

2001

Astrocytes: a driving force in brain signaling and encephalopathies

Aleksandar Jeremic
Iowa State University

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Astrocytes: A driving force in brain signaling and encephalopathies

by

Aleksandar Jeremic

**A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY**

Co-majors: Veterinary Anatomy; Neuroscience

**Program of Study Committee:
Srdija Jeftinija, Co-major Professor
Anumantha Kanthasamy, Co-major Professor
Lloyd Anderson
Walter Hsu
Mary Helen Greer
Donald Sakaguchi**

Iowa State University

Ames, Iowa

2001

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Co-major Professor

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Co-major Professor

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For the Co-major Program

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For the Co-major Program

To
my wife Jelena
and
Luka & Mina

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ABBREVIATION TABLE

α -MEM- α -minimum essential medium

ATP- adenosine 5` tri-phosphate

Ach-acetylcholine

Asp-aspartate

AO-acridine orange

BAPTA-AM-1,2-bis- ethane-N,N,N',N'-tetracetic acid-acetoxymethyl ester

Ca²⁺-calcium

[Ca²⁺]_i -intracellular calcium concentration

Cl⁻-chloride

CNS-central nervous system

DAG-1-Oleoyl-2-acetyl-sn-glycerol

D-AP5-D(-)-2-Amino-5-phosphonovaleric acid

EAA-excitatory amino acids

EGTA-ethylene glycol-bis-N,N,N',N'-tetraacetic acid

GFAP-glial fibrillary acidic protein

Glu-glutamate

HCO₃⁻-bicarbonate

Hepes-(N-[2-Hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid])

IP₃-inositol 1,4,5-trisphosphate

K⁺-potassium

MTT-[3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide

Na⁺-sodium

NPPB-5-nitro-2(3-phenylpropylamino)-benzoate

PCMPs- phenylsulfonic acid

PDC-L-*trans*-pyrrolidine-2,4-dicarboxylate

PI-propidium iodide

PNS- peripheral nervous system

PTX-pertussis toxin

RNase-ribonuclease

ROS-reactive oxygen species

SDF-1 α – stromal cell-derived factor 1 α

THC- Δ^9 -tetrahydrocannabinol

WIN-Win 55,212-2

ABSTRACT

For decades, astrocytes, the most numerous cell of the central nervous system, were considered to be passive supporters of nerve cell architecture and function, lacking excitability and having no role in signal integration. However, recent findings have progressively challenged that view, highlighting the active participation of astrocytes in synaptic transmission and information processing. This dissertation presents evidence for the major contribution of astrocytes in the modulation of neuronal excitability that is in large part mediated by the excitatory neurotransmitter glutamate. Neuroligands, ATP and SDF-1 α released glutamate from astrocytes that stimulated adjacent neurons by the NMDA-receptor activation. Furthermore, rapid bi-directional signaling loops between the neurons and glia have been revealed in experiments performed on co-cultures.

In addition to its fundamental physiological importance, this dissertation postulates a new role for astrocytes in brain pathology. Cell culture experiments revealed a direct toxic effect of neurotoxins, gp120, an HIV-1 envelope glycoprotein, and cannabinoids on astrocytes. A propensity of astrocytes to undergo apoptosis and to release excitotoxic glutamate in response to insult elicited by gp120 and cannabinoids suggests that, along with neurons, astroglia are an important site of action for neurotoxins in brain. Potential mechanisms of cannabinoid toxicity include formation of ceramide and free radicals and activation of several primary and secondary effector enzymes. An indirect contribution of astrocytes to the gp120 and cannabinoid-induced neuron injury is implied with this study. Potential mechanisms include disturbances of glia function and their ability to support

neurons and the astrocytes-mediated excitotoxins formation in the brain. This part of the dissertation gave well-deserved respect to the issue of cannabinoid and gp120 astrotoxicity because a better understanding of the pathogenesis of astrocytes in encephalopathies may have important therapeutic implications in the future.

CHAPTER 1. GENERAL INTRODUCTION

The mammalian nervous system is composed of more than 100 billion neurons surrounded by glial cells. Glial cells outnumber the neurons 10-50:1. A generally accepted view is that glial cells are nothing more than passively subservient to neurons. The development and advances in monitors of intracellular ion concentration open a new field of cellular physiological investigation. With these technical advances, studies show that glial cells are much more complex entities than had been appreciated.

The focus of this dissertation is twofold: first, the study of the effect of neuroligands, ATP and SDF-1 α , on astrocytes in order to further our understanding of astrocytes in brain function and second, the study of the effect of neurotoxins on astrocytes and the mechanism of astrototoxicity.

Thesis organization

This dissertation is composed of three journal papers. Each journal paper compromises a single chapter of the dissertation. The papers are preceded by a general introduction chapter containing a literature review, and followed by a general conclusion chapter consisting of a general discussion section and summary. References from all chapters are listed in a comprehensive listing at the end of the dissertation.

Literature Review

Neuroglia

Glial cells were first described in 1846 by German pathologist Rudolf Virchow who named them neuroglia (Greek, meaning nerve glue). In the vertebrates, glia can be divided into four major categories. In the peripheral nervous system (PNS) are the Schwann cells, and in the central nervous system (CNS) are the microglia, oligodendrocytes and astrocytes. Anatomists have divided normal astrocytes in the mature brain into two broad categories: fibrous astrocytes, which contains abundant intermediate filaments and are predominant in white matter, and protoplasmic astrocytes, which contain fewer filaments and are predominant in gray matter. The glial filaments are composed of glial fibrillary acidic protein (GFAP), a specific histochemical marker of astroglia. A third class known as reactive astrocytes appears following injury in the brain. An alternative view of glial subtypes has emerged from glial cell lineage studies *in vitro*. A compelling body of literature provided evidence that there are two lineages of astroglia cells in brain cell cultures designated as type I and type II astroglia (Raff et al., 1983).

For a long time, it had been widely believed that neuroglia primarily played a supportive role in the functioning of the nervous system. There are well-documented roles for glia in the supply of nutrients to neurons (Pentreath et al., 1986), establishment and maintenance of the blood-brain barrier (Abbott, 1991), neuronal guidance (Rakic, 1971) and the control of extracellular K^+ by locally removing K^+ released from active neurons (Kuffler et al., 1966). Also, glia control a variety of other important processes in the CNS,

like regulation of glycogen metabolism (Phelps, 1972), control of CNS development (Rakic, 1990), repair of neural damage (Gilmore et al., 1982), and uptake of transmitters (Schousboe, 1981; Kimelberg and Katz, 1996).

However, there is mounting evidence that glia in general, and astrocytes in particular, might play a much more central role in information processing in the brain (Barres, 1991; Kettenmann, 1996; Ullian et al., 2001). The two-way dialog, where astrocytes appear to communicate with neurons, leads to the possibility that astrocytes might play a key role in information processing (Attwell, 1994; Smith, 1994). This is further supported by the fact that astrocytes are targets of biologically active molecules. At the plasma membrane, both types of astroglia express numerous receptors for neurotransmitters, peptides, growth factors and hormones, like purinergic P2Y, histamine, bradykinin, adrenergic, and cholinergic receptors that regulate intracellular calcium levels (Murphy and Pearce, 1987; Dave and McCarthy, 1989; Finkbeiner, 1992; Hosli and Hosli, 1993; Giaume and Venance, 1996). A variety of ion channels are expressed in astroglia, voltage-gated sodium and calcium channels and ligand-gated channels GABA, AMPA, Kainate, and purinergic P2X as examples (Barres et al., 1990; Ritchie, 1992; Sontheimer, 1992). This indicates astrocyte capability to sense most of the signals sensed by neurons.

Similar to neuronal neurotransmitter receptors, astroglial receptors activate a number of second messenger systems including cyclic AMP (McCarthy and de Vellis, 1978), phosphoinositide metabolism (Pearce et al., 1986), intracellular calcium (Salm et al., 1990), and arachidonic acid (De George et al., 1986). Activation of these systems may, in turn, affect a number of physiological processes, including enzyme induction (Aizenman and de Vellis, 1987), proliferation (Morrison and de Vellis, 1981), membrane potential

(Bowman and Kimelberg, 1984), and neuromodulatory secretion (Shain and Martin, 1984). It is also established that astrocytes respond to stimuli with release of chemical transmitters, like aspartate, glutamate, D-serine and ATP (Parpura et al., 1994; Schell et al., 1995; Jeftinija et al., 1996; Wang et al., 2000).

Oligodendrocytes are glial cells that make and maintain myelin in the CNS. Other than supportive, their functions remain unknown. It is speculated that they might have an autocrine/paracrine function (Szuchet and Yim, 1990). Their counterparts in the PNS are the Schwann cells, which provide housing for axons within peripheral nerves. Similarly to oligodendrocytes, Schwann cells form myelin sheaths along the larger nerve fibers that allow saltatory conduction of nerve impulses, which increases the velocity of nerve conduction as much as 100 times. Beyond these basic functions, the Schwann cell is able to respond to nerve injury by helping to remove cellular debris, by facilitating the process of remyelination, and by promoting nerve fiber regeneration. Recent investigations documented a capability of Schwann cells to release the neurotransmitter glutamate in response to the neuroligands bradykinin and adenosine 5' tri-phosphate (ATP) that might play a role in glia-neuron signaling in the peripheral nervous system (Parpura et al., 1995a; Jeftinija et al., 1998).

Microglia, the other sub-type of neuroglia in the CNS, are often referred as sensors for pathological events in the brain. Their nature and identity have long been debated but it is now generally accepted that they are ontogenetically related to cells of the mononuclear phagocyte lineage, unlike all other cell types in the CNS. The most characteristic feature of microglial cells is the rapid activation in response to even minor pathological changes in the brain. They are a key factor in defense of the neural parenchyma against infectious diseases,

inflammation, trauma, ischemia, brain tumors and neurodegeneration (reviewed by Kreutzberg, 1996). Activated microglia can destroy invading microorganisms, remove debris, promote tissue repair by secreting growth factors and thus facilitate return to tissue homeostasis. Furthermore, microglia have receptors for CNS signaling molecules such as ATP, acetylcholine (Ach) and norepinephrine (Walz et al., 1993; Whitemore et al., 1993). Their ability to respond selectively to molecules involved in neurotransmission allows them, in their resting state, to monitor continuously the physiological integrity of their microenvironment and to react rapidly in the event of pathological disturbances.

Glutamate Receptors and Transporters

Glutamate is the major fast excitatory neurotransmitter in the mammalian central nervous system (Mayer and Westbrook, 1987). Upon stimulation glutamate is released from neural cells, astrocytes and neurons. Both type of cells express glutamate receptors. Two families of glutamate receptors have been cloned and characterized, G protein-coupled glutamate receptors also known as metabotropic receptors (mGluR) and an ion channel family of glutamate receptors. Until now eight receptors of the metabotropic class have been discovered and they have been classified into three groups based on their linkage to second messenger systems and their pharmacology. Each group has a distinct roles in the physiological and pathophysiological processes in the brain. Group I mGluR-receptors, which are coupled to $G_{q/11}$ proteins, stimulate IP_3/DAG formation and augment neurodegeneration mediated by the ionotropic glutamate receptors, whereas the Group II and Group III mGluR-receptors that modulate cAMP levels via $G_{i/o}$ proteins may be

neuroprotective (reviewed by Nicoletti et al., 1996). Modulation of synaptic transmission by mGluR-receptors has been also documented (Kilbride et al. 1998; Wittmann et al., 2001).

The ion channel family of glutamate receptors comprises 3 major subdivisions based on pharmacology and structural properties: NMDA (N-methyl-D-aspartate), AMPA (α -amino-3-hydroxyl-5-methylisoxazole-4-propionic acid), and Kainate subtypes of ionotropic glutamate receptors. The NMDA receptor is a high conduction channel (50 ps), permeable for both cations, Na^+ and Ca^{2+} , and blocked by magnesium at membrane potentials close to resting, endowing a voltage dependence to this ligand-gated ion channel (Mayer and Westbrook, 1987; Nicol et al., 1988). NMDA receptors are post-synaptic and play an important roles in the development of the CNS, plasticity and excitotoxicity (Rothman and Olney, 1986; Cotman et al., 1988). AMPA receptors are cation-permeable channels ($\text{Na}^+ / \text{K}^+ / \text{Ca}^{2+}$) that mediate chemical transmission at the majority of fast excitatory synapses in the CNS (Sheng and Lee, 2001). The role of kainate receptors is less clear however, a plethora of recent studies has altered this situation profoundly such that kainate receptors are now regarded as key players in the modulation of transmitter release, as important mediators of the postsynaptic actions of glutamate, and as possible targets for the development of antiepileptic and analgesic drugs (reviewed by Lerma et al., 2001).

The glutamate transporters in the plasma membranes of neural cells secure termination of glutamatergic synaptic transmission and keep glutamate levels below toxic concentrations. Five homologous types of the Na^+ / K^+ -dependent high-affinity glutamate transporters, termed GLAST (EAAT1), GLT1 (EAAT2), EAAC (EAAT3), EAAT4 and EAAT5, have been cloned recently from different species (reviewed by Gegelashvili and Schousboe, 1997). EAAC and EAAT5 are found exclusively in neurons (Yamada et al.,

1996; Torp et al., 1997), whereas GLAST and GLT1, the major contributors to glutamate uptake, are glia-specific transporters (Rothstein et al., 1996). GLT1 predominates quantitatively and is responsible for most of the glutamate uptake activity in the juvenile and adult brain.

Role of astrocytes in the control of synaptic transmission and neuronal excitability

For years, glial cells were assumed to be the silent cells that lacked excitability and had no role in signal integration in the brain. Recent findings challenged that view emphasizing the active contribution of astrocytes in synaptic transmission, both *in vitro* (Araque et al., 1998a; Ulian et al., 2001) and *in situ* (Pasti et al., 1997). A study by Newman et al. (1998) showed that Ca^{2+} elevations within glial cells of the retina can modulate information transfer from photoreceptors to retinal ganglion cells. This modulatory action provides compelling evidence that glial Ca^{2+} signals are intimately involved in the regulation of information processing in the nervous system. Astrocytes can transmit messages to surrounding cells by the direct release of signaling molecules such as the excitatory neurotransmitter glutamate that excites neurons (Nedergaard, 1994; Parpura et al., 1994; Jęftinija et al., 1996; Bezzi et al., 1998; Jeremic et al., 2001). In addition, astrocytes transmit messages to other cells by producing transcellular messengers including eicosanoids (Piomelli, 1994) and nitric oxide (Murphy et al., 1993).

Astrocytes may have opposite effects on the synapse; they can elicit a mGluR-dependent depression (Araque et al., 1998a) or an NMDA-receptor-dependent increase in neurotransmitter release (Araque et al., 1998b). Highly plastic, bi-directional signaling loops between astrocytes and neurons have been documented at both central (Pasti et al.,

1997) and peripheral synapses (Robitaille et al., 1998). Based on these insights, a new view emerged that the brain should no longer be regarded as a complex array of neuronal networks, but instead of integrated circuits of interactive neurons and glia (Bezzi and Volterra, 2001).

The role of glia and glutamate in the brain pathology

Damage to the central nervous system leads to cellular changes not only in the neural cells (neurons and glia) but also in endothelial cells and the cells of the immune system. Traumatic injury to the CNS results in a rapid response from resident astrocytes, a process often referred to as reactive astrogliosis or glial scarring. This neuroglial activation is a consistent feature in almost all forms of brain pathology and appears to reflect an evolutionary-conserved program which plays an important role for the repair of the injured nervous system (Raivich et al., 1999). It is still debated whether these reactions of glia to insult are beneficial or detrimental for the brain. It is believed that the reactive astrogliosis observed in most neurological disorders may regulate the removal of toxic compounds by damaged neurons and support neuronal growth by releasing trophic factors (Tacconi, 1998). However, it has also been suggested that astrocytes contribute to the decline of neurological function by accumulating and releasing excitotoxic amino acids after ischemia or oxidative stress, increasing in neuronal $[Ca^{2+}]_i$ and free radicals and releasing arachidonic acid and pro-inflammatory cytokines. (Shivachar et al., 1996; Ashner, 1998; Tacconi, 1998; Sastry and Rao, 2000).

Glutamate has gained additional attention due to recent observations showing that endogenous glutamate is toxic to neurons when it accumulates in the extracellular space in

the brain. Pathological activation of glutamate receptors is thought to be a final common pathway leading to neuronal damage in the course of many neurological diseases such as hypoxic-ischemic brain injury, epilepsy, Huntington's disease, Parkinson's disease, Alzheimer's disease, and HIV-associated dementia. Brain damage, during ischemia and other pathological events, is now thought to be partly due to the inappropriate release of EAAs such as L-Glu and L-Asp, which, through activation of EAA receptors, cause death of certain neurons (Choi, 1988; Faden et al., 1989). Reversal of glutamate transporters in an experimental paradigms that mimic severe ischemia led to extracellular glutamate accumulation and subsequent neuronal death in hippocampal slices (Rosi et al., 2000). Perhaps the most compelling argument implicating glutamate toxicity in induced neural injury is the finding that glutamate antagonists, in particular NMDA antagonists, are neuroprotective in a variety of paradigms (Rothman, 1983; Weiss et al., 1986; Goldberg et al., 1987).

Many of the external factors such as bacterial and viral infections, toxins and drugs of abuse induce apoptosis of neurons and glia *in vitro*. This is the prominent feature of many brain disorders *in vivo*. Recent investigations revealed a complex machinery of pro- and anti-apoptotic protein factors the balance of which determine the difference between life and death (Raghupathi et al., 2000). The effect of the brain injury on the expression patterns of survival promoting-proteins, such as Bcl-2, Bcl-xL and the extracellular-signal regulated kinase (ERK), or the death-inducing proteins and lipids, such as Bax, ceramide, c-Jun N-terminal kinase (JNK) and the caspase family of proteases have been documented (Ariga et al., 1999; Li and Yuan, 1999; Schwartz and Baron, 1999; Sastry and Rao, 2000).

Neuroligands

The research discussed in this dissertation has been focused on the short and long-term effect(s) of the neuroligands ATP, stromal cell-derived factor 1 α (SDF-1 α) and cannabinoids on neural cells, primarily astrocytes. We assayed changes in the $[Ca^{2+}]_i$, glutamate release and cell viability elicited by the neuroligands.

ATP The functional importance of intracellular ATP levels for the cellular metabolism has been recognized for many years. Recent investigations postulated a new role for ATP in modulation of neurotransmission, cardiac function and other biological processes (Burnstock, 1990; Gordon, 1986). It has been documented that ATP is released or co-released with acetylcholine and norepinephrine from nerves endings (Fyffe and Perl, 1984; Burnstock 1986). It has been suggested that ATP might also be an excitatory mediator in the CNS (Phillis and Wu, 1981). There are two classes of purinergic receptors (Burnstock 1990). One is the adenosine-sensitive P1-purinoreceptor, and the other is the ATP-sensitive P2-purinoreceptor. Activation of P2 receptors leads to an increase in $[Ca^{2+}]_i$ in a variety of excitable and unexcitable cells (El-Moatassim et al., 1992; Jahromi et al., 1992; Salter and Hicks, 1994). Two different mechanisms regulate the ATP-induced calcium transients. One involves the direct activation of the ionotropic family of P2X₁₋₇ receptors (Benham and Tsien, 1987). The other involves the activation of the metabotropic family of P2Y₁₋₁₁ receptors (Boyer et al., 1989). The P2Y receptor family is coupled to G_{q/11} proteins by which ATP induces activation of phospholipase C (PLC) and the subsequent breakdown of phosphoinositides (Pearce et al., 1989).

Cannabinoids The major psychoactive component of marijuana and its natural source plant, *Cannabis sativa*, is the Δ -9-tetrahydrocannabinol (THC). So far, two cannabinoid receptors have been cloned and characterized, CB₁ and CB₂. Radioligand binding studies confirmed the presence of CB₁ receptors in the brain (Axelrod et al., 1998), where they modulate adenylate cyclase and ion channel activity via G_{i/o} protein regulation (Tao and Abood, 1998). CB₂ receptors are found in the cells of the immune system and are also coupled to G_{i/o} proteins (Axelrod and Felder, 1998).

Arachidonylethanolamide (anandamide) was the first discovered endogenous ligand of CB₁ receptors (Devane et al., 1992). It mimics the pharmacological effects of THC. Studies showed that the synthesis and release of anandamide could be stimulated in cortical and striatal neurons, but not astrocytes, by the application of membrane-depolarizing agents such as ionomycin and high potassium (Di Marzo et al., 1994). Neurons and astrocytes were both found to re-uptake anandamide rapidly (Deutsch and Chin, 1993) by the saturable, selective, temperature-dependent and Na⁺-independent transporter, whose inhibition potentiates anandamide action (Beltramo et al., 1997). Recently, potent exogenous cannabinoid receptor agonists (Win 55,212-2; HU-210; CP-55940) that share similar pharmacological and physiological properties with the endogenous cannabinoid receptor ligands, have been synthesized (Felder et al., 1995).

In the nervous system cannabinoids exert most of their action through the specific interaction with abundant CB₁ receptors in neurons of specific brain regions (Pertwee et al., 1997). Cadogan (1997) showed that anandamide inhibits forskolin-stimulated cAMP formation in striatal slices. In hippocampal neurons, anandamide inhibits presynaptic N and P/Q type Ca²⁺ channels leading to the inhibition of transmitter release (Twitchell et al.,

1997). Anandamide stimulates arachidonic acid (AA) release from primary cultures of rat brain cortical astrocytes (Shivachar et al., 1996). Similarly, THC induces AA release from cultured hippocampal neurons (Chan et al., 1998). Furthermore, THC induces a delayed increase of $[Ca^{2+}]_i$ in primary hippocampal neurons (Chan et al., 1998). Cannabinoids effect on Ca^{2+} transients is also indirect as the R(+)-methanandamide enhances NMDA-elicited Ca^{2+} signals in cerebellar granule neurons through action at the CB_1 receptor (Netzeband et al., 1999). Cannabinoids also act on astrocytes by a non-cannabinoid receptor mechanism. The Venance's group (1995) found that anandamide blocked the propagation of astrocyte's Ca^{2+} waves evoked by glutamate released from neurons through the inhibition of gap junctions.

SDF-1 α and gp120 Chemokines, a large family of low molecular mass (8-10 kDa) cytokines, and their receptors are involved in cell migration during inflammation, in the establishment of the functional microenvironment and in organogenesis. They have been classified on the basis of the sequence of arranged cysteine groups into four subsets of chemokines reported to date: C, CC, CXC, and CX3C (Rollins et al., 1997). Most of the chemokines discovered to date belong to two major subfamilies: CXC (α -chemokines) or CC (β -chemokines). Chemokine receptors are members of the seven-transmembrane domain G-protein-coupled receptors (Bokoch, 1995). Chemokine receptors also act as co-receptors for entry of different HIV-1 strains into lymphocytes and macrophages. CCR5 has been shown to function as a co-receptor for macrophage-tropic HIV-1 strains (Alkhatib et

al., 1996), whereas CXCR4 act as a co-receptor for T cell-tropic HIV-1 strains (Bleul et al., 1996).

Although most chemokine receptors bind several chemokines, CXCR1 and CXCR4 are specific for only one physiological ligand. CXCR1 interacts with interleukin-8 (IL-8), whereas SDF-1 α is the only ligand identified so far for CXCR4 (Baggiolini et al., 1997). In contrast, the CC receptor subfamily interacts with the β -chemokines group: monocyte chemoattractant protein-1 (MCP-1), -1, -3, -4, macrophage-inflammatory protein-1 α (MIP-1 α) and RANTES. This group mainly attracts monocytes, whereas SDF-1 α is a neutrophil chemoattractant.

The activation signals after SDF-1 α stimulation are not well defined and various signal transduction pathways have been implicated through the association of CXCR4 receptor with G-proteins. Bajeto's group (1999) first showed that the CXCR4 receptor and its ligand SDF-1 α are expressed in cultured cortical type I astrocytes. The same group demonstrated the ability of SDF-1 α to increase $[Ca^{2+}]_i$ in cortical astrocytes and neurons and to inhibit cyclic AMP formation induced by forskolin treatment. It has been shown that SDF-1 α and gp120 induce activation of extracellular signal regulated kinases (ERK) in astrocytes and neurons (Lazarini et al., 2000; Bezzi et al., 2001). ERK activation is the neurotoxic or neuroprotective. Furthermore, SDF-1 α triggers CXCR4 receptor dimerization and activation of JAK / STAT pathway similar to the action of other cytokines (Vila-Coro et al., 1999).

HIV-1 viral products, implicated in neurotoxicity, include the proteins involved in viral replication: Tat, Nef and Rev, as well as envelope glycoproteins, gp120 and gp41

(Nath et al., 1998). Lipton's group (1991) has reported the first evidence that gp120 neurotoxicity was mediated by NMDA-receptor activation. Subsequent studies, incorporating a variety of complementary experimental approaches, provided additional evidence that gp120 has neurotoxic properties (Muller et al., 1992; Giulian et al., 1993; Yeung et al., 1995). gp120 appears to cause neurotoxicity indirectly following interactions with cells such as microglia and astrocytes (Lipton et al., 1994). In neurons, gp120 increases $[Ca^{2+}]_i$ by causing an influx of calcium through voltage- and receptor- operated calcium channels and release of calcium from intracellular pools (Dreyer et al., 1990). Many of the gp120 neurotoxic actions appear to be mediated through interactions with glia because, in glial cells, gp120 down regulates the production and release of endogenous neurotrophic substances and cytokines (Bagetta et al., 1996; Kong et al., 1996), inhibits glutamate uptake (Dreyer et al., 1995), and increases Na^+/H^+ exchange, K^+ conductance and tyrosine kinase activity (Benos et al., 1994; Bernardo et al., 1994; Bubien et al., 1995).

CHAPTER 2. ATP STIMULATES CALCIUM-DEPENDENT GLUTAMATE RELEASE FROM CULTURED ASTROCYTES

A paper published in the Journal of Neurochemistry

by

Aleksandar Jeremic, Ksenija Jeftinija, Jelena Stevanovich, Aleksandra Glavaski and Srdija Jeftinija

Abstract

ATP caused a dose-dependent, receptor-mediated increase in release of glutamate and aspartate from cultured astrocytes. Using calcium imaging in combination with HPLC, we found that increase in intracellular calcium coincided with increase in release of glutamate and aspartate. Competitive antagonists of P₂ receptors produced a block of the response to ATP. The increase in intracellular calcium and release of glutamate evoked by ATP were not abolished in low Ca²⁺-EGTA saline, suggesting involvement of intracellular calcium stores. Pretreatment of the glial cultures with an intracellular Ca²⁺ chelator abolished the stimulatory effects of ATP. Thapsigargin (1 μM), an inhibitor of Ca²⁺-ATPase of the Ca²⁺ pump of internal stores, significantly reduced the calcium transients and the release of aspartate and glutamate evoked by ATP. U73122 (10 μM), a phospholipase C inhibitor, attenuated the ATP-stimulatory effect on calcium transients and blocked ATP-evoked glutamate release in astrocytes. Replacement of extracellular sodium with choline failed to

influence ATP-induced glutamate release. Furthermore, the inhibitor of the glutamate transporters p-chloromercuri-phenylsulfonic acid and *L-trans*-pyrrolidine-2,4-dicarboxylate failed to impair the ability of ATP to stimulate glutamate release from astrocytes. However, an anion transport inhibitor, furosemide, and potent Cl⁻ channel blocker, 5-nitro-2(3-phenylpropylamino)-benzoate, reduced ATP-induced glutamate release. These results suggest that ATP stimulates EAA release from astrocytes through a calcium-dependent anion-transport sensitive mechanism.

Key words: astrocyte-neuron signaling, calcium transients, glutamate, purinergic receptors, transmitter release.

Introduction

Burnstock first showed in 1976 that extracellular adenosine 5'-triphosphate (ATP) can influence neurotransmission, cardiac function, muscle contraction and other biological processes, raising the issue that ATP can function as transmitter. It has been shown that ATP can be released or co-released with acetylcholine or norepinephrine from nerve endings (Fyffe and Perl, 1984; Burnstock, 1986). Extracellular ATP excites a subpopulation of rat dorsal horn neuron (Jahr and Jessel, 1983) and rat sensory neurons (Krishtal et al., 1988). It was also reported that ATP depolarized cultured hippocampal neurons and induced release of glutamate from the neurons (Inoue et al., 1992). The actions of ATP are mediated by P₂-purinoceptors which are present on many cell types including neurons and astrocytes (Phillis and Wu, 1981; Salter and Henry, 1985; Pearce et al., 1989). There is evidence that activation of P₂-purinoceptors leads to an increase in intracellular calcium in a

variety of excitable and unexcitable cells (O'Connor et al., 1991). Two mechanisms are involved in ATP-evoked intracellular calcium increase. First, ATP can activate nonselective cation channels, resulting in depolarization and subsequent opening of voltage-dependent Ca^{2+} channels (Friel, 1988). The increased calcium influx is also the result of direct activation of ATP-gated cation channels (Benham and Tsien 1987). Second, the extracellular ATP can stimulate the breakdown of inositol phospholipids and the resulting increase in inositol 1,4,5,-triphosphate (IP_3) is responsible in part for the elevation of cytosolic Ca^{2+} (Danziger et al., 1988).

There is an increasing body of evidence showing that neuroglia may be more actively involved in brain function than has been previously thought. It has been demonstrated that neuroglia have multiple roles in the nervous system which include the regulation of extracellular potassium, the uptake of excitatory amino acids (EAAs) and the release of neurotransmitter such as glutamate, aspartate and acetylcholine (Nicholls and Atwell, 1990; Barres, 1991; Kanai et al., 1993; Smith, 1994). Recent work from our laboratory shows that the release of the excitatory neurotransmitter glutamate from astroglial cells plays a key role in glia-neuron signaling (Parpura et al., 1994, Jeftinija et al., 1996).

Although it has been determined that extracellular ATP can stimulate Ca^{2+} influx and increase intracellular calcium in cultured astrocytes (Neary et al., 1991; Salter and Hicks, 1994), the consequences of induced calcium elevations in glia are unknown. Our previous study shows that the neuroligand bradykinin can induce the release of EAAs from cultured astrocytes in a Ca^{2+} -dependent manner (Parpura et al., 1994; Jeftinija et al., 1996). Furthermore, we demonstrated that ATP induced glutamate release from cultured Schwann

cells (Jeftinija et al., 1998). This evidence prompted us to propose that extracellular ATP might influence the functions of astrocytes. In this study, we demonstrate that ATP can evoke release of EAAs from cultured astrocytes and that intracellular Ca^{2+} plays a role in the release of EAAs.

Material and Methods

Cell Culture

Mixed cell cultures from neonatal rat cerebral cortex were established according to the previously described procedure (Jeftinija et al., 1996). Briefly, cortex was freshly dissected and tissue was enzymatically (papain 20i.u./ml; 1 hour at 37°C) and mechanically dissociated. The cells were plated into culture flasks and maintained at 37°C in a humidified 5% CO_2 /95% air atmosphere. Culture medium consisted of Eagle's MEM supplemented with 19% heat-inactivated FBS and 40mM glucose, 2mM L-glutamine and gentomicin.

The enriched astrocyte type-1 cultures were obtained by following the protocol previously described (Jeftinija et al., 1996). After mixed cultures reach confluence (9-12 days), the flasks were "preshaken" (260rpm) for 90min to remove microglia and dividing type I astroglia. Following preshake, the medium was replaced, and the flasks were incubated for 1 hour to equilibrate. The cultures were then shaken overnight (12-18h) at 260 rpm at 37°C. After this two sets of cells were present: adherent polygonal cells (mostly type I astroglia) and suspended cells. Cultures enriched in type I astroglia were obtained by trypsinising (0.25%) for 5 min the cells attached after the overnight shake. Trypsin was

inactivated by adding 5 ml of serum-supplemented BME (serum contains protease inhibitors) to each flask. Then the cell suspension was transferred to sterile 50-ml centrifuge tubes and spun at 100-x g for 10 min. Cells were resuspended in α -MEM and the number of viable cells was determined by trypan blue exclusion. Cells were plated on poly-L-lysine (1mg/ml; MW 100,000)-coated glass coverslips. The cells were maintained by completely changing the α -MEM medium every 2-3 days. All experiments were performed on cells that have been in culture for 3-5 days after re-plating.

The protocol used for acutely isolated astrocytes is a modification of procedure used for establishing the primary astrocyte cultures. Tissue from cortex or hippocampus were incubated with 2.5 % papain solution for 50 min. at 37° C. After the incubation tissue was mechanically dispersed by triturating through a fire-polished glass pipette 8-10 times and plated on the poly-L-lysine (1mg/ml, MW 100,000; Sigma) coated glass-bottomed dishes. Cells were incubated for 20 minutes at 37°C to allow them to adhere. A further 1-ml of α -MEM solution was added to each dish. The time from killing of the animal to the final addition of α -MEM and beginning of experiments were 1-2 hours. No obvious cell divisions were detected within 48 hours and cultures were considered to be acute cell cultures.

Immunocytochemistry (ICC):

Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enhanced DAB method (Jeftinija and Jeftinija., 1990). ICC was performed using antibodies raised against glial fibrillary acidic protein (GFAP; 1:5000, ICN Immunobiological) and the microtubule associated protein, MAP-2 (1:2000, Boehringer).

Staining was performed by exposing cultures to 0.04% 3-3'diaminobenzidine tetrahydrochloride (DAB, Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide dissolved in 0.1M sodium acetate. Controls were processed by omitting the specific antiserum.

Release methods and HPLC

The coverslips with glial cultures were mounted into a perfusion chamber and washed in normal HEPES saline for a period of 30 to 40 min to allow equilibration. Normal HEPES saline contains (in mM): NaCl 140, KCl 5, MgCl₂ 2, CaCl₂ 2, glucose 5 and HEPES 10 (pH 7.4). The volume of the chamber was about 50 μ l. Standard and test solutions were pumped into the perfusing system by a minipuls pump at a rate of 200 μ l/min. After equilibration, 200 μ l samples were collected every minute. Four control samples were collected for determination of basal concentrations of amino acids. Test substances were dissolved in the recording solution and delivered in known concentrations to the cultures. The amino acid content in the samples was determined by high-performance liquid chromatography (HPLC) with fluorescence detection. Prior to injection, aliquots of the samples were derivatized with o-phthalaldehyde (OPA) 2-mercaptoethanol reagent (Pierce). Chromatography was performed on a 15cm Microsorb-MV HPLC column (Rainin Instrument Co.) using a pH 5.9 sodium acetate methanol gradient. Basal rates of amino acids released were determined as the mean of the amino acids in 4 samples collected just prior to stimulation. In the experiments in which calcium was lowered to 0.2mM, Mg²⁺ was elevated to 2.5mM and 1mM EGTA was added.

Intracellular calcium imaging

The effect of experimental manipulation on glial $[Ca^{2+}]_i$ was evaluated by fluorescence ratio imaging technique. Cells were loaded with 5 μ M Fura-2 AM (Molecular Probes) for 40-60 minutes at 24°C. 1 μ l of 25% (w/w) of Pluronic F-127 (Molecular Probes) was mixed with every 4 nmol of Fura-2 AM to help dissolving of the ester into aqueous medium. After washing, cells were deesterified for 10 minutes at 24°C. All image processing and analysis were performed using an Attofluor system with Zeiss microscope. Background subtracted, rationed images (340/380 nm) were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz et al. (1985). Calibration was performed in situ according the procedure provided by the Attofluor, using the Fura-2 Penta K^+ salt as a standard.

Results

Morphology of cultured astrocytes

Phase-contrast microscopy indicated that glial cultures were enriched in type-1 astrocytes devoid of neurons. This was confirmed by immunocytochemistry which demonstrated that cultures lacked MAP-2 immunoreactivity but were more than 95% immunopositive for glial fibrillary acidic protein (GFAP). In parallel mixed cortical cultures containing neurons, MAP-2 immunoreactivity was reliably detected. Therefore, enriched astrocyte cultures in the current study were considered neuron-free and the predominant cell type in these cultures was GFAP-positive polygonal astrocyte type 1.

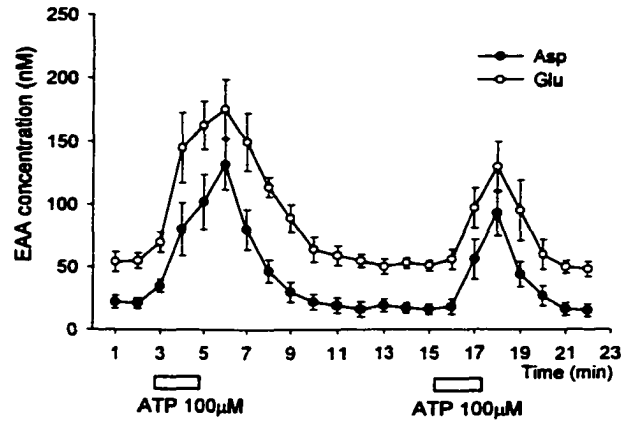
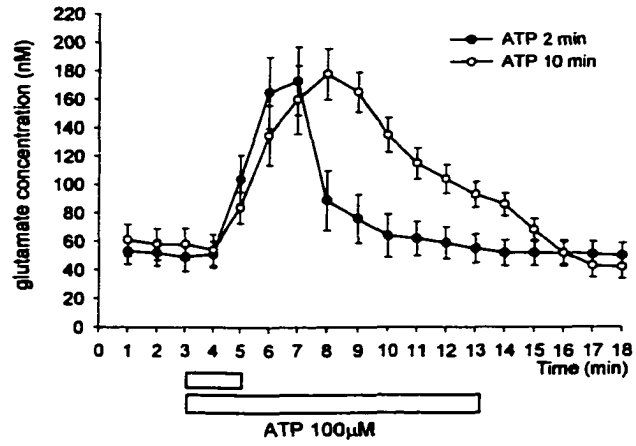
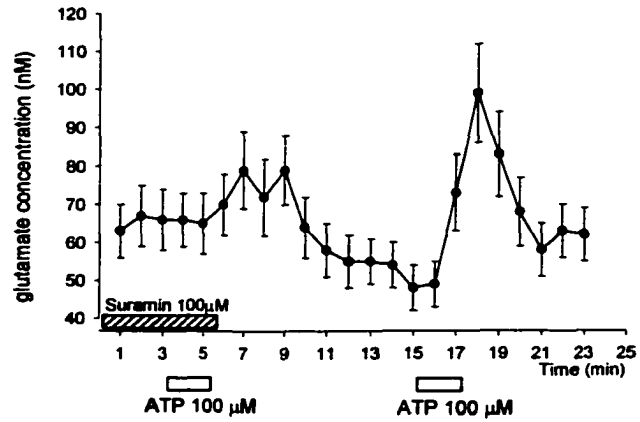
ATP evokes receptor-mediated release of glutamate from cultured astrocytes type I

The release of glutamate from primary glial cultures was assayed using HPLC on the superfusate. The basal release of aspartate and glutamate in to the superfusate produced levels of 25 ± 3 nM and 58 ± 4 nM, respectively (mean \pm SEM, n=12). Addition of ATP caused a concentration-dependent increase in release of excitatory amino acids (EAA) from astrocyte cultures. The threshold concentration of ATP was $1\mu\text{M}$ (data not shown).

Perfusion application of $10\mu\text{M}$ ATP for 2 min increased the concentration of aspartate and glutamate from 24 ± 5 nM to 38 ± 7 nM and from 54 ± 9 nM to 84 ± 13 nM, respectively (mean \pm SEM, n=6). Perfusion application of $100\mu\text{M}$ ATP for 2min to astrocyte culture resulted in a 212 ± 10 % increase of glutamate concentration and 407 ± 21 % increase of aspartate concentration (n=6, Fig. 1A). A second application of ATP 10 min after the first application resulted in an increase in the release of glutamate and aspartate that was at the level of 64% of that produced by the first application (Fig. 1A). A ten-minute application of ATP to astrocytes resulted in a peak increase in the release of glutamate followed by a decline to a plateau that was significantly higher than baseline release ($p < 0.001$, Student's t-test, n=6, Fig. 1B).

To investigate the type of purinergic receptors in cultured astrocytes the effects of adenosine were examined. Adenosine had no effect on release of glutamate from cultured astrocytes (data not shown). These results indicate that the ATP response is mediated by P_2 receptors. Suramin at a concentration of $100\mu\text{M}$ produced a block of the response to ATP (n=6; $P < 0.001$, Student's t-test, Fig. 1C). Suramin, a trypanocidal drug, is a competitive

Figure 1. Stimulatory effect of ATP on EAAs release from astroglia is receptor-mediated. The superfusate from astrocyte cultures was collected at one-minute intervals and levels of EAAs were measured using HPLC. (A) Addition of ATP (100 μ M) for 2 min causes a two-fold increase of glutamate concentration and fourfold increase of aspartate concentration from baseline levels. Repeated application of ATP ten minutes later produced an increase in EAA concentrations that were ~60% of the first application (B) Ten minutes application of ATP resulted in a twofold increase of glutamate concentration followed by sustained declining release of glutamate that was significantly higher than the control release. (C) Suramin (100 μ M), the P₂ receptor antagonist abolished the ATP-evoked glutamate release from astrocyte cultures.

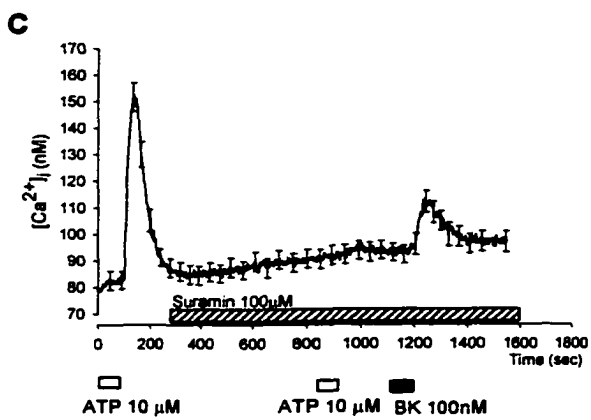
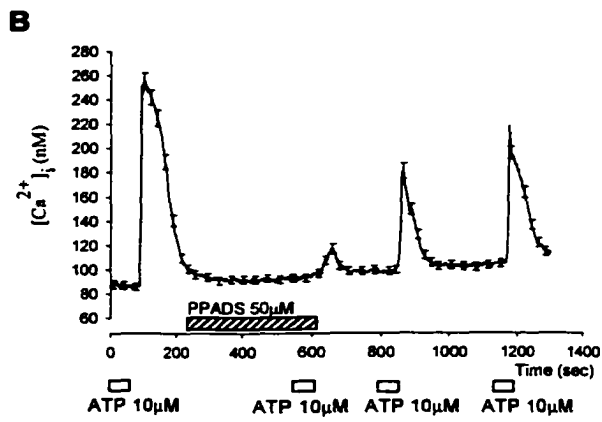
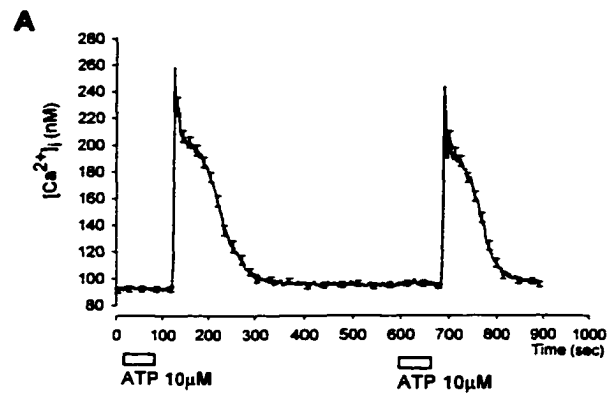
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antagonist at P₂ receptors (Dunn and Blakeley, 1988; Hoiting et al., 1990). The blockade of ATP-induced glutamate release by suramin was reversible (Fig. 1C). Similar results were obtained with pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium (PPADS), a selective P₂ antagonist (data not shown).

ATP mobilizes calcium in cultured astrocytes type I

To ask whether calcium might play a role in ATP-induced EAA release from cultured astrocytes, we used a fluorescence calcium imaging technique to monitor glial calcium levels. Cells were loaded with the membrane permeant calcium indicator fura-2 AM. In resting conditions, the cytoplasmic calcium level of glia was 91 ± 2 nM (n=201). ATP (10 μ M) raised the cytoplasmic level of free calcium in 97 % of cells tested (n=194 of 201). This increase of intracellular calcium reached the peak level of 207 ± 7 nM (n=189; Fig. 2A) about 80 seconds after the onset of ATP application and was sustained for several minutes. Repeated applications of ATP elevated calcium levels in astrocytes (Fig. 2A). The P₂ receptor antagonist PPADS (50 μ M) reversibly attenuated the stimulatory action of ATP on calcium mobilization. In the presence of the PPADS, the average resting calcium level of 92 ± 4 nM was increased by application of 10 μ M ATP to 114 ± 5 nM (n=74). However, after washout of the antagonist, ATP increased the cytoplasmic calcium level to 179 ± 5 nM (n=74), a value significantly greater than in the presence of the P₂ receptor antagonists ($p < 0.001$, ANOVA followed by Tukey's *post hoc* test, Fig. 2B). Similar results were obtained with suramin (Fig. 2C). The effect of suramin was selective, as effect of bradykinin was not influenced by application of suramin (Fig. 2C).

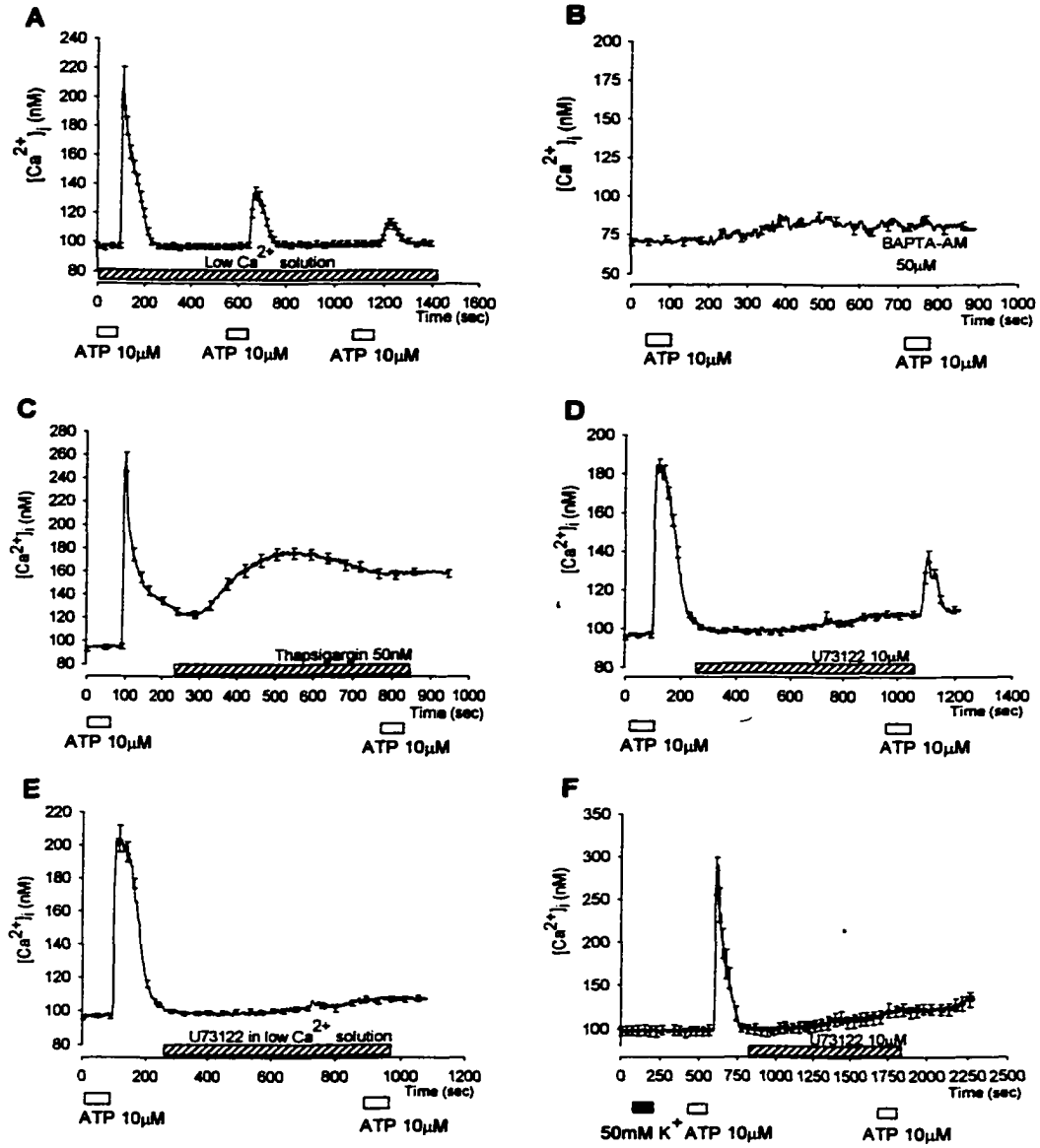
Figure 2. ATP-induced calcium transients requires activation of the P₂ receptor. Perfusion application of 10 μM ATP for 1 min reliably raised intracellular calcium in primary astrocyte cultures (A). PPADS (50 μM), a selective P₂ receptor antagonist reversibly attenuated the ATP stimulatory action on [Ca²⁺]_i in astrocytes (B). P₂ receptor involvement was further supported with 100 μM suramin, another P₂ receptor antagonist that selectively blocked the ATP but not bradykinin evoked calcium transients' (C).



Removal of external calcium from the bathing medium did not prevent the initial calcium mobilizing action of ATP. Cells were bathed in a calcium-depleted saline containing 0.2 mM calcium with the addition of 1 mM EGTA to yield an estimated free extracellular calcium level of 26 nM. We found that it was necessary to have some calcium in the bathing medium otherwise cells detached from the culture substrate. In this calcium-depleted saline, ATP elevated cytoplasm free calcium from 96 ± 2 nM to 210 ± 6 nM (n=121, Fig.3A). However, subsequent additions of ATP in calcium-depleted bathing medium had little calcium mobilizing action (n=26) and (n=18) for the second and third applications, respectively (Fig. 3A). These data suggest that ATP mobilize calcium from internal stores and that these stores must refill with calcium from the extracellular medium.

To evaluate contribution of internal calcium stores in ATP stimulatory effect on cultured astrocytes we used [1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetracetic acid-acetoxymethyl ester] (BAPTA-AM), an intracellular calcium chelator. Cultures pre-incubated in 50 μ M BAPTA-AM for 45 min did not respond to 10 μ M ATP application (n=87, Fig. 3B). Calcium mobilization from an internal store is further supported by observations using thapsigargin, an inhibitor of the Ca^{2+} -ATPase of internal calcium stores (Thastrup, 1990). Following the first application of ATP, thapsigargin (50 nM) was added to the culture to prevent reloading of internal stores with calcium. Addition of thapsigargin alone caused a significant increase in calcium from 120 ± 3 to 178 ± 5 nM (n=38; Student t-test, $p < 0.0001$, Fig. 3C). In the presence of thapsigargin, a second application of ATP failed to mobilize internal calcium (n=38; Fig. 3C) as compared to matched control cells

Figure 3. ATP mobilizes calcium from internal calcium stores. (A) The ATP stimulatory effect on calcium transients in astrocytes was preserved in low calcium solution suggesting that calcium is mobilized from internal stores. However, second and third addition of ATP had little effect on internal calcium levels. **(B)** BAPTA-AM (50 μ M), a cell permeable intracellular calcium chelator abolished the ATP evoked calcium transients. **(C)** Thapsigargin (50 nM), a Ca^{2+} -ATPase inhibitor completely blocked the increase in calcium induced by ATP application. **(D)** U73122 (10 μ M), a specific PLC inhibitor attenuated the ATP-stimulatory effect on calcium transients suggesting the involvement of PLC-IP₃ pathway. U73122 completely blocked the ATP stimulatory action only in the low Ca^{2+} solution pointing toward the contribution of ionotropic P₂ system and the extracellular calcium in the ATP-evoked calcium transients **(E)**. In acute cultures, U73122 completely blocked the ATP stimulatory effect implying that cells possess only metabotropic P₂ PLC-coupled receptors **(F)**.



which were incubated in the DMSO carrier saline, supporting the notion that ATP mobilizes calcium from an internal store.

To determine the type of internal calcium store that ATP acts on, we added ryanodine, caffeine-sensitive calcium store inhibitor. Ryanodine (10 μ M) did not affect the calcium mobilizing action of repeated applications of ATP (data not shown). Furthermore, the sustained presence of caffeine (20 mM) did not alter the ATP stimulatory effect in astrocytes (data not shown). Thus, we conclude that ATP predominantly mobilizes calcium from an ryanodine/caffeine-insensitive internal calcium store. This suggests that ATP's actions are mediated through an IP₃-sensitive calcium store.

ATP-induced calcium transient is blocked by U73122

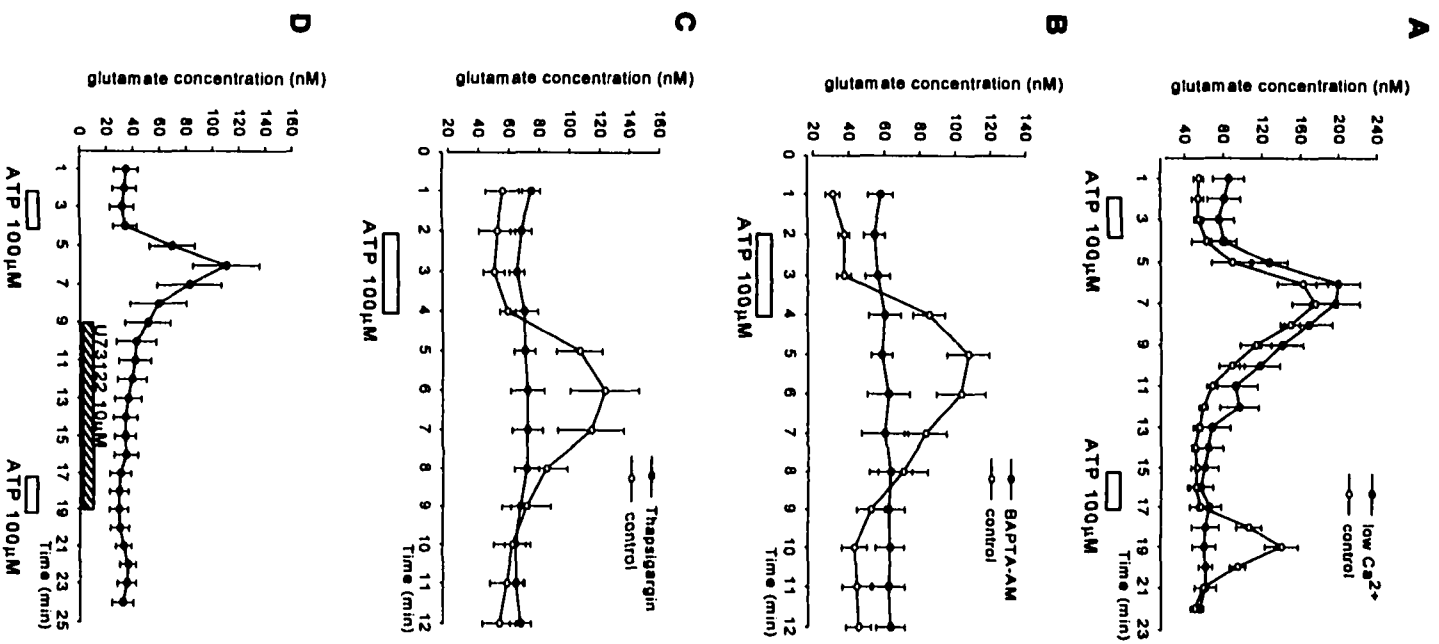
To examine the role of IP₃-sensitive calcium stores in ATP-evoked calcium transients we used U73122, a selective inhibitor of receptor-coupled phospholipase C (PLC) dependent processes (Smith, 1990). The effect of U73122 on the ATP-evoked calcium transients in astrocytes depended on the age of the culture. In primary astrocyte cultures (cells cultured for 12-15 days) the ATP-induced response was significantly smaller in cultures pretreated with 10 μ M U73122 (n=86, p<0.01, ANOVA followed by Tukey's *post hoc* test Fig. 3D). However, complete block of ATP-induced response in cultured astrocytes was achieved only in the low Ca²⁺-EGTA solution combined with U73122 (10 μ M) pretreatment (n=113, Fig. 3E). These results indicate the presence of both ionotropic and metabotropic components of ATP response in cultured astrocytes and by using P_{2X} receptor antibodies we determined the presence of both P_{2X2} and P_{2X3} receptors (data not shown). However,

response of the acutely isolated astrocytes (less than 48 hours in culture) to ATP was completely abolished by preincubation of the cells with 10 μM U73122, indicating presence of metabotropic P_2 receptor only (n=43, Fig. 3F). In order to identify neurons in acutely isolated cells, we took advantage of the observation that neurons respond promptly with a $[\text{Ca}^{2+}]_i$ increase to depolarization induced by 50 mM K^+ (Bezzi et al., 1998). Perfusion application of 50mM K was without effect (Fig. 3F). Culturing of the cells leads to expression of the P_2 ionotropic receptors which is in agreement with the studies showing different receptor expression patterns for acutely isolated astrocytes and cell in cultures (Kimelberg et al., 1997).

ATP induced release of glutamate does not depend on extracellular Ca^{2+}

Calcium-imaging data demonstrated that ATP induced increase in intracellular calcium does not depend on extracellular Ca^{2+} . To investigate whether calcium might play a role in ATP-induced EAAs release from astrocyte cultures, cells were bathed in a calcium-depleted saline containing 0.2 mM calcium with the addition of 1 mM EGTA to yield an estimated free extracellular calcium level of 26 nM. Consistent with the ability of ATP to mobilize intracellular calcium, the first ATP application stimulated the release of glutamate from glial cultures in calcium-depleted saline (n=6, Fig. 4A). The second ATP application failed to evoke glutamate release from cultures bathed in low Ca^{2+} solution but not in the control (Fig. 4A). This observation suggests that ATP-induced release of glutamate is not dependent on extracellular Ca^{2+} and therefore likely results from calcium release from intracellular stores.

Figure 4. Extracellular calcium is not necessary for ATP-evoked glutamate release from cultured astrocytes. **(A)** An increase in glutamate concentration in glia cell perfusate by the 100 μ M ATP application was not altered when cultures were bathed in low Ca^{2+} -EGTA solution. **(B)** In cultures incubated in 50 μ M BAPTA-AM for 30 min the ATP stimulatory effect was abolished. **(C)** A 30-min incubation of astrocytes in 1 μ M thapsigargin resulted in an increase in the baseline concentrations of glutamate and block of ATP-evoked increase in glutamate concentration. **(D)** A specific PLC inhibitor U73122 (10 μ M) diminished the ATP-evoked glutamate release from astrocytes.



In order to confirm that calcium is involved in glutamate release, we pretreated cultures with 50 μM BAPTA-AM, the membrane permeable Ca^{2+} chelator, for 30 min. The baseline level of glutamate was significantly elevated in superfusate from cultures exposed to BAPTA-AM ($P < 0.05$, Student's t-test, $n=6$, Fig. 4B) while ATP-induced release of glutamate was abolished ($n=6$, Fig. 4B). This result suggests that intracellular Ca^{2+} might be responsible for ATP-induced release of glutamate.

Involvement of an internal Ca^{2+} store in the ATP-evoked calcium mobilization and subsequent glutamate release were further supported in experiments using thapsigargin, a potent inhibitor of the Ca^{2+} -ATPase of the endoplasmic reticulum (Thastrup, 1990). Addition of thapsigargin (1 μM) alone raised glutamate baseline above the control glutamate concentrations ($P < 0.05$, Student's t-test, $n=6$, Fig. 4C). In the presence of thapsigargin, ATP failed to produce a change in glutamate concentration supporting the notion that ATP mobilizes calcium from an internal store (Fig. 4C). To determine the type of internal calcium store that ATP acts on, we added ryanodine, caffeine-sensitive calcium store inhibitor. Ryanodine (10 μM) did not affect the ATP-evoked increase in glutamate. Furthermore, the sustained presence of caffeine (20 mM) did not affect stimulatory action of ATP (data not shown). This suggests that ATP's actions are mediated through an IP_3 -sensitive calcium store.

To investigate the role of IP_3 -sensitive calcium store in ATP-evoked increase in intracellular Ca^{2+} and corresponding release of glutamate from cultured astrocytes we used PLC inhibitor U73122. Following the first application of ATP (100 μM) cultures were perfused with 5 μM U73122 solution for 10 min. We found that U73122 (5 μM) had no effect on basal levels of glutamate ($n=6$). In the presence of U73122, a second application

of ATP failed to produce a change in glutamate concentration (n=6, Fig. 4D) supporting the notion that ATP mobilizes calcium from an IP₃-sensitive calcium store. This is consistent with other studies, which have shown that ATP causes a phosphatidylinositol hydrolysis in oligodendrocytes and astrocytes (Pearce et al., 1989).

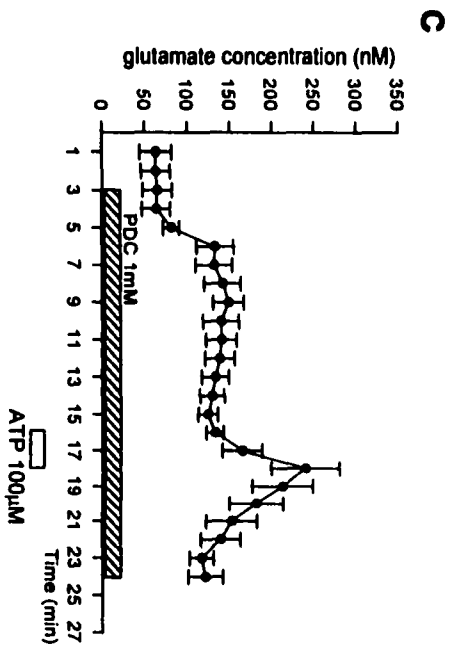
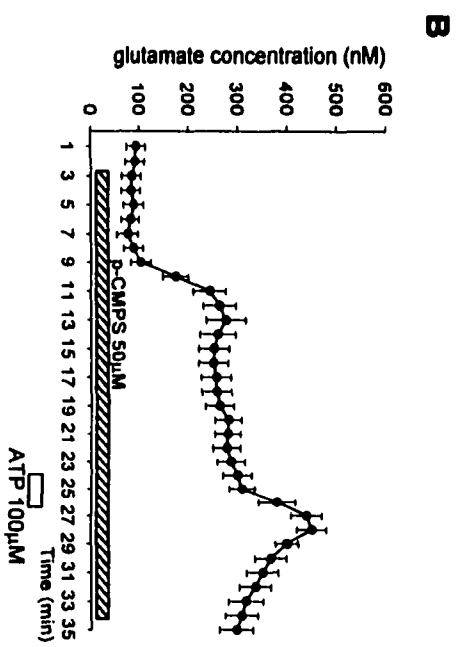
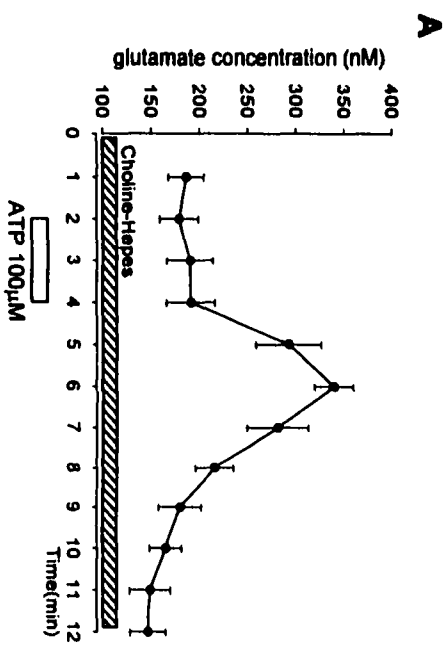
Inhibitor of glutamate uptake does not affect ATP-induced release of glutamate

Extracellular glutamate accumulation may be caused by inhibition of the uptake or reversal of the Na⁺-dependent glutamate transporter (Szatkowski et al., 1990). To examine the relationship between ATP-induced glutamate release and activity of Na⁺-dependent glutamate transporter, we preincubated cultures in Na⁺-free medium. In Na⁺-free medium extracellular NaCl was substituted with choline chloride. ATP-evoked release of glutamate or [Ca²⁺]_i was not influenced by pretreatment of the cultures with Na⁺-free medium (n=4, Fig 5A). These results suggest that ATP-induced glutamate release from cultured astrocytes is not Na⁺-dependent.

To determine whether membrane potential might regulate [Ca²⁺]_i and glutamate release from astrocytes, we exposed astrocyte cultures to high potassium saline. Following application of 50 mM K⁺ saline for 2 min we detected no increase in [Ca²⁺]_i and change of glutamate concentration (data not shown).

The possible role of glutamate transporters as mediators of ATP-induced glutamate release was further investigated using glutamate transport inhibitors p-chloromercuriphenylsulfonic acid (p-CMPS) and L-trans-pyrrolidine-2,4-dicarboxylate (PDC) (Balcar and Johnston, 1972; Balcar et al., 1987; Isaacson and Nicoll, 1993). Consistent with previous

Figure 5. ATP-induced glutamate release from primary astrocyte cultures is not mediated by transporter mechanism. **(A)** In cultures incubated in Na⁺-free medium, neither baseline nor ATP-evoked release of EAA was influenced by treatment. The glutamate transporter inhibitors p-CMPS (50 μM) and PDC (1mM) elevated the baseline concentration of glutamate but failed to block the stimulatory action of ATP on glutamate release **(B and C)**



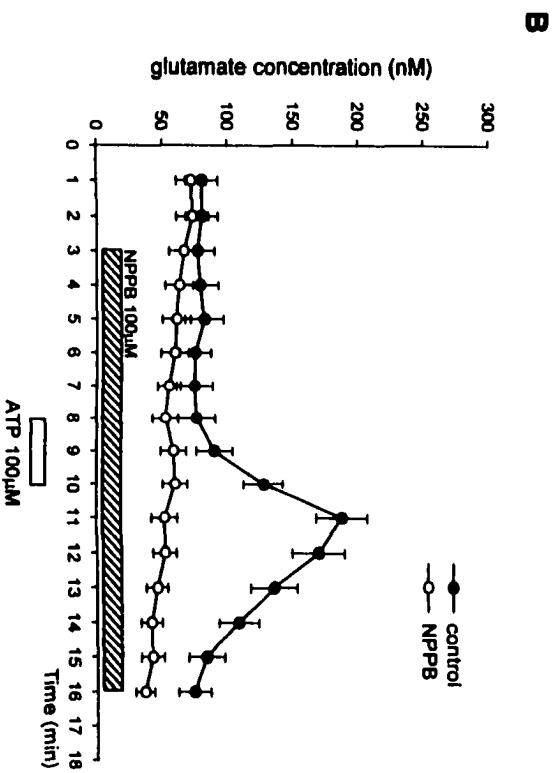
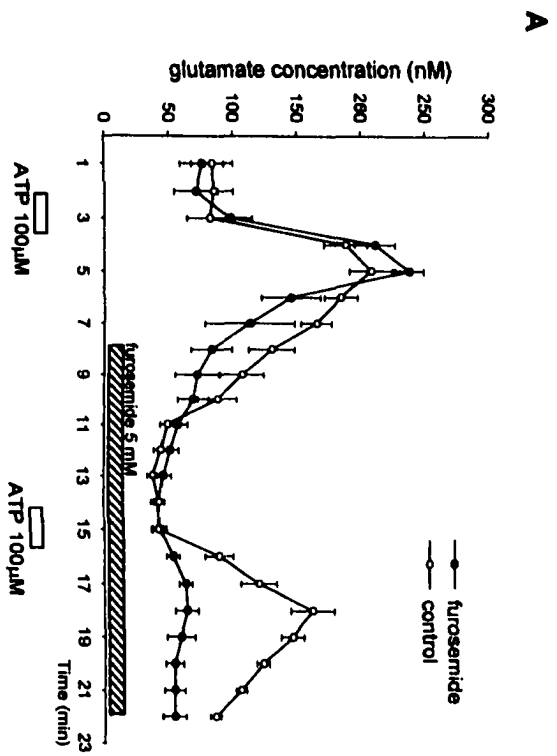
observations, p-CMPS (50 μ M) and PDC (1mM) produced an increase in the baseline concentration of glutamate in the perfusate (Fig. 5B and C). Both p-CAMP and PDC failed to impair the ability of ATP to stimulate glutamate release from astrocytes ($p < 0.001$, Student's t-test, $n=4$, Fig. 5B and C).

As we have previously shown that bradykinin-evoked release of glutamate from astrocyte cells is sensitive to the anion co-transport inhibitor furosemide (Parpura et al., 1994; Jefinija et al., 1996), we determined whether ATP-induced release of EAA from astrocytes has a similar sensitivity by incubating cultures in furosemide (5 mM). As illustrated in Figure 6A, ATP-induced release of glutamate was reduced in the presence of furosemide ($n=5$). In the presence of 5-nitro-2(3-phenylpropylamino)-benzoate (NPPB, 100 μ M), a potent Cl⁻ channel blocker in concentrations of $< 10\mu$ M (Wangemann et al., 1986; Diener and Rummel, 1989) and anion co-transport inhibitor in higher concentrations (Petersen and Cormann, 1987), the stimulatory effect of ATP on the release of glutamate was abolished in cultures pretreated with 100 μ M NPPB ($n=4$, Fig. 6B). NPPB in concentration of 10 μ M was without effect (data not shown). These data provide additionally support for the possibility that ATP-induced glutamate release from cultured astrocytes is sensitive to the anion transport inhibitors.

Role of ATP in neuron-to-glia signaling

To study possible role of ATP in neuron-glia interaction we determined whether ATP released by neurons acts on glial cells and further we determined whether glutamate released by ATP from glial cells is acting on neurons. Both of these experiments were

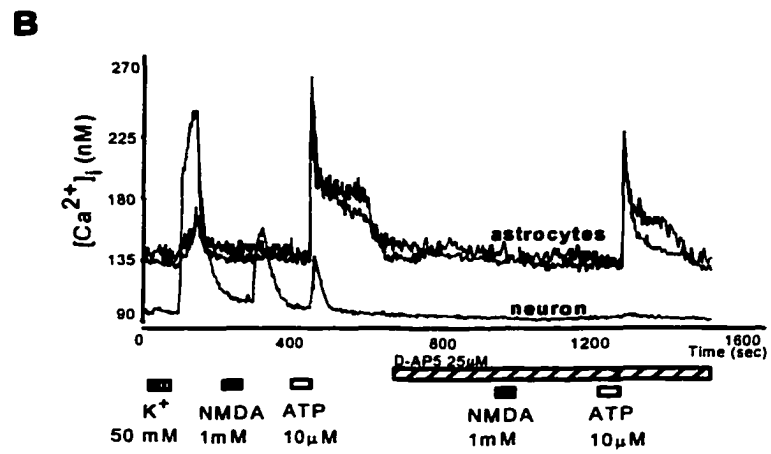
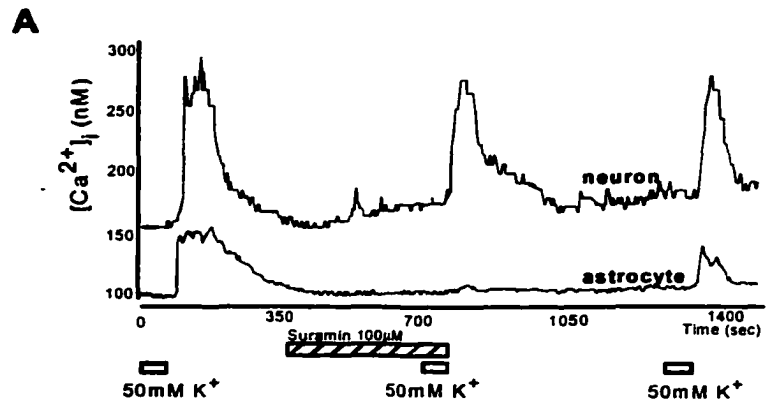
Figure 6. ATP-induced glutamate release from primary astrocyte cultures is sensitive to anion co-transport inhibitor and Cl⁻ channel blocker. **(A)** Bath application of 5 mM furosemide, an anion co-transporter inhibitor, attenuated the stimulatory effect of ATP on glutamate release from cultured astrocytes. **(B)** In the presence of NPPB (100μM), a potent Cl⁻ channels blocker, the stimulatory effect of ATP on the release of glutamate was abolished.



performed in mixed neuron-glia cultures. In order to identify neurons in mixed neuron-glia cultures, we took advantage of the observation that neurons responded promptly with a $[Ca^{2+}]_i$ increase to depolarization induced by 50 mM K^+ (Bezzi et al., 1998). To explore whether potassium-induced released ATP from neurons act on astrocytes to elevate calcium, we added suramin, a selective P_2 receptor antagonist. Suramin (100 μ M) abolished the potassium-induced stimulatory effect in about 30% of astrocytes in mixed cultures (n=12 of 37, Fig. 7A). Washing the cultures for 10min reversed the suramin effect (Fig. 7A).

To see whether ATP-induced glutamate released from astrocytes acts on neurons, we studied the effect of ATP on $[Ca^{2+}]_i$ in neurons co-cultured with astrocytes. When 10 μ M ATP was applied, calcium levels increased significantly in astrocytes (n = 46) and neurons (n = 26) after 15 and 50 sec, respectively (Fig. 7B). To determine whether ATP acts directly on neurons to elevate neuronal calcium levels or acts indirectly through glutamate released from astrocytes, we added 2-amino-5-phosphonopentanoic acid (D-AP5), a potent specific NMDA receptor antagonist. D-AP5 (25 μ M) abolished the ATP-induced calcium elevation in about 45% of neurons cultured with astrocytes (n=12 of 26), without altering the astrocyte response to ATP (Fig. 7B). These data suggest that ATP may elevate neuronal calcium indirectly by the action of glutamate, which is released from astrocytes in response to ATP.

Figure 7. ATP is a mediator of neuron to glia signaling (A) Bath application of 50 mM potassium directly stimulated changes in $[Ca^{2+}]_i$ in neuron and indirectly in glia in a mixed neuron-glia culture. Stimulatory effect of high potassium on astrocytes was abolished by suramin application. Horizontal bars indicate onset and duration of perfusion application of the compounds. The delay in the onset of the effect is due to the dead space of the perfusion system. **(B)** Stimulatory effect of ATP on neurons in the neuron-glia co-cultures is indirect one. Chart illustrates calcium changes in a neuron and two adjacent glia cells following application of 50mM K^+ , 1 mM NMDA and 10 μ M ATP prior to and during the application of 25 μ M D-AP5, a specific NMDA antagonist. Response of the neuron to NMDA and ATP was abolished in the presence of D-AP5.



Discussion

Our data demonstrate that the release from astrocytes of the excitatory amino acids, glutamate and aspartate, is induced by the neuroligand ATP. ATP caused the mobilization of calcium from internal stores in astrocytes within seconds of addition to cultures. This calcium elevation is sustained for several minutes. ATP releases glutamate and aspartate within one minute of delivery of ATP to the perfusion chamber. Thus, the ATP-induced calcium elevation is temporally coincident with the release of EAAs raising the possibility that it is required for EAA release. When calcium mobilization was blocked using BAPTA-AM or by removing external calcium during repeated ATP applications, ATP no longer stimulated the release of EAAs from astrocytes. Taken together these data demonstrate that ATP mobilizes internal calcium in astrocytes, which is both necessary and sufficient to stimulate the release of glutamate and aspartate.

A key issue to address is the nature of the release mechanism that is responsible for EAA liberation from astrocytes (Attwell, 1994; Smith 1994). This release mechanism may take at least three forms. Glutamate can be released through the reversal of a glutamate transporter. Both astrocytes and retinal Müller cells have been shown to release glutamate in a calcium-independent fashion in response to elevated $[K^+]_e$ due to the reversal of the normal glutamate uptake mechanism (Rutledge and Kimelberg, 1996; Szatkowski et al., 1990). This mechanism probably does not account for the release of EAAs in response to ATP in cortical astrocytes, since the action of ATP is calcium-dependent, glutamate release is not stimulated by depolarizing stimuli which would reverse the glutamate uptake carrier and p-CMPS and PDC do not block ATP-induced release of glutamate.

In cortical astrocytes hyposmotic medium causes cell swelling and the consequent release of glutamate, taurine and aspartate (Kimelberg et al., 1990; O'Connor and Kimelberg, 1993). An anion transport blocker, furosemide, reduces cell swelling dependent release from cortical astrocytes (Kimelberg et al., 1990). This raises the possibility that ATP may cause cell swelling which in turn stimulates EAA release from astrocytes. We therefore tested the action of furosemide on ATP-induced glutamate release. In astrocytes furosemide reduced ATP-stimulated glutamate release. In the presence of NPPB, a potent Cl^- channels in lower concentration and inhibitor of $\text{Cl}^-/\text{HCO}_3^-$, in higher concentration, the stimulatory effect of ATP on the release of glutamate was abolished only when anion transport-blocking concentrations of NPPB were used. These data provide additionally support for the possibility that ATP-induced glutamate release from cultured astrocytes is sensitive to the anion transport inhibitors.

A third potential mechanism of ATP-stimulated EAA release is through a membrane/vesicle release mechanism similar to that of neurons. Previously we have shown that some elements of the secretosome are present in the cultured astrocytes and that clostridial toxins influence neuroligand induced EAA release from cultured astrocytes (Jeftinija, 1997). Absence of some of the elements of secretory machinery does not rule out quantal membrane release mechanisms since muscle (Dan and Poo, 1992) and fibroblasts (Morimoto et al., 1995) have been demonstrated competent to release neurotransmitters in a quantal fashion. The nature of this calcium-dependent release apparatus from astrocytes remains to be defined. Bafilomycin, a V-ATPase inhibitor that dissipates the electrochemical proton gradient necessary for glutamate uptake in vesicles (Maycox et al., 1988), reduced the ability of astrocytes to release glutamate (Araque et al.,

2000), further supporting the hypothesis that vesicle exocytosis mediates glutamate release. Our finding that there are certain $[Ca^{2+}]_i$ changes that do not result in glutamate release from astrocytes suggests that certain $[Ca^{2+}]_i$ threshold should be reached for glutamate release. Recently it has been demonstrated that the calcium-regulated release of glutamate from astrocytes is engaged at physiological calcium levels (Parpura and Haydon, 2000).

The regulated release of neurotransmitters from glia may be a widespread property. Stimulation of motoneurons leads to the elevation of calcium in the Schwann cells that surround the presynaptic terminal (Jahromi et al., 1992). After denervation, Schwann cells have been shown capable of releasing acetylcholine (Dennis and Miledi, 1974). Depolarization of hippocampal astrocytes causes the calcium-dependent release of the amino acid taurine, but not glutamate (Philibert et al., 1988). Recently, we demonstrated that glial cells from the cerebral cortex release aspartate and glutamate in response to bradykinin (Parpura et al., 1994, Jęftinija et al., 1996). Since many neurotransmitters utilized by the nervous system can mobilize calcium in glia (Cornell-Bell et al., 1990; Glaum et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; McCarthy and Salm, 1991), it is possible that neurons have in addition to their fast neuron-neuron synaptic actions, a calcium mobilizing action on neighboring glia (Dani et al., 1992) which in turn leads to the release of other neurotransmitters from glia that may serve secondary roles in glia-neuronal transmission. In support of this possibility recent studies have demonstrated that glia can signal to neurons (Nedergaard, 1994; Parpura et al., 1994).

The specific roles for release of excitatory amino acids from glial cells remains to be elucidated. It has been demonstrated that ATP is a compound of the extracellular communication between astrocytes (Guthrie et al., 1999). The experiments reported here

demonstrate that neurons release a compound that is stimulatory to astrocytes and is blocked by selective purinergic receptor antagonists. This finding suggests a role for ATP as an effective compound of the neuron to astrocyte signaling. The release experiments reported here demonstrate that astrocytes release glutamate in response to ATP. Previously, Queiroz et al. (1997) reported that ATP release from astrocytes could be evoked by glutamate receptor activation. Therefore, there is a potential for formation of a positive feed-back loop between release of glutamate and ATP by astrocytes. Finally, our results with mixed cultures demonstrate that stimulatory effects of ATP on neurons are in part blocked by NMDA receptor antagonists suggesting an indirect effect through the astrocytes.

Given the important role of glutamate in the induction of long-term potentiation, it will be important to determine whether glia regulate synaptic plasticity, learning and memory by releasing the neurotransmitter glutamate and whether excessive glutamate release from glia also contributes to neurodegenerative disorders.

Acknowledgments

This work was supported by National Science Foundation Grant IBN 9604862

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CHAPTER 3. CANNABINOID-INDUCED ASTROTOXICITY

A paper submitted to *Glia*

by

Aleksandar Jeremic, Ksenija Jeftinija, Aleksandra Glavaski-Joksimovic, Jelena Stevanovic-Popovic, Srdija Jeftinija.

Abstract

Astrocytes account for more than half of the cells in the human brain and recently it has been shown that astrocytes integrate neuronal input and modulate synaptic transmission. The role of astrocytes in the pathophysiology of the brain, however, has been less investigated. Here we, for the first time, show that Δ^9 -tetrahydrocannabinol (THC), a psychoactive component of marijuana, produces a receptor-mediated stimulation of astrocytes and induces injury. Furthermore, using fluorescent dyes we show that astrocytes undergo apoptosis in response to cannabinoids. These data demonstrate a previously unknown property for astrocytes in the induction of cannabinoid-mediated astrotoxicity, suggesting that neurotoxicity may be initiated by nonneuronal signals and raising the possibility that astrocytes may actively participate in the neurotoxicity of cannabinoids.

Key Words: astrocytes, THC, calcium, apoptosis

Introduction

Marijuana, a widely used drug of abuse, has a long history of consumption for recreational and medical reasons. Marijuana is used therapeutically for several conditions, including treatment of pain and inflammation, nausea caused by AIDS and cancer chemotherapy and muscle spasms associated with multiple sclerosis (Adams and Martin, 1996; Hollister, 1986; Jaffe, 1990). The major psychoactive component of marijuana and its natural source plant *Cannabis sativa* is the Δ -9-tetrahydrocannabinol (THC). In humans, psychoactive cannabinoids elicit a variety of responses, like: euphoria, enhancement of sensory perception, antinociception, difficulties in concentration and impairment of memory that persist after withdrawal.

Two different cannabinoid receptors CB₁ and CB₂ have been found. Radioligand binding studies confirmed the presence of CB₁ receptors in the brain (Devane et al., 1988), where they modulate adenylate cyclase and ion channels activity via G_{i/o} protein regulation (Tao and Abood, 1998). CB₂ receptors are predominantly found in the cells of the immune system (Felder and Glass, 1998). Astrocytes express the mRNA's for CB₁ receptor determined by the Northern blot (Bouaboula et al., 1995a) and the CB₁ receptor determined by the Western blot (Sanchez et al., 1998a).

Although it is known that cannabinoids cause a reduction in neuron density in rodent's hippocampus (Landfield et al., 1988) and impair spatial memory in rats (Lichtman et al., 1995), marijuana toxicity has been underestimated. Only recently, a study showed that THC is toxic to cultured hippocampal neurons (Chan et al., 1998). It is known that prenatal exposure to THC affects gene expression and the activity of tyrosine hydroxylase during

early brain development (Hernandez et al., 1997). Although suggested, the role of astrocytes in the pathology of the nervous system has been difficult to study because astrocytes respond to an insult by cell division and repopulate the damaged area, forming glial scars.

In this study, we took advantage of methods to isolate astrocytes type I and then to culture them in a defined serum-free medium to regulate astrocyte proliferation. In light of new evidence which emphasises glia as a key player in the control of synapse formation and possible memory formation (Ullian et al., 2001), we undertaken a study to evaluate the deleterious effect of cannabinoids on astrocytes.

Materials and Methods

Cell cultures

Mixed neuron-glia and primary astrocyte cultures from neonatal rat cerebral cortex were established as described (Jeremic et al., 2001). Briefly, cortex was freshly dissected and tissue was enzymatically (papain 20 i.u./ml; 1 hour at 37°C) and mechanically dissociated. Cells were plated on poly-L-lysine (1 mg/ml; MW 100,000)-coated glass cover slips (for mixed cultures) or culture flasks and maintained at 37°C in a humidified 5% CO₂/95% air atmosphere (for primary astrocyte cultures). The cells were maintained by changing the medium every 2-3 days. Culture medium consisted of Eagle's MEM supplemented with 10% heat-inactivated FBS and 40 mM glucose, 2 mM L-glutamine and gentamicin. After the mixed cultures reached confluence (9-12 days), the flasks were "preshaken" (260 rpm) for 90 min to remove microglia and dividing type I astroglia. The cultures were then

shaken overnight (12-18 hours) at 260 rpm at 37°C. Cultures enriched in type I astroglia were obtained by trypsinizing (0.25%) the attached cells for 3 min. Trypsin was inactivated by adding 3 ml of Eagle's α MEM supplemented with the 10% heat-inactivated FBS (serum contains protease inhibitors). Astrocytes were plated on the poly-L-lysine (10 μ g/ml; MW 100,000)-coated glass cover slips. All experiments were performed on cells that had been in culture for 3-5 days after replating.

Organotypic cultures of brain slices were prepared as described (Gahwiler, 1981). Briefly, hippocampal slices of 250 μ m were obtained from 2 weeks old Sprague-Dawley rats. Acutely isolated slices were transferred to glass cover slips coated with chicken plasma that was coagulated by thrombin. Glass cover slips with the explant were placed into Petri dishes and were fed with the medium (25% horse serum, 25% Earls Balanced Salt Solution, 50% Basal Medium Eagles with glucose 6.4 mg/ml). Petri dishes were kept in an incubator at 36°C in 95% air and 5% CO₂.

Ca²⁺ imaging

The effect of experimental manipulation on glial [Ca²⁺]_i was evaluated by fluorescence ratio imaging technique. Cells were loaded with 5 μ M Fura-2 AM (Molecular Probes) for 40-60 minutes at 24°C. 1 μ l of 25% (w/w) of Pluronic F-127 (Molecular Probes) was mixed with every 4 nM of Fura-2 AM to help dissolving of the ester into aqueous medium. After washing, cells were deesterified for 10 min at 24°C. All image processing and analysis were performed using an Attofluor system with Zeiss microscope. Background subtracted, rationed images (340/380 nm) were used to calculate the [Ca²⁺]_i according to

Equation 5 of Grynkiewicz et al. (1985). Calibration was performed *in situ* according to the procedure provided by the Attofluor, using the Fura-2 Penta K⁺ salt as a standard.

Toxicity assay. Astrocyte cultures were plated on 24-well plates and maintained in serum-containing medium for 2-3 days. 24 hours before applying treatments, serum-containing media were replaced with serum-free QBSF-51 media (Sigma). In this media, astrocytes persist without a detectable change in astrocyte viability for at least 72 hours. Appropriate cannabinoid dilutions were made in absolute ethanol and a 500nM-1 μM concentrations of THC and WIN were added to cultured astrocytes for indicated periods of time. Vehicle in 0.1% concentration had no significant effect on the cell viability. At the end of incubation MTT was added to the QBSF-51 media (250 ng/ml) and incubated for 3 hours at 37° C. After incubation, the medium was removed and an insoluble formazan dye was solubilized with isopropanol/HCl solution. The absorbency of the converted dye was measured at 570 nm with background subtraction at 630 nm.

Fluorescent staining protocol

AnnexinV-FITC staining protocol: primary astrocyte cultures were incubated with 5 μM dye diluted in HEPES-buffer solution (in mM: NaCl 140, KCl 5, MgCl₂ 2, CaCl₂ 2, glucose 5 and HEPES 10; pH 7.4) for 15 min in the dark. Cultures were mounted in the perfusion chamber and examined with a Zeiss epifluorescence microscope using FITC filter set. Images were collected with a Nikon Coolpix digital camera and the fluorescence signal quantified with MetaMorph imaging software. Acridine orange/propidium iodide staining

protocol: 30 min before AO/PI staining, the cultures were incubated with 10 $\mu\text{g/ml}$ RNase A at 25° C. Additionally, cultures were incubated for 10 minutes in the dark with AO (5 μM) and PI (2.5 μM). Primary astrocyte cultures were washed twice in HEPES buffer solution and the cells were visualized under epifluorescence illumination: AO (480 nm for excitation and 510 nm barrier filter for emission); PI (540 nm and 610 nm excitation-emission filter set), with 40X oil immersion objective. The percentage of apoptotic cells was scored from each field and determinations were made in at least four separate cultures. The percentage of apoptotic cells was determined by nuclear morphology examination: cells with condensed and/or fragmented DNA were considered apoptotic, while the nuclei with diffusely and uniformly DNA distributions were considered as nonapoptotic.

A similar protocol was used for the determination of apoptotic astrocytes in mixed neuron-glia cultures. Prior to AO staining, mixed cultures were fixed in 4% formaldehyde for 30 min, permeabilized in solution containing: 0.5 % Triton X-100, 1 % BSA and 1 % horse serum for 25 min. Then, the cultures were incubated with monoclonal Anti-Glial Fibrillary Acidic Protein-Cy3 conjugated antibody (1: 200 dilution; Sigma, St. Louis, MO) for 4 hours at 25° C for astrocyte visualization.

For morphological analysis of apoptotic nuclei in organotypic cultures, cultures were prepared as described (Gahwiler, 1981). We implemented the same AO/antiGFAP-Cy3 staining protocol as for the mixed neuron-glia cultures. Images were obtained with the Leica confocal laser scanning microscope, using FITC filter set (excitation: 480 nm; emission: 520 nm) for AO staining and Cy3 filter set (excitation: 540 nm; emission: 610

nm). The same nuclear morphological criterion was used for apoptotic cell determination as was applied for the apoptotic nucleus determination in the primary astrocyte cultures.

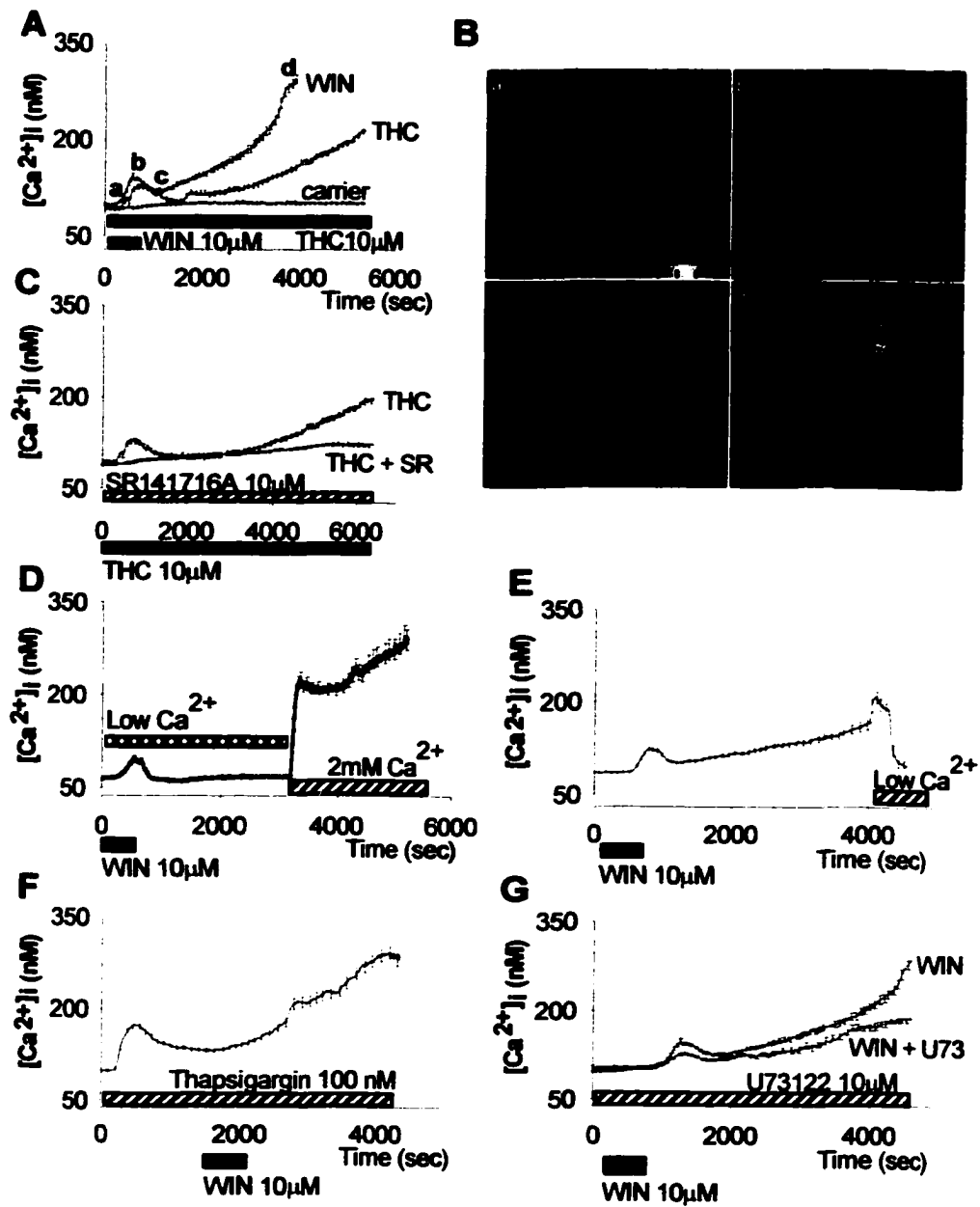
Results

Cannabinoids induce receptor-mediated biphasic calcium transients in astrocytes

Cover slips containing enriched astrocyte type I cells were mounted into a fast rate exchange perfusion chamber for imaging experiments (Jeremic et al., 2001). Using Fura-2 AM calcium imaging to examine the stimulatory effects of cannabinoids in astrocytes we determined that cannabinoids produced a biphasic calcium response. Perfusion application of Win 55,212-2 (WIN 10 μ M), a stable cannabinoid receptor agonist, for 10 min produced an increase in intracellular calcium, followed by return to the control level, and followed by a delayed and irreversible increase in intracellular calcium (n=34; Fig. 1A).

Δ^9 -tetrahydrocannabinol (THC 10 μ M), an active component of marijuana, elicited a similar pattern of calcium transients in astrocytes but with slower kinetics of the delayed increase (n=36; Fig. 1A). The recovery of the cultures from cannabinoid insult was not detected within the duration of the recording of 5000 sec. In addition, disappearance of the cell images was observed suggesting irreversible damage of membrane integrity and leakage of Fura-2 dye (Fig. 1B). A selective CB₁ receptor antagonist SR141716A (Rinaldi-Carmona et al., 1994), abolished the THC- induced calcium transients in astrocytes, suggesting that cannabinoids stimulate astrocytes via CB₁-receptor activation (n=32; Fig. 1C). Two different calcium pools contributed to the cannabinoids-induced biphasic response in astrocytes. Low extracellular Ca²⁺ did not affect the initial component of WIN-

Fig. 1. Cannabinoids induce biphasic calcium transients in the primary astrocyte cultures by a receptor-mediated mechanism. (A) Graph illustrates kinetics of calcium changes in astrocytes following the cannabinoid application. Perfusion application of the 10 μM THC induced a rapid increase in intracellular Ca^{2+} followed by recovery and by a delayed irreversible increase in intracellular Ca^{2+} (A). A more potent cannabinoid receptor agonist 10 μM WIN produced a greater response when applied for 10 min only (A, Bb). (B) Pseudocolour images (a-d) of the WIN-induced calcium transients in astrocytes are captured at the time marked on the graph (A). Scale bar, 25 μm . (C) Stimulatory effect of THC was abolished by pretreatment of the culture with the CB_1 -receptor antagonist, SR141716A (5 μM). (D and E) The rapid response of cells to cannabinoids (WIN 10 μM) was preserved in cells bathed in low calcium medium, while the delayed response was abolished in the absence of extracellular calcium. Replenishment of extracellular calcium resulted in a rapid increase in the intracellular calcium (D). Elimination of the extracellular calcium at the time of peak response to WIN resulted in an abrupt decrease in the response (E). (F) While the cannabinoid-induced rapid increase in intracellular calcium is abolished in cultures pretreated with thapsigargin (100nM), the delayed response was unaffected by thapsigargin pretreatment. (G) Both responses to cannabinoids were attenuated by the application of a PLC inhibitor. In the presence of U73122 (10 μM), a rapid response to WIN was decreased by $56 \pm 6 \%$ while the kinetics of the delayed response was slower.



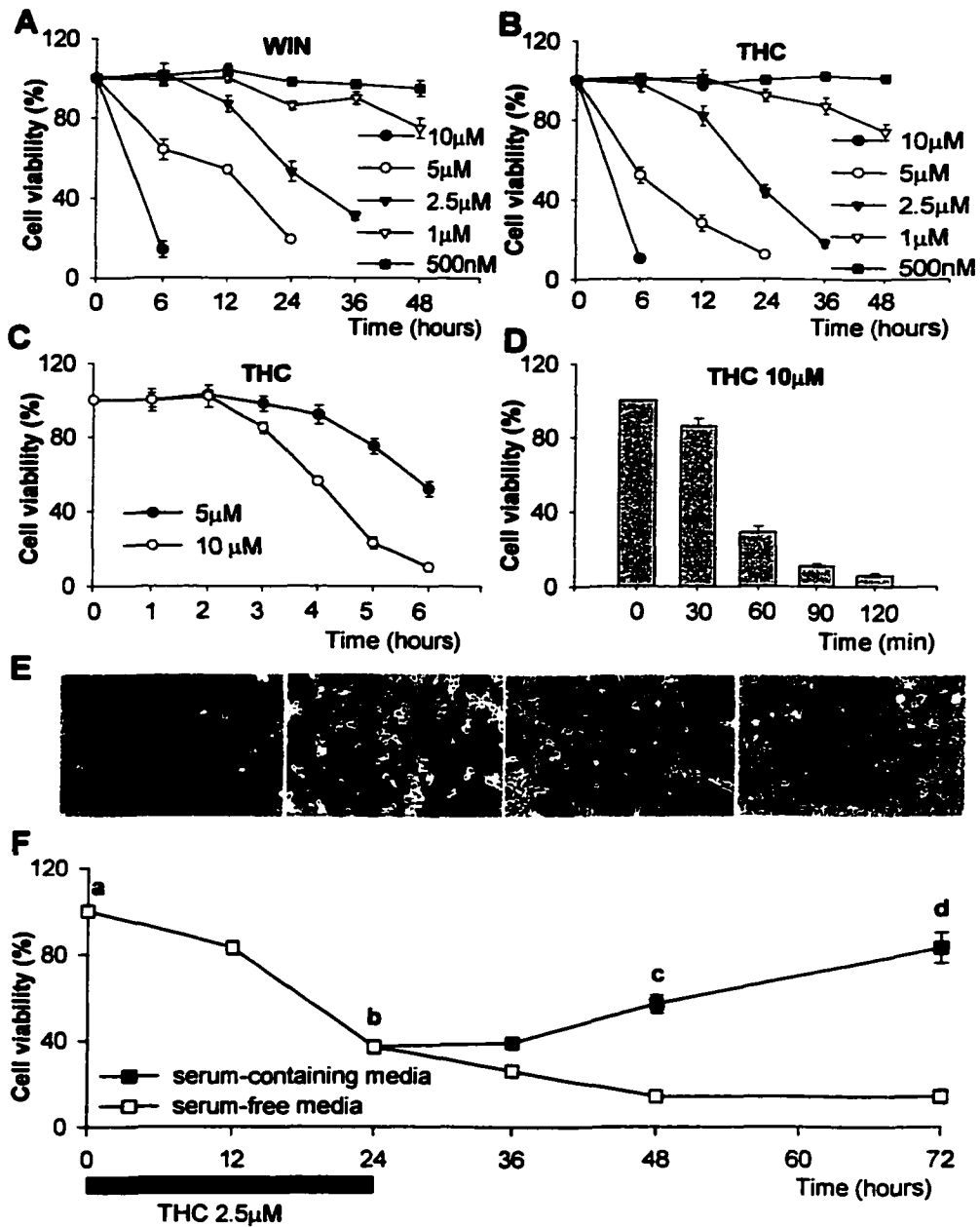
induced change in intracellular calcium while the delayed response was abolished (n=37; Fig. 1D and E). Pretreatment of cells with thapsigargin, a Ca^{2+} -ATP-ase inhibitor (Thastrup et al., 1990), blocked the cannabinoid-induced rapid increase in calcium while the delayed response was unaffected (n=41; Fig. 1F). These results suggest that the observed early increase in intracellular calcium induced by cannabinoids originated from the intracellular stores, while the irreversible calcium increase results from an extracellular calcium entry. Both responses to WIN application were attenuated in the presence of U73122, a phospholipase C (PLC) inhibitor (Smith et al., 1990), which confirms the role of receptor-coupled PLC dependent processes in the cannabinoid-induced calcium transients in astrocytes (n=45; Fig. 1G).

Cannabinoid-induced dose-dependent decrease in astrocyte viability is CB_1 receptor mediated

To determine the possible toxic effect of cannabinoids on the astrocytes as suggested by the Ca^{2+} imaging experiments (Fig. 1B), we employed MTT assay as a measure of cell viability (Hansen et al., 1989). Concentrations of WIN and THC as low as 1 μM were toxic to primary cortical astrocytes (n=8; Fig. 2A and B). This concentration is comparable to the THC-concentrations found in human plasma after consumption of a single marijuana cigarette (Chiang and Barnett, 1984). Cannabinoid-induced decrease in the cell viability was time- and concentration-dependent (Fig. 2A-C). Time course experiments showed that continuous application of 10 μM THC induced a significant decrease in cell viability 3 hours after treatment and a maximum of 90 % of the control after 6 hours (n=8; Fig. 2C). As concentrations of cannabinoids decreased, the time to

Fig. 2. Cannabinoid receptor ligand-induced astrotoxicity is a dose- and time-dependent.

(A and B) Continuous application of 10 μM WIN and THC induced a 90 % decrease in cell viability after 6 hours. In concentration of 5 μM , WIN and THC produced a 50 % decrease in astrocyte viability within 6 hours and a 90% decrease within 24 hours. As concentrations of cannabinoids decreased to 2.5 μM , no detectable decrease in cell viability was determined within the first 6 hours, but an 80-90 % decrease was recorded after 36 hours. At threshold concentration of 1 μM , both THC and WIN induced a measurable decrease in cell viability after 24 hours exposure and the maximum decrease in astrocyte viability by 20 % was detected after 48 hours. **(C)** To determine the onset and early course of the THC toxicity, astrocyte cultures were exposed to 5 and 10 μM THC in duration 1 to 6 hours, with the initial cannabinoid toxicity detected at 3 hours. **(D)** To determine the prolonged effect of short exposure to cannabinoids, cells were incubated with 10 μM THC for 30-120 min, followed by removal of the agonist and reincubation in the conditioned media for an additional 12 hours. Exposure of the astrocytes to 10 μM THC for 30 min resulted in a detectable damage in cells viability 12 hours later. **(E and F)** The cytotoxicity of cannabinoids on astrocyte cultures is reversible. To investigate the ability of astrocyte cultures to recover following a toxic insult, cultures were exposed to 2.5 μM THC for 24 hours that resulted in a 60 % decrease in cell viability. Replacement of the THC-containing medium with the cell growth-promoting medium resulted in a recovery of the cultures to the control level (E and F). Panels (E) illustrate the phase contrast micrography of astrocyte cultures obtained at the times indicated on the time course line of the histogram (F). Scale bar, 50 μM .



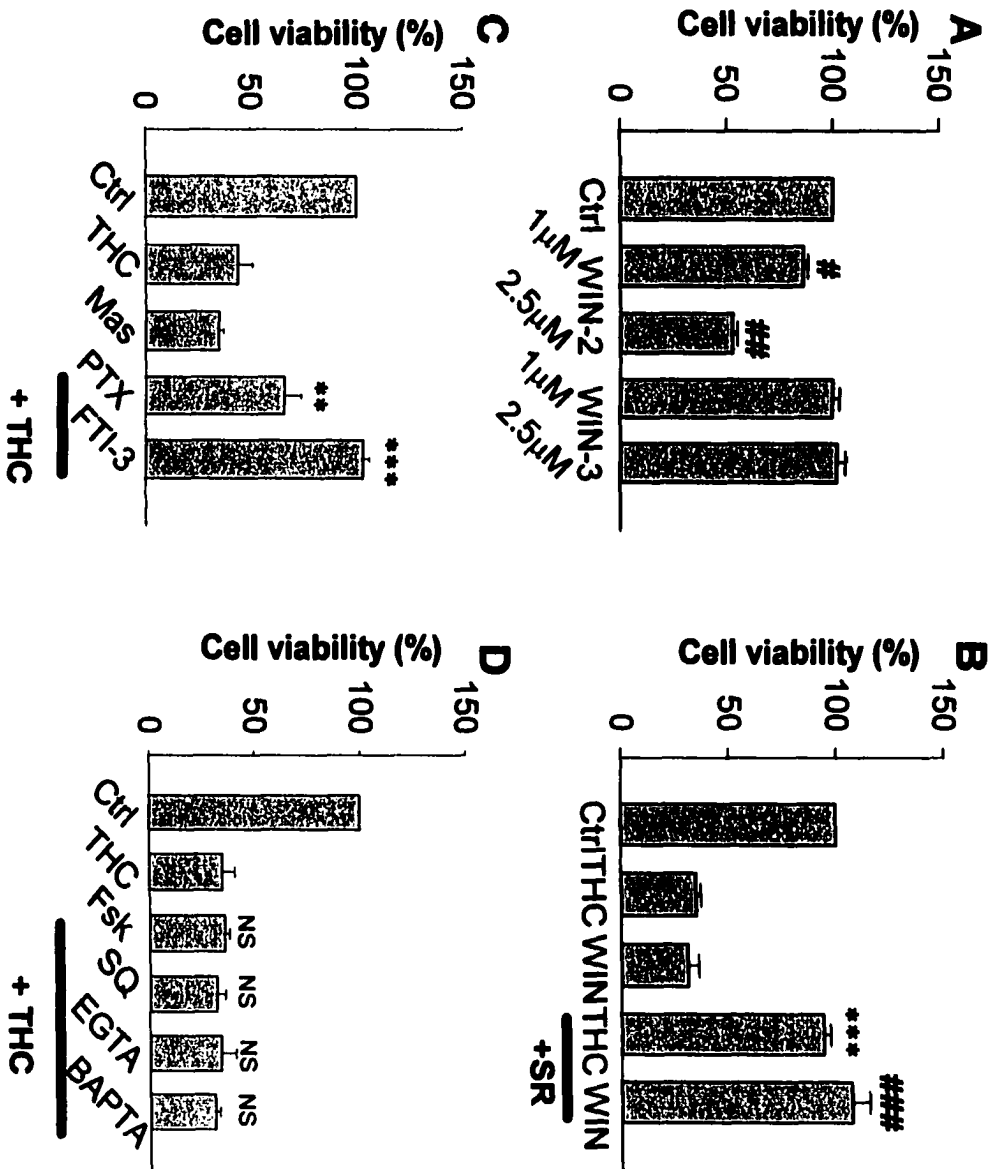
produce a detectable decrease in the cell viability was prolonged. At a threshold concentration of 1 μM both THC and WIN induced a measurable decrease in viability after 24 hours exposure (n=8; Fig. 2A and B). To determine the prolonged effect of short exposure to cannabinoids, THC was applied for 30-120 min at the concentration of 10 μM and the cell viability was determined 12 hours later. We observed that even 30 min incubation with THC was enough to initiate astrocyte death 12 hours later (n=6; Fig. 2D). The ability of astrocytes to recover from the cannabinoid insult was determined. Exposure of cells to 2.5 μM THC for 24 hours resulted in a 60 % decrease of astrocyte population (n=7; Fig. 2F). Replacement of the THC-containing medium with cell growth promoting medium resulted in recovery of the cultures to the control level (Fig. 2E and F).

To determine the possible role of CB_1 receptor in the cytotoxicity of cannabinoids, we used SR141716A, a CB_1 receptor antagonist. SR141716A (5 μM) abolished the THC- and WIN-induced astrocytes death (n=8; Fig. 3B). In addition, bath application of 1 μM and 2.5 μM S(-) Win 55,212-3, a less active enantiomer of R(+)Win 55,212-2, had no significant effect on cell viability ($p>0.1$, n=8; Fig. 3A).

Cannabinoids induce death of cortical astrocytes via primary and secondary effector enzyme activation

To test the possibility that THC-induced astrototoxicity results from inhibition of adenylyl cyclase activity by the activation of a pertussis toxin (PTX)-sensitive $G_{i/o}$ protein, we pre-treated cells with PTX (100 ng/ml) for 24 hours. PTX significantly protected cells against THC toxicity ($p<0.01$, n=8; Fig. 3C). In addition, our data suggested significant

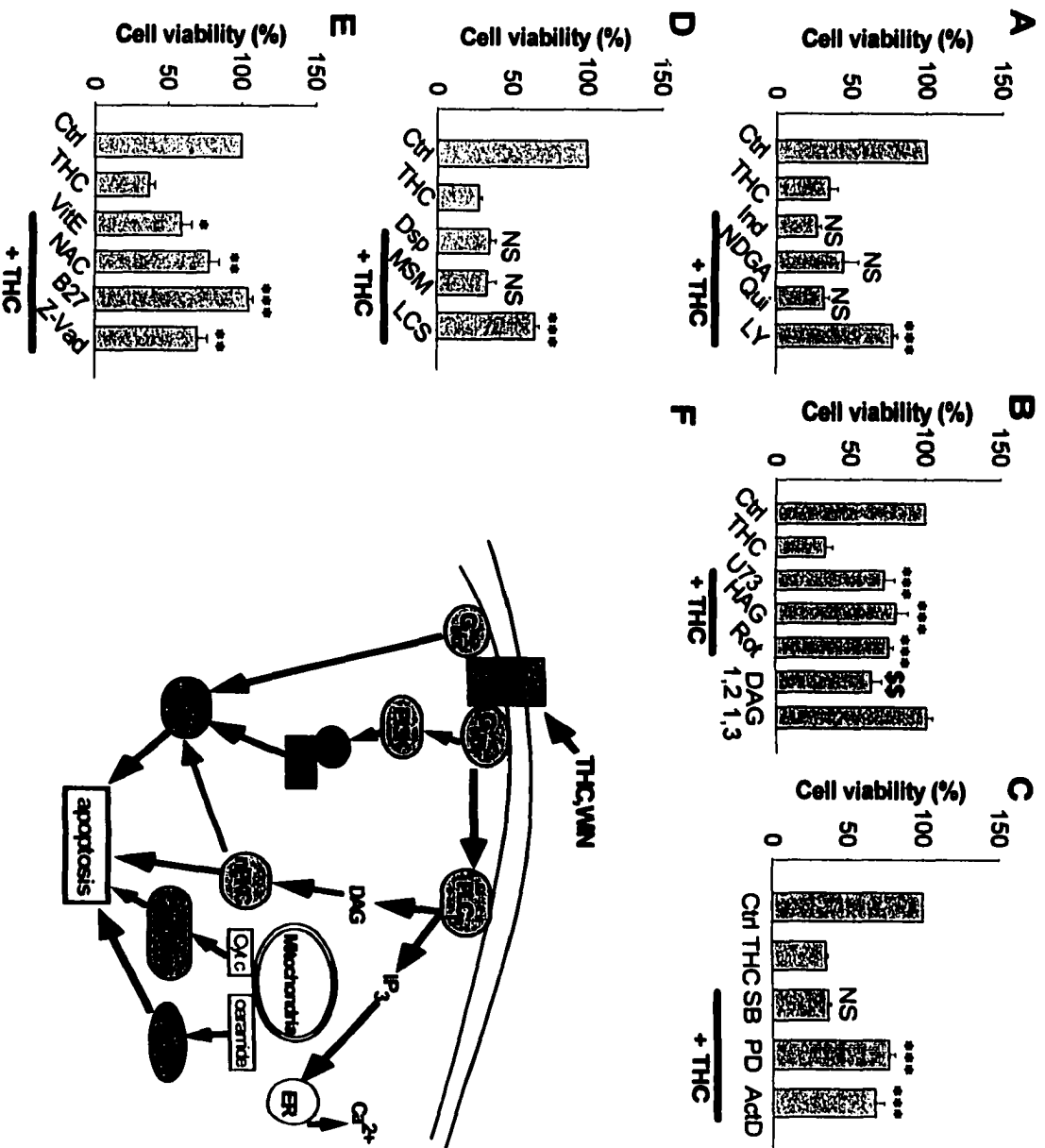
Fig. 3. Cannabinoid induced astrotoxicity is the CB₁ receptor-mediated. (A) While bath application of 1 μ M and 2.5 μ M R(+)-Win 55,212-2 (WIN-2) produced a significant decrease in cells viability ($p < 0.01$, $n = 8$), bath application of 1 μ M and 2.5 μ M S(-)-Win 55,212-3 (WIN-3), had no significant effect on cell viability ($p > 0.1$, $n = 8$). **(B)** A selective CB₁ receptor antagonist SR141716A (5 μ M) abolished the THC and WIN-induced astrotoxicity. **(C)** THC astrotoxicity is attributed to G-protein activation. Treatment of the cultures with 15 μ M mastoparan (Mas) resulted in decrease in cell viability to $35 \pm 2\%$ ($n = 8$). On the other hand 24 h pretreatment of cells with 100ng/ml pertussis toxin (PTX) and 5 μ M Ras farnesyltransferase inhibitor III peptide (FTI-3) protected the cultures against THC-induced astrotoxicity. **(D)** THC astrotoxicity is not attributed to the adenylyl cyclase-cAMP second messenger system and it is not extracellular calcium dependent. 10 μ M Forskolin (Fsk) and 100 μ M SQ22536 (SQ) did not significantly change the THC-induced toxicity ($p > 0.1$, $n = 8$). Maintenance of the astrocytes in the 3mM EGTA-supplemented serum-free medium or a loading the cells with 10 μ M BAPTA-AM were without effect on the THC-induced decrease in astrocyte viability.



contribution of small G-proteins like Ras to the THC-induced cell death, since the FTI-3, a potent Ras farnesyltransferase inhibitor peptide, abrogated THC toxicity (Fig. 3C). These results, in conjunction with the ability of mastoparan to trigger cell death (Fig. 3C), emphasize an important role of the G-protein signaling system in the cannabinoid-induced astrotoxicity. In contrast, neither the forskolin, an adenylyl cyclase activator, nor SQ22536, an adenylyl cyclase inhibitor, showed any significant effect on the THC-induced toxicity ($p > 0.1$, $n=8$; Fig 3D). These results uncouple the link between adenylyl cyclase activity and the receptor-mediated THC-induced astrotoxicity. Incubation of the astrocyte cultures in the low calcium medium or loading the cells with BAPTA-AM, an intracellular calcium chelator, did not rescue astrocytes from THC-induced cell death (Fig. 3D). These results suggest that cannabinoids induce a calcium-independent cell death, and the irreversible calcium entry along with a disappearance of the cell images observed upon cannabinoids application (Fig. 1A and B) represent an outcome of cannabinoids toxicity rather than the death-signaling pathway employed by cannabinoids.

It has been shown that cannabinoids could stimulate receptor mediated arachidonic acid (AA) release from cultured astrocytes (Shivachar et al., 1996). AA is the substrate for several major pathways. Cyclooxygenase (COX) produces prostaglandins and lipoxygenase produces leukotrienes. Both pathways generate free radicals (Yamamoto, 1991). The PLA₂ inhibitor, Quinacrine, and indomethacin, a COX inhibitor, blocked the THC-induced cell death in primary hippocampal neurons (Chan et al., 1998). However, both inhibitors failed to protect astrocytes against the toxic effect of THC (Fig. 4A), implying a different death-signaling pathway for astrocytes and neurons. Nordihydroguaiaretic acid (NDGA), a

Fig. 4 THC induces cell death via primary and secondary effector enzyme activation. **(A)** THC induced astrotoxicity is not attributed to the PLA₂-Cyclooxygenase pathway. Incubation with 0.5 μM Quinacrine (Qui) and 5 μM indomethacin (Ind) failed to protect astrocytes from the toxic effect of THC ($p > 0.05$, $n = 8$). 5 μM NDGA did not protect astrocytes as well. However 50 μM LY294002 (LY), a specific PI3-kinase inhibitor, significantly protected astrocytes against THC-induced astrotoxicity ($p < 0.001$, $n = 7$). **(B)** PLC and PKC inhibitors protected astrocytes against THC toxicity. 5 μM U73122 (U73) attenuated the cell death evoked by the THC. 10 μM 1-O-Hexadecyl-2-O-acetyl-sn-glycerol (HAG), significantly protected astrocytes from THC-mediated toxicity ($p < 0.001$, $n = 8$). 5 μM Rottlerin (Rot) induced similar protective effect in astrocytes. 1,2-DAG (2.5 μM), but not 1,3-DAG (2.5 μM), triggered a significant decrease in cell viability ($p < 0.01$, $n = 8$). **(C)** THC-induced astrotoxicity is associated with ERK activation. While 20 μM SB-203580 (SB) did not have any significant effect on THC-induced astrotoxicity ($p > 0.05$, $n = 8$), 20 μM PD98059 (PD) antagonized the toxic effect of THC. Incubation of cells with 1 μg/ml actinomycin D (ActD) abrogates the THC-induced astrotoxicity. **(D)** *De novo*-synthesized ceramide has a significant role in THC-induced astrotoxicity. Incubation of cells either with 10 μM Desipramine (Dsp) or 1 μM 3-O-methyl-sphingomyelin (MSM), had no significant effect on the THC induced astrotoxicity ($p > 0.1$, $n = 7$). However, 2.5 mM L-Cycloserine (LCS), an inhibitor of ceramide biosynthesis, significantly protected cells from THC toxicity ($p < 0.001$, $n = 8$). **(E)** Antioxidants, Vitamin E (50 μM), 250 μM N-acetyl-cysteine (NAC) and antioxidants solution 5% B-27 abrogated THC astrotoxicity. Z-Vad-FMK (50 μM), an inhibitor of caspases, protected astrocytes from the THC-induced cell death ($p < 0.01$, $n = 8$). **(F)** Cannabinoids induce astrocytes death through a hypothesized multiple signaling pathway. Activation of CB₁ receptor by cannabinoids induce a G_v_o-protein subunits dissociation and activation of multiple downstream signaling pathways: α subunit of G_v_o could mediate ERK activation via a PI3K / Ras-independent pathway (Jordan et al., 1999; Mochizuki et al., 1999), while the βγ dimer activates ERK through the PI3K / Ras / Raf cascade. Alternatively, βγ dimer may activate PLC_β, which in turn stimulates DAG and IP₃ formation. IP₃ triggers calcium release from intracellular stores while DAG activates a novel class of PKC's, that leads to apoptosis. In addition, cannabinoids induce caspases activation and *de novo* synthesis of ceramide that stimulates ROS overproduction.



selective lipoxygenase inhibitor, did not protect astrocytes as well (Fig. 4A). In contrast, LY294002, a specific phosphatidylinositol 3-kinase inhibitor (PI3K), blocked the THC-induced astrocytes death ($p < 0.001$, $n=7$; Fig. 4A). This result points to the possible activation of CB₁ receptor / Ras and G_{i/o} / PI3K / mitogen-activated protein kinase (MAP) signaling cascade in astrocytes elicited by cannabinoids. This was already observed as a primary signaling mechanism in the MAP cascade activation in other cells (Bouaboula et al., 1995b; Liu et al., 2000). As U73122, a selective inhibitor of PLC, abrogated calcium transient evoked by cannabinoids (Fig. 1G), we tested its ability to suppress cannabinoid-induced astrotoxicity. U73122 attenuated the astrocytes death evoked by the THC ($p < 0.001$, $n=8$; Fig. 4B). Furthermore, we observed a significant contribution of protein kinase C (PKC) to the cannabinoid-induced cell death as the 1-O-Hexadecyl-2-O-acetyl-sn-glycerol, an isotype-selective PKC inhibitor that inhibits PKC activation by diacylglycerols (Daniel et al., 1988), significantly protected astrocytes from the THC-mediated toxicity ($p < 0.001$, $n=4$; Fig. 4B). In same manner Rottlerin, a specific PKC δ inhibitor (Gschwendt et al., 1994), abrogated THC toxicity ($p < 0.001$, $n=4$; Fig. 4B). A PKC-dependent cell death was further supported by the ability of 1-Oleoyl-2-acetyl-sn-glycerol, a potent membrane-permeable DAG that activates PKC (Mori et al., 1982), to provoke cell death on its own (Fig. 4B).

The link between PKC activation and MAP cascade activation is well established. MAP kinase mediate diverse processes ranging from transcription of protooncogenes to apoptosis (Cobb, 1999). Cannabinoids elicited MAP kinase activation in many cells including C₆ glioma cell line and astrocytes (Galve-Roperh et al., 2000; Rueda et al., 2000; Sanchez et al., 1998b). These facts prompted us to evaluate the role of the MAP cascade in

these events. SB-203580, a selective inhibitor of p38 MAP kinase (Cuenda et al., 1995), did not have any significant effect on THC induced astrotoxicity ($p > 0.05$, $n = 8$; Fig. 4C). In contrast, a potent and selective inhibitor of extracellular signal-regulated kinase (ERK) cascade PD98059 (Dudley et al., 1995), was able to antagonize the toxic effect of THC on cultured astrocytes ($p < 0.001$, $n = 8$; Fig. 4C). Furthermore, actinomycin D, an inhibitor of transcription, significantly decreased THC-induced astrotoxicity ($p < 0.001$, $n = 8$; Fig. 4C), which suggests that THC may activate a transcription-dependent cell death.

An activation of the CB₁ receptor in astrocytes led to sphingomyelin hydrolysis and ceramide formation that stimulated glucose metabolism (Sanchez et al., 1998b). To determine a possible ceramide involvement in the initiation of apoptotic events in astrocytes by cannabinoids, we employed inhibitors of the ceramide generation pathways. Neither Desipramine, an acidic SM-ase inhibitor, nor 3-O-methyl-sphingomyelin, an inhibitor of neutral SM-ase, showed any significant protection of astrocytes against THC toxicity ($p > 0.05$, $n = 8$; Fig. 4D). However, L-Cycloserine, an inhibitor of serine palmitoyl transferase (Sundaram and Lev, 1984), the first committed step of ceramide synthesis *de novo*, significantly protected astrocytes from THC ($p < 0.001$, $n = 8$; Fig 4D). This result emphasizes a ceramide synthesis pathway as an important factor in the THC-induced apoptosis in astrocytes rather than ceramide generation through sphingomyelin hydrolysis.

There is mounting evidence to support a role of oxidative stress and reactive oxygen species (ROS) as important players in the chain of reaction leading to cell death. We examined the effect of the antioxidants, vitamins E and N-acetyl-cysteine, on the THC-induced astrocyte death. Both substances abrogated the cytotoxicity of THC (Fig. 4E). The full protection of cells against THC toxicity was achieved with the strong antioxidant

solution B-27 (Gibco) that contains mixture of five antioxidants: vitamin E and C, glutathione, superoxide dismutase and catalase (Brewer et al., 1993), thus supporting a role of free radicals on these processes (Fig. 4E). More importantly, Z-Vad-FMK, a cell permeable, broad spectrum caspase inhibitor, protected astrocytes from THC-induced cell death ($p < 0.01$, $n=8$; Fig. 4E).

Cannabinoids induce apoptosis in astrocytes

To discriminate between the types of cell death induced by cannabinoids fluorescent probes were used. Treatment of cultured primary astrocytes from rats with cannabinoids led to apoptotic body formation (panel b; Fig. 2E). AnnexinV-FITC conjugate is a phospholipid binding protein with high affinity for phosphatidylserine (PS). Externalization of PS is one of the earliest indicators of apoptosis. We observed a significant increase in the fluorescence intensity in cells treated with cannabinoids compared with control cells ($p < 0.001$, $n=4$; Fig. 5A-C).

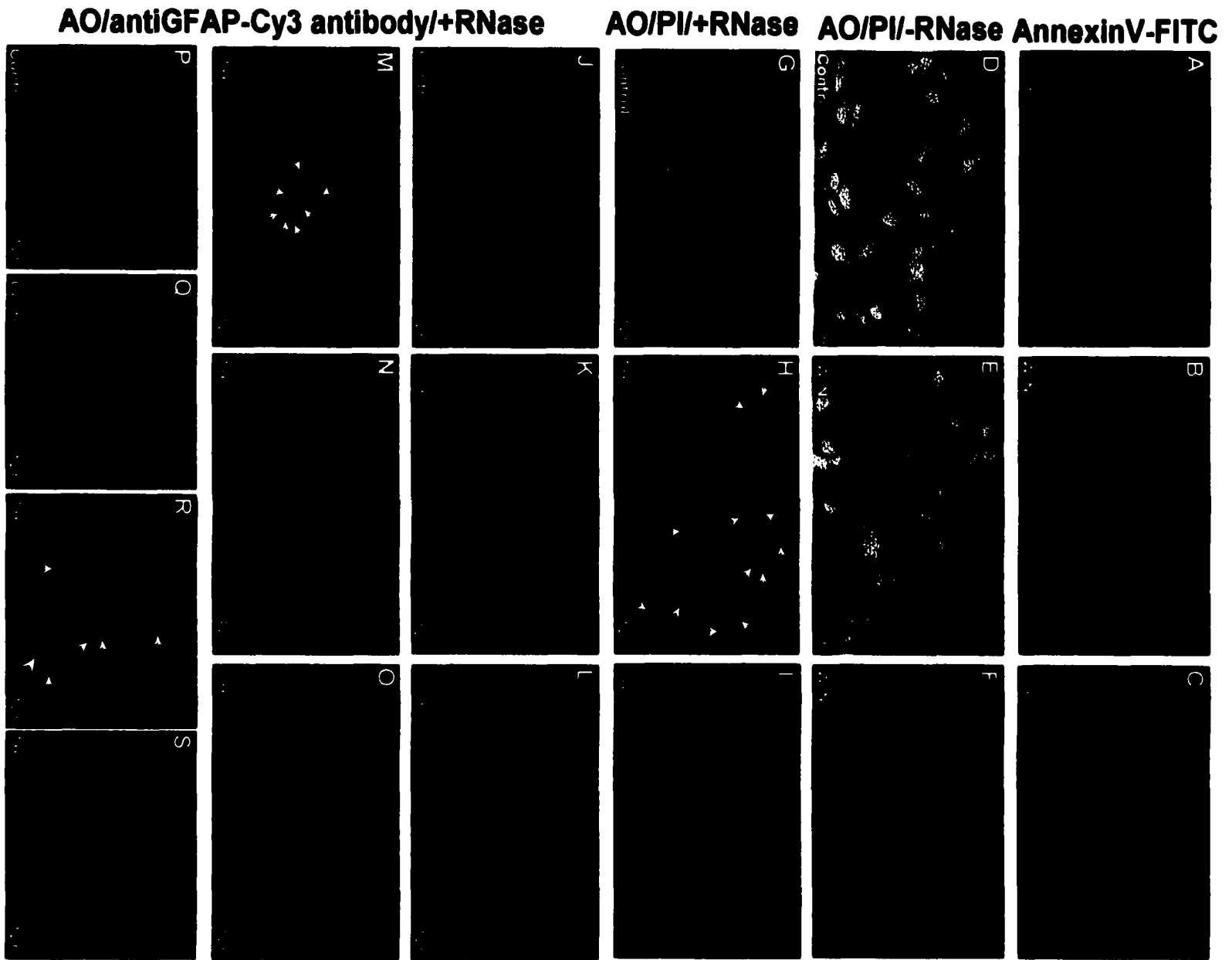
Acridine orange and propidium iodide fluorescent dyes discriminate between apoptotic and necrotic cell death. Acridine orange intercalates into double-stranded (intact) DNA emitting the green fluorescence, whereas when bound to single-stranded DNA or RNA, it fluoresces red (Darzynkiewicz et al., 1992). In the presence of RNase, cells fluoresce green (Fig. 5G) or a yellow in the absence of RNase due to the green-red spectra overlap (Fig. 5D), indicating viable cells. In addition to the increase in the amount of PS on the cell membrane, the early (6 hours) cannabinoid-induced apoptotic stage in astrocytes has been characterized by an increase in the RNA content as well. A change in RNA content was revealed by the marked shift in the fluorescence emission signal from a yellow

to an orange-red spectrum due to the increase in red fluorescence (Fig. 5D and E). Similar determination of cell's DNA and RNA contents using acridine orange staining has been recently employed (Vidotto et al., 1996). An increase in the RNA content in the early stage of apoptosis of astrocytes observed by fluorescent staining may be relevant to cannabinoid toxicity since we showed that THC activates a transcription-dependent cell death (Fig. 4C). The late apoptotic stage, 12 hours of the cannabinoid-induced cell death in primary astrocyte cultures, was characterized by a marked decrease in the cell body size and by a large increase in the number of cells with a condensed nucleus 76/124 (61 %) versus controls 7/147 (5 %) (n=4; Fig. 5G and H). Low PI fluorescence suggests that there is very limited necrosis in the 12 hour period (Fig. 5I). Apoptotic features similar to those that cannabinoids induced in a primary astrocyte cultures, were observed in the mixed neuron-glia cultures (Fig. 5M) and in the short-time (24 to 48 hours) cultured organotypic slices (Fig. 5R). This preparation recapitulates the types of cells normally found in the brain, i.e., neurons, astrocytes, microglia and macrophages.

Discussion

Cannabinoids induced a biphasic calcium response in cultured astrocytes. Initial, rapid increase in $[Ca^{2+}]_i$ was followed by decline to the control level and then by a steady and sustained increase. Both phases were CB_1 -receptor dependent and mediated by the PLC. The rapid component of the cannabinoid-induced calcium response originated from the intracellular calcium stores, while the delayed and sustained calcium increase resulted from the calcium influx. The sustained calcium elevation induced by the cannabinoids was

Fig. 5. Cannabinoids induce apoptosis in primary astrocyte, mixed neuron-glia and organotypic hippocampal cultures. (A-C) Fluorescent images of AnnexinV-FITC labeled cells, as early marker of apoptotic events in astrocytes, elicited by cannabinoids. Exposure of primary astrocyte cultures to 5 μ M WIN (B) and 5 μ M THC (C) for 6 hours resulted in 235 \pm 12 and 258 \pm 21 % increase in fluorescent intensity, respectively. (D-I) Cannabinoids induce nuclear condensation and increase in RNA content in astrocytes. Nuclear morphology was examined in enriched astrocyte cultures that were exposed to 5 μ M WIN (6 hours) and 5 μ M THC (12 hours) prior to 15-min exposure of cell-permanent DNA dye acridine orange (panel AO) and cell-nonpermanent DNA dye propidium iodide (panel PI). Cells were incubated with the nucleic acid dyes in the absence of RNase (D-F) and in the presence of RNase (G-I). Acridine orange intercalates into double-stranded DNA emitting the green fluorescent light (G). Nuclei that are stained with acridine orange in the absence of RNase emit yellow light due to the green and red fluorescence emission spectrum overlap (D). When the AO is bound to a single-stranded DNA or RNA, a fluorescent emission maximum of the dye is shifted to red spectra (Darzynkiewicz et al., 1992). In cells where the RNA content was not affected by the RNase treatment, cannabinoid-induced apoptosis was accompanied by an increase in RNA as revealed by the fluorescence emission shift toward the orange-red spectra (E). Cells exposed to the 5 μ M THC for 12h in the presence of RNase exhibit a marked decrease in cell body size compared with untreated cells and the loss of green fluorescence due to nuclear condensation which indicated a late apoptotic stage of cannabinoid-induced cell death. Arrows indicate representative examples of condensed chromatin (H). Propidium iodide can not permeate live cells, but stains necrotic cells with red fluorescence. Low fluorescence in PI panels illustrates very limited necrosis during a period of 12 hours (F and I). (J-O) THC induces apoptosis of astrocytes in mixed glia-neuron cultures. By combining AO staining and anti-GFAP-Cy3 conjugated antibody fluorescent staining, we visualized astrocytes in control conditions (J-L) and following the exposure to 5 μ M THC for 12 hours (M-O). Short arrows indicate representative examples of condensed chromatin of GFAP-positive cells, while long arrows indicate representative examples of condensed chromatin of other brain cells (M). (P-S) THC induces apoptosis of astrocytes in hippocampal organotypic cultures. We performed double staining to visualize GFAP-positive cells and to score apoptotic astrocytes. THC (5 μ M, 12hours) induce chromatin condensation in astrocytes indicated by short arrows and in other brain cells indicated by long arrows (R). Scale bars, 25 μ m.



accompanied with the disappearance of astrocytes from the visual field at the end of the calcium-imaging experiment, suggesting cannabinoid toxicity. We verified this assumption by the toxicity assay. Our results for the first time point to the toxic effects of cannabinoids on astrocytes, in both *in vitro* and *in situ* studies. In addition, we observed an ability of astrocytes to recover from the cannabinoid toxicity. The ability of astrocytes to repopulate might be of relevance to the pathology of the nervous system. It was documented that astrocytes respond to an insult by cell division and repopulate damaged area forming glial scars, a hallmark of many neurodegenerative changes in the brain (Raivich et al., 1999).

The CB₁ receptor is coupled to the G_{i/o} protein the activation of which modulates adenylate cyclase activity and cAMP levels. Survival of many cells depends on optimal cAMP levels (Holcomb et al., 1995). As expected PTX blocked cannabinoid toxicity. In addition, Ras peptide inhibitor abrogated THC toxicity, pointing to a major contribution of small G proteins in the mechanism of death. However, modulators of adenylyl cyclase activity had no significant effect on the cannabinoid toxicity, thus uncoupling the link between enzyme activity and cannabinoid toxicity.

G_{i/o} protein activation is the signal for the activation of many downstream signal transduction pathways, PLC and MAP cascade among others. PLC activation leads to generation of inositol-3-phosphates (IP₃) and diacylglycerols (DAG). DAG can activate PKC, which in turn can phosphorylate and thus activate channels, transcription factors like AP-1 and / or MAP cascade that can lead to apoptotic and non-apoptotic cell death in many cells including neural cells (Mohr et al., 1998; Murray et al., 1998). Two different PKC inhibitors were able to suppress THC-induced cell death, emphasizing the possible activation of a novel class of PKC's, calcium-independent isozymes, rather than the

activation of calcium-dependent conventional PKC isozymes. This agrees with the observed calcium-independent mechanism of cannabinoid-induced cell death. Contribution of DAG and DAG responsive PKC's in the THC-induced astrocyte death was further supported by the ability of membrane-permeable DAG that activates PKC to provoke cell death on its own.

Members of the ERK family are implicated in the cannabinoids toxicity because the selective ERK inhibitor, PD 098059, abrogated THC-induced cell death. In contrast, p38 MAP kinase inhibitor failed to protect astrocytes. It is known that ERK activation induces a proliferation of cells that depends on the duration of the stimulus (Marshall, 1998). However, recent investigations point to another role of the ERK cascade in the cell's fate, since the activation of ERK induced death of the hippocampal neurons (Murray et al., 1998) and astrocytes (Blazquez et al., 2000). A sustained ERK activation elicited by the THC induced apoptosis in C6 glioma cells in culture (Galve-Roperh et al., 2000). There are at least three different pathways that may activate ERK. (1) Activation of ERK cascade by α and $\beta\gamma$ -subunits released from heterotrimeric G_i and G_o -proteins (Luttrell et al., 1999; Mochizuki et al., 1999) and ERK activation by small G-proteins such as Ras (Fruman et al., 1998); (2) activation of ERK cascade by the calcium-independent PKC isoforms (Neary et al., 1999); (3) activation of ERK cascade by the ceramide-activated Raf-1 (Sanchez et al., 1998b).

Our results suggest that THC activates a $CB_1 / G_{i/o} / PI3K / Ras / ERK$ signaling cascade in astrocytes. We postulated a signaling pathway in which the CB_1 receptor activation induces G-protein subunits dissociation and subsequent $\beta\gamma$ subunits-mediated

PI3-kinase activation. Additionally, we observed a significant contribution of small G-proteins like Ras, the activation of which is mediated by PI3K (Fruman et al., 1998). Ras activation initiates Raf-1 protein kinase activation and subsequent MAP cascade activation (Morrison and Cutler, 1997). Legitimacy of the proposed death signaling pathway employed by the cannabinoids is further supported by the ability of THC to stimulate ERK activation, Raf-1 phosphorylation and Raf-1 translocation in astrocytes (Sanchez et al., 1998b). Because the inhibitor of the ceramide biosynthesis pathway protected astrocytes from THC toxicity, an alternative pathway for the mechanism of cannabinoid toxicity is suggested.

Utilization of the sphingomyelin pathway for induction of apoptosis has been recently demonstrated in many mammalian cells, including neural cells (Brugg et al., 1996; Jaffrezou et al., 1996). Ceramide generation through sphingomyelin hydrolysis by acid or neutral sphingomyelinase is usually considered the norm (Blazquez et al., 2000). A *de novo* synthesis pathway emerged as an alternative way for ceramide generation and an apoptotic stimulus in some cells: pancreatic, hematopoietic and astrocytes (Blazquez et al., 2000; Paumen et al., 1997; Shimabukuro et al., 1998). Our results indicate that the ceramide generated through the biosynthesis plays an important role in the THC-induced apoptosis in astrocytes rather than ceramide generated through the sphingomyelin hydrolysis. A link between ceramide formation and ROS generation has recently been suggested (Fernandez-Checa et al., 1998). We observed a significant contribution of free radicals to the toxicity of cannabinoids as antioxidants abrogated THC-induced cell death. Similar protection of hippocampal neurons with antioxidants against THC toxicity has been documented (Chan et al., 1998).

Caspases, a family of cysteine proteases, were implicated in THC toxicity because the broad caspase inhibitor Z-Vad-FMK protected astrocytes from death. Similarly anandamide, an endogenous cannabinoid receptor ligand, induced apoptosis in PC-12 cells via caspase-3 activation (Sarker et al., 2000). These results implicate mitochondria as an important link in a mechanism of cannabinoid toxicity since both, ROS overproduction and activation of caspases due to the mitochondria dysfunction is well established (Garcia-Ruiz et al., 2000; Kolesnick and Kronke, 1998; Zamzami et al., 1995).

In summary, these results for the first time point to the toxic effects of cannabinoids on astrocytes in both, *in vitro* and *in situ* studies. Activation of two kinases, PKC and ERK, promoters of cell proliferation in many cases, emerged as the major signaling pathway in the cannabinoid-induced astrotoxicity. Furthermore, our results suggest that cannabinoids activate a transcription-dependent cell death, a process that is, in many cases, tightly coupled to kinase activation. Finally, fluorescent staining revealed that cannabinoids induce apoptotic cell death in astrocytes. These data suggest that marijuana is more harmful to brain than previously thought an observation that might compromise clinical use of cannabinoids.

Acknowledgements

We thank Dr. L. L. Anderson, Dr. Gary Wenk, Dr. Frank Porreca, Dr. Bhanu Jena and Dr. Laszlo Urban for the helpful comments on the manuscript. National Science Foundation Grant IBN 9604862 supported this work.

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**CHAPTER 4. ASTROCYTE ACTIVATION AND APOPTOSIS
INDUCED BY gp120 AND SDF-1 α ARE MEDIATED BY CXCR4
RECEPTOR**

A paper to be submitted to the Journal of Neurophysiology

by

Aleksandar Jeremic, Ksenija Jeftinija and Srdija Jeftinija

Abstract

Astrocytes, one of the brain glial cells, may play important role in the pathogenesis of HIV-induced dementia and the neurotoxicity of drugs of abuse. The HIV envelope glycoprotein gp120 and the stromal cell-derived factor 1 α (SDF-1 α), a selective CXCR4 receptor agonist, produced chemokine receptor-mediated calcium transients and glutamate release in cultured astrocytes. Co-culturing neurons with glia increases the neuron's responsiveness to SDF-1 α stimulatory effects by approximately 8-fold. A selective NMDA receptor antagonist D-AP5 abolished the stimulatory effect of SDF-1 α on co-cultured neurons, suggesting that neuronal effects of SDF-1 α were astrocyte-mediated. In a similar manner, an anion co-transporter inhibitor, NPPB, abolished the SDF-1 α -induced calcium transients both in the neurons and in the glia in the mixed neuron-glia cultures, supporting the

hypothesis of astrocytic glutamate contribution to the α -chemokine stimulatory action. In addition to the transient effect on astrocytes, SDF-1 α and gp120 were toxic to astrocytes. Furthermore, we observed a synergy between cannabinoids and chemokines on the calcium transients in the astrocytes and the cell's viability. SDF-1 α or gp120 in combination with the cannabinoid receptor agonists enhance the kinetics of calcium transients elicited by cannabinoids and potentiate cannabinoid toxicity in the astrocytes. Coupling of two systems by the activation of two different receptors, CB₁ and CXCR4, whose signal transduction pathways converge at some point might explain the synergic action shown by chemokines and cannabinoids.

Introduction

There is mounting evidence that glia in general, and astrocytes in particular, might play a key role in information processing (Barres, 1991; Attwell, 1994; Smith, 1994; Kettenmann, 1996; Ullian et al., 2001). It has been demonstrated that astrocytes can transmit messages to surrounding cells by direct release of signaling molecules such as the excitatory neurotransmitter glutamate (Parpura et al., 1994; Jeftinija et al., 1996; Jeremic et al., 2001). Activation of glutamate receptors is thought to be a final common pathway leading to neuronal damage in the course of many neurological diseases such as hypoxic-ischemic brain injury, epilepsy, Huntington's disease, Parkinson's disease, Alzheimer's disease and HIV-associated dementia.

Progressive encephalopathy, known as AIDS dementia complex (ADC), in patients infected with human immunodeficiency virus type I (HIV-1) is the commonest form of

dementia in North Americans less than 60 years old. Numerous studies, incorporating a variety of complementary experimental approaches, provided compelling evidence that gp120, an HIV-1 envelope glycoprotein, has neurotoxic properties. It was shown that gp120 neurotoxicity was mediated by the N-methyl-D-aspartate (NMDA) receptor activation (Lipton et al., 1991). An indirect contribution of activated macrophages in the gp120-induced neuronal apoptosis has been documented (Kaul and Lipton, 1999). In addition to indirect pathways, recent investigations showed that gp120 IIIB, a glycoprotein of HIV-1 T-cell tropic strains that signal via the α -chemokine receptor CXCR4 (Davis et al., 1997) and stromal cell-derived factor 1 α (SDF-1 α), a selective CXCR4 receptor agonist, directly induce apoptosis in neurons (D'Souza and Harden, 1996; Berger et al., 1998; Hesselgesser et al., 1998; Meucci et al., 1998).

The CXCR4 receptor is abundant in the brain and alterations in the chemokine receptor expression along with reactive astrocytes (gliosis) in the hippocampus of ADC patients have been documented (van der Meer et al., 2000; Petit et al., 2001). The chemokines have been found in increased amounts in the brains of patients with HIV encephalitis (Klein et al., 1999). The progressive encephalopathy of HIV-infected individuals is quite variable in course and presentation. This could be attributable to differences in neurotoxicity among viral strains and / or to insults that result in increased extracellular glutamate, such as cerebral hypoxia-ischemia, hypoglycemia and drugs of abuse such as marijuana (Silverstein et al., 1990; Silverstein et al., 1991; Chan et al., 1998). For instance, glucocorticoids were found to exacerbate neurotoxicity of gp120 in primary hippocampal and cortical cultures (Brooke et al., 1997).

In this study, we tested the ability of CXCR4-receptor agonists, SDF-1 α and gp120 III_B, to stimulate astrocytes and to modulate cannabinoid-induced astrototoxicity. To our knowledge, this is the first study relating marijuana neurotoxicity and ADC.

Materials and Methods

Cell cultures. Mixed neuron-glia and primary astrocyte cultures from neonatal rat cerebral cortex or hippocampus were established as described (Jeftinija et al., 1996). Briefly, 1-3 day old Sprague-Dawley rats were quickly decapitated prior to removal of the brain according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. The cortex was freshly dissected and the tissue was enzymatically (papain 20 i.u./ml; 1 hour at 37°C) and mechanically dissociated. Cells were plated on poly-L-lysine (1 mg/ml; MW 100,000)-coated glass cover slips (for mixed cultures) or culture flasks and maintained at 37°C in a humidified 5% CO₂/95% air atmosphere (for primary astrocyte cultures). After mixed cultures reach confluence (9-12 days), the flasks were shaken overnight (12-18 hours) at 260 rpm at 37°C. Cultures enriched in type I astroglia were obtained by trypsinizing (0.25%) the attached cells for 3 min. Trypsin was inactivated by adding 3 ml of Eagle's α MEM supplemented with 10% heat-inactivated FBS (serum contains protease inhibitors). Astrocytes were plated on poly-L-lysine (10 μ g/ml; MW 100,000)-coated glass cover slips.

The protocol used for primary neuron cultures is a modification of the procedure used for preparing the mixed cultures. Tissue isolated from neonatal rat cerebral cortex or

hippocampus was enzymatically and mechanically dissociated. The cells were plated on poly-L-lysine (1mg/ml; MW 100,000)-coated glass cover slips. Neurons were cultured in serum-free medium consisting of Eagle's Neurobasal medium supplemented with 2% B-27 antioxidant solution (Gibco).

Acute cortical slices were prepared as described (Gahwiler, 1981). Briefly, cortical slices of 250 μm were obtained from 2 weeks old Sprague-Dawley rats. Acutely isolated slices were transferred to glass cover slips coated by chicken plasma that was coagulated by thrombin. Glass cover slips with the explant were placed in perfusion chamber for EAA content determination.

Ca²⁺ imaging. The effect of experimental manipulation on glial $[\text{Ca}^{2+}]_i$ was evaluated by the fluorescence ratio imaging technique. Cells were loaded with 5 μM Fura-2 AM (Molecular Probes) for 40-60 minutes at 24°C. After washing, cells were deesterified for 10 min at 24°C. All image processing and analysis were performed using an Attofluor system with Zeiss microscope. Background subtracted, rationed images (340/380 nm) were used to calculate the $[\text{Ca}^{2+}]_i$ according to Equation 5 of Grynkiewicz et al (1985). Calibration was performed *in situ* according the procedure provided by the Attofluor, using the Fura-2 Penta K⁺ salt as a standard. In the experiments in which we used low Ca²⁺ solution, calcium was lowered to 0.2mM while Mg²⁺ was elevated to 2.5mM and 1mM EGTA was added.

Release method and HPLC. Cover slips with astrocyte cultures or acute cortical slices were mounted into a perfusion chamber and washed in normal HEPES saline for a period of 20 to 30 min to allow equilibration. After equilibration, 200 μl samples were collected every minute. The amino acid content in the samples was determined by high-performance

liquid chromatography (HPLC) with fluorescence detection. Chromatography was performed on a 15cm Microsorb-MV HPLC column (Rainin Instrument Co.) using a pH 5.9 sodium acetate methanol gradient.

Toxicity assay. Cell viability was measured by the MTT assay. Astrocyte cultures were plated on 24-well plates and maintained in serum-containing medium for 2-3 days. 24 hours before applying treatments, the serum-containing media were replaced with serum-free media (QBSF-51 media, Sigma). In this medium astrocytes persist without a detectable change in astrocyte viability for at least 72 hours. Cultured astrocytes were incubated with agonists for indicated periods of time. At the end of incubation, MTT was added to the QBSF-51 media (250 ng/ml) and incubated for 3 hours at 37° C. After incubation, the medium was removed and an insoluble formazan dye was solubilized with isopropanol / HCl solution. Absorbance of the converted dye was measured at 570 nm with background subtraction at 630 nm. One hundred percent viability is defined as MTT conversion by cells treated with the carrier for the duration of the experiment.

Fluorescent staining protocol. AnnexinV-FITC staining protocol: primary astrocyte cultures were bathed in SDF-1 α -contained serum-free media (QBSF-51 media, Sigma) for indicated period of time and additionally incubated with the 5 μ M AnnexinV-FITC dye diluted in HEPES-buffer solution for 15 min in the dark. Cultures were washed and mounted onto a perfusion chamber and examined with a Zeiss fluorescence microscope using FITC filter set. Images were collected with a Nikon Coolpix digital camera and the fluorescence signal quantified with MetaMorph imaging software.

Acridine orange / propidium iodide staining protocol: cultures were bathed with SDF-1 α for the indicated period of time and loaded with dyes AO (5 μ M) and PI (2.5 μ M). Cells were visualized under fluorescence illumination: AO (480 nm for excitation and 510 nm barrier filter for emission); PI (540 nm and 610 nm excitation-emission filter set), with 40X oil immersion objective.

Statistical analysis. All graphs and bars represent the mean \pm SEM of four to six independent experiments. A statistical comparison between treatments was performed by One-way ANOVA followed by Neuman-Keuls *post hoc* test. Significance was established at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

SDF-1 α and gp120 induce Ca²⁺ transients and excitatory amino acids release from cultured type I astrocytes

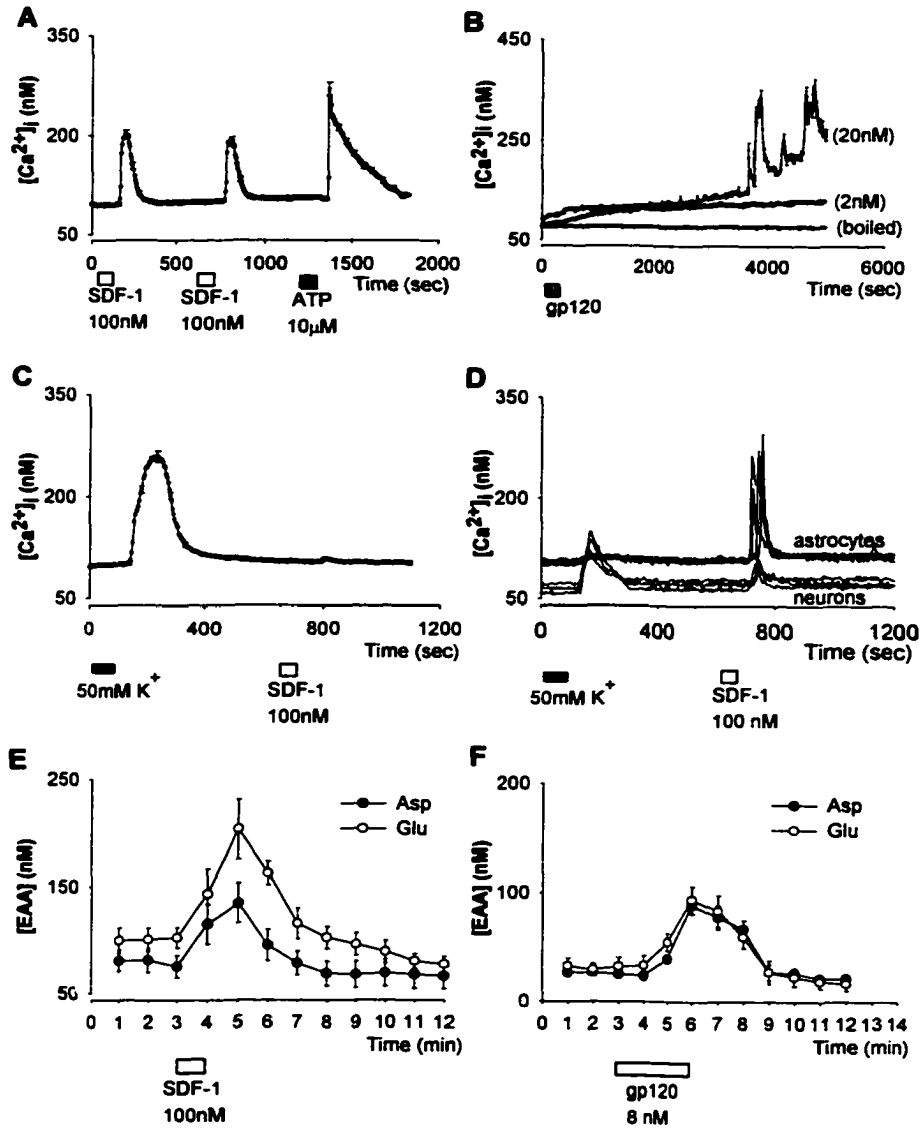
To determine the cell type responding to the application of SDF-1 α and gp120, experiments were performed on enriched astrocyte and neuron cultures. A brief perfusion application of SDF-1 α (100 nM, 1 min) produced a rapid increase in intracellular calcium in cultured astrocytes. This started approximately 90 sec after the beginning of the SDF-1 α application (Fig. 1A). SDF-1 α increased the level of free calcium from the basal 96 ± 2 nM to 196 ± 5 nM in 88 % of cells tested (n=180 of 204). The effect was reversible and a second application of SDF-1 α produced an increase in [Ca²⁺]_i that was 91 ± 6 % (n=178) of first application (Fig. 1A). The threshold concentration of SDF-1 α was 10 nM (data not shown). ATP, a P₂ receptor agonist, reliably elevated calcium in all astrocytes (n=108;

Jeremic et al., 2001), confirming functional responsiveness of astrocytes to SDF-1 α application (Fig. 1A).

Perfusion application of 20nM gp120 (3 min) produced an irreversible increase in intracellular calcium with two distinguishable stages; a slow developing increase that was followed by a large and spike-like increase in intracellular calcium (Fig. 1B). gp120, in threshold concentration of 2nM, induced a small and steady increase in intercellular calcium without the second stage detected with 20 nM gp120 (Fig. 1B). Heat inactivated gp120 was without any effect on calcium transients in astrocytes even at 50nM (Fig. 1B)

In contrast to astrocytes, neurons were much less sensitive to SDF-1 α application and only 9 % of neurons (n=8 of 85) responded with an increase in intracellular calcium (Fig. 1C). However, culturing neurons with glia markedly increased the number of neurons that responded to the SDF-1 α with elevation of intracellular calcium (Fig. 1D). 50 mM K⁺ was used as a nonselective depolarizing stimulus to functionally identify neurons (Morton and Davenport, 1992; Bezzi et al., 1998; Jeremic et al., 2001). In the mixed neuron-glia cultures, 71% (n=84 of 118) of cells functionally identified as neurons by application of 50mM K⁺ responded to SDF-1 α (Fig. 1D). Previously, we have shown that elevated internal calcium in astrocytes results in release of glutamate, which then stimulates adjacent neurons (Parpura et al., 1994). To see whether the SDF-1 α -induced calcium transients in astrocytes are accompanied by excitatory amino acids (EAA) release, we assayed changes in aspartate and glutamate concentration by HPLC on the superfusate from primary astrocyte cultures. Perfusion application of SDF-1 α (100nM, 1 min) provoked a significant increase in aspartate and glutamate concentration from 80 ± 9 to 135 ± 17 nM and from 100

Fig. 1 SDF-1 α and gp120 stimulates calcium transients and glutamate release from cultured astrocytes. (A) Perfusion application of 100nM SDF-1 α and 10 μ M ATP for 1 min reliably increased intracellular calcium in primary astrocyte cultures. (B) Application of 20nM gp120 for 3 minutes induced a slow increase in free intracellular calcium in primary astrocytes lasting 2000-3000 sec followed by a sharp oscillatory rise in intracellular calcium that stayed elevated throughout the experiment. 2nM gp120 induced a slow and steady increase in intracellular calcium till the end of experiment (B). Heat inactivated gp120 had no stimulatory effect on cultured astrocytes (B). (C) Application of 50mM K⁺ induced neuron depolarization and subsequent calcium influx in primary neuron cultures. In contrast, SDF-1 α had little stimulatory effect on calcium mobilization in neurons. (D) Co-culturing neurons with glia increase neuron responsiveness to the SDF-1 α stimulatory effect. (E) Addition of SDF-1 α (100nM) for 1 min in cultured astrocytes causes a one-fold increase in the aspartate and glutamate concentration above baseline level . (F) Perfusion application of gp120 (8nM) for 3 minutes stimulated aspartate and glutamate release from primary astrocyte cultures. Horizontal bars indicate onset and duration of perfusion application of the compound. The delay in the onset of the effect is due to the dead space of the perfusion system.



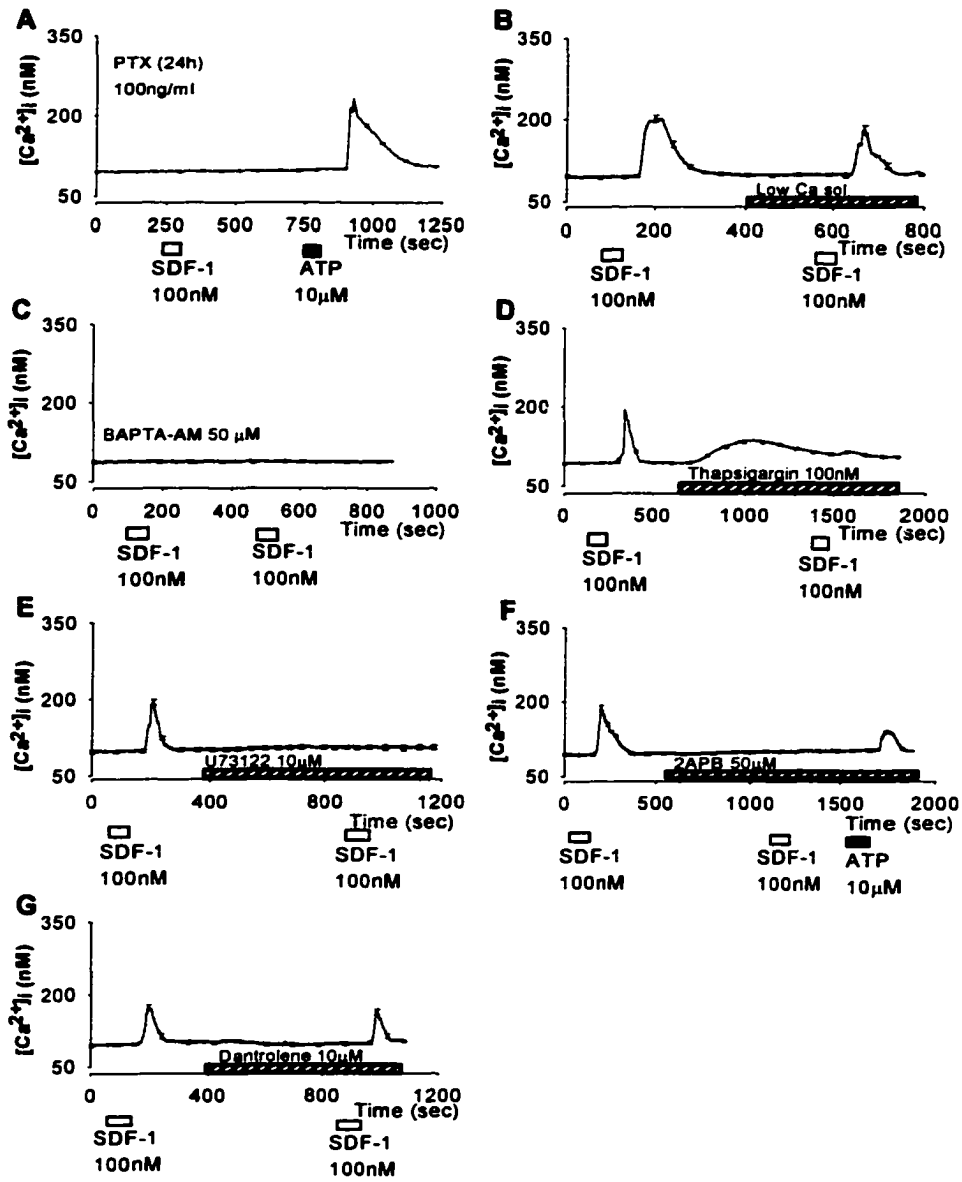
± 9 to 203 ± 27 nM, respectively ($n=7$, $p<0.05$, Student t test; Fig. 1E). In a similar manner, gp120 (8nM, 3min) stimulated EAA release from cultured astrocytes ($n=6$; Fig. 1F).

Role of IP₃-sensitive calcium stores in SDF-1 α -evoked calcium transients in cultured astrocytes

The CXCR4 receptor is coupled to the pertussis toxin (PTX)-sensitive G_{i/o} protein by activation of which this receptor exerts most of its biological action (Bokoch, 1995). We pre-treated cells with PTX (100ng/ml) for 24 hours and evaluated the stimulatory effect of SDF-1 α . As expected, PTX abolished the SDF-1 α effect on calcium transients in cultured astrocytes suggesting that SDF-1 α induce Ca²⁺ mobilization through the activation CXCR4 receptor / G_{i/o} protein signaling cascade (Fig. 2A). PTX action was selective as the stimulatory effect of ATP was not influenced by the PTX pre-treatment (Fig. 2A).

Removal of external calcium from bathing medium did not prevent the SDF-1 α mobilization action indicating that SDF-1 α mobilizes calcium from internal stores (Fig. 2B). We further tested the contribution of internal Ca²⁺ stores on the SDF-1 α stimulatory effect by pre-incubation of cells with 1,2-bis (2-aminophenoxy) ethane-N,N',N'-tetracetic acid-acetoxymethyl ester (BAPTA-AM), an intracellular calcium chelator. BAPTA abolished the stimulatory effect of SDF-1 α on astrocytes (Fig. 2C). Furthermore, in the presence of thapsigargin, an inhibitor of the Ca²⁺-ATPase of internal Ca²⁺ stores (Thastrup et al., 1990), a second application of SDF-1 α failed to mobilize internal calcium ($n=38$; Fig. 2D). Chemokine receptors activate different second messenger systems,

Fig. 2 SDF-1 α induces calcium transients in primary astrocyte cultures by the activation of PLC-IP₃ signal transduction pathway. **(A)** Pre-treatment of astrocytes with PTX (100ng/ml; 24 h) abolished the SDF-1 α stimulatory action, while it was without effect on the ATP-induced calcium transients in astrocytes. **(B)** SDF-1 α stimulatory effect was preserved in low calcium solution. **(C)** Pretreatment of cells with BAPTA-AM (50 μ M, 40 min) abolished the SDF-1 α -induced calcium increase. **(D)** Thapsigargin (100 nM) prevented reloading of internal stores with calcium and blocked the second SDF-1 α -induced calcium elevation. **(E)** U73122 (10 μ M) abolished the SDF-1 α stimulatory action. **(F)** Astrocytes pretreated with 50 μ M 2APB were insensitive to the SDF-1 α stimulatory action. Selectivity of antagonist was observed as 2APB erased the metabotropic component of the ATP response, leaving the ionotropic component of response only. **(G)** Dantrolene (10 μ M) failed to block the stimulatory action of SDF-1 α .



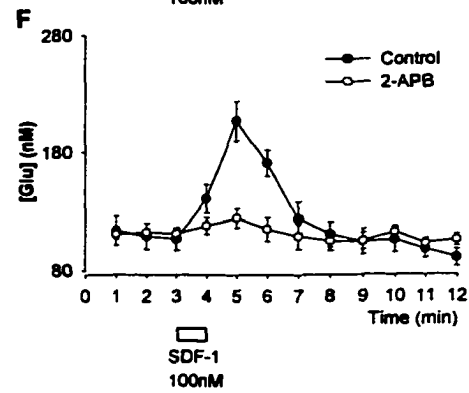
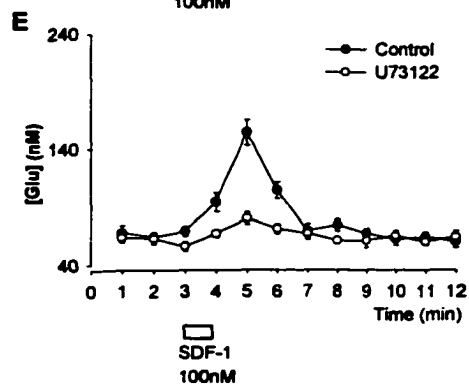
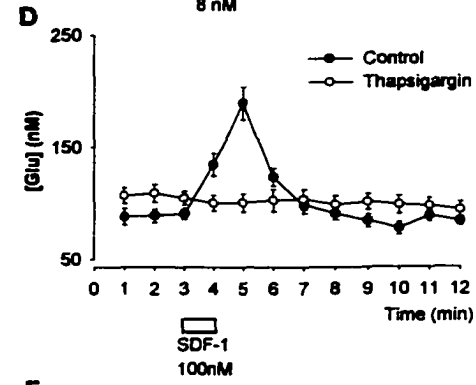
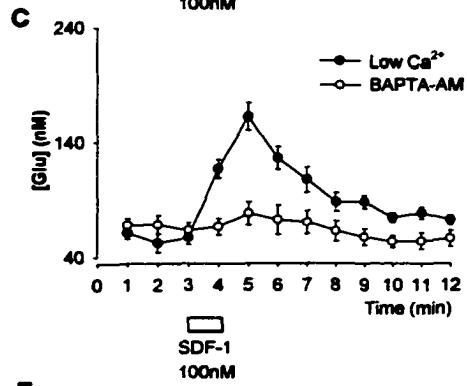
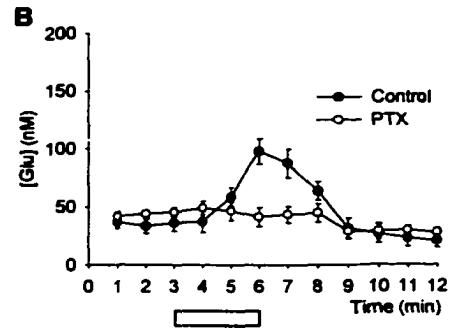
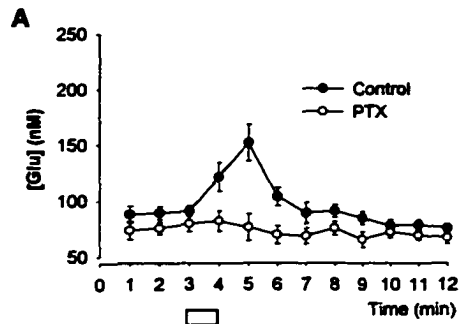
phospholipase C (PLC) among them. To examine the role of PLC, we used U73122, a selective inhibitor of the receptor-coupled PLC-dependent process (Smith et al., 1990). U73122 (10 μ M) blocked the SDF-1 α induced response in astrocytes, suggesting that activation of PLC precede the liberation of calcium from intracellular stores (Fig. 2E). To explore which stores are involved in SDF-1 α stimulatory action we used 2APB, a selective IP₃ receptor antagonist and dantrolene, a ryanodine receptor antagonist. 2-APB (50 μ M) abolished SDF-1 α action and significantly reduced ATP stimulatory effect, leaving only inotropic component of the ATP response (n=37, p<0.01; Fig. 2F). In contrast, dantrolene had no significant effect on the SDF-1 α mobilization effect (n=45, p>0.1; Fig. 2G).

SDF-1 α -induced release of glutamate depends on intracellular calcium

Because PTX blocked the SDF-1 α -induced calcium transients in astrocytes, we investigated whether toxin pre-treatment might influence the chemokine-induced EAA release from primary astrocyte cultures. PTX abolished the SDF-1 α - and gp120-induced glutamate release from astrocytes, suggesting that the G $\alpha_{i/o}$ subunits couple the CXCR4 receptor to the control of EAA release from these cells (n=4, Fig. 3A and B). This is consistent with recent *in situ* study, which showed that the SDF-1 α and gp120-induced glutamate release from astrocytes is CXCR4-receptor mediated (Bezzi et al., 2001).

Calcium-imaging data demonstrated that SDF-1 α mobilizes calcium from internal calcium stores by a PLC-IP₃ sensitive mechanism. To investigate whether a similar mechanism controls the SDF-1 α -induced glutamate release from astrocytes, cells were bathed in low- calcium saline containing 0.2mM calcium with addition of 1mM EGTA to

Fig. 3 SDF-1 α -induced EAA release from cultured astrocytes is mediated by the G $_{i/o}$ -PLC-IP $_3$ signaling pathway. **(A and B)** Pre-treatment of astrocyte cultures with pertussis toxin (100ng/ml; 24h) abolished the SDF-1 α and gp120-evoked glutamate release from astrocytes. **(C)** Stimulatory effect of SDF-1 α on astrocytes was preserved in low Ca $^{2+}$ -EGTA solution. In cultures incubated with the 50 μ M BAPTA-AM for 30 min, the SDF-1 α -induced glutamate release was abolished **(C)**. **(D)** Incubation of astrocytes in 1 μ M thapsigargin resulted in an increase in the basal glutamate concentrations and blockage of the SDF-1 α -evoked glutamate release. **(E)** A specific PLC inhibitor, U73122 (10 μ M), diminished glutamate release from astrocytes elicited by SDF-1 α . **(F)** A selective IP $_3$ -receptor antagonist, 2-APB, impaired the ability of SDF-1 α to stimulate EAA release from astrocytes.



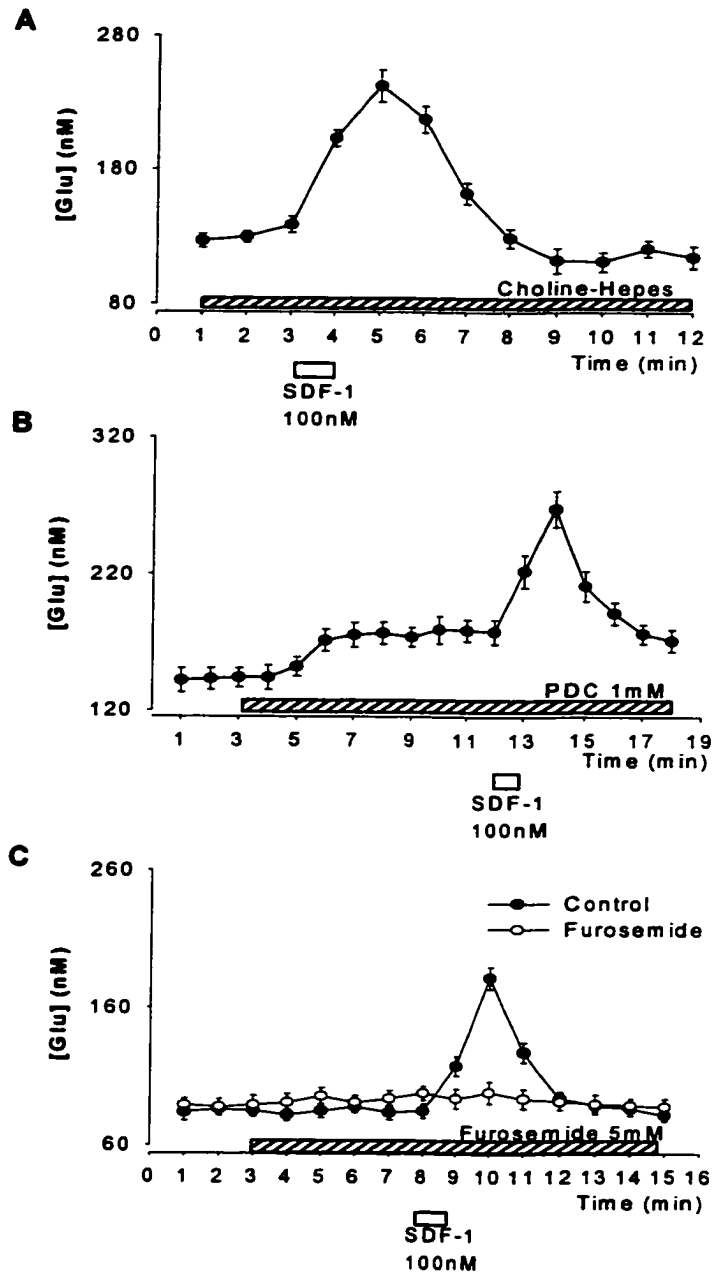
yield an estimated free extracellular calcium level of 26nM. In the absence of extracellular calcium, the stimulatory effect of SDF-1 α was preserved, suggesting that the SDF-1 α -induced release of glutamate is not dependent on extracellular calcium and therefore likely results in calcium release from intracellular stores (n=4, Fig. 3C). This hypothesis is further supported by the ability of BAPTA-AM, to block the SDF-1 α -induced glutamate release (n=4, Fig. 3C). Addition of thapsigargin, an inhibitor of Ca²⁺-ATP-ase of internal stores (Thastrup 1990), increased the basal glutamate levels above control levels (p<0.05, Student's t-test, n=4; Fig. 3D) and erased glutamate elevation induced by SDF1- α supporting the notion that SDF-1 α releases glutamate by the mobilization of calcium from internal stores (n=4, Fig. 3D).

To investigate the possible contribution of IP₃-sensitive calcium stores in the SDF-1 α stimulated glutamate release from astrocytes, we employed two pharmacological inhibitors of PLC-IP₃ signaling pathway. Both compounds, U73122, a PLC inhibitor and 2-APB, a selective IP₃-receptor antagonist, suppressed the release of glutamate elicited by SDF-1 α , emphasizing an important contribution of IP₃-sensitive calcium stores in the mechanism of release (n=4, Fig. 3E and F).

Glutamate transporters are not implicated in the SDF-1a-induced glutamate release from astrocytes

Extracellular glutamate accumulation may be caused by inhibition of the uptake or reversal of the Na⁺-dependent glutamate transporter (Szatkowski et al., 1990). To examine the relationship between SDF-1 α -induced glutamate release and activity of Na⁺-dependent

Fig 4. SDF-1 α -induced glutamate release from primary astrocyte cultures is not mediated by a glutamate transporter mechanism and it is anion-cotransport sensitive. (A) SDF-1 α stimulated release of glutamate from astrocytes in Na⁺-free solution. (B) The glutamate transporter inhibitor PDC (1mM) elevated the basal glutamate concentration but failed to block the stimulatory action of SDF-1 α on glutamate liberation from astrocytes. (C) Bath application of 5mM furosemide, an anion cotransporter inhibitor, abolished the SDF-1 α evoked glutamate release from astrocyte cultures.



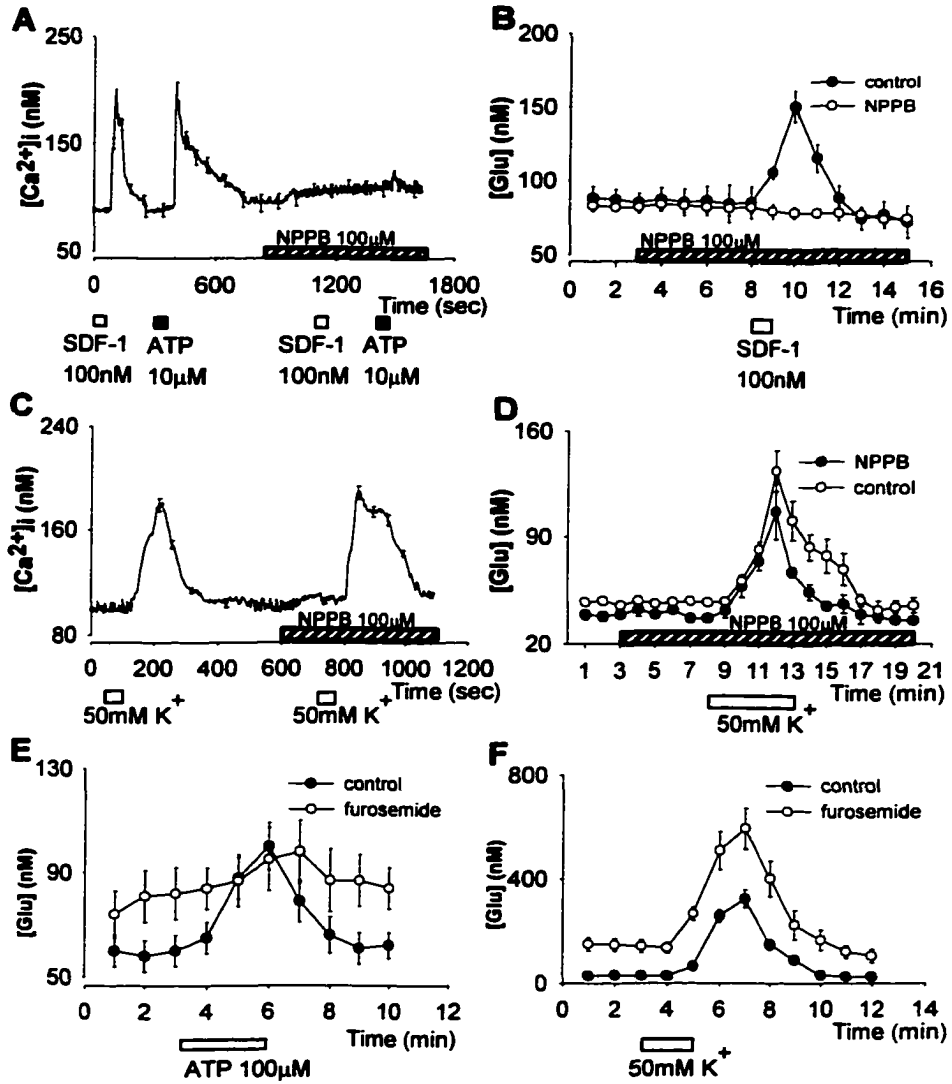
glutamate transporter, we pre-incubated cultures in Na⁺-free medium. In Na⁺-free medium extracellular NaCl was substituted with choline chloride. SDF-1 α -evoked release of glutamate and [Ca²⁺]_i were not influenced by pretreatment of the cultures with Na⁺-free medium suggesting that, in astrocytes, SDF-1 α stimulates a Na⁺-independent glutamate release (n=4, Fig. 4A). In consistency with previous observations, *L-trans*-pyroglutamate-2,4-dicarboxylate (PDC), a glutamate transport inhibitor (Isaacson and Nicoll, 1993), produced an increase in the baseline concentration of glutamate in the perfusate but failed to impair ability of the SDF-1 α to stimulate glutamate release from astrocytes, suggesting that glutamate transporter inhibitors do not mediate SDF-1 α -induced EAA release from these cells (p<0.01, Student's t-test, n=4, Fig. 4B).

Previously we have shown that neuroligand-evoked release of glutamate from astrocytes is sensitive to the anion co-transporter inhibitor furosemide (Parpura et al., 1994; Jeftinija et al., 1996, Jeremic et al., 2001). In this study, we tested whether SDF-1 α -induced release of EAA from astrocytes has a similar sensitivity by incubating astrocyte cultures in furosemide. Cultures bathed with furosemide (5mM) did not respond to SDF-1 α , implying that chemokines stimulate EAA release from astrocytes via anion-transport sensitive mechanism (n=4; Fig. 4C).

Anion co-transporter inhibitors selectively block glutamate release from astrocytes but not from neurons in cortical primary cultures and acute cortical slices

Swelling of astrocytes can induce release of glutamate and aspartate by the calcium-independent mechanism (Kimmelberg et al., 1990). It has been shown that anion transport

Fig. 5 Anion co-transporter inhibitors selectively block neuroligand-induced calcium increase and glutamate release from astrocytes with no effect on the depolarization-induced glutamate release from neurons in primary cultures or acute slices. **(A and B)** The SDF-1 α - and ATP-stimulatory effects on calcium transients and glutamate release in cultured astrocytes were abolished in the presence of 100 μ M NPPB. **(C and D)** NPPB (100 μ M) had no significant effect on the depolarization-induced calcium increase and glutamate release from primary neuron cultures. **(E and F)** Furosemide (5mM) selectively blocks ATP-induced release of glutamate from acute cortical slices **(E)**, without affecting the 50mM K⁺ (depolarization)-induced glutamate release in acute cortical slice preparations **(F)**.

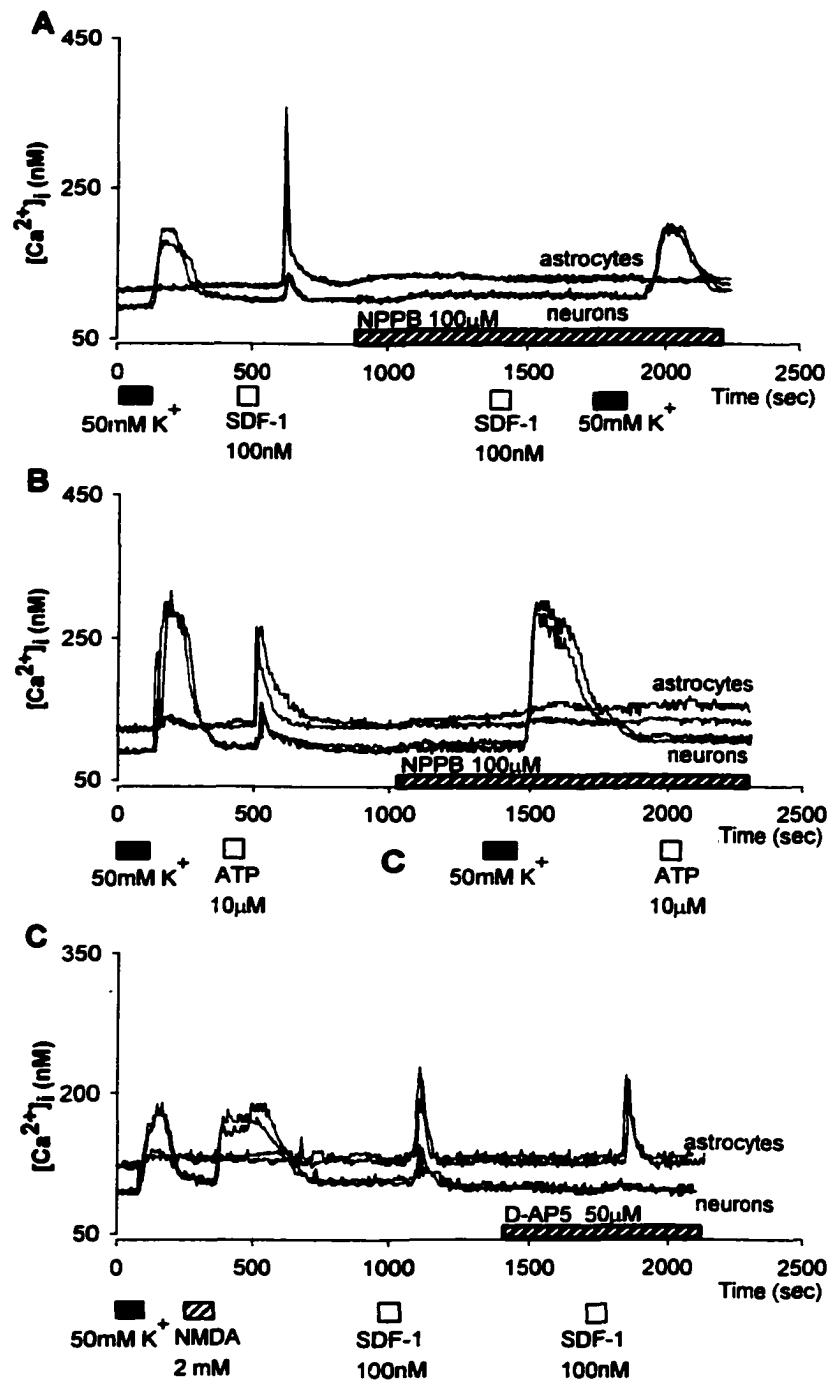


blocker, furosemide, reduced cell swelling-dependent release of EAA from cortical astrocytes (Kimelberg et al., 1990), thus raising the possibility that SDF-1 α may induce a cell swelling-dependent glutamate release from astrocytes. We therefore tested the actions of the anion cotransporter inhibitor and Cl⁻ channel blocker, furosemide and 5-nitro-2 (3-phenylpropylamino)-benzoate (NPPB), on the neuroligand-induced calcium transients and glutamate release from astrocytes (Fig. 5). NPPB is a potent Cl⁻ channel blocker at concentrations 10 μ M or less (Wangemann et al., 1986; Diener and Rummel, 1989) and a Cl⁻ / HCO₃⁻ co-transporter inhibitor at higher concentrations (Petersen and Cormann, 1987). At the concentration (10 μ M) at which it acts as a potent Cl⁻ channel blocker, NPPB failed to block the stimulatory effect of ATP and SDF-1 α (data not shown). Only when the higher anion-transport blocking concentration of NPPB was used were the stimulatory effects of SDF-1 α on astrocytes abolished (n=4, Fig. 5A and B). Our experiments revealed that the observed inhibition is selective. The co-transporter inhibitors, NPPB (only at 100 μ M) and furosemide, blocked the SDF-1 α -induced calcium changes and glutamate release from primary astrocyte cultures (Fig. 5A and B), whereas they had no significant effect on the depolarization-induced calcium transients and glutamate release from neurons in primary neuronal cultures (n=4, p>0.1; Fig. 5C and D) or in acute cortical slices (n=6, p>0.05; Fig. 5F).

Role for chemokines in the neuron-glia cross-talk

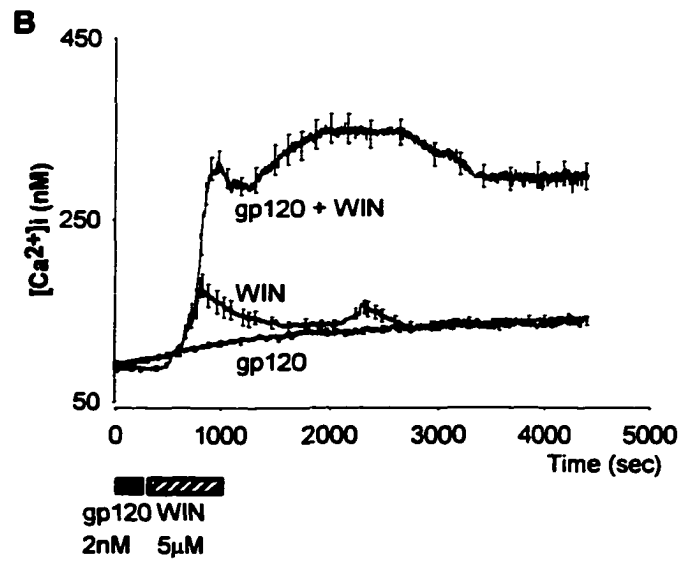
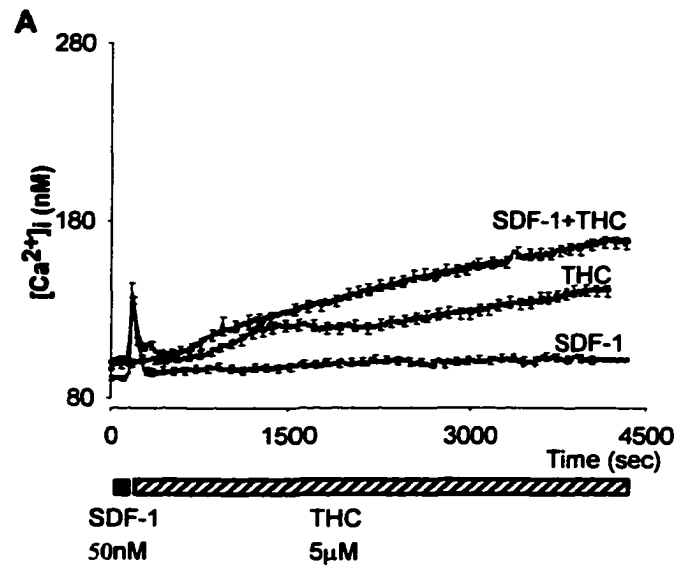
We implemented the anion-transport sensitive mechanism of glutamate release from astrocytes for the evaluation of SDF-1 α stimulatory effect on neural cells in mixed neuron-glia cultures (Fig. 6). Thus, we used 100 μ M NPPB as a selective pharmacological

Fig. 6 SDF-1 α and ATP stimulate glutamate-mediated elevation of neuronal calcium in mixed neuron-glia cultures. **(A)** NPPB (100 μ M) abrogated the SDF-1 α -induced calcium transients in glia and in neurons, whereas it had no significant effect on the depolarization-induced calcium influx in neurons. **(B)** The ATP- induced stimulatory effect on neuronal cells was suppressed in the presence of 100 μ M NPPB while the 50mM K⁺-induced calcium rise in neurons was not affected. **(C)** The stimulatory effect of SDF-1 α on neurons in neuron-glia co-cultures is indirect. The chart illustrates kinetics of calcium changes in two neurons and two adjacent glia cells following the application of SDF-1 α prior to and during the application of 50 μ M D-AP5. The response of the neurons to the SDF-1 α was abolished in the presence of D-AP5.



tool for the assessment of astrocytic glutamate contribution in the SDF-1 α -induced calcium mobilization in neurons. In mixed cultures, the hippocampal neurons were distinguished by their characteristic morphology, typical pyramidal shaped large soma giving rise to numerous processes. Functional identification of neurons was achieved by application of 50 mM K⁺. We have taken advantage of the observation that neurons responded promptly with a [Ca²⁺]_i increase to depolarization induced by 50 mM K⁺ (Morton and Davenport, 1992; Bezzi et al., 1998). Astrocytes, in mixed cultures, appeared as stellate cells that did not respond to application of 50mM K⁺. NPPB (100 μ M) abolished or significantly decreased SDF-1 α -induced calcium transients both in astrocytes 92 % (n= 24 of 26) and in neurons 52 % (n=14 of 27) without any significant effect on the depolarization-induced calcium transients in neurons (p>0.1, n=27; Fig. 6A). In similar manner, NPPB blocked the ATP-induced calcium transients in neurons (Fig. 6B). Those results raised the possibility that neuroligand-induced calcium elevation in neurons, at least in part, is indirect by the action of glutamate released from astrocytes in response to the SDF-1 α and ATP. We further tested this hypothesis by the employment of the specific NMDA antagonist D-AP5. D-AP5 (50 μ M) abolished the SDF-1 α -induced calcium elevations in 33% of neurons (n=11 of 33), significantly lowered their responses to the SDF-1 α in 36 % (p<0.05, n=12 of 33) and had no effect on 31 % of neuronal population in mixed neuron-glia cultures (p>0.05, n=10 of 33; Fig. 6C). Taken together these results suggest that glutamate released from astrocytes by SDF-1 α modulates neurons excitability through the NMDA receptor activation.

Fig. 7 SDF-1 α and gp120 augment cannabinoid-induced calcium transients in primary astrocyte cultures. (A) SDF-1 α (50nM, 1 min) augmented the stimulatory effect of 5 μ M THC on calcium transients in astrocytes. (B) gp120 (2nM, 3 min) and WIN (5 μ M, 10 min) application initiated a robust increase in intracellular calcium as compared to the effect of their solitary applications. The sustained calcium increase in cultures exposed to the combination of SDF-1 α or gp120 and cannabinoids was accompanied by the loss of regions of interest toward the end of recording, suggesting damage in cell`s membranes and loss of Fura-2 dye.



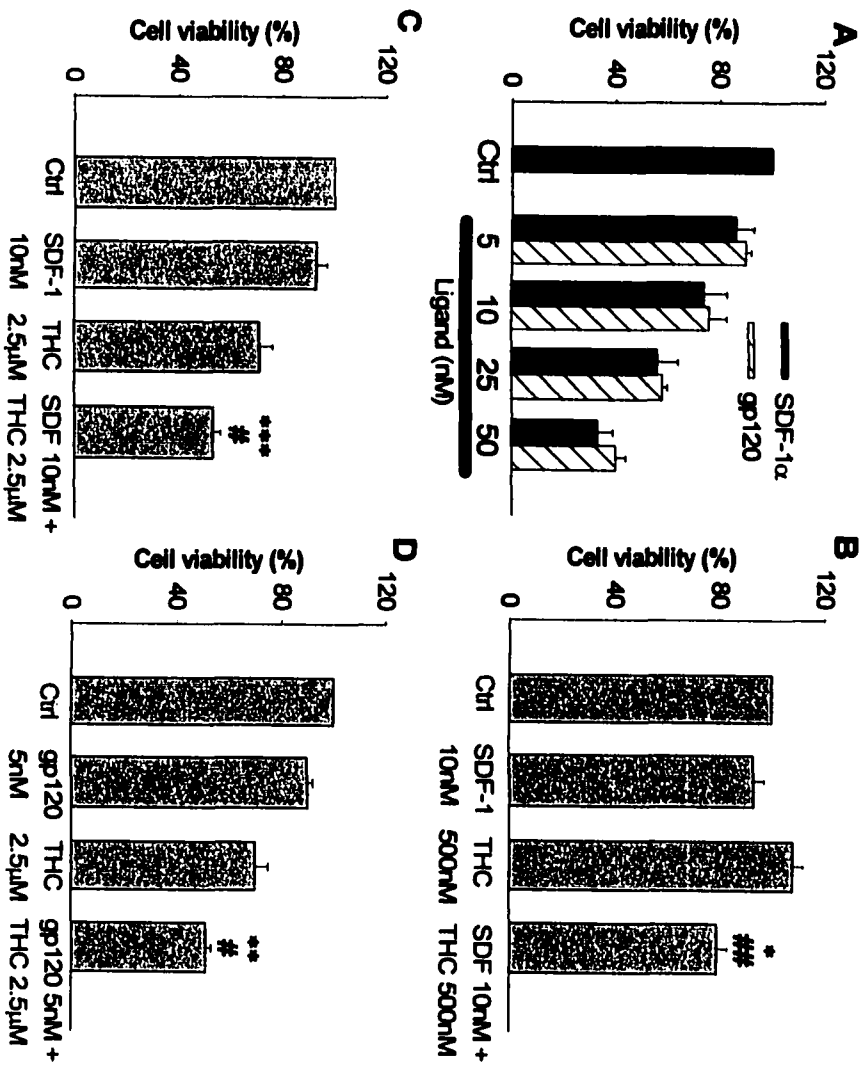
SDF-1 α and gp120 enhance cannabinoid-elicited calcium transients

Because the cannabinoid receptor CB₁ and the chemokine receptor CXCR4 are coupled to the same pertussis toxin sensitive G $\alpha_{i/o}$ protein, both may activate the same signaling events in cells. We tested ability of SDF-1 α , gp120 and cannabinoids to provoke synergetic action in astrocytes by the activation of their cognate receptors, CXCR4 and CB₁ (Fig. 7). Application of SDF-1 α and THC augmented the kinetics of THC-induced calcium increase in cultured astrocytes (Fig. 7A). In a similar fashion gp120 augmented the stimulatory effect of another CB₁ receptor agonist, Win 55,212-2 (WIN), on changes of intracellular calcium in cultured astrocytes (Fig. 7B). Recovery from the cannabinoid insult was observed in cultures treated only with WIN but not in those exposed to the combination of gp120 and WIN in which the calcium concentration remained elevated throughout the experiment (Fig. 7B). This sustained calcium elevation, initiated by the WIN application in cultures pre-treated with gp120, was accompanied with the disappearance of astrocytes from the visual field at the end of the experiment (data not shown).

SDF-1 α and gp120 induce apoptosis of astrocytes and exacerbate cannabinoid toxicity

An observed irreversible increase in the intracellular calcium and subsequent astrocyte's disappearance from the imaging field elicited by the SDF-1 α or gp120 in the presence of cannabinoids point to their toxicity (Fig. 7A and B). To determine the possible toxic effect of the SDF-1 α and gp120 on astrocytes we performed MTT assay as a measure of cell

Fig. 8 SDF-1 α and gp120 are toxic to cultured astrocytes. **(A)** Incubation of astrocytes for 24 hours with SDF-1 α or gp120 induced a dose-dependent (5-50 nM) decrease in cell viability as revealed by MTT assay. **(B and C)** SDF-1 α augmented cannabinoid-induced toxicity in primary astrocyte cultures. Incubation of astrocytes with SDF-1 α (10 nM, 12h) and THC (500nM, 12h) enhanced cannabinoid toxicity by decreasing the THC threshold concentration from 1 μ M to 500 nM (^{##} p<0.01 compared to THC treatment alone, ^{*} p<0.05 compared to SDF-1 α treatment alone, n=4; Fig. 8B). SDF-1 α (10nM, 12h) potentiates cannabinoid-induced cell death evoked by 2.5 μ M THC (12h) in cultured astrocytes ([#] p<0.05 compared to THC treatment alone, ^{***} p<0.001 compared to SDF-1 α treatment alone, n=4; Fig. 8C). **(D)** gp120 (5nM, 12h) potentiates the THC-induced astrotoxicity in primary astrocyte cultures ([#] p<0.05 compared to THC treatment alone, ^{**} p<0.01 compared to gp120 treatment alone, n=4; Fig. 8D).

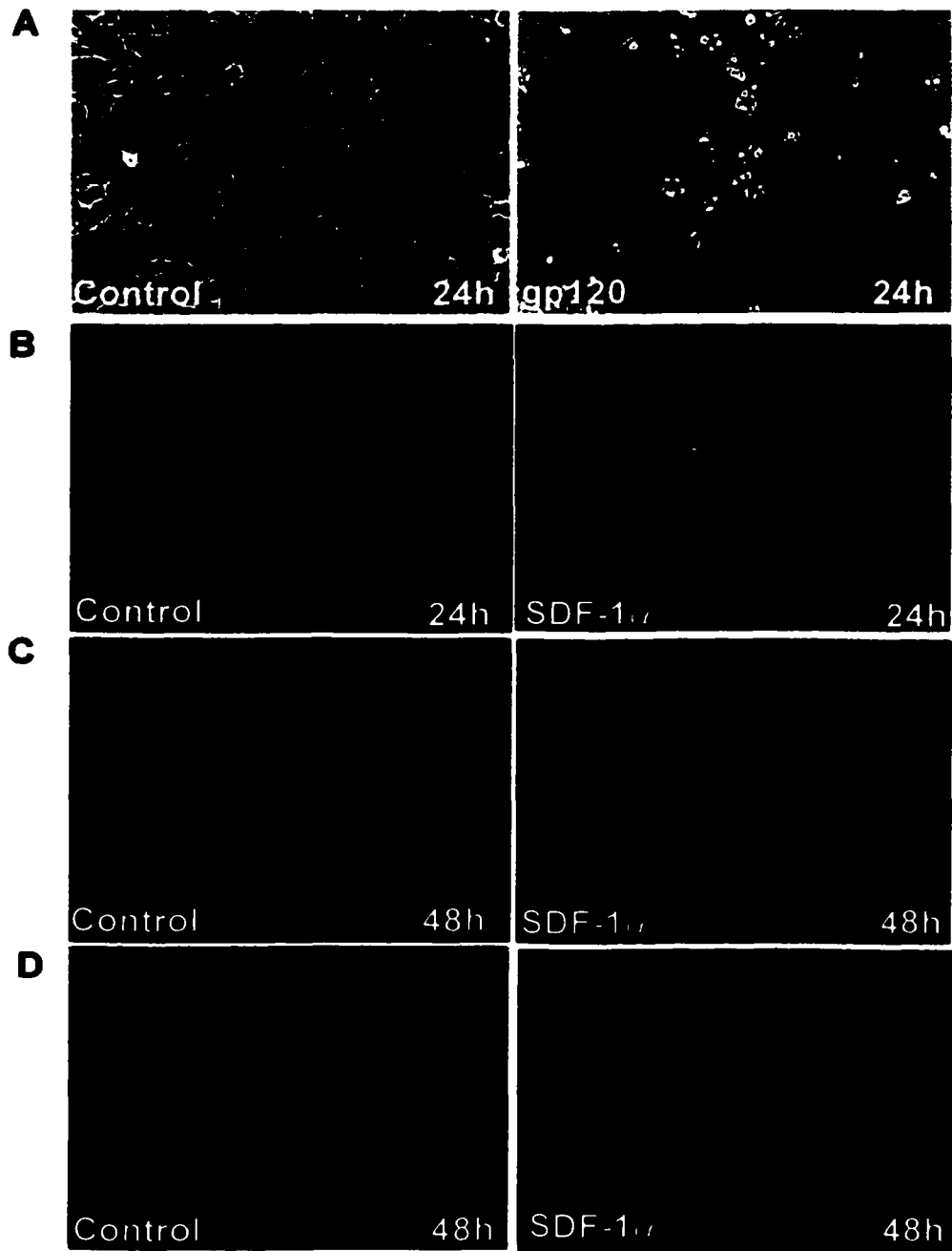


viability (Hansen et al., 1989). SDF-1 α and gp120 induced a dose-dependent decrease in cell viability during 24-hour exposure, with the initial toxicity detected at 5nM (n=6, Fig. 8A). THC, a psychoactive component of marijuana, was toxic for cultured astrocytes as well (n=4, Fig. 8C). Similarly, THC-induced death of hippocampal neurons has been recently documented (Chan et al., 1998). SDF-1 α and gp120 were found to contribute to the cannabinoid toxicity in astrocytes by decreasing the toxic threshold concentration of THC from 1 μ M to 500nM (Fig. 8B) and by increasing the potency of 2.5 μ M THC (p<0.05, n=4; Fig. 8C). In similar fashion, gp120 potentate cannabinoid toxicity (Fig. 8D). Co-application of gp120 (5nM) and THC (2.5 μ M) induced a significant decrease in astrocytes viability as compared to the effect of solitary applications (p<0.05, n=4; Fig.8D).

To discriminate between the types of cell death induced by SDF-1 α and gp120 in astrocytes, fluorescent probes were used. Treatment of primary astrocyte cultures with gp120 (20nM, 24h) led to disintegration of astrocyte cultures and characteristic apoptotic body formation (Fig. 9A). AnnexinV-FITC conjugate is a phospholipid binding protein with high affinity for phosphatidylserine (PS). Externalization of PS is one of the earliest indicators of apoptosis. We observed a significant increase in the fluorescence intensity in cells treated with the SDF-1 α (50nM, 24 h) as compared to control cells (p<0.01, n=5; Fig. 9B), indicating apoptosis of astrocytes.

For the assessment of possible necrotic events in astrocytes, cells were loaded with fluorescent dyes acridine orange (AO) and propidium iodide (PI). Acridine orange intercalates into double-stranded (intact) DNA and emit green fluorescence, whereas

Fig. 9 SDF-1 α and gp120 induce apoptotic / necrotic cell death in the primary astrocyte cultures. **(A)** gp120 (20 nM, 24h) application induced a disintegration of astrocyte cultures and characteristic apoptotic body formation as revealed by phase contrast microscopy. Scale bar, 100 μ m. **(B)** Fluorescent images of AnnexinV-FITC labeled cells, as early marker of apoptotic events in astrocytes elicited by SDF-1 α . Exposure of primary astrocyte cultures to 50 nM SDF-1 α for 24 hours resulted in a 93 % increase in fluorescent intensity from control values. **(C and D)** Nuclear morphology of astrocytes stained with the fluorescent dyes, a cell-permanent DNA dye acridine orange (Fig. 9C) and cell-nonpermanent DNA dye propidium iodide that stains only necrotic cell with red fluorescence (Fig. 9D). SDF-1 α (50nM, 48h) induced a marked decrease in the number of AO-stained fluorescent cells due to the SDF-1 α toxicity (Fig. 9C). An increase in a number of PI positive cells in the SDF-1 α treated primary astrocyte cultures illustrates that astrocytes entered a late (48 h) apoptotic / necrotic stage of chemokine-induced astrotoxicity (Fig. 9D).



bound to single-stranded DNA or RNA, it fluoresces red (Darzynkiewicz et al., 1992). Astrocyte cultures exposed to SDF-1 α (50nM, 48 hours) and stained with acridine orange afterwards exhibited a 67 % decrease in number of fluorescent cells compared to the control due to the SDF-1 α toxicity (Fig. 9C). In a cultures bathed with the SDF-1 α (50nM, 48 hours) and stained with the necrotic cell dye propidium iodide we observed a 56 % increase in number of PI positive cells compared to control cultures, suggesting a late (48 h) apoptotic / necrotic stage of the α -chemokine-induced astrotoxicity (Fig. 9D).

Discussion

Our data demonstrate that activation of the CXCR4 / G α_i signaling cascade in astrocytes by the SDF-1 α or gp120 induces calcium mobilization from internal calcium stores and releases glutamate. SDF-1 α stimulates calcium transients in astrocytes within seconds of addition to cultures. Similarly, SDF-1 α releases glutamate from cultured astrocytes within the 1st minute of agonist delivery to the perfusion chamber, an effect that temporally coincides with the SDF-1 α -induced calcium elevation, raising the possibility that it is required for the glutamate release. Involvement of intracellular calcium stores in the mechanism of the SDF-1 α -induced glutamate release from astrocytes is further supported by the fact that BAPTA-AM, an intracellular calcium chelator, and thapsigargin, an inhibitor of Ca²⁺-ATP-ase of internal stores, abolished the SDF-1 α -elicited calcium transients and glutamate release from astrocytes. These data demonstrate a capability of astrocytes to respond to the elevation of physiological calcium levels with glutamate

release, which is both necessary and sufficient to stimulate glutamate release from these cells (Jefinija et al., 1996).

U73212, a specific PLC inhibitor, blocked the SDF-1 α stimulatory effect implying an involvement of a receptor-coupled PLC-dependent process. Activation of PLC is often associated with the activation of G_q-coupled receptors. However, recent investigations point to an alternative-signaling pathway. It is established that the $\beta\gamma$ subunits of pertussis toxin-sensitive G_{i/o} can activate PLC (Exton, 1996), providing a signaling pathway by which the SDF-1 α may influence the activity of an enzyme. Cells of the CNS express two types of internal Ca²⁺ stores, one gated by IP₃ receptors and the others gated by ryanodine receptors. The role of the IP₃-sensitive calcium stores in the SDF-1 α -induced calcium mobilization and glutamate release from astrocytes was supported by the fact that the SDF-1 α stimulatory effect was abolished in the presence of 2APB, a selective IP₃ receptor antagonist but not in the presence of dantrolene, a ryanodine receptor antagonist. Thus SDF-1 α stimulates calcium mobilization from the IP₃-sensitive internal calcium stores and releases glutamate through the CXCR4 / G_{i $\beta\gamma$} / PLC _{β} / IP₃ signaling cascade activation.

Alternatively, glutamate can be released from astrocytes by the inhibition of uptake or reversal of the normal glutamate uptake mechanism (Szatkowski et al., 1990). This calcium-independent mechanism of glutamate release, was not implicated in the chemokine-induced EAA release from astrocytes because SDF-1 α required calcium to stimulate glutamate release, omission of Na⁺ from the perfusion solution did not alter the ability of SDF-1 α to stimulate EAA liberation from astrocytes, and the glutamate

transporter inhibitor, PDC was without effect on the SDF-1 α stimulatory action in astrocytes.

Recent studies have shown that activation of the PLC-IP₃ signaling pathway in astrocytes stimulates excitatory amino acid release from these cells via a calcium-dependent, anion-transport sensitive mechanism (Jeftinija et al., 1996; Jeremic et al., 2001). In this study, we show that this mechanism is selective because the anion co-transporter inhibitors NPPB (only in higher concentrations) and furosemide blocked the neuroligand-induced glutamate release from astrocytes but not the depolarization-induced glutamate release from neurons in both *in vitro* and *in situ* studies. Furthermore, anion transporter inhibitors blocked the neuroligand induced calcium release from intracellular stores in astrocytes with no effect on the depolarization-induced calcium influx in neurons. In conclusion, the anion co-transporter inhibitors blocked the release of glutamate from astrocytes by a calcium sensitive mechanism that is required for the SDF-1 α and ATP-induced glutamate release from astrocytes (Jeremic et al., 2001), further postulating a central role for calcium in the mechanism of EAA release from astrocytes.

The mechanism of NPPB and furosemide selectivity remains to be elucidated, nevertheless they were shown to be helpful pharmacological tools for the assessment of the astrocytic glutamate contribution in the SDF-1 α -induced calcium transients in neurons. Co-culturing neurons with glia increased the population of neurons that responded to SDF-1 α stimulatory effects by approximately 8-fold. An increase in neurons' responsiveness to SDF-1 α might be glia influence, since we have demonstrated that elevation of intracellular calcium in astrocytes is sufficient to induce the release of glutamate leading to an NMDA-

mediated increase in neuronal calcium (Parpura et al., 1994; Jeremic et al., 2001). In the mixed neuron-glia cultures NPPB (100 μ M) abolished or significantly decreased the SDF-1 α -induced calcium transients in more than 90% of glia and in 50% of neurons without affecting the depolarization-induced Ca²⁺ influx in neurons, supporting the hypothesis of astrocytic glutamate contribution to the α -chemokine stimulatory action in neurons. Glutamate involvement is further supported by the fact that the selective NMDA antagonist, D-AP5, blocked or significantly decreased the SDF-1 α -induced calcium transient in more than 70% of neurons without altering the astrocytes response to the SDF-1 α . These results agree with the recently performed *in situ* study which documents that SDF-1 α modulates neuronal electrical properties and synaptic transmission in rat cerebellum by a glutamate-sensitive mechanism (Limatola et al., 2000). Alternatively, a different CXCR4-receptor expression pattern might occur in neurons that are cultured alone versus neurons that are cultured in the presence of other brain cells. This might increase neuron susceptibility to the SDF-1 α . A direct activation of calcium transients in cultured neurons by SDF-1 α has been documented (Bajetto et al., 1999; Zheng et al., 1999).

In addition to the transient effect of SDF-1 α on the astrocytes' and neurons' excitability that may have possible physiological implications, prolonged applications of SDF-1 α and gp120 were toxic for astrocytes. This may have pathophysiological implications. Astrocytosis as well as neuronal and astrocyte apoptosis are prominent features of ADC (Ade-Biassette et al., 1995; Petito and Roberts, 1995), however, exact mechanism is still unknown. A toxicity assay confirmed the cytotoxicity of SDF-1 α and gp120 in the primary astrocyte cultures. Specifically, we observed that SDF-1 α and gp120

induce apoptosis in the cultured astrocytes in a 24 h period, whereas longer exposures (48h or latter) of cells to the SDF-1 α induce necrosis. Furthermore, we observed a synergy between cannabinoids and chemokines on the calcium transients and cells viability in the astrocytes. SDF-1 α or gp120 in combination with THC or WIN enhanced the kinetics of calcium transients elicited by cannabinoids and potentiated cannabinoid toxicity in the astrocytes. Coupling of two systems by the activation of two different receptors, CB₁ and CXCR4, whose signal transduction pathway converges at some point, might explain synergetic action shown by chemokines and cannabinoids. Because the CB₁ and CXCR4 receptor are coupled to the pertussis toxin-sensitive G_{i/o} protein that activates PLC, both receptors can initiate the same downstream signaling pathway. A similar hypothesis of the cannabinoid contribution to the HIV-mediated immune failure has been recently put forward (Gurwitz and Kloog, 1998).

In summary, our study revealed a significant contribution of the chemokine system in the modulation of neural cells' excitability that may have profound physiological implications. We argue that in addition to direct neuronal effect(s), SDF-1 α may induce calcium transients in neurons indirectly, through the NMDA receptor activation by the astrocytic glutamate, adding another member to the pool of neuroligands that exert a neuromodulatory role in the brain. In addition, we observed a synergy between cannabinoids, SDF-1 α and gp120 on the calcium transients and astrocytes' viability that may introduce neuron injury by the disturbances of glia function and their ability to support neurons. Alternatively, glia and chemokines / gp120 may induce neuronal injury through the release of glutamate and documented excitotoxicity (Lipton et al., 1991). It is likely that

both, direct and indirect pathways in conjunction could contribute to neuronal death.

Finally, we believe that cannabinoids, which are used to alleviate the nausea associated with HIV wasting, might influence the progression and severity of neural cells' injury induced by SDF-1 α and / or gp120 and in that way influence the course and presentation of AIDS dementia complex in these patients. Future analyses are needed to confirm this hypothesis, but in light of this evidence, the CXCR4-activated signaling pathway in astrocytes remains a plausible candidate for the HIV-1-induced neurodegeneration.

Acknowledgments

National Science Foundation Grant IBN 9604862 supported this work.

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CHAPTER 5. GENERAL CONCLUSIONS

This dissertation presents the results of investigations into the effects of neuroligands ATP, SDF-1 α and cannabinoids on astrocytes and the effect on astrocyte-neuron interaction. Understanding of the role of astrocytes in CNS function has been increasing in recent years. This research contributes to this body of knowledge by showing that endogenous neuroligands (ATP, SDF-1 α) and exogenous neuroligands (cannabinoids) affect astrocytes directly and neurons indirectly. These effects on astrocytes were stimulatory and toxic, strongly suggesting a significant role for astrocytes in the brain physiology and pathology.

General Discussion

Neuroligands elicit calcium transients in astrocytes primarily from internal stores

The Ca²⁺-based excitability of cultured astrocytes appears to be based on the regulated release of Ca²⁺ ions from intracellular stores (Cornell-Bell and Finkbeiner, 1991; Jeftinija et al., 1996; Jeremic et al., 2001). This view was confirmed since the ATP- and SDF-1 α -stimulatory effect in cultured astrocytes was blocked by BAPTA-AM, a cell-permeable calcium chelator and by thapsigargin, an inhibitor of the Ca²⁺-ATP-ase of internal stores (Thastrup et al., 1990). Both agonists were found to activate phospholipase C (PLC) since U73122, a selective inhibitor of receptor-coupled PLC-dependent processes

(Smith et al., 1990), blocked the response of astrocytes to ATP and SDF-1 α . Activation of PLC is often associated with activation of G_q-coupled receptors, a P2Y receptor for example. Alternatively, the $\beta\gamma$ dimer of the pertussis toxin-sensitive G_{i/o} can activate PLC (Exton et al., 1996), providing alternative signaling pathway by which SDF-1 α might influence the activity of the enzyme. Experiments with pertussis toxin documented that ATP and SDF-1 α stimulate PLC-mediated Ca²⁺ transients in astrocytes via G_q and G_{i/o} protein, respectively.

PLC activation leads to the generation of diacylglycerols (DAG) and inositol-3-phosphates (IP₃), which in turn activate and liberate calcium from IP₃-sensitive internal calcium stores. Cells of the CNS express two types of internal Ca²⁺ stores, one gated by IP₃ receptors and the other gated by ryanodine receptors (RyR). The scenario of IP₃-mediated calcium release was confirmed because 2APB, a selective IP₃ receptor antagonist, abolished SDF-1 α -induced Ca²⁺ transients in astrocytes and erased the metabotropic component of the ATP response, leaving only the ionotropic component. In contrast, dantrolene, a selective RyR antagonist, had no significant effect on the ATP and SDF-1 α stimulatory effects in astrocytes. Furthermore caffeine, a pharmacological agonist for RyR, failed to stimulate astrocytes (Jeftinija et al., 1996; Jeremic et al., 2001). Taken together, these results suggest that neuroligands predominantly mobilize Ca²⁺ in astrocytes from the ryanodine / caffeine-insensitive internal calcium stores.

Mechanism of the neuroligand-induced EAAs release from astrocytes

A major issue that is addressed in this dissertation is the mechanism responsible for the neuroligand-induced EAAs liberation from astrocytes. According to the literature, three different mechanisms control the EAAs release from astrocytes.

First, glutamate can be released from astrocytes by the reversal of the Na^+ -dependent (Kanner and Sharon, 1978) glutamate transporter that is stimulated with a depolarizing agent (Szatkowski et al., 1990). This probably does not account for the neuroligand-induced glutamate release from astrocytes because ATP and SDF-1 α require Ca^{2+} to stimulate EAAs release, glutamate transport inhibitors had no significant effect on the ligand-induced EAAs release and omitting Na^+ from the perfusion solution did not alter astrocytes' response to neuroligands. Furthermore, a brief application of strong depolarizing stimuli such as 50mM K^+ did not provoke any significant change in the basal astrocytic glutamate levels.

Second, a cell swelling-dependent release of EAAs from cortical astrocytes (Kimelberg et al., 1990) can account for the neuroligand-induced glutamate release. Furosemide, an anion transport blocker that reduces cell-swelling-induced EAAs release from astrocytes (Kimelberg et al., 1990), also reduces ATP- and SDF-1 α -stimulated glutamate release. Similarly NPPB, a potent Cl^- channel blocker at lower concentrations and $\text{Cl}^-/\text{HCO}_3^-$ cotransport inhibitor at higher concentrations (Wengemann et al., 1986; Petersen and Cormann, 1987), blocked the ATP- and SDF-1 α -induced glutamate release only when anion transport-blocking concentrations were used. This raises the possibility that ATP and SDF-1 α induce swelling of astrocytes, which in turn stimulates glutamate

release. However, the following facts hamper this hypothesis. Neuroligands, bradykinin and prostaglandin PGE₂ that stimulate glutamate release from glia by the same mechanism as ATP and SDF-1 α do not cause glia swelling (Parpura et al., 1995a; Bezzi et al., 1998). Furthermore, a review of the literature showed no single evidence that ATP and SDF-1 α evoke swelling in astrocytes or any other cell. On the contrary, ATP is involved in an autocrine process of regulatory volume decrease (RVD) by which cells can readjust their volume after transient swelling. Studies have shown that osmotic swelling of hepatocytes or epithelial cells stimulates release of intracellular ATP, which in turn stimulates P₂ receptors and activates Cl⁻ channels in a process essential for cell volume recovery (Okada et al., 2001; Roe et al., 2001). Finally, the research in this dissertation revealed that anion co-transporter inhibitors blocked the release of glutamate from astrocytes by a calcium sensitive mechanism that is required for the SDF-1 α and ATP-induced glutamate release (Jeremic et al., 2001), further supporting the central role for Ca²⁺ in the mechanism of EAA release from astrocytes.

The third potential mechanism for the neuroligand-induced glutamate release is a Ca²⁺-dependent exocytosis (Attwell 1994; Parpura et al., 1994). It is demonstrated that Ca²⁺ is both necessary and sufficient to stimulate the release of EAAs from astrocytes (Jeftinija et al., 1996). This dissertation supports the Ca²⁺-hypothesis of release because neuroligands stimulated glutamate release from astrocytes only when [Ca²⁺]_i was elevated. Furthermore, depletion of intracellular Ca²⁺ stores by thapsigargin or when Ca²⁺ mobilization was blocked by BAPTA-AM, neuroligands no longer stimulated the EAA release from astrocytes. Ca²⁺ imaging experiments revealed that there are certain [Ca²⁺]_i changes that do

not result in glutamate release from astrocytes suggesting that a certain $[Ca^{2+}]_i$ threshold should be reached for glutamate release. This agrees with recent study showing that Ca^{2+} -regulated release of glutamate from astrocytes is engaged at physiological Ca^{2+} levels (Parpura and Haydon, 2000). The nature of the Ca^{2+} -dependent glutamate release from astrocytes remains to be defined. Is this Ca^{2+} -dependent glutamate release from astrocytes vesicular? If so, is it similar to the neuronal vesicular release of transmitters?

The Ca^{2+} requirement for neuroligand-induced glutamate release from astrocytes supports exocytosis and existence of synaptic machinery in astrocytes supports vesicular transmitter release. The vesicular hypothesis of glutamate release from astrocytes gained respect from recent findings. SNARE proteins, syntaxin, synaptobrevin II and SNAP-23 (a homolog of SNAP-25) that are essential for vesicle fusion, are present in astrocytes (Parpura et al., 1995b; Jęftinija et al., 1997; Hepp et al., 1999). Clostridial toxins, which cleave SNARE proteins, blocked the Ca^{2+} -dependent glutamate release from astrocytes (Jęftinija et al., 1997; Bezzi et al., 1998; Araque et al., 2000). The potent secretagogue α -latrotoxin, which induces the exocytosis of vesicles at nerve terminals, also stimulates glutamate release from astrocytes (Parpura et al., 1995c). Bafilomycin A1, a toxin that inhibits the electrochemical proton gradient maintained by V-ATPase that is required for the vesicular uptake of neurotransmitters, greatly reduced glutamate release from astrocytes (Araque et al., 2000, Bezzi et al., 2001). Finally, electron microscopic analysis revealed the presence of dense-core and clear vesicles in cultured astrocytes which membranes are associated with synaptobrevin II and synaptophysin (Maienschein et al., 1999). These findings point to an existence of the vesicular apparatus that could control transmitter release from astrocytes in a process that is similar to the one in neurons.

Neuroligands ATP and SDF-1 α modulate excitability of neurons by glial glutamate

Astrocytes outnumber neurons in many brain regions and they are interconnected via gap junctions to form extensive networks. In light of evidence for neuron-glia interaction, it is possible that the degree of coupling between astrocytes could set a tone for the neuronal activity (Giaume and McCarthy, 1996). Ca^{2+} released from intracellular stores may initiate a wave of elevated cytosolic Ca^{2+} that propagates both within individual astrocytes and between astrocytes (Cornell-Bell et al., 1990; Dani et al., 1992). This Ca^{2+} wave, as it propagates through the network of astrocytes, interacts with adjacent neurons which in turn respond with increased neural Ca^{2+} (Parri et al., 2001).

It is known that ATP serves as an extracellular signal that coordinates the intracellular propagation of the Ca^{2+} wave (Guthrie et al., 1999), thus contributing to long-range Ca^{2+} signaling. This dissertation postulates a new role for ATP in the process of neurotransmission. The Ca^{2+} imaging experiments in mixed neuron-glia cultures provided evidence for the role of ATP in short-range Ca^{2+} signaling because the depolarization-induced release of ATP from neurons stimulated adjacent astrocytes. In addition, ATP was found to indirectly modulate neuronal excitability by the glial glutamate that induced the NMDA-receptor-mediated Ca^{2+} mobilization in the adjacent neurons, forming a putative bi-directional signaling loop. A positive feedback loop between the release of glutamate and ATP by astrocytes is also implied because Queiroz et al. (1997) have shown that ATP is released from astrocytes by glutamate receptor activation. Therefore, it is feasible to propose that ATP propagate signals in the brain both in an autocrine and in a paracrine fashion.

In a similar manner, SDF-1 α , a α -chemokine agonist, activated glia to release glutamate that stimulated neighboring neurons. This agrees with a recently performed *in situ* study which documents that SDF-1 α modulates neuronal electrical properties and synaptic function in rat cerebellum by a glutamate-sensitive mechanism (Limatola et al., 2000).

Astrocytes contribute to the neurotoxicity of THC and gp120

Damage to the CNS leads to cellular changes not only in the affected neurons but also in adjacent glial cells. Astrocytes respond to the insult rapidly with formation of glial scars (McGraw et al., 2001) or apoptosis (Raghupathi et al., 2000). Recently, brains of patients with AIDS have been shown to manifest neuronal injury and apoptotic-like cell death (Adle-Biassette et al., 1995; Petit and Roberts, 1995). The toxicity assay in this dissertation has documented death of astrocytes induced by THC, a psychoactive component of marijuana, and gp120, an HIV-1 envelope glycoprotein, which postulates their contribution to the brain injury elicited by drugs of abuse and HIV-1.

Both ligands induced apoptosis in the astrocytes in a time- and a dose-dependent manner, in the concentrations comparable to those found in the plasma of marijuana abusers and AIDS patients (Chiang and Barnett, 1984; Lipton, 1998). Distinct death-signaling pathways emerged for the toxicity of THC in the neurons and the astrocytes. Activation of the PLA2-COX system induced apoptosis in the cultured hippocampal neurons (Chan et al., 1998), whereas two proliferate systems, PKC and ERK, mediated cannabinoid-induced astrotoxicity. It is generally accepted that the activation of the ERK

cascade leads to cell proliferation (Derkinderen et al., 1999). However, recent investigations documented situations when ERK mediates cell cycle arrest (Pumiglia and Decker, 1997) and cell death (Murray et al., 1998). These studies define situation in which cell fate is controlled by the duration of the stimulus, rather than the stimulus *per se*. This view is supported by the recent study showing that prolonged activation of ERK cascade in C6 glioma cells by cannabinoids induces cell death (Galve-Roperh et al., 2000).

Mounting evidence suggests that cognitive dysfunction developing as a result of HIV-1 infection is mediated at least in part by generation of excitotoxins and free radicals in the brain. The mechanism involves excessive activation of the NMDA receptor-operated channels, with resultant excessive influx of Ca^{2+} and the generation of free radicals, leading to neuronal damage (reviewed by Lipton, 1998). Lipton et al. (1991) first documented that gp120 neurotoxicity was mediated by the NMDA-receptor activation. In this study, the CXCR4 receptor agonists, SDF-1 α and gp120 IIIB, were found to stimulate glutamate release from astrocytes providing the possible source for the HIV-1-induced excitotoxins formation in the brain. Furthermore, gp120 and SDF-1 α induced apoptosis in astrocytes may introduce neuron injury indirectly by the removal of the well-recognized supportive role of astrocytes. It is likely that both, direct and indirect pathways in conjunction could contribute to the SDF-1 α - and gp120-induced neuronal death.

The SDF-1 α - and gp120-induced astrocytes' death was exacerbated by cannabinoids in a synergy that may occur between gp120 and THC from marijuana, which is used in the clinical treatment of nausea associated with AIDS wasting. The exacerbation of gp120 and SDF-1 α astrotoxicity by cannabinoids may have a significant implication on

the course and presentation of dementia in the AIDS patients. A similar hypothesis of a cannabinoid contribution to the HIV-1-mediated immune failure has been recently put forward (Gurwitz and Kloog, 1998). Also, endocrine modulation of neurotoxicity of the gp120 has been recently reported (Brooke et al., 1997). Taken together these findings suggest that the simultaneous activation of putative neurochemical systems in the brain eventually causes neural damage and apoptosis that may affect the progression and severity of some CNS diseases.

Summary

ATP stimulates cultured type I astrocytes

The purpose of this study was to provide a mechanism that is responsible for the ATP-induced EAA release from astrocytes. Furthermore, we tested ATP as a signaling molecule in the process of neuron to glia communication. Application of 100 μ M ATP for two minutes evoked a two-fold increase in concentration of aspartate and four-fold increase of glutamate above base line. The EAAs release from astrocytes was preserved in low calcium saline suggesting involvement of intracellular calcium stores. Ca^{2+} requirement for the EAAs release from astrocytes is further supported by the fact that BAPTA-AM, a cell permeable intracellular chelator, and thapsigarin, an inhibitor of Ca^{2+} -ATP-ase of internal calcium stores, abolished the stimulatory effect of ATP on the cultured astrocytes. In agreement with release experiments, ATP mobilizes calcium in cultured type I astrocytes as revealed by a Ca^{2+} imaging technique. The stimulatory effect of ATP on calcium transients

and glutamate release in astrocytes is P2 receptor-mediated because suramin, a selective P2 receptor antagonist, blocked the response of cells to ATP.

Reversal of glutamate transporters does not account for the release of EAAs because omitting Na^+ from the perfusion solution or using glutamate transport inhibitors had no significant effect on the ATP-induced glutamate release. However, the ATP-induced glutamate release from astrocytes was sensitive to anion transport inhibitors, furosemide and NPPB, which raises the question of cell swelling-dependent EAA release from astrocytes.

Finally, a role for ATP as a mediator of neuron to glia signaling has been postulated. Using mixed neuron-glia cultures we observed that depolarization of neurons with 50mM K^+ directly stimulated a rise in the $[\text{Ca}^{2+}]_i$ in neurons and indirectly in adjacent glia by the ATP that is released from neurons. A putative, bi-directional link in neuron to glia communication was revealed by the fact that ATP elevates neuronal Ca^{2+} indirectly by the action of glutamate, which is released from astrocytes in response to ATP.

Cannabinoids and astrocytes

The objective of the second study was to evaluate any stimulatory effect of marijuana on brain cells. It was established that THC, the main psychoactive component of marijuana, and WIN 55,212-1 (WIN), a synthetic cannabinoid receptor ligand, potently stimulated astrocytes and induced injury in the same cells. Both cannabinoid ligands stimulated the CB_1 receptor-mediated biphasic Ca^{2+} response in astrocytes. Experiments with low Ca^{2+} saline revealed involvement of two different Ca^{2+} pools in the cannabinoid stimulatory action: the observed rapid increase in $[\text{Ca}^{2+}]_i$ induced by cannabinoids

originated from the intracellular stores, while the irreversible Ca^{2+} increase resulted from the extracellular calcium entry. U73122, a selective phospholipase C (PLC) inhibitor, attenuated the stimulatory effect of WIN, suggesting the role for the receptor-coupled PLC dependent processes in the cannabinoid-induced Ca^{2+} transients in astrocytes.

Besides the stimulatory effect of cannabinoids on the Ca^{2+} transients in astrocytes, cannabinoids induced apoptosis in astrocytes in both *in vitro* and *in situ* studies. A toxicity assay documented an activation of multiple signal transduction pathways in the cannabinoid-induced astrotoxicity. Activation of a novel class of PKC's and ERK was crucial in the cannabinoid toxicity. Contributions of pro-apoptotic factors, like the ceramide and the free radicals, have been also documented. Finally, the toxicity assay revealed the ability of astrocytes to recover from the cannabinoid insult as the viable astrocytes repopulated areas in which the THC irreversibly damaged the cells. This astrocyte feature mimics *in vivo* ability of glia to rapidly respond to brain injury with activation (astrogliosis) and proliferation forming glial scars, a hallmark of many neurodegenerative changes in the brain.

SDF-1 α and gp120 activate and kill astrocytes through CXCR4 receptor

In this study, the ability of CXCR4 receptor ligands, SDF-1 α and gp120 IIB, to stimulate Ca^{2+} transients and release glutamate from cultured astrocytes was tested. Both agonists were found to increase $[\text{Ca}^{2+}]_i$ and to release glutamate from astrocytes in a dose-dependent manner. The stimulatory effect of SDF-1 α and gp120 on astrocytes was abolished with pertussis toxin pre-treatment, implying activation of the CXCR4-receptor /

$G_{i/o}$ protein signaling cascade. U73122, a PLC inhibitor and 2APB, an IP_3 receptor antagonist, blocked the stimulatory effect of SDF-1 α in cultured astrocytes suggesting that α -chemokines mobilize Ca^{2+} and release glutamate by the activation of the PLC- IP_3 signaling pathway.

The SDF-1 α stimulated astrocytes primarily, whereas neurons were much less sensitive to the SDF-1 α . However, in the presence of glia, the number of neurons responding to the SDF-1 α increased by approximately 8-fold. Experiments performed on mixed neuron-glia cultures revealed that glia and glutamate indirectly contributed to the stimulatory effect of SDF-1 α in neurons. NPPB, an anion co-transport inhibitor that selectively block the release of glutamate from astrocytes, and D-AP5, a specific NMDA antagonist, abolished or significantly decreased SDF-1 α stimulatory effect in 50 % and 70 % of neuronal population, respectively.

To explore the factors that contribute to the AIDS dementia complex syndrome, toxicity of the ligands was evaluated. Prolonged applications of either SDF-1 α or gp120 (12h or more) were toxic for astrocytes. Cells exhibited characteristic features of apoptosis, formation of apoptotic bodies and externalization of the apoptotic marker phosphatidylserine (PS) as revealed by an increase in binding of AnnexinV-FITC conjugate.

Finally, we observed a synergy of SDF-1 α , gp120 and cannabinoids on the Ca^{2+} transients and the astrocytes' viability. Co-application of SDF-1 α or gp120 and THC or WIN enhances the kinetics of Ca^{2+} calcium transients elicited by cannabinoids and

furthermore potentiates cannabinoid toxicity in astrocytes, which implicates a possible role for cannabinoids and chemokines in the etiology of ADC.

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ACKNOWLEDGMENTS

I would like to thank my major professor Dr. Srdija Jefinija for his help, guidance and support that I received from him throughout this research.

I would also like to thank the members of my committee: Dr. Lloyd Anderson, Dr. Walter Hsu, Dr. Anumantha Kanthasamy, Dr. Mary Helen Greer and Dr. Donald Sakaguchi, for the suggestions and helpful comments regarding this study. I would like to express gratitude to Ksenija Jefinija for her input and expertise in this research. I want to thank Aleksandra Glavaski-Joksimovic for the support in this study.

I gratefully acknowledge the following organizations for their financial supports: National Science Foundation Grant IBN 9604862, Graduate College and Department of Biomedical Sciences.