Kinetic Analysis of the L-type Calcium Current in Enzymatically Dissociated Ferret Ventricular Myocytes

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Abstract. The L-type calcium current (I_{Ca-L}) was studied in single ferret ventricular myocytes using whole-cell recording with single patch pipettes. Voltage-clamp experiments were performed at room temperature with internal and external Na^+- and K^+-free Tyrode solutions in order to isolate I_{Ca-L}. For depolarizing steps eliciting small I_{Ca-L} the decay of the current is best described by one exponential. For depolarizing steps eliciting large I_{Ca-L} (i.e. between −10 and +30 mV), the decay of the current is best described by the sum of two exponentials with a calcium-dependent fast (T_f) time constant and a voltage-dependent slow (T_s) time constant. Experiments conducted with different external concentration of Ca^{2+} and Ba^{2+} suggested that the inactivation and the time course of reactivation of the current after a depolarizing pulse are dependent on calcium ions. This confirms previous observations in heart muscle and reveals the existence of a calcium-dependent regulation process of the L-type calcium current in enzymatically dissociated ventricular myocytes from ferret heart.

Key words: Whole-cell patch-clamp — Ferret heart — Ventricular myocytes — L-type calcium current — Inactivation — Reactivation

Introduction

The high level of resolution of the patch-clamp technique (Hamill et al. 1981) led to the discovery of two types of cardiac calcium currents: a low-threshold dihydropyridine-insensitive calcium current and a high-threshold dihydropyridine-sensitive calcium current also designed as I_{Ca-T} and I_{Ca-L} respectively, according to the classification proposed by Nilius et al. (1985).

Except in chick embryonic ventricular myocytes where I_{Ca-T} is twofold larger

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than I_{Ca-L} (Kawano and DeHaan, 1989), I_{Ca-L} is the major contributor to the calcium influx in the heart muscle and plays a key role in the regulation of contractility. Because of the crucial role played by I_{Ca-L}, considerable attention has been placed on the characteristics of the calcium influx through the dihydropyridine-sensitive calcium channels (Campbell and Giles 1990; Pelzer et al. 1990).

One of the main features of I_{Ca-L} is the presence of a time dependent decaying phase during a maintained depolarization. It was at first assumed that this decaying phase reflected a purely voltage-dependent inactivation process (Beeler and Reuter 1970; Ochi 1970; New and Trautwein 1972), in harmony with the Hodgkin-Huxley model (1952). In fact, it has been found that in many preparations calcium channel inactivation is partly regulated by membrane potential (for review see Eckert and Chad 1984).

In heart muscle several studies have presented evidence that I_{Ca-L} inactivates by a mechanism that is both calcium- and voltage-dependent (Kass and Sanguinetti 1984; Mentrard et al. 1984; Lee et al. 1985).

However, if the inactivation process has been well characterized the reactivation process (i.e. the recovery from inactivation) of the L-type calcium current following a step depolarization has been studied less extensively. The time of recovery is affected by different parameters such as the temperature, the frequency of stimulation, the holding potential, the intracellular concentration of calcium and the external charge carrier (Pelzer et al. 1990).

In the present experiments we recorded I_{Ca-L} from single ferret ventricular myocytes using the whole-cell patch-clamp technique. The kinetic characteristics of the inactivation and reactivation processes are described.

Materials and Methods

Dissociation procedure

Single ventricular myocytes from ferrets' hearts were obtained by an enzymatic dissociation procedure. Adult male ferrets (*Mustela putorius furo*; 1200–1500 g), previously heparined, were anaesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). The heart was rapidly removed and washed in a cold normal Tyrode solution. The aorta was cannulated for Langendorff perfusion and isolation of single cells was performed according to the method of Bouron et al. (1990a). In brief, the heart was first retrogradely perfused at 37 °C with a normal Tyrode solution. After the recovery of the contractile activity the heart was perfused for 4 min with a Ca^{2+}-free Tyrode solution containing 20 mmol/l taurine and 0.1 mmol/l EGTA, and then 2 min with the same solution without EGTA. Finally, the heart was perfused for 40–45 min with a recirculating enzyme-containing Tyrode solution. The right ventricle of the enzyme-digested heart was separated, washed, minced in the previous solution but without enzyme and gently shaken. The cells were then filtered through a 200 μm nylon mesh and gradually resuspended in a normal Tyrode solution and kept at room temperature with continuous

gassing (100% O_2). The protocol provides up to 80% of elongated-shaped viable myocytes (Bouron et al. 1990a).

Electrophysiological experiments

Measurements of calcium and barium currents were carried out with the whole-cell patch-clamp technique (Hamill et al. 1981). The isolated ventricular myocytes were transferred to an experimental chamber containing a Na^+- and K^+-free Tyrode solution and placed on the stage of an inverted microscope (Olympus IMT2, Olympus, Japan). Pipettes (1–3 MΩ) were pulled from capillary glass on a two-step vertical puller (Narishige PP-83, Narishige, Japan). All experiments were carried out at room temperature.

Calcium and barium currents were filtered at 3 kHz using a patch-clamp amplifier (Biologic, RK 300, France). Data acquisition and storage were carried out on a micro-computer (Epson PC AX 20) through a D/A-A/D converter (TM 40, Tecmar, USA) controlled by a software package (pClamp, Axon Instruments, USA).

The decay of the capacitive transient (induced by a depolarizing step of 10 mV from HP = −90 mV) was fitted to a single exponential function with a mean time of 0.75 ± 0.11 ms ($n = 21$). The cell capacitance (C_m) determined from the area under the capacitive transient was 182.02 ± 3.69 pF ($n = 21$). When series resistances were partially compensated (mean compensation 66%) the membrane capacitance could be charged with a time constant of 0.49 ± 0.9 ms ($n = 21$). Voltage error resulting from residual series resistance ($R_s = 2.69 \pm 0.27$ MΩ) ranged between 1.5 and 3.5 mV, indicating a good voltage homogeneity inside the cell during the time course of I_{Ca-L}. The cell membrane input resistance estimated from the positive slope of the current-voltage relationship of I_{Ca-L} was 67 ± 4.6 MΩ ($n = 11$).

I_{Ca-L} was elicited by 300 ms-duration voltage steps at a frequency of 0.1 Hz. The current amplitude was measured as the difference between peak inward current and the current at the end of the 300 ms-duration step.

The decay time constants of I_{Ca-L} were determined by least-squares fitting of one or two exponentials to the current trace (pClamp, Axon Instruments). Other specific protocols are described in the text or in figure legends. Statistical data are given as mean ± S.E.M.

Solutions

Normal Tyrode solution contained (in mmol/l): NaCl 120; KCl 5; $CaCl_2$ 1.8; $NaHCO_3$ 4; HEPES 20; glucose 11; pH 7.4. Ca^{2+}-free Tyrode solution contained (in mmol/l): NaCl 120; KCl 5; $MgCl_2$ 1.8; $NaHCO_3$ 4; HEPES 20; glucose 11; EGTA 0.1; taurine 20; pH 7.2. Enzyme-containing Tyrode solution contained (in mmol/l): NaCl 120; KCl 5; $MgCl_2$ 1.8; $CaCl_2$ 0.06; $NaHCO_3$ 4; HEPES 20; glucose 11; taurine 20; bovine serum albumin (BSA) 1 mg/ml; collagenase 1 mg/ml; elastase 0.06 mg/ml; pH 7.2.

I_{Ca-L} was characterized in the presence of a Na^+- and K^+-free Tyrode solution. All the external and internal sodium and potassium ions were equimolarly substituted by tetraethylamonium. The composition of the external solution was (in mmol/l): TEACl 130; $MgCl_2$ 1; $CaCl_2$ 1.3 or 10; HEPES 20; glucose 5.5; pH 7.2. The pipette solution contained (in mmol/l): TEACl 130; EGTA 1; ATPMg 5; HEPES 10; pH 7.2.

Collagenase extracted from *Clostridium histoliticum* and elastase of pig pancreas were obtained from Boehringer Mannheim France SA. All other chemicals and reagents were provided by Sigma Chimie France.

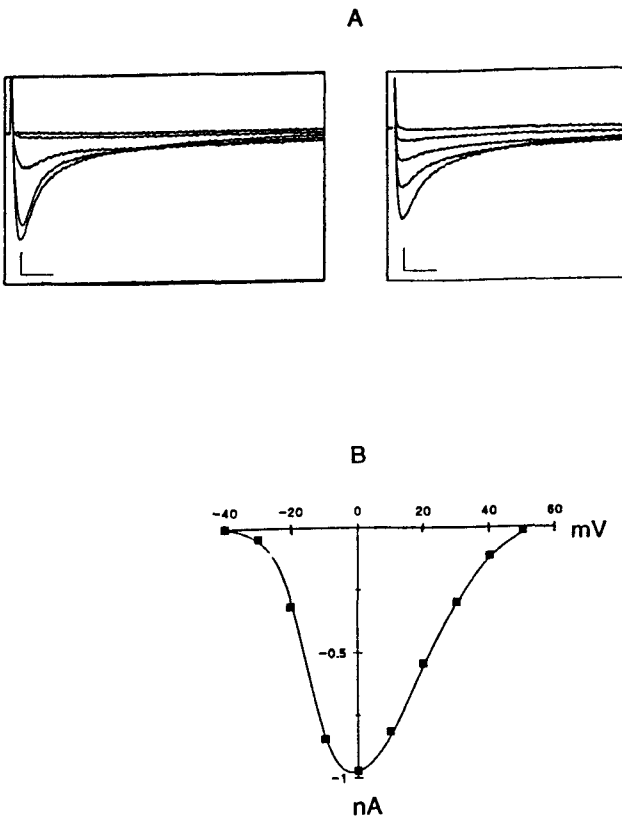

Figure 1. ***A.*** Current recordings elicited by 300 ms-duration steps from HP=−80 mV to −40, −30, −20, −10 and 0 mV (left panel) and to 10, 20, 30, 40 and 50 mV (right panel). Scale bars: 200 pA, 30 ms. ***B.*** Current-voltage relationship of I_{Ca-L}. Inward current amplitudes (determined as the difference between peak inward current and the current at the end of the 300 ms-duration step) are plotted against test potentials. HP=−80 mV.

Results

Current-voltage relationship

In the presence of 1 mmol/l external Ca^{2+}, the application of depolarizing pulses (in 10 mV step increments from HP = −80 mV) elicited an inward current for potentials more positive than −40 mV. The amplitude of the inward current increased with the amplitude of the depolarization, reaching a maximum value at 0 mV. For more positive step depolarizations, the amplitude of the current declined

and nearly disappeared at +50 mV (Fig. 1*A*). The corresponding current-voltage relationship is reported in Fig. 1*B*. The voltage dependence and the bell-shaped curve are comparable to that generally observed for I_{Ca-L} (Isenberg and Klockner 1982; Josephson et al. 1984; Boyett et al. 1988b).

Kinetic analysis

Inactivation of I_{Ca-L} during step depolarizations

For depolarizing pulses between −30 and +50 mV from HP = −80 mV the decaying phase of the current was fitted by one or two exponentials. For test pulses close to the activation threshold (−30/−20 mV) and for test pulses more positive than +40 mV (i.e. for depolarizing steps eliciting small I_{Ca-L} the decaying phase is described by one exponential with a time constant τ = 50 to 95 ms. For test pulses between −10 and +30 mV (i.e. for depolarizing steps eliciting large I_{Ca-L}) the decaying phase is best described by the sum of two exponentials with a fast (T_f) and a slow (T_s) time constant. Table 1 reports the mean values of T_f and T_s as a function of the test potential. T_f is weakly affected by the test potential whereas T_s is modified suggesting some voltage dependence. Increasing the external concentration of Ca^{2+} to 3 mmol/l did not significantly affect τ (64 − 121 ms, n = 4) nor T_s (T_s = 151.51 ± 27 ms, n = 4 at 0 mV) but strongly decreased T_f (Table 1).

These results suggest that during a step depolarization the decaying phase of I_{Ca-L} depends, at least partially, on the influx of Ca^{2+}. So, if the internal concentration of Ca^{2+} regulates the availability of the L-type calcium channels it should be possible to relate the amplitude of I_{Ca-L} during a test pulse to the amount of

Table 1. Values of the time constant of inactivation, T_s and T_f, as a function of the membrane potential and the external concentration of calcium.

	T_s (ms)	T_f(ms)	
Test potential	1 mmol/l	1mmol/l	3 mmol/l
(mV)	[Ca^{2+}]	[Ca^{2+}]	[Ca^{2+}]
−10	190.09 ± 20	21.3 ± 3.2	12.51 ± 6.8
0	152.23 ± 29.2	21.77 ± 4.99	10.83 ± 4.6
+10	117.18 ± 11.5	19.52 ± 2	8.72 ± 5.44
+20	142.25 ± 25	21.91 ± 3.8	14.02 ± 1.5
+30	169.82 ± 35	22.46 ± 3.9	23.89 ± 4.7

T_s: voltage-dependent slow time constant; T_f: calcium-dependent fast time constant. The L-type calcium current was induced by a 300 ms-depolarizing pulse from a HP = −80 mV to the voltage indicated. Data are mean ± S.E.M.

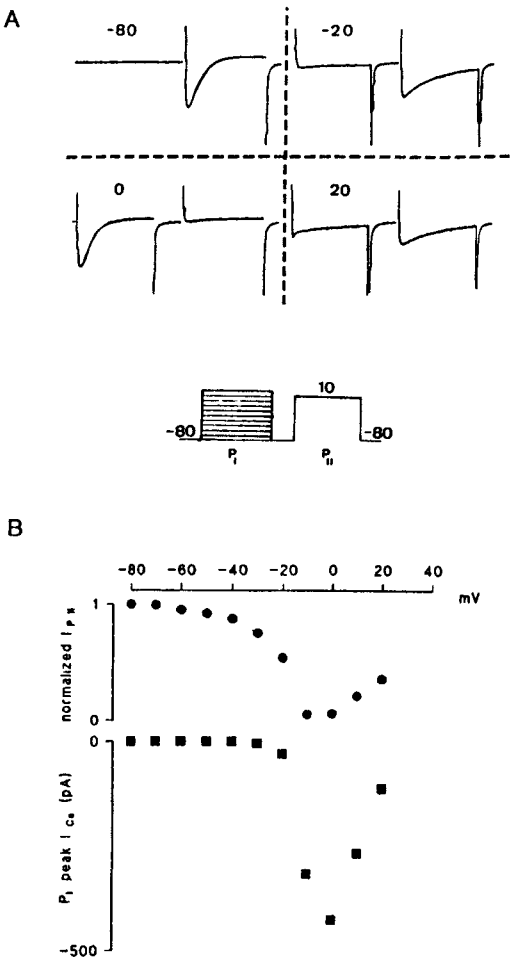

Figure 2. ***A.*** A pair (P_{I} and P_{II}) of 300 ms-duration pulses separated by a 100 ms interpulse was applied from a HP = −80 mV every 10 s (protocol in insert). Four representative current traces recorded from the same cell are shown; the test potential during P_{I} is indicated at the top. ***B.*** The normalized amplitude of the calcium current during P_{II} ($I_{P_{\mathrm{II}}}$) (defined as the ratio of $I_{P_{\mathrm{II}}}$ in the presence of a prepulse / $I_{P_{\mathrm{II}}}$ in the absence of a prepulse) is plotted against the voltage of P_{I} (filled circles). The resulting curve is compared to the coresponding current-voltage relationship of the calcium current elicited during P_{I} ($I_{P_{\mathrm{I}}}$, filled squares). Similar results were obtained in 3 other cells.

Ca^{2+} carried into the cell during a prepulse. For this, we employed the double-protocol of Brehm and Eckert (1978). In our conditions, a pair of 300 ms-duration pulses separated by a 100 ms interpulse was applied from a HP = −80 mV every

10 s. The test pulse (P_{II}) potential was held constant at +10 mV and the prepulse (P_I) potential varied from −80 to +20 mV by 10 mV increments. Results from a representative experiment are shown in Fig. 2*A* with the test potential during P_I indicated at the top. The normalized amplitude of the current during P_{II} ($I_{P_{II}}$) (defined as the ratio of $I_{P_{II}}$ in the presence of prepulse / $I_{P_{II}}$ in the absence of prepulse) is plotted against the voltage of P_I. The resulting curve (Fig. 2*B*, filled circles) shows that $I_{P_{II}}$ strongly decreases in the voltage range where I_{P_I} developed (filled squares). The inactivation was maximal at the voltage inducing the maximum current during P_I and then decreased as the current decreased. Thus, for short prepulses, the inactivation process seems partially dependent on the extent of calcium entry into the cell. Consistent with this interpretation is the observation that the inactivation is slower when 1 mmol/l Ba^{2+} is substituted for 1 mmol/l Ca^{2+}. Fig. 3*A* shows representative traces of currents elicited during a step depolarization to 0 mV from −50 mV. In the presence of Ba^{2+} (i.e. the condition designed to suppress the inhibitory action of Ca^{2+} on the current) the decaying phase is still present but is best fitted by one exponential (instead of 2) with a time constant of 120 ms, intermediate between T_f and T_s.

Steady-state inactivation of I_{Ca-L}

The steady-state inactivation of I_{Ca-L} was studied from a HP = −90 mV using a conventional two-pulse protocol consisting of a 3000 ms-duration conditioning prepulse (CP) followed after a 10 ms interval by a 300 ms-duration test pulse (TP) to 10 mV. The voltage of the CP varied between −60 and +40 mV by 10 mV increments. The ratio between the amplitude of I_{Ca-L} elicited during the TP preceded by a CP ($I_{Ca(test)}$) and the amplitude of I_{Ca-L} elicited by the TP in the absence of a CP ($I_{Ca(control)}$) as a function of the voltage during the CP is plotted in Fig. 3*B*. In the presence of 1 mmol/l Ca^{2+} (filled squares; $n = 4$) the inactivation is incomplete: even at positive potentials the maximum amount of inactivation reached a limiting value. The percentage of steady-state inactivation is 72.5% and 77% at +20 and +40 mV, respectively. The more complete inactivation at positive potentials observed in the presence of 10 mmol/l Ca^{2+} (Fig. 3*B* open squares, $n = 10$) can be accounted for by the influence of the calcium influx.

Reactivation of I_{Ca-L}

In Fig. 4 the time course of reactivation of the current was studied with a double-pulse protocol (Fig. 4*A*), as a function of the holding potential, of the nature of the divalent cation carrying the current and of the prepulse duration. Fig. 4*B* shows the time course of reactivation of the current carried by barium ions at two different HP. For HP = −70 mV (open circles), it is best fit by one exponential, the constant of which (T_r) is 750 ms and the time of recovery of one-half of control current $t_{1/2}$

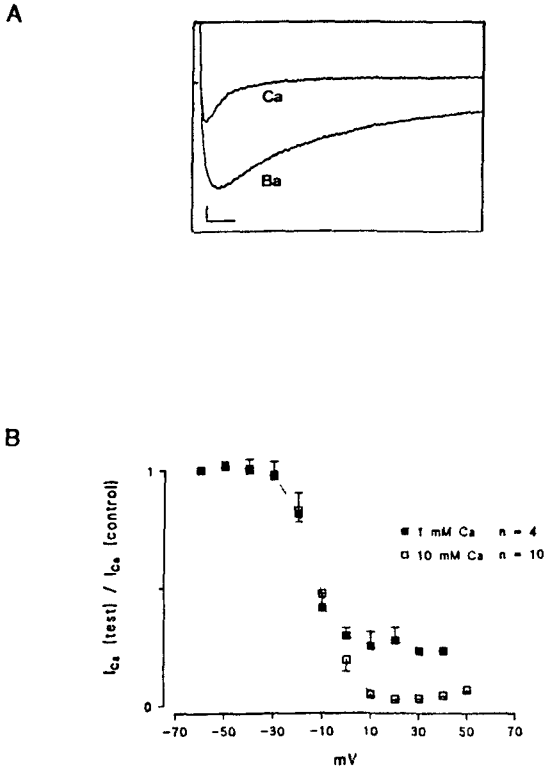

Figure 3. ***A.*** Current traces elicited during a step depolarization to 0 mV from a HP = −50 mV in the presence of 1 mmol/l Ca^{2+} or 1 mmol/l Ba^{2+} Scale bars 100 pA, 30 ms ***B*** Steady-state inactivation curves of I_{Ca-L} obtained in the presence of 1 mmol/l Ca^{2+} (filled squares) and 10 mmol/l Ca^{2+} (open squares), respectively In the two-pulse protocol used, the duration of the conditioning pulse (CP), the interpulse and the test pulse (TP) were 3000 ms, 10 ms and 300 ms, respectively The ratio between the amplitude of I_{Ca-L} elicited during the TP preceded by a CP ($I_{Ca(test)}$) and the amplitude of I_{Ca-L} elicited by the TP in the absence of a CP ($I_{Ca(control)}$) is plotted as function of the voltage during the CP In the presence of 1 mmol/l Ca^{2+}, the voltage of the CP was varied between −60 mV to +40 mV by 10 mV increments from a HP = −90 mV and the voltage of the TP was 10 mV In the presence of 10 mmol/l Ca^{2+} preliminary experiments have revealed the existence of 2 types of calcium currents (I_{Ca-L} and I_{Ca-L}) (Bouron 1991) So, in order to exclude the intervention of I_{Ca-L}, in 10 mmol/l Ca^{2+} the voltage of the CP varied between −40 mV to +50 mV from a HP = −40 mV, and the voltage of the TP was +20 mV The curves are plotted from the values obtained in 4 (in 1 mmol/l Ca^{2+}) and 10 (in 10 mmol/l Ca^{2+}) cells, respectively Mean ± S E M

is 37 5 ms For HP = −50 mV, the reactivation was slower with T_r = 935 ms and $t_{1/2}$ = 100 ms indicating that the reactivation process was faster at more negative

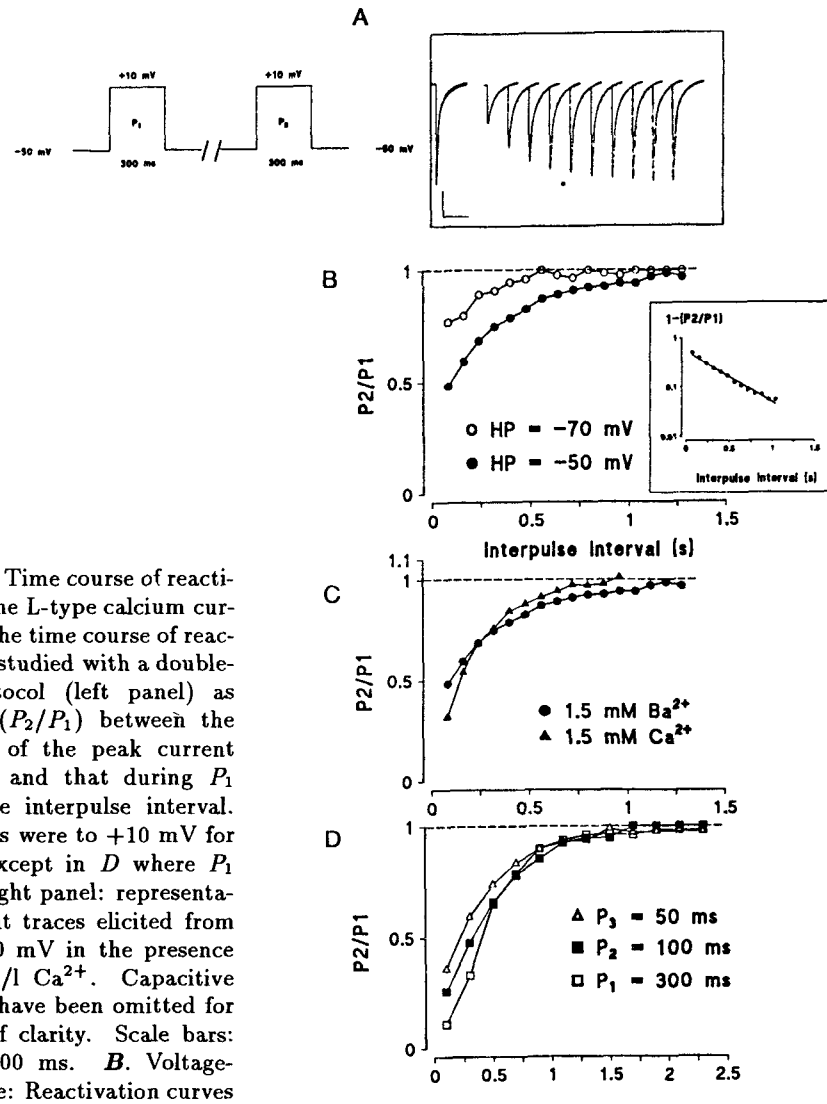

Figure 4. Time course of reactivation of the L-type calcium current. ***A***. The time course of reactivation is studied with a double-pulse protocol (left panel) as the ratio (P_2/P_1) between the amplitude of the peak current during P_2 and that during P_1 against the interpulse interval. Both pulses were to +10 mV for 300 ms (except in *D* where P_1 varies). Right panel: representative current traces elicited from a HP=−50 mV in the presence of 1 mmol/l Ca^{2+}. Capacitive transients have been omitted for the sake of clarity. Scale bars: 200 pA; 300 ms. ***B***. Voltage-dependence: Reactivation curves obtained with a HP of −70 mV (open circles) and −50 mV (filled circles) in the presence of 1.5 mmol/l Ba^{2+}. Inset: semi logarithmic plot of the inactivated portion ($1 - P_2/P_1$) of I_{Ca-L} against the interpulse interval. The time constant of reactivation was determined from the slope of the solid line. The current was elicited with the double protocol illustrated in *A* from a HP=−70 mV in the presence of 1.5 mmol/l Ba^{2+}. ***C***. Charge carrier-dependence: Reactivation curves obtained from a HP=−50 mV in the presence of 1.5 mmol/l Ba^{2+} (filled circles; same values as in Fig. 4*B*) and in 1.5 mmol/l Ca^{2+} (filled triangles). ***D***. Prepulse duration-dependence: Reactivation curves obtained from a HP=−50 mV and plotted for P_1 durations of 300 ms (open squares), 100 ms (filled squares) and 50 ms (open triangles). Similar results were obtained in 4 other cells.

potentials.

The rate of reactivation of the current depends on the charge carrier as shown in Fig. 4*C* (at HP = −50 mV). Compared to the former curve obtained in the presence of 1.5 mmol/l Ba^{2+} (T_r = 935 ms), the reactivation was faster in the presence of 1.5 mmol/l Ca^{2+} (T_r = 513 ms). Moreover, the reactivation curve exhibits an overshoot after 1 s which was more obvious when the calcium concentration was increased (data not shown).

The time course of reactivation of the current also varied with the prepulse duration. As shown in Fig. 4*D* for three different P_1 durations (300 ms, open squares; 100 ms, filled squares and 50 ms, open triangles) the shorter was P_1 the more restored was the current during P_2 because $t_{1/2}$ was 400, 310 and 225 ms, respectively.

Discussion

The ferret as an animal model

Until now, electrophysiological properties of the ferret heart have been poorly described. However, in papillary muscles, amperozide, which is a new psychotropic agent, exerts a blocking effect on I_{Ca-L} and emprophylline and theophylline, alone or in combination with terbutaline, potentiate I_{Ca-L} (Arlock 1988a,b). Experiments performed in enzymatically dissociated ventricular cells have revealed interesting properties: 1) application of acetylcholine exerts a negative inotropic effect due to the increase of a background K^+ current and in some cells a decrease of a Ca^{2+} current (Boyett et al. 1988a); 2) I_{Ca-L} is abolished by 9.6 μmol/l D600 and is regulated by the internal concentration of calcium (Boyett et al. 1988b); 3) recent experiments have shown the presence of three types of transient outward currents (I_{to}): a 4-amino-pyridine-sensitive I_{to}, a cadmium-sensitive I_{to} and a stilbene derivative-sensitive I_{to} (Bouron et al. 1991); and 4) in ventricular cells, two types of calcium current (I_{Ca-T} and I_{Ca-L}) are revealed in the presence of 10 mmol/l external Ca^{2+} (Bouron 1991) but only one type I_{Ca-L} is present at physiological concentrations of calcium (Bouron et al. 1990b).

Kinetic analysis of the L-type calcium current

In the presence of 1 mmol/l Ca^{2+} the ferret ventricular cells develop only one type of calcium current, of which the voltage dependence of activation corresponds to that of the L-type calcium current described in multicellular preparations (Arlock 1988a,b) and in single cells (Boyett et al. 1988b).

Inactivation process

In heart, it has been demonstrated that the calcium current inactivation process is controlled by both voltage- and calcium-dependent mechanisms (Kass and Sanguinetti 1984; Mentrard et al. 1984; Lee et al. 1985; Campbell et al. 1988; Hartzell and White 1989; Mazzanti et al. 1991). This has been confirmed in the present work under a number of different experimental conditions.

The analysis of the current decay during maintained depolarizations show that it is generally fit by a double-exponential function (between −10 and +30 mV). The fast time constant (T_f), whose values are comparable to that obtained in mammalian ventricular cells by Josephson et al. (1984), Tseng et al. (1987) and Tseng and Boyden (1989), shows a weak voltage dependence but decreases when the external calcium concentration and thus the calcium influx are increased. This calcium-dependence, which was also observed by substituting barium for calcium ions in the external solution, has already been reported (Josephson et al. 1984; Kass and Sanguinetti 1984; Lee et al. 1985; Hartzell and White 1989). Changing Ca^{2+} to Ba^{2+} produced a slowing of the rate of inactivation that lead to the hypothesis that the inactivation of I_{Ba} is primarily voltage dependent (Kass and Sanguinetti 1984; Hartzell and White 1989).

The calcium-dependent inactivation process was also demonstrated by the double-pulse protocol used in Fig. 2*A*. The non-monotonic inactivation curve can be correlated to the concomitant current-voltage relationship of the calcium current indicating some causal relation between the magnitude of calcium entry during a conditioning pulse and the degree of inactivation during a subsequent test pulse. This finding is in agreement with the experiments of Boyett et al. (1988b) showing that an increase in the internal concentration of calcium lead to a reduction of I_{Ca-L} in ferret ventricular myocytes. But, as generally found in similar double-pulse experiments (Mentrard et al. 1984; Lee et al. 1985), the upturn of inactivation is incomplete for high step depolarizations when the calcium entry becomes smaller and smaller. This could be accounted for by a contribution of the voltage-dependent inactivation as proposed by Lee et al. (1985).

In agreement with this are the curves of steady-state inactivation (Fig. 3*B*). However, for positive potentials, the incomplete inactivation observed in the presence of 1 mmol/l Ca^{2+} argues for a non-purely voltage-dependent process in the inactivation since more complete inactivation occurred in the presence of 10 mmol/l Ca^{2+}.

The existence of a calcium-dependent inactivation process has never been reported by single-channel studies. However, recently Yue et al. (1991) have shown that flux of calcium ions through an individual channel can modulate its own gating. But, as reported by Mazzanti et al. (1991) this experiment performed with a high concentration of calcium (160 mmol/l) does not explain the discrepancy

between single-channel and whole-cell studies performed at lower concentrations. Previous reports have suggested that the density of the L-type calcium channels would modulate the inactivation of I_{Ca-L} (Argibay et al. 1988; Mazzanti et al. 1991; Osaka and Joyner 1991); the current through one channel would be unable to establish the concentration necessary to block the pore but the current through groups of channels sufficiently close to one another would increase the internal concentration of Ca^{2+}, and then would modulate the gating of the channels (Mazzanti et al. 1991).

Reactivation process

As described in other cardiac preparations (Mentrard et al. 1984; Argibay et al. 1988; Campbell et al. 1988; Tseng 1988), the time course of reactivation was initially shown to be voltage-dependent since the time constant of reactivation was increased when the holding potential was more positive.

For a given HP the time course of reactivation was slower when Ba^{2+} was substituted for Ca^{2+} as previously shown in frog atrial trabeculae (Noble and Shimoni 1981), amphibian ventricular myocytes (Argibay et al. 1988) and mammalian ventricular myocytes (Tseng 1988), but not in mammalian multicellular preparations (Kass and Sanguinetti 1984). For short interpulses the reactivation of current was larger in Ba^{2+} than in Ca^{2+}, most likely because the barium current was not completely inactivated at the end of P_1; then the non-inactivated channels could be directly reopened by P_2. When the current was carried by Ca^{2+}, the major portion of the channels were inactivated at the end of P_1 and needed a delay for reactivation. Such an hypothesis, which implies that the channels can pass directly from an activated state to a resting state in a simple model such as that proposed by Sanguinetti et al. (1986), is strengthened by the results shown in Fig.4*D* where, in the presence of Ca^{2+}, the shorter is P_1 the larger is the reactivation at the onset of P_2.

The kinetics of the reactivation process seem faster in the presence of Ca^{2+} than in Ba^{2+} with regard to the time constant of reactivation suggesting that calcium ions play a role in the behaviour of the channel. In agreement with this is the overshoot observed in the presence of calcium (the present results; Argibay et al. 1988; Shimoni 1981; Tseng 1988) but not observed with Ba^{2+} and enhanced by high Ca^{2+}. Since it has also been reported that the overshoot is suppressed by buffering intracellular calcium with EGTA or BAPTA (Argibay et al. 1988; Tseng 1988), it seems likely that it is the intracellular calcium that accounts for the overshoot. The possible mechanisms reported to regulate the intracellular calcium concentration and then the reactivation process include an involvement of the sodium/calcium exchange mechanism (Argibay et al. 1988) and the sarcoplasmic reticulum Ca^{2+} release (Tseng 1988). From the present work the former hypothesis can be discarded since in our experimental conditions the sodium was omitted.

The possible intervention of outwards currents in the reactivation process (Shimoni 1981) can be ruled out because we used potassium-free solutions.

In conclusion, kinetic analysis of I_{Ca-L} has shown that during step depolarization between −10 and +30 mV the decay of the current develops with two time constants, T_f and T_s which are calcium- and voltage-dependent, respectively. This, and the evidence that calcium ions seem implicated in the reactivation process, suggest the existence of a calcium-dependent regulation process of I_{Ca-L} in enzymatically dissociated ferret ventricular myocytes.

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