MIN ZHOU AND HAROLD K. KIMELBERG
Center for Neuropharmacology and Neuroscience and Division of Neurosurgery, Albany Medical College, Albany, New York 12208

Received 12 May 2000; accepted in final form 28 August 2000

Zhou, Min and Harold K. Kimelberg, Freshly isolated astrocytes from rat hippocampus show two distinct current patterns and different [K+]o uptake capabilities. J Neurophysiol 84: 2746–2757, 2000. Whether astrocytes predominantly express ohmic K\(^+\) channels in vivo, and how expression of different K\(^+\) channels affects [K\(^+\)], homeostasis in the CNS have been long-standing questions for how astrocytes function. In the present study, we have addressed some of these questions in glial fibrillary acidic protein [GFAP(+)], freshly isolated astrocytes (FIAs) from CA1 and CA3 regions of P7–15 rat hippocampus. As isolated, these astrocytes were uncoupled allowing a higher resolution of electrophysiological study. FIAs showed two distinct ion current profiles, with neither showing a purely linear I-V relationship. One population of astrocytes had a combined expression of outward potassium currents (\(I_{Ko}\)) and inward sodium currents (\(I_{Na}\)). We term these outwardly rectifying astrocytes (ORA). Another population of astrocytes is characterized by a relatively symmetric potassium current pattern, comprising outward \(I_{Ko}\) and abundant inward potassium currents (\(I_{K\text{in}}\)), and a larger membrane capacitance (\(C_m\)) and more negative resting membrane potential (RMP) than ORAs. We term these variety rectifying astrocytes (VRA). The \(I_{K\text{in}}\) in 70% of the VRAs was essentially insensitive to Cs\(^+\), while \(I_{K\text{in}}\) in the remaining 30% of VRAs was sensitive. The \(I_{K\text{in}}\) of VRAs was most sensitive to 4-aminoypyridine (4-AP), while \(I_{K\text{in}}\) of ORAs was more sensitive to tetraethylammonium (TEA). ORAs and VRAs occurred approximately equally in FIAs isolated from the CA1 region (52% ORAs versus 48% VRAs), but ORAs were enriched in FIAs isolated from the CA3 region (71% ORAs versus 29% VRAs), suggesting an anatomical segregation of these two types of astrocytes within the hippocampus. VRAs, but not ORAs, showed robust inward currents in response to an increase in extracellular K\(^+\) from 5 to 10 mM. As VRAs showed a similar current pattern and other passive membrane properties (e.g., RMP, \(R_m\)) to “passive astrocytes” in situ (i.e., these showing linear I-V curves), such passive astrocytes possibly represent VRAs influenced by extensive gap-junction coupling in situ. Thus, our data suggest that, at least in CA1 and CA3 regions from P7–15 rats, there are two classes of GFAP(+) astrocytes which possess different K\(^+\) currents. Only VRAs seem suited to uptake of extracellular K\(^+\) via \(I_{K\text{in}}\) channels at physiological membrane potentials and increases of [K\(^+\)]o. ORAs show abundant outward potassium currents with more depolarized RMP. Thus VRAs and ORAs may cooperate in vivo for uptake and release of K\(^+\), respectively.

INTRODUCTION

With the application of the patch-clamp technique for studying astrocyte ion channels, a large repertoire of ion channels have been identified in astrocytes in primary cell culture, acute brain tissue slices, and in acutely dissociated cell preparations (Verkhratsky and Steinhauser 2000). These findings have altered the early concept that astrocytes only possess passive ohmic K\(^+\) conductances (Kuffler 1967). However, astrocytes displaying an ohmic I-V relationship, or “passive astrocytes,” have also been widely identified from different brain regions in situ, e.g., corpus callosum (Berger et al. 1991), hippocampus (D’Ambrosio et al. 1998; Steinhauser et al. 1992), spinal cord (Chvatal et al. 1995), and red nucleus (Akopian et al. 1997) using the patch-clamp technique. As a high percentage of these passive astrocytes showed positive staining for glial fibrillary acidic protein (GFAP), it has been suggested that these represent mature astrocytes (Chvatal et al. 1995; Steinhauser et al. 1994b).

Recently, D’Ambrosio et al. (1998) found a high percentage of passive astrocytes (72.5%) in the CA1 region as compared with CA3 (12.5%), which correlated with a more intensive cell coupling in the CA1 region. In another study, when ATP was omitted from the pipette solution, which should prevent the gating of gap junctions between coupled astrocytes in situ, no passive astrocytes were found in the CA1 region (Borody and Sontheimer 1997). These two studies suggest that the existence of passive astrocytes in situ is likely due to syncytial influences on the measured membrane current profiles.

In this study, we examined this question by studying the current properties of freshly isolated astrocytes (FIAs) from the CA1 and CA3 regions of rat hippocampus. In our recent studies, GFAP(+) FIAs showed different receptor expression profiles to GFAP(+) astrocytes in primary culture and the former seemed to better represent the in vivo situation (Cai and Kimelberg 1997; Kimelberg et al. 1997; Schools and Kimelberg 1999). By extension, the ion channel profiles analyzed in FIAs should better represent the properties of astrocytes in vivo. Additionally, FIAs provide an uncoupled astrocyte model, so that the voltage-clamp control should be largely improved and the ion channels kinetics can be studied at a higher resolution. Also, isolated cells allow a fast and complete exchange of extracellular solution which makes the observation of fast [K\(^+\)]o uptake possible.

We found two astrocyte types based on the membrane current profiles of process-bearing FIAs. Neither of them showed purely linear I-V curves. These two types of astrocytes also
exhibited differences in cell size, resting membrane potential, ion current pharmacology, and regional distribution. The values of both cell-membrane capacitance and resting membrane potential were bimodally distributed and corresponded to the results obtained in situ (D’Ambrosio et al. 1998), indicating that they are distinct astrocyte types. Only the VRA type of astrocyte, characterized by a resting membrane potential close to $E_{K}$ and expressing abundant inward potassium currents ($I_{Kin}$), was capable of channel-mediated $K^+$ uptake. By contrast, due to a more positive resting membrane potential and lack of $I_{Kin}$, the second type of astrocytes (outwardly rectifying astrocytes (ORAs)) failed to show $K^+$ uptake under the same condition.

**METHODS**

**Cell isolation**

Astrocytes were freshly isolated from rat hippocampus following the procedure of Tse et al. (1992), with modifications. Young rats (postnatal days 7 to 15) were anesthetized with 50% O$_2$-50% CO$_2$ and killed by decapitation. Their brains were dissected out and rinsed in Ca$^2+$-free bath solution (for composition, see next section). The hemispheres were quickly cut into slices (around 500-µm thick) in coronal orientation using a razor blade. Slice preparation was performed in Ca$^2+$-free bath solution at 6°C. The hippocampi were then dissected from the slices and incubated in the same solution for 1 h at room temperature, bubbled with 95% O$_2$-5% CO$_2$. This “rest” step is critical for obtaining astrocytes with the process-bearing morphologies shown in Fig. 1, A–C. After that, hippocampi were transferred for 30 min into normal aCSF solution containing 24 U/ml papain at 22°C, supplemented with 0.24 mg/ml cysteine as enzyme activator. After washing with Ca$^2+$-free bath solution, the hippocampi were stored in the same oxygenated Ca$^2+$-free bath solution at room temperature for at least 1 h for recovery. Right before the experiment, the CA1 or CA3 subregions were dissected out from a slice and triturated onto the recording chamber in standard bath solution using fire-polished Pasteur pipettes (tip diameter 200 µm) under microscopic observation. To wash away unattached cells and tissue debris, gravity perfusion (1–3 ml/min) of standard bath solution was switched on 5 min after trituration and was continued throughout the experiment. Recordings typically started 10 min after tissue trituration.

**Solution and drugs**

The standard bath solution contained 150 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM HEPES, and 10 mM D-glucose. The pH of the bath solution was adjusted to 7.4 with NaOH. In Ca$^2+$-free solution, CaCl$_2$ was substituted by 1 mM sodium pyruvate. The pipette solution was 140 mM KCl, 0.5 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM EGTA, 10 mM HEPES, 3 mM Mg-ATP, and 0.3 mM Na-GTP. For mRNA analysis, all solutions were prepared in sterile milliQ water and were autoclaved. Phosphate buffered saline (PBS) consisted of 137 mM NaCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, 0.5 mM MgCl$_2$, and 0.7 mM CaCl$_2$. 4-aminopyridine (4-AP), tetraethylammonium (TEA), tetrodotoxin (TTX), and all other reagents were purchased from Sigma (St. Louis, MO).

**Immunocytochemistry**

The procedure for GFAP staining of the freshly isolated cells has been described in detail in a previous report from our laboratory (Kimelberg et al. 1997). In brief, CA1 and CA3 regions of hippocampus were triturated after enzymatic treatment in standard bath solution onto coverslips precoated with 0.01% poly-D-lysine. After the cells had adhered to the coverslips (approximately 10 min), the attached cells were fixed by immersion in 4% paraformaldehyde solution for 30 min. The fixed cells were washed with cold PBS and then permeabilized with acetone at −20°C for 3 min. After washing in PBS, the cells were incubated in 3% normal goat serum (NGS) for 10 min. Following a rinse in PBS, the cells were incubated with 1:400 primary antibody (Rabbit anti-GFAP, DAKO, Carpinteria, CA) for 1 h. After further washing in PBS, the cells were incubated for 1 h with a secondary, fluorescein-conjugated goat antibody raised against rabbit IgG (Biosource, Camarillo, CA) at a 1:75 dilution. After a final wash, the coverslips were mounted onto slides with 50% glycerol in PBS and viewed in a Nikon Epi-fluorescence microscope. To determine nonspecific labeling the primary antibody was omitted.

**Electrophysiological recordings**

Membrane currents were measured by the patch-clamp technique in the whole-cell configuration. Current signals were amplified with an Axopatch 200B (Axon Instruments, Foster City, CA) and sampled by a TL-1 DMA Interface (Axon Instruments). Data acquisition was carried out by Pclamp 6.0.4 software (Axon Instruments) running on a Gateway 2000 Pentium II 233 computer. Low-resistance patch pipettes (3–5 MΩ) and cell harvesting pipettes were fabricated from borosilicate capillaries (OD: 1.2 or 1.5 mm, Warner Instrument, 137 mM NaCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, 0.5 mM MgCl$_2$, and 0.7 mM CaCl$_2$. 4-aminopyridine (4-AP), tetraethylammonium (TEA), tetrodotoxin (TTX), and all other reagents were purchased from Sigma (St. Louis, MO).
Hamden, CT) using a Flaming/Brown Micropipette Puller (model P-87, Sutter Instrument, Novato, CA). Membrane capacitances ($C_m$) and series resistances ($R_s$) were determined by a depolarizing test pulse from $-70$ to $-60$ mV (10 ms, filter at 10 kHz, sampling at 30 kHz). Input resistances ($R_i$) were estimated in the voltage-clamp mode from the currents induced by 10 mV hyperpolarizing steps. For slow drug application, drugs were delivered to the established whole-cell patches by gravity perfusion. For fast application, test solutions were rapidly applied to the established whole-cell patches through square tubes using the Perfusion Fast-Step System (SF-77, Warner Instrument) controlled by programmed data acquisition protocols. All experiments were performed at room temperature ($\sim 20$–$24^\circ$C).

**Single-cell RT-PCR for GFAP**

The single-cell reverse transcription polymerase chain reaction (RT-PCR) procedure for GFAP mRNA detection post recording has been described in detail in our previous study (Zhou et al. 2000). The primers for the first round (GFAP-1) and the second nested primers (GFAP-2) for rat cDNA encoding GFAP were designed to flank introns in genomic sequences according to the NCBI GenBank sequence data (accession number: L27219). The specificity of the oligos/primers used in this study and the sensitivity of the single-cell RT-PCR procedure has been previously assessed in our laboratory (Schools and Kimelberg 1999).

After membrane current recording, the same individual cell was harvested by applying gentle suction with a second pipette (diameter $\sim 10$ μm), which was silanized to avoid RNA adherence, baked to destroy RNases, and filled with 1 μl RNase-free milliQ water. The pipette contents were then expelled into a PCR tube filled with 10 μl milliQ water, and the tube was immediately placed in a $-80^\circ$C freezer.

RT-PCR for the cell was done on the same day after electrophysiological recording using a SuperScript One-Step RT-PCR system (Gibco BRL, Life Technologies) as previously described (Cai et al. 2000). The reverse transcription (RT) step was run at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 60 s; 40 cycles) with the GFAP-1 primer pair. Second-round amplification (denaturation at 94°C, annealing at 60°C, extension at 72°C; all 60 s; 35 cycles) was done with the nested primers, GFAP-2. First round product (1 μl) was used in each second-round reaction in a total volume of 50 μl. The PCR products were analyzed by 1.5% agarose gel electrophoresis. The PCR products gave the expected products of 437 base-pair (bp) size after second-round amplification.

**Data analyses**

Steady-state activation of $K^+$ currents was obtained by dividing maximum currents by the corresponding driving force, $V - E_k$, where $E_k$ is the $K^+$ equilibrium potential. Subsequently, data points were fitted by the equation

$$g/k_{\text{max}} = \left[1 + \exp\left(V/V_{0.5} - V/k_n\right)\right]^{-1}$$

with $V$ being the test potential, $V_{0.5}$ the voltage of half-maximum activation, and $k_n$ a slope factor.

Steady-state inactivation was fitted by a Boltzmann equation of the form

$$\Delta I/k_{\text{max}} = \left[1 + \exp\left(V - V_{g,0.5}/k_s\right)\right]^{-1}$$

where $I$ is the voltage-dependent current amplitude, $I_{\text{max}}$ maximum current, $V$ the prepulse potential, $V_{g,0.5}$ the voltage of half-maximum inactivation, and $k_s$ the slope factor. Significant differences between data were evaluated by the use of Student’s $t$ test. All the data are given as means $\pm$ SD. The level of significance was set at $P < 0.05$.

**RESULTS**

**Morphology, GFAP staining, and GFAP mRNA of FIAs from CA1 and CA3**

Figure 1, A–C, shows the morphology of freshly isolated astrocytes, as seen by phase contrast microscopy. Typically, the cell soma either had oval, elongated, or triangular shapes, with multiple, long, and bushy processes extending from the cell body. The morphology of astrocytes from CA1 and CA3 were not distinguishable.

The proportions of the different cell types in the fresh cell suspension based on morphology are summarized in Table 1. The process-bearing astrocytes shown in Fig. 1, A–C, amounted to $\sim 19$–25% of the total cells regardless of CA1 and CA3 regions, whereas 22% of the cells present in the suspension had a triangular appearance with several primary dendritic branches. The electrophysiological properties of these cells and absence of GFAP staining showed they were likely neurons (Zhou et al. 2000). Forty-three percent of the less-branched glia showed a small size and less-branched morphology. As we have described in another report, this population of cells was GFAP(−), but GFAP mRNA(+) (Zhou et al. 2000). The 14% remaining cells could not be classified into any of above morphologically defined cell populations due to atypical morphology, swelling, or general damage. We only studied process-bearing astrocytes for this report.

For GFAP staining, CA1 and CA3 regions were dissected out from 30 hippocampal slices obtained from postnatal day 8 to 15 animals ($n = 6$) and dissociated. After staining, we found a total of 254 isolated cells which preserved the same morphology as shown in Fig. 1, A–C. All of them stained positively for GFAP.

**TABLE 1. Proportion of different morphological cell types in CA1 and CA3 suspensions**

<table>
<thead>
<tr>
<th>Age of Rats</th>
<th>Regions</th>
<th>Process-Bearing Astrocytes (FIAs)</th>
<th>Less Branched Glia</th>
<th>Neurons</th>
<th>Unidentified Cells</th>
<th>Total Cells Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>P7</td>
<td>CA1</td>
<td>25%</td>
<td>46%</td>
<td>11%</td>
<td>18%</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>CA3</td>
<td>21%</td>
<td>45%</td>
<td>17%</td>
<td>16%</td>
<td>309</td>
</tr>
<tr>
<td>P15</td>
<td>CA1</td>
<td>21%</td>
<td>37%</td>
<td>31%</td>
<td>11%</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>CA3</td>
<td>19%</td>
<td>43%</td>
<td>28%</td>
<td>10%</td>
<td>571</td>
</tr>
<tr>
<td>Average, %</td>
<td></td>
<td>21.5</td>
<td>42.8</td>
<td>21.8</td>
<td>13.8</td>
<td></td>
</tr>
</tbody>
</table>

In each age group, the percentages of different cell types were calculated from the sum of 6 hippocampal CA1 or CA3 cell suspensions. In each suspension, at least three fields were counted and the number of cells divided into the different types according to their morphology. See text for description of different cell types. FIA, freshly isolated astrocytes.
GFAP antibody. An example of the GFAP immunoreactivity of these process-bearing cells is shown in Fig. 1E.

After patch-clamp recording, 25 similar process-bearing astrocytes were harvested for GFAP mRNA measurement by RT-PCR. Twenty-one out of 25 cells (84%) were GFAP mRNA positive \((n = 25)\) (Fig. 1F). These experiments together indicated that the process-bearing cells under study were both GFAP(+) and GFAP mRNA(+) astrocytes.

**Freshly isolated GFAP(+) astrocytes show two membrane current profiles**

The FIAs were found to exhibit two classes of membrane current profiles. One was characterized by a dominant expression of outward transient and sustained \(K^+\) currents, plus inward sodium currents (Fig. 2A). Because currents were mainly inducible in response to depolarizing voltage steps, and the \(I-V\) relationship showed a strong outward rectification (Fig. 2C), we term these cells outwardly rectifying astrocytes (ORA). These comprised 60% of the cells examined \((n = 80)\) from CA1 and CA3. The other type of astrocyte was characterized by a relatively symmetric inducible current pattern (Fig. 2B) and a variably rectifying \(I-V\) curve (Fig. 2D). We therefore term them variably rectifying astrocytes (VRA). These comprised the remaining 40% of cells recorded.

The ionic selectivity of both outward transient and sustained and inward sustained currents were determined from the reversal potentials of tail currents \((E_{rev})\). For outward currents, the membrane potential was stepped to +60 mV (both transient and sustained currents were found to be almost maximally activated at this voltage) for varied periods \((10, 50, 100\) ms), and then to different test potentials \((-120\) to +20 mV in 10 mV increments). A representative recording from a VRA is shown in Fig. 3A. As summarized in Table 2, the reversal potentials \((E_{rev})\) of outward currents were weakly dependent on prepulse duration. As the prepulse was prolonged to 100 ms, the tail currents still reversed at -74 mV. This \(E_{rev}\) is close to the theoretical potassium equilibrium potential calculated from pipette and bath solutions \([K^+]_i/[K^+]_o = 140/5\) mM \((E_K = -88\) mV), indicating that even though \(I_{K_d}\) were almost completely inactivated at this time, the outward currents were still dominated by potassium channels \((\text{presumably} I_{K_d})\). The involvement of nonspecific currents to the outward currents appears negligible as they will have an \(E_{rev}\) around 0 mV. The \(E_{rev}\) for inward sustained currents was also tested \((n = 5)\). As shown in Fig. 3B, the cell was first stepped to -180 mV for 50 ms and then to the same test potentials as for the outward currents. The mean \(E_{rev}\) of -84 ± 5 mV for inward sustained currents was very close to the \(E_K\) of -88 mV. These data demonstrate that both inward sustained and outward transient and sustained currents are predominantly carried by potassium. In this analysis, we also noticed that, in some VRAs, depolarization prepulse-evoked tail currents showed a time-dependent activation (Fig. 3A), implying the presence of endothelial \(I_{ha}\) like currents (Guatteo et al. 1996). A further clarification of these \(I_{ha}\) currents will need further study.

**Fig. 2.** Ion channel profiles of FIAs measured by whole-cell recordings. The cells were preconditioned at -110 mV for 300 ms to remove the inactivation of \(I_{K_a}\). Subsequently, the cells were stepped for 50 ms to test potentials ranging from -180 to +60 mV in 10 mV increments (for voltage commands, see shadowed inset). Two distinct astrocyte types, as defined by their ion channel profiles, were seen. A: representative recording of one type of astrocytes characterized by a dominant expression of outward transient \((I_{K_a})\) and sustained delayed rectifier potassium currents \((I_{K_d})\). This cell showed also initial inward transient sodium currents \((I_{Na})\). These \(I_{Na}\) currents are shown in an expanded scale in the inset below A. As the corresponding \(I-V\) curve showed a dominant outward rectification \((C)\), these type of cells were termed outward rectifying astrocytes (ORAs). B: the other astrocyte type was distinguished by a more symmetrical inducible potassium currents in response to de- and hyperpolarizing steps. The \(I-V\) curve of this type of astrocytes showed several regions of rectification \((D)\); therefore, we term them variably rectifying astrocytes (VRA). These two cells were all obtained from the CA1 region of a P8 rat. The membrane potentials measured ≤30 s after establishment of a whole-cell seal was -50 mV for recording A and -74 mV for recording B. The post recording RT-PCRs for GFAP mRNA are shown in Fig. 1F, lanes 4 and 6, for A and B, respectively.
The fast inward transients evoked from ORAs by depolarization steps typically activated when potentials were more positive than $-40 \text{ mV}$ and peaked at $-10 \text{ mV}$. The time to peak at $-10 \text{ mV}$ averaged $450 \pm 50 \mu s$ ($n = 20$). These currents were completely blocked by $100 \text{ nM TTX}$ ($n = 6$, Fig. 3, D–F). Application of $0.1 \text{ M TTX}$ also produced a $12 \pm 6 \text{ mV}$ hyperpolarization in resting-membrane potential in four ORA cells. Both the kinetics and TTX sensitivity of these sodium currents were similar to the astrocytes studied in situ by Bordey and Sontheimer (1997).

Different passive membrane properties between ORAs and VRAs

Resting membrane potentials (RMP) were measured in the current-clamp mode within $30 s$ after establishment of a stable whole-cell configuration, with a $140 \text{ mM KCl}$ pipette solution and $5 \text{ mM K}^+ \text{ bath solution}$. For all the cells, these RMPs spanned a wide range ($-25$ to $-85 \text{ mV}$). They were bimodally distributed and well fitted by a double Gaussian function, yielding two peak values of $-66$ and $-43 \text{ mV}$ (coefficient of correlation $r = 0.87$, Fig. 4A). These values closely match the two mean RMP figures found in VRAs ($-65 \pm 10 \text{ mV}$) and ORA ($-40 \pm 10 \text{ mV}$) astrocytes, respectively (Table 3). A wide variation in astrocyte RMPs is consistent with the in situ patch-clamp study by McKhann et al. (1997). Also, the two average peak RMP values found by FIAs were similar to the bimodal peak values of $-69$ and $-51 \text{ mV}$ found by D’Ambrosio et al. (1998) recorded in astrocytes of the hippocampal CA1 + CA3 regions in situ. A slightly lower value of the second peak of $-43 \text{ mV}$ for the FIAs perhaps reflects a developmental influence as 3–5 wk rats were used in the slice study. RMPs did not differ between the CA1 and CA3 regions for the two cell types. Input resistance ($R_{in}$) for ORAs ($5.0 \pm 3.5 \text{ G} \Omega$) was significantly higher than for VRAs ($0.6 \pm 0.3 \text{ G} \Omega$) in cells from both CA1 and CA3 (Table 3). However, although the $R_{in}$ values roughly followed a bimodal distribution,
Abundant $I_{Kohm}$ conductance exists in VRAs

The ion channel identities of different membrane current components can be assessed by their distinct pharmacology. We used prepulse activation/inactivation plus off-line digital subtraction strategies to isolated different current components. To isolate outward transient $I_{Ka}$ and sustained $I_{Kdr}$, prepulses at $-110$ and $-40$ mV (300 ms for each) prior to the test potentials were used, respectively (see shadowed inset for Fig. 5). With this strategy, we found that the current density of $I_{Ka}$ was lower in VRAs than in ORAs. The current density in ORAs was $63 \pm 12$ pA/pF ($n = 25$, peak response to a $+60$ mV step) versus $6 \pm 5$ pA/pF in VRAs ($n = 7$). We next used millimolar concentrations of 4-AP (Bordey and Sontheimer 1997, 1999; Tse et al. 1992) to test the sensitivity of $I_{Ka}$ seen in both cell types. As shown in Fig. 5, $B1–B3$, 4 mM 4-AP almost completely blocked $I_{Ka}$ in VRAs. At a +60-mV step, 4-AP blocked the peak $I_{Ka}$ current amplitude by 91 ± 15% compared with controls ($n = 4$). However, the same concentration of 4-AP showed only 63 ± 13% inhibition of $I_{Ka}$ in ORAs (Fig. 5, $A1–A3$, $n = 10$). From studies of recombinant K$^+$ channels, it is now clear that currents resembling $I_{Ka}$ can be mediated by a number of cloned K$^+$ channel subunits (see review by Coetzee et al. 1999). Heterogeneity of 4-AP-sensitive current has been recently reported in other cell types (Honjo et al. 1999) and also in astrocytes in situ (Bordey and Sontheimer 1999). The differential inhibitory effect of 4-AP on $I_{Ka}$ implies that a different subunit composition may exist between ORAs and VRAs.

To examine the contribution of $I_{Kdr}$ in outward sustained currents, we tested the sensitivity of outward sustained currents to the selective K$^+$ channel blocker TEA (Bordey and Sontheimer 1997; Tse et al. 1992). Sustained outward potassium currents were isolated by a set of depolarizing steps with a 300-ms prepulse at $-40$ mV to avoid activating $I_{Ka}$ (see shadowed inset in Fig. 5). TEA (15 mM) inhibited the outward sustained potassium currents in a voltage-dependent manner. At the +60 mV step, TEA blocked outward sustained potassium currents by $71 \pm 11\%$ in ORAs ($n = 6$; Fig. 5, $C1–C3$). The same concentration of TEA blocked only $25 \%$ ($n = 5$) of the sustained outward currents in VRAs (Fig. 5, $D1–D3$). These data suggest that in VRAs other current components besides $I_{Kdr}$ also contribute to the total outward sustained currents. The likely candidates are the unidentified leak potassium channels ($I_{Kohm}$, e.g., TREK, which was recently shown to be abundant in hippocampus (see review by Coetzee et al. 1999).

To further clarify the contribution of $I_{Kohm}$ to the sustained inward potassium currents in VRAs, we tested the sensitivity of inward potassium currents to extracellular 1 mM CsCl. At this concentration, $I_{Kir}$ currents observed in cultured astrocytes were almost completely blocked (Ransom and Sontheimer 1995). Sustained inward potassium currents were induced by a set of hyperpolarizing steps with a prepulse at 0 mV for 500 ms to remove the activation of $I_{Ka}$ (see shadowed inset in Fig. 6). We found that in 17 out of 32 VRAs, these currents did not show a typical $K_0^+$-like voltage- and time-dependent inactivation. Pharmacologically, only in 3 out 10 VRAs were these currents sensitive to 1 mM Cs$^+$ (Fig. 6, $A1–A3$), whereas another

---

**Fig. 4.** Fitting of RMP, $R_{in}$, and $C_m$ to double Gaussian models. A–C: RMPs, $R_{in}$s, and $C_m$s from 80 cells were plotted as a function of observed frequencies. The distributions of RMP and $C_m$ were clearly bimodal as they were well fitted by a double Gaussian function (smooth lines, with the coefficient of correlation $r = 0.87$ for A, and $r = 0.98$ for C), yielding a pair of RMP values of $-66$ and $-43$ mV, and two peak $C_m$ values of 10 and 30 pF, respectively. However, $R_{in}s$ could not be well fitted by this model (B).
7 VRAs showed only a weak sensitivity to Cs (inhibited by 20%, n = 7, Fig. 6, B1–B3). The noninactivating kinetics and the resistance of these inward potassium currents to Cs suggests an abundant coexistence of $I_{Kohm}$ in VRAs. Besides $I_{Kohm}$, other channels, e.g., ERG (Emmi et al. 2000) and $I_{ha}$ (Guatteo et al. 1996), are also likely involved. Therefore, inward currents of VRAs are likely composed of several $K_1$ components. For this reason, we term the current $I_{Kin}$ to cover this complexity. In summary, the pharmacological properties of $I_{Ka}$ and $I_{Kdr}$ are different between ORAs and VRAs. Notably, VRAs can possess abundant $I_{Kohm}$ or other Cs$^+$-insensitive $I_{Kin}$, which is similar to the properties of "passive cells" in situ (D’Ambrosio et al. 1998).

### TABLE 3. Passive membrane properties of FIAs

<table>
<thead>
<tr>
<th></th>
<th>CA1</th>
<th>CA3</th>
<th>CA1 + CA3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ORA</td>
<td>VRA</td>
<td>ORA</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−42 ± 12 (24)</td>
<td>−64 ± 15* (22)</td>
<td>−38 ± 13 (24)</td>
</tr>
<tr>
<td>C_m pF</td>
<td>9 ± 3 (24)</td>
<td>35 ± 12* (22)</td>
<td>10 ± 7 (24)</td>
</tr>
<tr>
<td>R_in GΩ</td>
<td>3.1 ± 0.2 (24)</td>
<td>0.6 ± 0.3* (22)</td>
<td>6.9 ± 5.4 (24)</td>
</tr>
</tbody>
</table>

Values are means ± SD with cell number in parentheses. *, significant differences between ORAs and VRAs at $P < 0.05$ (Student’s $t$ test).

A more limited voltage-clamp control occurs in VRAs compared to ORAs.

As noted in the INTRODUCTION, there has been controversy regarding whether there are any mammalian astrocytes in situ which show purely linear I-V curves. We found that no FIAs showed a linear I-V relationship. We therefore explored the possible reasons accounting for this difference between FIAs and some of the findings for astrocytes in situ.

In situ astrocytes are extensively connected by gap junctions. This leads to space-clamp problems and restricted voltage control. As discussed by Steinhauser et al. (1994a), poor space clamp makes the precise analysis of $I_{Na}$ kinetics impossible because $I_{Na}$ has a rapid activation and inactivation time course. Therefore, they studied astrocytic $I_{Na}$ exclusively by using freshly isolated astrocytes.

As VRAs showed a significantly larger size than ORAs (as reflected by $C_m$), this suggested that the smaller sized ORAs could be better clamped than the larger sized VRAs. To study this, we measured $I_{Ka}$, as it has a fast kinetics and is present in both astrocyte types. We analyzed the activation kinetics of $I_{Ka}$ for both VRAs and ORAs (Fig. 7, A–C) and fitted the $I_{Ka}$...
The CA1 region of P12 and P10 rats, respectively. (all at filled squares corresponding to the symbols used for the pulse as indicated by the vertical lines identified by open and filled circles and curves, the current amplitude values were taken close to the end of each 50-ms kinetics of both \( I_{K_a} \)). We found the half-maximum activation of \( I_{K_a} \) seen in ORAs and VRAs, showing that differences in voltage-clamp control mainly affect the activation kinetics of \( I_{K_a} \). In the same analysis, we found that the \( I_{K_a} \) amplitude reached a peak within 1.4 ± 0.3 ms (n = 12) in response to a +60 mV step in ORAs. In contrast, for VRAs the corresponding time was 6 ± 2 ms (n = 6), or four times longer than for ORAs. These analyses indicate that the different \( K_a \) activation kinetics between ORA and VRA could well be due to altered space-clamp control in these different sized astrocytic subpopulations.

Only VRAs showed a rapid response to a fast increase in [K\(^+\)]

We next addressed the question of whether these \( I_{K_{in}} \) channels seen in VRAs are capable of fast K\(^+\) uptake when [K\(^+\)]\(_o\) is rapidly increased. We directly tested the K\(^+\) uptake capabilities of both VRAs and ORAs by a fast change of [K\(^+\)]\(_o\) from 5 to 10 mM, a concentration likely to be achieved physiologically during intense neuronal activity. As shown in Fig. 8, only the VRA-type astrocytes (n = 3 for VRAs, n = 6 for ORAs) showed strong inward currents under these conditions. As the membrane potential was clamped at −70 mV in this experiment, this indicates that the \( I_{K_{in}} \) channels have a high conductance for K\(^+\) under these conditions. Further, the fast onset and offset kinetics in response to high K\(^+\) application suggests that VRAs are capable of rapidly removing K\(^+\) released from neurons, in agreement with a role of VRAs in [K\(^+\)]\(_o\) homeostasis.

Ion current profiles and occurrence of ORAs and VRAs in CA1 and CA3 regions

The distribution of the four ion current components, namely, \( I_{Na} \), \( I_{K_{in}} \), \( I_{K_{a}} \), and \( I_{K_{dr}} \), in the CA1 and CA3 regions of ORAs and VRAs is shown in Fig. 9A. There were no clear differences between CA1 and CA3 when the currents of the two types of FIAs were averaged. However, as shown in Fig. 9B, there were differences between ORAs and VRAs. One hundred percent of the ORA type astrocytes possessed \( I_{K_{in}} \) (n = 48), 92% (44/48) \( I_{K_{a}} \), and 90% (43/48) \( I_{Na} \). Inward potassium currents were observed in only 6 out of 48 ORAs, and all of these current amplitudes were <40 pA at −160 mV. As it is difficult to distinguish these small currents from nonspecific leak currents, so they were not registered as channel-mediated inward potassium currents. In contrast, all the VRAs possessed \( I_{K_{in}} \) 53% (17/32) had \( I_{K_{a}} \) and only one cell showed a detectable but very small \( I_{Na} \) (1/32) (Fig. 9B).

As shown in Fig. 9C, the occurrence of VRAs and ORAs showed regional differences between CA1 and CA3. In the CA1 region, ORA and VRA current patterns were recorded in essentially similar proportions of cells (52 versus 48%). However, ORAs were more frequently recorded than VRAs in FIAs prepared from the CA3 region (71 versus 29%).

### Activation Kinetics

activation kinetics by the Boltzmann equation (see methods, Eq. 1). We found the half-maximum activation of \( I_{K_a} \), \( V_{n,0.5} \) was 20 mV positive in VRAs as compared with ORAs without a change in slope (\( K_a \)) (Fig. 7C). These different \( V_{n,0.5} \) values corresponded well with the thresholds for \( I_{K_a} \) seen in ORAs and VRAs. When 100 nM TTX was added to block \( I_{Na} \), the \( I_{K_a} \) threshold in ORAs was −60 mV (n = 5, data not shown). This is very close to the value of −70 mV seen in cultured spinal cord astrocytes (Bordey and Sontheimer 1999). However, it was also 20 mV more negative than for VRAs (n = 3, data not shown). We also analyzed the steady-state inactivation of \( I_{K_a} \) by the Boltzmann equation (see methods, Eq. 2). As shown in Fig. 7, D–F, this analysis did not reveal any difference in both half-maximum inactivation, \( V_{n,0.5} \), and slope, \( K_a \) between ORAs and VRAs, showing that differences in voltage-clamp control mainly affect the activation kinetics of \( I_{K_a} \).
Observations of astrocytes isolated from older P20–30 rats showed that ORA and VRA remained the two astrocyte profiles (data not shown). However, some of the ORAs additionally expressed a more pronounced I_{Kin} but retained I_{Na} (n = 31, unpublished observations). A detailed analysis of acutely isolated astrocytes from older animals (P20–30) will be the subject of a future report.

**DISCUSSION**

Freshly isolated astrocytes

Acutely, or freshly isolated astrocytes (FIAs) as we term them, have been used on a limited basis to study astrocyte properties (reviewed in Barres et al. 1990; Kimelberg et al. 2000; Sontheimer 1994; Verkhratsky and Steinhaeuser 2000), especially in relation to the new findings on current and other properties of astrocytes determined in situ in slices. The FIAs offer the advantage of being able to clearly determine the properties of astrocytes without having to control for indirect effects, as is critical for work in slices. The widely used primary astrocyte cultures prepared from neonatal rodents have the same advantages but suffer from unpredictable changes occurring due to long-term culture (Kimelberg et al. 2000). The major potential disadvantages of the FIAs are alterations and damage during isolation and to what degree they are a selected sample of astrocytes in situ. The present studies, in showing a good correspondence in current profiles between FIAs and astrocytes recorded from the same region in slices, further support the use of FIAs as representative models.

**Heterogeneity and regional differences of GFAP(+) astrocytes subtype in hippocampus**

In this study, two types of GFAP(+) astrocytes were identified based on distinct ion channel profiles, passive membrane properties, and the good fit of their RMPs and C_m to a double Gaussian function. At this developmental stage (P7–P15), the major differences in the current profiles are the presence of I_{Na}...
(90%) but absence of $I_{\text{Kin}}$ in ORAs and presence of $I_{\text{Kin}}$ but absence of $I_{\text{Na}}$ in VRAs. In hippocampus, numerous GFAP(-) “complex” astrocytes also showed a similar current profile to ORAs (Seifert et al. 1997; Steinhäuser et al. 1994a; Zhou et al. 2000). However, both morphology and lack of GFAP staining distinguish them from ORAs. Furthermore, $I_{\text{Na}}$ in such complex glia almost disappears after P20 (Kressin et al. 1995), but it is continuously present in ORAs at least until P30 (unpublished observations).

We found essentially an equal frequency of occurrence of VRAs and ORAs in CA1. However, the ORAs were 2.4-fold more frequent than VRAs in the CA3 region (Fig. 9C). This agrees with the finding by D’Ambrosio et al. (1998) that astrocytes with different current profiles are anatomically segregated between the CA1 and CA3 regions with passive cells, which may correspond to our VRAs (see predominantly in CA1 region). However, such heterogeneity differs from the conclusions of Bordey and Sontheimer (2000). As astrocytes in CA1 and CA3 regions have a different structural relationship with neurons and blood vessels (Coyle 1978; McBain et al. 1990), these regional differences may reflect different physiological functions.

VRAs share several similarities with passive astrocytes recorded in situ

Passive astrocytes in situ are characterized by a linear $I-V$ relationship, low $R_m$, and an RMP close to $E_K$. However, whether these cells carry voltage-gated conductances is not clear (Chvatal et al. 1995). It is noteworthy that the VRAs are quite similar in many respects to the passive astrocytes recorded in situ. The VRAs show a relatively symmetrical potassium current pattern, the mean RMP of $-65 \pm 10$ mV is close to the $E_K$ and the average $R_m$ value of 0.6 GΩ is comparable to the low $R_m$ recorded for passive astrocytes in situ. Although VRAs showed large $I_{\text{Kin}}$ currents, 17 out of 32 VRAs (53%) failed to show time-dependent inactivation at potentials more negative than $-130$ mV (Fig. 6), which are the typical $I_{\text{Kin}}$ kinetics seen in cultured astrocytes (Ransom and Sontheimer 1995). This suggests an expression of other inward potassium current components besides $I_{\text{Kir}}$. Finally, 70% (7/10 cells) of $I_{\text{Kin}}$ in VRAs were less or insensitive to Cs$^+$, and TEA blocked only a fraction (30%) of sustained outward potassium currents in VRAs. These data together indicate a coexistence of abundant $I_{\text{Kohn}}$ and/or other K$^+$ channel-mediated currents with voltage-gated conductances in VRAs. Therefore, under the conditions in situ, especially the effects of extensive gap junction couplings (see next section), at least some of VRAs could represent the passive astrocytes recorded in situ.

Syncytia likely obscure the apparent activation of voltage-gated ion channels in situ

VRAs have an apparently large cell-surface area, as their mean $C_m$ value of 33.5 pF is not only 3.5 times larger than...
ORA-type astrocytes, but also 1.7 times larger than freshly isolated hippocampal pyramidal neurons (19.2 ± 6.6 pF, Siefert et al. 2000). This larger size is accompanied by a more positive \( I_{\text{ka}} \) activation threshold, half-maximal activation parameter (\( V_{n,0.5} \)), and a longer time to peak of maximal current activation as compared with the smaller ORAs. It is possible that the \( K^+ \) channel subunits that mediate \( I_{\text{ka}} \) are different between ORAs and VRAs as they showed different sensitivities to 4-AP. However, this difference is unlikely to account for the different \( I_{\text{ka}} \) activation kinetics between VRAs and ORAs. A number of cloned \( K^+ \) channels, including Kv1.4, Kv3.4, Kv4.1, Kv4.2, and Kv4.3, show the current kinetics resembling \( I_{\text{ka}} \) (Baldwin et al. 1991; Schrotter et al. 1991; Serodio et al. 1994, 1996; Stuhmer et al. 1989). The biophysical properties of \( I_{\text{ka}} \) seen in ORAs were very similar to Kv1.4, Kv4.1, Kv4.2, and Kv4.3 (\( V_{n,0.5} \approx -10 \) to \(-22 \) mV and \( V_{g,0.5} \approx -45 \) to \(-69 \) mV). VRA showed a similar \( V_{n,0.5} \) value of 8 mV to Kv3.4 (\( V_{n,0.5} = 13 \) to 19 mV), but a more negative \( V_{g,0.5} \) of \(-65 \) mV as compared with Kv3.4 (\( V_{g,0.5} = -20 \) to \(-32 \) mV). If Kv3.4 was a major participant in VRAs, VRAs should also have a distinct \( V_{g,0.5} \) around \(-23 \) to \(-32 \) mV. In our analysis, however, \( V_{g,0.5} \) showed no difference between VRAs and ORAs.

Because imperfect voltage clamp is an unavoidable limitation for whole-cell study, all the analyses generated from this study cannot be taken as absolute values. However, relative effects are reliable and our analyses indicate that the \( I_{\text{ka}} \) activation kinetics analyzed from VRAs were shifted to the positive compared to ORAs, likely caused by the difference in their cell sizes. It is reasonable to infer that the voltage-clamp control will be further reduced and activation of voltage-gated conductances will be further obscured by the coexistence of a large leak potassium conductance experienced in situ due to syncytial coupling. Thus, we speculate that the “passive” properties of astrocytes in situ likely result from their widespread electronic coupling.

**Functional implications**

The oldest and most frequently discussed role for the dominant \( K^+ \) conductances seen in astrocytes in the CNS is spatial buffering of \( K^+ \) released from active neurons (Orkand et al. 1966). In Müller cells and astrocytes, \( K_{\text{ir}} \) was suggested to be the most suitable channel for this role as it has a high open probability at the resting potential and the conductance increases with increasing \([K^+]_o \) (Newman and Frambach 1984; Ransom and Sontheimer 1995). Similarly, VRAs carried abundant \( I_{\text{kin}} \) and showed robust \([K^+]_o \) uptake (Fig. 8B). It is noteworthy that the \( I_{\text{kin}} \) in VRAs are at least partially mediated by \( K_{\text{sh}} \), suggesting an important role of these leak potassium channels in \( K^+ \) uptake. In astrocytes, these likely important functional channels, but their molecular identities and function in astrocytes have not yet been defined (Coetzee et al. 1999). Additionally, the channels participating in \( I_{\text{kin}} \) seem more complicated as another two currents, namely, \( I_{\text{na}} \) (Guatelli et al. 1996) and ERG (Emmi et al. 2000), have been identified in astrocytes, suggesting a further complexity in astrocyte \( K^+ \) channel expression. ORAs lacked \( I_{\text{ka}} \) and also failed to show any \([K^+]_o \) uptake capability (Fig. 8A). However, the finding that \( I_{\text{na}} \) is present in 90% of ORAs, and TTX induced a moderate hyperpolarization (12 mV) of membrane potential in ORAs (unpublished data) supports the idea that astrocyte \( Na^+ \) channels are open at the RMP, as described by Sontheimer et al. (1994, 1996) for cultured spinal cord astrocytes. If ORAs are coupling with VRAs in vivo, their depolarized RMP likely gate their abundant expressed outward voltage-gated potassium channels, particularly, \( K_a \), which has an activation threshold around \(-60 \) to \(-70 \) mV. Thus, open \( K_a \) channels could contribute to spatial buffering to release \([K^+]_o \) to an area where neuronal activity is less intense.

In conclusion, numerous studies have demonstrated that the study of astrocytes in situ is a powerful approach to address the questions of astrocyte properties and function in the CNS. FIA provides a useful additional single-cell model, allowing a study of astrocytes properties that are close to those exhibited in situ, but without having to account for the effects of the cell-cell coupling and influence of the surrounding neurons and other cells. Taking advantage of studying FIA, we demonstrated the existence of two distinct astrocyte types, but neither of them behaved precisely like the passive astrocytes described in situ.

We demonstrated that one type, VRAs, are capable of fast \([K^+]_o \) uptake, and the second type, ORAs, showed a more depolarized RMP and abundant expression of outward potassium currents and does not take up \([K^+]_o \), with a moderate physiological increase of \([K^+]_o \) from 5 to 10 mM. For future, these and other data from FIA can be integrated with in situ studies to further advance our understanding of the roles that astrocytes play in the CNS.

The authors thank Y. Goto for contributing to some of the experiments, Dr. M. W. Fleck, Dr. A. A. Mongin, and G.P. Schools for stimulating discussions and comments on the manuscript, and C. J. Charniga for excellent technical assistance.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-19492 to H. K. Kimelberg.

**REFERENCES**


