P2X$_7$ Receptor-Mediated Release of Excitatory Amino Acids from Astrocytes

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Astrocyte glutamate release can modulate synaptic activity and participate in brain intercellular signaling. P2X$_7$ receptors form large ion channels when activated by ATP or other ligands. Here we show that P2X$_7$ receptors provide a route for excitatory amino acid release from astrocytes. Studies were performed using murine cortical astrocyte cultures. ATP produced an inward current in patch-clamped astrocytes with properties characteristic of P2X$_7$ receptor activation: the current was amplified in low divalent cation medium, blocked by pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and more potently activated by 3′-O-(4-benzoyl)benzoyl ATP (BzATP) than by ATP itself. Measurement of current reversal potentials showed the relative BzATP-induced permeabilities to different substrates to be Na$^+$, 1 > Cl$^-$, 0.34 > N-methyl-D-glucamine, 0.27 > L-glutamate, 0.15 ≈ D-aspartate, 0.16. Astrocytes exposed to BzATP also became permeable to Lucifer yellow, indicating a large channel opening. Release of L-glutamate and D-aspartate through P2X$_7$ channels was confirmed using radiolabeled tracers. As with the inward current, release of glutamate and D-aspartate was induced by BzATP more potently than ATP, amplified in Ca^{2+}/Mg^{2+}-free medium, and blocked by PPADS or oxidized ATP. Efflux through P2X$_7$ channels is a previously unrecognized route of ligand-stimulated, nonvesicular astrocyte glutamate release.

Key words: D-aspartate; glutamate; P2Z; patch clamp; nonvesicular; purinergic

Introduction

Astrocyte glutamate release can modulate synaptic activity and participate in brain intercellular signaling (Araque et al., 2001; Bezzi et al., 2001; Nedergaard et al., 2002). Astrocyte glutamate release occurs by Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. Ca^{2+}-dependent release is triggered by ligands such as prostaglandins, ATP, and bradykinin as well as by the direct manipulation of astrocyte intracellular free Ca^{2+} (Parpura et al., 1994; Jeftinija et al., 1996; Bezzi et al., 1998; Sanzgiri et al., 1999; Jeremic et al., 2001). The route by which glutamate is released from astrocytes in response to intracellular Ca^{2+} elevations is not yet firmly established, but evidence suggests that vesicular release may contribute (Parpura et al., 1995; Araque et al., 2000).

Ca^{2+}-independent astrocyte glutamate release is less well characterized, but studies using brain slices suggest a continuous and rapid release of glutamate from astrocytes by a nonvesicular, Ca^{2+}-independent mechanism (Jabaudon et al., 1999). Ca^{2+}-independent astrocyte glutamate release can occur by reversal of glutamate uptake (Nicholls and Attwell, 1990; Longuemare and Swanson, 1995) and by opening of volume-sensitive anion channels (Kimelberg et al., 1990; Longuemare et al., 1999), but these processes are unlikely to be significant under normal conditions in the brain (Anderson and Swanson, 2000). In this study we

investigated the possibility that channel-forming P2X$_7$ receptors could contribute to Ca^{2+}-independent astrocyte glutamate release.

The P2X$_7$ receptor (also termed the P2Z receptor) was originally characterized by its reversible cell-permeabilizing effects (Dahlquist and Diamant, 1974; Steinberg and Silverstein, 1987; Di Virgilio, 1995). P2X$_7$ receptors form homomeric complexes that open large channels when activated by extracellular ATP (North and Surprenant, 2000). In some cell types, these channels are permeable to molecules up to 900 Da in size, whereas in others they are permeable only to smaller molecules or exhibit ion selectivity (Soltoff et al., 1992; Surprenant et al., 1996; Markwardt et al., 1997; Ralevic and Burnstock, 1998). P2X$_7$ receptors in the nervous system have been localized to microglia (Di Virgilio et al., 1999; Ferrari et al., 1999), neuronal processes (Brandle et al., 1998; Deuchars et al., 2001), Müller cells (Pannicke et al., 2000), Schwann cells (Colomar and Amedee, 2001), and astrocytes (Kukley et al., 2001; Panenka et al., 2001). ATP is an important mediator of astrocyte intercellular signaling (Guthrie et al., 1999; Cotrina et al., 2000; Wang et al., 2000), and activation of astrocyte P2X$_7$ receptors has been linked previously to mitogen-activated protein kinase activation, monocyte chemoattractant protein-1 expression, and purine release (Kukley et al., 2001; Panenka et al., 2001).

Mouse cortical astrocyte cultures were used to test the possibility that ATP could induce glutamate release from astrocytes by binding to P2X$_7$ receptors and inducing channel opening. ATP was found to induce an inward current, a permeabilization to Lucifer yellow, and an efflux of excitatory amino acids. Pharmacological characterization of these effects showed a pattern characteristic of P2X$_7$ receptors. These findings suggest a novel mechanism for ligand-mediated release of excitatory amino acids from

astrocytes. This work has been published previously in abstract form (Duan et al., 1999a).

Materials and Methods

Radiolabeled glutamate and D-aspartate were obtained from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), except as noted.

Cell cultures. Cultures were prepared from the cortices of 1-d-old mice (Simonsen Laboratories, Gilroy, CA) as described previously (Swanson et al., 1997a) in accordance with a protocol approved by the San Francisco Veterans Affairs Medical Center animal studies committee. Cortices were isolated, freed of meninges, dissociated with papain and trituration, and plated on glass coverslips or 24 well Falcon culture plates. At confluence (day 12–15), most of the cultures were treated for 48 hr with 20 μM cytosine arabinoside to prevent microglial proliferation. This medium was replaced with Eagle's minimal essential medium supplemented with 3% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 100 nM sodium selenate, 200 nM α-tocopherol, and 0.15 mM dibutyryl cAMP to induce the process-bearing morphology (Sensenbrenner et al., 1980; Swanson et al., 1997b). Each study was repeated on cells from at least two different batches of astrocyte cultures at 22–40 d *in vitro*. For studies measuring reversal potentials, the astrocytes were plated at low density so that single cells could be isolated for whole-cell recording to avoid space clamp problems attributable to gap junctions. In a subset of the cultures, cytosine arabinoside was omitted and the fetal bovine serum supplement was maintained at 10% to promote microglial survival and proliferation.

Reverse transcription-PCR. Total RNA was isolated from cultured mouse cortical astrocytes using the RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and evaluated by denaturing agarose gel electrophoresis. Reverse transcription (RT)-PCR was performed using the ProSTAR Ultra HF RT-PCR system (Stratagene, La Jolla, CA). The 5′ upstream primer was 5′-TCC ACC CTG TCC TAC TTT GG-3′ and the 3′ downstream primer was 5′-CTT GCA GAC TTT TCC CAA GC-3′. PCR used 35 cycles of 30 sec at 95°C, 30 sec at 55°C, 1 min at 72°C, and a final 10 min elongation step at 72°C.

Western blots and immunostaining. Homogenates were prepared from mouse brain cortices and from cortical mouse astrocyte cultures. Thirty micrograms of protein was run on 10% SDS polyacrylamide gels, electrophoretically transferred onto polyvinylidene fluoride membranes, and placed in a blocking solution containing affinity-purified polyclonal anti-P2X$_7$ receptor antiserum (Chemicon, Temecula, CA) at a 1:500 dilution for 12–18 hr at 4°C. In some cases the antiserum was preadsorbed with a fivefold molar excess of the peptide antigen. Antibody binding was visualized with a peroxidase-labeled anti-rabbit IgG and the 3–3′-diaminobenzidine method. Immunostaining of the astrocyte cultures was performed after fixation for 20 min in 4% paraformaldehyde. Cultures on glass coverslips were washed with a blocking buffer containing 10% goat serum and 0.1% Triton X-100 and then incubated with primary antibodies for 18 hr at 4°C. The primary antibodies were rabbit anti-P2X$_7$ (Chemicon) diluted 1:2000 along with either mouse anti-glial fibrillary acidic protein (GFAP; Chemicon) diluted 1:2000 to identify astrocytes or rat anti-mouse F4/80 (Serotec, Oxford, UK) diluted 1:500 to identify microglia. After washing to remove excess primary antibody, the cultures were incubated for 90 min at room temperature with fluorescence-tagged mouse monoclonal secondary antibodies (Molecular Probes, Eugene, OR): Alexa-fluor 488 anti-rabbit IgG for the P2X$_7$ antibody, Alexa-fluor 594 anti-rat IgG for the F4/80 antibody, and Alexa-fluor anti-mouse IgG for the GFAP antibody. Excess antibody was removed, the coverslips were mounted on glass slides, and cells were imaged using a Leica (Nussloch, Germany) TCS confocal microscope with 2 μm optical sections.

Whole-cell patch-clamp recording. Except as specified, the pipette solution contained (in mM): 100 *N*-methyl-D-glucamine chloride (NMDG-Cl), 3 Mg-ATP, 5 EGTA, and 10 HEPES. The external solution contained (in mM): 100 NaCl, 1 CaCl$_2$, 1.2 MgCl$_2$, 5 HEPES, and 10 glucose. For studies in low Ca^{2+}/Mg^{2+} external solutions, CaCl$_2$ was decreased to 0.3 mM and MgCl$_2$ to 0.1 mM. For both pipette and external solutions, the pH was adjusted to 7.2 with NMDG and the osmolarity was adjusted to 290 mOsm with sucrose. Recordings for the reversal potential (V_{rev}) deter-

minations began at least 15 min after establishing whole-cell patch configurations to allow a full exchange between cytoplasm and pipette solutions. Connection of the perfusion chamber to ground was established via a 3 M KCl agarose bridge. Liquid junction potentials (V_L values) were calculated with Clampex 7 software (Axon Instruments, Foster City, CA) and corrected by the equation $V_m = V_p - V_L$, where V_p is the command voltage (holding potential) and V_m is the actual potential difference across the membrane. Voltage pulse generation, data digitization, and data analysis were performed with a DigiData 1200 analog-to-digital/digital-to-analog interface with pClamp 7 software (Axon Instruments, Union City, CA). The "U-tube" solution change method (Duan and Cooke, 1999), which can change the solution surrounding a cell completely within 100–200 msec, was used to apply drugs. All drugs were added from concentrated, iso-osmolar, buffered (pH 7.2) stock solutions.

Glutamate and D-aspartate release. Studies were performed at 37°C in a balanced salt solution (BSS) as described previously (Longuemare and Swanson, 1995). The standard BSS contained (in mM): 135 NaCl, 3.1 KCl, 1.2 CaCl$_2$, 1.2 MgSO$_4$, 0.5 KH$_2$PO$_4$, 2 glucose, and 5.0 1,4-piperazinediethanesulfonic acid, adjusted to a final pH of 7.2 and to 280–320 mOsm. Ca^{2+} and Mg^{2+} were in some cases replaced by molar equivalents of Na$^+$. All drugs were added from concentrated, iso-osmolar, buffered (pH 7.2) stock solutions. Astrocytes were loaded with 0.17 μCi/ml [L-^{14}C(U)] glutamate (0.8 nmol/ml) or 0.34 μCi/ml [D-2,3-^{3}H]-aspartate (0.03 pmol/ml) for 60 min and then transferred to BSS. For studies using ^{14}C-glutamate, the cultures were preincubated for 30 min with 1 mM amino-oxyacetic acid and 0.5 mM methionine sulfoximine before adding ^{14}C-glutamate to inhibit the metabolism of glutamate to other radiolabeled metabolites (Farinelli and Nicklas, 1992). In some studies, the cultures were incubated with agents to assess other potential routes of excitatory amino acid efflux. L-Transpyrrolidine-2,4-dicarboxylic acid (PDC), used to block glutamate transporter reversal, was loaded into the cells by incubating the cultures in 50 μM PDC for 60 min after the preincubation with D-aspartate (Longuemare and Swanson, 1995). Thapsigargin and EGTA, used to assess the calcium dependency of D-aspartate release, were incubated with the cultures for 40 min in Ca^{2+}-free medium after the preincubation with D-aspartate. The calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) was added to the cultures as its acetoxy-methyl ester (AM) during the terminal 30 min of the D-aspartate preincubation.

The studies used 6 min incubations with purinergic receptor agonists. Incubations were terminated by the removal of the medium and lysis of the cells with 0.1N NaOH. Media and cell lysates were taken for scintillation counting to determine the percentage of radiolabeled substrate (^{14}C glutamate or ^{3}H D-aspartate) released in each culture well during the 6 min incubation period. Consistent with previous experience (Longuemare and Swanson, 1995), the total radiolabel loading (^{14}C or ^{3}H per milligram of protein) varied by <30% between studies, including studies using PDC preloading.

Lucifer yellow uptake. One-half of the cultures were preincubated with a 0.3 mM concentration of the irreversible P2X$_7$ antagonist oxidized ATP (Ox-ATP) (Murgia et al., 1993; Evans et al., 1995; North and Surprenant, 2000) for 2 hr in standard culture medium. The astrocytes on coverslips were then washed and transferred either to standard BSS or Ca^{2+}/Mg^{2+}-free BSS at 37°C. The cells were incubated for 5 min with 0.5 mg/ml Lucifer yellow, with or without 300 μM 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP). Incubations were terminated by washing into standard BSS, and fluorescence photomicrographs were obtained within 10 min using a laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) using 458 nm excitation. Five fields were randomly selected from each coverslip (four corner and one center) for image capture and measurement of whole-field fluorescence intensities. Whole-field fluorescence intensities were normalized to the mean intensity of the cultures treated with no BzATP in Ca^{2+}/Mg^{2+}-free BSS.

Cell survival. Rupture of astrocyte membranes was assessed by measuring the loss of lactate dehydrogenase (LDH) from the cultures. At designated time points the medium was removed and cells were solubilized in a solution of 3% Triton X-100, 0.02% bovine serum albumin, and 5 mM potassium phosphate buffer, pH 7.5. LDH activity in BzATP-
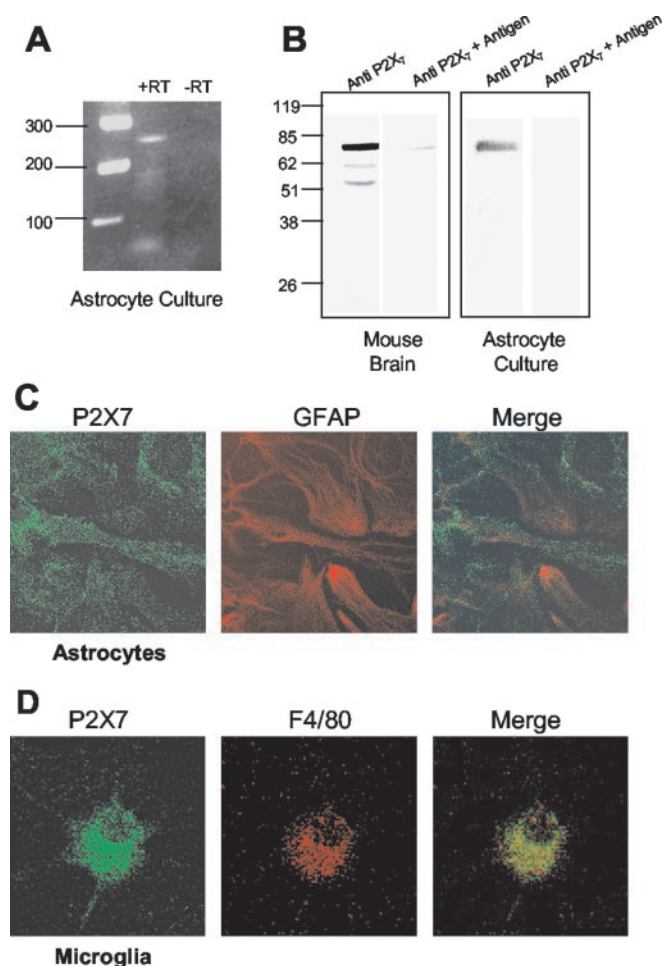
Figure 1. Astrocytes express P2X₇ receptors. *A*, RT-PCR showing P2X₇ mRNA expression in mouse astrocyte cultures. The expected product was 253 bp. +*RT* and −*RT* denote *lanes* with and without reverse transcriptase, respectively. The *left lane* shows molecular weight markers. *B*, Western blot showing P2X₇ mRNA expression in mouse astrocyte cultures and in mouse cortex. The expected P2X₇ band is at 72 kDa. Immunostaining is blocked by the coincubation of antibody with P2X₇ peptide epitope. *C*, Immunostaining of astrocyte cultures showed P2X₇ immunoreactivity over astrocyte surfaces surrounding GFAP intermediate filaments. *D*, Immunostaining of microglia (as a positive control) showed colocalization of P2X7 with the mouse microglia surface marker F4/80. The magnification in *D* is sixfold greater than in *C*. P2X₇ immunostaining in both astrocytes and microglia was blocked by coincubation of antibody with the P2X₇ peptide epitope (data not shown).

treated culture wells was measured by the method of Koh and Choi (1987) and expressed as percentage of activity in control wells from the same 24 well plate.

Statistical analysis. Each study was performed using astrocytes from at least two different culture preparation dates. *n* denotes the number of individual cells assessed in the patch-clamp studies or the number of culture wells assessed in the other studies. Statistical differences were determined by Student's *t* test for two-group comparisons or by one-way ANOVA with the Tukey test for multiple group comparisons.

Results

The murine cortical astrocyte cultures used for these studies form a confluent layer of process-bearing cells, of which >95% express the astrocyte-specific marker GFAP (Swanson et al., 1997a; Duan et al., 1999b). As shown in Figure 1, RT-PCR and Western blots confirm expression of the P2X₇ receptor in these cultures. Localization of P2X₇ receptors on the astrocyte cell membranes was confirmed by P2X₇ immunostaining in cultures that were costained for GFAP. A positive control for the P2X₇ immuno-

staining was prepared by promoting microglial proliferation in the cortical cultures (Di Virgilio et al., 1999; Colomar and Amedee, 2001). Microglia were identified by their characteristic morphology and by F4/80 epitope immunoreactivity (Perry et al., 1985) (Fig. 1*C*). P2X₇ immunoreactivity on both astrocytes and microglia was eliminated by omission of the anti-P2X₇ antibody or by adding excess P2X₇ target epitope peptide to the cultures with the antibody (data not shown).

ATP induces an inward current in astrocytes that is mediated by P2X₇ receptors

ATP-induced currents were studied in mouse astrocyte cultures by the whole-cell patch-clamp technique. The application of ATP at concentrations of ≥0.1 mM induced an inward current in 33 of 48 cells recorded. The current developed quickly and showed small desensitization (Fig. 2). The P2Y agonists UTP and 2-chloro ATP produced no detectable currents, suggesting that the effect of ATP was mediated by a P2X receptor. BzATP produced a current similar to that produced by ATP, but BzATP was a more potent agonist (Fig. 2). The EC₅₀ for BzATP was ~100 μM, whereas the EC₅₀ for ATP was ~300 μM. This difference suggests that a component of the ATP-induced inward current was carried either by P2X₁ or P2X₇ receptors, because these are the only receptor types known to be more potently activated by BzATP than by ATP (Bianchi et al., 1999; North and Surprenant, 2000). One way that P2X₇ receptors can be distinguished from P2X₁ receptors is by the amplified response of P2X₇ receptors in low divalent cation solutions (Di Virgilio, 1995; Ralevic and Burnstock, 1998; North and Surprenant, 2000). As shown in Figure 2, the inward currents induced by both ATP and BzATP were amplified in low Ca^{2+}, low Mg^{2+} solution. Under these conditions BzATP was again more potent than ATP, with the approximate EC₅₀ values being 50 and 300 μM, respectively.

The effects of receptor antagonists also suggested that the ATP response was mediated by P2X₇ receptors. Suramin, which is a potent P2X₁ antagonist with only weak activity at P2X₇ receptors (Chessell et al., 1997; Ralevic and Burnstock, 1998; Bianchi et al., 1999; North and Surprenant, 2000), reduced inward currents only at concentrations of >1 mM (data not shown). The nonspecific P2 receptor antagonist pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (Chessell et al., 1997) inhibited both ATP- and BzATP-induced currents (Fig. 3), providing a positive control for this response. Inhibition of the ATP- and BzATP-induced currents with 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (Fig. 3) is also consistent with, although not specific for, flux through an P2X₇-activated ion channel (Soltoff et al., 1993).

An additional distinguishing characteristic of P2X₇ receptors is that they gate channels permeable to molecules up to 900 Da in size. Lucifer yellow is a 457 Da fluorescent dye that has been shown previously to enter cells via P2X₇ receptors (Ballerini et al., 1996; Coutinho-Silva et al., 1999). As shown in Figure 4, astrocytes treated with 300 μM BzATP in Ca^{2+}/Mg^{2+}-free medium exhibited a rapid and large increase in Lucifer yellow permeability. This effect was diminished in standard medium and was blocked by preincubating the cultures with Ox-ATP (Fig. 4*B*). Ox-ATP (ATP dialdehyde) is a selective P2X₇ antagonist when used as an irreversible inhibitor (Murgia et al., 1993; Evans et al., 1995; North and Surprenant, 2000). The profile of BzATP > ATP ≫ UTP agonist potency, response amplification in low divalent cation medium, weak inhibition by suramin, large channel formation in response to BzATP, and irreversible blockade by Ox-ATP is unique to P2X₇ receptors.
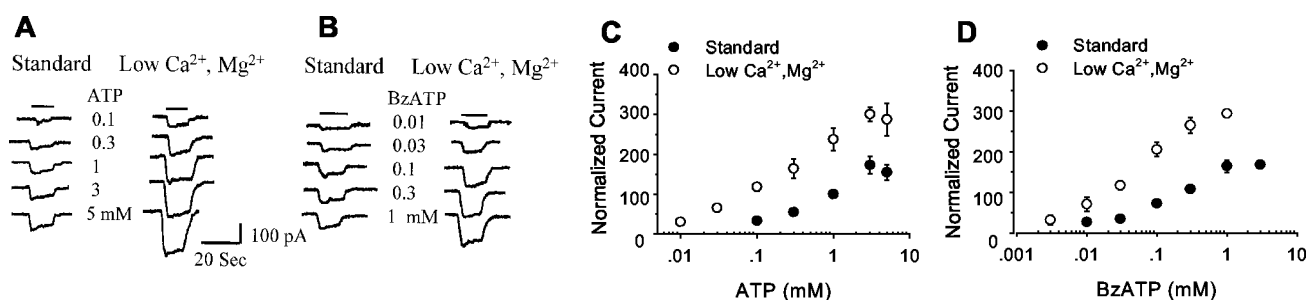
Figure 2. Whole-cell patch-clamp recordings showed inward currents during exposure to ATP or to the P2X$_7$ receptor-selective agonist BzATP. Representative recordings from single astrocytes are shown in *A* and *B*. Dose–response curves from averaged data (means ± SE) are plotted in *C* and *D* and expressed as the percentage of the 1 mM ATP-induced responses in normal divalent cation (standard) medium (*n* = 3–10 for each data point). Currents recorded in low Ca^{2+}, low Mg^{2+} medium were significantly greater than in normal medium at all ATP and BzATP concentrations tested (*p* < 0.05). External solution, 100 mM NaCl; internal solution, 100 mM NMDG-Cl; holding potential, −40 mV.
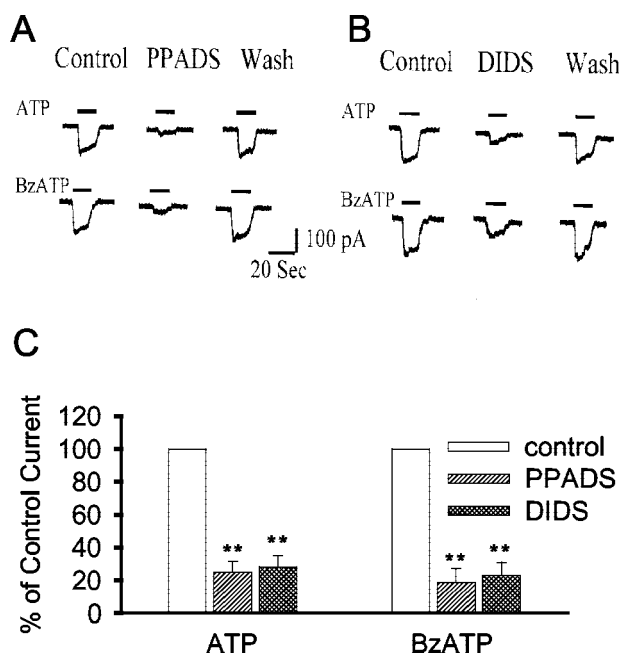

Figure 3. Currents induced by 1 mM ATP or 0.1 mM BzATP were reversibly inhibited by 20 μM PPADS, a P2 receptor antagonist, and by 1 mM DIDS. *Traces* in *A* and *B* are representative recordings from single patched astrocytes. *C* shows pooled data from five cells under each condition (means ± SE).**p < 0.01. Recordings were made with 100 mM NaCl in the external solution and 100 mM NMDG-Cl in the pipette. Holding potential, −40 mV.

### The astrocyte P2X$_7$ channel is permeable to glutamate

Permeability of the P2X$_7$-gated channel to glutamate and other ions was determined by constructing current–voltage curves in voltage-clamp configuration as illustrated in Figure 5; the reversal potentials of these currents are provided in Table 1. The relative BzATP-induced permeabilities to the different substrates, as calculated by the Goldman–Hodgkin–Katz equation, were Na$^+$, 1 > Cl$^-$, 0.34 > NMDG, 0.27 > L-glutamate, 0.15 ≈ D-aspartate, 0.16. Although the permeability to glutamate is less than that of Cl$^-$, the steep intracellular/extracellular gradient for glutamate provides a strong outward driving force for this anion (Erecinska and Silver, 1990; Anderson and Swanson, 2000). In the following experiments we directly measured ATP- and BzATP-induced release of glutamate from the astrocyte cultures.

P2X$_7$ receptor activation causes excitatory amino acid release from astrocyte cultures

Radiotracer loading of astrocytes was achieved by transporter-mediated uptake of ^{14}C L-glutamate or ^{3}H D-aspartate from the
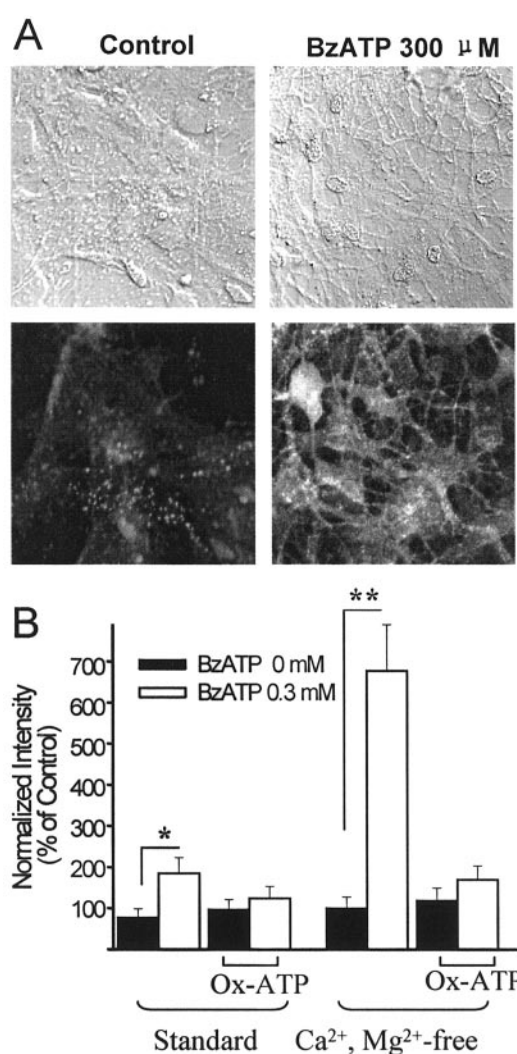
Figure 4. BzATP permeabilizes astrocytes to Lucifer yellow. *A, top*, Differential interference contrast images corresponding to the Lucifer yellow fluorescence images at the *bottom*. Cells were incubated for 5 min in Lucifer yellow in Ca^{2+}/Mg^{2+}-free BSS, with or without 300 μM BzATP. *B*, BzATP (0.3 mM) increased the Lucifer yellow fluorescence intensity in astrocytes; this effect was amplified in the Ca^{2+}/Mg^{2+}-free BSS. Pretreatment with the P2X$_7$ receptor antagonist Ox-ATP (0.3 mM) for 2 hr inhibited the effect of BzATP in both standard and Ca^{2+}/Mg^{2+}-free BSS. Data were normalized to the averaged intensity measured in cultures in the Ca^{2+}/Mg^{2+}-free solution. **p* < 0.05; **p < 0.01; *n* = 15 for each group. Error bars indicate SE.
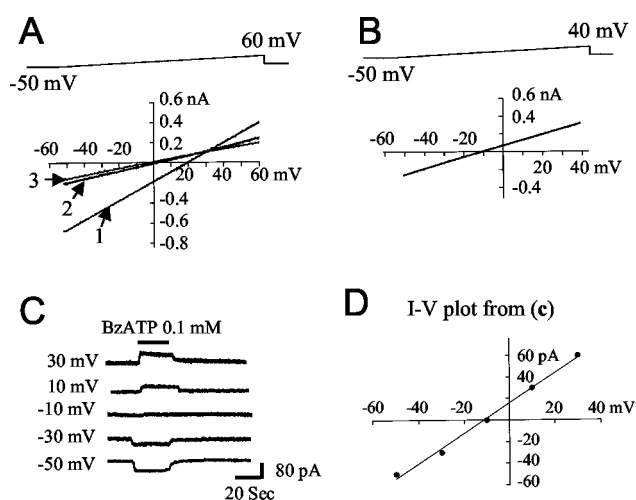
Figure 5. The reversal potentials of BzATP-induced currents under different external and internal ion conditions were determined by two methods. In *A* and *B*, 2 sec voltage ramp pulses were applied (*top*) and current–voltage curves obtained in the presence of 0.1 mM BzATP were subtracted by values obtained in the absence of BzATP. In *A*, the pipette contained 100 mM NMDG-Cl and the bath contained 100 mM NaCl (*1*), 100 mM NMDG-Cl (*2*), or 50 mM NMDG-Cl (*3*). In *B*, the pipette contained 100 mM NMDG-glutamate and the bath contained 100 mM NMDG-Cl. *C*, BzATP-induced currents were recorded under different holding potentials and plotted in *D*. The pipette contained 100 mM NMDG-glutamate and the bath contained 100 mM NMDG-Cl. Values obtained by these methods were in close agreement and are presented in Table 1.

Table 1. Reversal potentials of BzATP-induced inward currents

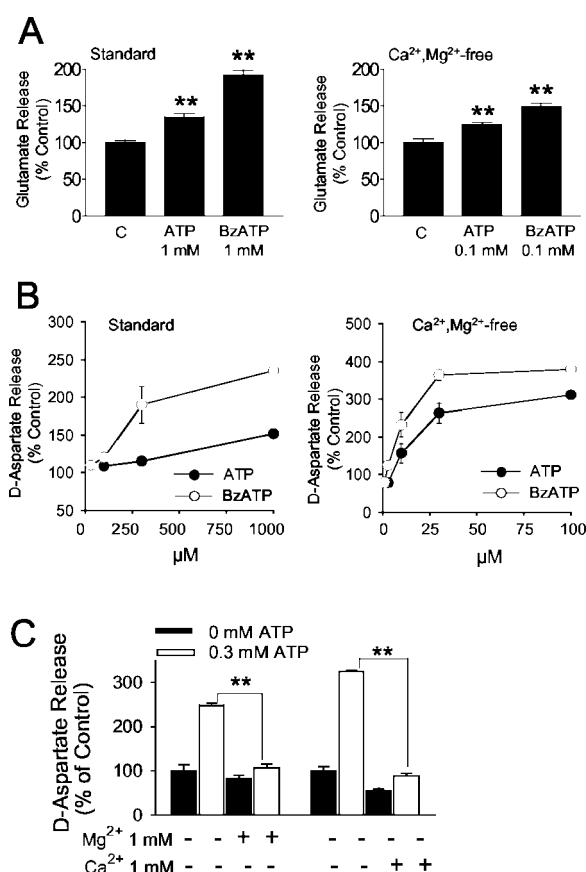| External solution[a]	Pipette solution[b]	Reversal potential
100 mM NaCl	100 mM NMDG-Cl	19.9 ± 0.5
100 mM NMDG-Cl	100 mM NMDG-Cl	0 ± 0.4
50 mM NMDG-Cl	100 mM NMDG-Cl	1.7 ± 0.5
100 mM NMDG-Cl	100 mM NMDG-L-glutamate[c]	−9.3 ± 0.7
100 mM NMDG-Cl	100 mM NMDG-D-aspartate[c]	−8.8 ± 0.3

Reversal potentials under each condition were determined using both methods described in Figure 3. Values are means ± SE; n = 6–16 cells. Osmolarity of both external and pipette solutions was adjusted to 290 mOsm with sucrose when needed.

[a]Also contained 5 mM HEPES.

[b]Also contained 0.3 mM CaCl₂, 0.1 mM MgCl₂, 5 mM HEPES, and 10 mM glucose.

[c]Also contained 2 mM NMDG-Cl.

Figure 6. ATP and BzATP induce an efflux of preloaded ¹⁴C glutamate and ³H D-aspartate. *A*, ¹⁴C glutamate release induced by 6 min incubations in standard BSS (*left*) or Ca²⁺/Mg²⁺-free BSS (*right*). BzATP produced a greater response than ATP under both conditions ($p < 0.05$), and the effects of both agonists were amplified in the Ca²⁺/Mg²⁺-free BSS. Cultures used for ¹⁴C glutamate studies were pretreated with methionine sulfoximine and amino-oxyacetic acid to inhibit the metabolism of glutamate to other ¹⁴C-labeled compounds (see Materials and Methods). *B*, ³H D-aspartate release induced by 6 min incubations in standard BSS (*left*) or Ca²⁺/Mg²⁺-free BSS (*right*). The dose–response curves indicate that BzATP is a more potent agonist than ATP and that the effects of both agonists are amplified in Ca²⁺/Mg²⁺-free medium. *C*, The effect of ATP on ³H D-aspartate release was amplified by the removal of both Mg²⁺ and Ca²⁺ but not by the removal of either cation alone. Values in each *panel* are means ± SE; n ≥ 6. +, With; −, without. **$p < 0.01$.

medium. Release of radiolabeled glutamate or D-aspartate under control conditions was typically 10–20% of the total radiolabel loaded when the cultures were placed in standard BSS and 25–35% when the cells were placed in Ca²⁺/Mg²⁺-free BSS. Astrocyte glutamate release was significantly increased by exposure to ATP or BzATP (Fig. 6*A*). As observed in the whole-cell patch-clamp studies, BzATP was a more potent agonist than ATP, and the responses to both agonists were markedly amplified in Ca²⁺/Mg²⁺-free BSS. Similar results were observed using D-aspartate as a tracer for glutamate flux (Fig. 6*B*). Under Ca²⁺/Mg²⁺-free conditions, which accentuate P2X₇-mediated responses, the EC₅₀ for BzATP was ∼10 μM, whereas the EC₅₀ for ATP was ∼25 μM. D-Aspartate is a glutamate analog that is taken up by plasma membrane glutamate transporters and has a permeability similar to glutamate at P2X₇ receptors (from Table 1), but that differs from L-glutamate in that it is not rapidly metabolized and not packaged by vesicular transporters (Naito and Ueda, 1985; Anderson and Swanson, 2000). The intracellular concentration of D-aspartate after loading was ∼10 nM (C. Anderson, unpublished observations), which is negligible compared with intracellular aspartate and glutamate concentrations (Erecinska and Silver, 1990). Because studies using glutamate and D-aspartate

showed similar results, most subsequent experiments were performed using ³H D-aspartate to avoid potential confounding effects of the agents required to prevent rapid glutamate metabolism.

To determine whether the response amplification observed in Ca²⁺/Mg²⁺ free medium was attributable specifically to the lack of Ca²⁺, experiments were performed using BSS prepared with Ca²⁺ and Mg²⁺ removed independently. As shown in Figure 6*C*, the effect of 0.3 mM ATP on D-aspartate release was substantially reduced by the presence of either 1 mM Mg²⁺ or 1 mM Ca²⁺, consistent with the properties of P2X₇ receptors (Chessell et al., 1997; Ralevic and Burnstock, 1998; Bianchi et al., 1999; North and Surprenant, 2000). Studies with other purinergic receptor agonists and antagonists also suggest that ATP-induced excitatory amino acid release occurs through astrocyte P2X₇ channels. As shown in Figure 7*A*, ATP-induced D-aspartate release was blocked by the nonselective P2 receptor antagonist PPADS, by the anion channel blocker DIDS, and by a 30 min preincubation with the irreversible, P2X₇-selective antagonist Ox-ATP. These agents were equally effective in Ca²⁺/Mg²⁺-free
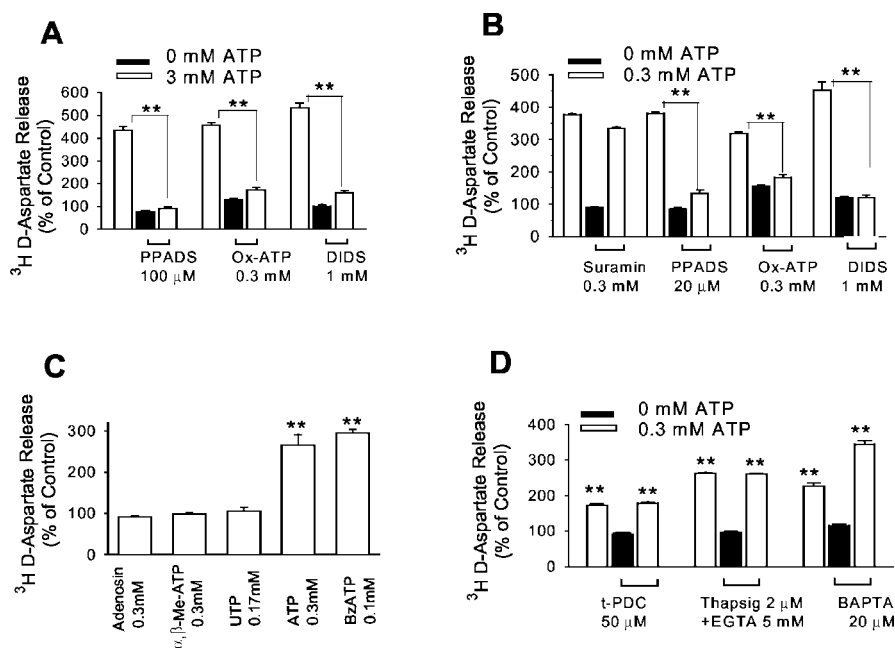
Figure 7. Pharmacology of ATP-induced D-aspartate release indicates action at P2X₇ receptors. *A*, Standard BSS. *B–D*, Ca²⁺/Mg²⁺-free BSS. *A*, ATP-induced D-aspartate release is blocked by DIDS, by the P2 receptor antagonist PPADS, and by a 2 hr preincubation with the irreversible P2X₇-selective inhibitor Ox-ATP. *B*, ATP was ~10-fold more potent in Ca²⁺/Mg²⁺-free medium than in standard medium (compare with *A*). The P2X₇ receptor inhibitors also blocked ATP-induced D-aspartate release in Ca²⁺/Mg²⁺-free medium, whereas the P2X₁ antagonist suramin was ineffective. *C*, D-Aspartate release was induced by BzATP and ATP but not by agonists of other purinergic receptor subtypes. *Adenosin*, Adenosine; *α,β-Me-ATP*, α,β-methylene ATP. *D*, ATP-induced release was not attenuated by preloading with PDC to block glutamate uptake reversal, by preloading the astrocytes with the calcium chelator BAPTA, or by depleting cell calcium with preincubation in Ca²⁺-free medium containing EGTA and thapsigargin (*Thapsig*). Preincubations with PDC, BAPTA-AM, and EGTA/thapsigargin were performed at 37°C for 40 min and followed by exchange with Ca²⁺/Mg²⁺-free BSS before the D-aspartate release assay. Values in each *panel* are means ± SE; $n \geq 6$. **$p < 0.01$.

medium, whereas the P2X₁-selective antagonist suramin was ineffective (Fig. 7*B*). ATP-induced release was not mimicked by other purinergic ligands, including the P1 agonist adenosine, the P2Y agonist UTP, and the potent P2X₁ agonist α,β-methylene ATP (Fig. 7*C*).

Evidence for ATP-induced glutamate or aspartate release by routes other than the P2X₇ channel was not observed. Release by Ca²⁺-dependent processes was rendered unlikely by the amplified response observed in Ca²⁺/Mg²⁺-free medium. In addition, ATP-induced release was not inhibited in astrocytes preincubated for 40 min in Ca²⁺-free medium containing the calcium chelator EGTA plus the calcium pump inhibitor thapsigargin (Fig. 7*D*). Similarly, preincubation for 40 min with a 20 μM concentration of the calcium chelator BAPTA-AM did not attenuate ATP-induced D-aspartate release (Fig. 7*D*). Inhibition of uptake reversal by preloading cultures with PDC (Longuemare and Swanson, 1995) also had no effect on ATP-induced release. Because extended activation of P2X₇ receptors causes cell lysis in some cell types, we also tested the possibility that excitatory amino acids were released because of the rupture of cell membranes by using intracellular LDH as an index of cell membrane integrity (Koh and Choi, 1987). LDH activity in the astrocyte cultures was not reduced at the end of the 6 min BzATP incubation period used for the radiotracer efflux studies in either standard BSS or Ca²⁺/Mg²⁺-free BSS at any BzATP concentration tested (Fig. 8). Intracellular LDH activity was also unchanged 24 hr after incubation with BzATP, excluding the possibility of delayed cell lysis or delayed leakage of LDH.

Discussion

Both glutamate and ATP serve as signaling molecules between astrocytes and neurons. The present findings suggest a mechanism by which glutamate can be released from astrocytes in response to extracellular ATP binding to P2X₇ receptors. Although the channel opened by P2X₇ ligand binding is not highly selective for glutamate, the strong driving force for glutamate release compared with other anions favors a significant glutamate efflux through the activated channel. Efflux through astrocyte P2X₇ channels is a previously unrecognized route of ligand-induced, nonvesicular astrocyte glutamate release.

ATP is a ligand for several purinergic receptor subtypes. A feature characteristic of P2X₇ receptors is that BzATP is a more potent and effective agonist than ATP (Ralevic and Burnstock, 1998; Bianchi et al., 1999; North and Surprenant, 2000). However, BzATP is also an agonist at several other P2X receptor subtypes, especially P2X₁, in which, as with P2X₇ receptors, it is significantly more potent than ATP itself. P2X₁ receptors can be pharmacologically distinguished from P2X₇ receptors by the fact that α,β-methylene ATP is a potent agonist, and suramin is a potent antagonist at the P2X₁ but not at the P2X₇ subtype (Bianchi et al., 1999; North and Surprenant, 2000). In addition, Ox-ATP (ATP dialdehyde) is an irreversible P2X₇ antagonist that does not irreversibly block P2X₁ or any other recognized P2X receptor subtype (Evans et al., 1995; North and Surprenant, 2000). The general P2X antagonist PPADS was initially thought to be relatively ineffective at P2X₇ receptors (Surprenant et al., 1996), but subsequent studies showed this to be an unreliable discriminator because of species differences (Chessell et al., 1998).

Another characteristic feature of P2X₇ receptors is the capacity to form large channels permeable to propidium and other dyes. However, P2X₂ and P2X₄ receptors can also form large pores with extended agonist exposure (Virginio et al., 1999), and
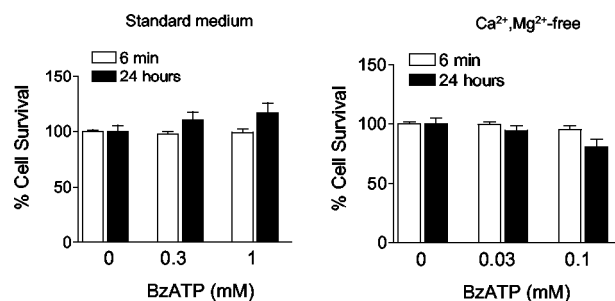
Figure 8. BzATP does not cause astrocyte cell lysis. Astrocytes were incubated with BzATP for 6 min in standard BSS (*left*) or Ca²⁺/Mg²⁺-free BSS (*right*). Cell survival was assessed by measuring intracellular LDH content in the cultures either immediately or 24 hr after the 6 min incubations. There was no significant change in LDH content under any of these conditions. Error bars indicate SE; $n = 8$.

$P2X_7$ channels do not have uniform permeability characteristics across all cell types (Steinberg and Silverstein, 1987; Nuttle and Dubyak, 1994). A more consistent feature of $P2X_7$ receptors is response amplification in low divalent cation medium; $P2X_7$ receptors are the only known P2X receptor subtype with this property (Ralevic and Burnstock, 1998; Bianchi et al., 1999; North and Surprenant, 2000).

In the present study, several lines of evidence were developed to establish that the ATP- and BzATP-induced D-aspartate release from astrocytes resulted from the activation of $P2X_7$ receptors: (1) BzATP and ATP produced an inward current that was amplified in low divalent cation medium, with BzATP being the more potent agonist. (2) BzATP-induced inward currents were carried by glutamate and D-aspartate. (3) BzATP rapidly permeabilized astrocytes to the large anionic dye Lucifer yellow. (4) Permeability to Lucifer yellow was strongly amplified in Ca^{2+}/Mg^{2+}-free medium and irreversibly blocked by the selective $P2X_7$ antagonist Ox-ATP. (5) BzATP induced a net efflux of glutamate and D-aspartate, with BzATP being the more potent agonist. (6) BzATP- and ATP-induced release of D-aspartate was amplified in low divalent cation medium. (7) BzATP- and ATP-induced D-aspartate efflux was irreversibly inhibited by Ox-ATP and not inhibited by 0.3 mM suramin. (8) Inward currents and D-aspartate efflux were not induced by α,β-methylene ATP, adenosine, or UTP. These data are all consistent with action at a $P2X_7$ receptor and are inconsistent with action at any other known receptor type.

Because $P2X_7$ channels in several cell types are permeable to large anionic dyes such as Lucifer yellow and fura-2 (Steinberg and Silverstein, 1987; Nuttle and Dubyak, 1994) it is not surprising that they are permeable to other anions as well. However, anion permeability of $P2X_7$ channels has not been extensively studied, and the literature suggests that permeability to anions, like cations, varies with cell type. In cultured mouse Schwann cells, a K^+-dependent Cl^- conductance has been observed with the activation of $P2X_7$ receptors (Colomar and Amedee, 2001). Coutinho-Silva and Persechini (1997) showed that $P2X_7$ pores in macrophages and J774 cell are permeable both to large cations (NMDG) and to anions (glutamate), whereas $P2X_7$ receptors in human B lymphocytes were reported to have negligible anion permeability (Bretschneider et al., 1995; Markwardt et al., 1997). The relative permeability of the $P2X_7$ channel to Na^+, $NMDG^+$, Cl^-, and glutamate estimated in the present study (Table 1) suggests that $P2X_7$ channels in astrocytes, although showing relative selectivity for Na^+ among tested ions, discriminate poorly between $NMDG^+$ and Cl^-. However, it remains possible that activation of $P2X_7$ receptors could simultaneously open both cation channels permeable to large cations such as NMDG and anion channels permeable to organic anions such as L-glutamate and D-aspartate.

Although the permeability of astrocyte $P2X_7$ channels to L-glutamate and D-aspartate was lower than that of Cl^- or Na^+ (relative permeabilities 0.15, 0.16, 0.34, and 1.0, respectively), radiotracer studies confirmed that a significant net efflux of L-glutamate and D-aspartate resulted from the activation of $P2X_7$ channels. Ion flux is determined not only by channel conductance but also by the ion concentrations and transmembrane ion gradient. Astrocyte intracellular glutamate is normally in the millimolar range, and astrocyte Na^+-dependent transporters maintain an intracellular/extracellular glutamate ratio of ~10,000:1 (Erecinska and Silver, 1990; Anderson and Swanson, 2000), which greatly exceeds the transmembrane gradient of the other

common anions and cations. The fraction of total ion flux attributable to glutamate is difficult to calculate, because the individual ion fluxes may not be independent of one another (Levitan and Garber, 1998). However, the radiotracer measurements indicate that full activation of astrocyte $P2X_7$ receptors could lead to a substantial glutamate efflux. The approximately threefold increase over basal glutamate release produced by maximally stimulating BzATP or ATP concentrations equates to the release of >30% of the intracellular glutamate pool within the 6 min observation interval.

Sustained activation of $P2X_7$ receptors leads to cell lysis in some cell types (Surprenant et al., 1996). The LDH release studies performed here showed that cell lysis is not the cause of excitatory amino acid efflux from astrocytes during $P2X_7$ receptor stimulation. The reason that $P2X_7$ receptor stimulation leads to cell lysis in some cell types but not others has not been established, but it may be related to the density of receptor expression. It may also be significant that $P2X_7$ receptors in the brain are exclusively monomeric, whereas $P2X_7$ receptors expressed elsewhere form homomeric multimers (Kim et al., 2001).

Previous reports have described a Ca^{2+}-dependent mechanism for ligand-induced glutamate release from astrocytes and Schwann cells (Parpura et al., 1994; Araque et al., 1998; Bezzi et al., 1998; Jeftinija and Jeftinija, 1998), including Ca^{2+}-dependent glutamate release induced by low micromolar ATP and blocked by suramin (Jeremic et al., 2001). The ATP-induced release described here was not blocked by suramin and was independent of Ca^{2+}, because it was not attenuated in cultures preincubated with the Ca^{2+}-pump inhibitor thapsigargin in Ca^{2+}-free medium or in cultures loaded with the Ca^{2+}-binding agent BAPTA. Quite the opposite, D-aspartate release was amplified severalfold in Ca^{2+}/Mg^{2+}-free medium. These findings may be reconciled by postulating that ligand-induced astrocyte release can occur by more than one mechanism. Alternatively, Ca^{2+}-dependent processes could act upstream of $P2X_7$ receptor activation. Because ATP functions as an autocrine and paracrine messenger (Wang et al., 1996; Cotrina et al., 1998; Guthrie et al., 1999; Queiroz et al., 1999), factors that induce astrocyte ATP release in one cell, such as elevated intracellular Ca^{2+} (Guthrie et al., 1999; Queiroz et al., 1999), could induce glutamate release by stimulating $P2X_7$ receptors on the same or nearby cells. In this way, ligands that cause an increase in intracellular calcium could indirectly lead to glutamate release through a process involving Ca^{2+}-dependent ATP release and subsequent, Ca^{2+}-independent activation of $P2X_7$ receptors.

Astrocyte $P2X_7$ receptors are expressed in brain astrocytes (Kukley et al., 2001; Panenka et al., 2001), and ATP is an important mediator of astrocyte intercellular signaling (Guthrie et al., 1999; Cotrina et al., 2000; Wang et al., 2000). Moreover, because glutamate can in turn induce ATP release from astrocytes (Queiroz et al., 1999), these reciprocal effects could contribute to a propagating signal. However, it should be noted that ATP is a weak $P2X_7$ agonist in the presence of normal extracellular Ca^{2+} and Mg^{2+} concentrations. This weak response to ATP is common to all $P2X_7$-mediated effects and all cell types on which $P2X_7$ receptors are expressed (North and Surprenant, 2000). Nevertheless, gene-deletion studies have demonstrated that $P2X_7$ receptors on macrophages are activated in vivo (Labasi et al., 2002). It is possible that $P2X_7$ receptor activation is achieved by transient, high ATP concentrations in confined spaces. Alternatively, the potency of ATP may be markedly increased in the presence of a coagonist or a priming stimulus (Le Feuvre et al., 2002). Given these considerations, the present findings indicate that astrocyte

P2X$_7$ receptors have the potential to conduct rapid nonvesicular glutamate release, but the physiological conditions in which astrocyte P2X$_7$ receptors are activated remain to be established.

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