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Sodium/Calcium Exchange in Rat Cortical Astrocytes

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Regulation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) by an Na/Ca exchanger was studied in primary cultured rat cortical astrocytes. $[Ca^{2+}]_{cyt}$ was measured by digital imaging in cells loaded with fura-2. The resting $[Ca^{2+}]_{cyt}$, ≈150 nM, was only slightly increased by reducing the extracellular Na^+ concentration ($[Na^+]_o$) to 6.2 mM, or by treating the cells with ouabain for 15 min (to raise cytosolic Na^+). Following treatment with ouabain, however, lowering $[Na^+]_o$ caused $[Ca^{2+}]_{cyt}$ to rise rapidly to ≈1300 nM. When Ca^{2+} sequestration in intracellular stores was blocked by thapsigargin, lowering $[Na^+]_o$ increased $[Ca^{2+}]_{cyt}$ to ≈1500 nM in the absence of ouabain. The low-$[Na^+]_o$-stimulated rise in $[Ca^{2+}]_{cyt}$ was abolished by removal of external Ca^{2+}, but was not blocked by the Ca^{2+} channel blocker verapamil, or by caffeine or ryanodine, which deplete an intracellular Ca^{2+} store responsible for Ca^{2+}-induced Ca^{2+} release. These data suggest that Na^+ gradient reduction promotes net Ca^{2+} gain via Na/Ca exchange. Normally, however, a large rise in $[Ca^{2+}]_{cyt}$ is prevented by sequestration of the entering Ca^{2+}; this buffering of cytosolic Ca^{2+} can be circumvented by blocking sequestration with thapsigargin, or overwhelmed by enhancing net Ca^{2+} gain by pretreating the cells with ouabain. The presence of Na/Ca exchanger protein and mRNA in the astrocytes was confirmed by Western and Northern blot analyses, respectively. Immunohistochemistry revealed that exchanger molecules are distributed in a reticular pattern over the astrocyte surface. We suggest that the Na/Ca exchanger plays a role in regulating both $[Ca^{2+}]_{cyt}$ and the intracellular stores of Ca^{2+} in astrocytes, and may thus contribute to the control of astrocyte responsiveness to neurotransmitters and neurotoxins.

[Key words: astrocytes, digital imaging, cell culture, Na/Ca exchange, endoplasmic reticulum, thapsigargin, sodium, calcium]

Recent evidence suggests that astrocytes play a much more significant role in the functioning of the mammalian nervous system than had been previously thought. In addition to such well-established functions as regulation of the extracellular K^+ concentration ($[K^+]_o$) and clearance of excitotoxic agents such as glutamate, astrocytes respond to a variety of neurotransmitters and neuromodulators, and they carry out complex intracellular and intercellular signaling (Cornell-Bell et al., 1990; Glaum et al., 1990; Jensen and Chiu, 1990; Barres, 1991; Charles et al., 1991; McCarthy and Salm, 1991). A central feature of these responses is a rise in the ionized cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). Within individual cells, $[Ca^{2+}]_{cyt}$ often undergoes a series of oscillations (Cornell-Bell et al., 1990; Jensen and Chiu, 1990; Charles et al., 1991). Moreover, the increase in $[Ca^{2+}]_{cyt}$ can propagate from cell to cell in cultures in which the cells are electrically coupled (Cornell-Bell et al., 1990; Charles et al., 1991). Such waves of $[Ca^{2+}]_{cyt}$ may propagate over many cells throughout an astrocyte syncytium both *in vitro* and *in vivo*. It has been suggested that the increase in $[Ca^{2+}]_{cyt}$ not only mediates the actions of neurotransmitters within individual cells (McCarthy and Salm, 1991), but may also serve as an intercellular signaling mechanism parallel to and independent of the electrical signals carried out by action potentials in neurons (Cornell-Bell et al., 1990).

A component of the astrocyte $[Ca^{2+}]_{cyt}$ response appears to be mediated by extracellular Ca^{2+} entry through voltage-gated Ca^{2+} channels (Barres et al., 1990; MacVicar et al., 1991). However, much of the response, including both oscillations and propagated waves (both intracellular and intercellular), require inositol 1,4,5-trisphosphate (IP_3) synthesis and the release of Ca^{2+} from IP_3-sensitive intracellular stores in the endoplasmic reticulum (ER) (Ahmed et al., 1990; Cornell-Bell et al., 1990; Glaum et al., 1990). Such intracellular Ca^{2+} stores in a variety of cell types accumulate Ca^{2+} via a specific ATP-dependent pump that is inhibited by compounds such as thapsigargin and cyclopiazonic acid (Seidler et al., 1989; Thastrup et al., 1990); Ca^{2+} is also released from ER stores by caffeine and ryanodine (Palade et al., 1987; McPherson et al., 1991). These stores undoubtedly play a crucial role in buffering transient increases in $[Ca^{2+}]_{cyt}$; nevertheless, in the steady-state, all Ca^{2+} that enters from the extracellular space must be extruded against a large electrochemical gradient. Two types of processes can mediate this net Ca^{2+} extrusion across the plasmalemma: an ATP-driven Ca^{2+} pump (Schatzmann, 1982) and Na/Ca exchange (Blaustein et al., 1991a,b). Moreover, the Na/Ca exchanger also mediates net Ca^{2+} influx upon reduction of the Na^+ electrochemical gradient. To understand the mechanisms that regulate $[Ca^{2+}]_{cyt}$, and therefore the magnitude of the cell's response to external stimuli, we

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must take into account Ca^{2+} sequestration into and release from internal stores as well as the activities of the plasmalemmal Ca^{2+} transport mechanisms.

The focus of this report is the plasmalemmal Na/Ca exchanger. Several studies have demonstrated that $[Ca^{2+}]_{cyt}$ in astrocytes is influenced by the Na^+ electrochemical gradient across the plasmalemma (reviewed in Finkbeiner, 1993): Ca^{2+} entry (Lazarewicz et al., 1977; Kim-Lee et al., 1992) and $[Ca^{2+}]_{cyt}$ (Blaustein et al., 1991; Cornell-Bell and Finkbeiner, 1991; Delumeau et al., 1991; MacVicar et al., 1991) are increased by removing external Na^+. Stimulus-evoked increases in $[Ca^{2+}]_{cyt}$ are also augmented when the Na^+ gradient is reduced (Cornell-Bell et al., 1990; Jensen and Chiu, 1990; Blaustein et al., 1991; Cornell-Bell and Finkbeiner, 1991). The effects of Na^+ gradient reduction on $[Ca^{2+}]_{cyt}$ are often small, however, and this has raised questions about the role of the Na/Ca exchanger in astrocytes (Jensen and Chiu, 1990).

In the present study, we used immunocytochemical methods to demonstrate, directly, the presence of an Na/Ca exchanger in cultured postnatal rat cortical astrocytes. We also employed computer assisted imaging of fura-2 fluorescence to examine the regulation of $[Ca^{2+}]_{cyt}$ in these cells. Our results indicate that the plasmalemmal Na/Ca exchanger is present in astrocytes and that it may play an important role in Ca^{2+} transport across the plasmalemma and in the regulation of ER Ca^{2+} stores.

Materials and Methods

Astrocyte cultures. Primary astrocyte cultures were prepared using a modification of the method of Booher and Sensenbrenner (1972). Cerebral hemispheres of newborn rats (<1 d old) were removed, placed in minimal essential medium with 20% fetal calf serum, cleaned of meninges, and trimmed to retain the neopallium. The tissue was then minced into small (1 mm^3) pieces, transferred to a 50 ml round-bottomed tube, and then mechanically disrupted by vortexing (75 sec). The cell suspension was then filtered through sterile nylon screening cloth with pores sizes of 80 μm (first sieving) and 10 μm (second sieving) to remove blood vessels and aggregated cells. The cell suspension was not digested with enzymes. The volume of the filtered cell suspension was then adjusted with medium (9 ml/brain) containing 10% fetal calf serum and seeded on 25 mm glass coverslips at a plating density of about 5 $\times$ 10^5 cells per coverslip. More than 95% of the cells were $GFAP^+$, and two types of astrocytes could be distinguished on the basis of their morphology, as described by Yarowsky and Krueger (1989): type-2 astrocytes were stellate, with round cell bodies; these cells were $GFAP^+$ and $A2B5^+$, and $GalC^-$. Type 1 astrocytes were epithelioid in shape, and were $GFAP^+$, and $A25B^-$ and $GalC^-$. Type 2 astrocytes sat on top of type 1 astrocytes, and accounted for fewer than 10% of the cells in these low-density cultures. The data described in this report all refer to type 1 astrocytes; comparable effects were observed, however, in type 2 astrocytes.

Fluorescent dye loading. Astrocytes, cultured on coverslips for 6–8 d, were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2, by incubating them for 45 min at 20–22°C in medium containing 5 μM fura-2/AM (the membrane-permeable acetoxymethyl ester). The coverslips were then transferred to a tissue chamber mounted on a microscope stage where they were superfused for 20–30 min with PSS to wash away the extracellular dye before study. The imaging experiments were carried out at 32–34°C.

Determination of $[Ca^+]_{cyt}$. Details of the methods used in this study have been published (Goldman et al., 1990; Goldman, 1991). Briefly, fura-2 fluorescence (510 nm emission excited by 380 and 360 nm illumination) from astrocytes, as well as background fluorescence, was imaged using a Nikon Diaphot inverted microscope equipped for epifluorescence microscopy. Video frames containing images of fura-2 fluorescence and the corresponding background images (fluorescence after removing cells from the field) were digitized using an Imaging Technology (Woburn, MA) series 151 image processor operating with an Everex 386 microcomputer. Normally, 32 consecutive video frames were averaged at video frame rates to improve the signal-to-noise ratio. The 380:360 nm fluorescence ratios of fura-2-loaded astrocytes were then calculated and calibrated to express $[Ca^{2+}]_{cyt}$ (Goldman et al., 1990). To simplify interpretation of the data, $[Ca^{2+}]_{cyt}$ measurements were limited to non-nuclear areas.

In a few experiments a Real Time Disk System (Applied Memory Technology, Tustin, CA) was used to acquire images at video frame rates (30 frames/sec). This enabled us to measure changes in $[Ca^{2+}]_{cyt}$ with a temporal resolution of 33 msec. Ratio images (380:360 nm) were calculated using background-subtracted 380 nm images acquired during exposures to low Na^+ lasting several seconds and 360 nm images acquired immediately before and after the periods in low Na^+. The latter 360 nm images were also used to verify that there was no significant change in dye signal or artifactual microscope movement during the exposure to low Na^+ (Goldman et al., 1990).

Materials and solutions for imaging experiments. Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Thapsigargin (TG) was purchased from LC Services (Woburn, MA). *N*-methyl-D-glucamine (NMDG), dimethylsulfoxide (DMSO), ouabain, and 5-HT (5-hydroxytryptamine) were purchased from Sigma (St. Louis, MO). All other reagents were analytical grade or the highest purity available.

The standard physiological salt solution (PSS) contained (in mM) NaCl, 140; KCl, 5.9; NaH_2PO_4, 1.2; $NaHCO_3$, 5; $MgCl_2$, 1.4; $CaCl_2$, 1.8; glucose, 11.5; and HEPES, 10 (titrated to pH 7.4 with NaOH). Although the principal buffer was HEPES, $NaHCO_3$ was included to sustain cellular HCO_3-dependent pH regulatory mechanisms (Boyarsky et al., 1993). For Ca^{2+}-free solutions the $CaCl_2$ was replaced by 1.8 mM $MgCl_2$ (total of 3.2 mM) and 0.5 mM EGTA was added. In the low-Na^+ (NMG) medium, the NaCl, was isosmotically replaced by *N*-methyl-D-glucamine (NMG), and pH was adjusted with HCl. Stock solutions containing 10 mM fura-2/AM were prepared in DMSO.

Membrane isolation from cultured astrocytes. Cultured astrocytes were washed with PBS (phosphate-buffered saline: 120 mM NaCl and 2.7 mM KCl, in 10 mM Na-phosphate buffer, pH 7.4). The cells were scraped into PBS (2 ml per 100 mm dish), and centrifuged at 3500 rpm in an SS-34 rotor (Sorvall RC-5B centrifuge, DuPont, Wilmington, DE). This and all subsequent preparative steps were carried out at 4°C. The pellet was resuspended in 10 mM HEPES-KOH, pH 7.0, containing 1 mM dithiothreitol (DTT) and 100 μM phenylmethylsulfonyl fluoride (PMSF). The suspension was gently stirred for 10 min at 4°C and then homogenized with a Polytron (Brinkmann, Westbury, NY) for 10 sec at 7000 rpm. Sucrose was then added to the homogenate to a final concentration of 250 mM, and the homogenate was centrifuged at 7000 $\times$ *g* for 10 min. The supernatant containing microsomal membranes was collected and centrifuged at 35,000 rpm in a Beckman L5-50 ultracentrifuge (70.1 Ti rotor) for 30 min. The pelleted microsomal membranes were used for immunoblotting. Protein concentrations were determined by the BCA protein assay reagent (Pierce, Rockford, IL), using bovine serum albumin (BSA) as a standard.

Synaptic plasma membranes (SPM) were prepared from the brains of freshly sacrificed Sprague–Dawley rats, as described by Salvaterra and Matthews (1980). Canine cardiac sarcolemmal membranes (Ambesi et al., 1991b) were generously supplied by Dr. G. Lindenmayer.

Immunoblotting. Proteins were solubilized in SDS buffer containing 20 mM dithiothreitol for 10 min at room temperature. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was calibrated with prestained protein molecular weight markers (Bio-Rad, Richmond, CA). Proteins were then transferred to a Nitro-Bind nitrocellulose membrane (MSI, Westboro, MA) as described by Towbin and Gordon (1984). The extent of transfer was checked by Ponceau-S staining. Membranes were blocked with 5% BSA in TBST (Tris-buffered saline with 0.1% Tween 20) for 6 hr at room temperature. The blots were then incubated overnight at room temperature with either preimmune serum or polyclonal antibodies (1:500 dilution in TBST) raised against the purified dog cardiac sarcolemmal Na/Ca exchanger (Ambesi et al., 1991a,b). The membranes were washed and incubated for 2 hr in TBST containing 0.25 μCi/ml ^{125}I-labeled Protein A (Amersham, Arlington Heights, IL), washed again, and exposed to Kodak (Rochester, NY) X-Omat film.

RNA blotting. Total RNA was prepared from cultured rat cortical astrocytes and from rat heart and brain as described (Chomczynski and Sacchi, 1987). Samples were electrophoresed for 3 hr at 90 V on a 1% agarose/6% formaldehyde gel, transferred to Nitro-Pure nitrocellulose membranes (MSI, Westboro, MA), and hybridized to a ^{32}P-labeled probe. The gel was calibrated with a 0.24–9.5 kb RNA ladder (GIBCO–Bethesda Research Labs, Gaithersburg, MD). The 800 bp cDNA insert

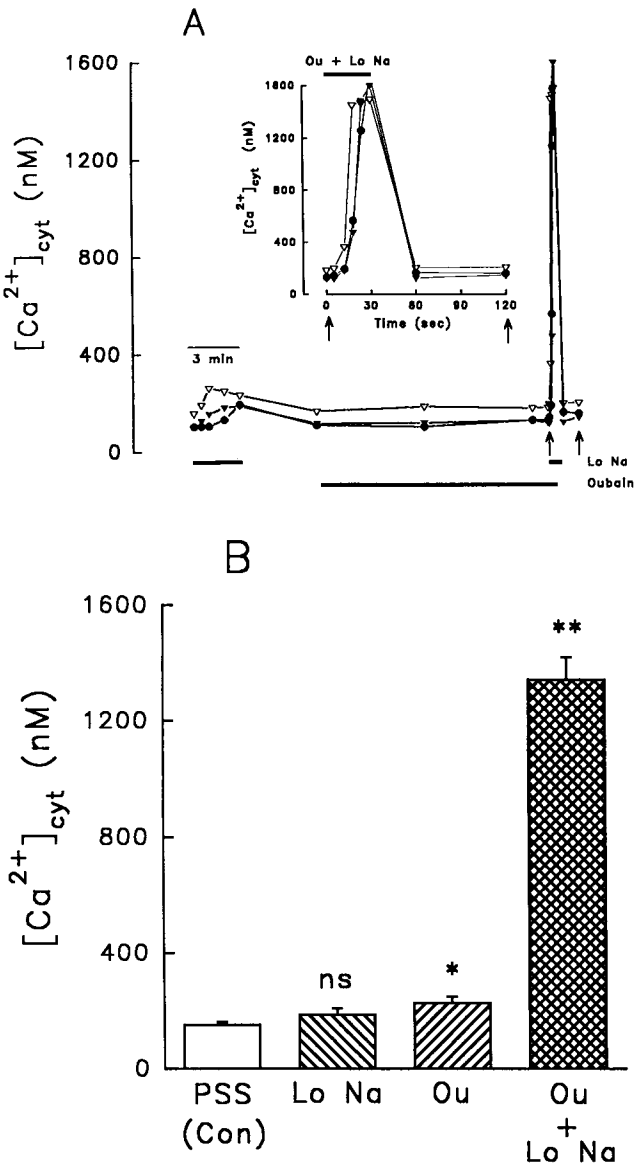

Figure 1. Effects of $[Na^+]_o$ reduction and of ouabain on $[Ca^{2+}]_{cyt}$ in unstimulated (resting) cultured rat cortical astrocytes. *A*, Time course of changes in $[Ca^{2+}]_{cyt}$ for three astrocytes (each represented by a different symbol) in the same field. The cells were preloaded with fura-2 and incubated in PSS for 25 min prior to the beginning of this record. In the main graph, reduction of $[Na^+]_o$ by replacing NaCl with NMG, and addition of 1 mM ouabain, are indicated by the *horizontal bars* below the graph. The *inset* shows the rapid response to low $[Na^+]_o$ in the presence of ouabain (delineated by the *vertical arrows*) on an expanded time scale. *B*, Composite data from a number of experiments similar to the one in *A*, showing $[Ca^{2+}]_{cyt}$ in astrocytes incubated in the control physiological salt solution [*PSS (Con)*; 98 cells from 18 preparations], in low-Na^+ medium with $[Na^+]_o$ = 6.2 mM (*Lo Na*; 38 cells from eight preparations), in PSS containing 1 mM ouabain for 15 min (*Ou*; 80 cells from 16 preparations), or transferred to low-Na^+ medium after a 15 min incubation in PSS containing 1 mM ouabain (*Ou + Lo Na*; 58 cells from 12 preparations). Error bars indicate SE. *ns*, not significantly different from control; *, $p < 0.01$ versus control; **, $p < 0.001$ versus control, *Lo Na*, and *Ou*.

corresponding to the 3′ coding sequence of the rat heart Na/Ca exchanger (Kofuji et al., 1992) was labeled with $dCT^{32}P$ by nick translation. Filters were prehybridized for a minimum of 6 hr at 42°C in hybridization buffer [50% deionized formamide, 5× standard saline citrate (SSC), 5× Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA] (Maniatis et al., 1982). Following hybridization with the cDNA probe for 16 hr at 42°C, the filters were washed using medium-stringency conditions (0.1× SSC at 37°C) and exposed to Kodak X-Omat film.

Immunofluorescence microscopy. The cultures were grown on 12 mm coverslips. The astrocytes were used for experiments 7 d after plating. The coverslips were rinsed in L15 medium, fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min at 4°C, washed in Tris-buffered saline (TBS: 150 mM NaCl, 100 mM Tris-HCl, 0.1% BSA, and 0.02% NaN_3, pH 7.2, at 4°C), and then permeabilized in 0.2% Triton X-100 in TBS for 15 min at 4°C. Nonspecific labeling was blocked by incubating the samples for 30 min in 10% normal donkey or goat serum (Jackson Immunoresearch, West Grove, PA) in TBS. An aliquot of affinity-purified polyclonal antibodies raised against the Na/Ca exchanger (Ambesi et al., 1991a; Juhaszova et al., 1994) was diluted 1:500 in TBS and applied to the coverslips for 17 hr at 4°C. The coverslips were then washed 3 × 5 min in TBS and labeled with FITC-conjugated, affinity-purified donkey anti-rabbit IgG, Fab_2 fragment (Jackson Immunoresearch) at 1:100 dilution in TBS. The coverslips were washed in TBS and mounted in Citifluor (City University, London, UK) to inhibit bleaching. The specimens were examined with an Olympus BH2 fluorescence microscope (Olympus Corp., Lake Success, NY) and photographed with Kodak TMAX 3200 film.

Statistical analysis and data presentation. Numerical data are presented as means ± SE of *n* single cells from one or more independent experiments. Where appropriate, Student's *t* test for paired or unpaired data was used to calculate the significance of the differences of the means.

Results

Intracellular Ca^{2+} in unstimulated astrocytes: effects of ouabain and of low $[Na^+]_o$

The mean intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in the non-nuclear regions of unstimulated cortical astrocytes was 153 ± 10 nM, with a range of 85–220 nM (Fig. 1). These $[Ca^{2+}]_{cyt}$ values are comparable to those obtained in some laboratories (e.g., MacVicar et al., 1991), albeit somewhat higher than those reported by others (e.g., Jensen and Chiu, 1990; Charles et al., 1991).

A common and convenient test for the presence of a plasmalemmal Na/Ca exchanger is to determine the effect of raising the cytosolic Na^+ concentration ($[Na^+]_{cyt}$) (e.g., with ouabain), or of lowering the external Na^+ concentration ($[Na^+]_o$), on resting $[Ca^{2+}]_{cyt}$. The effects of these manipulations on cortical astrocytes are illustrated in Figure 1. Time course data for three cells from a representative experiment are shown in Figure 1*A*, and composite results from a number of similar experiments are summarized in Figure 1*B*. Reduction of $[Na^+]_o$ from 146.2 mM to 6.2 mM (using NMG as a replacement) increased $[Ca^{2+}]_{cyt}$ only to 185 ± 21 nM on average, an insignificant rise. Exposure to 1 mM ouabain for 15 min did significantly increase $[Ca^{2+}]_{cyt}$, but only to 235 ± 23 nM.

These minimal effects of transplasmalemmal Na^+ gradient reduction on $[Ca^{2+}]_{cyt}$ have raised doubts about the contribution of Na/Ca exchange to Ca^{2+} homeostasis in astrocytes (Jensen and Chiu, 1990). Often overlooked, however, is the fact that a very large fraction (>99.9%) of the intracellular Ca^{2+} in most cells is buffered and/or sequestered (Blaustein, 1993; Fontana and Blaustein, 1993). Thus, large reductions in the Na^+ electrochemical gradient might, in fact, promote substantial net gain of Ca^{2+}, but less than 0.1% of this Ca^{2+} would be expected to appear as free Ca^{2+} unless the Ca^{2+} buffer systems are overwhelmed. Therefore, we attempted to challenge the astrocytes with an even larger change in the Na^+ electrochemical gradient. Following a 15 min exposure to 1 mM ouabain, the reduction of $[Na^+]_o$ resulted in a rapid, very large rise in $[Ca^{2+}]_{cyt}$ (Fig. 1*A*), to a mean of 1293 ± 29 nM (Fig. 1*B*). In mouse astrocytes, 1 mM ouabain reduces the Na^+ electrochemical gradient by

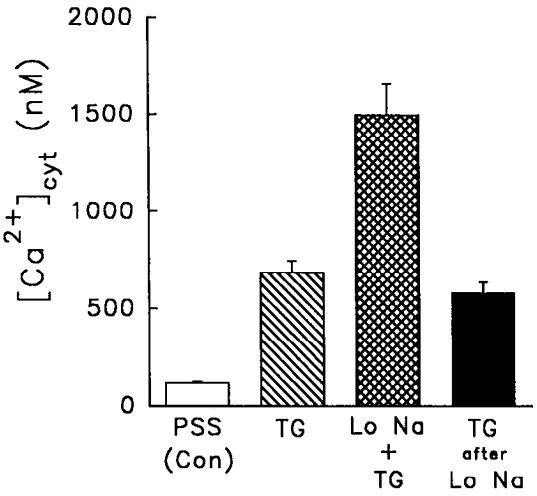

Figure 2. Effects of thapsigargin (*TG*; 30 nM) and subsequent reduction of $[Na^+]_o$ to 6.2 mM on steady $[Ca^{2+}]_{cyt}$. Following introduction of TG, $[Ca^{2+}]_{cyt}$ rose to a peak and then declined to the steady level indicated by the bar labeled *TG*. Upon reduction of $[Na^+]_o$, $[Ca^{2+}]_{cyt}$ rose to a new steady level (*Lo Na + TG*). $[Ca^{2+}]_{cyt}$ then declined when external Na^+ was added back (*TG after Lo Na*). The data are from a representative experiment and correspond to the means (±SE) from six cells. *Con*, PSS control.

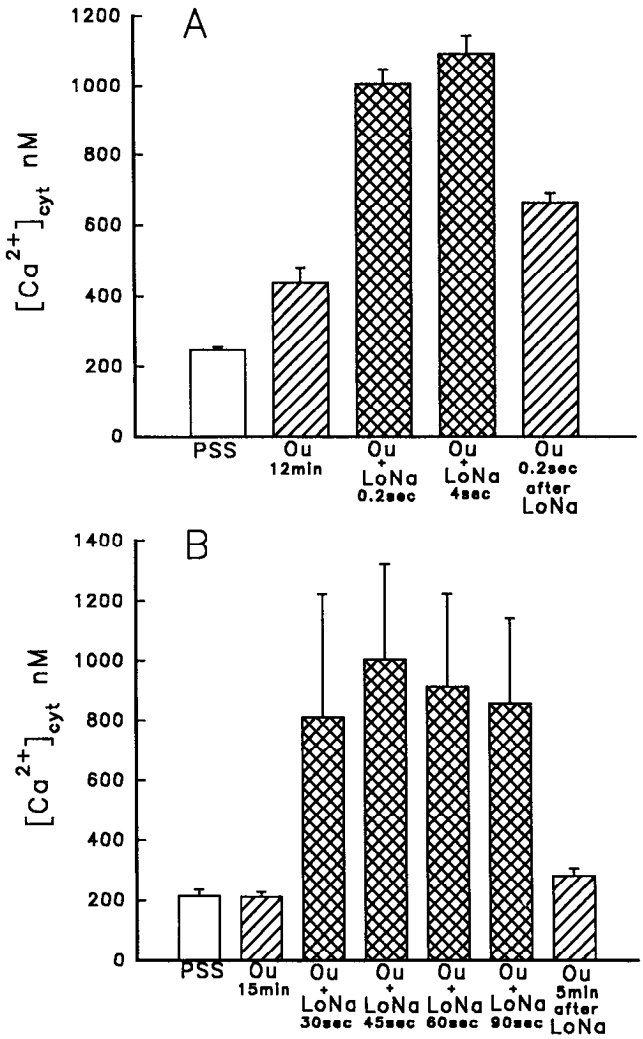

Figure 3. Time course of changes in $[Ca^{2+}]_{cyt}$ upon addition of 1 mM ouabain (*Ou*) to the superfusion medium and subsequent reduction of $[Na^+]_o$ to 6.2 mM (replacement by NMG; *LoNa*). *A*, Rapid time course data from an experiment in which the Real Time Disk system was used to record the data; four consecutive images were averaged to obtain each data point. Note that $[Ca^{2+}]_{cyt}$ rose rapidly (<0.2 sec) when $[Na^+]_o$ was reduced (*Ou + LoNa*), and recovered rapidly (<0.2 sec) when the Na^+ was replaced (*Ou 0.2 sec after LoNa*). *B*, Slower time course, from a different experiment, using standard image acquisition methods, shows that the $[Ca^{2+}]_{cyt}$ elevation is sustained for at least 90 sec in the low-Na^+ media. The data in *A* and *B* correspond to the means (±SE) from three and seven cells, respectively.

increasing $[Na^+]_{cyt}$ (by about 1 mM/min; Golovina et al., 1994) and depolarizing the cells (by about 1.2 mV/min; Walz, 1992). In preliminary experiments, we confirmed that 1 mM ouabain causes a similar depolarization of cultured rat astrocytes.

The data in Figure 2 support the view that the low-$[Na^+]_o$-induced rise in $[Ca^{2+}]_{cyt}$ is normally attenuated by intracellular Ca^{2+} sequestration in the ER. When 30 nM thapsigargin (TG), an ER Ca^{2+} pump inhibitor (Thastrup et al., 1990), was added to the superfusion fluid, $[Ca^{2+}]_{cyt}$ rose to a peak and then declined to a new, elevated steady level, presumably because of uncompensated leak of Ca^{2+} from the ER and increased plasmalemmal Ca^{2+} permeability (Takemura et al., 1989; Baro and Eisner, 1992; Parekh et al., 1993; Randriamampita and Tsien, 1993). With Ca^{2+} sequestration blocked, reduction of $[Na^+]_o$ in the absence of ouabain induced a further rise in $[Ca^{2+}]_{cyt}$ that was significantly larger than under control conditions (see Fig. 1).

This low-$[Na^+]_o$-induced increase in $[Ca^{2+}]_{cyt}$ was not a transient response. $[Ca^{2+}]_{cyt}$ rose rapidly (<0.2 sec; Fig. 3*A*), and generally remained elevated in the low-Na^+ media (Fig. 3*B*), in contrast to the transient Ca^{2+} responses observed with glutamate (Fig. 12 in Blaustein et al., 1991b). Furthermore, $[Ca^{2+}]_{cyt}$ declined rapidly (<0.2 sec) if the external Na^+ was reintroduced within a few min (Fig. 3*A,B*; see also Fig. 1*A*).

Dependence of the low-$[Na^+]_o$-evoked rise in $[Ca^{2+}]_{cyt}$ on Ca^{2+} influx and Ca^{2+} release from intracellular stores

The low-$[Na^+]_o$-induced rise in $[Ca^{2+}]_{cyt}$ was eliminated by removal of external Ca^{2+} (Fig. 4). It was not blocked, however, by the L-type Ca^{2+} channel blocker verapamil (10 μM; not shown), which completely blocks the Ca^{2+} entry induced by K^+ depolarization in mouse astrocytes (V. A. Golovina, personal communication). Thus, even in astrocytes depolarized by ouabain, the low-$[Na^+]_o$-induced Ca^{2+} entry is not mediated by Ca^{2+} channels. These characteristics are all consistent with the view that this external Ca^{2+}- and internal Na^+-dependent rise in $[Ca^{2+}]_{cyt}$ is mediated by an Na/Ca exchanger that can move sufficient amounts of Ca^{2+} across the astrocyte plasmalemma to alter $[Ca^{2+}]_{cyt}$ rapidly (Fig. 3*A*) and markedly, even though most of the entering Ca^{2+} is probably rapidly buffered (as noted above).

Whether all of this low-$[Na^+]_o$-induced rise in $[Ca^{2+}]_{cyt}$ was due to Ca^{2+} entry, or whether some Ca^{2+} was also derived from the Ca^{2+} stores in the ER—perhaps via Ca^{2+}-induced Ca^{2+} release (CICR)—is not clear from the preceding experiments. The fact that TG, either in the absence (Fig. 2) or in the presence of ouabain (not shown), does not prevent the rise in $[Ca^{2+}]_{cyt}$ in response to low $[Na^+]_o$ indicates that the TG-sensitive (and IP_3-releasable) Ca^{2+} store is not the source of this Ca^{2+}. Figure 5 shows that the low-$[Na^+]_o$-induced rise in $[Ca^{2+}]_{cyt}$ also is not due to CICR from a caffeine- and ryanodine-sensitive Ca^{2+}

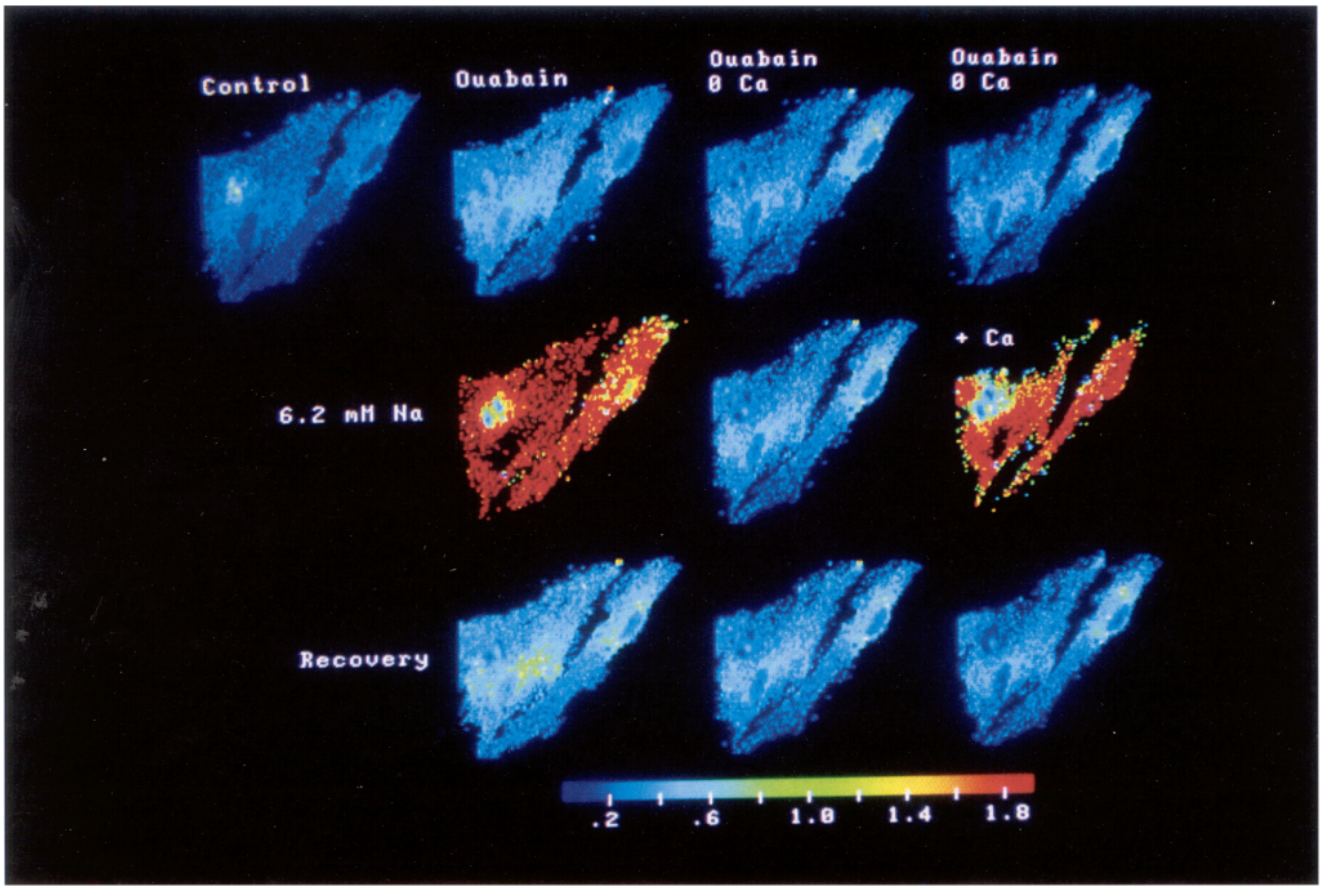

Figure 4. Pseudocolor images showing the changes in $[Ca^{2+}]_{cyt}$ in two astrocytes in response to ouabain, and subsequent reduction of $[Na^+]_o$ in the presence and absence of external Ca^{2+}. A 15 min incubation in PSS containing 1 mM ouabain raised $[Ca^{2+}]_{cyt}$ only slightly (*column 2, top*). Subsequent, brief reduction of $[Na^+]_o$ markedly and reversibly increased $[Ca^{2+}]_{cyt}$ in the presence of external Ca^{2+} (*columns 2 and 4*), but not in its absence (*column 3*). The scale at the bottom indicates the calculated free Ca^{2+} concentration (μM).

store: a large rise in $[Ca^{2+}]_{cyt}$ was observed in the presence of either 10 mM caffeine (Fig. 5*A*) or 10 μM ryanodine (Fig. 5*B*). Thus, most of the low-$[Na^+]_o$-induced rise in $[Ca^{2+}]_{cyt}$ is due to Ca^{2+} entry from the extracellular fluid, and does not come from intracellular Ca^{2+} stores.

Anomalous behavior of cells with a high resting $[Ca^{2+}]_{cyt}$

The cells in two preparations did not conform to the pattern described above. In these instances (Fig. 6), the control, resting $[Ca^{2+}]_{cyt}$ in all cells was substantially higher than in any of the other 18 preparations tested (Fig. 1): 338 ± 14 nM in one experiment (Fig. 6*A*), and 555 ± 23 nM in the other (Fig. 6*B*). Under these conditions of increased $[Ca^{2+}]_{cyt}$, we might anticipate increased Ca^{2+} sequestration as well. In both of these preparations, replacement of most of the external Na^+ by NMG, in the absence of ouabain, caused $[Ca^{2+}]_{cyt}$ to rise markedly and reversibly (Fig. 6). This suggests that the large $[Ca^{2+}]_{cyt}$ response to the removal of external Na^+ is a consequence of prior, partial Ca^{2+} loading (much of it, presumably, into the ER), and is not simply due to the ouabain, per se.

Identification of the astrocyte Na/Ca exchanger protein

The preceding observations demonstrate that reversal of the normal, inwardly directed Na^+ gradient rapidly drives Ca^{2+} into astrocytes. This physiological evidence supports the view that rat cortical astrocytes contain a plasmalemmal Na/Ca exchanger. To substantiate this, we probed the membrane proteins from cultured astrocytes with antiserum raised against the purified canine cardiac Na/Ca exchanger (Ambesi et al., 1991a).

Figure 7, *A* and *B*, shows Western blots of purified canine cardiac sarcolemmal proteins (lanes *A1* and *B1*) and microsomal membrane proteins from cultured rat astrocytes (lane *A2*). The polyclonal antiserum specifically cross-reacted with astrocyte membrane proteins as well as with cardiac sarcolemmal proteins of about 120 and 70 kDa. Rat brain synaptic plasma membrane peptide bands of similar molecular weight are also specifically labeled with this antiserum (Fig. 7, lane *B2*; see also Luther et al., 1992; Yip et al., 1992); in the experiment of Figure 7, lanes *B1* and *B2*, the antiserum did not cross-react with a 70 kDa peptide, which is believed to be a proteolytic fragment of the exchanger (Nicoll et al., 1990). The preimmune serum did not cross-react with any of these proteins (not shown; see Luther et al., 1992; Yip et al., 1992).

Identification of the Na/Ca exchanger message in astrocytes

Figure 8 shows a Northern blot of total RNA from freshly isolated rat heart (lane 1), from cultured rat astrocytes (lane 2), and from rat brain homogenate (lane 3). The blot was probed with an 800 bp cDNA that codes for the 3′ region of the Na/Ca exchanger from rat heart (Kofuji et al., 1992). Under medium-stringency conditions, the probe hybridized to a 7 kb mRNA from all three rat tissues, and all of these 7 kb signals

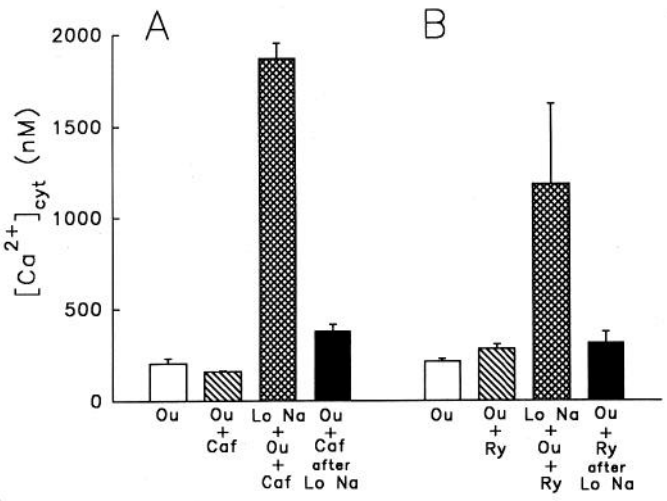

Figure 5. The effects of $[Na^+]_o$ reduction (to 6.2 mM; *Lo Na*) on $[Ca^{2+}]_{cyt}$ in ouabain-treated astrocytes in the presence of 10 mM caffeine (*A, Caf*) and 10 μM ryanodine (*B, Ry*). The cells were incubated in PSS containing 1 mM ouabain for at least 15 min prior to the treatments with the Ca^{2+} releasing agents and exposure to the low-$[Na^+]_o$ media. The recovery in PSS containing ouabain and the releasing agents (*Ou + Caf or Ry, after Lo Na*) is also shown. Each panel shows the responses of cells in a single preparation (*A*, three cells; *B*, seven cells); error bars indicate ±SE.

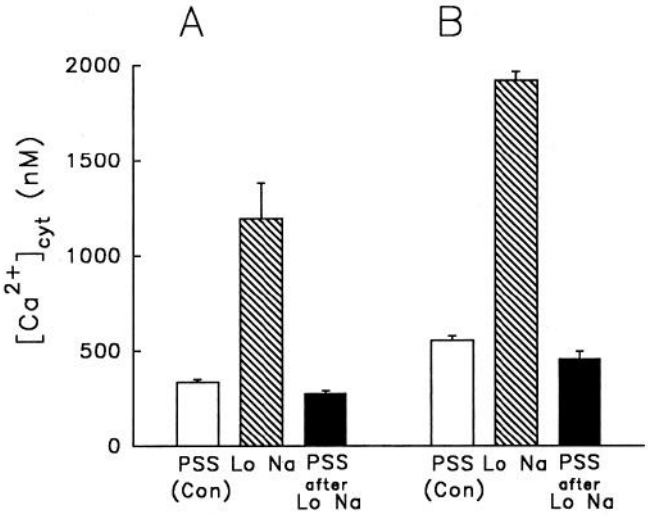

Figure 6. The effects of $[Na^+]_o$ reduction (*Lo Na*) on $[Ca^{2+}]_{cyt}$ in two astrocyte preparations (*A*, five cells; *B*, 10 cells) in which the initial resting $[Ca^{2+}]_{cyt}$ was unusually high (compare Fig. 1). The effects of $[Na^+]_o$ reduction (*Lo Na*) were reversible, and $[Ca^{2+}]_{cyt}$ returned to the control level when external Na^+ was replaced (*PSS after Lo Na*).

were strong. In addition, the probe hybridized to smaller (3.5 kb) and larger (about 14 kb) messages from the brain. A 7 kb mRNA has been identified as the full-sized message that encodes the canine cardiac Na/Ca exchanger (39); thus, our data suggest that the astrocyte exchanger is encoded by a similar-sized message. The larger and smaller messages from rat brain (also observed in human brain by Kofuji et al., 1992) may be the result of alternative splicing.

Localization of the Na/Ca exchanger in astrocytes by immunofluorescence microscopy

These studies demonstrate that rat cortical astrocytes have an Na/Ca exchanger that is similar in molecular size and immunological cross-reactivity to the Na/Ca exchanger of cardiac muscle. Thus, antiserum raised against the cardiac exchanger may be used with immunofluorescence techniques to determine the distribution of the Na/Ca exchanger in cultured astrocytes.

Affinity-purified anti-Na/Ca exchanger antibodies (Juhaszova et al., 1994) were employed to study the distribution of the exchanger on the surfaces of astrocytes. The astrocytes in Figure 9*A* were incubated with antibodies raised against the cardiac Na/Ca exchanger. All cells in the cultures were labeled, and the label was distributed over the entire surface of each of the cells. The labeling pattern appears to be diffuse (unorganized) in some areas. In other areas, however, the labeling gives the appearance of strings of beads arranged in a reticular network (Fig. 9*A*, arrows). The labeling is specific, as indicated by the absence of labeling when cells are incubated with similarly prepared preimmune serum (Fig. 9*B*).

Discussion

Astrocytes contain an Na/Ca exchanger

The experiments reported here demonstrate that cultured rat cortical astrocytes contain a plasmalemmal Na/Ca exchange mechanism that can mediate rapid changes in $[Ca^{2+}]_{cyt}$ when the Na^+ electrochemical gradient is altered. In this respect, astrocytes do not differ substantially from other cell types such as neurons, cardiac myocytes, smooth muscle cells, and many epithelial cells (Blaustein et al., 1991a). A specific, rather complex protocol is required to demonstrate increased $[Ca^{2+}]_{cyt}$ mediated by the Na/Ca exchanger. This explains previous reports of failure to observe increased $[Ca^{2+}]_{cyt}$ induced by lowered $[Na^+]_o$ alone. In order to observe a rise in $[Ca^{2+}]_{cyt}$, it is necessary to raise $[Na^+]_{cyt}$ (e.g., by inhibiting the Na pump) and to lower

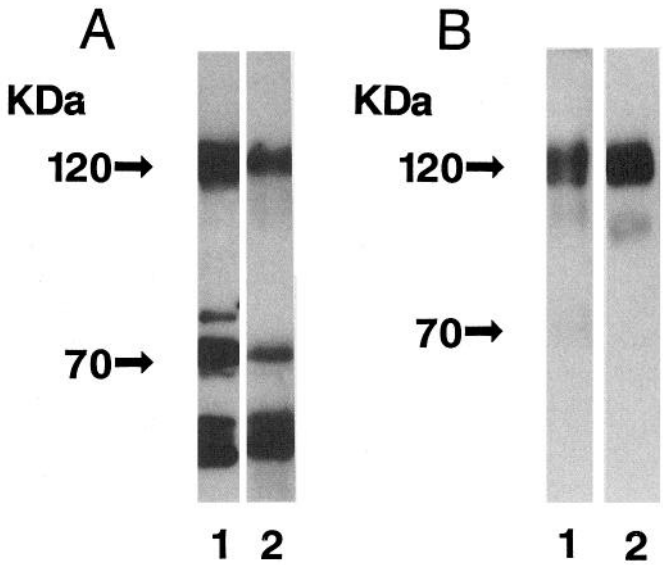

Figure 7. Western immunoblots of purified canine cardiac sarcolemmal proteins (*lanes A1 and B1*; 5 μg), cultured rat astrocyte microsomal membrane proteins (*lane A2*; 100 μg/lane) and rat brain synaptic plasma membrane (SPM) proteins (*lane B2*; 20 μg). *A* and *B* are from different experiments. The proteins were probed with antibodies raised against the purified canine cardiac sarcolemmal Na/Ca exchanger. The *arrows* at the left in *A* and *B* indicate the calculated positions of the 120 and 70 kDa bands.

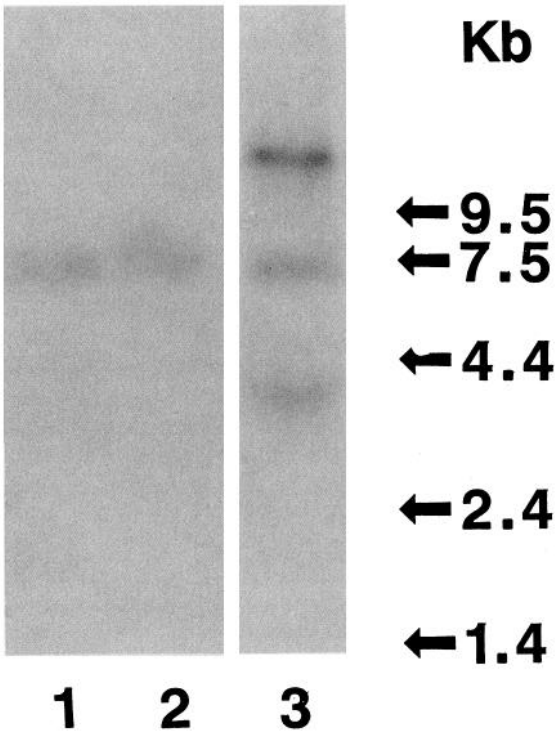

Figure 8. Northern blot of total RNA from freshly isolated rat heart (*lane 1*; 5 μg), cultured rat astrocytes (*lane 2*; 20 μg), and rat brain homogenate (*lane 3*; 20 μg). The RNA was probed with a cDNA containing 800 base pairs that codes for the 3′ region of the rat heart Na/Ca exchanger. The *arrows* at the right indicate the positions of the standards in kilobases (*Kb*).

extracellular [Na^+] simultaneously. Ouabain may contribute to this effect, not only by raising $[Na^+]_{cyt}$ (Golovina et al., 1994), but also by partially depolarizing the astrocytes (Walz, 1992) and thereby lowering the Na^+ electrochemical gradient even further. We suggest that these maneuvers promote net Ca^{2+} entry via the exchanger by reducing or reversing the Na^+ electrochemical gradient. Kinetically, high $[Na^+]_{cyt}$, low $[Na^+]_o$, and membrane depolarization would all be expected to accelerate Ca^{2+} influx and reduce Ca^{2+} efflux. Indeed, the large rise in $[Ca^{2+}]_{cyt}$ caused by these maneuvers is prevented by removal of extracellular Ca^{2+}. We emphasize that both increased $[Na^+]_{cyt}$ and lowered $[Na^+]_o$ are required for substantial increases in $[Ca^{2+}]_{cyt}$; either manipulation alone is ineffective. The reason is that the ER is able to buffer most of the Ca^{2+} entering in response to either elevated $[Na^+]_{cyt}$ or reduced $[Na^+]_o$ but is incapable of handling the very large amounts of Ca^{2+} entering in response to both maneuvers. This is supported by the observation that, in the presence of thapsigargin, an inhibitor of Ca^{2+} accumulation into IP_3-sensitive intracellular stores, lowering $[Na^+]_o$ induced a large increase in $[Ca^{2+}]_{cyt}$ similar in magnitude to that observed in the presence of ouabain.

In addition to the physiological results described above, we present biochemical evidence for the presence of an Na/Ca exchanger in cortical astrocytes. Northern blot analysis using a probe specific for the canine heart Na/Ca exchanger revealed a similar labeling pattern to that observed using RNA from rat heart and fetal rat brain. This suggests that Na/Ca exchangers in astrocytes have a similar molecular structure to those in heart and neurons. This conclusion is supported by Western blot data using antibodies raised against the heart Na/Ca exchanger; the antibody labeled similar bands in gels loaded with astrocyte, heart, and synaptic membranes. As both Northern and Western blot analyses indicate, Na/Ca exchangers from astrocytes are very similar in molecular structure to those in neurons, cardiac myocytes, and smooth muscle cells. This class of Na/Ca exchangers differs both structurally and functionally from the Na/Ca exchanger in photoreceptors (Reiländer et al., 1992). Fluorescence immunohistochemistry using antibodies raised against the heart Na/Ca exchanger revealed that virtually all astrocytes were labeled; however, the label was not uniformly distributed over the cell. Rather, at least in some areas, the exchanger was localized in reticular networks, possibly following underlying cytoskeletal structures. Distribution of the Na/Ca exchanger in similar reticular networks has been observed in smooth muscle cells, where it has been suggested that the plasmalemmal exchanger molecules may be concentrated in regions where the endoplasmic/sarcoplasmic reticulum (ER/SR) is closely apposed to the plasmalemma (Moore et al., 1993; Juhaszova et al., 1994). This may promote functional coupling between the ER/SR and the plasmalemma in terms of cell Ca^{2+} regulation (Blaustein, 1993; Moore and Fay, 1993).

Functional coupling of Na/Ca exchange to intracellular Ca^{2+} stores

A modest reduction in the net extrusion of Ca^{2+} via the Na/Ca exchanger does not substantially increase $[Ca^{2+}]_{cyt}$, suggesting that much of the Ca^{2+} entering the cell under these conditions is accumulated in intracellular stores. The observation that TG mimics the effect of ouabain in potentiating the action of reduced $[Na^+]_o$, and itself raises $[Ca^{2+}]_{cyt}$, suggests that an IP_3-sensitive Ca^{2+} store is largely responsible for this Ca^{2+} buffering activity. Further support for this idea comes from the observation that pretreatment of mouse cortical astrocytes with ouabain, K^+-free media, or reduced $[Na^+]_o$ greatly potentiates the magnitude of the Ca^{2+} transient induced by TG or cyclopiazonic acid (Golovina et al., 1993, 1994), which block Ca^{2+} uptake and lead to net Ca^{2+} leak from IP_3-sensitive stores. Thus, the initial consequence of reducing the effectiveness of Ca^{2+} extrusion via the Na/Ca exchanger is loading of the intracellular stores rather than a significant rise in $[Ca^{2+}]_{cyt}$, which might be cytotoxic (see below).

Release of Ca^{2+} from intracellular stores plays a role in a variety of cellular responses, including several that have been described in rodent astrocytes (Finkbeiner, 1993). For example, many neurotransmitters, including the excitotoxin glutamate, increase $[Ca^{2+}]_{cyt}$ in cultured astrocytes. Any such responses involving Ca^{2+} release from thapsigargin- (and IP_3-) sensitive stores should be greatly augmented when Ca^{2+} extrusion by the Na/Ca exchanger is compromised. For example, we have directly demonstrated that ouabain potentiates glutamate-induced increases in $[Ca^{2+}]_{cyt}$ in rat astrocytes (Blaustein et al., 1991b).

Recent studies have revealed that intercellular signaling takes places between cultured astrocytes by means of transient increases in $[Ca^{2+}]_{cyt}$ that propagate from cell to cell as waves of elevated $[Ca^{2+}]_{cyt}$. Such Ca^{2+}-mediated intercellular signaling may operate in parallel with the rapid interneuronal signaling mediated by action potentials and synaptic transmission. Charles et al. (1991) have demonstrated that such propagated Ca^{2+} waves require cyclical Ca^{2+} accumulation into and release from both IP_3 and Ca^{2+}-sensitive (caffeine/ryanodyne-sensitive) stores. Our

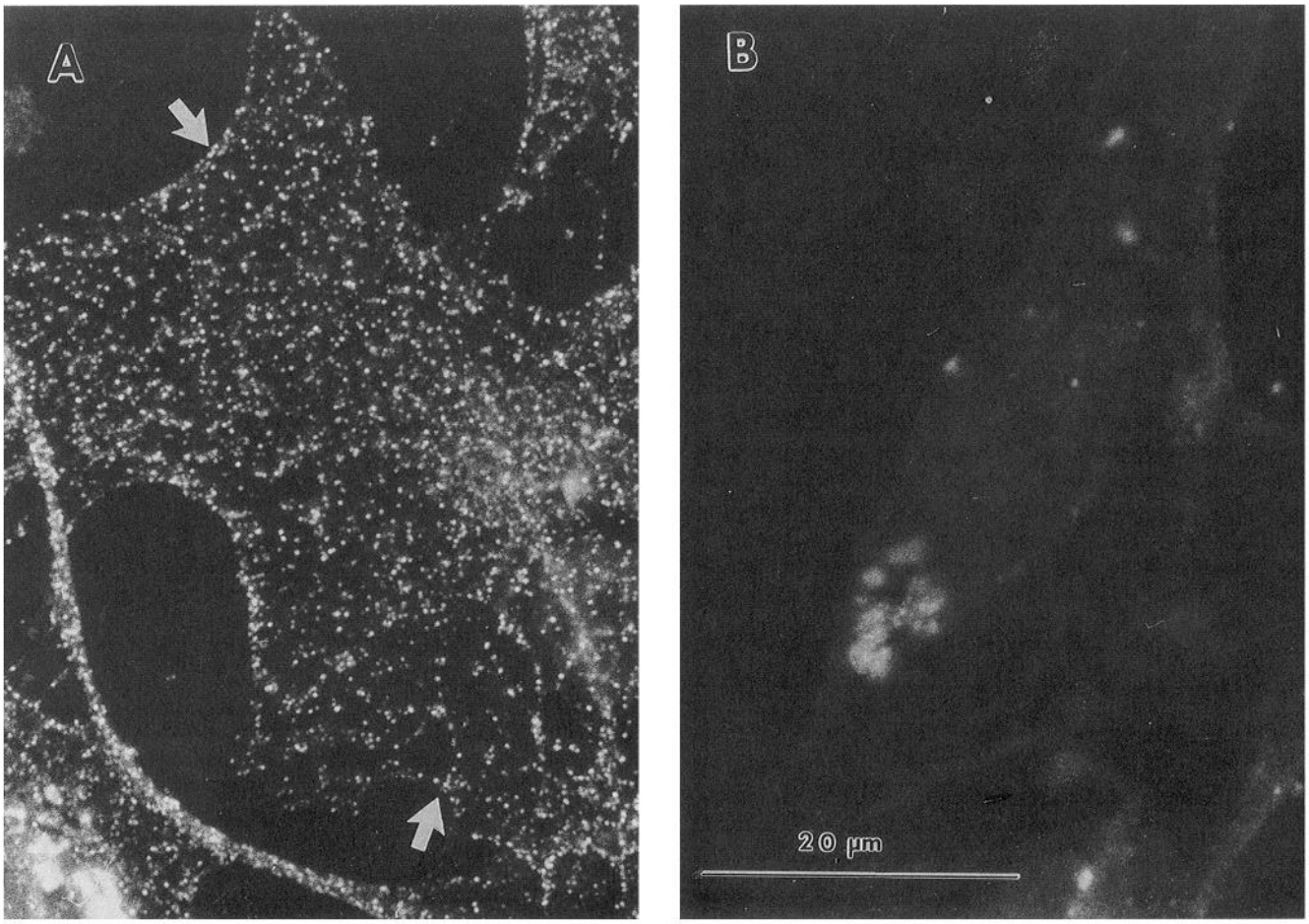

Figure 9. Immunochemical localization of Na/Ca exchangers in cultured rat brain astrocytes. The cell in *A* was probed with affinity-purified antibodies raised against purified canine cardiac sarcolemmal Na/Ca exchanger. The cell in *B* was probed with similarly prepared preimmune serum. The *arrows* in *A* point to regions of the cell in which the label has a distinct reticular pattern. Scale bar (in *B*), 20 μm (for *A* and *B*).

results suggest that the efficacy of Ca^{2+} extrusion by the Na/Ca exchanger might differentially affect loading of the IP_3-sensitive store and thus affect properties of the propagated Ca^{2+} waves such as magnitude, speed, or distance traveled.

Extracellular signals that might affect Ca^{2+} entry and loading of intracellular stores

Our data indicate that any of a variety of conditions that affect the maintenance of the transmembrane Na^+ electrochemical gradient will alter the efficacy of the Na/Ca exchanger and hence will affect both $[Ca^{2+}]_{cyt}$ and the amount of Ca^{2+} stored in and released from intracellular organelles. Conditions that depolarize the plasma membrane (e.g., elevated $[K^+]_o$ in the vicinity of active neurons) would increase net Ca^{2+} influx directly, by opening voltage-gated Ca^{2+} channels, and indirectly, by slowing Ca^{2+} extrusion via the (electrogenic) Na/Ca exchanger or by increasing Na^+ influx though noninactivated Na^+ channels. Recently, ouabain has been shown to be a vertebrate adrenocortical hormone (Hamlyn et al., 1991) but ouabain itself (Yamada et al., 1992), or an analog (Tymiak et al., 1993), may also be produced and secreted in the brain. Thus, inhibition of the Na^+ pump by endogenous ouabain may, via its indirect effect on Na/Ca exchange, play a physiological role in the control of intracellular Ca^{2+} stores in astrocytes and other brain cells.

Do astrocytes buffer Ca^{2+} in the extracellular space surrounding synapses?

Most central synapses are surrounded by astrocytes so that there is only a very limited extracellular space. Therefore, in view of the critical role of Ca^{2+} entry in neurotransmitter release (Katz, 1969), it is important to consider how the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is regulated in this very restricted space. Our evidence that the astrocyte Na/Ca exchanger can transport considerable amounts of Ca^{2+} across the plasmalemma, in both directions, and that it can help to modulate the amount of Ca^{2+} in astrocyte intracellular stores, raises a new possibility. Perhaps, astrocytes can act as sources and sinks for Ca^{2+}, so that they can help to buffer $[Ca^{2+}]_o$ in the synaptic clefts, much as they are thought to buffer $[K^+]_o$. For example, the astrocytes may extrude Ca^{2+} into the extracellular space when they are stimulated to release Ca^{2+} from intracellular stores and $[Ca^{2+}]_{cyt}$ rises transiently. The astrocytes may thereby help to replenish extracellular Ca^{2+} in the interstitial space surrounding synapses when the neurons are sufficiently active to deplete extracellular Ca^{2+} A related possibility is that the Na/Ca exchanger in the astrocytes may maintain the steady-state perisynaptic $[Ca^{2+}]$ at a lower level than that in the CSF in general. Thus, astrocytes, by controlling $[Ca^{2+}]_o$, may dynamically regulate the efficacy of adjacent synapses.

Possible role of astrocyte Na/Ca exchange system in neurodegeneration

Recent studies have suggested that $[Ca^{2+}]_{cyt}$ plays more than one critical role in cell survival. The central role of elevated $[Ca^{2+}]_{cyt}$ in cell death resulting from neurotoxins, ischemia, and tissue damage is well documented (Mattson et al., 1989; Stys et al., 1990; Siesjó, 1991; Choi, 1992). Indeed, it has been demonstrated that inhibition of the Na^+ pump by ouabain increases the susceptibility of neurons to excitotoxic death (Brines and Robbins, 1992). Thus, the failure of intracellular stores to buffer a Ca^{2+} load adequately (Fig. 1) when the Na/Ca exchanger is compromised (by reducing the Na^+ electrochemical gradient) may be the correlate of this ouabain-induced susceptibility to excitotoxicity. In contrast, $[Ca^{2+}]_{cyt}$ must be maintained above a threshold level for normal cell viability. According to the Ca^{2+} setpoint hypothesis (Franklin and Johnson, 1992) neurons with abnormally low $[Ca^{2+}]_{cyt}$, have an increased requirement for survival factors. Although astrocytes may be less sensitive than neurons to excitotoxic death induced by Ca^{2+} overload (Choi et al., 1987), it seems likely that, like neurons, astrocytes require that $[Ca^{2+}]_{cyt}$ be maintained within a certain critical range for optimum viability and function. The Na/Ca exchanger, coupled to releasable intracellular Ca^{2+} stores, is likely to play a central role in maintaining $[Ca^{2+}]_{cyt}$ within this range, both in the steady state and during active signaling by both neurons and astrocytes.

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