The Journal of Neuroscience, June 1993, 13(6): 2638-2650

Role of Calcium in Astrocyte Volume Regulation and in the Release of Ions and Amino Acids

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Primary astrocyte cultures exposed to hypotonic media undergo a rapid initial swelling followed by a regulatory volume decrease (RVD), which is associated with the release of ions and amino acids. The Ca^{2+} dependence of RVD was investigated. Using a method that measures extracellular electrical resistance to measure cell volume changes in substratum-attached cells, we found that when astrocytes were exposed to hypotonic media without calcium, RVD was abolished. The addition of $CaCl_2$ to astrocytes swollen in hypotonic media without calcium caused an almost immediate initiation of volume regulation, with an EC_{50} of approximately 0.1 mM $CaCl_2$. Swelling of astrocytes in hypotonic medium caused an increased influx of $^{45}Ca^{2+}$, which was partially blocked (60%) by 1 μM nimodipine, suggesting that voltage-gated L-type calcium channels were being opened. Previous work had shown that hypotonic media-induced swelling of astrocytes caused membrane potential depolarizations sufficient to open such channels (Kimelberg and O'Connor, 1988). By measuring intracellular free calcium with fura-2, we found that astrocytes swollen in hypotonic medium showed a rapid increase in $[Ca^{2+}]_i$, reaching a peak of approximately 600 nM, followed by a decrease to a sustained plateau (approximately 250 nM) mirroring the time course of volume regulation. The removal of extracellular calcium totally abolished, and the addition of 1 μM nimodipine partially abolished the elevated plateau, while neither affected the initial $[Ca^{2+}]_i$ peak. These data suggest that the initial peak of the hypotonic-induced rise in $[Ca^{2+}]_i$ is caused by release from intracellular stores and that the sustained elevated plateau is due to extracellular calcium influx.

The removal of extracellular calcium also abolished swelling-induced K^+(^{86}Rb) and $^{36}Cl^-$ efflux, but did not affect the swelling-induced release of ^{3}H-D-aspartate, or ^{3}H-taurine (data not shown). These data indicate that hypotonic-induced aspartate and taurine release is not necessary for RVD in astrocytes swollen by exposure to hypotonic media, since RVD is completely inhibited by the omission of external Ca^{2+}. The addition of 1 mM quinine HCl, which is known to block Ca^{2+}-activated K^+ channels, also abolished both volume regulation and $^{86}Rb^+$ efflux in hypotonic media-swollen astrocytes in the presence of medium calcium, but did not affect ^{3}H-D-aspartate efflux. We suggest that the swelling of astrocytes in hypotonic media which leads to a rapid membrane depolarization first opens voltage-gated calcium channels. Extracellular Ca^{2+} then enters the cell, leading to a sustained increase in intracellular free calcium ($[Ca^{2+}]_i$), triggering activation of Ca^{2+}-dependent ion channels and the release of K^+ and Cl^- followed by osmotically obligated water, thus leading to RVD. Although intimately associated with this process, swelling-induced release of amino acids, because of its independence of extracellular Ca^{2+}, does not seem to be involved in RVD.

[Key words: cell swelling, volume regulation, regulatory volume decrease, volume measurements, astrocytes, monolayer cultures, extracellular impedance, fura-2, intracellular calcium, potassium, chloride, aspartate]

Most vertebrate cells, when swollen by exposure to hypotonic media, are capable of returning to near normal volumes by a process known as regulatory volume decrease (RVD) (for recent reviews, see Chamberlin and Strange, 1989; Hoffmann and Simonsen, 1989; Grinstein and Foskett, 1990; Hoffmann and Kolb, 1991). RVD is achieved by the release of intracellular osmolytes (principally K^+ and Cl^-, and/or amino acids), causing osmotically obligated water to follow and allowing the cell to return toward its original volume. As in other vertebrate cells, mammalian glial cells respond to exposure to hypotonic media by rapidly swelling and then undergoing RVD, as first described by Kimelberg and Frangakis (1985) and Olson et al. (1986) for primary astrocyte monolayer cultures and for C_6 glioma cells by Kempski et al. (1983). For recent reviews of cell volume regulation in astrocytes and other cells in the mammalian nervous system, see Kimelberg (1991) and Ballanyi and Grafe (1988).

Understanding the intracellular signals that initiate and/or control RVD is of considerable interest, and both extracellular and intracellular Ca^{2+} has been shown to influence cell volume regulation in a number of cell types (Foskett and Spring, 1985; Cala et al., 1986; Eveloff and Warnock, 1987; Foskett and Melvin, 1989; McCarty and O'Neil, 1990; Pierce and Politis, 1990). As stated above, most vertebrate cell types accomplish volume regulation from hyposmotic stress by an efflux of K^+ and Cl^-. In some cases removal of extracellular Ca^{2+} blocks volume recovery by preventing KCl efflux (Davis and Finn, 1987). In other instances, changes in $[Ca^{2+}]_i$ alter volume recovery following hyposmotic stress by altering the pattern of osmolyte efflux (Grinstein et al., 1982; Cala et al., 1986). In some cell

Received Aug. 27, 1992; revised Dec. 7, 1992; accepted Dec. 26, 1992.

This work was supported by Grant NS 23750 to H.K.K, and Small Instrumentation Grant HL44035 ROE awarded to Albany Medical College for purchase of the Deltascan. We thank J. Albrecht and M. Aschner for critical reading of the manuscript.

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types loss of KCl is via separate K^+ and Cl conductances (Grinstein et al., 1982; Sarkadi et al., 1984; Hoffmann et al., 1986; Eveloff and Warnock, 1987; Hazama and Okada, 1988; Rothstein and Mack, 1990), while in others the K^+ movement is totally Cl^- dependent, suggesting the involvement of a coupled KCl system (Hoffmann et al., 1984; Pierce and Politis, 1990). Further evidence that Ca^{2+} affects K^+ or Cl^- channels is that quinine, a drug that inhibits Ca^{2+}-dependent K^+ transport and swelling-activated K^+ and Cl^- channels, blocks or reduces cell volume recovery (Grinstein et al., 1982; Foskett and Spring, 1985; Hoffmann et al., 1986; Hazama and Okada, 1988; Pierce and Politis, 1990; Banderali and Roy, 1992).

Examination of swelling-induced release of K^+, Cl^-, and amino acids such as glutamate, aspartate, and taurine in astrocytes is potentially relevant to many pathological states such as head trauma or stroke in the CNS where astrocytic swelling is an early and dominant event (Kimelberg, 1992). Clearance of K^+ has long been viewed as a major astrocyte function (Sykova, 1983; Walz, 1989), and increased extracellular levels of excitatory amino acids is thought to be one of the underlying problems in ischemic cerebral damage (Choi, 1988). Also, extracellular Ca^{2+} levels are known to fall during neuronal excitation and in a number of pathological states (Kimelberg and Ransom, 1986; Sykova, 1991), and some of this decrease in Ca^{2+} may be due to uptake into swollen astrocytes.

While efflux of amino acids has been shown to be associated with astrocytic swelling *in vitro* (Pasantes-Morales and Schousboe, 1988, 1989; Kimelberg et al., 1990b; Martin et al., 1990; Pasantes-Morales et al., 1990), its contribution to RVD has not been defined. Thus, for this reason and for its relevance to neurotransmitter release in general, we considered it important to determine the role of Ca^{2+} in both amino acid and KCl release and RVD. The present study thus had the following objectives: (1) to explore the involvement of calcium in hypotonic-induced astrocytic swelling and RVD, (2) to characterize the routes of swelling-induced calcium entry, (3) to determine if changes in $[Ca^{2+}]_i$ acts as an intracellular signal for volume regulation, and (4) to determine what contributions the release of K^+, Cl^-, and the amino acids aspartate and taurine make to volume regulation in primary astrocyte cultures.

Parts of these data have been presented in abstract form (O'Connor and Kimelberg, 1991).

Materials and Methods

Materials. All cell culture materials were from GIBCO (Grand Island, NY). Other chemicals were from Sigma (St. Louis, MO). All radiolabeled ions and amino acids were obtained from Amersham Corporation (Arlington Heights, IL). Fura 2/AM was obtained from Molecular Probes (Eugene, OR).

Astrocyte cultures. Primary astrocyte cultures were prepared from the cerebral cortex of neonatal rats after dissociation with a neutral protease as previously described by Frangakis and Kimelberg (1984), and grown in multi-well trays, on glass coverslips or on cell support film for the perfusion studies (Bellco Biotechnology, Inc., Vineland, NJ). Cultures were used upon reaching confluency, usually after 3–4 weeks, and showed ≥95% staining for the astrocytic-specific marker glial fibrillary acidic protein.

The control bathing medium for all experiments, unless otherwise noted, consisted of 122 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 10 mM D-(+)-glucose, and either 25 mM $NaHCO_3$ or HEPES. This isotonic media was maintained at pH 7.4 by bubbling with 5% CO_2 for the bicarbonate-buffered solution, or addition of NaOH to the HEPES-buffered solution. Hyposmotic media were made by removing specific amounts of NaCl from the initial recipe.

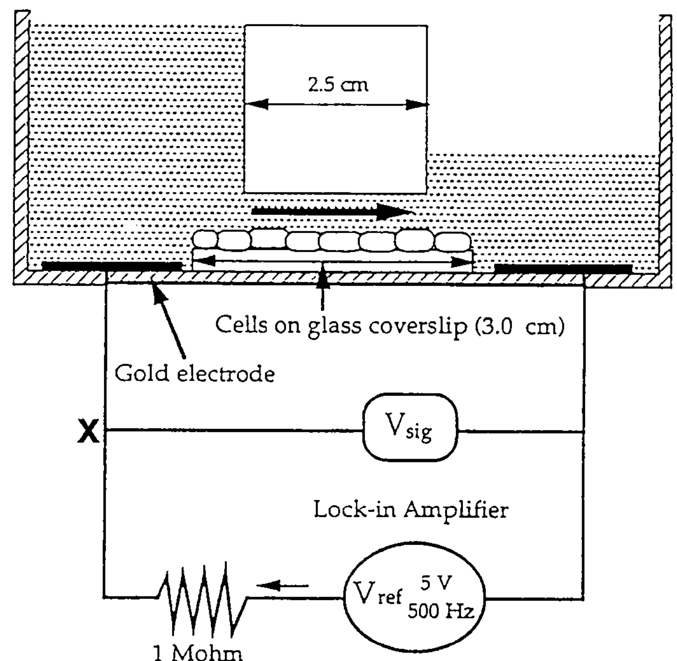

Figure 1. Schematic of the chamber used for electrical resistance measurements of volume changes in substratum-attached astrocytes. The lock-in amplifier both supplies a reference voltage and measures changes in the signal voltage at point *X*, thus acting as a voltage divider. The system is set up with a large external resistance (1 MΩ) much greater than the chamber resistance so that the current *i* is constant. The height of the fluid-filled channel above the cells is approximately 100 μm. Dimensions are not to scale (see Measurement of cell volume for details).

The osmolarity of all solutions was checked using a vapor pressure osmometer (Westcor Inc.).

Measurement of cell volume. A complete description detailing this method can be found in O'Connor et al. (1993), and this and other methods for measuring cell volume are reviewed in Kimelberg et al. (1992). Briefly, in this method cells are placed in a confined channel containing a salt solution and the electrical resistance of the channel measured using an applied alternating current (AC) (see Fig. 1). If the volume of the cells increases, then the volume of the solution within the channel available for current conduction decreases by the same amount, and consequently the measured resistance increases. This method allows for continuous measurements of volume changes in substratum-attached cultures. Gold-plated, low-impedance, inert electrodes are secured to the bottoms of two chambers, separated by the cell channel, with a dab of silicone grease. Leads from the gold electrodes, made of insulated copper wire, are soldered to the gold with pure indium and the connections covered in wax. The gold electrodes are connected through a large resistor (1 MΩ) to a lock-in amplifier (5301, EG&G Princeton Applied Research, Princeton, NJ) and a 500 Hz, 5 V signal is supplied to the system. The lock-in amplifier is used because it is able to resolve small voltage changes with high noise rejection. Apart from the solution in the channel, the two chambers are insulated from each other (Fig. 1).

The isosmotic, control bathing medium for the electrical resistance measurements consisted of 72 mM NaCl, 100 mM sucrose, 3.3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 10 mM D-(+)-glucose, and 25 mM HEPES, adjusted to pH 7.4 by addition of 1*N* NaOH. Hyposmotic media were made by removing sucrose. The replacement of part of the NaCl with sucrose had the important feature of maintaining the same electrolyte concentration in the respective iso- and hyposmotic buffers, resulting in almost identical resistivities for these two solutions. However, a small correction had to be made for a small decrease in solution conductance due to sucrose, which was corrected for by adding a small amount of water (see below). It is clearly critical to balance the test solutions to the same resistivity so that the resistance differences measured when the solutions are changed is only due to changes in cell volume. The resistivities of solutions were checked by placing two gold electrodes (described below) 1 cm apart and measuring the resistivity

directly with a 5 V, 500 Hz AC signal. The resistivities of paired isosmotic and hyposmotic solutions were then balanced to within 0.5% by adding small amounts of distilled water or NaCl.

To begin an experiment, a #1 coverslip (3.0 cm × 1.4 cm) on which a confluent monolayer of astrocytes was growing was placed in the channel and continuously perfused with isotonic solution at 25°C, the flow being driven by the height difference of solutions in the neighboring chambers (Fig. 1). An initial resistance was measured and normalized to 1.0, and subsequent changes in resistance were represented as a percentage change from the initial value. The chamber was designed to have a height of approximately 100 μm above the cells. This becomes convenient because, since we measure the percentage change in resistance, a 1% change in the measurement translates approximately directly to a 1 μm change in the average cell height of the monolayer. This linear relationship of normalized resistance to cell height only applies over a two- to threefold change in cell volume. To exchange solutions the isotonic buffer was 99% removed and replaced with an equal amount of the challenge buffer, with 90% of this amount being placed on one side of the channel and 10% on the other. It took approximately 3–5 sec for the new solution to bathe the cells completely. At the end of each experimental series, the coverslip was viewed under a microscope to confirm the astrocyte monolayer was intact, and the cell viability was assessed by trypan blue exclusion (0.08%; GIBCO).

$^{45}Ca^{2+}$ uptake studies. For these experiments, cells were grown in 12-well trays (Costar), the growth media aspirated off and the cells washed three times with the balanced salt solution (see above). The cells were then incubated in this medium for 30 min at 37°C. These media were then aspirated and 0.5 ml of the same isotonic or a hypotonic solution, warmed to 37°C and containing 0.5 μCi of ^{45}Ca (S.A., 2 Ci/μl; Amersham), was added to each well. The cells were then returned to the incubator. At the desired times this medium was aspirated from each well and each well was washed four times with 1 ml per wash of ice-cold 0.29 M mannitol solution, also containing 10 mM Tris nitrate and 0.5 mM $Ca(NO_3)_2$, pH 7.4 (Kimelberg et al., 1990b). The omission of Na^+ from the washing fluid prevents efflux of Ca^{2+} during the washing procedure by Na^+/Ca^{2+} exchange (due to lack of Na^+) and by ATP-dependent extrusion of Ca^{2+} (due to lowering of the temperature). A fifth wash with the above mannitol wash plus 1 mM EGTA was performed to remove any surface-bound calcium (Walz and Wilson, 1986). The cell monolayer from each well was then solubilized in 1 ml of 1*N* NaOH at room temperature for 20 min and cell protein was determined using the Pierce bicinchoninic acid reagent as modified for use in monolayer cell cultures (Goldschmidt and Kimelberg, 1989). $^{45}Ca^{2+}$ samples were measured by liquid scintillation counting.

Measurement of intracellular calcium. Measurements of $[Ca^{2+}]_i$ were made using the fluorescent calcium chelator fura-2 and a Deltascan I System for dual-wavelength microspectrofluorimetry (Photon Technology International Inc., Princeton, NJ) attached to an inverted Nikon Diaphot microscope. Astrocytes grown on 13.8 × 30 mm^2 glass coverslips were loaded with fura-2 by incubating the cells with 5 μM fura-2/AM for 30 min at 37°C in serum-free minimal essential medium. The astrocytes were then incubated in isotonic salt buffer (see above) without fura-2/AM for 30 min at 37°C to ensure complete hydrolysis of the ester. Fluorescent measurements were made at excitation wavelengths of 340 and 380 nm with the emission wavelength set at 500 nm. Cytosolic free calcium was determined as previously described by Cobbold and Rink (1987).

Efflux studies. Efflux studies were performed using a continuous perfusion method essentially the same as that described by Shain and Martin (1984), using cells grown to confluency on plastic cell support film. The cells were loaded, usually overnight, in growth media with 0.4 μCi of D-[2,3-^{3}H]-aspartic acid (S.A., 26 Ci/mmol) plus unlabeled D-aspartate for a final concentration of 100 μM aspartate, or 0.4 μCi of [1,2-^{3}H]-taurine (S.A., 35 Ci/mmol) plus unlabeled taurine for a final concentration of 50 μM taurine, or for 2 hr with 40–100 μCi of $^{86}Rb^+$ (for K^+) or 40–100 μCi of $^{36}Cl^-$. ^{3}H-D-aspartate was used as a nonmetabolizable analog of L-glutamate or L-aspartate. The support film was then carefully rolled into a cylinder, cells facing inward, and inserted into a 1 ml plastic syringe and continuously perfused with 37°C buffer using a gravity-driven system at a rate of 1 ml/min, and fractions were collected every minute. Four milliliters of Ecoscint (National Diagnostics, Manville, NJ) were added to each 1 ml fraction, which were then counted in a Packard Tri-Carb 1900TR Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT). Recent work in our laboratory has shown that after labeling the cells with both ^{51}Cr (chromium as $Na^{51}CrO_4$) and ^{3}H-D-aspartate, exposure to hypotonic medium causes increased loss of ^{3}H-D-aspartate but does not cause the appearance of ^{51}Cr in the effluent. This further supports the view that we are observing true efflux of ions and amino acids and that the released label is not due to loss or lysis of cells (H. K. Kimelberg, D. J. Bonville, and S. K Goderie, unpublished observations). Efflux of ions or amino acids was expressed as fractional release of the radioactivity in the cells at each time point. A computer program developed in our laboratories calculated the percentage efflux by adding back the effluxed radioactivity to each point, including the final radioactivity counted in the cell support film, thus correcting for loss of label in the cells over time.

Results

Effect of Ca^{2+} on RVD. The volume measurement experiments were done using the electrical resistance method described above. As noted in Materials and Methods, Figure 1 is a representation of the chamber used to make the volume measurements in the astrocytes. A recorded increase in the resistance means that the volume through the channel above the cells available for current flow has decreased by the same amount that the volume of the cell monolayer has increased. If the cells shrink or volume regulate after swelling, this would be recorded as a decrease in resistance. The upper trace in Figure 2*A* is an example of the response of astrocytes seen upon exposure to hyposmotic media (minus 100 mM sucrose; see caption and Materials and Methods). The astrocytes initially swell, as shown by the increase in normalized resistance, and reach a peak of swelling at approximately 3 min. The cells then begin to volume regulate (RVD) back to their initial volume (as shown by the subsequent decrease in resistance). Since the height above the resting astrocytes is approximately 100 μm, a 1% change in resistance would equal a 1 μm change in cell height, as described in Materials and Methods. The resting height of the astrocyte monolayer is normally 4–5 μm, as determined by this method (after wiping off the cells and remeasuring the resistance), and is also supported by the work of Parsons et al. (1989) using high-voltage electron microscopy and Shain et al. (1992) using confocal microscopy on primary astrocyte monolayers. The 3.5% increase in resistance would thus indicate an approximate 3.5 μm change in average cell monolayer height, or approximately a 70% increase in volume if cell volume is taken to approximate height (O'Connor et al., 1993). The bottom trace is the control response of astrocytes upon exposure to isotonic media.

In Figure 2*B*, astrocytes were exposed to hypotonic media that did not contain calcium throughout (as indicated) and it can be seen that the removal of extracellular calcium abolished volume regulation over the first 9 min. The addition of 1 mM quinine HCl to hypotonic media in the presence of calcium (lower nonregulating trace) also abolished volume regulation. Figure 2*B* also shows that the subsequent addition of 1 mM $CaCl_2$ to swollen astrocytes in calcium-free hypotonic media (indicated by arrowhead, upper trace) caused an almost immediate initiation of volume regulation. We have found in parallel experiments that the small final concentration of calcium and the small volume (2 μl) of concentrated calcium chloride (1 M) added to the hypotonic solution did not contribute to the decrease in measured resistance. Also, the addition of 2 μl of 1 M $MgCl_2$ or 1 M NaCl did not cause the initiation of volume regulation (data not shown). The addition of 1 μM nimodipine to hypotonic medium with calcium blocked RVD by approximately 75% (data not shown).

Figure 3*A* shows the dose dependency of astrocyte volume regulation on added calcium. As in the experiment shown in

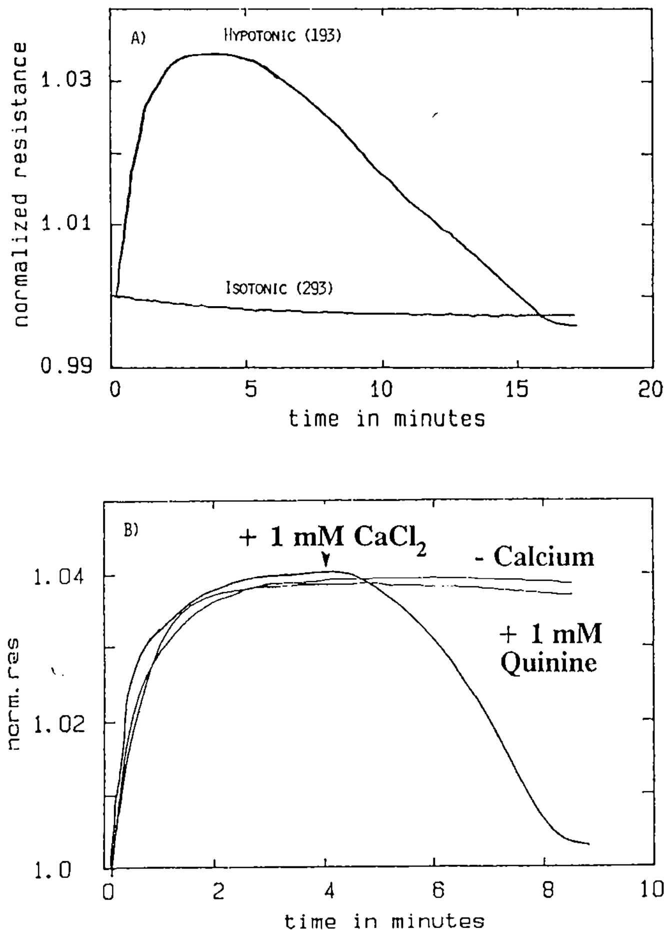

Figure 2. *A*, Volume changes of astrocytes, represented by the percentage change in normalized resistance, upon exposure to a hypotonic solution. Hypotonic solutions were made by removal of 100 mM sucrose from a 293 mOsm/kg solution in which 50 mM NaCl had been replaced by 100 mM sucrose. See Materials and Methods for further details. The system was first balanced in isotonic solution and the recorded resistance normalized to 1.0. The solution was replaced with hypotonic media (or the same media for the isotonic trace) and resistance changes continuously recorded. In the *bottom trace* astrocytes were exposed to isotonic buffer (293 mOsm/kg), showing that the background noise of the system was quite low and stable. When astrocytes were exposed to a hypotonic solution (193 mOsm/kg; *upper trace*) there was an increase in resistance indicating an increase in cell volume. The resistance peaked between 2 and 3 min with a return toward normal volume within 25 min, thus showing RVD. As described in the text (see Materials and Methods), the changes in normalized resistance are related to changes in average volume and also the height of the cell monolayer (O'Connor et al., 1993). For example, in the *top trace*, the 3.5% change in resistance corresponds approximately to a 3.5 μm change in cell height or a 70% increase in volume (see Effect of Ca^{2+} on RVD). *B*, Ca^{2+} dependency of astrocyte volume regulation as measured by the electrical resistance method. The removal of extracellular calcium (*− Calcium*) from hypotonic solution abolished volume regulation over the first 9 min. The addition of 1 mM quinine to the hypotonic solution with calcium also abolished RVD. In the *uppermost trace* astrocytes were exposed to hypotonic solution minus calcium, and at time = 4 min, 1 mM $CaCl_2$ was added as indicated, causing an almost immediate initiation of complete volume regulation.

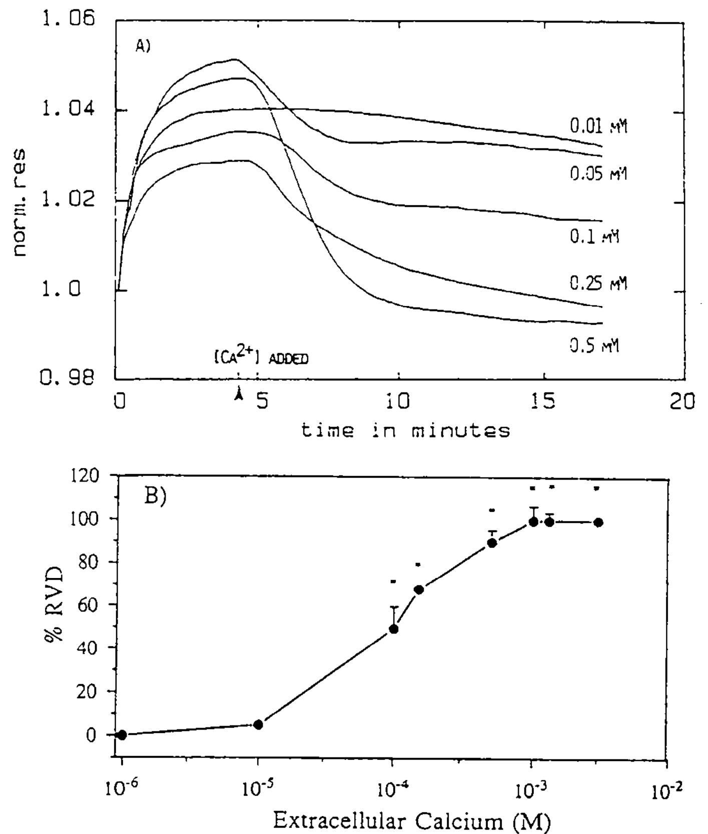

Figure 3. *A*, Dose dependency of RVD on extracellular calcium. Astrocytes were exposed to hypotonic solution minus calcium, and at time = 4 min varying concentrations of calcium were added to the external media. The addition of 0.1 mM $CaCl_2$ resulted in approximately a 50% return toward control volume while concentrations of >0.1 $CaCl_2$ resulted in full volume recovery. These responses were from the same coverslip of cells and between each hypotonic exposure there was a 15–20 min reexposure to normal isosmotic solution. The order of the experiments was as follows: 0.5, 0.1, 0.05, 0.01, 0.25 mM Ca^{2+}. *B* is a plot of the data from the experiments in *A*. The ordinate expresses the %RVD from peak volume to the resting plateau after volume regulation at approximately 15–20 min, versus added $[Ca^{2+}]_e$. The $[Ca^{2+}]_e$ that resulted in 50% volume regulation was approximately 100 μM. Each point shown is the mean ± SD from 2–10 experiments. *, $p < 0.05$.

Figure 2*B*, astrocytes were exposed to hypotonic media without calcium, and at time = 4 min varying concentrations of Ca^{2+} were added as indicated. All of these responses were from the same coverslip. Since this figure comprises successive experiments on the same coverslip, the differences in the initial peak swelling are likely due to the cells not having completely regained their intracellular level of osmolytes when the cells are placed back into isosmotic solution between successive exposures to hypotonic solutions (see caption for order of exposures). The addition of 0.1 mM $CaCl_2$ resulted in approximately a 50% return toward preswelling volumes while concentrations of >0.1 mM $CaCl_2$ resulted in full volume recovery. For these experiments, calcium stock solutions were made so that the same 2 μl sample size was added to the chamber to minimize the addition of water to the hypotonic solution, as this would affect its resistivity. Figure 3*B* is a graph of the dose dependency of astrocyte volume regulation on extracellular calcium. As can be seen the EC_{50} for Ca^{2+} was approximately 100 μM.

$^{45}Ca^{2+}$ uptake studies. Since RVD is dependent on extracellular calcium, we thought it likely that swelling causes increased calcium influx into the cell. Therefore, the effects of cell swelling on calcium influx were studied using $^{45}Ca^{2+}$. Figure 4*A* shows the time course for uptake of $^{45}Ca^{2+}$ by astrocyte cultures when exposed to hypotonic media, measured over 40 min. There is a delayed increase in $^{45}Ca^{2+}$ uptake after exposure to hypotonic medium. This technique measures net unidirectional influx of

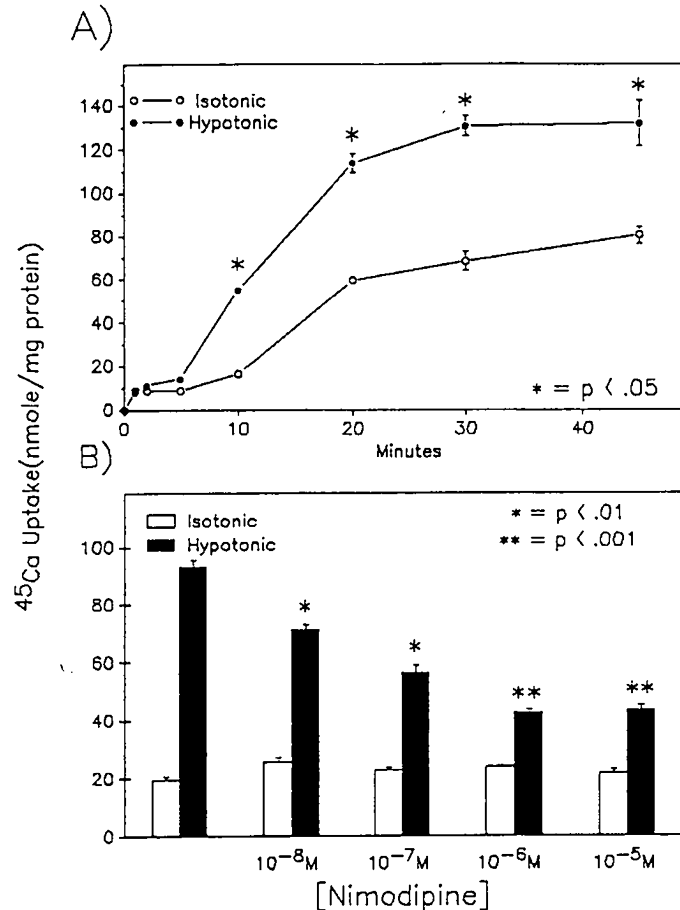

Figure 4. *A*, Time course of uptake of $^{45}Ca^{2+}$ into primary astrocyte cultures in isotonic and hypotonic media. The cells were exposed to media containing 0.5 μCi of $^{45}Ca^{2+}$ and then rapidly washed at the times shown. The cellular contents of $^{45}Ca^{2+}$ and cell protein were determined as described in Materials and Methods. As can be seen, the total $^{45}Ca^{2+}$ uptake significantly increased when astrocytes were exposed to hypotonic media (removal of 50 mM NaCl), as compared to isotonic controls. Student's *t* test analysis shows a statistical significance in $^{45}Ca^{2+}$ uptake in hypotonic versus isotonic media at all points including and past 10 min. *B* shows the effect of the voltage-sensitive calcium channel blocker nimodipine on $^{45}Ca^{2+}$ uptake in astrocyte cultures exposed to hypotonic media (minus 50 mM NaCl) measured after 20 min. Maximal blockage of $^{45}Ca^{2+}$ uptake occurred with 1 μM nimodipine (approximately 60%). Student's *t* test was used to indicate statistical significance. $n = 4$ wells ± SEM for data in both panels.

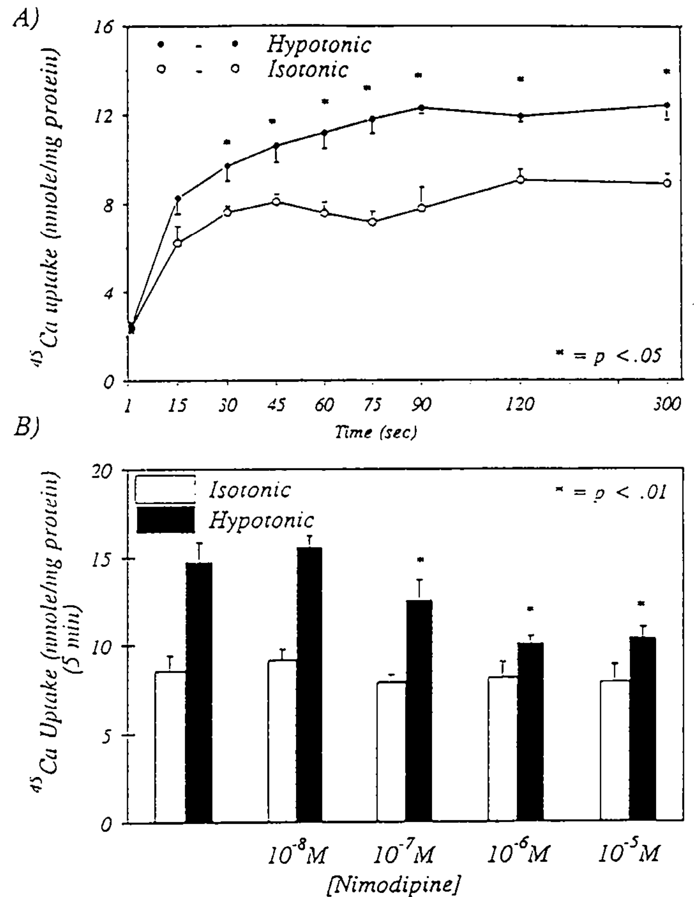

Figure 5. *A*, Swelling-induced increase in $^{45}Ca^{2+}$ uptake measured over 5 min. *B*, Effect of nimodipine on $^{45}Ca^{2+}$ uptake (nmol/mg protein) at 5 min. Maximal inhibition, approximately 66%, occurred with 1 μM nimodipine. Student's *t* test was used to indicate statistical significance. $n = 4$ wells ± SEM for data in both panels.

the tracer, and it is thought that the initial rise in $^{45}Ca^{2+}$ uptake is due to binding to the cell surface, the plateau represents entry of $^{45}Ca^{2+}$ into the cytosol, and the slow rise represents sequestration into the intracellular stores (Holtz et al., 1982; Meldolesi et al., 1988) (see Discussion).

Since an increase in $^{45}Ca^{2+}$ uptake during swelling was observed, the possible route(s) of entry for calcium into the cell was examined. Previous electrophysiology experiments had shown that swelling of the astrocytes caused a marked depolarization (Kimelberg and O'Connor, 1988), and a voltage-sensitive Ca^{2+} channel, resembling the L-type channel, has been described in cortical astrocytes (MacVicar, 1984; Barres et al., 1988, 1990; MacVicar and Tse, 1988; Albrecht and Lazarewicz, 1990). Therefore, the effects of voltage-sensitive Ca^{2+} channel blockers on the swelling-induced increase in $^{45}Ca^{2+}$ uptake were examined. From the data shown in Figure 4*B*, it can be seen that $^{45}Ca^{2+}$ uptake in hypotonic media over 20 min was partially blocked by increasing doses of nimodipine with a maximum block at 1 μM (approximately 60%). Nimodipine proved to be the most effective blocker while 1 μM nifedipine and 10 μM verapamil blocked between 40% and 50% of calcium influx (data not shown). This suggests that one route of entry for calcium during swelling is on L-type voltage-sensitive Ca^{2+} channels.

Since, as noted above, the initial plateau seen in our experiments is thought mainly to represent the initial entry of calcium into the cytoplasm, we examined the swelling-induced $^{45}Ca^{2+}$ uptake over the first 5 min in more detail. These data are shown in Figure 5*A*. $^{45}Ca^{2+}$ uptake was significant at all points past 15 sec ($p < 0.05$) and was also blocked approximately 66% by 1 μM nimodipine (Fig. 5*B*). The first 15 sec value probably represents binding of $^{45}Ca^{2+}$ to the cell surface, and therefore should not be affected by nimodipine, as was seen.

Measurement of intracellular free calcium. To complement our $^{45}Ca^{2+}$ uptake studies, and since $^{45}Ca^{2+}$ is likely to enter immediately all the intracellular pools, changes in intracellular free calcium ($[Ca^{2+}]_i$) were measured during swelling and volume regulation using the fluorescent probe fura-2. In these experiments, fura-2-loaded astrocytes were exposed to hypotonic media (minus 50 mM NaCl) and the change in intracellular free calcium recorded. As can be seen from Figure 6*A* the intracellular free calcium signal given by fura-2 rapidly increased during

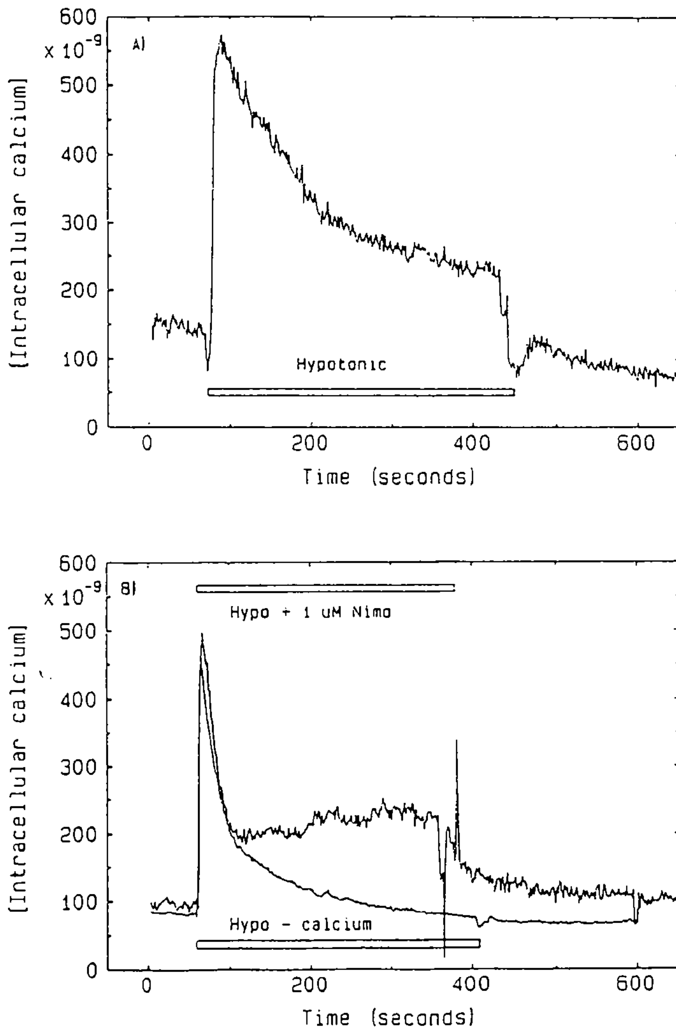

Figure 6. *A*, Effect of swelling of astrocytes on free $[Ca^{2+}]_i$ as measured with fura-2. Primary astrocyte cultures were grown on coverslips and loaded with 2.5 μM fura-2/AM for 30 min. Astrocytes were exposed to hypotonic media (minus 50 mM NaCl) for the period shown by the *open bar*. *B*, Astrocytes exposed to hypotonic media minus calcium (*lower trace*) showed a rapid and transient increase in $[Ca^{2+}]_i$ that quickly returned to baseline levels (within 120 sec) without a maintained plateau. Astrocytes exposed to hypotonic media with 1 μM nimodipine (*upper trace*) also showed a rapid and transient peak of $[Ca^{2+}]_i$ that returned to an intermediate plateau level (see Measurement of intracellular calcium). Each trace is a representative of 7–10 experiments.

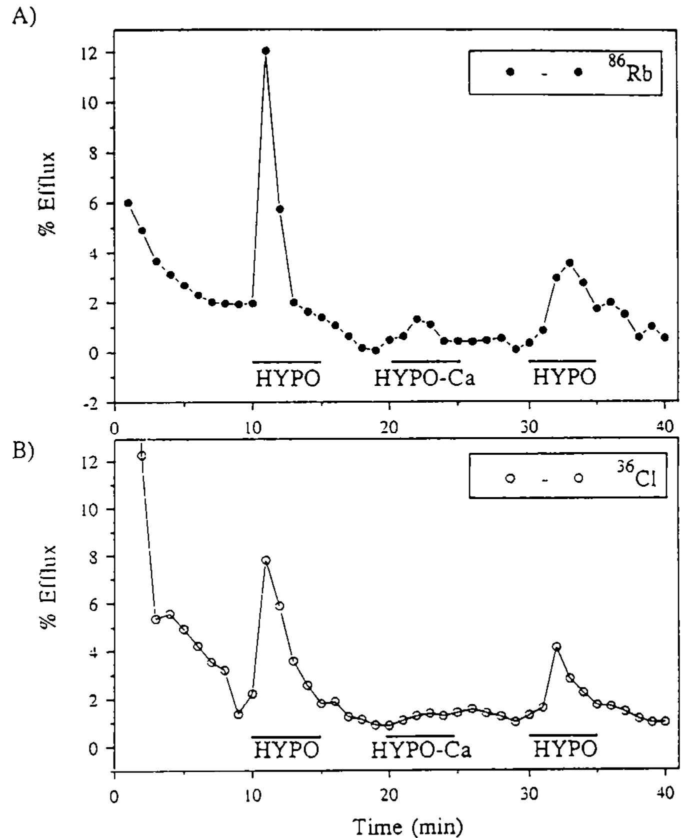

Figure 7. *A*, Hypotonic media–induced release of $^{86}Rb^+$ (a marker for K^+ transport) and $^{36}Cl^-$ (*B*). Astrocytes were loaded for 2 hr with 40 μCi of the appropriate radionuclide and then transferred to a perfusion chamber and perfused with the appropriate buffers at 1 ml/min. The perfusate was collected at 1 min intervals using a fraction collector, and the radioactivity in each sample determined by liquid scintillation counting. The data are expressed as a percentage of the remaining label left in the cell at each time point (see Materials and Methods). Exposure to hypotonic media caused the release of $^{86}Rb^+$ and $^{36}Cl^-$ (*first* and *third exposures*), while removal of extracellular calcium abolished the hypotonic-induced release of $^{86}Rb^+$ and $^{36}Cl^-$ (*middle exposures*).

exposure to hypotonic media, reaching a peak of approximately 580 nM, and then declined with an apparent exponential time course to an elevated plateau of approximately 250 nM. When the cells were returned to isotonic media a rapid return to resting $[Ca^{2+}]_i$ was observed. These results mirror the time course of both RVD (Fig. 2) and the membrane depolarization (Kimelberg and O'Connor, 1988) observed in astrocyte cultures during hypotonic-induced swelling. In Figure 6*B* (lower trace), cells were exposed to hypotonic media minus calcium. They also showed a rapid initial increase in their $[Ca^{2+}]_i$ level, but then rapidly returned to resting $[Ca^{2+}]_i$ levels without a sustained plateau. This finding suggests that the rapid initial increase in $[Ca^{2+}]_i$ results predominantly from swelling-activated release of intracellular Ca^{2+} stores and that the sustained plateau requires entry of extracellular Ca^{2+}. Astrocytes exposed to hypotonic media plus 1 μM nimodipine showed three phases of calcium kinetics (Fig. 6*B*, upper trace): first, a normal transient increase in $[Ca^{2+}]_i$; second, a rapid return toward baseline levels; and a third phase where before $[Ca^{2+}]_i$ reached baseline an increase to a sustained plateau was observed. We had expected to see a smaller sustained plateau with nimodipine since $^{45}Ca^{2+}$ uptake studies showed that nimodipine was capable of blocking 60% of swelling-induced calcium influx. However, the fall-off of the initial peak of $[Ca^{2+}]_i$ was always faster with nimodipine and the area under the curve within the first 50 sec after exposure to hypotonic solution was about one-third that in the absence of nimodipine (seen in five experiments). When control cells were exposed to isotonic media minus calcium (no extracellular calcium present plus 50 μM EGTA to chelate any calcium), or isotonic medium plus 1 μM nimodipine, the basal $[Ca^{2+}]_i$ level did not appreciably change over 5 min (data not shown).

Effects of Ca^{2+} on efflux of K^+ and Cl^-. Astrocytes, like most vertebrate cell types, accomplish volume regulation after hypotonic-induced swelling by releasing K^+ and Cl^- (Kimelberg and Frangakis, 1985). Since the removal of extracellular calcium abolished volume regulation, and volume regulation is accomplished by the release of ions, then it follows that the removal of extracellular calcium should also abolish ion release. We

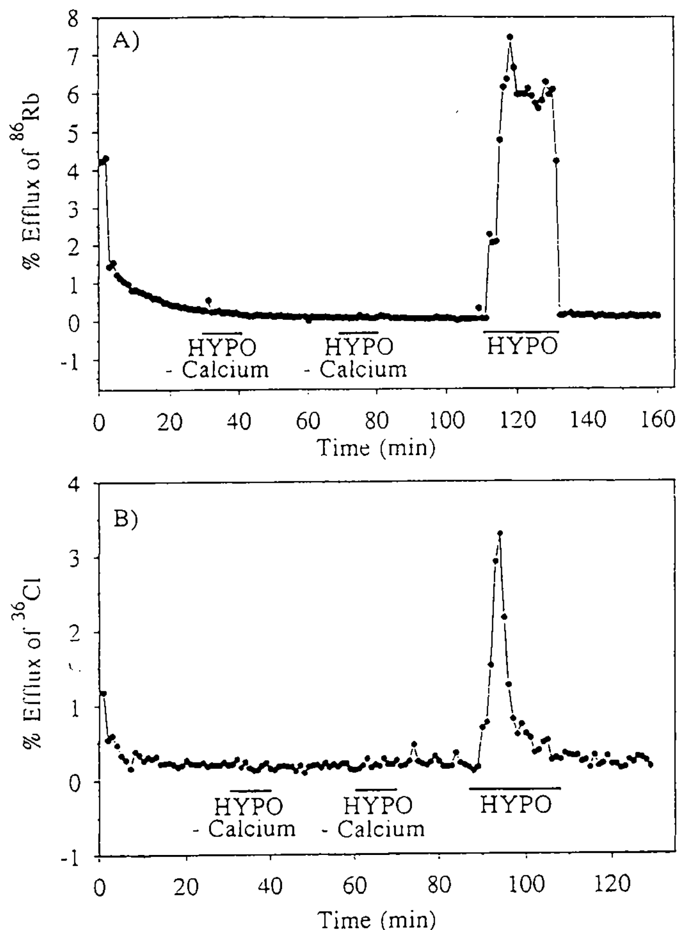

Figure 8. Astrocytes were loaded for 2 hr with 100 μCi of the appropriate radionuclide as in Figure 7: *A*, $^{86}Rb^+$; *B*, $^{36}Cl^-$. Efflux was also measured as in Figure 7 except that the order of addition varied such that the first two exposures were to hypotonic media without Ca^{2+} and were then followed by an exposure to hypotonic medium with normal Ca^{2+}. Cells were exposed to isotonic solutions containing normal Ca^{2+} between the exposures to hypotonic media.

examined the effect of removing extracellular calcium on swelling-induced $^{86}Rb^+$ efflux (using $^{86}Rb^+$ as a tracer for K^+ transport) and $^{36}Cl^-$ efflux. Astrocytes were loaded for 2 hr with 40 μCi of $^{86}Rb^+$ or $^{36}Cl^-$. Figure 7 shows that removal of extracellular calcium abolished the swelling-induced $^{86}Rb^+$ (*A*) and $^{36}Cl^-$ (*B*) efflux (middle responses). Calcium-dependent K^+ efflux may thus occur through calcium-activated potassium channels that are known to exist in astrocytes (Quandt and MacVicar, 1986) and calcium-dependent chloride efflux through calcium-activated Cl^- channels that have been seen in other cell types (Hoffmann et al., 1984; Pierce and Politis, 1990). The reason for the smaller third responses compared to the first response is unlikely to be due to loss of intracellular $^{86}Rb^+$ or $^{36}Cl^-$ since the efflux is expressed as a percentage of the amount left at each time point, which should correct for the progressively decreasing specific activity of $^{86}Rb^+$ and $^{36}Cl^-$ inside the cell. One possibility is that there may be a "desensitization" of K^+ channels or their regulatory processes for the third response, a residual compartmentation for K^+ and Cl^- that is relatively insensitive to hypotonic-induced release, or that subsequent exposures cause less swelling due to incomplete reaccumulation of the solutes lost during RVD. We thus employed a different order of addition, as shown in Figure 8: exposing the cells preloaded with $^{86}Rb^+$ or $^{36}Cl^-$ to Ca^{2+}-free media first. For these experiments astrocytes were loaded for 2 hr with increased radioactivity, namely, 100 μCi of $^{86}Rb^+$ or $^{36}Cl^-$ to increase the number of counts in the cells. As can be seen, no increased efflux of either $^{86}Rb^+$ or $^{36}Cl^-$ was observed when astrocytes were first exposed for two 10 min periods to hypotonic media minus Ca^{2+} buffer, between exposures to Ca^{2+}-containing isotonic medium. A large efflux was, however, observed upon a third exposure to hypotonic media with normal extracellular calcium present.

Effects of Ca^{2+} on efflux of aspartate and taurine. Primary astrocyte cultures swollen by exposing them to hypotonic media release both preloaded and endogenous glutamate, aspartate, and taurine (Pasantes-Morales and Schousboe, 1988; Kimelberg et al., 1990b; Pasantes-Morales et al., 1990). We therefore wished to determine how such release was also affected by the removal of medium calcium. Figure 9*A* shows efflux of preloaded ^{3}H-D-aspartate (a nonmetabolizable analog of glutamate) in response to successive exposures to hypotonic media (minus 50 mM NaCl). When astrocytes were exposed to hypotonic media, they initially showed a peak release of approximately 5–8% of their labeled aspartate pool. Subsequent hypotonic challenges consistently showed a decrease in this peak release. The reasons behind this apparent desensitization have not yet been explored (see comments on $^{86}Rb^+$ and $^{36}Cl^-$ efflux above). However, unlike the efflux of $^{86}Rb^+$ and $^{36}Cl^-$, the removal of extracellular calcium (third response) had no effect on the release of ^{3}H-D-aspartate. Since omission of calcium results only in a nominal calcium-free buffer, 2 mM EGTA was added to the calcium-free buffers to chelate any extracellular calcium present, and the effect of this type of solution is shown in Figure 9*B*. The presence of EGTA had no obvious effect on the hypotonic media–induced release, but there was a slow rise in the baseline efflux of ^{3}H-D-aspartate when the cells were exposed to isotonic media without Ca^{2+} plus EGTA (third exposure). We have also seen a slow increase in cell volume as measured by the impedance method when the astrocytes were exposed to isotonic media without Ca^{2+}, which further increased when EGTA was also present (data not shown). This finding supports the work by Olson et al. (1990), who showed that astrocyte cell volume was increased approximately 16% after exposure to isosmotic buffer containing Ca^{2+} but additionally EDTA, and the work of Martin et al. (1989), who showed increased release of ^{3}H-taurine in isosmotic Ca^{2+}-free EGTA-containing medium. One further possibility was that the increase in $[Ca^{2+}]_i$ observed using fura-2 during hypotonic exposure in calcium-free media, presumably due to release from intracellular stores (see Fig. 6*B*), was sufficient to support ^{3}H-D-aspartate release. In Figure 9*C*, astrocytes were loaded with 25 μM quin-2 for 1 hr to buffer intracellular calcium, with EGTA added to all calcium-free buffers. Again, this did not appear to affect the release of ^{3}H-D-aspartate due to exposure to hypotonic media. The removal of extracellular calcium and/or the buffering of intracellular calcium also did not affect hypotonic media–stimulated release of preloaded ^{3}H-taurine (data not shown). Since RVD in astrocytes is dependent on extracellular calcium, these data suggest that amino acid release is not a necessary component for RVD to occur, although it is clearly a consequence of astrocytic swelling.

Since we had found that 1 mM quinine, which is known to block calcium-activated K^+ channels, abolished volume regulation (see Fig. 2*B*), we also tested its effect on $^{86}Rb^+$ and ^{3}H-

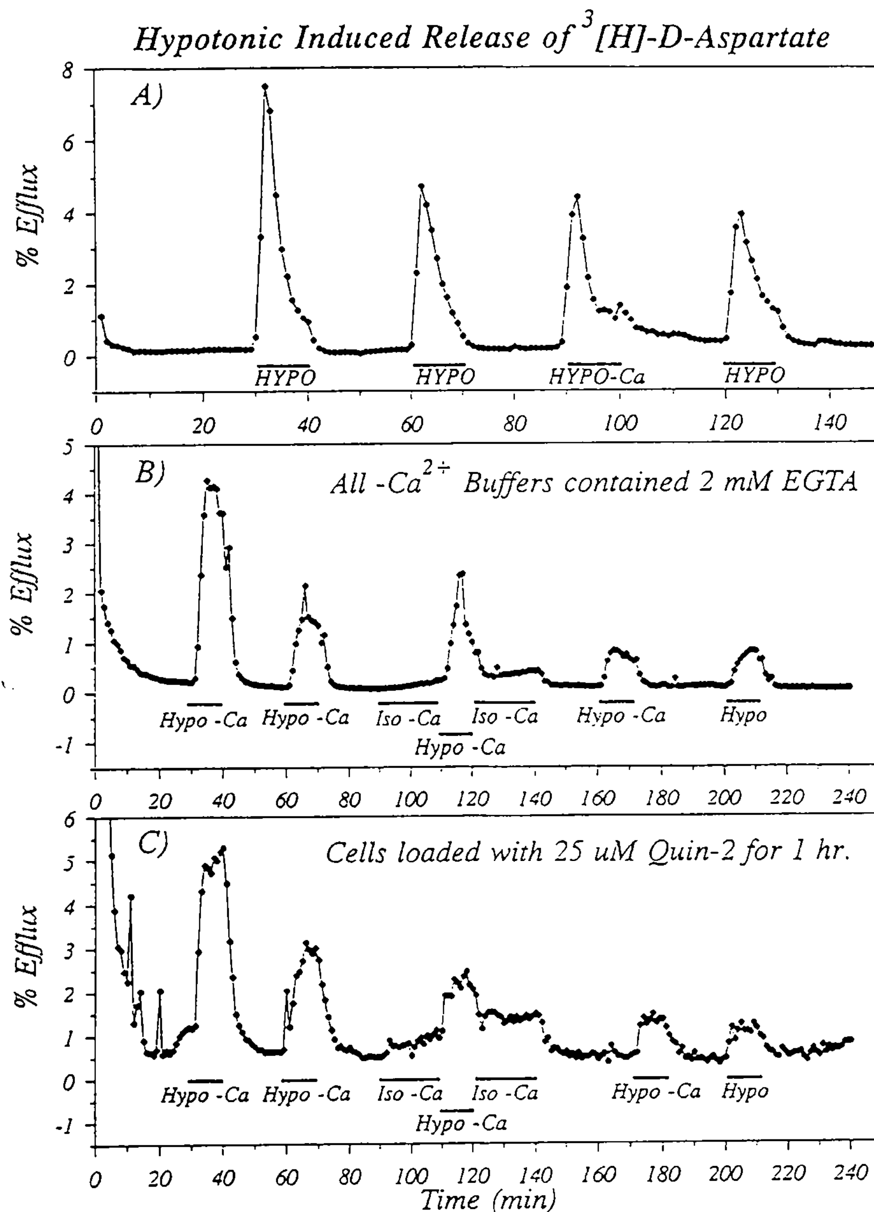

Figure 9. Calcium independence of hypotonic-induced efflux of ^{3}H-D-aspartate from primary astrocyte cultures. The cells, grown on cell support film, were incubated overnight with 0.4 μCi of ^{3}H-aspartic acid. *A*, The *third exposure* shows that removing extracellular calcium did not affect the hyposmotic (minus 50 mM NaCl)-induced release of ^{3}H-D-aspartate. *B*, Extracellular calcium was removed and 2 mM EGTA was also added to all hypotonic solutions to buffer any extracellular calcium that may have been present. Also, for the *third response*, cells were pre- and postexposed to an isotonic minus Ca^{2+} plus 2 mM EGTA solution. *C*, Extracellular calcium was removed and buffered with 2 mM EGTA, as in *B*, and the cells were also loaded with 25 μM quin-2 to buffer any intracellular free calcium.

D-aspartate release. As seen in Figure 10*A*, 1 mM quinine blocked the swelling-induced $^{86}Rb^+$ release, supporting the role of a calcium-activated K^+ efflux during astrocyte volume regulation, but had no effect on ^{3}H-D-aspartate release (Fig. 10*B*). This further supports our finding that amino acid release is not necessary for astrocyte volume regulation. The experiments in Figure 10 was also performed as a control for the necessary design of the volume measurement experiments using the resistance method, which requires the same ionic concentrations between iso- and hyposmotic solutions. The hypotonic buffers were made as usual, by the removal of 50 mM NaCl, but the isotonic control was made by adding 100 mM sucrose to the hypotonic buffer (as was done in the electrical resistance measurements). This experimental design allows us to maintain ionic concentrations (namely, NaCl) while altering the tonicity of the solutions, and thus shows that the release of amino acids and ions is swelling dependent and not due to a lowering of the extracellular Na^+ or Cl^- concentrations.

Discussion

Requirement of Ca^{2+} for volume regulation. Upon exposure to hypotonic solution, astrocytes in primary culture swell to a peak volume and then undergo RVD. A requirement for Ca^{2+} in controlling the RVD response has been shown in a number of cell types (Pierce and Politis, 1990). The data presented in this study indicate that hypotonic-induced cell volume regulation in astrocytes is dependent on extracellular calcium, since when calcium was removed from the extracellular medium astrocytes failed to exhibit RVD. Conversely, if calcium was added to swollen astrocytes in calcium-free media, volume regulation was almost immediately initiated.

McCarty and O'Neil (1990) reported that volume regulation after swelling was highly dependent on extracellular calcium in rabbit proximal straight tubule. They reported a half-maximal inhibition of RVD at 100 μM $CaCl_2$, as we found for astrocyte volume regulation. They also reported that if RVD was initially

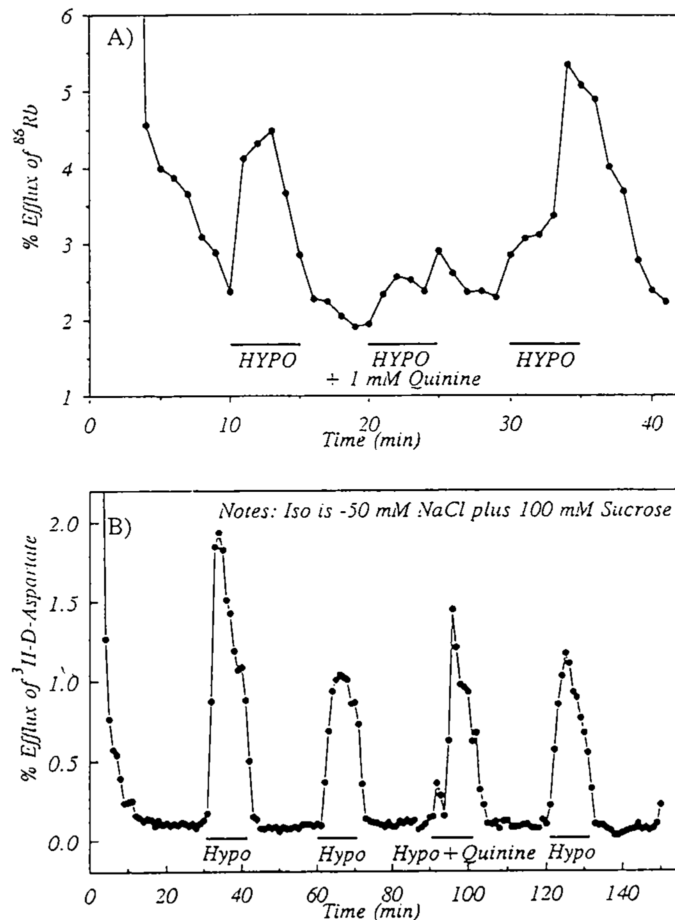

Figure 10. Effect of quinine on $^{86}Rb^+$ and ^{3}H-D-aspartate release. *A*, The addition of 1 mM quinine blocked hypotonic-induced $^{86}Rb^+$ release. *B*, The addition of 1 mM quinine did not affect hypotonic media–induced ^{3}H-D-aspartate release. The hypotonic buffer was made by removing 50 mM NaCl, while the isotonic control was made by adding 100 mM sucrose to the hypotonic buffer (as was done in the electrical resistance measurements).

blocked by reducing $[Ca^{2+}]_e$ or by the addition of verapamil during hypotonic swelling, volume regulation could only be restored by subsequently inducing calcium entry within the first 1 min or less of exposure to hypotonic media. They suggested a "calcium window" of < 1 min, during which RVD is sensitive to Ca^{2+}, and after that the Ca^{2+}-sensitive mechanisms for RVD undergo inactivation. We have found no such "calcium window" in astrocytes, as adding 1 mM $CaCl_2$ up to 10 min after exposure to hypotonic media minus calcium causes immediate initiation of RVD (data not shown).

Swelling-activated Ca^{2+} entry and rise in $[Ca^{2+}]_i$. Either extracellular or intracellular Ca^{2+} may be the signal that activates RVD following exposure to hypotonic solutions. In all cell types that report a calcium dependence of RVD, except for Ehrlich ascites tumor cells (Hoffmann et al., 1984) and lymphocytes (Grinstein et al., 1982), the source of calcium is extracellular, that is, requiring Ca^{2+} entry to activate RVD. Swelling-induced calcium uptake has been reported in renal proximal tubule cells (McCarty and O'Neil, 1991a,b), canine kidney cells (Rothstein and Mack, 1992), toad urinary bladder (Wong and Chase, 1986), and *Necturus* gallbladder (Foskett and Spring, 1985). Since we found that swelling-induced entry of $^{45}Ca^{2+}$ in astrocytes was partially blocked by the dihydropyridine Ca^{2+}-channel antagonist nimodipine, this indicates that one route of Ca^{2+} entry is through voltage-gated L-type channels. In previous electrophysiology experiments, we showed that exposing astrocytes to hypotonic solutions caused marked membrane potential depolarizations proportional to the degree of hypotonicity (Kimelberg and O'Connor, 1988), as also seen in other cells (Ubl et al., 1988). A voltage-sensitive Ca^{2+} channel, resembling the L-type channel, has been described in cortical astrocytes in isosmotic conditions when the cells were treated with dibutyryl cAMP (MacVicar, 1984, 1987; Barres et al., 1988, 1990; MacVicar and Tse, 1988). It is therefore of interest that we observed L-channel activity in swollen astrocytes that had not been treated with cAMP. Possibly, swelling activates the normally quiescent channels perhaps by increasing intracellular cAMP, as has been observed in other cells (Watson, 1989, 1990; Baquet et al., 1991). The depolarization observed in media made hypotonic by removal of 100 mOsm, as routinely used in this study, would be around 50 mV, reducing the membrane potential to approximately −20 mV, which would be sufficient to begin to open the voltage-sensitive calcium channels. Also we found that depolarizing the cells with raised K^+ in the medium (50 and 100 mM) led to an increase in the fura signal (data not shown).

However, since only 60% of the $^{45}Ca^{2+}$ entry was inhibited by nimodipine, other pathways must exist. One possible route for calcium entry could be via the electrogenic sodium–calcium $3Na^+/Ca^{2+}$ exchange system working in the reverse mode due to intracellular Ca^{2+} depletion and Na^+ gain as seen in astrocytes after exposure to low Ca^{2+} mimicking the "calcium paradox" phenomenon (Kim-Lee et al., 1992), and cell membrane depolarization. Since swelling increases release of intracellular Ca^{2+}, and thus presumably depletes internal stores, and this is known to lead to activation of Ca^{2+} channels, some of the increased $^{45}Ca^{2+}$ influx could also be via such a mechanism. Also, increased $^{45}Ca^{2+}$ uptake could be via Ca^{2+}/Ca^{2+} exchange.

A further additional route for swelling-activated Ca^{2+} entry could be through stretch-activated channels (SACs) in the plasma membrane. Cation-specific and Ca^{2+}-permeable channels sensitive to membrane stretch have been described in salamander choroid plexus epithelium (Christensen, 1987), porcine vascular endothelium (Lansman et al., 1987), renal proximal tubules (Filipovic and Sackin, 1991), *Xenopus* oocytes (Yang and Sachs, 1990), and murine skeletal myotubes (Franco and Lansman, 1990a,b). A SAC permeable to calcium has been reported in human retinal glial cells (Puro, 1991), but it was not determined if an increase in cell volume activated this channel. In whole-cell recordings from cultured astrocytes (Kimelberg et al., 1990a) nonspecific cation currents were seen upon exposure to hypotonic solutions, which are likely responsible for the marked depolarization of the membrane potential seen in swollen astrocytes (Kimelberg and O'Connor, 1988; Kimelberg and Kettenmann, 1990). These currents could include Ca^{2+}, but this was not determined. Further characterization of stretch-activated ion channels is necessary to elucidate their role in Ca^{2+} entry and K^+ and Cl^- efflux during volume regulation.

The extracellular calcium required to activate RVD is assumed to act as a source for the rise in $[Ca^{2+}]_i$. A swelling-induced increase in calcium influx, leading to a rise in intracellular calcium, has been reported in a number of cell types (Wong and Chase, 1986; Hazama and Okada, 1990; Rothstein and Mack, 1990; Wong et al., 1990). In our studies it was found that swelling in the presence of normal calcium was associated with an increase in intracellular free calcium concentration that

was characterized by a biphasic response. Using fura-2 we found that hypotonic media–induced swelling led to an initial transient increase in free $[Ca^{2+}]_i$, which was due to release of Ca^{2+} from intracellular stores since removal of $[Ca^{2+}]_e$ did not affect it. This release is stimulated by IP compounds and IP_3 increases have been reported to occur during RVD, at least in Ehrlich ascites cells (Hoffman and Kolb, 1992). In the absence of $[Ca^{2+}]_e$, $[Ca^{2+}]_i$ rapidly returned to baseline values, without the sustained plateau seen in the presence of normal $[Ca^{2+}]_e$. The sustained increase above baseline also appears to be partly due to Ca^{2+} entry since nimodipine, which blocked approximately 60% of the swelling-induced $^{45}Ca^{2+}$ entry, also caused a more rapid decline of the initial transient and a partial decrease of the sustained plateau (Fig. 7). That nimodipine did not abolish the plateau, as was seen upon removal of $[Ca^{2+}]_e$, might be expected as nimodipine did not block all calcium entry, as measured by influx of $^{45}Ca^{2+}$.

Release of ions. Volume regulation under hypotonic conditions is often due to the cellular loss of KCl with water. Both electroneutral cotransporters and electroconductive channels may be responsible for volume-sensitive KCl fluxes (Hoffmann and Simonsen, 1986). In some cells the K^+ and Cl^- conductances are separate, for example, in human lymphocytes (Grinstein et al., 1982), frog urinary bladder (Davis and Finn, 1987), human intestinal epithelial cells (Hazama and Okada, 1988), Ehrlich ascites cells (Hoffmann et al., 1984), and Chinese hamster ovary cells (Sarkadi et al., 1984). In other cell types, like *Necturus* gallbladder epithelial cells (Larson and Spring, 1984) and most red blood cells (Pierce and Politis, 1990), K^+ movements are completely Cl^- dependent, indicating cotransport.

Since the RVD process in astrocytes appeared completely sensitive to extracellular calcium, the possibility that volume-sensitive KCl efflux was sensitive to calcium was examined. The data indicated that release of both K^+ and Cl^- in RVD was dependent on $[Ca^{2+}]_e$. Calcium-activated K^+ channels have been reported in astrocytes (Quandt and MacVicar, 1986; Barres et al., 1990) and in astrocytic retinal Müller cells (Puro et al., 1991). These channels are activated by membrane depolarization and increases in intracellular Ca^{2+} concentration. A role for these channels in RVD was also supported by the finding that quinine, a known blocker of Ca^{2+}-activated K^+ channels, also blocked the swelling-induced release of $^{86}Rb^+$.

There is more evidence that activation of volume regulatory K^+ transport systems are controlled by cytosolic calcium than for Cl^- (Grinstein et al., 1982; Foskett and Spring, 1985; Hoffmann et al., 1986; Hazama and Okada, 1988; Pierce and Politis, 1990). Volume regulatory Cl^- conductances are activated in response to hyposmotic stress in Ca^{2+}-depleted lymphocytes (Grinstein et al., 1982) and Ca^{2+}-depleted human gut epithelial cells (Hazama and Okada, 1988). However, Ca^{2+}-activated Cl^- transport pathways have been reported in several cell types, including *Xenopus* oocytes (Miledi and Parker, 1984), tracheal epithelial cells (Frizzell et al., 1986), neurons (Mayer, 1985), and several other cell types (Hoffmann and Simonsen, 1989). Also, Hoffmann et al. (1986) showed that A23187, a calcium ionophore, caused increased Cl^- loss from hyposmotically stressed Ehrlich ascites cells. RVD involving Cl^- requires that there is adequate intracellular Cl^-. While Cl^- in cultured astrocytes is at 30–40 mM (Kimelberg, 1981), glial cells in guinea pig olfactory cortex showed a passive Cl^- distribution (Ballanyi et al., 1987). Perhaps *in vivo*, HCO_3^- efflux, or some other anion, also contributes to RVD in astrocytes.

Release of amino acids. Release of taurine, glutamate, aspartate, and other amino acids has been shown to occur during RVD in a number of vertebrate and invertebrate cell types (Gilles et al., 1991). Pasantes-Morales and Schousboe (1989) showed a hypotonic media–induced release of radiolabeled and endogenous taurine, and Kimelberg et al. (1990b) reported that exposure of primary astrocyte cultures to hypotonic media led to release of label after cells had been allowed to accumulate ^{3}H-L-glutamate, ^{3}H-D-aspartate, or ^{3}H-taurine, as well as release of endogenous glutamate and taurine as measured by HPLC. These findings led us (Kimelberg et al., 1990b) to suggest that swollen astrocytes may be an additional source of release for L-glutamate and L-aspartate, which could contribute to excitotoxic, amino acid–induced neuronal injury (Choi, 1988), since astrocytic swelling is an early event in a number of pathological states including traumatic head injury and cerebral ischemia (see reviews by Kimelberg and Ransom, 1986, and Kimelberg, 1992). While hypotonic conditions are only encountered *in vivo* in cases involving kidney failure, and astrocytic edema is likely due to a variety of other causes such as high $[K^+]_e$ or glutamate (Kimelberg, 1991, 1992; Kimelberg et al., 1993), we assume that the same degree of swelling will result in activation of the same processes. However, hyponatremia is often a problem in head-injured patients due to systemic effects of the injury. Resolution of astrocytic swelling on a time course of 1–2 hr, which is about sixfold longer than RVD, has been described after experimental closed-head injury in cats (Barron et al., 1988). Even under conditions of normal neuronal activity, accumulation of K^+ as KCl with H_2O and/or uptake of transmitters, such as glutamate, with Na^+, may be compensated for by Ca^{2+}-activated release of KCl to maintain normal cell volume.

We also examined whether the hypotonic-induced release of amino acids contributed to the RVD process in astrocytes, as has been suggested for other cell types (Law, 1991) and as has been suggested for taurine release in the brain (Thurston et al., 1980; Pasanates-Morales and Schousboe, 1988). Since RVD in our astrocytes was highly dependent on $[Ca^{2+}]_e$, then the release of amino acids should also be sensitive to $[Ca^{2+}]_e$ if such release contributes significantly to RVD. We found that removal of extracellular calcium and/or buffering of intracellular calcium had no effect on the swelling-induced release of ^{3}H-D-aspartate (Fig. 9) or ^{3}H-taurine (data not shown). Pasantes-Morales et al. (1990) have also reported that hypotonic-induced taurine release was unaffected by removal of extracellular calcium. Therefore, these data suggest that amino acid release during RVD, while occurring concomitantly, is not necessary for volume regulation in cultured astrocytes. One possible explanation is that because only 1–10% of the aspartate or glutamate pool is released during volume regulation, and because endogenous concentrations of these amino acids are quite low in the brain (Clarke et al., 1989), the release of aspartate or glutamate is not of a sufficient quantity to play an important role in volume regulation in astrocytes. Also, since taurine in the brain is approximately 2–5 mM, its role in volume regulation *in situ* has been questioned (Martin et al., 1990). Our data suggest that taurine does not contribute as an osmolyte even under marked hypotonic-induced swelling. However, in cultured astrocytes the apparent concentration of ^{3}H-taurine is 50–80 mM (Kimelberg et al., 1990b), so its apparent lack of contribution to RVD in cultured cells is surprising. The swelling-induced, Ca^{2+}-independent release of amino acids from astrocytes may contribute to the Ca^{2+}-independent release of glutamate seen during ex-

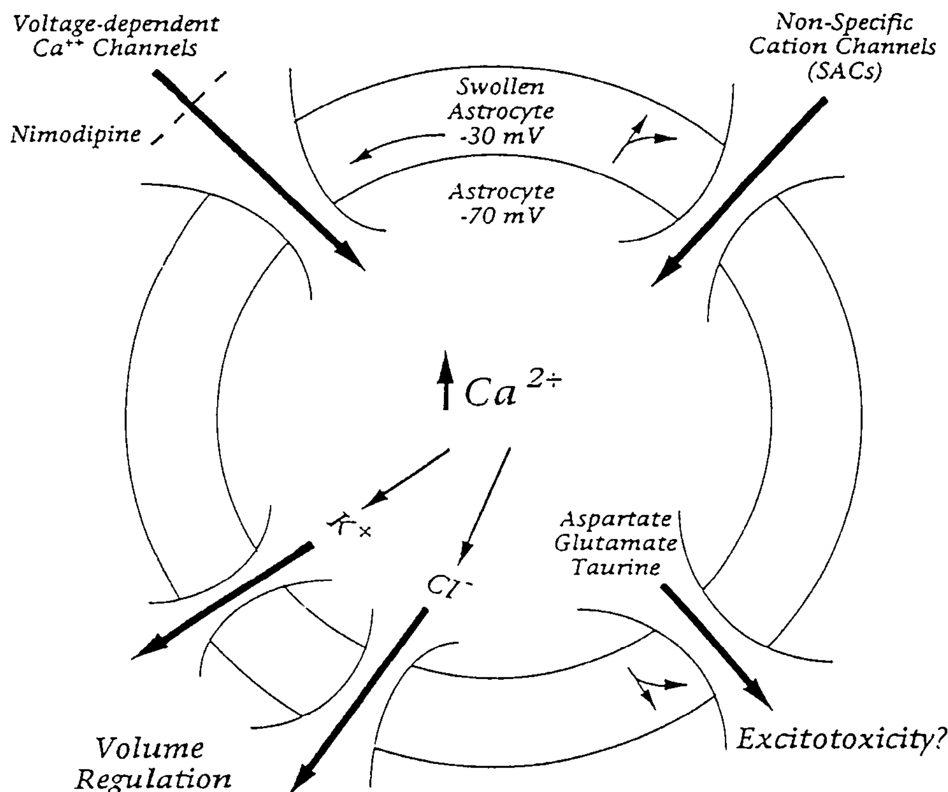

Figure 11. Possible roles of calcium involvement in volume regulation of astrocytes swollen by hypotonic media and different possible transport pathways for RVD. The sequence of events is suggested to occur as follows: astrocyte in hypotonic media → cell swelling due to water influx → membrane depolarization due to opening of nonspecific cation SACs → Ca^{2+} entry predominantly via voltage-gated channels → increased free $[Ca^{2+}]_i$ → activation of Ca^{2+}-dependent K^+ and Cl^- channels → efflux of ions → volume decrease. See last section of Discussion for further details.

perimental ischemia *in vivo*, which comes later and is smaller than the initial Ca^{2+}-dependent release (Katayama et al., 1991).

Model of the roles of Ca^{2+} in astrocyte RVD. Figure 11 is a proposed model depicting the possible roles of calcium in initiation of RVD in astrocytes swollen by exposure to hypotonic medium. In this model, swelling of astrocytes due to exposure to hypotonic media initially causes depolarization of the membrane potential, possibly through opening of nonspecific cation, swelling-activated channels (SACs). This depolarization then activates voltage-sensitive Ca^{2+} channels, allowing entry of calcium into the cell, since this entry can be partially blocked by voltage-sensitive calcium channel blockers. Other routes of Ca^{2+} entry also probably occur. Release from intracellular stores also causes the free $[Ca^{2+}]_i$ to increase transiently. However, it appears to be the sustained entry of extracellular Ca^{2+} that opens Ca^{2+}-dependent K^+ channels and Cl^- channels that allow KCl to leave the cell. Amino acids are also released, but by a mechanism that does not involve Ca^{2+} and therefore may be activated directly by tension in the membrane, or perhaps by the depolarization or by involvement of second messenger changes such as cAMP or phosphatidylinositol products (Watson, 1989, 1990; Baquet et al., 1991; Hoffmann and Kolb, 1991). The swelling-induced release of amino acids is inhibited by several anion transport blockers and is Na^+ independent, and in the case of glutamate is unaffected by glutamate uptake blockers, which argue against release being due to reversal of the uptake system (Kimelberg et al., 1990b). The release of intracellular ions, along with osmotically obligated water, results in the astrocytes' volume regulating back close to their initial volume.

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