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

Carl A.F. Rasmussen Jr., John L. Sutko, and William H. Barry

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 ($10^{-10}$ to $10^{-5}$ 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) Ca$^{2+}$ uptake, and/or an increase in SR Ca$^{2+}$ release that eventually depletes the SR of 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 Ca$^{2+}$ fluxes and Ca$^{2+}$ contents. Our data indicate an important role for the SR in excitation–contraction coupling in cultured chick embryonic ventricular cells and suggest that SR Ca$^{2+}$ is part of the rapidly exchanging 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 (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 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 Ca$^{2+}$ release during excitation–contraction coupling (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 ($i_{sc}$) in calf Purkinje fiber or rat ventricular muscle. In isolated SR, high concentrations of ryanodine ($10^{-4}$ M) stimulate Ca$^{2+}$ accumulation without altering the rate of ATP hydrolysis. This ryanodine-mediated increase in Ca$^{2+}$ accumulation occurs in a low density subset of SR that does not accumulate 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 Ca$^{2+}$ uptake and ATP hydrolysis, high concentrations of ryanodine also directly inhibit calcium-induced 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 Ca$^{2+}$ from SR in intact tissue and in isolated SR. This could account for a depletion of myocardial cell 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$^{2+}$]$_j$, 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-
gree to which caffeine 1) induces Ca\textsuperscript{2+} release from SR\textsuperscript{21,24,30-33}; 2) inhibits Ca\textsuperscript{2+} uptake by SR\textsuperscript{2,24}; 3) increases Ca\textsuperscript{2+} sensitivity of the contractile proteins\textsuperscript{34-36}; and/or 4) influences i

In the present work, we have investigated the effects of ryanodine and caffeine on cultured monolayers of chick embryo ventricular cells, a preparation in which changes in mechanical and electrical activity, and alterations in transsarcolemmal \textgreek{Ca}\textsuperscript{2+} fluxes and cell calcium contents can be readily measured. The findings are consistent with the hypothesis that caffeine enhances release and/or inhibits reuptake of Ca\textsuperscript{2+} by the SR, depleting SR Ca\textsuperscript{2+}. The results also demonstrate that the negative inotropic effect of ryanodine occurs without a significant decrease in total exchangeable Ca\textsuperscript{2+} 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 Ca\textsuperscript{2+} exchange in these cells is due to uptake and release of Ca\textsuperscript{2+} by the SR.

Materials and Methods

**Tissue Culture**

Spontaneously contracting chick embryo ventricular cell monolayer cultures were prepared under sterile conditions as previously described.\textsuperscript{40} Ventricles from 10-day-old chick embryo hearts were cut into 0.5 mm\textsuperscript{3} fragments, which were gently agitated in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free Hanks' solution (Gibco Laboratories, Grand Island, N.Y.). Cells were dissociated by 4 trypsinization cycles (0.025% w/v, trypsin) at 37\textdegree 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% Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-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, NaH\textsubscript{2}PO\textsubscript{4} 1.0, MgSO\textsubscript{4} 0.8, KCl 1.8, NaHCO\textsubscript{3} 26.2, CaCl\textsubscript{2} 0.87, and glucose 5.5). The culture medium final concentrations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were 1.1 mM. The cell suspension was diluted to 5 × 10\textsuperscript{4} cells/ml and placed in plastic petri dishes containing 25 mm circular glass coverslips. The cultures were incubated in a humidified 5% CO\textsubscript{2} and 95% air atmosphere at 37\textdegree 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 (t\textsubscript{w} = 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\textdegree C. To dampen vibrations the apparatus was supported by an air table. To assess contractility a 40× 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\textsuperscript{41} 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 M\textOmega. Cells were impaled using a PZ-577 Burleigh Inchworm Controller (Fishers, N.Y.) mounted on a conventional micromanipulator. Impalements were within 150 \mu m 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, \textgreek{dV/dt}\textsubscript{max}. Cell motion and membrane voltage signals were simultaneously displayed on a Honeywell multichannel 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 \textgreek{Ca}\textsuperscript{2+} in cultured chick embryo ventricular cells was determined using glass coverslips with attached monolayers as previously described.\textsuperscript{42} Cells were exposed to \textgreek{H}-leucine 24 hours prior to \textgreek{Ca}\textsuperscript{2+} experiments. The incorporation of \textgreek{H}-leucine into cell protein and the subsequent determination of \textgreek{H} counts enabled the normalization of \textgreek{Ca}\textsuperscript{2+} counts relative to milligrams of cell protein for each coverslip, after the relation between \textgreek{H} counts and protein concentration\textsuperscript{43} was determined in a sample of coverslips. For \textgreek{Ca} uptake determinations cells were preincubated for 10 minutes in a control solution at 37\textdegree C before being exposed for a specific time period to control or test solutions. The cells were then immersed in the same solution containing \textgreek{Ca}\textsuperscript{2+} (5 \muCi/ml) for the desired \textgreek{Ca} uptake time periods (0–120 minutes).

\textgreek{Ca}\textsuperscript{2+} efflux was determined in cells that had been labelled to steady state in \textgreek{Ca} 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, 45Ca cpm remaining in the cells after the 35-second
efflux period, fractional 45Ca efflux for each 5-second
period was calculated.

After coverslips were removed from the uptake or
efflux medium, they were washed for 8 seconds each in
two 80-ml volumes of balanced salt solution at 4°C.
This wash has been shown to remove more than 99%
of rapidly exchanging extracellular space marker
3H-EDTA.40 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 Eng-
land 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
45Ca cpm/nmol Ca2+ was determined. The calcium content
could then be calculated from the 45Ca cpm for each
sample as nmol Ca/mg protein. This calculated calcu-
lation 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 con-
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neous beat rate did not change significantly with ryan-
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to determine whether the negative inotropic effects
of ryanodine were associated with alterations in elec-
trical activity, simultaneous recordings of membrane
voltage, its first derivative, and cell motion were ob-
tained 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, ρ < 0.015) by ryan-
odine.

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 estab-
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presence of functional SR. Since ryanodine has been
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Statistical Analysis

Six ryanodine and 4 caffeine concentrations were
studied in mechanical activity experiments, with each
coverslip utilized for only 1 concentration. Therefore,
the 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 multi-
ple comparisons was applied.44 A t-test was also used to
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45Ca efflux for the first 5 seconds in caffeine
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**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...
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\(^{19}\) and Hess and Wier,\(^{28}\) 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 \(dV/dt\) 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\(^{25}\) and Purkinje fiber.\(^{27,28}\)

**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. Values for Ca(0) and k, determined by fitting the control 45Ca uptake data, were used in fitting the ryanodine 45Ca uptake data to calculate R. There was a significant reduction in R in ryanodine (3.00 ± 0.0002) compared to control (3.96 ± 0.13 nmol/mg protein/min, p < 0.0001). This finding suggests that the decrease in rapid 45Ca uptake is caused by a decrease in the content of the rapidly exchanging calcium compartment.

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

The effects of ryanodine on 45Ca efflux were measured next. After labelling paired coverslips in control 45Ca uptake medium, one of each pair was placed in ryanodine (10^-6 M) - 45Ca uptake medium for an additional 30 minutes. Fractional 45Ca 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^-1, mean ± 95% confidence intervals) and control (0.033 ± 0.003) were similar. Thus, a negative inotropic concentration of ryanodine decreased the rate but not the ultimate extent of labelling of the rapidly exchanging calcium compartment, without producing a detectable change in 45Ca efflux from that compartment.

**Calcium Flux and Content Changes With Caffeine**

The mechanism of the sustained negative inotropic effect of caffeine was investigated in 45Ca 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 45Ca-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 45Ca uptake and rapidly exchanging 45Ca content (Figure 7). We next measured the effects of caffeine on total exchangeable calcium content. Paired coverslips were exposed to control 45Ca medium or 10 mM caffeine - 45Ca 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 45Ca 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 45Ca efflux. After labeling to steady state for 120 minutes in 45Ca control solution with 1.8 mM [Ca^2+], 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 45Ca efflux (Figure 8a). The presence of Ca^2+ in the wash and efflux solutions could obscure the effect of caffeine on 45Ca efflux by causing SR Ca^2+ cycling during E-C coupling and Ca-Ca ex-

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Transient</th>
<th>Sustained</th>
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</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>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>106.4±8.2</td>
<td>108.4±7.8</td>
<td>89.5±7.0*</td>
</tr>
<tr>
<td>Overshoot (mV)</td>
<td>32.6±4.2</td>
<td>32.4±3.7</td>
<td>25.5±3.7*</td>
</tr>
<tr>
<td>Upstroke velocity (V/sec)</td>
<td>10.4±4.2</td>
<td>10.6±2.1</td>
<td>6.3±2.1*</td>
</tr>
<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>
<td>171.7±16.8</td>
<td>165.7±16.4</td>
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</tr>
<tr>
<td>APD-90</td>
<td>212.4±16.8</td>
<td>207.0±15.2</td>
<td>211.7±15.1</td>
</tr>
</tbody>
</table>

*Significant difference from control (p<0.05). Values are expressed as mean ± SEM (n = 6).
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 culture, 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 Ca\(^{2+}\)-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\(^{-7}\)-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 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 aquorin signal, and Hess and Wier suggested that enhanced release and/or decreased uptake of Ca\(^{2+}\) 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 Ca\(^{2+}\)-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 Ca\(^{2+}\) release resulting in electrogenic Na-Ca exchange and/or intracellular Ca\(^{2+}\) activated transient inward current. Mechmann and Pott have recently reported that abrupt exposure of sodium-loaded isolated myocytes to 5 mM caffeine abruptly releases Ca\(^{2+}\), producing an inward current which is probably electrogenic Na-Ca exchange. However, since caffeine can increase myofilament Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 ion developed more slowly than the decline in tension after exposure to caffeine. Also, the action potential and ion 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 Ca\(^{2+}\) uptake, leading to SR Ca\(^{2+}\) 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 Ca\(^{2+}\) 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$^{40}$ 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$^{40}$ rather than superficially bound.$^{58}$ Positively inotropic concentrations of ouabain or low [K$^+$], increase the calcium content of this rapidly exchanging calcium compartment.$^{42}$ Calcium channel blockers decrease the content of the rapidly exchanging calcium compartment.$^{50,59}$ Thus, the content of this rapidly exchanging intracellular calcium compartment seems to be modulated by Na-Ca exchange and $i_L$. The calcium content of this compartment is also reduced by metabolic inhibition, indicating a possible requirement for ATP in its maintenance.$^{60}$ Based on these observations, we have suggested that this rapidly exchanging compartment contains, at least in part, Ca$^{2+}$ within the SR.$^{45}$ 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,$^{61}$ inhibition of Ca$^{2+}$ reuptake into the SR alone could account for our caffeine results as has been suggested by Hess and Wier.$^{28}$ However, our finding of a greater effect of caffeine on $^{45}$Ca efflux in nominal zero [Ca$^{2+}$], (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$^{62}$ and rabbit$^{63}$ 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$^{2+}$. Caffeine also rapidly released $^{45}$Ca from actively loaded rabbit cardiac muscle SR.$^{64}$

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$^{2+}$. $^{28}$

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.$^{22,28,65,66}$ 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 ($t_{90} < 1$ 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.$^{50}$ These results are similar to ours and suggest that in adult rat heart the predominant source of contractile Ca$^{2+}$ is a rapidly

![Figure 8](image-url)

**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 zero Ca$^{2+}$, (•, n = 24) 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 = 36) for subsequent efflux periods.
exchanging intracellular compartment, probably including SR, and that caffeine releases Ca\textsuperscript{2+} from, inhibits reuptake by, and therefore depletes, this Ca\textsuperscript{2+} compartment.

Ca\textsuperscript{2+} abruptly released by caffeine from SR may be extruded by an electrogenic Na–Ca exchange\cite{55,57} or by slower Na\textsubscript{in}-independent mechanism, presumably a sarcoplasmic Ca-ATPase Ca\textsuperscript{2+} pump.\cite{55,56} Transsarcoplasmic Ca\textsuperscript{2+} extrusion after abrupt caffeine exposure can produce a transient increase in interstitial [Ca\textsuperscript{2+}] in intact tissue.\cite{14} Taken together, these data strongly support the hypothesis that Ca\textsuperscript{2+} in the SR is a part of the rapidly exchanging (t\textsubscript{1/2} < 1 minute) calcium compartment noted in \textsuperscript{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 transsarcoplasmic 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\cite{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\textsuperscript{2+} stores.\cite{66} This result is consistent with impaired release of Ca\textsuperscript{2+} from the SR induced by ryanodine. Impaired release of Ca\textsuperscript{2+} also could slow uptake of Ca\textsuperscript{2+}, since the SR would remain full and be less able to accumulate Ca\textsuperscript{2+} rapidly.\cite{11} This could account for our finding of a slowed initial rate of \textsuperscript{45}Ca uptake. However, impaired release of Ca\textsuperscript{2+} from SR might be expected to be manifest as slowed \textsuperscript{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\textsuperscript{2+} release are not reflected by transsarcolemmal \textsuperscript{45}Ca efflux measurements. Another possibility we are now addressing is that effects of ryanodine on \textsuperscript{45}Ca efflux are rate dependent. In any event, the present findings, while supporting the hypothesis that effects of ryanodine on \textsuperscript{45}Ca efflux are rate dependent, do not establish the precise mechanism of the negative inotropic effect of ryanodine.

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