ATP Inhibits NMDA Receptors after Heterologous Expression and in Cultured Hippocampal Neurons and Attenuates NMDA-Mediated Neurotoxicity

Stefanie Ortinau,[1]* Bodo Laube,[2]* and Herbert Zimmermann[1]

[1]Biozentrum der J. W. Goethe-Universität, Arbeitskreis Neurochemie, Zoologisches Institut, D-60439 Frankfurt am Main, Germany, and [2]Max-Planck-Institut für Hirnforschung, D-60439 Frankfurt am Main, Germany

We investigated the potential of ATP to inhibit heterologously expressed NMDA receptor subunit combinations, NMDA-induced currents in cultured hippocampal cells, and NMDA-induced neurotoxicity. The effect of ATP on diheteromeric NR1a/NR2A-D NMDA receptor (NR) combinations expressed in *Xenopus laevis* oocytes was studied by voltage-clamp recording. ATP strongly inhibited NMDA-induced inward currents only at the NR1a/NR2B receptor combination. At NMDA concentrations corresponding to the EC_{50} value (20 μM), ATP revealed an IC_{50} value of 135 μM. Mutation studies suggest that ATP exerts its inhibition via the glutamate-binding pocket of the NR2B subunit. Inosine 5′-triphosphate (ITP), GTP, and AMP also inhibited the recombinant NR1a/NR2B receptor, whereas UTP and CTP, ADP, or adenosine had no or only a small effect. Correspondingly, ATP inhibited NMDA-induced but not kainate-induced currents at cultured hippocampal neurons. An abundant expression of the NR2B subunit in the cultured neurons was verified by immunocytochemistry and blockade of NMDA-induced currents by the NR2B-selective antagonist ifenprodil. In addition we studied the role of ATP in NMDA-mediated neurotoxicity using cultured rat hippocampal cells. ATP exhibited a dose-dependent rescue effect when coapplied with the excitotoxicant NMDA, in contrast to ADP, AMP, and adenosine. The effect of ATP was mimicked by GTP and ITP but not by UTP and CTP. ATP had no effect on kainate-elicited neurotoxicity. Our results suggest that ATP can act as an inhibitor of NMDA receptors depending on receptor subunit composition and that it can attenuate NMDA-mediated neurotoxicity that is mediated neither by ATP nor by adenosine receptors.

Key words: **adenosine; ATP; glutamate; GTP; hippocampus; kainate; neurotoxicity; NMDA receptor; P2 receptor**

Introduction

In the CNS, extracellular ATP exerts a wide variety of functions, including presynaptic inhibition or facilitation of transmitter release, fast excitatory transmission, and signaling between astrocytes (Khakh, 2001; Robertson et al., 2001). ATP is co-released with a number of neurotransmitters (Khakh, 2001) and also released from activated glial cells (Haydon, 2001) and injured cells (Neary et al., 1996). Despite the wide distribution of the ionotropic P2X receptor subunits in the CNS (Nörenberg and Illes, 2000; Soto and Rubio, 2001), only scarce examples of direct ATP-mediated excitatory synaptic responses were reported (Khakh, 2001; Pankratov et al., 2002a). ATP-gated cation channels were recorded in subsets of neurons within brain slices from several brain regions (Khakh et al., 1997; Wong et al., 2000, Pankratov et al., 2002a). Although the ionotropic P2X receptors are preferentially activated by ATP, the similarly widely distributed metabotropic P2Y receptors can be activated by additional nucleotides such as ADP, UTP, and UDP (Ralevic and Burnstock, 1998).

Also GTP may be involved in extracellular signaling. GTP competitively inhibits ligand binding to NMDA and non-NMDA receptors (Monahan et al., 1988; Baron et al., 1989; Gorodinsky et al., 1993) and ligand-activated cellular responses (Baron et al., 1989; Paas et al., 1996). After *Xenopus* oocyte expression of the homomergic GluR1 subtype of the AMPA receptor or the heterodimeric NMDA NR1/NR2A receptor, GTP was found to block the agonist-elicited current responses in a dose-dependent and competitive manner (Paas et al., 1996). GTP could thus directly antagonize glutamate receptor-mediated neurotransmission. This raises the question of extracellular nucleotide availability. In contrast to ATP, the presence of GTP in the extracellular milieu has not yet been investigated. Although both GTP and ATP were found to be stored in cholinergic synaptic vesicles and in chromaffin granules, vesicular ATP concentrations were found to be sevenfold higher than vesicular GTP concentrations (Zimmermann, 1994). Because NMDA receptors play a central role in the physiology and plasticity of central synaptic transmission as well as in various acute and chronic disorders of the nervous system (Dingledine et al., 1999), we analyzed the inhibitory potential of ATP on NMDA receptors with varying subunit combinations.

NMDA receptors exist as heteromeric assemblies with essential glycine-binding NR1 subunits and varying contributions of the glutamate-binding NR2A-D subunits, and possibly also NR3 (Cull-Candy et al., 2001). The expression of these subunits varies between brain regions as well as during development and governs the functional specificity of NMDA receptor subtypes (Mori and

Received Feb. 20, 2003; revised March 24, 2003; accepted May 24, 2003.

This work was supported by Deutsche Forschungsgemeinschaft (SFB 269, A4 to H.Z.; La 1086/4-1 to B.L.) and the Fonds der Chemischen Industrie.

*S.O. and B.L. contributed equally to this work.

Correspondence should be addressed to Herbert Zimmermann, Biozentrum der J. W. Goethe-Universität, Arbeitskreis Neurochemie, Zoologisches Institut, Marie-Curie-Strasse 9, D-60439 Frankfurt am Main, Germany. E-mail: h.zimmermann@zoology.uni-frankfurt.de.

 0270-6474/03/234996-08$15.00/0

Mishina, 1995; Tovar and Westbrook, 1999; Cull-Candy et al., 2001; Janssens and Lesage, 2001). The induction of hippocampal glutamate toxicity has recently been attributed to the activation of extrasynaptic NMDA receptors predominantly composed of NR1/NR2B subunits (Hardingham et al., 2002). We therefore tested the effect of ATP also on NMDA-mediated currents in cultured hippocampal neurons and NMDA-induced neurotoxicity. Our results suggest that ATP can act as an inhibitor of NMDA receptors depending on receptor subunit composition and that it can attenuate NMDA-mediated neurotoxicity that is mediated neither by ATP nor by adenosine receptors.

Materials and Methods

Reagents. Nucleotides (ATP, ADP, AMP, GTP, UTP, ITP, CTP), adenosine, P^7,P^4-di(adenosine-5′) tetraphosphate (Ap_4A), 8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid (suramin), 2-methylthio ATP (2MeSATP), 3-*O*-(4-benzoyl)benzoyl ATP (BzATP), 1-amino-4-[[4-[[4-chloro-6-[[3 (or 4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid) (reactive blue 2), and NMDA were purchased from Sigma (Taufkirchen, Germany). Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), α,β-methylene ATP (α,β-meATP), α,β-methylene ADP (α,β-meADP), dizocilpine maleate (MK-801), [2S-(2α,3β,4β)]-2-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid (kainate), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), ifenprodil, and tetrodotoxin were obtained from RBI Sigma. Reagent grade liquids for HPLC were purchased from Merck (Darmstadt, Germany).

Neural cell culture. Hippocampi prepared from 1-d-old Wistar rats were incubated in Neurobasal medium with papain (1 mg/ml) (Sigma) and 0.2 mg/ml bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) for 20 min at 37°C. Tissue was dissociated by several passages through a fire-polished Pasteur pipette and centrifuged for 10 min (45 × *g*). Cells were plated on poly-L-lysine-coated glass coverslips (0.1 mg/ml) at a density of 100,000 cells per well or on poly-L-lysine-coated 24- or 96-well plates at a density of 120,000 or 20,000 cells per well, respectively. Cells were maintained in medium, greatly reducing astrocyte proliferation (Brewer et al., 1993) [neurobasal medium containing B27 supplement, GlutaMax-I Supplement (all from Life Technologies, Karlsruhe, Germany)] and penicillin/streptomycin (Sigma) at 37°C in a humidified incubator at 5% CO_2.

Electrophysiological analysis of recombinant receptors and cultured cells. Plasmid cDNAs of the NMDA receptor subunits, linarized by *Not*I (NR1a, NR2A, NR2B), *Xba*I (NR2C), and *Eco*RI (NR2D), respectively, were used for *in vitro* cRNA synthesis (Wittekindt et al., 2000). Procedures used for *Xenopus* oocyte isolation and maintenance for 2–7 d at 19°C, oocyte expression, and voltage-clamp recording of agonist responses in Mg^{2+}-free frog Ringer's solution at a holding potential of −70 mV have been described previously (Laube et al., 1997).

Cultured hippocampal cells [25–35 d *in vitro* (DIV)] were mounted under an inverted microscope (Axiovert 35, Zeiss, Jena, Germany) and superfused continuously (0.5 ml/min) at room temperature (21–25°C) with an extracellular bathing solution containing (in mM): 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 20 glucose, and 10 HEPES, adjusted to pH 7.2 with NaOH. Agonist-induced currents were recorded from neuronal somata in the whole-cell configuration of the patch-clamp technique and obtained with an EPC-9 amplifier (HEKA, Lambrecht, Germany) controlled by HEKA software, sampled at 20 Hz, and stored on disk (Laube et al., 1995). Effects of ATP on NMDA-induced currents were analyzed after superfusing the neurons with ATP for 5 sec before and during agonist application using NMDA at a concentration of 30 μM and glycine at 10 μM in the presence of 0.5 μM tetrodotoxin. The membrane potential was clamped at −70 mV in all experiments. Electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) with a Zeitz DMZ Universal Puller (Zeitz Instruments, Augsburg, Germany) to yield tip resistances of 2–6 MΩ. Series resistances after whole-cell formation (5–20 MΩ) were compensated for 50–90%. Patch pipettes contained (in mM): 120 CsCl, 20 tetraethylammonium chloride, 1 $CaCl_2$, 2 $MgCl_2$, 1 ATP, 11 EGTA, and 10 HEPES, pH 7.2. Drugs were applied with the bath solution using a microcapillary application system (DAD-12, Adams and List, Westbury, NY). Results represent means ± SEM. The significance of the data was evaluated using Student's *t* test and considered to be statistically significant at $p < 0.05$.

Immunocytochemistry. For immunolocalization, cells (33–37 DIV) were fixed in 100% ice-cold methanol for 7 min at −20°C. After they were washed several times in PBS, cells were blocked with 5% BSA in PBS for 20 min and exposed to primary antibodies in 1% BSA in PBS for 20 min: rabbit polyclonal anti-NMDAR1 (1:100) (Chemicon International, Hofheim, Germany); rabbit polyclonal anti-NMDAR2B (1:100) (Calbiochem, Bad Soden, Germany); mouse monoclonal anti-synaptobrevin II (1:200) (Synaptic Systems, Göttingen, Germany). After 20 min of incubation with secondary antibodies [fluorescein isothiocyanate-conjugated anti-mouse (1:200) and indocarbocyanine-conjugated anti-rabbit (1:400) (both from Dianova, Hamburg, Germany)] in 1% BSA containing PBS, cells were washed three times in PBS and mounted in Tris-HCl, glycerol with 2.5% 1,4-diazabicyclo-[2.2.2]-octane (Fluka, Neu-Ulm, Germany). Controls were performed in the absence of primary antibodies. Confocal microscopy was performed with a Zeiss LSM 5 Pascal (Zeiss, Oberkochen, Germany) confocal microscope and Zeiss objectives, using dual laser excitation.

Application of drugs in neurotoxicity assays. After 33–37 DIV, neurotoxicity was induced by exposing cells to 30 μM NMDA or 300 μM kainate in medium either alone or in combination with nucleotides, nucleosides, or glutamate and kainate antagonists. Antagonists were added to the cells 1 min before agonists, except for ifenprodil, which was added 5 min before NMDA. All agents were dissolved in water. Control cultures were treated with an equal volume of water. Neuronal death was quantified 24 hr later. Continued drug application was preferred to short-term application because exchange of medium and subsequent washing steps resulted in spontaneous neural death and increased data variance.

Cellular ELISA. Neuronal cell death was quantified in 96-well plate cell cultures using a mouse monoclonal antibody against the neuron-specific microtubule-associated protein MAP-2 (clone HM-2, 1:1000) (Sigma) and a cellular ELISA. After methanol fixation and application of the primary antibody, cells were incubated for 20 min with a secondary peroxidase-linked anti-mouse antibody (1:100) (Amersham Biosciences, Freiburg, Germany). This was followed by washing in PBS and 0.05 M phosphate-citrate buffer, pH 5.0, and incubation with *o*-phenylenediamine dihydrochloride (0.4 mg/ml) and 0.04% H_2O_2 for 10–20 min. The reaction was stopped by addition of 25 μl 3N HCl to 100 μl of reaction mixture. The absorbance was measured at 450 nm using a microtiter plate reader (Metertech, Nunc, Wiesbaden, Germany). In every experiment, six to eight wells were analyzed for each individual experimental condition.

Quantification of nucleotide hydrolysis. Hydrolysis of nucleotides added to intact cells was determined at 37°C in primary hippocampal cells seeded in 24-well plates (120,000 cells per well) or in 96-well plates (20,000 cells per well) after 33–37 DIV. Cells were incubated in 1 ml or 180 μl, respectively, of medium containing substrate nucleotides. Samples collected from the culture supernatants were boiled for 5 min, immediately chilled on ice, and centrifuged at 14,500 × *g* for 20 min at 4°C. Nucleotides and nucleosides were separated and quantified by HPLC (Jasco, Groβ-Umstadt, Germany) (Vollmayer et al., 2001). The absorbance at 260 nm was monitored continuously, and the nucleotide and nucleoside concentrations were determined from the area under each absorbance peak. HPLC data were analyzed using the Software Borwin, Version 1.21 (JMBS Developments, Le Fontanil, France).

Results

Effect of ATP on recombinant NMDA receptors

To investigate the inhibitory potential of ATP on NMDA receptors, we expressed heteromeric NR1a/NR2A, NR1a/NR2B, NR1a/NR2C, and NR1a/NR2D NMDA receptor subunit combinations in *Xenopus laevis* oocytes. The effect of ATP (500 μM) was analyzed by voltage-clamp recording at NMDA concentrations eliciting a half-maximal response of the respective subunit combination (Fig. 1*A*). A high inhibitory potency of ATP was ob-

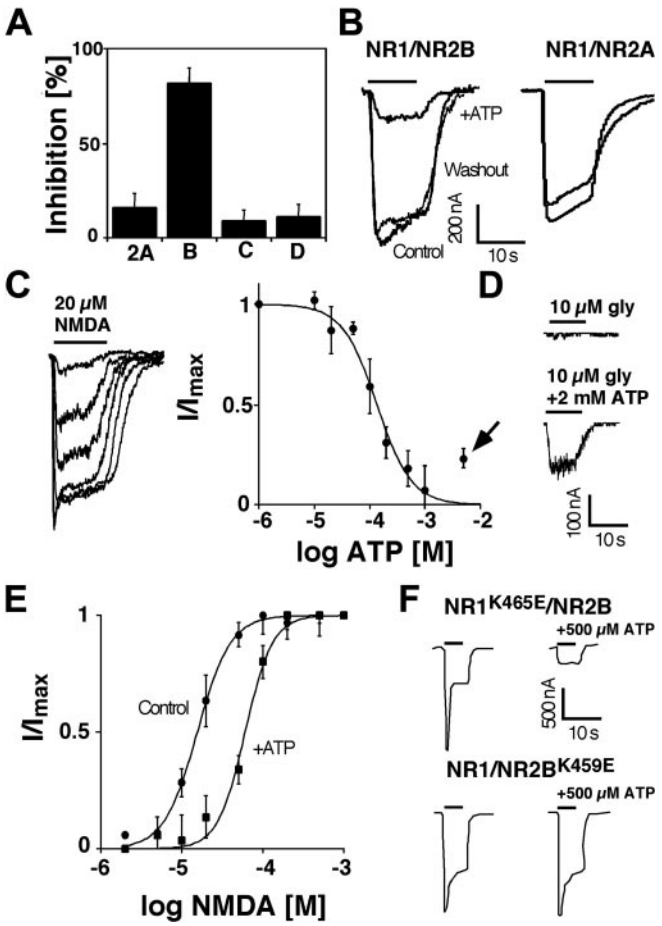

Figure 1. Effect of ATP on agonist-induced NMDA receptor currents of recombinant wild-type and mutant NMDA receptors. *A*, Bar diagram displaying the relative inhibition by 500 μM ATP of four different subunit combinations (NR1a/NR2A-D) expressed in *Xenopus* oocytes, at NMDA concentrations eliciting a half-maximal response of the respective subunit combination (for the NR1a/NR2A, NR1a/NR2B, and NR1a/NR2C combinations: 20 μM NMDA; for the NR1a/NR2D combination: 10 μM NMDA; $n = 3–6$). *B*, Reversible inhibition by 500 μM ATP of recombinant heteromeric NMDA receptors. Traces represent agonist-evoked responses of wild-type NR1a/NR2B and NR1a/NR2A receptors at 20 μM NMDA in the absence, in the presence, and after the washout of 500 μM ATP. *C*, Concentration-dependent inhibition of NR1a/NR2B receptor currents by ATP (IC_{50} value of 135 $\pm$ 14 μM; $n = 3$). At very high ATP concentrations the inhibitory effect of ATP is attenuated (arrow). *D*, Agonistic properties of high concentrations of ATP. Superfusion of NR1a/NR2B NMDA receptors with 10 μM glycine and 2 mM ATP elicited currents in the absence of NMDA. *E*, NMDA dose–response properties in the presence and absence (control) of 500 μM ATP (NR1a/NR2B) (17 $\pm$ 4.2 and 54 $\pm$ 8.3 μM, respectively; $n = 3$; $p < 0.05$). *F*, Agonist response properties of mutant NMDA receptors in the absence or presence of ATP (500 μM). Traces represent NMDA-evoked responses of mutant NR1^{K465E}/NR2B and NR1/NR2B^{K459E} receptors.

served with hetero-oligomers containing the NR2B subunit (82% inhibition). NR1a/NR2A, NR1a/NR2C, and NR1a/NR2D combinations were inhibited only to a small extent (<20%), indicating that ATP preferentially acts on NR2B subunit-containing channels. We therefore analyzed in more detail the inhibitory effect of ATP on recombinant NR1a/NR2B receptor subunit combinations.

Inward currents generated at saturating concentrations of NMDA (200 μM) and glycine (10 μM) were inhibited by 500 μM ATP in a reversible manner (Fig. 1*B*). Inhibition was enhanced by a factor of 2 when NMDA concentrations corresponding to the respective EC_{50} value (20 μM) were applied. The inhibition profile of ATP (500 μM) showed no dependence on the glycine concentration, suggesting that the inhibitory potency of ATP was mediated mainly by an interaction with glutamate efficacy and not via the glycine-binding site. The inhibition curve for the NR1a/NR2B combination in the presence of 20 μM NMDA and increasing concentrations of ATP revealed an IC_{50} value of 135 $\pm$ 14 μM (Fig. 1*C*). Interestingly, the inhibitory effect was attenuated at very high ATP concentrations. To further define the type of inhibition that ATP exerts on NR1a/NR2B receptor-mediated currents, we analyzed NMDA dose–response curves in the presence and absence of 500 μM ATP (Fig. 1*E*). We observed a decrease in the apparent NMDA affinity (EC_{50} value) from 17 $\pm$ 4.2 μM in the absence to 54 $\pm$ 8.3 μM in the presence of ATP, respectively. Neither NMDA nor ATP induced currents in noninjected oocytes.

To identify a putative binding determinant responsible for ATP interaction, we analyzed the conserved K465 and K459 residues within the NR1 (glycine) and NR2B (glutamate) agonist binding domains, respectively. These lysine residues are thought to be directly involved in ligand binding (Kuryatov et al., 1994; Laube et al., 1997; Wittekindt et al., 2001). In oocytes expressing mutant NR1a/NR2B^{K459E} heteromeric receptors, preapplication of 500 μM ATP followed by coapplication of 20 μM NMDA had no inhibitory effect (Fig. 1*F*), although ATP produced a potent block of the current response of recombinant wild-type NR1a/NR2B receptors (Fig. 1*C*). In contrast, ATP-mediated inhibition was maintained when the glycine-binding NR1a subunit was mutated in the homologous position (NR1a^{K465E}/NR2B). These results support the notion that ATP exerts its inhibition via the glutamate-binding pocket of the NR2B subunit of the NMDA receptor.

Partial agonist property of ATP

The attenuation of the inhibitory effect of ATP on NMDA-induced currents at very high concentrations (Fig. 1*C*) suggested that ATP may exert a partial agonist function. We therefore performed an additional series of experiments in the presence of glycine but absence of NMDA. Superfusion of NR1a/NR2B NMDA receptor-expressing oocytes with glycine alone (10 μM) elicited no detectable currents, but an NMDA receptor-specific activation was observed in the simultaneous presence of ATP (Fig. 1*D*). The highest ATP concentration analyzed (10 mM) yielded currents corresponding to 28 $\pm$ 5.9% ($n = 5$) of the value obtained with 200 μM NMDA.

Nucleotide specificity and inhibitors

To determine nucleotide specificity we analyzed the inhibitory potential of various nucleotides and adenosine (500 μM each) on NMDA-elicited (20 μM) currents at recombinant NR1a/NR2B receptors (Table 1). ADP and its nonhydrolyzable analog α,β-meADP revealed a potency of <20%, and adenosine had no effect. The purine nucleotides GTP and ITP and AMP reduced the currents to a similar extent as ATP (70–80%), whereas the pyrimidine nucleotides UTP and CTP induced no significant inhibition (<10%). 2MeSATP and α,β-meATP revealed an intermediate potency. The resulting rank order of potency was as follows: ATP > GTP > AMP > ITP > 2MeSATP > α,β-meATP > ADP > α,β-meADP > CTP > UTP > adenosine. In addition we analyzed a number of substances that had previously been shown to inhibit P2 receptors at a concentration of 100 μM (Ralevic and Burnstock, 1998). Reactive blue 2 (100 μM) inhibited the NMDA-induced (20 μM) inward current by 46 $\pm$ 8.8% (Table 1), confirming previous reports of an inhibitory effect of this protein dye also on NMDA receptors [references in Li (2000)]. No inhibition (<10%) was observed with 100 μM suramin or PPADS.

Table 1. Inhibition of the recombinant NR1a/NR2B NMDA receptor by nucleotides and inhibitors of P2 receptors

Compound	Inhibition (%)	*n*
ATP	82 ± 4.2	6
ADP	17 ± 2.9	3
AMP	72 ± 8	4
Adenosine	2 ± 1.8	5
GTP	77 ± 6.4	3
ITP	69 ± 7.5	3
CTP	5 ± 3.5	4
UTP	3 ± 1.8	3
2MeSATP	56 ± 8.1	3
α,β-meATP	38 ± 8.1	5
α,β-meADP	15 ± 4.7	3
Suramin (100 μM)	9 ± 3	4
PPADS (100 μM)	6 ± 3.6	5
Reactive blue 2 (100 μM)	46 ± 7.5	4

Inhibition was investigated after NR1a/NR2B cRNA injection into oocytes at NMDA and glycine concentrations of 20 and 10 μM, respectively. Values represent the percentage of current reduction determined at a holding potential of −70 mV. Unless indicated otherwise, compounds were applied at a concentration of 500 μM. Values are means ± SEM of *n* determinations.

Inhibition of agonist-induced NMDA receptor currents in cultured hippocampal neurons

The data reported so far suggest that ATP exerts its strong inhibitory effect selectively via the NR1a/NR2B NMDA receptor subunit combination. To investigate whether ATP was also able to interfere with NMDA-mediated currents in intact neurons expressing native hetero-oligomeric NMDA receptors, hippocampal neurons were cultured from newborn rats and analyzed after a period of 25–35 DIV. In ~35% of the neurons tested, application of 500 μM ATP induced a fast inward current that recovered after washout, in accordance with previous observations that ATP-mediated currents occur in hippocampal neurons (Wong et al., 2000). These currents could be inhibited up to 80% with the P2X antagonist PPADS (50 μM) (data not shown). To avoid simultaneous induction of ATP-mediated currents, we examined in a first set of experiments the effect of whole-cell currents evoked by NMDA or kainate in the presence of 50 μM PPADS (ineffective on NMDA-mediated currents at recombinant NMDA receptors; see above). As shown in Figure 2*A*, whole-cell peak inward currents of hippocampal neurons elicited by application of 30 μM NMDA were blocked in the presence of 500 μM ATP (73 ± 13.6%; $n = 3$). In contrast, currents evoked by 100 μM kainate showed only a slight decrease in the presence of 500 μM ATP (19 ± 6.5%; $n = 3$) (Fig. 2*C*). GTP (500 μM) inhibited both NMDA and kainate responses (65 ± 7 and 61 ± 12.4%, respectively; $n = 3$). To exclude the possibility that ATP antagonizes NMDA currents only in PPADS-sensitive neurons, we examined in addition neurons in which ATP failed to evoke an inward current. The inhibitory effect of 500 μM ATP was similar to that obtained in the presence of PPADS (61 ± 11%; $n = 8$). No difference was observed between cells cultured from 25 to 35 DIV. To further corroborate the NR2B subunit-selective inhibitory effect of ATP, we investigated the inhibitory potential of the well characterized NR2B-selective antagonist ifenprodil (Williams, 1993). Ifenprodil (10 μM) blocked NMDA-evoked currents to a similar extent as 500 μM ATP (68 ± 8%; $n = 5$) (Fig. 2*B*). This indicates a high contribution of NR2B-containing NMDA receptors in the cultures used. The inhibitory effect of ATP on NMDA-induced currents in cultured hippocampal neurons is in line with our findings at the recombinant NR1a/NR2B NMDA receptors and corroborates the inhibitory specificity of ATP at this NMDA subtype of glutamate receptor.

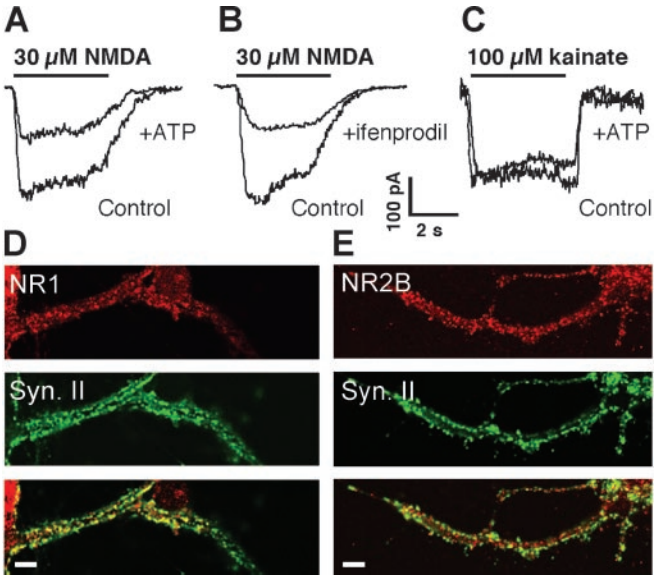

Figure 2. Inhibition of agonist-induced receptor currents in cultured hippocampal neurons and localization of the NMDA receptor subunits NR1 and NR2B. *A*, *B*, Whole-cell currents elicited by application of 30 μM NMDA were inhibited to a similar extent in the presence of 500 μM ATP (*A*) or 10 μM ifenprodil (*B*) (traces from the same neuron). *C*, Currents evoked by 100 μM kainate were only slightly affected by 500 μM ATP. *D*, *E*, Hippocampal cultures were double-labeled with antibodies against the NR1 (*D*) or NR2B (*E*) subunit (red) and the synaptic vesicle protein synaptobrevin II (Syn. II, green) (details of dendritic processes, confocal double-labeling cytochemistry). Scale bars, 5 μm.

Localization of NMDA receptor subunits

To verify the presence of NMDA receptor subunits in the culture system and to determine their cellular localization, we labeled primary hippocampal neurons with antibodies against the NR1 and NR2B subunits (Rao et al., 1998). This was compared with the localization of the synaptic vesicle protein synaptobrevin II. As revealed by confocal analysis, both NMDA receptor subunits were strongly expressed and distributed throughout the cell. At dendrites (Fig. 2*D*,*E*), both subunits exhibited an extensive punctuate pattern indicative of NMDA receptor clustering. The confocal images indicated an organellar intracellular localization of part of the receptors. The surface-located receptor clusters partially colocalized with the presynaptic marker synaptobrevin II.

ATP attenuates NMDA-mediated but not kainate-mediated neurotoxicity

Because glutamate exerts a neurotoxic effect via activation of NMDA receptors (Dingledine et al., 1999), we investigated the possibility that ATP could interfere with NMDA-mediated neurotoxicity in cultured hippocampal neurons (33–37 DIV). Compounds were added to hippocampal neurons in culture medium, and neuronal death was quantified after 24 hr by cellular ELISA. Both NMDA (30 μM) and kainate (300 μM) were cytotoxic for cultured hippocampal neurons, with 56 and 37% of neurons, respectively, surviving after 24 hr. These effects were prevented by coapplication of the respective antagonists (MK-801, 20 μM, or CNQX, 100 μM) (Fig. 3*A*). In addition to the open channel blocker MK-801, NMDA-induced neurotoxicity was also strongly attenuated by the NR2B-selective antagonist ifenprodil (Fig. 3*A*). The NMDA channel blocker MK-801 had no effect on kainate-induced cell death. ATP attenuated the cytotoxic effect of NMDA (30 μM) in a concentration-dependent manner. Neuronal rescue increased up to 54% at ATP concentrations between 10 and 1000 μM and decreased again at 5 and 10 mM concentrations

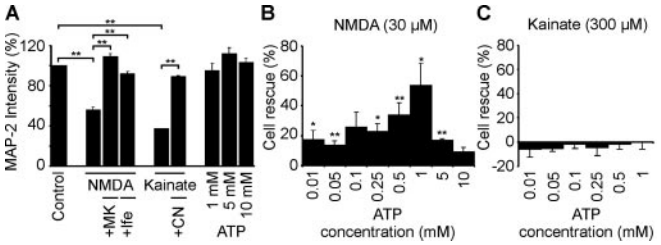

Figure 3. Neuroprotective function of ATP on NMDA- but not kainate-mediated neurotoxicity in hippocampal cultures (33–37 DIV) obtained from 1-d-old rats. *A*, NMDA (30 μM)- and kainate (300 μM)-induced neurotoxicity is inhibited by application of MK-801 (MK) (20 μM; $n = 10$) and ifenprodil (Ife) (10 μM; $n = 4$) or CNQX (CN) (100 μM; $n = 4$), respectively. ATP (1, 5, or 10 mM) alone had no neurotoxic effect ($n = 3$). Vehicle was added in control experiments. *B*, *C*, Comparison of the concentration-dependent rescue effect of ATP when applied together with NMDA ($n = 3$–5) (*B*) or kainate ($n = 3$) (*C*). The 100% value corresponds to the difference between MAP-2 immunoreactivity in NMDA-free controls and the MAP-2 immunoreactivity obtained after application of NMDA (*A*) (means ± SEM; *$p < 0.05$, **$p < 0.01$, compared with value after application of 30 μM NMDA).

(Fig. 3*B*). The decreased rescue effect at nonphysiologically high ATP concentrations could be attributable to its agonist property (Fig. 1*C*,*D*). Half-maximal rescue was obtained at ATP concentrations of ~300 μM. In contrast, ATP did not reduce kainate-induced (300 μM) neurotoxicity (Fig. 3*C*), suggesting that it did not exert a general neurotoxicity-antagonizing effect. Neural viability was not affected by application of ATP alone at concentrations up to 10 mM (±10% of control value; $n = 3$) (Fig. 3*A*). When 1 mM ATP was applied 3 hr before NMDA (30 μM), cell rescue was reduced to 21 ± 3.3% ($n = 11$). Application of ATP (1 mM) 3 hr after NMDA application (30 μM) yielded no rescue effect ($n = 11$). The following experiments were performed to further characterize the neuroprotective role of ATP and other nucleotides in hippocampal cultures.

Nucleotide specificity

For comparison with the results obtained with the heterologously expressed NR1a/NR2B receptor subunit combinations, we analyzed the efficacy of various additional nucleotides and of adenosine. ADP, AMP, and adenosine had no effect on neuron survival at concentrations between 10 and 1000 μM (Fig. 4*A*). This suggests that the effect of ATP was not mediated by its potential extracellular hydrolysis products. Although the purine nucleotides ITP and GTP also reduced NMDA-mediated cytotoxicity, the pyrimidine nucleotides UTP and CTP had no effect (Fig. 4*B*). None of the following substances revealed a protective effect on NMDA-treated hippocampal neurons (Fig. 4*C*): the two P2 receptor agonists (Ralevic and Burnstock, 1998) α,β-meATP and 2MeSATP (up to 1 mM), the dinucleotide Ap_4A (up to 1 mM), which has been shown to inhibit excitatory transmission in rat hippocampal neurons (Klishin et al., 1994); BzATP, which preferentially activates $P2X_7$ and $P2X_1$ receptors (Ralevic and Burnstock, 1998) (applied up to 250 μM); α,β-meADP, an inhibitor of ecto-5′-nucleotidase (Zimmermann, 2001) (data not shown). When applied alone at maximal concentrations, none of the substances affected neuron survival (data not shown).

Inhibitors of P2 receptors

The possible involvement of P2 receptor-mediated effects in the neurotoxicity assays was investigated by coapplication of the P2 receptor antagonists suramin, PPADS, or reactive blue 2. At a concentration of 100 μM, the substances have been widely used in the study of P2 receptors (Ralevic and Burnstock, 1998). When

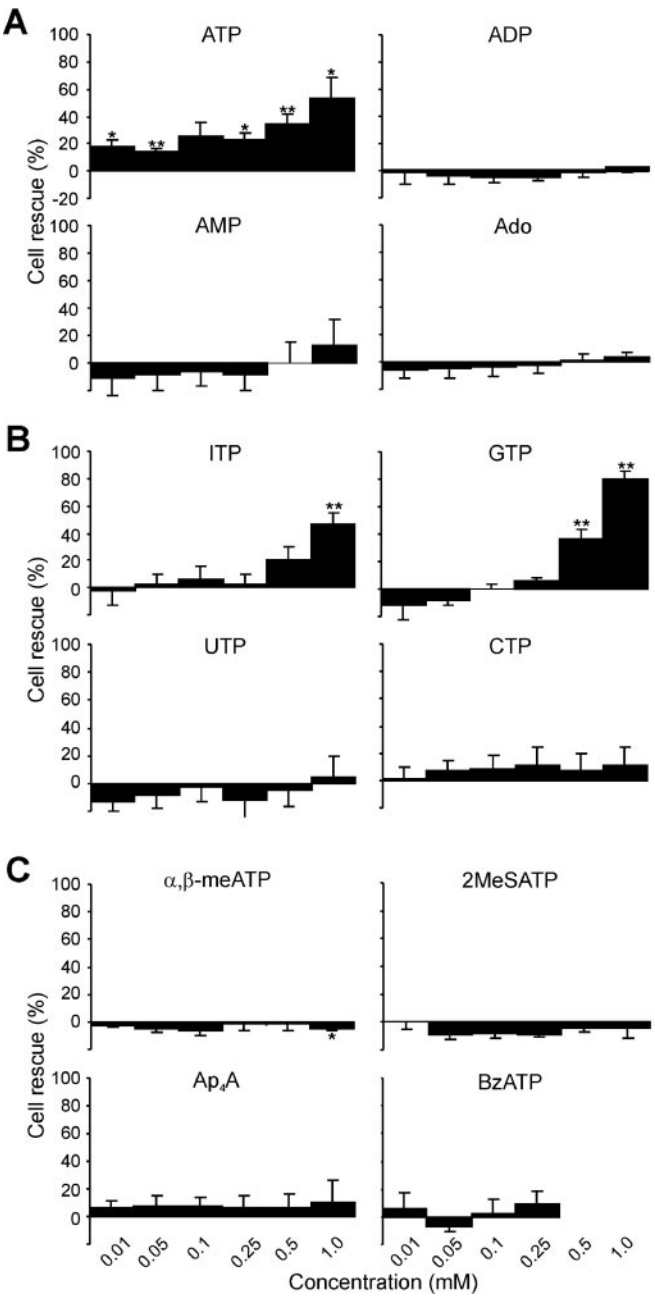

Figure 4. Comparison of potential agonists on NMDA-mediated cytotoxicity in hippocampal cultures. ATP, ADP, AMP, adenosine (Ado) (*A*), ITP, GTP, UTP, CTP (*B*), α,β-meATP, 2MeSATP, AP_4A, and BzATP (*C*) were applied at varying concentrations together with NMDA (30 μM). Cell rescue was quantified 24 hr after application of NMDA by cellular ELISA using MAP-2 immunoreactivity as for Figure 3, *B* and *C* (means ± SEM; $n = 3$–5; *$p < 0.05$, **$p < 0.01$, compared with value after application of 30 μM NMDA).

applied alone at concentrations of up to 100 μM, PPADS, suramin, and reactive blue 2 had a mild deteriorating effect on neuron survival (<15%). At a concentration of 100 μM, none of the three substances attenuated the rescue effect of ATP on NMDA-mediated (30 μM) neurotoxicity ($n = 3$; data not shown).

Stability of applied nucleotides

ATP-hydrolyzing ecto-enzymes are widely distributed in the CNS (Zimmermann, 2001). We therefore analyzed the potential of the cultured hippocampal cells to hydrolyze added nucleotides

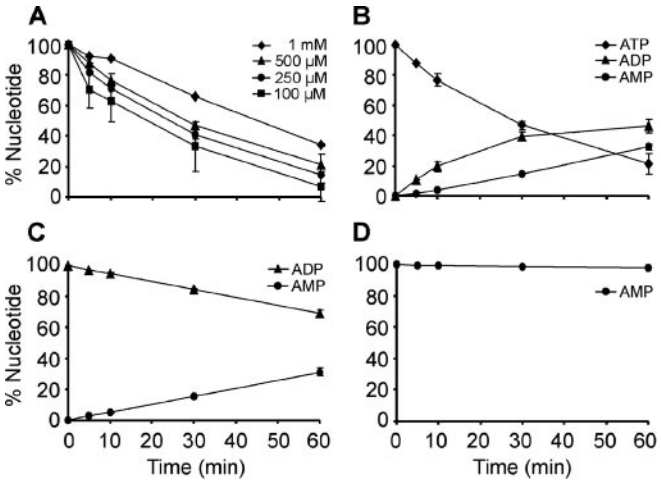

Figure 5. HPLC analysis of nucleotide hydrolysis by cultured hippocampal cells. Cells were grown in 24-well plates. *A*, Time-dependent degradation of ATP applied at concentrations between 100 and 1000 μM. *B–D*, Product analysis after application of 500 μM ATP (*B*), ADP (*C*), or AMP (*D*). Initial substrate and product concentrations are set at 100 and 0%, respectively (means ± range; $n = 2$).

and the formation of hydrolysis products. For ecto-nucleotidase analysis, cells were grown in 24-well plates, and nucleotides were quantified by HPLC. ATP was hydrolyzed by intact cultured cells, with the hydrolysis rate depending on the initial nucleotide concentration (Fig. 5*A*). At lower substrate concentration the hydrolysis rates decreased with time, presumably as a result of nonsaturating ATP concentrations. Reported K_m values for ecto-nucleotidases on intact cells are in the order of 100–200 μM (Zimmermann, 2001). Similar hydrolysis patterns were obtained for other nucleoside triphosphates (data not shown). When ATP was added as a substrate, ADP and AMP sequentially appeared as hydrolysis products, suggesting the presence of an ecto-apyrase (Fig. 5*B*). Identical product profiles were obtained at initial ATP concentrations from 100 to 1000 μM. ADP was hydrolyzed to AMP (Fig. 5*C*), but only 2% of the applied AMP was hydrolyzed after 60 min of incubation (Fig. 3*D*), suggesting that activity of ecto-5′-nucleotidase was essentially absent, in contrast to previous results obtained with acute hippocampal slices (Dunwiddie et al., 1997; Cunha et al., 1998). No nucleotide hydrolysis was obtained with the culture medium in the absence of cells, and nucleotide hydrolysis was not affected by the presence of NMDA 30 μM (data not shown). In addition we investigated the hydrolysis of ATP (1000 μM) in 96-well plates, corresponding to the culture conditions used in the neurotoxicity assays. Three hours after application, 22.0 ± 3.7% ($n = 6$) of the added nucleotide was left, corresponding to a remaining ATP concentration of 220 μM. At that ATP concentration a cell rescue of ~23% would be expected (Fig. 3*B*). Correspondingly, cell rescue was reduced to 21% when 1 mM ATP was applied 3 hr before NMDA (see above).

Discussion

Inhibition of NMDA receptors by ATP involves the NR2B subunit

We demonstrate that ATP directly inhibits the hetero-oligomeric NR1a/NR2B NMDA receptor. Similar to ifenprodil or haloperidol (Cull-Candy et al., 2001), ATP inhibits NR2B-containing hetero-oligomers, with little effect on NR1a/NR2A, NR1a/NR2C, and NR1a/NR2D. Although ADP had only a weak effect and adenosine had no inhibitory effect, AMP almost mimicked the potency of ATP on the NR1a/NR2B NMDA receptor subunit combination. Our results underpin the important role of the glutamate-binding NR2 subunit isoforms in determining the ATP sensitivity of NMDA receptors and indicate that native NMDA receptors containing different NR2 variants may differ in their ATP sensitivity. In striatal neurons, ATP (1 mM) failed to alter the synaptic current response to NMDA (10 μM) (Nörenberg et al., 1997).

This subunit selectivity is not paralleled by GTP or its hydrolysis products. Guanine nucleotides were previously observed to inhibit the binding of agonists and antagonists related to kainate and NMDA receptors in brain membrane preparations [references in Hood et al. (1990), Gorodinsky et al. (1993) and Aleu et al. (1999)] and to inhibit kainate- or glutamate-induced currents in intact neurons (Baron et al., 1989; Budson et al., 1991; Poulopoulou and Nowak, 1998) or also of heterologously expressed receptors (Paas et al., 1996). The inhibitory effect was not restricted to GTP but was observed also with GDP, GMP, cGMP, and a number of guanine nucleotide analogs. Guanosine 5′-[β-thio]diphosphate (GDPβS) was found to block NMDA receptor-induced seizures after intracerebroventricular administration (Baron et al., 1989). From the data reported in the literature and from our own analyses, we conclude that GTP can broadly act at AMPA/kainate and NMDA-mediated receptor responses. In contrast, ATP has no significant effect on AMPA/kainate receptors, and at NMDA receptors its inhibitory potency appears to be restricted to receptors containing the NR2B subunit.

The consensus nucleotide-binding motif

Within the N-terminal part of the chicken kainate-binding protein, a glycine-rich consensus nucleotide-binding motif (GxGxxG) was identified and implicated in GTP binding (Paas et al., 1996). The motif is also present in the mammalian AMPA, kainate, and NMDA receptor subunits. It is conserved in many adenine- and guanine-nucleotide-binding proteins (Saraste et al., 1990) and homologous also to the region of the NR2B subunit harboring K459 and assumed to be part of the ligand-binding pocket (Laube et al., 1997; Paas, 1998). Presumably this motive is generally involved in nucleotide binding at all glutamate receptors. The differential potency of the various purine and pyrimidine nucleotides and the underlying structural determinants for inhibiting individual receptor subtypes remain to be investigated.

ATP acts in a competitive manner to NMDA at the glutamate-binding site

Our experiments with recombinant NR1a/NR2B receptors suggest that ATP may act as a competitive antagonist at the glutamate-binding site of the NR2B subunit. The inhibitory effect is not mediated by changes in glycine efficacy or by blocking the ion channel. This is also supported by the observation that exchange of the positively charged K459 for a negatively charged glutamate in the NR2B subunit (but not of K465 in the homologous position in the glycine-binding NR1 subunit) abrogates the inhibitory effect of ATP. K459 presumably plays an important role in binding antagonists of the glutamate-binding site (Laube et al., 1997; Wittekindt et al., 2001) and/or in stabilizing the closed conformation of the glutamate-binding pocket, which is a prerequisite for the opening of the intrinsic ion channel (for review, see Madden, 2002). The antagonistic potential of AMP suggests that at least one free phosphate group is required for the inhibitory activity of purine nucleotides. Therefore, the side chain of K459 might play a role in stabilizing negative charges of

antagonists via ionic interactions, similar to findings with the competitive antagonists D-AP5 and conantokin G (Laube et al., 1997; Wittekindt et al., 2001). Binding at the glutamate-binding site may also explain the partial agonist potency of ATP at non-physiologically high concentrations.

ATP also inhibits NMDA-mediated effects in cultured neurons

When coapplied, ATP inhibited NMDA-mediated currents in cultured neurons but had only a small effect on kainate-induced currents. In contrast and in accordance with previous studies [references in Paas et al. (1996)], GTP inhibited both NMDA- and kainate-induced neuronal currents. The presence of the NR1a/NR2B NMDA receptor subunits in our culture system was verified both by the inhibitory effect of ifenprodil and by immunocytochemistry. We have not investigated the presence and abundance of additional subunits. It has previously been reported that both cellular localization and the abundance of ifenprodil- or haloperidol-sensitive NMDA receptor subunit combinations change with culture time (Tovar and Westbrook, 1999; Sinor et al., 2000) and that inhibition of NMDA-evoked whole-cell currents by haloperidol is reduced in long-term cultured cells (Sinor et al., 2000). Both our confocal analyses and the inhibitory potential of ifenprodil demonstrate that hippocampal neurons retain a high contribution of NR2B subunits under our culture conditions, compatible with the antagonistic potential of ATP.

In analogy with its effect on NMDA-mediated currents, coapplication of ATP resulted in an attenuation of NMDA-mediated death of cultured rat hippocampal neurons. This is noteworthy because the determination of neural death involved a long-term application protocol in which receptor desensitization, nucleotide degradation, and various intracellular reaction cascades leading to cell death were expected to be involved. Nevertheless, the efficacy of individual nucleotides in inhibiting recombinant NR1a/NR2B receptors and in attenuating NMDA-mediated neurotoxicity was very similar. The purine nucleotides ATP, ITP, and GTP were effective, whereas the pyrimidine nucleotides UTP and CTP, and ADP or adenosine had no or only a small effect. The ineffectiveness of UTP and CTP further excludes chelation of divalent cations or local pH effects (Dingledine et al., 1999) as mediators of NMDA receptor inhibition. Differences were observed with AMP and the P2 receptor agonists α,β-meATP and 2MeSATP. They had no rescue effect but revealed a significant inhibitory potential at the NR1a/NR2B receptor. This may result from differences in oligomeric structure and subunit composition between *in situ* NMDA receptors and recombinant receptors. In the neurotoxicity assays, the neuroprotective effect of ATP (1 mM) was still approximately two-thirds of that of ifenprodil, although ATP eventually became eliminated on prolonged application. This suggests that continued NMDA application can result in an impaired efficacy of NMDA, presumably because of desensitization (Lester and Jahr, 1992) or rundown (Rosenmund et al., 1995) of NMDA receptors.

The role of ATP in the hippocampus

According to our results, ATP can directly inhibit NMDA receptors of hippocampal neurons, in addition to its role as an extracellular source of the synaptic modulator adenosine (Dunwiddie et al., 1997; Cunha et al., 1998; Stone et al., 2000), as a co-substrate for ecto-protein phosphorylation (Fujii et al., 2002), or the previously observed direct excitatory effect via postsynaptic P2X receptors (Khakh, 2001; Robertson et al., 2001, Pankratov et al., 2002a). Furthermore, postsynaptically located P2Y receptors may modulate NMDA receptor-induced currents (Pankratov et al., 2002b; Wirkner et al., 2002). There is considerable evidence that the inhibitory effect of ATP on synaptic transmission in hippocampal slices is presynaptically mediated by the adenosine formed as a result of ecto-nucleotidase activity (Dunwiddie et al., 1997; Cunha et al., 1998; Masino et al., 2002). Nevertheless, open questions remain regarding the effects of ATP analogs mostly resistant to hydrolysis or of inhibitors of P2 and adenosine receptors that did not yield results consistent with the activation of known P1 and P2 receptors (Cunha et al., 1998; Ross et al., 1998; Mendoza-Fernandez et al., 2000).

Because in many of these experiments the individual effects of ATP had to be pharmacologically dissected, the net impact of ATP on hippocampal glutamatergic transmission remains difficult to discern. Presumably it depends on the degree of synaptic activation and the resulting effective extracellular nucleotide concentrations. Long-term potentiation in hippocampal slices by ATP occurred already at 400 nM, whereas concentrations of 50–150 μM temporarily depressed the potential (Wieraszko and Seyfried, 1989). With the exception of the $P2X_7$ receptor, the activation of which requires ATP concentrations >100 μM, the EC_{50} of ATP at recombinant P2X receptors ranges between 1 and 30 μM (Khakh, 2001). At the NR1a/NR2B receptor combination, the IC_{50} value of ATP at an NMDA concentration of 20 μM was 135 μM. Because the free intracellular concentration of ATP is >1 mM (Gribble et al., 2000) and vesicular ATP concentrations are in the high millimolar range (Zimmermann, 1994), NMDA receptors may become inhibited under conditions of potentiated or pathological ATP release such as epilepsy, trauma, or ischemia (Neary et al., 1996). This could involve spillover of glutamate (and ATP) to the NR1/NR2B subunit-containing extrasynaptic and potentially neurotoxic NMDA receptors (Hardingham et al., 2002). It therefore appears possible that ATP can contribute to inhibition of hippocampal glutamatergic transmission via inhibition of postsynaptic NMDA receptors.

References

Aleu J, Barat A, Burgos JS, Solsona C, Marsal J, Ramírez G (1999) Guanine nucleotides, including GMP, antagonize kainate responses in *Xenopus* oocytes injected with chick cerebellar membranes. J Neurochem 72:2170–2176.

Baron MD, Dudley MW, McCarthy DR, Miller FP, Reynolds IJ, Schmidt CJ (1989) Guanine nucleotides are competitive inhibitors of *N*-Methyl-D-aspartate and its receptor site both *in vitro* and *in vivo*. J Pharmacol Exp Ther 250:162–169.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal™, a new serum-free medium combination. J Neurosci Res 35:567–576.

Budson AE, Jackson PS, Lipton SA (1991) GDPβS antagonizes whole cell current responses to excitatory amino acids. Brain Res 548:346–348.

Cull-Candy S, Brickley S, Farrant M (2001) NMDA receptor subunits: diversity, development and disease. Curr Opin Neurobiol 11:327–335.

Cunha RA, Sebastiao AM, Ribeiro JA (1998) Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A(1) receptors. J Neurosci 18:1987–1995.

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51:7–61.

Dunwiddie TV, Diao LH, Proctor WR (1997) Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. J Neurosci 17:7673–7682.

Fujii S, Kato H, Kuroda Y (2002) Cooperativity between extracellular adenosine 5′-triphosphate and activation of *N*-methyl-D-aspartate receptors in long-term potentiation induction in hippocampal CA1 neurons. Neuroscience 113:617–628.

Gorodinsky A, Paas Y, Teichberg VI (1993) A ligand binding study of the interactions of guanine nucleotides with non-NMDA receptors. Neurochem Int 23:285–291.

Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG, Ashcroft FM (2000) A novel method for measurement of submembrane ATP concentration. J Biol Chem 275:30046–30049.

Hardingham GE, Fukunaga J, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405–414.

Haydon PG (2001) Glia: listening and talking to the synapse. Nat Rev Neurosci 2:185–193.

Hood WF, Thomas JW, Compton RP, Monahan JB (1990) Guanine nucleotide modulation of [^{3}H]TCP binding to the NMDA receptor complex. Eur J Pharmacol 188:43–49.

Janssens N, Lesage ASJ (2001) Glutamate receptor subunit expression in primary neuronal and secondary glial cultures. J Neurochem 77:1457–1474.

Khakh BS (2001) Molecular physiology of P2X receptors and ATP signaling at synapses. Nat Rev Neurosci 2:165–174.

Khakh BS, Humphrey PPA, Henderson G (1997) ATP-gated cation channels (P2X purinoceptors) in trigeminal mesencephalic nucleus neurones of the rat. J Physiol (Lond) 498:709–715.

Klishin A, Lozovaya N, Pintor J, Miras-Portugal MT, Krishtal O (1994) Possible functional role of diadenosine polyphosphates: negative feedback for excitation in the hippocampus. Neuroscience 58:235–236.

Kuryatov A, Laube B, Betz H, Kuhse J (1994) Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. Neuron 12:1291–1300.

Laube B, Kuhse J, Rundström N, Kirsch J, Schmieden V, Betz H (1995) Modulation by zinc ions of native rat and recombinant human inhibitory glycine receptors. J Physiol (Lond) 483:613–619.

Laube B, Hirai H, Sturgess M, Betz H, Kuhse J (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. Neuron 18:493–503.

Lester RAJ, Jahr CE (1992) NMDA channel behavior depends on agonist affinity. J Neurosci 12:635–643.

Li C (2000) Novel mechanisms of inhibition by the P2 receptor agonist PPADS of ATP-activated current in dorsal root ganglion neurons. J Neurophysiol 83:2533–2541.

Madden DR (2002) The structure and function of glutamate ion channels. Nat Neurosci Rev 3:91–101.

Masino SA, Diao LH, Illes P, Zahniser NR, Larson GA, Johansson B, Fredholm BB, Dunwiddie TV (2002) Modulation of hippocampal glutamatergic transmission by ATP is dependent on adenosine A(1) receptors. J Pharmacol Exp Ther 303:356–363.

Mendoza-Fernandez V, Andrew RD, Barajas-Lopez C (2000) ATP inhibits glutamate synaptic release by acting at P2Y receptors in pyramidal neurons of hippocampal slices. J Pharmacol Exp Ther 293:172–179.

Monahan JB, Hood WF, Michel J, Compton RP (1988) Effects of guanine nucleotides on *N*-methyl-D-aspartate receptor-ligand interactions. Mol Pharmacol 34:111–116.

Mori H, Mishina M (1995) Structure and function of the NMDA receptor channel. Neuropharmacology 34:1219–1237.

Neary JT, Rathbone MP, Cattabeni F, Abbracchio MP, Burnstock G (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci 19:13–18.

Nörenberg W, Illes P (2000) Neuronal P2X receptors: localisation and functional properties. Naunyn Schmiedebergs Arch Pharmacol 362:324–339.

Nörenberg W, Wirkner K, Illes P (1997) Effect of adenosine and some of its structural analogues on the conductance of NMDA receptor channels in a subset of striatal neurons. Br J Pharmacol 122:71–80.

Paas Y (1998) The macro- and microarchitecture of the ligand-binding domain of glutamate receptors. Trends Neurosci 21:117–125.

Paas Y, Devillers-Thiéry A, Changeux J-P, Medeville F, Teichberg VI (1996) Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. EMBO J 15:1548–1556.

Pankratov Y, Lalo U, Krishtal O, Verkhratsky A (2002a) Ionotropic P2X purinoreceptors mediate synaptic transmission in rat pyramidal neurones of layer II/III of somato-sensory cortex. J Physiol (Lond) 542:529–536.

Pankratov YV, Lalo UV, Krishtal OA (2002b) Role for P2X receptors in long-term potentiation. J Neurosci 22:8363–8369.

Poulopoulou C, Nowak LM (1998) Extracellular 3′,5′ cyclic guanosine monophosphate inhibits kainate-activated responses in cultured mouse cerebellar neurons. J Pharmacol Exp Ther 286:99–109.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.

Rao A, Kim E, Sheng M, Craig AM (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. J Neurosci 18:1217–1229.

Robertson SJ, Ennion SJ, Evans RJ, Edwards FA (2001) Synaptic P2X receptors. Curr Opin Neurobiol 11:378–386.

Rosenmund C, Feltz A, Westbrook GL (1995) Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. J Neurophysiol 73:427–430.

Ross FM, Brodie MJ, Stone TW (1998) Modulation by adenine nucleotides of epileptiform activity in the CA3 region of rat hippocampal slices. Br J Pharmacol 123:71–80.

Saraste M, Sibbald PR, Wittinghofer A (1990) The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci 15:430–434.

Sinor JD, Du S, Venneti S, Blitzblau RC, Leszkiewicz DN, Rosenberg PA, Aizenman E (2000) NMDA and glutamate evoke excitotoxicity at distinct cellular locations in rat cortical neurons *in vitro.* J Neurosci 20:8831–8837.

Soto F, Rubio ME (2001) Cloned P2X receptor subunits in cerebellum and hippocampus. Drug Dev Res 52:133–139.

Stone TW, O'Kane EM, Nikbakht MR, Ross FM (2000) Presynaptic P2 receptors? J Autonom Nerv Syst 81:244–248.

Tovar KR, Westbrook GL (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses *in vitro.* J Neurosci 19:4180–4188.

Vollmayer P, Koch M, Braun N, Heine P, Servos J, Israr E, Kegel B, Zimmermann H (2001) Multiple ecto-nucleotidases in PC12 cells: identification and cellular distribution after heterologous expression. J Neurochem 78:1019–1028.

Wieraszko A, Seyfried TN (1989) ATP-induced synaptic potentiation in hippocampal slices. Brain Res 491:356–359.

Williams K (1993) Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. Mol Pharmacol 44:851–859.

Wirkner K, Koles L, Thummler S, Luthardt J, Poelchen W, Franke H, Furst S, Illes P (2002) Interaction between P2Y and NMDA receptors in layer V pyramidal neurons of the rat prefrontal cortex. Neuropharmacology 42:476–488.

Wittekindt B, Betz H, Laube B (2000) Subunit-dependent inhibition of recombinant rodent *N*-methyl-D-aspartate receptors by HIV-1 glycoprotein 120 derived peptide. Neurosci Lett 280:151–154.

Wittekindt B, Malany S, Schemm R, Otvos L, Maccechini M-L, Laube B, Betz H (2001) Point mutations identify the glutamate binding pocket of the *N*-methyl-D-aspartate receptor as a major site of Conantokin-G inhibition. Neuropharmacology 41:753–761.

Wong AYC, Burnstock G, Gibb AJ (2000) Single channel properties of P2X ATP receptors in outside-out patches from rat hippocampal granule cells. J Physiol (Lond) 527:529–547.

Zimmermann H (1994) Signaling via ATP in the nervous system. Trends Neurosci 17:420–426.

Zimmermann H (2001) Ecto-nucleotidases. In: Handbook of experimental pharmacology. Purinergic and pyramidergic signaling (Abbracchio MP, Williams M, eds), pp 209–250. Heidelberg: Springer.