels. In addition to the proposed participation of N-type VSCC in glutamate exocytosis (based on the results with ω-CTx GVIA), ω-CTx MVIIC used on its own cannot differentiate which of the other two VSCC, P-type and Q-type, are coupled to glutamate exocytosis and in what proportion. To determine the contribution of P-type and Q-type VSCC in controlling glutamate release, it is necessary to use ω-Aga IVA in order to assess what proportion of depolarisation-evoked glutamate release and rises in $[Ca^{2+}]_c$ is due to influx of Ca^{2+} through P-type VSCC. In this respect, a concentration of ω-Aga IVA which only blocks P-type VSCC would be necessary (low nanomolar amounts). In separate experiments ω-CTx GVIA could be added with ω–Aga IVA to selectively block N-type VSCC and P-type VSCC respectively. Any glutamate release remaining after use of these two toxins could be tested for its sensitivity to block by ω-CTx MVIIC. ω-CTx MVIIC (at a concentration of the toxin which is selective for Q-type VSCC) added to ω-CTx GVIA/ω–Aga IVA-

treated synaptosomes would possibly indicate a participation of Q-type VSCC in glutamate exocytosis. By performing these 'toxin additivity' experiments, information about the relative contributions of N-type, P-type and Q-type VSCC in controlling glutamate release from cerebrocortical nerve terminals may be obtained.

Conclusions

- 30 mM KCl and 3 mM 4AP evoke comparable levels of Ca^{2+}-dependent glutamate release by different mechanisms of depolarisation; 4AP-mediated release is TTX-sensitive indicating that 4AP-evokes repetitive firing of Na^+ channels whereas KCl-evoked release is insensitive to TTX because rapid inactivation of Na^+ channels occurs with 'clamped' depolarisation. 4AP provides a more 'physiological' approach for evoking glutamate release from isolated nerve terminals.

- Despite their differing mechanism of action in causing synaptosomal depolarisation, KCl and 4AP both elicit glutamate release by mediating influx of Ca^{2+} through the common non-inactivating Ca^{2+} channels coupled to glutamate release.

- Transiently activated VSCC do not support glutamate release.

- L-type VSCC are not coupled to glutamate release in the rat cerebral cortex.

- The N-type VSCC blocker ω-CTX GVIA inhibits the KCl-evoked non-inactivating phase of Ca^{2+} influx and this leads to inhibition of glutamate release. Thus, N-type Ca^{2+} channels do, contrary to some earlier studies, contribute to the control of glutamate release in the cerebral cortex.

- ω-CTx MVIIC dose-dependently inhibits KCl-evoked and 4AP-evoked glutamate release with complete block at low μM amounts of ω-CTx MVIIC. This is due to blockade of one or more non-inactivating Ca^{2+} channel(s)

containing class A α_1 subunits; RNA encoding class A Ca^{2+} channel α_1 subunits can produce Ca^{2+} channels with a P-type Ca^{2+} channel phenotype and a Q-type Ca^{2+} channel phenotype. One or both of these Ca^{2+} channel types may control a major portion of glutamate releasefrom rat cerebral cortex nerve terminals.

- A complete and reversible inhibition of Ca^{2+}-dependent glutamate release by ω-CTx MVIIC presents the toxin as a useful tool/probe for assessing glutamate exocytosis.

CHAPTER FOUR

4 Presynaptic non-NMDA glutamate receptors facilitate glutamate exocytosis from cerebral cortex nerve terminals (synaptosomes)

Introduction

Glutamate receptors are found throughout the mammalian brain, where they constitute the major excitatory transmitter system. The longest-known and best-studied glutamate receptors are ligand-gated ion channels, also called ionotropic glutamate receptors, which are permeable to cations. They have traditionally been classified into three broad subtypes based upon pharmacological and electrophysiological data: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, kainate (KA) receptors and N-methyl-D-aspartate (NMDA) receptors. AMPA and KA glutamate receptors are termed non-NMDA receptors due to their lack of sensitivity to the agonist NMDA.

Molecular cloning has identified four closely related members of a family of AMPA-selective receptors, termed GluR1, GluR2, GluR3 and GluR4 (Boulter et al., 1990; Nakanishi et al., 1990), or GluR A, B, C and D (Keinanen et al., 1990). AMPA and glutamate act as partial agonists eliciting rapidly desensitising currents in expressed AMPA receptors whereas KA and domoate evoke maximal non-desensitising responses (Hollmann and Heinemann, 1994). Rapidly desensitising AMPA responses can be prevented and AMPA-mediated currents potentiated by use of cyclothiazide, a benzothiadiazide diuretic, (Partin et al., 1993; Wong and Mayer, 1993; Yamada and Tang, 1993; Rammes et al., 1994). Cloned and expressed AMPA receptor subunits can be antagonised by the non-competitive antagonist GYKI 52466 (Kessler et al., 1996). Immunostaining techniques show little evidence of a presynaptic location for cloned AMPA receptor subunits. Most immunostaining occurs at postsynaptic densities (Petralia and Wenthold, 1992; Hampson et al., 1992).

Cloned KA receptor subunits fall into 2 groups: 1) low-affinity KA subunits and 2) high-affinity KA subunits. Three low-affinity KA subunits have been identified, GluR5, GluR6 and GluR7 (Bettler et al., 1992; Egebjerg et al., 1991; Sommer et al., 1992; Lomeli et al., 1992). Expressed GluR5 and GluR6 subunits show the following agonist sensitivity profile: domoate > KA > glutamate (Hollmann and Heinemann, 1994). AMPA elicits no current (Seeburg, 1993). Domoate and

KA evoke markedly desensitising responses which can be prevented by treatment with concanavalin A (Wong and Mayer, 1993). Homomerically expressed GluR7 subunits produce no currents (Bettler et al., 1992). GluR6 subunits when expressed produce currents which are blocked by the novel compound NS-102 (Verdoorn et al., 1994). Low-affinity KA receptor subunits correlate closely to the low-affinity [^{3}H]kainate binding sites found on rat brain membranes (Young and Fagg, 1990) which selectively bind NS-102 (Johansen et al., 1993).

Two high-affinity KA subunits have been cloned termed KA1 and KA2 showing only 35 - 45% sequence homology with GluR 5 - 7 (Werner et al., 1991; Herb et al., 1992; Sakimura et al., 1992). KA1 and KA2 subunits only form functional receptors when expressed with GluR5 or GluR6 KA subunits. When GluR6 is co-expressed with KA2, functional channels with novel properties are seen; homomerically expressed GluR6 receptors are insensitive to AMPA whereas GluR6/KA2 channels are gated by AMPA in a non-desensitising manner (Seeburg, 1993). KA1 and KA2 subunits bind KA with close correlation to the high-affinity [^{3}H]kainate binding site on native rat brain membranes indicating that these sites may represent KA1 and KA2 subunits. KA2 subunits are highly expressed throughout the brain (Herb et al., 1992) and KA1 subunits are believed to have a presynaptic location in the hippocampus (Werner et al., 1991).

There is little evidence to suggest that NMDA receptors regulate glutamate release. However, presynaptic NMDA heteroreceptors regulate noradrenaline release (Wang et al., 1992) as well as dopamine release (Wang, 1991). In contrast to NMDA receptors, a few studies have indicated that presynaptic non-NMDA receptors modulate glutamate release. KA facilitates depolarisation-evoked Ca^{2+}-dependent glutamate release in a number of neuronal preparations (Ferkany et al., 1982; Poli et al., 1985; Terrian et al., 1991; Gannon and Terrian, 1991). AMPA and domoate also stimulate glutamate release (Terrian et al., 1991; Sherman et al., 1992), although glutamate release from hippocampal nerve terminals (synaptosomes) is not modulated by AMPA (Zhou et al., 1995) unless rapid desensitisation is prevented by using cyclothiazide (Barnes et al., 1994). Furthermore, AMPA-mediated facilitation of glutamate release from hippocampal synaptosomes is blocked by the AMPA receptor antagonist GYKI 52466 (Barnes et al., 1994) and it has been proposed that presynaptic AMPA receptors

composed of GluR 1 - 4 subunits modulate glutamate release in the hippocampus.

Curiously, as well as potentiating depolarisation-evoked Ca^{2+}-dependent glutamate release, KA and domoate appear to depress glutamate transmission in the hippocampus via a presynaptic mechanism (Barnes and Henley, 1994; Chittajallu et al., 1996). This response can be blocked by the novel GluR6 KA receptor antagonist NS-102 (Chittajallu et al., 1996) and it has been proposed that inhibitory presynaptic KA receptors containing GluR6 subunits exist in the hippocampus. These results are in stark contrast to results with hippocampal synaptosomes showing that both AMPA and KA elicit rises in cytosolic free Ca^{2+} levels, $[Ca^{2+}]_c$ (Malva et al., 1995b); an increase in $[Ca^{2+}]_c$ would not lead to inhibition of glutamate release.

A mechanism for presynaptic non-NMDA receptor modulation of glutamate release remains elusive. Classically, these ligand-gated ion channels conduct the cations Na^+, K^+ and Ca^{2+} (to differing degrees). This leads to membrane depolarisation. Early reports showed that KA depolarises the synaptosomal membrane potential (Pastuszko et al., 1984) and elicits rapid Ca^{2+} influx into nerve terminals (Pastuszko et al., 1984; Pastuszko and Wilson, 1985). Thus, presynaptic non-NMDA receptors activated by KA, may be similar to their postsynaptic counterparts, activation of which leads to Na^+/Ca^{2+} influx and membrane depolarisation.

As stated earlier, it would appear from recent findings (Barnes et al., 1994; Chittajallu et al., 1996) that AMPA and KA elicit opposing effects on glutamate exocytosis in the hippocampus by activation of presynaptic AMPA and KA receptors respectively. Studies addressing what effects non-NMDA glutamate receptor agonists have on glutamate release from cerebral cortex nerve terminals (synaptosomes) were performed. Using the K^+ channel blocker 4-aminopyridine (4AP) to depolarise cerebrocortical synaptosomes, which is thought to provide a correlate to depolarisation of nerve terminals *in vivo* (Nicholls and Coffey, 1994), it was observed that the non-NMDA glutamate receptor agonists AMPA, KA and domoate all facilitated the Ca^{2+}-dependent release of glutamate. AMPA responses appeared to be non-desensitising because they were as full as KA responses and were not modulated by the AMPA receptor desensitisation inhibitor cyclothiazide. Thus, it seems possible that AMPA may not be having its effects on an AMPA-type

receptor but instead may be activating a high-affinity KA-type receptor. The AMPA receptor antagonist GYKI 52466 did not significantly block KA-mediated responses (KA is the preferred agonist when studying AMPA receptors due to its non-desensitising responses) but the non-selective non-NMDA receptor antagonist CNQX and the selective GluR6 KA receptor antagonist NS-102 prevented the KA-mediated facilitation of 4AP-evoked glutamate release. Thus, KA, like AMPA, seems to be having its effects through activation of a high-affinity KA-type receptor. Furthermore, these antagonist studies strengthen the proposal that AMPA receptors are not involved in facilitation of glutamate release from cerebrocortical nerve terminals. KA and AMPA responses were not additive, thus, it is possible that AMPA and KA may activate a common presynaptic KA-type receptor, possibly containing GluR6 KA subunits.

Probing a mechanism of action of AMPA and KA, it was observed that KA significantly enhanced 4AP-evoked depolarisation of the synaptosomal plasma membrane potential. Thus, receptor activation may lead to a conductance of cations and membrane depolarisation. cAMP-dependent protein kinase (PKA) has been shown to modulate recombinant GluR6 KA receptor currents (Raymond et al., 1993). However, some investigative experiments showed that activators/inhibitors of PKA did not influence KA responses in nerve terminals.

Results and discussion

It has recently been reported that Ca^{2+}-dependent glutamate release from rat hippocampal synaptosomes can be facilitated by AMPA (only in the presence of cyclothiazide) (Barnes et al., 1994) and inhibited by KA (Chittajallu et al., 1996). Pharmacological analysis reveals that AMPA responses are blocked by GYKI 52466, an AMPA receptor -selective antagonist (Barnes et al., 1994), whereas KA-mediated inhibition of glutamate release from hippocampal synaptosomes can be prevented by the GluR6 KA receptor antagonist NS-102 (Chittajallu et al., 1996). This has led the authors to propose the presence of two distinct presynaptic non-NMDA glutamate receptors on hippocampal nerve terminals: AMPA receptors (GluR 1-4 subunits) that can facilitate glutamate release and KA receptors containing GluR6 subunits that can inhibit release. Studies with rat cerebral cortex synaptosomes showed that the three non-NMDA receptor agonists KA,

AMPA and domoate, at a concentration of 100 μM, all facilitated the Ca^{2+}-dependent release of glutamate evoked by 4AP (1 mM) (Fig.13). The level of facilitation of Ca^{2+}-dependent glutamate release was similar with all three agonists (Table 2.A) and amounted to a 32% increase over control 4AP-evoked glutamate release with KA, a 36% increase over control release with AMPA and a 27% increase over control release with domoate. Thus, contrasting to reports with hippocampal synaptosomes (Chittajallu et al., 1996), no inhibitory effects of non-NMDA glutamate receptor agonists were observed in cerebral cortex nerve terminals.

It has been reported that KA (at high μM concentrations) inhibits the nerve terminal plasma membrane acidic amino acid carrier causing Ca^{2+}-independent effects (Pocock et al., 1988). 4AP-evoked Ca^{2+}-independent gutamate release was not affected by any one of the three agonists (Table 2.B). This result indicates that at a concentration of 100 μM, non-NMDA receptor agonists do not significantly inhibit the plasma membrane glutamate transporter(s) in cerebrocortical nerve terminals.

AMPA acts as a partial agonist at AMPA receptors, eliciting rapidly desensitising responses which can be potentiated by use of the AMPA receptor desensitisation inhibitor cyclothiazide (Partin et al., 1993). In contrast, KA elicits a full non-desensitising response at AMPA receptors and because of this it is the agonist of choice when studying AMPA receptors. Results showed that AMPA facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release was not less than KA-mediated facilitation (Table 2.A). Thus, AMPA does not appear to be acting as a partial agonist and may not be evoking a rapidly desensitising response. This indicates that AMPA might not be having its effects on glutamate release through activation of an AMPA-type receptor. To further test this hypothesis, experiments were performed assessing whether or not AMPA responses could be modulated by the AMPA receptor desensitisation inhibitor cyclothiazide. Control glutamate release, evoked by addition of 4AP (1 mM) in the presence of 1 mM Ca^{2+}, was 26.1 nmol/mg protein/7 min (Fig.14.i). Preincubation of synaptosomes with cyclothiazide (100 μM) before 4AP addition did not alter control glutamate release (25.9 nmol/mg protein/7 min) (Fig.14.ii). The facilitation of 4AP-evoked glutamate release by AMPA (100μM), which amounted to some 8

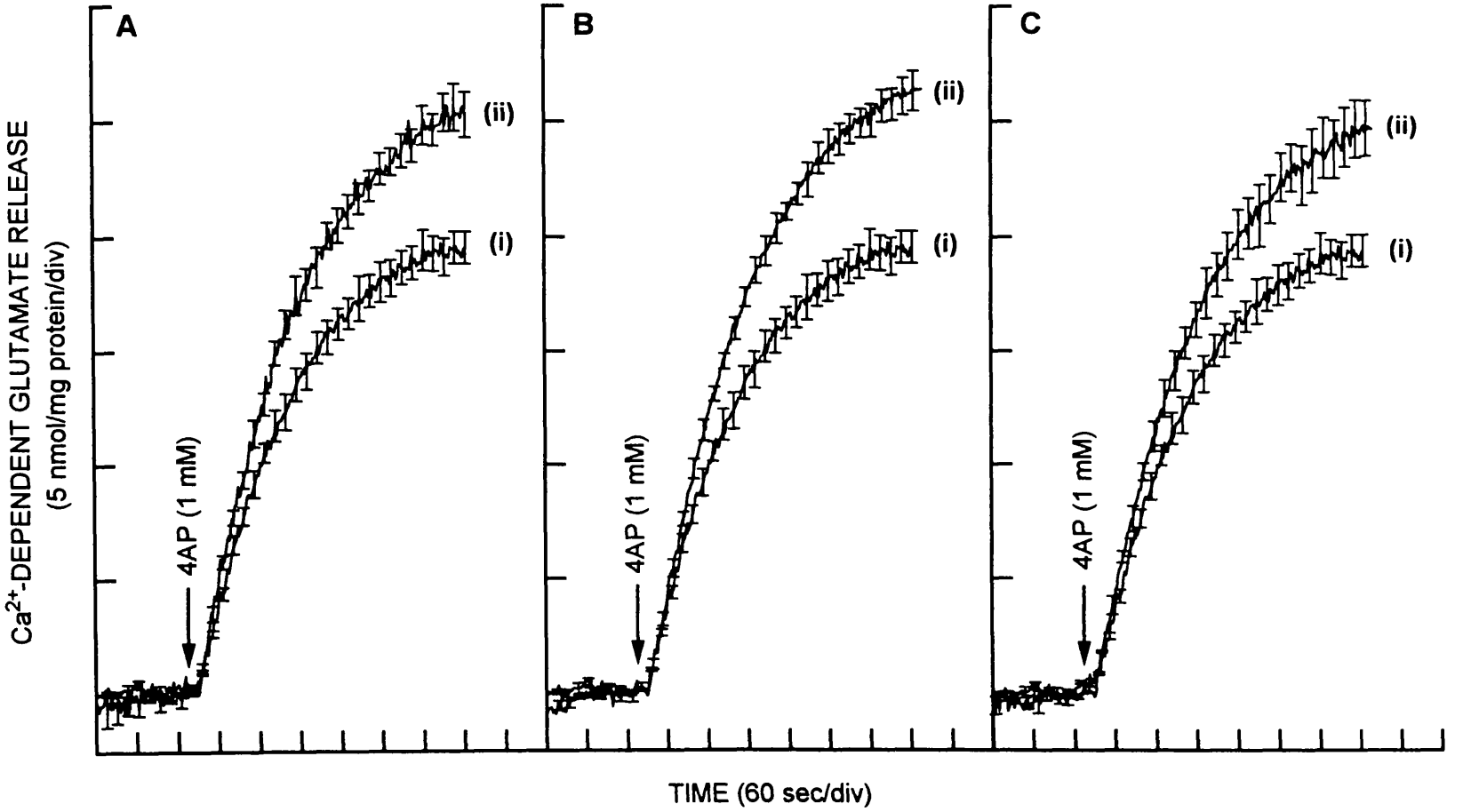

Fig.13. **KA, AMPA and domoate facilitate the Ca^{2+}-dependent release of glutamate evoked by 4AP.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Ca^{2+}-dependent glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM); (ii) Preincubation with KA (100 μM) for 30 s before 4AP addition. Ca^{2+}-dependent release was calculated as described in section 2.2.A of the Methods & Materials. (B) (i) Ca^{2+}-dependent glutamate release evoked by 4AP; (ii) Preincubation with AMPA (100 μM) for 30 s before 4AP addition. (C) (i) Ca^{2+}-dependent glutamate release evoked by 4AP; (ii) Preincubation with domoate (100 μM) for 30 s before 4AP addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three to four animals. Error bars are shown every 15 s for clarity.

Table 2.A. **Non-NMDA glutamate receptor agonists facilitate Ca^{2+}-dependent glutamate release evoked by 4AP (1mM)**

Ca^{2+}-DEPENDENT GLUTAMATE RELEASE (nmol/mg protein/7 min)			
Control	KA	AMPA	domoate
19.4 ± 0.5	25.6 ± 1*	26.4 ± 0.8*	24.7 ± 1.2*

*Significantly different from control ($p<0.05$, two-tailed Student's t-test)

Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A) was evoked by addition of 4AP (1mM) in the absence (Control) or presence of KA (100 μM), AMPA (100 μM) or domoate (100 μM), which were added for 30 s before 4AP addition. Ca^{2+}-dependent release was calculated by subtracting release determined in the absence of Ca^{2+} (presence of 200 μM EGTA) from release in the presence of 1mM Ca^{2+}. Data are the means ± SEM of independent experiments using synaptosomal preparations from three to four animals.

Table 2.B. **Non-NMDA glutamate receptor agonists do not alter Ca^{2+}-independent glutamate release evoked by 4AP (1mM)**

GLUTAMATE RELEASE (nmol/mg protein/7 min)			
Control	KA	AMPA	domoate
5.4 ± 0.3	5.5 ± 0.3*	6.0 ± 0.2*	5.1 ± 0.1*

*Not significantly different from control ($p>0.05$, two-tailed Student's t-test)

Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A) was evoked by addition of 4AP (1mM) in the absence of Ca^{2+} (presence of 200 μM EGTA) (Control). In some experiments, synaptosomes were preincubated with KA (100 μM), AMPA (100 μM) or domoate (100 μM) for 30 s before 4AP addition. Data are the means ± SEM of independent experiments using synaptosomal preparations from three to four animals.

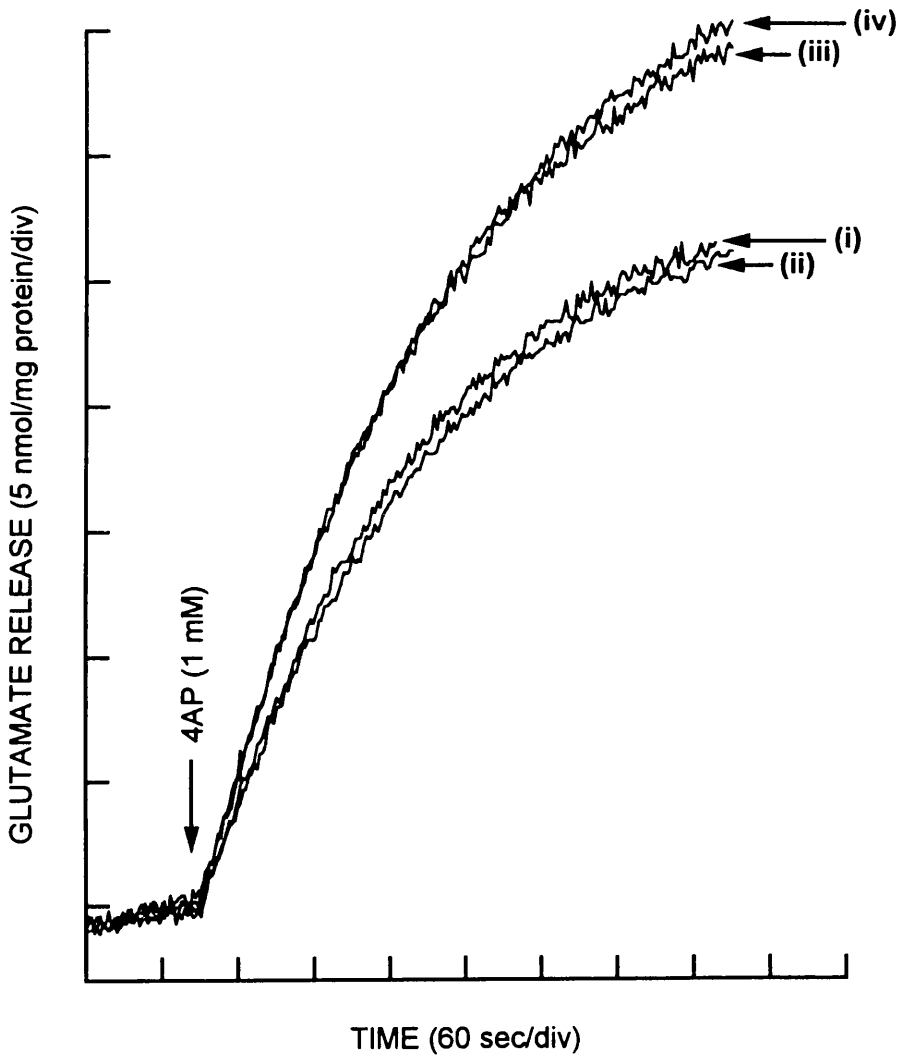

Fig.14. **Cyclothiazide does not modulate 4AP-evoked glutamate release or AMPA facilitation of 4AP-evoked glutamate release.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with cyclothiazide (100 μM) for 5 min before 4AP addition; (iii) Preincubation with AMPA (100 μM) for 30 s before 4AP addition; (iv) Preincubation with cyclothiazide (100 μM) and AMPA (100 μM) for 5 min and 30 s before 4AP addition respectively. Each trace is a single representative experiment.

nmol/7 min (Fig.14.iii) was not altered by preincubation with cyclothiazide prior to AMPA addition (Fig.14.iv). This latter result, together with results showing an equal facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release by KA and AMPA, (indicating that AMPA responses may be non-desensitising) strongly suggests that AMPA is not activating an AMPA-type glutamate receptor. In support of these functional release studies, there is little evidence from receptor localisation studies to suggest that cloned AMPA receptor subunits (GluR 1-4) have a presynaptic location; immunostaining occurs at postsynaptic densities (Petralia and Wenthold, 1992; Hampson et al., 1992).

As AMPA produces no currents in cloned homomerically expressed low-affinity KA receptor subunits (GluR 5 - 7) (Hollmann and Heinemann, 1994) it seems likely that the putative ionotropic glutamate receptor activated by AMPA which facilitates glutamate release from cerebral cortex synaptosomes, is a high-affinity KA receptor. In support of this proposal, it has been demonstrated that GluR5 or GluR6 KA receptor subunits when expressed with the high-affinity KA receptor subunit KA2, produce a high-affinity KA receptor channel which is gated by AMPA in a full agonist non-desensitising manner (Seeburg, 1993).

In contrast to AMPA, KA is a full non-desensitising agonist at AMPA receptors. Thus, the results in Fig.13.A showing that KA potentiates 4AP-evoked glutamate release, could be due to activation of an AMPA receptor or a KA receptor. One method of determining which receptor-type KA may be acting at, is to use specific antagonists of AMPA and KA receptors. In a set of experiments, control 4AP (1mM)-evoked glutamate release was 14.9 nmol/mg protein/5 min in the presence of 1mM Ca^{2+} , and preincubation of synaptosomes with 100 μM KA before 4AP addition facilitated glutamate release by some 4.5 nmol (19.45 nmol/mg protein/5 min) (Table 3). When synaptosomes were preincubated with the AMPA receptor antagonist GYKI 52466 (100 μM), control 4AP-evoked glutamate release appeared to be inhibited (11.98 nmol/mg protein/5 min) (Table 3). It is not clear whether this was a significant effect because GYKI 52466 caused severe quenching of the fluorescent signal during the glutamate release assay. However, preincubation with GYKI 52466 did not significantly antagonise KA-mediated potentiation of 4AP-evoked glutamate release; KA facilitated 4AP-evoked glutamate release by 3.5 nmol in the presence of GYKI 52466 (15.5

(* to ensure maximal block of non-NMDA glutamate receptors)

nmol/mg protein/5 min; cf. control release in the presence of GYKI 52466, 11.98 nmol/mg protein/5 min) (Table 3). Similar to GYKI 52466, the GluR6 KA receptor-selective antagonist NS-102 caused severe signal quenching, and preincubation with 100 μM NS-102 appeared to inhibit control 4AP-evoked glutamate release (10.65 nmol/mg protein/5 min) (Table 3). However, in contrast to GYKI 52466, preincubation with NS-102 completely prevented facilitation of 4AP-evoked glutamate release by KA (10.8 nmol/mg protein/5 min) (Table 3). Neither antagonist altered the Ca^{2+}-independent release of glutamate evoked by 4AP (data not shown). This observed NS-102-sensitive, KA-mediated potentiation of glutamate release is in complete contrast to studies with hippocampal synaptosomes showing that KA inhibits glutamate release in an NS-102 -sensitive manner (Chittajallu et al., 1996). As GYKI 52466, an AMPA receptor antagonist, failed to cause significant block of the KA response whilst NS-102, a specific GluR6 KA receptor antagonist, prevented KA-mediated facilitation of glutamate release, it is possible that KA facilitates glutamate release from cerebrocortical synaptosomes via activation of a 'putative' high-affinity KA receptor containing GluR6 subunits. Thus, like AMPA, KA does not seem to be mediating its effects on glutamate release through activation of an AMPA receptor containing GluR 1-4 subunits. In support of the proposal of a putative presynaptic high-affinity KA receptor that positively regulates glutamate exocytosis from cerebrocortical nerve terminals, there is evidence, at least in the hippocampus, to suggest that high-affinity KA receptor subunits have a presynaptic location (Werner et al., 1991).

To control for the severe quenching caused by NS-102 and GYKI 52466, the broad spectrum non-NMDA receptor antagonist CNQX was used to assess its effects on control 4AP-evoked glutamate release and KA facilitation of glutamate release. 4AP (1mM) evoked a Ca^{2+}-dependent release of 13.5 ± 0.64 nmol glutamate/mg protein/5 min (Fig.15.A.i). Addition of KA (100 μM) to synaptosomes before 4AP depolarisation facilitated Ca^{2+}-dependent release to 20.2 ± 0.97 nmol/mg protein/5 min (Fig.15.A.ii). Preincubation with the non-NMDA receptor antagonist CNQX (100 μM) before 4AP did not significantly alter Ca^{2+}-dependent glutamate release (12.7 ± 1.05 nmol/mg protein/5 min) (Fig.15.B.i). However, facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release by KA was blocked by CNQX; release in the presence of CNQX and KA was only 13.7 ± 1.08

Table 3. **KA-mediated facilitation of 4AP-evoked glutamate release is not blocked by the AMPA receptor antagonist GYKI 52466 but is prevented by the GluR6 KA receptor antagonist NS-102**

	GLUTAMATE RELEASE (nmol/mg protein/5 min)		
	Control	**GYKI 52466**	**NS-102**
4AP	14.9	11.98	10.65
4AP/KA	19.45	15.5	10.8

Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A) was evoked by 4AP (1mM) in the absence (Control) or presence of GYKI 52466 (100 μM) or NS-102 (100μM), which were added to synaptosomes 7 min before 4AP. In a separate set of experiments synaptosomes were preincubated with KA (100 μM) for 60 s before 4AP addition (4AP/KA) and release evoked in the absence (Control) or presence of GYKI 52466 (100 μM) or NS-102 (100μM), which were added to synaptosomes 7 min before 4AP. All experiments were performed in the presence of $CaCl_2$ (1mM). Data are the means of independent experiments using synaptosomal preparations from two animals.

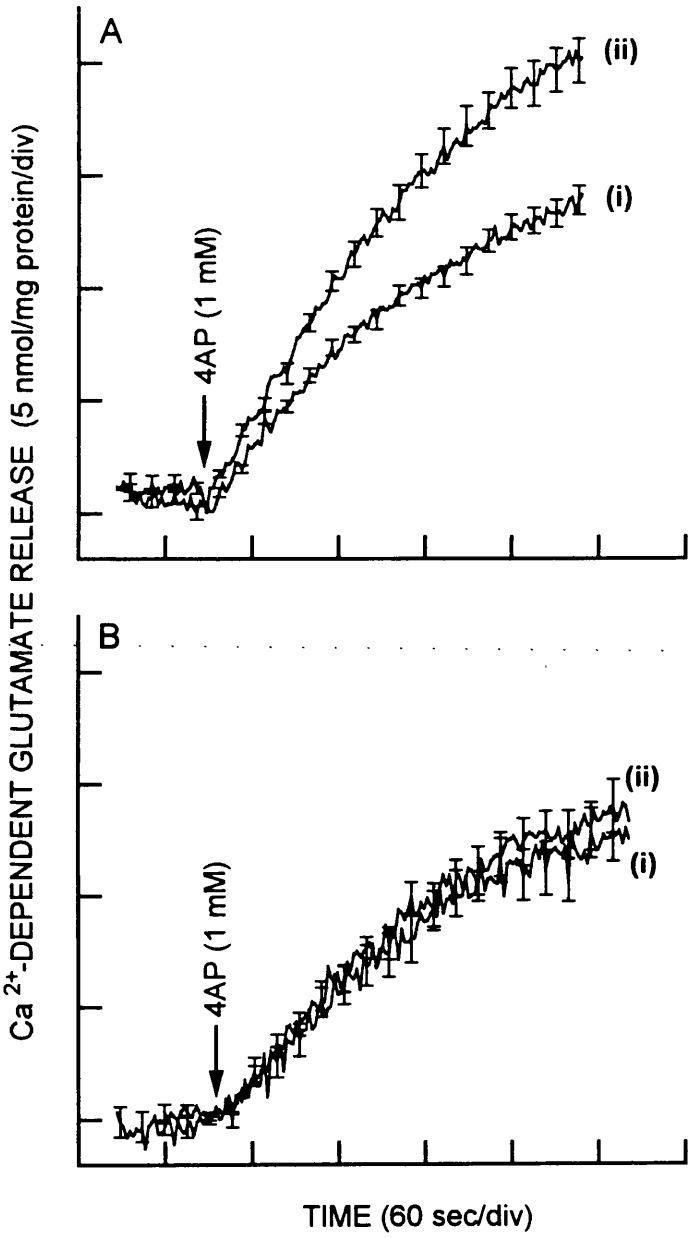

Fig.15. **CNQX, a non-NMDA glutamate receptor antagonist, blocks KA-mediated facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM); (ii) Preincubation with KA (100μM) for 30 s before 4AP addition. Net Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. (B) (i) Ca^{2+}-dependent glutamate release evoked by 4AP with CNQX (100μM) added 7 min prior to 4AP; (ii) Ca^{2+}-dependent glutamate release evoked by 4AP with CNQX (100μM) and KA (100μM) added 7 min and 30 s prior to 4AP respectively. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from five animals. Error bars are shown every 15 s for clarity.

glutamate/mg protein/5 min (Fig.15.B.ii) (cf. control glutamate release; 13.5 ± 0.64 nmol/mg protein/5 min). CNQX had no effect on 4AP-evoked Ca^{2+}-independent glutamate release (data not shown). These experiments with CNQX indicate that indeed KA is having its actions through a putative presynaptic non-NMDA receptor. However, CNQX did not alter control 4AP-evoked glutamate release, implying that the data from experiments with GYKI 52466 and NS-102 showing inhibition of control 4AP-evoked glutamate release by these antagonists (Table 3) may not be physiological and instead be due to the fluorescence quenching effects that occurred with these antagonists.

AMPA-mediated and KA-mediated facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release was not additive (Fig.16). AMPA (100 μM) facilitated 4AP-evoked glutamate release by 7 nmol/7 min and KA (100 μM) facilitated glutamate release by 6.2 nmol/7 min. When AMPA and KA were co-applied to synaptosomes, 4AP-evoked Ca^{2+}-dependent glutamate release was facilitated by 6.4 nmol/7 min. This result may indicate that AMPA and KA are acting through a common high-affinity KA receptor, which seems likely based on the observations that AMPA and KA are not acting through an AMPA-type glutamate receptor.

Classically, non-NMDA glutamate receptors are ligand-gated ion channels permeable to cations. AMPA/KA receptor activation leads to a conductance of Na^+ and Ca^{2+} (Brorson et al., 1992) causing membrane depolarisation. A mechanism of action for AMPA and KA modulation of glutamate release is not conclusive but there is data showing a slight depolarisation of the synaptosomal membrane potential by KA (Pastuszko et al., 1984). Furthermore, KA elicits a rapid increase in $^{45}Ca^{2+}$ uptake by crude rat brain synaptosomes (Pastuszko and Wilson, 1985; Pastuszko et al., 1984) and in hippocampal synaptosomes, KA and AMPA elicit rises in cytosolic free Ca^{2+} levels, $[Ca^{2+}]_c$ (Malva et al., 1995b). The synaptosomal membrane potential can be monitored by positively charged membrane potential-sensitive carbocyanine dyes such as $DiSC_2(5)$ (Enkvist et al., 1988). The dye becomes incorporated into the synaptosomal plasma membrane lipid bilayer. Upon depolarisation with 4AP, release of the dye from the membrane bilayer occurs, indicated experimentally as an increase in fluorescence. After $DiSC_2(5)$ equilibration, 4AP (1 mM) when applied to synaptosomes caused an increase in $DiSC_2(5)$ fluorescence of 3.1 ± 0.4 fluorescence units/2 min (Fig.17.i).

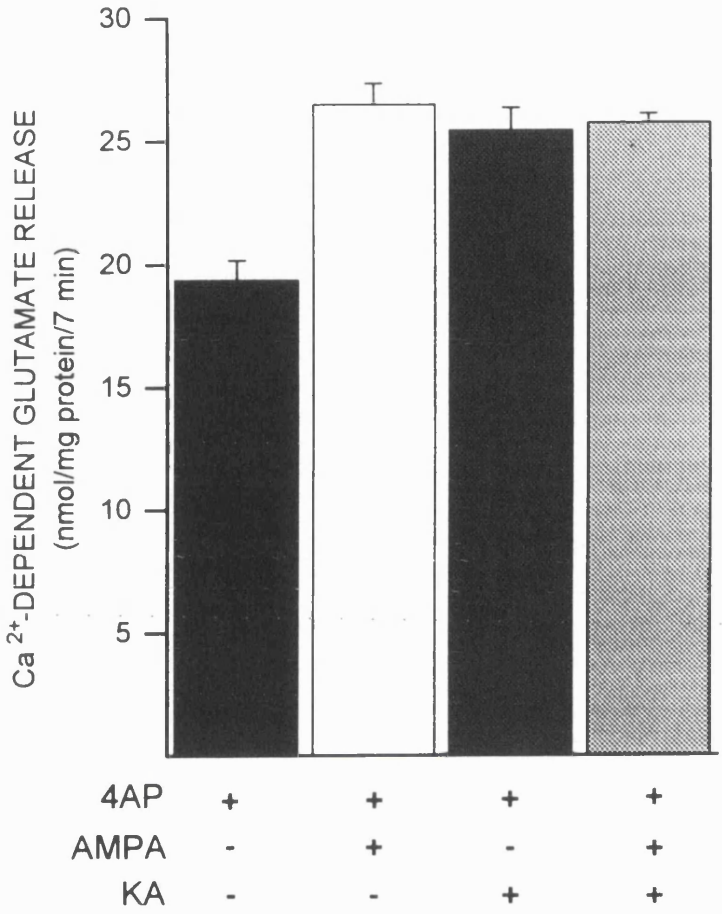

Fig.16. **AMPA-mediated and KA-mediated facilitation of the Ca^{2+}-dependent release of glutamate evoked by 4AP shows no additivity.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (1mM). Net Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. Where indicated, experiments were performed in the absence of AMPA and KA, presence of AMPA, presence of KA or presence of AMPA and KA. AMPA and KA were added for 30 s before 4AP addition and at a final concentration of 100 μM. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three to four animals.

Preincubation with KA (100μM) before 4AP addition potentiated the 4AP-mediated increase in fluorescence to 4.1 ± 0.5 fluorescence units/2 min (Fig.17.ii). This result showing that KA enhanced the 4AP-evoked depolarisation of the synaptosomal membrane potential seems to indicate that KA may be having its facilitatory effect on glutamate release by depolarising the synaptosomal plasma membrane. In support of this suggestion, it is known that activation of postsynaptic KA glutamate receptors depolarises neurons (Pook et al., 1993). A significant effect of KA on $DiSC_2(5)$ fluorescence in the absence of 4AP was not observed (data not shown). This may be because measurement of the synaptosomal plasma membrane potential with carbocyanine dyes is not a sensitive enough method for detecting changes in the resting membrane potential. However, depolarisation of the resting synaptosomal membrane potential by KA, albeit undetectable, could lower the threshold for voltage-dependent Na^+ channel activation in synaptosomes. Thus, when 4AP is applied to synaptosomes, by virtue of the mechanism of action of 4AP (blockade of K^+ channels leading to repetitive firing of TTX-sensitive Na^+ channels), a KA-mediated lowered threshold for Na^+ channel firing , could increase the population of Na^+ channels activated by 4AP. This could explain the enhanced 4AP-evoked depolarisation by KA.

In general, facilitation of glutamate exocytosis is likely to be the result of enhanced Ca^{2+} influx through VSCC linked to glutamate release. For example, in synaptosomes, activation of PKC by phorbol esters enhances 4AP-depolarisation and leads to a very large potentiation of Ca^{2+}-dependent glutamate release (Barrie et al., 1991; Coffey et al., 1993). Enhanced depolarisation by phorbol esters results in a moderate but significant increase in the 4AP-mediated rise in $[Ca^{2+}]_c$ (Coffey et al., 1993), which is indicative of enhanced Ca^{2+} influx through a Ca^{2+} channel linked to release. It is this increase in Ca^{2+} influx which leads to a potentiation of glutamate exocytosis. Experiments were performed using fura-2, to assess what effect KA and AMPA have on 4AP-evoked rises in $[Ca^{2+}]_c$. Elevation in $[Ca^{2+}]_c$ occurred after addition of 4AP (1 mM) to synaptosomes reaching a 'plateau' $[Ca^{2+}]_c$ of 286 ± 12 nM/2 min (Fig.18.i). Preincubation with KA (100 μM) before 4AP addition did not affect Ca^{2+} influx evoked by 4AP, 'plateau' $[Ca^{2+}]_c$ in the presence of KA being 285 ± 4 nM/2 min (Fig.18.ii). AMPA also had no effect on 4AP-evoked elevations in $[Ca^{2+}]_c$ (data not shown). The results may

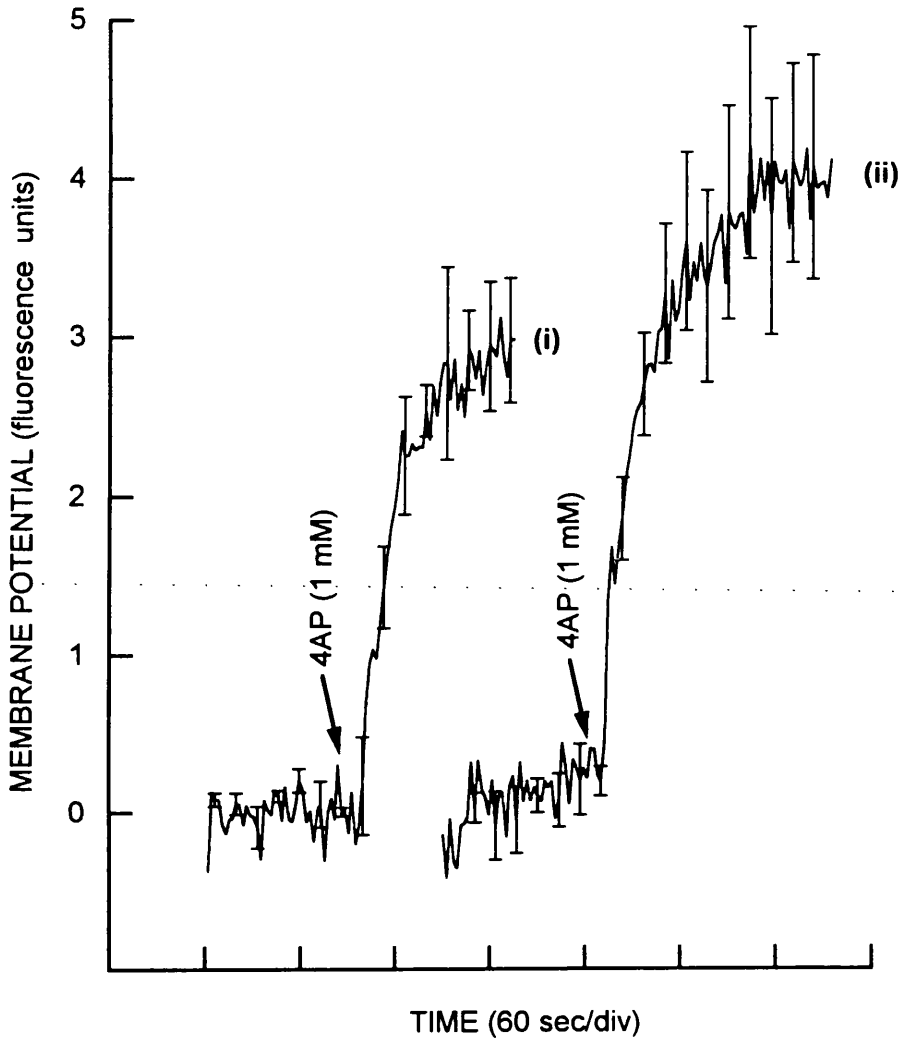

Fig.17. **KA enhances 4AP-evoked depolarisation of the synaptosomal membrane potential.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.5 and the synaptosomal membrane potential monitored with $DiSC_2(5)$. (i) Addition of 4AP (1mM) to synaptosomes; (ii) Preincubation with KA (100 μM) for 1 min before 4AP addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

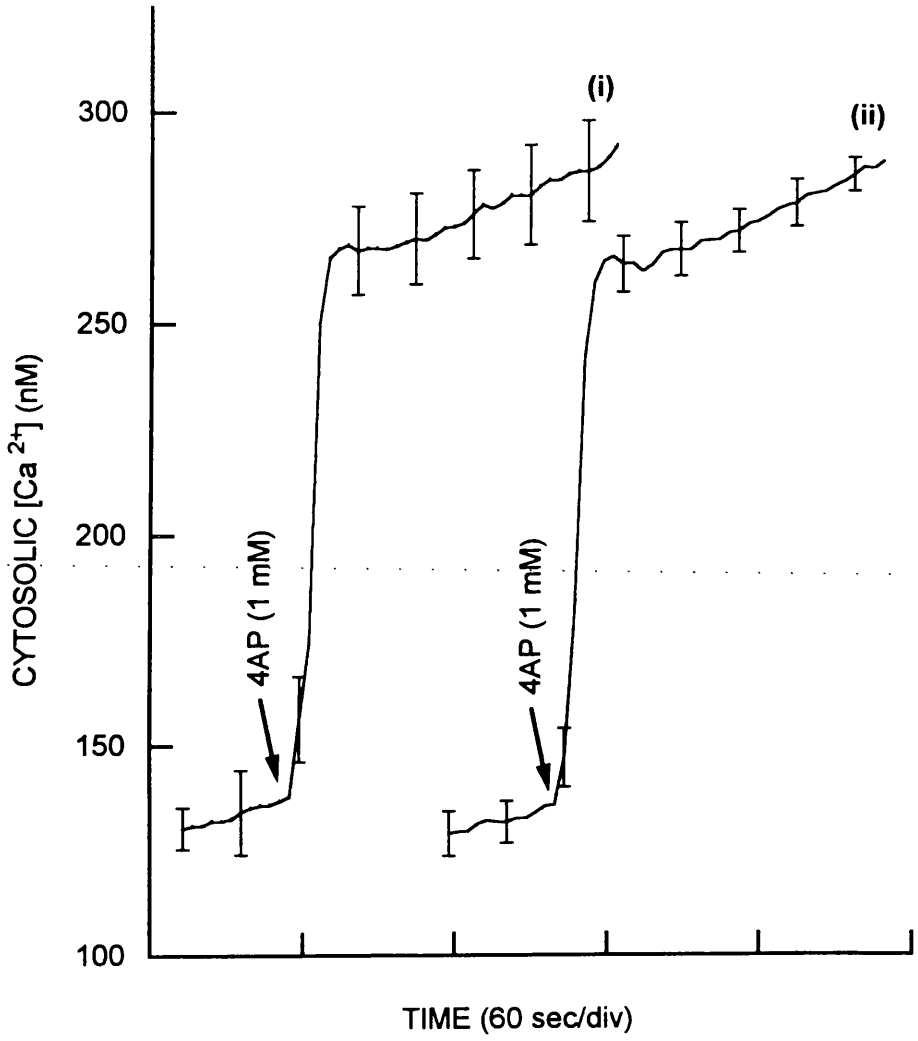

Fig.18. **KA has no effect on the 4AP-evoked increase in $[Ca^{2+}]_c$.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. (i) Elevation in $[Ca^{2+}]_c$ after addition of 4AP (1mM); (ii) Preincubation with KA (100μM) for 30 s before 4AP addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 30 s for clarity.

indicate that KA-mediated and AMPA-mediated facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release is not the result of increased Ca^{2+} influx into synaptosomes. This lack of effect seems confusing based on the idea that an enhanced 4AP-evoked depolarisation by KA would be expected to lead to an increased Ca^{2+} influx; previous studies show that an enhancement of the 4AP-evoked depolarisation of the synaptosomal plasma membrane potential results in increased Ca^{2+} entry into nerve terminals (Coffey et al., 1993). Furthermore, an increase in the transmitter release trigger (i.e. Ca^{2+}) seems to be the most physiologically relevant means by which to facilitate transmitter exocytosis. A direct interaction of a facilitatory ionotropic glutamate receptor with the transmitter release machinery itself (bypassing the VSCC linked to glutamate release) seems unlikely and would probably still incorporate a Ca^{2+} trigger. A possible explanation for the observed lack of effect of KA and AMPA on the 4AP-evoked elevation in $[Ca^{2+}]_c$ may take into account the experimental paradigm used to assess $[Ca^{2+}]_c$. Fura-2 reports overall, averaged population responses (with no indication of changes occurring at the level of an individual synaptosome/nerve terminal). Additionally, fura-2 does not reflect the localised influx of Ca^{2+} coupled to SSV exocytosis (Augustine and Neher, 1992; Sihra et al., 1992) and unless ionotropic receptor-mediated effects occur synchronously in a significant majority of nerve terminals, modulatory effects of KA and AMPA on $[Ca^{2+}]_c$ could be overlooked. The observation that KA-mediates a rapid increase in $^{45}Ca^{2+}$ uptake by crude rat brain synaptosomes (Pastuszko et al., 1984; Pastuszko and Wilson, 1985) may be misleading in relation to the putative presynaptic high-affinity KA receptor-mediated effects laid out in this chapter. This is because very high concentrations of KA (1 - 10 mM) were used in the abovementioned $^{45}Ca^{2+}$ studies. However, at these high concentrations, KA has been reported to have effects other than facilitation of glutamate exocytosis, i.e. Ca^{2+}-independent inhibition of the nerve terminal plasma membrane glutamate transporter occurs (Pocock et al., 1988). In the present studies, these potential effects of KA were purposely obviated by using low concentrations of KA.

There is little functional release data invoking a role of cAMP-dependent protein kinase (PKA) in the regulation of glutamate exocytosis. Despite this, in synaptosomes, forskolin, which activates adenylate cyclase, stimulates

translocation of the nerve terminal protein synapsin I from a particulate to a cytosolic fraction (Sihra et al., 1989). This translocation of synapsin I also occurs upon elevated external KCl depolarisation and 4AP depolarisation and has been linked to regulation of glutamate exocytosis. Thus, this stimulatory action of forskolin on synapsin I translocation indicates that PKA can be activated in nerve terminals and may be involved in regulation of glutamate release *in situ*. However, forskolin has no effect on KCl (30mM)-evoked glutamate release, indicating, at least *in vitro*, that PKA does not regulate glutamate release *per se*. Instead, nerve terminal PKA may be involved in the regulation of presynaptic receptors that modulate depolarisation-evoked glutamate release.

It is known that phosphorylation of ionotropic glutamate receptors by PKA can regulate their function. In hippocampal neurons, AMPA and KA responses are potentiated by the cAMP analogue cAMPS and depressed by Rp-cAMPS, an inhibitor of PKA (Wang et al., 1991). Cloned and expressed GluR6 KA receptors are directly phosphorylated by PKA, the result of which is a potentiation of receptor currents (Wang et al., 1993; Raymond et al., 1993). Results in this chapter show that AMPA and KA facilitate 4AP-evoked glutamate release from cerebral cortex synaptosomes possibly through activation of a high-affinity KA receptor containing GluR6 subunits. Experiments were performed using a sub-maximal concentration of KA (50 μM), to see whether KA facilitation of glutamate release could be positively or negatively regulated by PKA. To do this, inhibitors/activators of PKA were used in conjunction with KA. Control experiments indicated that 4AP-evoked glutamate release (Fig.19.A.i) was not modulated by preincubation of synaptosomes with the membrane-permeable cAMP analogue 8-Br-cAMP (250 μM) (Fig.19.A.ii). Furthermore, 4AP-evoked glutamate release (Fig.19.B.i) was not affected by attempts to inhibit synaptosomal PKA activity by preincubation with Rp-cAMPS (250 μM) (Fig.19.B.ii). The lack of effect of these agents on 4AP-evoked glutamate release confirms previous reports indicating that PKA probably does not regulate glutamate release *per se*. 4AP-evoked glutamate release (Fig.19.C.i) was facilitated by KA (50 μM) (Fig.19.C.ii) but 8-Br-cAMP did not modulate the KA response (Fig.19.C.iii). Similary, facilitation of 4AP-evoked glutamate release by 50 μM KA (Fig.19.D.ii) was not affected by preincubation with Rp-cAMPS before

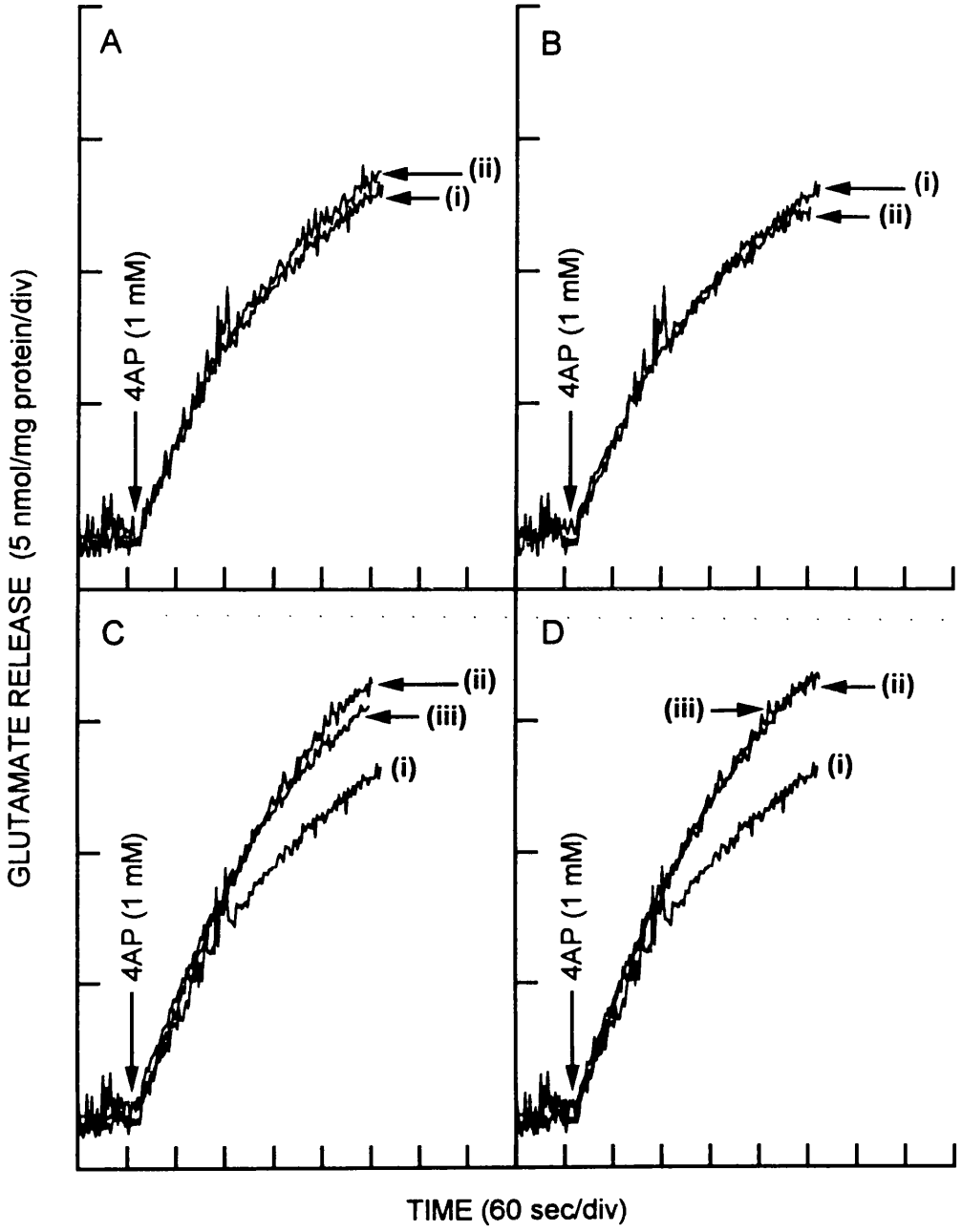

Fig.19. **KA responses do not appear to be modulated by cAMP-dependent protein kinase (PKA).** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with the cAMP analogue 8-Br-cAMP (250 μM) for 6 min before 4AP addition. (B) (i) 4AP-evoked glutamate release; (ii) Preincubation with the PKA inhibitior Rp-cAMPS (250 μM) for 6 min before 4AP addition. (C) (i) 4AP-evoked glutamate release; (ii) Preincubation with KA (50 μM) for 1 min before 4AP addition; (iii) Preincubation with 8-Br-cAMP (250 μM) and KA (50 μM) for 6 min and 1 min before 4AP addition respectively. (D) (i) 4AP-evoked glutamate release; (ii) Preincubation with KA (50 μM) for 1 min before 4AP addition; (iii) Preincubation with Rp-cAMPS (250 μM) and KA (50 μM) for 6 min and 1 min before 4AP addition respectively. Each trace is a single represenatative experiment.

KA addition (Fig.19.D.iii). These preliminary results indicate that the proposed presynaptic high-affinity KA receptor present on cerebral cortex nerve terminals which facilitates 4AP-evoked Ca^{2+}-dependent glutamate release may not be regulated by PKA. This is in contrast to cloned and expressed KA receptor subunits which can be regulated by PKA.

All of the results relating to glutamate release in this chapter have utilised an enzyme-linked assay of glutamate with continuous on-line fluorimetry (see Methods & Materials section 2.2.A). This involves the use of a 'glutamate trapping system' whereby glutamate dehydrogenase (GDH) and $NADP^+$ added to synaptosomes catalyse the oxidation of released glutamate by GDH, this being coupled to $NADP^+$ reduction to NADPH. NADPH fluorescence is monitored and provides a correlate to endogenously released glutamate. However, endogenous glutamate release can also be measured in the absence of a glutamate trap by using HPLC techniques (see Methods & Materials sections 2.2.B). As it has been reported that KA inhibits glutamate release from synaptosomes in the presence of a glutamate trap but stimulates glutamate release in the absence of the trap (albeit that the reported KA effects are on Ca^{2+}-independent glutamate release) (Pocock et al., 1988), experiments were performed to test this proposal. HPLC analysis of 4AP-evoked glutamate release and the effects of KA on this release are detailed in Fig.20. 4AP (1 mM) evoked the release of 8.41 ± 1.7 nmol glutamate/mg protein/2.5 min in the presence of added $CaCl_2$ (1 mM) and 3.23 ± 0.76 nmol glutamate/mg protein/2.5 min in the absence of 1mM Ca^{2+} but presence of EGTA (200 μM). Thus, calculated net 4AP-evoked Ca^{2+}-dependent glutamate release (Ca-dep) was 5.17 ± 0.94 nmol/mg protein/2.5 min. Preincubation of synaptosomes with KA (100 μM) before 4AP addition in the presence of Ca^{2+} facilitated glutamate release to 11.27 ± 1.7 nmol/mg protein/2.5 min. 4AP-evoked Ca^{2+}-independent glutamate release in the presence of KA was not significantly different to control Ca^{2+}-independent release (2.86 ± 0.61 nmol/mg protein/2.5 min). The calculated net 4AP-evoked Ca^{2+}-dependent glutamate release in the presence of KA was 8.41 ± 1.1 nmol/mg protein/2.5 min, thus, KA facilitated Ca^{2+}-dependent glutamate release by 3.24 nmol/2.5 min. This amounted to a 60% increase above control 4AP-evoked release. These HPLC studies indicate that irrespective of the presence or absence of a glutamate trapping

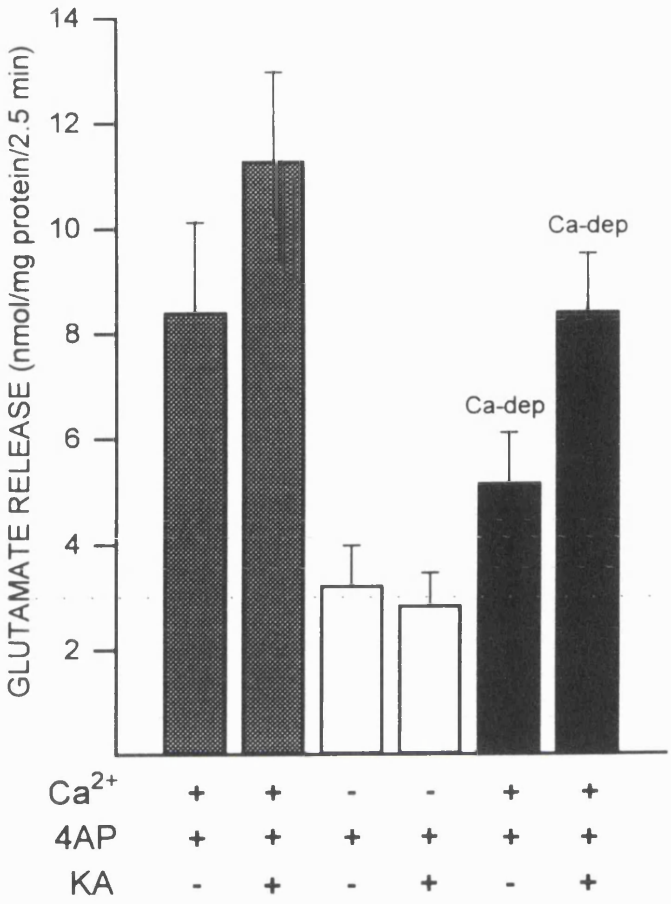

Fig.20. **KA facilitates 4AP-evoked Ca^{2+}-dependent glutamate exocytosis in the absence of a glutamate trap.** Percoll-purified synaptosomes (0.25 mg/ml) were incubated as described in the Methods & Materials section 2.2.B. Glutamate release, quantitated by HPLC analysis (Methods & Materials section 2.2.B), was evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM) or absence of 1mM Ca^{2+} (presence of 200 μM EGTA). Net Ca^{2+}-dependent release (Ca-dep) was calculated by subtracting release determined in the absence of 1mM Ca^{2+} (presence of EGTA) from release in the presence of $CaCl_2$ (1mM). Where indicated, synaptosomes were preincubated with KA (100 μM) for 30 s before 4AP addition. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals.

system, KA effects are always stimulatory and only on the Ca^{2+}-dependent component to 4AP-evoked glutamate release.

Conclusions

- In cerebral cortex synaptosomes, the non-NMDA receptor agonists AMPA, KA and domoate all facilitate the Ca^{2+}-dependent release of glutamate evoked by 4AP. This is in contrast to results in the hippocampus which show that synaptosomal Ca^{2+}-dependent glutamate release is potentiated by AMPA but inhibited by KA (Barnes et al., 1994; Chittajallu et al., 1996).

- AMPA responses are as full as KA responses and are not modulated by the AMPA receptor desensitisation inhibitor cyclothiazide. This indicates that AMPA is probably not mediating its actions through an AMPA-type receptor but instead via stimulation of a presynaptic high-affinity KA-type receptor.

- KA facilitation of glutamate release is blocked by CNQX and the GluR6 KA receptor antagonist NS-102, whereas the AMPA receptor antagonist GYKI 52466 does not antagonise KA effects. This indicates that, like AMPA, KA is stimulating a presynaptic high-affinity KA receptor possibly containing GluR6 KA subunits. Lending support to these pharmacological deductions, receptor localisation studies indicate that high-affinity KA receptors may have a presynaptic location (Werner et al.,1991).

- A lack of additivity of AMPA and KA facilitation of 4AP-evoked Ca^{2+}-dependent glutamate release may indicate that these agonists stimulate a common presynaptic high-affinity KA receptor.

- KA enhances 4AP-evoked depolarisation of the synaptosomal membrane potential. This suggests that the mechanism of action of AMPA/KA-mediated facilitation of 4AP-evoked glutamate release may be stimulation of a ligand-gated ionotropic glutamate receptor permeable to cations

leading to nerve terminal membrane depolarisation.

- Inhibitors/activators of PKA did not have any effect on KA facilitation of 4AP-evoked glutamate release. This suggests that in contrast to their postsynaptic counterparts, putative presynaptic high-affinity KA receptors may not be regulated by PKA.

CHAPTER FIVE

5 Inhibition of glutamate exocytosis from cerebral cortex nerve terminals (synaptosomes) by presynaptic $GABA_B$ receptors

Introduction

GABA is the primary inhibitory neurotransmitter in the brain producing inhibition through two different receptor types termed $GABA_A$ and $GABA_B$ on the basis of their pharmacological properties. At mammalian CNS synapses, $GABA_A$ receptors have a somatic location and produce powerful fast synaptic inhibition by directly increasing membrane Cl^- conductance (Stephenson, 1988). In contrast, $GABA_B$ receptors have a dual location being present on postsynaptic dendrites and presynaptic nerve terminals. $GABA_B$ receptors produce slower responses than $GABA_A$ receptors and are considered to be modulatory in action (Bowery, 1989). GABA is the endogenous ligand at both GABA receptor types.

The GABA analogue baclofen (β-*P* -chlorophenyl GABA) is used to distinguish the $GABA_B$ receptor from $GABA_A$ receptors. In this regard, although baclofen and GABA produce many similar effects, baclofen does not mimic all of the actions of GABA. Most GABA responses can be potently antagonised by the plant alkaloid bicuculline but baclofen effects are insensitive to bicuculline (Fox et al., 1978). Furthermore, the observation that GABA depresses noradrenaline release from mammalian nerve terminals through a mechanism that is insensitive to bicuculline but mimicked by baclofen (Bowery et al., 1980), together with [^{3}H]baclofen binding studies, led to the proposal of a novel GABA receptor termed the $GABA_B$ receptor, that is pharmacologically distinct from 'classical' bicuculline-sensitive $GABA_A$ receptors (Hill and Bowery, 1981). Some of the highest absolute concentrations of $GABA_B$ binding sites occur in the frontal cortex (Bowery et al., 1987). The guanyl nucleotide GTP inhibits $GABA_B$ receptor binding in a pertussis toxin-sensitive manner, suggesting that $GABA_B$ receptors are functionally coupled to inhibitory G-proteins, either G_i and/or G_o.

$GABA_B$ receptors are activated by baclofen in a stereospecific manner with (-) baclofen being equipotent to GABA, whereas (+)baclofen is >100 times less potent (Olpe et al., 1978; Haas et al., 1985; Hill and Bowery, 1981). Unlike $GABA_A$ receptors, no naturally occurring compounds have been identified that specifically antagonise $GABA_B$ receptors. Phaclofen (3-amino-2-(4-chlorophenyl)-

propylphosphinic acid, a phosphinic analogue of baclofen, is a weak but selective antagonist of $GABA_B$ receptors and seems to preferentially block postsynaptic $GABA_B$ receptors, suggesting that postsynaptic and presynaptic $GABA_B$ receptors may be pharmacologically distinguishable (Dutar and Nicoll, 1988). However, the most effective $GABA_B$ receptor antagonists to date are based on phosphinic analogues of GABA. One such compound, CGP 35348 (*P*-(3-aminopropyl)-*P*-diethoxymethylphosphinic acid) appears to be very selective for $GABA_B$ receptors (Olpe et al., 1990) having no action at $GABA_A$ receptors or any other transmitter system. Two novel phosphinic analogues of GABA, CGP 54626 and CGP 55845 are 15,000 times more potent than phaclofen and 5,000 times more potent than CGP 35348 at $GABA_B$ receptors. However, because they are relatively new compounds, little is known of their selectivity and their effect on synaptic responses has not been fully characterised.

$GABA_B$ receptors are coupled to a number of intracellular effector systems including inhibition of adenylyl cyclase via G_i and G_o, potentiation of receptor-mediated adenylyl cyclase activity, inhibition of voltage-sensitive Ca^{2+} channels (VSCC), and activation of K^+ channels (for review see Mott and Lewis, 1994). Ca^{2+} currents in dorsal root ganglion sensory neurons are inhibited by $GABA_B$ receptor activation via a direct suppression of VSCC (K^+ channels are not involved) (Dolphin et al., 1989). These $GABA_B$ receptor-mediated effects occur through a pertussis toxin-sensitive inhibitory G-protein (Dolphin and Scott, 1987), seemingly $G_o\,\alpha$ subunits (Menon-Johansson et al., 1993). Furthermore, inhibition of somatic Ca^{2+} currents in sensory neurons by G-protein coupled $GABA_B$ receptors does not appear to involve an intracellular second messenger, the effect being membrane-delimited instead (Dolphin et al., 1989). Similar to sensory neurons, $GABA_B$ receptor activation inhibits somatic Ca^{2+} currents in central neurons via coupling to a pertussis toxin-sensitive inhibitory G-protein (Wojcik et al., 1990; Scholz and Miller, 1991). In contrast to sensory neurons, inhibition of Ca^{2+} currents by (-) baclofen in spinal cord neurons is antagonised by activation of adenylyl cyclase using forskolin or by the cAMP analogue 8-Br-cAMP (Kamatchi and Ticku, 1990), suggesting that a second messenger may be involved in suppression of Ca^{2+} currents by (-)baclofen.

In addition to inhibition of Ca^{2+} channels, postsynaptic $GABA_B$ receptor

activation leads to membrane hyperpolarisation by increasing an inwardly rectifying K^+ conductance (Mott and Lewis, 1994). $GABA_B$ receptors that activate K^+ channels do so via coupling to G_o proteins (Andrade et al., 1986; Brown and Birnbaumer, 1990). Whether G-protein-mediated coupling of $GABA_B$ receptors to K^+ channels is direct or involves a diffusable second messenger remains to be conclusively demonstrated. However, cAMP does not appear to be involved (Innis et al., 1988).

A presynaptic effect of baclofen was first suggested in studies examining the antispastic effects of the drug. Systemic administration of (-)baclofen depresses mono- and polysynaptic reflex transmission in the spinal cord which appears to be mediated by a depression of transmitter release from excitatory nerve terminals (Pierau and Zimmermann, 1973). Electrophysiological studies support the existence of presynaptic $GABA_B$ receptors, based on findings that the suppressant action of (-)baclofen can be localised to specific afferent pathways such as the CA1 region of the hippocampus. In this area, (-)baclofen inhibits the Schaffer-Collateral-evoked excitatory responses with an IC_{50} of 3 - 4 μM (Lanthorn and Cotman, 1981; Potier and Dutar, 1993). Release studies support the existence of $GABA_B$ receptors, first demonstrated in guinea-pig cerebral cortex where (-)baclofen inhibits electrically-evoked glutamate release (Potashner, 1979). Presynaptic $GABA_B$ receptors inhibit K^+-evoked glutamate release from cerebral cortex synaptosomes in a phaclofen-insensitive, CGP 35348-sensitive manner (Pende et al., 1993; Raiteri et al., 1992; Bonanno and Raiteri, 1992). In the same synaptosomal preparation, however, (-) baclofen inhibits GABA and somatostatin release in a phaclofen-sensitive manner (Bonanno et al., 1989; Bonanno and Raiteri, 1992; Bonanno and Raiteri, 1993). Differing sensitivity to phaclofen and CGP 35348 in the cerebral cortex suggests that different neurotransmitters may be modulated by different subtypes of $GABA_B$ receptors.

Presynaptic $GABA_B$ receptors could potentially inhibit transmitter release by directly inhibiting a nerve terminal Ca^{2+} channel thereby reducing Ca^{2+} influx, or by activating a K^+ channel. An increase in K^+ conductance would suppress transmitter release by shunting invading action potentials thereby causing a decrease in Ca^{2+} influx. However, studies in the hippocampus show that Ba^{2+} (a K^+ channel blocker), which prevents (-) baclofen-induced postsynaptic

hyperpolarisation, does not affect the suppression of excitatory transmitter release produced by (-)baclofen (Thompson and Gahwiler, 1992), suggesting that either presynaptic $GABA_B$ receptors do not inhibit excitatory transmitter release by activating a K^+ conductance or that different K^+ conductances are activated by presynaptic and postsynaptic $GABA_B$ receptors.

Direct evidence relating a $GABA_B$ receptor-mediated inhibition of nerve terminal Ca^{2+} channels with depression of transmitter release has not yet been reported. Inhibition of somatic VSCC by (-)baclofen provides an indirect model of how Ca^{2+} channels on nerve terminals may be inhibited by $GABA_B$ receptors. That inhibition of nerve terminal VSCC may be the mechanism by which presynaptic $GABA_B$ receptors depress transmitter release is suggested by the observation that (-)baclofen depresses K^+-evoked transmitter release from synaptosomes (Bonanno et al., 1989) and inhibits K^+-evoked rises in cytosolic free Ca^{2+} levels, $[Ca^{2+}]_c$, in synaptosomes (Stirling et al., 1989; Tareilus et al., 1994).

Coupling of postsynaptic $GABA_B$ receptors to pertussis toxin (PTX)-sensitive G-proteins suggests that presynaptic $GABA_B$ receptors may also be coupled to a PTX-sensitive G-protein. However, experimental evidence relating presynaptic $GABA_B$ receptors with a PTX-sensitive G-protein is not conclusive. In whole brain or slice studies PTX blocks postsynaptic $GABA_B$ receptor responses whilst leaving presynaptic inhibition of transmitter release by (-)baclofen unaffected (Colmers and Williams, 1988; Dutar and Nicoll, 1988) or partially maintained (Potier and Dutar, 1993). In contrast to these studies indicating that presynaptic $GABA_B$ heteroreceptors may not be coupled to PTX-sensitive G-proteins, it has been demonstrated in cell cultures that (-)baclofen-mediated inhibition of hippocampal excitatory postsynaptic currents is blocked by PTX (Scholz and Miller, 1991) and long PTX treatment of cerebellar granule cells abolishes the inhibitory effect of (-)baclofen on glutamate release (Huston et al., 1993). Thus, it would appear that poor or slow access to presynaptic nerve terminals might explain a lack of effect of PTX in whole brain and slice studies. However, this may not be so because it has also been reported that presynaptic actions of (-)baclofen on excitatory transmission in hippocampal cell cultures are insensitive to PTX, whilst presynaptic $GABA_B$ autoreceptors regulating GABA release are PTX-sensitive (Thompson and Gahwiler, 1992). Thus, presynaptic

$GABA_B$ receptors may be coupled to PTX-sensitive and PTX-insensitive G-proteins.

In addition to G-proteins, second messenger systems have been implicated in the mechanism underlying inhibition of transmitter release by $GABA_B$ receptors. In cerebellar granule cells, cAMP analogues block inhibition of glutamate release by (-)baclofen (Travagli et al., 1991), suggesting that presynaptic $GABA_B$ receptors may be G-protein linked to inhibition of adenylate cyclase activity. In hippocampal cell cultures, activation of protein kinase C using phorbol esters has been reported to inhibit the presynaptic actions of (-)baclofen (Thompson and Gahwiler, 1992). Protein kinase C and adenylate cyclase may be involved in the modulation of transmitter release by $GABA_B$ receptors.

There have been a few reports demonstrating that (-)baclofen inhibits glutamate release from cerebrocortical nerve terminals (Pende et al., 1993; Raiteri et al., 1992; Bonanno and Raiteri, 1992). However, in these same studies a mechanism of action for (-)baclofen inhibition of glutamate release was not addressed. PhD studies were undertaken to characterise presynaptic $GABA_B$ receptor-mediated inhibition of glutamate release from cerebrocortical synaptosomes and to then investigate the mechanism that underlies modulation of glutamate release by presynaptic $GABA_B$ receptors. (-)Baclofen dose-dependently inhibited the Ca^{2+}-dependent release of glutamate evoked by 4AP whilst (+)baclofen had no significant effect. These effects of (-)baclofen were prevented by the $GABA_B$ receptor antagonist CGP 35348 but not by the $GABA_B$ receptor antagonist phaclofen. Similar to (-)baclofen, the endogenous $GABA_B$ receptor ligand GABA inhibited 4AP-evoked glutamate release in a CGP 35348-sensitive manner. (-)Baclofen-mediated inhibition of glutamate release was insensitive to pertussis toxin (PTX) indicating that a PTX-insensitive G-protein may be coupled to presynaptic $GABA_B$ receptors that inhibit excitatory transmitter glutamate release in the cerebral cortex. The mechanism underlying $GABA_B$ receptor-mediated inhibition of glutamate release was probed with the findings that (-)baclofen had no effect on the synaptosomal membrane potential or depolarisation of the membrane potential by 4AP. This finding argues against an action of presynaptic $GABA_B$ receptors on a terminal K^+ conductance. However, (-)baclofen caused a significant reduction in 4AP-evoked Ca^{2+} influx into

synaptosomes whilst having no effect on ionomycin-induced glutamate release. This result indicates that $GABA_B$ receptor activation may lead to an inhibition of the voltage-sensitive Ca^{2+} channels (VSCC) linked to glutamate release. Inhibition of nerve terminal Ca^{2+} channel(s) by presynaptic $GABA_B$ receptor activation may be a membrane-delimited effect with no intracellular second messenger involved. This is based on the finding that attempts at boosting synaptosomal cAMP levels did not prevent the inhibitory effect of (-)baclofen. Thus, unlike their postsynaptic counterparts, presynaptic $GABA_B$ receptors on excitatory nerve terminals may not be coupled to inhibition of adenylate cyclase activity. (-)Baclofen-mediated inhibition of glutamate release was reversed by activation of presynaptic protein kinase C with phorbol ester.

Results and discussion

Preincubation of cerebral cortex synaptosomes with a range of doses of the GABA analogue (-)baclofen (100nM - 100μM) inhibited Ca^{2+}-dependent glutamate release evoked by 4AP (1mM) (Fig.21.A). A maximal inhibition of 40% occurred at 50 - 100 μM (-)baclofen. The inhibition of glutamate release by baclofen was stereoselective as (+)baclofen did not produce any signifcant inhibition of glutamate release (data not shown). The IC_{50} value for (-) baclofen inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release, derived from a log. dose-response curve (Fig.21.B) was 4.5 μM. This is similar to IC_{50} values for (-) baclofen-mediated inhibition of K^+-evoked glutamate release from cerebral cortex synaptosomes (1.5 μM) (Bonanno and Raiteri, 1992) and (-)baclofen-mediated inhibition of excitatory hippocampal transmission (3.8 μM) (Lanthorn and Cotman, 1981). Ca^{2+}-independent glutamate release evoked by 4AP was not altered by (-) baclofen (50 μM) (Table 4.A), indicating that (-)baclofen only inhibits the Ca^{2+}-dependent component to 4AP-evoked glutamate release. This *in vitro* Ca^{2+}-dependent glutamate release is believed to represent exocytosis of SSVs containing glutamate *in situ*.

It has previously been shown that presynaptic $GABA_B$ heteroreceptors that limit the release of glutamate from nerve terminals are blocked by the $GABA_B$ receptor antagonist CGP 35348 but are insensitive to block by phaclofen, another $GABA_B$ receptor antagonist (Pende et al., 1993; Raiteri et al., 1992; Bonanno and

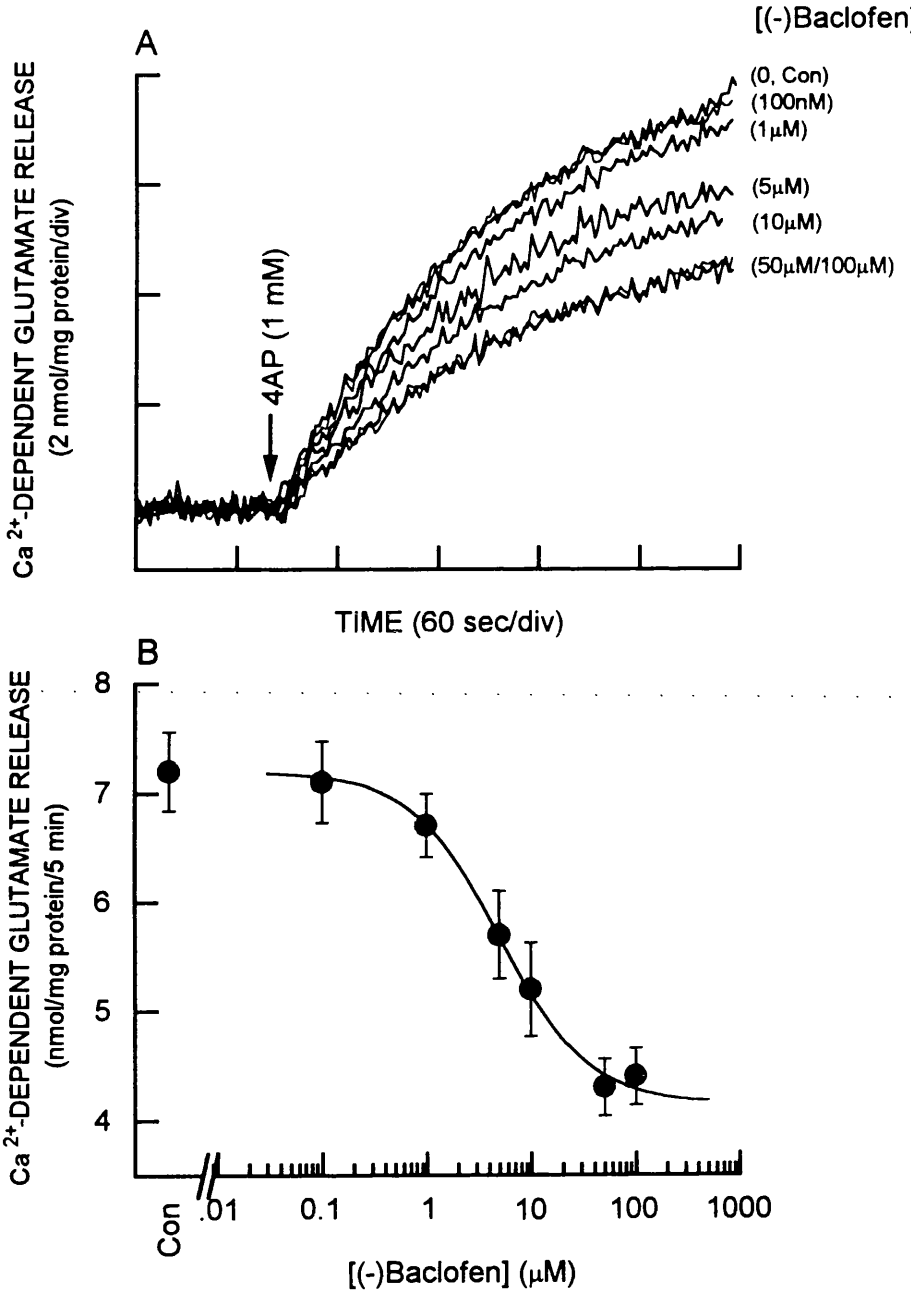

Fig.21.(-)Baclofen dose-dependently inhibits the Ca^{2+}-dependent release of glutamate evoked by 4AP. P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (1mM) in the absence (0, Con) or presence of a range of doses of (-)baclofen (100 nM - 100 μM), which were preincubated for 2 min before 4AP addition. Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. Each trace is the mean of independent experiments using synaptosomal preparations from three animals. (B) Log.dose-response curve for (-)baclofen inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release, fitted using a logistic function. Data are the means ± SEM of three independent experiments.

Table 4.A. **(-)Baclofen, GABA, CGP 35348 and phaclofen do not alter Ca^{2+}-independent glutamate release evoked by 4AP (1mM)**

	GLUTAMATE RELEASE (nmol/mg protein/5 min)
Control	1.22 ± 0.10
(-)baclofen	1.3 ± 0.15*
GABA	1.4 ± 0.13*
CGP 35348	1.23 ± 0.10*
phaclofen	1.3 ± 0.14*

*Not significantly different from control (p>0.05, two-tailed Student's t-test)

P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A) was evoked by addition of 4AP (1mM) in the absence of Ca^{2+} (presence of 200 μM EGTA) (Control). In some experiments, synaptosomes were pre-incubated with (-)baclofen (50 μM) or GABA (20 μM) for 2 min before 4AP addition, and CGP 35348 (200 μM) or phaclofen (200 μM) for 7 min before 4AP addition. Data are the means ± SEM of independent experiments using synaptosomal preparations from three animals.

Table 4.B. **(-)Baclofen does not alter Ca^{2+}-independent glutamate release evoked by KCl (15mM) or KCl (30mM)**

	GLUTAMATE RELEASE (nmol/mg protein/5 min)	
	KCl (15mM)	KCl (30mM)
Control	2.62 ± 0.13	3.25 ± 0.17
(-)baclofen	2.55 ± 0.13*	3.0 ± 0.15*

*Not significantly different from control (p>0.05, two-tailed Student's t-test)

P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A) was evoked by addition of KCl (15 mM) or KCl (30 mM) in the absence of Ca^{2+} (presence of 200 μM EGTA) and experiments performed in the absence (Control) or presence of (-) baclofen (50 μM) which was added 2 min before KCl. Data are the means ± SEM of independent experiments using synaptosomal preparations from three animals.

✳ Phaclofen is a weak antagonist at $GABA_B$ receptors and is often used at a concentration of 1 mM. In another set of experiments, phaclofen, at a concentration of 1 mM, failed to prevent the baclofen (20 μM)-mediated inhibition of glutamate release (data not shown).

Raiteri, 1992). Similar to these pharmacological findings, (-)baclofen inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release from cerebral cortex synaptosomes was prevented by CGP 35348 but not blocked by phaclofen. 4AP (1mM) evoked a Ca^{2+}-dependent release of 6.9 ± 0.20 nmol glutamate/mg protein/5 min (Fig.22). Addition of (-) baclofen (20 μM) to synaptosomes before 4AP depolarisation inhibited Ca^{2+}-dependent release to 4.7 ± 0.17 nmol/mg protein/5 min. Preincubation with CGP 35348 (200 μM) or phaclofen (200 μM) before 4AP did not significantly alter control Ca^{2+}-dependent glutamate release (CGP 35348, 7.1 ± 0.58 nmol/mg protein/5 min; phaclofen, 7.2 ± 0.40 nmol/mg protein/5 min) (Fig.22). However, inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release by (-) baclofen was blocked by CGP 35348. Release in the presence of (-)baclofen was 4.7 ± 0.17 nmol/mg protein/5 min and release in the presence of CGP 35348 and (-)baclofen was 6.7 ± 0.40 nmol/mg protein/5 min (Fig.22). Phaclofen did not antagonise the inhibitory effects of (-)baclofen. 4AP-evoked Ca^{2+}-dependent glutamate release in the presence of phaclofen and (-) baclofen was 4.9 ± 0.24 nmol/mg protein/5 min (cf. release in the presence of (-) baclofen, 4.7± 0.17 nmol/mg proten/5 min) (Fig.22). Neither antagonist had any effect on 4AP-evoked Ca^{2+}-independent glutamate release (Table 4.A). Thus, pharmacological analysis indicates that presynaptic phaclofen-insensitive, CGP 35348-sensitive $GABA_B$ receptors are probably present on glutamatergic nerve terminals, activation of which limit the release of glutamate.

It has been demonstrated in hippocampal slices that synaptically released GABA produces heterosynaptic depression of excitatory responses and the effect can be blocked by CGP 35348 (Isaacson et al., 1993), indicating that the response is produced by presynaptic $GABA_B$ receptors. Experiments were performed in order to confirm that the inhibition of 4AP-evoked glutamate release by (-)baclofen could be mimicked using the endogenous $GABA_B$ receptor ligand GABA. 4AP (1mM) evoked the release of 8.4 ± 0.40 nmol glutamate/mg protein/5 min in the presence of added $CaCl_2$ (1mM) (Fig.23.A.i) and preincubation with GABA (20 μM) before 4AP addition inhibited glutamate release to 6.0 ± 0.10 nmol/mg protein/5 min (Fig.23.A.ii). CGP 35348 blocked this GABA inhibition of glutamate release. Control 4AP-evoked glutamate release (8.4 ± 0.40 nmol/mg protein/5 min) (Fig.23.B.i) was inhibited by preincubation with GABA (20 μM) prior to 4AP

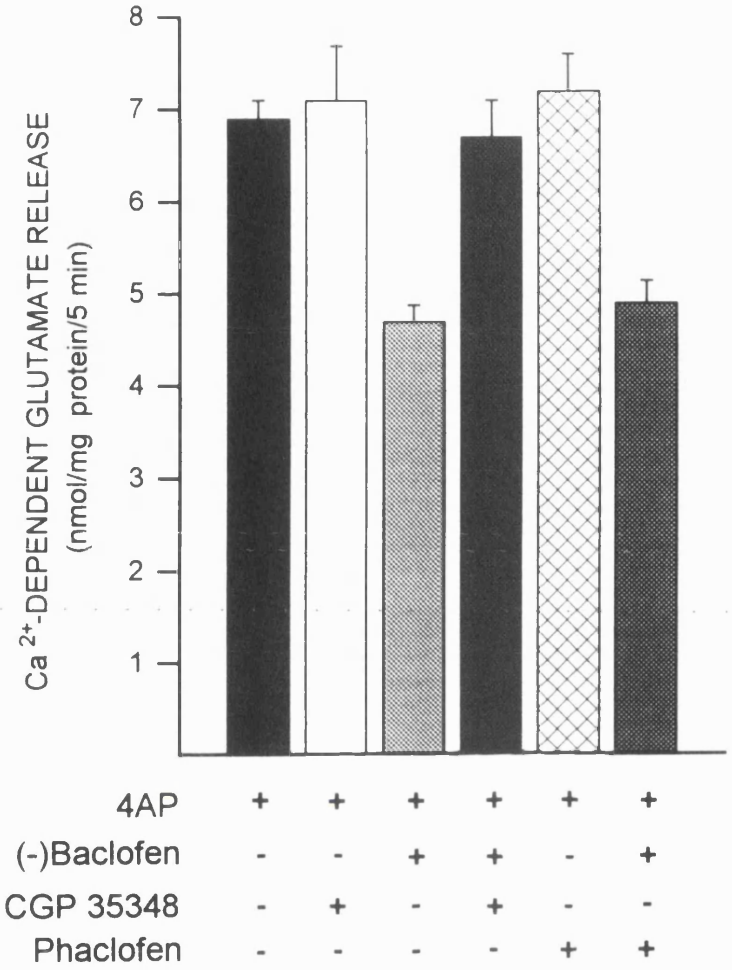

Fig.22. **(-)Baclofen-mediated inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release is prevented by the $GABA_B$ receptor antagonist CGP 35348 but is not blocked the $GABA_B$ receptor antagonist phaclofen.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (1mM). Where indicated, (-)baclofen (20μM) was added to synaptosomes for 2 min before 4AP addition, and CGP 35348 (200μM) or phaclofen (200μM) were added for 7 min before 4AP. Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals.

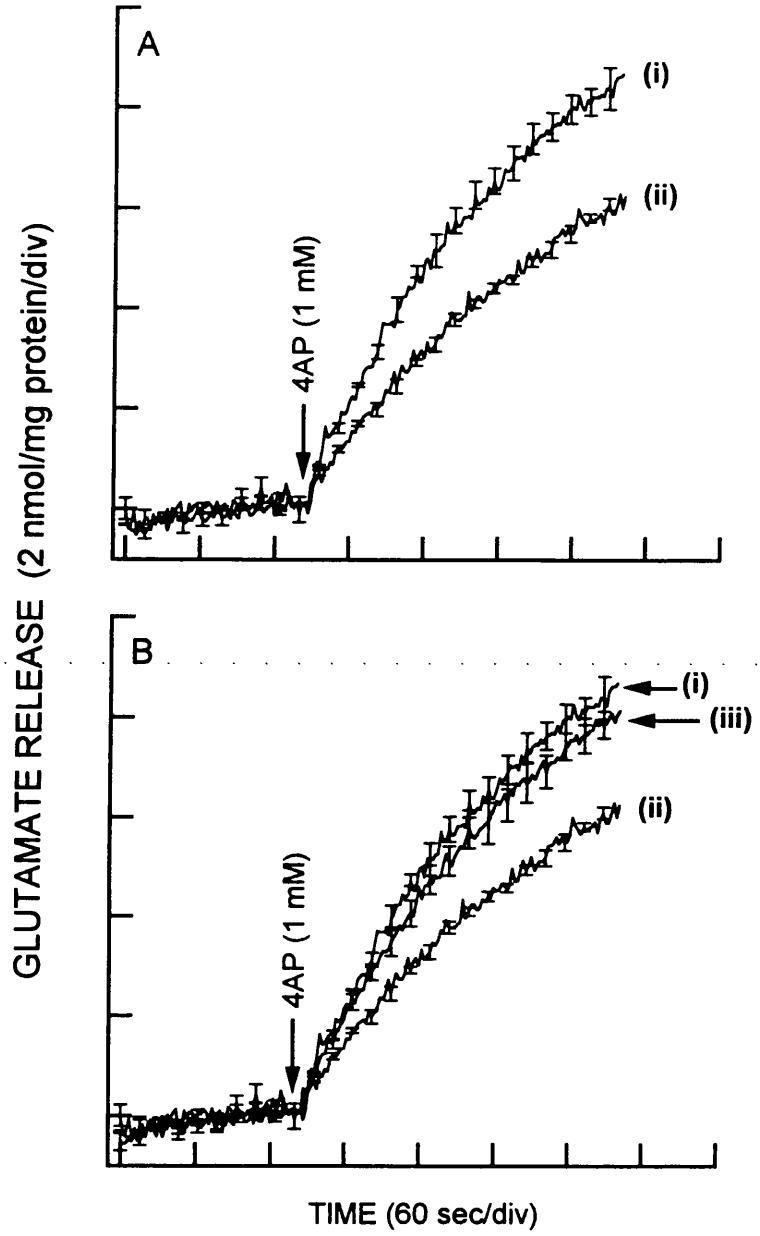

Fig.23. **GABA inhibits 4AP-evoked glutamate release in a CGP 35348-sensitive manner.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with GABA (20μM) for 2 min before 4AP addition. (B) (i) Glutamate release evoked by addition of 4AP (1mM); (ii) Preincubation with GABA (20μM) for 2 min before 4AP addition; (iii) Preincubation with CGP 35348 (200μM) and GABA (20μM) for 7 min and 2 min before 4AP addition respectively. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

✳ The PTX was presumed active, based on previous ADP-ribosylation studies (data not shown).

addition (6.0 ± 0.10 nmol/mg protein/5 min) (Fig.23.B.ii) and this GABA inhibition of glutamate release was blocked by preincubation with CGP 35348 (200 μM) before GABA addition (8.0 ± 0.30 nmol/mg protein/5 min) (Fig.23.B.iii). Similar to (-)baclofen, GABA (20 μM) had no effect on 4AP-evoked Ca^{2+}-independent glutamate release (Table 4.A). These results adopting a neurochemical approach to study of transmitter release complement ideas, based around electrophysiological data, that synaptically released GABA is capable of diffusing to $GABA_B$ heteroreceptors on excitatory terminals to produce a presynaptic depression of transmitter glutamate release (Isaacson et al., 1993).

There are conflicting reports as to whether presynaptic $GABA_B$ heteroreceptors that inhibit the release of excitatory amino acids are coupled to pertussis toxin (PTX)-sensitive G-proteins. Some reports indicate no PTX sensitivity of presynaptic $GABA_B$ receptors on excitatory nerve terminals (Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992), whilst others show partial PTX sensitivity (Potier and Dutar, 1993) or complete sensitivity (Huston et al., 1993). Experiments assessing the PTX sensitivity of (-)baclofen-mediated inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release were performed. Synaptosomes were incubated for 3 - 4 hours in the absence or presence of PTX (2 μg/0.5 mg synaptosomal protein). Control 4AP-evoked glutamate release was 7.2 ± 0.40 nmol/mg protein/5 min in the presence of added $CaCl_2$ (1mM) (Fig.24). Glutamate release from PTX-treated synaptosomes was not significantly different from control 4AP-evoked release (7.4 ± 0.40 nmol/mg protein/5 min) (Fig.24). Preincubation of control (minus PTX) synaptosomes with (-) baclofen (50 μM) before 4AP addition inhibited glutamate release to 5.0 ± 0.50 nmol/mg protein/5 min (Fig.24). PTX did not prevent inhibition of glutamate release by (-) baclofen. 4AP-evoked glutamate release in the presence of PTX and (-)baclofen was 5.1 ± 0.30 nmol/mg protein/5 min (Fig.24). The result indicates that inhibition of glutamate release from cerebral cortex nerve terminals by presynaptic $GABA_B$ heteroreceptors does not involve coupling to a PTX-sensitive inhibitory G-protein. Instead, a PTX-insensitive inhibitory G-protein may be involved. Using isolated nerve terminals, it is unlikely that this lack of effect of PTX is due to poor or slow access to the presynaptic nerve terminal as could be argued in studies adopting whole brain or brain slice techniques .

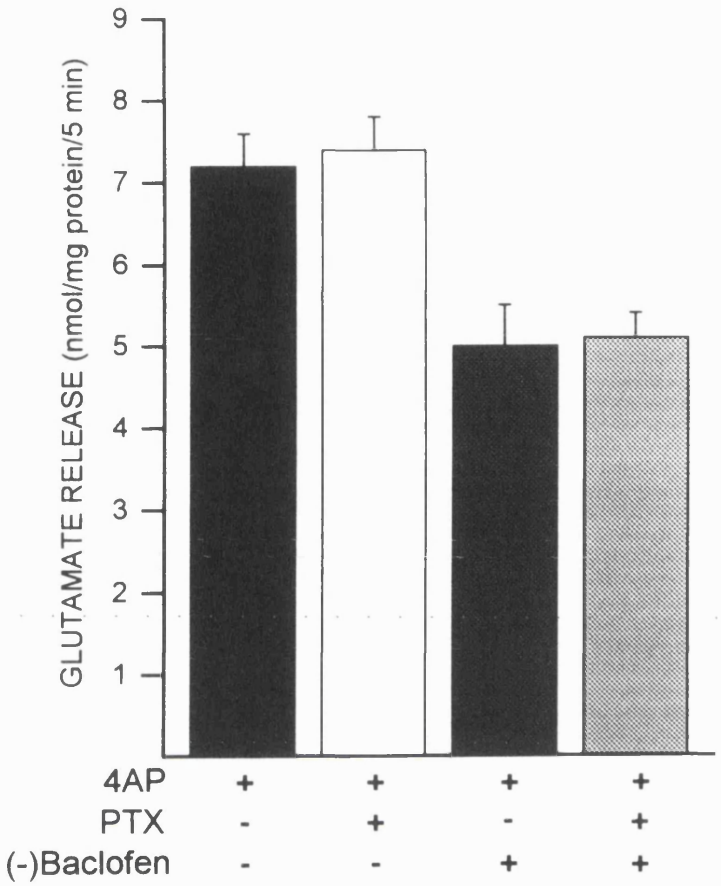

Fig.24. **(-)Baclofen-mediated inhibition of 4AP-evoked glutamate release is unaffected by treatment of synaptosomes with pertussis toxin (PTX).** P_2 synaptosomes (0.5 mg/ml) were incubated for 3 hours at 37°C in the absence or presence of PTX (2 μg), after which they were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM). Where indicated, (-)baclofen (50μM) was added to synaptosomes for 2 min before 4AP. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals.

As well as inhibition of 4AP-evoked glutamate release, (-)baclofen also inhibited the Ca^{2+}-dependent release of glutamate evoked by depolarisation of synaptosomes with elevated external KCl. KCl (15mM) evoked a Ca^{2+}-dependent release of 4.8 ± 0.17 nmol glutamate /mg protein/5 min (Fig.25). Preincubation with 50 μM (-)baclofen before 15 mM KCl inhibited Ca^{2+}-dependent release by 50% (2.4 ± 0.13 nmol/mg protein/5 min). However, this inhibitory effect of (-) baclofen decreased with stronger depolarisations. Ca^{2+}-dependent glutamate release evoked by 30 mM KCl (6.05 ± 0.50 nmol/mg protein/5 min) was inhibited only 18% by (-)baclofen (50 μM) (4.95 ± 0.37 nmol/mg protein/5 min) (Fig.25). Furthermore, at the strongest depolarising stimulus used, i.e. 60 mM KCl, control glutamate release (8.63 nmol/mg protein/5 min) was barely inhibited by preincubation with 50 μM (-) baclofen (8.46 nmol/mg protein/5 min) (Fig.25). K^+-evoked Ca^{2+}-independent glutamate release was not significantly altered by (-) baclofen (50 μM) (Table 4.B). It has been proposed that inhibitory effects of neurotransmitters are often most marked at intermediate levels of depolarisation, being less evident and even absent at large depolarisations (Bean, 1989). Electrophysiological data showing such phenomena have been interpreted as indicating that activation of receptors directly coupled to voltage-sensitive Ca^{2+} channels (VSCC) through an inhibitory G-protein results in the formation of a Ca^{2+} channel state that is more reluctant to open with applied voltage, but will open with strong depolarisation. The mechanism as to exactly how large depolarisations change a 'reluctant' Ca^{2+} channel to a 'willing' Ca^{2+} channel remains contentious; strong depolarisation may lead to dissociation of the G-protein from the Ca^{2+} channel, or cause a change in the gating properties of the Ca^{2+} channel (Dolphin, 1996). It is likely that presynaptic $GABA_B$ receptors are coupled to inhibitory G-proteins, and the loss of the inhibitory effect of (-)baclofen on glutamate release with stronger K^+ depolarisation could be explained using the 'reluctant versus willing Ca^{2+} channel model' described above. However, this model assumes that presynaptic G-protein linked $GABA_B$ receptors are negatively coupled to nerve terminal Ca^{2+} channels. Indeed, whether inhibition of a nerve terminal VSCC by $GABA_B$ receptor activation is responsible for depression of glutamate release has not yet been shown, although it has been reported that (-)baclofen inhibits K^+-evoked rises in $[Ca^{2+}]_c$ in synaptosomes (Stirling et al.,

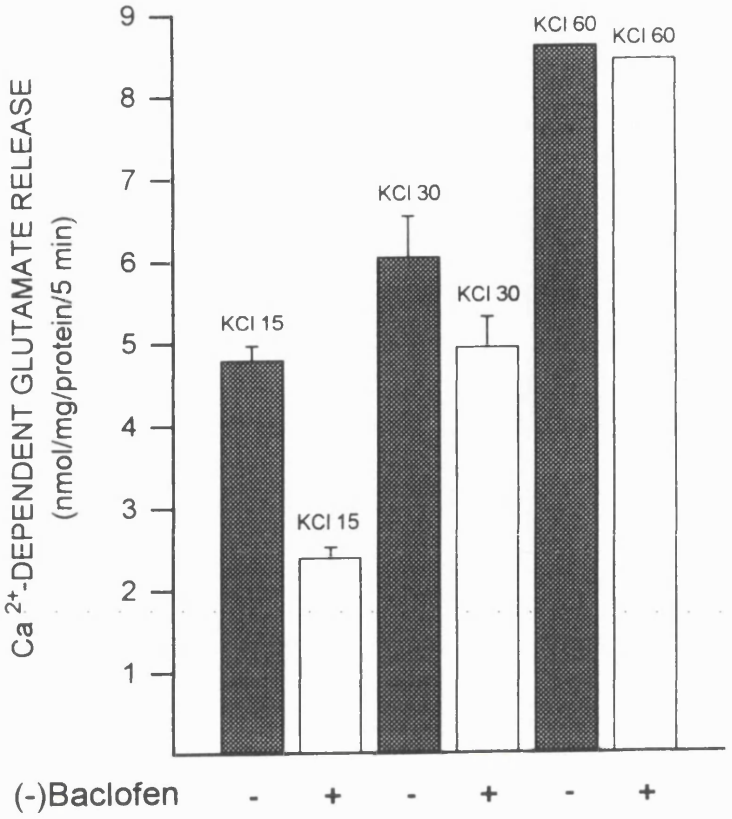

Fig.25. **Loss of the inhibitory effect of (-)baclofen when synaptosomes are depolarised with high external [K^+].** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of KCl (15mM), KCl (30mM) or KCl (60mM) in the absence or presence of (-)baclofen (50μM), which was added for 2 min before depolarisation. Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals, except the bars labelled KCl 60 which are single representative experiments.

1989; Tareilus et al., 1994).

Alternatively, it has been postulated that like postsynaptic $GABA_B$ receptors, presynaptic $GABA_B$ receptor activation could enhance activation of an *A* type K^+ current at the nerve terminal resting membrane potential (Saint et al., 1990). This could shorten the duration of invading action potentials, reduce depolarisation of the terminal membrane potential and thus suppress transmitter release via an indirect decrease in Ca^{2+} influx. Experiments were performed to investigate whether $GABA_B$ receptor activation by (-)baclofen and subsequent inhibition of glutamate release is the result of a direct inhibition of the VSCC linked to glutamate release or whether alteration of a nerve terminal K^+ conductance may be responsible. The synaptosomal membrane potential and depolarisation thereof was assessed with the membrane potential-sensitive dye $DiSC_2(5)$. After $DiSC_2(5)$ equlibration, 4AP (1mM) when applied to synaptosomes caused an increase in $DiSC_2(5)$ fluorescence of 1.6 ± 0.3 fluorescence units/3 min (Fig.26.A.i). Preincubation with 50 μM (-)baclofen before 4AP addition did not alter the resting membrane potential (data not shown) and produced no significant change in the 4AP-mediated increase in $DiSC_2(5)$ fluorescence (1.5 ± 0.1 fluorescence units/3 min) (Fig.26.A.ii). Similarly, KCl (15mM)-evoked depolarisation of the synaptosomal membrane potential (6.9 ± 0.54 fluorescence units/3 min) (Fig.26.B.i) was unaffected by preincubation with (-)baclofen prior to KCl addition (6.6 ± 0.60 fluorescence units/3 min) (Fig.26.B.ii). These results indicate that the observed inhibition of 4AP-evoked and KCl (15mM)-evoked Ca^{2+}-dependent glutamate release by (-)baclofen may not occur via a hyperpolarising effect of (-) baclofen on the synaptosomal membrane potential, i.e. it seems unlikely that $GABA_B$ receptor activation alters a nerve terminal K^+ conductance. Theoretically, enhancement of a resting nerve terminal *A* type K^+ conductance by (-) baclofen would cause a smaller depolarisation of the nerve terminal membrane potential upon arrival of an action potential , thus, the threshold for nerve terminal Na^+ channel activation would be raised resulting in a decreased Na^+ influx into the nerve terminal. A second line of experimental evidence that argues against modulation of a putative nerve terminal *A* type K^+ channel as a mechanism by which presynaptic $GABA_B$ receptors located on excitatory nerve terminals limit glutamate release was provided by experiments assessing Na^+ influx into

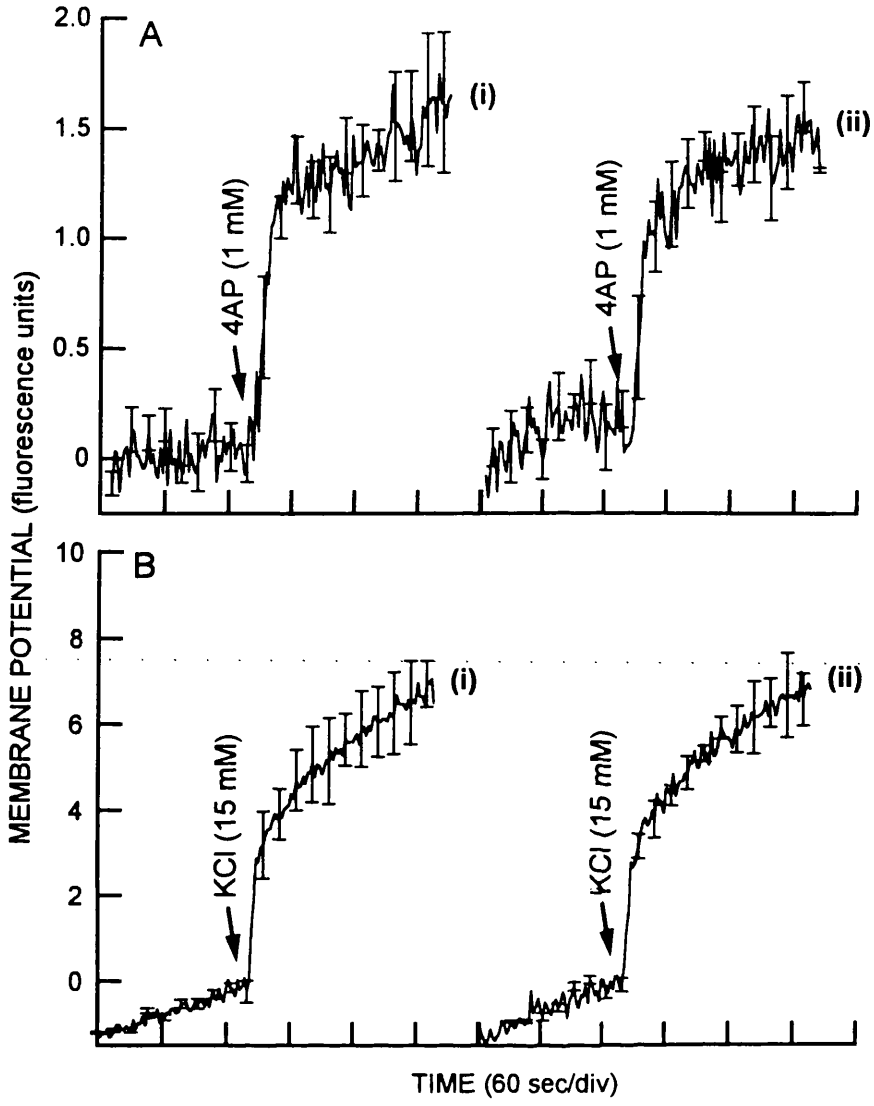

Fig.26. **(-)Baclofen does not alter 4AP-evoked or KCl-evoked depolarisation of the synaptosomal membrane potential.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.5 and the synaptosomal membrane potential monitored with $DiSC_2(5)$. (A) (i) Addition of 4AP (1mM) to synaptosomes; (ii) Pre-incubation with (-)baclofen (50μM) for 2 min before 4AP addition. (B) (i) Addition of KCl (15mM) to synaptosomes; (ii) Preincubation with (-)baclofen (50μM) for 2 min before KCl addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

synaptosomes. 4AP is thought to evoke glutamate release by inducing the repetitive firing of voltage-sensitive Na^+ channels (Nicholls and Coffey, 1994). Assessing cytosolic Na^+ with the Na^+-sensitive probe SBFI, it was observed that 4AP (1mM) elicited an increase in cytosolic Na^+ that was inhibited (83%) by preincubation with the voltage-sensitive Na^+ channel blocker tetrodotoxin (TTX) (2 μM) (Fig.27). Ca^{2+}-dependent glutamate release evoked by 4AP is potently inhibited by TTX (chapter three Fig.3.B), thus, it appears that SBFI is a good reporter of 4AP-mediated influx of Na^+ through TTX-sensitive Na^+ channels linked to glutamate release. Preincubation with (-)baclofen (50μM) before 4AP addition failed to alter the rise in cytosolic Na^+ levels (Fig.27). Thus, (-)baclofen may not affect 4AP-evoked influx of Na^+ through TTX-sensitive Na^+ channels linked to glutamate release. This result backs up the findings from $DiSC_2(5)$ membrane potential measurements showing no effect of (-)baclofen on the synaptosomal resting membrane potential or 4AP depolarisation thereof, and strengthens the proposal that (-) baclofen-mediated inhibition of glutamate release from cerebrocortical synaptosomes does not occur through alteration of a nerve terminal K^+ conductance.

It seems likely then that $GABA_B$ receptor activation inhibits glutamate release via a more direct inhibition of VSCC linked to glutamate release. This was found to be the case. Depolarisation-induced rises in nerve terminal free cytosolic Ca^{2+} levels, $[Ca^{2+}]_c$, resultant from extracellular Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (VSCC) can be assessed in synaptosomes with fura-2. Up to 95% of the K^+-evoked and 4AP-evoked 'plateau' phase of Ca^{2+} entry can be inhibited by preincubation of synaptosomes with the VSCC toxin ω-CTx MVIIC (2 μM) (chapter three Fig.11.A & Fig.12.A), which leads to complete inhibition of Ca^{2+}-dependent glutamate release. This near complete block of Ca^{2+} entry by ω-CTx MVIIC indicates that the majority of the depolarisation-evoked $[Ca^{2+}]_c$ 'plateau' measured with fura-2 is the result of Ca^{2+} influx through VSCC. Results in this chapter show that inhibition of 4AP-evoked and KCl (15 mM)-evoked Ca^{2+}-dependent glutamate release by (-) baclofen occurs through activation of a presynaptic $GABA_B$ receptor. In light of the finding that (-) baclofen probably does not mediate its inhibitory effects through alteration of a nerve terminal K^+ conductance, a reduction in the depolarisation-evoked ω-CTx MVIIC-sensitive

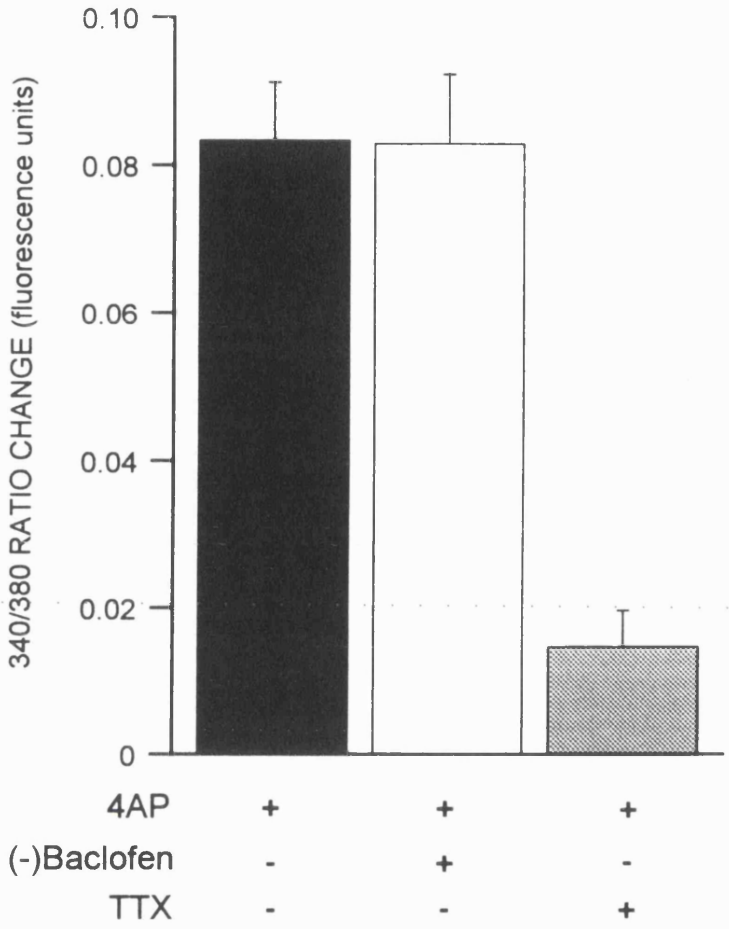

Fig.27. **(-)Baclofen does not alter the 4AP-evoked tetrodotoxin (TTX)-sensitive increase in cytosolic Na^+.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.4 and cytosolic Na^+ was assessed with SBFI. Changes in cytosolic Na^+ are shown as 340/380 ratio fluorescence. Synaptosomes were depolarised using 4AP (1mM), and where indicated (-) baclofen (50μM) or TTX (2μM) were added for 2 min and 5 min before 4AP respectively. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals.

$[Ca^{2+}]_c$ 'plateau' by (-)baclofen would indicate a $GABA_B$ receptor-mediated inhibition of a Ca^{2+} channel linked to glutamate release. In one set of experiments, elevation in $[Ca^{2+}]_c$ occurred after addition of 4AP (1mM) to synaptosomes (Fig.28.A.i), resulting in a $[Ca^{2+}]_c$ 'plateau' of 371 ± 8 nM/5 min (table 5). Preincubation of synaptosomes with (-)baclofen (50 μM) reduced the 4AP-evoked $[Ca^{2+}]_c$ 'plateau' (Fig.28.A.ii) by 23 nM (348 ± 8 nM/5 min, table 5). In a second set of experiments, KCl (15 mM) elicited a rise in $[Ca^{2+}]_c$ (Fig.28.B.i) with a characteristic Ca^{2+} 'spike' phase which decayed leaving a Ca^{2+} 'plateau' (332 ± 6 nM/5min, table 5). Preincubation with (-)baclofen had no effect on the KCl-evoked Ca^{2+} 'spike' but similar to 4AP, the $[Ca^{2+}]_c$ 'plateau' (Fig.28.B.ii) was inhibited by 19 nM (313 ± 5 nM/5 min, table 5). Interestingly, $[Ca^{2+}]_c$ studies also revealed that stronger depolarisation of synaptosomes with 30 mM KCl led to a rise in $[Ca^{2+}]_c$ (Fig.28.C.i) that was not significantly altered by preincubation with (-) baclofen (Fig.28.C.ii) (see table 5 for values). This last finding correlates well with the relative lack of inhibition of glutamate release produced by (-)baclofen when synaptosomes are strongly depolarised using high elevated external KCl, and strengthens the hypothesis that strong depolarisations may decouple G-protein linked receptors by facilitating the opening of Ca^{2+} channels to which the inhibitory G-protein is associated (Dolphin, 1996).

The reduction in the 4AP-evoked and KCl (15 mM)-evoked 'plateau' $[Ca^{2+}]_c$ phase by (-)baclofen may indicate that presynaptic $GABA_B$ receptor activation leads to a direct suppression of depolarisation-evoked Ca^{2+} entry through non-inactivating ω-CTx MVIIC-sensitive VSCC that are linked to the exocytosis of glutamate. A direct suppression of VSCC may be argued because my results also indicate that (-)baclofen does not appear to have a more indirect effect on glutamate release upstream of VSCC linked to glutamate release (membrane potential studies indicated that (-)baclofen does not alter a terminal K^+ conductance). Further evidence indicating that (-) baclofen indeed inhibits glutamate release via inhibition of VSCC linked to glutamate release came from experiments using ionomycin. Ionomycin evokes glutamate release by causing delocalised Ca^{2+} entry at non-specific points around the terminal plasma membrane, i.e. release does not occur via local Ca^{2+} entry through active zone VSCC coupled to SSV exocytosis. Ionomycin (5 μM) evoked the release of 6.67

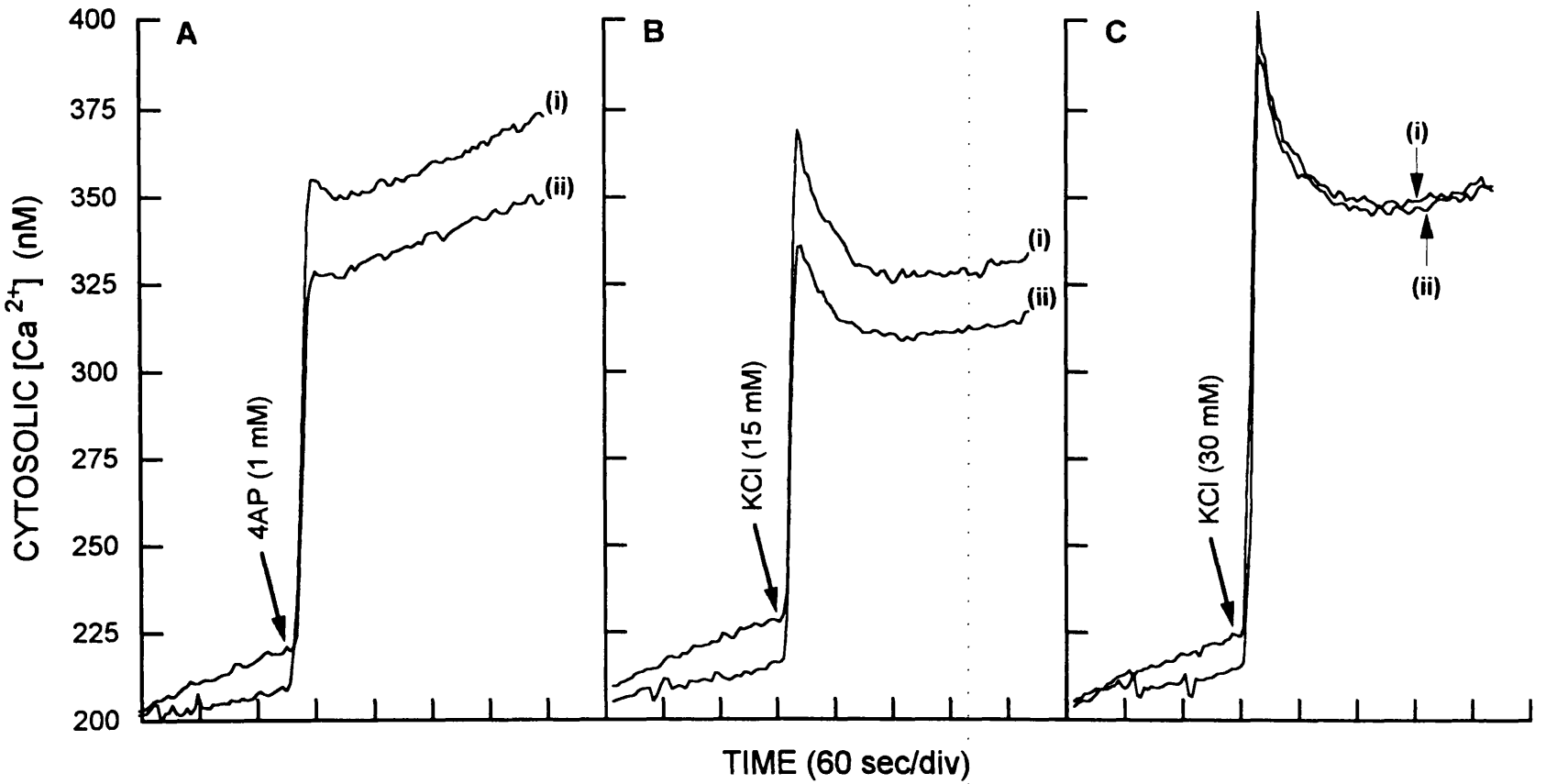

Fig.28. (-)Baclofen reduces the 4AP(1 mM)-evoked and KCl (15mM)-evoked increase in $[Ca^{2+}]_c$. P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. (A) (i) Elevation in $[Ca^{2+}]_c$ after addition of 4AP (1mM); (ii) Preincubation with (-)baclofen (50μM) for 2 min before 4AP addition. (B) (i) Elevation in $[Ca^{2+}]_c$ after addition of KCl (15mM); (ii) Preincubation with (-)baclofen (50μM) for 2 min before KCl (15mM) addition. (C) (i) Elevation in $[Ca^{2+}]_c$ after addition of KCl (30mM); (ii) Preincubation with (-)baclofen (50μM) for 2 min before KCl (30 mM) addition. Each trace is the mean of independent experiments using synaptosomal preparations from four animals (see table 5 for $[Ca^{2+}]_c$ mean values ± SEM).

Table 5. **(-) Baclofen reduces 4AP (1mM)-evoked and KCl (15mM)-evoked increases in $[Ca^{2+}]_c$**

	CYTOSOLIC $[Ca^{2+}]$ (nM/5 min)		
	4AP (1mM)	KCl (15mM)	KCl (30mM)
Control	371 ± 8	332 ± 6	353 ± 4
(-)baclofen	348 ± 8*	313 ± 5*	351 ± 6

*Significantly different from control ($p<0.05$, one-tailed Student's t-test)

P_2 synaptosomes were incubated as described in the Methods & Materials section 2.3 and $[Ca^{2+}]_c$ was assessed with fura-2. Synaptosomes were depolarised with 4AP (1mM), KCl (15mM) or KCl (30mM) in the absence (Control) or presence of (-) baclofen (50 μM) which was added 2 min before depolarisation. Data are the means ± SEM of independent experiments using synaptosomal preparations from four animals.

± 0.17 nmol glutamate/mg protein/5 min (Fig.29.i). Preincubation with (-)baclofen (50μM) before ionomycin addition did not alter glutamate release (6.70 ± 0.15 nmol/mg protein/5 min) (Fig.29.ii). This result strongly suggests that presynaptic $GABA_B$ receptors which limit the release of glutamate from cerebral cortex synaptosomes do so by inhibiting a Ca^{2+} channel linked to glutamate release. An interaction of presynaptic $GABA_B$ receptors downstream of a Ca^{2+} channel, e.g. at the transmitter release machinery, is unlikely because bypassing the VSCC linked to glutamate release with ionomycin abolishes the inhibitory action of (-)baclofen.

The present studies indicate that $GABA_B$ receptor activation by (-)baclofen mediates inhibition of 4AP-evoked glutamate release via a direct inhibition of a Ca^{2+} channel coupled to glutamate release (no K^+ channel involvement). However, from the literature, it is not clear whether a diffusable second messenger such as cAMP may be involved in presynaptic Ca^{2+} channel inhibition by $GABA_B$ receptors. Although postsynaptic $GABA_B$ receptors can be G-protein coupled to the inhibition of membrane-bound adenylyl cyclase activity (for review see Mott and Lewis, 1994), direct inhibition of somatic Ca^{2+} channels by $GABA_B$ receptor activation more commonly occurs by a membrane-delimited effect with no second messenger involvement (Dolphin et al., 1989). This may be true of the presynaptic $GABA_B$ receptors that directly inhibit VSCC linked to glutamate release. However, it has been demonstrated that the cAMP analogue CPT-cAMP blocks (-)baclofen-mediated inhibition of glutamate release from cultured cerebellar granule cells (Travagli et al., 1991), suggesting that there may be a class of presynaptic $GABA_B$ receptor that is G-protein coupled to inhibition of adenylate cyclase activity. Experiments were performed to assess whether the inhibitory effect of (-) baclofen on glutamate release from cerebral cortex nerve terminals could involve a decrease in synaptosomal cAMP levels. 4AP(1mM) evoked the release of 10.75 nmol glutamate/mg protein/5 min in the presence of 1mM Ca^{2+} (Fig.30.i). Preincubation with the membrane-permeable cAMP analogue 8-Br-cAMP (250 μM) prior to 4AP addition had no significant effect on glutamate release (10.52 nmol/mg protein/5 min) (Fig.30.ii). Addition of (-)baclofen (50 μM) to synaptosomes before 4AP inhibited glutamate release to 7.8 nmol/mg protein/5 min (Fig.30.iii). However, preincubation with 250 μM 8-Br-cAMP before (-) baclofen addition did not prevent inhibition of 4AP-evoked glutamate release by

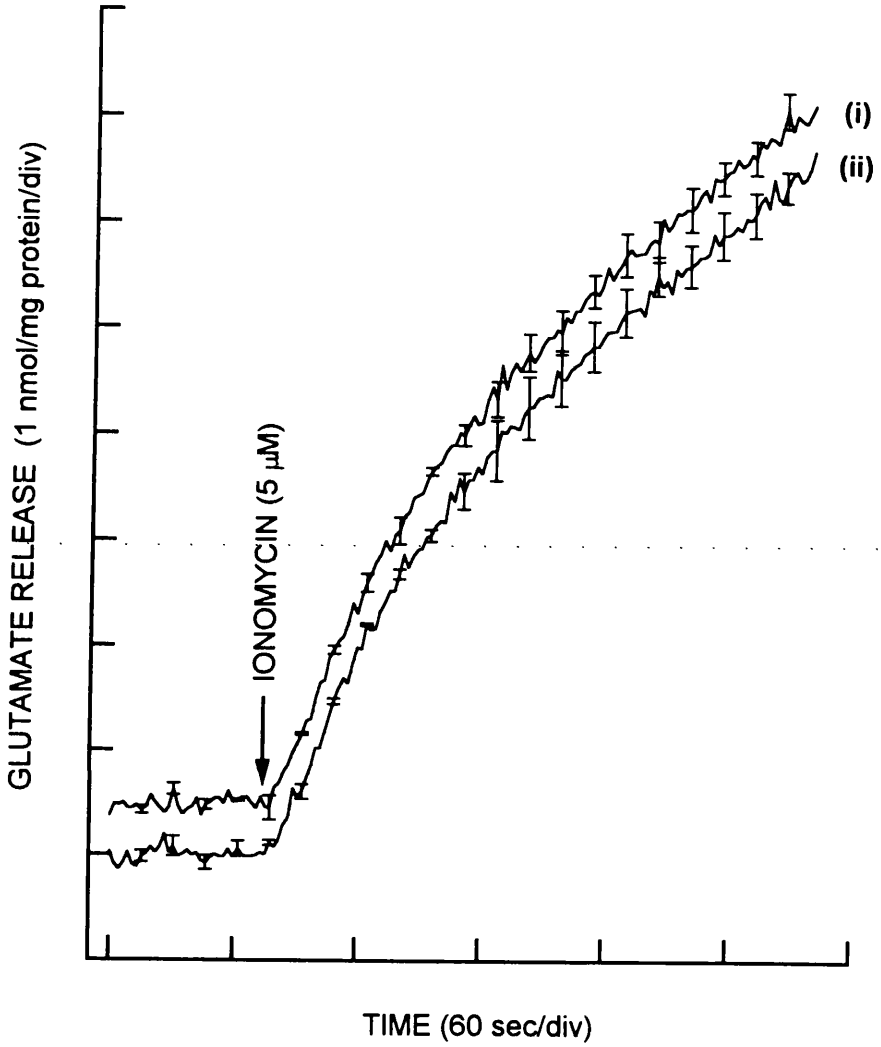

Fig.29. **(-)Baclofen does not inhibit ionomycin-evoked glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of ionomycin (5μM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with (-)baclofen (50μM) for 2 min before ionomycin addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s and the traces are offset for clarity.

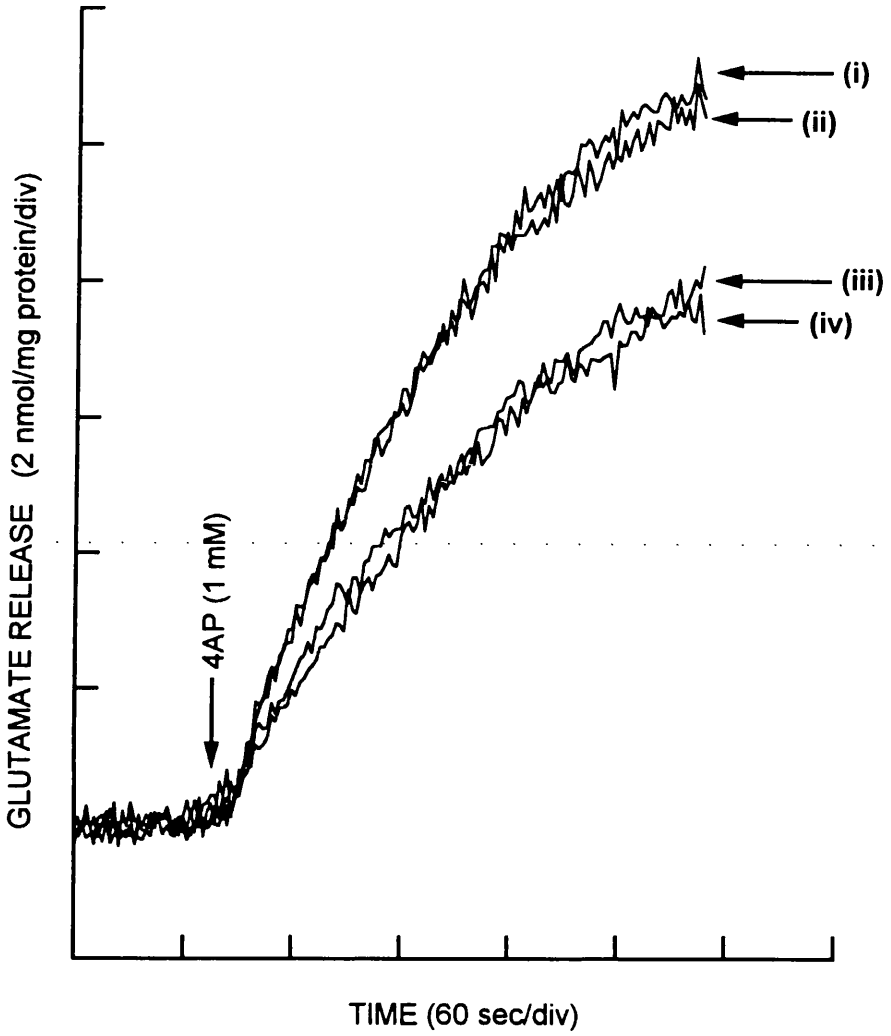

Fig.30. **A rise in synaptosomal cAMP levels does not prevent (-)baclofen-mediated inhibition of 4AP-evoked glutamate release.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with the membrane-permeable cAMP analogue 8-Br-cAMP (250 μM) for 7 min before 4AP addition; (iii) Preincubation with (-)baclofen (50μM) for 2 min before 4AP addition; (iv) Preincubation with 8-Br-cAMP (250 μM) and (-)baclofen (50μM) for 7 min and 2 min before 4AP addition respectively. Each trace is a single representative experiment.

(-)baclofen (7.6 nmol/mg protein/5 min) (Fig.30.iv). Thus, in contrast to (-) baclofen-mediated inhibition of K^+-evoked glutamate release from cerebellar granule cells, cAMP analogues do not appear to negate the inhibitory effects of (-)baclofen on 4AP-evoked glutamate release from cerebral cortex synaptosomes. Other synaptosomal studies show that inhibition of Ca^{2+}-dependent glutamate release from cerebral cortex synaptosomes by a presynaptic metabotropic glutamate receptor (mGluR) does not involve a reduction in presynaptic cAMP levels (Herrero et al., 1996; Sanchez-Prieto et al., 1996). Thus, it may be true that, in general, presynaptic inhibitory receptors that limit the release of glutamate from cerebral cortex nerve terminals, may do so without any involvement of a second messenger. Furthermore, the lack of effect of 8-Br-cAMP on (-)baclofen inhibition of glutamate release from cerebral cortex synaptosomes indicates that the mechanism underlying (-)baclofen-mediated inhibition may be comparable to inhibition of somatic voltage-sensitive Ca^{2+} channels (VSCC) by (-)baclofen, i.e. a membrane-delimited effect with no second messenger involved (at least not cAMP).

In the hippocampus, activation of protein kinase C (PKC) with phorbol ester has been reported to suppress the presynaptic inhibitory actions of (-)baclofen on glutamate release (Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992). Similarly, 12-*O*-tetradecanoylphorbol13-acetate (TPA), another activator of PKC, suppresses $GABA_B$ receptor-mediated inhibition of K^+ -evoked noradrenaline release and acetylcholine release from cerebellar slices (Taniyama et al., 1992). In cerebral cortex synaptosomes activation of PKC using phorbol ester or by stimulation of presynaptic facilitatory metabotropic glutamate receptors with 1S,3R-ACPD suppresses inhibition of 4AP-evoked and K^+-evoked Ca^{2+}-dependent glutamate release by presynaptic adenosine (A_1) receptors (Barrie and Nicholls, 1993; Budd and Nicholls, 1995). It has been proposed that suppression of presynaptic A_1 receptors by PKC could result from phosphorylation of the receptor itself, of the G-protein coupled to the receptor or the Ca^{2+} channel(s) inhibited by presynaptic A_1 receptors (Budd and Nicholls, 1995). A likely mechanism is via a PKC-mediated phosphorylation and inactivation of the inhibitory G_i protein, as has been reported in other systems (Katada et al., 1985). Experiments were performed to address whether stimulation of presynaptic PKC

with phorbol dibutyrate (PDBu) can also override (-)baclofen-mediated inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release from cerebral cortex nerve terminals. Electrophysiological data pertaining to this question would indicate that this is likely (Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992). A decoupling effect of PKC on presynaptic $GABA_B$ receptors would strengthen the argument that presynaptic facilitatory pathways utilising PKC may be able to override all presynaptic inhibitory transmitter systems that impinge upon glutamate releasing nerve terminals (Sanchez-Prieto et al., 1996), and that this could have important physiological consequences such as facilitating the 'strengthening' of excitatory synapses during learning and memory processes (Nicholls, 1992). 4AP (1mM) evoked a Ca^{2+}-dependent release of 7.0 ± 0.3 nmol glutamate/mg protein/5 min (Fig.31.A.i). Preincubation with PDBu (300 nM) before 4AP addition facilitated Ca^{2+}-dependent glutamate release by 45% (10.15 ± 0.2 nmol/mg protein/5 min) (Fig.31.A.ii). 4AP-evoked Ca^{2+}-dependent glutamate release (7.0 ± 0.3 nmol/mg protein/5 min) (Fig.31.B.i), was also attenuated to 4.3 ± 0.2 nmol/mg protein/5 min by preincubation with (-)baclofen (50 μM) prior to 4AP (Fig.31.B.ii). This inhibition of 4AP-evoked Ca^{2+}-dependent glutamate release by (-)baclofen (4.3 ± 0.2 nmol/mg protein/5 min) (Fig.31.C.i) was completely suppressed by pretreatment with 300 nM PDBu before (-)baclofen addition; i.e. there was no statistically significant difference between 4AP-evoked Ca^{2+}-dependent glutamate release in the presence of PDBu (10.15 ± 0.2 nmol/mg protein/5 min) (Fig.31.C.ii), and Ca^{2+}-dependent glutamate release in the presence of PDBu and (-)baclofen (9.5 ± 0.25 nmol/mg protein/5 min) (Fig.31.C.iii). PDBu also caused an increase in 4AP-evoked Ca^{2+}-independent glutamate release (control, 1.2 ± 0.1 nmol/mg protein/5 min; PDBu, 2.6 ± 0.1 nmol/mg protein/5 min). This effect on 4AP-evoked Ca^{2+}-independent glutamate release has been previously reported (Coffey et al., 1993) and is not thought to be mediated by PKC.

Potentiation of 4AP-evoked glutamate release by PKC is believed to be due to a PKC-mediated inhibition of a 4AP-insensitive/dendrotoxin-insensitive delayed rectifier K^+ channel (Barrie et al., 1991; Nicholls and Coffey, 1994). The relationship between this PKC-mediated potentiation of 4AP-evoked repetitive firing of Na^+ channels and the PKC-mediated decoupling of (-)baclofen inhibition is not resolved by the experiments shown in Fig.31. However, it seems likely that

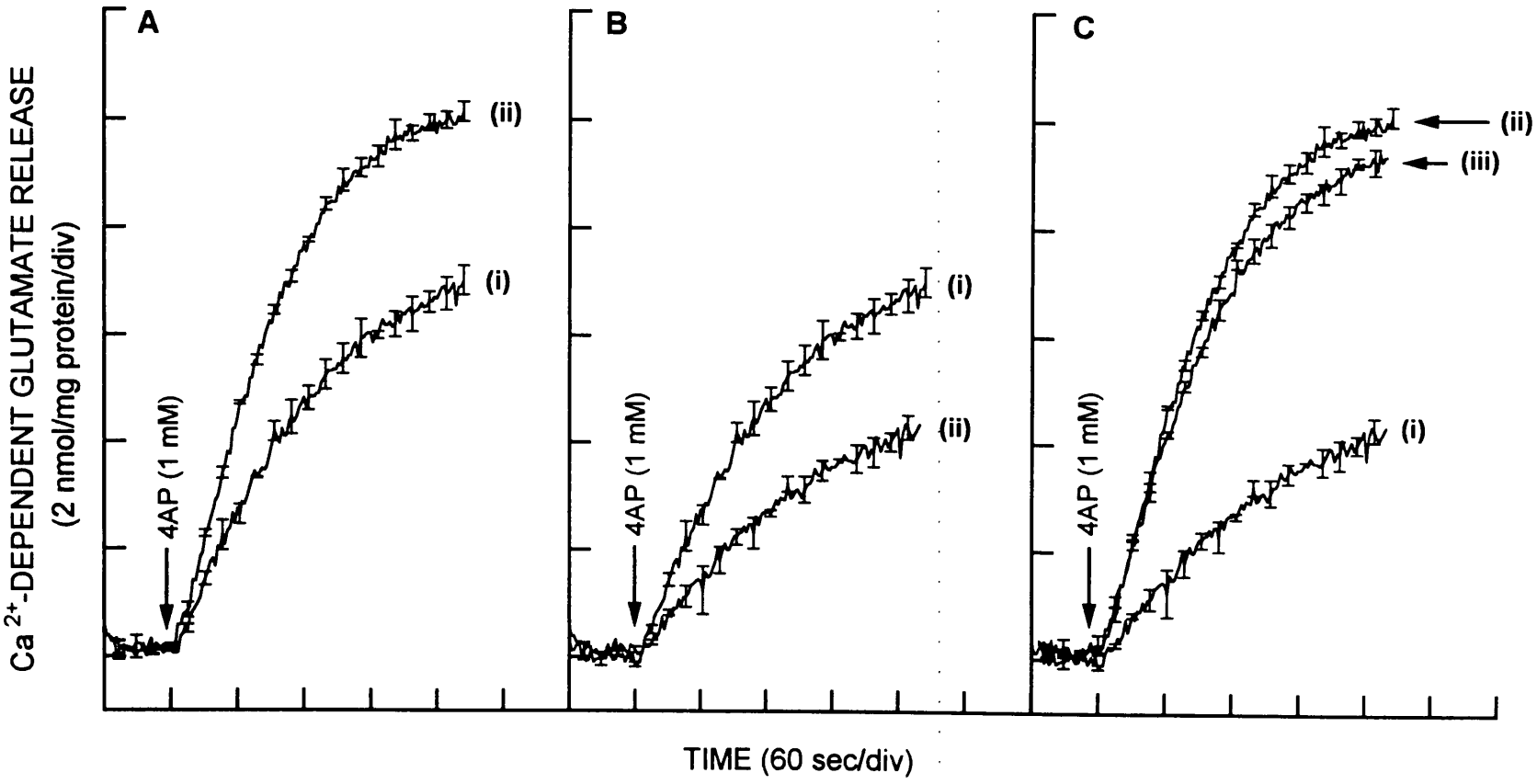

Fig.31. **Protein kinase C decouples (-)baclofen-mediated inhibition of the Ca^{2+}-dependent release of glutamate evoked by 4AP.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM); (ii) Preincubation with the PKC activator phorbol dibutyrate (PDBu) (300 nM) for 4 min before 4AP addition. Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods and Materials. (B) (i) Ca^{2+}-dependent glutamate release evoked by 4AP; (ii) Preincubation with (-)baclofen (50μM) for 2 min before 4AP addition. (C) (i) 4AP-evoked Ca^{2+}-dependent glutamate release with (-)baclofen (50μM) added 2 min prior to depolarisation; (ii) 4AP-evoked Ca^{2+}-dependent glutamate release with (PDBu) (300 nM) added 4 min prior to depolarisation; (iii) 4AP-evoked Ca^{2+}-dependent glutamate release with (PDBu) (300 nM) and (-)baclofen (50μM) added 4 min and 2 min prior to depolarisation respectively. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

potentiation of 4AP-evoked glutamate release and decoupling of presynaptic inhibitory receptors occurs by a dual action of presynaptic PKC at two distinct loci. This is based on the finding that adenosine receptor-mediated inhibition of glutamate release from cerebrocortical synaptosomes can be completely suppressed by a level of PKC activation that does not potentiate glutamate release (Budd and Nicholls, 1995). This has led to the suggestion that different presynaptic PKC isoforms may be responsible for: 1) facilitation of glutamate release via K^+ channel inhibition and 2) decoupling of presynaptic G-protein linked inhibitory receptors by PKC-mediated phosphorylation of the receptor, G-protein or Ca^{2+} channel involved (Budd and Nicholls, 1995).

To date, synaptosomal studies have provided direct evidence (without any postsynaptic attachments to confuse interpretation of presynaptic effects such as may occur in cell culture or brain slice studies) that stimulation of presynaptic PKC is able to suppress presynaptic inhibitory adenosine (A_1) receptors (Barrie and Nicholls, 1993; Budd and Nicholls, 1995), presynaptic inhibitory metabotropic glutamate receptors (mGluRs) (Vazquez et al., 1995b) and now reported in this thesis presynaptic inhibitory $GABA_B$ receptors. Stimulation of presynaptic PKC can be achieved through activation of presynaptic facilitatory transmitter systems, including metabotropic glutamate receptors (mGluRs) (Herrero et al., 1992; Coffey et al., 1994a), and so far it has been demonstrated that the mGluR agonist 1S,3R-ACPD can suppress adenosine receptor-mediated inhibition of glutamate release (Budd and Nicholls, 1995). It appears that the PKC second messenger system may represent a method for interaction between transmitter systems at the presynaptic level. Whether, like presynaptic A_1 receptors, presynaptic $GABA_B$ receptor-mediated inhibition of glutamate release can be suppressed by 'physiological' stimulation of PKC using mGluR agonists, not phorbol esters, remains to be demonstrated.

Conclusions

- (-)Baclofen and GABA inhibit glutamate exocytosis from cerebral cortex nerve terminals via stimulation of a presynaptic inhibitory $GABA_B$ heteroreceptor that is sensitive to antagonism by CGP 35348 but not by phaclofen. $GABA_B$ receptors are abundant in the cerebral cortex and the magnitude of the inhibitory effects observed with (-)baclofen suggest that the $GABA_B$ receptor represents a major inhibitory pathway present on a majority of cerebrocortical nerve terminals.

- The G-protein coupled to the presynaptic $GABA_B$ heteroreceptor is pertussis toxin-insensitive.

- No modulation of K^+ channels by (-)baclofen can be detected because no effect of (-)baclofen on average membrane depolarisation or Na^+ influx is seen during the repetitive firing of Na^+ channels induced by 4AP, thus, presynaptic inhibitory $GABA_B$ heteroreceptor activation may not alter a nerve terminal K^+ conductance. This finding is backed up by reports showing that alteration of a presynaptic K^+ conductance is not the mechanism by which other presynaptic inhibitory receptors mediate inhibition of glutamate exocytosis (Barrie and Nicholls, 1993).

- (-)Baclofen reduces the depolarisation-evoked non-inactivating phase of Ca^{2+} entry that is ω-CTx MVIIC-sensitive and linked to glutamate release. Together with a lack of effect of (-)baclofen on glutamate release when Ca^{2+} channels linked to release are bypassed using ionomycin, it appears that $GABA_B$ receptor activation limits glutamate exocytosis via a direct inhibition of voltage-sensitive Ca^{2+} channels linked to glutamate release.

- Postsynaptic $GABA_B$ receptors can be coupled to inhibition of adenylate cyclase activity, lowering cAMP levels. However, no intracellular second messenger that has been shown to be linked to postsynaptic $GABA_B$ receptors, i.e. cAMP, seems to be involved in the (-)baclofen-mediated

inhibition of presynaptic Ca^{2+} channels. This is based on the finding that the cAMP analogue 8-Br-cAMP (which may boost presynaptic cAMP levels) does not appear to counteract the (-)baclofen-mediated inhibition of glutamate release. Thus, PKA-mediated protein phosphorylation/ dephosphorylation may not be involved in $GABA_B$ receptor-mediated inhibition of Ca^{2+} channels and glutamate exocytosis. Instead, presynaptic G-protein-linked $GABA_B$ heteroreceptor-mediated inhibition of Ca^{2+} channel(s) may be a membrane-delimited effect. This would provide the nerve terminal with a fast negative control for glutamate exocytosis.

- As has been demonstrated with other transmitter systems, presynaptic $GABA_B$ receptor-mediated inhibition of glutamate release can be completely overridden by stimulation of presynaptic PKC, suggesting that PKC may through phosphorylation effects decouple a component of the $GABA_B$ receptor/G-protein/Ca^{2+} channel complex. This decoupling effect of presynaptic PKC might occur during synaptic plasticity, when it may be necessary to override the presynaptic inhibition of glutamate release that results from activation of presynaptic $GABA_B$ heteroreceptors.

CHAPTER SIX

6 Modulation of glutamate exocytosis by presynaptic inhibitory metabotropic glutamate receptors (mGluRs)

Introduction

Metabotropic glutamate receptors (mGluRs) are G-protein linked receptors which function to modulate the presynaptic release of glutamate (and other transmitters) and the postsynaptic sensitivity of neurons to glutamate excitation (for review see Schoepp, 1994). To date eight different mGluR subtypes (mGluR1 - mGluR8), along with various alternate splice versions of mGluR1 and mGluR5 have been cloned. These mGluR subtypes fall into three groups based on their pharmacological characteristics and second messenger coupling (see table 6 below).

Table 6. **Pharmacology of mGluRs**

Receptor (clone)	2nd messenger/ Cellular role	G-protein	Pharmacological characteristics	
			agonists	antagonists*
Group I (mGluR1, mGluR5)	↑ Phosphoinositide hydrolysis LTP/ LTD ↑ glutamate release	PTX-sensitive ?	1S,3R-ACPD (also group II agonist). DHPG (specific).	(S)-4C-PG(selective, competitive). (+)MCPG (non-selective).
Group II (mGluR2, mGluR3)	↓ cAMP ↓ glutamate release	PTX-sensitive	1S,3R-ACPD (also group I agonist). 1S,3S-ACPD and L-CCG-I (more selective for group II than group I). DCG-IV (potent, selective).	MCCG (presumed specific presynaptic antagonist based on electrophysiological studies). (+)-MCPG (non-selective).
Group III (mGluR4, mGluR6, mGluR7, mGluR8)	↓ cAMP ↓ glutamate release	PTX-sensitive	L-AP4 (specific group III agonist). L-SOP (non-selective).	MAP4 (selective in some systems; agonist activity at group II/group III in neurochemical systems).

*see Watkins and Collingridge, 1994.

In adult rat brain cerebrocortical nerve terminals (synaptosomes), activation of a presynaptic facilitatory mGluR with 1S,3R-ACPD in the presence of low concentrations of arachidonic acid (2 μM AA) enhances glutamate exocytosis elicited by submaximal concentrations of the K^+ channel blocker 4-aminopyridine (4AP) (Herrero et al., 1992; Coffey et al., 1994a). This facilitatory effect is thought

to occur by protein kinase C (PKC)-mediated inhibition of a presynaptic delayed-rectifier type of K^+ channel (Nicholls and Coffey, 1994). 1S,3R-ACPD-mediated mGlu receptor activation transiently elevates diacyglycerol (DAG) levels in synaptosomal membranes (Herrero et al., 1994). This response is not altered by AA (Vazquez et al., 1994) but the fatty acid is required for the phosphorylation of the major presynaptic PKC substrate MARCKS (myristoylated alanine-rich C-kinase substrate) (Coffey et al., 1994a) suggesting that the fatty acid acts to sensitise PKC to the DAG generated by the phospholipase C (PLC)-coupled group I mGluR (Sanchez-Prieto et al., 1996). A requirement of fatty acids in the potentiation of glutamate release by presynaptic mGluR activation has been observed in other brain preparations (Bramham et al., 1994; Lombardi et al., 1996; Collins et al., 1995; McGahon and Lynch, 1994). With regard to PKC-mediated facilitation of glutamate release from nerve terminals (Herrero et al., 1992; Coffey et al., 1994a) it appears as though presynaptic PKC acts like a coincidence detector in as much that potentiation of glutamate exocytosis by PKC requires two signals: presynaptic activation through PLC-coupled mGluR-mediated increases in DAG and activation by AA (or another 'putative' retrograde messenger). AA, generated postsynaptically as a consequence of NMDA receptor-mediated phospholipase A_2 (PLA_2) activity has been proposed as a retrograde messenger during synaptic plasticity (LTP) (Williams et al., 1989). As facilitation of glutamate exocytosis is transient and only occurs in the presence of AA, it has been proposed that this dual signal-mediated facilitation may serve a very specific physiological purpose. For example, during synaptic plasticity or long-term potentiation (LTP), which is an experimental model proposed to underlie the biochemistry of learning and memory processes, postsynaptically generated AA could diffuse to nerve terminals which have been conditioned and 'primed' with elevated DAG by mGluR activation. AA is known to enhance insertion of PKC into the plasma membrane. This would sustain mGluR-mediated presynaptic PKC activation (Sanchez-Prieto et al., 1996). A resulting PKC-mediated enhancement of glutamate exocytosis could then be involved in taking a conditioned synapse (Bortolotto et al., 1994) to a potentiated synapse.

As well as these positive-feedback mGlu-autoreceptors, negative-feedback mGlu-autoreceptors which limit the release of glutamate have been described.

Both 1S,3R-ACPD-sensitive/L-AP4-insensitive and L-AP4-sensitive subtypes of presynaptic mGluR have been demonstrated. L-AP4-sensitive presynaptic glutamate autoreceptors were initially described in the hippocampus where L-AP4 produces profound presynaptic depression at lateral perforant path-dentate gyrus synapses (Koerner and Cotman, 1981). Subsequent findings have shown that an L-AP4-insensitive /1S,3R-ACPD-sensitive glutamate receptor also inhibits hippocampal glutamate transmission by a presynaptic mechanism (Baskys and Malenka, 1991; Baskys, 1992; Vignes et al., 1995). Interestingly, these inhibitory effects in the hippocampus seem to be developmentally regulated showing maximal expression during the first three weeks postpartum whilst being negligible in adults (2-3 months old) (Baskys and Malenka, 1991). Subsequent studies using novel mGluR antagonists (Watkins and Collingridge, 1994) have demonstrated that the inhibitory effects of 1S,3R-ACPD in the hippocampus are selectively blocked by the group II mGluR antagonist MCCG, whilst L-AP4-mediated inhibition of hippocampal transmission is blocked by the group III mGluR antagonist MAP4 (Vignes et al., 1995). This pharmacological study strongly indicates the involvement of group II (1S,3R-ACPD-sensitive) and group III (L-AP4-sensitive) mGluRs in the presynaptic regulation of excitatory responses in the hippocampus; aided greatly by the development of mGluR group-specific antagonists (Watkins and Collingridge, 1994).

Consistent with the developmentally regulated depression of synaptic transmission by 1S,3R-ACPD and L-AP4 in the hippocampus, it has been demonstrated that a presynaptic mGluR(s) sensitive to L-AP4 and 1S,3R-ACPD inhibits the evoked release of glutamate from cerebrocortical synaptosomes (Vazquez et al., 1995a; Vazquez et al., 1995b; Sanchez-Prieto et al., 1996; Herrero et al., 1996), inhibition being present in young rats (1-3 weeks postpartum) but not in adult rats (Vazquez et al., 1995b). Part of this developmental change seems to incorporate a switch from an inhibitory mGluR to a presynaptic facilitatory mGluR in adult rats (Herrero et al., 1992; Vazquez et al., 1995b). It has been argued, based on results in both the hippocampus and cerebral cortex, that the control of excitatory synaptic transmission by presynaptic inhibitory mGlu-autoreceptors may be particularly important during the first three weeks of brain development (postpartum) when synaptic connections are unstable

and glutamate re-uptake mechanisms may not be completely developed (Baskys and Malenka, 1991; Sanchez-Prieto et al., 1996). Thus, presynaptic inhibitory mGluRs could have an important neuroprotective role at immature synapses, limiting accumulation of glutamate in the synaptic cleft.

These *in vitro* studies indicating that presynaptic inhibitory mGluRs on cerebral cortex nerve terminals may be developmentally regulated, are in contrast to studies with adult rat cerebral cortex *in vivo* and adult rat neocortical brain slices showing that L-AP4 and 1S,3R-ACPD differentially depress excitatory synaptic transmission by a presynaptic mechanism (Burke and Hablitz, 1994; Cahusac, 1994; Taylor and Cahusac, 1994). This suggests that presynaptic inhibitory mGluRs may be present on nerve terminals in the adult rat cerebral cortex. The specific mGluR subtypes that are located presynaptically and control glutamate release in the cerebral cortex remain to be determined. Antagonism of inhibitory responses is not observed using the novel non-selective mGluR antagonist (+)MCPG (Burke and Hablitz, 1994; Herrero et al., 1996).

In contrast to the hippocampus and cerebral cortex, presynaptic mGluR-mediated inhibition of glutamate release from striatal nerve terminals is not developmentally regulated (Lovinger, 1991; Lovinger et al., 1993; Lovinger and Lambert, 1993) and appears to be mediated exclusively by group II mGluRs (mGlu2 or mGlu3) (Lovinger and McCool, 1995). However, there are a few reports describing an L-AP4-mediated inhibition of striatal glutamate release (East et al., 1995; Calabresi et al., 1996) suggesting that in addition to a group II mGluR, group III mGluRs (mGlu4 or mGlu7) may be functionally present on glutamate-releasing nerve terminals in the striatum. The reasons for a discrepency in pharmacological findings relating to which subtypes of mGluR mediate presynaptic inhibition in the striatum are not clear.

Due to the contrasting reports about the effects of mGluR agonists in the cerebral cortex and striatum, studies were performed using adult rat cerebrocortical and striatal synaptosomes in order to see what effects the mGluR agonists 1S,3R-ACPD and L-AP4 have on glutamate exocytosis in these brain regions. 4AP-evoked Ca^{2+}-dependent glutamate release from adult rat striatal synaptosomes was inhibited by the group II mGluR agonist 1S,3R-ACPD but not by the group III mGluR agonist L-AP4. This is consistent with electrophysiological

data showing a non-developmentally regulated presynaptic depression of excitatory striatal synaptic transmission via activation of mGluR2 or mGluR3 (group II) (Lovinger and McCool, 1995). Contrary to previous reports (Herrero et al., 1992), in adult rat cerebrocortical synaptosomes a presynaptic facilitatory mGluR could not be demonstrated, i.e. 1S,3R-ACPD in the presence of low concentrations of arachidonic acid (2 μM AA) did not potentiate glutamate release evoked by submaximal concentrations of the K^+ channel blocker 4-aminopyridine (50 μM 4AP). Instead, 1S,3R-ACPD caused a small but significant inhibition of 4AP-evoked glutamate release. Further investigation of this inhibitory effect revealed that both 1S,3R-ACPD and L-AP4 attenuated KCl (15 mM)-evoked and 4AP (1mM)-evoked glutamate release from adult rat cerebrocortical synaptosomes. This may indicate the existence of group II and /or group III inhibitory mGlu-autoreceptors on adult cerebral cortex synaptosomes. These results in the adult rat cerebral cortex are not consistent with reports showing a developmental change from inhibition to facilitation in the presynaptic control of glutamate exocytosis by mGluRs (Vazquez et al., 1995b) but do complement electrophysiological findings showing presynaptic depression of excitatory responses by 1S,3R-ACPD and L-AP4 in the adult rat cerebral cortex (Burke and Hablitz, 1994; Cahusac, 1994; Taylor and Cahusac, 1994).

Results and discussion

In synaptosomes prepared from adult rat striatum, 4AP (1mM)-evoked a Ca^{2+}-dependent release of 7.36 ± 0.50 nmol glutamate/mg protein/5 min (Fig.32.A.i). Preincubation with the group II mGluR agonist 1S,3R-ACPD (100 μM) before 4AP addition inhibited Ca^{2+}-dependent release by 38% (4.59 ± 0.34 nmol/mg protein/5 min) (Fig.32.A.ii). 4AP-evoked Ca^{2+}-independent glutamate release (2.11 ± 0.70 nmol/mg protein/5 min, n=3) was not significantly altered by preincubation with 1S,3R-ACPD (100 μM) before 4AP addition (2.04 ± 0.39 nmol/mg protein/5 min, n=3). Thus, 1S,3R-ACPD only inhibited the Ca^{2+}-dependent component to 4AP-evoked glutamate release.

In contrast to 1S,3R-ACPD, L-AP4 did not attenuate glutamate release from striatal synaptosomes. Control 4AP-evoked glutamate release in the presence of 1mM Ca^{2+} (9.42 ± 0.40 nmol/mg protein/5 min) (Fig.32.B.i) was not

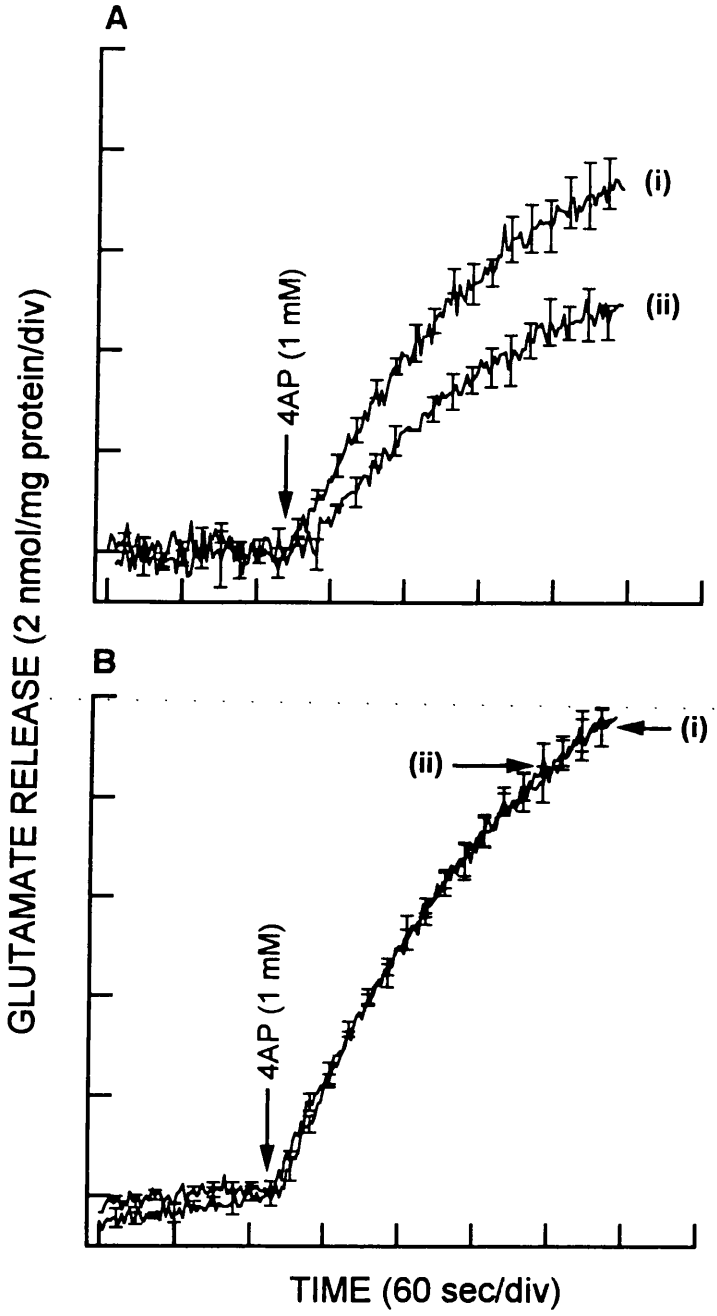

Fig.32. **1S,3R-ACPD, but not L-AP4, inhibits 4AP-evoked Ca^{2+}-dependent glutamate release from striatal synaptosomes.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (1mM); (ii) Preincubation with 1S,3R-ACPD (100 μM) for 2 min before 4AP addition. Net Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. (B) (i) Glutamate release evoked by 4AP (1mM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with L-AP4 (100 μM) for 2 min before 4AP addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

altered by preincubation with L-AP4 (100 μM) before 4AP addition (9.63 ± 0.13 nmol glutamate/mg protein/5 min) (Fig.32.B.ii). These release experiments complement electrophysiological findings indicating that 1S,3R-ACPD, but not L-AP4, depresses excitatory transmission at corticostriatal synapses by a presynaptic mechanism (believed to be inhibition of glutamate release) (Lovinger, 1991; Lovinger et al., 1993; Lovinger and Lambert, 1993). However, in contrast to the proposal that L-AP4 does not limit glutamate release from striatal nerve terminals, one neurochemical study has shown that the group III mGluR agonist L-AP4 potently inhibits 4AP (2 mM)-evoked glutamate release from striatal synaptosomes with an IC_{50} value of 0.2 μM (East et al., 1995). East and colleagues measured 4AP-evoked glutamate release using the same enzyme-linked assay described in the Methods and Materials section 2.2.A of this thesis, thus, it is not clear why opposing results with L-AP4 in striatal synaptosomes are seen.

1S,3R-ACPD is active at group I and group II mGluRs (see table 6). However, a role for group I mGluRs coupled to phosphoinositide metabolism, in presynaptic inhibition of striatal glutamate release seems unlikely since it has been shown that stimulation of group I mGluRs with 1S,3R-ACPD (in the presence of AA) facilitates glutamate release (Herrero et al., 1992; Coffey et al., 1994a). Furthermore, it has recently been demonstrated that the group II-specific mGluR agonists DCG-IV and LCCGI (see table 6) mimic the inhibitory actions of 1S,3R-ACPD in the striatum (Lovinger and McCool, 1995), which strongly indicates the presence of pharmacologically identifiable group II mGlu2 or mGlu3 autoreceptors in the striatum (Lovinger and McCool, 1995). The present glutamate release studies, using striatal synaptosomes, may strengthen the idea, based around electrophysiological data, that group II mGlu-autoreceptors, but not group III mGluRs, limit glutamate release in the striatum. It would seem appropriate to assess the effects of these selective group II mGluR agonists (DCG-IV and LCCGI) on 4AP-evoked glutamate release from striatal synaptosomes.

In the hippocampus, electrophysiological data shows that excitatory synaptic transmission is depressed by activation of presynaptic 1S,3R-ACPD-sensitive group II mGluRs and L-AP4-sensitive group III mGluRs (Vignes et al., 1995). Unlike striatal mGlu-autoreceptors, presynaptic depression of excitatory

transmission in the hippocampus by mGluR activation appears to be developmentally regulated, being present in young rats but negligible or absent in adult rats (Baskys and Malenka, 1991). However, in the cerebral cortex, a brain area in which glutamate transmission is generally less well characterised, data relating to whether presynaptic inhibitory mGluRs on cerebrocortical nerve terminals are developmentally regulated seems to be conflicting. On the one hand, electrophysiological studies indicate that presynaptic mGlu- autoreceptors may not be developmentally regulated. For example, it has been shown *in vivo* that 60% of all extracellular recordings of action potentials from adult rat neocortical neurons are depressed by pipette application of 1S,3R-ACPD with no effect on the postsynaptic level of firing, supporting a presynaptic site of action for 1S,3R-ACPD (Cahusac, 1994; Taylor and Cahusac, 1994). L-AP4 has similar effects. Furthermore, it has been shown that 1S,3R-ACPD and L-AP4 depress excitatory synaptic transmission in adult rat neocortical brain slices *in vitro* (Burke and Hablitz, 1994). The finding that the time interval between spontaneous postsynaptic potentials (PSP's) in adult rat neocortical slices is greatly increased by 1S,3R-ACPD has led to the proposal that 1S,3R-ACPD activates a presynaptic mGluR which depresses transmitter release (Burke and Hablitz, 1994). On the other hand, a few neurochemical studies have demonstrated that mGluR agonists do not inhibit K^+-evoked glutamate release from synaptosomes prepared from adult rat brain cerebral cortex (Herrero et al., 1992; Vazquez et al., 1995b). These same authors, report that the predominant mGluR present in adult rat cerebrocortical nerve terminals has facilitatory effects on glutamate release (Herrero et al., 1992; Coffey et al., 1994a; Vazquez et al., 1995b)). However, glutamate release from young rat cerebrocortical synaptosomes (1-3 weeks postpartum) is inhibited by 1S,3R-ACPD and L-AP4 (Vazquez et al., 1995a; Vazquez et al., 1995b; Sanchez-Prieto et al., 1996; Herrero et al., 1996) and the authors have proposed that developmental regulation of presynaptic mGluRs in the cerebral cortex exists, consistent with the developmentally regulated depression of synaptic transmission by L-AP4 and 1S,3R-ACPD in the hippocampus (Baskys and Malenka, 1991). They also propose that nerve terminals in the cerebral cortex go through a developmental switch from an inhibitory mGluR, which may play a specific neuroprotective role in the developing

brain, to a facilitatory mGluR in the adult brain (Vazquez et al., 1995b). Where inhibitory mGluRs might function to prevent excessive build up of glutamate in the synaptic cleft, they may not be required in adult nerve terminals which have a fully developed glutamate re-uptake mechanism. Instead the presynaptic facilitatory mGluR described in adult rat nerve terminals (Herrero et al., 1992) may be involved in the biochemical events of synaptic plasticity (Nicholls, 1992).

To add even more confusion to the role that presynaptic mGluRs play in glutamatergic synaptic transmission, some studies report no presynaptic effects of mGluR agonists (Prof D.Lodge - personal communication). The findings that presynaptic mGluRs can inhibit glutamate release (Burke and Hablitz, 1994) but also, under certain conditions, facilitate glutamate release (Herrero et al., 1992) suggests that two opposing mGluR-mediated pathways might exist in adult rat cerebral cortex nerve terminals. These opposing pathways may cancel each other out if concurrently stimulated. Concurrent stimulation could occur during experiments designed to assess the effects of mGluR agonists on glutamate transmission, if an agonist such as 1S,3R-ACPD which activates group I and group II mGluRs is used. 1S,3R-ACPD has, until recently, been the agonist of choice at group I/group II mGluRs and this could explain results showing a lack of inhibitory or facilitatory effects on glutamate transmission in the adult CNS. The development of novel group I-selective and group II-selective mGluR agonists may help to resolve the specific roles that group I and group II mGluRs play in presynaptic events.

Experiments were performed to assess whether mGluR agonists can facilitate and inhibit glutamate release from adult rat cerebrocortical synaptosomes. Initially, experiments were performed to address whether glutamate release evoked by depolarisation with a submaximal concentration of 4-aminopyridine (50 μM 4AP) is enhanced by activation of a presynaptic facilitatory mGluR, as previously described (Herrero et al., 1992). 4AP (50 μM) evoked the release of 3.63 ± 0.51 nmol glutamate/mg protein/5 min in the presence of 1 mM Ca^{2+} (Fig.33.A.i). Preincubation with 1S,3R-ACPD (100 μM) before 4AP addition appeared to inhibit release to 2.11 ± 0.40 nmol/mg protein/5 min (Fig.33.A.ii). Previous studies with adult cerebrocortical synaptosomes, have not reported any inhibitory effect of 100 μM 1S,3R-ACPD on 4AP-evoked glutamate

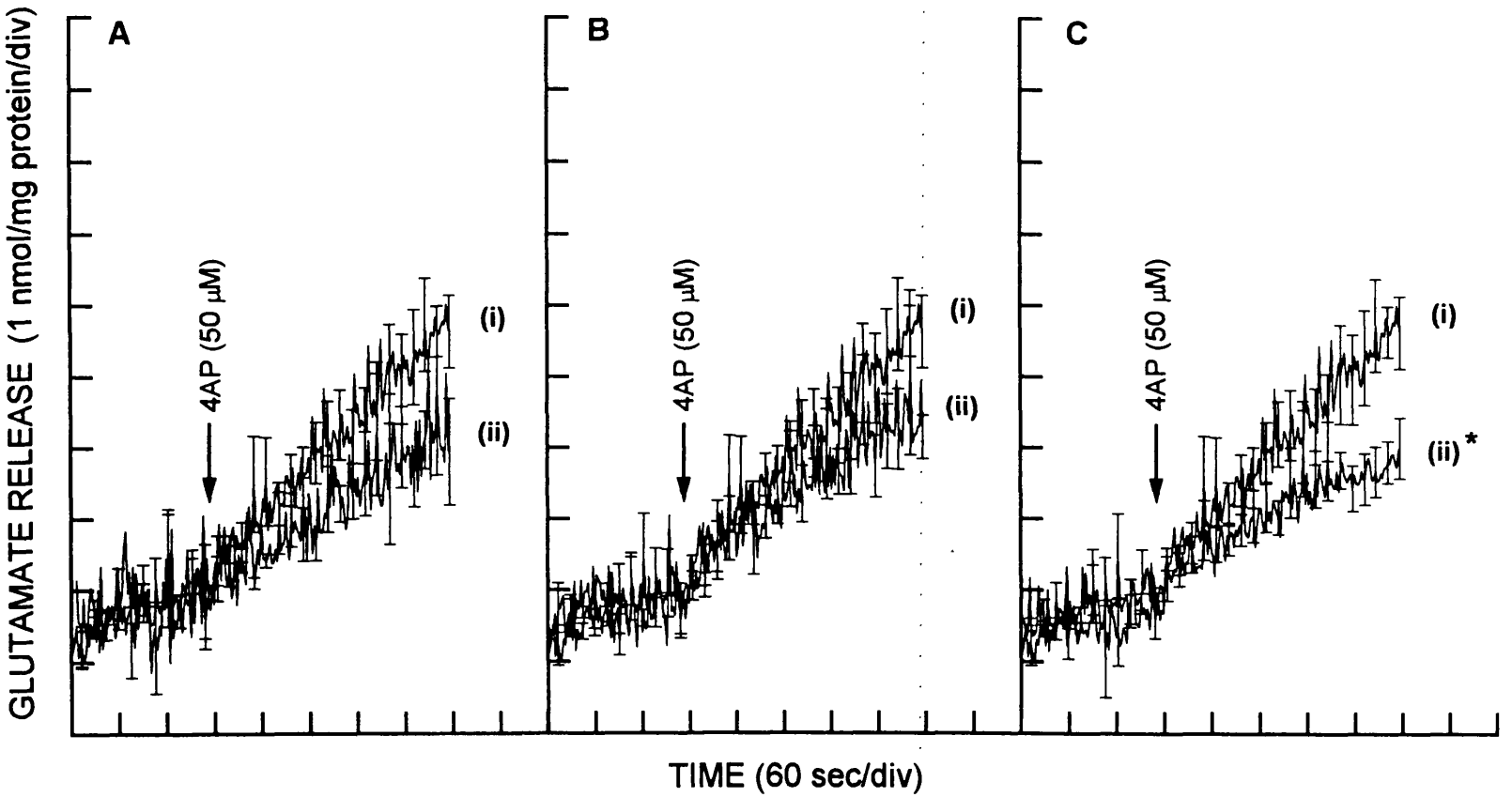

Fig.33. **1S,3R-ACPD in the presence of low concentrations of arachidonic acid (AA) does not not facilitate glutamate release evoked by a submaximal concentration of 4AP.** P_2 cerebrocortical synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of 4AP (50μM) in the presence of $CaCl_2$ (1mM); (ii) Preincubation with 1S,3R-ACPD (100 μM) for 60 s before 4AP addition. (B) (i) Glutamate release evoked by 4AP (50μM); (ii) Preincubation with AA (2 μM) for 30 s before 4AP addition. (C) (i) Glutamate release evoked by 4AP (50μM); (ii) Preincubation with 1S,3R-ACPD (100 μM) and AA (2 μM) for 60 s and 30 s prior to 4AP addition respectively. *Significantly different from (i) ($p<0.05$, two-tailed Student's t-test). Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

release (Herrero et al., 1992). Similar to 1S,3R-ACPD, preincubation with 2 μM AA before 4AP addition inhibited control glutamate release (3.63 ± 0.51 nmol/mg protein/5 min) (Fig.33.B.i) to 2.25 ± 0.56 nmol/mg protein/5 min (Fig.33.B.ii). This inhibitory effect of AA has been previously described and is consistent with an activation of presynaptic K^+ channels (Herrero et al., 1991). However, when AA (2 μM) is added to synaptosomes that have been 'primed' with 1S,3R-ACPD (1S,3R-ACPD mediates a transient increase in synaptosomal DAG) (Vazquez et al., 1994; Herrero et al., 1994), an enhancement of 4AP-evoked glutamate release has been observed, believed to be due to a synergistic stimulation of presynaptic PKC by AA and DAG (Herrero et al., 1992; Coffey et al., 1994a). Curiously, this enhancement of glutamate release by 1S,3R-ACPD and AA was not apparent in the present studies. Control 4AP-evoked glutamate release (3.63 ± 0.51 nmol/mg protein/5 min) (Fig.33.C.i) was significantly inhibited when synaptosomes were preincubated with 1S,3R-ACPD (100 μM) and AA (2 μM) for 60 s and 30 s prior to 4AP addition respectively (1.79 ± 0.30 nmol/mg protein/5 min) (Fig.33.C.ii). Further investigation of the observed inhibitory effects of 1S,3R-ACPD revealed that 1S,3R-ACPD and L-AP4 both powerfully inhibited Ca^{2+}-dependent glutamate release evoked by KCl (15 mM) and 4AP (1 mM). These depolarising stimuli were used in order to allow a comparison with the inhibitory effects of (-)baclofen on glutamate release from adult cerebrocortical synaptosomes to be made (see chapter five). Studies using cerebrocortical synaptosomes prepared from adult rat brain (two months old) revealed that 15 mM KCl evoked a Ca^{2+}-dependent release of 7.78 ± 0.39 nmol glutamate/mg protein/5 min (Fig.34.A.i). Preincubation with 1S,3R-ACPD (250 μM) before KCl addition inhibited Ca^{2+}-dependent glutamate release by 50% (3.9 ± 0.32nmol/mg protein/5 min) (Fig.34.A.ii). Similar to 1S,3R-ACPD, preincubation with L-AP4 (250 μM) inhibited control KCl-evoked Ca^{2+}-dependent glutamate release (7.78 ± 0.39 nmol/mg protein/5 min) (Fig.34.B.i) by 47% (4.10 ± 0.56 nmol/mg protein/5 min) (Fig.34.B.ii). KCl-evoked Ca^{2+}-independent glutamate release (3.1± 0.14 nmol/mg protein/5 min, *n*=3) was not significantly altered by preincubation with 1S,3R-ACPD (250μM) (3.0 ± 0.12 nmol/mg protein/5 min, n=3) or L-AP4 (250μM) (3.4 ± 0.28 nmol/mg protein/5 min, n=3). 1S,3R-ACPD and L-AP4 also inhibited glutamate release evoked by 4AP (1 mM). Preincubation with 100 μM or 250 μM 1S,3R-ACPD before 4AP addition

inhibited glutamate release by 10.1 ± 1.1 % and 28 ± 2.0 % respectively (Fig.35). L-AP4 (250 μM) also inhibited 4AP (1mM)-evoked glutamate release (data not shown). Ca^{2+}-independent glutamate release evoked by 4AP (1.62 nmol/mg protein/5 min) was not significantly altered by preincubation with 1S,3R-ACPD (250 μM) before 4AP addition (1.43 nmol/mg protein/5 min).

The present results show that L-AP4, which uniquely activates group III mGluRs, powerfully inhibits glutamate release from adult cerebral cortex nerve terminals *in vitro*. This may indicate that *in situ*, group III mGluRs are present on cerebrocortical nerve terminals. This L-AP4-mediated presynaptic inhibitory effect is markedly different to other synaptosomal studies demonstrating that L-AP4 does not inhibit glutamate release from adult rat nerve terminals but inhibits release from young rat nerve terminals (Vazquez et al., 1995a; Vazquez et al., 1995b; Sanchez-Prieto et al., 1996; Herrero et al., 1996). Thus, in contrast to Vazquez and co-workers who propose that group III presynaptic mGluRs are developmentally regulated, the present studies provide data to suggest that group III mGluRs may not be developmentally regulated and instead exert negative-feedback regulation of glutamate transmission in the adult mammalian CNS.

Similar to L-AP4, 1S,3R-ACPD strongly inhibited KCl-evoked and 4AP-evoked glutamate release from adult cerebrocortical synaptosomes. This may indicate that in addition to group III mGluRs, group II mGluRs may be functionally present on adult cerebral cortex nerve terminals. However, it has been previously demonstrated in adult cerebrocortical synaptosomes, that 1S,3R-ACPD does not inhibit KCl-evoked or 4AP-evoked glutamate release, instead mediating an AA-dependent facilitation of glutamate release via activation of a group I mGluR (Vazquez et al., 1995a; Vazquez et al., 1995b; Sanchez-Prieto et al., 1996; Herrero et al., 1996). The reasons for the discrepancy between PhD results indicating that 1S,3R-ACPD (in the presence of AA) does not facilitate 4AP-evoked glutamate release from adult rat synaptosomes, but 1S,3R-ACPD alone inhibits glutamate exocytosis, and studies which propose that facilitatory group I mGluRs, but not inhibitory group II mGluRs, are present on adult cerebral cortex nerve terminals (Sanchez-Prieto et al., 1996) are not readily clear. An explanation may be afforded based on the findings that presynaptic facilitatory mGluRs can be homologously desensitised by glutamate or submaximal stimulation of

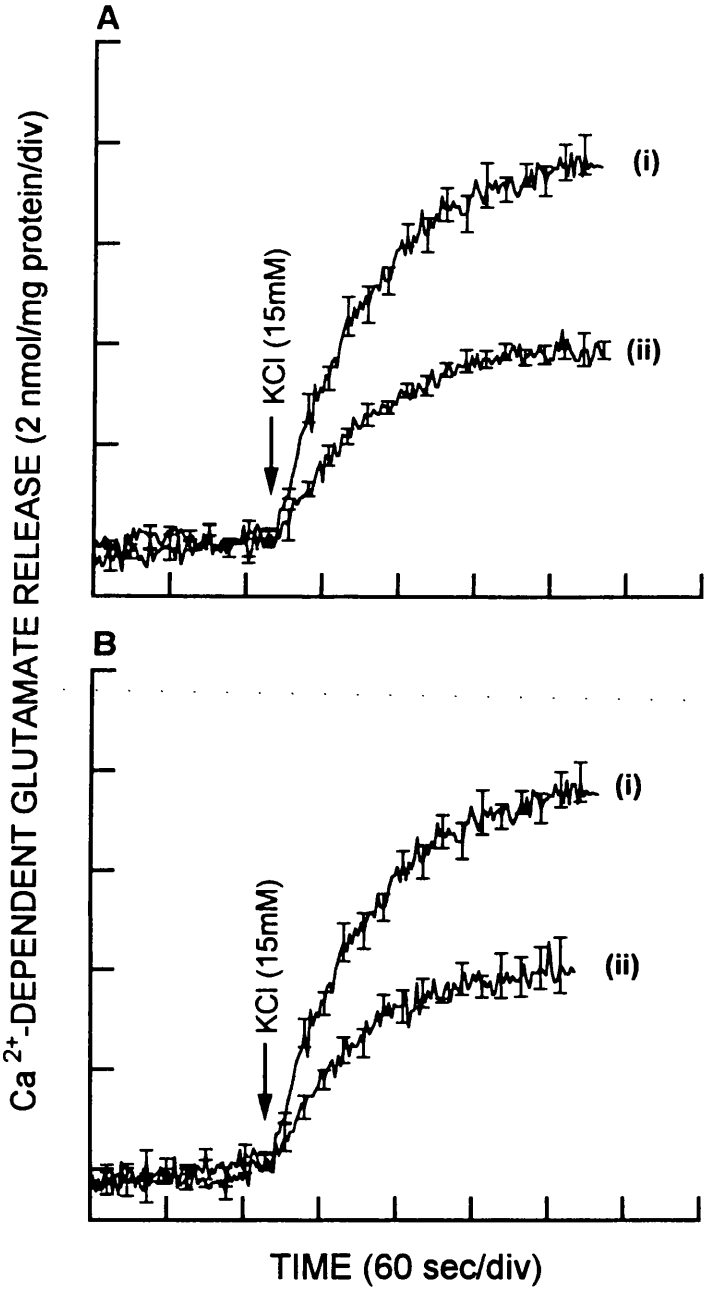

Fig.34. **1S,3R-ACPD and L-AP4 inhibit the Ca^{2+}-dependent release of glutamate evoked by KCl from adult rat cerebrocortical synaptosomes.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. (A) (i) Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), evoked by addition of KCl (15mM); (ii) Preincubation with 1S,3R-ACPD (250 μM) for 2 min before KCl addition. Net Ca^{2+}-dependent release was determined as described in section 2.2.A of the Methods & Materials. (B) (i) Ca^{2+}-dependent glutamate release evoked by KCl (15mM); (ii) Preincubation with L-AP4 (250 μM) for 2 min before KCl addition. Each trace is the mean ± SEM of independent experiments using synaptosomal preparations from three animals. Error bars are shown every 15 s for clarity.

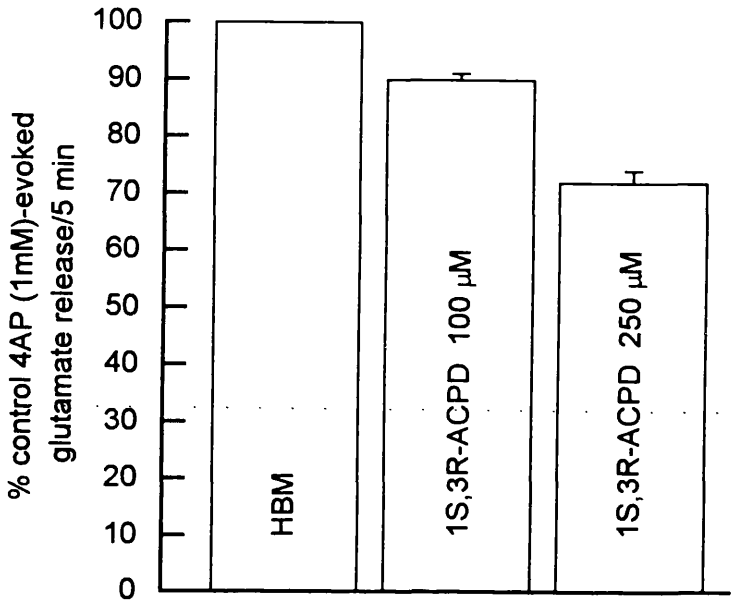

Fig.35. **1S,3R-ACPD inhibits 4AP-evoked glutamate release from adult rat cerebrocortical synaptosomes.** P_2 synaptosomes (0.5 mg/ml) were incubated as described in the Methods & Materials section 2.2.A. Glutamate release, assayed by on-line fluorimetry (Methods & Materials section 2.2.A), was evoked by addition of 4AP (1mM) in the presence of $CaCl_2$ (1mM). Where indicated, synaptosomes were preincubated with HBM vehicle, 1S,3R-ACPD (100 μM) or 1S,3R-ACPD (250 μM) for 2 min before 4AP addition. Each bar is the mean ± SEM of independent experiments using synaptosomal preparations from three animals.

synaptosomal PKC, whereas presynaptic inhibitory mGluRs activated by 1S,3R-ACPD are not subject to homologous desensitisation (Herrero et al., 1994; Vazquez et al., 1995a; Sanchez-Prieto et al., 1996). Thus, the lack of potentiation of 4AP-evoked glutamate release by 1S,3R-ACPD/AA in the present studies may be due to homologous desensitisation of the facilitatory mGluR. Desensitisation could have resulted from the presence of extrasynaptosomal glutamate or high basal PKC activity. With regard to the latter proposal, a set of experiments revealed that basal PKC activity was attenuated by the PKC inhibitor Ro 31-8220 (data not shown), thus, tonic PKC activity may have been high enough to elicit desensitisation effects. When using 1S,3R-ACPD to study presynaptic effects, both facilitatory group I mGluRs and inhibitory group II mGluRs will be stimulated. This may explain why in nerve terminals containing facilitatory mGluRs in a non-desensitised state, presynaptic inhibitory effects of 1S,3R-ACPD on glutamate release will not be observed (Herrero et al., 1992) because 1S,3R-ACPD through activation of facilitatory group I mGluRs might oppose and possibly suppress inhibitory group II mGluR effects. However, in the present studies it is possible that upon application of 1S,3R-ACPD to synaptosomes, the presynaptic inhibitory mGluR pathway is revealed because the presynaptic facilitatory mGluR may have been desensitised (possibly by high basal PKC activity) whereas the inhibitory mGluR is not desensitised. Thus, the proposed 1S,3R-ACPD-activated group II inhibitory mGluR, in the absence of any facilitatory responses, can be unmasked in the glutamate release assay, producing powerful inhibition of KCl-evoked Ca^{2+}-dependent glutamate release. Attempts to demonstrate that the group I presynaptic facilitatory mGluR may have been desensitised, resulting in the unmasking of a 1S,3R-ACPD-sensitive group II inhibitory mGluR may not be straightforward. However, glutamate release experiments could be performed in the presence of low amounts of the PKC inhibitor Ro 31-8220 in an attempt to reduce basal PKC activity. Lowered PKC activity may restore the facilitatory mGluR, so that when 1S,3R-ACPD is applied to synaptosomes activation of the facilitatory mGluR would oppose and possibly suppress the presynaptic inhibitory mGluR, resulting in loss of the observed inhibitory effect of 1S,3R-ACPD on KCl-evoked Ca^{2+}-dependent glutamate release. A simpler experiment that may definitively demonstrate the presence of an inhibitory group II mGluR on adult

cerebral cortex nerve terminals (in addition to proposed L-AP4-sensitive group III mGluRs) may be to obviate activation of group I mGluRs (which occurs with the use of 1S,3R-ACPD) by using the group II-selective mGluR agonist DCG-IV. Furthermore, antagonist studies using the group II antagonist MCCG and the group III antagonist MAP4 (see table 6) may be helpful in determining whether indeed glutamate release is inhibited by activation of both group II and group III presynaptic mGluRs. These antagonists have been successfully used in the hippocampus to show a role of both group II and group III mGluRs in the presynaptic depression of excitatory transmission seen with 1S,3R-ACPD and L-AP4 respectively (Vignes et al., 1995).

Similar to (-)baclofen-mediated inhibition of KCl (15 mM)-evoked Ca^{2+}-dependent glutamate release, 1S,3R-ACPD and L-AP4 both inhibited Ca^{2+}-dependent glutamate release by ~ 50%. Receptor localisation studies show that group II mGluRs (mGlu2 and mGlu3) and group III mGluRs (mGlu4 and mGlu7) are highly expressed in the cerebral cortex. The magnitude of the inhibitory effects observed with 1S,3R-ACPD and L-AP4 suggest that along with presynaptic $GABA_B$ heteroreceptors, group II and group III mGluRs may represent major inhibitory pathways present on a majority of adult cerebrocortical nerve terminals.

A mechanism of action for presynaptic inhibitory mGluRs remains to be conclusively demonstrated. It is possible that, like presynaptic adenosine (A_1) receptors (Barrie and Nicholls, 1993) and presynaptic $GABA_B$ receptors (chapter five), inhibition of glutamate release by presynaptic inhibitory mGluRs occurs as a result of suppression of the voltage-sensitive Ca^{2+} channels (VSCC) linked to glutamate exocytosis. The effect of 1S,3R-ACPD and L-AP4 on the KCl-evoked and 4AP-evoked increase in cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, in adult cerebrocortical synaptosomes needs to be investigated in order to determine whether indeed inhibition of Ca^{2+} channels is the mechanism by which mGluRs transduce the extracellular chemical signal into an inhibition of transmitter release.

Conclusions

- The group II mGluR agonist 1S,3R-ACPD, but not the group III mGluR agonist L-AP4, inhibits glutamate exocytosis from striatal nerve terminals (synaptosomes), complementing electrophysiological data which shows that group II mGluR agonists, but not group III mGluR agonists, depress glutamatergic transmission in the striatum (Lovinger and McCool, 1995).

- In adult cerebrocortical synaptosomes, both 1S,3R-ACPD and L-AP4 strongly inhibit glutamate exocytosis, suggesting that group II and group III mGlu-autoreceptors may be functionally present on adult nerve terminals in the cerebral cortex. These results are in contrast to neurochemical findings showing that presynaptic inhibitory mGluRs do not limit glutamate release from adult cerebral cortex synaptosomes, only being functionally present in young rats (Vazquez et al., 1995a; Vazquez et al., 1995b; Herrero et al., 1996).

- In contrast to previous studies with adult cerebrocortical synaptosomes (Herrero et al., 1992), 1S,3R-ACPD in the presence of AA does not enhance 4AP-evoked glutamate release. This may be due to rapid homologous desensitisation of the presynaptic facilitatory mGluR by basal PKC activity. Desensitisation of the group I facilitatory mGluR may unmask a previously undescribed group II inhibitory mGluR present on adult cerebrocortical nerve terminals.

CHAPTER SEVEN

7 Discussion

Isolated nerve terminals (synaptosomes) are commonly used to study transmitter release from the presynaptic digit. It is a simple preparation which can be rapidly made from brain tissue in ~ 30 min, providing a crude preparation, or one hour if a purification step is used to reduce contamination from myelin, free mitochondria and postsynaptic tissue. Once isolated, the nerve terminal retains the pathways for storage, release and re-uptake of fast acting transmitters like glutamate and GABA. Synaptosomes also maintain a membrane potential, a Ca^{2+} gradient out > in, cytosolic compartmentalisation and receptors capable of modulating the release of transmitter. The fact that nerve terminals dissociated from their axons remain biochemically viable for 6 - 8 hours *in vitro* provides strong evidence that *in situ* the nerve terminal largely operates autonomously from the cell soma, except for a slow replacement of proteins and membrane components which would occur physiologically by axonal transport.

Due to the fact that synaptosomes no longer contain an axon with a cell body, the nerve terminal membrane potential cannot be depolarised by a train of invading action potentials akin to depolarisation of the terminal *in situ*. Other methods of depolarisation are needed. Electrical stimulation methods have proved unsuccessful and the most common means of depolarisation employs the use of KCl which causes a 'clamped' membrane depolarisation. More recently, it has been discovered that blockade of terminal K^+ channels with dendrotoxin elicits depolarisation and transmitter release. However, dendrotoxin is expensive to routinely use and another far cheaper K^+ channel blocker, 4-aminopyridine (4AP), produces the same effect.

Both KCl and 4AP evoke glutamate release from synaptosomes with dual components. The majority of the glutamate released is dependent on physiological amounts of Ca^{2+} being present in the extracellular bathing medium. However, a significant proportion of the total amount of glutamate released is Ca^{2+}-independent and can be isolated by omitting Ca^{2+} from the bathing medium and mopping up any residual Ca^{2+} that may be present with the Ca^{2+} chelator EGTA. Ca^{2+}-independent release *in vitro* is thought to result from efflux of cytosolic glutamate via reversal of the nerve terminal plasma membrane

glutamate transporter. This normally carries Na^+ along with glutamate into the terminal, but prolonged depolarising conditions *in vitro* reverse this process. This release is not thought to be physiologically relevant, although this proposal is often questioned (Adam-Vizi, 1992).

The glutamate released by KCl and 4AP depolarisation that is Ca^{2+}-dependent is believed to be exocytotic, i.e. originating from SSV stores, as judged by its ATP requirement (ATP is needed to accumulate glutamate into SSVs), inhibition by bafilomycin A (a specific inhibitor of V-ATPases which drive the accumulation of type I transmitters into SSVs) and inhibition by tetanus-botulinum toxins (which cleave synaptobrevins involved in SSV exocytosis). Accepting that the Ca^{2+}-dependent release of glutamate evoked by biochemical depolarisation is exocytotic, the question remains as to whether *in vitro* biochemical depolarisation mimics the patterns of excitation-secretion coupling that occur from nerve terminal stimulation *in situ*. This seems unlikely, thus; while synaptosomal transmitter release data purporting to a modulation of glutamate release via presynaptic receptor activation may not be directly correlated with presynaptic receptor modulation *in situ*, isolated nerve terminals provide a working model.

Excitation-secretion coupling in the CNS is strictly dependent on Ca^{2+} entry into the nerve terminal through putative voltage-sensitive Ca^{2+} channels (VSCC). VSCC are classed into T-type, L-type, N-type, P-type, Q-type and possibly R-type based on electrophysiological characterisation of somatic and cloned/expressed VSCC. They all have distinctive characteristics such as activation threshold, inactivation kinetics, conductance, pharmacology and cellular distribution. VSCC play a central role in synaptic transmission and can be described as molecular switches for the electro-secretory transduction process to the postsynaptic neuron; their electrical activation triggers a cascade of reactions (currently not fully understood) which ultimately leads to release of transmitter(s). However, despite the central role that VSCC play in synaptic neurotransmission, a detailed understanding of this molecular key element has been hampered by the size of most nerve terminals in the CNS, i.e. terminals are usually only up to 1 μm in diameter, which is too small for conventional electrophysiological approaches. It is still therefore unclear whether the VSCC that were originally characterised in dorsal root ganglion and purkinje cell bodies (and more recently in hippocampal

✳ (see Verhage, 1994)

neurons and cerebellar granule neurons) are actually present in nerve terminals, being coupled to transmitter secretion. Identification of the molecular structure of somatic VSCC is making great progress whereas an understanding of the molecular make-up of VSCC in presynaptic membranes still remains to be tackled. The progress made in the cloning and expression of subunits for VSCC from neuronal and muscle tissue does allow, however, some insight into the molecular basis of VSCC function and regulation in general.

Information about the VSCC coupled to transmitter exocytosis has come from the use of VSCC toxins in biochemical studies of transmitter release or from electrophysiological investigations of synaptic transmission. Relatively selective inhibition of the putative VSCC present at presynaptic nerve terminals became possible for the first time with the discovery of the ω-toxins. These toxins are powerful pharmacological tools for blocking neurotransmitter release which may help to unravel which VSCC types are represented in nerve terminals. They may also play a key role in understanding the mechanisms involved in the modulation of VSCC by presynaptic receptors. The pharmacological criteria (by use of VSCC toxins) to discriminate between the different VSCC types electrophysiologically can be applied to the effect of these same toxins on transmitter release. Thus, comparisons/indications can be inferred as to which VSCC might be present in nerve terminals, related from a toxin-based classification of somatic VSCC.The assumption inherent in such an approach is that the pharmacological specificity is conserved between the soma and terminal. However, in general, some of these electrophysiologically-defined VSCC types may be preferentially located in nerve terminals and different types may be involved in the release of different types of transmitters.

Neurochemical studies have produced conflicting data on the role of certain types of VSCC involved in exocytosis. Blockade of electrophysiologically defined T-type, L-type, N-type, P-type and Q-type VSCC has been shown to inhibit exocytosis. These conflicts in neurochemical studies may be a result of the inherent problems associated with biochemical depolarisation; there is no consensus as to the type of depolarising agent used or the strength of depolarisation used and these could be important factors when studying presynaptic VSCC and their inhibition by toxins. In order to be able to measure

transmitter release *in vitro*, stimuli are often used which are rather different from those encountered under physiological conditions. For instance, a 'clamped' depolarisation with KCl (30mM) is certainly not physiological and under these conditions, VSCC that are not coupled to exocytosis of a specific transmitter *in situ* might be recruited. Artificially long depolarisations and thus Ca^{2+} channel open states, will produce prolonged Ca^{2+} influx, allowing Ca^{2+} to diffuse to cytosolic compartments that might not be reached physiologically. Overlapping cytosolic Ca^{2+} domains are thought occur *in situ* but only in a 'local' context. Overlap of distant intraterminal Ca^{2+} domains resultant from *in vitro* depolarising conditions may lead to repetitive, non-physiological exocytosis of SSVs. Thus, care must be taken when interpreting the so called vesicular recruitment phase of transmitter release *in vitro*. A possible remedy to this is to use a more physiological stimulus such as 4AP which blocks putative terminal K^+ channels. 4AP elicits a synaptosomal depolarisation which is thought to mimic physiological inputs into the nerve terminal via action potential propagation. Indeed electrophysiological recordings from presynaptic nerve endings at a specialised synapse in the auditory pathway called the calyx of Held supports the mechanism of action of 4AP (Forsythe, 1994). This mammalian nerve terminal albeit larger than conventional synaptic terminals, utilises glutamate as a transmitter and may give an insight into the physiology of excitatory synapses in the CNS. The terminal has a rapidly activating K^+ conductance that is blocked by 4AP and a rapid inward current carried by Na^+ which is sensitive to block by tetrodotoxin (TTX). These findings may confirm the mechanism by which 4AP is thought to depolarise mammalian CNS synaptosomes, i.e. blockade of K^+ channels which leads to the repetitive firing of TTX-sensitive Na^+ channels.

Glutamate release studies and $[Ca^{2+}]_c$ studies with the VSCC toxin ω-CTx MVIIC (Chapter three) provided no evidence to suggest that depolarisation with 1 mM 4AP or 30 mM KCl leads to a differential recruitment of VSCC coupled to glutamate exocytosis. This is based on the similar IC_{50} values calculated for ω-CTx MVIIC inhibition of 4AP-evoked and KCl-evoked elevations in $[Ca^{2+}]_c$ and glutamate release. This finding may indicate that comparisons <u>can</u> be made between scientific reports that have adopted use of either of these two stimuli when studying which presynaptic VSCC types control glutamate exocytosis.

Although ideally, use of a common depolarisation stimulus would be more helpful when trying to collate neurochemical data assessment of the VSCC linked to glutamate exocytosis.

It has been suggested that L-type VSCC occur in rat brain nerve terminals based on the observation that $^{45}Ca^{2+}$ uptake is inhibited by dihydropyridines (DHPs) which selectively block L-type VSCC (Turner and Goldin, 1985). However, this alone does not mean that L-type VSCC are coupled to SSV exocytosis. A parallel inhibition of glutamate release by DHPs would be required for such a statement to be made. A DHP-mediated inhibition of synaptosomal Ca^{2+} influx in the absence of a parallel release study could indicate inhibition of L-type VSCC possibly present on microsomes (which may be formed from postsynaptic membranes contaminating synaptosomal brain preparations). Indeed, DHP binding sites on brain microsomes have been observed (Cruz et al., 1987). Furthermore, there is little evidence for an inhibition of glutamate release by DHPs using synaptosomal preparations. The present studies add to this library of evidence by showing that glutamate release from synaptosomes prepared from the rat cerebral cortex is not inhibited by DHPs, strengthening the largely accepted view that class C/D L-type VSCC are probably not involved in SSV exocytosis of type I transmitters including glutamate and GABA. Inhibitory effects of DHPs on glutamate release from primary cell cultures (Huston et al., 1990) could be due to inhibition of a presynaptic L-type VSCC. However, due to the fact that in cerebellar granule cell cultures, glutamate releasing neurites are subject to modulatory inputs from postsynaptic cell bodies, it is equally possible that the L-type VSCC involved in inhibition of glutamate release could be located on the cell soma and blockade by DHPs could be exerting a more indirect inhibition of glutamate release.

It is generally believed that SSV exocytosis in the mammalian CNS is regulated by non L-type high voltage-activated (HVA) VSCC and that several types may co-exist in individual nerve terminals to control release of a single transmitter. The co-existence of different VSCC types in a nerve terminal is based around studies showing synergistic effects of VSCC toxins on glutamate release from rat brain synaptosomes (Dunlap et al., 1995). These synergy studies are only feasible if the toxins when applied alone have little effect or only a partial effect

on transmitter release. Such toxins as ω-CTx GVIA and ω-Aga IVA have been used to show the co-existence of N-type and P-type VSCC respectively on striatal glutamate releasing terminals (Turner et al., 1993). ω-CTx MVIIC is not a useful toxin for such studies because it causes complete inhibition of glutamate exocytosis from cerebrocortical synaptosomes (Chapter three). This is likely due to the fact that ω-CTx MVIIC is a far less discriminating ligand against mammalian HVA VSCC than ω-CTx GVIA and ω-Aga IVA. ω-CTx MVIIC is known to interact with N-type, P-type and Q-type VSCC in the mammalian CNS. This could suggest that ω-CTx MVIIC completely inhibits glutamate exocytosis due to a blocking action at all three of these VSCC subtypes, based on the findings that N-type, P-type and Q-type VSCC are implicated in the control of glutamate exocytosis in the hippocampus (Luebke et al., 1993; Nooney and Lodge, 1996). However, there is no rationale to suggest that glutamate transmission throughout the cerebrocortical circuitry is controlled by the same VSCC types that control hippocampal synaptic transmission. Even if this were the case, ω-CTx MVIIC would certainly not be able to answer the question of whether multiple VSCC types co-exist in an individual glutamatergic nerve terminal in the cerebral cortex. Although ω-CTx MVIIC is a reversible VSCC toxin and blocks exocytosis completely, making it a useful toxin for assessment of the general involvement of VSCC in a given process, its poor selectivity greatly compromises its utility for discriminating among types of VSCC. This probably reduces it s usefulness as a VSCC probe in the mammalian CNS as other VSCC toxins such as ω-CTx GVIA and ω-Aga IVA show far greater selectivity for specific VSCC types.

The HVA VSCC types controlling SSV exocytosis have been probed in synaptosomes, dissociated neurons and tissue slices and release has been monitored either by measuring the Ca^{2+}-dependent exocytosis of transmitters using HPLC analysis, enzyme-linked assay or [^{3}H]glutamate release studies. Alternatively in tissue slices, electrical responses in postsynaptic cells can indicate presynaptic effects. Toxin studies need to be performed using neurochemical preparations adopting biochemical measurements of release and electrophysiological analysis of synaptic transmission from brain slices, and only when all these techniques come to some common agreement can a strong role for a VSCC subtype in control of transmitter release *in situ* be proposed.

N-type VSCC blockers partially inhibit exocytosis of nearly all transmitter types in the mammalian CNS, suggesting that under physiological conditions *in situ* there may be certain events that require exocytosis to occur via Ca^{2+} influx through N-type VSCC. These conditions are currently unknown. There is much debate as to the role that N-type VSCC play in the exocytosis of fast acting transmitters glutamate and GABA whilst experimental data provide strong evidence to suggest a central role of N-type VSCC in the release of catecholamines. ω-CTx GVIA causes a small inhibition of glutamate release from hippocampal synaptosomes (Luebke et al., 1993). This finding is backed up by electrophysiological data from hippocampal slices that ω-CTx GVIA partially depresses excitatory synaptic transmission (Luebke et al., 1993; Nooney and Lodge, 1996). Together, the results strongly indicate that certain excitatory synapses in the CNS incorporate class B N-type VSCC for synaptic transmission. Interestingly, it has been demonstrated *in vitro* that syntaxin, a major ubiquitous nerve terminal protein involved in exocytosis, can alter the gating of several VSCC types including N-type VSCC (Bezprozvanny et al., 1995). This further strengthens the placement of N-type VSCC in nerve terminal membranes and it is reasonable to assume that syntaxin may play a role in the regulation of Ca^{2+} entry into nerve terminals through N-type VSCC.

The role that N-type VSCC play in controlling glutamate release in the cerebral cortex has been in doubt for some time. [^{3}H]Glutamate release studies showed that ω-CTx GVIA was ineffective at inhibiting glutamate release from cerebrocortical synaptosomes (Turner et al., 1992). However, it was later shown that ω-CTx GVIA inhibits a significant proportion of a non-inactivating phase of Ca^{2+} entry into cerebrocortical synaptosomes that is linked to the release of glutamate *in vitro* (Bowman et al., 1993). Together with the high density of [^{125}I]GVIA receptors present in the cerebral cortex, which are believed to correspond to N-type VSCC (Gohil et al., 1994; Filloux et al., 1994), it would appear as if there may be a rationale for the presence of presynaptically located N-type VSCC in the cerebral cortex. Indeed, N-type VSCC are coupled to release of catecholamines in the cerebral cortex. The finding in the present studies that ω-CTx GVIA blocks 20% of the KCl (30mM)-evoked non-inactivating phase of Ca^{2+} entry into cerebrocortical synaptosomes (Chapter three) is not completely

unexpected, based on a previous observation (Bowman et al., 1993). However, ω-CTx GVIA also inhibited Ca^{2+}-dependent glutamate exocytosis by 30 - 40 %. This is a novel finding and constitutes a significant proportion of glutamate exocytosis. The two results in parallel strongly suggest that a major population of glutamatergic nerve terminals in the cerebral cortex may contain N-type VSCC. Unfortunately, reading the literature no reference to electrophysiological rat neocortical slice studies showing inhibition of synaptic transmission by ω-CTx GVIA was apparent. Most electrophysiological studies of excitatory transmission are performed using hippocampal slices due to the well characterised synaptic circuitry in this brain region. A parallel electrophysiological study showing inhibition of excitatory synaptic transmission by ω-CTx GVIA in the cerebral cortex would further strengthen the neurochemical synaptosomal data described in this thesis indicating that presynaptic N-type VSCC may play a role in the release of glutamate in the cerebral cortex.

It is far more certain that P-type and/or Q-type VSCC play a major role in the control of glutamate exocytosis in all brain regions containing excitatory pathways. This is based on the huge amount of evidence showing that ω-Aga IVA, a P-type VSCC toxin, potently inhibits synaptic transmission in almost all regions of the brain that are rich in synaptic connections and inhibits Ca^{2+}-dependent glutamate release from synaptosomes prepared from cerebral cortex, striatum, hippocampus and cerebellum (for review see Olivera et al., 1994). The relatively novel toxin ω-CTx MVIIC blocks N-type, P-type and Q-type VSCC present on cell soma. However, the pharmacology of the toxin at presynaptic nerve terminals is unknown. The present studies show that ω-CTx MVIIC completely inhibited glutamate exocytosis in the cerebral cortex. Furthermore, they strongly indicate that putative N-type VSCC control glutamate release from nerve terminals. However, only 30 - 40% of glutamate release is blocked by ω-CTx GVIA, indicating that other VSCC types are involved in glutamate exocytosis. Experiments in cerebrocortical synaptosomes with ω-CTx MVIIC could, using a comparison of the pharmacology of ω-CTx MVIIC on cloned and somatic VSCC, be inhibiting the remaining proportion of glutamate release through a blockade of P-type or Q-type VSCC. ω-CTx MVIIC used alone cannot shed any light as to which of these VSCC controls glutamate release that is insensitive to inhibition by ω-CTx GVIA.

It is highly possible that both VSCC are involved. Studies with ω-Aga IVA used in concert with ω-CTx MVIIC might be helpful in determining whether the remaining proportion of release is linked to one or two VSCC types. These studies need to be done. Saying this, the question then arises of how many VSCC types might co-exist in an individual nerve terminal and be coupled to glutamate exocytosis. Are N-type and P/Q type VSCC present on common nerve terminals to exert dual and possibly even triple control of glutamate release, or are N-type VSCC found on one population of glutamate terminals in the cerebral cortex and P-type or Q-type on a different population; both scenarios are possible. The complexity and interplay between presynaptic VSCC subtypes and glutamate release in the cerebral cortex may be enormous and cannot be dissected using a heterogeneous population of isolated nerve terminals. Thus, the synaptosomal preparation can only provide a very basic deduction and the data described in this thesis may only propose that glutamate release from cerebrocortical nerve terminals can be controlled by putative class B N-type VSCC and by putative class A P/Q-type VSCC. This still only provides an indication as to which VSCC might be functionally present on glutamatergic nerve terminals, inferred from the pharmacology of the ω-toxins at electrophysiologically defined somatic VSCC types. However, VSCC-specific antibodies raised to the subunits that make up the VSCC protein complex are currently and successfully being developed for VSCC localisation studies. It is possible that the development of polyclonal antibodies raised to the various cloned and somatically defined VSCC subtypes may be of immense value in determining the native VSCC on glutamatergic nerve terminal (synaptosomal) membranes in different regions of the brain. This would really help to clarify the issue of whether or not the pharmacologically classified somatic VSCC are represented at the level of the presynaptic digit to perform the role of excitation-secretion coupling, granted that functional pharmacological transmitter release data and electrophysiological synaptic transmission results indicate roles for electrophysiologically defined N-type, P-type and Q-type VSCC in SSV exocytosis in the CNS. Such an analysis in synaptosomes could be performed relatively easily using immunoblotting techniques.

Another key question relating to the control of glutamatergic synaptic transmission is that of whether different presynaptic active zone VSCC types

correspond to different postsynaptic neurons containing a certain receptor type. For example, in the cerebral cortex, would presynaptic terminals releasing glutamate to poststynaptic neurons with NMDA, AMPA, kainate or metabotropic glutamate receptors, or differing combinations thereof, each have a markedly different characteristic spectrum of VSCC types at the corresponding presynaptic terminals? These questions need to be answered in order for us to have a more complete understanding of glutamatergic synaptic transmission in the CNS, but they can only be answered when electrophysiological advances allow simultaneous measurements of the electrical and release events at a single nerve terminal synapsing onto a postsynaptic neuron for which the receptor make-up is known. This technology may be far off but is surely one worth waiting for.

Finally, the question of whether neuronal VSCC blockers may have a clinical/therapeutic role must be considered. As discussed, the P/Q VSCC subfamily is thought to mediate the majority of glutamate and GABA exocytosis from central nerve terminals. However, toxins that target P-type and Q-type VSCC, i.e. ω-Aga IVA and ω-CTx MVIIC, have been shown to be lethal in low doses when systemically administered to rodents. This would in the case of ω-CTx MVIIC particularly, be due to the indiscriminate block of glutamatergic transmission at nearly all central synapses. This is paralleled by my studies showing that ω-CTx MVIIC totally abolishes glutamate exocytosis. Instead, the class B N-type VSCC blockers currently hold more therapeutic interest (Bowersox et al., 1994) as they do not produce lethal effects, perhaps due to their more specific and modulatory role in glutamate exocytosis. The ω-conopeptides derived from Conus species are reversible blockers of N-type VSCC and studies show that ω-CTx MVIIA is a good neuroprotectant in global or focal ischaemia due to such conditions as head trauma, cardiac arrest, coronary artery bypass surgery and stroke patients. ω-CTx MVIIA is not a potent blocker of glutamate release, instead selectively blocking noradrenaline release. From this finding, doubt is beginning to accumulate about the mechanisms involved in neuronal excitotoxicity. It is clear that excess glutamate release into the synaptic cleft leading to NMDA receptor-mediated Ca^{2+} entry into neurons, is not the only pathway that leads to neuronal death. The mechanisms are believed to be far more complex and multifactorial.

Moving on from the VSCC that control glutamate exocytosis, a major

modulation of these VSCC and thus of exocytosis itself may come from activation of presynaptic receptors. Most of my experiments were directed towards addressing the role that glutamate autoreceptors and presynaptic heteroreceptors may play in the modulation of glutamate release from presynaptic mammalian nerve terminals, with particular relevance to the cerebral cortex. Proof of the existence of most presynaptic glutamate receptor pathways remains ambiguous unlike our understanding of postsynaptic glutamate receptors. Evidence is largely based on inference from electrophysiological studies such as paired pulse facilitation (PPF) and recorded excitatory postsynaptic potentials/currents (EPSP s/EPSC s) or from neurochemical studies of transmitter release using synaptosomes, cell cultures and brain slices. Slices and cell cultures have the disadvantage over isolated nerve terminals in that electrophysiological data or release results purporting to an agonist-induced modulation of release need not be due to activation of putative presynaptic receptors but could be due to postsynaptic mechanisms. Conversely, synaptosomes have the distinct disadvantage that putative receptor effects alluded to using brain slice studies may be absent in correlate isolated nerve terminal studies possibly due to damage or loss of the receptor protein during the preparation of the synaptosomes. However, if results from electrophysiological data and neurochemical release studies relate closely in terms of pharmacology of the receptor then the evidence becomes stronger that presynaptic receptor mechanisms may be responsible for modulation of exocytosis. Still, due to the practical limitations of the synaptosomal preparation, there is little direct proof for the presynaptic localisation of receptor proteins in mammalian CNS nerve terminal membranes. This sets receptor studies at the level of the postsynaptic membrane and the presynaptic terminal apart.

Presynaptic receptors implicated in the self-regulation of transmitter release are often referred to as autoreceptors. This may be misleading because it is practically impossible, using any of the neuroscientific techniques currently available, to follow a single type of transmitter molecule, such as glutamate, and discriminate between glutamate originating from neighbouring or remote nerve terminals and glutamate secreted from the nerve terminal itself. Thus, an autoreceptor effect may not strictly imply self-regulation of release. If presynaptic glutamate receptors are located in the active zone region close to the VSCC

coupled to exocytosis, glutamate transmitter molecules originating from other nerve terminals might not be able to gain access to the largely impenetrable synaptic cleft, and thus the glutamate receptor will probably be activated by self-released glutamate. This is an autoreceptor in the purest sense of the word. However, it is highly possible that presynaptic glutamate receptors could be located on areas of the terminal membrane outside of the synaptic cleft region and thus would be a target for activation by glutamate released from other neighbouring nerve terminals. Strictly, this then would not be a self-regulating autoreceptor. Receptor localisation studies at an ultrastructural level may provide information that requires us to reassess the concept of an autoreceptor as well as providing *in situ* evidence to corroborate data indicating putative presynaptic receptor-mediated modulatory actions of many drugs. Currently, the only means of classifying many presynaptic receptors is inferred from the pharmacology of the presynaptic modulators at their better characterised postsynaptic receptors. There is no reason to assume that pharmacological agents at postsynaptic receptors will activate the same classes of receptors at presynaptic nerve terminals.

Few clear examples of direct induction of transmitter release by activation of presynapic receptors exist, more commonly their activation will modulate the stimulus-secretion coupling evoked biochemically by depolarisation with agents such as KCl or 4AP. Thus, presynaptic receptors may only be relevant in combination with the arrival of action potentials through the nerve terminal. With regard to this, PhD studies showed that AMPA, kainate and domoate did not elicit release of glutamate, but in combination with 4AP depolarisation these non-NMDA receptor agonists facilitated Ca^{2+}-dependent glutamate release possibly via modulation of the nerve terminal plasma membrane potential. The only definitive manner of determining whether the putative high-affinity KA receptor which facilitates glutamate exocytosis from cerebrocortical nerve terminals, does so via a receptor-mediated depolarisation of the nerve terminal plasma membrane potential would be through electrophysiological recordings from individual glutamatergic nerve terminals. Application of kainate, AMPA or domoate under these conditions would provide the answer to a question that has been posed for many years by those studying receptor modulation of glutamate release. Of course, as stated earlier, conventional electrophysiological recording techniques

are not possible due to the small size of the nerve terminal and so it remains to be seen whether these putative presynaptic KA receptors are similar to their cloned counterparts which have been electrophysiologically characterised.

Generally, few receptors have been found to potentiate transmitter exocytosis. Instead, most presynaptic receptor activation seems to inhibit exocytosis. One pathway that does potentiate glutamate exocytosis seems to involve stimulation of presynaptic protein kinase C (PKC). A presynaptic PKC isoform, as yet unidentified, when stimulated by phorbol ester or by the mGluR agonist 1S,3R-ACPD in the presence of arachidonic acid (Herrero et al., 1992) appears to inhibit a 4AP-insensitive subtype of K^+ channel, which leads to prolonged presynaptic Ca^{2+} channel openings (seen by an increased $[Ca^{2+}]_c$ in synaptosomes) and enhanced glutamate exocytosis. Likewise, the presynaptic facilitatory KA receptor proposed in the present studies could be mediating its effects through PKC, albeit by a different mechanism to that of PKC-mediated inhibition of a K^+ channel. When the KA receptor has ligand bound (KA, AMPA or domoate) in the absence of depolarisation, no significant change in the synaptosomal resting membrane potential or glutamate exocytosis may be observed, reflecting the fact that an ionotropic signal alone may not be sufficient to facilitate glutamate release. However, upon depolarisation of synaptosomes with 4AP, presynaptic PKC is stimulated (Coffey et al., 1993) and an as yet uncharacterised PKC isoform might phosphorylate the putative presynaptic KA receptor, resulting in enhanced current through the ion conducting pore region. This ionotropic effect may synergise with 4AP-evoked depolarisation, thus, enhancing the membrane depolarisation evoked by 4AP. As a result of this greater depolarisation, the VSCC coupled to glutamate exocytosis may be facilitated, leading to increased Ca^{2+} entry into synaptosomes (albeit undetectable with averaged population measurements of $[Ca^{2+}]_c$ using fura-2) and the observed potentiation of 4AP-evoked glutamate exocytosis with AMPA, kainate and domoate. This hypothesis describes a mechanism by which, under certain synaptic conditions, an ionotropic signal (arising extracellularly from receptor activation) and a metabotropic signal (arising intracellularly from PKC activation) may both be necessary for facilitation of glutamate exocytosis. Inhibitors/ activators of presynaptic PKC, used in concert with submaximal concentrations of

* (Wang et al., 1993)

AMPA, kainate or domoate, could reveal whether PKC-mediated phosphorylation may be part of the mechanism by which presynaptic KA receptors enhance 4AP-evoked depolarisation of the synaptosomal membrane potential and facilitate glutamate exocytosis.

Alternatively, cAMP-dependent protein kinase (PKA) could be another candidate for phosphorylating presynaptic KA receptors. This is based on the findings that GluR6 KA subunits have PKA consensus sites, and PKA enhances recombinant GluR6 KA receptor currents. It would also provide a functional role for adenylyl cyclase at the level of the presynaptic digit. Immunohistochemical localisation of the cyclase in rat brain hippocampal nerve terminals indicates that adenylyl cyclase may participate in modulation of glutamate release (Mons et al., 1995). However, positive modulators of adenylate cyclase activity such as forskolin have not been found to affect glutamate release from synaptosomes. Furthermore, the facilitatory effects of kainate on glutamate release were not modulated by inhibition of PKA, although these experiments were not performed exhaustively. Using a heterogeneous population of isolated nerve terminals, it may be difficult to obtain convincing evidence regarding whether or not presynaptic PKA or PKC mediate the observed facilitatory responses seen by activation of a putative presynaptic non-NMDA-type glutamate receptor sensitive to AMPA, kainate and domoate. Again, electrophysiological techniques at the level of the individual nerve terminal would probably allow for such an assessment. It is possible that the lack of effect of modulators of the PKA pathway in synaptosomes is unfruitful because physiological conditions *in situ* are not being mirrored with neurochemical *in vitro* conditions.

What are the physiological roles of kainate receptors? Very little is known about the possible cellular functions of native kainate receptors. Could they be presynaptic receptors involved in the regulation of glutamate release? If so, do they facilitate or inhibit glutamate release? Kainate has been proposed to inhibit K^+-evoked and 4AP-evoked [^{3}H]glutamate release from hippocampal synaptosomes via stimulation of a presynaptic kainate receptor (Chittajallu et al., 1996). These results are surprising when most neurochemical studies show kainate potentiates evoked glutamate release from nerve terminals in most brain regions, including the cerebral cortex (Chapter four) and also behaves as a potent

convulsant excitotoxin in the CNS (Mayer and Westbrook, 1987; Gaiarsa et al., 1994). Domoate has similar excitotoxic effects in the CNS (Debonnel et al., 1989b; Debonnel et al., 1989a) but Chittajallu et al (1996) also report that domoate and kainate reduce NMDA receptor-mediated synaptic responses in hippocampal slices by an effect that they deduce to be presynaptic. Two explanations could explain the differences seen between the results of Chittajallu et al (1996) showing inhibitory effects of kainate on glutamate release and the present data showing that non-NMDA receptor agonists only facilitate glutamate exocytosis. Firstly, the putative presynaptic KA receptors located on hippocampal nerve terminals and cerebral cortex terminals may be different subtypes. Secondly, whilst my experiments adopted the use of an enzyme-linked assay of endogenously released glutamate, Chittajallu et al (1996) label synaptosomes with exogenous [^{3}H]glutamate and then measure the release of [^{3}H]glutamate from the labelled nerve terminals as a marker for glutamate exocytosis. This method suffers from the problem that whilst [^{3}H]glutamate is taken up competently into the nerve terminal on the plasma membrane glutamate transporter, only a small quantity of label is accumulated into nerve terminal glutamatergic vesicles (Nicholls and Sihra, 1986). Thus, much of the labelled glutamate will remain in the cytosolic compartment. Upon depolarisation, [^{3}H]glutamate release will occur by vesicular exocytosis but a major proportion may result from efflux of cytosolic [^{3}H]glutamate via reversal of the glutamate transporter. This may underestimate vesicular glutamate release. Kainate is known to block the nerve terminal plasma membrane amino acid transporter (Pocock et al., 1988). If the hippocampus did not actually have any presynaptic kainate receptors, the predominant effect of kainate may be inhibition of glutamate re-uptake. This would reduce the amount of cytosolic [^{3}H]glutamate released via reversal of the glutamate transporter, upon depolarisation. Thus, [^{3}H]glutamate counts would be lower in the presence of kainate and an apparent inhibition of glutamate release by kainate would be observed in experiments using [^{3}H]glutamate as a marker for exocytosis. It does not seem likely that the hippocampus contains a lack of presynaptic kainate receptors because the effects of kainate on glutamate release reported by Chittajallu et al (1996) were blocked by the selective GluR6 kainate receptor antagonist NS-102. Thus, it is more likely that different subtypes of KA receptor

might modulate glutamate release in the hippocampus and cerebral cortex.

Even though ligand-binding studies and *in situ hybridisation* results show that KA receptor subunits are widespread, KA receptors have not been easily functionally detected in light of the fact that thay are probably found closeby to AMPA receptors but in far smaller numbers. Several of the fundamental questions surrounding the kainate receptor require analysis of the individual subunits, i.e. are the high-affinity kainate receptor subunits KA1 and KA2 part of molecular complexes that contain GluR 5 -7 subunits? Are the subunits localised at post-synaptic or presynaptic sites, or both? The present results show that Ca^{2+}-dependent glutamate release is facilitated from cerebrocortical synaptosomes by AMPA, kainate and domoate. Cyclothiazide, an AMPA receptor desensitisation inhibitor, did not modulate AMPA responses and kainate effects were blocked by CNQX and NS-102, but not blocked by GYKI 52466, an AMPA receptor antagonist. All this points towards a stimulation of a putative presynaptic high-affinity kainate receptor by the three non-NMDA receptor agonists AMPA, kainate and domoate. Furthermore, the block by NS-102 suggests that the receptor might contain GluR6 kainate receptor subunits. It is known that when GluR6 is expressed with KA2, functional GluR6/KA2 channels are formed that can be gated by AMPA in a non-desensitising manner. As AMPA had a maximal putatively non-desensitising effect on glutamate release (Chapter four), it is possible that KA2 subunits and GluR6 subunits make up the proposed putative presynaptic KA receptor. This is all hypothetical and some solid evidence as to the subunit structure of the putative presynaptic KA receptor might come from the use of subunit-specific antibodies. Antibodies selective for receptor subunits have been valuable tools in the analysis of the biochemical and distribution properties of several receptor families. Antibodies made to synthetic peptides corresponding to the carboxy terminal of GluR6 and KA2 (anti-GluR6 and anti-KA2) are highly specific for native GluR6 and KA2 subunits respectively (Wenthold et al., 1994). To address whether GluR6 and KA2 receptor subunits are represented in nerve terminals would involve immunoblotting with autoradiographic detection using anti-GluR6 and anti-KA2 antibodies to see if they bind to GluR6 and KA2 kainate receptor subunits that may be, based on thesis deductions, present in synaptosomal protein samples that have been dot-blotted or electrotransferred

* (see Bliss and Collingridge, 1993)

onto nitrocellulose membranes. If such an experiment proved positive, it is always possible that however pure the presynaptic membrane preparation may be, there is always going to be some postsynaptic attachment. This could contain postsynaptic kainate receptors made up of GluR6 or KA2 subunits and this may render the deductions from immunoblot studies relating to the presence of kainate receptor subunits on presynaptic membranes debatable.

The presence of a presynaptic autoreceptor that stimulates rather than inhibits glutamate release is unusual. Most transmitters are subject to a negative feedback control by autoreceptors that limit release by activation of presynaptic K^+ channels or inhibition of Ca^{2+} channels (see later). However, having only negative feedback control, through presynaptic mGlu-autoreceptors or $GABA_B$ and A_1 adenosine heteroreceptors, would prevent the glutamatergic nerve terminal from responding to high frequency stimuli (which are required for the induction of long-term potentiation (LTP) of excitatory synapses). Positive feedback control mechanisms that serve to override inhibitory inputs might serve as a necessary component to a mechanism that leads to the persistent enhancement of excitatory synaptic transmission. A presynaptic facilitatory high-affinity kainate receptor might serve such a purpose. This makes the assumption that presynaptic inhibitory receptors and presynaptic facilitatory receptors co-exist on a common population of glutamatergic nerve terminals. As yet, there is no convincing evidence to suggest that this may be the case, and opposing presynaptic receptor mechanisms could just as easily be present on distinct glutamatergic pathways in the cerebrocortical circuitry. Furthermore, at present, it remains a matter of debate as to whether or not the presynaptic digit and hence glutamate release is an important factor in the conditioning or induction phases of AMPA receptor-mediated LTP. It seems tempting, however, to assume that a presynaptic facilitatory glutamate receptor must play some role in synaptic plasticity of the glutamatergic synapse. Presumably, during a period of high frequency stimulation, endogenously released glutamate would feedback and permanently engage the putative facilitatory kainate receptor. However, some feedback regulatory mechanism would have to be in place to prevent a continuously activated receptor. This may be achieved in two ways. Firstly, the present data shows that the putative presynaptic facilitatory kainate receptor only enhanced evoked

✳ (see Seeburg 1993)

glutamate release. This indicates that activation of the receptor along with concurrent nerve terminal depolarisation by invading action potentials, is a prerequisite for potentiaton of transmitter release, i.e. receptor activation does not appear to be enough alone to directly alter exocytosis. In this respect, once postsynaptic activity upstream of the nerve terminal has quietened, action potential input into the nerve terminal will fall, silencing the synapse and the putative facilitatory kainate receptor. A second and important regulatory mechanism may be receptor desensitisation. It is known that cloned and expressed kainate receptor subunits produce currents that are rapidly desensitising upon application of glutamate. Also, native postsynaptic kainate receptors have only recently been dissected out from AMPA receptor responses in central neurons; novel AMPA receptor antagonists have enabled isolation of native kainate receptor responses which are rapidly desensitising. Presynaptic facilitatory kainate receptors may be subject to regulation via desensitisation. This could be homologous or heterologous desensitisation, and could occur through a PKC-mediated phosphorylation effect. It has been shown that PKC rapidly desensitises a facilitatory presynaptic mGluR in cerebral cortex nerve terminals (Herrero et al., 1994). Experiments manipulating the PKC pathway in synaptosomes might help to reveal whether the putative facilitatory kainate receptor is subject to regulation from PKC phosphorylation. Furthermore, it is possible that the observed effects of AMPA, kainate and domoate on 4AP-evoked Ca^{2+}-dependent glutamate release are not maximal responses, i.e. they may be desensitised responses. Rapid desensitisation of kainate-evoked and glutamate-evoked currents in cloned and expressed GluR 5 -7 kainate receptor subunits can be prevented by pretreatment with Concanavalin A (Con A) (Fletcher and Lodge, 1996). Use of Con A in synaptosomal experiments would be revealing in as much that it might strengthen the proposal that indeed the responses seen with AMPA, kainate and domoate on glutamate release are the result of activation of a presynaptic kainate receptor and it would also address the question of whether the observed responses with these drugs are in fact desensitising responses.

As stated earlier, most neurochemical data describe presynaptic modulation of transmitter release by inhibitory presynaptic receptors. Indeed, the present data show that (-)baclofen and GABA inhibit evoked glutamate release in a phaclofen-

insensitive, CGP 35348-sensitive manner (Chapter five). This complements electrophysiological data indicating that (-)baclofen and GABA depress EPSP's and neurochemical studies showing that they inhibit glutamate release from brain slices. All of these effects can be blocked by CGP 35348 but not by phaclofen. The electrophysiological data and neurochemical data correlate well and this strongly indicates that $GABA_B$ heteroreceptors are found on glutamatergic nerve terminals to limit the release of glutamate under certain conditions of synaptic activity. What is not clear from the literature is whether presynaptic $GABA_B$ heteroreceptors limit release by activation of K^+ channels, inhibition of Ca^{2+} channels or a combination of the two. Also, it is not readily clear whether the G-protein involved falls into the G_i /G_o category that are sensitive to pertussis toxin (PTX). In general, it is uncertain whether presynaptic G-proteins are the same as postsynaptic G-proteins. Presynaptic G-proteins need to be purified and cloned before these questions can be answered.

Experiments measuring $[Ca^{2+}]_c$ showed that (-)baclofen significantly reduced depolarisation-evoked Ca^{2+} entry into synaptosomes and this did not appear to be an indirect effect via alteration of a terminal K^+ channel. The majority of the literature relating to the mechanism of action of presynaptic $GABA_B$ receptors does seem to suggest that unlike their postsynaptic counterparts, presynaptic $GABA_B$ receptors do not alter K^+ conductances, instead having a more direct effect on a Ca^{2+} channel. It is impossible to show, using neurochemical techniques, that presynaptic $GABA_B$ receptors directly inhibit Ca^{2+} channels, as has been shown for postsynaptic $GABA_B$ receptors in dorsal root ganglion sensory neurons using electrophysiological approaches (Huston et al., 1990). Electrophysiological analysis of individual nerve terminal Ca^{2+} currents would be a prerequisite before it could be said that presynaptic $GABA_B$ receptors can directly inhibit VSCC as can occur with $GABA_B$ receptors located on cell bodies. Again, electrophysiological techniques cannot be performed on most central nerve terminals due to their small size.

The other contentious issue relating to presynaptic $GABA_B$ receptors on glutamate releasing terminals is whether or not they are coupled to PTX-sensitive G-proteins. PTX is used as a tool to study signal transduction through guanine nucleotide binding G-proteins. When a receptor-mediated response is blocked by

prior treatment with PTX the interpretation is straightforward; the receptor is most likely linked to a PTX-sensitive G-protein. However, when PTX does not have an effect, the lack of a G-protein-mediated response can be explained either by the G-protein mediating the physiological response being PTX-insensitive or that the toxin did not reach the area of interest in sufficient enough amounts. An elegant study by van der Ploeg et al (1991) looks at the PTX sensitivity of presynaptic A_1 adenosine receptors and they find a lack of effect of PTX on presynaptic adenosine responses when the toxin is administered *in vivo*. In the study they show that PTX equally ADP-ribosylates presynaptic G-proteins in two synaptosomal preparations where the first preparation is made from brain tissue that had been pretreated with PTX *in vivo* and the second preparation had been made from non-PTX treated brain tissue with PTX added directly to the synaptosomal medium. No difference with respect to PTX activity between these two synaptosomal preparations suggested to the authors that the lack of effect of PTX on presynaptic adenosine (A_1) responses was not due to poor diffusion of PTX to synaptic membranes. Instead, it was argued that the presynaptic adenosine receptor is not decoupled from its G-protein by PTX because the presynaptic A_1 receptor is coupled to a PTX-insensitive G-protein. Experiments with PTX and synaptosomes failed to show any decoupling of the (-)baclofen inhibitory effects on glutamate release. As PTX should not have any problem getting into isolated nerve terminals and being activated, this neurochemical data does support other electrophysiological studies showing that (-)baclofen depresses excitatory synaptic transmission in a PTX-insensitive manner (Colmers and Williams, 1988; Dutar and Nicoll, 1988). Postsynaptic $GABA_B$ receptors are believed to be coupled to PTX-sensitive G-proteins. Thus, a second difference between presynaptic and postsynaptic $GABA_B$ receptors that regulate excitatory synaptic transmission (on top of the putatively different ion channels that they modulate) may be that they are coupled to different G-proteins. This could explain the putatively differing signal transduction mechanisms that underlie pre- and postsynaptic $GABA_B$ receptor activation.

The precise physiological role that presynaptic $GABA_B$ receptors located on glutamatergic nerve terminals play in the CNS remains cloudy but it is thought that this receptor may provide an important target for therapeutic intervention in

the pathology of many CNS disorders. However, because postsynaptic $GABA_B$ receptors can have differing and opposing effects on neuronal activity compared to those located presynaptically on axon terminals, agonists and antagonists often produce complex and contradictory neuronal effects. Whilst (-)baclofen and CGP 35348 in an isolated nerve terminal preparation may be useful in indicating the presence of $GABA_B$ receptors on glutamatergic nerve terminals (Chapter five), these drugs have little therapeutic potential because they also have action at $GABA_B$ autoreceptors and postsynaptic $GABA_B$ receptors. Thus, in an *in situ* scenario, whilst glutamate exocytosis and excitatory transmission might be suppressed by activation of presynaptic $GABA_B$ heteroreceptors with (-)baclofen, concurrent disinhibition of GABAergic synapses through $GABA_B$ autoreceptor activation with (-)baclofen would cause neuronal excitation. Agonists and antagonists that are highly selective for 1) presynaptic $GABA_B$ autoreceptors, 2) presynaptic $GABA_B$ heteroreceptors and 3) postsynaptic $GABA_B$ receptors need to be developed and characterised before the $GABA_B$ receptor system can be seen as a therapeutic target for such CNS disorders as epilepsy. It is believed that novel selective agonists targetted towards $GABA_B$ receptors found on glutamate releasing nerve terminals could be of immense modulatory/preventative benefit to combat epileptiform bursting activity in susceptible brain regions (see Mott and Lewis, 1994).

It has very recently been reported that two $GABA_B$ receptor forms, designated $GABA_B$R1a and $GABA_B$R1b have been cloned (Kaupmann et al., 1997) and they seem likely to be the molecular correlates of native $GABA_B$ receptor proteins in the mammalian CNS. The two cloned $GABA_B$ receptors have similar M_r to native $GABA_B$ receptor proteins and are negatively coupled to adenylyl cyclase, with the rank order of the binding activities of antagonists and full agonists being identical to native rat cerebral cortex $GABA_B$ receptors. It is not yet known whether these two $GABA_B$ receptor clones correlate to postsynaptic or presynaptic $GABA_B$ receptor proteins, or both. Furthermore, as with the cloning of seven-transmembrane G-protein-linked metabotropic glutamate receptors (with which these novel $GABA_B$ receptor clones share homology), $GABA_B$ receptors may turn out to have a great deal of molecular diversity. Thus, the eventual cloning, expression, functional characterisation and receptor brain mapping of all of the

✳ (Schoepp and Conn, 1993)

subtypes of $GABA_B$ receptor that are found to exist in the mammalian CNS will be of enormous help in furthering our understanding of this important inhibitory receptor system. Furthermore, such knowledge would speed up the development of $GABA_B$ receptor subtype-specific ligands which may have important therapeutic potential in a number of CNS disorders where the $GABA_B$ receptor system has been implicated.

Our current understanding of presynaptic inhibitory glutamate autoreceptors that limit the release of glutamate from nerve terminals is less clear than knowledge of presynaptic $GABA_B$ heteroreceptors that inhibit glutamate exocytosis. All the current electrophysiological and neurochemical data points towards the presence of G-protein coupled metabotropic glutamate receptors (mGluRs) on terminal membranes. That is, except for one report of a presynaptic inhibitory ionotropic kainate-type glutamate receptor in the hippocampus (Chittajallu et al., 1996), which was discussed earlier. Unlike the $GABA_B$ receptors, eight different subtypes of mGluR (mGluR1 through mGluR8) have now been cloned and it would appear that presynaptic inhibitory mGlu-autoreceptors fall into group II or Group III mGluRs that are negatively linked to adenylate cyclase activity via a PTX-sensitive G-protein. It must be stated that like $GABA_B$ receptors, pre- and postsynaptic mGluRs exist and it is the postsynaptically located mGluRs that have been pharmacologically and biochemically characterised. Thus, it is not clear whether native presynaptic mGlu-autoreceptors mediate inhibition of glutamate release via a PTX-sensitive G-protein negatively linked to adenylate cyclase activity. Initial experiments addressing the PTX sensitivity of the observed inhibitory effects of 1S,3R-ACPD on glutamate release from adult rat striatal and cerebral cortex synaptosomes (Chapter six) might be helpful in comparing the G-proteins coupled to pre- and postsynaptic mGluRs.

In general, it is thought that the principle role of mGluRs is to alter the signal-to-noise ratio of glutamatergic transmission in the CNS. Spontaneous neuronal activity is supposed to constitute a noise. Presynaptic inhibitory mGlu-autoreceptors might reduce the noise and thereby generate signals. Under what physiological conditions presynaptic mGluRs are recruited into the synaptic transmission process is currently unknown; in principle they would appear to constitute negative feedback loops, like most other presynaptic receptors.

The present studies with striatal synaptosomes complement electrophysiological findings in the striatum that group II mGluRs suppress striatal excitatory transmission via an inhibition of glutamate release at corticostriatal synapses (Lovinger and McCool, 1995). Indeed a number of electrophysiological studies in the striatum have accumulated data in favour of a negative feedback on the release of glutamate that is exerted through mGlu-autoreceptors located on nerve terminals. This occurs at the corticostriatal synapses that are believed to be involved in striatal long-term potentiation (LTP) and long-term depression (LTD). However, the role that mGlu-autoreceptors play in striatal synaptic plasticity is not known. Whether they have a major or minor physiological role in striatal motor learning remains to be discovered. They may be central to certain pathologies of the striatal circuitry including Parkinson's disease and Huntingdon's disease. A greater understanding of dopamine receptor, ionotropic glutamate receptor and metabotropic receptor interactions at the postsynaptic level of the corticostriatal synapse may reveal that presynaptic mGlu-autoreceptors play a more subtle 'housekeeping' role in the striatal circuitry, co-ordinating the strength of signals arising from the cerebral cortex. In this way, the right amount of glutamate may be released into the synaptic cleft during repetitive stimulation of corticostriatal synapses. Repetitive stimulation is thought to be vital during the processes of LTP and LTD.

In the cerebral cortex and hippocampus, mGlu-autoreceptors have not been well characterised, particularly in adult brain. Due to the lack of evidence promoting the presence of mGlu-autoreceptors in adult rat hippocampus and cerebral cortex, and a few reports indicating their presence in young rats (Baskys and Malenka, 1991; Vazquez et al., 1995b) it has been proposed that presynaptic inhibitory mGlu-autoreceptors are developmentally regulated in the mammalian CNS. The present results show a profound inhibition of evoked glutamate release from adult rat cerebral cortex synaptosomes by the mGluR agonists 1S,3R-ACPD and L-AP4 (Chapter six). This suggests that glutamate release in adult cerebral cortex is subject to negative feedback regulation by heteroreceptors ($GABA_B$) and metabotropic glutamate autoreceptors. There is always a possibility that the mGluR agonists used in these studies could be acting on a presynaptic inhibitory receptor other than an mGluR. However, 1S,3R-ACPD and L-AP4 have been

shown to have no action at GABA receptors or adenosine receptors; the two other major inhibitory receptors that modulate glutamate release. Unfortunately, there is a lack of electrophysiological evidence in adult rat cerebral cortex slices to back up the present neurochemical findings which suggest that presynaptic inhibitory mGlu-autoreceptors exist on adult glutamatergic nerve terminals in this brain region. It also still remains to be convincingly demonstrated, either neurochemically or electrophysiologically, whether presynaptic mGlu-autoreceptors, like $GABA_B$ heteroreceptors, inhibit glutamate exocytosis via a direct suppression of presynaptic Ca^{2+} currents. Neurochemical assessment of the effects of mGluR agonists on depolarisation evoked Ca^{2+} entry into synaptosomes need to be performed to address this issue.

As with the presynaptic $GABA_B$ heteroreceptors present on glutamatergic nerve terminals, the development of very selective agonists at presynaptic inhibitory mGlu-autoreceptors could yield useful anticonvulsive and neuroprotective agents in the treatment of epilepsy and excitotoxic conditions. This is based on data implicating postsynaptic mGluRs in numerous brain pathologies including limbic seizures and loss of dentate granule cells through a synergistic interaction with NMDA receptors and Ca^{2+} influx (Schoepp and Conn, 1993). Indeed, one of the physiological roles of presynaptic inhibitory mGlu-autoreceptors in the cerebral cortex may be neuroprotection from cerebral ischaemic conditions that can occur in this brain region. In fitting with this proposal, group II mGlu2 and mGlu3 mRNA and group III mGlu4 and mGlu7 mRNA is abundant in the cerebral cortex and a significant proportion could encode mGluRs that are targetted to the presynaptic digit to function as neuroprotective agents amongst other important cellular roles. Receptor localisation studies at an ultrastructural level may provide definitive information as to the specific regions of the nerve terminal that mGluRs may be found. It has recently been shown in the hippocampus that group III mGlu7 receptors are located at presynaptic active zone regions, presumably to play a role in the negative feedback regulation of glutamate exocytosis (Shigemoto et al., 1996).

In summary, the present studies show that glutamate exocytosis can be modulated by a number of presynaptic receptors. Neurochemical data has been presented in this thesis which strongly suggest that glutamate autoreceptors, both

facilitatory and inhibitory, are functionally present on adult rat cerebrocortical nerve terminals, as well as inhibitory $GABA_B$ heteroreceptors. It is known that protein phosphorylation/dephosphorylation is a major post-translational modification pathway used by cells to alter the behaviour of a protein. The major classes of serine and threonine protein kinase - Ca^{2+}/Calmodulin-dependent-, Ca^{2+}/phospholipid-dependent- and cyclic AMP-dependent kinases, are present in nerve terminals (Sihra and Nichols, 1993). They effect the phosphorylation of a number of nerve terminal proteins upon depolarisation with KCl or 4AP *in vitro* (Sihra, 1993), and their activity, *in situ* within synaptosomes, can be determined by performing nerve terminal protein phosphorylation studies. In future work, such phosphorylation studies could be used to examine whether the positive- and negative-feedback regulation of glutamate release, observed through non-NMDA receptor agonists and mGluR/$GABA_B$ receptor agonists respectively, can be modulated by changes in kinase activity brought about by activators and inhibitors of the aforementioned kinase cascades. This would provide evidence of direct or indirect regulatory effects of protein phosphorylation/dephosphorylation on the receptor-mediated modulation of glutamate release.

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