Uptake of Calcium by Glycerinated Cardiac Slices

By Alan S. Fairhurst, Ph.D., Donald A. Palus, B.A., and Donald J. Jenden, M.D.

ABSTRACT

Partially glycerinated cardiac slices have been used to assess the effects of ryanodine and of mitochondrial and other inhibitors on calcium transport in systems that appear to retain a high degree of functional integrity. Strips of rabbit ventricle were extracted in 50% (w/v) glycerol for 24 hr at 4°C and slices 100 μ thick were cut on the freezing microtome. Such slices can bind 30 to 40 μmole of calcium/g of protein when incubated in medium containing 45Ca in the form of a Ca-EGTA buffer, binding being decreased with increasing times of glycerination. The concentrations of ATP and of Ca2+ required for half-maximal activity are 5 X 10^-6 M and 10^-7 M respectively; both ATP and creatine phosphate must be present to elicit maximal uptake. Calcium accumulation appears to be attributable to the sarcoplasmic reticulum rather than to mitochondria, and is inhibited by caffeine, Salicylan and ryanodine. Uptake is stimulated by oligomycin, but is not affected by azide, ouabain, carbachol, eserine, epinephrine or norepinephrine.

ADDITIONAL KEY WORDS: sarcoplasmic reticulum, calcium pump, ryanodine, caffeine, ouabain, oligomycin, norepinephrine, rabbit ventricle.

The occurrence of a calcium-binding system in cardiac muscle, analogous to that known to exist in skeletal muscle, has been demonstrated by the isolation of subcellular cardiac fractions consisting of remnants of the sarcoplasmic reticulum (vesicles) which can accumulate calcium in the presence of ATP and a suitable electrolyte environment (1, 2). Studies with preparations possessing a higher degree of structural integrity (3) have shown that cardiac fibers which have been extracted with 50% glycerol exhibit both contraction and relaxation when treated with ATP and creatine phosphate, and that the extent of relaxation decreases with increasing time of glycerination. It would thus appear that relaxation of the glycerinated cardiac fibers is related to the calcium-binding activity of the sarcoplasmic reticulum, and that this reticular activity is reduced by prolonged exposure to glycerol, as has been shown in skeletal muscle fibers (4). This study describes conditions under which calcium binding by slices of glycerol-extracted cardiac muscle may be demonstrated, together with the sensitivity of the binding process to various inhibitor agents.

Methods

Male rabbits weighing 2.5 to 3 kg were stunned and rapidly exsanguinated, and the heart was removed and placed in ice. Strips of left ventricular muscle approximately 3 x 3 x 13 mm were removed and mounted at rest length on a glass support and extracted for 24 hr at 4°C in a stirred solution containing 50% glycerol (w/v) and 100 mM histidine buffer at pH 7.0.

In preliminary experiments attempts were made to cut slices manually using a razor blade under the dissecting microscope. However, this technique proved unsatisfactory because it did not yield preparations of suitable uniformity and...
thickness and was quite tedious. Since in these experiments the cardiac muscle had been well glycerinated prior to slicing, the use of a standard histological freezing microtome was examined. Control experiments performed with slices dissected manually from glycerinated bundles before and after freezing disclosed that freezing did not decrease the calcium-binding activity of slices; the presence of this high concentration of glycerol presumably prevents the formation of ice crystals during freezing which might reduce the activity of the preparations.

In a typical experiment a glycerinated muscle bundle was frozen for 1 to 2 min on the bed of the microtome and 5 slices 100-μ thick and each containing about 750 μg of protein were cut. These slices were transferred to a solution containing 30 mM KCl, 30 mM KH₂PO₄, 1.6 mM ECTA [ethylene glycol bis (β-aminoethyl ether) tetraacetic acid], at pH 7.0 for 30 to 60 sec for removal of excess glycerol, and then to the incubation medium. Incubations were carried out with gentle continuous agitation at 24°C in small covered beakers in 1 ml of a solution of pH 7.0 containing 30 mM KCl, 30 mM KH₂PO₄, 5 mM MgCl₂, 5 mM ATP, 6 mM creatine phosphate, 1.6 mM EGTA and 0.64 mM total calcium chloride labeled with ⁴⁰Ca and having a specific activity of 10 mc/mm; 5 replicate slices were used for each experimental condition. In experiments with inhibitors, the slices were incubated for 5 min with the inhibitor prior to the addition of ATP and creatine phosphate. After incubation the slices were washed for approximately 30 sec in an identical solution except for the substitution of cold for radioactive calcium, and then dissolved at 100°C for 20 min in 1.75 ml of 0.125 N NaOH containing 0.57 mM ECTA. Aliquots (0.1 ml) of this digest were analyzed for protein (5) and 0.2-ml aliquots assayed for radioactivity in a liquid scintillation counter. Results were expressed as micromoles of calcium taken up per gram of protein and all averages were calculated as geometric means. In the experiments with inhibitors, the data were subjected to an analysis of variance to determine that significant differences existed between each inhibitor and control series. An analysis of covariance showed that the order in which slices were sectioned from the glycerinated myocardium exerted no significant influence either on the control uptake or on its sensitivity to the drugs.

The drugs used in this study were: caffeine, anhydrous, U.S.P.; eserine salicylate, U.S.P.; 1-epinephrine bitartrate and 1-norepinephrine bitartrate, Winthrop Laboratories; oligomycin and ouabain (Strophanthin-G), Sigma Chemical Co.; ryanodine, S. B. Penick and Co.; carbachol (carbamycholine chloride), K and K Laboratories; Salyrgan (sodium salicyl-[γ-hydroxymercuri-β-methoxypropyl]-amide-O-acetate), U.S.P.

**Results**

The time course of calcium uptake by cardiac muscle slices glycerinated for 24 hr under standard incubation conditions is shown in Figure 1; the maximal uptake was reached after 1 hr incubation, corresponding to the time required for maximal uptake in glycerinated rabbit psoas fibers of similar thickness.

With standard incubation conditions and a 1-hr incubation period, the effect of the length of the extraction period in glycerol on the capacity of the slices to bind calcium from the medium is as follows: The calcium binding capacity was somewhat higher after 2 days extraction than after 1 day, fell off markedly after 4 days extraction, and was essentially absent after 16 days glycerination. No attempt was made to determine activities for extraction times shorter than the standard 24 hr.
Table 1
Calcium Uptake of Glycerol-Extracted Muscle Slices as Influenced by Presence of ATP and Creatine Phosphate

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Calcium uptake (nmol/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP and ATP omitted</td>
<td>3.7</td>
</tr>
<tr>
<td>CP (6 mM)</td>
<td>5.8</td>
</tr>
<tr>
<td>ATP (5 mM)</td>
<td>7.8</td>
</tr>
<tr>
<td>CP (6 mM) plus ATP (5 mM)</td>
<td>39.0</td>
</tr>
</tbody>
</table>

Incubation conditions as in Figure 1 except that ATP and CP were varied as indicated. Incubation time was 1 hr.

preparation. Embry and Briggs (3) found that appreciably longer extraction times were necessary for the elimination of the relaxation response induced by creatine phosphate in glycerinated cardiac muscle fibers; however, their extractions were performed at —20°C.

Since Bowen and Martin (6) have shown that the supply of ATP diffusing to the interior of glycerol-extracted muscle becomes critical in fibers only slightly thicker than the slices used in this work, it was important to determine whether an ATP-restoring system was required in these cardiac slices to maintain the ATP concentration approximately constant throughout the slice. Table 1 summarizes an experiment in which calcium uptake by the slices was increased markedly by creatine phosphate when ATP was present, but was only slight in the presence of either ATP or creatine phosphate alone; this suggests that the maintenance of high ATP or low ADP levels or both underlies this effect. It is possible, however, that creatine phosphate is also required for some other function as suggested by studies on isolated cardiac vesicles (7). The ATP requirement is illustrated further by an experiment in which the concentration of added ATP was varied while creatine phosphate was maintained constant at 6 mM; the ATP concentration for half-maximal saturation is of the order of $5 \times 10^{-6}$M (Fig. 2).

The influence of the concentration of calcium ion on calcium uptake was studied while the total diffusible calcium was maintained constant, by varying the concentration of EGTA in the system. Table 2 shows that calcium uptake is maximal at a calcium ion concentration of $1.3 \times 10^{-7}$M and that the calcium-binding system is half-maximally activated at an ionized calcium level of approximately $10^{-5}$M.

![Figure 2](http://circres.ahajournals.org/)

The influence of ATP concentration on calcium uptake by glycerinated cardiac slices. Incubations for 1 hr as in Methods, with additions of ATP as indicated.
TABLE 2

<table>
<thead>
<tr>
<th>[EGTA] X 10^-7 M</th>
<th>[Ca^2+] X 10^-7 M</th>
<th>Calcium uptake (mmole/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>3.2</td>
<td>5.0</td>
<td>6.8</td>
</tr>
<tr>
<td>2.0</td>
<td>9.4</td>
<td>17.6</td>
</tr>
<tr>
<td>1.6</td>
<td>13.3</td>
<td>35.5</td>
</tr>
<tr>
<td>1.2</td>
<td>22.9</td>
<td>32.8</td>
</tr>
</tbody>
</table>

Total calcium was maintained constant at 0.64 mM and the concentration of EGTA was varied to control the ionized calcium levels. Incubation conditions as in Figure 1, with 1 hr incubation.

10^-7 M. The calcium concentration required for half-maximal uptake by isolated cardiac vesicles is around 6 X 10^-8 M (2); however, this value represents the total calcium chloride added, and the ionized calcium concentration would necessarily be lower than this figure.

The effects of the various inhibitor agents were examined when the steady state had been reached, after 1 hr incubation. Table 3 shows that calcium uptake by the slices is almost completely inhibited by the mercurial diuretic sodium salicyl-(γ-hydroxymercuri-β-methoxypropyl)-amide-O-acetate (Salyrgan), and that the alkaloid ryanodine and also caffeine have marked inhibitory effects. Oligomycin produced a marked stimulation of calcium uptake. Several other agents were tested but produced no significant effect on calcium accumulation. These included azide (5 X 10^-4 M), ouabain (10^-5 M), norepinephrine (3 X 10^-5 M), epinephrine (3 X 10^-5 M), carbachol (10^-5 M) and eserine (10^-4 M).

Discussion

We have established conditions under which glycerinated cardiac muscle slices of suitably uniform thickness can be conveniently prepared which are capable of concentrating calcium from incubation media containing very low concentrations of ionized calcium. This calcium binding is an energy-dependent process rather than a passive binding, since both ATP and an ATP-restoring system are required; the binding activity is decreased by extraction in glycerol for several days, indicating that the enzymatic system responsible for binding is damaged by prolonged extraction. The entities which might be involved in calcium binding in these slices are the sarcoplasmic reticulum and the mitochondria. Isolated heart mitochondria have been shown to accumulate calcium (8) but the concentration of calcium ions required for uptake in such studies was considerably higher than was found necessary in the present experiments. Also, studies with sodium azide show that calcium uptake by the cardiac slices is not affected by this agent, although azide is a potent inhibitor of calcium binding by isolated heart mitochondria; Salyrgan is a potent inhibitor of Ca^2+ uptake by cardiac slices, but does not inhibit calcium uptake by cardiac mitochondria (8). It may be tentatively concluded, therefore, that calcium binding in these studies is due to the activity of the cardiac sarcoplasmic reticulum rather than the mitochondria. The amount of calcium taken up by these cardiac slices is considerably smaller than the 200 to 300 μmole of calcium/g of protein taken up by glycerinated rabbit psoadic fibers under similar conditions (4). A lower level of bound calcium would be expected if the cardiac sarcoplasmic reticulum is responsible for calcium uptake.
binding in the present studies, since electron microscopic studies (9) have shown that cardiac muscle contains considerably less sarcoplasmic reticulum than does skeletal muscle; another factor contributing to the comparatively low uptake could be the lability of the cardiac reticulum, which has been described in subfractionation studies with cardiac muscle (1, 2). Attempts to locate sites of calcium accumulation by electron microscopic examination were unsuccessful because no definite deposits could be found, possibly due to loss of these small amounts of calcium during fixation procedures. Comparison of cardiac and skeletal systems with respect to the other variables studied in this report shows that these preparations from the two types of muscle exhibit comparable requirements for ATP and an ATP-restoring system as well as comparable concentrations of ATP and ionized calcium.

Of the results obtained with the various inhibitor agents, the marked inhibition produced by Salyrgan was not unexpected, since the calcium-binding systems of both skeletal and of cardiac vesicles require free sulfhydryl groups for activity and have been shown to be inhibited by this agent (10, 1). The alkaloid ryanodine is known to inhibit calcium accumulation in subcellular fractions of skeletal muscle (11, 12) and to modify skeletal muscle myosin B ATPase activity (23). The inhibition of calcium binding seen in these cardiac slices suggests that in the heart also the calcium-binding system is a site of action of the alkaloid. It is not yet clear, however, to what extent these in vitro findings explain the physiological action of ryanodine since the presence of the alkaloid induces flaccid paralysis in cardiac muscle but leads to rigor in skeletal muscle when injected in a dose of 0.2 mg/kg in dogs (13). The inhibitory effect of caffeine on calcium binding by the cardiac slices is in agreement with the concept that this agent acts on internal structures of muscle (14) and with results obtained with skeletal muscle fractions showing that caffeine induces the release of previously bound calcium from the particles (15). The marked stimulatory effect of oligomycin cannot be readily explained, in that this agent has been shown to have only inhibitory effects on the calcium transport systems of cardiac mitochondria (8), cardiac sarcoplasmic reticulum (16) and skeletal muscle vesicles (17). It is possible that oligomycin does not penetrate sufficiently to the calcium-binding elements within the cardiac slices, but does produce effects elsewhere within the tissue which then lead to more optimal binding activity by the reticulum. It is also possible that the properties of the intact sarcoplasmic reticulum differ sufficiently from those of the isolated vesicles to account for this stimulation.

The lack of effect of the other agents tested in this study could perhaps be explained by assuming that none of them penetrated to sites of action. If it can be assumed that adequate penetration did occur, however, the negative findings may be of some significance. The absence of an effect of ouabain might be expected since it has been shown that cardiac sarcoplasmic reticulum preparations are stimulated by ouabain only if isolated from a failing heart (18) or after damage by prior exposure to barbiturate (19). The possibility that acetylcholine acts as a transmitter linking the depolarization of the surface membrane with the activity of the sarcoplasmic reticulum has been suggested by the localization of acetylcholinesterase in reticular fragments of skeletal muscle (20) and in the longitudinal elements of the cardiac sarcoplasmic reticulum (21). The lack of effect of carbachol and of eserine in the present study would indicate, however, that calcium binding in these cardiac slices is not critically associated with cholinesterase activity, and is in agreement with the observation that carbachol does not elicit a contractile response when applied to skinned muscle fibers (22).

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References

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