Developmental aspects of cardiac Ca\textsuperscript{2+} signaling: interplay between RyR- and IP\textsubscript{3}R-gated Ca\textsuperscript{2+} stores

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The dominant mode of intracellular Ca\textsuperscript{2+} release in adult mammalian cardiomyocytes involves Ca\textsuperscript{2+} release via ryanodine receptors (RyRs), but it is less clear whether inositol 1,4,5-trisphosphate (IP\textsubscript{3})-gated Ca\textsuperscript{2+} release channels (IP\textsubscript{3}Rs) are also important during embryogenesis, play a significant role during early postnatal development. To address this question, we measured confocal two-dimensional Ca\textsuperscript{2+} dependent fluorescent intensities in acutely isolated neonatal and juvenile rat cardiomyocytes, either voltage-clamped or permeabilized, where rapid exchange of solution could be used to selectively activate the two types of Ca\textsuperscript{2+} release channel. Targeting RyRs with caffeine produced large and rapid Ca\textsuperscript{2+} signals throughout the cells. Application of ATP and endothelin-1 to voltage-clamped, or permeabilized, cells produced smaller and slower Ca\textsuperscript{2+} signals that were most prominent in subsarcolemmal regions and were suppressed by either the IP\textsubscript{3}-R-blocker 2-aminoethoxydiphenylborate or replacement of the biologically active form of IP\textsubscript{3} with its L-stereoisomer. Such IP\textsubscript{3}-R-gated Ca\textsuperscript{2+} release events were amplified by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) via RyRs since they were also reduced by compounds that block the RyRs (tetracaine) or deplete the Ca\textsuperscript{2+} pools they gate (caffeine, ryanodine). Spatial analysis revealed both subsarcolemmal and perinuclear origins for the IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release events RyR- and IP\textsubscript{3}R-gated Ca\textsuperscript{2+} signals had larger magnitudes in juvenile than in neonatal cardiomyocytes. Ca\textsuperscript{2+} signaling was generally quite similar in atrial and ventricular cardiomyocytes, although RyR- and IP\textsubscript{3}R-gated Ca\textsuperscript{2+} release mechanisms were found to be differentially expressed. The results suggest that an intermediate stage of Ca\textsuperscript{2+} signaling exists in developing cardiomyocytes, and that RyR open probability changes during postnatal development may play a role in regulating Ca\textsuperscript{2+} signaling.

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ing Ca\(^{2+}\) transients, in part, by local CICR. We found that developing cardiomyocytes have functional RyR- and IP\(_3\)-gated Ca\(^{2+}\) releases with unique pharmacology and spatial profiles. Maturation from neonatal to juvenile cardiac phenotype involved an upregulation of both types of Ca\(^{2+}\) release channel. The IP\(_3\)-mediated Ca\(^{2+}\) signaling was capable of generating both subsarcolemmal and perinuclear Ca\(^{2+}\) releases in developing cardiomyocytes, the former of which can directly contribute to CICR transients in the subsarcolemmal regions.

**MATERIALS AND METHODS**

Experiments were carried out in accordance with institutional and federal guidelines. Protocols were approved and supervised by Georgetown University Animal Care and Use Committee.

**Cell isolation.** For preparation of cardiomyocytes from neonatal and juvenile rats, with the juveniles first being deeply anesthetized, the animals were decapitated, the chest cavities were opened, and the hearts were removed. The excised hearts were retrogradely perfused at 37°C through the aorta, first for 2 min with Ca\(^{2+}\)-free Tyrode’s solution composed of (in mM) 137 NaCl, 5.4 K-glutamate, 1 MgCl\(_2\), 10 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH) and then with Ca\(^{2+}\)-free Tyrode’s solution supplemented with collagenase (0.8 mg/ml) and protease (0.1 mg/ml) for 4 min. The atrial and ventricular tissues were dissected and gently agitated to dissociate single cells. The freshly dissociated cells were plated onto glass coverslips, stored at room temperature in Tyrode’s solution containing 0.2 mM CaCl\(_2\), and used within 8 h in Ca\(^{2+}\) imaging experiments where the membrane potential was either voltage-clamped or abolished by membrane permeabilization. Cells that maintained their native elongated shape following the isolation procedure were selected for detailed examination over those that assumed a more globular appearance.

Drugs were dissolved in the external experimental solutions and applied rapidly using a concentration-clamp device (6). Rapid application of 100 \(\mu\)M ATP (Sigma, St. Louis, MO), 10 mM caffeine (Sigma), 100 nM endothelin-1 (Sigma), and 1 or 20 \(\mu\)M IP\(_{3}\) (D-myo-IP\(_{3}\); Calbiochem, Gibbstown, NJ) was used to probe Ca\(^{2+}\) stores. All experiments were carried out at room temperature (22–24°C).

**Whole-cell voltage-clamp procedures.** Some cells were voltage-clamped in the whole cell configuration (16) to hold the membrane potential steady at \(-70\) mV during recording of drug-induced Ca\(^{2+}\) transients, 2) measure membrane currents [(e.g., Na\(^{+}\)-Ca\(^{2+}\) exchanger current (Isaca) and Cl\(-\) current] that were activated during these interventions, and 3) subject the cells to repeated depolarizing pulses from \(-90\) to \(0\) mV to maintain and equilibrate intracellular Ca\(^{2+}\) stores. The voltage-clamped cells were dialyzed with a Ca\(^{2+}\)-buffered pipette solution containing (in mM) 110 CsOH, 110 aspartic acid, 5 NaCl, 20 Tris-Cl, 5 Mg-ATP, 0.2 Ca-AMP, 2 EGTA, 1 K-Fluo-4, 1.2 CaCl\(_2\), and 20 HEPES (titrated to pH 7.2 with CsOH) (56). With the consideration of the binding constants of Fluo-4 (\(K_b = 400\) nM) and EGTA (\(K_b = 100\) nM), the addition of 1.2 mM CaCl\(_2\) was calculated to buffer [Ca\(^{2+}\)]\(_i\), of the dialyzing solution at \(-100\) nM. Membrane currents were measured using a Dagan voltage-clamp amplifier using pClamp software. The conditioning pulses were initiated 3 min after rupture of the membrane, and fluorescence measurements were started 3 to 4 min later. The extracellular solution used during experiments contained (in mM) 137 NaCl, 5.4 K-glutamate, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 10 HEPES (titrated to pH 7.4 with NaOH).

**Permeabilization.** After being prestained with 20 \(\mu\)M Fluo-4 ace-toxymethyl ester (Fluo-4 AM), the cells were permeabilized with saponin (9, 63). First, the cells were suspended in a solution containing (in mM) 100 K-aspartate, 20 KCl, 0.5 EGTA, 0.75 MgCl\(_2\), and 10 HEPES (titrated to pH 7.2 with KOH). The cell surface membrane was permeabilized by adding 0.005% (wt/vol) saponin for 30 s. After 30 s, the bath solution was exchanged for a saponin-free simulated internal solution composed of 100 mM K-aspartate, 15 mM KCl, 5 mM KH\(_2\)PO\(_4\), 5 mM MgATP, 0.4 mM EGTA, 0.04 mM K\(_2\)Fluo-4, 0.2 mM CaCl\(_2\), ([Ca\(^{2+}\)]\(_i\) = 100 nM), 0.75 mM MgCl\(_2\), 10 mM phosphocreatine, 5 U/ml creatine phosphokinase, 8% dextran, and 10 mM HEPES (titrated to pH 7.2 with KOH).

**Two-dimensional confocal imaging.** Intracellular Ca\(^{2+}\) signals were measured with the fluorescent Ca\(^{2+}\)-indicator dye Fluo-4, which was used in conjunction with the nonfluorescent Ca\(^{2+}\)-chelator EGTA, as mentioned above. The cells were imaged using a Noran Odyssey XL rapid two-dimensional laser scanning confocal microscopy system (Noran Instruments, Madison, WI) attached to a Zeiss Axiosvert TV135 inverted microscope fitted with an \(\times\) 63 water-immersion objective lens. The excitation wavelength of the argon ion laser was set to 488 nm, and fluorescence emission was measured at wavelengths >515 nm. Cells were imaged confocally at 4–240 frames/s depending on the experiment.

The average resting fluorescence intensity (F\(_0\)) was calculated from several frames measured immediately before drug application. Images were filtered by 3 pixel \(\times\) 3 pixel averaging. The amplitudes of the Ca\(^{2+}\)-dependent cellular fluorescence signals were quantified as \(\Delta F/F_0 = F/F_0 - 1\), where F is the peak value and, like F\(_0\), was measured by integration over the entire area of each cell. Time quantifies the rate of rise of the Ca\(^{2+}\) signals and was calculated as 1.25 \times the time required for the transient to develop from 10% to 50% at the peak value (Fig. 1). Generally, the fluorescence images used in illustrations were normalized by dividing the images during activation [F(x,y)] by the average image before activation [F\(_d\)(x,y)]. (Figs. 1–5, 7, 8). The division was performed only provided F\(_d\)(x,y) exceeded a threshold that was chosen to generate a sharp transition from the color distributions of the cells to their surroundings, which are shown in black. The green-blue speckle seen in single frames before activation indicates the level of noise in the recordings, and thereby the type of low level signals that should be discarded as random fluctuations. In contrast, the signals of physiological interest were characterized by their larger amplitudes (yellow, orange, and red distributions) and systematic progression from frame to frame.

To reduce photobleaching, the laser beam that was used for excitation of Fluo-4 was electronically shuttered and triggered to open by the command of the pClamp program only during acquisition of data.

**Statistical analyses.** All data are presented as means \(\pm\) SE, n, where n is the number of cells examined. The unpaired Student’s t-test was used to evaluate differences between measurements from different populations of cells (e.g., neonatal vs. juvenile cardiomyocytes), whereas the paired t-test was used to compare different pharmacological interventions in the same population of cells (Figs. 5, B and D, and 6, C and D). Differences were considered statistically significant when \(P < 0.05\). Higher degrees of significance are indicated with two \((P < 0.01)\) or three \((P < 0.001)\) asterisks.

**RESULTS**

**RyR Ca\(^{2+}\) signals in isolated cardiomyocytes.** Excitation-contraction coupling in neonatal cardiomyocytes appears to be regulated by Ca\(^{2+}\) influx through the sarcolemma, with the L-type Ca\(^{2+}\) channel being the main source for trans-sarclemmal Ca\(^{2+}\) flux (2, 7, 14, 34, 55). However, although RyRs are functional in neonatal cardiomyocytes, Ry only reduces Ca\(^{2+}\) transients by \(-15\%\) in the day 1 neonatal rat heart versus 88% in juvenile cardiomyocytes, suggesting an increased reliance on RyR as a function of age (10, 11). Thus, to test the hypothesis that RyRs are upregulated as a function of maturation, we compared RyR-gated Ca\(^{2+}\) release in acutely isolated neonatal (days 1 to 2) and juvenile (days 8 to 10) cardiomyocytes, using the results from the widely studied adult cardio-
myocytes as a benchmark for full maturation of RyR-mediated Ca\(^{2+}\) signaling.

Freshly isolated cardiomyocytes were voltage-clamped and dialyzed with a Ca\(^{2+}\)-buffered internal solution, and conditioning pulses from -90 to -10 mV were applied at 5-s intervals to maintain the Ca\(^{2+}\) load of the SR throughout the experiments. In between conditioning pulses and with the cardiomyocytes being voltage-clamped at -70 mV to prevent activation of voltage-gated ion channels, RyR-mediated Ca\(^{2+}\) release was activated using rapid application of 10 mM caffeine, a RyR agonist, and changes in intracellular Ca\(^{2+}\) were detected using confocal Ca\(^{2+}\) imaging.

Figure 1A shows a comparison of the caffeine-activated Ca\(^{2+}\) transients in neonatal (b), juvenile (d), and adult (f) cardiomyocytes, with the normalized images recorded before activation being shown as well (a, c, and e, respectively), to give an indication of the spatial distribution of the caffeine-induced Ca\(^{2+}\) signal. The fluorescent signal Ca\(^{2+}\) transients of the neonatal (Neo), juvenile (Juv), and adult cardiomyocytes imaged at 120 Hz are shown in Fig. 1B. Comparisons of both the images (Fig. 1A) and the Ca\(^{2+}\) transients (Fig. 1B) indicate that maturation from neonate to adult is accompanied by larger and more uniformly distributed caffeine-activated Ca\(^{2+}\) transients. Indeed, analysis of the amplitude of the caffeine-induced Ca\(^{2+}\) transients (Fig. 1C) reveals that the magnitude of the caffeine Ca\(^{2+}\) transient increased with the age of the cardiomyocytes, with the neonates having the smallest amplitude (ΔF/F\(_{0}\) = 0.160 ± 0.022; n = 7) and significantly larger amplitudes being observed in both juveniles (Juv: ΔF/F\(_{0}\) = 0.483 ± 0.065; n = 3, P = 0.0003) and adults (ΔF/F\(_{0}\) = 0.705 ± 0.18; n = 2, P = 0.0005). Figure 1D shows that the time required for development of 50% of the full amplitude of the caffeine-induced Ca\(^{2+}\) transient was generally shorter in the juvenile (T\(_{\text{au50}}\) = 52 ± 14 ms; n = 3) and adult (T\(_{\text{au50}}\) = 61 ± 1 ms; n = 2) compared with the neonatal (T\(_{\text{au50}}\) = 220 ± 50 ms; n = 7) cardiomyocytes. The data demonstrate an increase in the velocity and magnitude of RyR-gated Ca\(^{2+}\) release as a function of development and support our hypothesis that RyRs are upregulated during postnatal development, with neonatal and juveniles representing two stages of progressive maturation.

**ATP-activated IP\(_{3}\)-Ca\(^{2+}\) signals in dialyzed developing cardiomyocytes.** Although IP\(_{3}\)-mediated Ca\(^{2+}\) signaling plays a large role in excitation-contraction coupling (ECC) of embryonic cardiomyocytes (43, 53), the role of IP\(_{3}\)-activated Ca\(^{2+}\) signaling in acutely isolated developing cardiomyocytes has never been studied before. The documented differences in the maturity of RyR-gated Ca\(^{2+}\) stores in neonatal and juvenile cardiomyocytes make these stages of development ideal to test whether parallel changes take place in IP\(_{3}\)-activated Ca\(^{2+}\) signaling, with IP\(_{3}\)-activated Ca\(^{2+}\) signaling perhaps having a reduced role as the role of RyR-gated Ca\(^{2+}\) signaling increases. To test this hypothesis, we compared the Ca\(^{2+}\) signals of acutely isolated neonatal and juvenile cardiomyocytes in response to activation of IP\(_{3}\) by rapid application of 100 μM ATP, which binds to extracellular purinergic receptors to activate the breakdown of PIP\(_{2}\) into IP\(_{3}\) (52). Similarly, 100 nM endothelin-1 (ET-1), which binds to specific cell surface receptors to generate IP\(_{3}\) (54) was tested as an alternative means to activate the IP\(_{3}\)-signaling pathway.

Ca\(^{2+}\)-buffered, voltage-clamped rat cardiomyocytes produced variable responses to ATP and ET-1, ranging from rapid whole cell Ca\(^{2+}\) transients to an increase in the diastolic [Ca\(^{2+}\)]c. Figure 2A shows representative normalized images of a voltage-clamped neonatal cardiomyocyte before (control) and after exposure to ATP, revealing that ATP activated an increase in Ca\(^{2+}\) fluorescence mostly confined to the subsarcolemmal area of the cell. The spatial profiles for ET-1 Ca\(^{2+}\) responses were virtually identical (not shown). As summarized in Fig. 2B, application of ATP generated an increase in Ca\(^{2+}\)-dependent fluorescence in both age groups with the neonatal cardiomyocytes producing a ΔF/F\(_{0}\) = 0.040 ± 0.004 (n = 9) and the juveniles a ΔF/F\(_{0}\) = 0.10 ± 0.04 (n = 6). Exposure of the same cells to ET-1 produced similar results, revealing an increase in the basal Ca\(^{2+}\) level of ΔF/F\(_{0}\) = 0.030 ± 0.004 (n = 6) in the neonates and ΔF/F\(_{0}\) = 0.040 ± 0.009 (n = 6) in the juveniles. Interestingly, maturation from the neonatal to the juvenile state appeared to be accompanied not only by an
increase in RyR-gated Ca\(^{2+}\) stores but also by maintenance, or slight upregulation, of the IP\(_3\)-gated Ca\(^{2+}\) stores. Although the differences in IP\(_3\)-mediated Ca\(^{2+}\) signaling only represent a trend, it was a surprising observation that we subjected to more extensive testing in permeabilized cells.

Figure 3 shows a representative example selected from a number of voltage-clamped Ca\(^{2+}\)-buffered juvenile cardiomyocytes where we compared the magnitude and time course of the ATP- and caffeine-activated Ca\(^{2+}\) transients. As shown in Fig. 3A, superimposing the whole cell Ca\(^{2+}\) fluorescence traces for the two interventions on the same graph reveals different kinetics and magnitudes to the caffeine and ATP responses. Tau\(_{0.5}\) of the caffeine-induced Ca\(^{2+}\) transient was shorter than that of ATP (26 ms vs. 240 ms). In addition, the amplitude of the caffeine-induced Ca\(^{2+}\) transient was larger than that of ATP (\(\Delta F/\Delta t = 0.70\) vs. 0.25). The cellular images shown in Fig. 3D, taken at the time points indicated in the corresponding traces in Fig. 3A, show that caffeine-induced rise in Ca\(^{2+}\) occurred simultaneously in both the peripheral and central regions of the cell (Fig. 3D, b-d). The appearance of the caffeine-activated Ca\(^{2+}\) signal at the top of the cell (Fig. 3D, b and c) corresponds to the area of the cell exposed to caffeine first. These data indicate that RyRs are distributed throughout the developing cardiomyocytes and agree with the findings of other researchers (15).

The ATP Ca\(^{2+}\) signal appears to develop first in the membrane-associated peripheral region (Fig. 3D, f and g), with delayed and incomplete invasion of the center of the cell (Fig. 3D, h). The subsarcolemmal origin of the response correlates with the data from neonatal cardiomyocyte shown in Fig. 2. The following, much larger magnitude of the ATP-activated Ca\(^{2+}\) transient, as well as the spread of the signal to some parts of the cell, suggests activation of the RyRs secondary to the primary IP\(_3\)-activated Ca\(^{2+}\)-release.

In cells where the caffeine- and ATP-induced Ca\(^{2+}\) transients were relatively large (Fig. 3A), we found that they were accompanied by significant transient inward currents (Fig. 3B; \(I_{NaCa}\)) of the type that in previous studies has been associated with activation of the Na\(^+\)-Ca\(^{2+}\) exchanger by elevation of \([Ca^{2+}]_i\) (4). Note that the initial Ca\(^{2+}\) fluorescence time course (Fig. 3A) is similar to that of the transient inward current (Fig. 3B), with both sets of traces revealing a bigger delay for the ATP-induced response. Despite the great disparity in the amplitudes of the cellular Ca\(^{2+}\) transients produced by the two interventions, the amplitudes of the associated inward currents were comparable (n = 3), suggesting that the Na\(^+\)-Ca\(^{2+}\) exchanger is quite sensitive to the localized subsarcolemmal Ca\(^{2+}\) transients evoked by ATP. The ATP-activated inward current was often followed by an outward maintained current that reversed at E\(_{Ca}\) and could be blocked by DIDS (unpublished data, Tufan and Morad). As summarized for three cells in Fig. 3C, the integral \(I_{NaCa}\) flux was larger for the caffeine-than for the ATP-activated Ca\(^{2+}\) release (1.47 ± 0.04 pC/pF vs. 0.25 ± 0.03 pC/pF), mirroring the larger magnitude of caffeine-induced Ca\(^{2+}\) fluorescence shown in Fig. 3A (Figs. 1 and 2). These results indicate that IP\(_3\)-induced Ca\(^{2+}\) release even contributes to cellular Ca\(^{2+}\) transients in highly Ca\(^{2+}\)-buffered cells exclusive of Na\(^+\) or L-type Ca\(^{2+}\) channel activation.

IP\(_3\)-R-gated Ca\(^{2+}\) signals in permeabilized cardiomyocytes. Although the voltage-clamped, Ca\(^{2+}\)-buffered cardiomyocytes provided insight into the magnitude of RyR- and IP\(_3\)-R-gated Ca\(^{2+}\) signals, one of the limitations in the previous set of experiments is the possibility that variability in the expression of extracellular receptors may limit the size and frequency of...
ATP- and ET-1-activated Ca\(^{2+}\) signals. Therefore, direct application of IP\(_3\) in permeabilized cells, although not representing a physiological response, was used to evoke Ca\(^{2+}\) signals and to compare the size of IP\(_3\)-R-gated Ca\(^{2+}\) stores in neonatal and juvenile cardiomyocytes, as well as the ability of IP\(_3\)-activated Ca\(^{2+}\) release to directly activate CICR.

As shown in Fig. 4, application of 20 \(\mu\)M IP\(_3\) activated Ca\(^{2+}\) transients in both acutely isolated neonatal (Fig. 4A) and juvenile (Fig. 4B) cardiomyocytes. The insets show normalized fluorescence images before (Fig. 4B, a and c) and after (Fig. 4B, b and d) application of IP\(_3\). As summarized in Fig. 4C, IP\(_3\) evoked significantly larger Ca\(^{2+}\) transients in juvenile than in neonatal cardiomyocytes (\(\Delta F/F_0 = 0.65 \pm 0.10, n = 18\) vs. \(\Delta F/F_0 = 1.45 \pm 0.36, n = 25, P = 0.07\)). The use of 1 \(\mu\)M IP\(_3\) instead of 20 \(\mu\)M IP\(_3\) produced fluorescence signals with similar amplitudes, but with delayed kinetics (data not shown).

To ensure that the observed effects of IP\(_3\) were due to IP\(_3\)-dependent Ca\(^{2+}\) release, myocytes were exposed to IP\(_3\) before and after incubation in solution containing the IP\(_3\) antagonist 2-aminoethoxydiphenylborate (2-APB). The traces and the corresponding images in Fig. 5A show that IP\(_3\) given before (Fig. 5A, a and b) and after washout of 2 \(\mu\)M 2-APB (Fig. 5A, e) produced an increase in the cytosolic [Ca\(^{2+}\)] that was absent when the cells were treated with 2-APB alone (Fig. 5A, c) and with IP\(_3\) in the presence of 2-APB (Fig. 5A, d). Average values from six cells (Fig. 5B) demonstrate that the Ca\(^{2+}\) signal produced by IP\(_3\) alone (\(\Delta F/F_0 = 0.20 \pm 0.04\)) was much larger than the Ca\(^{2+}\) signals that were measured after incubation with 2-APB when the cells were either exposed to IP\(_3\) (\(\Delta F/F_0 = 0.07 \pm 0.01; P = 0.02\)) or a recording was performed without intervention to ascertain the decay of the preceding Ca\(^{2+}\) signal and evaluate noise, bleaching, and the stability of the cells (\(\Delta F/F_0 = 0.07 \pm 0.01; P = 0.01\)).

Similarly, Fig. 5C shows sample records from a series of experiments where individual cells were found to respond more strongly to the naturally occurring D-IP\(_3\) (Fig. 5C, f and g) than to its stereoisomer L-IP\(_3\) (Fig. 5C, h and i). As illustrated in the Fig. 5D, the average Ca\(^{2+}\) signals produced by

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**Fig. 4.** Inositol (1,4,5)-trisphosphate (IP\(_3\))-triggered Ca\(^{2+}\) release in permeabilized neonatal (A) and juvenile (B) cardiomyocytes. A and B: cellular Ca\(^{2+}\) transients (F/F\(_0\)) upon extracellular application of 20 \(\mu\)M IP\(_3\) and normalized fluorescence images before (a and c) and after (b and d) application of 20 \(\mu\)M IP\(_3\) at the times indicated next to the traces. The histogram in C compares the average responses in neonatal and juvenile cardiomyocytes (confocal imaging at 120 frames/s; the nonvoltage clamped cells were stained in Fluo-4AM and then permeabilized and finally maintained in simulated internal solution; nonpaired t-test; same color scale as Fig. 1). *\(P \leq 0.05\).

**Fig. 5.** IP\(_3\)-gated Ca\(^{2+}\) signals in juvenile, permeabilized cardiomyocytes are reversibly suppressed by the IP\(_3\)-gated Ca\(^{2+}\) release channels (IP\(_3\)-R) antagonist 2-aminoethoxydiphenylborate (2-APB). The traces and the corresponding images in Fig. 5A show that IP\(_3\) given before (Fig. 5A, a and b) and after washout of 2 \(\mu\)M 2-APB (Fig. 5A, e) produced an increase in the cytosolic [Ca\(^{2+}\)] that was absent when the cells were treated with 2-APB alone (Fig. 5A, c) and with IP\(_3\) in the presence of 2-APB (Fig. 5A, d). Average values from six cells (Fig. 5B) demonstrate that the Ca\(^{2+}\) signal produced by IP\(_3\) alone (\(\Delta F/F_0 = 0.20 \pm 0.04\)) was much larger than the Ca\(^{2+}\) signals that were measured after incubation with 2-APB when the cells were either exposed to IP\(_3\) (\(\Delta F/F_0 = 0.07 \pm 0.01; P = 0.02\)) or a recording was performed without intervention to ascertain the decay of the preceding Ca\(^{2+}\) signal and evaluate noise, bleaching, and the stability of the cells (\(\Delta F/F_0 = 0.07 \pm 0.01; P = 0.01\)).

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20 μM D-IP₃ were greatly reduced when the cells were exposed to the same concentration of the L-isomer (ΔF/F₀: 0.20 ± 0.03 vs. 0.06 ± 0.01; n = 7; P = 0.0011). These control experiments show that the observed IP₃-activated Ca²⁺ transients are specific for IP₃Rs and IP₃.

**Contribution of RyR-gated Ca²⁺ release to IP₃-mediated Ca²⁺ signal.** To evaluate the contribution of RyR-gated Ca²⁺ release to the IP₃-activated Ca²⁺ signals, we performed experiments where Ca²⁺ release via RyRs was blocked by different interventions. Figure 6A shows changes in the IP₃-activated Ca²⁺ signal in a representative juvenile cardiomyocyte before (Fig. 6A, a and b, left) and after (Fig. 6A, c, right) incubation of the cardiomyocyte with 10 mM caffeine to deplete RyR-gated Ca²⁺ secondary to the initial large Ca²⁺ release as seen in the inset. Fig. 6B compares the accumulated results from atrial (Fig. 6B, a) and ventricular (v) cardiomyocytes from neonates (Neo) and juveniles (Juv). The Ca²⁺ signals in response to caffeine (red) were almost fourfold (3.9 ± 0.9) larger in juvenile than in neonatal cells, and similar increases were observed in response to IP₃ in the absence (green) or presence (orange; Fig. 6B) of caffeine. These observations are based on pooled data from atrial and ventricular cardiomyocytes (Fig. 4C) since the permeabilization studies generally showed no significant differences between these cell types when examined at the same age. The only departure from this pattern was found in the response to IP₃ alone, where the upregulation in juvenile cardiomyocytes cells surprisingly appeared to be limited to the ventricular cells. When normalized relative to the caffeine-induced Ca²⁺ signals, those evoked by IP₃ amounted to about half (53 ± 9%; n = 43; dashed line) and, overall, showed no significant increase in juveniles although in this age group the difference between atrial and ventricular cardiomyocytes was noticeable (Fig. 6C). Pretreatment with caffeine reduced the IP₃-induced Ca²⁺ signals to 39 ± 5% (n = 27), and similar reductions were found when the RyR-gated Ca²⁺ stores were depleted with 40 μM ryanodine or 1 mM tetracaine was added to block the gating of the RyRs (Fig. 6D).

These data, which are consistent with the findings from Ca²⁺-buffered voltage-clamped cardiomyocytes (Fig. 2), suggest that, although the maturation of juvenile cardiomyocytes is accompanied by an increase in both caffeine-sensitive RyR-gated Ca²⁺ stores and IP₃R-gated Ca²⁺ stores, a change in the relative contribution of the two signaling pathways is seen only as differential development of atrial versus ventricular cells.

**Spatial analysis of IP₃-activated Ca²⁺ signaling events.** The above results suggest that the IP₃-activated Ca²⁺ release directly activates the RyRs by CICR in both neonatal and juvenile cardiomyocytes, but it remains to be shown where in the cell this communication may occur and how it may correlate with the locations of the IP₃- and RyRs. Figure 7 illustrates the spatial development of IP₃-induced Ca²⁺ transients in representative neonatal (Fig. 7A) and juvenile (Fig. 7B) cardiomyocytes. Regions of interest in the cells were identified with numbers on the sketched cells, and the Ca²⁺ signals in these numbered regions were followed in the corresponding numbered traces (1–8), thereby mapping the time course of the IP₃-generated local Ca²⁺ responses. Labeled time marks on the traces correspond to the normalized cellular images (Fig. 7B, a–h). In both age groups, the IP₃-activated responses were first observed at subsarcolemmal locations (Figs. 7A, c, and B, b) and traveled along the periphery of the cell before variably moving into the center. This wave-like spread of the IP₃-generated Ca²⁺ signals near the cell surface suggests subsarcolemmal colocalization of IP₃Rs and RyRs and was characteristic of permeabilized cells, where failure of whole cell signal integration may be directly linked to the loss of membrane potential. The minimal involvement of the central portion of the cell (e.g., Figs. 7A region/trace 3, and B, region/trace 4) may be indicative of a spatial disconnect between IP₃Rs and RyRs in the center.

**Spatial analysis of IP₃-activated Ca²⁺ release after depletion of RyR-gated Ca²⁺ stores.** Although the strong IP₃-induced Ca²⁺ signals, which spread near the surface of neo-

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Fig. 6. Contribution of ryanodine receptors (RyR)-gated Ca²⁺ stores to the IP₃-triggered Ca²⁺ releases. A: cellular Ca²⁺ transients (ΔF/F₀) in a permeabilized juvenile cardiomyocyte upon extracellular application of 20 μM IP₃ (left), 10 mM caffeine (inset), and IP₃ after 3 min incubation in caffeine (right). Selected frames (a–c) show normalized fluorescence images at the times indicated. B: comparison of the average Ca²⁺ transients (ΔF/F₀) in atrial (a) and ventricular (v) cardiomyocytes from neonatal (Neo; open bars) and juvenile (Juv; hatched bars) rats. The Ca²⁺ transients were evoked by 10 mM caffeine (red) or 20 μM IP₃, either by itself (green) or after 3 to 4 min incubation with 10 mM caffeine (orange), 40 μM ryanodine (blue), or 1 mM tetracaine (purple). C: IP₃-activated Ca²⁺ transients normalized relative to the caffeine-induced transients measured in the same cells. D: suppression of IP₃-activated Ca²⁺ signals by pretreatment with caffeine, ryanodine, and tetracaine. Permeabilized cells are same color scale as Fig. 1; n values are indicated at each bar. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
natal and juvenile cells, are likely to reflect subsarcolemmal colocalization of IP$_3$Rs and RyRs, as found in adult atrial cells (31), it is unclear whether these signals may swamp the less conspicuous Ca$^{2+}$/H$^{+}$ release activity of functional IP$_3$Rs that may be present at central locations but may operate without the support of CICR from nearby RyR-gated Ca$^{2+}$/H$^{+}$ stores. Therefore, we measured the subcellular distribution of IP$_3$-induced Ca$^{2+}$/H$^{+}$ signals under conditions where the RyR-mediated component of the Ca$^{2+}$/H$^{+}$ release was inhibited.

Figure 8 shows the spatial development of an IP$_3$-induced Ca$^{2+}$/H$^{+}$ transient in a representative juvenile cardiomyocyte before and after incubation in caffeine to deplete and prevent refilling of the RyR-gated Ca$^{2+}$/H$^{+}$ stores. As shown in Fig. 8A, the whole cell Ca$^{2+}$/H$^{+}$ fluorescence changes were followed before (green) and after (orange) depletion of the RyR-gated Ca$^{2+}$/H$^{+}$ pools via long-term incubation (3 min) in 10 mM caffeine. Application of IP$_3$ (arrow) under control conditions produced a strong response (green trace) that had a rapid upstroke (Tau$_{50}$ = 12 ms) indicative of CICR and was regenerative with a period of $\sim$800 ms. The cellular response to IP$_3$ was greatly suppressed in the presence of caffeine (orange trace). To focus on the genesis of these Ca$^{2+}$/H$^{+}$ signals, we expanded the time scale of the first 40 ms of the recordings (Fig. 8C) and inspected associated consecutive frames recorded at 240 Hz (Fig. 8, D and E). Before the caffeine treatment, the IP$_3$-activated Ca$^{2+}$/H$^{+}$ transient was generally seen to originate at a single location near the cell surface (Fig. 8D, image d) and to spread steadily, primarily in the longitudinal direction of the cell (starting at Fig. 8D, frame f), eventually developing large cellular Ca$^{2+}$/H$^{+}$ transients with distributions

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Fig. 7. Spatial analysis of IP$_3$-mediated Ca$^{2+}$/H$^{+}$ signaling events in permeabilized neonatal (A) and juvenile (B) cardiomyocytes. Each panel shows a map that identifies 8 regions of interest, 8 traces (1–8) showing the time course of the fluorescence changes in these regions, and 8 normalized fluorescence images (a–h) that were measured at the times indicated by letters and arrow heads next to the traces. The vertical scale bars indicate $\Delta F/F_0$ values of 1 (A) or 4 (B; 20 mM IP$_3$; permeabilized cells; same color scale as Fig. 1).
Fig. 8. Time course of local IP$_3$-activated Ca$^{2+}$ signal before and after depletion of the RyR-gated Ca$^{2+}$ stores. A: cellular Ca$^{2+}$ changes ($\Delta F/F_0$) upon extracellular application (arrow) of 20 $\mu$M IP$_3$ before (green) and after (orange) 3-min incubation with 10 mM caffeine. The boxed regions of the traces and of the sample frame (B) were expanded to show details of the onset of the IP$_3$-induced responses on an expanded time scale (C) and as rapidly changing normalized fluorescence images recorded at 240 frames/s (D and E) at the times indicated in C (permeabilized juvenile cardiomyocyte; expanded color scale).

(continued)
releases during development show that both release systems are upregulated from the neonatal (days 1 to 2) to the juvenile (days 8-10; Fig. 6B) stage but that the IP3-mediated response with age overall changes little when normalized relative to CICR (Fig. 7C). Spatial analysis revealed Ry-sensitive IP3-mediated Ca2+ signals that are present throughout the cells at central (Fig. 8) and perinuclear (Fig. 9) locations but are most prominent in subsarcolemmal regions (Figs. 1–5 and 7) where they evoke CICR (Figs. 3, 4, and 6) and significant inward \( I_{\text{Ca}} \) (Fig. 3). Overall, our data indicate that developing cardiomyocytes have an intermediate Ca2+ signaling phenotype between the early postnatal and adult cardiomyocytes showing vestigial IP3-R- gated Ca2+ signaling in both atrial and ventricular myocytes capable of directly triggering CICR.

**RyR-gated Ca2+ release in postnatal developing cardiomyocytes.** Analysis of the caffeine-activated Ca2+ release pools in voltage-clamped, Ca2+-buffered cells suggests enhancement of the size and kinetics of RyR-gated Ca2+ stores during transition from neonatal to juvenile cardiomyocytes (Fig. 1). In permeabilized myocytes this enhancement was nearly fourfold (Fig. 6). The upregulation of the size of the RyR-gated Ca2+ stores is consistent with previous studies of developing cardiomyocytes (15, 45). The faster kinetics in juvenile versus neonatal cells reported here (Fig. 1D) may reflect an altered sensitivity or distribution of the RyRs (10).

**Characterization of IP3-R-gated Ca2+ release in postnatal developing cardiomyocytes.** Although other cellular studies of cardiac development have used cultured cardiomyocytes, to our knowledge this is the first study where IP3-R-gated Ca2+ signals were studied in acutely isolated cells. We pursued this strategy because cultured cardiomyocytes have been known to form spontaneously beating interconnected monolayers and drift to heterogeneous populations with variable functional and electrophysiological characteristics (39), dedifferentiation of cardiac structure (27, 38), and altered expression of IP3Rs (58) and other Ca2+ signaling proteins (48), all of which tend to complicate the interpretation of results and compromise physiological relevance. Nevertheless, it should also be recognized that, even without subsequent culturing, the enzymatic dispersion of cells appears to cause marked changes in the phosphoinositol-signaling pathways (57, 58), and it may be asked whether permeabilized cells may be better suited than dialyzed voltage-clamped cells to evaluate IP3-signaling by excluding confounding factors resulting from heterogeneity or enzymatic degradation of sarcolemmal ATP- and ET-1 receptors.

Application of ATP and ET-1 in voltage-clamped, Ca2+-buffered cells (Figs. 2 and 3) and IP3 in permeabilized cells (Figs. 4–9) produced results, ranging from a rise in the basal [Ca2+]i level to Ca2+ waves and transients throughout the cells at subsarcolemmal, central (Fig. 8), and perinuclear (Fig. 9) locations. Both voltage-clamp (Fig. 3) and permeabilization (Fig. 7) experiments revealed IP3-induced Ca2+ transients that followed a subsarcolemmal activation pathway. Although the IP3-R-gated Ca2+ signals were upregulated with age (Figs. 2 and 6), the magnitude of these responses was found to depend on prior exposure to compounds that targeted the RyR-gated Ca2+ stores (Figs. 6 and 8), indicating that the two types of Ca2+ signal are interdependent.

**Interplay between RyR- and IP3-R-gated Ca2+ stores.** The reduction in the IP3-mediated Ca2+ signals following prolonged exposure to caffeine (Fig. 6) suggests that Ca2+ release via IP3-R may stimulate CICR from nearby, functionally distinct, RyR-gated Ca2+ stores. Alternatively, some subcellular Ca2+ storage compartments may be gated by both Ry- and IP3-Rs. The first possibility is consistent with our observations that the IP3-mediated Ca2+ signals 1) generally displayed an initial slow rise that is followed by a fast caffeine-sensitive spike (Figs. 3A, 4, 6A, and 8); 2) propagated slowly under the membrane (Figs. 7 and 8); and 3) were suppressed equally by caffeine and Ry, both of which deplete the RyR-gated Ca2+ pools, and by tetracaine (Fig. 6), which is known to enhance the SR Ca2+ content by stabilizing RyRs in the closed state (13, 19, 37, 46). In addition, CICR is a promiscuous process that can be initiated by diverse Ca2+ triggers including Ca2+ channels within the same t-SR junctions, reverse mode Ca2+ transport by NCX (26), T-type Ca2+ current (47), or photolysis of caged Ca2+ (29), and, therefore, probably also to the IP3-induced Ca2+ signals. Our use of moderate and high concentrations of Ca2+ buffers was, in part, intended to reduce the free diffusion distance of Ca2+ (1) and thereby intercept the IP3-R-gated Ca2+ signals before they could trigger CICR. In this regard, we may have been successful in limiting the spread of CICR to the interior of the cells, but it would appear that higher concentrations of fast Ca2+ buffers, in combination with ultrastructural studies, would be required to quantify distances between IP3- and RyRs.

On the other hand, the alternative possibility that some IP3-gated Ca2+ pools may be partially emptied by prior exposure to caffeine does not necessarily require a high degree of colocalization of the two receptor types, as seen in adult atrial cells (31), only equilibration of Ca2+ between the subcompartments they gate. Such transfer of Ca2+ has been observed between SR and perinuclear stores, but it notably only occurs on the time scale of minutes (59). Finally, the open probability of IP3-Rs is steeply dependent on Ca2+, in the range from 10 to 100 nM (40), but our buffering of [Ca2+]i at 100 nM probably minimized such effects. Overall, present and published results...
favor the interpretation that the initial IP$_3$-mediated Ca$^{2+}$ release is boosted by local CICR via RyRs.

**Spatial profile of IP$_3$-R-gated Ca$^{2+}$ signaling.** In highly Ca$^{2+}$-buffered cells, application of ATP or ET-1 (Figs. 2 and 3) generated increases in the cytosolic Ca$^{2+}$ at the cell periphery that, in juvenile cells, infrequently triggered global Ca$^{2+}$ transients. In permeabilized cells, IP$_3$ produced RyR-sensitive global Ca$^{2+}$ transients that originated in subsarcolemmal regions and propagated to different degrees into the cell interior (Figs. 7 and 8). These IP$_3$-activated Ca$^{2+}$ transients in neonatal and juvenile cardiomyocytes have spatial Ca$^{2+}$ profiles similar to those of adult atrial cells (21, 28, 31, 32) where they may be explained by the existence of two regularly arranged populations of atrial RyRs, of which those near the cell surface colocalize with IP$_3$R in the junctional SR (28, 31). Our caffeine-activated Ca$^{2+}$ profiles (Figs. 1, 3, 6, and 9) suggest that functional RyRs are uniformly distributed throughout the cell in the developing cardiomyocytes. However, mapping of IP$_3$-activated CICR pathways in either voltage-clamped (Fig. 3) or permeabilized (Fig. 7) cells suggests that a similar spatial disconnect exists between the sarcolemma and the interior SR, as found in atrial cardiomyocytes (32). The presence of functional IP$_3$R throughout the cell (Fig. 8E), in combination with the predominant subsarcolemmal localization of IP$_3$R-gated Ca$^{2+}$ signals (Figs. 2–5 and 7), suggests that those in the interior may be relatively few or spatially dissociated from the centrally located RyRs.

Thus, in developing cardiomyocytes, IP$_3$R may have a closer spatial proximity to RyRs in the peripheral versus central and nuclear regions. The general absence of differences in RyR- and IP$_3$R-gated Ca$^{2+}$ between atrial and ventricular cardiomyocytes in the two age groups (Fig. 6) may indicate that developing cardiomyocytes are physiologically similar to atrial cardiomyocytes, with the distinctive ventricular physiology becoming more apparent after postnatal day 10. The finding that IP$_3$-triggered Ca$^{2+}$ signals of juveniles are larger in ventricular than in atrial myocytes (Fig. 6B) is surprising considering the importance of IP$_3$-mediated signaling in adult atrial cells (32).

**Role of IP$_3$-R-gated Ca$^{2+}$ signaling in postnatal cardiomyocytes.** There is evidence that IP$_3$-dependent Ca$^{2+}$ release can exert a positive inotropic effect in adult atrial and ventricular cells (8, 62), but it is generally found to be minor in nondiseased tissue, especially the ventricle. In our voltage-clamped and permeabilized cells, ATP- or IP$_3$-activated Ca$^{2+}$ transients suggest that IP$_3$ is capable of triggering Ca$^{2+}$ transients independent of depolarization of the membrane. In addition, the finding that IP$_3$-triggered Ca$^{2+}$ transients that were suppressed in the presence of caffeine, ryanodine, and tetracaine implicates a role for IP$_3$ in CICR in developing postnatal cardiomyocytes, where the resulting inward NCX current (Fig. 3) may contribute to membrane depolarization, as seen in pacing embryonic heart cells (23, 43) and in the genesis of arrhythmias (25).

The observation of a Ry-sensitive perinuclear IP$_3$-induced Ca$^{2+}$ signal (Fig. 9) is consistent with previous studies (20, 22, 30, 60) and may implicate Ca$^{2+}$-dependent nuclear transcription factors (25). Similarly, it has been suggested that the Ca$^{2+}$ that is released in response to IP$_3$ may be taken up preferentially by mitochondria that thereby influence the spread of the Ca$^{2+}$ signals (32) and are stimulated to synthesize ATP (17, 22, 24). These observations suggest that neurohumoral regulation of IP$_3$ production could affect the inotropic, chronotropic, and metabolic states of developing cardiomyocytes.

The prominent role of the IP$_3$-signaling pathways in cardiac diseases is generally characterized by activation of the renin-angiotensin and endothelin, enhanced synthesis of IP$_3$, and upregulation of IP$_3$Rs (25). In the present context, it may be relevant to emphasize the unexpected finding that IP$_3$ in juvenile rat ventricular cardiomyocytes generates strong subsarcolemmal Ca$^{2+}$ signals (Figs. 2, 3, 5, and 7), with the distribution typically seen in atrial cells (21, 28, 31, 32) where upregulation of IP$_3$R is associated with the relatively common human condition of atrial fibrillation (5, 61).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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DEVELOPMENTAL ASPECTS OF CARDIAC Ca\(^{2+}\) SIGNALING


