Ion Effects on Calcium Accumulation by Cardiac Sarcoplasmic Reticulum

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ABSTRACT The effects of monovalent cations on the active calcium-accumulating ability of cardiac sarcoplasmic reticulum were assessed. Grana prepared in an ion-free system accumulated calcium when ATP and Mg^{++} were present. Sodium ion and to a lesser extent lithium but not K^+ reduced the amount of calcium taken up. The reduction of calcium binding by Na^+ is not due to inhibition of uptake but to a rapid release of the radiocalcium bound. The amount of calcium released by sodium does not appear to be enough to explain contraction on the basis of sodium influx into muscle, but may be significant in the regulation of tension.

INTRODUCTION

The essential role for calcium in muscle contraction is well established. Calcium is generally considered to be a principal link between excitation and contraction (for review see reference 1). Precisely how free calcium ion concentration is regulated in the region of the contractile elements is not clear. The finding of an active calcium-accumulating system in muscle (2) has suggested a primary role for the sarcoplasmic reticulum in the regulation of free Ca^{++} concentration and thus in the regulation of tension (3, 4).

It is well known that tension development in cardiac muscle is responsive to changes in the ionic composition of the extracellular fluid. The sodium ion is of particular importance in this respect (5). It was of interest therefore to test the effects of sodium and other ions on a calcium-accumulating system from cardiac muscle in order to observe any effects that might give some insight into how the sarcoplasmic reticulum might regulate tension under ordinary circumstances.

The calcium pump located in the sarcoplasmic reticulum of cardiac muscle has been discussed recently (6). Although active preparations have been prepared (6–8) care was not taken in these studies to eliminate monovalent cation contamination so that precise effects of these ions could be measured. It is the purpose of this report to examine effects of monovalent cations on a

preparation of cardiac grana rendered as free as possible from contaminating ions.

METHODS

Preparation of Grana Rabbit hearts removed quickly from animals killed by a blow on the head were minced with scissors in a cold (4°C) homogenizing medium of 0.25 M sucrose and 0.1 mM Tris-HCl pH 6.8 (HM). The mince was homogenized by hand in 10 volumes of HM with all glass TenBroeck homogenizers. The homogenate was centrifuged at 1,000 *g* for 10 min in a Lourdes Model AA-C centrifuge (Lourdes Instrument Corp., Brooklyn, N.Y.). The pellet was washed with 2.5 volumes of HM and discarded. The supernate with added washings was centrifuged at 10,000 *g* for 20 min to remove mitochondria. The mitochondria-free supernate, checked by phase contrast microscopy, was spun at 78,000 *g* for 45 min in a Spinco Model L preparative ultracentrifuge at 2°C. This pellet was washed with 5 volumes of cold HM and then resuspended in HM in a volume equal to 10 times the weight of the original tissue in grams. This suspension (grana) contained 0.8 to 1.2 mg protein per ml as ascertained by the method described by Lowry et al. (9) using purified bovine serum albumin as standard. All preparations used were less than 24 hr old. Preparations older than 24 hr lost their ability to accumulate calcium.

Ion Content The amount of Ca^{++}, Na^{+}, and K^{+} in grana was determined by preparing the grana by a deproteinization method described by Rahill and Walser (10). Sodium and K^{+} were measured in an Eppendorf flame photometer (Brinkmann Instruments, Great Neck, N. Y.). The grana fraction contained 0.4 ± 0.02 μeq Na^{+} per mg protein and 0.2 ± 0.03 μeq K^{+} per mg protein. The calcium content of this preparation was measured by an EDTA titration method described by Walser (11). The amount of calcium determined by this technique was 0.12 ± 0.05 μmole per mg protein.

Calcium Uptake Grana suspensions (1.2 ml) were mixed with 10.8 ml of a solution containing 3 mM histidine-imidazole, pH 6.8, 20 μM ^{45}Ca (0.5 μc per ml) and other reagents that were used in the concentrations reported in the figures and tables. The mixture, containing 0.08 to 0.12 mg protein per ml, was incubated at 37°C for 10 min, then 5 ml aliquots were pipetted into a Millipore suction apparatus, connected to a vacuum line with a 25 mm diameter type HA Millipore filter pore size 0.45 μ. With this filter no protein was detected in the filtrate. Filtration of the entire 5 ml aliquot occurred within 2 sec. A total of 16 such tubes could be prepared and filtered at one time.

The trapped sediment on each filter was washed with 10 ml of the identical incubating solution without grana but with the same concentration of "cold" calcium substituted for ^{45}Ca. Whether cold calcium was included in the wash did not affect the degree of calcium binding, suggesting that the bound calcium was not readily exchangeable with the wash solution under these conditions. Experiments were always performed in duplicate. The filters were removed, dried, then placed in scintillation vials containing fluid as described by Loftfield and Eigner (12). Standards were prepared by placing 50 μl of ^{45}Ca of known activity on a Millipore filter, drying

it, then placing it in the scintillation vial as above. Counting was performed in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Company Inc., Downers Grove, Ill.). Counts per filter were in the range 5,000 to 100,000 per min. Controls containing all substances except grana were run simultaneously in each experiment, washed in the same manner, and represented the amount of ^{45}Ca bound nonspecifically to the filter paper. This amounted to less than 3 % of the experimental counts and was subtracted from each value when calculated. ATPase activity was measured on the filtrate by determining inorganic phosphorus (Pi) by the method of Fiske and Subbarow (13).

Rapid Addition Experiments To add substances rapidly to grana already loaded with ^{45}Ca, the following experiment was devised. Grana were incubated in a single tube at 37°C with ATP, Mg^{2+}, and ^{45}Ca with or without 3 mM oxalate in a volume of 17 ml. After 10 min three 5 ml aliquots were removed, mixed rapidly with 0.5

TABLE I
EFFECT OF OXALATE AND PHOSPHATE ON CALCIUM ACCUMULATION

Experimental conditions as in Methods.

Grana + 3 Mg^{2+} + 0.02 ^{45}Ca Cl_2 plus*	μMoles Ca^{2+} bound per g protein‡
0	0.8
3 ATP	5.1 (4-8)
3 ATP + 3 K-oxalate	40 (17-78)
3 ATP + 10 K-phosphate	30 (25-40)

*Preceding Nos. refer to final concentration in millimoles per liter.
‡ Means of five experiments; range of values in parentheses.

ml of either 1.0 M sucrose, NaCl, or KCl, and filtered. Mixing and filtering required no more than 3 sec in any instance, so that the test solution was in contact with the calcium-loaded grana no longer than this. Each filter was washed with 5 ml of 0.25 M sucrose.

Chemicals All salts were chlorides, analytical reagent grade (Fisher Chemical Co., Atlanta, Ga.). These salts were freed of other metals by passage through a Dowex A-1 column in the appropriate cationic form. Disodium ATP (Sigma Chemical Co., St. Louis, Mo.) was rendered sodium- and calcium-free by treatment with Dowex-50 in the H^+ form, and then by neutralizing the free acid with solid Tris (Trizma® Sigma) to pH 6.8.

RESULTS

Characterization of Calcium Uptake by Cardiac Grana

The value of 5 μmoles Ca^{2+} bound per gm protein in the absence of oxalate (Table I) may be compared with the value of 10 reported by Fanburg et al. (14) and with the calculated value of 12 reported by Lee et al. (15). In the presence of oxalate an average value of 40 in the present experiments agrees

with values obtained by Fanburg et al. Pyrophosphate also enhances calcium accumulation in this fraction in a manner similar to the enhancement seen with oxalate. Carsten's results (8) are in general agreement with those reported here. The above investigators all used an isolation medium of high ionic strength. It should be noted that the amount of Ca^{2+} uptake by cardiac grana is roughly 10-fold less than has been reported for skeletal muscle grana under similar conditions.

EFFECT OF ATP Varying concentrations of ATP were tested in the absence of oxalate (Fig. 1). ATP splitting was measured simultaneously. Maximum calcium uptake occurred at 0.3 mM ATP and did not increase at higher concentrations. As expected, the rate of ATP splitting was linear with ATP concentration but was not directly proportional to calcium uptake.

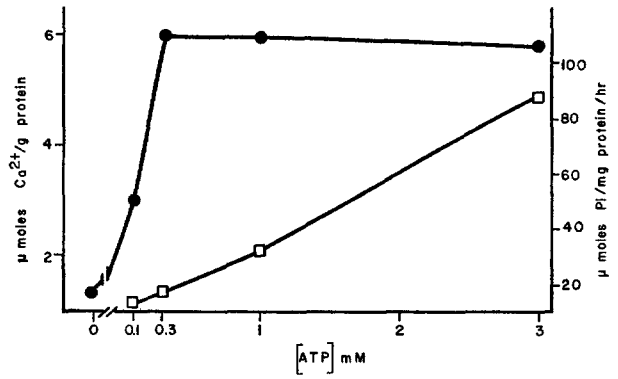

FIGURE 1. Effects of varying ATP concentrations on calcium uptake by cardiac grana in the absence of oxalate (filled circles). ATP hydrolysis (open squares) on right ordinate. Incubation conditions 37°C, 10 min. Other conditions as in Methods. Each point is the mean of four experiments.

When oxalate was present (Fig. 2), calcium uptake was severalfold higher and was maximal at 3 mM ATP. The curve followed the curve of ATP hydrolysis as previously shown (2).

RATE OF CALCIUM ACCUMULATION By filtering aliquots of a grana suspension at different times the rate of calcium uptake was determined as shown in Fig. 3. Initial uptake rates (<15 sec) appear to be independent of oxalate. Incubation periods beyond a minute show a steady increase in calcium uptake when oxalate is present whereas in the absence of oxalate, a relatively constant value is achieved after 5 min. In the experiments reported below, a 10 min incubation period was chosen arbitrarily to determine ion effects.

EFFECT OF IONS Table II shows the effects of incubating grana with ATP and Mg^{2+} in the presence and absence of Na^+, K^+, or Li^+. There is a

significant reduction of Ca^{++} uptake by Na^+ and Li^+, but not by K^+. The net reduction by Na^+ amounts to 2 μmoles Ca^{2+} per g of reticular protein. This effect seems to be a specific effect of Na^+ or Li^+ rather than one of ionic strength since all ions are present in the same concentrations.

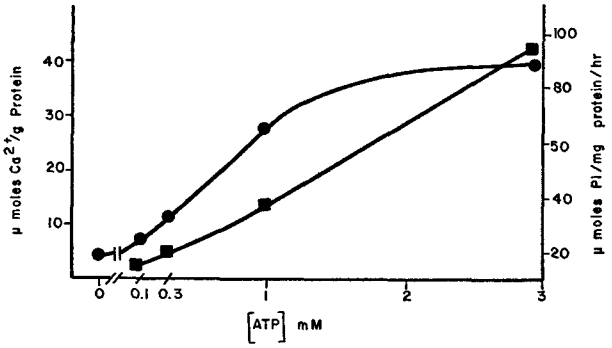

FIGURE 2. Effect of varying ATP concentrations on calcium uptake in the presence of 3 mM K oxalate. ATP hydrolysis on right ordinate. Mean of two experiments. Other conditions are the same as in Fig. 1.

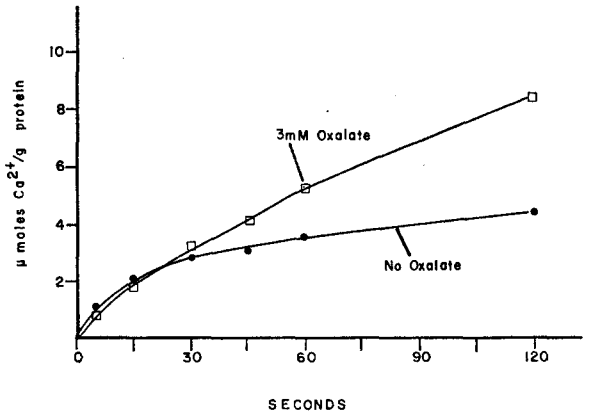

FIGURE 3. Rate of calcium accumulation by grana in the presence and absence of oxalate. These are the results of a single representative experiment in which 2.5 ml aliquots of a reaction mixture containing 0.02 mM $Ca^{45}Cl_2$, 3mM Tris-ATP, 3 mM $MgCl_2$, and 0.1 mg grana/ml were removed at various times, filtered, and washed. A similar reaction mixture with 3 mM K oxalate was run simultaneously.

In Fig. 4 the effects of graded concentrations of Na^+ and K^+ are presented. The effect with Na^+ becomes significant at 1 mM. There is no effect of K^+. At higher concentrations (200 mM) calcium uptake is depressed equally by both ions, presumably an ionic strength effect as previously reported (14). It is possible to calculate a rough "K_m" for the release effect. Half-maximal release of calcium occurs at 1.0 mM Na^+ (Fig. 4).

It is to be noted that grana washed in sodium and then recentrifuged and resuspended in potassium accumulated calcium to the same extent, demonstrating that the reduction in calcium binding by Na^+ is reversible (data not shown).

TABLE II
EFFECT OF NaCl, KCl, AND LiCl ON CALCIUM UPTAKE BY CARDIAC GRANA IN THE ABSENCE OF OXALATE
See Methods for details.

Conditions	Calcium uptake	
Grana + 3 Mg^{2+} + 3 ATP + 0.02 ^{45}Ca plus*	μMoles Ca^{2+} per g protein ± SEM (n in parentheses)	Relative binding‡
0§	4.45±0.39 (17)	1.0
100 KCl	4.85±0.80 (17)	1.07±0.04
100 LiCl	3.20±0.6 (4)	0.73±0.03
100 NaCl	2.44±0.30 (17)	0.59±0.01
No ATP	0.81±0.12 (12)	—
No ATP + 100 NaCl	0.61±0.21 (7)	—

* Preceding Nos. refer to final concentration in millimoles per liter.
‡ With each experiment when ions were tested, a control without ions was simultaneously run. The value obtained with ions is expresed as a ratio of the value obtained with the appropriate ion-free control. These ratios obtained with each experiment were then averaged and standard errors computed.
§ Na^+ + K^+–"free" controls contained less than 0.04 mM Na^+ and less than 0.02 mM K^+.

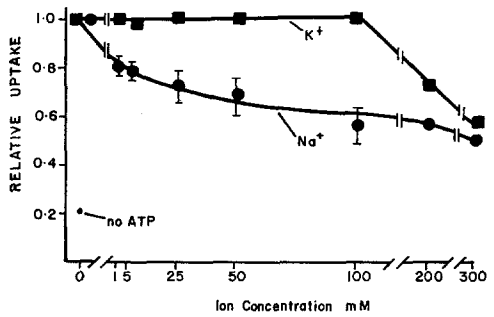

FIGURE 4. Effect of varying concentrations of Na^+ and K^+ on Ca^{2+} accumulation in the absence of oxalate or pyrophosphate. Relative uptake is calculated as in Table II. Ions were incubated for 10 min with 3 mM ATP, 20 μM ^{45}Ca, grana, 3 mM Mg^{2+}, and 0.25 M sucrose. Other details as in Methods. Na^+ n = 7, K^+ n = 5.

EFFECT OF ION COMBINATIONS An ATPase stimulated by Na^+ + K^+, but not by either ion alone, has been described in cardiac muscle (16, 17). The ultracentrifugal fraction in which this enzyme is found is not dissimilar from that of the preparation used in this report. Combinations of Na^+ + K^+ which would give maximal activation of the ATPase were tested on calcium binding (Table III). There was no additional effect of the ion combinations that could

not be explained by the effect of a single ion alone. In other words, the effect of sodium and potassium together on calcium binding can be explained by the effect of the Na^+ in the medium. There was no synergism when both ions were present. In addition, there was no enhanced ATP hydrolysis in the presence of $Na^+ + K^+$, nor was there inhibition by 10^{-4} M ouabain (data not shown) so that the operational requirements for the demonstration of this enzyme were not present. This result is similar to that obtained by Lee et al. (18) in skeletal muscle. Schwartz (17) has shown a requirement for desoxycholate in the

TABLE III
EFFECT OF VARYING COMBINATIONS OF Na^+ AND K^+ ON CALCIUM UPTAKE

See Methods.

Conditions	
Grana + 3 ATP + 3 Mg^{2+} + 0.02 ^{45}Ca plus*	μMoles Ca^{2+} bound per g protein‡
0	6.0
100 K^+ + 5 Na^+	4.9
100 K^+	6.7
5 Na^+	4.9
100 K^+ + 10 Na^+	4.5
10 Na^+	4.6
100 Na^+ + 5 K^+	4.4
100 Na^+	4.3
100 Na^+ + 10 K^+	4.22
100 Na^+	4.3
No ATP	0.96

* Preceding Nos. refer to final concentration in millimoles per liter.
‡ Means of five experiments on two separate preparations.

homogenizing medium to elicit $Na^+ + K^+$–dependent ATPase activity. Since we did not use desoxycholate, this perhaps explains our failure to demonstrate such activity in this preparation.

EFFECT OF RAPID ADDITION OF IONS The effect of sodium in reducing the amount of calcium taken up by the grana can be interpreted either as an inhibition of uptake, or as a release phenomenon. To solve this problem, sodium and other substances were exposed to calcium-loaded grana for less than 3 sec (see Methods). The results of these experiments are presented in Table IV. This table clearly shows that calcium already bound is rapidly released to a maximum value within 3 sec. The value obtained with rapid

Na^+ addition is no different from that obtained with incubation with 100 mM Na^+ for 10 min (Table II). Again, K^+ had no effect.

UPTAKE OF CALCIUM AND ION EFFECTS IN THE PRESENCE OF OXALATE Since previous studies in which oxalate was used did not show a distinct ion effect except at high ionic strengths, it was presumed that oxalate might load the granule with calcium in a form that sodium could not release. Accordingly, Na^+ and K^+ were added to grana that had been loaded previously with calcium in the presence of oxalate. Table IV shows that sodium produces the same absolute reduction of calcium uptake in oxalate-loaded as in oxalate-free granules. The data suggest a distinct pool of calcium (approximately 2 μmoles per g) that is susceptible to release by sodium but is independent of

TABLE IV
THE EFFECT OF RAPID ADDITION OF EITHER SUCROSE, NaCl, OR KCl TO A FINAL CONCENTRATION OF 100 mM TO GRANA THAT HAVE ALREADY ACCUMULATED ^{45}Ca*

Incubation conditions	Calcium μMoles per g protein ± SEM		
3 Mg^{2+} + grana + 0.02 ^{45}Ca plus‡	*Sucrose*	*NaCl*	*KCl*
3 ATP	5.1±0.2	3.04±0.3	5.2±0.18
3 ATP + 3 oxalate	49.6±0.5	47.5±0.6	50.2±0.7
0	0.81±0.07	0.74±0.09	0.87±0.08

$n = 5$.
* For experimental details see Methods.
‡ Preceding Nos. refer to final concentration in millimoles per liter.

oxalate storage. However, sodium had no effect on the small amount of calcium bound in the absence of ATP (Table II).

An explanation for the failure of others (8, 14, 19) to observe an ion effect is most likely afforded by the fact that the relatively high uptakes of calcium achieved in the presence of oxalate masked the inhibition noted here. As discussed below, the total amount of calcium (2 μmoles per g) that is released by sodium, not the per cent, is critical and perhaps physiologically significant.

DISCUSSION

By working with a system relatively free of Na^+ and K^+, the effects of these ions on calcium uptake by cardiac grana can be assessed. Sodium, and to a lesser extent lithium, but not potassium, causes a rapid release of some of the calcium accumulated by cardiac grana. The amount released by sodium is relatively constant in the presence or absence of oxalate and amounted to 1-2 μmoles per g of reticular protein. It is impossible to say whether the increased radioactivity in the preparation represents a net uptake of calcium or whether it represents ATP-dependent calcium exchange with that calcium already

present. However, in either instance the reduction in radioactivity after mixing with Na^+ or Li^+ must represent a net loss of calcium bound. This release may be due to an exchange of Na^+ with the bound ^{45}Ca. This "sodium-labile" calcium fraction was actively accumulated in the presence of ATP, but it seems unlikely that its release by Na^+ is energy-dependent. The increased amount of calcium that was taken up in the presence of oxalate does not appear to be sodium-labile as there was no increase in the total amount released when oxalate-loaded granules were treated with Na^+ (Table IV). The release of calcium by Na^+ is rapid and the limits of our present techniques do not permit a precise estimate of the rate except to say that it is within 3 sec.

Our grana fraction contains at least two calcium storage sites; i.e., one affected by Na^+, the other not. In the absence of oxalate, the sodium-labile fraction amounts to about a third of all calcium taken up. The calcium site, not affected by Na^+, stores calcium as a relatively inert pool not released by Na^+. Weber et al. (3) have proposed an interplay between two storage sites that may be similar to the situation here described. Our present experiments do not permit us to say whether the oxalate-stored calcium is available for sodium release at a slow rate; e.g., that calcium stored in the grana as oxalate or pyrophosphate can be converted to the sodium-labile fraction.

It is tempting to speculate that the Na^+ release effect is somehow tied in with the initiation of contraction and relaxation. For example, with depolarization in the transtubular system (T system) adjacent to the lateral vesicles (presumably the primary structure in our grana fraction and that intracellular structure that sequesters calcium), sodium would enter and release calcium accumulated by the lateral vesicles, thereby initiating contraction. The entering sodium, when it is removed presumably by a pump ATPase, would signal relaxation by reducing the concentration of sodium in the region of the lateral vesicle to a point at which it would no longer block calcium uptake. Before this proposal can be entertained there are some quantitative factors which must be considered.

Is the amount of calcium released by sodium (about 2 μmoles per g reticular protein) enough to initiate and sustain a contraction? Obviously, this question depends on the amount of sarcoplasmic reticulum present per unit of actomyosin. The amount of actomyosin present per gram of muscle is of the order of 200 mμmoles (3). The amount of sarcoplasmic reticulum per gram of muscle is much more difficult to obtain as no figures are available for cardiac muscle. Our yield of "reticular" protein with the method of isolation described is approximately 10 mg per g of muscle. This 10 mg undoubtedly represents more material than lateral vesicles and probably the actual figure is less (6). Nevertheless, it seems valid to use this figure in calculating the amount of calcium released per gram of heart muscle since it represents the amount of "calcium-accumulating protein" present in our assay system. When this figure

is used, entering sodium ion could release only 20 mμmoles of calcium per g of muscle which would account for less than 10% saturation of the actomyosin present. Microinjections of Na^+ into skeletal muscle (22, 23) have failed to produce visible contractions. Similar studies have not been performed on cardiac muscle. Although our data do not support a primary role for Na^+ entry as the initiator of contraction, it is entirely possible that entering Na^+ can influence tension. A recent study by Langer (20) concluded that Na^+ competes with calcium in the region of the sarcoplasmic reticulum as a potential explanation for the inotropic effect of a low external Na^+ concentration. His explanation is circumspect but supports previous observations by Luttgau and Niedergerke (21) that there is a primary role for Na^+, mediated through calcium in regulating tension in cardiac muscle. We feel our data are pertinent to these observations.

It is perhaps appropriate now to consider the endogenous intracellular sodium concentration in muscle reported to be in the millimolar range. It is not clear whether or not this intracellular Na^+ is in the ionic form in cardiac muscle. For Na^+-Ca^{++} competition to have any significance physiologically it must be postulated that this Na^+ inside the muscle cell in the resting state must be either not available for calcium competition (e.g. compartmentalized) or rendered nonionic by intracellular binding. There is little evidence to support either of these alternatives at present.

Our data do not support a primary role for Na^+ in the initiation of contraction in cardiac muscle. This conclusion follows a previous conclusion of Sandow's in skeletal muscle (24). Perhaps the finding of a clear-cut Na^+-Ca^{++} competition at the sarcoplasmic reticulum level albeit of small magnitude may be of value in the interpretation of the effect of drugs that perhaps produce their inotropic effects by alteration of membrane Na^+ transport; e.g., cardiac glycosides.

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