Modulation of Cellular Calcium Stores in the Perfused Rat Heart by Isoproterenol and Ryanodine

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SUMMARY. The inhibitory action of procaine on cellular calcium release was utilized to define a new cellular calcium pool which, under physiological conditions, is present only during catecholamine stimulation. Rat hearts labeled with \( { }^{45}\text{Ca}^{++} \) were perfused with medium containing procaine and EGTA at 23°C to remove extracellular calcium, and then cellular calcium was released by removal of procaine and restoration of calcium. By this method we have identified a cellular calcium pool (pool C) whose release is inhibited by procaine, but which does not require extracellular calcium for its release. Release of pool C can also be triggered by caffeine. [We have previously identified a cellular calcium pool (pool A) whose release is triggered by caffeine, inhibited by procaine, and which does require extracellular calcium for its release.] When hearts were labeled for 3 minutes with perfusate containing 1 mM \( { }^{45}\text{Ca}^{++} \), 48 ± 6 nmol Ca^+/g wet weight was found in pool A, but only 3 ± 1 nmol Ca^+/g in pool C. However, if isoproterenol was present during labeling, the hearts contained 72 ± 5 nmol Ca^+/g in pool A and 42 ± 6 nmol Ca^+/g in pool C. When calcium concentration in the labeling perfusate was varied, with and without isoproterenol, it was found that pool C does not begin to fill until pool A is almost full. The same effect was seen when excess cellular calcium uptake was induced by removing sodium from the perfusate. Ryanodine \((0.2 \, \mu\text{M})\) induced contractile failure \((t_c = 3.4 \pm 0.4 \text{ min})\) and depleted pool A in control hearts by 85%. Ryanodine also similarly depleted pools A and C in isoproterenol-treated hearts. When contractility was monitored at the same time as the hearts were labeled, a linear relationship between \(\frac{dP}{dt}\) and the sum of pools A and C was observed over a wide range of conditions. Pools A and C both selected strongly for calcium over barium. These observations suggest that both pools A and C are located in the sarcoplasmic reticulum and are intimately involved in the regulation of contractility. (Circ Res 53: 703-712, 1983)

ISOPROTERENOL and other catecholamines greatly enhance cardiac contractility by increasing the rate of both contraction and relaxation (Kukovetz et al., 1959; Sonnenblick, 1967; Hearse et al., 1978). The effect of isoproterenol is probably mediated through changes in Ca\(^{++}\) metabolism. A rise in cellular free Ca\(^{++}\) during systole upon isoproterenol addition has been observed in frog atrial trabeculae using aequorin (Allen and Blinks, 1978). The ability of the sarcoplasmic reticulum (SR) to sequester Ca\(^{++}\) is increased through a catecholamine/cAMP-mediated mechanism (Kirchberger et al, 1972; Schwartz et al., 1976; Lindemann et al., 1983). Catecholamines have also been shown to stimulate the rate of cellular Ca\(^{++}\) influx by electrophysiological measurements (Reuter and Scholz, 1977) and by a method which utilized the uptake of low levels of \( { }^{54}\text{Mn}^{++} \) (Hunter et al., 1981a).

Isoproterenol has recently been shown by our laboratory to enhance the steady state cellular Ca\(^{++}\) content of rat hearts (Hunter et al., 1981b). The increase of \( { }^{40}\text{Ca}^{++} \) was located in pool A, which is a cellular Ca\(^{++}\) pool whose release from the heart requires extracellular Ca\(^{++}\) (Hunter et al., 1981b; 1982) or caffeine (Hunter et al., 1982). Isoproterenol produced no change in the size of pool B, a cellular Ca\(^{++}\) pool whose release is not dependent on extracellular Ca\(^{++}\), but whose release is inhibited by cold (Hunter et al., 1981b). We separated pools A and B from extracellular Ca\(^{++}\) by perfusing hearts at 6°C after labeling with \( { }^{45}\text{Ca}^{++} \). Since the cold temperature inhibited cellular Ca\(^{++}\) efflux, the extracellular Ca\(^{++}\) could be washed away, leaving pools A and B in the heart. These were then released by reperfusing the hearts at 37°C. When we found that pool A required extracellular Ca\(^{++}\) for its release (Hunter et al., 1981b), we developed a more direct method for measuring it: labeled hearts were perfused at 23°C with solution containing EGTA (ethylene glycol bis (\(\beta\)-aminoethyl ether) \(N,N'\)-tetraacetic acid) to wash out extracellular Ca\(^{++}\) and pool B, and then pool A was released by restoring Ca\(^{++}\) to the perfusate (Hunter et al., 1982). By this method, we found that procaine had a strong inhibitory action on cellular Ca\(^{++}\) efflux (Hunter et al., 1982). It therefore became possible to check, using procaine, whether or not the cold washout technique had effectively inhibited the release of all cellular Ca\(^{++}\) pools. We report here the existence of a third cellular Ca\(^{++}\) pool (pool C) whose release is inhibited by procaine but which was not detectable by the cold washout technique because it was not retained by the heart during the
Cold perfusion. This pool appears to be of considerable significance: under physiological conditions it fills only when hearts are under β-adrenergic stimulation, and contractility is related to the sum of Ca++ in both pools A and C.

**Methods**

**Measurement of Cellular ⁴⁵Ca**

Hearts were excised from retired female breeder rats (Sprague-Dawley) and perfused through the aorta as described previously (Hunter et al., 1982). The basal perfusate contained 118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11 mM glucose, and 2.5 mM CaCl₂. It was gassed with 95% O₂ plus 5% CO₂ and warmed to 37°C. The pH was 7.4. After 5 minutes of perfusion, the heart was switched to one of the Ca ++ perfusates which were identical to the basal perfusate except they contained either 0.2, 1.0, 2.5, or 5.0 mM CaCl₂. A solution of 1 mM Ca ++ containing ⁴⁵Ca ++ (2 × 10⁶ cpm/ml) was delivered (20 ml/hour) by an infusion pump to the perfusate 4 cm above the heart. The heart was labeled for 3 minutes and the effluent perfusate collected. An aliquot of this was taken to measure the specific activity of the perfusate ⁴⁵Ca ++. Ventricular pressure was measured by a fluid-filled balloon (Hunter et al., 1982). Isoproterenol (−) (0.5 mM) was delivered to the perfusate (3 μl/min) by an infusion pump to give an average perfusate concentration of 0.3 μM. Ryanodine was a generous gift of Dr. E.F. Rogers (Merck, Sharp and Dohme).

Immediately after completion of the labeling period, the heart was switched to a perfusate identical to the basal perfusate except that Ca ++ was absent, and 0.5 mM EGTA and 20 mM sucrose were present (EGTA wash solution). The temperature of the wash solution was 23°C. Pool A ⁴⁵Ca ++ was released by switching the heart to the basal perfusate containing 2.5 mM Ca ++ (Hunter et al., 1982). For pool C measurement (see text), 10 mM procaine was present in the EGTA wash solution. Pool C ⁴⁵Ca ++ was released by switching the heart to the EGTA wash solution without procaine. The amount of ⁴⁵Ca ++ released each minute of washout was calculated as described previously (Hunter et al., 1982). When perfusion at 4°C was performed, an EGTA/MOPS wash solution was used. It was identical to the EGTA wash solution except 20 mM morpholinopropane sulfonic acid (adjusted to pH 7.4 with Tris) was present in place of 25 mM NaHCO₃.

**Labeling of Hearts with ⁴⁵Ca ++ with Zero Na**

After 5 minutes of perfusion with the basal medium at 37°C, the heart was switched to one of the following perfusates containing 0.2 mM ⁴⁵Ca ++: The "normal Na" perfusate (containing 125 mM NaCl), the "low Na" perfusate (containing 25 mM NaCl plus 100 mM choline Cl), the "zero Na" perfusate (containing 125 mM choline Cl) or the "zero Na", sucrose perfusate (containing 250 mM sucrose). In addition, each of the perfusates contained 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 11 mM glucose, 20 mM 4-morpholine propanesulfonic acid (adjusted to pH 7.4 with Tris), and 0.2 mM ⁴⁵Ca ++ (10⁶ cpm/ml). The perfusion was performed at 23°C for 2 minutes during which time the perfusate was gassed with 100% O₂. The labeling was stopped by perfusing the hearts with the EGTA wash solution containing 10 mM procaine, and pools A and C were measured as described above.

**Results**

**Measurement of Pool C**

To determine whether procaine protects against the loss of cellular ⁴⁵Ca ++ during the washout of the extracellular ⁴⁵Ca ++ in the cold, we performed the experiment shown in Figure 1. Two groups of isoproterenol-treated hearts were labeled for 3 minutes with 1 mM ⁴⁵Ca ++. The first group was perfused for 10 minutes at 4°C with the EGTA/MOPS wash solution not containing procaine (see Methods). Pool B was released by perfusing the hearts at 23°C with EGTA wash solution. Pool A then was released by perfusing the hearts with the basal medium containing 2.5 mM Ca ++. The size of the peaks was estimated by subtracting from each a baseline obtained by washout of ⁴⁵Ca ++-labeled hearts for 18 minutes at 4°C. Pool B contained 22 ± 4 nmol ⁴⁵Ca ++ /g wet weight of heart, and pool A contained 65 ± 13 nmol ⁴⁵Ca ++ /g. Immediately after labeling, the second group of hearts was perfused at 23°C for 15 seconds with the EGTA wash solution containing 10 mM procaine. They were then perfused at 4°C for 10 minutes with the EGTA/MOPS wash solution containing 10 mM procaine. The amount of ⁴⁵Ca ++ released from these hearts upon reperfusion at 23°C with the EGTA wash solution (without procaine) was 67 ± 4 nmol/g. That value is significantly different (P < 0.01) from the pool B ⁴⁵Ca ++ peak obtained from the group of hearts perfused in the cold without procaine. The amount of ⁴⁵Ca ++ in pool A in the procaine-perfused hearts was 76 ± 7 nmol/g, which is not significantly different (P > 0.1) from pool A in the hearts perfused without procaine. Procaine, therefore, appears to protect against a partial loss of cellular ⁴⁵Ca ++ during perfusion at 4°C.

The cellular ⁴⁵Ca ++ pool which was preserved by procaine was separated from pool B in the experiment shown in Figure 2. ⁴⁵Ca ++-labeled, isoproterenol-treated hearts were perfused with procaine at 4°C, as in the experiment of Figure 1. However, 10 mM procaine was also added to the EGTA wash solution for the initial 4 minutes of reperfusion at 23°C. Hearts then were perfused for an additional 4 minutes with the EGTA wash solution (without procaine), followed by a final perfusion with the basal medium. The results show that the procaine-sensitive increment of the "pool B" peak in Figure 1 was separated from pool B into a distinct peak (termed pool C). The separation was achieved because the release of pool B induced by rewarming to 23°C was not blocked by procaine, whereas the release of pool C remained procaine-sensitive at
Retention of cellular \(^{45}\)Ca\(^{++}\) during cold perfusion enhanced by procaine. Eight isoproterenol-treated hearts were labeled for 3 minutes with 1 nmol \(^{45}\)Ca\(^{++}\). Immediately after labeling, four of the hearts (O) were placed into ice cold EGTA/MOPS wash solution. The hearts were perfused for 10 minutes at 4°C with the EGTA/MOPS wash solution, and then at 23°C with the EGTA wash solution for four minutes, followed by a final 23°C perfusion with the basal medium containing 2.5 nmol Ca\(^{++}\). The other four hearts (●) were washed out in the same way as the first four, except for two changes: Immediately after labeling and before cooling, they were perfused for 15 seconds at 23°C with the EGTA wash solution containing 10 mM procaine; and 10 mM procaine was also present in the EGTA/MOPS wash solution.

23°C. The amount of \(^{45}\)Ca\(^{++}\) present in the three peaks was: pool B = 19 ± 3 nmol/g; pool C = 42 ± 6 nmol/g; and pool A = 72 ± 5 nmol/g.

When hearts not treated with isoproterenol were labeled with \(^{45}\)Ca\(^{++}\) and analyzed by the same washout procedure as used with the isoproterenol-treated hearts, a striking result was found (Fig. 2): Little if any \(^{45}\)Ca\(^{++}\) was found in pool C (3 ± 1 nmol/g), \(^{45}\)Ca\(^{++}\) in pool A (48 ± 6 nmol/g) was also significantly less (P < 0.01) than the amount of \(^{45}\)Ca\(^{++}\) present in isoproterenol-treated hearts. Pool B, however, was not significantly different (P > 0.1) at 22 ± 3 nmol/g.

When isoproterenol-treated hearts were labeled with 5 mM \(^{3}\)H-sucrose plus 1 mM \(^{45}\)Ca\(^{++}\), no peak of \(^{3}\)H-sucrose release was observed on removing procaine in experiments like that in Figure 2. This confirms the intracellular origin of pool C. The removal of procaine to induce pool C release did not cause any measurable contractile activity. Therefore, pool C release cannot result from an artifact associated with mechanical activity. The same is true for pool A: although release of pool A by 2.5 mM Ca\(^{++}\) is accompanied by contractile activity (Hunter et al., 1982), pool A can be released by a level of Ca\(^{++}\) (0.1 mM) too low for any measurable contractile activity (Hunter et al., 1981b). For the remaining experiments in this report, we are concerned only with pools A and C, since that is where the effect of isoproterenol is seen. Therefore, the procedure for measuring pools A and C was simplified in the following way: Extracellular \(^{45}\)Ca\(^{++}\) and pool B \(^{45}\)Ca\(^{++}\) were washed away simultaneously by perfusing the hearts at 23°C for 6 minutes with the EGTA wash solution containing 10 mM procaine. Then pool C was released by perfusion with the...
EGTA wash solution (without procaine) and pool A was released by perfusion with the basal medium. The elimination of the 4°C washout step only marginally altered the results: With 1 mM $^{45}$Ca$^{++}$ labeling, without the 4°C washout step, $^{45}$Ca$^{++}$ in pools A and C was 65 ± 6 and 51 ± 5 nmol/g for isoproterenol-treated hearts and 38 ± 6 and 2 ± 2 nmol/g for untreated hearts. When these four values are compared with the measurements obtained using the cold perfusion step (Fig. 2), the only difference of marginal significance (0.1 > P > 0.05) was in the size of pool A in untreated hearts.

Effect of Extracellular [Ca$^{++}$] on Pools A and C

As the extracellular [Ca$^{++}$] changes, there is a change in the size of both pools A and C. The data defining this relationship for isoproterenol-treated and untreated hearts are presented in Figure 3. Without isoproterenol treatment, little if any Ca$^{++}$ was accumulated in pool C. At the high level of 5 mM extracellular Ca$^{++}$, only 11 ± 3 nmol $^{45}$Ca$^{++}$/g was found in pool C, whereas pool A contained 67 ± 20 nmol $^{45}$Ca$^{++}$/g. In contrast, $^{45}$Ca$^{++}$ entered pool C of isoproterenol-treated hearts even when extracellular Ca$^{++}$ was 0.2 mM: pool C = 19 ± 4 nmol $^{45}$Ca$^{++}$/g and pool A = 50 ± 6 nmol $^{45}$Ca$^{++}$/g.

Contractility vs. Pools A and C

In Figure 4, the rate of ventricular pressure development (dP/dt), as measured with a ventricular balloon, is plotted against the size of pool A and against the sum of the sizes of pool A and pool C. Data were taken from isoproterenol-treated and untreated hearts in which the extracellular [Ca$^{++}$] was varied (see Fig. 3). When the amount of Ca$^{++}$ in pools A and C increased in response to increased extracellular [Ca$^{++}$] or to the addition of isoproterenol, dP/dt increased.

In addition to enhancing contractility, isoproterenol also increased heart rate from 192 ± 16 to 244 ± 21 beats/min and decreased the coronary flow of 10 ± 1 ml/min by 14 ± 6%. To test whether such changes in heart rate and coronary flow themselves produce an elevation in cellular Ca$^{++}$, the following experiment was done. Four rat hearts were stimulated to beat at 265 beats/min by externally applying 10-V, 10-msec pulses. As a result of stimulation, the coronary flow decreased by 17 ± 2%. During stimulation, the hearts were labeled for 3 minutes with $^{45}$Ca$^{++}$ added to the basal perfusate containing 1 mM Ca$^{++}$, and pools A and C were measured and found to be 48 ± 9 and 11 ± 2 nmol/g. That amount of pool A was not significantly different ($P > 0.1$) from pool A (38 ± 6 nmol/g) found in unstimulated hearts, but was significantly different ($0.01 < P < 0.05$) from pool A (50 ± 6 nmol/g) found in unstimulated hearts, but was significantly different ($0.01 < P < 0.05$).
0.02) from pool A (65 ± 6 nmol/g) found in isoproterenol-treated hearts. The increase of pool C caused by stimulation from 2 ± 2 to 11 ± 2 nmol/g was significant (P < 0.01), but was just 18% of the increase in the size of pool C produced by isoproterenol.

Release of Pool C by Caffeine

We have previously shown that caffeine (with no Ca++ addition) triggers the release of pool A, and that procaine inhibits the release triggered by caffeine (Hunter et al., 1982). In accordance with this, Figure 5 shows that only 3 ± 4 nmol/g of pool A 45Ca++ was released by 10 mM caffeine when 10 mM procaine was present. The hearts had been labeled for 3 minutes with 1 mM 45Ca++, so the size of pool A was 38 ± 6 nmol/g (see Fig. 3). If, however, isoproterenol-treated hearts were washed with the EGTA wash solution containing 10 mM procaine, addition of 10 mM caffeine caused the rapid release of 65 ± 7 nmol Ca++/g. That value is significantly different (P < 0.01) from the 3 ± 4 nmol 45Ca++ released from untreated hearts. The remaining 45Ca++ in both groups of hearts was then released by the basal perfusate containing 2.5 mM Ca++ and no procaine. The additional 45Ca++ released was 33 ± 7 nmol/g from the untreated hearts and 47 ± 9 nmol/g from the isoproterenol-treated hearts. The 45Ca++ remaining in the hearts after caffeine treatment was not releasable merely by removing procaine but required the addition of Ca++ (data not shown). This confirms that the 45Ca++ released by caffeine (plus procaine) from the isoproterenol-treated hearts came from pool C and not pool A.

Pool C Ca++ Uptake Induced by Removal of Na+

Substitution of choline Cl for NaCl in the basal perfusate resulted in an immediate loss of beating and an 80% loss of coronary flow. Since a poor coronary flow made temperature control of the perfused heart difficult, the experiments presented in this section all were done at room temperature. The perfusate concentration of Ca++ was 0.2 mM, and Ca++ was premixed into the perfusate, to avoid error in estimating perfusate specific activities introduced by changes in flow rate. A standard 2-minute labeling perfusion was used, at which time pools C and A were measured as described in Methods. The results are summarized in Figure 6. When NaCl in

![Figure 5](http://circres.ahajournals.org/...)

**Figure 5.** Release of pool C by caffeine. Four isoproterenol-treated hearts (●) and four untreated hearts (○) were labeled for 3 minutes with 45Ca++ added to the basal perfusate containing 1 mM Ca++. After a 6-minute washout period with the EGTA wash solution containing 10 mM procaine, the hearts were switched to the EGTA wash solution containing 10 mM procaine plus 10 mM caffeine. At 10 minutes, the hearts were then switched to the basal perfusate containing 2.5 mM Ca++.

![Figure 6](http://circres.ahajournals.org/...)

**Figure 6.** Enhancement of cellular 45Ca++ uptake by removal of extracellular Na+. The composition of the different perfusates and the labeling procedure with the low Na+ perfusates are described in Methods. Each group consisted of four hearts, and pool A (unshaded bars) and pool C (shaded bars) were measured as described in the legend to Figure 3. Ethanol (Etoh), ethanol containing 2 mM rotenone (rot), and ethanol containing 2 mM oligomycin (olig) were infused into the basal perfusate 2 cm above the hearts at 0.03 ml/min for a 2-minute period immediately before switching to the zero Na labeling perfusate.
the perfusate was 125 mM, 9 ± 2 nmol 45Ca++/g was found in pool A, and no measurable 45Ca++ was found in pool C. For an additional control, the effect of coronary flow on 45Ca++ uptake by hearts perfused with normal medium containing 125 mM NaCl was investigated by cross-clamping the perfusate delivery tube. When the coronary flow was decreased by 85% to 1.5 ml/min, uptake of 45Ca++ by pools A and C was not significantly changed (pool A = 8 ± 3 nmol/g) (P > 0.1). Substitution of 80% of the 125 mM NaCl in the perfusate with choline Cl resulted in a small increase of pool A from 9 ± 2 to 13 ± 3 nmol 45Ca++/g. (The difference is marginally significant at 0.05 < P < 0.1.) No uptake into pool C occurred. When 100% of the NaCl was replaced by choline Cl, pool A significantly increased (P < 0.01) to 26 ± 6 nmol 45Ca++/g, and 10 ± 6 nmol 45Ca++/g entered pool C. Upon perfusion with zero Na+, a large increase in cellular 45Ca++ resulted from blocking mitochondrial ATP synthesis with either rotenone [a respiratory inhibitor (Wainio, 1970)], or oligomycin [an inhibitor of oxidative phosphorylation (Lardy et al., 1958)]. The increase was mainly into pool C. 45Ca++ in pool C = 40 ± 6 nmol/g in rotenone-poisoned hearts and 28 ± 6 nmol/g in oligomycin-poisoned hearts. [Both values are significantly different (P < 0.01) from the value of 10 ± 6 nmol 45Ca++/g for pool C in unpoisoned hearts.] A similar selective increase of 45Ca++ into pool C occurred when the pH of the zero Na+ perfusate was elevated from 7.4 to 8. The effect of pH was more pronounced if sucrose was used in place of choline Cl to replace NaCl. When a zero Na+, sucrose (pH 8) perfusate was used, 41 ± 6 nmol 45Ca++/g accumulated in pool C. In Figure 7, the pool A and pool C 45Ca++ measurements obtained from each heart were measured during this series of experiments with Na+ removal are plotted independently, with the size of pool A and pool C on one axis and the sum of the sizes of pools A and C on the other. The results show that, under these conditions of low Na+, pool A saturated when the cell had around 30 nmol 45Ca++/g, and that pool C began to take up Ca++ only after pool A had neared saturation. We do not know why the maximum fill level for pool A induced by Na+ removal was lower than the maximum level of pool A found during normal Na+ perfusion (see Fig. 3).

Modulation of Cellular Ca++ by Ryanodine

Ryanodine is an alkaloid that is believed to be a selective inhibitor of skeletal and cardiac muscle SR Ca++ uptake (Hajdu, 1969; Fairhurst and Hasselbach, 1970; Naylor et al., 1970; Frank and Sleator, 1975a; Sutko and Willerson, 1980). When hearts from different species were tested, the rat heart was found to be most susceptible to ryanodine toxicity (Ciofalo, 1973; Sutko and Willerson, 1980). We have also found that ryanodine is a potent inhibitor of the perfused rat heart. Infusion of a 0.3 mM solution of ryanodine into the perfusate at 7 μl/ml (average perfusate [ryanodine] = 0.2 μM) induced a 50% loss of dP/dt by 3.4 ± 0.4 min (n = 4). This is in good agreement with the potency of ryanodine on rat hearts reported by others (Ciofalo, 1973; Sutko and Willerson, 1980). The effect of ryanodine on pool A was measured as follows: One minute after beginning the infusion with 0.2 μM ryanodine, 45Ca++ was also infused into the basal perfusate containing 2.5 mM Ca++. After 3 minutes of labeling with 45Ca++ plus ryanodine, pool A was measured as described previously (Hunter et al., 1982). In four ryanodine-treated hearts, there was just 7 ± 6 nmol 45Ca++/g in pool A, whereas pool A of five untreated control hearts contained 56 ± 6 nmol 45Ca++/g. The difference is significant (P < 0.01). By infusing isoproterenol in addition to ryanodine into the perfusate, the effect of ryanodine on both pools A and C was also investigated (Fig. 8). With 1 mM Ca++ in the labeling perfusate, pools A and C in four isoproterenol-treated control hearts contained 65 ± 7 and 56 ± 10 nmol 45Ca++/g, respectively. In four ryanodine-plus-isoproterenol-treated hearts, pools A and C contained just 8 ± 4 and 7 ± 10 nmol 45Ca++/g. Both the difference in pool A (± ryanodine) and the difference in pool C (± ryanodine) were significant (P < 0.01).

These very low measured values for pools A and C in ryanodine-treated hearts may be an underestimate of the actual steady state values of pools A and C. It is possible that ryanodine potentiated the leakage of pools A and C 45Ca++ from the cell during
the 6-minute washout of the extracellular $^{45}\text{Ca}^{++}$. In other words, ryanodine-induced loss of pools A and C $^{45}\text{Ca}^{++}$ may have occurred even when the normal mechanisms of release of pools A and C were blocked by including EGTA and procaine in the washout perfusate. To investigate this possibility, extracellular $^{45}\text{Ca}^{++}$ was washed out at 4°C to block ryanodine-induced loss of cellular $^{45}\text{Ca}^{++}$. In the experiment shown in Figure 9, one group of four rat hearts pretreated for 2 minutes with 0.2 $\mu\text{M}$ ryanodine in the perfusate was compared with a second group of untreated control hearts. Both groups of hearts were labeled for 2 minutes with the basal perfusate containing 0.2 $\mu\text{M}$ $^{45}\text{Ca}^{++}$ plus 0.3 $\mu\text{M}$ isoproterenol. Pool C contained 73 ± 4 nmol $^{45}\text{Ca}^{++}$/g and pools A plus C contained 20 ± 3 nmol $^{45}\text{Ca}^{++}$/g. Both the difference in pool B (± ryanodine) and the difference in pools A plus C (± ryanodine) were significant ($P < 0.01$).

**Selectivity of Pools A and C for Ca$^{++}$ over Ba$^{++}$**

Figure 10 shows the results of an experiment in which $^{45}\text{Ca}^{++}$ and $^{133}\text{Ba}^{++}$ uptake by pools A and C was measured. Eight hearts were perfused with the basal perfusate containing 1 mM Ca$^{++}$, 0.1 mM Ba$^{++}$, and 0.3 $\mu\text{M}$ isoproterenol. This ratio of Ca$^{++}$ to Ba$^{++}$ was chosen in order to maintain normal cardiac function. Four of the hearts were labeled for 3 minutes with $^{45}\text{Ca}^{++}$ and the other 4 hearts were labeled for 3 minutes with $^{133}\text{Ba}^{++}$. Pool C contained 73 ± 4 nmol $^{45}\text{Ca}^{++}$/g and 0.6 ± 0.7 nmol $^{133}\text{Ba}^{++}$/g. Pool A contained 81 ± 10 nmol $^{45}\text{Ca}^{++}$/g and 2.2 ± 0.3 nmol $^{133}\text{Ba}^{++}$/g. Expressed in another way, pool C selected for Ca$^{++}$ over Ba$^{++}$ by 12 to 1, and pool A selected for Ca$^{++}$ over Ba$^{++}$ by 4 to 1.
FIGURE 10. Comparison of $^{45}$Ca and $^{133}$Ba uptake by pools A and C. For 2 minutes, eight hearts were perfused with the basal perfusate containing 1 mM Ca++, 0.1 mM Ba++, and 0.3 µM isoproterenol. Four of the hearts were labeled with $^{45}$Ca for 3 minutes, and the other four were labeled with $^{133}$Ba for 3 minutes. Pools A and C were assayed as described in the legend to Figure 8. Release of $^{133}$Ba from the heart was measured in the same way as $^{45}$Ca release (see Methods).

Discussion

Even though pool C is released in the absence of extracellular Ca++, it is possible that its release is still mediated by a mechanism of Ca++-induced Ca++ release. This is suggested by recent studies on SR isolated from skeletal muscle (Yamamoto and Kasai, 1982a, 1982b). These authors have explained Ca++-induced Ca++ release in terms of the opening of a Ca++-gated cation channel. The affinity of the recognition site for Ca++ was greatly enhanced by caffeine and strongly inhibited by procaine. We have previously shown that caffeine triggers pool A release and that procaine inhibits its release (Hunter et al., 1982). In the present work, we have shown that procaine blocks the spontaneous release of pool C which occurs in the absence of extracellular Ca++ and that caffeine reverses the blockage (Fig. 5). However, under the same conditions, caffeine could not relieve the procaine inhibition of pool A release. Therefore, the triggering of pool C release could simply require a lower level of cellular free Ca++ than the triggering of pool A. The release of pool C in the absence of extracellular Ca++ would then require that sufficient cellular free Ca++ remained to trigger its release but not the release of pool A.

Interestingly, procaine-sensitive pool C release also took place at 4°C as well as 23°C (Fig. 1). It is possible that much of the loss at 4°C occurred within 1 minute after cooling the hearts, before beating was completely arrested. Therefore, for the experiment of Figure 1, in order to assure maximum inhibition of pool C release by procaine, beating was blocked by pre-perfusing the hearts for 15 seconds with procaine before cooling.

Pools A and C are probably located in the SR, as the Ca++-induced Ca++ release mechanism is contained there (Endo, 1977; Fabiato and Fabiato, 1977, 1979; Stephenson, 1981; Yamamoto and Kasai, 1982a). The data in Figure 2 show that pool A stores cellular Ca++ under normal conditions and that pool C may function as a backup reservoir for Ca++, called into play during catecholamine stimulation. However, Ca++ can also be forced into pool C in the absence of catecholamine stimulation, if enhanced Ca++ uptake is induced by removal of extracellular Na+ (Figs. 6 and 7). Since pool A accumulation of Ca++ takes preference over pool C, it is possible that the affinity of the uptake system for Ca++ is higher for pool A than for pool C. Alternatively, pool C may not take up Ca++ at all, but may receive Ca++ directly from pool A whenever Ca++ in pool A reaches some critical level. Finally, pools A and C could be a single pool, with the affinity of the cytosolic Ca++-induced Ca++ release site increasing as the pool Ca++ content increased. Pool C release in the absence of extracellular Ca++ would then be explained by a partial release of Ca++ from this pool. A graded rather than an all-or-nothing mechanism of Ca++-induced Ca++ release from SR of skinned rat heart fibers has been observed (Fabiato and Fabiato, 1979).

The size of dP/dt as shown in Figure 4 is correlated with the size of pools A and C. This correlation suggests that contractility may be in part regulated by the Ca++ levels of pools A and C. A study with skinned cardiac muscle fibers has shown that—all of the species tested—the rat has the greatest capacity for contraction dependent on Ca++-induced Ca++ release from SR (Fabiato and Fabiato, 1978). It could therefore be that the correlation between contractility and the level of Ca++ in pools A plus C will be less in hearts from other species. There appears to be a critical minimum amount of pool A Ca++ required for contractility, of about 20 nmol/g. This is the level of pool A found also in caffeine-poisoned rat hearts (Hunter et al., 1982). A saturation level of cellular Ca++ for contractility is further seen to be reached when the Ca++ in pools A and C was about 120 nmol/g. This is considerably larger than the 70 nmol/g of Ca++ needed in the cytoplasm to stimulate the myofibrils maximally (Solaro and Briggs, 1974). This suggests that not all the Ca++ in pools A and C is available for contraction at once. Furthermore, with isoproterenol-treated hearts and 2.5 mM extracellular Ca+++, the saturation level of 120 nmol/g Ca++ was exceeded by about 80 nmol/g. At that
level of extracellular Ca++, catecholamine stimulation has been shown to induce the phenomenon of oxygen wastage, believed to result from excessive cellular Ca++ cycling (Opie et al., 1979). A correlation between contractility of cardiac muscle preparations and Ca++ binding to the sarcosome isolated from the muscle has also been reported (Bers et al., 1981). This suggests that the amount of extracellular Ca++ available for influx per beat is also critically involved in the regulation of contractility. Significantly, isoproterenol—in addition to increasing the Ca++ in pools A and C—also increases the rate of extracellular Ca++ influx (Reuter and Scholz, 1977; Hunter et al., 1981a).

The use of extracellular Na+ removal to promote Ca++ influx (Figs. 6 and 7) has been demonstrated by other laboratories (Niedergerke, 1963; Glictsch et al., 1970; Wendl and Langer, 1977; Busselen and Van Kerkhove, 1978; Barry and Smith, 1982). Extracellular Na+ removal shifts the equilibrium of the reversible Na+/Ca++ exchanger to favor Ca++ influx over Ca++ efflux. The blockade of mitochondrial ATP synthesis resulted in enhanced Ca++ uptake following removal of extracellular Na+ (Fig. 6), possibly because the ATP-dependent sarcolemma Ca++ pump (Caroni and Carafoli, 1980) was partially inhibited by a decrease in the availability of ATP. Alternatively, the mitochondrial poisons may have prompted Ca++ influx by increasing the intracellular Na+ content by decreasing the amount of ATP available to the Na+/K+ ATPase. Barry and Smith (1982) have shown that inhibition of the Na+/K+ ATPase of cultured heart cells by ouabain increased tremendously the amount of radioactive Ca++ uptake induced by extracellular Na+ removal.

Previously, two other reports have found that catecholamines stimulate the rate of Ca++ uptake into heart tissue (Grossman and Furchgott, 1964; Meinertz et al., 1973). However, neither study found that catecholamines increased the extent of cellular Ca++ uptake. The present report has shown that isoproterenol increases the amount of cellular Ca++ in the beating rat heart by a factor of 2, and that most of the increased uptake is located in a pool of Ca++ (pool C) which, under physiological conditions, would be operative only during catecholamine stimulation. The study on frequency of stimulation suggests that frequency may also play a role in determining the amount of Ca++ in pool C.

Ryanodine has been shown here to decrease by as much as 85% the amount of Ca++ in pools A and C of the rat heart (Fig. 8). However, not all the Ca++ removed from pools A and C is lost from the cell. The experiment described in Figure 9 shows that as much as 29 of 45 nmol Ca++/g lost from pools A and C was retained in pool B. This suggests that Ca++ leaking from pools A and C passes through pool B and its way out of the cell. It is possible, however, that the measured size of pool B is not representative of the size of a pool spatially separate from the location of pools A and C, even without ryanodine: most pool B Ca++ could be located in the same place as A and C until the heart is reperfused with warm medium, at which time it leaks into the cytoplasm and is eventually extruded from the cell. Ryanodine would then act simply to increase the leak rate, putting into pool B what would otherwise be measured as pools A and C.

Our conclusion that ryanodine increases the leak rate of Ca++ from pools A and C supports further the postulate that pools A and C are the SR. That the SR is primarily affected by ryanodine is supported by studies in both cardiac (Nayler et al., 1970; Frank and Sleater, 1975a; Sutko and Willerson, 1980; Hilgemann et al., 1982) and skeletal muscle (Hajdu, 1969; Fairhurst and Hasselbach, 1970). However, there is controversy over how ryanodine alters Ca++ movements in the SR. A recent study with isolated SR vesicles from canine heart showed that high levels of ryanodine (0.3 nm) dramatically increased the rate of Ca++ uptake (Jones and Cala, 1981). This has lead to a proposal that ryanodine primarily acts in vivo as a blocker of SR Ca++ release (Sutko et al., 1979; Sutko and Willerson, 1980). Our work does not support this proposal but, instead, is in agreement with the more conventional view that ryanodine increases the leak of Ca++ from SR. This view is based on many studies, including the following. (1) Ryanodine (10 μM) strongly accelerated the beat-independent loss of cellular 45Ca++ from rat skeletal muscle and blocked the muscle’s ability to contract in the absence of extracellular Ca++ (Hajdu, 1969). (2) Ryanodine (1 nM) greatly accelerated the rest-induced decay of developed force in rat ventricle (Sutko and Willerson, 1980). The rest-induced decay relationship is believed to reflect the rate of loss of the SR Ca++ during rest (Allen et al., 1976). (3) Ryanodine (10-50 μM) increased by 30% the rate of 45Ca++ release from guinea pig atria (Frank and Sleator, 1975b) and canine papillary muscle (Naylor et al., 1970). (4) Ryanodine (10-100 μM) inhibited by 30% Ca++ uptake and stimulated by 30-200% Ca++ ATPase by SR vesicles isolated from cardiac (Naylor et al., 1970; Frank and Sleator, 1975b) and skeletal muscle (Fairhurst and Hasselbach, 1970; Fairhurst, 1973). The observed selectivity of pools A and C for Ca++ over Ba++ (Fig. 10) is further evidence that these pools are located in the SR. The affinity of the uptake system of isolated SR for Ca++ is much greater than for Ba++ (Vanderkooi and Martonosi, 1971; Batra and Daniel, 1971; Hurwitz et al., 1975).

This work was supported by a grant from the Surgical Associates of the University of Wisconsin Hospitals; also by a Grant-in-Aid from the American Heart Association and with funds contributed in part by the Wisconsin Affiliate. Address for reprints: Douglas R. Hunter, Department of Surgery, Clinical Science Center, 600 Highland Avenue, Madison, Wisconsin 53792.

Received November 1, 1982; accepted for publication September 20, 1983.
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INDEX TERMS: Calcium • Rat heart • Ryanodine • Contractility • Sarcoplasmic reticulum • Barium • Caffeine • Procaine

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Circulation Research/Vol. 53, No. 5, November 1983
Modulation of cellular calcium stores in the perfused rat heart by isoproterenol and ryanodine.

D R Hunter, R A Haworth and H A Berkoff

doi: 10.1161/01.RES.53.5.703

_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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