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Differences in isoproterenol stimulation of Ca^{2+} current of rat ventricular myocytes in neonatal compared to adult

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Abstract

The developmental changes in the isoproterenol stimulation of the L-type calcium current ($I_{Ca(L)}$) were studied in freshly isolated neonatal (3–5-day-old) and adult (2–3-month-old) rat ventricular myocytes using whole-cell voltage clamp (at room temperature). $I_{Ca(L)}$ was measured as the peak inward current at a test potential of +10 mV (or +20 mV) by applying a 300 ms pulse from a holding potential of −40 mV. The pipette solution was Cs^+-rich and Ca^{2+}-free. The external solution was Na^+-free and K^+-free. Isoproterenol stimulated $I_{Ca(L)}$ in a dose-dependent manner. The concentrations of isoproterenol for half-maximal effect were 6.8 nM in neonatal and 13.3 nM in adult. The maximal stimulation of $I_{Ca(L)}$ was 147 ± 14% in neonatal and 97 ± 7% in adult. The steady-state inactivation curves were not affected by isoproterenol, whereas the steady-state activation curve was shifted to the left in both neonatal and adult. Forskolin (10 μM) increased $I_{Ca(L)}$ by 105 ± 10% in neonatal and 90 ± 12% in adult. After stimulating $I_{Ca(L)}$ by forskolin, the addition of isoproterenol produced a further increase of $I_{Ca(L)}$ by 99 ± 27% in neonatal, but only by 19 ± 3% in adult. The presence of an inhibitor of cAMP-dependent protein kinase in the pipette did not affect this marked difference between neonatal (87 ± 23%) and adult (11 ± 8%). We conclude that, in rat ventricular myocytes, (1) stimulation of $I_{Ca(L)}$ by the β-adrenoceptor agonist, isoproterenol, is already fully developed in the neonatal stage and actually decreases during development; (2) there is evidence for a cAMP-independent stimulation of Ca^{2+} channels by isoproterenol, and this is greater in neonatal than in adult. We believe that the cAMP-independent pathway is the direct pathway mediated by $G_{s\alpha}$ protein.

Keywords: β-Adrenoceptor; Heart, neonatal; Ca^{2+} current; G protein; Voltage clamp, whole cell

1. Introduction

Calcium current through the slow (L-type) Ca^{2+} channels plays an important role in excitation-contraction coupling in cardiomyocytes (Morad and Cleemann, 1987; Callewaert, 1992). The modulation of this channel by β-adrenoceptor agonists and muscarinic acetylcholine agonists from sympathetic and parasympathetic nerves is a major mechanism for the regulation of heart rate and contractility (Levy, 1971; Watanabe and Besch, 1975). Binding of an agonist to a β-adrenoceptor activates a guanine nucleotide-binding protein (i.e., G_s protein). This protein stimulates adenylate cyclase and increases the level of cAMP. This process is followed by the phosphorylation of the $I_{Ca(L)}$ channel via the cAMP-dependent protein kinase (Vogel and Sperelakis, 1981; Kameyama et al., 1985; Fischmeister and Hartzell, 1987; Sumii and Sperelakis, 1995), leading to an increase in channel open probability, mean open time and channel availability (Tsien et al., 1986; Ochi and Kawashima, 1990). This is known as the slower indirect pathway for stimulation of $I_{Ca(L)}$. Muscarinic acetylcholine activation antagonizes the β-adrenergic stimulation of $I_{Ca(L)}$ through an inhibition of adenylate cyclase, which is mediated via the G_i protein (Hescheler et al., 1986).

However, the G_s protein by itself has been reported to stimulate the $I_{Ca(L)}$ channel directly (Yatani et al., 1987; Yatani and Brown, 1989; Pelzer et al., 1990). This is known as the faster direct pathway. Yatani et al. (1987) showed that the activated G_s protein α subunit ($^*G_{s\alpha}$) prolonged the survival of excised channels and stimulated the activity of channels incorporated into lipid bilayers. They showed that the product of $N P_o$ (where N is the number of functional channels in the bilayer and P_o is the open probability) was markedly increased when $^*G_{s\alpha}$ was

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added to the cis-compartment of the bilayer. It is accepted that the direct pathway for regulation of $I_{Ca(L)}$ is present in neurons (Holz IV et al., 1986; Dolphin and Scott, 1987; Bergamaschi et al., 1988). However, the presence of a direct pathway in cardiomyocytes is controversial. For example, it was reported that the G_s protein activated by β-adrenoceptor agonists was not coupled to the slow Ca^{2+} channels in frog, rat and guinea-pig cardiomyocytes (Hartzell et al., 1991; Huguet et al., 1992).

Several reports have focussed on the age-related changes in the effect of β-adrenoceptor agonists on the heart. For example, it was reported that the myocardial β-adrenoceptor responsiveness was decreased in healthy geriatric human subjects (Schulman et al., 1992; Stratton et al., 1992), and that there are developmental changes in β-adrenoceptor density. The number of β-adrenoceptors is higher in neonatal/young rat than in adult rat (Kojima et al., 1990), mouse (Chen et al., 1979), dog (Rockson et al., 1981) and rabbit (Feng et al., 1989). Similar findings were also reported in human atrial (Brodde et al., 1995) and ventricular (White et al., 1994) myocytes. However, to our knowledge, only one report (Osaka and Joyner, 1992) has been published about the effects of β-adrenoceptor agonists on $I_{Ca(L)}$ during postnatal development in mammalian ventricular cells using whole-cell voltage clamp. They reported that, in rabbit ventricular myocytes, the effect of the β-adrenoceptor agonist, isoproterenol, is greater in adult than in neonatal. Since this finding is opposite to the findings from the biochemical studies stated above (although this is not necessarily the same as functional activity of β-adrenoceptors), the postnatal developmental changes of the functional activity of β-adrenoceptors are still unclear.

This study was designed to test the functional activity of the β-adrenoceptor during postnatal development in rat ventricular cells using whole-cell voltage clamp. These developmental changes are important for understanding the regulation of developing hearts in vivo by the sympathetic nerves.

2. Materials and methods

2.1. Cell preparation

Freshly isolated single cells were prepared from ventricles of neonatal (3–5-day-old) and adult (2–3-month-old) Sprague-Dawley rats. The rats were decapitated under CO_2 anesthesia, and the hearts were removed and rinsed in oxygenated Tyrode solution, then immersed in Ca^{2+}-free Tyrode solution for 30 min in room temperature. The ventricles were dissected after the spontaneous beatings had ceased and small pieces of the ventricles were enzymatically digested for 20–40 min for neonatal and 60 min for adult in Ca^{2+}-free Tyrode solution (37°C) containing collagenase (0.3–0.5 mg/ml for neonatal and 1.0 mg/ml for adult, Wako, Japan). After incubation, the cells were mechanically dispersed in the modified KB (Kraftbruhe) solution using a Pasteur pipette. The cell suspension was stored in a refrigerator (4°C) until used.

2.2. Solution and drugs

The normal Tyrode solution contained (in mM): NaCl 143, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.5, Hepes 5 and pH adjusted to 7.4 with NaOH. The modified KB solution contained (in mM): K-glutamate 50, KOH 20, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 0.5, Hepes 10, and pH adjusted to 7.4 with KOH. The external test (Na^+-free and K^+-free) solution contained (in mM): tetraethylammonium chloride 150, $CaCl_2$ 1.8, $MgCl_2$ 0.5, 4-aminopyridine 3, Hepes 5, glucose 5.5, and pH adjusted to 7.4 with HCl. The pipette solution contained (in mM): CsOH 110, CsCl 20, L-glutamic acid 110, $MgCl_2$ 3, adenosine triphosphate disodium salt 5, creatine phosphate disodium salt 5, EGTA 10, Hepes 5, and pH adjusted to 7.2 with CsOH.

(−)-Isoproterenol hydrochloride (Sigma) was dissolved in distilled water (1 or 10 mM) with equimolar ascorbic acid, and was made fresh daily. Forskolin (Sigma) was dissolved in dimethyl sulfoxide to provide stock solution (25 or 50 mM). Maximal concentration of dimethylsulfoxide (0.2%) did not affect Ca^{2+} currents. The drugs were diluted by test solution to a given concentration. The cAMP-dependent protein kinase inhibitor peptide (Wiptide; 5–22) (Peninsula Labs.) was dissolved in the internal solution directly.

2.3. Whole-cell voltage clamp recording

Voltage-clamp recordings were performed in the whole-cell configuration of the patch-clamp method by using patch clamp amplifier (Axopatch-1D, Axon Instruments, USA) and fire-polished borosilicate glass pipettes (World Precision Instruments, USA) with resistances of 2–6 MΩ when filled with the pipette solution. The cell suspension was placed into a small chamber (1.4 ml, which contained external test solution) on the stage of an inverted microscope (Diaphoto, Nikon, Japan). Then they were constantly perfused with the external test solution at a speed of 1.8 ml/min.

The $I_{Ca(L)}$ were elicited from a holding potential of −40 mV to a test potential of +10 (or +20, depend on peak currents) for 300 ms every 15 s. The currents were abolished completely by 2 mM Co^{2+} and 1 mM Cd^{2+} consistent with the current being carried through a Ca^{2+} channel.

Current/voltage (I/V) curves were obtained by applying voltage steps in 10 mV increments (−40 mV to +60 mV) from a holding potential of −40 mV.

The steady-state inactivation curves were determined by using a double-pulse protocol: Conditioning pulse duration was 5 s and test pulse duration was 300 ms. The pulses

were applied to test potential of +10 mV from various conditioning pulse level (from −100 mV to +20 mV). There was a 5 ms interval to allow for resetting of the activation gate between the end of conditioning pulse and the beginning of the test pulse in which the membrane potential was returned to the holding potential of −80 mV.

For steady-state activation curves, the conductance (g_{Ca}) was calculated from the equation: $g_{Ca} = I_{Ca}/(V_m - V_{rev})$, where I_{Ca} is the peak current elicited by depolarizing test pulse to various potentials and V_{rev} is the reversal potential (obtained from the extrapolated I/V curves). Max g_{Ca} is the maximum Ca^{2+} conductance. The points for g_{Ca}/max g_{Ca} were plotted against the membrane potential as a relative amplitude.

Leak and residual capacitative currents were subtracted using currents elicited by small hyperpolarizing pulses (P/4 protocol). Current and voltage signals were filtered with cut-off frequency of 1 kHz and digitized by an A/D converter (TL-1, Axon Instruments) and sampled at 2.5 kHz. Current and voltage signals were stored in IBM-AT personal computer using the pCLAMP software (ver. 5.0 or 6.0, Axon Instruments). Membrane capacitance (C_m) was determined from the current amplitude elicited in response to a depolarizing and hyperpolarizing ramp voltage pulses at a rate of 0.8 V/s (first phase; depolarize to −30 mV from a holding potential of −50 mV, second phase; return to a holding potential of −50 mV) to avoid interference by any time-dependent ionic currents. Currents recordings included in this paper were obtained after $I_{Ca(L)}$ was stabilized (usually 5–10 min after breaking the membrane). All experiments were carried out at room temperature (23–25°C).

All data are presented as mean ± S.E.M. Statistical analyses were performed using Student's paired or unpaired t test and $P < 0.05$ was defined as significant.

3. Results

The membrane capacitance (pF) was 20.8 ± 0.8 in neonatal ($n = 52$) and 124.8 ± 7.0 in adult ($n = 56$). The basal Ca^{2+} current density (pA/pF) was 6.1 ± 0.4 ($n = 52$) in neonatal and 6.7 ± 0.3 ($n = 56$) in adult.

3.1. Dose-dependent effect of isoproterenol on $I_{Ca(L)}$

Fig. 1 shows the dose-response relationships for the stimulatory effect of isoproterenol on $I_{Ca(L)}$ in neonatal and adult rat ventricular cells. The maximal effect was obtained at ~0.1 μM isoproterenol in both groups. 10 μM isoproterenol produced a lower stimulation of $I_{Ca(L)}$ of 94 ± 17% ($n = 3$) in neonatal cells. Data points were fitted to the Hill equation, that is,

$$\text{stimulation}\,(\%) = \frac{[\text{drug}]^{n_H}}{[\text{drug}]^{n_H} + EC_{50}^{n_H}} \times E_{max}$$

where E_{max} is the maximum stimulatory effect, n_H is the Hill coefficient, EC_{50} is the concentration for half-maximal effect of the drug. The E_{max}, EC_{50} and n_H were respectively 146%, 6.7 nM and 1.6 in neonatal and 96%, 12.7 nM and 1.6 in adult. The value of E_{max} in adult is very close to the values reported previously in adult rat ventricular myocytes (Mitchell et al., 1983; Scamps et al., 1990). EC_{50} value is close to the value reported previously for rat (Scamps et al., 1990), but smaller than the value reported for rabbit (Osaka and Joyner, 1992). Thus, there are some species differences. Fig. 2 shows the effect of isoproterenol on $I_{Ca(L)}$ in neonatal and adult. After $I_{Ca(L)}$ were stabilized, the addition of isoproterenol (2 μM) increased the $I_{Ca(L)}$ by 147% ($a = 103$ pA; $b = 254$ pA) in neonatal (Fig. 2A) and 86% ($a = 1000$ pA; $b = 1860$ pA) in adult (Fig. 2B). The maximum effect of isoproterenol were obtained within 3–5 min in both groups.

3.2. Voltage-dependent effect of isoproterenol on $I_{Ca(L)}$

Fig. 3 shows the current/voltage relationships before and after application of isoproterenol (2 μM) in neonatal (Fig. 3A: $n = 6$) and adult (Fig. 3B: $n = 7$). After application of isoproterenol, the potential giving maximal $I_{Ca(L)}$ was shifted to the left from 19.2 ± 2.5 mV to 15.9 ± 2.3 mV ($P < 0.05$) in neonatal and from 9.7 ± 0.3 mV to 2.9 ± 0.7 mV ($P < 0.001$) in adult. Table 1 shows the changes of the time course of inactivation of $I_{Ca(L)}$ (at a test potential of +10 mV) after application of isoproterenol. In control condition, both fast and slow time constants (τ_f and τ_s) were longer in neonatal than in adult. The τ_f was shortened slightly after application of ISO in neonatal ($P < 0.05$) (τ_s was not changed), whereas it was

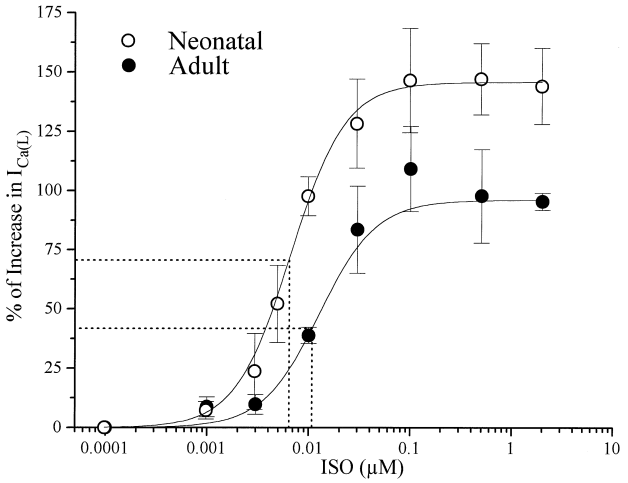

Fig. 1. Dose-response curves of isoproterenol on $I_{Ca(L)}$ in neonatal and adult rat ventricular myocytes. The ordinate represents the percent increase of $I_{Ca(L)}$ over control levels. Data points are mean ± S.E.M. These data were fitted to the Hill equation. The maximum stimulatory effect (E_{max}), Hill coefficient (n_H) and the concentration for half-stimulation (EC_{50}) by the drug were 146%, 6.7 nM and 1.6 in neonatal and 96%, 12.7 nM and 1.6 in adult, respectively.

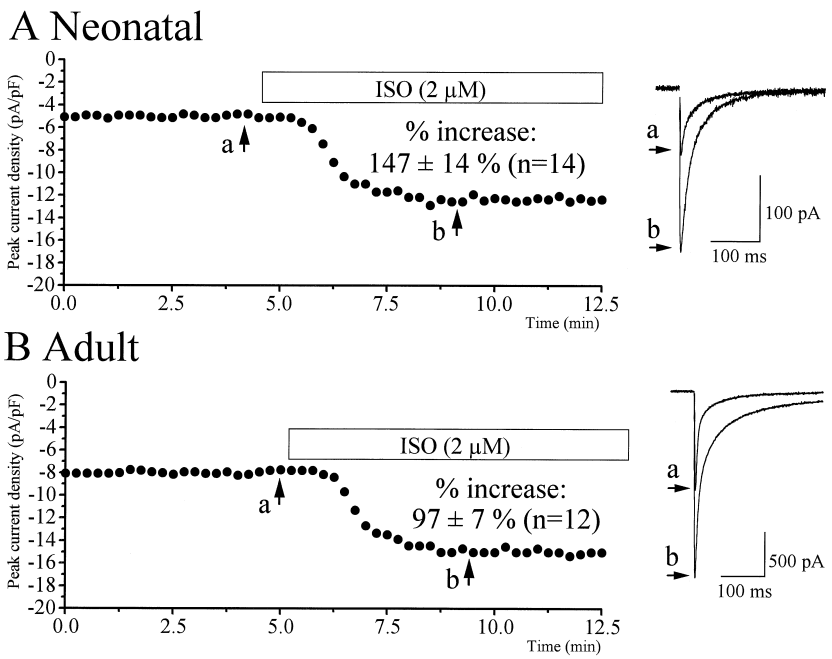

Fig. 2. Effect of isoproterenol on $I_{Ca(L)}$ in neonatal and adult rat ventricular myocytes. $I_{Ca(L)}$ was measured as the peak current at a test potential of +20 mV in neonatal and +10 mV in adult by applying 300 ms duration pulse from a holding potential of −40 mV. Time courses of the effect of ISO on 3-day-old neonatal (C_m = 20 pF) (panel A) and adult (C_m = 124 pF) (panel B) are shown. After $I_{Ca(L)}$ was stabilized, the application of isoproterenol (2 μM) increased the current by 147% (a = 103 pA; b = 254 pA) in neonatal (A) and 86% (a = 1000 pA; b = 1860 pA) in adult (B). Selected current traces are illustrated at points denoted. Summarized data are shown in panels A and B. There was significant difference between neonatal and adult.

prolonged in adult ($P < 0.001$). Even though these small differences were statistically significant, they may not be physiologically significant, especially since the possible spontaneous release of Ca^{2+} from the sarcoplasmic reticulum was not controlled. However, Josephson and Sperelakis (1991) reported that isoproterenol or cAMP speeds the kinetics of the Ca^{2+} channel gating charge movement.

To further clarify the characteristics of the effect of isoproterenol on $I_{Ca(L)}$, steady-state inactivation (f_∞) and activation (d_∞) were studied (Fig. 4). The f_∞ curves were obtained by a double-pulse protocol (inset in Fig. 4A). The peak current elicited by test pulses (from a prepulse of −100 mV) was normalized and plotted against the conditioning potential. These points were fitted to the Boltzmann equation: $1/\{1 + \exp[(V_m - V_h)/k]\}$, where V_m is the conditioning potential, V_h is the potential required for half-inhibition of current and k is the slope factor. In neonatal ($n = 6$), the values of V_h and k were −30 mV and 5.9 mV in control, and −31 mV (n.s.) and 5.8 mV (n.s.) after application of isoproterenol. In adult ($n = 7$),

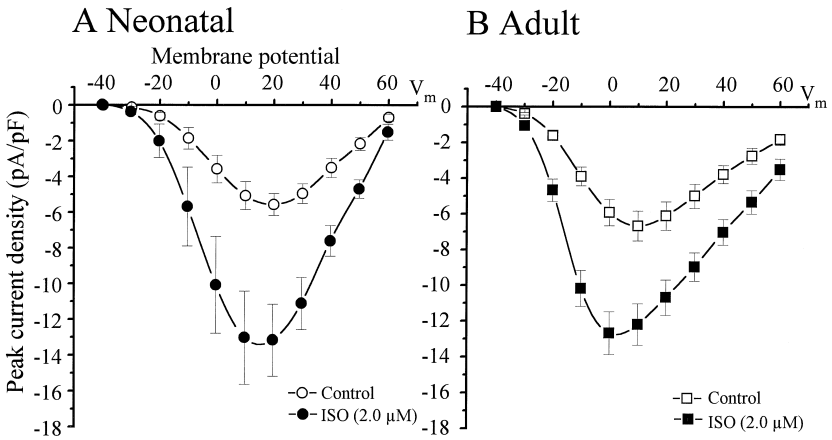

Fig. 3. Current/voltage relationships taken before (open symbols) and after (closed symbols) application of isoproterenol (2 μM) in neonatal (A) ($n = 6$) and adult (B) ($n = 7$) rat ventricular myocytes. The ordinate represents current density (pA/pF) obtained by normalizing the currents to the membrane capacitances. After application of isoproterenol, the potential for peak current was shifted to the left, slightly but significantly, from 19.2 ± 2.5 mV to 15.9 ± 2.3 mV ($P < 0.05$) in neonatal and from 9.7 ± 0.3 mV to 2.9 ± 0.7 mV ($P < 0.001$) in adult.

those values were −24 mV and 4.6 mV in control, −25 mV (n.s.) and 6.2 mV (n.s.) after isoproterenol. There were no significant differences in both V_h and k factors before and after isoproterenol.

The d_∞ curves were obtained from the method described in Section 2. The data were fitted to the Boltzmann equation. In neonatal ($n = 6$), the values of V_h and k were 5.2 ± 3.1 mV and 8.1 ± 0.7 in control, and 1.7 ± 2.9 mV ($P < 0.01$) and 8.0 ± 1.3 (n.s.) after isoproterenol. In adult ($n = 7$), those values were -8.3 ± 0.7 mV and 7.1 ± 0.3 in control and -14.0 ± 0.8 ($P < 0.001$) and 6.3 ± 0.4 ($P < 0.05$) after isoproterenol. Therefore the steady-state activation curve for adult was shifted to the left by 5.7 mV (Fig. 4B), whereas that for the neonate was shifted by only 3.5 mV (Fig. 4A).

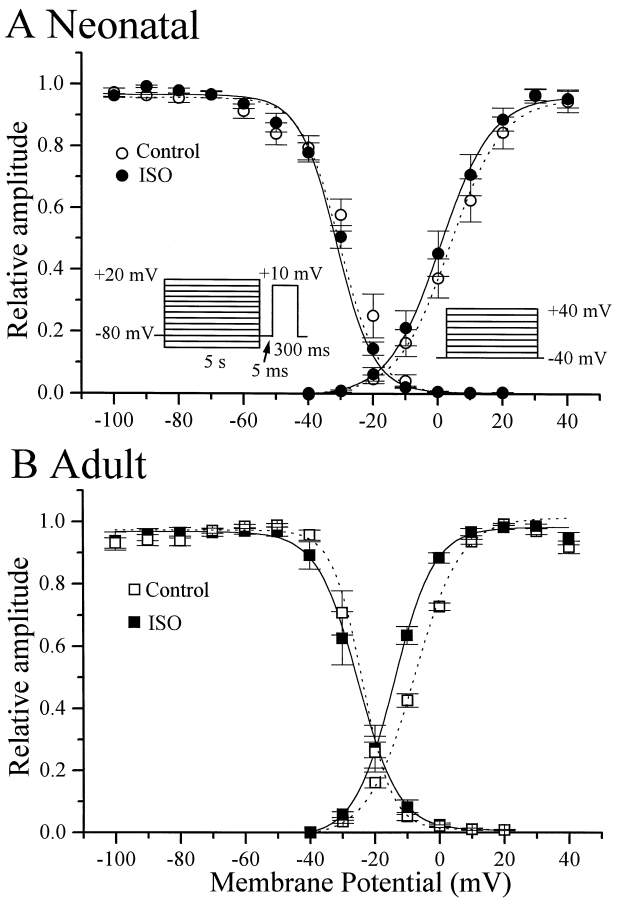

Fig. 4. Steady-state inactivation (f_∞) and activation (d_∞) curves of $I_{Ca(L)}$ before and after application of isoproterenol in rat ventricular myocytes. The f_∞ curves; in neonatal ($n = 6$), the values of the half-inactivation potential (V_h) and the slope factor (k) were −30 mV and 5.9 mV in control, −31 mV (n.s.) and 5.8 mV (n.s.) in the presence of isoproterenol. In adult ($n = 7$), those values were −24 mV and 4.6 mV in control, −25 mV (n.s.) and 6.2 mV (n.s.) in the presence of isoproterenol. The d_∞ curves; in neonatal ($n = 6$), the values of V_h and k were 5.2 ± 3.1 mV and 8.1 ± 0.7 mV in control, 1.7 ± 2.9 mV ($P < 0.01$) and 8.0 ± 1.3 mV (n.s.) in the presence of isoproterenol. In adult ($n = 7$), those values were -8.3 ± 0.7 mV and 7.1 ± 0.3 mV in control, -14.0 ± 0.8 mV ($P < 0.0001$) and 6.3 ± 0.4 mV ($P < 0.05$) in the presence of isoproterenol. Pulse protocols are given in the inset.

Table 1
Time course of inactivation (at +10 mV) of $I_{Ca(L)}$ before and after application of isoproterenol in neonatal and adult rat ventricular myocytes

		τ_f (ms)	τ_s (ms)
Neonatal ($n = 6$)	Control	9.8 ± 0.4	69.1 ± 6.6
	Isoproterenol	8.1 ± 0.9 [a]	57.5 ± 2.7
Adult ($n = 6$)	Control	6.3 ± 0.4	51.0 ± 2.4
	Isoproterenol	8.9 ± 0.5 [b]	63.3 ± 2.0 [b]

[a] $P < 0.05$, [b] $P < 0.001$ compared to control.

3.3. Effect of forskolin on $I_{Ca(L)}$

To investigate the activity of adenylate cyclase on $I_{Ca(L)}$ in neonatal and adult, we used forskolin, which is known as a direct activator of adenylate cyclase, to evaluate its activity. Fig. 5 shows the representative time courses and their current traces of the effect of forskolin on $I_{Ca(L)}$. After $I_{Ca(L)}$ were stabilized, the addition of forskolin (10 μM) increased the $I_{Ca(L)}$ by 103% ($a = 79$ pA; $b = 160$ pA) in neonatal (Fig. 5A) and 98% ($a = 787$ pA; $b = 1559$ pA) in adult (Fig. 5B). There were no significant differences in both groups.

3.4. Effect of isoproterenol on $I_{Ca(L)}$ in the presence of forskolin

Fig. 6 shows the representative time courses of isoproterenol responses in the presence of forskolin and their current traces. After $I_{Ca(L)}$ were fully stimulated by forskolin (10 μM), the addition of isoproterenol (0.5 μM) produced further stimulation by 97% ($a = 157$ pA; $b = 309$ pA) in neonatal (Fig. 6A) and only 24% ($a = 1559$ pA; $b = 1935$ pA) in adult (Fig. 6B). The effect of isoproterenol in the presence of forskolin in adult was close to the previous report in guinea pig (Shuba et al., 1993), showing that isoproterenol + forskolin produced further increase of $I_{Ca(L)}$ by 19%. Although the presence of the direct pathway of isoproterenol remains controversial, one possibility for explaining the greater effect of isoproterenol compared to forskolin could be that isoproterenol may activate both direct and indirect pathway, whereas forskolin can activate only the indirect pathway.

3.5. Effect of isoproterenol on $I_{Ca(L)}$ in presence of cAMP-dependent protein kinase inhibitor

To eliminate the indirect pathway for stimulation of the Ca^{2+} channel, we used some cells pre-incubated for 3–5 h (at room temperature) in a solution containing high concentration (30 μM) of the non-specific inhibitor of kinases, H-7. However, even under these conditions, forskolin (10 μM) could still stimulate $I_{Ca(L)}$ by $42 \pm 10\%$ ($n = 5$). Therefore, another approach was used. An inhibitor of cAMP-dependent protein kinase, namely PKI, was used to evaluate the direct pathway of isoproterenol

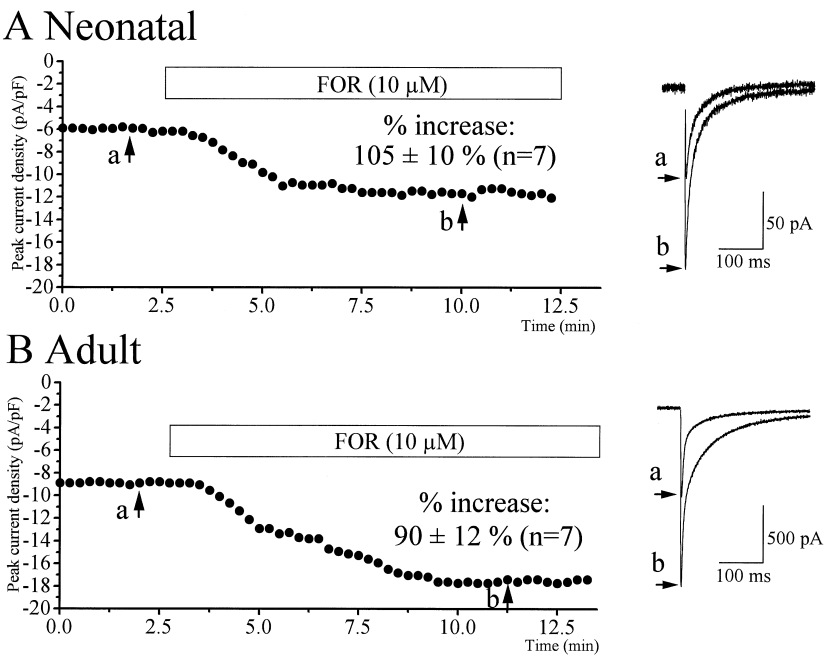

Fig. 5. Effect of forskolin on $I_{Ca(L)}$ in neonatal and adult rat ventricular myocytes. Time courses of the effect of forskolin in 3-day-old neonatal (*A*) (C_m = 14 pF) and adult (*B*) (C_m = 89 pF) are shown. After $I_{Ca(L)}$ was stabilized, the application of forskolin (10 μM) increased the currents by 103% (a = 79 pA; b = 160 pA) in neonatal (*A*) and 98% (a = 787 pA; b = 1559 pA) in adult (*B*). Selected current traces are illustrated at points denoted on the time course. Summarized data are shown in *A* and *B*. There was no significant difference between neonatal and adult.

stimulation (Fig. 7). The stimulation of the $I_{Ca(L)}$ by forskolin in the presence of 15 μM, 25 μM and 100 μM PKI in the pipette was 128 ± 30% (n = 3), 33 ± 15% (n = 6) and 10 ± 8% (n = 5), respectively, in the adult. In neonatal myocytes, the stimulation of the $I_{Ca(L)}$ by forskolin in the presence of 25 μM PKI in the pipette was 6.5 ± 6.5% (n = 4). Thus, even PKI could not completely suppress the effects of forskolin (even though the recording was started

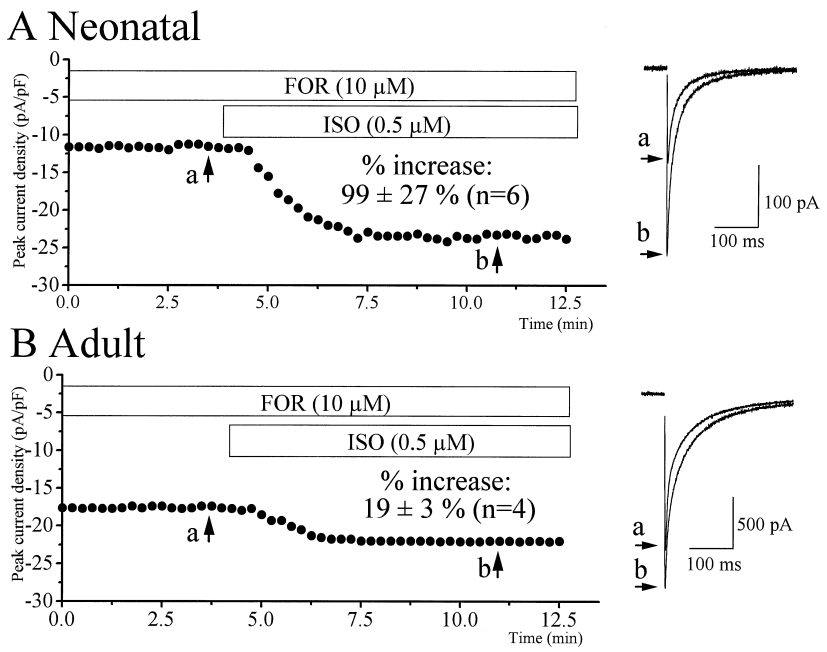

Fig. 6. Effect of isoproterenol on $I_{Ca(L)}$ in the presence of forskolin in neonatal and adult rat ventricular myocytes. Time courses of the effect of isoproterenol in the presence of forskolin on the peak $I_{Ca(L)}$ in 3-day-old neonatal (*A*) (C_m = 14 pF) and adult (*B*) (C_m = 89 pF) are shown. After stimulated $I_{Ca(L)}$ by forskolin (10 μM) was stabilized, the addition of isoproterenol (0.5 μM) produced a further increase of $I_{Ca(L)}$ by 97% (a = 157 pA; b = 309 pA) in neonatal (*A*) and only 24% (a = 1559 pA; b = 1935 pA) in adult (*B*). Selected current traces are illustrated at points denoted on the time course. Summarized data are shown in *A* and *B*. There was significant difference between neonatal and adult.

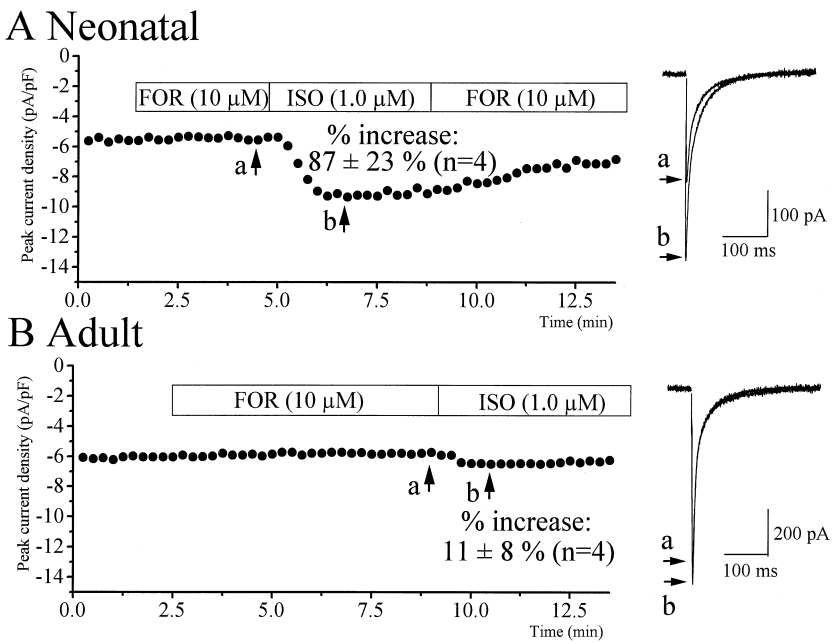

Fig. 7. Effect of isoproterenol on $I_{Ca(L)}$ in the presence of cAMP-dependent protein kinase inhibitor (PKI) in the pipette in neonatal and adult rat ventricular myocytes. Time courses of the effect of isoproterenol (1.0 μM) in the presence of PKI (25 μM) on the peak $I_{Ca(L)}$ in 5-day-old neonatal (*A*) (C_m = 35 pF) and adult (*B*) (C_m = 135 pF) are shown. After $I_{Ca(L)}$ was stabilized, the effects of forskolin and isoproterenol were tested. The addition of isoproterenol produced an increase of $I_{Ca(L)}$ by 73% (a = 238 pA; b = 411 pA) in neonatal (*A*) and only 11% (a = 776 pA; b = 862 pA) in adult (*B*). Selected current traces are illustrated at points denoted on the time course. Summarized data are shown in *A* and *B*. There was significant difference between neonatal and adult.

10 min or longer after breaking the membrane). We used 25 μM PKI, which is a higher concentration than that used in previous reports (Parsons and Hartzell, 1993; Kameyama et al., 1986). For analysis, we used only those cells which were stimulated by FOR by 30% or less in the presence of 25 μM PKI. Fig. 7 shows the representative data for isoproterenol stimulation in the presence of PKI. After $I_{Ca(L)}$ become stabilized in the presence of PKI, the effects of forskolin and isoproterenol were tested. The addition of isoproterenol produced further stimulation by 73% (a = 238 pA; b = 411 pA) in neonatal (Fig. 7A) and only 11% (a = 776 pA; b = 862 pA) in adult (Fig. 7B). The data from Figs. 6 and 7 are summarized in Fig. 8.

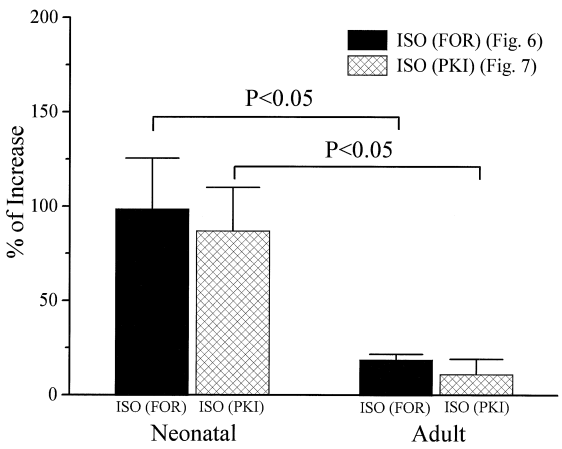

Fig. 8. Summarized data from Fig. 6 and Fig. 7. There were statistically significant differences in neonatal and adult in both experiments.

4. Discussion

The present study was designed to elucidate the postnatal changes in the β-adrenergic modulation of $I_{Ca(L)}$ in rat ventricular myocytes using whole-cell voltage clamp. The main findings can be summarized as follows. (1) The effect of isoproterenol on $I_{Ca(L)}$ was significantly greater in neonatal than in adult (as illustrated in Fig. 1). (2) The effect of forskolin on $I_{Ca(L)}$ did not change during this developmental period. (3) The additive effect of isoproterenol on $I_{Ca(L)}$ in the presence of forskolin was significantly greater in neonatal than in adult. (4) The effect of isoproterenol in the presence of cAMP-dependent protein kinase inhibitor (to inhibit phosphorylation by cAMP-dependent protein kinase) was also considerably greater in neonatal. These data suggest that the $I_{Ca(L)}$ response to β-adrenoceptor stimulation is greater in neonatal than in adult. As one possibility, there may exist the greater response of the cAMP-independent pathway in neonatal myocytes to explain this greater response to the β-adrenoceptor agonist. We believe that this is the direct pathway mediated by $G_{s\alpha}$ protein.

4.1. Developmental change in isoproterenol effect on $I_{Ca(L)}$

The number of β-adrenoceptors (density) in various species of animals (e.g., rat, mouse, rabbit and dog) increases during development from the fetal level to the neonatal/young level, and then decreases to the adult level (Chen et al., 1979; Rockson et al., 1981; Feng et al., 1989; Kojima et al., 1990; Tobise et al., 1994). The same tendency was also reported for human atrial (Brodde et al., 1995) and ventricular (White et al., 1994) myocytes. The present study showed that the effect of isoproterenol on $I_{Ca(L)}$ was greater in neonatal than in adult, in agreement with some previous biochemical reports showing that the β-adrenoceptor density was greater in neonatal than in adult rat (Kojima et al., 1990; Tobise et al., 1994). The greater ability of isoproterenol to stimulate $I_{Ca(L)}$ in neonatal may be explained, in part, by the greater number of β-adrenergic receptors. The present study focused on the postnatal developmental changes, while Masuda et al. (1996) reported on the perinatal developmental changes on the effect of isoproterenol on $I_{Ba(L)}$ in rat ventricular myocytes. They showed that isoproterenol had little or no effect on fetal days 15 and 18 (term in rat is 22 days), but had a marked stimulatory effect on $I_{Ba(L)}$ from fetal day 20 through neonatal day 10, and also showed that the time course of development of sensitivity to forskolin was similar to that of isoproterenol.

Roeske and Wildenthal (1981) observed that a decline in myocardial β-adrenoceptor density in mice from neonatal to adult stages coincident with cardiac sympathetic innervation and a rising concentration of tissue catecholamine. These observations support their contention that receptor downregulation occurs as an adaptive mechanism maintaining cardiovascular homeostasis as sympathetic innervation of the heart matures.

In contrast to our results, Osaka and Joyner (1992) reported that the efficacy of isoproterenol to stimulate $I_{Ca(L)}$ was significantly greater in *adult* than in *neonatal* myocytes, whereas the efficacy of forskolin to stimulate $I_{Ca(L)}$ was significantly greater in *neonatal* than in *adult* myocytes. They explained this discrepancy (i.e., the lesser effect of isoproterenol than that of forskolin in neonatal) by a high tonic activity of the G_i protein in early development.

In our new preliminary data, 100 μM carbachol (muscarinic acetylcholine agonist) inhibited the forskolin (10 μM)-stimulated $I_{Ca(L)}$ nearly completely ($98 \pm 2\%$) in neonatal rat ventricular myocytes and by only $18 \pm 3\%$ in adult. These results can be explained by at least two possibilities: (1) a decrease in the number (density) of muscarinic acetylcholine receptor in the ventricular myocardial cells of adult compared to neonatal (Nedoma et al., 1986; Kojima et al., 1990) and (2) the G_i system is weaker in adult than in neonatal (Osaka et al., 1993; Kumar et al., 1994).

4.2. Developmental changes in forskolin effect on $I_{Ca(L)}$

Forskolin directly interacts with the catalytic subunit of adenylate cyclase (without the requirement of G_s protein) leading to the stimulation of cAMP production, cAMP-dependent protein kinase activation and phosphorylation of the slow Ca^{2+} channel. There are some reports on the developmental changes of adenylate cyclase activity (i.e., the accumulation of cAMP by isoproterenol or forskolin). However, these reports are controversial and there are species' differences. Adenylate cyclase activity was reported to significantly increase in dog from neonatal to adult (Rockson et al., 1981; Vulliemoz et al., 1984), but did not change in rabbit (Hatjis, 1986). In contrast, adenylate cyclase activity was reported to decrease in rat (Tobise et al., 1994), rabbit (Kumar et al., 1994), human atrial (Brodde et al., 1995) and human ventricular (White et al., 1994) myocytes.

Even though 10 μM forskolin was reported to almost maximally stimulate adenylate cyclase (Osaka and Joyner, 1992; Hartzell and Budnitz, 1992), we used 100 μM forskolin to evaluate whether 10 μM was the saturating dose for stimulation of Ca^{2+} current. 100 μM forskolin stimulated Ca^{2+} current by $123 \pm 15\%$ ($n = 5$) in neonatal and $107 \pm 15\%$ ($n = 7$) (n.s.) in adult. These values are only a little greater than those obtained with 10 μM forskolin in both groups. Therefore, 10 μM forskolin was considered to be an almost saturating dose. Our results showed that there was a marked stimulation of $I_{Ca(L)}$ by 10 μM forskolin in the neonatal stages. In fact, the stimulation by forskolin was slightly greater in neonatal than in adult (but was not statistically significant). Thus, the catalytic subunit of adenylate cyclase and the cascade leading from adenylate cyclase to Ca^{2+} channel phosphorylation is already fully developed in the neonatal stage of rat ventricular myocytes.

4.3. Developmental changes in the direct pathway

Some possible reasons why β-adrenoceptor agonists have a smaller effect on $I_{Ca(L)}$ in adult than in neonatal are (1) the number of β-adrenoceptors decrease (Chen et al., 1979; Tanaka and Shigenobu, 1990; Tobise et al., 1994); (2) the activity of G_s protein is lowered (McMahon, 1989); (3) the activity of adenylate cyclase is reduced (Tobise et al., 1994; Kumar et al., 1994); (4) the degree of tonic inhibition of the channels via muscarinic acetylcholine receptor/G_i protein is increased (Kojima et al., 1988, 1990; Luetje et al., 1988; McMahon, 1989; Osaka et al., 1993; Kumar et al., 1994); (5) the direct stimulation pathway via $G_{s\alpha}$ protein is reduced.

We focussed our attention on the developmental change of the direct stimulation of $I_{Ca(L)}$ channels by isoproterenol via $G_{s\alpha}$ protein, i.e., the direct pathway. To evaluate the direct action of $G_{s\alpha}$ protein on $I_{Ca(L)}$ channels, we per-

formed two different types of experiments. One type was to determine the effect of isoproterenol after the adenylate cyclase was fully activated by forskolin. The other type was to determine the effect of isoproterenol after cAMP-dependent protein kinase was inhibited by PKI. The results of these experiments suggest that the direct pathway is present, and that this direct pathway may play a more important role in the β-adrenoceptor agonist stimulation of $I_{Ca(L)}$ in neonatal than in adult. However, caution must be exercised because, in our experimental condition, cAMP-dependent protein kinase was not completely inhibited by 25 μM PKI, and there are possibility of inadequate phosphorylation of Ca^{2+} channel even after applying the saturating dose of forskolin. Furthermore, Ca^{2+} channel activity is not only regulated by cAMP/cAMP-dependent protein kinase, but is regulated also by other kinases (such as protein kinase-C, cGMP-dependent protein kinase, calmodulin-dependent protein kinase and tyrosine kinase) and by phosphatases. Therefore, to prove the existence of the direct pathway, stimulation of $I_{Ca(L)}$ and of single-channel activity by exposure to $^{*}G_{s\alpha}$ should be demonstrated.

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