L- and T-Type Ca\(^{2+}\) Channels in Canine Cardiac Purkinje Cells
Single-Channel Demonstration of L-Type Ca\(^{2+}\) Window Current

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Canine cardiac Purkinje cells contain both L- and T-type calcium currents, yet the single Ca\(^{2+}\) channels have not been characterized from these cells. Additionally, previous studies have shown an overlap between the steady-state inactivation and activation curves for L-type Ca\(^{2+}\) currents, suggesting the presence of L-type Ca\(^{2+}\) “window” current. We used the on-cell, patch-clamp technique to study Ca\(^{2+}\) channels from isolated cardiac Purkinje cells. Patches contained one or more Ca\(^{2+}\) channels 75% of the time. L-type channels were seen in 69% and T-type channels in 73% of these patches. With 110 mM Ba\(^{2+}\) as the charge carrier, the conductances of the L- and T-type Ca\(^{2+}\) channels were 24.2±0.8 pS (n=9) and 9.0±0.5 pS (n=8), respectively (mean±SEM). With 110 mM Ca\(^{2+}\) as the charge carrier, the conductance of the L-type Ca\(^{2+}\) channel decreased to 9.7±1.2 pS (n=4), whereas the T-type Ca\(^{2+}\) channel conductance was unchanged. Voltage-dependent inactivation was shown for both L- and T-type Ca\(^{2+}\) channels, although for L-type Ca\(^{2+}\) channels with Ba\(^{2+}\) as the charge carrier, inactivation took at least 30 seconds at a potential of +40 mV. After channel inactivation was complete, L-type Ca\(^{2+}\) channel reopenings were observed following repolarizing steps into the window voltage range. Thus, our data identify both L- and T-type Ca\(^{2+}\) channels in cardiac Purkinje cells and demonstrate, at the single-channel level, L-type channel transitions expected for a window current. Window current may play an important role in shaping the action potential and in arrhythmogenesis. (Circulation Research 1992;70:456-464)

KEY WORDS • Ca\(^{2+}\) channels • window current • Purkinje cells • heart • inactivation

Cardiac tissues contain both L- and T-type Ca\(^{2+}\) channel currents.1-7 Studies of the voltage-gated properties of these channels have demonstrated overlap between their steady-state inactivation and activation relations, particularly for the L-type Ca\(^{2+}\) channel, suggesting the possibility that steady-state Ca\(^{2+}\) "window" currents exist.5,7-11 L-type Ca\(^{2+}\) window current has been proposed to have an important role in controlling the action potential shape,12,13 in some types of triggered arrhythmias,14,15 and possibly in the regulation of contraction.3,16

A window current is usually defined as a steady-state current that arises from voltage-gated channels within the narrow voltage range where the steady-state inactivation and activation relations overlap (see Figure 1). At potentials negative to the window, channels distribute between closed and inactivated states. For sufficiently long duration voltage steps positive to the window, the channels preferentially distribute into an inactivated state. Only within the window voltage range can a channel theoretically cycle between closed, open, and inactivated states, thus generating, in cells, a steady current. If no voltage range exists that permits both recovery from inactivation and activation, then a true steady-state Ca\(^{2+}\) current should not exist.

Previous attempts to demonstrate Ca\(^{2+}\) window current have focused on demonstrating a steady or persistent Ca\(^{2+}\) current under conditions when only time-independent currents were expected. Cohen and Lederer11 showed that the D600 sensitive L-type, steady Ca\(^{2+}\) current, measured 200 msec from the beginning of the depolarizing step, exhibited the voltage dependence predicted by the overlapping region of the steady-state inactivation and activation curves, supporting the concept of window current. McDonald and coworkers17 were able to record persistent L-type Ca\(^{2+}\) channel activity during 900-msec depolarizations. Cavallie and coworkers9 demonstrated persistent L-type Ca\(^{2+}\) channel activity, albeit rare, at holding potentials in the voltage range where the steady-state inactivation and activation curves overlapped, leading these authors to conclude that these channel openings represented window current. Similar whole-cell findings were shown for 1-second depolarizing steps by Hirano et al.3 Interpretation of these experiments, however, is complicated by the existence of a slowly inactivating component of the L-type Ca\(^{2+}\) current18 and by the complex single-channel behavior that occur near threshold voltage (see References 17 and 19). Currents or channel activity noted on depolarization into the window voltage range.

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could represent a slowly inactivating Ca\textsuperscript{2+} current as well as true window current.

The present study was designed to identify and characterize the types of Ca\textsuperscript{2+} channels in cardiac Purkinje cells and to test the hypothesis that L-type Ca\textsuperscript{2+} window current exists by analyzing the behavior of these Ca\textsuperscript{2+} channels under voltage-clamped conditions in which window but not slowly inactivating currents should exist. Purkinje cells were chosen for this study because previous work from our laboratory had shown that these cells contain both L- and T-type Ca\textsuperscript{2+} currents, with potential window voltage ranges for each.\textsuperscript{5} Because previous studies of single Ca\textsuperscript{2+} channels have not been reported for this preparation, we first characterized some properties of the L- and T-type Ca\textsuperscript{2+} channels. Subsequently, we showed that both channel types can be voltage inactivated. Once inactivated, we identify L-type Ca\textsuperscript{2+} channel reopenings on repolarization into the window voltage ranges, demonstrating the single-channel transitions necessary for this window current to exist.

Materials and Methods

Preparation

Single canine cardiac Purkinje cells were isolated enzymatically as described previously.\textsuperscript{20} The cells were stored in a dispersion solution (see below for contents) and were studied within 8 hours of isolation. Small aliquots of cells were added to the bath solution (see below for contents) in a perfusion chamber constructed on a glass coverslip and mounted on the stage of an inverted microscope (Diaphot, Nikon). The chamber was treated with polylysine (0.1 mg/ml) before each experiment to facilitate cell adhesion to the glass coverslip. All experiments were performed at room temperature (20–22°C).

Electrical Recordings

Single-channel recordings were made using the on-cell patch-clamp method of Hamill and coworkers.\textsuperscript{21} Glass suction pipettes were fabricated from hematocrit capillary tubes (Drummond Scientific Co.). Pipettes were pulled with a two-stage microelectrode puller (model PP-83, Narishige), coated with Sylgard 184 (Corning Medical), and fire polished to a final tip diameter of 1–1.5 \(\mu\)m by using a microforge (model MF-83, Narishige). When filled with pipette solutions (see below), the pipettes had resistances of 0.5–2 M\(\Omega\).

The bath solution was connected to ground via a 150 mM KCl/agar bridge and a Ag/AgCl half-cell electrode. Electrical contact with the pipette solution was via a chlorided silver wire. The electrode potential was adjusted to give a zero current between the pipette solution and the bath solution immediately before attempting to make a patch. Gigaohm seals between the pipette and single Purkinje cells were obtained by applying gentle suction to the pipette after contacting the cell membrane. Seal resistances for these experiments ranged between 10 and 100 G\(\Omega\).

Single-channel currents were studied in the on-cell patch configuration by using a patch-clamp amplifier (List EP-7, Darmstadt, FRG). Voltage-clamp protocols were generated and single-channel currents recorded using a 12-bit, A/D board (DAS-16, Metrobyte), under computer control (Zenith 386). Single-channel currents were filtered at 1 kHz with an eight-pole Bessel filter (model 902, Frequency Devices) and digitized at 10 kHz. Two voltage-clamp protocols were used. The first consisted of either 100- or 200-msec voltage steps from the holding potential repeated every 2 seconds. The second consisted of recording 200 msec of channel activity (e.g., sampling period) every 2 seconds while holding the patch at fixed potentials for long periods of time. Partial electronic capacitance compensation was used in all experiments. All data were stored on a hard disk for subsequent analysis.

Data Analysis

The data were capacity and leak corrected by subtracting an averaged null trace. By using the corrected tracings, an amplitude histogram was constructed and the open-channel amplitudes were estimated. The threshold to detect openings and closings was set at one half the estimated open-channel amplitude, and an open–closed table was constructed. These data were then used to create open-channel histograms, from which the actual open-channel current amplitudes were determined. Multiple simultaneous channel openings were not included in the analysis. To eliminate some of the error introduced by filtering, the current measured had to exceed threshold for at least three successive data points to be considered a valid opening. The standard deviation of the noise seen in most patches was less than or equal to 0.12 pA.

Solutions

The dispersion solution in which the cells were stored after isolation contained (mM) potassium glutamate 130, MgCl\textsubscript{2} 5.7, EGTA 0.1, and HEPES 10 (pH adjusted to 6.9 with KOH). The bathing solution contained (mM) potassium aspartate 140 and HEPES 10 (pH adjusted to...
7.4 with KOH). This solution depolarized the Purkinje cells to approximately 0 mV. The internal solution for the suction pipette contained (mM) either CaCl2 110 or BaCl2 110 with HEPES 10 (pH adjusted to 7.4 with tetraethylammonium hydroxide [TEA-OH]) and tetrodotoxin 0.012 (TTX; Sigma Chemical Co., St. Louis, Mo.). The TEA-OH and TTX were added to block K+ and Na+ channels, respectively. When used, Bay K 8644 (1–5 μM; Miles Pharmaceuticals) was added to the bathing solution (from a 10 mM stock of Bay K 8644 dissolved in absolute alcohol). The use of high divalent ion (Ca2+ or Ba2+) concentrations facilitates the identification of single-channel currents but also results in a shift to more positive voltages of the voltage-dependent properties of currents recorded from these cells.5

Where appropriate, data are reported as mean±SEM.

Results

Single Ca2+ channels of both the L and T type could be demonstrated easily in isolated canine Purkinje cells. Ca2+ channels were identified in approximately 75% of the first 35 successful patches. Of the patches with channels, 73% contained at least one T-type Ca2+ channel, 69% had at least one L-type Ca2+ channel, and 42% had both Ca2+ channel types.

L-Type Ca2+ Channels

Typical openings of an L-type Ca2+ channel during a range of voltage steps are shown in Figure 2. The pipette solution contained 110 mM BaCl2, and Bay K 8644 (5 μM) was added to the bath solution to prolong the channel openings. As the membrane patch was clamped to more positive potentials, the channel tended to open earlier and for longer durations. This is best demonstrated in Figure 3, where the probability that the channel shown in Figure 2 is open per sweep is plotted for consecutive voltage steps to the potentials shown. At the more positive potentials, the L-type Ca2+ channel tended to either open or remain closed for the
duration of the clamp step. The plot also shows that channel openings tended to occur in groups of sweeps.

The open-channel amplitude histograms and the current–voltage (I–V) relation for the open channel is shown in Figure 4. In all experiments, the I–V relation appeared linear. With Ba2+ (110 mM) as the charge carrier, the conductance of the L-type Ca2+ channel was 24.2±0.8 pS and the extrapolated reversal potential (estimated by linear regression) was 60.8±1.7 mV (n=9).

An estimate of the macroscopic currents resulting from a voltage step can be obtained by the ensemble average of single-channel recordings from many steps to the same voltage. The ensemble averages and their peak I–V relation for the channel from Figure 2 are shown in Figure 5. With Ba2+ as the charge carrier, little inactivation occurred during the 100-msec voltage-clamp steps. The corresponding I–V relation revealed a peak at approximately +30 mV.

Examples of L-type Ca2+ channel recordings, when Ca2+ (110 mM) is used as the charge carrier in the pipette, are shown in Figure 6. The corresponding ensemble average currents are shown below the sample tracings. Bay K 8644 was not used in this experiment. Again, channel openings are more frequent at the more positive voltage-clamp steps. In contrast to the experiments when Ba2+ was the charge carrier, the ensemble currents demonstrate marked inactivation during the 200-msec voltage-clamp step. With Ca2+ in the pipette, the conductance of the L-type Ca2+ channel was 9.7±1.2 pS and the extrapolated reversal potential was 70.2±6.0 mV (n=4).

**T-Type Ca2+ Channels**

Representative current recordings and ensemble averages obtained from T-type Ca2+ channels with Ba2+ (110 mM) as the charge carrier are shown in Figure 7. Compared with the L-type Ca2+ channels, the T-type Ca2+ channels opened at more negative potentials, tended to open early, inactivated completely during the 200-msec voltage-clamp steps to positive potentials regardless of whether the charge carrier was Ca2+ or Ba2+, and were unaffected in either conductance or kinetics by Bay K 8644. The open-channel amplitude histograms and I–V relation are shown in Figure 8. The I–V relation was linear. The conductance of the T-type channels was 9.0±0.5 pS (n=8) with Ba2+ (110 mM) as the charge carrier and 8.4 pS (n=2) with Ca2+ (110 mM) as the charge carrier. The extrapolated reversal potential with Ba2+ as the charge carrier was 48.5±13.9 mV, which was not statistically different from that obtained from Ba2+ current through the L-type Ca2+ channel. The large standard deviation of the reversal potential estimate is due in part to the fact that the open-channel conductance was determined over a relatively narrow voltage range that is far from the reversal potential, so the small variance in the slope (SEM=±0.5 pS) between patches yields a large variance in the extrapolated reversal potential.

**Inactivation**

T-type Ca2+ channels completely inactivate within 200 msec when clamped to positive potentials, as shown in Figure 7 (see also References 5, 6, 22, and 23), regardless of whether Ca2+ or Ba2+ is the charge carrier. In contrast, for the L-type Ca2+ channel when Ca2+ was the charge carrier, channel inactivation was incomplete by the end of a 200-msec voltage step, suggesting clear kinetic differences (see Figure 6). With Ca2+ as the
charge carrier, it is difficult to separate L-type from T-type Ca\(^{2+}\) channels because of the similarity of their conductances. With Ba\(^{2+}\) as the charge carrier, L- and T-type Ca\(^{2+}\) channel currents can be easily separated; however, the L-type Ca\(^{2+}\) channel revealed little inactivation during the 100-200-msec duration voltage-clamp steps we used (see Figure 3).

The L-type Ca\(^{2+}\) channel can be voltage inactivated, however, if given sufficient time. An experiment demonstrating L-type Ca\(^{2+}\) channel inactivation with Ba\(^{2+}\) as the charge carrier is shown in Figure 9. In this experiment, the membrane patch was voltage clamped to the potentials shown and 200 msec of single-channel data was recorded every 2 seconds. Activity from only one L-type Ca\(^{2+}\) channel was seen in this patch. The probability that the channel was open during each 200-msec sampling period is plotted. Channel activity developed after the potential across the patch was changed from -40 to +13 mV, and within about 20 seconds it disappeared. The channel could be in either a closed or inactivated state. However, with further depolarization of the patch to +30 and +40 mV, at which channel openings could be easily recorded (see panel B), no further channel activity could be elicited, indicating inactivation of the channel.

**L-Type Ca\(^{2+}\) Window Current**

If a window current exists for the L-type Ca\(^{2+}\) channel, then after inactivation of the channel at positive potentials, channel openings should return on repolarization into the window voltage range. The results of such an experiment are shown in Figure 10. This experiment is a continuation of the experiment shown in Figure 9. After inactivating the channel with a prolonged depolarization to +40 mV, the patch was repolarized to +13 mV. Channel activity returned as shown by the sample tracings in panel A. The patch was again clamped to +40 mV, and after about 30 seconds channel activity again ceased. The membrane was then repolarized to +25 mV, and no channel activity was noted. On further repolarization of the patch to +7 mV, channel activity again returned. Examples of the channel activity during this repolarization are shown in panel B. The difference in the pattern of channel reopenings seen after the repolarization steps to the two potentials (see panels A and B) does not imply a difference in channel behavior at the two potentials but is within the normal variability seen with a given L-type Ca\(^{2+}\) channel.

Protocols similar to that shown in Figure 10 were completed on nine patches containing L-type Ca\(^{2+}\) channels. From these experiments, L-type Ca\(^{2+}\) channel activity was identified on repolarization into the window voltage range in five patches. From these nine experiments, the probability of identifying an L-type Ca\(^{2+}\) channel reopening per sampling period (200 msec of acquired data every 2 seconds) was 0.024. Because channel openings were brief (the probability that the channel was open within a sampling period in which reopenings were identified was about 0.1), the probability that the channel was open during the repolarization steps was less than 0.002 (or less than 0.2%).

**Discussion**

**Single-Channel Properties**

It is interesting that both L- and T-type Ca\(^{2+}\) channels were observed frequently in these cells. Channels were seen in 75% of the successful patches. This is in sharp contrast to the results from guinea pig ventricular myocytes, in which L-type Ca\(^{2+}\) channel activity was noted in only about 12% of the patches.\(^5\) Using the single-channel conductances measured in this report, with the whole-cell L- and T-type Ca\(^{2+}\) currents reported by Hirano and coworkers\(^5\) under similar conditions, the minimum channel density for both channel types was calculated to be about six channels per square micrometer, a value comparable to that found for
L-type Ca\textsuperscript{2+} channels in guinea pig ventricular myocytes\textsuperscript{17} The fact that we observed T-type channels as frequently as L-type channels is consistent with the greater prevalence of T-type Ca\textsuperscript{2+} currents in Purkinje cells as opposed to ventricular cells\textsuperscript{7,22} Neither N-type\textsuperscript{25} nor B-type\textsuperscript{26} Ca\textsuperscript{2+} channels were identified in the isolated Purkinje cells.

The general properties of both Ca\textsuperscript{2+} channel types reported in this study (i.e., their conductance with Ba\textsuperscript{2+} or Ca\textsuperscript{2+} as the charge carrier, kinetics, and response to Bay K 8644) are in good agreement with those from cultured neonatal rat ventricular cells\textsuperscript{27} guinea pig ventricular myocytes\textsuperscript{1,9,19,22,28} calf sarcolemma membrane channels reconstituted in planar lipid bilayers\textsuperscript{26} and neuronal cells\textsuperscript{25,29,30} It is unlikely that the T channel represents Ca\textsuperscript{2+} transit through the Na\textsuperscript{+} channel because 1) the channels are not blocked by up to 100 μM TTX (unreported observation), 2) the single-channel kinetics differ from those of the Na\textsuperscript{+} channels seen in these cells\textsuperscript{31} and 3) the T channels activated and inactivated at potentials more positive than would be expected if the current was carried through Na\textsuperscript{+} channels.

**Figure 5.** Panel A: Ensemble averages for the L-type Ca\textsuperscript{2+} channel shown in Figure 2. The averages from clamp steps to 1, 11, 21, and 31 mV steps were constructed from 46, 40, 21, and 28 sweeps, respectively. Panel B: The peak current−voltage relation from the ensembles.

**Figure 6.** Sample recordings of L-type Ca\textsuperscript{2+} channels and their ensemble averages with 110 mM Ca\textsuperscript{2+} as the charge carrier. Voltage protocols are illustrated above the tracings. Note the decrease in channel current and more rapid decay of the ensemble currents compared with that seen when 110 mM Ba\textsuperscript{2+} is the charge carrier.
FIGURE 7. Sample recordings of T-type Ca\(^{2+}\) channels and the corresponding ensemble averages. Voltage protocols are illustrated above the tracings. Note the brief channel openings and rapid decay of the ensemble currents.

FIGURE 8. The open-channel amplitude histogram with the Gaussian fits and current–voltage relation for a typical T-type Ca\(^{2+}\) channel. In this patch, the T-type channel had a conductance of 9.2 pS and a reversal potential of +46 mV.
RESULTS

Inactivation

Results from experiments with a number of different cell types have consistently shown that the L-type Ca\(^{2+}\) current shows little inactivation over several hundred milliseconds when Ba\(^{2+}\) is the charge carrier.\(^{1,5,7,19,25,32}\) The data presented here are consistent with those observations. Our results also show that under these conditions (e.g., Ba\(^{2+}\) as the charge carrier), L-type Ca\(^{2+}\) channels can inactivate if given sufficient time. In our experiments, voltage-dependent inactivation required at least 30 seconds for a voltage step to +40 mV. These results are also in agreement with Yue and coworkers,\(^{32}\) who suggest that Ca\(^{2+}\)-facilitated inactivation results from calcium favoring occupancy of a closed state from which the channel can reopen rather than accelerating inactivation into an absorbing state. With Ba\(^{2+}\) as the charge carrier, depolarization favored channel occupancy in an inactivated state (see Figure 9).

In contrast, T-type Ca\(^{2+}\) channels were completely inactivated within 200 msec at positive potentials regardless of whether the charge carrier was Ca\(^{2+}\) or Ba\(^{2+}\). This agrees with results from whole-cell experiments with isolated canine Purkinje cells,\(^{6}\) rabbit sinoatrial cells,\(^{7}\) chick and rat sensory neurons,\(^{30}\) cultured 3T3 fibroblasts,\(^{23}\) and guinea pig ventricular cells.\(^{22}\)

L-Type Ca\(^{2+}\) Window Current

Hirano et al\(^{15}\) have studied L-type Ca\(^{2+}\) currents in whole-cell voltage-clamped isolated Purkinje cells with protocols similar to those used in this study. They were able to demonstrate recovery of an inward Ca\(^{2+}\) current when the cell was repolarized into the L-type Ca\(^{2+}\) window voltage range. This current responded to interventions that previously had been shown to affect L-type Ca\(^{2+}\) current. They concluded that L-type Ca\(^{2+}\) window current could be shown directly and that it could be separated from other processes, such as slow inactivation. The data we present here support and extend their conclusions. For a window current to exist, a voltage-gated ion channel must be able to cycle from an inactivated state to an open state within the appro-
private voltage range. Our data demonstrate that L-type Ca\(^{2+}\) channels can undergo these transitions. Although our results do not demonstrate whether the channel must first recover to a closed state before reopening, the results of Hirano and coworkers\(^{13}\) suggest that L-type Ca\(^{2+}\) channels cycle from an inactivated state to a closed state before reopening within the window.

In the present experiments we observed L-type Ca\(^{2+}\) channel reopenings when the patch was repolarized (from +40 mV) to the voltage range of 0 to +20 mV. This voltage range is close to the maximal overlap between the steady-state inactivation and activation curves for the L-type Ca\(^{2+}\) current with similar concentrations of divalent ions (110 mM Ba\(^{2+}\), see Reference 5) and positive to the window voltage range found with lower divalent ion concentrations.\(^{5.7-9,11}\) In principle, it would have been advantageous to study the probability of L-type Ca\(^{2+}\) channel reopenings throughout the window voltage range; however, channel reopenings were rare, with the probability of identifying an L-type Ca\(^{2+}\) channel on repolarization into the window voltage range was usually less than 20 pA. Thus, the delineation of the window voltage range is not easily suited to single-channel experiments but is better suited to whole-cell experiments in which the behavior of many channels is studied simultaneously. Our results do demonstrate clearly, however, that L-type Ca\(^{2+}\) channels can open on repolarization into the window voltage range.

In summary, our results show that 1) L- and T-type Ca\(^{2+}\) channels are present in relatively high densities in canine cardiac Purkinje cells, 2) both channel types can be voltage inactivated although with differing kinetics and ion dependencies, and 3) once inactivated, at least L-type Ca\(^{2+}\) channels can reopen on repolarization into their window voltage ranges.

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