Comparison of L-Type Calcium Channel Blockade by Nifedipine and/or Cadmium in Guinea Pig Ventricular Myocytes

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ABSTRACT
We tested the assumption that nifedipine blocks L-type calcium current $I_{Ca(L)}$ at $+10$ mV and unmasks $Na^+/Ca^{2+}$ exchange-triggered contractions in guinea pig isolated ventricular myocytes. Voltage-clamp pulses elicited $I_{Ca(L)}$ at $+10$ mV and evoked contractions in myocytes superfused with Tyrode’s solution (35°C). Nifedipine blocked $I_{Ca(L)}$ with an $IC_{50}$ of 0.3 μM; this decreased to 50 nM at a holding potential of $–40$ mV, indicating preferential block of inactivated L-type $Ca^{2+}$ channels. Use-independent block of $I_{Ca(L)}$ increased with concentration (10–100 μM) and application time when nifedipine was rapidly applied ($t_{1/2} = ~0.2$ s) during rest intervals (5–30 s). The fraction of use-dependent block of $I_{Ca(L)}$ diminished with increasing drug concentration. Nifedipine also accelerated $I_{Ca(L)}$ inactivation on the first test pulse. The combination of 30 μM nifedipine/30 μM Cd$^{2+}$ (Nif 30/Cd 30) was as effective as 100 μM nifedipine to suppress $I_{Ca(L)}$ on the first test pulse at $+10$ mV. The incidence of complete block of contractions, as for complete block of $I_{Ca(L)}$, increased as a function of nifedipine concentration and application time. Neither nifedipine nor Nif 30/Cd 30 affected $Na^+/Ca^{2+}$ exchange current at $+10$ to $+100$ mV. Contractions at $+100$ mV, although as large as those at $+10$ mV, were delayed in onset and resistant to nifedipine or Nif 30/Cd 30. We conclude that nifedipine-sensitive $I_{Ca(L)}$ triggers contractions at $+10$ mV, whereas nifedipine-resistant $Na^+/Ca^{2+}$ exchange current initiates those at $+100$ mV.

Contraction depends on calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) in mammalian heart cells (Fabiato, 1985). The usual trigger for CICR is $Ca^{2+}$ influx through the L-type $Ca^{2+}$ channel $I_{Ca(L)}$. Calcium entry through reverse mode operation of the Na$^+/Ca^{2+}$ exchanger (Nuss and Houser, 1992; Sham et al., 1992; Levi et al., 1994) and through T-type $Ca^{2+}$ channels $I_{Ca(T)}$ also can trigger CICR (Sipido et al., 1998). Calcium entry via T-type channels was a much less efficient trigger than entry via L-type channels; inasmuch as at comparable $Ca^{2+}$ influx, there was less $Ca^{2+}$ release from the SR with $I_{Ca(T)}$ than with $I_{Ca(L)}$.

The trigger function of reverse mode Na$^+/Ca^{2+}$ exchange $I_{Na/Ca}$ relative to $I_{Ca(L)}$ is debated. For example, reverse mode Na$^+/Ca^{2+}$ exchange is thought to account for phasic intracellular $Ca^{2+}$ transients and contractions observed at test potentials when $I_{Ca(L)}$ is reduced by $Ca^{2+}$ channel antagonists. In ventricular myocytes from rat (Wasserstrom and Vites, 1996), rabbit (Levi and Issberner, 1996), and guinea pig (Levi et al., 1996), nifedipine (10–32 μM) suppressed 90 to 99% of $I_{Ca(L)}$ but did not eliminate either the intracellular $Ca^{2+}$ transient or contractions. Other studies detected a trigger function of reverse mode $I_{Na/Ca}$, particularly at very positive potentials, have questioned the physiological role of $I_{Na/Ca}$-triggered release of SR $Ca^{2+}$. In cat ventricular myocytes, reverse mode $I_{Na/Ca}$ triggered phasic contractions in the presence of either 1 μM verapamil or nifedipine or 0.2 mM Cd$^{2+}$, yet a more important role of $I_{Na/Ca}$ was to load the SR with $Ca^{2+}$ (Nuss and Houser, 1992). Other studies interpreted similar results at $+80$ mV in rat ventricular myocytes to indicate that reverse mode $I_{Na/Ca}$ had slower kinetics to induce CICR (Sham et al., 1992). Verapamil (20 μM) or Cd$^{2+}$ (0.3 mM) only partially reduced contractions when $I_{Ca(L)}$ was suppressed at a test potential of $+2$ mV (Vornanen et al., 1994). They considered that reverse mode $I_{Na/Ca}$ contributed to triggering contraction at 35°C but not at 23°C because of the marked temperature dependence of $I_{Na/Ca}$. Although reverse mode Na$^+/Ca^{2+}$ exchange triggered intracellular $Ca^{2+}$ transients during steady-state block of $I_{Ca(L)}$ by either 10 μM nifedipine (Grantham and Cannell, 1996) or 20 μM nisoldipine (Sipido et al., 1997), its contribution during an action potential was estimated as small and inefficient. Model calculations indicate that $Ca^{2+}$ influx

ABBREVIATIONS: CICR, calcium-induced calcium release; SR, sarcoplasmic reticulum; $I_{Ca(L)}$, L-type calcium channel current; $I_{Ca(T)}$, T-type calcium channel current; $I_{Na/Ca}$, Na$^+/Ca^{2+}$ exchange current; DHP, dihydropyridine; Nif 30/Cd 30, 30 μM nifedipine plus 30 μM Cd$^{2+}$; E-C, excitation-contraction.

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through L-type channels would reduce Ca\(^{2+}\) entry through reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchange. This accords with Na\(^{+}\)/Ca\(^{2+}\) exchange having a variable efficiency such that it can provide a larger Ca\(^{2+}\) influx when \(I_{\text{Ca(L)}}\) is diminished by Ca\(^{2+}\) channel antagonists.

In general, those who report an important trigger function of reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchange have used rapid solution switching to deliver L-type Ca\(^{2+}\) channel antagonists; those who report a low triggering function have used steady-state conditions for \(I_{\text{Ca(L)}}\) block. Dihydropyridine (DHP) Ca\(^{2+}\) channel antagonists have been favored in these experiments because they are lipid soluble, very potent, and block the channel preferentially in the inactivated state. Channel block by DHPs is a function of drug concentration and assumed to be largely use-independent. However, L-type Ca\(^{2+}\) channel block by rapidly applied nifedipine in frog ventricular myocytes includes a small component of use-dependent block (Méry et al., 1996). That rapidly applied DHP antagonists may not be efficient blockers of L-type Ca\(^{2+}\) channels was raised in experiments with 20 \(\mu\)M nifedipine and 10 to 20 \(\mu\)M nifedipine (Sipido et al., 1995). In rat ventricular myocytes, nifedipine (10 \(\mu\)M) blocked \(I_{\text{Ca(L)}}\) by \(\sim 70\%\) in 2 min and completely at 4 min (Wasserstrom and Vites, 1996).

Consequently, we reexamined the use-independent and use-dependent components of DHP action on mammalian \(I_{\text{Ca(L)}}\). We tested nifedipine action on \(I_{\text{Ca(L)}}\) as a function of concentration, exposure time, and stimulus number to ascertain the extent and completeness of blockade. The inorganic ligand Cd\(^{2+}\) was used to standardize \(I_{\text{Ca(L)}}\) block. In some experiments, we also tested the effects of these compounds on contractions to evaluate the trigger functions of \(I_{\text{Ca(L)}}\) and \(I_{\text{Na/Ca}}\). A preliminary account of some of these findings has been presented (Shen et al., 1999).

Materials and Methods

Isolation of Ventricular Myocytes. Single ventricular myocytes were enzymatically isolated from the hearts of male and female guinea pigs (250–450 g) anesthetized with sodium pentobarbital (30 mg/kg i.p.) and anticoagulated with heparin (1000 I.U. i.p.). The heart was retrogradely perfused with Tyrode’s solution for 5 min at a rate of 8 to 10 ml/min through an aortic cannula in a Langendorff apparatus. The composition of Tyrode’s solution was 155 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 10 mM HEPES, and 20 mM glucose; pH was adjusted to 7.4 with NaOH. After disruption of the extracellular matrix with collagenase and protease, the enzymes were washed out by perfusion with 50 ml of Recovery solution. Recovery solution contained 130 mM potassium aspartate, 5 mM K\(_3\)ATP, 5 mM HEPES, and 20 mM glucose; pH was adjusted to 7.4 with KOH. The ventricles were removed and the cells were dispersed in Recovery solution and kept at 4°C for at least an hour. An aliquot of cell suspension was placed in a recording chamber (500-μl volume) mounted on the stage of an inverted microscope. After 10 min, it was superfused with Tyrode’s solution (2 ml/min); the glucose concentration was 10 mM for experiments. Temperature was 35°C.

Electrophysiology. An EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) was used to deliver voltage pulses in whole-cell mode. Voltage commands and current data acquisition were controlled by an IBM-compatible computer equipped with pClamp software (version 5.5; Axon Instruments, Burlingame, CA) and a Labmaster TL-1 interface (Axon Instruments). Glass capillary electrodes (1.1 mm i.d.; 1.3 mm o.d.) were filled with a pipette solution whose composition was 120 mM potassium aspartate, 30 mM KCl, 5 mM Na\(_3\)ATP, 10 mM MgCl\(_2\), and 5 mM HEPES; pH was adjusted to 7.3 with KOH. The resistance was 2 to 4 MΩ. In initial experiments, the pipette was filled with a Cs\(^{+}\)-rich solution with EGTA containing 135 mM cesium aspartate, 10 mM NaCl, 5 mM MgATP, 5 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.3 with CsOH. Accordingly, 10 mM CaCl\(_2\) was added to the bath solution. The pipette was connected to the amplifier by a Ag-AgCl wire, and the tip was gently pushed against the cell surface. Negative pressure was applied to the pipette interior until a gigahm seal was formed. After the electrode capacitance was compensated electronically in the cell-attached mode, the cell membrane was ruptured by additional negative pressure.

Drugs and Application. Calcium channel-blocking drugs were applied to myocytes by rapid superfusion from a reservoir via solenoid-controlled delivery. The time for complete solution change, estimated from the membrane current response to doubling the extra-cellular K\(^{+}\) concentration, was \(< 1\) s with a \(t_{1/2}\) of \(\sim 200\) ms. The applied solutions were warmed and the outlet of the rapid solution device brought within 50 μm of the cell. After recording conditions for the cell had stabilized, the rapid solution device was turned on and Tyrode’s solution, identical with the bath solution, applied to the cell. The temperature at the cell’s position transiently changed by 0.2–0.3°C when first switching on the Tyrode’s solution and by \(\pm 0.2\)°C when switching from Tyrode’s solution to a test solution. Nifedipine (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide and prepared fresh daily from the stock solution. All nifedipine solutions were protected from light during preparation, storage, and use.

Cell Contraction. A video-edge detector system (Crescent Electronics, Sandy, UT) tracked cell edge motion. A microscope-magnified (400 ×) cell image was continuously observed on a high-resolution TV monitor via a sequential scanning video camera attached to a side port of the microscope. The camera position was rotated so that the video monitor raster lines were parallel with the long axis of the cell. The video dimension analyzer monitored a selected raster line for light intensity differences between the end of the myocyte and the surrounding field. The signal from the detector was sent to a strip chart recorder and to a videocassette recorder for storage and off-line analysis.

Data Analysis. Steady-state \(I_{\text{Ca(L)}}\) block by nifedipine could be readily measured in the presence of Cs\(^{+}\)-containing solutions. In this case, the extent of block was taken as absolute peak inward current. When K\(^{+}\)-containing solutions were used, \(I_{\text{Ca(L)}}\) block by nifedipine was standardized against that caused by 0.1 to 0.3 mM Cd\(^{2+}\). The latter has been shown to block \(I_{\text{Ca(L)}}\) completely (Hobai et al., 1997). Measurements are reported as mean ± S.E.

Results

Concentration-Dependent Block of \(I_{\text{Ca(L)}}\) by Nifedipine in Steady State

Initially, we determined the concentration-dependent block of \(I_{\text{Ca(L)}}\) by nifedipine in the absence of K\(^{+}\)-currents (see Materials and Methods). Membrane voltage was stepped from −80 to −40 mV for 350 ms to inactivate the fast Na\(^{+}\) and the \(I_{\text{Ca(T)}}\) currents. A second voltage jump to +10 mV for 300 ms elicited \(I_{\text{Ca(L)}}\); the clamp protocol was repeated at 0.1 Hz. Block of \(I_{\text{Ca(L)}}\) by Cd\(^{2+}\) (0.1, 0.3, 1.0 mM) appeared complete because the peak of early inward current was positive after Cd\(^{2+}\) application for at least 2 min. The average Cd\(^{2+}\)-sensitive currents of eight cells are essentially equal and amounted to 854 ± 126 (0.1 mM), 857 ± 131 (0.3 mM), and 856 ± 133 pA (1 mM). The effect of 0.1 mM Cd\(^{2+}\) was taken as the standard for 100% block of \(I_{\text{Ca(L)}}\).

Nifedipine (0.1–100 μM) was cumulatively applied to the same myocyte; each concentration was present for at least 3 min. Half-maximal inhibition (IC\(_{50}\) of \(I_{\text{Ca(L)}}\) occurred at 0.3
μM nifedipine when the holding potential was −80 mV between test pulses (Fig. 1, filled squares). In steady state, nifedipine blocked \( I_{\text{Ca(L)}} \) by 94 ± 2.3 (30 μM) and 99 ± 0.8% (100 μM), respectively. When nifedipine was applied at a single rather than cumulative concentrations, \( I_{\text{Ca(L)}} \) block averaged 91 ± 3.5% for 30 μM (n = 5 cells) and 99 ± 4.0% with 100 μM nifedipine (n = 4 cells). A mixture of 30 μM nifedipine plus 30 μM Cd\(^{2+}\) (Nif 30/Cd 30) blocked \( I_{\text{Ca(L)}} \) by 97 ± 1.6% (n = 4 cells). The IC\(_{50}\) for nifedipine block of \( I_{\text{Ca(L)}} \) decreased to 50 nM when the holding potential was maintained at −40 mV between test pulses (Fig. 1, filled circles).

Steady-state inactivation of \( I_{\text{Ca(L)}} \) was determined in the absence and presence of 0.3 μM nifedipine (n = 4 cells). A 1-s command changed the conditioning potential from −80 to +10 mV in 10-mV steps. Afterward, a 10-ms return to −40 mV was inserted before applying a 200-ms jump to the test potential of +10 mV. Inactivation of \( I_{\text{Ca(L)}} \) was described by a Boltzmann relation (Fig. 2). Voltage-dependent inactivation of \( I_{\text{Ca(L)}} \) at 50% was shifted from −36 ± 3.3 (control) to −53 ± 1.6 mV (nifedipine). The slope factor was 14 ± 1.5 and 15 ± 1.5 in control and nifedipine, respectively. Steady-state block by nifedipine of \( I_{\text{Ca(L)}} \) in the presence of K\(^+-\)rich pipette solution was essentially the same as in the presence of Cs\(^+-\) rich pipette solution (vide infra).

Concentration- and Time-Dependent Block of \( I_{\text{Ca(L)}} \) by Rapidly Applied Nifedipine and/or Cadmium

Nifedipine Alone. In some studies of excitation-contraction (E-C) coupling (see the Introduction), the ability to suppress \( I_{\text{Ca(L)}} \) completely on the first test pulse after rapid application of a blocking agent has been emphasized. Thus, \( I_{\text{Ca(L)}} \) block should be maximal and complete on the first test pulse and be invariant during a train of test pulses. The protocol to test this hypothesis included two trains of 200-ms voltage-clamp pulses (−40 to +10 mV at 0.5 Hz) separated by a 10-s rest interval at −40 mV. The test-blocking agent was applied by rapid superfusion and present throughout the 10-s interval and the second train of test pulses. Membrane voltage was held at −40 mV to promote the blocking effect of nifedipine; the pipette solution was K\(^+-\)rich (see Materials and Methods).

The results in Fig. 3A illustrate the effects of nifedipine (30 and 100 μM) and Cd\(^{2+}\) (300 μM) in one ventricular myocyte. The nifedipine-sensitive \( I_{\text{Ca(L)}} \) was standardized against the complete block of \( I_{\text{Ca(L)}} \) that is caused by Cd\(^{2+}\). The control currents on the 1st and 10th test pulses are labeled “0”. The Cd\(^{2+}\)-sensitive \( I_{\text{Ca(L)}} \) was practically the same on the 1st (−1375 pA) and 10th (−1430 pA) test pulses (traces labeled “3”). Suppression of \( I_{\text{Ca(L)}} \) by 30 μM nifedipine (traces labeled “1”) was incomplete on the 1st test pulse, increased with successive test pulses, yet was still less than maximum on

\[ \text{Ordinate, } I_{\text{Ca(L)}} \text{ as fraction of maximum; abscissa, conditioning membrane potential. The curves are drawn according to Boltzmann relations; see text for details.} \]

\[ \text{Fig. 2. Steady-state inactivation of } I_{\text{Ca(L)}} \text{ in the absence (○) and presence of 0.3 μM nifedipine (●). The experimental protocol is shown as an inset. Ordinate, } I_{\text{Ca(L)}} \text{ as fraction of maximum; abscissa, conditioning membrane potential. The curves are drawn according to Boltzmann relations; see text for details.} \]

\[ \text{Fig. 3. Development of } I_{\text{Ca(L)}} \text{ block by nifedipine (30 and 100 μM) or 300 μM Cd}^{2+}; \text{ K}^{-}-\text{rich pipette solution. After 10 conditioning pulses (see inset at top of } A \text{ for protocol), nifedipine or } \text{ Cd}^{2+} \text{ was applied during a 10-s rest interval and 10 subsequent test pulses. A, membrane currents in one cell in control (○), 30 ( ), or 100 μM (2) nifedipine or 300 μM Cd}^{2+} \text{ (3) on the 1st (top) and 10th test pulse (bottom) after the 10-s interval. B, summary of results with unblocked } I_{\text{Ca(L)}} \text{ (a), use-dependent (b), and use-independent (c) block of } I_{\text{Ca(L)}}. \]
the 10th test pulse (Fig. 3A). With 100 μM nifedipine (traces labeled “2”), suppression of \( I_{\text{Ca(L)}} \) was greater than that by 30 μM on the 1st test pulse and almost equaled that by 300 μM Cd\(^{2+}\) on the 10th test pulse.

The results of eight experiments of this type are shown in Fig. 3B. \( I_{\text{Ca(L)}} \) is divided into three components: nonblocked, use-dependent, and use-independent block. The percentage block of \( I_{\text{Ca(L)}} \) on the first test pulse is attributed to use-independent inhibition (Fig. 3B, c). The difference of percent-block of use-dependent, and use-independent block. The percentage labeled “2”), suppression of use-independent block of \( I_{\text{Ca(L)}} \) is the nonblocked \( I_{\text{Ca(L)}} \) by nifedipine (Fig. 3B, a). For 30 μM nifedipine, use-independent inhibition of \( I_{\text{Ca(L)}} \) was 85 ± 4.4%, use-dependent inhibition of \( I_{\text{Ca(L)}} \) was 8%, and the nonblocked \( I_{\text{Ca(L)}} \) was 7%. Raising nifedipine to 100 μM yielded use-independent block of 95 ± 2.3%. For Cd\(^{2+}\), there is a greater use-independent inhibition of \( I_{\text{Ca(L)}} \) amounting to 99 ± 1.0% and a small use-dependent inhibition of 0.6%. The probability of completely blocking \( I_{\text{Ca(L)}} \) on the first test pulse after 10-s application is greatest with Cd\(^{2+}\), intermediate with 100 μM, and least with 30 μM nifedipine.

Our aim was to ascertain the optimal conditions to achieve complete \( I_{\text{Ca(L)}} \) block by nifedipine on the first test pulse because this is desirable for evaluating E-C coupling triggers. With the same protocol and standardization as previously used (Fig. 3), different concentrations of nifedipine (10, 30, 100 μM) were applied during selected rest intervals (5, 10, 15, 20, 30 s) plus test periods. These results are summarized in Fig. 4. Use-independent inhibition of \( I_{\text{Ca(L)}} \) progressively increased with nifedipine concentration. After 5-s application, use-independent block of \( I_{\text{Ca(L)}} \) averaged 70 ± 9.7, 80 ± 4.4, and 93 ± 3.6% at 10, 30, and 100 μM nifedipine, respectively. After 30-s application, 10, 30, and 100 μM nifedipine produced 93 ± 4.3, 93 ± 2.8, and 95 ± 2.5% use-independent inhibition of \( I_{\text{Ca(L)}} \), respectively. Thus, the likelihood of blocking \( I_{\text{Ca(L)}} \) completely on the first test pulse increased as a function of time as well as concentration of nifedipine. The decline of use-dependent fraction of block was monoexponential in the first 20 s with rate constants of –0.13 s\(^{-1}\) and –0.09 s\(^{-1}\) at 10 and 30 μM nifedipine, respectively. At 100 μM nifedipine, the use-dependent inhibition of \( I_{\text{Ca(L)}} \), declined at a rate of –0.04 s\(^{-1}\); this estimate is less reliable because use-dependent block varied from 6 to 4% at 5- to 20-s application times. However, at 100 μM nifedipine, the fraction of nonblocked \( I_{\text{Ca(L)}} \) was practically abolished an indication that under steady-state conditions (10th test pulse), 100 μM would completely suppress \( I_{\text{Ca(L)}} \).

**Nifedipine and Inactivation of \( I_{\text{Ca(L)}} \)**

Nifedipine increased the rate of \( I_{\text{Ca(L)}} \) inactivation (Lee and Tsien, 1983). We evaluated the hypothesis that some block of \( I_{\text{Ca(L)}} \), occurs during the first test pulse in experiments with 3 μM nifedipine. The voltage-clamp protocol was the same as described in Fig. 3. In control, \( I_{\text{Ca(L)}} \) inactivated in a biexponential manner with time constants (in milliseconds) \( \tau_1 \) and \( \tau_2 \) of 5.5 ± 0.4 and 51.3 ± 6.0 on the 1st test pulse and 5.2 ± 0.4 and 59.0 ± 3.3 on the 10th test pulse, respectively (\( n = 11 \) cells). In nifedipine, \( \tau_1 \) decreased by 20% to 4.4 ± 0.5 ms (1st test pulse; \( P = .006 \)) and by 33% to 3.5 ± 0.4 ms (10th test pulse; \( P = .002 \)). There was a tendency of \( \tau_2 \) to decrease in nifedipine on the 1st (46.8 ± 3.8 ms) and 10th (53.3 ± 8.2 ms) test pulses, respectively. However, these reductions were not statistically significant (\( P = .3 \) and .36, respectively). These averages are from all 11 cells in which \( \tau_1 \) and \( \tau_2 \) were reduced in seven cells. It was difficult to discern two phases of inactivation accurately at higher nifedipine concentrations. We measured the half-time for inactivation (\( t_{1/2} \)) in experiments with 30 μM nifedipine (\( n = 8 \) cells) and found that the control \( t_{1/2} \) was reduced from 11.5 ± 1.8 to 8.5 ± 1.2 ms on the 1st test pulse (\( P = .01 \)) and from 12.0 ± 2.2 to 8.5 ± 1.2 on the 10th test pulse (\( P = .01 \)).

**Test of Combination of Nifedipine and Cadmium**

Cadmium inhibited \( I_{\text{Ca(L)}} \) with an IC\(_{50}\) of ~2 μM (Hobai et al., 1997). The kinetics of block by Cd\(^{2+}\) is rapid (Lansman et al., 1986) and there is little use-dependent inhibition of \( I_{\text{Ca(L)}} \) at 10 s (Fig. 3). After 5-s application, Cd\(^{2+}\) inhibited 98 ± 2.0% of \( I_{\text{Ca(L)}} \) elicited on the first test pulse (\( n = 13 \)). The residual of 2% is less than that seen with nifedipine and was abolished at ≥10-s application intervals (<1%). Thus, 0.3 mM Cd\(^{2+}\) inhibited \( I_{\text{Ca(L)}} \) nearly completely on the first test pulse at ≥10 s. Because 0.3 mM Cd\(^{2+}\) inhibits \( I_{\text{Na/Ca}} \) by almost 50%, it cannot be used to distinguish the triggering roles of \( I_{\text{Ca(L)}} \) versus \( I_{\text{Na/Ca}} \) in E-C coupling (Hobai et al., 1997).

Inhibition of \( I_{\text{Na/Ca}} \) at 30 μM Cd\(^{2+}\) is predicted to be ≥3%, whereas suppression of \( I_{\text{Ca(L)}} \) is estimated at 88% by these authors. At 30 μM, Cd\(^{2+}\) inhibited \( I_{\text{Ca(L)}} \) by 83 ± 2.2 (\( n = 5 \)), 90 ± 2.2 (\( n = 6 \)), and 95 ± 1.3% (\( n = 4 \)) on the first test pulse after 5-, 10-, and 15-s rest intervals, respectively. Inhibition by 30 μM Cd\(^{2+}\) amounted to 96 ± 1.2% at the 10th test pulse, which was steady state (\( n = 15 \)). We tested the effects of Nif 30/Cd 30 to increase the use-independent inhibition of \( I_{\text{Ca(L)}} \). On the 1st and 10th test pulses after 10-s application of Nif 30/Cd 30 (Fig. 5A, traces labeled “1”), peak inward current was essentially the same as that seen after 0.3 mM Cd\(^{2+}\)
alone (Fig. 5A, traces labeled “2”). The inward shift of holding current at −40 mV with 0.3 mM Cd<sup>2+</sup> may result from I<sub>Na/Ca</sub> suppression. The end-of-pulse currents were the same in the presence and absence of the test agents. A summary of experiments after 10-s application is given in Fig. 5B. Nif 30/Cd 30 produced a use-independent inhibition of I<sub>Ca(L)</sub> of 95 ± 4.5% at 5-s, 97 ± 1.8% at 10-s, and 96 ± 4.1% at 15-s application. Thus, combining low concentrations of Cd<sup>2+</sup> and nifedipine produced use-independent inhibition of I<sub>Ca(L)</sub> at 10 s that was equivalent to that seen with 100 μM nifedipine.

**Relationship between Block of I<sub>Ca(L)</sub> and Contraction by Test Ligands**

Experiments at +10 mV. We repeated the experiments with ligands used to block I<sub>Ca(L)</sub> to ascertain the coupling between the L-type Ca<sup>2+</sup> current and contraction at a test potential of +10 mV. The protocol is shown in the upper portion of Fig. 6B. Six 200-ms conditioning pulses from −80 to 30 mV were applied at 1 Hz to maintain a relatively constant SR Ca<sup>2+</sup> content. During the 10-s pause, membrane voltage was held at −40 mV; a single 200-ms pulse to +10 mV elicited I<sub>Ca(L)</sub> and a contraction. Results from an experiment on a single ventricular myocyte are shown in Fig. 6. The control I<sub>Ca(L)</sub> and its corresponding contraction are shown in Fig. 6, A and B, respectively. The current and contraction obtained on the first test pulse after 10-s application of 0.3 mM Cd<sup>2+</sup> are indicated by the filled squares; both variables are completely suppressed. The records just after washout of Cd<sup>2+</sup> are not shown. Subsequently, 30 μM nifedipine was tested; I<sub>Ca(L)</sub> and its accompanying contraction were greatly, but not completely blocked (filled circles). Like 0.3 mM Cd<sup>2+</sup>, Nif 30/Cd 30 completely blocked the test I<sub>Ca(L)</sub> and its contraction on the first test pulse after 10 s. Test contraction recovered essentially completely after washout of Nif 30/Cd 30 (Fig. 6B, bottom). Figure 6A (bottom) shows amplified, superimposed current traces taken from the cell. A transient inwardly directed current is evident in the test of 30 μM nifedipine. In contrast, the current traces at +10 mV in either 0.3 mM Cd<sup>2+</sup> or Nif 30/Cd 30 do not show this inwardly directed transient. The inward shift of current in 0.3 mM Cd<sup>2+</sup> is consistent with suppression of I<sub>Na/Ca</sub> which is outward at +10 mV. The end-of-pulse currents at +10 mV are the same in 30 μM nifedipine and Nif 30/Cd 30.

A summary of all such experiments is shown in Fig. 7. The control I<sub>Ca(L)</sub> (999 ± 150 pA) diminished by 690 ± 100 pA on the first test pulse in 10 μM nifedipine (72 ± 5.5%); contractions decreased by 61 ± 6.8% from an initial value of 3.7 ± 0.50 to 1.6 ± 0.52 μm. At 30 μM, nifedipine reduced I<sub>Ca(L)</sub> from 1352 ± 249 pA at control to 265 ± 93 pA (83 ± 3.6% block) and contraction from 4.0 ± 0.53 to 1.0 ± 0.47 μm (79 ± 7.8% decrease) in nine cells. In three of these nine cells (33%), the contraction was abolished on the first test pulse. In the remaining six cells, the latency between the onset of I<sub>Ca(L)</sub> and contraction averaged 30 ± 6.5 ms in control and 30 ± 4.9 ms in 30 μM nifedipine, respectively. After 10 s in 100 μM nifedipine, the I<sub>Ca(L)</sub> decreased from 1052 ± 352 pA at control to 89 ± 28.6 pA (92 ± 2.1% block) and contraction diminished from 2.9 ± 0.20 to 0.14 ± 0.10 μm (96 ± 2.8% decrease) in 14 cells. Contractions on the first test pulse were eliminated completely in 12 of these 14 cells (86%). Adding Nif 30/Cd 30 significantly decreased I<sub>Ca(L)</sub> at control from 1395 ± 212 to 65 ± 21 pA (94 ± 2.2% block) and abolished contraction in six of seven cells (86%). The average contraction of all the cells was reduced from 4.4 ± 0.43 to 0.1 ± 0.09 μm (98 ± 2% decrease). The results indicate that the greater the inhibition of I<sub>Ca(L)</sub>, the more likely contraction will be abolished at +10-mV test potential.
Membrane Current and Cell Contraction at \( \approx +50 \text{ mV} \)

Reverse mode \( I_{\text{Na/Ca}} \) increases and \( I_{\text{Ca(L)}} \) decreases as membrane voltage becomes more positive. We tested the hypothesis that \( I_{\text{Ca(L)}} \) may be present at +50 mV. From a holding potential of -40 mV, membrane voltage was jumped in 10-mV steps to +100 mV at 0.33 Hz before and after addition of 100 \( \mu \text{M} \) nifedipine (\( n = 6 \) cells). The reversal potential of nifedipine-sensitive \( I_{\text{Ca(L)}} \) was 68 ± 2.7 mV, which is comparable to that seen with nisoldipine (Sipido et al., 1997).

We next evaluated the effects of 100 \( \mu \text{M} \) nifedipine (Fig. 8A) or Nif 30/Cd 30 (Fig. 8B) on membrane current and contraction at +50 and +100 mV with the same conditioning protocol as in Fig. 6. At +50 mV, the average change in membrane current was 4.0 ± 0.59 \( \mu \text{A} \) (\( n = 13 \) cells), essentially the same as at +10 mV (3.6 ± 0.21 \( \mu \text{A} \); \( n = 38 \); \( P = .42 \)). Initial membrane current at +50 mV shifted outward by 120 \( \mu \text{A} \) in 100 \( \mu \text{M} \) nifedipine (Fig. 8A, left, inset). In seven such experiments, nifedipine shifted peak membrane current at +50 mV outward by 212 ± 30.0 \( \mu \text{A} \) (\( P < .01 \)) yet the end-of-pulse current differed by only 14 ± 15.1 \( \mu \text{A} \) (\( P = .38 \)). There was no significant change in membrane currents at either -40 (14 ± 18.1 \( \mu \text{A} \); \( P = .48 \)) or -80 mV (9 ± 14.3 \( \mu \text{A} \); \( P = .81 \)). These results can be explained by block of inwardly directed \( I_{\text{Ca(L)}} \) at +50 mV with no effect on \( I_{\text{Na/Ca}} \). In the example shown in Fig. 8A, cell shortening decreased from 3.2 (control) to 1.9 \( \mu \text{m} \) in nifedipine; contraction latency increased from 45 to 75 ms. Nifedipine completely blocked contraction at +50 mV in only one cell. In the remaining six cells, cell shortening was reduced from 4.4 ± 0.67 to 2.5 ± 0.67 \( \mu \text{m} \) (43% decrease; \( P = .04 \)) and the latency to contraction onset was 67 ± 4.9 ms in 100 \( \mu \text{M} \) nifedipine compared with 48 ± 3.0 ms in control (\( P = .01 \)).

The combination of Nif 30/Cd 30 was tested in the remaining six cells. In the example shown (Fig. 8B, left), peak current shifted outward by 225 \( \mu \text{A} \). Control contraction amplitude and latency were 7.0 \( \mu \text{m} \) and 60 ms, respectively; these values changed to 2.2 \( \mu \text{m} \) and 100 ms in Nif 30/Cd 30. Nif 30/Cd 30 had the same effects on membrane current as 100 \( \mu \text{M} \) nifedipine. Thus, Nif 30/Cd 30 (\( n = 6 \)) did not change current at -40 mV (-12 ± 6.8 \( \mu \text{A} \); \( P = .72 \)) or at -80 mV (33 ± 18.1 \( \mu \text{A} \); \( P = .30 \)). However, peak current at +50 mV shifted outward by 218 ± 30.4 \( \mu \text{A} \) (\( P < .01 \)) and not at end of pulse (9 ± 26.7 \( \mu \text{A} \); \( P = .95 \)). Contractions ceased in three of six cells with Nif 30/Cd 30 and decreased from 3.5 ± 1.0 to 1.2 ± 0.70 \( \mu \text{m} \) (66% decrease; \( P = .01 \)) in the remainder. In these three cells, contraction onset was delayed from 53 ± 11.5 to 107 ± 11.5 ms by Nif 30/Cd 30 (\( P < .01 \)). Thus, at +50 mV, there is an inwardly directed \( I_{\text{Ca(L)}} \), whose block by nifedipine or Nif 30/Cd 30 not only decreased the extent of cell shortening but also delayed the onset of contraction. Addition of 5 mM Ni\(^{2+}\) shifted membrane current inward at +50 and -40 mV and outward at -80 mV. In six experiments with Ni\(^{2+}\), membrane current shifted inward by -238 ± 42.2 \( \mu \text{A} \) at -40 mV (\( P < .01 \)) and outward by 158 ± 46.5 \( \mu \text{A} \) at -80 mV (\( P < .01 \)). Thus, Ni\(^{2+}\) suppressed a current whose reversal potential is between -40 and -80 mV, consistent with its being \( I_{\text{Na/Ca}} \). Nickel had no effect on peak current at +50 mV (-33 ± 86.1 \( \mu \text{A} \); \( P = .19 \)) presumably because the outward shift from \( I_{\text{Ca(L)}} \) block was offset by an inward shift due to \( I_{\text{Na/Ca}} \) suppression. Evidence for the latter is the inward shift in end-of-pulse current by -273 ± 53.5 \( \mu \text{A} \) (\( P < .01 \)). Contractions were completely eliminated by 5 mM Ni\(^{2+}\) (\( n = 6 \) cells).
At +100 mV, current through L-type Ca\(^{2+}\) channels should be outward and carried by K\(^+\) (Lee and Tsien, 1983). Nifedipine (100 \(\mu\)M) shifted peak membrane current slightly inward by −35 pA at +100 mV (Fig. 8A, right). Neither contraction amplitude (3.2 \(\mu\)m) nor latency (70 ms) changed in nifedipine. On average, nifedipine shifted peak current inward by −180 ± 44 pA (n = 6 cells). With Nif 30/Cd 30 (Fig. 8B, right), current shifted inward by −100 pA. Cell shortening increased slightly from 5.8 to 6.0 \(\mu\)m, whereas latency remained constant at 70 ms. The Nif 30/Cd 30-sensitive current of −152 ± 38 pA (n = 6) was indistinguishable from that of nifedipine. Before drug addition, the average amplitude of contraction at +100 mV (4.9 ± 0.64 \(\mu\)m; n = 12) is larger than at +50 mV (4.0 ± 0.59 \(\mu\)m), and the latency to onset of the contraction at +100 mV (81 ± 4.5 ms) is greater than at 50 mV (50 ± 2.9 ms). Neither 100 \(\mu\)M nifedipine nor Nif 30/Cd 30 significantly changed contraction amplitude at +100 mV. In six cells, contraction amplitude averaged 4.8 ± 0.80 \(\mu\)m in control versus 4.3 ± 0.80 \(\mu\)m with 100 \(\mu\)M nifedipine (\(P = .12\)). In another six cells, cell shortening averaged 5.0 ± 1.0 and 5.3 ± 1.0 \(\mu\)m in control and Nif 30/Cd 30, respectively (\(P = .14\)). Contraction onset at +100 mV is slightly delayed by 100 \(\mu\)M nifedipine from 75 ± 6.2 to 88 ± 8.7 ms (\(P = .08\)) and by Nif 30/Cd 30 from 87 ± 6.7 to 95 ± 8.8 ms (\(P = .09\)), respectively.

**Discussion**

Rapidly applied nifedipine displayed use-dependent and use-independent components of \(I_{\text{Ca(L)}}\) block. The steady-state IC\(_{50}\) was 0.3 \(\mu\)M at a holding potential of −80 mV and 50 nM when the membrane was held at −40 mV (Lee and Tsien, 1983; Yamamoto et al., 1990). Depolarization promotes \(I_{\text{Ca(L)}}\) block because DHP affinity for receptor is greatest as L-type channels shift toward the inactivated state (Carmeliet and Mubagwa, 1998). The \(V_{1/2}\) for steady-state inactivation of \(I_{\text{Ca(L)}}\) shifted by 17 mV to more negative potentials in 0.3 \(\mu\)M nifedipine as predicted (Sunami et al., 1995; for review, see Carmeliet and Mubagwa, 1998).

**Use-Independent Block of \(I_{\text{Ca(L)}}\)** Fractional block of \(I_{\text{Ca(L)}}\) on the first test pulse increased with nifedipine concentration and application time, a finding favorable for studying contraction triggering mechanisms. Neutral DHPs rapidly partition into the plasma membrane lipid bilayer (Herbette et al., 1989), binding at hydrophobic amino acids −11 to 14 Å from the external plasma membrane surface (Bangalore et al., 1994) of the sixth transmembrane segments of domains III and IV in the L-type Ca\(^{2+}\) channel α1-subunit (Hockerman et al., 1997). Membrane partitioning did not limit kinetics of nifedipine action in frog ventricular myocytes (Méry et al., 1996).

**Use-Dependent Block of \(I_{\text{Ca(L)}}\) by Nifedipine.** Use-dependent block diminished as nifedipine concentration and time increased, and was least with Nif 30/Cd 30. Use-dependent block by nifedipine after 10 test pulses underestimates the magnitude of this component at steady state. DHPs exert use-dependent block of \(I_{\text{Ca(L)}}\) in mammalian and amphibian ventricular myocytes even when present during 3- to 15-min rest intervals (Lee and Tsien, 1983; Uehara and Hume, 1985; Sunami et al., 1995) and when rapidly applied at saturating concentrations (Levi and Issberner, 1996; Méry et al., 1996). Some block of \(I_{\text{Ca(L)}}\) by nifedipine (≤30 \(\mu\)M) appears during the first test pulse because the early inactivation phase, but not the second, decreased significantly. Our experimental conditions cannot distinguish it from inactivated-state block (for review, see Carmeliet and Mubagwa, 1998). DHP agonists and antagonists accelerate inactivation of \(I_{\text{Ca(L)}}\) or \(I_{\text{Na(Ca)}}\) (Lee and Tsien, 1983; Hess et al., 1985); this is attributed to a transition from mode 1 gating to mode 0 gating where the Ca\(^{2+}\) channel is stabilized and unavailable for opening (Hess et al., 1985). Nifedipine (Lee and Tsien, 1983), nisoldipine (Sanguinetti and Kass, 1984), and nifedipine (Álvarez and Vassort, 1992) accelerated \(I_{\text{Ca(L)}}\) inactivation during the first test pulse.

**Nifedipine Effects on \(I_{\text{Ca(L)}}\) and Contraction at +10 mV.** In steady state, nifedipine suppressed the slow inward current and contraction force by 50% at 0.3 and 0.5 \(\mu\)M, respectively (Bayer et al., 1977). We find contraction eliminated when either nifedipine or Nif 30/Cd 30 suppressed \(I_{\text{Ca(L)}}\) by ≥95% on the first test pulse after a 10-s rest exposure. The advantage of Nif 30/Cd 30 is the presence of ligands that use hydrophobic and hydrophilic pathways to their receptor sites. Both ligands are largely use-independent, unlike nifedipine combined with the verapamil analog D600 (Howarth and Levi, 1998); D600 is very use-dependent (for review, see Carmeliet and Mubagwa, 1998).

Nifedipine blocked \(I_{\text{Ca(L)}}\) less and contractions more on the first test pulse at +10 mV than that reported by other studies. The residual inward current in nifedipine at +10 mV (Fig. 3A) could be remaining \(I_{\text{Ca(L)}}\) (Sipido et al., 1995; Evans and Cannell, 1997). Alternatively, the current could be forward mode \(I_{\text{Na(Ca)}}\), which progressively diminishes as SR Ca\(^{2+}\) content decreases during the pulse train. Assuming complete \(I_{\text{Ca(L)}}\) block on the first test pulse, some studies have proposed that reverse mode \(I_{\text{Na(Ca)}}\) triggered the residual contraction or Ca\(^{2+}\) transient in nifedipine or verapamil (Vornanen et al., 1994; Levi and Issberner, 1996; Levi et al., 1996; Wasserstrom and Vites, 1996). Several lines of evidence implicate unblocked \(I_{\text{Ca(L)}}\) as the residual inward current at +10 mV. First, although 0.3 mM Cd\(^{2+}\) blocks \(I_{\text{Na(Ca)}}\) by 50% and \(I_{\text{Ca(L)}}\) completely, the early inward current sensitive to 100 \(\mu\)M nifedipine or Nif 30/Cd 30 (Fig. 5) was often the same as the Cd\(^{2+}\)-sensitive current on the first test pulse. Neither nifedipine nor Nf 30/Cd 30 inhibited \(I_{\text{Na(Ca)}}\) between +10 and +100 mV. Second, we observed similar results with 0.1 mM Cd\(^{2+}\), which blocks \(I_{\text{Ca(L)}}\) completely yet has a lesser effect on \(I_{\text{Na(Ca)}}\) (Hobai et al., 1997). Third, the likelihood of blocking \(I_{\text{Ca(L)}}\) completely on the first test pulse increased as nifedipine concentration rose from 30 to 100 \(\mu\)M. Fourth, the residual peak inward current decreased but was not delayed (Fig. 3A) in nifedipine. We ascribe this to a smaller \(I_{\text{Ca(L)}}\). Inward \(I_{\text{Na(Ca)}}\) is delayed because it follows the intracellular Ca\(^{2+}\) transient (Grantham and Cannell, 1996; Sipido et al., 1997). Nifedipine (20 \(\mu\)M), applied rapidly during a 9-s rest interval, delayed the time to peak of intracellular Ca\(^{2+}\) transients initiated by action potentials in ventricular myocytes from transgenic mice overexpressing the Na\(^+\)/Ca\(^{2+}\) exchanger (Yao et al., 1998). This delay indicated that Ca\(^{2+}\) influx from reverse mode \(I_{\text{Na(Ca)}}\) is slower than that by \(I_{\text{Ca(L)}}\). In other experiments with ventricular myocytes from transgenic mice overexpressing the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger, Ca\(^{2+}\) influx via the exchanger did not trigger CICR at −10 to +20 mV yet \(I_{\text{Ca(L)}}\)-triggered CICR caused forward mode \(I_{\text{Na(Ca)}}\) (Adachi-Akahane et al., 1997). When nisoldipine com-
pletely blocked $I_{Ca,L}$, the peak of the intracellular Ca$^{2+}$ transient was delayed >80 ms (Sipido et al., 1997), an effect attributed to triggering by reversed $I_{Na,Ca}$. In the latter experiments, conditioning pulses to +60 mV maintained SR Ca$^{2+}$ content, defined by caffeine-induced Ca$^{2+}$ transients. In contrast, the residual intracellular Ca$^{2+}$ transient on the first test pulse at +10 mV in 32 µM nifedipine/10 µM D600 was attributed to reverse mode $I_{Na,Ca}$, yet the time to peak of the Ca$^{2+}$ transient did not change (Howarth and Levi, 1998).

Our results question the assumption that addition of ≤30 µM nifedipine is sufficient to block all $I_{Ca,L}$ on the first test pulse at +10 mV so that simultaneous exposure to a ligand such as Ni$^{2+}$ is unable to block such channels further.

**Nifedipine Effects on Membrane Current and Contraction at +50 and +100 mV.** Reverse-mode Na$^+$/Ca$^{2+}$ exchange can trigger Ca$^{2+}$ release at voltages ≥50 mV because disabling the SR with caffeine (Sham et al., 1992) or ryanodine (Sipido et al., 1997) blocks Ca$^{2+}$ release transients at +80 mV (but see Adachi-Akahane et al., 1997; Mattiello et al., 1998).

Nifedipine (100 µM) or NiF 30/Cd 30 partially suppressed contractions evoked at +50 mV. Even at an overshoot potential of +50 mV, the onset and the initial component of contraction appears closely related to the inwardly directed $I_{Ca,L}$. The residual contraction was significantly delayed in onset when $I_{Ca,L}$ was suppressed at +50 mV (Nuss and Houser, 1992; Sham et al., 1992; Vornanen et al., 1994; Sipido et al., 1997). At +70 mV, the intracellular Ca$^{2+}$ transient was essentially unchanged in amplitude and time to peak (Sipido et al., 1997) as expected at the Ca$^{2+}$ reversal potential. At the action potential peak, Ca$^{2+}$ influx via $I_{Na,Ca}$ is ~10 to 30% of that by $I_{Ca,L}$ (Grantham and Cannell, 1996; Sipido et al., 1997; Litwin and Bridge, 1998). At +100 mV, contraction amplitudes are greater and their latencies longer than at +50 mV. The former indicates that the relation between voltage and contraction is not bell shaped when the pipette solution contains 10 mM Na$^+$ (Vornanen et al., 1994; Wasserstrom and Vites, 1996; Litwin and Bridge, 1998). Blockers of $I_{Ca,L}$ did not affect the amplitude or latency of contractions at +100 mV; this excludes $I_{Ca,L}$, and implicates reverse mode $I_{Na,Ca}$.

**Limitations.** L-type Ca$^{2+}$ channel block by nifedipine was indexed with Cd$^{2+}$. The implications of nonselective block by Cd$^{2+}$ have been presented (vide supra). A second limitation centers around the relative gain of triggering (Stern et al., 1999) and the synergy of triggers (Litwin et al., 1998). Gain (Ca$^{2+}$ release from SR/L-channel Ca$^{2+}$ influx) decreases as membrane voltage approaches $E_{Ca}$ (Wier et al., 1994; Stern et al., 1999), yet the relative efficacy of $I_{Na,Ca}$ increases as Ca$^{2+}$ influx is blocked by drugs (Cannell et al., 1995). In contrast, Ca$^{2+}$ entry via reverse mode $I_{Na,Ca}$ and $I_{Ca,L}$ could interact synergistically with the former amplifying the trigger effect of the latter (Litwin et al., 1998). Our findings at +10 mV do not accord with the synergy hypothesis because ≥95% $I_{Ca,L}$ block prevented contractions and contraction latency did not shift at <95% $I_{Ca,L}$ block. Nifedipine (100 µM) or NiF 30/Cd 30 delayed residual contractions at +50 mV and had no effect on contraction amplitude and latency at +100 mV. We assume reverse mode $I_{Na,Ca}$ could trigger Ca$^{2+}$ transients and contractions at these positive potentials as reported by other studies (Nuss and Houser, 1992; Sham et al., 1992; Sipido et al., 1997; Litwin et al., 1998). However, some studies have not detected this outcome (Adachi-Akahane et al., 1998; Mattiello et al., 1998). Nifedipine-resistant contractions at these positive potentials (Fig. 8) did not relax until repolarization such as can occur with tonic entry of Ca$^{2+}$ (Nuss and Houser, 1992; Adachi-Akahane et al., 1997). Experiments that disable the SR release mechanism are needed to distinguish between triggered and tonic nifedipine-resistant contractions at positive potentials. Finally, experiments with action potential-initiated contractions should provide evidence about the physiological role of Ca$^{2+}$ influx via the exchanger on E-C coupling.

**References**


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