Developmental Changes in the \( \beta \)-Adrenergic Modulation of Calcium Currents in Rabbit Ventricular Cells

Toshiyuki Osaka and Ronald W. Joyner

We studied the developmental changes in the \( \beta \)-adrenergic modulation of L-type calcium current (\( I_{\text{Ca}} \)) in enzymatically isolated adult (AD) and newborn (NB, 1–4-day-old) rabbit ventricular cells using the whole-cell patch-clamp method. \( I_{\text{Ca}} \) was measured as the peak inward current at a test potential of +15 mV by applying a 180–450-msec pulse from a holding potential of −40 mV with Cs⁺-rich pipettes and a K⁺-free bath solution at room temperature. In control, \( I_{\text{Ca}} \) density (obtained by normalizing \( I_{\text{Ca}} \) to the cell capacitance) was significantly higher in AD cells (5.5±0.2 [mean±SEM] pA/pF, \( n=65 \)) than in NB cells (2.6±0.1 pA/pF, \( n=60 \)). Isoproterenol (ISO, 1 nM–30 \( \mu \)M) increased \( I_{\text{Ca}} \) in a dose-dependent manner for both groups. The maximal effect (\( E_{\text{max}} \)) of ISO, expressed as percent increase in \( I_{\text{Ca}} \) over control levels, and the concentration for one half of the maximal effect (EC\(_{50}\)) were 203% and 51 nM, respectively, for AD cells and 111% and 81 nM, respectively, for NB cells. The effect of ISO (1 \( \mu \)M) on \( I_{\text{Ca}} \) was decreased as the test potential was increased from −10 to +40 mV. However, the ratio of the percent increase in \( I_{\text{Ca}} \) for AD versus NB cells was almost constant (2.09–2.45) at each test potential. Dose–response curves of forskolin (FOR, 0.3–50 \( \mu \)M) gave \( E_{\text{max}} \) and EC\(_{50}\) of 268% and 0.74 \( \mu \)M, respectively, for AD cells and 380% and 1.15 \( \mu \)M, respectively, for NB cells. After stimulating \( I_{\text{Ca}} \) by 10 \( \mu \)M ISO, the addition of 10 \( \mu \)M FOR produced a further increase in \( I_{\text{Ca}} \) of only 12±2% in AD cells (\( n=4 \)) but a further increase of 140±41% in NB cells (\( n=6 \)). FOR (10 \( \mu \)M) did not produce any increase in \( I_{\text{Ca}} \) for AD and NB cells after stimulating \( I_{\text{Ca}} \) by intracellular application of 200 nM cAMP. \( I_{\text{Ca}} \) density stimulated by 10 \( \mu \)M ISO (17.8±1.1 pA/pF, \( n=7 \)), 10 \( \mu \)M FOR (21.0±1.3 pA/pF, \( n=8 \)), or 200 \( \mu \)M cAMP (18.0±1.3 pA/pF, \( n=5 \)) was equivalent in AD cells, whereas \( I_{\text{Ca}} \) density stimulated by 10 \( \mu \)M ISO (5.8±0.6 pA/pF, \( n=9 \)) was significantly lower than that stimulated by either 10 \( \mu \)M FOR (13.8±1.5 pA/pF, \( n=7 \)) or 200 \( \mu \)M cAMP (13.4±0.7 pA/pF, \( n=7 \)) in NB cells. The \( I_{\text{Ca}} \) density for FOR or cAMP was significantly higher for AD than NB cells. Pretreatment of AD and NB cells with pertussis toxin markedly increased the ISO effect on \( I_{\text{Ca}} \) for NB cells, whereas the enhancement was relatively small for AD cells. We conclude that the effect of ISO to stimulate L-type \( I_{\text{Ca}} \) increases after birth in rabbit ventricular cells probably as a consequence of the reduction of tonic \( G_{i} \) inhibition of adenylate cyclase rather than the postnatal maturation of the \( \beta \)-receptor–adenylate cyclase system. (Circulation Research 1992;70:104–115)

The enhancement of Ca²⁺ current (\( I_{\text{Ca}} \)) through high-threshold (L-type) Ca²⁺ channels in cardiac cells by \( \beta \)-adrenergic agonists is the main process in regulating heart rate and excitation–contraction coupling by sympathetic nerve stimulation. Binding of an agonist to the \( \beta \)-adrenergic receptor activates a guanine nucleotide–binding protein (G protein), termed \( G_{\beta} \), which triggers the activation of adenylate cyclase and in turn increases cAMP.¹–³ The process is followed by the phosphorylation of the Ca²⁺ channel or one of its subunits through the activation of cAMP-dependent protein kinase,⁴–⁶ leading to an increase in channel open probability (\( P_{o} \)) and channel availability (\( P_{a} \)).⁷–¹⁰ The \( \beta \)-adrenergic stimulation of \( I_{\text{Ca}} \) is antagonized by muscarinic cholinergic stimulation through an indirect and/or direct inhibition of adenylate cyclase.¹¹,¹² The inhibitory responses are mediated by another G protein, termed \( G_{i},¹–³,¹³,¹⁴ \)
In recent years, increasing attention has been focused on age-related changes in the β-adrenergic receptor–adenylate cyclase system to elucidate sympathetic nerve regulation of cardiac muscle in postnatal developing hearts. Various results have been reported concerning age-related changes in β-adrenergic receptor density based on binding studies with radioligand β-antagonists. Thus, it has been reported that the maximal number of ligand binding sites \( (B_{\text{max}}, \text{femtomoles per milligram protein or tissue}) \) in cardiac muscle increases during the postnatal period in the rabbit\(^{15,16} \) and mouse,\(^ {17} \) whereas \( B_{\text{max}} \) decreases in the dog.\(^ {18} \) Both results have been observed in the rat.\(^ {19-21} \) In contrast to the inconsistency of age-related changes in β-receptor density, the stimulatory effect of β-agonists on adenylate cyclase activity in cardiac membranes has been demonstrated to increase with advancing age in most of the animal species examined.\(^ {19,22,23} \) It has also been reported that adenylate cyclase activity stimulated by 5′-guanylylimidodiphosphate \( \text{[Gpp(NH)p]} \) or NaF, whose effects are mediated by both \( G_{i} \) and \( G_{s} \) without the requirement of the receptor binding, increases during postnatal development.\(^ {22,23} \) Kojima et al\(^{24} \) have shown in rat ventricular membranes that cholina toxin substrates \( (G_{i}, \text{femtomoles per milligram tissue}) \) increase during the postnatal period.

To know how these age-related changes in the β-receptor–adenylate cyclase system modify the β-adrenergic modulation of L-type \( I_{\text{Ca}} \) is essential for understanding sympathetic nerve regulation of developing hearts. However, it is complicated to relate the results of the previous binding and biochemical studies to physiological responses of \( I_{\text{Ca}} \) to β-stimulation, because both cell shape and dimensions undergo postnatal changes in mammalian hearts. For example, newborn rabbit ventricular cells are smaller and have a surface-to-volume ratio two to three times as great as that of adult cells.\(^ {25} \) Surface density of β-receptor and intracellular levels of cAMP resulting from the activation of adenylate cyclase by β-agonists in intact cells may not necessarily be reflected by the age-related changes reported in the previous studies. In addition, it has been suggested that \( G_{i} \) has a tonic inhibitory influence on adenylate cyclase in heart cells, even in the absence of muscarinic agonists, based on the findings that pretreatment with pertussis toxin (PTX), which uncouples the interaction between \( G_{i} \) and muscarinic cholinergic receptors as well as other inhibitory receptors of adenylate cyclase,\(^ {26} \) increases the stimulatory effects of β-agonists on adenylate cyclase,\(^ {27,28} \) \( I_{\text{Ca}} \),\(^ {11} \) and contractility\(^ {29} \) in addition to the abolition of agonist-dependent inhibition of adenylate cyclase. This suggestion further complicates the understanding of age-related changes in β-adrenergic modulation of L-type \( I_{\text{Ca}} \) in mammalian hearts, since muscarinic cholinergic receptor density and \( G_{i} \) also undergo postnatal changes in mammalian hearts.\(^ {21,24,30-33} \) Direct measurement of \( I_{\text{Ca}} \) based on whole-cell voltage-clamp experiments should be required to clarify these points.

This study was designed to elucidate postnatal changes in β-adrenergic modulation of L-type \( I_{\text{Ca}} \) in mammalian hearts. We compared the effects of \((-\text{isoproterenol HCl (ISO)}\), a β-agonist, forskolin (FOR), a direct activator of adenylate cyclase,\(^ {33} \) and cAMP on L-type \( I_{\text{Ca}} \) in freshly isolated adult (AD) and newborn (NB, 1–4-day-old) rabbit ventricular cells under nearly identical experimental conditions using the whole-cell voltage-clamp technique. We also have compared the effect of PTX pretreatment on \( I_{\text{Ca}} \) response to ISO for both age groups to examine the difference in the contribution of \( G_{i} \) tonic inhibition to the ISO effect.

**Materials and Methods**

**Cell Preparation**

New Zealand White AD (2–3 kg) and NB (1–4-day-old) rabbits of either sex were used in the experiments. AD rabbits were first heparinized (1,000 units i.v.) and anesthetized with sodium pentobarbital (50 mg/kg i.v.). For NB rabbits, the same drugs were given intraperitoneally. Hearts were rapidly removed via thoracotomy with artificial ventilation, and the aorta was cannulated. Single ventricular cells were obtained from the AD and NB hearts by enzymatic dissociation as we described previously.\(^ {34} \) In brief, a dissected heart was mounted on a Langendorff apparatus and perfused with oxygenated, nominally Ca\(^ {2+} \)-free solution at a rate of 3–4 ml/min for 4 minutes at 36–37°C. Then, the heart was perfused with the same solution containing 0.14 mg/ml collagenase (Type I, Sigma Chemical Co., St. Louis, Mo.) and 0.07 mg/ml protease (Type XIV, Sigma). After 6–10 minutes of the enzyme treatment, the heart was perfused with a storage solution for 7–10 minutes at room temperature. The ventricle was cut into small pieces and triturated in the storage solution. The isolated cells were stored in the storage solution at 4°C.

**Solutions and Drugs**

The compositions of solutions used were as follows (mM): normal Tyrode’s solution: NaCl 148.8, KCl 4.0, CaCl\(_2\) 1.8, MgCl\(_2\) 0.53, NaH\(_2\)PO\(_4\) 0.33, HEPES 5.0, and glucose 5.0, pH 7.4 with NaOH; Ca\(^ {2+} \)-free solution: NaCl 100, KCl 10, K\(_2\)HPO\(_4\) 1.2, MgSO\(_4\) 5, taurine 50, glucose 20, and HEPES 10, pH 7.2 with KOH; storage solution: potassium glutamate 140, MgCl\(_2\) 5, EGTA 1, glucose 10, and HEPES 10, pH 7.4 with KOH; test solution: NaCl 130, CaCl\(_2\) 1.8, CsCl\(_2\) 20, MgCl\(_2\) 0.53, HEPES 5, glucose 5, tetrodotoxin 0.005–0.01 (Sigma), pH 7.4 with NaOH; pipette solution: CsOH 110, aspartic acid 90, CsCl 20, tetraethylammonium chloride 10, HEPES 5, EGTA 10, MgATP 5, Na\(_2\)-creatine phosphate 5, GTP(Tris) 0.4, and leupeptin (Sigma) 0.1, pH 7.4 with CsOH.

The drugs used in these experiments were ISO, carbamylcholine chloride (carbachol), FOR, cAMP, and PTX (Sigma). FOR was dissolved in anhydrous ethanol as a 20-mM stock solution, which was stored at −20°C. ISO and carbachol were dissolved in water.
to make 1-mM and 10-mM stock solutions, respectively, for each experiment. The stock solution for ISO contained 1 mM ascorbic acid. The drugs were diluted by the test solution to a given concentration.

In experiments involving pretreatment with PTX, the isolated cells from either AD or NB hearts were divided into two groups and incubated for 8–11 hours at room temperature (21–23°C) in the storage solution either with or without PTX (0.5–1.0 μg/ml).

**Experimental Procedure**

An aliquot of single ventricular cells in suspension was added in the chamber and allowed to settle to the bottom. The volume of the recording chamber was 0.2–0.3 ml. Solutions were superfused through the chamber at a rate of 2–3 ml/min. All the experiments were carried out at room temperature (21–23°C). Voltage-clamp experiments were performed in the whole-cell configuration with the patch-clamp method by use of an Axopatch-1B patch-clamp amplifier (Axon Instruments, Foster City, Calif.). Pipettes were pulled from borosilicate glass and, after fire-polishing, had a resistance of 1.0–2.0 MΩ when filled with the pipette solution. A tight seal (3–5 GΩ) was established in normal Tyrode’s solution. A pipette potential was maintained when applying a quick suction to the pipette, the external solution was changed to the test solution. The whole-cell configuration of the bath solution was obtained when filled with the pipette solution. A tight seal was established in normal Tyrode’s solution. A pipette potential was maintained when applying a quick suction to the pipette, the external solution was changed to the test solution. The complete change in the bath solution was obtained within 1 minute. For routine monitoring of ICa, the ventricular cell was depolarized every 10–20 seconds from a holding potential (Vh) of −40 mV to a test potential of +15 mV for 180–450 msec. To obtain current–voltage relations, a series of test steps of 180–450-msec duration was applied with 10-mV increments from a Vh of −40 mV. In our experimental conditions, all the K+ currents were eliminated by the use of Cs+ and tetraethylammonium chloride in the pipette and Cs+ in the external test solution. Na+ current was inactivated by using Vh of −40 mV, and possible residual Na+ current was blocked by tetrodotoxin. In some experiments we used N-methyl-D-glucamine instead of Na+ in the test solution. The replacement of Na+ with N-methyl-D-glucamine had no effect on the current elicited. Nifedipine (10 μM), an organic Ca²⁺ channel blocker, completely blocked the time-dependent current. The calcium currents elicited were filtered at a corner frequency of 1 kHz, digitized at 50–100 μsec intervals, and stored and analyzed on an IBM PC-AT computer with PCLAMP software (Axon Instruments). Membrane capacitance was measured using the calibrated capacity compensation circuit of the Axopatch voltage-clamp amplifier using a 10-mV hyperpolarizing voltage step from −80 mV. The values of cell capacitance obtained with this measurement gave a very close relation to those obtained with a conventional method of integrating the area beneath the capacitive transient and dividing that by the voltage step. Since the capacitance for AD cells (71.6±2.4 pF, n=70) was significantly greater than that for NB cells (16.1±0.6 pF, n=67), we expressed all the current data as current density (pA/pF) by normalizing the current to the cell capacitance. ICa density obtained 5–8 minutes after breaking the membrane was significantly higher for AD cells (5.5±0.2 pA/pF, n=65) than for NB cells (2.6±0.1 pA/pF, n=60), as we reported previously. Ventricular cells with a membrane capacitance of <100 pF were used for the experiments.

Statistical significance was determined by Student’s t test for paired or unpaired data, as appropriate. Values of p<0.05 were regarded as significant. Data are presented as mean±SEM in the text. Error bars in the figures represent SEM.

**Results**

**Dose-Dependent Effect of ISO on ICa**

The potentiation of ICa by β-adrenergic stimulation was examined quantitatively with various concentrations of ISO. Figure 1 shows superimposed current recordings (top panels) and time courses of ICa increase (bottom panels) produced by ISO in an AD cell (left panels) and an NB cell (right panels). ICa was measured as the peak inward current at a test potential of +15 mV by applying a 180-msec pulse from a Vh of −40 mV every 10 seconds. Because of the obvious differences in control ICa density, we expressed the vertical axis of the lower panel as percent increase in ICa over control levels. In the AD cell, after application of 0.1 μM ISO, ICa started to increase and reached a steady state (135% over the control level) within 4 minutes, and the ICa density returned to the control level with a similar time course, by subsequently washing out the ISO. The stimulatory effect of 0.1 μM ISO was much smaller in the NB cell. Thus, 0.1 μM ISO increased the NB ICa density, with a similar time course to the AD cell, by only 70% over the control level. An additional application of 1 μM ISO caused a further increase in the NB ICa density, but the stimulatory effect of 1 μM ISO on the NB ICa (126%) was still smaller than that of 0.1 μM ISO on the AD ICa.

The dose-dependent effect of ISO on ICa measured at a test potential of +15 mV is summarized in Figure 2. To avoid possible desensitization of the receptor to ISO, we did not apply repeatedly high doses of ISO (>0.3 μM) to the same cell. ISO caused a dose-dependent increase in the ICa density with an apparent threshold concentration of 1 nM for AD cells and of 10 nM for NB cells. The maximal effect (Emax) was obtained at ∼10 μM ISO for both groups. The continuous curves were derived from a nonlinear least-mean-squares regression of the means to the Michaelis equation. Emax of ISO to stimulate ICa (expressed as percent increase in ICa over control levels) and the concentration for one half of the maximal effect (EC50) obtained by this analysis were 203±5% and 51±8 nM, respec-
tively, for AD cells (n=6). For NB cells, \(E_{\text{max}}\) and \(EC_{50}\) were 111±2% and 81±5 nM, respectively (n=6). Thus, advancing age caused a twofold increase in the efficacy (\(E_{\text{max}}\)) of ISO to stimulate \(I_{Ca}\) with little change in the potency (\(EC_{50}\)).

Voltage-Dependent Effect of ISO on \(I_{Ca}\)

ISO has been reported to cause a marked voltage-dependent effect on \(I_{Ca}\) in frog ventricular cells, whereas the voltage dependence is reported to be much smaller for \(I_{Ca}\) from AD guinea pig ventricular cells. Therefore, the results obtained in the previous section might be due to different voltage dependencies of ISO on \(I_{Ca}\) for AD and NB cells. To check this possibility, the current–voltage relation before and after treatment by 1 \(\mu\)M ISO was examined in both AD and NB cells by applying a series of depolarization steps with a 10-mV increment from a \(V_h\) of -40 mV. Figure 3 shows superimposed current tracings in response to three different test potentials applied to an AD cell (left panels) and an NB cell (right panels). The averaged current–voltage relations obtained before and after ISO treatment are illustrated in Figure 4 for five AD cells (top left panel) and four NB cells (top right panel). In control conditions, the current–voltage relation for AD and NB cells was almost the same except for the different current density between the two groups, in agreement with our previous report. ISO (1 \(\mu\)M) produced an increase in \(I_{Ca}\) at each test potential without changing the apparent threshold potential and reversal potential for each group. The potential giving maximal \(I_{Ca}\) was shifted 5 mV in the negative direction in two of five AD cells and in one of four NB cells. To analyze the voltage-dependent effect of ISO on \(I_{Ca}\) quantitatively, we plotted the values of the percent increase in \(I_{Ca}\) at each test potential between -10 to +40 mV for both groups as shown in Figure 4, bottom panel. The stimulatory effect of ISO was greatest at -10 mV and decreased as the test potential was increased, as reported in frog ventricular cells. The ratio of the percent increase in \(I_{Ca}\) for AD versus NB cells was almost constant (2.09–

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Calcium current (\(I_{Ca}\)) recordings (top panels) and time courses of increase in \(I_{Ca}\) (bottom panels) before and after treatment of isoproterenol (ISO) from an adult (left panels) and a newborn (right panels) rabbit ventricular cell. The currents were elicited by depolarizing voltage steps from a holding potential of -40 mV to a test potential of +15 mV every 10 seconds for each cell. Ordinates in the top and bottom panels represent current density obtained by normalizing the current to the membrane capacitance and percent increase in the \(I_{Ca}\) over control levels, respectively. The current tracings illustrated in the top panels were recorded in control conditions (A) and in the presence of 0.1 \(\mu\)M ISO (B) for each cell, and in the presence of 1 \(\mu\)M ISO (C) for the newborn cell at a time indicated by these letters in the bottom panels. Vertical bars indicate the actual scale of the current. Membrane capacitances of the adult and newborn cells were 61.0 and 18.0 pF, respectively.

![Figure 2](https://example.com/fig2.png)

**Figure 2.** Graph showing dose–response curves of the effect of isoproterenol (ISO) on the calcium current (\(I_{Ca}\)) for adult (solid squares) and newborn (open triangles) rabbit ventricular cells. Ordinate represents percent increase in \(I_{Ca}\) over control levels. Data points show the mean values of the number of cells indicated near the symbols; vertical bars, representing the standard error of the mean, were shown when they were larger than the symbols. The continuous lines were derived from a nonlinear least-mean-squares regression of the means to the Michaelis equation: \(E_{\text{max}}=E_{\text{max}}(\text{ISO})/(EC_{50}+\text{ISO})\). Here, \(E_{\text{max}}\) is the maximal effect of ISO to stimulate \(I_{Ca}\) and \(EC_{50}\) is the concentration required for half maximal stimulation. \(E_{\text{max}}\) and \(EC_{50}\) were 203±5% and 51±8 nM, respectively, for adult cells (n=6) and 111±2% and 81±5 nM, respectively, for newborn cells (n=6). *Significant difference between adult and newborn values at each concentration of ISO at p<0.05.
2.45) at each test potential, suggesting that the voltage dependence of the effect of ISO on $I_{Ca}$ does not significantly change with advancing age.

**Dose-Dependent Effect of FOR on $I_{Ca}$**

To examine the effect of a direct stimulation of adenylate cyclase on $I_{Ca}$ for both groups, we investigated the effect of FOR, which is considered to interact with the catalytic unit of adenylate cyclase without requiring activation of $G_{s}$.

Figure 3 shows individual current tracings (top panels) and the time course of the change in current magnitude (bottom panels) to illustrate the stimulatory effect of FOR on $I_{Ca}$ for an AD and an NB cell. FOR (10 μM) caused a 200% increase in $I_{Ca}$ over the control level for the AD cell. In the NB cell, 10 μM FOR produced more than a 300% increase over control.

The dose-dependent effect of FOR on $I_{Ca}$ at a test potential of +15 mV is summarized in Figure 6. As was done for the experiments with ISO, the two dose–response curves were fitted using a nonlinear least-mean-squares regression of the means to the

**Figure 3.** Calcium current ($I_{Ca}$) recordings before treatment (CON) and after treatment of 1 μM isoproterenol (ISO) from an adult (left panels) and a newborn (right panels) rabbit ventricular cell elicited by depolarizing steps from a holding potential of $-40$ mV to test potentials (TP) indicated near the current tracings. Ordinate represents current density obtained by normalizing the current to the membrane capacitance. Vertical bars indicate the actual scale of the current. Membrane capacitances of the adult and newborn cells were 88.0 and 14.0 pF, respectively.

**Figure 4.** Graphs showing current–voltage relation for adult cells (n=5) (top left panel) and newborn cells (n=4) (top right panel) before treatment (CON, closed symbols) and after treatment of 1 μM isoproterenol (ISO, open symbols) and the voltage-dependent effect of 1 μM ISO on calcium current ($I_{Ca}$) (bottom panel) for both groups. Ordinates in the top and bottom panels represent current density obtained by normalizing the current to the membrane capacitance and percent increase in the $I_{Ca}$ over control levels, respectively. Data points are mean values; vertical bars, representing the standard error of the mean, are shown when they are larger than the symbols. *Significant difference between adult and newborn values at $p<0.05$. 

Downloaded from http://circres.ahajournals.org/ by guest on July 11, 2017
FIGURE 5. Calcium current \( (I_{Ca}) \) recordings (top panels) and time courses of increase in \( I_{Ca} \) (bottom panels) before and after treatment of 10 \( \mu \text{M} \) forskolin (FOR) from an adult (left panels) and a newborn (right panels) rabbit ventricular cell. The currents were elicited by depolarizing voltage steps from a holding potential of \(-40 \text{ mV} \) to a test potential of \(+15 \text{ mV} \) every 20 seconds for each cell. Ordinates in the top and bottom panels represent current density obtained by normalizing the current to the membrane capacitance and percent increase in the \( I_{Ca} \) over control levels, respectively. The current tracings illustrated in the top panels were recorded in control conditions (A) and in the presence of 10 \( \mu \text{M} \) FOR (B) for each cell at a time indicated by these letters in the bottom panels. Vertical bars indicate the actual scale of the current. Membrane capacitances of the adult and newborn cells were 70.0 and 20.0 pF, respectively.

Michaelis equation. The \( E_{\text{max}} \) and \( EC_{50} \) were 268\( \pm \)8\% and 0.74\( \pm \)0.09 \( \mu \text{M} \), respectively, for AD cells \( (n=6) \) and 380\( \pm \)10\% and 1.15\( \pm \)0.12 \( \mu \text{M} \), respectively, for NB cells \( (n=6) \). The percent increase caused by 10 \( \mu \text{M} \) FOR for NB cells \( (351\pm21\% \), \( n=7 \) \) was significantly greater than that for AD cells \( (232\pm17\% \), \( n=8 \) \). Thus, in contrast to the effect of ISO, the efficacy of FOR to stimulate \( I_{Ca} \) was greater for NB cells than for AD cells, whereas the potency was slightly higher for AD cells than for NB cells.

**Combined Effects of ISO and FOR on \( I_{Ca} \)**

To extend the results obtained in Figures 5 and 6, we studied two groups of AD and NB cells to which we first exposed each cell to 10 \( \mu \text{M} \) ISO and then added 10 \( \mu \text{M} \) FOR. Figure 7 shows current recordings (top panel) and the time course of increase in \( I_{Ca} \) (bottom panel) for an NB cell produced by ISO and FOR. As summarized in Figure 8, after increasing \( I_{Ca} \) with 10 \( \mu \text{M} \) ISO, the addition of 10 \( \mu \text{M} \) FOR caused a small additional increase \((12\pm2\% \) for AD cells \( (n=4) \) as reported in other cardiac cells.\(^{12,37}\) On the other hand, FOR produced a further marked increase in \( I_{Ca} \) \((140\pm41\% \) for NB cells \( (n=6) \) even when the \( I_{Ca} \) had already been increased by 10 \( \mu \text{M} \) ISO.

**Effect of cAMP on \( I_{Ca} \)**

We also examined the effect on \( I_{Ca} \) density for AD and NB cells of intracellular application of 200 \( \mu \text{M} \) cAMP, which has been reported to increase \( I_{Ca} \) maximally in both mammalian and amphibian heart cells.\(^{5,6,37}\) cAMP was dialyzed into the cell from the pipette. \( I_{Ca} \) density reached a steady state 7.3\( \pm \)1.7 and 4.5\( \pm \)0.5 minutes after breaking the membrane for AD \( (n=5) \) and NB \( (n=7) \) cells, respectively. An additional application of 10 \( \mu \text{M} \) FOR had no effect on the cAMP-stimulated \( I_{Ca} \) both for AD and NB cells, as shown in the top panels of Figure 9.

We summarize the \( I_{Ca} \) density obtained by 10 \( \mu \text{M} \) ISO, 10 \( \mu \text{M} \) FOR, and 200 \( \mu \text{M} \) cAMP for AD and NB cells in the bottom panels of Figure 9. There was no significant difference between \( I_{Ca} \) densities stimulated by 10 \( \mu \text{M} \) ISO \((17.8\pm1.1 \text{ pA/pF, } n=7) \), 10 \( \mu \text{M} \) FOR \((21.0\pm1.3 \text{ pA/pF, } n=8) \), and 200 \( \mu \text{M} \) cAMP \((18.0\pm1.3 \text{ pA/pF, } n=5) \) for AD cells, in agreement with the reports on AD guinea pig and frog ventricular cells.\(^{5,38}\) There was also no significant difference between \( I_{Ca} \) densities stimulated by 10 \( \mu \text{M} \) FOR \((13.8\pm1.5 \text{ pA/pF, } n=7) \) and 200 \( \mu \text{M} \) cAMP \((13.4\pm0.7 \text{ pA/pF, } n=7) \) for NB cells. However, the \( I_{Ca} \) density produced by 10 \( \mu \text{M} \) ISO \((5.8\pm0.6 \text{ pA/pF, } n=9) \) was significantly lower than that stimulated either by 10 \( \mu \text{M} \) FOR or 200 \( \mu \text{M} \) cAMP for NB cells. The ratio of AD versus NB \( I_{Ca} \) density was decreased from 2.1 in control to 1.5 in the presence of 10 \( \mu \text{M} \) FOR, because of the greater percent increase in \( I_{Ca} \) for NB cells than for AD cells by FOR. Similarly, the ratio was decreased to 1.3 in the presence of 200 \( \mu \text{M} \) cAMP. However, FOR-stimulated or cAMP-stimulated \( I_{Ca} \) density was still significantly higher for AD than for NB cells.
Effect of PTX Treatment on $I_{Ca}$

To examine the possibility that the smaller response of NB $I_{Ca}$ to ISO might be due to the greater influence of G, on the interaction between the β-receptor and adenylyl cyclase in NB cells, we pretreated AD and NB cells with PTX (0.5–1.0 μM/ml) for 8–11 hours at room temperature. Figure 10 shows $I_{Ca}$ recordings (top panel) and the time course of $I_{Ca}$ increase (bottom panel) produced by 0.1 μM ISO in an NB cell pretreated with PTX. In the PTX-treated cell, 0.1 μM ISO caused more than a 300% increase in $I_{Ca}$ density over control level. The effectiveness of PTX pretreatment was confirmed by the lack of the effect of 10 μM carbachol, a muscarinic agonist, on the ISO-stimulated $I_{Ca}$.

We summarize the effect of PTX on $I_{Ca}$ before and after application of 0.1 μM ISO for AD and NB cells in Figure 11. In AD cells, PTX pretreatment caused little change in the basal (in the absence of ISO) $I_{Ca}$ density ($4.5\pm0.5$ pA/pF for untreated cells, $n=5$; $5.0\pm0.4$ pA/pF for PTX-treated cells, $n=5$). In NB cells, on the other hand, basal $I_{Ca}$ density was significantly increased from $2.7\pm0.3$ pA/pF for untreated cells ($n=5$) to $3.8\pm0.4$ pA/pF for PTX-treated cells ($n=7$). The stimulatory effect of ISO on $I_{Ca}$ was

**FIGURE 6.** Graph showing dose–response curves of the effect of forskolin (FOR) on the calcium current ($I_{Ca}$) for adult (solid squares) and newborn (open triangles) rabbit ventricular cells. Ordinate represents percent increase in $I_{Ca}$ over control levels. Data points show the mean values of the number of cells indicated near the symbols; vertical bars, representing the standard error of the mean, were shown when they were larger than the symbols. The continuous lines were derived from a nonlinear least-mean-squares regression of the mean to the Michaelis equation: effect=$E_{max}*[FOR]/(IC50)+EC50$, where $E_{max}$ is the maximal effect of FOR to stimulate $I_{Ca}$ and $EC50$ is the concentration required for half-maximal stimulation. $E_{max}$ and $EC50$ were 268±8% and 0.74±0.09 μM, respectively, for adult cells (n=6) and 380±10% and 1.15±0.12 μM, respectively, for newborn cells (n=6). *Significant difference between adult and newborn values at the concentration of FOR at p<0.05.

**FIGURE 7.** Calcium current ($I_{Ca}$) recordings (top panel) and time course of increase in the $I_{Ca}$ (bottom panel) before and after treatment of 10 μM isoproterenol (ISO) and 10 μM ISO plus 10 μM forskolin (FOR) from a newborn rabbit ventricular cell. The currents were elicited by depolarizing voltage steps from a holding potential of -40 mV to a test potential of +15 mV every 20 seconds for each cell. Ordinates in the top and bottom panels represent current density obtained by normalizing the current to the membrane capacitance and percent increase in $I_{Ca}$ over control level, respectively. The current tracings illustrated in the top panel were recorded in control conditions (A), in the presence of 10 μM ISO (B), and in the presence of 10 μM ISO plus 10 μM FOR (C) at a time indicated by these letters in the bottom panel. Vertical bars indicate the actual scale of the current. Membrane capacitance of the cell was 15.0 pF.

**FIGURE 8.** Graph showing the combined effects of 10 μM isoproterenol (ISO) and 10 μM forskolin (FOR) on calcium current ($I_{Ca}$) for adult (n=4) and newborn (n=6) rabbit ventricular cells. Ordinate represents percent increase in $I_{Ca}$ over control levels. Filled and dotted bars indicate the effect of ISO alone and the additional effect of FOR on $I_{Ca}$, respectively, for the identical cell in each group. Vertical bars indicate the standard error of the mean. The solid triangle and the asterisk indicate significant differences between adult and newborn values of the ISO effect and the combination of ISO and FOR effect, respectively, at p<0.05.
enhanced by PTX pretreatment both for AD and NB cells. In AD cells the \( I_{\text{Ca}} \) density produced by 0.1 \( \mu \text{M} \) ISO was significantly greater for PTX-treated cells (15.1±1.4 pA/pF) than for untreated cells (11.0±1.0 pA/pF). The percent increase in \( I_{\text{Ca}} \) caused by ISO was increased, on average, from 152% to 205% by the PTX pretreatment. The enhancement of the ISO effect on \( I_{\text{Ca}} \) was much more prominent in NB cells. The ISO-stimulated \( I_{\text{Ca}} \) density was more than twofold greater for PTX-treated cells (11.6±1.1 pA/pF) than for untreated cells (4.7±0.4 pA/pF). The percent increase in \( I_{\text{Ca}} \) by 0.1 \( \mu \text{M} \) ISO was increased from 81% to 297% in the NB cells by the PTX pretreatment. The values of the ISO-stimulated \( I_{\text{Ca}} \) density in PTX-treated cells were very close to the values of the \( I_{\text{Ca}} \) density stimulated by either 10 \( \mu \text{M} \) FOR or 200 \( \mu \text{M} \) cAMP in untreated cells for each age group, indicating that after PTX pretreatment 0.1 \( \mu \text{M} \) ISO increased \( I_{\text{Ca}} \) density nearly maximally even in NB cells. Thus, the difference in the effects of ISO and FOR on NB \( I_{\text{Ca}} \) was eliminated by the PTX pretreatment.

**Discussion**

The present study was focused on the postnatal changes in the \( \beta \)-adrenergic modulation of L-type \( I_{\text{Ca}} \) of rabbit ventricular cells. Our results obtained in this study are summarized as follows: 1) The efficacy of ISO to stimulate \( I_{\text{Ca}} \) (expressed as percent increase over control levels) was significantly greater for AD cells than for NB cells. The potency and the voltage dependency of ISO for \( I_{\text{Ca}} \) were little changed between the two groups. 2) The efficacy of FOR to stimulate \( I_{\text{Ca}} \) was significantly greater for NB cells than for AD cells, whereas the potency was slightly higher for AD cells than for NB cells. 3) \( I_{\text{Ca}} \) density stimulated by a saturating dose of ISO was equivalent to that stimulated by either a saturating dose of FOR or cAMP for AD cells. On the other hand, the ISO-stimulated \( I_{\text{Ca}} \) density was significantly lower than either the FOR-stimulated or cAMP-stimulated \( I_{\text{Ca}} \) for NB cells. 4) PTX pretreatment markedly enhanced the ISO effect on \( I_{\text{Ca}} \) for NB cells, whereas the enhancement was relatively small for AD cells. To our knowledge, this is the first report of age-related changes in the \( \beta \)-adrenergic modulation of L-type \( I_{\text{Ca}} \) in mammalian heart cells.

It is well established that the stimulatory effect of ISO on L-type \( I_{\text{Ca}} \) in cardiac cells is primarily mediated by the activation of adenylate cyclase. Our results are consistent with a biochemical study by Schumacher et al.\(^2\) on the postnatal modulation of adenylate cyclase activity by \( \beta \)-stimulation. They demonstrated in membrane preparations of rabbit ventricle that maximal stimulation of adenylate cyclase by ISO, expressed as pico moles cAMP per minute per milligram protein, was 2.5-fold greater for AD membranes than for NB membranes (1, 7,
and 12 days old). The EC₅₀ values were slightly greater for AD membranes (0.258 μM) than for NB membranes (0.12–0.156 μM). The discrepancy between the values of the EC₅₀ for adenylate cyclase and IₐCa for AD preparations may be attributed, at least in part, to the difference in the concentrations of ISO required to saturate adenylate cyclase and IₐCa. Thus, the saturating concentration for adenylate cyclase in their data was one order of magnitude higher than that for IₐCa in the present study, in agreement with the finding observed in cardiac cells that IₐCa saturates at high concentrations of cAMP, and cannot reflect cAMP levels under such conditions. The saturation of IₐCa before the saturation of adenylate cyclase by ISO would result in a decrease in the EC₅₀ for IₐCa. Similar age-related increases in epinephrine-stimulated adenylate cyclase activity have been demonstrated in dog and rat ventricular membranes.

FOR is considered to interact with the catalytic unit of adenylate cyclase without the requirement of activation of Gₛ, leading to the stimulation of cAMP production and phosphorylation of calcium channels. The effectiveness of FOR to stimulate IₐCa for NB cells suggests that the catalytic unit of adenylate cyclase and the cascade leading from adenylate cyclase to Ca²⁺ channel phosphorylation may be almost mature at birth. Hatjis has reported that the catalytic unit of adenylate cyclase is already matured during the prenatal period, based on the observation that the percent increase in adenylate cyclase activity over basal activity by FOR was equivalent in fetal and AD rabbit cardiac membranes.

FOR has recently been reported to affect a number of systems by mechanisms that do not involve the activation of adenylate cyclase. Therefore, it is possible that the greater effect of FOR on IₐCa for NB cells, in comparison with the effect of ISO, might be attributed to a direct effect of FOR on NB IₐCa. However, such a possibility can be ruled out by the finding that 10 μM FOR did not increase IₐCa for NB cells or for AD cells when the cells were dialyzed by 200 μM cAMP. It has also been proposed that ISO could have a direct action on L-type Ca²⁺ channels, independent of adenylate cyclase, via the direct effect of Gₛ on the channel gating mechanism. However, it is also unlikely that the greater effect of ISO on AD IₐCa than ON IₐCa can be attributed to a larger contribution of the direct action to the ISO effect in AD cells, because a saturating dose of ISO, FOR, or cAMP increased IₐCa density to similar levels for AD cells, suggesting that the ISO effect was mostly mediated by the activation of adenylate cyclase.

The clear difference in Eₘₐₓ (efficacy) of ISO and FOR on IₐCa for NB cells, but not for AD cells, can most likely be explained by a less effective signal transduction from the β-receptor to adenylate cyclase in NB cells. Differential effects of ISO and FOR on cardiac contractility have been reported, showing that the positive inotropic effect of FOR was
significantly greater than that of ISO in NB rabbit papillary muscles but not in AD muscles.

The stimulatory effect of β-agonists on ICa is antagonized by muscarinic cholinergic stimulation through an indirect and/or direct inhibition of adenylate cyclase by Gi. PTX specifically uncouples the interaction between Gi and muscarinic cholinergic receptors as well as other inhibitory receptors of adenylate cyclase by the ADP-ribosylation of the α-subunit of G_i with little affecting the intrinsic properties of G_i. The toxin has been used as a tool to examine the mechanism of the antiadrenergic effect on adenylate cyclase by the inhibitory receptor agonists. Previous biochemical studies using PTX in heart cells as well as other preparations have suggested that Gi has a tonic inhibitory influence on adenylate cyclase even in the absence of the inhibitory receptor agonists, based on the observations that PTX pretreatment enhanced GTP-stimulated and/or β-agonist-stimulated adenylate cyclase activity in addition to the abolition of the agonist-dependent inhibition of adenylate cyclase. In agreement with the biochemical studies, Brown et al demonstrated in isolated adult guinea pig ventricular cells that PTX pretreatment increased the sensitivity of the contractile response to ISO without changing the E_max. Hescheler et al reported that PTX pretreatment caused an increase in the sensitivity of ISO to stimulate ICa in adult guinea pig ventricular cells by showing that 0.05 μM ISO, the half-maximal concentration for untreated cells, nearly maximally increased the ICa density for PTX-treated cells. A similar enhancement of the ISO effect on ICa was observed in our rabbit ventricular cells after PTX pretreatment. Thus, even in NB cells, 0.1 μM ISO increased ICa density close to the levels of 10 μM FOR-stimulated or 200 μM cAMP-stimulated ICa density for untreated cells. The results strongly suggest that the smaller response of ICa to ISO in NB cells than in AD cells is mainly attributed to the greater tonic inhibition of Gi on adenylate cyclase in NB cells than in AD cells. The tonic Gi inhibition would affect the ICa response to FOR but not to cAMP. The clear difference in the efficacy of ISO and FOR on NB ICa could be explained by a difference in the mode of Gi inhibition on ISO- and FOR-stimulated ICa. Thus, it has been shown in frog ventricular cells that the activation of G_i produced by acetylcholine or Gpp(NH)p, mainly reduces the efficacy of ISO to stimulate ICa, whereas the activation of Gi reduces the potency of FOR with little change in the efficacy. The stimulatory effect of a maximal dose of FOR on adenylate cyclase would overcome the tonic inhibition of adenylate cyclase by Gi in NB cells. Instead, the greater tonic inhibition of Gi in NB cells might account for the slightly lower potency of FOR for NB ICa than for AD ICa. Since PTX is believed to have no effect on Gi, it is also suggested that the β-receptor-Gi-adenylate cyclase pathway is functionally developed at birth in rabbit ventricular cells.

The precise mechanisms underlying the tonic Gi inhibition and its removal by PTX have still remained unclear. There has been accumulating evidence, in reconstitution studies in which purified receptors and G proteins were incorporated in phospholipid vesicles, suggesting that unoccupied receptors are not inactive but exhibit a basal agonist-independent activity responsible for the various effects of GTP observed on G protein–coupled effector functions in intact membranes including GTP-dependent activation or inhibition of adenylate cyclase. If it is the case in intact cells, the balance of functional G_i/G_i and/or stimulatory/inhibitory receptors would modify basal conditions of adenylate cyclase even in the absence of the agonists. The enhancement of the ISO effect by PTX pretreatment may be brought about through a shift of the G_i/G_i balance in favor of G_i where ICa becomes more responsive to ISO by blocking the interaction between the inhibitory receptors and Gi.

The greater tonic inhibition of Gi in NB cells may be attributed to the greater levels of Gi and/or the higher density of muscarinic receptors in younger animals. McMahon reported, in membrane preparations from rat hearts, that the levels of PTX-sensitive G proteins (G_i/G_o), measured by [32P]ADP-ribosylation with PTX (picomoles per milligram protein) and muscarinic receptor density, expressed as maximal number of [3H]QNB binding sites (femtomoles per milligram tissue), were twofold to threefold higher in NB membranes than in AD membranes. Luejt et al also demonstrated that the amount of α-subunit of Gi in rat ventricle decreases with age in parallel to the decrease in the levels of mRNA encoding the Gi subunit. It can be assumed that rabbit ventricular cells undergo similar age-related changes in Gi and/or muscarinic receptors, because the inhibitory effect of carbachol on ISO- or FOR-stimulated ICa is much greater in NB rabbit ventricular cells than in AD cells (authors’ unpublished data). Recently, the increase in Gi levels has also been proposed as a mechanism of the reduced inotropic effect of catecholamines on failing human hearts.

The E_max of FOR (percent increase in ICa) was significantly greater for NB cells than for AD cells, resulting in a decrease in the ratio of AD/NB ICa density from 2.1 in control to 1.5 in the presence of 10 μM FOR. Similarly, the AD/NB ICa ratio was decreased to 1.3 in the presence of 200 μM cAMP. However, the ICa density stimulated by either FOR or cAMP, in which the P_o and P_s of L-type Ca²⁺ channels are considered to increase almost maximally, was still significantly higher in AD cells than in NB cells. The results suggest that L-type Ca²⁺ channel density (number of channels per square micrometer of membrane) increases after birth in rabbit ventricular cells. Recently, Wibo et al reported, using a radioligand [3H](+)-PN 200-110, in rat ventricular tissue that L-type Ca²⁺ channel density increases along with the appearance and the differentiation of...
the transverse tubule (T tubule) and sarcoplasmic reticulum. Taking the development of T tubules into account, they estimated L-type Ca\textsuperscript{2+} channel density for AD and NB cells as 22 sites/μm\textsuperscript{2} and 13 sites/μm\textsuperscript{2}, respectively. The ratio of the channel density for AD versus NB rat ventricular cells (1.7) is in good agreement with the AD/NB ratio of I\textsubscript{Ca} density for rabbit ventricular cells (1.3–2.1) in our experiments.

It has been reported, in adult guinea pig ventricular cells, that in the absence of β-adrenergic stimulation (control conditions) a large portion of Ca\textsuperscript{2+} channels are in the dephosphorylated state, while the rest of the channels (20–30%), which have higher P\textsubscript{c} and/or P\textsubscript{r}, are phosphorylated, as reflected by a low basal adenylate cyclase activity (or basal cAMP level).\textsuperscript{60–62} According to Hatjis\textsuperscript{39} basal adenylate cyclase activity (picomoles cAMP per milligram protein per minute) in rabbit cardiac membranes increases with advancing age, in agreement with a greater tonic inhibition of G\textsubscript{i} on adenylate cyclase in NB cells. Therefore, we propose that the L-type Ca\textsuperscript{2+} channels for NB cells may be mostly dephosphorylated in basal conditions because of the lower intracellular cAMP levels caused by the greater G\textsubscript{i} inhibition of adenylate cyclase. That would produce a further decrease in the P\textsubscript{c} and/or P\textsubscript{r} of the Ca\textsuperscript{2+} channels, even compared with those for basal AD conditions, which in turn enables a greater percent increase in I\textsubscript{Ca} for NB than for AD cells in response to maximal stimulation. This assumption can be supported by the significant increase in basal I\textsubscript{Ca} density for NB cells, but not for AD cells, after pretreatment with PTX, which might be brought about through a greater increase in cAMP levels by removing the greater tonic G\textsubscript{i} inhibition of adenylate cyclase in NB cells.

Acknowledgments

We wish to thank Drs. H. Criss Hartzell and Douglas C. Eaton for their comments on this work. We are grateful to Ms. Dorothy Ellingsen for her help in the cell isolation.

References

4. Reuter H: Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 1983;301:569–574


42. Zankler BJ, Trube G, Ohno Shosaku T: Forskolin-induced block of delayed rectifying K+ channels in pancreatic beta-cells is not mediated by cAMP. Pflugers Arch 1988;411:613–619


KEY WORDS • developmental change • rabbit ventricular cell • calcium current • isoprotenerol • forskolin • cAMP • pertussis toxin
Developmental changes in the beta-adrenergic modulation of calcium currents in rabbit ventricular cells.

T Osaka and R W Joyner

*Circ Res.* 1992;70:104-115
doi: 10.1161/01.RES.70.1.104

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/70/1/104

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/