Slow Inactivation of L-Type Calcium Current Distorts the Measurement of L- and T-Type Calcium Current in Purkinje Myocytes

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ABSTRACT We have examined slow inactivation of L-type calcium current in canine Purkinje myocytes with the whole cell patch clamp technique. Slow inactivation is voltage dependent. It is negligible at −50 mV but can inactivate more than half of available i_{CaL} at −10 mV. There are two major consequences of this slow inactivation. First, standard protocols for the measurement of T-type current can dramatically overestimate its contribution to total calcium current, and second, the position and steepness of the inactivation versus voltage curve for i_{CaL} will depend on the method of measurement. Given the widespread attempts to identify calcium current components and characterize them biophysically, an important first step should be to determine the extent of slow inactivation of calcium current in each preparation.

INTRODUCTION

The Purkinje fiber of the cardiac conducting system has been a valuable experimental model for the study of inwardly directed membrane currents across the cardiac membrane (Gibbons and Fozzard, 1975; Kass and Tsien, 1976; Colatsky and Tsien, 1979).

More recently, the acute isolation of Purkinje myocytes has provided investigators with a relatively large cardiac cell largely devoid of T-tubules to investigate inward calcium currents (Sheets, January, and Fozzard, 1983; Gintant, Datyner, and Cohen, 1985). Recent investigations have demonstrated the presence of T and L type calcium currents in the Purkinje membrane (Tseng and Boyden, 1989; Hirano, Fozzard, and January, 1989). To separate the T and L type currents, the cell is first exposed to TTX and/or Na free solution (to remove the TTX-sensitive Na current). The cell is then voltage clamped either at a relatively hyperpolarized (−70 to −90 mV) or a relatively depolarized (−30 to −50 mV) potential, and a test pulse to a more positive potential applied and the corresponding test currents measured. Assuming that the

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only difference between current activated from the depolarized and hyperpolarized potentials is inactivation of T-type current, then the difference in test currents should yield a T-type current.

However, previous investigations in Purkinje fibers and in frog ventricular myocytes have reported the existence of a slow component of L-type Ca current inactivation (Kass and Scheuer, 1982; Schouten and Morad, 1989). This slow inactivation is reported to occur at more depolarized potentials than "normal" inactivation of i_{CaL}. Slow inactivation in the Purkinje fiber could be an artifact of the inadequate voltage clamp of the multicellular preparation or alternatively a real property of the Ca conductance.

In this study of Ca current in isolated Purkinje myocytes, where there are no restricted extracellular spaces, we find a slow component of L-type Ca current inactivation. We then ask two important questions: (*a*) does slow inactivation of L-type calcium current distort attempts to isolate and measure T-type calcium current?; and (*b*) does slow inactivation of L-type calcium current distort attempts to measure the voltage dependence of rapid inactivation of L-type calcium current?

We believe the answers to both these questions are important to the interpretation of all data thus far obtained on all preparations where T and/or L calcium currents have been studied and a slow component of i_{CaL} inactivation exists.

MATERIALS AND METHODS

We employed the whole cell patch clamp technique with the Axoclamp-2A in the switch clamp mode; switching frequency 10 kHz. Patch electrodes were pulled according to standard techniques and had an average resistance of 2.7 Mohms when filled with the pipette solution listed below. Their resistance usually increased by a factor of ~2 upon sealing. The experiments were performed on the stage of an inverted microscope at 26.5–27.5°C.

The pipette solution contained in mM: TEA 140, Cl 135, dextrose 10, K_4-BAPTA 5, HEPES 10, Mg-ATP 2 and Na-Creatinine Phosphate 3. The solution was buffered to pH 7.4 with TEA-OH.

The external solution contained in mM: TEA 146, Cl 160, Ca 10, dextrose 10, 4-AP 0.5, HEPES 10 (buffered to pH 7.4 with TEA-OH).

The preparation was the canine Purkinje myocyte. Isolated myocytes were prepared as described previously (Gintant et al., 1985; Cohen et al., 1987).

Measurement of i_{Ca}

The amplitude of i_{Ca} was measured as the difference between the peak of the calcium current and the magnitude of the current at the end of the test pulse (500 ms except in Fig. 1). TEA and 4-AP were added to the bathing solution and potassium removed from it and to a large extent from the pipette solution, to minimize overlap of and distortion of our Ca current measurements by K currents (Kass, Scheuer, and Malloy, 1982; Kenyon and Gibbons, 1979).

RESULTS

Slow Inactivation of i_{CaL} Exists in Purkinje Myocytes

We first investigated whether slow inactivation of i_{CaL} was present in canine Purkinje myocytes free of the restricted extracellular space of the Purkinje fiber. One sample result is illustrated in Fig. 1. The protocol is illustrated in the upper left hand portion of the figure. The preparation is first depolarized from −90 to −30 mV for either 500 ms or 5 s (to activate and inactivate i_{CaT}) and then a second further depolarization to 0 mV is applied (to activate i_{CaL}). If slow inactivation of i_{CaL} exists, one would expect that longer intervals between the depolarizing steps would result in smaller Ca currents recorded on depolarizing to 0 mV. As seen, there is substantially less Ca^{2+} current recorded at 0 mV after the more prolonged depolarization (5 s) to −30 mV. Similar protocols in 41 myocytes demonstrated that in ~50% (23 out of 41) of the myocytes, a slow inactivation process existed, occurring over several seconds.

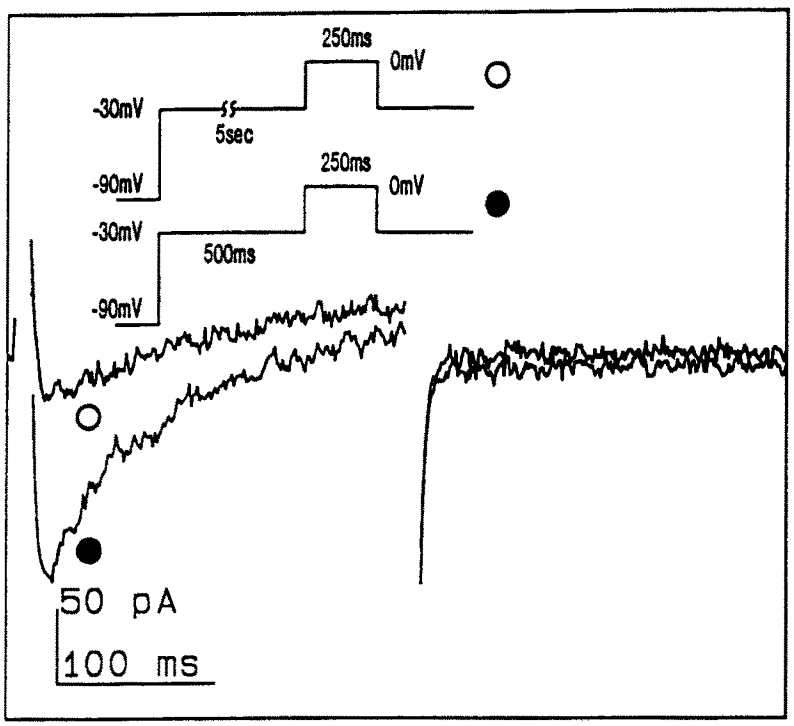

FIGURE 1. Slow inactivation of i_{Ca} in Purkinje myocytes. Calcium currents recorded after holding at −30 mV for 5 s (○) and after holding at −30 mV for 0.5 s (●). The calcium current after holding for 5 s is reduced markedly by slow inactivation of i_{Ca}.

Fig. 2 illustrates the protocol employed to characterize this slow inactivation of i_{CaL} in more detail and provides a plot of its time course. Individual records are shown in the inset. In this example, the cell was voltage clamped at −40 mV and a 1-s long voltage pulse to −90 mV applied. Then, on depolarizing the cell from −90 to −40 mV, a T-type Ca current is activated and inactivated during approximately the first 150 ms. The time period before the next depolarizing test voltage pulse to 0 mV is varied from 250 ms to 10 s, to correspondingly increase the amount of i_{CaL} inactivated by the slow inactivation process. The raw current records inset as *a–d* illustrate the current elicited upon depolarization to 0 mV after intervals of (*a*) 250 ms; (*b*) 1 s; (*c*) 3 s; and (*d*) 10 s between the depolarizing pulses. As seen, the peak Ca current thus elicited continues to decline with increasing intervals, clearly indicating that a second process independent of removal of T-type current is affecting the amplitude of i_{Ca} measured at that potential.

The recovery from slow inactivation was investigated by the protocol illustrated in Fig. 3. The cell was first voltage clamped at −30 mV (where slow inactivation is extensive), and repolarizing pulses to −90 mV applied for a variable time period to allow for recovery from slow inactivation. The preparation was then depolarized from −90 to −30 mV for 100 ms; this activates and inactivates T-type Ca current while

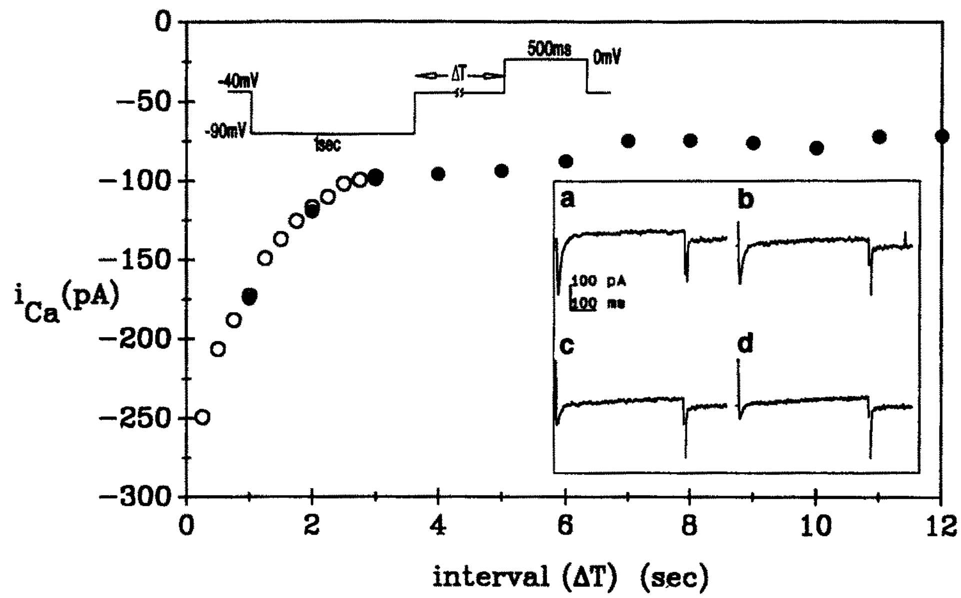

FIGURE 2. Time course of "slow" inactivation of i_{Ca}. From a holding potential of −40 mV, a Purkinje myocyte was hyperpolarized to −90 mV for 1 s. i_{Ca} was recorded at varying intervals after the hyperpolarization to −90 mV. Intervals were interleaved with short (250 ms increment ○) and long (1 s increment ●) intervals to define the time course of slow inactivation more accurately. Inset shows raw i_{Ca} recorded during the 500 ms step to 0 mV after intervals of (*A*) 250 ms; (*B*) 1 s; (*C*) 3 s; and (*D*) 10 s at −40 mV.

keeping slow inactivation of i_{CaL} to a minimum. From −30 mV the cell was depolarized to 0 mV and the L-type Ca current elicited taken as a measure of recovery of i_{CaL} from slow inactivation. The recovery from inactivation was well fit by a single exponential. In this example, $\gamma(-90\ \text{mV}) = 0.98$ s. The mean $\gamma(-90\ \text{mV}) = 0.91 \pm 0.13$ s (SEM, $n = 9$). A hyperpolarizing pulse to −90 mV for 1 s was used for recovering i_{CaL} from slow inactivation in the rest of this study. It allows most of i_{CaL} to recover while providing good recording viability for sustained protocols.

To obtain an estimate of the voltage dependence of slow inactivation of i_{CaL}, the following two protocols were performed. In the first, a 1-s hyperpolarizing pulse to

−90 mV from a holding potential of −30 mV was applied (refer to Fig. 7, protocol at right). As explained above, this pulse allows for recovery of most of i_{CaL} from slow inactivation. After this, depolarizing voltage steps of 500 ms duration and varying amplitude were applied, in an attempt to produce "normal" inactivation but minimal slow inactivation i_{CaL}. Then, a depolarizing test pulse to 0 mV was applied to elicit the calcium current as a measure of the "normal" inactivation of i_{CaL}. In the second protocol, voltage steps of 10-s duration and varying amplitude were applied from a holding potential of −30 mV (refer to Fig. 7, protocol at left). These longer steps produced both "normal" and slow inactivation of i_{CaL}. After the voltage step, a depolarizing test pulse to 0 mV was applied and the current elicited taken to be a measure of both "normal" and slow inactivation of i_{CaL}. The ratio of Ca current elicited with the test pulse after the long second pulse (to potential V) to that after the short second pulse (also to potential V) gave a measure of the voltage dependence of the slow inactivation process of i_{CaL}. Results of this set of experiments are shown in

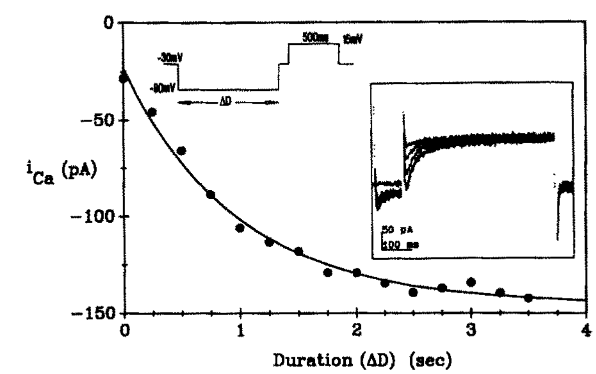

FIGURE 3. Time course of the recovery from "slow" inactivation. i_{Ca} was recorded at 0 mV after a variable duration hyperpolarization to −90 from −30 mV. A 100-ms interval at −30 mV inactivated any T-type calcium current (*inset*). The duration of this hyperpolarization was varied from 0 ms to 3.5 s in 250 ms increments. In this case the time constant for the recovery of i_{Ca} was 0.98 s. Inset shows raw i_{Ca} recorded following hyperpolarization for 0, 0.5, 1, 2, and 3 s.

Fig. 4. As seen, the more depolarized the voltage step, the greater the magnitude of slow inactivation of i_{CaL}. We may be underestimating the extent of the slow inactivation and its voltage dependence because in the first protocol, the 500-ms voltage step may be sufficient to produce some slow inactivation (see Fig. 3 for example).

Slow Inactivation of L-Type Calcium Current and the Measurement of T-Type Calcium Current

Measurements of the T-type Ca current are often made using voltage protocols similar to that shown in Fig. 5 *A*. In this example, the membrane is held to steady state at either −90 or −40 mV and then depolarized to −10 mV to elicit Ca current. The difference current (Fig. 5 *B*) is generally taken to be the T-type Ca current. The question we asked is whether this difference current is truly the equivalent of T-type Ca current. We have already demonstrated that slow inactivation of L-type calcium current occurs after depolarization of the holding potential. Thus, two events are occurring which affect the amplitude of i_{Ca} recorded from a depolarized holding potential. First, i_{CaT} activates and inactivates, and then i_{CaL} slowly inactivates. Thus, i_{Ca} recorded from a holding potential of −90 mV is equal to i_{CaL} + i_{CaT}, but i_{Ca} recorded from a holding potential of −40 mV is not i_{CaL} but i^*_{CaL}, that amount of L-type calcium current left after slow inactivation has occurred.

$$\text{Then, } i_{Ca90} - i_{Ca40} = i_{CaT} + i_{CaL} - i_{CaL^*} \neq i_{CaT}.$$

We next tried to measure the overestimate of i_{CaT} by using the protocols illustrated in Fig. 6. In the first protocol (Fig. 6 *G, open triangles*), the cell is initially held at −40 mV and a 1-s hyperpolarizing pulse to −90 mV applied (to allow recovery from slow inactivation of i_{CaL}). Then, 500 ms long depolarizing voltage pulses of varying amplitude were applied. The Ca current thus elicited contained both T + L components; i.e., i(△) = i_{CaT} + i_{CaL}. In the second protocol (Fig. 6 *G, open squares*), after the 1-s hyperpolarizing pulse to −90 mV, the voltage was stepped back up to −40 mV for a sufficient time to cause activation and inactivation of i_{CaT}, but short enough to cause minimal slow inactivation of i_{CaL}. After this short duration voltage step, 500 ms long depolarizing voltage pulses were applied. The Ca current thus elicited contained mostly the L component; i.e., i (□) = i_{CaL}. In the third protocol (Fig. 6 *G, filled squares*), the myocyte was maintained at −40 mV for a long time (usually 15 s). This allows both inactivation of i_{CaT} and slow inactivation of i_{CaL} to nearly reach a steady state. Then, 500 ms long depolarizing voltage pulses were applied. The Ca current thus elicited gives a best estimate of i^*_{CaL}, which is i_{CaL} left after slow inactivation has occurred; i.e., i (■) = i^*_{CaL}.

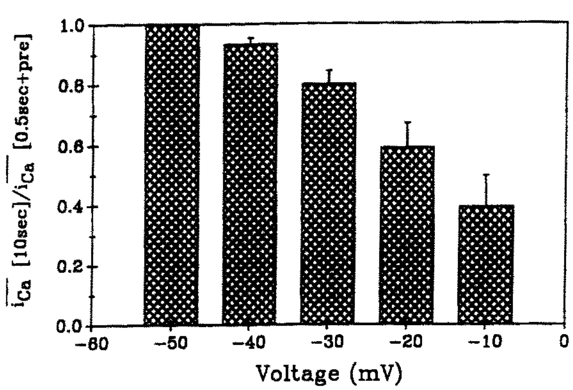

FIGURE 4. Voltage dependence of the magnitude of slow inactivation. The ratio of the peak amplitude of i_{Ca} (in response to a 500-ms pulse to +15 mV) after a 10 s period at −50, −40, −30, −20, and −10 mV divided by the amplitude of i_{Ca} after a 500 ms period at the same potentials (n = 13). The 500-ms pulse was preceded by a 1-s hyperpolarization to remove slow inactivation. The holding potential was −30 mV. Protocols are shown in Fig. 7.

Fig. 6 *A* illustrates the I-V relations for the first (△) (i = i_{CaT} + i_{CaL}) and third (■) (i = i^*_{CaL}) protocols described above. Fig. 6 *D* is the difference I-V relation, i.e., i_{CaT} + i_{CaL} − i^*_{CaL}; this represents the current lost due to slow inactivation of i_{CaL} as well as i_{CaT}. Fig. 6 *B* illustrates the I-V relations for the second (□) and third protocols (■) described above. Fig. 6 *E* is the difference I-V, i_{CaL} − i^*_{CaL}. This represents the component of i_{CaL} lost due to slow inactivation and therefore the extent of slow inactivation. Fig. 6 *C* illustrates the I-V relations for the first (△) and second (□) protocols described above and Fig. 6 *F* the difference current i_{CaT}. Comparing Fig. 6, *D* with *F*, gives an idea of the overestimate of i_{CaT}. In this cell, we found most of the difference current in 6 *D* was not true T-type current. This varied somewhat from cell to cell, but almost always indicated that an appreciable error in estimating the amplitude and kinetics of T-type calcium current could be made with standard protocols, due to slow inactivation of i_{CaL}.

Influence of Slow Inactivation of i_{Ca} *on the Measurement of the Voltage Dependence of "Normal" Inactivation of* i_{CaL}

The voltage dependence of "normal" inactivation of i_{CaL} is usually determined by measuring i_{CaL} elicited in response to a depolarizing test pulse, from a range of

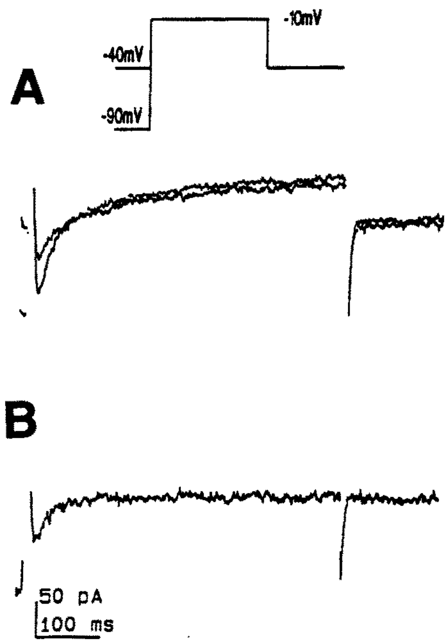

FIGURE 5. Difference current as a measure of T-type i_{Ca} in Purkinje myocytes. (*A*) Calcium currents recorded during a 250-ms pulse to −10 mV from holding potentials of −40 and −90 mV. (*B*) Difference current measured by subtracting the traces recorded at −90 mV from that recorded at −40 mV.

holding potentials where the cell has been held for a prolonged time period (Fig. 7, left hand side protocol [●]). "Normal" inactivation measured this way will be contaminated by slow inactivation of i_{CaL} which also occurs or is removed (with a voltage dependent magnitude and possibly time course) when the cell is held at potential V for a prolonged time period. A protocol to minimize the slow inactivation component is shown in Fig. 7 on the right hand side (○). The cell is first hyperpolarized to −90 mV for 1 s, to allow its recovery from slow inactivation of i_{CaL}. Then, voltage steps of 500 ms to varying potentials, V are applied. This activates and inactivates i_{CaT} (Hirano et al., 1989) but minimizes the magnitude of slow inactivation of i_{CaL}, and also allows "normal" inactivation of i_{CaL} to reach a steady state. The current in response to a depolarizing test pulse after this voltage step will reflect in large part the "normal" inactivation of i_{CaL}. Fig. 7 provides normalized f_∞ data for the

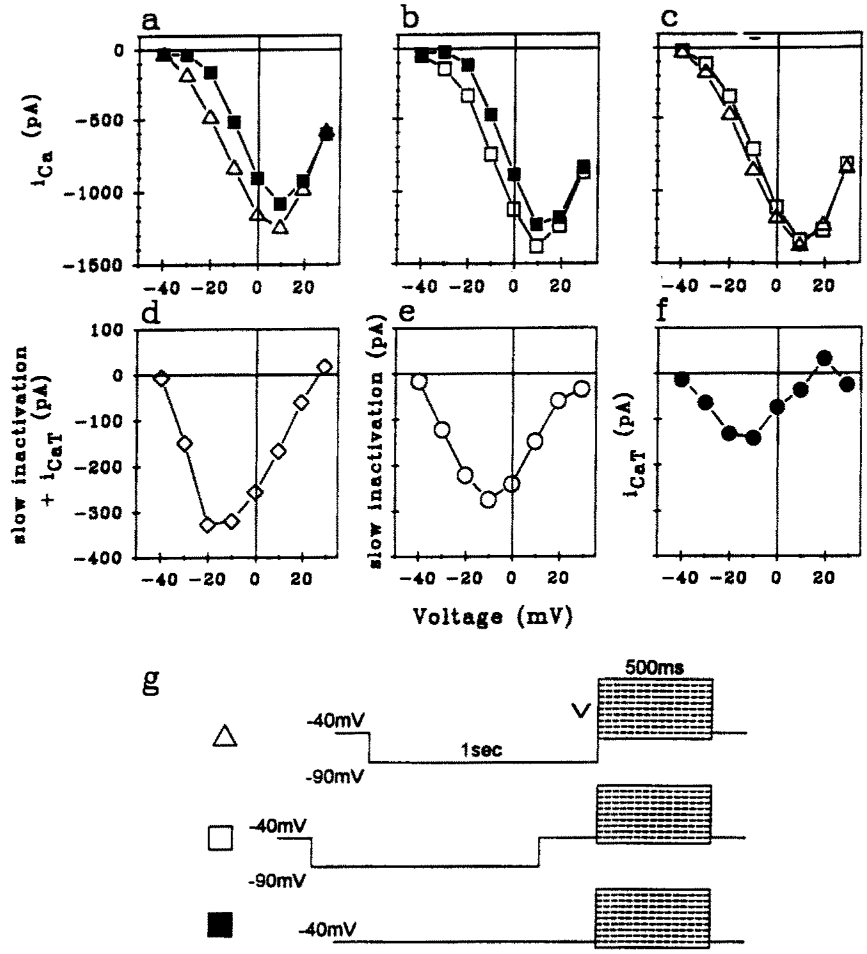

FIGURE 6. I-V relations as an estimate of T-type calcium current. Symbols used in *a–c* correspond to protocols shown in *g*. In each case protocol pairs were interleaved to minimize any influence of rundown. (*A*) Conventionally used I-V relations to define T-type calcium current. (*B*) I-V relations used to define slow inactivation. (*C*) I-V relations used to give a best estimate of T-type calcium current. (*D*) Difference I-V for (*A*) estimates slow inactivation + T-type calcium current. (*E*) Difference I-V for (*B*) estimates extent of slow inactivation. (*F*) Difference I-V for (*C*) estimates T-type calcium current. (*G*) Protocols: *open triangles* (△): elicits both T-type and L-type calcium current. *Open squares* (□): elicits only L-type calcium current (no slow inactivation). *Filled squares* (■): Elicits only L-type calcium current but with slow inactivation.

two protocols. It is clear that for the protocol that removes slow inactivation (○), the f_∞ curve is displaced to substantially more depolarized potentials with significant availability at potentials more positive than −30 mV. This availability at the more positive potentials is much reduced when slow inactivation is allowed to proceed. This basic observation was present in all 13 of our experiments of this type.

DISCUSSION

Our results demonstrate the existence of slow inactivation in canine Purkinje myocytes as has been previously reported for calf Purkinje fibers (Kass and Scheuer, 1982). As seen in Fig. 4, this slow inactivation process is voltage dependent, where almost 60% of i_{CaL} at −10 mV inactivates via slow inactivation, whereas <10% inactivates via this route at −40 mV.

The existence of this slow inactivation process alters the interpretation of previous experimental protocols designed to estimate T-type Ca current. In these previous studies in cardiac Purkinje myocytes (Tseng and Boyden, 1989; Hirano et al., 1989).

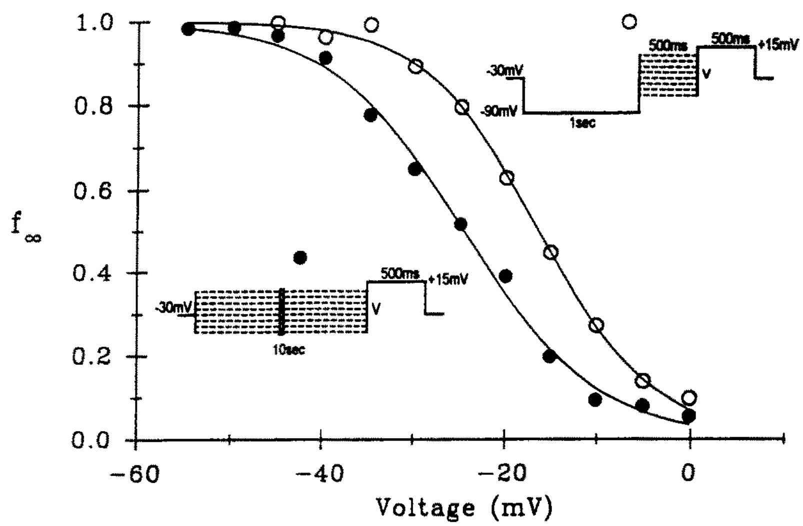

FIGURE 7. Voltage dependence of i_{Ca} inactivation. Calcium current recorded using the protocols indicated in the figure. In each case protocol pairs were interleaved to minimize any influence of rundown. (*Open circles*) Membrane was held for 500 ms at voltage indicated before a 500-ms test pulse to 15 mV to elicit i_{Ca}. (*Closed circles*) Membrane was held for 10 s at indicated voltage before a 500-ms test pulse to 15 mV to elicit i_{Ca}. f_∞ curve constructed using the data in *A*. Data for each protocol in *A* were normalized to give f_∞ curves for each protocol. The data were fitted using the equation $1/(1 + \exp((V - V_{1/2})/k))$ where V is the step voltage, (x axis in the plots). For the open circles, $V_{1/2} = -20.4$ mV, $k = 5.4$; for the *filled circles*, $V_{1/2} = -34.3$ mV, $k = 6.3$. The data points for −70 mV were excluded from the fit.

T-type calcium current was obtained as the difference in Ca current elicited by holding the membrane in the steady state at relatively depolarized or hyperpolarized potential. As demonstrated in Fig. 6, this protocol overestimates the T component by also including a component due to slow L-type inactivation. While the magnitude of T-type current is overestimated, there appears little question that another kinetically different component of calcium current does exist in Purkinje myocytes. T-type calcium current has also been reported in atrial (Bean, 1985), SA node (Hagiwara, Irisawa, and Kameyama, 1988) and ventricular (Tseng, Boyden, Robinson, and Hoffman, 1987) myocytes. The contribution of slow L-type inactivation to these measurements of i_{CaT} has yet to be determined, but certainly is a significant consideration in interpretation of these results.

Besides altering the interpretation of protocols designed to isolate i_{CaT}, slow inactivation of i_{CaL} implies that various protocols developed to obtain the voltage dependence of "normal" inactivation of i_{CaL} will yield different results. In principle, this is a steady state measurement, and so is best estimated by prolonged periods at various test potentials. However, a curve constructed in this way will contain two kinetically distinct components (i.e., due to "normal" and slow inactivation) the slower of which is much less relevant at physiologic heart rates. It is worth pointing out that since slow inactivation is more prominent at depolarized potentials, the relative availability of i_{CaL} at potentials positive to -50 mV declines as the duration of the prepulse is lengthened (see Fig. 7).

A number of hormones have been reported to alter i_{CaL} (Hirano et al., 1989) either increasing (e.g., β-agonists) or decreasing (e.g., ACh) the Ca influx. A report also exists suggesting that hormones can also alter i_{CaT} (Tseng and Boyden, 1989). It is worth reinvestigating these hormone actions recognizing that an alteration in the kinetics or magnitude of slow inactivation of i_{CaL} could alter measurements of the properties of either i_{CaT} or i_{CaL}.

In frog ventricular myocytes, slow inactivation of L-type calcium current appears to be largely voltage dependent and unaffected by $[Ca]_i$ or intracellular cAMP (Schouten and Morad, 1989). However, because this slow inactivation proceeds with a time course of tens of seconds (typically $\gamma \approx 40$ s) it may have different origins to that described in our study. Slow inactivation in pancreatic B cells appears to be more similar with average time constants of 2.75 s and significant inactivation between -40 and -30 mV (Hopkins, Satin, and Cook, 1991). These potentials are below the threshold for activation of L-type calcium current. Although there is slow inactivation of both L and T-type calcium current in other preparations the voltage range for this inactivation typically overlaps with that of activation of the current (Hennessy and King, 1985 [paramecium]; Bossu and Feltz, 1986 [rat sensory neurons]).

In cardiac tissues in general, it remains to be determined how alterations in extracellular and intracellular ionic constituents and second messengers alter this slow kinetic process as well as the prevalence of this process. The outcome of such studies would provide clues to the role of slow inactivation in controlling slow conduction during ischemia when extracellular K^+ and H^+ are increased and intracellular Ca^{2+}, H^+, and Na^+ are similarly elevated. Much remains to be done, but it is clear that a precise characterization of the myocardial cell's calcium conductance will require a detailed study of the kinetics and magnitude of this slow inactivation process.

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