Loss of $I_A$ Expression and Increased Excitability in Postnatal Rat Cajal-Retzius Cells

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Mienville, J.-M., Irina Maric, Dragan Maric, and John R. Clay. Loss of $I_A$ expression and increased excitability in postnatal rat Cajal-Retzius cells. J. Neurophysiol. 82: 1303–1310, 1999. Although an important secretory function of Cajal-Retzius (CR) cells has been discovered recently, the precise electrical status of these cells among other layer I neurons in particular and in cortical function in general is still unclear. In this paper, early postnatal CR cells from rat neocortex were found to express an inactivating K current whose molecular substrate is likely to be the Kv1.4 channel. Both electrophysiological and immunocytochemical experiments revealed that expression of this A-type current is down-regulated in vivo and virtually disappears by the second postnatal week. At this time, CR cells have become capable of evoked repetitive firing, and their action potentials are larger and faster, yet these electrical properties still appear incompatible with a role in cortical network function, as inferred from comparisons with other cortical neurons. Also at this time, a large proportion of CR cells display spontaneous spiking activity, which suggests the possibility of additional roles for these cells. We conclude that the loss of A channels along with an increase in Na channel density shape the changes in excitability of postnatal CR cells, in terms of both the patterns of evoked firing and the emergence of spontaneous spiking.

INTRODUCTION

Neocortical Cajal-Retzius (CR) cells represent a near perfect system for the developmental neurobiologist, owing to a relatively short life span and to reliable anatomic identifiers (Hestrin and Armstrong 1996; Marin-Padilla 1984; Mienville and Pesold 1999; Ramón y Cajal 1891; Retzius 1893), which may allow in extenso follow-ups of various parameters involved in the function and fate of these cells. Surprisingly, it is not until the past three years that papers on the physiology of these cells have begun to appear (Hestrin and Armstrong 1996; Mienville 1998; Mienville and Barker 1997; Mienville and Pesold 1999; Schwartz et al. 1998; Zhou and Hablitz 1996). Emerging from these reports is a repertoire of voltage- and ligand-gated ion channels that would make these cells akin to “regular” cortical neurons, were it not for the notable immaturity of some of their membrane electrical properties (Hestrin and Armstrong 1996; Mienville 1998; Mienville and Pesold 1999). The possibility that CR cells constitute a special type of glial cells has been raised in the past (see König and Schachner 1981; and Marín-Padilla 1984, for historical overviews). Although such an idea would not be incompatible with the above-mentioned repertoire of ion channels (Barres 1991), other features of CR cells including the presence of an axon, the expression of specific neuronal markers (König and Schachner 1981), and the ability to fire action potentials (APs) (Hestrin and Armstrong 1996; Zhou and Hablitz 1996) have definitively established their neuronal nature.

It is still unclear, however, whether CR cells participate in the same functions, including rapid transfer and processing of information, and are part of the same networks as those usually associated with cortical neurons. In that line of thought, Zhou and Hablitz (1996) did not detect any obvious difference in properties between postnatal CR cells and other layer I neurons, and Ekstrand et al. (1996) even proposed a role for CR cells in fast feed-forward inhibition. Against such roles, however, is the observation that J postnatal CR cells do not appear to have synaptic inputs (König and Marty 1981), consistent with their lack of spontaneous synaptic activity (A. Kriegstein, personal communication; J.-M. Mienville, personal observations), and 2) they disappear before full maturation of the cortex (Del Río et al. 1995; Mienville and Pesold 1999). Early on, Noback and Purpura (1961) expressed doubts as to a role of CR cells in electrical signaling and conjectured that their complex morphology may involve “some obscure functions.” As it turns out, light recently has been shed on such functions with the discovery that CR cells synthesize and secrete a large protein, Reelin, crucial for neuronal migration and proper cortical lamination (D’Arcangelo et al. 1995). Naturally, this per se does not exclude other roles for CR cells in connection with their possible synaptic outputs (see DISCUSSION). Meanwhile, one may ask the questions: is the firing capacity of CR cells compatible with the fast signaling required for higher cortical function? If not, could it be linked to Reelin secretion? If not, what could be its purpose? These are some of the questions addressed, from a developmental perspective, in the present paper. In connection with the marked increase in the excitability of late CR cells, we first provide anatomic and functional evidence for a postnatal down-regulation of an inactivating K current likely to be carried by Kv1.4 channels.

METHODS

All experiments were conducted with Fisher rat pups at three developmental stages corresponding to postnatal day (P) 1, 5, and 12–13. Animals were handled in accordance with guidelines set out by the National Institutes of Health.
Electrophysiology

In situ patch-clamp, single-pipette methods were used in the somatic whole cell and cell-attached recording configurations (Stuart et al. 1993). Brain slices were prepared, and CR cells were identified as previously described (Mienville 1998; Mienville and Barker 1997; Mienville and Pesold 1999). Any given experiment was performed on two to five slices from at least two littermates. For quantitative voltage-clamp experiments (i.e., Fig. 1), the extracellular solution was exactly as given by Mienville and Barker (1997) and was designed to prevent Na and Ca currents. Pipette solution contained (in mM) 146.7 KCl, 10 HEPES, and 3.3 KOH (pH 7.2). Evoked APs were studied in whole cell current clamp, whereas spontaneous firing was studied with cell-attached methods at a pipette potential of 0 mV; in both cases, the extracellular solution was regular Ringer (e.g., Mienville 1998). This solution was also used to fill on-cell pipettes, while a K-gluconate-based solution (Mienville 1998) was used for current-clamp experiments. During the latter, voltage responses to depolarizing current increments were obtained from a membrane potential of −80 mV maintained through constant DC injection. In this paper the term “suprathreshold” refers to a stimulus that evokes maximal AP frequency. APs were abolished by 1 µM tetrodotoxin (TTX; n = 9), suggesting the primary involvement of Na channels in their generation. Two cases displaying obvious “Ca shoulders” were eliminated from the analysis.

Signal amplification was through a List EPC-7 unit. Voltage- and current-clamp stimulation protocols, including P4 leak subtraction, as well as data acquisition and analysis, were provided by pCLAMP 6 (Axon Instruments, Foster City, CA). On-cell activity was stored on videotape (Instrutech, Port Washington, NY), and spikes were later counted with the PAT module of SES software (courtesy of Dr. John Dempster) after low-pass filtering at 500 Hz to avoid spurious counts due to membrane noise.

The drug 4-aminopyridine (4-AP; Sigma, St. Louis, MO) was dissolved directly in the extracellular solution and applied through the bath perfusion, while TTX (Sigma) was applied locally. Results are given as means ± SE. Student’s t-test and ANOVA were used when comparing two or more than two groups, respectively, unless otherwise noted.

RESULTS

**I_A progressively disappears in postnatal CR cells**

Our preceding work (Mienville and Barker 1997) had shown an increase in I_A functional expression by embryonic CR cells. The present results on postnatal CR cells demonstrate the inverse trend, i.e., a decrease in I_A functional expression (Fig. 1).
TABLE 1. Conductance-voltage parameters for $I_A$ and delayed rectifier currents recorded in CR cells at various postnatal stages

<table>
<thead>
<tr>
<th>Age</th>
<th>$D_{max}$</th>
<th>$V_{1/2}$</th>
<th>$k$</th>
<th>$n$</th>
<th>$D_{max}$</th>
<th>$V_{1/2}$</th>
<th>$k$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>977 ± 106</td>
<td>−16.9 ± 1.6</td>
<td>17.7 ± 0.7</td>
<td>14</td>
<td>516 ± 67</td>
<td>+5.3 ± 1.6</td>
<td>15.2 ± 0.6</td>
<td>14</td>
</tr>
<tr>
<td>P5</td>
<td>508 ± 77*</td>
<td>−14.7 ± 2.5</td>
<td>13.7 ± 0.6*</td>
<td>22</td>
<td>525 ± 41</td>
<td>+0.6 ± 0.4*</td>
<td>13.4 ± 0.2*</td>
<td>37</td>
</tr>
<tr>
<td>P13</td>
<td>165 ± 11*</td>
<td>−14.4 ± 2.4</td>
<td>11.8 ± 1.7*</td>
<td>4</td>
<td>584 ± 64</td>
<td>−0.7 ± 1.0*</td>
<td>12.5 ± 0.5*</td>
<td>18</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$ is number of cells. Parameters were obtained from Boltzmann fits [see legend of Fig. 1; $D_{max}$ (maximum density) substituted for $G_{max}$] to data from individual cells. CR, Cajal-Retzius; P1, postnatal day 1. * Differences from P1 significant at $P < 0.002$ (ANOVA followed by least significant difference test).

1, A–C). This decrease is manifested both in terms of the proportion of cells expressing $I_A$ (Fig. 1B), most P13 cells altogether lacking it (Fig. 1A, bottom traces), and in terms of current density in those older cells that still express it (Fig. 1C; Table 1). Simultaneous analysis of the delayed rectifier current indicates no change in maximum current density during this particular period (Fig. 1D; Table 1). A close look at the conductance-voltage ($G$-$V$) curves (Fig. 1, C and D) reveals subtle, development-related differences among the two other parameters, namely the potential of half-maximum activation, $V_{1/2}$, and the steepness of the curve, $k$ (Table 1). These differences could be due to a number of factors affecting channel function, including heteromeric assembly of main ($\alpha$) subunits, accessory ($\beta$) subunit expression, or posttranslational modifications. These aspects are beyond the scope of the present work and are not discussed further.

To obtain information on the molecular identity of CR cell $I_A$, we performed immunocytochemical experiments to investigate the expression of Kv1.4 and Kv4.2 proteins, two inactivating K channels widely expressed in brain (Seredio et al. 1994). Kv1.4 was found to be expressed by CR cells (Fig. 2) in all of four series of experiments. On the contrary, we were never able, during three separate experiments, to detect Kv4.2 in these cells, whereas this subunit clearly was expressed in P13 hippocampus (data not shown). These results are consistent with a previous study showing labeling of neocortex by Kv1.4 but not by Kv4.2, both channels being present in hippocampus (Sheng et al. 1992). Figure 2A shows Kv1.4 staining in P1 cortex. Staining is particularly intense on mostly horizontally oriented cells, consistent with the CR cell phenotype as also indicated by the presence of Reelin-immunopositive cells in sister sections (right inset). Despite robust evidence for an axonal localization of Kv1.4 in telencephalon (Sheng et al. 1992; Song et al. 1998), we find that the strongest labeling occurs on the proximal dendritic swelling of CR cells. In that respect, CR cells resemble dorsal cochlear nucleus neurons in which Kv1.4 was also localized to dendrites and somata (Juiz et al. 1996). At P5, Kv1.4 staining is still substantial in layer I (Fig. 2B), but labeling of CR cells appears somewhat fainter (inset). At P13, Kv1.4 staining is practically absent from layer I (Fig. 2C). Rare CR cells still present at this stage (bottom inset) (see also Mienville and Pesold 1999) show very faint staining for Kv1.4 antibody (top inset), whereas layer II/III cells are distinctly labeled. Concerning the latter, the limited

FIG. 2. Kv1.4 expression in developing CR cells. A: P1 cortex. Three cells whose proximal dendrite is heavily labeled with Kv1.4 antibody are magnified in the left inset (arrows). Other CR-like cells in layer I display various staining intensities. Note also the labeling of radial fibers in the cortical plate, possibly from migrating neurons expressing $I_A$ (Mienville and Barker 1997). Right inset shows 2 Reelin-positive cells from another P1 section. B: P5 cortex. Arrow points to a Kv1.4-labeled CR-like cell enlarged in the inset. Note the fainter staining in this cell. C: P13 cortex. Arrow points to a presumed CR cell (enlarged in top inset) that displays particularly weak Kv1.4 staining compared with layer II/III cells. Bottom inset shows a Reelin-immunopositive cell in another P13 section. Calibration bar: 30 $\mu$m for main panels; 20 $\mu$m for insets.
resolution of light microscopy does not permit conclusive localization of Kv1.4, but the label surrounding cell bodies is not incompatible with axon terminal staining, as seen in globus pallidus neurons (Song et al. 1998).

**Firing properties switch from single to repetitive during postnatal development**

Our primary goal here was to investigate possible relationships between developmental changes in $I_A$ expression and CR cell excitability. A striking result of this inquiry was the fundamental differences observed in terms of 1) the maximum number of APs triggered by a 250-ms pulse (Fig. 3), and 2) the waveform pattern of individual APs (Fig. 4). We will first address the first set of differences. Figure 3A depicts characteristic observations made at P1 and P13. At P1, 9/12 (75%) cells fired a single AP on suprathreshold stimulation, whereas at P12–13 only 1/16 (6%) cell fired a single AP. Intermediate proportions (7/17 cells; 41%) were noted at P5. The mean number of APs observed at each stage is reported in Fig. 3B. The maximum number attained was 7, which corresponds to a
frequency of 28 Hz. This is well below sustained frequencies observed in both mature and immature pyramidal neurons, which may range up to 50–100 Hz (McCormick and Prince 1987), and over an order of magnitude lower than those recorded in mature GABAergic interneurons, which may reach 600 Hz (Connors and Gutnick 1990; McCormick et al. 1985).

In an attempt to correlate AP number with $I_A$ expression, we coupled each current-clamp recording to a voltage-clamp recording consisting of a single pulse to $+40 \text{ mV}$ preceded by a prepulse to $-120 \text{ mV}$. Note that $+40 \text{ mV}$ is reasonably close to $E_{\text{Na}}$, so that contamination of $I_A$ by $I_{\text{Na}}$ was probably minimal. Indeed, the resulting traces closely resembled, in their development-related pattern, unsubtracted traces obtained in conditions of K current isolation (e.g., those displayed on the left of Fig. 1A). The ratio peak/steady-state current (P/SS) was thus used to provide an index of $I_A$ expression. Although a significant relationship was found at P5 between this ratio and AP number (Fig. 3C), no significant correlation was found at any other stage (not shown). This would make sense if the maturation of P5 cells showed greater variability (the so-called “range of talent”) for both parameters than P1 or P12–13 cells, for which correlations are expected to be “noisier.”

APs mature in postnatal CR cells but fail to acquire properties of adult cortical neurons

The next set of analyses examined changes in various parameters of individual APs during postnatal development of CR cells. As previously noted by Zhou and Hablitz (1996), APs increased in amplitude and decreased in duration (Fig. 4, A and B) in a manner consistent with an increase in Na channel density. From P1 to P12–13, amplitude as well as depolarization and repolarization rates increased (Fig. 4A), the latter two parameters apparently more so than AP amplitude because half-amplitude width as well as rise and fall times decreased (Fig. 4B). Zhou and Hablitz (1996) proposed the conclusion that layer I neurons, including CR cells, eventually acquire mature APs. Unless layer I differs widely from other cortical layers with respect to the mature properties of its constituents, we would have to disagree on the conclusion concerning CR cells. In our hands, the various AP parameters of CR cells were substantially different from those published for mature, non-layer I cortical neurons. For example, McCormick et al. (1985) and McCormick and Prince (1987) reported values in the range 80–100 mV for AP amplitude, 300–400 V/s for rate of rise, and 0.3–0.8 ms for half-amplitude width. Although their work was performed at 35–37°C, these values may be contrasted with those of P12–13 CR cells reported in Fig. 4, A and B. Interestingly, in the study of Hestrin and Armstrong (1996), the mean half-width for late postnatal CR cells (3.9 ms) was substantially larger than that for other layer I neurons (1.2–1.7 ms).

In view of likely relationships between repetitive firing and Na channel density (Lockery and Spitzer 1992), we tested for correlations between AP number and waveform parameters related to Na channel density. Significant correlations were only found with AP amplitude (Fig. 4C). This would be consistent with AP amplitude being a more accurate predictor of Na channel density than parameters involving time, which may also depend on distribution (e.g., somatic vs. neuritic).

Effects of 4-AP

To directly test the involvement of $I_A$ in the firing pattern of CR cells, we attempted to induce repetitive firing in P1 cells by blocking $I_A$ with 4-AP. Addition of 5 mM 4-AP to the bath perfusion virtually abolished $I_A$ as illustrated in Fig. 5A. Compared with a value of 2.50 ± 0.25 (mean ± SE) noted in control P1 cells, the P/SS ratio was 1.59 ± 0.06 in 4-AP, similar to the 1.55 ± 0.07 value for P12–13 CR cells, suggesting some degree of delayed rectifier inactivation in CR cells (Fig. 1A) (Mienville and Barker 1997). Elimination of $I_A$ did
not induce repetitive firing behavior (Fig. 5B), as neither the proportion of cells firing single spikes (25/31; 81%) nor mean AP number (1.5 ± 0.2) were different from control (P = 0.60; cf. results above). On the other hand, 4-AP did have substantial effects on individual AP parameters (Fig. 5, C and D). Specifically, amplitude, rise time and half-amplitude width were all increased. Because the rate of rise did not change, the increase in rise time and half-width appears as a mere consequence of the increase in amplitude. Ribera and Spitzer (1990) also observed an increase in spike overshoot on application of 4-AP. The increase in fall time was not significant (P = 0.11) despite the apparent lack of change in fall rate. This might be due to insufficient A/D resolution for fall rate with respect to sampling interval (software measures maximum dV/dr between 2 sampling points), as suggested by the very small SE of fall rates (Figs. 4A and 5C). These results nevertheless point to a lack of effect of IA on AP repolarization (see Fig. 5B).

Because there is no reason to suspect that 4-AP potentiates IA, it seems likely that one effect of IA is to dampen AP amplitude, owing to its fast activation partially overlapping with that of INa. Thus the developmental increase in AP amplitude may be related in part to the loss of IA. This raised the possibility that IA may interfere with AP threshold. In cultured neurons, it was shown that IA decreases both the threshold potential and rheobase for AP generation (Segal et al. 1984). The effect on threshold might be due to simultaneous and mutually antagonistic activations of IA and INa (compare threshold potentials for IA and AP in Figs. 1C and 6), whereas the effect on rheobase might stem from IA’s shunting conductance. Focusing on the former aspect, we found that the threshold potential for AP generation was significantly lower in P12–13 CR cells than in both P1 or P5 cells, yet it failed to change in P1 cells exposed to 4-AP (Fig. 6). This might be due to the tight dependence of threshold on INa density, which may hinder detection of IA’s effects.

Spontaneous firing occurs late in the development of CR cells

We have extended our initial observation of a lack of spontaneous firing in embryonic CR cells (Mienville 1998) to the perinatal period: spontaneous firing never occurred in P1 cells; it occurred in only 12/62 (19%) P5 cells, and in as many as 19/30 (63%) P12 cells (Fig. 7A). Consistent with these results, Schwartz et al. (1998) counted an average of 15% P1–8 CR cells displaying spontaneous Ca transients. Spontaneous firing frequencies were similar in P5 and P12 cells (Fig. 7B), and were well below maximally sustainable rates (see above).

DISCUSSION

Our previous work (Mienville and Barker 1997) showed an increased contribution of IA to the total K current evoked in embryonic day (E) 21 versus E18 CR cells. The present paper shows that this trend is reversed in postnatal CR cells, with a perinatal peak of IA expression subsequently falling down to a virtual loss at a time when few of these cells are remaining in cortex (Mienville and Pesold 1999). Such a decline is unlikely to reflect ongoing cell degeneration (Del Rio et al. 1995) because the delayed rectifier appears to remain functional, and other channels are even up-regulated in late CR cells (Mienville 1998; Mienville and Pesold 1999). Similar inverted-U patterns of K current expression have been described in non-mammalian muscle cells (Ribera and Spitzer 1991; Shidara and Okamura 1991).

Our immunocytochemical data strongly suggest that Kv1.4 is the molecular substrate of CR cell IA. First, we found that Kv1.4 antibody consistently stained CR cells; second, staining intensity seemed to closely follow the time course of IA expression. Third, Kv4.2 protein was never expressed by CR cells. Nevertheless, we cannot exclude that other IA-producing subunits, such as Kv4.1 (Serodio et al. 1994; Song et al. 1998), contribute to CR cell IA; this would imply that different subunits can be down-regulated simultaneously, which is not unprecedented (Tsaur et al. 1992). In rat hippocampus, Kv1.4
expression does not occur until P5 and subsequently increases toward adult intensities (Maletic-Savatic et al. 1995), which, taking our results into account, suggests varying spatiotemporal regulations of Kv1.4 in brain. At present, the mechanisms underlying such regulations can only be speculative, although promising leads already have been explored, as in the case of the down-regulation of Kv4.2 in hippocampus (Tsaur et al. 1992). CR cells thus may provide a unique mammalian model for future studies of the mechanisms involved in the differential regulation of ion channel expression.

The observed loss of $I_A$ expression has prompted us to investigate possible consequences on the excitability of CR cells. In view of the complexity deriving from the simultaneous (and opposite) changes in $I_A$ and $I_{Na}$ densities during CR cell development, we have initiated computer simulations based in part on a Goldman-Hodgkin-Katz formalism for K channel I-V curves (Clay 1998). Preliminary results (Mienville et al. 1999) indicate that up-regulation of $I_{Na}$ and downregulation of $I_A$ during the P1–P13 period are both required for repetitive firing to occur. Ribera and Spitzer (1990) observed the reverse phenomenon in developing amphibian neurons, in which $I_A$’s late emergence coincides with a switch from repetitive to single AP firing. Simulation studies performed in the same laboratory (Lockery and Spitzer 1992) also established that such a switch requires simultaneous manipulation of both $I_A$ and $I_{Na}$. These results can explain most of our present data, including the correlations between AP number and P/SS ratio (Fig. 3). The same model may also explain the correlation between the spontaneous frequency, and the possible consequences of CR cell spontaneous activity. Among the issues that remain to be resolved are the regulation of $I_A$ expression, the mechanisms that set firing frequency, and the possible consequences of CR cell spontaneous activity on the maturation of cortical circuits.

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