Glutamate-evoked release of ATP from cortical astroglial cells

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Ao meu pai

À minha mãe

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Summary

Excitatory amino acids are known to release adenyl compounds in the brain. Both the possibility that astroglial cells may release ATP in response to excitatory amino acids and the mechanism of that release were studied in astrocyte cultures derived from the brain hemispheres of newborn rats. ATP was measured with the luciferine-luciferase assay.

In primary cultures of astrocytes, glutamate receptor agonists did not increase the release of lactate dehydrogenase but elicited the release of ATP. There was a basal efflux of ATP, which was increased up to 19-fold by glutamate (100-1000 µM), N-methyl-D-aspartate (NMDA; 20-500 µM), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; 30-100 µM), kainate (20 µM) and (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (*trans*-ACPD; 100-1000 µM). The NMDA receptor-selective antagonist 2-amino-5-phosphonopentanoate (AP5, 100 µM) blocked the effect of NMDA but not the effects of AMPA, kainate and glutamate. The AMPA receptor-selective antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX; 30 µM) blocked the effect of AMPA and also of glutamate and NMDA but not the effect of kainate. The kainate receptor-selective antagonist γ-D-glutamyl-amino-methanesulfonate (GAMS; 30 µM) blocked the effect of kainate but not of glutamate.

The calcium ionophore ionomycin (5 µM) elicited release of ATP but only in the presence of external calcium. The release of ATP elicited by NMDA and kainate was abolished (or greatly reduced) by withdrawal of extracellular calcium as well as by cadmium (1 mM) and nicardipine (10 µM). In contrast, the release of ATP elicited by AMPA was not changed by these interventions; ATP release elicited by *trans*-ACPD was also not changed

by withdrawal of external calcium. α-Latrotoxin failed to elicit ATP release. Some putative blockers of ATP transporters or channels were tested on the ATP release elicited by glutamate receptor agonists. Only the two blockers of the cystic fibrosis transmembrane conductance regulator (CFTR), glibenclamide (100 µM) and diphenylamine-2-carboxylate (500 µM) changed the release of ATP. Both reduced the effect of AMPA without changing the effects of NMDA and kainate (only glibenclamide tested). Furthermore, lithium (1 mM) abolished the release of ATP evoked by glutamate and AMPA but only reduced the release elicited by NMDA and kainate.

The present study shows that cultured astroglial cells respond to activation of NMDA, AMPA, and kainate receptors with release of ATP. Two different mechanisms seem to be involved in the release of ATP from astroglial cells upon activation of ionotropic glutamate receptors: one involved in the NMDA- and kainate-induced release of ATP, the other in AMPA-induced release. The NMDA- and kainate-induced release of ATP requires an influx of calcium, is not due to neurone-like exocytosis, is not mediated by CFTR or a mechanism regulated by CFTR, and is reduced but not abolished by lithium. The AMPA-induced release does not require extracellular calcium may be mediated by CFTR or a mechanism regulated by CFTR, and is abolished by lithium.

By showing that astrocytes may release a neuroactive substance, namely ATP, with known modulatory and trophic actions in the central nervous system, this study supports the view that glial cells are intimately involved in the active control of neuronal activity and should be considered as integral modulatory elements of synapses.

Resumo

Vários estudos demonstraram que os aminoácidos excitatórios induzem a libertação de purinas (nomeadamente adenosina) no cérebro. Utilizando como modelo culturas primárias de astrócitos obtidas de cérebro de rato recém-nascido, estudou-se a possibilidade das células gliais, em particular os astrócitos, poderem libertar ATP no cérebro em resposta é estimulação com aminoácidos excitatórios bem como o mecanismo de libertação envolvido. O ATP libertado foi quantificado pelo método da luciferina-luciferase.

Em culturas primárias de astrócitos, nenhum dos agonistas dos receptores do glutamato testados aumentou a libertação da enzima lactato desidrogenase, embora o efluxo basal de ATP tenha sido aumentado (até cerca de 19 vezes) pelo glutamato (100-1000 µM), N-metil-D-aspartato (NMDA; 20-500 µM), ácido α-amino-3-hidroxi-5-metilisoxazol-4-propiónico (AMPA; 30-100 µM), kainato (20 µM) e pelo ácido (1S,3R)-1-amino-ciclopentano-1,3-dicarboxilico (trans-ACPD; 100-1000 µM). O antagonista selectivo dos receptores do NMDA, o ácido 2-amino-5-fosfono pentanóico (AP5, 100 µM), bloqueou o efeito do NMDA mas não modificou os efeitos do AMPA, kainato e glutamato. O antagonista selectivo dos receptores do AMPA, 2,3-dihidroxi-6-nitro-7-sulfamoil-benzo(f)quinoxalina (NBQX; 30 µM) antagonizou o efeito do AMPA e também os efeitos do glutamato e NMDA, mas não modificou a libertação de ATP induzida pelo kainato. O antagonista selectivo dos receptores do kainato, γ-D-glutamil-amino-metanosulfonato (GAMS; 30 µM), bloqueou o efeito do kainato mas não modificou significativamente o efeito do glutamato.

A ionomicina (5 μM), um ionóforo do cálcio, induziu a libertação de ATP mas apenas na presença de cálcio no meio extracelular. A libertação de ATP induzida pelo NMDA e pelo kainato foi abolida ou significativamente reduzida com a omissão de cálcio do meio extracelular, na presença de cádmio (1 mM) ou na presença de nicardipina (10 μM). Pelo contrário, a libertação de ATP induzida pelo AMPA não sofreu qualquer alteração pelas intervenções referidas e a libertação de ATP induzida pelo *trans*-ACPD também não sofreu alteração quando o cálcio extracelular foi omitido do meio. A α-latrotoxina não teve qualquer efeito sobre a libertação de ATP. Alguns compostos com potencialidade de bloquear transportadores ou canais permeáveis ao ATP foram testados sobre a libertação de ATP induzida pelos agonistas dos receptores do glutamato. Dos compostos testados apenas dois bloqueadores do CFTR (cystic fibrosis transmembrane conductance regulator), a glibenclamida (100 μM) e o ácido difenilamino-2-carboxílico (500 μM), modificaram a libertação de ATP. Ambos os compostos reduziram o efeito do AMPA, mas não modificaram o efeito do NMDA e kainato (apenas a glibenclamida foi testada). O lítio (1 mM) aboliu a libertação de ATP induzida pelo glutamato e AMPA e reduziu significativamente a libertação de ATP induzida pelo NMDA e kainato.

Os resultados obtidos demonstram que a activação dos receptores do NMDA, AMPA, e kainato induz a libertação de ATP de astrócitos em cultura. Dois mecanismos distintos parecem estar envolvidos na libertação de ATP: um envolvido na libertação de ATP induzida pelo NMDA e kainato, e um outro mecanismo envolvido na libertação de ATP induzida pelo AMPA. A libertação de ATP induzida pelo NMDA e kainato requer o influxo de cálcio, não envolve um processo de exocitose semelhante ao que ocorre nos neurónios, não é mediada pelo CFTR ou por um mecanismo regulado pelo CFTR e é reduzida pelo lítio. A libertação de ATP induzida pelo AMPA não é dependente do cálcio extracelular, podendo ser mediada pelo CFTR ou por um mecanismo regulado pelo CFTR e é abolida pelo lítio.

A demonstração de que os astrócitos libertam um composto neuroactivo, o ATP, com conhecidas acções moduladoras e tróficas no sistema nervoso central, vem corroborar com a tese de que as células gliais estão intimamente envolvidas no controlo da actividade neuronal e devem ser consideradas como elementos integrantes das sinapses.

Résumé

Il est resté démontré, en divers études, que les aminoacides excitateurs induisent la libération de dérivés de purines (nommément l'adénosine) dans le cerbère. En utilisant comme modèle des cultures primaires d'astrocytes obtenues à partir de cerbère de rat nouveau-né, on a cherché à déterminer la possibilité des cellules gliales, en particulier des astrocytes, pouvoir libérer ATP dans le cerbère et son mécanisme de libération, en réponse à l'estimulation avec des aminoacides excitateurs. L'ATP libéré a été quantifié par la méthode de luciférine-luciférase.

Aucun des agonistes des récepteurs du glutamate testés, a augmenté la libération de l'enzyme lacticodéshydrogénase, mais l'eflux basal de ATP a été augmenté (jusqu'à 19 fois, moins au plus) par le glutamate (100-1000 µM), N-méthyle-D-aspartate (NMDA; 20-500 µM), α-amino-3-hydroxy-5-méthylisoxasole-4-propionate (AMPA; 30-100 µM), kainate (20 µM) et par l'acide (1S,3R)-1-amino-ciclopentane-1,3-dicarboxilique (trans-ACPD; 100-1000 µM). L'antagoniste sélectif des récepteurs du NMDA, l'acide 2-amino-5-fosfonopentanoïque (AP5;100 µM), a bloqué l'effet du NMDA, mais n'a eu aucune influence sur les effets du AMPA, kainate et glutamate. L'antagoniste sélectif des récepteurs du AMPA, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX; 30 µM) a bloqué l'effet du AMPA bien que les effets du glutamate et NMDA, mais il n'a eu aucun effet sur la libération de ATP induite par le kainate. L'antagoniste sélectif des récepteurs du kainate, acide γ-D-glutamyl-amino-méthanesulfonique (GAMS; 30 µM), a bloqué l'effet du kainate, mais il n'a pas modifié significativement l'effet du glutamate.

L'ionomycine (5 µM), un ionophore du calcium, a induit la libération de ATP seulement en présence de calcium au milieu extracellulaire. La libération de ATP induite par le NMDA et par le kainate a été anéantie ou significativement réduite dans l'absence de calcium extracellulaire, en présence de cadmium (1 mM) ou en présence de nicardipine (10 µM). Au contraire, la libération de ATP induite par le AMPA n'a subi aucune altération par les interventions déjà référées et la libération de ATP induite par le *trans*-ACPD n'a pas subi aussi d'altération quand le calcium extracellulaire a été omis du milieu. La α-latrotoxine n'a eu aucun effet sur la libération de ATP. Parmi les divers composés testés avec potentiel d'empêché transporteurs ou canaux perméables à le ATP, seulement deux empêcheurs du CFTR (cystic fibrosis transmembrane conductance regulator), la glibenclamide (100 µM) et l'acide diphenylamine-2-carboxílique (500 µM) ont modifié la libération de ATP. Tous les deux composés ont réduit l'effet du AMPA, mais n'ont pas modifié l'effet du NMDA et du kainate (la glibenclamide, c'est la seule qui a été testée). Le lithium (1 mM) a anéanti la libération de ATP induite par le glutamate et AMPA et a réduit la libération de ATP induite par le NMDA et kainate.

Les résultats obtenus démontrent que l'activation des récepteurs du NMDA, AMPA and kainate induit la libération de ATP de astrocytes en culture. Deux mécanismes différents semblent être engagés dans la libération de ATP: l'un engagé dans la libération de ATP induite par NMDA et kainate et, un autre mécanisme engagé dans la libération de ATP induite par le AMPA. La libération de ATP induite par NMDA et kainate a besoin d'un influx de calcium; n'engage pas un procès d'exocytose semblable à ce qui pourvoit dans les neurones, n'est pas moyennée par le CFTR ou par un mécanisme régulé par le CFTR et est réduite par le lítio. La libération de ATP induite par le AMPA n'a pas besoin du calcium extracellulaire pouvant être moyennée par le CFTR ou par un mécanisme régulé par le CFTR et elle est anéantie par le lítio.

La situation qui démontre que les astrocytes libèrent un composé neuroactif, le ATP, avec des actions modulatrices et trophiques dans le système nerveux central, vient confirmer la thèse qui défend que les cellules gliales son intimement engagés dans le contrôle de l'activité neurale et doivent être considérés comme des éléments intégrants des synapses.

Abbreviations

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionate
AP5	($\pm$)-2-amino-5-phosphonopentanoic acid
ATP	adenosine-5'-triphosphate
cAMP	3',5'-cyclic adenosine phosphate
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	3',5'-cyclic guanosine phosphate
CNS	central nervous system
DIDS	4,4'-diisothiocyanato-stilbene-2,2'-disulfonate
DPC	diphenylamine-2-carboxylate
GABA	γ-aminobutyric acid
GAMS	γ-D-glutamyl-amino-methanesulfonic acid
GFAP	glial fibrillary acidic protein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanosulfonic acid]
INT	iodonitrotetrazolium
IP_3	inositol trisphosphate
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAP2a,b	microtubule associated protein-2a,b
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline
NMDA	N-methyl-D-aspartate
NO	nitric oxide
trans-ACPD	(1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid
VDCC	voltage-dependent calcium channels

Glial Cells

The number of neurones in the central nervous system (CNS) is enormous but they are exceeded in number 5-10 fold by cells collectively called neuroglia or glial cells. These cells were described for the first time in 1846 by Rudolf Virchow, but only in the beginning of this century the major glial populations: astrocytes, oligodendrocytes, and microglial cells were identified.

Glial cells were defined for a long time as neurone supporting cells, representing the CNS version of connective tissue cells. Over the last 30 years, the information obtained from the study of glial cell physiology has completely changed this view. Nowadays, glial cells are considered more than merely mechanical supportive cells. They are known to have an active role in the CNS, assisting nerve cells in the performance of their integrative and communicative functions. They are able to respond to extracellular messengers and to release neuroactive mediators, the same mediators neurones use to communicate. The present work demonstrates that astrocytes, like neurones, are the target of neurotransmitters and the source of a neuroactive compound, namely ATP.

The astrocyte

Classification

Astrocytes are a heterogeneous population of cells and, based on anatomic and morphologic criteria, it is possible to distinguish two major populations: the fibrous and the protoplasmic astrocytes. Fibrous astrocytes are found mainly in the white matter of the CNS. They have long thin processes that branch infrequently and contain abundant intermediate filaments. Protoplasmic astrocytes are found mainly in the grey matter of the CNS. They have a stellate form with multiple highly branched processes, which contain fewer intermediate filaments than fibrous astrocytes, have abundant cytoplasm and a nucleus that is larger than the nucleus of any the other type of glial cells.

Another approach to identify and classify astrocytes has emerged from *in vitro* studies, namely astrocytes in culture (also called astroglia). It is based on astroglia morphology and on antigenic phenotypes, using immunocytochemical methods.

Glial filaments are known to contain glial fibrillary acidic glycoprotein (GFAP) and the detection of GFAP is used to identify these cells. Astroglia are, thus, GFAP positive. Based on the morphology of GFAP positive cells and on the use of antibodies directed against astroglia antigens it was possible to identify two subtypes of astroglial cells, named type 1 and type 2 astroglia (Raff et al., 1983a). The monoclonal antibody A2B5, which binds to polysialogangliosides, was the first immunological cell marker used to distinguish between these two subtypes of astroglia. Type 1 astroglia are polygonal, GFAP positive and A2B5 negative cells. Type 2 astroglia are process bearing, GFAP positive, and A2B5 positive cells. Type 1 and type 2 astroglia were originally proposed to be the cell culture subtypes equivalent to fibrous and protoplasmic astrocytes, respectively (Miller and Raff, 1984). In spite of this putative equivalence, most investigators still prefer to use the terms protoplasmic

and fibrous to classify astrocytes *in vivo* and type 1 and type 2 to classify astrocytes in culture (Miller et al., 1989).

Astrocyte physiology

Astrocytes have an important role in homeostasis in the CNS. They regulate the composition of extracellular fluid and synaptic transmission; they remove the excess of K^+ ions that accumulate in the extracellular space following neuronal activity and take up and inactivate neurotransmitters.

Astrocytes also play an important role in neuronal development and neuronal plasticity (Rakic, 1981; Müller and Best, 1989). In the developing nervous system, they provide scaffoldings for the inward migration of young neurones to their final position and, in the adult, they form an elaborate framework in which neurones are deployed.

The intimate relationship between astrocyte processes and blood vessels (astrocyte end-feet are in contact with capillaries and arterioles) suggests that they may play important roles in neuronal energy supply and reserve (astrocytes are the main glycogen storage cells in the CNS). They also participate in the formation of the blood-brain barrier and in the regulation of blood flow during regional increases of neuronal activity (Janzer and Raff, 1987; Paulson and Newman, 1987).

Astrocytes and astroglia are extensively coupled through gap junctions resulting in the formation of a cellular syncytium (Bennett et al., 1991). Communication between astrocytes through gap junctions is though to underlie their ability to redistribute the excess of extracellular K^+ ions that occurs following neuronal activity. It has been suggested that gap junctions connecting astrocytes are of primary importance for regulation of glial metabolism

and distribution of glucose (Tabernero et al., 1996); regulation of cell volume and proliferation (Kimelberg and Kettenmann, 1990); and intracellular Ca^{2+} mediated communication between astrocytes (Cornell-Bell et al., 1990) and between astrocytes and neurones (Nedergaard, 1994).

Like neurones, astrocytes and astroglia express voltage-dependent ion-channels (Barres et al., 1990a), namely voltage-dependent K^+, Na^+ and Ca^{2+} channels (MacVicar, 1984; Barres et al., 1990a). In general, the density of K^+ channels in glial cells and in neurones is similar, while the densities of Na^+ and Ca^{2+} channels in astroglia are considerably lower than in neurones. Furthermore, the properties of astrocyte ion channels seem to be different from those of neurones; Na^+ channels in type 1 astroglia open at more negative potentials and more slowly than neuronal Na^+ channels in neurones (Barres et al., 1989).

Glial cells, in spite of the presence of Na^+ channels, are not able to generate action potentials; thus, the role of these channels in glia is still a matter of debate. It has been proposed that glial cells synthesise Na^+ channels and donate them to adjacent neurones, reducing the neuronal biosynthetic demands. Na^+-channels may also act as sensors to detect electric activity in neighbouring neurones or be coupled to Na^+/K^+-ATPase, generating the Na^+ gradient needed for the uptake of K^+ from the extracellular space (Sontheimer et al., 1996).

Astroglia responds to a variety of neurotransmitters (Bevan, 1990). Several studies have demonstrated that glial cells express one or several types of glutamate- and GABA-receptor gated ion channels (Bormann and Kettenmann, 1988). In addition to these ionotropic receptors, astrocytes in culture also express many metabotropic receptors coupled to adenylate cyclase, guanylate cyclase, phospholipases A_2 and C and nitric oxide synthase. Glial metabotropic receptors modulate the activity of ion channels and enzymes and, consequently, mediate cellular responses by changing intracellular levels of intracellular

messengers diacylglycerol, inositol trisphosphate (IP_3), Ca^{2+}, cAMP and cGMP (Murphy and Pearce, 1987; Teichberg, 1991).

Communication between glial cells and neurones

Glial cells as targets of substances released by neurones

Substances released from neurones including K^+, neurotransmitters, and metabolites (such as CO_2) may act on glial cells and influence glial metabolism. For instance, the K^+ released during neuronal activity causes a rapid astrocyte depolarisation and generates K^+ spatial-buffering currents within astroglia that lead to extracellular K^+ redistribution (Newman, 1986). An increase in extracellular K^+ also causes glial metabolic changes by stimulation of glycogenolysis (Reichenbach et al., 1993).

Astrocytes are also targets for neurotransmitters. For example, a rise in intracellular Ca^{2+} can be detected in glial cells after glutamate release from synaptic nerve terminals (Porter and McCarthy, 1996). This effect can be due to activation of astrocyte glutamate receptors or may be secondary to glutamate-receptor induced release of a mediator, namely NO. This possibility is supported by the observation that glutamate induces synthesis of NO in neurones by activation of NO synthase (Garthwaite et al., 1989a,b). Since NO is membrane soluble, it can diffuse into neurones and glia and activate their guanylate cyclase. An increase in cGMP may decrease astrocyte coupling through gap-junctions or modulate their resting membrane conductance (see de Vente et al., 1989).

Glial cells as modulators of neuronal activity

Control of neuronal microenvironment

Glial cells can modulate neuronal activity and synaptic transmission by controlling the concentration of ions and neurotransmitters in the extracellular fluid. For example, they take up the excess of K^+ that accumulates during neuronal activity. A decrease in the efficacy of K^+ removal causes K^+ accumulation and a consequent increase on neuronal excitability, altering spontaneous and impulse-mediated neurotransmitter release.

Additionally astrocytes may modulate neuronal excitability through interference with the extracellular Ca^{2+} concentration and pH. Astrocytes take up Ca^{2+} and changes in the efficacy of this system may have important consequences for the Ca^{2+}-dependent transmitter release (Barres, 1991). Through the operation of a Na^+-HCO_3^- transport system present in glia but not in neurones astrocytes regulate the extracellular pH with consequent changes on neuronal excitability (Ransom and Orkand, 1996).

Astrocytes located close to synapses regulate neurotransmission by participating in neurotransmitter inactivation, providing synaptic insulation, and preventing neurotransmitter diffusion to the nearby synapses. One of the best examples of glial regulation of synaptic transmission is glutamatergic transmission. Glial cells take up glutamate using high affinity carriers (Rothstein et al., 1994). Glutamate is subsequently transformed to glutamine, by glutamine synthetase; an enzyme synthesised by astrocytes but not by neurones (Derouiche and Frotscher, 1991). Glial glutamine serves to maintain levels of extracellular glutamine, which in turn can be used to replenish neuronal glutamate pools. Changes in the glial glutamate-uptake system, namely a decrease in glutamate uptake, would increase baseline glutamate concentration in the synaptic cleft causing either an enhancement of glutamatergic transmission or a decrease in synaptic efficacy due to glutamate receptor desensitisation.

Release of neuroactive mediators

Glial cells may also synthesise and release neuroactive mediators such as glutamate (Parpura et al., 1994) and this observation supports the existence of a glutamatergic bi-directional communication between neurones and astrocytes. Glutamate release may occur either by Ca^{2+}-dependent (Jeftinija et al., 1996) or Ca^{2+}-independent mechanisms (Szatkowski et al., 1990; Rutledge and Kimelberg, 1996). Glutamate released from glial cells may be the mediator of a feedforward mechanism that regulates neuronal activity and synaptic strength. For example, glutamate released from glial cells activates neuronal glutamate receptors and evokes glutamate release from neuronal cells (Parpura et al., 1994; Bezzi et al., 1998).

Astroglia also synthesises and releases glycine (co-agonist of glutamate receptors), quinolinic and homocysteic acids (agonists of glutamate receptors) and kynurenic acid (antagonist of glutamate receptors) which may modulate responses to glutamate (see Barres, 1991 for review). Glial cells also synthesise and release arachidonic acid (Stella et al., 1994). Arachidonic acid causes a sustained inhibition of the electrogenic glutamate uptake-system (Barbour et al, 1989) and may potentiate glutamate transmission (Williams et al., 1989).

Study of glial cells

Astrocytes are more difficult to study *in vivo* than neurones. The methods used to study neurones *in vivo*, such as electrophysiology, neurotransmitter histochemical tracking, or selective lesioning, are not easily applied to astrocytes *in vivo* or slices. Thus, in spite of

the abundance of astrocytes in the CNS, relatively few studies have addressed the role of these cells on normal brain functions.

In the early eighties some new techniques became available to be used in the study of glial functions. Among these new techniques are the methods to obtain purified cell cultures, the availability of specific cell markers to identify cell-types, the use of imaging techniques to measure intracellular Ca^{2+} changes and the patch-clamp technique to study ionic currents. Although some of these new techniques can be applied on intact systems such as brain slices, the study of astrocytes in this type of preparations still raises some problems. Slice preparations do not usually provide exposed membrane surfaces for patch-clamp measurements, or accessibility for the antibodies used in cell identification. Moreover, glial cells are connected through gap junctions, and the electrical properties of the syncytium they form raises difficulties in the control of membrane potential and the measurement of ionic currents. Thus, most of the knowledge of glial physiology came from *in vitro* studies, namely on cell cultures and acutely dissociated cells. Acute dissociation of cells has a serious disadvantage; it often shears off the astrocyte fine processes that may contain certain channel types or receptors. In culture, these processes may regrow, but it is not certain whether astroglial cells in culture have the same properties as astrocytes *in vivo*.

Astrocytes in culture

The study of astroglia has been simplified since it became possible to prepare cultures of glial cells from immature brain tissue with a higher efficacy (McCarthy and de Vellis, 1980). Another important step was the development of immunological markers to identify the cell types present in culture (Antanitus et al., 1975; Bock et al., 1977).

Primary cultures of astroglial cells, especially of cortical astrocytes (referred as cortical type 1 astrocytes), are used as standard preparations to study glial functions. The reason for their wide application is the fact that they are easily prepared. Since contamination with other cell types can be minimised by the use of appropriated conditions, almost pure cultures can be obtained. The few oligodendrocytes or their precursors, which appear on the top of the astroglial layer, can be removed by vigorous shaking (McCarthy and de Vellis, 1980). Contaminating microglial cells can either be removed by replating, or severely reduced in their proliferation, by adding lipopolyssacharide (LPS; Gebicke-Haerter et al., 1989).

The wide use of astroglia cell cultures allowed important advances in the knowledge of glial physiology, namely:

Receptor expression – More than 25 different types of receptors are known to be expressed by type 1 astrocytes, coupled to transducing systems that control ion channels to gene expression (Arenander et al., 1989a,b).

Lineage studies and cell differentiation – For example, it was shown that the stem cell O-2A progenitor cell has the potential to became a oligodendrocyte or a type 2 astrocyte depending on the culture medium (Raff et al., 1983b).

Neuronal-glial interactions – Based on results obtained in cell cultures, it is now accepted that interactions between neurones and glial cells occur in brain, particularly during development, that may be critical to normal brain function. The nature of such interactions was, for a long time, ignored or unknown due to the impossibility of their study *in situ*. Studies carried out with co-cultures of astrocytes and neurones confirmed the existence of neuronal-glia interactions, namely the ability of astroglia to support neurones in culture (Banker 1980; Unsicker et al., 1987). Astroglial cells grown in the presence of neurones also show marked morphological changes that appear to be a consequence of a direct contact between

astrocytes and neurones (Lerea and McCarthy, 1990), and also of the release of paracrine factors.

Although being a very convenient method to study the glial cells properties, the extrapolation of data obtained *in vitro* to *in vivo* has important limitations that must be considered.

First, astroglial cultures are generally prepared from immature brain tissue and the cells obtained do not mature to the same degree that occurs *in vivo*. It is possible to prepare cultures from mature brains, but the cells obtained most likely represent stem (immature) cells that still might be present in adult tissue. Since there are no specific markers of astrocytic maturation, it is prudent to consider cultured astroglia as immature cells with properties similar to the properties that astroblasts have *in vivo*.

A second limitation involves the existence of sub-populations of astroglia. Because there are no biochemical criteria that allow distinction different populations of astroglia cells, and because they have a homogeneous morphology, cultured astrocytes are thought to represent a single population of cells. However, receptor expression by different sub-populations of glial cells can vary considerably, depending on factors such as the culture conditions, developmental stage, or duration of cell maintenance in culture. Even within the same culture, different cells can express different sets of receptors (see Steinhäuser and Gallo, 1996). These observations reflect a high flexibility of glial cells with respect to receptor expression and suggest that, in pharmacological terms, astroglia in culture is a heterogeneous population of cells.

A third limitation is the lack of a clear relationship between the subtypes of astrocytes *in vitro* and astrocytes *in vivo*, in terms of homology. For instance, is not known if astrocytes that survive in cultures are only protoplasmic astrocytes, or if fibrous astrocytes also survive.

It is also uncertain whether the protoplasmic and fibrous phenotypes reflect intrinsic or environmental differences of astrocytes (see Barres, 1991).

Another limitation of these single cell-type cultures is the possibility that certain properties of astroglial cells depend on interactions with specific neuronal populations. For example, type 1 astrocytes in culture do not have processes while, *in situ*, they show many processes. This difference may be due to the lack of some "neuronal influence" since the presence of neurones induces the formation of processes by astroglia (Hatten, 1985).

The influence of neurones is also detectable in the membrane properties of astroglia. Neurones influence the expression of ion channels (Barres et al., 1990b; Corvalan et al., 1990), neurotransmitter receptors (Maderspach and Fajszi, 1983) and neurotransmitter inactivation systems by astroglia (Westergaard et al., 1991).

In conclusion, the development of astroglial cultures allowed the characterisation of these cells in terms of receptor expression, secretion of neurotrophic factors, ion fluxes, enzyme induction, protein synthesis, and phosphorylation, uptake of neurotransmitters, metabolic processes and lipid metabolism. However, the possibility of complex interactions between neurones and glial cells suggests caution in the extrapolation of data obtained from studies on astrocytes *in vitro* to the astrocyte *in vivo*.

Glutamate in the CNS

The amino acid glutamate is a ubiquitous transmitter in the CNS and the main neurotransmitter involved on the fast excitatory transmission. Aspartate and homocysteate may also mimic glutamate effects.

Glutamate does not cross the blood-brain barrier; it is synthesised within the brain. The pathways for glutamate synthesis include transamination, reduction of 2-oxoglutarate by glutamate dehydrogenase and deamination of glutamine by glutaminase (Nicholls, 1994).

In the neurones, glutamate is stored in synaptic vesicles and is mainly released by Ca^{2+}-dependent exocytosis (McMahon and Nicholls, 1991). Release of glutamate can also occur by Ca^{2+}-independent mechanisms: by reversal of the glutamate transporter (Attwell et al., 1993) or by a swelling-induced mechanism (Kimelberg et al., 1990). Once released glutamate is removed from the synaptic cleft by Na^{+}-coupled transporters present on glia cells and on neurones. Glial cells convert glutamate to glutamine by the enzyme glutamine synthase. Glutamine then diffuses to the cerebrospinal fluid where it is present at high concentrations (0.5 mM), and enters the neurone where it can be transformed to glutamate after hydrolysis by the mitochondrial glutaminase.

Two distinct classes of receptors mediate the effects of glutamate: the ionotropic and the metabotropic glutamate receptors. The ionotropic receptors are cationic channels,

permeable to Na^+, K^+ and (some of them) to Ca^{2+}. The metabotropic receptors are coupled to G-proteins and mediate changes on intracellular second messenger levels.

Ionotropic glutamate receptors

Ionotropic glutamate receptors have been classified into three subclasses named according to their selective agonists, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate), kainate, and NMDA (N-methyl-D-aspartate).

AMPA receptors - AMPA receptors were initially named quisqualate receptors. However, they were renamed AMPA since quisqualate was found to act also on metabotropic glutamate receptors and AMPA was a more selective agonist.

AMPA receptors are either homomeric or heteromeric oligomers, composed of multiple subunits. Up to now four different subunits have been cloned: GluR1, GluR2, GluR3, and GluR4 (Keinänen et al., 1990).

Native AMPA receptors show remarkable differences in functional properties, as consequence of differences on subunit composition. The GluR2 subunit determines the channel conductance and Ca^{2+} permeability. For example, homomeric receptors made of GluR1, GluR3 or GluR4 subunits show high Ca^{2+} permeability. In contrast, homomeric receptors made of GluR2 subunits, as well as heteromeric receptors having the GluR2 subunit, show low permeability to Ca^{2+} (Burnashev et al., 1992; Jonas et al., 1994). AMPA, glutamate and kainate are agonists of AMPA receptors and, with the exception of kainate, these agonists evoke rapid and desensitising responses (Trussell et al., 1988; Tang et al., 1989).

AMPA receptors have three separate binding sites at which agonists or antagonists can act: the agonist binding site, the desensitisation, and the intra-ion channel binding sites. The agonist binding site is also the binding site for the competitive antagonist 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline (NBQX; Sheardown et al., 1990) and 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione (YM90K; Ohmori et al., 1994).

Some drugs such as cyclothiazide, aniracetam or diazoxide prevent the AMPA and glutamate induced receptor-desensitisation (Yamada and Rothman, 1992; Patneau et al., 1993). The joro spider toxin (JSTX) and analogues bind to a site located within the channel and block ion flows (Blaschke et al., 1993; Iino et al., 1996). Some recently developed 2,3-benzodiazepines are also highly selective non-competitive antagonists at AMPA receptors and seem to interact with the desensitising site (Donevan and Rogawski, 1993; Palmer and Lodge, 1993). Among them are 1-(4-aminophenyl)-4-methyl-7, 8-methylenedioxy-5H-2, 3-benzodiazepine (GYKI 52466) and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-(3N-methylcarbamate)-2,3-benzodiaze-pine (GYKI 53655).

AMPA receptors mediate fast excitatory transmission in most of the synapses in the CNS. Several studies also suggest that Ca^{2+} entry through Ca^{2+}-permeable AMPA receptors plays a role in modulation of long-term synaptic function and participate in the regulation of synaptic plasticity in the hippocampus (Gu et al., 1996; Jia et al., 1996).

Kainate receptors – Although kainate is a potent agonist at AMPA receptors, it also activates a distinct class of ionotropic glutamate receptors, called kainate-preferring receptors or simply kainate receptors (Egebjerg et al., 1991).

Kainate receptors are made up of the GluR5, GluR6, GluR7, KA1, and KA2 subunits (Chittajallu et al., 1999). Kainate-evoked responses have been observed upon activation of homomeric receptors made up of GluR5 or GluR6 subunits (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). Homomeric assembly of GLU7, KA1 or KA2 subunits does

not form functional kainate receptors (Herb et al., 1992). Homomeric GluR5 or GluR6 channels are highly permeable to Ca^{2+} and are desensitised after a long exposition to kainate (Herb et al., 1992).

Pharmacological characterisation of kainate receptors has been difficult to establish because of the lack of selectivity of kainate and also because the desensitising kainate-receptor mediated response is easily masked by the non-desensitising AMPA-receptor mediated responses to kainate.

Some recent pharmacological tools may contribute to the pharmacological characterisation of kainate receptors, namely concanavalin A, a lectin that selectively prevents desensitisation of kainate receptor-mediated responses without affecting AMPA receptor-mediated responses (Partin et al., 1993); 5-nitro-6,7,8,9-tetrahydrobenzo(g)indole-2,3-dione-3-oxine (NS-102), an antagonist that selectively and reversibly blocks kainate responses with almost no effect on AMPA receptors (Johansen et al., 1993; Lerma et al., 1993); and 2S,4R-4-methylglutamate (SYM2081), a glutamate analogue that selectively desensitises kainate receptor-mediated currents (Zhou et al., 1997).

Kainate receptors have a widespread distribution throughout the CNS. Acting on presynaptic kainate receptors, kainate seems to exert modulatory actions that may vary according to the synapse; suppression *vs.* potentiation of synaptic transmission in CA1 and CA3 regions of the hippocampus, respectively (Malva et al., 1995, 1996; Chittajallu et al., 1996). It also has a potent convulsive action when applied *in vivo* probably due to the presynaptic action on mossy fibre terminals, leading to a massive glutamate release (Debonnel et al., 1989; Gaiarsa et al., 1994).

NMDA receptors - NMDA receptors are highly permeable to Ca^{2+} (Mayer and Westbrook, 1987), with slow gating kinetics (Lester et al., 1990), and are gated by Mg^{2+} in a voltage dependent manner (Nowak et al., 1984).

Molecular cloning studies have shown that the NMDA receptor is composed of two types of subunits, NMDAR1, and NMDAR2, the latter consisting of four homologous isoforms, NMDAR2A, NMDAR2B, NMDAR2C, and NMDAR2D (Hollmann and Heinemann, 1994). NMDAR1 is a fundamental subunit to form a functional NMDA receptor (Moriyoshi et al., 1991). In contrast, NMDAR2 receptor subunits will not form functional, homologous channels but rather functional heteromeric channels composed of NMDAR1 and one or more NMDAR2 subunits (Laurie and Seeburg, 1994).

NMDA receptors have several recognition sites, where agonists, modulators and non-competitive antagonists bind: the agonist site, the glycine site, the channel, and modulatory sites (such as the redox modulatory site, proton sensitive site, Zn^{2+} site, and the polyamine site; Sucher et al., 1996). Glutamate, ibotenate, quisqualate, and homocysteate are all potent NMDA receptor agonists and their effect is due to the binding at the glutamate/NMDA recognition site of the NMDA receptor. The NMDA-receptor competitive antagonists D-(-)-2-amino-5-phosphovalerate (AP5) and 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate (CGS-19755) also act on the glutamate/NMDA recognition site.

NMDA receptors are implicated in phenomena of synaptic plasticity, such as long-term potentiation and long-term depression in the hippocampus and neocortex (Kaczmarek et al., 1997). Under certain pathophysiological conditions such hypoglycaemia, ischemic insults, head trauma, or epileptic seizures, glutamate levels increase in the brain and can cause overactivation of NMDA receptors. Overactivation of NMDA receptors may lead to a variety of neurological disorders (Javitt and Zukin, 1991), or can cause activation of a cascade of cellular events that lead to neuronal cell death (Choi, 1988; Choi and Rothman, 1990).

Metabotropic glutamate receptors

Glutamate activates also G-protein coupled receptors, the metabotropic glutamate receptors. The gene family of metabotropic glutamate receptors comprises eight members identified as mGluR1-mGluR8. Although structurally related (some exhibiting more than 40 % homology), mGluR subtypes are highly heterogeneous in what concerns their agonist selectivity, signal transduction mechanisms and distribution in the CNS (Pin and Duvoisim, 1995).

Glutamate, quisqualate, and ibotenate activate metabotropic glutamate receptors. Furthermore, some glutamate analogues, such as 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and s-2-amino-4-phosphonobutyrate (L-AP4), are specific for mGluRs, while their potencies differ for each mGluR subtype. The eight mGluR subtypes have been classified into three subgroups based on the extent of homology of their amino acid sequences, agonist selectivity and associated signal transduction mechanisms (Nakanishi, 1992).

Group I receptors include $mGluR_1$ and $mGluR_5$. They are coupled to a Gq/11 - phospholipase C system and stimulate IP_3 formation and intracellular Ca^{2+} mobilisation (Pickering et al., 1993). Group II receptors include $mGlu_2$ and $mGlu_3$. They are negatively coupled to adenylyl cyclase and inhibit the formation of cAMP (Thomsen et al., 1992; Kemp et al., 1994). The response to mGlu stimulation is pertussis toxin-sensitive, thus suggesting the involvement of a Gi protein. The group III receptors include $mGlu_{4,6,7 \text{ and } 8}$. Like the mGluR of group II, they are also coupled to Gi but show a different pattern of agonist and antagonist selectivity (Prézeau et al., 1994; Bedingfield et al., 1995). Some mGluRs seem to be coupled to phospholipase D but the nature of the (novel?) receptor involved has not been elucidated (Holler at al., 1993).

Metabotropic glutamate receptors modulate neuronal excitability in the CNS.

The mGluR-mediated modulation seems to involve both direct excitatory postsynaptic effects (Charpak et al., 1990; Crépel at al., 1994) and inhibitory presynaptic effects at both excitatory and inhibitory synapses (see Nakanishi, 1994 for review).

Glutamate receptors on glial cells

Glial cells express ionotropic and metabotropic glutamate receptors (see Steinhäuser and Gallo, 1996). The existence of functional NMDA receptors is still questionable, but there is some indirect evidence suggesting that astrocytes express some of the NMDA receptor subunits (Aoki at al., 1994; Luque and Richards, 1995). NMDA-induced currents have been observed in Bergmann glia (Müller et al., 1993) and in hippocampal and cortical astrocytes (Steinhäuser et al., 1994; Porter and McCarthy, 1995). However, in these studies it was not possible to exclude an indirect effect of NMDA, via activation of neuronal NMDA-receptors with subsequent glutamate release. The presence of functional NMDA channels in glia has been more clearly demonstrated in a recent study on retinal Müller cells (Puro et al., 1996).

Glutamate may cause glial cell depolarisation (Bowman and Kimelberg, 1984), change of second messengers levels (Pin and Duvoisin, 1995; Porter and McCarthy, 1995), and release of autocrine and paracrine messengers that mediate glial-glial and glial-neuronal communication (Bezzi et al., 1998).

Glutamate also seems to cause long-term changes in glial-cell function or development, through modulation of gene expression (Sanchez and Ortega, 1994; Condorelli et al., 1993; Mack et al., 1994). For instance, glial glutamate receptors, activated by glutamate released during neuronal activity, may regulate glial proliferation and differentiation by modulating the expression of distinct sets of voltage-gated ionic channels (Gallo and Armstrong, 1995; Gallo et al., 1996).

Cellular communication via ATP in the CNS

Drury and Szent-Györgyi (1929) published the first report about potent actions of adenyl purines. Three decades later it was described that ATP was released from sensory neurones supplying the rabbit ear artery when submitted to antidromic stimulation (Holton, 1959). This observation was the base to propose ATP as a neurotransmitter. The role of ATP as neurotransmitter in the peripheral nervous system was supported in the coming years (Burnstock, 1972, 1990a). In the peripheral nervous system ATP is co-released with acetylcholine or noradrenaline, being a neurotransmitter involved in fast synaptic transmission (Burnstock, 1990b; von Kügelgen and Starke, 1991a). Furthermore, ATP also has excitatory effects on dorsal horn neurones (Evans et al., 1992; Gu and MacDermott, 1997) and in the CNS (see Inoue et al., 1996).

ATP can be rapidly metabolised in the synaptic cleft giving rise to adenosine which is a potent neuromodulator in both the peripheral and CNS (Ribeiro, 1995).

In addition to the short-term transmitter or modulator effects, purines may also participate in long-term effects (so called trophic effects). Purines seem to play important roles in embryonic development, growth, and cell proliferation and apoptosis. Together with growth factors, purines activate glial cells and neurones, and participate in modulation of immune response mechanisms in the CNS (see Burnstock, 1996; 1997 for review).

Purinergic receptors

The responses to extracellular purines are mainly due to ATP or one of its metabolic products, ADP, AMP, or adenosine. Receptors for ATP and other purines have been named purinergic receptors. These receptors are subdivided into two major classes: P_1 purinoceptors (also called adenosine receptors) and P_2 purinoceptors (also called receptors for nucleotides; Burnstock, 1978).

P_1-purinoceptors have been subdivided in A_1, A_{2A}, A_{2B} and A_3 subtypes (Fredholm et al., 1994). All are G-protein coupled receptors. P_2-purinoceptors receptors are further subdivided in two main classes: the ionotropic receptors (P_{2X}) and metabotropic receptors (P_{2Y}). P_{2X}-purinoceptors are ligand-gated ion channels, mediating Na^+ and Ca^{2+} influx (Bean, 1992). P_{2Y} receptors are G protein-coupled receptors often coupled to activation of phospholipase C and IP_3 formation, to cAMP generation and arachidonic acid mobilisation (see Windscheif, 1996 for review).

Recently, it has been demonstrated that some of the P_2-purinoceptors can also be activated by pyrimidine nucleotides and that there are P_2-purinoceptors activated by UTP (a pyrimidine nucleotide) but not by adenine nucleotides (Lazarowski and Harden, 1994). To overcome the nomenclature problems of having P_2 purinoceptors which are not activated by adenine nucleotides, P_2-purinoceptors are now called P2-receptors and include all receptors responsive to purine and/or pyrimidine nucleotides or dinucleotides (see Fredholm et al., 1997).

Role of ATP in the CNS

Contrasting with the strong evidence that ATP acts as an excitatory neurotransmitter at synapses in the peripheral nervous system very little is known about ATP effects in the CNS. In the hippocampus, ATP is released particularly during high frequency stimulation (Wieraszko and Ehrlich, 1994). In some CNS synapses, ATP acts as a fast excitatory neurotransmitter. In slices from medial habenula (Edwards et al., 1992; Spérlagh et al., 1995) and locus coeruleus (Illes et al., 1996), and in cultured neurones from the hippocampus (Inoue et al., 1992; 1995) ATP induces fast synaptic currents with pharmacological and electrophysiological properties consistent with the activation of receptors of the P_{2X} subtype.

ATP also regulates neurotransmitter release, an effect mediated by P_{2Y} receptors. In cortical brain slices, activation of P_{2Y} receptors causes an inhibition of noradrenaline (von Kügelgen et al., 1994a) and serotonin (von Kügelgen et al., 1997) and facilitation of glutamate release (Motin and Bennett, 1995). A P_{2Y}-receptor mediated inhibition of noradrenaline release was also described in hippocampal slices (Koch et al., 1997).

ATP can induce a different type of responses not mediated by P2-receptors. Extracellular ATP may be involved on phosphorylation of membrane proteins, an effect catalysed by ecto-protein kinases. This type of phosphorylation that seems to occur in a variety of cellular systems can cause, for example, stimulation of noradrenaline uptake into synaptosomes (Hardwick et al., 1989) or inhibition of nerve growth factor-induced neurite extension (Pawlowska et al., 1993).

ATP also acts on glial cells, stimulating Ca^{2+} influx, IP_3 formation, and mobilisation of intracellular Ca^{2+} (Kastritis et al., 1992; Pearce and Langley, 1994). Furthermore, a P_{2Y}-receptor mediated arachidonic acid mobilisation and stimulation of prostaglandin synthesis in astrocytes has been reported (Gebicke-Haerter et al., 1988; Bruner and Murphy, 1990).

Adenine nucleotides and nucleosides can cause long-term (trophic) effects both on neurones and glial cells, by influencing cell proliferation, growth and cytotoxicity (Abbracchio et al., 1996). ATP released during tissue injury is known to exert P2-receptor mediated morphogenic and mitogenic effects on astrocytes (Abbrachio et al., 1994; Neary et al., 1994). The fact neurones and glial cells both express P2-receptors and respond to ATP suggests that it may be a messenger involved in communication between astrocytes and neurones.

ATP sources and release mechanisms

ATP is present in millimolar concentrations (~3-5 mM) in the cytosol of all cell types. However, extracellular levels of the nucleotide are normally extremely low for two reasons. First, membrane permeability to ATP or MgATP (the predominant cytosolic form) is minimal. Second, ubiquitous ecto-ATPases and ectophosphatases rapidly and efficiently hydrolyse extracellular ATP (Ziganshin et al., 1994; Zimmermann, 1994). Thus, significant increases of extracellular ATP levels occur only transiently and in response to specific physiological and/or pathological stimuli.

In the CNS, release of ATP from synaptosomal preparations can be evoked by high KCl or veratridine (Potter and White, 1980). In slices of medial habenula (Sperlágh et al., 1995) and Schaffer collateral-comissural afferents in hippocampal slices (Wiercaszko et al., 1989) release of ATP may be elicited by electrical stimulation.

Release of adenine nucleotides and/or nucleosides evoked by glutamate is also a frequent observation in the CNS. Jhamandas and Dumbrille (1980) have shown that glutamate and aspartate can evoke release of [3H]-adenosine and [3H]-adenine nucleotides from the cortical surface *in vivo*. This observation has been confirmed by Perkins and Stone (1983) who studied the effect of glutamate and glutamate analogues on the release of [3H]-

purines. In cortex and hippocampus slices, activation of any of the three ionotropic glutamate receptors evokes release of adenosine (Hoehn and White, 1990a; Pedata et al., 1991; Craig and White, 1992).

Furthermore, it has been suggested that the type of adenyl compound released may be dependent on the type of ionotropic glutamate receptor involved. For example, in brain cortex slices, activation of NMDA receptors primarily releases a nucleotide, whereas activation of AMPA and kainate receptors releases adenosine (Craig and White, 1993).

ATP is released from neurones by exocytosis of secretory granules or vesicles that store it. This mechanisms is also involved in the release of ATP from certain types of secretory cells such as platelets, adrenal chromaffin cells, mast cells and basophils (see Dubyak and El-Moatassim, 1993). Furthermore, ATP release is a common observation due to membrane leakage of damaged cells after an ischemic insult and cell necrosis.

ATP can also be released from sites that apparently lack vesicular storage organelles and in the absence of irreversible cytolysis. For example, smooth muscle cells and endothelial cells release ATP upon activation of specific membrane receptors by a mechanism that is neither due to cell damage nor exocytosis (Yang et al., 1994). Recently, it has been shown that two members of the ABC (ATP-binding cassette) protein family, the P-glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR), are ATP-conductive channels (Abraham et al., 1993; Reisin et al., 1994; Schwiebert at al., 1995). This suggests that ATP release may be through a carrier or channel. Whether an intrinsic plasma membrane channel or carrier is a generalised mechanism involved in ATP release, particularly from non-neuronal cells, remains an open question.

The data available strongly support the view that in the brain, glutamate evokes release of adenyl compounds; however, several points deserve some clarification. First, which purine or purines is (are) released in first place upon activation of glutamate receptors. In most of the studies, only tritiated compounds or endogenous adenosine were measured

and the possibility that activation of glutamate receptors releases primarily endogenous ATP was never clearly demonstrated.

The second question raised concerns the source of these purines. Since ATP is a central transmitter, neurones are a likely source. Astroglial cells are also another possibility; they constitute most of the brain's mass, they express glutamate receptors and there is evidence that electrical stimulation evokes the release of [3H]-adenyl compounds from glial cells (Caciagli et al., 1988). However, the possibility of a glutamate receptor evoked release of endogenous ATP from astroglial cells and the question whether there is a preferential glutamate receptor involved on ATP release, has not been investigated.

Aims

In the present study the effect of glutamate and related agonists on the release of a defined nucleotide, ATP, from defined brain cells, namely cultured astrocytes derived from the cerebral hemispheres of the rat, was investigated. It was also examined whether any of the release mechanisms previously discussed mediate the release of ATP from primary cultures of astrocytes in response to glutamate receptor activation. The first approach was to verify whether the release of ATP was a consequence of cell lysis. The two other mechanisms discussed: the operation of a plasma membrane transporter or channel and the possibility of a Ca^{2+}-dependent plasma membrane/vesicle fusion mechanism, similar to neuronal regulated exocytosis, were also investigated.

Experimental Procedures

Cell cultures

Primary astroglial cultures were prepared from cerebral hemispheres of newborn Wistar rats as described by Keller et al. (1985). The brains were placed in ice-cold phosphate-buffered saline (PBS) containing 0.2% glucose and hemispheres were freed from meninges and blood vessels. After washing twice with ice-cold PBS, the hemispheres were cut into small pieces in "culture medium", i.e. Dulbecco's Modified Eagle Medium containing 1 g/l N-acetyl-alanyl-L-glutamine and supplemented with 10% foetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 10 ng/ml LPS (Gebicke-Haerter et al., 1989). Tissue from 2 to 4 hemispheres was then dissociated by trituration in 10 ml culture medium. The cell suspension was passed through a 50-µm pore nylon mesh and centrifuged at 200g for 5 min. The pellet was resuspended in medium and the suspension was centrifuged again at 200g for 5 min. Resuspension and centrifugation were repeated and the final pellet suspended in 1 ml medium. The number of cells (with 3.4 µm diameter) was determined and adjusted to approximately 200,000 cells/ml. One hundred microliter of this cell suspension was seeded on 5-mm diameter Thermanox coverslips (Nunc, Wiesbaden, Germany) placed in the wells of 96-well microtiter plates. The cultures were incubated at 37º C in a humidified atmosphere of 95% air and 5% CO_2.

The culture medium was replaced one day after the preparation and subsequently

twice a week unless stated otherwise. Confluent 13- to 16-day-old cultures were used for immunostaining and for release experiments. In some cases, cultures were incubated with botulinum toxin B for 16 h prior to the release experiment.

Immunocytochemistry

Immunocytochemistry was performed using antibodies against the glial fibrillary acidic protein (GFAP) and against the microtubule associated protein-2a,b (MAP-2a,b). The cultures were fixed in phosphate buffer (100 mM NaH_2PO_4, 50 mM NaCl, pH adjusted to 7.3) containing 4% formaldehyde and 4% sucrose. Non-specific binding was prevented by incubation with normal goat serum. The cells were then incubated with the primary antibody directed against the protein of interest.

GFAP was labelled with a rabbit polyclonnal anti-GFAP antiserum (1:400) at 4° C overnight. For detection of the immune complex a secondary antibody (anti-rabbit, 1:50) labelled with fluorescein isothiocyanate (FITC; Biozol, Eching, Germany) was used.

For immunostaining of MAP-2a,b a mouse monoclonal antibody (Sigma, München, Germany) was used at a concentration of 5 µg per ml. The resulting immune complex was detected with a biotinylated secondary antibody. The colour reaction was enhanced with the Vectastain ABC kit and 3,3'-diaminobenzidine as a substrate (Sigma).
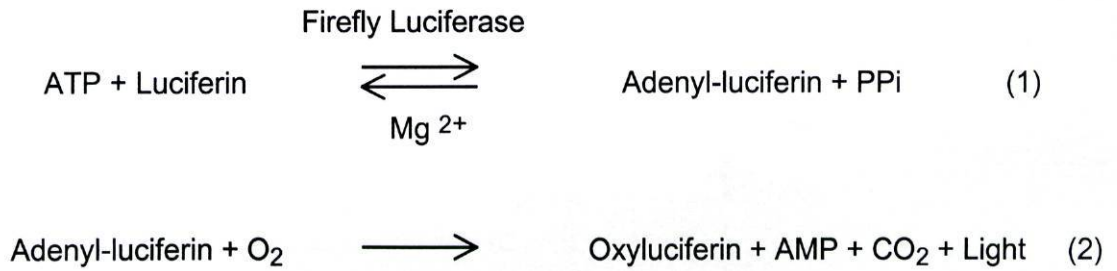
ATP release

Two coverslips were transferred to each of twelve 0.3-ml superfusion chambers and superfused at a rate of 0.6 ml/min with "superfusion buffer" at 25° C; preliminary experiments

showed that the basal efflux of ATP was lower, whereas the glutamate receptor agonist-evoked overflow of ATP was similar at 25º C as compared to 37º C. Unless stated otherwise, the buffer contained (mM) 110 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 1.0 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 1.0 sodium pyruvate and was gassed with a mixture of 95% O_2 and 5% CO_2. The pH was adjusted to 7.4 with NaOH (1 M). After 45 minutes of superfusion, 3-min fractions of superfusate were collected for 24 minutes. Glutamate receptor agonists, ionomycin and α-latrotoxin were added 54 minutes after onset of superfusion for the remaining 15 min of the experiment. For Ca^{2+}-free experiments, cells were superfused with Ca^{2+}-free buffer (the buffer had the same composition as described, except that $CaCl_2$ was replaced by NaCl) for 30 minutes before and during stimulation with the agonists or ionomycin. In experiments performed in the presence of cadmium, the buffer had the following composition (mM): 135 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 10 HEPES, 10 glucose, 1.0 sodium pyruvate, pH adjusted to 7.4 with NaOH (1 M). Glutamate receptor antagonists, cadmium, nicardipine, 4,4'-diisothio-cyanato-stilbene-2, 2'-disulfonate (DIDS), atractyloside, glibenclamide, diphenylamine-2-carboxylate (DPC) and lithium were present from the beginning of superfusion.

In some experiments, cells were lysed at the end of superfusion by incubation for 20 min in 300 µl of ice-cold water. After taking a 90 µl sample for protein determination (see below), the suspension was centrifuged at 4000g for 3 min, and a 100 µl aliquot was taken from the supernatant.

ATP was measured in the superfusates, and in some experiments in the supernatant of lysed cells, with the luciferin-luciferase technique using the ATP bioluminescence HS assay kit (Sigma). The general chemical reactions of the ATP bioluminescence HS assay kit are as follows:

$$\text{ATP + Luciferin} \xrightleftharpoons[\text{Mg}^{2+}]{\text{Firefly Luciferase}} \text{Adenyl-luciferin + PPi} \qquad (1)$$

$$\text{Adenyl-luciferin} + O_2 \longrightarrow \text{Oxyluciferin + AMP} + CO_2 + \text{Light} \qquad (2)$$
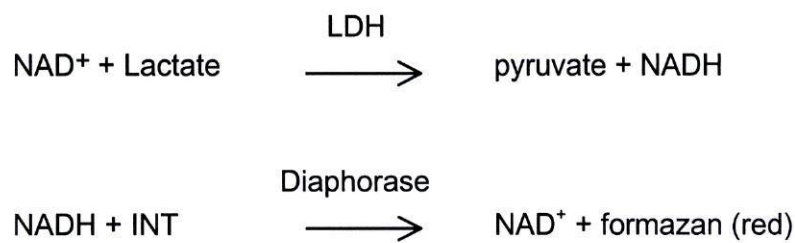
ATP is consumed and light is emitted when firefly luciferase catalyses the oxidation of D-luciferin. Reaction (1) is reversible and the equilibrium lies far to the right. Reaction (2) is essentially irreversible. When ATP is the limiting reagent, the light emitted is proportional to the ATP present. Drugs present throughout superfusion were also included in blank and calibration curve media. The protein content of the cells was determined at the end of experiments. For protein determination, cells were dissolved in NaOH 0.1 N and protein content was determined according to Lowry et al. (1951), using bovine albumin as standard.

The outflow of ATP was expressed as pmol per mg protein per minute, and cellular ATP as nmol per mg protein. The overflow of ATP induced by glutamate receptor agonists and ionomycin was calculated by subtraction of the basal outflow from the total outflow of ATP measured in the 15 min after addition of stimulant; the basal outflow was taken to be the average outflow in the 9 min before stimulant addition. Experiments with solvent instead of stimulant were evaluated in the same manner. None of the drugs present throughout superfusion changed the basal efflux of ATP.

Lactate dehydrogenase release

Cell damage was assessed quantitatively by measuring lactate dehydrogenase (LDH) release into the medium in the presence of glutamate receptor agonists. Two coverslips were

incubated under gentle shaking in 100 µl of "superfusion buffer" at 37° C in an atmosphere of 95% air and 5% CO_2 for two consecutive periods of 15 min each, in the first 15-min period without, in the second with glutamate receptor agonists. After 15 min, the medium was removed for determination of LDH activity and a second 100 µl sample of either superfusion buffer or buffer containing glutamate receptor agonists was added. The cells were incubated for another 15 min, and a second sample was withdrawn. The cells where then disrupted by addition of 10 µl of Triton X-100 at 9% (v/v), the mixture was centrifuged at 4000g for 10 min, and a final 100 µl aliquot was taken from the supernatant. LDH released in each period and after cell lysis was measured in aliquots of 50 µl with the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). The general chemical reactions of CytoTox 96 Assay are as follows:

$$NAD^+ + Lactate \xrightarrow{LDH} pyruvate + NADH$$

$$NADH + INT \xrightarrow{Diaphorase} NAD^+ + formazan\ (red)$$

The light absorbance at 490 nm is proportional to the amount of LDH released. Results were expressed as percentage of the total LDH released after cell lysis.

Materials

The following materials were used: (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) and α-latrotoxin (α-LTX) (Alexis, Grünberg, Germany); (±)-α-amino-3-

hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline (NBQX), glibenclamide, γ-D-glutamyl-amino-methanesulfonic acid (GAMS) (Research Biochemicals, Köln, Germany); ($\pm$)-2-amino-5-phosphonopentanoic acid (AP5), atractyloside sodium salt, botulinum toxin B, 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid dissodium salt (DIDS), ionomycin calcium salt, kainic acid, L-glutamic acid monosodium salt, lithium chloride, nicardipine hydrochloride and N-methyl-D-aspartic acid (NMDA; Sigma, Deisenhofen, Germany); cadmium chloride monohydrate (Merck, Darmstadt, Germany); diphenyl-amine-2-carboxylate (DPC) (Fluka, Neu-Ulm, Germany); Dulbecco's Modified Eagle Medium (Seromed, Biochrom, Berlin, Germany); foetal calf serum (Serva, Heidelberg, Germany); lipopolysaccharide from *Salmonella typhimurium* (LPS; Sebak, Aidenbach, Germany); penicillin G and streptomycin (Gibco, Eggenstein, Germany).

Glutamate receptor agonists, AP5, GAMS, cadmium, atractyloside and lithium were dissolved in the superfusion medium; nicardipine in dimethyl sulfoxide (final concentration 7 μM); ionomycin, DIDS and glibenclamide and NBQX in dimethyl sulfoxide (final concentration 14 μM); DPC in ethanol (final concentration 0.4%); α-latrotoxin in water.

Statistics

Results are given as arithmetic means ± S.E.M. from n observations. Differences between means were tested for significance by the Mann-Whitney test. A P-value less than 0.05 was taken to be statistically significant. For multiple comparisons with the same control, P values were adjusted according to Bonferroni.

Results

ATP release: *effect of glutamate receptor agonists*

The basal outflow of ATP in the 9 min before stimulation by glutamate receptor agonists averaged 0.60 ± 0.02 pmol per mg protein per min (n = 564).

Glutamate and the three receptor type-selective agonists NMDA, AMPA and kainate all caused significant and, where several concentrations were tested, concentration-dependent increases in the outflow of ATP (Fig. 1). *Trans*-ACPD, a selective agonist at metabotropic glutamate receptors and devoid of activity at ionotropic glutamate receptors (Palmer et al. 1989; Manzoni et al., 1990), also caused a concentration-dependent release of ATP from astrocytes (Fig. 1). The order of potency was AMPA > NMDA > trans-ACPD > glutamate. AMPA had the maximal effect.

Glutamate itself elicited a transient overflow of ATP at the lowest concentration (100 μM; Fig. 2A) but a long-lasting overflow at higher concentrations (Fig. 2B and C). The maximal acceleration of the outflow of ATP was 6-fold (at glutamate 1000 μM, Fig. 2C).
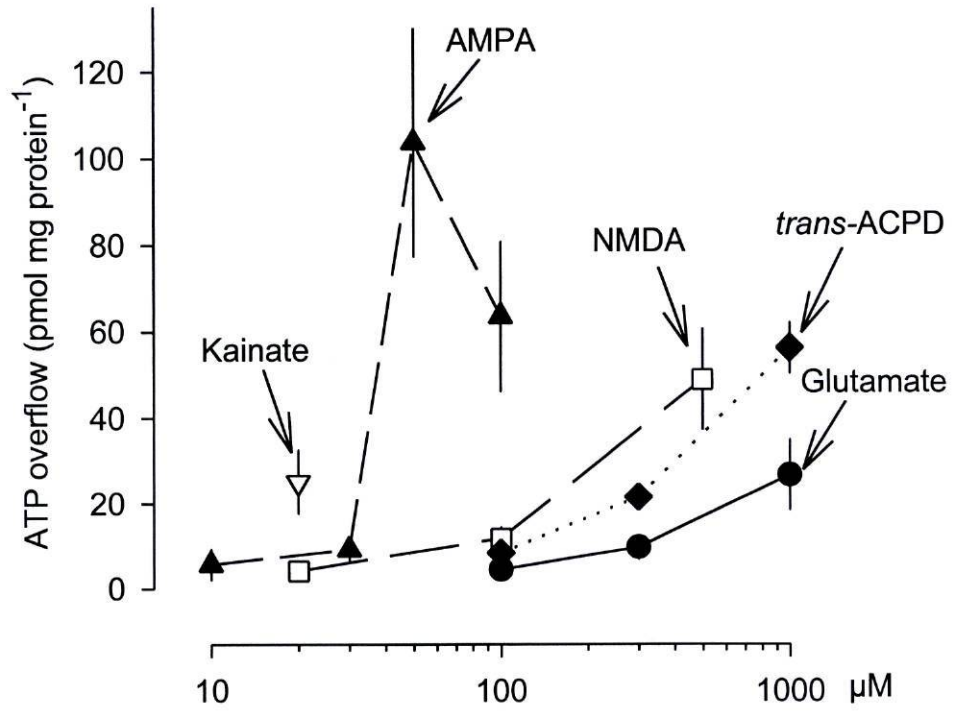
Figure 1. ATP release evoked by glutamate receptor agonists from superfused primary cultures of astrocytes: concentration response curves. Values are the means ± S.E.M from 6-21 observations.
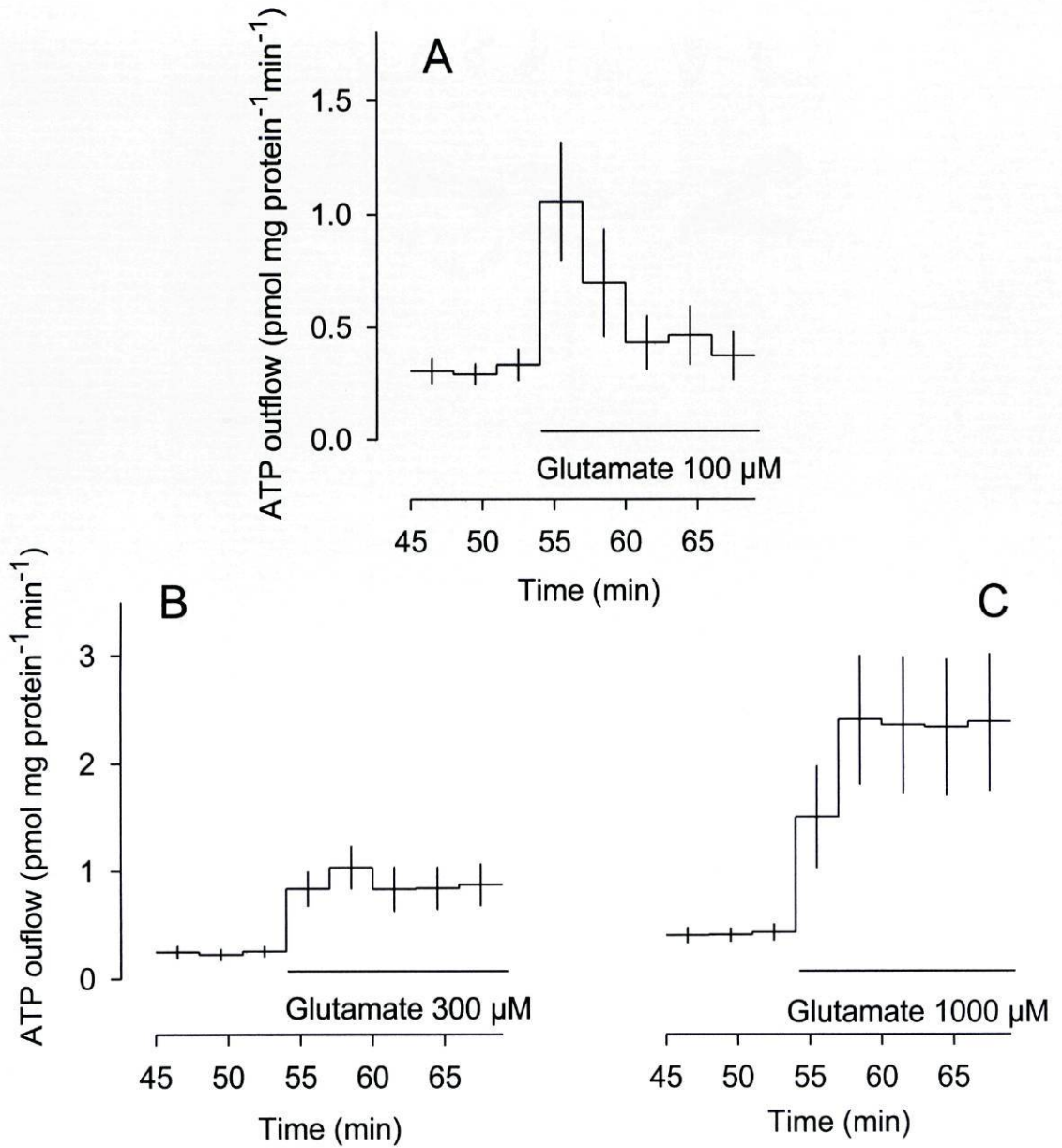
Figure 2. Time-course of the effect of glutamate on the outflow of ATP from superfused astrocytes. The agonist was added as indicated by the horizontal bar. Values are the means ± S.E.M. from 10-21 observations. Abcissae, minutes of superfusion.

The effect of NMDA was transient at the lowest concentration examined as shown for a concentration of 100 µM in Fig. 3A, but very variable at the highest concentration tested (500 µM; not shown). The maximal acceleration of ATP outflow was 19-fold (at NMDA 500 µM). AMPA caused long-lasting increases at all concentrations examined as shown for 30 µM in Fig. 3B. The maximal acceleration was 15-fold (AMPA 50 µM). The time course of the effect of kainate (20 µM) seemed to be intermediate between the transient increase elicited by NMDA and the maintained increase elicited by AMPA (Fig. 3C). The maximal acceleration was 5-fold. trans-ACPD, like AMPA, caused long-lasting increases at all concentrations tested as shown for trans-ACPD 300 µM (Fig. 3D). The maximal acceleration of ATP outflow was 7-fold (at 1000 µM trans-ACPD).

ATP release: *fraction released*

In some experiments with glutamate and NMDA, the ATP content of the cells was determined at the end of superfusion. It averaged 3.5 ± 0.3 nmol per mg protein (n=18). The outflow of ATP in the 9 min before agonist addition averaged 0.85 ± 0.13 pmol per mg protein per min (n=18), which was 0.03 ± 0.01 % of the cellular content per min. Glutamate (1000 µM) released 13.4 ± 1.9 pmol ATP per mg protein, which was 0.55 ± 0.14 % of the cellular content (n=6). NMDA (500 µM) released 11.7 ± 3.0 pmol ATP per mg protein, which was 0.32 ± 0.06 % of the cellular content (n=8).
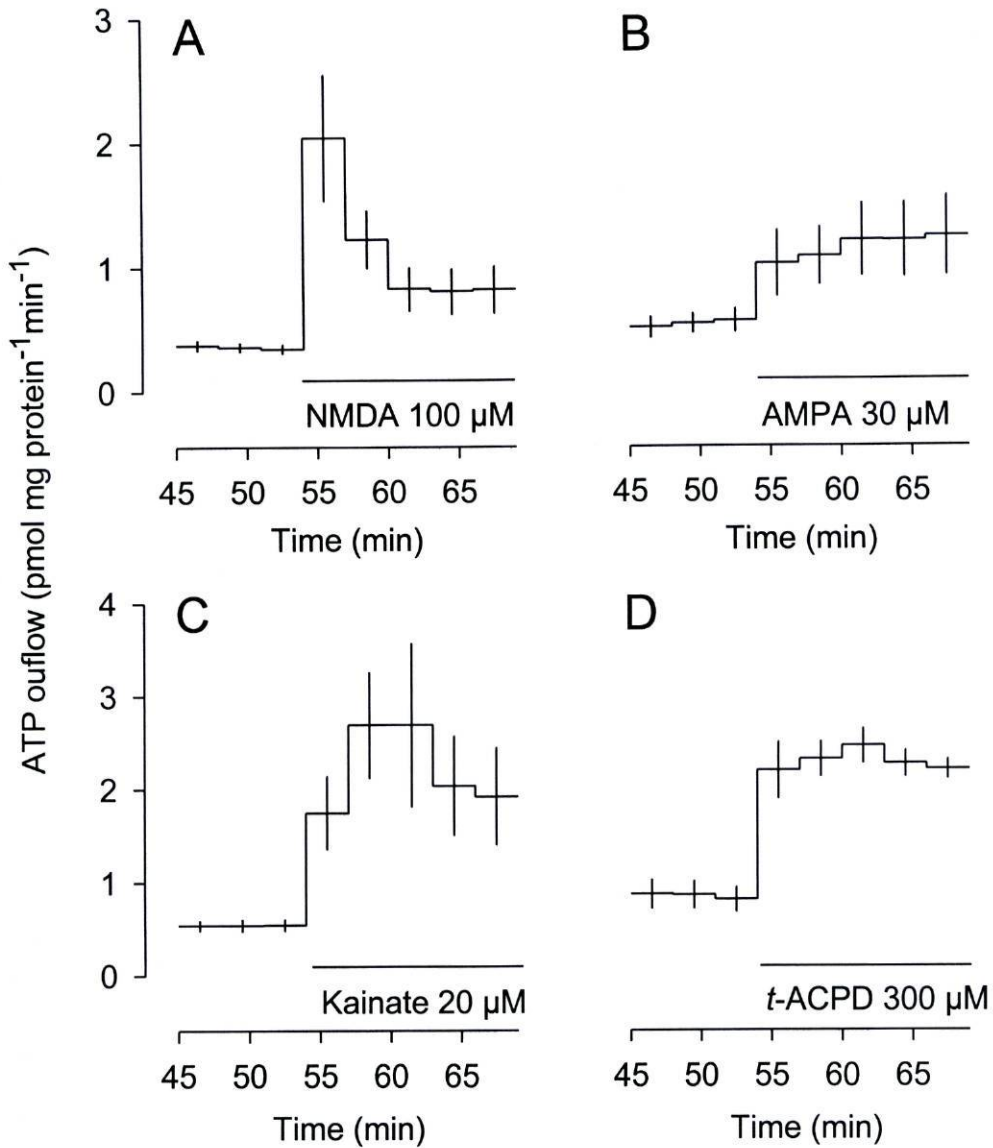
Figure 3. Time-course of the effect of glutamate receptor type-selective agonists on the outflow of ATP from superfused astrocytes. The agonists were added as indicated by the horizontal bar. Values are the means ± S.E.M. from 6-18 observations. Abcissae, minutes of superfusion.

ATP release: *possible contribution of neurones*

Immunocytochemistry demonstrated that most cells in culture were immunopositive for GFAP but some were positive for MAP2a,b, a specific marker for neurones (Fig. 4; cf. Hildebrand et al., 1997). Since we did not succeed in preparing cultures completely devoid of MAP2a,b positive cells, the release of ATP, evoked by glutamate receptor agonists, from cultures with different contents of MAP2a,b positive cells was compared. Cultures containing a lower number of MAP2a,b positive cells were prepared as described under Experimental Procedures with the exception that the culture medium was changed every day during the first 4 days of culturing. With this procedure, the number of MAP2a,b positive cells present in the cultures was reduced from 33.2% to 10.6% on average, i.e. by about 2/3 (Table 1). The release of ATP evoked by NMDA, AMPA and kainate did not differ between the two culture types (Table 1).
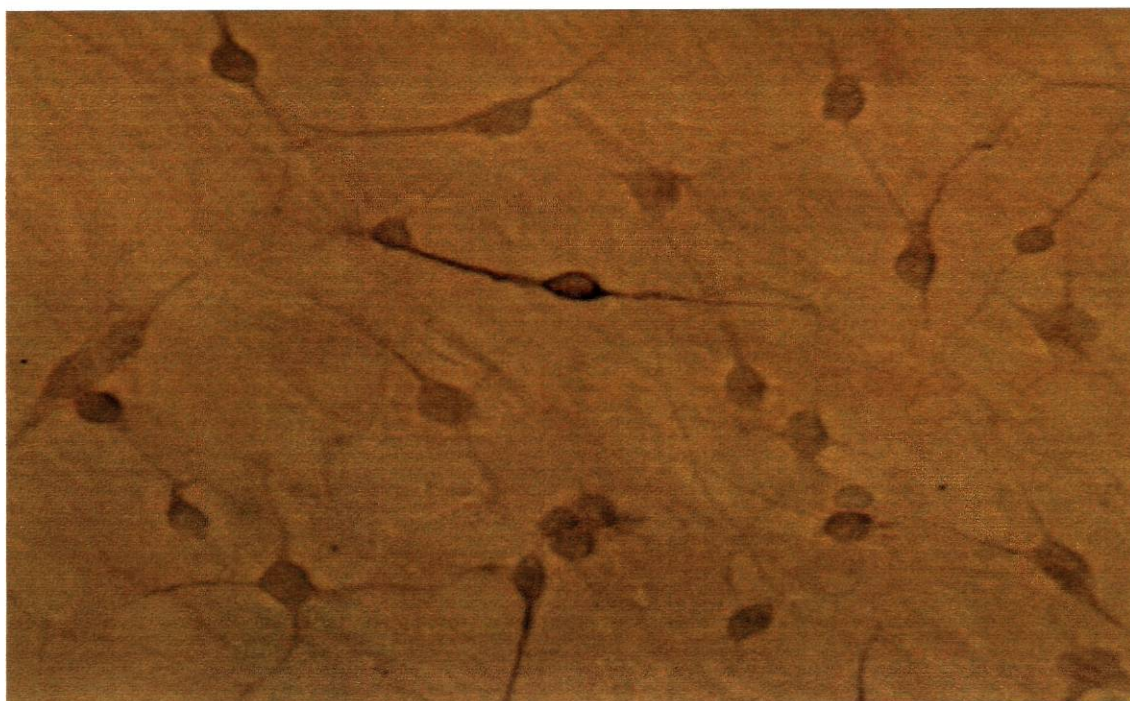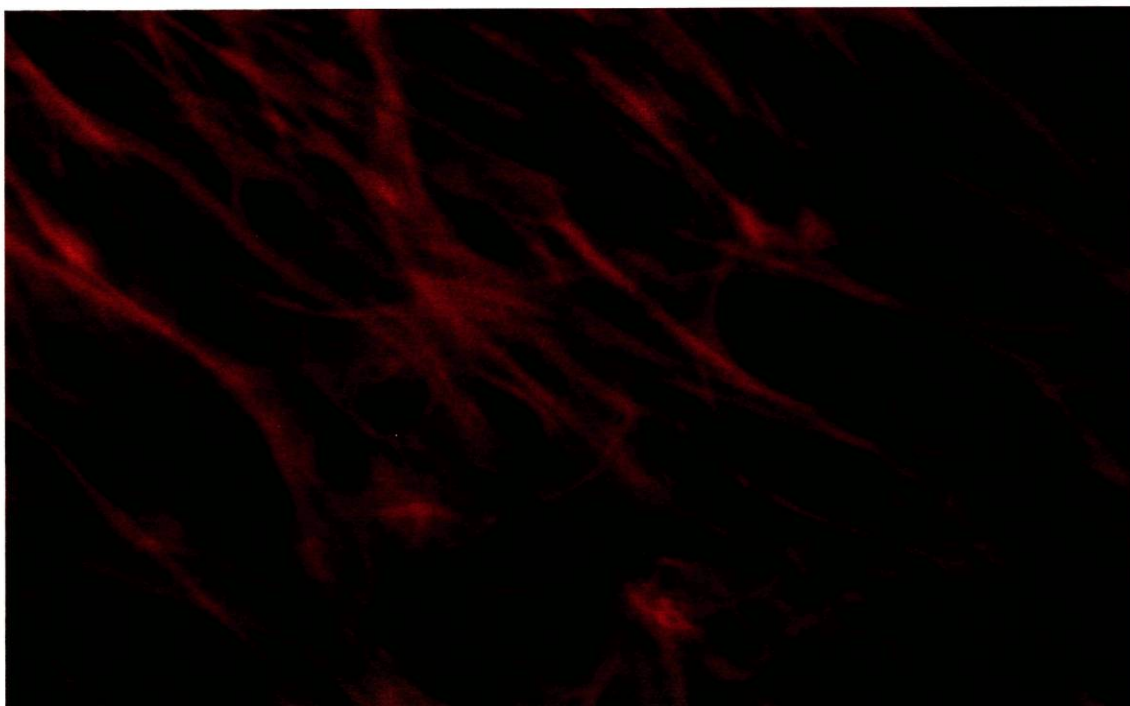
Figure 4. Glial fibrilary acidic protein (GFAP) immunofluorescence (upper panel) and phase contrast microscopy of microtubule associated protein-2a,b (MAP2a,b) positive cells (lower panel) in 15-day-old primary cultures of astrocytes prepared from brain hemispheres of newborn rats. Micrographs were obtained from the same field.

Table 1. Glutamate receptor agonist-evoked release of ATP from primary cultures of astrocytes containing different percentages of MAP2a,b positive cells.

| Agonist | (µM) | Agonist-evoked release of ATP (pmol per mg protein) | |
		33.2% MAP2a,b cells	10.6% MAP2a,b cells
NMDA	500	38.0 ± 3.3 (3)	37.9 ± 1.1 (3)
AMPA	30	18.3 ± 2.2 (6)	27.9 ± 3.6 (6)
Kainate	50	17.3 ± 4.8 (3)	17.6 ± 4.7 (3)

Cultures containing $33.2 \pm 4.3\%$ MAP2a,b positive cells were obtained as described in Experimental Procedures. Cultures with $10.6 \pm 2.3\%$ MAP2a,b positive cells were obtained in the same manner, but the medium was changed every day during the first 4 days in culture. The two kinds of cultures were run strictly in parallel. Cells were exposed to agonist from 54 min of superfusion onwards for 15 min. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow from total outflow observed from 54 to 69 min of superfusion. Means $\pm$ S.E.M. from (n) observations.

ATP release: *interaction with antagonists*

Three antagonists - AP5, NBQX, and GAMS - were tested against the agonists. They did not change the basal efflux of ATP. The NMDA receptor-selective antagonist AP5 (see Discussion for references), given at a concentration of 100 µM, abolished the effect of NMDA, did not significantly change the effects of AMPA and kainate, and also did not change the effect of glutamate (Table 2). NBQX is selective for AMPA receptors. At a concentration of 30 µM it prevented the effect of AMPA and did not significantly change that of kainate, although there was a tendency for a decrease. NBQX also abolished the increase in ATP outflow elicited by glutamate and, unexpectedly, the increase caused by NMDA (Table 2).

Finally, the kainate receptor-selective antagonist GAMS, given at a concentration of 30 µM, blocked the effect of kainate but did not change that of glutamate (Table 2).

Lactate dehydrogenase release

Release of the cytoplasmic enzyme LDH was examined in order to detect possible cell damage caused by glutamate. LDH was not discovered in the medium in superfusion experiments of the kind described so far. Therefore, a different protocol had to be used (see Experimental Procedures). Glutamate receptor agonists, at concentrations releasing ATP, failed to release LDH: when the agonists were added during the second of two 15-min incubation periods, release of LDH from astrocytes slightly declined rather than increased, as it did in controls without agonists (Table 3).

Table 2. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: interaction with antagonists.

Agonist	(µM)	Solvent	AP5 (100 µM)	NBQX (30 µM)	GAMS (30 µM)
		Agonist-evoked release of ATP (pmol per mg protein)			
—		0.2 ± 0.4 (67)	0.6 ± 0.8 (9)	-0.9 ± 0.4 (4)	-0.8 ± 0.9 (7)
Glutamate	100	4.4 ± 1.7 (17)	4.2 ± 1.9 (6)	—	—
	300	9.6 ± 2.8** (18)	22.4 ± 3.9** (5)	-3.1 ± 1.7†† (4)	30.8 ± 8.7** (6)
	1000	26.7 ± 8.3* (21)	21.9 ± 4.8** (7)	—	49.3 ± 13.3** (6)
NMDA	20	4.1 ± 0.9* (13)	-1.1 ± 1.4† (3)	—	—
	100	11.7 ± 2.7** (18)	-2.1 ± 1.0†† (5)	0.6 ± 1.4 (5)	—
	500	49.0 ± 11.8** (16)	7.2 ± 3.8*†† (5)	3.4 ± 0.4†† (3)	—
AMPA	10	5.6 ± 3.6 (8)	—	—	—
	30	9.1 ± 3.0** (10)	—	—	—
	50	103.6 ± 26.4** (8)	57.2 ± 23.5 (7)	—	—
	100	63.5 ± 17.3** (19)	—	-2.2 ± 0.3†† (4)	—
Kainate	20	25.1 ± 7.5** (19)	25.1 ± 6.5** (8)	12.1 ± 4.8* (5)	-0.1 ± 0.7† (5)
trans-ACPD	100	8.3 ± 1.8* (5)	—	—	—
	300	21.5 ± 1.8** (6)	—	—	—
	1000	56.3 ± 5.9** (6)	—	—	—

The antagonists AP5 (100 µM), NBQX (30 µM) or GAMS (30 µM) were present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. * and ** indicate significant differences from first line (no agonist; $P<0.05$ and $P<0.01$, respectively). † and †† indicate significant differences from effect of agonist in the absence of antagonist ($P<0.05$ and $P<0.01$, respectively).

Table 3. Release of lactate dehydrogenase (LDH) from astrocytes: effect of glutamate receptor agonists.

Drug present from 15-30 min	(µM)	LDH in incubation medium (% of total)		
		0-15 min	15-30 min	n
—		6.4 ± 1.2	4.3 ± 1.6	13
Glutamate	1000	5.7 ± 2.1	5.1 ± 2.2	6
NMDA	500	9.4 ± 2.3	6.6 ± 1.5	6
AMPA	30	5.5 ± 0.5	4.0 ± 0.8	5
Kainate	50	12.1 ± 2.4	5.1 ± 1.8	6
trans-ACPD	300	5.3 ± 1.2	4.3 ± 0.9	5

Cells were incubated in 100 µl buffer for two successive periods of 15 min (0-15 and 15-30 min). Glutamate receptor agonists were present during the second 15-min period. LDH release is expressed as a percentage of the total LDH released after cell lysis. Values are means ± S.E.M. from (n) observations.

ATP release: *effect of ionomycin*

The calcium ionophore ionomycin was tested to determine whether an increase in intracellular calcium was sufficient to release ATP. Ionomycin (5 µM) progressively increased the outflow of ATP from astrocytes over the 15 minutes of application in the presence of external calcium but not in its absence (Fig. 5). The overflow of ATP evoked by ionomycin was 21.9 ± 2.6 pmol per mg protein in the presence of calcium and 2.2 ± 0.9 pmol per mg protein its absence (n=17 and n=8 respectively; P<0.01).

ATP release: *omission of extracellular calcium*

Withdrawal of calcium increased the basal outflow of ATP (see Fig. 6). From 21 to 30 min of superfusion with nominally Ca^{2+}-free medium, i.e. in the 9 min before stimulation by glutamate receptor agonists, ATP outflow was 1.94 ± 0.15 pmol per mg protein per min, as compared to 0.72 ± 0.05 pmol per mg protein per min in the presence of calcium (n=64 and n=57, respectively; P<0.01).

Superfusion with Ca^{2+}- free buffer solution greatly reduced or abolished the overflow of ATP evoked by glutamate (1000 µM), NMDA and kainate and did not significantly change the overflow evoked by glutamate (300 µM), AMPA and *trans*-ACPD (Table 4). The loss of effect of NMDA in the absence of external Ca^{2+} is also shown in Fig. 6.
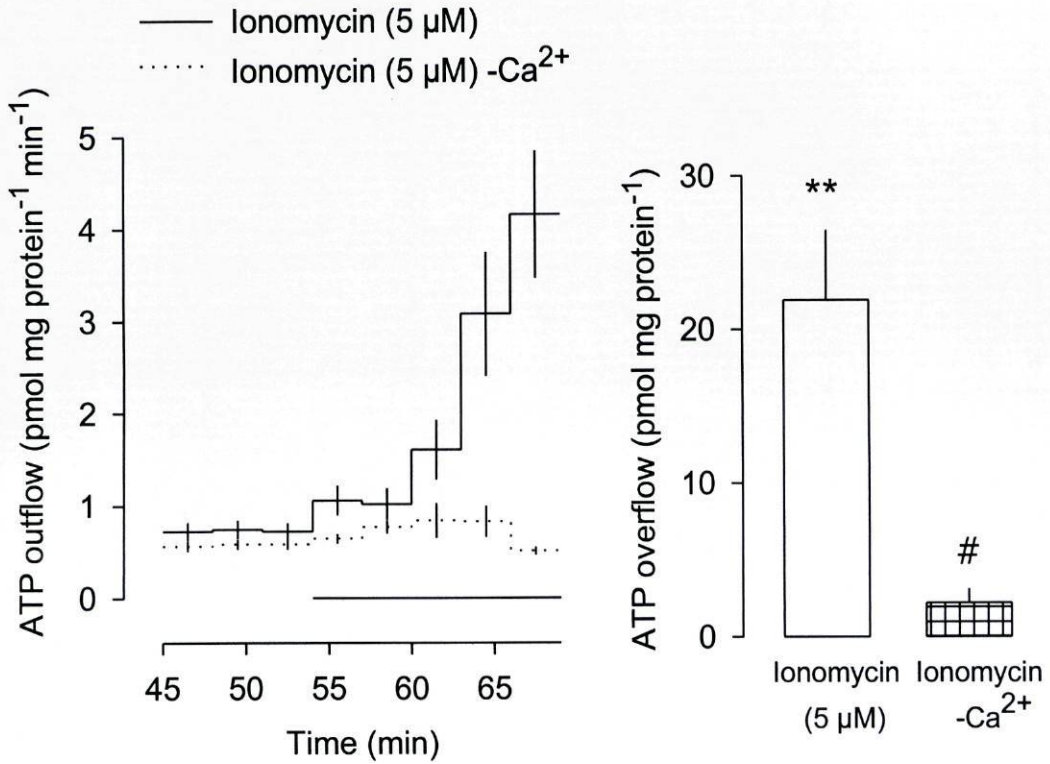
Figure 5. Release of ATP from superfused astrocytes elicited by ionomycin (5 µM). Ionomycin was added as indicated by the horizontal bar. In some experiments, calcium was omitted from the superfusion buffer 30 min before stimulation by ionomycin. Abcissae of left-hand panel, minutes of superfusion. Values are the means ± S.E.M. from 17 (ionomycin at normal Ca2+) and 8 (ionomycin and Ca2+ withdrawal) observations.

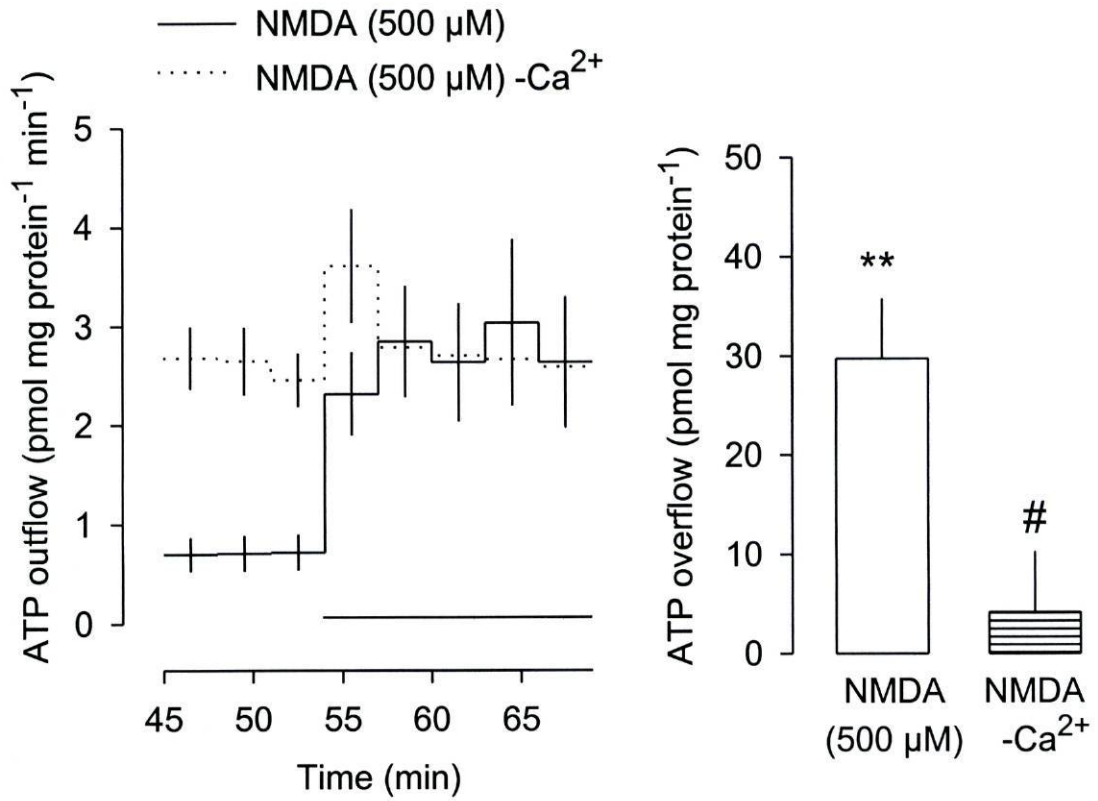**Figure 6.** NMDA-evoked release of ATP from superfused astrocytes: effect of calcium withdrawal. NMDA was added as indicated by the horizontal bar. Calcium was omitted from the superfusion buffer 30 min before stimulation with NMDA. Abcissae of left-hand panel, minutes of superfusion. Values are the means ± S.E.M. from 6 (NMDA at normal Ca^{2+}) and 11 (NMDA and Ca^{2+} withdrawal) observations.

Table 4. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of calcium withdrawal.

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		With Calcium	Without Calcium
-		-1.7 ± 0.8 (7)	2.7 ± 2.3 (10)
Glutamate	300	14.3 ± 4.9 ** (6)	14.5 ± 5.9 (6)
	1000	25.5 ± 3.9 ** (9)	10.9 ± 2.9 †† (8)
NMDA	500	29.8 ± 6.0 ** (6)	4.2 ± 6.1 † (11)
AMPA	10	23.5 ± 5.3 ** (5)	25.5 ± 4.3 ** (6)
	30	91.1 ± 3.2 ** (9)	75.0 ± 10.4 ** (9)
Kainate	50	19.4 ± 3.4 ** (12)	1.5 ± 1.7 †† (11)
trans-ACPD	300	26.9 ± 1.9 * (3)	26.6 ± 4.4 * (3)

Calcium was omitted from the superfusion buffer 30 minutes before stimulation with agonists. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. * and ** indicate significant differences from first line (no agonist; $P<0.05$ and $P<0.01$, respectively). † and †† indicate significant differences from effect of agonist in the presence of calcium ($P<0.05$ and $P<0.01$, respectively).

ATP release: *effect of blockers of voltage-dependent Ca2+channels*

Calcium can enter the cytoplasm of glial cells via ionotropic glutamate receptor channels (Finkbeiner, 1993; Porter and McCarthy, 1995) as well as through voltage-dependent Ca2+ channels (VDCCs; MacVicar, 1984).

In order to examine whether the release of ATP evoked by ionotropic glutamate receptor agonists required influx of Ca2+ through VDCCs, cadmium and nicardipine were tested againts the agonists. Like calcium withdrawal, cadmium, a non-selective blocker of VDCCs, at a concentration of 1 mM greatly reduced or abolished the overflow of ATP evoked by glutamate (1000 μM), NMDA and kainate and did not change the overflow evoked by glutamate (300 μM) and AMPA (Table 5). A similar pattern was observed with the selective L-type VDCC blocker nicardipine (10 μM, Table 6).

ATP release: *effect of α-latrotoxin and botulinum toxin B*

There is evidence that α-latrotoxin, an active compound of the black widow spider venom, elicits, whereas botulinum toxin B inhibits, exocytotic release of neurotransmitters from neurones and glutamate from astrocytes (see Discussion). However, addition of α-latrotoxin (3 nM; for 15 min) did not cause a significant increase in the outflow of ATP from astrocyte cultures (basal outflow over the 9 min before α-latrotoxin, 1.1 ± 0.2 pmol per mg protein per min; outflow over the 15 min in the presence of α-latrotoxin, 1.2 ± 0.3 pmol per mg protein per min; n=9).

Pre-treatment of astrocyte cultures with botulinum toxin B (100 nM) for 16 h did not significantly change the overflow of ATP evoked by NMDA (500 μM) or kainate (50 μM; Fig. 7).

Table 5. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of cadmium.

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		Solvent	Cadmium (1 mM)
-		0.2 ± 0.5 (26)	0.6 ± 0.5 (22)
Glutamate	300	10.3 ± 2.6 ** (14)	10.3 ± 5.3 (13)
	1000	15.3 ± 3.7 ** (12)	5.9 ± 2.5 † (11)
NMDA	500	41.1 ± 10.0 ** (7)	18.6 ± 3.2 **† (9)
AMPA	30	50.9 ± 10.4 ** (6)	37.2 ± 8.1 ** (8)
Kainate	50	19.1 ± 2.9 ** (8)	9.5 ± 2.3 **† (8)

Cadmium (1 mM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. ** indicates a significant difference from first line (no agonist; $P<0.01$). † indicates a significant difference from effect of agonist in the absence of cadmium (solvent; $P<0.05$ and $P<0.01$, respectively).

Table 6. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of nicardipine.

| Agonist | (µM) | Agonist-evoked release of ATP (pmol per mg protein) | |
		Solvent	Nicardipine (10 µM)
-		0.2 ± 0.4 (6)	0.4 ± 0.9 (9)
NMDA	500	21.4 ± 3.6 ** (11)	8.9 ± 1.9 **† (10)
AMPA	30	16.6 ± 3.6 ** (6)	24.0 ± 6.7 ** (6)
Kainate	50	14.7 ± 2.3 ** (9)	4.2 ± 1.4 †† (12)

Nicardipine (10 µM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. ** indicates a significant difference from first line (no agonist; $P<0.01$). † and †† indicate significant differences from effect of agonist in the absence of nicardipine (solvent; $P<0.05$ and $P<0.01$, respectively).

Figure 7. Release of ATP from superfused astrocytes elicited by NMDA and kainate: effect of botulinum toxin B. Agonists were added as indicated by the horizontal bar. Cultures were incubated with botulinum toxin B 16 h before superfusion. Abcissae of left-hand panel, minutes of superfusion. Values are the means ± S.E.M. from 8 observations with NMDA and 5 with kainate (control cultures); 12 observations with NMDA and 6 with kainate (cultures treated with botulinum toxin B).

ATP release: *interaction with atractyloside and DIDS*

The mitochondrial inner membrane possesses an ADP/ATP transporter that is blocked by atractyloside. Some other intracellular membranes, for example the endoplasmic reticulum, possess a voltage-dependent anion channel that is ATP permeable and blocked by DIDS but not by atractyloside (see Discussion). Atractyloside (10 µM) had no effect on ATP overflow evoked by AMPA (30 µM) (not shown). DIDS (100 µM), when present throughout superfusion, did not change the overflow of ATP evoked by NMDA (500 µM) or AMPA (30 µM; Fig.8).

ATP release: *interaction with glibenclamide and DPC*

Glibenclamide was tested for two reasons: because, at low concentrations (1 µM), it blocks the endothelial release of ATP in response to shear stress (Hasséssian et al., 1993); and because, at high concentrations (100 µM), it inhibits the cystic fibrosis transmembrane conductance regulator (CFTR; Sheppard and Welsh, 1993), which can also mediate, or regulate, cellular ATP release. The CFTR-mediated release of ATP is also blocked by DPC (Reisin et al., 1994).

Glibenclamide (1 µM) had no effect on the overflow of ATP from astrocytes evoked by NMDA (500 µM) or AMPA (30 µM; Table 7). Glibenclamide (100 µM), in contrast, although it did not change the overflow of ATP evoked by glutamate, NMDA and kainate, significantly reduced the overflow evoked by AMPA (Table 8). Similarly, DPC (500 µM) had no effect on the release of ATP evoked by NMDA but significantly reduced that evoked by AMPA (Table 9).

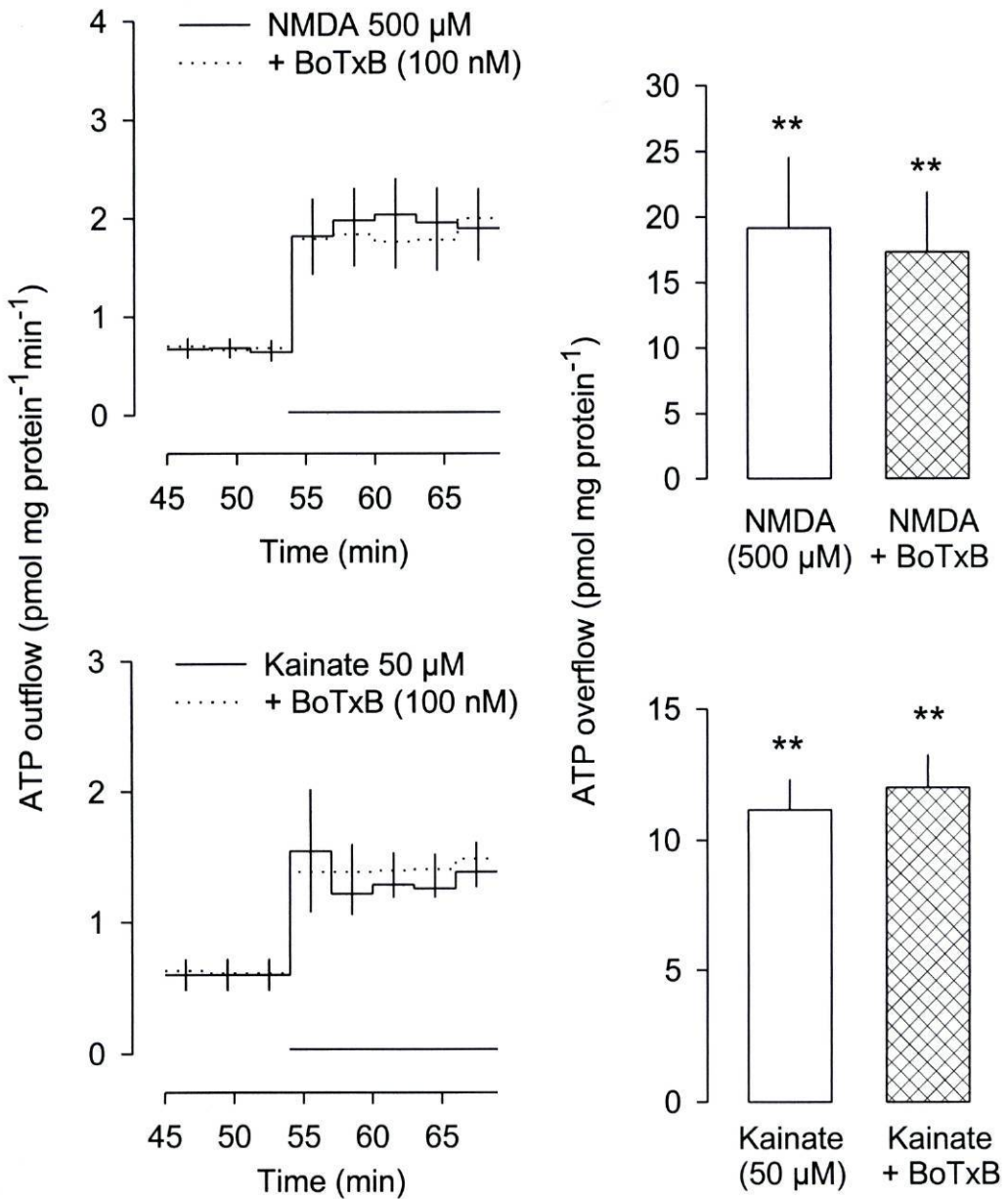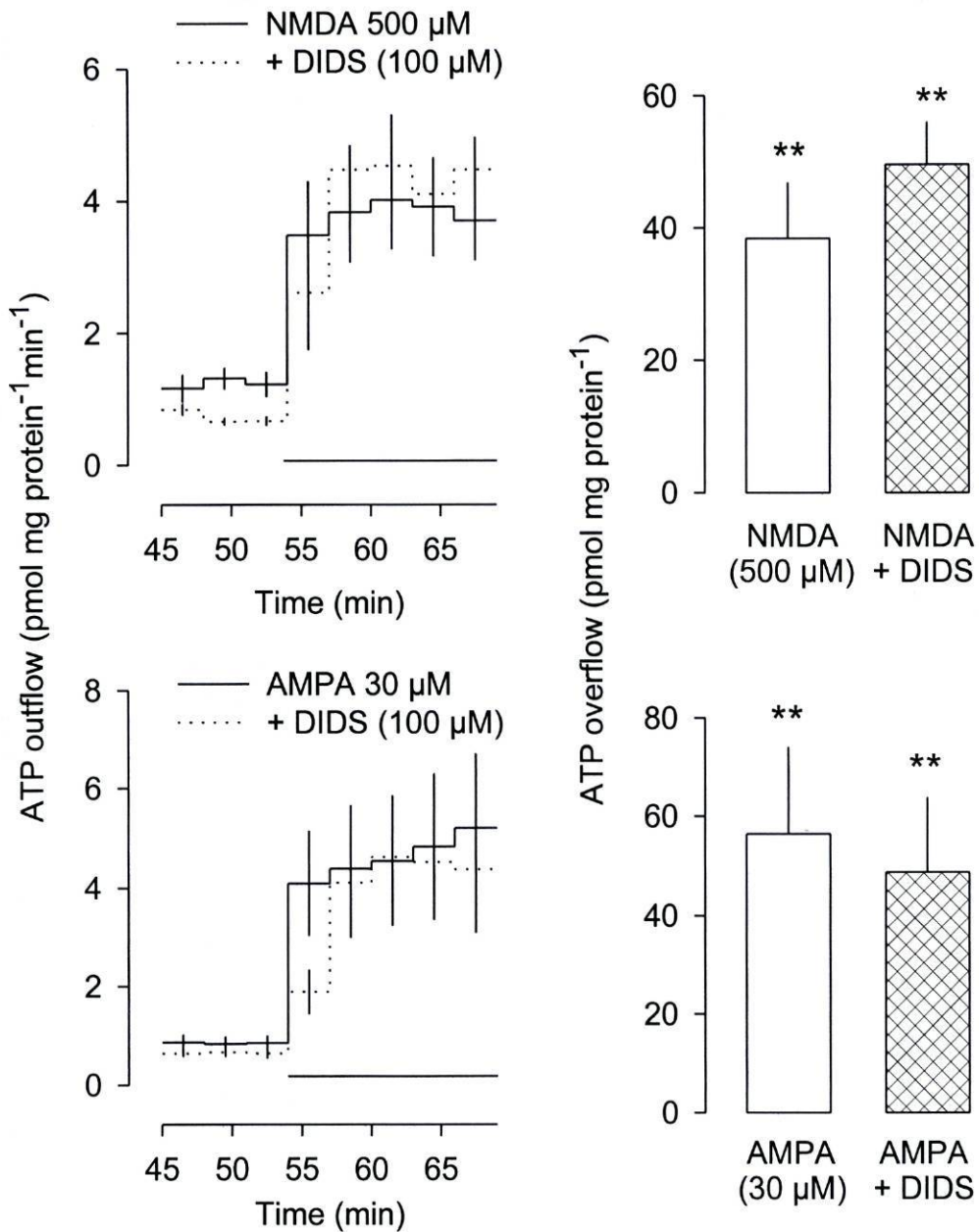**Figure 8.** Release of ATP from superfused astrocytes elicited by NMDA and AMPA: effect of DIDS (100 μM). Agonists were added as indicated by the horizontal bar. DIDS was present throughout superfusion. Abcissae of left-hand panel, minutes of superfusion. Values are the means ± S.E.M. from 6 observations with NMDA and 11 with AMPA (normal buffer); 4 observations with NMDA and 8 with AMPA (in the presence of DIDS 100 μM).

Table 7. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of glibenclamide (1 µM).

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		Solvent	Glibenclamide (1 µM)
NMDA	500	107.1 ± 12.1 (3)	87.0 ± 17.4 (3)
AMPA	30	38.5 ± 8.9 (3)	43.3 ± 9.9 (3)

Glibenclamide (1 µM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means $\pm$ S.E.M. from (n) observations.

Table 8. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of glibenclamide (100 µM).

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		Solvent	Glibenclamide (100 µM)
-		-1.2 ± 0.4 (6)	0.3 ± 0.9 (8)
Glutamate	300	6.1 ± 0.6 * (3)	4.5 ± 2.2 (3)
NMDA	500	36.2 ± 5.2 ** (6)	28.9 ± 6.7 ** (6)
AMPA	30	21.8 ± 3.2 ** (12)	8.5 ± 2.4 *†† (12)
Kainate	50	16.9 ± 2.9 ** (6)	13.2 ± 1.4 ** (5)

Glibenclamide (100 µM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. * and ** indicate significant differences from first line (no agonist; $P<0.05$ and $P<0.01$, respectively). †† indicates a significant difference from effect of agonist in the absence of glibenclamide (solvent; $P<0.01$).

Table 9. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of DPC.

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		Solvent	DPC (500 µM)
-		-0.3 ± 0.2 (3)	-0.4 ± 0.5 (5)
NMDA	100	28.9 ± 4.7 * (6)	27.8 ± 8.8 ** (5)
AMPA	30	26.9 ± 4.7 * (6)	13.0 ± 1.8 **†† (6)

DPC (500 µM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. * and ** indicate significant differences from first line (no agonist; $P<0.05$ and $P<0.01$, respectively). †† indicates a significant difference from effect of agonist in the absence of DPC (solvent; $P<0.01$).

ATP release: *interaction with lithium*

Lithium has been reported to block the release of ATP from guinea-pig ileal segments stimulated by α,β-methylene ATP or bethanechol (Katsuragi et al., 1996). At a concentration of 1 mM, lithium abolished the overflow of ATP evoked by glutamate and AMPA and greatly reduced the overflow evoked by NMDA and kainate (Table 10).

Table 10. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of lithium.

Agonist	(µM)	Agonist-evoked release of ATP (pmol per mg protein)	
		Solvent	Lithium (1 mM)
-		1.0 ± 1.2 (10)	-1.0 ± 1.2 (8)
Glutamate	300	7.4 ± 1.1 ** (6)	0.9 ± 1.2 †† (6)
NMDA	500	72.2 ± 9.7 ** (6)	24.2 ± 4.1 **†† (6)
AMPA	30	31.0 ± 4.7 ** (5)	-2.6 ± 3.2 †† (6)
Kainate	50	42.1 ± 7.9 ** (7)	16.2 ± 4.3 **†† (8)

Lithium (1 mM) was present throughout superfusion. Overflow of ATP evoked by glutamate receptor agonists was calculated by subtraction of basal outflow. Means ± S.E.M. from (n) observations. ** indicates a significant difference from first line (no agonist; $P<0.01$). †† indicates a significant difference from effect of agonist in the absence of lithium (solvent; $P<0.01$).

Discussion

The results obtained in the present work identify ATP as one adenyl compound that is released by excitatory amino acids from brain cells, and identify astrocytes as cells from which it may originate. The release of ATP from astroglia was elicited by glutamate and by selective agonists of each ionotropic glutamate receptor known, NMDA, AMPA and kainate as well trans-ACPD, a selective agonist of the metabotropic glutamate receptors devoid of activity at ionotropic receptors (Palmer at al., 1989; Manzoni et al., 1990).

The response to glutamate receptor agonists was variable; for example release of ATP elicited by NMDA was transient in some experiments (Fig. 3) but maintained in others (Fig. 6). Another example of variability is the large difference in ATP release between identically treated groups; for example, AMPA (30 µM) caused an averaged release of 18.3 pmol per mg protein in one group (Table 1) and 91.1 pmol per mg protein in another (Table 4). This variability can be explained by variations in the levels of receptor expression that may occur between different astrocyte cultures and even in different astrocytes within the same culture (Verkhratsky and Kettermann, 1996). To overcome the influences of such variability on the effects of drugs, drug-treated cultures where always compared with a strictly parallel control group.

NMDA, AMPA and kainate elicited the release of ATP from astroglial cells probably by acting through the receptors named after them. This view is supported by the fact that

these compounds released ATP at concentrations known to exert effects through NMDA, AMPA, and kainate receptors, respectively. Among those effects are the release of adenosine from rat brain cortex and hippocampal brain slices (Craig and White, 1993; Pedata et al., 1991) and the indirect adenosine-mediated inhibition of noradrenaline release from the rabbit brain cortex (von Kügelgen et al., 1993).

The effects of selective ionotropic glutamate receptor antagonists confirmed the view that NMDA, AMPA and kainate evoked the release of ATP through activation of the respective receptors; i) the NMDA receptor-selective antagonist AP5 (Evans et al., 1982) attenuated the effect of NMDA but not of AMPA and kainate; ii) the AMPA receptor-selective antagonist NBQX (Sheardown et al., 1990) abolished the effect of AMPA but not of kainate; iii) the kainate receptor-selective antagonist GAMS (Jones et al., 1984; Turski et al., 1985) blocked the effect of kainate.

Two observations on the effects of NMDA require additional comment. First, NMDA released ATP despite the presence of a physiological concentration of Mg^{2+}, in contrast to the classical NMDA receptor-mediated effects (Nowak et al., 1984). Resistance to Mg^{2+} has also been observed for the NMDA-induced release of adenosine from rat brain slices (Hoehn et al., 1990; Pedata et al., 1991) and for the NMDA-induced, adenosine-mediated inhibition of the release of noradrenaline in the rabbit brain cortex (von Kügelgen et al., 1993). A possible reason is a low membrane potential of the glial cells under our culture and superfusion conditions, alleviating the Mg^{2+} block of the NMDA receptor channel. An alternative explanation is the occurrence of spare NMDA receptors as it has been suggested for the NMDA-evoked adenosine release (Hoehn et al., 1990). According to this hypothesis, the block of Mg^{2+} can be overcome by the high concentrations of NMDA used. Second, the AMPA receptor antagonist NBQX prevented not only the release of ATP elicited by AMPA but also the release elicited by NMDA. Possibly, NBQX blocked the glycine site of the

astrocyte NMDA receptor since other quinoxaline derivatives have been shown to block the glycine site of NMDA receptors at low concentrations and thus block the NMDA-receptor mediated responses (Kessler et al., 1989).

Glutamate receptor agonists exert additional effects on glial cells, such as depolarisation and an increase in intracellular Ca^{2+}. In glial cells in situ, the effects seem to be mediated by NMDA, AMPA as well as by kainate receptors (Porter and McCarthy, 1995). Immunocytochemical studies on the distribution of cortical NMDA receptor have shown that cortical astrocytes express NMDA receptors (Conti et al., 1997). However, the presence of NMDA receptors in astroglial cells is still a matter of debate. In most of the studies on cultured glial cells, NMDA receptors mediating membrane depolarisation and an increase in Ca^{2+} have not been detected (see Teichberg, 1991). Recent reports suggest that glial cells can express functional NMDA receptors. For instance, in human cultured astrogial cells NMDA has been shown to stimulate release of prostaglandin E_2 (Mollace et al., 1995). The retinal Müller glial cells express one of the essential components of the NMDA receptor-channels, the NMDAR1 subunit, and are also responsive to NMDA even at resting membrane potential (Puro et al., 1996).

In the present study, glutamate, one of the "endogenous" ligands of glutamate receptors, also released ATP. However, it was not possible to identify clearly the receptor involved. The selective antagonism by NBQX, but not AP5 and GAMS (Table 2), suggests the involvement of AMPA receptor. However, NBQX also blocked the effect of NMDA and a simultaneous activation of AMPA and NMDA receptors can not be ruled out. Glutamate may also have acted at metabotropic receptors because trans-ACPD, a selective agonist of metabotropic glutamate receptors, also evoked ATP release in the present study.

Variability in the glutamate receptor involved has also been observed on glutamate evoked release of other adenyl compounds in the brain. For instance, glutamate evokes

release of adenosine from rat brain slices through NMDA as well as non-NMDA receptors, (Craig and White, 1993; Hoehn and White, 1990a). In rabbit cortical slices glutamate evokes adenosine release through non-NMDA receptors only (see von Kügelgen and Starke, 1995) whereas and in cortical synaptosomes, uptake of glutamate seems to be a prerequisite for the release (Hoehn and White, 1990b).

The occurrence of contaminating neurones in astroglial cultures has always been regarded as negligible. Neurones could be found immediately after seeding, however these neurones disappeared when the proliferating astroglial cells began to form a monolayer. Recently it has been demonstrated that some neuronal percursors do not turn into differentiated cells, but keep their ability to proliferate (Weiss et al., 1996; Eriksson et al., 1998). Neuronal precursors seem to be capable of survival and to proliferate in culture, as it has been demonstrated in primary cultures of astroglial cells prepared from neocortex of new-born rats (Hildebrand et al., 1997). The first neuronal cells appear shortly before confluence, when the glial monolayer is being formed. After confluence, these still undifferentiated cells increase in number and later become immunohistochemically positive for the neurone-specific marker microtubule associated protein 2a,b (MAP2a,b). In spite of the known interest of this finding, i.e. providing a model for the investigation of neuronal-glial interactions, it introduces another variable in the astroglial model.

In the present study, the observation that astroglial cultures prepared from the rat cerebral cortex may contain neurones has also been confirmed. However, the release of ATP elicited by glutamate agonists was not changed when the contamination by neurones was reduced by about 2/3, suggesting that neurones contributed little, if at all, to ATP release.

The role of Ca^{2+} in ATP release

Astrocytes *in situ* as well as in culture respond to glutamate receptor agonists with an increase in intracellular Ca^{2+} (Cornell-Bell et al., 1990; Porter and McCarthy, 1995). Both transmembrane influx and release of Ca^{2+} from intracellular stores can contribute (Glaum et al., 1990). Transmembrane entry predominates upon activation of NMDA and kainate receptors, whereas mobilisation from intracellular stores predominates upon activation of metabotropic glutamate receptors (Ahmed et al., 1990; Porter and McCarthy, 1995). AMPA receptors, although ionotropic receptors, can also trigger metabotropic transduction mechanisms including possibly the mobilisation of intracellular Ca^{2+} (Wang et al., 1997; Bezzi et al., 1998). From what is known about glial glutamate receptors and Ca^{2+}, therefore, a role of Ca^{2+} in the release of ATP from astrocytes would seem possible.

An increase in intracellular Ca^{2+} seems to be sufficient to evoke the release of ATP from astrocytes: the Ca^{2+} ionophore ionomycin elicited such release, and it did so only in the presence but not in the absence of external Ca^{2+}.

Calcium may cross the plasma membrane either through the ionotropic glutamate receptors themselves or through VDCCs that are opened by depolarisation (Barres et al., 1990b; Barres, 1991). The release of ATP evoked by NMDA and kainate from astroglial cells, was abolished or greatly reduced by withdrawal of extracellular Ca^{2+}, by cadmium and nicardipine. These findings support the following: i) Ca^{2+} is needed for the release of ATP triggered by NMDA and kainate receptor activation; ii) the Ca^{2+} involved comes from the extracellular space and; iii) Ca^{2+} influx occurs partly through VDCCs, mainly of the L-type.

In contrast, release of ATP evoked by AMPA was not reduced by withdrawal of external Ca^{2+} and was not changed by cadmium and nicardipine. A similar pattern was observed for the ATP release evoked by *trans*-ACPD. These findings exclude a major

contribution of Ca^{2+} entry to AMPA- and *trans*-ACPD-evoked ATP release. They do not, of course, exclude a role of Ca^{2+} mobilised from intracellular stores (see above).

Agonists at any of the three ionotropic glutamate receptors also release adenosine from brain tissue, and some of the adenosine may be formed from released ATP (Craig and White, 1993). In accord with the present results on glial ATP release, in rat brain slices the release of adenosine elicited by NMDA depended on extracellular Ca^{2+} whereas the release elicited by AMPA did not (Craig and White, 1993).

On the mechanism of ATP release

Once the role of Ca^{2+} on the release of ATP evoked by the glutamate receptor agonists from astroglial cells had been established, attempts were made to establish the mechanism(s) involved in the release of ATP from astroglial cells.

The release of ATP was not accompanied by release of the cytoplasmic enzyme LDH, indicating that there was no immediate major damage to the cells upon exposure to the excitatory amino acids. The fraction of cellular ATP that is released upon exposure to glutamate receptors agonists is also very low (less that 1%), lower that one's might expect to be observed as a consequence of cell lysis.

In order to explain the mechanism involved in the release of ATP from astrocytes, two other hypotheses have been considered and tested. One was the involvement of a plasma membrane carrier or channel. The other was the occurrence of a vesicular release similar to neuronal exocytosis. The possibility of a neurone-like exocytosis, the possible involvement of carrier molecules, and the effect of lithium will be discussed in turn.

Neurone-like exocytosis?

Astrocytes express cellubrevin, synaptobrevin II and syntaxin, three of the proteins involved in the pore formation during neural exocytosis (Parpura et al., 1995a).

A Ca^{2+}-dependent glutamate release, possibly of exocyotic nature, has been demonstrated to occur from astrocytes in primary culture, upon activation of bradykinin receptors (Jeftinija et al., 1996; Parpura et al., 1995b). In accord with a neurone-like exocytosis, botulinum toxin B and tetanus toxin, which proteolyses synaptobrevin II (Huttner, 1993), prevented the release of glutamate by bradykinin (Jeftinija et al., 1997).

The hypothesis of exocytotic glutamate release from astrocytes was also supported by the effect of α-latrotoxin, a component of the black widow spider venom. α-Latrotoxin binds to a receptor on nerve terminals and induces the release of neurotransmitter by directly stimulating the secretory machinery (Petrenko, 1993). One of the receptors for this toxin is a member of a protein family, the neurexins, which are transmembrane proteins that bind intracellulary to the synaptic vesicle protein, synaptotagmin (Ushkaryov et al., 1992). Interacting directly with synaptic proteins, α-latrotoxin bypasses the Ca^{2+}-requirement of transmitter release. α-Latrotoxin also evoked the release of glutamate from cultured astrocytes suggesting that astrocytes have a vesicle release machinery similar to the vesicular apparatus of the nerve terminal (Parpura et al., 1995a).

NMDA- and kainate-evoked release of ATP from astrocytes was Ca^{2+}-dependent. The possibility of an exocytotic mechanism was excluded since neither α-latrotoxin released ATP, nor did botulinum toxin B reduce the release of ATP elicited by NMDA and kainate.

Membrane passage by means of channels or transporters?

Besides cell damage and exocytosis, a transport molecule or channel is a third mechanism by which ATP might cross the astrocyte plasma membrane.

ATP is synthesised in mitochondria and is then transported across mitochondrial membranes to the cytoplasm. ATP is also necessary in other compartments of the cell such as the endoplasmic reticulum and Golgi apparatus. Furthermore, in nerve terminals ATP is also concentrated in vesicles being stored with neurotransmitters. To reach these compartments ATP must cross the membranes that surround them. This task is accomplished by several intracellular transporters or channels; the ADP/ATP transporter of the mitochondrial inner membrane, the ATP-permeable anion channel of the endoplasmic reticulum and other intracellular compartments, and the vesicular transporter that concentrates ATP within vesicles. Based on this knowledge the hypothesis that one of these systems could operate in the plasma membrane, and be responsible for the permeation of membrane to ATP, was considered.

Atractyloside blocks the ADP/ATP exchanger of the mitochondrial inner membrane (Ziegler and Penefsky, 1993) and the vesicular transporter (Gualix et al., 1996). It did not affect the release of ATP elicited by AMPA. DIDS is a chloride channel antagonist, which also blocks a pore-forming protein expressed for example in the endoplasmic reticulum and Golgi apparatus and involved in ATP transport (Clairmont et al., 1992; Guillén and Hirschberg, 1995; Shoshan-Barmatz at al., 1996). DIDS had no effect on ATP release evoked by NMDA and AMPA. These two observations exclude the possibility that these intracellular ATP transporters occur in the plasma membrane and are responsible for the release of ATP from astrocytes in response to glutamate receptor activation.

Furthermore, release of ATP from endothelial cells upon shear stress and from epithelial cells has been shown to occur by a glibenclamide sensitive mechanism. In blood vessel endothelia ATP release elicited by shear stress is blocked by a low concentration of glibenclamide (1 µM; Hasséssian et al., 1993). However, at a concentration of 1 µM glibenclamide did not influence ATP release evoked by NMDA and AMPA, from astroglial cells, which excludes this mechanism as well.

In contrast to these negative findings, a significant effect was obtained when the role of CFTR (which is involved in the release of ATP from epithelial cells) was examined. CFTR is a member of the ATP-binding cassette protein family which has been reported to serve as an ATP-conducting channel in the plasma membrane. CFTR has been shown to be expressed not only in epithelial cells but also in several cell types that release ATP such as muscle cells, erythrocytes and endothelial cells (Yamazaki and Hume, 1997; Sprague et al., 1998; Tousson et al., 1998). CFTR-mediated release of ATP is blocked by higher concentrations of glibenclamide (100 µM) as well as by DPC, but not by DIDS (Reisin et al., 1994; Schwiebert et al., 1995).

Both glibenclamide (100 µM) and DPC, while having no significant effect on the release of ATP evoked by NMDA and kainate significantly reduced the effect of AMPA. The effect of glibenclamide on AMPA evoked ATP release was not due to blockade of ATP-dependent K^+-channels, because these (like the endothelial release of ATP caused by shear stress) are also blocked by glibenclamide (1 µM; Quast and Cook, 1989), the concentration that did not change AMPA-evoked ATP release (see above).

Whether astrocytes in fact express CFTR or a similar transporter is not known. The present results suggest that this may be the case and that a molecule with a CFTR-like pharmacology may mediate the AMPA-induced, but not the NMDA- and kainate-induced, exit of ATP from astrocytes. Thus ATP release evoked by NMDA and kainate seems to be

mediated by a CFTR-independent mechanism. Release of ATP by other mechanisms besides CFTR-mediated transport has also been described in other systems, suggesting that ATP release can proceed through independent routes, even in the same cell type (Mitchell et al., 1998). For example, ocular cilliary epithelial cells release ATP by several mechanisms; hypotonic conditions induce the release of ATP by a mechanism that is not blocked by glibenclamide and DPC, thus not mediated by CFTR, but is sensitive to chloride channel blockers whereas ionomycin releases ATP through a mechanism that is neither mediated by CFTR nor sensitive to chloride channel blockers (Mitchell et al., 1998). Other mechanisms of ATP release, CFTR-independent, have also been shown to be physiological in cells either expressing or not the CFTR (Grygorczyk and Hanrahan, 1997; Watt et al., 1998).

The effect of lithium

Lithium blocks the release of ATP from smoth muscle cells elicited by activation of P_{2Y} and muscarinic receptors (Katsuragi et al., 1996). In the present experiments, lithium abolished the release elicited by AMPA and significantly reduced the release of ATP evoked by NMDA and kainate. What could be the mechanism of action of lithium and in what extent this result may help to clarify the mechanisms involved on AMPA, NMDA and kainate evoked ATP release?

The best-studied primary effect of lithium is inhibition of inositol monophosphatase and, hence, inhibition of the inositol phosphate signal transduction pathway because of depletion of inositol (Atack et al., 1995). In astrocytes, inositol phosphates are formed upon activation of metabotropic glutamate receptors (Steinhäuser and Gallo, 1996), but inositol phosphate formation upon activation of ionotropic glutamate receptors was never shown. In neurones, activation of all three ionotropic receptors leads to inositol phosphate formation

(Hartmann and Müller, 1993; Reis et al., 1995), probably due to activation of phospholipase C subsequent to an increase in intracellular Ca^{2+} (Baird and Naorski, 1990; Hartmann and Müller, 1993; Reis et al., 1995). On the basis of these findings one may speculate that release of ATP evoked by NMDA, AMPA and kainate might involve inositol phosphates, and that inhibition by lithium of inositol phosphate synthesis is the mechanism of blockade of the glutamate receptor-mediated ATP release. However, lithium exerts many other effects on cells (see Risby et al., 1991), and other mechanisms cannot be ruled out. Also, the mechanisms by which lithium influenced the release of ATP elicited by AMPA on the one hand and the release of ATP elicited by NMDA and kainate on the other hand might differ.

When one summarises the influence of various interventions on the release of ATP by the three selective ionotropic glutamate receptor agonists, the latter fall into two groups (Table 11). One comprises NMDA and kainate, the releasing effect of which depended on extracellular Ca^{2+}, was not changed by glibenclamide (100 µM) and DPC, and was reduced but not abolished by lithium. The other comprises AMPA only, the effect of which did not depend on extracellular Ca^{2+}, was reduced by glibenclamide (100 µM) and DPC, and was abolished by lithium. The effect of glutamate was intermediate.

Glutamate possibly elicits release of ATP from astrocytes through both NMDA and AMPA receptors; such a dual action may be responsible for its intermediate behaviour in the present study (Table 11).

Table 11. Glutamate receptor agonist-evoked release of ATP from superfused astrocytes: effect of Ca^{2+} withdrawal and drugs.

Effect of:	ATP release evoked by				
	Glutamate (µM)	NMDA (µM)	AMPA (µM)	Kainate (µM)	t-ACPD (µM)
Calcium withdrawal	300 → 1000 ↓	500 ↓	10 → 30 →	50 ↓	300 →
Cadmium (1000 µM)	300 → 1000 ↓	500 ↓	30 →	50 ↓	
Nicardipine (10 µM)		500 ↓	30 →	50 ↓	
Botulinum toxin B (0.1µM)		500 →		50 →	
Atractyloside (10 µM)			30 →		
DIDS (100 µM)		500 →	30 →		
Glibenclamide (1 µM)		500 →	30 →		
Glibenclamide (100 µM)	300 →	500 →	30 ↓	50 →	
DPC (500 µM)		100 →	30 ↓		
Lithium (1000 µM)	300 ↓	500 ↓	30 ↓	50 ↓	

↓ and → indicate, respectively, a decrease and no change of the release of ATP evoked by glutamate receptor agonists.

Conclusions

Cultured astroglial cells respond to activation of ionotropic glutamate receptors with release of ATP. They thus resemble other non-neuronal cells such as smooth muscle (Katsuragi et al., 1990; von Kügelgen and Starke, 1991b; Kurz et al., 1994) and endothelial cells (Yang et al., 1994) which release ATP upon activation of certain (although not glutamate) receptors. Neurones release ATP by exocytosis (see von Kügelgen et al., 1994b). Two different mechanisms seem to be involved in the release of ATP from astroglial cells upon activation of ionotropic glutamate receptors. The NMDA- and kainate-induced release of ATP requires an influx of Ca^{2+}, is not due to neurone-like exocytosis, is not mediated by CFTR or a mechanism regulated by CFTR, and is reduced (by an unknown mechanism) but not abolished by lithium. The AMPA-induced release does not require extracellular Ca^{2+}, may be mediated by CFTR or a mechanism regulated by CFTR, and is abolished (by an unknown mechanism) by lithium.

Purines are important modulators of neuronal functions (Zimmermann, 1994; Burnstock, 1997). They also may act as trophic factors for glial cells and neurones (Neary et al., 1996). The ability of astrocytes to release ATP and respond to ATP (King et al., 1996) suggests that ATP may act as an autocrine or paracrine messenger between these cells, and possibly between astrocytes and neurones.

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