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Analysis of the T-Type Calcium Channel in Embryonic Chick Ventricular Myocytes

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Summary. T-type calcium channels (I_T channels) were studied in cell-attached patch electrode recordings from the ventricular cell membrane of 14-day embryonic chick heart. All experiments were performed in the absence of Ca^{2+} with Na^+ (120 mM) as the charge carrier. I_T channels were distinguished from L-type calcium channels (I_L) by their more negative activation and inactivation potential ranges; their smaller unitary slope conductance (26 pS), and their insensitivity to isoproterenol or D600. Inactivation kinetics were voltage dependent. The time constant of inactivation was 37 msec when the membrane potential was depolarized 40 mV from rest (R + 40 mV), and 20 msec at R + 60 mV. The frequency histogram of channel open times (τ_o) was fit by a single-exponential curve while that of closed times (τ_c) was bi-exponential. τ_o was the same at R + 40 mV and R + 60 mV whereas τ_c was shortened at R + 60 mV. The open-state probability (P_o) increased with depolarization: 0.35 at R + 40 mV, 0.8 at R + 60 mV and 0.88 at R + 80 mV. This increase in P_o at depolarized potentials could be accounted for by the decrease in τ_c.

Key Words T-type calcium current · embryonic heart · single-channel recording · Na^+ through Ca^{2+} channel

Introduction

Three types of calcium channel, termed T-, N- and L-type, have been described in neuronal cells [14, 30]; two of these, T and L, have been identified by differences in their activation range and pharmacology in heart and other tissues (*see* refs. [1, 3, 34, 38] for reviews). In all adult cardiac preparations, the low-threshold, Ni^{2+}-sensitive, T-type current (I_T) has a lower current density than dihydropyridine-sensitive, L-type current (I_L), and makes only a minor contribution to total calcium current. In embryonic chick ventricle cells, by contrast, we found that I_T is roughly twice as large as I_L [22, 23]. Here we investigate the activation and inactivation kinetics and the conductance of the chick cardiac I_T channel, measured with cell-attached patch electrodes.

With physiological ionic concentrations, unitary calcium channel activity is difficult to record with on-cell patch electrodes because the channels have low conductance. With high concentrations (90–110 mM) of Ba^{2+} as the charge carrier, I_L and I_T channels have conductances of 16–25 pS and about 8 pS, respectively, in adult cells [3, 4, 14, 17, 29, 30, 33, 37]. When extracellular free Ca^{2+} is reduced to below about 10^{-6} M, both I_L and I_T channels become permeable to monovalent cations [16, 38] in both adult mammalian [18, 25] and 7-day embryonic chick [24, 26] ventricle cells. In this condition the conductance of I_L channels was 50–100 pS, i.e., 3–4 times greater than with Ba^{2+} as the charge carrier, and was sensitive to extracellular pH [31]. In the present study, we have focused on the properties of I_T channels in isolated ventricular cells of the 14-day chick embryonic heart. This is the first study in which cardiac I_T channel conductance and other parameters have been measured using Na^+ as the charge carrier and an alkaline pH to enhance channel conductance.

Materials and Methods

Cell Culture

White Leghorn chicken eggs were incubated for 14 days at 37.5°C. Single ventricular myocytes were dissociated from the trimmed apical ventricle and allowed to attach to the sulfonated surface of culture plates as described previously [12, 15, 22]. Isolated near-spherical cells with diameters of 13–18 μm were selected for experiments.

Solutions

Standard bath solution contained (in mM): KCl 2.5, NaCl 142, NaH_2PO_4 0.9, $CaCl_2$ 1.8, N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 10, dextrose 5, $MgSO_4$ 2.1, pH 7.4

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(adjusted with NaOH). The pipette solution contained (in mM): NaCl 120, ethyleneglycol-*bis*-(B-aminoethyl ether)N,N,N′-tetraacetic acid (EGTA) 10, Tris 10, tetraethylammonium (TEA) 20, tetrodotoxin (TTX) 0.0003, pH 8.3 (adjusted with NaOH). We chose these conditions to optimize the recording of Na current through calcium channels [25, 31, 32, 38]. The TTX was added to the bath and pipette solution to block Na-channels [15]; TEA served to block K^+ channels [19]; and the patch was exposed to a high pH to increase channel conductance [31].

Recording Methods

Calcium channel activity was recorded with cell-attached patch electrodes drawn from hard borosilicate glass capillaries (Corning 7052). Electrodes were coated with Sylgard (Dow Corning) and had resistances of 1.5–4 MΩ. Currents were recorded at room temperature (22 ± 1°C), low-pass filtered at 3 kHz, amplified by a List EPC-7, digitized with a sampling interval of 81 μsec and recorded on VCR tape through a Neurocorder (model DR-484). Signals were refiltered at 1 kHz (low-pass) and analyzed on an IBM PC-AT. For single-channel analysis, the threshold used to judge channel open state was set at half the unit current amplitude [11]. In separate experiments on nine 14-day cells with an electrode containing 120 mM KCl low-Ca^{2+} intracellular solution in whole-cell patch-clamp configuration, the zero-current potential (resting potential, R) recorded within 15 sec after rupture of the membrane patch was −92.2 ± 5.1 mV [35], which corresponded well with the resting potential of −90.9 ± 2.7 mV measured with high-resistance microelectrodes in electrically quiescent 14-day cell clusters [40]. In the present experiments, the patch potential (V_{patch}) was calculated as the difference between the resting potential (R) and the potential applied to the pipette ($V_{patch} = R - V_{pip}$). To elicit I_T channel activity, depolarizing voltage steps of 400 msec duration from a holding potential −20 mV from rest ($V_{patch} = R - 20$ mV ≈ −110 mV) to $R + 60$, $R + 70$, . . . $R + 140$ mV ($V_{patch} \approx -30$ to +50 mV) were applied to the patch electrode at 5-sec intervals. We found that steps to potentials more positive than $R + 140$ mV often damaged the cell or seal. I_L was activated from $R + 50$ mV ($V_{patch} \approx -40$ mV). Ensemble averaged currents were obtained by averaging 30–80 individual records from each patch at each potential. Capacitive and leakage currents were estimated from the average of 5–20 "blank" current responses having no openings. This average was subtracted from each current response before the responses were averaged. To obtain open-time and closed-time histograms of I_T, records were collected from five patches during the initial 50 msec of the test pulse.

The present study includes recordings from a total of 41 patches, 16 of which showed no channel activity. I_T channels were active in all of the remaining 25 patches (61%). I_L channel activity was rare, occurring in only three patches (7%).

Results

Distinguishing I_T from I_L

We found previously that the activation thresholds of I_T and I_L currents, measured in 14-day cells with whole-cell clamp technique were, respectively, −34 and −7 mV. These recordings were made with 1.8 mM Ca^{2+} in the bath [22, 23]. The steady-state inactivation curves had half-inactivation potentials of −60 mV for I_T and −18 mV for I_L. I_T was fully inactivated at −40 mV [23]. With Ca^{2+} exchanged for Na^+ at the external surface in the present experiments, we would expect the inactivation and activation curves for both I_T and I_L to shift in a negative direction [20]. Therefore, application of depolarizing pulses that brought V_{patch} into the range of −30 to +50 mV (≈R + 60 to R + 140 mV) from a holding patch potential near −40 mV (R + 50) would be expected to activate I_L channels but not I_T. Depolarizing pulses from a holding patch potential of −110 mV to V_{patch} = −60 to −30 mV would be expected to elicit predominantly I_T channel activity. From negative holding potentials, depolarizing pulses positive to about 60 mV from rest (R + 60) should activate both I_T and I_L channels.

In most of the successful recordings from chick ventricle cells, only a single channel type was seen, that decayed rapidly after the onset of a depolarizing pulse. However, in three favorable patches, two distinct types of channel behavior were apparent. Currents from the two channel types in a single patch are shown in Fig. 1*A–C*, activated from different holding potentials. With V_{pip} = 20 mV, V_{patch} was held at approximately −110 mV for 5 sec before stepping to V_{pip} = −40 mV ($V_{patch} = R + 40 \approx -50$ mV) to activate a channel with the features of I_T (Fig. 1*A*). As expected for a current that inactivates rapidly, channel activity is bunched near the beginning of the pulse and the channels have relatively long open dwell times. The number of channel openings declines markedly after about 200 msec. From the same holding potential, larger steps to V_{pip} = −80 mV ($V_{patch} = R + 80 \approx -10$ mV) activate an initial burst of the rapidly inactivating channels, followed in most cases by a flickering channel with shorter open dwell times, that remains active during the entire pulse (Fig. 1*B*). From R + 50 mV, steps to R + 80 mV elicit only the flickering channel that continues activity throughout the pulse (Fig. 1*C*). We interpret these records as showing that both I_T and I_L channels are activated at $V_{patch} \approx -10$ mV, when pulsed from a holding potential near −110 mV. Note that at the same test potential (R + 80 mV), the amplitude of I_T currents is smaller than that of I_L.

Conductance of the I_T Channel

In one of the patches in which both channels could be recorded, the amplitude of the unitary inward Na current through the two channel types decreased with depolarization (Fig. 2*A*). Amplitude histo-

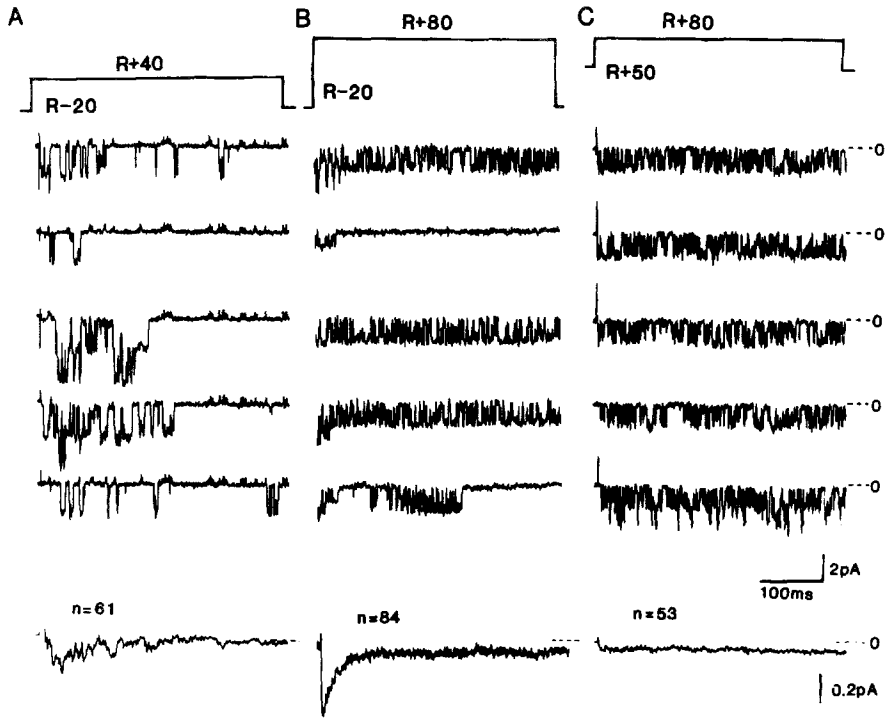

Fig. 1. Single-channel currents recorded with 120 mM Na^+ as charge carrier. Panels *A–C* show current records obtained from a single patch with three different stimulus protocols, as shown at the top of each panel; *R* is the resting potential, approximately −90 mV. The bottom trace in each column is an ensemble average of *n* records. (*A*) Channels open at a test potential *R* + 40 mV from a holding potential of *R* − 20 mV. The ensemble average current decays completely within 150 msec and shows no long-lasting component. These features are characteristic of the low-threshold channel (I_T) (*B*) At *R* + 80 mV 2–3 I_T channels open and close during the first 50–80 msec of each voltage pulse. The ensemble average trace shows the large initial component that decays rapidly to reveal a smaller current that lasts the duration of the pulse. (*C*) Channel activity in the high-threshold range was evoked at *R* + 80 mV test potential from a holding potential of *R* + 50 mV. The ensemble average shows that channel activity continues at a steady level throughout the length of the test pulse, as expected of I_L (patch #0013)

grams of I_T prepared from 46–84 pulses at each test potential (Fig. 2*B*) showed that the decrease in size of the channel openings was linear with depolarization (Fig. 2*C*). Moreover, steps from a negative holding potential (*R* − 20 mV, left panel, Fig. 2*A*) elicited smaller unitary currents with longer open dwell times than those from a more positive level (*R* + 50 mV, right panel, Fig. 2*A*). The slope conductances of the two channel types in the cell shown were 29 pS for I_T and 48 pS for I_L (Fig. 2*C*). The slope conductance of the I_T channels in seven additional patches (with no I_L activity) was 26 ± 5 pS (mean ± SD, *n* = 8).

I_T Channel Activity is not Blocked by D600

With chick ventricle cells in whole-cell clamp configuration, I_L was blocked by micromolar doses of D600 or nifedipine, while these agents had little or no effect on I_T [22, 23]. To confirm that the channels here defined as I_T in cell-attached patches were insensitive to I_L-channel blockers, 1 μM D600 was added to both the bath and pipette solution. In this condition, channel activity was readily stimulated from *R* − 20 mV to test potentials of *R* + 40 or *R* + 60 mV (Fig. 3*A* and *B*), and the ensemble averaged records had properties that corresponded to I_T current. In this same patch, steps from *R* + 50 to test potentials designed to elicit I_L channel activity were all blank (Fig. 3*C*).

Isoproterenol Does Not Enhance I_T Channel Activity

I_L channels are modulated by β-adrenergic intervention through cyclic AMP-dependent phosphorylation [7, 8, 21, 36]. However, the sensitivity of cardiac I_T channels to β-adrenergic stimulation is still controversial [2, 17, 27, 39]. Figure 4*A* shows channel activity evoked by steps from *R* − 20 mV to a test potential of *R* + 40 mV, before and after application 40 nM isoproterenol. In both conditions,

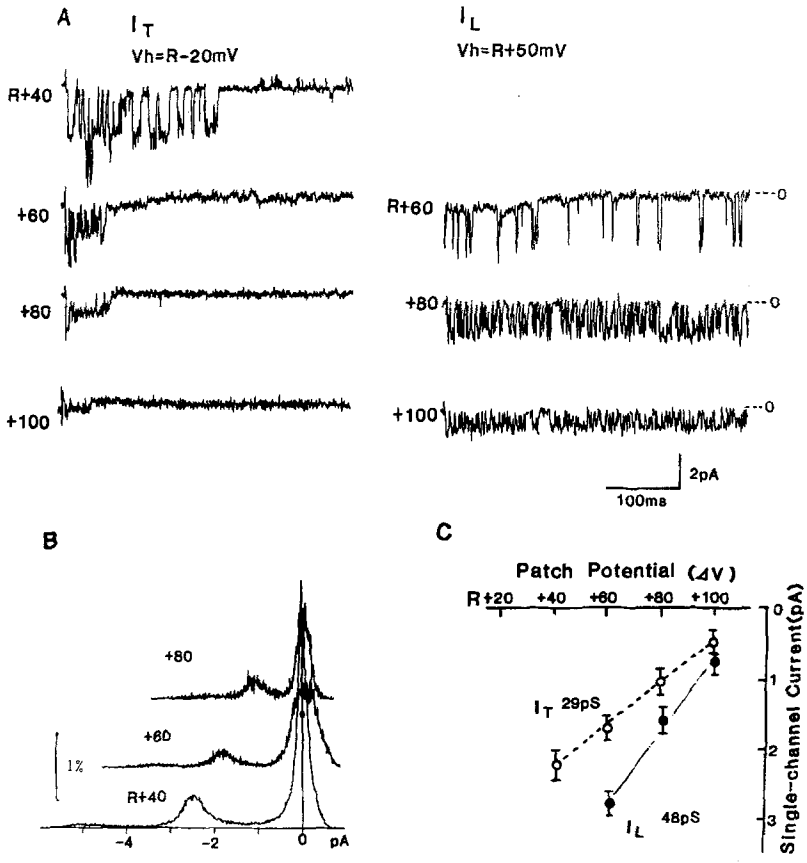

Fig. 2. Voltage dependence of I_T and I_L channels in the same patch. (*A*) left panel: I_T currents activated at four test potentials (40, 60, 80, and 100 mV depolarized from rest) from a holding patch potential of $R - 20$ mV. Right panel: I_L channel activity recorded from a holding potential of $R + 50$ mV. (*B*) Amplitude histograms of the I_T currents at $R + 40$, $R + 60$, and $R + 80$ mV. (*C*) Voltage dependence of the unitary currents, i/V, in this patch (#0013). Slope conductance of I_T is 29 pS (open symbols ± SD; from 64–84 traces), that of I_L is 48 pS (filled symbols; from 46–73 traces)

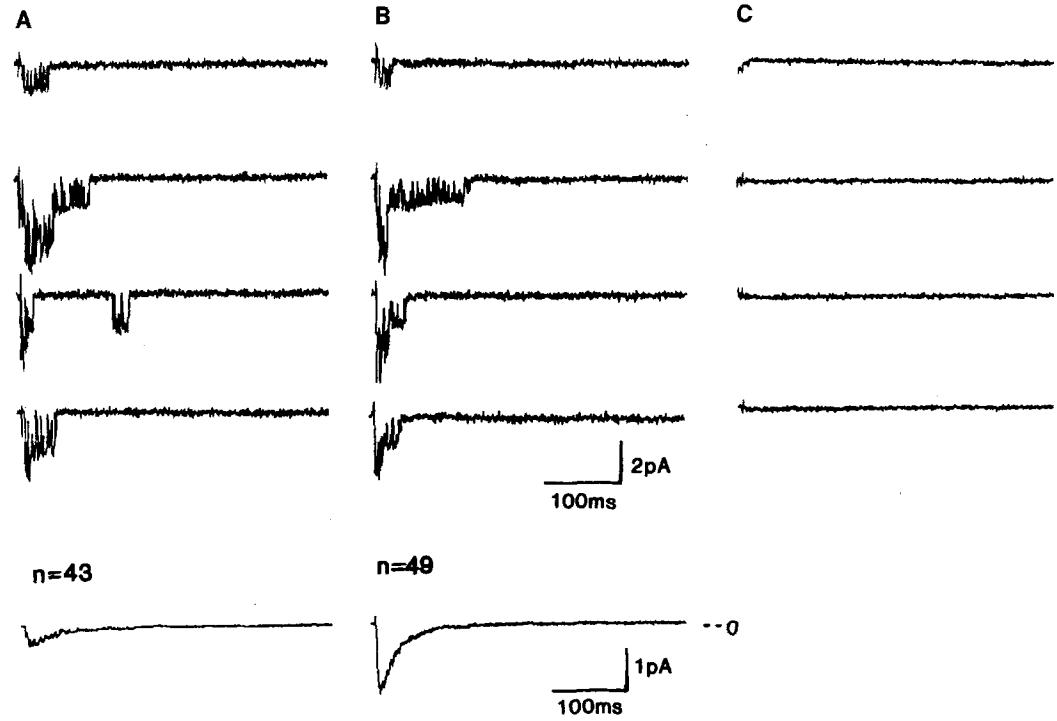

Fig. 3. Channel activity from a single patch in the presence of D600, in response to three different voltage protocols. (*A*) Holding potential $R - 20$ to test potential $R + 40$. (*B*) Holding potential $R - 20$ to test potential $R + 60$. Capacitive and leak currents averaged from 10 blank traces have been subtracted from each record. The bottom traces are ensemble averages of n records. (*C*) Holding potential $R + 50$ to test potential $R + 80$ elicits only blank records. D600 (1 μM) was present in both the pipette and the bath solution. The unitary current amplitude is 1.4 pA in *A*, 1.0 pA in *B* (patch #0011)

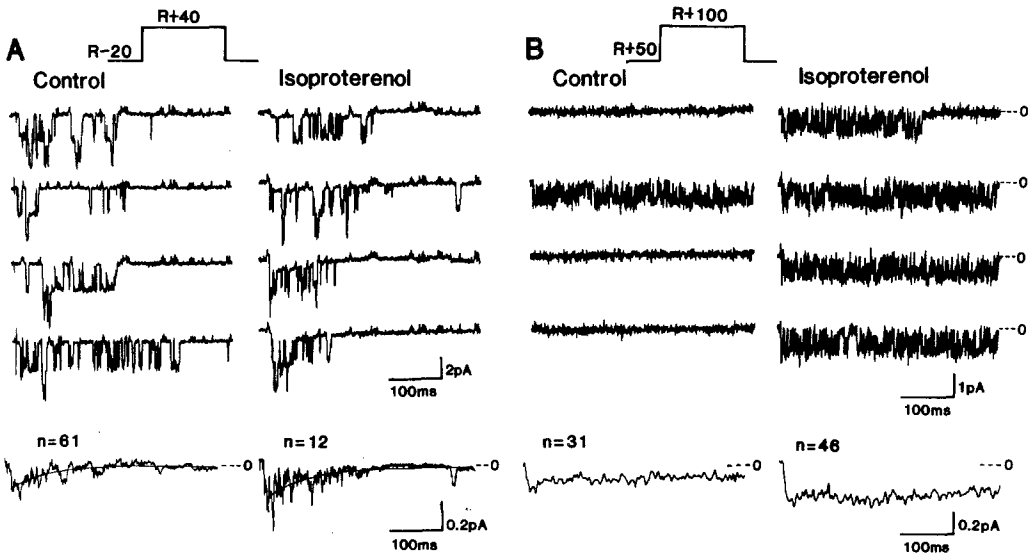

Fig. 4. Differential effect of isoproterenol on I_T and I_L in the same patch. I_T (*A*) and I_L (*B*) before (left) and after (right) 40 nM isoproterenol was added to the bath are shown. Four consecutive current records are shown, evoked by depolarizing voltage pulses as defined at the top of each panel. Ensemble averages are shown below. Bath application of isoproterenol increased the long-lasting I_L channel activity by 2.2-fold (*B*) as judged by integrating the area under the two curves, but increased I_T by only 16% (*A*). The averaged I_T currents were fit by single-exponential curves with peaks of −0.25 and −0.29 pA, and time constants of 14.1 and 14.3 msec, respectively, before and after the drug (patch #0013)

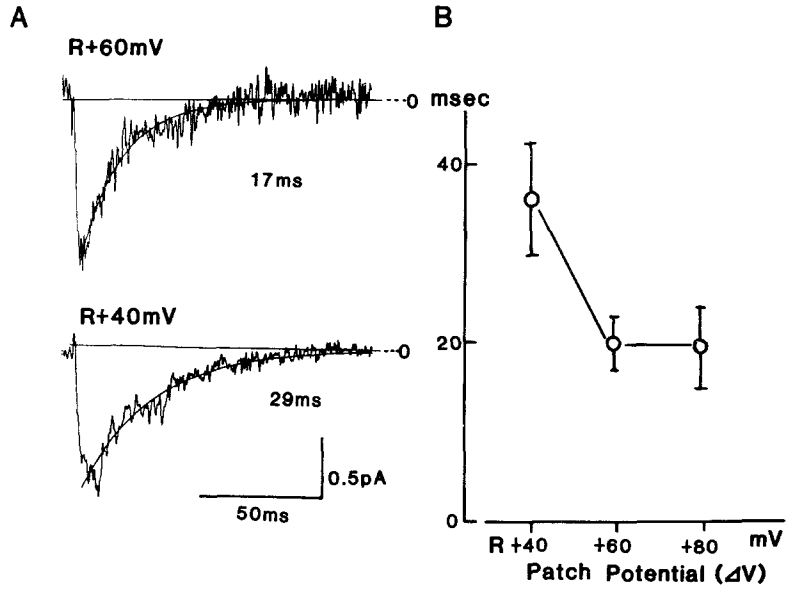

Fig. 5. Inactivation of the I_T channel. (*A*) I_T current recorded from a single patch at two different test potentials (patch #009). The traces shown at R + 60 and R + 40 mV are ensemble averages of 40 and 39 records, respectively. These averaged currents were fitted with single exponentials with time constants of 17 msec (R + 60 mV) and 29 msec (R + 40 mV). (*B*) Potential dependence of inactivation. Average inactivation time constants from pooled records are plotted at $V_{patch} \approx R$ + 40 mV (six patches), R + 60 mV (eight patches), and R + 80 mV (three patches). Each point represents the mean of approximately 200–400 individual pulse response records

channel activity ceases before the end of the 400-msec test pulse, and the openings have long dwell times characteristic of I_T at this potential. Isoproterenol had little effect. The averaged records with and without the drug could be fit with single-exponential curves with similar peaks and time constants. Furthermore, the ratio of I_T amplitude measured from the ensemble averages in the presence and absence of isoproterenol (10–80 nM) in the experiment depicted and in two other patches was 1.16, 1.01, and 1.05. In contrast, in a patch that contained an I_L channel, activity elicited by test potentials to R + 100 mV from R + 50 mV responded to application of 40 nM isoproterenol with a 2.2-fold increase in the ensemble average of the current records (Fig. 4*B*). We conclude that at a concentration of isoproterenol that more than doubled I_L activity, there was no appreciable increase in P_o of I_T.

Voltage Dependence of I_T Inactivation

The voltage dependence of I_T inactivation could be determined from ensemble averages of multiple records (Fig. 5*A*). The averaged I_T current reached a peak in 7–17 msec. The time course of inactivation at R + 40, R + 60, and R + 80 mV could be fit by

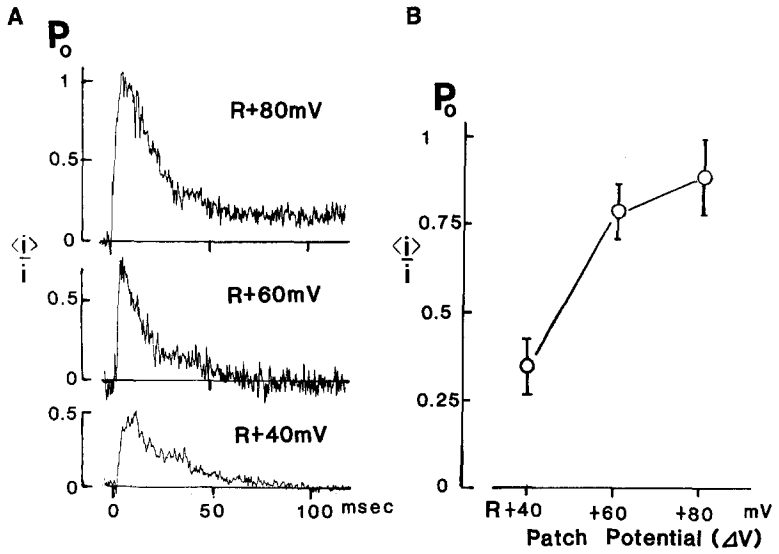

Fig. 6. Open probability (P_o) of the I_T channel. (*A*) P_o (= $\langle i \rangle / i$) as a function of time was calculated from the ensemble average of 80 records from a single patch (#002). (*B*) P_o (mean ± SD) from six patches plotted at each membrane potential. For the cell illustrated in *A*, peak $P_o = 1$ at $V_{patch} \approx -10$ mV

single-exponential curves with time constants of 37 ± 18 msec, 20 ± 7 msec, and 20 ± 11 msec, respectively (Fig. 5*B*).

Voltage Dependence and Time Course of Channel Open Probability

The probability of a channel being open at time t is given by $P_o(t) = \langle n(t) \rangle / N$, where $\langle n(t) \rangle$ is the ensemble mean number of channels open at time t and N is the number of active I_T channels in the patch. In an ensemble average of a series of records, as P_o approaches 1, the mean current per channel, $\langle i \rangle / N$ approaches the single-channel current, i/N. Measuring peak $\langle i \rangle / N$ from 80 records in a patch that contained two active I_T channels ($N = 2$), we determined that $P_o = \langle i \rangle / i = 0.51 \pm 0.15$ at $R + 40$ mV, 0.77 ± 0.14 at $R + 60$ mV and 1.08 ± 0.18 at $R + 100$ mV (Fig. 6*A*). P_o reached a maximum within 7–17 msec and then decreased with time at all voltages. The increase in mean peak P_o with depolarization of V_{patch}, from six similar patches is shown in Fig. 6*B*, where the average increase in P_o was from 0.35 to 0.88.

Frequency Histograms of the Open and Closed Times

The kinetics of channel opening and closing were analyzed during the initial 50 msec of each of the multiple test pulses in records from five patches. Each patch had a single active I_T channel and no I_L channel activity. In Fig. 7, the distribution of open times at $R + 60$ (Fig. 7*A*) and $R + 40$ mV (Fig. 7*B*), and of closed times at the same potentials (Fig. 7*C* and *D*) are shown. Individual I_T channel openings are relatively brief. The open-time histograms at the two potentials are similar. Both can be fit with a single exponential with time constants of 2.5 and 2.3 msec, respectively, (Fig. 7*A* and *B*), suggesting that activity at both potentials can be described in terms of a single open state. The closed-time histograms change appreciably with test potential. At $R + 60$ mV the single-exponential fit has a time constant of 0.5 msec (Fig. 7*C*), while at $R + 40$ mV the distribution is bi-exponential, with time constants of 1.3 and 12.5 msec (Fig. 7*D*), suggesting that the channel has more than one closed state.

Discussion

With either divalent or monovalent ions as charge carriers, cardiac cell membranes show two types of calcium channels with single-channel recording techniques. Using Na^+ as the charge carrier to increase channel conductance and for easier comparison with previous work on embryonic chick heart cells [22, 23, 24, 26], we have been able to distinguish these two channel types by differences in their unitary slope conductance (Fig. 2), their activation and inactivation voltage ranges (Fig. 1), and their differential sensitivity to D600 and the β-adrenergic agonist isoproterenol (Figs. 3 and 4). Although I_L channel activity was much rarer than we expected under the conditions employed, our results have confirmed those from adult mammalian cardiac cells. We have found that I_T channels in 14-day chick ventricle cells have a smaller conductance [3, 17, 29, 34, 39], a more negative potential activation range [2, 3], and little or no sensitivity to

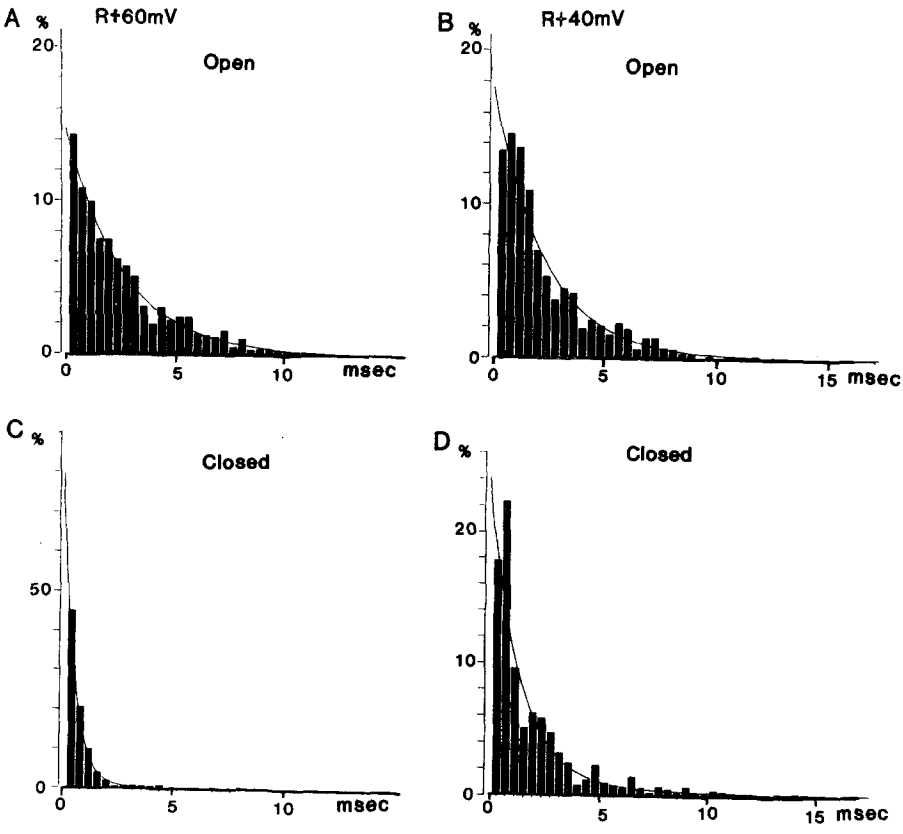

Fig. 7. Frequency histograms of open and closed time. Distribution of P_o at (*A*) R + 60 mV and (*B*) R + 40 mV. Average currents are fit with single-exponential curves with τ of (*A*) 2.5 msec and (*B*) 2.3 msec. Closed time (P_c) distribution at (*C*) R + 60 mV and (*D*) R + 40 mV. The distributions are fit with (*C*) a single exponential with $\tau \approx 0.5$ msec and (*D*) a double exponential with τ = 1.3 and 12.5 msec (records pooled from five patches)

isoproterenol [5, 17, 18, 20, 27, 39] as compared with I_L channels.

Single-Channel Conductance

I_T channels in embryonic ventricle had a mean unitary conductance of 26 pS with 120 mM Na^+ as the charge carrier. With physiological Ca^{2+} as the current carrier, single-channel Ca^{2+} currents are below the noise level of most recording systems. Therefore, in most previous studies, high concentrations of Ca^{2+} or Ba^{2+} were employed to record currents [4, 14, 17, 28, 30, 39]. In mammalian cardiac cells with 90–110 mM Ba^{2+} as the charge carrier, I_T unitary conductance was about 8 pS [17, 18], while that of I_L was 16–25 pS [4, 14, 17, 27, 29, 30, 33, 37]. The conductance of I_L channels for Na^+ (in the absence of divalent cations) was 50–100 pS, giving a Na^+/Ba^{2+} selectivity ratio of 3–4 [24–26, 31, 37, 38]. In 7-day embryonic chick heart cells, Levi and DeFelice [24] reported γ_L = 50–90 pS with Na^+ as the charge carrier, depending upon their interpretation of channel flickering. The variation in the conductance for monovalent cations from one study to another was large, in part at least, because upon removal of divalent cations, channel conductance became strongly dependent on extracellular pH [31, 32]. Although I_L channel activity was rare in the present experiments, the value we measured for I_L channel conductance is within the range reported by others. There are no measurements of γ_T with Na^+ as the charge carrier in mammalian cells, with which to compare our results directly. However, if the relative selectivity of the I_T channel for Na^+ and Ba^{2+} is 3–4, like that of the I_L channel, a reported value for Ba^{2+} conductance through I_T of 8 pS would predict a conductance of 24–32 pS for Na^+, which agrees well with our measured value of 26 pS.

The Effect of Isoproterenol

Stimulation of β-receptors of mammalian or amphibian cardiac tissue causes a dramatic increase in L-type calcium current [2, 7, 8, 13, 17, 20, 27, 36, 41] resulting from an enhancement of the mean channel open-state probability [17, 33]. However, β-receptor agonists have little [27] or no effect [2, 17, 20, 39] on I_T. Our results here confirm that 40 nM isoproterenol caused an approximate doubling of I_L channel activity, but had no effect on the channel that we identify as I_T (Fig. 4).

Kinetic Properties of I_T

L-type and T-type currents have been distinguished by differences in their activation and inactivation kinetics [14, 17, 37]. In neuroblastoma cells, two similar calcium currents have been termed type I and type II [42]. Inactivation of I_L channels is complex, in that the time course of current decay following a depolarizing step depends on both membrane potential and calcium concentration (*see* refs. [3, 14, 38] for review). By contrast, inactivation of I_T channels can be described in most systems by a single time constant that is unaffected by concentration of divalent cations, and depends solely on voltage [6, 9, 14, 17, 20]. Our results are consistent with those of previous workers in showing that I_T inactivation at the voltages tested was best fit with a mono-exponential curve with a time constant that varied with potential. Despite the obvious differences in the preparations (embryonic avian *versus* adult mammalian) and experimental conditions (monovalent *versus* divalent charge carrier, high *versus* normal external pH) even the absolute values of the time constants we recorded were of the same order as those seen before, when corrected for differences in temperature [2, 6, 9, 10, 14, 42].

Although our measurements of gating of I_T channels in the present study were limited to a narrow voltage range, the data suggested that I_T channels have one open state but more than one closed state. This result is consistent with the model of Fox et al. [14] derived from I_T channel behavior in neurons. The data show that the rate constant from closed state to open state changes with voltage but that from open to closed state does not, at least in the narrow voltage range between R + 40 mV and R + 60 mV. Within this range, the observed increase of P_o with more positive test potentials can be accounted for by the voltage dependence of the closed-open rate constant.

There are four major conclusions to be drawn from our results: (*i*) Ventricular cells from 14-day chick embryonic hearts contain T-type calcium channels with characteristic voltage ranges of activation and inactivation. (*ii*) Embryonic chick I_T channels are not modulated by β-adrenergic receptor binding or blocked by D600. (*iii*) The activation and inactivation kinetics of I_T channels in embryonic chick ventricle resemble those in other tissues. The data suggest that these channels have one open state and at least two closed states. (*iv*) The voltage-dependent increase of P_o can be explained by a decrease with depolarization in average duration of the closed state.

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