Energy-Linked Calcium Transport in Subcellular Fractions of the Failing Rat Heart

By John R. Muir, M.R.C.P., Naranjan S. Dholla, Ph.D., Josefina M. Ortexa, M.D., and Robert E. Olson, M.D.

ABSTRACT
The isolated rat heart perfused without substrate provides a good model in which to study biochemical changes in the failing myocardium. In this preparation, there is a decline in the ability of both the mitochondria and the sarcoplasmic reticulum to accumulate calcium in the absence of oxalate after 30 minutes of perfusion. Calcium binding by mitochondria fell from control levels of 47 ± 8 µmole/mg protein to 17.5 ± 4.9 µmole/mg protein and that by microsomes from 29.8 ± 5.1 µmole/mg protein to 15.2 ± 4.8 µmole/mg protein. This drop coincided with the start of the decline in myocardial contractility. The calcium uptake by the sarcoplasmic reticulum in the presence of oxalate decreased from control levels of 534.1 ± 32.0 to 160.5 ± 27.2 µmole/mg protein after 2 hours of perfusion, at which time the myocardial contractility had dropped to below 10% of control levels. This change in the calcium uptake by the sarcoplasmic reticulum is associated with an increase in its ATPase activity from 1.91 ± 0.21 to 3.00 ± 0.31 µmole P_i/mg protein/min over 2 hours of perfusion without substrate. This suggests that there is an uncoupling of the microsomal ATP-dependent calcium pump in these late stages of heart failure due to substrate lack. The change in calcium binding to reticulum occurred in association with the onset of contractile failure, whereas changes in calcium uptake in the presence of oxalate were delayed and probably represent irreversible disorganization of the intracellular membranes.

ADDITIONAL KEY WORDS: sarcoplasmic reticulum, heart failure due to substrate lack, calcium binding, mitochondria, calcium uptake, ATPase activity, oligomycin

The regulation of intracellular calcium concentration occupies a central position in current concepts of excitation-contraction coupling in skeletal muscle (1-3). The instantaneous concentration of calcium appears to be regulated by opposing forces related to the extent of depolarization of the T-system on the one hand and to an energy-linked calcium transport system located in the vesicles of the longitudinal system on the other (1, 4-7). It is possible that similar events occur in the myocardium because cardiac contractile proteins, like those of skeletal muscle, are sensitive to changes in the ionic strength of calcium (8, 9). Some workers have found a disappointingly low calcium-binding capacity in cardiac sarcoplasmic reticulum (8), although more recently higher activities have been reported (2, 10). It has therefore been suggested that the mitochondria may play an important role in regulating calcium concentrations in the cardiac cells, since they are capable of accumulating considerable amounts of calcium (11-13).

Recently, Gertz et al. (14) reported a
reduced level of calcium uptake and ATPase activity of sarcoplasmic reticulum isolated from spontaneously failing heart lung preparations, and Lee et al. (15) have also shown a decrease in both the rate and the total calcium uptake by the sarcoplasmic reticulum of ischemic dog heart muscle. In view of these findings, we decided to study the effect of heart failure induced by perfusion with substrate-free medium on the calcium transport and ATPase activity of the myocardial subcellular fragments of the isolated rat heart, a preparation demonstrated in this laboratory to be an excellent model in which biochemical and physiological events can be studied during the course of heart failure (16-18).

The degree of heart failure can be measured directly in terms of the myocardial contractility. It appears that the primary defect underlying cardiac failure in this system is a reduced rate of ATP formation, which is diminished by a lack of substrate rather than oxygen. A study in our laboratory of the changes in adenine nucleotides during the onset of cardiac failure in the rat heart perfused without substrate has demonstrated that it imitates the effects of hypoxia on a longer time scale.

In the present study, the term “calcium binding” is applied to the ability of microsomes or mitochondria to take up calcium in the absence of oxalate. It is understood that this convention employs an arbitrary meaning for binding, since it is likely that both binding and a small amount of accumulation occur under these conditions. On the other hand, for purposes of clarity, the term “calcium uptake” is employed in this paper to signify calcium accumulation by microsomes in the presence of 5 mM potassium oxalate.

**Materials and Methods**

Albino male rats weighing 250 to 325 g were decapitated, and the hearts were quickly removed and chilled in ice-cold oxygenated perfusion medium. The fat and connective tissue were trimmed away and the hearts were arranged for coronary perfusion by the Langendorff technique. The perfusion medium was a modified Krebs-Henseleit solution containing half of the original amount of Ca²⁺, i.e., 1.25 mM. It was equili-

brated with a gas mixture of 95% O₂ and 5% CO₂ and maintained at 37°C. In some of the experiments, the perfusion medium also contained 8 mM glucose. The contractile force and heart rate were monitored on a Sanborn recorder by a force displacement transducer.

In each experiment, two hearts were pooled after perfusion for the desired time and were immediately immersed in ice-cold homogenizing solution. The tissue was cut into small pieces and homogenized with 5 volumes of a solution containing 5 mM histidine, 5 mM potassium oxalate, 100 mM potassium chloride and 320 mM sucrose at pH 7.4. The oxalate was omitted from the homogenizing solution when hearts were being prepared for studies of calcium binding. The homogenate was centrifuged at 8000 × g for 20 minutes to remove the cell debris. The supernatant fluid was centrifuged at 8000 × g for 30 minutes to obtain the mitochondrial pellet and then for an additional hour at 37000 × g to obtain the sarcoplasmic reticulum pellet. The pellets were resuspended in 2 ml of the homogenizing solution for determination of calcium uptake in the presence of oxalate and in 1 ml of homogenizing solution for estimation of calcium binding.

The calcium uptake by the sarcoplasmic reticulum in the presence of oxalate was measured by using 0.2 ml of the resuspended reticulum in an incubation medium containing 2 mM histidine, 5 mM potassium oxalate, 150 mM potassium chloride, 5 mM magnesium chloride, 5 mM ATP, and 0.135 mM calcium chloride labeled with Ca⁴⁺. The pH of the medium was 7.4 and the total volume 4.0 ml. The final concentration of Ca⁴⁺ in the incubation mixture was 0.2 µC/ml. The reaction was started by adding ATP and was carried out for 20 minutes at 25°C with constant stirring. Aliquots removed at various times after the start of the reaction were filtered through a Swinney filter holder containing a 13 mm Millipore filter (type HA, size 0.45) using a syringe. The filtration was usually completed in 2 to 3 seconds. Filtrate in the amount of 0.05 ml was added to 15 ml of Bray’s solution (19) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Using a control, in which tissue was absent, the amount of calcium retained by the reticulum was calculated from the total free calcium in the reaction mixture and the percent isotopic Ca⁴⁺ retained. This is, of course, a minimum value, since the total free calcium pool was not measured chemically in these experiments. In most experiments, these data are presented as nμ moles Ca²⁺ taken up/mg protein/unit time.

Calcium binding was studied in a similar manner, except that potassium oxalate was
excluded from all solutions. The time course of
the incubation was shorter than that for the
measurement of calcium uptake, aliquots being
taken from the incubation flask at 15, 30, and 45
seconds and 1 and 2 minutes after the reaction
was started by adding ATP. In these experiments,
uptakes were generally complete between 15 and
30 seconds, but the values are expressed per 2
minutes, the total time of the experiment. The
subcellular fractions were characterized by study-
ing the influence of oligomycin (2.5 μg/ml) on
their ability to bind calcium. Oligomycin inhibits
ATP-driven calcium binding by mitochondria
(12) but has little or no effect on the calcium
binding of fresh sarcoplasmic reticulum (20). The
protein concentrations of the sample were
determined by either the Biuret reaction or by
Lowry's method (21) using bovine serum
albumin as a standard.

The ATPase activity of the reticulum was
measured by two methods. In the first, the
increase in inorganic phosphate content of the
filtrates from the calcium uptake studies was
measured by the Fiske-Subbarow method (22).
In addition, the initial rate of ATP breakdown by
a suspension of sarcoplasmic reticulum was
measured by following the decline in DPNH in a
system containing phosphoenol-pyruvate, py-
ruvate kinase and lactic dehydrogenase. In this
system, the ADP formed by hydrolysis of ATP is
rephosphorylated by phosphoenol-pyruvate. The
resulting pyruvate is then reduced to lactate by
lactic dehydrogenase and the decline in DPNH
was followed in an Eppendorf fluorometer at an
excitation wave length of 340 mμ. The final
composition of the reaction medium within the
cuvette was as follows: 61.5 mM Tris buffer pH
7.5; 30.8 mM KCl; 7.7 mM MgCl₂; 9.2 mM
phosphoenol-pyruvate; 46 mM DPNH; lactic
derhydrogenase, 8 μg/ml; pyruvate kinase, 8
μg/ml; and 3 mM ATP. The reaction was started
by adding 2 to 4 μg of sarcoplasmic reticulum.

Recent studies on the subcellular distribution of
adenine mononucleotide phosphatase (AMPase)
activity have indicated that this enzyme is
confined to the sarcoplasmic reticulum in the rat
myocardium (23). The AMPase activity of the
subcellular fractions was therefore used as a
marker for sarcoplasmic reticulum. It was esti-
mated by measuring the release of inorganic
phosphate from AMP by the subcellular fractions
in the presence of 0.05M glycine-sodium hydrox-
ide buffer (pH 8.5), 5 mM magnesium and 5 mM
AMP. The reaction was started by adding AMP
and stopped after 10 minutes with 10% trichlo-
racetic acid. The inorganic phosphate content was
measured by the Taussky-Schorr method (24).
Zero time levels were obtained by adding the
trichloracetic acid before the AMP. The AMPase
activity was finally expressed in terms of the
release of inorganic phosphate per minute per
milligram of protein.

For electron microscopic examination, the
pellet was fixed for 2 hours with osmium tetroxide
buffer at pH 7.4 (25) and was then dehydrated
with increasing concentrations of ethanol and
embedded in Epon 812. The sections were cut
with a glass knife using the Porter-Blum
ultramicrotome (MT-1 model), stained with
uranyl acetate and lead citrate (26, 27) and
examined with a Phillips 200 electron microscope.

Results

Yield of Subcellular Fractions.—The yield of
both the mitochondrial and the reticulum
pellets for pairs of hearts perfused without

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td>Fresh Weight and Yields of Mitochondrial and Reticulum for Rat Hearts Perfused for Ten Minutes and Two Hours Without Substrate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Perfusion time</th>
<th>Wet weight (g)</th>
<th>Mitochondrial yield (mg)</th>
<th>Reticulum yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min (12)</td>
<td>3.1±0.1</td>
<td>81±12</td>
<td>28±3</td>
</tr>
<tr>
<td>2 hours (10)</td>
<td>3.4±0.2</td>
<td>81±12</td>
<td>28±3</td>
</tr>
</tbody>
</table>

Values are means ± SE for pairs of rat hearts; number of experiments is in parentheses. The wet
weight of hearts perfused 2 hours has been corrected for the increased hydration over 2-hour
perfusion without substrate. The yield of fresh particles has been calculated by multiplying the
protein content by 5. The overall yields of mitochondria and reticulum are 10 ± 2% and 15 ±
2%, respectively, of that initially present in the heart. There is no significant difference between
the mitochondrial or reticulum yields after 10 minutes and after 2 hours of perfusion (P > 0.05
and P > 0.05).
Changes in heart rate, myocardial contractility, and calcium binding by the mitochondria and the sarcoplasmic reticulum during perfusion without substrate of the isolated rat heart. Mean and se are given; number of experiments is in parentheses. The drop in calcium binding by both the mitochondria and the reticulum is unlikely to have arisen by chance ($0.01 > P > 0.005; 0.05 > P > 0.025$).

Substrate for 10 minutes and 2 hours are shown in Table 1. The wet weights of the hearts perfused for 2 hours have been corrected for the increase in hydration that occurs during perfusion (18). The dry-to-wet weight ratio of rat hearts declines to 85% of the control value during this time. The fresh weight of mitochondria initially present in rat heart was calculated from values for cytochrome C and ubiquinone in whole rat heart (28) and in mitochondria (29); the ratios obtained indicate that rat heart contains 29 to 32% mitochondria by weight. The electron microscopic estimations of reticulum in muscle (30) were used to arrive at a figure of 5% fresh weight for cardiac reticulum. There was no significant difference in the yield of either mitochondria or reticulum from hearts perfused for 10 minutes and for 2 hours ($P > 0.05$ and $P > 0.05$), although the overall yields were low.

Myocardial Contractility, Heart Rate, and Calcium Binding by the Mitochondria and Sarcoplasmic Reticulum.—Isolated hearts were perfused for varying times with substrate-free medium. The myocardial contractile force, heart rate, and calcium binding by the mitochondria and by the sarcoplasmic reticulum are shown in Figure 1. The decline in contractile force after 30-minute perfusion and the negligible change in heart rate over the 2-hour period confirm results already reported from this laboratory (18). The calcium binding is expressed in terms of the accumulation of calcium per milligram of protein after 2 minutes of incubation. These conditions were uniformly followed because we have found that the calcium binding reaches a plateau 15 seconds after the addition of ATP.

### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>No.</th>
<th>Time (min)</th>
<th>Contractility (% control)</th>
<th>$\text{Ca}^{2+}$ binding (mumol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No substrate</td>
<td>8</td>
<td>10</td>
<td>100 ± 5</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>No substrate</td>
<td>8</td>
<td>30</td>
<td>95 ± 10</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>No substrate</td>
<td>8</td>
<td>60</td>
<td>54 ± 8</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Glucose added at 30 min</td>
<td>4</td>
<td>60</td>
<td>90 ± 10</td>
<td>31 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± se. The difference in contractility and calcium binding induced by addition of glucose at 30 minutes is highly significant ($P < 0.02$).
Calcium uptake and ATPase activity of the sarcoplasmic reticulum during perfusion without substrate of the isolated rat heart. The ATPase activity is expressed in terms of the release of inorganic phosphate from ATP over the 20-minute incubation period. The drop in the calcium uptake by the reticulum after 2 hour-perfusion is unlikely to have arisen by chance (P < 0.005).

Katz and Repke (9) have reported similar findings. The calcium-binding capacity of both the mitochondria and the sarcoplasmic reticulum fell after 30-minute perfusion without substrate, and these changes were unlikely to have arisen by chance (0.01 > P > 0.005; 0.05 > P > 0.025). When glucose was added to substrate-deprived hearts at 30 minutes, both contractility and the ability of the reticulum to bind calcium were restored, as shown in Table 2.

Calcium Uptake and ATPase Activity of the Sarcoplasmic Reticulum.—The ability of the sarcoplasmic reticulum to accumulate calcium in the presence of oxalate was not altered over the first 60 minutes of perfusion without substrate. However, after 2 hours of perfusion, the calcium uptake of the sarcoplasmic reticulum was reduced to a level approximately 30% of that found in control hearts (Fig. 2) (P < 0.005).

The ATPase activity of the sarcoplasmic reticulum, measured as the average increase in inorganic phosphate in the filtrate in which calcium uptake was observed over the 20-minute period, was unchanged by 2-hour perfusion without substrate (Fig. 2). However, when the true initial rate of ATPase activity of the sarcoplasmic reticulum was measured by ADP formation from ATP in the fluorimeter, there was a significant increase in the activity following the 2-hour perfusion period (P < 0.025) Table 3.

Influence of Glucose on the Calcium Uptake of Sarcoplasmic Reticulum.—When isolated rat hearts are perfused with medium containing 8 mM glucose, the contractile force does not change over a 2-hour period of perfusion (18). The calcium uptake of the sarcoplasmic reticulum was therefore compared in two groups of hearts perfused with and without addition of 8 mM glucose to the perfusate. The results are shown in Figure 3. It is apparent that the ability of the sarcoplasmic reticulum to accumulate calcium in the presence of oxalate is preserved when substrate is present in the perfusion medium over a 2-hour period.

Influence of Oligomycin on Calcium Accumulation.—As the various subcellular fractions separated by differential centrifugation are by no means pure (31), the effect of oligomycin, a known inhibitor of ATP-driven mitochondrial calcium transport (12), on the calcium uptake and binding by the different fractions was studied. Both the calcium uptake and binding by reticulum were unaffected by oligomycin, indicating that contaminating mitochondria were not contribut-

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**TABLE 3**

<table>
<thead>
<tr>
<th>Perfusion time</th>
<th>Activity (μmoles P1/mg protein/min)</th>
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<tbody>
<tr>
<td>0 (7)</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>2 hours (9)</td>
<td>3.0 ± 0.3</td>
</tr>
</tbody>
</table>

Rates were measured in the Eppendorf fluorimeter (see text for details). Values are means ± se; number of experiments is in parentheses. The difference between the two values was unlikely to have arisen by chance (P < 0.025).
Sarcoplasmic Reticulum:
Calcium Uptake
(mmol Ca++ /mg of protein)

10min 2 hr
perfused without glucose
300
500
250

10min 2 hr
perfused with glucose
750

FIGURE 3
Comparison of the calcium uptake by rat heart sarcoplasmic reticulum perfused for 10 minutes and 2 hours with and without glucose. Mean and SE are shown; number of experiments is in parentheses. Addition of substrate to the perfusion medium maintained the calcium uptake at control levels over the 2-hour perfusion.

ing to the calcium accumulation. On the other hand, as shown in Figure 4, the calcium binding by the mitochondrial fraction was inhibited approximately 85% by oligomycin, suggesting that the calcium binding in this fraction was, in fact, due to mitochondria. Oligomycin had similar effects on the calcium binding by the subcellular fractions isolated from hearts perfused for 2 hours without substrate.

AMPase Activity of 8,000 to 37,000 g Subcellular Fraction.—The AMPase activity of the sarcoplasmic reticulum fraction (8,000 to 37,000 g) was used as an enzymatic marker for this fraction. The mean activity for this fraction from ten pairs of control nonperfused hearts was 0.143 (SE ± 0.018) μmoles Pi/mg protein/min and for ten pairs of hearts perfused for 2 hours without substrate 0.187 (SE ± 0.018) μmoles Pi/mg protein/min. There was no significant difference between the two mean levels (P > 0.10).

Electron Microscopic Examination of Subcellular Fractions of the Perfused Heart.—The pellets of both the sarcoplasmic reticulum and the mitochondrial fractions of the hearts perfused for 10 minutes and 2 hours with substrate-free medium were fixed with osmium tetroxide, dehydrated with alcohol, and examined under the electron microscope. When compared with hearts perfused for 10 minutes, the sarcoplasmic reticulum from the 2-hour perfused hearts showed insignificant changes, possibly a slight increase in swelling.
FIGURE 5

Electron micrographs of the mitochondrial pellets from rat hearts perfused for 10 minutes (top) and 2 hours (bottom). The mitochondria are swollen and the cristae disrupted after 2-hour perfusion without substrate.

In contrast to the sarcoplasmic reticulum, the changes in the ultrastructure of mitochondria of the perfused heart were pronounced (Fig. 5). Mitochondria from hearts perfused without substrate were considerably swollen, and their cristae were disrupted.
Discussion

It has already been shown in this laboratory that the decline in myocardial contractility in the isolated rat heart perfused without substrate is associated with a drop in the ATP and a rise in the AMP content of the heart muscle (18). These data suggested that the failing contractility in this preparation reflected a failing rate of oxidative phosphorylation. If the control of intracellular calcium concentration by sarcoplasmic reticulum and possibly mitochondria is important in cardiac excitation-contraction coupling, a change in the ability of the various subcellular fractions to accumulate calcium might also occur under these conditions and contribute to the pathogenesis of failure. The results of the present study show that in the isolated heart failing from substrate lack, the calcium-binding capacity of both the sarcoplasmic reticulum and the mitochondria declines after 30 minutes. This change coincides with the onset of the decline in both the myocardial contractility and the ATP-AMP ratio in the heart (18). The calcium uptake in the presence of oxalate by the reticulum is also reduced, but the change occurs much later, when myocardial contractility is reduced to a negligible force after 2 hours of perfusion. The delayed onset of the defect in calcium uptake in the presence of oxalate is not unexpected, since this measure of calcium transport is less sensitive than calcium binding. Since the fall in contractility and calcium binding are coincident, it is possible that the fall in calcium binding contributes to the onset of cardiac failure in this preparation.

The decline in myocardial contractility in the isolated rat heart following perfusion without substrate can be reversed if glucose is added to the perfusate after 30 minutes to 1 hour (18). A similar reversibility in calcium binding by the reticulum has also been demonstrated. However, after 90 minutes, the changes become irreversible. It was suggested that the phase of irreversible myocardial failure is associated with irreversible alterations in intracellular membranes. The observation that the decline in reticular calcium uptake with oxalate coincides with irreversible myocardial failure supports this concept, since calcium uptake is dependent on the integrity of the reticular membrane (32, 33). It was also demonstrated that both myocardial contractility and calcium uptake can be maintained at normal levels in the isolated heart for up to 2 hours if glucose is added initially to the perfusion medium. The changes in the ultrastructure of the subcellular fragments following perfusion with substrate-free medium are also compatible with such a hypothesis.

Although the decline in the ability of the sarcoplasmic reticulum to accumulate calcium was not accompanied by a fall in the ATPase activity of the reticulum, measured in terms of the total inorganic phosphate released over a 20-minute period, this may reflect the insensitivity of this method of measuring initial ATPase rates. On the contrary, the initial rate of reticular ATPase activity was significantly elevated following 2 hours of perfusion without substrate, indicating an uncoupling of reticular ATPase activity from calcium uptake. Other investigators have also reported a similar uncoupling with trypsin and sodium oleate (34, 35), and during muscle maturation (36). The ability of trypsin (34) to uncouple ATPase activity from calcium uptake by the reticulum suggests that the reticular membrane must be intact if the energy released by the breakdown of ATP is to be utilized efficiently by the calcium transport system. Therefore, the uncoupling of the ATPase-calcium uptake system in the sarcoplasmic reticulum following 2-hour perfusion without substrate may again be due to disorganization of the intracellular membranes.

It is not likely that the observed decline in calcium accumulation by the subcellular fractions over the 2-hour period of perfusion was an artifact of particle isolation because (1) the yields of microsomal material from the hearts perfused for 10 minutes and 2 hours were the same, (2) the AMPase activity of the sarcoplasmic reticulum was unchanged over the perfusion time, and (3) addition of...
glucose prevented the change in calcium uptake.

The results presented here show that in myocardial failure due to substrate lack there is a decrease in calcium binding by mitochondria and sarcoplasmic reticulum after 30 minutes that correlates with the decline in myocardial contractility and in the ATP-AMP ratio in the heart muscle. When the failure becomes irreversible and not responsive to restoration of substrate, there is a decrease in calcium uptake by the sarcoplasmic reticulum and a rise in the microsomal ATPase. It is suggested that these latter changes in the final stage of failure are associated with irreversible alterations of the intracellular membranes.

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References

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