Comparison of Ca$^{2+}$, Sr$^{2+}$, and Mn$^{2+}$ Fluxes in Mitochondria of the Perfused Rat Heart

DOUGLAS R. HUNTER, HIROCHIKA KOMAI, ROBERT A. HAWORTH, MARK D. JACKSON, AND HERBERT A. BERKOFF

SUMMARY The amount of readily exchangeable Ca$^{2+}$ in mitochondria of an isolated working rat heart is less than 10 ng-ions/g heart. We therefore conclude that either no Ca$^{2+}$ enters mitochondria or that the Ca$^{2+}$ which does enter is removed continuously. Using Sr$^{2+}$ and Mn$^{2+}$, we obtained evidence that the mitochondrial Na$^+-$Ca$^{2+}$ exchanger was indeed operational in releasing metal from mitochondria of the heart. When Ca$^{2+}$ in the perfusate was replaced by Sr$^{2+}$, we found that a significant amount of Sr$^{2+}$ (approximately 100 ng-ions/g heart) entered mitochondria. When the heart then was returned to a Ca$^{2+}$-containing perfusate, over 80% of the Sr$^{2+}$ was washed out of mitochondria within 30 seconds. When low levels of Mn$^{2+}$ were added to the perfusate, we found that Mn$^{2+}$ accumulated in mitochondria irreversibly. This is evidence for the operation of the Na$^+$-Ca$^{2+}$ exchanger because Na$^+$ was found to release Ca$^{2+}$ and Sr$^{2+}$ but not Mn$^{2+}$ from isolated rat heart mitochondria. Our estimates indicate that when the Na$^+-$Ca$^{2+}$ exchanger is maximally operative, as in the Sr$^{2+}$-perfused heart, the flux of Sr$^{2+}$ through mitochondria is at most 10% of the total flux needed for the activation of contraction. The low level of Ca$^{2+}$ in the mitochondria of Ca$^{2+}$-perfused hearts suggests a much smaller flux of Ca$^{2+}$ through the mitochondria in this case. We therefore conclude that mitochondria play little if any role in the beat-to-beat regulation of normal Ca$^{2+}$ fluxes in the rat heart. Circ Res 47: 721–727, 1980

THE Ca$^{2+}$-sequestering activity of sarcoplasmic reticulum (SR) in heart is required for muscle relaxation (Harigaya and Schwartz, 1969; Kitazawa, 1976). The extent of the involvement of mitochondrial Ca$^{2+}$-uptake activity in heart is, however, unknown. The affinity of the mitochondrial Ca$^{2+}$ pump is too low to remove Ca$^{2+}$ effectively from troponin (Harigaya and Schwartz, 1969; Kitazawa, 1976). In fact, the observation that the kinetics of the mitochondrial Ca$^{2+}$-uptake system is sigmoidal (Vinogradov and Scarpa, 1972) suggests that mitochondria are designed not to compete with SR for removal of Ca$^{2+}$ from troponin. Thus, Ca$^{2+}$ uptake by mitochondria probably occurs only when intracellular Ca$^{2+}$ is sufficiently high to saturate the SR uptake system.

This conclusion is consistent with the observations made on skinned heart muscle fibers (Fabiato and Fabiato, 1975). They spontaneously twitch in the presence of an uncoupler when the concentration of free Ca$^{2+}$ is 1 μM or less. If, however, the level of free Ca$^{2+}$ is raised, relaxation then is inhibited by uncoupler. These results suggest that uncoupler-sensitive mitochondrial Ca$^{2+}$ uptake is required for relaxation of heart muscle when the free Ca$^{2+}$ concentration is >1 μM. In isolated fetal rat heart cells, intracellular Ca$^{2+}$ presumably does not have to reach these levels to support contraction, since such cells spontaneously beat in the presence of oligomycin plus uncoupler (Harary and Slater, 1965).

In the working heart, the question of whether mitochondria are involved in Ca$^{2+}$ fluxes under normal conditions has not been adequately answered. Accumulation of Ca$^{2+}$ by mitochondria under abnormal Ca$^{2+}$-over-loading conditions has been demonstrated conclusively (Shen and Jencks, 1972; Holland and Olson, 1975; Henry et al., 1977). However, reports in the literature dealing with Ca$^{2+}$ fluxes under normal conditions are conflicting. Unlike isolated fetal heart cells or skinned muscle fibers, the maintenance of contractile activity of the working heart is dependent on uncoupler-sensitive oxidative phosphorylation. Therefore, the observed potent inhibition of myocardial activity by uncoupler (Dhalla et al., 1975) cannot be taken as evidence for the involvement of mitochondrial Ca$^{2+}$ uptake. To study this problem, a direct approach of measuring the rate of labeling of the mitochondrial Ca$^{2+}$ pool by perfusate ⁴⁵Ca$^{2+}$ has been attempted. Previously this approach has given mixed results: both major (Dhalla et al., 1970; Horn et al., 1971; Patriarca and Carafoli, 1968) and minor (Harris, 1977) roles for mitochondrial Ca$^{2+}$ uptake have been inferred.

The methodology for measuring mitochondrial Ca$^{2+}$ has been subject to the criticism that artificial accumulation of Ca$^{2+}$ by mitochondria occurs after homogenization of the heart (Kitazawa, 1976). A further problem is that the method is dependent on the isolation of mitochondria. This process is
both time consuming—thereby increasing the likelihood of Ca\(^{2+}\) rearrangement, and selective—it isolates only subsarcolemmal and not interfibrillar mitochondria (Palmer et al., 1977). In this report we present a modified method in which the Ca\(^{2+}\) content of mitochondria in the whole unfractonated heart homogenate is assayed. This new method exploits the observation that Na\(^+\) releases Ca\(^{2+}\) from mitochondria (Crompton et al., 1976), allowing us specifically to assay mitochondrial Ca\(^{2+}\) in the homogenate.

**Methods**

**Isolated Rat Heart Perfusion**

Rats were anesthetized by intraperitoneal injection of thiamylal, sodium (about 100 mg/kg). The heart was excised and cannulas (polyethylene tubing, i.d. 1.40 mm) were placed in the aorta and pulmonary vein. The basal perfusate was Krebs-Henseleit (KH) bicarbonate (Krebs and Henseleit, 1932) containing 11 mM glucose, 2.5 mM Ca\(^{2+}\), and equilibrated with 95% O\(_2\) plus 5% CO\(_2\). The perfusate temperature was 37°C. A retrograde (Langendorff-type) perfusion was carried out for the first 5-10 minutes by perfusing through the aorta with the height of the reservoir 80 cm above the heart. The working heart perfusion mode was carried out according to the method of Neeley et al. (1967). The heart rate under the control condition was typically about 220 beats/min.

**Labeling of Heart**

After 5 minutes of perfusion in the working heart mode, the heart was switched to a KH glucose perfusate containing 70,000 cpn/ml perfusate of either \(^{48}\)Ca\(^{2+}\) (2.5 mM), \(^{85}\)Sr\(^{2+}\) (4.8 mM) plus 0.2 mM Ca\(^{2+}\), or \(^{54}\)Mn\(^{2+}\) (5 \(\mu\)M) plus 2.5 mM Ca\(^{2+}\). The length of time each working heart was perfused with label is given in the text. In label washout experiments, the heart was switched back to the basal perfusate.

**Homogenization of Heart**

The homogenization buffer (which was kept at 0°C) was 250 mM in sucrose, 5 mM in EGTA (K salt), and 10 mM in Tris (adjusted to pH 7.8 with HCl). Unless otherwise stated, 10 ml of this buffer were injected into the aortic cannula immediately after termination of perfusion. The heart was trimmed with scissors, leaving the ventricular area, which was cut in half and washed in homogenization buffer. It then was weighed, cut into about 10 pieces with a razor blade, and mechanically homogenized (2000 rpm) in the cold for 30 seconds with 4 ml of homogenization buffer in a 10-ml glass homogenizer tube (clearance, 0.007 in.). The total duration of the procedure was approximately 2 minutes.

**Selective Metal Release Assay**

A labeled heart homogenate (5 ml total volume) was added to a flask containing 15 ml of a medium which contained sucrose, 250 mm; 4-morpholine propanesulfonate (MOPS), 20 mM (adjusted to pH 7.1 with Tris); Pi (K\(^+\)), 2 mM; MgCl\(_2\), 2 mM; ATP (K\(^+\)), 2 mM; succinate (K\(^+\)), 5 mM; KCl, 40 mM; and EGTA (K\(^+\)) 5 mM, with bovine serum albumin (BSA), 0.7 mg/ml present. Rotenone (2.5 \(\mu\)M) was added to the flask and a sample was removed immediately and centrifuged to obtain the time zero supernatant. The remaining assay mixture was divided into three flasks; NaCl (12.5 mM) was added to one flask, A23187* (5 \(\mu\)M) to a second. The flasks were shaken continuously in a 30°C water bath and, after 10 minutes of incubation, samples were removed and centrifuged at 13,000 \(g\) for 2 minutes in a Brinkman microcentrifuge. Essentially all intact mitochondria were pelleted as judged by the absence of measurable pyruvate-malate oxidase activity in the supernatant. The amount of metal in the supernatants was determined by scintillation counting. The difference between the amount of metal in the supernatant from the 10-minute incubated homogenate and the time zero supernatant is the metal released spontaneously. The difference between the amount of metal in the Na\(^+\)-containing supernatant and the supernatant from the 10-minute incubated homogenate is the metal released by Na\(^+\). The metal not released by Na\(^+\) is equal to the difference between the A23187-containing supernatant and the Na\(^+\)-containing supernatant. These differences in many experiments were small in comparison to the amount of metal in the time zero supernatant, which contained between 70 and 90% of the metal in the A23187-containing supernatant. We therefore routinely counted each supernatant for 30 minutes.

**Fractionation of the Heart Homogenate**

A rat heart homogenate was diluted to 30 ml with homogenization buffer and centrifuged at 500 \(g\) for 10 minutes at 0°C. The supernatant was carefully poured off leaving the cell debris pellet. The supernatant then was centrifuged at 9000 \(g\) for 10 minutes at 0°C to obtain the mitochondrial pellet. The pellets were suspended with light homogenization in the homogenization buffer, and the selective metal release assay was performed. Part of the resuspended pellets was used for measurement of protein and cytochrome oxidase activity.

* A23187 is a divalent metal ionophore which has been shown to readily release essentially the complete mitochondrial divalent metal content (Reed and Lardy, 1972; Hunter and Haworth, 1979).
Standard Assays and Material

For cytochrome oxidase, the reaction mixture, kept at 30°C, was sucrose, 250 mM; MOPS, 20 mM (adjusted to pH 7.1 with Tris), tetramethyl-p-phenylene diamine, 0.2 mM; ascorbate, 20 mM; lysolecithin 3 mM; and cytochrome c 0.05 mM. A Beckman oxygen analyzer was used to measure oxygen consumption. Protein was determined by the biuret method. BSA, fraction V from Miles Laboratories, was dialyzed exhaustively at 0°C with a solution containing Tris-Cl, 10 mM (pH 7.8). Rat heart mitochondria were isolated as described above in the "Fractionation of the Heart Homogenate." ⁴⁶Ca²⁺, ⁸⁵Sr²⁺, and ⁵⁴Mn²⁺ were purchased from New England Nuclear. A23187 was a gift from Eli Lilly and Nuclear. A23187 was a gift from Eli Lilly.

Statistical Methods

The mean and standard deviation (sd) of measurements are reported. Significance of the difference between groups was determined by the unpaired Student's t-test.

Results

Assay of Mitochondrial Ca²⁺ in the Heart Homogenate

The amount of Ca²⁺ selectively released from the particulate material of the homogenate by Na⁺ is taken to be the mitochondrial Ca²⁺ content. The validity of this selective metal release assay (see Methods for details) is dependent on certain conditions. (1) Spontaneous release of Ca²⁺ from mitochondria (no Na⁺ addition) must be inhibited. This is accomplished by including inhibitors of the Ca²⁺-induced transition in the assay mixture. In a previous work (Hunter and Haworth, 1979), we demonstrated that, in the absence of Na⁺, the Ca²⁺-induced transition was responsible for spontaneous release of Ca²⁺ from mitochondria. (2) EGTA (5 mM) is included in the reaction mixture to eliminate all non-stored bound Ca²⁺ from the particulate material. Therefore any Ca²⁺ bound to membranes is not a source of error. In addition, EGTA prevents any reaccumulation of Ca²⁺ by mitochondria or SR, so we measure only release and not exchange or uptake. (3) The amount of mitochondrial Ca²⁺ must be small enough (<20 ng-ions/mg mitochondrial protein) to be released by Na⁺ within our standard 10-minute incubation period. If this condition is not met, it will be apparent from the fact that the total amount of stored Ca²⁺ in the homogenate will exceed the amount released by Na⁺. (4) There must be no significant non-mitochondrial Ca²⁺ stores in the homogenate which can also be released by Na⁺. That this is so has been tested directly using differential centrifugation and Sr²⁺. Sr²⁺-perfused rat hearts (see later for a full description of these experiments) accumulated significant amounts of Na⁺- releasable Sr²⁺ and therefore served as a good test of the assay. A similar test using Ca²⁺ itself was found to be impractical because of the barely detectable level of Na⁺- releasable Ca²⁺ (see later). The amount of protein, cytochrome oxidase activity, and Na⁺- releasable Sr²⁺ was measured in the cell debris and mitochondrial pellets (see Methods). Within these two pellets, we found that 19 ± 4% of the protein, 48 ± 2% of the cytochrome oxidase activity, and 43 ± 4% of the Na⁺- releasable Sr²⁺ (n = 7 experiments) was in the mitochondrial pellet. The specific activity of the mitochondrial pellet was therefore 3.95 times higher for cytochrome oxidase and 3.22 times higher for Na⁺- releasable Sr²⁺ than in the cell debris pellet. This demonstrates that Na⁺- releasable Sr²⁺ cofractionates with mitochondria. We therefore may conclude with confidence that the selective metal release assay gives an excellent measure of mitochondrial Sr²⁺ uptake in the perfused heart, and ipso facto of mitochondrial Ca²⁺ uptake when Ca²⁺ is measured.

In Figure 1 we demonstrate the success of the

![Figure 1](http://circres.ahajournals.org/)

**Figure 1** Selective Ca²⁺ release assay of rat heart mitochondria. Rat heart mitochondria (2.15 mg/ml) were added to a buffer (at 30°C), 250 mM in sucrose, 20 mM in MOPS (adjusted to 7.1 with Tris), 5 mM in succinate (K⁺), 2 mM in Pi (K), 1 mg/ml BSA and 6 μM in ADP. Five μM rotenone was added and then 15 μM ⁴⁶Ca²⁺. After 4 minutes, this solution was combined with the standard metal release assay mixture (total volume, 15 ml). Five milliliters of a heart homogenate were added (see Methods), and the selective metal release assay was performed. Three time points were taken instead of the standard single 10-minute point. The results are from two separate identical experiments.
selective metal release assay using isolated rat heart mitochondria preloaded with 10 ng-ions of $^{45}\text{Ca}^{2+}$-labeled Ca$^{2+}$/mg protein. For this experiment, a homogenized heart was added to the assay mixture so the exact conditions of our routine assay were duplicated. The results show that less than 10% of the mitochondrial $^{45}\text{Ca}^{2+}$ was released spontaneously, and 90% of it was released by Na$^+$ in 10 minutes.

**Preparation of Rat Heart Homogenate**

We found it is necessary to take precautions to prevent artifactual uptake of Ca$^{2+}$ by mitochondria during the homogenization period (Table 1). When 5 mM EGTA was present in the homogenization buffer, we obtained a value of approximately 200 ng-ions for mitochondrial Ca$^{2+}$ (Ca$^{2+}$ released by Na$^+$) in hearts perfused for 5 minutes with a $^{45}\text{Ca}^{2+}$-labeled perfusate. The same value for mitochondrial Ca$^{2+}$, however, was obtained from hearts not perfused with $^{45}\text{Ca}^{2+}$ but merely injected with an ice-cold $^{45}\text{Ca}^{2+}$-labeled perfusate. Since the beating stopped almost immediately after the injection, the uptake of Ca$^{2+}$ by the mitochondria of these hearts must have occurred by a beat-independent process, most likely during homogenization. This homogenization-induced uptake of Ca$^{2+}$ by mitochondria can be almost totally eliminated by injecting the ice-cold EGTA-containing homogenization buffer into the vascular space (Table 1). Mitochondria from hearts injected first with cold $^{45}\text{Ca}^{2+}$-labeled perfusate, then with homogenization buffer, contained only 10 ng-ions Ca$^{2+}$/g wet weight heart. This low value serves as a background control. All values of mitochondrial Ca$^{2+}$, Sr$^{2+}$, or Mn$^{2+}$ reported hereafter were obtained from hearts injected with cold EGTA-homogenization buffer.

**Ca$^{2+}$ in Mitochondria of Rat Heart**

The amount of $^{45}\text{Ca}^{2+}$-labeled mitochondrial Ca$^{2+}$ (Ca$^{2+}$ released by Na$^+$) in hearts perfused for 5 minutes with a $^{45}\text{Ca}^{2+}$-labeled perfusate was extremely small—17 ng-ions/g wet weight heart (Table 1). When this value is compared to the background control of 10 ng-ions/g wet weight heart, it is not significantly different. Therefore, we conclude that either little if any Ca$^{2+}$ enters rat heart mitochondria in vivo under normal conditions or that the Ca$^{2+}$ which enters is removed rapidly. A significant amount of Ca$^{2+}$ released spontaneously was found, however. We suspect that this pool of Ca$^{2+}$ was in the SR, since recent findings demonstrate that SR spontaneously releases Ca$^{2+}$ when reuptake is inhibited (Alonso et al., 1977; Kirchberger and Wong, 1978). In an attempt to determine whether the low value of mitochondrial Ca$^{2+}$ could result from a high rate of efflux, we undertook a study of mitochondrial Sr$^{2+}$ uptake in the working rat heart.

**Sr$^{2+}$ in Mitochondria of Rat Heart**

A rat heart continued to function if a perfusate containing 4.8 mM Sr$^{2+}$ plus 0.2 mM Ca$^{2+}$ replaced the standard 2.5 mM Ca$^{2+}$ perfusate (Wayne, 1966). The heart rate, however, slowed to around 120 and the pressure increased significantly. Results presented in Table 2 show that after 5 minutes of perfusion there was a considerable amount of Sr$^{2+}$ (103 ng-ions/g wet weight heart) in the mitochondria. When this value is compared to the background control of 20 ng-ions/g wet weight heart, the difference is highly significant. A second significant finding is also presented in Table 2—mitochondrial Sr$^{2+}$ was rapidly washed out. Just 30 seconds after the heart was switched back to a normal Ca$^{2+}$ perfusate, 80% of the mitochondrial Sr$^{2+}$ was gone. From these results it is clear that there is a highly active mitochondrial divalent metal release mechanism present in myocardial cells in vivo. One likely candidate for this activity is the Na$^+$-Ca$^{2+}$ exchanger (Crompton et al., 1976).

**Divalent Metal Specificity of the Mitochondrial Na$^+$-Ca$^{2+}$ Exchanger**

In Figure 2 we give evidence that Sr$^{2+}$ is released from isolated rat heart mitochondria by Na$^+$. The rate was a little slower than the rate of Ca$^{2+}$ release by Na$^+$. When Mn$^{2+}$ was studied, however, it was apparent that Na$^+$ cannot release it. This finding is important because it gives us one means of testing

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**Table 1 Distribution of Ca$^{2+}$ in Rat Heart Homogenate**

<table>
<thead>
<tr>
<th>Perfusion and homogenization conditions</th>
<th>Released* Ca$^{2+}$/g wet wt heart</th>
<th>Released spontaneously</th>
<th>Released by Na$^+$</th>
<th>Not released by Na$^+$</th>
<th>No. of hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Perfused 5 min with 2.5 mM $^{45}\text{Ca}^{2+}$</td>
<td>239 ± 47</td>
<td>193 ± 59</td>
<td>11 ± 10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2. Cold 2.5 mM $^{45}\text{Ca}^{2+}$ injected through heart</td>
<td>290 ± 21</td>
<td>209 ± 47</td>
<td>18 ± 22</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3. Cold 2.5 mM $^{45}\text{Ca}^{2+}$ injected through heart, then EGTA injected through heart</td>
<td>15 ± 7</td>
<td>10 ± 8</td>
<td>5 ± 3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>4. Perfused 5 min with 2.5 mM $^{45}\text{Ca}^{2+}$, then EGTA injected through heart</td>
<td>34 ± 10</td>
<td>17 ± 13</td>
<td>11 ± 10</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

* See Methods, "Selective Metal Release Assay," for details.
The results plotted are the Na\(^+\) release time points. Ninety-three percent of the Ca\(^{2+}\) was accumu-
lated in the absence of Na\(^+\), over the course of 10 minutes.

5% of the accumulated metals was released sponta-
neously by Na\(^+\). Rat heart mitochondria (0.3
mg/ml) were added to a buffer (at 30°C) identical to the
vascular and interstitial Ca, or artefactually low
because of failure to inhibit Na\(^+\)-Ca\(^{2+}\) exchanger is respon-
sible for mitochondrial metal release in the heart. This is no
surprise, since in Figure 2 we show that Na\(^+\) cannot
release Mn\(^{2+}\) from mitochondria.

When Mn\(^{2+}\) was removed from the perfusate and
the heart was perfused for an additional 10 minutes
without Mn\(^{2+}\), we found that Mn\(^{2+}\) was not lost
from the “not releasable by Na” pool (Table 3). We
conclude therefore that Mn\(^{2+}\) is not readily releas-
able from mitochondria in vivo. This is the pre-
dicted result if the Na\(^+\)-Ca\(^{2+}\) exchanger is respon-
sible for mitochondrial metal release in the heart.

Discussion

Our results show that to measure mitochondrial
Ca\(^{2+}\) content of a rat heart, the Ca\(^{2+}\) in the vascular
space must be removed before homogenization. Some earlier reports in the literature failed to take
this precaution (Patriarca and Carafoli, 1968; Dhalla et al., 1970; Harris, 1977). However, Henry
et al. (1977), in a study on Ca\(^{2+}\) accumulation after
low flow ischemia, did flush out rabbit hearts with
buffer before homogenization.

The question still remains of the extent to which
the values of mitochondrial divalent metal content
measured here, even with this precaution, may be
artefactually high because of incomplete removal of
vascular and interstitial Ca, or artefactually low
because of failure to inhibit Na\(^+\)-Ca\(^{2+}\) exchange.

The data obtained with Sr\(^{2+}\) and Ca\(^{2+}\) offer re-
assurance on this point. The observation of a high
level of Sr\(^{2+}\) in the mitochondria of hearts perfused
with Sr\(^{2+}\) gives assurance that, if it had been pres-
ent, a high level of Ca\(^{2+}\) would have been measured in the mitochondria of hearts perfused with Ca\(^{2+}\). The significance of the failure to measure Ca\(^{2+}\) is therefore enforced, and the observation cannot be explained away as an artefactual loss through uninhibited Na\(^{+}\)-Ca\(^{2+}\) exchange. Conversely, the low level of Ca\(^{2+}\) found in mitochondria validates the high level of Sr\(^{2+}\) found, and shows that it cannot have originated from incomplete removal of vascular or interstitial Sr\(^{2+}\).

We have attempted to determine whether Ca\(^{2+}\) enters mitochondria during normal myocardial activity. Our results show that no significant net accumulation of Ca\(^{2+}\) occurred in 5 minutes. However, we cannot conclude that no Ca\(^{2+}\) entered mitochondria during this period, since additional evidence presented here suggests that the Na\(^{+}\)-Ca\(^{2+}\) exchanger is active in releasing metal from mitochondria in the heart. But the amount of Ca\(^{2+}\) which could have passed through the heart mitochondria during this 5-minute period is extremely small when compared to the 50 ng-ions Ca\(^{2+}\)/g wet weight heart beat required for muscle contraction (Soloro and Briggs, 1974). We estimate from Figure 2 that the maximum rate of Na\(^{+}\) release of Ca\(^{2+}\) from mitochondria is 20 ng-ions/min per mg, in good agreement with the value found by Crompton et al. (1976). Using a value of 40 mg mitochondria/g wet weight heart (based on a comparison of cytochrome oxidase activities in the heart homogenate and isolated heart mitochondria) and 200 beats/min, we calculate that the maximum rate of mitochondrial Ca\(^{2+}\) release/beat per g wet weight heart is 4 ng-ions, which is roughly 10% of the Ca required for contraction. This is an upper limit based on the maximal activity of the mitochondrial Na\(^{+}\)-Ca\(^{2+}\) exchanger. In light of the negligible level of steady state mitochondrial Ca\(^{2+}\) reported here, we doubt that this Ca\(^{2+}\) release system can be working anywhere near maximally. The flux of Ca\(^{2+}\) through the mitochondria per beat is therefore likely to be much less than 4 ng-ions/g wet weight of heart.

The situation with the Sr-perfused heart is considerably different. Here we found an accumulation of over 100 ng-ions of readily exchangeable Sr\(^{2+}\)/g wet weight heart in mitochondria, and 80% of this was released within 30 seconds of perfusion with a Sr\(^{2+}\)-free medium. Assuming an exponential washout of Sr\(^{2+}\), this corresponds to an initial efflux rate of 8 ng-ions/min per mg mitochondrial protein. This value is close to the \(V_{\text{max}}\) of the Na\(^{+}\)-Ca\(^{2+}\) exchanger for Sr\(^{2+}\) (Fig. 2), suggesting that Na\(^{+}\)-dependent flux of Sr\(^{2+}\) through the mitochondria may be fully activated in hearts functioning on a Sr\(^{2+}\)-containing medium. The higher flux of Sr\(^{2+}\) through mitochondria would appear to indicate a higher level of cytosolic free Sr\(^{2+}\) in hearts working on Sr\(^{2+}\) than the level of cytosolic free Ca\(^{2+}\) in hearts working on Ca\(^{2+}\). Although the level of Sr\(^{2+}\) used was somewhat high (4.8 mm), no significant increase in mitochondrial Ca\(^{2+}\) was observed when perfusate Ca\(^{2+}\) was increased to this level (data not shown). A higher level of Sr\(^{2+}\) than Ca\(^{2+}\) in the cytosol could be the result of a greater influx of extracellular Sr\(^{2+}\) than Ca\(^{2+}\) during the action potential: Others have observed a substantial increase in duration of the slow current when Sr\(^{2+}\) was substituted for Ca\(^{2+}\) in the perfusate (Kohlhardt et al., 1973; Bass et al., 1975; Kawata and Hatae, 1977).

**Acknowledgments**

We thank Dr. David E. Green for his interest and support.

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**Table 3** Distribution of Mn\(^{2+}\) in Rat Heart Homogenate

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>Released(^*) spontaneously</th>
<th>Released by Na(^{+})</th>
<th>Not released by Na(^{+})</th>
<th>No. of hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfused 5 min with 5 (\mu M) (^{46})Mn(^{2+}) plus 2.5 mm Ca(^{2+})</td>
<td>210 ± 70</td>
<td>10 ± 10</td>
<td>280 ± 100</td>
<td>6</td>
</tr>
<tr>
<td>Perfused 10 min with 5 (\mu M) (^{46})Mn(^{2+}) plus 2.5 mm Ca(^{2+})</td>
<td>540 ± 80</td>
<td>30 ± 40</td>
<td>1210 ± 370</td>
<td>4</td>
</tr>
<tr>
<td>Perfused 10 min with 5 (\mu M) (^{46})Mn(^{2+}) plus 2.5 mm Ca(^{2+}), then perfused 10 min with 2.5 mm Ca(^{2+})</td>
<td>520 ± 100</td>
<td>90 ± 70</td>
<td>1820 ± 100</td>
<td>4</td>
</tr>
</tbody>
</table>

All hearts had cold EGTA-containing homogenization buffer injected into them.

\* See Methods, "Selective Metal Release Assay," for details.

**Table 4** Cofractionation of Mn\(^{2+}\) Not Released by Na\(^{+}\) with Cytochrome Oxidase Activity

<table>
<thead>
<tr>
<th>Pellet</th>
<th>Protein (mg)(*)</th>
<th>C.O. activity (automol[O]/min)</th>
<th>Mn(^{2+}) (pmol)</th>
<th>Ratio Mn(^{2+}) protein</th>
<th>Ratio Mn(^{2+}) C.O. activity</th>
<th>No. of hearts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell debris</td>
<td>57.8 ± 0.6</td>
<td>67.2 ± 0.6</td>
<td>410 ± 180</td>
<td>7.1</td>
<td>6.1</td>
<td>4</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>10.8 ± 0.8</td>
<td>31.0 ± 6.2</td>
<td>180 ± 80</td>
<td>16.7</td>
<td>5.8</td>
<td>4</td>
</tr>
</tbody>
</table>

Hearts were perfused for 10 minutes with the basal perfusate containing 5 \(\mu M\) \(^{46}\)Mn\(^{2+}\). They were homogenized and fractionated by differential centrifugation (see Methods). The selective metal release assay was performed on the pellets.

\* All units are per 1 g wet wt heart.
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