Cellular/Molecular

$P2X_7$ Receptors Mediate ATP Release and Amplification of Astrocytic Intercellular Ca^{2+} Signaling

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Modulation of synaptic transmission and brain microcirculation are new roles ascribed to astrocytes in CNS function. A mechanism by which astrocytes modify neuronal activity in the healthy brain depends on fluctuations of cytosolic Ca^{2+} levels, which regulate the release of "gliotransmitters" via an exocytic pathway. Under pathological conditions, however, the participation of other pathways, including connexin hemichannels and the pore-forming $P2X_7R$, have been proposed but remain controversial. Through the use of genetically modified 1321N1 human astrocytoma cells and of spinal cord astrocytes derived from neonatal Cx43- and $P2X_7R$-null mice, we provide strong evidence that $P2X_7Rs$, but not Cx43 hemichannels, are sites of ATP release that promote the amplification of Ca^{2+} signal transmission within the astrocytic network after exposure to low divalent cation solution. Moreover, our results showing that gap junction channel blockers (heptanol, octanol, carbenoxolone, flufenamic acid, and mefloquine) are antagonists of the $P2X_7R$ indicate the inadequacy of using these compounds as evidence for the participation of connexin hemichannels as sites of gliotransmitter release.

Key words: **ATP release; gap junction blockers; connexin hemichannels; Ca^{2+} waves; glia; astrocyte**

Introduction

Astrocytes "*in situ*" and "*in vitro*" respond to various neurotransmitters with increases in cytosolic Ca^{2+} (Porter and McCarthy, 1997), which then elicit release of "gliotransmitters" (e.g., ATP, glutamate) that modulate neuronal activity (Haydon, 2001; Newman, 2003). There is compelling evidence that the Ca^{2+}-dependent mechanism of transmitter release from astrocytes involves regulated exocytosis (Parpura et al., 1994; Coco et al., 2003; Bezzi et al., 2004). Several reports indicate that additional Ca^{2+}-independent mechanisms may also contribute to transmitter release from astrocytes; the identity of these pathways remains controversial, with conflicting evidence supporting the participation of connexin hemichannels and/or $P2X_7$ receptors in this process.

Although GABA and glutamate release through $P2X_7Rs$ was reported to occur in astrocytes (Wang et al., 2002; Duan et al., 2003), the only evidence favoring the participation of $P2X_7Rs$ in ATP release was provided by Ballerini et al. (1996). These authors showed that stimulation of rat astrocytes with a $P2X_7R$ agonist ([2′-3′-O-(4-Benzoyl-benzoyl) adenosine 5′-triphosphate (BzATP)) induced Lucifer yellow (LY) uptake and purine release, whereas the antagonist oxidized ATP (oATP) prevented these responses. However, in murine astrocytes, oATP failed to block ATP-induced ATP release, suggesting that the pore-forming $P2X_7R$ was not involved (Anderson et al., 2004).

The first report implicating hemichannels in the process of ATP release from glial (C6 glioma) cells was based on a direct correlation between the level of Cx43 expression and that of released ATP (Cotrina et al., 1998). Later, it was observed that only ATP-releasing astrocytes and Cx43-C6 cells displayed an inward current and uptake of propidium iodide that was potentiated by increasing Cx43 expression levels (Arcuino et al., 2002). In two recent studies (Stout et al., 2002; Stout and Charles, 2003), it was reported that a gap junction channel blocker prevented mechanically induced ATP release from astrocytes and from Cx43-C6 cells, whereas removal of extracellular Ca^{2+} potentiated this release. In contrast to these reports, however, is our observation that extracellular Ca^{2+} removal and/or forced Cx43 expression in a P2R-null astrocytoma cell line did not induce any significant LY uptake (Suadicani et al., 2004).

The identification of mechanisms mediating transmitter release from astrocytes is relevant not only for normal physiological processes but also for pathological states of the CNS. For instance, during ischemic insults (Harris and Symon, 1984) and seizures (Louvel and Heinemann, 1983), reduction of Ca^{2+} concentrations in the extracellular environment may trigger the release of transmitters from astrocytes. The aim of this study was to evaluate whether hemichannels and/or $P2X_7Rs$ participate in transmitter release from spinal cord astrocytes after exposure to low divalent cation solution. For this purpose, we used pharmacological inhibitors and mice lacking Cx43 or $P2X_7R$. We first evaluated whether gap junction channel blockers affected the properties of $P2X_7Rs$ and then analyzed the extent to which hemichannels and/or $P2X_7Rs$ participate in the amplification of intercellular Ca^{2+} wave transmission between wild-type (WT), Cx43-, and $P2X_7R$-null spinal cord astrocytes. Our results indicate that gap junction channel blockers are $P2X_7R$ antagonists, and that these receptors, but not Cx43 hemichannels, contribute to ATP release that amplifies astrocytic Ca^{2+} waves.

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Materials and Methods

Astrocyte cultures. Spinal cords were obtained from neonatal wild-type, Cx43-null (offspring of Cx43 heterozygous C57BL/6J-Gja1^{tm1Kdr}; The Jackson Laboratory, Bar Harbor, ME) and $P2X_7R$-null (C57BL/6; Pfizer, Groton, CT) mice. Animal colonies were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility at AECOM. Primary cultures of spinal cord astrocytes were prepared as described previously (Scemes et al., 2000) and grown in DMEM (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS; Invitrogen) and 1% Penicillin-Streptomycin (Mediatech, Cellgro, VA) at 37°C in a humidified 5% CO_2 incubator for 10–20 d. Cells were then trypsinized (0.05% trypsin for 1 min at 37°C), centrifuged, resuspended in DMEM, and seeded on MatTek dishes 2–4 d before experimentation.

Generation and maintenance of $P2X_7R$ clones. Clones stably expressing $P2X_7Rs$ were generated in the human astrocytoma 1321N1 cell line (European Collection of Cell Cultures, Salisbury, UK) as described previously (Suadicani et al., 2004). Briefly, 1321N1 cells grown in Petri dishes containing culture medium (DMEM, 10% FBS, and 1% Penicillin-Streptomycin) were plated on 35 mm dishes, grown to confluence, and transfected with 4–6 μg of the rat $P2X_7R$ cDNA (a gift from Dr. A. Surprenant, Institute of Molecular Physiology, University of Sheffield, Sheffield, UK) inserted in pcDNA3 vector using 6 μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, cells were transferred to 100 mm Petri dishes at low density (0.2–0.5 cell/mm^2) and maintained in selection medium [DMEM supplemented with 500 μg/ml G-418 (Invitrogen)] for 20–30 d at 37°C in a humidified 5% CO_2 incubator. Clones were then isolated, and the functional expression of the $P2X_7R$ was verified by the ability of the transfectants to respond to BzATP with Ca^{2+} transients and dye uptake (see below).

Intracellular calcium transients. Changes in cytosolic calcium levels were measured as described previously (Scemes et al., 2000; Suadicani et al., 2004). Briefly, cells plated on glass-bottomed dishes (MatTek, Ashland, MA) were loaded with the ratiometric Ca^{2+} indicator fura-2 AM (10 μM; Invitrogen, Eugene, OR) for 45 min at 37°C, and after several washes with DPBS (pH 7.4; Dulbecco PBS; Cellgro), cells were imaged on a Nikon TE2000 inverted microscope equipped with a CCD digital camera (Orca-ER; Hamamatsu Photonics, Hamamatsu, Japan) and a 20× panfluo objective (numerical aperture, 0.45; Nikon). Changes in fura-2 fluorescence intensities emitted at two excitation wavelengths (340 and 380 nm) were acquired at 1.0 Hz using the Lambda DG-4 (Sutter Instruments, Novato, CA) driven by a computer through Metafluor software (Universal Imaging, West Chester, PA). Values of intracellular Ca^{2+} levels determined from regions of interest placed on cells were obtained from fura-2 ratio images using an *in vitro* calibration curve. Noncumulative dose–response curves were obtained for the $P2X_7R$ agonist BzATP. An interval of 5 min after several washes with DPBS was maintained between the additions of increasing doses (10 nM to 1 mM) of the same agonist.

Dye uptake. Parental and $P2X_7R$-expressing 1321N1 cells plated on MatTek dishes were bathed for 5 min in DPBS containing the cell-impermeant (629 Da) dye YoPro-1 (5 μM; a cationic cyanine monomer that after binding to nucleic acids exhibits a 1800-fold fluorescence enhancement; Invitrogen), and the basal fluorescence intensity was measured from regions of interest placed on cells. To activate $P2X_7Rs$ and induce pore formation, cells were exposed to 200 μM BzATP in 5 μM YoPro control (DPBS, containing 1 mM Ca^{2+} and 492 μM $MgCl_2$) and low divalent cation solutions (LDPBS; containing zero Ca^{2+} and 43 μM $MgCl_2$, prepared by dissolving 1 mM $MgCl_2$ and 1 mM EDTA in 0 Ca^{2+}, 0 $MgCl_2$ DPBS; free-Mg^{2+} concentration was calculated using the MaxChelator software, http://www.stanford.edu/~cpatton/maxc.htlm), and the fluorescence intensity was measured after 5 min of exposure. To evaluate the effect of the $P2X_7R$ antagonist Brilliant Blue G (BBG) and of gap junction channel blockers on $P2X_7R$-mediated dye uptake, the $P2X_7R$-expressing cells were exposed to BzATP (100 or 200 μM) in 5 μM YoPro-1 low divalent cation solution in the absence and presence of each of these compounds, and the fluorescence intensity was measured after a 5 min exposure. After background subtraction, YoPro fluorescence intensity was normalized to values obtained from cells bathed in 5 μM YoPro low divalent cation solution. YoPro fluorescence was captured using an Orca-ER CCD camera attached to an inverted Nikon microscope equipped with a 20× dry objective and 488 nm filter set using Metafluor software.

ATP release measurements. The amount of ATP released by parental and $P2X_7R$-expressing 1321N1 cells exposed for 2 min to control (DPBS) and low divalent cation (LDPBS) solutions was determined using the Luciferin-Luciferase assay (Invitrogen). Total amount of ATP present in the bathing solutions was calculated according to standard curves and ATP concentration expressed as nM/10 μg protein. Distinct standard curves were generated by diluting ATP (50–5000 nM final concentrations) in each of the experimental solutions used.

Intercellular calcium waves. Spinal cord astrocytes plated on MatTek dishes were loaded with fura-2 AM (10 μM) for 45 min at 37°C and changes in fura-2 fluorescence intensities measured as described above. Intercellular calcium waves (ICWs) were triggered by focal mechanical stimulation of single cells in the center of the microscope field of view (460 × 367 μm), as described previously (Scemes et al., 1998). The properties of the ICW was analyzed in terms of efficacy (relative number of cells per field that responded with an increase in basal Ca^{2+} level) and of extent of spread, which was evaluated based on the efficacy in concentric tiers (~40 μm radius) set from the point of the stimulus (Suadicani et al., 2004). The glutamate antagonist MCPG [(*RS*)-α-Methyl-4-carboxyphenylglycine; Tocris Cookson, Ballwin, MO] and the ATP degrading enzyme apyrase (EC 3.6.1.5 grade III; Sigma, St. Louis, MO) were used to evaluate the participation of glutamate and ATP on the transmission of ICWs.

Western blotting. Whole-cell lysates were electrophoresed on 10% SDS-PAGE (Bio-Rad, Hercules, CA) and proteins transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After overnight incubation with blocking buffer (1× PBS with 5% nonfat dry milk) at 4°C, the membranes were incubated for 1 h at room temperature (RT) with polyclonal anti-Cx43 antibody (1:2000; a gift from Dr. E. L. Hertzberg, Albert Einstein College of Medicine, Department of Neuroscience, Bronx, NY) or polyclonal anti-$P2X_7R$ (1:500; Alomone Labs, Jerusalem, Israel) and monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000; Research Diagnostics, Flanders, NJ). After several washes in 1× PBS containing 0.5% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (1:2000; Vector Laboratories, Burlingame, CA) for 1 h at RT. Detection of bands was performed on x-ray film (Kodak, Rochester, NY) after incubation of the membranes with the enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK).

Chemicals. The following gap junction channel blockers were used: flufenamic acid (FFA), carbenoxolone (CBX), mefloquine (MFQ), octanol (OCT), and heptanol (HEP). The shorther chain alcohol, hexanol (HEXA), and the nonactive analog of CBX, glycyrrhizic acid (GCZ), were also used. We used BzATP as an agonist and BBG as an antagonist of the $P2X_7R$. With the exception of MFQ (kindly provided by Dr. M. Srinivas, Department of Biological Sciences, State University of New York State College of Optometry, New York, NY), all reagents were from Sigma.

Statistical analysis. All data are expressed as means ± SE. GraphPad Prism version 4 was used in the statistical analysis, and data sets were compared by one-way ANOVA followed by Newman–Keuls or Tukey's tests.

Results

Gap junction blockers are antagonists of and prevent ATP release through $P2X_7R$

Most evidence implicating connexin hemichannels in the release of transmitters from astrocytes has relied on the use of pharmacological compounds (gap junction channel blockers) that are nonspecific and have been shown to affect the activity of Na^+, K^+, and Cl^- channels as well as adenosine receptors (Gogelein et al., 1990; Farrugia et al., 1993; Ottolia and Toro, 1994; Poyraz et al., 2003; Weiss et al., 2003; Vessey et al., 2004). Whether some of the gap junction blockers that have been commonly used for such

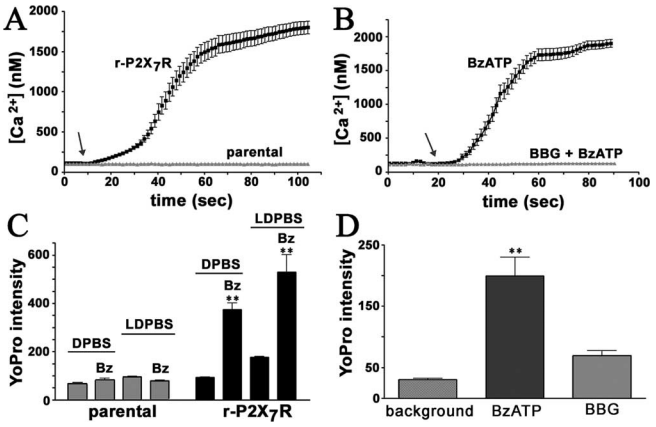

Figure 1. Functional characterization of $P2X_7R$ expressed in 1321N1 human astrocytoma cells. ***A***, Intracellular Ca^{2+} changes induced by bath application (arrow) of 200 μM BzATP in fura-2 AM loaded parental (white triangles) and $P2X_7R$-expressing 1321N1 (black squares) cells. ***B***, Increase in intracellular Ca^{2+} levels of $P2X_7R$-expressing 1321N1 cells induced by bath application (arrow) of 100 μM BzATP was prevented by 100 nM BBG (specific r-$P2X_7R$ antagonist). ***C***, YoPro-1 fluorescence intensity measured from parental and from $P2X_7R$-expressing 1321N1 cells bathed in control (DPBS) and in low divalent cation (LDPBS) solutions in the absence and presence of 200 μM BzATP. Note the characteristic potentiation of BzATP-induced YoPro-1 uptake in cells bathed in low divalent cation solutions. ***D***, BzATP-induced changes in YoPro-1 fluorescence intensity measured from $P2X_7R$-expressing 1321N1 cells bathed in low divalent cation solutions in the absence and in the presence of 100 nM BBG. The first bar shows background YoPro fluorescence measured from cells bathed in low divalent cation solution before the addition of BzATP. Data are expressed as mean ± SEM ($n = 80–120$ cells from at least 2 independent experiments; $^{**}p < 0.01$, ANOVA followed by Newman–Keuls test). Error bars represent SEM.

studies can also affect $P2X_7$ receptors has not been evaluated previously. To address this question, we tested whether these compounds could affect Ca^{2+} influx and cell permeabilization induced by $P2X_7R$ activation. To do this, we stably expressed the rat $P2X_7R$ in a cell line, the human 1321N1 astrocytoma, which is devoid of endogenous P2 receptor expression and is poorly coupled by Cx43 gap junction channels (Suadicani et al., 2004). The adequacy of this system for such studies was determined by analyzing the functional expression of these receptors. For that, we measured agonist induced Ca^{2+} influx and dye uptake in parental and $P2X_7R$-expressing cells, as well as tested the effects of a $P2X_7R$ antagonist on these events. Bath application of 200 μM $P2X_7R$ agonist BzATP induced an increase in intracellular Ca^{2+} in fura-2 AM-loaded $P2X_7R$ cells but not in parental cells (Fig. 1*A*). Moreover, Ca^{2+} influx induced by 100 μM BzATP was blocked after a 2 min preincubation with 100 nM BBG (Fig. 1*B*), a potent antagonist for the rat $P2X_7R$ (Jiang et al., 2000). Another characteristic of these ionotropic receptors, in addition to providing cation influx, is the induction of large pores in the cell membrane, which allow the diffusion of molecules up to 900 Da. To test for pore formation in $P2X_7R$-expressing cells, we measured uptake of the fluorescent dye YoPro-1 (Fig. 1*C*,*D*). As shown in Figure 1*C*, after BzATP (200 μM) application, an increase in intracellular YoPro fluorescence intensity in cells expressing $P2X_7R$ (black bars) was detected, which was not observed in parental cells (gray bars). As reported previously (Virginio et al., 1997), BzATP-induced influx of this 629 Da dye was potentiated when $P2X_7R$-expressing cells were bathed in low divalent cation solution (Fig. 1*C*). YoPro uptake induced by BzATP (100 μM) under these conditions was greatly reduced by BBG (100 nM) (Fig. 1*D*), further confirming the participation of $P2X_7Rs$.

FFA, CBX, MFQ, octanol, and heptanol are among the most common gap junction channel blockers used to verify the participation of connexin hemichannels in transmitter release from astrocytes and several other cell types, although they lack specificity. For instance, the nonsteroid fenamate drug, FFA, which at nanomolar concentration has an anti-inflammatory action, is a potent blocker of several ion channels (Gogelein et al., 1990; White and Aylwin, 1990; Wang et al., 1997; Schultz et al., 1999), including gap junctions, when used at micromolar levels (Srinivas and Spray, 2003). CBX is a glycyrrhetinic acid derivate that requires long exposure time and concentrations above 50 μM to block gap junctional communication (Davidson et al., 1986; Spray et al., 2002) and also affects other ion channels (Rouach et al., 2003; Vessey et al., 2004). Long chain alkanols (halothene) and alcohols (octanol and heptanol) have long been known to close gap junctions (Johnston et al., 1980) and also to reduce neuronal excitability probably by affecting membrane fluidity (Takens-Kwak et al., 1992).

To evaluate whether these compounds also display antagonistic activity against $P2X_7Rs$, we tested the effect on Ca^{2+} influx mediated by $P2X_7R$ activation. For that, we measured the effect of increasing concentrations of these blockers on the amplitude of intracellular calcium transients induced by bath application of a fixed maximal concentration of BzATP (100 μM), determined from the logistic dose–response curve for this agonist (Fig. 2*A*). With the exception of octanol and heptanol (which at 1.5 mM did not affect BzATP-induced Ca^{2+} influx) (Fig. 2*B*), all other compounds antagonized to different degrees the Ca^{2+} elevations in response to 100 μM BzATP (Fig. 2*A*). The calculated IC_{50} values (concentration inducing half-maximal inhibition) obtained from fitting the logistic curves displayed in Figure 2*A* were 655 nM for FFA, 175 nM for CBX, and 2.5 nM for MFQ, indicating that these drugs are ~100–1000 times more potent at blocking rat $P2X_7R$ than gap junction channels. Of note is the noncompetitive nature of the antagonism provided by CBX and MFQ, as indicated by the reduction of the maximal amplitude of BzATP-induced Ca^{2+} influx (50 and 70%, respectively). Figure 2, *C* and *D*, shows examples of the inhibitory effects of CBX and MFQ on intracellular Ca^{2+} mobilization induced by 100 μM BzATP. With the exception of FFA, the blockade provided by CBX and MFQ was irreversible, at least within 10 min after washout and a minimum of 2 min of pre-exposure was necessary for these compounds to block BzATP-induced Ca^{2+} rise. GCZ, a nonactive analog of CBX, and the short chain alcohol, hexanol (HEXA), did not affect BzATP-induced Ca^{2+} mobilization. In the presence of 100 μM GCZ or 3 mM HEXA, BzATP (100 μM) increased intracellular Ca^{2+} levels to 1668 ± 76 nM ($n = 52$ cells) and 1817 ± 74 nM ($n = 18$ cells), respectively; these values were not statistically different ($p > 0.05$; ANOVA) from those recorded in the absence of GCZ and HEXA (1846 ± 74 nM; $n = 23$ cells).

We next evaluated whether these compounds could also prevent YoPro-1 uptake in BzATP stimulated $P2X_7R$ cells bathed in low divalent cation solution. [Exposure to solutions with reduced divalent cation concentrations has been reported to increase the sensitivity of $P2X_7R$ to its ligands ATP and BzATP (Virginio et al., 1997; North and Surprenant, 2000) and also proposed to favor opening of hemichannels formed by certain connexins when exogenously expressed in *Xenopus* oocytes (Ebihara, 1996; Pfahnl and Dahl, 1999; Eskandari et al., 2002; Gomez-Hernandez et al., 2003)].

In these experiments, all gap junction channel blockers tested, including octanol and heptanol, were found to prevent the 22-fold increase in YoPro-1 uptake induced by 100 μM BzATP (Fig.

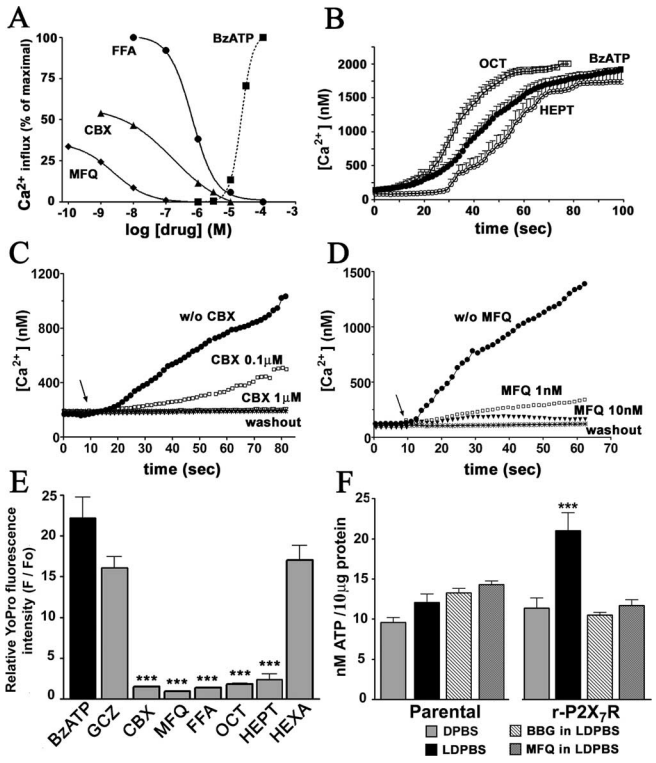

Figure 2. Gap junction channel blockers are antagonists of P2X$_7$R. ***A***, Dose–response for BzATP (squares) obtained from fura-2 AM loaded P2X$_7$R-expressing 1321N1 cells. Evidence for the nonsurmountable blockade of BzATP-induced Ca^{2+} influx by MFQ (diamonds), FFA (circles), and CBX (triangles) were obtained by using a fixed concentration of BzATP (100 μM) and varying the concentration of gap junction channel blockers. ***B***, Intracellular Ca^{2+} elevations induced by 100 μM BzATP in the absence and in the presence of 1.5 mM OCT or HEP. ***C*** and ***D*** show examples of the blockade provided by two doses of CBX and MFQ on BzATP-induced Ca^{2+} mobilization, respectively; note that after CBX and MFQ washout (10 min), no response to BzATP (100 μM) was recorded. Arrows indicate BzATP addition. ***E***, Bar histograms showing the mean relative YoPro fluorescence intensity (F/F_0) changes induced by 100 μM BzATP in the absence and presence of 100 μM GCZ, 50 μM CBX, 1 nM MFQ, 100 μM FFA (concentrations that produced at least 80% blockade of BzATP-induced Ca^{2+} influx) (Fig. 1), 1.5 mM OCT, 1.5 mM HEP, and 3 mM HEXA. ***F***, Bar histograms showing the mean values of ATP (nM/10 μg of total protein) present in the bathing solution of parental and P2X$_7$R-expressing 1321N1 cells exposed for 2 min to control and low divalent cation solutions in the absence and presence of BBG (100 nM) and MFQ (2 nM). Data are expressed as mean ± SEM (n = 100–210 cells from at least 2 independent experiments; *** $p < 0.001$, ANOVA followed by Newman–Keuls test). Error bars represent SEM.

2*E*); note in Figure 2*E* that neither GCZ nor HEXA affected BzATP-induced YoPro uptake. The possibility, however, that the effects of gap junction channels blockers on dye uptake are related to the influx through hemichannels [Cx43 is expressed in 1321N1 cells (Suadicani et al., 2004)] seems unlikely given that data presented in Figure 1*C* clearly show that only the P2X$_7$R expressing cells and not the parental cells display significant increment of YoPro-1 uptake in low divalent cation solutions.

The demonstrated ability of these active compounds to block P2X$_7$R-mediated dye uptake also suggested that they might prevent the efflux of transmitters through these receptors. To verify this possibility, we measured the amount of ATP present in the solutions bathing parental and P2X$_7$R-expressing 1321N1 cells. As shown in Figure 2*F*, the amount of ATP present in control solution bathing parental 1321N1 cells was not significantly different from that obtained from control solutions bathing P2X$_7$R-expressing cells. In contrast, when cells were exposed to low divalent cation solution, a twofold increase in the amount of ATP present in the bathing solution was obtained only for P2X$_7$R-expressing cells; this increase in ATP release from P2X$_7$R expressing cells bathed in low divalent solution was prevented by the P2X$_7$R antagonist BBG (100 nM) and by the gap junction channel blocker MFQ (2 nM) (Fig. 2*F*). These data, therefore, indicate that gap junction blockers are potent antagonists of the P2X$_7$R and prevent P2X$_7$R-mediated ATP release.

ATP release mediates the amplification of intercellular Ca^{2+} waves in spinal cord astrocytes

Given that it has been reported that glutamate and ATP are released after exposure of astrocytes to low divalent cation solution (Stout and Charles, 2003;Ye et al., 2003; Duan et al., 2003), we evaluated the extent to which ATP and glutamate contributed to amplification of ICW spread in spinal cord astrocytes. We first compared the efficacies (relative number of responding cells) and the distance traveled by mechanically induced ICWs in confluent cultures of wild-type spinal cord astrocytes bathed in control and in low divalent cation solutions and then analyzed the effects of the broad spectrum metabotropic glutamate receptor antagonist, MCPG, and of the ATP degrading enzyme, apyrase, in this process.

As illustrated in Figure 3, *A* and *B*, the ICWs induced in spinal cord astrocytes bathed in control solution spread to fewer cells and traveled shorter distances (efficacy, 0.39 ± 0.02; radius, 108.20 ± 4.27 μm; n = 97 fields, 18 experiments) than waves induced in astrocytes bathed in low divalent cation solution (efficacy, 0.71 ± 0.02; radius, 149.5 ± 3.15 μm; n = 35 fields, 6 experiments). The ATP degrading enzyme apyrase (50 U/ml), but not the glutamate antagonist MCPG, prevented ICW amplification induced by low divalent cation solution (Fig. 3*C*). In the presence of apyrase, the efficacy of ICW spread in astrocytes exposed to low divalent cation solution was reduced to 0.38 ± 0.04 (n = 25 fields, 3 experiments), a value similar ($p > 0.05$; ANOVA) to that obtained from cells bathed in control solution. In contrast, exposure of astrocytes to 1 mM MCPG, a concentration that has been shown to completely block glutamate-induced Ca^{2+} transients in these cells (Pasti et al., 1997), did not prevent ICW amplification; in this case, the efficacy (0.64 ± 0.04; n = 26 fields, 4 experiments) of ICW spread between cells bathed in low divalent cation solution containing the glutamate receptor antagonist was not significantly different ($p > 0.05$; ANOVA) from that observed in the absence of MCPG (efficacy, 0.71 ± 0.02; n = 35 fields, 6 experiments).

These data, therefore, indicate that ATP rather than glutamate mediates the amplification of the Ca^{2+} signal transmission between spinal cord astrocytes exposed to low divalent cation solution.

P2X$_7$Rs but not Cx43 hemichannels mediate ICW amplification

Astrocytes express a variety of P2 receptors, including the P2X$_7$R (Fumagalli et al., 2003), and are mainly coupled by Cx43 gap junction channels (Scemes et al., 2000). To determine whether Cx43 hemichannels and/or P2X$_7$R participate in the amplification of astrocytic ICW spread induced by low divalent cation solution, we used Cx43-null and P2X$_7$R-null spinal cord astrocytes. This strategy allowed testing the individual contribution of these two proteins to ICW spread without the use of pharmacological gap junction blockers, which are shown (Figs. 1, 2) to be antagonists of the P2X$_7$R.

Figure 4 depicts the relative number of WT, Cx43-, and P2X$_7$R-null astrocytes per tier (efficacy per tier) that participated

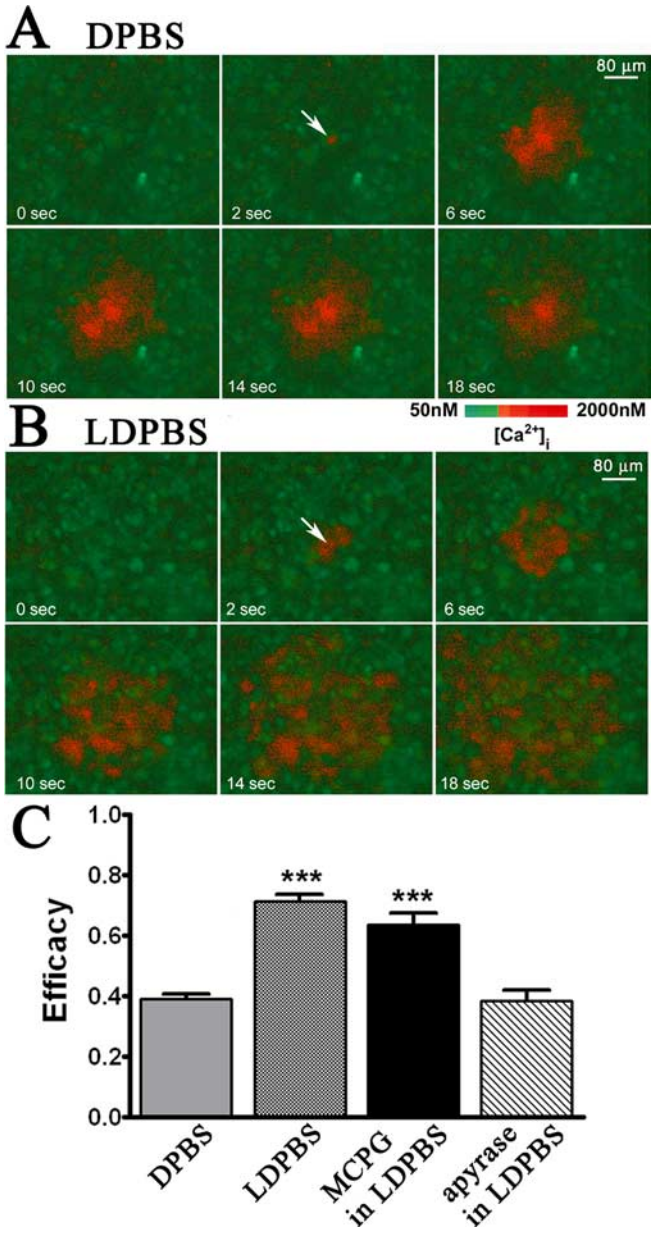

Figure 3. ATP mediates ICW amplification induced by low divalent cation solution. Pseudo-colored time-lapse images of mechanically induced ICW spread in confluent primary cultures of spinal cord astrocytes exposed to control (DPBS) (***A***) and low divalent cation solutions (LDPBS) (***B***). ***C***, Bar histogram showing the mean efficacy values of the Ca^{2+} signal transmission in cells bathed in control and low divalent cation solutions in the absence and presence of MCPG and apyrase. Note that only apyrase (50 U/ml) but not MCPG (1 mM) completely prevented the increase in the efficacy of ICW induced by low divalent cation solution. Error bars correspond to mean ± SEM. n = 25–97 fields; 3–18 experiments; *** $p < 0.001$ (ANOVA followed by Tukey's test).

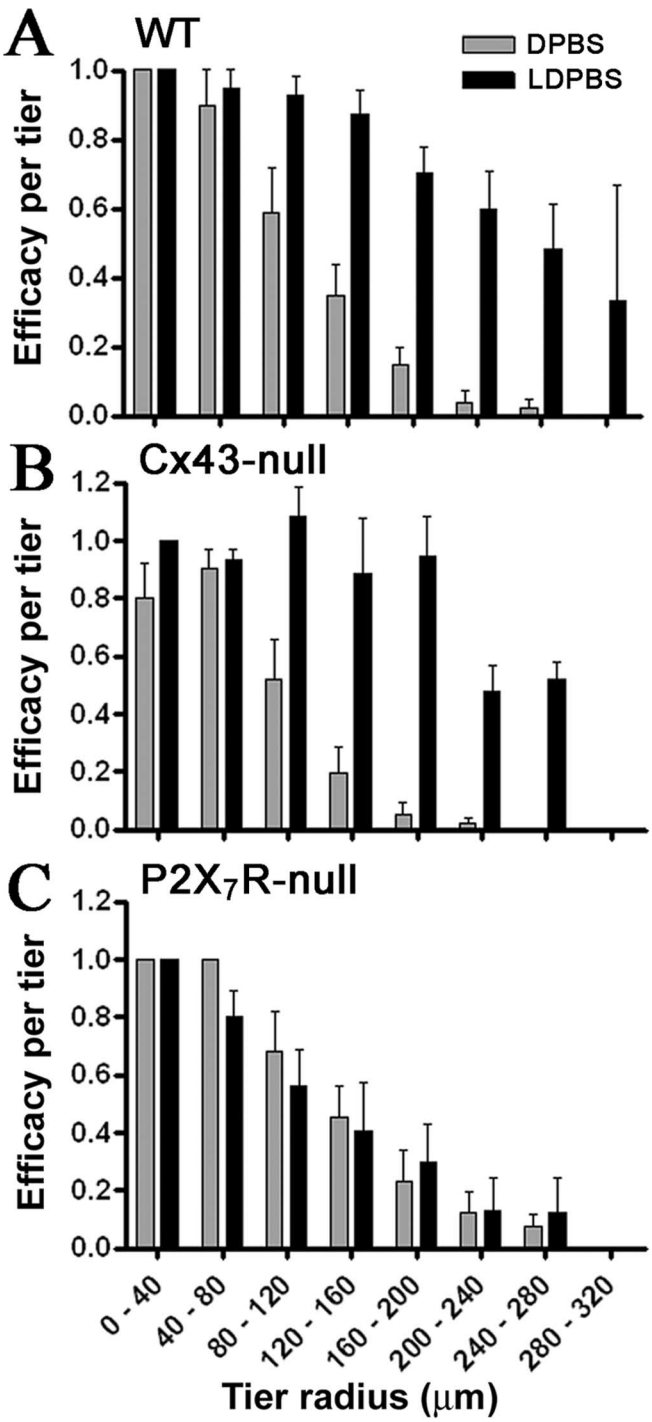

Figure 4. $P2X_7$ receptors contribute to ICW amplification induced by low divalent cation solution. Relative number of cells per tier (efficacy per tier) participating in the transmission of Ca^{2+} signals triggered by focal mechanical stimulation of single cells in confluent cultures of WT (***A***), Cx43- (***B***), and $P2X_7R$-null (***C***) spinal cord astrocytes bathed in control (gray bars) and low divalent cation (black bars) solutions. The increased relative number of participating cells per tier and extent of ICW transmission induced by low divalent cation solution is absent only in $P2X_7R$-null cells. Data are expressed as mean ± SEM (n = 5–6 fields from one representative experiment). Error bars represent SEM.

in ICW spread when cells were bathed in control (gray bars) and in low divalent cation (black bars) solutions. Exposure of WT cells to low divalent cation solution expanded the radius of astrocytic ICW spread and increased the number of participating cells (Fig. 4*A*). Note that for the same wave spread radius, particularly beyond 120 μm, reduction of extracellular divalent concentration of the bathing solution led to a threefold increase of the number of responding cells. Similarly to what was observed for WT astrocytes, exposure of Cx43-null astrocytes to low divalent cation solution significantly increased the distance of Ca^{2+} signal spread (Fig. 4*B*). In low divalent cation solution, ICWs spread farther (radius of 141.6 ± 3.86 μm) and with two times higher efficacy (0.68 ± 0.02) compared with ICWs recorded under control conditions (radius, 91.2 ± 4.35 μm; efficacy, 0.32 ± 0.02; n = 80 and 39 fields, 7–10 experiments). In contrast to what was observed for WT and Cx43-null astrocytes, exposure of $P2X_7R$-

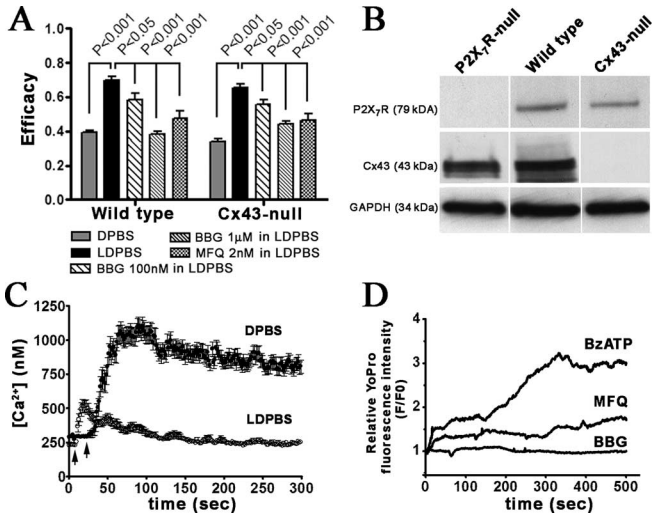

Figure 5. ***A***, Bar histograms showing the mean values of ICW efficacies obtained for WT and Cx43-null astrocytes exposed to control (DPBS) and low divalent cation (LDPBS) solutions in the absence and presence of BBG (100 nM and 1 μM) and MFQ (2 nM) ($n = 20–97$ fields, 3–18 experiments; p values from ANOVA followed by Tukey's test). ***B***, Representative Western blot showing the expression levels of $P2X_7R$, Cx43, and GAPDH obtained from whole-cell lysates of WT, Cx43-, and $P2X_7R$-null spinal cord astrocytes. ***C***, Intracellular Ca^{2+} changes induced by bath application (arrows) of 300 μM BzATP in fura-2 AM loaded WT spinal cord astrocytes bathed in control (DPBS; closed cirlces) and in low divalent cation solution (LDPBS; open circles). ***D***, Time course of relative YoPro-1 fluorescence intensity changes recorded from WT spinal cord astrocytes induced by 300 μM BzATP, in the absence and presence of BBG (100 nM) and MFQ (2 nM). Error bars represent SEM.

null astrocytes to low divalent cation solution did not lead to ICW amplification (radius, 123.7 ± 4.85 μm; efficacy, 0.50 ± 0.03; 24 fields, 4 experiments) (Fig. 4*C*). The amplification of the Ca^{2+} signal transmission observed in WT and Cx43 KO spinal cord astrocytes bathed in low divalent cation solution was significantly reduced by 100 nM $P2X_7R$ antagonist BBG and totally prevented by 1 μM BBG (Fig. 5*A*), which did not alter ICW efficacy under control conditions. [Electrophysiological experiments performed in collaboration with Dr. David C. Spray (Albert Einstein College of Medicine, Department of Neuroscience, Bronx, NY) indicated that BBG even at 10 μM did not affect Cx43 gap junction channels in Cx43 transfected N2A cells (our unpublished observations)]. Similarly, 2 nM MFQ, a gap junction channel blocker that, at this concentration, does not affect Cx43 channels (Cruikshank et al., 2004) but is a potent antagonist of the $P2X_7R$ (Figs. 1, 2) also attenuated ICW amplification observed in WT and Cx43-null cells (Fig. 5*A*).

Recently, Anderson et al. (2004) indicated that under normal divalent cation conditions, cultured cortical astrocytes displayed multiple mechanisms by which ATP induces ATP release, which were found to be sensitive to generic P2R receptor blockers, although insensitive to a $P2X_7R$ antagonist, oATP, and sensitive to some anion channel blockers. Moreover, when cortical astrocytes were bathed in 0 Ca^{2+} solutions in the absence of ATP stimulation, a distinct pathway sensitive to gap junction channel blockers was identified (Anderson et al., 2004). The results presented here describing that Ca^{2+} wave spread between spinal cord astrocytes bathed in divalent cation containing solution are insensitive to $P2X_7R$ antagonist BBG, whereas waves traveling between cells bathed in low divalent cation solution are affected by MFQ, are in agreement with those reported by Anderson et al. (2004). Missing from their studies, however, is the report of whether under 0 Ca^{2+} conditions oATP would also affect purine release from cortical astrocytes, as is shown here to be the case for BBG.

Because we have previously reported that expression of numerous genes is altered in astrocytes derived from Cx43-null mice (Iacobas et al., 2003), including some P2 receptors (Scemes et al., 2000; Suadicani et al., 2003), we evaluated by Western blot analysis whether the expression levels of Cx43 and $P2X_7R$ were altered in the $P2X_7R$- and Cx43-null spinal cord astrocytes. As shown in Figure 5*B*, deletion of Cx43 does not alter the expression levels of $P2X_7Rs$, and $P2X_7R$ deletion does not affect the expression levels of Cx43. Evidence for functional expression of $P2X_7R$ in cultured spinal cord astrocytes is provided in Figure 5*C*. Bath application of 300 μM BzATP induced a sustained increase in intracellular Ca^{2+} levels, which was prevented by bathing the cells in low divalent cation solution (Fig. 5*C*, left graph). The nonsustained Ca^{2+} transient observed under this low divalent cation condition is likely related to activation of metabotrobic P2Y receptors as a result of some contaminating ATP present in the BzATP solution (~95% purity; Sigma). In addition to inducing Ca^{2+} influx, BzATP (300 μM) promoted YoPro uptake (Fig. 5*D*, right graph), which was blocked by the $P2X_7R$ antagonist BBG (100 nM), as well as by MFQ (2 nM). Note that the amount of BzATP-induced YoPro-1 uptake in spinal cord astrocytes bathed in low divalent cation solution is much lower than that recorded from 1321N1 cells (compare Figs. 2*C* and 5*D*), which might indicate low expression of $P2X_7R$ in these cells.

Thus, these results provide strong evidence that $P2X_7R$ and not Cx43 hemichannels are involved in the amplification of ICW spread in spinal cord astrocytes exposed to low divalent cation solution. Furthermore, our results indicate that the use of mice with targeted deletions provide a more reliable, precise, and direct way to evaluate the contribution of hemichannels than the use of nonselective compounds such as the gap junction channel blockers.

Discussion

The data presented here support the conclusion that $P2X_7R$ and not Cx43 hemichannels participate in the amplification of Ca^{2+} signal transmission between spinal cord astrocytes under low divalent conditions by providing sites of ATP release. The identification of pathways mediating the release of ATP is highly relevant given that this triphosphate nucleotide is a ubiquitous extracellular messenger that affects diverse cellular functions including chemotaxis, cytokine and neurotransmitter release, cell proliferation, and apoptosis. Also, ATP is a potent messenger of the transmission of Ca^{2+} signals within and between astrocytes (van den Pol et al., 1992; Pearce and Langley, 1994); these astrocytic Ca^{2+} signals, in turn, modulate neuronal activity (Kang et al., 1998) and brain microcirculation (Zonta et al., 2003; Mulligan and MacVicar, 2004), by promoting regulated release of gliotransmitters (Parpura et al., 1995).

In addition to regulated exocytosis, other mechanisms of transmitter release from astrocytes have been proposed, including diffusion through gap junction hemichannels and the pore-forming $P2X_7$ receptors (Ballerini et al., 1996; Cotrina et al., 1998; Stout et al., 2002; Duan et al., 2003; Ye et al., 2003). The ability of cells to take up and release small molecules combined with the use of gap junction channel blockers have been considered adequate criteria for demonstrating involvement of connexin hemichannels (Saez et al., 2005). However, these are correlative studies with no direct demonstration of hemichannel participation in this process. For instance, the only biophysical evidence for opening of Cx43 hemichannels in mammalian cells (Contreras et al., 2003) indicated that activation of one to three unap-

posed Cx43 gap junction channels occurred after strong membrane depolarization (above +50 mV) and to display modest to low dependence on extracellular Ca^{2+}. Yet, under resting conditions, when Cx43 hemichannels have been shown to be electrophysiologically silent (Contreras et al., 2003), dye uptake from astrocytes and other cell types has been attributed to the diffusion through open hemichannels solely based on compounds that affect gap junction channels, blockers that lack specificity.

Clearly missing from the literature is an evaluation of whether gap junction channel blockers can affect other diffusion pathways such as the pore-forming $P2X_7R$. Our results indicate that indeed gap junction channel blockers are antagonists of the $P2X_7$Rs and thus cannot be used to discriminate between these two pathways. Based on the agonist dose–response curve for antagonist-pretreated cells, two types of antagonism were observed: a competitive antagonism provided by the fenamate FFA and a noncompetitive antagonism provided by the glycyrrhetinic acid derivative CBX and the quinine derivative MFQ. Moreover, our results showing that the long chain alcohols heptanol and octanol can block YoPro uptake without affecting BzATP-induced Ca^{2+} influx suggest that these compounds like calmidazolium and KN-62 (Blanchard et al., 1995; Virginio et al., 1997; Chessell et al., 1998; Michel et al., 2000) can differentiate between "channel" and "pore" forms of the $P2X_7R$. Differences in type of antagonism and potency of blockade observed here for the gap junction channel blockers are likely related to the distinct structures of these compounds. Compared with their action on gap junction channels, the blockade of $P2X_7R$ by FFA, CBX, and MFQ is either similar or more effective than what has been reported (Spray et al., 2002; Srinivas and Spray, 2003; Cruikshank et al., 2004).

It is worth mentioning here that the effects of gap junction channel blockers on $P2X_7R$ function described in this report extends beyond the two cell types analyzed, because we observed that these compounds cause similar blockade of YoPro uptake in other cell types, such as J774, a macrophage cell line, and in primary cultures of rat microglia and mouse cortical astrocytes (our unpublished observations).

By comparing the extent of ICW spread in wild-type, Cx43-, and $P2X_7R$-null spinal cord astrocytes exposed to low divalent cation solution, we provide strong evidence favoring the participation of the $P2X_7R$ in the amplification of Ca^{2+} signal transmission within the astrocytic network after exposure to low divalent cation solution. Such amplification is likely a result of the increased release of ATP through the pore-forming $P2X_7R$, as indicated from our results showing the presence of higher levels of ATP in solutions bathing $P2X_7R$-expressing than in parental 1321N1 cells.

Given that under normal conditions activation of these receptors requires a high concentration of agonist [half-maximal activation attained at 300 μM ATP (North and Surprenant, 2000)], their participation under physiological CNS states is unlikely to prevail. In fact, under normal conditions, other pathways have been proposed as sites of ATP release from astrocytes, including those mediated by Ca^{2+}-dependent and Ca^{2+}-independent mechanisms (Queiroz et al., 1999; Arcuino et al., 2002; Coco et al., 2003; Anderson et al., 2004). Moreover, a recent study performed on two $P2X_7-/-$ mouse lines indicated that, at least in the hippocampus of healthy mouse brains, these receptors are either not expressed or are expressed at undetectable levels (Sim et al., 2004), suggesting that they are unlikely candidates for gliotransmitter release under physiological situations. In contrast, under pathological conditions such as during inflammation, ischemia, spreading depression, and trauma, when upregulation of these receptors, changes in extracellular cation composition, and increased extracellular ATP levels have been reported (Kraig and Nicholson, 1978; Harris and Symon, 1984; Nilsson et al., 1993; Le Feuvre et al., 2002; Guerra et al., 2003; Narcisse et al., 2005), the participation of $P2X_7R$ might be expected to exacerbate the extent of cell damage. Indeed, evidence for the participation of $P2X_7R$ in the regulation of extracellular transmitter levels was provided from studies showing that ATP-evoked glutamate efflux from neuronal and non-neuronal cells was greatly attenuated in $P2X_7R$-null hippocampal slices (Papp et al., 2004) and from those showing that $P2X_7R$ antagonists greatly attenuated the extent of cell damage after acute spinal cord traumatic injury (Wang et al., 2004). Although our *in vitro* studies support the notion that $P2X_7R$ contributes sites for ATP release from spinal cord astrocytes under extremely low divalent cation conditions, this observation remains to be confirmed in different preparations, such as in brain slices of ischemic mice.

In conclusion, here, we provided evidence that several pharmacological tools widely used to study the role of connexin hemichannels in astrocytes also affect the $P2X_7R$. Rather, these results provide strong evidence that the $P2X_7R$, and not Cx43 hemichannels, represents the pathway of ATP release from cultured spinal cord astrocytes under low divalent cation solution. Furthermore, our results indicate that the use of mice with targeted deletions provide a more reliable, precise, and direct way to evaluate the contribution of hemichannels than the use of nonselective compounds such as the gap junction channel blockers.

References

Anderson CM, Bergher JP, Swanson RA (2004) ATP-induced ATP release from astrocytes. J Neurochem 88:246–256.

Arcuino G, Lin JH, Takano T, Liu C, Jiang L, Gao Q, Kang J, Nedergaard M (2002) Intercellular calcium signaling mediated by point-source burst release of ATP. Proc Natl Acad Sci USA 99:9840–9845.

Ballerini P, Rathbone MP, Di Iorio P, Renzetti A, Giuliani P, D'Alimonte I, Trubiani O, Caciagli F, Ciccarelli R (1996) Rat astroglial P2Z ($P2X_7$) receptors regulate intracellular calcium and purine release. NeuroReport 7:2533–2537.

Bezzi P, Gundersen V, Galbete JL, Seifert G, Steinhauser C, Pilati E, Volterra A (2004) Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. Nat Neurosci 7:613–620.

Blanchard DK, Hoffman SL, Djeu JY (1995) Inhibition of extracellular ATP-mediated lysis of human macrophages by calmodulin antagonists. J Cell Biochem 57:452–464.

Chessell IP, Michel AD, Humphrey PP (1998) Effects of antagonists at the human recombinant $P2X_7$ receptor. Br J Pharmacol 124:1314–1320.

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C (2003) Storage and release of ATP from astrocytes in culture. J Biol Chem 278:1354–1362.

Contreras JE, Saez JC, Bukauskas FF, Bennett MV (2003) Gating and regulation of connexin43 (Cx43) hemichannels. Proc Natl Acad Sci USA 100:11388–11393.

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95:15735–15740.

Cruikshank SJ, Hopperstad M, Younger M, Connors BW, Spray DC, Srinivas M (2004) Potent block of Cx36 and Cx50 gap junction channels by mefloquine. Proc Natl Acad Sci USA 101:12364–12369.

Davidson JS, Baumgarten IM, Harley EH (1986) Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochem Biophys Res Commun 134:29–36.

Duan S, Anderson CM, Keung EC, Chen Y, Chen Y, Swanson RA (2003) $P2X_7$ receptor-mediated release of excitatory amino acids from astrocytes. J Neurosci 23:1320–1328.

Ebihara L (1996) *Xenopus* connexin38 forms hemi-gap-junctional channels in the nonjunctional plasma membrane of *Xenopus* oocytes. Biophys J 71:742–748.

Eskandari S, Zampighi GA, Leung DW, Wright EM, Loo DD (2002) Inhibition of gap junction hemichannels by chloride channel blockers. J Membr Biol 185:93–102.

Farrugia G, Rae JL, Szurszewski JH (1993) Characterization of an outward potassium current in canine jejunal circular smooth muscle and its activation by fenamates. J Physiol (Lond) 468:297–310.

Fumagalli M, Brambilla R, D'Ambrosi N, Volonte C, Matteoli M, Verderio C, Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. Glia 43:218–303.

Gogelein H, Dahlem D, Englert HC, Lang HJ (1990) Flufenamic acid, mefenamic acid and niflumic acid inhibit single nonselective cation channels in the rat exocrine pancreas. FEBS Lett 268:79–82.

Gomez-Hernandez JM, de Miguel M, Larrosa B, Gonzalez D, Barrio LC (2003) Molecular basis of calcium regulation in connexin32 hemichannels. Proc Natl Acad Sci USA 100:16030–16035.

Guerra AN, Fistte PL, Pfiffer AZ, Qinchia-Rio BH, Brabhu U, Aga M, Delinger LC, Guadarrama AG, Abozeid A, Sommer JA, Proctor RA, Bertics PJ (2003) Purinergic receptor regulation of LPS-induced signaling and pathophysiology. J Endotox Res 9:1–8.

Harris RJ, Symon L (1984) Extracellular pH, potassium, and calcium activities in progressive ischaemia of rat cortex. J Cereb Blood Flow Metab 4:178–186.

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

Iacobas DA, Urban-Maldonado M, Iacobas S, Scemes E, Spray DC (2003) Array analysis of gene expression in connexin43 null astrocytes. Physiol Genomics 15:177–190.

Jiang LH, MacKenzie AB, North RA, Surprenant A (2000) Brilliant blue G selectively blocks ATP-gated rat $P2X_7$ receptors. Mol Pharmacol 58:82–88.

Johnston MF, Simon SA, Ramon F (1980) Interaction of anaesthetics with electrical synapses. Nature 286:498–500.

Kang J, Jiang L, Goldman SA, Nedergaard M (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. Nat Neurosci 1:683–692.

Kraig RP, Nicholson C (1978) Extracellular ionic variations during spreading depression. Neuroscience 3:1045–1059.

Le Feuvre R, Brough D, Rothwell N (2002) Extracellular ATP and $P2X_7$ receptors in neurodegeneration. Eur J Pharmacol 447:261–269.

Louvel J, Heinemann U (1983) Changes in $[Ca^{2+}]_0$, $[K^+]_0$ and neuronal activity during oenanthotoxin-induced epilepsy in cat sensorimotor cortex. Electroencephalogr Clin Neurophysiol 56:457–466.

Michel AD, Kaur R, Chessell IP, Humphrey PP (2000) Antagonist effects on human $P2X_7$ receptor-mediated cellular accumulation of YO-PRO-1. Br J Pharmacol 130:513–520.

Mulligan SJ, MacVicar BA (2004) Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. Nature 431:195–199.

Narcisse L, Scemes E, Zao Y, Lee SC, Brosnan CF (2005) The cytokine IL-1β transiently enhances $P2X_7$ receptor expression and function in human astrocytes. Glia 49:245–258.

Newman EA (2003) New roles for astrocytes: regulation of synaptic transmission. Trends Neurosci 26:536–542.

Nilsson P, Hillered L, Olsson Y, Sheardown MJ, Hansen AJ (1993) Regional changes in interstitial K^+ and Ca^{2+} levels following cortical compression contusion trauma in rats. J Cereb Blood Flow Metab 13:183–192.

North RA, Surprenant A (2000) Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40:563–580.

Ottolia M, Toro L (1994) Potentiation of large conductance K_{Ca} channels by niflumic, flufenamic, and mefenamic acids. Biophys J 67:2272–2279.

Papp L, Vizi ES, Sperlagh B (2004) Lack of ATP-evoked GABA and glutamate release in the hippocampus of $P2X_7$ receptor −/− mice. NeuroReport 15:2387–2391.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG (1994) Glutamate-mediated astrocyte-neuron signalling. Nature 369:744–747.

Parpura V, Liu F, Brethorst S, Jeftinija K, Jeftinija S, Haydon PG (1995) Alpha-latrotoxin stimulates glutamate release from cortical astrocytes in cell culture. FEBS Lett 360:266–270.

Pasti L, Volterra A, Pozzan T, Carmignoto G (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes *in situ*. J Neurosci 17:7817–7830.

Pearce B, Langley D (1994) Purine- and pyrimidine-stimulated phosphoinositide breakdown and intracellular calcium mobilisation in astrocytes. Brain Res 660:329–332.

Pfahnl A, Dahl G (1999) Gating of Cx46 gap junction hemichannels by calcium and voltage. Pflügers Arch 437:345–353.

Porter JT, McCarthy KD (1997) Astrocytic neurotransmitter receptors *in situ* and *in vivo*. Prog Neurobiol 51:439–455.

Poyraz D, Brau ME, Wotka F, Puhlmann B, Scholz AM, Hempelmann G, Kox WJ, Spies CD (2003) Lidocaine and octanol have different modes of action at tetrodotoxin-resistant Na^+ channels of peripheral nerves. Anesth Analg 97:1317–1324.

Queiroz G, Meyer DK, Meyer A, Starke K, von Kugelgen I (1999) A study of the mechanism of the release of ATP from rat cortical astroglial cells evoked by activation of glutamate receptors. Neuroscience 91:1171–1181.

Rouach N, Segal M, Koulakoff A, Giaume C, Avignone E (2003) Carbenoxolone blockade of neuronal network activity in culture is not mediated by an action on gap junctions. J Physiol (Lond) 553:729–745.

Saez JC, Retamal MA, Basilio D, Bukauskas FF, Bennett MV (2005) Connexin-based gap junction hemichannels: gating mechanisms. Biochim Biophys Acta 1711:215–224.

Scemes E, Dermietzel R, Spray DC (1998) Calcium waves between astrocytes from Cx43 knockout mice. Glia 24:65–73.

Scemes E, Suadicani SO, Spray DC (2000) Intercellular communication in spinal cord astrocytes: fine tuning between gap junctions and P2 nucleotide receptors in calcium wave propagation. J Neurosci 20:1435–1445.

Schultz BD, Singh AK, Devor DC, Bridges RJ (1999) Pharmacology of CFTR chloride channel activity. Physiol Rev 79:S109–S144.

Sim JA, Young MT, Sung HY, North RA, Surprenant A (2004) Reanalysis of $P2X_7$ receptor expression in rodent brain. J Neurosci 24:6307–6314.

Spray DC, Rozental R, Srinivas M (2002) Prospects for rational development of pharmacological gap junction channel blockers. Curr Drug Targets 3:455–464.

Srinivas M, Spray DC (2003) Closure of gap junction channels by arylaminobenzoates. Mol Pharmacol 63:1389–1397.

Stout C, Charles A (2003) Modulation of intercellular calcium signaling in astrocytes by extracellular calcium and magnesium. Glia 43:265–273.

Stout CE, Costantin JL, Naus CC, Charles AC (2002) Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem 277:10482–10488.

Suadicani SO, Pina-Benabou MH, Urban-Maldonado M, Spray DC, Scemes E (2003) Acute downregulation of Cx43 alters P2Y receptor expression levels in mouse spinal cord astrocytes. Glia 42:160–171.

Suadicani SO, Flores CE, Urban-Maldonado M, Beelitz M, Scemes E (2004) Gap junction channels coordinate the propagation of intercellular Ca^{2+} signals generated by P2Y receptor activation. Glia 48:217–229.

Takens-Kwak BR, Jongsma HJ, Rook MB, van Ginneken AC (1992) Mechanism of heptanol-induced uncoupling of cardiac gap junctions: a perforated patch-clamp study. Am J Physiol 262:C1531–C1538.

van den Pol AN, Finkbeiner SM, Cornell-Bell AH (1992) Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia *in vitro*. J Neurosci 12:2648–2664.

Vessey JP, Lalonde MR, Mizan HA, Welch NC, Kelly ME, Barnes S (2004) Carbenoxolone inhibition of voltage-gated Ca^{2+} channels and synaptic transmission in the retina. J Neurophysiol 92:1252–1256.

Virginio C, Church D, North RA, Surprenant A (1997) Effects of divalent cations, protons and calmidazolium at the rat $P2X_7$ receptor. Neuropharmacology 36:1285–1294.

Wang CM, Chang YY, Kuo JS, Sun SH (2002) Activation of $P2X_7$ receptors induced [^{3}H]GABA release from the RBA-2 type-2 astrocyte cell line through a Cl^-/HCO^{3-}-dependent mechanism. Glia 37:8–18.

Wang HS, Dixon JE, McKinnon D (1997) Unexpected and differential effects of Cl^- channel blockers on the Kv4.3 and Kv4.2 K^+ channels. Implications for the study of the I_{to2} current. Circ Res 81:711–718.

Wang X, Arcuino G, Takano T, Lin J, Peng WG, Wan P, Li P, Xu Q, Liu QS, Goldman SA, Nedergaard M (2004) $P2X_7$ receptor inhibition improves recovery after spinal cord injury. Nat Med 10:821–827.

Weiss SM, Benwell K, Cliffe IA, Gillespie RJ, Knight AR, Lerpiniere J, Misra A, Pratt RM, Revell D, Upton R, Dourish CT (2003) Discovery of nonxanthine adenosine A_{2A} receptor antagonists for the treatment of Parkinson's disease. Neurology 61:S101–S106.

White MM, Aylwin M (1990) Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. Mol Pharmacol 37:720–724.

Ye ZC, Wyeth MS, Baltan-Tekkok S, Ransom BR (2003) Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci 23:3588–3596.

Zonta M, Angulo MC, Gobbo S, Rosengarten B, Hossmann KA, Pozzan T, Carmignoto G (2003) Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci 6:43–50.