Properties of the Low Threshold Ca Current in Single Frog Atrial Cardiomyocytes

A Comparison with the High Threshold Ca Current

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ABSTRACT   The properties of the low threshold Ca current (I_{CaT}) in bullfrog (*Rana catesbeiana*) isolated atrial cardiomyocytes were studied using the whole-cell recording patch-clamp technique and compared with those of the high threshold Ca current (I_{CaL}). In 91% of atrial cells we observed both I_{CaT} and I_{CaL} when collagenase and trypsin were used to dissociate the cells. But when pronase was used, only 30% of the cells exhibited I_{CaT}. I_{CaT} was never found in ventricular cells. I_{CaT} could be investigated more easily when I_{CaL} was inhibited by Cd ions (50 μM). Its kinetics were unchanged by substituting Ba for Ca, or in the presence of high concentrations of Ba. Both I_{CaT} and I_{CaL} exhibited reduced inactivation after high depolarizing prepulses. I_{CaT} was found to be sensitive to dihydropyridines: 1 μM nifedipine decreased this current while 1 μM BAY K 8644 increased it; this occurred without significant variations in the steady-state inactivation curve. I_{CaT} was more sensitive than I_{CaL} to α_1-adrenergic and P_2-purinergic stimulations, while I_{CaL} was more sensitive to β-adrenergic stimulation. Isoproterenol was still able to increase I_{CaT} in the presence of high intracellular cAMP. Both currents were increased by 1 μM ouabain (although I_{CaL} only transiently) and decreased by 10 μM ouabain. It is concluded that the two types of Ca channels can be observed in bullfrog atrial cells and that they are specifically altered by pharmacological agents and neuromediators. This may have implications for cardiac behavior.

INTRODUCTION

Calcium entry through voltage-gated Ca channels is essential for several cellular functions including excitability, contraction, secretion, transmitter release, metabolic processes, and regulation of Ca channel kinetics themselves. In cardiac cells, two types of Ca channels have been identified: the predominant type activates around

−30 mV, inactivates slowly when Ba is the charge carrier, and has a large unitary conductance (Bean, 1985b; Nilius, Hess, Lansman, and Tsien, 1985; Hagiwara, Irisawa, and Kameyama, 1988). The current through these channels is commonly termed L-type Ca current (I_{CaL}) and corresponds to the slow inward current described many years ago by Rougier, Vassort, Garnier, Gargouil, and Coraboeuf (1969). I_{CaL} is more depressed by Cd than by Ni (Nilius et al., 1985; Mitra and Morad, 1986; Bonvallet, 1987; Bonvallet and Rougier, 1989; Hirano, Fozzard, and January, 1989a; but see Hagiwara et al., 1988). Its activity is modulated by α_1-adrenergic (Alvarez, Mongo, and Vassort, 1987), β-adrenergic (Brum, Osterreider, and Trautwein, 1984), and P_2-purinergic (Alvarez, Mongo, Scamps, and Vassort, 1990; Scamps, Legssyer, Mayoux, and Vassort, 1990) stimulation. I_{CaL} is sensitive to dihydropyridines (Bean, 1985b; Nilius et al., 1985; Mitra and Morad, 1986; Hagiwara et al., 1988; Bonvallet and Rougier, 1989; Hirano et al., 1989a).

The other cardiac Ca channel (I_{CaT}) has a low activation threshold, inactivates quickly even when Ba is the charge carrier, and has a smaller unitary conductance (Bean, 1985b; Nilius et al., 1985; Hagiwara et al., 1988; Droogmans and Nilius, 1989). In contrast to I_{CaL}, this current is more depressed by Ni than by Cd (Nilius et al., 1985; Mitra and Morad, 1986; Bonvallet, 1987; Bonvallet and Rougier, 1989; Hirano et al., 1989a) and is reported to be insensitive to dihydropyridines (Bean, 1985b; Nilius et al., 1985; Hagiwara et al., 1988; Bonvallet and Rougier, 1989; Hirano et al., 1989a; Bois and Lenfant, 1991; Charnet, Richard, Gurney, Ouadid, Tiaho, and Nargeot, 1991; but see Tseng and Boyden, 1989, and Akaike, Kostyuk, and Osipchuk, 1989). I_{CaT} is also reported to be insensitive to neurotransmitters (Bean, 1985b; Hagiwara et al., 1988; Tytgat, Nilius, Vereecke, and Carmeliet, 1988; Bonvallet and Rougier, 1989; Bois and Lenfant, 1991; Charnet et al., 1991). However, Mitra and Morad (1986) showed an increase in I_{CaT} by isoproterenol in guinea pig ventricular myocytes. Tseng and Boyden (1988) reported that β-stimulation increased I_{CaT} in canine ventricular myocytes but not in Purkinje cells, whereas α_1-adrenergic stimulation increased I_{CaT} in both cell types. In a subsequent paper, Tseng and Boyden (1989) confirmed the increase in I_{CaT} by α_1- but not by β-adrenergic stimulation in canine Purkinje cells. Furthermore, Tseng and Boyden (1991) suggested that activation of protein kinase C decreases I_{CaT} in cardiac Purkinje cells. In addition, Bonvallet and Rougier (1989) presented evidence that angiotensin II increases and atrial natriuretic factor decreases I_{CaT}. Thus, there seems to exist an agreement in the literature regarding the kinetics, selectivity, modulation, and pharmacology of I_{CaL}, but incomplete or contradictory results are reported for I_{CaT}. Even its presence in frog atrium remains a matter of debate (compare Bonvallet and Rougier, 1989, with Campbell, Giles, Hume, and Shibata, 1988a, and Campbell, Giles, and Shibata, 1988b).

Because each type of calcium channel might have different functional implications, an investigation of their pharmacological profiles would contribute to the understanding of their specialized physiological role. In this study, using the whole-cell patch-clamp technique, we investigated the characteristics and pharmacological properties of I_{CaT} in bullfrog atrial cells and compared them with those of I_{CaL} of atrial and ventricular cells.

MATERIALS AND METHODS

Single atrial and ventricular cells were dissociated from bullfrog hearts (Rana catesbeiana) by a method similar to that described by Arrio-Dupont and De Nay (1985). In brief, hearts were mounted in a Langendorff column and washed at 30°C with 100 ml of a standard Ringer solution (see below) containing only 50 μM CaCl₂ (low-Ca Ringer). Thereafter, 20 ml of low-Ca Ringer supplemented with 5 mM creatine, 1 μl/ml of nonessential amino acids and vitamins medium (MEM; Boehringer Mannheim Corp., Indianapolis, IN), 1 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 1.5 mg/ml collagenase (Boehringer Mannheim Corp.), and 0.4 mg/ml trypsin (Merck-Clévenot S.A., Nogent-sur-Marne, France), was recirculated for 45 min. Atria and ventricle were then separated, cut into small pieces, and agitated in the same Ringer without enzymes for 20 min. Supernatants were filtered through a nylon gauze (250 μm) and centrifugated at 900 revolutions/min for 5 min. Pellets of cells were then "diluted" in fresh Ringer, supplemented with MEM containing 0.9 mM CaCl₂, and stored in a refrigerator. Cells could be used for up to 36 h after isolation.

In some experiments, pronase E (1.5–1.75 mg/ml; Merck-Clévenot S.A.) was used instead of collagenase and trypsin. In these cases, the enzymatic dissociation period could be shortened to 20–25 min with a similar cell yield. However, as the low threshold Ca current (I_{CaT}) was often missing in pronase dissociation, this method was finally discarded. It is to be noted that in agreement with Dowell and Tarr (1988), single myocytes obtained from pronase dissociations were superior in overall viability and could be used for up to 72 h after isolation.

For electrophysiological recording, cells were left to lie in Petri dishes (type 1008; Falcon, Becton Dickinson and Co., Plymouth, UK) on the stage of an inverted microscope and perfused by gravity (0.65 ml/min) with a standard Ringer solution containing 20 mM CsCl, no KCl, and 1 μM tetrodotoxin (TTX; Sigma, L'Isle d'Abeau, Chesnes, France) to ensure complete blockade of K and Na currents. Cells were positioned at the extremity of one of six microcapillaries (Tygon microbore tubing, i.d. = 250 μm, o.d. = 750 μm; Norton Performance Plastics, Wayne, NJ) independently perfused by gravity (16 μl/min) allowing rapid changes (≤1 s) of the extracellular medium. Patch electrodes (1–3 MΩ) were made from glass capillaries (Microcaps; Drummond Scientific Co., Broomall, PA). A patch-clamp system (RK-300; Biologic, Claix, France) was used which permitted liquid junction potential compensation. After a gigaseal was obtained, the membrane patch was broken and whole-cell membrane capacitance and series resistance were measured and compensated. The intracellular solution in the patch electrode contained (mM): 120 CsCl, 5 K₂-EGTA, 4 MgCl₂, 5 Na₂-creatine phosphate, 3 Na₂-ATP, 0.4 Na₂-GTP, and 10 HEPES (pH 7.1, adjusted with KOH or CsOH). Under these experimental conditions, Ca currents can be measured as the difference between peak inward current and the current level at the end of the voltage-clamp pulse (Alvarez et al., 1990).

Voltage-clamp protocols were delivered by a SEN-7103 electronic stimulator (Nihon-Kohden, Tokyo, Japan) coupled to a S-8300 step-pulse generator (Nihon-Kohden). Membrane potential was routinely depolarized for 200 ms every 4 s from a holding potential (HP) of −100 to −50 mV for the study of the high threshold Ca current (I_{CaT}) or to 0 mV for the study of the low threshold Ca-current (I_{CaL}). In most ventricular cells studied, the HP was −80 mV. Availability curves for I_{CaT} and I_{CaL} were obtained from double-pulse protocols: every 4 s a 200-ms prepulse to different potentials was followed by a 200-ms pulse to −50 mV for I_{CaT} or to 0 mV for I_{CaL}. Prepulses and test pulses were separated by a 3-ms return to the holding potential. The amplitude of I_{Ca} elicited by the test pulse is plotted as a percentage of the I_{Ca} evoked in the absence of a prepulse. From prepulse depolarizations current-to-voltage relationships were obtained. Reactivation (recovery from inactivation) was analyzed by depolarizing the cell every 8 s from the HP to −50 mV for I_{CaT} or to 0 mV for I_{CaL}, returning to the HP for 13 intervals

from 3 ms to 3 s. Current recordings were stored on FM tape (DFR 3415; Sony, Clichy, France) and simultaneously displayed on a storage oscilloscope (VC-10; Nihon-Kohden).

Solutions and Drugs

The composition of the standard Ringer solution was (mM): 88.4 NaCl, 2.5 KCl, 23.8 NaHCO$_3$, 0.5 NaH$_2$PO$_4$, 1.8 MgCl$_2$, 1.8 CaCl$_2$, 5.0 glucose, and 5.0 Na$_2$-pyruvate; pH was adjusted to 7.4. Inorganic or organic compounds were added to this Ringer as indicated and all solutions were gassed with 95% O$_2$/5% CO$_2$. In a few experiments NaCl, NaHCO$_3$, and NaH$_2$PO$_4$ were substituted by 73.3 mM BaCl$_2$ and 10 mM HEPES; the pipette solution then contained 5 mM BaATP and 400 μM LiGTP to substitute all Na salts. All experiments were carried out at room temperature, 20–23°C.

The following drugs were used: isoproterenol, phenylephrine, and propranolol (Sigma Chemical Co.), nifedipine and BAY K 8644 both dissolved in ethanol (Bayer, Wuppertal, Germany), ATP and 3′, 5′ cAMP (Merck-Clévenot), prazosin (Pfizer, Orsay, France), and ouabain (BDH Chemicals Ltd., Dorset, UK).

Results were analyzed by a paired t test and are expressed as means and standard error of the mean.

RESULTS

Two Components of Calcium Current, I_{CaT} and I_{CaL}

In an atrial cell held at −100 mV, clamp steps above −70 mV activated a time-dependent inward current which became larger with increasing depolarizations. When the HP was set at −50 mV, clamp steps up to −35 mV did not activate any current. Depolarizations to more positive potentials elicited another inward current (Fig. 1 A). Fig. 1 B shows the voltage dependence of the averaged peak inward currents relative to cell membrane capacity obtained in most of the atrial cells. At −100 mV HP, an activation threshold of −75 mV and two maxima were clearly distinguished in the current–voltage (I-V) relationship. As TTX was present at a concentration of 1 μM (or 3 μM in some experiments), a concentration that induced a complete blockade of the Na current (see Hume and Giles, 1983), it follows that the maximum current at −30 mV in the I-V curve was probably not due to the inflow of Na through fast Na channels. Moreover, reducing Na to 30 mM (substituted by choline chloride) did not significantly affect the kinetics or the amplitude of the current elicited by membrane depolarizations up to −30 mV. However, the current evoked at 0 mV was increased by 15 ± 3% (n = 7) in Na-poor solutions. Finally, 3 mM MnCl$_2$ depressed the low and high threshold currents by ~40 and 60%, respectively (data not shown). These observations reinforce the view that the low and high threshold currents correspond to the T- and L-type Ca^{2+} currents, respectively.

In a series of experiments conducted on 34 atrial cells in which both currents were recorded over the full range of voltages, five cells exhibited I_{CaT} whose peak amplitude was larger than I_{CaL} (Fig. 1 C). Notice that in these five cells, I_{CaL} was much weaker and I_{CaT} slightly larger than in the other cells. Their shape under the microscope and their averaged membrane capacitance (68.6 ± 6.8 pF) were not different from other cells (see Table I). Finally, one cell exhibited only I_{CaT}; its peak amplitude was 1.4 pA/pF at −30 mV depolarization.

The characteristics of both I_{CaT} and I_{CaL} from a total of 156 cells are given in Table

I, which also compares I_{CaL} elicited in either atrial or ventricular cells. Membrane capacitance was the same in both cell types, indicating that we used atrial and ventricular cells of similar size. Peak I_{CaL} elicited by a depolarization to 0 mV had the same amplitude and kinetics in both cell types and was independent of the presence or absence of I_{CaT}. The maximal amplitude of I_{CaT} estimated at -30 mV was close to half-peak I_{CaL}. I_{CaT} kinetics were markedly accelerated by depolarizing to -30 mV instead of -50 mV, and were then similar to those of I_{CaL} at 0 mV.
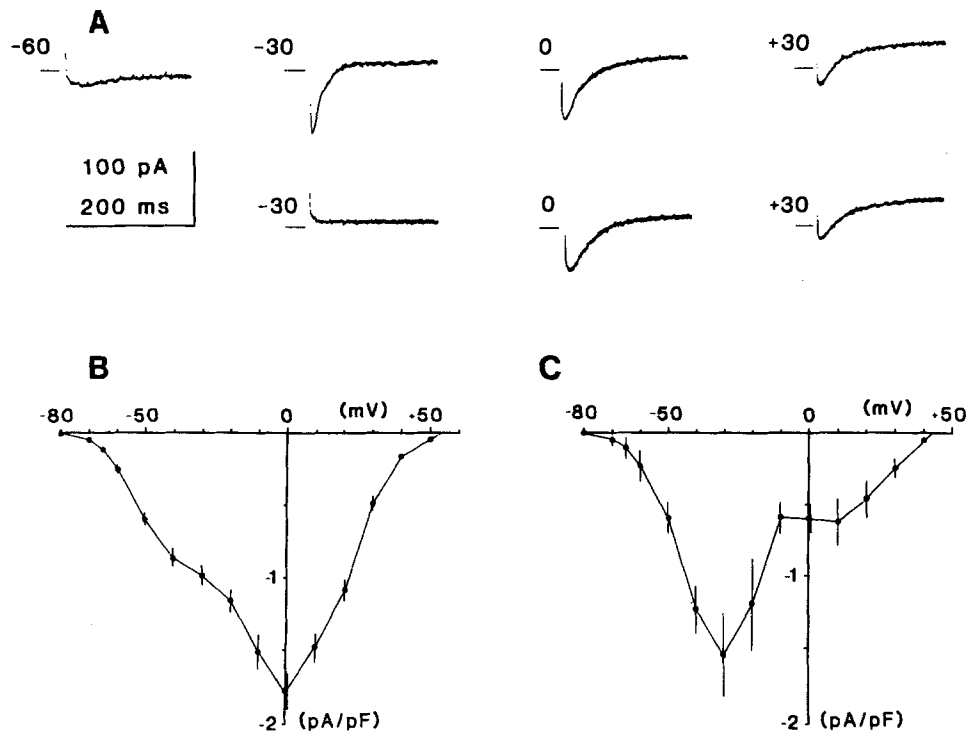

FIGURE 1.   Inward currents activated by depolarizing steps from in frog atrial cell. (A) Current recordings at HPs of -100 mV (top) and -50 mV ($bottom$) elicited in a cell by depolarizing pulses as indicated. Horizontal lines indicate zero current level. (B and C) Calcium current density–voltage relationships averaged on two pools of atrial cells which exhibit either $I_{CaT} < I_{CaL}$ (B; $n = 29$) or $I_{CaT} > I_{CaL}$ (C; $n = 5$). Results are expressed as means ± SE.

These two types of Ca currents, I_{CaT} and I_{CaL}, were found in 107 of 117 atrial cells when collagenase and trypsin were used for dissociating the cells. However, in pronase dissociations only 30% of atrial cells (4 of 12) presented both currents. Nevertheless, the characteristics of I_{CaT}, when present, were not affected by pronase.

In 35 ventricular cells examined, no inward currents were activated between -70 and -30 mV, indicating that I_{CaT} was absent, in agreement with the contention that ventricular cells exhibited only the high threshold current (see also Argibay, Fischmeister, and Hartzell, 1988).

TABLE I

Characteristics of the T- and L-Type Ca Currents

	I_{Ca}	Cm	Density	Time to peak	Time to half-inactivation
I_{CaT}	*pA*	*pF*	*pA/pF*	*ms*	*ms*
−50 mV (n = 78)	40.1 ± 2.3	79.8 ± 2.1	0.50 ± 0.03	13.3 ± 0.4	20.1 ± 0.6
−30 mV (n = 29)	67.8 ± 4.9	70.1 ± 2.6	0.97 ± 0.07	4.9 ± 0.3	14.6 ± 0.5
I_{CaL}					
Atrial (n = 69)	180.0 ± 15.7	81.1 ± 2.1	2.22 ± 0.17	5.4 ± 0.1	16.9 ± 0.3
Ventricular (n = 35)	188.0 ± 17.4	80.2 ± 3.3	2.34 ± 0.24	5.8 ± 0.1	16.3 ± 0.5

Notice that I_{CaL} peak and density were 215.8 ± 44.5 pA and 2.25 ± 0.47 pA/pF, respectively, in 14 atrial cells that did not show I_{CaT}. Amplitude, time to peak, and time to half-inactivation of I_{CaT} and I_{CaL} were estimated on traces elicited at −50 or −30 mV and at 0 mV, respectively, from a HP of −100 mV for atrial cells and −80 mV for ventricular cells.

When atrial cells were held at −50 mV, the threshold potential was more positive (−30 mV) and only one maximum was seen between 0 and +10 mV. The difference current obtained by subtracting peak current recorded for the same depolarizations applied from either −100 or −50 mV HP displayed a maximum at −30 mV (see Fig. 2). However, in ~70% of the cells, both atrial and ventricular, the amplitude of the inward current at potentials positive to −20 mV was significantly reduced when the HP was −50 instead of −100 mV. However, our standard double-pulse protocol did not demonstrate that this resulted from the voltage-dependent inactivation of I_{CaL} in these cases since identical steady-state inactivation curves were obtained at the two HPs. Such a reduction could be attributed to the slow inactivation of I_{CaL} as recently described by Schouten and Morad (1989). This phenomenon prevented us from
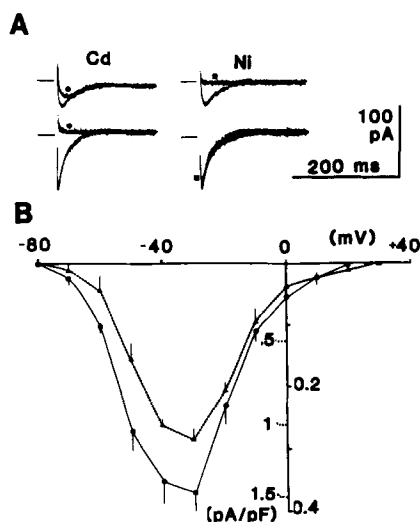
FIGURE 2. Effects of divalent cations on I_{CaT} and I_{CaL} in atrial cells. (*A*) The upper traces show the effects on I_{CaT} elicited by a 200-ms depolarizing step from −100 to −50 mV of Cd (50 μM) and Ni (50 μM) ions. The lower traces show the effects of the same two cations on I_{CaL} elicited by a depolarizating step from −100 to 0 mV. Horizontal lines at the left indicate zero current. Two different cells. (*B*) Current density–voltage relationships established in the presence of 50 μM Cd (●, *n* = 10, *solid line*) or in control solution for the current obtained by subtracting peak currents elicited by the same depolarization range from two HPs (−100 and −50 mV) averaged in four cells that did not exhibit slow inactivation (▲, *dotted line*).

estimating I_{CaT} at membrane potentials positive to -30 mV and from using such a high HP during pharmacological studies of I_{CaL}.

In the following studies, for routine monitoring of these currents, we measured I_{CaT} and I_{CaL} elicited by depolarizing pulses at -50 and 0 mV, respectively, form a HP of -100 mV. A depolarization to -50 mV allowed us to study changes in I_{CaT} without contamination of I_{CaL}, even under the influence of compounds that shifted the voltage dependence of I_{CaL} to more negative potentials (see below). Estimation of I_{CaL} at 0 mV in cells that possessed I_{CaT} was difficult. We chose to ignore the contribution of I_{CaT} to total inward current at 0 mV, since on the cells that did not show slow inactivation, the estimated I_{CaT} amplitude obtained from the difference current (see Fig. 2) was on average only 9% of total inward current ($n = 13$). Inward current at 0 mV in atrial cells will then be referred to as I_{CaL}.

Effects of Divalent Cations

Raising the extracellular Ca concentration to 3.6 mM significantly enhanced both currents: I_{CaT} and I_{CaL} were increased by 52 ± 5 and $31 \pm 5\%$, respectively ($P < 0.05$; $n = 6$).

In another series of experiments we studied the effects of the inorganic blockers Cd and Ni, as well as the use of Ba as charge carrier on I_{CaT} and I_{CaL}. Typical examples of the inhibitory effects of Cd and Ni ions are shown in Fig. 2 A. In the presence of 50 μM Cd ions, averaged I_{CaT} amplitude elicited by a -50-mV depolarization was $47.7 \pm 2.6\%$ ($n = 23$) of control. It was also noticeable that the I_{CaT} kinetics were slowed down. Time to peak current was increased from 12.7 ± 0.2 to 15.0 ± 0.5 ms and time of I_{CaT} half-inactivation (19.9 ± 0.9 ms at -50 mV) was significantly prolonged by $28.3 \pm 3.0\%$ ($n = 23$). The average I_{CaT} density–voltage relationship as shown in Fig. 2 B demonstrated that the reduction in peak current induced by Cd was seen at each potential; this occurred with a minor shift toward the left when compared with the I_{CaT} density–voltage relationship obtained after estimating I_{CaT} with the difference method in four control cells. Such an effect suggests that the Cd block is weakly voltage dependent.

Ni ions (50 μM) abolished I_{CaT} and slightly decreased I_{CaL} ($9.2 \pm 0.4\%$; $n = 8$). In the presence of 50 μM Ni the whole I_{CaL}-V relationship was shifted by ~ 10 mV toward hyperpolarizing potentials with a weak reduction in the maximal inward current; the threshold for the inward current was now -35 mV (not shown). A similar shift occurred on the availability curve. These effects were observed in the five cells investigated and were already reported by Mitra and Morad (1986).

The substitution of Ba^{2+} for Ca^{2+} significantly increased I_{CaL} by $64 \pm 8\%$ ($n = 6$) and markedly delayed its inactivation as already reported (Argibay et al., 1988); this left I_{CaT} amplitude and time course unaffected except in a few cells in which I_{CaT} amplitude was slightly reduced (not shown). Five experiments were performed in the total absence of Na ions, which were replaced by Ba^{2+} as is often done during single-channel patch-clamp experiments. Fig. 3 shows the currents elicited by depolarizing to -20 and $+20$ mV and the I-V relationships established under these conditions (73.3 mM Ba), as well as after adding 50 μM Cd ions. Except that the voltage dependence of both currents was shifted by ~ 30 mV in the depolarizing direction as expected from the screening effect of large divalent concentration,

roughly similar current kinetics were observed as with 1.8 mM Ba as the charge carrier (Fig. 3 A). Cd ions suppressed the large Ba current carried by L-type channels, but in the lower range of depolarizations left a significant current with typical T-type kinetics (Fig. 3 B). Furthermore, Ni ions (50 μM) abolished the Ba current carried through T-type channels (n = 3; see Fig. 8 B).

Inactivation and Recovery from Inactivation of I_{CaT} and I_{CaL}

The amplitude of I_{CaT} elicited by depolarizations to −50 mV was dependent upon the short-term previous history of the membrane potential. Prepulses of different magnitudes applied with different delays reduced I_{CaT} to different extents during the test pulse. A 200-ms prepulse to −50 mV fully suppressed the current during the test pulse applied after a 3-ms interval; however, after prepulse depolarization to +50

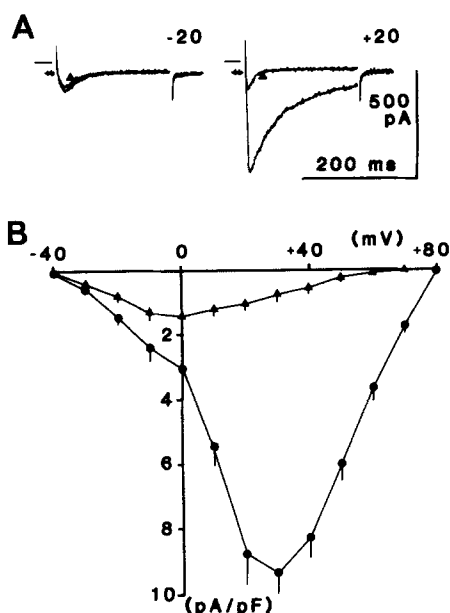

FIGURE 3. Inward currents in the presence of high Ba concentration and effects of Cd ions. (A) Representative recordings of currents elicited at −20 and +20 mV from a −100 mV HP in the presence of 73.3 mM Ba or after adding 50 μM Cd ions (▲); Cm = 70 pF. (B) I-V relationships established on five cells in high Ba solution (73.3 mM, ●) or after adding 50 μM Cd (▲). Peak Ba current amplitude was estimated by the algebraic difference between the peak inward current in Ba and the current recorded in the presence of Cd ions at the end of the 200-ms pulse of the same voltage.

mV, I_{CaT} partially recovered to reach ∼40% of its full value (Fig. 4 A). Its kinetics were not markedly changed. I_{CaT} recorded during the test pulse was markedly reduced when increasing the interval to 50 ms (Fig. 4 B). Finally, I_{CaT} was not significantly altered by increasing the prepulse from 200 to 500 ms (Fig. 4 C).

Fig. 5 A shows averaged inactivation curves for both Ca currents. On individual cells, the inactivation curve could be described in part by the theoretical Boltzmann distribution function, assuming that the decrease in availability from 1 to 0 when increasing prepulse amplitude is only sensitive to membrane depolarization. The mean values for the half-maximal potentials, V_f, and the slope factors, k, were −66.6 ± 0.5 mV and 6.4 ± 0.2 for I_{CaT} (n = 29) and −29.9 ± 0.9 mV and 7.1 ± 0.2 for I_{CaL} (n = 36), respectively. Inactivation curves for I_{CaT} were thus displaced ∼35

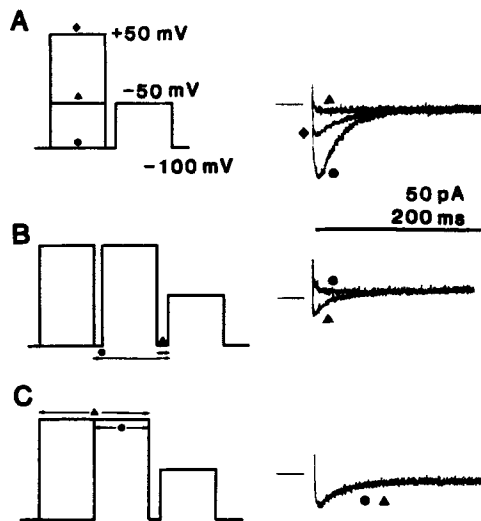FIGURE 4. Influence of the conditioning pulses on the current recorded during a test pulse to −50 mV. (A) Currents (●, ▲, ◆) recorded after prepulses of different amplitude: 0, −50, and +50 mV, respectively. (B) Currents (▲, ●) recorded after the same prepulse was applied 3 or 50 ms before the test pulse. (C) Currents (●, ▲) recorded after a prepulse of 200 or 500 ms duration. HP was −100 mV.

mV to more negative potentials and displayed a steeper voltage dependence when compared with I_{CaL} inactivation curves.

Both currents showed less inactivation with positive prepulse potentials. This is a well-known phenomenon for I_{CaL}, which was related to Ca-dependent Ca current inactivation (Mentrard, Vassort, and Fischmeister, 1984; Lee, Marban, and Tsien, 1985). A similar relationship with membrane potential was found for I_{CaT} inactivation. Increasing TTX concentrations up to 3 μM did not abolish I_{CaT} recovery after high prepulse potentials. This indicates that it was not an artefact due to I_{Na}. Nor was

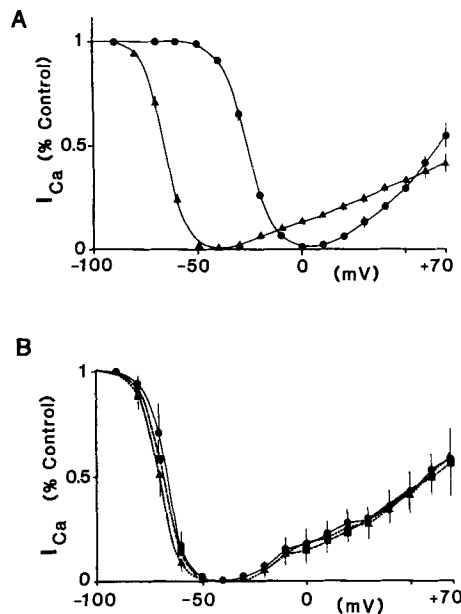FIGURE 5. Inactivation curves for I_{CaT} and I_{CaL}. (A) Voltage dependence of steady-state inactivation (availability) of I_{CaT} (▲) and I_{CaL} (●), obtained with the protocol described in Materials and Methods. Solid lines represent Boltzmann's fits of averaged values with $V_f = -66.2$ and -25.4 mV and $k = 5.2$ and 5.8 for I_{CaT} and I_{CaL}, respectively. (B) Voltage dependence of steady-state inactivation (availability) of I_{CaT} established with a 3-ms interval between the test pulse to −50 mV and the prepulse (200 ms) in control (●), 50 μM Cd (▲), and when Ba (1.8 mM) was the charge carrier (■); HP was −100 mV ($n = 4$). Curves fitted by the Boltzmann equation up to −50 mV.

it the result of a Ca-mediated inactivation of I_{CaT}, since similar availability curves for I_{CaT} were obtained in the presence of Cd or when Ba replaced Ca. Only a minor leftward shift (-4.6 ± 0.6 mV; $n = 5$) was determined on the availability curves of I_{CaT} when comparing I_{CaT} recorded in cells before and in the presence of 50 μM Cd (Fig. 5 B). Equimolar Ba substitution did not significantly affect the I_{CaT}/V relationship (not shown) or the availability curve (Fig. 5 B). Availability curves of I_{CaT} with similar characteristics including recovery from inactivation for high prepulses were
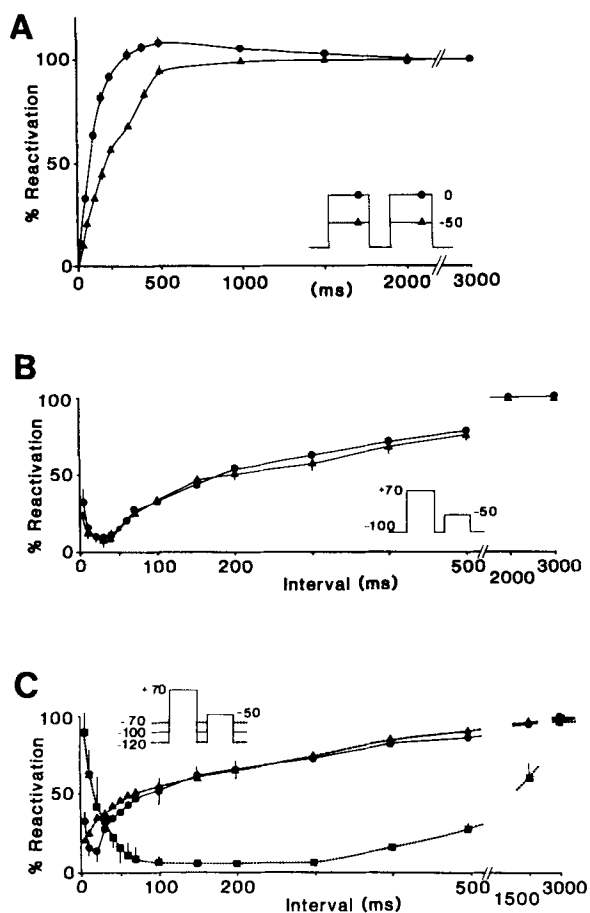

FIGURE 6. Reactivation curves for I_{CaT}. (A) Reactivation (recovery from inactivation) curves for I_{CaT} (▲) and I_{CaL} (●) obtained from an HP of -100 mV. Stimulation protocols were as described in Materials and Methods. (B) Reactivation (recovery from inactivation) curves established in control (●) or in the presence of 50 μM Cd and 1.8 mM Ba (▲) with a 200-ms conditioning prepulse to $+70$ mV followed at increasing intervals by a -50-mV test pulse; HP was -100 mV as indicated in the inset. Stimulation frequency was $1/8$ s ($n = 6$). (C) Reactivation (recovery from inactivation) curves established in the presence of 50 μM Cd and 1.8 mM Ba on three other cells held successively at -100 (●), -70 (■), and -120 mV (▲), otherwise using the same protocol as in B.

obtained in the high Ba (0 Na) solution and after the further addition of 50 μM Cd (not shown). The whole curves were, however, shifted to the right by ~30 mV; half-inactivation was -37.1 ± 1.5 or -40.6 ± 1.4 mV ($n = 5$) in 73.3 or in 73.3 mM Ba and 50 μM Cd, respectively. T-type currents recorded in Ba solutions at -20 mV showed similar time courses whether or not the test pulse was preceded by a prepulse as previously observed under control conditions.

I_{CaT} and I_{CaL} showed differences in their recovery characteristics (Fig. 6 A). Average

time of half-recovery from inactivation was 71.3 ± 0.9 ms for I_{CaL} ($n = 15$) and 171.0 ± 1.8 ms for I_{CaT} ($n = 10$). In agreement with the observations of Argibay et al. (1988), cells with I_{CaL} density <2.0 pA/pF exhibited an overshoot of up to 120% on the I_{CaL} reactivation curves. Such a phenomenon was never seen with I_{CaT}; rather, the slow recovery of I_{CaT} inactivation seemed to imply two kinetic processes as already characterized by Bossu and Feltz (1986).

In each ionic experimental condition, the I_{CaT} availability curve bent up. However, this apparent reduction of current inactivation with depolarization was obtained with a 3-ms interval between the high depolarizing prepulse and the test pulse and at −100 mV HP. On establishing a reactivation curve with a test pulse at −50 mV and a prepulse at +70 mV, the prolongation of the interval above 3 ms initially decreased current amplitude during the test pulse so that it was nearly abolished within 25 ms (Fig. 6 B; see also Fig. 4 B). Further increases in the interval between the conditioning pulse at high voltage and the test pulse allowed for full recovery from inactivation. Similar results were obtained in the presence of Cd ions and when Ba ions were the charge carrier as shown in Fig. 6 B. Recoveries from inactivation of both I_{CaT} and I_{CaL} were already shown to be voltage dependent (Argibay et al., 1988; Tseng, 1988; Tseng and Boyden, 1989; Hirano, Fozzard, and January, 1989b). Furthermore, for I_{CaT}, the time course of recovery did not depend on the ion carrier. In the presence of 50 μM Cd and 1.8 mM Ba, and after a 200-ms prepulse to +70 mV, some recovery from inactivation of I_{CaT} was seen only after at least 300 ms at −70 mV HP, while recovery was already significant after only 10 ms when HP was −120 mV (Fig. 6 C). Average times for half-recovery from inactivation were 65 ± 17, 90 ± 18, and 1,150 ± 237 ms at −120, −100, and −70 mV HP, respectively ($n = 3$). These values were independent of the charge carrier and of the presence or absence of Cd.

Fig. 6 shows that T-type current did not recover from inactivation in a simple manner regardless of whether Ca or Ba was the main charge carrier. The amplitude of the current during the test pulse depended on both the prepulse amplitude (see Fig. 5; cf. Fig. 6, A and B) and the interpulse duration (Fig. 6 B). Fig. 6 C further suggests that after 3 ms at a −100 mV interpulse HP, a significant part of the current had already deactivated when compared with the results at −70 mV HP, a situation in which the test pulse current was still 85% of peak current after 3 ms. Indeed, the decrease in the remaining I_{CaT} (deactivation) after a high depolarizing prepulse was strongly voltage dependent. I_{CaT} deactivated exponentially with a time constant of 29 ms at −70 mV. Deactivation of I_{CaT} was completed within 25 ms at −100 mV and within a few milliseconds at −120 mV. This was also true in the high-Ba solution; taking into account a 30-mV shift, deactivation was also completed in <10 ms at −100 mV. The deactivation rate was not dependent on the prepulse amplitude. These results are reminiscent of our previous results obtained with the double sucrose gap method on the slow inward Ca current (see Fig. 14 in Mentrard et al., 1984). They led us to reinvestigate the time dependence of inactivation at several voltages for both I_{CaT} and I_{CaL}.

The effects of increasing the prepulse duration on both I_{CaL} and I_{CaT} are illustrated in Fig. 7, at different voltages and when the current carrier was either Ca or Ba, using a 3-ms interpulse. Inactivation of I_{CaL} increased with prepulse duration. The effects were more marked at 0 mV prepulse potential and in the presence of Ca ions than at

higher prepotentials and in the presence of Ba ions (Fig. 7 A). When the prepulse potential was +40 mV, intermediate values were obtained in either Ca or Ba solutions, except that the initial reduction of I_{CaL} occurred more rapidly than with the two other prepulse voltages. These observations are in agreement with the fact that some part of the Ca current inactivation is mediated by Ca ions entering through the channel. The time dependence of I_{CaT} inactivation exhibited much more complex characteristics. A prepulse potential at −50 mV inactivated I_{CaT} within 200 ms. With larger prepulse depolarizations, inactivation was never completed (Fig. 7 B). A prepulse at +40 mV reduced I_{CaT} to 45% of its control amplitude within 20 ms; longer prepulses did not significantly change the inactivation level. A prepulse to +70 mV also reduced I_{CaT} by about half, but with a slower kinetic; however,
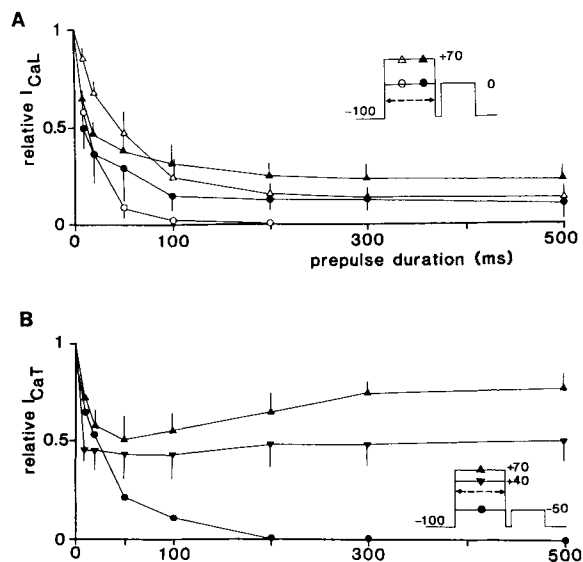

FIGURE 7. Effects of increasing prepulse duration on I_{CaT} and I_{CaL}. (A) Inactivation of I_{CaL} recorded during a depolarizing pulse at 0 mV by prepulse depolarizations to 0 mV (O, ●) or to +70 mV (△, ▲) whose durations increased up to 500 ms in control conditions (*open symbols*) or in 1.8 mM Ba (*closed symbols*) (n = 3–5). (B) Inactivation of I_{CaT} recorded during a depolarizing pulse to −50 mV by prepulse depolarizations to −50 (●), +40 (▼), and +70 mV (▲), whose durations increased up to 500 ms in control conditions (n = 3–5). Curves obtained in 1.8 mM Ba were identical to the ones obtained in control conditions at each of three potentials and are not shown for clarity.

prolonging it > 50 ms induced less inactivation. These effects were similar whether Ca or Ba was the charge carrier and in the presence or absence of Cd ions.

Effects of Dihydropyridines

BAY K 8644 at 1 μM increased both I_{CaT} and I_{CaL} under our experimental conditions (Fig. 8). However, there were significant differences in its action on the two current types. The effects of BAY K 8644 on I_{CaT} seemed to occur at lower concentrations and were less than on I_{CaL}; at 10 nM the increases in I_{CaT} and I_{CaL} were 1.5- and 1.4-fold the control value, respectively, instead of 1.8 and 2.7 at 1 μM (Fig. 8 B). The relative increase in I_{CaT} induced by 1 μM BAY K 8644 was the same whether HP was −100 or −80 mV; but I_{CaL}, which was increased twofold or more when HP was −100
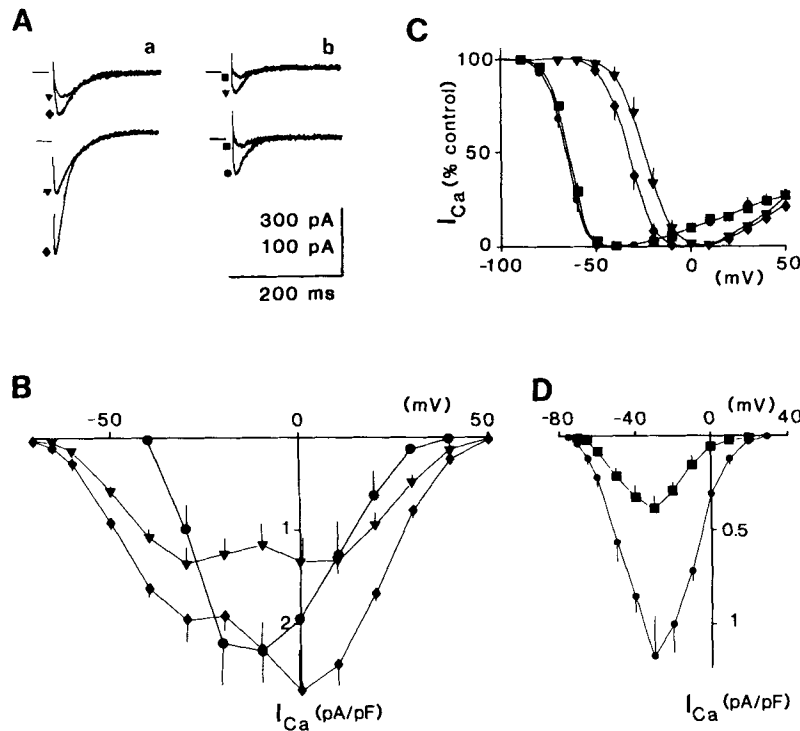
FIGURE 8. Effects of 1 μM BAY K 8644 on I_{CaT} and I_{CaL}. (*A*) The tracings (*a*) show recordings of I_{CaT} (*top*, elicited at −50 mV) and I_{CaL} (*bottom*, elicited at 0 mV), evoked from a HP of −100 mV in control conditions (▼) and after perfusion with BAY K 8644 at 1 μM (◆). The tracings (*b*) show the effects of 50 μM Cd on I_{CaT} elicited at −50 mV (*upper part*) and the effects of a further addition of BAY K 8644 (*lower part*) (▼, control; ■, Cd; ●, BAY K). (*B*) Current density–voltage relationships in the control solution (▼) and during the action of 1 μM BAY K 8644 (◆). Inward currents were increased over the whole range of potentials by BAY K 8644 when HP was −100 mV (*n* = 6). The addition of 50 μM Ni inhibited current below −40 mV and reduced the BAY K 8644 effect on I_{CaL} (●, *n* = 3). (*C*) Inactivation curves for I_{CaT} (■, ●) and I_{CaL} (▼, ◆) in control conditions (▼, ■) and under the action of BAY K 8644 (●, ◆) (*n* = 6). The normalization factors used to scale the curves after the effects of BAY K 8644 were 0.6 and 0.5 for I_{CaT} and I_{CaL}, respectively, as a consequence of the dihydropyridine-induced increased in currents elicited at −50 and 0 mV. (*D*) Current density–voltage relationships for I_{CaT} in atrial cells in the presence of 50 μM Cd (■) and after the addition of 1 μM BAY K 8644 (●) (*n* = 4).

mV, was inhibited when HP was −50 mV (not shown). Such an effect has already been reported by others (Sanguinetti, Krafte, and Kass, 1986; Kass, 1987). Note that holding the cell at either −80 or −50 mV should similarly affect the voltage-dependent kinetics of I_{CaT} or I_{CaL}, respectively, according to the voltage range of their availability curves. Fig. 8 *B* also shows that in the presence of BAY K 8644, peak I_{CaT} was not shifted, while peak I_{CaL} occurred at ∼10 mV lower depolarizations. This 10-mV leftward shift was also seen when HP was set at −50 mV (see also Sanguinetti et al., 1986). However, such a shift of I_{CaL} voltage dependence could not affect

estimation of I_{CaT} elicited at -50 mV since there was no current measurable below -40 mV in the presence of 50 μM Ni (Fig. 8 B). Note that Ni induced a further leftward shift and slightly reduced the BAY K 8644–enhanced I_{CaL}. Also, there was no current elicited by depolarizations below -40 mV in four other cells when Ba (1.8 mM) was the charge carrier in the presence of 50 μM Ni. BAY K 8644 significantly shortened the time of I_{CaT} half-inactivation by 24.7 $\pm$ 3.8% (from 21.2 $\pm$ 2.1 to 16.3 $\pm$ 2.3 ms; $P < 0.05$, $n = 9$) at 10 nM and by 23.6 $\pm$ 4.1% (to 17.4 $\pm$ 2.3 ms; $P < 0.05$, $n = 9$) at 1 μM. This was also true for I_{CaL} but only at 1 μM; time of I_{CaL} half-inactivation was decreased by 29.1 $\pm$ 8.2% (from 17.3 $\pm$ 1.0 to 12.0 $\pm$ 1.0 ms, $P < 0.05$, $n = 6$). The steady-state inactivation curve for I_{CaT} was not shifted (except by ~ 5 mV in one cell) under the action of 1 μM BAY K 8644, while I_{CaL} inactivation was significantly displaced by 10.9 $\pm$ 0.9 mV ($P < 0.01$, $n = 6$) toward more negative potentials (Fig. 8 C).

That BAY K 8644 increased I_{CaT} at every potential independent of its effects on I_{CaL} was reinforced by the following two observations. First, the fast inactivating T-type current was similarly increased by BAY K 8644 whether Ba or Ca ions were the charge carrier, without affecting current kinetics. Second, when I_{CaT} was recorded after inhibition of I_{CaL} by 50 μM Cd ions, the addition of the dihydropyridine agonist (1 μM) induced an increase in current amplitude with roughly the same magnification at each depolarizing test pulse (Fig. 8 D). As in control conditions, this increase was also unaffected by holding the cells at -80 mV. Typical current traces show that BAY K 8644 very markedly shortened time to peak and accelerated inactivation in the presence of Cd ions (Fig. 8 A, b). Note that the stimulatory effect of BAY K 8644 on peak I_{CaT} recorded in the presence of Cd ions was also much larger.

BAY K 8644 delayed the recoveries of inactivation of both I_{CaT} and I_{CaL}, the effect being more marked on I_{CaL} (Fig. 9, A and B). In addition, BAY K 8644 suppressed the overshoot in the recovery of inactivation curve of I_{CaL} when it occurred under control conditions. Time of I_{CaT} half-recovery of inactivation was increased by 54.5 $\pm$ 8.6 ms ($P < 0.01$, $n = 3$) and that of I_{CaL} by 117.5 $\pm$ 12.5 ms ($P < 0.01$, $n = 4$) with 1 μM BAY K 8644. In Fig. 9 C it is shown that the negative staircase of I_{CaT}, after an abrupt increase in stimulation rate, was only slightly enhanced in the presence of 1 μM BAY K 8644, also suggesting a weak use-dependent action of the drug on this current. On the other hand, I_{CaL} was markedly depressed in a use-dependent fashion by BAY K 8644 as reported in Fig. 9 C on a ventricular cell which showed a slight positive staircase of I_{CaL} under control conditions.

Nifedipine was used at 10 nM and 1 μM. Either increases or decreases in I_{CaT} and I_{CaL} were observed with nifedipine at 10 nM, but at 1 μM nifedipine decreased I_{CaT} and I_{CaL} to 82.5 $\pm$ 2.6 and 51.4 $\pm$ 4.1% ($P \leq 0.01$, $n = 7$) of their respective controls. Inactivation of both currents was also altered. Times of half-inactivation were decreased by 15.0 $\pm$ 3.6 and 22.7 $\pm$ 7.0% for I_{CaT} and I_{CaL}, respectively. The availability curve of I_{CaT} was not affected by nifedipine (1 μM), while a marked shift to more negative potentials (10.3 $\pm$ 0.7 mV; $P < 0.01$, $n = 7$) was found for I_{CaL}. The nifedipine-induced inhibition of I_{CaT} had similar relative magnitude when HP was less negative (-90 or -80 mV). All of these effects, including small increases in I_{CaT} amplitude induced by 10 nM nifedipine, were also observed when applying the dihydropyridine in the presence of Cd ions.

Effects of Neuromediators

The effects of β- and α_1-adrenergic and P_2-purinergic stimulations were investigated only on cells obtained from collagenase–trypsin dissociations; they are summarized in Table II. Under our experimental conditions, both I_{CaT} and I_{CaL} were sensitive to the three neuromediators. The agonistic effects of α_1-adrenergic and P_2-purinergic stimulations were even larger on I_{CaT} than on I_{CaL}; however, the increases were limited when compared with the large increase eventually elicited on I_{CaL} by β-adrenergic stimulation (Table II, Fig. 10 A, and see Fig. 11). In the presence of 1 μM propranolol, the α_1-adrenergic agonist phenylephrine (10 μM) was effective in increasing I_{CaT} by 48%, and although the effect was variable from cell to cell
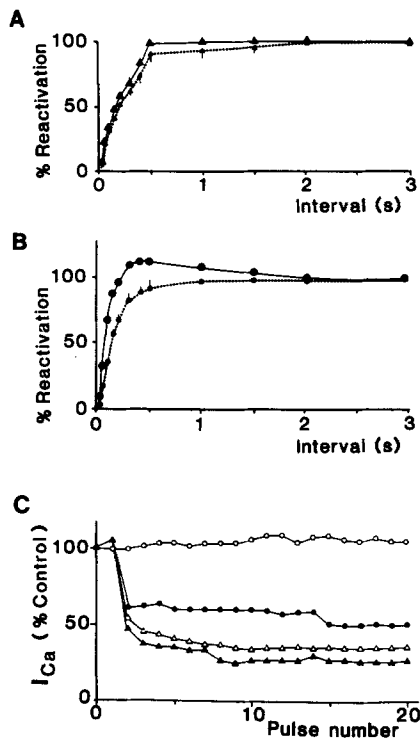

FIGURE 9. Effects of 1 μM BAY K 8644 on I_{CaT} and I_{CaL} characteristics. The effects of BAY K 8644 are shown (*dotted lines*) on reactivation curves for I_{CaT} (A: ▲, $n = 3$) and I_{CaL} (B: ●, $n = 4$). (C) Use-dependent effects of BAY K 8644 on I_{CaT} and I_{CaL}. In both control conditions and after the addition of BAY K 8644, stimulation was stopped and resumed after 1 min at a cycle length of 400 ms. Note that BAY K 8644 (1 μM, *closed symbols*) changed the staircase pattern of I_{CaL} (O, ●) from slightly positive to negative. The drug only slightly enhanced the negative staircase of I_{CaT} (△, ▲) after the increase in rate. HP $= -100$ mV and I_{CaT} and I_{CaL} were elicited at -50 and 0 mV, respectively, on a given cell.

(22–100% increase), it was seen in each of the seven cells studied. I_{CaL}, on the other hand, was increased at most by 20%, and in half of the cells this was only a transient effect. These effects were blocked by 0.1 μM prazosin in the three cells investigated. At 1 μM, ATP exerted a strong agonistic effect on I_{CaT}. This effect was seen in 12 of 17 cells. We already reported that ATP was not always active on I_{CaL} (Alvarez et al., 1990). As on I_{CaL}, the agonistic potency of ATP on I_{CaT} is limited to a very narrow range of concentrations; higher concentrations were less efficient or even reduced the current (Table II). No significant changes were detected in the kinetics of I_{CaT} or I_{CaL} under both α_1- and β-adrenergic or P_2-purinergic stimulations. The effects of the three neuromediators were rapidly reversible. Further results, obtained in the

TABLE II

Effects of α_1- and β-adrenergic and Purinergic Stimulations on I_{CaT} and I_{CaL}
in Frog Cardiac Cells

	Isoproterenol		Phenylephrine	ATP		
	0.1 μM	1 μM	10 μM	0.1 μM	1 μM	10 μM
I_{CaT}	133.0 ± 13.3‡ (5/8)	195.0 ± 27.5‡ (4/8)	148.2 ± 10.4* (7/7)	100 (0/4)	172.5 ± 26.3‡ (4/7)	121.5 ± 5.3‡ (3/4)
I_{CaT} (Cd)		218.0 ± 18.1* (11/12)	217.5 ± 31.1* (4/4)		222.8 ± 27.5* (8/10)	
I_{CaL}	463.6 ± 27.1* (14/14)	562.8 ± 23.2* (14/14)	120.1 ± 5.5‡ (9/9)	123.0 ± 6.9‡ (5/6)	140.0 ± 4.6‡ (5/6)	112.8 ± 3.7 (4/5)

Values are percent of control peak current amplitudes taking into account only cells that responded to the agonists (indicated in parenthesis). The experiments with phenylephrine were performed in the presence of 1 μM propranolol; the increase in I_{CaL} was transient in five of the nine cells and was estimated at peak. Currents were measured when depolarizing from a HP of -100 mV to -50 mV (I_{CaT}) or to 0 mV (I_{CaL}). In ventricular cells (eight with isoproterenol, two with phenylephrine, and two with ATP) HP was -80 mV. I_{CaT} (Cd) refers to Ca current recorded in the presence of 50 μM Cd in another series of experiments. Means ± SE. *$P \le 0.05$. ‡$P \le 0.05$.
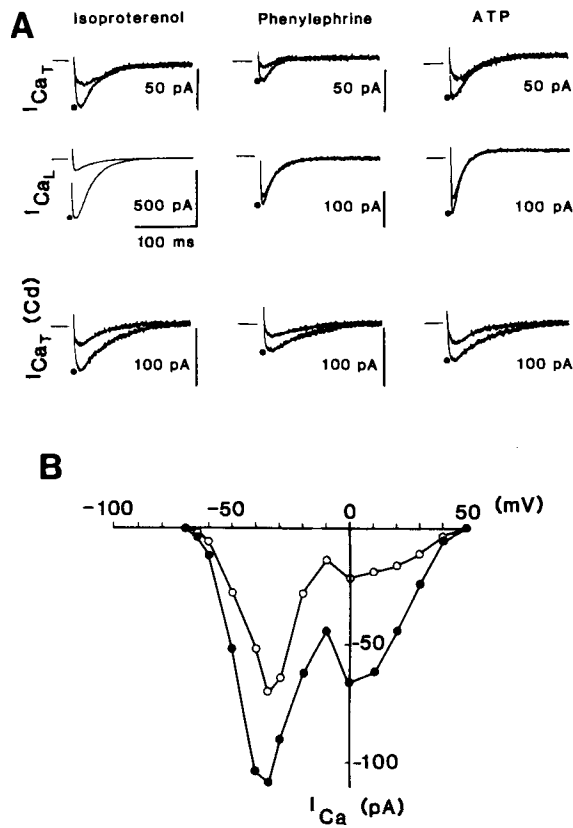
FIGURE 10. Modulation of I_{CaT} and I_{CaL} by neurotransmitters. (A) Effects of 1 μM isoproterenol and 10 μM phenylephrine in the presence of 1 μM propranolol and 1 μM ATP on I_{CaT} and I_{CaL} elicited by depolarizations to -50 or 0 mV from HP = -100 mV in control conditions (left) or on I_{CaT} in the presence of 50 μM Cd. Note that phenylephrine effect on I_{CaL} was maximal after 1 min (as shown here); I_{CaL} then recovered its control value within 2 min. Horizontal lines denote zero current level and filled circles indicate the presence of the neuromediators. (B) Current–voltage relationship established in control (○) and in the presence of 1 μM ATP (●) on an atrial cell that exhibited an unusually small I_{CaL}.

presence of Cd ions and reported in Fig. 10 A and Table II, also show that the amplitude of I_{CaT} could be modulated by neuromediators. Under this experimental condition essentially similar relative increases in I_{CaT} were observed with each of the three agonists. Fig. 10 B reports the effects of ATP obtained on one of the five atrial cells that exhibited an unusually small I_{CaL}. At each membrane depolarization both I_{CaT} and I_{CaL} were markedly increased by ATP.

The effects of β-adrenergic stimulation on I_{CaT} and I_{CaL} were further characterized in another experimental series by applying alternative depolarizations to −50 and 0 mV every 4 s from −100 mV HP. The results of such an experiment are shown on Fig. 11 A, which reports the relative increases in both currents after adding 1 μM
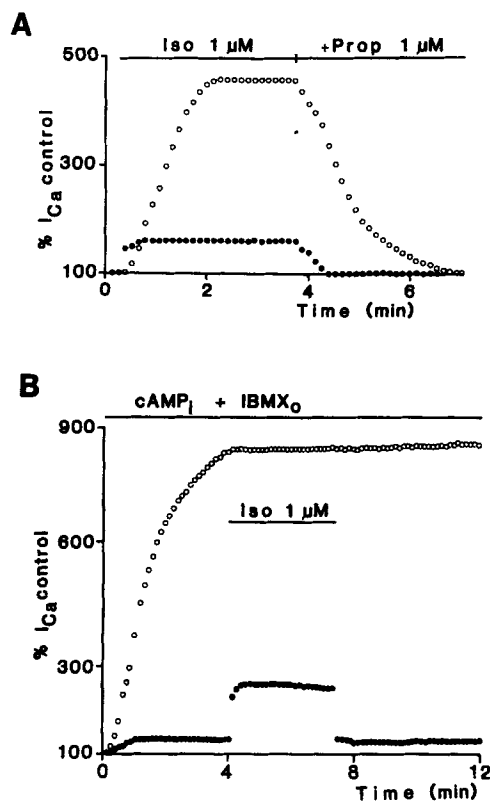

FIGURE 11. Effects of isoproterenol on I_{CaT} and I_{CaL}. (A) Relative increase in both I_{CaT} (●) and I_{CaL} (○) induced by the addition of 1 μM isoproterenol. The further addition of 1 μM propranolol antagonized these effects. I_{CaT} and I_{CaL} were 20 and 64 pA in control conditions at −50 and 0 mV, respectively. Cm = 58 pF. (B) Breaking the patch with a pipette containing 50 μM cAMP hardly increased I_{CaT} (●) as compared with the markedly enhanced I_{CaL} (○). The later addition of 1 μM isoproterenol did not affect I_{CaL} but increased I_{CaT}. The whole experiment was performed in the presence of 100 μM IBMX and 0.1 μM prazosin. I_{CaT} and I_{CaL} were 24 and 170 pA at −50 and 0 mV, respectively, just after breaking the patch (Cm = 100 pF).

isoproterenol. The isoproterenol-induced increase in I_{CaT} was much weaker and reached its maximum much faster than the I_{CaL} increase. Increases in both Ca currents were antagonized by 1 μM propranolol, with a much faster recovery of I_{CaT}. Prazosin was without effect. However, the β-adrenergic-induced increase in I_{CaT} was not related to a cAMP increase; evidence is provided in Fig. 11 B. When breaking the patch after a gigaseal was made with a pipette containing 50 μM cAMP, I_{CaL} increased markedly; there was also a small sustained increase in I_{CaT} which could be

TABLE III

Effects of Ouabain on I_{CaT} and I_{CaL} in Frog Cardiac Cells

	0.1 μM	1 μM (1 min)	1 μM (5 min)	10 μM
I_{CaT} ($n = 6$)	94.0 ± 8.3	127.3 ± 10.8	139.6 ± 14.9[‡]	82.5 ± 4.9[‡]
I_{CaL} ($n = 8$)	102.0 ± 2.0	137.6 ± 1.9*	88.8 ± 9.1	51.6 ± 2.1*

Values are percent of controls before the application of ouabain and are estimated from I_{CaT} and I_{CaL} elicited at −50 and 0 mV, respectively, from a HP of −100 mV but for three ventricular cells in which HP was −80 mV. Means ± SE. *$P \leq 0.01$. [‡]$P \leq 0.05$.

related to the usual "run up" of current during the stabilizing period. After I_{CaL} had reached its steady state, the further application of 1 μM isoproterenol (in the presence of 100 μM IBMX and 0.1 μM prazosin) had no effect on I_{CaL} but rapidly and significantly increased I_{CaT}. On wash-out of isoproterenol, I_{CaT} recovery was also very rapid. In this experimental series the isoproterenol-induced increases in I_{CaT} were 63.8 ± 10.0 and 62.8 ± 16.0% of I_{CaT} control, and their times to half-maximal amplitude were 13.5 ± 4.3 and 23.2 ± 5.3 s in control ($n = 8$) and cAMP-containing ($n = 5$) internal solutions, respectively. For comparison, on the same cells the increases in I_{CaL} were 405.6 ± 95.1 and 687.2 ± 120.6% of I_{CaL} control and times to half-maximal amplitude were 78.5 ± 11.1 and 66.0 ± 9.3 s on applying isoproterenol or after perfusing with cAMP, respectively.

Effects of Ouabain

It is well known that cardiac glycosides can, under some circumstances, increase (Marban and Tsien, 1982), decrease (Deslauriers, Ruiz-Ceretti, Schanne, and Payet, 1982), or exert a dual effect (Fischmeister, Brocas-Randolph, Lechene, Argibay, and Vassort, 1986) on I_{CaL}. The effects of ouabain on the two Ca currents in frog atrial cells are reported in Table III. At 1 μM, ouabain induced a sustained increase in I_{CaT} and exerted a biphasic effect on I_{CaL} as previously reported in frog ventricular cells (Fischmeister et al., 1986). Times of half-inactivation of both currents were significantly decreased with 1 and 10 μM ouabain, but without a clear relation to concentration. Time of I_{CaT} half-inactivation was reduced by 29.9 ± 9.5%, while that of I_{CaL} was reduced by 19.0 ± 5.3% ($n = 6$). In three of six cells the increase in I_{CaT} was preceded by oscillatory variations in amplitude (Fig. 12).
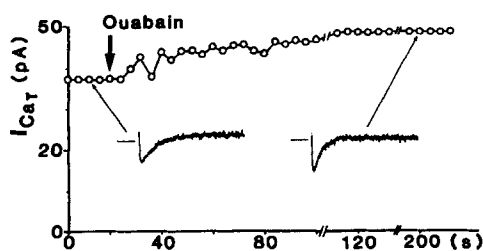

FIGURE 12. Effects of 1 μM ouabain on I_{CaT}. Note that after the beginning of ouabain perfusion, I_{CaT} showed transient variations in amplitude which stabilized within 1 min to a steady-state value greater than control. The inset shows current recordings. HP = −100 mV; I_{CaT} was evoked at −50 mV for 200 ms every 4 s.

DISCUSSION

The present results confirm that in bullfrog atrial, but not ventricular cells, two types of Ca currents, I_{CaT} and I_{CaL}, coexist, which can be recorded at physiological extracellular Ca concentrations. Following previous studies, several criteria were used to define the T-type Ca current. It activates and inactivates at relatively negative voltages, it is unchanged when substituting Ba for Ca; and it is partially inhibited by 50 μM Cd and blocked by 50 μM Ni but is insensitive to TTX. The possibility that the inward current recorded during depolarizations between −65 and −30 mV was a residual Na current was excluded because it appears unchanged when reducing Na to 30 mM and still exists when Ba is the only charge carrier, although within a different potential range as a result of surface charge screening. The most salient features of this work are the complex voltage dependence of I_{CaT} kinetics and the sensitivity of I_{CaT} to several pharmacological agents as well as to neuromediators.

Previous results obtained in cardiac cells isolated from the same (Bean, 1985a; Alvarez and Vassort, 1991) and another (Bonvallet, 1987; Bonvallet and Rougier, 1989) species of the same genus already suggested the existence of two Ca currents. On the other hand, Campbell et al. (1988a , b) found no evidence of the existence of I_{CaT} in the bullfrog atrial cells. As pointed out by Bonvallet and Rougier (1989), this could be due to species specificity or to the use of different recording techniques (dialyzed vs. nondialyzed cells). To these proposals we can add another one: the lack of recording I_{CaT} in bullfrog atrial cells might be related to the enzymatic dissociation procedure. Indeed, when we used pronase E, only 30% of atrial cells showed I_{CaT}, compared with 91% when collagenase and trypsin were used. A possible relationship between I_{CaT} and Ca paradox phenomenon might, a priori, be ruled out since the dissociation period in the low-Ca medium was 20–25 min when pronase was used, and 45 min with collagenase-trypsin. It has long been known that proteases affect ionic channels (see review by Narahashi, 1974). Thus, it has been shown that neuraminidase selectively enhances the transient Ca current in guinea pig myocytes (Yee, Weiss, and Langer, 1989). In squid giant axon, pronase specifically destroys the Na channel inactivation mechanism (Rojas and Armstrong, 1971). A pronase-derived alkaline proteinase b modifies the voltage dependence of the K channel from sarcoplasmic reticulum (Miller and Rosenberg, 1979). It has been recently shown in GH_3 cells that a protease-sensitive site within the Ca channel proteins must be phosphorylated to open the channel (Armstrong and Eckert, 1987). Finally, proteolytic enzymes such as trypsin and pronase markedly reduced the binding sites to indolizine sulfone, a new class of Ca antagonist (Chatelain, Beaufort, Meymans, and Clinet, 1991). It is then possible that pronase, in our case, or the rather long periods of enzymatic dissociation (up to 2.5 h) needed by Campbell et al. (1988a, b; see also Hume and Giles, 1981) might induce cellular changes leading to the disappearance of T-type channels. As for pronase, we cannot discard the possibility that collagenase and trypsin exert deleterious actions on Ca channels; however, 91% of atrial cells showed I_{CaT} when these enzymes were used. The small percent of atrial cells lacking I_{CaT} in these dissociations could also be related to the fact that these cells probably have their origins near the atrio-ventricular ring and are more "ventricular-like,"

having only I_{CaL} (Bonvallet and Rougier, 1989). As shown by Argibay et al. (1988) and the present results, frog ventricular cells do not exhibit I_{CaT}.

It is not easy to isolate I_{CaT} from I_{CaL} at every membrane potential. As already reported by others (e.g., Bean, 1989) Cd and Ni ions are far from being specific inhibitors of I_{CaL} and I_{CaT}, respectively; moreover, Cd block was shown to be weaker at more negative test potentials (Lansman, Hess, and Tsien, 1986). The difference I-V relationship method (at two HPs) is also not fully reliable since in many cells a HP of -50 mV (or even -60 mV) inactivates a substantial fraction of the inward current flowing at potentials positive to -20 mV (see also Schouten and Morad, 1989). Nevertheless, a reasonable picture can be formed on the basis of combining the results obtained with Cd, Ni, and the difference current method. In bullfrog atrial cells at physiological Ca concentrations, I_{CaT} and I_{CaL} activate at around -65 and -30 mV, respectively. Maximal I_{CaT} occurs around -35 mV and maximal I_{CaL} at 0 to $+10$ mV. The reversal potential for I_{CaL} is estimated to be near to $+50$ mV and that for I_{CaT} between $+20$ and $+30$ mV. These values are very similar to those reported by others in frog atrial and ventricular cells (Argibay et al., 1988; Campbell et al., 1988b; Bonvallet and Rougier, 1989; Alvarez and Vassort, 1991). I_{CaL} inactivates between -40 and 0 mV, with a potential for half-inactivation around -30 mV. At positive prepulse potentials, a facilitation of I_{CaL} occurs which was attributed to Ca-dependent Ca inactivation (Mentrard et al., 1984; Lee et al., 1985; Argibay et al., 1988). I_{CaT}, on the other hand, inactivates in the potential range from -80 to -50 mV, with a potential for half-inactivation around -65 mV; it also has a steeper voltage dependence than I_{CaL}. These observations are in full agreement with those of Droogmans and Nilius (1989) using single channel analysis. Ba substitution of Ca ions did not affect the inactivation time course of I_{CaT} and did not increase its amplitude. The voltage dependence of I_{CaT} availability remained unchanged in the presence of Ba. Reactivation kinetics (recovery from inactivation) of I_{CaT} was much slower and never showed an overshoot, as was commonly seen for I_{CaL}. These results indicate that inactivation of I_{CaT} is only voltage dependent as previously reported by others (Bean, 1985b; Bonvallet and Rougier, 1989; Hirano et al., 1989b).

Altogether the above reported characteristics of I_{CaT} confirm and extend previous results. T-type current amplitude and density in our cells were comparable to those reported by others in frog atrial cells (e.g., Bonvallet, 1987; Bonvallet and Rougier, 1989). Although variable, the ratio of I_{CaT} at -50 mV to I_{CaL} at 0 mV was 1:5 as an average, a value that was similar to that reported by Tseng and Boyden (1989) for canine ventricular myocytes. Peak I_{CaT} at -30 mV could be as large as half-peak I_{CaL}. On the other hand, a few cells exhibited very large I_{CaT} relative to I_{CaL}. They could be compared to embryonic cells (Kawano and de Haan, 1989) or to the sinus venosus cells (Bois and Lenfant, 1991), although they were not spontaneously beating. In most aspects our results obtained on I_{CaT} are similar to those reported in rabbit sinoatrial node cells by Hagiwara et al. (1988), in canine Purkinje cardiac cells by Hirano et al. (1989a), and in frog cardiac sinus venosus cells by Bois and Lenfant (1991). As indicated above, they documented that I_{CaT} kinetics were insensitive to Ca ions but depended on voltage (Bean, 1985b; Hirano et al., 1989b). However, our results show a specific characteristic of I_{CaT}: namely, that high predepolarizing steps did not fully inactivate I_{CaT} so that the availability curves bent up and the recoveries

from inactivation were biphasic. This remaining activation could be attributed to an additional, long-lasting open state induced by high depolarizations as early described for the Na current in nerve cells (Bezanilla and Armstrong, 1977) and recently reported for the cardiac L-type Ca channel (Pietrobon and Hess, 1990). Under their experimental conditions, the L-type current carried by Ba ions decayed exponentially with a time constant of ~ 100 ms. In our experiments (Fig. 6 C), after high depolarizations peak I_{CaT} decayed with increasing test pulse intervals; the time constant of decays was markedly voltage dependent, varying from a few milliseconds at -120 mV to 29 ms at -70 mV; it was not significantly changed in the presence of Ba at low or high concentrations, or in the presence of Cd ions. The activation that remains after a high depolarization might have a second origin. In a recent publication (Slesinger and Lansman, 1991), neuronal L-type Ca channels were shown to reopen upon return to resting potential, which suggests that some channels recover from inactivation by passing through an open state. Extending this complementary hypothesis, it can be proposed that high depolarizations allow both cardiac T- and L-type Ca channels to reopen from inactivation. Reopenings would not occur below -30 and $+10$ mV for T- and L-type channels, respectively; however, above these values reopenings would increase with increasing membrane depolarizations. Such reopenings also depend on the duration of the prepulse as particularly illustrated by the reduction in I_{CaT} inactivation which occurred during predepolarization to $+70$ mV lasting >50 ms (Fig. 7). In several aspects, including independence of ion carrier species and insensitivity to dihydropyridine Ca channel agonists, these results are comparable to the recently described double-pulse Ca^{2+} current facilitation in rat sympathetic neurons (Ikeda, 1991). This phenomenon thus represents a new characteristic of Ca currents.

A second specific observation relative to current kinetics and which also requires single-channel analysis is that Cd ions increased I_{CaT} time to peak and slowed its inactivation time course. The best available interpretation would be that Cd ions increased the first latency of openings of the T-type channel which has been proposed to control inactivation time course of the macroscopic T-type current (Droogmans and Nilius, 1989). Such a hypothesis would also account for the larger increase in peak current by BAY K 8644 in the presence of Cd, assuming that dihydropyridine antagonized the effects of Cd as suggested by the recovery of I_{CaT} inactivation kinetics under these conditions.

Another salient feature of this work is the finding that I_{CaT} was found to be more sensitive to dihydropyridines and neurotransmitters than previously reported (see, for example, Bean, 1985b; Nilius et al., 1985; Hagiwara et al., 1988; Bonvallet and Rougier, 1989; Hirano et al., 1989a; Tytgat et al., 1988; but see Tseng and Boyden, 1988, 1989, 1991), although a marked inhibition to 20% of I_{CaT} control by 1 μM niguldipine was recently communicated (Romanin, Seydl, Glossmann, and Schindler, 1992). A decrease in both currents (18% for I_{CaT} and 49% for I_{CaL}) is recorded with 1 μM nifedipine under our experimental conditions. However, as previously reported for the effects of nisoldipine on canine Purkinje cells (Tseng and Boyden, 1989), the steady-state inactivation of I_{CaT} was barely affected by nifedipine, while that of I_{CaL} was displaced by 10 mV toward more negative potentials. This suggests different mechanisms of action of this dihydropyridine on the two Ca currents. The same is

true with the dihydropyridine agonist BAY K 8644, which induced a 10-mV shift of I_{CaL} kinetics and increased or decreased I_{CaL} according to the holding membrane potential (Sanguinetti et al., 1986; Kass, 1987). Such an effect was not observed on I_{CaT}. The absence of shifts on the $I-V$ and availability curves and the fact that the increase in I_{CaT} was unchanged by varying HP between −100 and −80 mV suggest that the action of BAY K 8644 on I_{CaT} is not voltage dependent. Moreover, the fact that the I_{CaT}–availability curve was not shifted after addition of BAY K 8644, as with the inhibitory dihydropyridines, argues against the view that under such a condition the increase in I_{CaT} is artefactually related to I_{CaL} increase, since in such a case the I_{CaT}–availability curve would be shifted close to the I_{CaL}–availability curve. Our results reinforce the observations of Tseng and Boyden (1989) mentioned above, and of Mitra and Morad (1986), who noted that at high concentrations Ca agonists and antagonists also interact with the low threshold Ca current. Furthermore, Akaike et al. (1989) have shown that the low threshold Ca current in hypothalamic neurons is extremely sensitive to dihydropyridines and other organic blockers. They found, however, only a blocking effect of BAY K 8644 (at high concentrations, 10 μM) as already reported for I_{CaL} in cardiac cells (Bechem and Schramm, 1987). Finally, in a recent report (Wu, Wang, Karpinski, and Pang, 1992) it was shown that in neuroblastoma cells the effects of BAY K 8644 depend on the solvent; a 50% decrease of the T-type Ca current was observed in DMSO but not in ethanol with no shift of the availability curves in either case.

More surprising results were the specific responses of I_{CaT} and I_{CaL} to the neuromediators. As previously reported in frog cells, extracellular ATP increased I_{CaL} (Alvarez et al., 1990). The present results show that ATP also enhanced I_{CaT}. The effects were marked but not observed in every cell; they were obtained in a very narrow range of concentration. I_{CaT} was more sensitive to α_1-adrenergic stimulation, while I_{CaL} was more sensitive to β-adrenergic stimulation. Such a small increase in I_{CaL} after α_1-adrenergic stimulation has been previously reported in frog ventricular cells (Alvarez et al., 1987). As well, an increase in I_{CaT} by α_1 stimulation has been found in canine ventricular and Purkinje cells (Tseng and Boyden, 1988, 1989) and in smooth muscle cells of rat portal vein (Pacaud, Loirand, Mironneau, and Mironneau, 1987). Regarding the increase of I_{CaT} by β-adrenergic stimulation, our results are in agreement with those of Tseng and Boyden (1988) on canine ventricular myocytes and of Mitra and Morad (1986) in guinea pig ventricular cells, but differ from other reports on various cell types (Bean, 1985b; Tytgat et al., 1988; Bonvallet and Rougier, 1989). It should be noted that under our experimental conditions not only was I_{CaT} less sensitive to β stimulation than I_{CaL}, but the effects of β stimulation on I_{CaT} as well as those of the other neuromediators were not seen in every cell studied. These variations may arise from species and/or tissue specificity as well as from alterations in the state of the membrane following dissociation procedure. As we discussed above, T channels in frog atrial cells can be affected by the enzymatic dissociation to a larger extent than I_{CaL}. Whatever the case, with each of the three agonists the observed increases in I_{CaT} should not be artefacts related to increases in I_{CaL}: first, a depolarizing pulse to −50 mV was used to activate this current, well below I_{CaL} threshold, and second, similar relative increases were also observed after inhibition of I_{CaL} by Cd ions. The exact mechanisms of action of the

neuromediators on I_{CaT} require further investigation. Evidence exists in canine Purkinje cells that I_{CaT} can be transiently increased by raising intracellular Ca concentration (Tseng and Boyden, 1991). Such a mechanism might account for the weak increase in I_{CaT} seen in some experiments after adding intracellular cAMP (Fig. 11 B). However, it could hardly be involved in most aspects of this study on the positive effects of neuromediators as well as on the partial inactivation after high depolarizing prepulses, since very similar effects were observed when I_{CaL}, the major origin of intracellular Ca load, was inhibited by Cd (see Figs. 6 B, 8 D, and 10 A) or when I_{CaL} had been markedly increased by cAMP (Fig. 11 B). According to the rather fast changes in peak current, intracellular messengers might not be involved, but direct coupling by G proteins should be considered (Brown and Birnbaumer, 1988) as well as membrane perturbation similar to that obtained with alcohols or anesthetics (Mongo and Vassort, 1990).

Our experiments with ouabain confirm those of Fischmeister et al. (1986) on frog (*R. esculenta*) single ventricular cells and of Legrand, Deroubaix, Coulombe, and Coraboeuf (1990) on guinea pig ventricular myocytes. They indicate transient effects of cardiac glycosides on I_{CaL} while the increase in I_{CaT} was sustained. As postulated by Fischmeister et al. (1986), the inhibition of I_{CaL} by cardiac glycosides could be caused by an increase in free intracellular Ca (consecutive to Na-K pump inhibition) leading to a decrease in availability of I_{CaL}. The difference in behavior of I_{CaT} under the action of ouabain may then be related to the fact that I_{CaT} does not exhibit a Ca-dependent inactivation mechanism.

Physiological Consequences

The physiological role of I_{CaT} in heart cells remains uncertain. However, it might have major electrophysiological implications. From a mathematical simulation model, Nilius (1986) suggested that I_{CaT} plays an essential role in the pacemaker potential. I_{CaT} activation is considered to be responsible for the last part of the diastolic depolarization (Hagiwara et al., 1988). It has also been suggested that I_{CaT} participates in certain arrhythmias related to reentry due to slowly propagating action potential (Carmeliet, 1988) or to delayed after-depolarizations (Hirano et al., 1989a).

It is well known that ouabain and other cardiac glycosides may induce early and delayed after-depolarizations (Cranefield, 1975). Although the cellular basis for their initiation is not yet well established (for review, see January and Fozzard, 1988), we may suggest that ouabain-induced changes in I_{CaL} and I_{CaT} could, in part, contribute to the development of early and delayed after-depolarizations, respectively. It has been similarly shown that I_{CaL} plays a role in the early after-depolarizations induced by BAY K 8644 (January and Riddle, 1989).

α_1-Adrenergic stimulation whose responsivity is increased during myocardial ischemia and reperfusion contributes to the development of malignant ventricular arrhythmias. Receptor blockade has been shown to be effective in restoring sinus rhythm after such arrhythmias have been experimentally induced (Sheridan, Penkoske, Sobel, and Corr, 1980; Davey, 1986). Such arrhythmias may be consequent to an inward current activated by increased cytoplasmic calcium; the latter could be triggered following the activation of the phosphoinositol turnover by α_1-adrenergic

agonists. Arrhythmias will be facilitated by the fact that these agonists enhanced the T-type calcium current as described above. Furthermore, since P_2-purinergic agonists also increase phosphoinositol turnover (Legssyer, Poggioli, Renard, and Vassort, 1988) as well as facilitate I_{CaT}, it should be anticipated that P_2-purinergic stimulation could also lead to arrhythmia.

In conclusion, our study reports the existence of T-type Ca current on frog atrial cells. This current differs from the L-type Ca current by the voltage dependence of its kinetics, by the fact that Ba does not alter its kinetics, and by its lower sensitivity to Cd. Both currents are sensitive to pharmacological agents and neuromediators, but each with their own characteristics. In a few cells, T-type Ca current was larger than the L-type, but generally its maximal amplitude was about half; nevertheless, this suggests that it might have a major influence on electrical cell activity. Some atrial cells did not exhibit T-type Ca current under whole-cell patch-clamp conditions; in most cases this could be attributed to the enzymatic treatment during the dissociation procedure.

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