

# Modulation of the Conductance of Unitary Cardiac L-Type $\text{Ca}^{2+}$ Channels by Conditioning Voltage and Divalent Ions

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**ABSTRACT** The accompanying paper (Josephson, I. R., A. Guia, E. G. Lakatta, and M. D. Stern. 2002. *Biophys. J.* 83:2575–2586) examined the effects of conditioning prepulses on the kinetics of unitary L-type  $\text{Ca}^{2+}$  channel currents using  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  ions to determine the ionic-dependence of gating mechanisms responsible for channel inactivation and facilitation. Here we demonstrate that in addition to alterations in gating kinetics, the conductance of single L-type  $\text{Ca}^{2+}$  channels was also dependent on the prior conditioning voltage and permeant ions. All recordings were made in the absence of any  $\text{Ca}^{2+}$  channel agonists. Strongly depolarizing prepulses produced an increased frequency of long-duration (mode 2) openings during the test voltage steps. Mode 2 openings also displayed >25% larger single channel current amplitude (at 0 mV) than briefer (but well-resolved) mode 1 openings. The conductance of mode 2 openings was 26 pS for 105 mM  $\text{Ba}^{2+}$ , 18 pS for 5 mM  $\text{Ba}^{2+}$ , and 6 pS for 5 mM  $\text{Ca}^{2+}$  ions; these values were 70% greater than the conductance of  $\text{Ca}^{2+}$  channel openings of all durations (mode 1 and mode 2). Thus, the prepulse-driven shift into mode 2 gating results in a longer-lived  $\text{Ca}^{2+}$  channel conformation that, in addition, displays altered permeation properties. These results, and those in the accompanying paper, support the hypothesis that multiple aspects of single L-type  $\text{Ca}^{2+}$  channel behavior (gating kinetics, modal transitions, and ion permeation) are interrelated and are modulated by the magnitude of the conditioning depolarization and the nature and concentration of the ions permeating the channel.

## INTRODUCTION

Although it has been convenient to conceptualize ion channel gating as a simple two-state system (open and closed), most ligand-gated and voltage-gated ion channels display multiple conductance levels. For L-type  $\text{Ca}^{2+}$  channels, multiple conductance levels have been previously reported using cardiac myocytes (Chen and Hess, 1987), neurons (Church and Stanley, 1996), GH3 cells (Kunze and Ritchie, 1990),  $\text{Ca}^{2+}$  channel proteins reconstituted in bilayers (Ma and Coronado, 1988), and expressed  $\alpha 1$  subunits of the L-type  $\text{Ca}^{2+}$  channel (Gondo et al., 1998; Cloues and Sather, 2000). However, the conditions that may promote a given conductance level remain largely unknown. Moreover, the properties of conductance states may contain important information concerning permeation and gating mechanisms necessary for a further understanding of the structure of the L-type  $\text{Ca}^{2+}$  channel.

High-voltage prepulses have been shown to facilitate single  $\text{Ca}^{2+}$  channel activity by promoting a mode of  $\text{Ca}^{2+}$  channel gating (one that is characterized by openings of unusually long duration, as compared with the briefer, mode 1 openings) using  $\text{Ba}^{2+}$  ions (Pietrobon and Hess, 1990; Hirano et al., 1999) or  $\text{Ca}^{2+}$  ions as the charge carrier (Josephson et al., 2002). These prepulse-facilitated single  $\text{Ca}^{2+}$  channel currents resemble the long-duration (mode 2) type of gating originally described for L-type  $\text{Ca}^{2+}$  channels

during exposure to dihydropyridine agonists (Hess et al., 1984) or following  $\beta$ -adrenergic stimulation (Yue et al., 1990).

However, there is little information available concerning the conductance properties of mode 2 L-type  $\text{Ca}^{2+}$  currents, especially those recorded under more physiological conditions; that is, using a low concentration of  $\text{Ca}^{2+}$  ions as the charge carrier, and in the absence of L-type  $\text{Ca}^{2+}$  channel stimulation or agonists. In the present paper we focus on the conductance of unitary cardiac L-type  $\text{Ca}^{2+}$  channel currents displaying long-duration (mode 2) openings. We report that mode 2 L-type  $\text{Ca}^{2+}$  channel currents, recorded using  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  ions, are not only longer in duration, but also of greater conductance than briefer (but fully resolved) mode 1 openings. A preliminary report of some of these results has been presented in abstract form (Josephson et al., 2001c).

## MATERIALS AND METHODS

The methods, including solution preparation, isolation of the rat myocytes, and single L-type  $\text{Ca}^{2+}$  channel recording and analysis, are described in detail in the accompanying paper (Josephson et al., 2002).

## RESULTS

### Mode 2 openings are larger in amplitude

In the accompanying paper (Josephson et al., 2002) we have demonstrated that prepulse-dependent modulation of single L-type  $\text{Ca}^{2+}$  channel current gating can be characterized using a low concentration of  $\text{Ca}^{2+}$  ions, as well as  $\text{Ba}^{2+}$  ions. Voltage- and ion-dependent mode shifts were associated with a redistribution of the relative proportions, and

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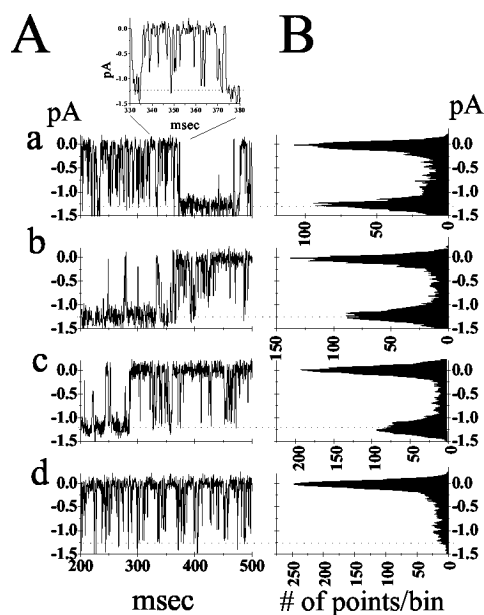


FIGURE 1 Mode 2, long-duration  $\text{Ca}^{2+}$  channel openings are larger in amplitude than briefer openings. Column A (*a–d*) shows representative single  $\text{Ca}^{2+}$  channel traces recorded with 105 mM  $\text{Ba}^{2+}$  ions during a test pulse to 0 mV, following a prepulse to +110 mV. Column B (*a–d*) shows the all-points amplitude histogram for each corresponding trace in column A. The dotted lines indicate the amplitude of the long (mode 2) openings. The inset above *Aa* shows a time-expanded portion of the trace; note that the briefer openings attain a fully resolved amplitude that is less than that of the long openings (indicated by the dotted line).

changes in the values, for the time constants describing the open channel dwell-times during subsequent test pulses. Depolarizing prepulses of moderate strength resulted in a shift toward briefer duration openings (mode 1 and mode 2  $\text{Ca}^{2+}$ ). Strong depolarizing prepulses increased the frequency of mode 2 openings. However, in addition to these modal shifts in gating kinetics, inspection of the single  $\text{Ca}^{2+}$  channel currents also suggested that there were differences in the amplitudes of prepulse-induced mode 2 openings as compared with briefer, mode 1 openings. The present paper focuses on the conductance of mode 2 openings.

Several methods were used to analyze the amplitudes of the mode 2 openings, as described below. First, a direct and unbiased method of event amplitude measurement is the all-points histogram (see Cloues and Sather, 2000). Therefore, following visual observation of the presence of multiple current levels we analyzed the single  $\text{Ca}^{2+}$  channel recordings using all-points histograms of the raw data.

Fig. 1 shows representative single  $\text{Ca}^{2+}$  channel traces recorded during a test pulse to 0 mV after a prepulse to +110 mV (column A, rows *a–d*) and the corresponding all-points-histogram for each trace (column B, rows *a–d*). The all-points histograms show a large peak at 0 pA, thus indicating the amount of time the channel was closed during each trace. In examples *a–c* the traces show one or more events displaying a long-duration, mode 2-type opening; the

open-amplitude is denoted by a dotted line. These mode 2 events correspond to the large peaks (at 1.25 pA) in their corresponding histograms. However, it should be noted that traces *a–c* (*Aa–c*) also display many openings that were briefer in duration, and smaller in magnitude than the mode 2 openings. The briefer events are also contained in the histograms (*Ba–c*). This is emphasized by trace *d*, which mainly shows briefer openings. Note that the majority of points in histogram of trace *d* (*Bd*) are distributed above the dotted line (i.e., at values <1.25 pA), demonstrating that briefer mode 1-type openings were of a smaller amplitude than mode 2 openings. The inset shows a time-expanded section of trace *Aa* that illustrates that the amplitudes of the briefer openings were fully resolved and not simply truncated by filtering.

For the second method of amplitude measurement, events were identified and analyzed using the 50% threshold method (see Materials and Methods in the accompanying article). Following event detection, correlations of event amplitude and duration were conducted. To eliminate the possibility that the full amplitude of brief (mode 1) openings were reduced as a result of a filtering artifact, we conservatively chose to limit the analysis of amplitudes to openings of 0.5 ms duration or greater. Given that the rise time of our recording system was 0.166 ms (at 2 kHz), the fraction of the maximum amplitude that is attained by an event ( $A_{\text{max}}/A_0$ ) is given by Colquhoun and Sigworth (1995) as

$$A_{\text{max}}/A_0 = \text{erf}(0.8860w/t_r) \quad (1)$$

Where erf() is the error function,  $w$  is the duration of the event (0.5 ms), and  $t_r$  is the rise-time of the filter. Thus, we estimate that channel events of 0.5 ms attain >99% of their amplitude after filtering.

In Fig. 2, scatterplots were used to visualize the relationship between the duration and the amplitude of  $\text{Ca}^{2+}$  channel openings during test pulses from all of the events recorded with a given prepulse potential. Most of the briefer-duration events were distributed symmetrically about an average amplitude. However, the longer-duration (mode 2) events attained amplitudes (indicated by the dotted horizontal line) that were significantly larger than the average amplitude of the briefer events. This observation was confirmed and quantified by analysis of single channel amplitude distributions. As shown in part A (using recordings made with 105 mM  $\text{Ba}^{2+}$  ions) panels *a* and *b* display the scatterplots of open time versus amplitude in the absence (*a*) and presence (*b*) of prepulses to +110 mV. The amplitude distributions of the test pulse currents were fit with a sum of two Gaussian functions, with average midpoints (and their proportions) of  $-1.13 \pm 0.1$  pA (14%) and  $-0.89 \pm 0.2$  pA (86%) in the absence of a prepulse (*c*), and  $-1.17 \pm 0.1$  pA (32%), and  $-0.92 \pm 0.1$  pA (68%), with a prepulse to +110 mV (*d*). Thus, the average amplitude of a long-duration mode 2 opening using 105 mM  $\text{Ba}^{2+}$  was ~27% greater than that of a shorter-duration opening (at 0 mV). In addi-

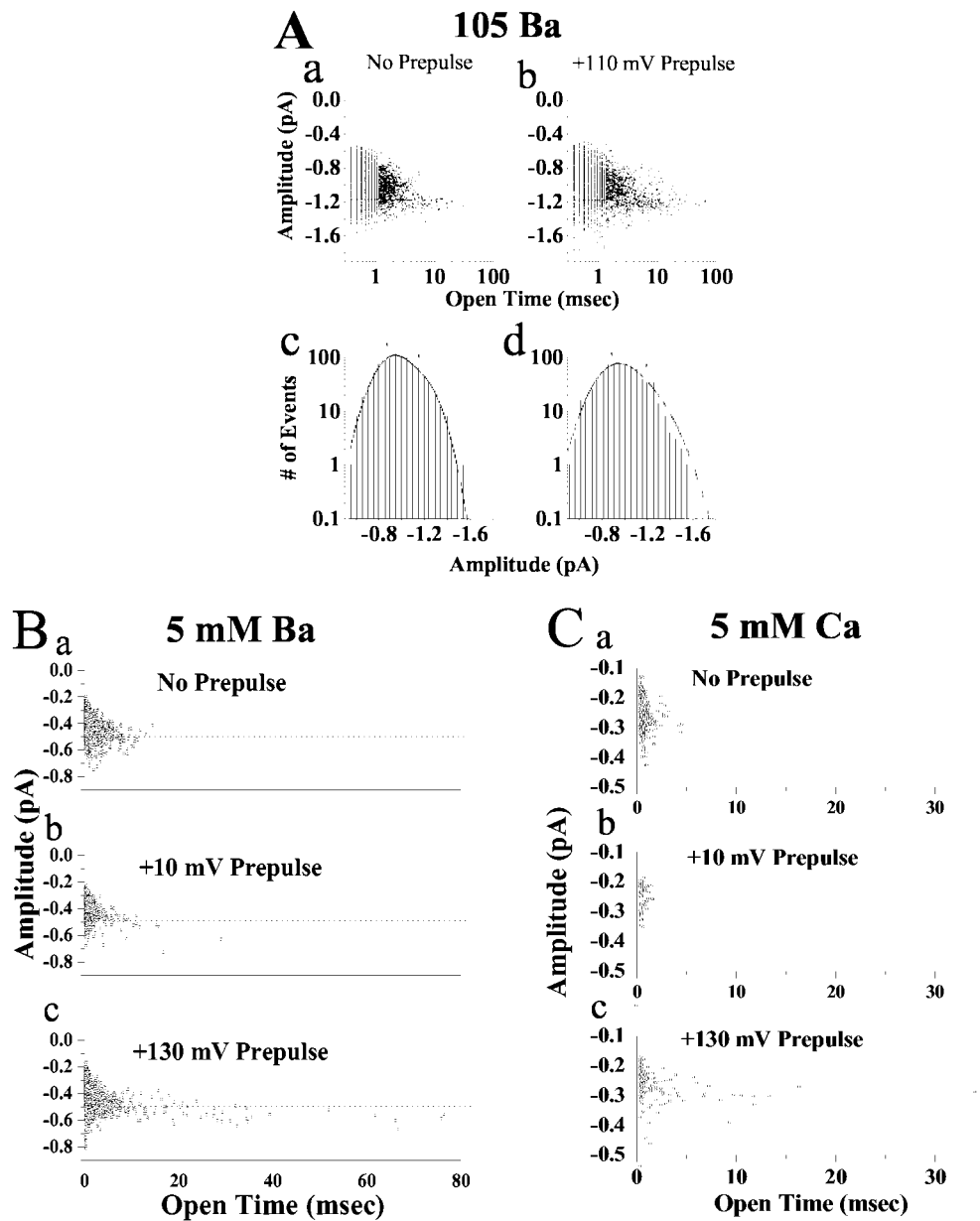


FIGURE 2 Correlation of event amplitude and open time of single Ca<sup>2+</sup> channel currents. (A) Scatterplot analysis (event amplitude vs. duration; *a* and *b*) and amplitude histograms (*c* and *d*) of open times for L-type Ca<sup>2+</sup> channel currents recorded during test pulses using 105 mM Ba<sup>2+</sup> ions in the absence of a prepulse (*left panels, a* and *c*) and following a prepulse to +110 mV (*right panels, b* and *d*). The dotted horizontal lines in *a* and *b* indicate the mean amplitude of the longer-duration events. Amplitude distributions *c* and *d* are fit with a sum of two Gaussian functions (midpoints (indicated by arrows):  $-1.17 \pm 0.074$  pA, and  $-0.92 \pm 0.057$  pA). (B) Scatterplot correlation of event amplitudes and duration for L-type Ca<sup>2+</sup> channel currents recorded during test pulses using 5 mM Ba<sup>2+</sup> ions in the absence of a prepulse (*a*), following a prepulse to +10 mV (*b*), and following a prepulse to +130 mV (*c*). The horizontal line indicates the mean amplitude of the longer-duration events. (C) Scatterplot analysis of amplitudes and open times for L-type Ca<sup>2+</sup> channel currents recorded during test pulses using 5 mM Ca<sup>2+</sup> ions in the absence of a prepulse (*a*), following a prepulse to +10 mV (*b*), and following a prepulse to +130 mV (*c*). The horizontal line indicates the mean amplitude of the longer-duration events.

tion, the scatterplot of panel *b* (with a prepulse to +110 mV) also demonstrates that the number of larger-amplitude mode 2 openings was greater than in the absence of a prepulse (panel *a*). This point is again reflected in the greater number

of large-amplitude events shown in the corresponding amplitude histogram (panel *d*).

The scatterplot analysis correlating event amplitude and open time was extended to single Ca<sup>2+</sup> channel currents

recorded with 5 mM  $\text{Ba}^{2+}$  (Fig. 2 *B*) and 5 mM  $\text{Ca}^{2+}$  ions (Fig. 2 *C*) ions. In both cases, the average of the longer-duration events (indicated by the dotted lines in the scatterplots) was larger in amplitude than the average of the shorter-duration events. In addition, the frequency of the larger-amplitude longer-duration events was increased by strong prepulses (Fig. 2 *B*, panel *c* and Fig. 2 *C*, panel *c*). The distributions of event amplitudes using 5 mM  $\text{Ba}^{2+}$  or 5 mM  $\text{Ca}^{2+}$  were also fit with a sum of two Gaussians (not shown). The averaged midpoints of the amplitude distributions (and their proportions) of the test pulse currents (following prepulses to +110 mV) for 5 mM  $\text{Ba}^{2+}$  were  $-0.56 \pm 0.08$  pA (21%) and  $-0.42 \pm 0.08$  pA (79%), and  $-0.30 \pm 0.1$  pA (39%) and  $-0.25 \pm 0.1$  pA (61%) for 5 mM  $\text{Ca}^{2+}$  ions.

In addition, a third method of amplitude analysis was applied using recordings made with 105 mM  $\text{Ba}^{2+}$ , 5 mM  $\text{Ba}^{2+}$ , or 5 mM  $\text{Ca}^{2+}$  ions. The single channel events lists were parsed into two groups by short-duration and long-duration events. The cutoff for the shorter events was  $<3.5$  ms for 105 mM  $\text{Ba}^{2+}$  and for 5 mM  $\text{Ba}^{2+}$ , and  $<2.0$  ms for 5 mM  $\text{Ca}^{2+}$  ions; these criteria for cutoff duration were more than five times the values for the fast time constants of the open-time distribution under each condition. For both ionic conditions, events shorter than 0.5 ms were excluded from the analysis to eliminate the possibility that the amplitudes of the shorter events were artifactually reduced by filtering. We chose this exclusion criterion because at a filtering frequency of 2 kHz events  $>0.5$  ms in duration are predicted to be  $>99\%$  of their unfiltered (actual) amplitude using a Gaussian filter (see Sakmann and Neher, 1983). The average amplitude of the short- and long-duration events was then calculated. For 105 mM  $\text{Ba}^{2+}$  ions (at test potential of 0 mV) the prepulse-induced long-duration opening amplitudes averaged  $-1.16 \pm 0.13$  pA, whereas the briefer openings averaged  $-0.91 \pm 0.22$  pA (statistically different at the  $p < 0.001$  level). Thus, the longer openings were 27% larger than the shorter openings for 105 mM  $\text{Ba}^{2+}$  ions. For 5 mM  $\text{Ba}^{2+}$  ions (at 0 mV), the prepulse-induced long-duration openings averaged  $-0.50 \pm 0.06$  pA, whereas the briefer openings averaged  $-0.39 \pm 0.03$  pA (statistically different at the  $p < 0.001$  level). Again, by this direct method, the longer openings were 28% larger than the shorter openings for 5 mM  $\text{Ba}^{2+}$  ions. Using the same method for 5 mM  $\text{Ca}^{2+}$  ions (at a test potential of  $-10$  mV), the average single channel current was  $-0.24 \pm 0.06$  pA for the briefer events, and  $-0.30 \pm 0.07$  pA ( $p < 0.001$ ) for the longer-duration openings, giving an increase of 25% for mode 2 openings.

### Increased conductance of mode 2 openings

To directly evaluate the conductance of mode 2 openings we measured the amplitudes of long-duration mode 2 openings that occurred near the end of a test pulse, and remained

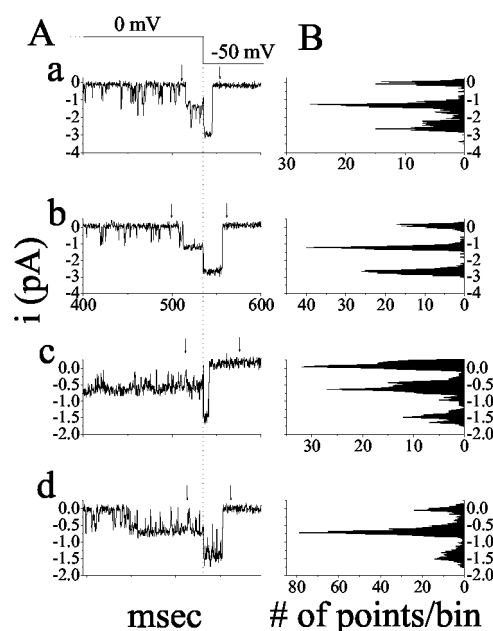


FIGURE 3 Mode 2 conductance measured by a tail current method using  $\text{Ba}^{2+}$  ions. (A) Representative recordings of L-type  $\text{Ca}^{2+}$  channel currents that displayed a mode 2 opening near the end of a test step to 0 mV (after a facilitating prepulse to +110 mV), that persisted immediately following repolarization to  $-50$  mV (single channel tail currents). Traces in *a* and *b* were recorded with 105 mM  $\text{Ba}^{2+}$  ions, and in *c* and *d* with 5 mM  $\text{Ba}^{2+}$  ions. Traces were corrected for leakage and capacity currents. The dotted line indicates the repolarization of the voltage step. (B) All-points histograms of the corresponding mode 2 openings shown in (A). The segment of the trace included in the histogram is bracketed by arrows.

open during and after repolarization of the test pulse to the holding potential. The mode 2 single  $\text{Ca}^{2+}$  channel “tail currents” had the advantage of yielding a slope conductance arising from individual, identifiable long-lasting openings of a single channel. As the deactivation of mode 2 is relatively slow, repolarization increases the electrochemical driving force on the permeating ions, and thus increases the single channel current amplitude. Examples of single mode 2  $\text{Ca}^{2+}$  channel tail currents are shown in Fig. 3 *A* (traces *a* and *b* were recorded with 105 mM  $\text{Ba}^{2+}$  ions; *c* and *d* with 5 mM  $\text{Ba}^{2+}$  ions). Accompanying each current trace is the all-points histogram (Fig. 3 *B*) showing the amplitude of the long opening during the test pulse, and following repolarization. The current amplitudes were measured as the midpoints obtained from Gaussian fits to the all-points histograms.

The voltage-dependence for the amplitudes of single  $\text{Ca}^{2+}$  channel currents recorded during mode 2 tail current openings are compared with  $\text{Ca}^{2+}$  channel openings of all durations (recorded during single test steps, and identified and analyzed by a 50% threshold method) in Fig. 4. It should be noted that “all openings” included mode 2 openings as well as briefer mode 1 openings; however, the frequency of mode 2 events in the absence of a facilitating



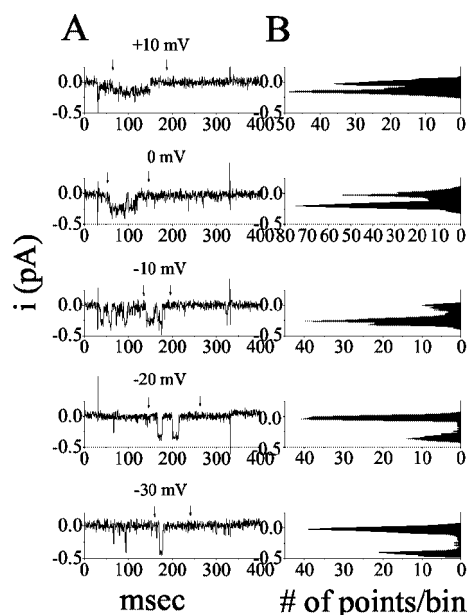


FIGURE 4 Mode 2 conductance measured using 5 mM Ca<sup>2+</sup> ions. (A) Representative examples of single L-type Ca<sup>2+</sup> channel currents displaying long-duration openings (mode 2) recorded during single steps to the potentials indicated from a HP of  $-50$  mV. The brief artifact at the beginning and end of the corrected trace denotes the onset and termination of the voltage step. (B) All-points histograms of the corresponding mode 2 opening shown in part A. The segment of the trace included in the histogram is bracketed by arrows.

prepulse was usually  $<5\%$  of the total number of openings. Part A displays results using 105 mM Ba<sup>2+</sup> ions, part B with 5 mM Ba<sup>2+</sup> ions. With 105 mM Ba<sup>2+</sup> ions, the slope conductance was  $25.7 \pm 1.2$  pS for mode 2, whereas a linear regression to the average data for all openings (single steps) gave a slope conductance  $14.5 \pm 0.5$  pS. For 5 mM Ba<sup>2+</sup> the slope conductance was  $18.2 \pm 0.6$  pS for mode 2, and was  $10.8 \pm 0.4$  pS for all openings. In addition to these differences in modal conductance, the apparent single channel reversal potentials were also different. For 105 mM Ba<sup>2+</sup> ions, the extrapolated apparent reversal potential was  $+47$  mV for mode 2 openings versus  $+65$  mV for all openings; for 5 mM Ba<sup>2+</sup> the extrapolated apparent reversal potential was  $+32$  mV for mode 2 versus  $+38$  mV for all openings.

As mode 2 tail current measurements were extremely rare with 5 mM Ca<sup>2+</sup> ions (due to decreased frequency of reopenings near the end of the test step), mode 2 openings were measured during single voltage steps by the all-points amplitude histogram method, as shown in Fig. 5. Part A displays examples of mode 2 openings occurring during single voltage steps to the test potentials indicated. Part B shows the corresponding all-points histograms, constructed using segments of the traces surrounding the openings (as indicated by the arrows). The all-points histograms were fit

with a sum of Gaussian functions to obtain the average amplitude of the mode 2 opening. With 5 mM Ca<sup>2+</sup> ions, a linear regression to the average amplitude data gave a slope conductance of  $6.1 \pm 0.3$  pS for mode 2, and  $3.6 \pm 0.2$  pS for openings of all durations (Fig. 5 C). The extrapolated apparent reversal potential for 5 mM Ca<sup>2+</sup> was  $+32$  mV for mode 2 versus  $+60$  mV for openings of all durations.

Thus, with Ca<sup>2+</sup> ions and Ba<sup>2+</sup> ions, facilitation by high-voltage prepulses produced longer-duration mode 2 openings that attained a significantly greater conductance than shorter-duration (but fully resolved) openings. The implications of this novel voltage-dependent change in Ca<sup>2+</sup> channel permeation (and gating kinetics) will be discussed subsequently.

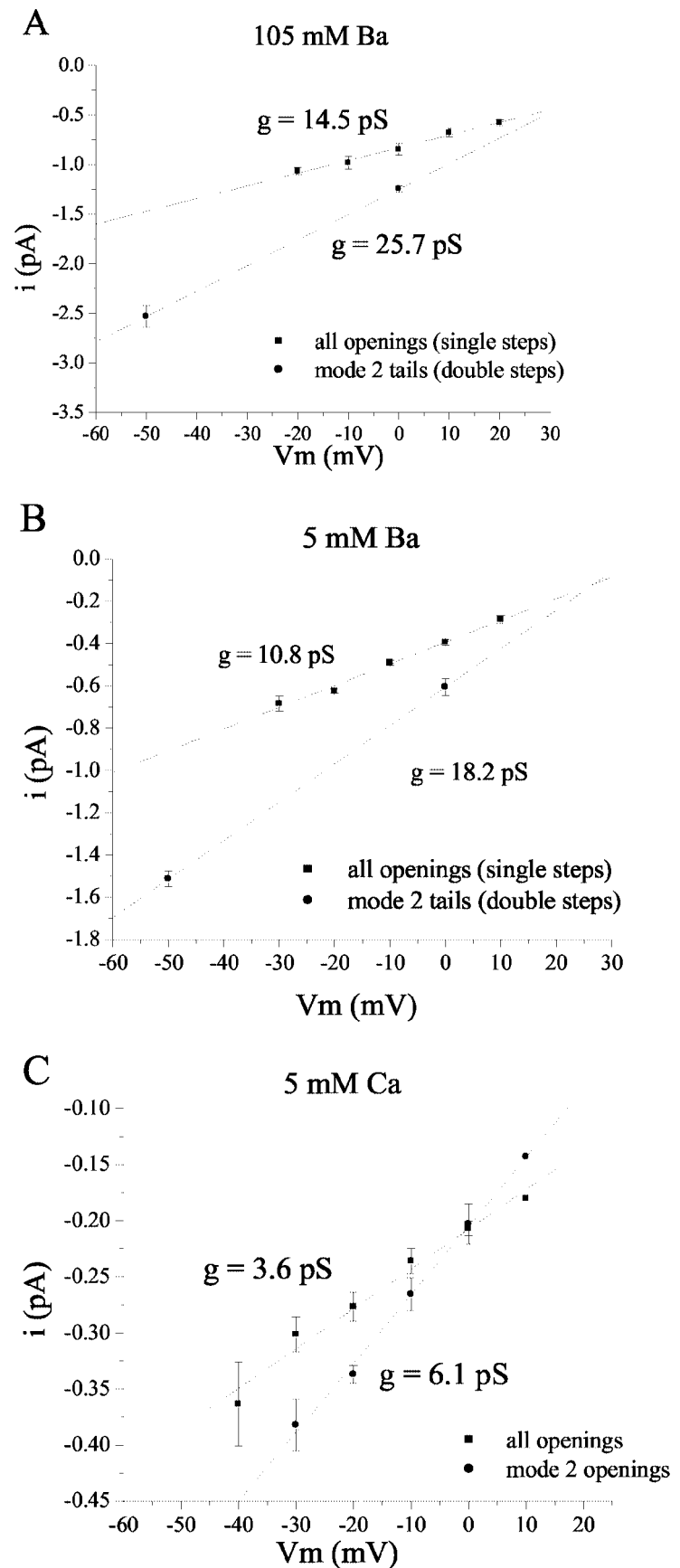
## DISCUSSION

The results of this paper and the accompanying paper (Josephson et al., 2002), demonstrate that multiple aspects of single L-type Ca<sup>2+</sup> channel behavior (gating kinetics, modal transitions, and single channel conductance) are influenced by the magnitude of the conditioning depolarization and the nature and concentration of the permeant ion. A novel and important feature of the present results is the demonstration that strong depolarization not only resulted in a shift to mode 2 long-openings using a low concentration of Ca<sup>2+</sup> ions, but also tended to temporarily “lock” the Ca<sup>2+</sup> channel in its highest conductance conformation.

Similarly, an early report on L-type Ca<sup>2+</sup> channels in smooth muscle cells (Caffrey et al., 1986) demonstrated that BayK8644, a Ca<sup>2+</sup> channel agonist that pharmacologically promotes mode 2 long openings, also increased the single Ca<sup>2+</sup> channel conductance by 25% (from 12 pS to 15 pS using 100 mM Ba<sup>2+</sup> ions), and an increase in single channel current amplitude with CPG (another DHP derivative that also promotes mode 2) has been reported for cardiac L-type Ca<sup>2+</sup> channels (Kokubun and Reuter, 1984). We have also found that another Ca<sup>2+</sup> channel agonist, FPL 64176, increases the single channel conductance to the same level as that of mode 2 openings (Josephson, personal observation).

The conductance of single cardiac L-type Ca<sup>2+</sup> channels has been reported over a wide range of values in previous studies, even in the absence of agonists and using the same divalent ion concentration (see Guia et al., 2001, for a review of the literature). In light of the present results, it seems plausible that contributing to at least a part of this range may be the variable number of mode 2 openings (as compared with mode 1) recorded in previous studies. The frequency of mode 2 openings (in the absence of voltage-facilitation or agonists) may be related to many factors, including species differences, endogenous intracellular levels of cyclic AMP or other second messengers, and the metabolic state of the myocytes. Moreover, mode 2 openings (that attain a stable amplitude level for an extended period of time) may have been favored in those previous

FIGURE 5 A comparison of mode 2 unitary L-type  $\text{Ca}^{2+}$  channel conductance with the conductance of openings of all durations. (A) 105 mM  $\text{Ba}^{2+}$ : single  $\text{Ca}^{2+}$  channel amplitudes were measured (as shown in Fig. 3) during mode 2 openings at a test potential of 0 mV (following a prepulse to +110 mV), and upon repolarization to -50 mV; the resulting all-points amplitude histograms were fit with a sum of Gaussian functions. The average mode 2 amplitudes (*circles*) gave a slope conductance (*dotted line*),  $g = 25.7 \pm 1.2$  pS ( $n = 4$  cells). The single  $\text{Ca}^{2+}$  channel conductance, obtained from event amplitudes detected of all openings during single voltage steps to the potentials indicated, was fit with a linear regression (*dotted line*) to the averaged amplitude data (*squares*,  $n = 5$  cells) yielding a conductance,  $g = 14.5 \pm 0.5$  pS. The two slope conductances were statistically different at the  $p < 0.01$  level. (B) 5 mM  $\text{Ba}^{2+}$ : single  $\text{Ca}^{2+}$  channel amplitudes were measured (as shown in Fig. 3) during mode 2 openings at a test potential of 0 mV (following a prepulse to +110 mV), and upon repolarization to -50 mV; the resulting all-points histograms were fit with a sum of Gaussian functions. The average mode 2 amplitudes (*circles*) gave a slope conductance,  $g = 18.2 \pm 0.6$  pS ( $n = 5$  cells). The single channel conductance, obtained from event detection of all openings during single voltage steps to the potentials indicated (*circles*), was fit with a linear regression (*dotted line*) to the average amplitude data and yields a conductance,  $10.8 \pm 0.4$  pS (*squares*,  $n = 7$  cells). The two slope conductances were statistically different at the  $p < 0.01$  level. (C) 5 mM  $\text{Ca}^{2+}$ : single  $\text{Ca}^{2+}$  channel mode 2 conductances were obtained by directly measuring amplitudes of mode 2 events at the test potentials indicated. The resulting all-points amplitude histograms were fit with a sum of Gaussian functions. The average mode 2 amplitudes (*circles*) were fit with a linear regression; the dotted line yields a conductance,  $g = 6.1 \pm 0.3$  pS ( $n = 5$  cells). The single channel conductance of all openings, obtained from event detection of all openings during single voltage steps to the potentials indicated, was fit with a linear regression to the average amplitude data (*squares*),  $g = 3.6 \pm 0.2$  pS ( $n = 6$  cells). The two slope conductances were statistically different at the  $p < 0.05$  level.



studies where Ca<sup>2+</sup> channel amplitude was measured by hand, thereby yielding a higher estimate of conductance.

In the present study mode 2 Ca<sup>2+</sup> channel openings not only displayed a larger slope conductance but, in addition, the extrapolations of their slope conductances to the zero current level gave apparent reversal potentials that were less positive than those obtained from the conductance measurements obtained from all openings. This finding raises the intriguing possibility that during mode 2 the Ca<sup>2+</sup> channel is temporarily less selective for divalent cations (i.e., Ca<sup>2+</sup> and Ba<sup>2+</sup>), and that monovalent cations having a less positive reversal potential (such as cesium ions in our experiments, or sodium ions physiologically) may be allowed to permeate the channel. To speculate further, this loss of selectivity may be facilitated by high-voltage prepulses that might have the effect of driving divalent cations (i.e., Ca<sup>2+</sup> ions) from their extracellular binding sites (perhaps in the pore region) that normally confer the divalent Ca<sup>2+</sup> ion selectivity to the Ca<sup>2+</sup> channel. Further experimentation using a variety of ionic conditions will be needed to test this novel hypothesis.

### Physiological relevance

A voltage- and time-dependent switch promoting a mode 2 behavior of the Ca<sup>2+</sup> channel would be a rapid and powerful mechanism to greatly enhance Ca<sup>2+</sup> influx during an ongoing train of cardiac action potentials (activity-dependent potentiation). With a 10- to 100-fold increase in mean open time (Josephson et al., 2002) and a 70% increase in conductance, this facilitory mechanism may have a profound effect on the local control of excitation-contraction coupling (see Stern, 1992), whether in directly producing Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum (SR) or in refilling the SR subsequent to Ca<sup>2+</sup> release.

Even in the absence of activity-dependent potentiation, voltage-induced long openings might play an important role during individual action potentials. The plateau phase of the cardiac ventricular action potential (of most mammalian species besides rat and mouse) remains relatively constant for >100 ms at positive potentials. With a physiological Ca<sup>2+</sup> ion concentration (1.8 mM) the voltage-dependence for activation of mode 2 would be shifted to less positive potentials, thus at plateau potentials long openings may be activated in a substantial number of Ca<sup>2+</sup> channels. As the deactivation of mode 2 is relatively slow compared to the briefer mode 1 gating (especially at depolarized potentials), mode 2 openings would also be occurring during the repolarization phase of the action potential. In addition, as repolarization progresses the driving force for Ca<sup>2+</sup> ion entry would also increase. The result of these factors would be a much larger influx of Ca<sup>2+</sup> ion during the later phases of the action potential than would otherwise occur in the absence of this facilitory mechanism.

Facilitation of Ca<sup>2+</sup> influx could also be potentiated by this voltage- and time-dependent mechanism in rat and mouse heart because in those species the high heart rate would activate mode 2 openings by summation over time, despite the very brief duration of each cardiac ventricular action potential. Thus, a late Ca<sup>2+</sup> influx would occur during the repolarization phase of the action potential due to the relatively slow rate of deactivation of the mode 2 openings. Along the same lines, an enhanced Ca<sup>2+</sup> influx produced by an augmentation of mode 2 activity during the abnormally rapid-firing, brief action potentials associated with ventricular fibrillation may contribute to Ca<sup>2+</sup> overload and further myocardial damage. In addition, frequency-dependent enhancement of mode 2 openings may have a role in modulating the activity of the sino-atrial nodal cells.

It also remains to be determined whether L-type Ca<sup>2+</sup> channels that are capable of displaying this facilitory behavior may be anatomically localized (with respect to the Ca<sup>2+</sup> release channels or other structures of the SR) to take functional advantage of this feature (e.g., in releasing Ca<sup>2+</sup> ions, or in refilling the SR). Finally, although the most studied functions of the L-type Ca<sup>2+</sup> current are in the electrogenesis of the cardiac action potential and in E-C coupling, we may also speculate that this voltage-dependent facilitation via mode 2 Ca<sup>2+</sup> channel openings is involved in other Ca<sup>2+</sup>-dependent signaling functions, such as activation of gene expression, or apoptosis.

In conclusion, the present results (which were obtained in the absence of any Ca<sup>2+</sup> channel agonists and using a low concentration of Ca<sup>2+</sup> ions and Ba<sup>2+</sup> ions) demonstrate that strong depolarization drives the native cardiac L-type Ca<sup>2+</sup> channel into a conformation that enables larger-amplitude, longer-duration openings. These findings suggest an intimate relationship of the voltage-sensing regions with the permeation-determining regions of the Ca<sup>2+</sup> channel. It will be of great importance to gain a further understanding of the properties of these long openings as they undoubtedly play a important role in the local control of E-C coupling (Stern, 1992) in normal, aging, and diseased hearts.

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### REFERENCES

- Caffrey, J. M., I. R. Josephson, and A. M. Brown. 1986. Calcium channels of amphibian stomach and mammalian aorta smooth muscle cells. *Biophys. J.* 49:1237–1242.
- Chen, C. F., and P. Hess. 1987. A complex new gating pattern detected in L-type Ca<sup>2+</sup> channels from guinea pig ventricular myocytes and mouse 3T3 fibroblasts. *J. Physiol.* 390:80.
- Church, P. J., and E. F. Stanley. 1996. Single L-type calcium channel conductance with physiological levels in chick ciliary ganglion neurons. *J. Physiol.* 496:59–68.
- Cloues, R., and W. Sather. 2000. Permeant ion binding affinity in subconductance states of an L-type Ca<sup>2+</sup> channel expressed in *Xenopus laevis* oocytes. *J. Physiol.* 524.1:19–36.

- Colquhoun, D., and F. J. Sigworth. 1995. Fitting and statistical analysis of single-channel records. In *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Press, New York.
- Gondo, N., K. Ono, K. Mannen, A. Yatani, S. Green, and M. Arita. 1998. Four conductance levels of cloned cardiac L-type  $\text{Ca}^{2+}$  channel  $\alpha 1$  and  $\alpha 1/b$  subunits. *FEBS Lett.* 423:86–92.
- Guia, A., M. D. Stern, E. G. Lakatta, and I. R. Josephson. 2001. Ion concentration-dependence of rat cardiac unitary L-type calcium channel conductance. *Biophys. J.* 80:2742–2750.
- Hess, P., J. B. Lansman, and R. W. Tsien. 1984. Different modes of  $\text{Ca}^{2+}$  channel gating behavior favored by dihydropyridine agonists and antagonists. *Nature.* 311:538–544.
- Hirano, Y., T. Yoshinaga, M. Murata, and M. Hiraoka. 1999. Prepulse-induced mode 2 gating behavior with and without B-adrenergic stimulation in cardiac L-type  $\text{Ca}^{2+}$  channels. *Am. J. Physiol. Cell Physiol.* 276:C1338–C1345.
- Josephson, I. R., A. Guia, E. G. Lakatta, and M. D. Stern. 2001. Voltage- and ion-dependent modulation of inactivation and facilitation of single L-type cardiac  $\text{Ca}^{2+}$  currents. *Biophys. J. (Annual Meeting Abstracts)* 2001:621a. (Abstr.).
- Josephson, I. R., A. Guia, E. G. Lakatta, and M. D. Stern. 2002. Modulation of the gating of unitary cardiac L-type  $\text{Ca}^{2+}$  channels by conditioning voltage and divalent ions. *Biophys. J.* 83:2575–2586.
- Kokubun, S., and H. Reuter. 1984. Dihydropyridine derivatives prolong the open state of  $\text{Ca}^{2+}$  channels in cultured cardiac cells. *Proc. Natl. Acad. Sci.* 81:4824–4827.
- Kunze, D. L., and A. K. Ritchie. 1990. Multiple conductance levels of the dihydropyridine-sensitive calcium channel in GH3 cells. *J. Membr. Biol.* 118:171–178.
- Ma, J., and R. Coronado. 1988. Heterogeneity of conductance states in calcium channels of skeletal muscle. *Biophys. J.* 55:415–424.
- Pietrobon, D., and P. Hess. 1990. Novel mechanism of voltage-dependent gating in L-type calcium channels. *Nature.* 346:651–655.
- Sakman, B., and E. Neher. 1983. *Single Channel Recording*. Plenum Press, New York.
- Stern, M. D. 1992. Theory of local control of cardiac excitation-contraction coupling. *Biophys. J.* 63:497–517.
- Yue, D., S. Herzig, and E. Marban. 1990. B-adrenergic stimulation of  $\text{Ca}^{2+}$  channels occurs by potentiation of high-activity gating modes. *Proc. Natl. Acad. Sci. U.S.A.* 87:753–757.