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Developmental Changes in the Calcium Currents in Embryonic Chick Ventricular Myocytes

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Summary. Using the patch-clamp technique, we recorded whole-cell calcium current from isolated cardiac myocytes dissociated from the apical ventricles of 7-day and 14-day chick embryos. In 70% of 14-day cells after 24 hr in culture, two component currents could be separated from total I_{Ca} activated from a holding potential (V_h) of −80 mV. L-type current (I_L) was activated by depolarizing steps from V_h −30 or −40 mV. The difference current (I_T) was obtained by subtracting I_L from I_{Ca}. I_T could also be distinguished pharmacologically from I_L in these cells. I_T was selectively blocked by 40–160 μM Ni^{2+}, whereas I_L was suppressed by 1 μM D600 or 2 μM nifedipine. The Ni^{2+}-resistant and D600-resistant currents had activation thresholds and peak voltages that were near those of I_T and I_L defined by voltage threshold, and resembled those in adult mammalian heart. In 7-day cells, I_T and I_L could be distinguished by voltage threshold in 45% (*S* cells), while an additional 45% of 7-day cells were nonseparable (*NS*) by activation voltage threshold. Nonetheless, in most *NS* cells, I_{Ca} was partly blocked by Ni^{2+} and by D600 given separately, and the effects were additive when these agents were given together. Differences among the cells in the ability to separate I_T and I_L by voltage threshold resulted largely from differences in the position of the steady-state inactivation and activation curves along the voltage axis. In all cells at both ages in which the steady-state inactivation relation was determined with a double-pulse protocol, the half-inactivation potential ($V_{1/2}$) of the Ni^{2+}-resistant current I_L averaged −18 mV. In contrast, $V_{1/2}$ of the Ni^{2+}-sensitive I_T was −60 mV in 14-day cells, −52 mV in 7-day *S* cells, and −43 mV in 7-day NS cells. The half-activation potential was near −2 mV for I_L at both ages, but that of I_T was −38 mV in 14-day and −29 mV in 7-day cells. Maximal current density was highly variable from cell to cell, but showed no systematic differences between 7-day and 14-day cells. These results indicate that the main developmental change that occurs in the components of I_{Ca} is a negative shift with embryonic age in the activation and inactivation relationships of I_T along the voltage axis.

Key Words T-type calcium current · embryonic heart · ion channel development · Na^+ through Ca^{2+} channels

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Introduction

Three different calcium currents have been found in various excitable membranes [5, 13, 36]. Two of these, L-type (or I_L) and T-type (T_T), are present in cardiac, smooth and skeletal muscle cells [5]. In the mammalian heart, I_L is the major component of the slow inward current that maintains the plateau phase of the cardiac action potential [7, 28], and its electrical and permeation properties have been studied extensively [32, 37]. The I_L channel has a unitary conductance near 20 pS with 90–110 mM Ba^{2+} as the charge carrier [13, 16] or 50–100 pS when passing Na^+ in the absence of divalent cations [23, 26, 27, 36]. The channel is modulated by β-adrenoceptor agonists [4, 21], it is blocked by phenylalkylamines such as verapamil or D600 [7], and it is sensitive to dihydropyridine antagonists such as nifedipine [4, 16] and agonists such as Bay K-8644 [4, 25]. An I_L-like channel has been described in neonatal mammalian heart cells [7, 31]. A similar channel appears early in muscle differentiation in ascidian embryos [34] and is prominent in the developing heart of the embryonic chick [22, 23, 27, 27*a*].

Much less is known about I_T. It is activated in a more negative voltage range than I_L [4]. I_T inactivates more rapidly, and is not affected by isoproterenol or verapamil [39, 40]. I_T is relatively insensitive to dihydropyridines, but is blocked with variable selectivity by Ni^{2+} at sub-millimolar concentrations [16, 38]. In adult preparations, the unitary conductance of I_T is only about one-third that of I_L [5, 13], and the whole-cell I_T current magnitude is only one-half to one-tenth that of I_L [16, 19, 20]. We have recently shown that 14-day embryonic chick ventricle cells have both I_L and I_T, with electrical and pharmacological properties that resemble those in adult mammalian cardiac preparations at both the whole-cell and single-channel levels [22, 23]. In these differentiating cells, the unitary conductance

of the I_T channel is also substantially smaller than that of I_L [23], but the I_T whole-cell current is roughly twice as large as I_L [22], apparently because I_L normally opens in a flickering mode with a low open-state probability [27*a*]. The aim of the present study was to explore the properties of I_T and I_L at earlier stages in the chick heart and to determine if they change with development.

Materials and Methods

Cell Cultures

White leghorn chicken eggs were incubated for 7 or 14 days at 37 ± 1°C. Single ventricular myocytes were isolated by a multiple-cycle dissociation procedure [8, 14] using trypsin or collagenase. Dissociated cells were suspended in medium 21212 overnight at 37 ± 1°C in an atmosphere of 85% N_2, 10% O_2, and 5% CO_2, while the cells became attached to the bottom of culture dishes. These dishes (Falcon 1008) were previously treated with 25 M sulfuric acid [14] and washed thoroughly, a procedure that promotes adherence while still allowing the cells to retain a spherical shape for up to 48 hr. Several minutes prior to experiments, the culture medium was replaced with tetraethylammonium (TEA) bath solution. Near-spherical single cells were selected for recordings.

Solutions

Culture medium 21212 consisted of: 25% M199, 2% heat-inactivated selected horse serum, 4% fetal calf serum, 68.5% K-free Ham's F-12 (all from GIBCO), penicillin-G (5 units/ml), and KCl (1.3 mM final concentration). TEA bath solution contained (in mM): TEACl 154, $CaCl_2$ 1.8, $MgCl_2$ 2, glucose 10, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, pH 7.4 (adjusted with CsOH), 300 nM tetrodotoxin (TTX). The pipette solution contained (in mM): CsCl 110, TEACl 20, $MgCl_2$ 4, $CaCl_2$ 0.068, ethyleneglycol-bis-(β-aminoethyl ether)N,N,N'-tetraacetic acid (EGTA) 11 or BAPTA (Molecular Probes, Eugene, OR) 5, HEPES 10, Na-adenosine 5'triphosphate (ATP) 5, Na-creatine phosphate 3, cyclic adenosine monophosphate (cAMP) 0.033, pH 7.1 (adjusted with CsOH). Free ionized Mg^{2+} and Ca^{2+} were calculated to be 7.6×10^{-4} M and $<10^{-9}$ M according to a program provided by Fabiato [12]. We chose the above ionic conditions to isolate calcium current from other currents [4, 26]. Inward sodium current through sodium channels was eliminated by substituting Na^+ with TEA^+ in the bath. TTX (300 nM) blocked Ca^{2+} flow through fast sodium channels [14]. Potassium currents were eliminated by internal Cs^+ and by internal and external TEA^+. Calcium-activated currents were minimized by buffering the pipette solution with 5 mM BAPTA or 11 mM EGTA. In these conditions we were able to record reproducible calcium currents, with no sign of contamination by other charge carriers. Calcium blockers applied were nifedipine, 2 μM [13], D600, 1 μM [28], and Ni^{2+}, 40–160 μM [16, 38].

Recording and Data Analysis

Experiments were done with patch electrodes in whole-cell clamp mode, at room temperature (21 ± 1°C), using methods described previously [14, 22, 23]. Hard borosilicate glass capillaries (Corning 7052) were used for fabricating recording electrodes (1.5-3 MΩ). Electrode-membrane seals of 20–80 GΩ were routinely achieved. Currents were digitized at 10 kHz, low-pass filtered at 3 kHz, amplified by a List EPC-7, and recorded with a Neurocorder (model DR-484) coupled to a VCR recorder, refiltered at 1 kHz (low-pass), and analyzed on an IBM PC-AT. Adequacy of voltage control has been extensively tested [14]. Currents were elicited with 400 msec voltage command (V_{com}) steps, applied at 3-sec intervals to $V_{com} = -70$ mV ($V_{com(-70)}$), -60 mV ($V_{com(-60)}$), . . . to $V_{com(80)}$ from two different levels of holding potential (V_h). Total calcium current (I_{Ca}) was activated from $V_{h(-80)}$. The high-threshold current, I_L, was elicited from $V_{h(-30)}$ or $V_{h(-40)}$. The low-threshold current, I_T, was obtained by subtracting I_L from I_{Ca} at each V_{com} as described [22]. We measured current amplitude by subtracting the current near the end of the command pulse (395 msec) from the initial inward peak. Activation threshold was defined as the V_{com} step that elicited a just-measurable current by that subtraction method. Steady-state inactivation was tested by applying a double-pulse voltage sequence to cells bathed in current blockers. For I_T, 2-sec conditioning pulses in the range -120 to -20 mV were applied to cells bathed in D600 or nifedipine, followed by a constant V_{com} step to -10 mV. For I_L, conditioning pulses in the range $V_h = -80$ to -10 mV were applied to cells exposed to Ni^{2+}, followed by a constant V_{com} step to 20 mV. The conditioning and V_{com} pulses were separated by a 3-msec step to -80 mV for I_T and -30 mV for I_L.

Computer software for data acquisition and analysis and the programmable stimulator (Challenger, series H) for application of voltage protocols were produced by W.N. Goolsby of our Computer Core facility. For Boltzmann analysis of steady activation and inactivation curves, data were fit using a Marquardt nonlinear least squares algorithm, with a convergence criterion of 10^{-6}. Averaged data are expressed as mean ± SD or mean ± SE as stipulated.

Criteria for adequacy of data were stringent. Cells were selected for patch clamping on the basis of size (13–18 μm diameter), near sphericity, and smooth appearance. Whole cell capacitance was 4–7 pF. Cells were discarded early in an experiment if the leak current was greater than 6 pA, or if the current activated by repeated steps from $V_{h(-80)}$ to $V_{com(0)}$ failed to stabilize in the first few minutes after rupturing the patch. Experiments generally lasted 20–30 min, but were aborted at any time if rundown reduced I_{Ca} to less than 80% of control values. In all cases included, currents were elicited repeatedly without appreciable rundown.

Results

Embryonic cells with an attached patch-electrode are fragile and difficult to maintain through all the voltage protocols and bath washes required for the experiments described here. Out of 1171 ventricle cells selected as suitable, partial data was obtained on 205. The results described here are based on experiments with a total of 87 cells (27 14-day, 50 7-day) in which complete voltage protocols and at least one bath change after application of a pharmacological agent were achieved.

14-Day Cells

Voltage Dependence

In about 70% of 14-day cells (19 out of 27 cells tried), two components of calcium current were separable

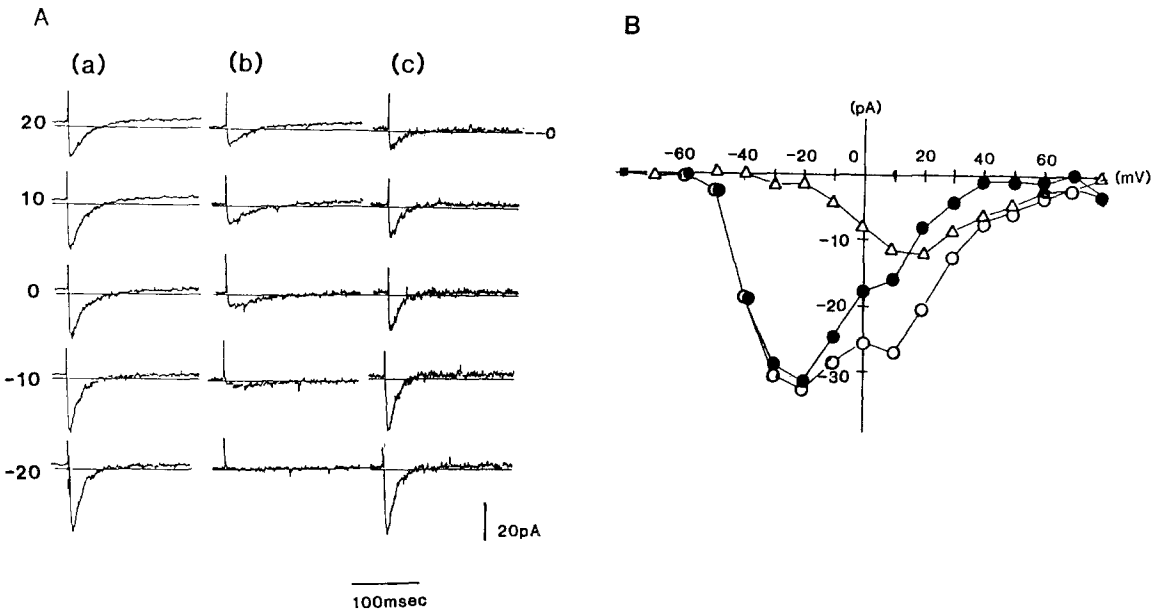

Fig. 1. Separation of I_L in 14-day cells by activation threshold potential. (*A*) Typical currents elicited by voltage steps from (column *a*) $V_{h(-80)}$ (= I_{Ca}) or (column *b*) $V_{h(-40)}$ (= I_L) to V_{com} shown at the left of each row of traces. Column *c* shows I_T obtained by subtraction of the current record in *b* from that in *a* at each potential. (*B*) Peak current-voltage relations of I_{Ca} (○), I_L (△), and I_T (●). (Cell #72)

Table 1. Electrical properties of I_T and I_L in 7-day and 14-day cells[a]

a) Separated by voltage subtraction	7-day (S)		14-day	
	I_T	I_L	I_T	I_L
Activation threshold (mV)	−34 ± 7	−12 ± 7	−34 ± 7	−7 ± 7
Peak voltage (mV)	−6 ± 10	21 ± 8	−4 ± 11	24 ± 7
Peak amplitude (pA)	−23 ± 13	−15 ± 8	−21 ± 13	−14 ± 14
n	27	27	19	19
b) Separated by drug sensitivity and voltage	7-day (*S* + *NS*)		14-day	
	Ni^{2+}	DHP/D600	Ni^{2+}	DHP/D600
Activation threshold (mV)	−28 ± 13	−5 ± 6	−33 ± 6	−4 ± 5
Peak voltage (mV)	4.5 ± 18	20 ± 8	−3 ± 15	23 ± 5
Peak amplitude (pA)	−20 ± 12	−11 ± 4	−20 ± 8	−13 ± 7
n	5	5[b]	8	7[c]

[a] Values represent mean and SD.
[b] Data pooled from three cells treated with D600 and two with nifedipine.
[c] Data pooled from four cells treated with D600 and three with nifedipine.

by their voltage threshold potential. These were termed *S* cells. The total inward current (I_{Ca}) elicited from $V_{h(-80)}$ (Fig. 1*A*, column *a*) reached a peak in 7–15 msec and decayed within 50–100 msec. The small high-threshold current elicited from $V_{h(-40)}$ (Fig. 1*A*, column *b*) reached a peak in 8–32 msec, and decayed more slowly than I_{Ca}. This current resembles I_L as described in adult heart [4, 19]. Subtracting I_L from I_{Ca} at each command potential gave the low-threshold difference current (column *c*), defined as I_T. From the *I–V* curves (Fig. 1*B*), the differences in voltage dependence of the two currents are apparent. The mean activation threshold potential, peak voltage, and peak amplitude of I_L and I_T in the 14-day *S* cells are listed in Table 1*a*. In eight 14-day cells, two current components were not separable by the potential at which they could be activated, either because the current elicited at the two holding potentials were essentially the same, or no current was activated at one or the other levels

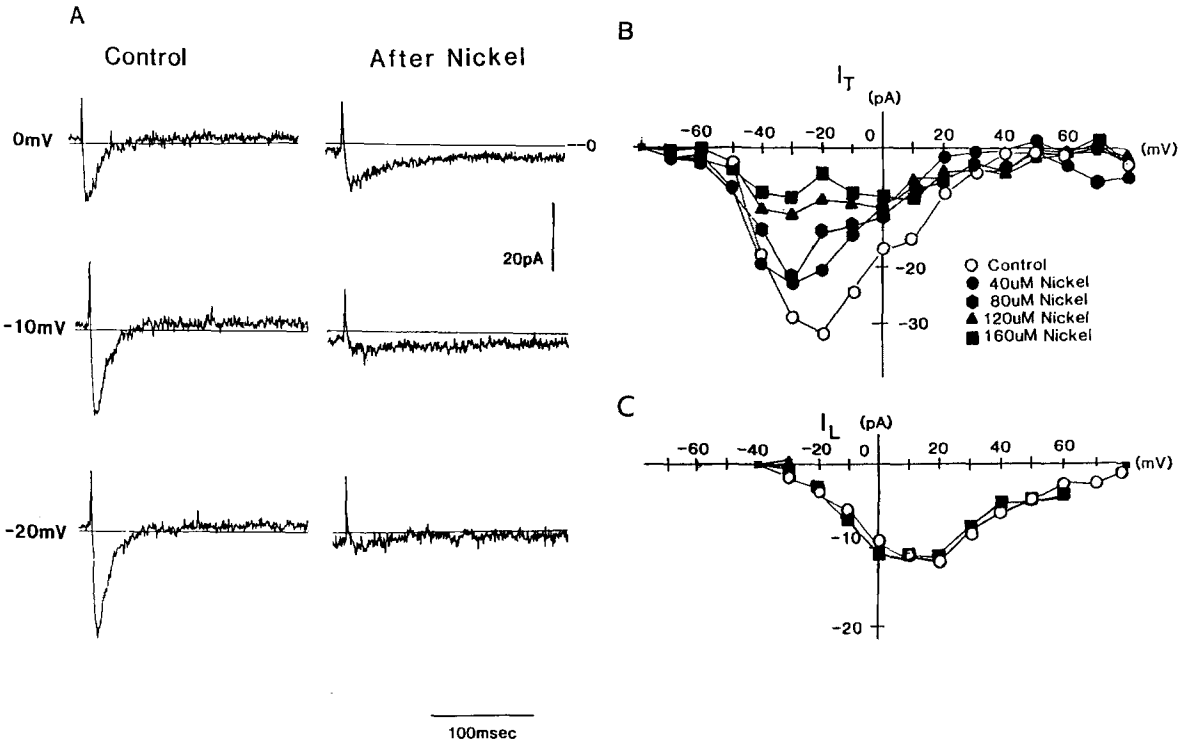

Fig. 2. The effect of Ni^{2+} on I_T and I_L. (*A*) The subtraction current, I_T, at the command potentials shown, in the absence (left) and presence (right) of 160 μM Ni^{2+}. (*B*) Current-voltage relations of peak I_T in control bath solution (○), and in the presence of increasing concentrations of Ni^{2+} from 40 (●) to 160 μM (■). (*C*) I_L elicited in the same cell by steps from $V_{h(-40)}$ in control bath solution, and in the presence of 160 μM Ni^{2+} (symbols same as in *B*). I_L is only slightly affected by Ni^{2+} at this concentration. (Cell #72)

of V_h. These are defined as nonseparable (*NS*) cells, and their properties are described in detail below.

Pharmacological Separation

To dissect the two currents pharmacologically, we applied the dihydropyridine antagonist, nifedipine, or the verapamil derivative, D600, to block I_L, and nickel ion to suppress I_T. Ni^{2+} blocked most of I_T (Fig. 2*A*) in a dose-dependent manner in the concentration range 40–160 μM, leaving mainly I_L as a Ni^{2+}-resistant current (Fig. 2*B*). Forty μM Ni^{2+} reduced peak I_T to 64% of control, while at 160 μM, blockade was to 14% of control ($n = 4$). At these same concentrations, Ni^{2+} had only a slight effect on I_L (Fig. 2*C*). Peak amplitude of I_L at $V_{com(20)}$ after exposure to 160 μM Ni^{2+} was 98% of control. Tetramethrin has also been reported to block I_T selectively [16, 38]. We applied this drug in the range 0.1–1 μM, but the results were highly variable from cell to cell, and in no case was there a marked difference in the effect on I_T and I_L ($n = 11$).

As in other preparations [4, 16, 19, 29] low doses of nifedipine or D600 substantially blocked I_L but had little or no effect on I_T (Fig. 3*A*). In a group of seven 14-day *S* cells, 1 μM D600 reduced maximal I_L to an average of 19% of control, while it decreased the subtraction current I_T only to 78% of control. The *I–V* curves (Fig. 3*B*) illustrate the control current from $V_{h(-80)}$ (I_{Ca}) in one of these cells (open circles) and that from $V_{h(-40)}$ (open triangles, I_L), both before and in the presence of D600. In the example shown, the *I–V* curves from $V_{h(-40)}$ and $V_{h(-80)}$ are both much reduced, and their peaks are shifted to the left after D600. Both curves are clearly composed of two components, one sensitive to and one resistant to D600. The D600-resistant components were almost completely blocked by Ni^{2+}, suggesting that these two components corresponded to the currents defined respectively as I_L and I_T, by voltage threshold. This impression is supported by comparing the shapes of the Ni^{2+}-resistant current activated from $V_{h(-80)}$ (filled circles) with I_L from $V_{h(-40)}$ in control bath solution (filled triangles, Fig. 3*C*). Additional evidence is listed in Table 1*b*, which shows that the activation voltage, peak voltage and peak amplitude of the currents defined by drug sensitivity were similar to those separated by voltage subtraction without drugs. In each case, the currents were cross-sensitive. That is, the Ni^{+}-resistant current

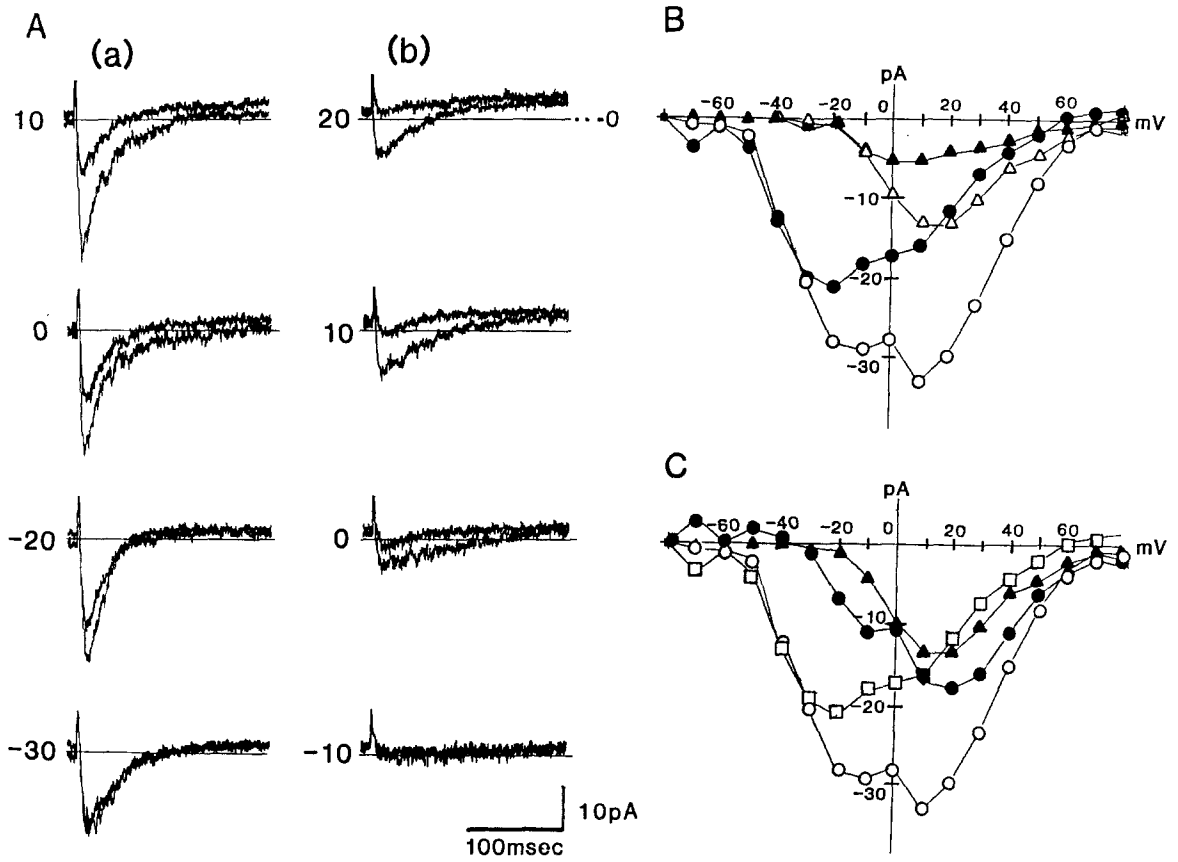

Fig. 3. Separation of I_T and I_L by threshold potential and D600. (*A*) Each pair of records shows total I_{Ca} ($V_{h(-80)}$, column *a*) and I_L ($V_{h(-40)}$, column *b*) at the command potential shown at the left of each trace, before (lower trace of each pair) and in the presence of 1 μM D600 (upper trace). (*B*) Peak *I–V* curves of the currents activated from the two holding potentials. Control I_{Ca} from $V_{h(-80)}$ (○); D600-resistant I_{Ca} from $V_{h(-80)}$ is mainly I_T (●); Control I_L from $V_{h(-40)}$ (△); in the presence of D600 from $V_{h(-40)}$ the residual current represents a small D600-resistant component of I_L plus that portion of I_T activated at positive potentials (▲). (*C*) Separation of D600-sensitive and Ni^{2+}-sensitive (D600-resistant) currents obtained by subtraction of currents before and after both drugs. Control I_{Ca} from $V_{h(-80)}$ (○); I_T activated from $V_{h(-80)}$ in D600 is the Ni^{2+}-sensitive current (□); current from $V_{h(-40)}$ that is D600-sensitive is mainly I_L (▲); Ni^{2+}-resistant current from $V_{h(-80)}$ is D600-sensitive and resembles I_L in its I/V curve (●). (Cell #69)

was blocked by D600, and the D600-resistant current was suppressed by Ni^+. Nifedipine (2 μM) had effects essentially identical to D600 (1 mM), and results with these two drugs have been pooled in Table 1. From these results we conclude that I_T and I_L in a majority of 14-day cells can be distinguished both by their electrophysiological and pharmacological properties.

7-Day Cells

Voltage Dependence

Applying the voltage protocols above, we found that only 27 out of 60 7-day ventricle cells (45%) had two distinct currents that were separable by V_h. In these 7-day *S* cells, the currents elicited from $V_{h(-80)}$ or $V_{h(-30)}$ (Fig. 4*A*), and the *I–V* curves of the separated currents (Fig. 4*B*) resembled those that were seen in 14-day cells. In the remaining 33 NS cells, I_{Ca} could not be separated into two components on the basis of V_h. The current activated from $V_{h(-30)}$ in 27 of these NS cells was less than half as large as the subtraction current, but contrary to the behavior of *S* cells, the activation threshold potential and peak voltage of the two components were not appreciably different (Fig. 4*C*). Cells with current that could be activated at both V_h levels were termed *NS* if subtraction yielded a current that was shifted by less than 10 mV from I_L on the voltage axis.

Pharmacological Separation

In essentially all (90%) of the 7-day cells tried (both *S* and *NS*), current was partially blocked by nifedipine, D600 and Ni^{2+}. In *S* cells, nifedipine (Fig. 5*A*

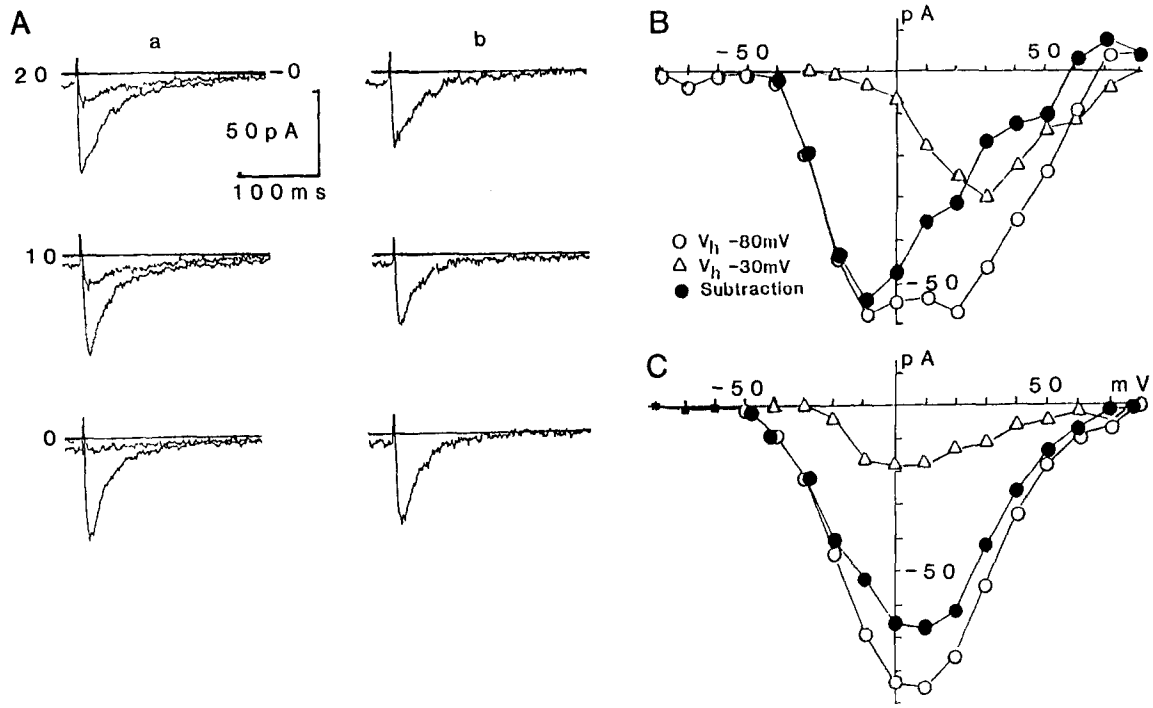

Fig. 4. Separation of I_T and I_L in 7-day cells by activation threshold potential. (*A*) (column *a*) Superimposed currents from an *S* cell elicited by voltage steps from $V_{h(-80)}$ (= I_{Ca}) and $V_{h(-30)}$ (= I_L) to V_{com} shown at the left. (Column *b*) I_T obtained by subtraction at each potential; cell #132. (*B*) Peak current-voltage relations of I_{Ca} (○), I_L (△), and I_T (●). (Cell #132) (*C*) Peak current-voltage relations from an *NS* cell in response to the same voltage sequence applied in *A* and *B*. (Cell #75)

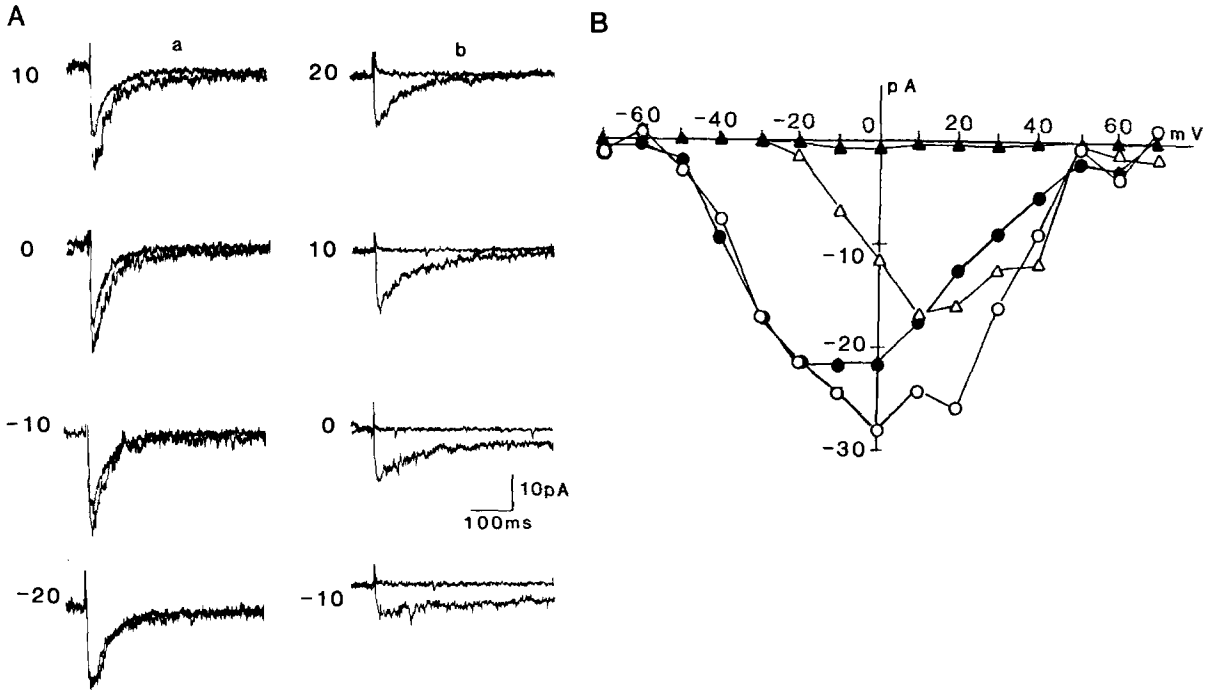

Fig. 5. The effect of nifedipine on I_T in a 7-day *S* cell. (*A*) Superimposed records of the subtraction current, I_T (column *a*), and I_L (column *b*) in the absence and presence of 2 μM nifedipine. (These currents have been leak-adjusted). (*B*) Current-voltage relations of peak I_T and I_L in control bath solution (open symbols), and in the presence of nifedipine (filled symbols). (Cell #136)

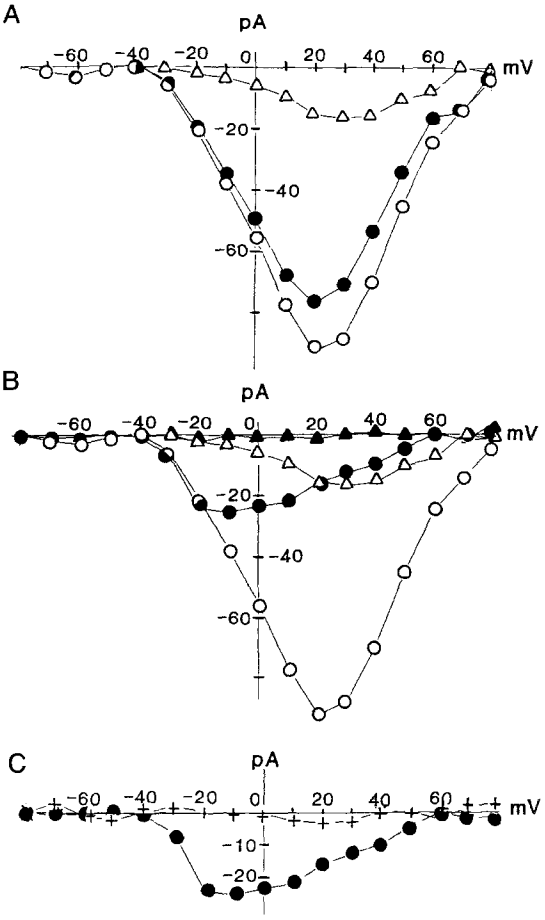

Fig. 6. Responses of a 7-day *NS* cell. (*A*) Nonseparability of I_T from I_L by V_h. Peak *I/V* curves of I_{Ca} elicited from $V_{h(-80)}$ (○); I_L activated from $V_{h(-30)}$ (△); and the subtraction current (●), showing little separation on the voltage axis. (*B*) Effect of D600. Control I_{Ca} and I_L, same as in *A* (open symbols); bath application of 1 μM D600 (filled symbols). The maximum D600-resistant component of I_{Ca} is about 40 mV negative to that of I_L. (*C*) Additive effect of D600 and Ni^{2+}. D600-resistant component of I_{Ca} ($V_h(-80)$) from *B* (●) is blocked by 80 μM Ni^{2+} (+). (Cell #82)

and *B*) or D600 were effective blockers of I_L, but had no appreciable effect on the subtraction current, I_T. Ni^{2+} suppressed I_T strongly but had little effect on I_L. As shown in Table 1*a* and *b*, the activation threshold, peak voltage, and amplitude of the Ni^{2+}-sensitive and DHP-sensitive currents were similar, respectively, to those of I_T and I_L, defined by V_h alone.

There are two alternative explanations for the inability to separate I_{Ca} into I_T and I_L by V_h in the 7-day *NS* cells. One possibility is that these cells contain only a single type of I_L-like calcium channel. The alternative is that both I_T and I_L channel types are present, but they have similar voltage dependencies in *NS* cells. To distinguish between these possibilities, we exposed *NS* cells to D600 or Ni^{2+}, and analyzed the residual drug-resistant current in each case. The *I–V* curve of the current activated from $V_h(-80)$ in the presence of 1 μM D600 revealed a D600-resistant component that was shifted 30–40 mV negative to that elicited from −30 mV (*compare* Fig. 6*A* and *B*), i.e., it resembled I_T. Moreover, like I_T, this current was blocked by Ni^{2+} (Fig. 6*C*). The *I–V* curve of the current activated from $V_{h(-30)}$ had the same shape as that of I_L, and was completely blocked by D600 (Fig. 6*B*, filled triangles). We tested the additive effects of Ni^{2+} and D600 (or nifedipine) in the opposite order (i.e., Ni^{2+} first) and obtained the same result. From these experiments, we conclude that *NS* cells have two different kinds of calcium channels, distinguishable by their drug sensitivities as corresponding to I_T and I_L, but that these channels do not differ in their voltage dependencies as they do in *S* cells. Cells defined as *S* and *NS* differ primarily in that the peak voltage of the D600-resistant, Ni^{2+}-sensitive current in *NS* cells is more positive than in *S* cells (Table 1*b*).

A small fraction of 7-day *NS* cells (11 of 60, 18%) had a substantial I_{Ca} current that could be elicited from $V_{h(-80)}$, but little or none (<5 pA) from $V_{h(-30)}$. In these cells, μM Ni^{2+} blocked I_{Ca} to 33% of control (Fig. 7*A*). Nifedipine had no effect on this current. Furthermore, the activation threshold and peak potential of I_{Ca} were near those of I_T in *S* cells. Thus, it appeared at first that these cells lacked I_L. However, in experiments in which external $[Ca^{2+}]$ was increased from 1.8 to 5 or 10 mM, or replaced by 1.8 or 3 mM Ba^{2+}, we found that I_{Ca} from $V_{h(-80)}$ increased markedly, and that a long-lasting current could be elicited from $V_{h(-30)}$ (Fig. 7*B*). This current was blocked by D600 or nifedipine, confirming that these *NS* cells also contained both I_T and I_L channels.

Voltage Dependence of Inactivation

We obtained steady-state inactivation and activation curves for I_T and I_L using both drug resistance and voltage dependence to isolate the two currents. For the I_T inactivation relationship, the membrane potential of cells exposed to nifedipine or D500 was held for 2-sec steps at 10 mV increments between −120 and −10 mV before stepping to $V_{com(-10)}$. At this potential, I_T was maximally activated from conditioning potentials negative to about −70 mV, with little contamination by any residual I_L (Fig. 8*A*, left). Inactivation of I_L was determined with steps between $V_{h(-80)}$ and $V_{h(+10)}$, to $V_{com(+20)}$, in the presence of Ni^{2+} (Fig. 8*A*, right). The peak amplitudes were normalized and plotted against V_h (Fig. 8*B* and *C*). Because it was impossible to subject these cells to the multiple washes required for switching from one

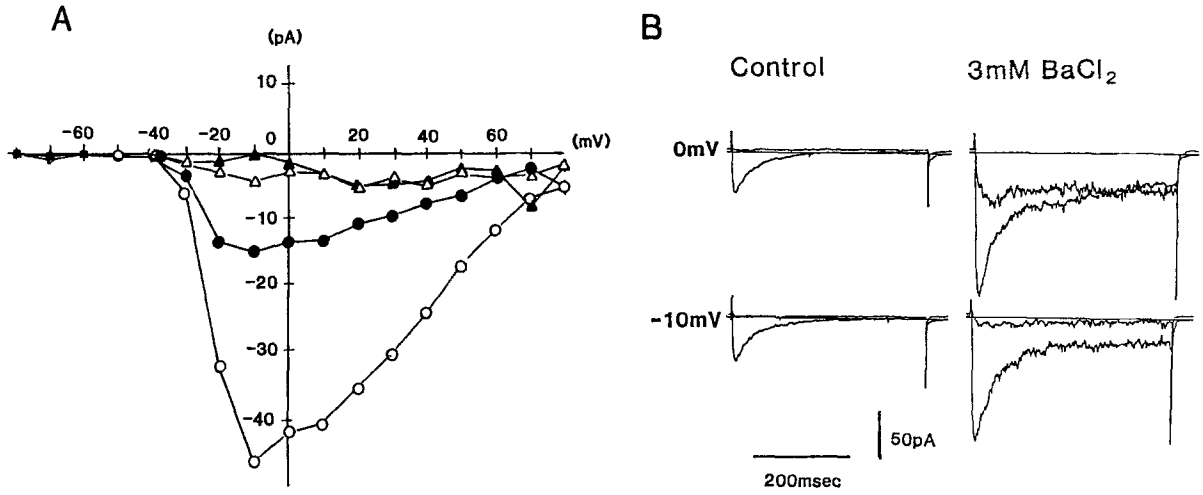

Fig. 7. Responses of a 7-day *NS* cell. (*A*) Peak *I/V* curves of currents from $V_{h(-80)}$ (circles) and $V_{h(-30)}$ (triangles) in control bath solution (open symbols) and after application of 80 μM Ni^{2+} (filled symbols). (Cell #64). (*B*) Superimposed currents from V_h = −80 and −30 mV, in control bath solution containing 1.8 mM $CaCl_2$ (left) and after replacement with 3 mM $BaCl_2$ (right). (Cell #118)

Table 2. Activation and inactivation parameters of I_T and I_L in 7-day and 14-day ventricle cells[a]

	7-day		14-day	
	I_T	I_L	I_T	I_L
Inactivation $V_{1/2}$ (mV)	−49.0 ± 1.3	−17.7 ± 0.9	−59.7 ± 1.0	−18.0 ± 0.8
Slope factor, s (mV)[b]	9.8 ± 1.2	5.1 ± 0.9	5.6 ± 0.1	8.1 ± 0.6
n	6	4	5	5
Activation $V_{1/2}$ (mV)	−27.5 ± 1.3	−3.3 ± 2.0	−38.3 ± 1.3	−1.7 ± 1.1
Slope factor, s (mV)[b]	4.3 ± 1.2	7.1 ± 1.6	6.3 ± 1.1	7.5 ± 8.8
n	7	6	5	5

[a] Values represent mean and SE.
[b] mV/e-fold change.

drug to another, the I_T and I_L inactivation curves were obtained from different populations of cells. As shown in Fig. 8*A* and *B*, I_T was completely inactivated at potentials positive to −30 mV; the half-inactivation potential ($V_{1/2}$) was near −60 mV (Table 2). The curve for I_L was about 40 mV more positive ($V_{1/2}$ = −18 mV). This degree of clean separation, and the steepness of the two curves account for their separability by the voltage subtraction method. In 7-day cells with pooled data from *S* and *NS* cells (Fig. 8*C*), $V_{1/2}$ of I_L was nearly identical to that of 14-day cells, but the curve for I_T was more positive ($V_{1/2}$ = −49 mV) and less steep. At −40 mV, where I_L was fully available, it would have been contaminated by I_T (in the absence of Ni^{2+}) by as much as 30%. To see how this overlap was related to the separability of 7-day cells by voltage threshold, we calculated the I_T inactivation curves separately for two *S* and two *NS* cells. Those curves showed half-inactivation potentials at −52 and −43 mV, respectively (Fig. 9). The I_T inactivation curve for 7-day *S* cells has about the same slope as that of 14-day cells and is separated from it by 34 mV. The I_T curve for *NS* cells is less steep (s = 8.4) and about 10 mV more positive, thus accounting for the inability to separate I_T and I_L by the voltage subtraction method in these preparations.

Voltage Dependence of Activation

The voltage-activation relationships of I_L in 14-day and 7-day cells (in the presence of Ni^{2+}) were virtually superimposable (Fig. 10). $V_{1/2}$ of the two curves

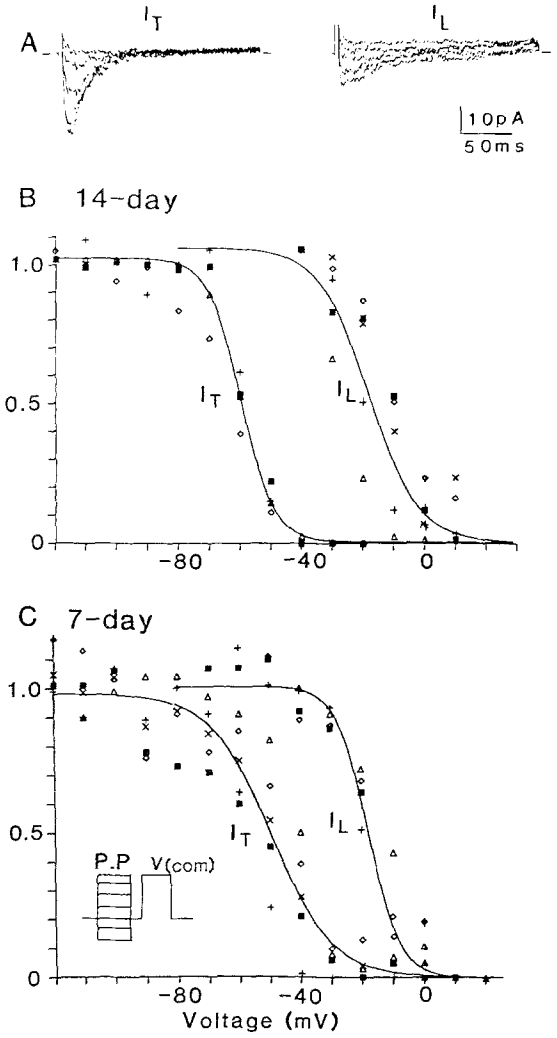

Fig. 8. Steady-state inactivation of DHP-resistant (I_T) and Ni^{2+}-resistant (I_L) current. I_T was activated by a double-pulse protocol (inset in *C*), consisting of 2-sec prepulses at 10 mV increments between $V_{h(-120)}$ and $V_{h(-10)}$, each separated by 3 msec from a 200-ms step to $V_{com(-10)}$, in cells bathed in 2 μM nifedipine or 1 μM D600. I_L was elicited by conditioning steps between $V_{h(-80)}$ to $V_{h(+10)}$ to $V_{com(+20)}$, with 80 μM Ni^{2+} in the bath. (*A*) I_T and I_L currents from a 14-day cell. (Left) I_T elicited from conditioning pulses of −80, −70, −60, −50 and −40 mV to $V_{com(-10)}$; (right) I_L from −40 to 0 mV, to $V_{com(+20)}$. (*B*) Pooled data from four 14-day cells. The continuous curves are derived from the Boltzmann relation: $Y_\infty = [(1 + \exp(V_m - V_{1/2})/s]^{-1}$. (*C*) Pooled data from six 7-day cells, values of $V_{1/2}$ and s are given in Table 2

was −2 and −3 mV, respectively (Table 1*b*). The activation curve for I_T (in nifedipine) was 36 mV more negative ($V_{1/2}$ = −38 mV) than I_L in 14-day cells (n = 5), but only 24 mV more negative in 7-day cells ($V_{1/2}$ = −27.5 mV: n = 7). These data again indicate that while the electrical properties of I_L remain relatively constant during the second week of cardiac development, those of I_T change.

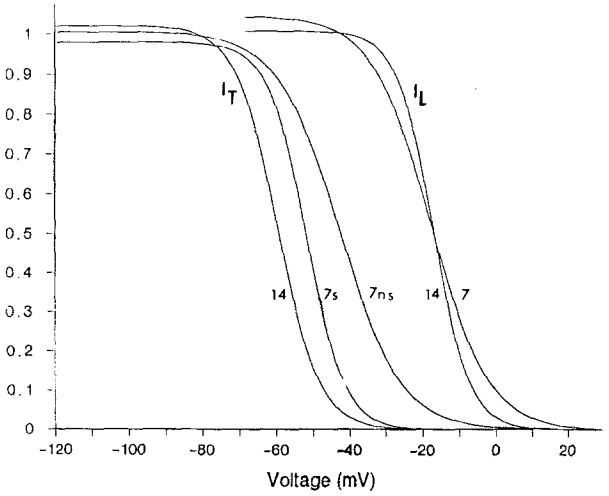

Fig. 9. Comparison of the voltage-dependence of steady-state inactivation of I_T and I_L in 14-day cells, and in 7-day *S* and *NS* cells. Curves fitted to 14-day and pooled 7-day I_L are taken from Fig. 8. Parameters for the I_T curves are:

	$V_{1/2}$ (mV)	s
7-day NS	−42.8 ± 1.8	8.4 ± 1.6
7-day *S*	−51.9 ± 0.8	5.1 ± 0.7
14-day	−59.7 ± 1.0	5.6 ± 0.9

Discussion

Embryonic Chick Cells Contain Two Components of I_{Ca}

In the present work, we have defined I_L as a calcium-selective current [19, 32, 37] that is blocked by micromolar concentrations of D600 or nifedipine [4, 7, 16, 19], is unaffected by submillimolar concentrations of Ni^{2+} [16], and has activation and steady-state inactivation ranges centered near 0 and −25 mV, respectively [7, 16, 36]. In contrast, I_T is sensitive to Ni^{2+} [16, 38], insensitive to micromolar concentrations of D600 or nifedipine [39, 40], and has activation and steady-state inactivation ranges centered positive to −40 mV and negative to −60 mV, respectively [16, 36]. We show that at the end of 14 days of development in the embryonic chick heart, more than 70% of the ventricle cells have clearly distinguishable I_T and I_L components, in confirmation of our previous report [22], and that these currents are also present in many cells as early as seven days of development. Finally, we demonstrate that the voltage dependence of I_T undergoes substantial developmental changes between 7 and 14 days of incubation, while that of I_L remains relatively unchanged.

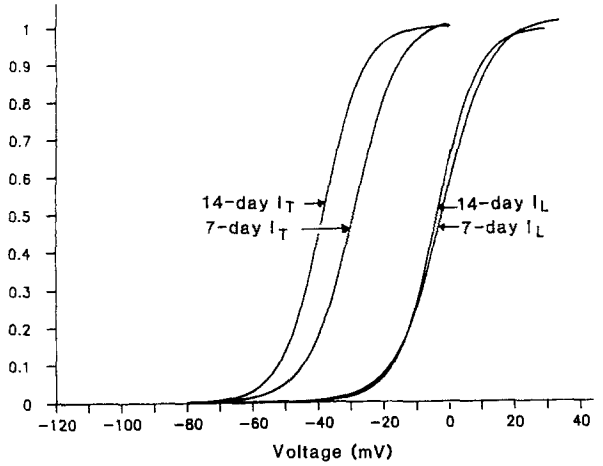

Fig. 10. Voltage-dependence of activation of I_T and I_L in 14-day and 7-day cells. I_T was activated from $V_{h(-80)}$ with 2 μM nifedipine in the bath; I_L was elicited from $V_{h(-30)}$ in the presence of 80 μM Ni^{2+}. Curves were fitted to data from five 14-day cells and seven 7-day cells. Parameters are shown in Table 2

Developmental Changes in Calcium Current

When the ventricle first forms during embryonic development, it contracts spontaneously and has slow pacemaker-like action potentials. As the heart develops, the maximum diastolic potential (MDP), shifts negatively to about −90 mV [10, 11], the current density of the excitatory sodium current increases about eightfold [14], and there is a marked slowing of the inactivation kinetics of the inward rectifier current, I_{K1} [11]. We have only recently begun to understand the variety of modes of embryonic differentiation of ionic currents in excitable cells [6, 9–11, 33, 35]. Some channels have adult electrical and pharmacological properties when the current first appears during organ differentiation. No appreciable changes take place during development except for increases or decreases in magnitude, i.e., current density. This appears to be the case with the excitatory sodium current, I_{Na} [14]. Similarly, a calcium current that resembles I_L appears in muscle lineage-specific blastomeres shortly after the neurula stage in ascidian embryos [34], indicating that the development of L-type calcium channels may be one of the earliest steps in muscle differentiation. In mammalian muscle I_L already has fully differentiated properties at neonatal stages, increasing only in magnitude with postnatal development [2, 3]. In contrast, a nifedipine-resistant, rapidly inactivating calcium current, equivalent to I_T, which is present with widely varying magnitude in neonatal skeletal muscle cells, disappears completely during 3–4 weeks of postnatal development [3]. An equally dramatic change takes place in the cardiac inward rectifier current, I_{K1}, which undergoes a 10-fold slowing of its inactivation kinetics during the first two weeks of cardiogenesis in the chick embryo [33].

Our findings here with I_T and I_L in embryonic chick ventricle cells represent the first study of I_{Ca} at two different embryonic stages. In both 7-day and 14-day cells, a current with the distinctive properties of I_L was generally present, though in some 7-day cells it was necessary to increase extracellular calcium or replace it with barium to reveal this current. At both stages, I_L was blocked by dihydropyridines or by phenylalkylamines, was unaffected by Ni^{2+}, and was activated by small depolarizing steps from a positive holding potential. In contrast, I_T appeared to undergo clearcut developmental changes between 7 and 15 days of incubation. At the earlier stage, these channels were already distinguishable by their selective sensitivity to Ni^{2+} and lack of response to nifedipine or D600. But their activation and inactivation parameters were both located in a depolarized voltage range by comparison with the condition in 14-day cells (Figs. 9 and 10) or in adult mammalian cardiac tissues [16, 19].

T-type channels appear to resemble I_L channels in that they are permeable to monovalent cations in the absence of divalent ions [18] and they are blocked by micromolar Ca^{2+} in a voltage-dependent manner [15, 37]. These traits suggest that the permeation properties of I_T, like those of I_L, result from the characteristics of the calcium-binding sites within the pore [32, 37]. The mechanism of the leftward shift of the steady-state inactivation and (to a lesser extent) the activation curves of I_T during the second week of development could result from a global change in internal or external surface potential affecting the field through the pore [13]. Calcium channels are sensitive to changes in the internal ionic milieu and especially to divalent ions that have large effects on charge screening. As expected, alterations in $[Ca^{2+}]_i$ [19] or $[Mg^{2+}]_i$ [1] modulate Ca-channel activity. This appears to be an unlikely mechanism for the shift in I_T, however, because such changes would be expected to affect other channels as well, such as I_L and I_{Na}. A more likely explanation, though wholly speculative, is that a change occurs during development either in surface charges localized near the T-channels, or in the sensitivity of the voltage sensor itself. Treatments that affect the inner aspect of the channel protein can modify its voltage dependence and kinetics [17, 24]. Alternatively, modification of a protonation site at the external mouth of the channel [30] could shift the voltage relations.

Comparison of Calcium Currents in Embryonic Chick and Adult Mammalian Hearts

The $V_{1/2}$ of the activation curves of I_L and I_T in chick ventricle cells were nearly the same as those in adult rabbit SA node: $V_{1/2}$ for the two curves were near 0 and −30 mV [16]. The steady-state inactivation relation of I_L was also similar between chick and adult mammalian heart: $V_{1/2}$ was −18 mV in embryonic cells (Fig. 10), −22 mV in guinea pig ventricle myocytes [41], −25 mV in rabbit SA node cells [16], and −20 to −31 mV (depending on external $[Ca^{2+}]$) in canine Purkinje cells [19]. However, the half-inactivation potential of I_T in the mammalian preparations was more negative, near −70 mV [16, 19, 41] than that of even the 14-day chick cells (−60 mV, Fig. 10), suggesting that the negative shift in $V_{1/2}$ of I_T between 7 and 14 days may continue during later development and maturation.

The current density of I_T in 14-day chick ventricle cells was 4.2 pA/pF [22]. In both the 7-day and 14-day cells used in the present study, I_T at its peak potential was, on average, 1.5 times larger than maximal I_L, presumably because I_L normally functions in a flickering mode with a low open-state probability [27*a*]. This is in striking contrast to the condition of adult mammalian heart cells, where I_T characteristically makes only a small contribution to total I_{Ca}. For example, in canine atrial cells, I_T was only about one-tenth as large as I_L [4] and in ventricle cells from dog and guinea pig heart it made an even smaller contribution [4, 41]. The adult preparation in which the relative magnitude of I_T is largest is the rabbit SA node cell, but even there I_T had only about one-fifth the current density of I_L [16]. The significance of the predominance of I_T in embryonic cardiac cells is not clear. But a hint as to a possible molecular basis for the larger embryonic I_T may come from the role of surface glycoproteins in regulating ionic currents. Yee et al. [41] found that in 25–50% of guinea-pig ventricle cells, treatment with neuraminidase sufficient to remove a substantial fraction of the surface sialic acid, caused a threefold increase in peak I_T with no enhancing effect on I_L, and without affecting the kinetics of either current or the position of their steady-state inactivation curves on the voltage axis. The significance of this is that the sialic acid content of embryonic chick ventricular tissue (14 nmol/mg protein [28*a*]) is much lower than that measured in adult guinea-pig ventricle (100 nmol/mg protein [41]).

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