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RELEASE OF GLUTAMATE, NORADRENALINE AND ADENOSINE FROM RAT PARIETAL CORTICAL SLICES

by

Yushan Wang

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

at

Department of Pharmacology

Dalhousie University

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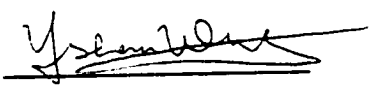

Yushan Wang (author)

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ABSTRACT

The roles of protein kinase C (PKC) and Li^+ in N-methyl-D-aspartate (NMDA)-evoked releases of adenosine and noradrenaline (NA) were studied. In the absence of Mg^{2+}, activation of PKC by phorbol 12-myristate 13-acetate (PMA) potentiated the release of adenosine evoked by 20μM NMDA and the release of [^{3}H]NA evoked by 100μM NMDA. The potentiating effects of PMA on NMDA-evoked releases of adenosine and NA were reversed by inhibition of PKC with GF109,203X (GFX). GFX by itself had no effect on NMDA-evoked release of either adenosine or NA, suggesting that endogenous PKC does not play a role in NMDA-evoked releases. Li^+, at concentrations of 1.5mM or 10mM, potentiated 300μM NMDA-evoked release of NA but had no effect on the release of adenosine. In the presence of Mg^{2+}, NMDA-evoked release of NA was essentially abolished, whereas adenosine release persisted. Under these conditions, PMA or Li^+ did not permit NMDA-evoked release of NA to occur, nor did they increase NMDA-evoked adenosine release. Taken together, these results indicate that PKC does not play an essential role in NMDA-evoked release of either adenosine or NA. However, activation of PKC potentiates the release of adenosine and NA evoked by submaximal concentrations of NMDA, but not by relieving the voltage-sensitive Mg^{2+} block of NMDA receptors.

Inflammation may play an important role in various neurological disorders, including the central actions of bacterial and viral infections. Here we show that exposure of rat parietal cortical slices to lipopolysaccharide (LPS) triggers very rapid (<2.5 min) releases of glutamate, NA and adenosine. Detoxified LPS is ineffective. LPS-evoked release of NA appears to be partly due to the released glutamate acting at its ionotropic receptors. LPS-evoked release of glutamate does not require extracellular Ca^{2+} and is tetrodotoxin (TTX)-insensitive. The glutamate transport blocker L-trans-pyrrolidine-2,4-dicarboxylate (tPDC) decreases glutamate release, suggesting that release might occur via the reversal of glutamate transporter(s). These findings raise the possibility that LPS releases glutamate from non-neuronal cells rather than from neurons. It is not known exactly how LPS releases glutamate. Glutamate release is not mimicked by IL-1β, IL-6, TNF-α, or a combination of IL-1β and TNF-α, suggesting that it is not mediated by these cytokines. Inhibition of COX-1 with resveratrol, COX-2 with NS-398, or both COX-1 and COX-2 with indomethacin does not diminish LPS-evoked glutamate release. Thus prostaglandins do not appear to be involved in LPS-evoked glutamate release. Inhibition of eNOS with L-NIO, iNOS with L-NIL, nNOS with 7-NI, or non-selective inhibition of NOS with L-NAME has no effects on LPS-evoked glutamate release, indicating that NO is not involved. Finally, scavenging free radicals with SOD and catalase or with α-tocopherol does not prevent LPS from releasing glutamate. These findings raise the possibility that similar inappropriate excitations may participate in other neurological disorders such as AIDS dementia. Indeed, the HIV glycoproteins gp120 and gp41 also caused very rapid releases of glutamate, NA and adenosine from rat cortical slices. HIV gp41 is much more effective than gp120 at releasing glutamate and NA while both glycoproteins are equally effective at releasing adenosine

LIST OF ABBREVIATIONS

AA	arachidonic acid
AMP	adenosine 5'-monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate
APV	D(-)-2-amino-5-phosphonopentanoic acid
BBB	blood brain barrier
p-BPB	p-bromophenacylbromide
7-Cl-KYN	7-chlorokynurenic acid
CNS	central nervous system
COX	cyclooxygenase
DHK	dihydrokainate
DMSO	dimethyl sulfoxide
EAA	excitatory amino acids
EAAT	excitatory amino acid transporters
GDH	glutamate dehydrogenase
GFX	3-[1-(3-dimethylamino-propyl)-indol-3-yl]-3-(indol-3-yl)-maleimide, GF109,203X
iGluR	ionotropic glutamate receptor
mGluR	metabotropic glutamate receptor
HPLC	high performance liquid chromatography
i.c.v.	intracerebroventricular
IL	interleukin
IP_3	inositol 1,4,5-trisphosphate
LPS	lipopolysaccharide, endotoxin
LT	leukotriene
LTD	long-term depression
LTP	long-term potentiation
MK-801	dizocilpine maleate
MS	multiple sclerosis
NA	noradrenaline

L-NAME	N^G-nitro-L-arginine methyl ester
NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide disodium
NDGA	nordihydroguaiaretic acid
NGF	nerve growth factor
7-NI	7-nitroindazole
L-NIL	N^6-(1-iminoethyl)-L-lysine
L-NIO	N^5-(1-iminoethyl)-L-ornithine
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial NOS
iNOS	immunological NOS
NSAID	non-steroidal anti-inflammatory drug
nNOS	neuronal NOS
PAF	platelet activating factor
PAG	phosphate-activated glutaminase
PDE	phosphodiesterase
PG	prostaglandin
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PTK	protein tyrosine kinase
PTX	pertussis toxin
tPDC	L-trans-pyrrolidine-2,4,-dicarboxylate
SOD	superoxide dismutase
DL-TBOA	D,L-threo-β-benzyloxyaspartate
THA	D,L-threo-β-hydroxyaspartate
TNF-α	tumor necrosis factor α

Trolox	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TTX	tetrodotoxin
TXA_2	thromboxane A_2

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CHAPTER 1

GENERAL INTRODUCTION

Excitatory Amino Acids in the Central Nervous System

Glutamate and aspartate are the major excitatory neurotransmitters in the central nervous system (CNS) (Monaghan *et al.*, 1989). Through interactions with their specific membrane receptors, these excitatory neurotransmitters play roles in many physiological functions such as cognition, learning, memory and sensation (Gasic and Hollmann, 1992; Bliss and Collingridge, 1993). In addition, they are also involved in the plasticity of synaptic connections in the nervous system (Lipton and Kater, 1989). However, excessive glutamate release and consequent over stimulation of excitatory amino acid (EAA) receptors during neurological stress, such as ischemia, trauma, and epileptic seizures, can lead to excitotoxicity, and ultimately, neuronal cell death (Barnett et al., 1995). Furthermore, glutamate excitotoxicity has been implicated in the genesis of various neurodegenerative diseases including Alzheimer's and Huntington's diseases (Lipton and Rosenberg, 1994; Akiyama et al., 2000), multiple sclerosis (Pitt et al., 2000) and AIDS dementia (Lipton, 1994).

Physiological Release of Glutamate

Glutamate satisfies the four major criteria for neurotransmitters: a) presynaptic localization, b) Ca^{2+}-dependent release, c) existence of specific antagonists and, d) existence of mechanisms to terminate its actions (Fonnum, 1984). Under physiological conditions, glutamate is released into the synaptic cleft through a Ca^{2+}-dependent process in response to depolarization of the presynaptic nerve terminal (Hudspith, 1997). Similar to the release of other neurotransmitters, Ca^{2+} entry via voltage-sensitive Ca^{2+} channels is critical in the glutamate release process (Hicks and Conti, 1996). Although multiple

types of Ca^{2+} channels are involved, N-type and P-type channels are believed to be the most important in regulating neuronal glutamate release under physiological conditions (Dunlap et al., 1995; Nicholls et al., 1996). After release, glutamate binds to its specific receptors in order to exert its physiological functions.

Excitatory Amino Acid Receptors

Based on molecular structures, agonist and antagonist profiles and signal transduction pathways, EAA receptors are currently divided into two distinct classes: ionotropic and metabotropic glutamate receptors (Fillenz, 1995; Hudspith, 1997; Ozawa et al., 1998). The ionotropic receptors (iGluRs) contain cation-specific ion channels and are further subdivided into three major groups: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-mehtyl-5-isoxazolepropionate (AMPA) and kainate receptors. On the other hand, the metabotropic glutamate receptors (mGluRs), which contain three subtypes, are coupled to GTP-binding proteins (G-proteins) and modulate the production of intracellular second messengers (Collinngridge and Lester, 1989; Ozawa et al., 1998). The classification of EAA receptors and some basic characteristics of the individual receptor subtypes are summarized in Table 1-1.

Table 1-1. Classification of Glutamate Receptors

Receptor Subtypes		Subunit Genes	Agonists	Antagonists	Transduction Pathways
iGluRs	**NMDA**	NR1 NR2A- NR2D	<u>Agonist site:</u> NMDA Glutamate Aspartate Ibotenate Quinolinate <u>Glycine site:</u> Glycine D-serine	<u>Channel:</u> MK801 APV PCP Mg^{2+} <u>Glycine site</u> 7-Cl-KA CNQX	$\uparrow[Ca^{2+}]i$ $\uparrow[Na^{+}]i$
	AMPA	GluR1-4	AMPA Quisqualate Glutamate	NBQX, CNQX, GYKI53655	$\uparrow[Na^{+}]_i$ ($\uparrow[Ca^{2+}]_i$?)
	Kainate	GluR5-7 KA1- KA2	Kainate Domoate Glutamate Quisqualate	NBQX, CNQX, NS-102	$\uparrow[Na^{+}]_i$
mGluRs	**Group 1**	mGlu1 mGlu5	Quisqualate, ibotenate, ACPD	(+)-MCPG	↑PLC
	Group 2	mGlu2 mGlu3	DCG-IV, ACPD	MCCG	↓cAMP
	Group 3	mGlu4 mGlu6-8	L-Ap4	MHP4	↓cAMP

Non-NMDA (AMPA and Kainate) Receptors

Non-NMDA receptors were first identified electrophysiologically as receptors being preferentially activated by quisqualate or kainate (Doble, 1999), and therefore were termed "quisqualate receptors". However, aside from its agonist properties toward quisqualate receptors, quisqualate was later found to also activate metabotropic glutamate receptors (Perouansky and Grantyn, 1989). Hence, the term "quisqualate receptors" was replaced by "AMPA receptors", since AMPA is a selective agonist for these receptors (Ozawa et al, 1998). AMPA and kainate receptors are often collectively called non-NMDA receptors (Doble, 1999).

Non-NMDA receptors are distributed ubiquitously throughout the CNS, although regional differences are conspicuous. It has been reported that AMPA receptors are most highly enriched in all fields of the hippocampus and dentate gyrus (Monaghan and Cotman, 1982; Nielsen et al., 1988, 1990). AMPA receptor channels consist of homomeric or heteromeric assemblies of GluR1-GluR4 subunits (Seeburg, 1993), while kainate receptor channels consist of multimeric assemblies of GluR5-GluR7 and KA1 and KA2 subunits (Lerma, 1998; Chittajallu et al, 1999) (Table 1-1).

Non-NMDA receptor channels have been considered to be permeable only to Na^+ and K^+, and activation of these channels results in rapid membrane depolarization (Patneau and Mayer, 1991). The rapid activation and deactivation kinetics of these receptor channels renders them suitable for providing fast excitatory synaptic transmission throughout the CNS, and their generally very low Ca^{2+} permeability ensures that this glutamate-activated excitation does not trigger longer term biochemical processes evoked by an increase in intracellular Ca^{2+} concentrations (Partin et al., 1993;

Hudspith, 1997). On the other hand, native AMPA receptors with high Ca^{2+} permeability have been reported in a variety of cells in the CNS (Itazawa et al., 1997). The Ca^{2+}-permeable AMPA receptors are involved in excitatory synaptic transmission in a population of hippocampal and neocortical non-pyramidal and spinal dorsal horn neurons (Gu et al., 1996).

Non-NMDA receptors may play important roles in the pathogenesis of neurological diseases. Pharmacological studies have demonstrated that, while NMDA receptor antagonists are protective in moderate but not severe ischemia (Buchan et al., 1991), non-NMDA receptor antagonists, such as NBQX, are effective in preventing delayed CA1 cell death following severe ischemia (Sheardown et al., 1990; Buchan et al., 1991). It has been reported that following severe transient forebrain ischemia, GluR2 gene expression is preferentially reduced in CA1 hippocampal neurons at a time point that preceded their degeneration (Pellegrini-Giampietro et al., 1992, 1994). Since the Ca^{2+} permeability of AMPA receptors is increased by a reduced expression of GluR2, the result suggests that the increased Ca^{2+} entry caused by a switch in AMPA receptor subunit gene expression is a mechanism underlying delayed death of CA1 neurons (Ozawa et al., 1998). Moreover, non-NMDA receptors seem to play a role in neurodegenerative diseases. For instance, it has been shown that the HIV non-structural protein *tat* dose- dependently induced depolarization of cultured human fetal neurons and in human and rat brain slices, in a manner similar to those produced by kainate, a non-NMDA receptor agonist (Cheng et al., 1998). The excitatory responses of *tat* can be blocked by non-NMDA receptor antagonists (Magnuson et al., 1995; Nath et al., 1996). Taken together, these results suggest that non-NMDA receptor activation might be

involved in some of the neurological symptoms seen in AIDS dementia. Similarly, non-NMDA receptor involvement in other neurodegenerative diseases such Alzheimer's disease, Parkinson's disease, and Huntington's disease has also been postulated (Ozawa, et al., 1998).

NMDA Receptors

NMDA receptor channels are among the first glutamate receptors characterized in the mammalian CNS (Danysz and Parsons, 1998). NMDA receptors can be reconstituted *in vitro* as heteromeric combinations of the NR1 subunit and one of the four NR2 subunits (NR2A-2D) (Seeburg, 1993; Sucher et al., 1996). The exact stoichiometry of these subunit assemblies is not clear. Although previous data indicated that NMDA receptors are pentameric (Brose et al., 1993; Ferrer Montiel and Montal, 1996), recent findings suggest that NMDA receptors may in fact be tetrameric assemblies (Laube et al., 1998). In any event, the complexity of the NMDA receptor assemblies may underline the diversity of NMDA receptor functions involved in both physiological and pathophysiological conditions (Hrabetova et al., 2000). In response to cell membrane depolarization, NMDA receptors undergo conformational changes to open the receptor channels to permit Ca^{2+} and Na^{+} influx through the channel (Ziff, 1999). Most subsequent postsynaptic events are direct consequences of Ca^{2+} entry (Hudspith, 1997).

Properties

The gating properties of NMDA receptor channels are complex and subject to modulation at several different sites (Hudspith, 1997). In addition to its binding site for

EAAs such as NMDA or glutamate, the receptor has a second binding site for glycine, which facilitates the actions of glutamate or NMDA (Johnson and Ascher, 1987). In fact, NMDA receptor responses in transfected *Xenopus* oocytes diminish quickly when extracellular glycine is reduced to a negligible level (Kleckner and Dingledine, 1988), suggesting that glycine not only facilitates NMDA responses, but also is essential for NMDA receptor functions. In addition, pharmacological studies have demonstrated that NMDA responses are completely blocked by specific glycine site antagonists, such as 7-chlorokynurenic acid (7-Cl-KYN), suggesting that the glycine-binding site is a prerequisite for NMDA receptor activation (Kemp et al., 1988). On the other hand, under normal conditions, externally added glycine is not needed for NMDA receptor activation. This can be easily explained by the fact that experimental solutions are generally contaminated by low concentrations of glycine, which are already high enough to facilitate NMDA receptor activation (Benveniste et al., 1990, Molnar and Erdo, 1996). Therefore, it is widely accepted that glycine is a co-agonist for the NMDA receptors (Danysz and Parsons, 1998).

Another important feature of the NMDA receptor channels is that they are blocked by physiological concentrations of Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984). Single channel studies have shown that NMDA receptor conductance is dramatically slowed by the presence of 1mM Mg^{2+} in experimental solutions (Nowak et al., 1984; Aschner and Nowak, 1988). In addition, NMDA-evoked release of noradrenaline from rat cortical slices is abolished in the presence of physiological concentrations of Mg^{2+} (Craig and White, 1992). However, this blockade of NMDA receptors by Mg^{2+} is voltage-dependent and can be removed by membrane depolarization

(Mayer and Westbrook, 1987). Therefore, the rapid activation of non-NMDA receptors by glutamate released into the synaptic cleft and the subsequent depolarization of the post-synaptic membrane will diminish the inhibition of the NMDA receptors by Mg^{2+} and lead to their activation. These events are likely to occur in neuronal synapses, since AMPA and NMDA receptors are apparently co-localized at postsynaptic membranes on dendrites (Bekkers and Stevens, 1989; Jones and Baughman, 1991; Doble, 1999; Takumi et al., 1999).

Modulation by protein phosphorylation

Besides facilitation by glycine and blockade by Mg^{2+}, NMDA receptors are subject to modulation by protein phosphorylation (Lau and Huganir, 1995; Westphal et al., 1999; Soderling and Derkach, 2000). In fact, NMDA receptors form complexes in the postsynaptic density with other proteins such as PSD95, Ca^{2+}-calmodulin, SAP102, α-actinin and the tyrosine kinase family members (Yu et al., 1997; Kennedy, 1998; Salter, 1998). It has been shown that infusion of protein tyrosine kinases (PTKs) phosphorylates the intracellular C-terminal tyrosine residues of NMDA receptors, and thus potentiates the current through NR1-NR2A or NR1-NR2B recombinant channels (Zheng et al., 1998). Protein kinase C (PKC) also plays important roles in potentiating NMDA receptor activities (Raymond et al., 1994; Vallano, 1998). The facilitatory effects of metabotropic glutamate receptors on NMDA-evoked responses are mediated by the activation of PKC (Aniksztejn *et al.*, 1992; Kelso *et al.*, 1992). PKC activation by phorbol esters has also been shown to enhance NMDA currents in various systems including rat trigeminal neurons (Chen and Huang, 1991) and oocytes expressing total rat

brain mRNA (Kelso *et al.*, 1992). The mechanisms underlying the potentiation of PKC on NMDA-evoked responses remain to be elucidated. In electrophysiological studies, Chen and Huang (1992) proposed that PKC potentiates NMDA-activated currents mainly by reducing the voltage-dependent Mg^{2+} block of NMDA receptor channels. Moreover, reduction of the Mg^{2+} block on NMDA receptors by stretch-induced injury in cultured rat neuronal cells is partially restored by pretreatment with the PKC inhibitor calphostin C (Zhang *et al.*, 1996), suggesting that PKC acts, at least in part, through reducing the Mg^{2+} block. In contrast, Wagner and Leonard (1996) showed that PKC-activation fails to induce any change in the sensitivity of NMDA receptors to Mg^{2+} block in *Xenopus* oocytes expressing cloned NMDA receptors. Similar conclusions have been drawn using mouse striatal neurons (Murphy *et al.*, 1994) and primary cultures of cerebellar granule neurons of the rat (Patel *et al.*, 1995). Therefore, to solve the discrepancies between data obtained by these studies, it is necessary to directly evaluate the relationship between PKC-dependent subunit phosphorylation and electrophysiological changes in NMDA receptor conductance.

Functions

NMDA receptors are ubiquitously distributed throughout the CNS, and their activation is essential for many physiological functions of the brain, such as development, learning and memory and neuronal outgrowth (Vallano, 1998; Contestabile, 2000). At resting membrane potentials, NMDA receptors are blocked by physiological concentrations of Mg^{2+}, and this prevents them from being activated by their agonists. For this reason, they are not thought to be involved in the early phase of fast excitatory

neurotransmission (Doble, 1999). However, NMDA receptors may become involved in secondary glutamatergic synaptic neurotransmission following AMPA receptor activation (Morales and Goda, 1999). Such a phenomenon has been well characterized within the CNS, such as long-term potentiation (LTP) in the hippocampus, long-term depression (LTD) in the cerebellum, and stimulus-dependent plasticity in the visual cortex (Collingridge and Singer, 1990; Bear and Abraham, 1996). Both LTP and LTD play important roles in learning and memory (Collingridge and Singer, 1990; Kullmann et al., 1996; Isaac et al., 1999).

Role in excitotoxicity

NMDA receptors have been implicated in a variety of neurological disorders. Over-activation of NMDA receptors in certain pathological conditions such as brain ischemia, head trauma, or epileptic seizures triggers a cascade of cellular events that can eventually lead to neuronal cell death (Montal, 1998; Nicholls and Budd, 1998). In addition, NMDA receptor over-activation has been shown to be involved in several inflammatory neurodegenerative diseases including encephalitis, Alzheimer's disease (Lipton and Rosenberg, 1994), multiple sclerosis (Pitt et al., 2000) and AIDS dementia (Lipton, 1994).

Glutamate neurotoxicity is predominantly mediated by the excessive entry of Ca^{2+} resulting from over-activation of glutamate receptors, among which, NMDA receptors play a major part. It has been shown that either competitive or non-competitive NMDA receptor antagonists attenuate neuronal cell death induced by *in vitro* hypoxia or ischemia (Choi and Rothman, 1990). In addition, non-neuronal cells transfected with recombinant

NR1/NR2A or NR1/NR2B undergo cell death when exposed to high concentrations of glutamate, and NMDA receptor antagonists protect those cells against glutamate toxicity (Anegawa, et al., 1995, Raymond et al., 1996). Furthermore, in rats injected with antisense oligonucleotides to mRNAs coding for NMDA receptor subunits, the magnitude of brain lesions following experimental cerebral ischemia is reduced dramatically (Wahlstedt et al., 1993).

Neurodegenerative diseases are a major cause of morbidity in the elderly and thus, an important issue in public health (Doble, 1999). Recent research indicates that brain excitotoxicity might play an important role in the genesis and progression of these diseases. It has been demonstrated that β-amyloid peptides, which represent a hallmark in Alzheimer's disease (Ashall and Goate, 1994), sensitize cultured neurons to excitotoxic cell death (Mattson et al., 1992). Therefore, the formation of extracellular β-amyloid may render neighboring healthy neurons vulnerable to excitotoxicity, and provide a mechanism whereby excitotoxic damage may promote neurodegeneration (Doble, 1998). Importantly, β-amyloid peptides stimulate microglia to release the neurotoxin NTox (Giulian, 1997). Although its chemical identity remains controversial, NTox is not structurally related to glutamate or quinolinate (Giulian, 1997; 1999). NTox can kill neurons in co-cultures of microglia and neurons by a mechanism that is prevented by NMDA receptor antagonists (Giulian, 1997). These reports suggest that a direct link exists between NMDA receptor mediated excitotoxicity and Alzheimer's disease.

The role of NMDA receptors in AIDS dementia has been investigated extensively in the past (Lipton, 1994; 1996). Pharmacological studies indicate that competitive or

non-competitive NMDA receptor antagonists protect against HIV glycoprotein gp120-induced neuronal cell death (Lipton, 1992; Savio and Levi, 1993). In addition, gp120 has been shown to interact directly with NMDA receptor channels to promote noradrenaline release from rat brain synaptosomes and hippocampal slices (Pittaluga et al., 1996). Furthermore, gp120 increases extracellular glutamate formation by both inhibiting the uptake and increasing the release of glutamate from cultured rat astrocytes (Vesce et al., 1997). Therefore, it is possible that gp120 stimulates the release of glutamate, which then acts at NMDA and non-NMDA receptors to promote neuronal cell death in the brain.

The involvement of NMDA receptors in other neurodegenerative diseases such as amyotophic lateral sclerosis, Parkinson's disease, Huntington's disease and schizophrenia has also been reported (Hicks and Conti, 1996; Doble, 1999). In fact, it has been suggested that NMDA receptor activation and subsequent Ca^{2+} accumulation in the intracellular space might serve as a final common pathway for the progression of many neurodegenerative diseases (Lipton and Rosenberg, 1994).

Metabotropic Receptors

Three metabotropic receptor families consisting of eight different subtypes have been identified (Table 1-1). Activation of group I receptors leads to stimulation of phospholipase C (PLC) and subsequent formation of inositol trisphosphate (IP_3), which in turn stimulates the release of Ca^{2+} from intracellular stores (Masu et al., 1991; Abe et al., 1992; Aramori and Nakanishi, 1992). On the other hand, activation of group II or group III metabotropic glutamate receptors leads to the inhibition of adenylyl cyclase. Group II

mGluRs show strong inhibition of forskolin-induced cAMP formation (Tanabe et al., 1992, 1993), whereas maximal inhibition of cAMP formation is somewhat smaller for the group III receptors. All these effects of group II and group III receptors on cAMP inhibition are strongly inhibited by pertussis toxin (PTX), suggesting that the G-proteins coupled to these receptors belong to the Gi family (Duvoisin et al., 1995).

Unlike ionotropic glutamate receptors, metabotropic glutamate receptors are not generally thought to be involved in fast synaptic neurotransmission (Pin and Duvoisin, 1995; Toms et al., 1996). However, a detailed understanding of the physiological roles of metabotropic receptors has been impeded by the lack of potent and specific agonists and antagonists. Available data suggest that metabotropic receptors are involved in the regulation of neuronal excitability, presynaptic inhibition, and synaptic plasticity (Nakanishi, 1994; Bortolotto et al., 1999).

Roles of metabotropic glutamate receptors in glutamatergic synaptic transmission have been demonstrated in several synapses. For instance, mGluR agonists induce a slow depolarization accompanied by an increase in firing in many neurons, including those of the hippocampus, neocortex and cerebellum (Ozawa et al., 1998). These excitatory effects are probably due to direct actions of mGluR agonists on K^+ channels (Nakanishi, 1995; Ozawa et al., 1998). In addition, mGluR activation has been shown to broaden the action potentials in hippocampal neurons by slowing repolarization (Hu and Storm, 1991).

In addition to the direct excitatory postsynaptic effects, mGluR activation suppresses both excitatory and inhibitory transmission at synapses by presynaptic mechanisms (Nakanishi, 1994). Several studies have shown that mGluRs are localized at

presynaptic terminals (Bradley et al., 1996; Shigemoto et al., 1996; Yokoi et al., 1996). These presynaptic mGluRs may play important roles in the regulation of synaptic functions. Besides their capability to serve as autoreceptors to modulation glutamate release (Herrero et al., 1992; Scanziani et al., 1997), they are also able to induce plasticity due to presynaptic mechanisms (Kobayashi et al., 1996). However, the mechanisms underlying mGluR-mediated presynaptic inhibition are not fully understood. It has been suggested that inhibition of presynaptic channels is responsible for the suppression of transmitter release by mGluR agonists (Ozawa, 1998). Activation of mGluRs has been shown to inhibit both L-type (Chavis et al., 1994) and N-type (Ikeda et al., 1995) Ca^{2+} channels in a pertussis toxin dependent manner. In addition, fluorometric measurement of presynaptic Ca^{2+} transients in hippocampal slices has provided direct evidence that inhibition of presynaptic Ca^{2+} influx accounts for the suppression of transmitter release caused by mGluR activation (Yoshino and Kamiya, 1995).

Compared with ionotropic receptors, the possible role of mGluRs in mediating LTP and LTD is much less clear. Most experimental results generated from studies with specific agonists and antagonists suggest that mGluRs are not directly involved in mediating LTP and LTD. Rather, they play modulatory roles by regulating NMDA and non-NMDA receptor functions (Ozawa et al., 1998; Bortolotto et al., 1999).

Investigations into the roles of metabotropic glutamate receptors in mediating neurological disorders have been hindered by the fact that mGluRs influence neuronal activities in a complicated manner. Because different receptor subtypes couple to different second messenger systems and produce a variety of consequences, and available agonists and antagonists are rarely selective towards a particular mGluR subtype, it is

difficult to test the roles of individual receptor subtypes in mediating neurotoxicity. Nevertheless, it has been reported that neuronal cell death caused by glutamate was attenuated by the group II (mGluR2, mGluR3)-selective agonist DCG-IV (Bruno et al., 1994, 1995; Buisson et al., 1996), but not by the group III (mGluR3, mGluR6-mGluR8) agonist L-AP4 (Pizzi et al., 1996), indicating that group II receptors may be responsible for this effect. Since group II receptors mediate presynaptic inhibition at many synapses, the inhibition of glutamate release may account for this protective effect.

Uptake of Excitatory Amino Acids

Unlike other neurotransmitters (e.g. acetylcholine), which are converted into inactive compounds by extracellular enzymes as a means of their termination of actions, glutamate is not generally metabolized in normal synaptic functioning (Kanner, 1994; Trotti et al., 1998). Instead, the excitatory actions of glutamate are rapidly terminated by its efficient removal from the synapse through glutamate uptake systems existing in both glia and neurons (Nicholls and Attwell, 1990; Seal and Amara, 1999). It is generally believed that transporters in glia (e.g. astrocytes) perform more important functions in maintaining extracellular glutamate levels than those in neurons (Kanner, 1994; Hasson, et al., 2000).

Five distinct glutamate transporter families have been cloned and characterized (EAAT1 - EAAT5, Table 1-2) by molecular cloning techniques in mammalian CNS (Palmada and Centelles, 1998; Gegelashvili and Schousboe, 1998; Tanaka, 2000). All subtypes are shown to transport L-glutamate, L-aspartate, and D-aspartate with high affinity (Seal and Amara, 1999). Biochemical studies show that EAA transport is

thermodynamically coupled to the inward movement of two or three Na^+ (Schwartz and Tachibana, 1990) and a proton (Zerangue and Kavanaugh, 1996), and to the outward movement of a K^+ (Szatkowski et al., 1990).

Under pathological conditions, however, glutamate transporters may lose their abilities to maintain glutamate homeostasis (Takahashi et al., 1997). After traumatic or ischemic injury to the CNS, dramatic increases in extracellular glutamate levels have been observed in various animal species (Choi and Rothman, 1990). Importantly, in a recent study, Rossi et al. (2000) showed that the glutamate release in severe ischemic conditions in hippocampus is due to the reversal of glutamate transporters in astrocytes (Rossi et al., 2000). In addition, reversed operation of glutamate transporters has been reported in many oxidative-associated neurodegenerative diseases including amyotropic lateral sclerosis (Rothstein et al., 1992), Alzheimer's disease (Masliah et al., 1996) and AIDS dementia (Vesce et al., 1997). The role of glutamate transporters in neuroinflammation will be discussed later in this Chapter.

Table 1-2. Classification of excitatory amino acid transporter

Subtype	Species	Distribution	CNS cell type	Inhibitors
EAAT1	Human	CNS, peripheral tissues	Neurons, glia	THA, tPDC, DL-TBOA
	Rat (GLAST)	CNS	Glia	THA, tPDC
EAAT2	Human	CNS, placenta	Glia	THA, tPDC, DHK DL-TBOA
	Rat (GLT1)	CNS	Glia	THA, tPDC, DHK
EAAT3	Human	CNS, peripheral tissues	Neurons	THA, tPDC
	Rat (rEAAC1)	CNS, peripheral tissues	Neurons	THA, tPDC
	Rabbit (EAAC1)	CNS, peripheral tissues	Neurons	THA
EAAT4	Human	CNS (cerebellum)	Neurons	THA, tPDC
	Mouse	CNS (cerebellum)	Neurons	THA, tPDC
EAAT5	Human	Retina	Neurons, glia	THA, tPDC
	Salamander	Retina		THA

Inflammatory Responses in the CNS

Inflammation is an effective defensive response of the body to injury or infection and is essential for survival. In the periphery, inflammatory responses bring fluids, proteins and inflammatory cells such as macrophages from the blood to the site of infection (Perry et al., 1999). These cells and proteins then initiate immune responses to prevent harm and to eliminate pathogens such as bacteria or viruses. However, if the stimulus is strong enough to overcome the defensive ability of the body, tissue damage may occur, leading to the impairment of tissue function.

The complex cascade of peripheral inflammatory reactions has been studied extensively in the past. The gram-negative bacterial endotoxin lipopolysaccharide (LPS) has been widely used to stimulate the immune/inflammatory responses both in vivo and in vitro (Wan et al., 1994; Montero-Menei et al., 1996). LPS consists of polysaccharides combined with a lipid A component, the latter being responsible for many, but not all, of its biological actions (Sweet and Hume, 1996). Peripherally, LPS interacts with high affinity CD14 and other lower affinity receptors (CD11c/CD18) on polymorphonuclear leukocytes (PMNs), B- and T- lymphocytes, monocytes, tissue macrophages, endothelial or smooth muscle cells, and mast cells (Leal-Berumen et al., 1994, 1996). Interaction between LPS and its receptors leads to the release of inflammatory mediators, such as cytokines, prostaglandins (PGs), thromboxane A_2 (TXA_2), leukotrienes, C5a, and reactive species. These various products result in the sepsis syndrome manifested as fever, hypothermia, tachycardia, tachypnea, oliguria, acidosis and hypotension, and can ultimately produce septic shock and multi-organ failure (Feuerstein et al., 1998). Activation of mast cells and macrophages can play important roles in evoking immediate

responses, by releasing preformed/newly synthesized substances such as histamine, leukotrienes and TNF (Marshall et al., 1996).

Compared with the peripheral immune/inflammatory system, the CNS has been considered isolated and protected from most immune/inflammatory processes. However, there is mounting evidence that the CNS is affected by both central and peripheral inflammatory reactions (de Vries et al., 1997). Indeed, many neurodegenerative disorders, including trauma and stroke damage, Alzheimer's disease, Parkinson's disease, AIDS dementia, as well as neurological pathologies associated more directly with specific bacterial infections of the CNS, appear to involve inflammatory processes (Perry et al., 1993; Yeung et al., 1995; Feuerstein et al., 1997). In many cases, it is not clear whether central inflammation is the cause of, or a response to, damage in the CNS. There is little doubt that neuronal cell death can trigger an inflammatory response in the brain. However, activation of an inflammatory response in the brain certainly has the capacity to cause pathological responses, including neuronal damage.

Under normal circumstances, the brain is protected from the ravages of full-blown peripheral inflammatory reactions, due to the presence of the blood brain barrier (BBB). However, the integrity of the BBB becomes disrupted in whole or in part shortly after serious systemic (peripheral) inflammatory responses occur (De Vries et al., 1997), thus exposing the CNS to systemic inflammatory mediators and cellular components of the inflammatory response such as activated neutrophils. In the brain, the microglia perform functions as resident macrophages. Once activated in response to traumatic injury or pathogens, they proliferate, migrate and perform macrophage functions, such as producing cytokines (Gonzalez-Scarano and Baltuch, 1999), prostanoids, and nitric oxide

(Minghetti and Levi, 1998). Cytokines in turn can amplify the inflammatory responses by recruiting cells to the site of injury. In addition, perisynaptic astrocytes can become reactive, acquiring some macrophage-like qualities, while losing their normal abilities to regulate extracellular concentrations of K^+ and glutamate (Aschner et al., 1999).

Role of Glial Cells in Brain Inflammation

There are four major types of glial cells in the brain; macroglia, microglia, schwann cells and oligodendrocytes (Verkhratsky and Steinhauser, 2000). The macroglia are further divided into ependymoglia, astrocytes and myelinating glia (Laming et al., 2000). Historically, glial cells were not believed to perform important functions in the brain due to their lack of excitability (Kuffler, et al., 1966). However, recent research indicates that they are important effector cells in many pathological conditions of the brain (Minghetti and Levi, 1998). Due to their reactivity to a wide range of stimuli, they play crucial roles in host defense, and facilitate neuroprotection and repair processes. Upon stimulation with immunogens, these cells are able to release various inflammatory mediators such as cytokines, prostanoids and nitric oxide (Minghetti and Levi, 1998). Moreover, astrocytes perform important functions by maintaining K^+ and glutamate homeostasis in the brain (Turecek and Trussell, 2000).

Microglia and brain inflammation

Microglia are generally considered to be resident macrophages of the CNS. Evidence from immunocytochemical studies using macrophage-specific markers has shown that monocytes enter the developing CNS and give rise to microglia (Perry and

Gordon, 1991). Although microglia represent about 10-20% of total cells in the brain, their roles in normal function remain largely unknown (Minghetti and Levi, 1998). It is generally believed that these cells are quiescent under normal brain functioning. However, under pathological conditions, they become quickly activated to perform macrophage-like functions. A striking feature of these cells is their ability to synthesize and release a variety of inflammatory mediators, including cytokines, free radicals and prostanoids (Minghetti and Levi, 1998). These substances, alone or in concert with factors derived from other brain cells such as neurons and astrocytes, may play crucial parts in host defense or in the establishment or maintenance of brain damage (McGeer and McGeer, 1995; Moore and Thanos, 1996; Nadeau and Rivest, 2000). The role of these individual inflammatory mediators in the pathogenesis of brain inflammation and neurodegenerative diseases will be discussed later in this section.

Numerous studies have shown that immunogens such as the HIV glycoproteins which are associated with AIDS dementia, and β-amyloid peptides which are associated with Alzheimer's disease, are toxic to neurons only in the presence of glia (Barger and Harmon, 1997; Corasaniti et al, 1998). Moreover, microglia are likely infected by the HIV virus (Wiley et al., 1986). Taken together, these findings indicate that microglia are first attacked by those immunogens associated with the virus and then secrete various inflammatory mediators, which play major roles in the subsequent neurotoxicity. The secretory products include the aforementioned inflammatory mediators and possibly other toxic substances (Koutsilieri et al., 1999). Importantly, LPS releases glutamate from cultured microglia (Patrizio and Levi, 1994) and macrophages (Klegeris and McGeer, 1997). Moreover, the HIV glycoprotein gp120 and the β-amyloid peptides

Aβ1-41 and Aβ1-40 apparently release NTox from cultured microglia (Giulian, 1997), which can activate NMDA receptors (Giulian, 1999). These findings suggest that microglia-derived excitotoxic substances may play an important role in the pathogeneses of these neurodegenerative diseases.

Astrocytes and brain inflammation

Astrocytes represent the largest population of cells in the brain, and they perform important functions by maintaining K^+ and glutamate homeostasis. As discussed in the first part of this Chapter, astrocytes possess high affinity Na^+-dependent glutamate transporters, which uptake glutamate from the extracellular space to keep the perisynaptic glutamate concentrations low (Gegelashvili and Schousboe, 1997; 1998). Among the five cloned glutamate transporters, at least one subtype (EAAT2) is astrocyte-specific and plays a major part in glutamate removal from synaptic clefts (Lehre and Danbolt, 1998; Kojima et al., 1999). Within astrocytes, glutamate is metabolized to glutamine, catalyzed by the enzyme glutamine synthetase, which is rich in astrocytes but not present in neurons (Tansey et al., 1991). Glutamine is then released from astrocytes and recovered by presynaptic terminals, where it is converted into glutamate and recycled as a neurotransmitter (Hertz et al., 1999). In addition, astrocytes are rich in phosphate-activated glutaminase (PAG), an enzyme responsible for the hydrolysis of glutamine to glutamate, and thus can synthesize glutamate (Hertz et al., 1999). Therefore, the intracellular concentration of glutamate in astrocytes is at least 10,000 times higher than that found extracellularly in the immediate vicinities of synapses (Doble, 1999). Thus, it is not surprising that under certain pathological conditions, astrocytes will have the

potential to cause massive release of glutamate, which can lead to subsequent excitotoxicity. It has been shown that under brain ischemic conditions, glutamate is released mainly through reverse operation of glutamate transporters in astrocytes (Rossi et al, 2000).

In addition to their capacity to take up glutamate from the perisynaptic space, astrocytes also express numerous ion channels and transmitter receptors (Verkhratsky and Steinhauser, 2000). Functional metabotropic glutamate receptors and non-NMDA receptors have been identified in cultured astrocytes (Sontheimer et al., 1988; Usowicz et al., 1989). In contrast, reliable evidence for functional NMDA receptor expression in astrocytes is still lacking (Verkhratsky and Steinhauser, 2000), although it has been reported that NMDA receptors seem to be involved in the regulation of proliferation in cultured retinal Müller cell-like glial cells (Uchihori and Puro, 1993). Activation of these glutamate receptor subtypes may have important physiological and pathological implications. It has been shown that activation of AMPA receptors could increase the intracellular concentrations of Ca^{2+} (Nedergaard, 1994; Parpura et al., 1994), which plays a major role in neuron-glia signaling (Araque et al., 1998). Importantly, co-activation of non-NMDA receptors and mGluRs releases glutamate from cultured rat astrocytes through a mechanism that involves the formation of prostaglandin E_2 (PGE_2) (Bezzi et al., 1998). The latter is a well-known inflammatory mediator in the brain. In addition, PGE_2 has been shown to exert its neuromodulatory activities through the release of glutamate from astrocytes in cultured hippocampal cells (Sanzgiri et al., 1999). On the other hand, activation of mGluRs inhibits glutamate release from cultured astrocytes

independent of glutamate transporters (Ye and Sontheimer, 1999). The apparent paradoxical results of mGluR activation on glutamate release await further elucidation.

A unique feature of astrocytes is their ability to swell under certain pathological conditions (Aschner et al., 1999). As shown by electron microscopic studies, astrocytic swelling occurs in numerous pathological states (Dietrich et al., 1994), as well as in response to a number of neurotoxins (Aschner and Aschner, 1992; Aschner and LoPachin, 1993). The mechanisms of astrocytic swelling appear complex and involve alterations of several ion transporters such as the Cl^-/HCO_3^- and Na^+/H^+ exchange transporters (Kempski et al., 1988). It is noteworthy that the HIV glycoprotein gp120-induced increases in intracellular Ca^{2+} in cultured astrocytes are completed blocked by the amiloride analogue methyl isobutyl amiloride, which blocks the Na^+/H^+ exchanger (Holden et al., 1997). This suggests that gp120-induced neurotoxicity may involve an astrocytic swelling mechanism.

Inflammatory Mediators in the CNS

Numerous inflammatory mediators have been implicated in mediating the pathologies in brain inflammation; these include NO, oxygen free radicals, prostanoids, cytokines and chemokines (Xiao and Link, 1998). The following section will briefly review the roles of some of these substances in brain inflammation.

NO and oxygen free radicals

During the past decade, NO has emerged as an important mediator of physiological and pathophysiological processes because of its wide range of biological

effects and its production in a variety of cell types and tissues. In the brain, NO can originate from neurons, glia, or endothelial cells lining cerebral blood vessels (Bruhwyler et al., 1993; Snyder et al., 1998). Following its generation by NO synthase (NOS), NO readily diffuses beyond its site of origin to influence adjacent cells (Griffith and Stuehr, 1995). Under physiological conditions, NO serves as a second messenger in neuronal signal transduction following activation of excitatory amino acid receptors such as NMDA receptors in the CNS (East and Garthwaite, 1991; Clementi, 1998). However, excessive production of NO can be neurotoxic (Brüne et al., 1998; Wada et al., 1998; Floyd, 1999). Recent studies show that NO promotes glutamate release from rat synaptosomal preparations (Sequeira et al., 1997; McNaught and Brown, 1998), suggesting that a direct link between NO formation and excitotoxicity might exist in the brain.

Three major isoforms of NOS have been identified in the body; endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutive and Ca^{2+}/calmodulin-dependent, and inducible or immunological NOS (iNOS), which is Ca^{2+}/calmodulin-independent (Bredt and Synder, 1994; Dawson and Dawson, 1996, Murphy, 2000). Astrocytes display all three isoforms of NOS, and activated microglia express iNOS (Murphy et al., 1993). Numerous studies have shown that NO production in the brain is elevated under pathological conditions such as cerebral ischemia (Strijbos, 1998), ALS (Chou et al., 1996), MS (Lin et al., 1993), Alzheimer's disease (McGeer and McGeer, 1995; Heales et al., 1999) and Parkinson's disease (Ara et al., 1998).

A variety of mechanisms for NO neurotoxicity have been postulated, both through direct interactions with cell surface and internal molecules and through chemical interactions with superoxide to form other potentially toxic intermediates (Xiao and Link, 1998). Peroxynitrite ($ONOO^-$) formed by the interaction of NO with superoxide anion is believed to be the major molecule responsible for NO neurotoxicity. It has been shown that $ONOO^-$ is a potent oxidant and can cause cell death (Beckman, 1994; Ducrocq et al., 1999; Torreilles et al., 1999). Moreover, the $ONOO^-$ scavenger uric acid has been reported to be neuroprotective (Hooper et al., 1997). These data suggest that $ONOO^-$ plays an important role in mediating NO neurotoxicity. On the other hand, NO is not always toxic to neurons, since neuroprotective effects of NO production in neurodegenerative diseases have also been reported (Zidek and Mašk, 1998).

Both microglia and astrocytes also possess the ability to produce free radicals upon stimulation by inflammatory factors (Murphy, 2000). Enhanced production of superoxide has been demonstrated in experimental models of traumatic brain injury (Kontes and Wei, 1986), and in macrophages stimulated by LPS (Martinez and Moreno, 2000) or by β-amyloid peptides (Klegeris and McGeer, 1997). Oxygen free radicals have been implicated in a variety of neurodegenerative disorders including brain ischemic damage, seizure disorders, and Alzheimer's disease (Jesberger and Richardson, 1991). They have been postulated to mediate brain excitotoxic damage (Pellegrini-Giampietro et al., 1988; Coyle and Puttarcken, 1993; Ciani et al., 1996). Free radical scavengers have been shown to protect against excitotoxicity caused by excitotoxins such as glutamate in the striatum in vivo (Miyamoto and Coyle, 1990; Schulz et al., 1995). Furthermore, enhanced glutamate release by free radicals has been reported in brain synaptosomes

(Gilman et al., 1994) and cultured astrocytes (Volterra et al., 1994). It is possible that glutamate, nitric oxide and free radicals may act in a sequential as well as a reinforcing manner, leading to neurodegeneration.

Prostanoids

Polyunsaturated fatty acids are integral structural components of membrane phospholipids, where they play important roles in maintaining the structural and functional characteristics of cell membranes. However, these fatty acids are not permanently attached to cell membranes. They can be liberated and metabolized into various bioactive substances (Katsuki and Okuda, 1995). Arachidonic acid (AA) is one of the most important bioactive fatty acids in the brain. Besides its ability to be bioactive, it also serves as a precursor for a number of other bioactive substances such as prostaglandins (PGs), leukotrienes (LTs) and thromboxanes (TXs) (Balsinde et al., 1999). Three enzyme superfamilies are involved in the metabolism of AA, namely, phospholipase A_2 (PLA_2), cyclooxygenases (COX-1 and COX-2), and lipoxygenases (Farooqui et al., 1997; Tang et al., 1997; Dubois et al., 1998). All of these enzymes play important roles in the regulation of brain functions (Balsinde et al., 1999). A simplified scheme of AA metabolism is shown in Figure 1-1.

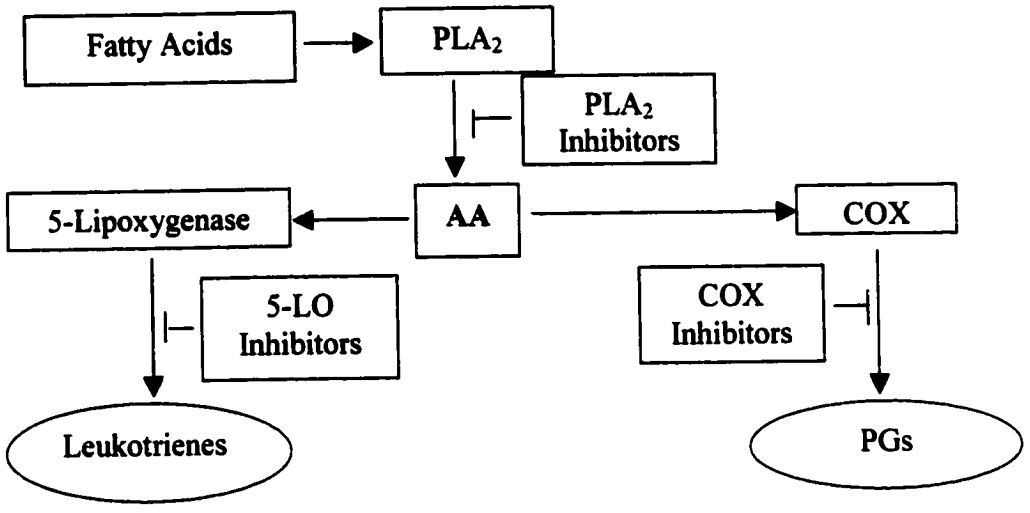


Fig. 1-1. Simplified Metabolism of Arachidonic Acid

The significance of the AA cascade in cell signaling in the nervous system was first demonstrated by Piomelli et al. (1987), who showed that AA or its metabolites suppress neurotransmitter release from axon terminals of *Aplysia* sensory neurons. Subsequent studies showed that these substances are able to modulate the activities of protein kinases and ion channels (Budd and Nicholls, 1998), to modulate neurotransmitter release (Peterson et al., 1995), and to enhance neurotransmission (Collins and Davies, 1998). However, under inflammatory conditions, excessive formation ot AA and its metabolites, especially PGs, may promote neurodegeneration and neuronal cell death (Minghetti and Levi, 1998). In fact, a history of the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to be associated with a decreased risk of developing Alzheimer's disease (Breitner et al., 1994). Recent studies

indicate that releases of AA and/or its metabolites are increased after LPS stimulation of cultured macrophages (Balboa et al., 1999; Patel et al., 1999). Activities of PLA_2 and COX are upregulated under inflammatory conditions (Lacroix and Rivest, 1998; Pistritto et al., 1998; Balsinde et al., 1999). Moreover, increased levels of PGE_2 in the brain have been observed in patients with AIDS dementia (Griffin et al., 1994) and Alzheimer's disease (Pasinetti and Aisen, 1998). These data suggest that AA and/or PGs may be involved in the pathogenesis of brain inflammatory diseases.

The roles of AA and its metabolites in the pathogenesis of neurodegenerative diseases are complex because, in some instances, individual prostanoids may exert opposite effects on common targets (Phipps et al., 1991). In addition, under inflammatory conditions, the activation of the AA cascade is often accompanied by the generation of a broad range of other molecules, including cytokines and free radicals, which have been shown to play important roles in inflammation (Katsuki et al., 1995). Nevertheless, it has been shown that AA and its metabolites may contribute to cell damage by perturbing neuronal cell membranes, by affecting the activity of ion channels, by inhibiting glutamate uptake or by altering mitochondrial respiratory activities (Katsuki and Okuda, 1995). Significantly, co-activation of both non-NMDA and metabotropic glutamate receptors promotes the release of glutamate from cultured astrocytes and rat cortical slices via the formation of PGs, since COX inhibitors abolish while PGE_2 mimics this glutamate release process (Bezzi et al., 1998). Moreover, in an *in vivo* microdialysis study, Monda et al. (1998) showed that extracellular glutamate and aspartate levels in rat frontal cortex were increased after i.c.v. injection of PGE_1. Therefore, it seems possible

that AA and its metabolites may exert their neurotoxic activities, at least in part, through the release of EAAs from glial cells.

On the other hand, there is increasing evidence that prostanoids are not always toxic in some instances. In fact, PGE_2 and PGI_2 have been recently reported to protect cultured neurons from several kinds of noxious conditions, including hypoxia/ reoxygenation and glutamate toxicity (Cazevieille et al., 1993; 1994; Akaike et al., 1994). Moreover, PGs, in particular PGE_2, could protect neurons indirectly, i.e., through the regulation of inflammatory and immune responses occurring in many brain pathologies. Indeed, PGE_2, in addition to its well-known pro-inflammatory activity, can limit the activation of macrophages and microglia, and regulate functions of T and B cells (Weissmann, 1993; Phipps et al., 1991). Therefore, further research to determine the exact roles of these presumably pro-inflammatory substances is warranted.

Cytokines

Cytokines are a diverse group of polypeptides, which comprises interleukins, chemokines, tumor necrosis factors, interferons, and growth and cell stimulating factors (Rothwell, 1999). Cytokines perform important functions in the brain, such as controlling neuronal and glial activation, proliferation, differentiation and survival. Because of these functions, they can influence neuronal and glial plasticity, as well as development and regeneration of the nervous system (Martiney et al., 1998; Muñoz-Fermández and Fresno, 1998). On the other hand, uncontrolled or excessive production of these cytokines may cause damage to the CNS (Rothwell and Liheshi, 1994; Rothwell and Hopkins, 1995; Rothwell, 1997). This aspect of pro-inflammatory cytokines on CNS

has received most attention because of its obvious clinical implications (Fontana et al., 1984; Vitkovic et al., 2000).

In response to inflammatory stimulations, the expression of pro-inflammatory cytokines in the brain increases rapidly (Hopkins and Rothwell, 1995). A variety of inflammatory compounds can induce the synthesis and release of pro-inflammatory cytokines. These compounds include, but are not limited to, prostaglandins, viral, bacterial or parasitic components and products of tissue damage (Pan et al., 1997). It has been demonstrated that excessive production of pro-inflammatory cytokines can contribute to the clinical manifestations of many neurodegenerative and infectious diseases (Arwin et al., 1996; Merrill and Benveniste, 1996).

The major sources of proinflammatory cytokines (IL-1, IL-6 and TNF-α) in the brain appear to be astrocytes and activated microglia (Giulian et al., 1986; Sawada et al., 1989; Chung and Benveniste, 1990; Morganti-Kossmann et al., 1992). However, neurons also have the ability to produce these cytokines (Breder et al., 1993), suggesting that neurons may also play a role in the actions of these cytokines in the CNS. Upon release from their sites of origin, these cytokines exert their actions by binding to their specific receptors (Pan et al., 1997; Loddick et al., 1998), which exist on the cell surface of glial cells as well as neurons (Aschner, 1998).

Inflammatory cytokines can trigger a cascade of cytokines in the CNS, normally first acting on glia followed by glia-derived cytokines acting on neurons (Muñoz-Fermández and Fresno, 1998). For example, TNF-α mostly induces IL-1 and IL-6. IL-1 then triggers the releases of other growth factors or neurotrophins (Norris et al., 1994). TNF-α production can also be induced by other cytokines such as IL-1β and interferon γ

(Sharif et al., 1993; Nitta et al., 1994). A striking feature of the production of inflammatory cytokines is synergism among their inducers. For example, interferon γ or IL-1β alone may not induce the production of TNF-α but may cause a marked release of TNF-α when administered together (Pan et al., 1997). Moreover, LPS-induced release of TNF-α is enhanced by interferon γ in cultured astrocytes (Chung et al., 1991). Besides their abilities to induce the production of other cytokines, inflammatory cytokines have been demonstrated to stimulate the releases of neurotoxic substances such as glutamate, NO and AA from cultured astrocytes and microglia (Giulian and Vaca, 1993; Piani and Fontana, 1994). These data suggest that inflammatory cytokines may play a role in mediating excitotoxicity in the CNS. On the other hand, inflammatory cytokines enhance the release of nerve growth factor (NGF) and other neurotrophic molecules capable of promoting the survival and repair of brain tissue (Friedman et al., 1990; Brenneman et al., 1992), thereby facilitating neuronal survival and recovery. These apparent paradoxical actions of inflammatory cytokines reveal the complexity of the inflammatory system in the CNS.

Adenosine in the CNS and Its Role in Inflammation

The nucleoside adenosine is an important inhibitory neuromodulator in the CNS (Phillis and Wu, 1981; Marangos and Boulenger, 1985; Greene and Haas, 1991). Because there is no direct evidence that adenosine is released from synaptic vesicles, it is generally not recognized as a neurotransmitter (Dunwiddie, 1985).

Adenosine Receptors and Their Functions in the CNS

Adenosine exerts its functions in the CNS by interacting with its specific cell membrane receptors. Four subtypes of adenosine receptors are cloned and characterized to date; A_1, A_{2A}, A_{2B}, and A_3 receptors (Pathbone et al., 1999). All of these receptors are metabotropic and thus linked via G proteins to adenylyl cyclase, guanylyl cyclase, ion channels or phospholipases (Table 1-3) (Collis and Hourani, 1993; Sweeney, 1997).

In general, the affinity of adenosine A_2 receptors for adenosine is lower than that of A_1 receptors, and their stimulation elevates, while activation of both A_1 and A_3 receptors reduces, the concentration of intracellular cAMP (Jacobson, 1998; Svenningsson et al., 1999). In addition to their inhibitory effects on adenylyl cyclase, and contrary to A_2 receptors, both A_1 and A_3 receptors are also characterized by their stimulatory effect on phospholipase C (PLC) (Linden, 1995), and therefore they are able to increase the intracellular levels of IP_3, resulting in Ca^{2+} mobilization within the cell.

Table 1-3. Classification of adenosine receptors

Subtype	Agonists	Antagonists	Distribution	Transduction Pathways
A_1	CPA, CHA, CCPA	CPX, CPT	Hippocampus, cortex, superior colliculus, cerebellum	↓AC, ↑PLC-PKC, ↑K^+ conductance, ↓Ca^{2+} conductance
A_{2A}	CGS-21680	KF 17837, SCH-58261	Striatum, smooth muscle	↑AC, ↑IP_3
A_{2B}	NECA	XAC, Alloxazind	Striatum, hippocampus, cortex, platelet	↑AC
A_3	IB-MECA, DBXRM	I-ABOPX, BW-A1433	Throughout the brain	↑PLC, ↑IP_3

Adenosine A_1 Receptors

The majority of adenosine's inhibitory effects are through the activation of A_1 receptors (Khakh and Kennedy, 1998). A_1 receptors are distributed ubiquitously throughout the brain, with the highest receptor density found in the hippocampus and cortex (Goodman and Snyder, 1982). Their primary cellular locations are believed to be both pre- and post-synaptic (Deckert and Jorgensen, 1988), however, they can also be found on dendrites (Rivkees et al., 1995). Electrophysiological studies show that the inhibitory effects of adenosine A_1 receptor stimulation have both pre- and post-synaptic components. Pre-synaptic activation of A_1 receptors reduces Ca^{2+} influx through the inhibition of voltage-gated Ca^{2+} channels (Yawo and Chuhma, 1993), thus inhibiting neurotransmitter release (Lupica et al., 1992). On the other hand, activation of post-synaptic A_1 receptors enhances the inward K^+ currents (Segal, 1992) and voltage-dependent, GABA-independent Cl^- conductance (Mager et al., 1990), leading to decreased excitability of the cell. Other effects of A_1 receptor activation include sleep induction, antinociception and autonomic control of cardiac function, among others (Sawynok, 1998; Nyce, 1999).

Pathophysiologically, the role of A_1 receptors in neurodegeneration, especially in brain ischemic damage, has been extensively investigated (Abbracchio and Cattabeni, 1999; Von Lubitz, 1999). Several studies have shown that A_1 receptor densities are reduced in conditions of cerebral ischemia and stroke (Daval et al., 1989; Onodera et al., 1989; Nagasawa et al., 1994). Such a loss of A_1 receptors may have important implications in the subsequent neuronal death after ischemia, since this will cause disinhibition of glutamate release, leading to excessive activation of glutamate receptors

(Fredholm and Dunwiddie, 1988; Katchman and Hershkovitz, 1993). In fact, it has been suggested that A_1 receptor loss and the subsequent disinhibition of the excitatory afferent input may constitute one of the key mechanisms underlying the mechanisms of ischemic damage (Von Lubitz et al., 1995). Although discrepancies exist, numerous studies have shown that adenosine, acting at its A_1 receptors, is protective against ischemic damage in various regions of the brain (Rudolphi et al., 1992; Sweeney, 1997). In whole animal models of stroke, administration of A_1 receptor agonists reduces brain damage and improves global neurological scores, while antagonists exacerbate brain damage and worsen neurological outcomes (Simpson et al., 1992; Von Lubitz et al., 1994). Moreover, in vitro studies show that A_1 receptor agonists attenuate damage to neurons in culture (Mori et al., 1992; Daval and Nicolas, 1994; Volonté et al, 1999) while A_1 antagonists potentiate damage (Lobner and Choi, 1994) in hypoxic conditions. Therefore, it seems possible that adenosine and its analogues, acting at A_1 receptors, have beneficial effects against ischemic damage in the brain (Bischofberger et al., 1997).

Adenosine A_2 Receptors

The highest densities of adenosine A_{2A} receptors are found in the striatum (Rosin et al., 1998; Moreau and Huber, 1999), although both A_2 receptor subtypes (A_{2A} and A_{2B}) are also present on smooth muscle fibers and endothelial cells of cerebral blood vessels (Kalaria and Harik, 1986; Svenningsson et al., 1999). Adenosine A_{2A} receptors are also expressed on astrocytes and microglia in the CNS and on macrophages in the periphery (Fiebich et al., 1996a,b). On striatopallidal neurons, A_{2A} receptors are co-localized with dopamine D_2 receptors on the same cell (Johansson et al., 1997) where they can influence

each other's functions. It has been postulated that the antagonistic interactions between A_{2A}/D_2 and A_1/D_1 receptors in the ventral and dorsal striatum are at least partly responsible for the motor stimulant effects of adenosine receptor antagonists and for the motor depressant actions of adenosine receptor agonists (Ribeiro, 1999).

Contrary to A_1 receptors, the neuronal actions of adenosine A_2 receptors are excitatory (Sebastiao and Ribeiro, 1996). Activation of A_{2A} receptors results in Ca^{2+}-dependent glutamate and acetylcholine release in the striatum (Popoli et al., 1995; Mori et al., 1996) that may involve voltage gated Ca^{2+} channels. Therefore, it is not surprising that A_{2A} antagonists protect against, while their agonists exacerbate excitotoxic damage in the brain, especially in the striatum (Ongini et al., 1997; Jones et al., 1998; Chen et al., 1999). Similar to the effects of A_1 receptor agonists, the neuroprotective effects of A_{2A} receptor antagonists consist of several components that may involve reduction of glutamate release (Simpson et al., 1992), possible attenuation of ischemia-induced aberrations in A_{2A} receptor-mediated modulation of striatal dopamine D_2 receptors (Von Lubitz et al., 1995), and prevention of microglial activation, the latter process requiring a concordant activation of A_2 and A_1 receptors (Schubert et al., 1997). Besides its role in brain ischemic damage, A_{2A} receptors have also been implicated in sleep promotion (Satoh et al., 1998). Significantly, adenosine, through activation of A_{2A} receptors on neutrophils and glia, can act as an endogenous anti-inflammatory agent (Cronstein, 1994). This aspect of adenosine actions will be discussed later in this Chapter.

Although adenosine A_{2B} receptors are widely distributed throughout the brain, the functions of this receptor subtype in the CNS remain virtually unknown (Feoktistov and Biaggioni, 1997). It has been suggested that A_{2B} receptors may be involved in

stimulating glutamate release from rat cerebral cortex (Phillis et al., 1993). In addition, it has been shown that this receptor subtype contributes to the accumulation of cAMP in primary astrocytes culture (Peakman and Hill, 1994), and mediates the increase of IL-6 mRNA in human astroglioma cells (Fiebich et al., 1996a,b), suggesting a role of this receptor subtype in inflammatory responses.

A_3 Receptors

Adenosine A_3 receptors are distributed throughout the brain, but at a density that is significantly lower than that of other adenosine receptor subtypes (Ji et al., 1994). A_3 receptors are mainly expressed on microglia (Fiebich et al., 1996a,b) and in vascular smooth muscles (Zhao et al., 1997). The existence of this receptor on neurons has also been reported (Linden, 1994; Dunwiddie et al., 1997).

Despite extensive research in the past, the roles of A_3 receptors in the CNS remain largely unknown, probably due to their relatively low affinity for adenosine (Von Lubitz, 1997; Von Lubitz et al., 1999a). Available data indicate that adenosine A_3 receptors may play important roles in the generation of ischemic brain damage. For example, activation of A_3 receptors in the hippocampus results in the desensitization of A_1 receptor-mediated inhibition of excitatory synaptic transmission (Dunwiddie et al., 1997), suggesting that activation of A_3 receptors may be detrimental during ischemic brain damage. Indeed, acute pre-ischemic treatment (15 min before ischemia) with the A_3 receptor agonist IB-MECA dose-dependently enhances mortality and neuronal damage in the cortex, hippocampus, and striatum of gerbils at 7 days after the ischemic insult (Von Lubitz, 1997). However, chronic administration of IB-MECA (60 days before ischemia)

produces dramatically opposite effects, i.e. a striking improvement of both survival and neuronal preservation (Von Lubitz et al., 1999b). Furthermore, it has been shown that, A_3 agonists are cytoprotective at low concentrations whereas they induce apoptosis in cultured astrocytes at high concentrations (Yao et al., 1997; Rathbone et al., 1999). These apparent paradoxical effects of A_3 receptors remain to be further investigated.

Importantly, A_3 receptors are also expressed on microglia, which are activated during inflammation (Minghetti and Levi, 1998). This may imply that A_3 receptors play a role in brain inflammation. This aspect of A_3 receptor function will be discussed later in this Chapter.

Adenosine Release in the CNS

As discussed above, adenosine, by acting at its specific receptors, modulates many functions in the CNS. In order for adenosine to act at its extracellular receptors, it must first get into the extracellular space. Although it is not certain as to whether adenosine itself is transported out of cells or whether it is largely formed extracellularly from adenosine 5'-monophosphate (AMP) and other nucleotides (Latini et al., 1995; Cunha et al., 1996), there is no doubt that adenosine levels increase rapidly in circumstances where the supply:demand ratio for oxygen decreases, such as in the case of ischaemia and hypoxia (Meghji and Newby, 1990; Pedata et al., 1993) and CNS injury (McAdoo et al., 2000). It is also known that such pathological conditions release EAAs from neurons and glia (Barnett et al., 1995). Moreover, activation of EAA receptors releases adenosine from slices of parietal cortex (Hoehn and White, 1990a; White, 1996) and hippocampus (Arens et al., 1992; Latini et al., 1999). Therefore, it is conceivable

that, under conditions of ischemia and hypoxia in the brain, EAAs are first released, which then act at their specific glutamate receptors (mainly the ionotropic subtypes) to release adenosine from various cell types of the brain. On the other hand, the adenosine released during low level NMDA receptor activation may provide an inhibitory threshold against further EAA receptor mediated neurotransmission and therefore, protect the brain against further excitotoxic damage (Craig and White, 1991; White et al., 1993; Manzoni et al, 1994).

Characterization studies show that glutamate-evoked adenosine release is through the activation of NMDA and non-NMDA receptors in rat cortical slices (Hoehn and White, 1990a,b). However, the release of adenosine from synaptosomes evoked by glutamate is mediated by glutamate transport, not by receptors (Hoehn and White, 1990c). In addition, glutamate-evoked adenosine release from rat parietal cortical slices is not diminished by inhibition of ecto-5'-nucleotidase, a key enzyme that catabolizes nucleotides to adenosine (Lloyd et al., 1993; Latini et al., 1995). Adenosine release thus appears to be due to the release of adenosine *per se* rather than the release of a nucleotide (Hoehn and White, 1990a). More detailed studies show that activation of non-NMDA receptors releases adenosine as such, whereas activation of NMDA receptors releases nucleotides, which are then converted into adenosine extracellularly (Craig and White, 1993). Furthermore, glutamate-evoked adenosine release in the CNS has been found to be either partly or totally Ca^{2+}-independent (Jhamandas and Dumbrille, 1980; Hoehn and White, 1990a).

Subsequent studies compared NMDA-evoked releases of adenosine and [^{3}H]noradrenaline from rat cortical slices. NMDA-evoked [^{3}H]NA release is virtually

abolished by the voltage-sensitive Na^+ channel blocker tetrodotoxin (TTX), whereas adenosine release is not (Hoehn et al., 1990), suggesting that NMDA-evoked release of NA, but not of adenosine, requires propagated action potentials. NMDA is 33-times more potent in releasing adenosine than releasing NA (Hoehn *et al.*, 1990). Moreover, partial non-competitive antagonism of NMDA-evoked adenosine release by Mg^{2+}, MK801 or the glycine site antagonist 7-chlorokynurenic acid can be overcome by high concentrations of NMDA (Hoehn *et al.*, 1990; Craig and White, 1991). These results support the concept that spare receptors exist for NMDA-evoked adenosine release but not for NA release.

Role of Adenosine in Inflammation

In addition to its role as a potent inhibitory neuromodulator in the CNS, adenosine has recently been implicated as a potent regulator of inflammatory responses and as such, may be useful as a pharmacological agent in the therapy of inflammatory diseases (Cronstein, 1994; Cronstein et al., 1995; Flamand et al., 2000). Extracellular adenosine formation is stimulated during periods of ischemia and over-excitation, as well as in inflammation (Cronstein, 1994). By acting at its specific receptors, adenosine may modulate inflammatory responses. Indeed, elevating extracellular adenosine levels decreases toxic shock in animal models and is now being developed as a therapeutic strategy for this disorder in humans (Firestein et al., 1994; Gadangi et al., 1996). Endogenous adenosine also depresses LPS-stimulated TNF-α synthesis by cultured monocytes (including macrophages) (Eigler et al., 1997), suggesting that extracellular adenosine could play an important role in controlling inflammatory responses.

Among the various adenosine receptor subtypes, A_{2A} receptors are believed to be the primary subtype responsible for its anti-inflammatory effects (Sullivan and Linden, 1998). Functional and binding assays indicate that A_{2A} receptors are widely expressed in various inflammatory cells, including lymphocytes (Varani et al., 1997), neutrophils (Martini et al., 1991), eosinophils (Yukawa et al., 1989), basophils (Hughes et al., 1987), monocytes/macrophages (Eigler et al., 1997), mast cells (Suzuki et al., 1998), and on vascular endothelial tissues (Bouma et al., 1996). The anti-inflammatory effects of methotrexate, sulphasalazine and the phosphodiesterase (PDE) IV inhibitor, rolipram, appear to be mediated, at least in part, by promoting the activity of endogenous extracellular adenosine at A_{2A} receptors on macrophages, microglia and neutrophils (Cronstein, 1995; Eigler et al., 1997; Sullivan et al., 1999). In addition, adenosine, acting at A_{2A} receptors, inhibits arachidonic acid release from neutrophils stimulated with LPS or TNF-α (Flamand et al., 2000).

The anti-inflammatory effects of adenosine have also been explored in animal models of inflammation *in vivo*. For instance, it has been demonstrated that the selective adenosine A_{2A} receptor agonist, 2-cyclohexylmethyl-idenehydrazinoadenosine (WRC-0470) decreased pleocytosis and reduced blood brain barrier permeability in a rat model of endotoxin-stimulated meningitis (Sullivan et al., 1999). In addition, in animal models of ischemia and reperfusion, adenosine acting as an agonist at A_{2A} receptors reduces reperfusion injury in part by decreasing the inflammatory response (Jordan et al., 1997).

Although A_{2A} receptors are primarily responsible for the anti-inflammatory effects of adenosine, other receptor subtypes have also been implicated in inflammatory responses. For example, it has been reported that adenosine, acting at lower affinity A_{2B}

receptors, promotes the formation of neuroprotective cytokine IL-6 by human astroglioma cells in culture (Yamada and Hatanaka, 1994; Fiebich et al., 1996b). In addition, the specific A_3 receptor agonist 2-chloro-N^6-(3—iodobenzyl)adenosine-5'-N-methyluronamide (Cl-IB-MECA) reduces platelet-activating factor-stimulated neutrophil adherence to coronary endothelium; and this effect is blocked by the A_3 antagonist MRS-1220 (Jordan et al., 1999), suggesting that A_3 receptors may also be involved in the modulation of inflammatory responses.

Based on the evidence above, it can be concluded that, adenosine, acting at A_{2A} (and possibly A_{2B} or A_3) receptors, plays a very important role in decreasing inflammatory responses. It is possible to ease inflammatory responses by promoting endogenous adenosine release from various sources in animal models.

Research Proposals

Modulation of NMDA-Evoked Releases of Adenosine and [^{3}H]NA by Protein Kinase C and Li^+ in Rat Parietal Cortical Slices

Activation of NMDA receptors releases both adenosine and [^{3}H]NA from rat parietal cortical slices, and NMDA-evoked release of [^{3}H]NA is abolished by physiological concentrations of Mg^{2+} (1.2 mM) (Hoehn et al., 1990). It has been suggested that activation of protein kinase C removes the Mg^{2+} block of the NMDA channels, thus allowing NMDA responses to proceed (Chen and Huang, 1992). The primary objective of this study is to determine whether activation of PKC will potentiate

NMDA-evoked releases of [^{3}H]NA and adenosine. If so, whether or not PKC's effect is due to the removal of the Mg^{2+} block will be investigated. Besides PKC, the effect of lithium on NMDA-evoked releases of [^{3}H]NA and adenosine will also be investigated.

Immediate Excitatory Responses Caused by Bacterial and Viral Immunogens in Rat Parietal Cortical Slices

1. Does the bacterial endotoxin LPS release glutamate, [^{3}H]NA and adenosine from rat cortical slices? If so, are glutamate receptors involved in LPS-evoked releases of these substances? Preliminary experiments showed that LPS evokes very rapid (< 2.5 min) releases of the neurotransmitters glutamate and [^{3}H]NA, and of the neuromodulator adenosine from rat parietal cortical slices. The possible involvement of ionotropic glutamate receptors (NMDA and non-NMDA) in LPS-evoked releases of these substances will be determined using specific receptor antagonists.

2. What are the cellular sources of LPS-evoked release of glutamate? LPS-evoked release of glutamate from cultures of microglia has been reported previously (Patrizio and Levi, 1994), and the HIV glycoprotein gp120 has been shown to promote the rapid release of glutamate from cultured astrocytes (Vesce et al., 1997). These findings raise the possibility that LPS might release glutamate from non-neuronal cells in the cortical slices. In the present study, we will determine if LPS-evoked releases of glutamate, [^{3}H]NA and adenosine are Ca^{2+} dependent. We will also see if propagated action potentials are involved in these release processes. Finally, we will use the non-selective

glutamate transporter inhibitor, tPDC to see if LPS-evoked release of glutamate is mediated through the reversal of glutamate transporters.

3. Do prostanoids mediate LPS-evoked releases of glutamate, [^{3}H]NA and adenosine? Bezzi et al. (1998) showed that co-activation of both non-NMDA and metabotropic glutamate receptors promotes the release of glutamate both from cultured astrocytes and from cortical and hippocampal slices through a mechanism that involves prostaglandins (Bezzi et al., 1998). It has been shown that LPS triggers the release of PGE_2 in macrophages (Balboa et al., 1999). This raised the possibility that LPS might promote the rapid formation of PGE_2, which in turn triggers the release of glutamate from astrocytes in brain slices. We will determine the effects of various COX inhibitors, lipoxygenase inhibitors as well as phospholipase A_2 inhibitors on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices.

4. Do cytokines play a role in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine? Inflammatory cytokines are released when glial cells are activated in the brain. TNF-α and IL-1β have been shown to exacerbate excitotoxicity in the brain (Mattson and Mark, 1996, Feuerstein et al., 1998). We will determine whether TNF-α and IL-1β, or a combination of the two, releases glutamate, [^{3}H]NA and adenosine from cortical slices. If they do, we will examine the effects of specific antibodies against these cytokines on LPS-evoked releases.

5. Are NO and oxygen free radicals involved in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine? Both NO and oxygen free radicals have been implicated in excitotoxicity in the brain (Ciani et al., 1996). We will examine the effects of the non-selective NOS inhibitor L-NAME, as well as selective inhibitors of individual NOS isoforms on LPS-evoked releases of glutamate, NA and adenosine from cortical slices. The effects of oxygen free radical scavengers superoxide oxidase (SOD) and catalase, and the antioxidants t-α-tocopherol and Trolox will also be examined.

6. Do other immunogens release glutamate, NA and adenosine from cortical slices? The fact that LPS evokes very rapid releases of the neurotransmitters glutamate and NA, and of the neuromodulator adenosine raises the possibility that similar responses may occur with other immunogens such as the HIV glycoproteins gp120 and gp41, which are specifically associated with AIDS dementia. We will investigate if different concentrations of gp120 or gp41 release glutamate, NA and adenosine from cortical slices.

CHAPTER 2

GENERAL METHODS

Materials

Animals. Male Sprague-Dawley rats weighing 250-350 g were obtained from Charles River Canada Ltd., St. Constant, Quebec, Canada. They were housed in groups of 2 per cage and maintained on a 12 hour light/12 hour dark cycle at room temperature in the Animal Care Facility of the Sir Charles Tupper Medical Building, Dalhousie University. Rats were housed for a minimum of 5 days prior to use and were fed standard chow and tap water ad libitum.

Drugs and Chemicals. Adenosine, N-Methyl-D-Aspartate (NMDA), lipopolysaccharide (LPS, endotoxin, from Escherichia coli, serotype 0127:B8), 1-octanesulfonic acid, glutamate, superoxide dismutase (SOD), catalase, d-α-tocopherol, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), indomethacin, nordihydroguaiaretic acid (NDGA), p-bromophenacylbromide (p-BPB) and lithium chloride (LiCl) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA), 3-[1-(3-dimethylamino-propyl)-indol-3-yl]-3-(indol-3-yl)-maleimide (GF109,203X, GFX), N^G-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole (7-NI), resveratrol, NS-398, dizocilpine maleate (MK-801), D(-)-2-amino-5-phosphonopentanoic acid (APV), and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX) were obtained from Research Biochemicals Inc. (Natick, MA, USA). N^6-(1-iminoethyl)-L-lysine (L-NIL) and N^5-(1-iminoethyl)-L-ornithine (L-NIO) were purchased from Tocris Cookson Inc. (Ballwin, MO, USA). IL-1β, IL-6, and TNF-α were purchased from Reprotech Inc. (Rocky Hill, NJ, USA). HIV-1_{IIIB} gp120 and HIV-1_{HXB2} gp41 recombinant viral proteins

were purchased from Advanced Biotechnologies Inc. (Columbia, Maryland, USA). Chloroacetaldehyde was obtained from ICN (Plainview, NY, USA). L-[7-^{3}H] noradrenaline, Solvable™, and Ecolite™ were obtained from Du Pont-New England Canada Inc. (Markham, Ontario, Canada). Acetonitrile was purchased from BDH (Dartmouth, Nova Scotia, Canada). All other chemicals were obtained from commercial sources. MK-801, indomethacin, p-BPB and d-α-tocopherol were initially dissolved in ethanol and then diluted 1,000 folds in Krebs-Henseleit medium. PMA, GFX, 7-NI and NS-398 were initially dissolved in dimethyl sulfoxide (DMSO) and then diluted 100 folds in Krebs-Henseleit medium. All other drugs were dissolved in Krebs-Henseleit medium. Krebs-Henseleit medium was freshly prepared from stock solutions that were made in H_2O that had been distilled and then passed through a Millipak 40 sterile purification system.

In experiments involving indomethacin, p-BPB, d-α-tocopherol, PMA, GFX, or NS-398, controls were superfused with an identical concentration of their respective solvents.

Methods

Preparation of Cortical Slices. All procedures involving animals were approved by the Dalhousie University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. Adult male Sprague Dawley rats were killed by decapitation in a guillotine and their brains rapidly excised into ice cold Krebs-Henseleit bicarbonate medium containing (mM) NaCl, 111; $NaHCO_3$, 26.2; NaH_2PO_4, 1.2; KCl, 4.7; $CaCl_2$, 1.8; $MgCl_2$, 1.2; and glucose, 11, gassed with 95% O_2-5% CO_2 to maintain a

pH of 7.4. An ethanol-cleaned razor blade was used to dissect the whole brain essentially as described previously (Hoehn and White, 1990a). The lateral 1 to 1.5-mm portion of parietal cortex was removed from both hemispheres of the brain with a recessed tissue slicer. Slices (0.4mm) of parietal cortex were prepared on a McIlwain tissue chopper and rapidly placed into ice-cold Krebs-Henseleit bicarbonate medium. Adjacent slices were placed alternately into each of two tissue baths so that each bath contained six slices from both sides of the brain, weighing a total of about 80 mg.

Superfusion of Cortical Slices. The superfusing baths were constructed from modified 3 ml syringes as described before (Hoehn and White, 1990a). The internal environment of each tissue bath was contained by two rubber syringe plungers which had their tip surfaces leveled, and their internal volumes were adjusted to 0.5 ml by plunger movement. Sixteen-Gauge size needles, from which both the sharp tips and plastic ends had been removed and these ends smoothed by filing, were used to puncture through the center of the rubber plungers. Tygon® tubing (wall diameter of 1/32 in.; internal diameter of 1/32 in. for the inlet and outlet, Fischer Scientific Company, Pittsburg, PA, USA) was attached to these needles thereby making up the superfusion fluid inlet and outlet lines. The needles and tubing were sealed and secured to the rubber plungers with Tube and Tile Silicon Sealant (Canadian Tire Corp. Ltd., Toronto, Ont.). The slices rested on nylon mesh screens (plain square 425 μm, B&S.H. Thompson Co. Ltd., St. Laurent, Que.) so as to completely cover their surfaces. These screens were supported 2 mm above the bottom surface of the tissue baths by cylindrical platforms constructed from 1 ml plastic Finnpipette® syringe tips (Fisher Scientific Company, Pittsburg, PA, USA).

Tissue baths were immersed in a 36°C circulating water bath and superfused using a Minipulse 2 peristaltic pump. The pump utilized PVC Manifold Tubing (Gilson Medical Electronics Inc., Middleton, WI, USA) with an internal diameter of 0.76 mm and was joined to superfusion tubing using tubing connectors (Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Three-way stopcock permitted switching between different superfusion solutions. Two baths were run in parallel and were assigned in alternate experiments to either "test" or "control" observations. After an initial 5-min superfusion period, slices were labeled with [^{3}H]NA by superfusion for 10 min with oxygenated Krebs-Henseleit bicarbonate medium containing freshly-prepared 10^{-7} M [^{3}H]NA at 36°C. Superfusion was continued with Krebs-Henseleit bicarbonate medium for a further 90 min before collection of 10 (for the study of NMDA-evoked releases) or 24 (for the study of LPS-evoked releases) serial 2.5-min fractions. After collection of 3 or 4 samples to determine basal release, the superfusing medium was switched to medium containing drugs (either NMDA or LPS) for 10 min unless specified, after which the superfusing buffer was switched back to normal Krebs-Henseleit buffer and the collection continued until the end of the experiment. For studies with two identical periods of stimulation by LPS, slices were first exposed to LPS for an initial 5 min after the basal collection of samples, the slices were allowed to recover in normal Krebs-Henseleit buffer for 30 min, and the second stimulation by LPS was delivered for another 5 min. Sample collection continued for 10 min after the second stimulation. To study the effects of drugs on NMDA- or LPS-evoked releases, drugs were introduced into the superfusing buffer 20 min (unless specified) prior to NMDA or LPS stimulation.

After collection of all fractions, the slices were carefully removed from the tissue baths for determination of wet tissue weight (approximately 80-100 mg) and then solubilized in 1 ml of Solvable™ to determine tissue [^{3}H]NA content.

Detection of Adenosine Release. The adenosine content in the superfusate was determined by the method of Wojcik and Neff (1983) with modification, essentially as described previously (MacDonald and White, 1985; Craig and White, 1992). Immediately following collection, samples (0.5 ml) of superfusate were deproteinated with 250 µl of 0.15 M $Ba(OH)_2$ and 250 µl of 0.15 M $ZnSO_4$, mixed and centrifuged at 11,600 g for 4 min in a benchtop microcentrifuge (Beckman II microcentrifuge). The supernatants (600 µl) were carefully removed and added to 75 µl of 4.5% chloroacetaldehyde in 1.5 ml microcentrifuge tubes, which were then tightly sealed and boiled for 20 min to form 1-N^6-ethenoadenosine. Samples were allowed to cool to room temperature and then stored overnight at 4°C prior to assay (stable for > 1 week, Wojcik and Neff, 1983). Adenosine standards were prepared from stock solutions made in Millipore water and stored at −15°C. These were thawed daily and diluted with appropriate Krebs-Henseleit medium with or without drugs. Standards were processed simultaneously and identically to the superfusate fractions.

The fluorescent product of adenosine, 1-N^6-ethenoadenosine, was assayed using high-performance liquid chromatography (HPLC) with fluorescence detection (Craig and White, 1992). Aliquots (100 µl) of the derivatized samples were injected through a Waters 712 WISP Autosampler into a pump solvent delivery system (Waters model 501) attached to a Waters 4 µm C_{18} reversed-phase radial-pak cartridge (Nova-Pak C_{18}, 5 mm

I.D.) contained in a compressible Radial-Pak RCM 8 × 10 cartridge holder (Waters model 80100). The mobile phase consisted of 50 mM acetate buffer (pH 4.5), 2.2 mM 1-octanesulfonic acid and 18% acetonitrile, which were run at a flow rate of 0.7 ml/min. The ethanol-adenosine peaks were detected with a fluorescence detector (Waters model 420 AC with excitation at a wavelength of 290 nm and a long pass emission filter at 420 nm) and were quantitated by direct comparison of peak heights to those of known concentrations of adenosine standards. Standards exhibited a strict linear relationship with respect to the concentration of adenosine to a maximum of 250 pmol per 100 µl. adenosine retention time was approximately 3 min and detection limit was 1-2 pmol per 100 µl injection.

Adenosine release for each fraction was expressed as pmol/min/g of cortex, to give the rate of release. "Evoked adenosine release" (above basal) was obtained by subtracting the basal rate of release immediately preceding exposure to the releasing agent from every other sample. "Total evoked adenosine release" was the cumulative amount of the evoked adenosine release during the entire period of each exposure to the releasing agent and was expressed as nmol/g of cortex.

Determination of [^{3}H]NA Release. After removal of 0.5 ml of the superfusate for determination of adenosine, 0.5 ml of sample was placed into scintillation vials containing 10 ml of Ecolite™ and the dpm of [^{3}H]NA released was determined with a Beckman model LS5801 scintillation counter. The slices were weighed and then solubilized in 1 ml of Solvable™. Tissue [^{3}H]NA contents were determined by scintillation spectrometry in 14 ml of Ecolite™. The rate of [^{3}H]NA release was

standardized as the percent of tissue content at the beginning of the fraction collection period and was expressed as percent of content per min. "Evoked [^{3}H]NA release" was obtained by subtracting the basal rate of release in the sample immediately preceding exposure to the releasing agent from every other sample and was expressed as percent of content per min. "Total evoked [^{3}H]NA release" was determined as the percent of [^{3}H]NA content released during the entire period of exposure to the releasing agent (Hoehn et al., 1990).

Detection of Glutamate Release. Glutamate content in the superfusing medium was measured by a specific fluorescence assay (excitation and emission at 335 and 430 nm, respectively) performed in a Perkin-Elmer fluorescence spectrophotometer (Nicholls and Sihra, 1986). Superfusates (0.5 ml) of each fraction were transferred to a 1.5 ml microcentrifuge, boiled for 10 min and frozen at –20°C for overnight before assayed. At the time of the assay, samples were thawed at room temperature, centrifuged for 5 min and 0.25 ml of supernatant were transferred to a reaction mixture containing glutamate dehydrogenase (GDH, 40 U/ml) and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$, 1 mM) in HEPES buffer (in mM: NaCl 123; KCl 4.7; NaH_2PO_4 1.2; $CaCl_2$ 1.8; $MgCl_2$ 1.2, glucose 11; and HEPES 10, pH 7.4). Glutamate oxidation by GDH to α-ketoglutarate led to the formation of NADPH with fluorescence emission, which can be measured by the specific fluorescence assay (Nicholls and Sihra, 1986).

Rate of glutamate release was calculated by referring to standard curves constructed for various concentration of glutamate, which were treated identically with the samples (in the linear range 0.25 – 4.0 nM), and expressed as nmol/g/min. "Evoked

glutamate release" (above basal) was obtained by subtracting the basal rate of release immediately preceding exposure to the releasing agent from every other sample. "Total evoked glutamate release" was the cumulative amount of the evoked glutamate release during the entire period of each exposure to the releasing agent and was expressed as μmol/g of cortex.

Statistical Analysis. Paired Student's *t* tests were conducted on the total glutamate, adenosine and [^{3}H]NA releases from the two treatment groups.

CHAPTER 3

MODULATION OF NMDA-EVOKED RELEASES OF ADENOSINE AND [^{3}H]NA BY PROTEIN KINASE C AND LITHIUM IN RAT PARIETAL CORTICAL SLICES

ABSTRACT

The roles of protein kinase C (PKC) and lithium (Li^+) in NMDA-evoked releases of adenosine and [^{3}H]noradrenaline (NA) from slices of rat parietal cortex were studied. In the absence of Mg^{2+}, the PKC activator phorbol 12-myristate 13-acetate (1.0 μM PMA) did not release either adenosine or [^{3}H]NA but it potentiated the release of adenosine evoked by 20 μM NMDA and the release of [^{3}H]NA evoked by 100 μM NMDA. The potentiating effects of PMA on NMDA-evoked releases of adenosine and [^{3}H]NA were reversed by the PKC inhibitor GF109,203X (1.0 μM GFX). GFX by itself had no effect on NMDA-evoked release of either adenosine or [^{3}H]NA, suggesting that endogenous PKC does not play a role in NMDA-evoked releases. Li^+ (10 mM) by itself did not release either adenosine or [^{3}H]NA from rat cortical slices. However, at concentrations of 1.5 mM or 10 mM, it potentiated 300 μM NMDA-evoked [^{3}H]NA release from rat cortical slices. Unlike PMA, various concentrations of Li^+ had no effect on NMDA-evoked adenosine release. In the presence of Mg^{2+}, NMDA-evoked release of [^{3}H]NA was essentially abolished, whereas adenosine release persisted. Under these conditions, PMA or Li^+ did not permit NMDA-evoked release of [^{3}H]NA to occur, nor did they increase NMDA-evoked adenosine release. Taken together, these results indicate that PKC does not play an essential role in NMDA-evoked release of either adenosine or NA. However, activation of PKC potentiates the release of adenosine and NA evoked by submaximal concentrations of NMDA, but not by relieving the voltage-sensitive Mg^{2+} block of NMDA receptors. In addition, the potentiating effect of Li^+ on NMDA-evoked [^{3}H]NA release was not related to PKC activation, since GFX did not reverse the effect of Li^+. In conclusion, activation of PKC will have the effect of

increasing the inhibitory threshold provided by released adenosine when only a few NMDA receptors are activated, and of promoting and accelerating excitatory responses when most of the available NMDA receptors become activated. The observation that Li^+ potentiates NMDA-evoked [^{3}H]NA release may have relevance to the toxic action of Li^+ in the treatment of biopolar depression.

INTRODUCTION

Glutamate and aspartate are the major excitatory neurotransmitters in the CNS, and activation of EAA receptors releases both adenosine and NA from rat cortical slices (Hoehn *et al.*, 1990; Craig and White, 1991; see Chapter 1 for details). Moreover, the actions of EAA receptor agonists are subject to modulation by second messengers (see Chapter 1).

Protein kinase C is a Ca^{2+}- and phospholipid-dependent enzyme highly concentrated in the brain (Nishizuka, 1986). Its activity is very important in mediating neurotransmitter release and synaptic plasticity (Hollingsworth *et al.*, 1985; Harvey and Collingridge, 1993; Ohtani *et al.*, 1995). The facilitatory effects of metabotropic glutamate receptors on NMDA-evoked responses are mediated by the activation of PKC (Aniksztejn *et al.*, 1992; Kelso *et al.*, 1992). PKC activation by phorbol esters has also been shown to enhance NMDA currents in various systems including rat trigeminal neurons (Chen and Huang, 1991) and oocytes expressing total rat brain mRNA (Kelso *et al.*, 1992). In addition, NMDA-evoked adenosine release from rat cortical slices is potentiated when M_3 muscarinic receptors are activated (Semba and White, 1997). M_3 muscarinic receptors are G protein coupled receptors that activate PKC. This raises the possibility that the potentiating effect of M_3 agonists on NMDA-evoked adenosine release might be mediated by the activation of PKC. The present study was undertaken to investigate the possible role of PKC in NMDA-evoked adenosine and NA release from rat cortical slices, and whether PKC activation affects NMDA-evoked adenosine and NA release in the cortex.

Li^+ is one of the most clinically efficacious drugs used to treat bipolar depression. Although its exact mechanism of action is still not clear, several hypotheses have been advanced (Lenox et al., 1998). The most well known hypothesis has been the inositol depletion theory (Berridge et al., 1982; Berridge and Irvine, 1989). This is based on the observation that Li^+ inhibits inositol monophosphatases and inositol polyphosphate 1-phosphatase activities, resulting in the accumulation of certain inositol phosphates and the ultimate reduction of IP_3 in the brain (Berridge, 1989). Recent studies have shown that therapeutic concentrations of Li^+ acutely increase the intracellular levels of IP_3 through the activation of NMDA receptors in monkey and mouse cerebral cortical slices (Dixon and Hokin, 1994; Dixon et al., 1992, 1994). Other studies have shown that Li^+ augments *fos* protooncogene expression mediated by activation of PKC coupled receptors in rat brain and PC12 cells (Divish et al., 1991; Krivanek, 1993), suggesting a role for PKC in mediating Li^+'s effects.

Since Li^+ is able to modulate the activity of PKC (Krivanek, 1993; Manji et al., 1993; Watson and Lenox, 1996), it seemed possible that Li^+ might have an effect on NMDA-evoked releases of adenosine and [^{3}H]NA from rat cortical slices. Therefore, we examined the possibility that Li^+ might potentiate NMDA-evoked adenosine or [^{3}H]NA release from rat cortical slices, and if so, whether PKC activation plays a role.

MATERIALS AND METHODS

Preparation and superfusion of rat brain cortical slices were performed as described in Chapter 2. After collection of three samples to determine basal release, the superfusing medium was switched for 10 min to medium containing NMDA or a mixture of NMDA and drugs, after which the superfusing buffer was switched back to Krebs-Henseleit buffer for the final three fractions. In experiments conducted in "0 Mg^{2+}", slices were superfused for 65 min before sample collection with medium from which $MgCl_2$ has been omitted.

RESULTS

Effects of PKC inhibition with GFX on NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. GFX is a cell-permeable protein kinase inhibitor that is structurally similar to staurosporin and inhibits PKC by acting as a competitive inhibitor of the ATP-binding site of PKC. GFX is a highly selective and potent inhibitor of PKC compared with staurosporin (Toullec *et al.*, 1991; Davis *et al.*, 1992).

By itself, GFX (1.0 μM) did not affect NMDA-evoked adenosine release (Fig. 3-1A), nor did it have any effect on NMDA-evoked [^{3}H]NA release (Fig. 3-1B). These results indicate that endogenous PKC activity does not mediate the NMDA-evoked release of either adenosine or [^{3}H]NA.

Effects of PKC activation with PMA on NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. PMA (1.0 μM) by itself did not release either adenosine or [^{3}H]NA (Fig. 3-2), indicating that activation of PKC by itself does not

promote the release of either adenosine or [^{3}H]NA from rat cortical slices. However, in the absence of Mg^{2+}, pretreatment of cortical slices with 1.0 μM PMA increased 2-fold the maximum rate of adenosine release evoked by 20 μM NMDA (Fig. 3-3A). The total amount of NMDA-evoked adenosine release was also increased 2-fold by PMA (Fig. 3-3A). This low concentration of NMDA (20 μM) did not release [^{3}H]NA (Fig. 3-3B).

At 50 μM NMDA, PMA no longer had a potentiating effect on NMDA-evoked adenosine release (Fig. 3-4A), indicating that PMA was only effective at submaximal concentrations of NMDA. From the same cortical slices, 50 μM NMDA did not release [^{3}H]NA either in the presence or absence of PMA (Fig. 3-4B).

When NMDA concentration was increased to 100 μM, which is supra-maximal for adenosine release while sub-maximal for [^{3}H]NA release (Hoehn and White, 1990c), PMA had no effect on NMDA-evoked adenosine release (Fig. 3-5A), similar to the results obtained with 50 μM NMDA. However, the evoked release of [^{3}H]NA was enhanced by 1.0 μM PMA at this NMDA concentration. The maximum rates of [^{3}H]NA release were increased 2.5-fold by 1.0 μM PMA (Fig. 3-5B). The total amount of [^{3}H]NA released was increased 1.9-fold (Fig. 3-5B).

The potentiation of NMDA-evoked [^{3}H]NA release by PMA was only present at sub-maximal concentrations of NMDA (i.e., 100 μM), and disappeared when the NMDA concentration was increased to 1 mM (Fig. 3-6B).

Fig. 3-1. Effect of PKC inhibition with 1.0 μM GFX on 100 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course of NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA. GFX was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***GFX by itself did not affect NMDA-evoked adenosine or [^{3}H]NA release.***

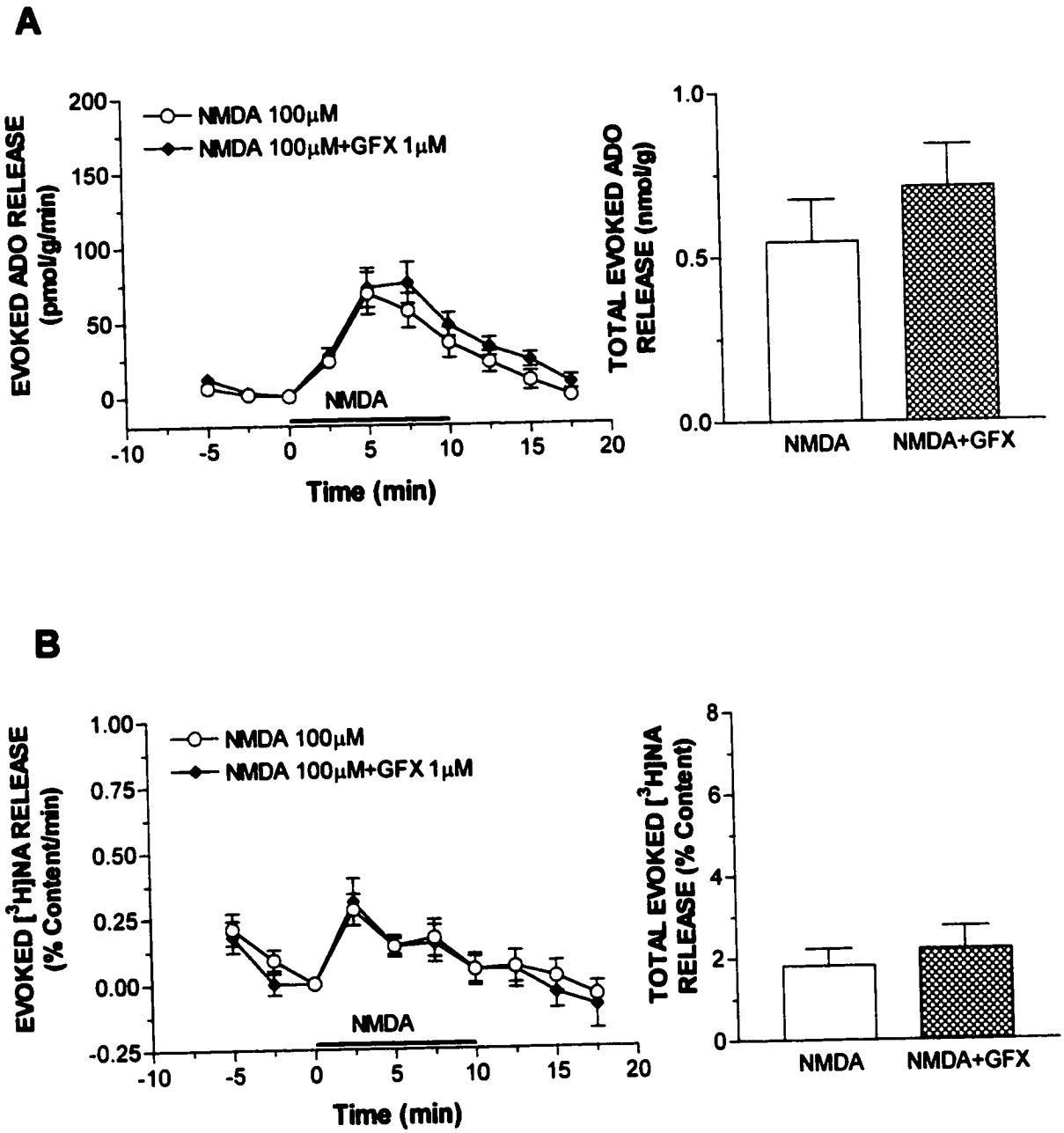

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 100μM
NMDA 100μM+GFX 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+GFX
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 100μM
NMDA 100μM+GFX 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+GFX

Fig. 3-1

Fig. 3-2. Effect of PKC activation with 1.0 μM PMA on basal adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA and NMDA were present from 0 to 10 min. Values are mean ± S.E.M. from 3 experiments. ***PMA itself does not release adenosine or [^{3}H]NA from rat cortical slices.***

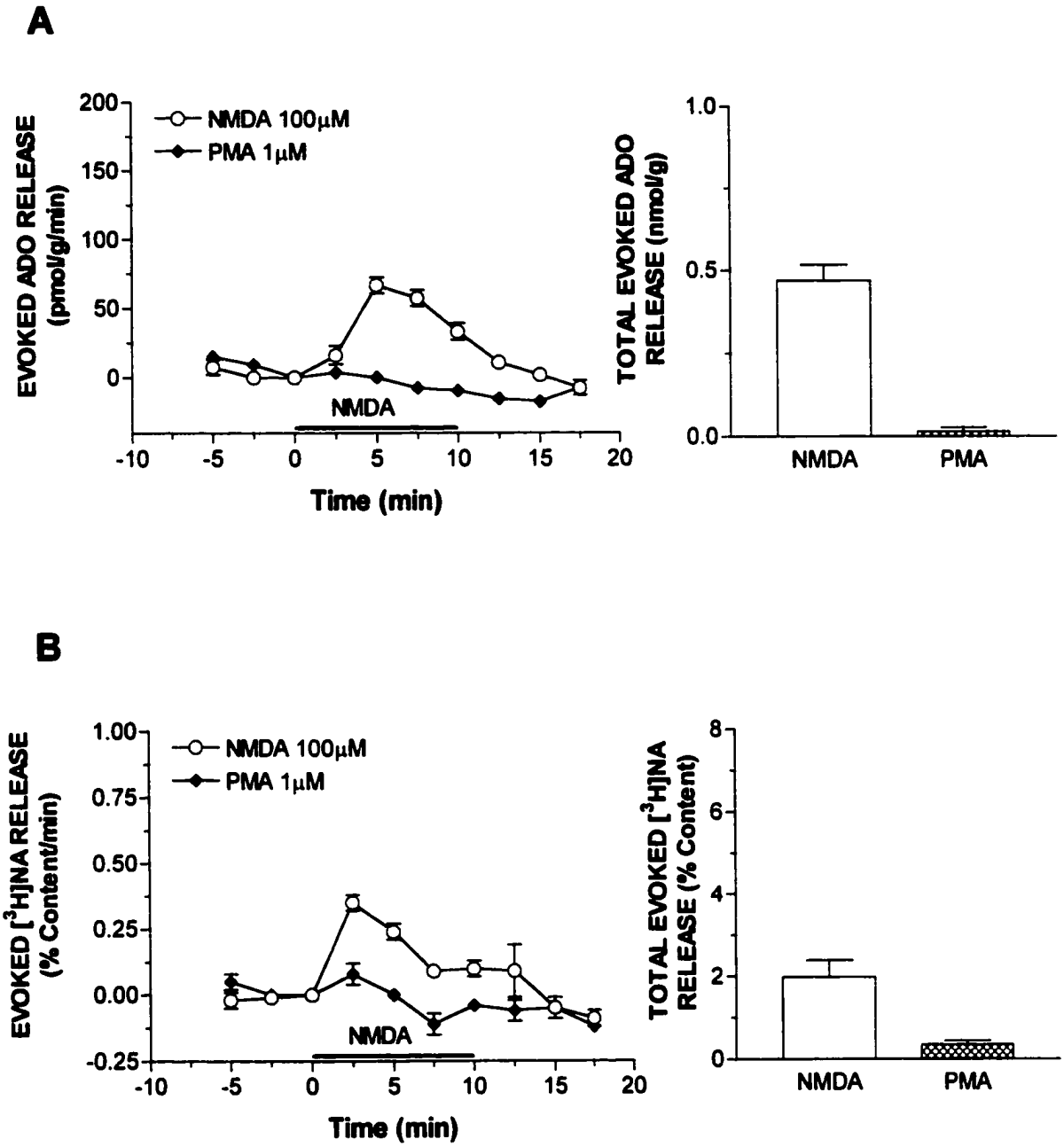

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 100μM
PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 100μM
PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
PMA

Fig. 3-2

Fig. 3-3. Effect of PKC activation with 1.0 μM PMA on 20 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 5 experiments. ***Activation of PKC with 1 μM PMA significantly potentiated 20 μM NMDA-evoked ADO release from rat cortical slices in the absence of extracellular Mg^{2+}.*** * Significantly different from control ($P < .05$, paired *t* test).

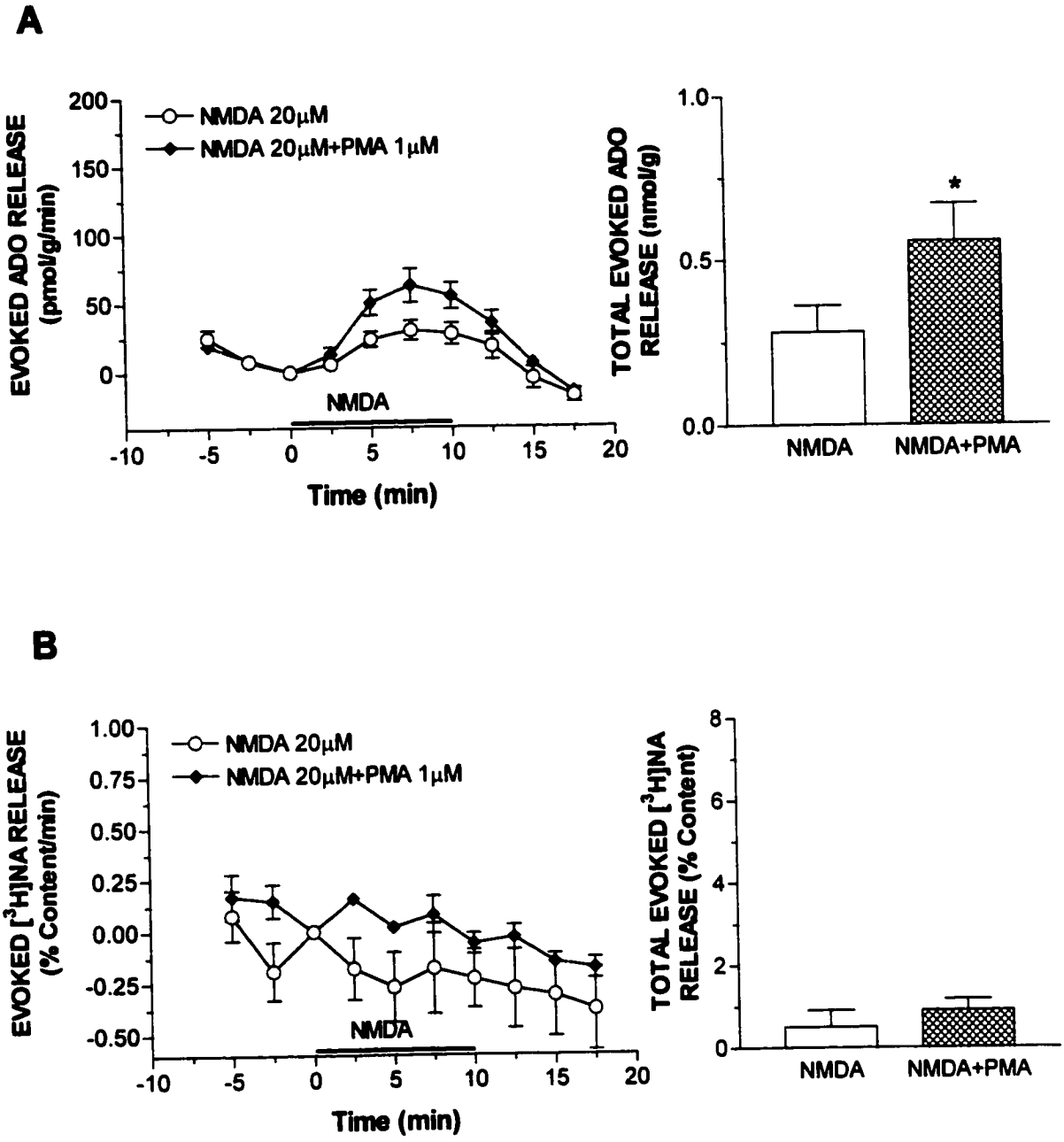

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 20μM
NMDA 20μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 20μM
NMDA 20μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+PMA

Fig. 3-3

Effects of GFX on PMA potentiated release of [^{3}H]NA evoked by NMDA in the absence of Mg^{2+}. We tested whether the potentiating effect of PMA on NMDA-evoked adenosine and [^{3}H]NA release could be blocked by the specific PKC inhibitor GFX. Co-administration of GFX with PMA had no effect on 100 μM NMDA-evoked adenosine release (Fig. 3-7A). This is consistent with the observation that PMA had no effect on adenosine release evoked by maximal concentrations of NMDA. However, the facilitatory effect of PMA on 100 μM NMDA-evoked release of [^{3}H]NA was reversed by co-administration with 1.0 μM GFX (Fig. 3-7B). Similarly, the potentiating effect of PMA on 20 μM NMDA-evoked release of adenosine was also diminished by GFX (Fig. 3-8A). Taken together, these results suggest that the potentiating effects of PMA on submaximal concentrations of NMDA-evoked releases of adenosine and [^{3}H]NA are through the specific activation of PKC.

Effects of PKC activation with PMA on NMDA-evoked adenosine and [^{3}H]NA release in the presence of Mg^{2+}. Previously, it has been shown that activation of PKC removes the Mg^{2+} block of the NMDA channel, thus permitting NMDA activation to proceed (Chen and Huang, 1992). Therefore, we tested if activation of PKC could remove the Mg^{2+} block of NMDA receptors to allow [^{3}H]NA release to occur. In agreement with a previous report (Hoehn *et al.*, 1990), NMDA–evoked [^{3}H]NA release was essentially abolished whereas adenosine release persisted in the presence of 1.2 mM Mg^{2+} (Fig. 3-9). PMA did not potentiate the release of adenosine (Fig. 3-9A), nor did it permit the release of [^{3}H]NA evoked by 300 μM NMDA to occur (Fig. 3-9B). These results indicate that activation of PKC does not potentiate NMDA-evoked adenosine or [^{3}H]NA release through removal of the Mg^{2+} block of NMDA receptors.

Fig. 3-4. Effect of PKC activation with 1.0 μM PMA on 50 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 5 experiments. ***Activation of PKC with 1 μM PMA did not potentiate 50 μM NMDA (which is supramaximal for evoking adenosine release) evoked adenosine release from rat cortical slices.***

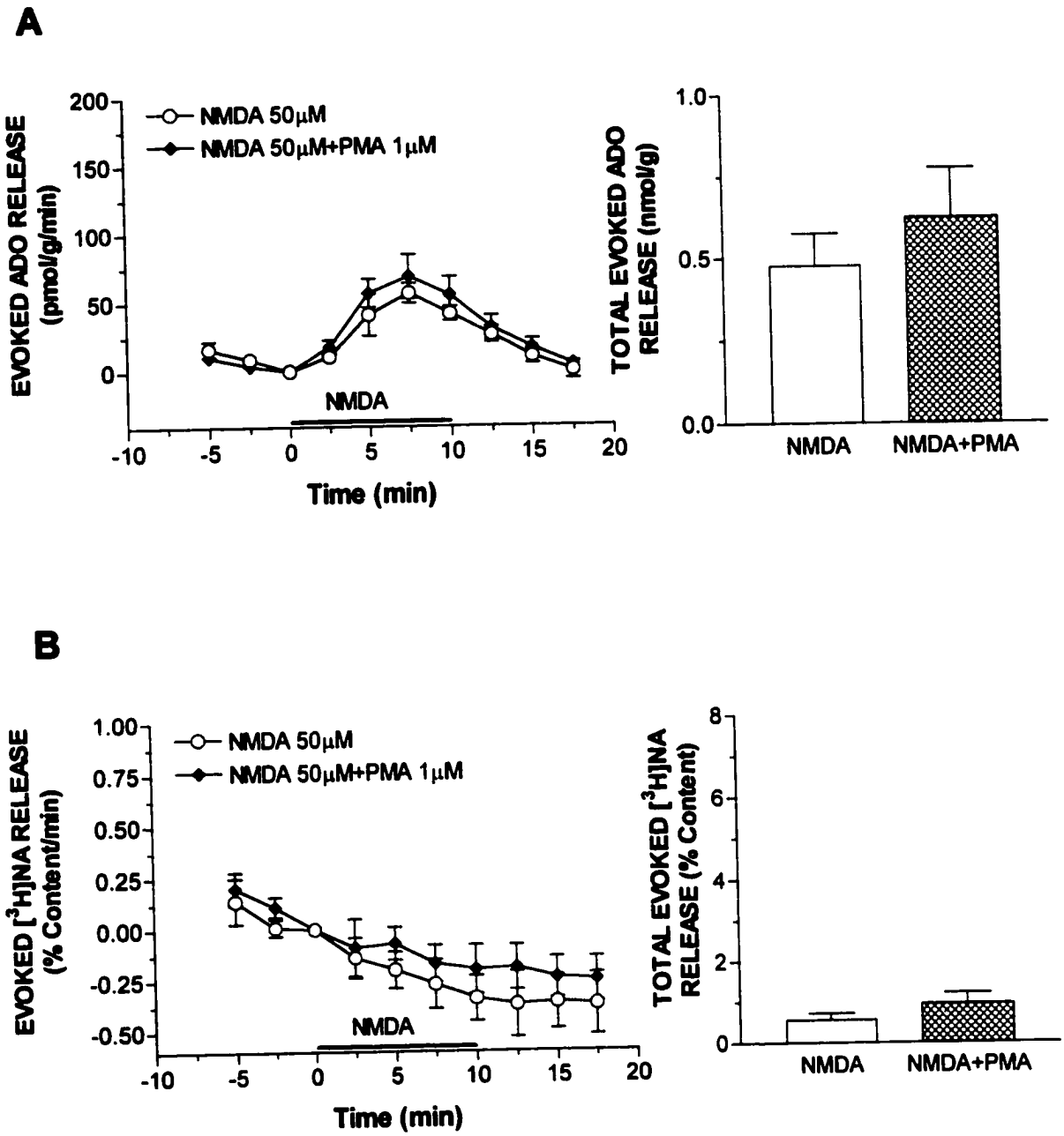

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 50μM
NMDA 50μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 50μM
NMDA 50μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+PMA

Fig. 3-4

Fig. 3-5. Effect of PKC activation with 1.0 μM PMA on 100 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 8 experiments. ***Activation of PKC with 1 μM PMA significantly potentiated 100 μM NMDA-evoked release of [^{3}H]NA from rat cortical slices, while it had no effect on adenosine release evoked by the same concentration of NMDA.*** * Significantly different from control ($P < .05$, paired *t* test).

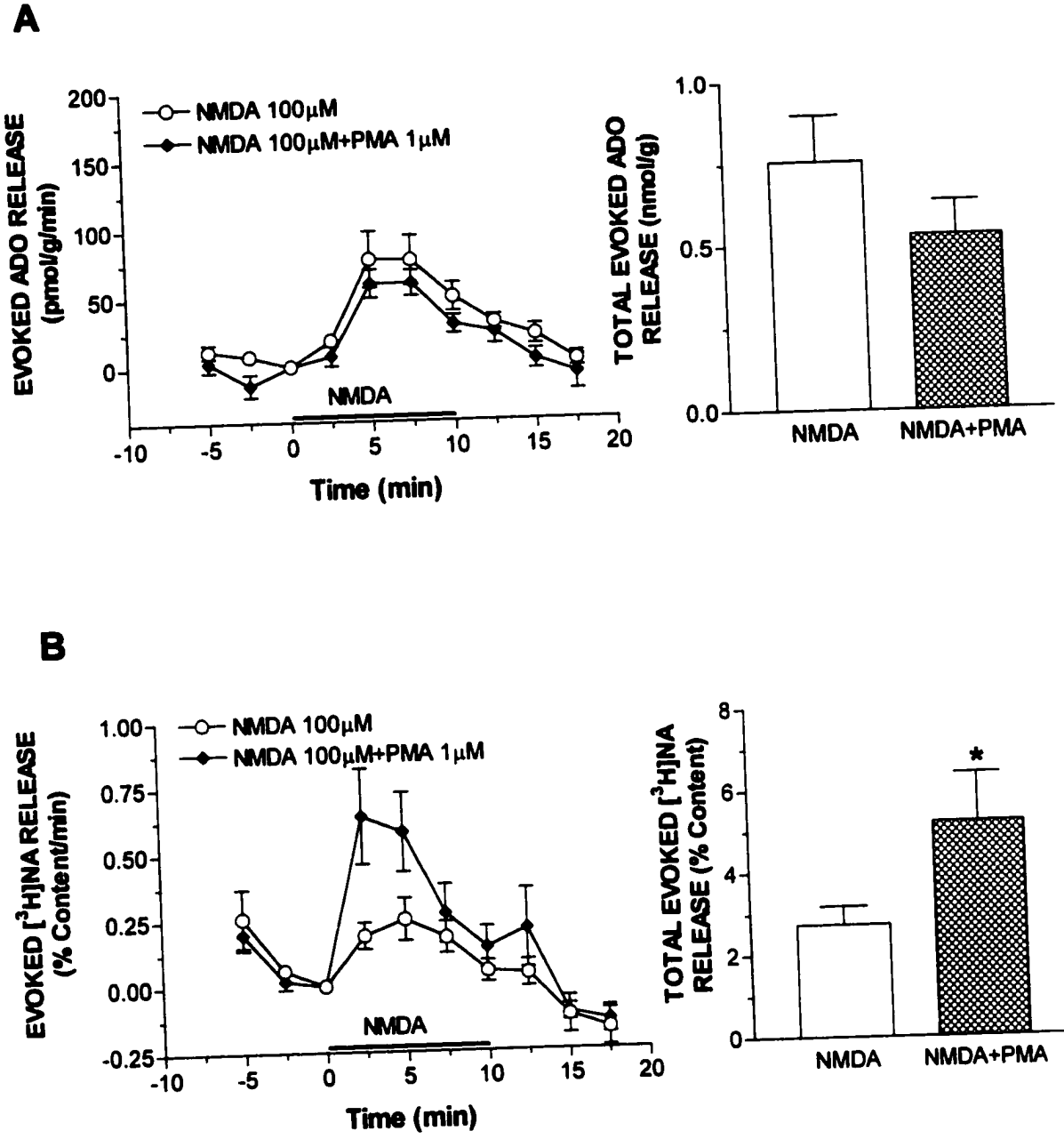

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 100μM
NMDA 100μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 100μM
NMDA 100μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
NMDA
NMDA+PMA

Fig. 3-5

Fig. 3-6. Effect of PKC activation with 1.0 μM PMA on 1 mM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 5 experiments. ***Activation of PKC with 1 μM PMA did not potentiate 1 mM NMDA evoked release of either adenosine or [^{3}H]NA from rat cortical slices in the absence of extracellular Mg^{2+}.***

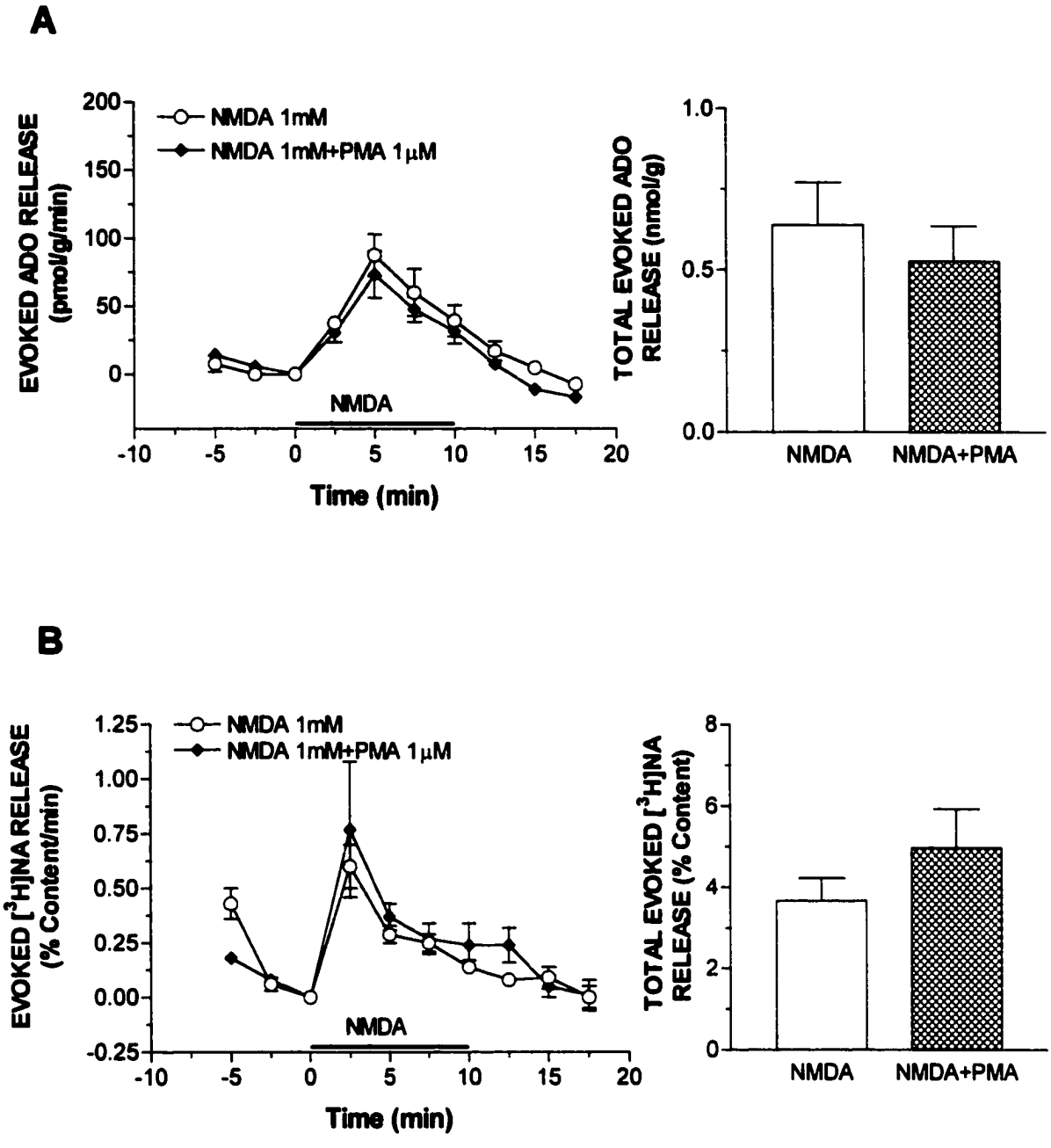

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 1mM
NMDA 1mM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 1mM
NMDA 1mM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+PMA

Fig. 3-6

Fig. 3-7. Effect of 1.0 μM GFX on PMA's effects on 100 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. GFX and PMA were present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***The potentiating effect of PMA on 100 μM NMDA-evoked [^{3}H]NA release was reversed by the PKC inhibitor GFX (1 μM).*** * Significantly different from control ($P < .05$, paired *t* test).

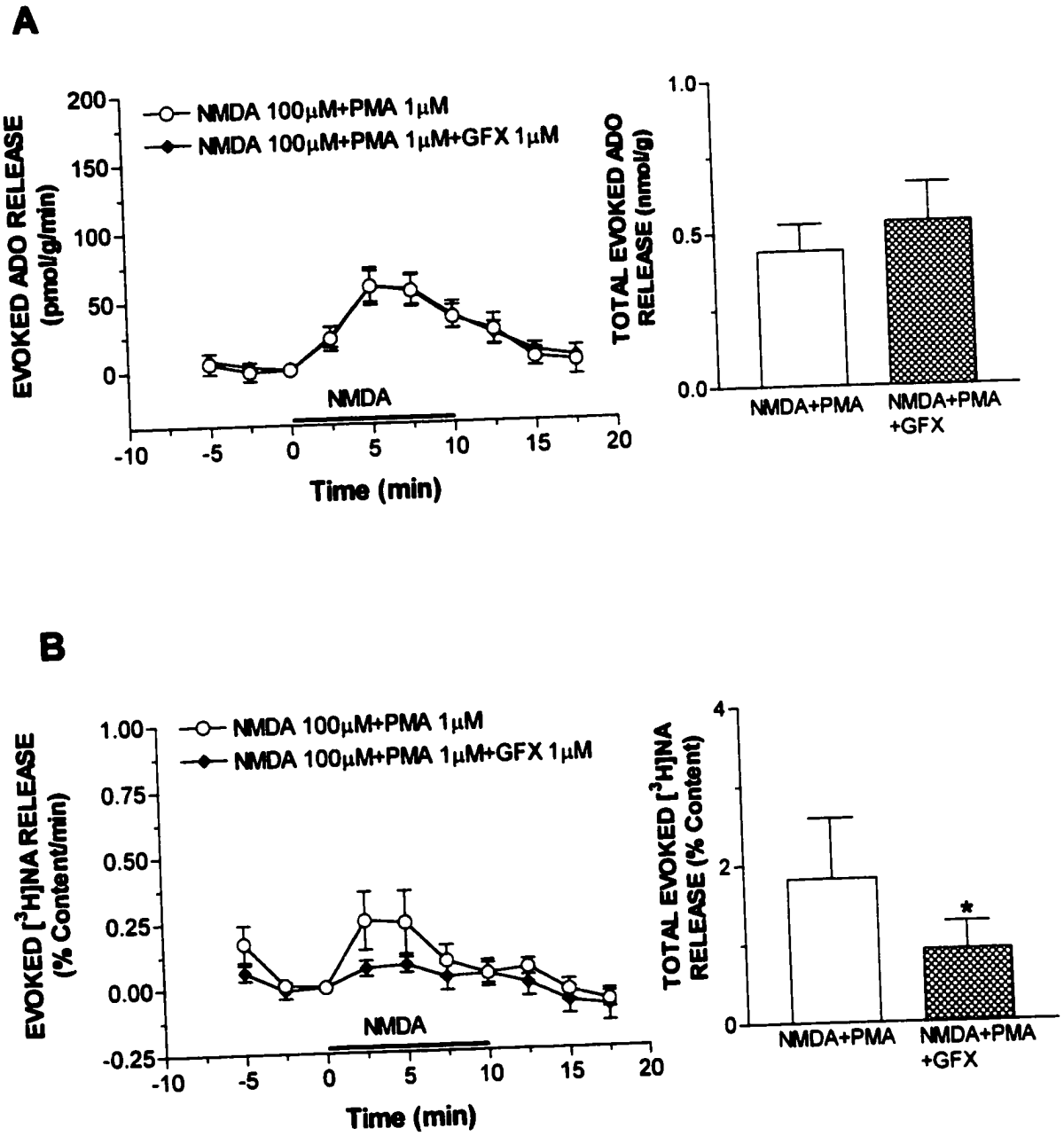

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 100μM+PMA 1μM
NMDA 100μM+PMA 1μM+GFX 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA+PMA
NMDA+PMA +GFX
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 100μM+PMA 1μM
NMDA 100μM+PMA 1μM+GFX 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA+PMA
NMDA+PMA +GFX

Fig. 3-7

Fig. 3-8. Effect of 1.0 μM GFX on PMA's effects on 20 μM NMDA-evoked adenosine (ADO) release in the absence of Mg^{2+}. Left panel shows the time course of NMDA-evoked adenosine release. Right panels represents the total amount of adenosine release evoked by NMDA. GFX and PMA were present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***The potentiating effect of PMA on 20 μM NMDA-evoked adenosine release was reversed by the PKC inhibitor GFX (1 μM).*** * Significantly different from control ($P < .05$, paired *t* test).

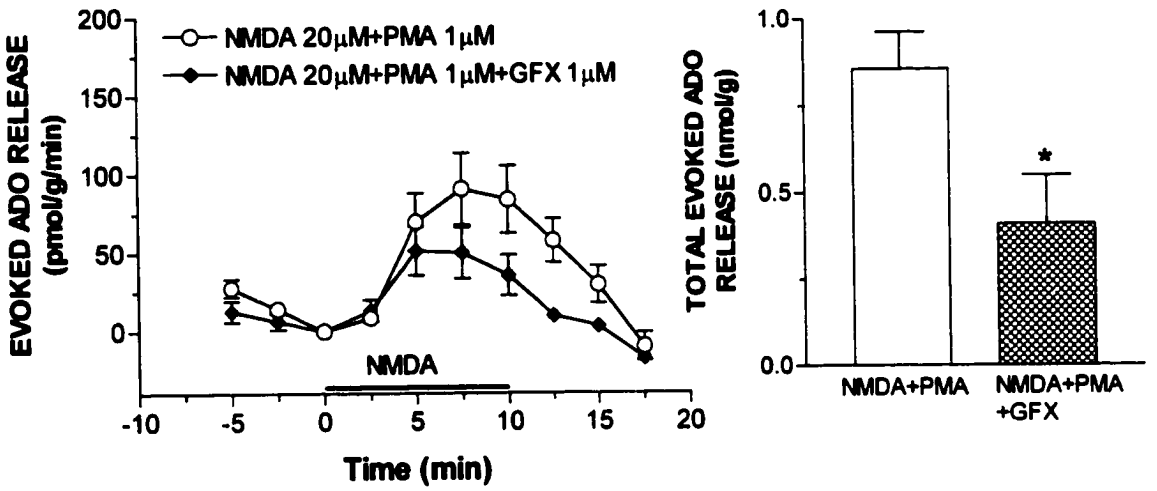

EVOKED ADO RELEASE (pmol/g/min)
200
150
100
50
0
NMDA 20μM+PMA 1μM
NMDA 20μM+PMA 1μM+GFX 1μM
NMDA
-10
-5
0
5
10
15
20
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
1.0
0.5
0.0
*
NMDA+PMA
NMDA+PMA +GFX

Fig. 3-8

Fig. 3-9. Effect of PKC activation with 1.0 μM PMA on 300 μM NMDA-evoked adenosine and [^{3}H]NA release in the presence of 1.2 mM Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. PMA was present from 15 min before exposure to NMDA until the end of the experiment. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 5 experiments. ***In the presence of extracellular Mg^{2+}, activation of PKC with PMA did not potentiate NMDA-evoked release of adenosine, nor did it allow NMDA-evoked release of [^{3}H]NA to occur.***

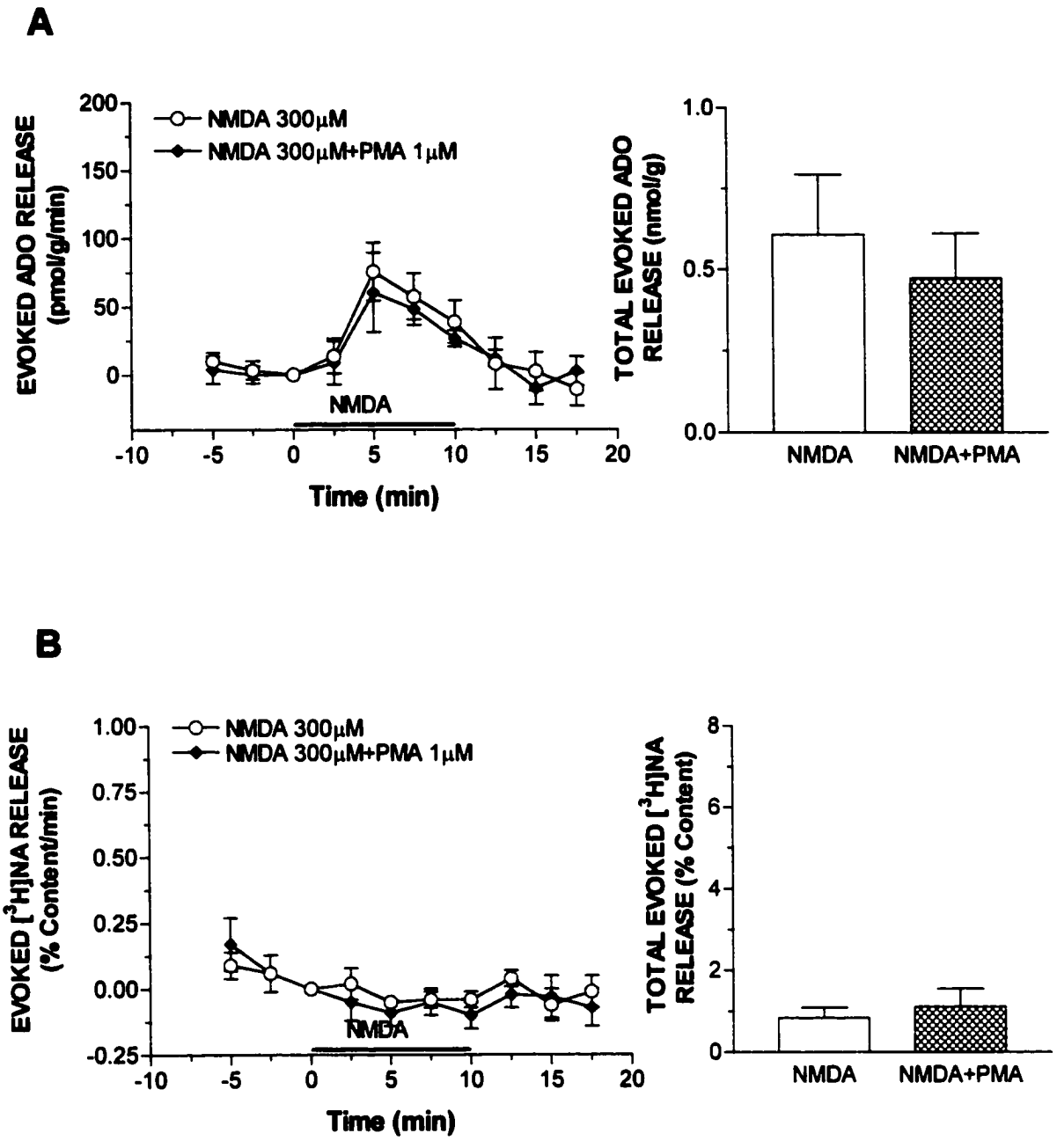

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 300μM
NMDA 300μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+PMA
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 300μM
NMDA 300μM+PMA 1μM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+PMA

Fig. 3-9

Effect of Li^+ on NMDA-evoked adenosine and [^{3}H]NA release in the absence of extracellular Mg^{2+}. By itself, 10 mM Li^+ had no effect on the basal release of either adenosine or [^{3}H]NA. However, when slices were superfused with Li^+ for 10 min before exposure to 300 μM NMDA, both total evoked release and the maximal rate of evoked release of [^{3}H]NA were enhanced about 5 fold (Fig. 3-10B). In contrast, pretreatment with Li^+ had no effect on NMDA-evoked release of adenosine (Fig. 3-10A).

Although commonly used by many investigators (Divish et el., 1991; Lee et el., 1992; Dixon and Hokin, 1994; Dixon et al., 1994), the Li^+ concentration (10 mM) employed in the above experiments was in the toxic range. To investigate whether therapeutic concentrations of Li^+ can also potentiate NMDA-evoked [^{3}H]NA release, we tested the effects of 1.5 mM Li^+ on 300 μM NMDA-evoked [^{3}H]NA and adenosine release from rat cortical slices. As shown in Figure 3-11, both the total NMDA-evoked release and the maximal rate of evoked release of [^{3}H]NA were increased significantly in the presence of 1.5 mM Li^+ (Fig. 3-11B), although to a lesser extent than with 10 mM Li^+. Similar to the effect of 10 mM Li^+, 1.5 mM Li^+ did not potentiate NMDA-evoked adenosine release (Fig. 3-11A).

Because there are spare receptors for NMDA-evoked adenosine release but not for NA release, the potency of NMDA at releasing adenosine is 33-fold greater than for releasing NA (Hoehn et al., 1990). Moreover, activation of PKC can only potentiate [^{3}H]NA and adenosine release evoked by submaximal NMDA concentrations. Since 300 μM NMDA is submaximal for evoking [^{3}H]NA release while supramaximal for evoking adenosine release, we tested the effect of 1.5 mM Li^+ on adenosine release evoked by 20 μM NMDA, which is submaximal for evoking adenosine release. As shown in Figure 3-

12A, Li^+ had no effect on adenosine release evoked by 20 μM NMDA. At this NMDA concentration, no [^{3}H]NA release was evoked either by NMDA alone or by the combination of NMDA and Li^+ (Fig. 3-12B).

Effect of Li^+ on NMDA-evoked [^{3}H]NA and adenosine release in the presence of extracellular Mg^{2+}. To test if Li^+'s potentiating effect on NMDA-evoked [^{3}H]NA release was due to removal of the Mg^{2+} block at the NMDA receptor channels, experiments were performed in the presence of physiological concentrations of extracellular Mg^{2+} (1.2 mM). In the presence of extracellular Mg^{2+}, neither 300 μM NMDA alone nor the combination of NMDA and 10 mM Li^+ released [^{3}H]NA from the slices (Fig. 3-13B). Similar to results in the absence of extracellular Mg^{2+}, Li^+ did not potentiate 300 μM NMDA-evoked release of adenosine (Fig. 3-13A). These data suggest that the effect of Li^+ on NMDA-evoked release of [^{3}H]NA was not due to the removal of the Mg^{2+} block of the NMDA receptors.

Effect of protein kinase C inhibition with GFX on Li^+'s potentiating effect on NMDA-evoked [^{3}H]NA release. It has been reported that both acute and chronic treatment with Li^+ alters PKC activity in various animal species (Krivanek, 1993; Li et al., 1993; Watson and Lenox, 1996). Therefore, we investigated the possible involvement of protein kinase C activity in mediating the potentiating effect of Li^+ on NMDA-evoked [^{3}H]NA release. As shown in Figure 3-14B, GFX (1 μM) did not block the potentiating effect of 1.5 mM Li^+ on 300 μM NMDA-evoked release of [^{3}H]NA, nor did it affect NMDA-evoked adenosine release (Fig. 3-14A). This suggests that the potentiating effect of Li^+ on NMDA-evoked NA release is not mediated by the activation of PKC.

Fig. 3-10. Effect of 10 mM Li^+ on 300 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. Li^+ was introduced to the medium 15 min before exposure to NMDA. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***Li^+ (10 mM) significantly potentiated 300 μM NMDA-evoked release of [^{3}H]NA, while it had no effect on the release of adenosine evoked by the same concentration of NMDA.*** * Significantly different from control (NMDA alone).

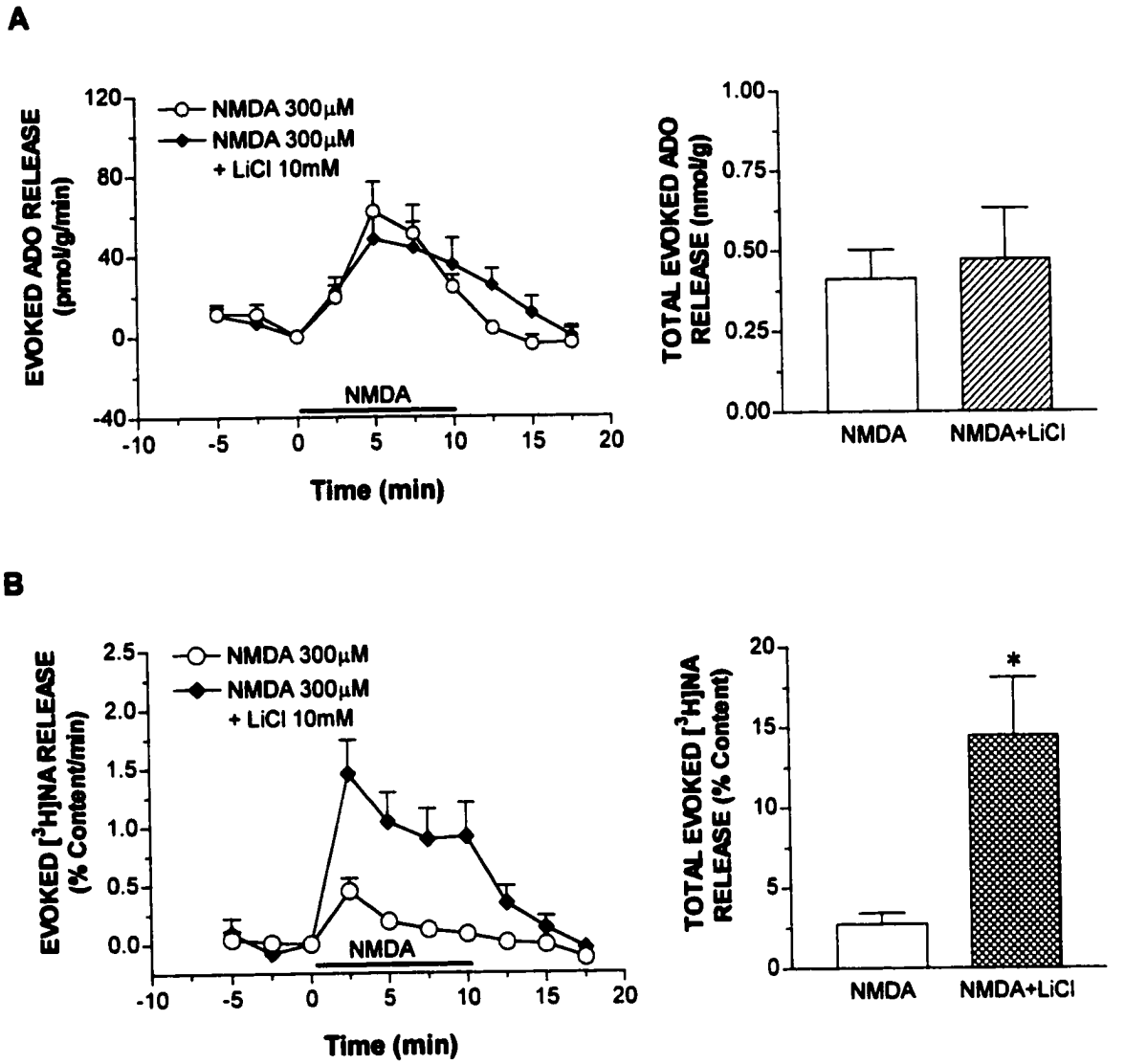

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 300μM
NMDA 300μM + LiCl 10mM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+LiCl
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 300μM
NMDA 300μM + LiCl 10mM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
NMDA
NMDA+LiCl

Fig. 3-10

Fig. 3-11. Effect of 1.5 mM Li^+ on 300 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. Li^+ was introduced to the medium 15 min before exposure to NMDA. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. Note that the scales for NA release differ from that in Fig 3-10. ***Li^+ (1.5 mM) significantly potentiated 300 μM NMDA-evoked release of [^{3}H]NA, while it had no effect on the release of adenosine evoked by the same concentration of NMDA.*** * Significantly different from control (NMDA alone).

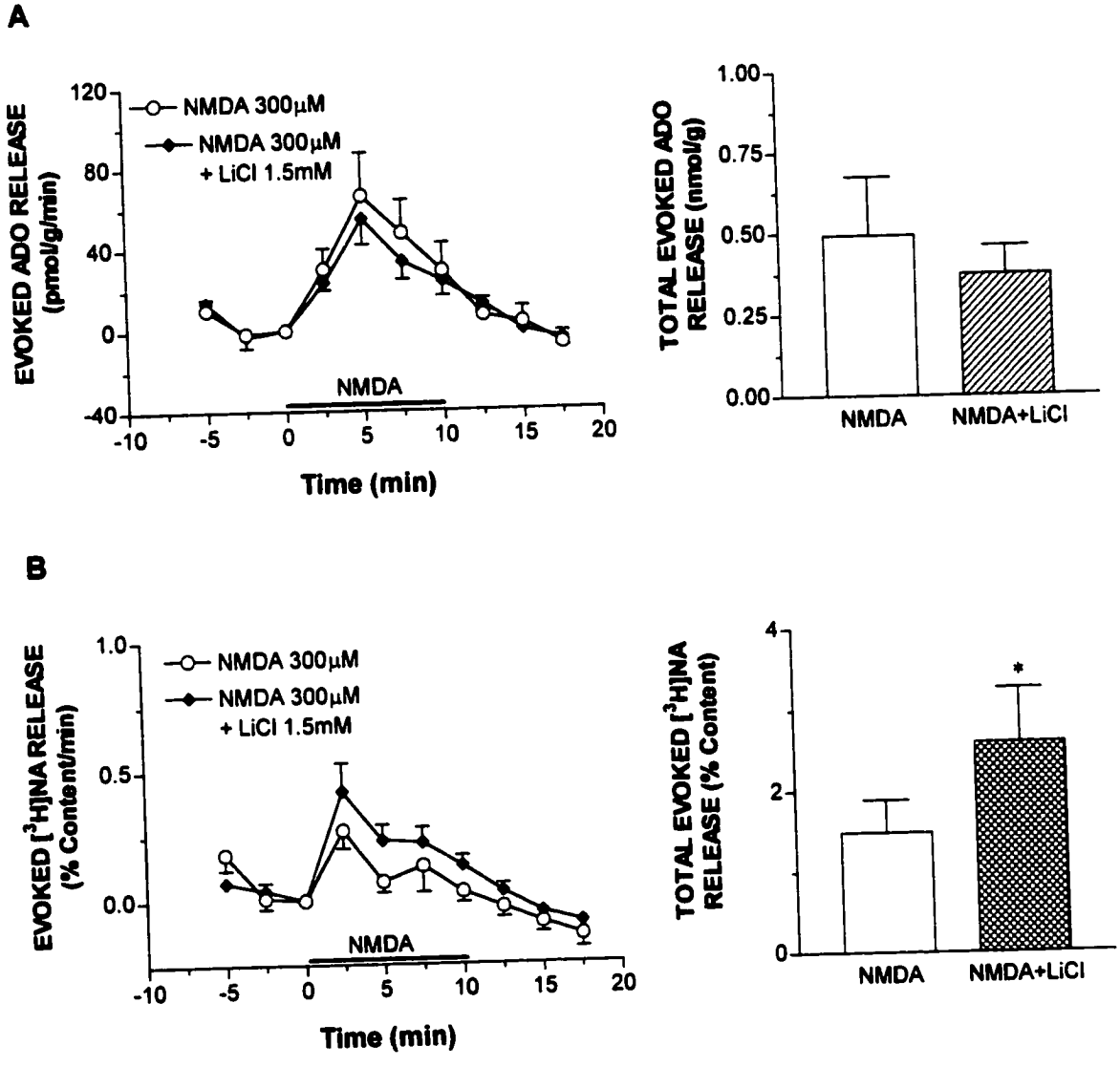
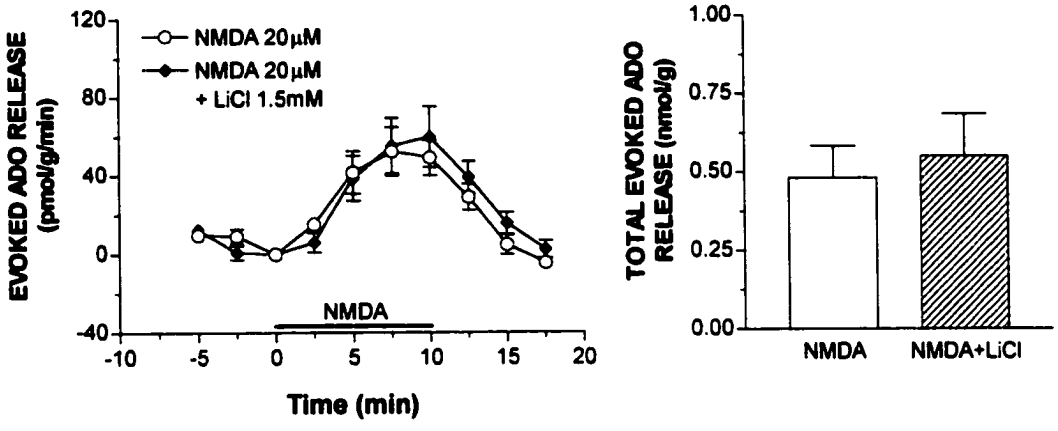

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 300μM
NMDA 300μM + LiCl 1.5mM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+LiCl
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 300μM
NMDA 300μM + LiCl 1.5mM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+LiCl

Fig. 3-11

Fig. 3-12. Effect of 1.5 mM Li^+ on 20 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. Li^+ was introduced to the medium 15 min before exposure to NMDA. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***Li^+ (1.5 mM) had no effect on 20 μM NMDA-evoked release of either adenosine or [^{3}H]NA from rat brain cortical slices.***

A


EVOKED ADO RELEASE (pmol/g/min)
120
80
40
0
-40
NMDA 20μM
NMDA 20μM + LiCl 1.5mM
NMDA
-10
-5
0
5
10
15
20
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
1.00
0.75
0.50
0.25
0.00
NMDA
NMDA+LiCl

B

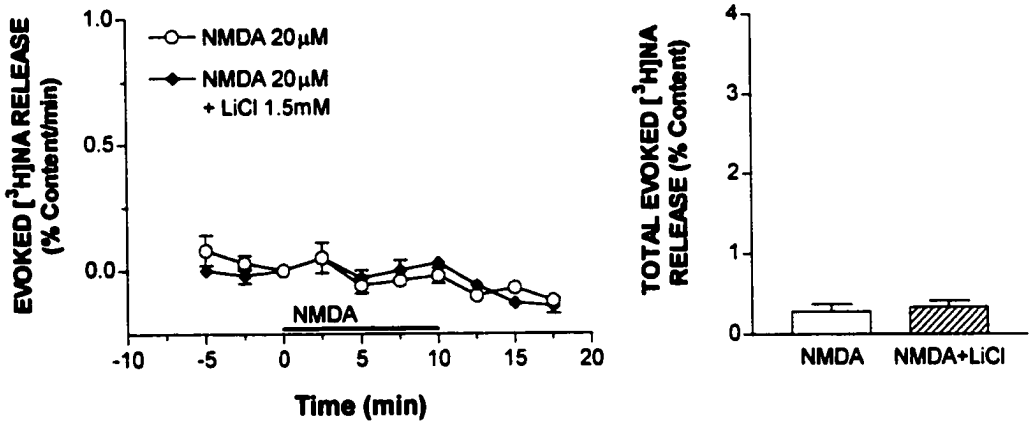

EVOKED [3H]NA RELEASE (% Content/min)
1.0
0.5
0.0
NMDA 20μM
NMDA 20μM + LiCl 1.5mM
NMDA
-10
-5
0
5
10
15
20
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
4
3
2
1
0
NMDA
NMDA+LiCl

Fig. 3-12

Fig. 3-13. Effect of 10 mM Li^+ on 300 μM NMDA-evoked adenosine and [^{3}H]NA release in the presence of 1.2 mM Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA-evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA from rat cortical slices. Li^+ was introduced to the superfusion medium 15 min before exposure to NMDA. NMDA was present from 0 to 10 min. Values are mean ± S.E.M. from 3 experiments. ***Li^+ (10 mM) had no effect on NMDA-evoked release of adenosine, nor did it permit NMDA-evoked [^{3}H]NA release to occur.***

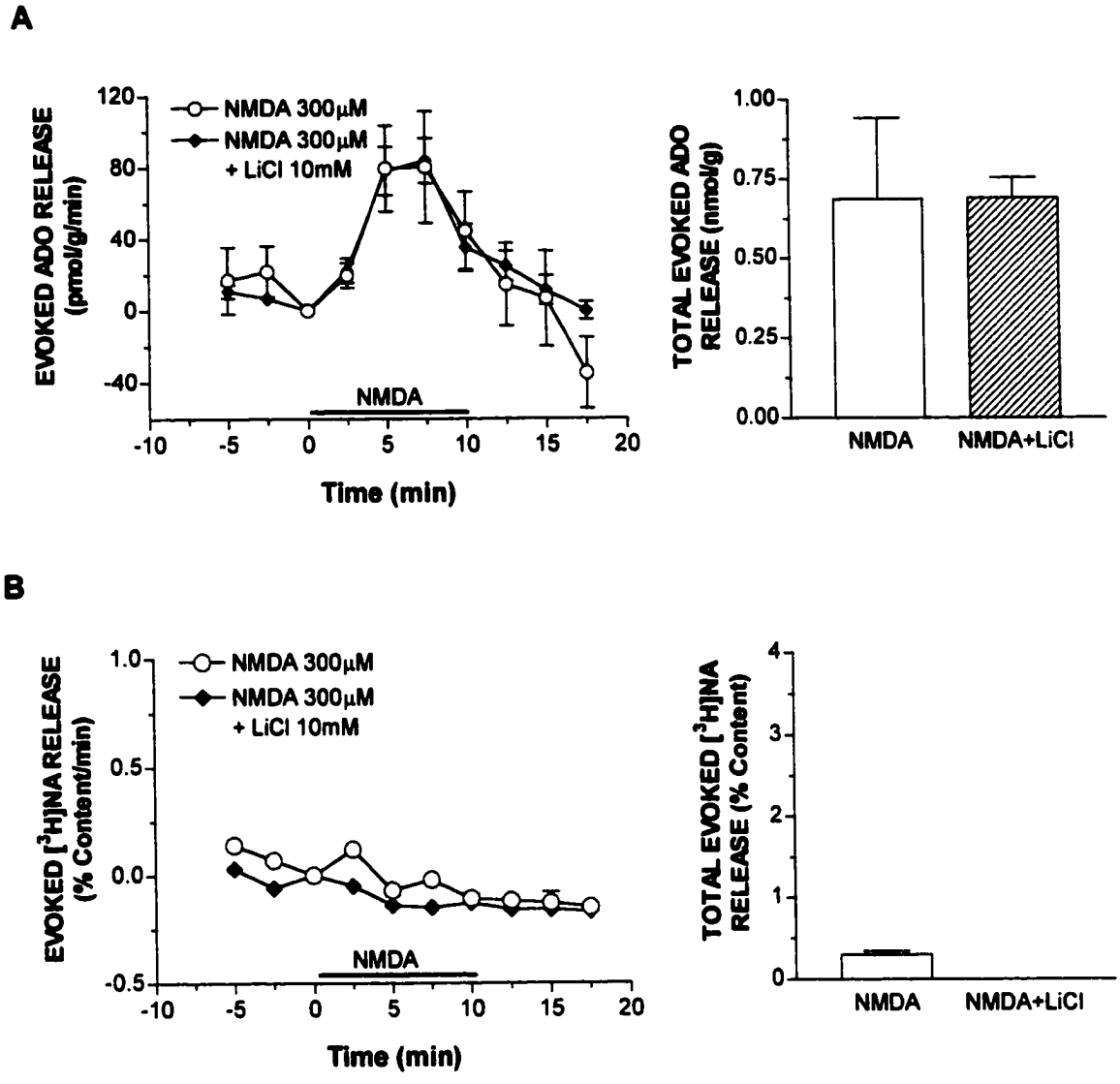
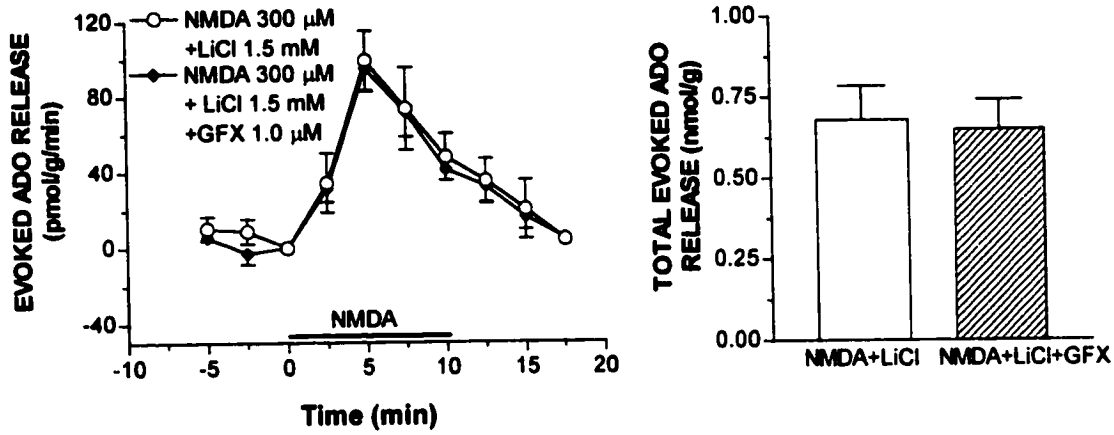

A
EVOKED ADO RELEASE (pmol/g/min)
NMDA 300μM
NMDA 300μM + LiCl 10mM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA
NMDA+LiCl
B
EVOKED [3H]NA RELEASE (% Content/min)
NMDA 300μM
NMDA 300μM + LiCl 10mM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA
NMDA+LiCl

Fig. 3-13

Fig. 3-14. Effect of the PKC inhibitor GFX on Li^+'s effects on 300 μM NMDA-evoked adenosine and [^{3}H]NA release in the absence of Mg^{2+}. (A) Release of adenosine (ADO), (B) release of [^{3}H]NA. Left panels show the time course for NMDA – evoked releases. Right panels represent the total amount of adenosine or [^{3}H]NA release evoked by NMDA. GFX (1 μM) and Li^+ (1.5 mM) were present from 10 min before exposure to NMDA until the end of the experiment. NMDA (300 μM) was present from 0 to 10 min. Values are mean ± S.E.M. from 6 experiments. ***The PKC inhibitor GFX did not reverse the potentiating effect of 1.5 mM Li^+ on NMDA-evoked release of [^{3}H]NA from rat cortical slices.***

A


EVOKED ADO RELEASE (pmol/g/min)
NMDA 300 µM +LiCl 1.5 mM
NMDA 300 µM + LiCl 1.5 mM +GFX 1.0 µM
NMDA
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
NMDA+LiCl
NMDA+LiCl+GFX

B

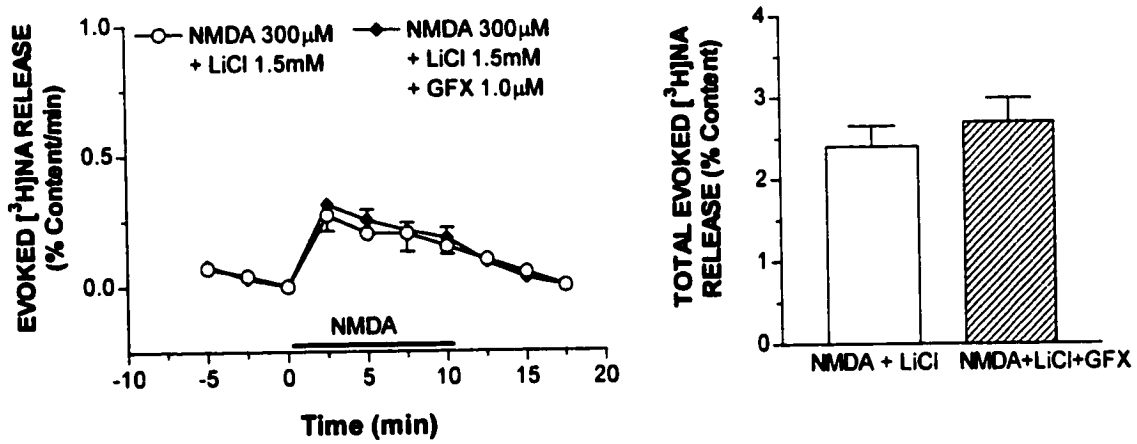

EVOKED [3H]NA RELEASE (% Content/min)
NMDA 300µM + LiCl 1.5mM
NMDA 300µM + LiCl 1.5mM + GFX 1.0µM
NMDA
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
NMDA + LiCl
NMDA+LiCl+GFX

Fig. 3-14

DISCUSSION

Effect of PKC Activation on NMDA-Evoked Releases of Adenosine and [^{3}H]NA

In accordance with previous reports (Hoehn *et al.*, 1990; Craig and White, 1991), the present study showed that, in the absence of Mg^{2+}, high concentrations of NMDA (100 μM) released both adenosine and [^{3}H]NA, but low concentrations of NMDA (20 μM) released only adenosine. In the presence of 1.2 mM Mg^{2+}, NMDA-evoked [^{3}H]NA release was abolished whereas adenosine release persisted. These results support the concept that there are spare receptors for NMDA-evoked adenosine release but not for [^{3}H]NA release (Hoehn *et al.*, 1990; Craig and White, 1991).

Endogenous protein kinase C appears to be required for NMDA-evoked increases in cytosolic Ca^{2+} in rat striatal neurons (Murphy *et al.*, 1994). However, the selective protein kinase C inhibitor GFX did not diminish NMDA-evoked adenosine and [^{3}H]NA release in the present study, suggesting that endogenous protein kinase C activity does not play an essential role in these release processes. Using the two-electrode voltage-clamp technique, Kelso *et al.* (1992) showed that protein kinase inhibitors do not depress NMDA-activated currents expressed in *Xenopus* oocytes, also suggesting that PKC does not play an essential role in NMDA receptor function.

Although PKC does not appear to be involved in NMDA-evoked release of either adenosine or [^{3}H]NA in rat cortical slices, activation of PKC by PMA did potentiate their releases evoked by submaximal concentrations of NMDA, suggesting a role for PKC in modulating these releasing processes. The potentiating effect of PMA on NMDA-evoked NA release appears to relate specifically to activation of PKC because it was reversed by the specific PKC inhibitor GFX.

The mechanisms underlying the potentiation of PKC on NMDA-evoked responses remain to be elucidated. In electrophysiological studies, Chen and Huang (1992) proposed that PKC potentiated the NMDA-activated currents mainly by reducing the voltage-dependent Mg^{2+} block of the NMDA receptor channels. Moreover, reduction of the Mg^{2+} block on NMDA receptors by stretch-induced injury in cultured rat neuronal cells is partially restored by pretreatment with the PKC inhibitor calphostin C (Zhang *et al.*, 1996), suggesting that PKC acts, at least in part, through reducing the Mg^{2+} block. However, in the present study, PMA did not potentiate NMDA-evoked adenosine or [^{3}H]NA release from rat cortical slices in the presence of physiological concentrations of Mg^{2+} (1.2 mM). This does not support the hypothesis that activation of PKC potentiates NMDA responses by removing the Mg^{2+} block of NMDA receptors in rat cortical slices. In accordance with our observations, Wagner and Leonard (1996) showed that PKC-activation fails to induce any change in the sensitivity of NMDA receptors to Mg^{2+} block in *Xenopus* oocytes expressing cloned NMDA receptors. Similar conclusions have been drawn using mouse striatal neurons (Murphy *et al.*, 1994) and primary cultures of cerebellar granule neurons of the rat (Patel *et al.*, 1995). PMA did not increase the maximal NMDA-evoked releases of either adenosine or [^{3}H]NA from rat cortical slices. Murphy *et al.* (1994) also showed that activation of PKC did not increase maximal NMDA-evoked elevations of intraneuronal Ca^{2+} in cultured striatal neurons.

The results of the present study can be explained if activation of PKC increases the agonist affinity of NMDA receptors. This would increase responses when activation of NMDA receptors is submaximal but have no effect on responses to maximal NMDA receptor activation. It is also possible that activation of PKC exerts its effects at some

site following NMDA receptor activation, perhaps on the transduction mechanisms that promote adenosine and NE release. However, these transduction mechanisms cannot include PKC activation consequent to NMDA receptor activation, because neither the release of adenosine nor the release of [^{3}H]NA is diminished by PKC inhibitors.

Our current findings indicate that activation of PKC by the phorbol ester PMA potentiated adenosine release at low levels of NMDA receptor activation while it potentiated [^{3}H]NA release at higher levels of NMDA receptor activation with no effect on adenosine release. This could have important functional implications. Adenosine, released during low levels of NMDA receptor activation, provides an inhibitory threshold that must be overcome in order for excitatory NMDA-mediated processes to proceed maximally (Craig and White, 1992). When only a few NMDA receptors are activated, PKC will increase extracellular adenosine and thus elevate the inhibitory threshold against excitatory neurotransmission that might play a role in modulating normal physiological processes such as learning and memory. This would provide even more selectivity for these essential excitatory processes. However, when the presynaptic release of glutamate is very high, large numbers of NMDA receptors will become activated and the inhibitory threshold provided by adenosine will be overcome. Under these circumstances, activation of PKC will promote the excitatory actions of NMDA receptor activation (e.g. NA release) without producing a corresponding increase in the inhibitory threshold (e.g. adenosine release). This will have the effect of accelerating NMDA-mediated excitatory responses and promoting processes such as learning and memory.

Effect of Lithium on NMDA-Evoked Release of Adenosine and [^{3}H]NA

Although lithium has been used for decades to treat bipolar depression, its mechanism(s) of action is (are) still unresolved (Lenox et al., 1998). Several mechanisms have been proposed, including interference with the inositol cycle (Berridge and Irvine, 1989), modulation of receptor coupled G protein functions (Li et al., 1991, 1993), and modulation of protein kinase C activities (Divish et al., 1991; Krivanek, 1993; Manji et al., 1993). In the present study, we showed that Li^+ at therapeutic concentrations potentiated NMDA-evoked release of [^{3}H]NA but not of adenosine from rat parietal cortex in the absence of Mg^{2+}. In an effort to explain the possible mechanism of this potentiation, we investigated the role of protein kinase C in Li^+'s effect. However, our results indicate that PKC does not appear to be involved in Li^+'s actions. It remains possible that the Li^+-induced increases in NMDA-evoked NA release are mediated by alterations in the inositol phosphate cycle, rather than by activation of PKC. Indeed, increases in inositol phosphate content by acute treatment with therapeutic concentrations of lithium have been reported (Dixon et al., 1992; Lee et al., 1992; Dixon and Hokin, 1994). Unfortunately, the role of inositol phosphate cycle in lithium's potentiating effect on NMDA-evoked [^{3}H]NA release could not be tested in our slice preparations, because none of the available IP_3 receptor antagonists penetrate cell membranes at the time of performing these experiments.

Li^+ apparently stimulates the release of glutamate from cerebral cortex in a time- and concentration- dependent manner (Dixon et al., 1994; Hokin et al., 1996). However, this cannot explain Li^+'s potentiating effect on NMDA-evoked [^{3}H]NA release from rat cortical slices in our study, because Li^+ by itself did not release either adenosine or

[^{3}H]NA. In addition, the potentiation of NMDA-evoked release of [^{3}H]NA does not appear to be due to the removal of the Mg^{2+} block of NMDA receptors because, in the presence of Mg^{2+}, addition of Li^+ to the superfusion medium did not allow NMDA-evoked release of [^{3}H]NA to occur.

The observation that Li^+ potentiated NMDA-evoked NA release from rat brain cortical slices may have some relevance to the acute toxic effects of Li^+ in the treatment of bi-polar depression.

CHAPTER 4

IMMEDIATE EXCITATORY RESPONSES CAUSED BY THE BACTERIAL ENDOTOXIN LPS AND HIV GLYCOPROTEINS IN RAT BRAIN CORTICAL SLICES

PART I

THE BACTERIAL ENDOTOXIN LIPOPOLYSACCHARIDE CAUSES INAPPROPRIATE EXCITATION IN RAT BRAIN CORTEX

ABSTRACT

The bacterial endotoxin lipopolysaccharide (LPS) has been widely used to stimulate immune/inflammatory responses both systemically and in the CNS. Here, we show that exposure of parietal cortical slices from adult rats to LPS triggered very rapid (<2.5min) and sustained releases of the neurotransmitters glutamate and noradrenaline (NA), and of the neuromodulator adenosine. The responses to LPS declined rapidly following removal of the LPS and exhibited no tachyphylaxis to repeated exposures to LPS. The detoxified form of LPS had no effect. LPS-evoked release of [^{3}H]NA, but not of glutamate or adenosine, appears to be partly due to the released glutamate acting at ionotropic receptors on the noradrenergic axons present in the cortical slices. LPS appears to release glutamate, which then acts at non-NMDA receptors to remove the voltage-sensitive Mg^{2+} block of NMDA receptors, thus permitting NMDA receptors to be activated and NA release to proceed. Omission of Ca^{2+} from the beginning of experiments or blockade of Na^+-channels by TTX had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices. These results suggest that LPS-evoked release of glutamate likely originates from non-neuronal cells in the slices. Finally, LPS-evoked releases of glutamate and [^{3}H]NA were diminished by the non-selective glutamate transporter blocker tPDC, indicating that glutamate is released via the reversal of the high-affinity, Na^+-dependent glutamate transporters. It seems possible that rapid, inappropriate excitation may occur in the immediate vicinity of gram-negative bacterial infections in the brain. If similar inappropriate excitations are also triggered by those immunogens specifically associated with Alzheimer's disease (β-amyloid) or AIDS dementia (gp120 and gp41), they might explain some of the acute, transient neurological and psychiatric symptoms associated with these disorders.

INTRODUCTION

In the past, the central nervous system (CNS) was considered isolated and protected from most immune/inflammatory processes. However, there is mounting evidence that the CNS is affected by both central and peripheral inflammatory reactions (de Vries et al., 1997; Xiao et al., 1998). Indeed, many neurodegenerative disorders, including Alzheimer's disease, AIDS dementia and multiple sclerosis, as well as neurological pathologies associated more directly with specific bacterial infections of the CNS, appear to involve inflammatory processes (Perry et al., 1993; Yeung et al., 1995; Feuerstein et al., 1997). In many cases, it is not clear whether central inflammation is the cause of, or a response to, damage in the CNS. There is little doubt that neuronal cell death can trigger an inflammatory response in the brain. However, activation of an inflammatory response in the brain also has the capacity to cause pathological responses, including neuronal damage (Mattson and Mark, 1996).

Although clearly less robust than the peripheral inflammatory responses, the brain does possess its own inflammatory system. Microglia, which are largely quiescent (inactive), can be activated to perform macrophage-like functions. Astrocytes can also become reactive and possess characteristics of peripheral macrophages (Perry et al., 1993). The bacterial endotoxin LPS has been widely used to stimulate the immune/inflammatory responses both systemically and in the CNS *in vivo* and *in vitro* (Wan et al., 1994; Monter-Menei et al., 1996). Significantly, LPS activates macrophages to release various cytokines, which participate in inflammatory responses (Zhao and Schwartz, 1998). There is evidence that LPS may promote glutamate release in the CNS (Wan et al., 1994; al-Shabanah et al., 1996; Mascarucci et al., 1998). In the present

study, we show that LPS triggers the rapid releases of the neurotransmitters glutamate and noradrenaline and of the neuromodulator adenosine from slices of rat parietal cortex. Adenosine may function as a neuroprotectant in the CNS (Cronstein, 1995).

MATERIALS AND METHODS

Materials, preparation of cortical slices, and measurement of glutamate, [^{3}H]NA and adenosine are described in Chapter 2. After collection of three samples to determine basal release, the superfusing medium was switched to medium containing LPS for either 10 or 40 min, after which the superfusing buffer was switched back to normal Krebs-Henseleit buffer and the collection continued until the end of the experiment. The experimental procedure for studies with two identical periods of stimulation has been described in Chapter 2.

RESULTS

LPS triggers the rapid releases of glutamate, [^{3}H]NA and adenosine from cortical slices. LPS (1 and 5μg/ml) did not release the neurotransmitters glutamate and NA, or the neuromodulator adenosine from slices of rat parietal cortex (data not shown). LPS (10 and 20μg/ml) evoked very slight and inconsistent releases of glutamate, NA and adenosine (Fig. 4-1). However, LPS (50μg/ml) triggered very rapid (<2.5 min) and sustained releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex (Fig. 4-2 A, B and C). Their releases reached peak values within 7.5 min after exposure to LPS and were sustained while LPS was present in the superfusion medium (Fig. 4-2, 40-min treatment). Upon removal of LPS, the releases of glutamate, [^{3}H]NA and

adenosine rapidly returned to their basal levels (Fig. 4-2, 10 min treatment), indicating that these responses are readily reversible. The slices responded to repeated exposures to LPS without any apparent tachyphylaxis (Fig. 4-3). Finally, the detoxified form of LPS failed to release glutamate, [^{3}H]NA or adenosine (Fig. 4-3), suggesting that LPS exerts its actions specifically through the biologically active, lipid A portion of the molecule.

Role of NMDA and non-NMDA ionotropic receptors in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Activation of either NMDA or non-NMDA (AMPA and kainate) receptors rapidly releases both NA and adenosine from rat brain cortical slices (Hoehn and White, 1990a, b; Craig and White, 1993). This raised the possibility that the LPS-evoked releases of NA and adenosine might result from released glutamate acting at its receptors. Therefore, we examined the effects of the non-competitive NMDA receptor antagonist MK-801 (3 μM) and a high concentration of the competitive NMDA antagonist APV (500 μM) on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine. LPS-evoked [^{3}H]NA release was decreased significantly by both MK-801 (Fig. 4-4B) and APV (Fig. 4-5B). However, neither MK-801 nor APV significantly reduced LPS-evoked releases of glutamate (Fig. 4-4A and Fig. 4-5A) or adenosine (Fig. 4-4C and Fig. 4-5C), indicating that these release processes were not likely mediated by the activation of NMDA receptors.

Fig. 4-1. Concentration-response curves for LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Slices were exposed to various concentrations of LPS for 5-min and the total evoked releases were calculated for each stimulation. Values are Mean ± S.E.M. from 3 experiments.

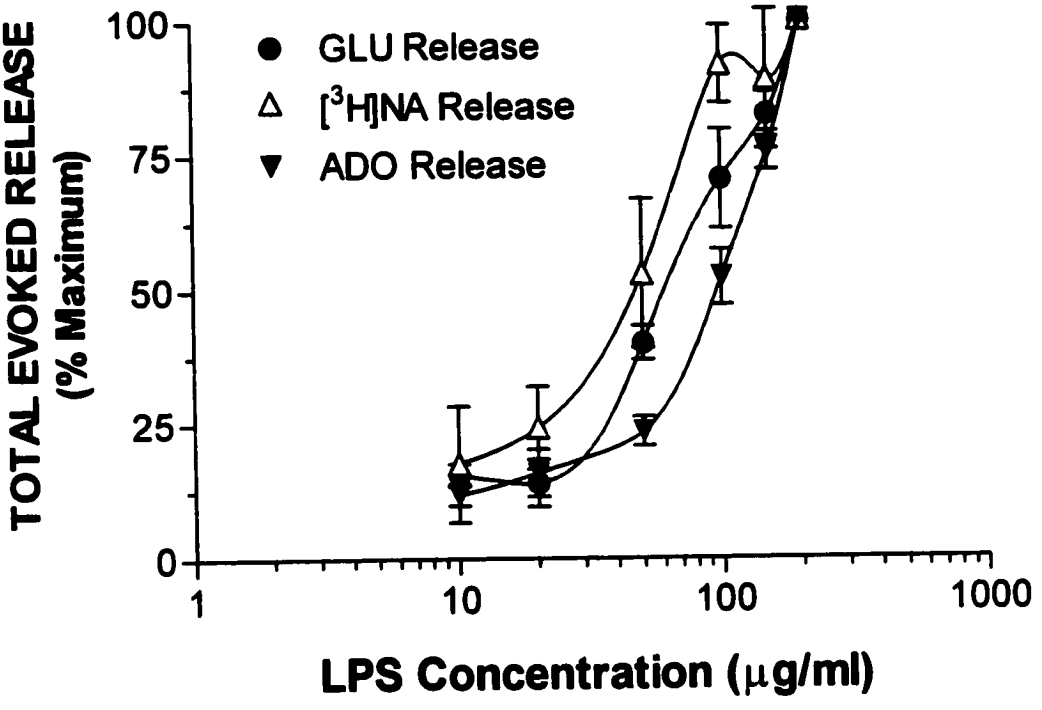
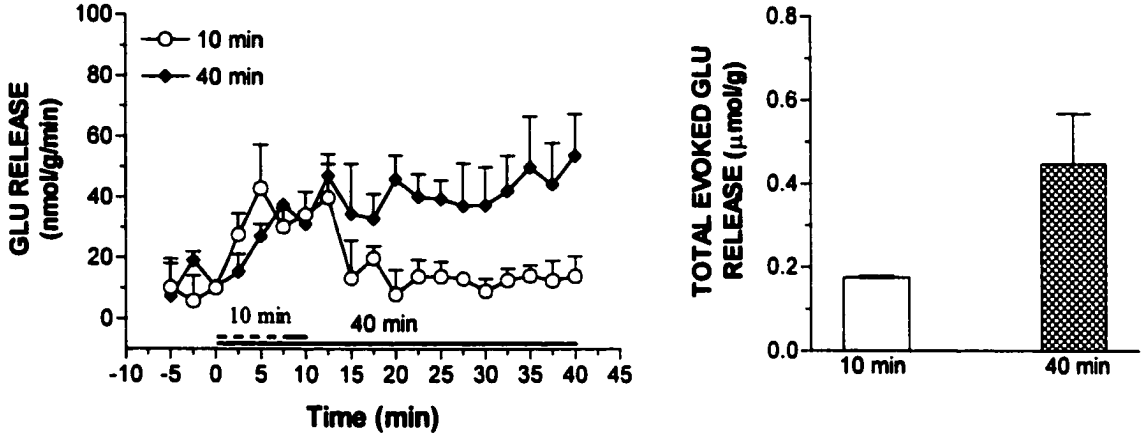

TOTAL EVOKED RELEASE
(% Maximum)
100
75
50
25
0
1
10
100
1000
LPS Concentration (μg/ml)
GLU Release
[3H]NA Release
ADO Release

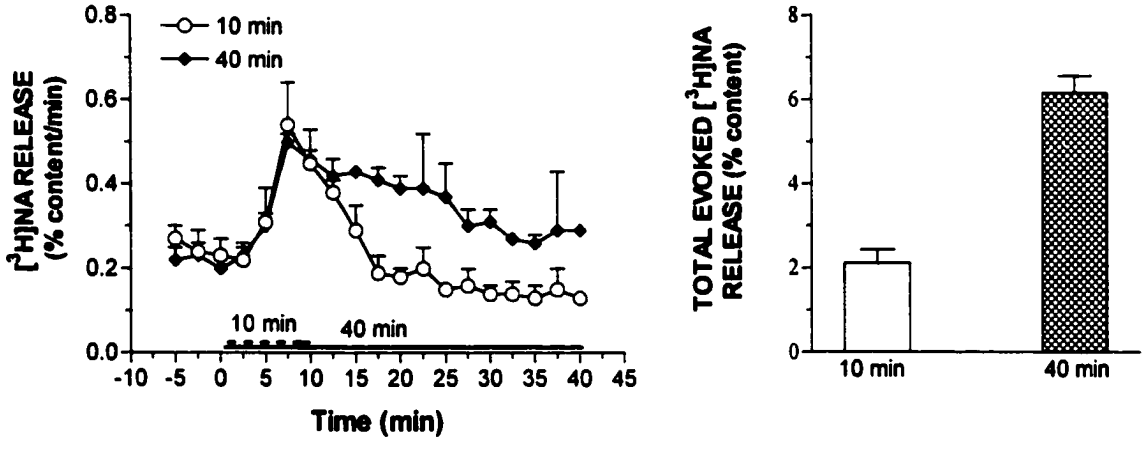
Fig. 4-1

Fig. 4-2. LPS (50 μg/ml) triggers rapid (<2.5 min) releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracelluar Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses for LPS-evoked releases. Right panels (bar graphs) represent total evoked releases. Values are mean ± S.E.M. from 4 experiments. ***Stimulation with LPS for either 10 min or 40 min induced rapid releases, which reached maximums at 7.5 min after exposure and persisted during exposure to LPS. The rates of release of glutamate, [^{3}H]NA and adenosine rapidly returned to basal levels when LPS was withdrawn after 10 min exposure.***

A


GLU RELEASE (nmol/g/min)
10 min
40 min
Time (min)
TOTAL EVOKED GLU RELEASE (μmol/g)
10 min
40 min

B


[3H]NA RELEASE (% content/min)
10 min
40 min
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% content)
10 min
40 min

C

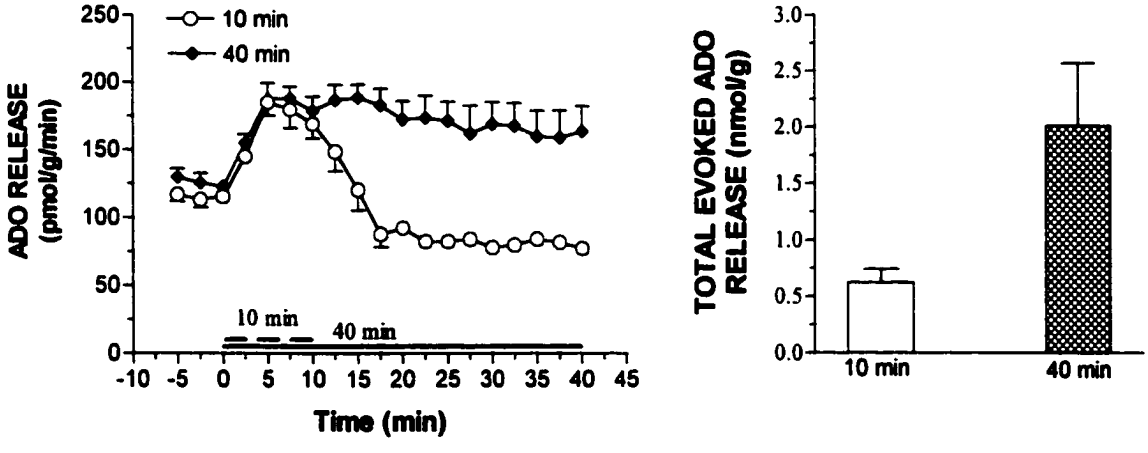
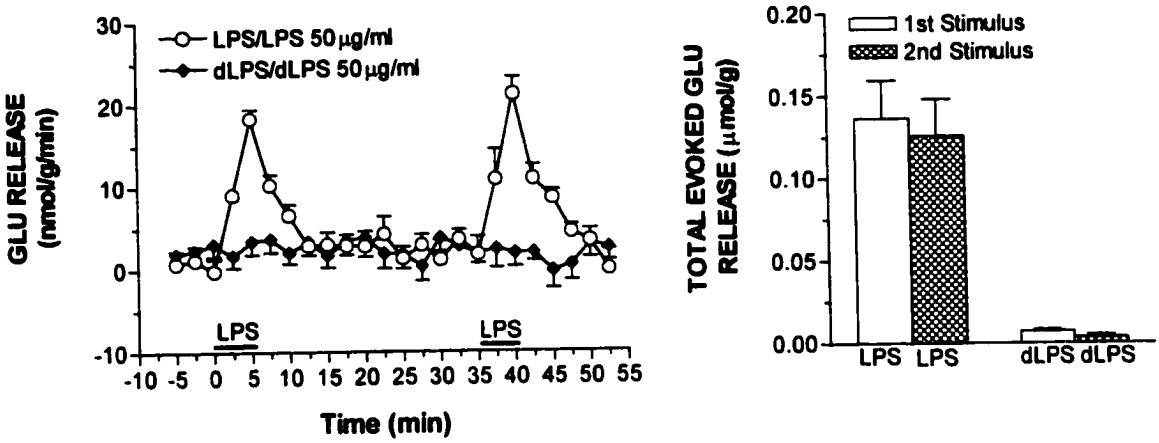

ADO RELEASE (pmol/g/min)
10 min
40 min
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
10 min
40 min

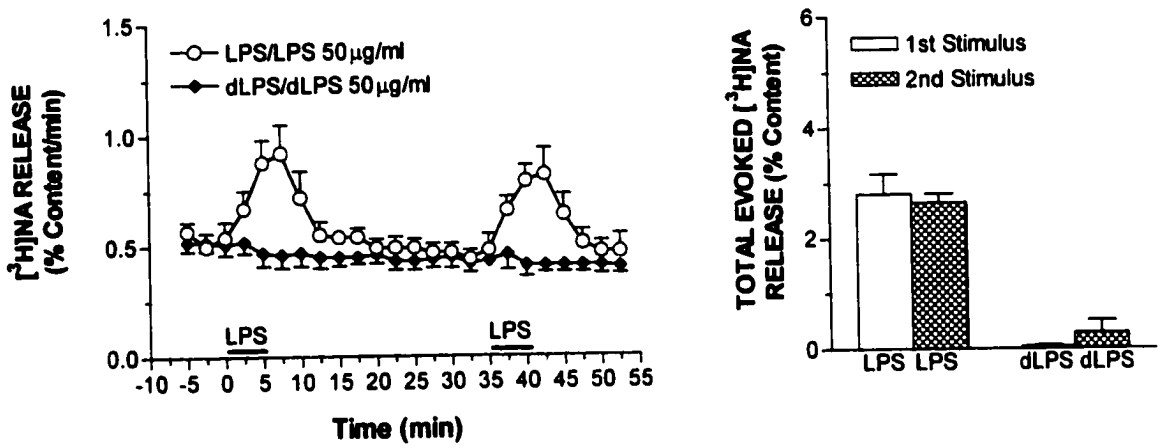
Fig. 4-2

Fig. 4-3. Effect of LPS (50 μg/ml) and detoxified LPS (50 μg/ml) on glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for release after repeated exposure to LPS for 5 min each. Right panels (bar graphs) represent total evoked releases. Values are mean ± S.E.M. from 3 experiments. ***No tachyphylaxis was observed for LPS-evoked releases of glutamate, NA and adenosine. Detoxified LPS caused no release. There were no differences in total evoked releases between the first and the second exposures to LPS.***

A


LPS/LPS 50 µg/ml
dLPS/dLPS 50 µg/ml
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (µmol/g)
LPS LPS
dLPS dLPS

B


LPS/LPS 50 µg/ml
dLPS/dLPS 50 µg/ml
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS
dLPS dLPS

C

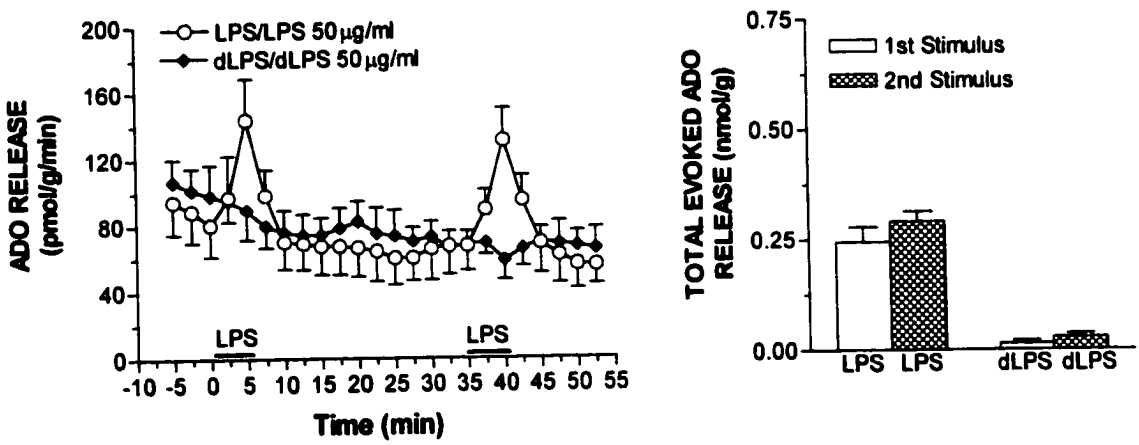
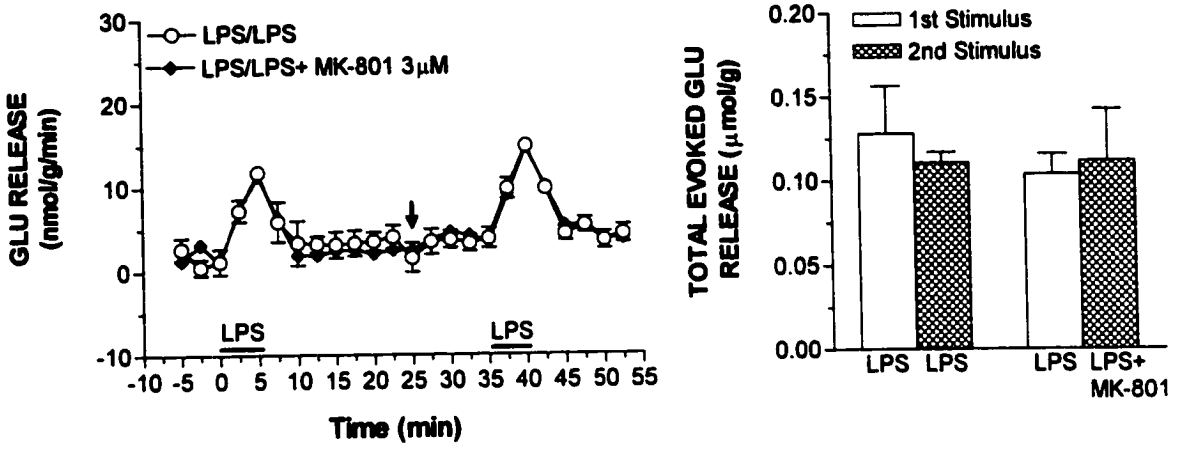

LPS/LPS 50 µg/ml
dLPS/dLPS 50 µg/ml
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
dLPS dLPS

Fig. 4-3

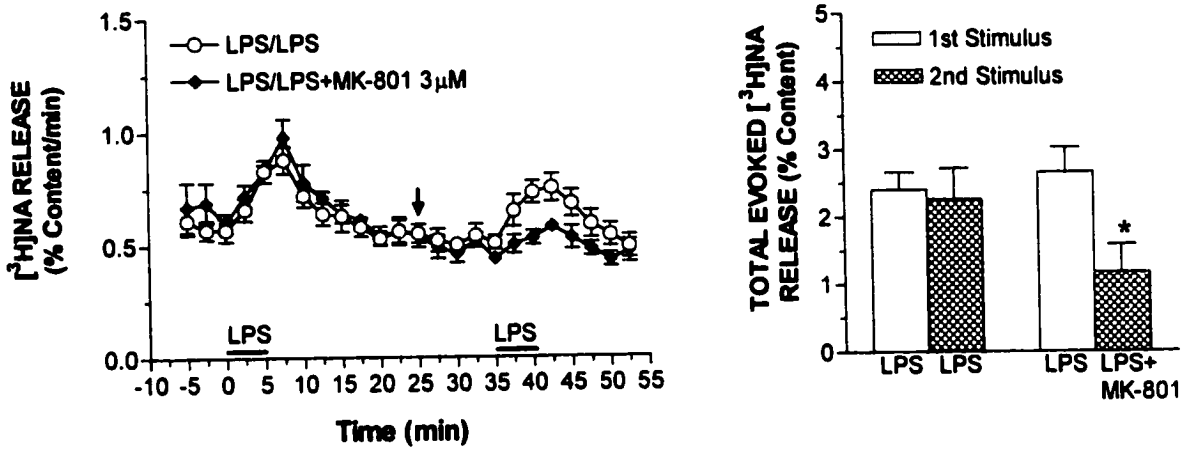
Significantly, LPS-evoked [^{3}H]NA release was observed even in the presence of a physiological concentration of Mg^{2+} (1.2 mM), a situation that have been shown previously to abolish the NMDA-evoked release of NA (Hoehn et al., 1990). The most likely explanation for this is that LPS releases glutamate, which then acts at non-NMDA receptors to relieve the Mg^{2+}-block of NMDA receptors, and thus permits NMDA receptor activation to take place. To test this hypothesis, we studied the effect of the non-NMDA receptor antagonist NBQX (10 μM) on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine. In the presence of Mg^{2+}, NBQX reduced the LPS-evoked release of [^{3}H]NA by approximately 50% (Fig. 4-6B). However, in the absence of Mg^{2+}, LPS-evoked release of [^{3}H]NA was not significantly decreased by NBQX (Fig. 4-6B). Finally, the combined blockade of both NMDA and non-NMDA receptors with MK-801 and NBQX, respectively, did not produce additive inhibition of LPS-evoked [^{3}H]NA release (Fig. 4-7B). NBQX had no effect on LPS-evoked glutamate (Fig. 4-6A) or adenosine (Fig. 4-6C) release either in the presence or in the absence of Mg^{2+}, nor did the combination of MK-801 and NBQX affect their releases (Fig. 4-7A and 4-7C). Taken together, these results suggest that LPS releases glutamate, which then acts at non-NMDA receptors to remove the voltage-sensitive Mg^{2+} block of NMDA receptors, thus permitting NMDA receptors to be activated and NA release to proceed.

Fig. 4-4. Effect of the non-competitive NMDA receptor antagonist MK-801 (3 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine. Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when MK-801 was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***MK-801 significantly decreased the release of [^{3}H]NA evoked by LPS but had no effect on the releases of glutamate or adenosine.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t*-test.

A


LPS/LPS
LPS/LPS+ MK-801 3μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+ MK-801

B


LPS/LPS
LPS/LPS+MK-801 3μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
LPS LPS
LPS LPS+ MK-801

C

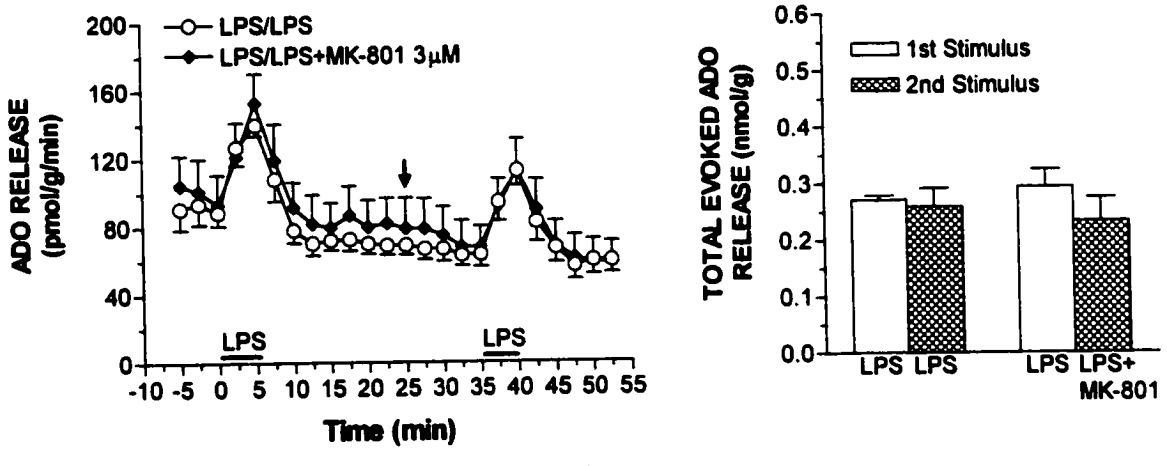

LPS/LPS
LPS/LPS+MK-801 3μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
LPS LPS+ MK-801

Fig. 4-4

Fig. 4-5. Effect of the competitive NMDA receptor antagonist APV (500 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when APV was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***APV significantly decreased the release of [^{3}H]NA evoked by LPS but had no effect on the releases of glutamate and adenosine.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t*-test.

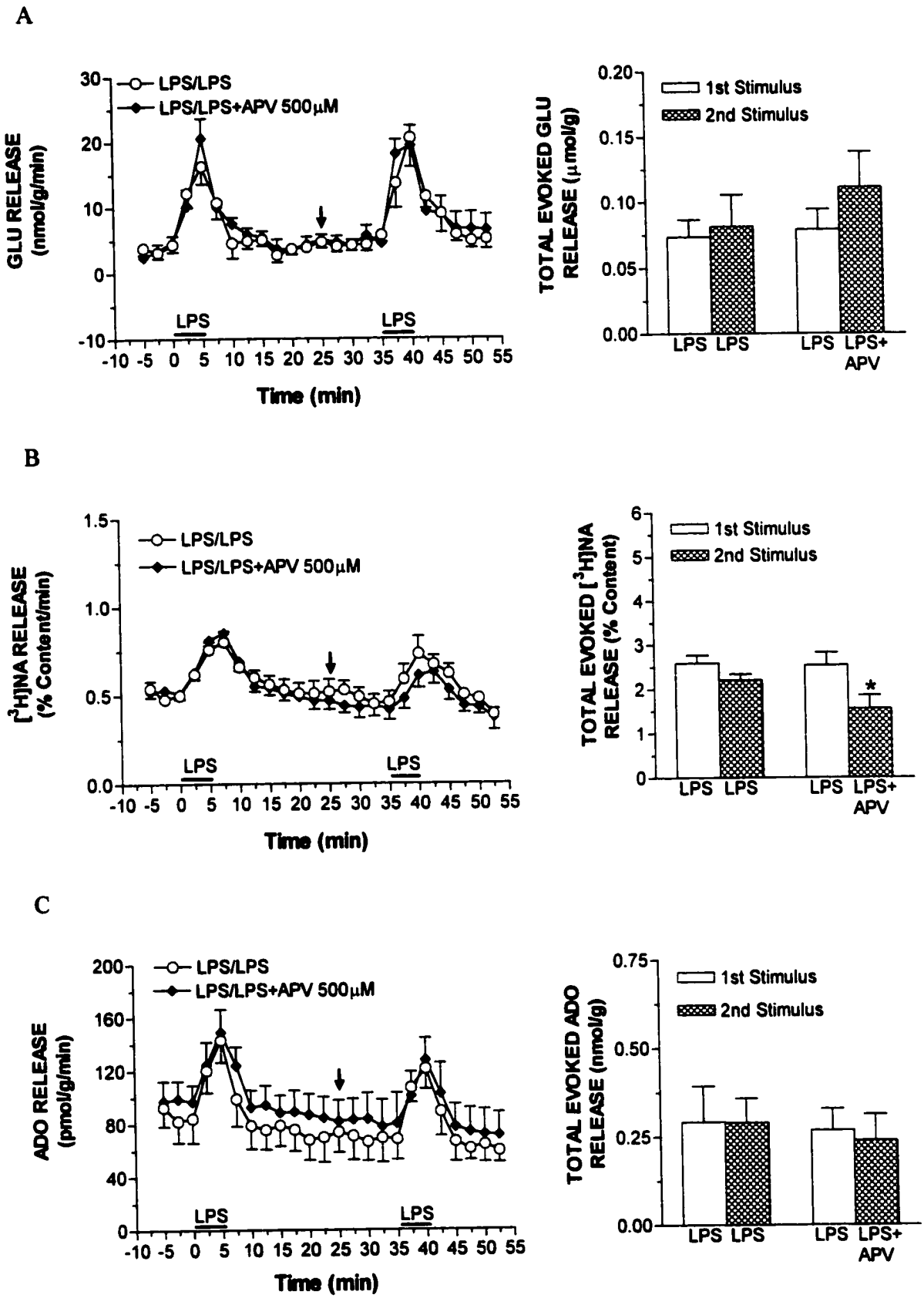
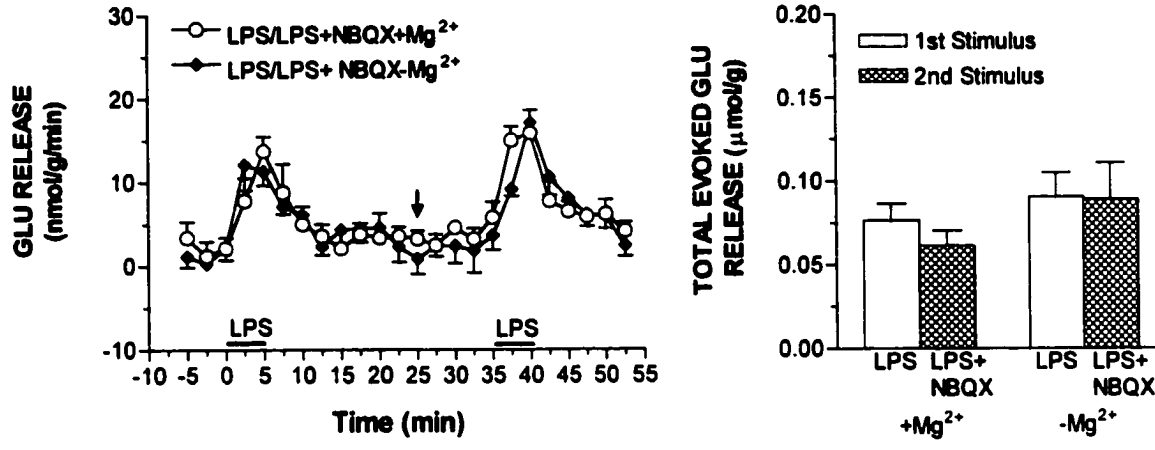

A
LPS/LPS
LPS/LPS+APV 500μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+ APV
B
LPS/LPS
LPS/LPS+APV 500μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
LPS LPS
LPS LPS+ APV
C
LPS/LPS
LPS/LPS+APV 500μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
LPS LPS+ APV

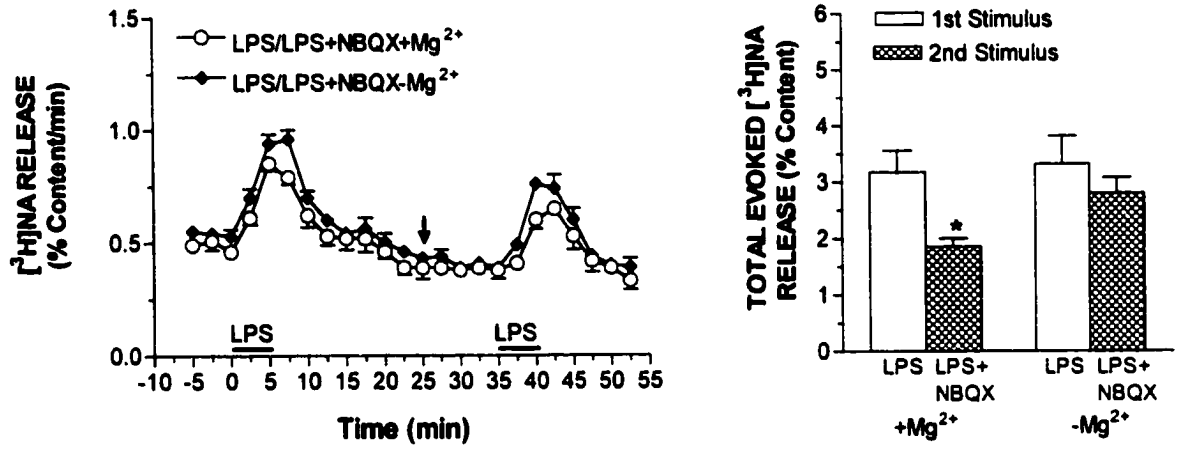
Fig. 4-5

Fig. 4-6. Effect of the non-NMDA receptor antagonist NBQX (10 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine releases from slices of rat parietal cortex in the presence and absence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses of LPS-evoked releases and the effects of NBQX. Arrows indicate the time when NBQX was introduced into the superfusion medium. Right panels represent the total evoked releases. Values are mean ± S.E.M. from 4 experiments. ***In the presence of extracellular Mg^{2+}, NBQX significantly decreased the total LPS-evoked release of [^{3}H]NA but had no effect on the releases of glutamate or adenosine. In the absence of extracellular Mg^{2+}, NBQX had no effect on LPS-evoked [^{3}H]NA release, nor did it affect the releases of glutamate or adenosine.*** * $p < 0.05$ compared with the first stimulation (LPS alone), Student's paired *t*-test.

A


LPS/LPS+NBQX+Mg2+
LPS/LPS+ NBQX-Mg2+
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS+ NBQX
LPS LPS+ NBQX
+Mg2+
-Mg2+

B


LPS/LPS+NBQX+Mg2+
LPS/LPS+NBQX-Mg2+
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
LPS LPS+ NBQX
LPS LPS+ NBQX
+Mg2+
-Mg2+

C

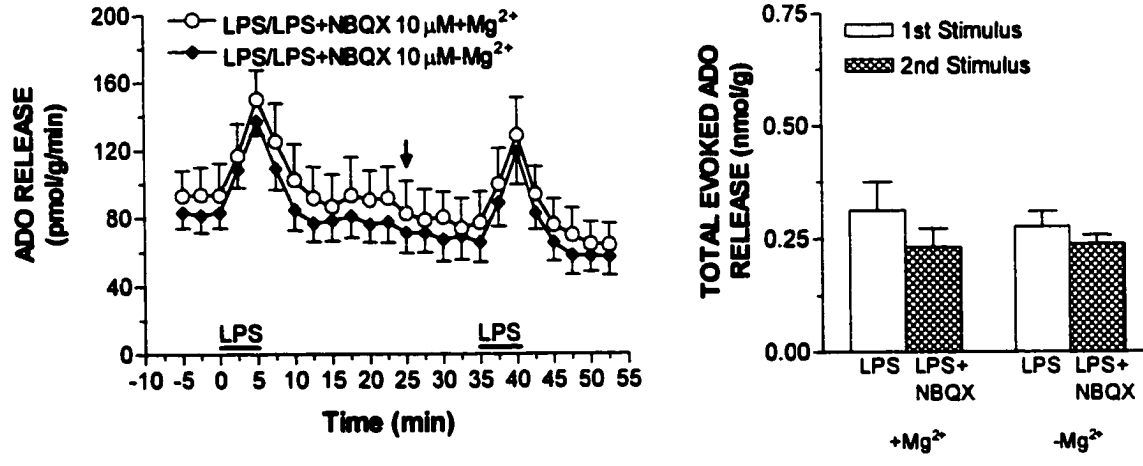
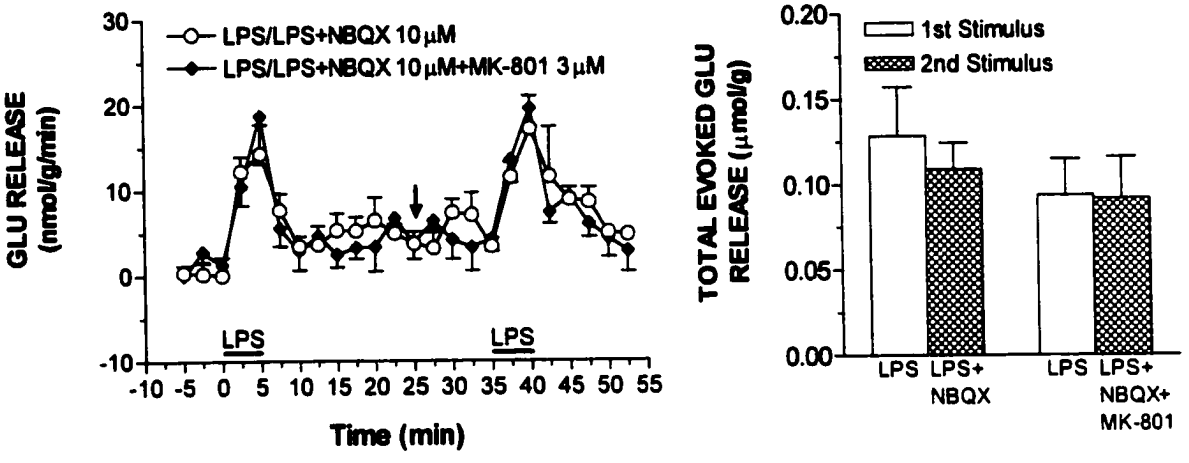

LPS/LPS+NBQX 10 μM+Mg2+
LPS/LPS+NBQX 10 μM-Mg2+
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS+ NBQX
LPS LPS+ NBQX
+Mg2+
-Mg2+

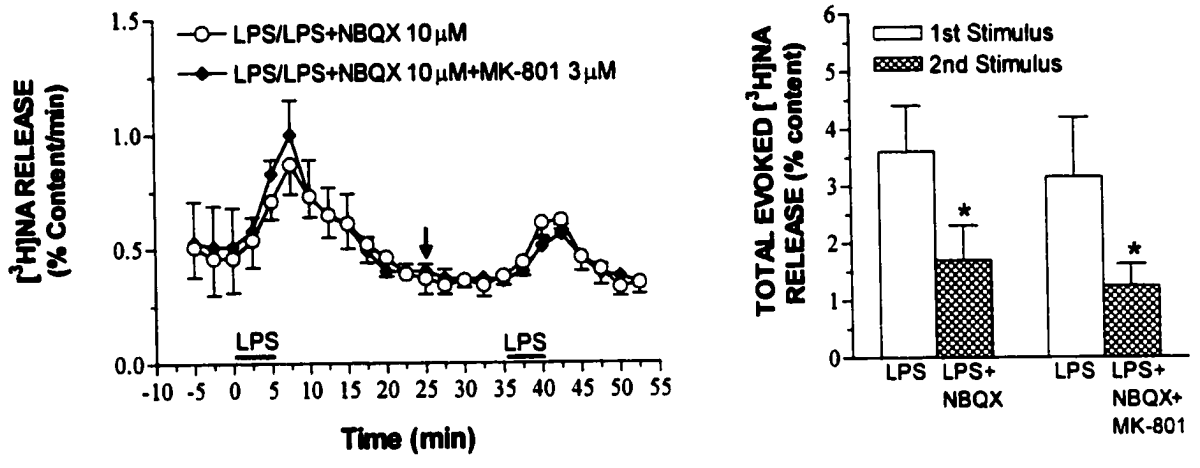
Fig. 4-6

Fig. 4-7. Effect of the non-NMDA receptor antagonist NBQX (10 μM) and the combination of NBQX and MK-801 (3 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine releases from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses of LPS-evoked releases and the effects of NBQX and MK-801. Arrows indicate the time when MK-801 or NBQX was introduced into the superfusion medium. Right panels represent the total evoked releases. Values are mean ± S.E.M. from 4 experiments. ***NBQX significantly decreased the total LPS-evoked release of [^{3}H]NA but had no effect on the releases of glutamate or adenosine. The combination of the two antagonists did not produce additive inhibition of [^{3}H]NA release, nor did it reduce LPS-evoked releases of glutamate or adenosine.*** * $p < 0.05$ compared with the first stimulation (LPS alone), Student's paired *t*-test.

A


LPS/LPS+NBQX 10μM
LPS/LPS+NBQX 10μM+MK-801 3μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
TOTAL EVOKED GLU RELEASE (μmol/g)
1st Stimulus
2nd Stimulus
LPS
LPS+ NBQX
LPS
LPS+ NBQX+ MK-801

B


LPS/LPS+NBQX 10μM
LPS/LPS+NBQX 10μM+MK-801 3μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% content)
1st Stimulus
2nd Stimulus
LPS
LPS+ NBQX
LPS
LPS+ NBQX+ MK-801

C

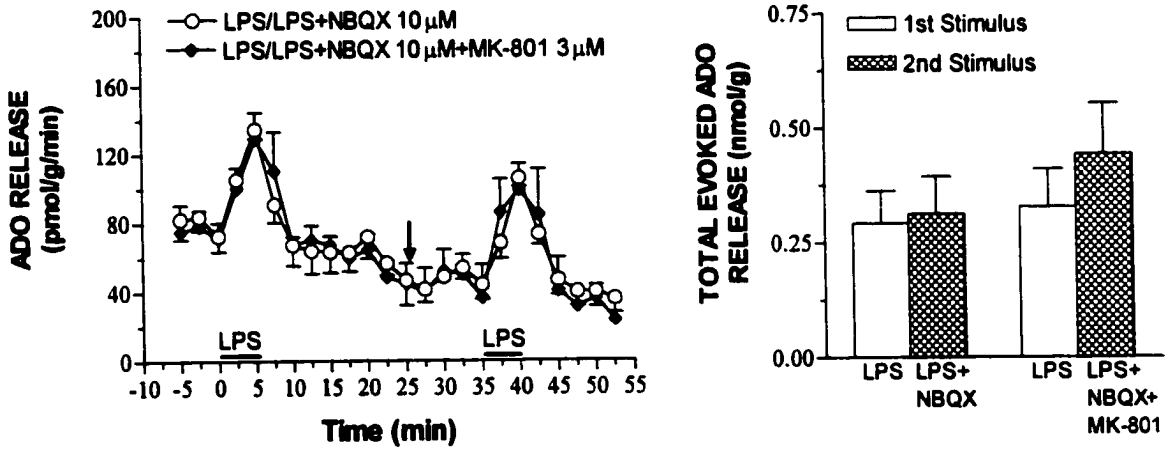

LPS/LPS+NBQX 10μM
LPS/LPS+NBQX 10μM+MK-801 3μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
1st Stimulus
2nd Stimulus
LPS
LPS+ NBQX
LPS
LPS+ NBQX+ MK-801

Fig. 4-7

Cellular sources of LPS-evoked releases of glutamate, [^{3}H]NA and adenosine. LPS-evoked release of [^{3}H]NA likely originates from noradrenergic axons present in the cortex. However, the cellular source(s) of LPS-evoked glutamate release (neuronal, microglial, astrocytic) remain unclear. To examine the possible cellular site(s) of glutamate formation, the involvement of Ca^{2+} and propagated action potentials in LPS-evoked releases was tested. Omission of Ca^{2+} from the beginning of the experiment had no effects on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices (Fig. 4-8), indicating that extracellular Ca^{2+} is not required for these release processes. Moreover, LPS-evoked releases of glutamate, [^{3}H]NA or adenosine are not diminished when Na^+-channels are blocked by TTX (Fig. 4-9), indicating that LPS-evoked releases of these substances do not involve the propagation of action potentials.

Numerous reports show that glutamate transporters can be reversed during pathological conditions such as ischemia (Rossi et al., 2000) and central inflammation (Vesce et al., 1997). Therefore, we tested the effect of the non-selective glutamate transporter blocker 2,4-*trans*-PDC (tPDC) on LPS-evoked glutamate release. As shown in Figure 4-10, LPS-evoked releases of glutamate and [^{3}H]NA were significantly diminished by 1 mM tPDC (Fig. 4-10A,B), suggesting that glutamate release might occur via reversal of the high affinity, Na^+-dependent glutamate transporters. The decrease in LPS-evoked release of [^{3}H]NA is probably due to the decreased release of glutamate, which acts at its ionotropic receptors to release NA. Interestingly, tPDC by itself increased basal adenosine release (Fig. 4-10C, left panel), but had no effect on total LPS-evoked adenosine release (Fig. 4-10C, right panel). The reason for the elevation of basal adenosine release is not clear.

Fig. 4-8. Lack of Ca^{2+} dependence of LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses of LPS-evoked releases and the effects of Ca^{2+}. Right panels represent the total evoked releases. Ca^{2+} was omitted from the beginning of the experiment in one bath. After the first stimulation, Ca^{2+} was re-introduced into the superfusion medium, and the total evoked releases were compared between the first and the second stimulation. Values are mean ± S.E.M. from 4 experiments. ***Omission of Ca^{2+} had no effect on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine.***

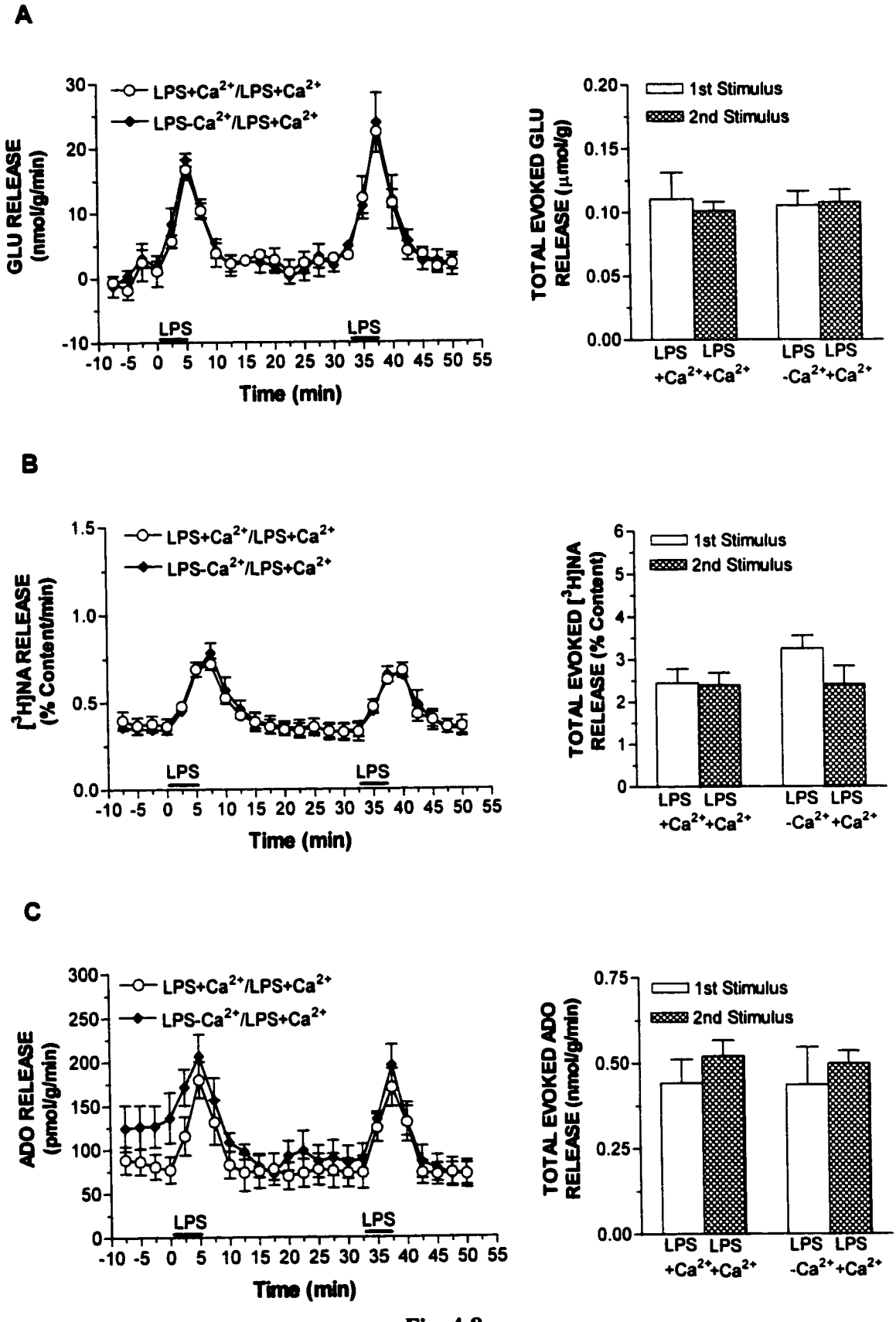
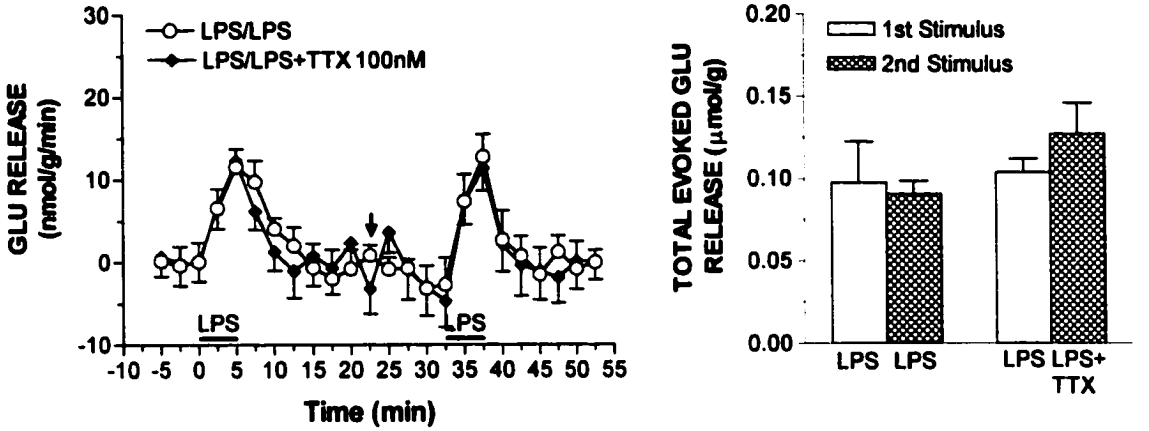

A
GLU RELEASE (nmol/g/min)
LPS+Ca2+/LPS+Ca2+
LPS-Ca2+/LPS+Ca2+
LPS
LPS
Time (min)
TOTAL EVOKED GLU RELEASE (μmol/g)
1st Stimulus
2nd Stimulus
LPS +Ca2+ LPS +Ca2+
LPS -Ca2+ LPS +Ca2+
B
[3H]NA RELEASE (% Content/min)
LPS+Ca2+/LPS+Ca2+
LPS-Ca2+/LPS+Ca2+
LPS
LPS
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
1st Stimulus
2nd Stimulus
LPS +Ca2+ LPS +Ca2+
LPS -Ca2+ LPS +Ca2+
C
ADO RELEASE (pmol/g/min)
LPS+Ca2+/LPS+Ca2+
LPS-Ca2+/LPS+Ca2+
LPS
LPS
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g/min)
1st Stimulus
2nd Stimulus
LPS +Ca2+ LPS +Ca2+
LPS -Ca2+ LPS +Ca2+

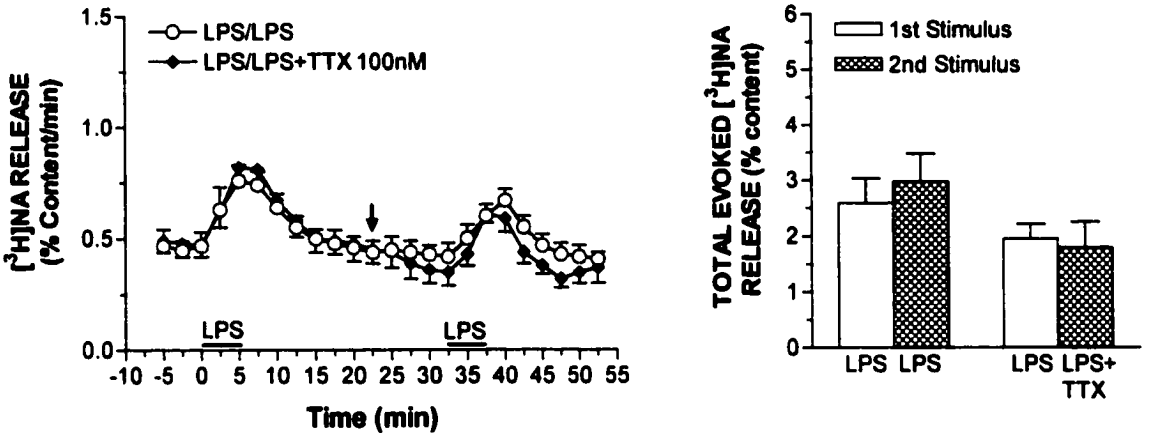
Fig. 4-8

Fig. 4-9. Effect of the Na^+-channel blocker TTX (100 nM) on LPS (50μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine. Left panels show the time courses of LPS-evoked releases and the effects of TTX. Arrows indicate the time when TTX was introduced into the superfusion medium. Right panels represent the total evoked releases. Values are mean ± S.E.M. from 4 experiments. ***TTX had no effect on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine, suggesting that propagated action potentials are not required for releases.***

A


LPS/LPS
LPS/LPS+TTX 100nM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+ TTX

B


LPS/LPS
LPS/LPS+TTX 100nM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% content)
LPS LPS
LPS LPS+ TTX

C

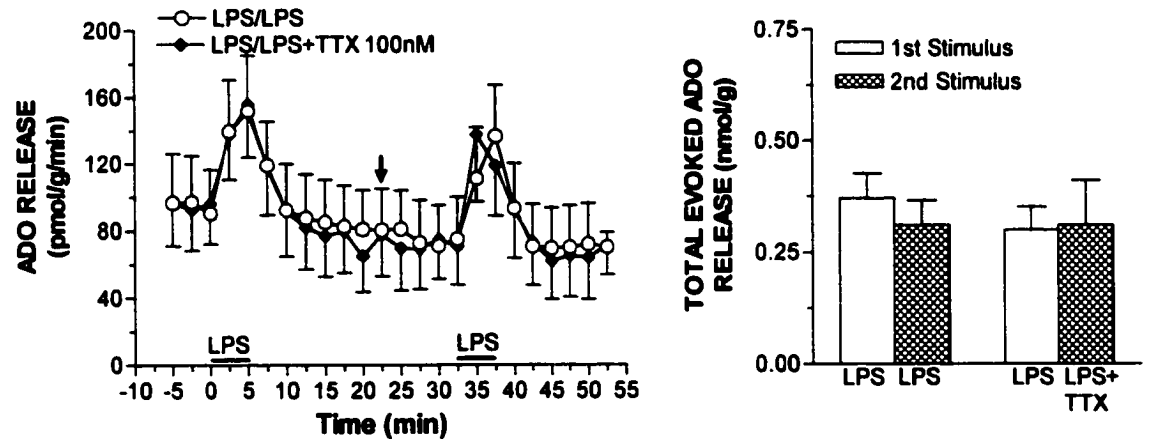

LPS/LPS
LPS/LPS+TTX 100nM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
LPS LPS+ TTX

Fig. 4-9

Fig. 4-10. Effect of the non-selective glutamate transporter blocker 2,4-*trans*-PDC (tPDC, 1 mM) on LPS (50 μg/ml)-evoked releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses of LPS-evoked releases and the effects of tPDC. tPDC was introduced into the superfusion medium 30 min before the second stimulation. Right panels represent the total evoked releases. Value are means ± S.E.M. from 4 experiments. ***tPDC significantly diminished LPS-evoked releases of glutamate and [^{3}H]NA, suggesting that LPS-evoked glutamate release is, at least in part, via the reversal of glutamate transporters. tPDC elevated basal release of adenosine but had no effect on LPS-evoked adenosine release.*** *$p< 0.05$, compared with the first stimulation (LPS alone), Student's *t*-test.

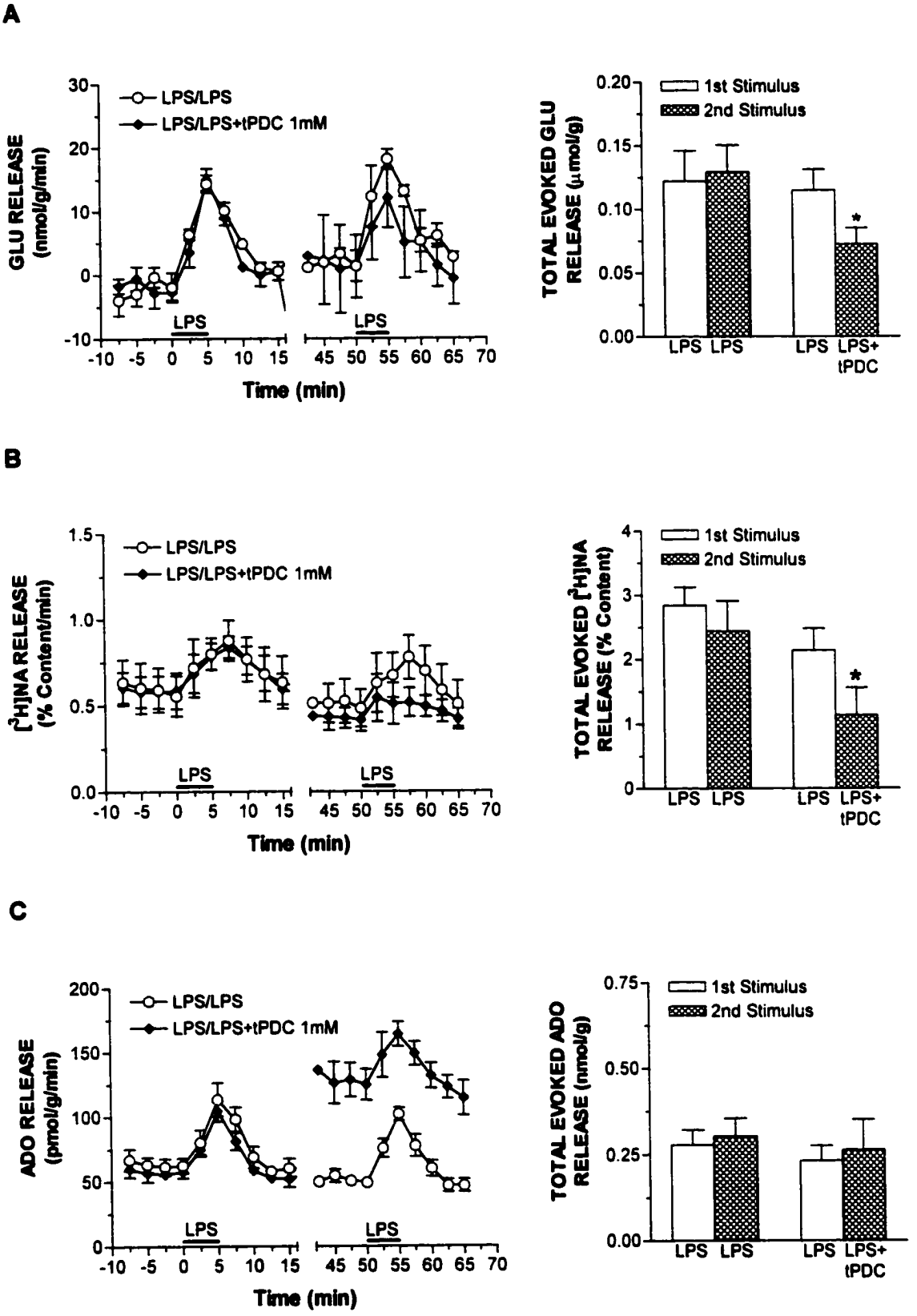

A
GLU RELEASE (nmol/g/min)
LPS/LPS
LPS/LPS+tPDC 1mM
LPS
LPS
Time (min)
TOTAL EVOKED GLU RELEASE (µmol/g)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ tPDC
B
[3H]NA RELEASE (% Content/min)
LPS/LPS
LPS/LPS+tPDC 1mM
LPS
LPS
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ tPDC
C
ADO RELEASE (pmol/g/min)
LPS/LPS
LPS/LPS+tPDC 1mM
LPS
LPS
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ tPDC

Fig. 5-10

DISCUSSION

The bacterial endotoxin LPS (50 μg/ml) triggered very rapid (<2.5 min) and sustained releases of glutamate, [^{3}H]NA and adenosine from adult rat brain cortical slices. To the best of our knowledge, these are the most rapid responses to LPS ever observed in the brain (MacNeil et al, 1996; Klegeris and McGeer, 1997; Pistritto et al, 1998) and much too rapid to involve gene transcription and protein synthesis. The speed of onset of action is all the more remarkable when one considers that LPS has a molecular weight of 1-4 million and would have difficulty penetrating the cortical slices. These rapid responses to LPS observed in the present study occurred in slices of supposedly normal, integrated parietal cortex from adult rats, rather than from cultures of neurons, glia or arbitrary mixtures of the two, which may not reflect normal, integrated brain tissue. Nor were extraneous inflammatory cell types, which may not reside in normal, integrated brain tissue introduced into the preparation (Skaper et al., 1996).

The concentration of LPS (50 μg/ml) used was relatively high. However, LPS concentrations in the immediate vicinity of gram-negative bacteria would be very high. In any event, the LPS-evoked responses observed in this study are unlikely to result from non-specific effects or acute damage to brain cells for the following reasons. First, LPS-evoked releases of glutamate, NA and adenosine were all readily reversible, rapidly returning to basal levels when LPS was withdrawn. Secondly, the slices responded to repeated exposures to LPS without any apparent tachyphylaxis. Finally, the detoxified form of LPS (50 μg/ml) failed to release glutamate, NA or adenosine, suggesting that LPS exerts its actions specifically through the biologically active lipid portion of the molecule (Rietschel et al., 1997).

LPS-evoked release of [^{3}H]NA was decreased significantly by both the non-competitive NMDA receptor antagonist MK-801 and by the competitive NMDA receptor antagonist APV, suggesting that the LPS-evoked release of [^{3}H]NA is partly due to the activation of brain NMDA receptors. This release occurred even when extracellular Mg^{2+} was present in the medium. Subsequent studies with the non-NMDA receptor antagonist NBQX, alone and in combination with MK-801, indicate that LPS appears to release glutamate, which then acts at non-NMDA receptors to relieve the Mg^{2+} block of NMDA receptors present on noradrenergic axons, and thus permits NMDA receptor activation to occur. In contrast, neither NMDA nor non-NMDA ionotropic receptors appear to mediate LPS-evoked release of either glutamate or adenosine. In the case of adenosine, this is surprising because the activation of either NMDA or non-NMDA ionotropic receptors has been shown to release adenosine from cortical slices (Craig and White, 1993). It seems that LPS-evoked adenosine release involves processes other than those mediated by released glutamate. It is possible that LPS-evoked releases of glutamate and adenosine are mediated by other factors associated with immune/inflammatory responses, such as cytokines and/or prostanoids, which are released by LPS (Hua et al., 1996; Cannon et al., 1998; Martiney et al., 1998; Zhao and Schwartz, 1998).

It seems likely that the NA release evoked by LPS originates from noradrenergic axons present in the cortex, since it is blocked by NMDA receptor antagonists, and there is no clear evidence that NMDA receptors are expressed in glial cells (Verkhratsky and Steinhauser, 2000). However, the observation that LPS-evoked release of [^{3}H]NA is not Ca^{2+}-dependent would argue against this. In Ca^{2+}-dependence studies, although Ca^{2+} was

omitted from the beginning of the experiments, the involvement of intracellular Ca^{2+} mobilization could not be excluded. Unfortunately, intracellular Ca^{2+} scavengers could not be used in our experiments in order to maintain the viability of slices. The cellular sites of origin for glutamate and adenosine release are much less clear. LPS-evoked release of glutamate from cultures of microglia has been reported previously (Patrizio and Levi, 1994), and the AIDS viral coat protein gp120 has been shown to promote the rapid release of glutamate from cultured astrocytes (Vesce et al., 1997). These findings raise the possibility that the glutamate released in our study may have originated from glial, rather than neuronal, elements in the cortical slices. Indeed, LPS-evoked glutamate release is Ca^{2+}-independent, and it is not blocked by the Na^{+}-channel blocker TTX. These results suggest that glutamate release evoked by LPS is not neurogenic. Moreover, LPS-evoked glutamate release is diminished when glutamate transporters are blocked with tPDC, suggesting that release might occur via the reversal of high affinity, Na^{+}-dependent glutamate transporters. Although present in microglia and neurons, these glutamate transporters are particularly abundant in astrocytes, where they normally maintain low extracellular glutamate levels in the immediate vicinities of synapses. Preliminary research in our laboratory indicates that LPS causes the rapid release of glutamate from isolated and cultured rat brain astrocytes (Hardy and White, unpublished observations). These findings support the possibility that LPS acts directly on astrocytes to promote the release of glutamate, apparently via high affinity glutamate transporters. Because astrocytes are closely associated with synapses, the released glutamate could act at synaptic glutamatergic receptors and hence cause non-neurogenic and inappropriate excitation of the postsynaptic elements.

The present study demonstrates a direct link between inflammation and rapid excitation in the cortex. Because of its inflammatory rather than neuronal origins, this excitation is inappropriate and hence pathological. The triggering of this inappropriate excitation, especially in the immediate vicinity of a gram-negative bacterial infection in the brain, could cause some of the transient but serious neurological/psychiatric symptoms associated with these infections. If similar rapid, inappropriate excitatory events are triggered by other immunogens, such as β-amyloid in Alzheimer's disease, HIV coat proteins gp120 and gp41 in AIDS dementia, and myelin basic protein in multiple sclerosis, this could account for some of the acute but transient neurological and psychiatric symptoms observed with these disorders. In fact, there is evidence that gp120 does promote the activation of NMDA receptors in hypothalamic slices (Raber et al., 1997). In extreme cases, this excessive and inappropriate excitation might even lead to subsequent excitotoxicity and neurodegeneration (Mattson and Mark, 1996).

The observation that LPS also triggers a rapid release of adenosine has important implications. Numerous studies have shown that adenosine, acting at specific adenosine A_{2A} receptors on macrophages and neutrophils, decreases inflammatory responses (Cronstein, 1995; Eigler et al., 1997). In addition, extracellular adenosine can act at neuronal A_1 receptors to decrease both the release of glutamate from neurons and glutamate's excitatory actions postsynaptically (Dolphin and Archer, 1983; Pasquini et al., 1987). Drugs that promote the formation of extracellular adenosine and/or potentiate its cellular actions may provide protection against this inappropriate excitation produced by immunogens in the brain.

PART II

MECHANISMS OF LPS-EVOKED RELEASES OF GLUTAMATE, [^{3}H]NA AND ADENOSINE FROM RAT BRAIN CORTICAL SLICES

ABSTRACT

The possible mechanisms of LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices were investigated in this study. Inhibition of nitric oxide synthase (NOS) with the nNOS inhibitor 7-NI, the eNOS inhibitor L-NIO, the iNOS inhibitor L-NIL, or the non-specific NOS inhibitor L-NAME had no effect on LPS-evoked release of glutamate, suggesting that NO formation does not play a role in LPS-evoked release of glutamate in cortical slices. However, LPS-evoked release of [^{3}H]NA was significantly diminished by both L-NIO and L-NIL, as well as by L-NAME, suggesting that NO may be involved in this process. Pretreatment with L-NAME also reduced the release of adenosine evoked by LPS. Scavenging free radicals with the combination of superoxide dismutase (SOD) and catalase, or pretreatment of slices with the antioxidant d-α-tocopherol or its water soluble form (Trolox) failed to modulate LPS-evoked releases of glutamate, [^{3}H]NA or adenosine, indicating that neither oxygen free radicals nor $ONOO^-$ are involved in these releasing processes. Inhibition of cyclooxygenase (COX) with the non-selective COX inhibitor indomethacin and the selective COX-2 inhibitor NS-398 had no effects on LPS-evoked release of glutamate. Because the selective COX-1 inhibitor resveratrol produces fluorescence, the role of COX-1 on LPS-evoked glutamate release could not be assessed. LPS-evoked release of [^{3}H]NA was diminished by both resveratrol and NS-398, as well as by indomethacin, suggesting that both isoforms of COX may be involved. NS-398 reduced, whereas resveratrol increased, the release of adenosine evoked by LPS, indicating a complex role for COX. Inhibition of lipoxygenase with NDGA reduced LPS-evoked release of [^{3}H]NA, while it increased LPS-evoked release of adenosine, indicating a differential

regulation of these processes. Inhibition of PLA_2 with p-BPB had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine. Finally, IL-1β, TNF-α, or a combination of both did not release glutamate, [^{3}H]NA or adenosine, suggesting that these cytokines do not mediate LPS-evoked release.

INTRODUCTION

Exposure of rat parietal cortical slices to the bacterial endotoxin LPS results in very rapid releases of the neurotransmitters glutamate and NA and of the neuromodulator adenosine. LPS-evoked release of [^{3}H]NA, but not of glutamate or adenosine, appears to be partly due to the released glutamate acting at ionotropic receptors on the noradrenergic axons present in the cortical slices (See Part I, this Chapter). Numerous studies have shown that LPS releases various inflammatory mediators, including NO, oxygen free radicals, PGs and cytokines, from the brain (Hopkins and Rothwell, 1995; Satta et al., 1998; Balboa et al., 1999; Patel et al., 1999 Yamada et al., 1999). Moreover, these inflammatory mediators are associated with neurodegenerative diseases (Gilman et al., 1994; Sequeira et al., 1997; McNaught and Brown, 1998; Monda et al., 1998, see Chapter 1 for review). The current study was undertaken to investigate the possible involvement of these mediators in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat brain cortical slices.

MATERIALS AND METHODS

Materials, preparation of cortical slices, and measurement of glutamate, [^{3}H]NA and adenosine are described in Chapter 2.

RESULTS

Effect of NOS inhibitors on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. In agreement with the results of Part I, repeated exposure of slices to LPS did not reveal any tachyphylaxis (Fig. 4-11). This allowed us to assess the effects of various drugs on LPS-evoked releases of these substances by applying drugs before the second stimulation. As shown in Figure 4-11, pretreatment of the slices with 50 μM of the non-selective NOS inhibitor L-NAME, which has been commonly used by others to inhibit NOS activity (Jones et al., 1995), had no effect on LPS-evoked release of glutamate (Fig. 4-11A). However, the rates of release and the total LPS-evoked releases of [^{3}H]NA and adenosine were significantly decreased by L-NAME (Fig. 4-11B & C). These data indicate that NO formation may be involved in LPS-evoked releases of NA and adenosine. To test this, we tried to measure NO formation by measuring its metabolite, nitrite, using Griess Reagent (protocol provided by Sigma). Unfortunately, nitrite could not be detected in the superfusate due to low sensitivity of this method. In order to assess the roles of different NOS isoform inhibitors on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine, we tested the effects of the nNOS-inhibitor 7-NI, the eNOS inhibitor L-NIO and the iNOS inhibitor L-NIL, at concentrations that have been used by others to selectively inhibit NOS isoform activities (Jones et al., 1995; Cox and Johnson, 1998). Pretreatment of slices with the nNOS inhibitor 7-NI (50 μM) had no effect on LPS-evoked releases of glutamate and adenosine in cortical slices (Fig. 4-12A, C). Although 7-NI appeared to shift the time course of LPS-evoked [^{3}H]NA release slightly to the right, it had no effect on total LPS-evoked release of [^{3}H]NA (Fig. 4-12B). In contrast, both the eNOS inhibitor L-NIO (100 μM)

and the iNOS inhibitor L-NIL (10 μM) significantly decreased LPS-evoked [^{3}H]NA release (Fig. 4-13B). The eNOS inhibitor L-NIO appeared to increase the basal release of glutamate (Fig. 4-13A); however, because the other bath contained L-NIL, a comparison between the two was not possible. Neither of these inhibitors had any effects on LPS-evoked releases of glutamate and adenosine (Fig. 4-13A & C). These findings indicate that NO formation is not involved in LPS-evoked release of glutamate. In contrast, LPS-evoked releases of NA and adenosine appear to involve the formation of NO.

Effect of free radical scavengers on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Oxygen free radicals have been implicated in excitatory neurotransmitter release and associated neurodegeneration (Pellegrini-Giampietro et al., 1988; Jesberger and Richardson, 1991). Therefore, we examined the effects of free radical scavengers on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. As shown in Figure 4-14, although the combination of SOD and catalase appears to decrease the basal release of glutamate (Fig. 4-14A, left panel), it did not affect LPS-evoked releases of glutamate, [^{3}H]NA or adenosine (Fig. 4-14). Since both SOD and catalase are cell impermeable free radical scavengers, which only break down free radicals in the extracellular space (Jesberger and Richardson, 1991), we tested the effects of the cell permeable antioxidant d-α-tocopherol and the water soluble compound Trolox on LPS-evoked releases. Neither drug had any effects on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine (Fig. 4-15; Fig. 4-16). These results suggest that oxygen free radicals are not involved in LPS-evoked releases of glutamate, NA or adenosine from rat cortical slices.

Fig. 4-11. Effects of the non-selective NOS inhibitor L-NAME (50 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when L-NAME was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 5 experiments. ***Pretreatment of slices with L-NAME caused a small but significant decrease in LPS-evoked [^{3}H]NA release. L-NAME also diminished LPS-evoked release of adenosine from rat cortical slices.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t* test.

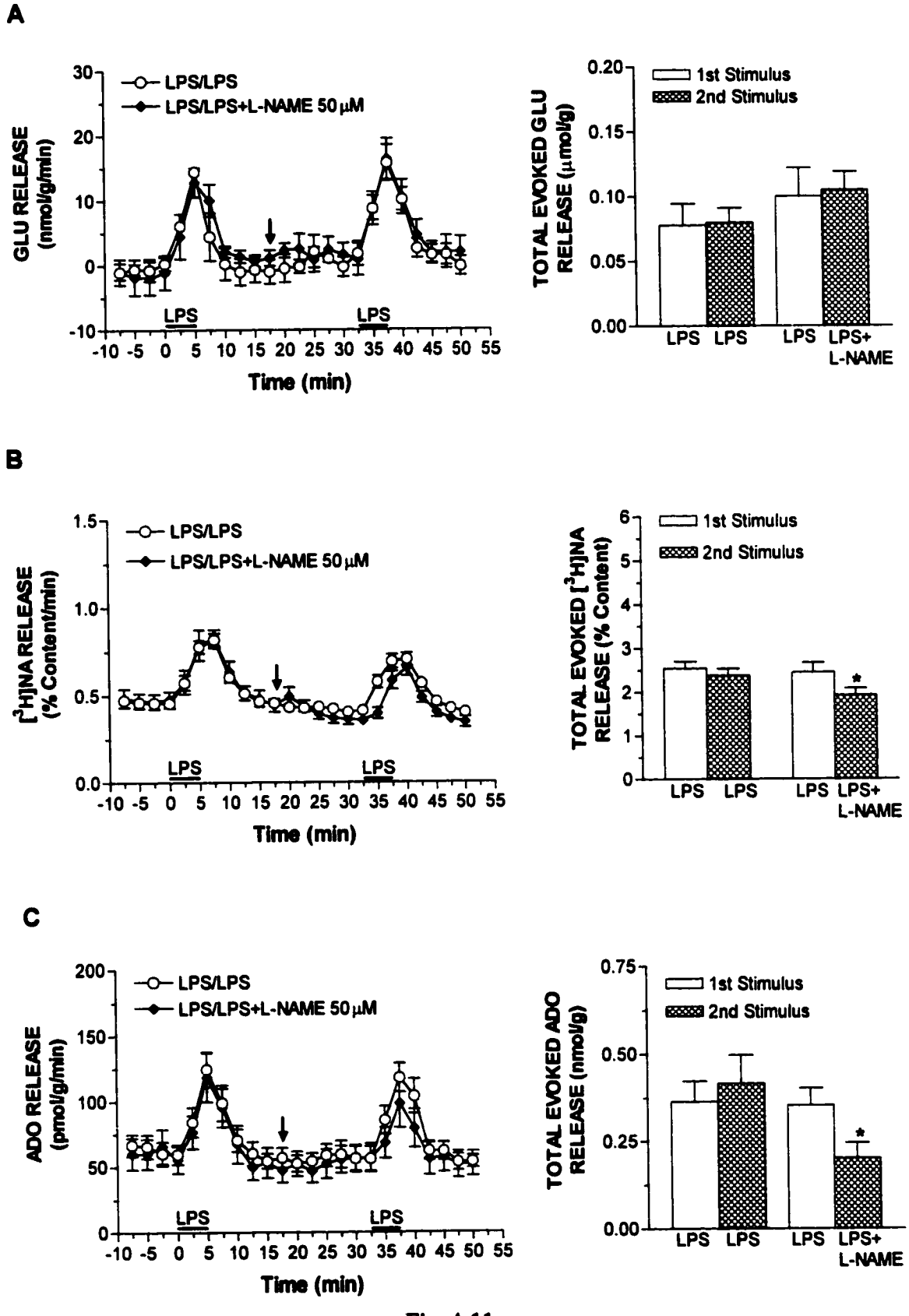

A
LPS/LPS
LPS/LPS+L-NAME 50 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS LPS LPS+ L-NAME
B
LPS/LPS
LPS/LPS+L-NAME 50 μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS LPS LPS+ L-NAME
C
LPS/LPS
LPS/LPS+L-NAME 50 μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS LPS LPS+ L-NAME

Fig. 4-11

Fig. 4-12. Effects of the nNOS inhibitor 7-NI (50 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-course for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when 7-NI was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Although 7-NI appeared to shift the time course of LPS-evoked [^{3}H]NA release to the right, it did not affect the total evoked release of [^{3}H]NA. 7-NI had no effect on LPS-evoked release of glutamate and adenosine.***

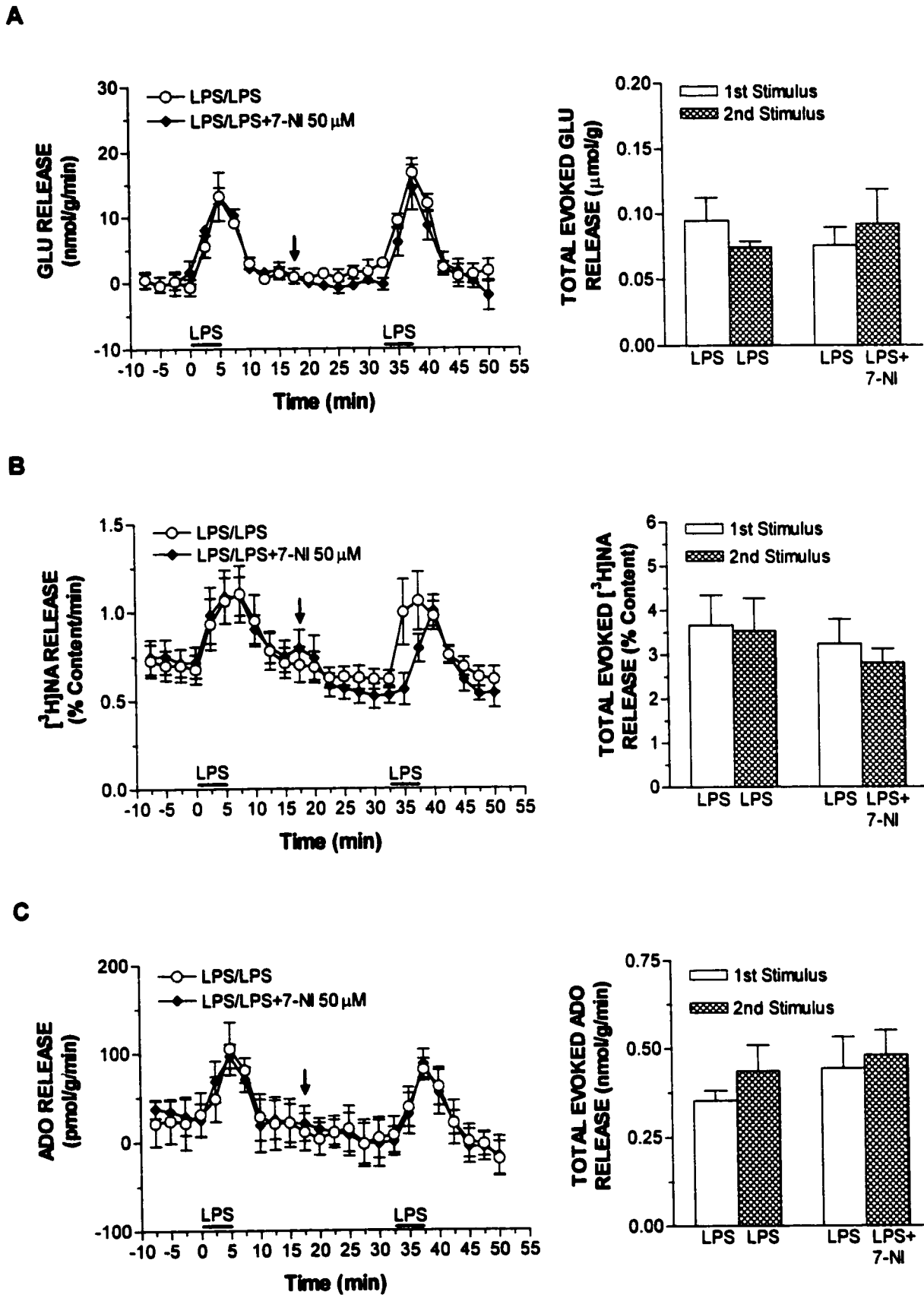

A
LPS/LPS
LPS/LPS+7-NI 50 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+ 7-NI
B
LPS/LPS
LPS/LPS+7-NI 50 μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS
LPS LPS+ 7-NI
C
LPS/LPS
LPS/LPS+7-NI 50 μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g/min)
LPS LPS
LPS LPS+ 7-NI

Fig. 4-12

Fig. 4-13. Effects of the iNOS inhibitor L-NIL (10 μM) and the eNOS inhibitor L-NIO (100 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when L-NIL or L-NIO was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Both L-NIL and L-NIO decreased LPS-evoked release of [^{3}H]NA but had no effect on LPS-evoked releases of glutamate and adenosine.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t* test.

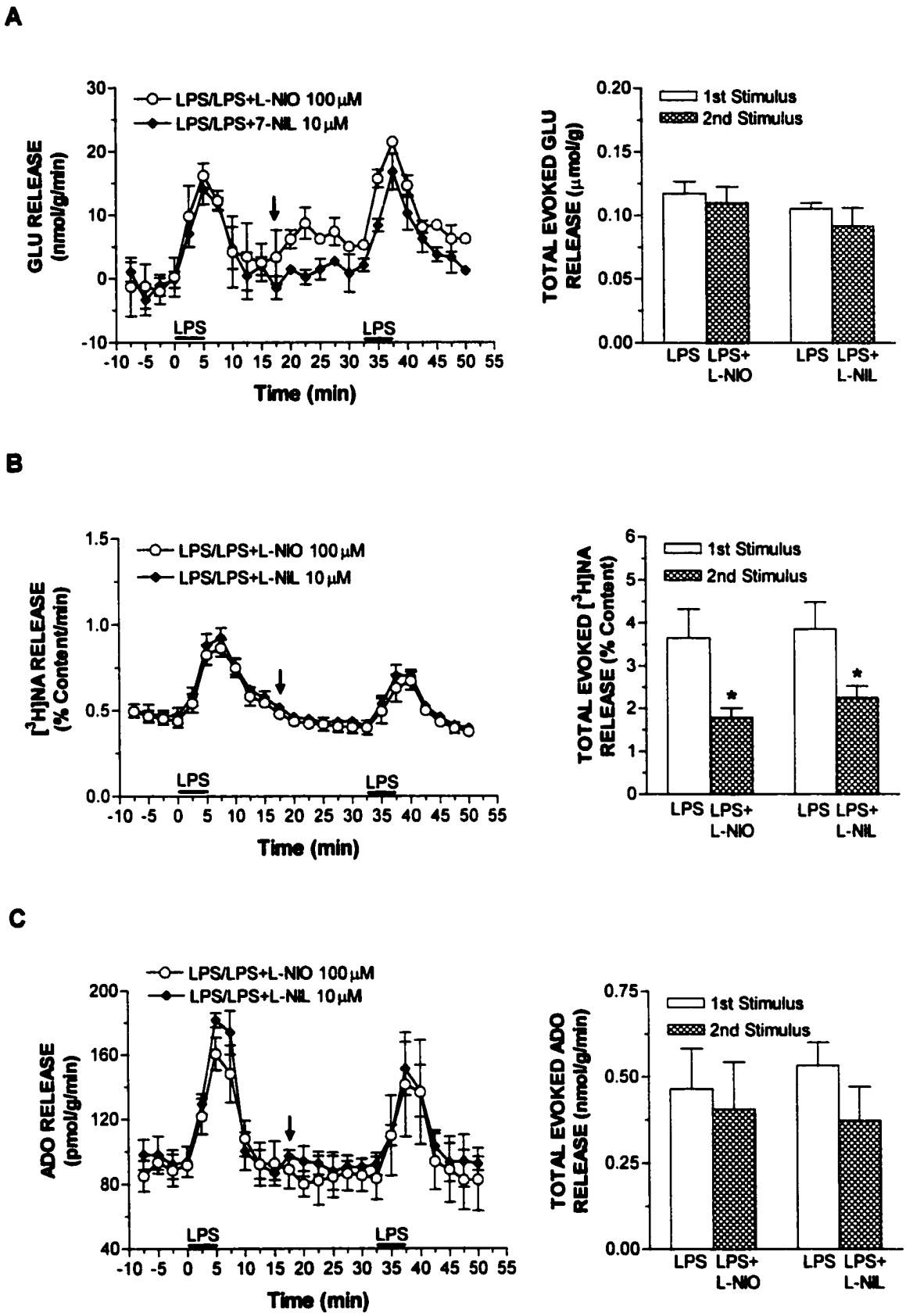

A
LPS/LPS+L-NIO 100 μM
LPS/LPS+7-NIL 10 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS+ L-NIO
LPS LPS+ L-NIL
B
LPS/LPS+L-NIO 100 μM
LPS/LPS+L-NIL 10 μM
[3H]NA RELEASE (% Content/min)
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
C
LPS/LPS+L-NIO 100 μM
LPS/LPS+L-NIL 10 μM
ADO RELEASE (pmol/g/min)
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g/min)

Fig. 4-13

Fig. 4-14. Effects of the combination of SOD (250 u/ml) and catalase (3250 u/ml) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when SOD and catalase were introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Although the combination of SOD and catalase appears to decrease the basal release of glutamate, it had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices.***

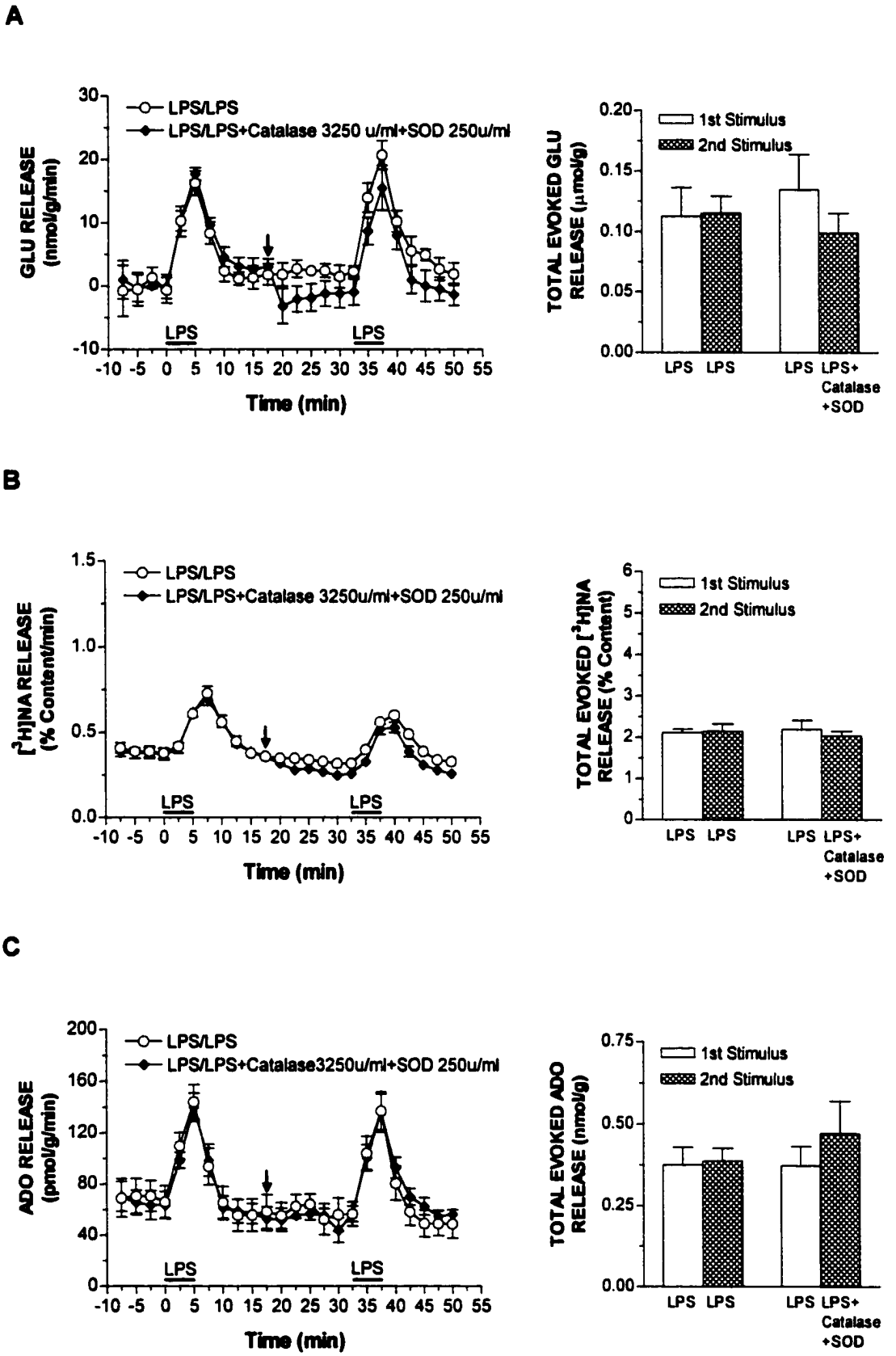

A
LPS/LPS
LPS/LPS+Catalase 3250 u/ml+SOD 250u/ml
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS LPS LPS+ Catalase +SOD
B
LPS/LPS
LPS/LPS+Catalase 3250u/ml+SOD 250u/ml
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS LPS LPS+ Catalase +SOD
C
LPS/LPS
LPS/LPS+Catalase3250u/ml+SOD 250u/ml
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS LPS LPS+ Catalase +SOD

Fig. 4-14

Fig. 4-15. Effects of the antioxidant t-α-tocopherol (200 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when tocopherol was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***D-α-Tocopherol had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices.***

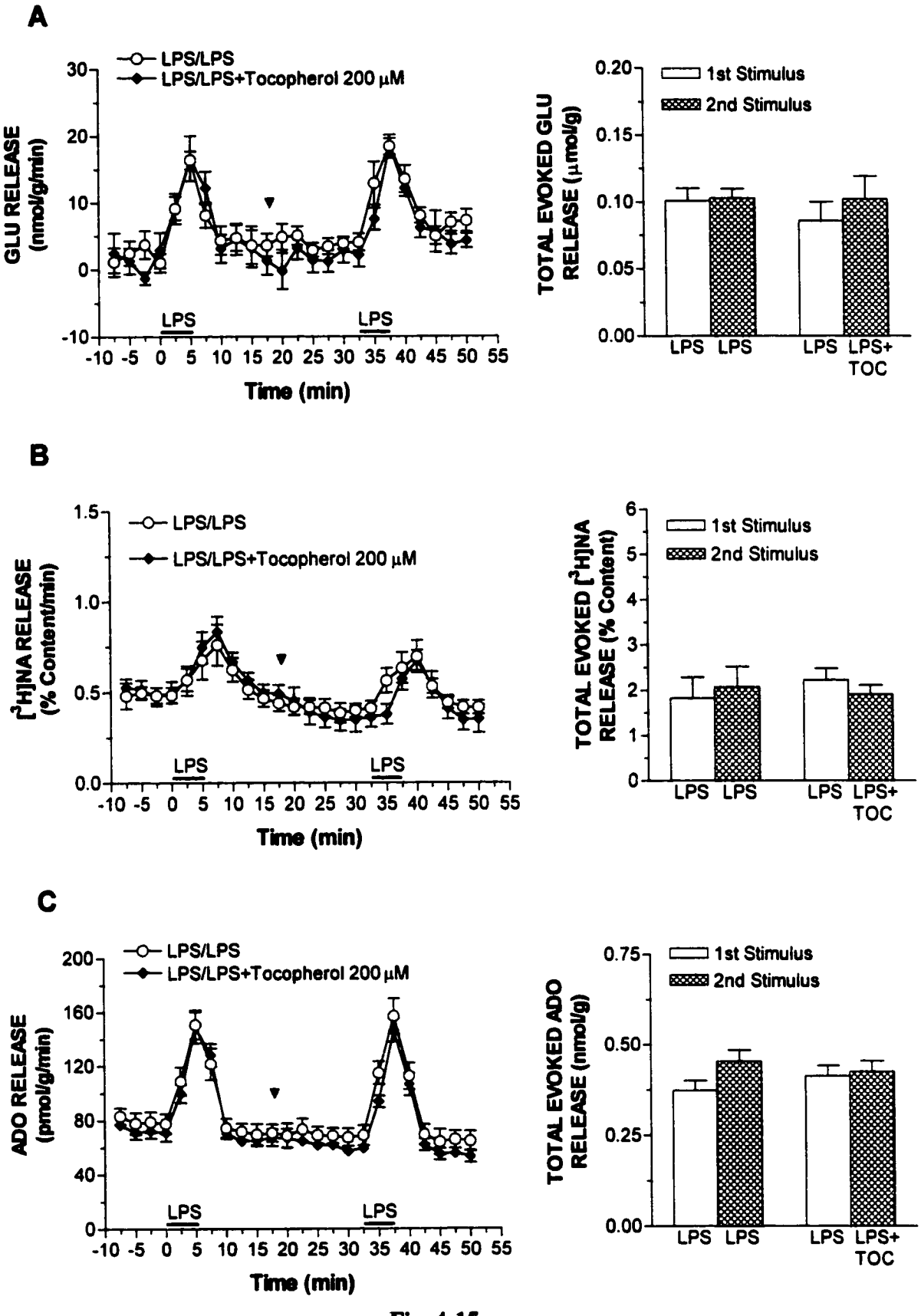

A
LPS/LPS
LPS/LPS+Tocopherol 200 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+ TOC
B
LPS/LPS
LPS/LPS+Tocopherol 200 μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS
LPS LPS+ TOC
C
LPS/LPS
LPS/LPS+Tocopherol 200 μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
LPS LPS+ TOC

Fig. 4-15

Fig. 4-16. Effects of the water soluble antioxidant Trolox (200 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when Trolox was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Trolox had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices.***

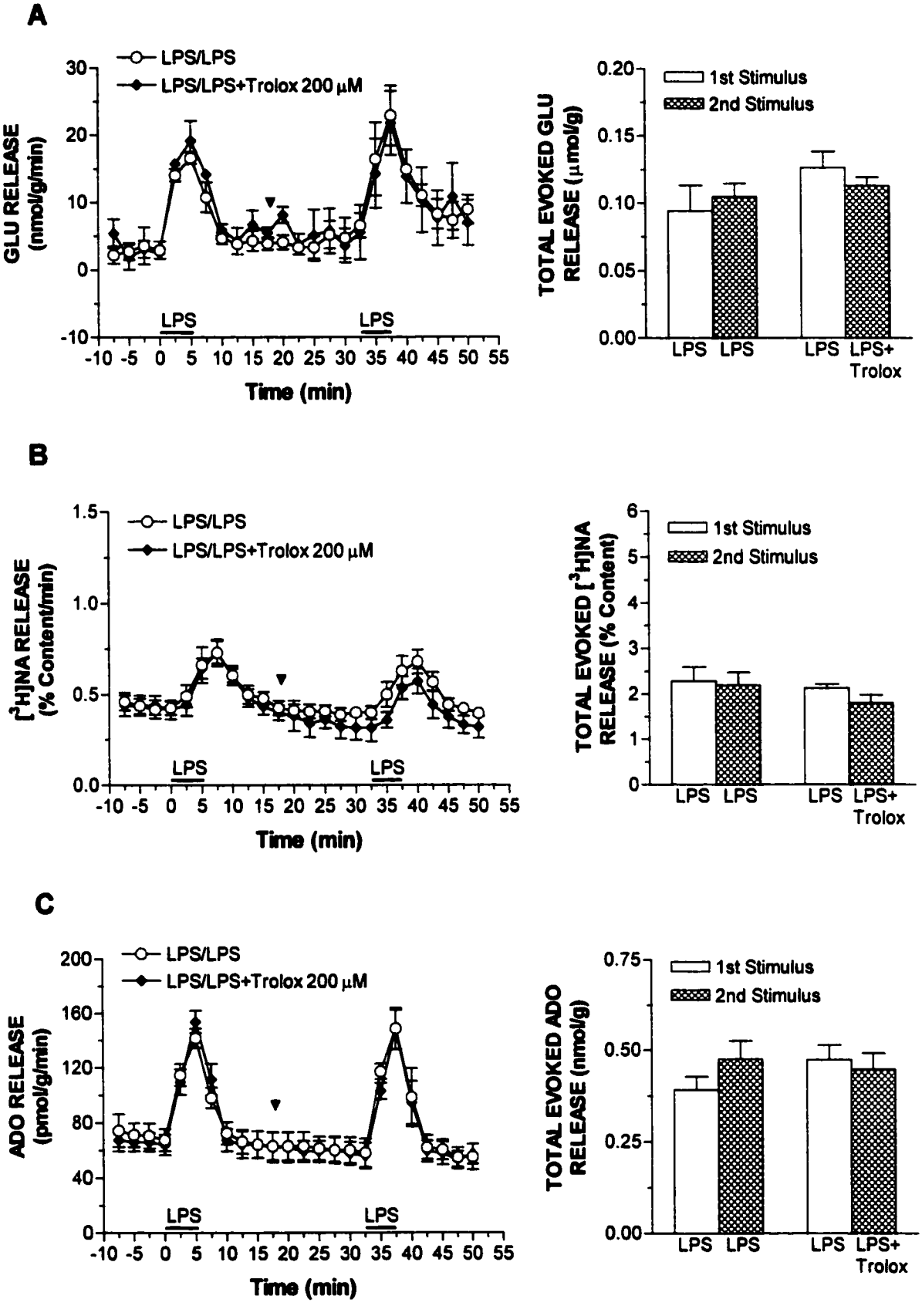

A
LPS/LPS
LPS/LPS+Trolox 200 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS LPS LPS+ Trolox
B
LPS/LPS
LPS/LPS+Trolox 200 μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
LPS LPS LPS LPS+ Trolox
C
LPS/LPS
LPS/LPS+Trolox 200 μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS LPS LPS+ Trolox

Fig. 4-16

Effect of COX inhibitors on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. To test whether or not prostaglandins are involved in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices, we studied the effects of various inhibitors of cyclooxygenases. The concentrations of drugs used in this section were comparable to those used by others under similar experimental conditions. Pretreatment of slices with the non-selective COX inhibitor indomethacin (100 μM) had no effect on LPS-evoked releases of glutamate or adenosine (Fig. 4-17A &C). However, the rate of release and the total LPS-evoked release of [^{3}H]NA were both significantly decreased by indomethacin (Fig. 4-17B). Indomethacin also tended to decrease the basal release of [^{3}H]NA (Fig. 4-17B, left panel), but with no statistical difference. In order to assess the roles of different COX isoforms in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine, we tested the effects of selective COX-1 and COX-2 inhibition on LPS-evoked releases. Pretreatment of slices with 10 μM NS-398, which is a highly selective COX-2 inhibitor (Futaki et al., 1994), had no effect on LPS-evoked release of glutamate in cortical slices (Fig. 4-18A). However, inhibition of COX-2 with NS-398 significantly decreased total evoked releases of both [^{3}H]NA and adenosine (Fig. 4-18B &C). The COX-1 inhibitor resveratrol (100 μM) produces fluorescence and, therefore, interferes with the glutamate assay. Hence, an evaluation of the effect of this drug on LPS-evoked glutamate release was not possible. Inhibition of COX-1 with resveratrol or inhibition of both COX-1 and COX-2 with a combination of resveratrol and NS-398 dramatically diminished LPS-evoked release of [^{3}H]NA (Fig. 4-19A). In contrast to the effect of COX-2 inhibition on adenosine release, both resveratrol and a combination of resveratrol and NS-398 significantly increased LPS-evoked release of

adenosine (Fig. 4-19B). These findings suggest that prostaglandins may play positive roles in LPS-evoked [^{3}H]NA release, and no role in glutamate release. The possible role of prostaglandins in LPS-evoked release of adenosine appears to be complex.

Effects of PLA_2 and lipoxygenase inhibition on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. In addition to prostaglandins, other metabolites of arachidonic acid, such as leukotrienes, have also been implicated in brain inflammation and associated neurodegeneration (Tang et al., 1997). Therefore, we examined the effects of inhibitors of PLA_2 and lipoxygenase on LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Drug concentrations used in this section were comparable to those used by others to inhibit their respective enzymes (Peterson et al., 1995). Inhibition of PLA_2 with 10 μM p-BPB did not affect LPS-evoked releases of glutamate, [^{3}H]NA or adenosine (Fig. 4-20), suggesting that arachidonic acid synthesis itself is not likely involved in LPS-evoked releases of these substances. Because the lipoxygenase inhibitor NDGA itself produces fluorescence, it interfered with the glutamate assay. Thus we could not examine the effect of this drug on LPS-evoked release of glutamate. However, inhibition of lipoxygenase with 10 μM NDGA decreased LPS-evoked release of [^{3}H]NA significantly (Fig. 4-20B). In contrast, NDGA increased LPS-evoked release of adenosine (Fig. 4-20C).

Fig. 4-17. Effects of the non-selective COX inhibitor indomethacin (100 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when indomethacin (Indo) was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Indomethacin significantly diminished LPS-evoked [^{3}H]NA release but had no effect on LPS-evoked glutamate or adenosine release. Indomethacin also tended to decrease basal release of [^{3}H]NA.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t* test.

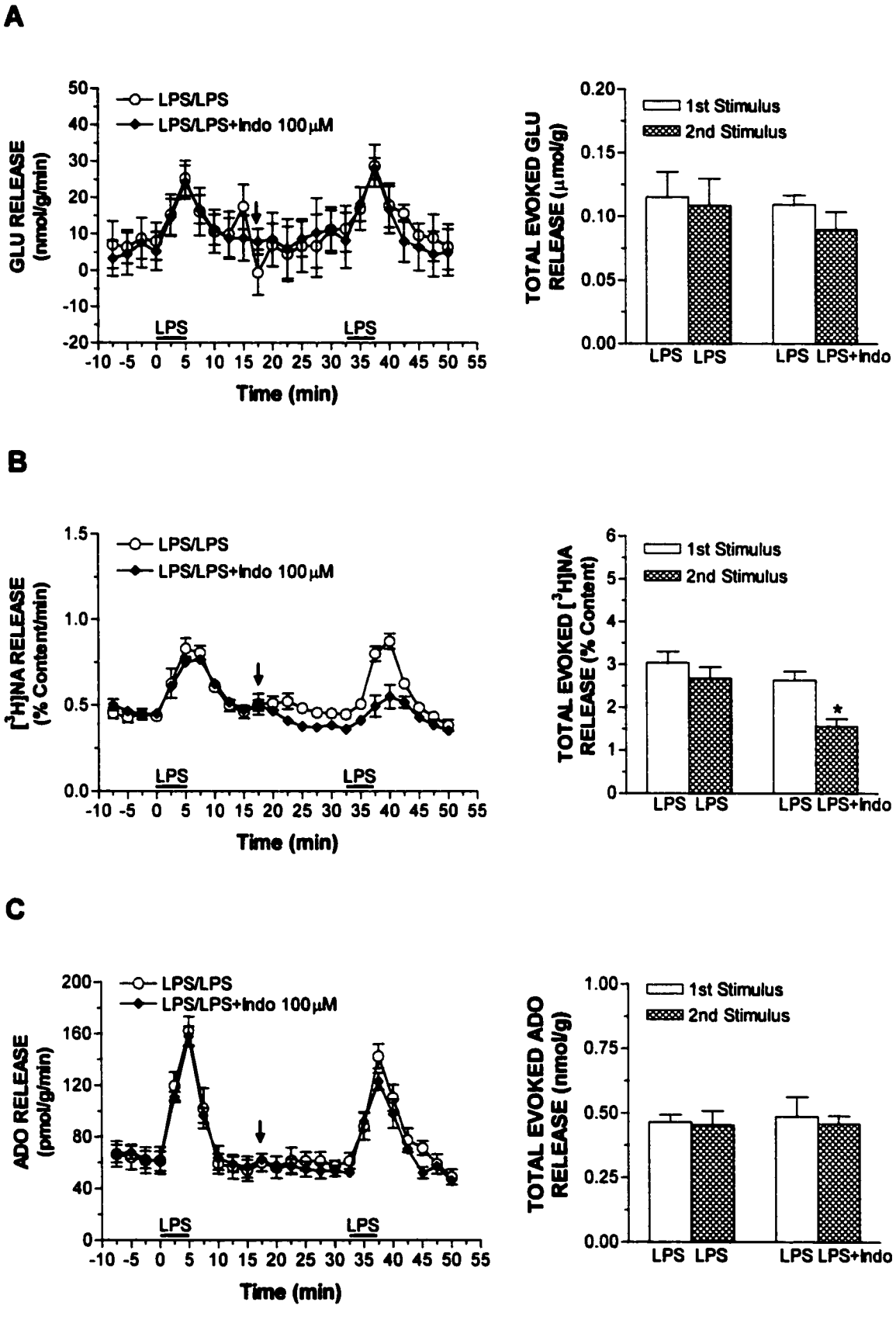

A
LPS/LPS
LPS/LPS+Indo 100μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
LPS LPS
LPS LPS+Indo
B
LPS/LPS
LPS/LPS+Indo 100μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% Content)
*
LPS LPS
LPS LPS+Indo
C
LPS/LPS
LPS/LPS+Indo 100μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)
LPS LPS
LPS LPS+Indo

Fig. 4-17

Fig. 4-18. Effects of the COX-2 inhibitor NS-398 (10 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when NS-398 was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***NS-398 significantly decreased LPS-evoked releases of [^{3}H]NA and adenosine but had no effect on LPS-evoked release of glutamate from rat cortical slices.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t* test.

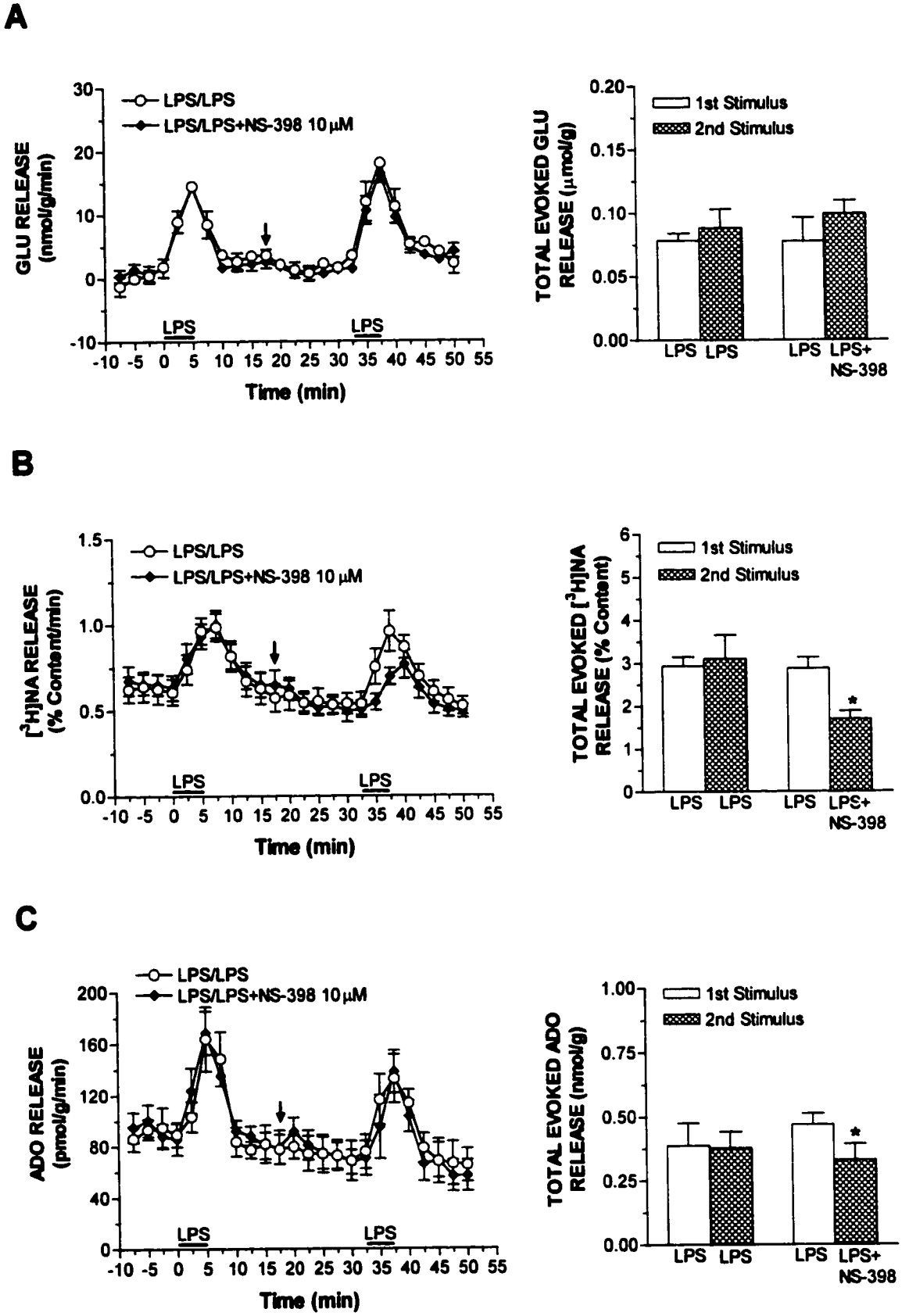
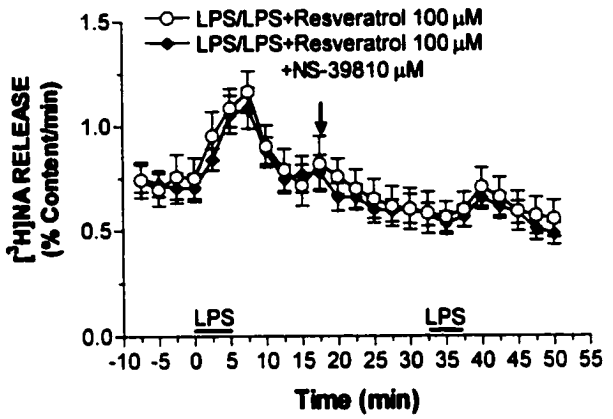
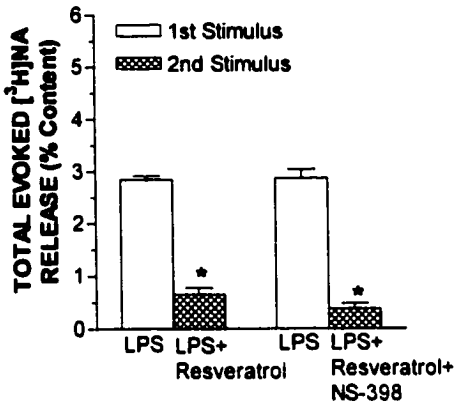

A
LPS/LPS
LPS/LPS+NS-398 10 μM
GLU RELEASE (nmol/g/min)
LPS
LPS
Time (min)
TOTAL EVOKED GLU RELEASE (μmol/g)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ NS-398
B
LPS/LPS
LPS/LPS+NS-398 10 μM
[3H]NA RELEASE (% Content/min)
LPS
LPS
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ NS-398
C
LPS/LPS
LPS/LPS+NS-398 10 μM
ADO RELEASE (pmol/g/min)
LPS
LPS
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
1st Stimulus
2nd Stimulus
LPS LPS
LPS LPS+ NS-398

Fig. 4-18

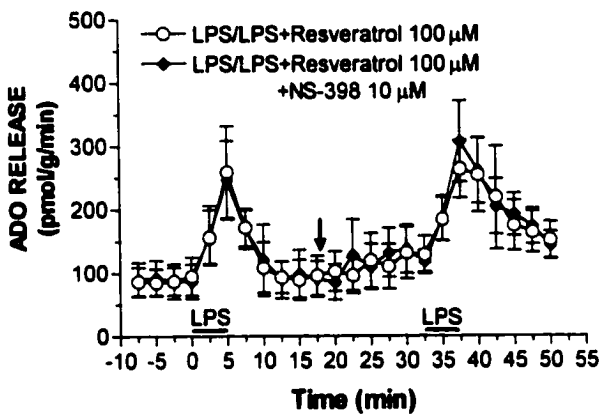
Fig. 4-19. Effects of the COX-1 inhibitor resveratrol (100 μM) and the combination of resveratrol and NS-398 on LPS (50 μg/ml)-evoked [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of [^{3}H]NA, (B) release of adenosine (ADO). Left panels show the time-courses for releases after repeated exposure to LPS for 5 min each. Arrows indicate the time when resveratrol or resveratrol + NS-398 was introduced into the superfusion medium. Right panels represent total evoked releases after LPS stimulation. Values are mean ± S.E.M. from 4 experiments. ***Resveratrol alone or in combination with NS-398 significantly decreased LPS-evoked [^{3}H]NA release while they increased LPS-evoked adenosine release from rat cortical slices.*** * $p<0.05$ compared with the first stimulation (LPS alone), Student's paired *t* test.

A


LPS/LPS+Resveratrol 100 μM
LPS/LPS+Resveratrol 100 μM
+NS-39810 μM
[3H]NA RELEASE
(% Content/min)
LPS
LPS
Time (min)


1st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA
RELEASE (% Content)
LPS
LPS+
Resveratrol
LPS
LPS+
Resveratrol+
NS-398

B


LPS/LPS+Resveratrol 100 μM
LPS/LPS+Resveratrol 100 μM
+NS-398 10 μM
ADO RELEASE
(pmol/g/min)
LPS
LPS
Time (min)

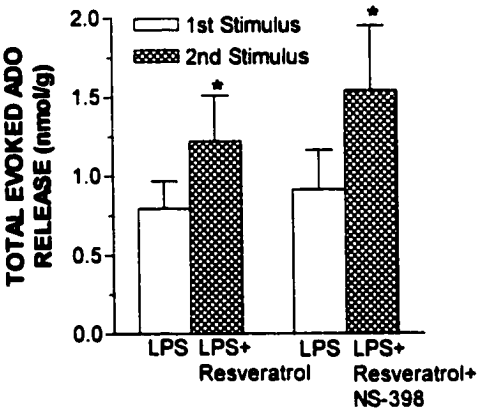

1st Stimulus
2nd Stimulus
TOTAL EVOKED ADO
RELEASE (nmol/g)
LPS
LPS+
Resveratrol
LPS
LPS+
Resveratrol+
NS-398

Fig. 4-19

Fig. 4-20. Effects of the PLA_2 inhibitor p-BPB (10 μM) and the lipoxygenase inhibitor NDGA (10 μM) on LPS (50 μg/ml)-evoked glutamate, [^{3}H]NA and adenosine release from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Values are mean ± S.E.M. from 4 experiments. ***BPB had no effect on LPS-evoked releases of glutamate, [^{3}H]NA or adenosine. NDGA significantly decreased LPS-evoked release of [^{3}H]NA while it increased LPS-evoked release of adenosine from rat cortical slices.*** * $p<0.05$ compared with LPS alone, Student's paired *t* test.

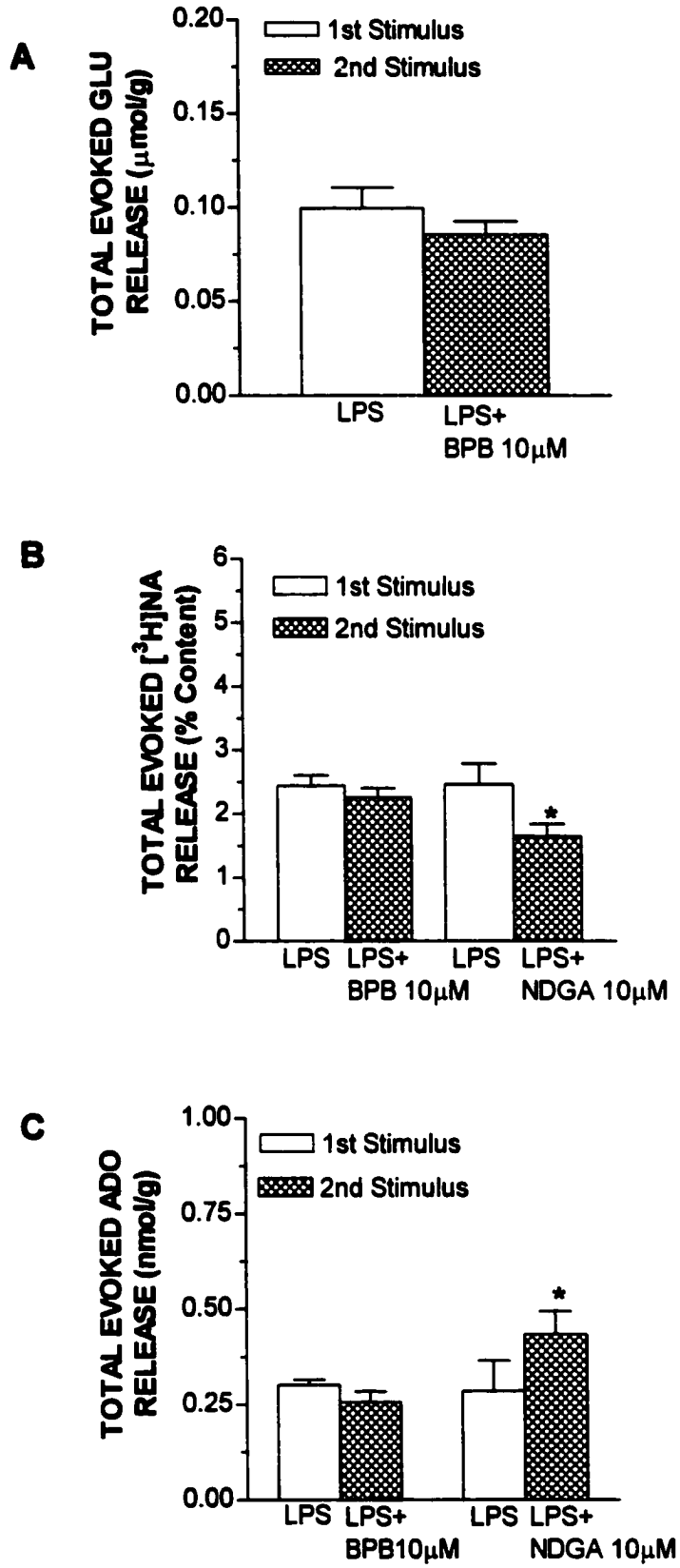

A
TOTAL EVOKED GLU RELEASE (μmol/g)
0.20
0.15
0.10
0.05
0.00
1st Stimulus
2nd Stimulus
LPS
LPS+ BPB 10μM
B
TOTAL EVOKED [3H]NA RELEASE (% Content)
6
5
4
3
2
1
0
1st Stimulus
2nd Stimulus
*
LPS
LPS+ BPB 10μM
LPS
LPS+ NDGA 10μM
C
TOTAL EVOKED ADO RELEASE (nmol/g)
1.00
0.75
0.50
0.25
0.00
1st Stimulus
2nd Stimulus
*
LPS
LPS+ BPB10μM
LPS
LPS+ NDGA 10μM

Fig. 4-20

Effects of inflammatory cytokines on the releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Cytokines have been implicated in neurodegeneration and neurotoxicity (Giulian and Vaca, 1993) on one hand and neuroprotection on the other (Cheng et al., 1994). Therefore, we investigated the roles of inflammatory cytokines in mediating the releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Compared to 50 μg/ml LPS, 10 ng/ml IL-1β, 50 ng/ml TNF-α, or 20 ng/ml IL-6 did not release glutamate, [^{3}H]NA or adenosine (Fig. 4-21). It has been shown that synergism between cytokines may amplify their neurotoxicity (Skaper et al., 1995). Therefore, we tested whether a combination of 50 ng/ml TNF-α and 10 ng/ml IL-1β might release glutamate, [^{3}H]NA or adenosine from rat cortical slices. As shown in Figure 4-21, the combination of IL-1β and TNF-α also did not release glutamate, [^{3}H]NA or adenosine from rat cortical slices.

Fig. 4-21. Effects of IL-1β (10 ng/ml), IL-6 (20 ng/ml) and TNF-α (50 ng/ml) on the releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Slices were exposed to 50 μg/ml LPS or different concentrations of cytokines for 5 min. Total evoked releases were obtained as described in Chapter 2. Values are means ± S.E.M. from 4 experiments. C***ompared with 50 μg/ml LPS, these cytokines did not release glutamate, [^{3}H]NA or adenosine from rat cortical slices.***

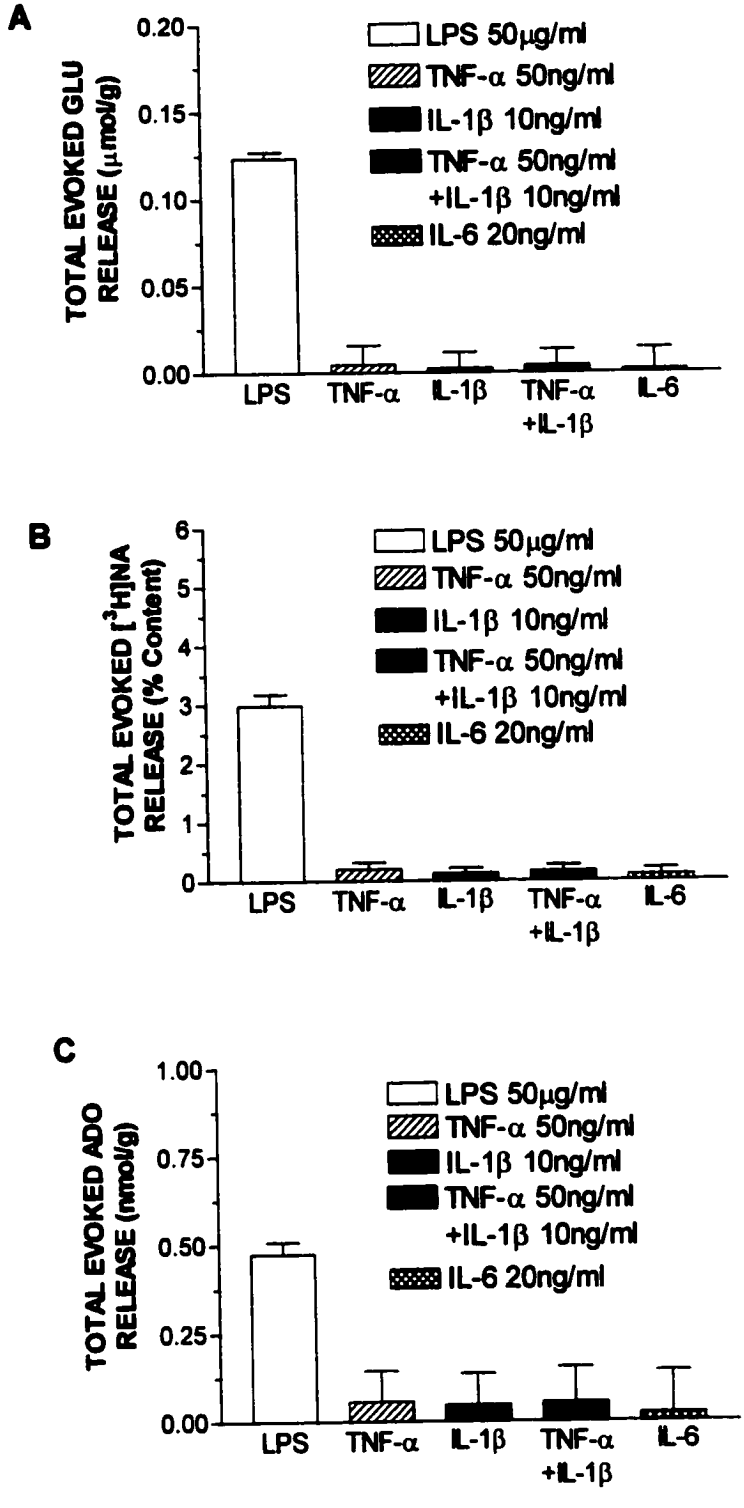

A
TOTAL EVOKED GLU RELEASE (μmol/g)
0.20
0.15
0.10
0.05
0.00
LPS 50μg/ml
TNF-α 50ng/ml
IL-1β 10ng/ml
TNF-α 50ng/ml +IL-1β 10ng/ml
IL-6 20ng/ml
LPS
TNF-α
IL-1β
TNF-α +IL-1β
IL-6
B
TOTAL EVOKED [3H]NA RELEASE (% Content)
6
5
4
3
2
1
0
LPS 50μg/ml
TNF-α 50ng/ml
IL-1β 10ng/ml
TNF-α 50ng/ml +IL-1β 10ng/ml
IL-6 20ng/ml
LPS
TNF-α
IL-1β
TNF-α +IL-1β
IL-6
C
TOTAL EVOKED ADO RELEASE (nmol/g)
1.00
0.75
0.50
0.25
0.00
LPS 50μg/ml
TNF-α 50ng/ml
IL-1β 10ng/ml
TNF-α 50ng/ml +IL-1β 10ng/ml
IL-6 20ng/ml
LPS
TNF-α
IL-1β
TNF-α +IL-1β
IL-6

Fig. 4-21

DISCUSSION

Roles of nitric oxide in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Three major isoforms of NOS have been identified in the body; endothelial NOS (eNOS) and neuronal NOS (nNOS), which are Ca^{2+}/calmodulin-dependent, and inducible or immunological NOS (iNOS), which is Ca^{2+}/calmodulin-independent (Bredt and Synder, 1994). In the present study, none of the NOS inhibitors employed affected LPS-evoked release of glutamate, suggesting that NO is not likely involved in this process. However, the non-selective NOS inhibitor L-NAME significantly decreased LPS-evoked releases of [^{3}H]NA and adenosine, indicating that NO formation may be involved in LPS-evoked release of [^{3}H]NA and adenosine. The eNOS inhibitor L-NIO and the iNOS inhibitor L-NIL also diminished LPS-evoked release of [^{3}H]NA. eNOS is widely distributed in vascular endothelial cells as well as in astrocytes (Gabbott and Bacon, 1996), making it a likely candidate for mediating the increased NO production after LPS stimulation in the cortex. The observation that the nNOS inhibitor 7-NI had no effect on LPS-evoked release of [^{3}H]NA is surprising because LPS-evoked [^{3}H]NA release is partly due to the activation of NMDA receptors, and NMDA receptor activation activates nNOS (Jones et al., 1995). One possible explanation for this is that the concentration of the nNOS inhibitor 7-NI (50 μM) may be too low to completely inhibit nNOS activity under our experimental conditions. However, in subsequent studies 100 μM 7-NI also did not affect LPS-evoked [^{3}H]NA. In addition, even lower concentrations of 7-NI (27 μM) have been used to inhibit nNOS in brain slices (Litt et al., 1999). Therefore, the concentration of the inhibitor does not seem

to contribute to this observation. Finally, the observation that the iNOS inhibitor also decreased LPS-evoked released [^{3}H]NA is unexpected. Although brain iNOS activity is elevated following systemic treatment with LPS in the rat (Satta et al., 1998), iNOS is not expressed in normal brain. This observation could merely reflect the poor selectivity of this drug.

LPS-evoked release of [^{3}H]NA is partly due to the released glutamate acting at both NMDA and non-NMDA ionotropic glutamate receptors (see Chapter 3). NO donors enhance while NOS inhibitors diminish NMDA-evoked [^{3}H]NA release from rat hippocampal slices (Jones et al., 1995). Moreover, NMDA-evoked [^{14}C]GABA and [^{3}H]acetylcholine releases from rat striatal slices were partly blocked by the NOS inhibitor nitroarginine (Hanania and Johnson, 1998), suggesting that NMDA evoked neurotransmitter release is at least in part mediated by the endogenous formation of NO. Finally, Hall et al. (1998), using in vivo microdialysis, showed that the NMDA-evoked excitatory activities in rat locus coeruleus were abolished by the non-selective NOS inhibitor L-NAME, also suggesting a role for NO formation in NMDA responses in the brain. Thus, it seems likely that LPS evokes the release of glutamate, which then acts at ionotropic glutamate receptors to activate NOS and produce NO, which in turn mediates [^{3}H]NA release from noradrenergic axons present in the cortex.

The non-selective NOS inhibitor L-NAME also diminished LPS-evoked release of adenosine, suggesting that NO formation is involved in LPS-evoked adenosine release. The precise mechanism by which NO mediates the LPS-evoked release of adenosine remains unclear because, unlike the LPS-evoked release of NA, LPS-evoked adenosine release is not triggered by released glutamate acting at its ionotropic receptors (see Part

I). The most significant observation was that the activation of NOS does not appear to play an essential role in the LPS-evoked release of glutamate.

Role of free radicals in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Oxygen free radicals have been shown to increase the release of excitatory amino acids from rat hippocampal slices (Pellegrini-Giampetro et al., 1988). Free radicals also inhibit glutamate uptake from cultured rat astrocytes (Volterra et al., 1994), and are associated with glutamate release in traumatic brain injury (Globus et al., 1995). In addition, reactive oxygen species can combine with NO to form the highly reactive and toxic free radical $ONOO^-$ (Beckman, 1994; Ducrocq et al., 1999). However, scavenging free radicals by the combination of SOD and catalase did not diminish the LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices in the present study. Both SOD and catalase are cell impermeable free radical scavengers (Jesberger and Richardson, 1991). However, the antioxidant d-α-tocopherol and its water soluble form Trolox, both of which are cell permeable and are known to block the process that propagates the peroxidation cascade along a membrane (Borek, 1988), did not affect the LPS-evoked releases of glutamate, [^{3}H]NA, or adenosine. Taken together, these results indicate that oxygen free radicals are not likely involved in LPS-evoked releases of these substances. Moreover, the involvement of NO in LPS-evoked [^{3}H]NA release does not appear to be mediated by $ONOO^-$ formation, since scavenging free radicals had no effect on LPS-evoked release of [^{3}H]NA.

Roles of prostanoids in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. There is mounting evidence that brain levels of AA and its associated metabolites are elevated under inflammatory conditions (Farooqui et al., 1997). However, their roles as inflammatory mediators in various neurodegenerative diseases remain controversial (Katsuki and Okuda, 1995). For instance, although PGE_2, the predominant form of PGs in the brain (Kaufmann et al., 1997), is considered a proinflammatory molecule, it suppresses LPS-evoked IL-1β secretion (Caggiano and Kraig, 1999) and downregulates iNOS expression (Minghetti et al., 1998) in cultured microglial cells. Importantly, co-activation of non-NMDA and metabotropic glutamate receptors releases glutamate from cultured rat astrocytes and cortical slices via the formation of prostaglandins (Bezzi et al., 1998). Moreover, elevated levels of brain glutamate and aspartate have been observed in the rat following i.c.v. injection of PGE_1 (Monda et al., 1998).

Two isoforms of COX exist. COX-1 is constitutively expressed in most cells and tissues, whereas COX-2 is up-regulated under inflammatory conditions (Kaufmann et al., 1997). However, recent evidence shows that COX-2 is also constitutively expressed in the brain (Yasojima et al., 1999), and its activity is increased shortly after brain ischemia (Planas et al., 1999). In the present study, neither indomethacin, which inhibits both COX isoforms, nor NS-398, which inhibits COX-2, affected glutamate release evoked by LPS, suggesting that PGs are not involved in LPS-evoked release of glutamate from rat cortical slices. Similarly, inhibition of PLA_2 or lipoxygenases did not alter the LPS-evoked release of glutamate. Taken together, these findings indicate that AA, PGs and leukotrienes do not appear to play a part in LPS-evoked glutamate release.

LPS-evoked release of [^{3}H]NA was diminished by both the COX-1 inhibitor resveratrol and the COX-2 inhibitor NS-398, as well as by the non-selective COX inhibitor indomethacin, suggesting that both COX isoforms may be involved in LPS-evoked release of [^{3}H]NA. The effect of resveratrol was very profound, nearly abolishing LPS-evoked [^{3}H]NA release. Resveratrol has been shown to strongly inhibit superoxide and hydrogen peroxide production induced by LPS in macrophages (Martinez and Moreno, 2000), raising the possibility that its actions might be mediated by diminishing free radical formation rather than by inhibition of COX-1. However, this seems unlikely because LPS-evoked release of [^{3}H]NA does not appear to be mediated by free radicals (see previous section, p169). Inhibition of lipoxygenase with NDGA also diminished LPS-evoked release of [^{3}H]NA, suggesting that leukotrienes may also be involved in this release process.

The observation that inhibition of either COX or lipoxygenase decreased LPS-evoked [^{3}H]NA release suggests that PGs and leukotrienes are involved in mediating LPS-evoked release of [^{3}H]NA from rat cortical slices. Alternatively, inhibition of these enzymes may cause an accumulation of arachidonic acid, which then might inhibit LPS-evoked [^{3}H]NA release. However, this possibility seems unlikely, since inhibition of PLA_2 with p-BPB, which should decrease arachidonic acid levels, had no effect on LPS-evoked [^{3}H]NA release. Recently, Combs et al. (2000) reported that the neuroprotective effects of certain NSAIDs were mediated by direct interaction with the ligand activated nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). However, this process takes a relatively long time (18 to 72 h), whereas the actions of the COX inhibitors in the present study took only a few minutes.

LPS-evoked release of [^{3}H]NA is partly due to the released glutamate acting at both NMDA and non-NMDA ionotropic glutamate receptors (see Part I, this Chapter). NMDA receptor activation has been shown to release PGs from rat spinal cord (Ding and Yaksh, 1999). Moreover, glutamate release evoked by co-activation of non-NMDA and metabotropic glutamate receptors is mediated by the release of prostaglandins (Bezzi et al., 1998). Thus, it seems possible that LPS evokes the release of glutamate, which then acts at ionotropic and metabotropic glutamate receptors to promote the formation of PGs and leukotrienes, which in turn mediate [^{3}H]NA release from noradrenergic axons present in the cortex.

Interestingly, inhibition of COX-1 with resveratrol and lipoxygenase with NDGA increased, whereas inhibition of COX-2 with NS-398 decreased, LPS-evoked release of adenosine. These results are not likely due to the buildup of arachidonic acid, as inhibition of PLA_2 with p-BPB had no effect on LPS-evoked adenosine release. Rather, they suggest that PGs and leukotrienes, formed after LPS stimulation, might modulate the release of adenosine. In addition to inhibiting COX-1, resveratrol is an antioxidant (Martinez and Moreno, 2000). However, free radicals do not appear to be involved in LPS-evoked adenosine release (see previous section, p169), so that inhibition of COX-1 appears to be the most likely explanation for resveratrol's action on LPS-evoked adenosine release. The mechanism for the opposing effects of COX-1 and COX-2 inhibition on LPS-evoked adenosine release remains to be elucidated. COX-1 and COX-2 are expressed in neurons, glia, and endothelium (Yasojima et al, 1999). Perhaps COX-1 and COX-2, located in different cell types, regulate LPS-evoked adenosine release differentially in the brain.

The findings that resveratrol and NDGA potentiate LPS-evoked adenosine release may have important implications. Numerous studies have shown that adenosine, acting at specific adenosine A_{2A} receptors on macrophages and neutrophils, decreases inflammatory responses (Cronstein, 1995; Eigler et al., 1997). In addition, extracellular adenosine can act at neuronal A_1 receptors to decrease both the release of glutamate from neurons and glutamate's excitatory actions postsynaptically (Dolphine and Archer, 1983; Pasquini et al., 1987). Drugs, such as resveratrol and NDGA, which promote the formation of extracellular adenosine, may provide protection against inappropriate excitation produced by LPS.

Roles of cytokines in LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Inflammatory cytokines have been implicated in various neurodegenerative disorders (Rothwell, 1999). TNF-α and IL-1 have been shown to stimulate the releases of neurotoxic substances such as glutamate, NO and arachidonic acid from astrocytes and microglia (Giulian and Vaca, 1993; Piani and Fontana, 1994). However, in the present study, LPS-evoked releases of glutamate, [^{3}H]NA or adenosine from rat cortical slices were not mimicked by the cytokines (TNF-α, IL-1β or IL-6), alone or in combination, suggesting that these cytokines are not involved.

CHAPTER 5

IMMEDIATE EXCITATORY RESPONSES CAUSED BY THE HIV GLYCOPROTEINS GP41 AND GP120 IN RAT PARIETAL CORTICAL SLICES

ABSTRACT

In the previous chapter, we showed that the bacterial endotoxin LPS causes very rapid releases of the neurotransmitters glutamate and NA and of the neuromodulator adenosine from rat parietal cortical slices. This raised the possibility that similar responses might occur with immunogens specifically associated with viruses, such as the HIV glycoproteins which are associated with AIDS dementia. Here we show that exposure of rat parietal cortical slices to the HIV glycoproteins gp120 and gp41 triggered very rapid releases of the neurotransmitters glutamate and [^{3}H]NA, and of the neuromodulator adenosine. HIV gp41 was much more effective than gp120 at releasing glutamate and [^{3}H]NA, while both glycoproteins were equally effective at releasing adenosine. The responses to gp120 and gp41 declined rapidly to basal levels following the removal of these glycoproteins. There appeared to be no tachyphylaxis after repeated stimulation with gp41. Preincubation of slices with the glucocorticoid corticosterone for 90 min did not potentiate gp41 or gp120-evoked responses. Finally, the combination of different concentrations of these two glycoproteins did not produce supra-additive effects. It seems possible that rapid, inappropriate excitation may occur in the immediate vicinity of HIV infections in the brain. This might explain some of the acute, transient neurological and psychiatric symptoms associated with AIDS dementia.

INTRODUCTION

Approximately one third of AIDS patients develop dementia, characterized by the impairment of cognition and motor performance (Lipton, 1996; 1998). Although it is generally believed that neurons are relatively resistant to neuro-invasive HIV strains, it is well established that macrophages and microglia are infected by HIV-1 (Mucke et al., 1995). The HIV glycoproteins gp120 and gp41 are important structural proteins in the envelope of the virus; gp120 is shed by the virus while gp41 remains bound to the envelope (Lipton, 1996). Importantly, gp120 promotes extracellular glutamate formation by both inhibiting uptake and increasing release of glutamate from cultured rat astrocytes (Vesce et al., 1997). In addition, gp120 potentiates NMDA-evoked noradrenaline release from brain preparations *in vitro* (Pittaluga et al., 1996). Finally, gp41 has been shown to induce nitric oxide formation and neuronal cell death in mixed cultures of rat neurons (Adamson et al., 1996).

The gram-negative bacterial endotoxin lippopolysaccharide (LPS) evokes very rapid releases of the neurotransmitters glutamate and noradrenaline and of the neuromodulator adenosine from rat cortical slices (see Chapter 4). This raised the possibility that similar excitatory responses may occur with the HIV coat proteins gp120 and gp41. This study was undertaken to test this.

MATERIALS AND METHODS

Preparation and superfusion of rat brain cortical slices were performed as described in Chapter 2.

RESULTS

The HIV glycoproteins gp41 and gp120 trigger the rapid releases of glutamate, [^{3}H]NA and adenosine from cortical slices. Neither gp120 nor gp41 at concentrations of 100 pM, 200 pM or 1 nM released glutamate, [^{3}H]NA or adenosine. However, 10 nM of either gp120 or gp41 triggered very rapid (<2.5 min) releases of the neurotransmitters glutamate and [^{3}H]NA, and of the neuromodulator adenosine from slices of rat parietal cortex (Fig. 5-1). HIV gp41 (10 nM) released more glutamate (Fig. 5-1A) and [^{3}H]NA (Fig. 5-1B) than did gp120, while both glycoproteins were equally effective at releasing adenosine (Fig. 5-1C). The responses to gp120 and gp41 declined rapidly to basal levels following the removal of these glycoproteins, indicating complete reversibility. Moreover, there appeared to be no tachyphylaxis after repeated stimulation with 10 nM gp41 (Fig. 5-2). Several studies have shown that gp120 is excitotoxic (Müller et al., 1996). However, the observation that gp41 was much more effective than gp120 at releasing glutamate and [^{3}H]NA is consistent with previous findings that gp41 at a concentration of 100 nM produced neuronal cell death while gp120 at the same concentration did not (Adamson et al., 1996).

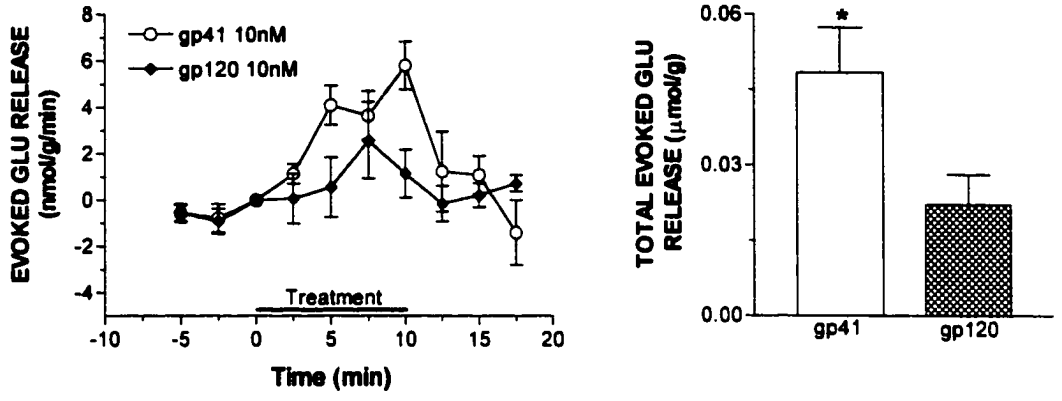
Effect of corticosterone on gp41-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. It has been reported that corticosterone can exacerbate gp120-induced neurotoxicity in cultured rat neurons (Iyer et al., 1998; Yusim et al., 2000). Therefore, we examined the effect of 1 μM corticosterone on gp41-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. Preincubation of cortical slices with 1 μM corticosterone for 90 min did not potentiate the releases of glutamate, [^{3}H]NA or adenosine evoked by either gp41 (Fig. 5-3) or gp120 (Fig. 5-4),

suggesting that stress hormones do not intensify gp120 or gp41-evoked releases under our experimental conditions.

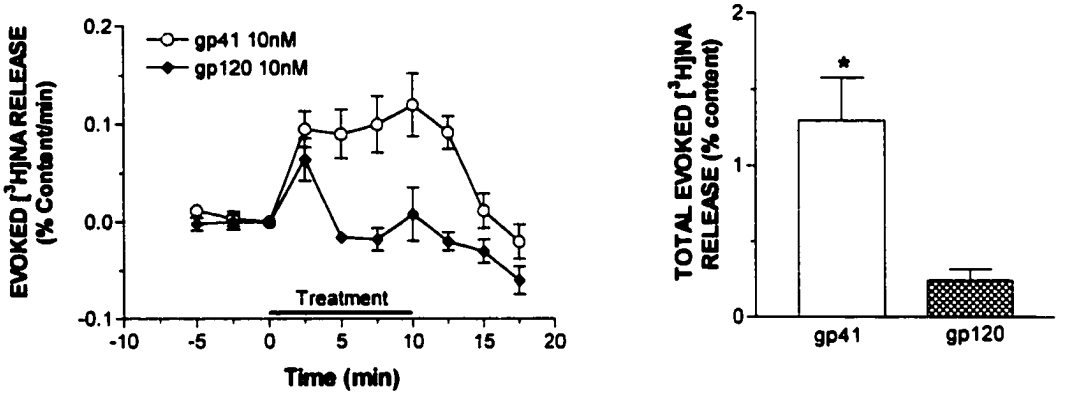
Effect of a combination of gp41 and gp120 on glutamate, [^{3}H]NA and adenosine release from rat cortical slices. Because both gp41 and gp120 are structural proteins of the HIV virus, they might interact with each other at their sites of action to exacerbate each other's actions. Therefore, we tested the effect of the combination of these two proteins on the releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. As shown in figure 5-5, the combination of 10 nM gp41 with 10 nM gp120 had no supra-additive effects on releasing these substances.

Fig. 5-1. The HIV glycoproteins gp41 (10 nM) and gp120 (10 nM) triggered very rapid (<2.5 min) releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time course for gp41- and gp120-evoked releases. Right panels represent total evoked releases. Values are mean ± S.E.M. from 6 experiments. ***Basal glutamate release was 1.76±1.87 nmol/g/min; basal [^{3}H]NA release was 0.59±0.10 %/min; basal adenosine release was 72.8±14.6 pmol/g/min. Gp41 was more effective than gp120 at releasing glutamate and [^{3}H]NA, while both glycoproteins were equi-effective at releasing adenosine.*** * $p<0.05$ significantly greater than release evoked by gp120.

A


gp41 10nM
gp120 10nM
EVOKED GLU RELEASE (nmol/g/min)
Treatment
Time (min)
TOTAL EVOKED GLU RELEASE (µmol/g)
gp41
gp120

B


gp41 10nM
gp120 10nM
EVOKED [3H]NA RELEASE (% Content/min)
Treatment
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% content)
gp41
gp120

C

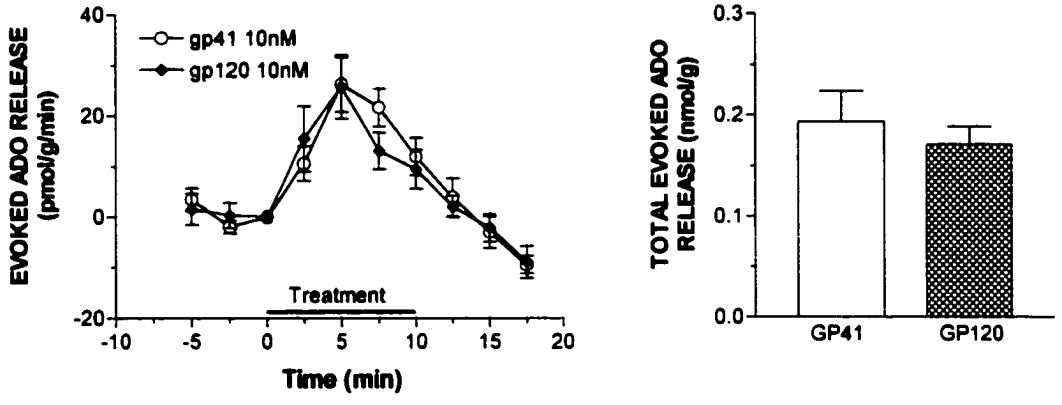

gp41 10nM
gp120 10nM
EVOKED ADO RELEASE (pmol/g/min)
Treatment
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
GP41
GP120

Fig. 5-1

Fig. 5-2. Effect of repeated stimulation with gp41 (10 nM) on the releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA and, (C) release of adenosine (ADO). Slices were first exposed to gp41 for 10 min, and allowed to recover for 30 min. The second stimulation was delivered for another 10 min. Total evoked releases were compared between the first and the second stimulation. Values are mean ± S.E.M. from 3 experiments. ***No tachyphylaxis was observed after repeated stimulation with gp41.***

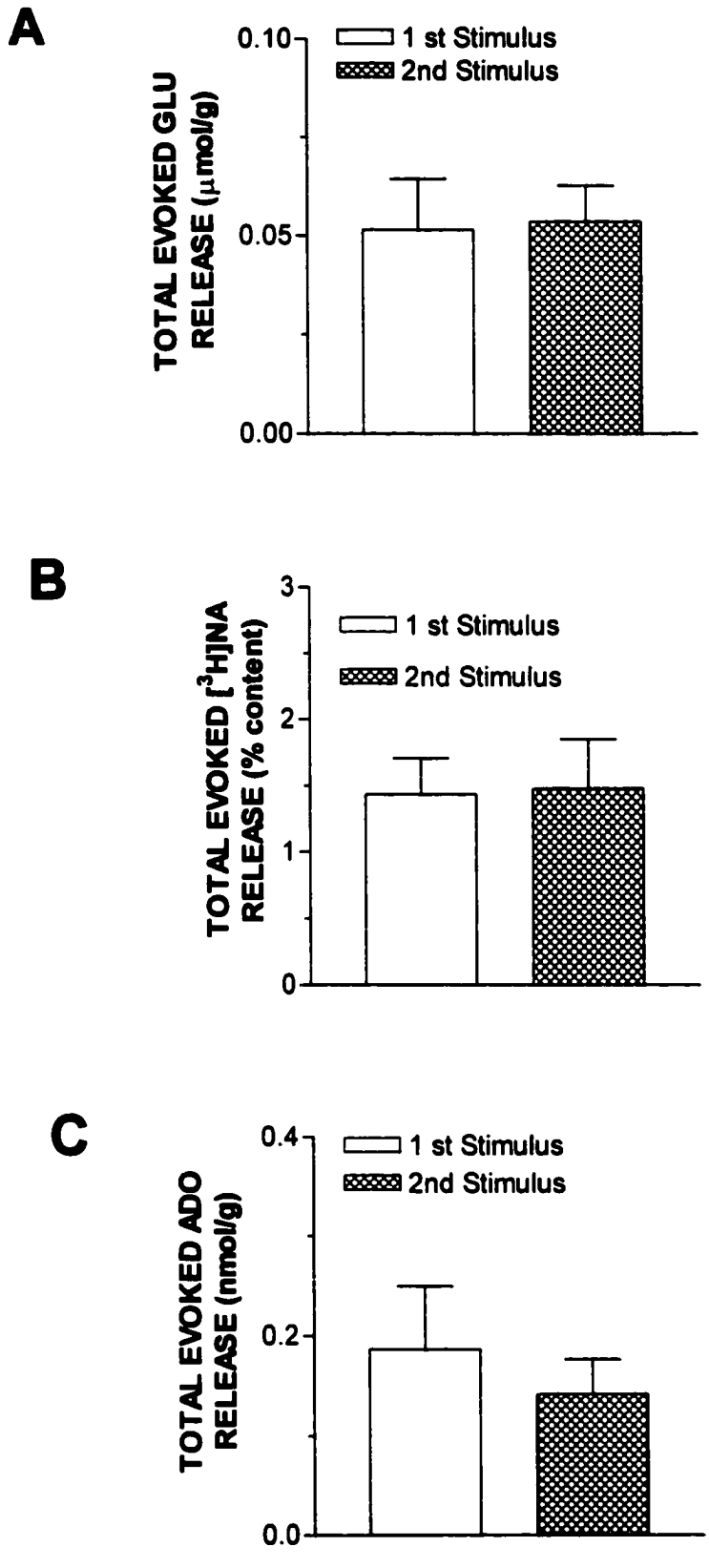

A
0.10
0.05
0.00
1 st Stimulus
2nd Stimulus
TOTAL EVOKED GLU RELEASE (μmol/g)
B
3
2
1
0
1 st Stimulus
2nd Stimulus
TOTAL EVOKED [3H]NA RELEASE (% content)
C
0.4
0.2
0.0
1 st Stimulus
2nd Stimulus
TOTAL EVOKED ADO RELEASE (nmol/g)

Fig.5-2

Fig. 5-3. Effect of corticosterone (1 μM) on gp41 (10 nM)-evoked releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate, (B) release of [^{3}H]NA, (C) release of adenosine. Left panels show the time course for gp41-evoked releases. Right panels represent total evoked releases. Values are mean ± S.E.M. from 6 experiments. ***Preincubation of cortical slices with 1 μM corticosterone (CORT) for 90 min did not potentiate the releases of glutamate, [^{3}H]NA or adenosine evoked by gp41.***

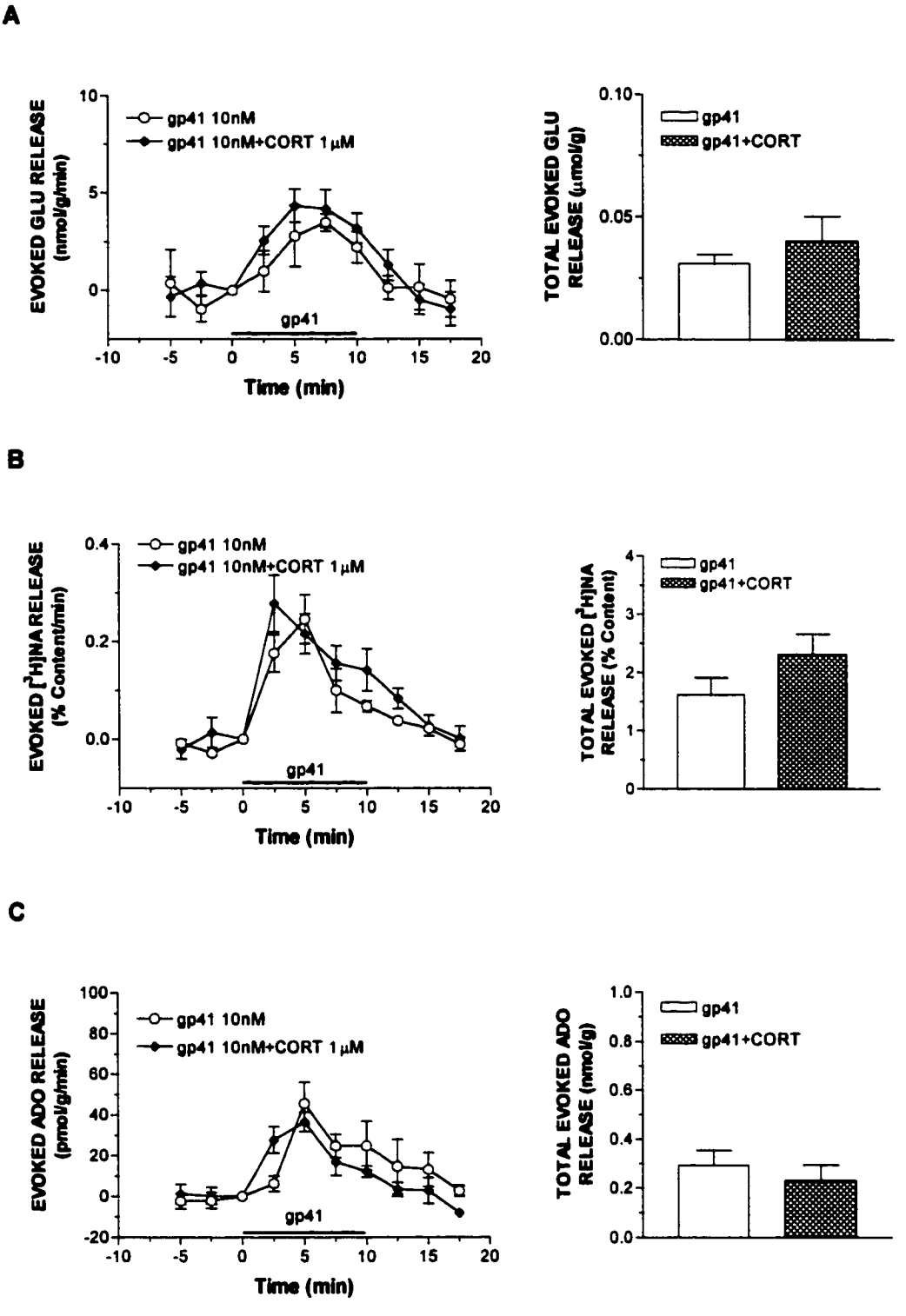

A
EVOKED GLU RELEASE (nmol/g/min)
gp41 10nM
gp41 10nM+CORT 1 μM
gp41
Time (min)
TOTAL EVOKED GLU RELEASE (μmol/g)
gp41
gp41+CORT
B
EVOKED [3H]NA RELEASE (% Content/min)
gp41 10nM
gp41 10nM+CORT 1 μM
gp41
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
gp41
gp41+CORT
C
EVOKED ADO RELEASE (pmol/g/min)
gp41 10nM
gp41 10nM+CORT 1 μM
gp41
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
gp41
gp41+CORT

Fig. 5-3

Fig. 5-4. Effect of corticosterone (1 μM) on gp120 (10 nM)-evoked releases of glutamate, [^{3}H]NA and adenosine from slices of rat parietal cortex in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Values are mean ± S.E.M. from 3 experiments. ***Preincubation of cortical slices with 1 μM corticosterone (CORT) did not potentiate the releases of glutamate, [^{3}H]NA or adenosine evoked by gp120.***

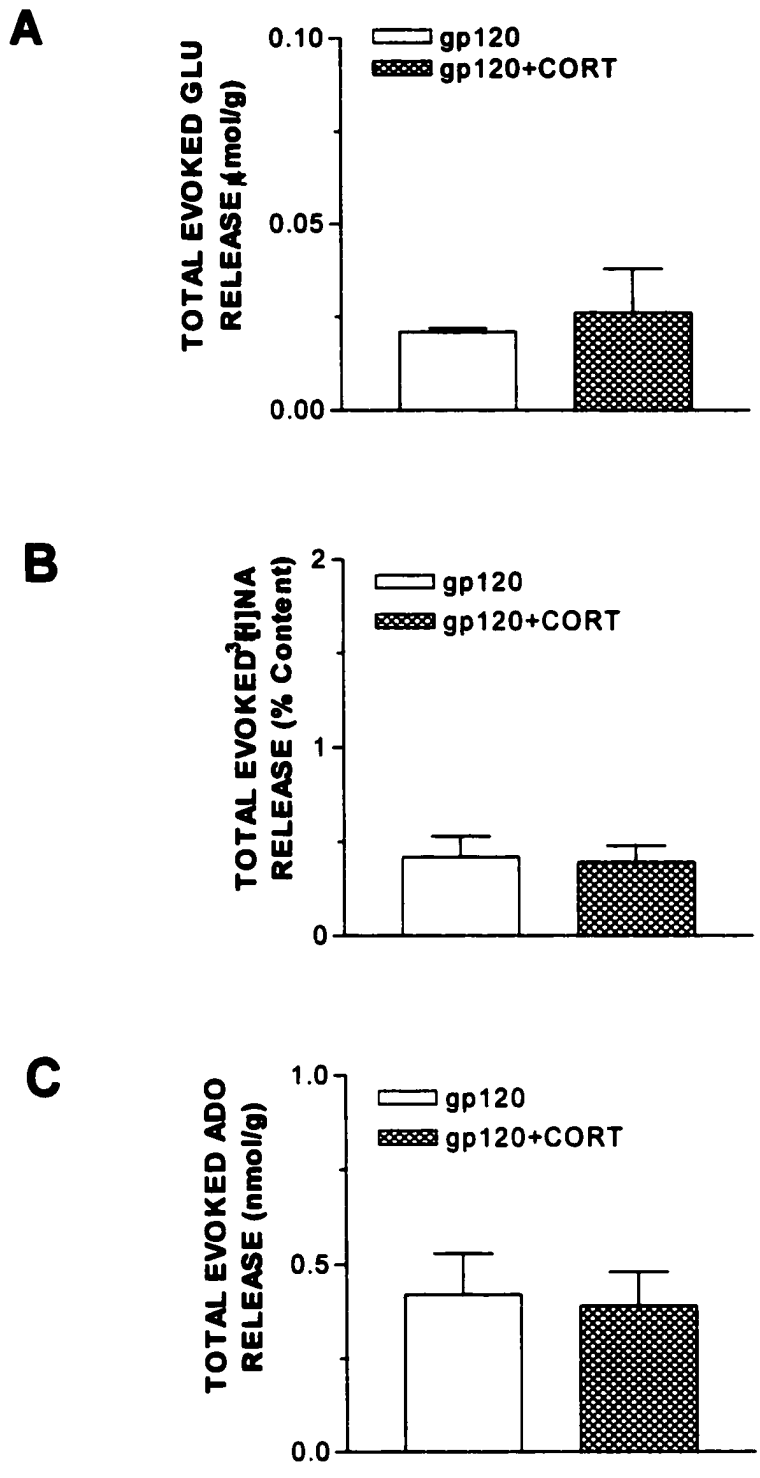

A
TOTAL EVOKED GLU RELEASE (nmol/g)
0.10
0.05
0.00
gp120
gp120+CORT
B
TOTAL EVOKED [3H]NA RELEASE (% Content)
2
1
0
gp120
gp120+CORT
C
TOTAL EVOKED ADO RELEASE (nmol/g)
1.0
0.5
0.0
gp120
gp120+CORT

Fig. 5-4

Fig. 5-5. Releases of glutamate, [^{3}H]NA and adenosine by the combination of gp41 (10 nM) and gp120 (10 nM) from rat parietal cortical slices in the presence of extracellular Mg^{2+}. (A) Release of glutamate (GLU), (B) release of [^{3}H]NA, (C) release of adenosine (ADO). Left panels show the time courses for gp41- and gp120-evoked releases. Right panels represent total evoked releases. Values are mean ± SEM from 6 experiments. ***Combination of 10 nM gp41 and 10 nM gp120 had no supra-additive effects on releases.***

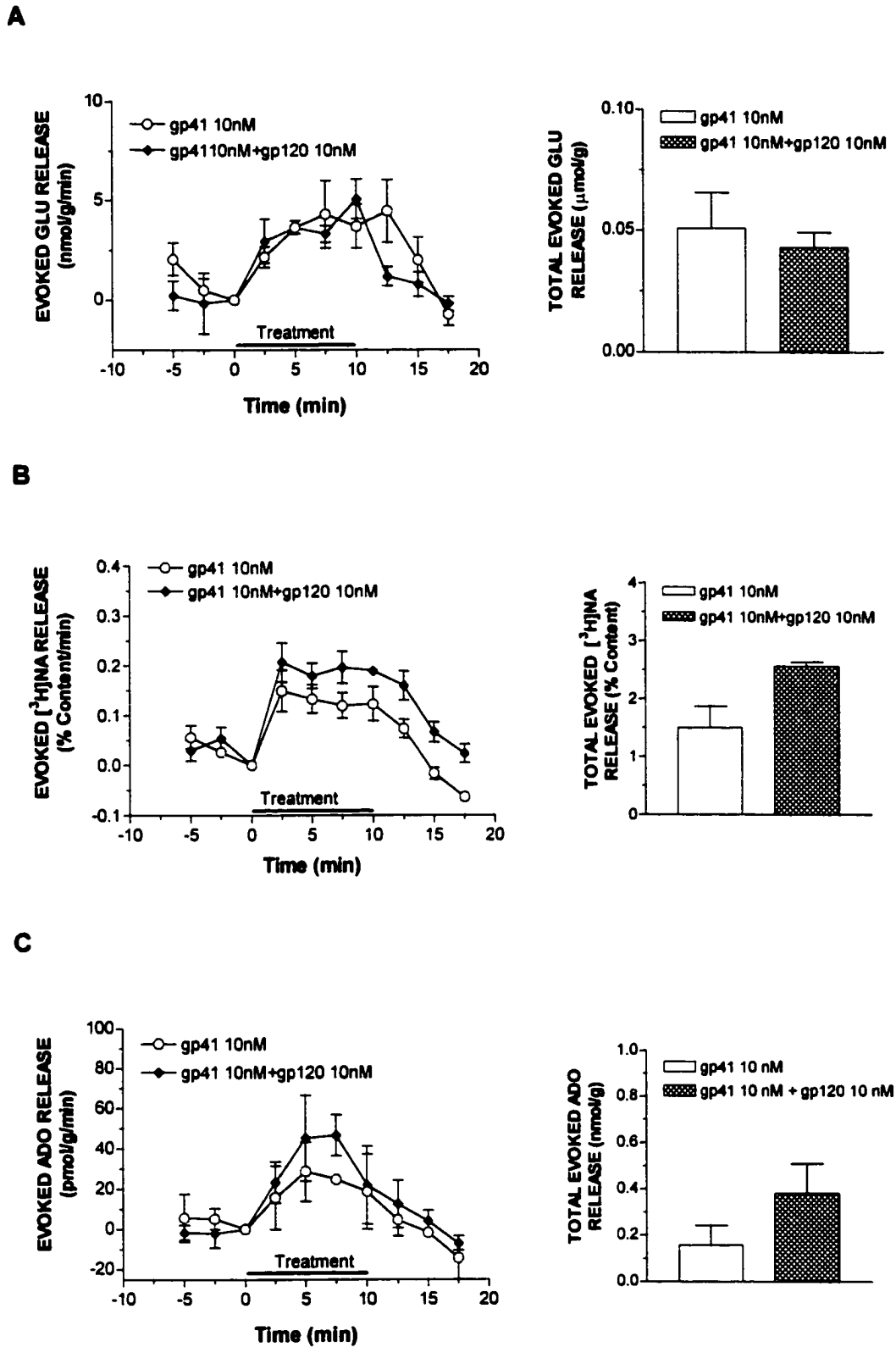

A
EVOKED GLU RELEASE (nmol/g/min)
gp41 10nM
gp4110nM+gp120 10nM
Treatment
Time (min)
TOTAL EVOKED GLU RELEASE (µmol/g)
gp41 10nM
gp41 10nM+gp120 10nM
B
EVOKED [3H]NA RELEASE (% Content/min)
gp41 10nM
gp41 10nM+gp120 10nM
Treatment
Time (min)
TOTAL EVOKED [3H]NA RELEASE (% Content)
gp41 10nM
gp41 10nM+gp120 10nM
C
EVOKED ADO RELEASE (pmol/g/min)
gp41 10nM
gp41 10nM+gp120 10nM
Treatment
Time (min)
TOTAL EVOKED ADO RELEASE (nmol/g)
gp41 10 nM
gp41 10 nM + gp120 10 nM

Fig. 5-5

DISCUSSION

The present study showed that the HIV glycoproteins gp120 and gp41 evoked very rapid releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices. These release processes were too rapid to involve gene transcription and protein synthesis. In addition, these rapid responses to gp120 and gp41 occurred in slices of supposedly normal, integrated parietal cortex from adult rats, rather than from cultures of neurons, glia, or arbitrary mixtures of the two.

The concentrations of the glycoproteins (10 nM) used were relatively high. However, even higher concentrations of gp41 and gp120 have been used by others to study the effects of these glycoproteins on iNOS expression and neuronal death in mixed cultures of rat neurons (Adamson et al., 1996). In any event, glycoprotein concentrations in the immediate vicinity of the HIV virus could be very high. Finally, the HIV glycoprotein-evoked responses observed in the present study are unlikely to result from nonspecific effects or acute damage to brain cells for the following reasons. First, these responses returned to basal values when either gp120 or gp41 was withdrawn from the superfusing medium. Secondly, repeated stimulation with gp41 showed no tachyphylaxis. The observation that the HIV glycoproteins also trigger a rapid release of adenosine may have important implications. Although adenosine can sometimes act at specific A_{2A} receptors to produce neuronal excitation (Ongini et al., 1997), extracellular adenosine in the cortex acts predominantly at neuronal A_1 receptors to decrease both the release of glutamate from neurons and glutamate's excitatory actions postsynaptically (Dolphin and Archer, 1983; Pasquini et al., 1987). Extracellular adenosine also acts at specific adenosine A_{2A} receptors on macrophages and neutrophils to decrease

inflammatory responses (Cronstein, 1995; Eigler et al., 1997). Thus drugs that promote the formation of extracellular adenosine and/or potentiate its cellular actions may provide some protection against this inappropriate excitation produced by HIV glycoproteins in the brain.

The cellular sites of action for these glycoproteins are not clear. [^{3}H]NA likely arises from noradrenergic synaptic terminals in the cortex. However, gp120 promotes the rapid release of glutamate from cultured astrocytes (Vesce et al., 1997), raising the possibility that the glutamate released in our study may have originated from glial, rather than neuronal, elements in the cortical slices. Because this rapid excitation in cortical slices is triggered by HIV coat glycoproteins rather than by neurotransmission, this excitation is inappropriate and hence pathological. This inappropriate excitation could cause some of the transient but serious neurological/psychiatric symptoms associated with AIDS dementia. Furthermore, the fact that gp41 was more effective at releasing glutamate than gp120 suggest that gp41 might play a greater role in mediating inappropriate excitation in the immediate vicinity of HIV infections in the brain.

CHAPTER 6

SUMMARY, SIGNIFICANCE AND FUTURE WORKS

Summary

Inflammation in the CNS has received broad attention in recent years. However, most studies have focused on the role of inflammation in chronic neurodegenerative conditions. Few have examined the acute effects of inflammatory substances such the gram-negative bacterial endotoxin LPS in the brain. This study demonstrates for the first time that LPS and the HIV glycoproteins gp41 and gp120 trigger very rapid (< 2.5 min) releases of the neurotransmitters glutamate and NA and of the neuromodulator adenosine from rat brain parietal cortex. The speed of onset of LPS- and glycoproteins-evoked responses is very rapid, too rapid to involve gene transcription and protein synthesis. LPS appears to release glutamate from non-neuronal cells via the reversal of glutamate transporters. The released glutamate then acts at its ionotropic NMDA and non-NMDA receptors existing on noradrenergic axons to promote the formation of NO and PGs, which in turn promote the release of NA. Some of the characteristics of LPS-evoked releases of glutamate, [^{3}H]NA and adenosine from rat cortical slices are summarized in Table 6-1.

The most significant observations of this study are that LPS triggers the rapid release of glutamate from non-neuronal cells via the reversed operation of high-affinity Na^+-dependent glutamate transporters, and that the formation of NO, oxygen free radicals, PGs and cytokines do not appear to mediate LPS-evoked release of glutamate from rat brain parietal cortex. Therefore, the precise mechanism by which LPS releases glutamate remains to be investigated.

Table 6-1. Characteristics of LPS-evoked releases of glutamate, NA and adenosine from rat cortical slices*

	Effects on Releases		
	Glutamate	**NA**	**Adenosine**
Ionotropic GluRs			
NMDA antagonists (MK-801, APV)	-	↓**	-
Non-NMDA antagonist (NBQX)	-	↓**	-
Nitric Oxide (NO)			
Non-selective NOS inhibitor (L-NAME)	-	↓	↓
Prostanoids			
Non-selective COX inhibitor (indomethacin)	-	↓	-
COX-1 inhibitor (Resveratrol)		↓	↑
COX-2 inhibitor (NS-398)	-	↓	↓
PLA_2 inhibitor (p-BPB)	-	-	-
Lipoxygenase inhibitor (NDGA)		↓	↑
Free Radicals			
Scavengers (SOD, catalase)	-	-	-
Antioxidants (α-tocopherol, Trolox)	-	-	-
Cytokines			
Not mimicked by:			
TNF-α	-	-	-
IL-1β	-	-	-
IL-6	-	-	-
Propagated Action Potentials			
TTX	-	-	-
Glutamate Transporters			
Non-selective transporter inhibitor (PDC)	↓	↓***	-

* **All responses to LPS are reversible and do not exhibit tachyphylaxis**

** **Due to released glutamate's actions at its ionotropic receptors**

*** **Probably secondary to block of LPS-evoked glutamate release**

Significance

This thesis demonstrates that LPS triggers rapid excitatory actions in rat parietal cortex. Because these responses are not triggered by neuronal inputs but by a molecule that resides on the exterior of gram negative bacteria such as E. coli, they are abnormal and inappropriate. The triggering of this inappropriate excitation in the immediate vicinity of gram-negative bacterial infections in the brain could cause some of the transient but serious neurological symptoms associated with these infections, such as delirium and cognitive dysfunctions. In the extreme situation, LPS might even cause localized excitotoxicity and neuronal cell death. Gram positive bacterial cell wall components have been shown to promote the formation of microglial chemokines (Prinz et al., 1999), raising the possibility that similar inappropriate excitations might also occur immediately adjacent to gram positive bacteria in the brain. The AIDS HIV viral coat glycoproteins gp120 and gp41 also promote the releases of glutamate, NA and adenosine from slices of rat brain parietal cortex. These findings raise the possibility that similar inappropriate excitation might occur near viral infections in the brain. Finally, there is evidence that β-amyloid (1-40), which has been associated with Alzheimer's disease, can trigger the formation of superoxide anions and the release of glutamate from peritoneal macrophages (Klegeris and McGeer, 1997). Assuming that similar responses can be triggered in the brain's microglia and astrocytes, these might cause some of the acute neurological and psychiatric symptoms that occur in Alzheimer's disease. Perhaps many other as yet unknown substances that are not normally found in brain parenchyma can trigger similar excitatory responses, possibly resulting in dysfunctions such as delirium

and/or thought disorders. If so, these non-neurogenic and inappropriate excitations may be more common than we have previously thought.

Future Studies

1. To further investigate the mechanisms of LPS-evoked release of glutamate from rat cortical slices.

Is activation of PKC or PTK involved in LPS-evoked release of glutamate? PKC and PTK are important second messengers that transduct signals in various cells upon activation of a variety of receptors. It has been shown that inhibition of either PKC or PTK by their specific inhibitors decreases LPS-induced production of TNF-α from human monocytes (Shapira et al., 1994; Shames et el., 1999). Therefore, it is possible that LPS-evoked release of glutamate is through the activation of PKC and/or PTK. To test this, specific enzyme inhibitors (e.g. GFX for PKC, genistein for PTK) can be used to see whether or not LPS-evoked release of glutamate can be diminished.

2. To investigate the mechanisms of gp120- and gp41-evoked releases of glutamate, NA and adenosine from rat parietal cortical slices.

Preliminary research in this study showed that the HIV glycoproteins gp120 and gp41 also evoked very rapid releases of the neurotransmitters glutamate and NA and of the neuromodulator adenosine from rat brain cortical slices. Future research in this area would be to address the mechanisms of these release processes. For example, what are the cellular source(s) of gp120- and gp41-evoked releases? Are NMDA and non-NMDA receptors involved in these releases? Do NO, free radicals, PGs or cytokines play roles in these release?

3. To examine if other immunogens such as β-amyloid proteins also evoke similar responses in rat brain cortical slices.

The observation that both LPS and the HIV glycoproteins trigger very rapid releases of glutamate, NA and adenosine from rat brain cortical slices raises the possibility that similar responses might occur with other immunogens such as β-amyloid proteins, which are specifically associated with Alzheimer's disease. If similar responses occur, this could explain some of the acute but transient neurological symptoms seen in patients with Alzheimer's disease. To test this hypothesis, we performed preliminary experiments with the β-amyloid peptides Aβ1-42, Aβ1-40 and Aβ25-35. The results were ambiguous and further experiments could not be done due to the high cost of these peptides. However, further experiments should be proposed to examine this hypothesis.

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