Effects of Ryanodine and Caffeine on Contractility, Membrane Voltage, and Calcium Exchange in Cultured Heart Cells

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To investigate the mechanisms of action of ryanodine and caffeine, changes in mechanical and electrical activity caused by these agents were correlated with alterations in $^{45}$Ca fluxes and cell Ca contents in chick embryo ventricular cell monolayer cultures. Ryanodine $(10^{-10} - 10^{-8} \text{M})$ irreversibly decreased contraction amplitude by 10–70% relative to control in a concentration-dependent manner with minimal effects on electrical activity. Ryanodine caused a slight decrease in rapid $^{45}$Ca uptake, but no change in total exchangeable calcium content or rapid $^{45}$Ca efflux. Caffeine (1–20 mM) caused a transient (less than 10 seconds) 5–12% increase in contraction amplitude followed by a sustained 9–76% decrease in contraction amplitude and a 10 mV decrease in diastolic membrane voltage. Caffeine caused a decrease in rapid $^{45}$Ca uptake, a decrease in total exchangeable calcium content, and an increase in rapid $^{45}$Ca efflux. These results suggest that caffeine produces a decrease in sarcoplasmic reticulum (SR) $\text{Ca}^{2+}$ uptake, and/or an increase in SR $\text{Ca}^{2+}$ release that eventually depletes the SR of $\text{Ca}^{2+}$, presumably accounting for the negative inotropic effect. The ryanodine effects on contraction are more difficult to account for solely in terms of alterations of transsarcolemmal $\text{Ca}^{2+}$ fluxes and $\text{Ca}^{2+}$ contents. Our data indicate an important role for the SR in excitation–contraction coupling in cultured chick embryo ventricular cells and suggest that SR $\text{Ca}^{2+}$ is part of the rapidly exchanging $\text{Ca}^{2+}$ compartment noted in $^{45}$Ca flux studies. (Circulation Research 1987;60:495–504)

Ryanodine and caffeine influence the inotropic state of cardiac muscle at least in part by altering the uptake and release of calcium ion ($\text{Ca}^{2+}$) by the sarcoplasmic reticulum (SR). The neutral alkaloid ryanodine decreases contractility in the nanomolar to micromolar concentration range in cardiac muscle, and in different species the relative sensitivity to ryanodine parallels the apparent relative dependence on SR $\text{Ca}^{2+}$ release for the development of contraction. It has been hypothesized that ryanodine exerts its negative inotropic effect on cardiac muscle by decreasing SR $\text{Ca}^{2+}$ release during excitation–contraction (E–C) coupling. In support of this view, negative inotropic concentrations of ryanodine decrease the aequorin light transient in dog Purkinje fiber, but do not inhibit the slow inward current ($i_{\text{si}}$) in calf Purkinje fiber or rat ventricular muscle. In isolated SR, high concentrations of ryanodine $(10^{-4} \text{M})$ stimulate $\text{Ca}^{2+}$ accumulation without altering the rate of ATP hydrolysis. This ryanodine-mediated increase in $\text{Ca}^{2+}$ accumulation occurs in a low density subset of SR that does not accumulate $\text{Ca}^{2+}$ in the absence of ryanodine. Although these results could also be due to a ryanodine-induced increase in the efficiency of coupling between SR $\text{Ca}^{2+}$ uptake and ATP hydrolysis, high concentrations of ryanodine also directly inhibit calcium-induced $\text{Ca}^{2+}$ release from canine cardiac SR and in skinned rat ventricular cells. Other recent studies have suggested that under some conditions ryanodine may cause release of $\text{Ca}^{2+}$ from SR in intact tissue and in isolated SR. This could account for a depletion of myocardial cell $\text{Ca}^{2+}$ noted in earlier work, but how this occurs and the importance of these various effects in producing the negative inotropic response is not clear.

The inotropic effects of caffeine (1–20 mM) have been studied in multiple cardiac muscle preparations and a variety of responses have been documented. The effects of caffeine on contractility are dependent on concentrations of caffeine and [Ca$^{2+}$]$_{\text{i}}$, rate and duration of caffeine exposure, species, and age. Frog atrial trabeculae, toad ventricle, and kitten papillary muscle display positive inotropic responses. With abrupt exposure to caffeine, adult rat ventricle develops a transient contracture followed by a negative inotropic effect. However, with slower exposure to caffeine only the negative inotropic effect is seen. Newborn rat ventricle and dog Purkinje fiber and papillary muscle exhibit a transient positive inotropic response followed by a sustained negative inotropic response after abrupt exposure to caffeine. These differences in inotropic responses undoubtedly reflect the relative de-
sensitivity of the contractile proteins; 36-39
release from Sr-28>29l37<2i.24.3o-33.34 inhibits Ca²⁺ release from the SR, depleting SR Ca²⁺ content. Furthermore, the experiments indicate that these cultured cell monolayers have functional SR that plays an important role in E-C coupling and that a considerable component of rapid Ca²⁺ exchange in these cells is due to uptake and release of Ca²⁺ by the SR.

Materials and Methods

Tissue Culture

Spontaneously contracting chick embryo ventricular cell monolayer cultures were prepared under sterile conditions as previously described.40 Ventricles from 10-day-old chick embryo hearts were cut into 0.5 mm² fragments, which were gently agitated in Ca²⁺- and Mg²⁺-free Hanks’ solution (Gibco Laboratories, Grand Island, N.Y.). Cells were dissociated by 4 trypsinization cycles (0.025% w/v, trypsin) at 37°C. Cell suspensions from each dissociation cycle were placed in 20 ml of cold trypsin inhibitor solution (50% heat-inactivated fetal calf serum and 50% Ca²⁺- and Mg²⁺-free Hanks’ solution) and centrifuged at 1,000 rpm for 10 minutes. The pellet was resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% medium 199 with Hanks' salts, 0.1% penicillin-streptomycin solution, and 54% balanced salt solution (in mM: NaCl 116, NaH₂PO₄ 1.0, MgSO₄ 0.8, KCl 1.18, NaHCO₃ 26.2, CaCl₂ 0.87, and glucose 5.5). The culture medium final concentrations were K⁺ 4, Na⁺ 143, and Ca²⁺ 1.1 mM. The cell suspension was diluted to 5 x 10⁵ cells/ml and placed in plastic petri dishes containing 25 mm circular glass coverslips. The cultures were incubated in a humidified 5% CO₂ and 95% air atmosphere at 37°C for 3 days. By 2–3 days in culture, confluent monolayers developed in which approximately 80% of the cells exhibited spontaneous synchronous contractions. Plastic microspheres were added to the cultures 1 day prior to contractility experiments. The microspheres settled onto the monolayer and became attached to cell surfaces. Their movement with cell contraction provided a stable image for contraction amplitude measurement.

Contractility Measurements

A glass coverslip with attached ventricular cell monolayer was continuously superfused at a rate of 0.96 ml/min in a chamber with an inlet port attached via a manifold to syringe pumps containing superfusion solutions. Switching the active pump permitted the fluid superfusing the cells to be rapidly changed (τ₀ = 2 seconds). The chamber was placed on the stage of an inverted phase contrast microscope (Leitz Diavert) that was enclosed in a Lucite box in which the temperature was maintained at 37°C. To dampen vibrations the apparatus was supported by an air table. To assess contractility a 40 x objective was used to magnify a portion of the monolayer. The image of a microsphere was observed with a low light-level television camera (Dage 650 SSX) connected to a video motion detector that monitored a selected raster line segment to provide position data every 16 milliseconds. The motion detector analog voltage output was calibrated to indicate microns of cell motion.

Electrophysiological Measurements

Voltage measuring microelectrodes were prepared from 1.2 mm, o.d. capillary tubing with extruded fiber (Frederick Haer & Co., Brunswick, Me.) and filled with 3M KCl by capillary action. The microelectrodes had resistances of 70–100 MΩ. Cells were impaled using a PZ-577 Burleigh Inchworm Controller (Fishers, N.Y.) mounted on a conventional micromanipulator. Impalements were within 150 μm of the region where cell motion was being monitored. Transmembrane voltage was measured as the voltage difference between the intracellular microelectrode and the bath ground Ag-AgCl electrode using a WPI M-707 electrometer (New Haven, Conn.) and amplified with a Honeywell Accudata, 122 DC amplifier. A Honeywell Accudata 132 differentiator was used to determine the maximum upstroke velocity of the action potential, dv/dtₘₜ. Cell motion and membrane voltage signals were simultaneously displayed on a Honeywell multi-channel oscilloscope and recorded by a Honeywell 1858 ultraviolet recorder and a Hewlett Packard 3968 FM tape recorder.

Calcium Fluxes and Contents

Uptake or efflux of ⁴⁵Ca in cultured chick embryo ventricular cells was determined using glass coverslips with attached monolayers as previously described.42 Cells were exposed to ³H-leucine 24 hours prior to ⁴⁵Ca experiments. The incorporation of ³H-leucine into cell protein and the subsequent determination of ³H counts enabled the normalization of ⁴⁵Ca counts relative to milligrams of cell protein for each coverslip, after the relation between ³H counts and protein concentration was determined in a sample of coverslips. For ⁴⁵Ca uptake determinations cells were preincubated for 10 minutes in a control solution at 37°C before being exposed for a specific time period to control or test solutions. The cells were then immersed in the same solution containing ⁴⁵Ca (5 μCi/ml) for the desired ⁴⁵Ca uptake time periods (0–120 minutes).

Ca²⁺ efflux was determined in cells that had been labelled to steady state in ⁴⁵Ca uptake medium for 120 minutes. Individual coverslips were washed for 15 seconds in nonlabelled solution to remove extracellular...
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calcium content only after labeling to steady state. This wash has been shown to remove more than 99% of rapidly exchanging extracellular space marker 3HEDTA. The cells were then scraped off the cover slip and placed in 2 ml of a solution containing 1% sodium dodecyl sulfate and 10 mM Na2B4O7, which produced complete cell dissolution within 2 hours. A 1.6-ml aliquot of the dissolved cell mixture was placed in 15 ml Aquasol liquid scintillation fluid (New England Nuclear, Boston, Mass.). Simultaneous counting of 45Ca and 3H counts in the dissolved cells from each coverslip was performed using a liquid scintillation spectrometer (Packard, model 3330). Fifty microliters of 45Ca uptake medium was placed in a 1.6 ml sample of dissolved cells not exposed to any isotope. From the 45Ca cpm observed from this sample and the known Ca2+ concentration of the uptake medium, the fraction of the 45Ca cpm for each sample as nmol Ca/mg protein. This calculated calcium content is equivalent to the true exchangeable calcium content only after labeling to steady state, which requires 120 minutes.

Solutions and Materials
Superfusion solutions (pH 7.35) contained 1% fetal calf serum and (in mM) NaCl 137, KCl 4.0, MgCl2 0.5, CaCl2 1.8, and HEPES 5. In the ryanodine experiments the solutions contained 20 mM glucose. In the caffeine experiments the control solutions contained 25 mM glucose. Caffeine (1–20 mM) replaced glucose on a mole-for-mole basis. The spontaneous beat rate and contraction amplitude remained stable in control superfusion solution for at least 3 hours. Ryanodine was obtained from Merck Sharpe & Dohme (West Point, Penn.) and S.B. Penick and Co. (Lyndhurst, N.J., Lot #704-RWP-1). Caffeine-sodium benzoate (50:50 w/w mixture) was obtained from Sigma Chemical Co., St. Louis, Mo.

Statistical Analysis
Six ryanodine and 4 caffeine concentrations were studied in mechanical activity experiments, with each coverslip utilized for only 1 concentration. Therefore, a t test of the log of the ratio of drug effect relative to control was used to test for significance at the 0.01 level. This relatively stringent significance level was used because of the number of drug concentrations studied. In addition, in the analysis of the biphasic electrical and mechanical inotropic effects of caffeine, analysis of variance (ANOVA) was used. If the F-ratio determined by ANOVA suggested that means differed at the 0.05 level, the Newman-Keuls method for multiple comparisons was applied. A t test was also used to determine the significance of the differences in fractional 45Ca efflux for the first 5 seconds in caffeine relative to control, in the presence and absence of extracellular Ca2+.

Results

Inotropic and Electrical Effects of Ryanodine
Prior to these experiments the relative dependence of E-C coupling in cultured chick embryo ventricular cells on Ca2+ release from SR had not been established, although results obtained in cultured chick embryo ventricular cell aggregates have indicated the presence of functional SR. Since ryanodine has been proposed to act specifically on SR, its inotropic effects were examined first. A decrease in contraction amplitude was noted 2–10 minutes after exposure to ryanodine, and the maximal decrease for a given concentration was achieved by 15–30 minutes. After 30 minutes of superfusion with ryanodine, return to control solution for up to 70 minutes was not associated with reversal of the negative inotropic effect. Figure 1 shows the concentration dependence of the negative inotropic effects of ryanodine. The decrease in contraction amplitude reached a plateau and became significantly different from control in the 10−7–10−5 M ryanodine concentration range (p < 0.01). The spontaneous beat rate did not change significantly with ryanodine concentrations of 10−10–10−8 M.

To determine whether the negative inotropic effects of ryanodine were associated with alterations in electrical activity, simultaneous recordings of membrane voltage, its first derivative, and cell motion were obtained in 5 experiments. A representative example is shown in Figure 2. There were no significant changes in action potential characteristics relative to control, while the contraction amplitude was significantly reduced (55.4 ± 2.0% of control, p < 0.015) by ryanodine.

FIGURE 1. Contraction amplitude-log ryanodine concentration relation. The contraction amplitude observed after 30 minutes of exposure to ryanodine is expressed as a percentage of the control amplitude. Each filled circle represents the mean ± SEM. The number of experiments at each concentration is indicated in parentheses.
**Inotropic and Electrical Effects of Caffeine**

Figure 3 shows an example of the inotropic effects of abrupt exposure to 20 mM caffeine. After a change to superfusate containing caffeine, there was a transient upshift of the end diastolic cell position associated with an increase in spontaneous beat rate and an increase in contraction amplitude. As shown, the increase in contraction amplitude followed the upshift of the end diastolic cell position and increase in rate by 4–8 beats. After several more beats, this transient phase was followed by a sustained downshift of the end diastolic cell position, a decrease in the beat rate, and a decrease in contraction amplitude. This biphasic response was seen at all caffeine concentrations tested. After resuming superfusion with control solution, there was a return to precaffeine control amplitude and

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**Figure 2.** Effects of ryanodine \(10^{-6}\) on electrical and mechanical activity of cultured chick embryo ventricular cells. Each panel shows simultaneous recordings of the first derivative of the membrane voltage (upper trace and left upper scale, volts/sec), cell motion (middle trace and right scale, microns), and membrane voltage (lower trace and left lower scale, millivolts). The membrane voltage zero reference for both panels is shown on the membrane voltage calibration (lower left scale). Left panel: control recordings; Right panel: recordings obtained 8 minutes after exposure to ryanodine \(10^{-6}\) M.

**Figure 3.** The time course of the effects of caffeine (20 mM) on the mechanical activity of cultured ventricular cells. The vertical lines are at 10-second intervals. The fast paper speed is 10 times the slow. At the left the cell motion signal during superfusion with control solution is shown. The change from control to a superfusion solution containing 20 mM caffeine is indicated below the motion tracing. After approximately 45 seconds superfusion with control solution was resumed as indicated below the motion tracing. The break in the recording during the second control period was 150 seconds.
beat rate. Figure 4 shows the caffeine concentration dependence of both the maximum transient and sustained contraction amplitude changes. With greater caffeine concentrations the transient increase in contraction amplitude was larger and became significantly different from control at 20 mM caffeine ($p < 0.01$). The sustained decrease in contraction amplitude was significant for caffeine concentrations of 10 and 20 mM when compared to control ($p < 0.01$). The increase in beat rate observed during the transient positive inotropic phase was statistically significant relative to control at each caffeine concentration ($p < 0.01$), but the decrease in beat rate observed during the sustained negative inotropic phase was not significant relative to control.

To determine the electrophysiological correlates of these changes in contraction amplitude and beat rate, we simultaneously recorded the effects of exposure to 20 mM caffeine on electrical and mechanical activity in 6 experiments (Figure 5 and Table 1). No change in membrane voltage was detected that would account for the increase in beat rate observed during the transient positive inotropic phase of caffeine effect. It is assumed however, that there is an acceleration of the diastolic depolarization rate, a more negative threshold voltage, and/or a more positive maximum diastolic voltage in the dominant pacemaker cell of the culture at a site different from the recorded cell. Consistent with the findings of Clusin and Hess and Wier, during the sustained negative inotropic phase of caffeine effect there was diastolic membrane depolarization ($p < 0.01$). Also, late in the sustained phase the action potential amplitude and $dV/dt_{max}$ decreased relative to control (Table 1), ($p < 0.01$). Thus, both the electrical and mechanical effects of caffeine in chick embryo ventricular cells are similar to those described in canine ventricular muscle and Purkinje fiber.

**Calcium Flux and Content Changes With Ryanodine**

The electrical and mechanical effects of ryanodine and caffeine suggested that the SR plays a significant role in E-C coupling and is a site of action for both agents in these cells. The effects of these drugs on $^{45}$Ca uptake, content, and efflux were determined next.

Since the inotropic effects of ryanodine were fully developed within 30 minutes of exposure, cells were preincubated in control or ryanodine ($10^{-6}$ M) containing solutions for 30 minutes at 37°C. The cells were then transferred to solutions containing $^{45}$Ca for the desired uptake period (0-80 seconds). Figure 6 shows that ryanodine decreased $^{45}$Ca labeling of the rapidly exchanging calcium compartment. The data were fit to a single exponential equation, $Ca(t) = Ca(0) + R/k (1 - e^{-kt})$, where $Ca(t)$ is the calculated $^{45}$Ca content at time $= t$, $Ca(0)$ is the "background" $^{45}$Ca content at time $= 0$, $R$ is the uptake rate and $k$ is the efflux rate.
constant.\textsuperscript{40,48} Values for Ca(0) and k, determined by fitting the control \textsuperscript{45}Ca uptake data, were in fitting the ryanodine \textsuperscript{45}Ca uptake data to calculate R. There was a significant reduction in R in ryanodine (3.00 ± 0.13 mmol/mg protein/min) when compared to control (3.96 ± 0.13 mmol/mg protein/min, p < 0.0002).

To consider the effects of ryanodine on the rapidly exchanging calcium content, the total exchangeable calcium content was determined. Paired coverslips were exposed to control \textsuperscript{45}Ca medium for 120 minutes, and then one of each pair was placed in ryanodine (10\textsuperscript{-6} M) - \textsuperscript{45}Ca medium for 30 minutes, while the other remained in control \textsuperscript{45}Ca medium for the additional 30 minutes. Exposure to ryanodine did not alter the total exchangeable calcium content (ryanodine: 4.90 ± 0.13 vs. control: 5.06 ± 0.19 [n = 6] mmol/mg protein).

The effects of ryanodine on \textsuperscript{45}Ca efflux were measured next. After labelling paired coverslips in control \textsuperscript{45}Ca uptake medium, one of each pair was placed in ryanodine (10\textsuperscript{-6} M) \textsuperscript{45}Ca uptake medium for an additional 30 minutes. Fractional \textsuperscript{45}Ca efflux was measured in unlabelled control or ryanodine containing solutions. Using a single exponential to fit the data by the standard nonlinear regression model, the rate constant for ryanodine (0.037 ± 0.014 sec\textsuperscript{-1}, mean ± 95% confidence intervals) and control (0.033 ± 0.003) were similar. Thus, a negative inotropic concentration or ryanodine decreased the rate but not the ultimate extent of labelling of the rapidly exchanging calcium compartment, without producing a detectable change in \textsuperscript{45}Ca efflux from that compartment.

### Calcium Flux and Content Changes With Caffeine

The mechanism of the sustained negative inotropic effect of caffeine was investigated in \textsuperscript{45}Ca flux experiments. Cells were either equilibrated for 5 minutes in control solution, followed by 5 minutes in caffeine containing solution or equilibrated for 10 minutes in control solution. The control and caffeine treated cells were then immersed in \textsuperscript{45}Ca-labelled control or caffeine solutions for the desired uptake period. In a concentration-dependent manner 1–20 mM caffeine decreased the initial rate of rapid \textsuperscript{45}Ca uptake and rapidly exchanging \textsuperscript{45}Ca content (Figure 7). We next measured the effects of caffeine on total exchangeable calcium content. Paired coverslips were exposed to control \textsuperscript{45}Ca medium or 10 mM caffeine – \textsuperscript{45}Ca medium for 120 minutes. The total exchangeable calcium content in 10 mM caffeine (4.04 ± 0.10 [n = 24] nmol/mg protein) was significantly reduced relative to control (5.02 ± 0.10 [n = 24] nmol/mg protein, p < 0.001). This finding suggests that the decrease in rapid \textsuperscript{45}Ca uptake is caused by a decrease in the content of the rapidly exchanging calcium compartment.

Since the content of the rapidly exchanging calcium compartment is decreased by caffeine, we hypothesized that abrupt exposure to caffeine might increase rapid \textsuperscript{45}Ca efflux. After labeling to steady state for 120 minutes in \textsuperscript{45}Ca control solution with 1.8 mM \textsuperscript{2+}Ca, cells were washed for 15 seconds in unlabelled control solution to remove extracellular tracer, and then efflux was measured. During the first 5 seconds after caffeine exposure there was a small but significant (p < 0.01) increase in fractional \textsuperscript{45}Ca efflux (Figure 8a). The presence of \textsuperscript{2+}Ca in the wash and efflux solutions could obscure the effect of caffeine on \textsuperscript{45}Ca efflux by causing SR \textsuperscript{2+}Ca cycling during E-C coupling and Ca--Ca ex-

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### Table 1. Effects of Caffeine (20 mM) on Electrical Activity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Transient</th>
<th>Sustained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation voltage (mV)</td>
<td>-73.7±5.6</td>
<td>-75.8±5.0</td>
<td>-64.0±4.1*</td>
</tr>
<tr>
<td>Maximum diastolic voltage (mV)</td>
<td>-84.9±3.1</td>
<td>-85.0±3.2</td>
<td>-74.8±2.1*</td>
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<tr>
<td>Amplitude (mV)</td>
<td>106.4±8.2</td>
<td>108.4±7.8</td>
<td>89.5±7.0*</td>
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<td>Overshoot (mV)</td>
<td>32.6±4.2</td>
<td>32.4±3.7</td>
<td>25.5±3.7*</td>
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<tr>
<td>Upstroke velocity (V/sec)</td>
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<td>10.6±2.1</td>
<td>6.3±2.1*</td>
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<tr>
<td>Diastolic depolarization rate (mV/sec)</td>
<td>30.0±7.8</td>
<td>33.7±7.9</td>
<td>28.8±7.6*</td>
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<td>APD-50 (msec)</td>
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<td>165.7±16.4</td>
<td>149.1±9.3</td>
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<td>APD-90</td>
<td>212.4±16.8</td>
<td>207.0±15.2</td>
<td>211.7±15.1</td>
</tr>
</tbody>
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*pSignificant difference from control (p < 0.05). Values are expressed as mean ± SEM (n = 6).
change. Therefore, after Ca labeling the effect of caffeine on efflux was also studied in nominal zero [Ca], (Figure 8b). In nominal zero [Ca], caffeine caused a greater increase in fractional Ca efflux relative to control than did in the presence of extracellular Ca. These results support the hypothesis that caffeine promotes rapid Ca release from the SR.

Discussion

Functional Role of Sarcoplasmic Reticulum in Chick Embryo Ventricular Cells

As in mammalian myocardial cells, intact avian myocardial cells have well-developed SR. During embryonic maturation, SR can be seen after 45 hours of incubation. SR development parallels that of myofibrils with a mature appearance occurring before 15 days. However, when ventricular cells dissociated from 10-day-old embryo hearts are grown in layer cultures, they tend to lose their adult-like properties and show anatomical characteristics of less mature ventricles. Although it has been recognized that these cultured cell layers retain an ability to synthesize SR Ca2+-ATPase, the role of the SR in E-C coupling has not been clear.

Ryanodine partially and irreversibly inhibited contraction in cultured chick embryo ventricular cells in a concentration-dependent manner with a plateau at 10-7 to 10-5 M, an effect comparable to that observed in mammalian cardiac muscles, which have functional SR. Negatively inotropic concentrations of ryanodine have minimal effect on electrical activity in this cultured cell preparation. This is consistent with the observations that ryanodine only slightly prolongs the action potential in guinea pig papillary muscle and dog Purkinje fiber and does not decrease i in dog Purkinje fiber or isolated adult rat ventricular cells.

The ryanodine results suggest that the SR is functionally important in these cultured ventricular cells, and the caffeine data provide further support for this hypothesis. Caffeine (1-20 mM) exerted a biphasic inotropic effect with a transient increase in contraction amplitude and beat rate, and upshift of the end diastolic cell position followed by a sustained decrease in contraction amplitude and beat rate and downshift of the end diastolic cell position. Both these types of effects have been ascribed to altered SR function induced by caffeine. In canine Purkinje fibers, the caffeine-related transient increase in contractile force is associated with an increase in the second component of the aequorin signal, and Hess and Wier suggested that enhanced release and/or decreased uptake of Ca by the SR may be involved. This hypothesis is supported by the work of Sheu et al who found an increase in quin2 fluorescence in dissociated rat ventricular myocytes after abrupt exposure to caffeine and by Sutko et al who noted an initial increase in resting force and Ca2+-dependent phosphorylase activity after exposure of rat myocardium to 20 mM caffeine. Also consistent are the results of Eisner and Valdeolmillos, who observed an initial increase in aequorin light emission in sheep cardiac Purkinje fibers with exposure to caffeine.

In experiments with voltage clamped chick embryo ventricular cell aggregates, abrupt exposure to caffeine resulted in a Na+-sensitive transient inward current and brief asynchronous twitches that have been ascribed to SR Ca2+ release resulting in electrogenic Na-Ca exchange and/or intracellular Ca2+ activated transient inward current. Mechmann and Pott have also recently reported that abrupt exposure of sodium-loaded isolated myocytes to 5 mM caffeine abruptly releases Ca2+, producing an inward current which is probably electrogenic Na-Ca exchange. However, since caffeine can increase myofilament Ca2+ sensitivity at least as rapidly as it diffuses into skinned rat heart trabeculae, the initial increase in contractility could also be due in part to enhanced myofilament Ca2+ sensitivity.

Several studies have considered the mechanism of the sustained decrease in contractility after caffeine exposure. In canine Purkinje fibers, Hess and Wier observed that the decrease in i developed more slowly than the decline in tension after exposure to caffeine. Also, the action potential and i were unchanged by caffeine during the sustained decrease in peak tension in canine papillary muscle. Since the negative inotropic effect of caffeine was associated with a decrease in both components of the aequorin signal, Hess and Wier proposed that caffeine caused impaired SR Ca2+ uptake, leading to SR Ca2+ depletion and thus a reduced calcium transient. Thus, the effects of ryanodine and both the transient and sustained effects of caffeine in these monolayers of cultured cells, support a role for the SR in E-C coupling in this preparation.

Effects of Ryanodine and Caffeine on Calcium Exchange

To characterize further how ryanodine and caffeine alter Ca2+ availability for contraction, inotropic and
electrical effects were correlated with changes in unidirectional $^{45}$Ca fluxes and cell calcium contents. Previous work by Barry and Smith\(^\text{40}\) has shown that $^{45}$Ca uptake by cultured monolayers of chick embryo ventricular cells can be separated into a rapidly exchanging calcium compartment that labels within 1–2 minutes and a slowly exchanging calcium pool that labels within 2 hours. Each compartment accounts for about 50% of the total exchangeable calcium content. $^{45}$Ca within the rapidly exchanging calcium compartment is not displaceable by 1 mM La, and thus the compartment seems to be intracellular\(^\text{40}\) rather than superficially bound.\(^\text{58}\) Positively inotropic concentrations of ouabain or low $[K^+]_o$ increase the calcium content of this rapidly exchanging calcium compartment.\(^\text{52}\) Calcium channel blockers decrease the content of the rapidly exchanging calcium compartment.\(^\text{30,59}\) Thus, the content of this rapidly exchanging intracellular calcium compartment seems to be modulated by Na-Ca exchange and $i_h$. The calcium content of this compartment is also reduced by metabolic inhibition, indicating a possible requirement for ATP in its maintenance.\(^\text{60}\) Based on these observations, we have suggested that this rapidly exchanging compartment contains, at least in part, $Ca^{2+}$ within the SR.\(^\text{45}\) The present results support this hypothesis.

Caffeine had marked effects on $^{45}$Ca fluxes and cell calcium content. Abrupt exposure to caffeine in the presence or absence of extracellular $Ca^{2+}$ resulted in enhanced rapid $^{45}$Ca efflux. This suggests that the rapid onset of both the positive inotropic effect and upshift of the end diastolic cell position are due to enhanced release, as well as diminished reuptake of $Ca^{2+}$ by the SR, making more $Ca^{2+}$ available to the contractile apparatus. If it is assumed that at rest there is some basal turnover of $Ca^{2+}$ between the SR and the myoplasm as a result of continuous or phasic $Ca^{2+}$ release and reuptake,\(^\text{61}\) inhibition of $Ca^{2+}$ reuptake into the SR alone could account for our caffeine results as has been suggested by Hess and Wier.\(^\text{28}\) However, our finding of a greater effect of caffeine on $^{45}$Ca efflux in nominal zero $[Ca^{2+}]_i$, (Figure 8), a situation in which SR $Ca^{2+}$ turnover is reduced by the absence of E-C coupling, suggests that there is in fact a caffeine-mediated increase in SR $Ca^{2+}$ release. Studies using cardiac muscle vesicular preparations also support the contention that caffeine releases $Ca^{2+}$ from the SR. In SR from dog\(^\text{62}\) and rabbit\(^\text{63}\) cardiac muscle, caffeine reduced net $Ca^{2+}$ accumulation. Since calcium-stimulated ATPase was increased, it was proposed that caffeine exaggerated passive $Ca^{2+}$ efflux from SR by making the SR membrane more permeable to $Ca^{2+}$.\(^\text{63}\) Caffeine also rapidly released $^{45}$Ca from actively loaded rabbit cardiac muscle SR.\(^\text{64}\)

In our experiments, prolonged exposure to caffeine also decreased rapid $^{45}$Ca uptake and total exchangeable calcium content. In sum, these observations are consistent with the proposal that the sustained negative inotropic effect of caffeine is due to SR $Ca^{2+}$ depletion due to enhanced release and/or decreased uptake of $Ca^{2+}$.\(^\text{28}\)

The results reported in other studies of the influence of caffeine on tracer $Ca^{2+}$ movement in intact heart tissue have been variable, probably reflecting diffusion limitations of some of these preparations and species differences.\(^\text{22,28,65,66}\) Notably, however, in isolated rat heart perfused in zero $[Ca^{2+}]_o$, sudden exposure to caffeine produced a contraction and released $^{45}$Ca from a rapidly exchanging intracellular calcium compartment ($i_o < 1$ minute). When caffeine was added to the $^{45}$Ca labelling perfusate, contractile failure and a decrease in the calcium content of this rapidly exchanging compartment was observed.\(^\text{50}\) These results are similar to ours and suggest that in adult rat heart the predominant source of contractile $Ca^{2+}$ is a rapidly exchangeable intracellular calcium compartment.\(^\text{50}\) The data also (Figure 8) suggests a role for ATP in maintaining the calcium content of the rapidly exchanging compartment.

![Figure 8](http://circres.ahajournals.org/)

**FIGURE 8.** Effects of abrupt exposure to caffeine (20 mM) in the presence (top) or absence (bottom) of extracellular $Ca^{2+}$ on rapid fractional $^{45}$Ca efflux. Fractional $^{45}$Ca efflux is plotted as a function of time. At 0 seconds cells were removed from the wash solution and placed in control efflux solution for the first 5 seconds of efflux measurement. In the top panel after 5 seconds (vertical arrow), efflux was either continued in control solution (○, n = 36) or changed to control plus 20 mM caffeine (●, n = 36) for subsequent efflux periods. In the bottom panel, cells were removed from a nominal zero $[Ca^{2+}]_o$ wash and were placed in nominal zero $[Ca^{2+}]_o$ control solution for the first 5 seconds of efflux measurement. After 5 seconds (vertical arrow) efflux was either continued in zero $[Ca^{2+}]_o$ (○, n = 20) or changed in zero $[Ca^{2+}]_o$ plus 20 mM caffeine (●, n = 24) for subsequent efflux periods.
exchanging intracellular compartment, probably including SR, and that caffeine releases Ca\(^{2+}\) from, inhibits reuptake by, and therefore depletes, this Ca\(^{2+}\) compartment.

Ca\(^{2+}\) abruptly released by caffeine from SR may be extruded by an electrogenic Na--Ca exchange\(^{55,57}\) or by slower Na\(^-\)independent mechanism, presumably a sarcosomal Ca-ATPase Ca\(^{2+}\) pump.\(^{55,56}\) Transsarcosomal Ca\(^{2+}\) extrusion after abrupt caffeine exposure can produce a transient increase in interstitial [Ca\(^{2+}\)] in intact tissue.\(^{14}\) Taken together, these data strongly support the hypothesis that Ca\(^{2+}\) in the SR is a part of the rapidly exchanging (\(t_{1/2} < 1\) minute) calcium compartment noted in \(^{45}\)Ca flux studies in intact myocardial cells.

The negative inotropic effects of ryanodine are difficult to interpret only in terms of the observed alterations in transsarcosomal calcium fluxes and calcium contents. It does appear that a negative inotropic effect of ryanodine can occur without a significant decrease in total (and thus presumably also SR) calcium content. This finding is consistent with the results of Bers et al\(^{13}\) who found a negative inotropic effect of ryanodine in rabbit ventricular papillary muscle without a decrease in the magnitude of the maximal cold contracture, presumably indicating normal SR Ca\(^{2+}\) stores.\(^{68}\) This result is consistent with impaired release of Ca\(^{2+}\) from the SR induced by ryanodine. Impaired release of Ca\(^{2+}\) also could slow uptake of Ca\(^{2+}\), since the SR would remain full and be less able to accumulate Ca\(^{2+}\) rapidly.\(^{11}\) This could account for our finding of a slowed initial rate of \(^{45}\)Ca uptake. However, impaired release of Ca\(^{2+}\) from SR might be expected to be manifest as slowed \(^{45}\)Ca efflux from the rapidly exchanging (presumably SR) compartment, and this was not observed.

We do not at present have an explanation for this. It is possible that alterations in SR Ca\(^{2+}\) release are not reflected by transsarcosomal \(^{45}\)Ca efflux measurements. Another possibility we are now addressing is that effects of ryanodine on \(^{45}\)Ca efflux are rate dependent. In any event, the present findings, while supporting an important role for the SR in E-C coupling in these cells, do not establish the precise mechanism of the negative inotropic effect of ryanodine.

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**Key Words** • excitation-contraction coupling • electrophysiology • Ca flux • caffeine • ryanodine • sarcoplasmic reticulum • cultured heart cells
Effects of ryanodine and caffeine on contractility, membrane voltage, and calcium exchange in cultured heart cells.
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_Circ Res._ 1987;60:495-504
doi: 10.1161/01.RES.60.4.495

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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