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Astrocyte Na^+ Channels Are Required for Maintenance of Na^+/K^+-ATPase Activity

H. Sontheimer,[1,2] E. Fernandez-Marques,[1] N. Ullrich,[3] C. A. Pappas,[1] and S. G. Waxman[1,2]

[1]Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510, [2]Neuroscience Research Center, VA Hospital, West Haven, Connecticut 06516, and [3]Interdepartmental Neuroscience Graduate Program, Yale University School of Medicine, New Haven, Connecticut 06510

Astrocytes *in vitro* and *in situ* have been shown to express voltage-activated ion channels previously thought to be restricted to excitable cells, including voltage-activated Na^+, Ca^{2+}, and K^+ channels. However, unlike neurons, astrocytes do not generate action potentials, and the functional role of voltage-activated channels in astrocytes has been an enigma. In order to study the function of Na^+ channels in glial cells, we carried out ion flux measurements, patch-clamp recordings, and ratiometric imaging of $[Na^+]_i$ during blockade of Na^+ channels on rat spinal cord astrocytes cultured for 7–10 d. Acute blockade of astrocyte Na^+ channels by TTX had multiple effects: (1) TTX reduced, in a dose-dependent manner, Na^+/K^+-ATPase activity measured as unidirectional influx of $^{86}Rb^+$; (2) TTX depolarized astrocyte membrane potential at a rate of approximately 1 mV/min; (3) TTX (100 μM) reduced $[Na^+]_i$; and (4) prolonged exposure to micromolar TTX induced astrocyte death. All these effects of TTX could be mimicked by ouabain or strophanthidin, specific blockers of the Na^+/K^+-ATPase. The effects of TTX and ouabain (or strophanthidin) were not additive. These results suggest that TTX-blockable Na^+ channels in glial cells serve functions that do not require their participation in action potential electrogenesis; in particular, we propose that glial Na^+ channels constitute a "return" pathway for Na^+/K^+-ATPase function, which permits Na^+ ions to enter the cells to maintain $[Na^+]_i$ at concentrations necessary for activity of the Na^+/K^+-ATPase. Since astrocyte Na^+/K^+-ATPase is believed to participate in $[K^+]_o$ homeostasis in the CNS, the coupling of Na^+ flux through voltage-activated Na^+ channels to ATPase activity may provide a feedback loop that participates in the regulation of K^+ ion levels in the extracellular space.

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Correspondence should be addressed to Harald Sontheimer, Ph.D., Department of Neurology, Yale University, School of Medicine, 333 Cedar Street, LCI 704, New Haven, CT 06510.

Numerous studies on glial cells *in vitro* and *in situ* have demonstrated that astrocytes can express voltage-activated ion channels with features similar to channels expressed in excitable cells (for review, see Barres et al., 1990; Chiu 1991; Ritchie, 1992). While the importance of glial K^+ channels in controlling $[K^+]_o$ homeostasis has been demonstrated *in vitro* and *in situ* (Orkand, 1977; Newman, 1984, 1986; Ballanyi et al., 1987), the functional roles of voltage-activated Ca^{2+} and Na^+ channels in glial cells are less well understood. Although action potential-like responses mediated by Ca^{2+} (MacVicar, 1984) or Na^+ channels (Sontheimer et al., 1992) can be elicited in cultured astrocytes under certain experimental conditions, most glial cells *in vitro* (Sontheimer and Waxman, 1992) and *in situ* (Ransom and Goldring, 1973; Somjen, 1975; Walz and MacVicar, 1988; Sontheimer and Waxman, 1993) lack the ability to fire action potentials. Recent patch-clamp recordings from astrocytes in hippocampal brain slices demonstrated that astrocytes *in situ,* as previously shown *in vitro*, can express voltage-activated Na^+ channels (Sontheimer and Waxman, 1993). Furthermore, astrocytes *in situ* express Na^+ channel immunoreactivity (Black et al., 1989), thus decreasing concern that these channels are only seen in cell culture. Nevertheless, these observations have not delineated the functional roles of glial Na^+ channels.

It is now clear that some subclasses of astrocytes, namely, process-bearing astrocytes (Barres et al., 1988, 1989b; Sontheimer et al., 1991a, 1992), can express TTX-sensitive (TTX-S) Na^+ channels with kinetic features comparable to channels expressed in most neurons. In contrast, flat, non-process-bearing astrocytes, which typically represent the majority of cells in such cultures (~95%), express TTX-resistant (TTX-R) Na^+ channels (Bevan et al., 1985; Nowak et al., 1987; Sontheimer and Waxman, 1992; Sontheimer et al., 1992) with kinetic features that differ from those of neuronal Na^+ channels (Barres et al., 1989b; Sontheimer and Waxman, 1992). Recent molecular cloning approaches provide further evidence that these astrocytes may express a unique "glial form" of TTX-R Na^+ channel (Gautron et al., 1992), termed Na-g. The functional role of Na-g is not known.

Na^+ channels, both TTX-S and TTX-R, require large changes in the transmembrane voltage to be fully activated. It is questionable whether glial cells ever experience voltage changes sufficient for voltage-dependent gating of Na^+ channels, which requires depolarizations in excess of 40 mV in most cells. At least four factors mitigate against action potential electrogenesis in astrocytes, despite the presence of voltage-activated Na^+ channels. (1) Most glial cells express Na^+ channels at only low den-

sities (<1 channel/μm^2; Table 1 in Sontheimer, 1992). (2) The high gK:gNa ratio of astrocytes retards electrogenesis (Sontheimer et al., 1991b). (3) As a result of coupling to adjacent cells via gap junctions (Connors et al., 1984; Kettenmann and Ransom, 1988; Mobbs et al., 1988; Dermietzel et al., 1991) each astrocyte within the syncytium is "clamped" close to the population potential, inhibiting large depolarizations. (4) In the few astrocytes that express high densities of Na^+ channels (2–8 channels/μm^2; Sontheimer and Waxman, 1992; Sontheimer et al., 1992), most Na^+ channels are inactivated due to a mismatch between steady-state inactivation and resting potential (Sontheimer and Waxman, 1992).

Given these concerns, we set out to investigate whether voltage-activated Na^+ channels in glial cells could serve functions that do not require their participation in action potential electrogenesis. We chose to study spinal cord astrocytes, which express Na^+ channels in uniquely high densities *in vitro* (2–8 channels/μm^2), about 1–2 orders of magnitude higher than in other astrocytes (Sontheimer et al., 1992). The data presented here provide evidence that a TTX-inhibitable Na^+ conductance exists in these astrocytes at their resting potential, and that Na^+ ion fluxes mediated by Na^+ channels are important for operation of the astrocyte Na^+/K^+-ATPase. Blockage of this "return pathway" for Na^+ ions reduces $[Na^+]_i$, and inhibits ATPase activity, and prolonged exposure (30–120 min) leads to cell death, suggesting that the presence of Na^+ channels is vital for these cells.

Materials and Methods

Cell cultures

Astrocytes. Cultures were obtained from neonatal Sprague–Dawley rats as described elsewhere (Black et al., 1993). In brief, rat pups were deeply anesthetized by CO_2 narcosis and decapitated. Spinal cords were dissected free from mid-cervical to lower lumbar levels and the meninges removed. The cords were minced and incubated in an enzyme solution containing Earle's salts, 30 U/ml papain (Worthington), 0.5 mM EDTA, and 1.65 mM L-cysteine for 30 min at 37°C. The tissue was triturated in complete medium [Earle's minimal essential medium containing 10% fetal bovine serum (Hyclone), penicillin/streptomycin (500 U/ml each) and 20 mM glucose] containing trypsin inhibitor and bovine serum albumin (BSA) (each 1.5 mg/ml), and the cell suspension was plated onto polyornithine/laminin-coated 12 mm circular glass coverslips at a density of 2.5×10^5/ml. The cells were maintained at 37°C in a 5% $C0_2$, 95% air atmosphere and were fed every second day with complete media.

C6 glioma cells. C6 rat astrocytoma cell lines were purchased from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium plus 10% heat-inactivated fetal calf serum (GIBCO). Cells were plated on uncoated 12 mm glass coverslips and were used for uptake studies 2 d after plating.

DRG neurons. Dorsal root ganglion (DRG) neurons were prepared as previously described (Roy and Narahashi, 1992). In brief, 7-d-old Sprague–Dawley rats were anesthetized with sodium pentobarbital (i.p., 0.4 ml at 3 mg/ml). The vertebral column was removed and cut longitudinally, generating two hemisections that were placed in sterile Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). The ganglia were isolated from the spinal lumen and incubated for 20 min in trypsin (2 mg/ml, 36°C). Cells were rinsed twice with PBS and were mechanically isolated by trituration, and were plated on polylysine-coated 12 mm glass coverslips.

Cell identification

Two morphologically distinguishable subtypes of astrocytes (Sontheimer et al., 1992) were used for recordings. (1) *Pancake astrocytes* are round, non-process-bearing cells with large somata. Their unique morphology makes them easy to identify. (2) *Stellate astrocytes* have round, generally small somata and extensive processes that are often arborized. These cell types were initially identified immunohistochemically as glial fibrillary acidic protein (GFAP)-positive astrocytes [e.g., as described by Black et al. (1993) and Sontheimer et al. (1991b, 1992)]. A2B5 antibodies failed to distinguish the two subtypes of astrocytes reliably, suggesting that they may differ from the astrocyte lineage in optic nerve (Miller and Szigeti, 1991; Sontheimer et al., 1992; Black et al., 1993). Flux studies were obtained from astrocyte populations grown on glass coverslips or 24-well plates. Those cell populations were used after 7–10 d *in vitro* (DIV) and were >98% $GFAP^+$ astrocytes. They contained approximately 5–10% astrocytes with stellate, process-bearing morphology and 90–95% flat, non-process-bearing astrocytes. The time window of 7–10 DIV was selected for study because our previous electrophysiological studies indicated that spinal cord astrocytes express highest densities of Na^+ channels during this time period (Sontheimer et al., 1992).

Electrophysiology

Current and voltage recordings were obtained using the whole-cell mode of the patch-clamp technique (Hamill et al., 1981) in both voltage-clamp and current-clamp mode. Patch pipettes were made from thin-walled borosilicate glass (World Precision Instruments, TW150F-40; o.d. 1.5 mm, i.d. 1.2 mm). Electrodes were filled with a solution containing, in mM, KCl, 145; $MgCl_2$, 1; $CaCl_2$, 0.2; EGTA, 10; HEPES, 10; pH adjusted to 7.4 using Tris. Recordings were made on the stage of an inverted microscope (Nikon Diaphot) equipped with Hoffman modulation contrast optics and epifluorescence. Cells were continuously superfused with bicarbonate-buffered saline solution at room temperature, allowing for rapid (<20 sec) exchange of the bath volume. The bath solution contained, in mM, NaCl, 122.6; KCl, 5.0; $MgSO_4$, 1.2; $CaCl_2$, 1.0; Na_2HPO_4, 2.0; NaH_2PO_4, 0.4; $NaHCO_3$, 25.0; Na_2SO_4, 1.2; glucose, 10.5; bubbled with 5:95% CO_2:O_2. Additional recordings were also obtained in HEPES-buffered solution, and these yielded similar results, but were not included in the analysis.

Current and voltage recordings were obtained using an Axopatch-1D amplifier (Axon Instruments). Voltage signals were low-pass filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices), and then digitized on line at 1 kHz using a Labmaster TL-125 digitizing board (Axon Instruments) interfaced with an IBM-compatible computer (Gateway 486, Gateway 2000 Inc.). Current signals were filtered at 3 kHz and digitized at 100 kHz using the above equipment. Cell capacitance was measured as described previously (Sontheimer et al., 1991b). Data acquisition was performed using either AXOTAPE or PCLAMP (Axon Instruments). Data were analyzed using a scientific graphing program (ORIGIN, MicroCal), and slopes of voltage changes were obtained from linear regression analysis. Na^+ current densities were derived from peak currents by dividing peaks by membrane capacitance. Peak Na^+ currents were evoked in response to a step from −110 mV to −10 mV.

$^{86}Rb^+$ flux studies

$^{86}Rb^+$ was used to measure Na^+/K^+-ATPase activity. $^{86}Rb^+$ ions are taken up by the Na^+/K^+-ATPase like K^+ ions, but have the advantage of a much longer half-life than $^{42}K^+$ (Kimelberg and Mayhew, 1975). Thus, measurement of the unidirectional ouabain-sensitive $^{86}Rb^+$ uptake provides a quantitative method for assaying Na^+/K^+-ATPase activity. Astrocytes used for flux studies were grown in 24-well trays and were used after 7–10 DIV. The growth media were removed by aspiration and the cells were washed three times with a normal balanced salt solution containing, in mM, NaCl, 137; KCl, 5.3; $MgCl_2$, 1; $CaCl_2$, 3; dextrose, 25; HEPES, 10; pH 7.2. A 0.5 ml aliquot of the same buffer containing 5 μCi of $^{86}Rb^+$ was added to each well (at 37°C). Following a 15 min incubation with the isotope, the media were aspirated from each well and cells were washed three times with 0.5 ml of ice-cold 0.29 M mannitol containing 10 mM Tris nitrate and 0.5 mM $Ca(NO_3)_2$ at pH 7.4. The cell monolayer from each well was then solubilized in 0.5 ml of 1 *N* NaOH at room temperature for 20 min. The cellular content of the isotope was measured by liquid scintillation counting (counted by Cerenkov radiation), and cell protein was determined using the bicinchoninic acid protein assay (Smith et al., 1985) following the modifications described by Goldschmidt and Kimelberg (1989) for cultured cells. Counts were normalized as dpm/μl•μg protein. $^{86}Rb^+$ fluxes were determined in the presence and absence of the following drugs: TTX (100 nM to 100 μM; Sigma), strophanthidin (1–5 mM; Sigma), ouabain (1–5 mM; Sigma), 4-aminopyridine (4-AP; 2 mM; Sigma), and Ba^{2+} (1 mM; Sigma), and combinations of these drugs. For comparison of experiments under these various conditions, values were normalized to

their untreated controls, and changes in flux were expressed as percentage of control.

Viability studies

Cell viability was examined by monitoring the cells' ability to exclude trypan blue (0.03%; Sigma; procedure as described by Sigma, *Biochemicals and Organic Compounds*, 1992, pp 1330–1331). Cell counts were also made on sister cultures, paired with the ones used for flux studies, which were cultured and treated identically. For each condition, cells from at least 10 randomly selected regions of at least three wells were counted under phase contrast at 200× magnification using an Olympus CK2 microscope, and the numbers of viable and nonviable cells were determined in each region. Using this protocol cell viability was assayed for untreated control cells and for cells maintained in the presence of 100 nM to 100 μM TTX or 1–5 mM strophanthidin for 0, 5, 15, 60, and 120 min. To assure that those trypan blue exclusion studies were a valid indication for cell viability, we additionally studied cell viability using a new two-color fluorescence cell viability assay (Molecular Probes, "Live/Dead") that permits the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability, intracellular esterase activity and plasma membrane integrity (Moore et al., 1990). The concentrations of fluorophores used were ethidium homodimer at 2 μM and calcein acetoxymethyl ester (AM) at 1 μM.

Ratiometric imaging of $[Na^+]_i$ using SBFI

Cytosolic free Na^+ was measured using of a recently developed indicator for Na^+, SBFI (sodium-binding benzofuran isophthalate; Harootunian et al., 1989). Astrocytes were cultured as described above but on 24 × 24 mm square coverslips that doubled as the bottom of a flow-through perfusion chamber. The membrane-permeable acetoxymethyl ester form of SBFI (SBFI/AM; Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM. Before introduction to cells the dye was mixed 1:1 with 25% Pluronic F-127 (Molecular Probes). Final concentration of the dye was 5 μM, and cells were loaded for 90 min at room temperature. Recordings were obtained on the stage of a Nikon Diaphot microscope using a commercially available ratiometric imaging setup (Georgia Instruments). Excitation light was provided by a two-channel monochrometer containing separate xenon arc lamps and defraction gradients for both wavelengths used (340:385 nm). Excitation light was passed to the cells through a dichroic reflector (400 nm) and emitted light above 450 nm was collected. Fluorescence signals were amplified by an image intensifier (GenIISyS) and collected with a CCD camera (CCD72, Dage) interfaced to a frame grabber (Matrox). Custom software controlled both excitation source and collection of images, and mean pixel intensity of cells of interest was stored to disk separately for 340 and 385 nm. To account for fluorescence rundown, the fluorescence ratio 340:385 was used to assay cytosolic free Na^+. Cells were continuously perfused at room temperature at a rate of 2 ml/min using the same saline as used for electrophysiology. Full exchange of the bath fluid required 12 sec. For calibration of fluorescence signals cells were superfused with saline containing 10 mM Na^+ with all residual Na^+ replaced by *N*-methyl-D-glucamine, in the presence of the ionophore gramidicidin D (5 μM in DMSO). Cells did not tolerate gramidicidin well, and thus our attempts to calibrate them were limited to a single Na^+ concentration at the end of every experiment. Prior to application of gramidicidin, we applied a 0 Na^+ bath solution that had been demonstrated to deplete intracellular Na^+ completely in other cells (Negulescu et al., 1990). Assuming that this represented the maximal effect, we expressed our data as percentage of 340:385 ratio as compared to the same ratio in 0 Na^+. Our limited attempts to calibrate absolute values assumed that in 0 Na^+ bath solution $[Na^+]_i$ was also close to 0, and that over the range of 0–15 mM the 340:385 ratio was linear as previously described for gastric cells and lymphocytes (Harootunian et al., 1989; Negulescu et al., 1990). Long-term recordings were not possible for two reasons. (1) We observed a rapid bleach of SBFI dye, which limited the total length of recordings to 20–30 min. (2) TTX had to be applied at high concentrations (100 μM) as the K_d for Na^+ channel block in these cells was 1 μM. The flow rate of our system required 1 mg of TTX for a 10 min application. Graphing of data was obtained as described for electrophysiology data.

Data analysis and statistics

Voltage recordings were obtained by on-line digitizing of data as described above. For analysis, these traces were exported to ORIGIN (MicroCal Inc.), a scientific graphing and data analysis program. Slopes of voltage changes were derived from linear fits, and mean values and standard deviation were derived from the fitted values. Flux studies were analyzed as described above. Mean values of changes in flux rates were determined and graphed to represent mean and SEs. All statistical tests were done using a commercially available statistics program INSTAT (GraphPad Inc.). Whenever two groups of data were compared we used either Student's *t* test (for data with normal distribution) or the Mann-Whitney test (Wilcoxon; for non-normal data) and the uncorrected *p* values of these tests are given. In those events where multiple groups of data were compared we used a one-way analysis of variance (ANOVA) test to determine significance. *P* values were corrected using the Bonferroni method, and whenever corrected *p* values were given, this is indicated in the text.

Results

Astrocytes cultured from rat spinal cord were used to study functional roles of astrocyte Na^+ channels because these cells can express Na^+ channels in higher densities than observed in most other glial cells (Sontheimer et al., 1992). Whole-cell Na^+ currents in these cells often exceed 5 nA and channel densities have been estimated to be between 2 and 8 channels/μm^2 (Sontheimer et al., 1992). These cultures contain two morphologically distinguishable astrocyte subtypes that express Na^+ channels that differ in their sensitivity to TTX (Sontheimer and Waxman, 1992). Process-bearing *stellate astrocytes* comprise ~5% of all cells in these cultures and express TTX-S Na^+ channels (K_d = 5 nM) that resemble Na^+ channels expressed in most excitable cells (Hille, 1992) in terms of their kinetics, steady-state activation and steady-state inactivation. The majority of cells (~95%) are non-process-bearing *pancake astrocytes* and these express TTX-R "glial" Na^+ channels (K_d = 1 μM), which differ in their steady-state activation and steady-state inactivation curves from TTX-S channels (Sontheimer and Waxman, 1992).

Effects of TTX on membrane potential

Using patch-clamp methods (Hamill et al., 1981), whole-cell current-clamp recordings were obtained from both astrocyte types to search for voltage-activated Na^+ currents. Voltage recordings were then obtained in current-clamp mode (in the absence of any holding currents) to determine whether blockage of Na^+ channels with TTX influences the cells' resting potential.

In astrocytes that expressed voltage-activated Na^+ currents in high densities (>50 pA/pF), as assessed by their whole-cell currents, application of 1–100 μM TTX resulted in an immediate and transient hyperpolarization of the membrane by 3–10 mV, bringing the potential closer to the K^+ equilibrium potential (E_K = −84 mV under the imposed ionic conditions; Fig. 1*a*, arrow). This transient hyperpolarization was followed by a much slower, persistent depolarization that was observed in both stellate and pancake astrocytes. Astrocytes that expressed Na^+ currents at lower channel densities (5–30 pA/pF) did not show a transient hyperpolarization upon TTX application, but showed the slow depolarization following exposure to TTX (Fig. 1*b*). This slow depolarization persisted during continued TTX application with a mean rate of 1.04 mV/min (N = 9; SD 0.35). The depolarization was reversible upon removal of TTX, but only if the membrane had not reached values more positive than −65 mV (Fig. 1*a*). If the membrane depolarized beyond this level it continued to "run down" the membrane potential at an accelerated rate even upon termination of TTX (Fig. 1*b*, arrow).

In contrast, in astrocytes that did not show measurable Na^+ currents (I_{Na} < 1 pA/pF), membrane potential was not affected

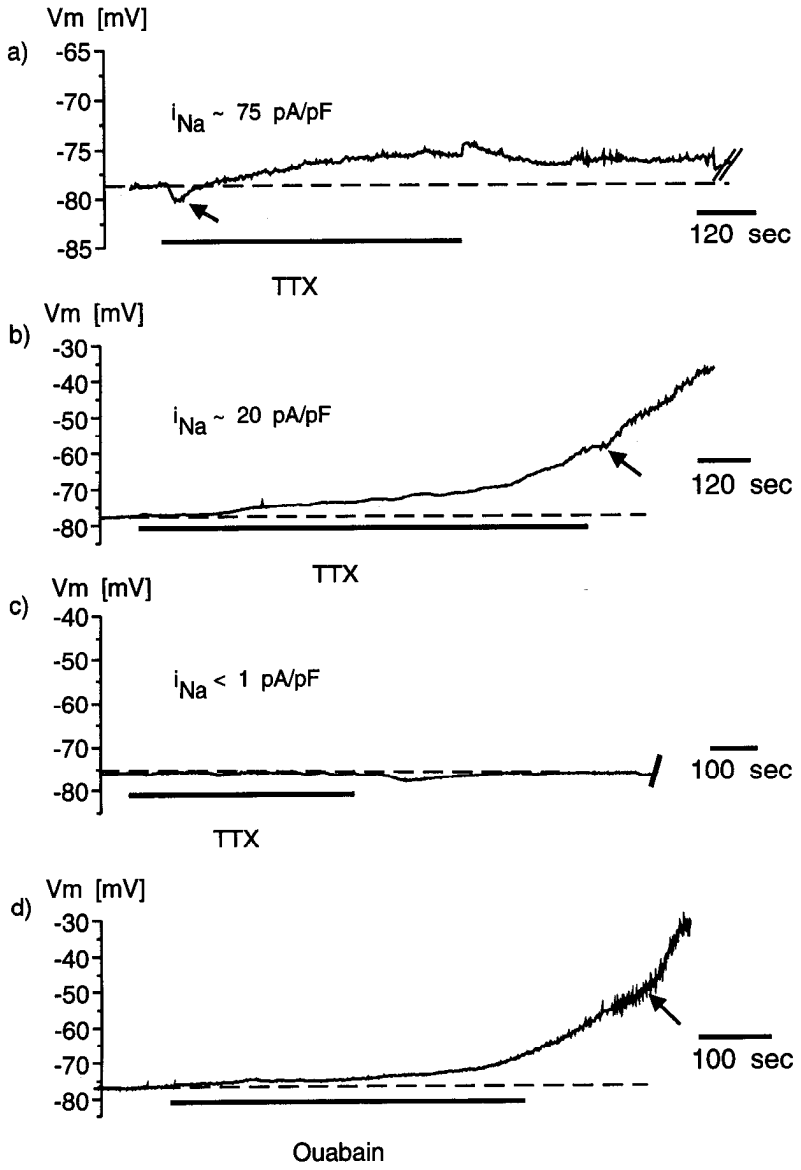

Figure 1. Effects of TTX and ouabain on resting potential of representative cultured spinal cord astrocytes. TTX (1 μM) and ouabain (1 mM) were applied for the periods indicated by the *horizontal bars*. *a*, Example of an astrocyte that had comparably large Na^+ current density (I_{Na} ~ 75 pA/pF). In cells with I_{Na} of >50 pA/pF, TTX application evoked a transient hyperpolarization (*arrow*) preceding a prolonged depolarization. *b*, Example of a cell that expressed Na^+ currents at a lower density of 20 pA/pF. Note that in cells where membrane potential depolarized to levels more positive than −65 mV, rundown continued at accelerated rate (*arrow*) after removal of TTX from the bath. *c*, Example of an astrocyte that did not show measurable Na^+ currents. Potential is unchanged during application of TTX. *d*, Application of ouabain to a representative astrocyte. Ouabain-induced depolarization was similar in time course and magnitude to TTX-induced depolarization (*b*).

by application of TTX (1–100 μM; Fig. 1*c*). Stable recordings could be obtained for up to 90 min during which membrane potential did not change in these cells.

The transient hyperpolarization following TTX application (Fig. 1*a*), observed in cells that expressed Na^+ channels in high densities, suggests that a significant resting Na^+ conductance exists, large enough to contribute a few millivolts to the cells' resting potential. Indeed, steady-state Na^+ current activation (m_∞) and steady-state Na^+ current inactivation (h_∞) kinetics in these astrocytes show overlapping regions where both m_∞ and h_∞ are >0, close to the cells' resting potential (Sontheimer and Waxman, 1992), suggesting the existence of a resting Na^+ conductance. These "window currents" suffice to explain the hyperpolarization that we observed, but do not account for the slow depolarization observed in Na^+ current-expressing astrocytes. The slow time course of the depolarization suggested that it could be mediated by an ion transporter rather than by ion channels.

To determine whether changes in Na^+/K^+-ATPase activity could reproduce this depolarization, we examined the effects of ouabain, a specific inhibitor of the Na^+/K^+-ATPase, on these astrocytes. As shown in Figure 1*d*, ouabain produced a slow depolarization that was qualitatively similar to the one observed following application of TTX in all cells studied. The mean rate of this depolarization was 1.5 mV/min (SD 0.74, $N = 5$), similar to the rate of depolarization induced by TTX. As with TTX, when the membrane depolarization reached levels more positive than −65 mV, rundown of the membrane potential continued at an accelerated rate. The similarity in the effects of TTX and ouabain suggested that the depolarizations induced by these drugs might be mediated by the same pathway, namely, by changes in the activity of the Na^+/K^+-ATPase.

$^{86}Rb^+$ influx as a measure of Na^+/K^+-ATPase activity

To test the possibility that blockage of Na^+ channels may have altered ATPase activity, we assayed Na^+/K^+-ATPase activity more directly, by measuring unidirectional influx of $^{86}Rb^+$, which is taken up by Na^+/K^+-ATPase in the same manner as K^+ (Kimelberg and Mayhew, 1975) but has a significantly longer half-life than $^{42}K^+$. Thus, unidirectional flux rates of $^{86}Rb^+$ can

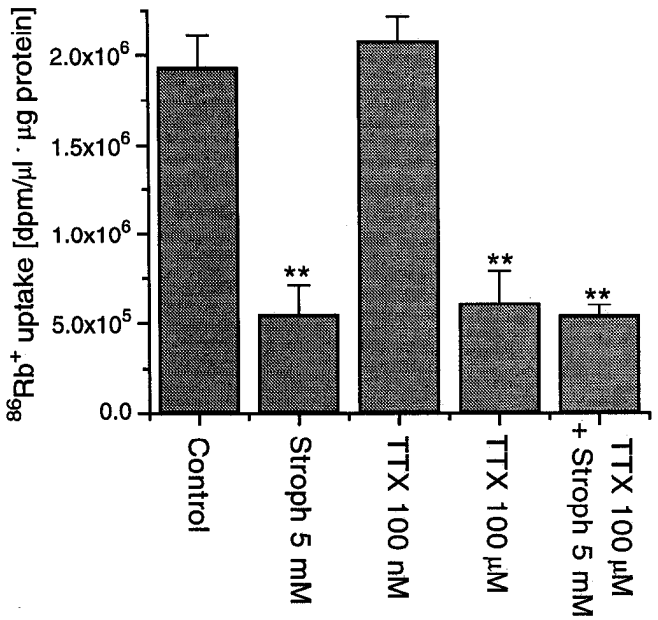

Figure 2. Representative experiment showing alteration of Na^+/K^+-ATPase activity by TTX and strophanthidin measured as unidirectional influx of $^{86}Rb^+$. $^{86}Rb^+$ uptake was determined in spinal cord astrocytes following 5 min incubation with the isotope in combination with TTX, strophanthidin or TTX and strophanthidin in combination at the concentrations indicated (significant changes in flux: **, $p < 0.01$, Bonferroni p values from ANOVA). Strophanthidin, 100 µM TTX, as well as both drugs in combination showed similar reduction in $^{86}Rb^+$ uptake, while 100 nM TTX did not alter $^{86}Rb^+$ uptake significantly.

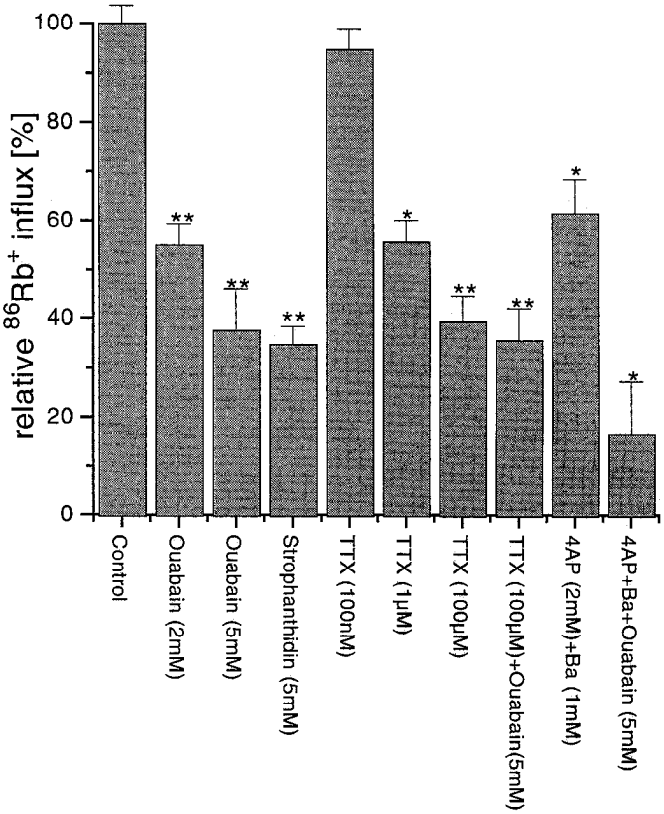

Figure 3. Dose-dependent reduction of $^{86}Rb^+$ uptake by ouabain and TTX. The effects of ouabain and strophanthidin on $^{86}Rb^+$ uptake were compared, at different concentrations, to effects of TTX or TTX in combination with ouabain. To determine the relative contribution of K^+ channel-mediated $^{86}Rb^+$ influx, 4-AP in combination with Ba^{2+} was applied and relative $^{86}Rb^+$ uptake determined in the presence of the drugs. $^{86}Rb^+$ uptake rates in the presence of these drugs were normalized to untreated control cells, and the results of all experiments obtained with the given drug were pooled and mean and SEM were plotted. N values were, from left to right, 56, 21, 6, 29, 31, 4, 48, 15, 6, and 4. Effects that differed significantly from control as determined by ANOVA analysis: **, $p < 0.001$, Bonferroni p value; *, $p < 0.01$ from t test, but Bonferroni p values from ANOVA analysis were not significant because of smaller N numbers. Ouabain and TTX inhibited $^{86}Rb^+$ uptake in a dose-dependent manner. 4-AP + Ba^{2+} reduced $^{86}Rb^+$ uptake by approximately 40%, demonstrating that the residual TTX- and ouabain-resistant influx is mediated by K^+ channels.

serve as a measure of Na^+/K^+-ATPase activity. $^{86}Rb^+$ can enter cells via at least two pathways: (1) by diffusion through K^+ channels, and (2) by active transport through the Na^+/K^+-ATPase. $^{86}Rb^+$ flux rates were determined in untreated astrocytes and compared to flux rates in astrocytes exposed to either TTX (100 nM to 100 µM; Figs. 2, 3), ouabain (1–5 mM; Fig. 3), strophanthidin (1–5 mM; Figs. 2, 3), or TTX in combination with either ouabain or strophanthidin (Figs. 2, 3) for 15 min. The results from a representative experiment are summarized in Figure 2.

Strophanthidin reduced $^{86}Rb^+$ fluxes in a dose-dependent manner, with the most pronounced inhibition, up to 80%, observed at 5 mM concentration. The low sensitivity of the astrocyte Na^+/K^+-ATPase is in agreement with previous observations (Sweadner, 1979) demonstrating that astrocytes express an isoform of the Na^+/K^+-ATPase that is relatively resistant to both ouabain and strophanthidin (K_d of 1 mM for strophanthidin (Sweadner, 1979). We studied in more detail the dose dependence of Na^+/K^+-ATPase inhibition (measured by $^{86}Rb^+$ flux) by ouabain, strophanthidin, and TTX; mean values resulting from these experiments were plotted in Figure 3. Ouabain inhibition of $^{86}Rb^+$ influx ranged from 87% to 32% of control (2 mM ouabain: influx decreased to 54.9 ± 4.4%, N = 21; 5 mM ouabain: 37.6 ± 8.5%, N = 6) and was similar to inhibition by strophanthidin (5 mM: 37.4 ± 5.4%, N = 12).

As shown in Figures 2 and 3, TTX also inhibited $^{86}Rb^+$ influx in a manner similar to strophanthidin and ouabain. TTX reduced $^{86}Rb^+$ flux in a dose-dependent manner, with maximal inhibition at 100 µM TTX (the highest concentration studied; Fig. 3) that resulted in a reduction of $^{86}Rb^+$ fluxes by 61% (±5.2%, N = 48; $p < 0.001$). This was almost indistinguishable to the reduction observed by strophanthidin ($p < 0.001$ compared to control, $p = 0.49$ compared to TTX, ANOVA Bonferroni corrected p values). At lower TTX concentrations, TTX effects were smaller, and, in most experiments, 100 nM TTX did not show any effect (95 ± 4%, N = 31; $p = 0.54$), as shown for one representative experiment in Figure 2 and for the mean of 31 experiments in Figure 3.

To investigate whether the effects of TTX and strophanthidin were mediated by separate pathways, or whether both effects were mediated by the Na^+/K^+-ATPase, we studied whether the effects were additive. The combined application of TTX and strophanthidin did not decrease $^{86}Rb^+$ fluxes below the level observed with either one of the drugs alone (Fig. 2). This was also true for the combined application of 100 µM TTX and 5 mM ouabain (Fig. 3), which resulted in an inhibition of $^{86}Rb^+$ influx of 64.3 ± 6.4% (N = 15) that did not differ significantly from application of TTX alone (61 ± 5.2%, N = 48; $p = 0.74$),

suggesting that TTX and strophanthidin affected the same pathway for $^{86}Rb^+$ entry, namely, the Na^+/K^+-ATPase.

Inhibition of $^{86}Rb^+$ entry by TTX or strophanthidin was always incomplete, leaving 20–50% of influx (mean 39%) unaccounted for. However, since $^{86}Rb^+$ also permeates K^+ channels, it is likely that channel-mediated $^{86}Rb^+$ entry can account for the residual $^{86}Rb^+$ accumulation. Indeed, application of a cocktail known to block astrocyte K^+ channels (2 mM 4-AP in combination with 1 mM Ba^{2+}) reduced $^{86}Rb^+$ influx by 38.4 ± 6.8%, $N = 6$), and combining the latter cocktail with ouabain resulted in an almost complete inhibition of $^{86}Rb^+$ influx (83.6 ± 10.9%, $N = 4$). $Na^+/K^+/2Cl^-$ transport has been demonstrated to be expressed in cultured cortical astrocytes (Kimelberg and Frangakis, 1985; Walz and Hinks, 1985), and this transporter would be able to also account for $^{86}Rb^+$ influx. Applying furosemide (1 mM) or bumetanide (0.1 mM), which are commonly used inhibitors for $Na^+/K^+/2Cl^-$ uptake, resulted in variable but typically small (<20% of control) effects on $^{86}Rb^+$ uptake, suggesting that indeed, Na^+/K^+-ATPase and K^+ channel–mediated diffusion represent the main entry pathways for $^{86}Rb^+$.

C6 glioma and DRG cells

To investigate whether the proposed relationship between Na^+ influx through voltage-activated ion channels and Na^+/K^+-ATPase activity is unique to astrocytes, or whether it is applicable more widely, we obtained $^{86}Rb^+$ influx data like those described above, for C6 glioma cell lines and acutely dissociated DRG neurons. In our electrophysiological recordings we observed that cultured C6 glioma cells did not express detectable Na^+ channels, consistent with a previous report (Wang et al., 1992). In contrast to cultured astrocytes, 15 min of incubation of C6 glioma cells with 100 μM TTX failed to alter the relative rate of $^{86}Rb^+$ influx in these cell lines (Fig. 4; mean, 107.8 ± 41.3%, $N = 5$; $p = 0.8$). Acutely dissociated DRG neurons expressed both TTX-S and TTX-R voltage-activated Na^+ channels in our patch-clamp recordings (not shown), consistent with previous reports (Kostyuk et al., 1981; Caffrey et al., 1992; Roy and Narahashi 1992). However, as in C6 glioma cells, and unlike in astrocytes, incubation with 100 μM TTX, which inhibited Na^+ currents in our electrophysiological recordings, did not alter $^{86}Rb^+$ influx rates in these cells (97.3 ± 31.5%, $N = 4$; $p = 0.97$). Thus, the coupling between Na^+ channel–mediated Na^+ ion influx and Na^+/K^+-ATPase activity appears to be specific for astrocytes.

Effect of TTX on astrocyte viability

In all mammalian cells studied to date, the Na^+/K^+-ATPase contributes significantly in maintaining the ionic transmembrane gradients, and thus its function is essential for cell survival. The above results suggest that in spinal cord astrocytes Na^+ channels constitute a return pathway that is important for operation of ATPase. To assess how vital this "Na^+ return pathway" is for astrocytes, we asked whether astrocytes would tolerate chronic exposure to either TTX or strophanthidin. Cell survival was assayed by monitoring the cells' ability to exclude trypan blue, and the percentage of viable cells after a given drug exposure time was determined.

Inhibition of the astrocyte ATPase by 5 mM strophanthidin, and exposure of cells to TTX both lead to significant cell death. While untreated control cultures did not show evidence of significant cell death when monitored over a 2 hr period, strophanthidin reduced the percentage of viable cells to 3.5% within

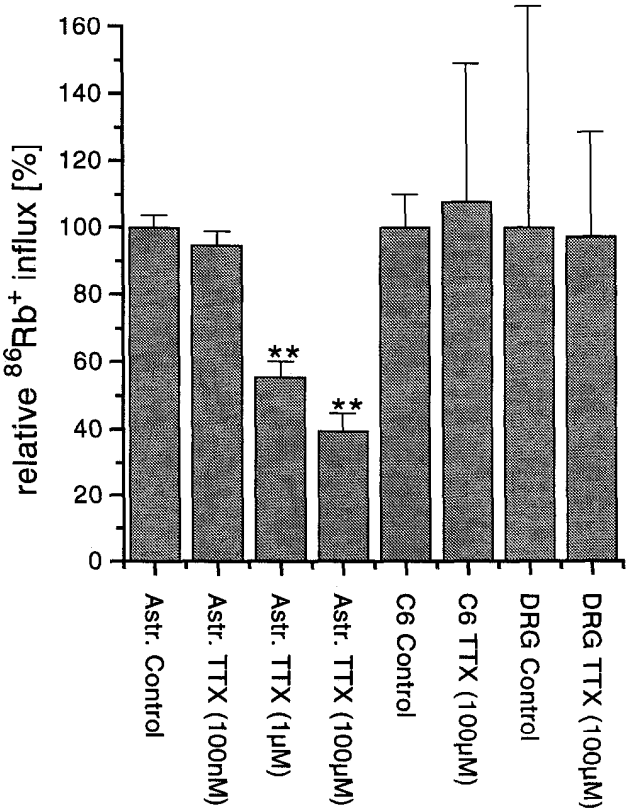

Figure 4. TTX reduction of $^{86}Rb^+$ uptake is specific for astrocytes. $^{86}Rb^+$ uptake was determined in cultures of rat spinal cord astrocytes, rat C6 glioma cell lines, and rat DRG neurons. Uptake was normalized to untreated control cells for the three preparations. TTX effects were significant only in astrocytes (**, $p < 0.01$), whereas TTX effects did not alter $^{86}Rb^+$ significantly in C6 glioma cells or DRG neurons.

60 min (Fig. 5). Exposure of cells to TTX had similar effects (Fig. 5), although the effectiveness of TTX was dependent on the concentration used (Fig. 6). Incubation with 10 μM TTX reduced the percentage of viable cells to 54% within 60 min and only 19% of the cells survived after a 120 min treatment with 10 μM TTX (Fig. 5). Even larger effects were observed at 100 μM TTX, which reduced cell survival to 8.27% (Fig. 6). TTX effects were substantially smaller in 1 μM TTX (87% viable at 60 min), and 100 nM TTX did not reduce cell viability significantly (Fig. 6; $p = 0.91$).

To assure the validity of the assay used to determine cell survival, we obtained additional experiments using a recently described cytotoxicity assay ("Live/Dead," Molecular Probes, Inc.) that utilizes fluorescence dyes to discriminate between live an dead cells. This assay identifies dead cells by a red fluorescent fluorophore ethidium homodimer, which is excluded from live cells. Viable cells are identified by a yellow-green (fluorescein) stain generated by the enzymatic hydrolysis of calcein-AM that only occurs in live cells as a result of esterase activity. Results from those studies are straightforward to monitor, as cells light up differentially under the fluorescence microscope as yellow (=live) or red (=dead) cells. Representative examples of cells studied with this assay are illustrated in Figure 7. Untreated control cells typically showed >98% live (yellow) cells (Fig. 7*a*). By contrast, astrocytes treated with 100 μM TTX for 60 min showed substantial numbers of dead (red) cells (Fig. 7*b*), as

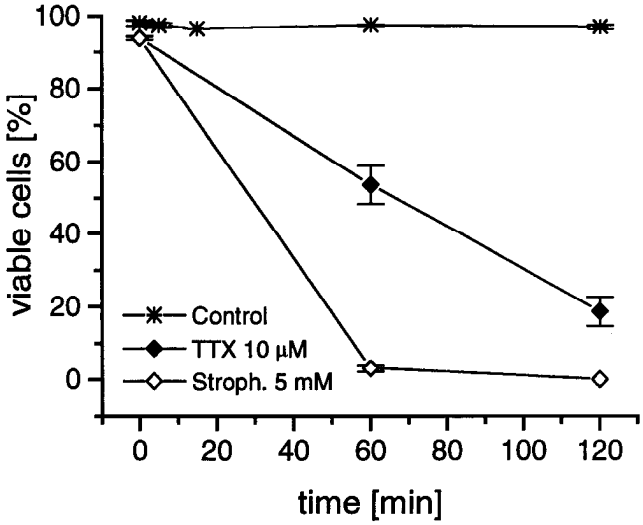

Figure 5. Reduced astrocyte viability in TTX and strophanthidin. Cell viability was assayed by determining the percentage of cells that maintained the ability to exclude trypan-blue. Cells were incubated for up to 2 hr in various concentrations of TTX and strophanthidin: 10 randomly selected cell samples for each drug (cell number range, 55–285 cells) were counted at 5, 60, and 120 min and the percentage of viable cells determined.

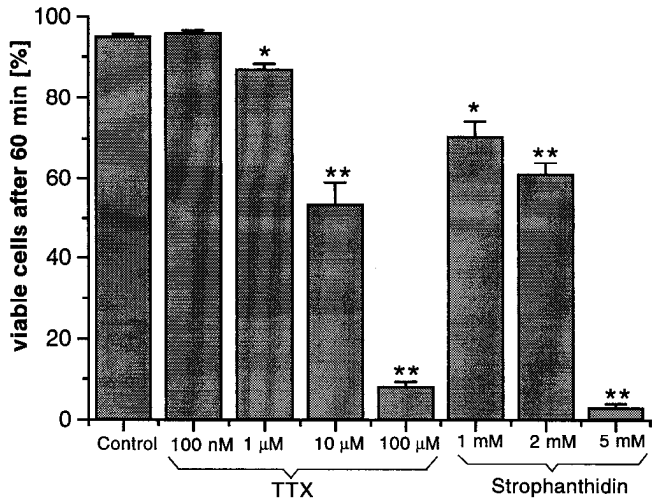

Figure 6. TTX and strophanthidin induce astrocyte death in a dose-dependent manner. Cell viability, as assayed by cells' ability to exclude trypan blue, was determined after 60 min incubation with TTX or strophanthidin at the concentrations indicated (significant changes: *, $p < 0.05$; **, $p < 0.01$).

similarly observed in astrocytes treated with 5 mM ouabain (Fig. 7*c*). In contrast to these effects of TTX on astrocyte survival, neither DRG neurons nor C6 glioma cells were significantly affected by identical treatment with 100 μM TTX.

These cell survival experiments demonstrate that fluxes of Na^+ ions through TTX inhibitable Na^+ channels are essential for the survival of a significant percentage of spinal cord astrocytes in culture, and further support the hypothesis that these channels, in astrocytes, provide a vital return pathway for Na^+/K^+-ATPase function.

Recording of $[Na^+]_i$ by quantitative fluorescence ratio imaging

As the coupling of Na^+ channels to ATPase function can only be mediated by $[Na^+]_i$, we expected that $[Na^+]_i$ would decrease following Na^+ channel inhibition by TTX, which in turn would result in a depletion of Na^+ as substrate for ATPase function. To test whether this was indeed the case, we used the Na^+-sensitive ratiometric indicator SBFI to determine by quantitative digital ratio imaging whether $[Na^+]_i$ in astrocytes changes in response to TTX. Superfusion of TTX (100 μM) in most cells (9 of 12) resulted in a reversible decrease in the 340:385 nm ratio indicating a decrease in $[Na^+]_i$ (Fig. 8). Due to the high concentrations needed for Na^+ channel block and since bleaching of SBFI did not allow studies in excess of 20–30 min, we were limited to a 10 min TTX challenge. However, in all instances where TTX was effective, the 340:385 ratio was still declining after 10 min of TTX exposure and prior to the switch back to control solution. We compared TTX effects to the decrease of $[Na^+]_i$ following incubation with Na^+-free saline, which in other cell types was shown to deplete $[Na^+]_i$ completely within minutes (Negulescu et al., 1990). Na^+-free saline reversibly decreased the 340:385 ratio signal in all cells tested (12 of 12; Fig. 8). This effect was more rapid and its magnitude always exceeded the effects of TTX. To achieve some indication as to absolute Na^+ concentrations, we attempted to calibrate our recordings by application of 10 mM Na^+–saline in the presence of the ionophore gramidicidin (5 μM) at the end of every experiment. Unfortunately, cells did not tolerate the ionophore well and SBFI bleached rapidly, and thus we were not able to obtain more complete calibration. Thus, to quantify effects of TTX on $[Na^+]_i$, we determined the relative decrease in 340:385 ratios in the presence of TTX and in the presence of Na^+-free saline. TTX effects after 10 min were 31.6% (SD 5.6%, $N = 9$) of those in Na^+-free solution. Assuming that in Na^+-free solution $[Na^+]_i$ approached 0 (Negulescu et al., 1990), and assuming that the endpoint calibration with 10 mM Na^+ reflected a true calibration value, and furthermore assuming a linear relationship for 340:385 ratios over the concentration range studied (0–15 mM; Negulescu et al., 1990), recordings were calibrated to absolute concentration values. Resting $[Na^+]_i$ was thus determined to be 10.1 mM (SD 3.6, $N = 12$) and TTX within 10 min dropped $[Na^+]_i$ by 4 mM (SD 2.5, $N = 9$), suggesting a rate of 0.4 mM/min. While these absolute values may be flawed by the limitations to our calibration procedures and depend on the assumptions made above, the qualitative change of $[Na^+]_i$ clearly demonstrates that TTX results in a drop in $[Na^+]_i$.

Discussion

The data presented here provide evidence for the existence of a small resting Na^+ conductance in spinal cord astrocytes that can be abolished by 100 μM TTX. Since blockade of Na^+ channels not only depolarized the cells, but also significantly reduced ATPase activity as measured by unidirectional ouabain-sensitive influx of $^{86}Rb^+$, and reduced $[Na^+]_i$, our results suggest that channel-mediated Na^+ entry and Na^+/K^+-ATPase activity are functionally interdependent.

Based on our observations, we suggest the following model to explain the coupling of Na^+ channel–mediated Na^+ fluxes and Na^+/K^+-ATPase (Fig. 9): a small, but finite open probability exists for astrocyte Na^+ channels at the resting potential [both m_∞ and h_∞ are >0 (Sontheimer and Waxman, 1992)], leading to a small, steady influx of Na^+ ions that follow the electrochemical gradient. This influx of Na^+ ions is sufficient to maintain cytoplasmic Na^+ at concentrations required to

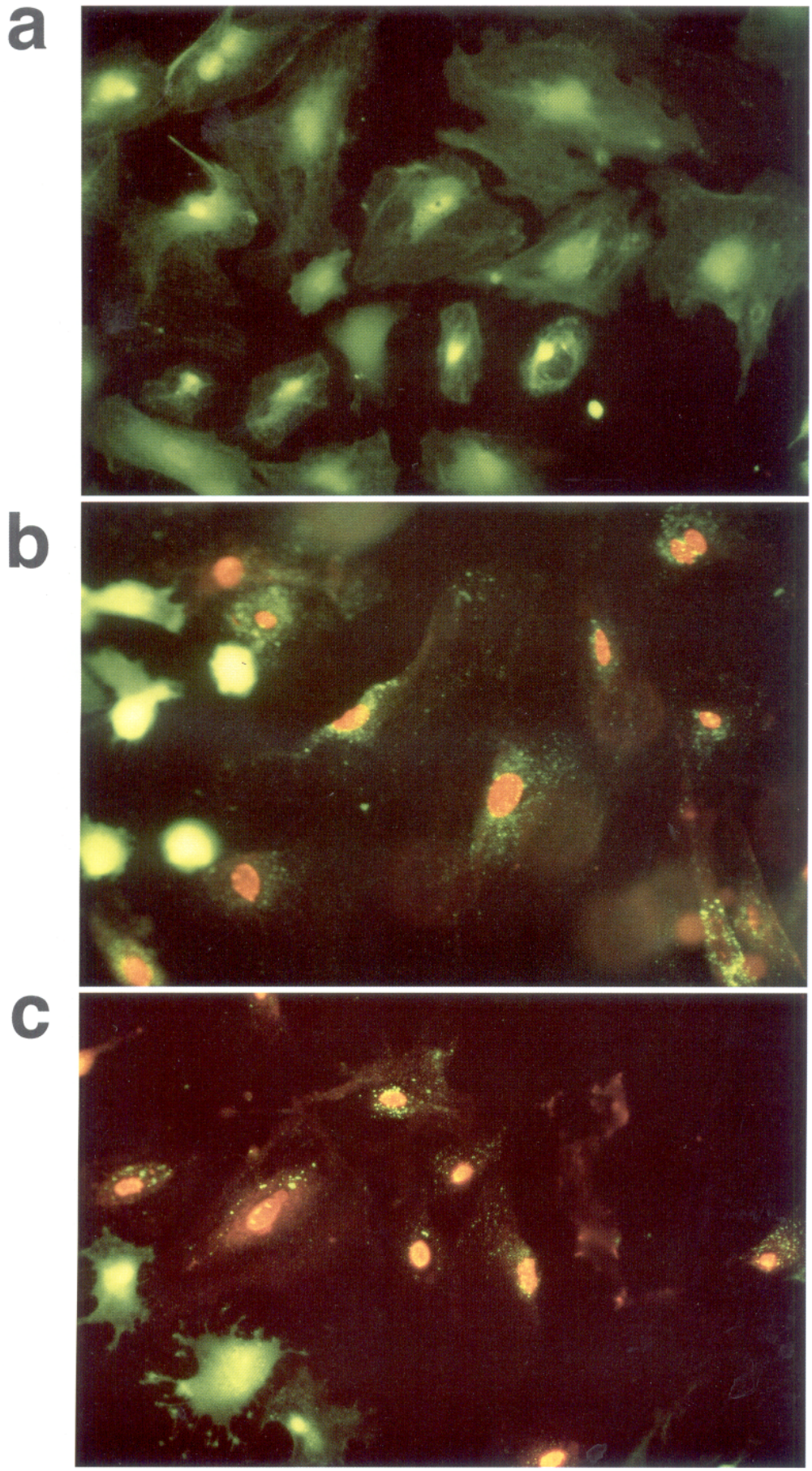

Figure 7. Astrocyte viability studied using calcein-AM/ethidium homodimer ("Live/Dead") cytotoxicity assay. Cells were treated with two fluorophores that selectively distinguish live (*yellow*) and dead (*red*) cells. Untreated cells incubated for 30 min with fluorophores did not contain dead cells (*a*). By contrast, cells treated in addition with 100 μM TTX (*b*) or 5 mM ouabain (*c*) showed a significant number of dead (*red*) cells.

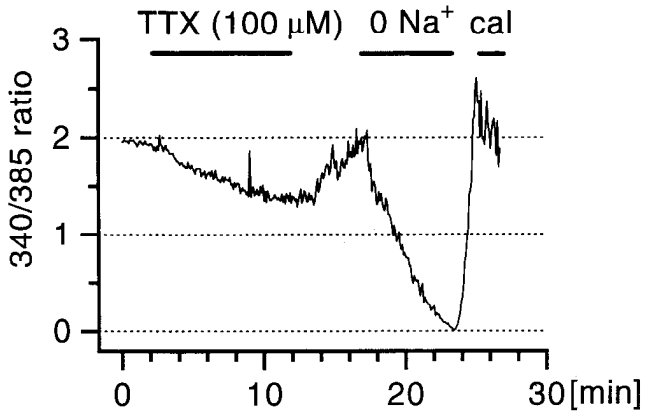

Figure 8. Ratiometric measurements of $[Na^+]_i$ using SBFI. The relative 340:385 nm ratio of SBFI fluorescence was obtained from cells loaded with the Na^+-sensitive dye SBFI as described in Materials and Methods. Application of TTX resulted in a significant and reversible decrease in the 340:385 ratio, indicating a drop of $[Na^+]_i$. Changing the bath solution to an Na^+-free solution resulted in a similar, although more pronounced, drop in $[Na^+]_i$. Calibration with 10 mM Na^+ in the presence of the ionophore gramicidin (*cal*) indicated that resting $[Na^+]_i$ in this cell was close to 10 mM.

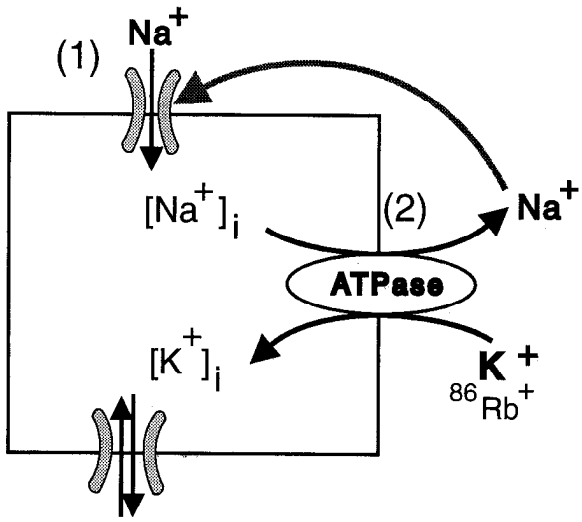

Figure 9. Simplified cell model to explain Na^+ channel–Na^+/K^+-ATPase coupling in spinal cord astrocytes. Na^+ channels function to maintain intracellular Na^+ ($[Na^+]_i$) at levels that provide a substrate for Na^+/K^+-ATPase. TTX blocks Na^+ channels (*1*), while strophanthidin and ouabain interfere with activity of the ATPase (*2*).

maintain Na^+/K^+-ATPase activity. Studies on Na^+/K^+-ATPases from numerous tissues (Stein, 1986) including glial cells (Kettenmann et al., 1987) show that low micromolar concentrations of $[Na^+]_i$ are required to allow the Na^+/K^+-ATPase to function, and it has been demonstrated that Na^+/K^+-ATPase activity in cultured astrocytes depends on $[Na^+]_i$ (Walz and Hinks, 1986). A K_m for $[Na^+]_i$ of 10 mM has been shown for Na^+/K^+-ATPase activation in batch isolated glial cells (Kimelberg et al., 1978). Blockage of Na^+ channels by TTX inhibits the replenishment of intracellular Na^+ ions, and through the continued operation of the Na^+/K^+-ATPase, $[Na^+]_i$ is depleted. Our imaging data using the Na^+-sensitive indicator SBFI indicate that within 10 min $[Na^+]_i$ is reduced by about 30–40% of control. $[Na^+]_i$ continued to decline throughout the period of TTX application (Fig. 9), and as we were limited to relatively short TTX applications (10 min), we were not able to record long enough to see a complete depletion of $[Na^+]_i$. However, from our data we extrapolated that complete depletion of $[Na^+]_i$ would be expected after 20–40 min. As a consequence, Na^+/K^+-ATPase activity is reduced due to a lack of substrate. This is reflected in the reduction of unidirectional influx of $^{86}Rb^+$, which is a direct measure of Na^+/K^+-ATPase activity. Inhibition of the Na^+/K^+-ATPase by ouabain or strophanthidin, as expected, also leads to a reduction in K^+ influx associated with a slow depolarization. Ouabain-induced depolarizations have been reported in numerous cell types, and are consistent with the breakdown of the ionic gradients for Na^+ and K^+ ions in the absence of ATPase function. Ouabain inhibition of Na^+/K^+-ATPase in glial cells has previously been demonstrated (Kimelberg et al., 1979b; Tang et al., 1980; Hertz, 1986), and in cultured cortical astrocytes results in membrane depolarizations of magnitude and rate [0.9 mV/min (Kimelberg et al., 1979b; Hertz, 1986); 1.2 mV/min (Harold and Walz, 1992)] similar to those that we observed in spinal cord astrocytes after exposure to TTX and strophanthidin.

The Na^+ influx through Na^+ channels is small and does not always contribute significantly to the resting conductance. Whether the resting potential is influenced by TTX blockage depends on the relative contributions of Na^+ and K^+ conductance. If the K^+ conductance exceeds the Na^+ conductance by severalfold, as typically is the case for astrocytes (Sontheimer et al., 1991b, 1992; Sontheimer, 1992) blockage of the Na^+ conductance would not be expected to alter the resting potential significantly, as observed in those cells that had Na^+ current densities of <30 pA/pF (these cells did, however, display the slow depolarization presumably related to the breakdown of the ionic gradients following inhibition of the Na^+/K^+-ATPase). We only observed a contribution of resting Na^+ conductance to resting potential in those cells where Na^+ currents were expressed in high densities (typically >50 pA/pF).

The dependence of ATPase activity on Na^+ ion entry through TTX-inhibitable Na^+ channels is supported further by our observation that TTX exposure reduced astrocyte viability. Over 80% of astrocytes died during a 2 hr exposure to TTX, strophanthidin, or ouabain. According to our proposed model, astrocyte death was caused as a consequence of the inhibition of ATPase activity by a lack of cytoplasmic Na^+ ions. Although we are not certain as to the cause of cell death, it is likely to be Ca^{2+} mediated. Voltage-dependent Ca^{2+} channels have been described for cultured astrocytes (MacVicar, 1984; Barres et al., 1989a), and these would be activated by the depolarization following the inhibition of the Na^+/K^+-ATPase. Strophanthidin and ouabain exerted their inhibitory effects directly on the pump; TTX, in contrast, acted by abolishing the Na^+ channel–mediated "return" flux of Na^+ ions necessary to maintain the $[Na^+]_i$ substrate required for ATPase activity. These results are consistent with those of Ibata et al. (1971), who observed, using electron microscopy, glial cell injury following exposure to low-Na^+ medium. Interestingly, TTX exposure has also been shown to reduce viability of cultured rat spinal cord neurons (Bergey et al., 1981) but failed to affect viability of cultured DRG neurons. In these studies, effects were most pronounced at early stages of *in vitro* development. TTX-induced neuronal death has been interpreted differently, and has led to the speculation that neurons require the ability to fire action potentials to survive in culture (Bergey et al., 1981). However, the interpretation

suggested for spinal cord astrocytes here, namely, that Na^+ channels are important for Na^+/K^+-ATPase function, may also explain the observed neuronal cell death following chronic TTX treatment.

We included two additional cell preparations in our study: C6 glioma cell lines and DRG neurons. In both preparations, TTX was ineffective in inhibiting Na^+/K^+-ATPase as judged by $^{86}Rb^+$ influx, whereas ouabain and strophanthidin, as expected, reduced $^{86}Rb^+$ influx substantially. Since C6 glioma cells lack Na^+ channels (Wang et al., 1992), this observation was not surprising, and served as a control to exclude the possibility that TTX was affecting, in a less specific way, pathways other than Na^+ channels. The results obtained from DRG neurons are more intriguing. These cells express both TTX-S and TTX-R Na^+ channels (Kostyuk et al., 1981; Caffrey et al., 1992; Roy and Narahashi 1992). The inability of TTX to reduce $^{86}Rb^+$ influx rates in DRG neurons suggests that the proposed interdependence of Na^+ channel and ATPase function does not exist for DRG cells. Macroscopic Na^+ current properties in these neurons differ substantially from those observed in spinal cord astrocytes. Most notably, "window currents" are almost absent in DRG neurons, as m_∞ and h_∞ do not overlap significantly; thus, DRG neurons do not have a standing Na^+ conductance at rest (Roy and Narahashi 1992). Such a "standing" Na^+ conductance, however, is required by the proposed model, and its absence explains why a link between Na^+ channel activity and ATPase function is absent in these neurons. These results are also compatible with the previous observation that the viability of DRG neurons is not affected by chronic TTX treatment (Bergey et al., 1981).

The proposed model may still have more general applicability, and may explain the expression of voltage-activated Na^+ channels in other nonexcitable cells, for example, Schwann cells (Chiu et al., 1984; Gray and Ritchie, 1985; Howe and Ritchie, 1990) or fibroblasts (Munson et al., 1979), where a functional role of Na^+ channels has not yet been defined.

Some astrocytes, approximately 20% of stellate and 15% of pancake cells did not display measurable Na^+ currents (resolution threshold ~ 1 pA/pF), and were able to maintain a stable resting potential in the presence or absence of TTX. We concluded that these cells lack a resting Na^+ conductance, at least one mediated by TTX-inhibitable, time- and voltage-dependent Na^+ channels. *In vitro* studies on other astrocyte preparations indicated that even larger percentages of astrocytes lack expression of voltage-activated Na^+ channels [mouse brain, 70% (Nowak et al., 1987); rat hippocampus, 50–60% (Sontheimer et al., 1991b); rat optic nerve, 50–70% (Barres et al., 1988; Sontheimer et al., 1991a)], and the percentage of cells that do express Na^+ channels decreases with time *in vitro* and *in vivo* (Minturn et al., 1991; Sontheimer et al., 1991a,b, 1992). In the absence of Na^+ channels, such cells must have other mechanisms that maintain $[Na^+]_i$ at concentrations sufficient to support ATPase activity. Astrocytes can express numerous Na^+-dependent ion carriers or cotransporters, for example, Na^+/HCO_3^+ exchanger (Astion et al., 1989, 1991; Newman and Astion 1991), Na^+/Glu-cotransporter (Hertz, 1979; Brew and Attwell, 1987; Barbour et al., 1988; Nicholls and Attwell, 1990; Sarantis and Attwell, 1990), Na^+/KCl^- cotransport (Walz and Hinks 1986), and Na^+/H^+ exchanger (Kimelberg, 1979; Kimelberg et al., 1979a; Walz and Hertz, 1984). Activity of these transporters would result in a net influx of Na^+ ions, and could provide a route for Na^+ influx sufficient to maintain ATPase activity. Walz and Hicks (1986) proposed an Na^+ cycle for cultured mouse cortical astrocytes where the Na^+/K^+-ATPase is fueled by Na^+ ions entering by means of furosemide-sensitive Na^+/KCl^- cotransport. It is presently not known which Na^+-dependent carriers or transporters are expressed in spinal cord astrocytes. We only observed small changes with application of furosemide or bumetanide, indicating that Na^+/KCl^- cotransport is weak. However, glutamate was highly potent in stimulating $^{86}Rb^+$ influx indicative of the presence of Na^+-glutamate transporters (H. Sontheimer, unpublished observations). The latter, however, would not be effective as an Na^+ pathway under normal conditions in the absence of the neurotransmitter. Na^+/K^+-ATPase activity would be thus dependent on Na^+ channels. The heterogeneity of astrocytes with respect to Na^+ channel density (Sontheimer, 1992) could represent a compensatory mechanism, ensuring the presence of a return pathway for Na^+/K^+-ATPase in cells that express varying densities of Na^+-dependent ion carriers and cotransporters. Since our present study was limited to spinal cord astrocytes at 7–10 DIV, it is unclear whether the proposed relationship of Na^+ channel priming of the Na^+/K^+-ATPase exists also in more mature astrocytes. It is possible that this mechanism is only functional during periods in which cells do not yet express other Na^+ dependent carriers at sufficient density. If true, this would explain the loss of Na^+ channels in astrocytes cultured for prolonged periods.

Under physiological conditions, $[K^+]_o$ is tightly regulated in the CNS to assure neuronal excitability, and one of the major functions of astrocytes in the CNS is believed to be the maintenance of this K^+ homeostasis (Kuffler et al., 1966; Orkand et al., 1966; Somjen, 1975; Orkand, 1977; Newman, 1986, 1993). K^+ ions that rapidly accumulate in the extracellular space following nerve activity are taken up by astrocytes via at least two independent mechanisms: (1) entrance into glial cells through K^+ ion channels (Newman 1984; Newman, 1986; Orkand, 1977; Orkand et al., 1966) and (2) Na^+/K^+-ATPase–mediated K^+ uptake (Tang et al., 1980; Ballanyi et al., 1987). The latter mechanism, to operate effectively, requires a sufficient supply of intracellular Na^+, and it has previously been shown that Na^+/K^+-ATPase in astrocytes depends on and can be stimulated by increases in $[Na^+]_i$ (Walz and Hinks, 1986). In batch-isolated glial cells a K_m for $[Na^+]_i$ of 10 mM has been shown for Na^+/K^+-ATPase activation (Kimelberg et al., 1978), and thus, if $[Na]_i$ is around 5–15 mM, as our SBFI imaging measurements indicate, then it is uniquely poised to activate the Na^+/K^+-ATPase maximally (see Kimelberg et al., 1993, for review). Since astrocyte membrane potential depends strongly on $[K^+]_o$ (Kuffler et al., 1966; Orkand et al., 1966; Orkand, 1977) even small increases in $[K^+]_o$ will depolarize astrocytes. Due to the resulting increased open probability of Na^+ channels (Sontheimer and Waxman, 1992), channel-mediated influx of Na^+ ions would be enhanced and would result in a stimulation of the Na^+/K^+-ATPase–mediated uptake of K^+ ions. Thus, the model proposed here for spinal cord astrocytes, which couples Na^+ channel–mediated influx to Na^+/K^+-ATPase activity, would not only provide a vital pathway for Na^+/K^+-ATPase function under resting, steady-state conditions, but could also operate in a "feedback" manner to facilitate the Na^+/K^+-ATPase–mediated removal of $[K^+]_o$ following intense neuronal activity.

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