Ionic Mechanism of Electroresponsiveness in Cerebellar Granule Cells Implicates the Action of a Persistent Sodium Current

EGIDIO D’ANGELO, GIOVANNA DE FILIPPI, PAOLA ROSSI, AND VANNI TAGLIETTI

Istituto di Fisiologia Generale e Istituto Nazionale per la Fisica della Materie, Pavia Unit, I-27100 Pavia, Italy

D’Angelo, Egidio, Giovanna De Filippi, Paola Rossi, and Vanni Taglietti. Ionic mechanism of electroresponsiveness in cerebellar granule cells implicates the action of a persistent sodium current. J. Neurophysiol. 80: 493–503, 1998. Although substantial knowledge has been accumulated on cerebellar granule cell voltage-dependent currents, their role in regulating electroresponsiveness has remained speculative. In this paper, we have used patch-clamp recording techniques in acute slice preparations to investigate the ionic basis of electroresponsiveness of rat cerebellar granule cells at a mature developmental stage. The granule cell generated a Na+-dependent spike discharge resistant to voltage and time inactivation, showing a linear frequency increase with injected currents. Action potentials arose when subthreshold depolarizing potentials, which were driven by a persistent Na+ current, reached a critical threshold. The stability and linearity of the repetitive discharge was based on a complex mechanism involving a N-type Ca2+ current blocked by ω-CTX GVIA, and a Ca2+-dependent K+ current blocked by charubotoxin and low tetraethylammonium (TEA; <1 mM); a voltage-dependent Ca2+-independent K+ current blocked by high TEA (>1 mM); and an A current blocked by 2 mM 4-aminopyridine. Weakening TEA-sensitive K+ currents switched the granule cell into a bursting mode sustained by the persistent Na+ current. A dynamic model is proposed in which the Na+ current-dependent action potential causes secondary Ca2+ current activation and feedback voltage- and Ca2+-dependent afterhyperpolarization. The afterhyperpolarization reprimed the channels inactivated in the spike, preventing adaptation and bursting and controlling the duration of the interspike interval and firing frequency. This result reveals complex dynamics behind repetitive spike discharge and suggests that a persistent Na+ current plays an important role in action potential initiation and in the regulation of mossy fiber-granule cells transmission.

INTRODUCTION

Neurons use numerous voltage- and Ca2+-dependent channels to generate ionic current flows and regulate membrane potential. Although some channels are involved primarily in generating action potentials, others influence subthreshold responses and rhythmicity (Llinas 1988; Schwindt 1992), eventually determining the electroresponsive properties relevant to neuronal signal processing. The granule cells form the largest neuronal population in the mammalian brain and regulate information transfer along the major afferent systems to the cerebellum (Ito 1984). In the past decade, numerous investigations have reported voltage-dependent membrane currents in granule cells in culture (Cull-Candy et al. 1989; Fagni et al. 1991; Hochberger 1987; Kofuji et al. 1996; Randall and Tsien 1995; Stewart et al. 1995; Surmeier et al. 1996) and in situ (Bardoni and Belluzzi 1993; D’Angelo et al. 1994; Rossi et al. 1994). These studies have shown a transient tetrodotoxin (TTX)-sensitive Na+ current, multiple high-voltage-activated (HVA) Ca2+ currents, and multiple K+ currents, including a fast-activating and a delayed-rectifier K+ current, a Ca2+-dependent K+ current, and an inward rectifier K+ current. However, their relationship with the intrinsic excitability of persistent Na+ currents in controlling spike initiation (Alonso and Llinas 1989; Alzheimer et al. 1993; Azouz et al. 1996; Gutfreund et al. 1995; Taylor 1990).

In this paper, we identify the role of Na+, Ca2+, and K+ currents in the discharge of mature granule cells, complementing previous knowledge on voltage-dependent conductances to provide a cohesive functional framework. Granule cell firing was sustained by Na+ currents (D’Angelo et al. 1995, 1997). Action potentials arose when subthreshold depolarizing potentials, which were driven by a persistent Na+ current, reached a critical threshold. The repetitive discharge was stabilized by a repolarizing feedback based on Ca2+- and voltage-dependent K+ currents, which reprimed the channels inactivated in the spike and prevented the granule cell from Na+-dependent bursting. This result reveals complex dynamics behind fast repetitive spike discharge and suggests that a persistent Na+ current plays an important role in action potential initiation. The implications of this finding for mossy fiber-granule cell signal transduction are discussed.

METHODS

Cerebellar slices (250 μM thick) were obtained from 21- to 26-day-old rats (Wistar strain, day of birth = P1) as reported previously (D’Angelo et al. 1995, 1997). The rats were decapitated after halothane (Alrich) anesthesia. Krebs solution for slice cutting and recovery contained (in mM) 120 NaCl, 2 KCl, 1.2 MgSO4, 26 NaHCO3, 1.2 KH2PO4, 2 CaCl2, and 11 glucose. This solution was equilibrated with 95% O2-5% CO2 (pH 7.4). The slices were maintained at room temperature before being transferred to a 1.5-ml recording chamber mounted on the stage of an upright microscope (Zeiss Standard-16). The preparations were superfused at a rate of 5–10 ml/min with a Krebs solution to which 10 μM bicuculline had been added and were maintained at 30°C with a Peltier feedback device (HCC-100A, Dagan, Minneapolis, MN).

Bicuculline increased input resistance by 1.5–2.5 times and enhanced spike activation by current injection (n = 8, not shown), consistent with tonic γ-aminobutyric acid (GABAγ) receptor activation by ambient GABA (Brickley et al. 1997). More importantly for this study, bicuculline reduced spontaneous inhibitory synaptic activity (see RESULTS).
TABLE 1. Action potential properties in rat cerebellar granule cells

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Overshoot</th>
<th>Undershoot</th>
<th>HW</th>
<th>Rising Rate</th>
<th>Falling Rate</th>
<th>dV/dt Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bridge</td>
<td></td>
<td>12.4 ± 9.2</td>
<td>-56.4 ± 9.7</td>
<td>0.65 ± 0.26</td>
<td>196 ± 106</td>
<td>-128.3 ± 67.2</td>
<td>1.53</td>
</tr>
<tr>
<td>Patch-clamp fast PPR</td>
<td>10</td>
<td>9.8 ± 11</td>
<td>-55.0 ± 4.6</td>
<td>0.79 ± 0.16</td>
<td>151.1 ± 54.6</td>
<td>-94.5 ± 33.9</td>
<td>1.59</td>
</tr>
<tr>
<td>Patch-clamp fast WCR</td>
<td>8</td>
<td>11.6 ± 8.5</td>
<td>-53.6 ± 5.4</td>
<td>0.92 ± 0.29</td>
<td>155.3 ± 27.9</td>
<td>-88.8 ± 29.9</td>
<td>1.74</td>
</tr>
</tbody>
</table>

Values are means ± SD. The overshoot, undershoot, and rising and falling rate of granule cell spikes were compared in different recording conditions. Recordings obtained using a bridge amplifier did not significantly differ from those obtained using a patch-clamp amplifier in the fast current-clamp mode. In the fast patch-clamp current-clamp mode, no significant difference was observed between the perforated-patch (PPR) and whole cell recording (WCR) configuration. Statistical significance has been tested at the 0.05 probability level.

**Patch-clamp recordings**

Patch-clamp recordings (Edwards et al. 1989) were taken from granule cells in the internal granular layer of rat cerebellar slices using the "blind patch" approach. Most of these recordings were made by using an Axopatch 200-A amplifier for current-clamp recordings in the fast mode. Patch pipettes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, Germany) and had 8–12 MΩ resistance before a seal was formed (seal resistance was usually >20 GΩ). The pipette solution used for whole cell recordings (in mM) 126 K-glucuronate, 4 KCl, 4 NaCl, 1 MgSO₄, 0.02 CaCl₂, 0.1 bis-(o-aminophenoxo)-N,N,N’,N’-tetraacetic acid (BAPTA), 15 glucose, 3 ATP, and 5 N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES; pH was adjusted to 7.2 with KOH). In some cases Cs⁺ was used instead of K⁺ to reduce permeation through K⁺ channels [it contained (in mM) 78 Cs₂SO₄, 4 CsCl, 4 NaCl, 1 MgSO₄, 0.02 CaCl₂, 0.1 BAPTA, 15 glucose, 3 ATP, and 15 HEPES; pH was adjusted to 7.2 with CsOH]. These solutions reproduced the cytoplasmic Ca²⁺ buffer concentration (0.03–0.3 mM) (Simon and Llinas 1985), and the resting Ca²⁺ levels measured in central neurons (usually 50–100 nM) (for the granule cells, see Marchetti et al. 1995). In other experiments, the perforated-patch technique was used to prevent cytoplasmic washout and maintain the endogenous Ca²⁺ buffer (Horn and Marty 1988). The pipette solution used for perforated-patch recordings contained (in mM) 80 K₂SO₄, 8 NaCl, 15 glucose, and 5 HEPES (pH adjusted to 7.2 with KOH) and nystatin 100 µg/ml. Solutions for both whole cell and perforated-patch recordings maintained a chloride reversal potential of ~65 mV as recently measured in mature rat cerebellar granule cells (Brickley et al. 1996).

Because patch-clamp amplifiers may influence the excitability response (Magistretti et al. 1996), patch-clamp current-clamp recordings performed using the Axopatch-200A amplifier in fast mode were compared with those obtained with a conventional microelectrode amplifier (Axoclamp-2A in bridge mode). As shown in Table 1, the action potentials recorded with the patch-clamp amplifier did not significantly differ from those recorded using the microelectrode amplifier. It also should be noted that firing remained unchanged when using the perforated-patch instead of whole cell recording configuration (see Figs. 1 and 2).

In the cell-attached configuration, electrode capacitance was cancelled carefully before obtaining electrical access to the cell to allow for electronic compensation of pipette charging during subsequent current-clamp recordings (D’Angelo et al. 1995). In those experiments in which the patch-clamp amplifier was used, the current transients elicited by 10-mV hyperpolarizing pulses from the holding potential of ~70 mV in voltage-clamp mode showed a monoeponential relaxation with time constant τα = 66 ± 27 µs in whole-cell recordings (mean ± SD), n = 14; τα = 93 ± 31 µs in perforated-patch recordings, n = 10) and were used to estimate series resistance (Rₛ = 21.1 ± 8.7 MΩ in whole cell recordings, n = 14; Rₛ = 35 ± 12 MΩ in perforated-patch recordings, n = 10), input resistance (Rᵢ = 2 ± 0.7 GΩ in whole cell recordings, n = 14; Rᵢ = 2.2 ± 0.8 GΩ in perforated-patch recordings, n = 10), and input capacitance (Cᵢ = 3 ± 0.5 pF in whole cell recordings, n = 14; Cᵢ = 2.8 ± 0.6 pF in perforated-patch recordings, n = 10). Depending on the high Rₛ:Rᵢ ratio (Rᵢ:Rₛ > 60), bridge balancing in current-clamp recordings proved to be of little effect and was not routinely used either in the whole cell or the perforated-patch recording configuration (D’Angelo et al. 1995).

The data were sampled with a TL-1 DMA Interface (sampling time = 50–250 µs for current-clamp recordings, 10 µs for voltage-clamp recordings) and analyzed with pClamp software (Axon Instruments, Foster City, CA). Membrane potential was measured relative to an agar-bridge reference electrode and was not adjusted for liquid-junction potentials (usually <5 mV). HW denotes duration of action potentials at half-amplitude. Data are reported as means ± SD, and statistical comparisons were done using Student’s t-test.

**Solutions and drugs**

The control and test solutions were applied locally through a multibarrel pipette. The perfusion of the control solution was commenced before seal formation, and was maintained until switching to the test solutions. Bicuculline, TTX, tetraethylammonium chloride (TEA), and 4-aminopyridine (4-AP) were obtained from Sigma. The conotoxin ω-CTX GVIA was obtained from Peninsula Laboratories (Belmont, CA), charbdotoxin (ChTX) and apamine from Alomone (Tel-Aviv, Israel), and BAPTA tetrapotassium salt from Molecular Probes (Eugene, OR). The glutamate receptor antagonists D-2-amino-5-phosphonovaleric acid (APV), 7-chlorokynurenic acid (7-CI-Kyn), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris Cookson (Bristol, UK). Stock solutions were prepared for all drugs and stored frozen at −20°C. The drugs were diluted to their final concentration in the appropriate Krebs solution before use.

**Results**

Patch-clamp recordings (n = 91) were performed in the internal granular layer of laminae IV–IX of acute slices obtained from the rat cerebellar vermis at P21–P26, when migration is concluded (Altman 1972) and the granule cells show homogeneous electrophysiological properties (D’Angelo et al. 1995, 1997).

**General properties of electroresponsiveness**

The granule cells showed a resting membrane potential of −62.4 ± 11.1 mV (n = 36) and generated no spontaneous action potentials at rest (Brickley et al. 1996; D’Angelo et al. 1995, 1997). The subthreshold voltage response to pulse current injection usually showed no time-dependent inward rectification, with smaller potential changes and faster mem-
brane charging occurring at hyperpolarized than depolarized membrane potentials (Fig. 1A; a V-I plot demonstrating inward rectification is reported in the inset). When injected with a depolarizing current of sufficient intensity, the granule cell generated repetitive spikes at an apparent threshold of $-41.2 \pm 3.1$ mV (measured as the 1st point in the rising phase; $n = 36$). The spike frequency usually decreased by $<20\%$ during 800 ms of repetitive discharge, and frequencies as high as 150 Hz have been measured (Fig. 1B). The current-frequency plots could be interpolated with a straight line with a slope of $11.3 \pm 3.1$ spikes s$^{-1}$ pA$^{-1}$ ($n = 7$; $R^2 = 0.95 \pm 0.05$; Fig. 1B). These properties coincided with those reported previously (D’Angelo et al. 1995) and are considered further later in the paper.

The granule cells maintained their repetitive discharge after conditioning at different membrane potentials over a wide subthreshold voltage range (from $-100$ mV to threshold, Fig. 1C). In five of the six cells in which this experiment was performed, the spike frequency was higher for conditioning at $-50$ mV than at $-80$ mV, presumably reflecting $A$-current inactivation (see further). The time during which the repetitive discharge remained stable varied from cell to cell, ranging from seconds up to tens of seconds (Fig. 1D). The repetitive discharge eventually failed as spike afterhyperpolarization (AHP) decreased and the action potential generating mechanism was inactivated.

The action potential arose with a small prepotential ($3–6$ mV) deriving from subthreshold depolarizing potentials that were occasionally observed in isolation (Fig. 2A). Isolated subthreshold depolarizing potentials occurred mainly with low stimulus intensities or when firing was fatigued by intense and prolonged current injection. The subthreshold depolarizing potentials sometimes appeared as afterdepolarizations after an action potential (Fig. 2A) and sustained spike clustering (Fig. 2B). Nonetheless, the subthreshold depolarizing potentials were neither as prominent nor as regular as in other central neurons (e.g., Alonso and Llinas 1989; Azouz et al. 1996; Gutfrudend et al. 1995). Finally, we noted that the subthreshold depolarizing potentials were independent from synaptic activity, as they were measured in the presence of bicuculline and persisted when glutamate receptor blockers were added to the bath (100 $\mu$M APV + 50 7-CI-Kyn + 10 $\mu$M CNQX, $n = 5$; not shown).

The action potential overshoot typically peaked at $\sim 10$ mV, and the undershoot at around $-55$ mV (Table 1). The action potential therefore developed mostly above the membrane potential range in which inward rectification was seen (see Fig. 1A, inset). An individual action potential is shown...
enlarged in Fig. 2C, together with its first time-derivative, dV/dt. The action potential half-width was between 0.5 and 1 ms. The depolarization rate ranged from 90 to 280 V/s, being ~1.5 times faster than the repolarization rate. As can be seen in Figs. 1 and 2, no significant difference in the major firing properties nor in action potential characteristics (Table 1) were found using either the whole cell or the perforated-patch recording technique, or using different current-clamp amplifiers (see METHODS). The experiments reported in the following text were performed using a patch-clamp amplifier in the whole cell recording mode.

Ionic currents involved in action potential generation

Both action potentials and subthreshold depolarizing potentials were blocked by 1 μM TTX (Fig. 3A), being therefore generated by Na⁺ currents. After TTX application, membrane depolarization in the 10-mV subthreshold region was inhibited (Fig. 3B), unmasking a strong repolarizing reaction causing outward rectification in V-I plots (Fig. 3C).

After TTX action, the application of the K⁺ channel blocker, 20 mM TEA, disclosed a slow high-threshold spike (HTS; Fig. 3A). The apparent threshold measured at the flexus point was ~33.3 ± 7 mV (n = 7). HTS was blocked by 5 μM ω-CTX GVIA (n = 4; Fig. 3A), which has been shown to block >95% of the Ca²⁺ current in mature granule cells (Ica) (Rossi et al. 1994), or 1 mM Ni²⁺ (n = 5, not shown). HTS was thus a Ca²⁺ action potential with threshold and pharmacological sensitivities corresponding to those of the N-type current of granule cells (D’Angelo et al. 1997). It should be noted that HTS activated at a higher threshold than TTX-sensitive action potentials and subthreshold depolarizing potentials, indicating that Ica was activated during the action potential but did not contribute to setting it off. The application of 20 mM TEA also removed the outward rectification (Fig. 3C; n = 6), which therefore depended on TEA-sensitive K⁺ currents.

Na⁺ channel-dependent transient and persistent depolarizations

The depolarizing action of Na⁺ currents was investigated by injecting step-current pulses after block of Ca²⁺ channels with 1 mM Ni²⁺ and of K⁺ channels with 20 mM TEA and 4 mM 4-AP (in these experiments Cs⁺-containing patch-pipettes were used; n = 5; Fig. 4). Inward rectification usually observed at low potentials was absent in these recordings, reflecting the block of inward rectifier K⁺ channels (Kofuji et al. 1996; Surmeier et al. 1996). The Na⁺-dependent response started at around ~55 mV with a slow depolarizing ramp leading to spike activation at ~45 mV (Fig. 4A). The spike was followed by a depolarizing plateau rising membrane potential toward ~30 mV. With stronger current injection, the plateau depolarization increase proportionately to the stimulus (Fig. 4B). The steady-state depolarization showed, consequently, a nonlinearity in the ~55/30 mV region (Fig. 4C). After 1 μM TTX application, all active responses disappeared, and the steady-state depolarization became linear over the whole membrane potential range (Fig. 4, B and C). This result indicated that the Na⁺ current comprised a transient (t-Ica) and a persistent (p-Ica) TTX-sensitive component, as reported in other neurons (Llinas 1988; Schwindt 1992). By activating below spike threshold, p-Ica probably provided the depolarizing drive for TTX-sensitive subthreshold depolarizing potentials (cf. Figs. 3, A and B, and 8, A and B, and see DISCUSSION).

Role of the Ca²⁺ current

Ca²⁺ currents have complex effects on neuronal excitability (Llinas 1988; Schwindt 1992), which vary according to the type of Ca²⁺ and Ca²⁺-dependent channels involved. Ca²⁺-channel block (with 5 μM ω-CTX GVIA) had three main effects on granule cell excitability. First, both the spike overshoot and undershoot were significantly reduced (Fig. 5A; Table 2). Spike reduction, which occurred as early as
FIG. 3. Ionic currents involved in action potential generation. A: application of 1 µM tetrodotoxin (TTX) blocked granule cell spikes as well as subthreshold depolarizing potentials (inset). Subsequent 20 mM tetraethylammonium (TEA) application revealed a high-threshold spike (HTS) that was blocked by 5 µM α-CTX GVIA. All tracings were obtained by 9-pA step current injection from the holding potential of −90 mV. HTS activates at higher threshold than TTX-sensitive action potentials. B: action potential generation was enhanced by a TTX-sensitive depolarizing current, generating a ramp-like subthreshold depolarizing potential (arrowhead). Application of 1 µM TTX abolished the ramp, reducing the efficiency of the injected depolarizing current. Note that responses reaching membrane potentials lower than −60 mV remained similar before and after TTX. C: after action potential block by 1 µM TTX, current injection at different intensities revealed a strong outward rectification in the threshold membrane potential region. This outward rectification was largely removed by 20 mM TEA. Note HTS activation at high stimulus intensity.

the first spike in a train, suggested that the action potential was enhanced by the Ca²⁺ current. AHP reduction is likely to result from the reduced activation of a Ca²⁺-dependent K⁺ current (I KCa, see further). Inactivation of the spike generating mechanism probably due to the pronounced depolarization explains the marked spike adaptation observed at the highest stimulus intensities (Fig. 5A, bottom right). Second, subthreshold voltage responses to current injection increased with marginal changes in inward rectification (Fig. 5B). This effect was associated with a reduction in the current needed to generate spikes, and with a reduction of first spike delay. Finally, the I-f relationship shifted to the left and lost its linearity, decreasing steepness as the injected current increased (Fig. 5C). The nature of Ca²⁺-dependent effects is further considered later in the paper.

Role of TEA-sensitive K⁺ currents

The role of repolarizing currents was investigated by using K⁺ channel blockers. In cerebellar granule cells, TEA is known to reduce I KCa at 1 mM (Fagni et al. 1991). A voltage-dependent K⁺ current, I KV, also is blocked by TEA in granule

FIG. 4. Na⁺ channel-dependent transient and persistent depolarizations. Voltage responses generated by Na⁺ channels were elicited from −87 mV by step current injection (as indicated below tracings) after blocking Ca²⁺ channels (with 1 mM Ni²⁺) and K⁺ channels (with 20 mM TEA and 4 mM 4-aminopyridine [4-AP]), and using Cs⁺ as the main intracellular cation. A: current injection generated a slow depolarizing ramp in the subthreshold region (arrowhead). Ramp triggered a spike at −45 mV. Spike was followed by a depolarizing plateau. B: TTX blocked both the transient and persistent Na⁺-dependent responses. Persistent response arose between −55 and −30 mV. C: steady-state membrane depolarizations elicited by step-current injection in B were measured taking the average over the last 200 ms of tracings (at this time the spike was subsided). Persistent Na⁺-dependent depolarization caused a marked nonlinearity in the V-I plot between −55 and −30 mV (●). After TTX application, the V-I plot became linear over the whole membrane potential range (○).
FIG. 5. Actions of ω-CTX GVIA. A: voltage responses were elicited from –90 mV using different step-current intensities (as indicated beside the tracings) in control and during ω-CTX GVIA application (5 μM for 5 min), respectively. Note the enhanced subthreshold membrane potential charging, the decreased spike overshoot and undershoot, the increased firing rate, and the adaptation occurring at high current intensity during ω-CTX GVIA application. Inset: action potentials recorded in control (○) and during ω-CTX GVIA application ( ● ) on enlarged scale (20 mV, 1 ms). B and C: voltage-current plot and current-frequency plot, respectively, in control (○) and during ω-CTX GVIA application ( ● ) in the same cell as in A. Plots illustrate the higher reactivity of the cell (steeper V-I plot, left-shifted t-I plot) during ω-CTX GVIA application.

cells (Cull-Candy et al. 1989). TEA concentrations required to block I_KV are usually higher than those required to block I_KCa (see Rudy 1988; Storm 1990).

The application of 0.5 mM TEA to six neurons reduced the spike AHP and increased firing frequency (Fig. 6; Table 2). These actions largely overlapped with those of ω-CTX GVIA, suggesting that I_KCa activation mediates the effects of Ca^2+ channel opening (and consequent Ca^2+ influx) during the spike. We also noted that the application of 0.5 mM TEA slightly but significantly increased the spike overshoot (Fig. 6; Table 2), probably involving a partial I_KV inhibition (Storm 1990).

Increasing TEA to 1 mM changed firing to a rhythmic mode, consisting of slow depolarizing waves generating bursts of spikes showing amplitude adaptation (Fig. 6). Using stimulations just sufficient to reach threshold, the burst frequency was 2–5 Hz. At 5–20 mM TEA, the amplitude and duration of the slow depolarizing waves increased, as did spike adaptation in the burst. Thus at 1 mM or more, the effects of TEA differed from those reflecting I_KCa block, indicating an additional block of I_KV.

**Pharmacology of Ca^2+-dependent channels**

The results reported above suggest that the I_Ca/I_KCa system makes a significant contribution to AHP. The nature of the Ca^2+-dependent K+ channels was further investigated using ChTX and apamine, two toxins that are known to inhibit different channel types selectively in neurons. ChTX primarily blocks large conductance Ca^2+-dependent K+ channels (Miller et al. 1985) of the same class as those reported in granule cells in culture (Fagni et al. 1991). Indeed, the application of 5–50 mM ChTX (n = 5; Fig. 7A; Table 2) produced effects similar to those of 0.5 mM TEA (cf. Fig. 6) or ω-CTX GVIA (cf. Fig. 5A). Conversely, application of the bee venom 0.1–1 μM apamine, which blocks small-conductance Ca^2+-dependent K+ channels (Blatz and Magleby 1986), proved ineffective (n = 5; Fig. 7B).

**TABLE 2. Action potential changes during TEA, ω-CTX GVIA, and ChTX application**

<table>
<thead>
<tr>
<th></th>
<th>Overshoot</th>
<th>Undershoot</th>
<th>HW</th>
<th>Rising Rate</th>
<th>Falling Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEA</td>
<td>6</td>
<td>0.09 ± 0.09*</td>
<td>–0.61 ± 0.14†</td>
<td>0.340 ± 0.21</td>
<td>0.07 ± 0.1</td>
</tr>
<tr>
<td>ω-CTX-GVIA</td>
<td>5</td>
<td>–0.28 ± 0.2*</td>
<td>–0.71 ± 0.31†</td>
<td>0.22 ± 0.18*</td>
<td>–0.26 ± 0.25</td>
</tr>
<tr>
<td>ChTX</td>
<td>4</td>
<td>–0.14 ± 0.12</td>
<td>–0.63 ± 0.28*</td>
<td>0.18 ± 0.06*</td>
<td>–0.06 ± 0.07</td>
</tr>
</tbody>
</table>

Application of tetraethylammonium (TEA, 0.5 mM), ω-CTX GVIA (5 μM), or chariibotoxin ChTX; 50 nM) broadened the spike reducing the falling rate and the undershoot, suggesting a common action on I_KCa. Moreover, ω-CTX GVIA reduced the overshoot, suggesting an involvement of I_KV in early spike repolarization. Data are reported as percent changes compared with control, and statistical significance of the changes is indicated (*P < 0.05; †P < 0.01). Data without symbols were considered not significant.
Evidence for A-current activation

The slow and sometimes concave membrane potential trajectory preceding the first spike in a train (Fig. 7C) and the increased firing frequency after conditioning at depolarized membrane potentials (see Fig. 2B) or AHP reduction (e.g., after application of ω-CTX GVIA or TEA; see Figs. 5C and 6A) suggested the involvement of a fast-activating K⁺ A-current, Iₐ (Connor and Stevens 1971). Indeed, application of the Iₐ blocker 2 mM 4-AP reduced the delay before the first spike (Fig. 7C). At the same time, however, the spike AHP was reduced and the firing pattern converted into bursts (2 cells) or slow spikes (3 cells), in a similar fashion to TEA. This is probably because 4-AP blocks both Iₜᵥ and Iₐ currents in granule cells (Bardoni and Belluzzi 1993).

Ionic nature of bursting

Bursting induced by 1 mM TEA was investigated by using Ca²⁺ and Na⁺ channel blockers. Figure 8A shows that Ca²⁺ channel block reduced the slow depolarizing wave of the burst, which was therefore a HTS (cf. Fig. 3A). At the same time, spike adaptation in the burst was reduced. Nonetheless, bursting persisted in an apparent relationship with subthreshold depolarizing potentials. Similar effects were observed in four cells using 5 μM ω-CTX GVIA and in three cells using 1 mM Ni²⁺. In all these cells, both subthreshold depolarizing potentials and action potentials were blocked by 1 μM TTX (Fig. 8A).

A similar result was observed in a further four granule cells to which 1 mM TEA had been applied. In these cells, no slow waves or bursts were clearly discernible and firing consisted of broad action potentials (HW = 4.6 ± 0.5 ms; n = 4) that sometimes were clustered into doublets or triplets (Fig. 8B). In these cells, Ca²⁺ channel block (with 5 μM ω-CTX GVIA) narrowed the spikes (HW = 3.9 ± 0.3 ms; n = 3; P < 0.05), which therefore were mixed Ca²⁺/Na⁺ mediated spikes. The remaining subthreshold depolarizing potentials were blocked by 1 μM TTX, as were the action potentials (Fig. 8B).

These experiments revealed that Na⁺ currents generated slow rhythmicity when repolarizing systems were weakened. Iₜᵥ, although increasing and prolonging the bursts, was not critical in generating slow rhythmicity (indeed, HTS activated at a higher threshold than Na⁺-dependent depolarizations and did not demonstrate any rhythmicity when the Na⁺ current had been previously blocked, cf. Fig. 3A).

Finally, we attempted to determine whether N-methyl-D-aspartate (NMDA)-receptor–mediated currents were involved in supporting bursting (Hochman et al. 1994; Silva et al. 1991). However, bursting in cerebellar granule cells persisted after NMDA receptor block with 100 μM APV + 50 μM 7-Cl-Kyn (D’Angelo et al. 1995), indicating that the NMDA currents were not involved (Fig. 8C).

Discussion

The granule cell of the mature rat cerebellum generated Na⁺ channel-dependent action potentials and subthreshold depolarizing potentials, involving a transient and a persistent Na⁺ current. Spikes drove Ca²⁺ and K⁺ channel activation generating a feedback repolarization, ensuring the stability of repetitive spike discharges and enabling the granule cell to perform an almost linear input-output conversion of injected currents over a wide dynamic range. The virtual absence of spike frequency adaptation and the persistence of the repetitive spike discharge during prolonged current injection suggest that the granule cell is more sensitive to the amplitude of the stimulus than to its rate of change (Schwindt 1992; Schwindt et al. 1997). This property would ensure that voltage- and frequency-dependent transformations of mossy fiber impulses operated by synaptic conductances and inward rectification during synaptic transmission (D’Angelo et al. 1995) are translated reliably into a proportional spike dis-
FIG. 7. Actions of charibdotoxin (ChTX), apamine, and 4-AP. A: application of 50 nM ChTX increased the initial action potential frequency. Afterward the repetitive discharge showed a marked adaptation. B: application of 500 nM apamine did not change the action potential discharge. C: application of 1 mM 4-AP decreased 1st-spike latency, decreased AHP, and increased firing frequency and the tendency to spike clustering.

Nature and function of membrane currents

In this paper, we have demonstrated the contribution of several voltage- and Ca\(^{2+}\)-dependent currents in generating the electrical response of mature rat cerebellar granule cells. Owing to the high efficiency of potential transmission along the dendrites (electrotonic length is \(L < 0.04\), and voltage attenuation is \(<1\%\) ) (D’Angelo et al. 1993, 1995; Silver et al. 1992), the role of membrane currents could be explained largely without invoking electrotonic effects.

1) TTX-sensitive Na\(^{+}\) currents generated the spike, as well as subthreshold depolarizing potentials, reflecting the involvement of transient and persistent Na\(^{+}\) currents (\(t-I_{Na}\) and \(p-I_{Na}\), respectively; see Fig. 4). Because of the electrotonic compactness of the granule cell, return currents from the dendrites are unlikely to play a significant role in generating subthreshold activity (cf. Mainen and Sejnowsky 1996), suggesting that persistent kinetics indeed reflect intrinsic Na\(^{+}\) channel properties. We noted that persistent Na\(^{+}\)-dependent depolarizations did not vanish at high potentials (cf. Fig. 4), as it would otherwise be expected from a window current (a persistent current generated where the voltage dependence of activation and inactivation of transient Na\(^{+}\) currents overlap). Therefore, a window current is insufficient to explain \(p-I_{Na}\), and a specific channel or channel gating mechanism generating persistent Na\(^{+}\) currents should be involved (Alzheimer et al. 1993; Taylor 1993). Coexistence of \(t-I_{Na}\) and \(p-I_{Na}\) in granule cells did not emerge from previous granule cells studies (e.g., Cull-Candy et al. 1989), suggesting that further voltage-clamp experiments are needed to clarify the nature of granule cell Na\(^{+}\) currents.

2) A Ni\(^{2+}\)- and \(\omega\)-CTX GVIA-sensitive Ca\(^{2+}\) current (\(I_{Ca}\)) enhanced the spike AHP and played a primary role in stabilizing repetitive spike discharge (see following paragraph). \(I_{Ca}\) activated during the spike but did not contribute to its initiation, consistent with fast high-threshold activation kinetics. As suggested by the long duration of HTS in high TEA, \(I_{Ca}\) inactivation is likely to be slow. Its sensitivity to \(\omega\)-CTX GVIA, the high-threshold, fast activation, and slow inactivation indicated that \(I_{Ca}\) corresponded to the N-type HVA current reported in mature granule cells (Rossi et al. 1994). Unexpectedly, the effects of Ca\(^{2+}\)/channel block extended to low potentials. Although a divalent cation such as Ni\(^{2+}\) may exert a blocking action on leakage currents that are not Ca\(^{2+}\)-dependent (Friedman and Gutnick 1989), this seems less likely to occur with the highly selective peptidic toxin \(\omega\)-CTX GVIA. At present, the action mechanism of Ca\(^{2+}\)/channel blockers on subthreshold responses remains to be elucidated.

3) A Ca\(^{2+}\)-dependent K\(^{+}\) current (\(I_{KCa}\) ), blocked by submillimolar TEA, by 5–50 nM ChTX, or after Ca\(^{2+}\)/channel block, enhanced spike repolarization and AHP. On account of its TEA and ChTX sensitivity, and considering the fast AHP time course, \(I_{KCa}\) probably was generated by large-conductance Ca\(^{2+}\)-dependent K\(^{+}\) channels, which indeed have been recorded from granule cells in culture (Fagni et al. 1991). Because neither delayed AHP or activity-dependent spike frequency adaptation were observed (Llinas 1988; Schwindt 1992; Storm 1990) nor the channel blocker apamine (Blatz and Magleby 1986) was effective, small-conductance Ca\(^{2+}\)-dependent K\(^{+}\) channels are unlikely to have been activated.

4) A TEA-sensitive Ca\(^{2+}\)-independent K\(^{+}\)-current, \(I_{KV}\), increased the efficiency of the \(I_{Ca}/I_{KCa}\) system in repolarizing the spike and generating AHP as well as in preventing bursting. The potential involvement in early spike repolarization (Storm 1990) and the sensitivity to high TEA concentrations suggested that \(I_{KV}\) was related to a delayed-rectifier K\(^{+}\) current (Bardoni and Belluzzi 1993; Cull-Candy et al. 1989).
5) A 4-AP-sensitive current, corresponding to the fast-activating $K^+$ current ($I_{A}$) of granule cells, delayed spike activation (Bardoni and Belluzzi 1993; Cull-Candy et al. 1989). According to its gating and kinetic properties, $I_{A}$ should activate/inactivate in the spike, deinactivate in the AHP and reactivate slowly in the interspike interval (Connors and Stevens 1971). A weakening of this mechanism helps to explain the increased firing frequency observed when AHP was reduced (preventing $I_{A}$ deinactivation) or when the membrane was conditioned at a depolarized potential (causing $I_{A}$ inactivation).

6) Finally, the granule cells expressed an inward-rectifier $K^+$ current ($I_{IR}$). Considering the time-independent nature of subthreshold voltage responses, $I_{IR}$ should be similar to that measured in mice (Kofuji et al. 1996; Surmeier et al. 1996) but probably differs from that in turtle granule cells (Gabbiani et al. 1994). Because $I_{IR}$ activation occurred below the spike AHP, $I_{IR}$ should not be a primary determinant of firing or subthreshold depolarizing potentials.

A current set comprising $t-I_{Na}/p-I_{Na}$, $I_{Ca}$, $I_{KCa}$, $I_{KV}$, $I_{A}$, and $I_{IR}$ accounted for the major aspects of mature granule cell electroresponsiveness. A M-like current also has been reported in granule cells in culture (Watkins and Mathie 1996), although evidence for this current in situ is still lacking. We finally note that persistence of immature properties, mostly concerning transient R-type $Ca^{2+}$ currents (D’Angelo et al. 1997; Randall and Tsien 1995), may increase the initial firing frequency in some granule cells.

**Ionic dynamics of repetitive firing**

The dynamics of conductance changes during repetitive firing are summarized in Fig. 9A. Subthreshold depolarizing potentials and spikes were driven by $p-I_{Na}$ and $t-I_{Na}$, respectively, followed by secondary $I_{A}$ activation. $I_{KV}$ and $I_{KCa}$ then generated a feedback repolarization and AHP. During AHP, inactivation was removed from $I_{A}$ and $t-I_{Na}$. The $I_{Ca}/I_{KCa}$-dependent control of AHP was involved in maintaining a linear output frequency over a wide input range. At low intensities, the $I_{Ca}/I_{KCa}$ system should indirectly ensure linearity by controlling $I_{A}$ repriming, which in turn regulates the duration of the interspike interval (Connors and Stevens 1971). At high intensity, the $I_{Ca}/I_{KCa}$ system should ensure linearity by preventing the firing process from being inactivated by excessive depolarization. Both the $I_{Ca}/I_{KCa}$ system and $I_{A}$ concurred to delay the first spike and may therefore have a role in regulating the timing of granule cell discharge.

**Ionic dynamics of bursting**

The weakening of membrane repolarizing systems switched the granule cell into a slow rhythmic bursting mode. Rhythmic bursting was initiated by subthreshold depolarizing potentials generated by Na$^+$ currents, probably exploiting the relatively low threshold and persistent kinetics of $p-I_{Na}$ (Fig. 9B). The initial Na$^+$-dependent depolarization boosted $Ca^{2+}$ channel-dependent HTS and a spike burst. The burst should terminate when the repolarizing action of (unblocked) $K^+$ currents prevailed.

In granule cells, the conversion of a repetitive discharge into bursts depended on the favorable balance of protracted inward currents against $K^+$ currents. A related mechanism has been proposed to explain the difference between fast spiking, regular-spiking, and intrinsically bursting neocortical neurons (Connors and Gutnick 1990; McCormick et al. 1985), in which the effective outward current decreases as...
K⁺ channels are segregated in partially uncoupled dendrites (Mainen and Sejnowsky 1996). It is not presently known whether bursting in granule cells is a mere pharmacological effect or if it can be induced by neuromodulators inhibiting the repolarizing systems (Rudy 1988).

Functional implications of the persistent Na⁺ current

The present analysis of electroresponsiveness suggests that p-I_{Na} drives the subthreshold depolarizing potentials and sustains spike clustering. Because p-I_{Na} generates a noninactivating depolarization, the transient nature of subthreshold depolarizing potentials is likely to depend on activation of repolarizing K⁺ currents (cf. Figs. 3 and 4). Indeed, both subthreshold depolarizing potentials and spike clustering were accentuated by weakening K⁺ currents, generating a slow rhythmicity (Fig. 8). It should be noted that subthreshold oscillations in cortical neurons are more marked and regular than those observed in granule cells (Alonso and Llinas 1989; Azouz et al. 1996; Gutfreund et al. 1995). This may depend on the expression of Na⁺ currents or K⁺ currents [a time-dependent inward rectifier, I_{L} (Alonso and Llinas 1989); a M current, I_{M} (Gutfreund et al. 1995)] different from those of granule cells.

By contrasting repolarizing currents in the threshold region, p-I_{Na} drives membrane potential to threshold for self-regenerative depolarization. This mechanism should be particularly effective in promoting spike initiation at low stimulus intensities, extending granule cell discharge toward low frequencies. p-I_{Na} also may play an important role in strengthening excitatory postsynaptic potential-spike coupling (Lipowsky et al. 1996), determining the reliability of spike initiation during synaptic transmission. This effect may be enhanced by a regenerative interaction of p-I_{Na} with the NMDA current, which increases its amplitude and protracts its kinetics during membrane depolarization (D’Angelo et al. 1995). A similar regenerative interaction with the NMDA current has been proposed for Ca²⁺ currents in immature granule cells (D’Angelo et al. 1997).

Conclusions

The almost linear conversion of the input current into a related action potential discharge was performed by cerebellar granule cells at the expense of a complex mechanism involving the interaction of numerous voltage- and Ca²⁺-dependent currents. The two critical processes appeared to be spike initiation, which depended on the prevalence of a persistent Na⁺ current over repolarizing currents, and feedback repolarization triggered by the spike, which depended on voltage- and Ca²⁺-dependent K⁺ currents. By repriming the spike generating mechanism and I_{A}, feedback repolarization ensured the stability and linearity of firing. Channel modulation by endogenous agonists may change electroresponsiveness, exploiting the intrinsic bursting capability of the granule cell to control information transfer into the cerebellar cortex. These observations provide a basis for investigating changes in granule cell electroresponsiveness associated with modulation and plasticity and for a mathematical simulation of the neuron, which will be set out in a forthcoming paper.

This work was supported by grants from the Instituto Nazionale della Materia, Ministero dell’Università e della Ricerca Scientifica e Tecnologica, and Telethon (Grant E464) of Italy.

Address for reprint requests: E. D’Angelo, Istituto di Fisiologia Generale, Via Forlanini 6, I-27100 Pavia, Italy.

Received 28 October 1997; accepted in final form 17 March 1998.

REFERENCES


BRECILEY, S. G., CULL–CANDY, S. G., AND FARRANT, M. Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting


