β2-Adrenergic receptor agonists stimulate L-type calcium current independent of PKA in newborn rabbit ventricular myocytes

Leon P. Collis,1 Shekhar Srivastava,1 William A. Coetzee,1 and Michael Artman2

1Department of Pediatrics, Program in Pediatric Cardiology, New York University School of Medicine, New York, New York; and 2Department of Pediatrics, University of Iowa Carver College of Medicine, Iowa City, Iowa

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Collis LP, Srivastava S, Coetzee WA, Artman M. β2-Adrenergic receptor agonists stimulate L-type calcium current independent of PKA in newborn rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol 293: H2826–H2835, 2007. First published August 24, 2007; doi:10.1152/ajpheart.00101.2007.—Selective stimulation of β2-adrenergic receptors (β2-ARs) in newborn rabbit ventricular myocardium invokes a positive inotropic effect that is lost during postnatal maturation. The underlying mechanisms for this age-related stimulatory response remain unresolved. We examined the effects of β2-AR stimulation on L-type Ca2+ current (ICa,L) during postnatal development. ICa,L was measured (37°C; either Ca2+ or Ba2+ as the charge carrier) using the whole-cell patch-clamp technique in newborn (1 to 5 days old) and adult rabbit ventricular myocytes. Ca2+ transients were measured concomitantly by dialyzing the cell with indo-1. Activation of β2-ARs (with either 100 nM zinterol or 1 μM isoproterenol in the presence of the β1-AR antagonist, CGP20712A) stimulated ICa,L twofold in newborns but not in adults. The β2-AR-mediated increase in Ca2+ transient amplitude in newborns was due exclusively to the augmentation of ICa,L. Zinterol increased the rate of inactivation of ICa,L and increased the Ca2+ flux integral. The β2-AR inverse agonist, ICI-118551 (500 nM), but not the β1-AR antagonist, CGP20712A (500 nM), blocked the response to zinterol. Unexpectedly, the PKA blockers, H-89 (10 μM), PKI 6-22 amide (10 μM), and Rp-cAMP (100 μM), all failed to prevent the response to zinterol but completely blocked responses to selective β1-AR stimulation of ICa,L in newborns. Our results demonstrate that in addition to the conventional β1-AR/CAMP/PKA pathway, newborn rabbit myocardium exhibits a novel β2-AR-mediated, PKA-insensitive pathway that stimulates ICa,L. This striking developmental difference plays a major role in the age-related differences in inotropic responses to β2-AR agonists.

MATERIALS AND METHODS

Cell isolation. Ventricular myocytes were isolated from the hearts of New Zealand White rabbits at two different age groups: 1 to 5 days old (both sexes) and adult males (>150-day old; left ventricle only). Rabbits were heparinized (500 IU/kg) and anesthetized using pentobarbital sodium (60 mg/kg body wt). Isolation involved retrograde Langendorff perfusion with a collagenase-based digestion described previously (3). Myocytes were stored in Krebs bicarbonate solution (3) at room temperature until used. All experiments were performed within 8 h of cell isolation and were performed in accordance with the recommendations from the National Institutes of Health “Guiding Principles in the Care and Use of Animals” and approved by the Institutional Laboratory Animal Care and Use Committee at New...
York University School of Medicine. A total of 30 newborn and 6 adult rabbits were used for myocyte studies. Individual numbers of preparations (preps) are presented in RESULTS.

**Electrophysiology.** Adult and newborn ventricular myocytes were patch clamped in whole cell mode at 37°C. Micropipettes (1–2 MΩ for adult, and 4–5 MΩ for newborn) were used. Ica,L was measured by using the methods previously described (49) with slight modifications. Seal and cell rupture was achieved in standard Tyrode solution, and myocytes were voltage clamped to −80 mV and perfused with the modified Ica,L bath solution. Currents were measured with a patch-clamp amplifier (Axopatch 200B, Axon Instruments) and low-pass filtered (−3 dB at 1 kHz). The offset potential was corrected by zeroing the potential before touching the surface of the cell with the pipette tip. Cell capacitance, access resistance, and membrane seal were zeroing the potential before touching the surface of the cell with the pipette tip. Cell capacitance, access resistance, and membrane seal resistance were measured using a +5-mV step pulse (pClamp v.8.1, Axon Instruments) at a holding potential of −80 mV. Access resistances between 2 and 10 MΩ were deemed adequate. Cell capacitance and series resistance were compensated, the latter to at least 70%. Clamp potentials were offset to correct for a shift in calculated junctional potential of −0.5 mV (Clampex v.8.1; Axon Instruments). Ica,L was measured with a prepulse from −80 to −50 mV for 200 ms to inactivate T-type Ca2+ current (Ica,T) and Na+ current (INa). A step-pulse series from −50 to −50 mV (Δ10 mV; 0.1 Hz) was used to assess the shift in voltage dependence of peak Ica,L, whereas a single pulse from −50 mV to −10 mV (0.05 Hz) was used to examine the effect of the drug on peak Ica,L with time. Steady-state activation parameters in newborn myocytes were extracted from conditioning steps using a double-voltage protocol (Δ10 mV; 0.01 Hz; Fig. 3A, top). Inactivation parameters were extracted from a test pulse to −10 mV that followed the conditioning pulses after a 5-ms step to −80 mV (Fig. 3A, top). Time dependence of inactivation was assessed in newborn myocytes, using the voltage protocol shown (0.1 Hz; Fig. 3B, top). At the close of the experiment, 10 μM nifedipine was applied to block Ica,L. This component was numerically subtracted from the Ica,L traces.

**Concomitant measurements of Ca2+ transients and Ica,L.** The bath solution consisted of Tyrode buffer with 1.8 mM Ca2+ and equimolar CsCl replacing KCl. Newborn (1–5 day old) ventricular myocytes were patch clamped in whole cell mode with 3–5-MΩ pipettes. Background fluorescence was assessed after gigaseal formation but before rupture. Fluorescence measurements were obtained using Delta Ram fluorescence system (Photon Technology International, Princeton, NJ). The bath solution consisted of Tyrode buffer with 1.8 mM Ca2+ and equimolar CsCl replacing KCl. Newborn (1–5 day old) ventricular myocytes were patch clamped in whole cell mode with 3–5-MΩ pipettes. Background fluorescence was assessed after gigaseal formation but before rupture. Fluorescence measurements were obtained using a Delta Ram fluorescence system [Photon Technology International (PTI)]. After 10 min of dialysis, 10 cycles (0.2 Hz) of step clamps (−50 mV for 200 ms, and then −10 mV for 200 ms) were used. Fluorescence was recorded as the ratio, F = F0/Frest (Felix; v.1.42, PTI). The relative change in fluorescence (ΔF/F0: F1 = peak, F0 = resting) was assessed before and after application of zinterol and nifedipine (10 μM). Peak Ca2+ transient was averaged over the last five cycles at steady state.

**Solutions.** Tyrode saline contained (in mM) 137 NaCl, 5.4 KCl, 10 HEPES, 1 MgCl2, 0.33 Na2HPO4, and 1.8 CaCl2 adjusted with NaOH to pH 7.4. Ica,L bath solution contained (in mM) 140 tetraethylammonium (TEA), 6 CsCl, 5 HEPES, 2 CaCl2, 1 MgCl2, 10 glucose, 0.5 niflumic acid, pH adjusted to 7.4 with TEA-OH. Pipette solution for recording Ica,L contained (in mM) 125 CsCl, 20 HEPES, 10 Mg-ATP, 5 β-APTA (tetracessamine salt), 0.3 GTP (Tris salt), buffered to 7.2 with CsOH. Mg-ATP and GTP were included to minimize rundown during whole cell patch clamp. Rundown was negligible, where 95 ± 6%, 87 ± 4%, and 80 ± 3% (n = 4) of initial peak Ica,L remained after 10, 30, and 40 min, respectively. Most experiments were completed after 20 min of postureture. To block the influence of sarcoplasmic reticulum Ca2+ release on β2-AR stimulation, 10 μM ryanodine was included in the pipette. The pipette solution for concomitant measurement of Ica,L and Ca2+ transients contained (in mM) 130 K+glutamate, 9 KCl, 10 NaCl, 1 MgCl2, 5 MgATP, 10 HEPES, and 0.01 indol-1-K+ pentapotassium salt (Molecular Probes), pH adjusted to 7.2 with KOH.

**Drug application.** The β-AR agonists, zinterol (gift of Bristol-Myers Squibb) and isoproterenol (Iso, 1 μM; Sigma-Aldrich), were added for 3 min. The β-blockers, CGP20712A (CGP; 500 mM; Sigma-Aldrich) and ICI-118551 (ICI; 500 mM; Sigma-Aldrich) were applied for 10 min before β-AR stimulation. The membrane-permeable PKA blocker N-(2-[p-bromocinnamyl]amino)ethyl)-5-isouquinoline-sulfonamide (H8; 10 μM; Sigma-Aldrich) and the Ca2+/calmodulin kinase II (CaMKII) blocker, KN93 (5 μM; Calbiochem), were added for 20 min before stimulation with zinterol. The PKA blockers, adenosine 3′,5′-cyclic phosphorothioate-Rp (Rp-cAMP; 100 μM; Calbiochem) and PK1 6-22 amide (PKI; 10 μM; Calbiochem), were dialyzed intracellularly via the patch pipette for at least 5 min before β-AR stimulation. IBMX (100 μM; 5 min; Sigma-Aldrich) was used to inhibit phosphodiesterases (PDEs), and 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (8-CPT-cAMP; 100 μM; Calbiochem) was used to increase intracellular cAMP concentration. ADP ribosylation of Gα, was catalyzed with pertussis toxin (PTX; 200 ng/ml at 37°C for 3 h). In some experiments, equimolar BaCl2 replaced CaCl2 in the bath solution.

**Statistics.** Results are presented as means ± SE (unless stated otherwise). Statistical significance was determined by Student’s t-test for paired data (unless stated otherwise) and a one-way ANOVA for current-voltage (I-V) relationships. A value of P < 0.05 was considered significant.

**RESULTS**

**Stimulation of β2-ARs augments Ica,L in newborns but not adults.** To investigate the amplitude and kinetics of Ica,L, all ancillary currents were blocked and 2 mM Ca2+ was used as a charge carrier with heavily buffered [Ca2+2]. Under these conditions, zinterol increased nifedipine-sensitive Ica,L almost twofold in newborn myocytes (Fig. 1A and Table 1; P < 0.05; 10 preps). The effect of zinterol was reversed with washout (Fig. 1A, top). In contrast to newborns, zinterol had no effect on peak Ica,L in adult rabbit myocytes (Fig. 1A; control: 17.3 ± 0.7; zinterol: 16.3 ± 0.8 pA/pF; n = 5; 3 preps). The presence of minor residual inward current with 10 μM nifedipine in the adult suggests newborn myocytes are more sensitive to nifedipine and that 10 μM nifedipine cannot completely block all Ica,L in adult myocytes. Mean cell capacitance increased from 22.7 ± 1.3 (n = 30) in newborns to 131.0 ± 8.6 pF (n = 7) in adult myocytes. There was also a twofold increase in Ica,L density postnatally (Fig. 1A). In addition, the adult I-V plot for Ica,L showed a +10-mV shift in voltage dependence of peak Ica,L relative to the newborn (Fig. 1A; I-V curves). In newborn myocytes, zinterol induced a slight hyperpolarizing shift in the voltage dependence of peak Ica,L (Fig. 1A; I-V curve) with no change in the reversal potential for Ica,L (∼+50 mV). In newborn myocytes, the time constants of inactivation of Ica,L were significantly reduced by zinterol, using both biexponential or monoexponential fits (Table 1; P < 0.05). In addition, zinterol increased the integral of peak Ica,L and corresponding [Ca2+]i flux (in amol/pF) by twofold (Table 1). Thus, despite faster inactivation, the total Ca2+ influx through L-type channels was more than doubled in the presence of zinterol.

**Zinterol induces a concomitant increase in Ca2+ transient and Ica,L in newborns. Ica,L and Ca2+ transients were recorded simultaneously in newborn myocytes to verify that the zinterol-induced increase in Ica,L leads to a functional increase in [Ca2+2]. Zinterol augmented peak Ica,L from 3.5 ± 0.3 to 5.2 ± 0.7 pA/pF (P < 0.05; n = 5; 4 preps) and increased the amplitude of the Ca2+ transient by 25.1 ± 4.8% (Fig. 1B; ΔF

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Fig. 1. β2-Adrenergic receptor (AR) stimulation of Ca\(^{2+}\) transients in newborn (Nb) myocytes is mediated by L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)). A, left: representative I\(_{\text{Ca,L}}\) traces during stimulation with 100 nM zinterol (Zint) in Nb and adult (Ad) cardiac myocytes, recorded at −10 and 0 mV, respectively. Current was nifedipine (Nif) sensitive. A, right: current-voltage (I-V) plots of peak I\(_{\text{Ca,L}}\) density in Nb (n = 6) and Ad (n = 6) cardiac myocytes. In Nb myocytes, the increase in current density with Zint was voltage dependent (2-factor ANOVA with replication; P < 0.05; n = 6). B: peak I\(_{\text{Ca,L}}\) and Ca\(^{2+}\) transients recorded concomitantly from Nb myocytes dialyzed with indo-1. C: representative Ca\(^{2+}\) transients at peak I\(_{\text{Ca,L}}\) (−10 mV; 200 ms) in Nb myocytes exposed to Nif and Zint plus Nif. D: representative Ca\(^{2+}\) transients measured with voltage clamp to +50 mV (200 ms) before and after Zint application. Arbitrary units are fluorescence ΔF/ΔF\(_0\) units. Voltage-clamp protocol for all transients was a 200-ms prepotential step to −50 mV, followed by a 200-ms step to either −10 or +50 mV. V\(_{\text{m}}\), test potential.

ratio units: 1.0 ± 0.1 to 1.25 ± 0.1; P < 0.05; n = 5). Basal I\(_{\text{Ca,L}}\) was significantly lower compared with the control data in Fig. 1A due to the reduced Ca\(^{2+}\) buffering that was necessary for recording Ca\(^{2+}\) transients. Under these nominal Ca\(^{2+}\) buffering conditions, a monoeponential curve was the best fit for the inactivation profile for I\(_{\text{Ca,L}}\). As with the data in Fig. 1A, \(\tau_1\) was significantly decreased with zinterol (basal: 25.5 ± 1.7; zinterol: 22.0 ± 1.2 ms; P < 0.05; n = 5). To examine whether the zinterol-mediated increase in I\(_{\text{Ca,L}}\) accounted for this observed increase in Ca\(^{2+}\) transient, I\(_{\text{Ca,L}}\) was blocked with nifedipine or by voltage clamping to the apparent reversal potential for I\(_{\text{Ca,L}}\) (+50 mV) in newborn myocytes. After a 3-min perfusion with 10 μM nifedipine, I\(_{\text{Ca,L}}\) was completely blocked (Fig. 1C). This current shows a negligible amount of residual inward and outward current, possibly from incomplete inactivation of I\(_{\text{K,\text{Ca,L}}}\), I\(_{\text{Ca,T}}\), and K\(^+\) current. This was partly due to an abbreviated prepulse of only 200 ms at −50 mV that was necessary to prevent hypercontraction of newborn myocytes. These residual inward currents were also observed at +50 mV (Fig. 1D), suggesting this is not I\(_{\text{Ca,L}}\). At 0 mV and in the presence of nifedipine, the amplitude of the Ca\(^{2+}\) transient was reduced by 23.6% [Fig. 1C; ΔF (ratio units): 1.2 ± 0.4 to 0.9 ± 0.3; n = 4; P < 0.05]. This was coupled with a 6% decrease in the level of diastolic fluorescence (Fig. 1C; 1.05 ± 0.3 to 0.97 ± 0.3 ΔF units; n = 5; P < 0.05). Subsequent application of zinterol in nifedipine-treated myocytes failed to increase the amplitude of the Ca\(^{2+}\) transient [Fig. 1C; ΔF (ratio units): 0.9 ± 0.3 to 1.1 ± 0.3; n = 4; P > 0.05]. At +50 mV, I\(_{\text{Ca,L}}\) was absent (Fig. 1D), yet the Ca\(^{2+}\) transient amplitude was increased by 51.6% (−10 mV: 1.1 ± 0.1 ΔF; +50 mV: 1.7 ±
Table 1. Effects of zinterol on $I_{\text{Ca,L}}$ in newborn ventricular myocytes

<table>
<thead>
<tr>
<th>Peak $I_{\text{Ca,L}}$ parameters</th>
<th>n</th>
<th>Control</th>
<th>Zinterol</th>
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<tr>
<td>Peak $I_{\text{Ca,L}}$, pA/pF</td>
<td>12</td>
<td>-9.2±0.9</td>
<td>-16.2±2.3*</td>
</tr>
<tr>
<td>Inactivation $\tau_{\text{fast}}$, ms</td>
<td>12</td>
<td>16.7±2.7</td>
<td>15.3±2.4*</td>
</tr>
<tr>
<td>Inactivation $\tau_{\text{slow}}$, ms</td>
<td>12</td>
<td>163±50.2</td>
<td>101±35*</td>
</tr>
<tr>
<td>Inactivation $\tau_{\text{mono}}$, ms</td>
<td>5</td>
<td>23.3±8.8</td>
<td>21.1±2.4*</td>
</tr>
<tr>
<td>$Ca^{2+}$ flux, amol/pF; $f_{I_{\text{Ca,L}}}$ dt</td>
<td>5</td>
<td>1.0±0.1</td>
<td>2.3±0.2*</td>
</tr>
<tr>
<td>$\tau_{\text{recovery}}$, ms*</td>
<td>5</td>
<td>57.8±2.0</td>
<td>79.8±6.7*</td>
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</tbody>
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Steady state parameters

**Activation**

<table>
<thead>
<tr>
<th>$V_{\text{m}}$, mV</th>
<th>slope (k)</th>
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<tr>
<td>-16.2±0.1</td>
<td>5.2±0.1</td>
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**Inactivation**

<table>
<thead>
<tr>
<th>$V_{\text{m}}$, mV</th>
<th>slope (k)</th>
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<tr>
<td>-30.5±0.3</td>
<td>4.5±0.2</td>
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</table>

Values are means ± SE; n, number of myocytes. L-type Ca$^{2+}$ current ($I_{\text{Ca,L}}$) was recorded at a holding potential of -10 mV. Inactivation time constants and Ca$^{2+}$-flux integrals were extrapolated from recordings of peak $I_{\text{Ca,L}}$. Monoeponential and biexponential plots were fitted to the inactivation time course of $I_{\text{Ca,L}}$ in order to evaluate $\tau_1$ and $\tau_{\text{fast/slow}}$, respectively (OriginPro 7.0, OriginLab). Intracellular Ca$^{2+}$ concentration flux = $f_{I_{\text{Ca,L}}}$ dt/dz, where $z$ = valency and $F$ = Faraday’s constant (49), normalized to $C_m$. Steady-state parameters were extracted from Boltzmann curves fitted to data in Fig. 3A. $V_{\text{m}}$, Voltage at half-maximal conductance. *P < 0.05 for control vs. zinterol.

*Extracted from exponential curves in Fig. 3B.

Zinterol increases $I_{\text{Ca,L}}$ in newborn myocytes by β2-AR stimulation. In newborns, the nonselective β-AR agonist, Iso (1 μM), increased $I_{\text{Ca,L}}$ to a similar magnitude as zinterol (peak $I_{\text{Ca,L}}$ control: 8.0±0.8; Iso: 13.4±1.9 pA/pF; n = 6; P < 0.05; 4 preps), although this increase was less than that observed in adult myocytes (peak $I_{\text{Ca,L}}$ control: 16.1±0.9; Iso: 41.3±4.5 pA/pF; P < 0.05; n = 4). In newborns, β2-AR stimulation using ISO in combination with the β1-AR blocker, CGP (500 nM) increased $I_{\text{Ca,L}}$ to a similar extent with zinterol alone (Fig. 2, A and C; n = 4; 2 preps) but failed to stimulate $I_{\text{Ca,L}}$ in adult myocytes (Fig. 2A; n = 4; 3 preps). This provides additional evidence that activation of β2-ARs fails to stimulate Ca$^{2+}$-channels in the adult and confirms that CGP is an effective blocker of β1-ARs. CGP alone did not alter basal current significantly (peak $I_{\text{Ca,L}}$: control: 15.9±1.5; CGP: 14.1±2.0 pA/pF; n = 4; P < 0.05; 2 preps).

Zinterol mediated its effect on $I_{\text{Ca,L}}$ via activation of β2-ARs. Evidence for this assertion is as follows: the increase in $I_{\text{Ca,L}}$ was blocked by the β2-AR inverse agonist ICI (Fig. 2, B and C; peak $I_{\text{Ca,L}}$ control: 11.2±2.2; zinterol: 9.6±1.5 pA/pF; n = 4; P < 0.05; 3 preps). The β1-AR blocker, CGP, failed to block the effect of zinterol in newborn myocytes (Fig. 2, B and C; peak $I_{\text{Ca,L}}$: control: 8.1±2.1; zinterol: 12.7±4.2 pA/pF; n = 4; P < 0.05; 2 preps). Interestingly, ICI by itself did not significantly alter basal $I_{\text{Ca,L}}$ (peak $I_{\text{Ca,L}}$ control: 10.3±1.7; ICI: 9.7±1.5 pA/pF; P > 0.05; n = 5; 3 preps), suggesting there is little constitutive β2-AR stimulation of $I_{\text{Ca,L}}$ in newborn rabbit myocytes. This result also implies that ICI exhibits negligible inverse agonist activity as previously suggested (14).

Zinterol affects the voltage-dependence of $I_{\text{Ca,L}}$ in newborn myocytes. The negative shift of peak $I_{\text{Ca,L}}$ with zinterol was further studied by analyzing the voltage dependence of steady-state activation and inactivation in newborn myocytes. Zinterol shifted the steady-state activation and inactivation curves by -5.0 and -5.6 mV, respectively (Fig. 3A and Table 1; n = 5; P < 0.05; 3 preps), whereas the slope factor (k) was not significantly affected (Table 1). The window current (overlap of inactivation and activation curves) was similar for both control and zinterol, indicating that the fraction of steady-state current relative to maximum conductance was not affected.

The time course of recovery from inactivation was significantly, though only mildly, prolonged by zinterol (Fig. 3B and Table 1; 3 preps). This was possibly a secondary effect of increased Ca$^{2+}$ influx with zinterol stimulation. β2-AR but not β2-AR stimulation is mediated by PKA in the newborn. Selective stimulation of β1-ARs in newborn myocytes was achieved using Iso (1 μM) in ICI-pretreated cells (Fig. 4A). The effect of Iso on peak $I_{\text{Ca,L}}$ was 18.2% less in the presence of ICI than with ISO alone, suggesting ISO is an agonist for both β1-ARs and β2-ARs in the newborn. As with selective β2-AR stimulation, a slight negative shift in voltage dependence of the I-V relationship was observed. β1-AR stimulation of $I_{\text{Ca,L}}$ in newborns was completely blocked at all potentials by intracellular dialysis of the PKA-specific inhibitor, Rp-cAMP (100 μM; Fig. 4B). The I-V curve shows that this block was not due to a shift in the voltage dependence of peak $I_{\text{Ca,L}}$ (Fig. 4B, right), and Rp-cAMP did not affect basal $I_{\text{Ca,L}}$ (Fig. 4, A and B). However, Rp-cAMP failed to prevent the increase in peak $I_{\text{Ca,L}}$ by zinterol ($I_{\text{Ca,L}}$ increased by 66 ± 16%; Fig. 4, C and D; n = 7; 5 preps). The I-V relationship shows that this increase was not mediated by a shift in voltage dependence of peak $I_{\text{Ca,L}}$. To eliminate the possibility of limited blockade of PKA by Rp-cAMP, additional PKA inhibitors were tested. Intracellular dialysis with the peptide, PKI (10 μM), and bath application of the synthetic PKA inhibitor, H89 (10 μM; 20 min exposure), did not prevent the zinterol-mediated increase in $I_{\text{Ca,L}}$ (Fig. 4D). However, H89 and PKI did reduce mean basal $I_{\text{Ca,L}}$ by 26% and 23%, respectively (Fig. 4C; P < 0.05; unpaired t-test; 4 and 3 preps, respectively). Prolonged exposures (>30 min) of H89 decreased basal $I_{\text{Ca,L}}$ by 42.1% to 4.4 ± 0.7 pA/pF (n = 4; P < 0.05; 2 preps), suggesting either a role for constitutive PKA at rest or possibly a secondary effect of the H89 and PKI compounds. These data indicate that in newborn myocytes, there is a substantial PKA-independent component to stimulation of $I_{\text{Ca,L}}$ by zinterol.

The effect of zinterol is not Ca$^{2+}$ or CaMKII dependent. To investigate whether changes in [Ca$^{2+}$], during voltage clamp regulate the β2-AR response, we used Ba$^{2+}$ (2 mM) as a charge carrier. In the newborn, switching to Ba$^{2+}$ exhibited a mild slowing of inactivation for both time constants (Fig. 5A; $\tau_{\text{fast}}$: 9.9±0.8 to 23.7±1.3; $\tau_{\text{slow}}$: 79.3±3.2 to 89.5±2.1 ms; n = 4; 2 preps). In the adult, whereas $\tau_{\text{fast}}$ decreased by a similar magnitude with Ba$^{2+}$, $\tau_{\text{slow}}$ was markedly prolonged ($\tau_{\text{fast}}$: 19.4±1.8 to 35.6±2.4 ms; $\tau_{\text{slow}}$: 69.5±5.7 to 114.2±6.8
Fig. 2. The effect of Zint is mediated by β2-ARs. A: representative peak \( I_{\text{Ca,L}} \) traces in CGP20712A (CGP)-treated Nb and Ad cardiac myocytes during stimulation with isoproterenol (Iso). B: representative peak \( I_{\text{Ca,L}} \) in a Nb myocyte during application with Zint following pretreatment with CGP or ICI-118551 (ICI). C: effect of various β-adrenergic interventions on \( I_{\text{Ca,L}} \) in Nb myocytes, expressed as percent increase in \( I_{\text{Ca,L}} \) relative to basal \( I_{\text{Ca,L}} \). Each intervention had a significant effect on \( I_{\text{Ca,L}} \) (\( P < 0.05 \)) compared to basal values (represented by the horizontal dashed line). **\( P < 0.05 \), β-AR agonist vs. β-AR agonist plus β-AR blocker. Error bars are means ± SE. \( I_{\text{Ca,L}} \), Ca\(^{2+} \) current. \( n \), Number of experiments.

\[ \text{ms}; \; n = 3; \; P < 0.05; \; \text{Fig. 5A, inset}. \] In the newborn, zinterol stimulated Ba\(^{2+} \) current (\( I_{\text{Ba,L}} \)) similar to \( I_{\text{Ca,L}} \) (92 ± 24%; Fig. 5, B and D; \( n = 4; \; 2 \) preps) and decreased the inactivation time constants (\( \tau_{\text{fast}} \); 23.7 ± 1.3 to 21.3 ± 1.0; \( \tau_{\text{slow}} \); 81.2 ± 2.1 to 61.6 ± 5.0 ms; \( n = 4; \; P < 0.05 \)). This suggests that the β2-AR response is not mediated by Ca\(^{2+} \) influx through Ca\(^{2+} \) channels.

Ca\(^{2+} \)/CaMKII has been implicated as an alternative pathway to PKA in β-AR signaling in cardiac myocytes (40). To examine the possible role of CaMKII in the newborn, we used the CaMKII inhibitor KN93 (5 μM). Interestingly, KN93 reduced basal \( I_{\text{Ca,L}} \) in the newborn myocyte (Fig. 5D; \( P < 0.05 \); \( n = 6; \; 3 \) preps). The inactive analog of KN93 (KN92; 5 μM) also reduced basal \( I_{\text{Ca,L}} \) (Fig. 5D; \( P < 0.05 \); \( n = 4; \; 2 \) preps), suggesting a nonspecific interaction between the Ca\(^{2+} \) channel and these compounds. Nevertheless, both KN93 and KN92 failed to prevent or augment the effect of zinterol on \( I_{\text{Ca,L}} \), suggesting that CaMKII is not involved in mediating the β2-AR effects on \( I_{\text{Ca,L}} \) in newborn myocytes.

**Downstream mediators of β2-AR stimulation.** To assess whether Gi restricts β2-AR signaling in the newborn myocyte, myocytes were incubated with PTX, which catalyzes the ADP-riboseylation of the α-subunits of Gi. PTX treatment augmented the zinterol-mediated increase in \( I_{\text{Ca,L}} \) by 68 ± 48% (Fig. 5, C and D; \( P < 0.05 \); unpaired t-test; \( n = 7; \; 5 \) preps) but did not affect the level of basal \( I_{\text{Ca,L}} \) (Fig. 5D).

Myocytes were exposed to a saturating concentration of the PDE blocker, IBMX (100 μM), to maximally increase cAMP (1). IBMX increased basal peak \( I_{\text{Ca,L}} \) significantly (Fig. 5, C and D; \( P < 0.05 \); \( n = 4; \; 2 \) preps). With this concentration of IBMX, zinterol did not further increase the amplitude of peak \( I_{\text{Ca,L}} \) (Fig. 5, C and D; \( n = 4; \; 2 \) preps), suggesting saturation of the cAMP pathway. This effect was replicated by dialyzing the cells with 100 μM 8-CPT-cAMP (Fig. 5, C and D; \( P < 0.05 \); \( n = 4 \)).

**DISCUSSION**

**Developmental change in the effect of selective β2-AR stimulation on \( I_{\text{Ca,L}} \).** This study was motivated by the observation that cardiac myocytes from newborn, but not adult, rats exhibit a robust positive inotropic response to β2-AR agonists (26). To characterize the potential mechanisms for this developmental change, we measured the effects of β2-AR stimulation on \( I_{\text{Ca,L}} \) using the selective β2-AR agonist, zinterol, and the nonselective β-AR agonist, Iso, in the presence of the β1-AR blocker, CGP. Stimulation of β2-ARs using either protocol significantly increased \( I_{\text{Ca,L}} \) in freshly isolated newborn rabbit myocytes but had no effect on \( I_{\text{Ca,L}} \) in adult myocytes. This also demonstrated the efficacy of CGP as a β1-AR blocker, by preventing any stimulatory action of Iso in the adult. In the newborn, CGP failed to block the effect of zinterol, which supports the claim that 100 nM zinterol acts exclusively on β2-ARs. A well-documented β2-AR inverse agonist, ICI (14), also blocked any stimulatory action of zinterol. The relative response to Iso was greater in adults than in newborn myocytes. This is consistent
with a relative lower density of β-ARs at birth in the rabbit (36) and an increased efficacy of Iso to stimulate ICa,L with age (32).

In addition to our present findings in rabbits, β2-AR stimulation has been previously shown to increase contractility in the newborn rat (26, 34) and newborn mouse (8). However, there is a lack of information on the specific Ca\(^{2+}\) sources and transport pathways involved in this process. We have shown that increased ICa,L plays a major role in the augmented response to β2-AR agonists in newborn rabbits. The diminished responsiveness to β2-AR-mediated stimulation of Ca\(^{2+}\) current (ICa) in the adult has been observed in a number of species, including mouse (42), rat (6, 27), and dog (30). Thus the age-related changes in β2-AR responsiveness may be a general developmental phenomenon that is not necessarily exclusive to the species that we chose for these studies (rabbit).

**Stimulation of Ca\(^{2+}\) transient amplitude by β2-AR agonists is attributable to increased ICa,L.** Zinterol increased the Ca\(^{2+}\) transient amplitude in newborn ventricular myocytes. This increase was not secondary to β2-AR-mediated changes in action potential duration (APD), since experiments were performed under voltage-clamp conditions. We have previously shown that newborn cardiac myocytes are particularly sensitive to APD (13, 16). In the newborn, Ca\(^{2+}\) transients had a larger amplitude at +50 versus −10 mV, suggesting a significant role for reverse-mode Na\(^+-Ca\(^{2+}\) exchange (NCX)-dependent Ca\(^{2+}\) influx (18). To eliminate the possibility of β2-AR regulation of NCX in the newborn, we used nifedipine and/or voltage clamp to +50 mV to block L-type Ca\(^{2+}\) channels. In both cases, the response to zinterol was inhibited, indicating that the effect on Ca\(^{2+}\) transient was attributable solely to the increase in ICa,L and not from Ca\(^{2+}\) influx via NCX. Therefore, at a constant duration of depolarization, β2-AR agonists enhance Ca\(^{2+}\) transient amplitude primarily through increased ICa,L.

We have previously shown that under basal conditions, the Ca\(^{2+}\) transient in the newborn rabbit is relatively less reliant on Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel as either a direct source of "activator" Ca\(^{2+}\) or as the "trigger" for Ca\(^{2+}\) release from intracellular stores (13, 17). In addition, there is a significantly diminished fractional release from the sarcoplasmic reticulum in the newborn on a beat-to-beat basis (3). In fact, reverse-mode NCX current can provide sufficient Ca\(^{2+}\) influx to support contraction in newborn myocytes, and there is evidence that contraction and relaxation at birth are predominately controlled by NCX (16, 17). However, β2-AR-mediated regulation of NCX remains controversial, and nonselective β2-AR stimulation in the rabbit heart does not regulate NCX activity (12). Thus, if the newborn heart is more dependent on NCX and NCX is not regulated by adrenergic stimulation, how does the newborn increase cardiac contractility during stress?

**The effects of β2-AR stimulation on the kinetics of ICa,L.** Contrary to previous reports (29, 44, 48), β2-AR stimulation decreased both τ\(_{\text{fast}}\) (Ca\(^{2+}\) dependent) and τ\(_{\text{slow}}\) (voltage dependent) of ICa,L. The former could have resulted from greater influx of Ca\(^{2+}\) ions, as a consequence of an increase in ICa,L amplitude with zinterol. This correlates with the shift in steady-state inactivation toward hyperpolarized levels as a result of the negative shift in steady-state activation. This leftward shift in activation curves has been attributed to the phosphorylation of
residues within the voltage sensor of the Ca\textsuperscript{2+} channel (22), the result of which would facilitate the opening of Ca\textsuperscript{2+} channels earlier in depolarization. These data, together with the lack of effect of zinterol on the reversal potential and threshold of activation, indicate that zinterol augments $I_{Ca,L}$ by increasing the single-channel open probability, similar to the effect seen with phosphorylation of Ca\textsuperscript{2+} channels by PKA (23, 48).

In addition, the rate of inactivation for peak $I_{Ba}$ was also increased by zinterol for both time constants. The fast component here was not due to Ca\textsuperscript{2+}-dependent inactivation since Ba\textsuperscript{2+} was the charge carrier. The implies that $\beta_{2}$-AR stimulation can accelerate the voltage-dependent inactivation of $I_{Ca,L}$, which differs from the effect seen during PKA phosphorylation of $I_{Ca,L}$ (48). The basis of this effect remains unclear, although a decrease in $\tau_{slow}$ was reported in newborn rabbit myocytes during nonselective $\beta$-AR stimulation (24). Interestingly, $\tau_{slow}$ was increased markedly by Ba\textsuperscript{2+} in the adult but only mildly augmented in the newborn. Differences in the inactivation of Ba\textsuperscript{2+} currents have been attributed to the alternative splicing of exons in the $\alpha_{1C}$-subunit of the Ca\textsuperscript{2+} channel (37). A developmental shift (postnatal to adult) from the IVS3A to the IVS3B mRNA isoforms of the $\alpha_{1C}$-subunit has recently been identified in the rabbit (18). Although this switch in isoforms has been attributed to a downregulation of $I_{Ca,L}$-dependent EC coupling (5), as seen in the newborn, it could also confer properties such as localization and interaction with down-stream regulators of adrenergic signaling.

Although zinterol accelerated the inactivation rate of $I_{Ca,L}$, more Ca\textsuperscript{2+} entered the newborn myocytes in the presence of zinterol. In fact, the estimated Ca\textsuperscript{2+} flux value in the presence of zinterol was similar to that for adult rabbit myocytes under basal conditions using the same voltage step and extracellular Ca\textsuperscript{2+} concentration (49). This helps to explain the increase in Ca\textsuperscript{2+} transient amplitude observed.

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**Fig. 4.** $\beta_{2}$-AR-mediated stimulation of $I_{Ca,L}$ does not depend on activation of PKA in Nb myocytes. A: representative traces of peak $I_{Ca,L}$ (left) and $I-V$ curves (right) in Nb myocytes pretreated with ICI and Iso to stimulate $\beta_{2}$-ARs exclusively. B: as above, but myocytes were dialyzed with 100 $\mu$M Rp-cAMP to inhibit PKA. C: representative traces of peak $I_{Ca,L}$ (left) and $I-V$ curves (right) showing effect of Zint after dialysis with Rp-cAMP. Error bars are means $\pm$ SE. *$P < 0.05$; $n=4$ experiments for all plots. D: summary data showing the effect of the PKA blockers, H89 (10 $\mu$M; bath applied), PKI (10 $\mu$M; dialyzed), and Rp-cAMP (100 $\mu$M; dialyzed) on $\beta_{2}$-AR stimulation of peak $I_{Ca,L}$ in Nb myocytes ($n$, number of experiments). *$P < 0.05$ (PKA blocker only vs. PKA blocker + Zint). Errors bars are means $\pm$ SE. **$P < 0.05$ (basal $I_{Ca,L}$ vs. PKA blocker only).**
explains the observed differences in prevented cyclases (35). Interestingly, intracellular dialysis of BAPTA (20, 41). However, we found that peak been previously reported in newborn rabbit cardiac myocytes exemplified by the Ca\(^{2+}\) is calcium-dependent. This is further illustrated by the Ca\(^{2+}\)-transient experiments, where Ba\(^{2+}\) as the initial charge carrier and then substituted with 2 mM Ba\(^{2+}\). Inset: comparison of peak \(I_{\text{Ca,L}}\) and Ba\(^{2+}\) current (\(I_{\text{Ba}}\)) in the Ad cardiac myocyte. B: representative recording of peak \(I_{\text{Ba}}\) (2 mM Ba\(^{2+}\)) in a Nb myocyte before and after treatment with Zint. C: representative peak \(I_{\text{Ca,L}}\) with and without Zint after pretreatment with shown interventions. Incubation with pertussis toxin (PTX: 200 ng/ml; 3 h) did not alter control \(I_{\text{Ca,L}}\) but enhanced the response to Zint. A representative trace of Zint stimulation in a cell without PTX is shown for reference. In addition, cells were either incubated for IBMX (100 \(\mu\)M) or dialyzed with 100 \(\mu\)M 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) before treatment with Zint. D: summary of the influence of various pharmacological interventions on the response of peak \(I_{\text{Ca,L}}\) to Zint in Nb myocytes. Error bars are means ± SE. *P < 0.05, control vs. Zint. **P < 0.05, basal \(I_{\text{Ca,L}}\) for treatment vs. basal \(I_{\text{Ca,L}}\) for control. ‡ P < 0.05, unpaired t-test; Zint (n = 12 experiments) vs. treatment + Zint (see text for sample sizes).

with zinterol since the zinterol-mediated increase in [Ca\(^{2+}\)], is \(I_{\text{Ca,L}}\) dependent.

Modulation of \(\beta_{2}\)-AR responses by intracellular Ca\(^{2+}\). An increase in peak \(I_{\text{Ca}}\) density during postnatal maturation has been previously reported in newborn rabbit cardiac myocytes (20, 41). However, we found that peak \(I_{\text{Ca,L}}\) in newborn cells was twofold greater than that reported in previous studies, which also used freshly isolated myocytes (20, 41). BAPTA enhances peak \(I_{\text{Ca,L}}\) and reduces \(I_{\text{Ca,L}}\) inactivation compared with EGTA as an intracellular Ca\(^{2+}\) buffer (35), which likely explains the observed differences in \(I_{\text{Ca,L}}\) amplitude between our data and those of previous investigators. This is further exemplified by the Ca\(^{2+}\)-transient experiments, where nominal Ca\(^{2+}\) buffering significantly reduced basal peak \(I_{\text{Ca,L}}\). The degree of Ca\(^{2+}\) buffering and therefore the level of [Ca\(^{2+}\)] can regulate cell signaling by modulating Ca\(^{2+}\)-sensitive adenylyl cyclases (35). Interestingly, intracellular dialysis of BAPTA prevented \(\beta_{2}\)-AR-stimulated phosphorylation of the S1928 residue of the \(\alpha_{1c}\)-subunit of adult cardiac Ca\(^{2+}\) channels (19). However, this phosphorylation event was not coupled to functional changes in \(I_{\text{Ca,L}}\) magnitude. We show that in both high and nominal Ca\(^{2+}\) buffering, zinterol augmented \(I_{\text{Ca,L}}\) by a similar magnitude. This suggests that modifications in free [Ca\(^{2+}\)], near Ca\(^{2+}\) channels influence the kinetics of the Ca\(^{2+}\) current but does not affect signaling by \(\beta_{2}\)-ARs. Indeed, Ba\(^{2+}\) currents were augmented by a similar magnitude by zinterol, suggesting that Ca\(^{2+}\) entering through Ca\(^{2+}\) channels does not regulate \(\beta_{2}\)-AR-mediated events. This contrasts with studies where Ba\(^{2+}\) enhances PKA-mediated increases in \(I_{\text{Ca,L}}\), presumably through relieving inhibition on Ca\(^{2+}\)-sensitive adenylyl cyclases (35, 47) and/or inhibition of calmodulin (39). In regard to \(\beta_{2}\)-AR regulation, newborn myocytes appear to lack this Ca\(^{2+}\) feedback mechanism, particularly since PKA is not involved.

Downstream mediators of \(\beta_{2}\)-AR signaling. Few prior studies have attempted to identify the underlying mechanisms for the enhanced response to \(\beta_{2}\)-AR agonists in the newborn heart, and the results to date are inconclusive. In adult myocytes, \(\beta_{2}\)-AR stimulation of \(I_{\text{Ca,L}}\) is unmasked by PTX treatment, suggesting that under control conditions, \(G_{i}\) plays a role in restricting \(\beta_{2}\)-AR downstream signaling (42). Differences in \(\beta_{2}\)-AR responsiveness among species and developmental stages have been attributed to the degree of \(\beta_{2}\)-AR-\(G_{i}\) coupling (43). Here we have shown that PTX exposure augments \(\beta_{2}\)-AR...

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stimulation of $I_{\text{Ca,L}}$ in newborn myocytes. Therefore, $G_{\text{i}}$ is still an active component of $\beta_2$-AR signaling in the newborn and does not account for the enhanced responsiveness to $\beta_2$-AR agonists under basal conditions. Indeed, $G_{\text{i}}$ expression has been shown to be greater in the newborn heart relative to the adult heart (34).

We have also demonstrated that in the newborn, $I_{\text{Ca,L}}$ is maximally activated by inhibition of PDEs and with the dialysis of a cAMP analog. A saturating dose (1) of the nonselective PDE inhibitor, IBMX, increased $I_{\text{Ca,L}}$ by threefold in the newborn and blocked further stimulation by zinterol. We obtained a similar result by dialyzing myocytes with 8-CPT-cAMP, a cAMP analog. These results suggest that either cAMP upregulation interferes with $\beta_2$-AR signaling in cardiac myocytes or that $\beta_2$-AR agonists are ineffective after maximal cAMP stimulation. Indeed, $\beta_2$-AR stimulation has been shown to robustly increase intracellular cAMP in newborn cardiac myocytes (8, 26, 34). Although the possibility of $\beta_2$-AR-cAMP coupling remains disputed (26, 27, 34), pharmacological data presented here suggest that $\beta_2$-ARs stimulate $I_{\text{Ca,L}}$ in a cAMP-dependent, but PKA-independent, manner.

In adult rabbit ventricular myocytes, the CaMKII complex has been implicated as a mediator of L-type Ca$^{2+}$ channel function (2). The effects of chronic stimulation of $\beta_2$-ARs on EC coupling have also been shown to be mediated by CaMKII, wherein the cAMP/ PKA pathway is desensitized and the CaMKII pathway becomes sensitized (40). However, blockade of CaMKII did not modulate the effect of $\beta_2$-AR activation on $I_{\text{Ca,L}}$ in newborn myocytes. The effects of chronic $\beta_2$-AR stimulation in newborn myocytes remain to be investigated.

$\beta_2$-ARs but not $\beta_1$-ARs stimulate $I_{\text{Ca,L}}$ independently of PKA in newborn cardiac myocytes. $\beta_1$-AR-mediated stimulation of $I_{\text{Ca,L}}$ depends on activation of PKA (23). This is consistent with the previous observation that PKA blockade inhibited $\beta_1$-AR-mediated chronotropy in newborn cardiac myocytes (8). Our present results indicate a PKA-independent component to the coupling between $\beta_2$-ARs and L-type Ca$^{2+}$ channels in newborns. In particular, Rp-cAMP, although able to prevent $\beta_1$-AR stimulation, failed to suppress the $\beta_2$-AR-mediated increase in $I_{\text{Ca,L}}$, even after 10 min of intracellular dialysis. The PKA blockers H89 and PKI significantly reduced basal $I_{\text{Ca,L}}$, suggesting that either PKA is constitutively active in the newborn or that they had a nonspecific action on $I_{\text{Ca,L}}$, as described previously for H89 (9, 33). Constitutively active PKA may be the result of the higher [Ca$^{2+}$]i buffering used in this study (47). However, Rp-cAMP did not reduce basal $I_{\text{Ca,L}}$, and the discrepancy remains. It is possible that PKI and H89 were more effective at blocking PKA when constitutively active. For example, PKI is a competitive peptide blocker of the catalytic domain of PKA, whereas Rp-cAMP is a competitive antagonist of cAMP for PKA. Constitutively active PKA may be a result of the catalytic domain outnumbering the regulatory domain or becoming activated independently of cAMP (21). In this case, Rp-cAMP would be less effective.

With the use of a nonselective $\beta$-AR agonists, a PKA-independent component to $\beta$-AR signaling has been previously identified in newborns but was absent in adult rabbit ventricular myocytes (24). Our data suggest that this component is coupled to the $\beta_2$-AR pathway, whereas $\beta_1$-AR-mediated stimulation of $I_{\text{Ca,L}}$ requires activation of PKA. This is further verified by the fact that stimulation of newborn cardiac $\beta_1$-ARs, but not $\beta_2$-ARs, is able to phosphorylate PKA-dependent substrates (10). It remains to be determined whether the PTX-augmented component to newborn $\beta_2$-AR signaling is dependent on PKA. PKA-independent pathways in $\beta_2$-AR signaling have been previously demonstrated in cardiac myocytes (8) and in other cell types (4, 7). CaMKII (40) has been implicated in this PKA-independent signaling. However, we have shown that blockade of this pathway does not suppress the effect of $\beta_2$-AR-mediated stimulation of $I_{\text{Ca,L}}$ in newborn rabbit myocytes. Stimulation of $I_{\text{Ca,L}}$ may involve a direct interaction between $G_{\text{i}}$, the $\beta_2$-AR and the L-type Ca$^{2+}$ channel, although this proposal is controversial (7, 46). More recently, the cAMP-dependent Rap-GTP exchange factor (Epac) has been implicated in $\beta_2$-AR signaling in mouse myocytes (31), which might serve as a possible pathway that is PKA independent. Other possible molecular mechanisms have been proposed for the robust stimulation of $I_{\text{Ca,L}}$ via $\beta_2$-ARs, although they remain inconclusive. For instance, the main consensus site for PKA-dependent phosphorylation, S1928, is not required for $\beta_2$-AR (11) or $\beta_2$-AR-mediated increases in Ca$^{2+}$ currents (19). One novel alternative is via ahnak, a $I_{\text{Ca,L}}$ effector protein that binds to the $\beta$-subunit of the Ca$^{2+}$ channel. PKA phosphorylates and relieves the inhibition of ahnak, similar to the mechanism of phospholamban (15). If PKA is not involved in $\beta_2$-AR signaling during sympathetic stimulation in the newborn, the question remains as to what are the sources and sites of phosphorylation, if any, for downstream targets of $\beta_2$-ARs.

In summary, our results demonstrate that the developmental difference in the inotropic response to $\beta_2$-AR receptor agonists is attributable to an increase in $I_{\text{Ca,L}}$ that occurs in newborns but not in adults. Although the precise cellular mechanisms remain to be determined, it appears that this response in newborns does not require activation of PKA. This pathway may play an important role in providing additional inotropic support to the newborn heart under conditions of increased sympathetic stimulation.

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REFERENCES


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