ABSTRACT
The transport of calcium through the membranes of the sarcoplasmic reticulum plays an important role in the relaxation-contraction cycle of the heart. To study and characterize this system, fragmented sarcotubular vesicles from dog hearts were prepared by ultracentrifugation. The fraction between 15,000 and 40,000 g was partially purified by gradient centrifugation. Calcium accumulation by the vesicles in the presence of ATP, Mg, 45Ca, and oxalate was studied. The rate of calcium uptake increased with the ATP concentration and was not affected by changes in K or Na concentration nor by substitution of isosmotic amounts of sucrose for 75% of the Na ions. Splitting of ATP was increased upon substitution of sucrose. In aged preparations of vesicles, ouabain or acetylstrophanthidin decreased the rate of their calcium accumulation and partially reversed the inhibition of myofibrillar ATPase by the vesicles. It is suggested that the partial inhibition of calcium uptake increases the concentration of free calcium, and thus makes more calcium available for contraction of the myofibril.

ADDITIONAL KEY WORDS
endoplasmic reticulum
ionic medium
sucrose
microsomes
ATP splitting
calcium accumulation
dogs, rabbits

The precise mode of action of intracellular calcium as yet is not explained, but it becomes increasingly apparent that there are several sites of calcium binding in the cell. One of these seems to be the cell membrane and another the endoplasmic reticulum, often called the sarcotubular vesicles; each is associated with a different mechanism of calcium action. In the mammalian heart there exists an active mechanism of calcium accumulation located in the sarcotubular vesicles (1-3). In view of the role of calcium in myocardial contraction and the inotropic effect of digitalis on the contracting myocardium, the problem confronts us whether the inotropic effect of digitalis compounds can be associated with changes in the calcium level of the sarcotubular vesicles.

The availability of a method to prepare partially purified, highly active sarcotubular vesicles from cardiac tissue (1) made it feasible to study the effect of cardiac glycosides on the "calcium pump" of these vesicles. In this report, the calcium accumulation and ATP splitting in preparations of vesicles from dog hearts and the inhibition of myofibrillar ATPase in the presence of the vesicles have been investigated and the action of digitalis compounds on these preparations has been studied. ATP splitting was measured because it is directly related to myofibrillar contraction (4) and requires calcium under certain conditions (2, 5). Calcium uptake of sarcotubular vesicles as well as inhibition of myofibrillar ATPase by sarcotubular vesicles was determined as a measure of relaxation (6). The results indicate that the digitalis compounds under certain conditions depress the rate of calcium accumulation and partially reverse the inhibition of myofibrillar ATPase.
Materials and Methods

Preparation of vesicles. The method of preparation of the cardiac granules or vesicles is that described previously (1) with some modifications, primarily to adapt it to a different laboratory animal. In a typical experiment, a large (22 kg) dog was anesthetized with 200 mg of thiamylal (Surital, Parke Davis) iv, and exsanguinated from the femoral artery. The chest cavity was quickly opened and the heart excised. The heart was opened and placed in crushed ice. As soon as the heart was cold, it was cut into small pieces and dissected free of fat and blood vessels. The tissue (100 to 190 g) was homogenized in 200 to 380 ml (2 volumes) of ice-cold 0.3 M sucrose containing 2.0 mM ascorbic acid and 0.02 M Tris buffer, pH 7.2. The dissecting and homogenizing were carried out in a cold room. The homogenate was centrifuged in a Lourdes centrifuge for 20 min at 15,000 × g (11,000 rpm). The supernatant was filtered through gauze and centrifuged for 90 min at approximately 40,000 × g (21,000 rpm) in a Spinco Model L-2 ultracentrifuge using rotor no. 30. The pellet was suspended in 5 ml of 0.08 M NaCl-0.005 M sodium oxalate and homogenized by hand in a tissue grinder coated with teflon. Further purification was accomplished by gradient centrifugation in sucrose as described previously (1, 7). Protein concentration was determined by the method of Lowry et al. (8).

Assay for Calcium Uptake. To measure the rate of calcium uptake, incubation mixtures of 10 ml were prepared containing 5 × 10–3 M sodium oxalate, 5 × 10–3 M MgCl2, 5 × 10–3 M ATP (free of calcium) (9), 0.1 M NaCl, 0.01 M KCl, 0.01 M histidine buffer (pH 7.2), 1 × 10–4 M CaCl2, 0.1 µC 46Ca and 0.08 mg granule protein of the vesicle preparation/ml. The vesicles stored in sucrose on ice were brought to 0.8 mg protein/ml in 40% sucrose before addition to the incubation vessel. The final sucrose concentration in the incubation mixture was 4%. The incubation mixtures were immediately deproteinized in trichloroacetic acid and assayed for inorganic phosphate (11).

Assay for Inhibition of Myofibrillar ATPase. Rabbit skeletal myofibrils were prepared according to the method of Perry (12). Three measurements of ATPase activity were made; (A) in myofibrils with granules; (B) in myofibrils without granules; and (C) in granules alone. The reaction was measured in a standard medium (7) for 8 min at 22 to 25°C; it was started by addition of ATP and stopped by addition of 3 ml 10% trichloroacetic acid. The percent inhibition of the myofibrils by the vesicles is given by:

\[
1 - \frac{A-C}{B} \times 100.
\]

Additions of digitalis compounds to the basic medium were made to A, B, and C. All experiments were made in triplicate.

Aging of Vesicles. Artificial aging of granules was achieved by diluting the granule suspensions obtained by gradient centrifugation to 1.60 mg protein/ml with 0.08 M NaCl-0.005 M sodium oxalate. The suspensions were kept in ice for specified, accurately timed periods. The aging process was terminated by addition of sucrose to 40%.

Materials. The purification of ATP (Sigma) and of sucrose (Mann’s) with ion exchange resins has been described (1, 9). Ouabain was obtained either from Calbiochem, Nutritional Biochemicals or Sigma. Acetylstrophanthidin was a gift from Eli Lilly Company.

Results

On the average, 56 mg of vesicular protein was obtained from 100 g of dog myocardium after gradient centrifugation. This yield was considerably higher than previously obtained from rabbit hearts (1). Upon thorough examination with the electron-microscope, no intact mitochondria were found in the rabbit heart vesicles (1) prepared in the same manner as the dog heart vesicles described here. To test further for the presence of mitochondria in the vesicles from dog hearts, calcium uptake was measured in the presence of 5 mM sodium azide which inhibits the uptake of calcium by mitochondria without affecting calcium uptake by sarcotubular vesicles (13). Since there was no inhibition in the presence of sodium azide, these preparations must be essentially free of mitochondria.

The vesicles from the dog heart were more active than those from rabbit hearts. At a calcium concentration of 1 × 10–4 M, the calcium uptake often reached 1.25 µmole/mg protein in 8 min, uptake being limited at this point by the amount of calcium available. That the vesicles had an even greater capacity...
Calcium uptake (a) and ATP splitting by sarcotubular vesicles (b); •, 0.1 mM CaCl₂; ○, 0.2 mM CaCl₂.

for accumulating calcium was shown by their greater uptake when the calcium concentration was raised to 2 × 10⁻⁴ M (Fig. 1). In the absence of oxalate, calcium uptake was only 0.03 μmole/mg protein, similar to the uptake by bovine cardiac vesicles (3) and much lower than in rabbit skeletal muscle (14). Upon substitution of phosphate (0.1 mM) for oxalate, calcium uptake reached only 0.3 μmole/mg protein. The calcium uptake in the presence of oxalate was consistently high in the vesicles stored in sucrose over a period of 3 weeks, still showing some uptake after 4 weeks (longest time tested).

The calcium accumulation by the vesicles as a function of the ATP concentration is shown in Figure 2. As it is desirable for maximum activity to have the ATP in the form of Mg-ATP (15), the Mg concentration was kept the same as the ATP concentration. Between 1 and 10 mM, calcium uptake increased with an increase in the ATP concentration. The most rapid calcium uptake was obtained at an ATP concentration of 10 mM. In contrast to skeletal muscle vesicles (14), high concentrations of ATP were not inhibitory. Below 1 mM ATP concentration, calcium precipitated from solution as evidenced by low counts in the blanks without vesicles. To avoid inaccurate results due to possible insolubility of calcium salts and to insure high Ca uptake, 5 mM ATP was used throughout this investigation.

The effect of varying the concentration of Na and K ions was explored in another series of experiments. The following combination of concentrations was used: 0, 10, 30, 60, 100 and 120 mM NaCl each in combination with 0, 10, 30, 60 and 100 mM KCl (100 mM KCl was tested only up to 60 mM NaCl). The 16-min uptake was chosen for comparison. In the

Calcium accumulation as a function of ATP and Mg concentration. ATP and Mg concentration were varied simultaneously. Incubation time was 4 min.

Calcium uptake (a) and ATP splitting (b) in various ionic media; •, 0.12 mM NaCl; ○, 0.12 mM KCl; △, 172 mM sucrose, 24 mM NaCl, 10 mM KCl, 5 mM Na oxalate; A, 240 mM sucrose, 5 mM Na oxalate.

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particular preparation used, 0.8 μmole Ca was taken up per mg protein in 16 min. Whether the experiment involved a change in the Na-K ratio or in ionic strength, or in both, there was no significant change in calcium uptake with one exception: in the absence of both NaCl and KCl, uptake was decreased to 0.54 μmole of calcium/mg protein. Because otherwise no significant differences were found, the results are not given in graphic form. There was no significant change in the rate of calcium uptake by vesicles in a medium that contained either no KCl or no NaCl (Fig. 3). Furthermore, when isosmotic amounts of sucrose were substituted for 75% of the 120 mM sodium ion concentration, calcium uptake was unchanged; again no significant uptake occurred without oxalate.

On complete substitution with sucrose, the ATPase activity decreased simultaneously with the rate of calcium uptake, but it was increased on substitution of 75% of the Na with sucrose (see Fig. 3).

Addition of ouabain or of acetylstrophanthidin, chosen because of their relatively high solubility in water, in a large number of experiments in concentrations from 10^{-4} to 10^{-9} M usually showed no effect on Ca uptake. However, in preparations that were 3 or 4 weeks old and that showed a gradual decline in Ca uptake, this activity was often further decreased by 10% or more in the presence of 10^{-8} M ouabain. It was therefore attempted to artificially age the vesicles without disrupting them physically.

To accomplish this, suspensions of vesicles were diluted to 1.60 mg protein/ml with Na oxalate-NaCl. Figure 4 shows a typical experiment on the time course of aging, giving the 16-min Ca uptake of aliquots aged for various time intervals. In a series of 10 separate experiments, a decrease in the rate of Ca uptake of 6 to 18% with an average of 10% in the presence of 10^{-8} M ouabain or acetylstrophanthidin was observed in 8 experiments in which vesicles were aged sufficiently to show a Ca uptake between 0.3 and 0.7 μmole Ca/mg protein per 16 min, while 2 showed no change. In a second series of experiments, the rate of calcium uptake decreased in 9 out of 12 experiments, 2 showing no change, 1 an increase. The decrease amounted to 8 to 21% for the 8-min incubation period in the presence of 10^{-8} M ouabain or acetylstrophanthidin with an average of 12% inhibition. These calculations are based on raw counts obtained. In Figure 5a, 4 experiments, those in which data for all ouabain concentrations from 10^{-6} to 10^{-9} M were obtained simultaneously, are averaged. In the data presented in Figure 5, the average 8-min decrease is close to 30% due to the method of calculation. The inhibition was even greater for the 2-min incubation period (see Fig. 5b).
Inhibition of myofibrillar ATPase with fresh sarcotubular vesicles was 85 to 90%. The effect of ouabain became apparent only when the inhibiting activity of the vesicles declined, usually within a few days. In the range of 15 to 60% inhibition, 10^{-7} and 10^{-4} M ouabain further reduced the inhibition. Results for ouabain and acetylstrophanthidin are given in Table 1. Three additional experiments showed no change. Addition of ouabain to either fibrils or granules alone never produced a change in their ATPase activity. Inhibition of myofibrillar ATPase by 0.1 M EGTA, used in the place of vesicles, was not affected by ouabain or by acetylstrophanthidin.

**Discussion**

The present work further characterizes the process of calcium uptake by cardiac sarcotubular vesicles. The preparations from dog heart were more active than those previously obtained from rabbit hearts and showed little loss of activity when stabilized in sucrose, even over a period of 4 weeks. Inhibition of myofibrillar ATPase, on the other hand, showed a decline within 3 to 7 days and thus appears to be a more sensitive method than calcium uptake for measuring the activity of the vesicles under the conditions employed. The rate of Ca uptake in dog heart sarcotubular vesicles was as high as in sarcotubular vesicles from rabbit skeletal muscle (9) and so was the inhibition of myofibrillar ATPase. ATP is necessary for Ca uptake with the magnitude of uptake dependent on the concentration of ATP and magnesium. The requirement for high concentrations of ATP appears to be a fundamental difference between heart and skeletal muscle, and earlier statements as to low calcium accumulation in heart muscle (16) may have to be revised.

In view of a reported increase in 45Ca uptake and increased tension development of perfused papillary muscle upon lowering the Na concentration to 25% (17), it was surprising to see that replacement of 75% Na ions by isosmotic amounts of sucrose had no effect on Ca uptake by the vesicles (Fig. 3), while nearly complete substitution of Na produced only a slightly decreased initial rate of uptake. These results would indicate that the Ca:Na ratio...
antagonism (18) in cardiac muscle is not located in the part of the sarcotubular membrane that is concerned with active Ca uptake or is not directly concerned with the action of the calcium pump as far as calcium accumulation goes.

In spite of the insensitivity of the calcium uptake to changes in the relative concentrations of Na* and K*, the vesicular ATPase activity showed a maximum at a Na* concentration of about 29 mM (Fig. 3). Activation of the ATPase of the sarcotubular membranes by small amounts of Na* and K* has been demonstrated before (19, 20).

The basic mechanism of the positive inotropic effect of therapeutic doses of cardiac glycosides is still unknown. When the sarcotubular vesicles became identified with the storage of calcium controlling the relaxation-contraction cycle (21), it became imperative to investigate the role of the isolated sarcotubular vesicles in the action of cardiac glycosides. We have demonstrated a decrease in the ability of the vesicles to accumulate calcium and to inhibit myofibrillar ATPase in the presence of rather low concentrations of ouabain. In particular, the initial rate of calcium uptake was depressed. In the inhibition of relaxation, the primary effect of ouabain must be on the vesicles rather than on the fibrils, and relates to their ability to regulate the level of free calcium. In physiological terms, calcium accumulation by the vesicles may be considered as the basis for relaxation and decreased accumulation signifies contraction. Ouabain or acetylstrophanthidin could act in a way to make more free calcium available, and thereby increase cardiac contractility. The observation that the ouabain sensitivity was dependent on the functional state of the vesicles, as well as the extremely low concentration of ouabain and of acetylstrophanthidin in relation to calcium, exclude a simple binding of calcium to these compounds as explanation. A direct effect on the membrane would be more likely; such an effect would consist in delaying transport of calcium into the vesicles or accelerating leakage of calcium from the vesicles.

Finally, my findings are in agreement with a report that states that the inhibition of the syneresis of myofibrils caused by addition of fragmented sarcotubular vesicles (so-called "relaxing factor") was diminished by cardiac glycosides under certain conditions (22). The conditions were passage of electrical impulses of low frequency and voltage which partially reversed the inhibition of myofibrillar syneresis by the vesicles. The reversal was potentiated by $10^{-6}$ to $5 \times 10^{-7}$ M strophanthidin. These experiments were carried out at a vesicle concentration approximately 10 times that used in my experiments. In this case also, the action of the glycosides consisted in inhibiting the activity of the vesicles, and it manifested itself only under certain conditions. Partial inhibition of Ca uptake by cardiac glycosides in relaxing factor preparations in the presence of phosphate has recently been reported (23).

In view of the observations reported in this paper, it is proposed that a possible mechanism for the inotropic action of digitalis compounds in heart muscle contraction may be found in decreased calcium accumulation by sarcotubular vesicles and decreased myofibrillar ATPase inhibition. The former event would increase the concentration of free calcium, and thereby increase the amount of calcium available to the actomyosin system for contraction as evidenced here by greater myofibrillar ATPase activity.

References


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