Synaptic Activation of Ca$^{2+}$ Action Potentials in Immature Rat Cerebellar Granule Cells In Situ

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D’Angelo, Egidio, Giovanna De Filippi, Paola Rossi, and Vanni Taglietti. Synaptic activation of Ca$^{2+}$ action potentials in immature rat cerebellar granule cells in situ. J. Neurophysiol. 78: 1631–1642, 1997. Although numerous Ca$^{2+}$ channels have been identified in cerebellar granule cells, their role in regulating excitability remained unclear. We therefore investigated the excitable response in granule cells using whole cell patch-clamp recordings in acute rat cerebellar slices throughout the time of development (P4–P21, n = 183), with the aim of identifying the role of Ca$^{2+}$ channels and their activation mechanism. After depolarizing current injection, 46% of granule cells showed Ca$^{2+}$ action potentials, whereas repetitive Na$^+$ spikes were observed in an increasing proportion of granule cells from P4 to P21. Because Ca$^{2+}$ action potentials were no longer observed after P21, they characterized an immature granule cell functional stage. Ca$^{2+}$ action potentials consisted of an intermediate-threshold spike (ITS) activating at −60 to −50 mV and sensitive to voltage inactivation and of a high-threshold spike (HTS), activating at above −30 mV and resistant to voltage inactivation. Both ITS and HTS comprised transient and protracted Ca$^{2+}$ channel-dependent depolarizations. The Ca$^{2+}$ action potentials could be activated synaptically by excitatory postsynaptic potentials, which were significantly slower and had a proportionately greater N-methyl-D-aspartate (NMDA) receptor-mediated component than those recorded in cells with fast repetitive Na$^+$ spikes. The NMDA receptor current, by providing a sustained and regenerative active current injection, was critical for activating the ITS, which was not self-regenerative. Moreover, NMDA receptors determined temporal summation of impulses during repetitive mossy fiber transmission, raising membrane potential into the range required for generating protracted Ca$^{2+}$ channel-dependent depolarizations. The nature of Ca$^{2+}$ action potentials was considered further using selective ion channel blockers. N-, L-, and P-type Ca$^{2+}$ channels generated protracted depolarizations, whereas the ITS and HTS transient phase was generated by putative R-type channels (R$_{ITS}$ and R$_{HTS}$, respectively). R$_{HTS}$ channels had a higher activation threshold and were more resistant to voltage inactivation than R$_{ITS}$ channels. At a mature stage, most of the Ca$^{2+}$-dependent effects depended on the N-type current, which promoted spike repolarization and regulated the Na$^+$-dependent discharge frequency. These observations relate Ca$^{2+}$ channel types with specific neuronal excitable properties and developmental states in situ. Synaptic NMDA receptor-dependent activation of Ca$^{2+}$ action potentials provides a sophisticated mechanism for Ca$^{2+}$ signaling, which might be involved in granule cell development and plasticity.

INTRODUCTION

The granule cells of mature rat cerebellum generate a repetitive spike discharge, regulating information transfer along the mossy fiber pathway (D’Angelo et al. 1995). However, the excitable response at early developmental stages may have different properties and involve different channels than at a mature stage. Immature neurons can generate Ca$^{2+}$ action potentials (Baccaglini and Spitzer 1977; McCleskey 1994; Spitzer 1991), involving specific Ca$^{2+}$ channel currents (McCobb et al. 1989; Yaari et al. 1987). Specific Ca$^{2+}$ channel expression indeed has been suggested at an immature stage of granule cell development (Rossi et al. 1994).

The Ca$^{2+}$ channels of granule cells have been characterized extensively in cell culture, revealing high-voltage activated (HVA) Ca$^{2+}$ channels of the L, N, and P type. In addition to these, other channels also may be expressed, namely the Q- and R-type channels (Amico et al. 1995; Bossu et al. 1994; Ellinor et al. 1993; Forti et al. 1994; Pearson et al. 1995; Randall and Tsien 1995; Sather et al. 1993; Tottene et al. 1996; Zhang et al. 1993). These channels have different gating and kinetic properties and coexist in granule cells. Their role in regulating granule cell excitability remained elusive, however, because no correlation with functional states in situ has been established. By using current-clamp patch-clamp recordings in cerebellar slices, we found that a large proportion of granule cells of the developing cerebellum generated Ca$^{2+}$ action potentials with complex time course and voltage dependence due to the opening of L-, P-, N-, and putative R-type channels. At a mature stage, the Ca$^{2+}$ action potentials disappeared, and N-type Ca$^{2+}$ channels participated in regulating Na$^+$-dependent spike discharge.

Granule cells are activated synaptically by mossy fibers (Eccles et al. 1967) involving glutamate receptors of the N-methyl-D-aspartate (NMDA) type, which, at an early developmental stage, account for most of the synaptic current (D’Angelo et al. 1993). We found that synaptic activation of the NMDA receptors, by providing a protracted and voltage-dependent current injection, was critical for sustaining Ca$^{2+}$ action potential generation.

METHODS

Cerebellar slices (250 μm thick) were obtained from 4- to 21-day-old rats (Wistar strain, day of birth = P1) as reported previously (D’Angelo et al. 1993, 1995). The rats were anesthetized with halothane (Aldrich) before being killed by decapitation. Krebs solution for slice cutting and recovery contained (in mM) 120 NaCl, 2 KCl, 1.2 MgSO$_4$, 26 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 2CaCl$_2$, and 11 glucose. This solution was equilibrated with 95% O$_2$-5% CO$_2$ (pH 7.4). Slices were maintained at room temperature before being transferred to the recording chamber (1.5 ml) 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 glycine and 10 μM bicuculline were added, and
maintained at 30°C with a feed-back Peltier device (HCC-100A, Dagan, Minneapolis, MN).

**Whole cell current-clamp recordings**

The experimental technique was substantially similar to that used in our previous paper (D’Angelo et al. 1995). Granule cells in lobules IV–IX were recorded in the whole cell patch-clamp configuration (Edwards et al. 1989) using the “blind-patch” approach. Recordings were performed with an Axopatch-1D or an Axopatch 200-A (fast current-clamp mode) amplifier. The data were sampled with a TL-1 DMA Interface (sampling time = 250 μs for current-clamp recordings, 10 μs for voltage-clamp recordings) and analyzed with pClamp software (Axon Instruments, Foster City, CA). Mossy fiber stimulation was performed with a bipolar tungsten electrode (Clark Instruments, Pangbourne, UK) via a stimulus isolation unit. The stimulating electrode was placed over the mossy fiber bundle, and stimuli were applied at the frequency of 0.1 Hz or in 500-msec trains of 5, 10, or 50 Hz. In some experiments, a second stimulating electrode was placed in proximity of the Purkinje cell layer to test granule cell antidromic excitation.

Patch pipettes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, Germany) and had 8–12 MΩ resistance before a seal was formed with a filling solution containing (in mM) 126 K-glucuronate, 4 NaCl, 1 MgSO₄, 0.02 CaCl₂, 0.1 bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), 15 glucose, 3 ATP, 5 2-hydroxyethylpiperazine-N',N'-2-ethanesulfonic acid (HEPES; pH was adjusted to 7.2 with KOH). This solution buffered intracellular Ca²⁺ at 100 nM, similar to the resting Ca²⁺ concentration measured in granule cells (Marchetti et al. 1995). After a giga-seal was formed (seal resistance was usually >20 GΩ), the electrode capacitance was canceled carefully before repatching the patch to allow for the electronic compensation of pipette charging during subsequent current-clamp recordings (D’Angelo et al. 1995). Once in the whole cell configuration, the current transients elicited by 10-mV hyperpolarizing pulses from the holding potential of −70 mV in voltage-clamp mode showed a monoexponential relaxation (time constant = 81 ± 27 μs, n = 40), and were used to estimate series resistance (21.1 ± 8.7 MΩ, n = 40) and input resistance and capacitance. Depending on the high-input-to-series resistance ratio, bridge balancing in current-clamp recordings, 10⁻⁸ times, the reversibility (or irreversibility) in drug action, and the apparent saturation of the effects at the concentrations used in the present experiments are consistent with observations reported in experiments in cell culture (e.g., Amico et al. 1995; Pearson et al. 1995; Randall and Tsien 1995). Any residual Ca²⁺ action potentials could be blocked with 1 mM Ni²⁺ at the end of the experiments.

The glutamate receptor antagonists b-2-amino-5-phosphonovaleric acid (APV), 7-chlorokynurenine acid (7-Cl-kyn), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris Cookson (Bristol, UK) and prepared as reported previously. The stimulating electrode was placed over the mossy fiber bundle, and stimuli were applied at the frequency of 0.1 Hz (D’Angelo and Taglietti 1995). A fiber bundle, and stimuli were applied at the frequency of 0.1 Hz. The stimulating electrode was placed over the mossy fiber bundle, and stimuli were applied at the frequency of 0.1 Hz (D’Angelo et al. 1995). A second stimulating electrode was placed in proximity of the Purkinje cell layer to test granule cell antidromic excitation.

**Quantification of Ca²⁺ channel blockage**

To quantify the effect of Ca²⁺ channel blockers, we estimated the ionic current generating ITS, iₜₘ. This could be done considering that the current injected through the microelectode (iᵢ) divided into a capacitive (iₑ) and an ionic (iₜₘ) component and that iₑ is a leakage current (iₑ) as well as the voltage-sensitive current (iₜₘ) giving rise to the action potential (Jack et al. 1975). It follows the equation

\[ iₜₘ = iₑ(iᵢ + iₑ) \] (1)

where \( iₑ = Cₑ \frac{dV}{dt} \) and \( iᵢ = V/Rₑ \) could be measured directly from voltage tracings (\( Cₑ \) and \( Rₑ \) values were obtained as explained above, and \( V \) indicates membrane potential), and \( iₑ \) was known. Because the ITS rising phase was slow, the relative contribution of \( iₑ \) was taken into account in our measurements, although this term often is neglected (cf. Jack et al. 1975; McCormick et al. 1992) in several cells recorded under pharmacological Na⁺ and K⁺ current blockage (1 μM TTX, 20 mM TEA, 4 mM 4-AP) — the cells had \( Cₑ = 5.7 \pm 1.4 \) pF and \( Rₑ = 3.9 \pm 0.3 \) GΩ and were maintained at a holding potential of −79.7 ± 3.5 mV — the maximum inward current involved in ITS generation was \( iₜₘ = 2.9 \pm 0.4 \) pA/pF at −26 ± 5 mV. The ITS was consistent with Ca²⁺ current measurements previously performed in immature granule cells in situ (Rossi et al. 1994). It should be noted that \( iₑ \) depended on the effectiveness of the regenerative process and
The subthreshold membrane charging of granule cells was smooth with no humps or afterpotentials. Granule cells with ITS/HTS had an almost linear subthreshold voltage response, whereas granule cells with a fast repetitive firing showed marked inward rectification (Fig. 1, A and B), as demonstrated in the voltage-current plots in Fig. 1C. Consequently, $R_m$ was larger in granule cells with ITS/HTS than with fast repetitive firing over most of the subthreshold membrane potential range, although $R_m$ values were similar close to the threshold for action potential activation (Table 1). $C_m$ was also larger in granule cells with ITS/HTS than with fast repetitive firing, probably reflecting morphological rearrangement during development (Altman 1972). The larger $R_m$ and $C_m$ accounted for passive membrane transients being slower in granule cells with ITS/HTS than with fast repetitive firing (Fig. 1, A and B, Table 1).

The ionic nature of granule cell action potentials was investigated using specific ion channel blockers. The Na$^+$ channel blocker TTX (1 μM) blocked the fast repetitive action potentials (Fig. 2B, $n = 9$). TTX-resistant action potentials started at a relatively low threshold (ITS) and generated a fast repetitive spike discharge. Note also the marked inward rectification in the subthreshold voltage response in B, but not in A. In this and following figures, the current-clamp protocol is shown at the bottom. C: subthreshold voltage-current relationships of the cells in A and B, documenting the different intensity of inward rectification. Membrane voltage values were measured at 600 ms, where the tracings attained a steady level.

### RESULTS

Patch-clamp recordings were performed on 183 neurons in the internal granular layer of rat cerebellar slices from P4 to P21. The neurons did not display spontaneous firing either during seal formation or after having established the whole cell recording configuration and had the low membrane capacitance and high-input resistance typical of granule cells (Table 1) (D’Angelo et al. 1995). When recorded from near rest (−70 mV), the granule cells showed two basic firing patterns on depolarizing current injection. Forty-six percent of granule cells showed characteristic action potentials comprising components with different threshold and time course (Fig. 1A). The principal component consisted of a broad action potential (HW > 30 ms) activating at a threshold between −60 and −50 mV, which is intermediate between low- and high-threshold spikes in thalamic (Jahnsen and Linas 1984) and inferior olivary neurons (Linas and Yarom 1981). By analogy, this granule cell action potential has been termed intermediate-threshold spike, or ITS. When stimulus intensity was sufficiently high, ITS could prime a high-threshold spike at above −30 mV (HTS; cf. the two uppermost tracings in Fig. 1A). Because these excitable responses were not usually recorded beyond P21, they were related to an immature stage of granule cell development. Other granule cells fired fast spikes (HW < 2 ms) repeatedly at high-frequency but did not show any ITS (Fig. 1B), like the cells recorded at a mature developmental stage (D’Angelo et al. 1995). Electrophysiologically mature granule cells were observed occasionally as early as P5 and became more frequent thereafter (4% at P4–P9, 22% at P10–P21). Some granule cells (24%) showed firing patterns including both the ITS/HTS and fast repetitive action potentials, whereas other granule cells (18%) did not show any excitable response (not shown).

### Table 1. Passive membrane properties of granule cells

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<tr>
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<th>ITS and HTS</th>
<th>Fast Repetitive Spikes</th>
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<tbody>
<tr>
<td>$V_r$ (mV)</td>
<td>−61.8 ± 11.9</td>
<td>−59.4 ± 15.1</td>
</tr>
<tr>
<td>$C_m$ (pF)*</td>
<td>5.9 ± 2.4</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>$R_m$ (GΩ)</td>
<td></td>
<td></td>
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<tr>
<td>Low range*</td>
<td>4.1 ± 1.3</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>High range</td>
<td>4.4 ± 0.9</td>
<td>3.9 ± 1.6</td>
</tr>
<tr>
<td>$r_m$ (ms)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low range*</td>
<td>24.4 ± 6.4</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>High range*</td>
<td>25.7 ± 7.1</td>
<td>12.3 ± 3.2</td>
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*Statistically significant difference of the mean values at $P < 0.01$. Values are means ± SD.

Passive membrane properties in granule cells generating intermediate threshold spiking (ITS) and high threshold spiking (HTS; $n = 34$) or fast repetitive spikes ($n = 36$). $V_r$ is resting membrane potential, $R_m$ and $C_m$ are membrane input resistance and capacitance, respectively. $C_m$ has been estimated from current transients in the voltage-clamp mode (see Methods). $R_m$ has been measured as the cord resistance in VI plots (see Fig. 1C) in a high (just subthreshold for action potential generation) and low (below −80 mV) voltage range. In both ranges, $r_m$ has been calculated as the $R_m C_m$ product. $R_m$ values were similar close to the threshold for action potential activation (Table 1). $C_m$ was larger in granule cells with ITS/HTS than with fast repetitive firing, probably reflecting morphological rearrangement during development (Altman 1972). The larger $R_m$ and $C_m$ accounted for passive membrane transients being slower in granule cells with ITS/HTS than with fast repetitive firing (Fig. 1, A and B, Table 1).
potentials (ITS and HTS) were blocked by 1 mM Ni$^{2+}$ (Fig. 2A, n = 10), indicating that they had been generated by Ca$^{2+}$ channel currents. Therefore ITS and HTS were Ca$^{2+}$ action potentials, whereas Na$^+$ action potentials sustained the fast repetitive firing. It should be noted that TTX caused appreciable modifications in HTS in 6 of 10 granule cells that did not show repetitive firing (cf. Fig. 2A) and that Ca$^{2+}$ and repetitive Na$^+$ action potentials coexisted in another 5 granule cells (not shown).

In the presence of 1 μM TTX, local perfusion of a solution containing the Ca$^{2+}$ chelator 5 mM EGTA and no added Ca$^{2+}$ abolished both ITS and HTS (n = 6). In addition to bearing out the Ca$^{2+}$-dependence of both ITS and HTS, this ruled out any noticeable contribution of persistent TTX-bearing out the Ca$^{2+}$ containing the Ca$^{2+}$ stimulation (Fig. 3).

**Synaptic activation of Ca$^{2+}$ action potentials**

In 15 of 17 granule cells generating Ca$^{2+}$ action potentials, EPSPs and characteristic EPSP/action potential complexes (comprising ITS and HTS) were observed after mossy fiber stimulation (Fig. 3A). EPSPs were activated with a delay of 1–1.5 ms, compatible with monosynaptic granule cell activation. Similarly as in granule cells with fast repetitive Na$^+$ spikes (Fig. 3B), transition between neighboring levels in the EPSP and EPSP/Ca$^{2+}$ action potential complex occurred with small changes in the stimulus intensity, suggesting that an increasing number of mossy fiber synapses was recruited. If the response to the minimal stimulus intensity is assumed to correspond to unitary synaptic events (see D’Angelo et al. 1995; Silver et al. 1996), the unitary EPSP should have an amplitude of 8.8 ± 2.4 mV from the holding membrane potential of −71.4 ± 2.3 mV (n = 6). Higher levels of synaptic activation then would correspond to the recruitment of up to four mossy fiber synapses, in good agreement with the morphological evidence that granule cells make an average of four synapses with as much as different mossy fibers (Eccles et al. 1967). Accordingly, 2.6 ± 1.1 (n = 8) mossy fiber synapses had to be activated synchronously to generate ITS. Despite using stimulus intensities higher than those effectively activating mossy fibers, stimulation close to the Purkinje cell layer failed to elicit any antidromic spikes (n = 11), suggesting that the ascending axon in granule cells generating Ca$^{2+}$ action potentials was inefficient in propagating excitation.

EPSPs in granule cells generating Ca$^{2+}$ action potentials comprised a NMDA and a non-NMDA receptor-mediated component. The NMDA component was slower than passive membrane transients, whereas the non-NMDA component and passive transients decayed at a similar rate (Fig. 3C).
Excitatory postsynaptic potential (EPSP) amplitude and time course in granule cells generating ITS and HTS or fast repetitive spikes (the number of observations is indicated in parentheses). Unless otherwise indicated, the measurements are relative to the holding membrane potential of −70 mV. The relative contribution of the N-methyl-D-aspartate (NMDA) receptor-mediated component is expressed as percent of control EPSP amplitude at the same membrane potential. HW denotes duration of EPSPs or spikes at half amplitude. † Data from D’Angelo et al. (1995) and unpublished observations. ‡ Unitary amplitude from −75 mV. †† Statistically significant difference of the mean values at P < 0.01.

EPSPs showed a marked voltage dependence, increasing in size and slowing down the more the membrane was depolarized (Fig. 3D), as expected from voltage-dependent increase and slowing down of the NMDA current during membrane depolarization (D’Angelo et al. 1994b, 1995). It should be noted that the EPSPs were slower in granule cells generating Ca2+ than Na+ action potentials (Fig. 3, A and B, Table 2), probably due to a larger membrane time constant and a greater contribution of the NMDA relative to non-NMDA component (D’Angelo et al. 1993).

Requirements for Ca2+ action potential generation

ITS activation showed a marked dependence on the intensity of injected current pulses. Moreover, a brief current injection failed to prime ITS activation, whereas ITS could be activated as the pulse duration was increased (Fig. 4A). This observation indicates that ITS is not self-regenerative and that ITS generation requires a protracted supporting current, which must be provided by synaptic channels during mossy fiber transmission. Blocking the NMDA receptors precluded ITS activation (Fig. 4B). The residual depolarization decayed close to passive membrane transients as expected from a non-NMDA EPSP. The higher efficiency of NMDA than non-NMDA receptors in generating ITS apparently was related to the slow regenerative inward current produced by NMDA receptors during depolarization (D’Angelo et al. 1994b) (see Fig. 3D). Repetitive stimulation at frequencies between 5 and 50 Hz generated ITS and HTS followed by a protracted depolarization (Fig. 4C). At these frequencies, a doublet of EPSPs resulted in a saturation of the response, which was maintained by any subsequent input. Blocking NMDA receptors revealed that non-NMDA EPSPs were too short and small to determine effective temporal summation and sustain Ca2+ channel-dependent responses. Results similar to those shown in Fig. 4 have been obtained in six other granule cells to which glutamate receptor antagonists have been applied, revealing a primary role for the NMDA receptors in driving Ca2+ action potential generation.

Voltage-inactivation in ITS and HTS

In addition to having distinct activation thresholds, ITS and HTS showed distinct voltage inactivation. ITS could be activated from low holding potentials (usually less than −60 mV, Fig. 5A, bottom) but was inactivated at potentials higher than −50 mV (Fig. 5A, top). This mechanism resulted in rebound ITS activation on return to a membrane potential around −50 mV after a period of hyperpolarization at around −80 mV, as illustrated in Fig. 5A and observed in another eight granule cells. Conversely, HTS still could be activated from −50 mV. Therefore ITS had much more pronounced voltage inactivation than HTS. Differential ITS and HTS voltage inactivation also could be evidenced during synaptic transmission. ITS, but not HTS synaptic activation, failed when the cell was held at around −50 mV for 5 s (Fig. 5B). The voltage dependence of ITS inactivation occurred in the range of membrane potentials between −90 and −50 mV, as observed using different conditioning membrane potentials (n = 7, Fig. 5C, left). Paired stimulation experiments demonstrated that inactivation developed during the ITS, causing refractoriness in ITS reaction. After a condition-

![Figure 4](http://jn.physiology.org/)

**FIG. 4.** Requirements for Ca2+ action potential activation. **A:** ITS showed graded activation with protracted current pulses of increasing intensity (left). ITS could not be activated as current pulse was shortened (middle). Short current pulses proved ineffective despite their intensity was higher than that used in protracted current pulses (right). **B:** ITS and HTS were activated during low-frequency mossy fiber stimulation (0.1 Hz). After NMDA receptor blockage with APV + 7-CI-Kyn, the ITS/HTS complex could no longer be elicited, and EPSP decay only slightly deviated from passive membrane discharge (○, ○, ○, ○). Note that the non-NMDA EPSP had nearly the same amplitude as control EPSP and crossed ITS threshold. **D:** during high-frequency mossy fiber stimulation (12 Hz), temporal summation sustained an intense protracted depolarization. After APV + 7-CI-Kyn application, a dramatic reduction in temporal summation precluded any protracted depolarization, uncovering non-NMDA EPSP depression along the trains. Same cell (P14), holding potential (−80 mV), and scale in A–C.

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**TABLE 2. Properties of granule cell EPSPs**

<table>
<thead>
<tr>
<th>Component</th>
<th>ITS and HTS</th>
<th>Fast Repetitive Spikes*</th>
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<tr>
<td>Amplitude, mV</td>
<td>8.8 ± 2.4 (8)</td>
<td>11.4 ± 2.1 (11)†</td>
</tr>
<tr>
<td>ttp, ms‡</td>
<td>15.2 ± 8.4 (6)</td>
<td>7.2 ± 2.6 (5)</td>
</tr>
<tr>
<td>HW, ms‡</td>
<td>133 ± 54 (6)</td>
<td>43 ± 25 (5)</td>
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<tr>
<td>NMDA amplitude, %‡</td>
<td>68 ± 17 (6)</td>
<td>29 ± 11 (9)</td>
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* Data from D’Angelo et al. (1995) and unpublished observations. †† Statistically significant difference of the mean values at P < 0.01.
ing ITS at \(-80 \text{ mV}\), refractoriness was removed in \(\sim 500 \text{ ms}\) \((n = 3; \text{Fig. 5C, right})\).

**Action of \(K^+\) channel blockers on ITS and HTS**

To assess the role of repolarizing currents in the \(Ca^{2+}\) action potentials, we used the broad spectrum \(K^+\) channel blocker, TEA. Application of 20 mM TEA could modify ITS (Fig. 6A), although this effect was observed inconstantly and was statistically not significant \((8 \pm 13\% i_{max} \text{ change}; n = 9; \text{NS})\). Therefore the ITS time course should be determined largely by \(Ca^{2+}\) channel and passive membrane properties. Conversely the application of TEA as low as 1 mM increased HTS, revealing that HTS was limited severely by repolarizing \(K^+\) conductances. A marked HTS broadening was observed as the TEA concentration was increased \(\leq 20 \text{ mM}\) (Fig. 6B). The differential action of TEA on ITS and HTS could be explained if we consider that effective activation of TEA-sensitive \(K^+\) currents occurs at around \(-30 \text{ mV}\) (Bardoni and Belluzzi 1993; Gabbiani et al. 1994), thus not significantly affecting the ITS time course. At more than \(-30 \text{ mV}, K^+\) currents rapidly increase, limiting the development of HTS. The comparison of ITS and HTS after TEA application also shows that ITS had faster inactivation than the HTS current.

Among TEA-insensitive \(K^+\) currents, the transient \(I_h\) current and \(Ca^{2+}\)-dependent \(K^+\) currents (see Bardoni and Belluzzi 1993) may be activated during the action potentials. Application of the \(I_h\) blocker 4-AP (2 mM; Fig. 6C) had, however, no remarkable effect on ITS \((-6 \pm 7\% i_{max} \text{ change}; n = 4, \text{NS})\). The peptidic toxin apamine (500 nM; Fig. 6D) was also ineffective \((+2 \pm 6\% i_{max} \text{ change}; n = 5, \text{NS})\), ruling out the involvement of TEA-insensitive \(Ca^{2+}\)-dependent \(K^+\) channels in action potential repolarization.

**Pharmacological identification of ITS components**

Recent findings demonstrate the coexistence of multiple HVA \(Ca^{2+}\) current components in cerebellar granule cells in culture (Amico et al. 1995; Pearson et al. 1995; Randall and Tsien 1995). Indeed, ITS was inhibited by 10 \(\mu M\) nifedipine \((n = 14)\), by the *Agelenopsys Aperta* peptidic toxin 3–30 \(nM\) \(\omega\)-Aga IVA \((n = 13)\), and by the *Conus Geographus* peptidic toxin 5 \(\mu M\) \(\omega\)-CTX GVIA \((n = 15)\), which are known to block L, P, and N channels, respectively (Fig. 7, A and B). All these \(Ca^{2+}\) channel antagonists reduced both a transient and a protracted ITS component, as shown below. ITS was not affected at all by 500 \(\mu M\) amiloride \((-2 \pm 3\% i_{max} \text{ change}; n = 6, \text{NS}; \text{Fig. 4C})\) as expected from previous studies showing that low-voltage activated channels are not expressed in granule cells (see, for instance, Randall and Tsien 1995; Rossi et al. 1994).

The sequential application of 5 \(\mu M\) \(\omega\)-CTX GVIA, 10 \(\mu M\) nifedipine, and 300 \(nM\) \(\omega\)-Aga IVA showed \(N_-, L_-\), and P-type channel coexpression in granule cells (Fig. 7B). In eight experiments, \(i_{max}\) changed by \(-14 \pm 11\%, -37 \pm 14\%, -52 \pm 21\%, \text{respectively, and } i_{tg}\) by \(-51 \pm 18\%\).
The protracted depolarizations in ITS were reduced after 5 μM ω-CTX GVIA, 10 μM nifedipine, and 300 nM ω-Aga IVA coapplication, so that R_{RTS} (which could be blocked reversibly by 5 μM ω-CTX MVIC) appeared narrower than ITS. Exponential fitting showed that R_{RTS} decay conformed to passive membrane discharge (Fig. 8A). Therefore the putative R_{RTS} current should inactivate more rapidly than L-, N-, P-type currents, as well as of passive membrane discharge (time constant \( \tau_{\text{max}} = 32.2 \pm 9.6 \) ms, \( n = 5 \)), and largely accounted for the transient component in ITS.

The role of L-, N-, and P-type currents was investigated further in experiments in which coapplication of 5 μM ω-CTX GVIA, 10 μM nifedipine, and 300 nM ω-Aga IVA almost fully prevented ITS generation (–96 ± 16% \( i_{\text{max}} \) change, \( P < 0.01 \), \( n = 7 \); Fig. 8B). Subsequently, the R_{RTS} component (which decayed conforming to passive membrane discharge and was reduced reversibly by 5 μM ω-CTX GVIA) could be restored by increasing the injected current intensity (–42 ± 18% \( i_{\text{max}} \) change \( P < 0.01 \), \( n = 7 \)). These observations suggested that L-, N-, and P-type currents boosted regenerative activation in the transient ITS component.
Figure 8C shows differential voltage inactivation in the ITS components. In control recordings, changing the holding membrane potential from −85 to −65 mV caused a greater inactivation in the transient ITS component than in the subsequent protracted depolarization (−54 ± 12% \( i_{max} \) vs. −14 ± 9% \( i_{p} \) change, \( n = 5, P < 0.01 \)). This difference was even larger when ITS elicited from −85 mV were compared with ITS elicited from −58 mV (−97 ± 6% \( i_{max} \) vs. −23 ± 8% \( i_{p} \) change, \( n = 5, P < 0.01 \)). After perfusing a solution containing 5 \( \mu \)M \( \omega \)-CTX GVIA, 10 \( \mu \)M nifedipine, and 300 nM \( \omega \)-Aga IVA, the transient ITS component still showed voltage-dependent inactivation. The transient \( R_{ITS} \) was therefore more voltage inactivated than protracted \( L-, P-, \) and \( N\)-type currents.

**HTS pharmacology**

The pharmacology of HTS was investigated from a membrane potential of around −50 mV in the presence of 20 mM TEA. The application of 5 \( \mu \)M \( \omega \)-CTX GVIA markedly and irreversibly inhibited HTS (Fig. 9, \( n = 15 \)). The residual action potential could activate repeatedly, suggesting that the residual \( \mathrm{Ca}^{2+} \) current is incompletely voltage inactivated at potentials as high as −30 mV. A subsequent application of 10 \( \mu \)M nifedipine, 300 nM \( \omega \)-Aga IVA, and 5 \( \mu \)M \( \omega \)-CTX MVIIC reduced the efficiency of depolarizing pulses (Fig. 6, \( n = 5 \)), as expected from the action of these drugs on protracted depolarizations (see previous section). Neither of these drugs, however, could suppress the residual action potentials, which were blocked finally by 1 mM Ni\(^{2+} \) (\( n = 12 \)). The constant observation of a conspicuous HTS component insensitive to \( \omega \)-CTX GVIA, nifedipine, \( \omega \)-Aga IVA, and \( \omega \)-CTX MVIIC suggested activation of putative \( R\)-type channels different from those involved in ITS, which we shall define as \( R_{HTS} \).

**\( \mathrm{Ca}^{2+} \) channel activation in \( \mathrm{Na}^+ \) spikes**

Activation of \( \mathrm{Ca}^{2+} \) channels in granule cells with fast repetitive \( \mathrm{Na}^+ \) spikes was investigated at P19–P21, when the probability of recording cells with a mature firing pattern was high. As noted in Fig. 2B, these granule cells did not show any ITS or HTS after \( \mathrm{Na}^+ \) spike blockage by 1 \( \mu \)M TTX. However, a HTS was revealed on applying 20 mM TEA. This HTS was inhibited strongly by 5 \( \mu \)M \( \omega \)-CTX GVIA (Fig. 10A, \( n = 9 \)), and subsequent application of 10 \( \mu \)M nifedipine, 300 nM \( \omega \)-Aga IVA, and 5 \( \mu \)M \( \omega \)-CTX MVIIC had no noticeable effect.

The role of \( \mathrm{Ca}^{2+} \) currents in the fast \( \mathrm{Na}^+ \)-dependent spiking discharge was tested by application of 5 \( \mu \)M \( \omega \)-CTX
FIG. 9. HTS pharmacology. In this and similar experiments, membrane potential was maintained at around −50 mV for 5 s to inactivate the transient ITS component before activating ITS (inset). Experimental tracings illustrate sequential application of 5 μM ω-CTX GVIA, 10 μM nifedipine + 300 nM ω-Aga IVA + 5 μM ω-CTX MVIIC, and 1 mM Ni²⁺ in solutions containing 1 μM TTX and 20 mM TEA. A conspicuous HTS remained after coapplication of the organic Ca²⁺ channel blockers. Full HTS block was obtained with Ni²⁺ perfusion (note that no regenerative membrane depolarizations were observed despite the current intensity was increased). Cell recorded at P13.

GVIA. ω-CTX GVIA caused a reduction in spike afterhyperpolarization (AHP; n = 5; Fig. 10B). As expected from the experiments on HTS inhibition shown in Fig. 10A, no noticeable enhancement of ω-CTX GVIA action was observed following a further application of 1 mM Ni²⁺. The N-type channel enhancement of AHP probably depended on the activation of Ca²⁺-dependent repolarizing currents (Gabbiani et al. 1994). In association with AHP reduction, ω-CTX GVIA increased spike frequency (n = 5; Fig. 10C), indicating an important role for the N-type channels in controlling granule cell coding properties during repetitive discharge.

DISCUSSION

We have investigated the excitable and synaptic properties of granule cells in the internal granular layer of developing rat cerebellum using patch-clamp whole cell recordings in acute slice preparations (Edwards et al. 1989). During the first three postnatal weeks, when migration and major developmental changes take place (Altman 1972), nearly 50% of granule cells generated Ca²⁺ action potentials. The Ca²⁺ action potentials depended on L-, N-, P-, and putative R-type Ca²⁺ channel activation, concurring to generate depolarizations with different threshold, kinetics, and voltage sensitivity. These properties were no longer observed after the third postnatal week, when granule cells show fast repetitive Na⁺-dependent firing (D’Angelo et al. 1995), and Ca²⁺- dependent regulation of excitability depends prominently on N-type channels. Therefore, consistent with changes in Ca²⁺ currents (Rossi et al. 1994), the Ca²⁺ action potentials appeared as a property of granule cells at immature developmental stage. The Ca²⁺ action potentials could be activated synaptically by mossy fiber stimulation, revealing a critical role for NMDA receptors in Ca²⁺ action potential generation.

Synaptic activation of Ca²⁺ action potentials

Ca²⁺ action potentials consisted of a spike termed intermediate threshold (ITS), because it activates at potentials (−60/−50 mV) laying between those typical of low-thresh-

FIG. 10. Ca²⁺ channel activation in Na⁺ spikes. A: after having blocked Na⁺-dependent firing with 1 μM TTX, a HTS was revealed by 20 mM TEA application. This HTS was inhibited strongly by 5 μM ω-CTX GVIA. A set of tracings is shown from a hyperpolarized holding potential to demonstrate the absence of ITS. − − −, obtained after a subsequent application of 10 μM nifedipine + 300 nM ω-Aga IVA + 5 μM ω-CTX MVIIC. B: spike afterhyperpolarization was reduced by 5 μM ω-CTX GVIA. No greater inhibition was obtained by subsequent 1 mM Ni²⁺ application (− − −). C: application of 5 μM ω-CTX GVIA increased the firing frequency. Cells recorded at P19-P20.
old spike (LTS) and HTS (Jahnsen and Llinas 1984; Llinas and Yarom 1981; McCormick et al. 1992), and of a HTS, activating at more than −30 mV. Both ITS and HTS comprised transient and protracted Ca\(^{2+}\) channel-dependent depolarizations. ITS was less affected by repolarizing K\(^+\) currents, and ITS transient phase showed more marked voltage- and time-dependent inactivation than HTS. In inferior olivary (Llinas and Yarom 1981) and thalamic relay neurons (Jahnsen and Llinas 1984), LTS is somatic, whereas HTS originates from remote dendritic regions. Dendritic channel activation (Spruston et al. 1995) may generate local current flows, causing an apparent increase in spike threshold and inducing repetitive spike activation. However, the granule cell is electrotonically compact and behaves like a lumped soma-dendritic compartment (D’Angelo et al. 1993, 1995; Silver et al. 1992, 1996), so that Ca\(^{2+}\) channel location is unlike to remarkably influence the generation of Ca\(^{2+}\) action potentials.

The granule cells did not generate spontaneous membrane potential oscillations. Oscillatory activity was not observed in the cell-attached or perforated-patch configuration (E. D’Angelo, G. De Filippi, P. Rossi, and V. Taglietti, unpublished observation), ruling out the possibility that autorhythmicity was disrupted by cytoplasmic changes caused by the pipette solution. The natural stimulus for Ca\(^{2+}\) action potential activation probably is provided by mossy fiber activity. Synaptic activation of Ca\(^{2+}\) action potentials depended on NMDA receptors, although non-NMDA receptors contributed to synaptic depolarization. The requirement for NMDA receptor activation was related to the slow kinetics and regenerative behavior of the NMDA current, which increases and lasts longer the more the membrane is depolarized (D’Angelo et al. 1994b). Thus the NMDA receptors provided the protracted inward current required to sustain the transient phase of ITS, which was not self-regenerative. During repetitive mossy fiber activity, NMDA receptor sustained temporal summation of impulses at a frequency as low as 5 Hz, raising membrane potential into the range required for generating protracted Ca\(^{2+}\) channel-dependent depolarizations.

An important determinant of Ca\(^{2+}\) action potential generation was the granule cell membrane potential. On the one hand, ITS was inactivated strongly by membrane depolarization, and ITS repriming required several hundred milliseconds at a rather negative membrane potential (typically lower than −65 mV). On the other hand, HTS still could be activated from membrane potentials causing ITS inactivation (see Fig. 5). Another critical factor was the pattern of mossy fiber stimulation. Our present as well as previous results (D’Angelo et al. 1995) suggest that a small number (usually 1–4) of mossy fibers could be recruited by increasing the stimulus intensity (see Fig. 4). Accordingly, ITS generation should require synchronous activity in at least two mossy fibers, and the recruitment of additional mossy fibers should activate the whole ITS/HTS complex. During repetitive mossy fiber stimulation, the response saturated, rendering the granule cell refractory to any subsequent input. Response saturation might be prevented by the repolarizing action of Golgi cell inhibitory postsynaptic potentials (Brickley et al. 1996; E. D’Angelo, P. Rossi, Armano, and V. Taglietti, unpublished observations), allowing ITS recovery from inactivation and subsequent rebound ITS activation (cf. Fig. 5A). Therefore inactivating properties may play an important role in relating ITS to inhibitory activity in Golgi cell fibers (Eccles et al. 1967; Shimono et al. 1976).

**Multiple Ca\(^{2+}\) channels in ITS and HTS**

The analysis of ITS and HTS inhibition by nifedipine, ω-CTX GVIA, ω-Aga IVA, and ω-CTX MVIIC suggested a selective involvement of L-, N-, P-, and putative R-type Ca\(^{2+}\) channels. Dissection of Ca\(^{2+}\) currents in culture also has suggested the expression of Q-type channels (Randall and Tsien 1995), an α\(_{1A}\) subunit-related channel that has been expressed previously in Xenopus oocytes (Sather et al. 1993). A distinctive property of the Q channel is the irreversible high-affinity block by ω-CTX MVIIC. In ITS, however, the ω-CTX MVIIC block was readily reversible and may affect a fraction of the R\(_{ITS}\) current. It should be noted that a putative R-type current has been reported in granule cells in culture; this current was blocked partially and reversibly by ω-CTX MVIIC (Ellinor et al. 1993). Ellinor’s et al. (1993) R-type current showed activation threshold, voltage-dependent inactivation, and decay kinetics compatible with R\(_{ITS}\) and with the transient non-L, non-N, non-P current component specific to immature granule cells (Rossi et al. 1994). R\(_{ITS}\) may be composite itself, including the G\(_2\) and G\(_3\) channels that have been identified recently in granule cells in culture (Pietrobon et al. 1996; Tottene et al. 1996). The insensitivity to amiloride differentiates R\(_{ITS}\) from T-type channels expressed in other immature neurons (McCobb et al. 1989; Yaari et al. 1987), although similarities of R-type and T-type channel functional properties have been reported (Carbone et al. 1996). Not only an ITS but also an HTS component may reflect activation of R-type Ca\(^{2+}\) channels. R\(_{HTS}\) showed poor voltage inactivation and high apparent activation threshold (more than −30 mV), therefore differing from R\(_{ITS}\). R\(_{HTS}\) also differed from Q-type channels, which are inactivated almost completely at −50 mV and are sensitive to ω-Aga IVA and ω-CTX MVIIC (see Carbone et al. 1996).

The multiple Ca\(^{2+}\) channels provided Ca\(^{2+}\) action potentials with complex regulatory properties. The N-, L-, and P-type channels generated protracted depolarizations, whereas the R\(_{ITS}\) and R\(_{HTS}\) channels generated transient depolarizations with different activation thresholds. The activation mechanism of ITS was particularly interesting. R\(_{ITS}\) channels had more pronounced voltage-dependent inactivation than the other channels, allowing selective modulation of ITS generation depending on the cell membrane potential. Moreover, N-, L-, and P-type channel currents enhanced regenerative R\(_{ITS}\)-channel activation, reinforcing a cooperative mechanism initiated by the NMDA channel current at the synapse. In addition to having different gating and kinetics properties, the multiple Ca\(^{2+}\) channels provide targets for chemical modulation (Amico et al. 1995; Haws et al. 1993) suitable for changing the efficiency of ITS and HTS generation.

**Development of granule cell electroresponsiveness**

The N-type channels play a relevant role in granule cell migration (Komuro and Rakic 1993) and are maintained...
Development of mossy fiber transmission

Simultaneous to action potential changes, major changes occurred in mossy fiber activation of granule cells. Granule cells generating Ca\(^{2+}\) action potentials showed slower EPSPs than those generating Na\(^+\) action potentials. The slower EPSP time course probably was related to a greater membrane time constant, depending both on the absence of inward rectification and a greater membrane capacitance. Moreover, contribution of a slow NMDA relative to a fast non-NMDA receptor-mediated component was greater in granule cells generating Ca\(^{2+}\) than Na\(^+\) action potentials, probably reflecting the developmental changes in synaptic currents reported previously (D’Angelo et al. 1993). Changes in NMDA current kinetics accompanying developmental substitution in NMDA receptor subunits (Ebralidize et al. 1996; Takahashi et al. 1996; Vallano et al. 1996) also may contribute to the observed differences in synaptic depolarization, although a correlation between expression of synaptic and membrane channels remains to be demonstrated.

Conclusions

Synaptic transmission at the mossy fiber synapse activated Ca\(^{2+}\) action potentials in granule cells at an immature developmental stage. This excitable response required NMDA receptor activation and involved the opening of L-, N-, P-,

and putative R-type Ca\(^{2+}\) channels. The cooperative interaction of NMDA and Ca\(^{2+}\) channels in regulating granule cell synaptic excitation may be extended by considering that NMDA and Ca\(^{2+}\) channels are the two major pathways for Ca\(^{2+}\) influx through the neuronal membrane. Synaptic activation of Ca\(^{2+}\) action potentials, by amplifying NMDA receptor-mediated Ca\(^{2+}\) influx, may generate a network-dependent signal suitable for influencing granule cell development and plasticity (Garthwaite 1994; Spitzer 1991). At a mature stage, most of the Ca\(^{2+}\) channel-dependent effects were due to N-type channels, which promoted Na\(^+\) spike repolarization regulating granule cell spike-frequency coding. An increasing proportion of functionally mature granule cells helped explaining the improvement of mossy fiber efficiency to relay information to Purkinje cells observed in vivo during the first three postnatal weeks (Puro and Woodward 1977; Shimon et al. 1976).

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