The Activity of the Na+/Ca2+ Exchanger Largely Modulates the Ca2+ Signal Induced by Hypo-Osmotic Stress in Rat Cerebellar Astrocytes. The Effect of Osmolarity on Exchange Activity

Héctor ROJAS1, Magaly RAMOS1, Gustavo BENAIM2, Carlo CAPUTO1, and Reinaldo DiPOLO1

1Laboratorio de Fisiología Celular, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 21827, Caracas 1020A; and 2Laboratorio de Señalización Celular, Centro de Biociencias y Medicina Molecular, Instituto de Estudios Avanzados (IDEA), Apartado 17606, Caracas 1015A, Venezuela

Abstract: We recently demonstrated that rat cerebellar Type-1 astrocytes express a very active Na+/Ca2+ exchanger highly co-localized with ryanodine receptors (RyRs), which in turn play a key role in glutamate-induced Ca2+ signaling through a calcium-induced calcium release (CICR) mechanism. In this work we have explored whether the Na+/Ca2+ exchanger has any role in the Ca2+ signal induced by hypo-osmotic stress in these cells, using microspectrofluorometric measurements with Fura-2, pharmacological tools, and confocal microscopy image analysis. We present evidence for the first time that the increase in [Ca2+]i in rat cerebellar Type-1 astrocytes, resulting from moderate hypotonic shock, is mediated by Ca2+ release from ryanodine-operated Ca2+ stores, and that the magnitude of the intracellular Ca2+ signal induced by hypotonicity in the short term (up to 240 s) is small and controlled by the activity of the Na+/Ca2+ exchanger operating in its extrusion mode. With longer times in the hypotonic medium, intracellular Ca2+ store depletion leads to Ca2+ entry through store-operated Ca2+ channels. We found it interesting that the activity of the Na+/Ca2+ exchanger measured during this reverse mode operation (Ca2+ entry in exchange for internal Na+) was found to be greatly increased in hypotonic solutions and decreased in hypertonic ones. The buffering of the [Ca2+]i rise induced by hypo-osmotic stress may prevent excessive increases in [Ca2+]i, which otherwise might impair the normal function of this glial cell.

Key words: Na+/Ca2+ exchange, hypo-osmotic stress, [Ca2+]i release, ryanodine receptors, cerebellar astrocytes.

Glial cells constitute almost 50% of total brain cell volume, and astrocytes account for about half of all glial cells [1]. In astrocytes, intracellular Ca2+ concentration ([Ca2+]i) may change in response to different stimuli, including neurotransmitters, peptides [2], and hypotonic shock [3]. Astrocyte swelling is considered a major factor in brain damage associated with post ischemic brain edema and brain injury, hyponatremia, and hepatic encephalopathy [4–6]. It produces a regulatory volume decrease (RVD) that is associated with the release, among others, of glutamate, aspartate, and taurine [7, 8], concomitant with a decrease in glutamate uptake. This leads to a reduced clearance capacity of excitatory amino acid in the extracellular medium, leading to further cell damage [9]. Several mechanisms have been proposed to explain the increase in [Ca2+]i, associated with astrocyte swelling, including [Ca2+]i release from intracellular stores and Ca2+ entry from the extracellular medium [3]. However, the exact mechanism may vary, depending on the glial cell type. For instance, it is known that glutamate-induced swelling and glutamate-induced [Ca2+]i increase are observed only in Type-1 and not in Type-2 astrocytes [10–12].

Using microspectrofluorometric measurements and confocal microscopy image analysis, we recently demonstrated that in rat cerebellar Type-1 astrocytes (i) the [Ca2+]i, and the Ca2+ flux balance under resting conditions are mainly due to the operation of a very active Na+/Ca2+ exchanger; (ii) both Na+/Ca2+ exchanger and ryanodine receptors (RyRs) are highly colocalized; (iii) Ca2+ entry via reverse Na+/Ca2+ exchange markedly increases [Ca2+]i, by a calcium-induced calcium release mechanism (CICR); and (iv) [Ca2+]i increase induced by L-glutamate is the consequence of the activation of the reverse Na+/Ca2+ exchange, as a result of Na+ entry through the electrogenic glutamate transporter [13, 14].
In the present work we have investigated the role of the Na’/Ca2+ exchanger in modulating the hypo-osmolarity-induced increase in [Ca2+]i in rat cerebellar Type-1 astrocytes. Using microspectrofluorometric measurements with Fura-2, pharmacological tools, and confocal microscopy image analysis, we present evidence for the first time that in rat cerebellar astrocytes, the large increase in [Ca2+]i observed in hypotonic media is a consequence of Ca2+ release from RyR-operated intracellular Ca2+ stores, and that the magnitude of the intracellular Ca2+ signal induced by a moderate hypo-osmotic shock (170 mosm/l) is largely controlled in the short term (<240 s) by the activity of the Na’/Ca2+ exchanger operating in its extrusion mode (external Na’-dependent Ca2+ extrusion). At longer times, due to the continuous operation of the exchanger, depletion of the intracellular Ca2+ stores promotes Ca2+ entry through store-operated calcium channels (SOCC), which are blocked by specific inhibitors and quenched by Mn2+. It is interesting that the activity of the Na’/Ca2+ exchanger measured during its operation in the reverse mode (Ca2+ entry in exchange for internal Na’) was found to be greatly increased in hypotonic solutions and decreased in hypertonic ones, an effect that persists after regulatory volume regulation (RVR). The buffering of the [Ca2+]i rise as a consequence of the operation of the Na’/Ca2+ exchanger during hypo-osmotic stress may prevent excessive increases in the [Ca2+]i, which otherwise might impair the normal function of this glial cell.

METHODS

Extra cellular solutions were prepared with deionized ultrapure (18 MΩ) water (Milli-Q, Millipore, Bedford, MA). Ryanodine, thapsigargin (Tg), carbonyl cyanide, 4-(trifluoromethoxy)phenylhydrazone (FCCP), dimethyl sulfoxide (DMSO), D-mannitol, glucose, and Heps were from SIGMA Co. (St.Louis, MO); 4-chloro-m-Cresol (4-Cmc) was from Fluka Chemical Corp. (RonKonKoma, NY); 2-aminoethoxydiphenyl borate (2-APB) and 3,5-bis(trifluoromethyl)pyrazole derivative (BTP2) were from Calbiochem (La Jolla, CA). Endothelin-3 was from American Peptide Co. (Sunnyvale, CA). Fura-2/AM, Fluo-3/AM, Rhod-2/AM, SBFI/AM, and 2’4’-dichlorobenzamil hydrochloride (DCB) were from Invitrogen Molecular Probes (Eugene, OR). NaCl, LiCl, KCl, MgCl2, MnCl2, and CaCl2 were from Merck (Darmstadt, Germany). Fluorescent dyes and all insoluble reagents were dissolved in DMSO (0.1% final concentration).

Cell dissociation and identification of Type-1 astrocytes. Cerebellar cells were dissociated following the method of [15] modified as reported [16]. In brief, 5- to 8-day-old Sprague-Dawley rats were anaesthetized, and sacrificed by hebearding in accordance with Institutional Animal Care and Use Committee guidelines. Their brains were extracted and immersed in a solution containing a high K’/high Na’ ratio (in mM: Na2SO4, 81.4; K2SO4, 30; MgCl2, 5.8; Glucose, 20.4; Heps, 10; Phenol red, 0.5%; [pH 7.4] at 4°C). The cerebellum vermis was dissected and minced into small (2–3 mm) pieces, then immediately transferred to the same solution with 1 mg/ml of protease (type XIII, Sigma Co., MO) and kept at 35–37°C for 6 min. The pieces were washed thoroughly with the high K’/high Na’ solution to remove the protease and then transferred to a Na’-0Ca solution containing trypsin inhibitor (1 mg/ml soybean Type-I-S, Sigma Co., MO) and BSA (1 mg/ml, Sigma Co., MO). The tissue was dissociated by gentle trituration with a fire-polished pipette. Subsequently, the cells were centrifuged at 600 × g for 4 min and washed with Tyrode containing no Ca2+. This last procedure was repeated three times. Dissociated cells were resuspended in a DMEM culture medium (Dulbeco’s Modified Eagle Medium; Gibco BRL, Rockville, MD). The cells (200 µl) were plated onto 22 × 40 mm glass coverslips (0.15 mm thick, Warner Instruments Corp., Hamden, CT) containing poly-L-lysine (100 µg/ml, Cat. # 1524, SIGMA Co., MO) and placed in 35 mm diameter culture plates. The plates were covered with the DMEM medium containing 70 ng/ml of growth factor (mNGF, Cat. # 1524, SIGMA Co., MO) and placed in the incubator until used (3–5 days later).

We used four methods to distinguish Type-1 astrocytes from Purkinje cells and other glial cells present in our preparation: (1) morphologically: Type-1 protoplasmic astrocytes were morphologically recognized under phase contrast optics [17]. These were phase-dark, with a flattened, irregularly polygonal, epithelioid look [11, 12]; Type-2 astrocytes have a stellate morphology with radially distributed fine processes [17, 18]. They could also be distinguished from classical fusiform cells; (2) immunologically: immunocytochemistry of glial cells was carried out using an anti-GFAP (antiglial fibrillary acidic protein; 4) monoclonal antibody. The cells were washed three times with a PBS solution at pH 7.4 and permeabilized with methanol at −20°C for 5 min, fixed with 2% glutaraldehyde in PBS for 15 min, and then treated with 2% BSA for 15 min. Different preparations were exposed to the anti-GFAP monoclonal antibody (1:1,000) for 60 min. Lastly, the cells were incubated for 30 min at room temperature with FITC-conjugated antirabbit IgG or 1 h at room temperature with the antirabbit IgG conjugated to Alexa-Fluo 546 (1:50), (1.5 µg/ml; Invitrogen Corp., Carlsbad, CA). The fluorescence of GFAP-immunoreactive astrocytes was examined using an epi-fluorescence microscope. This procedure was repeated on culture days 1, 3, 5, 10, 14, and 21. More than 95% of all Type-1 astrocytes that were identified by morphological procedures gave positive immunological, pharmacological, and functional responses. Cellular viability was measured on the same days as the GFAP test using a commercial market kit: Calcein-AM and Ethidium homodimer-1 (kit
viability/cytotoxicity [Invitrogen Corp., Carlsbad, CA]). More than 80% of the cells in our primary coculture were viable; (3) pharmacologically: more than 95% of the cells morphologically identified as Type-1 astrocytes responded to nanomolar concentrations of Endothelin-3, with a fast transient rise in \([\text{Ca}^{2+}]_\text{i}\) followed by a plateau higher than the resting fluorescence [13, 19]. Other glial cells with the morphological appearance of Type-2 astrocytes or oligodendrocytes did not respond to this agonist [13, 20]; and (4) functionally: in this work we have added a new criterion for identifying a given glial cell as a Type-1 astrocyte, i.e., the ability of these glial cells to respond with a rise in \([\text{Ca}^{2+}]_\text{i}\) upon activation of the reverse \(\text{Na}^+/{\text{Ca}^{2+}}\) exchange (increase in intracellular \(\text{Ca}^{2+}\), after a removal of the external \(\text{Na}^+\) in the presence of external \(\text{Ca}^{2+}\)). In 75 cells with the morphological appearance of typical Type-1 astrocytes (from two different cultures, 4 days old), we found that more than 90% of them responded to pulses of 0Na, Ca solution with an increase in the \(\text{Ca}^{2+}\)-dependent Fluo-3 signal, which was completely reversible upon the readdition of external \(\text{Na}^+\) [14].

**Intracellular \(\text{Ca}^{2+}\) and \(\text{Na}^+\) measurements.** Determination of \([\text{Ca}^{2+}]_\text{i}\), was carried out in Type-1 astrocytes, loaded for 45 min (34–36°C) with either the fluorescent dye Fura-2/AM or Fluo-3/AM dissolved in standard Tyrode solution (see solutions and extracellular perfusion). The final concentration of both Fura-2/AM and Fluo-3/AM was 8 \(\mu\)M. Measurement of the \(\text{Na}^+\)-dependent fluorescent signals was achieved with the \(\text{Na}^+\) fluorescent probe SBFI by preincubating the cells in standard Tyrode solution with SBFI/AM (20 \(\mu\)M) for 90 min (34–36°C). Coverslips with the loaded cells were placed in an open experimental chamber (RC-27, Warner Instruments Corp., Hamden, CT), mounted on an Eclipse T300 Nikon (Japan) inverted microscope connected to a HyperSwitch model fluorescence imaging apparatus (IonOptix Co., Milton, MA). For Fura-2 and SBFI measurements, the light from a xenon lamp was filtered, alternating 340 and 380 nm interference filters. The resultant fluorescence was passed through a 400 nm dichroic mirror, filtered at 510 nm, and collected using an intensified CCD camera. Fluorescence signals and images were taken at a rate of 33 ms/frame. The images were digitalized and analyzed using the IonOptix software. The \(\text{Ca}^{2+}\) concentration was calculated according to the formula [21]:

\[
[\text{Ca}^{2+}]_i = \frac{K \times (R - R_{\text{min}})/(R_{\text{max}} - R) \times S_f/S_b}{S_f^2/S_b^2}
\]

where \(R\) is the measured fluorescence ratio. The values of \(R_{\text{max}}\) and \(R_{\text{min}}\) and the constant \(S_f/S_b\) (fluorescence of free and \(\text{Ca}^{2+}\) bound Fura-2 at 380 nm) were calculated in vitro using variable \(\text{Ca}^{2+}\)/EGTA ratios to give different \([\text{Ca}^{2+}]_i\) (Kit #1 Invitrogen Corp., Carlsbad, CA). The Fura-2 dissociation constant \(K\) for the Fura-\(\text{Ca}^{2+}\) complex was taken as 225 nM. All calculations and graphics were carried out with Origin (OriginLab Corp., Northampton, MA) and SigmaPlot (SPSS Inc., Chicago, IL). Experimental values are given as mean ± SEM.

**Solutions and extracellular perfusion.** The experimental chamber was superfused with a peristaltic pump at a rate of about 1 ml/min (36°C) with standard Tyrode solution (control solution) containing (mM): NaCl, 145; KCl, 4; MgCl_2, 2; CaCl_2, 2; Glucose, 10; and Hepes, 10 (pH 7.4). Osmolality was adjusted to 300 ± 1.5 mosm/l. The extracellular solutions are presented in Table 1. The control solution is identified as Iso-NaCa (300 mosm/l). Test solutions are Hypo-NaCa, 0Na0Ca, Na0Ca, 0NaCa (230 mosm/l), and Hyper-NaCa; 0Na0Ca, and 0NaCa (400 mosm/l). In all 0Ca solutions, CaCl_2 was substituted by MgCl_2 containing 100 \(\mu\)M EGTA. The control and test solutions were delivered close to the cells (200 \(\mu\)m) via temperature-controlled pressure ejection from a 100 \(\mu\)m I.D. carbonated pipette, as previously described [13]. Since Na\(^{2+}/\text{Ca}^{2+}\) exchanger in rat cerebellar astrocytes has high-temperature sensitivity, all experiments were carried out at 36–37°C [16]. All Iso, Hypo, and Hyper solutions contained 100 mM Na\(^+\) to avoid \(\text{Ca}^{2+}\) entry through the reverse Na\(^+/{\text{Ca}^{2+}}\) exchanger working in its reverse mode (see Fig. 1a and [14]). Osmolality was varied by the addition of D-mannitol.

**Detection of sub cellular \(\text{Ca}^{2+}\) signal by using microscope laser-scanning confocal.** Sub cellular \([\text{Ca}^{2+}]_\text{c}\) was

### Table 1. External solutions.

<table>
<thead>
<tr>
<th></th>
<th>Iso (300 mosm/l)</th>
<th>Hypo (230 mosm/l)</th>
<th>Hyper (400 mosm/l)</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>100</td>
<td>0 Na0Ca</td>
<td>Na0Ca 0Na0Ca</td>
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<td>KCl</td>
<td>4</td>
<td>4 Na0Ca</td>
<td>0 Na0Ca 4 Na0Ca</td>
</tr>
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<td>MgCl₂</td>
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<td>5 Na0Ca</td>
<td>5 Na0Ca 5 Na0Ca</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
<td>0 Na0Ca</td>
<td>0 Na0Ca 0 Na0Ca</td>
</tr>
<tr>
<td>Hepes</td>
<td>5</td>
<td>5 Na0Ca</td>
<td>5 Na0Ca 5 Na0Ca</td>
</tr>
<tr>
<td>Glucose</td>
<td>11</td>
<td>11 Na0Ca</td>
<td>11 Na0Ca 11 Na0Ca</td>
</tr>
<tr>
<td>Mannitol</td>
<td>63</td>
<td>63 Na0Ca</td>
<td>360 Na0Ca 360 Na0Ca</td>
</tr>
</tbody>
</table>

All concentrations are in mM. External pH = 7.4, Temperature 36°C.
monitored in individual cells by using time-scan confocal microscopy. Type-1 astrocytes were incubated with Rhod-2/AM (10 µM) for 50 min at 37°C in Tyrode solution. The coverslip with the loaded cells was placed in an open superfusion chamber on the laser-scanning confocal microscope stage (LSCM, Nikon C1), mounted on a Nikon Eclipse TE-2000-U inverted microscope, equipped with a Nikon 100/1.40 PlanApo VC oil-immersion objective, coupled to a C1-LU2 unit with a neon (543 nm) laser. These laser units were controlled by a D-eclipse C1 interface. Data were acquired with the Nikon control program C1 confocal microscope EZ-C1 and analyzed with the ImageJ program (Rasband, W.S., Image J, National Institute of Health, Bethesda, MD). Digital images of 512 × 512 pixels, 0.127 µm/pixel, were obtained using the same microscope settings and acquisition parameters.

RESULTS
Effect of hypo- and hypertonic solutions on cell volume of Type-1 cerebellar astrocytes. External Na+-dependence of Ca2+ entry via reverse Na+/Ca2+ exchange
To evaluate the volume changes of Type-1 astrocyte exposed to external solutions of different osmolarities, we measured the Ca2+-independent fluorescence emission of Fura-2 at 360 nm (isosbestic point). Figure 1a shows that a short (about 60 s) osmotic changes either in hypertonic or hypotonic solutions, the fluorescence change reaches a plateau indicating a fast shrinkage or swelling of the astrocyte, respectively. Figure 1b shows a typical experiment in which a hypotonic shock was applied to the astrocyte for a prolonged time (>240 s). In this experiment, the rapid decrease in the 360 nm Fura-2 intensity is followed by a slow recovery to its original level, indicating the presence of regulatory volume decrease. Figure 1c shows the Ca2+ concentration dependence of the reverse Na+/Ca2+ exchange. The numbers in parentheses represent different cells. The points are mean ± SE.

Fig. 1. (a) Effect of short exposure to hyper- and hypotonic media on Fura-2 fluorescence at its isosbestic point (360 nm). Notice that for short (about 60 s) osmotic changes either in hypertonic or hypotonic solution, the fluorescence change reaches a plateau indicating a fast shrinkage or swelling of the astrocyte, respectively. (b) Effect of prolonged exposure of hypotonic media. Notice that the decrease in fluorescence slowly recovers to its original level, indicating the presence of regulatory volume decrease. (c) Effect of extracellular [Na+] on the normalized Fluo-3 fluorescence in the presence of 2 mM Ca2+. Observe that below a value of 100 mM external [Na+] there is a significant activation of the reverse Na+/Ca2+ exchange. The numbers in parentheses represent different cells. The points are mean ± SE.

Since the aim of the present work is to investigate the role of the Na+/Ca2+ exchanger, if any, on the modulation of the intracellular Ca2+ signal induced by hypo-osmotic shock, and since Type-1 rat cerebellar astrocytes express a very active Na+/Ca2+ exchanger [13, 14], it is critical
to experimentally determine the lowest values of the extracellular [Na+] that must be present in the extracellular solutions to avoid Ca^{2+} entry occurring through the reverse Na^{+}/Ca^{2+} [13, 14] exchange. Figure 1c shows the Fluo-3 fluorescence against the resting fluorescence as a function of the extracellular [Na^+]. In these experiments, isosmolar solutions (300 mosm/l), were prepared containing 2 mM Ca^{2+} and diminishing amounts of NaCl, replaced by D-mannitol. The experiments clearly show that in the range between 100 and 150 mM NaCl, no increase in [Ca^{2+}] is observed, indicating no significant activation of the reverse Na^{+}/Ca^{2+} exchange. However, for values of extracellular [Na^{+}] below 100 mM, a significant increase in [Ca^{2+}], is found. Therefore in all experimental solutions used in this work (Iso, Hyper, and Hypo) the external [Na^{+}] was set at 100 mM.

**Effect of hyper-osmolarity and hypo-osmolarity changes on [Ca^{2+}]. Role of Na^{+}/Ca^{2+} exchange on the magnitude and decay of the hypo-osmotic–induced Ca^{2+} signal**

Figure 2a shows a modest increase in the [Ca^{2+}], of a Type-1 cerebellar astrocyte, caused by its exposure to a hypo-osmotic (230 mosm/l) medium containing 0Na0Ca, completely blocking the Na^{+}/Ca^{2+} exchange in its forward and reverse modes. Changing the external medium by only 70 mosm/l gave a modest increase in [Ca^{2+}]. (10–15 nM), which reversed switching back to the Iso-NaCa medium. The experiment of Fig. 2b shows the effect of imposing a much larger hypotonic shock (∆osmolarity = 170 mosm/l) from a hypertonic medium on [Ca^{2+}]. The run started in the Iso-NaCa medium with a basal [Ca^{2+}], of 95 nM; changing the medium to a Hyper-NaCa (400 mosm/l; see Table 1 and discussion) and then to a Hyper-0Na0Ca caused no changes in the [Ca^{2+}]. A subsequent change to a Hypo-0Na0Ca medium induced a large increase in [Ca^{2+}], with an initial slow spontaneous decay in the 0Na0Ca medium, followed by a rapid decrease when Na^{+} was re-admitted to the external solution (Hypo-0Na0Ca). The increase in [Ca^{2+}], caused by a ∆osmolarity = 170 mosm/l in the absence of both external Na^{+} and Ca^{2+} (Hypo-0Na0Ca solution) in 50 cells was 270 ± 25 nM. In all cells studied, a hypertonic condition causes no effect on the level of [Ca^{2+}]. It has been shown that in astrocytes the magnitude of the Ca^{2+} response is osmolarity-dependent and maximal at 205 mosm/l [3]. Because the lowest limit of osmolarity we can induce in our astrocytes is 230 mosm/l (100 mM external Na^{+} see Fig. 1c) without activating the reverse Na^{+}/Ca^{2+}, to obtain a reasonable Ca^{2+} response we decided to induce the hypotonic shock from a hyper-osmotic level of 400 mosm/l.

To explore the mechanisms that control the hypo-osmotic–induced Ca^{2+} signals, astrocytes were subject to consecutive cycles of Hypo-0Na0Ca and Hypo-Na0Ca solutions. Figure 3 shows a representative experiment of several astrocytes (n = 35 cells) in which, after inducing an intracellular Ca^{2+} response similar to that shown in Fig. 2b, the cell was exposed to the Hypo-Na0Ca, which caused a rapid decline of the Ca^{2+} signal. The solution changes from Hypo-0Na0Ca to Hypo-Na0Ca and back to Hypo-Na0Ca were repeated three times. The experiment clearly shows that the [Ca^{2+}], increase in the hypotonic medium depends on the presence of external Na^{+} and, more important, that its magnitude decreases progressively after each period in the Hypo-Na0Ca. The result strongly suggests that the Ca^{2+} increase is due to the release from intracellular stores and that a large fraction of the released Ca^{2+}, is extruded to the extracellular medium.
Fig. 3. The effect of consecutive cycles of: Hyper-NaCa to Hypo-0Na0Ca to Hypo-Na0Ca on the level of \([\text{Ca}^{2+}]\). Notice the progressive disappearance of the hypotonic rise in \([\text{Ca}^{2+}]\) in the Hypo-0Na0Ca medium (see text for explanation). The arrows indicate changes in the external solutions. Observe the rapid return of the \([\text{Ca}^{2+}]\) upon the addition of external Na⁺.

by the Na⁺/Ca²⁺ exchanger operating in its forward mode. The importance of this mechanism, energized by extracellular Na⁺, in determining the magnitude of the hypotonichypotonic–dependent Ca²⁺ signal is demonstrated in Fig. 4. The initial part of Fig. 4a confirms that no \([\text{Ca}^{2+}]\) increase is generated upon changing solutions from an Iso-NaCa to a Hyper-NaCa. Following the application of a Hypo-NaCa solution, a small, but significant change in the \([\text{Ca}^{2+}]\), is produced, which is greatly increased when the activity of the Na⁺/Ca²⁺ exchange is stopped by the Hypo-0Na0Ca solution. The experiment also shows that, in the absence of external Na⁺, the decay of the \([\text{Ca}^{2+}]\) signal is markedly slowed down. Figure 4b presents evidence that the large and rapid increase in the \([\text{Ca}^{2+}]\), caused by changing from the Hypo-NaCa to the Hypo-0Na0Ca, is due to an inhibition of the Na⁺/Ca²⁺ exchanger activity. The experiment clearly shows that treatment of the cell with the known Na⁺/Ca²⁺ exchange inhibitor DCB, 50 µM in the presence of external Na⁺ and Ca²⁺, causes an abrupt increase in the \([\text{Ca}^{2+}]\), which slowly decays to a plateau.

Figure 5 shows a representative experiment of a series of astrocytes \((n = 15)\), showing that the Na⁺/Ca²⁺ exchanger may also control the rise in \([\text{Ca}^{2+}]\), induced by procedures other than hypotonic shock. In these experiments, carried out in normal iso-osmotic medium, the astrocytes were subjected to a large intracellular calcium release by inhibiting the calcium ATPase of the endoplasmic reticulum with Tg, opening the ryanodine channels with the agonist 4-CmC, and inhibiting the mitochondrial Ca²⁺ uptake by the mitochondrial inhibitor FCCP. Figure 5a shows that when the Na⁺/Ca²⁺ exchanger is inhibited with Iso-0Na0Ca, the above treatment causes a marked and rapid increase in \([\text{Ca}^{2+}]\), from a level of 100 nM to about 800 nM \((\Delta[\text{Ca}^{2+}] = 700 \text{nM})\). This increase is reversed upon exposure to Na0Ca. Figure 5b shows that when the Na⁺/Ca²⁺ exchanger is fully activated by the Iso-Na0Ca solution, a similar Ca²⁺ release treatment causes only a small increase in \([\text{Ca}^{2+}]\), to about 140 nM, from 112 nM, \((\Delta[\text{Ca}^{2+}] = 28 \text{nM})\), an increase 30 times smaller than that shown in Fig. 5a.

In rat cerebellar astrocytes, \([\text{Ca}^{2+}]\), increases resulting from hypo-osmolarity involve Ca²⁺ release from ryanodine-dependent intracellular Ca²⁺ stores

Because most of the \([\text{Ca}^{2+}]\) increment resulting from hypo-osmolarity occurs in the absence of external cal-
Fig. 5. The effect of an abrupt release of Ca\textsuperscript{2+} from most Ca\textsuperscript{2+} intracellular stores by the addition of a cocktail of 10 \(\mu\)M Tg + 250 \(\mu\)M 4-CmC + 5 \(\mu\)M FCCP. (a) In a Na\textsuperscript{+}- and Ca\textsuperscript{2+}-free medium (Iso-0Na0Ca; complete inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger). (b) In a Na\textsuperscript{+}-containing Ca\textsuperscript{2+}-free medium (Iso-Na0Ca; full activation of the forward Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger). Notice that in (a) the Ca\textsuperscript{2+} release cocktail largely increases the [Ca\textsuperscript{2+}] up to 800 nM, from a baseline of about 100 nM, as compared with only 30 nM in (b).

Fig. 6. The effect of a hypotonic medium lacking both Na\textsuperscript{+} and Ca\textsuperscript{2+} (Hypo-0Na0Ca) to inhibit the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in the continuous presence of 5 \(\mu\)M FCCP. (a) In an astrocyte in which FCCP causes a modest but significant increase in [Ca\textsuperscript{2+}]. (b) In an astrocyte in which FCCP causes no change in the [Ca\textsuperscript{2+}]. Observe that in these two patterns of astrocyte behaviour, a pulse of hypotonicity in 0Na0Ca produces the same rise in [Ca\textsuperscript{2+}], similar to that found in most astrocytes. This suggests that the rise in [Ca\textsuperscript{2+}] originates from an intracellular Ca\textsuperscript{2+} reservoir different from the mitochondrial compartment. Notice too that after a peak of [Ca\textsuperscript{2+}] in Hypo-0Na0Ca, the [Ca\textsuperscript{2+}] decreases at a much slower rate than in the presence of external Na\textsuperscript{+} (see Figs. 2b, 3, and 9).
ryanodine-sensitive calcium stores are involved in the hypo-osmolarity–induced increase in \([Ca^{2+}]\). Figure 7a shows a typical experiment (85% of all cells \(n = 23\)) in which changing the extracellular medium from a Hyper-NaCa medium to a Hypo-0Na0Ca solution causes an increment in \([Ca^{2+}]\), of about 200 nM, which is rapidly reverted by reperfusion again with the Hyper-NaCa medium. After this, the cell was treated with a sufficiently high concentration of ryanodine (50 µM) to block RyRs and therefore made unable to release \(Ca^{2+}\) from the endoplasmic reticulum [24, 25]. In this condition, a second test with the hypotonic 0Na0Ca solution caused only a small increase in \([Ca^{2+}]\). This small increment in \([Ca^{2+}]\), was abolished by the addition of a Hypo-Na0Ca medium.

Figure 7b shows a complementary experiment in which the high concentration of ryanodine was added from the beginning of the experiment in the Hyper-NaCa medium. In this case, ryanodine caused a small increment in \([Ca^{2+}]\), most probably diffusion effects with a lower concentration of ryanodine, which activates the RyRs before blockade by the higher concentration. In any case, exposure to the Hypo-0Na0Ca solution failed to induce any increment in \([Ca^{2+}]\), thus indicating an involvement of ryanodine-dependent \(Ca^{2+}\) stores in the generation of the hypo-osmolarity–induced increment in \([Ca^{2+}]\).

We recently showed that, under isotonic conditions, the low affinity \(Ca^{2+}\)-sensitive dye Rhod-2 accumulates within intracellular \(Ca^{2+}\) stores, particularly in the endoplasmic reticulum (ER) where it is highly colocalized with thapsigargin receptors [14]. Therefore confocal microscopy experiments were carried out to obtain evidence of the ER origin of the \(Ca^{2+}\) involved in the increase in \([Ca^{2+}]\), induced by a hypo-osmolarity shock. Since from the above experiments with FCCP, the mitochondria do not seem to be a source of \(Ca^{2+}\), during a hypo-osmotic shock, and to minimize the presence of a Rhod-2 signal coming from the mitochondria, the subsequent experiments were carried out in the presence of 5 µM FCCP. Panel (a) of Fig. 8 shows two images obtained at the same confocal plane (near the middle of the cell) in the Hyper-NaCa medium (image on the left), and after a 300 s exposure to the Hypo-0Na0Ca medium (image on the right). Notice that after this relatively long exposure the cell volume was the same as at the beginning of the experiment, indicating that RVD had already occurred. The image on the left shows that Rhod-2 is internally compartmentalized in clusters, suggesting significant accumulations of \(Ca^{2+}\) within intracellular stores (in the ER). The image on the right shows that exposure to the Hypo-0Na0Ca solution causes a great variation in the distribution of the Rhod-2 fluorescence, especially in the disappearance of the cluster pattern. Fluorescence redistribution is better appreciated in the lower graphs, panel (b) representing three-dimensional plots of the pixel gray-value intensity, represented in pseudocolor, of the two preceding images.

Similar results were obtained with 6 additional cells. In order to quantify the results, we calculated the mean pixel intensity of equivalent confocal images corresponding to different cells in the two experimental conditions. The mean percent decrease in fluorescence in the Hypo-0Na0Ca medium was 35.9 ± 6.1% \((n = 7)\) relative to the control in Hyper-NaCa. If indeed hypotonicity is releasing \(Ca^{2+}\) from ryanodine sensitive \(Ca^{2+}\) stores from the ER, then the Rhod-2 fluorescence cluster pattern in the Hypo-0Na0Ca medium (panel a, right) should not disappear in the presence of a high ryanodine concentration that blocks \(Ca^{2+}\) release from the ER. Panels (c) and (d) show a representative experiment \((n = 12)\), similar to that shown in (a) and (b), but carried out in the presence of 50 µM ryanodine. It is clear that under this condition the Rhod-2 cluster pattern in the ER remains unaltered.

Figure 9 summarizes the results of several experiments in which the magnitude of the increase in \([Ca^{2+}]\),
Na+/Ca2+ Exchange and Ca2+ during Hypotonic Stress in Cerebellar Astrocytes

The Journal of Physiological Sciences
Vol. 58, No. 4, 2008

Fig. 8. Confocal microscopy of Rhod-2 stained astrocytes bathed in Hyper-NaCa (t = 0 s) and Hypo-0Na0Ca media (t = 300 s). The two images in panel (a) were obtained at the same confocal plane in the Hyper-NaCa medium (left image) and 300 s later in the Hypo-0Na0Ca medium, both in the presence of 5 µM FCCP. Panel (b) represents three-dimensional plots of the pixel gray-value-intensity of the two images of panel (a). The red fluorescence shows the distribution and intensity of Rhod-2, which are clearly different in the two media, more highly localized in the hyper than in the hypo medium. Panel (c) shows single confocal images obtained at about the same focal plane under the Hyper-NaCa (t = 0 s) and Hypo-0Na0Ca (t = 300 s) in the presence of 50 µM ryanodine. Notice the persistence of the Rhod-2 cluster patterns in the two osmotic conditions. Panel (d) shows three-dimensional plots of the Rhod-2 fluorescence intensity distribution obtained from the images of panel (c). Observe that in the presence of ryanodine, the peak Rhod-2 fluorescence intensity is much the same under the two osmotic conditions.

Fig. 9. Summary of several experiments on the short-term (<120 s) effect of different osmotic and ionic compositions on [Ca2+], in single cerebellar Type-1 astrocytes. Ordinate: net change in [Ca2+], in nM. Abscissa: labels of the solutions Iso, Hyper, and Hypo with the compositions in Na+ and Ca2+. Notice the lack of effect of hypotonic medium on [Ca2+]i as compared to that of a Hypo-NaCa or Hypo-0Na0Ca. Observe that the large increment in [Ca2+], in the Hypo-0Na0Ca medium is completely inhibited by 50 µM ryanodine.

induced by osmotic changes was determined under different experimental conditions. The figure shows first, that no changes in [Ca2+]i occur when switching from the Iso-NaCa control (300 mosm/l; see Table 1) to the Hyper-NaCa medium (400 mosm/l); second, a small but significant increment in [Ca2+]i occurs when going from a Hyper-NaCa to a Hypo-NaCa medium (230 mosm/l; ∆[Ca2+]i = 45 ± 13 nM [n = 30]); third, a much larger increment occurs going from a Hyper-NaCa to a Hypo-0Na0Ca medium (Δ[Ca2+]i = 270 ± 25 nM, n = 50); and fourth, all increments from Hyper- to Hypo-osmotic conditions were totally abolished in the presence of high ryanodine concentration (50 µM, n = 10).

The above experiments, and in particular those summarized in Fig. 9, strongly suggest that in the presence of extracellular Na+ (a functional Na+/Ca2+ exchanger in its extrusion mode), the exchanger largely buffers the increment in [Ca2+]i induced by hypo-osmotic stress. With the exchanger efficiently pumping out the Ca2+ released from ryanodine-dependent Ca2+ stores, one would expect the stores to be depleted, and this should result in the opening of store-operated calcium channels (SOCC). In agreement with this, Fig 10a shows that if the hypotonic treatment is prolonged for some minutes in a solution containing external Na+ and Ca2+, a progressive increase in [Ca2+], occurs is totally dependent on extracellular Ca2+. Figure 10b shows that this calcium entry during prolonged hypotonic stress is completely blocked by
BTP-2, a specific inhibitor of SOCC [26]. Further confirmation that this calcium entry occurs through the SOCC was obtained in experiments such as that shown in Fig. 11, using either Mn2+ (part a), a divalent cation which permeates the store-operated channels [27] and completely quenches the Fura-2 signal, or 2-APB (part b), another known blocker of the SOCC in cerebellar astrocytes [14, 28], which also blocks Ca2+ entry under prolonged hypo-osmotic shock in Na+- and Ca2+-containing medium. Observe that the rise in [Ca2+] shown in (a) is completely abolished by BTP2.

**Fig. 10.** (a) Effect of a prolonged (minutes) hypo-osmotic stress in the continuous presence of external Na+ and Ca2+ on [Ca2+]. Notice the small increment in [Ca2+] in the Hypo-NaCa in the first 120 s followed by a progressive rapid rise in [Ca2+], which is totally dependent on extracellular Ca2+. (b) Effect of BTP-2 (10 µM), an inhibitor of the SOCC on [Ca2+], during a prolonged hypo-osmotic shock in Na+-and Ca2+-containing medium. Observe that the rise in [Ca2+] shown in (a) is completely abolished by BTP2.

**Fig. 11.** (a) Effect of a prolonged (minutes) hypo-osmotic stress in the continuous presence of external Na+ and Ca2+ on [Ca2+] and its quenching by 10 mM Mn2+. (b) Effect of 2-APB (80 µM), an inhibitor of the SOCC, on [Ca2+] during a prolonged hypo-osmotic shock in Na+- and Ca2+-containing medium. Observe that the rise in [Ca2+] shown in (a) is completely abolished by 2-APB.

**Effect of hyper- and hypotonic treatments on the activity of the Na+/Ca2+ exchanger**

Since much of this work is related to the modulator role of the Na+/Ca2+ exchanger on the intracellular Ca2+ signals induced by hypotonic shocks, we decided to explore if, and how, changes in the tonicity of the external medium altered the activity of this counter transporter. Na+/Ca2+ exchange activity was measured as the rate of increase in intracellular Ca2+ on reversing the Na+ gradient by eliminating extracellular Na+ in the presence of 2 mM Ca2+. We have previously shown that in this preparation, the increase in [Ca2+] upon the removal of extracellular Na+ in the presence of 2 mM reflects the activity of the Na+/Ca2+ exchanger working in its reverse mode, since this effect is completely abolished by the exchange inhibitors KB-R7943 and SEA-0400 [13, 14]. We have therefore used these criteria to measure the activity of the Na+/Ca2+ exchanger under hyper and hypo tonic conditions to prevent any release of calcium from ryanodine-dependent Ca2+ stores induced by hypo-osmotic shock, and to eliminate any calcium-induced calcium release (CICR) in response to Ca2+ entry through the reverse Na+/Ca2+ exchange [14], the experiments were carried out in the presence of a high concentration (50 µM) of ryanodine. Figure 12a shows a representative experiment in eight astrocyte studies in which switching from the hypertonic medium (Hyper-NaCa) to the Hyper-0NaCa medium activates the reverse Na+/Ca2+ exchange, causing a ∆[Ca2+] increase of about 50 nM during a perfusion period of 100 s, which was reversed after the readmission of the Hyper-NaCa medium. In contrast, activation of the reverse Na+/Ca2+ exchange in the hypotonic medium (Hypo-0NaCa) for a similar
Na⁺/Ca²⁺ Exchange and Ca²⁺i during Hypotonic Stress in Cerebellar Astrocytes

The Journal of Physiological Sciences
Vol. 58, No. 4, 2008

Fig. 12. The effect of hyper- and hypotonic shocks on the activity of the Na⁺/Ca exchanger working in its reverse modality (Ca²⁺ entry in exchange for internal Na⁺). (a) The continuous presence of 50 µM ryanodine reversal of the Na⁺ gradient (0NaCa medium) in a hypotonic solution causes a modest increment in [Ca²⁺]. A similar treatment in a hypotonic medium causes a much larger increase in [Ca²⁺], and therefore in exchange activity. (b) Summary of 8 different experiments on the effect of hyper- and hypotonic shocks on the activity of the Na⁺/Ca²⁺ exchange working in its reverse mode (Hyper-0NaCa and Hypo-0NaCa). Ordinate: rate of rise in [Ca²⁺] in nM/s. Black rectangle: in the presence of the hypotonic medium. White rectangles: in the presence of the hypertonic medium. The number in parentheses refers to different astrocytes (n = 8). Values refer to mean ± SE. (c) Control experiment using the Na⁺ sensitive probe SBFI (340/380 nm ratio) to follow changes in intracellular Na⁺ during hyperosmotic- or hypo-osmotic shock. Notice that in our conditions, Hyper-NaCa and Hypo-NaCa, no significant changes in intracellular Na⁺ are observed.

Our previous laboratory work have shown that in rat cerebellar Type-1 astrocytes, the Na⁺/Ca²⁺ exchanger activity is responsible for the resting [Ca²⁺], as well as for the resting Ca²⁺ balance fluxes [13]. Moreover, the intracellular calcium signaling, induced by physiological concentrations of the excitatory amino acids L-glutamate and L-aspartate, is the result of Ca²⁺ entry through the reverse Na⁺/Ca²⁺ exchange, which is greatly amplified by a CICR mechanism involving RyRs and ryanodine sensitive Ca²⁺ stores [14].

In this work we have explored how the activity of the Na⁺/Ca²⁺ exchanger modulates the hypo-osmotic–induced [Ca²⁺], increase observed in Type-I cerebellar astrocytes. Of particular importance was the experimental determination of the minimum level of extracellular Na⁺ that could be present in the test solutions without an activation of Ca²⁺ entry through the reverse Na⁺/Ca²⁺ exchange, which is greatly amplified by a CICR mechanism involving RyRs and ryanodine sensitive Ca²⁺ stores [14].

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DISCUSSION

Astrocyte swelling occurs in different types of brain injuries and is accompanied, among others, by volume regulatory osmolyte fluxes [29], stimulation of glycogen synthesis [30], proliferation and gliosis [31], increases in protein tyrosine nitration [32], and elevation in the intracellular [Ca²⁺] [3, 29]. The Ca²⁺ signaling triggered by astrocyte swelling is thought to impair astrocyte function by effects on gene expression, protein phosphorylation, and intervention in Ca²⁺ signal-mediated receptor function [3].

period (100 s) caused a Δ[Ca²⁺] of 293 nM, or six times larger than that in the hypertonic medium. Figure 10b summarizes the results obtained with eight different cells in which the rate of increase of Δ[Ca²⁺] was measured both in the hyper- and hypotonic media. The rate of increase in [Ca²⁺] upon activation of the reverse Na⁺/Ca²⁺ exchange in the Hypo-0NaCa was 6.5 times higher than in the Hyper-0NaCa media. As a control, Fig. 12c shows that this result is not the consequence of changes in intracellular [Na⁺], which could activate the reverse exchange, since the fluorescence signal from the Na⁺ probe (SBFI) measured at 340/380 ratio was not significantly modified in the hypotonic medium.
Δosmolarity = 70 mosm/l from the iso-osmotic media. It is known that the incubation of brain astrocytes in hypertonic media (405 mosm/l) does not change the [Ca²⁺], and that the maximum peak in [Ca²⁺], occurs at 205 mosm/l ([3], or a Δosmolarity = 200 mosm/kg). Since a change of 70 mosm/l is significantly lower than that reported for maximal [Ca²⁺], response (see Fig. 2, a and b), in the present experiments a larger Δosmolarity (= 170 mosm/l) was achieved by starting the experiment from a hypertonic solution of 400 mosm/kg (see Fig. 2, a and b). Critical evidence that the Na⁺/Ca²⁺ exchanger largely contributes to pumping out the intracellular Ca²⁺ released during a hypotonic shock is the reason why the increase in [Ca²⁺], in response to pulses of hypertonic solution in 0Na0Ca is rapidly reversed when Na⁺ is readmitted in the external medium (see Figs. 2, 3, and 7). Moreover, the progressive decrease of the [Ca²⁺], response during consecutive pulses of hypotonic 0Na0Ca solution indicates that a depletion of intracellular Ca²⁺ stores is involved in this phenomenon.

One could argue that the progressive decrease in the hypotonic intracellular Ca²⁺ signal is related to the time course of the RVD (see Fig. 1b). An argument against this explanation is that after the first hypotonic pulse in Hypo-0Na0Ca, returning to Hypo-NaCa for a period long enough to complete the RVD, a second hypotonic pulse in 0Na0Ca increases [Ca²⁺], to a value close to the first one (experiments not shown). Further evidence that the intracellular Ca²⁺ release by the hypotonic shock is rapidly pumped out by the Na⁺/Ca²⁺ exchanger is the observation that first, the magnitude of the hypotonic-induced [Ca²⁺], increase is substantially smaller in the hypotonic medium containing Na⁺ than that in the absence of external Na⁺ (see Fig. 4a); second, when the exchanger is inhibited by DCCB in the hypotonic medium containing Na⁺, a rapid increase in [Ca²⁺], is observed (see Fig. 4b) as a consequence of blocking the exchanger to a halt; and third, a pharmacological procedure (Tg + 4 CmC + FCCP) intended to release near all the intracellular Ca²⁺ stores, including ER and mitochondria, induces a large rise in [Ca²⁺], if the forward operation of the Na⁺/Ca²⁺ exchange is inhibited by removing external Na⁺. This is in marked contrast with the much smaller [Ca²⁺], increase observed in the presence of a fully operating forward exchange in the presence of external Na⁺ (see Fig. 5, a and b).

In several preparations there is evidence of an intimate association between the Na⁺/Ca²⁺ exchanger and intracellular Ca²⁺ stores [33]. This association has been well established in smooth muscle cells where the exchanger is in close proximity to the sarcoplasmic reticulum (SR), where Ca²⁺ release from the SR through the RyRs is intimately coupled to its extrusion through the Na⁺/Ca²⁺ exchanger. RyRs and ryanodine Ca²⁺ stores have been clearly demonstrated in astrocytes [13, 34–39]. It has been recently shown, using Ca²⁺ imaging confocal microscopy and immunocytochemistry techniques, that in Type-1 rat cerebellar astrocytes, RyRs, ryanodine Ca²⁺ stores, and the plasma membrane Na⁺/Ca²⁺ exchanger are highly colocalized and that Ca²⁺ released by a CICR mechanism is rapidly removed by the forward operation of the Na⁺/Ca²⁺ exchanger [14]. An involvement of ryanodine-sensitive Ca²⁺-induced-Ca²⁺-release has been previously demonstrated in human epithelial cells [40] and in S40 transformed rabbit corneal epithelial cells [41] when exposed to hypotonic medium. The experiments presented in this work show that the intracellular Ca²⁺ released during moderate hypo-osmotic stress comes from ryanodine-sensitive Ca²⁺ stores. The arguments in favor of this proposition are as follows: (i) in the presence of the mitochondrial blocker FCCP, the intracellular Ca²⁺ signal induced by a hypo-osmotic shock is similar to that in the absence of FCCP (see Fig. 6a); (ii) with concentrations of ryanodine sufficient to block RyRs, no effect of hypo-tonicity on [Ca²⁺], is observed. Similar results were obtained with 10 μM ruthenium red, a concentration known to block ryanodine receptors (experiments not shown) [24]; and (iii) Confocal Rhod-2 fluorescence images show a significant decrease of the fluorescence intensities associated with well-defined Ca²⁺ store compartments, upon the induction of a hypotonic shock in the presence of FCCP, and these images also show that this decrease in Rhod-localized fluorescence is prevented by a high [ryanodine] (see Fig. 8). This strongly suggests that the ER is the source of the Ca²⁺ released during a hypo-tonicity shock. It has been reported that in cultured rat cerebral astrocytes, ryanodine does not block the hypo-osmotic–induced Ca²⁺ response [3]. The most plausible explanation for this discrepancy, as compared to the present results, is that the concentration of ryanodine used in those studies (0.5 μM) is too low to completely block the ryanodine-sensitive Ca²⁺ channels [24]. An additional strong argument in favor of the idea that the ER is the source of Ca²⁺ during hypo-osmotic shock is that a prolonged exposure of the astrocyte to a hypotonic medium induces a Ca²⁺ entry from the extracellular medium, which is completely inhibited by two known SOCC inhibitors (BTP2 and 2-APB) and quenched by Mn²⁺. An involvement of ryanodine-sensitive Ca²⁺-induced-Ca²⁺-release has been previously demonstrated in human epithelial cells [40] and S40-transformed rabbit corneal epithelial cells [41] when exposed to hypotonic medium. Although the mechanism by which a hypotonic shock induces a liberation of Ca²⁺ from ryanodine-sensitive Ca²⁺ stores is unknown, it has been proposed that cytoskeleton alterations during cell volume changes may play a role. In fact, there is evidence of a reorganization of microfilaments following hypotonic cell exposure [42, 43]. It is of interest that changes in the cytoskeleton have been shown to affect the activity of the Na⁺/Ca²⁺ exchanger [27].

An interesting finding reported in this work is the
effect of osmolarity on the activity of the Na"/Ca"+ exchanger. We have previously shown that the Na"/Ca"+ exchanger working in its forward and reverse modes is responsible for most of the flux balance under resting physiological conditions [13]. From the measured values of the resting [Ca"2+], and [Na"+], in this preparation [13], the Na"/Ca"+ exchange equilibrium potential (E_{Na,Ca} = 3E_{Na} – 2E_{Ca}) is close to –69 mV. For a normal resting potential of about –85 mV, the exchanger will favor Ca"2+ extrusion. Therefore calcium entry upon the removal of external Na"+ (see Fig. 12a) should reflect the activity of the Na"/Ca"+ working in its reverse mode. This is also confirmed by the rise in [Ca"2+] in the absence of external Na"+ being completely abolished by KB-R7943, an inhibitor of the reverse exchange [13]. Our independent results confirm recent work showing that in transfected CHO cells, the activity of the Na"/Ca"+ exchanger is sensitive to osmotically-induced volume changes being stimulated in hypotonic media and inhibited in hypertonic media [44]. The direct consequence of this effect for the case of astrocytes is that in hypotonic media the Na"/Ca"+ exchanger will be more active in pumping out intracellular Ca"2+, which is released during the hypotonic shock. The mechanism by which osmolarity changes affect the Na"/Ca"+ exchange activity is unknown. Several observations demonstrate that alterations in cell volume are associated with changes in the amount and organization of actin filaments [42, 45]. Changes in F-actin were seen as early as 1 min after osmotic challenge and were not completely reversed even after full volume recovery [46]. One way in which actin filaments could regulate transporter activity would be by direct interaction in such a way that changes in this interaction might up- or down-regulate the transporter activity [47]. It is also known that hypotonic swelling largely stimulates ascorbate efflux from cerebellar astrocytes, significantly decreasing its intracellular concentration [48]. Furthermore, it has been reported that the activity of the Na"/Ca"+ exchanger is much lower in ascorbate-rich astrocytes than in ascorbate-poor cells [49]. A speculative possibility is that the decrease in [ascorbate] during hypo-osmotic shock may de-inhibit the Na"/Ca"+ exchanger, thus increasing its activity in hypotonic media. This hypothetical mechanism might also contribute to cellular osmo-regulation and the scavenging of reactive oxygen species accumulated in the extracellular medium during ischemia or hypotonic shock.

In summary, the major finding of this work is that in rat cerebellar Type-1 astrocytes, a moderate short-term hypotonic shock causes a release of calcium from ryanodine Ca"2+-dependent stores and that the activity of the Na"/Ca"+ exchanger, in its forward mode, determines the initial magnitude of the rise in [Ca"2+]. The intimate co-localization of RyRs and the Na"/Ca"+ exchanger [14] seems to be essential for avoiding large increases in [Ca"2+], during early hypo-osmosis, which might impair the normal function of the astrocyte. Critical evidence that the hypo-osmotic treatment liberates Ca"2+ from ryanodine operated Ca"2+ stores, which is later pumped out by the operation of the Na"/Ca"+ exchanger, is that prolonged hypotonic stress opens SOCC as a consequence of Ca"2+ depletion in the endoplasmic reticulum. Although in this work we have focused on the effect of short-term hypotonic stress on [Ca"2+], and its origin and the role of the Na"/Ca"+ exchanger in modulating that response, further studies are required to elucidate the long-term effects of hypotonic stress, both on the role of the SOCC in ER refilling and on the regulation of the Na"/Ca"+ exchange activity.

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