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Dihydropyridine- and ω-Conotoxin–Sensitive Ca^{2+} Currents in Cerebellar Neurons: Persistent Block of L-Type Channels by a Pertussis Toxin–Sensitive G-Protein

Christine M. Haws,[a] Paul A. Slesinger,[b] and Jeffry B. Lansman

Department of Pharmacology, School of Medicine, University of California, San Francisco, California 94143-0450

The inhibition of high-threshold Ca^{2+} channel currents by activated G-proteins was studied in mouse cerebellar granule cells making use of the hydrolysis-resistant GTP analog GTP-γ-S. When individual granule cells were internally dialyzed with GTP-γ-S, the high-threshold Ca^{2+} current decreased to ~20% of its initial value within ~2 min. The GTP-γ-S–resistant current was reduced further by the subsequent addition of either ω-conotoxin or dihydropyridine antagonist, indicating that both N- and L-type Ca^{2+} channels carried the remaining current. Continuous exposure to the dihydropyridine agonist +(*S*)-202-791 caused a rapid increase in the GTP-γ-S–resistant current. The L-type current evoked by the agonist subsequently decreased to the level observed prior to adding the drug following a time course similar to the initial inhibition of the total high-threshold current. A second application of the drug at a later time failed to increase the current a second time, indicating a persistent blockade of the agonist-evoked L-current. Pretreating cells with pertussis toxin prevented the initial inhibition of the total whole-cell Ca^{2+} channel current as well as the subsequent inhibition of the agonist-evoked L-current. The results show that a pertussis toxin–sensitive G-protein produces a persistent inhibition of L-type Ca^{2+} channels in these central neurons.

[Key words: Ca^{2+} channel, dihydropyridine, ω-conotoxin, G protein, granule cell, cerebellum]

There is considerable evidence for the existence of different types of Ca^{2+} channels in neurons (reviewed by Miller, 1987; Tsien et al., 1988; Bean, 1989; Hess, 1990). Because different types of Ca^{2+} channels may underlie specific functions, such as the release of neurotransmitters, the pharmacological characterization of neuronal Ca^{2+} currents is important for establishing functional roles for different Ca^{2+} channel subtypes. Two classes of substances have proved useful in characterizing high-threshold Ca^{2+} currents in neurons. The 1,4-dihydropyridines selectively inhibit a class of L-type Ca^{2+} channels (Fox et al., 1987). ω-Conotoxin (fraction GVIA), a 27 amino acid toxin from the marine snail *Conus geographus,* was originally found by McCleskey et al. (1987) to block neuronal N-type Ca^{2+} current as well as dihydropyridine-sensitive L-type Ca^{2+} channel currents. Subsequent work, however, indicated that ω-conotoxin did not block neuronal L-type Ca^{2+} channels and that it could be considered a specific inhibitor of N-type Ca^{2+} channels, which are insensitive to dihydropyridines (Aosaki and Kasai, 1989; Plummer et al., 1989). Similar pharmacologic components of high-threshold Ca^{2+} current have been found in mammalian central neurons (Mogul and Fox, 1991; O'Dell and Alger, 1991; Regan et al., 1991).

A wide variety of neurotransmitters and hormones inhibit neuronal Ca^{2+} currents, and it is thought that this is a general mechanism for controlling the release of neurotransmitters at presynaptic terminals (reviewed by Carbone and Swandulla, 1989). The inhibitory effects of neurotransmitters on Ca^{2+} currents in peripheral neurons are mediated by specific GTP-binding proteins that are thought to act either directly by an interaction with the channel protein or through the generation of diffusible second messengers (reviewed by Rosenthal et al., 1988; Dolphin, 1990). In light of the diversity of Ca^{2+} channel subtypes in mammalian neurons, it is important to determine the types of Ca^{2+} channels that may be targets for inhibitory G-proteins. Neurotransmitter receptors coupled to G-proteins have been shown to inhibit N-type Ca^{2+} channels in peripheral neurons (Hirning et al., 1988; Kasai and Aosaki, 1989; Lipscombe et al., 1989; Plummer et al., 1991), although one report suggests an additional inhibitory effect of G-protein–coupled neurotransmitters on L-type Ca^{2+} channels (Bley and Tsien, 1990). There is less information on the types of Ca^{2+} channels that are inhibited by G-proteins in central neurons.

Dissociated cultures of cerebellar cells are enriched in granule cells and have been useful for studying ionic conductances and synaptic transmission (Hirano et al., 1986; Carboni and Wojcki, 1987; Hockberger et al., 1987; Huck and Lux, 1987; Zhu and Chuang, 1987; Cull-Candy et al., 1988) as well as the cellular mechanisms of development of central neurons (reviewed in Burgoyne and Cambray-Deakin, 1988). We have shown previously that cerebellar granule cells possess only high-threshold Ca^{2+} currents that have a large component carried by dihydropyridine-sensitive Ca^{2+} channels, making these cells a suitable preparation for studying L-type Ca^{2+} channels in central neurons (Slesinger and Lansman, 1991a,b). Here we analyze in more detail the dihydropyridine- and ω-conotoxin-sensitive components of the high-threshold Ca^{2+} current in cerebellar granule cells. Making use of the hydrolysis-resistant GTP analog GTP-γ-S, we asked whether G-protein–mediated channel inhibition involves a specific pharmacologic component of Ca^{2+} current. Our results provide evidence showing that a pertussis toxin–

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Correspondence should be addressed to Dr J. B. Lansman at the above address.

[a] Present address: Department of Psychology, Stanford University, Stanford, CA.

[b] Present address: Howard Hughes Medical Institute, UCSF, San Francisco, CA.

sensitive G-protein produces a persistent inhibition of L-type Ca^{2+} channels.

Some of these results have appeared as an abstract (Haws and Lansman, 1990).

Materials and Methods

Tissue culture. Cultures of dissociated cerebellar cells were prepared following a modification of the procedure of Hatten and Sidman (1978) as described previously (Slesinger and Lansman, 1991a). Cerebellar cells were plated at a density of 0.05–0.5 × 10^6 cells/ml on glass coverslips precoated with 25 μg/ml poly-L-lysine (Sigma). Cultures were kept in a humidified atmosphere of 5% CO_2, 95% air at 37°C in a medium containing Minimal Essential Medium (MEM) with Earle's basal salts and 2 mM glutamine. MEM was supplemented with 10% horse serum, 25 mM KCl, and 0.06–0.2% glucose. Recordings were made within 2–3 d of plating unless otherwise indicated.

Solutions. The intracellular solution (Cs-aspartate) contained (in mM) 120 aspartate, 1 $MgCl_2$, 10 HEPES, 15 glucose, 20 NaCl, and was titrated with CsOH to pH 7.4. To minimize Ca^{2+} current washout, 5 mM bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA; Molecular Probes), 2 mM magnesium-5'-adenosine triphosphate (Mg-ATP, Sigma), and 1 mM adenosine 3':5'-cyclic-monophosphate (cAMP, Sigma) were included in the intracellular solution (Byerly and Yazejian, 1986; Chad and Eckert, 1986; Armstrong and Eckert, 1987). Ca^{2+} channel currents in granule cells rapidly wash out if cAMP is not included in the intracellular solution (Slesinger and Lansman, 1991a). The osmolarity was adjusted to ~330 mOsm by adding glucose. The bathing medium contained 20 mM $BaCl_2$ (Aldrich Chemicals) adjusted to ~330 mOsm with tetraethylammonium chloride (TEA-Cl; Sigma), 10 mM HEPES, and 10 mM glucose. The pH was adjusted to 7.4 by adding TEA-OH. Dihydropyridines were prepared as 10 mM stock solutions in 100% ethanol and kept at −20°C in a light-tight container. Fresh test solutions were prepared before each experiment.

Dihydropyridines (Sandoz) and ω-conotoxin (Peninsula Labs) were applied by perfusing the cell locally with a second electrode having a tip diameter of ~10 μm. The perfusion electrode was lowered into the bath and positioned 10–50 μm from the granule cell after the establishment of a stable recording. Perfusing cells with a bathing solution containing 0.1% ethanol or the bathing solution alone had no effect on the recorded Ca^{2+} channel currents. In experiments with pertussis toxin, cultures of cerebellar cells were preincubated with 200 ng/ml of the toxin (List Biological Laboratories) for 6–8 hr. Treating cells with pertussis toxin had no obvious effects on either the amplitude or the kinetics of the Ca^{2+} currents recorded from cerebellar granule cells when compared with recordings from cells from parallel cultures.

Electrophysiological methods. Individual coverslips were placed in a recording chamber mounted on a Nikon phase-contrast microscope. Whole-cell currents were recorded following the method described by Hamill et al. (1981). Patch electrodes were made from Boralex hematocrit glass (Rochester Scientific) and had resistances of 3–7 MΩ with Cs-Asp in the pipette and 20 $BaCl_2$/TEA in the bath. Current signals were recorded with a List EPC-7 amplifier with a 0.5 GΩ feedback resistor in the head stage. Voltage command pulses were generated and current responses simultaneously digitized and stored on a laboratory computer (PDP 11/73, Indec Systems, Sunnyvale, CA). Currents were filtered with an eight-pole, low-pass Bessel filter at 2–20 kHz (−3 dB) and sampled at 5–100 kHz. The potential between pipette solution and bath was zeroed before making a seal.

The membrane seal resistance ranged from 5 to 50 GΩ. Membrane capacitance and ~10–70% of the series resistance were compensated electronically after a whole-cell recording was established. Single test pulses were delivered at 0.1–0.2 Hz. All current traces shown were corrected for linear leak and capacity current by subtracting the averaged current response to four voltage steps of one-fourth the amplitude of the test pulse after appropriate scaling. All recordings were done at room temperature (21–24°C).

Results

Pharmacological components of high-threshold Ca^{2+} current in cerebellar granule cells

Figure 1 shows the effects of ω-conotoxin and dihydropyridines on the high-threshold Ca^{2+} channel current in cerebellar granule cells. Ca^{2+} channel current was isolated by using Cs^+ as the major intracellular cation in the patch electrode to block currents through K^+ channels and a Na^+-free bathing solution containing 20 mM Ba^{2+} as the charge carrier through Ca^{2+} channels. The high-threshold Ca^{2+} currents shown in Figure 1*A*, were evoked by a voltage step to +20 mV from a holding potential of −90 mV. Figure 1*Ai* shows the ω-conotoxin–sensitive component of the high-threshold current. Exposing the cell to 15 μM ω-conotoxin reduced the high-threshold current by roughly half. In a number of recordings from different cells, 15 μM ω-conotoxin inhibited the high-threshold current by 45 ± 18% of control (mean ± SD, n = 11). While low concentrations of the toxin produced less inhibition, higher concentrations did not reduce the current further. Figure 1*Aii* shows the high-threshold Ca^{2+} channel current before and after applying the toxin with the current records scaled so the peak amplitudes are the same. There was little difference in the time course of the current before and after adding the toxin. Thus, the ω-conotoxin–sensitive channels do not produce a component of current with very different kinetics from those channels not blocked by the toxin.

Figure 1*Ai* also shows that the current not blocked by the toxin is carried in large part by L-type channels. Figure 1*Ai* shows that dihydropyridine agonist +(*S*)-202-791 produced a large increase in the Ca^{2+} current, consistent with an enhancement of current flowing through L-type Ca^{2+} channels (Hess et al., 1984). Figure 1*B* shows the relationship between the peak current and the potential of the test pulse, first in the presence of ω-conotoxin, and then after applying the dihydropyridine agonist. The results shown in Figure 1, *A* and *B*, indicate the high-threshold Ca^{2+} current is carried by both N- and L-type Ca^{2+} channels as distinguished pharmacologically by the sequential application of ω-conotoxin and a specific L-channel agonist.

Figure 1, *C* and *D*, confirms the existence of an L component of the high-threshold Ca^{2+} channel current in cerebellar granule cells. Figure 1*Ci* shows a recording of the current evoked by a test pulse to +20 mV before and after adding 1 μM of the dihydropyridine antagonist −(*R*)-202-791. The large reduction in the current indicates a substantial fraction is carried by L-type channels. Figure 1*Cii* shows the currents recorded before and after adding the antagonist scaled so that the peak amplitudes are the same. Note that the antagonist enhanced the rate of decay, consistent with the possibility that the antagonist blocks the open channel (Cohen and McCarthy, 1987; cf. Hess et al., 1984). Similar results were obtained with nitrendipine, which blocked ~33% of the high-threshold current in two experiments (data not shown). Figure 1*D* shows the relationship between the peak current and the test pulse potential measured before and after adding the antagonist. The reduction of the current in the presence of −(*R*)-202-791 occurred over a wide range of test pulse potentials. Figure 2 summarizes the results from a number of recordings from different cells in which the dihydropyridine- and ω-conotoxin-sensitive components of the high-threshold Ca^{2+} channel current were measured. In cerebellar granule cells, dihydropyridine antagonists and ω-conotoxin blocked roughly equivalent fractions of high-threshold Ca^{2+} channel current. We estimate from the results shown in Figure 2 that only 5–10% of the high-threshold current would be carried by channels resistant to both drugs (Regan et al., 1991). Micromolar concentrations (20 μM) of the multivalent metal ions Cd^{2+} and Gd^{3+} inhibited virtually all the high-threshold current, suggesting that

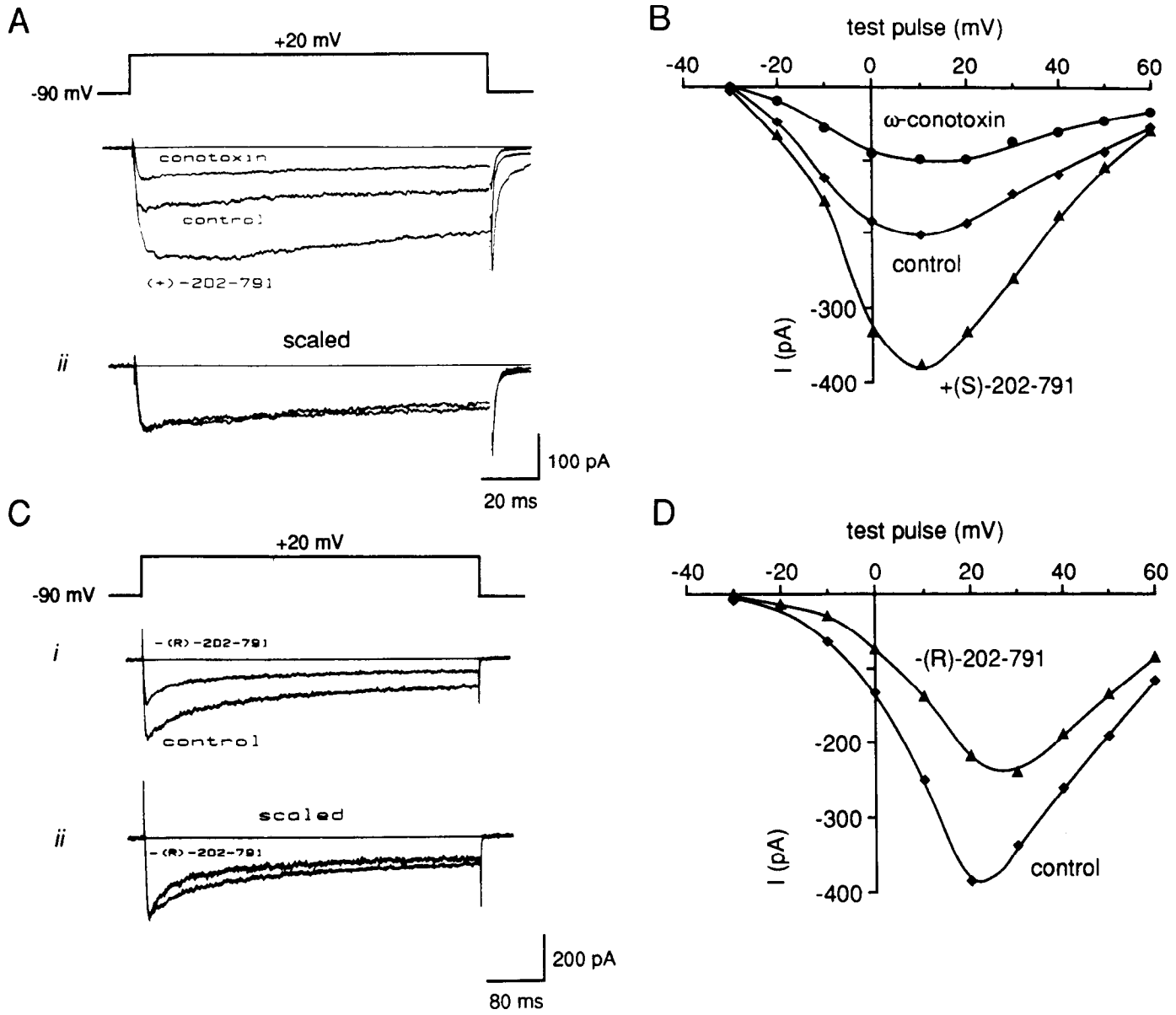

Figure 1. Pharmacological components of high-threshold Ca^{2+} channel current in mouse cerebellar granule cells. *A*, N and L components revealed by the application of ω-conotoxin and dihydropyridine agonist. *i*, Whole-cell currents carried by Ba^{2+} evoked by a voltage step from a holding potential of −90 mV to a test potential of +20 mV lasting 160 msec. After obtaining the control response, 15 μM ω-conotoxin was applied to the cell via a second perfusion pipette. The reduction of current shows the contribution of ω-conotoxin–sensitive N-type channels. After applying conotoxin, 1 μM of the dihydropyridine agonist +(*S*)-202-791 was added to the bath, which produced a large increase in the whole-cell current. *ii* shows the currents before (control) and after applying ω-conotoxin scaled by a constant so that the peak amplitudes are the same. *B*, Peak current–voltage relations for the control current and after exposing the cell to ω-conotoxin and then +(*S*)-202-791. *C*, The L component of current shown by application of the dihydropyridine antagonist −(*R*)-202-791 (*i*). Same voltage step protocol as *A* except that the test pulse was 500 msec in duration. *ii*, The current in the absence and presence of dihydropyridine antagonist scaled so that the peak amplitudes are the same. Note the faster decay of the whole-cell current in the presence of antagonist. *D*, Peak current–voltage relations for the current in the absence and presence of −(*R*)-202-791.

the N and L components of Ca^{2+} channel current are not easily separated by using metal ion blockers.

Pharmacologic components of the GTP-γ-S–resistant high-threshold Ca^{2+} channel current

We examined the inhibitory actions of the hydrolysis-resistant analog of GTP, GTP-γ-S, on the high-threshold Ca^{2+} channel current in granule cells under conditions that would minimize the contribution of second messenger pathways such as those involving cAMP and intracellular Ca^{2+}. Figure 3 compares Ca^{2+} channel currents evoked by a test pulse to +30 mV in a cell perfused with either GTP or GTP-γ-S. In cells perfused with GTP, the current recorded in response to voltage steps more positive than +30 mV turned on rapidly and then decayed to a non-zero level by the end of the 160 msec test pulse. Including 500 μM GTP-γ-S in the patch electrode filling solution instead of GTP altered the time course of the inward Ca^{2+} channel current. Ca^{2+} channel current turned on more slowly and the initial decay characteristic of current elicited with strong depolarizations was reduced. Figure 4 shows the time to peak

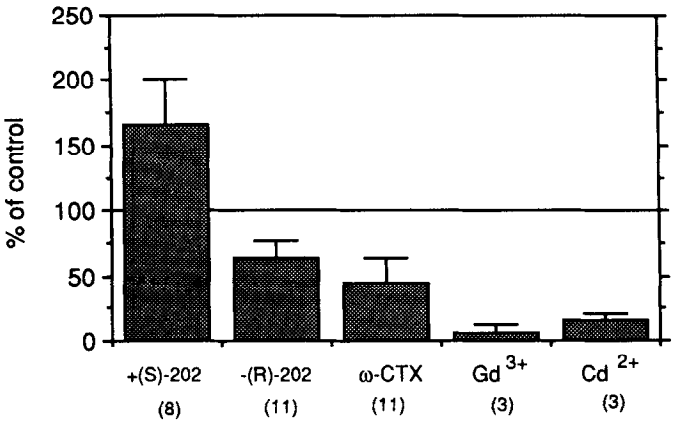

Figure 2. Pharmacological sensitivity of the high-threshold Ca^{2+} current in cerebellar granule cells expressed as a percentage of the control current. *Numbers at the bottom* indicate the number of recordings. Error bars indicate the SD from the mean. *ω-CTX*, ω-conotoxin.

current measured in cells perfused with either GTP (open circles) or GTP-γ-S (solid circles) plotted as a function of the potential of the test pulse. GTP-γ-S slows channel opening, consistent with its effects on Ca^{2+} currents in peripheral neurons (Dolphin and Scott, 1987).

Figure 3 also shows the effects of including either 500 μM GTP or 500 μM GTP-γ-S in the standard intracellular solution on the amplitude of the peak Ca^{2+} channel current measured as a function of time after establishing a whole-cell recording. Peak current measured shortly after the start of the whole-cell recording, ranged from ~50 to 300 pA. The numbered records in Figure 3*A* indicate the time after the start of the whole-cell recording with the first record obtained immediately after breaking into the cell and the third record ~2 min later. Note that the peak current measured in response to the test pulse in recordings with GTP in the electrode increased with time, while the current recorded with GTP-γ-S in the electrode decreased substantially with time. Figure 3*B* shows the peak current from seven cells elicited by test pulses to +30 mV applied once every 10 sec. The currents are normalized to their peak amplitudes. With GTP in the electrode (open circles), the current evoked in response to a fixed test pulse increased during the first ~1 min of the recording, but did not change appreciably over the nest ~3–4 min. With GTP-γ-S in the electrode (solid symbols), the amplitude of the current gradually declined to a steady level within 2–3 min after the start of the whole-cell recording to ~20% of its initial value.

The inhibition of Ca^{2+} channel current with GTP-γ-S in the patch electrode occurred even though the electrode filling solution contained 5–10 mM BAPTA to maintain intracellular free Ca^{2+} in the nanomolar range (Neher, 1986). In three other experiments, the electrode filling solution contained the catalytic subunit of cAMP-dependent protein kinase or 100 μM leupeptin, an inhibitor of calcium-dependent proteases (cf. Chad et al., 1987). Neither of these substances altered the inhibition of current produced by GTP-γ-S, suggesting it involves a mechanism independent of cAMP-dependent phosphorylation or leupeptin-sensitive proteases.

We examined the effects of ω-conotoxin and dihydropyridine antagonists on the GTP-γ-S–resistant current to discern the extent to which GTP-γ-S inhibits a specific pharmacologic component of the high-threshold Ca^{2+} channel current. Figure 5 shows that ω-conotoxin blocked roughly half of the remaining

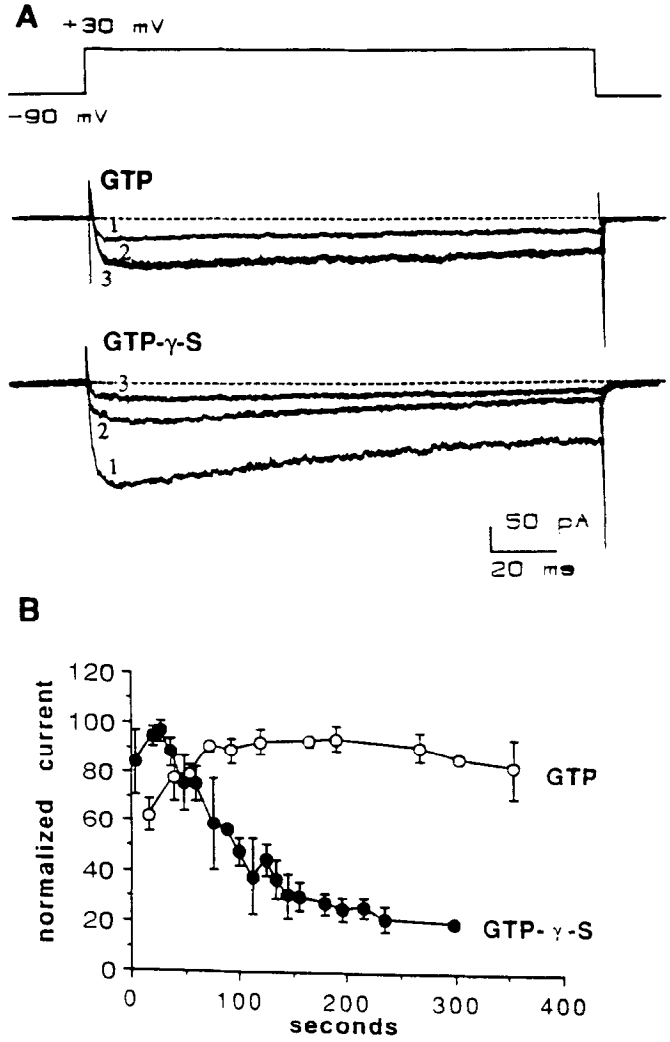

Figure 3. Effects of internal GTP-γ-S on the high-threshold Ca^{2+} channel current in cerebellar granule cells. *A*, Records showing the effects on the high-threshold current of perfusing granule cells with either GTP (*top*) or GTP-γ-S (*bottom*). Currents were elicited by voltage steps to +30 mV from a holding potential of −90 mV. Pulse duration was 160 msec. *B*, Time course of the change in the amplitude of high-threshold Ca^{2+} current after the start of the whole-cell recording in cells perfused with GTP (n = 6; *open symbols*) or GTP-γ-S (n = 7; *solid symbols*). Currents were recorded during a step depolarization to +30 mV from a holding potential of −90 mV. Currents sampled at 5 kHz and filtered at 2 kHz.

current in cells dialyzed with GTP-γ-S, suggesting a large residual N component. Figure 5 also shows that the dihydropyridine antagonist reduced the GTP-γ-S–resistant current, as expected if a significant fraction of the resistant current is carried by L-type channels. These results indicate that the GTP-γ-S–resistant current has both N and L components. The subsequent experiments focused on the contribution of L-type Ca^{2+} channels to the GTP-γ-S–resistant current as revealed by the application of the specific L-channel agonist +(*S*)-202-791.

Persistent block of L-type Ca^{2+} channel current in the presence of GTP-γ-S

Figure 6*A* shows the L component of high-threshold Ca^{2+} channel current evoked by exposure to dihydropyridine agonist in a granule cell dialyzed with GTP-γ-S. When there was no further reduction in the amplitude of the Ca^{2+} channel current, the cell

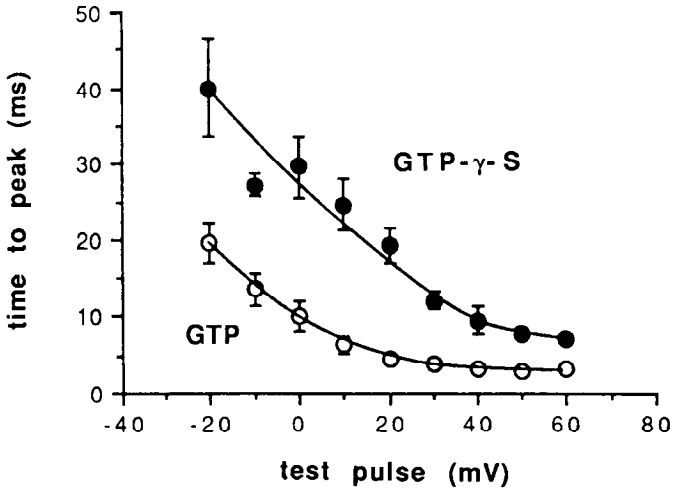

Figure 4. Effect of internal GTP (*open circles*) or GTP-γ-S (*solid circles*) on the time to peak current evoked in response to voltage steps to various membrane potentials. Error bars indicate the SD from the mean.

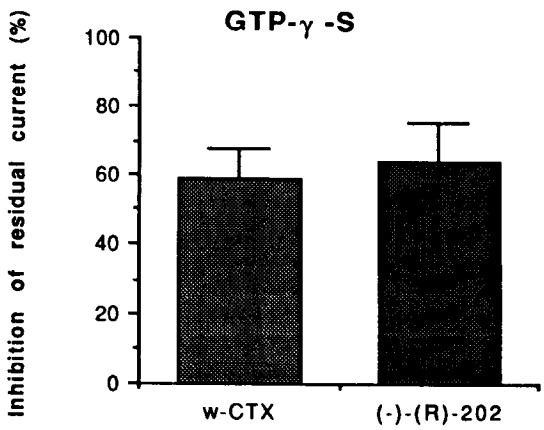

Figure 5. Effect of ω-conotoxin (*ω-CTX*) or the dihydropyridine antagonist −(*R*)-202-791 on the GTP-γ-S–resistant current. After the high-threshold Ca^{2+} channel current had declined to a steady level during intracellular dialysis with GTP-γ-S, either 15 μM ω-conotoxin or 1 μM −(*R*)-202-791 was applied via a second perfusion pipette.

was exposed to the dihydropyridine agonist +(*S*)-202-791 by positioning the tip of a second glass pipette containing ~1 μM of the drug close to the cell. Figure 6*B* shows the change in the amplitude in the current evoked in response to a test pulse of fixed amplitude before and after adding dihydropyridine agonist. Before adding the agonist, the current decreased to ~50% of its initial value. Adding the agonist (indicated by the bar in the figure) promptly increased the amplitude of the inward current. The increase in the Ca^{2+} channel current produced by the agonist, however, was not maintained during the time of exposure to the drug, but decreased within 2 min to the level observed prior to adding the agonist. Moreover, there was no discernible change in the time course of the current before adding the agonist (Fig. 6*A*, trace 1) and after the agonist-activated L-current had decreased to a steady level (trace 3). Note that the slow tail current at the end of the voltage step, which is characteristic of the response to dihydropyridine agonist (Hess et al., 1984), is also inhibited during the time of exposure to the agonist. In the presence of GTP-γ-S, evidently, the agonist evokes an L-current that becomes completely inhibited in the presence of the drug.

Figure 6*B* shows that exposing the cell to the dihydropyridine agonist a second time after the current returned to its initial amplitude failed to increase the current again. The solid bars in Figure 6*B* indicate the time during which the perfusion pipette containing +(*S*)-202-791 was positioned near the cell. In several other experiments, we were unable to produce a second response to dihydropyridine agonist. Because the interval between the application of the first and second dose was less than ~2–3 min in these experiments, we cannot exclude the possibility that the response to dihydropyridine agonist desensitizes in cells perfused with GTP-γ-S but recovers with a very slow time course. The results, nevertheless, indicate that GTP-γ-S produces a persistent inhibition of the L-type Ca^{2+} channel current evoked by the dihydropyridine agonist. Similar results were obtained in recordings from four other cells.

One possible explanation for the transient activation of the L-type current by agonist in the presence of GTP-γ-S is that the agonist activates a population of silent channels that subsequently become susceptible to the inhibitory effects of GTP-γ-S. This interpretation is suggested by the observation that the rate of inhibition of the agonist-activated current is similar to the rate of inhibition produced initially by GTP-γ-S. Figure 6*B* (inset) shows the average time course of the decrease of the agonist-activated current in the presence of GTP-γ-S (data from five experiments). Both the inhibition of the initial current after the start of the whole-cell recording (dotted line, from Fig. 3) and inhibition of the agonist-activated component following the addition of +(*S*)-202-791 decreased within ~1 min. The rate of the initial inhibition of the current does not appear to reflect the time course of GTP-γ-S diffusion into the cell, since the small enhancement of current with GTP in the patch electrode occurs much more rapidly. A similar small, rapid increase in current also occurs with GTP-γ-S in the electrode, consistent with the fast phase of the change in current amplitude arising from the diffusional exchange of the cell interior with the electrode filling solution.

To test for the involvement of a G-protein–coupled mechanism, cultures of cerebellar neurons were pretreated with pertussis toxin, which ADP ribosylates the α-subunit of specific G-proteins, preventing coupling to the physiological receptor (Casey and Gilman, 1988). Figure 7 shows that pretreating granule cells with pertussis toxin prevented the inhibition of Ca^{2+} channel current by intracellularly applied GTP-γ-S, as well as the subsequent inhibition of the L-type current evoked by dihydropyridine agonist. In cells pretreated with pertussis toxin, the current gradually increased, rather than decreased, after the start of the whole-cell recording to a steady level within about a minute. The records in Figure 7*A* show the current before (trace 1) and shortly after (trace 2) exposing the cell to +(*S*-202-791. Exposing the cell to the agonist produced a large increase in the current, which had the characteristic slowly deactivating tail current at the end of the pulse. Moreover, the response was maintained during the continuous exposure of the cell to the agonist (trace 3). As shown in Figure 7*B*, removing the perfusion pipette from the vicinity of the cell caused the amplitude of the current to fall back toward control levels. A second exposure to the agonist also elicited a maintained response, although in this experiment it was somewhat smaller than the first response. In granule cells pretreated with pertussis toxin, the agonist produced a maintained increase in the high-

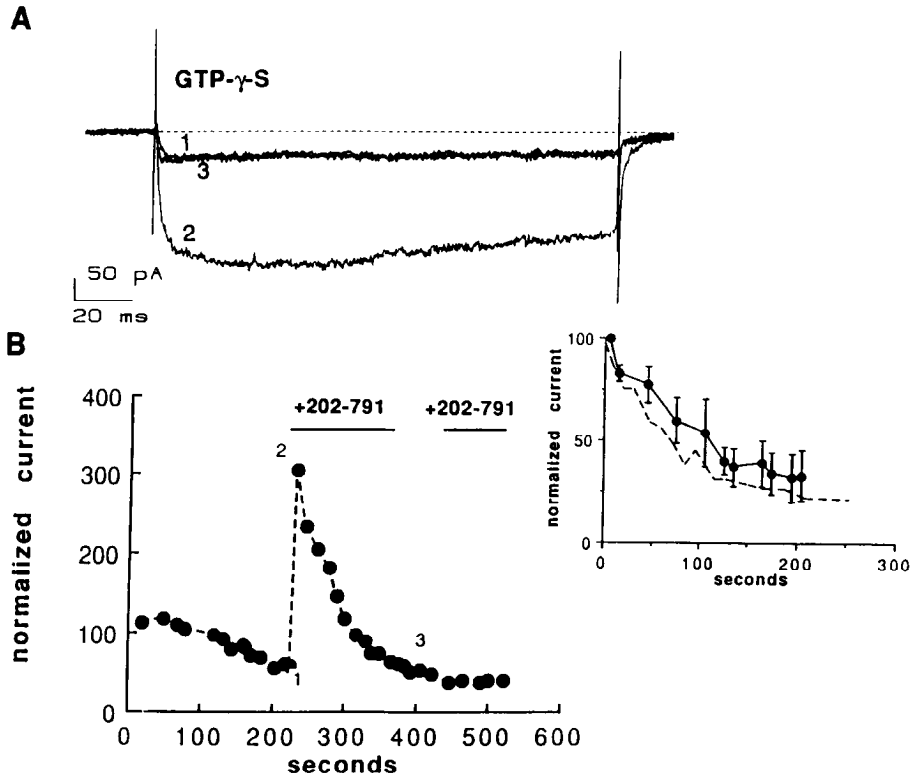

Figure 6. Effect of the dihydropyridine agonist +(*S*)-202-791 on high-threshold Ca^{2+} channel current in cells perfused with 500 μM GTP-γ-S showing the specific and persistent blockade of the dihydropyridine agonist–activated L-current. *A*, Ca^{2+} channel currents recorded before (*1*), shortly after (*2*), and ~3 min after (*3*) adding the agonist from a second perfusion pipette. *B*, Plot of peak current produced in response to a fixed test pulse recorded at different times after the start of a whole-cell recording. The *bars* indicate the time during which the second pipette containing 1 μM +(*S*)-202-791 was positioned next to the cell. *Inset*, Average time course of the decrease of the agonist-activated current in cells perfused with 500 μM GTP-γ-S. *Dotted line* represents the average time course for the inhibition of the initial high-threshold current after the start of the whole-cell recording.

threshold Ca^{2+} current in five out of five cells tested. The results show that pretreating cells with pertussis toxin blocks the inhibition of the total high-threshold Ca^{2+} current produced by intracellular GTP-γ-S as first described by Dolphin and Scott (1987), but also blocks the inhibition of the L-current evoked by exposure to dihydropyridine agonist.

Although the contribution of cAMP-dependent mechanisms should have been minimal in these experiments, activation of other protein kinases, such as protein kinase C (Rane and Dunlap, 1986) or calmodulin-dependent kinase may be part of divergent pathways involved in the inhibition of Ca^{2+} channel current. In preliminary experiments, we tested the effects of H7, a relatively nonselective kinase inhibitor, and the specific protein kinase C inhibitor PKCI19-31 (Rane et al., 1989) and found little effect on the inhibition of the current evoked by the agonist in the presence of GTP-γ-S in three cells.

Changes in the effects of GTP-γ-S on high-threshold Ca^{2+} currents during granule cell development in tissue culture

During the course of these experiments, we noticed that the decrease in the amplitude of the high-threshold Ca^{2+} channel current in the presence of intracellular GTP-γ-S depended on the length of time the cells had been in culture. As cells developed in tissue culture, there was a progressive lessening of the ability of GTP-γ-S to inhibit Ca^{2+} channel current. Cells that had been in culture longer than 7 d were virtually resistant to inhibition by GTP-γ-S. Figure 8*A* shows an example of the high-threshold Ca^{2+} current from a cell that had been in culture 7 d recorded at various times after the start of the whole-cell recording with GTP-γ-S in the electrode. The numbers next to each trace correspond to the time after the start of the recording (Fig. 8*B*). The current increased, rather than decreased, during the first minute of the recording and its amplitude remained constant over the next ~5 min. Figure 8*B* compares the time course of the amplitude of the current evoked in response to a fixed test pulse in cells perfused with GTP-γ-S that had been in culture 7–9 d (solid symbols) or 1–2 d (dotted line).

The failure of GTP-γ-S to inhibit Ca^{2+} current in cells that had been in culture for more than ~7 d appeared to involve a gradual change in the sensitivity of the high-threshold current to GTP-γ-S with time in culture. We cannot rule out the possibility that this change involved a change in the relative numbers of granule cells in our cultures and recordings from older cells were not from granule cells. Several observations, however, suggest this is not the case. Cell capacitance remained relatively constant up to ~14 d *in vitro*, showing no abrupt change as might be expected for a single type of cell. In support of this, the older cells from which recordings were made had the characteristic bipolar morphology of granule cells, which differs from other cerebellar cells that generally have much larger and more complex dendrites.

If older cells are resistant to inhibition during internal perfusion with GTP-γ-S, we asked whether the response to dihydropyridine agonist was also resistant to inhibition. Figure 9*A* shows examples of currents recorded before and after adding the agonist. Figure 9*B* shows the amplitude of peak Ca^{2+} channel current elicited by a voltage step from −90 mV to +30 mV as a function of time after the start of the whole-cell recording. As expected for older cells, there is no inhibition of current during the first minute and a half of the recording. Dihydropyridine agonist was applied from the perfusion pipette during the period marked by the bar. In contrast to the response to agonist in younger cells, which was completely inhibited in the constant presence of drug within a minute (Fig. 6), the response to agonist was maintained for more than 5 min. In addition to being insensitive to inhibition to GTP-γ-S, the response to dihydro-

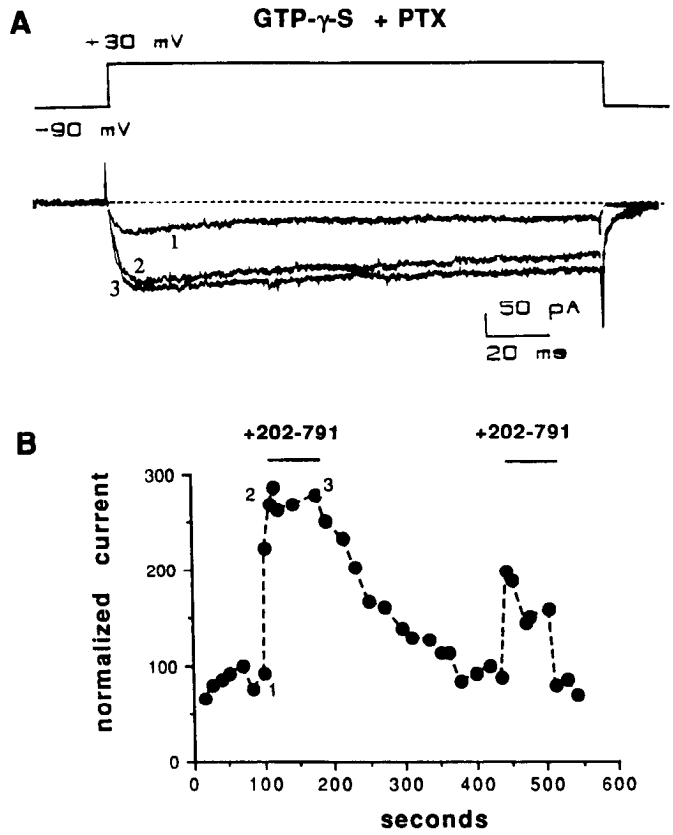

Figure 7. Pretreating cells with pertussis toxin (200 ng/ml) for 6–8 hr inhibits the persistent blockade of L-type current. *A,* Records of high-threshold Ca^{2+} channel current measured before (*1*), shortly after (*2*), and ~1.5 min after adding +(*S*)-202-791. *B,* Time course in the change in the peak amplitude of the current. *Bars* indicate the time during which the cell was exposed to the dihydropyridine agonist. Note that the response to agonist is maintained during constant exposure to the agonist and a second, but smaller, response is elicited by a second exposure to the agonist.

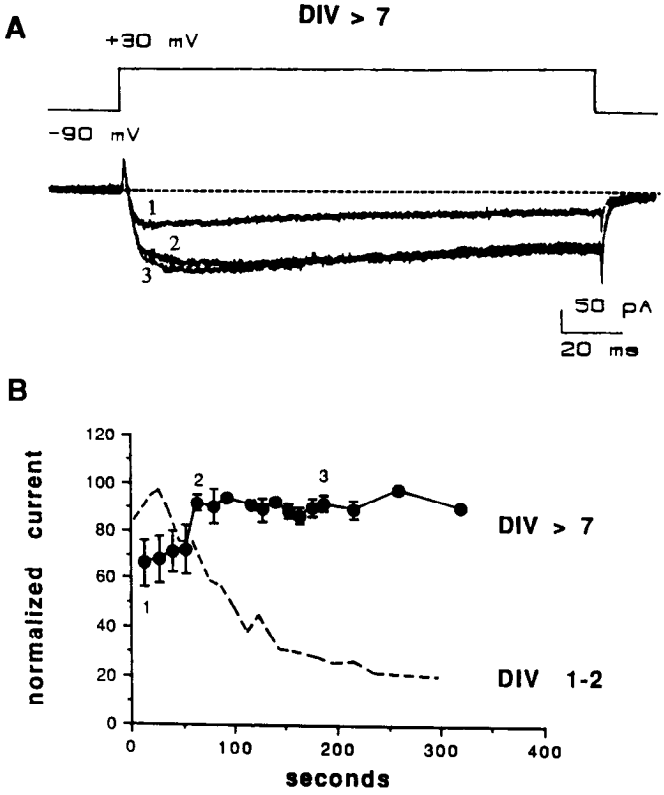

Figure 8. Changes during time in culture in the response to intracellular perfusion with GTP-γ-S. *A,* Records of Ca^{2+} channel current from cells that had been in culture 7 d. The patch electrode contained 500 μM GTP-γ-S. The records shown were measured ~15 sec (*1*), 1.5 min (*2*), and 3.5 min (*3*) after establishing the whole-cell recording. Note that the currents increased to a steady level ~1.5 min with no indication of inhibition. *B,* The amplitude of the current produced in response to a fixed test pulse at various times after the whole-cell recording was established. Currents are normalized to maximum current. *Solid symbols* represent cells that had been in culture 7–9 d; *dotted line,* the response of cells that had been in culture 1–2 d. Currents sampled at 5 kHz and filtered at 2 kHz.

pyridine agonist in older cells was not as large as that produced by the agonist in younger cells. A 1 μM concentration of +(*S*)-202-791 increased the Ca^{2+} current 371 ± 57% in young cells (n = 4) and 240 ± 48% in older cells (n = 7).

Discussion

The results presented in this article show that a pertussis toxin–sensitive G-protein inhibits L-type Ca^{2+} channels in mouse cerebellar granule cells. This conclusion is based on the complete and apparently persistent block of a dihydropyridine agonist–evoked Ca^{2+} current in cells internally perfused with GTP-γ-S. Moreover, the initial inhibition of the total high-threshold Ca^{2+} current by GTP-γ-S, as well as the subsequent persistent inhibition of the agonist-evoked L-current, are both prevented by pretreating the cells with pertussis toxin. Thus, L-type Ca^{2+} channels may be important regulatory targets for inhibitory G-proteins in central neurons. Although these experiments were designed to minimize the contribution of second messenger–activated pathways, we cannot exclude the possibility that at least part of the inhibition of L-type Ca^{2+} channels involves such mechanism, in addition to a direct inhibitory action of an activated G-protein.

Plummer et al. (1989) reported that GTP-γ-S reduced the high-threshold current in rat superior cervical ganglion cells primarily by inhibiting N-type channels, since ω-conotoxin and GTP-γ-S inhibited roughly the same fraction of high-threshold current. We find, however, that while GTP-γ-S inhibits ~80% of the high-threshold Ca^{2+} current in granule cells, ω-conotoxin inhibits only ~45% of the current in the absence of GTP-γ-S. Thus, GTP-γ-S inhibits substantially more high-threshold Ca^{2+} channel current in cerebellar granule cells than can be accounted for by the block of ω-conotoxin–sensitive Ca^{2+} channels. The large component of high-threshold Ca^{2+} channel current inhibited by GTP-γ-S relative to the ω-conotoxin–sensitive component can be accounted for by inhibition of current carried by L-type Ca^{2+} channels. Activation of G-proteins by intracellular GTP-γ-S can, apparently, inhibit both N and L-type Ca^{2+} channels, and the extent of inhibition of a particular type of channel may differ in different types of neurons.

Our results are consistent with those of Pollo et al. (1991) who found that GTP-γ-S inhibits either ω-conotoxin- or dihydropyridine-sensitive high-threshold Ca^{2+} currents in rat sensory neurons. Pollo et al. (1991) also found that the extent of inhibition produced by either ω-conotoxin or the dihydropyridine antagonist nifedipine was similar in cells perfused with either GTP or GTP-γ-S. The inhibition of both N- and L-type

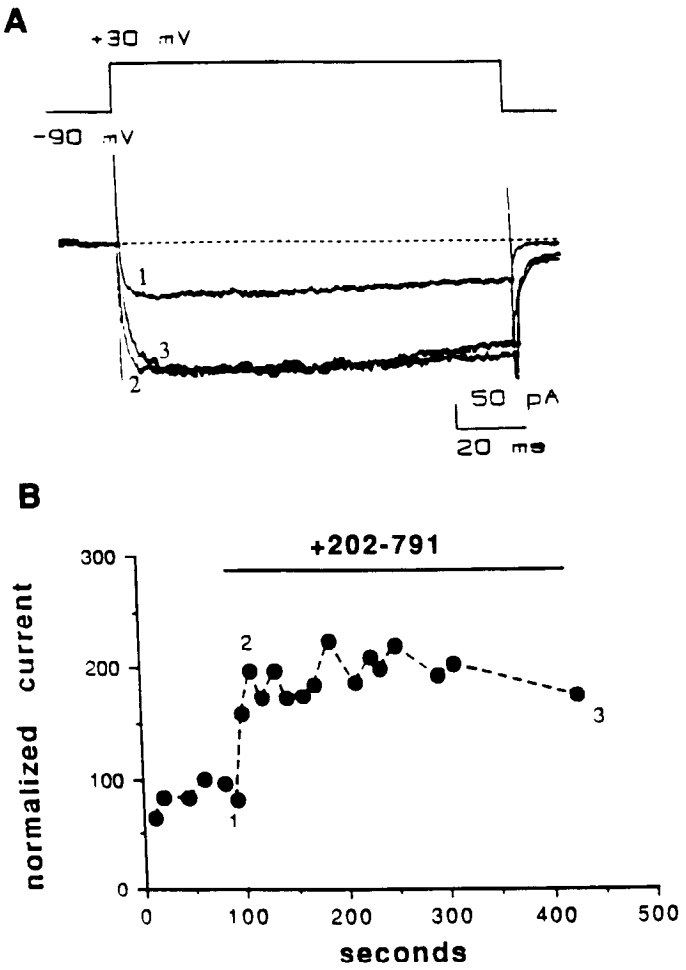

Figure 9. The effect of 1 μM +(*S*)-202-791 on a cell perfused with 500 μM GTP-γ-S that had been in culture ~7 d. The drug was added during the period marked by the *bar* from a second pipette placed close to the cell under study. *A*, Examples of Ca^{2+} channel current before (*1*), shortly after (*2*), and ~6 min after constant exposure to agonist. *B*, Time course of the response to +(*S*)-202-791. Currents sampled at 5 kHz and filtered at 2 kHz.

Ca^{2+} channel current by intracellular GTP-γ-S suggests that the binding site for the inhibitory G-protein is distinct from or only weakly associated with the dihydropyridine receptor of the neuronal L-type Ca^{2+} channel or that different G-proteins are targeted to distinct channels. Other reports, however, have suggested that G-proteins interact directly with the dihydropyridine receptor of L-type channels causing changes in the agonist/antagonist properties of specific dihydropyridines (Dolphin and Scott, 1989). We found no evidence, however, showing that intracellular GTP-γ-S alters the agonist/antagonist properties of dihydropyridines (see also Plummer et al., 1989). By contrast, biochemical studies have indicated direct coupling between G-proteins and the dihydropyridine receptor (Bergamaschi et al., 1988; Meucci et al., 1988), but the effects of such coupling on channel function are not well understood.

The results show that the initial inhibition of total high-threshold current and the subsequent inhibition of the agonist-activated L-current follow a remarkably similar time course. The time course appears to be slower than can be accounted for by the diffusional exchange of the cell interior with the electrode filling solution. One possible explanation for these findings is that the agonist recruits silent channels, which then become susceptible to the inhibitory action of intracellular GTP-γ-S. This interpretation is supported by the similar rates of inhibition of the initial current and the agonist-activated current, which are comparable to the intrinsic rate of GDP release that limits G-protein activation of GTP-γ-S (Breitwieser and Szabo, 1988). An alternative mechanism is that the dihydropyridine receptor desensitizes to the agonist in the presence of GTP-γ-S. If this were the case, the decrease of the agonist-evoked L-current might be expected to be kinetically distinguishable from the initial inhibition of the total current. The similar time courses of these two processes, however, and the absence of any discernable recovery of agonist sensitivity over several minutes would support a mechanism in which L-type channels are recruited by the agonist and then become inhibited by an activated G-protein.

Developmental changes in the response of high-threshold Ca^{2+} current to GTP-γ-S

The results show that GTP-γ-S fails to inhibit the Ca^{2+} channel currents in granule cells that had been allowed to develop in tissue culture. Cerebellar granule cells in culture show many of the features of development *in vivo* (Hockberger et al., 1987; reviewed by Burgoyne and Cambray-Deakin, 1988). The granule cells in 7-d-old mice are in the process of migrating from the internal to the external granule cell layer, a process that takes up to 21 d to occur. It is interesting to speculate that the loss of responsiveness to GTP-γ-S reflects a developmental mechanism involving changes in the types of Ca^{2+} channels that are expressed. Further studies aimed at identifying the Ca^{2+} channel subtypes that are expressed during postnatal development of the cerebellum are clearly required. The results on tissue cultured cells, nonetheless, point out that particular stages of development may be associated with the expression of specific types of Ca^{2+} channels that differ in their response to physiological neuromodulators acting through GTP-binding proteins. Neuronal growth and the generation of specific neuronal connections by Ca^{2+}-dependent mechanisms controlling growth cone motility may involve such developmental changes acting at the level of the expression of specific types of Ca^{2+} channels.

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