

Roles of Two Types of Anion Channels in Glutamate Release from Mouse Astrocytes Under Ischemic or Osmotic Stress

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KEY WORDS

astrocyte; anion channels; glutamate release; osmotic stress; swelling; ischemia

ABSTRACT

Astrocytes release glutamate upon hyperexcitation in the normal brain, and in response to pathologic insults such as ischemia and trauma. In our experiments, both hypotonic and ischemic stimuli caused the release of glutamate from cultured mouse astrocytes, which occurred with little or no contribution of gap junction hemichannels, vesicle-mediated exocytosis, or reversed operation of the Na-dependent glutamate transporter. Cell swelling and chemical ischemia activated, in cell-attached membrane patches, anionic channels with large unitary conductance (~400 pS) and inactivation kinetics at potentials more positive than +20 mV or more negative than -20 mV. These properties are different from those of volume-sensitive outwardly rectifying (VSOR) Cl⁻ channels, which were also expressed in these cells and exhibited intermediate unitary conductance (~80 pS) and inactivation kinetics at large positive potentials of more than +40 mV. Both maxi-anion channels and VSOR Cl⁻ channels were permeable to glutamate with permeability ratios of glutamate to chloride of 0.21 ± 0.07 and 0.15 ± 0.01 , respectively. However, the release of glutamate was significantly more sensitive to Gd³⁺, a blocker of maxi-anion channels, than to phloretin, a blocker of VSOR Cl⁻ channels. We conclude that these two channels jointly represent a major conductive pathway for the release of glutamate from swollen and ischemia-challenged astrocytes, with the contribution of maxi-anion channels being predominant. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Extracellular space (ECS) in the brain normally constitutes ~24% of the total brain volume, but may decrease to 12–17% during repetitive electrical stimulation (Nicholson, 2005; Sykova, 2004) or even to ~5% in ischemia due to the increase in intracellular space (ICS) resulting from astrocyte swelling (Nicholson, 2005). Swelling of brain tissue, or brain edema, occurs as a result of a number of different pathological conditions such as stroke, traumatic injury, brain tumors, some systemic infections, liver failure, and

kidney failure, and represents a significant cause of morbidity and mortality (Kimelberg, 2004, 2005; Kimelberg et al., 2004; Unterberg et al., 2004). Brain edema initiates astroglial swelling (Kimelberg, 2004, 2005), which is currently considered to be the main cause of the release of glutamate (Kimelberg et al., 2004; Pasantés-Morales et al., 2002; Phillis and O'Regan, 2003) and is adequately modeled by astrocytic primary cultures. Putative glutamate-releasing pathways currently considered include exocytosis, transporters (such as Na⁺-coupled glutamate transporter operating in a reverse mode), and conductive release via an ion channel such as a gap junction hemichannel or a chloride channel (Evanko et al., 2004; Parpura et al., 2004; Phillis and O'Regan, 2003). In the present study, we focused on the mechanism of glutamate release via swelling-induced anion-conductive pathways.

Cultured astrocytes exhibit high levels of macroscopic chloride conductance in response to osmotic swelling (Crepel et al., 1998; Lascola and Kraig, 1996; Olson and Li, 1997; Olson et al., 2004; Parkerson and Sontheimer, 2004). Consistent with a role for chloride channels in glutamate release, chloride channel blockers inhibited the hypotonicity- and ischemia-induced glutamate release from cultured astrocytes (Haskew-Layton et al., 2005; Kimelberg et al., 1990; Mongin and Kimelberg, 2002; Mongin et al., 1999; Rutledge et al., 1998), from brain slices (Basarsky et al., 1999; Bothwell et al., 2001), as well as in animal models of global and focal ischemia (Estevez et al., 1999; Feustel et al., 2004; Kimelberg et al., 2000, 2003; Phillis et al., 1997, 1998; Seki et al., 1999). Volume-sensitive outwardly rectifying (VSOR) chloride channels (Nilius et al., 1997; Okada, 1997; Strange et al., 1996) are believed to be the main mediator of swelling-induced glutamate release in the brain (Kimelberg, 2004, 2005). However, this

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attractive hypothesis is poorly supported by electrophysiological data. Moreover, detailed studies of excitatory amino acid release suggested an involvement of at least two different types of anion-transporting pathways (Franco et al., 2001; Mongin et al., 1999; Mongin and Kimelberg, 2002; Pasantes-Morales et al., 2002). The identities and relative contributions of these putative pathways remain obscure. Osmotic stimulation is known to activate not only VSOR but also some other chloride channels, including the maxi-anion channel, in cells of various origins (Sabirov and Okada, 2004a, 2005; Strange et al., 1996). In the present report, we demonstrate that stimulation of astrocytes with osmotic stress and chemical ischemia activates the glutamate-conducting maxi-anion channel, which constitutes a major pathway for the release of glutamate observed in these conditions. Contribution of glutamate-permeable VSOR Cl^- channels was smaller than that of the maxi-anion channel.

MATERIALS AND METHODS

Cells

The experimental protocol was approved in advance by the Ethics Review Committee for Animal Experimentation of the National Institute for Physiological Sciences. Astrocytes were obtained from neonatal mouse brain cortex as previously described (Zhang et al., 2004), with some modifications. The primary culture medium for mouse astrocytes was Eagle's MEM supplemented with L-glutamine (Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10% fetal bovine serum, and 2.2 g/L NaHCO_3 . Astrocytes were obtained from 2–3-day old Slc:ddy mouse pups. Briefly, the pups were anaesthetized with halothane, the brains were dissected from the skulls, and the cerebellum, olfactory bulbs, and meninges were removed. The remaining cerebral cortex was first washed in ice-cold phosphate-buffered saline (PBS) and minced in ice-cold Leibovitz's L-15 medium (Life Technologies, Rockville, MD). The tissue was then dissociated in papain (10 U/mL; Worthington Biochemical Corporation, Lakewood, NJ) dissolved in Earle's balanced salt solution (Life Technologies) for 20 min at 37°C. The supernatant was removed and 5 mL of fresh MEM containing 0.05 mg/mL DNase I was added. After shaking for 2 min, the tissue was washed with fresh MEM three times, resuspended in new medium, and triturated. The cells were passed through a 70 μm nylon filter, plated in culture flasks at a density of $\sim 2.5 \times 10^5$ cells/cm², and cultured in a humidified 5% CO_2 /95% air environment. After 12 h, the medium was replaced and cells were subsequently fed every 2–3 days with new MEM. After becoming confluent, the cells were shaken briefly to remove the microglia growing on the surface of the astrocytes. The cells were then trypsinized (0.25% trypsin, 0.02% EDTA) and transferred to new dishes for further culturing. Immunohistochemical tests showed that $\sim 93\%$ of the cultured cells were positive for anti-GFAP (glial fibrillary acidic protein) antibody (Dako, Carpinteria, CA).

Solutions and Chemicals

The standard Ringer solution contained (in mM) 135 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 Na-HEPES, 6 HEPES, and 5 glucose (pH 7.4, 290 mosmol/kg- H_2O). Hypotonic bath solution was made by reducing the concentration of NaCl in this solution to 100 mM (210 mosmol/kg H_2O). In inside-out, outside-out, and on-cell experiments we used standard Ringer solution in the bath. The pipette solution for inside-out and on-cell experiments was standard Ringer solution. The pipette solution for outside-out experiments contained (in mM) 120 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 HEPES (pH 7.4 adjusted with NaOH), and 10 EGTA (pCa 7.6; 275 mosmol/kg- H_2O). For whole-cell current measurements, the isotonic bath solution contained (in mM) 100 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 Na-HEPES, 6 HEPES, and 5 glucose, (pH 7.4, 290 mosmol/kg- H_2O adjusted with 80 mM mannitol). Hypotonic bath solution was made by omitting mannitol from this isotonic solution. The pipette solution for whole-cell experiments contained (in mM) 125 CsCl, 2 CaCl_2 , 1 MgCl_2 , 3 Na-ATP, 5 HEPES (pH 7.4 adjusted with CsOH), and 10 EGTA (pCa 7.65; 275 mosmol/kg- H_2O). For measurements of glutamate permeability of maxi-anion channels, the low- Cl^- bath solution was prepared by replacing 135 mM NaCl in standard Ringer solution with 135 mM Na-glutamate. In some experiments, a Cl^- -free solution containing (mM) 146 Na-glutamate and 5 HEPES (pH 7.4 adjusted with NaOH) was used. For measurements of glutamate permeability of the VSOR Cl^- channels, the low- Cl^- pipette solution was prepared by replacing 100 mM CsCl in the whole-cell pipette solution with 100 mM Cs-glutamate. In on-cell single VSOR Cl^- channel experiments, cells were bathed in hypotonic high- K^+ solution containing (in mM) 100 KCl, 1 MgCl_2 , 2 CaCl_2 , 5 Na-HEPES, 6 HEPES, and 5 glucose (pH 7.4, 218 ± 4 mosmol/kg- H_2O). The pipette was filled with a solution containing (in mM) 100 CsCl, 1 MgCl_2 , 2 CaCl_2 , and 5 HEPES (pH 7.4 adjusted with CsOH, 208 ± 3 mosmol/kg- H_2O). In some experiments, the CsCl concentration was reduced to 30 mM, or CsCl was replaced with equimolar TEA-Cl. For chemical ischemia experiments, 10 mM 2-deoxy-D-glucose (2DG), 5 mM NaCN, and 1 mM iodoacetic acid were added to the standard Ringer solution devoid of glucose (pH was adjusted to 7.4).

GdCl_3 was stored as a 50-mM stock solution in water and added directly to Ringer solution immediately before each experiment. 5-Nitro-2-(phenylpropylamino)-benzoate (NPPB), glibenclamide, 4-acetamino-4'-isothiocyanostilbene (SITS), arachidonic acid, indomethacin, nordihydroguaiaretic acid (NDGA), clotrimazole, DL-threo- β -benzoyloxyaspartate (TBOA), phloretin, tamoxifen, brefeldin A (BFA), carbenoxolone, and 1-octanol were purchased from Sigma-Aldrich. Bafilomycin A1 was purchased from WAKO (Osaka, Japan) and BAPTA/AM from DOJINDO (Mashiki, Japan). The drugs were added to Ringer solution immediately before use from stock solutions in DMSO. DMSO did not have any effect, when added alone at a concentration less than 0.1%. L-Glutamate dehydrogenase (E.C. 1.4.1.3), nicotinamide adenine dinucleotide (NAD), and its reduced

form, NADH (all from Sigma-Aldrich), were dissolved in Ringer solution.

Osmolality of all solutions was measured using a freezing-point depression osmometer (OM802, Vogel, Kevelaer, Germany).

Electrophysiology

Patch electrodes were fabricated from borosilicate glass capillaries using a laser micropipette puller (P-2000, Sutter Instrument, Novato, CA) and had a tip resistance of 2–3 M Ω when filled with pipette solution. Fast and slow capacitative transients were routinely compensated for. For whole-cell recordings, the access resistance did not exceed 5 M Ω and was always compensated for by 80%. Membrane currents were measured with an EPC-9 patch-clamp system (Heka-Electronics, Lambrecht/Pfalz, Germany). The membrane potential was controlled by shifting the pipette potential (V_p). Currents were filtered at 1 kHz and sampled at 3 kHz. Data acquisition and analysis were done using Pulse + PulseFit (Heka-Electronics). Whenever the bath Cl^- concentration was altered, a salt bridge containing 3 M KCl in 2% agarose was used to minimize variations of the bath electrode potential. Liquid junction potentials were calculated using pCLAMP 8.1 (Axon Instruments, Foster, CA) algorithms and were corrected either on- or off-line when appropriate. All experiments were performed at room temperature (23–25°C).

Assay of Glutamate Release

Astrocytes for glutamate release measurements were cultured in 5.5 cm Petri dishes until confluence. The cells were washed three times with 2 mL Ringer solution and then incubated with 2.5 mL of the appropriate test solution at 37°C for various intervals of time as indicated. Gentle rocking at 6 rpm (Rotator 4631, Lab-Line Instruments, Melrose Park, IL) ensured even distribution of the released glutamate. Extracellular solution samples (2 mL from each dish) were collected for the fluorometric glutamate release assay. When required, drugs were added to the extracellular solution to give the final concentrations as indicated. The samples were incubated at room temperature for 40 min with 200 μL of 11.2 mg/mL NAD and 20 μL of 15 mg/mL L-glutamate dehydrogenase, and the fluorescence of NADH was measured with a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) using a 340 nm filter for excitation and a 410–600 nm filter for emission, respectively. The relationship between glutamate concentration and NADH fluorescence was tested using 0.5–5 μM glutamate dissolved in standard Ringer, hypotonic or ischemic bath solution and was found to be linear over this range. The fluorescence of 5 μM NADH was roughly equal to that observed in the 5 μM glutamate calibration assay, indicating that the glutamate-dependent NAD conversion to NADH was complete. All the drugs used were checked for effect on the glutamate release assay. Gd^{3+} ions had no effect on the enzymatic assay. NPPB, SITS,

phloretin, BAPTA/AM and the cocktail of indomethacin, NDGA, and clotrimazole considerably increased the background fluorescence, while tamoxifen decreased it. In these cases, the calibration procedure was carried out in the presence of the drugs.

Cell Membrane Integrity Assay

Astrocytes were cultured in 96-well plates until confluence. The cells were washed three times with 100 μL Ringer solution and then incubated with 100 μL of the test solution at 37°C for 45 min. Lactate dehydrogenase (LDH) release was measured using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI) according to the manufacturer's manual.

Data Analysis

Single-channel amplitudes were measured by manually placing a cursor at the open and closed channel levels. Background currents were subtracted and the mean patch currents were measured at the beginning (first 25–30 ms) of current traces in order to minimize the contribution of voltage-dependent current inactivation. By dividing the macro-patch current by the single-channel current amplitude, the NP_o value was calculated, where P_o and N represent the open channel probability and the number of active channels, respectively. The maximal NP_o values were averaged for all the patches tested and presented as the mean $NP_o \pm \text{SEM}$. For single maxi-anion channels in asymmetric conditions, the reversal potentials were calculated by fitting I–V curves to a second-order polynomial (Sabirov and Okada, 2004b). For whole-cell VSOR currents, the reversal potentials were measured directly from the ramp current-to-voltage relationships. The permeability ratio for glutamate (Glu) was calculated from the Goldman–Hodgkin–Katz (GHK) equation:

$$E_{\text{rev}} = -RT/F \ln[(P_{\text{Cl}}[\text{Cl}]_o)/(P_{\text{Cl}}[\text{Cl}]_i + P_{\text{Glu}}[\text{Glu}]_i)],$$

where E_{rev} is the reversal potential when Glu was present on the intracellular side at a concentration of $[\text{Glu}]_i$ (the intracellular side was low- Cl^- bath solution for maxi-anion channels and low- Cl^- pipette solution for whole-cell VSOR currents). $[\text{Cl}]_o$ and $[\text{Cl}]_i$ are the Cl^- concentrations on the extracellular and intracellular sides, respectively (see corresponding solutions for respective experimental conditions). P_{Cl} and P_{Glu} are the permeability coefficients of Cl^- and glutamate, respectively.

Data were analyzed in Origin 6.1 and OriginPro 7.0 (MicroCal Software, Northampton, MA). Pooled data are given as means \pm SEM of observations (n). Statistical differences of the data were evaluated by ANOVA and the paired or unpaired Student's t test where appropriate and considered significant at $P < 0.05$.

In all figures, the membrane potential (V_m) is indicated according to the following convention: $V_m = V_p$ (the pipette potential) for whole-cell and outside-out experiments, and

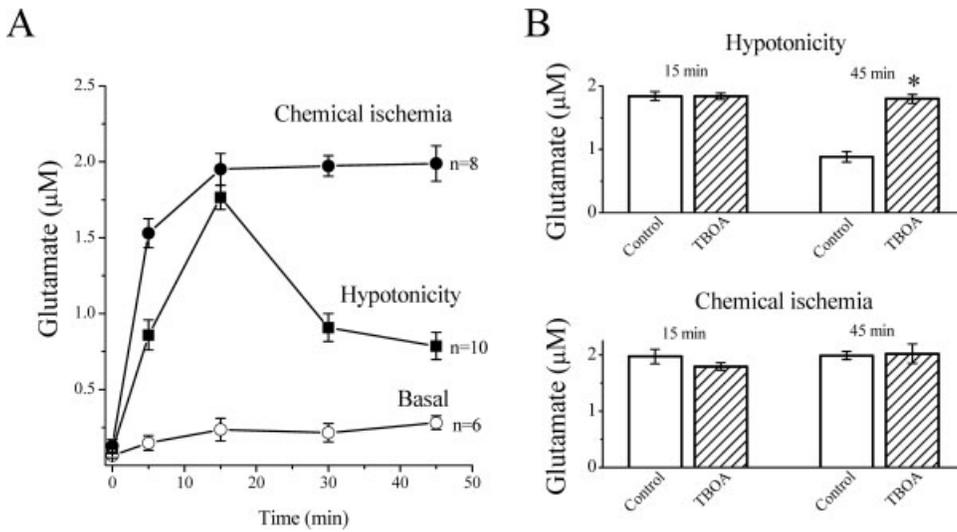


Fig. 1. Glutamate release from astrocytes induced by hypotonic and ischemic stress. **A:** The time course of change in the extracellular glutamate concentration in control basal (\circ), hypotonic (\blacksquare), and ischemic (\bullet) conditions. **B:** Effects of a glutamate transporter blocker, TBOA (100 μ M), on the net release of glutamate from astrocytes subjected to hypotonic (top panel) or ischemic (bottom panel) stress for 15 or 45 min. $n = 5$ for each group. *Significantly different from the control value at $P < 0.05$.

$V_m = -V_p$ for inside-out experiments. For on-cell records, applied voltages are represented as $-V_p$ values.

RESULTS

Hypotonic and Ischemic Stimulation Induced Net Glutamate Release from Astrocytes

The basal glutamate release from astrocytes was relatively low in control Ringer solution (the composition; see Materials and Methods) and did not exceed $0.28 \pm 0.05 \mu\text{M}$ over a 45-min incubation time (Fig. 1A, open circles). In contrast, when the cells were transferred to hypotonic solution (210 mosmol/kg- H_2O), which was prepared by reducing the NaCl concentration from 135 to 100 mM in Ringer solution, or ischemic solution, which was prepared by replacing glucose with 2DG and by adding a mitochondrial respiration inhibitor NaCN (5 mM) and a glycolysis inhibitor iodoacetic acid (1 mM) to Ringer solution, the extracellular glutamate concentration rapidly reached a level of 1.7–2 μM within 15 min (Fig. 1A, filled symbols). In hypotonic solution, the extracellular glutamate concentration rise was transient; the extracellular glutamate reached a maximum level and then gradually decreased. In contrast, in ischemic conditions the extracellular glutamate concentration remained at the maximum level up to at least 45 min of incubation. To exclude the possibility that the glutamate release was caused by cell membrane damage, we examined cell integrity using the LDH assay method. In these experiments, the LDH release was $2.90\% \pm 0.32\%$ ($n = 5$), $2.69\% \pm 0.73\%$ ($n = 15$), and $3.40\% \pm 1.08\%$ ($n = 15$) in control, hypotonic, and ischemic conditions, respectively. These figures are not statistically different from each other, indicating that the net glutamate release observed was not due to cell damage caused by hypotonic or ischemic stress.

Astrocytes are known to express sodium-dependent excitatory amino acid transporters (EAAT), which may mediate the release of glutamate when functioning in a reverse mode (Rossi et al., 2000). In our experiments, a

specific inhibitor of Na^+ -dependent glutamate uptake, TBOA, did not affect the level of net glutamate release assessed at 15 min for either type of stimulation (Fig. 1B). However, when this drug was tested with a 45-min incubation time, the glutamate release induced by hypotonicity was not reduced and remained at a level as high as that observed at 15 min (Fig. 1B, top panel). This result suggests that the secondary decrease in glutamate concentration seen in Figure 1A (closed squares) was due to a secondary active uptake of the released glutamate by Na^+ -glutamate cotransporters, which are functioning in a non-reverse mode under hypotonic stress. These cotransporters apparently do not function under ischemic conditions since the glutamate release was not affected by TBOA in the case of ischemia stress (Fig. 1B, bottom panel).

In the following pharmacological experiments, we used a time point of 15 min when the net glutamate release was least affected by the secondary active uptake system. Astrocytes are known to express connexins. Furthermore, gap junction hemichannels have been demonstrated to be present in the plasma membrane of astrocytes and have been suggested to play a role in glutamate release from these cells (Ye et al., 2003). Since the absence of divalent cations is a prerequisite for hemichannel opening (Ye et al., 2003), however, we assume that 2 mM Ca^{2+} and 1 mM Mg^{2+} present in the extracellular space effectively blocked the astrocytic hemichannels in our experiments. Actually, carbenoxolone and 1-octanol, which are known to block the hemichannels in astrocytes (Ye et al., 2003), did not have any significant effects on the net glutamate release from astrocytes induced by cell swelling (top panel) or by chemical ischemia (bottom panel), as shown in Fig. 2. These results suggest that gap junction hemichannels are not involved in glutamate release under the present experimental conditions.

Vesicle-mediated exocytotic release of glutamate has been suggested to occur in astrocytes (Evanko et al., 2004; Parpura et al., 2004). However, BFA, an inhibitor of vesicular transport, did not have any effect on the net

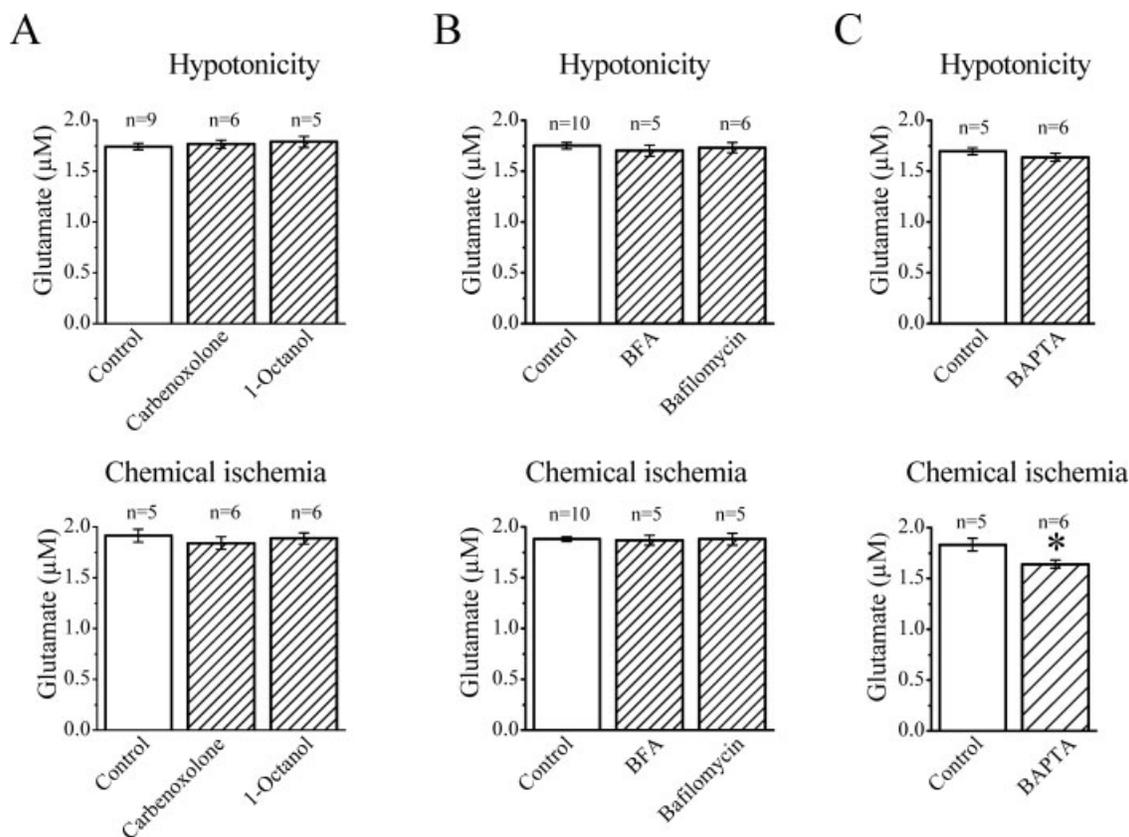


Fig. 2. Insensitivity of hypotonicity- and ischemia-induced glutamate release from astrocytes to inhibitors of gap junction hemi-channels and exocytosis. **A:** Effects of the gap junction hemichannel blockers carbenoxolone (100 μM) and 1-octanol (2 mM) on the net glutamate release from astrocytes subjected to hypotonic stress (top panel) or chemical ischemia (bottom panel) for 15 min. Carbenoxolone and 1-octanol were added to the test solutions during stimulation. **B:** Effects of exocytosis inhibitors, BFA (5 μM) and bafilomycin A1 (5 μM) on the net glutamate release from astrocytes subjected to hypotonic stress (top panel) or chemical

(bottom panel) for 15 min. BFA was applied for 120 min before and throughout the stimulation. Bafilomycin A1 was applied for 90 min before and throughout the stimulation. Mean values for drug-treated groups are not significantly different from those for control, as determined by the ANOVA test. **C:** Effect of BAPTA on the net glutamate release from astrocytes subjected to hypotonic stress (top panel) or chemical ischemia (bottom panel) for 15 min. BAPTA/AM (50 μM) was applied for 20 min before, and throughout, the stimulation. *Significantly different from the control value at $P < 0.05$.

release of glutamate observed upon stimulation by hypotonicity or chemical ischemia (Fig. 2B). Bafilomycin A1, an inhibitor of vesicular proton pumps, was also ineffective (Fig. 2B). Therefore, we conclude that vesicle fusion-mediated exocytosis does not contribute significantly to glutamate release as measured in these experimental conditions. Chelating the intracellular Ca^{2+} with BAPTA/AM had no effect on the hypotonicity-induced release of glutamate and only slightly reduced the glutamate release upon chemical ischemia (Fig. 2C). This result suggests that Ca-dependent processes have minor, if any, contribution to the net glutamate release in our experimental conditions.

Hypotonic and Ischemic Stimulation Induced Activation of Large-conductance Events in Cell-attached Patches on Astrocytes

In the cell-attached (on-cell) configuration, no unitary anion channel events were observed in astrocytes per-

fused with control Ringer solution (Fig. 3A, Control), although cationic channel events were rarely observed at the holding potential of 0 mV. When the isotonic bath solution was replaced with hypotonic solution, we could consistently observe single-channel events of large amplitude (Fig. 3A, Hypotonicity). The channel events had mean unitary amplitudes of 10.5 ± 0.7 pA ($n = 10$) and -6.0 ± 0.8 pA ($n = 10$) at +25 and -25 mV, respectively. Similar events with mean unitary amplitudes of 10.3 ± 1.2 pA ($n = 12$) and -6.0 ± 0.8 pA ($n = 10$) at +25 and -25 mV, respectively, were also observed when the astrocytes were stimulated with chemical ischemia (Fig. 3A, Chemical ischemia). The unitary I-V relationship for these events exhibited slightly outward rectification (Fig. 3B) and had a reversal potential of -5.1 ± 0.7 mV. The mean slope conductances for channels activated by hypotonicity were 409 ± 18 and 241 ± 20 pS at positive and negative potentials, respectively. The channels activated by chemical ischemia had mean slope conductance of 430 ± 20 and 272 ± 19 pS for outward and inward currents, respectively.

The time course of channel activation is illustrated in Fig. 4. In control basal conditions, we observed channel

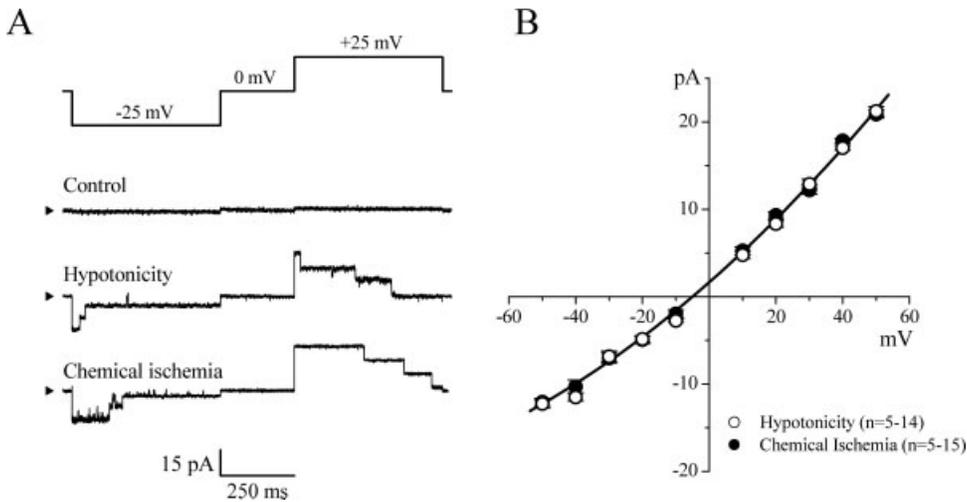


Fig. 3. Activation of maxi-anion channels by hypotonic or ischemic stimulation in cell-attached membrane patches. **A:** Representative current recordings in control conditions (Control) and upon hypotonic (Hypotonicity) and ischemic (Chemical ischemia) stimulation. The applied pulse protocol is shown at the top. Arrowheads indicate the zero current level. **B:** Current-to-voltage relationships for single-channel events recorded in hypotonic (○) and ischemic (●) conditions. Data were collected from four different patches in hypotonic and seven patches in ischemic conditions. The solid line is a polynomial fit to cumulative data for hypotonic and ischemic experiments.

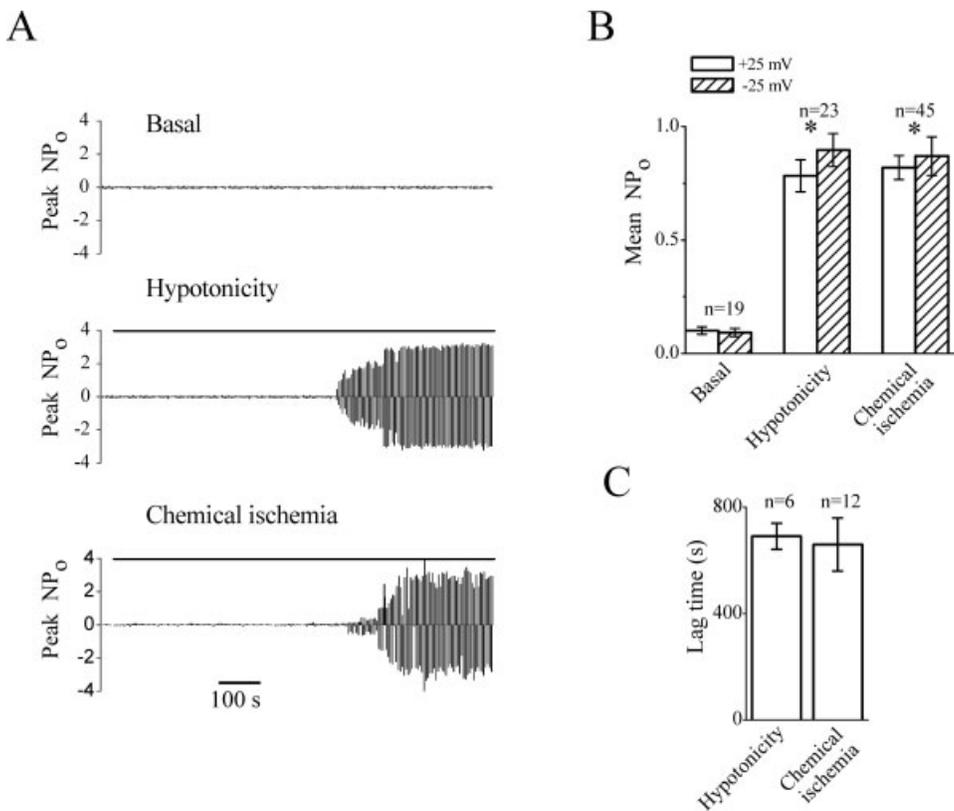


Fig. 4. Kinetics of hypotonic or ischemic activation of the maxi-anion channel in cell-attached patches. **A:** Time course of NP_o recorded from astrocytes exposed to isotonic Ringer solution (Basal) and to hypotonic (Hypotonicity) and ischemic (Chemical ischemia) solutions during application of alternating pulses (0.5-s duration, 5-s interval) from 0 to ± 25 mV. **B:** Mean NP_o calculated as the maximal NP_o divided by the total patches tested in control isotonic conditions (Basal) and upon hypotonic (Hypotonicity) or ischemic (Chemical ischemia) stimulation at +25 mV (open columns) and -25 mV (hatched columns). **C:** The mean lag time of maxi-anion channel activation (defined as the time from the beginning of stimulation to the opening of the first channel) after the application of hypotonic or ischemic stimulation.

events in only 1 out of 19 cells tested. In contrast, hypotonic stimulation caused activation of channels with large amplitude in 6 out of 23 patches tested, whereas chemical ischemia induced channel activation in 12 out of 45 patches tested. The mean NP_o value was significantly higher under hypotonic and ischemic conditions compared with under basal conditions (Fig. 4B). Channel activation occurred after a lag time, which varied from patch to patch and ranged from 6.8–16.6 min for hypotonic stimulation and from 7.6–15.9 min for chemical ischemia. Mean values, however, did not differ significantly for hypotonic and ischemic stimulation (Fig. 4C). It should be noted

that no other types of channel activity, such as VSOR-like (characterized by intermediate single-channel conductance and outward rectification), CFTR-like (8–10 pS with no rectification), or Ca^{2+} -activated Cl^- channel-like (with voltage-dependent activation at positive potentials and inactivation at negative potentials) activity, were observed in the cell-attached mode under these experimental conditions. The relatively low incidence of channel-containing patches (about 25–30% in these experiments) may reflect either low density of maxi-anion channels or an uneven distribution of these ion channels over the astrocyte cell surface.

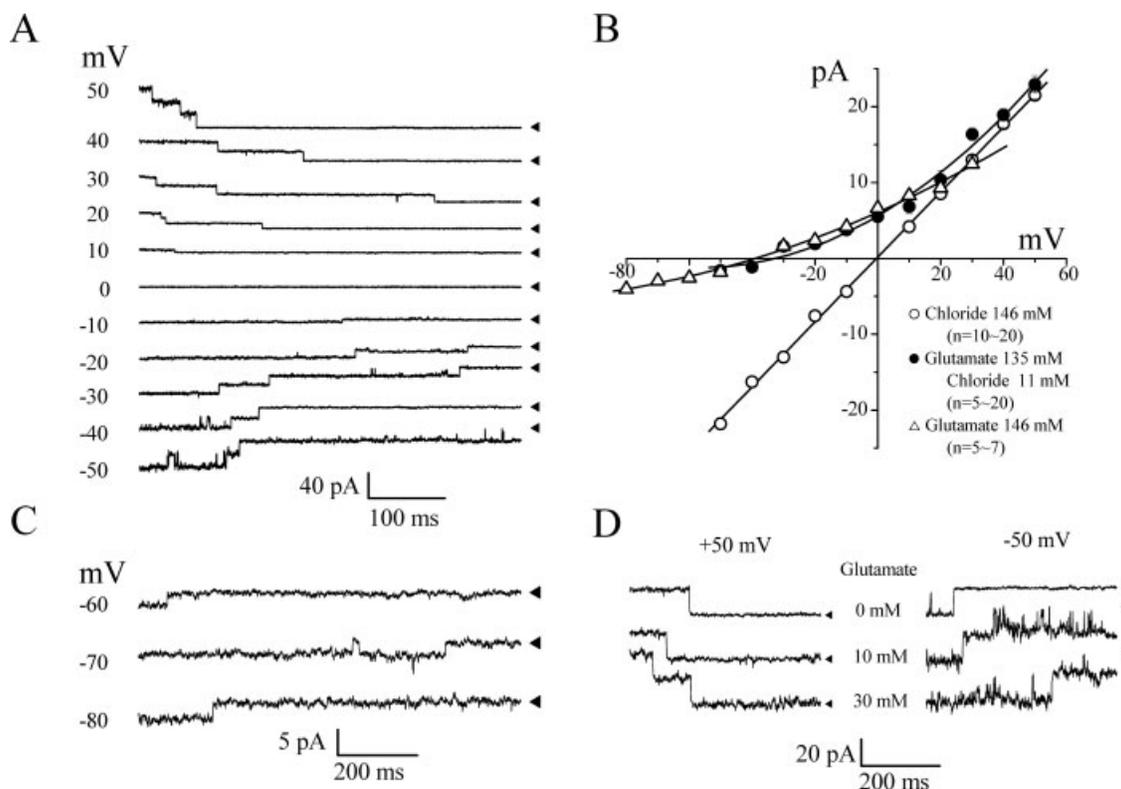


Fig. 5. Glutamate permeability of the maxi-anion channel in excised inside-out patches. **A:** Representative current traces recorded at different voltages from a patch containing three active channels. The pipette was filled with normal Ringer solution, and the maxi-anion channels were activated by excising the patch into normal Ringer solution. The holding potential was 0 mV. Currents were elicited by application of step-pulses (500 ms) from -50 to $+50$ mV in 10-mV increments. Arrowheads indicate the zero current level. **B:** Unitary I-V relationships for the maxi-anion channel in symmetrical chloride conditions (○), in asymmetrical chloride conditions in which 135 mM chloride in the bath (intracellular) solution was replaced with 135 mM glutamate (●), and in asymmetrical conditions in which all chloride in the bath (intracellular) solution was replaced with 146 mM glutamate (△). The solid line for

symmetrical conditions is a linear fit, which gives a slope conductance of 403.9 ± 1.7 pS. The solid lines for asymmetrical conditions are polynomial fits, which give a reversal potential of -33.5 ± 1.4 mV for 135 mM glutamate plus 11 mM chloride and -38.9 ± 1.2 mV for 146 mM glutamate. The error bars are not shown when smaller than size of the symbols. **C:** Glutamate currents through astrocytic maxi-anion channel. Traces were recorded in asymmetrical conditions in which all chloride in the bath (intracellular) solution was replaced with 146 mM glutamate (same as for open triangles in B). **D:** Effect of glutamate on maxi-anion channel currents. The representative current traces recorded in response to 500 ms test pulses from 0 to $+50$ mV (left traces) and -50 mV (right traces).

Maxi-anion Channels Constituted a Major Pathway for Glutamate Release from Astrocytes under Hypotonic or Ischemic Stress

When recorded in the excised inside-out mode, large-conductance single-channel events displayed voltage-dependent inactivation at positive potentials greater than $+20$ mV and negative potentials less than -20 mV (Fig. 5A). The unitary I-V relationship was linear (Fig. 5B) with a slope conductance of 403.9 ± 1.7 pS and a reversal potential of 0 mV in symmetrical conditions, with normal Ringer solution in both the bath and the pipette. The reversal potential shifted to -33.5 mV (Table 1) when 135 mM NaCl in the bath solution was replaced with 135 mM Na-glutamate. This value is less than the theoretical Nernst potential for Cl^- , which is -66.3 mV in these conditions (Table 1). This result indicates that the maxi-channel is anion-selective with a permeability ratio of glutamate to Cl^- of 0.21. This value is close to $P_{\text{glutamate}}/P_{\text{Cl}} = 0.22$ and 0.20, which were the values obtained for the maxi-anion channel in mammary C127 cells (Sabirov et al., 2001) and rat cardiomyo-

cytes (Dutta et al., 2004), respectively. Very similar inward currents and a reversal potential of -35.5 ± 0.6 mV ($n = 6-12$ from 3 different patches) were obtained in the experiments using a holding potential of -35 mV (close to the reversal potential in these experimental conditions). Therefore, we concluded that the inward currents on Fig. 5B (filled circles) do not reflect a build-up of Cl^- on the underside of the membrane at the holding potential of 0 mV.

To record the currents carried by glutamate, we replaced Ringer's bath solution with 146 mM Na-glutamate. This maneuver caused a shift of the reversal potential to -38.9 mV (Fig. 5B, open triangles), yielding a permeability ratio of glutamate to Cl^- of 0.22 (Table 1). This value is close to that obtained by partial chloride replacement (Fig. 5B, filled circles). Importantly, small but clearly discernible single-channel events were observed at potentials more negative than -50 mV (Fig. 5C). The current amplitude was insensitive to a 10-times reduction of HEPES concentration in the bath ($n = 7$). A possible contribution of the cationic flux from the pipette could be ruled out, because we found no difference in the inward current

TABLE 1. Chloride Equilibrium Potentials, Experimentally Measured Reversal Potentials and Permeability Ratios for Maxi-Anion and VSOR Cl⁻ Channels

Channel	Solutions (pipette/bath)	LJ (mV)	Cl (out)/Cl (in)	Calculated E_{Cl} (mV)	Measured E_{rev} (mV)	$P_{glutamate}/P_{Cl}$
Maxi-anion	Solution 1/2	0 ¹	146/11	-66.3	-33.5 ± 1.4	0.21 ± 0.07
Maxi-anion	Solution 1/3	0 ¹	146/0	- ²	-38.9 ± 1.2	0.22 ± 0.01
VSOR	Solution 4/5	4.9	111/31	-32.7	-22.5 ± 0.7	0.15 ± 0.01

Solution 1 is normal Ringer solution; solution 2 is Ringer solution containing 135 mM Na-glutamate in place of 135 mM NaCl; solution 3 contained 146 mM Na-glutamate and 5 mM HEPES at pH 7.4; solution 4 is the whole-cell pipette solution in which 100 mM CsCl was replaced with 100 mM Cs-glutamate; solution 5 is the hypotonic bath solution for whole-cell recordings (see Materials and Methods for the detailed compositions). LJ is liquid junction potential (see Materials and Methods). Cl (out) and Cl (in) correspond to the intracellular and extracellular chloride concentrations, respectively. Theoretical chloride equilibrium potential (E_{Cl}) was calculated from the Nernst equation using respective activities. Permeability ratio ($P_{glutamate}/P_{Cl}$) was calculated from equation 1 (see Materials and Methods).

¹The offset was zeroed in symmetrical conditions with solution 1 in the pipette and in the bath.

²No chloride was present in the bath.

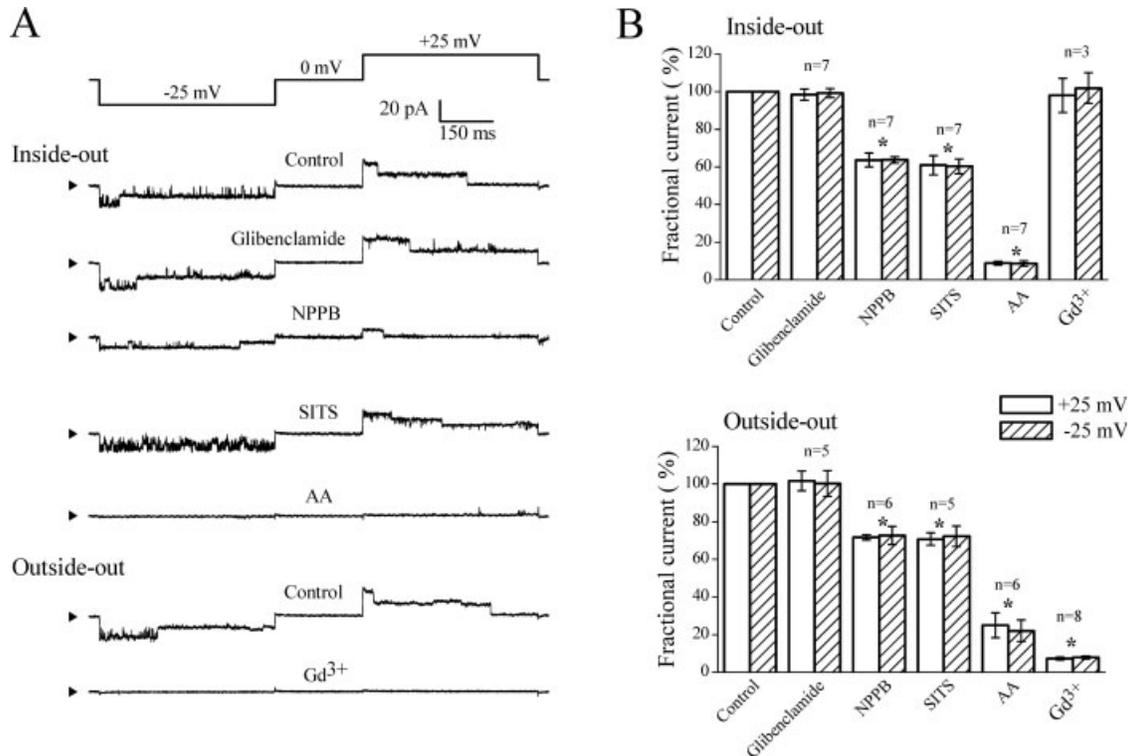


Fig. 6. The pharmacological profile of the maxi-anion channel in excised patches. **A:** Representative single-channel current traces recorded from excised inside-out and outside-out patches in the absence (control) or presence of glibenclamide (200 μ M), NPPB (100 μ M), SITS (100 μ M), arachidonic acid (AA, 20 μ M), or Gd^{3+} (50 μ M), during application of step pulses (the protocol shown at the top of traces). Arrowheads indicate the zero current level. **B:** Effects of drugs on mean currents in

excised macro-patches containing multiple maxi-anion channels. Currents were recorded at +25 mV (open columns) and -25 mV (hatched columns). Data are normalized to the mean current measured before application of drugs and after correction for the background current. Each column represents the mean \pm SEM (vertical bar). * $P < 0.05$ compared with control.

measured at -80 mV ($n = 7$, data not shown) when all monovalent cations in the pipette solution were replaced with TEA^+ . Since no other anion except glutamate was present in the bath, it is concluded that this inward current was carried by glutamate. As shown in Fig. 5D, single-channel amplitude did not change significantly when 10 or 30 mM glutamate was added to the Ringer's bath solution both at +50 mV ($n = 14$) and at -50 mV ($n = 13$), indicating that glutamate ions do not interfere with the chloride flux. No visible effect of 10–30 mM glutamate on the voltage-dependent gating was observed at both positive and negative potentials (Fig. 5D). These data imply that glutamate ions do not exert a competitive block on the astrocytic maxi-anion channel.

In the inside-out mode, the maxi-anion channel was insensitive to glibenclamide (200 μ M), a potent inhibitor of CFTR and VSOR Cl⁻ channels as well as of ATP-sensitive K⁺ channels. In contrast, as shown in Fig. 6, a carboxylate analog Cl⁻ channel blocker, NPPB (100 μ M), prominently decreased the single-channel current amplitude in symmetrical Cl⁻ conditions. A stilbene-derivative Cl⁻ channel blocker, SITS (100 μ M), caused a profound flickery block of inside-out single-channel events activated after excision. Also, arachidonic acid (20 μ M) completely abolished channel activity detected in the inside-out mode. The bath application of Gd^{3+} (50 μ M) had no effect on channel activity in inside-out patches, whereas it rapidly blocked channel activity in outside-out patches

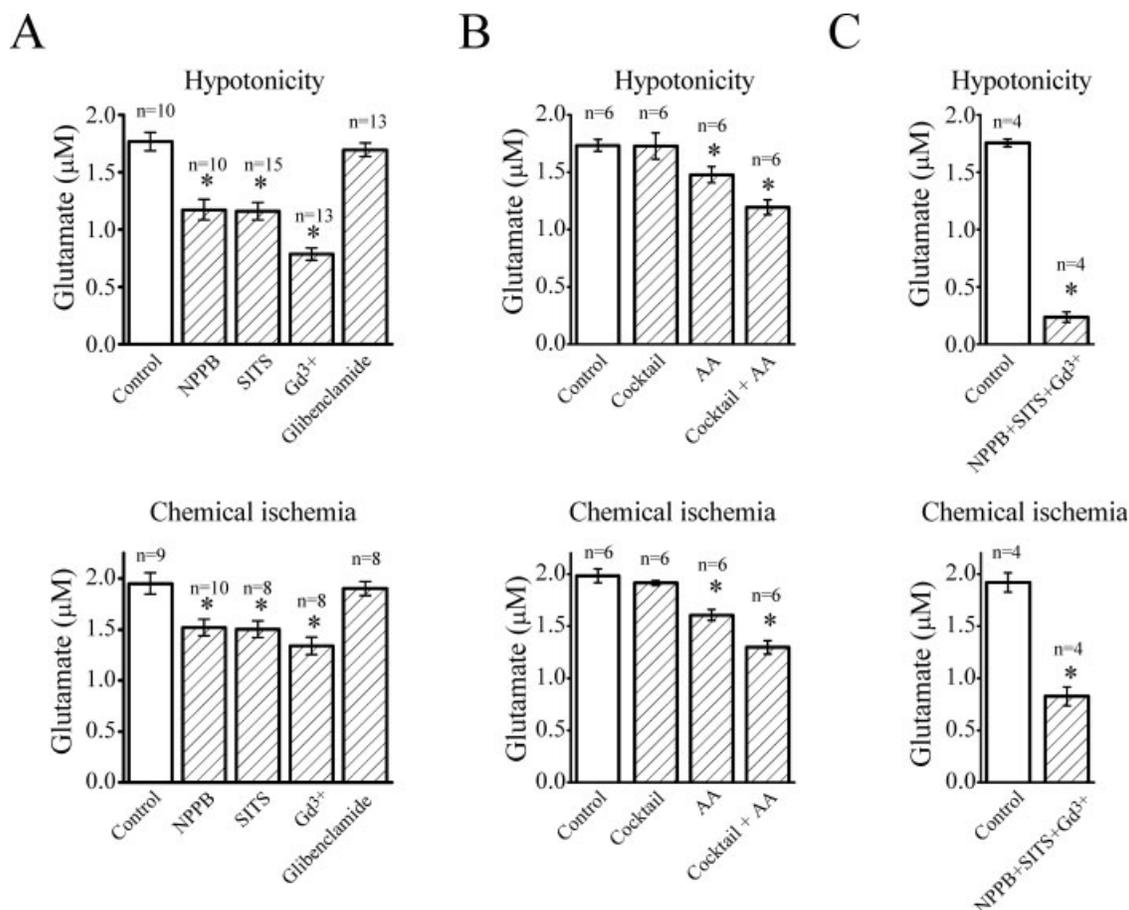


Fig. 7. The pharmacological profile of the net glutamate release induced by hypotonic or ischemic stimulation. The cells were stimulated for 15 min either by hypotonicity (top panels) or chemical ischemia (bottom panels). **A:** Effects of NPPB (100 μ M), SITS (100 μ M), Gd^{3+} (50 μ M), and glibenclamide (200 μ M). **B:** Effects of arachidonic acid (20 μ M). In these

experiments, the arachidonate metabolizing pathways were suppressed by a cocktail of three oxygenase blockers, indomethacin, NDGA, and clotrimazole (all at 20 μ M). **C:** Effects of combined application of NPPB, SITS, and Gd^{3+} at the same concentrations as in A. * $P < 0.05$ compared with control.

(Fig. 6A). Effects of these drugs on the macro-patch currents activated after patch excision are summarized in Fig. 6B.

The pharmacological properties described earlier are very close to those of the maxi-anion channel in mammary C127 cells (Dutta et al., 2002; Sabirov et al., 2001) and in neonatal cardiomyocytes in primary culture (Dutta et al., 2004). Since the maxi-anion channel in these cells as well as in astrocytes can conduct glutamate, we hypothesized that this channel could be involved in the release of glutamate under hypotonic and ischemic conditions. To test this hypothesis, we first tested the effect of two maxi-anion channel blockers, NPPB and Gd^{3+} , on the glutamate currents measured at -80 mV. In the inside-out mode, NPPB (100 μ M) added to the bath solution caused a prominent decrease in the single-channel current amplitude from -4.0 ± 0.1 ($n = 7$) to -1.6 ± 0.2 pA ($n = 9$). Gd^{3+} (50 μ M) added to the pipette solution completely abolished the glutamate currents in 14 patches tested (data not shown). We next studied the effects of maxi-anion channel inhibitors on the net glutamate release from astrocytes. As shown in Fig. 7, NPPB, SITS, and Gd^{3+} significantly reduced the release of glutamate from both astrocytes stimulated by

hypotonicity (top panel) and astrocytes stimulated by chemical ischemia (bottom panel). Similar to patch-clamp experiments in outside-out mode, the most effective inhibitor was gadolinium ion, whereas NPPB and SITS had moderate suppressive effects and glibenclamide was ineffective. Arachidonic acid only slightly suppressed the net glutamate release induced by hypotonicity and chemical ischemia (Fig. 7B), suggesting that oxygenase-mediated arachidonate metabolism might occur at a high rate in astrocytes. Therefore, in a separate set of experiments, we suppressed the downstream metabolic pathways of arachidonic acid by using a cocktail of oxygenase inhibitors, consisting of 20 μ M each of NDGA, indomethacin, and clotrimazole (Dutta et al., 2002, 2004). The cocktail itself did not have any effect on glutamate release. When added in the presence of the inhibitory cocktail, arachidonic acid had a prominent suppressive effect on the net release of glutamate due to osmotic or ischemic stress (Fig. 7B). These results are a strong indication that the maxi-anion channel is a major contributor to the mass release of glutamate from astrocytes. However, even a very effective inhibitor of the maxi-anion channel, gadolinium, did not completely abolish the net glutamate release that we

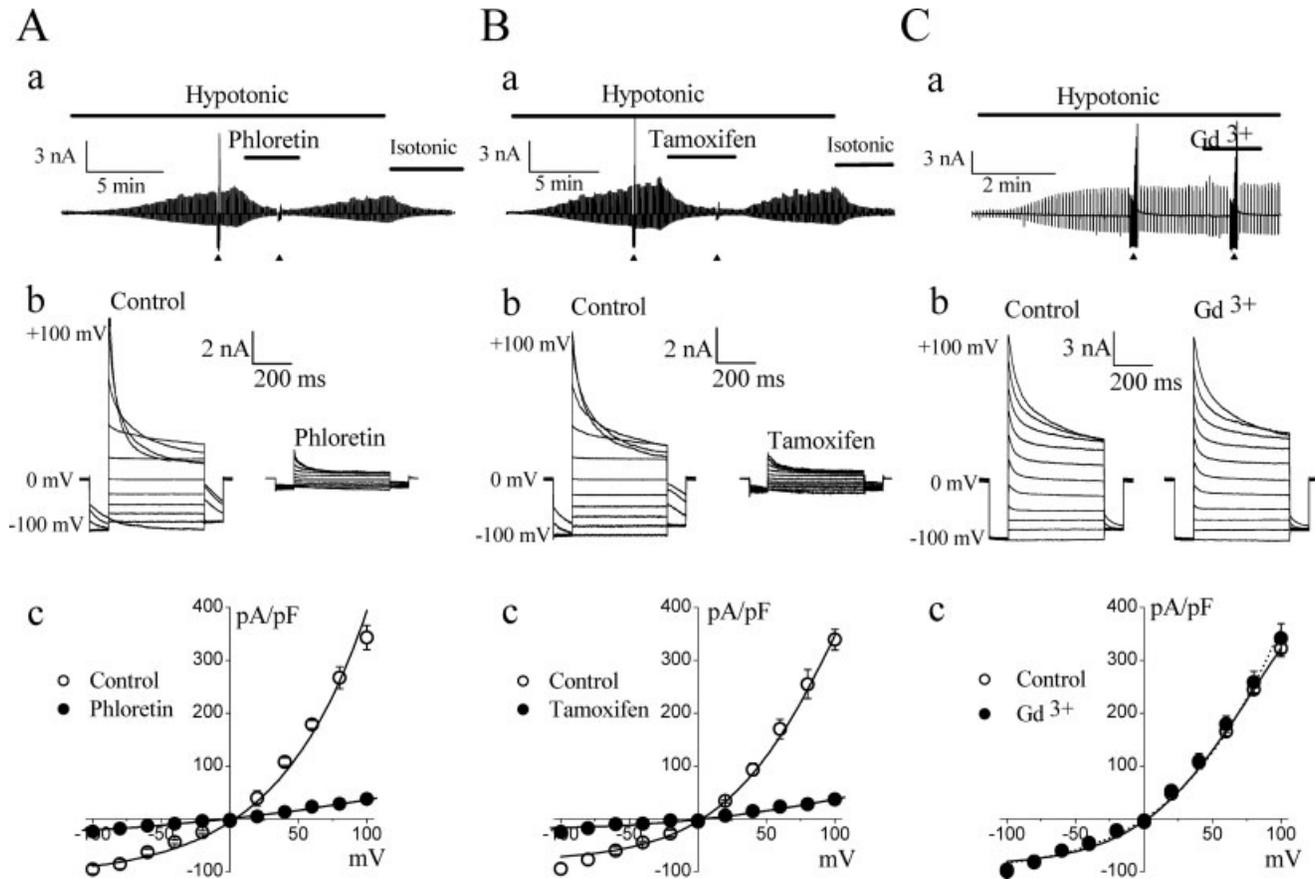


Fig. 8. The pharmacological profile of whole-cell VSOR chloride currents activated by a hypotonic challenge. Effects of 100 μM phloretin (A), 50 μM tamoxifen (B), and 50 μM Gd^{3+} (C) on the VSOR currents recorded from astrocytes in the whole-cell configuration. (a) Representative records during application of alternating pulses from 0 to ± 50 mV (every 5 s); horizontal bars indicate bath application of hypotonic solution, drugs, or isotonic solution. (b) Expanded traces of current responses at the time points indicated by arrowheads in (a). The holding potential

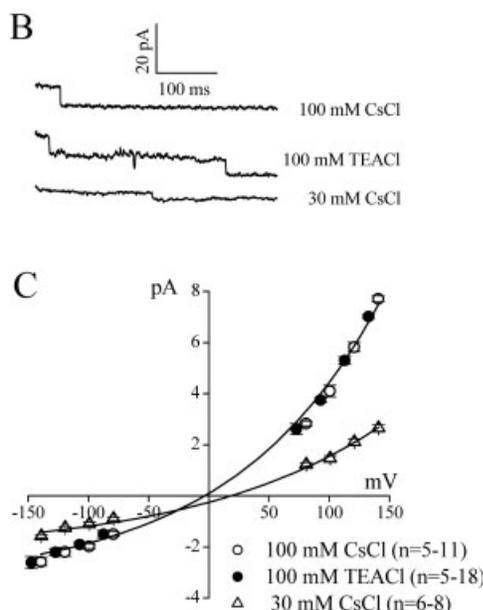
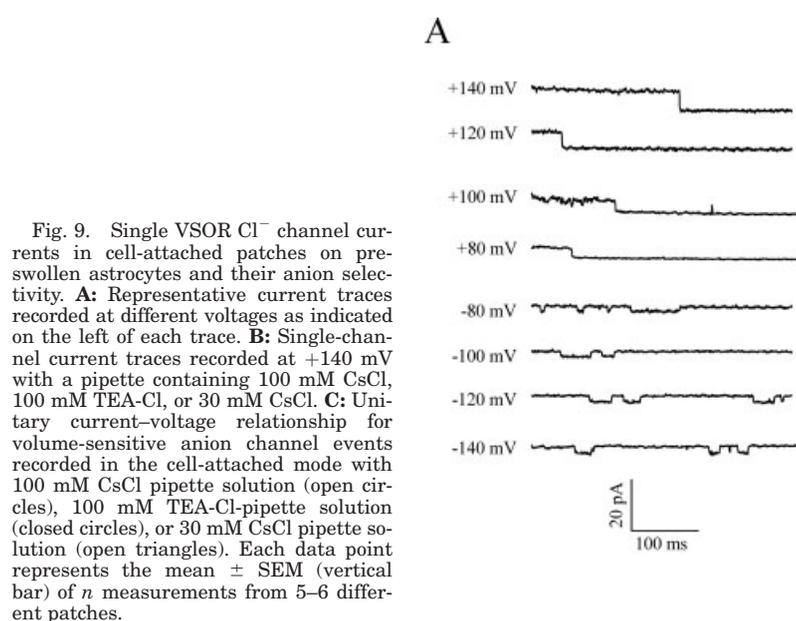
was 0 mV. After a pre-pulse to -100 mV (100 ms), currents were elicited by application of step-pulses (500 ms) from -100 to $+100$ mV in 20-mV increments. (c) Instantaneous current-to-voltage relationships measured at the beginning of test-pulses in (b) before (control, \circ) and during the application of drugs (\bullet). Currents are normalized by the respective cell capacitances; $n = 6$ for all data points. The E_{rev} values for A, B, and C are 3.5, 3.4, and 3.0 mV, respectively, and are close to the equilibrium potential for Cl^- ($+3.8$ mV).

observed, but suppressed it by only $55.5\% \pm 3.0\%$ in hypotonic conditions and by $31.3\% \pm 4.3\%$ in ischemic conditions. This implies that some other pathways might also be involved. Indeed, a combined application of three different drugs, NPPB, SITS, and Gd^{3+} , suppressed the total glutamate release induced by hypotonicity and chemical ischemia by $86.5\% \pm 2.6\%$ and $57.9\% \pm 4.7\%$ (Fig. 7C), respectively. These results suggest that NPPB or SITS are affecting both maxi-anion channels and some other glutamate-permeable pathway, presumably VSOR chloride channels.

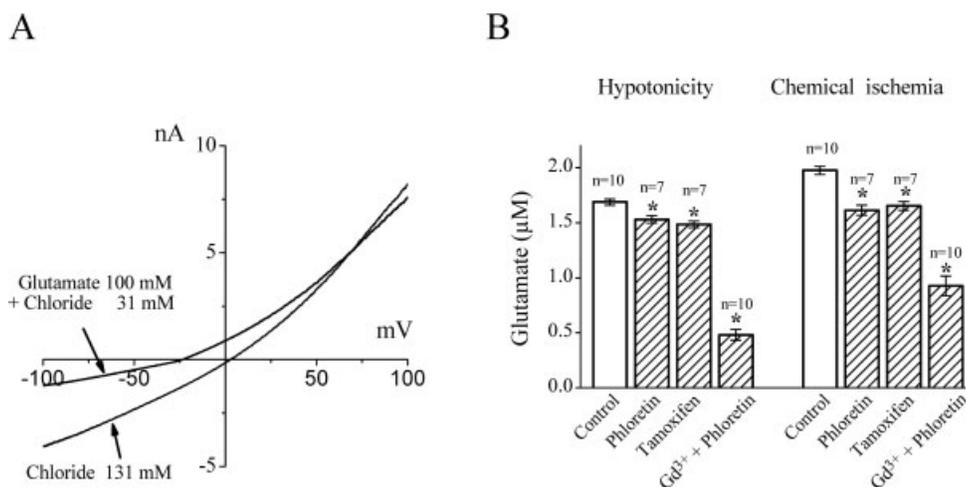
VSOR Anion Channels Constituted a Minor Pathway for Glutamate Release from Astrocytes in Hypotonic or Ischemic Stress

Consistent with earlier observations (Crepel et al., 1998; Lascola and Kraig, 1996; Olson and Li, 1997; Olson et al., 2004; Parkerson and Sontheimer, 2004), cultured astrocytes studied in the conventional whole-cell configuration responded to swelling induced by a hypotonic challenge

(72%) with activation of VSOR Cl^- currents (Fig. 8A, a). This current exhibited prominent outward rectification (Figs. 8A–C, b and c) and inactivation at positive potentials larger than $+40$ mV (Figs. 8A–C, b). As shown in Fig. 8A, the swelling-induced whole-cell current was potently inhibited by a bisphenol, phloretin (100 μM), which selectively blocks VSOR Cl^- currents but has no effect on Ca^{2+} -activated and cAMP-activated Cl^- currents at concentrations equal or lower than 100 μM (Fan et al., 2001). The outward current at $+100$ mV was inhibited by $88.9\% \pm 1.1\%$ and the inward current at -100 mV was inhibited by $75.0\% \pm 2.5\%$ (Fig. 8A, b and c). Note that maxi-anion channels, the currents of which could potentially contaminate whole-cell currents, should have been completely inactivated at ± 100 mV. An anti-estrogen, tamoxifen (50 μM), which is another widely used VSOR channel blocker (Zhang et al., 1994), also exerted a potent inhibitory effect on the whole-cell currents at both positive ($89.0\% \pm 0.5\%$ inhibition at $+100$ mV) and negative ($74.2\% \pm 3.2\%$ inhibition at -100 mV) voltages (Fig. 8B, b and c). The VSOR Cl^- current in astrocytes was insensitive to Gd^{3+} (50 μM) added to the extracellular solution (Fig. 8C).



Cell-attached patches never exhibited VSOR-like channel activity when giga-seals were formed before applying osmotic stress. This is consistent with the previous observation that VSOR Cl^- channels can be monitored only in membrane patches formed after cell swelling (Okada, 1997). In our experiments, when cells were swollen in hypotonic high- K^+ solution prior to seal formation for ~ 5 –10 min, a high level of single-channel activity was observed in the on-cell mode. The single-channel events displayed outward rectification with an outward slope conductance of 81.1 ± 2.5 pS and an inward slope conductance of 18.1 ± 2.7 pS (Figs. 9A,C, open circles) and were insensitive to the replacement of Cs^+ with TEA^+ in the pipette solution (Figs. 9B,C). Reducing the pipette CsCl concentration from 100 to 30 mM reduced the channel amplitude (measured at +140 mV) to $34\% \pm 2\%$ (Figs. 9B,C) and caused a positive shift of the reversal potential of about 20 mV. These results are consistent with anionic selectivity of this channel. Thus, outward rectification,



anion selectivity, and time-dependent inactivation reproduce the phenotype of the VSOR anion channels observed earlier in Intestine 407 cells (Okada et al., 1994; Okada, 1997; Ternovsky et al., 2004; Tsumura et al., 1996) and other cell types (Jackson and Strange, 1995; Nilius et al., 1997; Sabirov et al., 2000; Strange et al., 1996). To assess the contribution of VSOR chloride channels to net glutamate release, we first confirmed their permeability to glutamate using the whole-cell recording mode. In these experiments, Cl^- ions in the pipette were reduced from 131 to 31 mM by replacement with glutamate. This maneuver resulted in a shift of the reversal potential from $+2.5 \pm 0.5$ mV ($n = 6$), which is close to the equilibrium potential for Cl^- ($+3.8$ mV at 25°C), to -22.5 ± 0.7 mV ($n = 5$; Fig. 10A). This shift is significantly less than that expected from the theoretical Nernst potential for Cl^- , which is -32.7 mV in these conditions, suggesting that the channel is, to some extent, permeable to glutamate. We estimated the permeability ratio of glutamate to Cl^-

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to be 0.15 (Table 1). This value is close to the value obtained by Schmid et al. (1998) in cultured pancreatic acinar cells ($P_{\text{glutamate}}/P_{\text{Cl}} = 0.12$) and by Levitan and Garber (1998) in a myeloma cell line ($P_{\text{glutamate}}/P_{\text{Cl}} = 0.17$), but somewhat higher than the value of $P_{\text{glutamate}}/P_{\text{Cl}} = 0.057$ obtained by Carpaneto et al. (1999) for the N2A neuroblastoma cell line. Thus, we conclude that the VSOR Cl^- channel can permit the passage of glutamate anions.

We next tested the sensitivity of glutamate release from astrocytes to VSOR channel blockers. In these experiments, phloretin (100 μM) and tamoxifen (50 μM) exhibited weak but statistically significant suppressive effects on the net release of glutamate induced by hypotonicity ($9.3\% \pm 2.0\%$ and $12.0\% \pm 1.8\%$, respectively) and by chemical ischemia ($18.4\% \pm 2.3\%$ and $16.3\% \pm 2.0\%$, respectively) (Fig. 10B). The fact that two effective blockers of the VSOR Cl^- channel only partially suppressed the total glutamate release strongly suggests that the VSOR Cl^- channel is a significant, but not the main, glutamate-release pathway activated by osmotic and ischemic stress in the plasma membrane of cultured astrocytes. Blocking both maxi-anion and VSOR Cl^- channels by combined application of gadolinium (50 μM) and phloretin (100 μM) inhibited $71.5\% \pm 3.0\%$ of swelling-induced and $49.4\% \pm 4.4\%$ of ischemia-induced glutamate release. Thus, jointly, the two channels may account for 2/3 of the total glutamate release due to osmotic stimulation and for about half of the total glutamate release resulting from ischemic stress.

DISCUSSION

In the present study, we followed the dynamics of extracellular glutamate using a fluorometric NAD^+/NADH -coupled enzymatic assay. The extracellular glutamate concentration rapidly reached a level of $\sim 2 \mu\text{M}$ within the first 15 min and then fell to about 50% of its maximal value after 45 min of incubation in hypotonic conditions. This decrease was due to the Na^+ -dependent glutamate transport functioning in the normal inward direction and was abolished by a selective inhibitor of this system, TBOA. This system was apparently not functional under chemical ischemia, as the time course of the net glutamate release under these conditions was TBOA-insensitive. Also, there seemed to be little or no contribution of vesicle-mediated exocytosis to the net glutamate release, as specific inhibitors and chelation of intracellular Ca^{2+} did not significantly affect the observed net release of glutamate. Although NPPB and gadolinium were shown to inhibit connexin hemichannels (Eskandari et al., 2002), contribution of this channel to glutamate release would be unlikely in our experimental conditions for the following reasons: First, the extent of NPPB-induced suppression of glutamate release (Fig. 7A) was essentially the same as that by SITS, which was shown to have no inhibitory effect on gap junction hemichannels (Eskandari et al., 2002). Second, net glutamate release was not significantly affected by carbenoxolone and 1-octanol that were reported to block the hemichannels in astrocytes (Ye et al., 2003). Third, the present experiments were performed in the presence of

millimolar concentrations of divalent cations that should block hemichannel opening (Ye et al., 2003). Therefore, as in earlier studies (Haskew-Layton et al., 2005; Kimelberg et al., 1990; Mongin and Kimelberg, 2002; Mongin et al., 1999; Rutledge et al., 1998), we postulated that electrogenic channel-mediated pathways are the main mediators of swelling- and ischemia-induced glutamate release from cultured astrocytes.

As expected, cell swelling and chemical ischemia activated an anionic conductance in cell-attached membrane patches on cultured astrocytes. The major single-channel events displayed properties different from those of VSOR Cl^- channels. They had a very large unitary conductance of ~ 400 pS (compared to 50–80 pS for normal VSOR Cl^- channels, see Okada, 1997) and inactivated at moderate positive and negative potentials of more than ± 20 mV. These swelling-activated channels could be distinguished pharmacologically from VSOR Cl^- channels by their insensitivity to phloretin and glibenclamide and inhibition by Gd^{3+} . These biophysical and pharmacological properties are very similar to those described in our recent studies of maxi-anion channels in mammary C127 cells (Dutta et al., 2002; Sabirov et al., 2001), in kidney macula densa cells (Bell et al., 2003), and in neonatal cardiomyocytes (Dutta et al., 2004), where they mediate stimulated release of ATP (Sabirov and Okada, 2004a, 2005). Similar channels have been detected previously in cultured astrocytes (Dermietzel et al., 1994; Jalonen, 1993; Nowak et al., 1987; Sonnhof, 1987), although their function in these cells was not understood. Consistent with our previous observations (Sabirov et al., 2001; Sabirov and Okada, 2004b), the astrocytic maxi-anion channel was permeable to glutamate with a $P_{\text{glutamate}}/P_{\text{Cl}}$ of around 0.2 and could therefore serve as a mediator for the stimulated release of glutamate from cultured astrocytes. Consistent with this hypothesis, the swelling- and ischemia-induced release of glutamate was greatly suppressed by blockers of maxi-anion channels such as NPPB, SITS, Gd^{3+} , and arachidonate. This result strongly suggests that the maxi-anion channel is the major contributor to the release of glutamate from cultured astrocytes. It should be noted that although the employed anion channel blockers are not very specific, Gd^{3+} ions are known to block only maxi-anion channels, but neither VSOR nor CFTR Cl^- channels. However, even the strongest inhibitor of the maxi-anion channel, Gd^{3+} , inhibited only about half of the total glutamate release in hypotonic conditions and about one third of the release induced by chemical ischemia (see Fig. 7). This result suggests that another pathway is also involved in the observed release of glutamate.

This second pathway is likely to be the VSOR Cl^- channel. In the present study, we confirmed the expression of this type of channel in our preparations. The phenotype of the whole-cell current resembled the phenotype observed by other authors (Nilius et al., 1997; Okada, 1997, 2004; Strange et al., 1996). The astrocytic VSOR Cl^- channel has not been described at the single-channel level. In our experiments, cell-attached patches did not exhibit VSOR-like channel activity if giga-seals were formed before induction of osmotic swelling. This fact does not necessarily imply an

involvement of exocytotic insertion of channel proteins to the plasma membrane, but may suggest an important role of membrane unfolding, which cannot be attained in tight-sealed membrane patches, in VSOR channel activation (Okada, 1997). Our results indicate that the astrocyte VSOR Cl^- channel exhibits a single-channel conductance of intermediate values, with an outward slope conductance of around 81 pS and an inward one of around 18 pS. These values are similar to those observed in C6 glioma cells (Jackson and Strange, 1995) in human epithelial Intestine 407 cells (Okada et al., 1994; Ternovsky et al., 2004; Tsumura et al., 1996;) and in endothelial cells (Sabirov et al., 2000). Unlike tamoxifen-insensitive epithelial (Okada, 1997) and neuronal (Inoue et al., 2005) VSOR Cl^- channels, the channel in astrocytes was effectively blocked by tamoxifen. Tamoxifen sensitivity has also been demonstrated for VSOR currents in the STG-1 glioma cell line (Ransom et al., 2001).

The astrocytic VSOR Cl^- channel was glutamate-permeable (with a $P_{\text{glutamate}}/P_{\text{Cl}^-}$ of ~ 0.15) and could potentially mediate the release of glutamate. Indeed, two effective blockers of the VSOR Cl^- channel, phloretin and tamoxifen, tangibly suppressed the net release of glutamate in both hypotonic and ischemic conditions. However, the inhibition was rather mild and could account for only 9–12% of total glutamate release due to osmotic stress and 16–18% of the release due to chemical ischemia. Therefore, although the VSOR Cl^- channel does contribute to the release of glutamate from cultured astrocytes, this contribution is relatively small compared to that of the maxi-anion channel.

It should be noted that the net glutamate release measured after 15- or 45-min exposure to hypotonic or ischemic stress should be composed of not only the efflux but also the influx due to reuptake systems. If the inhibitors of anion channels used modify the rate of glutamate reuptake, this might distort, to a certain extent, our quantitative evaluation of relative contributions of VSOR versus maxi-anion channels for net glutamate release. To make more accurate evaluation, initial rates of glutamate efflux must be measured in future studies.

The involvement of at least two different types of anion-transporting pathways in the swelling-induced release of chloride and taurine (Stutzin et al., 1999) and of taurine and aspartate (Franco et al., 2001; Mongin et al., 1999; Pasantes-Morales et al., 2002) has been postulated for astrocytes based on the differential sensitivity of the two pathways to inhibitors of anion transport and phosphorylation. The relative contribution of these putative pathways could vary depending on experimental conditions. Thus, phloretin inhibited $\sim 80\%$ of aspartate release due to mild osmotic stress, but only $\sim 40\%$ (Mongin and Kimelberg, 2002) or 60% (Haskew-Layton et al., 2005) of the release due to stronger hypotonic stimulation. Tamoxifen inhibited only $\sim 30\%$ when added to the hypotonic solution and $\sim 80\%$ when an additional 10-min pretreatment was used (Rutledge et al., 1998).

Our results suggest that the maxi-anion channel is more efficient than the VSOR Cl^- channel in transporting glutamate. A structural basis for this difference could be

related to the different radii of the pores of these two channels: ~ 1.3 nm for the maxi-anion channel (Sabirov and Okada, 2004b) compared to ~ 0.63 nm for the VSOR Cl^- channel (Ternovsky et al., 2004). A larger inward current (around -10 pA at -40 mM: Fig. 3) with higher glutamate permeability through the single maxi-anion channel compared to that through the single VSOR Cl^- channel (around -1 pA at -40 mM: Fig. 9) suggests that a single maxi-anion channel is at least one order of magnitude more efficient in transporting glutamate than a single VSOR Cl^- channel.

What is the number of active channels that would be sufficient for the observed net glutamate release? Assuming an intracellular glutamate concentration of 10 mM (Kimelberg et al., 1990; Sonnewald et al., 1997) and an intracellular Cl^- concentration of 30 mM (Bekar and Walz, 2002), and using the current amplitudes and permeability ratios measured above at room temperature, it can be estimated that at -40 mV, 0.67 pA current was carried by glutamate. Therefore, a single astrocytic maxi-anion channel in the fully open state may transport $\sim 6.3 \times 10^6$ glutamate ions/s, whereas a single VSOR Cl^- channel may transport $\sim 4.7 \times 10^5$ glutamate ions/s. In our experiments performed at 37°C , cells in one 5.5-cm Petri dish released 2.5 μM glutamate into 2.5 mL extracellular volume over 15 min, which corresponds to a rate of around 1.4×10^6 glutamate ions/s/cell in response to both cell swelling and chemical ischemia. Provided these anion channel current amplitudes and permeability ratios at 37°C are not largely different from those at room temperature, thus, even a single maxi-anion channel or several (three or more) VSOR Cl^- channels would be sufficient for the observed rate of glutamate release. This underestimates the actual number of functioning channels however, since the open channel probability is usually less than 1 (e.g. around 0.1–0.2 at -40 mV for the maxi-anion channel: Sabirov et al., 2001). Astrocytes are likely to possess a much larger number of glutamate-release channels: e.g. 2,000–3,000 as judged from the whole-cell VSOR currents of Fig. 8 or 25–50 as judged from the maxi-anion channel-like whole-cell currents obtained in astrocytes (Liu et al., unpublished observations) using conditions reported previously (Dutta et al., 2004; Sabirov et al., 2001). However, only a few of them are activated during osmotic and ischemic stimulation. This is perhaps because the cell volume of astrocytes rapidly returns to its original value through the highly efficient regulatory volume decrease (RVD) mechanism in these cells (Pasantes-Morales et al., 2000), which would cause the closure of the activated volume-regulated channels after cell volume recovery.

In conclusion, we identified two anion channels, the maxi-anion channel and the VSOR Cl^- channel, which are active at normal range of membrane potentials and jointly represent a major conductive pathway for the release of glutamate from swollen and ischemia-challenged astrocytes, with the contribution of maxi-anion channels being predominant. Our results suggest that maxi-anion channels may play a critical role in brain pathologies such as trauma and ischemia and may represent a prospective target for therapy. Under physiological conditions, maxi-anion

channels may function in astrocytes as a regulated glutamate- and possibly ATP-releasing (see Sabirov and Okada, 2004a, 2005) pathway for two-way cell-to-cell communication, which is essential for information processing in the brain (Fields and Stevens-Graham, 2002). It should be noted, however, that small but significant fraction of the total glutamate release remained even in the presence of combinations of the two channel blockers, suggesting that some other unidentified pathways for glutamate release still remain to be discovered in astrocytes under chemical ischemia or hypoosmotic stress. Glutamate-permeable P2 \times 7 receptor channels expressed in astrocytes might represent one of them (Duan et al., 2003; Fellin et al., 2006).

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