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EXCITATORY AMINO ACID-EVOKED RELEASE OF ENDOGENOUS ADENOSINE
FROM RAT NEOCORTEX

by

Katja Hoehn, M.D.

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
at
Dalhousie University
Halifax, Nova Scotia
September, 1990

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This thesis is dedicated to my parents, Ingrid and Erwin Hoehn, and my brother, Felix Hoehn.

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ABSTRACT

Excitatory amino acids (EAAs) are neurotransmitters in the neocortex; they have also been implicated in excitotoxic neuronal death during hypoxia/ischemia, hypoglycemia, and seizures. It has been suggested that the inhibitory neuromodulator, adenosine, protects against EAA-mediated neurotoxicity. A: Both K^+ and glutamate released adenosine from cortical slices; NMDA, kainate and quisqualate also evoked receptor-mediated release of adenosine. K^+ -evoked adenosine release was diminished in Ca^{2+} -free medium, while EAA agonist-evoked release was not, although kainate appeared to release adenosine from separate Ca^{2+} -dependent and independent pools. K^+ -evoked adenosine release was partly mediated indirectly through the release of an endogenous EAA acting at NMDA receptors; [3H]NA release was not. NMDA was 33 times more potent at releasing adenosine than [3H]NA. NMDA-evoked [3H]NA release was abolished by TTX, indicating that action potentials were essential; TTX only decreased adenosine release by 35%. Block of adenosine release by the uncompetitive antagonist, Mg^{2+} , was overcome by high concentrations of NMDA; block of [3H]NA release was not overcome by high NMDA concentrations. These results suggest that maximal adenosine release requires activation of only a small fraction of available NMDA receptors (i.e. there are spare receptors for NMDA-evoked adenosine release). Released adenosine may not be neuroprotective against NMDA-mediated excitotoxicity; however, it may provide an inhibitory threshold that must be overcome before NMDA-mediated neurotransmission can proceed maximally. Adenosine may help to maintain the selectivity of NMDA-mediated processes. B: The EAA receptors mediating adenosine release are not located on presynaptic terminals because EAA agonists did not release adenosine from cortical synaptosomes. However, L-glutamate did release adenosine but this was mediated by the Na^+ -dependent transport of glutamate into the synaptosomes and not by EAA receptors. Unlike cortical slices, adenosine released from synaptosomes by glutamate was derived from a released nucleotide (not ATP or cyclic AMP). This non-receptor-mediated process was not observed in intact cortical slices, possibly because it is overwhelmed by receptor-mediated release. Nevertheless, this adenosine arises directly from glutamatergic nerve terminals so that it would be in an appropriate location to act at inhibitory presynaptic receptors and inhibit further release of glutamate.

LIST OF ABBREVIATIONS

ACPD: trans-1-amino-cyclopentyl-1,3-dicarboxylate

ADP: adenosine 5'-diphosphate

AMP: adenosine 5'-monophosphate

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANAPP₃: arylazido aminopropionyl ATP

ANOVA: analysis of variance

APB: 2-amino-4-phosphonobutyric acid

APH: 2-amino-7-phosphonoheptanoic acid

APV: 2-amino-5-phosphonovaleric acid

ATP: adenosine 5'-triphosphate

CHA: N⁶-cyclohexyladenosine

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CNS: central nervous system

CPP: 3-((-)-2-carboxypiperazin-5-yl)propyl-1-phosphonate

cyclic AMP: cyclic adenosine 3',5'-adenosine monophosphate

DGG: γ -D-glutamyl-glycine

DNQX: 6,7-dinitroquinoxaline-2,3-dione

DPCPX: 1,3-dipropyl-8-cyclopentylxanthine

dpms: disintegration per minute

EAA: excitatory amino acid

EGTA: ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid

g: gram

G-protein(s): GTP binding protein(s)

GABA: γ -amino-butyric acid

GMP: guanosine 5'-monophosphate

HA-966: 1-hydroxy-3-aminopyrrolidone-2
HPLC: high performance liquid chromatography
5-HT: 5-hydroxytryptamine
IBMX: 3-isobutyl-1-methylxanthine
LTP: long-term potentiation
MK-801: (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
maleate
min: minute(s)
NECA: 5'-N-ethylcarboxamidoadenosine
NMDA: N-methyl-D-aspartate
NA: noradrenaline
NO: nitric oxide
PCP: phencyclidine
PI: phosphatidylinositol
PIA: N⁶-1-phenyl-2-propyladenosine
PNS: peripheral nervous system
pS: picosiemens
s: second(s)
SNK: Student-Newman-Keuls
TTX: tetrodotoxin

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INTRODUCTION

The past few years have witnessed an explosion of knowledge concerning excitatory amino acid (EAA) neurotransmitters. It is now recognized that the amino acid, glutamate, is the major excitatory neurotransmitter in the vertebrate CNS (Watkins and Evans, 1981; Collingridge and Lester, 1989; Watkins et al., 1990). Acting at specific N-methyl-D-aspartate (NMDA) and non-NMDA EAA receptors, glutamate directly depolarizes neurons. Non-NMDA receptors mediate fast excitatory neurotransmission at most central synapses (Collingridge and Lester, 1989). NMDA receptors, on the other hand, possess unique pharmacological and physiological properties and play pivotal roles in synaptic plasticity in the developing and mature CNS (Mayer and Westbrook, 1987; Cotman and Iversen, 1987). Recent insight into NMDA receptor involvement in memory and learning has generated enormous interest. Although EAA receptors are critically involved in normal brain functioning, their overstimulation can mediate neuronal cell death (Rothman and Olney, 1987; Koh et al., 1990). In fact, EAA receptors have been implicated in such diverse neuropathologies as hypoxic/ischemic and hypoglycemic damage, seizure disorders, trauma, and neurodegenerative disorders such as Huntington's and Alzheimer's diseases (Rothman and Olney, 1987; Faden et al., 1989).

The inhibitory neuromodulator, adenosine, has also received considerable attention (Dunwiddie, 1985; Snyder, 1985; Stone, 1989). Adenosine, acting at specific extracellular adenosine A₁ and A₂ receptors both pre- and post-synaptically, exerts a number of important inhibitory effects in the CNS. These include anticonvulsant, sedative, and antinociceptive actions (Maitre et al., 1974; Dunwiddie and Worth, 1982;

Sawynok et al., 1989). Of particular relevance for the present study, adenosine inhibits the firing of central neurons (Phillis et al., 1975) and decreases the release neurotransmitters, including glutamate and aspartate (reviewed by Phillis and Wu, 1981; Fredholm and Dunwiddie, 1988). Moreover, the central stimulatory actions of the methylxanthines, attributed largely to their ability to block adenosine receptors (Fredholm, 1980; Daly et al., 1981), suggest that adenosine exerts a continuous inhibitory tone on central neurons. It has recently been suggested that adenosine may also act as an endogenous antiexcitotoxic agent against EAA receptor-mediated toxicity (Dragunow and Faull, 1988).

This thesis is concerned with EAA-evoked release of endogenous adenosine in the neocortex. The INTRODUCTION will briefly outline current knowledge of EAAs and adenosine in the CNS, highlighting aspects relevant to the present study. Special emphasis will be placed on the neocortex, on adenosine release, and on the interactions between EAAs and adenosine. For further details the reader may refer to recent reviews of adenosine (Dunwiddie, 1985; Snyder, 1985; Daly, 1985; Fredholm et al., 1987; Williams, 1987, 1989; Fredholm and Dunwiddie, 1988; Dragunow and Faull, 1988; Stone, 1989) and EAAs (Mayer and Westbrook, 1987; Johnson and Koerner, 1988; Cotman et al., 1988; Stone and Burton, 1988; Collingridge and Lester, 1989; Wroblewski and Danysz, 1989; Monaghan et al, 1989; Choi and Rothman, 1990; Watkins et al., 1990; Young and Fagg, 1990, MacDonald and Nowak, 1990).

I. EAAs IN THE CNS

1. Glutamate neurophysiology.

The excitatory amino acids, glutamate and aspartate, occur in high concentrations in brain. In human cerebral cortex, for example, the concentration of glutamate is 8.6-10.8 $\mu\text{mol/g}$ wet tissue weight. (Perry et al., 1971). This high concentration is not surprising in light of the central role of glutamate in metabolic processes including the synthesis of proteins, peptides, purines, fatty acids, γ -aminobutyric acid (GABA), and coenzymes such as glutathione and folic acid (Watkins and Evans, 1981). The concept that glutamate might be a neurotransmitter initially met with resistance because it was difficult to dissociate its role as an intermediary metabolite from its role as a putative neurotransmitter. It has now become recognized that glutamate, which acts at all three of the major EAA receptor subtypes (NMDA, kainate and quisqualate) largely satisfies the requirements for a neurotransmitter (Watkins and Evans, 1981; Fonnum, 1984).

The following section will briefly review some of the evidence supporting a neurotransmitter role for EAAs, particularly glutamate, in the neocortex. The preparations used in the present study, cortical slices and synaptosomes, contain terminals of corticocortical pathways (which may be either association pathways or callosal pathways) and terminals of afferent pathways to the cortex, but not terminals of corticofugal fibres. Thus the following section will focus on evidence supporting a neurotransmitter role for glutamate and/or aspartate in corticocortical pathways and in afferent pathways to the cortex. At the same time some of the techniques which have been used to identify putative

amino acid pathways in other brain regions will be described.

A. Evidence that glutamate is a neurotransmitter in the neocortex.

One criterion for acceptance of a substance as a neurotransmitter is its presynaptic localization (Werman, 1966). A number of strategies have been employed to demonstrate a releasable pool of EAAs in the cerebral cortex. Barbaresi et al. (1987) have shown retrograde labelling of callosal and association neurons in cat sensory cortex with D-[³H]aspartate. This nonmetabolizable analogue is thought to be taken up selectively by axon terminals that release glutamate and/or aspartate, and to be transported in a retrograde fashion to the neuronal soma. An immunochemical technique has been employed to label neurons rich in glutamate. These are presumed to represent a "neurotransmitter pool" of glutamate (Hepler et al., 1988). Conti et al. (1988) have used this antiserum in combination with horse-radish peroxidase to show that a large proportion of association and callosal neurons in the cat somatic sensory areas are immunoreactive for glutamate. They have suggested that these neurons use glutamate as a neurotransmitter.

A second criterion for neurotransmitter classification is the demonstration of release by physiological stimuli (Werman, 1966). Ca²⁺-dependent release of glutamate and/or aspartate has been demonstrated. Cortical slices and synaptosomes release endogenous glutamate and/or aspartate in vitro (Potashner, 1978; McMahon et al., 1989). In vivo release from sensory cortex following peripheral sensory stimulation has also been described (Abdhu-Ghani et al., 1979). Hicks et al. (1985) have shown that glutamate and aspartate are released from visual cortex in vivo following stimulation of corticocortical association pathways to this

area, suggesting that EAAs mediate synaptic transmission within visual corticocortical pathways.

A third criterion requires that the extracellular action of the putative neurotransmitter can be rapidly terminated (Werman, 1966). Glutamate is removed from the extracellular space by a Na^+ -dependent, high-affinity uptake system (see INTRODUCTION, section I.1B). High affinity uptake of glutamate and aspartate into cortical slices and synaptosomes has been demonstrated (Bennet et al., 1972; Benjamin and Quastel, 1976). Further, Fonnum et al. (1981) have demonstrated a small (27%) but significant decrease in [^3H]aspartate uptake into cortical synaptosomes following ablation of the contralateral hemicortex, suggesting that there is an uptake of [^3H]aspartate into terminals from the contralateral cortex.

The final and most fundamental criterion to be satisfied is that responses to the applied neurotransmitter candidate must mimic synaptically evoked responses (Werman, 1966). At first glance, it would appear that identity of action should be easy to demonstrate in the cerebral cortex. The powerful excitatory effects of iontophoretic application of glutamate to cerebral cortex in vivo have been known since 1963 (Krnjevic and Phillis, 1963). Moreover, EAA receptors are particularly abundant in the neocortex. Indeed, the neocortex is one of the three brain regions with the highest density of the NMDA subtype of EAA receptors (Monaghan and Cotman, 1985). Nevertheless, this criterion has been difficult to establish, not only in the cortex, but also in other brain regions. The poor selectivity of antagonists used in early studies and the absence of an antagonist to distinguish between kainate and quisqualate receptors have presented problems. Moreover, this criterion

requires not only so-called pharmacological identity as shown by studies with antagonists, but also a demonstration that the currents activated by the putative transmitter match exactly the synaptically-activated currents. This is difficult to demonstrate due to the mixed agonist profiles of glutamate and aspartate, the avid uptake mechanisms for these amino acids, and the technical difficulties inherent in using voltage clamp techniques to analyze synaptically activated currents.

In spite of these difficulties, evidence that both NMDA and non-NMDA receptors mediate neurotransmission in the neocortex is strong (Thomson et al., 1989a). Both NMDA and non-NMDA antagonists inhibit stimulation-induced synaptic excitation in visual cortex (Hicks et al., 1981; Hicks and Guedes, 1981). Intracellular recording from neocortical slices has revealed that electrical stimulation of white matter evokes an excitatory postsynaptic potential which has all the characteristic features of an NMDA-mediated synaptic potential in terms of its voltage relationship, Mg^{2+} -sensitivity, and sensitivity to antagonists (Thomson et al., 1985). NMDA receptors are involved in the induction of long-term potentiation (LTP; a model for learning and memory) in rat visual and frontal cortex (Artola and Singer, 1987; Sutor and Hablitz, 1989). However, the identity of the endogenous EAA(s) mediating either neurotransmission or LTP is not yet clear. Thomson (1986,1988) concluded that glutamate could be the endogenous transmitter mediating short latency excitatory postsynaptic potentials exhibiting conventional voltage relations, although the endogenous EAA agonists, aspartate, cysteate, or cysteine sulfonate are also possible candidates. The longer latency, NMDA-mediated, excitatory postsynaptic potential may be mediated by the above candidates or by L-

homocysteate (Thomson, 1988; Zeise et al., 1988). The evidence to date primarily favours glutamate as the endogenous neurotransmitter.

B. Glutamate uptake. Glutamate is removed from the extracellular environment by a carrier-mediated active transport process. Both a high-affinity Na^+ -dependent amino acid transport and a Na^+ -independent low-affinity transport have been described (Bennet et al., 1972; 1973). The high-affinity transporter has been characterized extensively (reviewed by Erecińska, 1987). This carrier cotransports glutamate and two Na^+ ions, with K_m and V_{max} values reported to be between 10 and 40 μM and 2-8 nmol/min/mg protein, respectively (Erecińska, 1987). L-Glutamate and L- and D-aspartate are high affinity substrates, while D-glutamate is a poor substrate (Takagaki, 1976; Benjamin and Quastel, 1976). However, the ability of D-glutamate to inhibit the high-affinity uptake of L-glutamate suggests that it does interact with the high-affinity transporter (Benjamin and Quastel, 1976; Takagaki, 1976; Balcar et al., 1977). In their review, Watkins and Evans (1981) suggested that uptake of L-glutamate might cause depolarization. Uptake-mediated depolarization has recently been demonstrated in synaptosomal preparations and is accompanied by stimulation of Na^+, K^+ -ATPase activity and an increase in O_2 consumption (Erecińska, 1989; McMahon et al., 1989). A glial counterpart of the neuronal transporter has also been characterized (Henn et al., 1974; Erecińska and Silver, 1986).

2. EAA receptors.

Actions of EAAs are mediated via specific EAA receptors which have been subdivided on the basis of electrophysiological, pharmacological, and anatomical studies (Watkins and Evans, 1981; Table 1). Until recently,

only three subtypes were recognized and were named after the specific agonists, N-methyl-D-aspartate, kainate and quisqualate (Watkins and Evans, 1981; Fagg, 1985). The NMDA receptor has been most extensively studied, largely due to the availability of selective antagonists. 2-Amino-5-phosphonovaleric acid (APV) and 2-amino-7-phosphonoheptanoic acid (APH), as well as the more recently described and more potent antagonist, 3-((-)-2-carboxypiperazin-5-yl)propyl-1-phosphonate (CPP), competitively antagonize responses to NMDA (Monaghan et al., 1989; Collingridge and Lester, 1989). Uncompetitive antagonists for this receptor include Mg^{2+} , (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), phencyclidine (PCP), and Zn^{2+} (see INTRODUCTION, section I.3). No known antagonists differentiate between the kainate receptor and the classical ionotropic quisqualate (now renamed AMPA) receptor. However, these receptors are blocked by non-selective EAA antagonists such as γ -D-glutamyl-glycine (DGG; Fagg et al., 1986; Mayer and Westbrook, 1987). The quinoxalinediones, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX), were described by Honoré and colleagues (Fletcher et al., 1988; Honoré et al., 1988) and represent the first reasonably potent and selective antagonists for the kainate and the ionotropic quisqualate receptor.

Each of the three classically recognized EAA receptors is directly linked to a receptor-gated ion channel. The kainate- and quisqualate-gated channels are characterized by small primary conductances (5-15 pS) and mediate fast synaptic potentials (MacDermott and Dale, 1987; Collingridge and Lester, 1989). NMDA receptor ligands, in contrast, gate a channel with a primary conductance of about 50 pS. Excitatory

Table 1. Excitatory amino acid receptor classification.*

	NMDA	KAINATE	QUISQUALATE (renamed AMPA)	METABOTROPIC	AP4
Agonists	NMDA	kainate domoate	AMPA quisqualate ibotenate	ACPD quisqualate	L-AP4 (=L-APB)
Antagonists	D-AP5(=D-APV) D-AP7(=D-APH) CGS19755 <u>non-competitive, (glycine site):</u> HA-966 7-chlorokynurenate	CNQX DNQX	CNQX DNQX	---	---
Nonspecific Antagonists	DGG	DGG	DGG	---	---
Channel Blockers	MK-801 PCP Ketamine	---	---	---	---
Ions Conducted	Na ⁺ /K ⁺ /Ca ²⁺	Na ⁺ /K ⁺	Na ⁺ /K ⁺	---	---

*See text for details. Adapted from "Receptor Nomenclature Supplement" to Trends Pharmacol., Elsevier Science Publishers, Ltd., January 1990.

postsynaptic potentials mediated by NMDA are characteristically slow (Winkles et al., 1990). Non-NMDA receptor-associated channels conduct only Na^+ and K^+ , whereas NMDA receptor-associated channels conduct Na^+ , K^+ and Ca^{2+} (MacDermott and Dale, 1987; Mayer and Westbrook, 1987). Although Ca^{2+} may carry only a small component (possibly less than 10%) of the current activated by NMDA (Pumain et al., 1987; Collingridge and Lester, 1989), Ca^{2+} permeability is pivotal in physiological and pathophysiological processes mediated by NMDA receptors.

Quantitative autoradiographic techniques using L- $[\text{}^3\text{H}]$ glutamate (Monaghan et al., 1983) and subtype-specific ligands support the existence of distinct EAA receptor subtypes (Cotman et al., 1987; Young and Fagg, 1990). NMDA and ionotropic quisqualate receptors colocalize in most brain regions, whereas kainate receptors are more abundant in areas not having a high density of NMDA receptors (Cotman et al., 1987). Interestingly, areas which contain a high density of NMDA receptors are those involved in memory and learning and those which are particularly susceptible to ischemic damage (eg. hippocampal CA1 region and neocortex). Kainate receptors are abundant in the hippocampal CA3 region, the inner layers of the cortex, and the hypothalamus and reticular nucleus of the thalamus (Cotman et al., 1987; Young and Fagg, 1990). In the neocortex, NMDA and quisqualate receptors are concentrated primarily in the superficial layers whereas kainate receptors are concentrated primarily in the deeper layers (Cotman and Monaghan, 1986). It may be noted that the hippocampal CA3 region, which contains the highest density of kainate receptors, is the most sensitive to kainate-induced neurotoxicity. In addition, LTP in this region, unlike LTP in other hippocampal regions, is NMDA antagonist-

insensitive and pertussis toxin-sensitive (Ito et al., 1988).

It has recently been recognized that quisqualate activates a receptor coupled to the generation of inositol 1,4,5-triphosphate and the mobilization of intracellular Ca^{2+} , in addition to the classical ionotropic receptor. This receptor, which is linked to phosphatidylinositol (PI) hydrolysis and referred to as the metabotropic receptor, was first described in cultured striatal neurons (Sladeczek et al., 1985) and rat hippocampal slices (Nicoletti et al., 1986). Neocortical slices (Godfrey et al., 1988), cultured astrocytes (Nicoletti et al., 1990), and *Xenopus* oocytes injected with rat brain messenger RNA (Sugiyama et al., 1987; Horikoshi et al., 1989) all express this novel receptor. Sugiyama and colleagues have studied this receptor in injected *Xenopus* oocytes and in cultured hippocampal cells using voltage-clamp and Fura-2 intracellular Ca^{2+} monitoring techniques. They demonstrated that PI hydrolysis and the subsequent mobilization of intracellular Ca^{2+} are mediated directly through a pertussis toxin-sensitive GTP binding protein (G-protein) and are not caused by ion fluxes (Sugiyama et al., 1987; Furuya et al., 1989).

The metabotropic quisqualate receptor has a pharmacological profile different from the classical ionotropic quisqualate receptor. It is not blocked by either the nonselective EAA antagonist, DGG, or by more selective non-NMDA antagonists such as CNQX (Sladeczek et al., 1988). There are no known effective antagonists for this receptor, although L-2-amino-4-phosphonobutyric acid (L-APB) has been reported to block the metabotropic receptor in certain preparations (Schoepp and Johnson, 1988; but see Sugiyama et al., 1989). Other agonists for this receptor include glutamate and ibotenate (Watkins et al., 1990). Monaghan et al. (1989)

have proposed that the metabotropic receptor be named the ACPD receptor in light of the observation that trans-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) is the most selective (McLennan and Lui, 1982) and potent (Desai and Conn, 1990) agonist for this receptor.

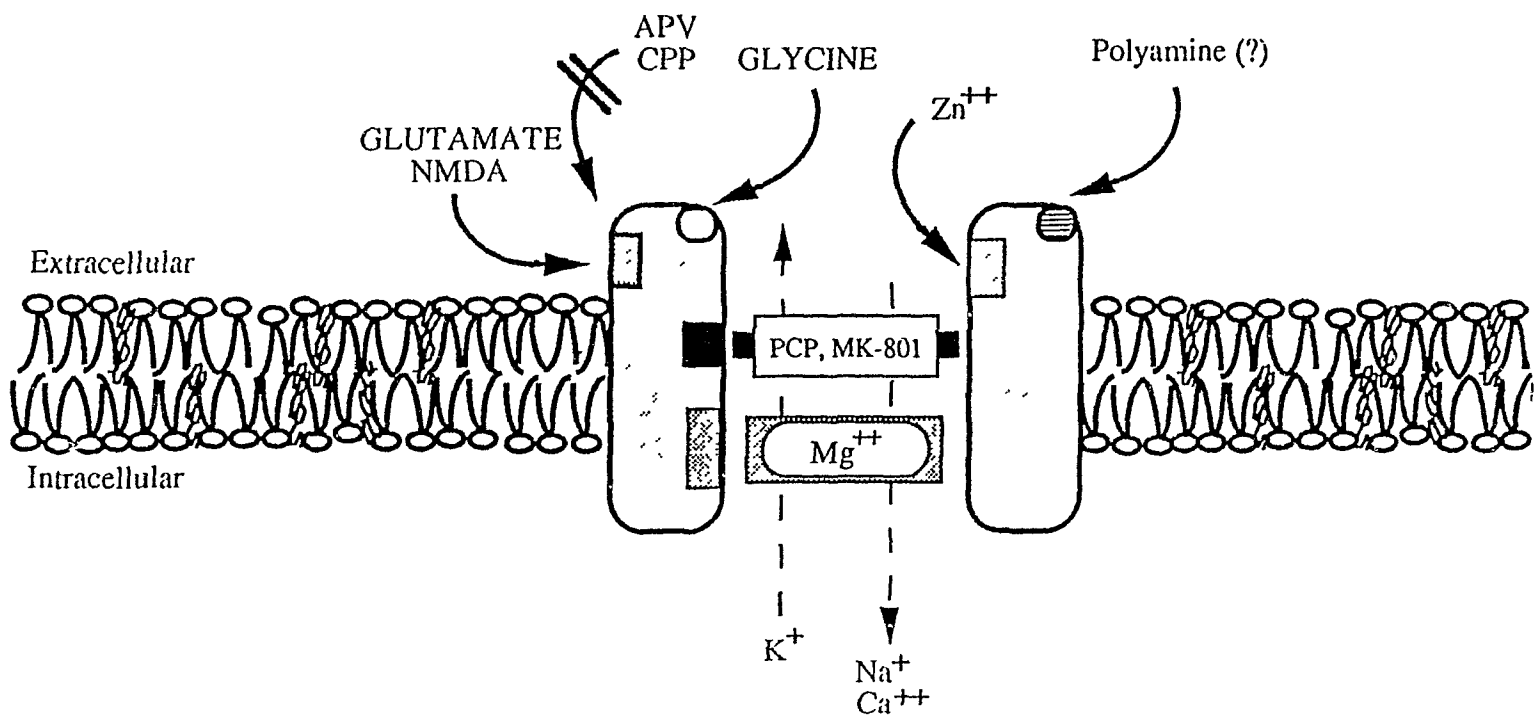
In addition to the four EAA receptors described above, it has been proposed that yet another EAA receptor may exist, which does not recognize the classical EAA agonists, NMDA, kainate and quisqualate. This receptor, named after the glutamate analogue L-APB, is characterized by the ability of L-APB to inhibit excitatory neurotransmission in the spinal cord (Davies and Watkins, 1979, 1982) and hippocampus (Koerner and Cotman, 1981). It has been suggested that the site of action of L-APB is presynaptic because it has no effect on responses to exogenously applied EAA agonists (Collingridge and Lester, 1989). This is supported by the observation that L-APB increases paired-pulse facilitation and decreases the K^+ -evoked release of endogenous glutamate and aspartate but not GABA in the olfactory cortex (Anson and Collins, 1987). It has not been possible to identify the proposed L-APB receptor in binding studies (Young and Fagg, 1990). Although the potent inhibition by L-APB of Cl^- -dependent [3H]glutamate binding was initially thought to delineate the physiologically-described L-APB receptor, it has become clear that Cl^- -dependent, L-APB-sensitive [3H]glutamate binding represents transport sites for glutamate into vesicles and does not correspond to the presynaptic APB receptor (Collingridge and Lester, 1989).

3. Modulatory sites on the NMDA receptor.

The NMDA receptor is a large macromolecular complex possessing multiple regulatory sites. In this sense it has been compared to the

Figure 1. A model of the NMDA receptor. Adapted from Foster and Fagg (1987). See text for details.

Figure 1



GABA-benzodiazepine receptor-ion-channel complex. In addition to the agonist recognition site, the NMDA receptor-ion-channel complex (Fig. 1) includes: (a) a site within the receptor-associated ion channel where physiological concentrations of Mg^{2+} block in a voltage-dependent manner; (b) a site outside the ion channel where glycine, in a strychnine-insensitive manner, acts as a co-agonist for NMDA receptor activation; (c) the "PCP site" within the ion channel, mediating uncompetitive inhibition by PCP and MK-801; (d) a site mediating the antagonistic actions of Zn^{2+} , and possibly; (e) a site through which the polyamines spermine and spermidine enhance NMDA receptor activation. Multiple regulatory sites on the NMDA receptor-ion channel complex may impart the special properties and fine regulation of activation required for learning, memory and synaptic plasticity in neural development (see INTRODUCTION, section I.6). A failure of regulation of NMDA receptor activation at any of these sites could form the basis of neuropathological conditions such as Huntington's disease and Alzheimer's disease (see INTRODUCTION, section III).

A. The Mg^{2+} site. The block of NMDA receptor-associated ion channels by Mg^{2+} has been characterized extensively. Nowak et al. (1984) demonstrated that Mg^{2+} imparts a characteristic voltage-dependence on NMDA conductances. Using patch-clamp techniques, they showed that in Mg^{2+} -free medium, the current-voltage relation of NMDA channels is linear with a reversal potential near 0 mV. In the presence of Mg^{2+} , however, the NMDA current-voltage relation exhibits a distinctive region of negative slope conductance which occurs at voltages more negative than 30-40 mV (Nowak et al., 1984). The Mg^{2+} block is virtually complete at -60 to -70 mV in 1 to 2 mM Mg^{2+} , and is gradually and progressively alleviated upon

depolarization (Ascher and Nowak, 1987). Thus activation of NMDA receptor-mediated conductances requires at least two concurrent processes: (1) the presence of an agonist at the recognition site, and (2) sufficient postsynaptic depolarization to relieve the channel blockade. These two requirements for NMDA receptor-ion channel activation have been proposed to impart "Hebbian" properties on the NMDA receptor (Collingridge, 1987; Cotman et al., 1988; Collingridge and Lester, 1989), because Hebb (1949) originally proposed that synaptic strengthening is contingent upon concurrent pre- and postsynaptic activity. These properties imparted on the NMDA receptor by Mg^{2+} may underlie its pivotal role in various forms of synaptic plasticity (Cotman et al., 1988).

B. The glycine site. The presence of glycine may constitute a third requirement for activation of NMDA receptors. Glycine, via an independent action at a strychnine-insensitive site on the NMDA receptor, increases the frequency of channel opening (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). [3H]Glycine binding correlates with the binding of other ligands for the NMDA receptor but is distinct from that of [3H]strychnine (Young and Fagg, 1990). Glycine has been reported to increase [3H]glutamate binding to the NMDA agonist recognition site (Kessler et al., 1989a; Kloog et al., 1990) and accelerate recovery of NMDA receptor-mediated responses following desensitization (Mayer et al., 1989). Most importantly, there is increasing evidence from electrophysiological and binding studies that glycine is actually a co-agonist at the NMDA receptor and that it is absolutely required for NMDA receptors to be activated (Kleckner and Dingledine, 1988; Kloog et al., 1990). The functional significance of the glycine requirement for NMDA

receptor activation is not clear since synaptic cleft concentrations of glycine are not known. If glycine concentrations are in the micromolar range, glycine sites would be saturated in vivo. Indeed, it has been difficult to demonstrate the effects of exogenous glycine in most in vitro studies (reviewed by Thomson, 1989; but see Thomson et al., 1989b). However, it was recently reported that D-serine, a glycine site agonist, increases cyclic GMP formation in the rat cerebellum in vivo; this effect is blocked by antagonists of the glycine site and by uncompetitive NMDA antagonists (Wood et al., 1989a). These findings suggest that certain strychnine-insensitive glycine sites may not be saturated in vivo and that the glycine requirement for NMDA receptor activation may be functionally significant.

C. The PCP site. Another modulatory site of the NMDA receptor is characterized by the actions of the uncompetitive antagonists, PCP and MK-801. Electrophysiological and neurochemical studies have demonstrated that these molecules are selective uncompetitive antagonists and act at a site within the NMDA receptor-associated channel which is distinct from the Mg^{2+} site (Wong et al., 1986; Lodge and Johnson, 1990). Inhibition of the NMDA channel by these antagonists exhibits limited voltage-dependence and is "use-dependent" in that both onset and termination of the block require the presence of an agonist at the NMDA recognition site (Wong et al., 1986; MacDonald et al., 1987; Lodge and Johnson, 1990). Binding of [3H]MK-801 to the modulatory PCP site is enhanced by NMDA agonists and by glycine and is blocked by NMDA antagonists such as APV (Foster and Wong, 1987; Ransom and Stec, 1988a).

D. The Zn^{2+} site. Another divalent cation, Zn^{2+} , uncompetitively

blocks responses to NMDA (Peters et al., 1987; Westbrook and Mayer, 1987). This action is mediated via a site separate from the Mg^{2+} site which, due to lack of voltage-dependence, is thought to be outside the membrane electric field (Mayer et al., 1988). Although Zn^{2+} antagonizes NMDA responses, it increases responses to quisqualate and kainate (Koh and Choi, 1988). Endogenous Zn^{2+} , which is released from brain tissue in a Ca^{2+} -dependent manner upon stimulation (Assaf and Chung, 1984), may therefore influence which receptor subtypes are preferentially activated by glutamate.

E. The polyamine site. The most recently described modulatory site of the NMDA receptor is the "polyamine site" through which spermine and spermidine enhance [3H]MK-801 binding (Ransom and Stec, 1988a; Williams et al., 1988). The neuroprotective vasodilator, ifenprodil, and the spider toxin, argiotoxin₆₃₆ may act as NMDA antagonists via this site (Lehmann et al., 1990). Preliminary evidence suggests that polyamines enhance the effects of maximal concentrations of glutamate and glycine, but that they are not necessary for NMDA responses (Lehmann et al., 1990). This suggests that, unlike glycine, they are neuromodulators rather than co-agonists.

4. Second messenger involvement in NMDA receptor-mediated effects.

There is little evidence that NMDA receptors in adult brain are directly coupled to second messenger systems. A link between NMDA receptors and G-proteins was initially inferred from the finding that NMDA-displaceable [3H]glutamate binding is reduced by guanine nucleotides (Monahan et al., 1988; Collingridge and Lester, 1989). However, it was subsequently demonstrated that guanine nucleotides are extracellular

competitive inhibitors of NMDA at the receptor recognition site and do not act intracellularly, as would be expected if G-proteins were involved (Baron et al., 1989). NMDA receptors have been linked to a myriad of second messenger effects in the cerebellum. In this region, NMDA receptors enhance cyclic GMP formation, release arachidonic acid, activate phospholipase C, increase the expression of the c-fos proto-oncogene (Wroblewski and Danysz, 1989), and release nitric oxide (NO) (Garthwaite et al., 1988). These effects all appear to be initiated by the influx of cations through the NMDA-associated channel. This is supported by the observation that Mg^{2+} blocks NMDA-activated Ca^{2+} influx, cyclic GMP formation, and arachidonic acid release in cultured cerebellar granule cells (Wroblewski and Danysz, 1989). Similarly, NMDA-stimulated arachidonic acid release from striatal neurons in culture is potently inhibited by Mg^{2+} (Dumuis et al., 1988). Recent evidence suggests that NMDA-stimulated elevation of cyclic GMP is secondary to NO formation, which, in turn, is secondary to Ca^{2+} influx through NMDA receptor-operated ion channels (Bredt and Snyder, 1989; Garthwaite et al., 1989).

In regions of the brain other than the cerebellum, NMDA has a weak stimulatory effect on PI hydrolysis. Maximal reported stimulation of PI hydrolysis by NMDA is 10%, 40%, and 25%, in hippocampal slices (Morrisett et al., 1990), primary cultures of striatal neurons (Sladeczek et al., 1985), and rat forebrain synaptoneurosomes (Recasens et al., 1987), respectively. The significance of this small effect is not clear. In addition to this small stimulatory effect, NMDA inhibits PI hydrolysis stimulated by other agonists. In hippocampal (Baudry et al., 1986) and cortical (Godfrey et al., 1988; Noble et al., 1989) slices NMDA markedly

inhibits PI hydrolysis stimulated by some agonists (eg. carbachol, K^+ depolarization, histamine, and 5-hydroxytryptamine (5-HT) while it either has no effect (Baudry et al., 1986; Noble et al., 1989) or inhibits only slightly (Godfrey et al., 1988) the response to noradrenaline.

The mechanism whereby NMDA inhibits agonist-stimulated PI hydrolysis has been controversial. Godfrey et al. (1988) concluded that the most likely mechanism for NMDA-mediated inhibition of agonist-stimulated PI hydrolysis is neurotoxicity due to excessive cation influx through the NMDA receptor-associated channel. In contrast, initial studies by Gonzales and Moerschbacher (1989a) suggested that this NMDA effect is mediated independently of the NMDA receptor-associated ion channel in so far as it appeared at first to be insensitive to Mg^{2+} . The most compelling explanation, put forth by Morrisett et al. (1990), is that NMDA-mediated inhibition of carbachol-stimulated PI hydrolysis is a direct result of depolarization due to the influx of Na^+ via the NMDA receptor-associated channel and that it is not a result of neurotoxicity. Their explanation is based on the following observations: (1) Na^+ but not Ca^{2+} ions are required for the NMDA-mediated inhibition of PI turnover (Baudry et al., 1986; Gonzales and Moerschbacher, 1989b; Morrisett et al., 1990); (2) Mg^{2+} and several structurally distinct ligands of the PCP site within the NMDA receptor-associated ion channel block the inhibitory effect of NMDA (Gonzales and Moerschbacher, 1989b; Morrisett et al., 1990); (3) tetrodotoxin (TTX) does not block NMDA inhibition (Morrisett et al., 1990); (4) K^+ or veratridine-induced depolarizations in the presence of NMDA antagonists mimic the effect of NMDA (Morrisett et al., 1990); and (5) concentrations of NMDA required to depolarize, as determined by

reversible inhibition of electrophysiological responses to Schaffer collateral stimulation, correspond to those which inhibit carbachol-stimulated PI hydrolysis (Morrisett et al., 1990). Taken together, these results strongly suggest that the influx of Na^+ through NMDA receptor-associated channels depolarizes neurons and that this inhibits stimulated PI hydrolysis. The reversibility of electrophysiologic effects produced by the same concentrations of NMDA and the lack of a Ca^{2+} requirement preclude the possibility that the inhibition of PI hydrolysis is a neurotoxic effect of NMDA. Although the significance of the inhibition of agonist-stimulated PI hydrolysis by NMDA not yet clear, one study has demonstrated that this effect is enhanced following kindling (Morrisett et al., 1989).

5. Evidence for NMDA receptor heterogeneity.

There is considerable interest in the possibility that there may be subtypes of NMDA receptors. Although independent studies have presented several lines of evidence for NMDA receptor subtypes, a correspondence between the subtypes characterized by different groups has yet to be established.

Stone and colleagues (Perkins and Stone, 1983b; Stone and Burton, 1988) proposed the subdivision of NMDA receptors into NMDA1 and NMDA2 subtypes on the basis of regional differences in the relative potency of quinolinate and NMDA to act as agonists and in the ability of kynurenate to act as an antagonist. They suggested that quinolinate acts primarily on NMDA2 receptors in the neocortex, striatum, and hippocampus, and that NMDA1 receptors in the spinal cord and cerebellum are relatively insensitive to quinolinate (Perkins and Stone, 1983b). However,

Collingridge and Lester (1989) have raised a number of potential problems with this subdivision of NMDA receptors. Primarily, they point out that quinolinate hyperpolarizes spinal cord but not cortical neurons via an action not mediated by NMDA receptors (Martin and Lodge, 1985). If quinolinate is indeed a mixed agonist, then the subtype classification proposed by Stone and his colleagues may be invalid.

A second subtype classification, put forward by Costa and his colleagues (Fadda et al., 1987; reviewed by Wroblewski and Danysz, 1989), divides NMDA into ionotropic (G_{o1}) receptors which mediate increases in cerebellar cyclic GMP formation and "metabotropic" (G_{p1}) receptors which mediate PI hydrolysis in the cerebellum. The proposed "metabotropic" NMDA receptor (G_{p1}) is distinct from the quisqualate metabotropic receptor in that it is inhibited by Mg^{2+} , APV, and PCP. These authors suggest the "metabotropic" NMDA receptor is also different from the ionotropic NMDA receptor on the basis of selective enhancement of "metabotropic" but not ionotropic NMDA responses by Ni^{2+} and Co^{2+} (Fadda et al., 1987; Wroblewski and Danysz, 1989). Further evidence is needed to support a distinction between the NMDA receptor which mediates ion fluxes and the NMDA receptor which increases PI hydrolysis in the cerebellum.

The most compelling evidence to date for NMDA receptor heterogeneity was presented by Monaghan et al. (1988a, 1988b). This group showed that the distribution of NMDA-displaceable [3H]glutamate binding is not identical to the distribution of binding of the competitive antagonist, [3H]CPP. [3H]Glutamate binding is higher in the striatum and septum, whereas [3H]CPP binding is higher in the inner cerebral cortex and thalamus (Monaghan et al., 1988a). In agreement with this, agonists are better

displacers of [^3H]glutamate binding in striatum and septum, and competitive antagonists are better displacers in the cortex and thalamus. Interestingly, glycine also exhibits a difference between these anatomical regions. In antagonist-preferring regions (cortex and thalamus) glycine causes a greater percent increase in [^3H]glutamate binding than it does in agonist-preferring regions (striatum and septum; Monaghan et al., 1988a). While glycine increases agonist binding it decreases antagonist binding. These observations present two possibilities. It is possible that there are distinct NMDA receptor populations. Another interpretation, preferred by Monaghan and colleagues, is that the NMDA receptor has interconvertible conformations, with glycine converting the antagonist-preferring to the agonist-preferring conformation (Monaghan et al., 1988a, 1988b, 1989).

Preliminary results of a number of groups (reviewed by Lehmann et al., 1990) suggest that analogous interconvertible agonist- and antagonist- preferring sites may exist for the glycine recognition site. Each conformation may correspond to a specific conformation of the glutamate site. Many additional questions are raised by these preliminary findings. Ultimately, the question of whether distinct subtypes of NMDA receptors exist, or whether differences in binding sites represent alternative conformations of the gene products, will likely be solved using molecular biological approaches.

6. Synaptic plasticity and the NMDA receptor.

NMDA receptors are thought to be critically involved in memory acquisition and developmental plasticity. A fundamental role for NMDA receptors in normal CNS development is suggested by the ability of NMDA antagonists to block the ocular dominance shift in kitten visual cortex

(Kleinschmidt et al., 1987; Rauschecker and Hahn, 1987). Moreover, NMDA receptors are crucial for the induction of LTP, an increase in synaptic efficacy that is thought to underlie learning and memory (Collingridge and Bliss, 1987). NMDA antagonists block the induction of LTP in hippocampal slices (Harris et al., 1984) and intraventricular administration of the NMDA antagonist, APV, prevents both the induction of LTP and learning of a spatial discrimination task (Morris et al., 1986).

Another form of enhanced synaptic activity involving NMDA receptors is kindling. Kindling refers to the progressive development of seizure activity in response to repeated electrical stimulation of certain brain regions at an initially subconvulsant level (Goddard et al., 1969). Following kindling, a previously-absent NMDA receptor-mediated component of transmission can be detected in the dentate gyrus (Mody and Heinemann, 1987). It is interesting that NMDA receptors express the enhanced synaptic efficacy in kindling. This contrasts with LTP, where NMDA receptors are critically involved in the induction of LTP, but where the enhanced synaptic efficacy is expressed entirely by non-NMDA receptors (Muller et al., 1988).

7. Kainate and quisqualate interactions.

Since Watkins and Evans (1981) outlined the basis for distinguishing between the so-called "non-NMDA" receptors, evidence that kainate and quisqualate act at distinct receptors has continued to accumulate. There are striking differences between the regional distribution (see INTRODUCTION, section I.2) and the selectivity of [^3H]kainate and [^3H] α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) binding. The agonist order of potency for displacing [^3H]kainate binding is domoate >

kainate > quisqualate > glutamate, whereas for [^3H]AMPA binding it is AMPA = quisqualate > glutamate > kainate (Young and Fagg, 1990). The molecular target sizes for [^3H]AMPA and [^3H]kainate binding sites are 51.6kD and 76.6kD respectively (Honoré and Nielsen, 1985; Honoré et al., 1986). Moreover, differences in electrophysiological responses to kainate and quisqualate have been reported (eg. Perouansky and Grantyn, 1989; Agrawal and Evans, 1986).

While evidence for differences in kainate and quisqualate binding sites and electrophysiological responses has accumulated, complex interactions between kainate and quisqualate have been recognized. At low concentrations, quisqualate depresses kainate-induced currents (Kiskin et al., 1986; Mayer and Vyklicky, 1989; Perouansky and Grantyn, 1989; Rassendren et al., 1989) and kainate-induced neurotransmitter release (Gallo et al., 1989; Pin et al., 1989). There is also overlap in the pharmacology of the binding sites. Kainate inhibits [^3H]AMPA binding with relatively high affinity, and quisqualate (but not AMPA) inhibits [^3H]kainate binding (Monaghan et al., 1989). Studies with the new non-NMDA antagonists, DNQX and CNQX, present another apparent paradox. These antagonists are approximately five times more potent at inhibiting [^3H]AMPA binding than [^3H]kainate binding, and yet they are equipotent in inhibiting electrophysiological responses to kainate and quisqualate (Honoré et al., 1988).

A number of hypotheses have been put forth to explain the interactions between kainate and quisqualate receptors. Although there is as yet no consensus, the most cogent hypothesis is that kainate acts at two distinct sites, corresponding to the high and low affinity

[³H]kainate binding sites (Monaghan et al., 1989; Collingridge and Lester, 1989; Young and Fagg, 1990). The vast majority of kainate effects observed in biochemical and electrophysiological studies are thought to be mediated by the ionotropic quisqualate receptor and to correspond to the low affinity [³H]kainate binding site (Foster, 1988; Verdoorn and Dingledine, 1988; Monaghan et al., 1989; Young and Fagg, 1990). This hypothesis implies that one receptor mediates the effects of AMPA, the non-metabotropic effects of quisqualate, and most effects of kainate. This is consistent with the ability of DNQX and CNQX to antagonize quisqualate and kainate responses with the same potency. The high affinity [³H]kainate site, according to this hypothesis, mediates only a few select effects of kainate. These effects include the selective, high-affinity activation by kainate of afferent C fibres (Agrawal and Evans, 1986) and the neurotoxic effects of kainate, which are observed most potently in the CA3 hippocampal region, an area enriched in high-affinity [³H]kainate sites (Monaghan et al., 1989; Young and Fagg, 1990).

Although the above hypothesis may explain most experimental findings, a number of questions remain unresolved. For example, it is difficult to reconcile the reported differences in the average unitary conductances activated by kainate and quisqualate (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987) with the suggestion that most kainate responses are mediated through ionotropic quisqualate receptors. Moreover, analysis of whole cell currents activated by kainate and quisqualate in cultured striatal (Tse et al., 1989) and superior colliculus (Perouansky and Grantyn, 1989) has revealed that the ratios of maximal current amplitudes activated by kainate and quisqualate varies

from cell to cell and that the currents activated by these agonists have clearly distinguishable properties. This argues against a common receptor molecule mediating most effects of kainate and quisqualate. Also, if kainate primarily activates quisqualate receptors, how can this be reconciled with the observation that kainate responses characteristically do not desensitize while quisqualate responses do (Kiskin et al., 1986; Mayer and Vyklicky, 1989; Kushner et al., 1988; Perouansky and Grantyn, 1989)? It appears likely that, as knowledge of kainate and quisqualate responses increases, the above hypothesis will require revision.

II. PURINES IN THE CNS

Drury and Szent-Gyorgyi first described the potent inhibitory effect of purines in the cardiovascular system in 1929. The extracellular actions of adenosine did not receive much further attention until Sattin and Rall (1970) observed that the methylxanthines, caffeine and theophylline, acted as antagonists of certain extracellular actions of adenosine in the CNS. Many of the arguments initially raised against glutamate as a neurotransmitter were also brought forth against adenosine. It seemed implausible that such an ubiquitous intermediary metabolite should be released to act extracellularly. Nevertheless, it is now recognized that adenosine is an important inhibitory neuromodulator in the CNS (see reviews cited on p. 2).

1. Sources of extracellular adenosine and ATP.

In order for purines to act extracellularly, they must be released from cells. Extracellular adenosine may originate either from the release of adenosine per se or from the extracellular metabolism of a released

nucleotide (Pearson, 1985; Henderson, 1985). ATP release has been demonstrated in the CNS (see INTRODUCTION, section II.6). In the peripheral nervous system (PNS) there is evidence that ATP may be co-stored and co-released with other neurotransmitters (reviewed by White, 1988). ATP, released either in combination with other neurotransmitters or by itself in the CNS, may act at extracellular P_2 -purinoceptors (see INTRODUCTION, section II.2) or, following metabolism by a series of ecto-nucleotidases (Pearson, 1985; Henderson, 1985), provide a source of extracellular adenosine. There is also some evidence that cyclic AMP may be released from cells in the CNS (Rosenberg and Dichter, 1989) and ecto-phosphodiesterases have been described which could convert released cyclic AMP to adenosine extracellularly (Rosberg et al., 1975; Selstam and Rosberg, 1976).

Alternatively, adenosine may be released per se. Unlike ATP, there is no evidence that adenosine is stored in synaptic vesicles and secreted as such from nerves. Instead, a bidirectional, facilitated diffusional transporter for nucleosides has been described in various tissues including nerves (reviewed by Arch and Newsholme, 1978; Wu and Phillis, 1984; Paterson et al., 1985; Deckert et al., 1988) and cultured astrocytes (Hertz, 1978). Uptake of adenosine via this transporter plays an important role in terminating the extracellular actions of adenosine when the extracellular concentration exceeds the intracellular concentration. Under normal conditions, the intracellular free adenosine concentration has been estimated to be 1-4 nmole/g of tissue (Rubio et al., 1975; Winn et al., 1980). When intracellular adenosine concentrations become elevated, however, the nucleoside transporter appears to facilitate the

efflux of adenosine (Wohlhueter and Plagemann, 1982; Jonzon and Fredholm, 1985; White and MacDonald, in press). Release of adenosine via the nucleoside transporter could provide an important source of extracellular adenosine in situations such as ischemia and hypoxia where intracellular adenosine levels are increased.

The most likely immediate intracellular precursors of releasable adenosine appear to be 5'AMP and S-adenosylhomocysteine, with less likely sources being nucleic acids and adenine (reviewed by Arch and Newsholme, 1978; Nakamura et al., 1983; Phillis and Wu, 1981; Stefanovich, 1988). The dephosphorylation of 5'AMP to adenosine probably predominates under normal conditions, although S-adenosylhomocysteine derived from the demethylation of S-adenosylmethionine may be an important precursor for adenosine during seizures (Dragunow, 1988).

Following uptake, adenosine is either phosphorylated by adenosine kinase to form AMP or deaminated by adenosine deaminase to form inosine (Arch and Newsholme, 1978; Wu and Phillis, 1984). Reported K_m values for adenosine deaminase (between 6 and 60 μM) are higher than those for adenosine kinase (between 0.4 and 5.8 μM ; Arch and Newsholme, 1978); this is consistent with in vitro studies showing that the principal metabolic fate of radiolabelled adenosine in brain slices is its intracellular phosphorylation to nucleotides (reviewed by Wu and Phillis, 1984). Endogenous levels of intracellular adenine nucleotides have been estimated to be 100 to 1000 times greater than those of adenosine (Winn et al., 1980).

2. Classification of purine receptors.

The classification of extracellular purine receptors has been outlined in several recent reviews (Daly, 1985; Stone, 1981a, 1985, 1989; Williams, 1987; White, 1988) and is summarized in Table 2. In 1978 Burnstock proposed the division of purine receptors into P_1 and P_2 subtypes, with P_1 designating a site where adenosine is more potent than ATP and where methylxanthines antagonize, and P_2 designating a site where ATP is more potent than adenosine. P_1 (adenosine) receptors have been further subdivided into A_1 and A_2 receptors on the basis of opposing inhibitory and stimulatory effects on adenylate cyclase, respectively (Van Calker et al., 1979). In light of evidence that adenosine receptors may act independently of the adenylate cyclase effector system, the P_1 receptor classification has recently been revised (Stone, 1985; Hamprecht and Van Calker, 1985) to reflect only the relative potencies of adenosine analogues and the stereoselectivity for the isomers of N^6 -1-phenyl-2-propyladenosine (PIA; see Table 2). Further subclassifications of P_1 receptors have been proposed (Daly et al., 1983; Ribeiro and Sebastiao, 1986; Bruns et al., 1986; Munshi et al., 1988) but these have not yet gained general acceptance.

Difficulties with the A_1 and A_2 subclassification of adenosine receptors have arisen due to observations of different orders of potency of agonists in different tissues (Stone, 1989). Although there are a number of antagonists for adenosine receptors, their usefulness in distinguishing between subclasses has been limited. The classical adenosine antagonists, caffeine and theophylline, do not differentiate between A_1 and A_2 adenosine receptors. Moreover, they have relatively low

Table 2. Purine receptor classification.*

	P ₁ (Adenosine)		P ₂ (ATP)	
	A ₁	A ₂	P _{2X}	P _{2Y}
Potency Order	R-PIA>NECA R-PIA/S-PIA>10	NECA>R-PIA R-PIA/S-PIA<10	α , β methATP> β , γ methATP> >ATP=2meththioATP	2meththioATP>>ATP> α , β methATP= β , γ methATP
Antagonists	caffeine, theophylline 8-phenyltheophylline DPCPX	caffeine, theophylline 8-phenyl- theophylline 15943A	ANAPP3 desensitization by α , β methATP	---
Effector Pathways	cAMP ↓ K ⁺ channel (G protein) Ca ²⁺ channel (G protein) (PI metabolism?)	cAMP ↑	intrinsic cation channel	PI metabolism

*See text for details. Compiled from the following sources: Hamprecht and Van Calker, 1985; Stone, 1985; Burnstock and Kennedy, 1985; White, 1988; "Receptor Nomenclature Supplement" to Trends Pharmacol., Elsevier Science Publishers, Ltd., January 1990.

potencies and exhibit nonspecific effects such as phosphodiesterase inhibition and intracellular Ca^{2+} mobilization (Daly et al., 1981). The use of more potent 8-phenyl-substituted antagonists (Bruns et al., 1983; Jacobson et al., 1986) has made it possible to discriminate between the effects at adenosine receptors and those due to phosphodiesterase inhibition. More selective A_1 and A_2 antagonists have now also been developed. DPCPX (1,3-dipropyl-8-cyclopentylxanthine) is a highly selective A_1 antagonist (Bruns et al., 1987; Lee and Reddington, 1986), whereas the triazoloquinazoline, CGS 15943A, has an approximately 8-fold preference for A_2 over A_1 receptors (Williams et al., 1987; Williams and Jarvis, 1988). These more selective antagonists may help to clarify the subclassification of adenosine receptors. Ultimate elucidation of the nature of adenosine receptor subtypes awaits molecular biological approaches. Advances towards the sequencing of adenosine receptors are exemplified by the recent solubilization, purification, and reconstitution of the A_1 receptor (Nakata, 1989; Linden and Munshi, 1989) and the development of a high-affinity photoaffinity ligand for the A_2 receptor (Barrington et al., 1989).

P_2 receptors have been divided into P_{2x} and P_{2y} subtypes, as proposed by Burnstock and Kennedy (1985) and illustrated in Table 2. This classification is based on the order of potency of ATP and its analogues, the sensitivity to antagonism by arylazido aminopropionyl ATP (ANAPP₃) and desensitization following prior exposure to α,β -methylene ATP. P_{2x} receptors are excitatory and mediate smooth muscle contraction in the vas deferens and urinary bladder of rats and guinea pigs, whereas P_{2y} receptors are inhibitory and mediate relaxation of the guinea pig taenia coli and

the rabbit portal vein (Burnstock and Kennedy, 1985).

3. Actions of extracellular adenosine.

A. Second messengers. Adenosine receptors were originally classified on the basis of inhibition or stimulation of adenylate cyclase (Van Calker et al., 1979; Londos et al., 1980). Although adenosine modulation of adenylate cyclase activity has been extensively studied in the brain (reviewed by Daly, 1985; Stone, 1989), the extent to which changes in cyclase activity mediate the pre- and postsynaptic actions of adenosine in the CNS is not clear (Fredholm and Dunwiddie, 1988; Fredholm et al., 1988). Indeed, there is evidence that the presynaptic (Fredholm and Lindgren, 1987; Dunér-Engstrom and Fredholm, 1988) and postsynaptic (Trussell and Jackson, 1987) inhibitory effects of adenosine are independent of cyclic AMP.

More recently, modulation by adenosine of PI hydrolysis has been demonstrated in the CNS. In the neocortex of the guinea pig and mouse, adenosine analogues potentiate (Hollingsworth et al., 1986) or inhibit (Kendall and Hill, 1988) histamine-H1 receptor-mediated PI hydrolysis, respectively, without affecting responses to noradrenaline and carbachol. The adenosine receptor which mediates inhibition or potentiation of histamine-stimulated PI hydrolysis appears to be distinct from the adenosine receptor which mediates cyclic AMP accumulation (Hollingsworth et al., 1986; Alexander et al., 1989). Although in the cortex adenosine receptor stimulation has no effect on basal PI hydrolysis (Hollingsworth et al., 1986; Kendall and Hill, 1988), adenosine has been reported to inhibit basal PI hydrolysis in striatum (Petcoff and Cooper, 1987; but see El-Etr et al., 1989a). Moreover, adenosine potentiates carbachol- or

noradrenaline-stimulated PI hydrolysis in this region (El-Etr et al., 1989a). 2-Chloroadenosine also potentiates α_1 -adrenergic stimulation of PI hydrolysis in glial cultures derived from the striatum and mesencephalus, but not from cortex (El-Etr et al., 1989b). The physiological significance of these interactions between adenosine and other neurotransmitters on PI hydrolysis is unclear. There is some evidence that protein kinase C may be involved in adenosine-mediated inhibition of neurotransmitter release at the neuromuscular junction (Branișteanu et al., 1989), and it is plausible that PI turnover may be involved in some of the central actions of adenosine.

G-proteins link receptor activation with second messenger systems. There is abundant evidence that A_1 receptors are associated with G-proteins. Thus GTP influences the binding of agonists to A_1 receptors in studies using brain membranes or autoradiography (Goodman et al., 1982; Snyder, 1985). Studies with pertussis toxin, which inactivates certain G-proteins by catalyzing their ADP ribosylation, corroborate the association between A_1 receptors and G-proteins. Pertussis toxin blocks the hyperpolarizing effects of adenosine on hippocampal CA1 pyramidal cells (Fredholm et al., 1989; Stratton et al., 1989; Zgombick et al., 1989), suggesting that this postsynaptic effect of adenosine involves a G-protein. The link between G-proteins and presynaptic adenosine A_1 effects is not as clear. Although pertussis toxin blocks adenosine inhibition of glutamate release in the cerebellum (Dolphin and Prestwich, 1985), it has been reported to inhibit (Stratton et al., 1989) or not affect (Fredholm et al., 1989) adenosine-mediated depression of hippocampal excitatory postsynaptic potentials, a primarily presynaptic

effect. The differences between the findings of these two groups may be accounted for by the higher hippocampal concentration of pertussis toxin achieved by Stratton et al. (1989); this group injected the toxin directly into the hippocampus. It is possible that the presynaptic effects of adenosine are mediated via a G-protein which is less sensitive to pertussis toxin than the G-protein that mediates the postsynaptic effects.

Although evidence for a link between adenosine A_1 receptors and G-protein(s) is strong, the nature of the G-protein(s) associated with A_1 receptors is not clear. It is possible that adenosine A_1 receptors are linked via the same or different G-protein(s) to several different effector systems (Fredholm and Dunwiddie, 1988). By analogy, it has been demonstrated using recombinant biochemical techniques, that one subtype of M2 muscarinic receptor can interact via a pertussis toxin-sensitive G-protein(s) with two different effector systems (Ashkenazi et al., 1987). Adenosine A_1 receptors may be associated with G_i and G_o , G-proteins linked to inhibition of adenylate cyclase (Gilman, 1984), and receptor-linked activation of phospholipase C (Bradford and Rubin, 1986), respectively. They may also be linked to G_o , a G-protein that mediates receptor-linked changes in ion conductances (Dunlap et al., 1987), in so far as adenosine's effects on K^+ (Böhm et al., 1986; Kurachi et al., 1986; Trussell and Jackson, 1987) and Ca^{2+} channels (Scott and Dolphin, 1989; Gross et al., 1989) are pertussis toxin sensitive while they appear to be independent of changes in adenylate cyclase activity.

B. Electrophysiological studies. Electrophysiological studies have been conducted to characterize the ionic currents affected by adenosine. Ca^{2+} , K^+ , Na^+ and Cl^- conductances have all been implicated in adenosine's

actions. Effects of adenosine on Ca^{2+} currents have been established. Adenosine inhibits regenerative Ca^{2+} -dependent action potentials (Ca^{2+} spikes) in the hippocampus (Proctor and Dunwiddie, 1983) and adenosine analogues inhibit somatically recorded Ca^{2+} currents (probably N type) in hippocampal (Madison et al., 1987) and sensory neurons (Scott and Dolphin, 1989; Gross et al., 1989). Adenosine also increases postsynaptic K^+ conductances in the hippocampus (Segal, 1982; Haas and Greene, 1988; Trussell and Jackson, 1985) and striatum (Trussell and Jackson, 1985; 1987) and this effect is mediated by a G-protein independently of changes in adenylate cyclase activity (Trussell and Jackson, 1987). Interestingly, adenosine A_1 receptors mediating the increase in K^+ conductance and A_1 receptors mediating the decrease in adenylate cyclase activity in the striatum appear to share a common pool(s) of pertussis toxin-sensitive G-proteins with $5\text{-HT}_{1\text{A}}$ receptors (Zgombick et al., 1989).

Although adenosine's effects on somatic Ca^{2+} and K^+ currents are well documented, attempts to identify the currents involved in adenosine's presynaptic effects have yielded conflicting results. The initial discovery that adenosine inhibits Ca^{2+} uptake into synaptosomes (Ribeiro et al., 1979; Wu et al., 1982) has not been reproduced in more recent studies (Barr et al., 1985; Dunwiddie, 1985; Michaelis et al., 1988; Garritsen et al., 1989). Michaelis et al. (1988) have suggested that the initial event implicated in presynaptic inhibition produced by adenosine is an increase in the membrane K^+ conductance rather than a primary effect on a Ca^{2+} conductance. Voltage-dependent Na^+ channels have been implicated in that the adenosine analogue, CHA, at high concentrations, inhibits $^{22}\text{Na}^+$ uptake by rat brain synaptosomes (Simoes et al., 1988). Definitive

identification of the conductance changes mediating adenosine's presynaptic actions awaits further study.

Most recently, Cl^- conductances have been added to the list of conductances possibly modulated by adenosine. Schubert and colleagues (Schubert and Mager, 1989; Mager et al., 1989) have observed that adenosine-mediated increases in conductance in cultured hippocampal neurons and synaptic modulation in hippocampal slices both appear to depend on Cl^- ions, in so far as the adenosine effects could be inhibited by replacing Cl^- with gluconate, by blocking the membrane chloride pump, or by blocking chloride channels. It is possible that adenosine may affect a variety of different conductances depending on the tissue and species.

Electrophysiological studies have also addressed the relative importance of the pre- versus the postsynaptic actions of adenosine in mediation inhibition in the CNS. Phillis et al. (1979a) concluded that adenosine acted presynaptically because it did not affect membrane resistance or the threshold for action potential generation in intracellular recordings of neocortical neurons. Subsequent studies in the hippocampus, however, suggested that postsynaptic effects do occur. In this region, relatively high concentrations of adenosine caused a postsynaptic hyperpolarization which was associated with an approximately 30% decrease in input resistance in about 50% of neurons (Siggins and Schubert, 1981; Segal, 1982). Nevertheless, the primary mechanism of adenosine inhibition in the hippocampus appears to be presynaptic, since adenosine depresses hippocampal synaptic responses by 95-100% while reducing responses to exogenous glutamate by only 0-30% (Proctor and

Dunwiddie, 1987). It is possible that, in addition to its recognized pre- and postsynaptic effects, adenosine influences dendritic conductances. Although this possibility was originally dismissed by Proctor and Dunwiddie (1987), Schubert and Mager (1989) have recently presented evidence for adenosinergic "tuning" of dendritic membrane conductances via extrasynaptic receptors.

C. Neurotransmitter release. Adenosine decreases neurotransmitter release in the PNS and CNS. In the CNS, adenosine inhibits the release of noradrenaline (Harms et al., 1978; Jackisch et al., 1985; Fredholm and Lindgren, 1987), dopamine (Michaelis et al., 1979; Harms et al., 1979; Wood et al., 1989b), acetylcholine (Jhamandas and Sawynok, 1976; Harms et al., 1979; Corrieri et al., 1981; Pedata et al., 1983, 1986), and 5-HT (Harms et al., 1979; Feuerstein et al., 1988). Interestingly, adenosine was reported to decrease the release of the inhibitory neurotransmitter, GABA, in some studies (Harms et al., 1979; Hollins and Stone, 1980a) but not others (Dolphin and Archer, 1983; Limberger et al., 1986). Regional differences in adenosine's effects are suggested by the observations that adenosine inhibits 5-HT release in the hippocampus but not in the caudate nucleus (Feuerstein et al., 1988); it also inhibits acetylcholine release from purified striatal but not neocortical cholinergic nerve terminals (Richardson et al., 1987). In contrast, no regional differences have been observed in adenosine's effects on glutamate and aspartate release. Thus adenosine inhibits EAA release from hippocampal slices (Dolphin and Archer, 1983; Corradetti et al., 1984; Fastbom and Fredholm, 1985; Burke and Nadler, 1988), cultured cerebellar granule cells (Dolphin and Prestwich, 1985; Drejer et al., 1987) and synaptosomes from hippocampus

(Hernandez et al., 1989), cerebellum (Clark and Dar, 1989), and neocortex (Arvin et al., 1989). Although the precise intracellular mechanism(s) whereby adenosine inhibits neurotransmitter release is/are not yet clear, the inhibition appears to be mediated via the A₁ receptor subtype and is independent of alterations in adenylate cyclase activity (Fredholm and Dunwiddie, 1988).

D. Behavioral actions. Adenosine and its analogues produce pronounced behavioral effects (reviewed by Barraco, 1985). Injected intraperitoneally, adenosine analogues are anticonvulsant (Maitre et al., 1974; Dunwiddie and Worth, 1982) and provoke sedation, hypotension, hypothermia (Dunwiddie and Worth, 1982; Snyder et al., 1981) and sleep (Radulovacki, et al., 1982). In many cases, methylxanthines alone cause effects opposite to those of adenosine analogues (Snyder et al., 1981), suggesting that endogenous adenosine may exert a tonic inhibitory influence on behaviour. There is compelling evidence that adenosine acts as an endogenous anticonvulsant (reviewed by Dragunow, 1986, 1988). Adenosine and its analogues are also antinociceptive (reviewed by Sawynok and Sweeney, 1989) and adenosine may mediate morphine analgesia at the spinal level (Sawynok et al., 1989).

It has been suggested that many of the behavioral effects of peripherally administered adenosine analogues may be indirectly mediated by actions outside the CNS (Phillis and Wu, 1981). Indeed, Brodie et al., (1987) found that systemic injections of R-PIA, 2-chloroadenosine, or NECA, at concentrations up to 1000 times the concentrations required to observe behavioral effects, failed to depress hippocampal evoked responses in vivo. Moreover, using autoradiography and microdialysis, these authors

demonstrated that peripherally-administered R-PIA does not enter the brain in significant concentrations. Nevertheless, behavioral effects including decreased locomotor activity, anticonvulsant actions and sleep have been observed following central administration of adenosine analogues (Feldberg and Sherwood, 1954; Barraco et al., 1983, 1984), and behavioral effects of peripherally-administered adenosine analogues are generally only blocked by antagonists that cross into the CNS (Nikodijevic et al., 1990).

4. Actions of extracellular ATP.

In contrast to the fairly well-established neurotransmitter role of ATP in the PNS (reviewed by Gordon, 1986; White, 1988), the possible actions of extracellular ATP in the CNS have not received much attention. Holton and Holton (1954) first proposed that ATP might be an excitatory transmitter in excitatory afferents to the spinal cord, but this idea gained little support until the 1970s when Phillis et al. (1975, 1979a) demonstrated that iontophoretically applied ATP excites neocortical neurons. Whereas the central excitation by ATP was once attributed to its chelation of extracellular Ca^{2+} (Krnjevic, 1974), it has now been demonstrated that ATP excites subpopulations of spinal cord neurons (Jahr and Jessel, 1983; Fyffe and Perl, 1984; Salter and Henry, 1985) in a methylxanthine-insensitive manner, suggesting that ATP might mediate low-threshold primary afferent inputs to the spinal dorsal horn.

The mechanism whereby ATP excites central neurons is not clear. Studies in the PNS have determined that excitatory P_{2x} receptors are linked directly to an ion channel, whereas inhibitory P_{2y} receptors modulate an apamin-sensitive K^+ channel (White, 1988, Table 2). In addition, a presumably P_{2y} receptor-mediated, ATP-induced phospholipase C activation

and mobilization of Ca^{2+} has been described (Sasakawa et al., 1989; van der Merwe et al., 1989). In the CNS, ATP-induced PI hydrolysis has been observed in astrocytes where it is thought to mediate the G-protein-sensitive induction of prostaglandin synthesis (Gebicke-Haerter et al., 1988; Pearce et al., 1989). The significance of these actions of ATP on astrocytes is not yet clear. In addition to acting at P_2 purinoceptors, ATP may act as a substrate for an ecto-protein kinase (Ehrlich et al., 1986) or it may act at P_1 purinoceptors following its rapid extracellular metabolism to adenosine.

It has been proposed that extracellular ATP might play a role in long-term alterations in signal transduction mediated by repetitive stimulation (Ehrlich et al., 1988; Wieraszko and Seyfried, 1989). Indeed, Wieraszko and Seyfried (1989) have observed that exogenously applied ATP has a biphasic effect on synaptic efficacy in the hippocampus, causing an increase of up to 100% in the size of the population spike in CA1 at a concentration of 400 nM. These findings raise the possibility that ATP might be involved in synaptic plasticity that occurs during learning and memory. This is still highly speculative, however, particularly in light of the recent report by Stone and Cusack (1989) that stable ATP analogues have no effect on evoked or spontaneous neuronal activity in the same region of the hippocampus.

5. Localization of sites of adenosine action. The distribution of adenosine receptors may indicate specific sites of adenosine action in the CNS. Specific, high-affinity radioligands have facilitated the study of A_1 adenosine receptor distribution in membrane homogenates and tissue sections (Bruns et al., 1980a; Schwabe and Trost, 1980; Lewis et al.,

1981; Goodman and Snyder, 1982). A₂ receptor distribution has been studied by combining the nonselective adenosine agonist, [³H]5'-N-ethylcarboxamidoadenosine (NECA) with pretreatment with the alkylating agent, N-ethylmaleimide (Reddington et al., 1986) or with low concentrations of A₁ receptor agonists to eliminate binding to A₁ receptors (Bruns et al., 1986; Jarvis et al., 1989). These techniques have revealed that the hippocampus, cortex, and cerebellum possess a particularly high density of A₁ receptors, (Lewis et al., 1981; Goodman and Snyder, 1982), whereas A₂ receptors are abundant in the striatum and olfactory tubercle (Reddington et al., 1986; Jarvis et al., 1989).

The association of adenosine receptors with specific neuronal populations has also been investigated. Of particular interest is the finding that adenosine receptors are localized on EAA nerve terminals. In the cerebellum, biochemical (Wojcik and Neff, 1983a) and autoradiographic studies (Goodman et al., 1983) have localized adenosine receptors, probably of the A₁ subtype, to the axon terminals of excitatory granule cells. Adenosine receptors also occur on excitatory projections to the dentate gyrus and superior colliculus in rats, as indicated by a decrease in adenosine receptors in these regions following lesioning of the perforant path (Dragunow et al., 1988), or enucleation (Goodman et al., 1983; Geiger, 1986), respectively. Moreover, there is evidence for the occurrence of A₁ adenosine receptors on terminals of Schaffer collaterals to the CA1 region of the hippocampus, although these may constitute only about 10-30% of A₁ receptors in this region (Onodera and Kogure, 1988; Deckert and Jorgenson, 1988). The presence of adenosine receptors on terminals of these putatively EAA neurons (Canzek et al.,

1981; Fonnum, 1984; Otterson and Storm-Mathisen, 1986) is consistent with the finding that adenosine inhibits glutamate release from synaptosomes prepared from various brain regions (see INTRODUCTION, section II.3C). However, it has not been possible to demonstrate adenosine receptors on certain putative EAA terminals. Cortical ablation does not decrease adenosine receptor binding in the thalamus or striatum (Goodman et al., 1983; but see Alexander and Reddington, 1989) even though cortical projections to these areas are putatively glutamatergic (Fonnum, 1984; Otterson and Storm-Mathisen, 1986).

In contrast to glutamatergic nerve terminals, anatomical studies have not provided evidence for adenosine receptors on monoaminergic nerve terminals. Murray and Cheney (1982) were unable to demonstrate a decrease in N⁶-cyclohexyl[³H]adenosine (CHA) binding sites in hippocampal membranes of rat or guinea pig brain following lesioning of dopaminergic, noradrenergic or serotonergic inputs. Destruction of dopaminergic or serotonergic inputs to the rat neostriatum also does not decrease adenosine A₁ or A₂ receptor binding in this region (Alexander and Reddington, 1989). The lack of anatomical evidence for adenosine receptors on dopaminergic terminals is difficult to reconcile with the finding that [³H]dopamine release from striatal synaptosomes is modulated by adenosine, adenosine analogues, adenosine uptake blockers and by adenosine deaminase (Michaelis et al. 1979). Anatomical and biochemical evidence supports the existence of adenosine receptors on cholinergic nerve terminals in the neostriatum, in that both A₁ and A₂ receptor binding decreases following intrastriatal kainic acid injection (Alexander and Reddington, 1989), and adenosine inhibits acetylcholine release from

purified striatal cholinergic nerve terminals (Richardson and Brown, 1987; Richardson et al., 1987). In addition to their presynaptic localization on some neuronal populations, A₁ adenosine receptors have been localized to postsynaptic (Onodera and Kogure, 1988; Deckert and Jorgenson, 1988) and extrasynaptic regions (Tetzlaff et al., 1987) as well as on glial cells (Hösli and Hösli, 1988). Indeed, the original A₁-A₂ classification was defined using cultured glia (Van Calker et al., 1979).

Adenosine receptor distribution studies have been complemented with studies of the distribution of other putative markers of purinergic function in the CNS. Regional heterogeneity of distribution of adenosine uptake sites has been demonstrated using radiolabelled nucleoside transport inhibitors, [³H]nitrobenzylthioinosine and [³H]dipyridamole (reviewed by Wu and Phillis, 1984; Deckert et al., 1988). Likewise, the distribution of 5'-nucleotidase (Scott, 1967; Schubert et al., 1979; Lee et al., 1986), adenosine deaminase activity (Geiger and Nagy, 1986) and immunoreactivity (Yamamoto et al., 1987; Nagy et al., 1988), and even the distribution of adenosine-containing neurons as determined by immunohistochemistry (Braas et al., 1986), have all been studied. It is uncertain to what extent the distribution of these various markers, particularly the latter two, reflects regions where adenosine acts as a neuromodulator. There are some reports of correlations between putative markers for purinergic systems. For example, the distribution of sites labelled by [³H]nitrobenzylthioinosine correlates with adenosine deaminase immunoreactivity (Nagy et al., 1985). However, reports of poor correlations are also abundant. Thus there is no apparent correlation between A₁ receptor localization and ecto-5'nucleotidase distribution

(Goodman and Snyder, 1982; Lee et al., 1986; Fastbom et al., 1987).

Perhaps, a more direct approach to identifying regions where adenosine might act as a neuromodulator is to study the distribution of adenosine release. The regional distribution of adenosine and ATP release from synaptosomes from various brain regions has been determined (Potter and White, 1980; White and MacDonald, in press). Although K^+ - or veratridine-evoked adenosine release does not correlate with the distribution of other known markers of purinergic systems, basal (unstimulated) adenosine release correlates fairly well with the reported distribution of A_1 receptor binding. Further studies of regional distribution of release, possibly examining neurotransmitter-evoked release from more intact brain preparations, may help to identify regions where endogenous adenosine acts extracellularly.

6. Purine release in the CNS.

A. Evidence for release. In the 1970s, Shimizu et al. (1970), studying the intracellular formation of cyclic AMP following labelling of nucleotide precursors with ^{14}C -adenine, discovered that depolarization with K^+ , ouabain and veratridine increased the release of radiolabel into the medium. This observation led to numerous in vitro studies of depolarization and electrical stimulation-induced release of radiolabelled purines (reviewed by Phillis and Wu, 1981; Stone, 1981a; Dunwiddie, 1985). McIlwain and his colleagues demonstrated Ca^{2+} -dependent release of labelled adenosine derivatives from cortical slices and synaptosomal beds (Pull and McIlwain, 1972; Kuroda and McIlwain, 1974). Subsequently, release was demonstrated in vivo (Sulakhe and Phillis, 1975; Schubert et al., 1976; Jhamandas and Dumbrille, 1980) and in response to a number of additional

stimuli including ischemia (Berne et al., 1974) and exposure to morphine (Phillis et al., 1979b; Stone, 1981b; but see Jhamandas and Dumbrille, 1980) and the EAA agonists, glutamate, aspartate, kainate, NMDA, and quinolinic acid (Pull and McIlwain, 1975; Jhamandas and Dumbrille, 1980; Perkins and Stone, 1983c). Release of radiolabelled purines from glia has also been reported (Caciagli et al., 1988).

In the above studies, the release of radiolabel into perfusates was determined following loading of tissues with radiolabelled adenosine or adenine. The inherent assumption in these studies is that the radiolabelled purine is taken up and released from the same pool and in the same manner as the endogenous substance. There is evidence that the above assumptions may not be correct. Indeed, the concentration and identity of the prelabelling agent as well as the length of loading time may influence the characteristics of release. Thus Jhamandas and Dumbrille (1980) found that the concentration of [^3H]adenine used to load the cortex ($0.2\ \mu\text{M}$ versus $200\ \mu\text{M}$) affected the consistency of L-glutamate-evoked ^3H release, leading them to postulate that a high concentration of [^3H]adenine might label a pool other than the "synaptic" releasable pool. Bender et al. (1981) reported that the release of [^3H]adenine derivatives was Ca^{2+} -dependent following a loading period of 30 s, but was Ca^{2+} -independent following a 15 min loading period. Finally, Pull and McIlwain (1972) found that released radiolabelled purines were of higher specific activity than the collective value for the acid extracted adenine derivatives of the tissue, suggesting the existence of separate intracellular pools of purines. Other disadvantages of studies using radiolabelled purines are that quantitation of release is often precluded

because the specific activities of the releasable pools are not known and the identity of the released purine is difficult to determine (White, 1985a).

More recently, methods have been developed whereby endogenous adenosine release can be determined using reversed phase HPLC with ultraviolet (Fredholm and Sollevi, 1981) or fluorescence (Wojcik and Neff, 1982) detection. Employing the latter technique, Wojcik and Neff (1983b) measured Ca^{2+} -dependent, K^+ -evoked release of endogenous adenosine from striatal slices. Similarly, the release of endogenous adenosine has been characterized in synaptosomal preparations of whole brain (MacDonald and White, 1985) and spinal cord (Sweeney et al., 1987a) and in spinal cord in vivo (Sweeney et al., 1987b). Zetterström et al. (1982), using in vivo microdialysis, estimated the extracellular concentration of endogenous adenosine in the striatum to be 1-2 μM . Similar studies of endogenous adenosine levels in the striatum have revealed marked increases in the extracellular adenosine concentration following hypoxia/ischemia (Zetterström et al., 1982; Hagberg et al., 1986, 1987; Van Wylen et al., 1986), K^+ depolarization (Van Wylen et al., 1986; Ballarín et al., 1987), and hypoglycemia (Butcher et al., 1987). In the neocortex, increased interstitial adenosine concentrations have been demonstrated in hypoxemia (Phillis et al., 1988) and systemic hypotension (Park et al., 1988).

In addition to endogenous adenosine release, the release of endogenous ATP in the CNS has been demonstrated (reviewed by White, 1985a, 1985b). White (1977, 1978) developed a method whereby ATP release can be directly and continuously monitored in vitro using a luciferin-luciferase assay. He demonstrated Ca^{2+} -dependence of K^+ - (but not

veratridine-) evoked release of ATP from synaptosomes from rat brain. Release was subsequently demonstrated in synaptosomes from various brain regions (Potter and White, 1980), from dorsal spinal cord (White et al., 1985) from purified cholinergic nerve terminals (Richardson and Brown, 1987), from neocortex following electrical stimulation in vitro (Wu and Phillis, 1978), and from primary cultures of neurons following depolarization with K^+ or veratridine (Zhang et al., 1988). More recently, Ca^{2+} -dependent release of ATP has been observed during high frequency stimulation of Schaffer collaterals in hippocampal slices (Wieraszko et al., 1989). This release appears to arise presynaptically in that it is not blocked by the non-specific EAA antagonist, kynurenic acid, and is not mimicked by glutamate application.

B. Release as a nucleotide or a nucleoside? In studies in which extracellular adenosine is measured, this adenosine could arise either from adenosine released per se or from the release of a nucleotide which is subsequently metabolized extracellularly to adenosine. In light of the evidence that ATP is released in the CNS (see previous section) and that enzymes capable of converting extracellular ATP to adenosine exist in the CNS (Manery and Dryden, 1979; MacDonald and White, 1985; Nagy et al., 1986), ATP represents a possible source for extracellular adenosine. In addition, cyclic AMP is released from brain (Rosenberg and Dichter, 1989) and extracellular phosphodiesterases have been demonstrated in brain and other tissues (Rosberg et al., 1975; Selstam and Rosberg, 1976; Rosenberg and Dichter, 1989), suggesting that released cyclic AMP may be a source for extracellular adenosine. It is clear that measurement of extracellular adenosine is not, in itself, sufficient to establish that

release of this purine occurs as the nucleoside.

Early studies to address the question of whether adenosine is released as a nucleoside attempted to do so either by comparing the identity of the intracellular labelled purines and the identity of the released labelled purines (Kuroda and McIlwain, 1974), or by comparing the identity of labelled purines released in response to depolarizing stimuli to those released by hypoosmotic shock (Fredholm and Vernet, 1979). In the first instance, Kuroda and McIlwain found that, although nucleotides constituted 53-58% of the labelled purines in guinea pig cortical synaptosomes, more than 87% of the labelled purines released in response to K^+ or electrical depolarization were nucleosides. In the second instance, only 6% of purines released from superfused hypothalamic synaptosomes by depolarization were nucleotides, whereas 75% of purines "released" by hypoosmotic shock were nucleotides. Although these studies have been cited as evidence for nucleoside release (Fredholm and Hedquist, 1980; Phillis and Wu, 1981; Snyder, 1985), they do not resolve the key question of whether metabolism from nucleotide to nucleoside occurs just before or just after release.

More recent studies have addressed this question by characterizing release in the presence of ecto-5'-nucleotidase inhibitors. The first evidence that adenosine itself might be released arose from studies of veratridine-induced accumulation of cyclic AMP in guinea pig cortical slices (Pons et al., 1980). Subsequent studies of in vivo and in vitro K^+ -and veratridine-evoked radiolabelled purine release by Barberis and colleagues (Daval and Barberis, 1981; Barberis et al., 1984) led to similar conclusions. This contrasts with a study of the release of

endogenous adenosine from rat brain synaptosomes by MacDonald and White (1985). These investigators found that, whereas K^+ -depolarization results primarily in nucleoside release, veratridine releases approximately equal amounts of nucleotide and nucleoside. These contrasting observations may be attributed to the characteristics of radiolabelled purine release versus endogenous adenosine release or to species differences. Nevertheless, it is clear that adenosine can, to a certain extent, be released in its own right, and it is likely that the proportion of adenosine released as such depends on the nature of the stimulus.

C. Neuronal and non-neuronal source(s) of released purines.

It is also not clear whether adenosine or nucleotide release in the brain arises specifically from neurons or whether it arises from other cell types. Attempts to identify the neurons from which adenosine is released have met with limited success. Wojcik and Neff (1983b) demonstrated decreased K^+ -evoked release of endogenous adenosine from striatal slices following kainic acid lesioning, suggesting that some of the adenosine release in the striatum arises from intrinsic neurons. Pedata et al. (1989) showed that radiolabelled purine release from cortical slices is decreased following cholinergic or noradrenergic denervation but not following destruction of serotonergic neurons. This suggests that purine release may arise, in part, from cholinergic or noradrenergic inputs to the cortex. However, it is possible that destruction of these inputs affected the handling of the radiolabelled purines at sites other than the degenerated nerves; therefore these results should be interpreted with caution.

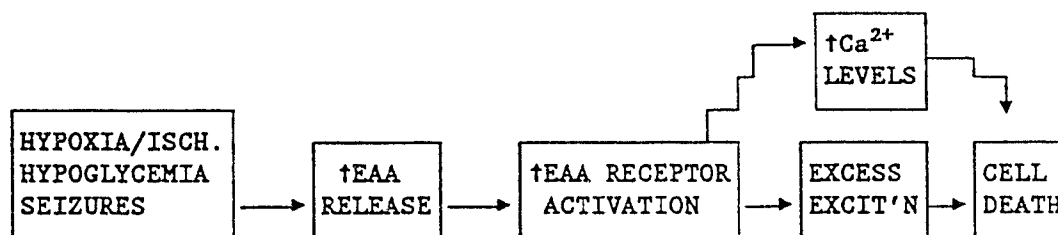
Synaptosomal studies demonstrating the release of adenosine per se

and of ATP presumably from nerve terminals, suggest a neuronal source for released purines (MacDonald and White, 1985; White, 1977, 1978). However, it is possible that contaminating glial elements contribute to release from synaptosomal preparations (Henn et al., 1976). Most of the ATP released from whole brain synaptosomes does not appear to originate primarily from catecholaminergic nerves because release is unaffected when these nerves are destroyed by pretreatment with intraventricular 6-hydroxydopamine (Potter and White, 1982). Richardson and Brown (1987) have shown that ATP is released from affinity-purified cholinergic synaptosomes following exposure to K^+ or veratridine, suggesting that cholinergic nerve terminals do release ATP. However, pretreatment with botulinum toxin A does not appreciably diminish ATP release from whole brain synaptosomes, suggesting that cholinergic nerve terminals contribute only a small fraction of the ATP released from this preparation (White et al., 1980).

Non-neuronal cells also constitute a possible source for released purines. Pearson and Gordon (1979) have demonstrated a selective release of radiolabelled purine derivatives from cultured aortic vascular endothelial cells, raising the possibility that central vascular elements might contribute to purine release. Additionally, Lewin and Bleck (1979) demonstrated the release of radiolabelled purine derivatives from cultured astrocytoma cells. More recently, the uptake (Bender and Hertz, 1986) and electrically stimulated release (Caciagli et al., 1988) of purine derivatives from cultured glial cells has been claimed. Moreover, endogenous adenosine is released from chick glial cells when they are metabolically poisoned (Meghji et al., 1989) and this may reflect a

release that occurs under more physiological conditions. Thus, neither vascular elements nor glial cells can be excluded as possible sources for released adenosine. Further, although release of both adenosine and ATP occurs from presynaptic terminals, the possibility that release also arises from other parts of the neuron has not been excluded. Indeed, it is possible that, unlike the more traditional neurotransmitters, adenosine can be released from cell bodies of neurons, and from glia and vascular elements in the CNS as well as from presynaptic terminals.

III. THE EXCITOTOXIC HYPOTHESIS



1. Evidence for the excitotoxic hypothesis.

The excitotoxic hypothesis is represented schematically above. It was proposed by Olney et al. (1971) who showed that the order of potency of EAA agonists for producing excitation and toxicity were parallel. This hypothesis has now gained widespread acceptance and has evolved to encompass a number of neuropathologies including hypoxia/ischemia, hypoglycemia, epilepsy, and possibly Huntington's and Alzheimer's diseases. It is supported by four main lines of evidence:

1. Extracellular concentrations of EAAs rise markedly during ischemia, hypoglycemia, and convulsions. Extracellular aspartate and glutamate concentrations increase three- and eight- fold respectively

following ischemia (Benveniste et al., 1984). Similar increases in extracellular EAA concentrations occur in hypoglycemic (Wieloch, 1986) and epileptic tissue (eg. Dodd et al., 1980, but see Lehmann et al., 1986). The increase in extracellular EAA concentrations is most likely a result of augmented release, although decreased uptake of glutamate and aspartate into synaptosomes (Silverstein et al., 1986) and glia (Drejer et al., 1985), possibly due to inhibition by released arachidonic acid (Barbour et al., 1989), may also contribute.

11. Exogenous EAAs, acting at both NMDA and non-NMDA receptors induce cytopathology with patterns resembling those observed following ischemia, hypoglycemia or seizures. The hippocampus and neocortex, regions which are particularly susceptible to ischemic and hypoglycemic damage (Wieloch, 1986) and in which seizure activity is readily initiated (Meldrum, 1987), contain much higher densities of EAA receptors than areas less vulnerable (Monaghan and Cotman, 1985; Meldrum, 1987). Exogenously applied EEA agonists cause neuronal cell death by acting at both NMDA and non-NMDA receptors in vivo and in vitro (Sloviter and Dempster, 1985; Siman and Card, 1988; Frandsen et al, 1989; Koh et al., 1990). Central injections of glutamate and aspartate, but not acetylcholine or GABA, mimic the pattern of hippocampal cell death observed in epilepsy and in hypoglycemia (Sloviter and Dempster, 1985; Wieloch, 1986). Although the cytopathologies induced by administration of the various EAA agonists and by ischemia/hypoxia, hypoglycemia, and seizures are similar, there are some differences in the patterns of distribution of toxicity which may be due to differences in the sites of release of EAAs and in the accompanying metabolic changes (Wieloch, 1986).

iii. EAA antagonists are anticonvulsant and block ischemia and hypoglycemia-induced neuronal death. There is a good correlation between the antineurotoxic and antiexcitatory activities of various agents (Olney, 1988). Competitive and uncompetitive NMDA antagonists are anticonvulsant (Meldrum, 1986) and protect in both in vivo and in vitro models of ischemia (Simon et al., 1984; Foster et al., 1988) and hypoglycemia (Wieloch, 1985). In some cases, the simultaneous block of non-NMDA receptors provides additional protection against seizures (Meldrum, 1986, 1988) and against ischemia (Mosinger and Olney, 1989). Although a large number of laboratories have reported protection by NMDA antagonists against ischemia, a number of negative results have also been reported in models of global ischemia (see Choi and Rothman, 1990 for a review).

iv. EAA receptor activation causes intracellular Ca^{2+} accumulation, resulting in Ca^{2+} -mediated damage. Excess loading of Ca^{2+} in neuronal cytoplasm is thought to underlie cell death (Siesjö, 1988). The protective effect of removal of extracellular Ca^{2+} in vitro argues strongly for a role for Ca^{2+} in EAA-mediated toxicity (Choi, 1987; Murphy et al., 1988). There are a number of different mechanisms whereby EAAs can increase intracellular Ca^{2+} . Ca^{2+} entry directly via the NMDA receptor-associated ion channel appears to be the most important (Garthwaite and Garthwaite, 1987; Rothman and Olney, 1987). However, Ca^{2+} influx through voltage-dependent Ca^{2+} channels may contribute to injury following activation of non-NMDA receptors (Murphy et al., 1988; Weiss et al., 1989). Finally, metabotropic quisqualate receptors, which mobilize intracellular Ca^{2+} , may trigger quisqualate-induced neurotoxicity in the hippocampus (Garthwaite and Garthwaite, 1989).

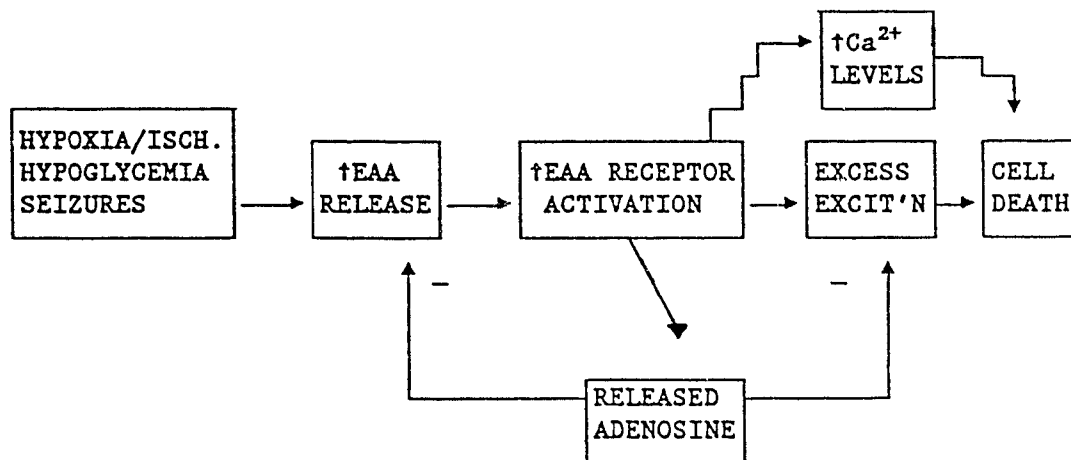
It is possible that other neuropathologies such as neurodegenerative diseases and trauma may also involve EAAs (Maragos, et al., 1987; Faden, 1989). The endogenous NMDA receptor ligand, quinolinate, has been linked with the etiology of Huntington's disease (Rothman and Olney, 1987) and it was recently demonstrated that glutamate elicits antigenic changes in hippocampal neurons comparable to those found in the neurofibrillary tangles characteristic of Alzheimer's disease (Mattson, 1990). NMDA antagonists reduce traumatic brain injury, suggesting that they may be beneficial in the treatment of acute head injury (Faden et al., 1989). Thus EAAs, in addition to their participation in physiological neurotransmission, may mediate a wide range of neuropathologies.

2. Evidence that adenosine is neuroprotective.

It has been proposed that adenosine may act as an endogenous antiexcitotoxic agent (Dragunow and Faull, 1988). In vivo, 2-chloroadenosine, and CHA protect against ischemic cell death in the hippocampus of rats (Evans et al., 1987) and gerbils (Daval et al., 1989), respectively. Conversely, theophylline enhances ischemic cell death in the hippocampus (Rudolphi et al., 1987) while chronic caffeine-induced upregulation of adenosine A₁ receptors decreases ischemic neuronal damage in this region (Rudolphi et al., 1989). In vitro, adenosine and 2-chloroadenosine protect against hypoxia and hypoglycemia-induced injury (Goldberg et al., 1988). Endogenous adenosine may also act as an anticonvulsant, as suggested by the observation that adenosine antagonists prolong seizures and adenosine agonists are potent anticonvulsants (Dragunow, 1988).

As illustrated below, adenosine may act either presynaptically or

postsynaptically to protect against excitotoxicity. A primarily presynaptic action of adenosine is suggested by the observation that adenosine protects against hypoxia- or ischemia-induced cortical neuronal injury in vitro, but not against the toxicity of exogenously applied glutamate (Goldberg et al., 1988). However, adenosine analogues do protect against kainate and quinolinate-induced injury in vivo (Arvin et al., 1989; Connick and Stone, 1989). Thus, although presynaptic effects of adenosine on neurotransmitter release likely predominate, postsynaptic effects of adenosine via inhibition of neuronal firing and decreased Ca^{2+} entry may be important mechanisms of protection in vivo.



Excitotoxicity occurs under conditions in which the supply of O_2 and/or glucose is limited. In addition to adenosine's pre- and postsynaptic effects on neurons to decrease the demand of compromised tissue for O_2 and glucose, adenosine might also protect by increasing O_2 and glucose delivery. Via adenosine A_2 receptors, presumably on astrocytes, adenosine increases glycogenolysis in mouse cerebral cortex slices (Magistretti et al., 1986). A_2 receptors may also mediate

vasodilatation (Berne et al., 1974) and antithrombosis (Fredholm and Sollevi, 1987) which would promote blood flow to the hypoxic/ischemic region. Therefore, adenosine released during metabolic stress may, through a variety of mechanisms, restore the balance between energy supply and demand.

IV. RESEARCH PROPOSAL

The present study was undertaken in order to address the following questions:

A. General questions concerning adenosine release. (i) What is the time course of endogenous adenosine release evoked by K^+ and EAA agonists? In light of the report that radiolabelled purine release occurs following rather than during exposure to K^+ (Hollins and Stone, 1980b), a clear determination of the time course of adenosine release evoked by K^+ and EAAs was essential. To this end, fractions were collected at intervals of 2.5 min. (ii) Is the adenosine released by K^+ and EAA agonists derived from the extracellular conversion of a released nucleotide or from the release of adenosine itself? Studies were conducted with inhibitors of ecto-5'nucleotidase to determine whether adenosine is released as such or whether released adenosine is derived from the extracellular metabolism of a released nucleotide (see also INTRODUCTION, section II.6.B). (iii) Is EAA- and K^+ -evoked adenosine release from slices Ca^{2+} -dependent? Ca^{2+} -dependence was assessed in order to compare the release of adenosine with the release of classical neurotransmitters.

B. Questions concerning adenosine release evoked by EAAs. The study of EAA-evoked adenosine release has both physiological and

pathophysiological relevance. The important role of EAAs in excitatory neurotransmission in the neocortex and the evidence that adenosine may act as an inhibitory neuromodulator in this region have been outlined. The possible pathophysiological relevance of EAA-evoked adenosine release was presented in section III of the INTRODUCTION. Specific questions concerning EAA-evoked adenosine release were: (i) Do glutamate, NMDA, kainate and quisqualate release endogenous adenosine from rat cortical slices? The time course and total amount of adenosine release evoked by these agonists were determined. (ii) Is release by various EAA agonists mediated through EAA receptors? The involvement of EAA receptors was assessed using appropriate available antagonists. (iii) Does adenosine release evoked by K^+ and EAA agonists depend on propagated action potentials? TTX-sensitivity of EAA-evoked adenosine could determine the role of action potentials in the release. (iv) Do EAAs release adenosine by acting at EAA receptors on nerve terminals? This question was addressed in studies of EAA-evoked release of adenosine from cortical synaptosomes.

MATERIALS

I. ANIMALS

Male Sprague-Dawley rats weighing 225-250 g were supplied by Canadian Hybrid Farms, Kentville, Nova Scotia. They were housed in groups of 2-6 at the Animal Care Facilities of the Sir Charles Tupper Medical Building, Dalhousie University, for at least six days prior to use. Animals were maintained on a light:dark cycle of 12hr:12hr and fed standard rat chow and tap water ad lib.

II. DRUGS AND CHEMICALS

Adenosine, α,β -methylene adenosine 5'-diphosphate (α,β -methylene ADP) (sodium), D,L-APB, D,L-APV, D-aspartic acid, adenosine 5'-triphosphate (ATP) (disodium), cyclic adenosine 3',5'-adenosine monophosphate (cyclic AMP) (sodium), bovine serum albumin, choline chloride, dihydrokainic acid, dipyridamole, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), L-glutamic acid, D-glutamic acid, DGG, guanosine 5'-monophosphate (GMP) (sodium), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-isobutyl-1-methylxanthine (IBMX), kainic acid, LiCl, crystalline D-luciferin, firefly lantern extract (FLE-50), NMDA, 1-octanesulfonic acid, quisqualic acid, and Tris (hydroxymethyl)aminomethane (Trizma Base) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chloroacetaldehyde was purchased from ICN Biomedicals Inc. (Plainview, N.Y., U.S.A.), TTX from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.), DNQX from Tocris Neuramin (Essex, U.K.), and MK-801 from Research Biochemicals (Natick, MA, U.S.A.).

L-Noradrenaline [$7\text{-}^3\text{H}$], Protosol, Aquasol-2 and Econofluor were purchased from Du Pont-NEN Canada Inc., (Markham, Ont., Canada). All other chemicals were supplied by British Drug Houses (Dartmouth, Nova Scotia, Canada) including NaCl, KCl, MgCl_2 , CaCl_2 , NaHCO_3 , NaH_2PO_4 , Na_2CO_3 , NaOH, $\text{Ba}(\text{OH})_2$, ZnSO_4 , acetic acid (glacial), acetonitrile, Folin and Ciocalteu reagent, glucose, potassium sodium tartrate, sodium acetate and sucrose.

III. PREPARATION OF SOLUTIONS

Solutions were prepared in distilled and Millipore-filtered H_2O . Drugs added to slice or synaptosomal preparations were dissolved in Krebs-Henseleit medium. MK-801 was initially dissolved in ethanol which was diluted 1000-fold with Krebs-Henseleit medium. In experiments with MK-801, controls were exposed to an identical concentration of ethanol.

METHODS

I. INCUBATED SLICES

1. Isolation of parietal cortex.

Rats were decapitated with a guillotine and their brains were rapidly removed to ice-cold Krebs-Henseleit bicarbonate medium containing 111 mM NaCl, 26.2 mM NaHCO₃, 1.2 mM NaH₂PO₄, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, and 11 mM glucose gassed with 95%O₂-5%CO₂ to maintain a pH of 7.4. During dissection, brains were continuously bathed in ice-cold Krebs-Henseleit bicarbonate medium. Brains were initially divided with a razor blade into three transverse sections as described by Glowinski and Iversen (1966) using the optic chiasm and mamillary bodies as guides (Fig. 2). The lower portion of the middle section was removed by a horizontal cut at the level of the rhinalis fissure according to the method of H. Robertson (personal communication). The remaining section was divided into right and left halves from which the outer 1-1.5mm portion of cortex including both grey and white matter but not including noncortical tissue, was sliced with a recessed tissue slicer. This yielded sections of parietal cortex as outlined by Zeman and Innes (1963), which differed from the section of cortex used by Glowinski and Iversen (1966). Parasagittal slices (0.4 mm) were prepared from parietal cortex from both sides of the brain using a McIlwain tissue chopper.

2. Incubations.

The slices (15-20), weighing about 100 mg, were placed in a 1.5 ml microfuge tube (polypropylene eppendorf®, Brinkmann Instruments Co., Westbury, NY, U.S.A.) containing 1 ml of Krebs-Henseleit bicarbonate medium warmed to 37°C and gassed with 95%O₂-5%CO₂ to maintain a pH of 7.4.

Figure 2. Dissection of the rat brain. A: Ventral aspect illustrating division of the cerebrum into 3 parts using the optic chiasm and the mamillary bodies as guides. Cortex was isolated from the middle section (B) only. B: Coronal section illustrating the division at the level of the rhinalis fissure. The region of the brain below this cut was removed prior to isolation of cortex with a recessed cortex slicer as described in the text.

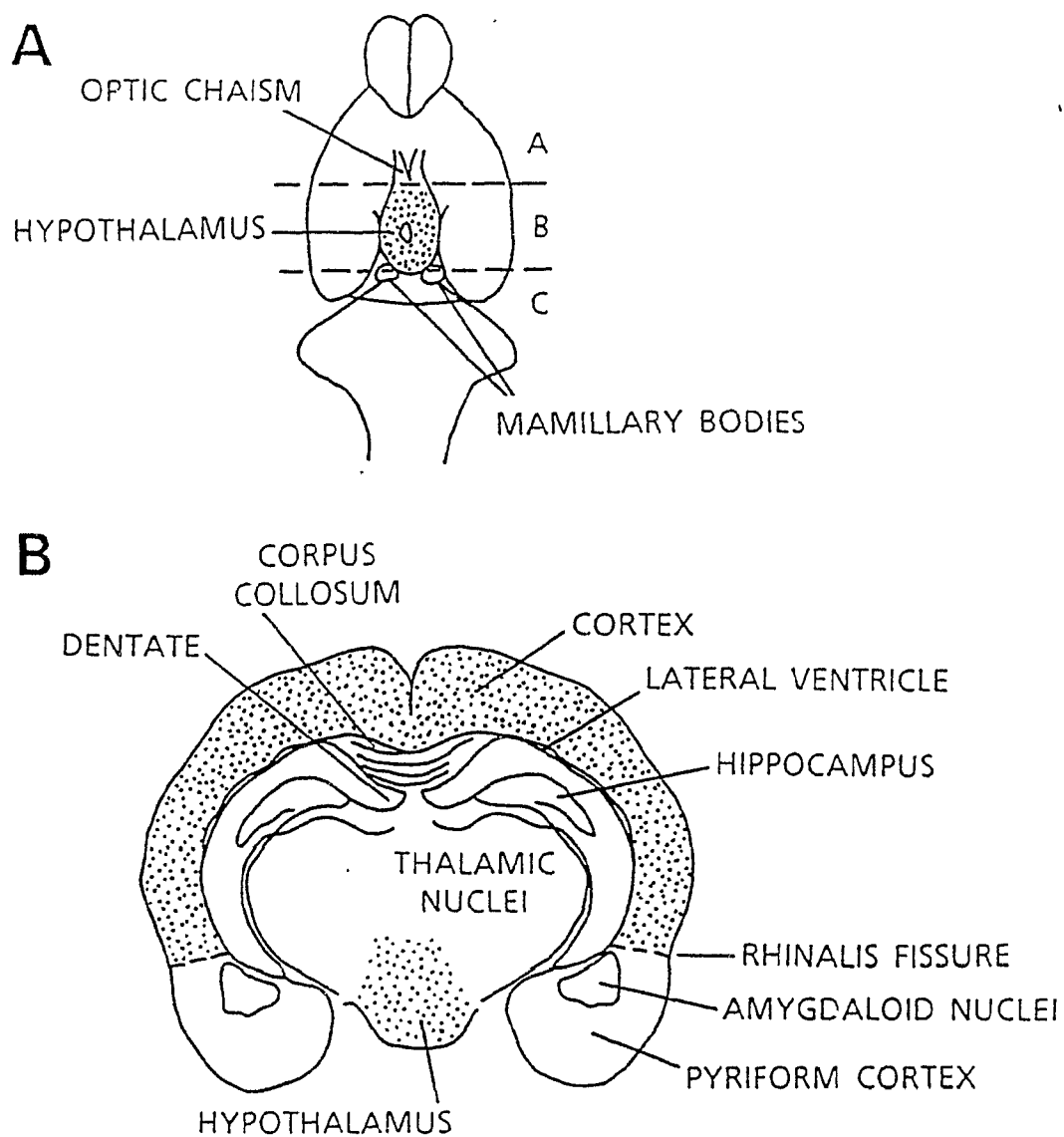


Figure 2

Medium was exchanged at 10 min intervals by gentle aspiration with a pipette, followed by rapid replacement with fresh medium. After an initial 130 min incubation period, slices were exposed for 10 min to releasing medium containing either 30 mM KCl or glutamate. In high KCl medium, molarity was maintained by a compensating decrease in NaCl concentration. Successive 10-min samples of incubation medium were collected and assayed for adenosine content.

3. Adenosine assay.

Adenosine was assayed by a modification of the method of Wojcik and Neff (1982) essentially as described by MacDonald and White (1985). Superfusate, (0.5 ml) was deproteinated by addition of 0.25 ml of 0.3 M ZnSO_4 and 0.25 ml of 0.3 M $\text{Ba}(\text{OH})_2$, mixing, and centrifugation for 4 min at top speed in a benchtop microfuge (Beckman). To 425 μl of supernatant were added 75 μl of 4.5% chloroacetaldehyde, and the tubes were placed in a boiling water bath for 20 min to form the etheno-derivative of adenosine. Detection and quantitation of adenosine was performed by high performance liquid chromatography (HPLC) with fluorescence detection either immediately after cooling or after storage at 4°C. Refrigerated samples were stable for at least one week (Wojcik and Neff, 1983b) but were routinely assayed not more than three days after collection.

Aliquots (100 μl) were injected with a Waters model U6K injector into a Waters model 6000A solvent delivery system attached to an Alltech model C-18 reverse-phase column (10 μm) and chromatographed with a solvent system containing 50 mM acetate buffer (pH 4.5), 2 mM 1-octanesulfonic acid, and 15% acetonitrile at a flow rate of 2 ml/min. Etheno-adenosine peaks were detected with a Waters model 420 AC fluorescence detector with

excitation at a wavelength of 280 nm and a longpass emission filter at 399 nm. The retention time was about 3 min. Stock solutions of adenosine standards were prepared in distilled and Millipore-filtered H₂O and stored at -15°C. These were thawed daily, diluted appropriately in Krebs-Henseleit medium and treated identically to the samples. The amount of adenosine in the samples was quantitated by comparison of peak heights with the standards. Peak heights exhibited a strict linear relationship with respect to the concentration of adenosine. Total adenosine was expressed as pmol adenosine/g cortex/10 min.

4. Statistics.

Differences in the rate of "control" and "test" release of adenosine immediately following exposure to an agonist were analysed by two-tailed Student's *t* tests for unpaired data.

II. SUPERFUSED SLICES

1. Preparation of slices.

Slices of parietal cortex were prepared as described above except that 0.4 mm slices were cut in a coronal rather than parasagittal direction, resulting in slices with approximate dimensions of 0.4 mm X 1-1.5 mm X 10 mm. Adjacent slices were placed alternately into each of two baths so that each bath contained six slices, three from each side of the brain, weighing a total of about 70 mg. This procedure permitted the use of paired statistical analyses for the slices in the two baths.

2. Superfusion apparatus.

The superfusion chambers, illustrated in Fig. 3, were modifications of those described by Marien et al. (1983). Each system consisted of a

Figure 3. Cortical slice superfusion chamber. Volume (0.5ml) was set by movement of plungers at the inflow and outflow ends of the plastic chamber. Slices covered the top of a nylon mesh screen (425 μm mesh size). The nylon mesh was supported by a cylindrical platform which was constructed from the collar of a Finn timer pipette tip (200-1000 μl , Fisher Scientific Ltd.). See text for further details.

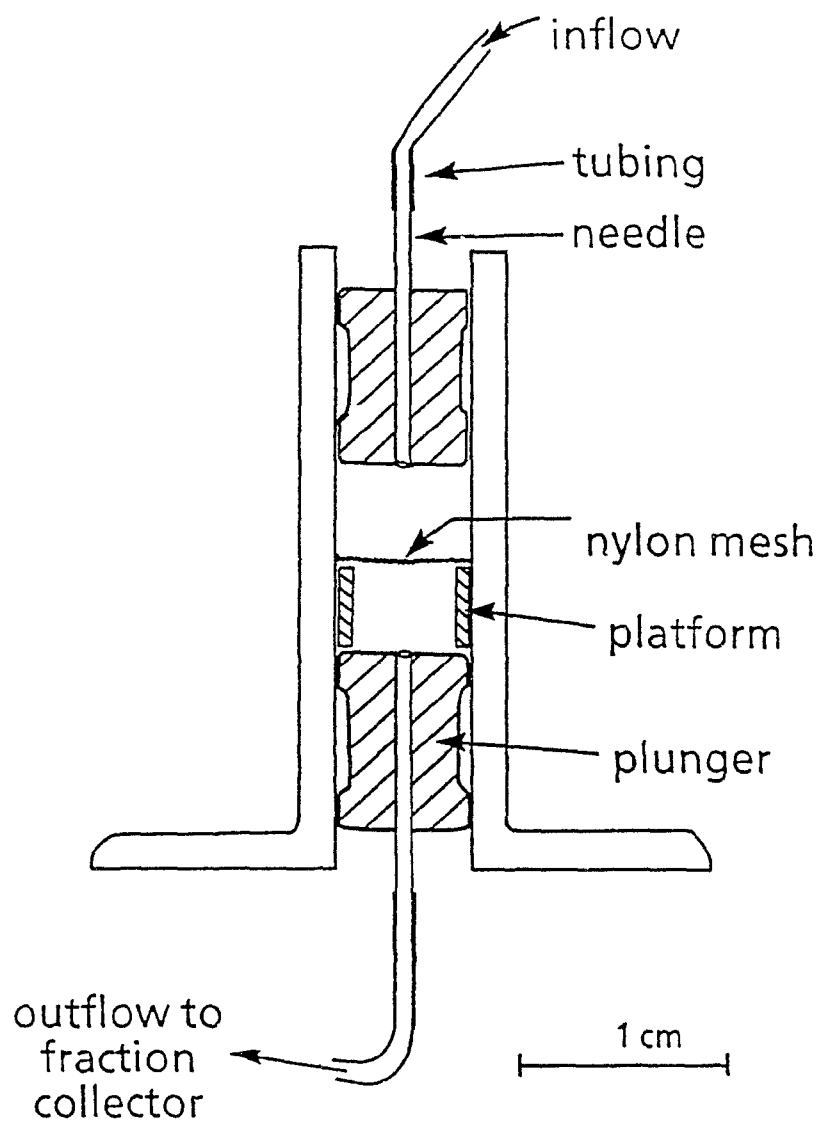


Figure 3

3-ml plastic syringe adjusted to a 0.5-ml volume by rubber plungers into the centre of which were glued 16-gauge needles. Slices were placed so that they covered a nylon mesh (425 μ m) supported by a cylindrical plastic platform at the centre of the bath. Both baths were immersed in a circulating water bath at 36°C. Krebs-Henseleit bicarbonate medium, at 36°C and gassed with 95%O₂-5%CO₂, was pumped from top to bottom by a Gilson Minipulse 2 peristaltic pump at the rate of 0.75 ml/min. The pump tubing was PVC Manifold Tubing (Gilson Medical Electronics Inc., Middleton WI, U.S.A.) with an internal diameter of 0.76 mm. The remainder of the tubing (Tygon®, Fisher Scientific Company, Pittsburgh, Penn., U.S.A.) had a wall diameter of 1/32 in. The internal tubing diameters were 1/32 in. and 3/32 in. up to the inflow and beyond the outflow of the bath, respectively. A three-way stopcock allowed switching between superfusion solutions. The total tubing void volume of 0.5 ml beyond the stopcock was accounted for by inserting a 40 s delay between switching of the stopcock and collection of samples. Samples from the two baths were collected either manually or with a LKB HeliRak fraction collector (Bromma, Sweden) into glass test tubes, and were immediately deproteinated and derivatized for amnosine assay and/or placed into scintillation vials.

3 Superfusion protocol.

Two baths were run in parallel and assigned in alternate experiments to either "control" observations (stimulating agent alone) or "test" observations (stimulating agent plus the drug or ionic condition to be tested). After an initial 65-min superfusion period, 10 serial 2.5-min fractions were collected. After collection of three samples to determine basal release, superfusing medium was switched for 10 min to medium

containing EAA agonists or 30 mM KCl. In high KCl medium, molarity was maintained by a compensating decrease in NaCl concentration. Concentrations of NMDA, kainate, and quisqualate (between 50 μ M and 500 μ M) were chosen to conform to concentrations used in other studies of EAA-evoked neurotransmitter release from brain slices or cultures (Lehmann and Scatton, 1982; Ferkany and Coyle, 1983; Weiss, 1988; Clow and Jhamandas, 1989; Harris and Miller, 1989; Jaffé and Vaello, 1989). In some experiments, "test" slices were superfused with medium from which $MgCl_2$ or $CaCl_2$ had been omitted (without substitution) or medium containing test drugs prior to exposure to releasing medium. At the end of the experiment the slices were removed from the tissue baths for determination of wet tissue weight.

In early experiments with [3H]noradrenaline, in which the release of [3H]noradrenaline but not adenosine was determined, slices were preincubated for 30 min in 10 ml of continuously gassed Krebs-Henseleit bicarbonate medium containing freshly-prepared 10^{-7} M L-noradrenaline [$7-^3H$] (specific activity 14.2 Ci/mmol, Du Pont-NEN Canada) at 37°C. The slices were then placed in the superfusion baths and the remainder of the experiment was conducted as described above.

In experiments in which release of both adenosine and [3H]noradrenaline was determined, slices were initially superfused with Krebs-Henseleit medium for 5 min. Slices were then labelled with [3H]noradrenaline by superfusion for 10 min with oxygenated Krebs-Henseleit bicarbonate medium containing freshly prepared 10^{-7} M L-noradrenaline [$7-^3H$] (specific activity 13.1 Ci/mmol, Du Pont-NEN Canada) at 36°C. Superfusion was continued with Krebs-Henseleit bicarbonate medium for a

further 65 min before collection of 10 serial 2.5 min samples.

4. Adenosine assay.

Samples and standards were deproteinated with ZnSO_4 and Ba(OH)_2 and derivatized with chloroacetaldehyde as described above (Methods, Section I.3). To improve the sensitivity of detection of adenosine, the samples and standards in Krebs-Henseleit bicarbonate medium were concentrated by evaporation to dryness under N_2 (N-Evap, Organomastics Ass. Inc., South Berlin, MA.) and reconstituted with 120 μl of H_2O before aliquots (100 μl) were injected into the HPLC for detection and quantitation of adenosine as described above (METHODS, Section I.3). In some experiments, a different HPLC pump (Waters 501) and column (4 μm reverse-phase Waters model 80100 in a compression module) were used. In these experiments, comparable retention times and chromatography were achieved by increasing the buffer concentration of octanesulfonic acid to 2.2 mM and decreasing the solvent flow rate to 0.8 ml/min.

Evoked adenosine release was expressed as pmol adenosine released/g cortex/min to give the net rate of release above baseline. This was obtained by subtracting the value of the sample immediately preceding exposure to the releasing agent from every other sample. Total evoked adenosine release was the amount released during the entire 17.5 min period following exposure to the releasing agent and was expressed as nmol/g cortex.

Initial experiments in which α,β -methylene ADP and GMP were present to inhibit ecto-5'-nucleotidase showed that these drugs produced large chromatographic peaks which interfered with adenosine chromatography. It was found that these peaks were diminished but not abolished following

$\text{Ba}(\text{OH})_2/\text{ZnSO}_4$ precipitation, probably because the nucleotides were incompletely precipitated by a single deproteination. Therefore, for these experiments, the procedure was modified to incorporate a second $\text{Ba}(\text{OH})_2/\text{ZnSO}_4$ precipitation on 0.6 ml of the original supernatant.

In superfusion experiments in which both adenosine and [^3H]noradrenaline were determined at the same time, the amount of supernatant combined with 75 μl of chloroacetaldehyde before boiling and concentration under N_2 was increased from 425 μl to 600 μl in order to achieve larger adenosine peaks on the HPLC.

5. Quantitation of [^3H]noradrenaline release.

Aliquots of superfusate (1 ml) were placed into scintillation vials containing 10 ml Aquasol-2 and the dpms of [^3H]noradrenaline released were determined with a Beckman Model LS5801 scintillation counter. The slices were weighed and then solubilized in 1 ml Protosol. Tissue [^3H]noradrenaline contents were determined by scintillation spectrometry in 14 ml Econofluor. The rate/min of [^3H]noradrenaline release was standardized as the percent of total tissue [^3H]noradrenaline content at the beginning of the sample collection period. The rate of evoked [^3H]noradrenaline release was obtained by subtracting the percent release/min in the sample immediately preceding exposure to the releasing agent from every other sample and was expressed as percent content/min. Total evoked [^3H]noradrenaline release was determined as the percent of [^3H]noradrenaline content released during the entire 17.5 min period following exposure to the releasing agent. It has been reported that under similar conditions, greater than 90% of the radioactivity recovered in the medium coeluted with noradrenaline as determined by HPLC (Daniell

and Leslie, 1986).

6. Concentration-response determination for NMDA-evoked release of adenosine and [³H]noradrenaline.

For construction of concentration-response curves, standardization to eliminate inter-experimental differences in the absolute amount of [³H]noradrenaline or adenosine was obtained by stimulating one of the two baths in each experiment with 3 mM NMDA. The total amount of [³H]noradrenaline or adenosine released by a "test" concentration was then expressed as a percent of the amount released by 3 mM NMDA in each experiment.

7. Statistics.

Differences in total evoked release between paired "test" and "control" slices were analysed by two-tailed Student's t tests for paired data.

III. SYNAPTOSOMES

1. Preparation of synaptosomes (P₂).

For each preparation, two rats were decapitated and their brains were rapidly removed to ice-cold 0.32 M sucrose buffered to pH 7.4 with 5 mM HEPES. Parietal cortices were isolated as described above (section I.1) with the exception that, rather than using a recessed tissue slicer, cortices were trimmed of any noncortical tissue using the underlying lateral ventricle and corpus callosum as a guide. Crude P₂ synaptosomal fractions were prepared essentially according to the method of Gray and Whittaker (1962) as described previously by MacDonald and White (1985). Cortices from two brains were homogenized in 6.5 ml ice-cold HEPES-

buffered sucrose with a teflon-glass homogenizer (15 strokes at 800 rpm, clearance, 0.25 mm). The homogenate was centrifuged for 10 min at 1000g in an SS-34 rotor in a Sorvall RC2-B refrigerated centrifuge. The supernatant was decanted, diluted to 40 ml with 0.32 M sucrose and centrifuged for 20 min at 12,000Xg. The resulting P_2 pellets were resuspended in 40 ml of 0.32 M sucrose and recovered by centrifugation for 30 min at 20,000Xg. The final P_2 pellets were resuspended in 5 ml of Krebs-Henseleit bicarbonate medium gassed with a 95%O₂-5%CO₂ mixture to maintain a pH of 7.4. For Ca²⁺-free or Mg²⁺-free experiments, the pellets were resuspended in medium identical to Krebs-Henseleit except that CaCl₂ or MgCl₂ were omitted. The suspension was preincubated for 30 min at 37°C with periodic gassing before studies of adenosine release were conducted.

In experiments to determine the Na⁺-dependence of release, P_2 pellets were resuspended in a Tris-buffered medium containing 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 11 mM glucose, 20 mM Tris-HCl (pH 7.4), and either 111 mM NaCl, 111 mM LiCl, 111 mM choline chloride, or 222 mM sucrose. For these experiments, the 30-min preincubation at 37°C was omitted.

2. Purification of synaptosomal, myelin, and mitochondrial fractions on sucrose density gradients.

P_2 pellets from 4 rat cortices were resuspended in 12 ml of 0.32 M HEPES-buffered sucrose (pH 7.4); 2 ml were layered onto each of six sucrose density gradients consisting of 5 ml of 0.8 M sucrose on 5 ml of 1.2 M sucrose (Gray and Whittaker, 1962). The gradients were centrifuged for 60 min at 150,000 g in an International B60 centrifuge (rotor SB283). The synaptosomes at the 0.8:1.2 M sucrose interface were removed in 3 ml using a syringe and needle with curved tip. The pooled material was

diluted to 40 ml with ice-cold 0.32 M HEPES-buffered sucrose and centrifuged for 30 min at 20,000g. The myelin-containing material at the 0.32 M:0.8 M sucrose interface and the mitochondrial pellet at the bottom of the 1.2 M sucrose were treated similarly. The resulting pellets were finally suspended in 5 ml of Krebs-Henseleit medium and preincubated at 37°C for 30 min before studies of adenosine release were performed.

3. Release of adenosine.

Synaptosomes were centrifuged at top speed in a bench-top centrifuge at room temperature for 15 min. The pellet was resuspended with 5 ml of Krebs-Henseleit medium at 37°C, giving a final concentration of 2-3 mg protein/ml. In experiments with IBMX, the synaptosomal suspension was divided equally immediately prior to centrifugation, and one of the two resulting pellets was resuspended in 2.5 ml of Krebs-Henseleit medium containing 1 mM IBMX, whereas the other was resuspended in 2.5 ml of IBMX-free medium. Purified synaptosomal, myelin or mitochondrial fractions were also resuspended with 2.5 ml of Krebs-Henseleit medium, giving final concentrations of about 4, 2, and 2 mg protein/ml, respectively. In experiments to determine the Na⁺-dependence of release, the 30-min preincubation at 37°C and the subsequent centrifugation and resuspension were omitted.

Aliquots (350 μ l) of this suspension were immediately added to 15 μ l volumes of drugs to be tested in 1.5 ml microfuge tubes. Tube contents were mixed and incubated for 10 min at 37°C, after which the incubation was terminated by centrifugation for 4 min at top speed in a Beckman Microfuge II microcentrifuge. A 250- μ l aliquot of supernatant was removed and assayed for adenosine.

4. Adenosine assay.

Adenosine was assayed as described above (METHODS, Section I.3) with the following modifications. Supernatant (250 μ l) was deproteinized with 125 μ l of 0.3 M ZnSO_4 and 125 μ l of 0.3 M Ba(OH)_2 , mixed, centrifuged, and derivatized with chloroacetaldehyde. Aliquots (100 μ l) of unconcentrated sample were injected into the HPLC using either a Waters model U6K injector or a Waters automatic injector (WISP Model 712). Adenosine release was expressed as pmoles/mg protein/10 min. Evoked adenosine release was calculated by subtracting basal extrasynaptosomal adenosine (which consists of background contents of adenosine in the medium following synaptosomal resuspension and basal release during the 10 min incubation period) from the amount of extrasynaptosomal adenosine following exposure to drug for 10 min.

5. ATP release.

Synaptosomes (P₂) were prepared from two rat cortices as described above. P₂ pellets were resuspended in Krebs-Henseleit medium to a final concentration of about 1.5 mg protein/ml. Synaptosomal suspensions were preincubated at 37°C for at least 30 min prior to assay, and periodically gassed with 95% O₂-5%CO₂. ATP release was detected by monitoring the light produced by the ATP-luciferin-luciferase reaction with an Aminco "Chem Glow" chemiluminometer, as described previously by White (1977, 1978). For each trial, an aliquot of the synaptosomal suspension (500 μ l) was placed into a 6 X 50 mm cylindrical cuvette and 20 μ l of fortified luciferin-luciferase enzyme mixture were added. The enzyme mixture contained equal volumes of synthetic D-luciferin (5 mg/ml, stored at -20°C) and crude firefly extract (50 mg FLE 50 in 2 ml H₂O, prepared daily).

The contents were mixed and the cuvette was placed in the chamber of the chemiluminometer. Background light emission was monitored until a fairly constant level was attained (about 2 min). ATP release evoked by drugs (injected in 10 μ l volumes) was monitored continuously with an Aminco photomultiplier and recorded with a pen recorder. The amount of ATP released was quantitated by comparison of the maximum light produced (maximum peak height) with that produced by injection of known ATP standards into the synaptosomal/enzyme suspension. ATP standards were prepared in distilled and Millipore-filtered H₂O, stored at -15°C and thawed daily to be diluted appropriately. It had been shown previously that the maximum peak height of the light response is related linearly to the concentration of ATP in the suspension, and that the assay system is specific for ATP (White, 1978). Results were expressed as maximum ATP concentration/mg of protein.

6. [³H]Noradrenaline release.

To determine [³H]noradrenaline release from synaptosomes, P₂ synaptosomal pellets were resuspended in 5 ml Krebs-Henseleit bicarbonate medium containing freshly prepared 10⁻⁷ M L-noradrenaline [³H] (specific activity 13.1 Ci/mmol, Du Pont-NEN Canada) at 37°C for 30 min and periodically gassed with 95% O₂-5%CO₂. Following loading, synaptosomes were centrifuged at top speed in a bench-top centrifuge at room temperature for 15 min, resuspended in Krebs-Henseleit bicarbonate medium and immediately recentrifuged for another 15 min. The remainder of the experiment was as described above (METHODS Section III.3). Adenosine release was monitored simultaneously from the same synaptosomal suspensions. At the end of the experiment, supernatant (250 μ l) was either

assayed for adenosine or placed into scintillation vials containing 10 ml Aquasol-2 for determination of dpms ^3H as described previously (METHODS, Section II.5). The dpms in an aliquot (350 μl) of the synaptosomal suspension were also determined.

$[^3\text{H}]$ Noradrenaline release was expressed as a percent of the total $[^3\text{H}]$ noradrenaline content of the synaptosomal suspension/10 min. Evoked $[^3\text{H}]$ noradrenaline release was calculated by subtracting the basal extrasynaptosomal $[^3\text{H}]$ noradrenaline (which consists of background contents of $[^3\text{H}]$ noradrenaline in the medium following synaptosomal resuspension and basal release during the 10-min incubation period) from the amount of extrasynaptosomal $[^3\text{H}]$ noradrenaline following exposure to drug for 10 min.

7. Protein assay.

Aliquots of the synaptosomal suspensions were stored at -20°C until assayed for protein content. After thawing to room temperature, the suspensions were diluted 1:50 in H_2O and protein was assayed by the method of Hartree (1972) using a Turner spectrophotometer. This represents a modification of the Lowry method and yields a linear standard curve when absorbances are read at a wavelength of 650 nm. Crude synaptosomal (P_2) and purified fractions (synaptosomal, myelin, mitochondrial) protein concentrations were determined by linear regression analysis from the standard curves, multiplied by the appropriate dilution factors and expressed as mg of protein/ml.

8. Statistics.

Results were analysed using either a two-tailed Student's t tests for paired data (2 treatment groups) or randomized block analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) for post-hoc

analysis (more than 2 treatment groups).

RESULTS

I. K^+ AND GLUTAMATE-EVOKED ADENOSINE RELEASE FROM INCUBATED SLICES

1. Release of adenosine evoked by K^+ .

Depolarization by exposure to 30 mM K^+ released endogenous adenosine from rat cortical slices incubated in a bath (Fig. 4). Release peaked by 10 min exposure to K^+ but remained elevated for a further 10 min following removal of the releasing medium, returning to baseline by about 40 min following exposure.

2. Involvement of NMDA receptors in glutamate-evoked adenosine release.

Exposure to glutamate (5 mM) also released adenosine (Fig. 5). This release peaked during exposure to glutamate, but remained elevated for a further 20 min following removal of glutamate. To determine whether the NMDA receptor subtype was involved in glutamate-evoked adenosine release, the effect of the competitive NMDA antagonist, D,L-APV (Mayer and Westbrook, 1987; Monaghan et al., 1989), on release was determined. APV (100 μ M) had no effect on basal adenosine release (Fig. 5). APV, added 20 min prior to exposure to glutamate and present for the remainder of the experiment, antagonized evoked adenosine release during exposure to glutamate, but failed to antagonize the late phase of release observed following exposure to glutamate. The total amount of adenosine released by glutamate was decreased by approximately 50% in the presence of APV, indicating that NMDA receptors are involved in glutamate-evoked release of adenosine.

Figure 4. Time course of K^+ -evoked adenosine (ADN) release from bath-incubated slices. K^+ (30 mM) was present from 0 to 10 min. Each value represents mean \pm SEM of 5 experiments.

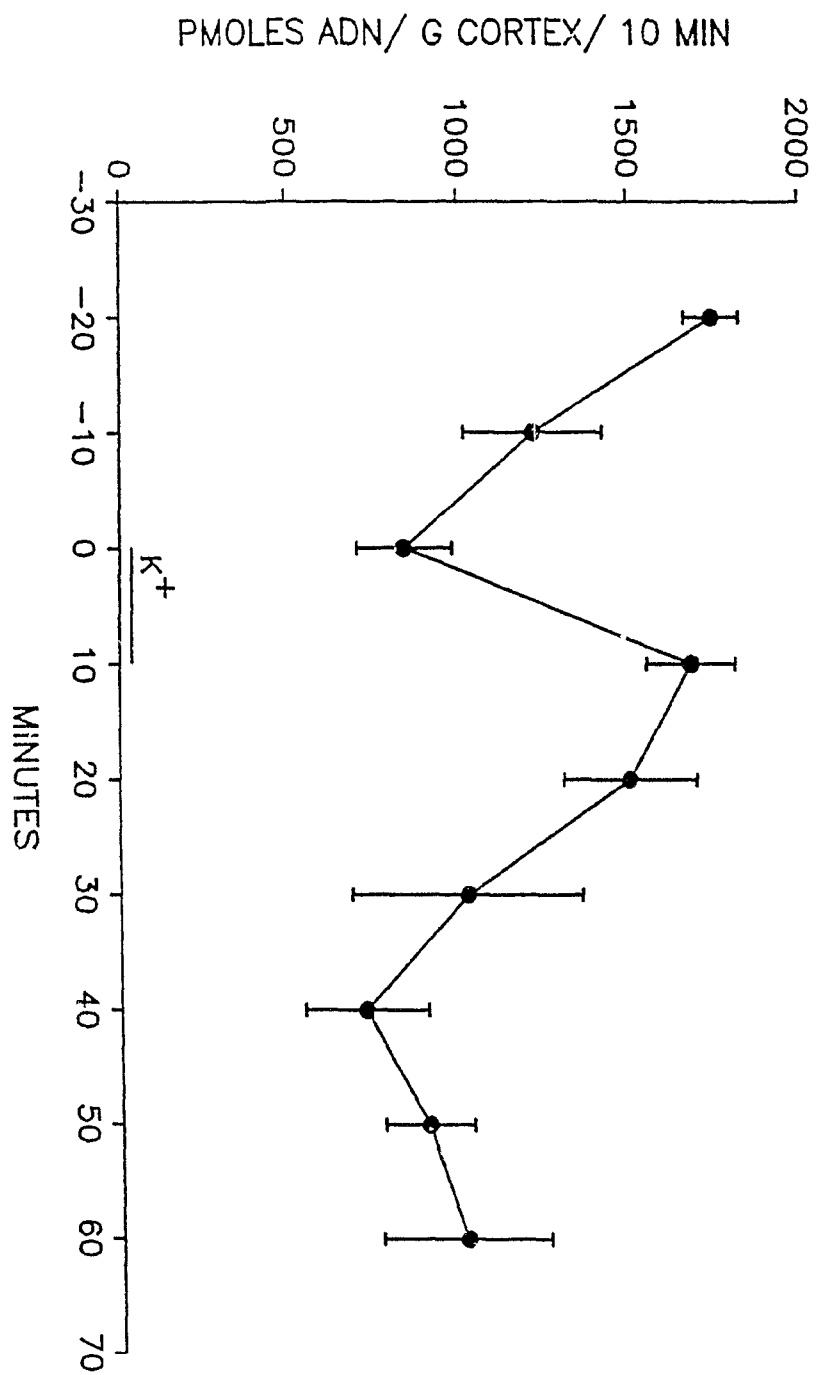


Figure 4

Figure 5. Time course and effect of APV on glutamate-evoked adenosine (ADN) release from bath-incubated slices. Glutamate (GLU, 5 mM) was present from 0 to 10 min. APV (100 μ M) was present from -20 min until the end of the experiment. Values are means \pm SEM from 6 and 4 experiments for glutamate alone, and glutamate and APV, respectively. Values for APV are from one experiment. *Significantly different from glutamate alone ($P < 0.05$, unpaired t test).

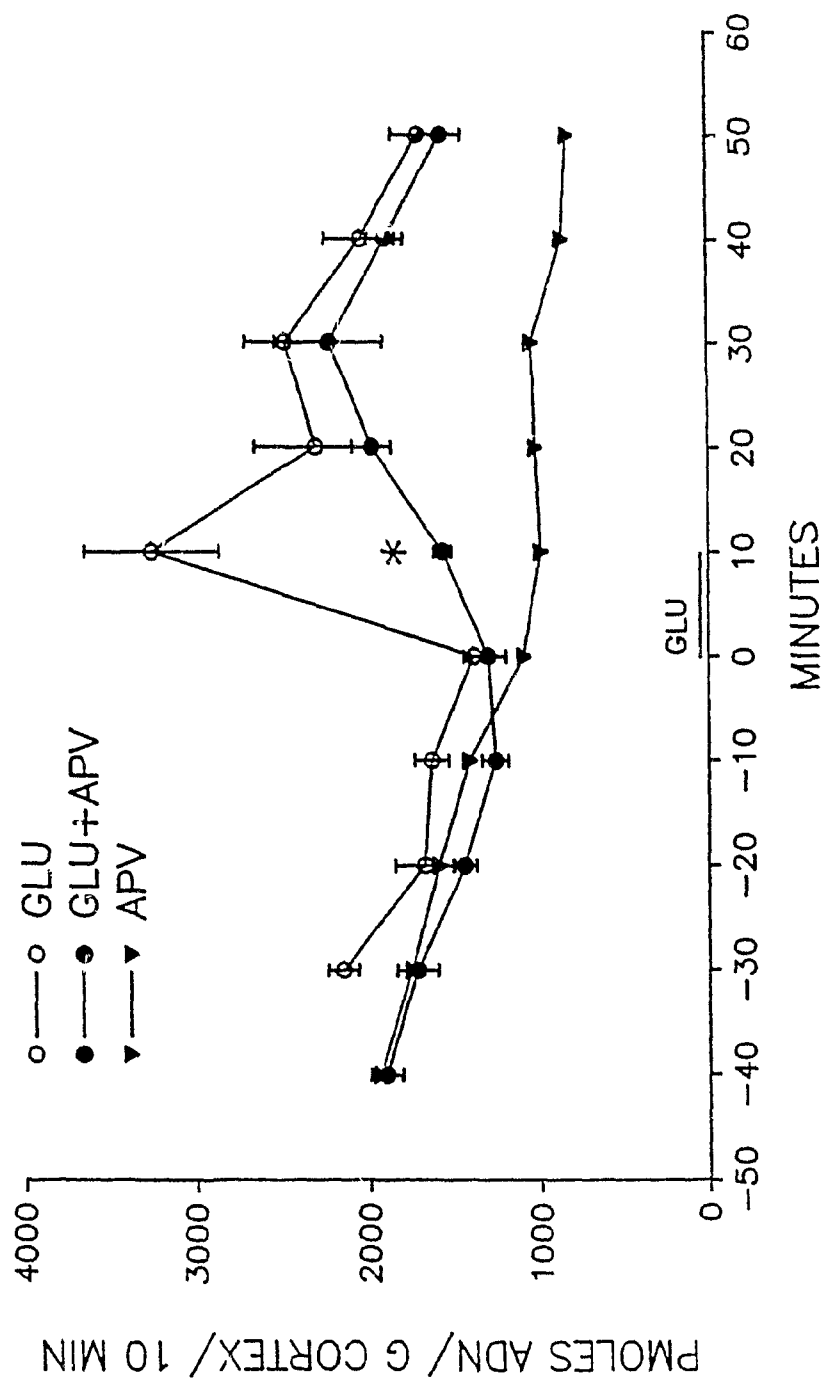


Figure 5

II. INVOLVEMENT OF EXCITATORY AMINO ACID RECEPTORS IN K^+ AND EXCITATORY AMINO ACID-EVOKED ADENOSINE RELEASE FROM SUPERFUSED SLICES

1. Release of adenosine evoked by K^+ and glutamate.

To achieve greater temporal resolution of adenosine release following exposure to releasing agents and to allow more rapid and complete exchange of medium, subsequent experiments were conducted with continuously superfused cortical slices. Exposure to K^+ (30 mM) released endogenous adenosine from superfused rat cortical slices (Fig. 6A). The rate of adenosine release was increased by 2.5 min following exposure to K^+ and reached a maximum at 7.5-10 min. The maximum rate of adenosine release at 10 min of exposure to K^+ (965 ± 119 pmol/g of cortex/min) was at least four times the basal rate of release (226 ± 22 pmol/g of cortex/min, 14 experiments).

Glutamate (1-10 mM) also released adenosine in a concentration-dependent manner (Fig. 6B). At the higher concentrations of glutamate, the rate of adenosine release was increased by 2.5 min and reached maximum by 7.5 min of exposure to glutamate. The maximum rate of 5 mM glutamate-evoked adenosine release ($1,014 \pm 80$ pmol/g of cortex/min) was about five times the basal rate of release (213 ± 11 pmol/g of cortex/min, 15 experiments).

2. Effect of the glutamate uptake blocker, dihydrokainate, on glutamate-evoked release of adenosine.

Glutamate's action at receptors in slice preparations may be attenuated because it is rapidly taken up from the extracellular space (Garthwaite, 1985). Dihydrokainate (500 μ M), which inhibits the uptake of glutamate (Johnston et al., 1979) and enhances glutamate's actions in

Figure 6. A: Time course of K^+ -evoked adenosine release from superfused slices. K^+ (30 mM) was present from 0 to 10 min; n=14. B: Time course for glutamate-evoked adenosine release from superfused slices. Glutamate (GLU) was present from 0 to 10 min.; 1 mM (Δ -- Δ , n=3); 2.2 mM (Δ -- Δ , n=7); 5 mM (\bullet -- \bullet , n=15); 10 mM (O--O, n=4).

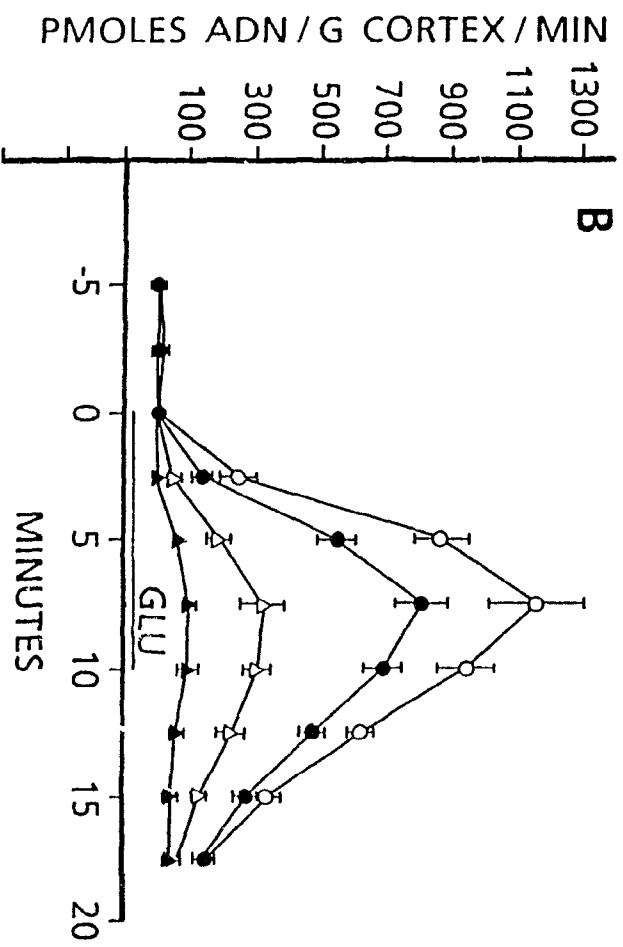
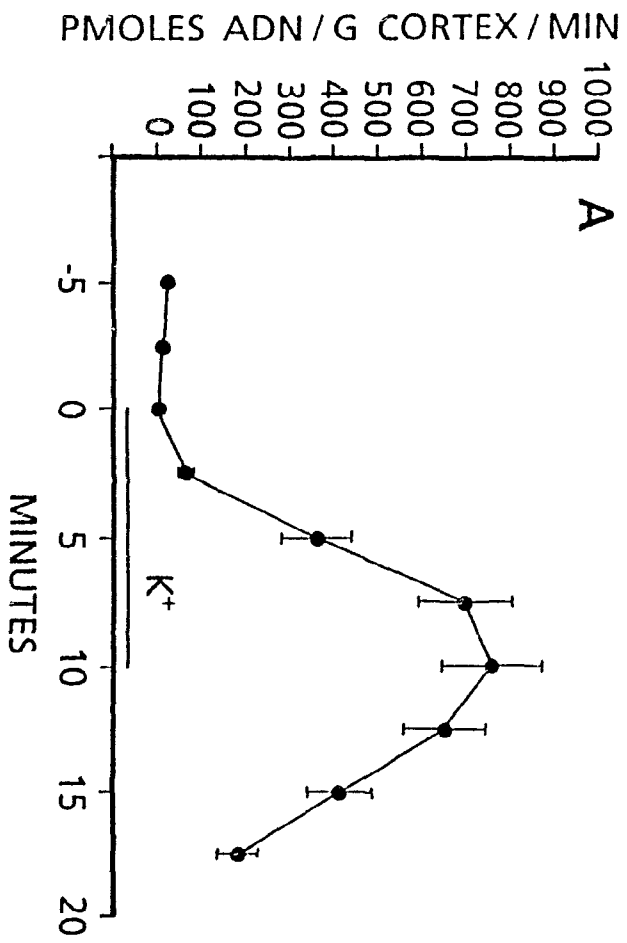


Figure 6

various systems (Lodge et al., 1979, 1980; Sawada et al., 1985), potentiated glutamate-evoked adenosine release (Fig. 7), indicating that uptake of glutamate probably attenuates the ability of glutamate to release adenosine from superfused cortical slices.

3. Contribution of extracellular nucleotides to adenosine release

Because ectoenzymes capable of converting released nucleotides to adenosine are present in rat brain (Manery and Dryden, 1979; MacDonald and White, 1985; Nagy et al., 1986), it was possible that adenosine detected in the superfusate resulted from the degradation of released nucleotide and not from the release of adenosine itself. To test this, release was determined in the presence of α,β -methylene ADP (0.5 mM) and GMP (5 mM) to inhibit ecto-5'-nucleotidase. This combination has been shown previously to block conversion of nucleotide to adenosine by rat brain synaptosomes by about 90% (MacDonald and White, 1985). When ecto-5'-nucleotidase was inhibited, basal adenosine release was diminished by 41% (Table 3), indicating that a significant proportion of basal adenosine release was derived from the extracellular metabolism of released nucleotide. In contrast, neither K^+ - nor glutamate-evoked adenosine release was decreased when ecto-5'-nucleotidase was inhibited, indicating that K^+ and glutamate probably released adenosine per se (Table 3).

4. Receptor-mediated release of adenosine by NMDA, kainate, and quisqualate.

Exposure of slices to 500 μ M NMDA, kainate or quisqualate released endogenous adenosine from superfused rat cortical slices (Fig. 8). The rate of adenosine release was increased within 2.5 min of exposure to the agonists.

Figure 7. Effect of the glutamate (GLU) uptake blocker, dihydrokainate (DHK), on glutamate-evoked adenosine (ADN) release from superfused slices. Glutamate (0.5 mM) was present from 0 to 10 min. DHK (0.5 mM) was present from 15 min prior to exposure to glutamate until the end of the experiment. Values are the means \pm SEM from 4 experiments. Inset: Histograms represent the total amount of adenosine released.

*Significantly different from control ($p < 0.05$, paired t test).

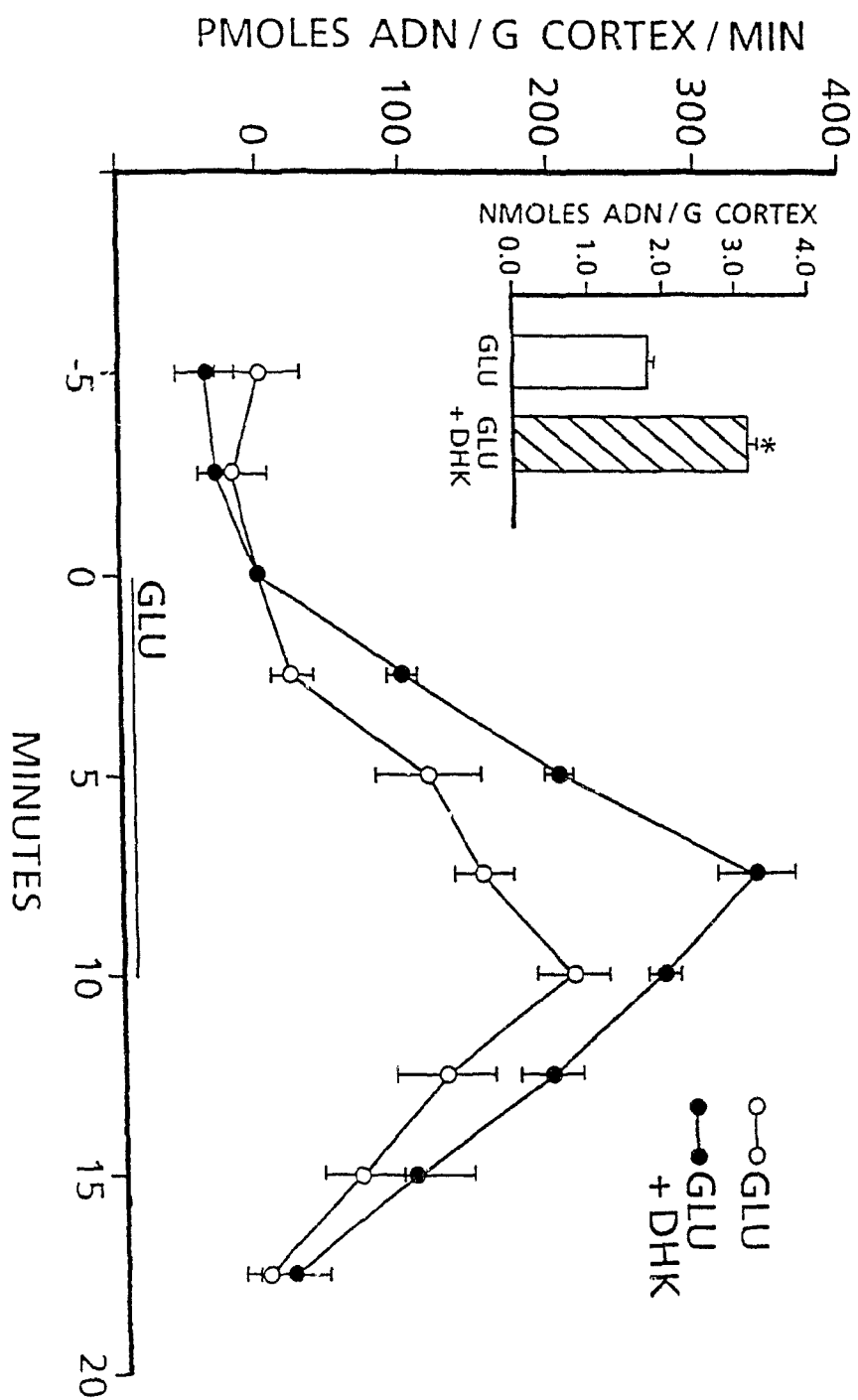


Figure 7

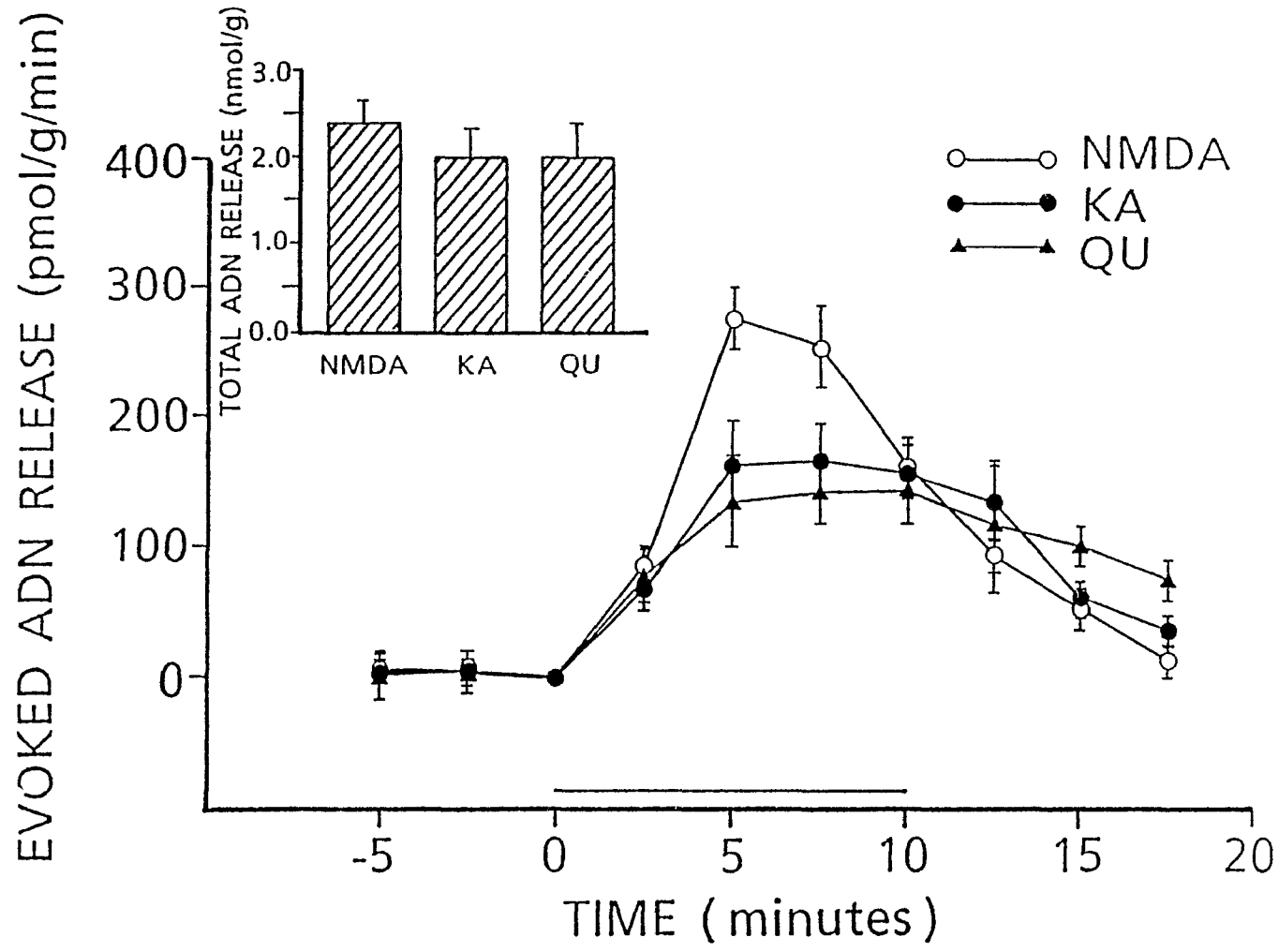
TABLE 3. Effect of inhibition of ecto-5'-nucleotidase on basal and evoked release of adenosine from cortical slices.

	ADENOSINE RELEASE (nmol/g cortex)		
	CONTROL	5'-NUCLEOTIDASE INHIBITORS	% CONTROL
Basal (7)	0.61 \pm 0.06	0.36 \pm 0.03*	59
K ⁺ (30 mM) (3)	6.18 \pm 0.36	6.24 \pm 0.53	101
Glutamate (1.5 mM) (3)	3.58 \pm 1.28	3.34 \pm 1.19	93

Slices were exposed to K⁺ or glutamate in the presence and absence of 0.5 mM α,β -methylene ADP and 5 mM GMP to inhibit 5'-nucleotidase. 5'-nucleotidase inhibitors were present from 10 min prior to exposure to K⁺ or glutamate until the end of the experiment. Data are means \pm SEM of the number of experiments given in parentheses. *Significantly different from respective control ($p < 0.05$, paired t test).

Figure 8. Time course of adenosine (ADN) release evoked by NMDA (500 μ M, n=10), kainate (KA, 500 μ M, n=5) and quisqualate (QU, 500 μ M, n=4) from superfused slices. Agonists were present from 0 to 10 min. Values are means \pm SEM. INSET: Histograms represent the total amount of adenosine released by the respective agonists (500 μ M).

Figure 8

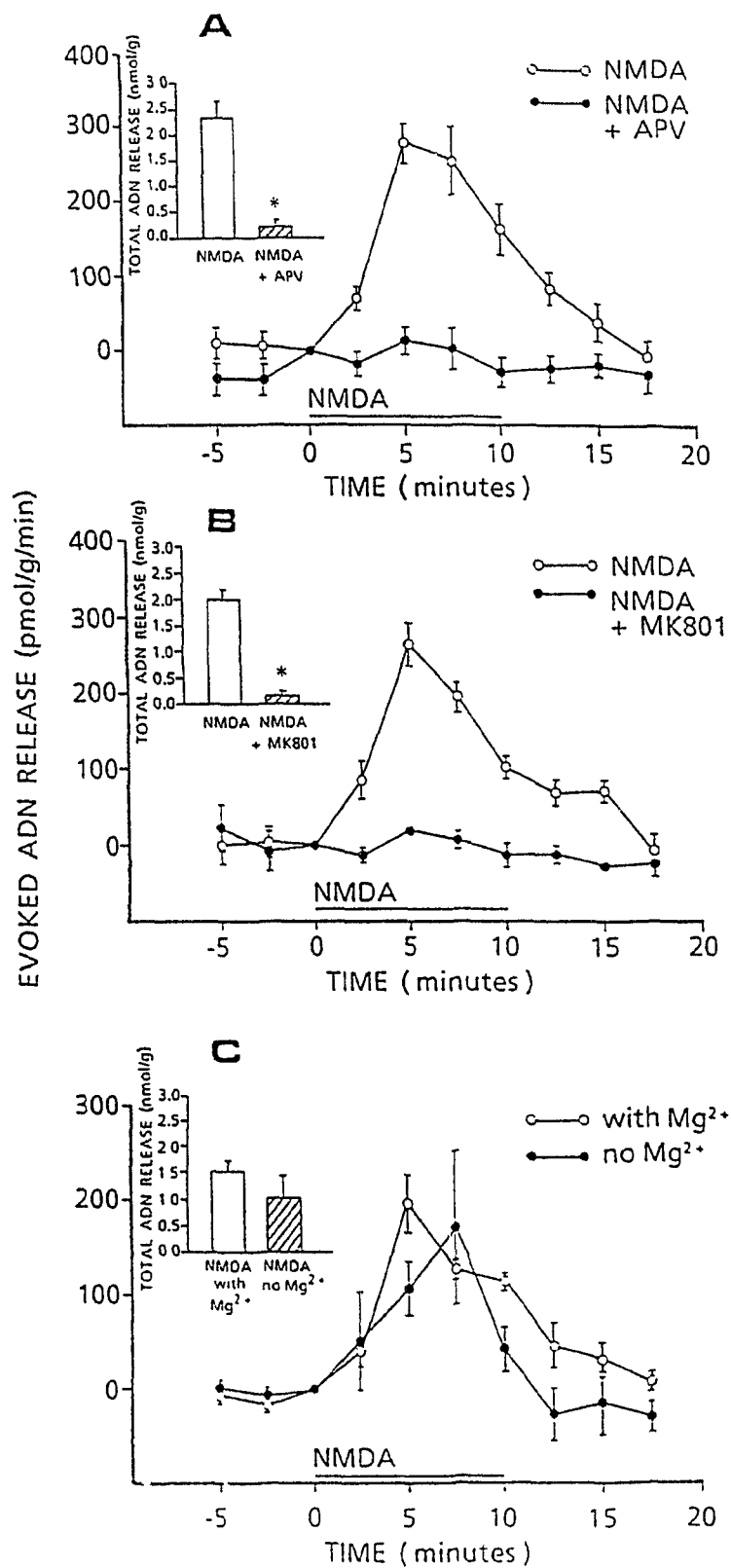


To determine whether NMDA released adenosine by acting at specific NMDA receptors, the effects of the competitive NMDA receptor antagonist, D,L-APV, and the uncompetitive NMDA receptor antagonist, MK-801 (Wong et al., 1986), on NMDA-evoked adenosine release were examined. APV was present from 7.5 min prior to exposure to NMDA, whereas MK-801, which characteristically exhibits a slow onset and use-dependence of block (Wong et al., 1986), was present 20 min prior to exposure to NMDA. Neither APV (1 mM) nor MK-801 (3 μ M) affected basal adenosine release. However, both APV and MK-801 virtually abolished NMDA-evoked adenosine release (Fig. 9, A and B), indicating that NMDA-evoked adenosine release was mediated through an action of NMDA at its receptor.

Mg^{2+} blocks NMDA receptor-associated ion channels in an uncompetitive, voltage-dependent manner (Nowak et al., 1984), and potentiation of NMDA receptor-mediated events in the absence of Mg^{2+} has been demonstrated (Collingridge and Lester, 1989). Mg^{2+} -free medium did not alter the basal rate of adenosine release (the mean rate of adenosine release between -7.5 and 0 min was 325 ± 18 pmol/g cortex/min versus 325 ± 10 pmol/g cortex/min in the presence and absence of Mg^{2+} , respectively). Surprisingly, NMDA-evoked release of adenosine was not potentiated in the absence of Mg^{2+} (Fig. 9C). Although there appeared to be a slight delay in NMDA-evoked adenosine release in the absence of Mg^{2+} , this did not occur in all experiments (see for example Fig. 35B) and was not statistically significant.

The lack of potentiation of NMDA-evoked adenosine release in the absence of Mg^{2+} raised the possibility that the slices may have been partially depolarized, which would relieve the voltage-dependent Mg^{2+} block

Figure 9. Effect of NMDA receptor antagonists on NMDA-evoked adenosine release from superfused slices. NMDA (500 μ M) was present from 0 to 10 min. A: D,L-APV (1 mM) was present from 7.5 min prior to exposure to NMDA until the end of the experiment. Values are the means \pm SEM from 5 experiments. B: MK-801 (3 μ M) was present from 20 min prior to exposure to NMDA until the end of the experiment. Values are the means \pm SEM from 4 experiments. C: Both "control" and "test" slices were superfused for an initial 45 min with Mg²⁺-free Krebs-Henseleit medium. At -20 min Mg²⁺ (1.2 mM) was restored to "control" slices. Values are the means \pm SEM from 5 experiments. INSETS: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).



(Mayer and Westbrook, 1987; Nowak et al., 1984). If the slices were indeed depolarized, then hyperpolarizing conditions should restore the Mg^{2+} -sensitivity of NMDA-evoked release of adenosine. To test this, slices were hyperpolarized by superfusion with medium in which the KCl concentration was reduced from 4.7 mM to 1 mM. Surprisingly, NMDA-evoked adenosine release in hyperpolarizing medium was not increased, but was in fact markedly decreased in the absence of Mg^{2+} (Fig. 10), suggesting that the lack of enhancement of NMDA-evoked adenosine release in Mg^{2+} -free medium was not due to depolarization of the tissue.

To determine whether adenosine release evoked by kainate was receptor-mediated, experiments were conducted with the EAA receptor antagonists, DGG (Fagg et al., 1986; Mayer and Westbrook, 1987) and DNQX (Fletcher et al., 1988; Honore et al., 1988), which have been shown to block non-NMDA receptors. Neither DGG nor DNQX affected basal adenosine release (the mean basal rate of adenosine release in the first 3 samples following exposure to DNQX was 170 ± 20 pmol/g cortex/min in the absence and 173 ± 18 pmol/g cortex/min in the presence of DNQX, not significant by paired t test, $n=10$). The nonspecific EAA receptor antagonist, DGG (2 mM), diminished 100 μ M kainate-evoked adenosine release by 40% (Fig. 11A). Likewise, the non-NMDA receptor antagonist, DNQX (10 μ M), decreased 50 μ M kainate-evoked adenosine release by 39% (Fig. 11B). Although DNQX also noncompetitively inhibits NMDA responses via an action at the glycine modulatory site of the NMDA receptor (Kessler et al., 1989b), it is unlikely that this played a part here because neither APV nor MK-801 diminished adenosine release evoked by kainate (Table 4). Adenosine release evoked by quisqualate (50 μ M) was also diminished by 49% by 10 μ M

Figure 10. Effect of hyperpolarization with 1 mM K^+ on NMDA-evoked release of adenosine (ADN) from superfused slices. KCl concentration in the Krebs-Henseleit medium of both "with Mg^{2+} " and "no Mg^{2+} " was reduced from 4.7 to 1 mM throughout the experiment. Following preparation of slices, "no Mg^{2+} " slices were superfused with Krebs-Henseleit medium from which Mg^{2+} had been omitted. NMDA (500 μ M) was present from 0 to 10 min. Values are means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

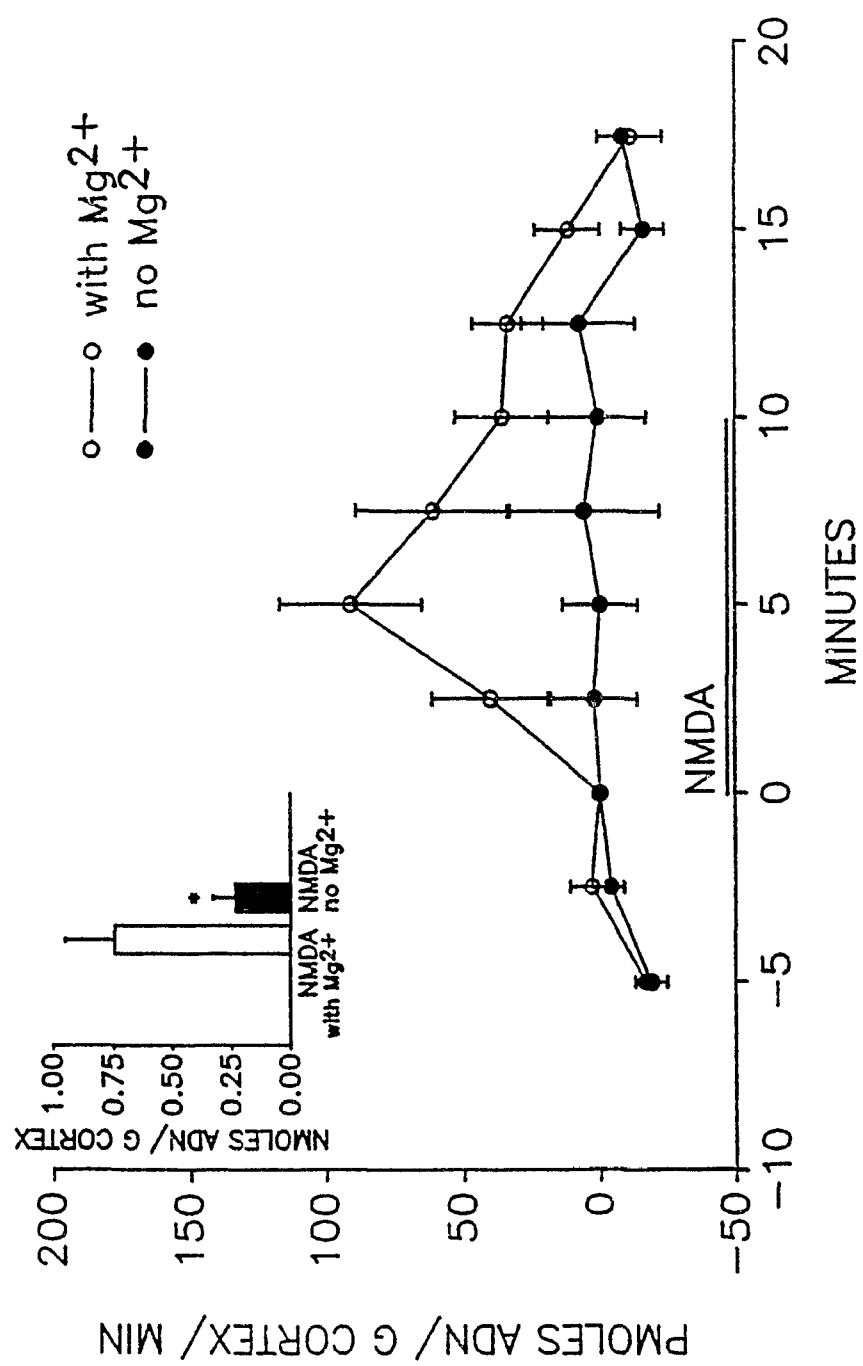


Figure 10

Figure 11. Effects of the nonspecific EAA receptor antagonist, DGG, and the non-NMDA receptor antagonist, DNQX, on kainate-evoked adenosine (ADN) release from superfused slices. **A:** Kainate (KA, 100 μ M) was present from 0 to 10 min. DGG (2 mM) was present from 7.5 min prior to exposure to kainate until the end of the experiment. Values are the means \pm SEM from 5 experiments. **B:** Kainate (50 μ M) was present from 0 to 10 min. DNQX (10 μ M) was present from 20 min prior to exposure to kainate until the end of the experiment. Values are the means \pm SEM from 5 experiments. **INSETS:** Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

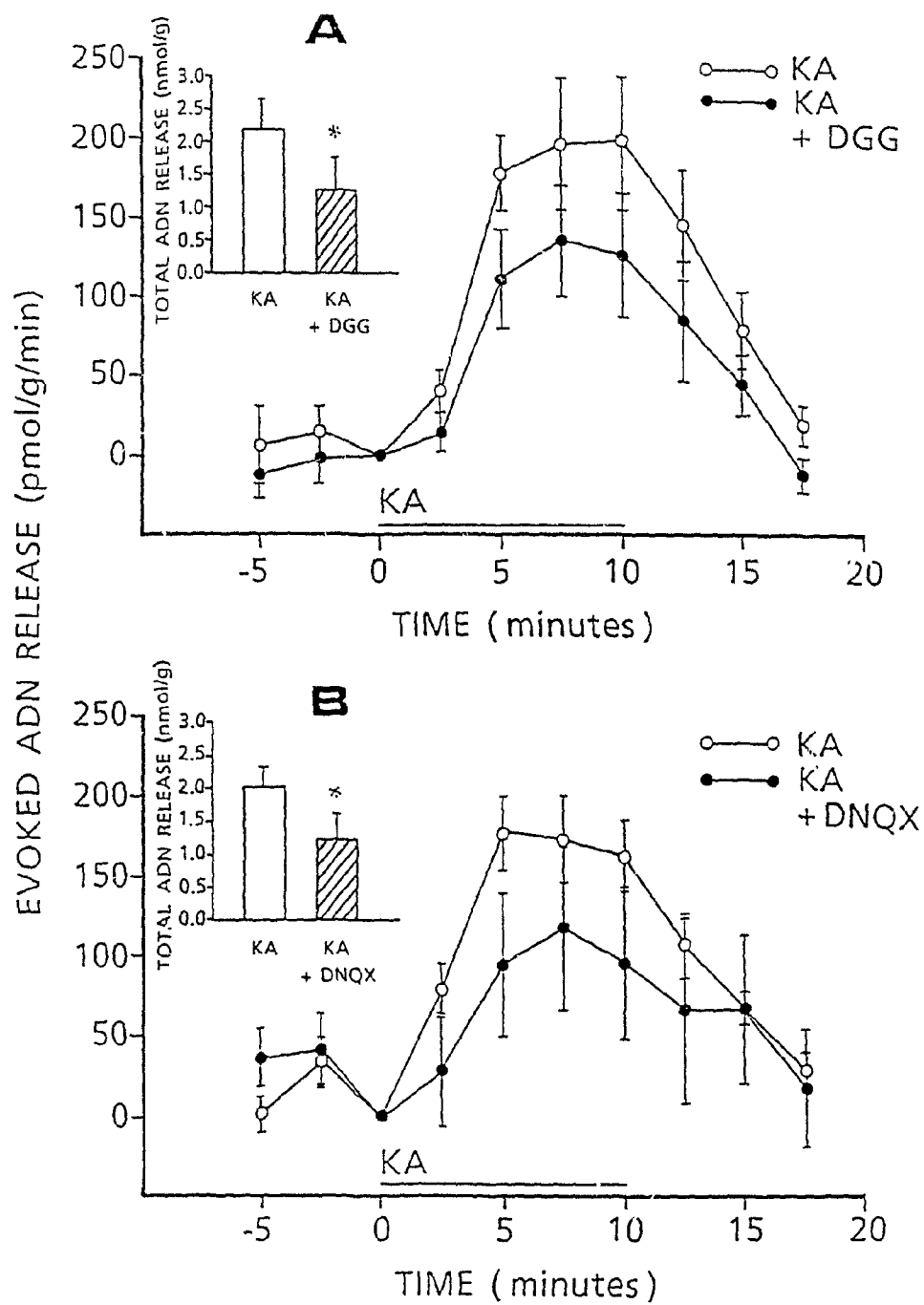


Figure 11

TABLE 4. Effects of the NMDA antagonists, APV and MK-801, on kainate-evoked release of adenosine from cortical slices.

ANTAGONIST		ADENOSINE RELEASE (nmol/g cortex)
A.		
Kainate (100 μ M)	-	1.88 ± 0.32
Kainate (100 μ M)	APV (1 mM)	1.64 ± 0.38 (NS)
B.		
Kainate (50 μ M)	-	1.56 ± 0.46
Kainate (50 μ M)	MK-801 (3 μ M)	1.40 ± 0.41 (NS)

Slices were exposed to kainate in the presence and absence of either APV (A) or MK-801 (B) in separate experiments. APV or MK-801 was present from 7.5 or 20 min, respectively prior the exposure to kainate until the end of the experiment. Data are means \pm SEM of 3 experiments. (NS) No significant difference from release in the absence of antagonist (paired t test).

of the non-NMDA antagonist, DNQX (Fig. 12).

5. Involvement of NMDA and non-NMDA receptors in glutamate-evoked adenosine release.

To determine whether glutamate-evoked adenosine release was mediated through NMDA receptors, the effects of the competitive NMDA receptor antagonist, APV (1 mM), and the uncompetitive NMDA receptor antagonist, MK-801 (3 μ M), on glutamate-evoked adenosine release were determined. APV and MK-801 decreased glutamate-evoked adenosine release by 50 and 55% respectively (Fig. 13), indicating that NMDA receptors mediated at least 50% of glutamate-evoked adenosine release. However, Mg^{2+} -free medium, did not augment glutamate-evoked adenosine release (Table 5). The lack of Mg^{2+} -sensitivity was also seen for NMDA-evoked adenosine release, where it was investigated in detail.

The effects of the addition of the non-NMDA antagonist, DNQX, on glutamate-evoked adenosine release in the presence of NMDA receptor blockade by MK-801 (3 μ M) was determined. DNQX (10 μ M) diminished glutamate-evoked adenosine release by a further 66% (Fig. 14) and 50 μ M DNQX virtually abolished glutamate-evoked adenosine release (Fig. 15), suggesting that non-NMDA receptors also mediated part of the glutamate-evoked release of adenosine.

6. Involvement of NMDA but not non-NMDA receptors in K^+ -evoked adenosine release.

Because elevated extracellular K^+ releases both glutamate and aspartate from rat brain slices (Crowder et al., 1987; Burke and Nadler, 1988; Connick and Stone, 1988a, 1988b), it seemed possible that part of the K^+ -evoked release of adenosine might be mediated indirectly via the

Figure 12. Effects of the non-NMDA receptor antagonist, DNQX, on quisqualate-evoked adenosine (ADN) release from superfused slices. Quisqualate (QU, 50 μ M) was present from 0 to 10 min. DNQX (10 μ M) was present from 20 min prior to exposure to quisqualate until the end of the experiment. Values are the means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released.

*Significantly different from control ($p < 0.05$, paired t test).

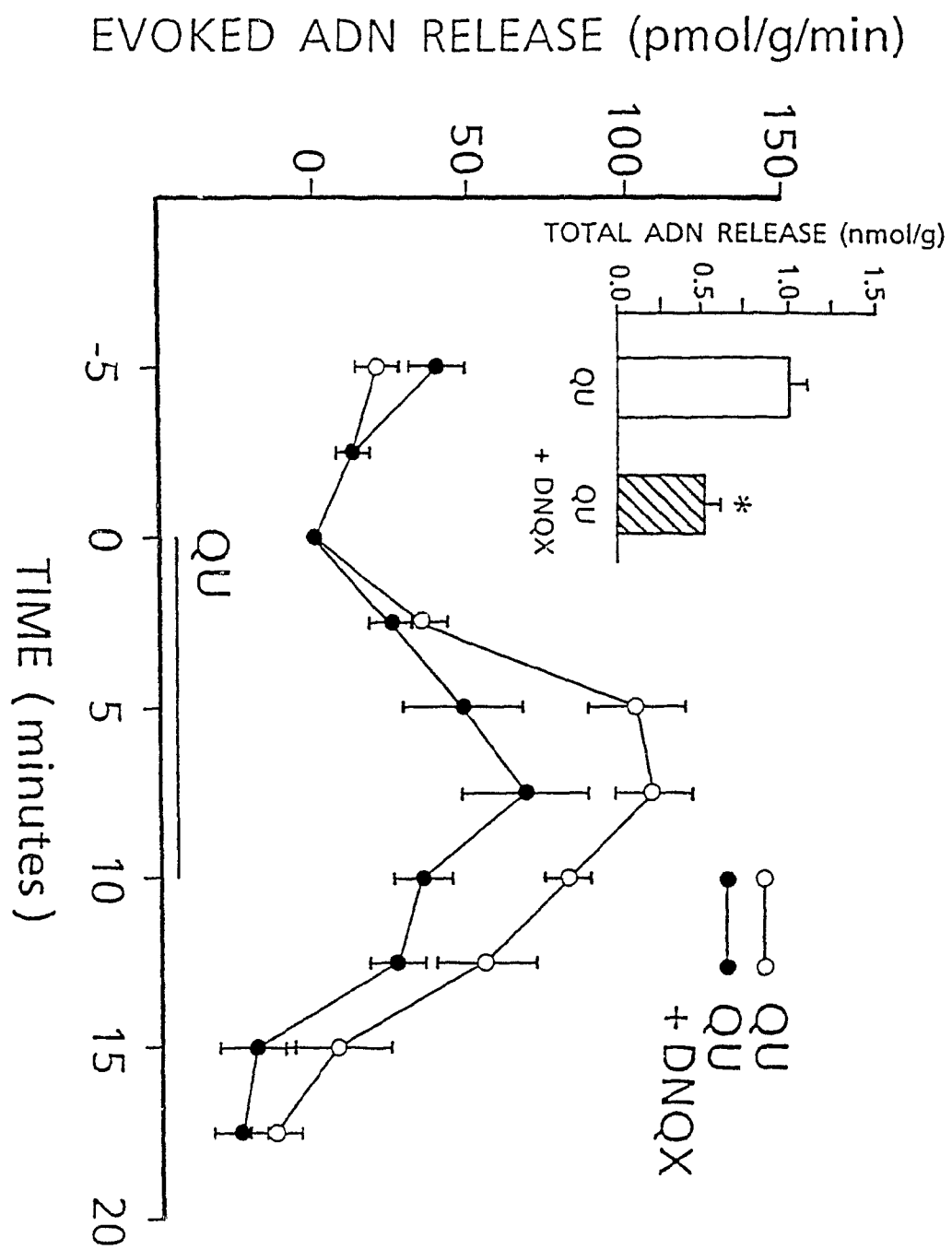


Figure 12

Figure 13. Effect of NMDA antagonists on glutamate-evoked adenosine (ADN) release from superfused slices. Glutamate (GLU, 1.5 mM) was present from 0 to 10 min. A: APV (1 mM) was present from 7.5 min prior to exposure to glutamate until the end of the experiment. Values are the means \pm SEM from 7 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test). B: MK-801 (3 μ M) was present from 20 min prior to exposure to glutamate until the end of the experiment. Values are the means \pm SEM from 3 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

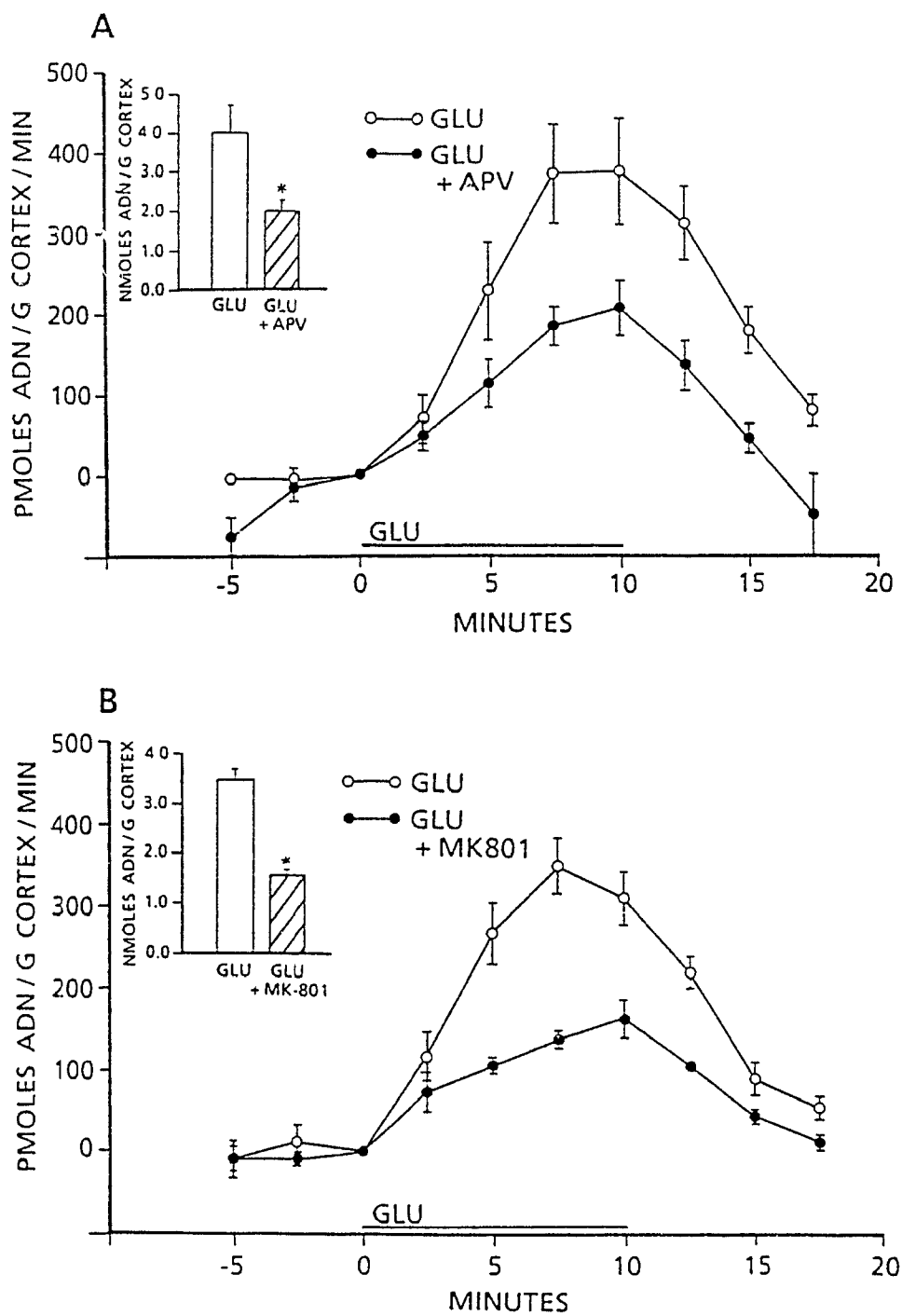


Figure 13

TABLE 5. Effect of Mg^{2+} -free medium on glutamate-evoked release of adenosine from cortical slices.

	ADENOSINE RELEASE (nmol/g cortex)	
	1.2 mM Mg^{2+}	Mg^{2+} -FREE
Glutamate (5 mM)	4.41 ± 0.52	4.48 ± 0.56 (NS)

Slices were prepared in 1.2 mM Mg^{2+} -containing medium. Both "control" and " Mg^{2+} -free" slices were then superfused with Mg^{2+} -free medium until 20 min prior to exposure to glutamate, when Mg^{2+} (1.2 mM) was restored to "control" slices. Values are means \pm SEM from 3 experiments. (NS) No significant difference from release in the presence of Mg^{2+} (paired t test).

Figure 14. Effect of the non-NMDA antagonist, DNQX (10 μ M), on glutamate-evoked adenosine (ADN) release from superfused slices in the presence of MK-801. Glutamate (GLU, 1.5 mM) was present from 0 to 10 min. DNQX and MK-801 (3 μ M) were present from 20 min prior to exposure to glutamate until the end of the experiment. Values are the means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

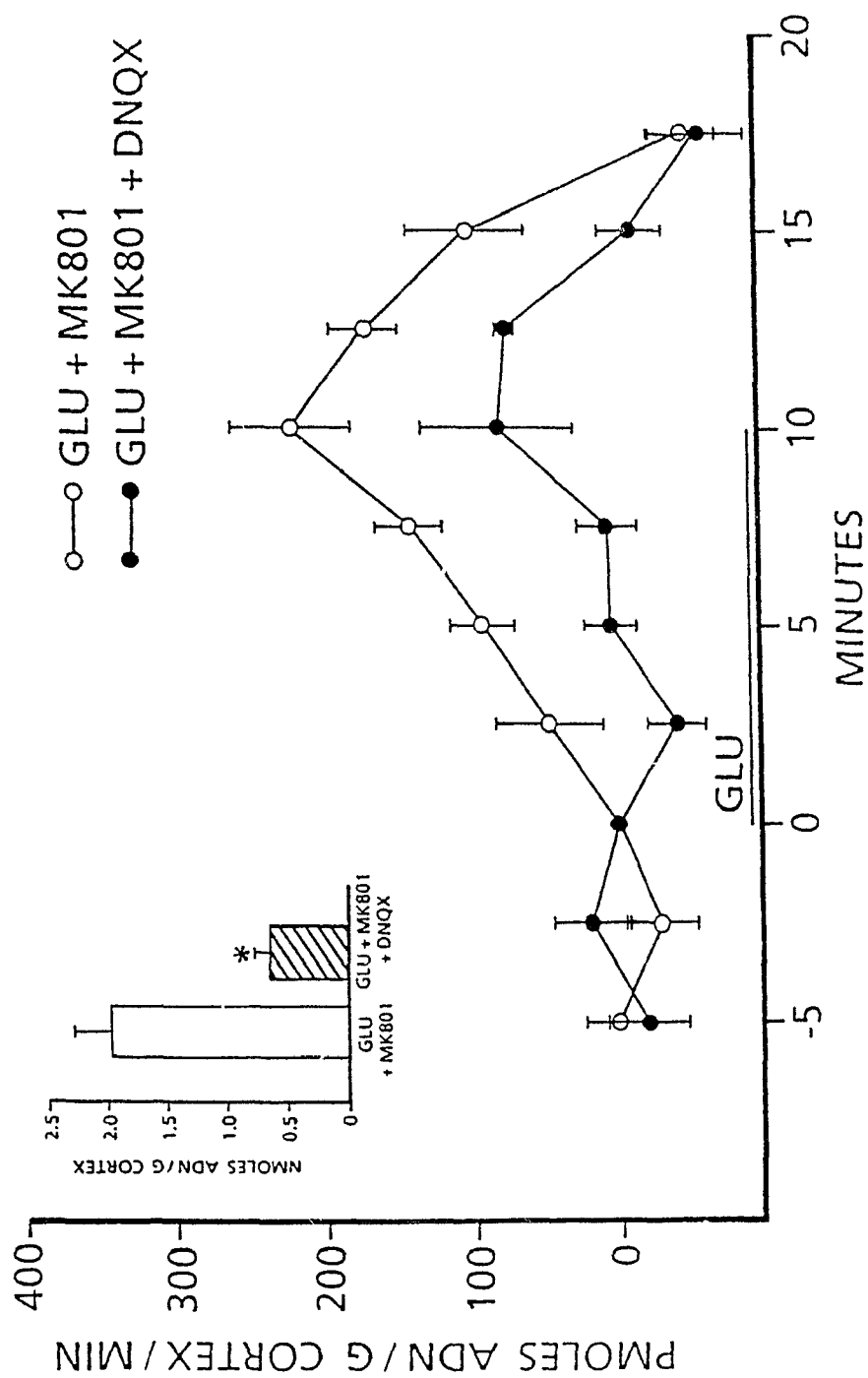


Figure 14

Figure 15. Effect of the non-NMDA antagonist, DNQX (50 μ M), on glutamate-evoked adenosine (ADN) release from superfused slices in the presence of MK-801. Glutamate (GLU, 1.5 mM) was present from 0 to 10 min. DNQX and MK-801 (3 μ M) were present from 20 min prior to exposure to glutamate until the end of the experiment. Values are the means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

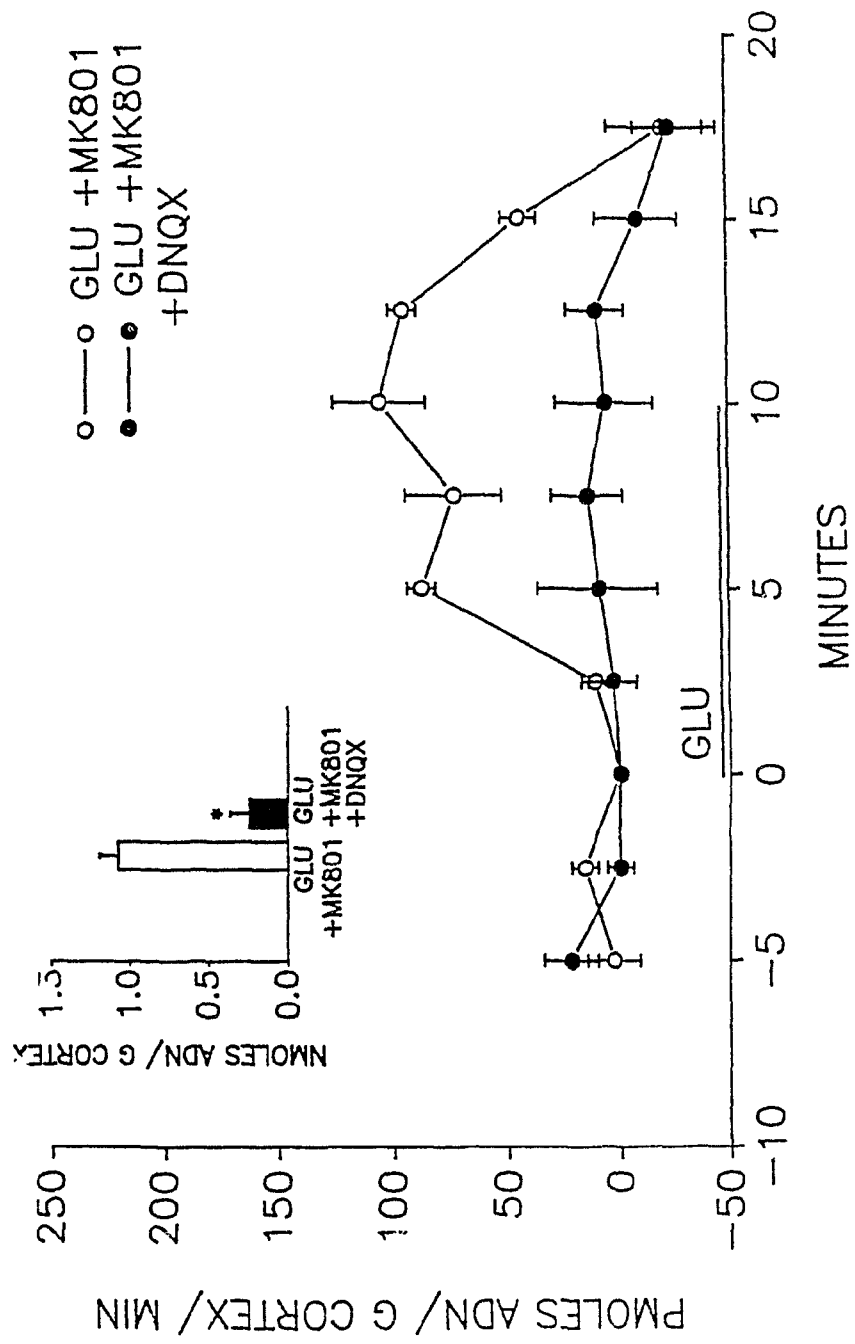


Figure 15

release of an endogenous EAA. Indeed, K^+ -evoked adenosine release was diminished by 31 and 32% when NMDA receptors were blocked by either APV (1 mM) or MK-801 (3 μ M), respectively (Fig. 16), suggesting that a substantial proportion of K^+ -evoked adenosine release was mediated through NMDA receptors. The addition of 10 μ M DNQX in the presence of MK-801 did not further diminish K^+ -evoked adenosine release (Fig. 17), indicating that actions of released EAAs at non-NMDA receptors probably did not contribute to adenosine release evoked by K^+ depolarization.

7. Lack of involvement of NMDA receptors in K^+ -evoked [3 H]noradrenaline release.

The possibility that a proportion of K^+ -evoked release of [3 H]noradrenaline might also be mediated indirectly through NMDA receptors was investigated. K^+ -evoked [3 H]noradrenaline release was not diminished, but was in fact increased by 31% in the presence of 3 μ M MK-801 (Fig. 18), indicating that, unlike K^+ -evoked adenosine release, K^+ -evoked [3 H]noradrenaline release is not secondary to released EAAs acting at NMDA receptors.

8. TTX-sensitivity of adenosine release evoked by K^+ and EAA agonists.

To determine whether propagated action potentials were involved in the adenosine release evoked by K^+ and glutamate, release was studied during blockade of voltage-dependent Na^+ channels by TTX. TTX (1 μ M) did not affect basal release of adenosine (not shown). However, TTX diminished K^+ -evoked adenosine release by 44% (Fig. 19), indicating that a substantial proportion of K^+ -evoked adenosine release is mediated indirectly through propagated action potentials. Similarly, TTX diminished glutamate, NMDA, and kainate-evoked adenosine release by 21,

Figure 16. Effect of NMDA antagonists on K^+ -evoked adenosine (ADN) release from superfused slices. K^+ (30 mM) was present from 0 to 10 min. A: APV (1 mM) was present from 7.5 min prior to exposure to K^+ until the end of the experiment. Values are the means \pm SEM from 6 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test). B: MK-801 (3 μ M) was present from 20 min prior to exposure to K^+ until the end of the experiment. Values are means \pm SEM from 6 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

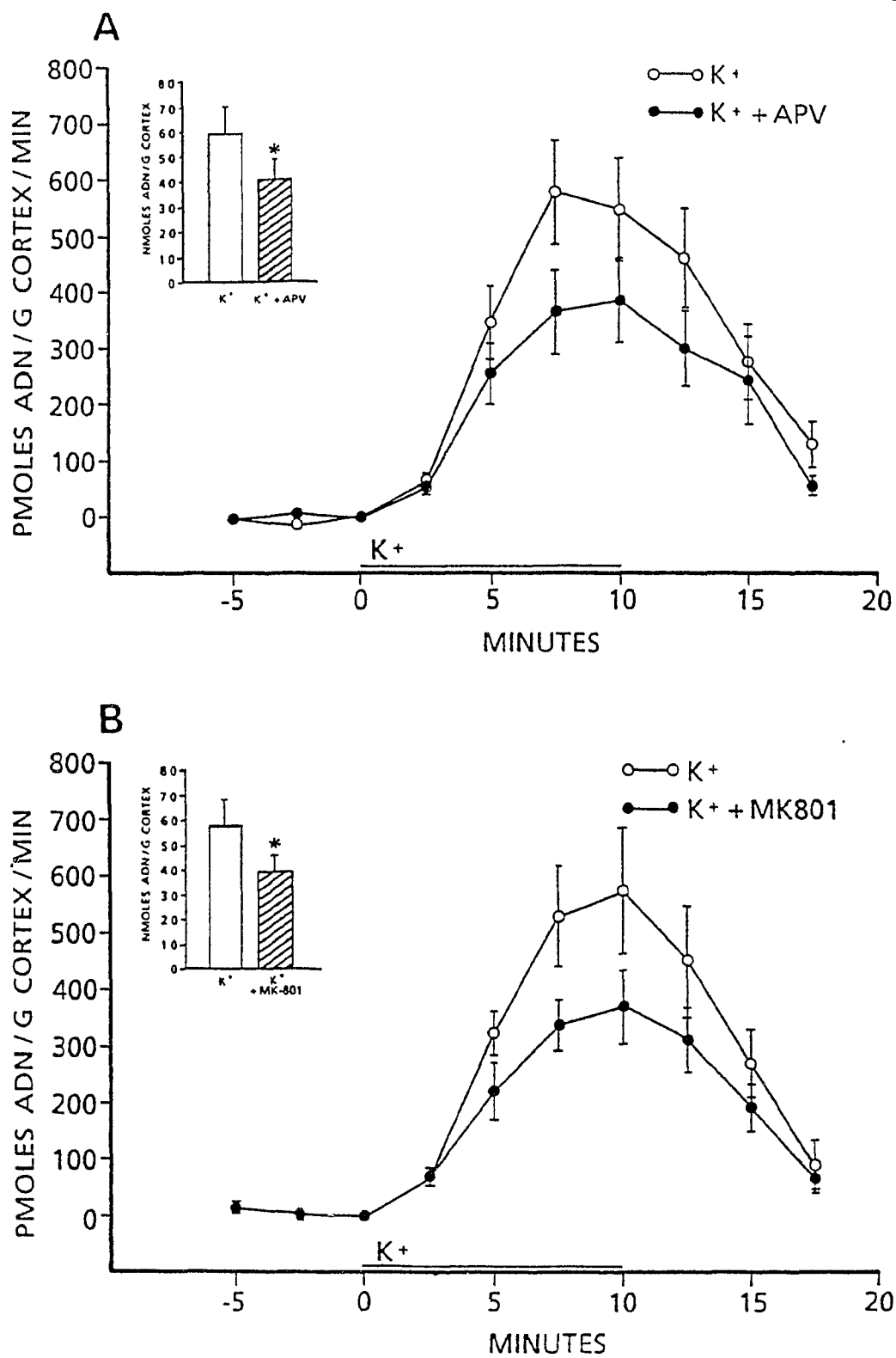


Figure 16

Figure 17. Effect of the non-NMDA antagonist, DNQX (10 μ M), on K^+ -evoked adenosine (ADN) release from superfused slices in the presence of MK-801. K^+ (30 mM) was present from 0 to 10 min. DNQX and MK-801 (3 μ M) were present from 20 min prior to exposure to K^+ until the end of the experiment. Values are the means \pm SEM from 3 experiments. INSET: Histograms represent the total amount of adenosine released.

Figure 17

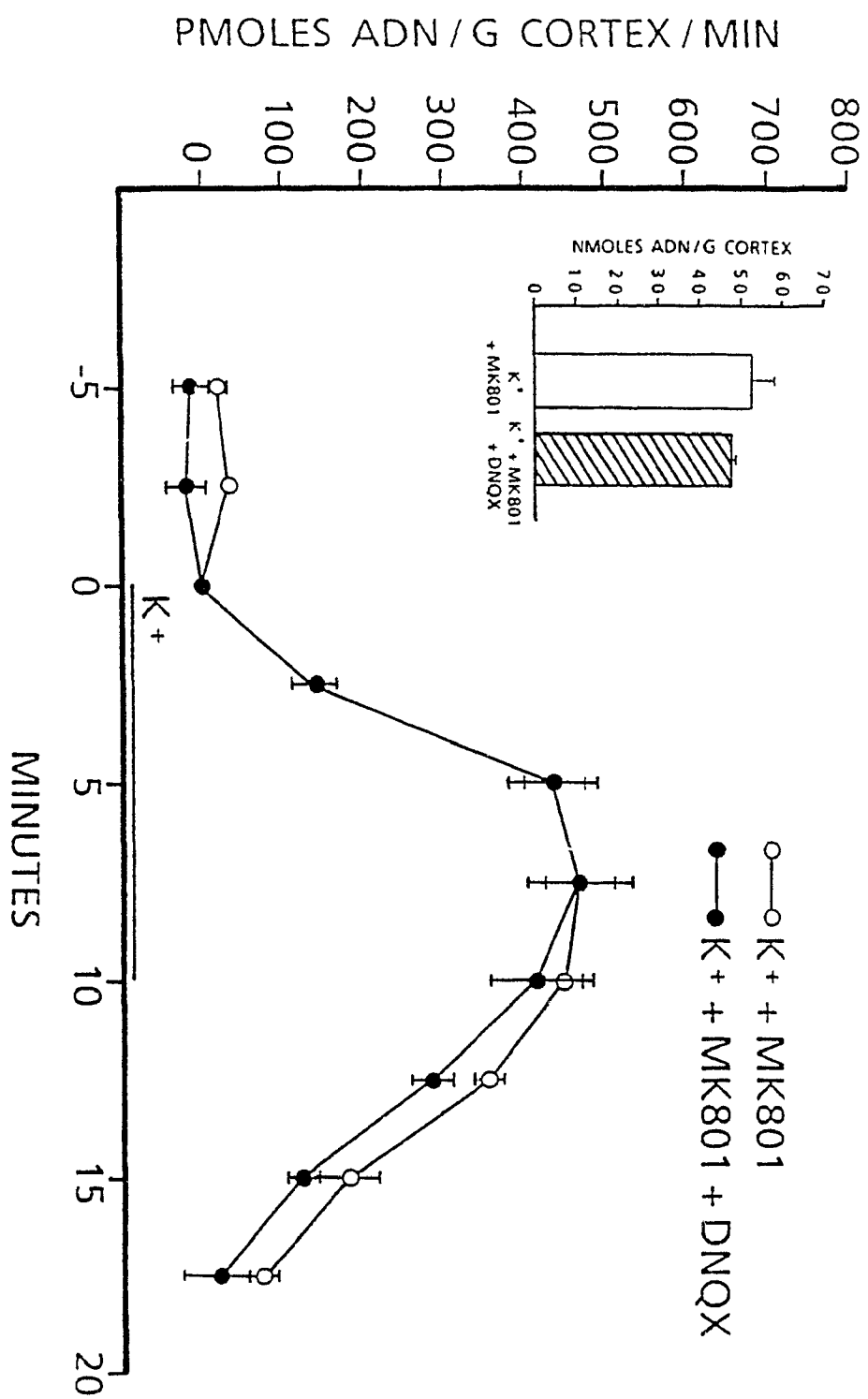


Figure 18. Effect of the NMDA antagonist, MK-801, on K^+ -evoked [3H]noradrenaline ([3H]NA) release from superfused slices. Slices were labelled with [3H]noradrenaline by incubation for 30 min in 10 ml of oxygenated Krebs-Henseleit bicarbonate medium containing 10^{-7} M [3H]noradrenaline as described in METHODS, Section II.3. Slices were then placed in the superfusion baths and the remainder of the experiment was conducted as for adenosine release. K^+ (30 mM) was present from 0 to 10 min. MK-801 (3 μ M) was present from 20 min prior to exposure to K^+ until the end of the experiment. Values are the means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

Figure 18

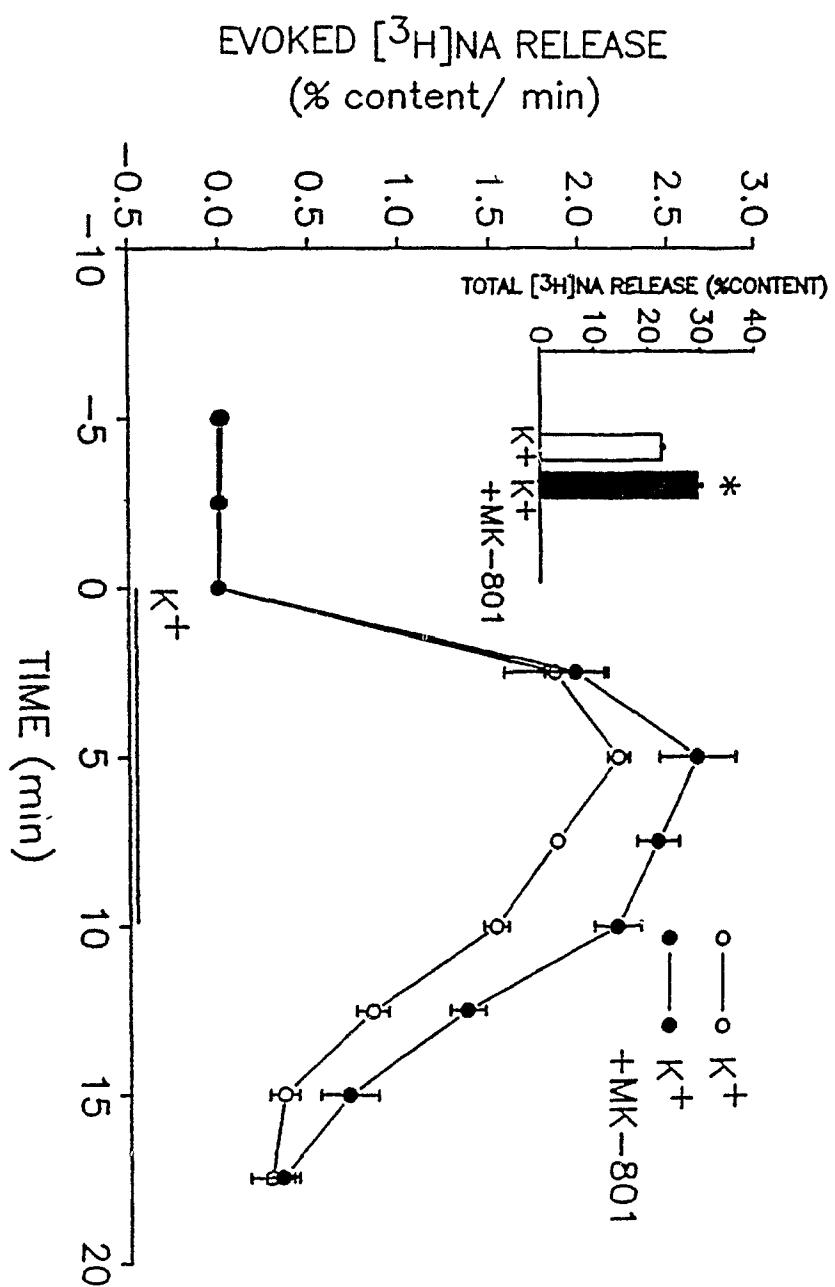
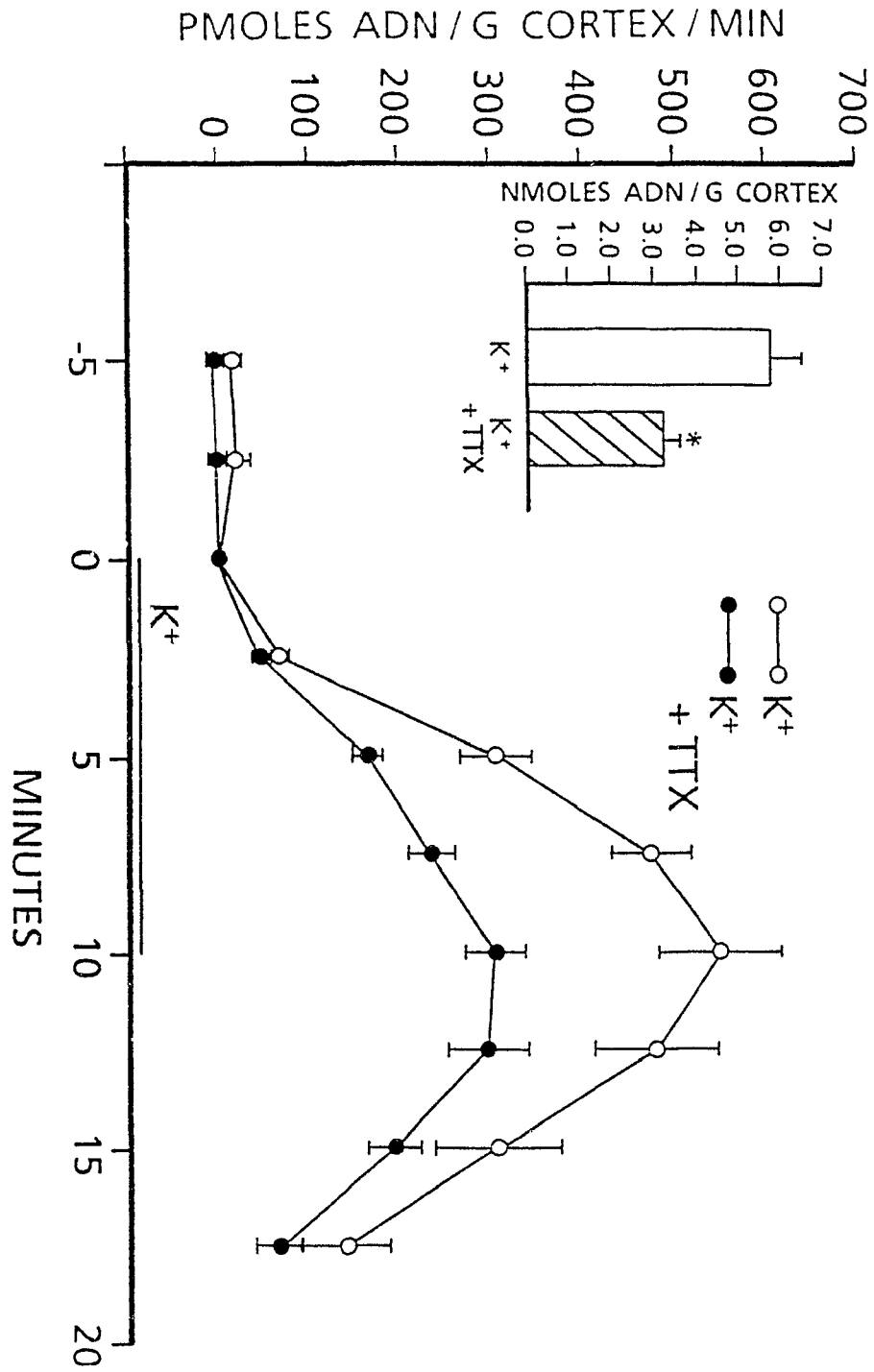


Figure 19. Effect of TTX on K^+ -evoked adenosine (ADN) release from superfused slices. K^+ (30 mM) was present from 0 to 10 min. TTX ($1 \mu M$) was present from 20 min prior to K^+ exposure until the end of the releasing period. Values are means \pm SEM from 4 experiments. **INSET:** Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

Figure 19



40, and 19%, respectively (Figs. 20 and 21), indicating that propagated action potentials were also involved, to some extent, in release evoked by these EAA receptor agonists.

9. Ca^{2+} -dependence of adenosine release.

A. Ca^{2+} -dependence of K^{+} -evoked adenosine release. The basal (unstimulated) rate of adenosine release was increased in Ca^{2+} -free medium from 215 ± 45 to 422 ± 45 pmol/g of cortex/min ($p < 0.05$, paired t test, 9 experiments). However, K^{+} -evoked release of adenosine was diminished in Ca^{2+} -free medium (Fig. 22), thus resembling the evoked release of classical neurotransmitters. The rate of K^{+} -evoked adenosine release peaked earlier in Ca^{2+} -free medium, but was less sustained than in the presence of Ca^{2+} .

It seemed possible, considering the elevation in basal adenosine release that occurred in the absence of Ca^{2+} , that the diminished K^{+} -evoked release could have been due to depletion of a releasable adenosine pool, or to damage of the slices as a result of preincubation in Ca^{2+} -free medium. To test this, Ca^{2+} was restored after the initial K^{+} depolarization and the slices were exposed for another 10 min to elevated K^{+} . Slices that originally had been incubated in the absence of Ca^{2+} exhibited a marked increase in evoked adenosine release during the subsequent depolarization in the presence of Ca^{2+} which was, in time course as well as in quantity, comparable to control K^{+} -evoked release in the presence of Ca^{2+} (Fig. 22). Therefore, it would appear that adenosine had not been depleted nor had the slices been damaged as a result of preincubation in the absence of Ca^{2+} . Slices that had been depolarized initially in the presence of Ca^{2+} showed a marked decrease in K^{+} -evoked release during a second K^{+} depolarization, suggesting that the initial K^{+}

Figure 20. Effect of TTX on glutamate-evoked adenosine (ADN) release from superfused slices. Glutamate (GLU, 1.5 mM) was present from 0 to 10 min. TTX (1 μ M) was present from 20 min prior to glutamate exposure until the end of the releasing period. Values are means \pm SEM from 5 experiments. INSET: Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

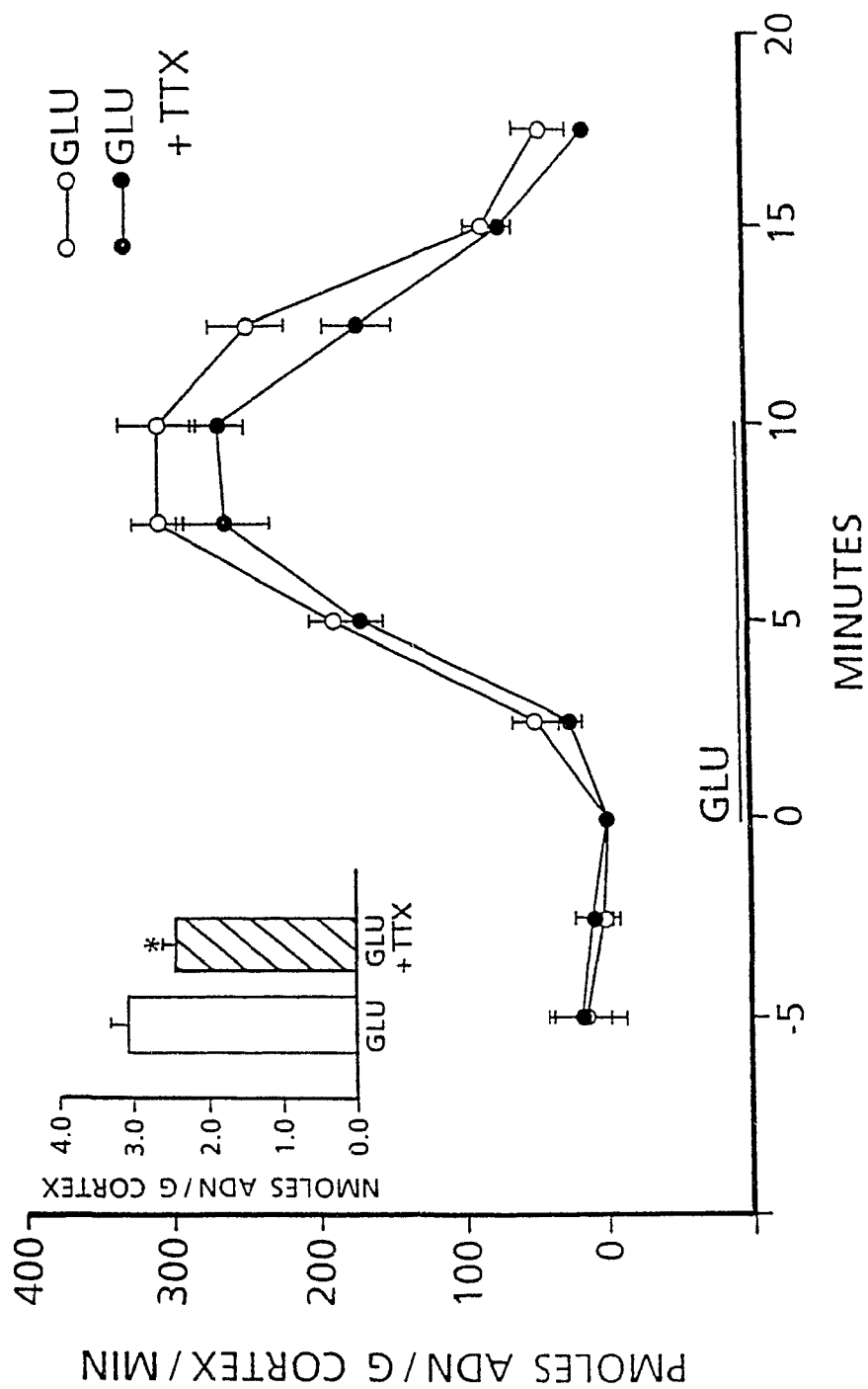


Figure 20

Figure 21. Effect of TTX on NMDA-and kainate-evoked adenosine (ADN) release from superfused slices. TTX ($1\ \mu\text{M}$) was present from 20 min prior to exposure to NMDA or kainate (KA) until the end of the experiment. A: NMDA ($500\ \mu\text{M}$) was present from 0 to 10 min. Values are means \pm SEM from 5 experiments. **INSETS:** Histograms represent the total amount of adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

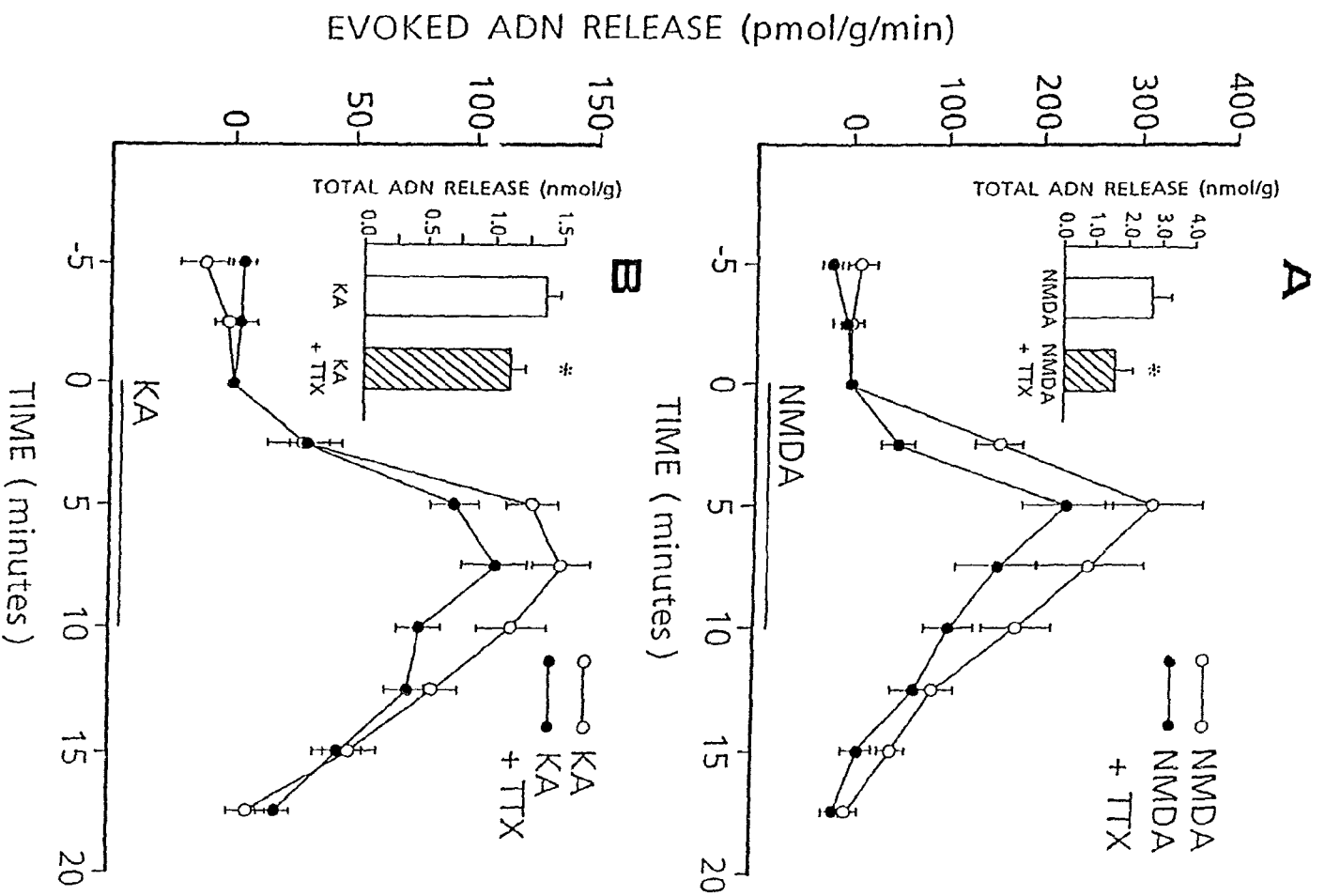


Figure 21

Figure 22. Ca^{2+} -dependence of K^+ -evoked release of adenosine (ADN) from superfused slices. Slices were exposed to K^+ (30 mM) from 0 to 10 min and from 45 to 55 min. Control slices were perfused with Ca^{2+} -containing medium for the entire experiment. "Test" slices were perfused with Ca^{2+} -free medium from the beginning of the experiment until after the first K^+ depolarization. At 17.5 min, Ca^{2+} was restored to "test" slices and was present for the remainder of the experiment. Values are the means \pm SEM from 5 experiments. TOP: Control slices (O--O, Ca^{2+} present throughout); "test" slices (\bullet -- \bullet , Ca^{2+} absent during first K^+ depolarization). BOTTOM: Histograms represent the total amount of adenosine released during the first (S1) and second (S2) stimulation periods. *Significantly different from control slices ($p < 0.05$, paired t test). $^+$ Significantly different from corresponding S1 ($p < 0.05$, paired t test).

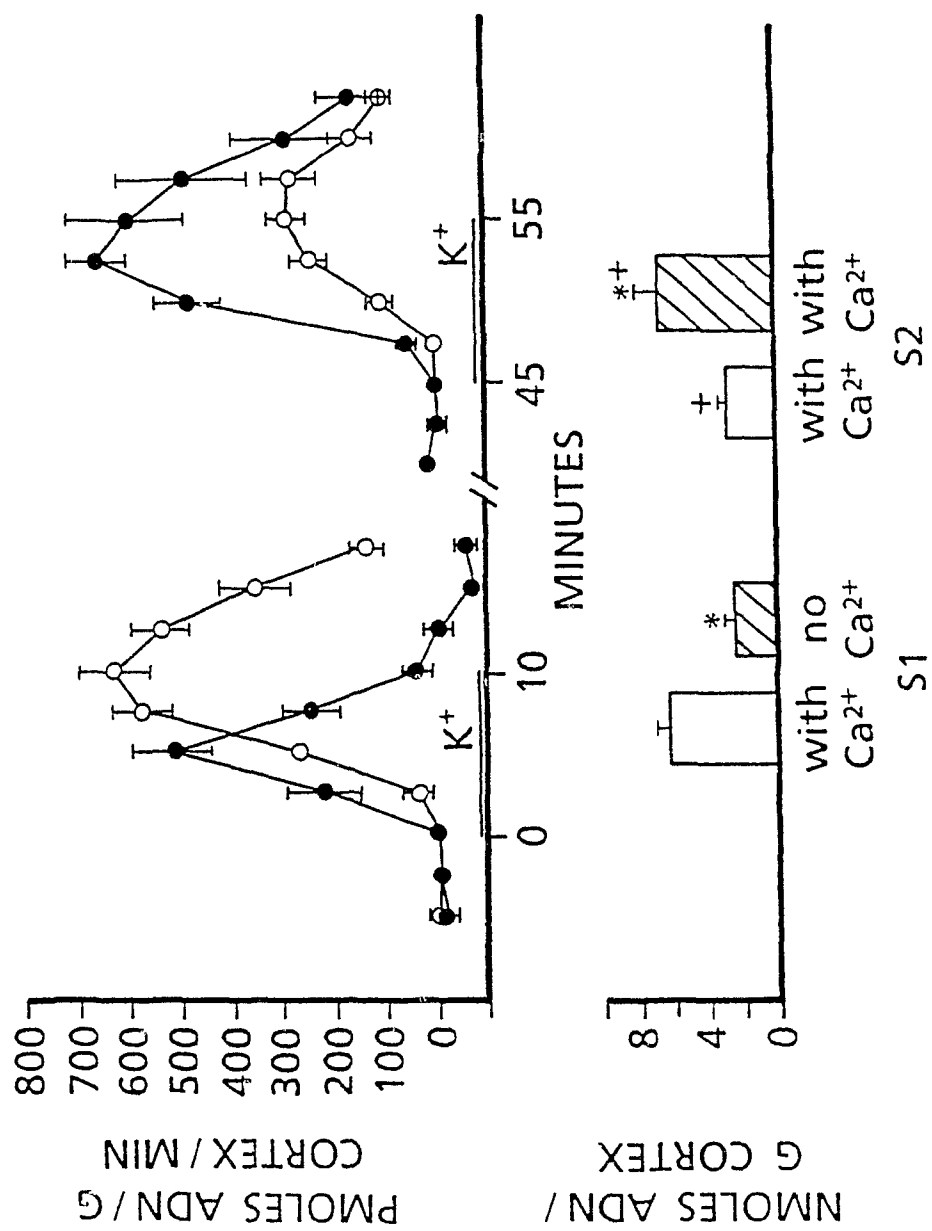


Figure 22

depolarization may have depleted the releasable pool of adenosine.

B. Ca^{2+} -dependence of glutamate-evoked adenosine release. In contrast with K^+ -evoked adenosine release, glutamate-evoked adenosine release was not Ca^{2+} -dependent (Fig. 23). There was, however, a change in the time course for adenosine release in the absence of Ca^{2+} , which was similar to that seen with K^+ -evoked release. Thus, the rate of glutamate-evoked release of adenosine at 2.5 and 5 min was greater in the absence than in the presence of Ca^{2+} . Following restoration of Ca^{2+} , a second exposure to glutamate resulted in a comparable time course and amount of adenosine release from both sets of slices. Release was diminished during the second exposure to glutamate as compared with the first exposure, which may have been due to depletion of the releasable pool of adenosine or to desensitization of EAA receptors. (Bernstein and Fisher, 1985; Krishtal et al., 1988; but see Hori and Carpenter, 1988).

C. Ca^{2+} -dependence of NMDA-evoked adenosine release. Similarly to glutamate-evoked adenosine release, there was no difference in the total amount of adenosine release evoked by NMDA in the presence or absence of Ca^{2+} (Fig. 24). Moreover, there was no difference in the time course of NMDA-evoked adenosine release in the absence of Ca^{2+} , indicating that NMDA-evoked adenosine release was not Ca^{2+} -dependent. A second stimulation by NMDA of slices which had initially been stimulated in the presence of Ca^{2+} was virtually ineffective at releasing adenosine (Fig. 24). However, when slices which had originally been stimulated in the absence of Ca^{2+} were exposed to NMDA for a second time in the presence of Ca^{2+} , they released an amount of adenosine equal to approximately 50% of the initial release. The observation that an initial NMDA exposure in the absence of Ca^{2+}

Figure 23. Ca^{2+} -dependence of glutamate-evoked release of adenosine (ADN) from superfused slices. Slices were exposed to glutamate (GLU, 5 mM) from 0 to 10 min and from 45 to 55 min. Control slices were perfused with Ca^{2+} -containing medium for the entire experiment. "Test" slices were perfused with Ca^{2+} -free medium from the beginning of the experiment until after the first glutamate exposure. At 17.5 min, Ca^{2+} was restored to "test" slices and was present for the remainder of the experiment. Values are the means \pm SEM from 4 experiments. TOP: Control slices (O--O, Ca^{2+} present throughout); "test" slices (\bullet -- \bullet , Ca^{2+} absent during first glutamate exposure). *Significantly different from control slices ($p < 0.05$, paired t test). BOTTOM: Histograms represent the total amount of adenosine released during the first (S1) and second (S2) stimulation periods. [†]Significantly different from corresponding S1 ($p < 0.05$, paired t test).

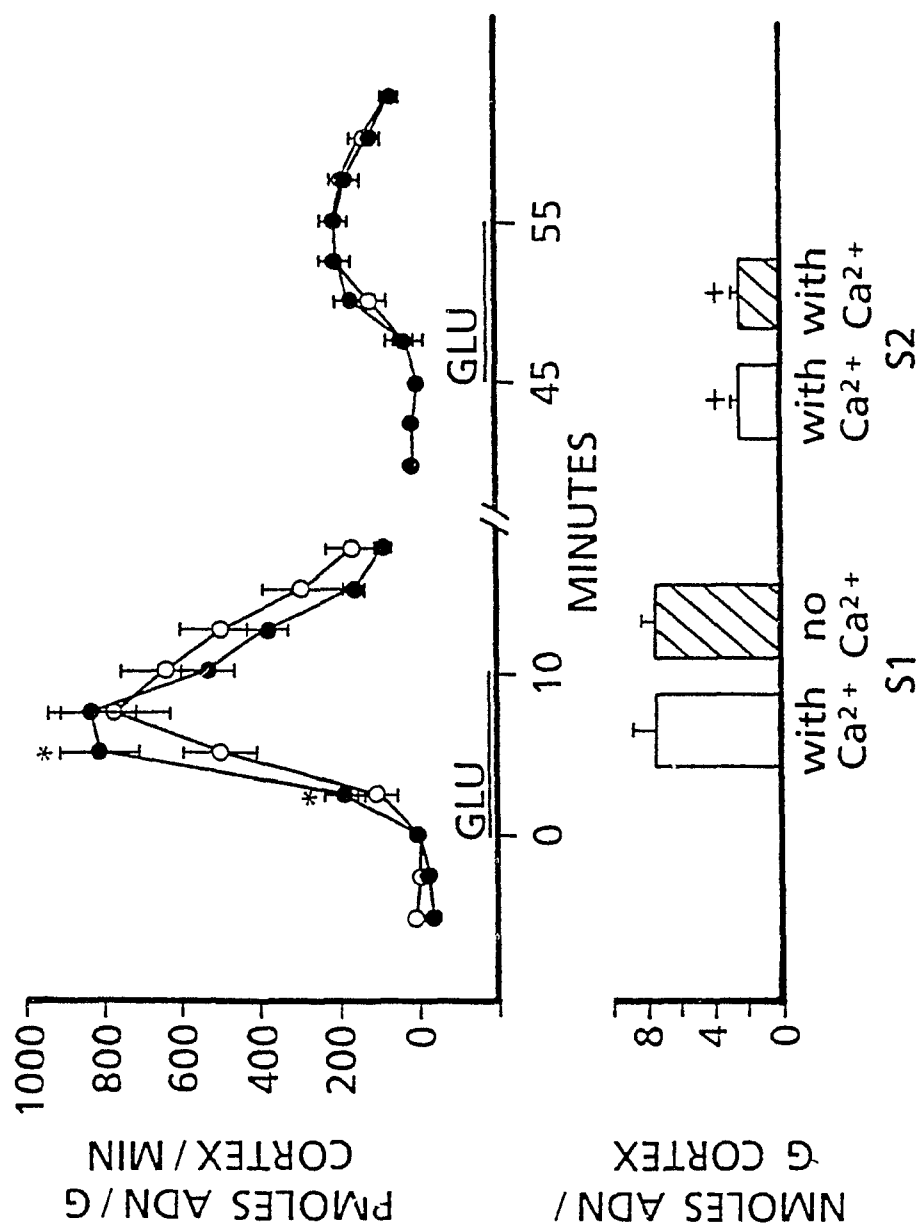
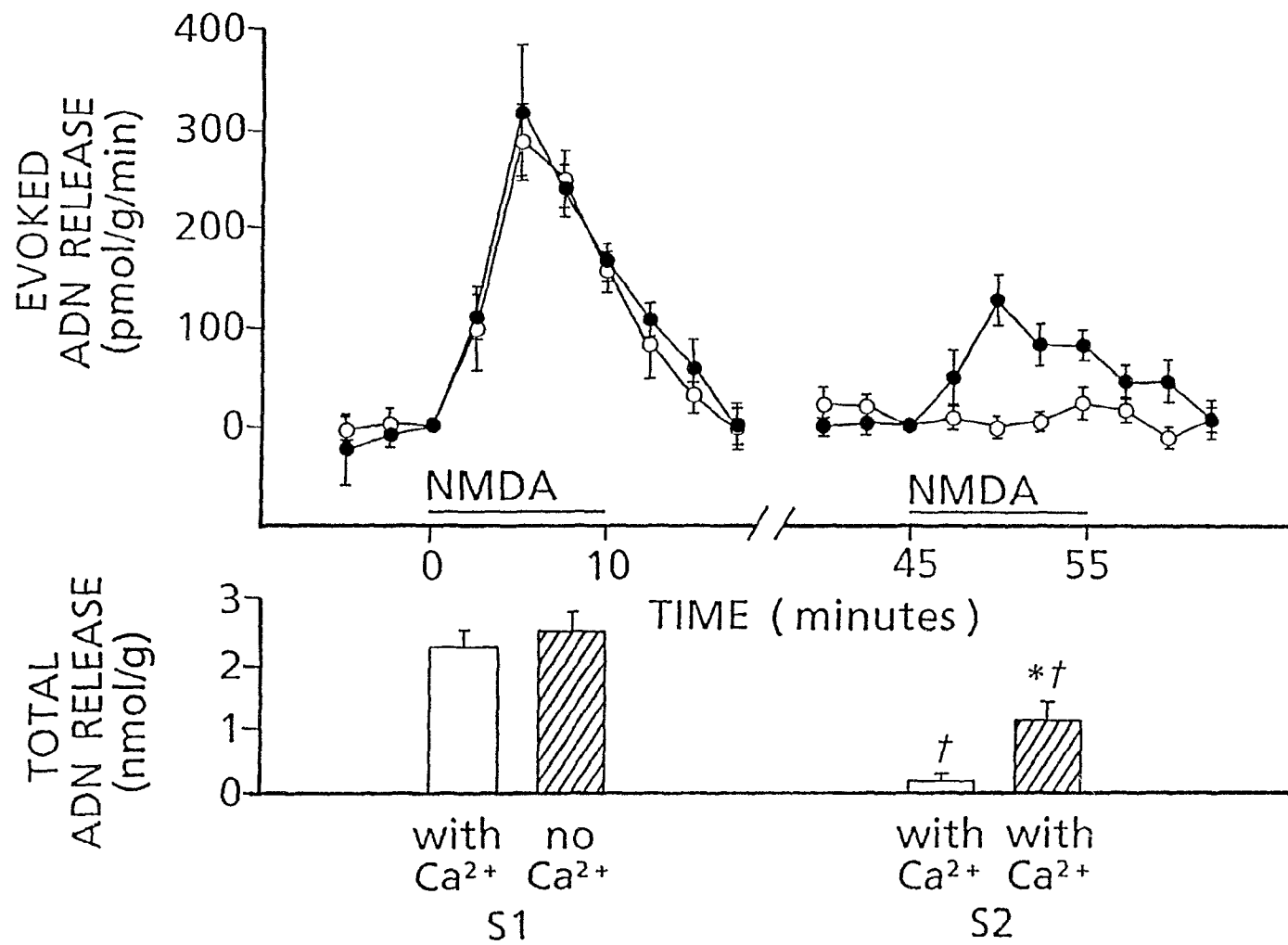


Figure 23

Figure 24. Ca^{2+} -dependence of NMDA-evoked release of adenosine (ADN) from superfused slices. Slices were exposed to NMDA ($500\ \mu\text{M}$) from 0 to 10 min and from 45 to 55 min. Control slices were perfused with Ca^{2+} -containing medium for the entire experiment. "Test" slices were perfused with Ca^{2+} -free medium from the beginning of the experiment until after the first NMDA exposure. At 17.5 min, Ca^{2+} was restored to "test" slices and was present for the remainder of the experiment. Values are the means \pm SEM from 4 experiments. TOP: Control slices (O--O, Ca^{2+} present throughout); "test" slices (\bullet -- \bullet , Ca^{2+} absent during first NMDA exposure). BOTTOM: Histograms represent the total amount of adenosine released during the first (S1) and second (S2) stimulation periods. *Significantly different from control slices ($p < 0.05$, paired t test). † Significantly different from corresponding S1 ($p < 0.05$, paired t test).

Figure 24



protected against loss of NMDA-evoked adenosine release during a second stimulation suggests two possible explanations. NMDA may release adenosine from a Ca^{2+} -dependent pool and also from a separate Ca^{2+} -independent pool. Alternatively, it is possible that desensitization of NMDA receptors, which has been demonstrated in a number of studies (Fagni et al., 1983; Krishtal et al., 1986; Kushner et al., 1988; Lehmann et al., 1988) is somehow prevented in the absence of Ca^{2+} (Mayer and Westbrook, 1987).

D. Ca^{2+} -dependence of quisqualate-evoked adenosine release. As with NMDA and glutamate-evoked adenosine release, quisqualate-evoked adenosine release also appeared to be Ca^{2+} -independent, in so far as the total amount of adenosine released was unaffected by the absence of extracellular Ca^{2+} (Fig. 25). However, quisqualate-evoked adenosine release resembled glutamate-evoked adenosine release in that the rate of adenosine release evoked by quisqualate in the absence of Ca^{2+} reached its maximum earlier and was less sustained than in the presence of Ca^{2+} . During a second 10-min stimulation, quisqualate was ineffective at releasing adenosine either from slices which had been previously stimulated in the absence of Ca^{2+} but to which Ca^{2+} had been restored, or from control slices stimulated in the presence of Ca^{2+} for a second time. The absence of adenosine release by a second exposure to quisqualate may be due to depletion of a releasable pool of adenosine, or to quisqualate receptor desensitization (Kiskin et al., 1986; Mayer and Vyklicky, 1989; Perouansky and Grantyn, 1989).

E. Ca^{2+} -dependence of kainate-evoked adenosine release. Kainate-evoked adenosine release also appeared to be Ca^{2+} -independent (Fig. 26A), in so far as the total amount of adenosine released by kainate was

Figure 25. Ca^{2+} -dependence of quisqualate-evoked release of adenosine (ADN) from superfused slices. Slices were exposed to quisqualate (QU, 500 μM) from 0 to 10 min and from 45 to 55 min. Control slices were perfused with Ca^{2+} -containing medium for the entire experiment. "Test" slices were perfused with Ca^{2+} -free medium from the beginning of the experiment until after the first NMDA exposure. At 17.5 min, Ca^{2+} was restored to "test" slices and was present for the remainder of the experiment. Values are the means \pm SEM from 3 experiments. TOP: Control slices (O--O, Ca^{2+} present throughout); "test" slices (\bullet -- \bullet , Ca^{2+} absent during first quisqualate exposure). BOTTOM: Histograms represent the total amount of adenosine released during the first (S1) and second (S2) stimulation periods. [†]Significantly different from corresponding S1 ($p < 0.05$, paired t test).

Figure 25

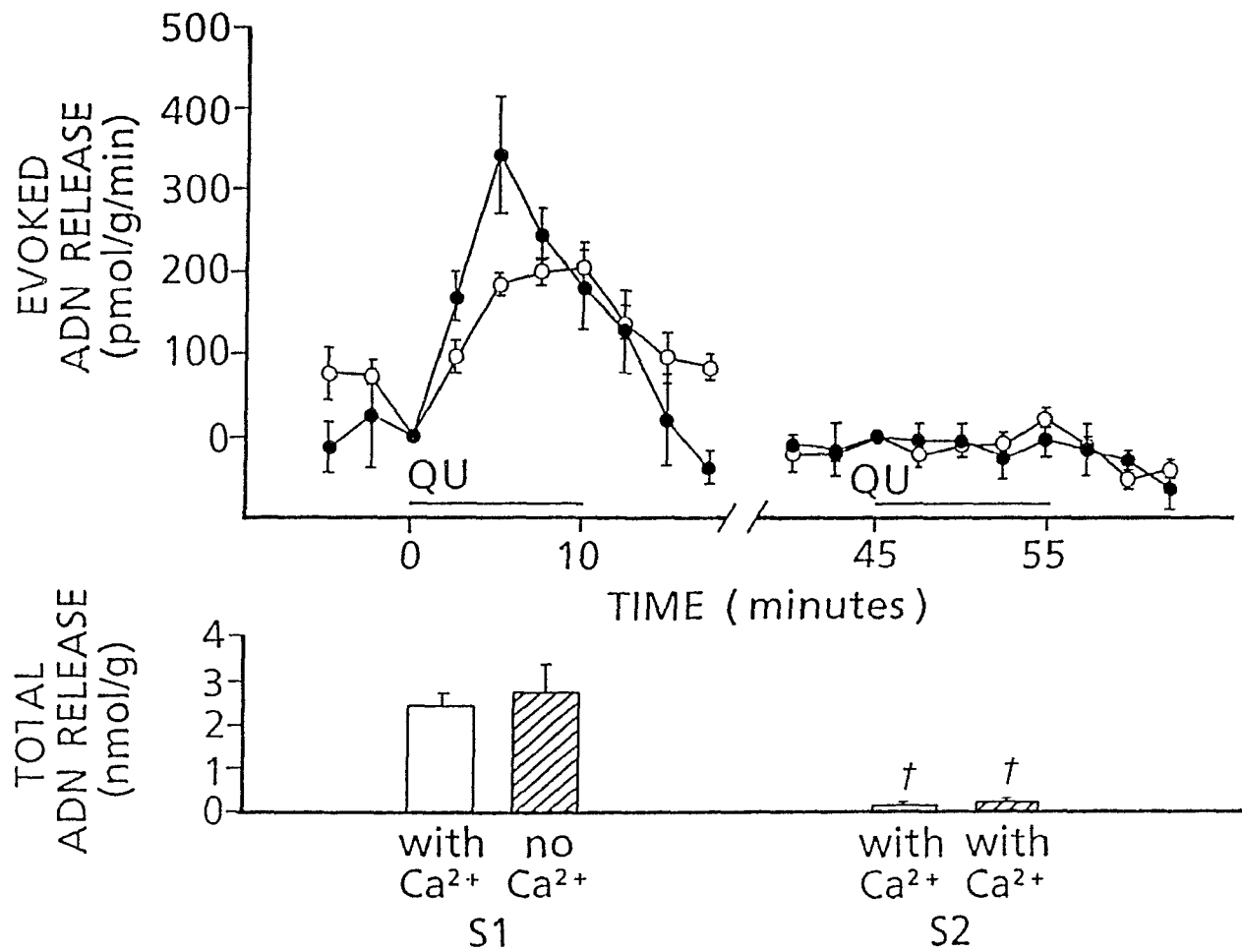


Figure 26. Ca^{2+} -dependence of kainate-evoked release of adenosine (ADN) from superfused slices. Slices were exposed to kainate (KA, 100 μM) from 0 to 10 and from 45 to 55 min. Histograms on the bottom of each graph represent the total amount of adenosine released during the first (S1) and second (S2) stimulation periods. A: Control slices were perfused with Ca^{2+} -containing medium for the entire experiment. "Test" slices were perfused with Ca^{2+} -free medium from the beginning of the experiment until after the first kainate exposure. At 17.5 min, Ca^{2+} was restored to "test" slices and was present for the remainder of the experiment. Values are the means \pm SEM from 4 experiments. Control slices (O--O, Ca^{2+} present throughout); "test" slices (\bullet -- \bullet , Ca^{2+} absent during first kainate exposure). B: Slices were perfused for the entire experiment with Ca^{2+} -free medium. Values are the means \pm SEM from 4 experiments. *Significantly different from control slices ($p < 0.05$, paired t test). [†]Significantly different from corresponding S1 ($p < 0.05$, paired t test).

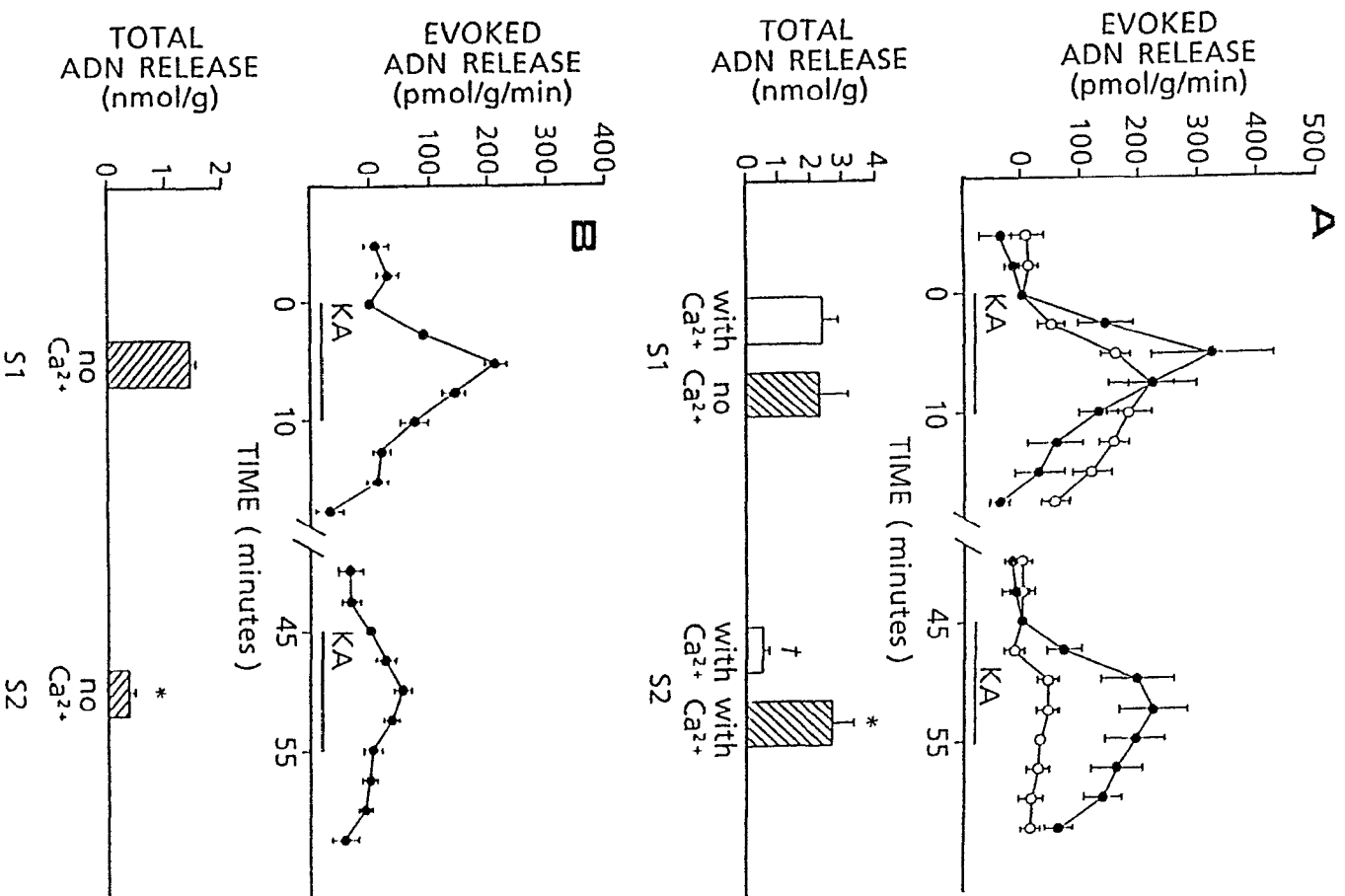


Figure 26

unaffected by the absence of extracellular Ca^{2+} . However, the rate of kainate-evoked release of adenosine in the absence of Ca^{2+} was initially faster but less sustained than release evoked in the presence of Ca^{2+} (Fig. 26A).

Slices which had been stimulated in the presence of Ca^{2+} showed a marked decrease in kainate-evoked adenosine release during a second stimulation in the presence of Ca^{2+} . The decrease in kainate-evoked adenosine release during the second stimulation is probably not a result of receptor desensitization, because it is generally believed that kainate receptors do not desensitize (Fagni et al., 1983; Kiskin et al., 1986; Kushner et al., 1988; Mayer and Vyklicky, 1989). Following restoration of Ca^{2+} to slices previously stimulated in the absence of Ca^{2+} , a second exposure to kainate resulted in adenosine release which was remarkably comparable, both in time course as well as in total quantity, to release evoked when Ca^{2+} was present during the first exposure to kainate (compare the closed circles in the right panel of Fig. 25A with the open circles in the left panel). The total amount of kainate-evoked adenosine release observed during the second stimulation with Ca^{2+} restored was not decreased from release observed during the initial stimulation in the absence of extracellular Ca^{2+} . However, when slices were subjected to two consecutive 10-min kainate stimulations in the absence of Ca^{2+} , release of adenosine during the second stimulation was decreased substantially (Fig. 26B), suggesting that the Ca^{2+} -independent pool of releasable adenosine had been depleted. The above results suggest that there may be 2 separate pools of adenosine which can be released by kainate, one of which is Ca^{2+} -dependent and the other Ca^{2+} -independent.

III. NON-RECEPTOR-MEDIATED, GLUTAMATE-EVOKED ADENOSINE RELEASE FROM SYNAPTOSOMES

1. Glutamate-evoked adenosine release from synaptosomes.

The location of the EAA receptors involved in adenosine release from superfused cortical slices is not known. To investigate the possibility that these receptors might be located on presynaptic terminals in the cortex, the same region of parietal cortex from which slices had been prepared was used to study adenosine release from synaptosomes. L-Glutamate released endogenous adenosine from P_2 synaptosomal suspensions in a concentration-dependent manner (Fig. 27A; Table 7). Although the amount of adenosine released by glutamate was small in comparison to the basal extrasynaptosomal value, it was comparable to the amount of adenosine released by 30 mM KCl (130 ± 11 pmol of adenosine/g of cortex/ 10 min in six experiments). It was previously determined that the high background levels of adenosine in synaptosomal suspensions are due primarily to adenosine released during synaptosomal resuspension and only a small proportion of basal adenosine is released during the 10-min incubation period in the absence of a releasing agent (MacDonald and White, 1985).

To determine whether release arose from the synaptosomes or from mitochondrial or myelin contaminants of the P_2 preparation (Gray and Whittaker, 1962), purified synaptosomal, myelin and mitochondrial fractions were prepared and exposed to L-glutamate. L-Glutamate released adenosine from the synaptosomal fraction, but did not release adenosine from either the myelin or mitochondrial fraction (Fig. 27B). These results justify the use of P_2 synaptosomal suspensions in subsequent

Figure 27. Concentration-response relationship of L-glutamate-evoked adenosine (ADN) release from synaptosomes (P_2). Evoked release values are the means \pm SEM from 5 experiments. Basal extrasynaptosomal adenosine was 941 ± 85 pmol/mg of protein. B: Synaptosomal (SYN), mitochondrial (MIT), and myelin (MYE) fractions were isolated on sucrose density gradients, and adenosine release evoked by L-glutamate ($300 \mu\text{M}$) was determined. Histograms represent the means \pm SEM from 3 experiments. *Significantly different from release by synaptosomal fraction ($p < 0.05$, randomized block ANOVA with SNK test).

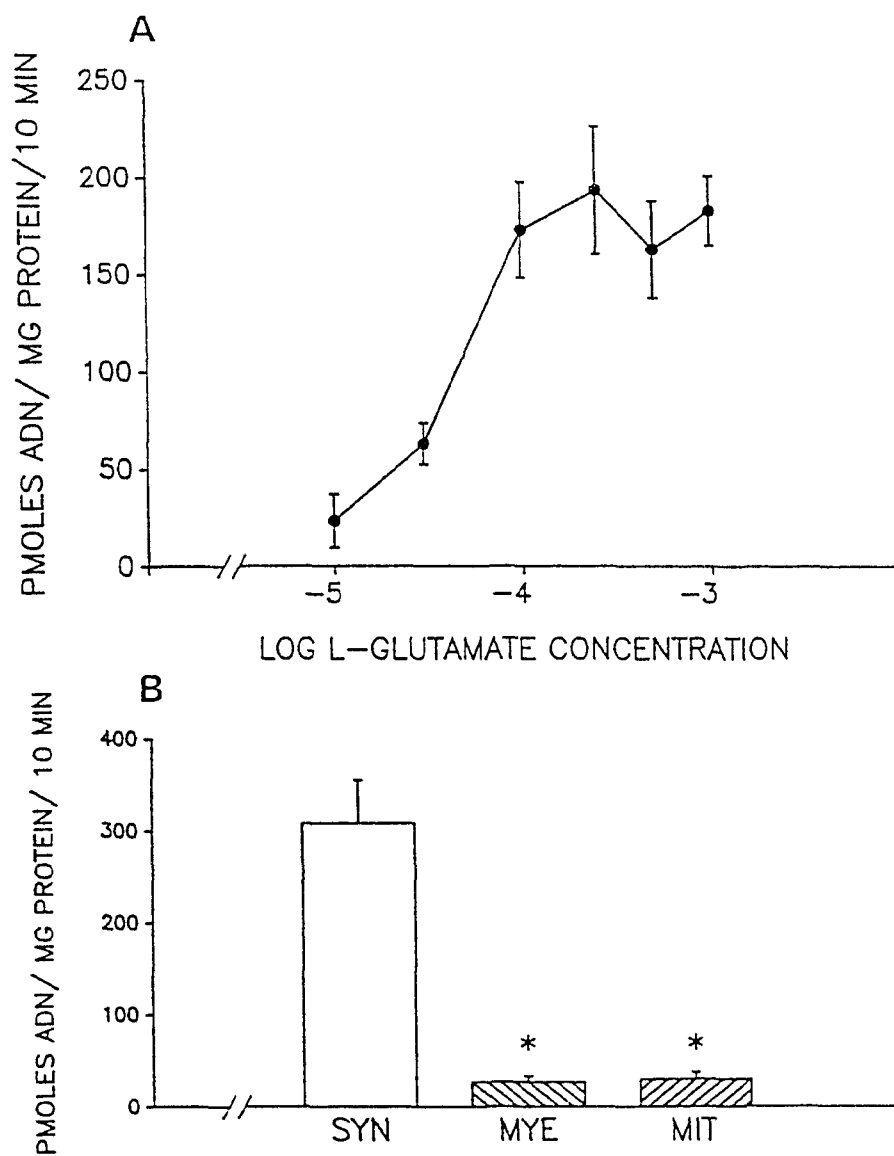


Figure 27

experiments.

2. Effects of excitatory amino acid agonists and antagonists on adenosine release from synaptosomes.

It was expected that the release of adenosine evoked by L-glutamate was mediated by activation of EAA receptors on the synaptosomes. To determine if this was the case, experiments were conducted with the uncompetitive NMDA receptor antagonist, MK-801, and the competitive non-NMDA receptor antagonist, DNQX. Surprisingly, neither MK-801 (3 μ M) nor DNQX (10 μ M) antagonized L-glutamate-evoked adenosine release (Table 6A and B), suggesting that neither NMDA nor non-NMDA receptors mediated this release.

It has been reported that Mg^{2+} inhibits NMDA receptor-mediated increases in intracellular Ca^{2+} in cortical synaptosomes but not in cortical slices (O'Shaughnessy and Lodge, 1988). Thus it was possible that Mg^{2+} might modulate glutamate-evoked adenosine release from cortical synaptosomes, even though such a modulation was not evident in the slice preparation (Table 5). However, glutamate-evoked adenosine release was not enhanced in the absence of Mg^{2+} (Table 6A).

Release of adenosine was also not evoked by the specific EAA receptor agonists, NMDA, kainate, or quisqualate in the presence of Mg^{2+} (Table 7A). In three experiments in the absence of Mg^{2+} , NMDA also failed to release adenosine (Table 7B). APB, which has been claimed to act at a novel subclass of presynaptic glutamate receptors in the CNS (Anson and Collins, 1987; Forsythe and Clements, 1988), also did not release adenosine (Table 8). Moreover, APB did not antagonize L-glutamate-evoked release of adenosine (Table 8). The above results indicate that the release of

TABLE 6. Effect of EAA antagonists on L-glutamate-evoked release of adenosine from cortical synaptosomes.

ANTAGONIST		ADENOSINE	
		TOTAL EXTRASYNAPTOSOMAL (pmol/mg protein)	EVOLED (pmol/mg protein/ 10 min)
A.			
-	-	906 ± 33	-
-	Mg ²⁺ (1.2 mM)	956 ± 30	-
L-Glutamate(500 μM)	-	1052 ± 54	146 ± 22
L-Glutamate(500 μM)	MK-801(3 μM)	1025 ± 64	120 ± 35 (NS)
L-Glutamate(500 μM)	Mg ²⁺ (1.2 mM)	1132 ± 59	176 ± 30 (NS)
L-Glutamate(500 μM)	Mg ²⁺ (1.2 mM) + MK-801(3 μM)	1120 ± 54	164 ± 32 (NS)
B.			
-	-	1163 ± 341	-
L-Glutamate(100 μM)	-	1302 ± 365	138 ± 26
L-Glutamate(100 μM)	DNQX(10 μM)	1310 ± 360	146 ± 22 (NS)

Synaptosomes were incubated for 10 min in the presence and absence of glutamate and EAA antagonists. In (B) synaptosomes were incubated in Mg²⁺-containing medium whereas in (A) they were preincubated in Mg²⁺-free medium and in some cases Mg²⁺ (1.2 mM) was added during the 10 min incubation with L-glutamate. Values are the means ± SEM of adenosine accumulation from 3 experiments in (A) and 4 in (B). (NS) No significant difference from release in the absence of antagonist (paired t test).

TABLE 7. Effects of NMDA, kainate and quisqualate on adenosine release from cortical synaptosomes.

AGONIST	Mg ²⁺ (1.2 mM)	ADENOSINE	
		Total Extrasynaptosomal (pmol/mg protein)	Evoked (pmol/mg protein/10 min)
A.			
-	+	916 ± 213	-
NMDA (500 μM)	+	913 ± 123	-4 ± 12
Kainate (500 μM)	+	905 ± 117	-11 ± 7
Quisqualate (500 μM)	+	927 ± 123	12 ± 2
L-Glutamate (500 μM)	+	1039 ± 119*	123 ± 13
B.			
-	-	744 ± 44	-
-	+	753 ± 41	-
NMDA (500 μM)	-	758 ± 45	14 ± 2
NMDA (500 μM)	+	761 ± 46	8 ± 5

Synaptosomes were incubated for 10 min in the presence and absence of EAA agonists. In (A) synaptosomes were incubated in 1.2 mM Mg^{2+} -containing medium whereas in (B) they were preincubated in Mg^{2+} -free medium and in some cases Mg^{2+} (1.2 mM) was added during the 10 min incubation with NMDA. Values are the means \pm SEM of adenosine accumulation in the medium from (A) 4 and (B) 3 experiments. *Significantly different from control ($p < 0.05$, randomized block ANOVA with Student-Newman-Keuls test).

TABLE 8. Effect of D,L-APB on total extrasynaptosomal adenosine and on L-glutamate-evoked release of adenosine from cortical synaptosomes.

DRUGS	ADENOSINE	
	Total Extrasynaptosomal (pmol/mg protein)	Evoked (pmol/mg protein/10 min)
-	863 ± 26	-
APB (500 μM)	841 ± 43(NS) ¹	-23 ± 16
L-Glutamate (100 μM)	1009 ± 58	145 ± 32
L-Glutamate (100 μM) + APB (200 μM)	1020 ± 52	157 ± 33(NS) ²

Synaptosomes were incubated for 10 min in the presence and absence of L-glutamate and/or APB. Values are the means ± SEM of adenosine accumulation in the medium from four experiments. (NS)¹ No significant difference from the total extrasynaptosomal adenosine in the absence of APB (paired t test). (NS)² No significant difference from release in absence of APB (paired t test).

adenosine evoked by L-glutamate was not mediated by activation of specific EAA receptors on the cortical synaptosomes.

3. Role of glutamate uptake in glutamate-evoked adenosine release from synaptosomes.

Because the L-glutamate-evoked adenosine release from synaptosomes did not appear to be receptor-mediated, it seemed possible that another mechanism, namely the uptake of L-glutamate into synaptosomes via the Na^+ -dependent, acidic amino acid carrier (Erecińska, 1987), might mediate adenosine release. To test this, the effect of dihydrokainate, which is a competitive nontransportable inhibitor of high-affinity glutamate uptake (Johnston et al., 1979), on the L-glutamate-evoked release of adenosine was investigated. Dihydrokainate (0.5 mM) diminished 100 μM glutamate-evoked adenosine release by 59%, and 1 mM dihydrokainate virtually abolished release evoked by 50 μM glutamate (Fig. 28A), suggesting that glutamate uptake was indeed involved in L-glutamate-evoked adenosine release.

High-affinity glutamate uptake into synaptosomes is Na^+ -dependent (Bennet et al., 1972; Erecińska, 1987). To determine the possible role of Na^+ in L-glutamate-evoked release of adenosine, studies were conducted in which extracellular NaCl was substituted by LiCl, choline chloride, or sucrose. In the absence of Na^+ , adenosine release evoked by L-glutamate was virtually abolished (Fig. 28B). This, and the above findings with dihydrokainate, strongly suggest that the Na^+ -dependent amino acid transport system is involved in glutamate-evoked release of adenosine from cortical synaptosomes.

Uptake of glutamate involves both transport across the cell membrane

Figure 28. A: Effect of the inhibitor of glutamate uptake, dihydrokainate (DHK), on L-glutamate-evoked adenosine (ADN) release from synaptosomes. Histograms represent the means \pm SEM of evoked release from 6 experiments with 100 μ M L-glutamate and 4 experiments with 50 μ M L-glutamate. *Significantly different from respective control ($p < 0.05$, paired t test). B: Effects of substitution of NaCl with LiCl, choline chloride, or sucrose on the release of adenosine evoked by 300 μ M L-glutamate. Histograms represent the means \pm SEM of evoked release from 5 experiments. *Significantly different from release in the presence of Na^+ ($p < 0.05$, randomized block ANOVA with SNK test).

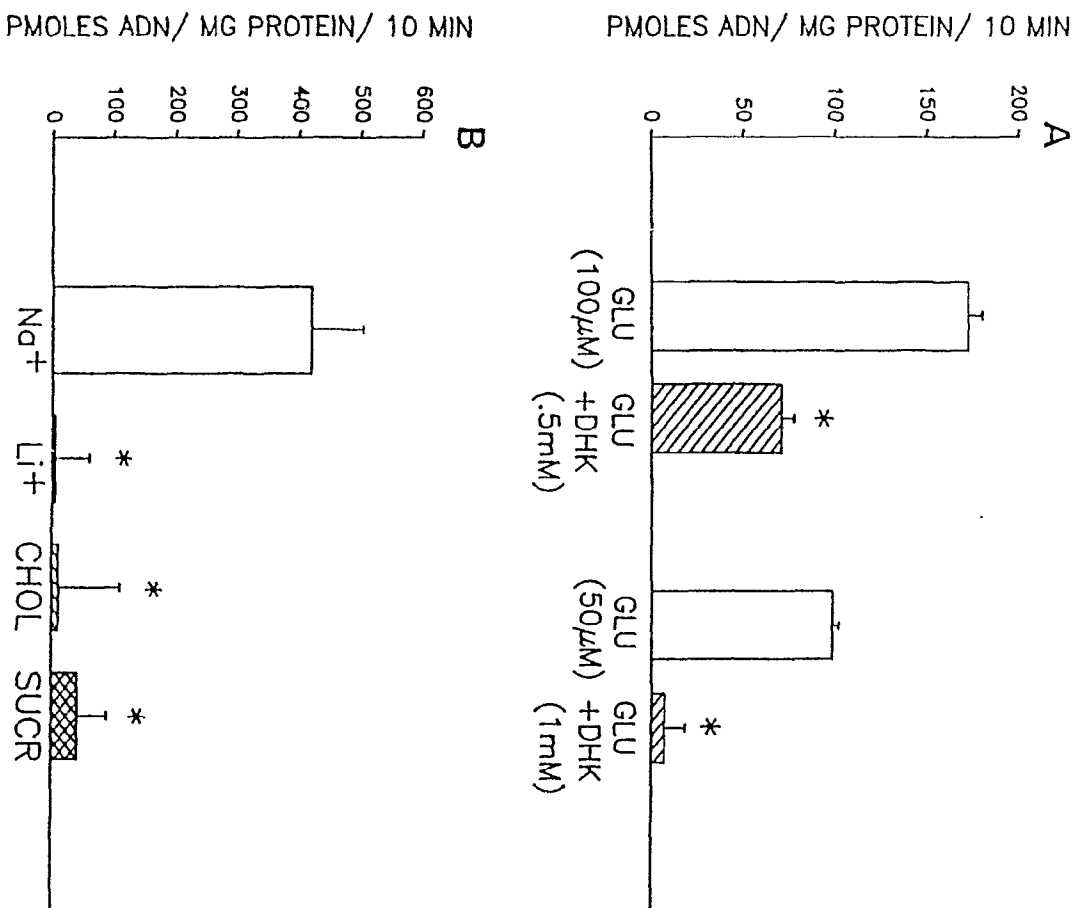


Figure 28

and subsequent metabolism. The nonmetabolizable analogues, D-aspartate and D-glutamate (Davies and Johnston, 1976; Takagaki, 1976), were used to differentiate between transport and metabolism as the mediator of glutamate uptake-evoked adenosine release. D-aspartate, which is a good substrate for the high-affinity acidic amino acid transporter (Erecińska, 1987), released adenosine in a concentration-dependent manner, although it appeared to be somewhat less potent than L-glutamate (Fig. 29A). D-Glutamate, a poor substrate for high-affinity uptake (Balcar and Johnston, 1972), was correspondingly much less potent than L-glutamate in releasing adenosine (Fig. 29B). Dihydrokainate (0.5 mM) decreased adenosine release evoked by D-glutamate (1 mM) from 91.1 ± 13 pmol/mg of protein/10 min to 0.9 ± 3.9 pmol/mg of protein/10 min ($p < 0.05$, paired t test), indicating that D-glutamate-evoked adenosine release probably occurred as a consequence of transport on the high-affinity carrier. The above results strongly suggest that the Na^+ -dependent, high-affinity transport of acidic amino acids across the synaptosomal membrane, rather than the subsequent metabolism of the amino acids, mediates adenosine release.

4. Ca^{2+} -dependence of glutamate-evoked adenosine release from synaptosomes.

Experiments were conducted in Ca^{2+} -free medium with and without the addition of the chelating agent EGTA (1 mM) to determine whether glutamate-evoked release of adenosine from synaptosomes was Ca^{2+} -dependent. Basal (unstimulated) extrasynaptosomal adenosine accumulation was increased in Ca^{2+} -free medium and increased further when EGTA was added (Table 9), consistent with previous results with synaptosomes prepared from whole rat brain (MacDonald and White, 1985). However, L-glutamate-evoked adenosine release was not diminished when Ca^{2+} was omitted from the

Figure 29. A: D-Aspartate-evoked release of adenosine (ADN) from synaptosomes. Values are the means \pm SEM from 6 experiments in which both D-aspartate-evoked and L-glutamate-evoked adenosine release were determined from the same synaptosomal preparations. Basal extrasynaptosomal adenosine was 777 ± 43 pmol/mg of protein. B: D-Glutamate-evoked release of adenosine from synaptosomes. Values are the means \pm SEM from 7 experiments in which both D-glutamate-evoked and L-glutamate-evoked adenosine release were determined from the same synaptosomal preparations. Basal extrasynaptosomal adenosine was $1,017 \pm 53$ pmol/mg protein.

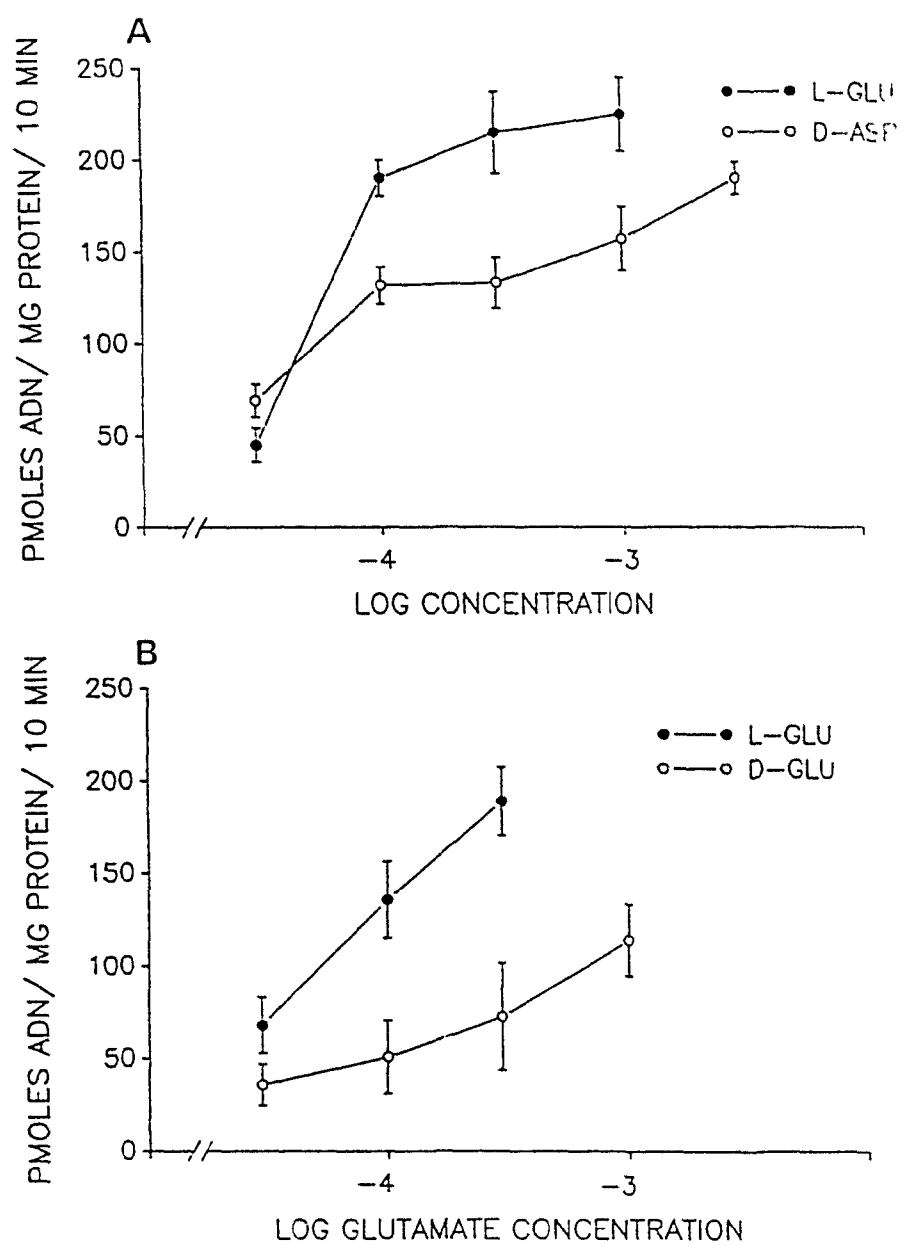


Figure 29

TABLE 9. Ca^{2+} -dependence of L-glutamate-evoked adenosine release from cortical synaptosomes.

	ADENOSINE	
	Total Extrasynaptosomal (pmol/mg protein)	Evoked (pmol/mg protein/10 min)
CONTROL		
Ca^{2+} (1.8 mM)	200 ± 16	-
Ca^{2+} - free	$468 \pm 42^*$	-
Ca^{2+} - free + 1 mM EGTA	$573 \pm 39^*$	-
GLUTAMATE (100 μM)		
Ca^{2+} (1.8 mM)	346 ± 37	146 ± 34
Ca^{2+} - free	$595 \pm 40^*$	127 ± 32 (NS)
Ca^{2+} - free + 1 mM EGTA	$812 \pm 34^*$	239 ± 33 (NS)

Synaptosomes were prepared in Ca^{2+} -free medium, and Ca^{2+} was restored to 1.8 mM or 1 mM EGTA was added to chelate residual Ca^{2+} during the 10 min incubation in the presence or absence of glutamate. Values are the means \pm SEM from 6 experiments. *Significantly different from extrasynaptosomal accumulation in the presence of Ca^{2+} ($p < 0.05$, randomized block ANOVA with Student-Newman-Keuls test). (NS) No significant difference from release in the presence of Ca^{2+} (randomized block ANOVA with Student-Newman-Keuls test).

incubation medium either in the presence or absence of EGTA (Table 9), indicating that Ca^{2+} is not required for L-glutamate-evoked release of adenosine.

5. Role of voltage-sensitive Na^+ channels in glutamate-evoked adenosine release from synaptosomes.

Voltage-dependent Na^+ channels have been implicated in the GABA uptake-mediated release of [^3H]noradrenaline from hippocampal synaptosomes, in so far as release is decreased in the presence of TTX (Bonanno et al., 1989). However, TTX (1 μM) had no effect on 100 μM L-glutamate-evoked release of adenosine from rat cortical synaptosomes (Table 10), indicating that voltage-sensitive Na^+ channels are not involved in L-glutamate-evoked adenosine release from synaptosomes.

6. Effect of a high concentration of DNQX on glutamate uptake-mediated adenosine release from synaptosomes.

A concentration (10 μM) of DNQX which is several times the IC_{50} for inhibition of kainate and AMPA binding to rat cortical membranes (Honore et al., 1988), decreased non-NMDA receptor-mediated adenosine release from slices (Figs. 10 and 11) and decreases kainate and quisqualate-induced depolarizations (Fletcher et al., 1988). At this concentration DNQX did not affect L-glutamate uptake-evoked adenosine release from synaptosomes (Table 6B). However, 50 μM and 100 μM DNQX decreased L-glutamate uptake-evoked adenosine release from synaptosomes by 21% and 51%, respectively (Fig. 30), suggesting that at these very high concentrations, DNQX may interfere with glutamate transport.

TABLE 10. Effect of TTX on L-glutamate-evoked release of adenosine from cortical synaptosomes.

DRUGS	ADENOSINE	
	Total Extrasynaptosomal (pmol/mg protein)	Evoked (pmol/mg protein/10 min)
-	813 \pm 54	-
TTX (1 μ M)	836 \pm 50	-
L-Glutamate (100 μ M)	993 \pm 55	180 \pm 10
L-Glutamate (100 μ M) + TTX (1 μ M)	1027 \pm 47	190 \pm 18 (NS)

Synaptosomes were incubated for 10 min in the presence and absence of glutamate and/or TTX. Values are the means \pm SEM of adenosine accumulation in the medium from four experiments. (NS) No significant difference from release in the absence of TTX (paired t test).

Figure 30. Effect of high concentrations of DNQX on L-glutamate-evoked release of adenosine (ADN) from synaptosomes. Histograms represent the means \pm SEM of evoked release from the stated number of experiments. *Significantly different from control ($p < 0.05$, paired t test).

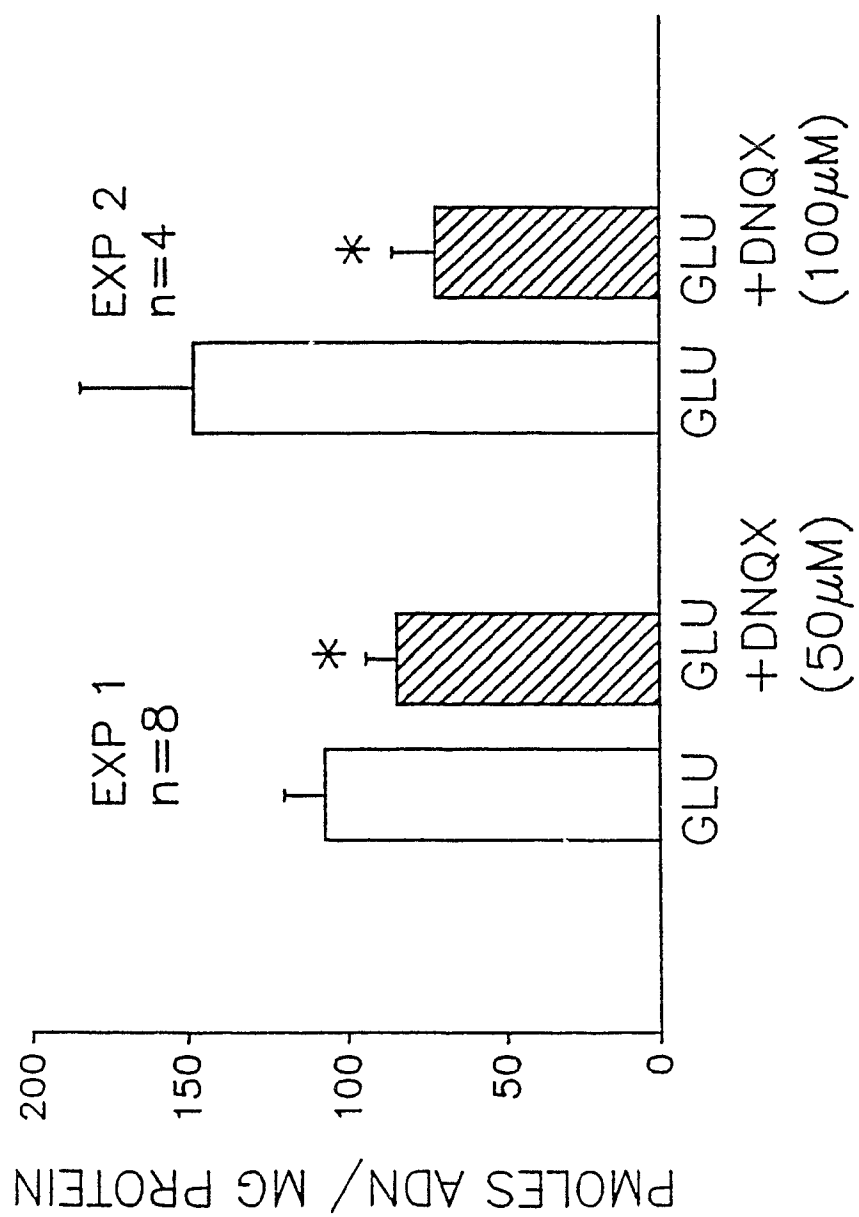


Figure 30

7. Role of the nucleoside transporter in adenosine release from synaptosomes.

The K^+ -evoked release of adenosine from whole brain synaptosomes appears to occur via efflux on the bidirectional nucleoside carrier (White and MacDonald, in press). To determine whether L-glutamate-evoked adenosine release from synaptosomes occurs via the nucleoside transporter, studies were conducted in the presence and absence of the nucleoside transport inhibitor, dipyridamole. Dipyridamole (120 μ M) decreased 30 mM KCl-evoked adenosine release from cortical synaptosomes (Fig. 31), indicating that this was mediated in part by the nucleoside transporter and confirming previous results with synaptosomes prepared from whole rat brain (White and MacDonald, in press). However, 100 μ M L-glutamate-evoked adenosine release was not diminished, but was actually increased, in the presence of dipyridamole (Fig. 31), suggesting that glutamate-evoked release of adenosine from synaptosomes does not occur via the nucleoside transporter.

8. Nature of the purine released from synaptosomes by L-glutamate.

A. Does L-glutamate release a nucleotide from synaptosomes? The finding that inhibiting the nucleoside transporter with dipyridamole increased the levels of extrasynaptosomal adenosine evoked by glutamate suggested that glutamate-evoked adenosine release may have been derived from the extracellular metabolism of a released nucleotide. Ectoenzymes capable of converting released nucleotides to adenosine are present in rat brain (Manery and Dryden, 1979; Nagy et al., 1986), and adenosine derived extrasynaptosomally from a released nucleotide would be prevented from re-entering the synaptosomes when the nucleoside transporter is inhibited by

Figure 31. Effect of the inhibitor of nucleoside transport, dipyridamole (DPR, 120 μ M), on adenosine (ADN) release evoked by K^+ (30 mM) or L-glutamate (GLU, 100 μ M) from synaptosomes. Histograms represent the means \pm SEM from 6 experiments. *Significantly different from corresponding control ($p < 0.05$, paired t test).

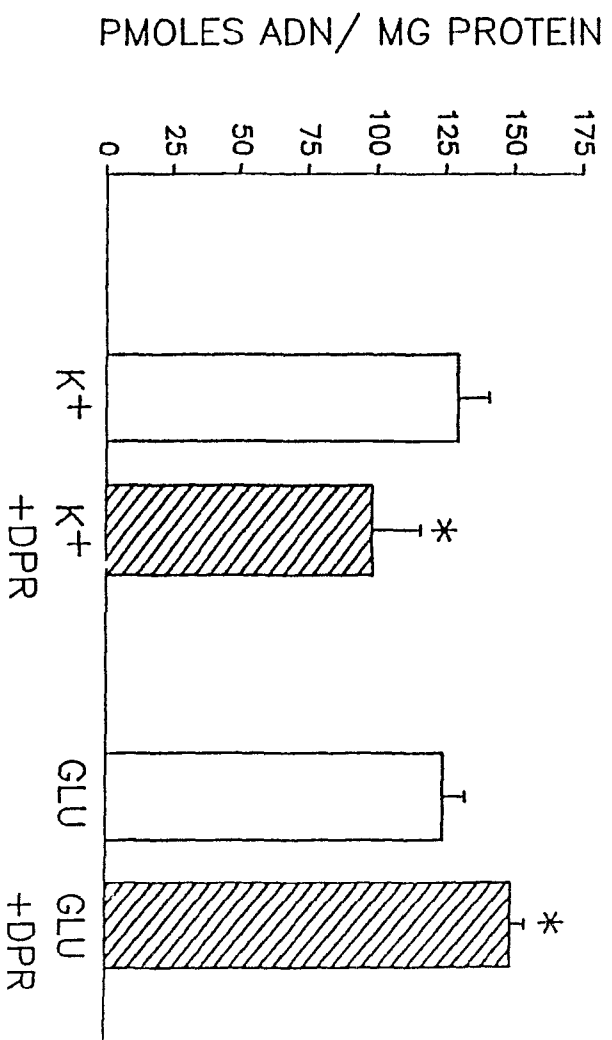


Figure 31

dipyridamole.

To determine whether glutamate-evoked adenosine release did indeed arise from a released nucleotide, release was determined in the presence of α,β -methylene-ADP (0.5 mM) and GMP (5 mM) to inhibit ecto-5'-nucleotidase. This treatment has been shown previously to inhibit the extrasynaptosomal metabolism of AMP to adenosine by about 90% (MacDonald and White, 1985). When ecto-5'-nucleotidase was inhibited, glutamate-evoked adenosine release was virtually abolished (Fig. 32A), indicating that the L-glutamate-evoked release of adenosine from synaptosomes is derived largely from the extracellular metabolism of released nucleotide(s).

B. Does L-glutamate release ATP from synaptosomes? It was possible that the nucleotide released by L-glutamate could have been ATP, because depolarization of cortical synaptosomes has been shown to release ATP (Potter and White, 1980). To test this possibility, L-glutamate-evoked release of ATP from cortical synaptosomes was determined (Fig. 32B). Whereas depolarization by 30 mM K^+ released ATP from the synaptosomes, exposure to L-glutamate (1 mM) did not. Thus, it is unlikely that glutamate-evoked adenosine release is derived from released ATP.

C. Does L-glutamate release cyclic AMP from synaptosomes? There is evidence that cyclic AMP may be released from rat neocortex (Rosenberg and Dichter, 1989) and that in neocortex and some other tissues, cyclic AMP may be metabolized by an extracellular cyclic AMP phosphodiesterase to AMP (Rosberg et al., 1975; Selstam and Rosberg, 1976; Rosenberg and Dichter, 1989). The AMP derived from cyclic AMP extracellularly could then be metabolized by ecto-5'-nucleotidases to adenosine, thus providing

Figure 32. A: Effect of inhibition of ecto-5'-nucleotidase with α,β -methylene ADP (0.5 mM) and GMP (5 mM) on 100 μ M L-glutamate (GLU)-evoked adenosine (ADN) release from synaptosomes. Histograms represent the means \pm SEM from 4 experiments. *Significantly different from control ($p < 0.05$, paired t test). B: Release of ATP from rat cortical synaptosomes evoked by K^+ and L-glutamate. Tracing shown is representative of those obtained in three separate experiments.

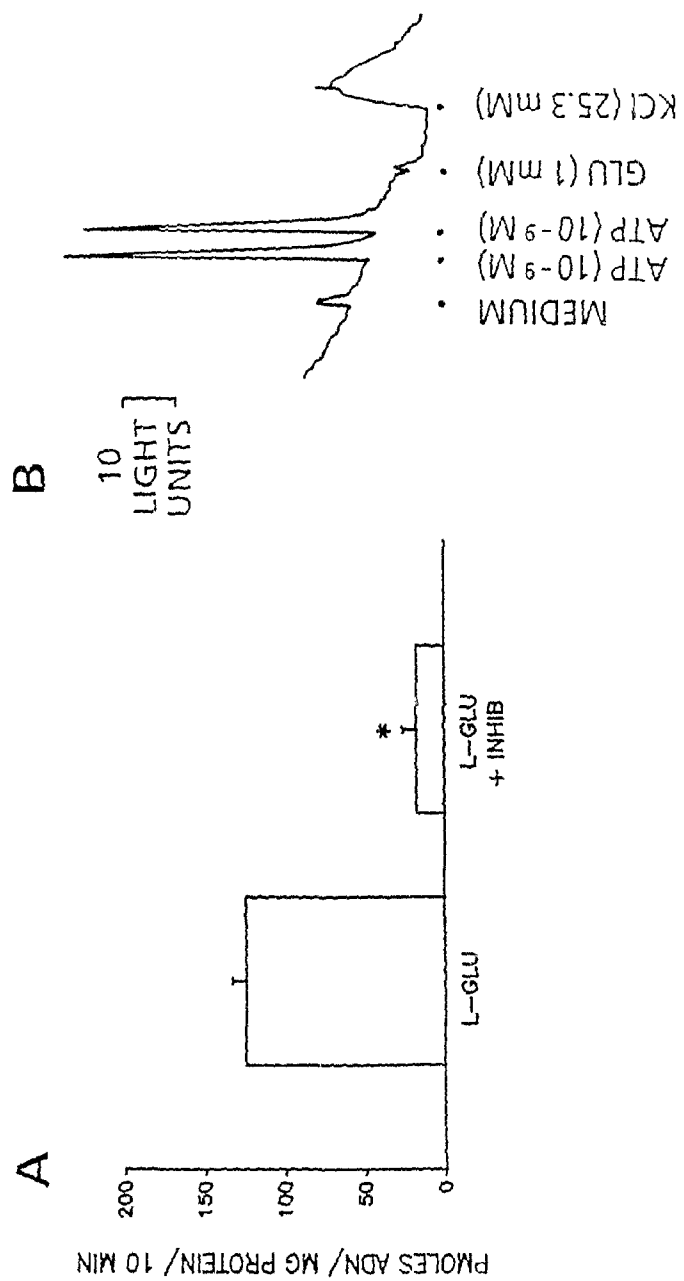


Figure 32

a potential source of extracellular adenosine. To examine the possibility that adenosine released by L-glutamate was derived from the extracellular metabolism of cyclic AMP, studies were conducted with the phosphodiesterase inhibitor, IBMX. In the absence of IBMX, virtually all of 1 μ M exogenous cyclic AMP added to the synaptosomal suspension was recovered as adenosine and no peak corresponding to cyclic AMP could be detected (Fig. 33), indicating that the synaptosomal suspension was capable of metabolizing added cyclic AMP to adenosine. IBMX (1 mM) inhibited the conversion of added cyclic AMP to adenosine by 45% and a peak corresponding to cyclic AMP became detectable. However, IBMX had no effect on the amount of adenosine detected in the medium following exposure of synaptosomes to L-glutamate and no peak corresponding to cyclic AMP was detected (Fig. 33). These results indicate that the adenosine detected extrasynaptosomally following exposure to glutamate probably was not derived from the extracellular metabolism of released cyclic AMP.

IV. EFFECTS OF K^+ AND EXCITATORY AMINO ACID AGONISTS ON RELEASE OF $[^3H]$ NORADRENALINE FROM SYNAPTOSOMES

To determine if either glutamate transport into cortical synaptosomes or activation of EAA receptors on cortical synaptosomes might evoke the release of a classical neurotransmitter, studies were conducted in which synaptosomes were preloaded with $[^3H]$ noradrenaline. Synaptosomes were exposed for 10 min to 30 mM KCl or EAA agonists and the release of $[^3H]$ noradrenaline was determined. As a control, glutamate-evoked adenosine release was also assessed from the same synaptosomal preparation in

Figure 33. Effect of inhibition of phosphodiesterase with IBMX (1 mM) on synaptosomal conversion of exogenous (added) cyclic AMP and on L-glutamate (GLU)-evoked adenosine (ADN) release from synaptosomes. Representative HPLC tracings are shown above the corresponding histograms to illustrate the simultaneous detection of adenosine (retention time of 3.30-3.31) and cyclic AMP (retention time of 2.24). Values are the means \pm SEM from 4 experiments. The histograms represent evoked adenosine release (total adenosine accumulation extrasynaptosomally 10 min following addition of either exogenous cyclic AMP or 100 μ M glutamate minus the corresponding basal extrasynaptosomal adenosine), whereas the HPLC tracings show the total amount of extrasynaptosomal adenosine present after 10 min. Basal extrasynaptosomal adenosine values were 808 ± 21 pmol/mg of protein and 836 ± 31 pmol/ mg of protein in the absence and presence of IBMX respectively. *Significantly different from corresponding control ($p < 0.05$, paired t test).

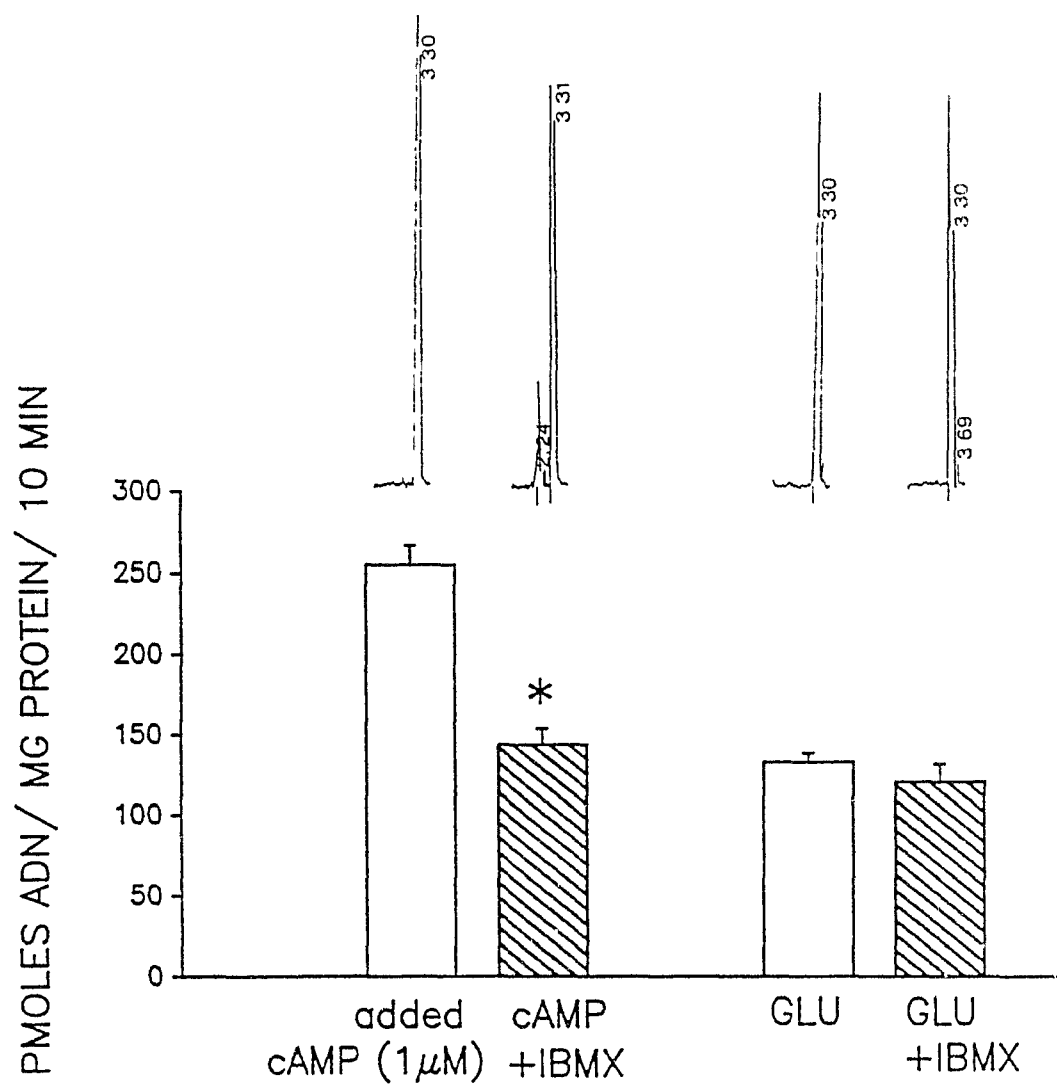


Figure 33

parallel with [^3H]noradrenaline release. In Mg^{2+} -free medium, glutamate (500 μM) released adenosine from the synaptosomes but did not release [^3H]noradrenaline (Table 11), indicating that glutamate transport into synaptosomes does not release [^3H]noradrenaline.

NMDA and quisqualate also did not release [^3H]noradrenaline from synaptosomes in Mg^{2+} -free medium, indicating a lack of NMDA and quisqualate receptor-mediated release of [^3H]noradrenaline from synaptosomes. Kainate evoked a small but statistically significant release of [^3H]noradrenaline, representing only about 1% of the total [^3H]noradrenaline contents of the synaptosomal suspension (Table 11). On the other hand, K^+ -depolarization released approximately 6% of the total synaptosomal [^3H]noradrenaline contents, indicating the presence of a substantial releasable pool of [^3H]noradrenaline in the synaptosomes.

V. A COMPARISON OF NMDA-EVOKED RELEASE OF ADENOSINE AND [^3H]NORADRENALINE FROM CORTICAL SLICES

1. Effects of MK-801 on the release of [^3H]noradrenaline and adenosine.

Release of both [^3H]noradrenaline and adenosine by 500 μM NMDA was determined simultaneously from slices of parietal cortex (Fig. 34). The maximum rate of [^3H]noradrenaline release occurred in the first 2.5 min fraction following exposure to NMDA, whereas the maximal rate of adenosine release occurred later. MK-801 (3 μM), an uncompetitive NMDA receptor antagonist acting at the NMDA receptor-associated ionic channel (Wong et al., 1986; Halliwell et al., 1989), virtually abolished NMDA-evoked release of both [^3H]noradrenaline and adenosine (Fig. 34), suggesting that release by NMDA of both [^3H]noradrenaline and adenosine requires influx of

TABLE 11. Effects of NMDA, kainate, quisqualate, L-glutamate and K⁺-depolarization on [³H]noradrenaline release from cortical synaptosomes.

	[³ H]NORADRENALINE	
	Total Extrasynaptosomal (% of content)	Evoked (% of content/10 min)
Control	15.7 ± 0.5	-
NMDA (500 μM)	16.1 ± 0.5	0.5 ± 0.1
Kainate (500 μM)	16.8 ± 0.5*	1.1 ± 0.1
Quisqualate (500 μM)	16.1 ± 0.5	0.4 ± 0.1
L-Glutamate (500 μM)	16.2 ± 0.5	0.6 ± 0.1
K ⁺ (30 mM)	21.6 ± 0.6*	5.9 ± 0.3

Synaptosomes were incubated for 10 min in the presence of releasing agents. Values are means ± SEM of [³H]noradrenaline accumulation in the medium from 7 experiments and are expressed as a percent of the total amount of [³H]noradrenaline present in the synaptosomal suspension. Total extrasynaptosomal [³H]noradrenaline at 0 min (before incubation) was 9.5 ± 4% content. In each experiment, L-glutamate released adenosine from the synaptosomal suspension. *Significantly different from control (p < 0.05, randomized block ANOVA with Student-Newman-Keuls test).

Figure 34. Effect of the uncompetitive antagonist, MK-801 ($3 \mu\text{M}$), on NMDA-evoked release of (A) [^3H]noradrenaline ([^3H]NA) and (B) adenosine (ADN) from the same slices in Mg^{2+} -free medium. MK-801 was present from 20 min prior to exposure to NMDA until the end of the experiment. NMDA ($500 \mu\text{M}$) was present from 0 to 10 min. Values are means \pm SEM from 4 experiments. INSETS: Histograms represent the total amount of [^3H]noradrenaline or adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

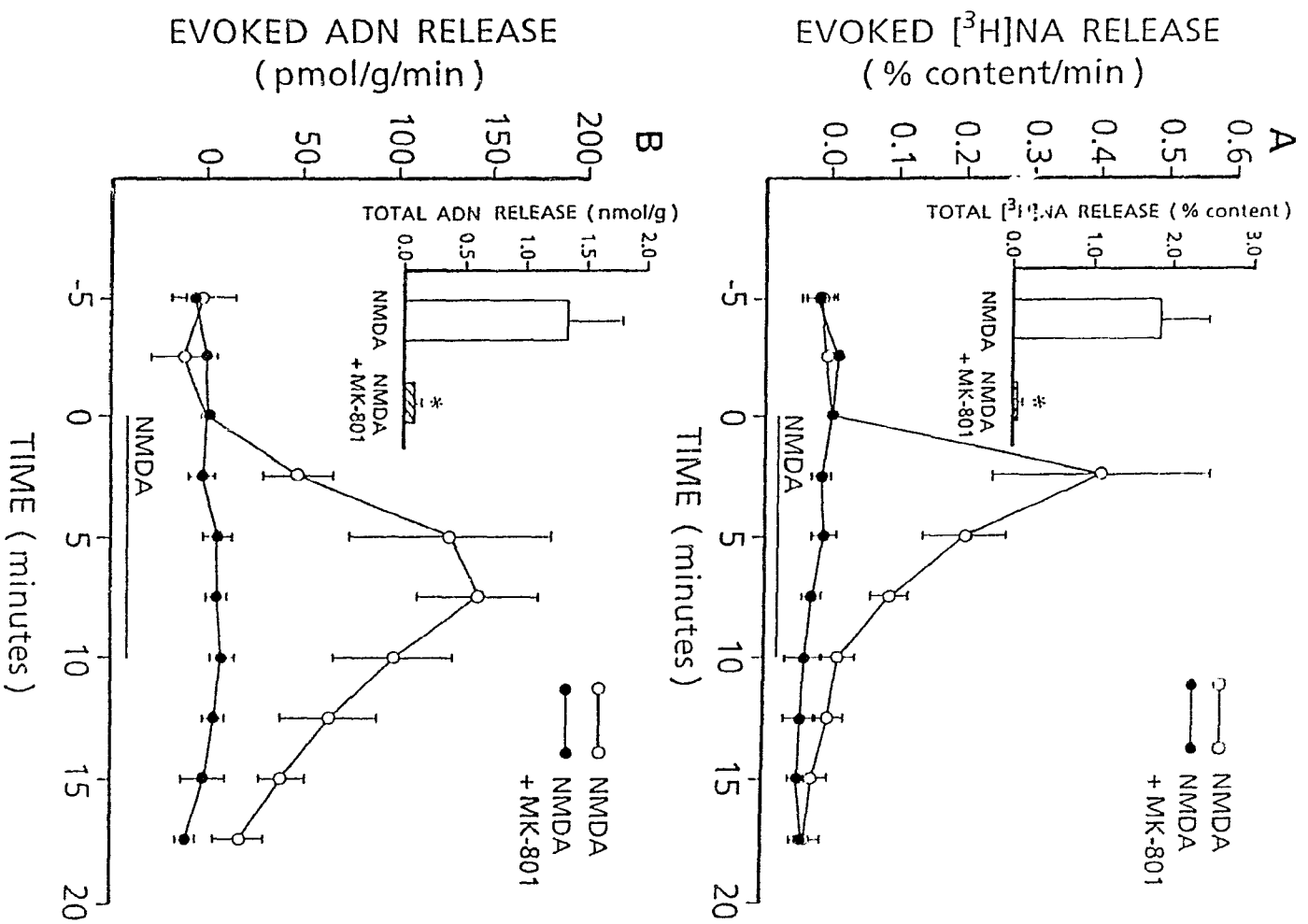


Figure 34

cations through the NMDA receptor-linked ionic channel.

2. Effects of Mg^{2+} on the release of [3H]noradrenaline and adenosine.

In order to verify that the lack of Mg^{2+} block of NMDA-evoked adenosine release (Fig. 9) was not due to partial depolarization of the cortical slices, the effect of Mg^{2+} -free medium on NMDA-evoked adenosine and [3H]noradrenaline release was assessed concurrently. In agreement with previous studies (Keith et al., 1988; Fink et al., 1989), NMDA was virtually ineffective at releasing [3H]noradrenaline in the presence of 1.2 mM Mg^{2+} but a marked release was observed when Mg^{2+} was omitted from the superfusing medium (Fig. 35A). This indicates that the slices were not depolarized and confirms the observations in hyperpolarizing medium (Fig. 10). In contrast, 500 μM NMDA released adenosine in the presence of Mg^{2+} and this release was not increased, but was decreased by 33%, when Mg^{2+} was omitted from the perfusing medium (Fig. 35B). Although statistically significant, the decrease in NMDA-evoked adenosine release in the absence of Mg^{2+} was not an entirely consistent finding in that it occurred in only 10 of the 14 experiments.

3. Effect of partial depolarization with K^+ on NMDA-evoked release of [3H]noradrenaline and adenosine in Mg^{2+} -containing medium.

The observation that NMDA-evoked release of adenosine was actually decreased rather than increased in the absence of Mg^{2+} suggested that Mg^{2+} may be necessary for adenosine release. Such a Mg^{2+} requirement for adenosine release might offset any effect that removal of Mg^{2+} might have on NMDA-evoked receptor activation. To test this, 500 μM NMDA-evoked adenosine release in the presence of Mg^{2+} was determined when the voltage-sensitive Mg^{2+} block of the NMDA-ion channel was partially alleviated by

Figure 35. Effect of Mg^{2+} -free medium on NMDA-evoked release of (A) [3H]noradrenaline ([3H]NA) and (B) adenosine (ADN) from the same slices. NMDA (500 μM) was present from 0 to 10 min. Values are means \pm SEM from 14 experiments. **INSETS:** Histograms represent the total amount of [3H]noradrenaline or adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

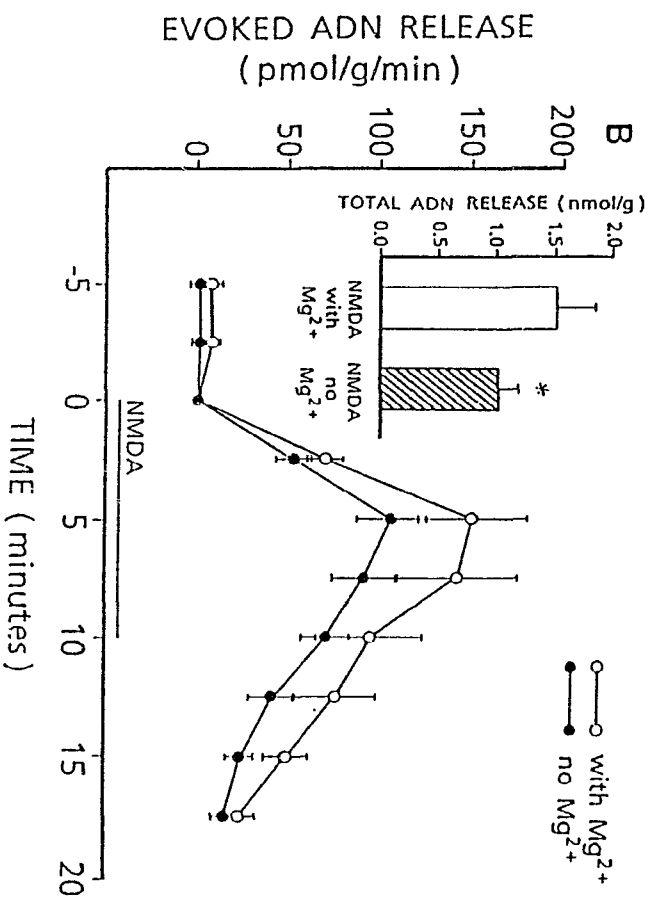
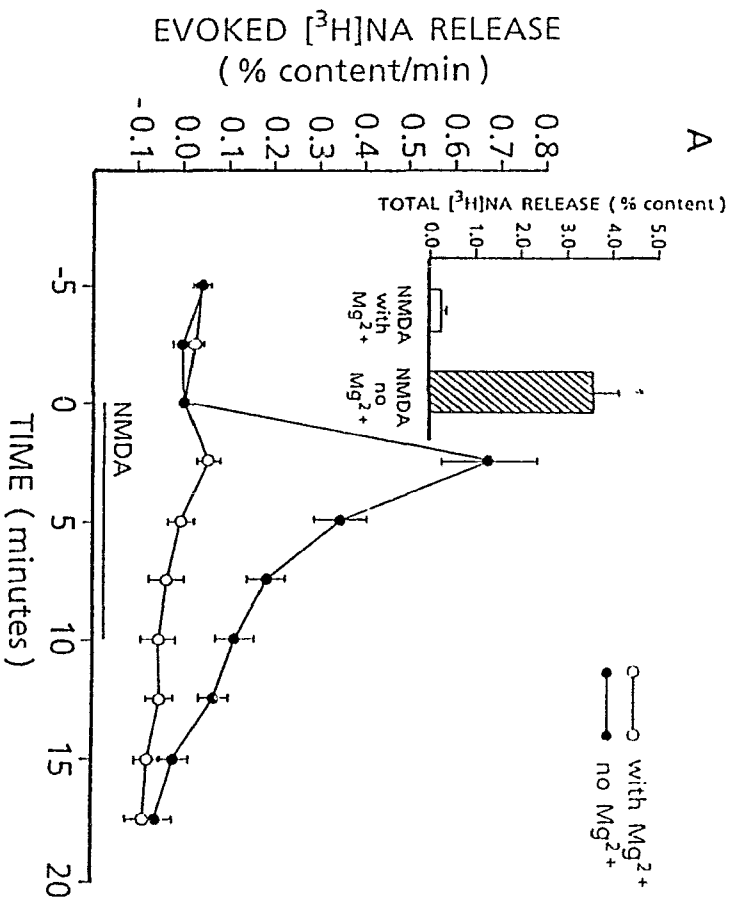


Figure 35

depolarization with 12 mM K^+ . Partial depolarization with 12 mM K^+ augmented NMDA-evoked [3H]noradrenaline release in Mg^{2+} -containing medium, indicating that the Mg^{2+} block of the NMDA receptor had indeed been partially alleviated (Fig. 36A). However, this did not augment NMDA-evoked adenosine release (Fig. 36B). These findings, where the Mg^{2+} concentration was kept constant, indicate that the lack of enhancement of NMDA-evoked adenosine release in the absence of Mg^{2+} observed previously was not due to an effect of Mg^{2+} -free medium either on the adenosine release process per se or on the amount of endogenous adenosine available for release.

4. TTX-sensitivity of NMDA-evoked release of [3H]noradrenaline and adenosine.

To determine the role of propagated action potentials in 500 μM NMDA-evoked release of [3H]noradrenaline and adenosine in Mg^{2+} -free medium, release was determined during blockade of voltage-dependent Na^+ channels by TTX. TTX (1 μM) virtually abolished NMDA-evoked release of [3H]noradrenaline while diminishing NMDA-evoked adenosine release by only 33% (Fig. 37, A and B), indicating that propagated action potentials are essential for NMDA-evoked [3H]noradrenaline release but contribute only slightly to adenosine release.

It has been reported that spontaneously occurring Na^+ -action potentials alleviate the Mg^{2+} blockade of NMDA-evoked [3H]dopamine release from neurons in culture (Boksa et. al, 1989). It seemed possible that the voltage-sensitive Mg^{2+} block of NMDA receptors mediating adenosine release from cortical slices may have been alleviated by spontaneously occurring action potentials and/or by the tonic release of some transmitter that

Figure 36. Effect of depolarization with 12 mM K^+ on NMDA-evoked release of (A) [3H]noradrenaline ([3H]NA) and (B) adenosine (ADN) from the same slices in the presence of 1.2 mM Mg^{2+} . K^+ was present from 30 min prior to exposure to NMDA until the end of the experiment. NMDA (500 μM) was present from 0 to 10 min. Values are means \pm SEM from 5 experiments. INSETS: Histograms represent the total amount of [3H]noradrenaline or adenosine released. *Significantly different from control ($p < 0.05$, paired t test).

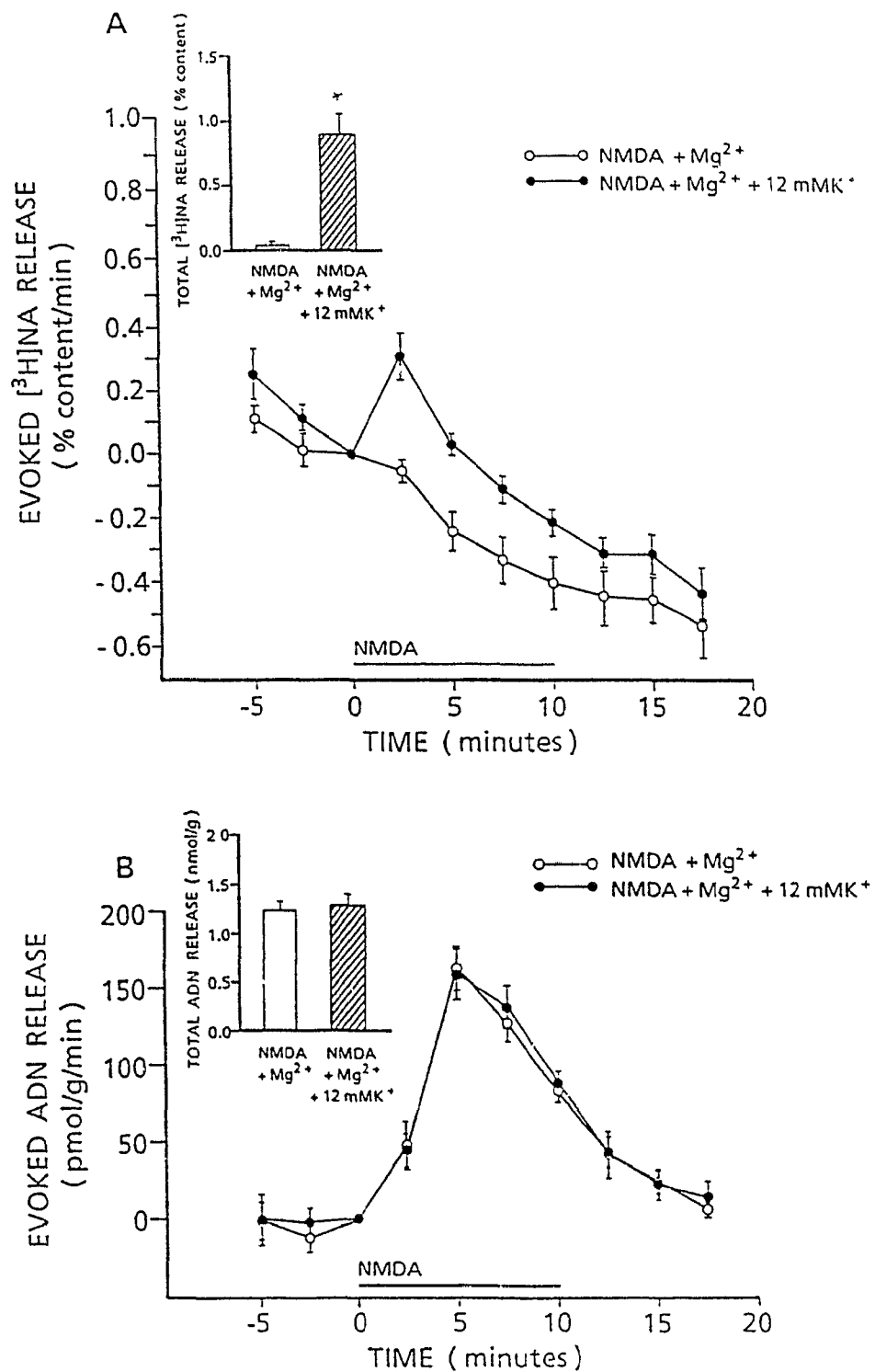


Figure 36

Figure 37. Effect of TTX on NMDA-evoked release of (A) [^3H]noradrenaline ([^3H]NA) and (B) adenosine (ADN) from the same slices in the absence of Mg^{2+} . TTX ($1\text{ }\mu\text{M}$) was present from 20 min prior to exposure to NMDA until the end of the experiment. NMDA ($500\text{ }\mu\text{M}$) was present from 0 to 10 min. Values are means \pm SEM from 5 experiments. INSETS: Histograms represent the total amount of [^3H]noradrenaline or adenosine released.

*Significantly different from control ($p < 0.05$, paired t test).

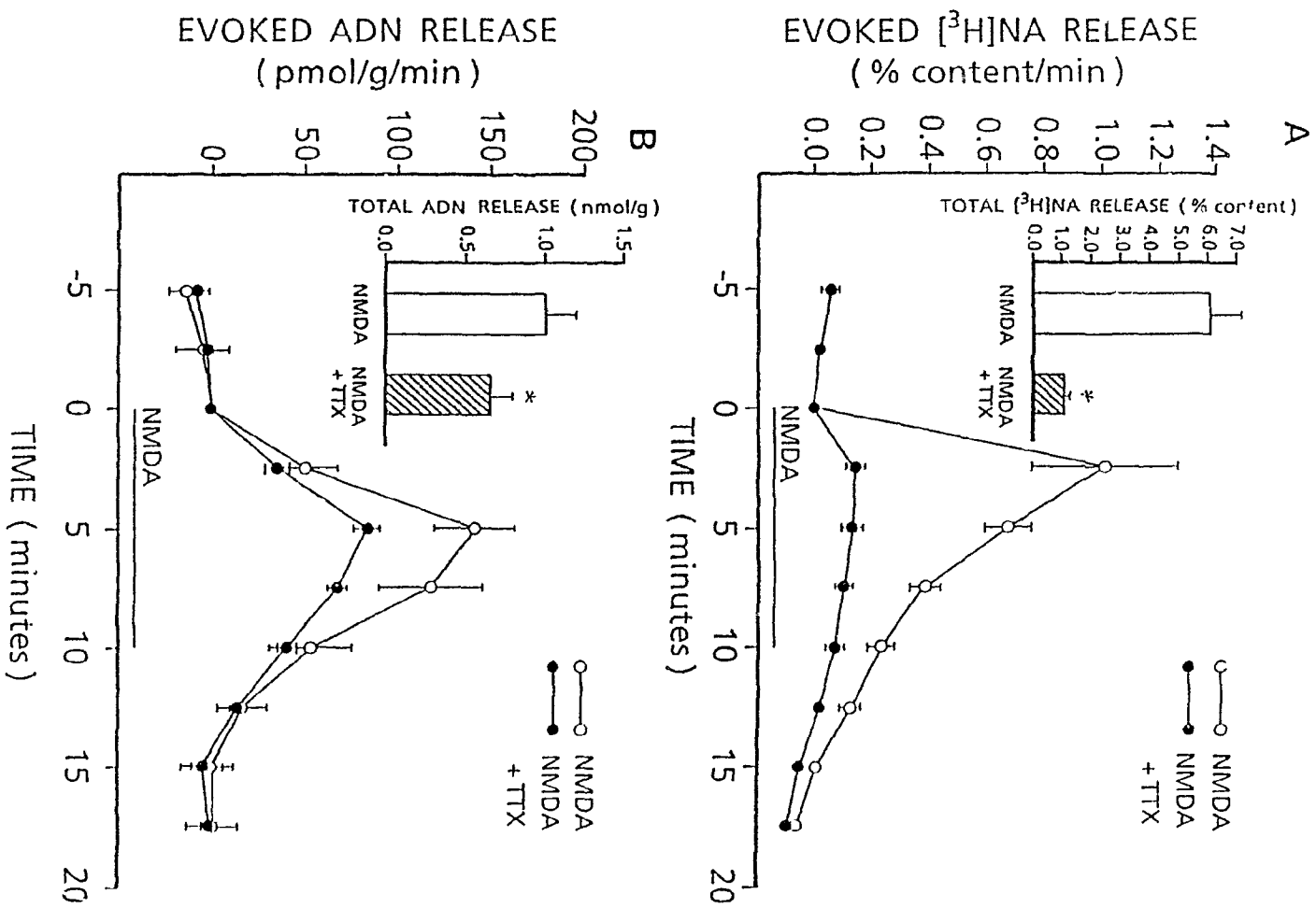


Figure 37

partially depolarizes the nerves which release adenosine. To test this, the effect of Mg^{2+} on 500 μM NMDA-evoked adenosine release was studied in the presence of TTX to block voltage-sensitive Na^+ -channels and prevent the generation of action potentials. In the presence of TTX (1 μM), Mg^{2+} -sensitivity of 500 μM NMDA-evoked adenosine release was not observed (Fig. 38), indicating that the lack of Mg^{2+} sensitivity was not due to spontaneous action potentials in the slices.

5. Concentration-response relationship of NMDA-evoked release of adenosine and [3H]noradrenaline

The concentration of NMDA employed (500 μM) was selected because it had been shown in previous studies to be submaximal for releasing [3H]noradrenaline from cortical slices (Keith et al., 1988; Fink et al., 1989). Detailed determination of the concentration-response relationships for NMDA-evoked adenosine and [3H]noradrenaline release had been avoided because the paired bath system would only provide one data point relative to a standard response per experiment. At this point, however, the observations that NMDA-evoked adenosine release was not reduced by the uncompetitive antagonist Mg^{2+} , but was blocked by the uncompetitive antagonist MK-801 (3 μM) and by 1mM of the competitive antagonist D,L-APV (Fig. 9), raised the possibility that release was mediated by an unique subtype of NMDA receptor which is unaffected by Mg^{2+} . To test whether NMDA-evoked adenosine release might occur at an NMDA receptor subtype with a different affinity for NMDA than the NMDA receptor mediating [3H]noradrenaline release, concentration-response relationships for NMDA-evoked adenosine and [3H]noradrenaline release were determined.

Fig. 39 shows that NMDA was 33 fold more potent in releasing

Figure 38. Effect of Mg^{2+} -free medium on NMDA evoked release of adenosine (ADN) from superfused slices in the presence of TTX. TTX ($1 \mu M$) was present from 20 min prior to exposure to NMDA until the end of the experiment. NMDA ($500 \mu M$) was present from 0 to 10 min. Values are means \pm SEM from 4 experiments. INSET: Histograms represent the total amount of adenosine released.

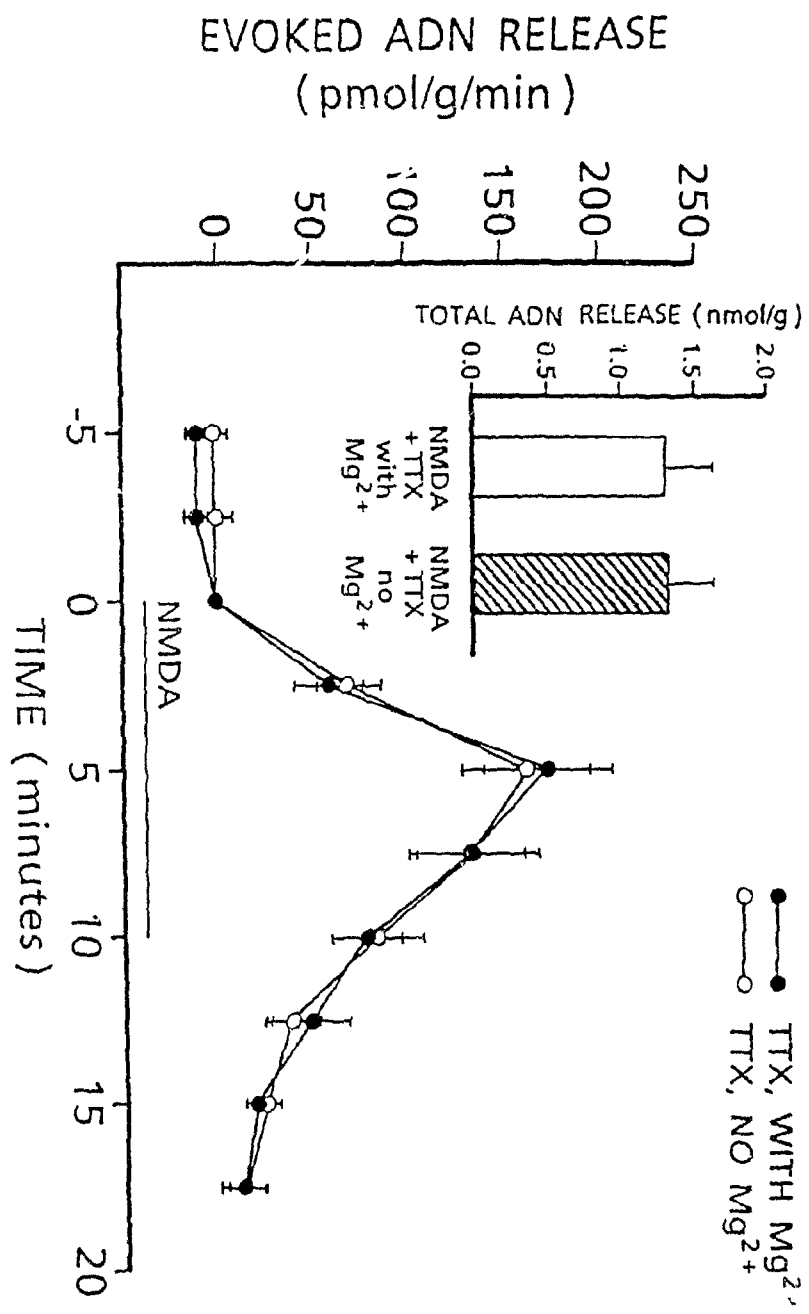


Figure 38

Figure 39. Concentration-response relationship of NMDA-evoked release of [^3H]noradrenaline ([^3H]NA) and adenosine (ADN) from the same slices in Mg^{2+} -free medium. Values represent the total amount of [^3H]noradrenaline or adenosine released by the "test" concentration of NMDA and are expressed as a percent of the total amount released by 3 mM NMDA in that experiment (see methods for details). Values are means \pm SEM from 4 experiments for all values except 30 μM NMDA where values are from 5 experiments.

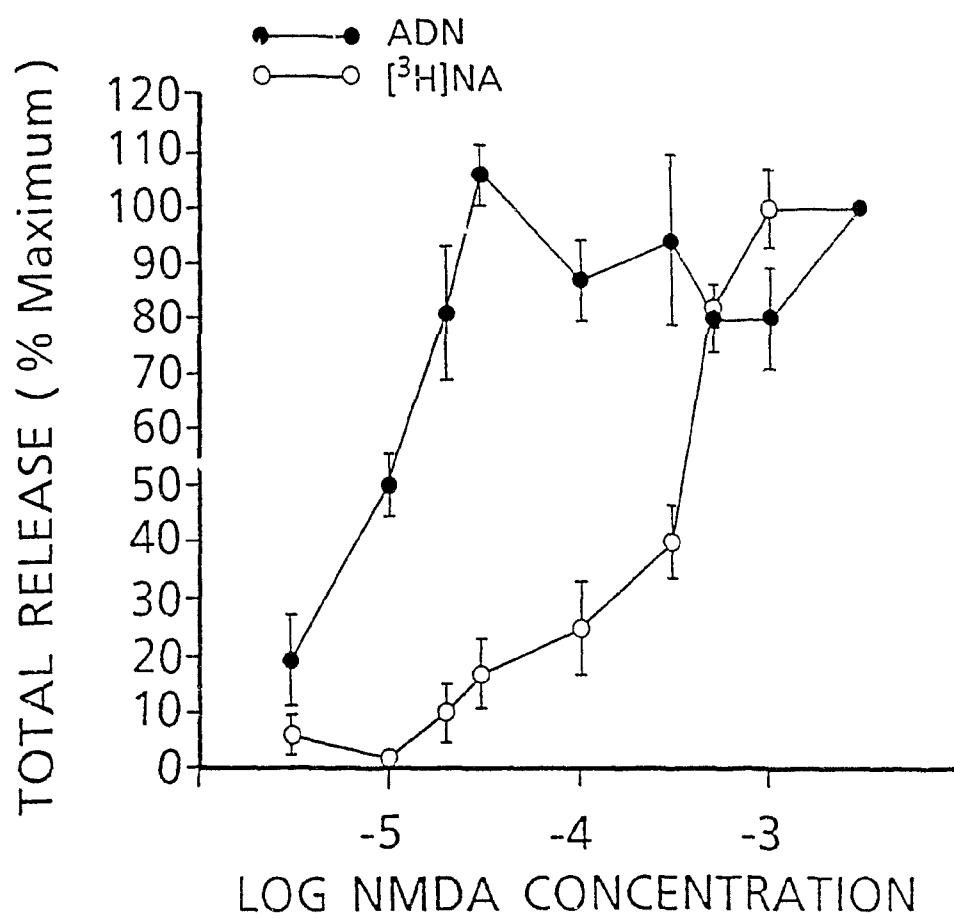


Figure 39

adenosine (EC_{50} 10 μ M) than it was in releasing [3 H]noradrenaline (EC_{50} 330 μ M). Whereas maximal release of [3 H]noradrenaline occurred at 500 to 1000 μ M NMDA, maximal adenosine release occurred at 30 μ M NMDA, a concentration at which [3 H]noradrenaline release was only 20% maximum. At a concentration of NMDA that was half-maximal for adenosine release (10 μ M), virtually no [3 H]noradrenaline release was seen.

The observation that maximal adenosine release occurs at NMDA concentrations far lower than those which elicit [3 H]noradrenaline release makes it unlikely that adenosine, released during NMDA receptor activation, will prevent excessive NMDA receptor stimulation and consequently be an effective endogenous antiexcitotoxic agent against NMDA receptor-mediated excitotoxicity as has been suggested previously (Dragunow and Faull, 1988). The difference in potency and Mg^{2+} sensitivity observed between NMDA-evoked adenosine and [3 H]noradrenaline release raised the possibility that NMDA may release adenosine by acting at a high-affinity subtype of NMDA receptor which is also Mg^{2+} -insensitive. However, these results could also be explained if maximal adenosine release occurs when only a small fraction of the NMDA receptors are activated (ie. there are spare receptors for NMDA-evoked adenosine release). In this case, uncompetitive antagonism of NMDA receptors by Mg^{2+} would be overcome as long as not all of the NMDA channels are blocked by Mg^{2+} .

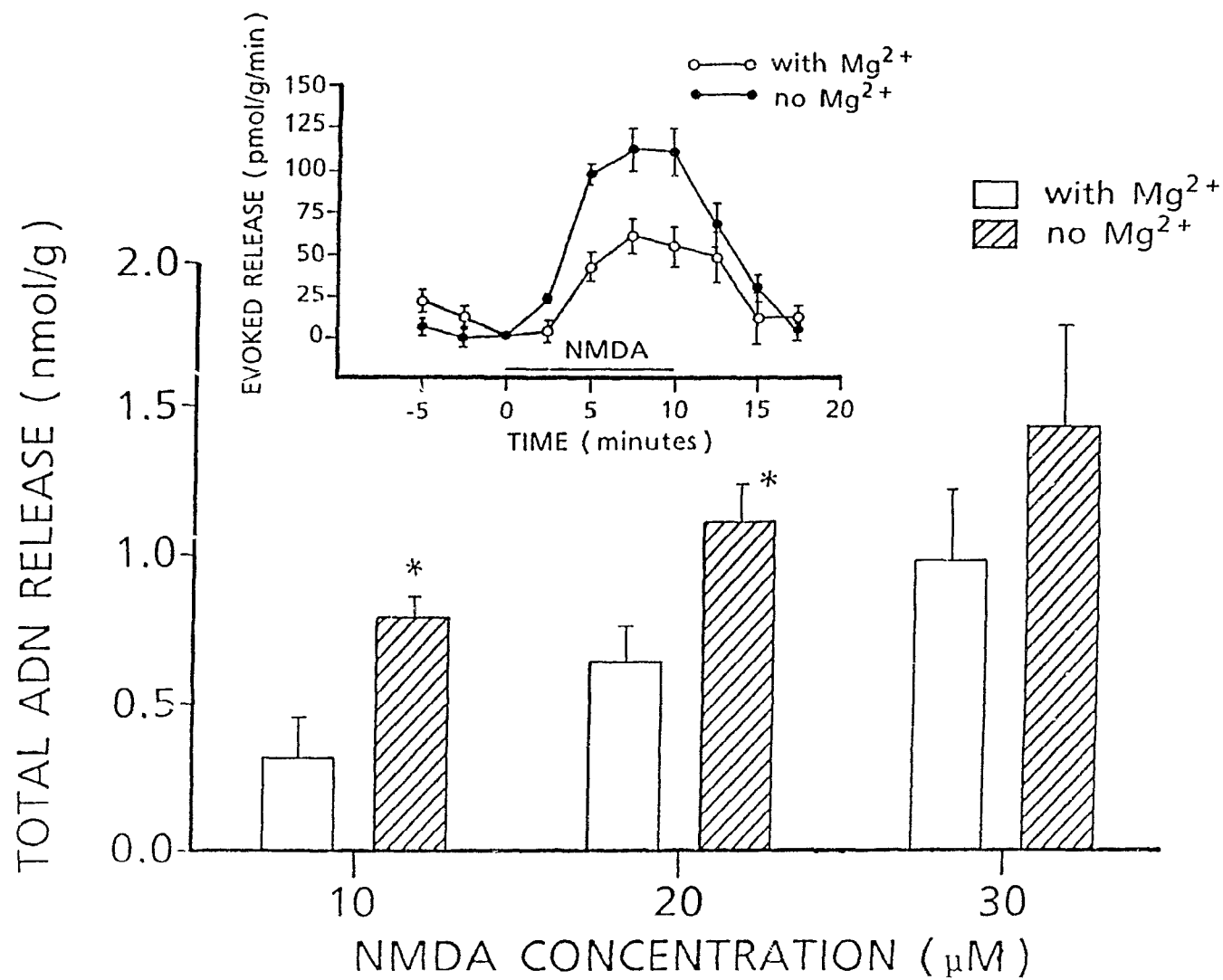
6. Mg^{2+} -sensitivity of NMDA-evoked adenosine release.

If the NMDA receptors which release [3 H]noradrenaline are identical to those which release adenosine, then Mg^{2+} should inhibit adenosine release evoked by submaximal concentrations of NMDA. When the effect of Mg^{2+} on NMDA-evoked adenosine release was re-examined using concentrations

of NMDA which were submaximal for NMDA-evoked adenosine release, NMDA-evoked adenosine release exhibited the expected inhibitory modulation by Mg^{2+} (Fig. 40). Thus, release evoked by 10 and 20 μM NMDA was increased 2.5- and 1.8-fold, respectively in the absence of Mg^{2+} . These results are consistent with the notion that only a relatively small proportion of NMDA receptors must be activated for adenosine release to proceed maximally. This contrasts with [3H]noradrenaline release where, even at very high concentrations of NMDA (3 mM), release was blocked by Mg^{2+} (total evoked release in the presence of Mg^{2+} was $0.33 \pm 0.20\%$ of content versus $3.34 \pm 0.69\%$ in Mg^{2+} -free medium, significant by paired t test, $n=4$). Although other explanations for these findings are possible, the simplest and most conservative explanation for the difference in potency of NMDA at releasing adenosine and [3H]noradrenaline and the ability of high concentrations of NMDA to overcome the competitive block of NMDA receptors by Mg^{2+} is that there are spare NMDA receptors for NMDA-evoked adenosine release.

Figure 40. Effect of Mg^{2+} -free medium on adenosine (ADN) release by submaximal concentrations of NMDA. Histograms represent the total amount of adenosine released. Values represent means \pm SEM from 5 experiments. *Significantly different from control ($p < 0.05$, paired t test). INSET: Time course of adenosine released by NMDA ($20 \mu M$) in the presence and absence of Mg^{2+} .

Figure 40



DISCUSSION

I. K^+ AND EAA RECEPTOR-MEDIATED RELEASE OF ADENOSINE FROM CORTICAL SLICES

1. K^+ and glutamate-evoked adenosine release from cortical slices.

K^+ released endogenous adenosine from cortical slices and this release occurred during exposure to elevated K^+ . This contrasts with the finding of Hollins and Stone (1980b), that K^+ -evoked release of radiolabelled purines from rat cortical slices in the presence of dipyridamole did not occur during exposure to elevated K^+ , but occurred in the first post- K^+ sample, regardless of whether K^+ was present for 2 or 10 min. It is possible that radiolabelled purines are released from a different intracellular purine pool than the pool of endogenous adenosine which is released. Alternatively, it is possible that dipyridamole, which has been shown to inhibit evoked adenosine release in a number of studies (Fredholm et al., 1980; Jonzon and Fredholm, 1985; White and MacDonald, in press), may have altered the profile of purine release observed by Hollins and Stone (1980b). Since endogenous adenosine release does occur during depolarization when dipyridamole is absent, the argument (Stone, 1981a; Snyder, 1985; Dunwiddie, 1985) that adenosine does not resemble a classical neurotransmitter because it is released only after removal of the depolarizing agent is not valid.

Although endogenous adenosine release occurred during K^+ stimulation, it was, nevertheless, slower in onset than release of the classical neurotransmitter, [3H]noradrenaline. Whereas K^+ -evoked [3H]noradrenaline release reached maximum in the first 2.5 min fraction following exposure to K^+ (or NMDA), adenosine release reached maximum later. Electrical

stimulation of hippocampal slices also results in a delayed release of [^3H]purines relative to [^3H]noradrenaline (Jonzon and Fredholm, 1985), and a delayed K^+ -evoked release of [^3H]purines relative to [^3H]GABA has been reported (Stone et al., 1981). It is possible that the slower onset of adenosine release in comparison to other neurotransmitters reflects the time required for intracellular adenosine formation prior to its release or the involvement of processes mediated by second messengers.

Glutamate also released adenosine from superfused cortical slices in a concentration-dependent manner, consistent with demonstrated glutamate-evoked [^3H]purine release from slices (Pull and McIlwain, 1975). Although relatively high concentrations of glutamate were required to release adenosine, these concentrations were consistent with those necessary to elicit other actions of glutamate in slices (Luini et al., 1981; Lehmann and Scatton, 1982; Marien et al., 1983; Garthwaite, 1985; Snell and Johnson, 1986). The hypothesis that glutamate's action at its receptor is masked by high-affinity glutamate uptake (Garthwaite, 1985) was supported in the present study by the potentiation by dihydrokainate, a blocker of high-affinity glutamate uptake (Johnston et al., 1979), of glutamate-evoked release of adenosine.

2. Contribution of extracellular nucleotides to release of adenosine from cortical slices.

The basal release of adenosine was decreased by inhibitors of ecto-5'-nucleotidase, indicating that much of this release occurred in the form of a nucleotide that was subsequently converted extracellularly to adenosine. This finding is in agreement with the observations that basal release of adenosine from whole brain and spinal cord synaptosomes is also

derived to a large extent from a released nucleotide (MacDonald and White, 1985; Sweeney et al., 1987a), that α,β -methylene ADP diminishes basal release of [^3H]adenosine from cat basal ganglia in vivo (Barberis et al., 1984), and that dipyridamole increases the basal release of [^3H]adenosine from neocortical synaptosomes (Daval and Barberis, 1981). The identity of the released nucleotide is not known, although possible candidates are ATP, ADP, AMP or cyclic AMP.

In contrast with basal adenosine release, K^+ -evoked adenosine release was not diminished when ecto-5'-nucleotidase was inhibited, indicating that adenosine itself was released. This is in agreement with the finding that α,β -methylene ADP does not alter the K^+ -evoked release of purines from [^3H]adenosine-labelled rabbit retina (Perez et al., 1986). A previous study of K^+ -evoked adenosine release from synaptosomes determined that inhibitors of ecto-5'-nucleotidase decreased K^+ -evoked adenosine release from synaptosomes by 33% (MacDonald and White, 1985). It is possible that the contribution of nucleotides to K^+ -evoked adenosine release from whole brain synaptosomes, but not from cortical slices, reflects differences in release from isolated nerve terminals versus release from intact neurons and/or glia present in slices.

Glutamate also released adenosine itself and not a nucleotide from slices. This agrees with the observation by Pons et al. (1980) that glutamate-evoked accumulation of cyclic AMP, thought to be mediated indirectly through released adenosine (Bruns et al., 1980b), is not decreased in the presence of inhibitors of ecto-5'-nucleotidase. It may be significant that adenosine release evoked by glutamate occurs in the form of adenosine per se and not as a nucleotide. ATP, which is also

released from the cortex during depolarization (Wu and Phillis, 1978; Potter and White, 1980) can exert excitatory actions when applied iontophoretically in the CNS (Phillis et al., 1979a). If the glutamate-evoked increase in extracellular adenosine was derived from released ATP, the latter nucleotide might elicit excitation as a result of an action at P_2 -purinoceptors and stimulate rather than inhibit neuronal activity.

3. Receptor-mediated release of adenosine from cortical slices by NMDA, kainate, and quisqualate.

NMDA, kainate and quisqualate released endogenous adenosine from superfused rat cortical slices, consistent with observations from in vivo studies that kainate, quisqualate and NMDA release radiolabelled purines from rat cortex (Jhamandas and Dumbrille, 1980; Perkins and Stone, 1983c) and that kainate releases endogenous adenosine from the hippocampus (Lehmann et al., 1987). Both APV and MK-801 effectively blocked NMDA-evoked adenosine release, indicating that release was mediated through NMDA receptors. In further support of this conclusion is the apparent lack of nonspecific effects of these NMDA receptor antagonists, in so far as they did not affect kainate-evoked adenosine release. The observation that 500 μ M NMDA-evoked adenosine release was not modulated by physiological Mg^{2+} concentrations, a finding which seemed initially to be inconsistent with NMDA receptor mediation of adenosine release, will be discussed later (DISCUSSION, Section IV).

Kainate and quisqualate released adenosine by acting at non-NMDA receptors. Thus kainate-evoked adenosine release was diminished by the nonspecific EAA receptor antagonist, DGG (Fagg et al., 1986; Mayer and Westbrook, 1987), and both kainate and quisqualate-evoked adenosine

release were antagonized by the more specific non-NMDA receptor antagonist, DNQX (Fletcher et al., 1988; Honoré et al., 1988). The lack of block of kainate-induced adenosine release by the specific NMDA receptor antagonists, MK-801 and APV, indicates the lack of either direct or indirect NMDA receptor involvement in kainate-evoked adenosine release. In contrast to the present finding, Perkins and Stone (1983c) reported a 23% inhibition of 100 μ M (but not 500 μ M) kainate-evoked [3 H]purine release from rat cortex in vivo by the NMDA antagonist, APH. This may represent differences between release of endogenous adenosine versus [3 H]purines or possibly nonspecific effects of APH. Alternatively, a small indirect NMDA receptor component of kainate-evoked adenosine release may occur in vivo.

The observation that DNQX antagonizes quisqualate-evoked adenosine release suggests that quisqualate releases adenosine primarily by acting at the ionotropic quisqualate/AMPA receptor and not the metabotropic quisqualate receptor, although a small effect via the metabotropic quisqualate receptor cannot be ruled out. An action of quisqualate at the ionotropic receptor is supported by the preliminary observation that AMPA (100 μ M) also released adenosine from rat cortical slices in one experiment (results not shown).

4. Involvement of NMDA and non-NMDA receptors in glutamate-evoked adenosine release from cortical slices.

Both NMDA and non-NMDA receptors were involved in glutamate-evoked adenosine release. Thus the NMDA antagonists, APV and MK-801, inhibited glutamate-evoked adenosine release from cortical slices by about 50% and this release was further diminished by the addition of the non-NMDA antagonist, DNQX. In incubated slices APV antagonized release during, but

not following, exposure to glutamate, an observation not confirmed in superfused slices. The reason for the lack of antagonism by APV of the delayed release of adenosine following exposure to glutamate in bath-incubated slices is not clear, but it could represent a non-NMDA receptor-mediated component or a glutamate uptake-mediated component.

The involvement of both NMDA and non-NMDA receptors in glutamate-evoked adenosine release from superfused cortical slices is in agreement with the findings of Jhamandas and Dumbrille (1980) that glutamate-evoked release of radiolabelled purine derivatives from rat cortex *in vivo* is diminished by both 1-hydroxy-3-aminopyrrolidone-2 (HA-966), an uncompetitive antagonist acting at the strychnine-insensitive glycine site on the NMDA receptor (Collingridge and Lester, 1989), and glutamic acid diethyl ester. These results are consistent with the involvement of both NMDA and non-NMDA receptors in neocortical synaptic transmission (Thomson et al., 1989a) and excitotoxic pathology (Rothman and Olney, 1987; Frandsen et al., 1989; Koh et al., 1990).

5. Involvement of NMDA receptors in K^+ -evoked release of adenosine but not [3H]noradrenaline from cortical slices.

Interestingly, K^+ -evoked adenosine release was not entirely due to direct depolarization, but was mediated in part indirectly through an action of a released endogenous EAA at NMDA receptors. Thus, K^+ -evoked adenosine release was decreased by both APV and MK-801, which are structurally unrelated and act at different loci on the NMDA receptor-ion channel complex (Foster and Fagg, 1987), but not by the non-NMDA antagonist, DNQX. The Ca^{2+} dependence of K^+ -evoked release of adenosine (see DISCUSSION, section I.7) may be due to a Ca^{2+} requirement for the

release of an endogenous EAA, which could, in turn, evoke a Ca^{2+} -independent release of adenosine. The involvement of NMDA but not non-NMDA receptors suggests that NMDA receptors may be more readily accessible to the site for release of the EAA, or that the endogenous EAA released by K^+ may have a preference for NMDA over non-NMDA receptors. In this regard, aspartate (Collingridge and Lester, 1989), homocysteic acid (Do et al., 1986; 1988), and N-acetylaspartylglutamate (Westbrook et al., 1986; Zollinger et al., 1988; but see Whitemore and Koerner, 1989), which preferentially act at NMDA receptors and are released from neocortex, are possible candidates. Quinolinic acid is another possible candidate (McLennan, 1984; Perkins and Stone, 1983a; Stone and Burton, 1988).

The observation that K^+ -evoked release of adenosine may be mediated indirectly through the release of an EAA raises the possibility that K^+ -evoked events observed in other studies could also be secondary to EAA release. Indeed, similar observations have been reported for K^+ -induced ion fluxes. Luini et al. (1981, 1983) found that the order of potency of several EAA antagonists in blocking K^+ -evoked Na^+ efflux from slices of rat brain correlated with their order of potency in blocking NMDA, but not non-NMDA receptors. Similarly, K^+ -evoked stimulation of Ca^{2+} uptake into hippocampal slices (Crowder et al. 1987) and K^+ -evoked Na^+ efflux from hippocampal neurons (Ransom and Stec, 1988b) are inhibited by NMDA antagonists.

In contrast to K^+ -evoked adenosine release and K^+ -stimulated ion fluxes, [^3H]noradrenaline release by K^+ is not mediated indirectly through a released EAA. Thus MK-801 did not inhibit, but actually potentiated K^+ -evoked [^3H]noradrenaline release. The reason for the increase in K^+ -evoked

[³H]noradrenaline release in the presence of MK-801 is not clear. Although nonspecific effects of MK-801, such as the block of voltage-gated K⁺ channels, have been reported (Rothman, 1988), they are unlikely to be responsible because the concentrations of MK-801 for these effects are 33- to 330- fold higher than those used in the present study. Nevertheless, it appears that K⁺-evoked [³H]noradrenaline release, unlike K⁺-evoked adenosine release, is not mediated indirectly through the release of an endogenous EAA acting at NMDA receptors. Similarly, K⁺-evoked release of [³H]acetylcholine from striatal slices is only minimally decreased (16%) by 100 μ M APV (Lehmann and Scatton, 1982) and K⁺-evoked GABA release is not inhibited by either APV (1mM) or APH (1mM) (Crowder et al., 1987).

6. TTX-sensitivity of adenosine release evoked by K⁺ and EAA agonists from cortical slices.

K⁺-evoked adenosine release, and to a lesser extent also glutamate-evoked adenosine release, appeared to be mediated in part by propagated action potentials in the cortical slices in so far as release was diminished by 1 μ M TTX. Jhamandas and Dumbrille (1980) reported that glutamate-evoked purine release from cortex in vivo was unaffected by 16 μ M TTX but was diminished by 80 μ M TTX, a concentration much higher than is normally required to inhibit action potentials. However, these authors found no TTX sensitivity of K⁺-evoked purine release, possibly because the K⁺ concentration in their study (56 mM versus 30 mM in the present study) produced more direct depolarization. In agreement with the TTX-sensitivity of glutamate-evoked adenosine release, release evoked by NMDA and by kainate was also diminished by TTX, indicating some involvement of propagated action potentials in release by these agents as well.

7. Ca^{2+} -dependence of adenosine release evoked by K^+ and EAA agonists from cortical slices.

Basal (unstimulated) adenosine release from cortical slices and synaptosomes was increased in the absence of Ca^{2+} . There are reports of similar increases in the spontaneous efflux of [^{14}C]purines, glutamate, GABA, and [^3H]GABA (Pull and McIlwain, 1973; Valdés and Orrego, 1978; Villanueva et al., 1988; Minc-Golomb et al., 1988) but not [^3H]aspartate (Minc-Golomb et al., 1988) from brain slices in Ca^{2+} -deficient medium.

K^+ -evoked adenosine release was diminished in the absence of Ca^{2+} . Ca^{2+} -dependence has been reported previously for K^+ -evoked release of endogenous adenosine from whole brain synaptosomes (MacDonald and White, 1985) and rat striatal slices (Wojcik and Neff, 1983b), as well as for the release of radiolabelled purines from rat cortex in vivo (Jhamandas and Dumbrille, 1980) and rabbit retina in vitro (Perez et al., 1988). In the present study, the adenosine release that persisted in the absence of Ca^{2+} may be due to residual extracellular Ca^{2+} in the slice or it may represent a separate, Ca^{2+} -independent, releasable pool of adenosine. Evidence in favour of the latter possibility is the altered time course for release in the absence of Ca^{2+} and the apparent lack of depletion of the Ca^{2+} -dependent pool following depolarization in Ca^{2+} -free medium. Bender et al. (1981) have proposed the existence of independent releasable pools of purines on the basis of their observation that veratridine-evoked release of purine derivatives from neocortical synaptosomes was Ca^{2+} -dependent following a 30 s loading period with [^3H]adenosine and Ca^{2+} -independent following a 15 min loading period.

Omitting Ca^{2+} from the medium had no effect on the total amount of

adenosine released by EAA agonists, although the time courses for release differed in some cases (see below). The lack of Ca^{2+} -dependence of EAA-evoked adenosine release suggests that the mechanism of adenosine release may differ fundamentally from the mechanism of release of classical neurotransmitters. Indeed, there is evidence that adenosine may be released via a bidirectional nucleoside transport system (Jonzon and Fredholm, 1985; White and MacDonald, in press) rather than via a neurosecretory mechanism. The observation that a tonic inhibitory purinergic tone persists in low Ca^{2+} medium in hippocampal slices (Fowler, 1988; Haas and Greene, 1988) suggests that adenosine released in a Ca^{2+} -independent manner may be of physiological relevance.

In agreement with the current finding is the previous observation that theophylline-sensitive, glutamate-evoked increase of cyclic AMP in rat cortical slices, thought to be mediated by released adenosine (Newman and McIlwain, 1977; Bruns et al., 1980b; Pons et al., 1980), is not Ca^{2+} -dependent (Shimizu et al., 1974). However, Jhamandas and Dumbrielle (1980) reported Ca^{2+} -dependence of glutamate-evoked release of radiolabelled purines from rat cortex *in vivo*. Also, Perez and Ehinger (1989) recently reported that, whereas quisqualate-evoked release of radiolabelled purine derivatives was not significantly reduced at either low (25 μM) or high (100 μM) quisqualate concentrations, release of purines evoked by low (25 μM), but not high (100 μM) concentrations of kainate or NMDA was diminished in Ca^{2+} -free, EGTA-containing medium. The reason for these differences is not clear, although it is possible that radiolabelled purines are released from an intracellular pool distinct from the source for endogenous adenosine release. However, the possibility that lower

concentrations of kainate and NMDA might also evoke a Ca^{2+} -dependent release of adenosine from cortex cannot be excluded.

Although the total amount of adenosine released in the absence of Ca^{2+} was diminished in the case of K^+ and unaffected in the case of glutamate, NMDA, kainate, and quisqualate, the initial rate of release evoked by K^+ , glutamate, kainate, and quisqualate was actually increased in low Ca^{2+} . The reason for this increase is not clear. Alterations in membrane properties in response to reduced external Ca^{2+} concentrations have been described (Hille, 1968) and it is possible that enhanced membrane excitability in the absence of Ca^{2+} may account for both the increased basal adenosine release and the increased initial rate of evoked release. Alternatively, the different time course for adenosine release in the absence of Ca^{2+} could indicate that adenosine is released from separate Ca^{2+} -dependent and Ca^{2+} -independent pools. If there are indeed separate, Ca^{2+} -dependent and Ca^{2+} -independent releasable pools of adenosine, then the observation that the initial rates of evoked release in Ca^{2+} -free medium are actually increased suggests that the presence of Ca^{2+} antagonizes adenosine release from the Ca^{2+} -independent pool. The possibility that Ca^{2+} may antagonize release from a Ca^{2+} -independent pool has been proposed previously for the Ca^{2+} -independent release of endogenous GABA, glutamate, and aspartate (Nadler et al. 1977).

A second stimulation by EAA agonists in the presence of Ca^{2+} released markedly less adenosine than was released by the first agonist stimulation. The most likely explanations for the decreased adenosine release during the second stimulations are either desensitization of EAA receptors or depletion of Ca^{2+} -dependent pools of adenosine. The question

of EAA receptor desensitization is somewhat controversial. However, all reports except one (Hori and Carpenter, 1988) indicate that NMDA (Fagni et al., 1983; Krishtal et al., 1988; Kushner et al., 1988; Lehmann et al., 1988) and quisqualate (Kiskin et al., 1986; Mayer and Vyklicky, 1989; Perouansky and Grantyn, 1989) receptors desensitize, whereas kainate receptors do not (Fagni et al., 1983; Kiskin et al., 1986; Kushner et al., 1988; Mayer and Vyklicky, 1989), although a decrement in electrical responses to kainate when applied at intervals less than 20 min has been reported (Perouansky and Grantyn, 1989).

Restoration of Ca^{2+} to slices which had been stimulated previously in the absence of Ca^{2+} failed to restore a second quisqualate-evoked release of adenosine. The observed decrement of quisqualate-evoked adenosine release may be due, at least in part, to quisqualate receptor desensitization. In contrast, during a second exposure with Ca^{2+} restored, NMDA released significantly more adenosine from slices previously stimulated in the absence of Ca^{2+} than from slices previously stimulated in the presence of Ca^{2+} . It is possible that the absence of Ca^{2+} during the initial stimulation somehow protected against desensitization of the NMDA receptors. Indeed, Ca^{2+} -dependent inactivation of NMDA responses has been described (Mayer and Westbrook, 1985). It is also possible that NMDA releases adenosine from separate Ca^{2+} -dependent and independent pools.

When slices which had been previously exposed to kainate in the absence of Ca^{2+} were re-exposed to kainate in the presence of Ca^{2+} , the time course and total amount of adenosine for the second release was remarkably similar to that observed from slices during an initial stimulation in the presence of Ca^{2+} . Because there is evidence that

kainate receptors do not desensitize (see references above), the release of adenosine from separate Ca^{2+} -dependent and Ca^{2+} -independent pools appears to be the most likely explanation for these observations. In further support of this are the findings that the pools could apparently be depleted independently of each other. Thus when Ca^{2+} was present throughout, kainate-evoked release of adenosine was greatly diminished during the second exposure to kainate. It is unlikely that Ca^{2+} -dependent excitotoxicity by kainate can explain the reduced release of adenosine observed during the second exposure. When Ca^{2+} was absent during both the first and second exposures to kainate, release of adenosine was also greatly diminished during the second exposure. This contrasts with the observation that release of adenosine was not diminished from slices which had initially been stimulated in the absence of Ca^{2+} and were then re-exposed to kainate in the presence of Ca^{2+} . The above findings are most compatible with the possibility that kainate releases adenosine from separate Ca^{2+} -dependent and -independent pools in the cortex.

II. NON-RECEPTOR-MEDIATED GLUTAMATE-EVOKED RELEASE OF ADENOSINE BUT NOT $[^3\text{H}]$ NORADRENALINE FROM CORTICAL SYNAPTOSOMES

1. Lack of involvement of EAA receptors in glutamate-evoked adenosine release from cortical synaptosomes.

The location of the EAA receptors involved in adenosine release from superfused cortical slices is not known. To determine whether EAA receptors mediating glutamate-evoked adenosine release are located on presynaptic terminals, the same region of parietal cortex from which slices had been prepared was used for the preparation of synaptosomes.

L-Glutamate released endogenous adenosine from rat parietal cortical synaptosomes; this release arose from the synaptosomes and not from myelin or mitochondrial contaminants of the crude P₂ synaptosomal preparation. However, in contrast to L-glutamate-evoked adenosine release from cortical slices which is mediated by both NMDA and non-NMDA receptors (see previous section), L-glutamate-evoked release of adenosine from synaptosomes was not receptor-mediated. Thus, L-glutamate-evoked release of adenosine from synaptosomes was not blocked by the NMDA antagonist MK-801 or by the non-NMDA antagonist, DNQX, at concentrations that effectively block these receptors in cortex (Wong et al., 1986; Fletcher et al., 1988) and markedly diminished glutamate-evoked adenosine release from rat cortical slices in the present study.

Adenosine release was not evoked by EAA agonists such as NMDA, kainate, or quisqualate. Moreover, the putative presynaptic APB-sensitive excitatory amino acid receptor (Monaghan et al., 1989) did not appear to be involved in L-glutamate-evoked adenosine release, as APB neither released adenosine nor antagonized L-glutamate-evoked adenosine release. It is unlikely that adenosine release reflected nonspecific damage to the synaptosomes caused by glutamate. In other systems, the neurotoxic effects of glutamate have been attributed to activation of excitatory amino acid receptors (Rothman and Olney, 1987), whereas L-glutamate-evoked release of adenosine was not receptor-mediated. Furthermore, L-glutamate appears to have a selective effect, in so far as it did not release ATP from cortical synaptosomes.

2. Role of glutamate uptake in glutamate-evoked adenosine release from cortical synaptosomes.

Because excitatory amino acid receptors did not appear to mediate L-glutamate-evoked release of adenosine from synaptosomes, it was hypothesized that the uptake of L-glutamate might mediate adenosine release. The suggestion that glutamate uptake might release adenosine has been made previously (Bruns et al., 1980b). The observations that inhibiting high-affinity glutamate uptake with dihydrokainate blocked glutamate-evoked release of adenosine, and that glutamate-evoked adenosine release, like the high-affinity transport system for glutamate (Bennet et al., 1972; Erecińska, 1987), was Na^+ -dependent, strongly favour the suggestion that glutamate uptake mediates adenosine release from cortical synaptosomes. Although it is possible that the Na^+ requirement reflects an effect on adenosine release rather than on glutamate uptake, no evidence for a Na^+ dependency for the adenosine release process per se has been reported to date.

L-Glutamate uptake involves both its transport across the cell membrane and its subsequent intrasynaptosomal metabolism. D-Aspartate, which is a good substrate for the high-affinity acidic amino acid transporter but is not metabolized (Davies and Johnston, 1976; Erecińska, 1987), released adenosine. Moreover, D-glutamate, which has a low affinity for the high-affinity L-glutamate transporter (Balcar and Johnston, 1972), was correspondingly much less effective than either L-glutamate or D-aspartate at releasing adenosine. Although the existence of a single, low-affinity component for D-glutamate uptake (Takagaki, 1976; Benjamin and Quastel, 1976) has led some investigators to conclude

that D-glutamate is not transported by the high-affinity carrier, the modulation of its effects by dihydrokainate in the present and other studies (Lodge et al., 1979, 1980) suggests that D-glutamate may, in fact, be a low-affinity substrate for the high-affinity L-glutamate carrier. Release of adenosine by both D-aspartate and D-glutamate suggests that the Na^+ -dependent, high-affinity transport of acidic amino acids across the synaptosomal membrane, rather than the subsequent metabolism of the amino acids, mediates adenosine release.

High-affinity glutamate transport is coupled to the influx of Na^+ , which depolarizes synaptosomes (Erecińska, 1989; McMahon et al., 1989) and stimulates both synaptosomal Na^+, K^+ -ATPase activity and O_2 consumption (Erecińska, 1989). Adenosine release following glutamate transport may be due to depolarization or to activation of Na^+, K^+ -ATPase. A similar process appears to be involved in the release of glutamate from guinea pig cortical synaptosomes induced by D-aspartate uptake (McMahon et al., 1989). Although GABA uptake-induced release of [^3H]noradrenaline from rat hippocampal synaptosomes has been demonstrated (Bonanno et al., 1989), glutamate uptake into cortical synaptosomes did not release [^3H]noradrenaline in the present study. Glutamate may be transported in a population of synaptosomes different from those which transport [^3H]noradrenaline. It is also possible that [^3H]noradrenaline release requires a stronger depolarization than is required for glutamate uptake-mediated adenosine release from synaptosomes. Moreover, L-glutamate transport-mediated adenosine release from cortical synaptosomes is Ca^{2+} -independent, whereas GABA uptake-induced release of [^3H]noradrenaline from rat hippocampal synaptosomes (Bonanno et al., 1989) and D-aspartate

transport-mediated release of glutamate (McMahon et al., 1989) are both Ca^{2+} -dependent. Furthermore, GABA uptake-mediated [^3H]noradrenaline release is TTX sensitive (Bonanno et al., 1989), whereas glutamate transport-mediated adenosine release is not TTX sensitive and thus does not appear to involve the activation of voltage-sensitive Na^+ channels.

3. Nature of the purine released by L-glutamate from cortical synaptosomes.

Glutamate uptake does not appear to release adenosine per se by efflux on the bidirectional nucleoside transporter, as has been shown for the K^+ - and veratridine-evoked release of adenosine from whole brain synaptosomes (MacDonald and White, 1985) and for K^+ -evoked adenosine release from cortical synaptosomes in the present study, in so far as release was augmented rather than diminished by the nucleoside transport inhibitor, dipyridamole. Because adenosine derived extrasynaptosomally from a released nucleotide would be prevented from re-entering the synaptosomes when the nucleoside transporter is inhibited by dipyridamole, the augmentation of extrasynaptosomal adenosine concentrations by dipyridamole is consistent with the possibility that glutamate transport releases a nucleotide that is subsequently metabolized extracellularly to adenosine. This is supported by the observation that inhibition of ecto-5'-nucleotidase with α,β -methylene ADP and GMP virtually abolished the extrasynaptosomal accumulation of adenosine evoked by glutamate.

The identity of the nucleotide that is released and subsequently metabolized to adenosine extrasynaptosomally remains unclear, although it does not appear to be either ATP or cyclic AMP. L-Glutamate failed to release detectable amounts of ATP from cortical synaptosomes. Moreover,

inhibition of a substantial proportion of cyclic AMP phosphodiesterase with IBMX did not reduce the amount of adenosine detected in the incubation medium following exposure of synaptosomes to L-glutamate, indicating that adenosine detected extrasynaptosomally following exposure to L-glutamate was not derived primarily from the extracellular metabolism of released cyclic AMP. By the process of elimination, it would appear that L-glutamate released either ADP or AMP from cortical synaptosomes but final identification requires further investigation.

4. Release of [^3H]noradrenaline from cortical synaptosomes.

Although K^+ released [^3H]noradrenaline from synaptosomes, indicating the presence of a releasable pool, glutamate, NMDA and quisqualate did not release [^3H]noradrenaline. This suggests that neither EAA receptor-mediated nor glutamate transport-mediated [^3H]noradrenaline release occurs from cortical synaptosomes. These findings are in agreement with the observations by Fink et al. (1989) that NMDA does not release [^3H]noradrenaline or [^3H]5-HT from rat cortical synaptosomes. Interestingly, in the present study, kainate evoked a small (1% of total [^3H]noradrenaline content) but statistically significant release of [^3H]noradrenaline from the synaptosomes. The amount of [^3H]noradrenaline released by kainate may be small relative to the amount released by K^+ because only a small subpopulation of nerve terminals in the cortex possesses these receptors. However, glutamate is an effective agonist at kainate receptors (Collingridge and Lester, 1989) and thus should also have released [^3H]noradrenaline if kainate-evoked [^3H]noradrenaline release was receptor-mediated. It is therefore not clear whether kainate-evoked [^3H]noradrenaline release represents a kainate receptor-mediated effect or

whether this represents a nonspecific effect of kainate.

5. Do EAA receptors exist on cortical presynaptic terminals?

The absence of EAA receptor-mediated release of adenosine or [^3H]noradrenaline from synaptosomes (except for possibly a small kainate receptor-mediated release of [^3H]noradrenaline) raises the question as to whether there are excitatory amino acid receptors on cortical presynaptic terminals or synaptosomes. The possible existence of kainate receptors on synaptosomes has been a contentious issue. Although a number of groups have reported that millimolar kainate stimulates a Ca^{2+} -dependent release of glutamate from synaptosomes (Pastuszko et al., 1984; Poli et al., 1985; Arvin et al., 1989), Pocock et al. (1988) have presented convincing data demonstrating that the kainate-mediated release of glutamate from neocortical synaptosomes is not a receptor-mediated event, but occurs as a result of kainate's inhibition of the glutamate transporter. Arvin et al. (1989), however, maintain that kainate does release glutamate by acting at receptors on neocortical synaptosomes and indicate that they have evidence (as yet unpublished) that different methodologies might explain the differences between their results and those of Pocock et al. (1988).

There are relatively few other reports of excitatory amino acid receptor-mediated events in cortical synaptosomal preparation. The lack of NMDA receptor-mediated [^3H]noradrenaline release observed in the present study is consistent with previous reports (Fink et al., 1989). However, O'Shaughnessy and Lodge (1988) have reported enhancement of Ca^{2+} mobilization in cortical synaptosomes by both NMDA and quisqualate. An apparently quisqualate receptor-mediated Ca^{2+} -independent release of

glutamate, in the absence of receptor-mediated release of glutamate by either NMDA or kainate, has also been reported (Barrie and Nicholls, 1988; McMahon et al., 1989). Thus, there is limited evidence for the existence of EAA receptors on cortical synaptosomes. Nevertheless, adenosine release from cortical synaptosomes was not evoked by activation of EAA receptors in the present study. Quisqualate and NMDA receptors mediating Ca^{2+} mobilization (O'Shaughnessy and Lodge, 1988) and quisqualate receptors mediating glutamate release (Barrie and Nicholls, 1988) may be located on a subpopulation(s) of synaptosomes distinct from those that release adenosine or [^3H]noradrenaline. Alternatively, it is possible that presynaptic excitatory amino acid receptors are not functionally coupled to the release of adenosine or [^3H]noradrenaline. A less likely possibility is that EAA receptors are somehow inactivated during the preparation of synaptosomes, although it is not clear why such inactivation should occur in some studies but not in others.

III. CELLULAR SOURCE(S) OF RELEASED ADENOSINE

The cellular source(s) of EAA-evoked adenosine release is not clear. The observation that NMDA, kainate and quisqualate do not release adenosine from cortical synaptosomes suggests that receptor-mediated release observed in cortical slices probably does not originate from the immediate presynaptic portion of nerves. However, adenosine could arise from other neuronal processes and cell bodies. The relatively small TTX-sensitivity of EAA receptor-mediated adenosine release suggests that release through EAA receptors is not primarily mediated through neuronal circuits and that its cellular site of origin is quite close to the EAA

receptors mediating its release. Moreover, there is evidence for the existence of non-NMDA receptors on glia (MacVicar et al., 1988; Backus et al., 1989; Usowicz et al., 1989), and field stimulation-induced release of radiolabelled purine derivatives from glial cultures has been reported (Caciagli et al., 1988), suggesting that glia could be potential sources of the adenosine released by kainate or quisqualate in the present study. Unlike responses to kainate and quisqualate, responses to NMDA have generally been reported to be absent in astrocytes in primary culture (Backus et al., 1989; Usowicz et al., 1989; Kimelberg et al., 1989), with the exception of a reported NMDA response which occurred only following prior exposure to kainate (Enkvist et al. 1988). Thus it is unlikely that adenosine released by NMDA in the present study arises from astrocytes.

Although presynaptic terminals are the most likely source of adenosine released following glutamate transport into synaptosomes, one should bear in mind that synaptosomal preparations are contaminated with glial elements (Henn et al., 1976) which, like neurons, possess high-affinity Na^+ -dependent transport systems for glutamate (Henn et al., 1974; Erecińska, 1987). A possible contribution by glia to glutamate uptake-mediated release of adenosine and to the consequent modulation of synaptic activity at glutamatergic synapses should also be considered.

IV. A COMPARISON OF NMDA-EVOKED RELEASE OF ADENOSINE AND [^3H]NORADRENALINE FROM CORTICAL SLICES

1. TTX-sensitivity of NMDA-evoked adenosine and [^3H]noradrenaline release from cortical slices.

NMDA (500 μM) released both adenosine and [^3H]noradrenaline from rat

cortical slices. Propagated action potentials were essential for NMDA-evoked [^3H]noradrenaline release in so far as TTX virtually abolished NMDA-evoked release of [^3H]noradrenaline, as has been reported previously (Fink et al., 1989). Together with the observation that NMDA does not release [^3H]noradrenaline from cortical synaptosomes (present study and Fink et al., 1989), this suggests that the NMDA receptors which mediate [^3H]noradrenaline release in the cortex are not located on noradrenergic nerve terminals. In contrast, NMDA-evoked adenosine release was relatively TTX-insensitive, suggesting that propagated action potentials were not primarily involved. This observation is not unique to adenosine, in that TTX-insensitivity has also been found for NMDA-evoked release of somatostatin from cortical neurons (Tapia-Arancibia and Astier, 1989) and for the striatal release of [^3H]GABA (Weiss, 1988) and dopamine in some studies (Roberts and Anderson, 1979; Clow and Jhamandas, 1989) but not others (Snell and Johnson, 1986; Carter et al., 1988). The TTX-insensitivity of NMDA-evoked adenosine release suggests that it is not primarily mediated through neuronal circuits and that its cellular site of origin is quite close to the NMDA receptors mediating its release. However, the observation that NMDA does not release adenosine from synaptosomes suggests that release probably does not arise from the immediate presynaptic portion of nerves.

2. Possible explanations for the lack of Mg^{2+} block of 500 μM NMDA-evoked adenosine release.

As pointed out previously (DISCUSSION, Section I.3), NMDA-evoked adenosine release is blocked by both the competitive NMDA antagonist, APV, and the uncompetitive NMDA receptor-channel blocker, MK-801, findings

which strongly suggest that NMDA-evoked adenosine release is mediated by activation of the NMDA receptor-associated ion channel complex. Surprisingly, 500 μ M NMDA-evoked adenosine release was actually decreased in the absence of Mg^{2+} , a finding which appears inconsistent with mediation of adenosine release through activation of classical NMDA receptors. The reason for the small decrease in adenosine release in the absence of Mg^{2+} is not clear but it suggests that Mg^{2+} may be involved at some point in the adenosine release process.

There are several possible explanations for the lack of enhancement of NMDA-evoked adenosine release in Mg^{2+} -free medium. It seemed possible that the slices might be partially depolarized, which would alleviate the voltage-dependent block by Mg^{2+} of the NMDA receptor-associated ionic channels (Nowak et al., 1984). However, the inability to unmask the Mg^{2+} block of NMDA-evoked adenosine release even in hyperpolarizing medium (1 mM K^+), argues against partial depolarization of the slices as an explanation for these findings. This conclusion is further supported by the observation that, in agreement with previous reports (Keith et al., 1988; Fink et al., 1989), physiological concentrations of Mg^{2+} (1.2 mM) blocked NMDA-evoked [3H]noradrenaline release from cortical slices, while adenosine release was not diminished. Thus the slices in the present study did not appear to be depolarized and this cannot explain the lack of enhancement of NMDA-evoked adenosine release in Mg^{2+} -free medium.

It was possible that Mg^{2+} is required for adenosine release per se and that this requirement counteracted the inhibitory effect of Mg^{2+} on the NMDA receptor-associated ionic channels. In agreement with a previous study using hippocampal slices (Schmidt and Taylor, 1988), partial

depolarization with 12 mM K^+ augmented NMDA-evoked [3H]noradrenaline release, indicating that the Mg^{2+} block of the NMDA receptor had been diminished. However, partial depolarization with K^+ did not augment NMDA-evoked adenosine release. Under these conditions, the concentration of extracellular Mg^{2+} was maintained constant. Therefore, an effect of Mg^{2+} -free medium either on the adenosine release process per se or on the amount of endogenous adenosine available for release cannot explain the lack of enhancement of NMDA-evoked adenosine release in Mg^{2+} -free medium.

A third possibility arises from the preliminary observation that NMDA-evoked [3H]dopamine release from mesencephalic cultures is only Mg^{2+} -sensitive when TTX is present to block ongoing synaptic activity (Boksa et al., 1989). Presumably spontaneously generated action potentials tonically release a transmitter which partially depolarizes the nerves that release [3H]dopamine and alleviates the voltage-dependent Mg^{2+} block of the NMDA receptors. However, TTX did not unmask the Mg^{2+} block of NMDA-evoked adenosine release in the present study, indicating that the lack of block was not a result of ongoing synaptic activity-induced depolarization.

3. Concentration-response relationships of NMDA-evoked release of adenosine and [3H]noradrenaline.

The concentration of NMDA used (500 μM) was chosen because it had been shown to be submaximal in releasing [3H]noradrenaline from cortical slices (Keith et al., 1988; Fink et al., 1989). However, when the concentration-response relationships for NMDA-evoked release of adenosine and [3H]noradrenaline were determined, it was found that NMDA was 33 times more potent at releasing adenosine than [3H]noradrenaline. The EC_{50} for

NMDA-evoked [^3H]noradrenaline release (330 μM) is comparable to previously reported EC_{50}s of about 100 μM or more in cortex (Keith et al., 1988; Fink et al., 1989) and hippocampus (Jones et al., 1987), but differs from higher potencies of NMDA-evoked [^3H]noradrenaline release from hippocampal slices reported by others (Ransom and Deschenes, 1988; Schmidt and Taylor, 1988). Differences in potency of NMDA could reflect differences in slice dimensions and perfusion parameters. The 10-min exposure of NMDA in the present study may have led to tissue concentrations substantially lower than the applied concentrations (Müller et al., 1988) due to diffusional barriers and uptake of NMDA (Skerrit and Johnston, 1981; Garthwaite, 1985). Thus, to compare the relative potency of NMDA it was imperative that the release of both [^3H]noradrenaline and adenosine were measured in the same perfusate. This revealed that maximal adenosine release occurred when [^3H]noradrenaline release was less than 20% of maximum, and half-maximal adenosine release occurred at a concentration of NMDA (10 μM) that was ineffective at releasing [^3H]noradrenaline.

4. Evidence that there are spare receptors for NMDA-evoked adenosine release.

The differences in potency of NMDA-evoked adenosine versus [^3H]noradrenaline release raised two additional possible explanations for the lack of Mg^{2+} effect on NMDA-evoked adenosine release. It is possible that NMDA releases adenosine and [^3H]noradrenaline through actions at two distinct NMDA receptor subtypes with different affinities for NMDA and different Mg^{2+} -sensitivities. There have been previous reports of Mg^{2+} -insensitive, NMDA receptor-mediated events (Schmidt et al, 1987; Ransom and Stec, 1988b; Gonzales and Moerschbaecher, 1989a), although the

possibility that these were due to partial depolarization of the preparations was not excluded. Moreover, some investigators have suggested that there may be subtypes of NMDA receptors (see INTRODUCTION, section I.5).

However, it is not necessary to invoke NMDA receptor subtypes to explain the current findings. A more conservative explanation for the observed differences in potency for NMDA-evoked release of [^3H]noradrenaline and adenosine is that maximal adenosine release requires activation of only a small fraction of the available NMDA receptors. In this sense, one can consider that there are spare receptors for NMDA-evoked adenosine release. This would explain the ability of high agonist concentrations to overcome the uncompetitive antagonism of NMDA receptors by Mg^{2+} , as long as not all of the NMDA channels are blocked by Mg^{2+} . If the NMDA receptors which release [^3H]noradrenaline are identical to those which release adenosine, then Mg^{2+} should inhibit adenosine release evoked by submaximal concentrations of NMDA. Indeed Mg^{2+} did inhibit NMDA-evoked adenosine release at submaximal concentrations of NMDA (10 and 20 μM). Furthermore, MK-801, which uncompetitively antagonizes NMDA-mediated responses by occupying a separate site from Mg^{2+} in the NMDA-channel, exhibits the same characteristics as Mg^{2+} . Thus MK-801 blocked both NMDA-evoked release of [^3H]noradrenaline and adenosine at a relatively high concentration (3 μM) in the present study, but at a lower concentration (300 nM) MK-801 inhibited the release of [^3H]noradrenaline without affecting adenosine release (observation by Constance G. Craig in Dr. White's laboratory; Hoehn, Craig and White, in press). Spare NMDA receptors have also been suggested as a possible explanation for the

finding that the glycine site antagonist, HA-966, at low concentrations produces a parallel shift in the NMDA dose-response curve for NMDA neuronal excitotoxicity, but decreases the maximal response to NMDA at higher concentrations (Patel et al., 1990).

There are a number of striking similarities between NMDA-evoked adenosine release observed in the present study and NMDA-induced decreases in carbachol-stimulated PI turnover in cortical (Gonzales and Moerschbaecher, 1989b) and hippocampal (Morrisett et al., 1990) slices. These include a lack of Ca^{2+} -dependence, minimal involvement of propagated action potentials, blockade by ligands acting at the PCP site in the NMDA receptor-associated ion channel, and a similar potency of NMDA in ting both responses (Gonzales and Moerschbaecher, 1989b; Morrisett et al., 1990). Most remarkably, Mg^{2+} does not affect the maximal inhibition achieved by NMDA of carbachol-stimulated PI turnover, although Mg^{2+} antagonizes the NMDA effect at submaximal NMDA concentrations (Gonzales and Moerschbaecher, 1989b; Morrisett et al., 1990). Although the authors did not raise the possibility of spare receptors for NMDA inhibition of carbachol-stimulated PI hydrolysis, their observations strongly support the existence of spare receptors for this NMDA-mediated effect.

Morrisett et al. (1990) concluded that NMDA inhibits carbachol-stimulated PI hydrolysis by depolarization following Na^+ influx through the NMDA receptor-associated ion channel. They found that removal of Na^+ from the medium blocked the NMDA effect and that K^+ depolarization mimicked it. The remarkable similarities between NMDA-evoked adenosine release and NMDA-induced inhibition of carbachol-stimulated PI turnover suggests that NMDA-evoked adenosine release may be initiated by the same event (ie. Na^+

influx through the NMDA channel). Further, the parallelism between these two NMDA responses raises the possibility that they may be causally related. It is unlikely that released adenosine mediates the NMDA-induced decrease in carbachol-stimulated PI hydrolysis, because adenosine does not inhibit carbachol-stimulated PI hydrolysis in guinea pig (Hollingsworth et al., 1986) or mouse (Kendall and Hill, 1988) cortical slices. However, species differences in the ability of adenosine to stimulate or inhibit agonist-induced PI hydrolysis have been reported (Alexander et al., 1989), and the possibility that adenosine mediates NMDA inhibition of carbachol-stimulated PI hydrolysis in rat cortex cannot be excluded. The alternative possibility, that NMDA inhibition of tonic muscarinic stimulation of PI hydrolysis in cortical slices causes adenosine release, is also not likely because Pedata et al. (1988) have reported that atropine does not affect basal [^3H]purine release from rat cortical slices.

As discussed above, NMDA-evoked release of adenosine is largely TTX-insensitive whereas [^3H]noradrenaline release is blocked by TTX. The differential sensitivity to NMDA could be explained if maximal adenosine release is triggered by a relatively small influx of Na^+ through the NMDA-receptor channel, whereas [^3H]noradrenaline requires enough cation influx to depolarize the nerves sufficiently to activate the voltage-sensitive Na^+ -channels and, consequently, generate propagated action potentials in the noradrenergic nerves. A relatively small amount of Na^+ influx through NMDA receptor-associated channels might initiate activation of some intracellular second messenger which triggers adenosine release. In any case, the above observations are entirely consistent with the possibility that there are spare NMDA receptors for adenosine release in the cortex,

but definitive proof awaits studies with irreversible NMDA-receptor antagonists which act at the agonist recognition site, if and when they become available. Although there may be other explanations for these findings, the concept of spare receptors appears to be the simplest.

The observation that maximal adenosine release occurs at NMDA concentrations which apparently produce very little generation of action potentials (as seen with TTX-sensitive [^3H]noradrenaline release) may have important physiological and pathophysiological implications. It seems unlikely that released adenosine will provide much protection against excessive NMDA neurotransmission as has been suggested previously (Dragunow and Faull, 1988) because adenosine release appears to be maximal at low, rather than high, levels of NMDA receptor activation. However, adenosine, released during submaximal activation of NMDA receptors in vivo, could act presynaptically to decrease further EAA release (Dolphin and Archer, 1983; Corradetti, et al., 1984; Fastbom and Fredholm, 1985; Burke and Nadler, 1988) and postsynaptically to hyperpolarize neurons (Segal, 1982; Proctor and Dunwiddie, 1987). In this sense, released adenosine could provide a tonic inhibitory threshold which must be overcome in order for NMDA-mediated excitatory neurotransmission to proceed maximally. Released adenosine, like extracellular Mg^{2+} (Collingridge and Bliss, 1987), may serve to maintain the selectivity of NMDA-mediated processes such as learning, memory and synaptic plasticity in the cortex.

V. PROPOSALS FOR FUTURE RESEARCH

1. Does another uncompetitive NMDA antagonist, Zn^{2+} , support the existence of spare receptors for NMDA-evoked adenosine release?

Zn^{2+} , like Mg^{2+} and MK-801, blocks NMDA responses in an uncompetitive manner (see INTRODUCTION, section I.3D). If Zn^{2+} antagonizes NMDA-evoked release of adenosine and [^3H]noradrenaline, and if antagonism of adenosine but not [^3H]noradrenaline release can be overcome by high concentrations of NMDA, this would provide further evidence for the existence of spare NMDA receptors for NMDA-evoked adenosine release.

2. Does glycine modulate NMDA-evoked adenosine and [^3H]noradrenaline release?

A glycine site on the NMDA receptor-associated ion channel augments NMDA responses in a strychnine-insensitive manner (see INTRODUCTION, section I.3B). Because glycine is required for NMDA receptor activation (Kleckner and Dingledine, 1988; Thomson, 1989), the existence of spare receptors for NMDA-evoked release of adenosine but not [^3H]noradrenaline could be due to higher endogenous glycine concentrations at the NMDA receptors which mediate adenosine release than at those which mediate [^3H]noradrenaline release. If this is the case, addition of exogenous glycine (in the presence of strychnine to block strychnine-sensitive glycine receptors) to the superfusing medium might increase the potency of NMDA at releasing [^3H]noradrenaline.

If adding glycine does not affect either NMDA-evoked release of adenosine or [^3H]noradrenaline, this would suggest that endogenous glycine levels are sufficient to saturate the glycine site on the NMDA receptor, as has been reported in most in vitro studies (Taylor et al., 1988;

Fletcher and Lodge, 1988; Keith et al., 1989; but see Thomson et al., 1989b). In this case, experiments could be conducted with the glycine site antagonist, 7-chlorokynurenate, which blocks NMDA responses uncompetitively (Kemp et al., 1988). If 7-chlorokynurenate, like Mg^{2+} and MK-801, inhibits both NMDA-evoked release of adenosine and [3H]noradrenaline at low NMDA concentrations and if inhibition of adenosine but not [3H]noradrenaline release can be overcome by high concentrations of NMDA, this would further support the existence of spare NMDA receptors for NMDA-evoked adenosine release. Although antagonism by 7-chlorokynurenate is uncompetitive with respect to NMDA, it is competitive with respect to glycine (Kemp et al., 1988). For this reason, it would be expected that the inhibition by 7-chlorokynurenate of NMDA-evoked adenosine and [3H]noradrenaline release could be reversed by the addition of exogenous glycine. This would verify the specificity of the 7-chlorokynurenate block at the glycine site of the NMDA receptor.

3. Is NMDA-evoked adenosine release Na^+ -dependent?

The remarkable similarity between NMDA-evoked adenosine release from rat cortical slices and NMDA-mediated inhibition of carbachol-stimulated PI hydrolysis in rat cortical slices has been discussed (see DISCUSSION, section IV.4). NMDA-mediated inhibition of carbachol-stimulated PI hydrolysis is abolished when TRIS is substituted for Na^+ in the medium (Morrisett et al., 1990). The Na^+ -dependence of NMDA-evoked adenosine release should be investigated as this could provide further insight into the mechanism of NMDA-evoked adenosine release. Since NMDA-evoked adenosine release does not appear to be Ca^{2+} -dependent, it is likely that influx of Na^+ through the NMDA receptor-associated channel initiates events

leading to adenosine release.

4. Does released adenosine mediate NMDA inhibition of carbachol-stimulated PI hydrolysis in rat cortical slices?

In light of the similarities between NMDA-evoked adenosine release and NMDA-mediated inhibition of carbachol-stimulated PI hydrolysis, the possibility that adenosine released by NMDA might mediate inhibition of carbachol-stimulated PI hydrolysis, although unlikely (see section IV.4 in DISCUSSION), should be explored. This possibility could be tested in rat cortical slices by determining whether NMDA-mediated inhibition of carbachol-stimulated PI hydrolysis can be blocked by an adenosine receptor antagonist, such as 8-phenyltheophylline.

5. Does adenosine released by NMDA inhibit further NMDA receptor activation by exogenous NMDA?

Previous studies have shown that some effects of applied NMDA or NMDA agonists are modulated by adenosine (Connick and Stone, 1989) while others are not (Brooks and Stone, 1988; Goldberg et al., 1988). It is possible that adenosine released by NMDA inhibits further NMDA receptor activation. This question could be addressed by studying NMDA-evoked adenosine and [³H]noradrenaline release in the presence of an adenosine antagonist such as 8-phenyltheophylline. If modulation of release by endogenous adenosine is not observed, adenosine agonists could be applied to establish whether P₁-purinoceptors are capable of modulating NMDA-mediated responses.

6. Does released adenosine inhibit further release of EAAs?

The possibility that adenosine released following NMDA receptor activation inhibits further release of EAAs could be addressed by

simulating hypoxic/ischemic conditions in the cortical slice preparation. Preliminary studies with NMDA antagonists would be necessary to determine whether NMDA receptors mediate hypoxic/ischemic adenosine release in cortical slices in vitro as has been shown in the striatum in vivo (Hagberg et al., 1986). The possibility that NMDA receptor activation under these conditions also releases [^3H]noradrenaline could be examined. If NMDA receptors mediate hypoxia/ischemia-evoked release of adenosine and/or [^3H]noradrenaline in vitro, one could ask whether adenosine antagonists increase this release. Results of these experiments, together with results of experiments with applied NMDA, could indicate whether endogenous adenosine, released following NMDA receptor activation, acts pre- or post-synaptically to decrease further NMDA receptor activation.

7. What is the nature of the purine released by NMDA?

It was shown that glutamate-evoked adenosine release occurs primarily in the form of adenosine per se and not as a nucleotide, and that about 50% of glutamate-evoked adenosine release is mediated by NMDA receptors. However, studies should be conducted with inhibitors of ecto-5'-nucleotidase to verify that NMDA-evoked adenosine release also occurs as adenosine per se and is not due in part to the release of a nucleotide which subsequently is converted extracellularly to adenosine. In addition, studies with dipyrindamole or nitrobenzylthioinosine, inhibitors of the nucleoside transporter, could determine whether NMDA-evoked adenosine release occurs on the bidirectional nucleoside transporter (see references in INTRODUCTION, section II.1). Similar studies should also be conducted with non-NMDA agonists.

8. Are non-NMDA agonists more potent at releasing adenosine than at releasing [^3H]noradrenaline?

The high potency of NMDA at releasing adenosine as compared to [^3H]noradrenaline raises the possibility that the concentrations of kainate and quisqualate used in the present study, which were chosen on the basis of reports of kainate- and quisqualate-evoked release of other neurotransmitters (references in METHODS, section II.3), may also be supramaximal for evoking adenosine release. This possibility should be examined by determining the concentration-response relationship for release of adenosine and [^3H]noradrenaline by non-NMDA agonists. If non-NMDA agonists are also much more potent at releasing adenosine than [^3H]noradrenaline, then this would indicate that adenosine might not be an effective neuroprotectant against non-NMDA receptor-mediated neurotoxicity. This might also suggest that only a small fraction of non-NMDA receptors need to be activated in order to elicit maximal adenosine release and that there are spare receptors for non-NMDA receptor-mediated adenosine release in the cortex.

9. What is the Ca^{2+} -dependence of adenosine released by low concentrations of EAA agonists?

Although results from the present study indicate that the total amount of adenosine released by EAA agonists is unaffected by removal of extracellular Ca^{2+} , these experiments were conducted before the concentration-response relationship of NMDA-evoked adenosine release was determined. Because Perez et al. (1989) have shown that NMDA- and kainate-evoked release of radiolabelled purine derivatives is Ca^{2+} -independent at high NMDA or kainate concentrations (100 μM) but Ca^{2+} -

dependent at low concentrations (25 μ M), the Ca^{2+} -dependence of EAA-evoked adenosine release from cortical slices should be tested using concentrations of NMDA and non-NMDA agonists which are submaximal for adenosine release.

10. Does the presence of extracellular Ca^{2+} influence the nature of the purine released?

In the present study, experiments in which slices were exposed to two consecutive stimulations by K^+ or kainate suggested the existence of separate Ca^{2+} -dependent and independent releasable pools of adenosine. The nature of these pools is unknown. Bender et al. (1981) have also proposed, on the basis of their observations of veratridine-evoked labelled purine release from cortical synaptosomes, that there are Ca^{2+} -independent releasable pools of purines. These authors have suggested that release of a nucleoside might be Ca^{2+} -dependent whereas release of a nucleotide might be Ca^{2+} -independent. The possibility that K^+ or kainate stimulation in Ca^{2+} -free medium releases a nucleotide, whereas K^+ or kainate stimulation in Ca^{2+} -containing medium releases adenosine per se could be investigated using inhibitors of ecto-5'-nucleotidase.

11. Does activation of the metabotropic quisqualate receptor also release adenosine?

Since completion of the experiments with quisqualate, it has been established that quisqualate acts on two separate receptor populations, the ionotropic quisqualate/AMPA receptor, and the metabotropic quisqualate receptor (see INTRODUCTION, section I.2). In light of this new knowledge, it should be determined whether the more specific agonist for the metabotropic quisqualate receptor, trans-ACPD (Collingridge and Lester,

1989), releases adenosine. Studies should also be conducted with the more specific agonist for the quisqualate/AMPA receptor, AMPA, to confirm that activation of this receptor releases adenosine. Studies with antagonists of the metabotropic quisqualate receptor could also be performed when these become available.

12. Does kainate release adenosine by acting at its own receptor or via an action at the ionotropic quisqualate receptor?

There is evidence suggesting that kainate, AMPA and quisqualate all activate the same receptor, although kainate does not desensitize this receptor whereas quisqualate and AMPA do (references in INTRODUCTION, section 1.7). In light of evidence that quisqualate activates both the ionotropic and the metabotropic quisqualate receptors (see INTRODUCTION, section 1.2), future experiments should be conducted with AMPA instead of quisqualate to preclude effects mediated through the metabotropic quisqualate receptor. If additivity of adenosine release can be observed in the presence of maximal concentrations of AMPA and kainate, this would indicate that these agonists act at different receptors to release adenosine. On the other hand, if a low concentration of AMPA inhibits kainate-evoked adenosine release, this would suggest that these agonists release adenosine by acting at the same receptor. In fact, inhibition by quisqualate or AMPA of kainate-induced currents or neurotransmitter release has been observed in other studies (references in INTRODUCTION, section, 1.7).

13. Can glutamate uptake-mediated adenosine release be demonstrated in the cortical slice preparation?

In synaptosomes, glutamate transport releases adenosine. However,

the receptor-mediated component of glutamate-evoked adenosine release appears to predominate over the glutamate transport-mediated component in cortical slices in so far as block of the glutamate transporter with dihydrokainate augments rather than inhibits glutamate-evoked adenosine release from slices. Further, glutamate-evoked adenosine release from slices is virtually abolished by a combination of MK-801 and DNQX. However, synaptosomal studies suggested that high concentrations (50 to 100 μM) of DNQX also block glutamate uptake. Therefore, the possibility remains that a portion of glutamate-evoked adenosine release from slices might be mediated by glutamate transport. This could be tested by examining the effect of dihydrokainate on glutamate-evoked adenosine release following blockade of receptors with MK-801 (3 μM) and DNQX (10 μM). If after receptor blockade, dihydrokainate blocks rather than augments release, this would suggest that a component of glutamate-evoked adenosine release is mediated via glutamate uptake. The effect of inhibitors of ecto-5'-nucleotidase on the remaining portion of glutamate-evoked adenosine release following receptor blockade could also be determined. Inhibition by ecto-5'-nucleotidase of remaining adenosine release following receptor blockade would support the existence of the glutamate transport-mediated component of adenosine release in the slice preparation.

14. Do EAAs release adenosine from primary cultures of neurons or glia?

Glia may contribute to EAA-evoked adenosine release from slices or synaptosomes (references in DISCUSSION, section III). Studies of EAA-evoked adenosine release from primary cultures of astrocytes or neurons could determine whether EAA-evoked adenosine release occurs from these

preparations.

VI. SUMMARY AND CONCLUSIONS

Table 12 contrasts glutamate-evoked adenosine release from rat parietal cortical slices with glutamate-evoked adenosine release from synaptosomes prepared from the same region. Whereas glutamate releases adenosine from cortical slices by acting at both NMDA and non-NMDA receptors, EAA receptor-mediated release does not occur in synaptosomes. In contrast, the uptake of L-glutamate releases adenosine from synaptosomes, but uptake-mediated release is not observed in cortical slices. L-Glutamate transport into synaptosomes releases a nucleotide whereas EAA receptor-mediated adenosine release from slices occurs primarily in the form of adenosine per se. Neither EAA receptor-mediated nor L-glutamate uptake-mediated adenosine release requires the presence of extracellular Ca^{2+} . However, an effect of extracellular Ca^{2+} on adenosine release is observed in cortical slices, in that the initial rate of adenosine release evoked by K^+ , glutamate, kainate or quisqualate from slices is increased in Ca^{2+} -free medium. Experiments in which slices were exposed to EAA agonists for two consecutive periods suggest the presence of separate Ca^{2+} -dependent and Ca^{2+} -independent releasable pools of adenosine, especially in the case of kainate-evoked release. While propagated action potentials are involved to some extent in EAA-evoked adenosine release from slices, propagated action potentials are not involved in glutamate uptake-mediated adenosine release from synaptosomes.

Glutamate-evoked adenosine release from presynaptic terminals is shown diagrammatically in Fig. 41. Glutamate is taken up by a

Table 12. Comparison of glutamate-evoked adenosine release from slices versus synaptosomes from rat parietal cortex.

	SLICES	SYNAPTOSOMES
GLUTAMATE RELEASES	+	+
RECEPTOR-MEDIATED	+	-
MEDIATED THROUGH GLUTAMATE UPTAKE	-	+
RELEASE OF ADENOSINE <u>PER SE</u>	+	-
RELEASE OF NUCLEOTIDE	-	+
Ca ²⁺ -DEPENDENT	-	-
ACTION POTENTIAL INVOLVEMENT	+/-	-

Figure 41. Scheme for L-glutamate-evoked adenosine release from presynaptic terminals in the rat cortex. L-Glutamate (GLU), in combination with Na^+ , is taken up into a glutamatergic nerve terminal via a high-affinity transporter. This influx of Na^+ results in release of ADP and/or AMP by a mechanism which may involve depolarization and/or stimulation of the Na^+/K^+ -ATPase. Release of ADP and/or AMP is Ca^{2+} -independent and does not involve voltage-sensitive Na^+ channels. Released ADP and/or AMP is metabolized extracellularly by ecto-5'-nucleotidase to adenosine and the latter can then act on both presynaptic A_1 adenosine receptors on the glutamatergic terminal or on postsynaptic adenosine receptors.

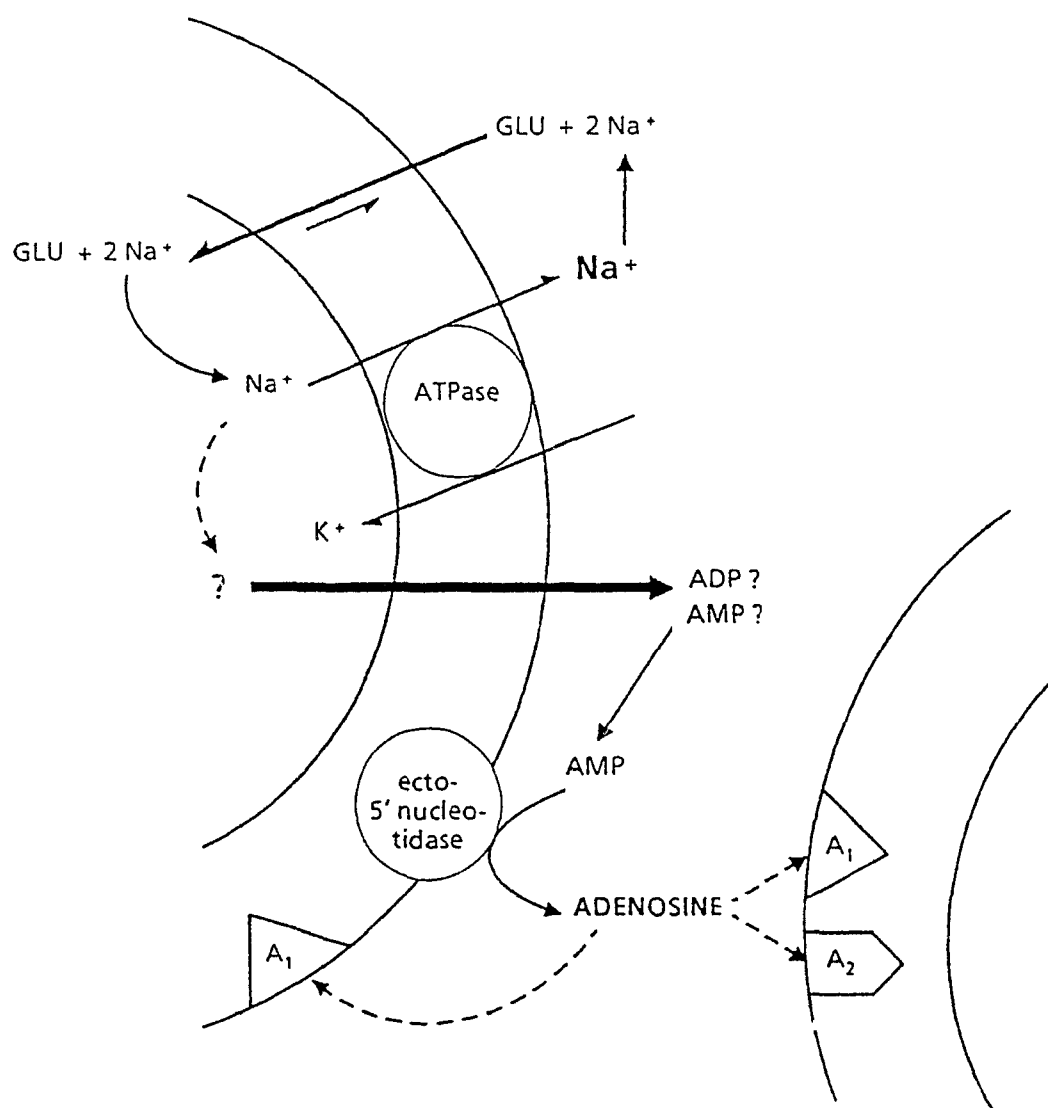


Figure 41

Na⁺-dependent, high-affinity transport system into the presynaptic terminal. This results in the Na⁺-dependent release of a nucleotide (not ATP or cyclic AMP but possibly ADP or AMP) which is subsequently converted to adenosine extracellularly. There is both anatomical and biochemical evidence for the existence of presynaptic A₁ adenosine receptors on glutamatergic nerve terminals (references in INTRODUCTION, sections II.3.C and II.5) which, when activated, diminish the release of glutamate.

In slices of rat parietal cortex, glutamate receptor-mediated release of adenosine appears to predominate over glutamate transport-mediated release. Thus, dihydrokainate, which blocked glutamate uptake-mediated release of adenosine from synaptosomes, potentiated glutamate receptor-mediated release of adenosine from slices, presumably by increasing the availability of extracellular glutamate to act at its receptors. These results suggest that the glutamate uptake-mediated release of adenosine in cortical slices is relatively small in comparison to the receptor-mediated component. Nevertheless, glutamate uptake-mediated release of adenosine may be functionally important in vivo because it should occur directly from glutamatergic nerve terminals. Thus glutamate uptake-mediated adenosine release from glutamatergic nerve terminals could result in a relatively high concentration of extraneuronal adenosine in the immediate vicinity of the inhibitory presynaptic adenosine receptors where it might inhibit further release of glutamate. The recently described aspartate uptake-mediated release of glutamate from cortical synaptosomes (McMahon et al., 1989) suggests that there may be an amplification mechanism that promotes glutamate release from glutamatergic nerve terminals. Glutamate uptake-mediated release of

adenosine could provide a negative feedback mechanism to counteract excessive glutamate amplification and the possible development of excitotoxicity.

Table 13 contrasts NMDA-evoked release of endogenous adenosine and [^3H]noradrenaline from rat cortical slices. Concentration-response studies revealed that NMDA is 33-fold more potent at releasing adenosine than [^3H]noradrenaline. This observation might be interpreted as evidence that adenosine release is mediated by a high affinity subtype of NMDA receptor. However, it is probably not necessary to invoke a new subtype of NMDA receptor to explain these findings. Another, more conservative, explanation is that maximal NMDA-evoked adenosine release requires activation of only a small fraction of available NMDA receptors (ie. there are spare NMDA receptors for NMDA-evoked adenosine release). The existence of spare receptors is supported by the findings that the block of NMDA-evoked adenosine release by two uncompetitive antagonists, Mg^{2+} and MK-801, can be overcome by high concentrations of NMDA (see Table 13).

The physiological and pathological implications of the observation that maximal NMDA-evoked adenosine release occurs at NMDA concentrations far lower than those which produce [^3H]noradrenaline release have been discussed. It appears unlikely that adenosine, released during NMDA receptor activation, will be an effective endogenous antiexcitotoxic agent against excessive NMDA receptor stimulation as was suggested at the outset of the present study (see INTRODUCTION, section III). However, released adenosine may provide an inhibitory threshold which must be overcome for NMDA receptor activation to proceed maximally and thereby maintain the selectivity NMDA receptor activation necessary for memory, learning and

Table 13. Summary of NMDA-evoked release of adenosine and [^3H]noradrenaline from cortical slices.

A. DOSE-RESPONSE CHARACTERISTICS (Mg^{2+} -FREE MEDIUM)

	ADENOSINE RELEASE	[^3H]NA RELEASE
EC_{50}	$10\mu\text{M}$	$330\mu\text{M}$
EC_{100}	$30\mu\text{M}$	$500\text{-}1000\mu\text{M}$

B. ACTION POTENTIAL INVOLVEMENT (Mg^{2+} -FREE MEDIUM)

NMDA	TTX	ADENOSINE RELEASE	[^3H]NA RELEASE
$500\mu\text{M}$	$1\mu\text{M}$	\downarrow (35%)	$\downarrow\downarrow\downarrow$ (82%)

C. Mg^{2+} -SENSITIVITY

NMDA	Mg^{2+}	ADENOSINE RELEASE	[^3H]NA RELEASE
$10\mu\text{M}$	1.2mM	$\downarrow\downarrow$ (60%)	ND
$20\mu\text{M}$	1.2mM	$\downarrow\downarrow$ (45%)	ND
$500\mu\text{M}$	1.2mM	\uparrow (50%)	$\downarrow\downarrow\downarrow$ (92%)
$3000\mu\text{M}$	1.2mM	\uparrow (52%)	$\downarrow\downarrow\downarrow$ (90%)

D. MK-801 SENSITIVITY (Mg^{2+} -FREE MEDIUM)

NMDA	MK-801	ADENOSINE RELEASE	[^3H]NA RELEASE
$500\mu\text{M}$	$3\mu\text{M}$	$\downarrow\downarrow\downarrow$ (93%)	$\downarrow\downarrow\downarrow$ (99%)
$500\mu\text{M}$	$0.3\mu\text{M}$	\leftrightarrow (3% NS)	$\downarrow\downarrow$ (66%)

Numbers in parenthesis are the % change in release in 4-6 separate experiments. All results are significantly different from control ($p < 0.05$, paired t test) except where non-significance is indicated (NS). ND; not determined. Experiments with $0.3\mu\text{M}$ MK-801 were conducted by Constance G. Craig, in Dr. White's laboratory.

synaptic plasticity. It remains to be determined whether non-NMDA agonists also evoke maximal adenosine release at concentrations below those which produce maximal excitation and whether adenosine released following activation of non-NMDA receptors might be protective. Although the protective role of endogenous adenosine against EAA receptor-mediated excitotoxicity is questionable, at least in the case of NMDA receptor-mediated effects, this does not preclude a possible therapeutic benefit from the administration of adenosine agonists to augment purinergic inhibition and decrease excitotoxic damage. Protection by exogenous adenosine against excitotoxicity has, in fact, been demonstrated in a number of in vivo studies (Evans et al., 1987; Connick and Stone, 1989; Daval et al., 1989). It is hoped that future research will increase our understanding of the mechanisms of EAA-evoked adenosine release in vivo under physiological and pathophysiological conditions and clarify the precise role of the adenosine released by EAAs. A fundamental understanding of these processes should ultimately be of benefit in developing new approaches to the therapy of certain CNS pathologies.

APPENDIX

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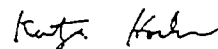
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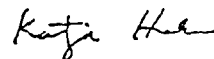
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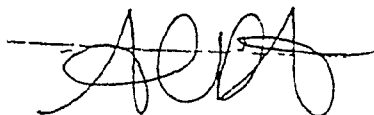


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