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Passive Glial Cells, Fact or Artifact?

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Abstract. Astrocytes that are recorded in acute tissue slices of rat hippocampus using whole-cell patch-clamp, commonly exhibit voltage-activated Na^+ and K^+ currents. Some reports have described astrocytes that appear to lack voltage-activated currents and proposed that these cells constitute a subpopulation of electrophysiologically passive astrocytes. We show here that these cells can spontaneously change during a recording unmasking expression of previously suppressed voltage-activated currents, suggesting that such cells do not represent a subpopulation of passive astrocytes. Superfusion of a low Ca^{2+}/EGTA solution was able to reversibly suppress voltage-activated K^+ currents in cultured astrocytes. Currents were restored upon addition of normal bath Ca^{2+}. These effects of Ca^{2+} on both outward and inward K^+ currents were dose- and time-dependent, with increasing concentrations of Ca^{2+} (from 0 to 800 μM) leading to a gradual unmasking of voltage-dependent outward and inward K^+ currents. The transition from an apparently passive cell to one exhibiting prominent voltage-activated currents was not associated with any changes in membrane capacitance or access resistance. By contrast, in cells in which low access resistance or poor seal accounted for the absence of voltage-activated currents, improvement of cell access was always accompanied by changes in series resistance and membrane capacitance.

We propose that spillage of pipette solution containing low Ca^{2+}/EGTA during cell approach in slice recordings and/or poor cell access, lead to a transient masking of voltage-activated currents even in astrocytes that express prominent voltage-activated currents. These cells, however, do not constitute a subpopulation of electrophysiologically passive astrocytes.

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Key words: Patch-clamp — Slices — Astrocyte — Voltage-activated channels — Potassium current

Introduction

Glial cells were initially described as electrophysiologically passive cells lacking any voltage-activated ion channels (Kuffler & Potter, 1964; Kuffler, 1967). However, numerous reports have since demonstrated that cultured astrocytes express a variety of voltage-activated Na^+, K^+, Ca^{2+} and Cl^- channels. Moreover, several studies have confirmed the presence of Na^+ and various types of voltage-activated K^+ channels in astrocytes in acute hippocampal slices (Berger, Schnitzer & Kettenmann, 1991; Steinhauser et al., 1992; Sontheimer & Waxman, 1993; Steinhauser et al., 1994; Bordey & Sontheimer, 1997; D'Ambrosio et al., 1998) and whole mount retina (Reichelt et al., 1993; Clark & Mobbs, 1994). Some of these studies have reported the existence of two classes of astrocytes. These include cells that express predominantly voltage-activated channels, so-called complex cells, and cells that seem to lack time- or voltage-activated channels consequently appearing to behave ohmic in response to current or voltage changes, so-called passive cells.

In over 300 recordings of astrocytes in rat hippocampal slices, we found little evidence for purely passive glial cells. This is not to say that all recordings yielded identical current complements. Indeed, channel expression changes markedly during development (Bordey & Sontheimer, 1997), with channels showing stage-specific expression patterns. For example, differentiated astrocytes in slices from animals older than postnatal day (P)14 are always characterized by expression of inwardly rectifying K^+ channels (Kir), whereas a significant percentage of more immature, probably dividing astrocytes lack expression of Kir but instead expressed large out-

wardly rectifying K^+ currents. Nevertheless, essentially every cell exhibited expression of some voltage-activated K^+ currents.

In light of the discrepancy of our findings with reports from other laboratories (Berger et al., 1991; Steinhauser et al., 1992; Steinhauser et al., 1994; D'Ambrosio et al., 1998) we set out to reinvestigate whether passive glial cells can be identified in rat hippocampal slices, and if so, whether they indeed lack voltage-dependent currents or whether currents may be modulated in ways to curtail their activation.

Our data suggest that cells that initially appear to be electrophysiologically passive show a transient masking of voltage-dependent currents, most likely caused by low extracellular Ca^{2+} due to spillage of EGTA buffered pipette solution into the extracellular space. Upon washout of EGTA and restoration of physiological Ca^{2+} these cells display prominent voltage-activated channels. We therefore suggest that the presence of passive cells in slice recordings could be artifactual and caused by spillage of a 0 Ca^{2+}/EGTA/high K^+ solution from the patch pipette, and the slow restoration of extracellular Ca^{2+} concentration $[Ca^{2+}]_o$ causes the unmasking of voltage-dependent K^+ currents.

Materials and Methods

Cell Culture

Primary spinal cord astrocyte cultures from P_0–P_1 Sprague-Dawley rat pups were obtained as previously described (MacFarlane & Sontheimer, 1997). Pups were anesthetized by hypothermia and then decapitated and spinal cords were dissected from midcervical to lumbar regions. Tissue was excised in filter sterilized Complete Saline Solution (CSS) containing the following in mM: 137 NaCl, 5.3 KCl, 1 $MgCl_2$, 25 Glucose, 10 N-[2-Hydroxyethyl]piperazine-N′-[2]etanesulfonic acid] (Hepes), 3 $CaCl_2$, adjusted to pH = 7.2 by NaOH. Tissue was then stripped of meninges and blood vessels, minced, and incubated for 20 min at 37°C and 95% O_2/5% CO_2 in CSS with 0.5 mM (ethylenedinitrilo)tetraacetic acid (EDTA), 1.65 mM L-cysteine, and 30 U/ml papain (Worthington, Freehold, NJ). Enzyme solution was aspirated and tissue was rinsed with culture medium consisting of Earle's Minimal Essential Media (EMEM) (GIBCO, Grand Island, NY) supplemented with 20 mM glucose, 10% Fetal Calf Serum (FCS; HyClone, Logan, UT), 500 U/ml of penicillin/streptomycin, 1.0 mg/ml trypsin-inhibitor and 1.0 mg/ml BSA. Tissue was dissociated by trituration with a fire-polished Pasteur pipette. Cells were plated on poly-ornithine/laminin coated 12 mm glass coverslips (MacAlaster Bicknell, New Haven, CT) at a density of 1.0×10^6 cells/ml. Cells were maintained at 37°C and 95% O_2/5% CO_2 in culture medium changed every 3–4 weeks. Cultures were >95% positively immunoreactive for glial fibrillary acidic protein, an astrocyte-specific cytoskeletal protein (rabbit monoclonal, INCSTAR, Stillwater, MN). Coverslips with cultured cells were transferred to a recording chamber mounted on the stage of an inverted Nikon Diaphot microscope equipped with Hoffman Modulation Contrast Optics.

Slice Preparation

Methods used for preparation of thin hippocampal slices were as previously described (Bordey & Sontheimer, 1997). Briefly, 20- to 35-day-old Sprague Dawley rats were anesthetized using pentobarbital (50 mg/kg) and decapitated. The brain was quickly removed and placed in ice-cold (4°C) calcium-free artificial cerebrospinal fluid (ACSF-Ca free) containing (in mM): NaCl, 116; KCl, 4.5; $MgCl_2$, 0.8; $NaHCO_3$, 26.2; glucose, 11.1; Hepes, 5, which was continuously oxygenated with 95% O_2/5% CO_2. The brain was hemisected, and a block of tissue containing the hippocampus was glued (cyanoacrylate glue) to the stage of a Vibratome. Transverse hippocampal slices (150 μm thick) were cut in cold oxygenated ACSF-Ca-free and transferred to a beaker filled with ACSF-Ca free at room temperature. After a recovery period of at least 1 h in ACSF-Ca free, slices were placed in a flow-through chamber mounted on the stage of an upright microscope (Nikon Optiphot2) equipped with a ×40 (2 mm working distance), water immersion objective and Nomarksi optics.

Electrophysiology

Recordings were obtained using the whole-cell patch-clamp technique (Edwards et al., 1989). Patch pipettes were pulled from thin-walled borosilicate glass (o.d., 1.55 mm; i.d., 1.2 mm. WPI, TW150F-40) on a PP-83 puller (Narishige, Japan). Pipettes had resistances of 2–3 MΩ for culture recordings and 4–7 MΩ for slice recordings when filled with the following solution (in mM): KCl, 145; $CaCl_2$, 0.2; $MgCl_2$, 1.0; ethylene glycol-bis (-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10; Hepes (sodium salt), 10; pH adjusted to 7.2 with tris (hydroxymethyl)aminomethane (Tris). The chambers was continuously perfused with the following bath solution for culture recordings: 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.6 Na_2HPO4, 0.4 $NaHPO_4$, 10.5 Glucose, 32.5 Hepes, 1.2 $CaCl_2$, adjusted to pH = 7.4 (NaOH) and with oxygenated ACSF (*see* composition above) containing 1.8 mM $CaCl_2$ for slice recordings. The appropriate $[Ca^{2+}]_o$ was obtained by adding varying Ca^{2+} concentrations to a solution containing 1 mM EGTA. Voltage-clamp recordings were performed using either an Axopatch 200A or 1-B amplifiers (Axon Instruments, Foster City, CA) for slice and culture recordings, respectively. Current signals were low-pass filtered at 5 kHz and digitized on-line at 25-100 kHz using a Digidata 1200 digitizing board (Axon Instruments) interfaced with an IBM-compatible computer system for both in situ and in vitro recordings.

Amphotericin B was used for perforated patch recordings (Rae et al., 1991). A stock solution of amphotericin B (6 mg/100 μl) was made daily in dimethylsulfoxide (DMSO), was dissolved and ultrasonicated in the pipette solution (4 μl/ml). To improve the gigaseal formation tips of electrodes were dipped into pipette solution for 1–2 sec and then back filled with the amphotericin-containing solution.

Data Analysis

Data acquisition, storage and analysis were done using PClamp version 6 (Axon Instruments). For all measurements, capacitance compensation and series resistance compensation (60–80%) were used to minimize voltage errors. Settings were determined by compensating the transients of a small (5 mV) 10 msec hyperpolarizing voltage step; the capacitance reading of the amplifier was used as value for the whole-cell capacitance. Membrane resistance (Rm) was evaluated from the average response to 50 hyperpolarizing (10 mV) current pulses (20 msec). Alternatively, for leak current subtraction the membrane resistance was determined with Clampfit (Axon Instruments) and a subsequent leak subtraction was performed off-line. Peak currents were determined using Clampfit, and statistical values (mean ± SEM, with *n*, being the number of cells tested) were evaluated with a statistical graphing and curve-fitting program (Origin, MicroCal).

All chemicals were from Sigma (St. Louis, MO) unless otherwise stated.

Results

Whole-cell patch-clamp recordings were obtained from 73 glial cells in rat hippocampal slices ranging from postnatal days 18 to 30. The majority of these cells showed a combination of voltage-activated outward and inward currents, similar to those previously described (e.g., Bordey & Sontheimer, 1997). However, 11 cells showed little time or voltage-dependence in our initial recordings as illustrated for one representative cell in Figure 1*A* (without leak-subtraction) and 1*D* (after off-line leak-subtraction). These currents were elicited by stepping the cell membrane from −160 to 80 mV (10 mV increment) from a holding potential of −80 mV. We would have ordinarily discarded these cells for further analysis. However, several investigators have classified such cells as a subpopulation of passive glial cells (Berger et al., 1991; Steinhauser et al., 1992; Steinhauser et al., 1994; D'Ambrosio et al., 1998) indicating a lack of voltage-activated currents. In 7 of 11 cells we observed a spontaneous transition from this apparent passive electrophysiological behavior to behavior that is characteristic of complex glial cells (Sontheimer & Waxman, 1993; Bordey & Sontheimer, 1997), namely, exhibiting prominent voltage-activated currents within 3–10 min of recording. The data illustrated in Fig. 1*B* (non-leak subtracted) and 1*E* (off-line leak subtracted) show recordings from the same cell as in Fig. 1*A* and *D*, 3 min later. The cell now shows prominent voltage-dependent outward and inward potassium currents and small inward Na^+ currents like those previously described for hippocampal astrocytes in situ (e.g., Bordey & Sontheimer, 1997). This change also resulted in a transition of the current-voltage (*I–V*) curve from linear (Fig. 1*C*, filled circles) to voltage-dependent (Fig. 1*C*, open circles). *I–V* curves were established by plotting current amplitudes at the end of each voltage step (indicated by the symbol above each family of traces in Fig. 1 *A* and *B*) as a function of applied voltage. The cell membrane capacitance and series resistance changed only slightly from 18 to 22 pF and from 16 to 13 MΩ respectively, and was insufficient to account for the observed appearance of voltage-activated currents by changes in uncompensated series resistance error.

In the following experiments we examined in further detail some cells that initially lacked voltage-activated currents, and mechanisms involved in the spontaneous unmasking of currents during the experiment. Our initial hypothesis was that the observed changes were primarily due to changes in the extracellular ionic environment when approaching the cell. In fact, to obtain cell recordings in slices, intracellular pipette solution is consistently

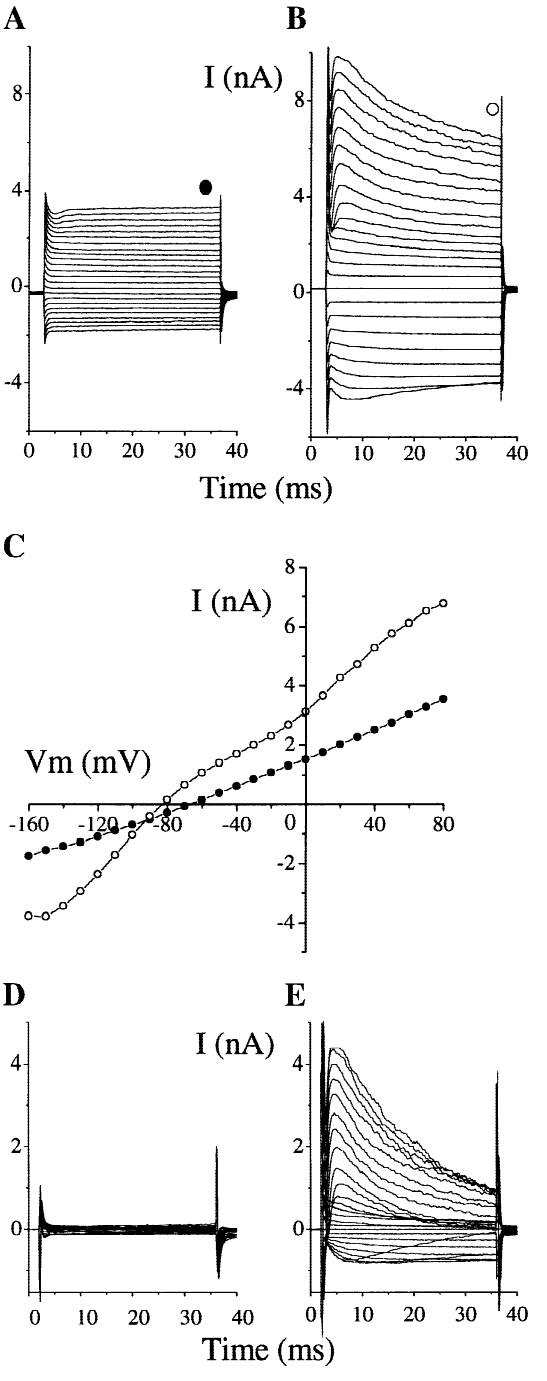

Fig. 1. An unexpected transition from a passive to a complex cell. (*A* and *B*) Whole-cell recordings of the same astrocyte in a hippocampal slice, spaced by 3 min. The cell spontaneously transformed from a passive cell lacking time- or voltage-dependent ion channels to a complex cell expressing voltage-activated ion channels. The cell membrane was stepped from −160 to 80 mV from a holding potential of −80 mV. (*C*) Current-voltage (*IV*) curves of the traces in A (filled circles) and in B (open circles). (*D* and *E*) Same recordings as in A and B, respectively, after off-line leak subtraction.

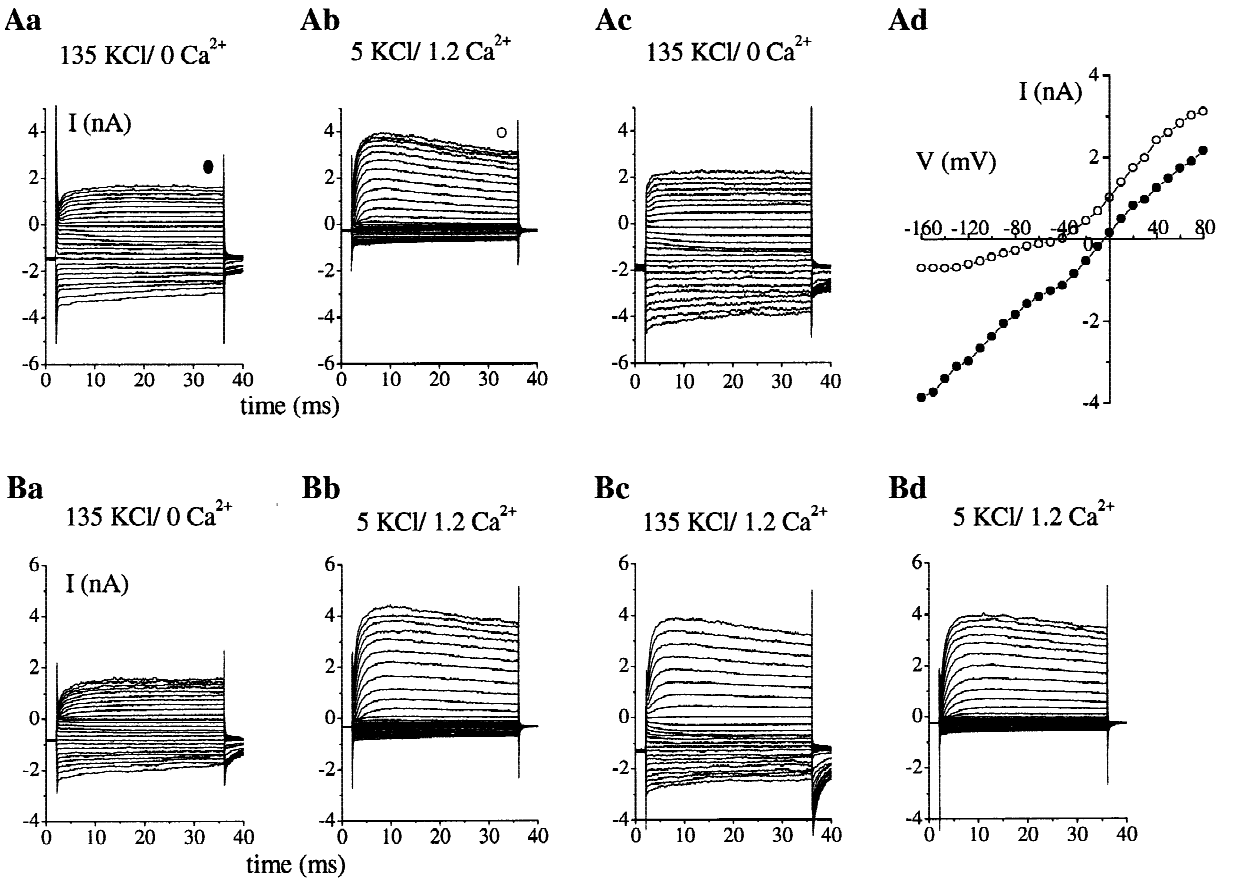

Fig. 2. Differential effects of 135 K^+/0 Ca^{2+} and 135 K^+/1.2 Ca^{2+} solutions. (*A* and *B*) Whole-cell recordings from the same spinal cord astrocyte in culture after stepping the cell membrane from −160 to 80 mV (10 mV increment steps) from a holding potential of −80 mV. (*Aa*) Whole-cell recordings in a 135 KCl/0 Ca^{2+} bath solution. (*Ab*) Recording from the same cell as in *A* after applying a 5 KCl/1.2 mM Ca^{2+} solution. (*Ac*) Application of a 135 KCl/0 Ca^{2+} bath solution on the same cell as in *Aa*. (*Ad*) Current amplitudes measured at the end of the depolarizing pulse plotted against the membrane potential for recordings in *Aa* (filled circles) and *Ab* (opened circles). (*Ba*) Whole-cell recordings in a 135 KCl/0 Ca^{2+} bath solution. (*Bb*) Restoration of a control 5 KCl/1.2 mM Ca^{2+} solution leads to restoration of outward currents and decrease of inward K^+ currents as observed for recordings in *A*. (*Bc*) Application of a 135 KCl/1.2 Ca^{2+} bath solution on the same cell as in *Ba*. (*Bd*) Restoration of a control solution with 5 KCl/1.2 Ca^{2+}.

expelled by positive pressure in order to prevent clogging of the pipette. This would extrude high K^+ and 0 Ca^{2+}/EGTA into the extracellular space surrounding the cell. To test this hypothesis we obtained recordings in cultured glial cells affording the opportunity to readily control the composition of the extracellular milieu.

Application of Intracellular Pipette Solution

Figure 2*A* shows representative non-leak subtracted current traces recorded by whole-cell patch-clamp from a cultured spinal cord astrocyte in the external presence of pipette solution, e.g., containing 135 K^+/0 Ca^{2+} buffered with EGTA (Fig. 2*Aa*). Application of voltage steps ranging from −160 to 80 mV from a holding potential of −80 mV activated currents with relatively little voltage dependence. As a result, the plot of steady-state outward and inward currents as a function of membrane potential was almost linear (Fig. 2*Ad*, open circles). However, when regular extracellular solution, e.g., 5 KCl/1.2 Ca^{2+} was restored (Fig. 2*Ab*), the cell exhibited much more pronounced voltage-activated currents more typical of those previously reported for cultured astrocytes (Sontheimer et al., 1992). The *I–V* curve obtained in a 135 K^+/0 Ca^{2+} solution changed from linear to outwardly rectifying (Fig. 2*Ad*, filled circles) upon restoration of physiological extracellular solution.

Changes in K^+ current amplitude is not unexpected since we changed the K^+ equilibrium potential (from −86 to 2 mV). The changes in apparent voltage dependence, however, cannot be explained by this shift alone. These recordings bear some resemblance to the spontaneous transition observed in brain slice recordings (Fig. 1*A* and *B*), specifically with regards to the unmasking of outwardly rectifying K^+ currents. However, they do not mimic the observed spontaneous appearance of inwardly rectifying K^+ currents. To further resolve this difference,

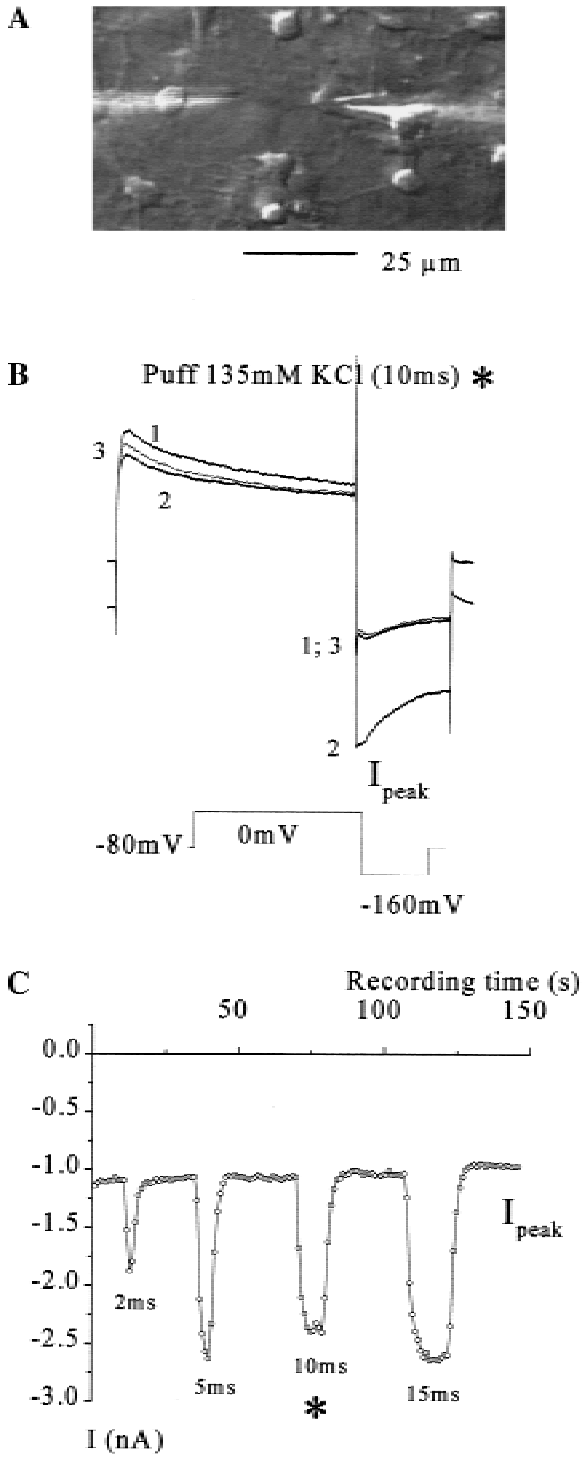

Fig. 3. Application of high K^+ solution on spinal cord astrocytes by pressure application through patch pipette. (*A*) Photograph of a recorded cell showing the patch pipette on the right and the pressure application pipette on the left. (*B*) Whole-cell recordings were obtained after stepping the cell membrane from 0 to −160 mV from a holding potential of −80 mV. This protocol was repeated every 5 sec. Four successive pressure applications of high KCl were performed while the cell was bathed in control solution (5 mM KCl/1.2 mM CaCl). Trace 2 corresponds to a recording when the cell was exposed to a high KCl (135 mM) solution applied by pressure for 10 msec. Trace 1 and trace 3 (recovery) correspond to recordings in control conditions before and after the 5 ms-pressure application. (*C*) The peak current amplitude (I_{peak}) measured at −160 mV is plotted as a function of the recording time.

we separately investigated the effects of high K^+ and 0 Ca^{2+}, reasoning that following superfusion of cells in brain slices with pipette solution, K^+ and Ca^{2+} concentration may be restored to their original levels with a very different time course.

In an initial attempt, we simply repeated experiments outlined in Fig. 2*A* on a different cell (Fig. 2*Ba* and *Bb*) but following exposure to normal extracellular solution cells were superfused by pipette solution that contained normal extracellular Ca^{2+} concentrations, namely 1.2 mM (Fig. 2*Bc*). As seen with high K^+ and Ca^{2+} free pipette solution, this led to a substantial increase in inward K^+ currents, but there was no significant effect on the voltage-dependence of outward K^+ currents. These effects were fully reversible (Fig. 2*Bd*).

To study the time course of this effect in greater detail, we placed a second patch pipette containing 135 mM KCl/1.2 mM Ca^{2+} pipette solution in close proximity to a cell under whole-cell recording (Fig. 3*A*) and expelled the solution by moderate pressure application. This essentially resulted in a reversible, large increase in inward currents (Fig. 3*B,* (1) control, (2) in high K^+, (3) recovery). Such an application was repeated every 5 sec and the resulting changes in inward currents were plotted as a function of recording time in Fig. 3*C.* The observed changes in inward K^+ current amplitude were rapid and fully reversible. Specifically, 10–15 msec pressure pulses were sufficient to fully activate inward K^+ currents.

Effect of low $[Ca^{2+}]_o$

Changes in $[Ca^{2+}]_o$ have been shown to alter the gating properties of potassium channels in other cell types (Green & Andersen, 1991; Hille, 1992). To test whether changes in the free extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) in the concentration range that one would expect to occur in the vicinity of a cell in slices during patch-clamp recordings can induce changes in K^+ current amplitudes, we recorded from cells superfused with regular bath solution containing 5 mM K^+ while varying $[Ca^{2+}]_o$ from 0 to 1.2 mM. An example of a cell recorded in regular bath solution containing 1.2 mM Ca^{2+} is shown in Fig. 4*Aa*. The cell exhibited voltage-dependent outward and inward currents. However, when $[Ca^{2+}]_o$ was lowered to 250 μM (Fig. 4*Ab*) both outward and inward currents were greatly suppressed and the *I–V* curve changed from voltage-dependent (filled squares) to al-

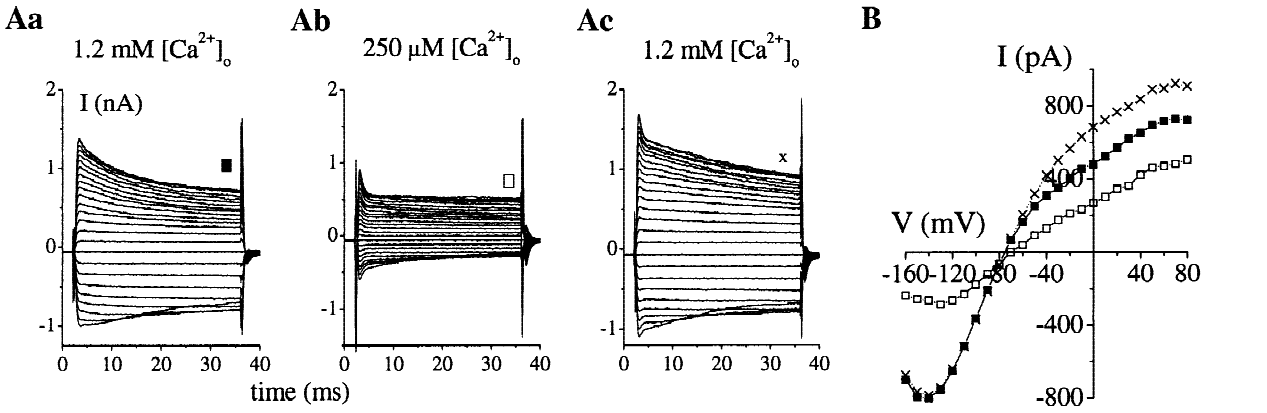

Fig. 4. Reversible inhibition of K^+ currents by low $[Ca^{2+}]_o$. (*A*) Whole-cell recordings from a spinal cord astrocyte in culture after stepping the cell membrane from −160 to 80 mV from a holding potential of −80 mV, in two different conditions. (*Aa*) The cell is bathed in a control solution (5 KCl/1.2 mM Ca^{2+}). (*Ab*) Application of a low $[Ca^{2+}]_o$ of 250 μM leads to the disappearance of transient outward currents and decrease of the inward currents. (*Ac*) Full recovery of currents after restoration of a control $[Ca^{2+}]_o$. (*B*) Current amplitudes measured at the end of the depolarizing pulse plotted against the membrane potential for recordings in *Aa* (filled squares), *Ab* (opened squares), and *Ac* (cross).

most linear (open squares) (Fig. 4*B*). In slices, the transition from 0 $[Ca^{2+}]_o$ to physiological $[Ca^{2+}]_o$ during the seal formation, may occur relatively slowly as EGTA needs to wash out of the extracellular space. To more closely mimic these conditions, cultured astrocytes were successively exposed to increasing $[Ca^{2+}]_o$ ranging from 0 to 800 μM (in 200 μM increments). To monitor the effects of a gradual increase in $[Ca^{2+}]_o$ on outward and inward current amplitudes, the cell membrane was stepped to 0 mV and then to −160 mV from a holding potential of −80 mV. This two-step voltage protocol was applied every 5 sec and peak outward (filled squares) and inward (open circles) currents were plotted as a function of time in Fig. 5*B*. Application of 200 μM $[Ca^{2+}]_o$ induced a decrease of the inward current amplitude without any effect on the outward current amplitude (trace number 2). Subsequent application of 400 μM $[Ca^{2+}]_o$ led to a decrease in both outward and inward currents (trace 3); in 600 μM $[Ca^{2+}]_o$ both outward and inward current amplitudes increased. At 800 μM $[Ca^{2+}]_o$, inward and outward currents were largely inhibited leaving only non-voltage-dependent currents intact. Over time, however, voltage-dependent outward and inward currents recovered partially. These experiments suggest that progressive increases in $[Ca^{2+}]_o$ to physiological values have marked and differential effects on outward and inward currents in astrocytes. The observed changes suggest that in our slice recordings, progressive increase in $[Ca^{2+}]_o$ surrounding the cell may have been largely responsible for the initial masking and ultimate revelation of voltage-activated currents.

Incomplete Cell Access

Proper cell access and low series resistance are essential to achieve reliable whole-cell voltage-clamp. Even under the best conditions, adequate voltage control cannot be assured due to inherent limitations of the whole-cell patch-clamp technique. We noticed that in slice recordings greater care than in culture recordings has to be exercised to assure proper access to cells. It is possible that in slices poor cell access is due to partial clogging of pipette tips after they were forced through tissue. While series resistance is one important parameter to use in assessing whether adequate cell access has been obtained, an independent measure would be desirable to assess the quality of voltage-clamp in tissue slices. In Fig. 6 we describe how the expression of voltage-activated inwardly rectifying K^+ currents which are abundantly expressed in differentiated astrocytes (Brodey & Sontheimer, 1997) can be used to evaluate the quality of the achieved voltage control. Figure 6*A* and *B* shows recordings obtained from an astrocyte in the hippocampal brain slice. Application of negative voltage steps (−10 mV increment) from a prepulse potential of 0 mV, activates inward currents at potentials greater −120 mV that show a characteristic time-dependent inactivation. This inactivation is due to voltage-dependent Na^+ block and has been well characterized in astrocytes (Ransom & Sontheimer, 1995; Ransom, Sontheimer & Janigro, 1996) and other cell types (Dousmanis & Pennefather, 1992; Sakmann & Trube, 1992). Initially, upon establishment of whole-cell recordings, little inwardly rectifying K^+ current was recorded (Fig. 6*A*). However, application of further suction to the pipette, over a time period of 5 min, showed appearance of inward currents with rapid and pronounced inactivation (Fig. 6*B*), characteristic of inwardly rectifying K^+ currents. This change was also associated with a significant increase in membrane capacitance from 15 to 58 pF and a decrease in series resistance (Rs) from 28.5 to 9.8 MΩ with 65% compensation in both cases. Changes in the current in-

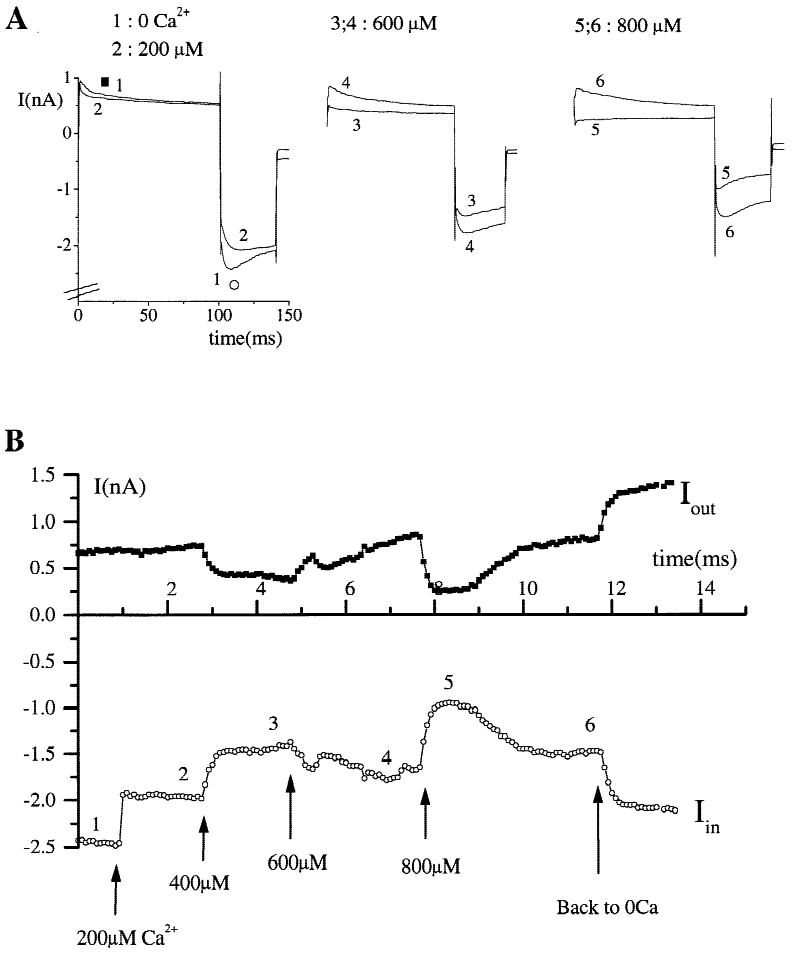

Fig. 5. Time- and concentration-dependent modulation of K^+ currents by $[Ca^{2+}]_o$. (*A*) Whole-cell recordings in 0 $[Ca^{2+}]_o$. When adding increasing concentrations of Ca^{2+} ranging from 0 to 800 μM (200 μM increments), the cell membrane was repeatedly stepped from 0 mV to −160 mV every 5 sec. Progressive addition of Ca^{2+} changed the biophysical profile of the recorded cell. (*B*) For the same cell as in *A*, plots of the inward current amplitude were measured at −160 mV (opened circles) and the peak outward current (filled squares) as a function of the recording time.

activation are more readily visible in the inset to Fig. 6*B* superimposing a single voltage step to −160 mV. Due to the voltage-dependent block of these currents by Na^+, the magnitude of this block increases with more negative voltages hence resulting in a decrease in the steady-state component of this current (I_{ss}). This is not the case in the recording in Fig. 6*A* in which we argue that here proper voltage control was not achieved due to poor cell access.

The characteristic Na^+-dependent inactivation thus appears to be a very sensitive feature allowing us to monitor the quality of recordings in astrocytes. To more convincingly demonstrate this observation, and to rule out modulatory effects by cell dialysis, we obtained perforated patch-clamp recordings from cultured astrocytes over extended period of times. While amphotericin ionophores were inserted in the membrane patch, an increase in cell access and a decrease in access resistance was accompanied with an increase in the Na^+-dependent block of Kir currents (Fig. 6*Ca* and *Cb*). Cm (pF) and uncompensated Rs (MΩ) values were: 28.7, 26 (Fig. 6*Ca*, 18 min) and 36, 17 (Fig. 6*Cb*, 44 min), respectively.

As can be seen in Figs. 6*Ca–Cb*, as time progressed this resulted in much reduced steady-state currents (I_{ss}) and much enhanced difference in the Kir current amplitudes at the beginning and at the end of a −180 mV hyperpolarized voltage step (I_{delta}) values. Following this observation, we analyzed 59 recordings of astrocytes recorded in hippocampal slices and measured I_{delta} (Fig. 6*C*), and plotted these values (representative of the current inhibited by Na^+ ions) as a function of the peak current (I_{peak}) measured at −180 mV. These astrocytes fell along a regression line with a coefficient of 0.97 (Fig. 6*D*). In those cells where access was initially poor but subsequently improved as in the example of Fig. 6*A* and *Ca*, it can be demonstrated that the initial ratio of I_{peak}/I_{delta} falls far away from the regression line (marked as *A* in Fig. 6*D*) but as access has improved this ratio falls onto the regression line (marked as *B* in Fig. 6*D*). Thus in those cells in which Kir is expressed, its voltage-dependent block by Na^+ can be used as a means to assess whether adequate voltage control and/or proper cell access had been achieved.

Discussion

We show that a subpopulation of recordings from astrocytes in acute rat hippocampal brain slices appears to

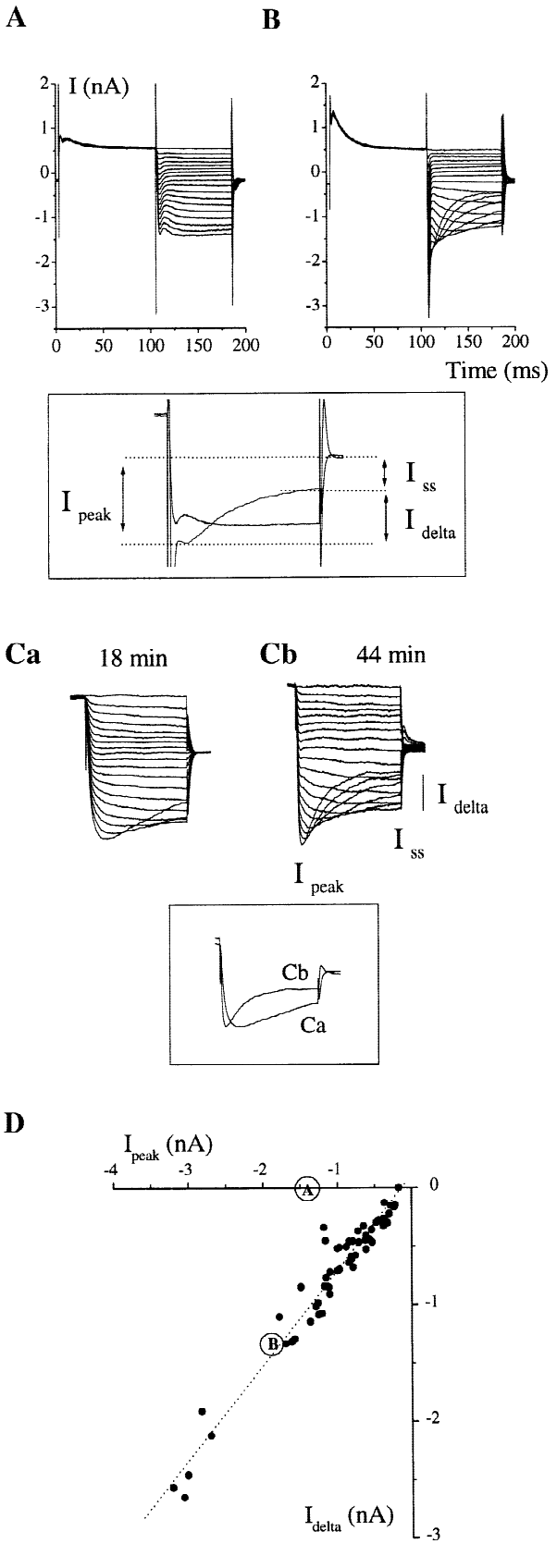

Fig. 6. Voltage-dependent inhibition of Kir by Na^+ as indicator of voltage-clamp quality. (*A*) and (*B*) Whole-cell traces of a spinal cord astrocyte after stepping the cell from 0 to −160 mV (10 mV increments) from a pre-pulse to 0 mV. This protocol activates inwardly rectifying K^+ currents (Kir) in astrocytes. The recordings in *A* displayed Kir currents with virtually no inactivation while 6 min later, recordings in *B* from the same cell showed Kir currents with a large inactivation. Associated with this change, the cell membrane capacitance increased from 15 pF to 58 pF and the series resistance (Rs) decreased from 28.5 to 9.8 MΩ (65% compensation for both recordings). The inset under Fig. 6*A* and *B* shows two superimposed traces from *A* and *B* for a single voltage step to −160 mV and illustrated the magnitude increase of the Na^+-dependent inactivation of Kir currents. *Ca* and *Cb* represent successive whole-cell recordings from the same cell at different times (same scale). This cell has been recorded by using the perforated patch-clamp technique. A slow and progressive development of the Kir inactivation appears over time concomitant with a decrease in the series resistance. The inset under Fig. 6 *Ca* and *Cb* shows two superimposed traces from *Ca* and *Cb* for a single voltage step to −180 mV. The amplitude difference (I_{delta}) between the Kir peak current measured at −160 mV (I_{peak}) and the steady-state current (I_{ss}), corresponds to a measure of the Na^+-dependent inactivation of Kir current. (*D*) I_{peak} was plotted as a function of I_{delta} (filled circles) for 59 cells recorded in hippocampal brain slices. The resulting curve could be fit by a straight line with a coefficient of correlation of 0.97. Measures obtained from recordings in *A* and *B* were plotted as A and B. The cell whose traces in *A* shows little Kir inactivation lies outside the linear regression (A). However this same cell (traces in B) falls onto this linear regression after improved cell access (B).

initially lack voltage-activated currents, e.g., appear passive, but can spontaneously show prominent outward and inward K^+ currents after a few minutes into a recording. Using cultured cells we show that a similar unmasking of voltage-gated currents can be the result of changes in the extracellular ionic milieu, specifically changes in K^+ and Ca^{2+}. Pipette solution used in patch-slice recordings often contains high K^+ and very low Ca^{2+} buffered with EGTA. This solution is often expelled during electrode approach onto cells by positive pressure applied to the pipette to prevent pipette clogging. We argue that leakage of pipette fluid may be at least partially responsible for the masking of voltage-activated currents in astrocytes. In cells where these conditions were remedied, voltage-activated currents were always recorded suggesting that astrocytes in rat hippocampal slices express a combination of voltage-activated currents. No subpopulation of cells that would be properly described as passive was identified.

A second finding in our study pertains to criteria used to assess proper cell access during recordings, a prerequisite for sufficient voltage-clamp in whole-cell recordings. The typical criteria used to determine if the cell access is good enough to allow proper recordings are the series resistance and the cell membrane capacitance, and these values are typically monitored throughout a recording. In Fig. 6, an example is illustrated where a cell that initially lacked the typical Na^+-dependent inactivation of Kir currents could be induced to show large Na^+-dependent inactivation of Kir currents upon further suction to the patch pipette affording better access. We concluded that this change was due to improved access to the cell because of a concomitant increase in Cm and decrease in Rs. Differentiated astrocytes abundantly express Kir currents. Since one of their biophysical features is the highly voltage-dependent inhibition by extracellular Na^+ ions, the presence or absence of this phenomenon can be used as a biological indicator for appropriate voltage control. The utility of this approach was tested in perforated patch-clamp recordings where we observed that improved access resulted in better voltage-clamp as indicated by enhanced Na^+-dependent inactivation (block) of Kir currents in these cells. In every recorded cell that showed such currents, the ratio of peak Kir current amplitude *vs.* the Na^+-inactivation block suggested a linear relationship in all cells that showed proper clamp. Any outlying cells from this linear regression was an immediate indication of an incomplete access for these recorded cells. These cells could be ''rescued'' by application of further suction to the patch-pipette resulting in lower Rs and higher Cm values. It is very possible that the membrane is folded inside the pipette by a suction that is too strong. In fact, it has been reported that a spontaneous seal with a planar configuration will lead to higher current amplitudes than in suction-induced patches (Bohle & Benndorf, 1994).

In contrast to cells with poor access, cells that showed a spontaneous transition from passive to complex cell with no obvious explanation was not accompanied by significant changes in series resistance or membrane capacitance. For these cells we propose that superfusion by pipette solution transiently masked voltage-activated currents. While it is impossible to unequivocally determine the reason for this spontaneous transition, several lines of evidence support the notion that buffered $[Ca^{2+}]_o$ and high K^+ are predominantly responsible for the initial masking of voltage-activated currents.

We initially recorded cells in high KCl/0 Ca^{2+} solution and switched to normal bath solution. Under these conditions inward currents including the holding current increased probably due to the elevated K^+ driving force. In addition these cells showed a loss of the transient outward current leading to a linear *I–V* relationship typical of passive cells. These conditions only partially explain the changes we observed in slices. Similarly, application of a high KCl/0 Ca^{2+} or a high KCl solution by bath or pipette did not mimic the spontaneous switch from passive cell to a complex cell with higher current amplitudes shown in Fig. 1. During the actual whole-cell formation, a change in the extracellular potassium concentration ($[K^+]_o$) might be buffered fast enough to only transiently affect the membrane of the recorded cell since as shown in Fig. 3 the effects of a high KCl/0 Ca^{2+} solution were extremely fast and fully reversible. We suggest that the effects of low Ca^{2+}/EGTA may be much more persistent than high K^+ and that the washout of EGTA may take several minutes. Indeed, our experiments that varied $[Ca^{2+}]_o$ while maintaining K^+ at 5 mM, closely mimic the recordings obtained in slices (Fig. 4).

Several previous studies suggested that low $[Ca^{2+}]_o$ has marked effects on ion channels some of which can be prevented by a high $[K^+]_o$ (Grissmer & Cahalan, 1989; Armstrong & Miller, 1990; Hille, 1992). This may explain why our cell culture experiments with 0 $[Ca^{2+}]_o$/high K^+ were insufficient to mimic the recordings observed in slices whereas 0 $[Ca^{2+}]_o$/normal K^+ was sufficient. The increases in nonvoltage dependent currents, initially observed in the 0 Ca^{2+} solution, are consistent with previous reports showing that A-type K^+ channels disappear upon removal of external Ca^{2+} and are replaced by a steady, nonselective leak conductance (Grissmer & Cahalan, 1989; Armstrong & Miller, 1990). Gradual increases in $[Ca^{2+}]_o$ in normal $[K^+]_o$ (5 mM) most closely mimicked the recordings in slices suggesting that a slow washout of EGTA in slices may be affecting these recordings. If such a slow restoration of time or voltage-activated potassium currents would also occur during establishment of the whole-cell recordings in slices it would explain the observed spontaneous transition from passive to complex cell.

Previous studies that reported the presence of two populations of astrocytes in mouse hippocampal slices

referred to these as passive or complex cells based on whether they expressed or lacked voltage-activated currents (Berger et al., 1991; Steinhauser et al., 1992; Steinhauser et al., 1994; D'Ambrosio et al., 1998). It is conceivable that under the conditions described here, voltage-activated currents may have been masked in some cells making them appear passive. Alternatively, however, it is possible that other differences account for the differences in recordings. Most intriguingly, species specific differences may account for some of the discrepancies. Walz and Kimelberg (1985) reported a dramatic species difference with regards to resting K^+ fluxes in mouse *vs.* rat astrocytes. In these studies, under resting conditions based on $^{42}K^+$ flux data, mouse astrocytes were 75-fold more K^+ permeable than rat astrocytes (Walz & Kimelberg, 1985). If this difference is also maintained in vivo, it is conceivable that due to this enhanced resting K^+ permeability of mouse astrocytes, voltage-activated currents are masked or even absent in some mouse astrocytes. However, while most studies reporting passive and complex cells were obtained in mouse, at least one study shows passive glial cells in rat hippocampus (D'Ambrosio et al., 1998). These authors argue that within the hippocampus subpopulations of cells exist, and that particularly in CA3 more passive cells can be found than in CA1 of rat hippocampus. We have recorded from 23 cells in CA3 of rat hippocampus and did not find any passive astrocytes that completely lacked voltage-activated currents. Also in an unrelated study we recorded from astrocytes in the cortex of mice bearing experimental brain tumors (Ullrich et al., 1998). These cells were outside the tumor margins and displayed typical voltage-activated outward and inward currents as we reported for rat astrocytes. In addition, Clark and Mobbs (1994) recorded from astrocytes in the retina of rabbit and found no evidence for a subpopulation of passive cells. Clearly further investigations are needed to study possible regional and species variations in greater detail. In light of the findings in the present study we favor the notion that passive glial cells observed in some studies may have shown a masking of voltage-activated channels possibly caused by changes in extracellular ionic milieu, specifically by highly buffered $[Ca^{2+}]_o$.

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