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RESEARCH PAPER

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Carbenoxolone inhibits volume-regulated anion conductance in cultured rat cortical astroglia

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Keywords: swelling-induced chloride conductance, astroglial cells, gap-junction inhibitor, excitatory amino acid release, neuroprotection

Abbreviations: AQP4, aquaporin-4; BSA, bovine serum albumin; CBX, carbenoxolone; CNS, central nervous system; Cx, connexin; DME medium, dulbecco's modified eagle medium; EAA, excitatory amino acid; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; panx-1, pannexin-1; PBS, phosphate buffer solution; PBST, PBS containing 0.05% tween 20; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; RVD, regulatory volume decrease; TIRFM, total internal reflection microfluorimetry; VRAC, volume-regulated anion channels

Accumulating evidence indicate that the gap-junction inhibitor carbenoxolone (CBX) regulates neuronal synchronization, depresses epileptiform activity and has a neuroprotective action. These CBX effects do not depend solely on its ability to inhibit gap junction channels formed by connexins (Cx), but the underlying mechanisms remain to be elucidated. Here we addressed the questions whether CBX modulates volume-regulated anion channels (VRAC) involved in the regulatory volume decrease and regulates the associated release of excitatory amino acids in cultured rat cortical astrocytes. We found that CBX inhibits VRAC conductance with potency comparable to that able to depress the activity of the most abundant astroglial gap junction protein connexin43 (Cx43). However, the knock down of Cx43 with small interfering RNA (siRNA) oligonucleotides and the use of various pharmacological tools revealed that VRAC inhibition was not mediated by interaction of CBX with astroglial Cx proteins. Comparative experiments in HEK293 cells stably expressing another putative target of CBX, the purinergic ionotropic receptor P2X7, indicate that the presence of this receptor was not necessary for CBX-mediated depression of VRAC. Finally, we show that in COS-7 cells, which are not endowed with pannexin-1 protein, another astroglial plasma membrane interactor of CBX, VRAC current retained its sensitivity to CBX. Complementary analyses indicate that the VRAC-mediated release of excitatory amino acid aspartate was decreased by CBX. Collectively, these findings support the notion that CBX could affect astroglial ability to modulate neuronal activity by suppressing excitatory amino acid release through VRAC, thereby providing a possible mechanistic clue for the neuroprotective effect of CBX in vivo.

Introduction

A large body of in vitro and in vivo evidence indicates that physiological activity of astroglial syncytium is crucial for the homeostatic regulation of ions and neurotransmitters in the perineuronal milieu to ensure proper neuronal network activity (reviewed in refs. 1 and 2). It has been widely demonstrated that the dynamic control of the extracellular levels of the excitatory amino acid (EAA) glutamate is mediated by a tuned control of astroglial release and uptake mechanisms.[3] Numerous evidence suggest that glutamate can be released from astrocytes both in vitro and in situ through exocitotic mechanism controlled by increase in intracellular calcium (Ca^{2+}).[4-7] Under ischemic conditions, however, the astroglial transport of EAA mediated by plasma-membrane proteins such as channels and transporters has been described as adjunctive modality whereby these transmitters can modulate neuronal signaling (reviewed in ref. 8). At present four different transmembrane-proteins mediated mechanisms have been claimed to participate to non-exocytotic glutamate release in astroglia: (1) Reverse operation of glutamate transporters.[9,10] (2) The gap junction hemi-channels formed by exameric assembly of proteins called connexins (Cx).[11,12] (3) The purinergic ionotropic receptor called P2X7 which, upon swelling or prolonged agonist stimulation, gates the passage of small molecules such as glutamate and ATP.[13,14] (4) Volume-regulated anion channels (VRAC), which open upon cell swelling and are involved in the process of regulatory volume decrease (RVD).[15-18]

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Among the glutamate-permeable pathways identified so far in astroglia VRAC is the only one of which the molecular identity is still elusive.[19] This fact has hampered the possibility to develop specific pharmacological tools to address its exact contribution to extracellular glutamate dynamics observed in injuries of the central nervous system (CNS) that promote neurodegeneration and in which abnormally high levels of glutamate have a pathogenetic role.[20] In fact, targeting swelling-induced astroglial glutamate release through VRAC has been proposed as a valid therapeutic approach to limit neuronal damage occurring as a result of hypoxia/ischemia and stroke.[21-24]

Carbenoxolone (CBX), the succinyl ester of glycyrrhizic acid, is a well-known molecule that in the CNS is able to inhibit some subtypes of gap junction proteins.[25] Numerous studies in vivo and in vitro support the notion of a neuroprotective role for CBX through modulation of gap junction activity in various different CNS injuries such as ischemia, stroke and epilepsy.[26-28] Recent evidence, however, have revealed that CBX is also able to modulate other astroglial transmembrane proteins that could be involved in its neuroprotective action. In particular, the gap-junction-related protein called pannexin-1 (panx-1) and the P2X7 receptor were reported to be inhibited by micromolar concentrations of CBX.[14,29] Here we have addressed the question whether neuroprotective low concentrations of CBX were also able to depress VRAC activity and the associated EAA release. We found that in cultured rat cortical astrocytes micromolar concentrations of CBX inhibit VRAC activated by hypotonic challenge. By reducing the expression of connexin43 (Cx43)—the most abundant gap-junction protein in cultured astrocytes—with small interfering RNA (siRNA) oligonucleotides, we show that VRAC depression is not due to interaction of CBX with this Cx. The CBX action is not mimicked by Cx-inactive structural analog glycyrrhizic acid, and it is not affected by the connexin-modulating agent quinine, or by the specific P2X7 antagonist Brilliant Blue G. Complementary experiments in HEK293 stably transfected with the purinergic ionotropic receptor P2X7 indicate that this receptor was not involved in CBX-induced depression of VRAC. Finally, by comparative experiments we demonstrate that panx-1, which is also expressed in cultured astroglia, in not involved in VRAC blockage by CBX. Importantly, in all conditions CBX-mediated inhibition of VRAC is paralleled by depression of D-aspartate release reinforcing the tenet that EAA release by hypotonic challenge is mediated by CBX-sensitive VRAC. The possible functional relevance of this novel effect of CBX is discussed. A preliminary account of these results has been presented in abstract form.[30]

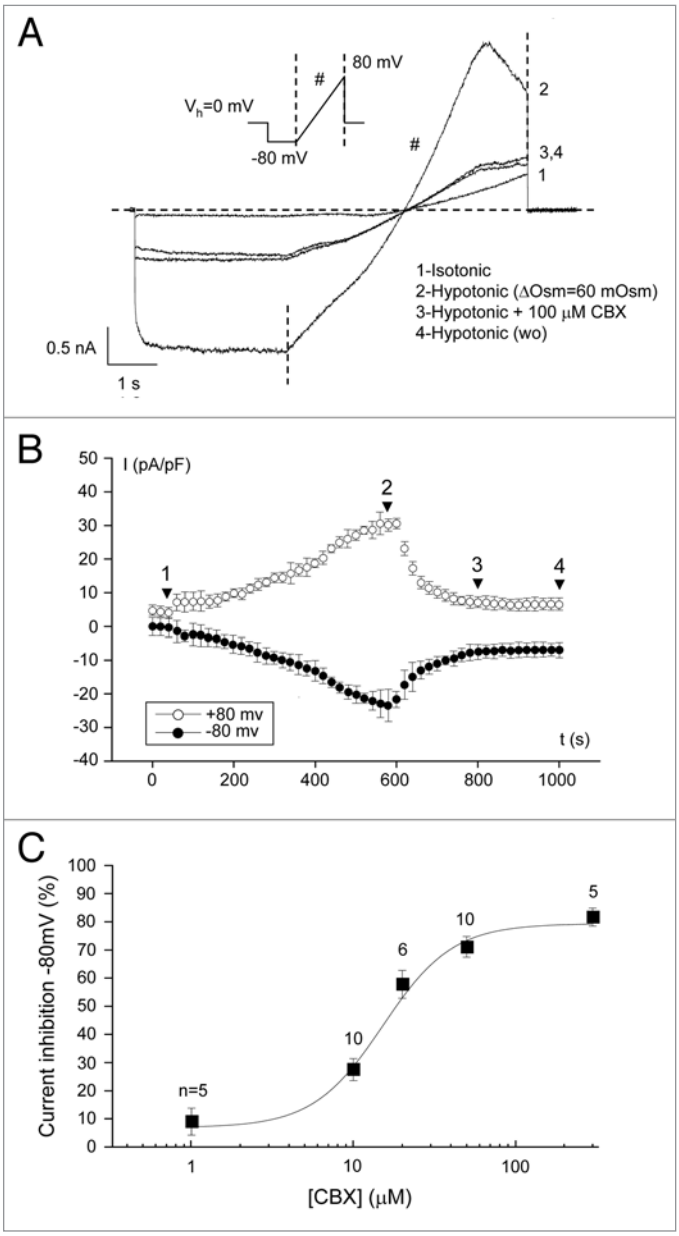

Figure 1. Carbenoxolone inhibits volume-regulated anion channels. (A) Representative current traces mediated by volume-regulated anion channels (VRAC) elicited with a stimulation protocol shown in the inset under extracellular isotonic solution (1), at the maximal current level following hypotonic challenge (2), after addition of carbenoxolone (CBX) to the hypotonic saline (3) and prolonged washout with hypotonic solution (4). (B) Time course of mean VRAC current amplitudes at +80 and -80 mV obtained as in (A) (n = 7). Numbers denote time points of current traces in (A). (C) Dose-response curve fitted with Hill equation. Numbers above each data point represent the number of cells measured at every concentration. Horizontal dashed line denotes the zero-current level. #depicts the ramp component of the stimulation.

Results

Carbenoxolone strongly inhibits the hypotonicity-evoked chloride conductance in rat cultured cortical astrocytes. In the first set of experiments we investigated whether carbenoxolone (CBX) was able to affect the Cl^- conductance described as principal efflux pathway underlying the swelling-induced astrocytic glutamate release.[8,38,39] Astrocytes were clamped at the holding potential (V_h) of 0 mV, next to the astrocyte zero-current potential under our experimental conditions, and stimulated with voltage ramps of 1-s duration from -80 to 80 mV, after a 1 s-long step potential to -80 mV (**Fig. 1A**, inset). Whole-cell membrane conductance recorded in extracellular isotonic saline (**Fig. 1A**) was small, and increased upon exposure to hypotonic

solution (ΔOsm = 60 mOsm). The biophysical properties of the hypotonicy-activated ramp currents overlapped those mediated by volume-regulated anion channels (VRAC) previously described in cultured astroglia.[38,39] Following full activation of VRAC, exposure to CBX (100 μM) added to the hypotonic saline caused a strong inhibition of ramp currents. The time course of mean current densities recorded at -80 mV and +80 mV reveals that the onset of current blockage was rapid occurring within 15 sec of CBX challenge but reached the value of steady-state inhibition after ~4 min (**Fig. 1B**). Notably, current suppression appeared to be irreversible as even prolonged washout (5 min) with hypotonic solution did not cause any significant current recovery. To address the potency of CBX effect, astrocytes were challenged with CBX at various concentrations (range 1–300 μM). The data points of normalized inhibition of hypotonicity-evoked VRAC current at -80 mV were fitted with Hill equation to obtain the dose-response curve (**Fig. 1C**). The best fitting depicts the IC_{50} at 15.4 ± 2.1 μM and the Hill coefficient of 2 ± 0.5.

In order to analyze the voltage and time dependencies of CBX effect, Cl^- currents were elicited with a voltage step protocol (**inset to Fig. 2A**) composed of a family of voltage steps from V_h 0 mV from -80 to 60 mV in 20 mV increments delivered every 10 sec. Voltage step currents were obtained by digital subtraction of current traces recorded before and after 5–7 min of hypotonic challenge preceding (**Fig. 2A**) and following 3–4 min exposure to CBX in hypotonic saline (**Fig. 2B**). As previously shown, VRAC currents activated instantaneously at all voltages and did not inactivate at potentials between -40 and +40 mV. At membrane potentials positive to +40 mV the evoked currents displayed a typical time-dependent inactivation, whose rate and extent became larger at more depolarized potentials.[18,38,39] Astrocyte exposure to a maximal-effective concentration of CBX (50 μM) caused a rapid depression of the hypotonicity-induced voltage-step currents (**Fig. 2B**). Current-voltage relationship (I-V) of peak (**Fig. 2C**) and steady-state current densities (**Fig. 2D**) revealed that inhibition by CBX was voltage and time independent (inset to **Fig. 2D**). Finally, we compared the effect of CBX with that of glycyrrhizic acid (GLA, 50 μM), which is a structurally related analog of CBX, but is inactive at Cxs.[25] In a given astrocyte VRAC current was unaffected by GLA (50 μM) but was strongly depressed by CBX (**Fig. 2E and F**).

Carbenoxolone impedes activation of hypotonicity-evoked chloride current but does not alter water permeability. In order to verify whether CBX action could also affect VRAC activation, ramp currents were recorded in cells bathed in isotonic saline containing CBX for 1 min before the hypotonic challenge in the presence of CBX (**Fig. 3A**). Because there is some evidence that in other cell types soluble factors which are necessary for activation of VRAC are lost during intracellular dialysis from recording pipette,[40,41] this set of experiments was performed within 120 sec from the delivery of the first ramp stimulation, a time period during which VRAC activation by hypotonicity still occurs (see **Fig. 1B**). When astrocytes were bathed with isotonic solution in the presence of CBX, subsequent challenge with hypotonic saline did not cause any significant increase in membrane conductance.

We next asked whether this effect could be due to an indirect action of CBX mediated by its blockage of water permeability. To address this issue astroglial water transport was measured by TIRF analysis. Astrocytes were perfused with isotonic saline and with hypotonic solution in the absence (**Fig. 3B**, left) and in the presence (**Fig. 3B**, right) of CBX (50 μM). The results clearly indicate that the temporal kinetics (inset to **Fig. 3B**) of cell swelling was not significantly affected by CBX, thus strongly suggesting that water permeability was not altered by CBX.

The VRAC-mediated chloride current is not affected by connexin and P2X7 receptor modulating agents. To gain further insight into the mechanism of CBX blockage of VRAC conductance, we verified whether other agents able to modulate Cx proteins could reproduce the action of CBX. In a given astrocyte, whereas quinine, a broad-spectrum inhibitor of various connexins,[42] but also an activator of Cx43 hemichannels in cultured astrocytes,[43] did not alter control currents and VRAC, CBX caused a robust decrease of hypotonicity-evoked Cl^- current (**Fig. 4B**). The same result was obtained when using 2-aminoethoxydiphenyl borate (2-APB), a blocker of junctional conductance through Cx30 and Cx26,[44] which are expressed in astrocytes in vivo (data not shown).[45,46]

Because it has been reported that CBX can depress the ionotropic purinergic receptor P2X7,[14] which is functionally expressed in cultured astrocytes[13,36,47,48] we investigated whether the inhibitor of P2X7, Brilliant Blue G (BBG), could mimic the CBX action (**Fig. 4C and D**). The results indicate that in a given astrocyte VRAC current was not affected by BBG (100 μM) but it was completely depressed by CBX. Collectively, these results show that CBX action is independent of its ability to inhibit Cx and P2X7 activities.

VRAC inhibition by CBX is independent of connexin43 expression. To address more carefully the involvement of Cx proteins in CBX effect on VRAC we performed electrophysiological experiments in astrocytes in which connexin43 (Cx43) was knocked down by small-interfering RNA technology (siRNA). Western blot analysis of total protein extracts of cultured astrocytes after 6 days of transfection with control oligos (CTsiRNA) and oligos able to downregulate Cx43 (Cx43siRNA) revealed a strong decrease of the specific band for Cx43 (at 43 kDa) in Cx43siRNA cells (**Fig. 5A**, left). Comparative immunofluorescence analysis of subconfluent astrocytes transfected with CTsiRNA and Cx43siRNA (**Fig. 5A**, right images) confirmed that in Cx43siRNA astrocytes Cx43 expression was very low.

We next addressed the differences in VRAC activity and CBX sensitivity in CTsiRNA and Cx43siRNA astrocytes (**Fig. 5B and C**). Transfection with CTsiRNA and Cx43siRNA did not affect basal conductance and left unaltered VRAC responses to hypotonicity and CBX sensitivity (**Fig. 5D and E**). These findings strongly suggest that the level of Cx43 expression in cultured astrocytes is not relevant for the effect of CBX on VRAC.

The ionotropic receptor P2X7 is not involved in CBX blockade of VRAC. The pharmacological analysis suggested that P2X7 activity is not involved in CBX inhibition of VRAC in cortical astrocytes. However, that observation did not rule out the possibility that CBX interaction with P2X7 affects VRAC sensitivity

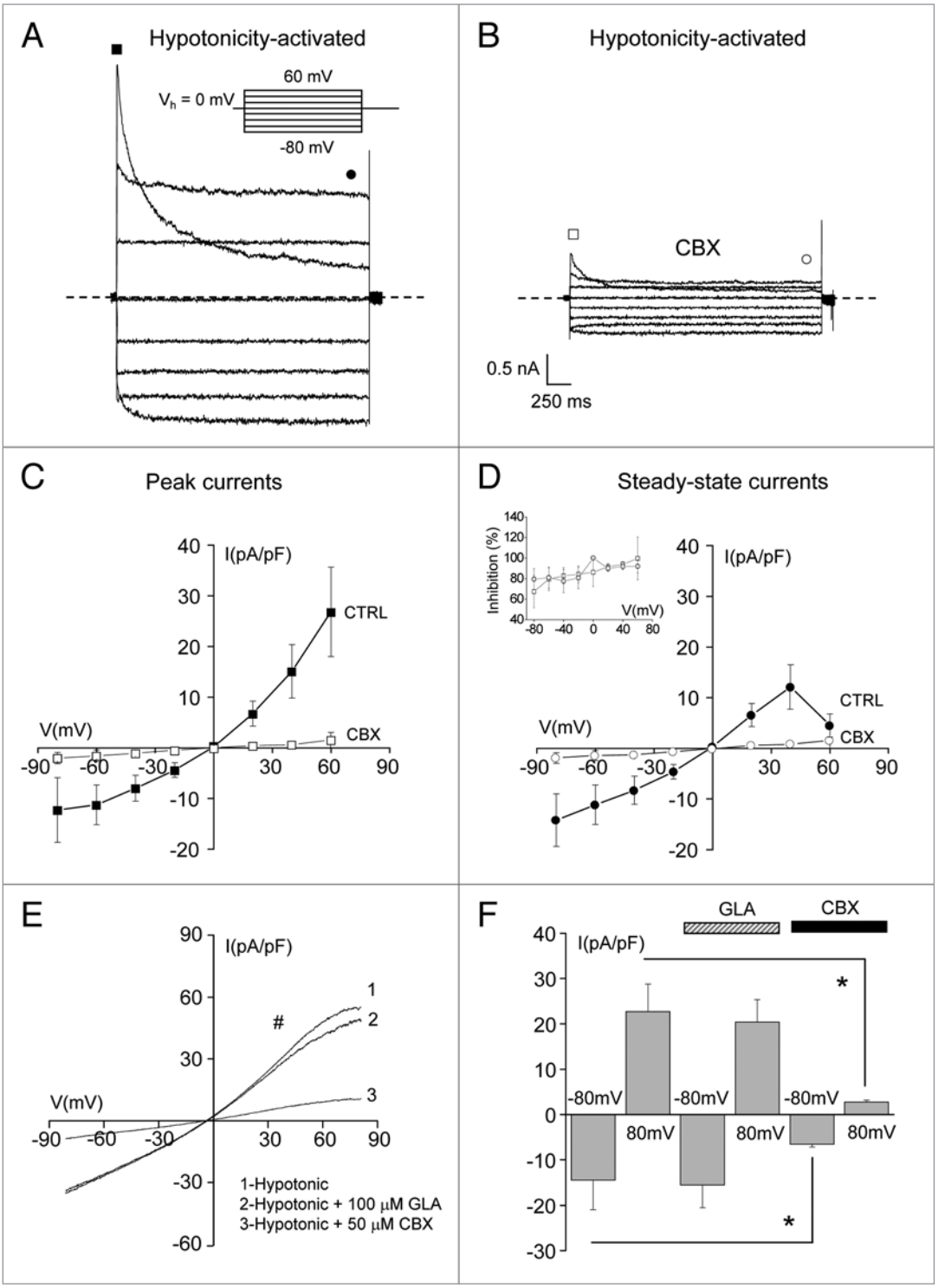

Figure 2. CBX action on VRAC activity is voltage independent. (A) Representative step currents evoked with a voltage-step protocol (inset). Current traces are those obtained by digital subtraction of currents after maximal activation of VRAC with hypotonic saline and those elicited in isotonic condition. (B) Current traces of astrocyte in A after maximal blockage with CBX (50 μM). (C) Current-voltage relationship (I-V) of mean peak currents elicited in several astrocytes (n = 9) before (■) and after (□) CBX challenge. (D) I-V plot of mean steady-state currents before (●) and following (○) CBX administration to the cells in (C). In the inset the percentage of inhibition of peak and steady-state currents is shown. (E) Representative ramp currents in an astrocyte stimulated as in Figure 1A upon hypotonicity (1), after challenge with the structural analogue of CBX, glycyrrhizic acid, added to the hypotonic saline (GLA, 100 μM) (2), and hypotonic saline plus CBX (3). (F) Histogram of hypotonicity-activated current density at -80 and +80 mV in astrocytes challenged as in (E). Data are means ± SE (n = 14). Dashed lines denote the zero-current levels. *p < 0.05 compared to hypotonic solution alone. #in (E) depicts the ramp component of the stimulation shown in Figure 1A.

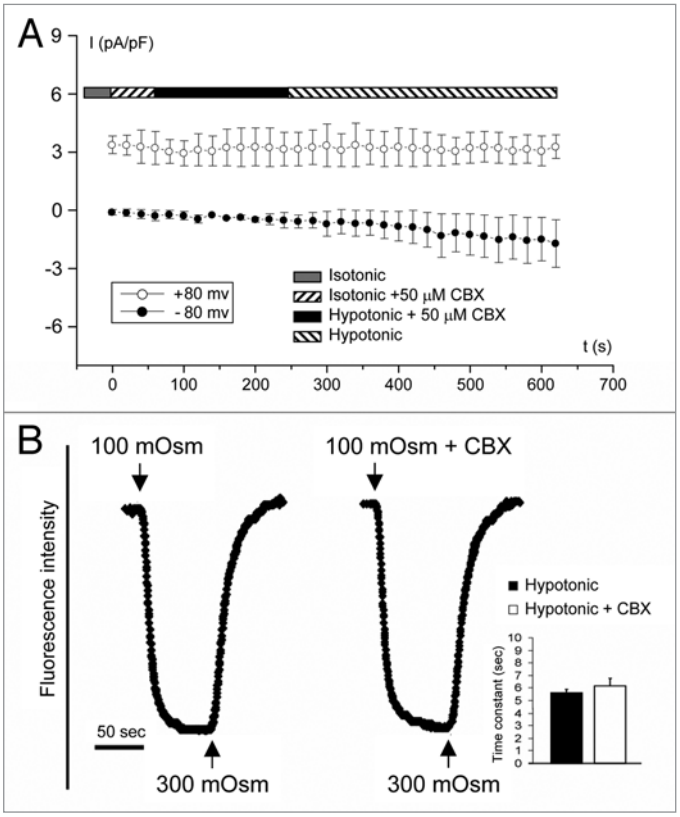

Figure 3. CBX effect is not due to diminution of water movement. (A) Time course of mean VRAC current amplitudes at +80 and -80 mV elicited as in Figure 1B but with CBX challenge initiated 1 min before hypotonic stimulation. (B) Typical kinetics and time constant histogram (inset) of the single exponential fitting of cell swelling in response to the hypotonic shock in the absence and in the presence of CBX. The histograms are means ± SE of separate sets of measurements (n = 6).

to hypotonicity. To address this issue we took advantage of the fact that VRAC are expressed ubiquitously (reviewed in ref. 49) and investigated whether the effect of CBX could be related to P2X7 expression. To answer this question the effect of CBX on VRAC current was studied in the HEK293 cell line untransfected and stably transfected with recombinant rat P2X7/GFP. Western blot analysis (**Fig. 6A**) of total proteins from cultured astrocytes and HEK293 stably transfected with recombinant rat P2X7/GFP revealed two specific bands in astrocytic sample (see GFAP signal) corresponding to P2X7 receptor and a higher band at the expected size for P2X7/GFP in transfected HEK293, thereby confirming protein expression in both samples.

To verify functional expression of P2X7 we first recorded ramp currents inuntransfected HEK293 and P2X7/GFP-transfected cells challenged with the P2X7 agonist BzATP (**Fig. 6B**). As expected, large ramp currents were elicited by BzATP only in P2X7-bearing cells. Hypotonic challenge caused a rapid increase in membrane conductance in untransfected and P2X7-transfected

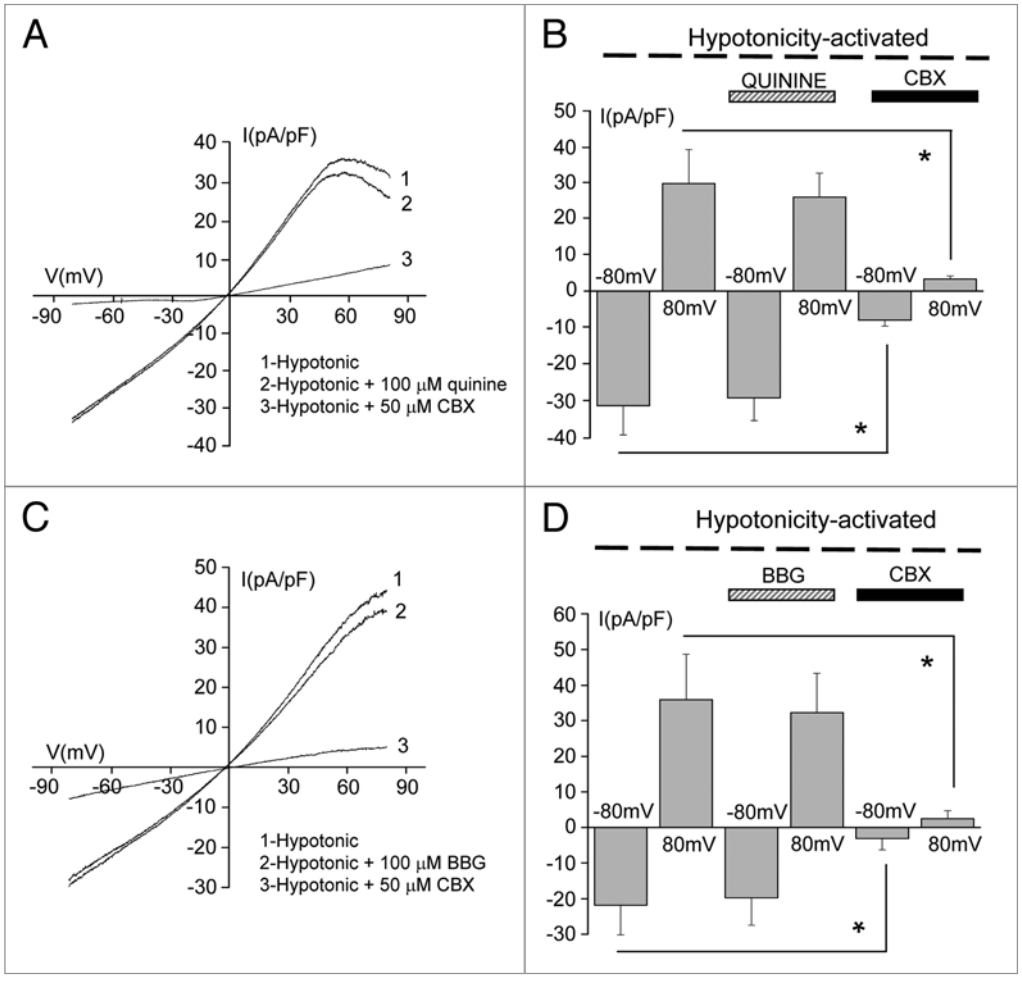

Figure 4. CBX effect is not mimicked by agents affecting connexins and P2X7. (A) Representative ramp currents in a single astrocyte upon hypotonicity (1), after addition of quinine to the hypotoncic solution (100 μM) (2) and hypotonic saline plus CBX (3). (B) Histogram of hypotonicity-activated current density at -80 and +80 mV in astrocytes stimulated as in (A). Data are means ± SE (n = 8). (C) Representative ramp currents in an astrocyte following hypotonic stimulation (1), after addition of the specific P2X7 blocker Brilliant Blue G (BBG, 100 μM) to the hypotonic saline (2) and hypotonic solution plus CBX (3). (D) Histogram of hypotonicity-activated current density at -80 and +80 mV in astrocytes stimulated as in (C). Data are means ± SE (n = 6). *p < 0.05 compared to hypotonic solution alone.

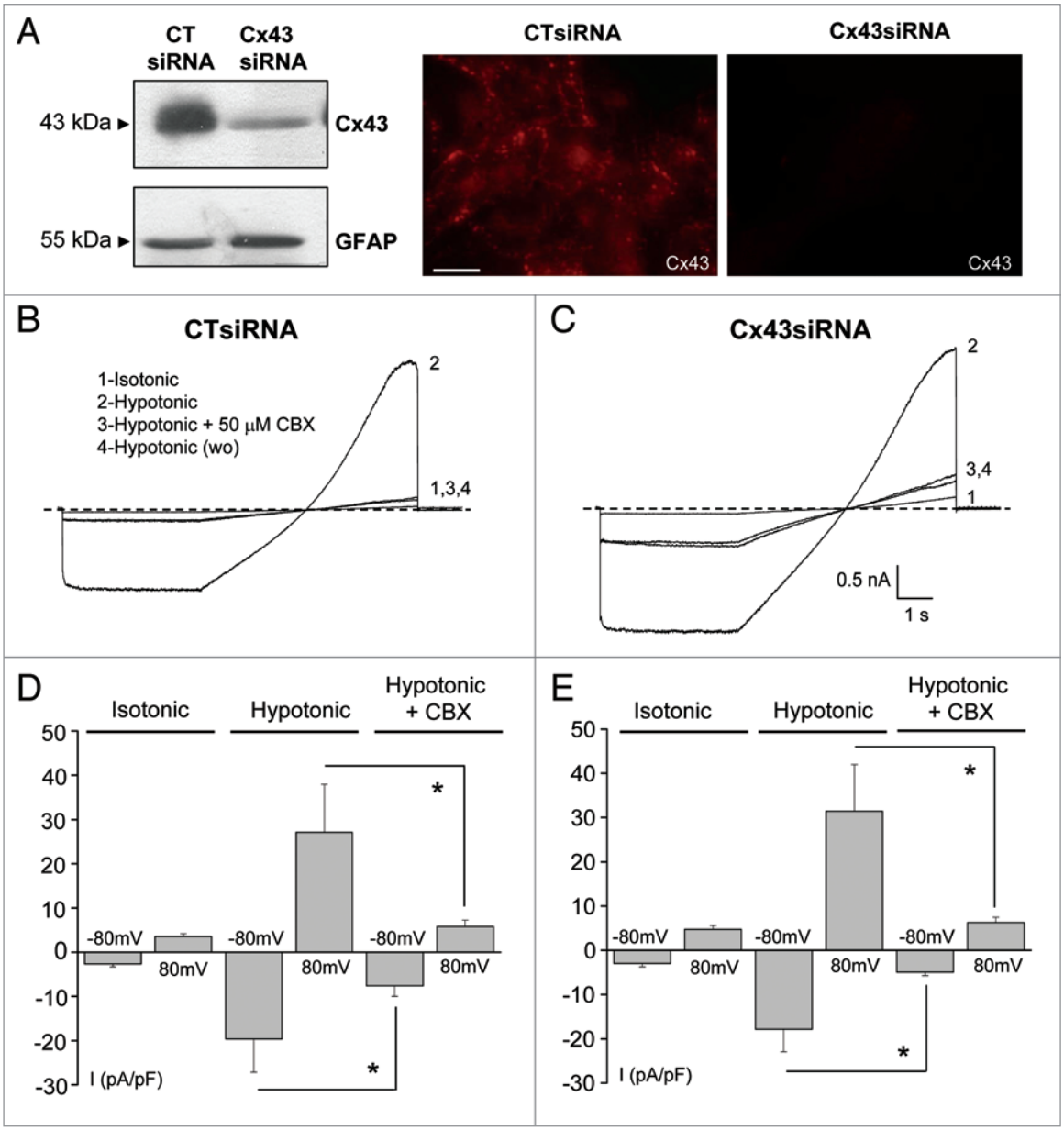

Figure 5. CBX action in astrocytes is not affected by downregulation of Cx43. (A) Left: Representative western blot of primary cultured astroglial cells after 6 days of transfection with scramble small-interfering oligos (CTsiRNA) or oligos for downregulating the expression of Cx43 (Cx43siRNA). In the same blot GFAP was also probed. Right: Immunofluorescence photomicrographs of astrocytes transfected with CTsiRNA or Cx43siRNA immunostained for Cx43 at day 6 post-transfection. Primary antibody against Cx43 was revealed with Alexa Fluor 595-conjugated secondary antibody. Calibration bar: 20 μM. (B) Representative current traces elicited as in Figure 1A in a CTsiRNA-transfected astrocyte at day 6 bathed with isotonic saline (1), following hypotonic challenge (2), after addition of CBX to the hypotonic solution (3) and upon washing out with hypotonic saline (4). (C) Representative current traces in Cx43siRNA-transfected astrocyte at day 6 stimulated as in (B). (D) Histogram of hypotonicity-activated current density at -80 and +80 mV in CTsiRNA-transfected astrocytes stimulated as in (B). Data are means ± SE (n = 9). (E) Histogram of hypotonicity-evoked current density at -80 and +80 mV in Cx43siRNA-transfected astrocytes stimulated as in (C). Data are means ± SE (n = 11). Dashed lines denote the zero-current levels. *p < 0.05 compared to hypotonic solution alone.

cells (**Fig. 6C and D**). Hypotonicity-evoked currents reversed at ~0 mV and displayed similar voltage dependencies. Importantly, they were strongly inhibited by the specific VRAC inhibitor DCPIB (10 μM, data not shown),[18] and were depressed by CBX with identical efficacy. The maximal current density at -80 mV and +80 mV upon hypotonicity before and after exposure to CBX revealed that the current magnitudes in untransfected and P2X7/GFP-tranfected HEK293 cells were comparable (**Fig. 6E and F**). These findings suggest that HEK293 possess VRAC-like current and indicate that the presence of P2X7 is not necessary for CBX action on hypotonicity-evoked Cl^- conductance.

CBX blockage of VRAC is independent of expression of connexin-related protein pannexin-1. Panx-1 is the first cloned member of a novel protein family called pannexins

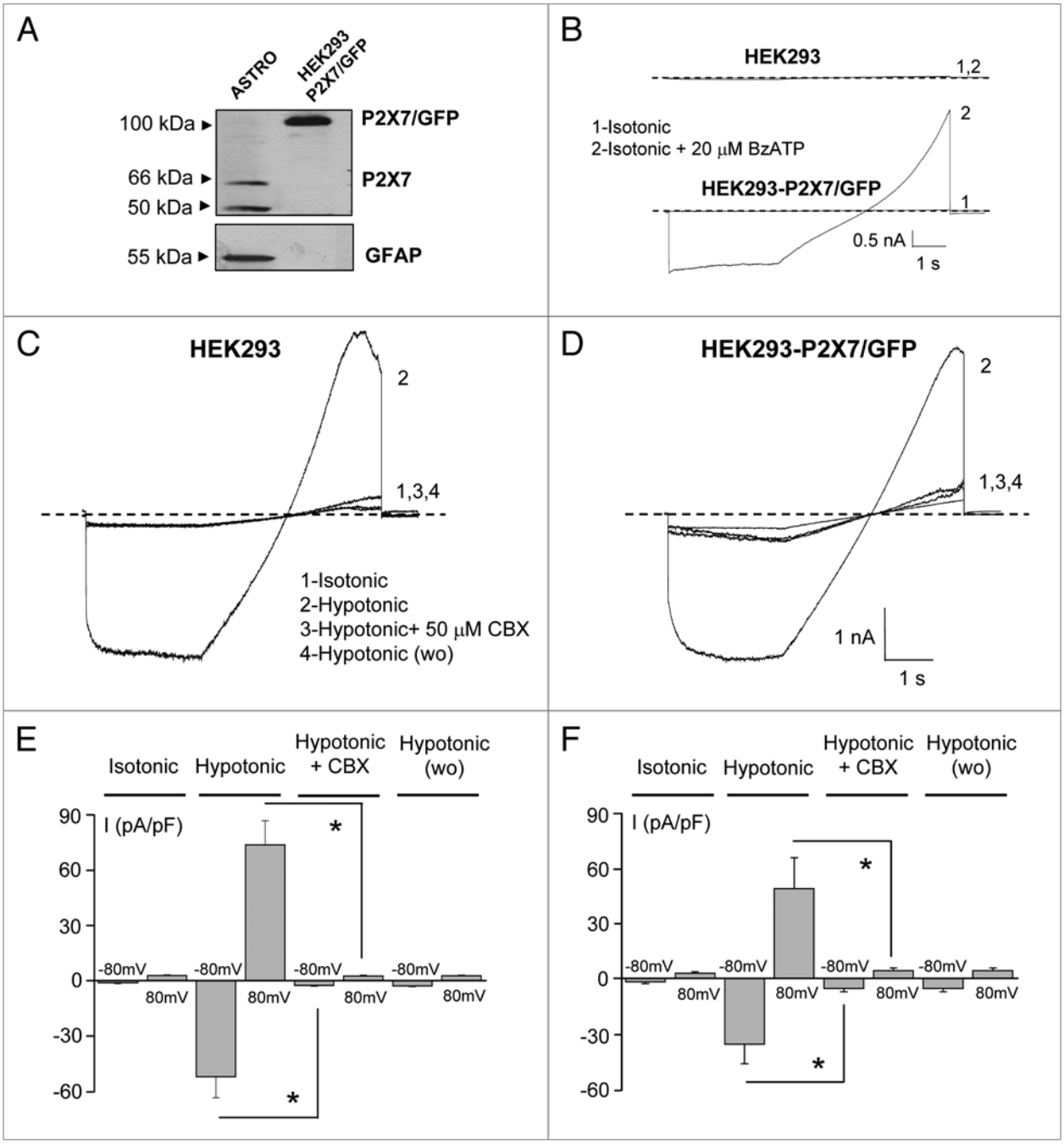

Figure 6. CBX effect is independent of the presence of P2X7 receptor. (A) Western blotting depicting the presence of P2X7 receptor in cultured astroglial cells (left) and in HEK293 cells stably expressing the recombinant rat P2X7/GFP fusion protein. The specific astroglial protein GFAP was also probed. (B) Representative current traces elicited as in Figure 1A in the absence (1) or presence of the P2X7 receptor agonist BzATP (2) in an untransfected HEK293 cell (above) and HEK293 cell expressing the P2X7/GFP fusion protein (below). (C) Representative current traces elicited as in Figure 1A in an untransfected HEK293 cell bathed with isotonic saline (1), following hypotonic challenge (2), after addition of CBX to the hypotonic saline (3) and upon washing out with hypotonic solution (4). (D) The same stimulation as in (C) in a HEK293 cell expressing the P2X7/GFP fusion protein. (E) Histogram of hypotonicity-activated current density at -80 and +80 mV in untransfected HEK293 cells stimulated as in (C). Data are means ± SE (n = 5). (F) Histogram of hypotonicity-activated current density at -80 and +80 mV in HEK293 cells stably expressing the P2X7/GFP fusion protein challenged as in (D). Data are means ± SE (n = 8). Dashed lines denote the zero-current levels. *p < 0.05 compared to hypotonic solution alone.

that are structurally related to connexins (reviewed in ref. 50). Interestingly, panx-1 forms a voltage sensitive channel which is expressed in brain astrocytes and is inhibited by CBX.[51,52] To verify whether CBX inhibition of VRAC could be due to its action on panx-1 we compared CBX effect in cultured astroglia with that produced in a cell line that displays VRAC activity but does not express panx-1. By using affinity-purified anti-panx-1 antibody, we performed western blot analysis of total protein

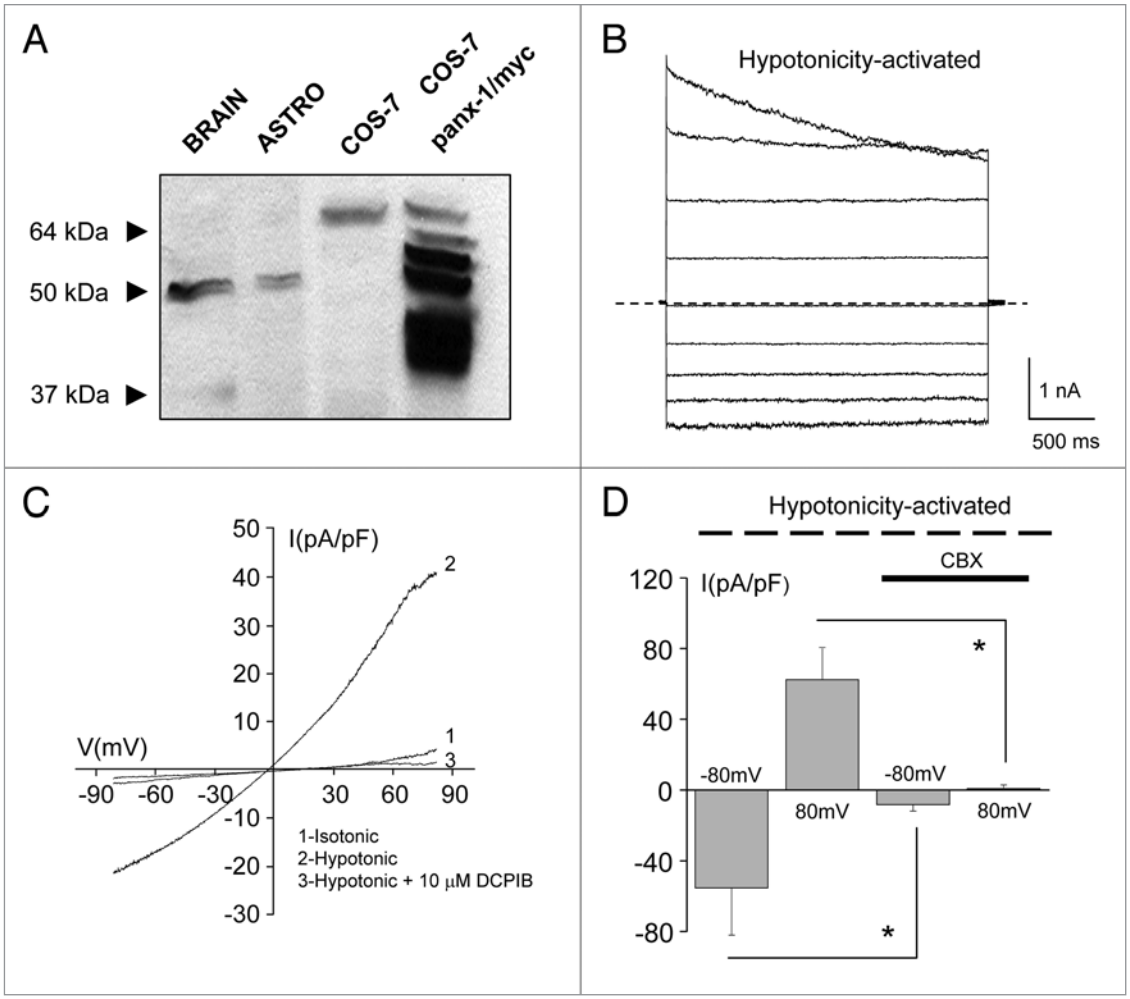

Figure 7. The connexin-related protein pannexin-1 is not involved in CBX effect on VRAC. (A) Representative immunoblotting of lysates from whole brain (brain), primary cultured astrocytes (astro) and the COS-7 cell line untransfected (COS-7) and transfected with panx-1/myc construct (COS-7 panx-1/myc) probed with an antibody against pannexin-1 denoting the specific band for the mature form of the protein at ~50 kDa in whole brain, cultured astroglia and transfected COS-7 but not in untransfected cells. (B) Representative voltage-step traces from -80 to 80 mV in 20 mV increments depicting hypotonicity-activated currents in an untransfected COS-7 cell. (C) Representative ramp current traces in an untransfected COS-7 cell in isotonic saline (1), upon hypotonic challenge (2) and after adding the specific VRAC inhibitor DCPIB to the hypotonic saline (3). (D) Histogram of hypotonicity-activated current density at -80 and +80 mV in untransfected COS-7 cells exposed to CBX. Data are means ± SE (n = 7). Dashed line denotes the zero-current level. *p < 0.05 compared to hypotonic solution alone.

extracts from brain, cultured cortical astrocytes and COS-7 cells (**Fig. 7A**). The specificity of the antibody was tested in panx-1/myc transfected COS-7 cells. The results show that one band of ~50 kDa corresponding to the mature form of panx-1,[53] was present in whole brain lysate, and in cultured astrocytes and panx-1/myc-transfected COS-7 protein extracts, but not in untransfected COS-7 cells. Other specific bands corresponding to different forms of panx-1/myc protein were identified by the antibody only in transfected COS-7 cells.[53] Next, we verified whether panx-1 was involved in CBX inhibition of VRAC-mediated Cl^- conductance by analyzing the sensitivity of hypotonicity-evoked currents to CBX in untransfected COS-7 cells. In these cells, voltage step stimulation upon hypotonicity (ΔOsm = 60 mOsm) evoked currents that displayed time and voltage dependencies (**Fig. 7B**) comparable to those of astroglial VRAC and were completely depressed by the VRAC inhibitor DCPIB (10 μM) (**Fig. 7C**). Of note, challenge with CBX strongly inhibited the hypotonicity-activated conductance (**Fig. 7D**). Collectively, these data indicate that COS-7 cells display a hypotonicity-induced current whose properties overlap those of VRAC current in cultured astrocytes. They also show that the presence of panx-1 is not essential for the inhibitory effect of CBX on the hypotonicity-induced Cl^- current in COS-7 cells.

CBX inhibits hypotonicity-induced [^{3}H]D-aspartate release from cultured astrocytes independently of the presence of Cx43 and P2X7. There is evidence that VRAC activation mediates swelling-induced glutamate and aspartate release from cultured astrocytes.[18] Moreover, recent work described the ability of CBX to block swelling-evoked glutamate efflux in cultured hippocampal astrocytes.[54] Other indirect evidence suggest that volume-sensitive organic osmolyte release may involve more than one permeability pathway.[55,56] Thus, in the final set

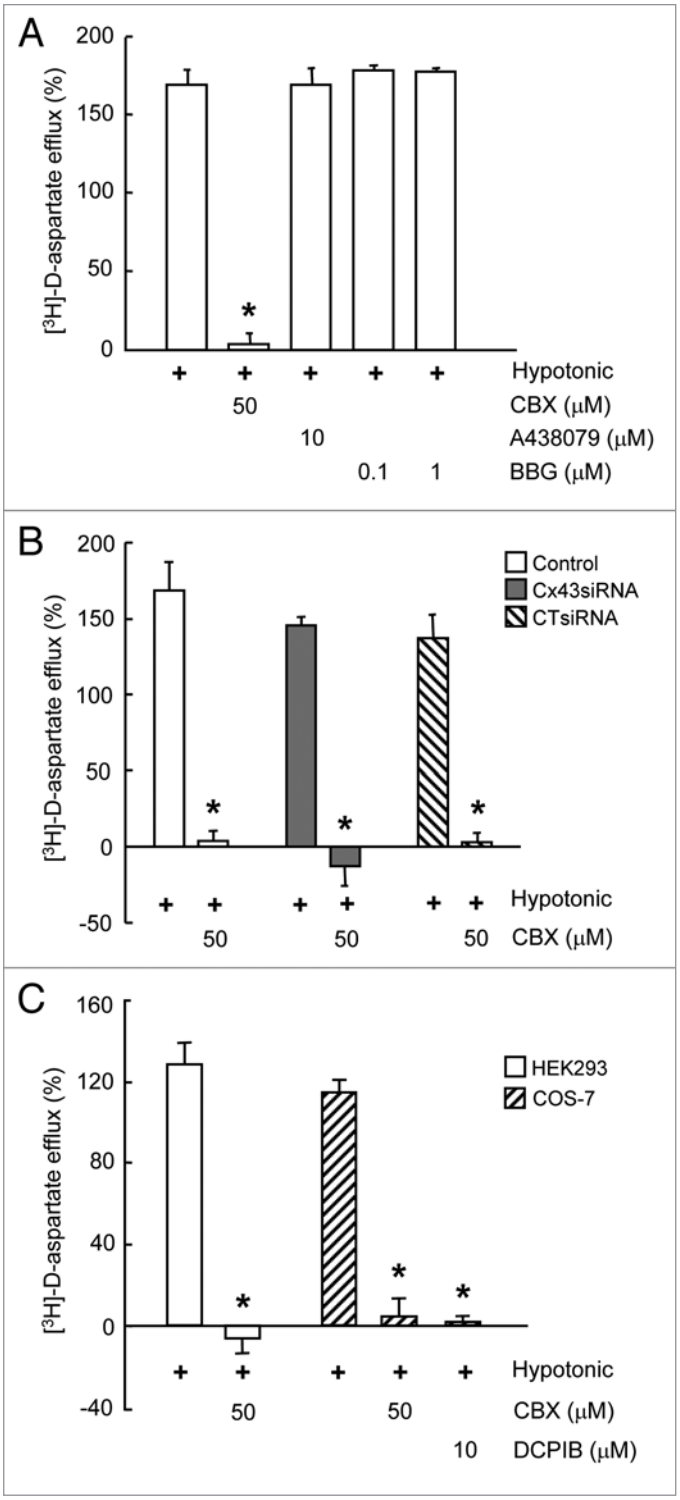

Figure 8. CBX depresses aspartate release in cultured astrocytes, HEK293 and COS-7 cells. (A) Histogram of swelling-activated [^{3}H] D-aspartate efflux from cultured astrocytes in the presence of CBX and various P2X7 receptor antagonists. (B) Histogram of CBX-sensitive swelling-activated [^{3}H]D-aspartate efflux in control astrocytes, and CTsiRNA- or Cx43siRNA-transfected astrocytes. (C) Histogram of CBX-sensitive hypotonicity-activated [^{3}H]D-aspartate efflux in HEK293 and COS-7 cells. Bars represent percent variations of [^{3}H]D-aspartate release from basal levels. Challenge with hypotonic solution (250 mOsm) was for 2 min; CBX, BBG, A438079 and DCPIB were added 10 min before and throughout hypotonic challenge. Data are means ± SE of 3–6 independent experiments performed in duplicate. *$p < 0.05$ compared to hypotonic solution alone.

of experiments we verified whether CBX-mediated depression of hypotonicity-activated Cl^- conductance was paralleled by [^{3}H]D-aspartate release in cortical astroglia in primary culture (**Fig. 8A and B**). As reported in previous studies,[18] hypotonicity induced [^{3}H]D-aspartate efflux from astrocytes. The hypotonicity-evoked efflux was completely blocked by 50 μM CBX but not by the selective antagonists of P2X7 purinergic receptors BBG (0.1 or 1 μM)[57] or A438079 (10 μM).[58] To address the role of Cx43 expression in CBX depression of swelling-induced [^{3}H]D-aspartate we applied the same experimental paradigm to cultured astrocytes after 6 days of transfection with siRNA oligos for Cx43. The hypotonicity-evoked [^{3}H]D-aspartate efflux was not significantly different in untransfected, CTsiRNA- and Cx43siRNA-transfected astroglia (**Fig. 8B**). In agreement with electrophysiological experiments, hypotonic solution-evoked [^{3}H]D-aspartate efflux from astrocytes was completely abolished by 50 μM CBX in the three experimental conditions. Taken together, these data show that CBX is able to block swelling-evoked [^{3}H]D-aspartate from astrocytes independently of variations in expression level of Cx43. Of note, experiments performed in untransfected HEK293 and COS-7 cells showed that [^{3}H] D-aspartate release by hypotonicity was strongly depressed by CBX and by the specific VRAC inhibitor DCPIB (**Fig. 8C**). These results indicate that the molecular machinery for swelling-evoked EAA is present and functional in these cell lines and suggest that VRAC current is involved in D-aspartate release also in such cells. Altogether, the data reveal that the presence of P2X7 receptor and panx-1 is not necessary for the CBX-mediated blockade of hypotonicity-induced EAA release.

Discussion

The main finding of this study is the demonstration that the putative relative selective inhibitor of gap junction proteins CBX depresses hypotonicity-activated current mediated by volume-regulated anion channels (VRAC). We show that CBX action is not abolished by the downregulation of the expression of the major connexin protein present in astrocytes, Cx43.[59,60] We provide evidence that another target of CBX, the ionotropic purinergic receptor P2X7 is not involved in the blockage of VRAC activity.[14] To our knowledge, this is the first study reporting that VRAC are a molecular target of CBX.

It was recently reported that in cultured astrocytes CBX suppressed the release of excitatory amino acids evoked by hypotonicity.[54] Here, we present direct evidence that this CBX action is likely due to inhibition of VRAC current. A strong depression of [3H]D-aspartate by CBX was also depicted in cell lines (HEK293 and COS-7) which displayed CBX-sensitive VRAC-like current. Of note, this latter observation denotes that swelling-induced amino-acid release is not restricted to specific cell types but, at least under some experimental conditions, depends on the ability of the cells to activate VRAC. However, only the

uncovering of the molecular identity of VRAC will allow this question to be solved definitively by permitting the manipulation of protein expression.

The possibility that the hypotonicity-induced current is not mediated by VRAC but rather by other proteins, which allow ion fluxes and are known to be modulated by CBX, can be ruled out for several reasons: (1) Although Cx43 expression was not fully abrogated by its specific knocking down, the densities of hypotonicity-evoked current in CTsiRNA- and Cx43siRNA-treated astrocytes were not significantly different. Moreover, quinine, which activates Cx43 hemichannels in astrocytes,[43] did not affect the hypotonicity-evoked current. The possibility that hemichannels formed by other connexins such as Cx26 and Cx30, which are less abundantly expressed in astrocytes in vivo,[45,46] could participate to this current is unlikely because 2-APB, previously shown to inhibit gap-junctional currents through these connexins,[44] was ineffective in depressing the hypotonicity-induced current. (2) The density of the hypotonicity-evoked, CBX-sensitive current was not modified by the presence of the purinergic P2X7 receptor or by its pharmacological inhibition. (3) The CBX sensitivity of hypotonicity-induced current was also observed in a cell line that does not express panx-1, a member of the pannexin family of membrane proteins related to connexins.[61] This is noteworthy because panx-1 is expressed in astrocytes in vitro and in situ,[62,63] display mechano-sensitivity,[64] and is strongly inhibited by CBX when expressed in heterologous system.[52]

It could be envisaged that CBX inhibits the large-conductance anion channel previously identified in cultured astroglial cells,[65,66] which is also activated upon hypotonicity.[67] However, this possibility can be ruled out because such maxi-channel is activated by strong hypotonicity (delta >100 mOsm) and is depressed by the lanthanide gadolinium (Gd^{3+}) but not by phloretin.[68,69] The hypotonic stimulation used here was relatively low and Gd^{3+} (50 μM) was totally ineffective in blocking the CBX-sensitive current (our unpublished observation). By contrast, the finding that DCPIB, at a concentration known to inhibit VRAC specifically,[70] depressed the CBX-sensitive hypotoncity-activated current strongly supports the notion that indeed this current is mediated by VRAC (our unpublished observation, but see Benfenati et al.[39]).

The molecular mechanism through which CBX exerts its action on VRAC is unknown. CBX action appears to be voltage insensitive. Similar voltage independency has been reported for phloretin and DCPIB.[69,70] From our study we cannot exclude that CBX effect is mediated by an indirect action on a molecular component that remains to be identified and which is part of or modulates VRAC. In another cellular context CBX was described to suppress voltage-gated Ca^{2+} channels.[71] Whether intracellular Ca^{2+} elevation plays a role in VRAC activity in astrocytes is still uncertain.[72,73] Under our experimental conditions cultured astrocytes do not express voltage-gated Ca^{2+} channels.[31] However, we previously showed that in cultured astroglia hypotonicity caused an increase in intracellular Ca^{2+} level through the activation of the Ca^{2+} permeable, cationic channel TRPV4.[74] Preliminary single-cell microfluorometry experiments revealed that hypotonicity-induced intracellular Ca^{2+} rise was not affected by CBX. Moreover, upon removal of extracellular Ca^{2+} CBX continued to inhibit VRAC (our unpublished observations). Another mechanistic explanation is that because of its lipophilic nature CBX acts at an intracellular site. Notably, CBX was recently reported to elicit mitochondrial depolarization in neurons and astrocytes in primary culture.[75] In response to ischemic challenge astroglial cells show rapid mitochondrial depolarization (reviewed in ref. 76) and inhibition of mitochondrial respiration by hypoxic/ischemic insults was shown to inhibit volume-regulated anion channels in neurons.[77] Whether such mitochondrial regulation by CBX plays a role in the described action of CBX on astrocytic VRAC warrants further investigation.

Our results strongly support the tenet that CBX-mediated depression of VRAC is coupled to marked reduction of D-aspartate release. The dissection of the pathways involved in the release of EAA as glutamate and aspartate from swelling astroglial cells and the definition of the mechanisms that can modulate them is relevant especially in pathophysiological settings.[78] The features of the swelling-induced EAA release pathways in astroglia remains to be fully elucidated.[16,23,55,73,79] This may partly reflect the fact that, at least under some experimental conditions, the release is mediated by the co-operation of diverse swelling-sensitive permeability mechanisms which show different properties.[67] As stated above, under our experimental conditions the maxi-anion channel does not contribute to the hypotonicity-induced Cl^- current. This result is indirectly supported by previous work revealing that in mouse cultured astroglial cells CBX was ineffective in attenuating swelling-induced glutamate release through maxi-anion channels.[67] Moreover, the D-aspartate release was completely suppressed by CBX at the same concentration shown to inhibit VRAC activity. Finally, in COS-7 cells the CBX-sensitive D-aspartate efflux evoked by hypotonic challenge was also depressed by the VRAC inhibitor DCPIB. Although we cannot rule out completely the contribution of other channels/transporters, these results strongly suggest that in vitro VRAC current plays a critical role in astroglial D-aspartate release abrogated by CBX.

The here-described inhibition of VRAC by CBX may provide an additional mechanistic clue for the pharmacological actions of CBX in vivo. CBX controls spontaneous electrical oscillations of neuronal network.[80,81] Astrocytes have been suggested to play a critical role in such synchronous oscillatory activity,[82] but in mixed neuronal-astroglial cultures in which astrocytes lacked of Cx43, CBX continued to depress neuronal network activity.[83] Moreover, it was recently reported that CBX suppresses long-term potentiation (LTP) in mouse hippocampal slices through blockage of glutamate-sensitive NMDA-evoked currents.[84] However, CBX does not alter intrinsic membrane properties of hippocampal neurons[85,86] and does not affect the neuronal glutamate release.[83] Of note, significant receptor-dependent astroglial glutamate release was shown to occur in situ through volume-sensitive anion channels.[17]

The role of CBX in pathological setting is controversial. CBX was shown to have a strong anticonvulsant activity in rodent seizure models.[87,88] In a mouse model of perinatal global ischemia, CBX was shown to reduce delayed neuronal cell death through a mechanism apparently linked to gap-junction channel

inhibition.[27] CBX administration was reported also to depress glutamate release from microglial cells protecting neuronal cells following transient global ischemia.[89] However, in vitro CBX had opposite effects exacerbating neuronal death in response to various pathological insults.[75,90] Finally, in a mouse model of essential tremor, CBX suppressed tremor.[91] Interestingly, this CBX effect was mimicked by another putative gap junctional channel blocker mefloquine, which has also been demonstrated to strongly depress VRAC in vitro.[92]

Glutamate-mediated excitotoxic damage is critical in brain ischemia.[93] Several lines of evidence indicate that glutamate release from astroglial cells plays a major role during the ischemic event (reviewed in ref. 8). In addition to VRAC at least three other mechanisms may be implicated in the rise of extracellular glutamate upon ischemia. The reverse mode of Na^+-dependent glutamate transporter was reported to be a major pathway for glutamate release in experimental model of ischemia in situ and in vivo.[94,95] By contrast the significance of glutamate exit through P2X7,[13] and hemichannels formed by opening of unopposed Cx proteins[13] is still controversial (reviewed in ref. 96). The release through VRAC was envisaged to be the predominant mechanism causing excitotoxicity in the ischemic core and penumbra.[22,23] However, those results were not conclusive because of the use of unspecific molecular tools to block VRAC. Hence, the individuation of novel pharmacological agents able to interfere specifically with VRAC-mediated EAA release would be of great relevance because it would allow to determine conclusively their contribution to ischemic damage.[78] Our results obviously do not meet such requirement because CBX has diverse molecular targets. However, they provide insights into the mechanism of CBX action and define a novel molecular frame for the design and synthesis of innovative pharmacological tools useful to limit neurodegeneration in brain ischemia.

Materials and Methods

Cell culturing. Primary cultures of pure cortical rat astrocytes were prepared as previously described.[31] Briefly, after removing the meninges, the cerebral cortices of one- to two-day-old pups (P0-P2) were mechanically dissociated and placed in cell culture flasks containing DME-glutamax medium supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all products purchased from Gibco-Invitrogen, Milan, Italy). Culture flasks were maintained in a humidified-atmosphere incubator at 37°C and 5% CO_2 for three to five weeks. The culture medium change was replaced every three days. Before the medium change, flasks were gently shaken for 5 minutes in order to detach microglial cells that seeded on top of the astrocyte monolayer. Immunostaining for the glial fibrillary acidic protein (GFAP) and the flat, polygonal morphological phenotype of the cultured cells indicated that more than 95% were type-1 cortical astrocytes.[31]

Small interfering RNA synthesis and transfection. RNA duplexes of 21 nucleotides with a sequence identical to that of rat Cx43 were chemically synthesized by Dharmacon Research, Inc., Lafayette, CO, USA. Control (CT) siRNA made of scrambled sequence of Cx43 siRNA was used to monitor non-specific effects of RNA interference method.[32] The selected siRNA sequences were submitted to a BLAST search to avoid the possible targeting of other homologous genes. To allow incorporation of siRNAs into astrocytes, 2–3-weeks old cultured astrocytes were seeded the day before transfection at the density of 30,000 cells/cm^2 in DME medium containing 10% FBS. Transient transfection of siRNAs was carried out as previously described using oligofectamine (Invitrogen, Milan, Italy) in DME medium in which serum and penicillin/streptomycin were omitted.[33] For each experiment, specific silencing was confirmed by western blot analysis. To prepare the cells for electrophysiological recordings, 5 days after transfection medium was replaced with DME medium containing penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively). Astrocytes were maintained for 6 hours in this medium before their enzymatic dispersion (trypsin-EDTA) in Petri dishes (33-mm diameter) at a density of 1 x 10^4 per dish. The day after (day 6 from transfection) cells were used for patch clamp experiments. Control experiments in untrasfected and astrocytes that were transfected with oligofectamine alone revealed that the transfection procedure did not modify the apparent channel activity.

Immunofluorescence. Astrocytes plated in Petri dishes were fixed with 4% p/v paraformaldehyde in 0.1 M phosphate buffer saline (PBS) for 15 min. After blocking with 3% bovine serum albumin (BSA) in PBS for 15 min at room temperature, cells were incubated overnight at 4°C with rabbit anti-Cx43 affinity-purified antibody (Sigma, Milan, Italia) diluted 1:300 in blocking solution in which 0.1% Triton X100 was added. The next day astrocytes were incubated with Alexa Fluor 595-conjugated donkey anti-rabbit antibody (Molecular Probes-Invitrogen, Carlsbad, CA, USA) diluted 1:1,000 in blocking solution containing 0.1% Triton X100. Astroglial cells were visually examined with a Nikon inverted epifluorescence microscope, and images were captured with a digital camera (Nikon Coolpix 4500).

Electrophysiology. Current recordings were obtained with the whole-cell configuration of the patch clamp technique as previously described.[31] Patch pipettes were prepared from thin-walled borosilicate glass capillaries to have a tip resistance of 2–4 MΩ when filled with the standard internal solution. Membrane currents were amplified (amplifier List EPC-7, Darmstadt, Germany), filtered at 2 kHz (-3 dB) and acquired at a sample rate of 5 kHz on a microcomputer for off-line analysis (pClamp 6, Axon Instrument, Foster City, CA, USA and Origin 6.0, MicroCal, Northhampton, MA, USA). Because of the large amplitude of the currents often measured, the access resistance (below 10 MΩ) was corrected for to 60–80%. Reference electrode was an agar bridge filled with 150 mM NaCl. Experiments were carried out at 20–24°C. Current densities were calculated by dividing the current values measured at each membrane potential by the cell capacitance derived from the correction of the capacitive transients of the recorded cells by means of the analogical circuit of the patch-clamp amplifier.

Solutions and chemicals. Salts and other chemicals were of the highest purity grade (Sigma, Milan, Italy). For electrophysiological experiments the standard bath saline was (mM) 140

NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 glucose, pH 7.4 with NaOH and osmolarity adjusted to ~315 mOsm with mannitol. In order to isolate Cl^- current the external bath perfusion, called isotonic saline, was (mM): 122 CsCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 TES, 5 glucose, pH 7.4 with CsOH and osmolarity adjusted to ~320 mOsm with mannitol. The intracellular (pipette) solution was composed of (mM): 126 CsCl, 2 $MgCl_2$, 1 EGTA, 10 TES, pH 7.2 with CsOH and osmolarity ~300 mOsm with mannitol. Hypotonic extracellular solution of ~260 mOsm was obtained by omitting mannitol. Osmolality was measured with a vapor-pressure osmometer (Wescor 5500, Delcon, Italy). The different solutions containing the pharmacological agents were applied with a gravity-driven, local perfusion system at a flow rate of ~200 μl/min positioned within ~200 μm of the recorded cell. Quinine, glycyrrhizic acid and DCPIB (all from Sigma) were prepared in DMSO at concentrations 1,000-fold higher than those used in the perfusion saline. A438079 (Tocris), CBX (Sigma) and BBG (Sigma) were dissolved in water at a concentration of 50 mM and aliquots were kept at -20°C.

Water transport measurement. Water transport studies were performed using a total internal reflection microfluorimetry (TIRFM)-based assay, as previously described.[34] Rat astrocytes were cultured on 20-mm round glass coverslips and used before confluence. Cells were washed several times in PBS, and incubated 20 minutes with 1 μM calcein-AM, an aqueous-phase fluorophore (Molecular Probes, Eugene, OR, USA). Coverslips were then mounted in a custom perfusion chamber that allows rapid solution exchange. The experiments were performed at 10°C. For osmotic water permeability measurements, PBS was rapidly exchanged with hypo/hyperosmotic treatment by reducing/increasing the NaCl concentration of PBS. Cell swelling, in response to osmotic gradient, results in cytosolic fluorophore dilution and decreased fluorescence signal while cell shrinkage produces increased fluorescence.[35] Osmotic properties of cultured astrocytes were evaluated from the time course of total internal reflection fluorescence (TIRF) in response to osmotic gradients. The kinetics of osmotic volume changes were characterized by comparing the time constant for cell swelling, obtained from the experimental data, fitted to a single exponential function.[35]

Stable expression of P2X7 receptor. Cultures of the human embryonic kidney cell line HEK293 were grown in DME/F12 medium supplemented with 10% FBS and gentamycin/glutamine (5 mg/ml and 2 mM, respectively) and maintained in an incubator with humidified atmosphere at 37°C with 5% CO_2. HEK293 cells were stably transfected with the plasmid (pcDNA3) containing the full-length rat P2X7 fused with the green fluorescent protein (P2X7/GFP) as previously described.[36]

Transient expression of pannexin-1. Pannexin-1-myc construct was a generous gift of Prof. Gerhard Dahl (Department of Physiology and Biophysics, University of Miami, USA). COS-7 cells were cultured in DME medium supplemented with 10% FBS, nonessential amino acid cocktail (0.1 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml), grown in 100-mm Petri dishes and maintained in a humidified-atmosphere incubator at 37°C with 5% CO_2. For expression in mammalian cells, the day before transfection, COS-7 cells were re-plated in 35-mm Petri dishes at a density of 2–5 x 10^4 per dish, maintained in supplemented DME medium and transfected with the pannexin-1-myc construct by using the DEAE-dextran method (Sigma-Aldrich, Milan, Italy).[37]

Immunoblot analysis. Rabbit anti-pannexin antibody was provided by Gerhard Dahl, Department of Physiology and Biophysics, University of Miami, USA. Transfected and untransfected COS-7 and HEK293 cells, or 3- to 4-week-old astrocytes in primary culture were washed twice with ice-cold PBS, harvested in lysis buffer (in mM: 50 Tris-HCl, 100 NaCl, 1 EGTA at pH 7.4, 0.5% sodium deoxycholate, 1% Triton) and scraped off. The lysate was centrifuged at 14,000 x g for 30 min at 4°C. Rat brain were homogenized at 4°C in a 5 ml solution containing 7.5 mM sodium phosphate (pH 7.0), 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, and complete protease inhibitor cocktail (Roche Diagnostics). After a 10-min 1,000 x g spin at 4°C, 1% Triton X-100, 0.5% sodium deoxycholate, and 150 mM NaCl were added for 30 min at 4°C. Protein concentration was determined in the supernatant (Bio-Rad Laboratories, Hercules, CA, USA). Ten to 25 μg of total protein content were separated on 12% SDS-polyacrylamide gel, electrotransferred onto a nitrocellulose membranes (Bio-Rad Laboratories), blocked in 5% fat free milk in PBS containing 0.05% Tween 20 (PBST) and probed overnight with the primary antibody against Cx43 (1:1,000), or GFAP (1:500, Sigma), or P2X7 (1:100, Alomone Labs) and panx-1. Membranes were subsequently washed with PBST and incubated with IgG horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Milan, Italia), and developed with the enhanced chemiluminescence detection system (ECL-Plus, Amersham Biosciences Europe, Milano, Italy).

[^{3}H]D-aspartate release. The presence of excitatory amino acid transporters on astroglial cells, HEK293 or COS-7 cells was exploited in order to load these cells with [^{3}H]D-aspartate. Briefly, 3- to 4-week-old untransfected astrocytes, astrocytes transfected with CTsiRNA or Cx43 siRNA (at day six following oligonucleotide tranfection), HEK293 or COS-7 cells grown to confluence in flasks were used. Cells were incubated (30 min at 37°C) with [^{3}H]D-aspartate (0.06 μmol/L) in 5 mL standard HEPES medium (in mM: 122.5 NaCl, 3.6 KCl, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 4.5 glucose and 10 HEPES, adjusted to 310 mOsm with mannitol and pH 7.4 with NaOH), transferred to parallel superfusion chambers maintained at 37°C (100 μg protein/chamber), stratified on Millipore filters (Millipore Corp., Bedford, MA, USA) and superfused (0.5 mL/min) with standard medium. After 30 min of superfusion, superfusate fractions were collected in 5 min samples (from B1 to Bn) until the end of the experiment. After 40 min of superfusion, cells were exposed (2 min) to hypotonic solution (250 mOsm, obtained by omitting mannitol from standard HEPES medium) and then reperfused with standard medium. When used, CBX, BBG A438079 and DCPIB were added 10 min before the challenge with hypotonic solution. At the end of superfusion, the radioactivity of filters on which cells were layered and of superfusate samples was determined by liquid scintillation counting. The efflux of radioactivity in each fraction was calculated as a percentage of the total radioactivity present at the onset of the fraction considered (fractional release).

The mean tritium fractional release in B_1 and B_2 fractions was taken as the 100% control value for each chamber. Tritium efflux in B_n fractions was evaluated as the percent variation of tritium fractional release with respect to the corresponding control value. The hypotonicity-evoked tritium efflux (in the presence or absence of the drugs) was measured by subtracting the area under the curves of percent variations of tritium fractional release in control chambers from the area under the curve of the percent variations in treated chambers; in each experiment, two chambers superfused with standard medium were used as controls.

Statistical analysis. Currents elicited with families of voltage steps or voltage ramps were analyzed with Clampfit of pClamp 6 suite (Axon Instruments, Foster City, CA, USA) and Origin (Microcal, Northampton, MA, USA). Data are expressed as mean ± standard error (SE) of several cells (n) in the different conditions. Because of possible differences in cell size, membrane currents have been normalized and are shown as current densities. The statistical evaluation was performed with two-tailed Student's t-test and a p value <0.05 was taken as statistically significant.

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