# The Dihydropyridine-sensitive Calcium Channel Subtype in Cone Photoreceptors

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ABSTRACT High-voltage activated Ca channels in tiger salamander cone photoreceptors were studied with nystatin-permeabilized patch recordings in 3 mM $Ca^{2+}$ and 10 mM $Ba^{2+}$. The majority of Ca channel current was dihydropyridine sensitive, suggesting a preponderance of L-type Ca channels. However, voltage-dependent, incomplete block (maximum 60%) by nifedipine (0.1–100 μM) was evident in recordings of cones in tissue slice. In isolated cones, where the block was more potent, nifedipine (0.1–10 μM) or nisoldipine (0.5–5 μM) still failed to eliminate completely the Ca channel current. Nisoldipine was equally effective in blocking Ca channel current elicited in the presence of 10 mM $Ba^{2+}$ (76% block) or 3 mM $Ca^{2+}$ (88% block). 15% of the $Ba^{2+}$ current was reversibly blocked by ω-conotoxin GVIA (1 μM). After enhancement with 1 μM Bay K 8644, ω-conotoxin GVIA blocked a greater proportion (22%) of $Ba^{2+}$ current than in control. After achieving partial block of the $Ba^{2+}$ current with nifedipine, concomitant application of ω-conotoxin GVIA produced no further block. The P-type Ca channel blocker, ω-agatoxin IVA (200 nM), had variable and insignificant effects. The current persisting in the presence of these blockers could be eliminated with $Cd^{2+}$ (100 μM). These results indicate that photoreceptors express an L-type Ca channel having a distinguishing pharmacological profile similar to the $\alpha_{1D}$ Ca channel subtype. The presence of additional Ca channel subtypes, resistant to the widely used L-, N-, and P-type Ca channel blockers, cannot, however, be ruled out. Key words: nifedipine • nisoldipine • presynaptic • L-type Ca channel

## INTRODUCTION

The importance of calcium as a regulator of a variety of cellular processes is firmly established. Voltage-gated calcium channels, referred to as Ca channels in this report, provide a major regulated avenue for calcium influx, making the study of these ion channels and their critical role in neurotransmission of considerable interest. Typically, neuronal presynaptic Ca channels are located at the distal ends of axons, often rendering these channels inaccessible for study via conventional voltage clamp techniques. Within the vertebrate central nervous system, the photoreceptor output synapse provides a unique model for the study of presynaptic Ca channels (Attwell, 1990). Because these channels are located within or electrotonically near the somal (inner segment) membrane of the photoreceptor, the study of presynaptic Ca channels by whole-cell voltage clamp methods is possible.

The increased use of naturally occurring toxins in pharmacological investigations of Ca channels has revealed several subtypes of high voltage–activated (HVA)[1] Ca channels in neural membranes (Olivera et al., 1994). Whereas biophysical criteria confirm numerous HVA subtypes (Nowycky et al., 1985*a*), this approach has not kept pace with the pharmacological advances in this field.

Photoreceptor Ca channels have been considered to be of the HVA subtype (Bader et al., 1982; Corey et al., 1984; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Lasater and Witkovsky, 1991). Previous studies have used dihydropyridines to define the presence of L-type Ca channels in cones (Maricq and Korenbrot, 1988; Barnes and Hille, 1989) and ω-conotoxin GVIA to define the lack of N-type Ca channels (Lasater and Witkovsky, 1991). However, rigorous pharmacological examination of the cone Ca channel has not been performed. The purpose of the present study was to investigate in more detail the pharmacology of these Ca channels with nystatin-permeabilized patch recordings using cones in retinal slices and isolated cones, and using $Ba^{2+}$ or $Ca^{2+}$ as charge carriers. This study comes at

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[1] *Abbreviation used in this paper*: HVA, high voltage activated.


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a time when the role of L-type Ca channels at the cone photoreceptor output synapse faces a challenge: High concentrations of dihydropyridines do not completely eliminate synaptic transmission from photoreceptors, and an additional calcium-permeable channel type (e.g., cGMP–gated channels) has been proposed to play a role at this synapse (Rieke and Schwartz, 1994). To establish whether multiple Ca channel subtypes exist at this synapse, and to define modulatory pathways that influence photoreceptor Ca channels and synaptic transmission (Barnes et al., 1993; Kurenny et al., 1994), the identity of the Ca channels must be determined. In this report we assess the identifiability of the presynaptic HVA Ca channel subtype involved in regulation of the cone photoreceptor synapse.

## METHODS

Two cone preparations were used in this study: retinal slices and isolated cells. Slices were made according to the methods of Werblin (1978). Larval tiger salamanders were killed by decapitation, the eyes were removed, and the anterior portion of the eye was cut away. A 1 mm by 1 mm piece of the eyecup was cut free and set, ganglion cell side down, upon a filter (Millipore Corp., Bedford, MA). After the cells adhered to the filter, the sclera was removed under saline, and the tissue and filter were chopped with a razor blade into 150-μm slices. These were rotated through 90° to allow viewing of the retinal cross-section under a microscope (model UM-2; Diaphot, Nikon, Inc., Melville, NY) equipped with a ×40 immersion objective (Carl Zeiss, Inc., Thornwood, NY). Isolated cone photoreceptors retaining outer segments were obtained from retinas with trituration after treatment with papain (0.5 mg/ml, 15 min, 20°C) and were visualized in a Nikon Diaphot microscope. In both forms, the cones were recorded from under constant, bright microscope illumination, except during applications of dihydropyridines. Patch electrodes, coated with Sylgard (Dow Corning, Midland, MI) and fire polished to resistances of 2.5–6 Mohm, were filled with a solution containing (in mM) 100 CsCl, 3.5 $MgCl_2$,1.5 ATP $Na_2$, 10 HEPES, and 1 EGTA, pH 7.2, and sonicated with 150 μg/ml nystatin (Korn et al., 1991). Before back-filling with this solution, the pipette tip was briefly dipped in filtered, nystatin-free solution. Whole-cell access, judged by the appearance of membrane capacitance currents with time constants reflecting access resistances in the 10–30 Mohm range, typically occurred within 5 min, and the series resistance was compensated by 40–90% (Axopatch 1D and Axopatch 200). Cones had capacitances ranging from 6 to 48 pF (16.5 ± 9.2 pF, mean ± SEM, $n = 70$), similar to values reported previously for cones in slices (26 ± 9 pF; Merchant and Barnes, 1991) and enzymatically isolated cones (20 ± 10 pF; Barnes and Hille, 1989). Filtered signals were digitized with an interface (Indec Systems, Sunnyvale, CA) for storage in a 386 computer running BASIC-FASTLAB acquisition software.

For cell isolation, the bathing solution contained (in mM) 90 NaCl, 2.5 KCl, 3 $CaCl_2$, 10 HEPES, and 8 *d*-glucose at pH 7.6. To isolate Ca channel currents, the bath contained (in mM) 10 TEABr, 65 NaCl, 5 CsCl, 10 HEPES, 10 $BaCl_2$, 2.5 KCl, and 8 glucose at pH 7.6. Experiments using a Ca external solution contained the following (in mM): 10 TEACl, 75 NaCl, 5 CsCl, 10 HEPES, 3 $CaCl_2$, 2.5 KCl, and 8 glucose at pH 7.6. Ca channel agonists and antagonists were diluted in external bathing solution from concentrated stocks and applied via gravity-driven bath flow with suction pump removal. Complete or massive block of the peak Ca channel current could be rapidly achieved with the divalent cation cadmium (100 μM). Nifedipine and Bay K 8644 were diluted from 100-mM or 10-mM stocks, respectively, in DMSO. DMSO as high as 0.1% was found not to affect $Ba^{2+}$ currents when applied alone to six cells. A 10-mM stock of nisoldipine was made up in 95% ethanol and diluted in $Ba^{2+}$ or $Ca^{2+}$ external solution (Rieke and Schwartz, 1994), or prepared at 10-mM stock in DMSO. Cytochrome *c* (0.01% wt/vol), which did not affect Ca channel currents when applied alone ($n = 4$), was used in conjunction with ω-agatoxin IVA. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) with the exceptions of nisoldipine, which was a gift of Miles Inc. (Kankakee, IL), and agatoxin IVA, which was obtained from Peptides International Inc. (Louisville, KY) and Research Biochemicals International (Natick, MA).

Block was judged to have reached steady state when current magnitudes, in response to repeated, equivalent depolarizing steps, overlapped for at least five repetitions (usually 15 s). Percentage block was defined as $[1 - (I_{test})/(I_{control})] \cdot 100$, and was assessed at the peak of the control I–V relation. Cadmium-subtracted current magnitudes were usually averaged over the last 20–30 ms of each voltage step. An exception to this was made in slices where the voltage dependence of nifedipine block was most evident during the transient phase of the evoked current (see Fig. 1). All statistical data are presented as mean ± SEM unless otherwise noted and analyzed using an independent Student's *t* test where appropriate. Statistical significance was considered with $P < 0.05$.

## RESULTS

### *Dihydropyridine Sensitivity of the Ca Channel Current*

We characterized a Ca channel subtype in cones by using the dihydropyridine antagonist, nifedipine, at several concentrations in retinal slices bathed in 10 mM $Ba^{2+}$. Barium currents exhibited a modest transient component, apparently because of contamination from currents in other types of channels (Barnes and Deschênes, 1992). Up to 60% of the peak Ca channel current was blocked in a voltage-dependent manner by nifedipine (0.1–100 μM). Even at 10–100 μM, concentrations shown to affect other types of ion channels (Jones and Jacobs, 1990), a component of Ca channel current was resistant to block. Fig. 1 *A* shows Ca channel currents evoked by test depolarizations to −5 mV in control and in the presence of nifedipine and $Cd^{2+}$. The complete I–V relations under these conditions are shown in Fig. 1 *B*. Voltage-dependent block was evident when currents were tested in 10 μM nifedipine, with the greatest degree of block of the inward current evoked from a holding potential of −40 mV.

Concentration-response data were pooled from 43 nifedipine-treated cells in retinal slices, for three holding potentials, as shown in Fig. 1 *C*. Marked voltage-

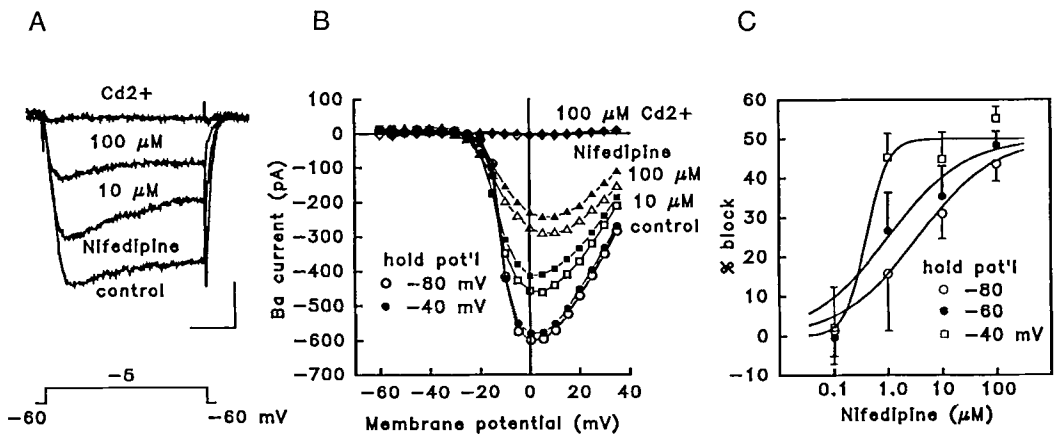


FIGURE 1. Nifedipine actions are dose and voltage dependent. (*A*) Leak-subtracted $Ba^{2+}$ currents from a cone in a retinal slice elicited during steps to −5 mV from a holding potential of −60 mV in control, nifedipine (10 and 100 μM), and $Cd^{2+}$ (100 μM). Scale bars, 30 ms and 150 pA. (*B*) Current–voltage relations from the same cell in *A*, in control, nifedipine (10 and 100 μM), and $Cd^{2+}$ (100 μM). These leak-corrected data were generated from holding potentials of −40 (*closed symbols*) and −80 mV (*open symbols*). (*C*) Pooled nifedipine concentration-response data from 43 cones in slices. Data are means ± SEM with 9–17 observations per point. Half-maximal block occurred at 3.5, 1, and 0.4 μM for holding potentials of −80, −60, and −40 mV, respectively. Note that at the 100 μM nifedipine concentration, significant $Ba^{2+}$ current remains unblocked.

dependent inhibition of the $Ba^{2+}$ current by nifedipine was evident at 1 μM. The particularly strong block at 1 μM that occurred in cells held at −40 mV could have been due, at least in part, to compromised membrane voltage control of cones in slices.

In subsequent experiments with isolated cone photoreceptors, where voltage control was better and where penetration of nifedipine was not an issue, we observed less transience in the control barium currents. We found in isolated cones that 10 μM nifedipine antagonized a greater proportion of the control current (64.6 ± 3.3%, $n$ = 12) than in recordings in slices (36.1 ± 6.1%, $n$ = 17) when cells were held at −60 mV ($P$ = 0.02, independent $t$ test). There was, however, no difference at 1 μM nifedipine ($P$ = 0.45).

High sensitivity to dihydropyridines is considered diagnostic for L-type Ca channels (Hille, 1992). In cardiac, smooth muscle, and brain cells, dihydropyridines, including nifedipine, block L-type Ca channels at submicromolar concentrations (Janis and Triggle, 1991). Fig. 2 illustrates that in isolated cones, where both membrane potential and concentrations of nifedipine are controlled more reliably, 0.1 μM nifedipine produced modest but voltage-dependent block of the peak $Ba^{2+}$ current. In six such experiments, the average block (taken at the peak of the I–V relation) was 49.7 ±

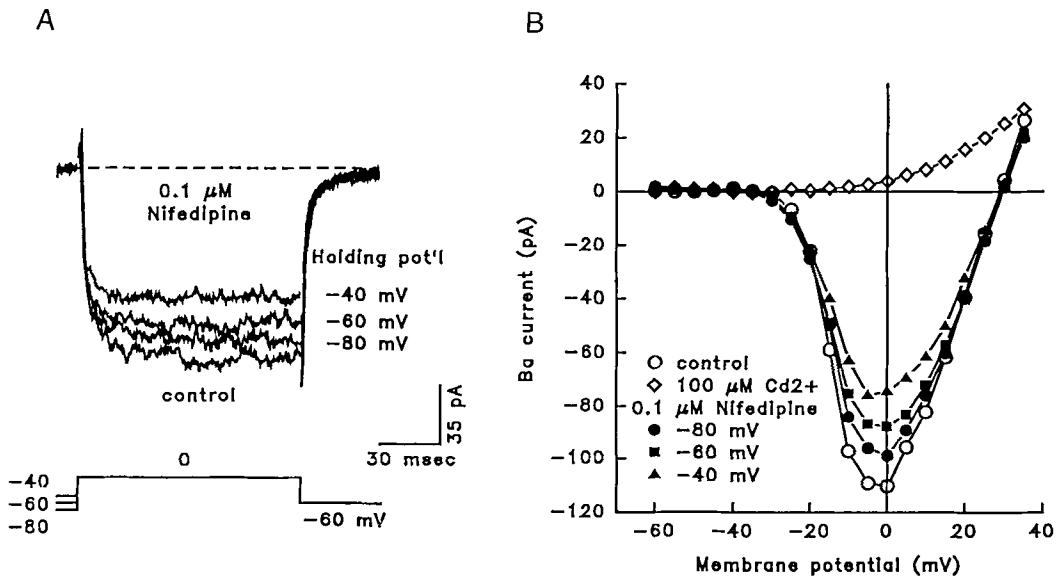


FIGURE 2. Low concentrations of nifedipine induce modest voltage-dependent block of Ca channel currents in isolated cones. (*A*) Leak-corrected current records demonstrating voltage-dependent block of the peak Ca channel current by 0.1 μM nifedipine in an isolated cone. Control sweeps at the holding potentials indicated were similar, and the record obtained from −60 mV is shown for clarity. (*B*) Current–voltage relations from the cell in *A*. Voltage dependence of nifedipine block is most evident near the peak. Data for control and $Cd^{2+}$ (100 μM) responses are provided from −60 mV holding potential for clarity.

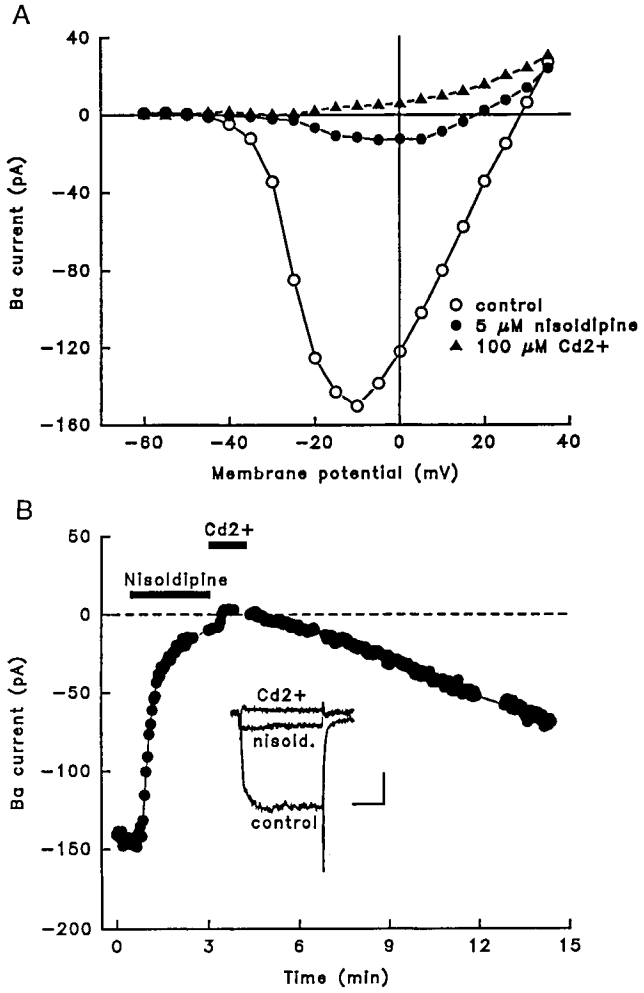


FIGURE 3. Nisoldipine is a potent but incomplete blocker of Ca channel currents. (*A*) Leak-corrected current–voltage relation from an isolated cone in control (10 mM $Ba^{2+}$, *open circles*), nisoldipine (5 μM, *closed circles*), and $Cd^{2+}$ (100 μM, *closed triangles*). Note that the nisoldipine-resistant current fraction was abolished in $Cd^{2+}$. (*B*) Time course of current block by nisoldipine. Peak current was elicited every 3 s with voltage steps from −60 to −10 mV and plotted against time. When steady-state block was achieved (horizontal bar indicates drug application), $Cd^{2+}$ was added and blocked the remaining current. Full recovery from nisoldipine block was not observed in six cells tested. Current records taken before (control), at peak nisoldipine block, and in $Cd^{2+}$ are shown in the inset (scale bars, 40 ms and 50 pA).

8.3%, 21.4 ± 5.2%, and 21.8 ± 5.6% for cells held at −40, −60, and −80 mV, respectively. Compared with cones in slices held at −60 mV, significantly more block was observed in isolated cones treated with 0.1 μM nifedipine ($P = 0.04$, independent $t$ test).

We verified the actions of nifedipine by using a second dihydropyridine antagonist, nisoldipine, on Ca channel currents in isolated cones. Fig. 3 illustrates the current–voltage relation and time course of block in an experiment where nisoldipine (5 μM), upon reaching a steady state, incompletely blocked the $Ba^{2+}$ current. The remaining current was abolished with 100 μM $Cd^{2+}$. In six cells, nisoldipine (5 μM) blocked 76.2 ± 8.1% of the control $Ba^{2+}$ current, which was significantly more ($P = 0.02$, $t$ test) than nifedipine (46.1 ± 2.9%, $n = 5$) used at the same concentration (see Fig. 8). Fig. 3 *B* also shows that, unlike nifedipine, reversal of nisoldipine block was slow and incomplete over the time scale tested. Since some dihydropyridine effects seem sensitive to the type of solvent used (Wu et al., 1992*a*; Wu et al., 1992*b*), we compared the magnitude of nisoldipine block when ethanol was used to solubilize the drug, as described above, with that when DMSO was used. From the same holding potential (−60 mV), 5 μM nisoldipine (solubilized in DMSO) produced 88.8 ± 1.6% block ($n = 4$), which was indistinguishable ($P = 0.27$) from the experiments in which ethanol had been used.

Complete block of cone Ca channels by 5 μM nisoldipine was recently reported when $Ca^{2+}$ (3 mM) was used as the charge carrier (Rieke and Schwartz, 1994). In addition to using $Ba^{2+}$, we assessed nisoldipine block of cone Ca channels using a $Ca^{2+}$-containing (3 mM) external solution. Fig. 4 shows data from two such experiments. In Fig. 4 *A*, the current–voltage relations reveal a small, $Cd^{2+}$-sensitive current that persisted after bath application of nisoldipine. In a different cone shown in Fig. 4 *B*, the time course of dihydropyridine block is illustrated with peak current plotted against time under the conditions indicated. Typically, a small current remained after steady-state block was reached, which was completely abolished in $Cd^{2+}$. In nine cells tested, 88.0 ± 3.2% of the control $Ca^{2+}$ current (in 3 mM $Ca^{2+}$) was blocked by nisoldipine, with the remaining current abolished in $Cd^{2+}$ (100 μM). This degree of nisoldipine block was not significantly different from experiments performed in 10 mM external $Ba^{2+}$ ($P = 0.16$, $t$ test). Thus, dihydropyridine-resistant, $Cd^{2+}$-sensitive current was consistently observed in cones, and this was independent of the charge carrier or the channel antagonist. Note, however, that these observations do not necessarily imply the presence of an additional Ca channel type, as discussed below.

### *Conotoxin GVIA Sensitivity of the Ca Channel Current*

From the preceding results it is evident that even high concentrations of dihydropyridine antagonists fail to abolish completely the $Cd^{2+}$-sensitive peak Ca channel current in cone photoreceptors. This suggests that additional Ca channel subtypes could contribute to the total inward current observed under our recording conditions. To characterize these additional current components, we used the Ca channel antagonist, ω-conotoxin GVIA, a peptide toxin whose blocking actions define the presence of N-type Ca channels (Olivera et al.,

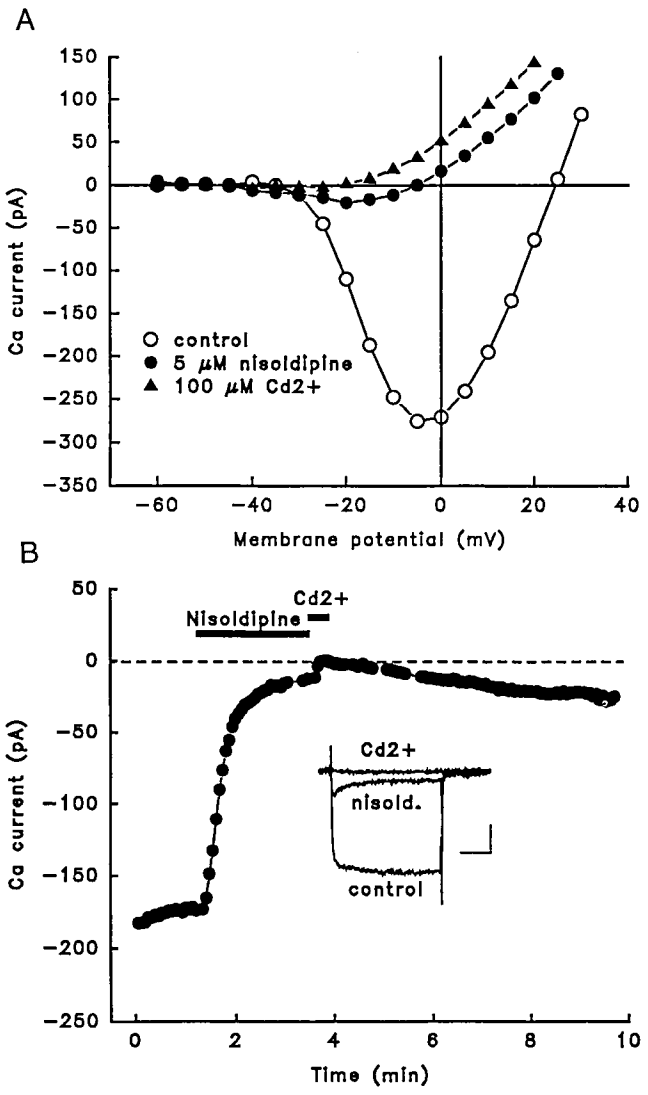


FIGURE 4. Charge carrier does not influence nisoldipine block. (*A*) Leak-subtracted current–voltage relations from an isolated cone recorded in 3 mM external $Ca^{2+}$. The nisoldipine-insensitive current was abolished in $Cd^{2+}$. (*B*) Time course of nisoldipine block is illustrated in another cell where the record was not interrupted to obtain I–V relations. Peak current was elicited every 4 s with voltage steps from −60 to 0 mV and plotted against time. As observed with $Ba^{2+}$-containing media, steady-state nisoldipine block remained incomplete when $Ca^{2+}$ was the charge carrier. Nisoldipine inhibition of the current recovered very slowly. Leak-corrected current records taken before, at peak block, and in $Cd^{2+}$ (average of five sweeps) are shown in the inset (scale bars, 30 ms and 50 pA).

1994). Fig. 5 illustrates partial block of Ca channel current by 1 μM conotoxin GVIA in a cone bathed in 10 mM $Ba^{2+}$, and the almost total abolition of the remaining inward current in the presence of 100 μM $Cd^{2+}$. In 10 isolated cone photoreceptors, 1 μM conotoxin GVIA reversibly inhibited the Ca channel current by 15.4 ± 2.9%. Reversibility also ruled out channel rundown as a mistaken interpretation of current block.

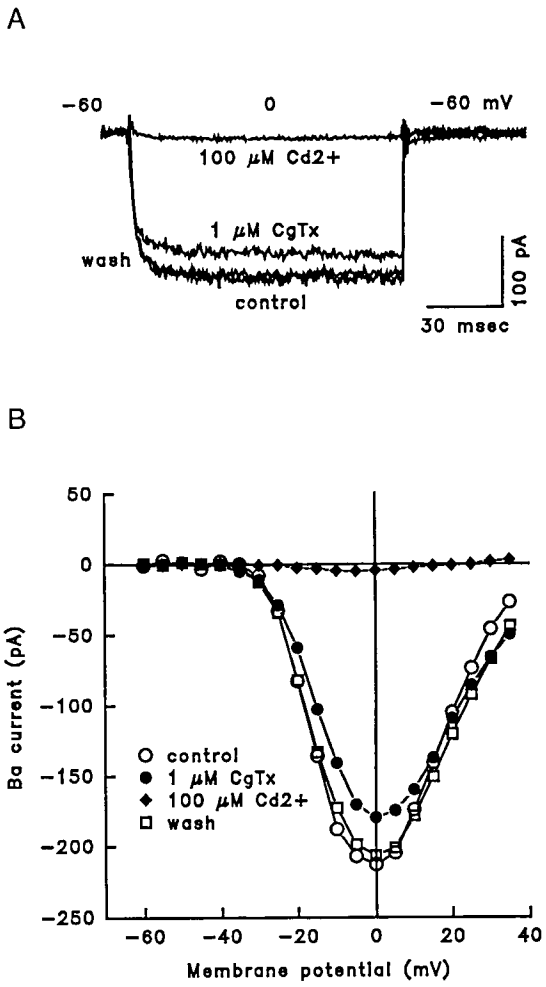


FIGURE 5. Block of Ca channel currents by conotoxin GVIA. (*A*) $Ba^{2+}$ currents recorded from an isolated cone at 0 mV in the presence and absence of 1 μM conotoxin GVIA (CgTx). Holding potential was −60 mV. (*B*) Leak-subtracted I–V relations for the data in *A*, including one obtained in 100 μM cadmium.

### *Agatoxin Sensitivity of the Ca Channel Current*

To reveal additional Ca channel subtypes, we used the P-channel blocker ω-agatoxin IVA, which we found to variably reduce $Ba^{2+}$ current in cone photoreceptors (3.9 ± 4.8%, $n = 9$; see Fig. 8). The concentration used (200 nM) is considered to be saturating for this class of channel (Mintz et al., 1992; Mintz and Bean, 1993). When inhibitory effects of agatoxin IVA were detected, they were always reversible (six of nine cells).

### *Dihydropyridines and Conotoxin GVIA Interact with the Same Channels*

The evidence presented so far conventionally suggests the presence of L- and N-type Ca channels in cones. To establish the specificity of conotoxin GVIA action on the HVA Ca channel current, we used the dihydropyridine agonist Bay K 8644, which has been shown to in-

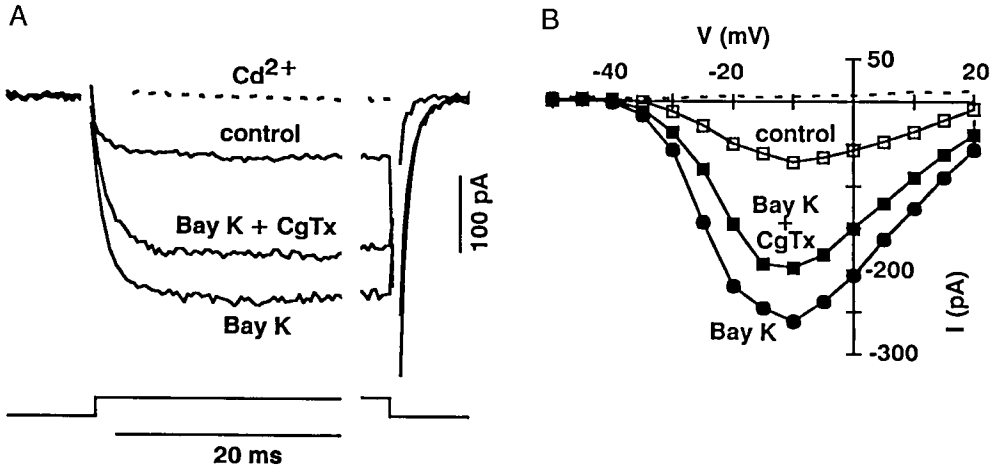


FIGURE 6. Block of Bay K 8644–enhanced Ca channel currents by conotoxin GVIA. (*A*) $Ba^{2+}$ currents recorded from an isolated cone at −10 mV in control, 1 μM Bay K 8644, then with 1 μM conotoxin GVIA added to the Bay K, and finally in 100 μM $Cd^{2+}$. Drugs were applied in the order given above, and the holding potential was −60 mV. The first 22 ms and last 4 ms of each 110 ms depolarization are shown. (*B*) Leak-corrected I–V relations are shown from the data in *A* for control (*open squares*), 1 μM Bay K 8644 (*closed circles*), 1 μM Bay K 8644 plus 1 μM conotoxin GVIA (*closed squares*), and 100 μM $Cd^{2+}$ (*dashed line*).

crease the burst time of L-type Ca channels (Nowycky et al., 1985*b*). In doing so, Bay K enhances current elicited during depolarizing voltage steps and also prolongs the tail currents. Fig. 6 presents the manifestation of these actions in cone photoreceptors. Bay K 8644 (1 μM) increased the peak Ca channel current about fourfold and increased the time constant of tail current deactivation from <2 ms to >5 ms. In addition, activation midpoints were typically shifted in the hyperpolarizing direction in the presence of the dihydropyridine agonist (−7.7 ± 1.8 mV, $n$ = 6). The effect of conotoxin GVIA on the Bay K–prolonged tail current, a defined and isolated component of L-type Ca channel activity, has been examined previously (Plummer et al., 1989; Regan et al., 1991). In the presence of Bay K 8644, conotoxin GVIA blocked a disproportionately large amount of current. In the experiment shown, conotoxin GVIA blocked almost the same amount of current in the presence of Bay K 8644 (65 pA) as was present in control (80 pA). Overall, Bay K 8644 increased current elicited by the depolarizing test step by 413 ± 67% ($n$ = 8). Conotoxin GVIA (1 μM) blocked a slightly greater proportion of current (22.7 ± 4.2%, $n$ = 8) in Bay K 8644–treated cells than in control (15.4 ± 2.9%, $n$ = 10, $P$ = 0.16, $t$ test). If conotoxin GVIA blocked specifically the Bay K–insensitive current component, we would have expected a 4% blocking efficiency (15%/413%) in the presence of Bay K. Since conotoxin GVIA block was slightly greater in the presence of Bay K than in control, these data suggest that conotoxin GVIA was in fact blocking the Bay K-enhanced current.

In contrast, however, conotoxin GVIA had a less obvious effect on the Bay K–slowed tail current. For example, for the cell illustrated in Fig. 6, a single exponential was fit to the control tail current (1.4 ± 0.5 ms, measured in response to five test voltages) while two exponentials were used in Bay K (1.1 ± 0.3 and 5.3 ± 1.4 ms, six test voltages). The fast time constant reflects an upper limit for unmodified channel deactivation, which may be faster but cannot be accurately resolved because of the limited speed of the voltage clamp. Conotoxin GVIA reduced the fast tail current component (time constant of 1.7 ± 0.1 ms, four test potentials) more than the Bay K–slowed tail current component in this cell. In four of six cells tested in this manner with Bay K and conotoxin GVIA in combination, block of slow tail current was difficult to detect, whereas, in two other cells, reduction of the slow tail current was evident.

Further experiments showed that the blocking actions of dihydropyridines and conotoxin GVIA are not additive. Fig. 7 provides an example of an experiment where two Ca channel blockers, nifedipine (1 μM) and conotoxin GVIA (1 μM), as well as $Cd^{2+}$ (100 μM), were used in combination. As this figure illustrates, the simultaneous application of nifedipine and conotoxin GVIA resulted in no further suppression of the peak current, which was completely abolished in $Cd^{2+}$. However, conotoxin GVIA applied alone reversibly inhibited the current. In seven experiments performed in this manner, conotoxin GVIA actually increased current by 0.6 ± 7.0% in the presence of nifedipine, whereas, either before application of nifedipine or after washing out the effects nifedipine, conotoxin GVIA applied alone blocked 15.4 ± 2.9% of the current ($P$ < 0.03, $t$ test). Although agatoxin IVA by itself inhibited

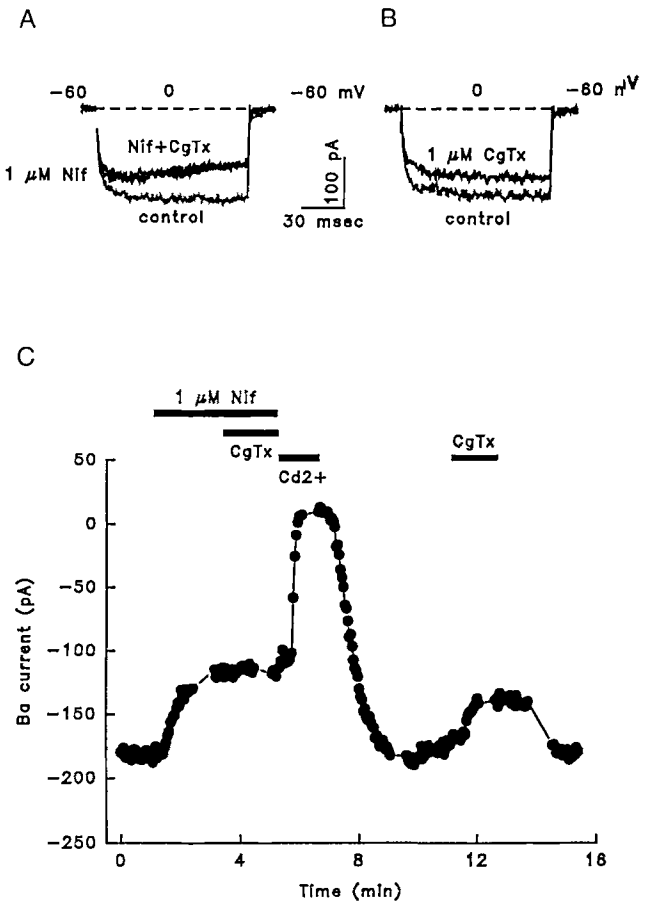


FIGURE 7. Block of Ca channel currents by nifedipine and conotoxin GVIA is not additive. (*A*) $Ba^{2+}$ currents recorded from an isolated cone at 0 mV in the presence and absence of 1 μM nifedipine and 1 μM nifedipine plus 1 μM conotoxin GVIA (CgTx). Holding potential was −60 mV. (*B*) Current records from the same cell before and during application of 1 μM conotoxin GVIA in the absence of nifedipine. Current records in *A* and *B* were leak subtracted; the dashed line represents the zero current level. (*C*) Peak current amplitudes were evoked with steps from −60 to 0 mV every 3 s and plotted against time to illustrate the time course of nifedipine, conotoxin GVIA, and $Cd^{2+}$ block in the cell shown in *A* and *B*. The effects of Ca channel antagonists were fully reversible.

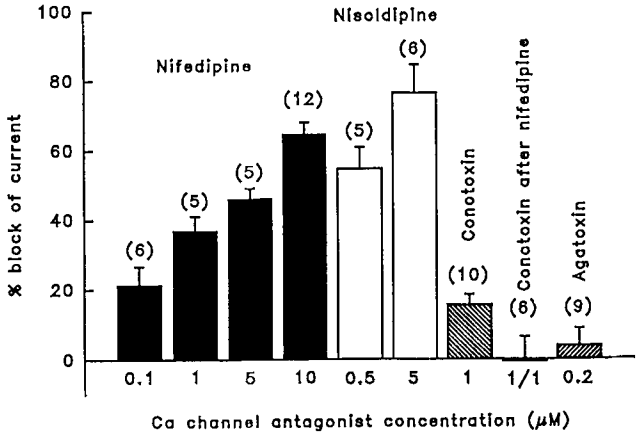


FIGURE 8. Summary of Ca channel antagonist effects on isolated cone $Ba^{2+}$ currents. Inhibitory actions of each Ca channel antagonist on control current are shown. In the case of conotoxin GVIA application to nifedipine-treated cells, control current was taken as current remaining after nifedipine block. Numbers in parentheses represent the number of cells in each group. Holding potential was −60 mV in each case. Values were averaged from currents evoked at the peak of the current–voltage relation for each cell (typically measured at values of −10 to +10 mV).

current in six of nine cells tested (see above), when applied in combination with nifedipine and conotoxin GVIA, there was never a reduction observed (increase of 4.5 ± 13.1%, $n = 4$, data not shown).

Fig. 8 summarizes the results of Ca channel blockade by the antagonists used in this study in isolated cones bathed in 10 mM $Ba^{2+}$ and held at −60 mV. These data underscore the inability of the two dihydropyridine antagonists, used at several concentrations, to antagonize completely the Ca channel currents in cones. Pooled data from experiments using conotoxin GVIA and agatoxin IVA are also shown, as are the effects of conotoxin GVIA applied in the presence of nifedipine.

## DISCUSSION

This study sought to characterize the calcium current of cone photoreceptors using pharmacological tools applied to isolated cells and cells in retinal slices. The principal finding of this study is that the cone Ca channel is an atypical L-type channel, as characterized by incomplete block of current at diagnostic (submicromolar) concentrations of dihydropyridine antagonists. We confirmed that whole-cell Ca channel current is entirely of the HVA type, as no low voltage–activated current was evident when more negative holding potentials were used (Barnes and Hille, 1989). When tested in $Ba^{2+}$- or $Ca^{2+}$-containing media, we confirmed that the majority of the Ca channel current was carried in dihydropyridine-sensitive Ca channels (Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Lasater and Witkovsky, 1991; Rieke and Schwartz, 1994), which suggests conventionally that the major Ca channel subtype in cones is L-type. However, ~ 20% of the HVA current was resistant to high concentrations (5 μM) of nisoldipine, and ~40% was resistant to yet higher concentrations (100 μM) of nifedipine. The resistant current could be blocked with $Cd^{2+}$ (100 μM), specifying it as a Ca channel current, and we discuss below the likelihood that it truly represents an additional Ca channel subtype in cone photoreceptors.

### *L-Type Ca Channels Account for the Majority of Cone Ca Channel Current*

Previous studies have shown that Ca channels in vertebrate photoreceptors are sensitive to dihydropyridine

agonists and antagonists (Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Lasater and Witkovsky, 1991; Rieke and Schwartz, 1994). The present study extends these observations by examining the inhibitory effects of dihydropyridine antagonists in cones over a broad range of concentrations, at several membrane potentials, and in circumstances where Ca channel current was carried by $Ba^{2+}$ or $Ca^{2+}$. These experiments show that block by nifedipine occurs at lower concentrations as the holding potential becomes more depolarized, a hallmark of the voltage-dependent Ca channel interactions described for dihydropyridines (Bean, 1984; Hille, 1992). However, cone L-type channels exhibit atypical dihydropyridine pharmacology in that they are much less sensitive to L-type channel blockers than the prototypical cardiac or smooth muscle Ca channel (Janis and Triggle, 1991). In addition, only minimal differences in blocking efficacies were noted for nisoldipine in 3 mM $Ca^{2+}$ and 10 mM $Ba^{2+}$, allowing extension of our interpretations to a wider range of existing experimental reports concerning photoreceptor Ca channels and calcium-dependent synaptic transmission.

When probed with the Ca channel blocker conotoxin GVIA to define pharmacologically the presence of N channels (Nowycky et al., 1985*b*), we found that ~15% of the peak cone Ca channel current was sensitive to this toxin. By some criteria, this suggests that cone photoreceptors express N-type Ca channels, but we reject this conclusion for several reasons:

(*a*) Unlike other neuronal cell types where the actions of conotoxin GVIA have been irreversible, the effects of conotoxin GVIA in our hands were completely reversible. Previous studies have shown that, in some instances, conotoxin GVIA can reversibly inhibit L-type Ca channel currents (Aosaki and Kasai, 1989; Mynlieff and Beam, 1992; Wang et al., 1992), while others have indicated that N-type channels can also be reversibly blocked by conotoxin GVIA (Plummer et al., 1989; Boland et al., 1994). The potency and reversibility of conotoxin GVIA block of N-type Ca channels is, in some cell types, a function of the divalent cation type and its concentration (Boland et al., 1994; Elmslie et al., 1994). Increased divalent concentrations speed the rate of conotoxin GVIA dissociation, similar to what we observed during current recovery from nisoldipine block in the presence of 3 mM $Ca^{2+}$ or 10 mM $Ba^{2+}$.

(*b*) When Bay K 8644 increased Ca channel current during test depolarizations by several hundred percent, conotoxin GVIA blocked a greater proportion of Ca channel current than it had in control. This indicates either that conotoxin GVIA blocked Bay K–activated L-type Ca channels or that Bay K activated another type of Ca channel in cones that is sensitive to conotoxin GVIA. Since it is unprecedented that dihydropyridine agonists activate channels other than L-type, we prefer the former interpretation. It should be noted, however, that conotoxin GVIA did not consistently affect to the expected degree the Bay K–enhanced tail currents, a current component conventionally defined as carried in L-type Ca channels (Plummer et al., 1989; Regan et al., 1991; Mintz, 1994). Some variability observed in the tail current block could have been accounted for by inadequate temporal resolution of the fast component of Ca channel deactivation, but clamp speed should have been fast enough to resolve the Bay K–slowed tail current. The use of higher concentrations of conotoxin GVIA (10 μM, as used by Williams et al., 1992) might help resolve this seemingly paradoxical observation.

(*c*) The appearance of "inactivation" during treatment with dihydropyridines might be interpreted to mean that inactivating N-type Ca channels were revealed when the L-type Ca channels were blocked. On the other hand, the slow inactivation could reflect time- and voltage-dependent block of L-type Ca channels by dihydropyridines during the test step (Bean, 1984; Hille, 1992).

Distinct Ca channel populations could have been revealed during block of the test current with application of several blockers at the same time. Application of nifedipine and conotoxin GVIA (or nifedipine, conotoxin GVIA, and agatoxin IVA) was made at high concentrations to several cells. Nifedipine occluded the conotoxin GVIA effect since none of the nifedipine-insensitive current was inhibited by conotoxin GVIA under these conditions. This result is at variance with the notion that conotoxin GVIA sensitivity implies the presence of N-type channels, but is consistent with the pharmacological profile of the expressed D-class L-type Ca channel ($\alpha_{1D}$ subunit functionally expressed in oocytes with $\alpha_{2b}$ and $\beta_2$ subunits; Williams et al., 1992). This clone, reversibly sensitive to conotoxin GVIA and agatoxin IVA, is thus a good candidate for the type of Ca channel in cones.

One possible explanation for the surprisingly strong occlusion by nifedipine of the effects of conotoxin GVIA is that cones could express different Ca channel phenotypes with varying characteristics of L- and N-type Ca channels. For example, suppose that $\alpha_{1D}$-like channels, weakly sensitive to conotoxin GVIA and potently sensitive to dihydropyridines, coexist with a population of $\alpha_{1C}$-like L-type channels, which are conotoxin GVIA insensitive and, for the sake of this argument, less sensitive to dihydropyridines than the $\alpha_{1D}$-like channels. It would then be feasible that nifedipine, which causes a 40% block at 1 μM, could completely occlude the actions of conotoxin GVIA, and that, in the presence of Bay K, conotoxin GVIA would exhibit a greater degree of block than in control. These possibilities offer reasonable explanations as molecular biological techniques have identified several additional Ca channels, some of

which have yet to be characterized pharmacologically (Snutch et al., 1990; Sather et al., 1993). These additional Ca channels may represent channels with pharmacological profiles common to several previously characterized Ca channels. Indeed, pharmacological overlap between P-, Q-, and O-type Ca channels has been extensively documented (Olivera et al., 1994), and the molecular diversity of L-type Ca channels is well known (Tsien et al., 1991). Nevertheless, it remains possible that these channels in cone photoreceptors retain features of L-, N-, and P-type Ca channels, and that an appropriate pharmacological, physiological, or molecular biological "classification" is not yet available. These results emphasize the limitations in defining Ca channel subtypes with existing Ca channel ligands, particularly in neurons of lower vertebrates (Olivera et al., 1994).

Species differences remain a significant contributor to this discussion because conotoxin GVIA was found to be without effect when tested on the Ca channel current in turtle cone photoreceptors (Lasater and Witkovsky, 1991). In addition, the recording methods themselves may play a role in affecting the population of Ca channels seen in cone cells. For example, this study used perforated-patch techniques to study photoreceptor Ca channel pharmacology, and it remains a possibility that some Ca channel subtypes could be more susceptible to washout than others, an effect that would alter the subtype repertoire whether one used ruptured- or permeabilized-patch techniques. Owing especially to the intact cytosol of these permeabilized cones, it remains possible that the effects of dihydropyridines, particularly at high concentrations, were mediated in part via interactions with other cellular functions, such as those involving calmodulin, cAMP phosphodiesterase, or protein kinase C, among a host of other possibilities (Zernig, 1990).

*Dihydropyridine-resistant Ca Channel Current*

It was also evident from our results that a proportion of cone HVA Ca channel current was insensitive to nifedipine and nisoldipine, even when these antagonists were applied at high nonspecific concentrations, after ample time had elapsed to exclude very slow actions of the antagonists, and when the blockers were tested from several holding potentials. The insensitivity of this current to these antagonists could be due to their lack of efficacy in cone photoreceptors, although, for example, nisoldipine shows very high efficacy in L-type Ca channel block compared with other dihydropyridine antagonists in other preparations (Janis et al., 1987). Thus, while our data do not exclude the possibility of an additional dihydropyridine-resistant HVA Ca channel component in cone photoreceptors, a more parsimonious explanation is that dihydropyridines are not fully efficacious in this cell type, and that even at concentrations shown to be saturating or nonspecific elsewhere, significant Ca channel current remains. This observation underscores what may be a distinguishing feature of the cone photoreceptor L-type Ca channel.

The presence of dihydropyridine-resistant Ca current in cones has relevance to questions about the presynaptic mechanisms of calcium-dependent neurotransmission at this synapse. In contrast to the report of Rieke and Schwartz (1994), we find that dihydropyridine Ca channel antagonists do not eliminate cone Ca channel current entirely, whether elicited under conditions of high $Ba^{2+}$ (10 mM) or physiological $Ca^{2+}$ (3 mM). There are subtle differences in the techniques used in these studies; for example, Rieke and Schwartz (1994) used voltage ramps to measure Ca current–voltage relations and included niflumic acid in the perfusing saline to inhibit Ca-activated Cl-current, although this drug may have weak effects on the calcium current itself (Barnes and Deschênes, 1992). It is unlikely that the voltage-dependent, dihydropyridine-resistant current we report is carried in cGMP-gated channels, which are not activated by depolarization. Our observation that $\sim 20\%$ of the calcium current in isolated cones persists in the presence of high concentrations of dihydropyridines (and that even more persists in the semi-intact preparation, the retinal slice), may provide an alternative explanation for observed synaptic transmission in the presence of high nisoldipine concentrations at this synapse (Rieke and Schwartz, 1994).

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