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Blockade of Ca^{2+} and K^+ Currents in Bag Cell Neurons of *Aplysia californica* by Dihydropyridine Ca^{2+} Antagonists

Jeanne M. Nerbonne[a] and Alison M. Gurney[b]

Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125

The effects of dihydropyridine calcium antagonists on whole-cell Ca^{2+} and K^+ currents in the neurosecretory bag cells of the marine mollusc *Aplysia californica* have been investigated. Nifedipine and nisoldipine blocked bag cell Ca^{2+} currents with effects similar to those seen previously on Ca^{2+} currents in cardiac muscle: both compounds appeared to interact with Ca^{2+} channels when they were closed, open, and inactivated. Also, as seen in cardiac cells, nifedipine apparently binds with higher affinity to Ca^{2+} channels when they are inactivated than when they are either closed or open. Nifedipine and nisoldipine also inhibited 2 outward K^+ currents in bag cells: the "delayed rectifier" (I_K) and the "A" (I_A) currents. Half-maximal blockade of Ca^{2+} currents occurred at ~1.4 μM nifedipine, compared to ~3–5 μM for half-maximal blockade of I_K and I_A. The effects of these compounds on bag cell Ca^{2+} and K^+ currents are interpreted and discussed here in terms of the "modulated receptor" model of drug action. In contrast, however, no measurable effects of nifedipine or nisoldipine were seen on Ca^{2+} (and/or K^+) currents in several vertebrate neuronal cell types. Our results suggest that there are likely to be structural and/or conformational variations in Ca^{2+} channels in different cells, tissues, and/or species and also that, in some cells, Ca^{2+} and K^+ channels might be structurally similar. These findings also suggest, therefore, that if dihydropyridine binding is used to identify Ca^{2+} channels, care should be taken to ensure that binding correlates closely with the Ca^{2+} channels of interest.

Numerous studies of dihydropyridine (DHP) Ca^{2+} antagonists (Fleckenstein, 1983; Janis and Triggle, 1983; Nayler, 1983; Triggle and Swamy, 1983) have characterized binding properties in cardiac and smooth muscle (de Pover et al., 1983; Holck et al., 1983; Pang and Sperelakis, 1983) and effects on Ca^{2+} currents (Kass and Tsien, 1975; Ehara and Kaufmann, 1978; Kanaya et al., 1983; Kass, 1983; Lee and Tsien, 1983; Morgan et al., 1983; Hess et al., 1984; Sanguinetti and Kass, 1984a, b; Bean, 1984, 1985; Hume, 1985; Uehara and Hume, 1985). As a result, it is generally assumed that DHP antagonists and agonists interact with a common receptor, thought to be the Ca^{2+} channel or a closely related structural site (Glossmann et al., 1982; Holck et al., 1983; Miller and Freedman, 1984).

In some neuronal tissues, saturable, high-affinity DHP binding sites have also been demonstrated (Glossman et al., 1982; Gould et al., 1982, 1985; Marangos et al., 1982, 1984; Yamamura et al., 1982; Curtis and Catterall, 1983; Miller and Freedman, 1984), although it is not clear that these sites reflect the presence of functioning voltage-sensitive Ca^{2+} channels (Glossmann et al., 1982; Miller and Freedman, 1984). DHP Ca^{2+} antagonists have been shown to block Ca^{2+} currents in snail neurons (Nishi et al., 1983) and K^+-induced Ca^{2+} influx in neuronal cell lines (Takahashi and Ogura, 1983; Freedman et al., 1984; Ogura and Takahashi, 1984) and rat brain synaptosomes (Turner and Goldin, 1985). The DHP Ca^{2+} agonist BayK 8644 (Schramm et al., 1983) reportedly enhances Ca^{2+} influx in chromaffin cells (Garcia et al., 1984), increases K^+-evoked transmitter release from PC-12 cells (Shalaby et al., 1984), and alters Ca^{2+} channel gating in dorsal root ganglion (DRG) neurons (Nowycky et al., 1985a). In other cases, however, DHP effects on Ca^{2+} currents and/or influx in neurons have not been detected (Takahashi and Ogura, 1983; Ogura and Takahashi, 1984; Shalaby et al., 1984; Boll and Lux, 1985; Litzinger et al., 1985). The significance, therefore, of the presence of high-affinity DHP binding sites on neurons remains uncertain. As DHP Ca^{2+} antagonists reportedly alter other (e.g., K^+) ionic currents in some preparations (Kass and Tsien, 1975; Nishi et al., 1983; Hume, 1985), it seems possible that binding studies reveal interactions at sites distinct from, or in addition to, voltage-dependent Ca^{2+} channels. Indeed, it has been demonstrated in the transverse tubule system of skeletal muscle that the majority of DHP binding sites (although voltage-dependent) are not voltage-sensitive Ca^{2+} channels (Schwartz et al., 1985). It seems possible that a similar situation might also exist in neuronal preparations.

Quantitative studies of Ca^{2+} antagonists are often complicated by the fact that Ca^{2+} currents can "run down" during electrophysiological experiments (Kostyuk, 1981; Byerly and Hagiwara, 1982) and DHP effects are often not readily reversible, perhaps owing to lipid solubility and uptake into membranes (Pang and Sperelakis, 1983). As a result, effects due to current rundown and channel blockade can be difficult to distinguish. The photolability of nifedipine, the lack of activity of its photoproduct (Ebel et al., 1978; Morad et al., 1983; Sanguinetti and Kass, 1984a; Nerbonne et al., 1985; Gurney et al., 1985), and

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Correspondence should be addressed to Jeanne M. Nerbonne, Department of Pharmacology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110.

[a] Present address: Department of Pharmacology, Washington University Medical School, 660 So. Euclid Ave., St. Louis, MO 63110.

[b] Present address: Department of Pharmacology, St. Thomas's Hospital Medical School, London SE1 7EH, U.K.

the fact that photoconversion is complete within 100 μsec (Morad et al., 1983) make this molecule a "reversible" antagonist (Sanguinetti and Kass, 1984a). Previously, we demonstrated that nearly complete reversal of the nifedipine-induced suppression of Ca^{2+} currents is provided by single, 1 ms light flashes in frog atrial fibers (Nerbonne et al., 1985) and in cultured rat ventricular myocytes (Gurney et al., 1985). The latter study also provided insights into the mechanisms of the nifedipine-induced suppression of Ca^{2+} currents that are not revealed using more conventional approaches. The experiments described here extend this work to neuronal Ca^{2+} currents. The aims were: (1) to characterize the blocking potency of nifedipine on neuronal Ca^{2+} currents; (2) to evaluate the selectivity of the observed effects; and (3) to compare the efficacy and specificity of current suppression in neurons and cardiac muscle.

A preliminary account of this work has appeared (Gurney and Nerbonne, 1984).

Materials and Methods

Aplysia californica (obtained from Mr. John Scotese, Alacrity Marine Supply, Marina Del Rey, CA) were maintained in the laboratory in well-aerated, filtered artificial seawater (ASW) tanks at 14 or 20°C. Animals kept at 20°C were fed reconstituted seaweed daily. For cell isolations, abdominal ganglia were removed and dissociated using described procedures (Kaczmarek and Strumwasser, 1981). Briefly, ganglia were incubated for 20 hr at 20°C in culture medium (see below) containing 1.25% by weight of the neutral protease, Dispase (Godo Shusei Co., Japan). After rinsing in fresh culture medium, bag cell clusters were dissected free from the ganglia, pleurovisceral connectives, and adjoining connective tissue, and individual cells were obtained by gentle trituration of the clusters. Cells were plated on collagen-coated glass coverslips in 35 mm culture dishes and maintained at 10–14°C; within hours, cells adhered to the substrate and some began to extend processes. Cells maintained at 10°C, although more fragile than those kept at 14°C, permitted better voltage-clamp control and faster intracellular dialysis, evidently because processes were absent or very short. Isolated bag cells (on coverslips) were transferred to a thermostatted recording chamber (Chabala et al., 1985), mounted on the stage of an inverted microscope (Diavert, Leitz), for recordings. Although some cells were lost during this manipulation, surviving cells were amenable to patch-clamp techniques. In the experiments here, cells were used within 72 hr of plating; current records were obtained at 14–15°C.

Published methods were used to prepare primary cultures of neonatal rat superior cervical ganglion (Mains and Patterson, 1973), neonatal rat dorsal root ganglion (Fields et al., 1978), and embryonic (8 d) chick ciliary ganglion (Nishi and Berg, 1977) neurons.

Data collection and analyses. The whole-cell recording method (Hamill et al., 1981) was employed to record membrane ionic currents. The voltage-clamp circuit was provided by the Dagan (model 8900) patch clamp/whole-cell clamp with either a 1 GΩ (Ca^{2+} currents) or a 100 MΩ (K^+ currents) feedback resistor. The tip diameters of the recording pipettes were 1.0–1.5 μm; resistances were 0.3–1 MΩ in ASW. The fast, inward Na^+ current (I_{Na}) was blocked by the addition of 5 μM TTX to the bath. The slow, inward Ca^{2+} current (I_{Ca}) was revealed by using pipettes filled with 480 mM CsCl; apparently through intracellular dialysis, Cs^+ effectively blocked all outward K^+ currents. It was frequently possible to monitor the rate of intracellular Cs^+ dialysis as the time course of washout of depolarization-induced outward K^+ currents (after obtaining whole-cell recordings). With 25–35 μm cells and 1 μm pipettes, it often required 10–15 min to suppress (completely) the K^+ currents. Seal resistances were in the range of 1–3 GΩ. Series resistance compensation was employed and checked at regular intervals to ensure adequate clamping during all experiments. If the series resistance changed, recordings were stopped; data were discarded unless it was clear when, during the experiment, the resistance had increased.

Inward Ca^{2+} currents were evoked by depolarizations from holding potentials negative to −30 mV. Recordings of I_{Ca} were stable and could generally be continued for 30–60 min with no measurable changes in I_{Ca} properties; thus, the general problem of Ca^{2+} current "rundown" (Kostyuk, 1981; Byerly and Hagiwara, 1982) was not a serious complication in these experiments. Kinetic analyses of the waveforms of the Ca^{2+} currents were performed by first-order exponential fits to all but the first 3 msec of the rising phases of the currents (Bean, 1984, 1985; Gurney et al., 1985). Although this approach is an oversimplification, as it implies 1 type of Ca^{2+} channel, reasonably good fits are obtained, apparently because 1 channel type predominates under the experimental conditions employed here (Bean, 1985; Boll and Lux, 1985; Nilius et al., 1985; Nowycky et al., 1985b). Under other conditions, we have evidence for the presence of at least one additional type of Ca^{2+} channel (Nerbonne and Gurney, unpublished observations). In some cells at relatively large depolarizations, an additional slow inward current, similar to the late, slow Ca^{2+} current seen previously in molluscan neurons (Adams et al., 1980), was also observed; we have not considered this current here. Outward K^+ currents were measured when the CsCl in the pipettes was replaced by 480 mM KCl and with 5 μM TTX and 10 mM $NiCl_2$ (1–2 mM $CaCl_2$) in the bath; uncontaminated K^+ current records were obtained during step depolarizations from holding potentials negative to −30 mV.

Linear leakage currents were subtracted before displaying and storing the data. Experimental parameters, e.g., holding potential, voltage steps, flashlamp trigger, etc., were controlled with an IBM Personal Computer interfaced with a Tecmar Labmaster (model 20009, Tecmar Corp.). Current records were filtered at 3 kHz and stored digitally. The software used in data acquisition and analysis has been described (Kegel et al., 1985). When the capacitances of the pipette and membrane were compensated before the patch was ruptured, bag cell membrane capacitance was determined from the integral of the capacitive current (in response to small voltage steps).

Solutions. The medium used for the bag cell cultures had the following composition (mM): NaCl, 470; KCl, 14; $MgSO_4$, 28; $MgCl_2$, 27; $CaCl_2$, 11; $NaHCO_3$, 13; HEPES, 15; glucose, 10, with nonessential amino acids (Gibco, 100×), essential amino acids (Gibco, 50×), vitamins (Gibco, 100×), and antibiotics (penicillin/streptomycin, Gibco, 100×) added at 1× (pH 7.8). The medium was sterile-filtered (0.23 μM) and stored at 4°C. For recordings, the bath solution was ASW containing the following (mM): NaCl, 480; KCl, 10.4; $MgCl_2$, 55; $CaCl_2$, 11; Tris, 10, pH 7.8. Outward currents were measured in a low (1–2 mM) Ca^{2+} bath solution with 10 mM $NiCl_2$, which suppressed $I_{Ca} > 95\%$; Ca^{2+} could not be removed completely from the bath, as the cells became leaky. Pipettes contained (mM): KCl, 480; EGTA, 10; glucose 10; Tris, 10, pH 7.8. To measure inward currents and suppress outward ones, the KCl in the pipettes was replaced with 480 mM CsCl.

Recordings of I_{Ca} from vertebrate neurons were made in bath solutions of the following composition (mM): NaCl, 140; $MgCl_2$, 1; $CaCl_2$, 10; HEPES, 5; glucose, 5, pH 7.3; TTX was added at 2 μM to suppress I_{Na}. For Ca^{2+} current recordings the pipettes were filled with filtered (0.2 μM) solutions of (mM): CsCl, 140; HEPES, 5; glucose, 5; EGTA, 10, pH 7.3. For studies of K^+ currents, the CsCl was replaced with 140 mM KCl and I_{Ca} was suppressed by the addition of 5 mM $CoCl_2$ to a low (1 mM) Ca^{2+} bath solution.

Stock solutions of nifedipine, nisoldipine, and BayK 8644, at 25 mM, were prepared in dimethyl sulfoxide (DMSO); the stocks were stable in the dark at 4°C for at least 3 months. Test solutions were prepared by serial dilutions of the stocks with the bath solution. The DMSO concentrations in these experiments never exceeded 0.1%, a concentration that by itself did not measurably affect ionic currents; at higher concentrations (>1%), DMSO selectively suppressed K^+ currents in this preparation for reasons that are not clear.

Results

In normal ASW, with KCl in the pipettes, both inward and outward currents were observed in response to step depolarizations from holding potentials negative to −40 mV in single voltage-clamped *Aplysia* bag cells. With CsCl in the pipettes, outward K^+ currents were suppressed and inward currents were measured separately. With 5 μM TTX in the bath, to suppress I_{Na}, depolarizing voltage steps from holding potentials negative to −30 mV revealed only I_{Ca}; in the presence of 5–10 mM $NiCl_2$ in low (1–2 mM) Ca^{2+} ASW, I_{Ca} was suppressed 90–95%. As shown in Figure 1*A*, I_{Ca} rose to a peak within a few milliseconds of the start of the voltage step and inactivated very slowly. The activation and inactivation kinetics were somewhat slower than I_{Ca} recorded from these cells using 2 microelectrode voltage-

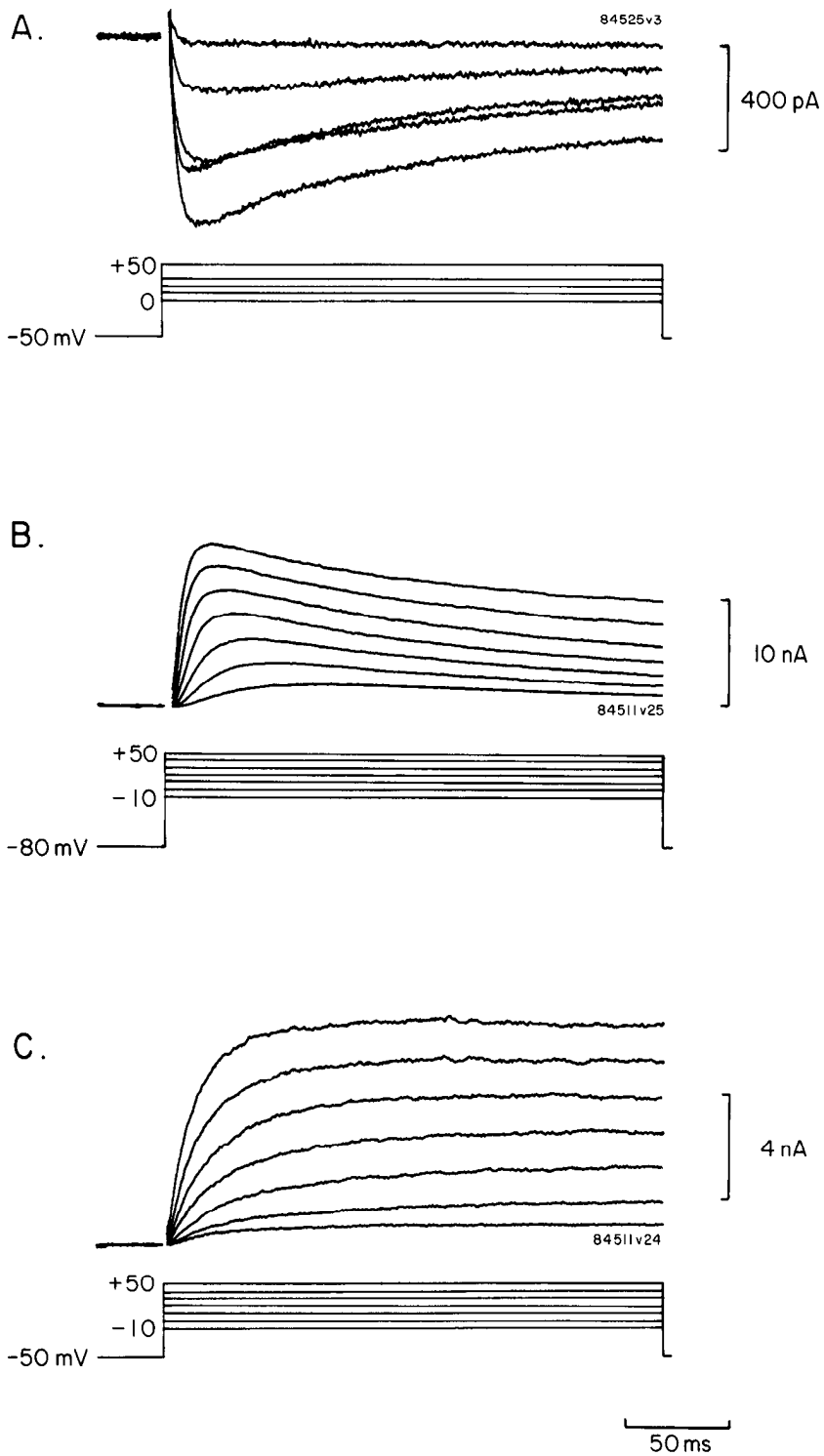

Figure 1. Whole-cell Ca^{2+} (*A*) and K^+ (*B, C*) currents recorded from isolated *Aplysia californica* bag cells. *A*, Ca^{2+} currents (I_{Ca}) were evoked by depolarizations to various test potentials from a holding potential of −50 mV; 140 mM CsCl in the patch pipette blocked K^+ currents, while 5 μM TTX in the bath suppressed I_{Na}. *B*, Outward K^+ currents were evoked by depolarizations from −80 mV. The pipette contained 140 mM K^+, and I_{Ca} was suppressed by lowering the Ca^{2+} in the bath to 1 mM and adding $NiCl_2$ (10 mM). At this holding potential, the total outward current comprises the transient (I_A) and the delayed (I_K) K^+ currents. *C*, Outward currents were recorded under the same conditions as in *B*, except that the holding potential was −50 mV. At this potential, I_A was inactivated and only the slower, noninactivating, I_K was observed.

clamp techniques (Kaczmarek and Strumwasser, 1984); these differences presumably result from more effective blockade of outward K^+ currents using the "whole-cell" method (Hamill et al., 1981) with Cs^+ in the pipettes. In this configuration, I_{Ca} was apparently well isolated from all outward currents, including the relatively large (in these cells) Ca^{2+}-dependent K^+ current(s) (Kaczmarek and Strumwasser, 1984).

When I_{Na} (5 μM TTX) and I_{Ca} (low Ca^{2+} and 10 mM $NiCl_2$) were blocked, and pipettes contained KCl, outward currents were recorded during step depolarizations from holding potentials negative to −30 mV (Fig. 1, *B* and *C*). At least 2 outward currents were evident: the fast-activating, fast-inactivating current, termed the "A" current (I_A) (Hagiwara et al., 1961; Connor and Stevens, 1971a, b), and a slower-activating, noninactivating current, the "delayed" K^+ current (I_K). These 2 currents, previously characterized in bag cells (Kaczmarek and Strumwasser, 1984), can be separated on the basis of their voltage sensitivities (Strong, 1984), as I_A is inactivated at holding potentials positive to ~−50 mV (compare Fig. 1, *B* and *C*). The waveforms of outward K^+ currents in normal ASW, evoked by depolarizations

from holding potentials −40 to −80 mV, were different: the steady-state amplitudes of total outward currents and the rates of current activation were increased. Similar observations, made previously (Kaczmarek and Strumwasser, 1984), were attributed to the presence of Ca^{2+} activated-K^+ currents (as well as I_{Ca}) in normal ASW. Ca^{2+}-dependent currents have not been evaluated here; studies of K^+ currents were performed with 5–10 mM $NiCl_2$ in low (1–2 mM) Ca^{2+}-ASW, and only Ca^{2+}-independent K^+ currents have been considered.

Suppression of I_{Ca} *by nifedipine*

At concentrations greater than ~200 nM, nifedipine reduced the amplitude of I_{Ca} in voltage-clamped bag cells. This effect was reversible on superfusion of drug-free ASW, although in many cases washing did not provide complete recovery; similar findings have been observed previously in cardiac tissue (Sanguinetti and Kass, 1984a; Gurney et al., 1985). Irradiation during washing, however, usually led to complete reversal of the blockade attributed to nifedipine. The percentage block of I_{Ca} by nifedipine could be determined experimentally, therefore, in 2 ways: (1) the reduction in current amplitude following wash-on and (2) the recovery of current amplitude after washing and irradiation. Both methods were used and, although they gave similar results, light-induced recovery was the method of choice, as the intervals between test and control trials were shorter and the experiments were less likely to be influenced by current "rundown" (Kostyuk, 1981; Byerly and Hagiwara, 1982). The fractional inhibition of I_{Ca} amplitude by nifedipine as a function of concentration is shown in Figure 2*A*. The concentration producing 50% inhibition (EC_{50}) was found by interpolation to be ~1.4 μM. This value, although approximately 3-fold higher than that found in cardiac tissue (Lee and Tsien, 1983; Gurney et al., 1985; Nerbonne et al., 1985), is similar to findings in isolated snail neurons (Nishi et al., 1983). Pre-irradiated solutions of nifedipine at concentrations to 10 μM (in the presence and absence of light) had no measurable effects on I_{Ca}.

In the absence of nifedipine, I_{Ca} was routinely observed on depolarizations to test potentials more positive than −10 mV, from holding potentials between −40 and −60 mV. I_{Ca} amplitude varied as a function of test potential and reached a maximum at +30 mV, as shown in Figure 2*B*, where data from several cells, normalized to the current amplitude measured at +30 mV, are shown. Nifedipine reduced I_{Ca} amplitude equally at all test potentials and at all nifedipine concentrations (0.5–5.0 μM) examined. These findings are comparable to the lack of voltage dependence to the steady-state block of Ca^{2+} current amplitude by nifedipine in cardiac muscle (Gurney et al., 1985; Nerbonne et al., 1985).

Effects on I_{Ca} *kinetics*

When I_{Na} was blocked, except for the first 3–5 msec, the rising phase of I_{Ca} could be accurately resolved and fit by a single exponential. This treatment assumes the presence of a single type of Ca^{2+} channel, which is contrary to the available evidence in neuronal and cardiac cells (Carbone and Lux, 1984; Bean, 1985; Boll and Lux, 1985; Nilius et al., 1985; Nowycky et al., 1985b), including bag cells (J. M. Nerbonne and A. M. Gurney, unpublished observations). Nevertheless, reasonably good fits are obtained assuming this simple model (Bean, 1985; Gurney et al., 1985), presumably because the experimental conditions employed favor the activation of predominantly 1 type of Ca^{2+} channel (Carbone and Lux, 1984; Bean, 1985; Boll and Lux, 1985; Nowycky et al., 1985b). In addition, this approach appears reliable as dihydropyridines apparently only interact with 1 type of Ca^{2+} channel (Bean, 1985; Boll and Lux, 1985; Nilius et al., 1985; Nowycky et al., 1985b). The first-order rate constants for I_{Ca} activation estimated from these fits are apparently voltage-dependent, increasing with increasing depolarization (Fig. 2*C*). In the presence of 0.5–5 μM nifedipine, the rates of Ca^{2+} current activation were not altered (Fig. 2*C*); these results are similar to previous findings in cardiac muscle (Lee and Tsien, 1983;

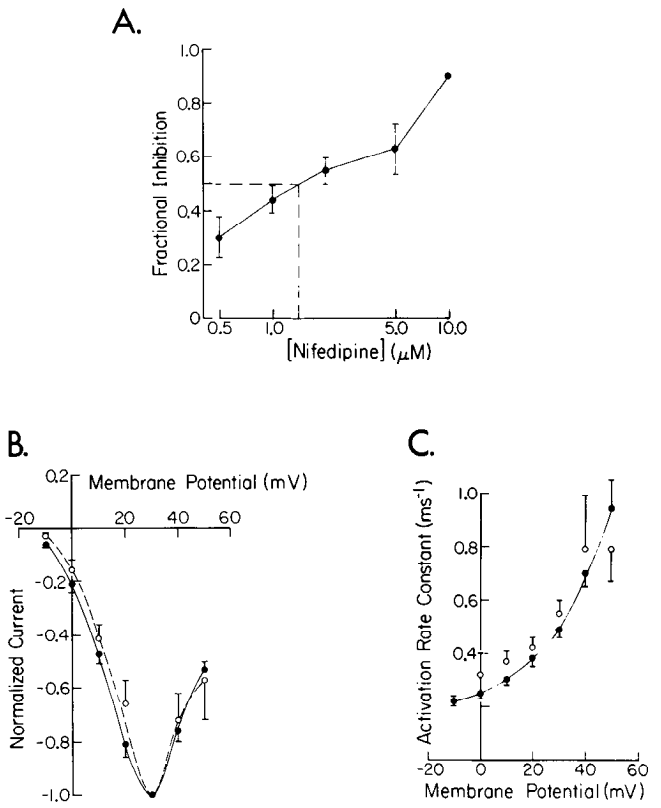

Figure 2. I_{Ca} and nifedipine. *A*, Relationship between the fractional inhibition of peak I_{Ca} amplitude and nifedipine concentration. As discussed in the text, it is possible to determine the percentage inhibition of I_{Ca} amplitude by nifedipine in 2 ways: (1) suppression of I_{Ca} (from control) after addition of nifedipine, or (2) recovery of I_{Ca} amplitude following irradiation and photoremoval of nifedipine. Although we have used both methods, the data plotted here were obtained in experiments exploiting the latter protocol. Mean values from the results obtained on 3 (0.5 μM), 4 (1 μM), 9 (2 μM), 5 (5μM), and 2 (10 μM) cells are shown. Similar results were obtained in experiments utilizing the first approach outlined above. Interpolation provided an estimate of the EC_{50} value of 1.4 ± 0.4 μM for the suppression of I_{Ca} amplitude, which reflects blockade of closed Ca^{2+} channels. *B*, Current versus voltage relationships for peak I_{Ca} amplitude as a function of test potential. For each cell analyzed, peak current amplitude at each test potential was normalized against I_{Ca} measured during the voltage step to +30 mV (the voltage at which peak I_{Ca} amplitude was maximal). The data plotted here are the mean values of normalized data from 10 (nifedipine) and 27 (control) cells. The relationship was similar in the absence (●) and in the presence (○) of 2 μM nifedipine, revealing that the suppression of I_{Ca} amplitude by nifedipine was independent of test potential. *C*, I_{Ca} activation rate constants, estimated from single-exponential fits to all but the first 3 msec of the rising phase of the current, are plotted as a function of the test potential in the absence (●) and presence (○) of 2 μM nifedipine; holding potential was −50 mV. Mean values from results obtained in 16 (2 μM nifedipine) and 30 (control) cells are plotted; these data have not been normalized. The rate constants apparently increased with depolarization, but nifedipine did not measurably alter the rates at any test potential.

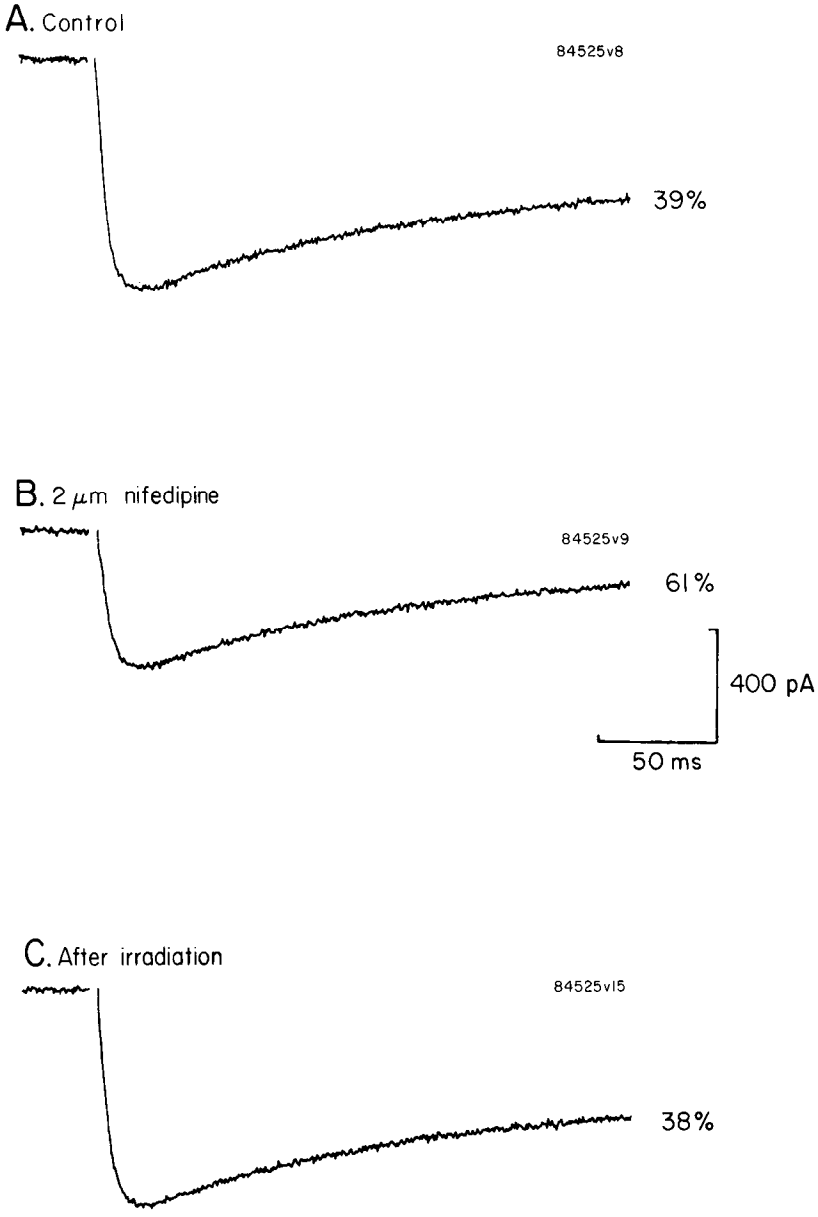

Figure 3. Nifedipine accelerated the decay phase of I_{Ca}. Currents were evoked, from a holding potential of −50 mV, by step depolarizations to + 30 mV at 5 sec intervals. I_{Ca} decayed to around 40% of its peak value in ~200 msec before the addition of nifedipine (*A*) and after its removal by irradiation (*C*). In the presence of 2 μM nifedipine (*B*), I_{Ca} decayed to around 60% of the peak current in the same period.

Gurney et al., 1985).Thus, the observed reduction in I_{Ca} amplitude(s) in the absence of any measurable effect on the rate(s) of I_{Ca} activation is consistent with the notion that nifedipine acts on closed Ca^{2+} channels, thereby reducing the fraction of channels that are available to open during depolarization. An alternative interpretation of these data would be that nifedipine blocks Ca^{2+} channels as fast as they open, i.e., equilibrating on a time scale much faster than the rate of channel opening. This possibility seems less likely than the interpretation favoring an effect on closed Ca^{2+} channels primarily because other data (see below) reveal that the rate of block of open Ca^{2+} channels is much slower than the rate of channel opening.

Although nifedipine was without measurable effect on the kinetics of current activation, the rate of I_{Ca} decay was accelerated as compared to controls (Fig. 3). This effect can be accounted for if nifedipine, in addition to interacting with closed Ca^{2+} channels, also affects open channels; i.e., nifedipine, by blocking open Ca^{2+} channels, causes an increase in the apparent rate of I_{Ca} inactivation. It is also possible, however, that nifedipine directly increases the normal rate of I_{Ca} inactivation. Although it is not possible to eliminate this possibility unequivocally, we find that the observed acceleration in the apparent rate of I_{Ca} inactivation increases linearly with nifedipine concentration (over the range 0.5–5 μM) as expected for open-channel block. In addition, these observations are similar to previous findings in cardiac cells (Gurney et al., 1985). In that case, we interpreted the effect in terms of the "modulated receptor" model (Hille, 1977; Hondeghem and Katzung, 1977, 1984) of drug action (Gurney et al., 1985); similar observations, made previously by others, have also been interpreted in this context (Bean, 1984; Sanguinetti and Kass, 1984b; Uehara and Hume, 1985). In heart cells, nifedipine accelerated the decay of whole-cell Ba^{2+} currents; there were no measurable effects on the decay rates when Ca^{2+} carried inward current, presumably because the already rapid rate of Ca^{2+}-dependent inactivation obscured the slow development of open-channel block (Gurney et al., 1985).

In bag cells, however, the normal rate(s) of I_{Ca} inactivation is slow; indeed, the waveforms of I_{Ca} in bag cells more closely resemble Ba^{2+} (than Ca^{2+}) currents in cardiac myocytes (Fig. 1*A*; Gurney et al., 1985). Thus, with Ca^{2+} as the current carrier, blockade of open bag cell Ca^{2+} channels by nifedipine was revealed. The concentration dependence for this effect is similar to that for suppression of I_{Ca} amplitude (Fig. 2*A*); the effect was half-maximal at ~2 μM nifedipine. Closed- and open-channel block of Ba^{2+} currents in heart cells by nifedipine also displayed similar concentration dependences (Gurney et al., 1985).

In cardiac muscle, we and others (Bean, 1984; Hondeghem and Katzung, 1984; Sanguinetti and Kass, 1984b; Gurney et al., 1985; Uehara and Hume, 1985) have described specific effects of DHP Ca^{2+} antagonists that can be attributed to blockade of inactivated Ca^{2+} channels. In order to examine this possibility in bag cells, we evaluated the effects of nifedipine on steady-state inactivation of I_{Ca}. An experimental protocol was selected in which depolarizing prepulses of varying amplitudes and durations were delivered from a holding potential of −50 mV, prior to a test pulse to +30 mV (Fig. 4). The fractional inactivation produced during the prepulse was measured as the ratio of the amplitude of the test current after the prepulse to the amplitude of the current evoked by depolarization directly from −50 mV. A 1.25 sec prepulse was determined (experimentally) to provide substantial, yet reversible, inactivation at potentials positive to −20 mV. Increasing the duration of the prepulse to 2.5 sec had little effect on the results, suggesting that the inactivation measured here was at, or close to, the steady state. Although longer prepulses could have been used routinely to ensure that we were indeed measuring "steady-state" inactivation (Bean, 1984; Gurney et al., 1985), these frequently resulted in irreversible inactivation of I_{Ca}. The 1.25 sec prepulse, therefore, was experimentally optimum to provide (near) "steady-state" inactivation of I_{Ca} without the additional complication of "rundown" of the current (which is accelerated by depolarization). Nifedipine caused the inactivation profile to shift to more hyperpolarized potentials (Fig. 4), implying that nifedipine interacts with inactivated Ca^{2+} channels; a greater fraction of inactivated channels is present, therefore, at any given membrane potential, in the presence of nifedipine than in controls. The observed shifts were smaller than those seen in cardiac cells (Bean, 1984; Sanguinetti and Kass, 1984b; Gurney et al., 1985; Uehara and Hume, 1985): 2 μM nifedipine caused a −10 mV shift in the steady-state inactivation curve here, as compared to a −15 to −20 mV shift in the presence of 0.5 μM nifedipine in ventricular cells (Gurney et al., 1985).

The following equation, which describes the availability of channels for opening as a function of membrane potential (Hodgkin and Huxley, 1952), was fit to the curves in Figure 4, using the algorithm of Marquardt (1963):

$$I = 1/[1 + \exp(V - V_h)/k] \quad (1)$$

where I is the relative amplitude of the test current at membrane potential, V; V_h is the midpoint voltage of the curve; and k is the slope factor. Assuming that the EC_{50} for the suppression of I_{Ca} amplitude reflects the dissociation constant, K_R, for binding to resting channels, then the dissociation constant, K_I, for binding to inactivated channels can be estimated by substituting the values of k = 9 mV (from the fit to the control curve in Fig. 4) and K_R = 1.4 μM into the following equation (Bean et al., 1983):

$$-\Delta V_h = k \ln[(1 + N/K_I)/(1 + N/K_R)] \quad (2)$$

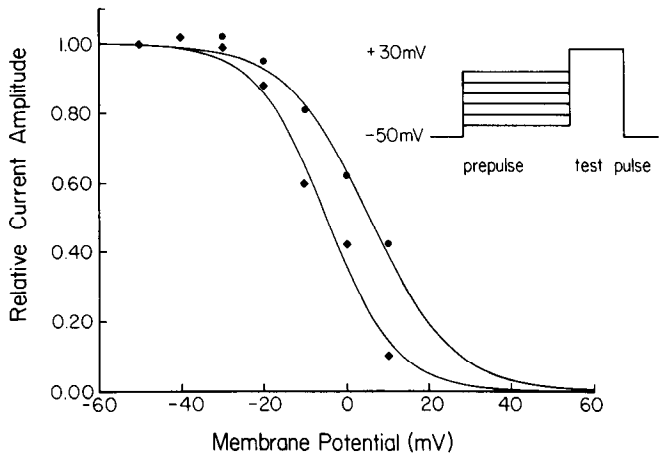

Figure 4. Effect of nifedipine on Ca^{2+} channel availability. The experimental protocol is illustrated in the inset. From a −50 mV holding potential, 1.25 sec prepulses to various potentials were applied before stepping to the test potential of +30 mV. The amplitudes of the test currents have been normalized against the amplitude of I_{Ca} evoked directly from −50 mV and are plotted as a function of the prepulse potential in the absence (●) and in the presence (◆) of 2 μM nifedipine. The smooth curves drawn through the points are the best fits of Eq. (1) to the experimental data. After washing and irradiation, the control curve was recovered.

where ΔV_h is the shift in the inactivation curve produced by nifedipine at concentration N. A shift of −10 mV produced by 2 μM nifedipine provided an estimate for K_I of 314 nM. Thus, as seen in heart cells (Bean, 1984; Sanguinetti and Kass, 1984b; Gurney et al., 1985; Uehara and Hume, 1985), it appears that the affinity of nifedipine for Ca^{2+} channels that are inactivated is greater than that for Ca^{2+} channels that are either closed or open.

Recovery of I_{Ca} *by irradiation*

In contrast to previous results in cardiac tissue (Gurney et al., 1985; Nerbonne et al., 1985), flash-induced recovery of I_{Ca} was not efficient in bag cells. Single flashes were ineffective at completely reversing the blockade (Fig. 5, *A, B*) at nifedipine concentrations in the 0.5–5 μM range, corresponding to ~20–80% inhibition of I_{Ca} amplitude. As continuous, steady illumination provided complete recovery of the current (Fig. 5*C*), however, there appears to be no "irreversible" block of I_{Ca}. Flash effects were also dependent on flash timing (Fig. 5): flashes presented late in the voltage step (Fig. 5*B*) resulted in little recovery during the same trial, although current amplitude increased further in the subsequent trial, to the level seen when the flash was presented prior to the voltage step (Fig. 5*A*). These results are consistent with the "modulated receptor" model also, as revealing the reversal of the blockade of inactivated channels (at late times during the voltage step). Removal of nifedipine from inactivated channels would not provide an immediate increase in I_{Ca} amplitude, although, following membrane repolarization (accompanied by the return of some inactivated channels to the closed state), more channels become available for opening during subsequent depolarizations.

Suppression of I_K *and* I_A

Whole-cell K^+ currents recorded from isolated bag cells were also suppressed by nifedipine at concentrations greater than ~0.5 μM (Fig. 6). This effect was also reversed by irradiation;

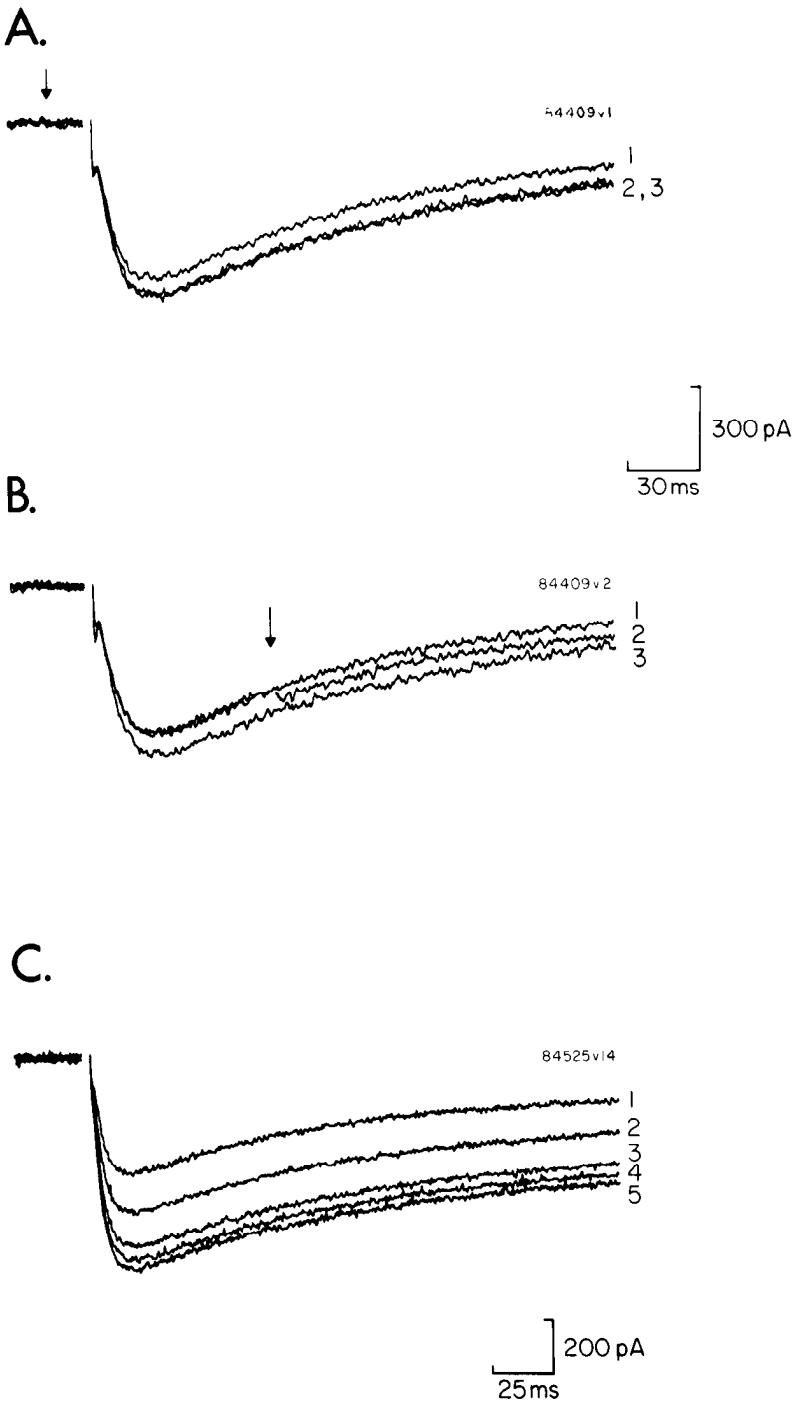

Figure 5. Effects of irradiation on the nifedipine-induced suppression of I_{Ca}. *A* and *B*, Three superimposed Ca^{2+} currents, evoked by step depolarizations to +30 mV from a holding potential of −50 mV, recorded at 5 sec intervals; currents are labeled in the order in which they were recorded. Records were obtained in the presence of 2 μM nifedipine; flashes from a xenon-short arc flashlamp, duration ~1 msec (Nerbonne, 1985), were presented, at the times indicted by the *arrows*, during the second sweep only. When the flash was delivered before the voltage step (*A*), a small increase in the current was observed and the third sweep superimposed on the second. When the flash was presented late during the voltage step (*B*), the resulting increase in current was very small, but a further enhancement of I_{Ca} amplitude was apparent on the subsequent sweep. *C*, Five consecutive Ca^{2+} currents, evoked at 5 sec intervals by step depolarizations to + 30 mV from −50 mV, are superimposed and labeled in the order in which they were recorded. Sweep 1 was recorded in the presence of 2 μM nifedipine. Steady illumination was begun immediately following the first sweep and continued until after sweep 5 was recorded. During continuous irradiation, I_{Ca} gradually increased as a result of photodestruction of nifedipine. The peak amplitude in sweep 5 was close to that of the control current, before addition of nifedipine (not shown).

continuous illumination was required, and flashes provided only partial recovery (~10%/flash). Irradiation alone had no measurable effects on the kinetics or amplitudes of K^+ currents and pre-irradiated solutions of nifedipine, in the presence or absence of light, did not alter the K^+ currents.

The effects of nifedipine on I_K and I_A were analyzed when Ca^{2+}-dependent K^+ currents were suppressed (10 mM $NiCl_2$ in low Ca^{2+} ASW). When I_A was inactivated at holding potentials positive to −50 mV (Strong, 1984), depolarizations evoked only I_K. The amplitudes of I_K were then measured as the steady-state currents at the end of 200 msec depolarizations from −50 mV. As shown in Figure 6*B*, nifedipine reduced the amplitudes of

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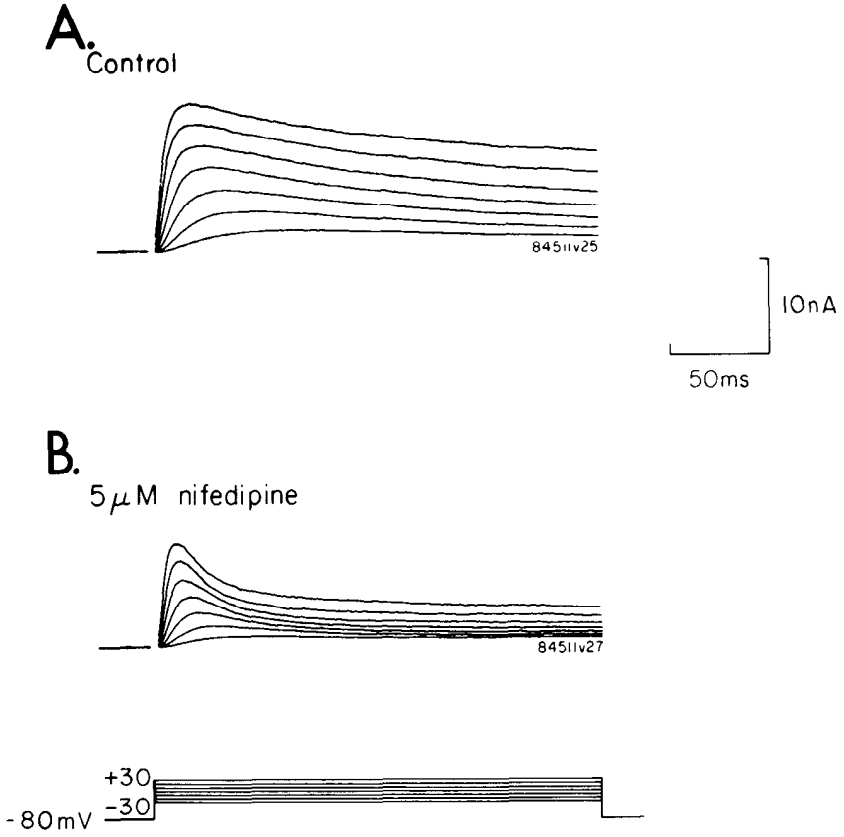
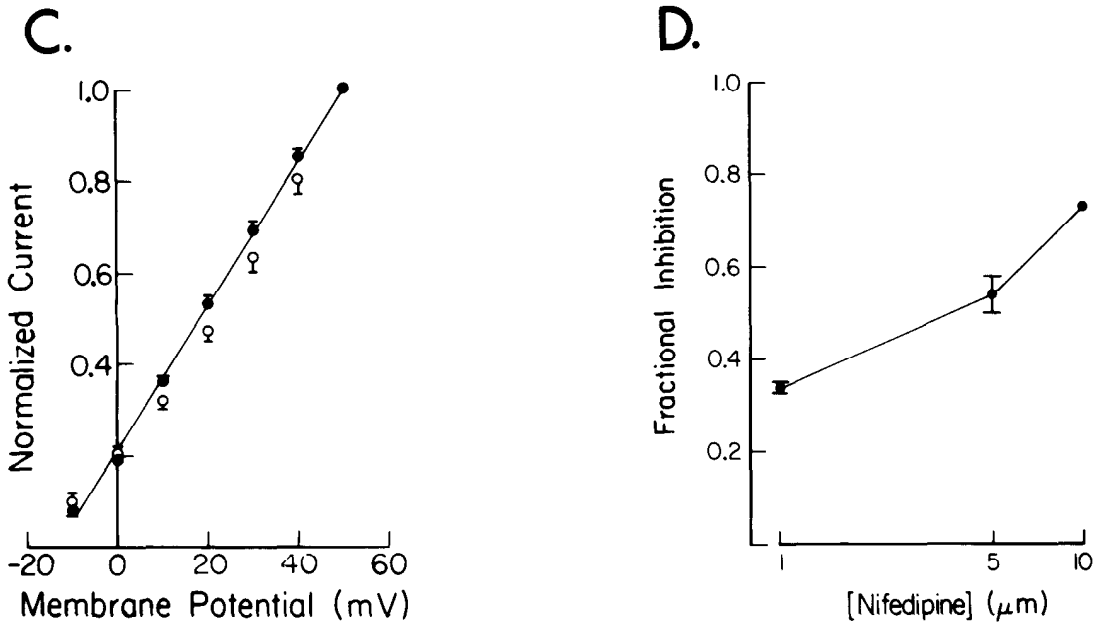
Figure 6. Effects of nifedipine on outward K^+ currents. *A* and *B*, Same series of outward currents evoked by step depolarizations, from a holding potential of −80 mV, to various test potentials. From this holding potential, the records comprise 2 overlapping currents, I_A and I_K. Total outward K^+ current amplitudes were suppressed in the presence of 5 μM nifedipine (*B*) as compared with control currents, measured prior to exposure to





nifedipine (*A*). Both the peak (predominantly I_A) and the steady-state (predominantly I_K) currents were reduced by 5 μM nifedipine, although the effect on the steady-state current was larger (*B*). *C*, Relationship between the steady-state amplitudes of I_K, measured at the end of 200 msec depolarizing voltage steps from a −50 mV holding potential, and the test potential in the absence (●) and in the presence (○) of 5 μM nifedipine. The current amplitudes in each case have been normalized to the amplitude of the current evoked by depolarization to +50 mV. The relationship was unchanged by nifedipine, implying that the nifedipine-induced suppression of current amplitude was independent of test potential. *D*, Fractional inhibition of I_K amplitude, measured at the end of a 200 msec voltage step to +40 mV from a −50 mV holding potential, plotted as a function of nifedipine concentration. Interpolation provided an estimate of the EC_{50} for this effect of ~3.5 μM.

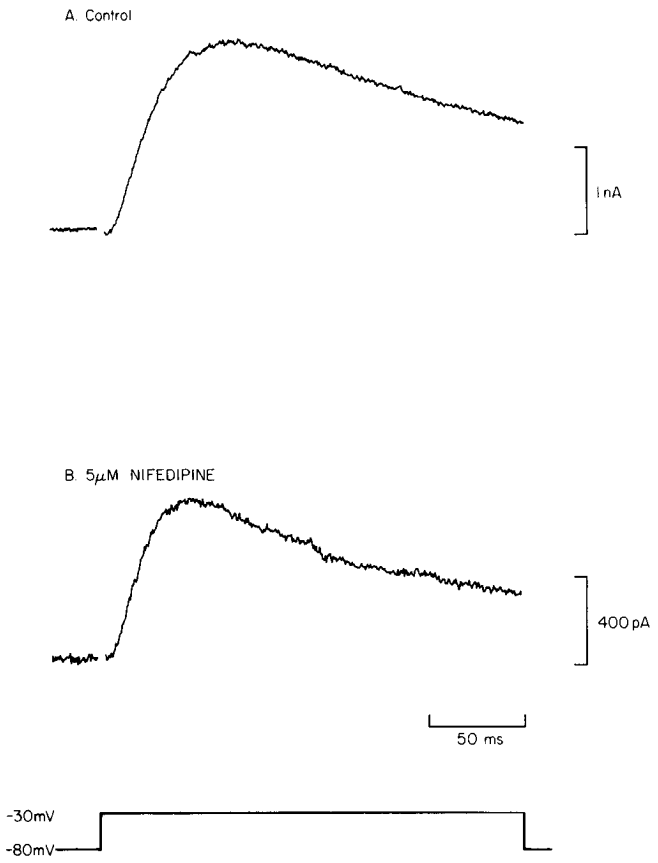


Figure 7. Effects of nifedipine on I_A. Currents were evoked by step depolarizations to −30 mV from a holding potential of −80 mV. At this potential, there was minimal contamination from I_K (see also Strong, 1984). Compared to the control current (*A*), I_A recorded in the presence of 5 μM nifedipine (*B*) was reduced in amplitude and the rate of I_A decay following the peak was faster. The rising phase of I_A, however, appeared to be unaltered by nifedipine.

I_K similarly at all membrane potentials tested; the absence of voltage-dependent block is similar to the present results on I_{Ca} and to previous results on Ca^{2+} currents in cardiac cells (Lee and Tsien, 1983; Gurney et al., 1985).

Apparent rate constants for I_K activation were measured at various test potentials from single-exponential fits to the rising phases of currents evoked from −50 mV. Although the rate constants varied with test potential, there were no measurable effects of nifedipine on the rates of I_K activation; these data are consistent with the notion that nifedipine blocks closed I_K channels and effectively reduces the fraction of channels available for opening on depolarization. The EC_{50} for this effect was ~3.5 μM (Fig. 6*D*); although higher than the EC_{50} for blockade of closed Ca^{2+} channels, this value is comparable to that reported for blockade of K^+ currents in snail neurons (Nishi et al., 1983). This result is, however, in sharp contrast to findings in frog atrial cells, where not only was the EC_{50} for suppression of I_K by nisoldipine ~3 times greater than that found here, but, also, approximately 1000-fold higher concentrations were required to block I_K than were necessary to suppress I_{Ca} (Hume, 1985). In our previous studies in cardiac cells (Gurney et al., 1985; Nerbonne et al., 1985), we did not observe any effects of the DHPs on K^+ currents, although we did not evaluate concentrations greater than ~2 μM.

In controls, I_K does not inactivate during 200 msec depolarizations, and in the presence of nifedipine (1–10 μM), I_K remained noninactivating at all test potentials examined. Thus, although nifedipine suppressed current amplitudes, it had *no* measurable effect on the kinetics of I_K. It seems unlikely, therefore, at least under the conditions employed here, that nifedipine blocks open I_K channels. Although not studied extensively, blockade of inactivated I_K channels also did not appear to be significant.

Nifedipine was slightly less effective at reducing the peak amplitude of the outward current, predominantly I_A, than it was at suppressing I_K amplitude, and ~5 μM was required for half-maximal blockade of I_A; the effect of nifedipine on I_A amplitude did not vary with the test potential. As I_A is activated at more negative potentials than I_K, during depolarizations to −30 mV from holding potentials negative to −50 mV, I_A is large and can be measured with minimal contamination from I_K (Strong, 1984). The effects of nifedipine on I_A could therefore be studied in isolation using voltage steps to −30 mV from a holding potential of −80 mV. As seen previously for I_{Ca} and I_K, nifedipine (5 μM) did not alter the rate of activation of I_A at −30 mV; higher concentrations were not tested. These results imply that nifedipine also blocks closed I_A channels. In contrast to the results obtained for I_K, the apparent rate of inactivation of I_A *was* increased (~40%) in the presence of 5 μM nifedipine (Fig. 7). We interpret these results as also consistent with the "modulated receptor" model, i.e., that nifedipine also blocks open I_A channels. Blockade of inactivated I_A channels by nifedipine, although not evaluated quantitatively, however, did not appear to be significant.

Thus, nifedipine appears to block K^+ currents in a manner similar to Ca^{2+} currents, although there does not appear to be a high-affinity interaction with open I_K channels or with inactivated I_K and I_A channels. Interestingly, in frog atrial cells, blockade of I_K by nisoldipine appears to be relieved at positive membrane potentials (Hume, 1985). This finding is in contrast to the effects on I_{Ca} seen here and to those seen previously in cardiac cells (Bean, 1984; Gurney et al., 1985; Hume, 1985; Uehara and Hume, 1985), as the suppression of I_{Ca} is greater at positive potentials and is relieved at negative membrane potentials. The observation that the block of I_K is not increased at positive potentials (Hume, 1985) is, however, consistent with our findings in bag cells that interactions of nifedipine with inactivated K^+ channels does not appear to be significant.

Effects of other dihydropyridines

Results similar to those described above were obtained with the (DHP) Ca^{2+} antagonist nisoldipine; the same concentrations were tested and nisoldipine displayed similar blocking potency. Nisoldipine suppressed I_{Ca} in a voltage-independent manner, had no effect on the rate of I_{Ca} activation, accelerated I_{Ca} decay, and caused a hyperpolarizing shift in the steady-state I_{Ca} inactivation curve. Nisoldipine is also light sensitive (Sanguinetti and Kass, 1984a), and complete reversal of I_{Ca} blockade could be effected by steady illumination; as with nifedipine, however, single flashes provided only partial recovery. Nisoldipine also effectively decreased the amplitudes of I_K and I_A, and these effects were similarly reversed by irradiation. It is interesting to note, however, that the molecule produced on photolysis of nisoldipine also displayed K^+ current blocking activity in bag cells. This is in contrast to the observed lack of physiological activity of the DHP photoproducts (from both nifedipine and nisoldipine) on Ca^{2+} currents, both in bag cells and in cardiac tissue (Morad et

al., 1983; Sanguinetti and Kass, 1984a; Gurney et al., 1985; Nerbonne et al., 1985).

In contrast to the results obtained with the Ca^{2+} antagonists, we found that the Ca^{2+} agonist, BayK 8644, which reportedly affects Ca^{2+} channels in cardiac and smooth muscle cells (Schramm et al., 1983) and in some neurons (Boll and Lux, 1985; Nowycky et al., 1985a, b), was without effect on bag cell Ca^{2+} or K^+ currents; concentrations up to 25 μM at a variety of membrane potentials were examined. Interestingly, however, prior exposure of isolated bag cells to BayK 8644 (1–10 μM) antagonized the inhibitory effects of nifedipine (and nisoldipine) on bag cell Ca^{2+} and K^+ currents. Although we have not investigated this phenomenon in detail, the result does imply that either the agonist binds to the same site(s) as the antagonists or that it, in some other way, protects against the effects of the antagonists. It should also be noted that although BayK 8644 affected single Ca^{2+} channels in DRG neurons, no effects on whole-cell Ca^{2+} currents were observed (Nowycky et al., 1985a). It may be that BayK 8644 acts as a Ca^{2+} agonist only when high extracellular K^+ is present (causing depolarization) and/or when high concentrations of Ba^{2+} (~100 mM) are used to carry inward current through the Ca^{2+} channels (Boll and Lux, 1985; Nowycky et al., 1985a, b). Although we interpret our results as suggestive of the presence of a binding site that is not coupled to changing Ca^{2+} influx, it is certainly also possible, as suggested previously (Nowycky et al., 1985a), that either the loss of a requisite intracellular component (which regulates Ca^{2+} influx) or the dialysis of the cell interior eliminates the responses to Bayk 8644 in whole-cell recordings.

Effects of nifedipine in other neuronal cells

The effects of nifedipine were also tested on I_{Ca} recorded from superior cervical ganglion (SCG), DRG, dissociated from neonatal rats, and ciliary ganglion neurons, isolated from 8-d embryonic chicks. In each case, I_{Ca} was evoked by depolarizations to potentials more positive than −20 mV, from holding potentials of −40 to −80 mV in the presence of 5 μM TTX to suppress I_{Na} and 140 mM Cs^+ (in the pipettes) to block outward K^+ currents. At concentrations to 5 μM, nifedipine had no measurable effects on either the amplitudes or kinetics of I_{Ca} in any of these cell types. As, in cardiac cells (Bean, 1984; Sanguinetti and Kass, 1984b; Gurney et al., 1985; Hume, 1985; Uehara and Hume, 1985), Ca^{2+} channel blockade is more pronounced at depolarized holding potentials, we also studied the effects of nifedipine on the availability of Ca^{2+} channels at various potentials in cultured SCG neurons using the experimental protocol described above for bag cells. At concentrations to 5 μM, nifedipine did not cause any measurable voltage shift in the availability of Ca^{2+} channels for opening. We also attempted to study the effects of varying the holding potential, using a protocol similar to that described by Bean (1984). The membrane was held at −20 mV, where I_{Ca} was inactivated compared with I_{Ca} evoked from −80 mV; under these conditions, however, substantial irreversible inactivation occurred. This irreversible inactivation of I_{Ca} was both time- and voltage-dependent, and when nifedipine (0.5 μM) was added to the solution perfusing the experimental chamber, no further effects on I_{Ca} amplitudes were revealed. We find no clear evidence, therefore, that nifedipine affects whole-cell Ca^{2+} currents in these neuronal preparations. In each of the vertebrate neuronal cells examined here, nifedipine was similarly without effects on K^+ currents, recorded during step depolarizations from −50 or −80 mV.

Discussion

The results presented here demonstrate that the DHP Ca^{2+} antagonists nifedipine and nisoldipine suppress whole-cell Ca^{2+} and K^+ currents in isolated bag cells. The properties of both compounds are similar, and half-maximal binding concentrations are ~1.4 μM and ~3.5 μM for I_{Ca} and I_K, respectively. These compounds are therefore less effective at blocking I_{Ca} in bag cells than in cardiac muscle (EC_{50} ~0.5 μM) (Lee and Tsien, 1983; Morad et al., 1983; Sanguinetti and Kass, 1984a; Gurney et al., 1985; Nerbonne et al., 1985); the difference is only a factor of 3, however, suggesting that the DHP binding sites in these cells might have structural similarities. The blocking potencies in bag cells are, however, nearly identical to those seen previously in snail neurons (Nishi et al., 1983), suggesting that DHP binding sites in these cells are likely to be quite similar. In bag cells and snail neurons (Nishi et al., 1983), I_{Ca} and I_K are affected by similar concentrations of nifedipine. If these effects reflect something about the structure(s) of the various ion channels, then it might be that, in invertebrate neurons, Ca^{2+} and K^+ channels share some common structural components. Ca^{2+} antagonists also reportedly affect ionic currents distinct from I_{Ca} (Kass and Tsien, 1975) in Purkinje fibers, and high concentrations suppress I_K in isolated frog atrial cells (Hume, 1985). These results could also suggest a similarity between Ca^{2+} and K^+ channels in these cells. Comparative biochemical/molecular analyses of channel proteins should provide more insight (than is provided by the electrophysiological data) into the similarities and differences in the various channel types in the same, as well as in different, cells.

The effects of the DHPs on whole-cell currents (I_{Ca}, I_K, and I_A) in bag cells are most simply interpreted within the framework of the "modulated receptor" model (Hille, 1977; Hondeghem and Katzung, 1977, 1984), an approach used previously (Bean, 1984; Sanguinetti and Kass, 1984; Gurney et al., 1985; Uehara and Hume, 1985) to model the effects of DHPs on cardiac Ca^{2+} channels. In some respects, the modulated receptor model is consistent with one that assumes DHPs alter channel gating, i.e., shifting channels between different "modes" of gating where openings (or closings) are more (or less) probable (Hess et al., 1984; Nowycky et al., 1985a). There are, however, some important distinctions. In the "modes" of gating model, interconversions between the different gating modes are slow, i.e., on the time scale of seconds, and it has been proposed that DHPs preferentially increase the probability of either gating mode "0" (antagonist) or "2" (agonist) (Hess et al., 1984; Nowycky et al., 1985a). According to this model, antagonists promote gating mode "0", by reducing channel availability, i.e., decreasing the probability of channel opening; agonists in contrast, promote gating mode "2", characterized by long-lasting openings and very brief channel closings (Hess et al., 1984). Some DHPs, e.g., nitrendipine, are observed to promote both mode "0" and mode "2"; this result is interpreted as revealing the partial agonist behavior of this "Ca^{2+} antagonist" (Hess et al., 1984). Our previous results in cardiac cells (Gurney et al., 1985) revealed that the interconversions between nonconducting (nifedipine-bound) and conducting (nifedipine-free) states occurred on the time scale of milliseconds. We favored, therefore, the more conventional approach to interpreting these data (Gurney et al., 1985); we suggested that DHPs alter the number of kinetic states available to a channel, as described by the modulated receptor model. In this model, interactions with various states of Ca^{2+} or K^+

channels are possible and are distinguished. The suppression of Ca^{2+} and K^+ current amplitudes arises primarily from effects on closed channels, acceleration of the rates of I_{Ca} and I_A decay are accounted for by effects on open channels, and the hyperpolarizing shift in the steady-state inactivation curve for Ca^{2+} channels reveals an additional effect on inactivated Ca^{2+} channels. The data also reveal that nifedipine interacts more effectively with Ca^{2+} channels when they are inactivated than when they are either closed or open. Although it certainly remains a possibility that some other model could be invoked to explain the data, we have not done this, as our data are more consistent with the modulated receptor model (Hille, 1977; Hondeghem and Katzung, 1977, 1984) than with the gating mode model (Hess et al., 1984; Nowycky et al., 1985a). Recent mechanistic studies of Ca^{2+} channel gating (Cavalié et al., 1986) have also questioned the validity of the gating mode model.

In contrast to the results on bag cells, we find that DHPs are ineffective at suppressing whole-cell Ca^{2+} (and K^+) currents in (rat) SCG, (chick) ciliary ganglion, and (rat) DRG neurons. Although these results appear to conflict with a report of BayK 8644 interaction with 1 type of Ca^{2+} channel in chick DRG neurons, in that study, effects were seen only in single-channel (not in whole-cell) current records (Nowycky et al., 1985a). In addition, Boll and Lux (1985), report that BayK 8644 has variable (agonist and antagonist) effects and nifedipine is ineffective on Ca^{2+} currents in these cells. Similarly, in mouse DRG neurons, DHPs do not affect voltage-sensitive Ca^{2+} influx (Litzinger et al., 1985). Although the reasons for the variability in DHP effects are not immediately obvious, and the implications of these combined observations are not completely clear, we can conclude that no DHP antagonist effects could be seen on vertebrate neuronal Ca^{2+} or K^+ currents under any of the experimental conditions employed here. The observed differences could simply reflect different experimental protocols: for example, it may be that high extracellular K^+ (or high Ba^{2+}) are necessary to reveal effects on some neuronal Ca^{2+} channels. It is certainly also possible that higher concentrations of nifedipine ($\gg 5\ \mu M$) would be needed to affect whole-cell currents than single-channel Ca^{2+} currents. Alternatively, it might be that the differences reveal: the absence of (or the presence of altered) DHP binding sites; the presence of quite distinct (chemically or conformationally) Ca^{2+} channels; or, perhaps, different connections between Ca^{2+} antagonist binding and Ca^{2+} influx. Although it is not possible to determine from our data whether one or more of these explanations is most likely, it is possible to conclude that either these various studies reveal distinct sites (or mechanisms) of DHP action or that the properties of the sites (or mechanisms) are different (or have changed) because of the particular experimental paradigms employed. Our results do indeed reveal that DHP antagonists can interact with similar affinities at sites distinct from Ca^{2+} channels in bag cells; the agonist, BayK 8644, although not directly affecting bag cell Ca^{2+} or K^+ currents, does afford protection against the antagonists. In view of these observations, the variability of DHP effects in different cells and/or under different experimental conditions, and the observed differences between DHP binding and the presence of functional Ca^{2+} channels (Schwartz et al., 1985), it seems clear that efforts aimed at identification of voltage-dependent Ca^{2+} channels using DHP binding as an assay would best be conducted in preparations in which corresponding biochemical and electrophysiological data can both be obtained in the same preparation and under very similar or identical experimental conditions.

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