Pharmacological Dissection of Multiple Types of Ca^{2+} Channel Currents in Rat Cerebellar Granule Neurons

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The diversity of Ca^{2+} channel types in rat cerebellar granule neurons was investigated with whole-cell recordings (5 mM external Ba^{2+}). Contributions of five different high-voltage-activated Ca^{2+} channel current components were distinguished pharmacologically. Nimodipine-sensitive L-type current and ω -CTx-GVIA-sensitive N-type current contributed 15 and 20% of the total current, respectively. The bulk of the remaining current (46%) was inhibited by ω -Aga-IVA. The current blocked by this toxin was further subdivided into two components, P-type and Q-type, on the basis of differences in their inactivation kinetics and sensitivity to ω -Aga-IVA. P-Type current was noninactivating during 0.1 sec depolarizations, half-blocked at about 1–3 nM ω -Aga-IVA, and contributed ~11% of the total current; Q-type current was prominently inactivating, half-blocked at ~90 nM ω -Aga-IVA, and comprised 35% of the total current. Both P- and Q-type currents were potently inhibited by the *Conus magus* toxin ω -CTx-MVIIIC. A current component resistant to all of the aforementioned blockers (R-type) displayed more rapid inactivation than the other components and constituted 19% of the total current. The Q-type current, the largest of the current components in the granule neurons, resembles currents that can be generated in *Xenopus* oocytes by expression of cloned α_{1A} subunits.

[Key words: calcium channel, calcium current, cerebellum, granule neuron, ω -conotoxin-GVIA, ω -Aga-IVA]

The diversity of Ca^{2+} channels in vertebrate neurons is of fundamental interest because voltage-gated Ca^{2+} entry through these channels controls a wide variety of physiological functions, encompassing neurotransmitter release, membrane excitability, neurite outgrowth, bioenergetics, and gene expression (Reuter, 1983; Hille, 1992). The existence of more than one type of voltage-gated calcium channel in vertebrate neurons has been appreciated for some time (Carbone and Lux, 1984; Armstrong

and Matteson, 1985; Fedulova et al., 1985; Nowycky et al., 1985). Biophysical and pharmacological analysis has led to the description of several classes of Ca^{2+} channels, usually referred to as L-, N-, T-, and P-type (Nowycky et al., 1985; Fox et al., 1987a,b; Bean 1989; Llinás et al., 1989; Hess, 1990). These channel types differ considerably in their responsiveness to neuromodulators (Tsien et al., 1988; Bean, 1989), their distribution among various types of neurons (Miller and Fox, 1990; Tsien et al., 1991; Mintz et al., 1992b; Snutch and Reiner, 1992), and their localization in different regions within individual neurons (Westenbroek et al., 1992). The variety of Ca^{2+} channel types allows many possibilities for multiplicity of function (Hille, 1992).

The full extent of Ca^{2+} channel diversity remains incompletely understood. On one hand, molecular cloning uncovers an ever-increasing number of Ca^{2+} channel subunits with a wide distribution in the CNS (Perez-Reyes et al., 1990; Tsien et al., 1991; Snutch and Reiner, 1992; Miller, 1993). On the other hand, pharmacological evidence points to the possible existence of additional types of Ca^{2+} channels outside of the T, N, L, and P classification (Regan et al., 1991; Artelejo et al., 1992; Mintz et al., 1992b; Swartz et al., 1993; Forti et al., 1994). While both lines of investigation suggest additional complexity, the match-up between the cloned subunits and Ca^{2+} channels in neurons is incomplete. Some Ca^{2+} channel α_1 subunits appear in good correspondence with established channel types. For example neuronal L-type channel currents are generated by class C and D α_1 subunits (Mikami et al., 1989; Williams et al., 1992a) and N-type Ca^{2+} channels arise from the class B α_1 (Williams et al., 1992b). Other α_1 s, however, are more difficult to identify as known calcium channel types in nerve cells. A leading example is the class A α_1 subunit (also known as brain-I or BI), the first α_1 subunit to be isolated from nervous tissue (Mori et al., 1991; Starr et al., 1991). Transcripts for α_{1A} abound in the mammalian brain, particularly in cerebellar Purkinje and granule cells (Mori et al., 1991; Fujita et al., 1993). This has led to the presumption that this subunit is equivalent to the P-type channel, the dominant Ca^{2+} conductance in the Purkinje neurons (Snutch and Reiner, 1992; but see Tsien et al., 1991). However, the expression of α_{1A} in oocytes (Sather et al., 1993; Stea et al., 1994) or in a mammalian cell line (Niidome et al., 1994) produces a phenotype different from the P-type current, suggesting that α_{1A} can also generate a novel non-P-type Ca^{2+} channel.

We have undertaken a study of the classes of Ca^{2+} channel in rat cerebellar granule cells, the most abundant neuron in the mammalian CNS (Ramón y Cajal, 1904). Granule cells give rise to parallel fiber synapses onto the dendrites of Purkinje neurons,

Received Aug. 17, 1994; revised Oct. 31, 1994; accepted Nov. 4, 1994.

We thank Beverly Wendland, Felix Schweizer, Karl Deisseroth, and Haruhiko Bito for preparation of cerebellar granule cells, George Miljanich (Neurex) for providing gifts of ω -CTx-MVIIIC and ω -CTx-GVIA, A. J. G. Baxter (Fisons PLC) for providing FPL 64176, and Michael Adams (UC Riverside) for a gift of ω -Aga-IVA and helpful discussions. We are grateful to Ilya Bezprozvanny, Karl Deisseroth, William Sather, David Wheeler, and Ji-Fang Zhang for comments on the MS. This work was supported by NIH Grant NS24067 (R.W.T.) and an NIMH-Silvio Conte Center for Neuroscience Research. A.R. is a Lucille P. Markey Visiting Fellow.

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synapses whose strength undergoes modification during long-term depression and other forms of synaptic plasticity (see Ito, 1989, for review). Granule cells are electrically compact (Silver et al., 1992; Randall and Tsien 1993), express a range of different α_1 subunits, and survive well in tissue culture. All of these traits proved favorable for the analysis of Ca^{2+} channels in our experiments. Using a series of potent channel inhibitors, we have defined five readily distinguishable components of Ca^{2+} channel current, including L-, N-, and P-type Ca^{2+} channels with conventional properties, as well as additional components, Q-type and R-type. Together, Q-type and R-type currents constitute half of the peak inward Ca^{2+} channel current. The Q-type current is the largest component in the granule cells and displays electrophysiological and pharmacological properties that match up closely with the α_{1A} subunit expressed in oocytes.

Some of the results have been presented in abstract form (Randall et al., 1993a).

Materials and Methods

Cell preparation and culture. Cerebellar neurons were prepared using minor modifications of a procedure for preparation of hippocampal cultures (Malgaroli and Tsien, 1992). Cerebella were removed from the brains of decapitated 2–5-d-old rat pups of either sex. Following removal of the dura, the cerebellum was cut into small ($\sim 1 \text{ mm}^3$) pieces and digested with trypsin (type XI, Sigma, 10 mg/ml) for 4 min at 24°C in a Ca^{2+} - and Mg^{2+} -containing, HEPES-buffered salt solution supplemented with 0.5 mg/ml DNAase (type IV Sigma). This treatment was followed by trituration with fire-polished Pasteur pipettes in a high Mg^{2+} , 0 Ca^{2+} , bicarbonate-buffered saline. Following three centrifugation/resuspension steps, the cells were plated on square No. 1 coverslips (VWR), previously coated with Matrigel (Collaborative Research Inc.). The cells were maintained in culture under 5% CO_2 at 37°C , in minimal essential medium (5.3 mM K^+ , GIBCO), supplemented with glucose 5 gm/liter, transferrin 100 mg/liter, insulin 25 mg/liter, glutamine 300 mg/liter, cytosine arabinoside 2–5 μM , and 10% fetal calf serum (Hyclone). Cultures were fed after 2 d by replacement of 50–70% of the culture medium and sometimes were fed a second time after 5–6 d *in vitro*.

Electrophysiology. Prior to recording, dishes containing a single coverslip were removed from the incubator, the culture medium being replaced with Tyrode solution containing (mM) NaCl 119, KCl 5, CaCl_2 2, MgCl_2 1, glucose 30, HEPES-NaOH 25 (pH 7.3), 313 mOsm. The coverslip was broken into numerous pieces and a single shard placed in the recording chamber, while the remaining shards were kept in Tyrode at room temperature until used or discarded. In control experiments we were unable to detect any change in the size or properties of the Ba^{2+} currents from cells that were maintained in Tyrode at room temperature for < 10 min or more than 5 hr. Standard patch clamp recordings (Hamill et al., 1981) (Axopatch 1D amplifier, Axon Instruments) were made with fire-polished patch-clamp pipettes, fabricated from 1.2 mm diameter, thick-walled borosilicate glass (Clark Electromedical) with a horizontal electrode puller (Sutter Instruments). The pipettes were filled with CsMeSO_4 108, MgCl_2 4.5, EGTA 9, ATP 4, GTP 0.3, HEPES 24 (pH 7.3 with CsOH), 292 mOsm, and showed resistances of 2–8 M Ω (typically 5 M Ω). For recordings of Ba^{2+} currents, the external solution contained TEA-Cl 160, BaCl_2 5, HEPES- CsOH 10 (pH 7.3), 313 mOsm. Gigaseals (> 5 G Ω) were formed by gentle mouth suction, following which fast capacitance transients were neutralized. The whole-cell configuration was achieved using a combination of further suction and application of 200 μsec , 2 V DC pulses. Series resistances were < 20 μM (typically 8–10 M Ω) and were typically not compensated. In control experiments, the recorded currents were essentially the same with and without series resistance compensation, presumably because the cell capacitance and total current magnitudes were relatively small.

For the analysis of toxin actions on voltage-activated Ba^{2+} currents, we adopted a standard protocol of applying a 105 msec duration step to a test potential of 0 mV every 10 sec, the standard holding potential being -80 mV . Each test pulse was preceded by a +10 mV pulse of identical length that was used to monitor series resistance, capacitance, and input (leak) resistance, and to allow subsequent leak subtraction. In

the solutions used to acquire Ba^{2+} currents, the leak resistance varied between 6 and 20 G Ω .

Data storage and analysis. Occasionally, current and voltage signals were recorded on digital tape and digitized following appropriate filtering off line. Usually, however, data were directly filtered (1.5 to 8 kHz, 8-pole Bessel filter, Frequency Devices) and digitized (3.33 to 25 kHz) within the pCLAMP acquisition environment (Axon Instruments). Leak subtraction and analysis were performed by custom-written programs (AXOBASIC), used in conjunction with a spreadsheet program (QUATTRO PRO, Borland). All data are presented as mean $\pm 1 \text{ SEM}$.

Rundown correction. Corrections for rundown of non-L-, non-N-type Ba^{2+} current were based on direct observations of time-dependent changes in the amplitude of this current, which decreased from normalized values of 1.0 to 0.907 over a 5 min period under our whole-cell recording conditions (see Fig. 5A), with no detectable change in waveform. A straight line provided a very close fit to the rundown data (while of no theoretical significance), and was used to correct data points at various times to what would have been expected if rundown had not occurred (see Fig. 5B). Thus, with rundown correction, the magnitude of a drug-sensitive current at 5 min would be estimated as $[(I_{\text{drug}}/0.907) - I_{\text{control}}]$. This kind of correction was extended to estimates of the waveform of drug-sensitive currents, making the assumption that various components of the non-L-, non-N-type current undergo rundown at the same rate. This was supported empirically by the finding that the time course of the non-L-, non-N-type current did not change with rundown. Stated in another way, the experimentally determined rundown correction factor was the same at all points during a depolarizing test pulse.

Drug application. Drug-containing solutions were delivered by gravity feed from a linear array of glass capillaries, positioned near the recorded cell. In addition, the recording chamber was continuously perfused at $\sim 1 \text{ ml/min}$, with a peristaltic pump connected to a small antechamber. Bath solution was removed by continuous suction. The efficiency of the cell superfusion was verified by comparison of the blockade of Ba^{2+} current produced by 1 μM Cd^{2+} , applied either via the glass capillaries, or by slow bath perfusion. All drugs were stored as 100–5000 $\times$ stock solutions at -20°C . Cytochrome *c* (0.1 mg/ml) was included in all recording solutions to block nonspecific peptide binding sites; in control experiments, this agent was shown to have no effect on Ba^{2+} currents ($n = 6$).

Toxin preincubation experiments. The preincubation experiments (see Fig. 7) were carried out by incubating granule cells for the prescribed period in Tyrode solution at 24°C in the presence of cytochrome *c* and the relevant toxin. Sham-preincubated cells were subjected to the same procedure in the absence of toxin, but in the presence of cytochrome *c*. For any particular experiment, the sham- and toxin-preincubated cells were obtained from the same cell culture, and all recordings were made on the same day. To avoid any possibility of reversal of the effects of the toxin preincubation, the preincubating concentration of toxin was included in the recording solutions at all times.

In a similar fashion to toxin preincubation experiments, tests of effects of elevation of intracellular cyclic AMP (cAMP) were performed on two groups of cells. For the treated group, preincubation time was > 30 min in Tyrode solution supplemented with 1 mM 8-Br-cAMP and 10 μM forskolin, and the recording solution contained 0.1 mM 8-Br-cAMP and 2 μM forskolin. The control group were preincubated in Tyrode solution without additions.

Fine chemicals. Nimodipine was provided by Dr. A. Scriabine (Miles Pharmaceuticals, West Haven, CT), FPL 64176 by Dr. A. J. G. Baxter (Fisons PLC, Loughborough, UK), ω -CTx-MVIIIC by George Miljanich (Neurex Corp, Menlo Park, CA). Purified ω -Aga-IVA was a generous gift from Dr. M. E. Adams (UC Riverside). Synthetic ω -Aga-IVA was obtained from Peptides International (Louisville, KY). ω -CTx-GVIIA was obtained from Peninsula Laboratories (Belmont, CA) or as a gift from Neurex Corporation. 8-Br-cAMP was obtained from Boehringer USA and forskolin from Research Biochemicals Inc. (Natick, MA).

Results

Whole-cell recordings were made from more than 1000 cultured rat cerebellar granule cells maintained for 1–11 d *in vitro* (DIV). The cell bodies of granule cells were identified by their round or oval shape and their small size (4–6 μm) and low capacitance ($5.26 \pm 0.08 \text{ pF}$, mean $\pm \text{SEM}$). Cells with this morphology comprise $\sim 90\%$ of the neurons in our cerebellar cultures.

Figure 1. Properties of global Ba^{2+} currents in cerebellar granule cells. *A*, Activation of Ba^{2+} currents with increasingly large depolarizing steps ($V_t = 40$ mV to 0 mV, $V_h = -80$ mV). *B*, Peak Ba^{2+} current plotted against test potential ($V_h = -80$ mV). Peak current values were normalized by maximal peak current (I_{max} , almost always at $V_t = 0$ mV) for each cell. Data pooled from six cells. *C*, Dependence of Ba^{2+} current on holding potential. Holding potential (V_h) varied from -100 mV to -20 mV in 10 mV increments, and was maintained for 10 sec before the test pulse ($V_t = 0$ mV). Note that currents are larger and exhibit increasingly prominent decay at more negative holding potentials. *D*, Steady-state inactivation data pooled from four cells. Peak currents were normalized by the largest current observed in the holding potential range -100 to 20 mV. Standard errors smaller than the symbols are not shown. Smooth curve is a Boltzmann function, $h_\infty = [1 + \exp\{(V - V_{1/2})/k\}]^{-1}$, with $V_{1/2} = -52.4$ mV, $k = 13.8$ mV. *E*, Development of Ba^{2+} current amplitude with increasing periods *in vitro*. Mean amplitude of peak Ba^{2+} current recorded in the absence of any Ca^{2+} channel inhibitors (open symbols). Number of cells used for pooled data varied from 6 (8 DIV) to 35 (6 DIV). Also shown are mean values of total cell capacitance (filled symbols). Numbers of cells varied from 8 (4 DIV) to 132 (6 DIV). Some of the capacitance determinations were carried out in the presence of Ca^{2+} channel inhibitors.

Properties of global Ca^{2+} channel current

In recordings from cells bathed in Tyrode solution with 2 mM Ca^{2+} (data not shown), a depolarizing step from -80 mV to 0 mV evoked a rapidly activating and inactivating TTX-sensitive Na^+ current (102.7 ± 9.9 pA/pF), followed by a more prolonged Cd^{2+} -sensitive Ca^{2+} current (7.0 ± 2.2 pA/pF). Upon replacement of Tyrode solution with a sodium-free extracellular solution containing 5 mM Ba^{2+} and 1 μ M TTX, the Na^+ current was blocked, leaving an inward Ba^{2+} current with the characteristics illustrated in Figure 1. The Ba^{2+} current was entirely high-voltage activated (HVA), significant inward current not being observed until the depolarizing step reached -40 mV (Fig. 1*A,B*). The onset of the inward current became considerably faster as the depolarization was increased. Maximal inward current was observed at a test potential (V_t) of 0 mV. The I/V relationship (Fig. 1*B*, data pooled from six cells) shows a decrease in peak current with further depolarization. As expected for HVA channels, 20 μ M Cd^{2+} reversibly blocked $\sim 95\%$ of the Ba^{2+} current. Evidence for low-voltage activated (LVA) Ba^{2+} current such as that carried by T-type channels (Carbone and Lux, 1984; Now-

ycky et al., 1985; Bean, 1989; Hess, 1990) was found in $< 1\%$ of the cells studied.

The inactivation properties of the Ba^{2+} current were complex, as previously reported in mouse cerebellar granule neurons (Slesinger and Lansman, 1991a,b). The waveform of the Ba^{2+} current displayed considerably less decay during the test pulse as the holding potential (V_h) was varied from -100 mV towards 0 mV (Fig. 1*C*). The steady-state voltage dependence of inactivation (Fig. 1*D*) showed some deviation from a simple Boltzmann relationship (smooth curve). These findings might be explained if the total Ba^{2+} current were comprised of multiple components (but see also Slesinger and Lansman, 1991b).

The electrophysiological properties of the cells undergo developmental changes with the number of days *in vitro*. The inward Ba^{2+} current evoked by a step from -80 mV to 0 mV increased rapidly over the first 5 DIV while the total cell capacitance remained largely unchanged (Fig. 1*E*). A similar increase in the size of the sodium current was also found (data not shown). In the experiments described below we have focused on cells > 5 DIV.

Figure 2. Effects of L- and N-type channel inhibitors on granule cell Ba^{2+} current. **A**, Plot of peak Ba^{2+} current versus time for an experiment in which 10 μM nimodipine, 1 μM ω -CTx-GVIA, and 10 μM Cd^{2+} were sequentially applied to a 5 DIV granule neuron. **B**, Examples of Ba^{2+} currents (averages of three successive sweeps) taken from the same experiment. Sweeps taken at times indicated by *a–d* in **A**. **C**, Subtractive determinations of the nimodipine-sensitive (L-type) and ω -CTx-GVIA-sensitive (N-type) current waveforms.

Pharmacologically defined components of the total Ba^{2+} current

In order to determine the calcium current complement of these cells, we carried out a pharmacological dissection of the global Ba^{2+} current with agents known to block L- and N-type channels (Fig. 2). We first applied the dihydropyridine (DHP) compound nimodipine (10 μM) a selective blocker of the L-type calcium channels (Bean, 1989; Hess, 1990; Regan et al., 1991; McCarthy and TanPiengco, 1992). This compound promptly decreased the Ba^{2+} current by 10.1 ± 1.0 pA/pF or about 18%. This blockade could be rapidly reversed upon removal of the drug (data not shown). Next, in the continued presence of nimodipine, N-type channels were inhibited by means of ω -CTx-GVIA, a cone snail toxin with considerable selectivity for N-type channels in other neurons (Aosaki and Kasai, 1987; Plummer et al., 1989; Regan et al., 1991; Boland et al., 1994). Application of 1 μM ω -CTx-GVIA rapidly and irreversibly blocked an additional fraction of Ba^{2+} current, typically 20–25%. The remainder of the current (average, $60.8 \pm 3.2\%$) was reversibly inhibited by application of 10 μM Cd^{2+} , a generally effective blocker of HVA Ca^{2+} channels.

Tests of the effectiveness of channel blockade

Since more than half of the total Ba^{2+} current remains unblocked in the combined presence of nimodipine and ω -CTx-GVIA, it appears that channels other than L- or N-type make a substantial contribution to the global Ba^{2+} current in cerebellar granule neurons, as has been demonstrated in other neurons (Regan et al., 1991). However, the validity of this conclusion depends on the completeness of the pharmacological blockade by the various inhibitors. This was tested by experiments like those illustrated in Figure 3. The effectiveness of the blockade of L-type channels by 10 μM nimodipine was assessed by means of FPL 64176, a

powerful agonist on L-type channels (McKechnie et al., 1989), that acts at a locus independent of the DHP binding site (Zheng et al., 1991). As expected from its known actions in other systems (Zheng et al., 1991; Sather et al., 1993), FPL 64176 strongly affected Ca^{2+} channel currents in the granule neurons (Fig. 3A), greatly increasing the total current during the depolarization and prolonging the tail current upon membrane repolarization (trace b, right). The stimulatory actions of FPL 64176 were completely abolished by 10 μM nimodipine (Fig. 3A, bottom) ($n = 6$). This indicates that nimodipine has completely blocked the L-type current.

The effectiveness of 1 μM ω -CTx-GVIA in achieving a complete blockade of N-type channels was tested by comparing effects of this compound applied successively at 1 and 3 μM (Fig. 3B). The Ba^{2+} current in 3 μM ω -CTx-GVIA (c) showed no greater degree of block than that in 1 μM toxin (b) relative to the control record in the absence of toxin (a). As illustrated in Figure 3B (right), this was a consistent finding ($n = 6$ cells). In comparison to the average of currents sensitive to 1 μM ω -CTx-GVIA (a, b), the average of the additional toxin-sensitive current, blocked at the higher dose (b–c) was essentially zero. Thus, 1 μM ω -CTx-GVIA appears sufficient to yield a saturating block of current carried by N-type channels. This point was confirmed in another series of experiments that demonstrated that the rate of block but not its magnitude was increased as the ω -CTx-GVIA concentration was increased from 0.3 to 1 to 3 μM (data not shown). We conclude that block of L- and N-type Ca^{2+} channels is virtually complete under the experimental conditions of Figure 2. This brings to the fore the question of what type(s) of channel(s) carry the remaining current.

Non-L-, non-N-type current can be divided into ω -Aga-IVA-sensitive and -insensitive components

The large current (46.8 ± 1.61 pA/pF) that remains in the presence of nimodipine and ω -CTx-GVIA (Fig. 2B) is high-voltage

Figure 3. L- and N-type channels are completely blocked by nimodipine and ω -CTx-GVIA. **A**, Application of FPL 64176, a potent stimulator of L-type channels, as a test of the effectiveness of 10 μ M nimodipine in blocking L-type channels. FPL 64176 enhancement of Ba^{2+} current amplitude (upper graph) is completely prevented in the presence of 10 μ M nimodipine (lower graph). **Right**, representative traces (averages of three sweeps), taken at the points indicated on the graphs. FPL 64176 greatly prolongs tail current in the absence of nimodipine but has no effect in its presence. **B**, Effects of successive applications of 1 μ M and 3 μ M ω -CTx-GVIA. Nimodipine (10 μ M) present throughout. **Right**, average current blocked by 1 μ M ω -CTx-GVIA (*a-b*), compared with the current further blocked by 3 μ M ω -CTx-GVIA (*b-c*). Data pooled from six cells. Note that scatter in *a* and *b* largely reflects cell-to-cell variations in magnitude of ω -Aga-IVA-sensitive current (see Fig. 10 for indication of consistency in the degree of inactivation).

activated, completely and reversibly blocked by Cd^{2+} , and decays by $34.8 \pm 0.8\%$ during a 105 msec depolarization (data from 202 typical cells). These properties are reminiscent of the current supported by class A α_1 subunits expressed in *Xenopus* oocytes (Mori et al., 1991; Sather et al., 1993). While the α_{1A} current is not reduced by DHPs or ω -CTx-GVIA (Mori et al., 1991), it can be blocked by the funnel web spider toxin ω -Aga-IVA and the cone snail toxin ω -CTx-MVIIC (Sather et al., 1993). ω -Aga-IVA is a well-known and very potent blocker ($K_d \sim 1$ nM) of P-type channels in cerebellar Purkinje cells (Mintz et al., 1992a,b; Mintz and Bean, 1993; but cf. Sutton et al., 1994). Figure 4A illustrates the action of 1 μ M ω -Aga-IVA, applied in the presence of nimodipine and ω -CTx-GVIA. The spider toxin produced a promptly developing decrease in the non-L-type, non-N-type current. When analyzed by subtraction, as demonstrated in Figure 4C, the waveform of the current blocked by this dose of ω -Aga-IVA (*b-c*) was invariably decaying in nature.

The current remaining in the presence of micromolar doses of nimodipine, ω -CTx-GVIA and ω -Aga-IVA (Fig. 4B, trace *c*) has been designated as R-type (Ellinor et al., 1993; Zhang et al., 1993). This terminology carries no presumption that R-type current is generated by a single class of channels; this remains to be determined. As described previously, this residual component is also resistant to the cone snail toxin ω -CTx-MVIIC (see also p. 21). R-type current (*c*) decays more rapidly and more completely than the current blocked by ω -Aga-IVA (*b-c*). Further consideration of the characteristics of R-type current will be taken up below.

Concentration-dependent kinetics of ω -Aga-IVA action

The dose and time dependence of ω -Aga-IVA block were characterized in an extensive series of experiments (Fig. 5). Nimodipine (10 μ M) and ω -CTx-GVIA (1 μ M) were present throughout the recordings. Measurements of the peak current amplitude from individual cells were normalized to their value before exposure to ω -Aga-IVA, time aligned, and averaged. Figure 5A

compares the time-dependent decrease in Ba^{2+} current amplitude in cells exposed to 1.5 nM ω -Aga-IVA and in a control group subjected to a sham solution change but no agatoxin. The toxin block is small but significantly greater than rundown in the control group. Figure 5B shows the block at 1.5 nM toxin after correction for rundown as estimated from the control group (see Materials and Methods for details). Raising the toxin concentration over the range between 1.5 nM and 3 μ M greatly increased the rapidity and extent of block (Fig. 5B). At 3 μ M ω -Aga-IVA, block reached a steady state within 30 sec. A similar dose dependence of the rate of blockade was observed when individual cells were subjected to two successive applications of ω -Aga-IVA, at concentrations of 15 nM and then 1 μ M ($n = 6$, data not shown). The dose dependence of the rate and completeness of ω -Aga-IVA-induced block was not detectably different when shorter test pulses were applied at a higher frequency (0.4 or 2 Hz, e.g., Fig. 8A). Furthermore, the inhibitory effect of 300 nM ω -Aga-IVA we observed in the absence of any test pulses was indistinguishable from that seen with frequent test pulses to monitor the time course of the onset of block ($n = 5$, data not shown).

Figure 5C shows the maximal degree of block plotted against toxin concentration (filled symbols). Also included are data for the actions of 100 nM purified ω -Aga-IVA (open diamond) and for the toxin preincubation experiments described later in the paper (cross, open circle). A saturating degree of block is achieved at the highest agatoxin concentrations (1, 2, or 3 μ M ω -Aga-IVA), with an unblocked fraction of 0.234, which consists of R-type current (Fig. 4B). Allowing for this residuum, the dose dependence of the agatoxin block could be fitted reasonably well with a one-to-one binding relationship (dashed curve) with an IC_{50} of 28 nM. This value is considerably higher than the ~ 1 nM IC_{50} of P-type current in Purkinje neurons, but somewhat lower than the ~ 100 nM IC_{50} reported for currents generated by α_{1A} subunit expression in *Xenopus* oocytes (Sather et al., 1993).

Figure 4. Effect of ω -Aga-IVA on non-L-, non-N-type current. **A**, Time course of an experiment in which $1 \mu\text{M}$ ω -Aga-IVA was applied, following inhibition of L- and N-type current with $10 \mu\text{M}$ nimodipine and $1 \mu\text{M}$ ω -CTx-GVIA. **B**, Averages of three traces obtained at the times shown (a–c) in **A**. **C**, Subtractive determination of the waveform of the ω -Aga-IVA-sensitive current from the sweeps shown in **B**.

Concentration dependence of the waveform of the ω -Aga-IVA-sensitive current

The characteristics of the ω -Aga-IVA-sensitive current in cerebellar granule neurons were examined in greater detail in order to see if components with properties more reminiscent of the ω -Aga-IVA-sensitive currents previously described in Purkinje neurons and oocytes could be identified. These previously described currents differ markedly in their time course of inactivation during a depolarizing step. P-Type current in Purkinje neurons shows virtually no decay during depolarizations lasting up to several seconds (Regan, 1991; Llinás et al., 1992; Mintz et al., 1992a,b; Usowicz et al., 1993). In contrast, expression of the α_{1A} subunit in *Xenopus* oocytes can generate a current that inactivates substantially during the first 0.1 sec of a test depolarization (Sather et al., 1993).

Indeed, we found that a small fraction of the total ω -Aga-IVA-sensitive current in the granule neurons exhibited the non-decaying waveform of P-type current. As illustrated in Figure 6A, the waveform of the current blocked by ω -Aga-IVA depended critically on the dose of toxin. This is shown for data sets corrected for rundown, but it was also true if the rundown correction were omitted. As illustrated in the left-most record, the toxin-sensitive current derived by subtracting currents before and after a 5 min exposure to 1.5 nM ω -Aga-IVA was essentially nondecaying. The degree of decay markedly increased, however, as the toxin concentration was elevated into the micromolar range. This trend is documented quantitatively in Figure 6B, which shows pooled results for the degree of decay during the

105 msec depolarization. The fractional decay shows a graded increase over the range between 1.5 nM and $3 \mu\text{M}$, reaching a saturating level of about 30%. These results are not consistent with all-or-nothing block of either a single component of current, or of two components with different waveforms but equal toxin sensitivity. In either case, the fractional decay should be the same at all toxin doses. The observations are also inconsistent with what would be expected if ω -Aga-IVA were to act as an open channel blocker (e.g., like D600 on L-type Ca^{2+} channels). If this were the case, the degree of block would deepen during the test pulse, and the waveform of the toxin-sensitive current would show a time-dependent increase rather than a sustained or decaying time course as actually found.

Subdivision of the ω -Aga-IVA-sensitive current into two components

The data in Figure 6B was compared to a theoretical relationship based on the premise that the total ω -Aga-IVA-sensitive current is comprised of two components: one that is nondecaying and highly toxin sensitive (possibly like P-type current in cerebellar Purkinje cells), and another component, henceforth designated as “Q-type,” that is decaying and less susceptible to toxin blockade (perhaps like currents that can be generated by α_{1A} subunits in oocytes). Let us assume that the nondecaying P-type current contributes a fraction P , while the remainder $(1 - P)$ of the total ω -Aga-IVA-sensitive current is Q-type, and that ω -Aga-IVA blocks each of the components in a one-to-one manner (Mintz and Bean, 1993), without changing their individual time course of decay (Mintz et al., 1992a,b; Sather et al., 1993). Then the percentage of decay of the overall ω -Aga-IVA difference current will show the following dose dependence:

$$\% \text{ decay} = f/[1 + P(T + K_Q)/(1 - P)(T + K_P)] \quad (1)$$

where f is the fractional decay of the Q-type current during the 105 msec test pulse, T is the toxin concentration, K_P is the dissociation constant of the P-type current, and K_Q is the dissociation constant of the Q-type current (see figure legend for details). The solid curve in Figure 6B is Equation 1 with $f = 35.2\%$, $P = 0.244$, $K_P = 1 \text{ nM}$, and $K_Q = 89.1 \text{ nM}$. The theoretical curve provides a good fit. Thus, the data appears consistent with expectations if the ω -Aga-IVA-sensitive current were approximately one-fourth P-type and three-fourths Q-type.

The predicted toxin dependence of the percentage decay is strongly influenced by f , P , and K_Q . It is consistent with a range of values for K_P , ranging between about 1 and 3 nM. The theoretical relationship for $K_P = 5 \text{ nM}$ (dashed curve, Fig. 6B) predicts somewhat more decay than that observed at the lowest toxin concentrations.

The predictions of the two-component hypothesis can be compared with the dose-response relationship in Figure 5C. The solid curve, generated with the same set of parameter values as for the solid curve in Figure 6B, provides a good fit to the pooled data for the ω -Aga-IVA-sensitive current. The data points for block by 1.5 and 5 nM ω -Aga-IVA lie somewhat above the curve as would be expected if toxin block at these low concentrations had not achieved steady state at 5 min. Thus, the set of data in Figure 5C is consistent with the two component hypothesis (Fig. 6), although it is not sufficient by itself to exclude a one-component scheme.

Recovery from ω -Aga-IVA-induced block

Following removal of ω -Aga-IVA, block of P-type current in cerebellar Purkinje cells is slowly reversed, with a time constant

Figure 5. Dose dependence of the kinetics and magnitude of ω -Aga-IVA-induced block. **A**, Effect of application of 1.5 nM ω -Aga-IVA (filled triangles, $n = 8$ cells), compared with rundown determined in the absence of ω -Aga-IVA (open circles, $n = 8$). Nimodipine (10 μ M) and 1 μ M ω -CTx-GVIA were present throughout, and solution changes were made at $t = 0$ (switch to ω -Aga-IVA-containing solution or sham solution change). Data from individual cells were normalized to the period -40 to 0 sec and averaged. Error bars are not shown when smaller than symbol. The solid line represents the best straight line fit to the open symbols and provides an empirical measure of rundown. **B**, Dose-dependent blockade of non-L-, non-N-type Ca^{2+} channel currents by ω -Aga-IVA. The graph illustrates the time course of the actions of synthetic ω -Aga-IVA in the dose range 1.5 nM (least block, slowest rate) to 3000 nM (most block, fastest rate). The curves represent data (acquired at 0.1 Hz) from between 8 and 29 cells. Data from each cell was normalized to the pre- ω -Aga-IVA current level ($t = -40$ to 0 sec) before averaging. Pooled data at each ω -Aga-IVA concentration terminates at the conclusion of the ω -Aga-IVA application and has been corrected for the amount rundown demonstrated by the control cells shown in A (see Materials and Methods for details). Data for 100 nM ω -Aga-IVA was recorded from a particular batch of neurons several months after the results for all other doses had been obtained and is somewhat out of line with other data sets. This was due to a paucity in R-type current (current resistant to nimodipine, ω -CTx-GVIA, and ω -Aga-IVA), as verified by direct application of 600 nM toxin to the same batch of cells (data not shown). In a similar vein, small variations in the density and degree of decay of non-L-, non-N-type current were observed in comparisons between cell preparations separated by periods of several months. **C**, Dose-response relationship for ω -Aga-IVA block. Abscissa plots current remaining after exposure to ω -Aga-IVA. Synthetic ω -Aga-IVA unless otherwise stated. Filled circles represent isochronal data ($t = 5$ min) for applications of ω -Aga-IVA in the range between 1.5 and 15 nM, and steady-state data for ω -Aga-IVA applications in the range between 30 and 3000 nM. The cross shows the result of a preincubation experiment with 5 nM ω -Aga-IVA (Fig. 9A). The open circle depicts the result of a preincubation experiment with 100 nM ω -Aga-IVA (Fig. 9B,C). The open diamond is the average amount of block seen at steady state with 100 nM purified ω -Aga-IVA ($n = 8$). The data represented by the filled symbols are fit with two curves. The dashed line is a one-to-one binding curve with a K_d of 28 nM and a saturating level of block at 0.234. The residual toxin-insensitive component represents R-type current (Fig. 4A, trace c). The solid line represents the sum of two one-to-one binding curves, combining to give a saturating level of block at 0.235. The K_d and amplitude of the two binding sites are 1.0 nM and 19.5%, and 89.1 nM and 57.0%, respectively.

Figure 6. Dose dependence of the decay of currents sensitive to ω -Aga-IVA. **A**, Average waveforms of currents sensitive to ω -Aga-IVA over a range of concentrations. Each trace represents averages of normalized ω -Aga-IVA-sensitive currents evoked by 105 msec steps from -80 to 0 mV. Individual ω -Aga-IVA-sensitive currents were determined by subtracting the pre-Aga-IVA current from the (rundown-corrected) current in the presence of ω -Aga-IVA (see Materials and Methods). Shown are waveforms at 1.5 nM (8 cells), 15 nM (11 cells), 100 nM (28 cells), or 1000 nM (15 cells). Error bars shown at occasional points represent the standard error of the mean. **B**, The degree of decay of the drug-sensitive current (inset) plotted against the ω -Aga-IVA concentration. The data is fit with two curves of the form $\% \text{ decay} = f/[1 + P([\omega\text{-Aga-IVA}] + K_Q)/(1 - P)([\omega\text{-Aga-IVA}] + K_P)]$. Variables defined in text. The only fixed parameter in the fits was K_P , which was set equal to 1 nM (solid line) or 5 nM (dashed line). The parameters for the solid line are $K_P = 1$ nM, $K_Q = 89.1$ nM, $P = 0.244$, $f = 35.2\%$, and for the dashed line are, $K_P = 5$ nM, $K_Q = 122$ nM, $P = 0.256$, $f = 36.3\%$.

of ~ 50 min (Mintz and Bean, 1993). The slowness of the off-rate is consistent with a binding site with very high affinity for toxin. In contrast, a more rapid recovery from ω -Aga-IVA block ($\tau \sim 4$ – 8 min) was observed for α_{1A} expressed in *Xenopus* oocytes (Sather et al., 1993). In the cerebellar granule neurons, a considerable portion of the toxin-sensitive current was restored over the course of a ~ 7 min recovery period (Fig. 7A). This was corroborated in an extensive series of experiments characterizing the removal of the toxin effect following application of 0.1 , 1 , or 3 μM ω -Aga-IVA. In all cases, a partial recovery of inward Ba^{2+} current was seen upon withdrawal of toxin. Representative data are illustrated in Figure 7 for 1 μM ω -Aga-IVA. The recovery proceeded along a roughly exponential trajectory with a time constant of ~ 4 min and an estimated maximal amplitude of about 23% of the current originally blocked by ω -Aga-IVA. The time constant and amplitude of the recovery did not vary in any obvious way with the preceding level of ω -Aga-IVA. Pooling results obtained from fits to the average of each of the three groups gave a mean time constant of 439 ± 67 sec and a level of recovery of $29.2 \pm 5.5\%$ of the current originally blocked by ω -Aga-IVA. If recovery data from each group was rundown corrected before fitting (data not shown), the time con-

stant increased to 688 ± 201 sec ($n = 3$), and the magnitude of recovery rose to $56.7 \pm 10.6\%$ ($n = 3$). In a subset of cells ($n = 2$, open circles), a second application of ω -Aga-IVA caused a redevelopment of block with a very similar time course to that observed during the original initial toxin exposure. This confirmed that some of the channels initially blocked by ω -Aga-IVA had recovered from inhibition during washout.

We examined the averaged waveforms of the recovered ω -Aga-IVA-sensitive current to find out if they were decaying or nondecaying (Fig. 7B). The toxin-sensitive current in an individual cell was obtained by subtracting the current signal recorded just before the removal of toxin from the current at the end of recovery period. In comparison to current blocked by the original toxin application (block), which decayed by $19.5 \pm 1.4\%$, the recovered current (wash) decayed by $26.5 \pm 4.5\%$, a significantly greater degree ($p < 0.01$). Furthermore, the waveform of the current blocked by a second toxin application (reblock) also declined to a larger extent than the current originally blocked in the same cells (Fig. 7C, $n = 2$). Taken together, these results support the idea that the recovery from block and subsequent reblock involve the decaying Q-type current to a greater extent than the nondecaying P-type current.

Figure 7. Characterization of the reversal of ω -Aga-IVA-induced block. **A**, Reversal of 1 μ M ω -Aga-IVA-induced block following removal of ω -Aga-IVA (10 μ M nimodipine and 1 μ M ω -CTx-GVIA present throughout). Filled symbols represent pooled data from seven cells. The data from individual cells were resynchronized 20 sec prior to toxin removal (gaps between groups of data points are due to slight differences in the time of toxin exposure among the individual cells). Data has not been corrected for rundown to avoid any possible overemphasis of recovery. Open symbols represent data from two cells in which toxin was reapplied following washout. The data from these cells was renormalized (right ordinate) and shown on a scale to match up with the average Ba^{2+} current in the larger population of cells at the time immediately before the second application of ω -Aga-IVA. **B**, A comparison of the waveform of the current blocked by the initial ω -Aga-IVA application (a–b) with that recovered by prolonged toxin washout (d–c). Averaged waveforms from the cells represented by filled symbols in **A**. **C**, A comparison of the waveforms of the currents blocked by initial toxin exposure and by toxin reapplication (e–f). Data for the two cells depicted with open symbols in **A**.

Recovery from ω -Aga-IVA-induced block is hastened by strong depolarizing pulses

The application of a series of strong depolarizing pulses speeds recovery from ω -Aga-IVA block, both for P-type current in cerebellar Purkinje cells (Mintz et al., 1992a,b) and currents supported by α_{1A} expressed in *Xenopus* oocytes (Sather et al., 1993). This was also found for the ω -Aga-IVA-sensitive currents described here. Pooled results from 53 experiments showed a restitution of $58.1 \pm 2.8\%$ (range 21 to 105%) of the current originally blocked by ω -Aga-IVA (100–3000 nM), following a combination of toxin washout and strong depolarizing pulses. The percentage recovery of current showed little dependence on the previous dose of toxin or on the magnitude of the toxin-sensitive or -insensitive current (data not shown). In the absence of ω -Aga-IVA, application of strong pulses did not appreciably alter the nimodipine- and ω -CTx-GVIA-insensitive current ($n = 5$, data not shown).

Toxin block could be largely reversed even in the maintained presence of ω -Aga-IVA by application of a series of very strong depolarizing pulses (Fig. 8A). The time course of the subsequent redevelopment of the inhibitory effect of toxin agrees very well with original onset of block. Figure 8B illustrates the recovery from block after toxin has been removed. The effects of separate groups of depolarizing pulses are cumulative and greatly accelerate the recovery relative to that observed later in the same cell in the absence of strong depolarizing pulses. Even after recovery reaches a near steady state following removal of toxin, a delayed application of strong depolarizations promotes further reversal of toxin blockade (Fig. 8C). The waveform of the component that is restored by strong depolarizations (knockoff) is clearly

decaying, like the current recovered during the previous washoff period (washoff). Thus, Q-type current seems to contribute to both of these difference currents.

Preincubation experiments with ω -Aga-IVA

The results up to this point suggest that the bulk of the current blocked by ω -Aga-IVA in rat cerebellar granule neurons is significantly different in waveform and ω -Aga-IVA sensitivity from the P-type current as originally defined in rat cerebellar Purkinje neurons (Mintz et al., 1992a,b; Mintz and Bean, 1993). We have called this Q-type current. However, a smaller fraction (~25%) of the overall ω -Aga-IVA-blocked current appears to be non-decaying in waveform and quite sensitive to ω -Aga-IVA, like the P-type current of Purkinje neurons. It is reasonable to ask if the apparent difference between the magnitudes of Q-type and P-type currents is genuine, and not simply due to incomplete equilibration at low toxin concentrations over the span of a typical experiment. Accordingly, we performed additional experiments to test for ω -Aga-IVA block during preincubation periods lasting > 1 hr prior to the initiation of whole-cell recording.

Figure 9A compares the pooled behavior of two groups of granule neurons, one subjected to sham incubation (cytochrome *c* but no ω -Aga-IVA, the other incubated with 5 nM ω -Aga-IVA, a concentration well above the IC_{50} for blockade of P-type current in Purkinje neurons (~1 nM). Recording was carried out in standard solutions containing 10 μ M nimodipine and 1 μ M ω -CTx-GVIA, with ω -Aga-IVA absent or present just as in the respective preincubation solutions. There was no significant difference in the current magnitudes among the control and toxin-preincubated groups ($p > 0.1$). Thus, no evidence was obtained

Figure 8. Relief of ω -Aga-IVA block by large depolarizations. *A*, Temporary relief of ω -Aga-IVA block of Ca^{2+} channel current induced by a train of strong depolarizations (vertical lines), applied in the maintained presence of the toxin. Ba^{2+} current was assayed every 500 msec with 11.2 msec test pulses to 0 mV, and relief of toxin block was achieved by application at 1 Hz of 30 pulses of 50 msec duration to +150 mV. *B*, Use of strong depolarizing pulses to greatly accelerate the recovery from block following ω -Aga-IVA removal. Voltage-dependent relief of toxin block was followed by a reapplication of ω -Aga-IVA and a prolonged washout. In this experiment, test pulses were of the standard frequency (0.1 Hz), amplitude (to 0 mV), and duration (105 msec) described in Materials and Methods. Knockoff was achieved using five cycles of a standard protocol (arrows). Each arrow represents a train of 20 pulses (+100 mV for 50 msec) at 1 Hz; recovery was assessed with a solitary test pulse, applied 10 sec following each train. *C*, Relief of toxin block with strong depolarizing pulses following partial recovery from toxin block due to toxin washout. Standard test pulse protocol (0.1 Hz, 105 msec duration, 0 mV) was used to monitor partial recovery; strong pulse protocol identical to that in *B*. *D*, Averages of normalized waveforms of difference currents in experiments like that illustrated in *C*. The upper trace ("wash off") represents the average of the currents recovered during wash off of 1–3 μM ω -Aga-IVA (*b-a*). The lower trace ("knock off") represents the waveform of the current subsequently recovered by the application of strong depolarizing pulses (*c-b*) to a subset of these cells ($n = 10$).

for any major contribution of a current component with the nanomolar sensitivity of previously defined P-type channels. Presumably, the variability in current density from one cell to the next was too large to allow detection of a small degree of block like that seen with acute application of 5 nM toxin to individual cells (Fig. 5A).

Additional experiments were undertaken to test the effects of preincubation with 100 nM ω -Aga-IVA (Fig. 9B–D). The non-L-, non-N-type current averaged 66.5 ± 11.2 pA/pF in the con-

Figure 9. Preincubation experiments as a test for steady-state block by ω -Aga-IVA. *A*, Comparison of the magnitudes of non-L-type, non-N-type current in cells preincubated for 1–5 hr in Tyrode containing cytochrome *c* and 5 nM ω -Aga-IVA or in sham-preincubated cells (Tyrode plus cytochrome *c*). The symbols indicate data from individual cells contributing to the group mean and standard error as illustrated. *B*, Effect of a 0.5 to 2.2 hr preincubation with 100 nM ω -Aga-IVA on the magnitude of subsequently recorded Ba^{2+} currents. Pooled results for amplitudes of non-L-, non-N-type current (left), current blocked by acute application of 3 μM ω -Aga-IVA (center), and current spared by acute toxin application (right). *C*, Time course of the block of non-L-, non-N-type current induced by acute application of 3 μM ω -Aga-IVA. Cells were either sham preincubated (squares) or preincubated and maintained in 100 nM ω -Aga-IVA (circles). *D*, Averages of normalized waveforms of currents blocked by 3 μM ω -Aga-IVA in the two groups shown in *C*.

trol group of mock preincubated cells, and 28.4 ± 1.8 pA/pF in the neurons that had been preincubated for 0.5–2.2 hr with 100 nM ω -Aga-IVA, a significant difference ($p < 0.004$) (Fig. 9B). Both groups of cells were subsequently exposed to 3 μM ω -Aga-IVA to determine how much ω -Aga-IVA-sensitive current had been left unblocked by the prior preincubation. The acute reduction in current averaged 52.1 pA/pF in the control group and 16.4 pA/pF in the toxin-preincubated cells ($p < 0.01$; Fig. 9B) and proceeded with a similar time course in both cases (Fig. 9C). This confirms that the preincubation with toxin was effective in strongly reducing the ω -Aga-IVA-sensitive current, while leaving a substantial fraction unblocked (16.4/52.1 or ~31%). Following the acute application of 3 μM ω -Aga-IVA, the residual currents are not detectably different ($p > 0.35$), as expected with only R-type current left unblocked.

Relative contributions of the Ca^{2+} channel components

Figure 10 shows the contributions of the various Ca^{2+} channel components to the total whole-cell Ca^{2+} channel current, based

Figure 10. Comparison of pharmacologically dissected current components. *A*, Pharmacologically defined current components from individual cells were normalized to peak current, averaged, then scaled by their mean peak current density. Error bars are shown for individual time points. L-Type currents dissected with 10 μ M nimodipine ($n = 23$); N-type currents determined with 1 μ M ω -CTx-GVIA ($n = 25$); P-type + Q-type current ($n = 64$) obtained with 1–3 μ M ω -Aga-IVA following blockade of N- and L-type currents. R-Type current ($n = 26$) is estimated as the current that remains unblocked in the combined presence of 10 μ M nimodipine, 1 μ M ω -CTx-GVIA, 3 μ M ω -Aga-IVA. *B*, Pooled results for mean current density of the four current components shown in *A*. The number above each bar denotes the percentage contribution that the current makes to the global Ba^{2+} current. The bar denoting ω -Aga-IVA sensitivity is divided into fractions corresponding to the amount of P-type (shaded) and Q-type (unshaded) current as determined by the analysis illustrated in Figure 6. The asterisk in *A* marks the time course and estimated magnitude of the corresponding P-type current (waveform determined by application of 1.5 nM ω -Aga-IVA in Figure 6A, smoothed by Hanning filter).

on pooled data from a large number of cerebellar granule neurons. Individual components of Ba^{2+} current were defined by the pharmacological procedures described in the preceding sections. The mean time course of each component during a 105 msec depolarization was determined by normalizing the individual drug-sensitive current components (illustrated for individual examples in Figs. 2, 4) to an amplitude of unity before averaging

across the population of cells. The relative magnitudes of the components (*B*) were obtained by dividing the peak drug-sensitive current amplitude by cell capacitance before averaging across different cells. The average time course was scaled by the mean current density (*B*) to produce each of the current waveforms in (*A*).

As defined by 10 μ M nimodipine, L-type current averaged 10.1 ± 1.0 pA/pF ($n = 27$) and showed a nondecaying time course as expected from recordings in other systems. The ω -CTx-GVIA-sensitive N-type current displayed a mean amplitude of 13.8 ± 1.2 pA/pF ($n = 50$). The partially decaying waveform (decrease of $30.3 \pm 3.1\%$ from peak in 105 msec) is typical of N-type currents in other neurons (Kongsamut et al., 1989; Plummer et al., 1989; Regan et al., 1991). The ω -Aga-IVA-sensitive current averaged 32.5 ± 3.0 pA/pF, or 46% of the total current. This was further subdivided into noninactivating P-type current and inactivating Q-type current in a $\sim 1:3$ ratio, as determined by the analysis in Figure 6. The contribution of P-type current is indicated in Figure 10 by the asterisk (*A*) and the shaded bar (*B*). According to this analysis, P-type current was 8 pA/pF, or 11% of the total current, while the Q-type current was 24 pA/pF in amplitude or 35% of total current. The R-type current, the remaining component, accounts for 13.1 ± 1.0 pA/pF or about 19% of the total Ba^{2+} current.

Because P-type current appears to be essentially nondecaying, its relative contribution to the total current will increase progressively during a sustained depolarization. Currents recorded in the presence of nimodipine and ω -CTx-GVIA decayed to $16.9 \pm 1.6\%$ of their peak value at the end of a 2.1 sec pulse to 0 mV. This residual current is comparable in magnitude to the P-type current, which comprises about 17% of the total non-L-, non-N-type current.

Pharmacological analysis with ω -CTx-MVIIC

The cone snail toxin ω -CTx-MVIIC provides another pharmacological tool for separation of Ca^{2+} channel currents. It has been shown to block nondecaying P-type channel current in cerebellar Purkinje cells (Hillyard et al., 1992), and decaying currents supported by class A α_1 subunits expressed in *Xenopus* oocytes (Sather et al., 1993). N-Type current is also susceptible to block by ω -CTx-MVIIC (Swartz et al., 1993; Grantham et al., 1994; J.-F. Zhang, P. T. Ellinor, and R. W. Tsien, unpublished observations), although this is of no consequence if ω -CTx-GVIA is already present. Figure 11A illustrates the actions of ω -CTx-MVIIC on non-L-, non-N-type Ba^{2+} current in a typical cerebellar granule cell. Almost 70% of the Ca^{2+} channel current remained in the presence of 10 μ M nimodipine and 1 μ M ω -CTx-GVIA. Application of ω -CTx-MVIIC (5 μ M) caused a further slow reduction of this current ($\tau = 55$ sec), leaving a residual current amounting to less than one-fifth of the original current in the absence of inhibitors. On average, ω -CTx-MVIIC blocked $71 \pm 3\%$ ($n = 13$) of the nimodipine and ω -CTx-GVIA-insensitive Ba^{2+} current. The waveform of the ω -CTx-MVIIC-sensitive current (Fig. 11B) was very similar to that of the current sensitive to high doses of ω -Aga-IVA (that is, P-type + Q-type). Likewise, the waveform of the nimodipine and ω -CTx-GVIA-insensitive current remaining in the presence of ω -CTx-MVIIC was very similar to that remaining after 1–3 μ M ω -Aga-IVA. Furthermore, pooled results for the magnitudes of the ω -CTx-MVIIC-sensitive and -insensitive components (Fig. 11C) were in excellent agreement with the ω -Aga-IVA-sensitive and -insensitive components in Figure 10. Thus, both pharma-

Figure 11. Block of non-L-, non-N-type current by ω -CTX-MVIIC. **A**, Effect of 5 μM ω -CTX-MVIIC after prior block of L- and N-type currents by nimodipine and ω -CTX-GVIA. The current spared by ω -CTX-MVIIC was reversibly inhibited by application of 100 μM Ni^{2+} . **B**, Averages of normalized waveforms of currents blocked ($n = 13$) and spared ($n = 22$), by application of 5 μM ω -CTX-MVIIC. Current waveforms were scaled to mean peak current density (see **C**). All results obtained in the continuous presence of nimodipine and ω -CTX-GVIA. **C**, Mean peak current density of ω -CTX-MVIIC-sensitive (P + Q-type) and ω -CTX-MVIIC-insensitive (R-type) currents.

cological procedures are equivalent to the extent of isolating the R-type current.

Long applications of low doses of ω -CTX-MVIIC block the Q-type current

The α_{1A} subunit expressed in *Xenopus* oocytes is rather sensitive to ω -CTX-MVIIC ($\text{IC}_{50} < 150 \text{ nM}$), although the block is relatively slow ($t_{1/2} \sim 40 \text{ min}$ at 150 nM) (Sather et al., 1993). This raises the question of whether ω -CTX-MVIIC-mediated block of Q-type current is comparably potent. Slowly developing effects of toxin would be difficult to examine in granule cells because Ca^{2+} channel activity runs down during continuous whole-cell recording (e.g., Fig. 5A). We circumvented this problem by preincubating neurons with a lower concentration of toxin before commencement of whole-cell recording. The pharmacology of granule cells preincubated with 0.5 μM ω -CTX-MVIIC for 2–4 hr was compared with the behavior of cells subjected to a sham preincubation. Figure 12 illustrates the impact of the preincubation on the total Ba^{2+} current and the component blocked by acutely applied 5 μM ω -CTX-MVIIC. Representative records from individual cells (**A**) and pooled data from many cells (**B**) are shown. Preincubation with 0.5 μM ω -CTX-MVIIC lowers the total Ba^{2+} current to slightly more than half its control value. The block of Ba^{2+} current by acute application of 5 μM ω -CTX-MVIIC, prominent in the sham-incubated cell (*left traces*), is almost entirely occluded by the toxin preincubation (*right traces*). This is a very consistent finding, as indicated by the pooled data. In six cells, the acutely ω -CTX-MVIIC-sensitive current

Figure 12. Block of P- and Q-type currents by preincubation with low doses of ω -CTX-MVIIC. **A**, Current traces from exemplar cells showing effect of 2–4 hr preincubation with 500 nM ω -CTX-MVIIC on Ba^{2+} current in subsequent whole-cell recordings and its response to acute application of 5 μM ω -CTX-MVIIC. While ω -CTX-MVIIC produced a typical inhibition (30 pA/pF) in a sham preincubated cell (*left*), it was almost ineffective at reducing Ba^{2+} current following preincubation with 500 nM ω -CTX-MVIIC (*right*). **B**, Pooled data comparing eight sham preincubated cells and six cells preincubated with 500 nM ω -CTX-MVIIC. *Left*, The magnitude of global Ba^{2+} current following sham preincubation (*open bar*) or ω -CTX-MVIIC preincubation (*shaded bar*). *Right*, The mean reduction of inward current produced by acute application of 5 μM ω -CTX-MVIIC in the same cells. Note that nimodipine and ω -CTX-GVIA were not present.

was $29.3 \pm 3.8 \text{ pA/pF}$ in sham-treated cells, and only $3.0 \pm 0.5 \text{ pA/pF}$ in the cells preincubated with toxin. Thus, preincubation with 0.5 μM ω -CTX-MVIIC effectively occludes the effect of the 10-fold higher toxin concentration. Indeed, the waveform of the extra current blocked by the higher dose of ω -CTX-MVIIC was sustained rather than decaying, consistent with the idea that it arose from blockade of P-type channels that were spared by the toxin preincubation (Hillyard et al., 1992).

Comparison of fast gating characteristics of various current types

There are appreciable differences between P-, Q-, and R-type currents with respect to their rates of inactivation during a maintained depolarization, while Q- and N-type channels are similar. This leads to the question of whether the various channel types also differ with regard to activation and deactivation kinetics. We approached this question by characterizing the rapid gating properties of toxin-sensitive components, evoked by brief depolarizing test pulses and studied on a fast time scale. Depicted

Figure 13. Activation and deactivation kinetics of drug-sensitive currents. Examples of drug-sensitive current components recorded at high time resolution. Representative records illustrating $1 \mu\text{M}$ ω -CTX-GVIA-sensitive current recorded in the continuous presence of nimodipine (left); current sensitive to $2 \mu\text{M}$ ω -Aga-IVA, recorded in the presence of nimodipine and ω -CTX-GVIA (center); current sensitive to $50 \mu\text{M}$ Cd^{2+} , recorded in the presence of nimodipine and ω -CTX-GVIA (right). Each trace shows the difference between an average of six to eight predrug sweeps and an average of six to eight sweeps at steady-state block in an individual experiment. The current amplitudes at 0 mV ranged from 120 to 240 pA but are shown normalized to allow comparison of kinetics. Data points were acquired every 40 sec, but for clarity, only every other point (open circles) is shown. The smooth curves are single exponential fits of the rising phase and tail current with time constants as indicated. Fits to the rising phase were made to a set of data points of greater magnitude than that indicated by the arrow. All data points in the tail current were used to fit the exponential decay corresponding to current deactivation.

in Figure 13 are examples of (A) the ω -CTX-GVIA-sensitive current (N-type), (B) the ω -Aga-IVA-sensitive current (P-type + Q-type), and (C) the nimodipine- and ω -CTX-GVIA-insensitive, Cd^{2+} -sensitive current (P-, Q-, + R-type). The mean values obtained from a number of different cells for the exponential time constants for activation at 0 mV were $0.93 \pm 0.09 \text{ msec}$ (N), $1.15 \pm 0.05 \text{ msec}$ (P+Q), and $1.22 \pm 0.06 \text{ msec}$ (P+Q+R). The time constants of deactivation at -80 mV , obtained by fitting the tail current decay to a single exponential, were $259 \pm 40 \mu\text{sec}$ (N), $229 \pm 11 \mu\text{sec}$ (P+Q), and $240 \pm 21 \mu\text{sec}$ (P+Q+R). In no case was there an additional slow exponential component of significance, consistent with the absence of T-type current, which is known to deactivate much more slowly than HVA currents (Armstrong and Matteson, 1985). The values for onset and decay time constants are very similar to those reported for L-type, N-type, and non-L-, non-N-type currents in a variety of central neurons lacking T-type currents (Regan et al., 1992; Brown et al., 1993). Taken together, these results suggest that Q-type and R-type channels are similar to N-type channels with regard to activation-deactivation kinetics. This is not surprising, in light of the overall structural homology among non-L-type channels in regions of the α_1 subunit known to underlie voltage-dependent gating (Tsien et al., 1991), but it discourages the use of tail current analysis as a means of component dissection.

Tests of effects of elevation of intracellular cyclic AMP

Additional experiments were undertaken to determine if the relative magnitudes of Q-type and P-type currents were subject to modulatory control. In the extreme case, one might imagine some sort of reciprocal interconversion between these compo-

Figure 14. Maneuvers that raise intracellular cAMP levels fail to alter inactivation kinetics. **A**, Examples of currents from control (left) or forskolin and 8-bromo-cAMP-treated cells (right) (see Materials and Methods). In each case, the larger current (a,c) is the global Ca^{2+} channel current during a 105 msec test depolarization to 0 mV . The smaller currents (b,d) were recorded following application of $10 \mu\text{M}$ nimodipine and $1 \mu\text{M}$ ω -CTX-GVIA. **B**, Pooled data from control cells (filled circles, $n = 14$) and forskolin and 8-bromo-cAMP-treated cells (filled squares, $n = 15$). Global current density (left), density of nimodipine and ω -CTX-GVIA-insensitive current (center), and the degree of decay of the nimodipine and ω -CTX-GVIA-insensitive current (right). The symbols represent data from individual neurons yielding the values for mean and standard error indicated for each group.

nents. This question was prompted by consideration of the experiments of Fournier et al. (1993). They described Ba^{2+} currents in *Xenopus* oocytes injected with mRNA prepared from rat cerebellum that strongly resemble the ω -Aga-IVA-sensitive current we observed in granule cells. Their current was promptly activated at strong depolarizations and displayed $\sim 40\%$ decay during a 400 msec pulse to $+10 \text{ mV}$. It was not enhanced by Bay K 8644 or reduced by ω -CTX-GVIA, but it was strongly inhibited by crude venom from *Agenolopsis aperta*, the source of ω -Aga-IVA and FTX. The resemblance to currents in granule neurons is not surprising, given the great abundance of these cells in the cerebellum. Fournier et al. (1993) found that elevation of cyclic AMP increased the expressed currents by nearly twofold in amplitude, and caused a marked slowing of their inactivation (possible changes in the sensitivity to *A. aperta* venom were not reported). These observations led us to ask if similar changes occurred in the granule neurons and if they might represent a conversion of decaying Q-type current to nondecaying P-type current. Accordingly, we compared the behavior of groups of granule cells subjected to sham treatment or exposed to 8-bromo-cyclic AMP plus forskolin (Fig. 14). If anything, under conditions of elevated cyclic AMP the global current was marginally smaller ($p > 0.03$), and slightly less current remained after exposure to nimodipine and ω -CTX-GVIA ($p > 0.02$). Most importantly, the percentage decay during the 105 msec test depolarization was not different in control and under conditions that raise cyclic AMP (35% vs. 36%, $p > 0.6$). Thus, we failed to see any hint of an interconversion between Q-type and P-type channels under the conditions of these experiments.

Discussion

Five distinguishable components of Ca^{2+} channel current

This article presents evidence that Ca^{2+} channel currents in cerebellar granule neurons are comprised of at least five components, referred to as L-, N-, P-, Q-, and R-type currents. The individual components were distinguished on the basis of their pharmacological properties and, to some extent, their voltage-dependent gating. Kinetics were studied with Ba^{2+} as the charge carrier to avoid secondary complications due to Ca^{2+} -induced current changes. Each of the five currents may be categorized as high voltage-activated (HVA); there was little or no low-voltage activated current such as that carried by T-type Ca^{2+} channels (Fig. 1). This array of currents is more diverse than previously reported at the whole-cell level for cerebellar granule neurons or any other mammalian neuron described thus far. However, the diversity is not totally unexpected, given the multiplicity of Ca^{2+} channel α_1 subunits. Indeed, as we discuss below, the individual components correspond well with Ca^{2+} channel currents derived from the expression of various α_1 subunits.

Together, L- and N-type currents accounted for about one-third of the total Ca^{2+} channel current (Figs. 2, 4). Their contributions were assessed by use of nimodipine (10 μM) and $\omega\text{-CTx-GVIA}$ (1–3 μM), respectively. Control experiments indicated that the blockade was complete in both cases, as expected from results in other systems (Fig. 3). Thus, the remaining two-thirds of the total current must be attributed to other types of Ca^{2+} channels. After elimination of L- and N-type currents, application of micromolar concentrations of $\omega\text{-Aga-IVA}$ blocked almost half of the overall Ca^{2+} channel current (Fig. 4). The $\omega\text{-Aga-IVA}$ -sensitive current was of particular interest because it differed significantly from classical P-type current. As originally defined in cerebellar Purkinje cells (Llinás et al., 1989; Regan, 1991; Llinás et al., 1992), P-type current is virtually noninactivating with 1 sec depolarizations (Usowicz et al., 1992) and half-blocked at ~ 1 nM $\omega\text{-Aga-IVA}$ (Mintz et al., 1992a,b; Mintz and Bean, 1993). In contrast, in granule neurons, the current inhibited by $\omega\text{-Aga-IVA}$ was prominently inactivating (Figs. 4, 6) and was 50% blocked at a dose around 30 nM. These conclusions were based on extensive analysis of the time and dose dependence of $\omega\text{-Aga-IVA}$ block over a wide range of toxin concentrations (Fig. 5); 100 nM $\omega\text{-Aga-IVA}$ failed to block completely, even with toxin exposures lasting for > 1 hr (Fig. 9). At the very least, this indicates the diversity of currents that can be targeted by $\omega\text{-Aga-IVA}$ in various neurons.

P- and Q-type currents: two components of $\omega\text{-Aga-IVA}$ -sensitive current

One of the main points of this study was the subdivision of the $\omega\text{-Aga-IVA}$ -sensitive current in granule neurons into two distinct components. The key observation was that the waveform of the $\omega\text{-Aga-IVA}$ difference current was strongly dose dependent (Fig. 6). This finding runs contrary to expectations for a single class of channels whose contribution is simply scaled down by toxin block, as previously found for $\omega\text{-Aga-IVA}$ -sensitive currents (Mintz et al., 1992a,b; Mintz and Bean, 1993; Sather et al., 1993; Niidome et al., 1994). More detailed analysis revealed that a fraction of the total $\omega\text{-Aga-IVA}$ -sensitive current is a component with classical P-type properties, while the bulk of it arises from a novel current that we have called Q-type. The distinction between P-type and Q-type was based on differences in the waveforms of these components and their sensitivities to

$\omega\text{-Aga-IVA}$ (Fig. 6). The P-type component showed no decay during 0.1 sec depolarizing pulses and was potently blocked by $\omega\text{-Aga-IVA}$, with an IC_{50} in the low nanomolar range (Fig. 6). It recovered very slowly from $\omega\text{-Aga-IVA}$ block, as might be expected for a high affinity interaction (Fig. 7). In contrast, the Q-type current showed $\sim 35\%$ inactivation during 0.1 sec depolarizations, and was less sensitive to $\omega\text{-Aga-IVA}$, with an IC_{50} of ~ 90 nM (Fig. 6). Q-Type current displayed a relatively rapid, albeit partial, recovery upon removal of the $\omega\text{-Aga-IVA}$, with a time constant of ~ 5 min (Fig. 7). In certain other respects, the P-type and Q-type currents were quite similar. For example, in both cases $\omega\text{-Aga-IVA}$ block could be reversed by application of very strong depolarizations (Fig. 8). Both currents were completely blocked by $\omega\text{-CTx-MVIIC}$ at concentrations no more than 0.5 μM (Figs. 11, 12).

As described in this study, the P-type current in granule neurons exhibits properties very similar to those of the P-type current as originally defined in cerebellar Purkinje cells (Llinás et al., 1989, 1992; Mintz et al., 1992a,b; Usowicz et al., 1992). Likewise, the Q-type current in granule neurons strongly resembles currents that can be generated by expression of the α_{1A} subunit in *Xenopus* oocytes (Sather et al., 1993; Stea et al., 1994) or in stably transfected baby hamster kidney cells (Niidome et al., 1994).

These results have relevance to the functional contribution of α_{1A} subunits in neurons. Given the close resemblance between currents generated by α_{1A} in oocytes and Q-type current in cerebellar granule neurons, it seems likely that α_{1A} underlies the Q-type current. It is also possible that α_{1A} can generate P-type channels as well (Sather et al., 1993; Stea et al., 1994). Indeed, differences in gating kinetics between P- and Q-type might arise from alternative splicing or variability in the identity of the β subunit (Stea et al., 1994). In combination, Q-type and P-type currents are considerably larger than other types of Ca^{2+} currents in granule neurons. Since these neurons greatly outnumber all other neurons in the cerebellum (Ramón y Cajal, 1904), it is to be expected that α_{1A} transcripts should be so abundant in the cerebellum (Snutch et al., 1989).

The finding that Q-type and P-type channels can coexist in a single cell largely excludes the possibility that these distinct channel phenotypes arise solely from general differences in membrane or cellular environment. It will be interesting to learn more about the regulatory mechanisms that give the P-type current such prominence in cerebellar Purkinje cells while allowing Q-type current to outweigh the P-type current by a 3:1 ratio in cerebellar granule neurons. So far, we have found that the balance between these currents is not significantly affected by acutely raising intracellular cyclic AMP (Fig. 13).

R-type current

R-Type current contributed 15–20% of the total current in the granule neurons. It was isolated as the current remaining in the presence of nimodipine, $\omega\text{-CTx-GVIA}$, and either or both $\omega\text{-Aga-IVA}$ and $\omega\text{-CTx-MVIIC}$ (Figs. 4, 10, 11; Ellinor et al., 1993; Zhang et al., 1993). The robustness of the pharmacological distinction between Q-type current and R-type current is supported by various findings. First, prolonged preincubation with 0.5 μM $\omega\text{-CTx-MVIIC}$ occludes the effects of an acute application of 5 μM toxin (Fig. 12A), consistent with a saturating effect of the toxin, and presumably, complete inhibition of Q-type current. Second, close agreement exists between R-type currents determined with $\omega\text{-Aga-IVA}$ (Fig. 4) or $\omega\text{-CTx-MVIIC}$

Table 1. Pharmacological actions of the agents used in this study on various Ca²⁺ channel currents

	L (α_{1C}/α_{1D})	N (α_{1B})	P ($? \pm \alpha_{1A}$)	Q (α_{1A})	R (α_{1E})
Nimodipine	blocks 1,2	$\leftrightarrow$ 2,3,4	$\leftrightarrow$ 5,6	$\leftrightarrow$ 7,8	$\leftrightarrow$ 8,9,10
ω -CTx-GVIA	$\leftrightarrow$ 2,3,4,11	blocks 3,4,11,12	$\leftrightarrow$ 13	$\leftrightarrow$ 8,14	$\leftrightarrow$ 15,16
ω -Aga-IVA	$\leftrightarrow$ 17,18	$\leftrightarrow$ 17,18	blocks IC ₅₀ ~ 1 nM 8,17	blocks IC ₅₀ ~ 90 nM 7,8	$\leftrightarrow$ 8,9,10
ω -CTx-MVIIC	$\leftrightarrow$ 19	blocks 19,20,21	blocks 19	blocks 8	$\leftrightarrow$ 8,9,10,16

$\leftrightarrow$, no effect. Numbers indicate representative, not exhaustive, references to studies: 1, McCarthy and TanPiengco, 1992; 2, Regan et al., 1991; 3, Aosaki and Kasai, 1987; 4, Plummer et al., 1988; 5, Mintz et al., 1992a; 6, Usowicz et al., 1992; 7, Sather et al., 1993; 8, present study; 9, Ellinor et al., 1993; 10, Zhang et al., 1993; 11, Jones and Marks, 1989; 12, Williams et al., 1992b; 13, Mintz et al., 1992b; 14, Mori et al., 1991; 15, Soong et al., 1993; 16, Williams et al., 1994; 17, Mintz and Bean, 1993; 18, Brown et al., 1994; 19, Hillyard et al., 1992; 20, Grantham et al., 1994; 21, Swartz et al., 1993.

(Fig. 11). Third, the magnitudes of nimodipine-, ω -CTx-GVIA- and ω -CTx-MVIIC-insensitive currents were not significantly different in the absence or presence of ω -Aga-IVA (100 nM), further indicating that ω -Aga-IVA fails to block R-type current. Fourth, the properties of the pharmacologically dissected R-type current were quite distinct from L-, N-, P-, or Q-type channels with regard to rapidity of inactivation and sensitivity to block by Ni²⁺ (Figs. 10, 11; Zhang et al., 1993). Currents with R-type properties have also been found in AtT20 cells and both cultured and acutely dissociated hippocampal neurons (A. Randall, unpublished observations). In all of these systems, studies would greatly benefit from the availability of a selective blocker for R-type current. Since the R-type current has been isolated thus far by exclusion of other currents, one must be particularly alert to the possibility that it may be generated by more than one type of channel.

The R-type current shares a number of characteristics with the current generated by α_{1E} subunits expressed in *Xenopus* oocytes or HEK293 cells (Soong et al., 1993; Williams et al., 1994), which also lacks responsiveness to DHPs and ω -CTx-GVIA, while displaying sensitivity to both Ni²⁺ and Cd²⁺. There is a particularly striking resemblance with α_{1E} in HEK293 cells (Williams et al., 1994) with respect to voltage dependence, speed of activation and inactivation, and the relative sizes of currents carried by Ba²⁺ and Ca²⁺. In light of the overall similarities, and the presence of α_{1E} transcripts in rat cerebellar granule neurons (Soong et al., 1993), it seems reasonable to attribute the R-type current to α_{1E} .

Assessment of the validity of the pharmacological analysis

Table 1 provides an overview of the known pharmacological effects of nimodipine, ω -CTx-GVIA, ω -Aga-IVA, and ω -CTx-MVIIC. Based on work in other neurons and in expression systems for Ca²⁺ channel α_1 subunits, as well as our own experiments, these agents can be used to block some type or types of channel completely, while fully sparing others. Insofar as possible, we performed control experiments to check that this was the case (e.g., Fig. 3). As a further precaution, we arranged the order of application of inhibitors so as to apply the most thoroughly characterized and possibly most selective agents first.

Nimodipine was used to pharmacologically dissect the L-type current with the expectation that it would exert a negligible effect on N-, P-, Q-, and R-type channels. At 10 μ M, nimodipine appeared to block L-type channels completely (~15% of the global Ca²⁺ channel current); this was verified by use of the L-type stimulator FPL 64176 (Fig. 3A). Previous work has demonstrated the effectiveness of nimodipine in blocking neuronal L-type channels (Regan et al., 1991; McCarthy and TanPiengco, 1992), and at ≤ 10 μ M nimodipine or nifedipine, the absence of block of N-type channels (Aosaki and Kasai, 1987; Plummer et al., 1988; Regan et al., 1989; Brown et al., 1994), P-type channels (Mintz et al., 1992a,b; Usowicz et al., 1992), or channels derived from α_{1A} (Mori et al., 1991; Sather et al., 1993) or α_{1E} (Ellinor et al., 1993; Soong et al., 1993; Zhang et al., 1993). The possibility of nonselective nimodipine effects on P- or Q-type channels was not supported by preincubation experiments. These showed that prior blockade of P- and Q-type channels with ω -CTx-MVIIC failed to decrease the current subsequently inhibited by nimodipine (data not shown). The ineffectiveness of nimodipine in blocking channels other than L-type is further supported by the time course of the nimodipine-sensitive current, which shows slower activation than the other currents (Fig. 10), as expected from comparisons between the behavior of α_{1C} and other subunits in oocytes (e.g., Sather et al., 1993). Furthermore, the nimodipine-sensitive current contained no hint of the decaying waveforms of N-, Q-, and R-type channels. While we feel satisfied that nimodipine was effective in targeting L-type current, we cannot exclude the possibility that the L-type current may be further subdivided by more detailed analysis (see Forti and Pietrobon, 1993; Bossu et al., 1994).

ω -CTx-GVIA was used to block the N-type current (~20% of the total Ca²⁺ channel current). ω -CTx-GVIA produced maximal block of N-type current at 1–3 μ M (Fig. 3B), and we have no reason to think that it affected the other components. This toxin acts as an essentially irreversible inhibitor of N-type channels in other neurons (e.g., Aosaki and Kasai, 1987; Fox et al., 1987; Bean, 1989; Jones and Marks, 1989; Kongsamut et al., 1989; Plummer et al., 1989), and α_{1B} subunits expressed in human embryonic kidney cells (Williams et al., 1992b; Grantham et al., 1994). Previous studies in oocytes indicate that this toxin

lacks effectiveness in blocking P-type channels in Purkinje neurons (Mintz et al., 1992b) and currents derived from α_1 subunits other than α_{1B} , including α_{1A} (Mori et al., 1991; Stea et al., 1994) and α_{1E} (Soong et al., 1993; Williams et al., 1994).

In general, ω -Aga-IVA and ω -CTx-MVIIC were applied after L- and N-type currents had already been blocked. Our experiments support the idea that both of these agents exert effects on more than one type of channel (Table 1). ω -Aga-IVA block of Q-type as well as P-type current must be considered, particularly at ω -Aga-IVA concentrations ≥ 20 nM (Figs. 5, 6; Sather et al., 1993). ω -CTx-MVIIC is actually a relatively nonselective agent, capable of blocking N-type channels in central neurons (Swartz et al., 1993), α_{1B} channels expressed in HEK cells (Grantham et al., 1994), or *Xenopus* oocytes (Zhang, Ellinor, and Tsien, unpublished observations), P-type channels (Hillyard et al., 1992), as well as Q-type channels (Zhang et al., 1993; this paper). While ω -CTx-MVIIC cannot be used as a selective inhibitor of Q-type channels, it can be used in combination with nimodipine for isolation of R-type current (Fig. 11).

Comparisons with other studies in cerebellar granule neurons

Single channel recordings in rat cerebellar granule cells have revealed three novel classes of Ca^{2+} channels (G1, G2, G3) that are different in pharmacological or biophysical properties from T-, L-, N-, or P-type channels (Forti et al., 1994). This provides an independent indication of the degree of Ca^{2+} channel diversity in these neurons. It seems likely that at least some of the single channel activity is related to Q-type and R-type currents, but further work will be needed to establish their exact relationship (see also Bossu et al., 1994).

Q- and R-type currents have not been reported in earlier whole-cell recordings from granule cells. While early studies found little or no Ca^{2+} channel current (Hirano et al., 1986; Hockberger et al., 1987; Cull-Candy et al., 1989), more recent work points to a dominance of L-type currents (Huston et al., 1990; De Waard et al., 1991; Marchetti et al., 1991; Slesinger and Lansman, 1991a,b; Haws et al., 1993). Current sensitive to ω -CTx-GVIA has also been reported (De Waard et al., 1991; Haws et al., 1993; Sutton et al., 1994). There are various explanations for why Q- and R-type currents may not have been detected previously. The recent availability of potent toxins has certainly been helpful in bringing their contributions into focus. Furthermore, the expression of Q-type current in granule cells may vary with culture conditions. The extracellular K^+ concentration may be an important factor. In previous electrophysiological studies, culture media containing elevated K^+ (usually 25 mM, Gallo et al., 1987) were used to enhance survival of granule cells, while in our experiments, the granule neurons were maintained in media containing 5 mM extracellular K^+ . While this did decrease long-term neuronal survival (Randall et al., 1993b), it also greatly enhanced the degree of synaptic connectivity, in line with previous comparisons between granule cells grown in high and low K^+ with respect to levels of enzymes, neurotransmitter receptors, neurotransmitter release and, most importantly, depolarization-induced Ca^{2+} entry (Moran and Patel 1989; Peng et al., 1991; Pearson et al., 1992; Zhao and Peng, 1993). It is also noteworthy that sympathetic neurons cultured in elevated K^+ have been found deficient in their expression of non-L-type channels (Murrell and Tolkovsky, 1993). An intriguing implication of these results is that the expression of Q-type (and possibly N-type) channels might go hand in hand with the formation of functional synapses.

Possible function of Q-type current

The Q-type current was the largest single component in the granule neurons, raising questions about its possible functional role. R. D. Randall et al. (1994) have studied transmission between granule neurons and large Purkinje-like neurons in cerebellar cultures, and have found that a combination of nickel, nifedipine and ω -CTx-GVIA failed to abolish evoked EPSCs. At 100 nM, ω -Aga-IVA largely blocked transmission. This would be consistent with participation of P-type channels, or with involvement of Q-type channels, given the steep power-law dependence of neurosecretion on Ca^{2+} entry (Wheeler et al., 1994; Wu and Saggau, 1994). As additional information, we have found that ω -CTx-MVIIC greatly depresses action potential-dependent synaptic transmission in granule cell cultures (Randall et al., 1993). Further studies with lower doses of ω -Aga-IVA would be helpful.

Acutely dissociated hippocampal neurons from area CA3 display a substantial amount of nimodipine-, ω -CTx-GVIA-, and ω -Aga-IVA-resistant current with the waveform and ω -CTx-MVIIC sensitivity expected for Q-type current (Swartz et al., 1993; A. Randall and R. W. Tsien, unpublished observations). Furthermore, glutamatergic synaptic transmission between CA3 and CA1 neurons is dominated by a Ca^{2+} entry pathway whose pharmacology matches that of the Q-type current (Wheeler et al., 1994). The Q-type current of hippocampal neurons and cerebellar granule cells is modulated by activation of certain neurotransmitter receptors, including GABA_B and metabotropic glutamate receptors (Randall et al., 1993c). Thus, modulation of Q-type channels may contribute to the overall regulation of synaptic strength produced by activation of presynaptic signaling systems (Wheeler et al., 1994).

The Q-type current also appears to be present in non-neuronal excitable cells. AtT20 cells, a cell line derived from mouse anterior pituitary, display a current component with a time course and pharmacological profile similar to Q-type current in granule neurons (Randall and Tsien, 1994). In all of these systems, new-found knowledge of the diversity of Ca^{2+} channel subtypes leads immediately to questions about their function and regulation. It will be interesting to find out to what extent the various subtypes may be differentially modulated (see Wu and Saggau, 1994).

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