Modulation of the Gating of Unitary Cardiac L-Type Ca^{2+} Channels by Conditioning Voltage and Divalent Ions

Ira R. Josephson, Antonio Guia, Edward G. Lakatta, and Michael D. Stern
Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224 USA

ABSTRACT Although a considerable number of studies have characterized inactivation and facilitation of macroscopic L-type Ca^{2+} channel currents, the single channel properties underlying these important regulatory processes have only rarely been examined using Ca^{2+} ions. We have compared unitary L-type Ca^{2+} channel currents recorded with a low concentration of Ca^{2+} ions with those recorded with Ba^{2+} ions to elucidate the ionic dependence of the mechanisms responsible for the prepulse-dependent modulation of Ca^{2+} channel gating kinetics. Conditioning prepulses were applied across a wide range of voltages to examine their effects on the subsequent Ca^{2+} channel activity, recorded at a constant test potential. All recordings were made in the absence of any Ca^{2+} channel agonists. Moderate-depolarizing prepulses resulted in a decrease in the probability of opening of the Ca^{2+} channels during subsequent test voltage steps (inactivation), the extent of which was more dramatic with Ca^{2+} ions than Ba^{2+} ions. Facilitation, or increase of the average probability of opening with strong predepolarization, was due to long-duration mode 2 openings with Ca^{2+} ions and Ba^{2+} ions, despite a decrease in Ca^{2+} channel availability (inactivation) under these conditions. The degree of both prepulse-induced inactivation and facilitation decreased with increasing Ba^{2+} ion concentration. The time constants (and their proportions) describing the distributions of Ca^{2+} channel open times (which reflect mode switching) were also prepulse-, and ion-dependent. These results support the hypothesis that both prior depolarization and the nature and concentration of permeant ions modulate the gating properties of cardiac L-type Ca^{2+} channels.

INTRODUCTION

Although inactivation and facilitation of whole-cell L-type Ca^{2+} currents have been thoroughly studied (see McDonald et al., 1994, for a review), the single channel mechanisms underlying these important regulatory processes remain less clear. Moreover, previous studies concerning single L-type Ca^{2+} channel gating properties have used Ba^{2+} ions (Pietrobon and Hess, 1990; Hirano et al., 1999), a high concentration of Ca^{2+} ions with Ba^{2+} ions (Imredy and Yue, 1994), and/or the addition of an L-type Ca^{2+} channel agonist (Yue et al., 1990; Imredy and Yue, 1994) to increase the amplitude and/or duration of the channel openings. Such nonphysiological interventions may produce marked differences in Ca^{2+} channel behavior, including alterations in the single Ca^{2+} channel conductance (see Guia et al., 2001) and the voltage-dependent kinetics of channel gating.

The L-type Ca^{2+} channel is activated by membrane depolarization, and subsequent Ca^{2+} ion influx through the channel is self-limited during a sustained depolarization. In myocardial cells, as in other types of excitable cells, regulation of Ca^{2+} influx through L-type Ca^{2+} channels is achieved by controlling channel opening and closing in response to membrane potential and prior Ca^{2+} ion entry (Brehm and Eckert, 1978; Brown et al., 1981; Josephson et al., 1984; Lee et al., 1985; Hadley and Hume, 1987; Yue et al., 1990; Imredy and Yue, 1994). Thus, the inactivation of the L-type Ca^{2+} channel during depolarization results from both voltage-dependent and ion-dependent mechanisms (Brehm and Eckert, 1978; Brown et al., 1981; Josephson et al., 1984; Lee et al., 1985; Hadley and Hume, 1987). On the single channel level, ion-dependent inactivation has been characterized as a shift of gating from relatively frequent, brief openings (mode 1) to a lower open probability, termed "mode Ca" (Imredy and Yue, 1994).

Conversely, several types of facilitation are known to produce an enhancement of the L-type Ca^{2+} current (see Dolphin, 1996, for a review). Of these, strong conditioning depolarization has been shown to result in a (Ca^{2+}-independent) increase, or facilitation of the macroscopic cardiac L-type Ca^{2+} current that is related to an increase in the number of long-duration (mode 2) openings of the Ca^{2+} channel (Pietrobon and Hess, 1990).

Therefore, in the present paper and in the accompanying paper (Josephson et al., 2002) we have compared the effects of a nearly physiological concentration of Ca^{2+} ions, with a range of concentrations of Ba^{2+} ions (in the absence of any Ca^{2+} channel agonists), to investigate the single channel mechanisms involved in prepulse-dependent and ion-dependent inactivation and facilitation of the L-type Ca^{2+} channel. In the accompanying paper (Josephson et al., 2002) we report that in addition to alterations in gating, strong prepulses modulate the conductance of the Ca^{2+} channel, suggesting a more intimate association between these channel functions than was previously thought. Together with the results of the accompanying paper, these results suggest that the molecular mechanisms involved in prepulse-mediated alterations in both gating and conductance of single L-type

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Address reprint requests to Dr. Ira Josephson, Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, 5600 Nathan Shock Drive, Baltimore, MD 21224. Tel.: 410-558-8644; Fax: 410-558-8150; E-mail: josephsoni@grc.nia.nih.gov.

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Ca^{2+} channels are strongly influenced by the nature and concentration of the divalent ionic species permeating the channel. A preliminary report of some of the results has been presented in abstract form (Josephson et al., 2001).

MATERIALS AND METHODS

Myocyte preparation

Cells were isolated in accordance with National Institutes of Health guidelines for the care and use of animals. Male Sprague-Dawley rats (250–300 g, 2–3 months old) were anesthetized with pentobarbital (80–100 mg/kg, i.p.) and their hearts were removed via a transverse incision over the diaphragm. The hearts were washed in a nominally calcium-free modified Krebs solution (in mM: 120 NaCl; 5.4 KCl; 1.6 $MgSO_4$; 1 NaH_2PO_4; 20 $NaHCO_3$; 5.6 glucose; 5 taurine; gassed with 95% O_2, 5% CO_2) and then suspended and perfused via the aorta (constant pressure, 100 cm H_2O) on a heated (37°C) Langendorff apparatus. The hearts were cleared of extracellular calcium by nonrecirculating retrograde perfusion of the same solution for 5 min, then switched to a recirculating solution of similar content with the addition of protease (0.02 mg/ml, type XIV, Sigma Chemical Co., St. Louis, MO) and collagenase (1 mg/ml; type B, 220–230 U/mg, Boehringer-Mannheim, Indianapolis, IN, or type 2, Worthington, Lakewood, NJ), and, after 3 to 4 min in the enzymes, 50 μM $CaCl_2$ was added to the perfusate. At the end of the first digestion, the ventricles were chopped into several chunks and then placed into fresh Krebs solution containing 100 μM $CaCl_2$ and collagenase (1 mg/ml). This second digestion was allowed to proceed in a shaker (60–70 rpm) at 37°C until a satisfactory yield was obtained (10–15 min). The second digestion was quenched by filtering the supernatant for centrifugation at 500 $\times$ *g* and three subsequent washes with a modified Tyrode's solution (in mM: 137 NaCl; 4.9 KCl; 15 glucose; 1.2 $MgSO_4$; 1.2 NaH_2PO_4; 20 HEPES; NaOH, pH 7.4) with successively increasing calcium concentrations (250, 500, 1000 μM). The cells were allowed to settle from the supernatant for 10 min between washes. Cells were stored at room temperature in a similar Tyrode's solution containing 1 mM $CaCl_2$. The myocytes isolated in this manner were relaxed and rod-shaped, with clear sarcomeric striations and smooth, clean membranes.

Chemicals and solutions

All chemicals used in the cell isolation procedure were purchased from Mallinckrodt Chemicals Co. (Paris, KY), except for HEPES (ICN Biochemicals Inc., Aurora, OH) and $MgSO_4$ (Mallinckrodt Baker Inc., Phillipsburg, NJ). Chemicals used for physiological recordings were purchased from Sigma except for sucrose (ICN) and NaOH (Mallinckrodt). Pentobarbital (Sigma) was dissolved 30 mg/ml in a 10% ethanolic aqueous solution.

Single channel recording and analysis

We have previously demonstrated that unitary L-type Ca^{2+} channel currents can be reliably recorded with a low concentration of Ca^{2+} ions permeating the channel, and in the absence of channel agonists (Guia et al., 2001). Recording of unitary L-type Ca^{2+} channels was performed as previously described (Guia et al., 2001). Aliquots of cells were placed in a 0.1 ml bath mounted on the stage of a conventional inverted microscope. At least 10 min was allowed for the cells to attach to the coverslip on the bottom of the bath. The cells were then perfused with a high potassium depolarizing solution (HiK) at an approximate rate of 2–3 ml/min. The HiK solution (in mM: 120 potassium aspartate; 25 KCl; 10 HEPES; 10 glucose; 2 $MgCl_2$; 1 $CaCl_2$; 2 EGTA; 6 KOH, pH 7.2, 290 mOsm) was used to depolarize the cells to near 0 mV so that V_m was equal to $-$Vpatch. The free calcium concentration in the HiK solution was calculated to be ~80 nM. To allow stabilization in their new milieu, the cells were perfused with HiK for at least 20 min before unitary current measurements were conducted. All experiments were performed at room temperature (22.5–23.5°C).

Borosilicate pipettes made from Corning 7052 glass (1.5 OD, 0.86 ID, Model 5968, A-M Systems, Inc., Carlsborg, WA) were pulled in 3 or 4 heating cycles using a horizontal Flaming-Brown pipette puller (model P-97, Sutter Instrument Co., Novato, CA) or a CO_2 laser-based puller (model P-2000, Sutter Instrument Co.) to yield tips ~1 μm in diameter. The pipette tips were firepolished (model MF-83, Narishige Instrument Lab., Tokyo, Japan) to produce 8 to 15 MΩ tip resistances when filled with the pipette solutions, and were painted with a thick layer of silicone elastomer (Sylgard, Dow-Corning 184, Essex Brownell, Fort Wayne, IN, polymerized under a heat gun) to within 100 μm of the tip. Pipettes were filled with a solution containing $BaCl_2$ or $CaCl_2$ of the desired concentration, 10 mM CsCl and 5 mM 4-aminopyridine to block K^+ currents, 10 mM HEPES, and TEA-OH to pH 7.4, with sucrose added to maintain normal osmolarity. Pipettes were stored in a covered container and were back-filled with pipette solution and used immediately. Seal resistances of 50 to >300 GΩ were obtained by applying slight pressure with the pipette tip on the membrane, then applying gentle suction inside the pipette using a gas-tight glass syringe. For each seal, the pipette potential was offset to 0 mV with the pipette positioned near the membrane before initiating a seal. Formation of a stable seal was usually accomplished within a 20 to 30 s after the pipette potential was nulled. No other corrections were made for junction potentials. Membrane and pipette capacitances were corrected electronically. The noise at a bandwidth of 5 KHz was measured and only seals quieter than 250 fA RMS were used.

Current amplification was accomplished with an Axopatch 200B patch clamp (Axon Instruments Co., Burlingame, CA) and recorded on a computer hard disk using PClamp software (v. 6 and v. 8, Axon Instruments Co.) via a Digidata 1200A signal acquisition system. Data were filtered at 2 kHz ($-$3 dB, 4-pole Bessel) and digitized at 10 kHz sampling rate. A 100 ms prepulse that varied from $-$50 to +130 mV (in 20 mV increments) was immediately followed by a 300 or 400 ms test voltage step to $-$10 or 0 mV. These double voltage-step protocols were applied at a rate of 0.5 Hz (allowing for complete recovery between runs), from a holding potential (HP) of $-$50 mV. The entire 10-step double-pulse protocol was repeated 100–200 times, or until channel rundown was observed.

Each file from a series of repeated protocols was parsed and transposed into 10 files, using software developed in the laboratory. Each of these 10 files contained the episodes recorded at a given prepulse potential. The current traces were corrected for leakage and capacity currents by subtraction of an average of episodes devoid of single channel activity during the test voltage step (null sweeps). The identification of single channel opening and closing transitions using a 50% amplitude threshold (set constant for each experiment) was accomplished using Fetchan 6.0/PClamp (Axon Instruments). The rise time of our recording system (0.166 ms at 2 kHz) limited resolution of kinetic events to those lasting >0.2 ms. Thus, events shorter than 0.2 ms were not included in the kinetic analysis. The number of active Ca^{2+} channels in a given patch (*N*) was estimated by the maximum number of overlapping currents recorded upon repolarization following a prestep to +130 mV (maximal activation, and synchronization of mode 2 openings) and the probability of opening was calculated by dividing by *N*. Data were pooled from myocytes from multiple rat hearts; the total number of events analyzed was 111,500 for 105 mM Ba^{2+}, 54,826 for 10 mM Ba^{2+}, 30,345 for 5 mM Ba^{2+}, 12,714 for 2 mM Ba^{2+}, and 5,464 for 5 mM Ca^{2+}. The analysis of the probability of opening, open-time distributions and their exponential fits, amplitude distributions and their Gaussian fits, and scatterplots of amplitude versus duration were done using a modified version of pSTAT (PClamp, Axon Instruments). Data are reported as means $\pm$ SEM. Testing for statistical significance was accomplished using an analysis of variance (ANOVA), Dunnett's method, or Student's paired *t*-test, as was appropriate.

In this and the accompanying paper (Josephson et al., 2002) we use the "modal" nomenclature developed by Hess et al. (1984) and Yue et al. (1990) in describing the single L-type Ca^{2+} channel currents. Thus, relatively frequent, brief-duration openings are referred to as "mode 1," and relatively infrequent, longer-duration openings are referred to as "mode 2."

RESULTS

Voltage prepulses modulate the gating of single Ca^{2+} channels: Ca^{2+} versus Ba^{2+} ions

Fig. 1 *A* displays representative current traces of single L-type Ca^{2+} channel activity (corrected for leakage and capacity currents) using 105 mM Ba^{2+} ions in the patch pipette solution, and recorded during test voltage steps to −10 mV from a holding potential of −50 mV. The test voltage steps were preceded by either no prepulse (−50 mV) in column *a*, a prepulse to +10 mV in column *b*, or a prepulse to +110 mV in column *c*. It is evident from an examination of the current traces that the pattern of Ca^{2+} channel activity was influenced by the relatively brief prepulse (100 ms in duration). In the absence of a prepulse (*a*) the Ca^{2+} channel openings were quite frequent and were nearly time-invariant during the test pulse. Following a prepulse to +10 mV (*b*) the channel openings during the test step were less frequent than in (*a*), consistent with a partial inactivation of the current at this moderate level of prepulse depolarization. Most striking, however, was the increased activity of the Ca^{2+} channel following strong predepolarization to +110 mV (*c*). The signature of this prepulse-mediated facilitation of the Ca^{2+} channel activity is an enhancement in the number of long-duration (mode 2-type) channel openings upon the return to the test voltage.

A similar, but slightly different, pattern of behavior is observed when a lower concentration of Ba^{2+} ions permeates the Ca^{2+} channel. Fig. 1 *B* displays representative single Ca^{2+} channel activity recorded with 5 mM Ba^{2+} ions in the pipette solution, using identical protocols as shown in Fig. 1 *A*. Under these conditions, the channel openings

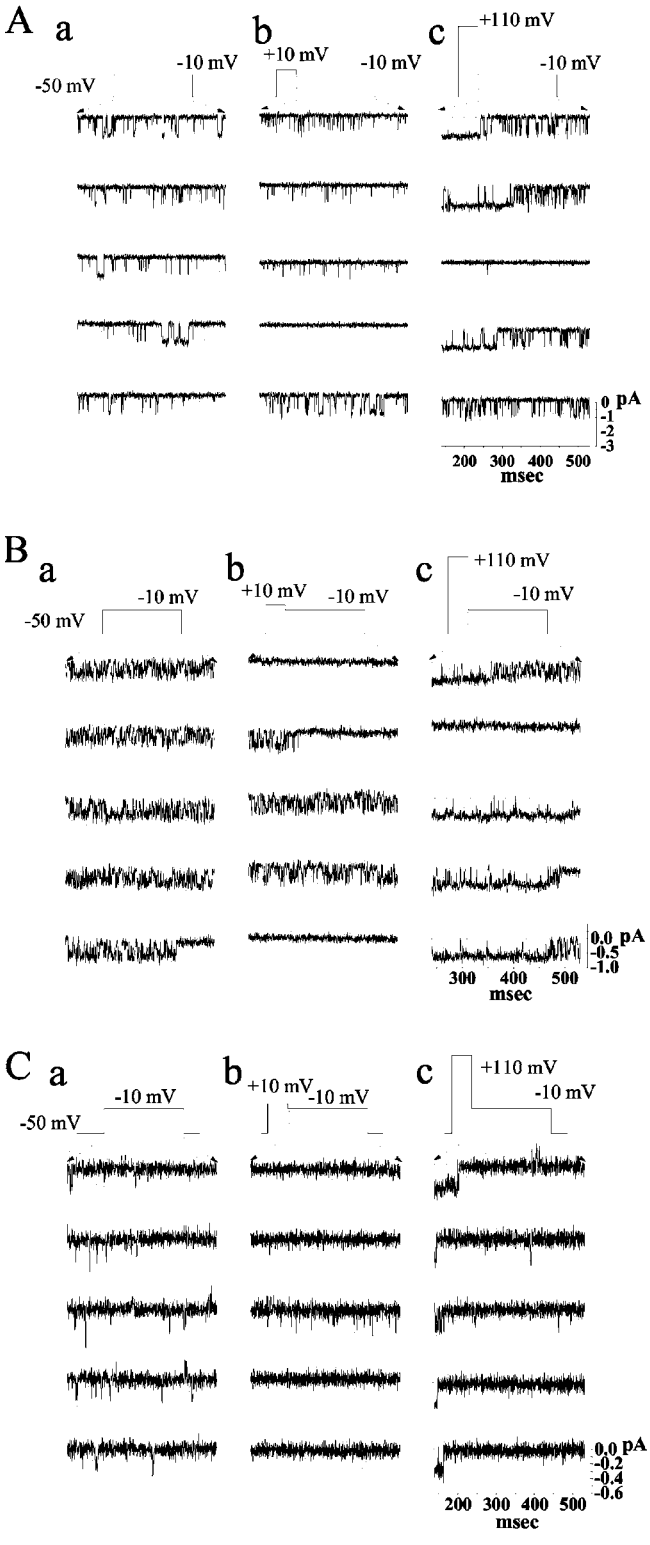

FIGURE 1 Effects of voltage prepulses on single L-type Ca^{2+} channel currents during test steps using 105 mM Ba^{2+}, 5 mM Ba^{2+}, and 5 mM Ca^{2+} ions. (*A*) Representative single L-type Ca^{2+} channel currents recorded during 400-ms test pulses to −10 mV in the absence of a 100-ms prepulse (*a*, *left column*), following a 100-ms prepulse to +10 mV (*b*, *middle column*) or a 100-ms prepulse to +110 mV (*c*, *right column*). The pipette contained 105 mM Ba^{2+} ions. The holding potential was −50 mV, the cell resting potential was zeroed as described in the Methods. The patch contained one active channel. The current traces were corrected for capacity and leakage currents. (*B*) Examples of the effects of prepulses on single L-type Ca^{2+} channel currents recorded using 5 mM Ba^{2+} ions in the patch pipette. The voltage-step protocol was identical to that shown in Fig. 1, except the test step was 300 ms in duration. The currents recorded in response to a test pulse to −10 mV are shown in the absence of a prepulse (*a*, *left column*), following a prepulse to +10 mV (*b*, *middle column*) or a prepulse to + 110 mV (*c*, *right column*). The patch contained one active channel. Note the expanded current calibration as compared with part *A*. (*C*) The effects of prepulses on single L-type Ca^{2+} channel currents recorded with 5 mM Ca^{2+} ions in the patch pipette. Voltage protocols as described in part *A*. The currents recorded in response to a test pulse are shown in the absence of a prepulse (*a*, *left column*), following a prepulse to +10 mV (*b*, *middle column*) or a prepulse to + 110 mV (*c*, *right column*). The patch contained one active channel. Note the expanded current calibration as compared with parts *A* and *B*.

during the test step, which were frequent in the absence of a prepulse (*a*), were strongly reduced by a prepulse to +10 mV (*b*), and were strongly enhanced by a prepulse to +110 mV (*c*). The facilitation with strong predepolarization was more pronounced with this lower Ba^{2+} concentration (5 mM), with some reopenings lasting the entire duration of the sweep.

In contrast, the substitution of Ca^{2+} ions for Ba^{2+} ions in the pipette solution resulted in a substantial overall decrease in Ca^{2+} channel opening and reopening frequency, as well as dramatic changes in the prepulse-mediated behavior of the channel. Fig. 1 *C* shows examples of the L-type Ca^{2+} channel currents (recorded during a test step to −10 mV) using 5 mM Ca^{2+} ions, in the absence of a prepulse (*a*), following a prepulse to +10 mV (*b*), and after strong depolarization to +110 mV (*c*). With 5 mM Ca^{2+}, prepulses to +10 mV resulted in a marked decrease in the number of Ca^{2+} channel openings. Upon strong predepolarization, long-duration single Ca^{2+} currents were observed upon return to the test potential (*c*); however, the subsequent frequency of reopening was reduced as compared with Ba^{2+} ions (compare with Fig. 1, *A* and *B*).

The probability of opening during the test pulse: Ca^{2+} versus Ba^{2+} ions

To examine the voltage-dependent effects of conditioning prepulses on the overall activity of the Ca^{2+} channel currents we analyzed the averaged probability of opening during an ensemble of test steps (P_{avg}), as a function of the permeant divalent ion. Fig. 2 *A* presents the voltage-dependent effects of 100-ms prepulses on the probability of opening of the single L-type Ca^{2+} channel currents, averaged over multiple test steps. For comparison, data are presented from experiments using 105 mM Ba^{2+} (*open squares*; n = 1000 episodes, 6 cells), 10 mM Ba^{2+} (*open triangles*; n = 650 episodes, 6 cells), 5 mM Ba^{2+} (*open circles*; 300 episodes, 5 cells), 2 mM Ba^{2+} (*open diamonds*; n = 660 episodes, 7 cells) or 5 mM Ca^{2+} ions (*closed circles*; n = 400 episodes, 6 cells) in the patch pipette solution. As can be seen in Fig. 2 *A*, the P_{avg} during the test pulse decreased with increasing prepulse potential, and reached a minimum at +30 to +50 mV for 105 mM Ba^{2+}, +30 mV for 10 mM Ba^{2+}, +10 mV for 5 mM Ba^{2+}, −10 mV for 2 mM Ba^{2+}, and +30 mV for 5 Ca^{2+} mM ions. The shifting of the U-shaped inactivation/facilitation curve on the voltage axis is caused by the effects of screening of membrane surface charges by the type and concentration of divalent ion in the recording solution (e.g., Wilson et al., 1983). Similar amounts of surface charge-related shifts in the threshold potential for Ca^{2+} channel activation were also noted during the prepulse; the threshold for Ca^{2+} channel activation was −40 to −30 mV for 2 mM Ba^{2+}, −30 to −20 mV for 5 mM Ba^{2+}, −10 to 0 mV for 105 mM Ba^{2+}, and −30 to −20 mV for 5 mM Ca^{2+}.

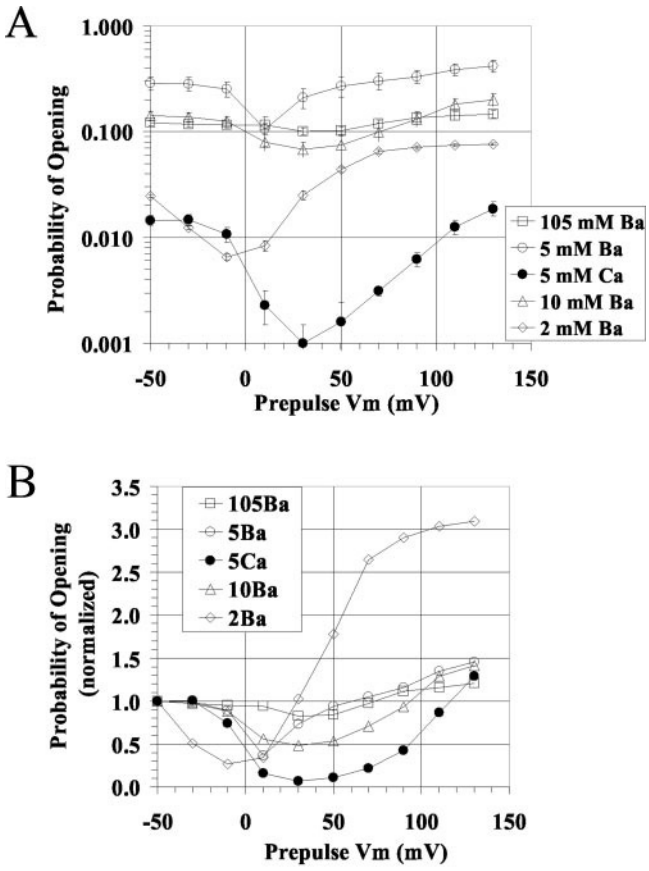

FIGURE 2 The voltage-dependent effect of prepulses on the probability of Ca^{2+} channel opening during test steps. (*A*) The different symbols represent pooled data from experiments conducted using 105 mM Ba^{2+} (*open squares*; n = 1000 episodes, 6 cells), 10 mM Ba^{2+} (*open triangles*; n = 650 episodes, 6 cells), 5 mM Ba^{2+} (*open circles*; n = 300 episodes, 5 cells), 2 mM Ba^{2+} (*open diamonds*; n = 660 episodes, 7 cells), and 5 mM Ca^{2+} (*closed circles*; n = 400 episodes, 6 cells). Plotted (logarithmically) is the probability of opening (the sum of all open times detected divided by total episode time) averaged over test voltage steps that were preceded by a prepulse to the indicated potential (means ± SEM). Note the greater averaged probability of opening (at all potentials) for Ba^{2+} ions (*open symbols*) compared with Ca^{2+} ions (*closed circles*). (*B*) Averaged probability of opening data shown in *A* were normalized to their values at −50 mV (without a prepulse) and replotted. Values <1.0 represent inactivation, and values >1.0 represent facilitation of the single Ca^{2+} channel currents.

Prepulses to more positive potentials produced a return of the test pulse P_{avg} to its initial value, and higher depolarizing prepulses resulted in a further increase or facilitation of the test pulse P_{avg} to values above those recorded without a prepulse. It is interesting to note that this U-shaped curve appears to be similar to that usually recorded using whole-cell Ca^{2+} channel currents during double-pulse protocols (e.g., Josephson et al., 1984); however, in the macroscopic analysis of inactivation only the magnitude of the peak current is plotted, whereas Fig. 2 presents the entire test pulse P_o, averaged over multiple episodes of single Ca^{2+} channel recordings.

To compare the extent of prepulse-mediated inactivation or facilitation of P_{avg} recorded with Ba^{2+} or Ca^{2+} ions, the data from Fig. 2 *A* were normalized to their P_{avg} obtained at −50 mV, and are presented in Fig. 2 *B*. Normalization of the data was advantageous for several reasons. First, as noted above, changes in external divalent ion concentrations are well-known to produce a voltage-shift in Ca^{2+} channel gating parameters due to alterations in surface potential (Wilson et al., 1983) that results in changes in P_o at the test potential. Therefore, the transmembrane potential "sensed" by the Ca^{2+} channel gating voltage sensors during the test potential was actually shifted to more negative potentials as a function of increasing divalent ion concentration. Second, the intrinsic variability of activity among Ca^{2+} channels made the comparison of absolute values of P_o between groups problematical. Third, our method for determining the number of active channels (*N*) present might underestimate *N*, and therefore overestimate P_o.

The normalized plot (Fig. 2 *B*) shows that the maximal extent of inactivation of P_{avg} (over moderate prepulse potentials) was greatest with 5 mM Ca^{2+} (94%); and inactivation with Ba^{2+} ions followed in order of increasing concentration: 2 mM Ba^{2+} (74%), 5 mM Ba^{2+} (63%), 10 mM Ba^{2+} (52%), 105 mM Ba^{2+} (18%). The maximal degree of facilitation (following a +110 mV or +130 mV prepulse) was (in decreasing order): 2 mM Ba^{2+} (341%), 5 mM Ba^{2+} (46%), 10 mM Ba^{2+} (41%), 5 mM Ca^{2+} (29%), and 105 mM Ba^{2+} (20%). Each of the preceding measurements was found to be statistically significant at the $p < 0.01$ level, when compared with their control values (in the absence of a prepulse). Thus, the degree of prepulse-induced inactivation decreased with increasing Ba^{2+} ion concentration, and the degree of facilitation also decreased with increasing Ba^{2+} ion concentration. Stated differently, the Ba^{2+} currents that inactivated most during the test pulse also displayed the greatest amount of facilitation at high prepulse potentials. This interesting finding will be addressed further in the Discussion.

A more detailed analysis was then conducted to determine by what single channel mechanism(s) the averaged probability of opening (P_{avg}) was being altered by the application of depolarizing prepulses. First, the probability of channel opening (P_o) of each test pulse was calculated to determine the cumulative fraction of Ca^{2+} channel activity during the episode. Episodic diaries of the P_o during the test pulse were then plotted to decipher the pattern of activity that contributed to the prepulse-mediated inactivation or facilitation of the Ca^{2+} channel. Fig. 3 *A* displays P_o diaries from episodes recorded using 105 mM Ba^{2+} ions, without a prepulse (*a*), following a prepulse to +20 mV (*b*), and following a prepulse to +110 mV (*c*). Inspection of the diaries reveals that a prepulse to +20 mV (*b*) results in only a slight overall reduction of P_o, and an increase in the number of null episodes (those test pulses without Ca^{2+} channel opening). However, the prepulse to +110 mV produced an increase in P_o of the active sweeps.

For comparison with the experiments using 105 mM Ba^{2+}, an analogous series of double-voltage step experiments were conducted using 5 mM Ba^{2+} ions. The single Ca^{2+} channel currents recorded during the second, test pulse with 5 mM Ba^{2+} are shown in Fig. 3 *B*. As shown in the diary, using 5 mM Ba^{2+} ions (a 21-fold reduction in ion concentration, as compared with 105 mM Ba^{2+}) prepulses to +10 mV (*b*) resulted in a more substantial reduction of P_o, and an increased number of null episodes. In addition, prepulses to +130 mV (*c*) resulted in an even greater enhancement of P_o in individual episodes.

The effects of prepulses on the test pulse diaries of P_o obtained using 5 mM Ca^{2+} (Fig. 3 *C*) were even more dramatic. In this nearly physiological condition, the P_o of the Ca^{2+} channel (which in the absence of a prepulse (*a*) was ~10-fold lower than that recorded using Ba^{2+} ions) was further dramatically reduced following a prepulse to +10 mV (*b*), with most episodes displaying no channel openings. Nevertheless, prepulses to +130 mV (*c*) produced episodes with P_o occasionally greater than in the absence of a prepulse. However, the number of null traces was also greater at +130 mV than in the absence of a prepulse.

Thus, it is apparent from inspection of the P_o diaries that the number of null test pulse traces (i.e., without channel openings) increased with increasing prepulse depolarization, using Ba^{2+} ions as well as Ca^{2+} ions, as shown in Fig. 4. The ratio of the number of active traces (i.e., containing one or more openings) divided by the total number of traces provides a measure of Ca^{2+} channel availability. By this method, the availability of the Ca^{2+} channels during the test pulse can be seen to decrease with increasing prepulse potential, but then increase again at higher prepulse potentials. The increase at the higher prepulse potentials reflects the contribution of traces containing one or more long-duration openings.

The prepulse-induced reduction in availability was most marked using 5 mM Ca^{2+} ions, and was less prevalent with Ba^{2+} ions. However, the prepulse-mediated reduction in availability was inversely related to Ba^{2+} ion concentration, with 2 mM Ba^{2+} having a greater effect on availability than 5 mM Ba^{2+}, which was greater than 105 mM Ba^{2+}. Also, note that the maximum reduction in availability occurred at a prepulse potential of +10 mV for 2 mM Ba^{2+}, +30 mV for 5 mM Ba^{2+}, +30 to +50 for 5 mM Ca^{2+}, and +70 mV in 105 mM. The relative shift in potential most probably was caused by changes in surface potential produced by the permeant ions (Wilson et al., 1983).

It is important to bear in mind that the relatively short-duration prepulse (100 ms) used throughout this study was chosen because it does not produce complete (i.e., absorbing) voltage-dependent inactivation, thus allowing a determination of the relative ion concentration-dependent effects

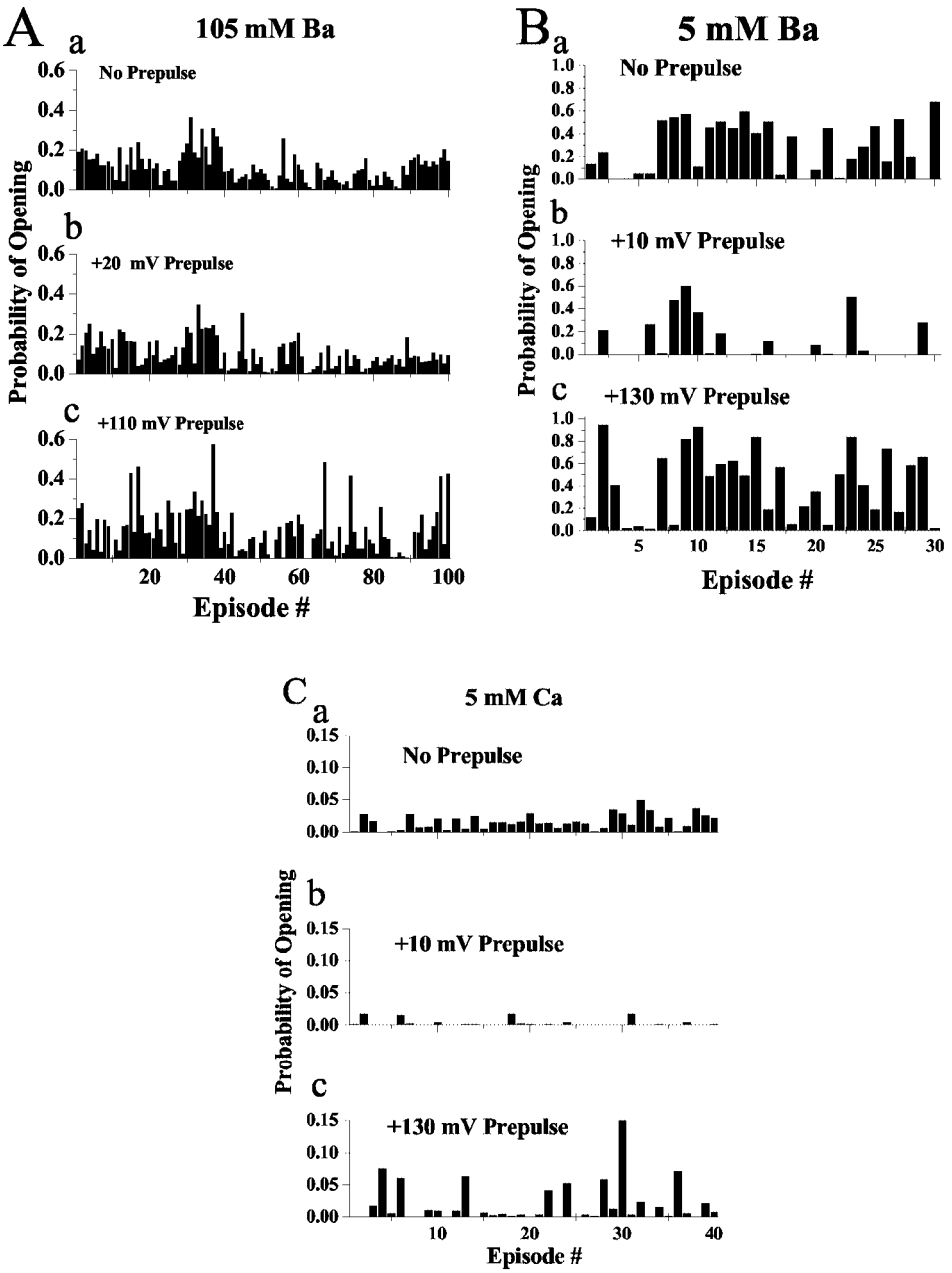

FIGURE 3 Prepulse-dependent effects on episodic diaries of the probability of opening of Ca^{2+} channels during a test pulse. (*A*) Recorded using 105 mM Ba^{2+} ions, during a test pulse (0 mV) in the absence of a prepulse (*a*, *top row*), with a prepulse to +20 mV (*b*, *middle row*), or with a prepulse to +110 mV (*c*, *bottom row*). (*B*) Recorded using 5 mM Ba^{2+} ions, during a test pulse (0 mV) in the absence of a prepulse (*a*, *top row*), with a prepulse to +10 mV (*b*, *middle row*), or with a prepulse to +130 mV (*c*, *bottom row*). (*C*) Recorded using 5 mM Ca^{2+} ions, during a test pulse (0 mV) in the absence of a prepulse (*a*, *top row*), with a prepulse to +10 mV (*b*, *middle row*), or with a prepulse to +130 mV (*c*, *bottom row*).

on inactivation and facilitation. Thus, the rebound in availability at high prepulse potentials reflects the decrease in null traces due to an increase in those traces displaying long-openings.

Given the relative decrease in Ca^{2+} channel availability at high prepulse potentials (as compared with the absence of a prepulse) how does strong conditioning depolarization produce a facilitation of the test pulse, P_{avg}, to values larger than those obtained in the absence of a prepulse? Further examination of the P_o diaries revealed that following a strong prepulse some of the episodes with test pulse activity display a P_o that was greater than those without a prepulse. Thus, the increase in P_o during these highly active sweeps is greater than the decrease in the proportion of active sweeps, resulting in an increase or facilitation of the average P_o following strong prepulses.

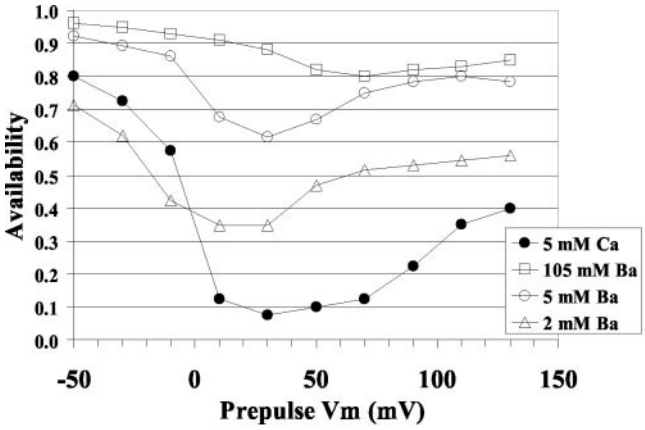

FIGURE 4 The availability of single Ca^{2+} channel currents during the test pulse, plotted as a function of prepulse potential. Mean availability data from experiments using Ba^{2+} ions are shown by the open symbols (105 mM, squares (1000 episodes, 6 patches); 5 mM, circles (300 episodes, 5 patches); 2 mM, triangles (660 episodes, 7 patches)); and those using 5 mM Ca^{2+} are shown by the closed circles (400 episodes, 6 patches). Availability is defined as the fraction of episodes displaying Ca^{2+} channel openings.

The extent of the prepulse-dependent facilitation on the P_{avg} of the Ca^{2+} channel during the test pulse is even more evident after correcting for the effects of the prepulse on Ca^{2+} channel availability. The resulting corrected P_{avg} (after removal of null sweeps caused by either voltage-dependent, or ion-dependent inactivation that lasted for the entire test pulse, data not shown) emphasizes the voltage-dependent activation of the prepulse-dependent facilitation of P_{avg}. The increase, or facilitation of the corrected test pulse P_{avg} with a prepulse to +130 mV (as compared with no prepulse) was 36% for 105 mM Ba^{2+}, 46% for 5 mM Ba^{2+}, 258% for 5 mM Ca^{2+}, and 392% for 2 mM Ba^{2+} ions ($p < 0.01$).

Distributions of Ca^{2+} channel open times are prepulse- and ion-dependent

We next investigated the single channel gating mechanisms underlying the prepulse-induced inactivation and facilitation of the test pulse P_o. To address this question, analyses of the distributions of Ca^{2+} channel open-times as a function of prepulse potential were performed with data recorded using 105 mM Ba^{2+} (Fig. 5 *A*), 5 mM Ba^{2+} (Fig. 5 *B*), or 5 mM Ca^{2+} ions (Fig. 5 *C*). Representative distributions of the open-times are displayed in log-log plots to better visualize the long-duration events, and were fit with a sum of three exponentials. A sum of three exponentials provided a better fit than the sum of two exponentials, as judged by comparing the goodness-of-fit criteria (the "F"-value calculated from the sum of squared errors for the two models in pSTAT).

The extracted fit parameters (fast time constant, τ_1; medium time constant, τ_2; and slow time constant, τ_3) of the exponential functions and their respective proportions are presented in Table 1. Averaged test pulse time constants are displayed for the pooled data using 5 mM Ca^{2+} (6 cells), 5 mM Ba^{2+} (5 cells), and 105 mM Ba^{2+} (6 cells) at three prepulse potentials: −50 mV (no prepulse), +10 mV or +30 mV (the prepulse potential yielding maximal inhibition), and +110 mV or +130 mV (yielding maximal facilitation). A similar pattern of time constants as a function of prepulse potential was found for 5 mM Ca^{2+}, 5 mM Ba^{2+}, and 105 mM Ba^{2+}; however, the values for τ_1 and τ_2 with 5 mM Ca^{2+} ions were consistently much smaller than those found for Ba^{2+} ions. Under all three ionic conditions moderate depolarization produced a decrease, and high depolarization produced an increase in the values for the three time constants.

Also shown in Table 1 is the prepulse-dependence of the proportion of each time constant; a comparison of these values gives the relative contributions of the short-, medium-, and long-duration openings under each condition. As can be seen, there was a greater contribution of short-duration (τ_1) events in 105 mM Ba^{2+} as compared with 5 mM Ba^{2+}. However, both 105 mM Ba^{2+} and 5 mM Ba^{2+} displayed a prepulse-dependent decrease (with moderate depolarization), and increase (with strong depolarization) in the relative number of long-duration (τ_3) events. A more striking dependence on prepulse potential is seen with 5 mM Ca^{2+} ions permeating the channel; in this case the relative numbers of short-, medium-, and long-duration events are all decreased at moderate prepulse potentials, whereas the relative numbers of medium- and long-duration events at high prepulse potentials are markedly increased.

The relative contribution of each kinetic component to the overall distribution of Ca^{2+} channel open times, as a function of prepulse potential and permeating ion, is presented in Fig. 6. The data are plotted as the product of the average time constant of each component (in ms) and its average proportion (yielding a dimensionless fraction between 0 and 1). Part *A* shows the results using 105 mM Ba^{2+} ions (6 cells); part *B* using 5 mM Ba^{2+} ions (5 cells), and part *C* using 5 mM Ca^{2+} ions (6 cells). These plots present the fraction of the total Ca^{2+} current conducted by short-, medium-, and long-duration events as a function of prepulse potential.

For 105 mM Ba^{2+} ions (Fig. 6 *A*) a large fraction of the current (73%) is carried by short (τ_1) openings (*open columns*) with prepulses to −50 mV, +30 mV, and +130 mV. The fraction carried by medium-duration (τ_2) openings (*hatched columns*) is an increasing function of prepulse potential. The fraction carried by long-duration (τ_3) openings (*solid columns*) decreases with moderate prepulses (+30 mV), but then increases with strong prepulses (+130 mV). With a prepulse to +130 mV a substantial fraction of the total current (27%) is carried by long (τ_3) openings.

For 5 mM Ba^{2+} ions (Fig. 6 *B*) a similar pattern for the kinetic proportions of the currents was obtained. However, as compared with 105 mM Ba^{2+}, with 5 mM Ba^{2+} ions a

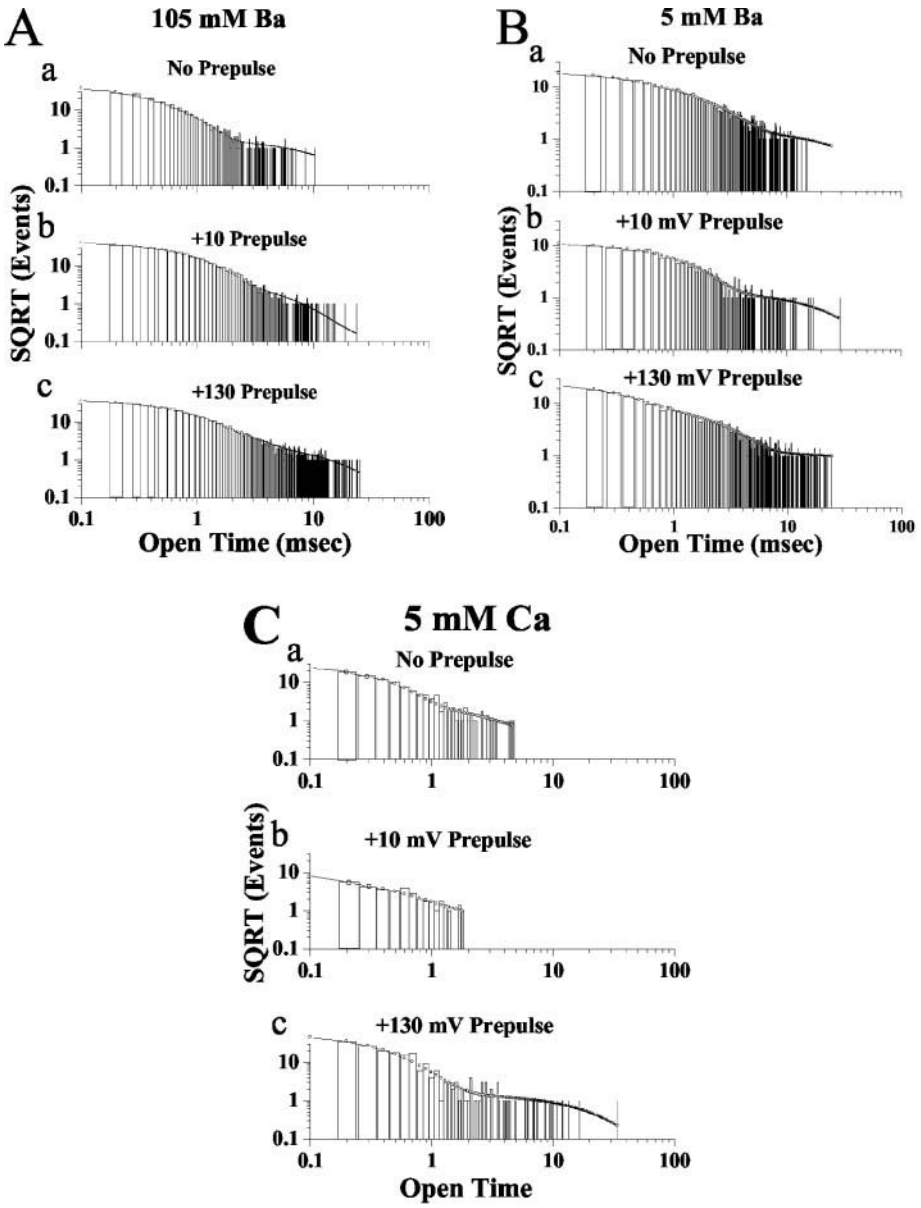

FIGURE 5 Analysis of the distribution of Ca^{2+} channel open-times during a test pulse, as a function of prepulse potential. The distributions of the open-times (during test steps to −10 mV) are displayed in logarithmic plots to visualize the long-duration events, and then fit with a sum of three exponentials in the absence of a prepulse (*a*, *top row*), with a prepulse to +10 mV (*b*, *middle row*), or with a prepulse to +130 mV (*c*, *bottom row*). Examples shown were recorded using 105 mM Ba^{2+} ions (*A*); 5 mM Ba^{2+} ions (*B*); or 5 mM Ca^{2+} ions (*C*).

larger fraction of the current is carried by medium-duration openings (τ_2) at all prepulse potentials (*hatched columns*). In addition, moderate prepulses (+10 mV) resulted in a decrease in both short- (τ_1) and medium-duration (τ_2) openings, compared with no prepulse (−50 mV). Strong prepulses (+130) produced an increased contribution of both short and medium durations. Similar to the case of 105 mM Ba^{2+}, with 5 mM Ba^{2+} the long-duration (τ_3) component (*solid columns*) was decreased with moderate prepulses (+10 mV), and then increased with strong prepulse (+130 mV).

For 5 mM Ca^{2+} ions (Fig. 6 *C*), in the absence of a prepulse (at −50 mV) a large proportion of the total Ca^{2+} current (52%) was composed of long-duration (τ_3) events (*solid columns*). With moderate prepulses to +10 mV all three components were strongly inhibited, consistent with the conspicuous decrease in the probability of opening due to Ca^{2+}-dependent inactivation. However, with high prepulses (to +130 mV) there is a large increase in the relative contributions of medium- (τ_2) and long-duration (τ_3) events (74% of total current). Thus, even though the relative frequency of τ_3 events is lower than τ_1 or τ_2 events, they

TABLE 1 Open-time constants (in milliseconds) and their proportions

	5 mM Ca^{2+}			5 mM Ba^{2+}			105 mM Ba^{2+}		
	τ_1 (Pro)	τ_2 (Pro)	τ_3 (Pro)	τ_1 (Pro)	τ_2 (Pro)	τ_3 (Pro)	τ_1 (Pro)	τ_2 (Pro)	τ_3 (Pro)
Prepulse									
None*	0.107 (0.835)	0.407 (0.135)	5.304 (0.03)	0.543 (0.58)	1.756 (0.383)	5.499 (0.037)	0.474 (0.932)	1.478 (0.047)	4.890 (0.020)
Moderate†	0.046 (0.896)	0.385 (0.101)	2.319 (0.003)	0.162 (0.61)	0.815 (0.364)	2.572 (0.026)	0.419 (0.891)	1.099 (0.107)	3.469 (0.002)
Strong‡	0.093 (0.307)	0.433 (0.593)	8.266 (0.100)	0.30 (0.493)	1.863 (0.455)	12.081 (0.051)	0.459 (0.879)	2.942 (0.102)	13.951 (0.019)

*−50 mV for 5 Ca^{2+}, 5 Ba^{2+}, 105 Ba^{2+}.
†+10 mV for 5 Ca^{2+}, 5 Ba^{2+}, +30 mV for 105 Ba^{2+}.
‡+130 mV for 5 Ca^{2+}, 105 Ba^{2+}, +110 mV for 5 Ba^{2+}.

conduct a surprisingly large fraction of the total Ca^{2+} current, due to their markedly increased open-duration.

DISCUSSION

The present study is the first to describe the inhibitory and facilitory effects of a wide range of conditioning voltages on single cardiac L-type Ca^{2+} channel currents with a near-physiological level of Ca^{2+} ions permeating the channel, and in the absence of any Ca^{2+} channel agonists. We have found marked ion-dependent alterations in Ca^{2+} channel gating kinetics with both prepulse-induced inhibition (following moderate depolarization) and facilitation (following strong depolarization). An additional dimension of the study is that we have characterized the concentration-dependent effects of Ba^{2+} ions in modulating single Ca^{2+} channel gating in response to voltage prepulses. These findings support the hypothesis that both the conditioning voltage and the permeating divalent cations alter the gating properties of the L-type Ca^{2+} channel.

The present results reveal that the amount and timing of Ca^{2+} influx into the myocardial cell are exquisitely fine-tuned by the utilization of these opposing facilitory and inhibitory mechanisms, as each of these predominates over a different range of potentials and manifests different kinetics. A "U-shaped" curve for the voltage-dependence of inactivation (where the curve reaches a minimum at moderately depolarizing prepulses, and then increases at higher prepulse depolarization) has traditionally been one of the hallmarks of Ca^{2+}-dependent inactivation of the macroscopic L-type Ca^{2+} channel current. It has been widely thought that the U-shaped curve reaches a minimum over prepulse potentials that elicit the maximum Ca^{2+} current, and that inactivation is relieved at higher potentials because I_{Ca} was diminished as E_{Ca} was approached as the prepulse voltage was increased. However, the present results point to a more complex single channel mechanism to explain the U-shape of inactivation. Thus, at moderately depolarizing prepulse potentials Ca^{2+} channel availability is at its lowest value, due to a combination of voltage-dependent and Ca^{2+}-dependent inactivation. Following higher prepulse potentials, test pulse Ca^{2+} channel availability may still be low, but this inhibition is overcome by an increase in mode 2 openings of active sweeps. Therefore, the U-shape is derived as a product of the decreasing Ca^{2+} channel availability and the increasing activation of mode 2 with increasing prepulse potential.

The interaction between these two opposing regulatory mechanisms of L-type Ca^{2+} channels, inactivation and facilitation, can be observed most clearly when Ca^{2+} ions are permeating the channel. Under these conditions, a moderate predepolarization produces a marked decrease in the test pulse P_o due to a reduction in the frequency of openings of all durations. Conversely, strong predepolarization elicits longer-duration openings upon return to the test potential. The mode 2 openings are more noticeably prolonged with Ba^{2+} ions permeating the channel. The simplest explanation to account for this effect is that Ba^{2+} ion binds, but with a lesser affinity, to a site within the Ca^{2+} channel protein that normally leads to Ca^{2+}-dependent closure of the channel. This idea was originally proposed to explain the relative slowing of macroscopic Ba^{2+} current inactivation in neurons (Brown et al., 1981) and in native myocytes (Josephson et al., 1984), and has more recently been applied to results obtained using cloned cardiac Ca^{2+} channels (Ferreira et al., 1997).

An additional novel finding of the present study is that the maximal amount of prepulse-induced inactivation (reduction of P_o) was found to decrease with increasing Ba^{2+} ion concentration, and that the degree of prepulse-induced facilitation (increase in P_o) also decreased with increasing Ba^{2+} ion concentration. In other words, divalent ion conditions that favored the more rapid development of inactivation also promoted the greatest amount of facilitation of the single Ca^{2+} currents.

It is well known from previous whole-cell experiments that the initial component of the biphasic inactivation of the Ba^{2+} current becomes slower as the Ba^{2+} ion concentration is increased (see McDonald et al., 1994, for a review). This peculiar behavior of the macroscopic L-type Ca^{2+} current when using Ba^{2+} ions as the charge carrier is opposite to that observed when using Ca^{2+} ions as the charge carrier. With Ca^{2+} ions permeating the Ca^{2+} channel the fast, or initial, phase of the inactivation of the whole-cell current becomes even more rapid as the Ca^{2+} ion concentration is increased. Indeed, this relationship is held as one of the

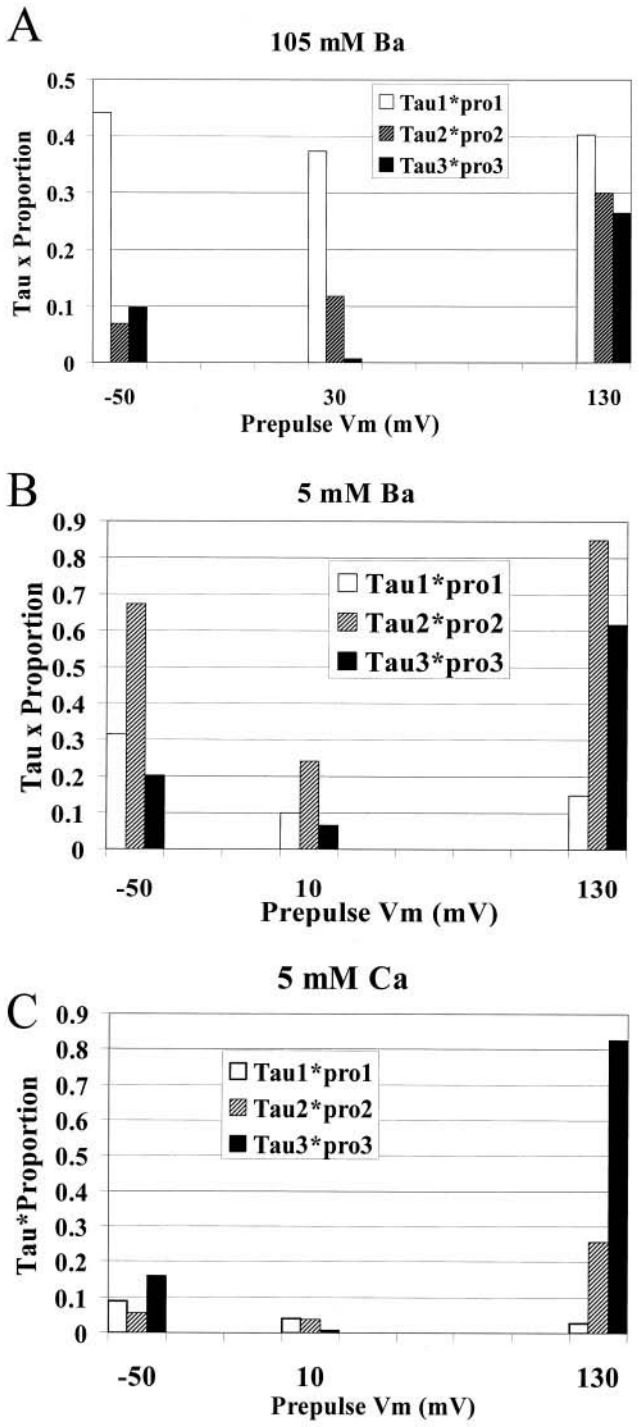

FIGURE 6 The relative contribution of each kinetic component to the overall distribution of Ca^{2+} channel open times, as a function of prepulse potential and permeating ions. The data are plotted as the product of the average time constant of each component (in ms), and its average proportion (a dimensionless fraction between 0 and 1). (*A*) Results using 105 mM Ba^{2+} ions; (*B*) using 5 mM Ba^{2+} ions; and (*C*) using 5 mM Ca^{2+} ions.

signatures of Ca^{2+}-dependent inactivation of the Ca^{2+} current. Taken together, this very different behavior of the inactivation of the macroscopic Ca^{2+} and Ba^{2+} currents suggests that at least two divalent cation binding sites may be involved to produce these characteristics. One site (site 1), which may be responsible for initiating the ion-dependent inactivation, may have a much lower affinity for Ba^{2+} ions than for Ca^{2+} ions. That would, of course, explain the relatively slower initial inactivation of the macroscopic Ba^{2+} current, as compared to the Ca^{2+} current. A second site (site 2), perhaps located on the extracellular side of the Ca^{2+} channel or in the outer region of its pore, may be involved in determining the reopening frequency of the Ca^{2+} channel. This site may be sensitive to the external divalent ion concentration in a manner such that a higher ion concentration produces a greater number of reopenings of the Ca^{2+} channel during a depolarization. The reopening frequency is a major determinant of burst length, and therefore of the time course for the decay of the ensemble, or macroscopic Ca^{2+} current. The product of the relative contributions of these two mechanisms (and the relative affinities of these two hypothetical sites to Ba^{2+} and Ca^{2+}) can then explain the very different inactivation behavior of the currents. For Ba^{2+} ions, the affinity for site 2 would be greater than for site 1, and therefore the current inactivation would be slower with increasing Ba^{2+} ion concentration. Conversely, for Ca^{2+} ions the affinity for site 1 would be greater than for site 2, thus the current inactivation would be faster with increasing Ca^{2+} ion concentration.

As the principal point of Ca^{2+} influx into myocardial cells, the L-type Ca^{2+} channel has evolved to possess numerous control mechanisms to insure efficient regulation over the influx of this crucial ion. At the single L-type Ca^{2+} channel level, a moderate-depolarizing prepulse, with its attendant Ca^{2+} influx, leads to a diminution of the subsequent Ca^{2+} channel activity. This alteration in behavior has been characterized as a shift from a gating mode displaying mostly relatively brief channel openings ("mode 1") into a gating mode of very low opening probability, termed "mode Ca" (Yue et al., 1990; Imredy and Yue, 1994). The molecular locus of this mechanism, the action of prior Ca^{2+} influx to inhibit subsequent Ca^{2+} influx, involves a calmodulin-binding site that has been implicated in Ca^{2+}-dependent inactivation of the Ca^{2+} channel (Zuhlke et al., 1999). Previous single channel studies assumed that Ca^{2+}-dependent inactivation did not significantly affect the gating charge movement and, therefore, the Ca^{2+} channel voltage sensor(s) (Imredy and Yue, 1994); however, more recent work suggests that gating currents (and therefore transitions among the closed states) are indeed affected by Ca^{2+} influx (Shirokov, 2000). In light of this, revised models of Ca^{2+} channel gating and modal interconversion are needed that take into account these newer findings.

The molecular locus for the high-voltage facilitation of the cardiac L-type Ca^{2+} channel remains somewhat obscure. It was originally suggested that an additional conformational change of one or more of the channel's voltage-

sensing S4 regions, or other (as yet unknown) voltage-sensing moieties of the channel, may be responsible for transducing the conformational change produced by strong depolarizing prepulses (Pietrobon and Hess, 1990). In support of this view, several gating current studies have identified a component of Ca^{2+} channel charge movement occurring at very positive voltages in native myocytes (Bean and Rios, 1989; Josephson and Sperelakis, 1992) and for cloned human cardiac L-type Ca^{2+} channels (Josephson and Varadi, 1996; Josephson, 1997). Additionally, the coexpression of a β subunit with the α1C of the Ca^{2+} channel is required for voltage-dependent facilitation (Kamp et al., 2000) and also enhances the occurrence of long-duration single Ca^{2+} channel currents (Constantin et al., 1998). However, it is interesting to note that, in contrast to neuronal N- and P/Q-type Ca^{2+} channels, the facilitation of the cardiac (α1C) Ca^{2+} channel by strong depolarization appears to be independent of a G protein pathway (Kamp et al., 2000).

It has previously been observed in whole-cell recordings of L-type Ca^{2+} channel currents (especially when using Ba^{2+} ions as the charge carrier) that the decay of the tail currents elicited upon repolarization of the voltage step could be described by a fast and a slow component (McDonald et al., 1994). Although it has sometimes been explained as the deactivation of a second open state of the Ca^{2+} channel, or even related to the Na^{+}-Ca^{2+} exchange current, the present results would indicate that this slow component of deactivation reflects the closure of mode 2 openings at the return voltage. Interestingly, a similar time-dependent recruitment of mode 2-like channel openings, and a slow deactivation time course of the macroscopic current, have recently been reported for *Shaker* K^{+} channels (Olcese et al., 2000). This finding for K^{+} channels suggests that time-dependent modal conversion may be a general feature of many types of voltage-dependent ion channels.

In the present study we found that a (lower) Ba^{2+} ion concentration that produced a greater degree of inactivation also produced a greater degree of facilitation. This relationship may imply the possibility that these two processes may be linked. In this regard, it may be noted that a feature of high prepulse-dependent facilitation of the L-type Ca^{2+} channel is the relatively long time-dependence (on the order of tens to hundreds of milliseconds) for its activation and deactivation (Pietrobon and Hess, 1990; Hirano et al., 1999). This suggests that following the rapid movements (on the order of milliseconds) of the voltage sensors, additional (and much slower) conformational rearrangements of the Ca^{2+} channel occur during prolonged depolarization, which may permit the long duration openings (mode 2) upon return to the test potential. By analogy, other examples of slow conformational gating changes are the long time course for the development of voltage-dependent inactivation of the Ca^{2+} current and for the associated negative shift in the availability of Ca^{2+} channel gating charge movement (Shirokov, 2000; Josephson, 1996). It is tempting to speculate that the slow deactivation of mode 2 during repolarization of the action potential is a mechanism designed to increase Ca^{2+} ion influx at a time and voltage when mode 1 openings have ceased.

In summary, we have demonstrated, using a low concentration of Ca^{2+} ions and a range of Ba^{2+} ion concentrations, that the application of a conditioning prepulse produces voltage- and ion-dependent alterations in the probability of opening, availability, and gating kinetics of the subsequent single L-type Ca^{2+} channel currents. A greater understanding of these microscopic mechanisms for modulation of inactivation and facilitation of the Ca^{2+} currents will provide a more comprehensive and accurate portrayal of the complex behavior of the L-type Ca^{2+} channel and its role in the local control of cardiac E-C coupling (Stern, 1992).

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