Developmental Changes in Modulation of Calcium Currents of Rabbit Ventricular Cells by Phosphodiesterase Inhibitors

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**Background** We have previously shown major differences in β-adrenergic and muscarinic modulation of L-type calcium currents (I\textsubscript{Ca}) in newborn and adult rabbit heart. However, little is known about developmental changes in modulation of I\textsubscript{Ca} by phosphodiesterases (PDEs), which also regulate intracellular cAMP concentration by its hydrolysis.

**Methods and Results** Enzymatically isolated adult and newborn (1- to 3-day-old) rabbit ventricular myocytes were used to study the effects of PDE inhibitors on I\textsubscript{Ca} measured by the whole-cell patch-clamp method. 3-Isobutyl-1-methyl-xanthine (IBMX), a nonselective PDE inhibitor, increased I\textsubscript{Ca} in a dose-dependent manner for both groups. The maximal effect of IBMX, expressed as percentage increase in I\textsubscript{Ca} over control levels, was greater for newborn myocytes than for adult myocytes, but the effects of IBMX applied alone were observed only at concentrations >10 μmol/L. The concomitant use of 0.1 μmol/L isoproterenol produced a significant potentiation of the IBMX effect on I\textsubscript{Ca}, with a significant additive effect of IBMX in newborn myocytes even at 0.05 μmol/L IBMX. The concomitant use of a subthreshold concentration of IBMX (0.1 μmol/L) did not potentiate the dose dependence of adult I\textsubscript{Ca} on isoproterenol but did markedly potentiate the dose dependence of newborn I\textsubscript{Ca} on isoproterenol. The E\textsubscript{max} and EC\textsubscript{50} of isoproterenol in the presence of 0.1 μmol/L IBMX on newborn I\textsubscript{Ca} were 235% and 8 nmol/L, respectively, whereas the E\textsubscript{max} and EC\textsubscript{50} of isoproterenol in the absence of IBMX on newborn I\textsubscript{Ca} were 111% and 81 nmol/L, respectively. The addition of 50 μmol/L IBMX to 10 μmol/L isoproterenol markedly increased the newborn I\textsubscript{Ca} density up to a level equivalent to that reached with 200 μmol/L cAMP in the pipette (14.9±1.2 versus 13.4±0.7 pA/pF). Our data suggest that the inhibition constant (K\textsubscript{i}) of IBMX for inhibiting PDEs that participate in the regulation of I\textsubscript{Ca} is much lower in newborn than in adult myocytes. Milrinone 1 μmol/L, a selective PDE III inhibitor, increased the 0.1 μmol/L isoproterenol-stimulated I\textsubscript{Ca} of adult myocytes but had no significant additive effect for the 0.1 μmol/L isoproterenol–stimulated I\textsubscript{Ca} of newborn myocytes. Rolipram 1 μmol/L, a selective PDE IV inhibitor, increased the 0.1 μmol/L isoproterenol–stimulated I\textsubscript{Ca} for newborn myocytes but had no significant additive effect for the 0.1 μmol/L isoproterenol–stimulated I\textsubscript{Ca} for adult myocytes.

**Conclusions** These results suggest that the most important PDE isozyme for regulation of I\textsubscript{Ca} of rabbit myocytes changes from PDE IV to PDE III during the postnatal period. (Circulation. 1994;90:469-478.)

**Key Words** calcium • 3-isobutyl-1-methyl-xanthine • phosphodiesterase • milrinone • rolipram

**C**alcium current (I\textsubscript{Ca}) through high-threshold (L-type) Ca\textsuperscript{2+} channels plays an important role of regulating action potential duration as well as excitation-contraction coupling in cardiac myocytes. The modulation of this channel by β-adrenergic agonists and muscarinic acetylcholine agonists from sympathetic and parasympathetic nerve stimulation is a major mechanism for the regulation of heart rate and contractility.

β-Adrenergic agonists enhance I\textsubscript{Ca} in cardiac myocytes by binding of an agonist to a β-adrenergic receptor, which then activates a guanine nucleotide–binding protein, G\textsubscript{β}. This protein then stimulates the activity of adenyl cyclase and produces an increase in the level of intracellular cAMP.\textsuperscript{1} This process is followed by the phosphorylation of the Ca\textsuperscript{2+} channel or its subunits by a cAMP-dependent protein kinase, leading to an increase in channel open probability and channel availability.\textsuperscript{2,3} Muscarinic cholinergic stimulation antagonizes the β-adrenergic stimulation of I\textsubscript{Ca} through an indirect and/or direct inhibition of adenyl cyclase, with this inhibitory response mediated by another G protein, G\textsubscript{i}.\textsuperscript{4}

We recently described postnatal developmental changes of β-adrenergic and muscarinic modulation of I\textsubscript{Ca}\textsubscript{6} in rabbit ventricular myocytes. In summary, the efficacy of isoproterenol to stimulate I\textsubscript{Ca} is lower in newborn (1- to 3-day-old) than in adult rabbit ventricular myocytes, and the efficacy of carbachol to inhibit isoproterenol-stimulated or forskolin-stimulated I\textsubscript{Ca} is much greater in newborn than in adult myocytes. Pertussis toxin treatment of newborn myocytes increased the levels of basal I\textsubscript{Ca}, produced a much stronger response to isoproterenol, and completely blocked the actions of carbachol. These data and previous biochemical studies\textsuperscript{7,9} suggest that a tonic G\textsubscript{i} inhibition of adenyl cyclase is present in newborn myocytes and that the overall balance between influences of G\textsubscript{i} and G\textsubscript{β} on the activity of adenyl cyclase seems to favor G\textsubscript{i} effects in newborn myocytes and G\textsubscript{β} effects in adult myocytes.

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The intracellular cAMP concentration is determined not only by the rate of cAMP production by adenylyl cyclase but also by the rate of hydrolysis by phosphodiesterases (PDEs). Age-related differences of inotropic effects of PDE inhibitors have been reported, with amrinone or milrinone, inhibitors of cGMP-inhibited PDE, having a much weaker inotropic effect on newborn papillary muscle than on adult papillary muscles. Kithas et al\textsuperscript{12} reported postnatal developmental changes in the activity of PDE isozymes in both the soluble and the particulate fractions of rabbit heart muscle.

According to the current guidelines for PDE nomenclature,\textsuperscript{13} cAMP is hydrolyzed to 5'-AMP by at least four classes of PDE isozymes: (1) a Ca-calmodulin–dependent PDE (PDE I), which hydrolyzes both cAMP and cGMP and whose cAMP hydrolytic activity is dependent on Ca\textsuperscript{2+} and calmodulin; (2) a cGMP-stimulated PDE (PDE II), which hydrolyzes both cAMP and cGMP with low affinity (high $K_a$) and whose cAMP hydrolytic activity is stimulated by cGMP; (3) a cGMP-inhibited PDE (PDE III), which hydrolyzes cAMP with high affinity (low $K_a$) and whose cAMP hydrolytic activity is inhibited by cGMP; and (4) a cAMP-specific PDE (PDE IV), which hydrolyzes only cAMP with high affinity ($K_a$) and is also referred to as a cGMP-insensitive or rolipram-sensitive PDE.

The regulation of cAMP hydrolysis is less well documented, and it remains unclear whether and how these different types of PDE contribute to the regulation of I\textsubscript{Ca}.\textsuperscript{14–18} In addition, not all of these PDE types may be functional in cardiac myocytes. Bode et al\textsuperscript{19} were able to separate four PDE types from homogenates of whole rat ventricle, but homogenates produced from isolated ventricular myocytes lacked activity of the calmodulin–dependent PDE I. Furthermore, there are no reports about developmental changes in the role of PDEs in the regulation of I\textsubscript{Ca}. This study was designed to examine postnatal changes in the modulation of L-type I\textsubscript{Ca} in mammalian hearts by inhibitors of PDE enzymes. We compared the effects of 3-isobutyl-1-methyl-xanthine (IBMX, a nonselective PDE inhibitor), milrinone (a selective PDE III inhibitor), and rolipram (a selective PDE IV inhibitor) on L-type I\textsubscript{Ca} in freshly dissociated adult and newborn rabbit ventricular myocytes under nearly identical experimental conditions using the whole-cell voltage clamp technique.

**Methods**

**Cell Preparation**

New Zealand White adult (1.5 to 2.5 kg) and newborn (1- to 3-day-old) rabbits of either sex were used in the experiments. Adult rabbits were first heparinized (1000 U IV) and anesthetized with sodium pentobarbital (50 mg/kg IV). For newborn rabbits, the same drugs were given intraperitoneally. Hearts were rapidly removed via thoracotomy with artificial ventilation, and the aorta was cannulated. Single ventricular myocytes were obtained from adult and newborn hearts by enzymatic dissociation as we previously described.\textsuperscript{20} In brief, hearts were mounted on a Langendorff apparatus and perfused sequentially with normal Tyrode’s solution for 5 to 6 minutes, nominally Ca\textsuperscript{2+}-free Tyrode’s solution for 5 to 8 minutes, the same solution containing 0.05 to 0.12 mg/mL collagenase (Yakult Co) and 0.01 mg/mL protease (type XIV, Sigma Chemical Co) for 5 to 9 minutes, and a storage solution for 5 minutes at a rate of 3 to 4 mL · g\textsuperscript{-1} · min\textsuperscript{-1} at 33°C to 34°C. The ventricle was cut into small pieces, pipetted, and filtered by a mesh. The isolated myocytes were kept in the storage solution at 4°C.

**Solutions**

The compositions of solutions used were as follows (in mmol/L): normal Tyrode’s solution, NaCl 148.8, KCl 4.0, CaCl\textsubscript{2} 1.8, MgCl\textsubscript{2} 0.53, NaHPO\textsubscript{4} 0.33, HEPES 5, glucose 5, pH 7.4 with NaOH; Ca\textsuperscript{2+}-free Tyrode’s solution, NaCl 100, KCl 10, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 5, taurine 50, glucose 20, HEPES 10, pH 7.1 with NaOH; storage solution, potassium glutamate 120, MgCl\textsubscript{2} 5, taurine 20, EGTA 1, glucose 10, HEPES 10, pH 7.4 with KOH; test solution, NaCl 130, CaCl\textsubscript{2} 1.8, CsCl 20, MgCl\textsubscript{2} 0.53, HEPES 5, glucose 5, pH 7.4 with NaOH; and pipette solution, CsOH 110, aspartic acid 90, CsCl 20, HEPES 5, EGTA 10, tetraethylammonium chloride 10, MgATP 5, Na\textsubscript{2} creatine phosphate 5, GTP (Tris) 0.4, leupeptin (Sigma Chemical Co) 0.1, pH 7.2 with CsOH. Pipettes had resistances of 1.5 to 3 MΩ for adult ventricular myocytes and 2.5 to 6 MΩ for newborn ventricular myocytes before seal formation and access resistances of 2.5 to 10 MΩ during the data recordings.

The drugs used in the experiments were isoproterenol (1 HCl, IBMX), milrinone, and milrinone from Sigma. Rolipram was a gift from Berlex Laboratories. Solutions for isoproterenol were freshly prepared from stock solution as described previously.\textsuperscript{5} IBMX, milrinone, and rolipram were dissolved in DMSO at a concentration at least 1000 times the test solution.

**Data Acquisition**

Electrophysiological recordings were conducted using the whole-cell configuration of the patch-clamp technique with an Axopatch-1B patch amplifier (Axon Instruments) as we previously described with compensation for series resistance. For routine monitoring of I\textsubscript{Ca}, the ventricular myocyte was depolarized every 10 seconds from a holding potential of -40 mV to a test potential of +15 mV for 180 milliseconds. No leakage correction was required. This test voltage is based on the peak current of the I-V relations for both control and isoproterenol-stimulated I\textsubscript{Ca} as previously published.\textsuperscript{5,20} We showed previously that currents elicited with this protocol are stable over long periods of time and are completely blocked by nitrendipine.\textsuperscript{20} The elicited I\textsubscript{Ca} was filtered at a corner frequency of 2 kHz, digitized at 200-microsecond intervals, and stored and analyzed on an IBM-compatible computer with pCLAMP software (Axon Instruments). I\textsubscript{Ca} was measured as the peak inward current. All experiments were performed at room temperature (21°C to 23°C). Ventricular myocytes with a membrane capacitance <100 pF were used for the experiments. Cells that had “rundown” of I\textsubscript{Ca} were excluded from the analysis by accepting data only from myocytes in which I\textsubscript{Ca} was stable in the control condition and reached a stable effect with the drugs tested.

Statistical analysis was performed with SYSTAT for Windows (SYSTAT). Statistical significance between two groups was determined by Student’s t test for paired or unpaired data, as appropriate. When more than two groups were compared, statistical significance was determined by one-way ANOVA. When a significant $F$ value was detected, then Tukey’s two-tailed test was performed to determine a significant difference between each group. Values of $P<.05$ were regarded as significant. Data are presented as mean±SEM.

**Results**

**Dose-Dependent Effects of IBMX on Basal I\textsubscript{Ca} Current**

The potentiation of I\textsubscript{Ca} by inhibition of PDEs was examined quantitatively with various concentrations of IBMX for both adult and newborn rabbit ventricular myocytes. Fig 1 shows superimposed I\textsubscript{Ca} current records
in adult (left) and newborn (right) ventricular myocytes. In both adult and newborn myocytes, 10 µmol/L IBMX was the apparent threshold concentration to increase $I_{Ca}$. In the adult cell, after application of 100 µmol/L IBMX, $I_{Ca}$ reached a steady state (122% over the control level) within 10 minutes (inset in lower left of Fig 1), and the $I_{Ca}$ density returned to control levels by subsequently washing out IBMX (data not shown). The stimulatory effect of 100 µmol/L IBMX on newborn $I_{Ca}$ (212% over the control level) was larger than on the adult $I_{Ca}$, with a similar time course.

The dose-dependent effect of IBMX on basal $I_{Ca}$ is summarized in Fig 2. Dose-response relations are presented here and in subsequent figures as percent increase in $I_{Ca}$ because the basal current densities in the present experiments were about twice as great for the adult myocytes (4.21±0.21, n=27) as for the newborn myocytes (2.10±0.11, n=36), similar to values we have presented previously. IBMX caused a dose-dependent increase in $I_{Ca}$ density with a threshold concentration of about 10 µmol/L for both adult and newborn myocytes ($P<.05$ from basal $I_{Ca}$). The effect of IBMX to stimulate basal $I_{Ca}$ at concentrations above 30 µmol/L was significantly greater in newborn than in adult myocytes ($P<.05$). The stimulatory effect of IBMX on $I_{Ca}$ reached maximum at the concentration of about 100 µmol/L for adult myocytes and 300 µmol/L for newborn myocytes, respectively. The continuous curves were derived from nonlinear least-mean-squares regression of the means to the Michaelis equation. The maximal effect ($E_{max}$) of IBMX to stimulate $I_{Ca}$ (expressed as percent increase in $I_{Ca}$ over control levels) and the concentration for one half of the maximal effect ($E_{EC50}$) obtained by this analysis were 159±29% and 71±33 µmol/L, respectively, for adult and 317±31% and 64±20 µmol/L, respectively, for newborn myocytes. Unlike the maximal effect of isoproterenol, which increases $I_{Ca}$ by 203% for adult and 111% for newborn, the maximal effect of IBMX to stimulate basal $I_{Ca}$ was greater in newborn than in adult.

**Dose-Dependent Effects of IBMX on Isoproterenol-Stimulated $I_{Ca}$ Current**

Ventricular myocytes in situ receive β-adrenergic stimulation through circulating catecholamines in the blood and through sympathetic innervation, which stimulates cAMP production by activating adenylyl cyclase. We investigated the effects of IBMX with concomitant application of isoproterenol, since the potency and efficacy of IBMX to stimulate $I_{Ca}$ might be different under conditions of higher cAMP production because the four types of PDE isozymes have different $K_m$ and
Vmax for cAMP and different K1 or IC50 for PDE inhibitors.21-22 Fig 3 (top) shows superimposed IcA current recordings and time courses of IcA peak current (insets) in an adult (left) and a newborn (right) cell before and after application of 0.1 μmol/L isoproterenol and then the application of 0.1 μmol/L isoproterenol plus 0.1 μmol/L IBMX. In the adult cell, 0.1 μmol/L isoproterenol alone increased IcA from 3.4 to 9.1 pA/pF, and the addition of 0.1 μmol/L IBMX had no significant additive effect (9.1 to 9.3 pA/pF). In contrast to the adult cell, the stimulatory effect of 0.1 μmol/L isoproterenol alone on newborn IcA was smaller (2.9 to 4.8 pA/pF), but the additive effect of 0.1 μmol/L IBMX was much greater (4.8 to 8.2 pA/pF).

The lower panels of Fig 3 summarize dose-response relations of the effects of IBMX on IcA with concomitant application of 0.1 μmol/L isoproterenol. Both drugs were applied simultaneously in most of the experiments to minimize the rundown effect on IcA. The application of 0.1 μmol/L isoproterenol without IBMX increases IcA by 120% in adult ventricular myocytes and by 70% in newborn myocytes,3 and these data are included as two horizontal dotted lines in Fig 3. The continuous curves were derived from nonlinear least-mean-squares regression of the means to the Michaelis equation, with the isoproterenol-stimulated current in the absence of IBMX at each age being set as the baseline for that age. The Emax of IBMX to stimulate isoproterenol-stimulated IcA (expressed as percent increase in IcA over the 0.1 μmol/L isoproterenol alone) and the concentration for half-maximal stimulation (EC50) obtained by this analysis were 110±7% and 1.11±0.36 μmol/L for adult and 192±17% and 0.07±0.03 μmol/L for newborn, respectively. With concomitant submaximal β-adrenergic stimulation, the EC50 of IBMX to stimulate IcA decreased about 3 log units for newborn and about 70 times less for adult compared with IBMX alone.

**Dose-Dependent Effects of Isoproterenol with 0.1 μmol/L IBMX on IcA**

We examined whether a subthreshold concentration of IBMX had an effect on the isoproterenol dose-response curve on IcA in adult and newborn myocytes. Fig 4 summarizes the dose-dependent effect of isoproterenol with and without a subthreshold concentration of IBMX (0.1 μmol/L) on IcA in adult (left) and newborn (right) myocytes. The isoproterenol dose-response curves for adult and newborn myocytes without IBMX (thin line and open squares) from our previous work3 were superimposed for comparison.

In adult myocytes, the percent increase in IcA stimulated by low concentrations of isoproterenol (0.01 and 0.1 μmol/L) with 0.1 μmol/L IBMX seemed slightly higher than that by isoproterenol alone; however, it did not reach statistical significance. Emax and EC50 of isoproterenol with 0.1 μmol/L IBMX to stimulate IcA for adult myocytes were 187±15% and 31±15 nmol/L, respectively. Those of isoproterenol alone to stimulate IcA for adult myocytes were 203±5% and 51±8 nmol/L, respectively. In contrast to adult myocytes, the stimulatory effect of isoproterenol at all concentrations tested on newborn IcA was markedly potentiated by 0.1 μmol/L IBMX (P<.01). Emax and EC50 of isoproterenol with 0.1 μmol/L IBMX to stimulate IcA for newborn myocytes were 235±6% and 8±1 nmol/L, respectively. Those of isoproterenol alone to stimulate IcA for newborn myocytes were 111±2% and 81±5 nmol/L. The addition of 0.1 μmol/L IBMX not only potentiated the efficacy two times but also increased the potency of isoproterenol to stimulate newborn IcA by one log unit.

**Effects of IBMX on IcA Increased by a Supramaximal Dose of Isoproterenol**

We previously reported that in newborn myocytes the maximum isoproterenol-stimulated IcA density was significantly lower than either the maximum forskolin-stimulated or 200 μmol/L cAMP (in the pipette)–stimulated IcA for newborn myocytes. The lower stimulatory effect of isoproterenol in newborn myocytes may be partly explained by the tonic inhibition by G1 protein of adenylyl cyclase, and subsequent smaller production of cAMP by adenylyl cyclase after pretreatment by pertussis toxin of newborn myocytes markedly enhanced the isoproterenol effect on IcA.5,6 However, the intracellular cAMP concentration is determined by the balance of cAMP production by adenylyl cyclase and its hydrolysis by PDE. To evaluate the relation between cAMP production by maximum β-adrenergic stimulation and cAMP hydrolysis by PDE, we studied adult and newborn myocytes in which we first exposed each cell to 10 μmol/L isoproterenol and then added 50 μmol/L IBMX. Fig 5 shows current recordings and time course of IcA peak current (top) of an adult (left) and a newborn (right) cell before and after application of 10 μmol/L isoproterenol and then the application of this drug plus 50 μmol/L IBMX after reaching the steady state of IcA with 10 μmol/L isoproterenol. IcA increased
Fig 3. Calcium current (I<sub>Ca</sub>) recordings (top) and time courses of I<sub>Ca</sub> peak current (insets) before and after treatment with 0.1 μmol/L isoproterenol (ISO) and 0.1 μmol/L isoproterenol plus 0.1 μmol/L 3-isobutyl-1-methyl-xanthine (IBMX) from adult (AD) (left) and newborn (NB) (right) rabbit ventricular myocytes. The currents were elicited by the same depolarizing voltage steps as in Fig 1. Ordinates represent the I<sub>Ca</sub> density (pA/pF) obtained by normalizing the current to the membrane capacitance. The current tracings illustrated in the top panels were recorded in the control condition (A), in the presence of 0.1 μmol/L isoproterenol (B), and in the presence of 0.1 μmol/L isoproterenol plus 0.1 μmol/L IBMX (C) under stable conditions at a time indicated by these letters in the insets. Membrane capacitances of the adult and newborn myocytes were 46.4 and 12.3 pf, respectively. Experiment A930203A1 (left) and A930121N2 (right). Bottom, Dose-response curves of the effects of IBMX with concomitant application of 0.1 μmol/L isoproterenol on the calcium current (I<sub>Ca</sub>), for adult (A, left) and newborn (B, right) rabbit ventricular myocytes. Ordinate represents percent increase in I<sub>Ca</sub> over control levels. Numbers beside data points indicate the number of experiments for that data point. Data points show the mean values, and vertical bars represent SEM. Two horizontal lines, which represent the percent increase by 0.1 μmol/L isoproterenol in adult and newborn ventricular myocytes from the values we previously published, were added to demonstrate the effect of 0.1 μmol/L isoproterenol without IBMX on I<sub>Ca</sub>, E<sub>ca</sub> and EC<sub>50</sub> for the additive effect of IBMX to 0.1 μmol/L isoproterenol were 110±7% and 1.11±0.36 μmol/L, respectively, for adult myocytes and 192±17% and 0.07±0.03 μmol/L, respectively, for newborn myocytes.

from 4.6 to 13.7 pA/pF in the adult cell and 2.2 to 4.9 pA/pF in the newborn cell with the application of 10 μmol/L isoproterenol. IBMX 50 μmol/L had only a small additive effect on the adult I<sub>Ca</sub> (13.7 to 15.1 pA/pF) but had a marked additive effect on the newborn I<sub>Ca</sub> (4.9 to 13.5 pA/pF). The bottom panels of Fig 5 summarize the effects of 10 μmol/L isoproterenol alone versus the combination of 10 μmol/L isoproterenol plus 50 μmol/L IBMX for six adult (left) and seven newborn (right) myocytes. The I<sub>Ca</sub> density produced by external application of 10 μmol/L forskolin or 200 μmol/L cAMP in the pipette from our previous report is added for comparison. As we previously showed, the results obtained with 200 μmol/L cAMP in the pipette represent the maximal obtainable I<sub>Ca</sub> current due to increases in cAMP, since the addition of 10 μmol/L forskolin under these conditions did not further increase I<sub>Ca</sub>, which might be expected from the fact that 200 μmol/L cAMP is far above the physiological concentration.14,24 The adult I<sub>Ca</sub> density stimulated by 10 μmol/L isoproterenol was 17.1±3.0 pA/pF. IBMX 50 μmol/L had no significant additive effect (19.0±3.1 pA/pF) on 10 μmol/L isoproterenol-stimulated adult I<sub>Ca</sub>. There was no significant difference in adult I<sub>Ca</sub> density values among 10 μmol/L isoproterenol, 10 μmol/L isoproterenol plus 50 μmol/L IBMX, 10 μmol/L forskolin (n=8), and 200 μmol/L cAMP in the pipette (n=5). In contrast to adult myocytes, the stimulatory effect of 10 μmol/L isoproterenol (6.6±0.9 pA/pF) is significantly smaller than that of 10 μmol/L forskolin (13.8±1.5 pA/pF, P<.01, n=7) or cAMP (13.4±0.7 pA/pF, P<.01, n=7) in the pipette, as we previously showed.5 The addition of 50 μmol/L IBMX markedly increased the I<sub>Ca</sub> density to 14.9±1.2 pA/pF, which is comparable to the effect of 10 μmol/L forskolin or intracellular application of 200 μmol/L cAMP.

Effects of Milrinone and Rolipram on I<sub>Ca</sub> Increased by a Low Dose of Isoproterenol

To extend the results obtained with IBMX, a nonselective PDE inhibitor, we studied the effects of milrinone, a selective inhibitor of cGMP-inhibited PDE (PDE
III), and rolipram, a selective inhibitor of cGMP-insensitive PDE (PDE IV), on 0.1 μmol/L isoproterenol–stimulated I\textsubscript{Ca} current. Our preliminary results showed that 30 to 100 μmol/L milrinone or rolipram was required to increase I\textsubscript{Ca} in adult and newborn myocytes when applied without isoproterenol (data not shown). Milrinone and rolipram at such high concentrations lost their selectivity as inhibitors of PDE III or PDE IV.\textsuperscript{21,25-27} Therefore, we used 1 μmol/L milrinone or 1 μmol/L rolipram along with the application of 0.1 μmol/L isoproterenol on both adult and newborn rabbit ventricular myocytes. Milrinone 1 μmol/L and rolipram 1 μmol/L have been shown to act as selective PDE III and PDE IV inhibitors, respectively.\textsuperscript{21,25-27}

Fig 6 shows superimposed current recordings with 1 μmol/L milrinone (top) and 1 μmol/L rolipram (bottom) in adult (left) and newborn (right) ventricular myocytes. Milrinone 1 μmol/L caused a 70% additional increase in I\textsubscript{Ca} over 0.1 μmol/L isoproterenol–stimulated current for the adult cell, whereas rolipram caused no significant increase in I\textsubscript{Ca} over 0.1 μmol/L isoproterenol–stimulated current for the adult cell. In contrast to the effects on adult myocytes, 1 μmol/L milrinone resulted in only a small additional increase in newborn I\textsubscript{Ca} over the effect produced by 0.1 μmol/L isoproterenol alone, and 1 μmol/L rolipram resulted in an 80% additional increase in newborn I\textsubscript{Ca} over that produced by 0.1 μmol/L isoproterenol alone.

Fig 7 summarizes the effects of 0.1 μmol/L isoproterenol, 0.1 μmol/L isoproterenol with 1 μmol/L milrinone, or 0.1 μmol/L isoproterenol with 1 μmol/L rolipram on I\textsubscript{Ca} for adult and newborn rabbit ventricular myocytes. Rolipram 1 μmol/L significantly enhanced the effect of 0.1 μmol/L isoproterenol on I\textsubscript{Ca} for newborn myocytes (P<.005, n=9), whereas 1 μmol/L milrinone had no significant additional effect for newborn myocytes (n=6). The increase in I\textsubscript{Ca} for adult myocytes by 0.1 μmol/L isoproterenol plus 1 μmol/L milrinone was significantly greater than that by 0.1 μmol/L isoproterenol alone (P<.05, n=6), whereas 1 μmol/L rolipram had no significant additional effect to 0.1 μmol/L isoproterenol for adult myocytes (n=6).

**Discussion**

The present study was focused on postnatal changes in PDE modulation of L-type I\textsubscript{Ca} of rabbit ventricular myocytes. Our results obtained in this study using IBMX, a nonselective PDE inhibitor, are summarized as follows:

1. The efficacy of IBMX to stimulate basal I\textsubscript{Ca} (expressed as percent increase over control current level) was significantly greater for newborn myocytes than for adult myocytes, whereas the potency of IBMX for newborn was almost the same as that for adult.

2. The efficacy and potency of the additional effect of IBMX when applied concomitantly with 0.1 μmol/L isoproterenol to stimulate I\textsubscript{Ca} were significantly greater for newborn myocytes than for adult myocytes. The potency of IBMX to stimulate I\textsubscript{Ca} was increased by concomitant use of 0.1 μmol/L isoproterenol with much greater potentiation in newborn than in adult.

3. The efficacy and potency of isoproterenol to stimulate I\textsubscript{Ca} were markedly potentiated by concomitant use of 0.1 μmol/L IBMX in newborn myocytes, whereas in adult myocytes there was no significant potentiation by 0.1 μmol/L IBMX of the response to isoproterenol.

4. The addition of 50 μmol/L IBMX markedly enhanced newborn I\textsubscript{Ca} after application of a supramaximal concentration of isoproterenol (10 μmol/L) up to a level equivalent to that reached with 200 μmol/L cAMP in the pipette. There was no significant additive effect of 50 μmol/L IBMX on 10 μmol/L isoproterenol–stimulated adult I\textsubscript{Ca}.

It is well established that I\textsubscript{Ca} as well as contractility of cardiac muscles is modulated by cAMP-dependent...
Figure 5. Top, Calcium current (I_{Ca}) recordings and time course of peak I_{Ca} from an adult (left) and a newborn (right) rabbit ventricular cell before and after treatment with 10 μmol/L isoproterenol (ISO) and 10 μmol/L isoproterenol plus 50 μmol/L 3-isobutyl-1-methylxanthine (IBMX). The currents were elicited by same depolarizing voltage steps as in Fig 1. Ordinate represents the I_{Ca} density obtained by normalizing the current to the membrane capacitance. Membrane capacitances of the adult and newborn cells were 80.2 and 15.1 pF, respectively. Experiments A940118A2 and A920707N3. Bottom, Bar graphs showing I_{Ca} density obtained in control (CTL) conditions and in the presence of 10 μmol/L isoproterenol and of 10 μmol/L isoproterenol plus 10 μmol/L IBMX from six adult and seven newborn rabbit ventricular myocytes. I_{Ca} density in the presence of externally applied 10 μmol/L forskolin (FOR) (n=8 for adult, n=7 for newborn) and of 200 μmol/L L-arginine (L-Arg) in the pipette (n=5 for adult, n=7 for newborn) from data previously reported was added for comparison. *Significant difference (P<.01) between each pair of groups compared. Error bars indicate SEM.

Increase in cAMP concentration activates cAMP-dependent protein kinase (PK-A) by binding its regulatory subunits. Activated PK-A phosphorylates various intracellular proteins, including L-type calcium channel, phospholamban, and troponin I. Phosphorylation of L-type Ca^{2+} channels by PK-A leads to an increase in the mean probability of channel opening and channel availability.

Some of the PDE inhibitors are reported to increase I_{Ca} by elevating the intracellular cAMP concentration through inhibiting hydrolysis of cAMP by PDE. Therefore, the effect of PDE inhibitors to stimulate I_{Ca} depends on the increase in cAMP concentration achieved by inhibition of cAMP breakdown and does not directly depend on the extent of inhibition of PDE activity. The potency of IBMX to increase intracellular cAMP concentration would be greater when the concentration or the production rate of cAMP is higher, which would thus affect the potency of this drug to stimulate I_{Ca} and contractility of cardiac muscles. Actually, without the application of isoproterenol, IBMX had a very low potency to stimulate I_{Ca} in both newborn and adult myocytes in our experiments, whereas, with the concomitant application of 0.1 μmol/L isoproterenol, a marked potentiation of the IBMX effect was demonstrated both in newborn and adult, with much greater potentiation in newborn than in adult.

The application of 0.1 μmol/L IBMX did not significantly potentiate the isoproterenol-stimulated current at any concentration we tested in adult myocytes, whereas 0.1 μmol/L IBMX significantly potentiated the isoproterenol-stimulated current at all concentrations we tested in newborn myocytes (Fig 4). These results clearly show that 0.1 μmol/L IBMX is below the threshold concentration to inhibit PDE of adult myocytes and above the threshold concentration to inhibit PDE of newborn myocytes. IBMX potentiated the 0.1 μmol/L isoproterenol-stimulated I_{Ca} from 1 μmol/L in adult and from 0.05 μmol/L in newborn (Fig 3, bottom). Therefore, PDEs of newborn myocytes, which modulate I_{Ca}, seem at least 10 times more sensitive to IBMX than those of adult. In other words, the effective K_{i} value of IBMX for PDEs that regulate I_{Ca} seems to be much lower in newborn myocytes than in adult myocytes.

The similar low potency of IBMX to stimulate I_{Ca} in both newborn and adult myocytes compared with the
higher potency (newborn>adult) for increasing the isoproterenol-stimulated I_c and the distinctly lower estimated K_i value of IBMX in newborn than in adult myocytes suggest that the basal level of cAMP concentration for newborn myocytes is much lower than for adult myocytes. This does not necessarily mean that the actual concentrations of cAMP are lower in newborn than in adult myocytes, because the cAMP dose-response relation for I_c may not be the same in newborn as in adult. I_c or single Ca^{2+} channel activity is regulated by interaction of phosphorylation by protein kinase and dephosphorylation by phosphatase of subunits of its channel. Therefore, a difference in the activity of protein kinases as well as that of phosphatases between newborn and adult cardiac myocytes could influence the dose-response of cAMP for I_c and change the potency of cAMP between newborn and adult. We have recently shown that microsyrin, a potent phosphatase 1 and 2A inhibitor, has much greater stimulatory effect on newborn I_c than adult ones, which suggests that phosphatase activity to regulate I_c may be much higher in newborn myocytes than in adult myocytes.

There are several possibilities to explain this distinctly different K_i value of IBMX between the PDE for adult versus newborn myocytes. One is that there may be a developmental change in the characteristics of PDEs concerning sensitivity to IBMX. Artman et al. reported that the K_i value of piroximone to cytosolic low-K_a cGMP-inhibitable PDE differs significantly between adult and immature rabbit heart, although the K_i of piroximone for adult was found to be 30 times lower than that for newborn. The second possibility is that the
distribution of types of PDE isozymes in newborn rabbit heart might be quite different from that in adult.12 Although IBMX is reported to be a nonselective PDE inhibitor with similar IC50 or Ki values to each PDE isozyme,26,24 the overall Ki value to IBMX might be different between newborn and adult if there were a large difference of PDE isozymes between adult and newborn heart (discussed below). Another possibility is that IBMX may be acting other than as a PDE inhibitor, which could influence the sensitivity or estimated Ki value of IBMX by increasing the IC50. IBMX has a strong antagonistic effect on A1-adenosine receptors, resulting from a structural homology between adenosine and IBMX, which have a common ring structure. Adenosine has been reported to antagonize the stimulatory effect of isoproterenol on ICa.25 However, this side action of IBMX is unlikely to explain the stimulatory effect on ICa in our results. The amount of adenosine a single cell produces is unlikely to be sufficiently high to activate adenosine receptors, especially because the myocytes are constantly superfused at a relatively high rate of 2.5 mL/min through 0.3 mL tissue bath.15 Furthermore, we have shown that 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX), a strong A1-adenosine receptor antagonist that does not inhibit PDE, was ineffective in potentiating the isoproterenol effect on ICa (unpublished data). These results indicate that the effects of IBMX were not due to the action of adenosine receptor antagonism.

Effects of Milrinone and Rolipram on ICa for Adult and Newborn

Milrinone 1 μmol/L had a significant additional effect on the 0.1 μmol/L isoproterenol-stimulated ICa for adult and no additional effect for newborn. Rolipram 1 μmol/L had no additional effect on 0.1 μmol/L isoproterenol-stimulated ICa for adult but a marked additional effect for newborn. Since 1 μmol/L milrinone and 1 μmol/L rolipram can be used as selective inhibitors of PDE III and PDE IV, respectively,21,25-27 PDE III seems to be the dominant isozyme that regulates ICa for adult rabbit ventricular myocytes, whereas PDE IV seems to be the dominant isozyme that regulates ICa for newborn myocytes. These results correlate very well with the biochemical results from adult and newborn rabbit hearts reported by Kithas.12 They analyzed the postnatal change in cytosolic PDE isozyme activity as well as the subcellular distribution of high-affinity cAMP PDEs in rabbit ventricular myocardium and demonstrated that in the soluble fraction of rabbit ventricular muscle, cGMP-inhibitable, milrinone-sensitive PDE activity is much higher in adult than in newborn, whereas cGMP-insensitive, rolipram-sensitive PDE is the dominant type in the soluble fraction of newborn rabbit ventricular muscle. It is interesting that PDE IV is the dominant isozyme in frog heart,24 which is evolutionarily primitive, and in newborn heart, which is developmentally immature.12

The ineffectiveness of milrinone for stimulating newborn ICa may explain, at least in part, the reported10,11 weaker inotropic effect of this cardiotonic PDE III inhibitor in newborn heart than in adult heart. Since ICa is the major source of Ca2+ influx into ventricular myocytes and influxed Ca2+ is taken up and released by sarcoplasmic reticulum, a weaker ability of a PDE III inhibitor to increase ICa may result in a weaker inotropic response to milrinone for newborn myocytes. The weaker inotropic response by PDE III inhibitors for newborn hearts might also be explained by the immaturity of sarcoplasmic reticulum in newborn heart.36,37 In rabbit or canine hearts, the PDE from the membrane-bound fraction, which represents basically the sarcoplasmic reticulum fraction, consists largely of PDE III,12,38,39 which is the target of cardiotonic PDE III inhibitors. According to Kithas,12 this membrane-bound PDE III activity increases fivefold during maturation.

Clinical Relevance

Our results have several important points of clinical relevance in treating heart failure during the neonatal period, especially during the postoperative low cardiac output state of congenital heart disease. The decreased β-adrenergic stimulation of ICa that we have reported,2 as well as the decreased modulation of contractility for newborn rabbit heart reported by others,40 has demonstrated that, in newborn hearts, the isoproterenol responses were much less effective than direct adenyl cyclase stimulation with forskolin. However, we have now shown that the addition of 50 μmol/L IBMX to isoproterenol increased the ICa density up to the equivalent level obtained with forskolin or intracellular application of 200 μmol/L cAMP. Also, additions of low concentrations of either IBMX or rolipram significantly potentiated the effect of isoproterenol for newborn ICa. We have also done experiments with measurements of developed isovolumic pressure in Langendorff-perfused adult and newborn rabbit hearts and have shown that 0.1 μmol/L IBMX strongly potentiates the inotropic effect of isoproterenol in newborn but not in adult rabbit hearts (unpublished data). Therefore, the concomitant use of β-adrenergic stimulation and PDE inhibitors has a potential to overcome the weaker response of β-adrenergic stimulation on ICa as well as on contractility for newborn hearts. Our data predict that PDE IV inhibitors, not PDE III inhibitors, which are used as cardiotonic drugs for adult hearts, may be the most effective in achieving an additive effect on newborn contractility.

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