Cardiac Calcium Channels in Planar Lipid Bilayers

L-Type Channels and Calcium-permeable Channels Open at Negative Membrane Potentials

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ABSTRACT Planar lipid bilayer recordings were used to study Ca channels from bovine cardiac sarcolemmal membranes. Ca channel activity was recorded in the absence of nucleotides or soluble enzymes, over a range of membrane potentials and ionic conditions that cannot be achieved in intact cells. The dihydropyridine-sensitive L-type Ca channel, studied in the presence of Bay K 8644, was identified by a detailed comparison of its properties in artificial membranes and in intact cells. L-type Ca channels in bilayers showed voltage dependence of channel activation and inactivation, open and closed times, and single-channel conductances in Ba^{2+} and Ca^{2+} very similar to those found in cell-attached patch recordings. Open channels were blocked by micromolar concentrations of external Cd^{2+}. In this cell-free system, channel activity tended to decrease during the course of an experiment, reminiscent of Ca^{2+} channel "rundown" in whole-cell and excised-patch recordings. A purely voltage-dependent component of inactivation was observed in the absence of Ca^{2+} stores or changes in intracellular Ca^{2+}. Millimolar internal Ca^{2+} reduced unitary Ba^{2+} influx but did not greatly increase the rate or extent of inactivation or the rate of channel rundown. In symmetrical Ba^{2+} solutions, unitary conductance saturated as the Ba^{2+} concentration was increased up to 500 mM. The bilayer recordings also revealed activity of a novel Ca^{2+}-permeable channel, termed "B-type" because it may contribute a steady background current at negative membrane potentials, which is distinct from L-type or T-type Ca channels previously reported. Unlike L-type channels, B-type channels have a small unitary Ba^{2+} conductance (7 pS), but do not discriminate between Ba^{2+} and Ca^{2+}, show no obvious sensitivity to Bay K 8644, and do not run down. Unlike either L- or T-type channels, B-type channels did not require a depolarization for activation and displayed mean open times of >100 ms.

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/88/07/0027/28 $2.00
Volume 92 July 1988 27–54

INTRODUCTION

Planar bilayers provide a cell-free system for studying Ca channel function under a wide range of experimental conditions. For example, bilayer recordings have been used to characterize Ca channels in membranes that are difficult to approach with patch-clamp methods, including brain synaptosomes (Nelson et al., 1984; Nelson, 1986), skeletal muscle transverse tubule (t-tubule) membranes (e.g., Affolter and Coronado, 1985; Coronado and Affolter, 1986; Rosenberg et al., 1986), ciliary membranes of *Paramecia* (Erlich et al., 1984), and sarcoplasmic reticulum membranes (Smith et al., 1985, 1986; Suarez-Isla et al., 1986). For other Ca channels, such as cardiac L-type Ca channels, bilayer recordings can be used to complement cell-attached patch recordings. Because there is easy access to the intracellular side of the membrane, a wide range of intracellular, extracellular, and membrane conditions are possible.

Patch-clamp studies of cardiac L-type Ca channels have provided insights into mechanisms of ion permeation (Hess and Tsien, 1984; Hess et al., 1986; Lansman et al., 1986), channel gating (Reuter et al., 1982; Cavalie et al., 1983, 1986; Hess et al., 1984; McDonald et al., 1986), and channel modulation by neurotransmitters and drugs (Cachelin et al., 1983; Brum et al., 1984; Hess et al., 1984; Kameyama et al., 1985; Tsien et al., 1986). Difficulties in studying cardiac L-type Ca channels in planar bilayers might be anticipated since their activity rapidly and irreversibly disappears from patches excised into simple salt solutions (Cavalie et al., 1983; Nilius et al., 1985; see also Fenwick et al., 1982; Armstrong and Eckert, 1987). Contrary to such expectations, L-type channel activity has been successfully recorded in planar bilayers after incorporation of cardiac sarcolemmal membranes (Ehrlich et al., 1986; Rosenberg et al., 1986). Also, dihydropyridine-sensitive Ca channels from skeletal muscle t-tubule membranes have been recorded in planar lipid bilayers and similar channel activity is recovered in the bilayers after the solubilization and purification of dihydropyridine-binding proteins (Flockerzi et al., 1986; Smith et al., 1987; Talvenheimo et al., 1987).

In this article, we show that the cardiac L-type Ca channel retains its fundamental properties when studied in the cell-free system. Activation and inactivation are strongly voltage dependent. The channel conductance for Ba^{2+} and Ca^{2+} and its sensitivity to block by external Cd^{2+} are similar to those in the intact cell. Even in the absence of Ca^{2+}, the channel shows a time-dependent inactivation during maintained depolarizations. Channel activity can be seen for some time despite the removal of soluble enzymes or nucleotides, but it tends to disappear spontaneously and irreversibly during the course of a bilayer recording.

We took advantage of the cell-free system to investigate channel properties under conditions not feasible in intact cells. We recorded current-voltage relations with symmetrical Ba^{2+} and examined the dependence of the conductance on the Ba^{2+} concentration. We also studied the effects of high concentrations of internal Ca^{2+} (Ca_i) on the conductance and gating properties of these Ca channels. As expected from models of the mechanism of ion permeation through L-type Ca channels (Hess and Tsien, 1984; Almers and McCleskey, 1984), inward Ba^{2+} currents were reduced by millimolar Ca_i. However, the channel still opened at $Ca_i > 1$ mM, contrary to expectations of models of Ca_i-dependent inactivation (see Eckert and Chad, 1984,

for review). High Ca_i did not appear to affect the rate of channel inactivation during a depolarization, nor did it appear to influence the rate of channel rundown.

In addition to the L-type Ca channels, we have recorded the activity of a divalent-selective channel that differs widely from the L-type or T-type Ca channels previously described in cardiac cells (Bean, 1985; Nilius et al., 1985; Mitra and Morad, 1986). The activity of this channel in planar lipid bilayers could be confused with L-type channel activity, so we compare the functional properties of the two different channel types under the same experimental conditions. A preliminary report of some of this work has been presented in abstract form (Rosenberg and Tsien, 1987).

METHODS

Preparation of Cardiac Sarcolemmal Vesicles

Sarcolemmal membrane fragments from calf ventricular muscle were prepared as described by Slaughter et al. (1983). Hearts were obtained from freshly killed animals at a local slaughterhouse, immediately immersed in ice-cold Tyrode's solution, and transported to the laboratory at 4°C. All subsequent manipulations were carried out in the cold, all solutions were kept on ice, and all centrifugations were at 0–4°C. Ventricular muscle was trimmed free of fat and thick connective tissue, and cut into 1–2-cm^3 pieces. These were homogenized in 2 vol of ice-cold mannitol buffer (250 mM mannitol, 70 mM Tris-SO_4, pH 7.4), first in a Waring blender (5-s burst at top speed) and then in a Polytron homogenizer (20 s, setting 6.5, PT 35 probe; Brinkmann Instruments Co., Westbury, NY). The homogenate was centrifuged at 14,000 *g* for 20 min. The supernatants were discarded, and the pellets were resuspended in the original volume of mannitol buffer with the homogenizer (three bursts of 30 s, setting 6.5, PT 35 probe). This homogenate was centrifuged at 14,000 *g* for 20 min. The supernatants were decanted and retained, and the pellets were rehomogenized and centrifuged as above (three bursts with the homogenizer, 14,000 *g* for 20 min). The supernatants were combined and centrifuged at 45,000 *g* for 30 min. The pellets were resuspended in a volume of mannitol buffer equal to ~50% of the original weight of muscle. This suspension was layered over 0.64 M sucrose, 20 mM imidazole-HCl, pH 7.4, and centrifuged in a rotor (60 Ti, Beckman Instruments, Inc., Fullerton, CA) at 45,000 rpm (145,000 *g*) for 90 min. The turbid band at the sucrose-mannitol interface was collected, diluted threefold in 160 mM NaCl, 20 mM MOPS-NaOH, pH 7.4, and centrifuged at 140,000 *g* for 45 min. The pellets were resuspended in the NaCl buffer at a protein concentration of ~3 mg/ml (Lowry assay; see Peterson, 1977). The suspension was divided into 0.2-ml aliquots, flash-frozen in liquid nitrogen, and stored at −70°C for up to 10 mo.

Planar Lipid Bilayers

Planar lipid bilayers were formed from decane solutions of bovine brain phosphatidylethanolamine (10 mg/ml) and bovine brain phosphatidylserine (10 mg/ml) (Mueller and Rudin, 1969) across a 200-μm-diam hole in a polystyrene partition. Lipids were obtained from Avanti Polar Lipids, Inc., Birmingham, AL, and were used without further purification. Membrane thinning was monitored optically. The bilayers had resistances of 30–100 GΩ and capacitances of 80–120 pF. At the time of bilayer formation, aqueous solutions contained 50 mM NaCl, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4.

After bilayer formation, $BaCl_2$ or $CaCl_2$ was added to the *cis* chamber (to 100 mM from 1-M stock, unless otherwise noted), 1 μM Bay K 8644 (from a 5-mM stock in ethanol) was

added to both chambers, and sarcolemmal vesicles (10–30 μg protein/ml) were added to the *cis* chamber. After stirring, the solution volumes were adjusted so that the level in the *cis* chamber was slightly higher than that in the *trans* chamber, and the bilayer was broken and re-formed in an effort to transport membrane vesicles into the vicinity of the bilayer by bulk flow. Channel activity usually appeared within 5 min.

Voltage clamp of the bilayers was achieved with a patch-clamp amplifier and headstage, modified for extra capacity compensation (V. Pantani and associates, Yale University, New Haven, CT). Voltages were defined as *trans* minus *cis*. The *trans* chamber was held negative with respect to the *cis* chamber, and a depolarization resulted in characteristic channel openings. Thus, the *trans* chamber represents the cellular interior, consistent with conventional incorporation of outside-out vesicles (Jones et al., 1980). Any channels that might have become incorporated with the opposite orientation would experience a very positive holding potential and would therefore be in the inactivated state. Currents flowing from *cis* to *trans* represent "inward" currents and are shown as downward transitions in the recordings. Unless otherwise noted, currents were filtered at 200 Hz (−3 dB, eight-pole Bessel low-pass filter), digitized at 1,000 Hz, and stored for later analysis on a computer (PDP 11/23, Digital Equipment Corp., Marlboro, MA). Capacitative transients were minimized at the time of data recording with analog capacity compensation with three time constants. For further subtraction of linear leakage and remaining capacitative currents at the time of analysis, current records lacking channel activity were averaged and subtracted. Analog capacity compensation could not prevent amplifier and analog-to-digital converter saturation for periods of 10–50 ms after voltage transitions, and these periods appear as noiseless lines in the recordings shown. Channel openings that occurred within the saturation period were registered as opening at the time the current signal escaped from saturation, resulting in an underestimation of the open interval. However, because the number of openings per sweep is large, the net effect is expected to be minimal. Channel openings lasting up to the point of repolarization were drawn as if closing occurred at the end of the pulse. These events were excluded from open-time analysis.

Analysis of unitary current amplitudes were determined by eye using cursors projected on the computer display. Opening and closing events were determined automatically using a discriminator set at 50% of the unitary current amplitude. Open or closed intervals that were terminated by the end of a pulse (or the end of a sweep if there was no pulse) were excluded. Histograms of open and closed times were fitted by single or double exponentials using a Levenberg-Marquardt routine for the minimization of χ^2 (kindly provided by Dr. R. S. Kass, University of Rochester Medical Center, Rochester, NY). The open-channel probability during each sweep was calculated from the cumulative area between the current signal and a computer-fitted baseline.

Patch-Clamp Recordings

Single ventricular myocytes were obtained from adult guinea pigs by enzymatic dissociation as described (Lee and Tsien, 1984, and references therein). Unitary Ca channel activity was recorded from cell-attached patches as described by Hess et al. (1986).

RESULTS

Comparison of L-Type Ca Channel Activity in Bilayers and Intact Cells

Fig. 1 shows a direct comparison of Ca channel activity recorded from an intact cardiac myocyte and from a bilayer after incorporation of cardiac sarcolemmal vesi-

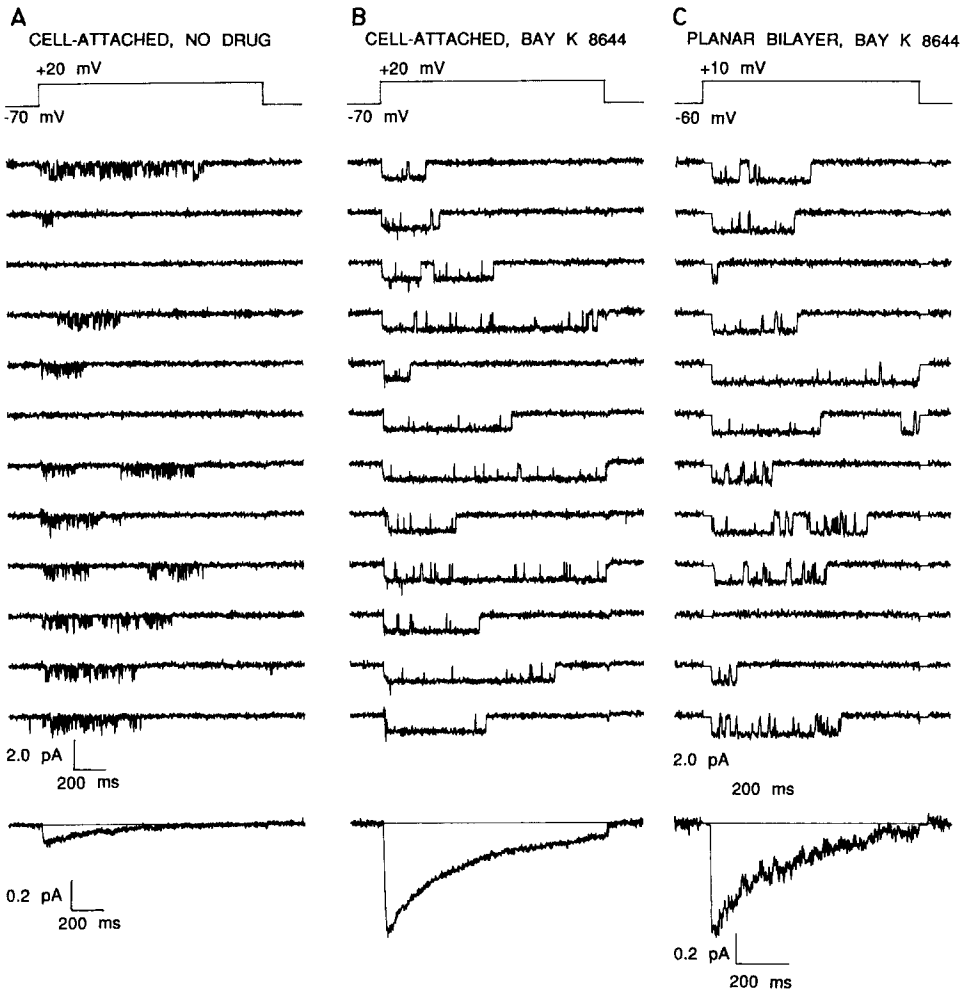

FIGURE 1. Comparison of Ba^{2+} currents through L-type Ca channels in a cell-attached patch and a planar lipid bilayer. (*A* and *B*) Sequential records obtained from a cell-attached patch on a guinea pig ventricular cell before (*A*) and after (*B*) the addition of 5 μM Bay K 8644 to the bath. The patch was held at −70 mV for 3 s between pulses. The bath solution contained 140 mM K-aspartate, 10 mM K-EGTA, and 10 mM HEPES-KOH (pH 7.5). The pipette solution contained 110 mM $BaCl_2$ and 5 mM HEPES–TEA-OH. Linear leak and capacity currents have been subtracted. The bottom records show the averaged current from 303 (*A*) or 344 (*B*) individual records. Cell G44E. (*C*) Consecutive records of a Ca channel recorded in a planar lipid bilayer after incorporation of calf cardiac sarcolemmal vesicles. The intracellular solution (*trans;* see Methods) contained 50 mM NaCl, 1 mM Na-EDTA, and 10 mM HEPES-NaOH (pH 7.4), and the extracellular (*cis*) solution contained 100 mM $BaCl_2$, 50 mM NaCl, 1 mM Na-EDTA, and 10 mM HEPES-NaOH (pH 7.4). Both solutions contained 1 μM Bay K 8644. The bilayer was held at −60 mV for 2 s between pulses. Linear leak and capacity currents have been subtracted. The bottom trace shows the averaged current from 42 individual records. Bilayer J6K4.

cles. In the cell-attached recording (Fig. 1 *A*), depolarizing pulses from a holding potential of −70 mV to a test potential of +20 mV elicited bursts of brief unitary inward Ba^{2+} current of ~1.2 pA amplitude. The individual openings within a burst were poorly resolved at the filter cutoff frequency of 500 Hz, which approximated the recording conditions in the bilayer experiments. The bursts of openings were all grouped toward the beginning of the pulse. As a consequence, the mean current (bottom trace in Fig. 1 *A*) reached a maximum shortly after the onset of depolarization and then decayed to zero during the maintained voltage-clamp pulse. Thus, even when Ba^{2+} carries the current, cardiac L-type Ca channels may inactivate completely during a very long depolarization (cf. Cavalie et al., 1986).

Exposure to this cell to 1 μM Bay K 8644, a dihydropyridine Ca channel agonist (Schramm et al., 1983), changed the gating pattern of the channel dramatically (Fig. 1 *B*). The individual openings within a burst were markedly prolonged. The opening probability during a burst approached unity, leading to an approximately fivefold increase of the mean current (bottom trace in Fig. 1 *B*). The channel kinetics and the sensitivity to Bay K 8644 clearly identified this channel as an L-type Ca channel (Hess et al., 1984; Kokubun and Reuter, 1984; Ochi et al., 1984; Nilius et al., 1985).

We used the characteristic gating pattern produced by Bay K 8644 to identify L-type Ca channel activity in bilayers (Fig. 1 *C*). Both the unitary channel activity and the kinetics of the mean current in the bilayer recordings were very similar to those in the intact cell (compare panels *B* and *C*). The conductances of the channels were very similar, and under matched experimental conditions, the current-voltage (*I-V*) relations of the channels in the bilayers and in intact cells were virtually identical (Rosenberg et al., 1986). We therefore conclude that the channel activity recorded from the bilayer results from an L-type Ca channel incorporated into the bilayer from the sarcolemmal vesicles. The next several sections of this report are a detailed comparison of the properties of the L-type channels in the bilayers and in intact myocytes.

Stability of L-Type Ca Channel Activity in the Bilayers

The ability to record L-type Ca channel activity in a cell-free system was somewhat unexpected, because unitary activity of this channel type has been shown to disappear quickly after excision of a cell-attached patch into simple solutions similar to those used in bilayer recording. Such irreversible loss of unitary activity was found in L-type channels from cardiac (Cavalie et al., 1983; Nilius et al., 1985) as well as noncardiac cells (e.g., Fenwick et al., 1982; Armstrong and Eckert, 1987) and is believed to be the basis for the irreversible decrease ("rundown," "washout") of macroscopic Ca^{2+} currents in most cells that are studied under conditions of intracellular dialysis (Fenwick et al., 1982; Byerly and Hagiwara, 1982; Chad and Eckert, 1986).

Indeed, we found that L-type Ca channel activity in the bilayer was much more labile than that reported for other types of biological channels incorporated into artificial membranes (e.g., Na channels: Krueger et al., 1983; Moczydlowski et al., 1984; Green et al., 1986; K channels: Coronado et al., 1980; Latorre et al., 1982; Cl channels: Hanke and Miller, 1983; other types of Ca channels: Nelson et al., 1984;

Ehrlich et al., 1984; Smith et al., 1985, 1986; Coyne et al., 1987). Fig. 2 shows two patterns of disappearance of L-type Ca channel activity from bilayers. In the example illustrated in Fig. 2, *A* and *B*, the channel very abruptly failed to open after ~3 min of activity. The plot of the open probability vs. time demonstrates that the

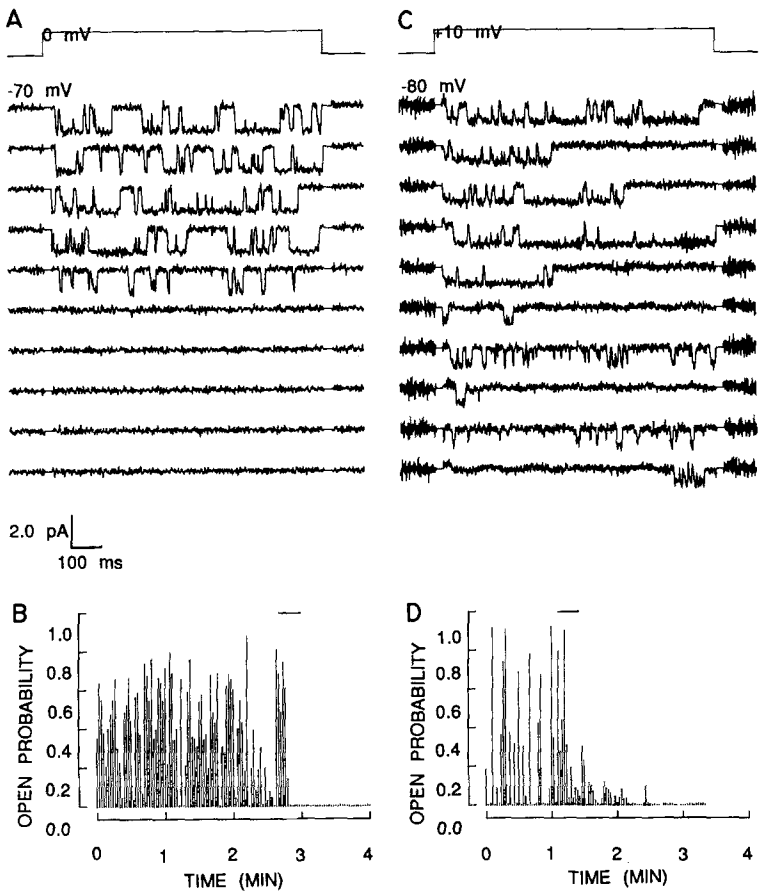

FIGURE 2. Decline of Ca channel activity during the course of bilayer recording. (*A* and *C*) Consecutive records obtained at intervals of 2 s from two different planar lipid bilayers. (*B* and *D*) Channel open probability during the depolarizing pulses, plotted on a sweep-by-sweep basis for the duration of each experiment. The horizontal bars within panels *B* and *D* indicate the region from which the sweeps displayed in *A* and *C* were obtained. The excess noise at −80 mV in *C* is from acoustical vibration and does not affect the analysis of the data during the pulse to +10 mV. *A* and *B*: bilayer J5P; *C* and *D*: bilayer J4AA1.

opening probability was well maintained, with very few blank sweeps and no sign of a graded decline before the sudden loss of channel activity.

A different pattern of channel disappearance can be seen in Fig. 2, *C* and *D*. Here, the channel open probability remained high for the first 1.2 min, during

which activity was characterized by the typical long openings and brief closings. Suddenly, the gating pattern changed to one with much briefer openings and longer closings, reducing the maximum opening probability to 0.2–0.3. Activity continued at this diminished level for ~1 min, after which channel activity disappeared completely.

The decline of channel activity illustrated in panels *C* and *D* was the more commonly observed pattern and was most similar to patterns generally seen in excised patches from intact cells (e.g., Fenwick et al., 1982; McCleskey, E. W., P. Hess, and R. W. Tsien, unpublished data). The time at which channel rundown started was quite variable; stable periods of high channel activity lasted from just a few sweeps to a maximum of 5–10 min. A systematic study of the "lifetime" of a channel in the bilayer was made difficult by the need for repetitive depolarizations, which tended to shorten the lifetime of a bilayer.

Thus, the phenomenon of rundown was also present in L-type Ca channels incorporated into lipid bilayers. However, since functional channels were observed, it seems that for many channels, rundown was very slow during the preparation and storage of the membrane vesicles, and was initiated only after the channel was inserted into the bilayer. Furthermore, the rundown process in the cell-free system cannot be attributed to Ca^{2+}-dependent processes, because the inward currents were carried by Ba^{2+} and the internal solution contained 1 mM EDTA.

Activation and Steady State Inactivation of L-Type Ca Channels in Bilayers

In the following figures, we show results obtained during the period of maintained high activity of L-type Ca channels in bilayers. As shown in Fig. 1 *C*, the channel activates and inactivates during a depolarizing voltage pulse. The voltage dependence of channel activation and inactivation is shown in Fig. 3. The activation curve (open symbols) was obtained by plotting the open probability as a function of the test potential (the holding potential was −80 mV). The steady state inactivation curve (filled symbols) was obtained by plotting the open probability as a function of the holding potential when channel availability was tested by a pulse to +10 mV. All the values were normalized by the appropriate maximum in each bilayer. There are no data between +60 and +120 mV, near the reversal potential, where unitary activity was difficult to measure. The points on the activation curve at the two most positive potentials were obtained from outward (Na^+) currents through the channel (see Rosenberg et al., 1986). There was a narrow range of voltages around 0 mV where both activation and steady state inactivation were nonzero. Within this voltage range, continuous channel activity was observed with steady depolarizations (records not shown), but the steady state open probability was only a fraction of the peak value obtained with depolarizing pulses.

The results in Fig. 3 can be compared with the voltage dependence of activation and steady state inactivation obtained in intact cells with ~100 mM Ba^{2+} as the external charge carrier (Reuter et al., 1982; McDonald et al., 1986; Kokubun et al., 1987; Nilius, B., P. Hess, and R. W. Tsien, manuscript in preparation). Estimates of the midpoint potential for activation is 15 mV in Fig. 3, compared with ~10 mV (Reuter et al., 1982), −10 mV (McDonald et al., 1986), or +25 mV (Nilius et al., manuscript in preparation) for cell-attached patches. The midpoint potential for

steady state inactivation is −30 mV in Fig. 3 and in cell-attached patches (McDonald et al., 1986; Kokubun et al., 1987). Thus, the positions of the activation and inactivation curves are similar in planar bilayers and cell-attached patches. However, the voltage dependence of activation and inactivation is less steep in the artificial membranes.

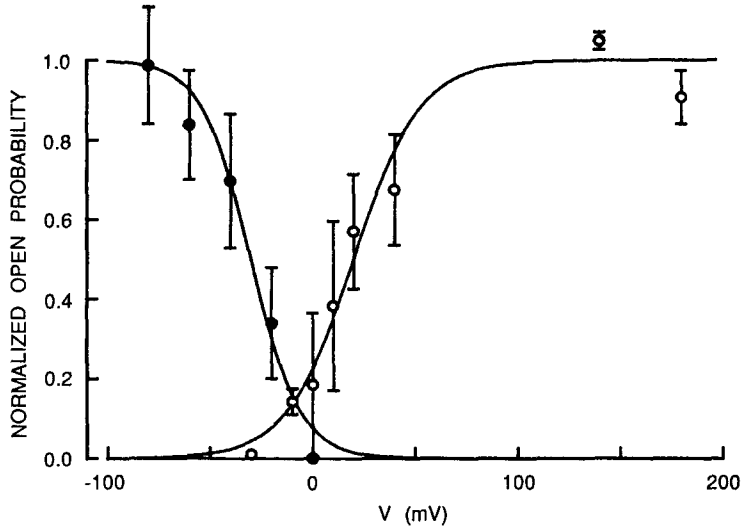

FIGURE 3. Voltage dependence of activation (○) and steady state inactivation (●) of L-type Ca channels in planar lipid bilayers. For activation, the open probability was determined for each sweep containing channel activity; null sweeps were excluded from the analysis. The open probability (means ± SEM) was normalized to 0.825, the average of the values at 140 and 180 mV, and plotted as a function of the test potential. The smooth curve is drawn according to the equation $P_{open} = 1/\{1 + \exp[(V_{½} - V)/K]\}$, where $V_{½} = 18$ mV and $k = 16$ mV. The bilayer was held at −80 mV for 2 s between pulses. Bilayer J4Y5; these data were previously published (Rosenberg et al., 1986). For inactivation, the open probability was determined for each sweep, including null sweeps, and values of means ± SEM are plotted as a function of the holding potential. The bilayer was held at each holding potential for 2 s and depolarized to a test potential of +10 mV. Values were normalized to 0.254, the value at a holding potential of −80 mV. The smooth curve is drawn according to the equation $P_{open} = 1/\{1 + \exp\cdot[(V - V_{½})/K]\}$, where $V_{½} = -30$ mV and $k = 18$ mV. Bilayer J5V3.

Single-Channel Kinetics of the L-Type Channel in Bilayers

Practically all of the resolved openings of the L-type Ca channel in the bilayer were of the kind promoted by Bay K 8644 ("mode 2"; see Hess et al., 1984). This is to be expected, since short openings of the L-type channel (mode 1 gating) have a mean duration of <1 ms (Cavalie et al., 1983; Cachelin et al., 1983; Hess et al., 1984) and were severely attenuated by our recording protocol (200-Hz cutoff frequency, 1-ms sampling interval). Thus, histograms of open- and closed-time durations are usually consistent with the presence of one open state and two closed states, as proposed for gating promoted by Bay K 8644 (Kokubun and Reuter, 1984; Ochi et al., 1984;

Hess et al., 1984). In the experiment illustrated in Fig. 4, the mean duration of the open state was 14 ms at a test potential of +10 mV (Fig. 4 *A*). In a total of eight experiments, the mean open time at this potential averaged 13.0 ± 2.3 ms, compared with 18 ms for the channels in cell-attached patches treated with Bay K 8644 (Hess et al., 1984). The closed-time distribution (Fig. 4 *B*) was fitted by the sum of two exponentials, with time constants of 2 and 26 ms (2.9 ± 0.85 and 22.5 ± 2.6 ms, $n = 7$). This kinetic analysis ignored transitions to the inactivated state(s), because such transitions resulted in channel closures that normally outlasted the duration of

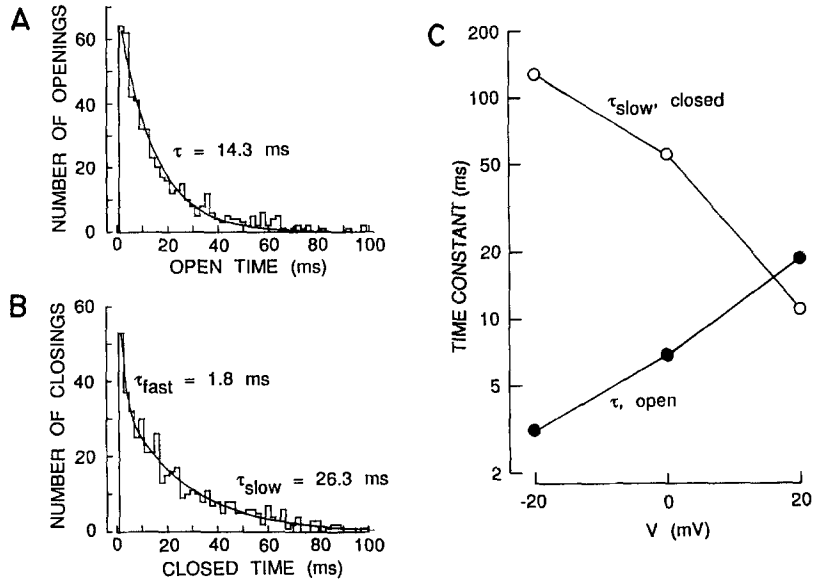

FIGURE 4. Open- and closed-time kinetics of L-type Ca channels recorded in planar lipid bilayers in the presence of Bay K 8644. (*A*) Histogram of channel open lifetimes. Holding potential (HP) = −60 mV; test potential (TP) = +10 mV. The smooth curve is a single-exponential decay (τ = 14.3 ms) obtained by minimizing χ^2. Bilayer J6H1. (*B*) Histogram of channel closed times. HP = −60 mV, TP = +10 mV. The curve is a double-exponential decay obtained by minimizing χ^2; τ_{fast} = 1.8 ms; τ_{slow} = 26.3 ms. Bilayer J6H1. (*C*) Voltage dependence of channel open and closed times. Exponential fits of the open- and closed-time distributions were obtained and the τ and τ_{slow} values are plotted as a function of the test potential. HP = −60 mV. Bilayer J4E1.

the depolarization (e.g., Fig. 1 *C*) and thus were excluded from the analysis. The 2.9-ms time constant is similar to the 2.1-ms time constant from intact cells (Hess et al., 1984) but the 26-ms time constant was not observed in intact cells.

Both the open-time and the closed-time durations were voltage dependent (Fig. 4 *C*). The open-time duration increased from <5 ms at weak depolarizations to 20–25 ms near +20 mV. In other experiments, in which channel openings were evoked by depolarizations >150 mV, the mean open times were ~30–50 ms. The slower time constant of the closed-state distribution also decreased dramatically as the

membrane was depolarized, contributing further to the increase in the open probability at positive potentials.

Pharmacology of L-Type Ca Channels in Bilayers

The pharmacological effects of Bay K 8644 on the L-type channel in the bilayer were very similar to those observed in intact cells (see Fig. 1). We have tried to extend the comparison of drug effects to include dihydropyridine and non-dihydropyridine Ca channel antagonists. Addition of D-575 (10 μM) to the internal solution gave an immediate and sustained decrease of the channel activity in two experiments (data not shown). Similar decreases in opening probability were observed upon addition of nimodipine (6 μM) to the external solution (three experiments; data not shown). These results are consistent with the pharmacology expected for L-type channels, although they cannot be regarded as strong evidence, since removal of the drugs was not considered feasible, given the limited lifetime of the channels in the bilayers, and spontaneous rundown could not be excluded as a contributing factor to the decrease of channel activity.

Block of L-Type Ca Channels by External Cd^{2+}

A key property of Ca channels is their susceptibility to block by polyvalent metal ions like Cd^{2+} (e.g., Hagiwara and Byerly, 1981). Cardiac L-type channels are among the most Cd^{2+}-sensitive Ca channel types, with a K_I in the low micromolar range (Lee and Tsien, 1983; Nilius et al., 1985; Lansman et al., 1986). Fig. 5 shows the effect of external Cd^{2+} on a Ca channel in a planar lipid bilayer. Cd^{2+} block was apparent as (*a*) a concentration-dependent reduction of the inward single-channel current carried by Ba^{2+}, and (*b*) a concentration-dependent increase in the noise level of the open channel current (Fig. 5 *A*). This is consistent with a blocking mechanism in which the blocking and unblocking transitions are too fast to be fully resolved. It is the expected result for Cd^{2+} block of cardiac L-type channels in bilayer recordings, since the average dwell time of a Cd ion in the channel is ~1 ms at 0 mV (Lansman et al., 1986). The steady state block was quantified by measuring unitary current levels (obtained after digitally filtering the records) as a function of the Cd^{2+} concentration (Fig. 5 *B*). Superimposed on the data points is a curve that describes a one-to-one interaction between a blocking ion and the pore:

$$i = i_{(Cd=0)} \cdot \{1/(1 + [Cd^{2+}]/K_{I,app})\},$$

where $K_{I,app}$ is the apparent dissociation constant for Cd^{2+} in the presence of the 100 mM Ba^{2+} used in these experiments. The value of $K_{I,app}$ = 36 μM is in very good agreement with that predicted from results in intact cardiac cells over a range of Ba^{2+} and Cd^{2+} concentrations (e.g., $K_{I,app}$ = 20 μM at 50 mM Ba^{2+}; Lansman et al., 1986). Thus, cardiac L-type Ca channels in bilayers are much more Cd^{2+}-sensitive than the Ca channels from synaptosomes incorporated into bilayers (Nelson, 1986); for these, $K_{I,app}$ = 4.1 mM Cd^{2+} with 250 mM Ba^{2+}.

Discrimination between Ba^{2+} and Ca^{2+} as Charge Carriers

Selectivity of the cardiac L-type Ca channel for different permeant ions appears to result mainly from different affinities of these ions to channel-binding sites (Hess

and Tsien, 1984; Almers and McCleskey, 1984). Thus, because Ca^{2+} binds more tightly than Ba^{2+}, as judged by the ability of Ca^{2+} to block Ba^{2+} currents (Hess and Tsien, 1984; Hess et al., 1986), the mobility of Ca^{2+} is limited and the conductance for Ca^{2+} is lower than for Ba^{2+} (Hess et al., 1986). Fig. 6 shows that this essential property of the channel is maintained in the bilayer. At negative potentials, where the *I-V* relation is approximately linear, the Ba^{2+} conductance is 23 pS (Fig. 6 *A*), whereas the Ca^{2+} conductance over the same voltage range is only ~7 pS (Fig. 6 *B*).

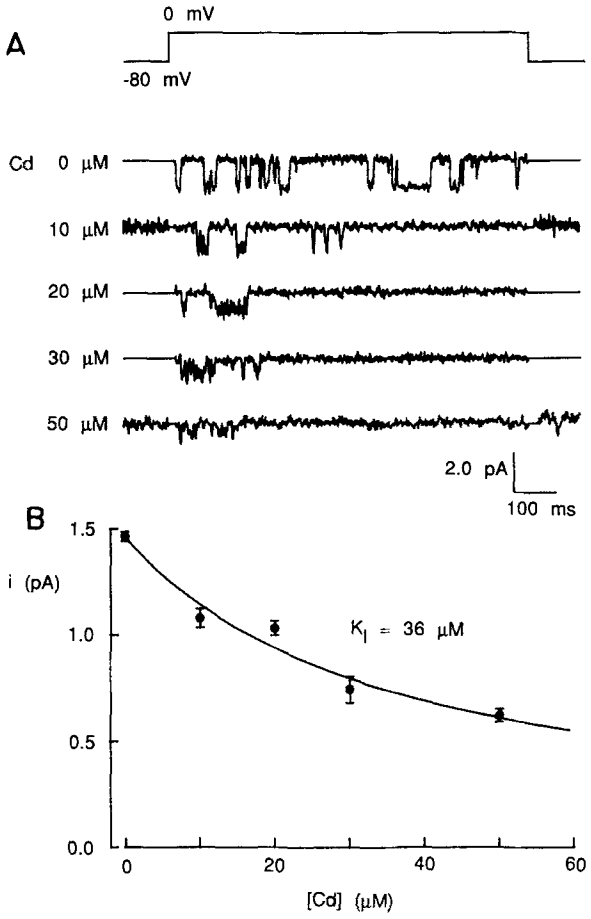

FIGURE 5. Block of inward Ba^{2+} currents by external Cd^{2+}. (*A*) Records of inward currents carried by 100 mM external Ba^{2+} obtained in the presence of the Cd^{2+} concentration shown. HP = −80 mV; TP = 0 mV. Bilayer J5Q. (*B*) Plot of the unitary current magnitude as a function of the external Cd^{2+} concentration. Unitary current amplitudes were determined by eye after digital low-pass filtering the records to smooth rapid fluctuations due to flickery block. The smooth curve is drawn according to the equation $i = 1.46\ \text{pA} \cdot [1/(1 + [Cd^{2+}]/K_{I,app})]$, where $K_{I,app}$ = 36 μM was determined by a nonlinear least-squares fit of the data. Bilayers J5Q and J5R.

Reversal potentials cannot be measured accurately because of the small currents and the shallow *I-V* relation in that voltage range. Nevertheless, it is clear that the currents reverse at very positive potentials, confirming the high divalent-to-monovalent selectivity of this type of Ca channel (Reuter and Scholz, 1977; Lee and Tsien, 1984; Hess et al., 1986).

The large outward currents obtained at potentials beyond the reversal potential were carried by internal Na^+. At any given potential, the outward Na^+ currents in the presence of external Ba^{2+} were much larger than with external Ca^{2+} (compare

panels *A* and *B* of Fig. 6). This is the most direct evidence yet available at the single-channel level that monovalent permeability is strongly dependent on decreasing channel occupancy by divalent ions (Fukushima and Hagiwara, 1985; Hess et al., 1986). Evidently, since Ca^{2+} shows higher affinity for the pore than Ba^{2+} (and therefore greater occupancy), it blocks outward Na^+ movement to a greater extent.

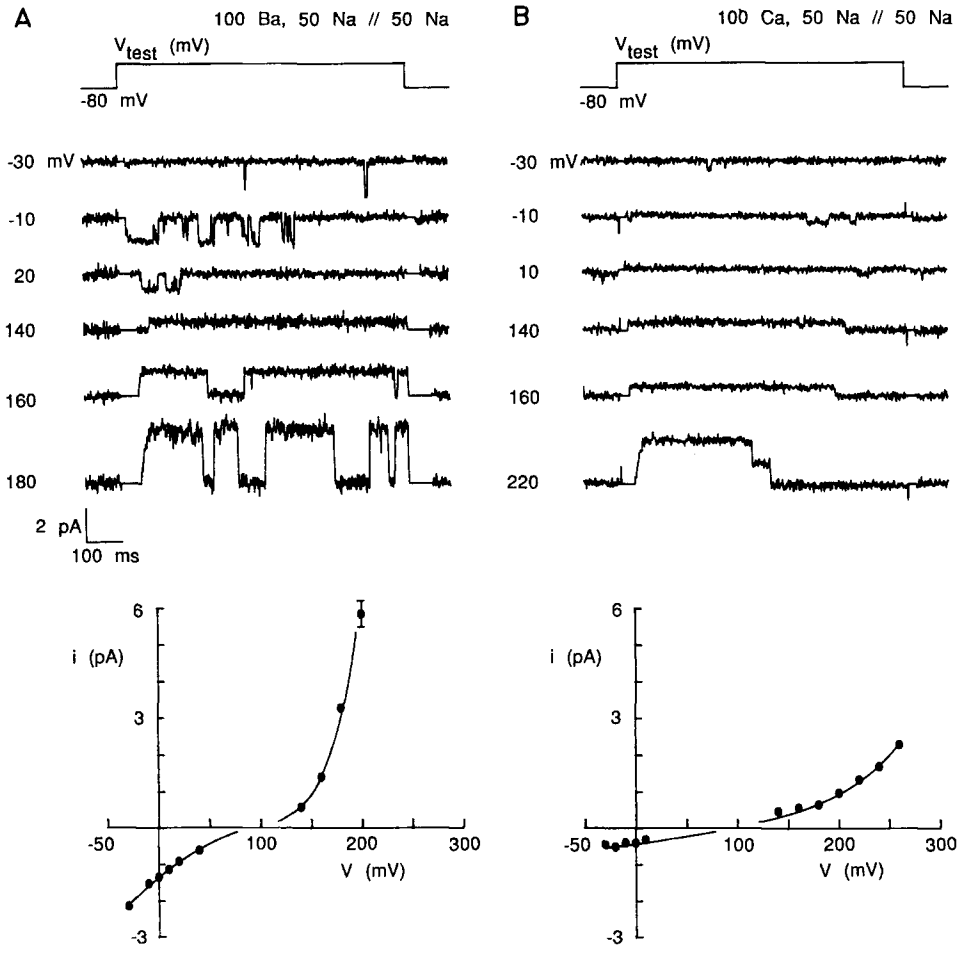

FIGURE 6. Comparison of single-channel currents in the presence of 100 mM Ba^{2+} or Ca^{2+} in the external solution. (*Top*) Records of inward Ba^{2+} (*A*) or Ca^{2+} (*B*) currents and outward Na^+ currents were obtained at the test potentials indicated. HP = −80 mV. (*Bottom*) Plots of the unitary currents as a function of the test potential. Bilayers J4Y5 (*A*) and J5T (*B*).

Effects of Internal Ca^{2+} on Inward Ba^{2+} Current

The competitive antagonism between Ca^{2+} and Ba^{2+} as external charge carriers has been studied extensively (Hess and Tsien, 1984; Lansman et al., 1986). However, investigations of the effects of Ca_i have not been feasible in intact cells. Such experiments are of interest both as tests of models of ion permeation and as probes of the

mechanisms of channel inactivation, which in cardiac cells is mediated in part by increases in Ca_i (Mentrard et al., 1984; Kass and Sanguinetti, 1984; Lee et al., 1985; Nilius and Roeder, 1985; Bechem and Pott, 1985).

Fig. 7 shows block of unitary inward Ba^{2+} currents at 0 mV by addition of Ca^{2+} to the internal Na^+. The block was apparent as a concentration-dependent reduction of the single-channel current level without an increase in open-channel noise (Fig. 7 *A*). A plot of unitary current against Ca_i (Fig. 7 *B*) is fitted by a curve for blocking ion binding to one site with a $K_{I,app}$ value of 4 mM. Lansman et al. (1986) found that external Ca^{2+} blocked Ba^{2+} currents with a $K_{I,app}$ of ~15 mM. Thus, under asymmetric ionic conditions (Ba_o vs. Na_i), Ca^{2+} was a more potent blocker from the inside than from the outside, even when the smaller currents carried by Ca^{2+} itself at these low concentrations are taken into account (see Hess et al., 1986).

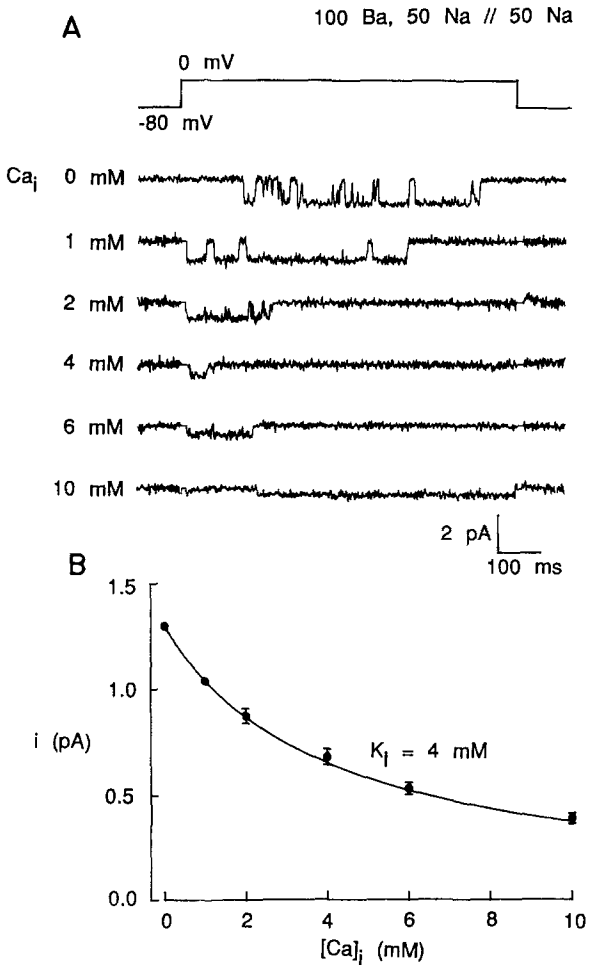

FIGURE 7. Block of inward Ba^{2+} currents by internal Ca^{2+}. (*A*) Records obtained with 100 mM external Ba^{2+} and the concentration of internal Ca^{2+} shown. HP = −80 mV; TP = 0 mV. (*B*) Unitary current is plotted as a function of the concentration of internal Ca^{2+}. Unitary currents were determined by eye from the original leak-subtracted recordings. The smooth curve is drawn according to $i = 1.3\ \text{pA} \cdot [1/(1 + [Ca^{2+}]/K_{I,app})]$, where $K_{I,app} = 4$ mM, as determined by a nonlinear least-squares fit of the data. Bilayers J5U1 and J5U2.

The higher potency of Ca^{2+} at the inner surface is open to different interpreta-

tions. One explanation is that the pore is fundamentally asymmetric, even though this is not apparent in experiments with symmetrical Ba^{2+} solutions (see Rosenberg et al., 1986, and Fig. 9 below). Alternatively, the difference in apparent affinities might reflect the asymmetry in charge carriers, rather than any structural asymmetry in the pore itself. The $K_{I,app}$ for Ca^{2+} increases with the occupancy of the blocking site by competing Ba ions (see, e.g., Lansman et al., 1986). With Ba^{2+} present only in the external solution, a pore with at least two binding (blocking) sites with equal affinities must have a lower Ba^{2+} occupancy at the internal site, favoring block by internally applied Ca^{2+}.

In addition to influencing channel permeation, Ca_i might be expected to alter channel gating, particularly channel inactivation. Inactivation of macroscopic L-type Ca^{2+} currents in cardiac preparations has both voltage- and Ca^{2+}-dependent components (Mentrard et al., 1984; Kass and Sanguinetti, 1984; Lee et al., 1985). The mechanism underlying Ca^{2+}-dependent inactivation is unclear, but a leading

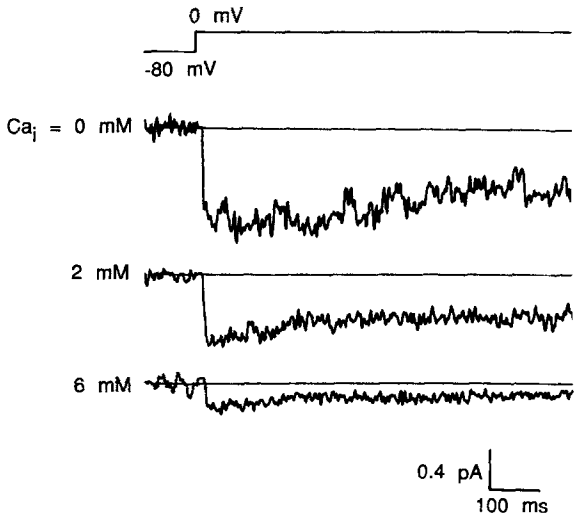

FIGURE 8. Lack of effect of high internal Ca^{2+} on averaged channel kinetics. Records were collected before the addition of Ca^{2+} (*A*) and after 2 mM (*B*) and 6 mM (*C*) $CaCl_2$ were added to the internal solution. The averages shown were constructed from 14 (*A*), 24 (*B*), and 30 (*C*) individual records. Current and time scales are the same for all averaged currents. Bilayer J5U2.

hypothesis (Standen and Stanfield, 1982; Eckert and Chad, 1984) is that Ca^{2+} binds to a site located at the internal side of the channel and thus causes the channel to inactivate. Such a model can reproduce the time course of inactivation observed in macroscopic current recordings (Standen and Stanfield, 1982; Eckert and Chad, 1984), if it is assumed that the binding of Ca^{2+} to the receptor has a dissociation constant of ~ 1 μM. This model predicts that at $Ca_i \gg 1$ μM, the channel should be completely inactivated.

Contrary to the prediction of the model, the data in Fig. 7 clearly show that L-type Ca channel activity could still be recorded with Ca_i as high as 10 mM. Thus, even in very elevated Ca_i, the level of steady state inactivation did not appear to be affected. Furthermore, averaged currents in the presence of 0, 2, and 6 mM Ca_i (Fig. 8) show that elevated Ca_i did not accelerate the rate of inactivation during the depolarizing pulses. The averaged currents have a smaller amplitude in the presence

of high Ca_i, largely because of the reduction in the size of the unitary currents, but the time course of the current was not affected to any great extent. Also, the rate of rundown of Ca channels was not affected by the presence of Ca_i. For example, in the experiment illustrated in Fig. 8, the L-type Ca channel was highly active for >7 min in the continued presence of millimolar Ca_i.

These results exclude models in which Ca^{2+}-dependent inactivation arises from a direct interaction between Ca^{2+} and a high-affinity binding site on the channel itself. However, our experiments do not rule out (*a*) interaction of Ca_i with a low-affinity binding site on the channel that is accessible only when the channel is open and is screened by permeating Ba^{2+}, or (*b*) an indirect action of Ca^{2+} involving an intermediary or regulatory component that is lost in the preparation of membrane vesicles or in the incorporation into the planar bilayer, but is not essential for channel activation, permeation, or pharmacological sensitivity (see also Brown et al., 1986).

Ba^{2+} Conductance in Symmetrical Ba^{2+} Solutions

Previous evidence supports the idea that L-type Ca channels are multi-ion pores (Hess and Tsien, 1984; Almers and McCleskey, 1984). Such pores should display a decrease of conductance when concentrations of permeant ions are raised symmetrically to the point of filling all the ion-binding sites (Hille and Schwarz, 1978). This theoretical prediction prompted us to measure the conductance of L-type channels with a range of Ba^{2+} concentrations. Fig. 9 *A* shows *I-V* plots obtained with symmetrical solutions containing 10 or 500 mM Ba^{2+}. The *I-V* relations are essentially linear, as previously described (Rosenberg et al., 1986). The conductance increased from 16 pS in 10 mM Ba^{2+} to 26 pS in 500 mM Ba^{2+}. Values close to 26 pS were obtained with Ba^{2+} concentrations ranging from 50 to 500 mM (Fig. 9 *B*); there was no indication of a decline in conductance within this range. In this respect, our results are roughly consistent with the Ba^{2+} dependence of the conductance of Ca channels from rabbit skeletal muscle (Ma and Coronado, 1987). Higher concentrations of Ba^{2+} were difficult to test for technical reasons (see legend to Fig. 9).

These results do not provide direct confirmation of the idea that the Ca channel is a single-file pore with multiple binding sites for divalent cations, but they do not exclude it either. Channel conductance decreases are to be expected only when the channel is filled with ions >90% of the time (Hille and Schwarz, 1978). For example, K^+ concentrations >1 M are required in order to observe a decrease in the conductance of the delayed rectifier K channel from squid axons (Wagoner and Oxford, 1987). Near-complete occupancy of Ca channels may require even greater Ba^{2+} concentrations because of very strong repulsive electrostatic interactions between doubly charged ions in the pore (see Hess and Tsien, 1984; Almers and McCleskey, 1984). A subtle decline in conductance would have been difficult to resolve; the predicted conductance decrease should be most prominent around 0 mV, where elementary currents cannot be measured accurately, and should be small at strong driving forces, where most of the current measurements were made.

A Second Type of Unitary Ba^{2+} Conductance in the Bilayer

In addition to the L-type channel activity described, we have recorded unitary Ba^{2+} currents with distinctly different properties. This additional activity is shown along

with L-type openings in the same bilayer in Fig. 10. During the depolarizing pulse to +20 mV, L-type openings were ~1.3 pA in amplitude; on this time scale, which is compressed fourfold compared with the previous figures, the ~20-ms openings appeared as brief spikes. As expected, L-type openings were restricted to the duration of the depolarizing voltage pulse. In contrast, openings of the second type of

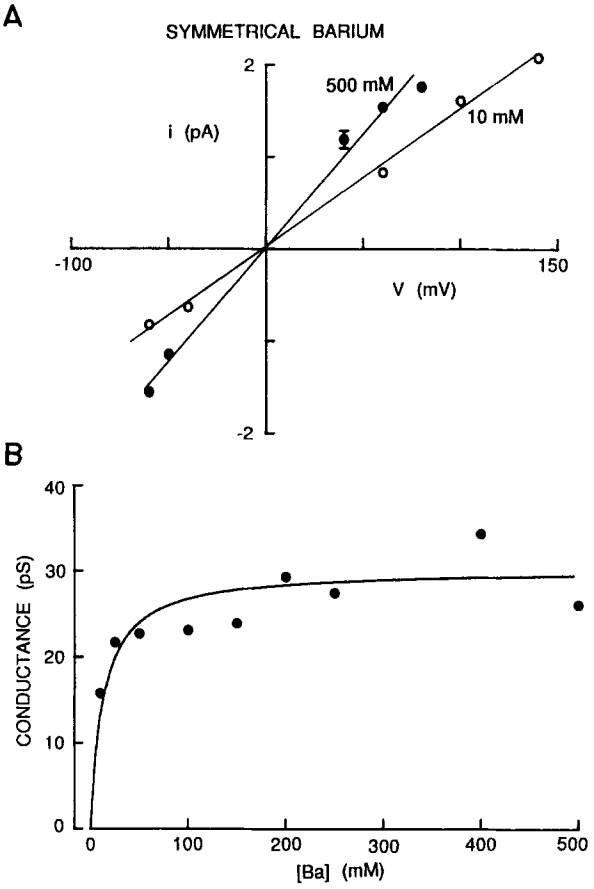

FIGURE 9. Dependence of the conductance of L-type Ca channels on the concentration of Ba^{2+} in symmetrical solutions. Channel activity was first obtained in the presence of 10–100 mM $BaCl_2$ in the external solution only, and then $BaCl_2$ was added to one or both chambers from a 1-M stock solution to achieve the desired symmetrical concentrations. As a result, concentrations >500 mM were difficult to achieve with this method. (*A*) The magnitude of unitary currents obtained in the presence 10 or 500 mM $BaCl_2$ (with 50 mM NaCl and 10 mM HEPES-NaOH) on both sides of the bilayer are plotted as a function of test potential. The lines represent least-squares fits of the data. The conductances are 15.8 pS (10 mM) and 26 pS (500 mM). Bilayers J5AB1 (10 mM) and J5Y (500 mM). (*B*) Channel conductance, obtained from the slopes of *I-V* plots in symmetrical solutions, is plotted as a function of the Ba^{2+} concentration on both sides of the bilayers. The smooth curve is drawn according to the equation $g = g_{max} \cdot [1/(1 + K_m/[Ba^{2+}])]$, where g_{max} = 30.2 pS and the K_m = 12.9 mM as determined by a linear least-squares fit of an Eadie-Hofstee plot of the data (not shown).

channel were several hundred milliseconds long and were seen in isolation at a holding potential of −60 mV. The elementary currents at −60 mV were smaller than L-type channel currents at +20 mV, despite the larger driving force for Ba^{2+} influx. We have named this second channel the "B-type" channel because it contributes a steady background of unitary activity even at negative holding potentials. Although the two channel types were sometimes observed in the same bilayer (Fig. 10), their

appearance was not correlated. The frequency of appearance in bilayer recordings was roughly similar for both types of channels, with no systematic differences among individual membrane preparations.

B-type and L-type channels were easily distinguished on the basis of their conductance and voltage-dependent kinetics. B-type channel activity was seen in the absence of voltage pulses at constant holding potentials ranging from −100 to 0 mV (Fig. 11 *A*). A plot of unitary current against holding potential (Fig. 11 *B*) shows that conductance of the B-type channel increased from 7 pS at 0 mV to 10 pS at −100 mV. At all potentials, B-type unitary currents were smaller than openings of L-type channels, which displayed a slope conductance of 23 pS near 0 mV. The reversal potential (E_{rev}) of the B-type channel was within the range of +30 to +50 mV, which indicates that it is much less selective for Ba^{2+} over Na^+ than the L-type channel, whose E_{rev} was near +100 mV under the same ionic conditions. Further-

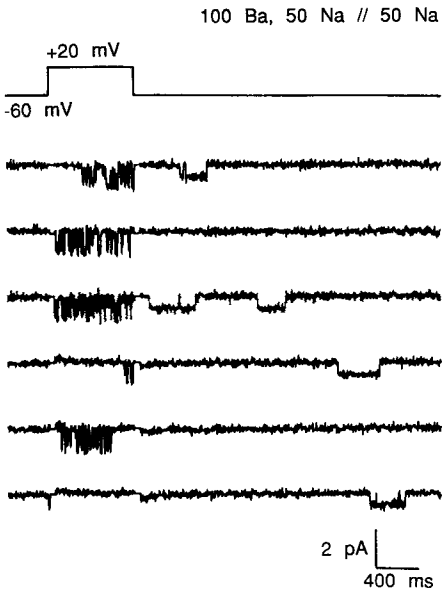

FIGURE 10. Ba^{2+} currents carried by B-and L-type channels in the same planar lipid bilayer. L-type channels open during the pulse to +20 mV, while B-type channel activity is seen most clearly during the period when the membrane was held at −60 mV. The low-pass filter cutoff was set to 100 Hz, and the records were digitized at 250 Hz. Bilayer J7C1.

more, the *I-V* relation of the B-type channel is very similar with Ca^{2+} or Ba^{2+} as the external charge carrier (compare circles and triangles in Fig. 11 *B*). This is in contrast to L-type channels, which display a conductance for Ba^{2+} threefold larger than their conductance for Ca^{2+} (Nilius et al., 1985; Hess et al., 1986; see Fig. 6).

The voltage dependence of the open probability of the B-type channel is displayed in Fig. 12. The open probability was at a maximum at −70 mV and gradually declined at more positive and more negative potentials. This voltage dependence was the result of changes in both open- and closed-time durations. As Fig. 13 illustrates, open times were distributed monoexponentially over a wide span of holding potentials. The exponential time constants varied from 76 ms at −100 mV (*A*) to >300 ms at −60 mV (*C*). This change in the mean open time was a major factor in the increase in open probability over the negative potential range.

The closed times showed a complicated dependence on membrane potential (not shown). At −100 mV, the closed-time distribution was fitted by two exponential components with time constants of ~5 and ~50 ms, leading to the burst-like appearance of the channel openings in Fig. 11 *A* (bottom trace). A decrease in the number

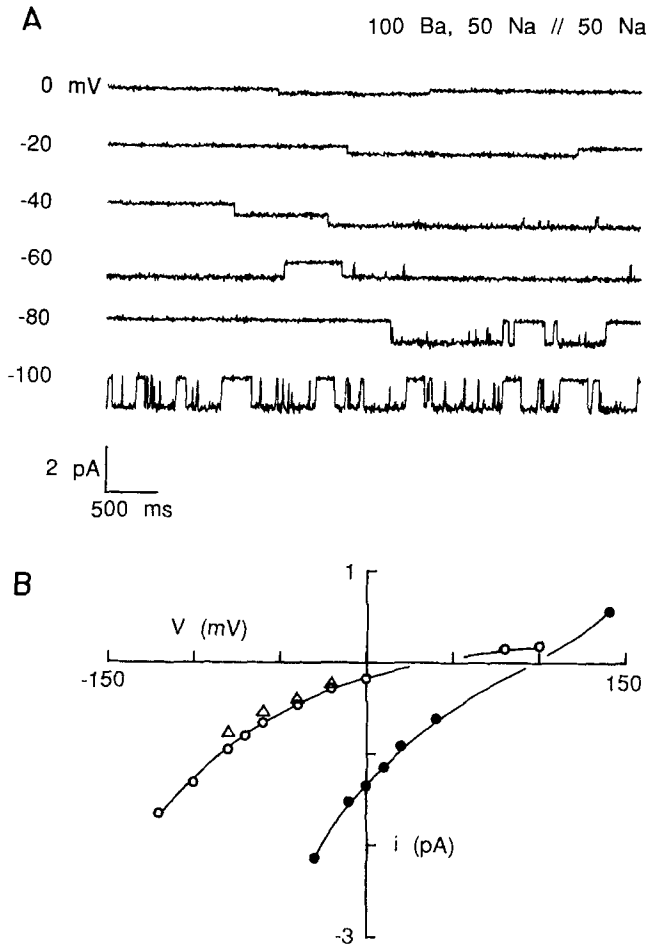

FIGURE 11. (*A*) Openings of B-type channels recorded at steady holding potentials indicated; no depolarizing pulses were applied. The low-pass filter cutoff was 100 Hz, and the records were digitized at 200 Hz. Bilayer J6E1. (*B*) *I-V* relationships for L-type and B-type channels. Unitary currents of an L-type channel (●) with 100 mM external Ba^{2+}, plotted against test potential. Same data as in Fig. 6 *A*. Unitary currents of B-type channels with 100 mM external Ba^{2+} (○) or Ca^{2+} (△) are plotted vs. voltage. Bilayers J6E2 (○) and J7C (△). Smooth curves were drawn by eye.

of closings with depolarizations to −80 and −60 mV contributed to the increase in open probability over this range. As the potential approached 0 mV, the closings became extremely long, and thus contributed to the overall reduction in channel open probability.

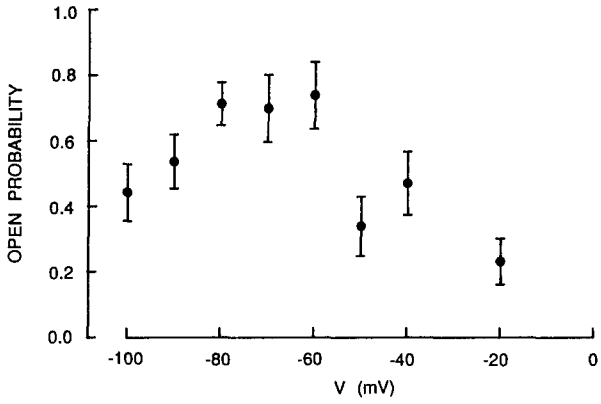

FIGURE 12. Voltage dependence of B-type channel open probability. The open probability was calculated for all sweeps (including null sweeps) and the means ± SE were plotted as a function of the steady voltage. No depolarization was applied. The low-pass filter was set at 100 Hz, and the records were digitized at 200 Hz. Bilayer J6E2.

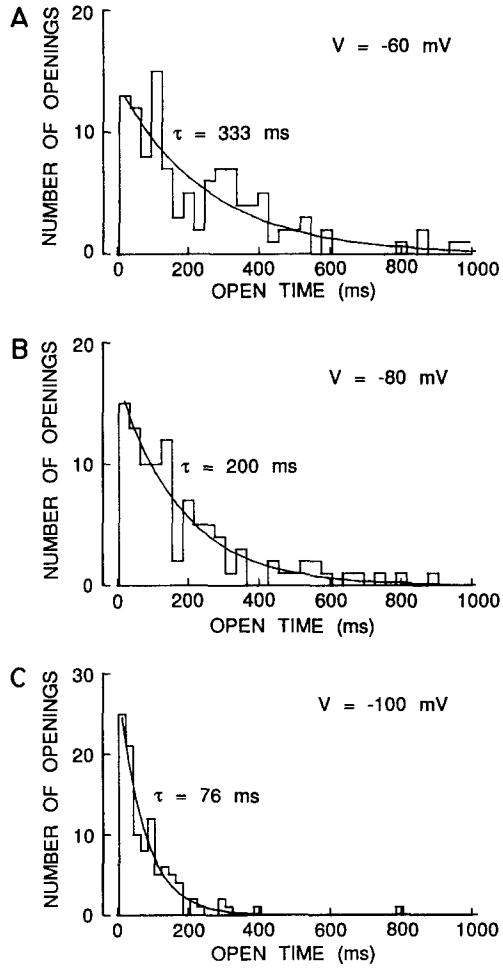

FIGURE 13. Voltage dependence of B-type channel open times. Histograms of channel open durations at −100 (*A*), −80 (*B*), and −60 (*C*) mV are shown. The smooth curves are single-exponential decays obtained by minimizing χ^2. Bilayers J6E2 (*A*) and J5AC3 (*B* and *C*).

B-type channels were most often recorded in the presence of Bay K 8644 because this was necessary to resolve the activity of L-type channels also present in the bilayers (see above). However, in two experiments, B-type channel openings were recorded in the absence of any drug. Subsequent addition of Bay K 8644 appeared to have no effect on the kinetic and conductance properties of the B-type channels, in contrast to its marked effects on L-type channels (see above).

Another important difference between B-type and L-type channels is that the activity of the B-type channels showed no sign of decrease during the course of a bilayer recording. In none of the 25 experiments in which B-type channels were recorded did the activity decrease before the bilayer broke.

DISCUSSION

These experiments demonstrate that L-type channels from cardiac sarcolemmal membranes retain several key characteristics when studied in planar bilayers, including (*a*) voltage- and time-dependent activation and inactivation, (*b*) single-channel conductance and selectivity for divalent cations over monovalent cations and selectivity among divalent cations, (*c*) sensitivity to organic and inorganic Ca channel blockers, and (*d*) rundown of activity following incorporation of channels in the bilayer. As a cell-free system, the bilayers proved advantageous for studying Ca channel properties with millimolar levels of cytoplasmic Ca^{2+} or Ba^{2+}, conditions beyond the capability of conventional cell-attached patch recordings.

Survival of L-Type Ca Channel Activity in Planar Bilayers

An important result of these experiments is the finding that L-type Ca channels retain their functional properties during the preparation of membrane fragments and display activity in planar bilayers despite the absence of intracellular nucleotides, soluble enzymes, or Ca ions. This behavior is noteworthy, given that activity of L-type channels is known to disappear within minutes when membrane patches are excised from intact cells. We have considered various interpretations of the ability of channels to remain functional in the present experiments. One idea is that only a small percentage of the total number of Ca channels remain viable in the membrane vesicles and that these account for the activity seen in bilayer recordings. This seems unlikely since the number of active Ca channels roughly matched the number of active batrachotoxin-modified Na channels in parallel experiments with the same membrane preparations (data not shown). Another interpretation is that preparing membrane vesicles at 0–4°C and storing them at −70°C prevents activity of membrane-associated proteases and other enzymes that may contribute to channel rundown after disruption of the membrane. Once the channel is incorporated into the planar bilayer, any enzyme activity of this sort may be further forestalled by dilution of membrane proteins in the large lipid phase. Finally, the degree of phosphorylation of Ca channels may be a factor in their survival (Chad et al., 1986). The cardiac Ca channels in our membrane preparations may be predominantly phosphorylated, judging by their exceptionally high probability of opening immediately after incorporation, which is only reached in intact cells after treatment with agents that promote phosphorylation by cyclic AMP–dependent protein kinase (Cachelin et al.,

1983; Brum et al., 1984; Bean et al., 1984; Kameyama et al., 1985; Tsien et al., 1986).

Why channel activity runs down after incorporation is not known. The disappearance of channel activity in our bilayer recordings is sometimes abrupt and sometimes gradual (Fig. 2), just as we have seen in patches excised from intact cells (McCleskey, E. W., and P. Hess, unpublished observations). It is possible that channel disappearance is due to a thickening of the bilayer during the course of recording, but this seems unlikely, given the observation that the B-type channels do not disappear during recordings that in general last longer than those of the L-type channels. Alternatively, dephosphorylation of the L-type channels may be the cause for either the gradual decline or the abrupt disappearance of activity. However, we note that if a membrane-associated phosphatase is involved, its activity does not require intracellular Ca^{2+}.

Inactivation of Cardiac L-Type Ca Channels

Our experiments demonstrate inactivation and recovery from inactivation of cardiac L-type Ca channels in the complete absence of Ca ions, releasable Ca^{2+} stores, or ATP. This confirms the idea that inactivation of these channels can take place in a purely voltage-dependent manner (Kass and Sanguinetti, 1984; Lee et al., 1985; Hadley and Hume, 1987), and argues against the idea that inactivation and its removal depend strictly on channel dephosphorylation and rephosphorylation (Chad and Eckert, 1986). With Ba^{2+} as a charge carrier, the rate of inactivation in bilayers is similar to that in intact cardiac cells (Fig. 1). In bilayer recordings, as in cell-attached patch recordings of single Ca channels in both the presence and the absence of Bay K 8644, the rate of inactivation varied considerably from channel to channel (Cavalie et al., 1986), and therefore is not likely to be only a manifestation of the partial antagonistic effects of Bay K 8644 (Kobubun et al., 1987). Much faster inactivation is seen in intact cells when Ca^{2+} is the charge carrier, consistent with the contribution of an additional Ca^{2+}-mediated mechanism of inactivation (Mentrard et al., 1984; Kass and Sanguinetti, 1984; Bechem and Pott, 1985; Lee et al., 1985; Nilius and Roeder, 1985; see Tsien, 1983, for a review including results from other cells).

The bilayer recordings allowed us to look for effects of millimolar Ca_i on Ca channel inactivation. If Ca^{2+} triggered inactivation by interacting with a high-affinity binding site on the cytoplasmic side of the channel molecule itself, one would expect little or no Ca channel current under these conditions. Instead, we found that Ca channel activity remained clear even with 10 mM Ca_i. The average Ba^{2+} influx through the channel was reduced with millimolar Ca^{2+} (Fig. 8), but this was largely accounted for by a decrease in the unitary current, as expected from Ca^{2+}-Ba^{2+} interactions within the pore. If Ca_i interacted only with the open state to trigger inactivation, then one would expect an increase in the rate of inactivation during a pulse. However, we found no indication of such an increase or of reduced channel availability in pulses following those with a high level of channel activity.

How can these results be reconciled with the large body of evidence demonstrating Ca_i-dependent inactivation of cardiac L-type Ca channels? One possibility is that the Ca_i dependence involves a cytoplasmic factor or a membrane component that is

loosely associated with the Ca channel and is lost during the process of membrane preparation or incorporation—for example, a Ca^{2+}-dependent phosphatase (Chad and Eckert, 1986; Chad et al., 1986). If such a component is responsible for Ca_i-dependent inactivation, it cannot be a prerequisite for "rundown," since this can be seen even in the planar bilayer.

Distinctive Properties of B-Type Ca Channels

Although our experiments were directed at studying L-type channels, they also provided a very clear demonstration of a hitherto unrecognized channel that we call

TABLE I

Comparison of B-, L-, and T-Type Channel Properties

	B-type*	L-type	T-type
Reversal potential (ionic conditions, in//out)	~40 mV (100 Ba, 50 Na//50 Na)	~100 mV* (100 Ba, 50 Na//50 Na)	~75 mV‡ (115 Ba//120 Cs)
Selectivity ratio§ (P_{Ba}/P_{Na}) or (P_{Ba}/P_{Ca})	~10	~1,200	~300
Conductance (100 mM Ba^{2+}) (voltage range used)	7–10 pS (−40 to 0 mV)	23–25 pS*‖ (−30 to +10 mV)	8 pS¶ (−30 to +10 mV)
Conductance (100 mM Ca^{2+}) (voltage range used)	7–10 pS (−40 to 0 mV)	8 pS*‖ (−30 to +10 mV)	8 pS¶ (−30 to +10 mV)
Activation voltage range	−100 to −60 mV	−20 to +30 mV‡,¶	−50 to −20 mV‡,¶
Inactivation gating	No	Yes‖	Yes‡,¶
Responds to Bay K 8644	No	Yes*,‡,¶,**	No¶
Greatest open time constant in Bay K 8644 (voltage used)	>300 ms (−60 mV)	~20 ms*,** (20 mV)	~10 ms‡,¶ (0 mV)
"Washout"	No	Yes*,‖,**	No**

*This article.

‡Bean (1985).

§Based on the equation

$$E_{rev} = \frac{RT}{F} \ln \left\{ \frac{4 \cdot P_{Ba} \cdot \gamma^{o}_{Ba} \cdot Ba_o \cdot (1 + E_{rev}F/RT) + P_{Na} \cdot \gamma^{o}_{Na} \cdot Na_o}{P_{Na} \cdot \gamma^{i}_{Na} \cdot Na_i} \right\},$$

where $\gamma^{o}_{Ba} = 0.25$; $Ba_o = 100$ mM; $\gamma^{o}_{Na} = 0.70$, $Na_o = 50$ mM; $\gamma^{i}_{Na} = 0.80$, $Na_i = 50$ mM; $\gamma^{i}_{Cs} = 0.74$, $Cs_i = 120$ mM, derived from constant-field theory and the assumption of zero surface potential difference; see Lee and Tsien (1984).

‖Reuter et al. (1982); Cavalie et al. (1983, 1986); McDonald et al. (1986); Hess et al. (1986).

¶Nilius et al. (1985).

**Hess et al. (1984).

"B-type." B-type channels are very easy to study in planar bilayer recordings; they appear to be successfully incorporated about as often as L-type channels and display long, easily resolved openings that can be recorded over a very broad range of holding potentials, even in the absence of depolarizing pulses. B-type channel activity may also be present in other recordings from cardiac sarcolemmal membranes incorporated in planar bilayers, although the identification of distinct channels has not yet been made (see Ehrlich et al., 1986, Fig. 1; Ashley et al., 1986).

Table I summarizes features of B-type channels that distinguish them from L-type channels, as studied in bilayers or cell-attached patch recordings, or from T-type channels in patch recordings. The absence of clear T-type Ca channel activity in our bilayer recordings was not surprising, since their openings are small and brief enough to be missed, given the limited resolution of recordings from relatively large bilayers. Several points of contrast between B-type and L-type channels have already been mentioned above. Comparison of B-type and T-type channels also shows considerable differences between these channels with respect to selectivity, voltage dependence, inactivation, and mean open time.

Possible Cellular Localization of B-Type Channels

Activity of B-type channels in cell-attached or excised patches has not been reported in the many articles studying Ca channels in heart cells, although those experiments involved rapid pulses to depolarized potentials where B-type channel activity is difficult to resolve. We attempted to record B-type channel activity in cell-attached and excised patches of guinea pig ventricular myocytes, using a slow time base and steady negative holding potentials in order to maximize the probability of detecting B-type channels, but we were unable to find corresponding unitary events in ~15 patch recordings in the presence or absence of Bay K 8644. In the absence of direct evidence for B-type channels in intact cells, a variety of possible cellular origins can be considered. B-type channels might come from myocardial membranes other than the sarcolemma itself, such as the T system, the sarcoplasmic reticulum, or other organelles. However, it should be pointed out that the membrane vesicle preparation is strongly enriched in surface membrane markers and that B-type channel activity was not seen in other fractions preferentially enriched in sarcoplasmic reticulum or mitochondrial fragments.

A more likely possibility is that B-type channels originate from surface membranes of endothelial cells, which constitute a major contaminant of cardiac sarcolemma preparations (Tomlins et al., 1986). Finally, it is possible that B-type Ca channels exist in cardiac sarcolemmal membranes, but at a functional density low enough to escape detection in the limited number of cell-attached patch recordings we carried out. They could have been missed in earlier experiments, which did not focus on negative holding potentials where B-type channel activity is seen most clearly. These possibilities could be distinguished by a careful search for B-type Ca channel activity in patches from cardiac myoctyes and arterial endothelial cells.

This work was supported by National Institutes of Health grant HL-13306, by Miles Pharmaceuticals, and by a fellowship from the Muscular Dystrophy Association to R. L. Rosenberg.

Original version received 21 September 1987 and accepted version received 12 February 1988.

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