# Calcium-Dependent Inactivation of L-Type Calcium Channels in Planar Lipid Bilayers

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ABSTRACT Intracellular $Ca^{2+}$ can inhibit the activity of voltage-gated Ca channels by modulating the rate of channel inactivation. $Ca^{2+}$-dependent inactivation of these channels may be a common negative feedback process important for regulating $Ca^{2+}$ entry under physiological and pathological conditions. This article demonstrates that the inactivation of cardiac L-type Ca channels, reconstituted into planar lipid bilayers and studied in the presence of a dihydropyridine agonist, is sensitive to $Ca^{2+}$. The rates and extents of inactivation, determined from ensemble averages of unitary $Ba^{2+}$ currents, decreased when the calcium concentration facing the intracellular surface of the channel ($[Ca^{2+}]_i$) was lowered from ~10 μM to 20 nM by the addition of $Ca^{2+}$ chelators. The rates and extents of $Ba^{2+}$ current inactivation could also be increased by subsequent addition of $Ca^{2+}$ raising the $[Ca^{2+}]_i$ to 15 μM, thus demonstrating that the $Ca^{2+}$ dependence of inactivation could be reversibly regulated by changes in $[Ca^{2+}]_i$. In addition, reconstituted Ca channels inactivated more quickly when the inward current was carried by $Ca^{2+}$ than when it was carried by $Ba^{2+}$, suggesting that local increases in $[Ca^{2+}]_i$ could actuate $Ca^{2+}$-dependent inactivation. These data support models in which $Ca^{2+}$ binds to the channel itself or to closely associated regulatory proteins to control the rate of channel inactivation, and are inconsistent with purely enzymatic models for channel inactivation.

## INTRODUCTION

Voltage-gated Ca channels regulate $Ca^{2+}$ entry by activating in response to membrane depolarization, and then inactivating to a long-lasting closed state. $Ca^{2+}$ entry through voltage-gated Ca channels plays a key role in the cardiac action potential and the development of the myocardial contraction, and Ca channels are important targets for modulation by neurotransmitters, enzymes, and cardiotonic drugs. Inactivation of L-type Ca channels in most cells depends on both transmembrane voltage and the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (e.g., Brown et al., 1981; Kass and Sanguinetti, 1984; Lee et al., 1985; Gutnick et al., 1989; Satin and Cook, 1989; Giannattasio et al., 1991), and reversible $Ca^{2+}$-dependent inactivation is observed in excised membrane patches (Romanin et al., 1992). $Ca^{2+}$-dependent inactivation of voltage-gated Ca channels may be a negative feedback process that helps regulate $Ca^{2+}$ entry under physiological and pathological conditions. For example, hypoxic and ischemic insult to myocardium results in an increase of the $[Ca^{2+}]_i$ (Steenbergen et al., 1987) which may promote L-type Ca channel inactivation (Yue et al., 1990), and help control the onset of slow conduction and ventricular fibrillation (Janse and Wit, 1989).

Enzymatic and nonenzymatic mechanisms have been proposed to describe the process of $Ca^{2+}$-dependent inactivation in a variety of cell types. In an enzymatic mechanism, increases in $[Ca^{2+}]_i$ activate $Ca^{2+}$-dependent phosphatases in the vicinity of the Ca channel which dephosphorylate the channel and cause it to enter an inactivated state (Chad and Eckert, 1986; Armstrong et al., 1991; Hadley and Lederer, 1991). An important feature of this model is that channels must be rephosphorylated for subsequent voltage-dependent activation. In nonenzymatic mechanisms, $Ca^{2+}$ is proposed to bind directly to the channel protein or to an associated regulatory protein to promote inactivation via an allosteric interaction (Sherman et al., 1990; Yue et al., 1990; Imredy and Yue, 1992). Consistent with this model is the observation that increases in $[Ca^{2+}]_i$ cause L-type Ca channels to enter a long-lasting, but nonabsorbing, closed state (Yue et al., 1990). In addition, Johnson and Byerly (1993) propose that elevated $[Ca^{2+}]_i$ modulates Ca channel inactivation via allosteric interactions between the cytoskeleton and Ca channels.

Mechanistic characterization of $Ca^{2+}$-dependent inactivation of L-type Ca channels could be facilitated by simultaneous access to intra- and extracellular environments of the channel. Whole-cell and cell-attached patch-clamp techniques have been successful in describing the fundamental properties of L-type Ca channels as well as identifying the important players in Ca channel regulation, but they are limited in their experimental access to and control of the intracellular environment. Although the excised patch technique provides improved access to the intracellular environment (e.g., Yatani et al., 1987; Kaibara and Kameyama, 1988; Armstrong et al., 1991; Romanin et al., 1992), the activity of L-type Ca channels in these patches rapidly decreases with time ("run down") (Fenwick et al., 1982).

An alternative to these techniques is to study Ca channel activity following incorporation into planar lipid bilayers where both the intracellular and extracellular $[Ca^{2+}]$ can be

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controlled. Since the cellular cytoplasm is biochemically removed during membrane preparation, the internal concentrations of ATP, GTP, and other enzyme cofactors can be controlled, thus limiting the participation of many membrane-bound enzymes that become incorporated into the bilayers along with the channels (Chung et al., 1991; Wang et al., 1993). Although "rundown" of channel activity has been a problem with this technique, the activity of L-type Ca channels in planar lipid bilayers can be sustained for longer times if activated stimulatory G-protein ($G_{s\alpha}$) is present in the intracellular chamber, or if phosphorylation of sarcolemmal components is promoted by treating membranes with the catalytic subunit of cAMP-dependent protein kinase (PKA) prior to incorporation (Yatani et al., 1987; Imoto et al., 1988; Wang et al., 1993). Unless otherwise noted, all experiments described in this paper were done in the presence of activated $G_{s\alpha}$.

In this paper we describe, for the first time, the effects of $Ca^{2+}$ on the inactivation of dihydropyridine-modified L-type Ca channels from cardiac sarcolemma inserted into planar lipid bilayers (Ehrlich et al., 1986; Rosenberg et al., 1986). Although earlier studies did not observe $Ca^{2+}$-dependent inactivation in this system (Rosenberg et al., 1988; see Discussion), we report here that increases in $[Ca^{2+}]_i$ and permeating $Ca^{2+}$ could reversibly cause a decrease in channel open probability and an increase in the rate of channel inactivation during voltage pulses. This indicates that a component responsible for $Ca^{2+}$-dependent inactivation is closely associated with or is part of the Ca channels and can be reconstituted into the planar lipid bilayers. Preliminary reports of some of these results have been described (Hirshberg et al., 1990; Rosenberg and Haack, 1993).

## MATERIALS AND METHODS

### Membrane preparation

Cardiac sarcolemma were prepared from porcine left ventricle by homogenization, differential centrifugation, and a one-step sucrose gradient fractionation as described (Rosenberg et al., 1988).

### Planar lipid bilayers

Planar lipid bilayers (100–150 $\mu$m diameter) were formed from decane solutions of either 1-palmitoyl-2-oleoyl phosphatidylethanolamine (15 mg/ml) and 1-palmitoyl-2-oleoyl phosphatidylserine (5 mg/ml) or 1-palmitoyl-2-oleoyl phosphatidylethanolamine (12 mg/ml) and 1-palmitoyl-2-oleoyl phosphatidylserine (8 mg/ml) as described (Rosenberg and Chen, 1991). Lipids were obtained from Avanti Polar Lipids, Inc. (Pelham, AL) and were used without further purification

### Vesicle fusion

At the time of bilayer formation, aqueous solutions contained 50 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Na, pH 7.0, as described (Rosenberg et al., 1988). The final concentration of the dihydropyridine agonist (+)-202–791 (5 mM in ethanol) (Sandoz, Basel, Switzerland) included in both chambers was 1 $\mu$M. (+)202–791 was included to increase channel open times (Hess et al., 1984), because the signal-to-noise properties of planar lipid bilayers does not permit recording of the small amplitudes and brief open times of unmodified L-type Ca channels. Because it was important to reduce "rundown" and extend periods of individual channel activity in order to create ensemble averages of single-channel activity that reflected characteristics of channel inactivation, recombinant $G_{s\alpha}$, activated with guanosine 5′-*O*-(3-thiotriphosphate), was added to the *trans* (intracellular) chamber at a concentration of 5–6 nM in order to enhance and prolong channel activity in the bilayers (Yatani et al., 1987; Imoto et al., 1988; Wang et al., 1993). $BaCl_2$ and sarcolemmal vesicles were added to the *cis* chamber. In a few experiments, sarcolemmal vesicles were treated with adenosine 3′,5′-cyclic monophosphate-dependent protein kinase (PKA; Sigma; dissolved in 0.325 M dithiothreitol, aliquots stored at −80°C for up to 4 months) prior to incorporation, as previously described (Wang et al., 1993), except that sarcolemmal membranes were incubated with 10 mM $MgCl_2$, 0.2 mM adenosine-5′-*O*-(3-thiotriphosphate) and 250 units/ml catalytic subunit of PKA for 60 min at 30°C (a unit is defined as the amount of protein necessary to catalyze the transfer of 1 pmol of phosphate from ATP to hydrolyzed and partially dephosphorylated casein per min at pH 6.5 at 30°C). The solution level in the *cis* chamber was made slightly higher than in the *trans* chamber, and the bilayer was broken and immediately reformed in order to transport vesicles to the vicinity of the bilayer. Channel incorporation occurred spontaneously, usually within 5 min. L-type Ca channels were identified by their rapid activation during voltage depolarizations, current amplitude (1.2–1.5 pA at 0 mV in 100 mM external $Ba^{2+}$), open and closed times, and tendency to inactivate during long depolarizations.

### Data acquisition

Voltage clamp of the bilayers, recording of the currents, and analog and digital leak and capacitance compensation were performed as previously described (Rosenberg and Chen, 1991). Voltages were defined as *trans* minus *cis*, and currents from *cis* to *trans* are shown as inward (downward) transitions. Thus, the *trans* chamber represents the cellular interior, consistent with the conventional incorporation of outside-out vesicles. Bilayers were held at −60 to −70 mV for 5.2 s between 800-ms test pulses to 0 mV. Depolarizations caused large capacitive transients that saturated the amplifier and analog-to-digital converter; these appear as noiseless periods in the records and ensemble averages. Channel activity appeared spontaneously as inward current transitions. Currents were filtered at 200 Hz (−3 dB, 8-pole Bessel lowpass), digitized at 1 KHz, and stored in computer memory for later analysis.

Any channels that might have become incorporated into the bilayers with the opposite orientation would experience a large positive holding potential, and would probably be in the inactivated state (Campbell et al., 1988). Even if inactivation at positive potential was incomplete (Campbell et al., 1988), the open probability of a channel with the reverse orientation would be much smaller than for those with the normal orientation. If inactivation of channels with reverse orientation was incomplete, then robust activity at the holding potential would be expected. The very high levels of activity in most recordings, especially early in each experiment before rundown occurred, and the lack of activity at the negative holding potentials, provide evidence that the channels recorded had the expected orientation.

### Free $Ca^{2+}$ concentrations

Additions of $BaCl_2$, $CaCl_2$, HEDTA, and BAPTA (Tsien, 1980) as required for each experiment, were made from appropriate stock solutions. The concentration of free $Ca^{2+}$ was calculated (Schoenmakers et al., 1992) with published stability constants (Martell and Smith, 1974).

### Data analysis

Data analysis was performed as described (Rosenberg and Chen, 1991) using an in-house analysis program. Ensemble averages were formed by summation of leak-subtracted recordings, and were subjected to an additional step of digital Gaussian filtering at 40 or 100 Hz (Colquhoun and

Sigworth (1983); see figure legends). Changes in the rate of channel inactivation are observed as changes in the rate of decay and extent of inactivation of the averaged current during the depolarization. A nonlinear regression using the Marquardt-Levenberg algorithm (SigmaPlot version 5, Jandel Scientific) was used to fit the ensemble averages to the equation for a single-exponential inactivating component and a variable noninactivating pedestal: ($I = Ae^{-t/\tau} + B$). The time constants ($\tau$) and percent noninactivating component [$B/(A + B)$] are reported in the figure legends.

## RESULTS

L-type Ca channel activity has been successfully recorded in planar lipid bilayers following incorporation of cardiac sarcolemmal membranes (Ehrlich et al., 1986; Rosenberg et al., 1986). Dihydropyridine agonists are necessary in these experiments to overcome technical difficulties due to the small current amplitudes and brief open times of L-type Ca channels and the electrical noise associated with the large areas of planar lipid bilayers. Reconstituted channels are virtually identical to those in cell attached patches with regard to conductance, ion selectivity, voltage-dependence, and pharmacological sensitivity (Rosenberg et al., 1986, 1988; Ehrlich et al., 1986). In this paper, the inactivation of single Ca channels was examined by forming ensemble averages of large numbers of single-channel recordings, approximating the behavior of large numbers of individual channels. Changes in the rate of channel inactivation were observed as changes in the rate of decay of the averaged current during the depolarization.

### Characteristics of voltage-dependent inactivation of reconstituted L-type Ca channels

Before characterizing the effect of $[Ca^{2+}]_i$ on the inactivation of reconstituted, dihydropyridine-modified L-type Ca channels, we studied the voltage-dependent form of inactivation when $[Ca^{2+}]_i$ was below 20 nM. Inactivation of $Ba^{2+}$ currents through reconstituted L-type Ca channels can be seen in both single-channel recordings (Fig. 1 *A*) and in ensemble averages (Fig. 1 *B*) during an 800-ms voltage pulse from a holding potential of −60 to 0 mV. In these single-channel recordings, inactivation is seen as long-lasting closed events during the depolarization (Fig. 1 *A*; second, third, and sixth records). This inactivation was clearly reversible; although the channel inactivated during many of the depolarizations, it was available for activation following a 5.2-s period at −60 mV.

In ensemble averages obtained from a large number of depolarization-evoked unitary events, channel inactivation was observed as a time-dependent decay. In an effort to quantify single channel inactivation rates for numeric comparisons, the rates and extents of channel inactivation were determined by fitting ensemble averages with a single-exponential inactivating component and a variable noninactivating pedestal (see Materials and Methods and figure legends). In this example (Fig. 1 *B*), the inactivation is thought to be purely voltage-dependent, because $[Ca^{2+}]_i$ was buffered to approximately 20 nM with 1 mM HEDTA and because the external charge-carrying divalent cation was $Ba^{2+}$.

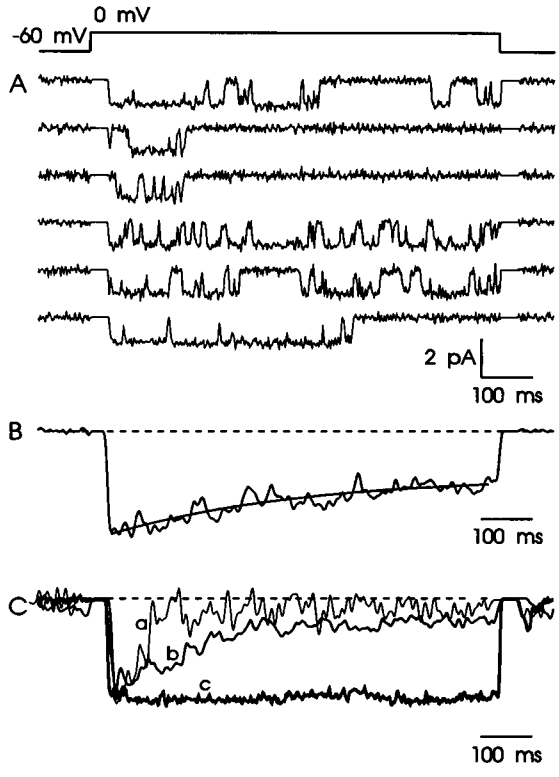


FIGURE 1 Example of typical dihydropyridine-modified L-type Ca channel activity in a planar lipid bilayer. Channel activity was recorded using 100 mM $Ba^{2+}$ as the charge carrier and 1 mM HEDTA to lower $[Ca^{2+}]_i$ to 20 nM. Channel openings were triggered by the membrane depolarization indicated. (*A*) Sequential leak-subtracted recordings; channel openings are shown as downward transitions (R05061). (*B*) The ensemble average (from 75 depolarizations) from the experiment illustrated in *A* after additional digital filtering at 40 Hz. The smooth line shows the single-exponential decay to a variable pedestal that best fit the ensemble average (see Materials and Methods). The time constant of the inactivating component was 374 ms, and the pedestal was 45% of the peak value. (*C*) Ensemble averages, scaled to the same "peak" inward current, from three additional experiments (R05031, R07054, and Y08231), illustrating the channel-to-channel variability observed in the rate of voltage-dependent inactivation. For averages *a* and *b*, the time constants of the inactivating components and the size of the noninactivating pedestals as a percent of the maximal averaged currents were 46 ms/7% and 175 ms/20%, respectively; average *c* had no inactivating component. Ensemble averages show rapid channel activation, such that the maximal channel open probability occurs within the first 30–50 ms following the depolarization, during the period of amplifier and analog-to-digital converter saturation. Channels showed essentially zero open probability during the interpulse interval at −60 mV, as illustrated by the low level of activity during the 100 ms before the pulse. In addition, channels deactivated very rapidly at the end of the depolarization, as determined by the lack of "tail" current following repolarization and the 30–50 ms of amplifier and A-to-D converter saturation.

Fig. 1 *C* shows that there was substantial channel-to-channel variability in the inactivation rate, even when ionic and membrane lipid conditions were identical. Some Ca channels showed essentially no voltage-dependent inactivation during the 800-ms depolarization (average *c*), so that the

ensemble average remained fairly constant during the depolarization. However, other channels showed rapid voltage-dependent inactivation (average *a*), and the ensemble average decayed to less than 10% of its maximal value during the pulse, with a decay time constant of approximately 50 ms. Most channels showed an intermediate rate of voltage-dependent inactivation (average *b*, see also Fig. 1 *B*), decaying to 20–40% of the maximal value during the 800-ms pulse with time constants of 100–200 ms.

The channel to channel variability of voltage-dependent inactivation rates was observed independent of whether stimulatory G-protein was present in the intracellular chamber. Similar variability in channel inactivation was seen following incorporation of L-type Ca channels from membranes treated with the catalytic subunit of PKA, suggesting that this variability could be a characteristic of individual channels, although differential phosphorylation and G-protein interactions could play a role in this heterogeneity of voltage-dependent inactivation rates (see Discussion). Because of this channel-to-channel heterogeneity, each channel served as its own control for studying the effect of $Ca^{2+}$ on the rate of inactivation. In addition, we found that, under these conditions, inactivation rates of reconstituted L-type Ca channels remained constant throughout an experiment unless intracellular $Ca^{2+}$ was changed (data not shown).

## Inactivation of reconstituted L-type Ca channels is reversibly accelerated by increased $[Ca^{2+}]_i$

In order to determine if the reconstituted channels manifested $Ca^{2+}$-dependent inactivation, we examined the rate of inactivation of single L-type Ca channels exposed to different $[Ca^{2+}]_i$. Fig. 2, *A–C*, shows unitary $Ba^{2+}$ currents from the same single L-type Ca channel under three different intracellular ionic conditions: (*A*) ambient $[Ca^{2+}]_i$ (in the absence of $Ca^{2+}$ buffer; approximately 10 $\mu$M), (*B*) after reducing $[Ca^{2+}]_i$ to 20 nM with 1 mM HEDTA, and (*C*) after increasing $[Ca^{2+}]_i$ to approximately 15 $\mu$M by the addition of $CaCl_2$. Ensemble averages of the channel activity in each of these conditions are compared in *D*; chelation of internal $Ca^{2+}$ (average *b*) caused a modest (30%) increase in the peak averaged current and a substantial (threefold) slowing of the rate of channel inactivation (compare averages *a* and *b*). Both effects were reversed by a subsequent increase in $[Ca^{2+}]_i$ (average *c*).

In order to focus on the inactivation rate, ensemble averages were scaled so that the maximum of the ensemble average was the same for each condition (Fig. 3 *A*). At high $[Ca^{2+}]_i$, the channel tended to inactivate almost completely during the depolarization (averages *a* and *c*) but when $[Ca^{2+}]_i$ was buffered to very low concentrations, inactivation was slowed. In this experiment, the time constant for the inactivating component changed from 222 ms in 10–15 $\mu$M $[Ca^{2+}]_i$ to > 500 ms in 20 nM $[Ca^{2+}]_i$. We were able to perform both increases and decreases in $[Ca^{2+}]_i$ on single channels in eight separate experiments (Table 1); in five of eight experiments we obtained results similar to those shown in Fig. 2, with 2–5-fold changes in the rate of channel inactivation following changes in $[Ca^{2+}]_i$. In the remaining three experiments changes in $[Ca^{2+}]_i$ did not affect the rate of channel inactivation.

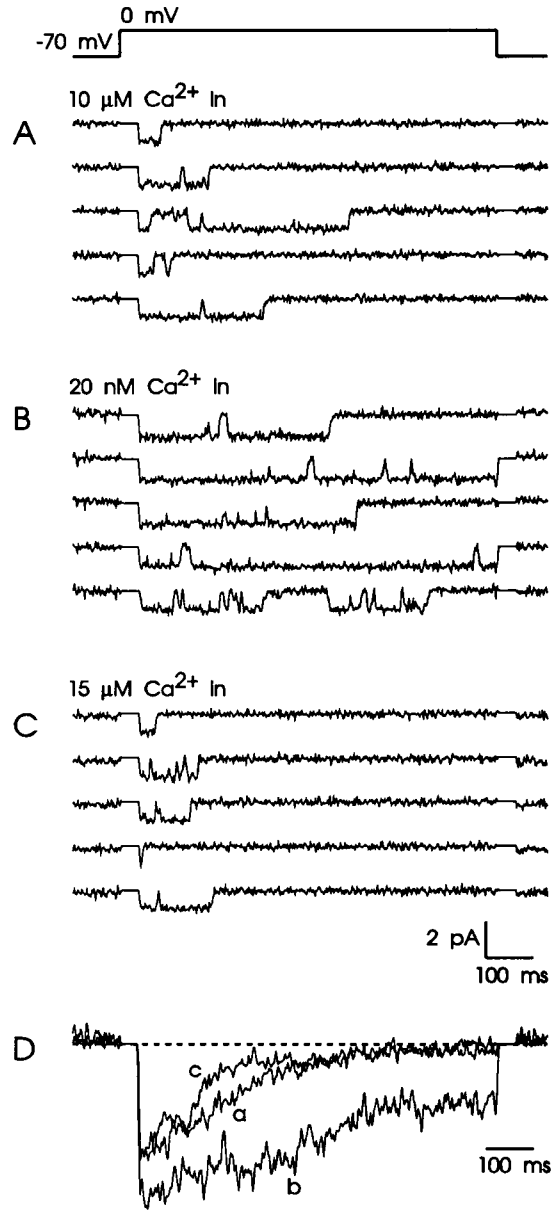


FIGURE 2 $Ca^{2+}$-dependent inactivation of reconstituted L-type Ca channels. Channel activity was recorded with 100 mM $Ba^{2+}$ as the charge carrier. (*A–C*) Selected recordings from a single reconstituted L-type Ca channel (R08092) exposed to three different $[Ca^{2+}]_i$. (*D*) Ensemble averages from *A–C* were digitally filtered at 100 Hz. The data show the results with ambient (~10 $\mu$M) internal $Ca^{2+}$ (*A* and *D*, average *a*; 29 sweeps), following addition of 1 mM HEDTA to lower $[Ca^{2+}]_i$ to 20 nM (*B* and *D*, average *b*; 31 sweeps) and following addition of $CaCl_2$ to the internal solution, raising free $[Ca^{2+}]_i$ to 15 $\mu$M (*C* and *D*, average *c*; 37 sweeps).

In addition, we have observed similar changes in the rate of inactivation following either an increase or decrease in $[Ca^{2+}]_i$ in an additional 11/15 experiments (Table 2). In the 3/15 remaining experiments we saw no effect on the rate of inactivation following changes in $[Ca^{2+}]_i$.

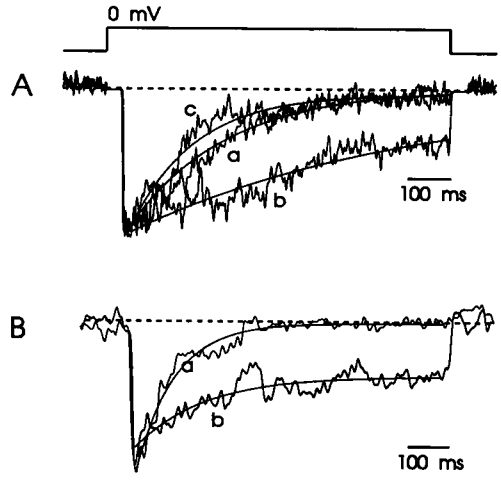


FIGURE 3 $Ca^{2+}$-dependent inactivation was similar for $G_{s\alpha}$-modulated and PKA-modulated L-type Ca channels. Channel activity was recorded using 100 mM $Ba^{2+}$. (*A*) Ensemble averages from a single L-type Ca channels in the presence of intracellular $G_{s\alpha}$ and with three different internal solutions (shown in Fig. 2 *D*) all scaled to the same peak inward "current" and digitally filtered at 100 Hz; average *a*, ambient (10 $\mu$M) internal $Ca^{2+}$; average *b*, following addition of 1 mM HEDTA to lower $[Ca^{2+}]_i$ to 20 nM in the internal solution; average *c*, following addition of $CaCl_2$ to the internal solution, raising free $Ca^{2+}$ to 15 $\mu$M. The smooth line shows the single-exponential decay to a variable pedestal that best fit the data (see Materials and Methods). The time constants of the inactivating components and the percent(s) of the peak current that were noninactivating were 222 ms and 0% for average *a*, 673 ms and 0% for average *b*, and 146 ms and 3% for average *c*. Holding potential was −70 mV. (*B*) Ensemble averages from L-type Ca channel (J23720) following incorporation of membranes treated with the catalytic subunit of PKA and $Mg^{2+}$-ATP (Wang et al., 1993) with two different internal solutions; average *a*, ambient (10 $\mu$M) internal $Ca^{2+}$; average *b*, following addition of 1 mM HEDTA to lower $[Ca^{2+}]_i$ to 20 nM. Averages were scaled to the same maximal value and filtered at 40 Hz. The time constant and pedestal were 92 ms and 1% for average *a*, and 169 ms and 43% for average *b*. The holding potential was −65 mV.

In order to test whether the observed $Ca^{2+}$-dependence of inactivation required the exogenous $G_{s\alpha}$, we recorded L-type Ca channels whose activity was instead sustained by treatment with PKA (Wang et al., 1992). As shown in Fig. 3 *B*, channels incorporated from membranes that were treated with the catalytic subunit of PKA and recorded in the absence of $G_{s\alpha}$ also responded to changes in $[Ca^{2+}]_i$. In three of three experiments, chelation of $[Ca^{2+}]_i$ by 1 mM BAPTA caused the rate of inactivation to decrease.

## Acceleration of inactivation of reconstituted L-type Ca channels is dependent on changes in $[Ca^{2+}]_i$ and not on changes in $[Ba^{2+}]_i$

In order to determine if changes in the rate of channel inactivation were specific for $[Ca^{2+}]_i$, we changed intracellular free barium concentration ($[Ba^{2+}]_i$). In the absence of HEDTA, changing $[Ba^{2+}]_i$ from approximately 2 $\mu$M (ambient) to 10 mM did not change the rate of Ca channel inactivation (three of three trials; data not shown). In the presence of 1 mM HEDTA, changing $[Ba^{2+}]_i$ from <20 nM to 1 mM also failed to modulate the rate of channel inactivation

**TABLE 1 Channel inactivation rates with different $[Ca^{2+}]_i$**

| Experiment | $[Ca^{2+}]_i$ ~10 $\mu$M | 20 nM | 15 $\mu$M |
|---|---|---|---|
| J28042 | 104 | 192 | 51 |
| J28050 | 41 | 227 | 112 |
| J28070 | 192 | 370 | 184 |
| R08092 | 222 | 673 | 146 |
| Y07151 | 266 | 69* | 168 |
| J28047 | 320 | 319 | 320 |
| J28062 | 107 | 80 | 76 |
| Y08111 | 124 | 115 | 47‡ |

Each line reports the time constant of the inactivating component in ms for individual experiments. $[Ca^{2+}]_i$ was lowered from ambient (~10 $\mu$M) to 20 nM by the addition of 1 mM HEDTA to the intracellular chamber, and then increased to 15 $\mu$M by subsequent addition of intracellular $Ca^{2+}$. In 5/8 experiments the time constant for the inactivating component increased when the $[Ca^{2+}]_i$ was lowered and reversibly decreased when the $[Ca^{2+}]_i$ was increased.

* Although in this experiment the time constant for inactivation decreased, the extent of inactivation decreased from 100 to 44%; ‡ Although chelation of intracellular $Ca^{2+}$ in this experiment did not affect the rate of inactivation, a subsequent increase in $[Ca^{2+}]_i$ caused an increase in the rate of inactivation.

**TABLE 2 Summary of the results for single step changes in $[Ca^{2+}]_i$**

| Experiment | Effect on rate of inactivation | Number of trials |
|---|---|---|
| Chelation of $[Ca^{2+}]_i$ | Decreased rate | 6/9 |
| | Increased rate | 1/9 |
| | No effect | 2/9 |
| Addition of $\mu$M $[Ca^{2+}]_i$ | Increased rate | 5/6 |
| | No effect | 1/6 |

in seven of eight trials (but accelerated inactivation in one of eight trials, data not shown). Because HEDTA chelation of $Ba^{2+}$ causes the release of bound $Ca^{2+}$, addition of intracellular $Ba^{2+}$ is associated with small changes in the $[Ca^{2+}]_i$, from 0.1 to 0.5 $\mu$M. These results suggest that increases in the $[Ca^{2+}]_i$ below a final $[Ca^{2+}]_i$ of 0.5 $\mu$M do not significantly modulate L-type Ca channel inactivation in our system. This observation was consistent with that of Romanin et al., (1992) who reported that a 50% reduction in the inactivation of $Ba^{2+}$ currents through cardiac L-type Ca channels in inside-out patches occurs at ~4 $\mu$M $[Ca^{2+}]_i$.

## Permeating $Ca^{2+}$ increases channel inactivation rates

Several studies support the hypothesis that the site(s) available for interaction with $Ca^{2+}$ important for $Ca^{2+}$-dependent inactivation are close to or part of the channel (Yue et al., 1990; Imredy and Yue, 1992). In an attempt to further evaluate this hypothesis, we tested whether permeating $Ca^{2+}$ could increase the rate of inactivation of a single, reconstituted Ca channel, despite a well defined, $Ca^{2+}$-buffered intracellular solution. An increase in the rate of inactivation would suggest a proximity of the $Ca^{2+}$-dependent inactivation site(s) and the pore of the channel. Thus, we examined

whether changing the concentration of $Ca^{2+}$ in the extracellular chamber from a $Ca^{2+}$-free solution (7.5 mM $Ba^{2+}$) to a $Ca^{2+}$-rich solution (7.5 mM $Ba^{2+}$ plus 75 mM $Ca^{2+}$) could affect the rate of channel inactivation when the internal solution contained the rapid, pH-insensitive $Ca^{2+}$ buffer BAPTA (1 mM) (Tsien, 1980). The shift from 7.5 mM $Ba^{2+}$ to 7.5 mM $Ba^{2+}$ plus 75 mM $Ca^{2+}$ changes the dominant permeant ion from $Ba^{2+}$ to $Ca^{2+}$. If one assumes simple competition at a single site (Moczydlowski, 1986) and uses a $K_m$ of 14 mM for $Ca^{2+}$ and a $K_m$ of 28 mM for $Ba^{2+}$ (Hess et al., 1986), then in [7.5 mM $Ba^{2+}$ + 75 mM $Ca^{2+}$] at least 80% of the current was carried by $Ca^{2+}$. If, instead, the permeating ion was determined by simple competition at the external site of a multi-site pore in which each site has a $K_m$ of 0.3 $\mu$M for $Ca^{2+}$ and 17 $\mu$M for $Ba^{2+}$ (Hess and Tsien, 1984), then approximately 99% of the current was carried by $Ca^{2+}$. In our experiments, the unitary currents in 7.5 mM $Ba^{2+}$ were approximately 0.5 pA at 0 mV, as expected from the hyperbolic conductance-concentration relationship for $Ba^{2+}$ (Fig. 4 *A* (Hess et al., 1986)). The unitary currents in [7.5 mM $Ba^{2+}$ + 75 mM $Ca^{2+}$] were also approximately 0.5 pA at 0 mV (Fig. 4 *B*) due to the excess of $Ca^{2+}$ over $Ba^{2+}$ available for the permeation ion binding sites (Lansman et al., 1986), and the characteristic lower conductance for $Ca^{2+}$ (Hess et al., 1986).

As shown in Fig. 4, *A–C*, currents carried predominantly by $Ca^{2+}$ inactivated faster and more completely than when current through the same channel was carried exclusively by $Ba^{2+}$. Fig. 4 *C* shows ensemble averages for $Ba^{2+}$ currents (average *a*) and mostly $Ca^{2+}$ currents (average *b*). There was a large (7.8-fold) increase in the rate of inactivation when $Ca^{2+}$ carried the current compared to when $Ba^{2+}$ is the current-carrying ion. Similar results were obtained in a total of three of six experiments when intracellular $Ca^{2+}$ was buffered with BAPTA, and in one of two trials using 1 mM HEDTA instead. In the remaining experiments changes in the extracellular [$Ca^{2+}$] did not affect the rate of channel inactivation.

The rate of channel inactivation due to increases in external [$Ca^{2+}$] was not simply due to a large change in extracellular divalent ion concentration. Fig. 4 *D* shows that in the presence of intracellular $Ca^{2+}$ buffer, changes in external [$Ba^{2+}$] failed to modulate the rate of inactivation (seven of seven trials). Similar results were obtained in the absence of $Ca^{2+}$ buffer (three of three trials). These results indicated that changes in surface charge screening or binding were not responsible for the $Ca^{2+}$-dependent inactivation observed in our system following the addition of external $Ca^{2+}$.

Thus, both increases in the permeating $Ca^{2+}$ concentration and global $[Ca^{2+}]_i$ could speed inactivation of reconstituted Ca channels (Figs. 2–4). In addition, we could observe both of these effects on a single reconstituted Ca channel. An example of such an experiment is shown in Fig. 5, which compares the rates of Ca channel inactivation under three different conditions: (*a*) 7.5 mM external $Ba^{2+}$ and ambient (10 $\mu$M) $[Ca^{2+}]_i$, (*b*) following addition of 1 mM HEDTA to the internal chamber, and (*c*) addition

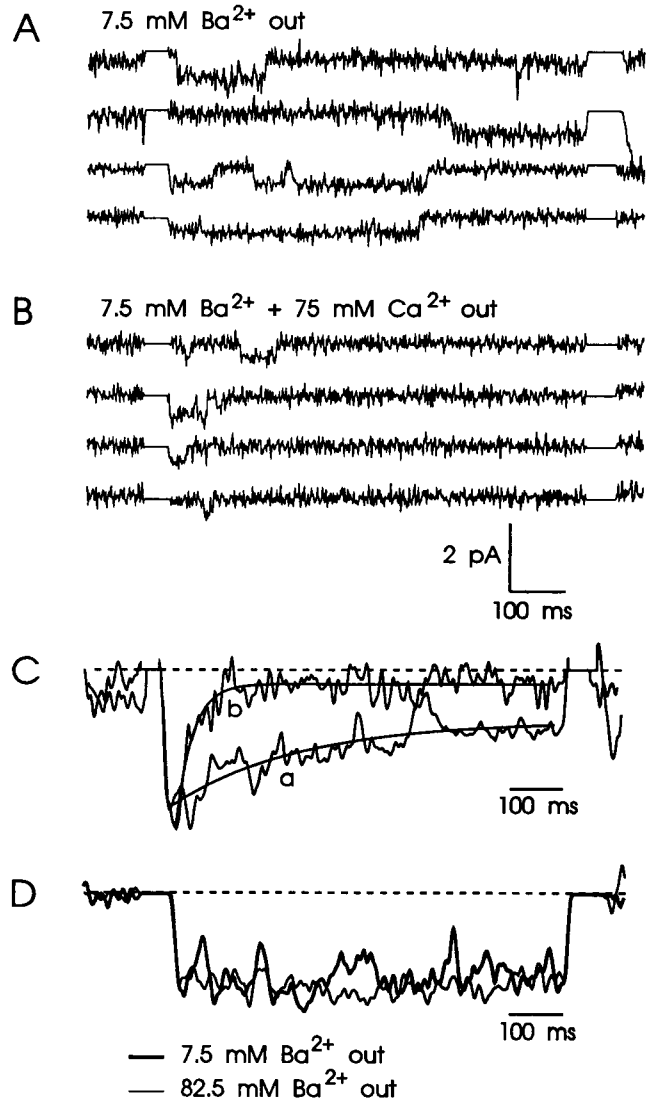


FIGURE 4 $Ca^{2+}$ flux through L-type Ca channels increases the rate of channel inactivation. Internal solution contained 1 mM BAPTA. (*A*) Selected recordings of single channel events with 7.5 mM $Ba^{2+}$ in the external solution (unitary current ~0.5 pA; R24044). (*B*) Recordings obtained after $CaCl_2$ was added to the external solution to a final concentration of 75 mM so that 80–99% of the channel current was carried by $Ca^{2+}$ instead of $Ba^{2+}$ (depending on the permeation model, see text; unitary current also ~0.5 pA). (*C*) Ensemble averages of $Ba^{2+}$ currents (average *a*; 40 sweeps; 40-Hz filtering) and after the addition of external $Ca^{2+}$ (average *b*; 39 sweeps, 40-Hz filtering). The scaling on the two averages was identical. The smooth line shows the fit of the data to a single-exponential decay to a variable pedestal. The time constant of the inactivating component and the pedestal were: average *a*, 242 ms and 38%; average *b*, 31 ms and 9%. (*D*) Changes in external [$Ba^{2+}$] and the rate of $Ba^{2+}$ flux do not change channel inactivation rates (R25082). Internal solution contained 1 mM HEDTA. Single channel events were first recorded with 7.5 mM external $Ba^{2+}$ (unitary current 0.55 pA, not shown), and used to form the bold-line ensemble average (13 sweeps; 40-Hz filtering). $BaCl_2$ was added to the external solution to a final total concentration of 82.5 mM. The unitary current increased to 1.2 pA, as expected (not shown). The fine-line ensemble average was formed from 19 sweeps and was scaled to the same maximal value as the bold-line average. If, instead of this arbitrary scaling, a factor of 2.2 was used to scale the average in 7.5 mM $Ba^{2+}$ (representing the change in unitary current), the ensemble average in 82.5 mM $Ba^{2+}$ was approximately 40% larger than the one from 7.5 mM $Ba^{2+}$, suggesting an effect of $Ba^{2+}$ concentration on channel activation.

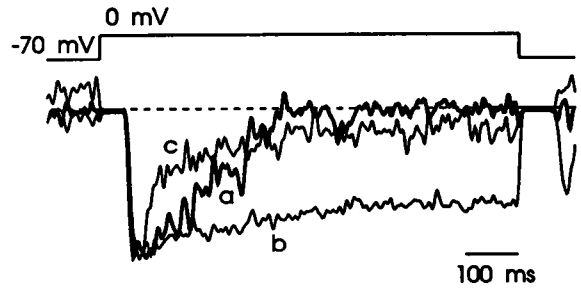


FIGURE 5 Changes in both permeating $Ca^{2+}$ and intracellular $Ca^{2+}$ concentrations can regulate channel inactivation. Ensemble averages from a single L-type Ca channel (R25061) with three different internal and external solutions: (*a*) 7.5 mM $Ba^{2+}$ outside and ambient (10 $\mu$M) $Ca^{2+}$ inside (10 sweeps); (*b*) 7.5 mM $Ba^{2+}$ outside and 20 nM $Ca^{2+}$ inside (1 mM HEDTA inside; 26 sweeps); (*c*) 7.5 mM $Ba^{2+}$ and 75 mM $Ca^{2+}$ outside and 20 nM $Ca^{2+}$ inside (41 sweeps). In this experiment the time constant of the inactivating component and the pedestal were, average *a*, 145 ms and 16%; average *b*, 283 ms and 63%; average *c*, 80 ms and 0%. Thus, chelation of global $[Ca^{2+}]_i$ lowered the rate of inactivation as shown in Figs. 2 and 3, and then changing the permeant ion from $Ba^{2+}$ to (mostly) $Ca^{2+}$ increased the rate of inactivation as seen in Fig. 5. All averages were scaled to the same maximal value, and filtered at 40 Hz.

of 75 mM $Ca^{2+}$ to the external solution. In this experiment, the rate of inactivation of $Ba^{2+}$ currents was reduced twofold by chelation of global $[Ca^{2+}]_i$, and was then increased 3.5-fold by changing the external solution so that $Ca^{2+}$ was the major permeating ion.

## DISCUSSION

We report here the characteristics of $Ca^{2+}$-dependent inactivation of single dihydropyridine-modified L-type Ca channels reconstituted into planar lipid bilayers. Our goals were to determine whether $Ca^{2+}$-dependent inactivation could occur in the absence of soluble cellular components or nucleotides, and to differentiate between enzymatic and nonenzymatic mechanisms for cardiac L-type Ca channel inactivation. In addition, we wanted to describe the characteristics of $Ca^{2+}$-dependent inactivation of reconstituted channels in relation to cardiac L-type Ca channels in native membranes.

Reconstitution of L-type Ca channels into planar lipid bilayers has several advantages for studying the mechanism of $Ca^{2+}$-dependent inactivation. One advantage is the ability to prolong channel activity by the addition of stimulatory G-protein ($G_{s\alpha}$) to the intracellular chamber. Ca channel activity in excised patches disappears very quickly due to "rundown," and the control of "rundown" by the addition of kinases, phosphatase inhibitors, and G-proteins has a variable rate of success. $G_{s\alpha}$ may be less likely to reduce rundown of Ca channels in excised patches because cytoplasmic gel attached to the cytoplasmic face of excised patches may limit the access of G-proteins to the surface of the channels. It is possible that preparation of membrane vesicles may reduce the contribution of a cytoplasmic "plug" near the channels, thus enhancing the ability of exogenously added $G_{s\alpha}$ to prolong channel activity in bilayers with a relatively high success rate.

An additional advantage of this approach is that it provides improved access to both the intracellular and extracellular surface of the channel and enables functional and biochemical isolation of channel proteins from soluble intracellular modulators. Whole-cell and cell-attached patch recordings were less appealing, because they do not permit sufficient control of the intracellular environment in the immediate vicinity of the Ca channels. In addition, the bilayer approach offers the potential for solubilization and isolation of Ca channels from associated regulatory proteins (e.g., Flockerzi et al., 1986; Smith et al., 1987; Hamilton et al., 1991), and may provide a means for the direct comparison of channel modulatory processes from channels in different tissues.

Changes in $[Ca^{2+}]_i$ associated with electrical activity are proposed to range from global increases of hundreds of nanomolar (e.g., Lipscombe et al., 1988), to local increases in $[Ca^{2+}]_i$ (near Ca channels or $Ca^{2+}$ release pools) of more than 100 $\mu$M (Smith and Augustine, 1988; Llinas et al., 1992). In neurons and heart cells, it is thought that the localized increases in $[Ca^{2+}]_i$ are most important for neurotransmitter release, release of $Ca^{2+}$ from intracellular stores, or other cellular responses (Smith and Augustine, 1988; Lederer et al., 1990; Llinas et al., 1992). Our results demonstrate that global increases in $[Ca^{2+}]_i$ (in the 10–20 micromolar range) (Figs. 2 and 3) can elicit $Ca^{2+}$-dependent inactivation of L-type Ca channels recorded in cell-free planar lipid bilayers. These results were consistent with those of Romanin et al., (1992), who showed that cardiac L-type Ca channel activity in excised inside-out membrane patches could be reversibly modulated by $[Ca^{2+}]_i$. Thus, our results showing an effect of $[Ca^{2+}]_i$ in the 10–20 $\mu$M range (but not at ~1 $\mu$M) indicates that the reconstituted Ca channels respond to $[Ca^{2+}]_i$ within the range expected for increases in localized $[Ca^{2+}]_i$ near Ca channels in intact cells.

Although our experimental results do not rule out the possible involvement of accessory proteins in the mechanism of $Ca^{2+}$-dependent Ca channel inactivation seen in our system, two observations argue against the dephosphorylation-rephosphorylation model as the only mechanism for channel inactivation and recovery from inactivation. First, reconstituted L-type Ca channels were available for activity following both $Ca^{2+}$- and voltage-dependent inactivation in the absence of intracellular ATP (Figs. 1 and 2), a condition that makes rephosphorylation extremely unlikely. Second, the inactivation rates from ensemble averages were reversibly modulated as a function of $[Ca^{2+}]_i$ despite the absence of ATP (Figs. 2 and 3). If inactivation resulted from dephosphorylation of the channel, it should have become permanently silent in our recordings. However, our experiments do not rule out a role for dephosphorylation in the inactivation of Ca channels in native cells studied in the absence of DHP agonists.

In addition, our results do not argue against a regulatory role for Ca channel dephosphorylation. There is substantial evidence for stimulation of Ca channel activity by cAMP-dependent protein kinase (for review see Hille, 1992), and the

reversal of this stimulation by phosphoprotein phosphatases (Kameyama et al., 1986). However, our results argue against the equivalence of the dephosphorylated and inactivated states. The dephosphorylation of L-type Ca channels by $Ca^{2+}$-dependent and $Ca^{2+}$-independent phosphatases is a modulatory event that renders them less likely to be activated (e.g., Kameyama et al. (1986); for review, see Trautwein and Hescheler (1990)). Dephosphorylation of L-type Ca channels may participate in very fast Ca channel inactivation in intact cells (inactivation time constants of 20–40 ms), but our results suggest that direct, nonenzymatic mechanisms also play a role.

In intact cells, the size of elevated $Ca^{2+}$ "domains" resulting from influx of $Ca^{2+}$ are estimated to be 50 nm in radius (Smith and Augustine, 1988). In our experiments, the domain of high $[Ca^{2+}]_i$ probably has a similar radius; although $Ca^{2+}$ current amplitudes are larger with 75 mM $Ca^{2+}$ in the external solution than under physiological conditions, 1 mM BAPTA (a fast, high affinity, $Ca^{2+}$ buffer) will buffer $[Ca^{2+}]_i$ more efficiently. Since the Ca channel molecule itself has a radius of approximately 15–20 nm (Leung et al., 1988), our results demonstrating that permeating $Ca^{2+}$ can increase the rate of channel inactivation (Figs. 4 and 5) indicate that the sites important for $Ca^{2+}$-dependent inactivation are in the pore or close to the internal mouth of the Ca channel. However, because the domain of elevated $[Ca^{2+}]_i$ is likely to be larger than the channel molecule, $Ca^{2+}$ influx through a single Ca channel could trigger inactivation of nearby channels (Imredy and Yue, 1992) via direct interactions with the Ca channel itself or interactions with nearby accessory proteins.

Physiologically, both $Ca^{2+}$- and voltage-dependent mechanisms probably play a role in the inactivation of cardiac Ca channels. $Ca^{2+}$ influx in cardiac myocytes sustains the prolonged plateau of the cardiac action potential (lasting ~100–200 ms), despite the activation of delayed rectifier potassium channels. Thus, a substantial number of L-type Ca channels must be active >100 ms after the upstroke of the action potential. The rate of inactivation of $Ba^{2+}$ currents through cardiac L-type Ca channels reconstituted into planar lipid bilayers varied between ~40 and ~250 ms (average = ~150 ms) when $[Ca^{2+}]_i$ was 10–15 $\mu$M (see Table 1), suggesting that channel inactivation rates of reconstituted Ca channels are in the range that would be expected physiologically. $Ca^{2+}$-dependent inactivation of $Ca^{2+}$ currents observed in cardiac cells occurs with time constants between 10 and 70 ms (e.g., Kass and Sanguinetti, 1984; Mentrard et al., 1984; Bechem and Pott, 1985; Yue et al., 1990; Hadley and Lederer, 1991; Mazzanti et al., 1991). One possible explanation for the slower inactivation kinetics observed in our experiments could be due to the use of $Ba^{2+}$ as the charge carrier (e.g., Kass and Sanguinetti, 1984; Cavalie et al., 1986; Kaibara and Kameyama, 1988; Yue et al., 1990). In our experiments, when $Ca^{2+}$ was the charge carrier, 1 mM BAPTA was present intracellularly. Thus, the combination of the nature of the current carrying ion and the ability to increase $[Ca^{2+}]_i$ may be important for generating the rapid rates of $Ca^{2+}$-dependent inactivation observed in cardiac cells. An alternative hypothesis is that differences in the lipid environment or the loss of a soluble regulatory component may be responsible for the slower $Ca^{2+}$-dependent inactivation kinetics of reconstituted channels.

Previous results from reconstituted L-type Ca channels, focusing on the interaction of permeant ions within the pore of the channels, did not show any increase in inactivation rates in the presence of millimolar internal $Ca^{2+}$ concentrations (Rosenberg et al., 1988). Our present results demonstrate that not all reconstituted L-type Ca channels are sensitive to $[Ca^{2+}]_i$ (see Tables 1 and 2). The variability in $Ca^{2+}$-sensitivity could be due to the presence of multiple subtypes of L-type Ca channels, or the loss of accessory proteins important for channel inactivation from a subset of the channels. Thus, previous experiments could have failed to detect an effect of $[Ca^{2+}]_i$ because of the relatively few experimental tests performed with elevated $[Ca^{2+}]_i$.

One interesting characteristic of the voltage-dependent inactivation of reconstituted L-type Ca channels was the considerable channel-to-channel variability of inactivation rates. This observation is not unique to the bilayer system. In 1986, Cavalie et al., observed quantitative patch-to-patch variability in Ca channel gating behavior when examining $Ba^{2+}$ currents through voltage-activated cardiac Ca channels in cell-attached patches (see also Mazzanti et al. (1991)). Monoexponential fits of the declining phases of the ensemble mean currents varied from 90 to 1400 ms (Cavalie et al., 1986). Risso and DeFelice (1993) have also observed patch-to-patch variability in channel kinetics for both $Ba^{2+}$ and $Ca^{2+}$ currents in chick ventricle cells. In addition, functional diversity among neuronal L-type Ca channels has also been reported (Forti and Pietrobon, 1993). Thus, the channel-to-channel variability observed in our system may reflect subtle structural or functional differences associated with individual L-type Ca channels in the heart.

One limitation of our approach was that we were unable to distinguish between direct $Ca^{2+}$ binding to the channel protein as opposed to binding to a closely associated modulatory protein. In the future, it could be possible to identify the need for accessory proteins by reconstituting $Ca^{2+}$-dependent inactivation from solubilized and biochemically isolated Ca channel subunits. Alternatively, this may be accomplished by the reconstitution of ion channel proteins obtained from *in vitro* translations of cloned channels (Rosenberg and East, 1992; Shen et al., 1993).

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