Effects of ATP on the Interaction of Ca^{++}, Mg^{++}, and K^{+} with Fragmented Sarcoplasmic Reticulum Isolated from Rabbit Skeletal Muscle

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ABSTRACT Fragmented sarcoplasmic reticulum isolated from skeletal muscle of the rabbit has a cation-binding capacity of about 350 μeq/g of protein at neutral pH. The same binding sites bind Ca, Mg, K, and H ions and, consequently, the selective binding of Ca induced by ATP releases an amount of the other cations equivalent to the Ca taken up. At pH values below 6.2, an increasing number of binding sites are associated with H^{+}, and ATP induces exchange of Ca mostly for H^{+}. At pH values above 6.2, the binding sites exist in the form of Mg and K, and Ca is bound in exchange for these cations. The total bound Ca + Mg + K, expressed in microequivalents of cations bound per gram of protein, is approximately constant at various pCa values, which indicates a stoichiometric exchange of Ca for the other cations. To accomplish the same degree of exchange of Ca for other cations bound, in the absence of ATP, concentrations of free Ca^{++} of about 1000-fold higher than those needed in the presence of ATP are required in the medium. We cannot distinguish between a mechanism whereby Ca actively transported into a compartment of the microsomal vesicles containing also the binding sites is bound passively to these sites in exchange for Mg, K, and H and another in which ATP selectively increases the affinity of surface-binding sites for Ca. Irrespective of the mechanism of accumulation, the Ca retained does not contribute to the activity of the cation in the membrane fraction. Caffeine (10 mM) has no effect on the binding of Ca, but releases a more labile fraction of Ca, which presumably accumulates in excess of the bound Ca. Procaine (5 mM) antagonizes the effect of caffeine. Acetylcholine and epinephrine have no effect on the binding of Ca.

The contraction-relaxation cycle evoked in a muscle cell by depolarizing the plasma membrane is probably regulated by a transient increase in the concentration of free calcium ions in equilibrium with the myofibrils (1–4). Ebashi (5, 6), Hasselbach (7, 8), Weber (9–11) and their coworkers have shown that

ATP[1] induces the microsomal fraction isolated from skeletal muscle to bind Ca avidly. This fraction is composed of fragmented membranes of the tubular sarcoplasmic reticulum (and presumably of the transverse tubular system) which ramifies extensively within the muscle cell (12, 13). Peachey (13) estimated recently that the surface areas of the membranes of the sarcoplasmic reticulum and of the transverse tubules of a muscle cell 100 μ in diameter are, respectively, 50 and 7 times the area of the sarcolemma. This extensive membranous network with a high affinity for Ca is considered adequate to maintain the Ca associated with actomyosin in muscle at rest below the level required to activate the contractile mechanism (10, 14, 15). Stimulation of the muscle cell releases Ca from the sarcoplasmic reticulum by a mechanism not yet understood, and the Ca so released triggers contraction by interacting with the myofibrils until it is reabsorbed by the sarcoplasmic reticulum (16).

We have conducted studies on the isolated microsomal fraction of rabbit skeletal muscle (also referred to as microsomal vesicles or relaxing factor) to determine the nature of the interaction of the reticular membranes with Ca. We have been particularly concerned with the state of Ca accumulated by the isolated membrane fragments since it is of fundamental importance to know whether this Ca is accumulated by an active transport process or merely by adsorption at binding sites of the lipoprotein membranes. Although results of some experiments conducted in the presence of oxalate suggest that isolated microsomal vesicles transport Ca against an activity gradient at concentrations of external Ca of the order of 10^{-7} M (7, 11), the concentration of free Ca in the vesicles estimated from the solubility product of calcium oxalate is less than 1 % of the total Ca accumulated. In the absence of oxalate, the concentration of free Ca in the vesicles may be higher, but there is no good estimate of the magnitude of the value. We also have no information on the magnitude and polarity of the electrical potential which may exist across the isolated vesicular membranes or across the tubular membranes *in situ*. Therefore, one cannot speak with assurance of an active transport process in this system, which is poorly defined with respect to parameters germane to the definition of active transport (17).

We have shown previously (18) that binding sites do exist in these membranes which could account for an accumulation of cations, including Ca, of about 350 μeq/g of protein at neutral pH. We have now investigated the interaction of Ca, Mg, and K with the microsomal fraction from the skeletal muscle of the rabbit under conditions for selective uptake of Ca. There is an interdependence of the binding of the three cations at binding sites of the fragmented reticular membranes which suggests that the cations compete for the same binding sites and that about 80 % of the Ca binding induced by ATP

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*-tetraacetic acid.

occurs by displacing Mg and K from the binding sites of the membranes. Caffeine, which causes contracture of muscle and potentiates the twitch presumably by releasing Ca from the sarcoplasmic reticulum (16, 19, 20, 43), does not release Ca adsorbed at binding sites of fragmented sarcoplasmic reticulum, but mobilizes a more labile fraction of Ca retained by these structures.

METHODS

Preparation of Biological Material The microsomal fraction of rabbit skeletal muscle was isolated by differential centrifugation. Muscle tissue obtained from the back and legs of adult rabbits weighing about 3 kg was ground in a meat grinder and then was suspended in 4 volumes of 100 mM NaCl–5 mM imidazole solution of

TABLE I

TOTAL LIPID AND PHOSPHOLIPID CONTENT OF MICROSOMES FROM SKELETAL MUSCLE OF RABBIT

Treatment of microsomal fraction	Percentage of dry weight		Ratio of chemical fraction to nitrogen		Percentage of phospholipid in lipid fraction	Dry weight / Nitrogen weight
	Total lipid	Phospho-lipid	Total lipid	Phospho-lipid		
None	44	27	3.7	2.3	62	8.5
Washed once with 0.6 M KCl	45	27	3.9	2.4	62	8.6

pH 6.8 at 0°C. This suspension was homogenized in a Waring Blendor for 30 sec, and the homogenate was centrifuged at 39,100 *g* for 1 hr. The supernatant solution was discarded and the combined pellets were resuspended in a solution of NaCl-imidazole as above and homogenized for 30 sec. This suspension was centrifuged at 7970 *g* for 20 min, and the supernatant solution was collected and centrifuged again at 7970 *g* for 10 min. The microsomal fraction was isolated from the supernatant solution obtained after the 10 min centrifugation. This fraction consists of the material which sediments during centrifugation for 1 hr at 39,100 *g* and is the one designated "relaxing factor." Routinely this fraction was washed once with 50 volumes of 100 mM KCl–1 mM imidazole solution of pH 6.8 at 0°C and stored in suspension in it at a protein concentration of 10 to 20 mg/ml. The yield varied from one preparation to another, but was usually about 200 mg/100 g of muscle.

Relaxing factor isolated by this procedure has a lipid content of about 44% (Table I) and presumably represents a relatively homogeneous membranous fraction, free from myosin and actomyosin, since extracting the fraction obtained with 0.6 M KCl does not increase its lipid content or its ability to bind Ca in the presence of ATP. Dr. A. Weber, who first suggested this procedure to us, recently reported additional properties of the preparation (11).

Actomyosin from skeletal muscle of rabbit was prepared from homogenized muscle extracted for 24 hr with Weber-Edsall solution, as described previously (23).

Determinations of Lipid, Phospholipid, and Dry Weight in Microsomes The lipids of the microsomal fraction were extracted by a modification of the procedure of Schneider (24). Samples of microsomal suspensions containing 3–6 mg of nitrogen were transferred to tared 12 ml conical centrifuge tubes, and 5 ml of 10% trichloroacetic acid at 0°C were added to each tube. The contents of the tubes were then mixed and the tubes were kept in ice for 30 min. The precipitate was stirred occasionally with a glass rod. At the end of this period, the tubes were centrifuged in an International table centrifuge at top speed until well packed pellets formed (5–10 min). The supernatant fluid was discarded, and the pellets were washed twice with 10 ml of water each time by resuspending them in the water and centrifuging as before.

Series of microsomal samples treated as described above were either dried at 105°C for 15 hr and their dry weight determined, or their lipids were extracted in ethanol and a 3:1 mixture of ethanol-ether (24). 3 ml samples of the extracts were quickly digested in 25 ml Kjeldahl flasks with 0.5 ml of 10 *N* sulfuric acid. Two drops of 30% hydrogen peroxide were added toward the end of the digestion after the samples had been allowed to cool somewhat; then the Kjeldahl flasks were rinsed with water. The digestion was then allowed to proceed until all the water had evaporated, and the samples were clear. The total time for the digestion to go to completion is 35–45 min.

The digested samples were analyzed for inorganic phosphate by the method described by Taussky and Shorr (25). From the total phosphate extracted with the alcohol and ether treatment from the microsomal samples, we calculated the amount of phospholipid in the original microsomes by multiplying the weight of phosphate extracted by a factor of 25 (26).

The total lipid content of microsomes was determined from the difference in dry weights of the microsomes before and after extracting the lipids.

Binding of Cations by Microsomes We studied the ability of the microsomal fraction to bind cations in the absence and in the presence of ATP. The uptake of cations in the absence of ATP we designate "passive binding" and the uptake in excess of the passive binding induced by ATP we shall refer to as "active binding" without implying that such binding is necessarily an active transport process; i.e., a transport of the ions against an electrochemical gradient.

Samples of washed relaxing factor containing 10–15 mg of protein were transferred to 13 ml polypropylene tubes containing the cations in the chloride form and buffers at 23°C as indicated in the figure legends. The volume in the tubes was made up to 10 ml and after 10 min the tubes were centrifuged for 15–30 min at 0° to 5°C in a Spinco model L centrifuge at 105,300 *g*. In studies of the effect of ATP on the binding of Ca, Mg, and K by relaxing factor, ATP was added to the polypropylene tubes immediately before centrifugation. The supernatant solutions were collected, and the pellets and internal walls of the tubes were rinsed with deionized water. Cations trapped in the pellets in conjunction with supernatant solution were removed by resuspending the pellets in 9 ml of 0.25 M sucrose and centrifuging the suspensions in the Spinco centrifuge for 30 min as before. The cations in the washing solutions were measured, and corrections for cations trapped in the pellets in conjunction with washing fluid were applied. The pellets collected were resuspended in 7.5 ml

of deionized water, and samples of this suspension were taken for analyses of protein, Ca, Mg, K, and phosphate.

The passive and active binding of cations by relaxing factor are rapid reactions which proceed to completion during the time required for centrifuging the microsomes (6, 18). Washing the microsomal pellets with a nonionic solution (sucrose in this case) after the cations are taken up allows those cations that may have been maintained in the vesicular structures against a chemical gradient to leak out of the vesicles, and only those cations associated with nondiffusible anions within the vesicles or in the vesicular membranes are retained.

Analysis of Cations The concentrations of cations in the microsomal suspensions and in the supernatant solutions were determined by absorption spectroscopy with a Perkin-Elmer absorption spectrophotometer, model 303. The protein-free supernatant solutions were analyzed directly. The cations in the microsomal suspensions were first extracted with trichloroacetic acid (final concentration 5%), and their concentrations were subsequently determined. Interferences of phosphate and Na^+ or K^+ with the analyses were obviated by the presence in samples and standards of 0.5% La^{+++} and approximately equal concentrations of Na^+ or K^+ (27). Conditions were chosen to facilitate analyses of all cations of interest in any one sample.

Analysis of Phosphate We determined the amount of ATP hydrolyzed by relaxing factor from the concentration of inorganic phosphate appearing in the supernatant solution after centrifuging the microsomal suspensions immediately after adding ATP. The concentrations of inorganic phosphate were determined by the method described by Taussky and Shorr (25). Inorganic phosphate was also determined by the method of Chen et al. (28) in extracts of microsomal pellets prepared by precipitating the proteins with cold 5% trichloroacetic acid. In these extracts the concentration of phosphate was too low to be determined by the Taussky and Shorr technique.

Analyses of Protein These were performed by the biuret method (29) standardized by Kjeldahl analysis of nitrogen.

Superprecipitation The rate and extent of superprecipitation of actomyosin suspensions at 23°C were determined in a volume of 3 ml from turbidity measurements ot the suspensions made with a DU Beckman spectrophotometer at 550 mμ in 1 cm cells (22). We adapted a magnetic stirring device to the spectrophotometer to permit constant mixing of the suspension while the superprecipitation reaction proceeded. It consists of a small electric motor attached to the bottom of the cell compartment of the spectrophotometer to drive a magnetic bar inside the cell. The reaction was initiated by adding 100 μl of 30 mM ATP, and, at specified times, 100 to 300 μl of various reagents (caffeine, 50 mM; procaine, 50 mM; and adrenaline, 5×10^{-4} M) were also added, as indicated in Fig. 9.

Reagents The ATP, phosphocreatine, and creatine phosphokinase were obtained from Sigma Chemical Company. The ATP and phosphocreatine were used in the disodium form. Contaminating Ca in ATP was not removed except in experiments whose results are summarized in Fig. 9. The procedure for removing Ca from ATP has been described elsewhere (23). All other compounds were also of reagent grade.

RESULTS

Lipid Content of Microsomes The lipid and phospholipid contents of the microsomal fraction of rabbit skeletal muscle as isolated by our procedure are summarized in Table I. In terms of dry weight, the fragmented sarcoplasmic membranes contain 44 % lipid, 62 % of which is phospholipid; thus the phospholipid fraction accounts for 27 % of the total dry weight of the microsomal fraction. The ratios of lipid, phospholipid, and dry weight to total nitrogen are 3.7, 2.3, and 8.5, respectively. These values were reproducible from preparation to preparation even when the preparations were washed several times with 0.1 M KCl. Values reported by different investigators (30–32) vary, but are consistent in indicating that the membranous fragments isolated in the microsomal fraction of muscle are mainly a lipoprotein complex in which a large fraction of the lipid is phospholipid; this observation has also been made for other cellular membrane systems (33). Washing the microsomal fraction with 0.6 M KCl solubilized 5–15 % of the total protein, but apparently a proportional amount of lipid was also lost by this treatment since the composition of the material sedimenting was similar to that of the microsomal fraction washed with 0.1 M KCl (Table I). These observations suggest that contamination of the microsomal preparations with myosin and actomyosin was relatively small. Since the microsomal preparations treated with 0.6 M KCl did not show any increase in the active binding of Ca over those washed with 0.1 M KCl, we conducted the following binding studies with microsomes washed with 0.1 M KCl.

Effect of ATP on the Binding of Ca, Mg, and K by the Microsomal Fraction The microsomal fraction of skeletal muscle of rabbit binds Ca, Mg, and K and the binding of each of these cations is influenced by the concentration of H^+ and other cations in the medium (18).

In the absence of ATP and Ca (pCa > 8) (pCa = −log of molar concentration of free Ca^{++}), 290–300 μeq of Mg/g of protein are bound by the relaxing factor if the equilibrating medium contains 3.8 mM Mg and 10 mM K, at pH 6.9. This Mg is displaced by Ca as the concentration of Ca in the equilibrating medium is increased; at a Ca concentration of about 2.0 mM (a ratio of free Mg to free Ca of 1.6 to 1.8) the Ca displaced 50 % of the Mg originally bound (Fig. 1).

If in addition to Ca, we also added ATP (1.0 mM) to the medium, a concentration of Ca of the order of 10^{-6} M sufficed to displace 50 % of the Mg. Both in the absence and in the presence of ATP, 150–165 μeq of Ca/g of protein were associated with the microsomes, but since in the absence of ATP a concentration of free Ca of about 2.0 mM was required in the medium to give this degree of Ca binding, whereas the same amount of Ca was bound by the

relaxing factor at a free Ca concentration of only 10^{-6} M, it is evident that the displacement of Mg and the binding of Ca are enhanced by ATP.

There are also about 20 μeq of K/g of protein associated with the microsomes under the conditions specified above (Fig. 1). This fraction of bound K decreased only slightly, when increasing amounts of Ca were retained by the relaxing factor, and presumably is bound very tightly. On the average for each Ca bound one Mg was displaced and consequently the total cations bound re-

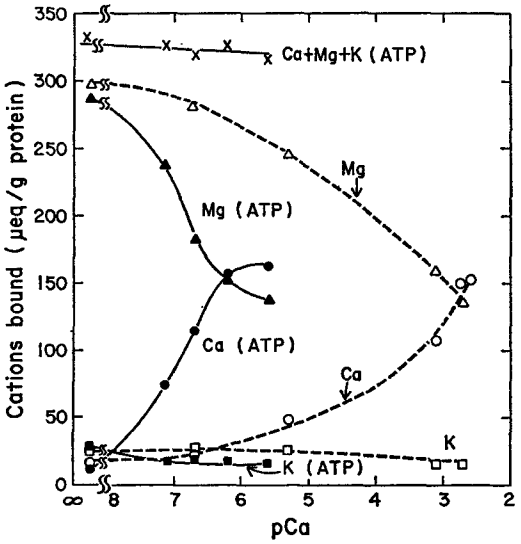

FIGURE 1. Binding of Ca, Mg, and K by microsomes from rabbit skeletal muscle at various concentrations of free Ca in the presence (solid lines) and in the absence (broken lines) of ATP. Samples containing about 10 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 3.8 mM Mg, 10 mM KCl, 10 mM imidazole of pH 6.9, 1.0 mM EGTA, and Ca to give the pCa values plotted on the abscissa. The suspensions (total volume of 10 ml) were then centrifuged for 20 min at 105,400 *g*, and the pellets were washed once with 0.25 M sucrose, as described in the text. When ATP was also present it was added just before centrifugation (final concentration of ATP was 1.0 mM).

mained approximately constant (Fig. 1) for the binding of Ca, Mg, and K in the presence of ATP.

We observed also a competition between Ca and K in the presence of ATP similar to that between Ca and Mg. In these experiments the concentration of K in the equilibrating media was adjusted to 110 mM to induce about 50 % of the binding sites of the microsomal membranes to be associated with K before studying the effect of Ca on the binding of K and Mg (Fig. 2). Calcium is bound maximally at pCa of 5–6, and K is displaced maximally also in this same range of Ca concentration. Fig. 2 also shows that the displacement of K by Ca parallels a similar effect of Ca on Mg bound. Thus, the amounts of

K and Mg bound decrease from 134 μeq of K/g of protein without added Ca to 54 μeq of K/g of protein at pCa of 5, and from 210 μeq of Mg/g of protein without added Ca to 112 μeq of Mg/g of protein at pCa of 5. The total amount of cations bound, i.e. Ca plus Mg plus K, remains constant at 350–365 μeq/g of protein even though the amount of Ca retained by the microsomal fraction increases from 20 μeq of Ca/g of protein in the absence of added Ca to about 180 μeq of Ca/g of protein at a pCa of 5–6.

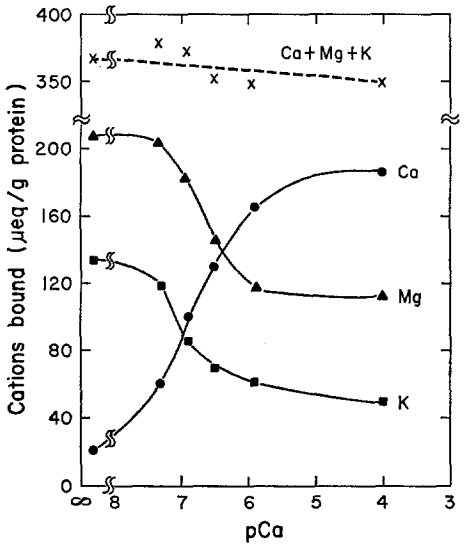

FIGURE 2. The effect of various concentrations of free Ca in the presence of 1.0 mM ATP on the binding of Mg and K by microsomes from rabbit skeletal muscle at high concentration of K (110 mM). Samples containing about 10 mg of microsomal protein from rabbit skeletal muscle were equilibrated, centrifuged, and washed as described in Fig. 1. The equilibrating solutions of a final volume of 10 ml contained 3.8 mM Mg, 110 mM K, 10 mM imidazole at pH 6.9, 1.0 mM EGTA, and Ca to give the pCa values plotted on the abscissa.

The values for the pCa were calculated from the measured concentrations of total Ca in the supernatant solutions after centrifuging the microsomal fraction for 15–25 min at 105,400 *g* and from the concentrations of EGTA in the media which we maintained at 1.0 mM. For these calculations we used a dissociation constant for CaEGTA of 10^{-11} (34) corrected for the pH of the experiments. The ATP present does not change the concentration of free Ca significantly, since the nucleotide exists mostly in the form of MgATP under the conditions of the experiments.

The relaxing factor retains maximal amounts of Ca in the presence of ATP when the pCa in the medium is 5–6. This value corresponds to a concentration of free Ca in the medium needed for optimum active binding of this cation by

relaxing factor higher than that reported by Weber, Herz, and Reiss (14) who reported maximal binding of Ca at pCa values of 6–7. We separated the microsomal fraction from the assay medium containing ATP, Mg, and Ca by centrifugation, and it is doubtful that the rate of diffusion of ATP from the supernatant solution into the tightly packed pellet was sufficient to maintain the active transport system for Ca operating during the procedure. Therefore, Ca that may have accumulated in the microsomal vesicles against an activity gradient probably diffused out during centrifugation and raised the concentration of free Ca^{++} in the medium, whereas Weber et al. (14) determined the pCa of the medium on filtrates of the microsomal suspension and thus could

TABLE II

EFFECT OF WASHING RABBIT SKELETAL MUSCLE MICROSOMES WITH 0.25 M SUCROSE ON BINDING OF CALCIUM AND MAGNESIUM

No. of washes	Calcium bound	Magnesium bound
	(μeq/g protein)	
0	160	215
1	154	212
2	152	210
3	148	205

Samples of muscle microsomes containing 15 mg of protein were equilibrated for 10 min at 23°C in solutions containing 3.8 mM Mg, 10 mM K, 0.15 mM Ca, and 10 mM imidazole at pH 7.0. ATP was added to a final concentration of 1.0 mM, and the suspensions were centrifuged for 20 min at 105,400 *g*. The pellets obtained were then washed with 0.25 M sucrose, as described in the text.

measure the free Ca^{++} in the medium before loss of Ca from the vesicles. Consistent with this supposition is the fact that the values for Ca binding reported by Weber et al. are higher than those measured by our technique. We shall analyze the inferences of these differences in more detail in the Discussion.

A significant aspect of our results is that even after the ATP in the pellet is depleted or after all the ATP added to the microsomal suspension containing Mg and Ca is hydrolyzed and the microsomes are then centrifuged and washed several times with a nonionic solution (0.25 M sucrose), a fraction of Ca corresponding to 80–100 μmoles/g of protein remains associated with the membranous fraction (Table II). We believe that this Ca is adsorbed at fixed binding sites of the lipoprotein membranes and that the competition between the various cations depends on their relative affinities for and accessibility to the binding sites (see Discussion).

Dependence of Binding of Ca and Mg on pH We observed previously that the passive binding of Ca and Mg by skeletal muscle microsomes depends on pH in a manner suggesting that H^+ competes with Ca^{++} and Mg^{++} for the

binding sites of the microsomes (18). Fig. 3 shows that the active binding of Ca by this muscle fraction also depends on pH in the pH range of 4.9 to 7.9, optimum Ca uptake occurring at about pH 6.4.

Comparison of the binding of Ca, Mg, and K at various pH values in the presence (Fig. 3 *a*) and in the absence (Fig. 3 *b*) of ATP shows that the dependence of the binding of Ca on pH in the two instances differs; i.e., whereas the passive binding of Ca increases with increasing pH, the active binding of

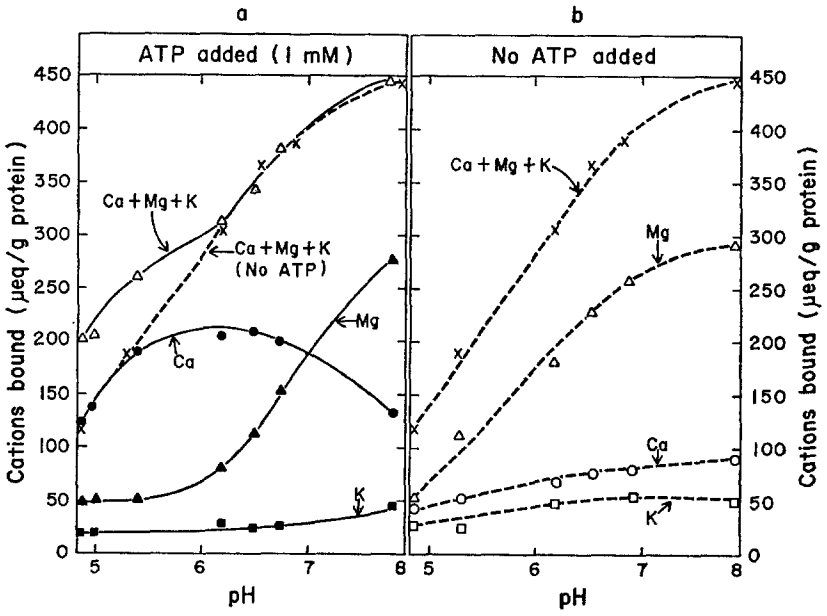

FIGURE 3. The effect of pH on the binding of Ca, Mg, and K by microsomes from rabbit skeletal muscle in the presence (*a*, solid lines) and in the absence (*b*, broken lines) of 1.0 mM ATP. Samples containing 10 mg of microsomal protein were equilibrated, centrifuged, and washed as described in Fig. 1. The final volume of the equilibrating solutions was 10 ml and contained 3.8 mM Mg, 15 mM K, 0.2 mM Ca, and 10 mM Tris-maleate buffer adjusted to pH values between 4.9 and 8.0.

Ca is optimal at pH of about 6.4 and decreases on either side of this pH value. The differences in the binding of Mg and K in the presence and absence of ATP can be explained in terms of displacement of these ions by Ca bound in the presence of ATP in excess of Ca passively bound when no ATP is present. Thus, at maximal binding of Ca induced by ATP, the binding of Mg and K is depressed (Fig. 3 *a*). Above pH 6.2, the exchange of Ca for Mg and K is stoichiometric if expressed on an equivalent basis. This is shown in Fig. 3 *a* where we plotted the total Ca plus Mg plus K bound at various pH values in the presence and in the absence of ATP. Above pH 6.2, the two curves coincide which indicates that ATP neither creates new binding sites for Ca nor pro-

motes an uptake of Ca that is not accompanied by a release of an equal number of equivalents of Mg plus K.

At pH values below 6.2 the binding of Ca induced by ATP does not occur in exchange for Mg and K exclusively. At these lower pH values an increasing number of binding sites of the lipoprotein membrane fragments are in the hydrogen form, and ATP apparently induces binding of Ca at these sites in exchange for H. This exchange is suggested by the increase in total Ca plus Mg plus K bound in the presence of ATP over the total of these cations bound in the absence of ATP at pH values below 6.2 (Fig. 3 *a*).

The binding of Ca by the microsomes in the absence of ATP increased from 42 μeq/g of protein at pH 4.9 to 90 μeq/g of protein at pH 8.0. This relatively high passive binding of Ca in the absence of ATP occurred because the concentration of free Ca^{++} in the medium was relatively high, about 10^{-4} M. We could not regulate the concentration of free Ca^{++} in the medium by EGTA because the affinity of this compound for Ca varies with pH. Instead of controlling the concentration of free Ca^{++} in the assay medium with a Ca-EGTA buffer, we added relatively high concentrations of Ca (0.1 to 0.2 mM) to assure a sufficient supply of this cation to the relaxing factor.

Dependence of the Binding of Ca on Mg and K We showed previously that the affinity constants for the passive binding of Ca and Mg by the microsomal fraction of skeletal muscle of rabbit are about 1000-fold higher than those for Na and K (18) and that these cations interact with the same sites of the microsomal membranes. The active binding of Ca by these membranes is modified by K and Mg (Figs. 4 and 5). In Fig. 4 we plotted the binding of Ca at various pCa values in the presence of 10 and 110 mM K. Increasing the concentration of K from 10 mM to 110 mM decreases the passive and active binding of Ca. The effect is more pronounced on the active than on the passive binding which suggests that K affects the mechanism by which ATP induces the selective binding of Ca in addition to competing with Ca for the binding sites. The effect of K above 10 mM is always one of decreasing the apparent affinity and capacity of the membranes for Ca.

Magnesium is required for active binding of Ca by the microsomes (6, 7). In the presence of 110 mM K and 1 mM ATP at pH 6.7, 4 mM Mg are required for maximal binding of Ca (Fig. 5). Increasing the concentration of Mg up to 15 mM has no further effect on the binding of Ca, but the amount of Mg bound by the microsomes increases with increasing concentrations of Mg above 4 mM. In this range of Mg concentration (i.e., 4–15 mM), the binding of Mg displaces K from the binding sites of the microsomal membranes and presumably also H since less K is displaced than Mg is bound (Fig. 5). The insensitivity of the Ca bound to the concentrations of Mg above 4 mM probably reflects a very stable interaction between the binding sites of the membranes and Ca in the

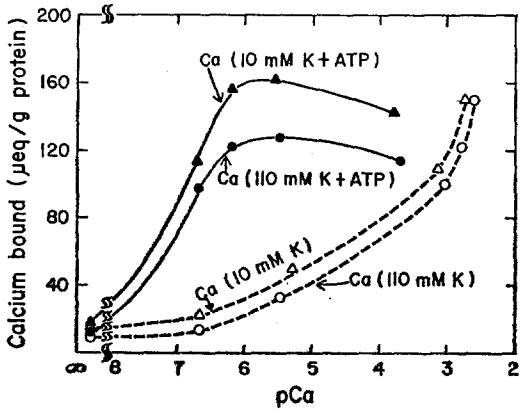

FIGURE 4. The effect of K on the binding of Ca by microsomes from rabbit skeletal muscle at various pCa values in the presence (solid lines) and in the absence (broken lines) of 1.0 mM ATP. Samples containing 10 mg of microsomal protein were equilibrated, centrifuged, and washed as described in Fig. 1. The final volume of the equilibrating solutions was 10 ml and contained 3.8 mM Mg, 10 mM imidazole at pH 6.9, 1.0 mM EGTA, and sufficient Ca to give the pCa plotted on the abscissa. The concentration of K was either 10 mM or 110 mM as indicated.

presence of Mg. It will be recalled that the affinity of Mg for the binding sites is about 1000-fold higher than that of K and it would be expected, therefore, that 15 mM Mg would compete to a greater extent with Ca for the binding sites than does 110 mM K which, as noted above, significantly decreases the active binding of Ca. This expectation is warranted only if we could assume

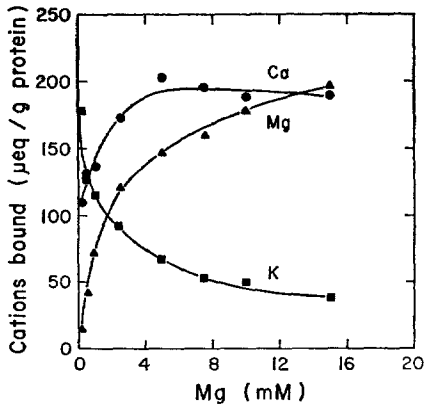

FIGURE 5. Dependence of the binding of Ca by microsomes from rabbit skeletal muscle on Mg in the presence of 1.0 mM ATP. Samples of 10 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 110 mM K, 1.0 mM Ca-EGTA, 10 mM imidazole at pH 6.7, and Mg, as indicated on the abscissa. After adding ATP to a final concentration of 1.0 mM (final volume of 10 ml), tubes were immediately centrifuged, and the microsomal pellets obtained were washed as described in the text.

that the effects of Mg and K on the binding of Ca were explained entirely in terms of competition of these ions with Ca for the binding sites, but, although such an explanation seems valid for passive binding, it apparently is not valid for active binding of the cation.

Shifts of Cations Associated with the Microsomes Induced by Various Concentrations of ATP We anticipated that ATP would be an effective competitor with the binding sites of the microsomal membranes for Mg and Ca since it complexes these cations very avidly (35). Fig. 6 *b* shows that the amount of Mg bound by the microsomes decreases as the concentration of ATP increases above 10^{-4} M. In these experiments we added 1.0 mM of EGTA to the medium to reduce the concentration of free Ca and the amount of Ca bound to minimal values. The effect of ATP on the shift of the cations associated with the microsomal membranes is, therefore, limited to Mg and K which compete with each other for the same binding sites (18). Therefore, reducing the concentration of free Mg^{++} in the assay medium by increasing the concentration of ATP causes a larger number of binding sites to be associated with K. This interdependence of the binding of Mg and K appears in Fig. 6 *b* as a decrease in bound Mg and an increase in bound K as the concentration of ATP is increased. The effect is particularly significant above 1 mM ATP when the concentration of Mg is 2.5 mM.

Fig. 6 *a* shows the results of similar experiments, except that Ca is present in addition to Mg and K. The binding of Ca induced by ATP causes a decrease in the binding of Mg and K at concentrations of ATP as low as 10^{-5} M. Maximal uptake of Ca (195 μeq/g of protein) occurs at a concentration of ATP slightly higher than 10^{-4} M. At this concentration of ATP, K binding is at a minimum (30 μmoles/g of protein). Concentrations of ATP higher than 1 mM decrease the binding of both Ca and Mg presumably by decreasing the concentration of these cations in the free form in the medium. The concentration of free K in the medium remains essentially constant because of the relatively high concentration of K (60 mM) and the low affinity of ATP for the monovalent cation (36). Consequently, more K is associated with the microsomal membranes as the concentrations of free Ca and Mg in the medium are decreased by ATP (Fig. 6 *a* and *b*).

The total cations bound when no Ca is added to the medium decrease with increasing concentration of ATP from 325 μeq/g of protein in the absence of ATP to 240 μeq/g of protein at 5.0 mM ATP (Fig. 6 *b*). However, if Ca is also present, ATP has only a slight effect on the total value of Ca plus Mg plus K bound by the microsomes presumably because, although the concentration of the free cations in the medium is decreased by ATP, the nucleotide also promotes the active uptake of Ca and thus prevents a reduction in the total cations bound (Fig. 6 *a*).

Binding of Inorganic Phosphate in Conjunction with Calcium The results reported above indicate that, at least under certain conditions, the Ca bound by the microsomal membranes exchanges stoichiometrically for Mg and K and that no anion is transported with Ca. We tested, however, whether the presence of phosphate in the medium at concentrations expected to be produced by the hydrolysis of ATP affects the fraction of Ca bound. If we replaced ATP by inorganic phosphate we observed that no Ca was taken up in excess

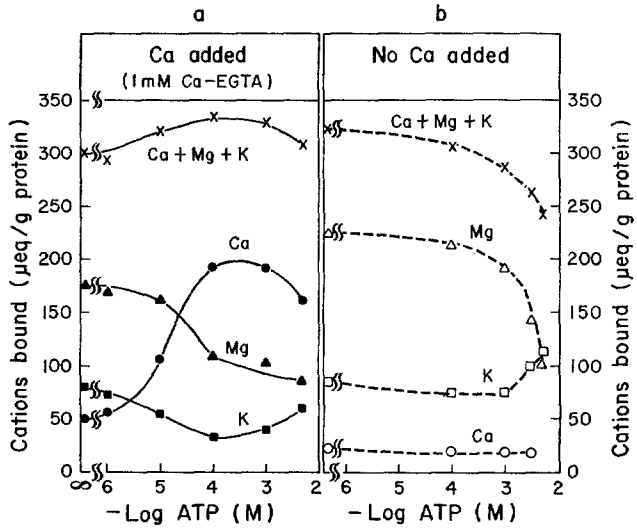

FIGURE 6. Effect of varying concentrations of ATP on the binding of Mg and K by microsomes from skeletal muscle of rabbit in the presence (*a*, solid lines) and in the absence (*b*, broken lines) of Ca. Samples of microsomes containing about 15 mg of protein were equilibrated for 10 min at 23°C in a final volume of 9 ml containing 2.5 mM Mg, 60 mM K, 10 mM imidazole at pH 6.9, and either 1.0 mM Ca-EGTA (*a*) or 1.0 mM EGTA (*b*). ATP was added as indicated on the abscissa just before centrifugation. Final volume was 10 ml.

of the amount originally present in the membranes even at phosphate concentrations of 5.0 mM. A comparison of the effectiveness of ATP and phosphate in promoting Ca uptake by the microsomal fraction is shown in Fig. 7. The values in parentheses represent the concentration of ATP (mM) remaining in the supernatant solution after centrifugation as determined from the original concentration of ATP and the inorganic phosphate liberated. It will be noted that an original concentration of ATP of the order of 10^{-4} M induces maximal Ca uptake even though all the ATP is hydrolyzed before the microsomes are centrifuged. Since ADP does not support Ca uptake by the relaxing factor (6, 11), it would appear that it is either the transient presence or the hydrolysis of ATP that causes the increase in the binding of Ca by the microsomes. We

have, however, not excluded the possibility that resynthesis of ATP by the myokinase system supplies additional ATP.

The amount of phosphate bound is not significantly different when either ATP or phosphate is added to the media; only 5–10 μmoles of Pi/g of protein were detected at all concentrations of ATP or phosphate studied (Fig. 7). Thus, the fraction of bound Ca studied by us (80–100 μmoles/g of protein) was not in the form of $Ca_3(PO_4)_2$. The $Ca_3(PO_4)_2$ that might form when the microsomes are in suspension either is not retained by the microsomal mem-

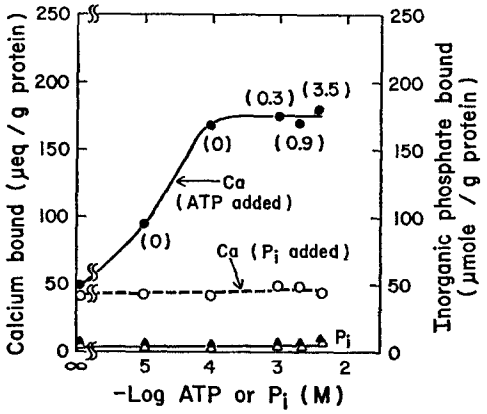

FIGURE 7. Comparison of the effects of ATP and inorganic phosphate (Pi) on the binding of Ca and Pi by microsomes from skeletal muscle of rabbit. Samples of microsomes containing 15 mg of protein were equilibrated for 10 min at 23°C in solutions containing 3.8 mM Mg, 15 mM K, 1.0 mM Ca-EGTA, 10 mM imidazole at pH 6.5, and ATP or Pi (in the form of KH_2PO_4 solution adjusted to pH 6.5). The ATP or Pi was added just before centrifugation (final volume of 10 ml) to give final concentrations of up to 5.0 mM. The pellets obtained were washed once with 0.25 M sucrose. Values in parentheses represent the concentration of ATP at the end of centrifugation. Triangles represent the binding of Pi by the microsomal fraction when ATP (solid triangles) or Pi (open triangles) was added.

branes or vesicles or if it is retained, it is subsequently removed by the washing procedure before the cations and phosphate bound are measured.

Effects of Caffeine and Other Agents on the Binding of Ca and Mg by Microsomes

Caffeine increases the outflux of Ca in amphibian (20) and mammalian skeletal muscles (46). It is thought that the increased outflux of Ca reflects a release of Ca by the sarcoplasmic reticulum induced by caffeine. Such release has been observed in the microsomal fraction of skeletal muscle of rabbit and frog by Herz and Weber (37), but the mechanism of action of caffeine is not clear. We investigated, therefore, the effect of caffeine on the fraction of Ca which we believe to be adsorbed at binding sites of the fragmented membranes of sarcoplasmic reticulum, i.e. that fraction of Ca retained by the microsomal

fraction after the ATP in the medium is removed and the microsomes have been washed to remove Ca trapped with supernatant solution and also Ca which may have been maintained in the microsomal vesicles against its activity gradient.

Caffeine does not affect the Ca bound to the membranes. Fig. 8 shows that the level of Mg bound is also unchanged by concentrations of caffeine up to 10 mM. The Ca bound referred to in Fig. 8 as bound "activity" is that fraction of Ca which was bound in response to ATP and which remains in the microsomal fraction after the ATP is removed from the medium by the washing

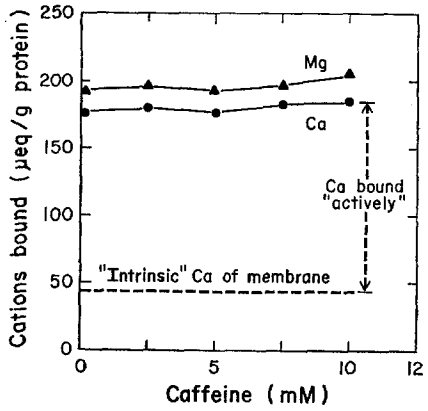

FIGURE 8. The effect of caffeine on the binding of Ca and Mg by microsomes from skeletal muscle of rabbit in the presence of 2.0 mM ATP. Samples of microsomes containing 15 mg of protein were equilibrated at 23°C for 10 min in solutions containing 3.8 mM Mg, 10 mM K, 0.15 mM Ca, caffeine as indicated on the abscissa, and 10 mM imidazole at pH 6.8. ATP was added just before centrifugation. "Intrinsic Ca" refers to Ca associated with the membranes isolated by the procedure described in the text. Calcium bound "actively" refers to the binding of Ca induced by ATP.

procedure described under "Methods." It represents the Ca retained in the membranes in excess of that in the controls containing only the "intrinsic" Ca.

Herz and Weber (37) observed that caffeine releases Ca bound by the relaxing factor of skeletal muscle of rabbit only if maximal amounts of the cation are retained before adding caffeine and that under these conditions only 20–40 % of the Ca retained is released. It occurred to us that we did not observe release of Ca by caffeine because the Ca retained by the microsomal fraction in our experiments is equivalent to the Ca which Weber and Herz found to be insensitive to caffeine. The results reported below suggest that this is in fact the case.

A suspension of actomyosin containing 0.5 mg of actomyosin/ml, 2.0 mM Mg, and 50 mM KCl superprecipitates when 1.0 mM ATP is added. If the actomyosin suspension also contains relaxing factor, as indicated in Fig. 9, adding ATP does not cause superprecipitation unless Ca in excess of the Ca

capacity of the relaxing factor in the medium is also present. In the absence of relaxing factor, there is apparently enough Ca in the medium to support superprecipitation as measured by the increase in turbidity of the suspension, but if the medium contains relaxing factor, it binds this Ca and inhibits the reaction.

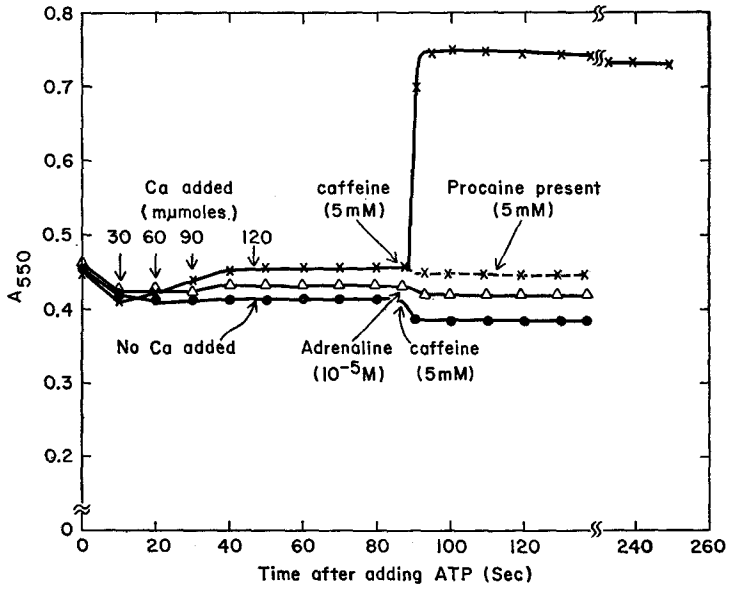

FIGURE 9. Effect of caffeine, procaine, and adrenaline on the superprecipitation of actomyosin suspensions relaxed by the microsomal fraction from rabbit skeletal muscle. Actomyosin (0.5 mg of protein/ml) was suspended in 3.0 ml of solutions in a 1 cm light path cuvette containing 50 mM KCl, 2.0 mM Mg, 10 mM imidazole at pH 7.0, 10 mM phosphocreatine, 0.5 mg of creatine phosphokinase, and 1.2 mg of relaxing factor protein. ATP (0.1 ml of 30 mM) was added at zero time, and the effect of 5 mM caffeine was tested in each of a series of assay mixtures after adding 0, 30, 60, 90, and 120 mμmoles of Ca. Effect of caffeine is shown for zero Ca (●–●–●) and for 120 mμmoles of added Ca (×–×–×). Effect of caffeine with intermediate concentrations of Ca was identical to that with no Ca added. The degree of superprecipitation is reflected by the increase in absorbance at 550 mμ. The effects of 5.0 mM procaine added before caffeine and of 10^{-5} M adrenaline are also shown. In both instances 120 mμmoles of Ca were added before testing the compounds.

With this experimental approach we determined how much Ca was required to saturate the relaxing factor. If at various stages before reaching the saturation value, we added caffeine (5 mM) to the medium, no change in the turbidity of the suspensions was observed, other than that indicated by a slight decrease in optical density caused by dilution of the suspension (Fig. 9), until the Ca added was within 15–30 % of the saturation value for the relaxing factor in the suspension. After this value of Ca was added, 5 mM of caffeine induced a rapid superprecipitation (Fig. 9). The percentage of saturation of relaxing factor with respect to Ca required for the effect of caffeine varied

from experiment to experiment, but we never observed an effect unless the relaxing factor was at least 70 % saturated (i.e., unless at least 70 % of the Ca that would induce superprecipitation in the absence of caffeine was added).

The values for Ca added indicated in Fig. 9 refer to millimicromoles of Ca added per 1.2 mg of relaxing factor protein in the cuvette. In these experiments, caffeine induced superprecipitation only when 120 mμmoles of Ca/1.2 mg of relaxing factor protein were present, i.e. 100 μmoles of Ca/g of protein, which corresponds to the highest values that we obtained by the centrifugation method (usually 80–100 μmoles/g of protein). Determination of the maximal Ca uptake by relaxing factor used in these experiments by the filtration method or from the amount required to cause superprecipitation of the actomyosin without caffeine gave values of 120–130 μmoles of Ca/g of protein.

The effect of caffeine can be obviated if procaine (5.0 mM) is added before caffeine (Fig. 9). Procaine was not always effective to the same extent in this respect, and occasionally we observed a response to caffeine in the presence of procaine which was, however, delayed and proceeded at a relatively slow rate once it started. Feinstein (21) has reported that the two compounds act as competitive inhibitors in frog sartorius muscle.

The validity of these results is questionable since caffeine was effective only if added when the relaxing factor was nearly filled to capacity and, therefore, we could not be certain that sufficient contaminating Ca had not been added with the caffeine solution to trigger the superprecipitation reaction. This now appears unlikely because the effect of caffeine can be prevented by adding procaine before caffeine (Fig. 9). Neither caffeine nor procaine affects the superprecipitation reaction in the absence of relaxing factor.

Fig. 9 shows in addition that epinephrine (10^{-5} M) does not affect the system which responds to caffeine. Acetylcholine was also tested in this system in concentrations up to 10^{-3} M with negative results.

DISCUSSION

Our results show that ATP increases the amount of Ca adsorbed at binding sites of the lipoprotein membranes isolated in the microsomal fraction of skeletal muscle of rabbit. These sites also bind Mg and K, but their apparent affinity differs for each cation and depends on the concentration of other cations in the medium. ATP influences the binding of the three cations by sarcoplasmic reticulum, but it selectively increases the binding of Ca by this muscle-cell fraction. We cannot assume that the selective uptake of Ca by isolated fragmented sarcoplasmic reticulum induced by ATP is an active transport process without further evidence that the movement of Ca into the microsomal phase occurs against an electrochemical gradient, and this cannot be determined at present since there is no information on the polarity and magnitude of the transmembrane potential which may exist across the limiting membranes of the isolated microsomal vesicles. On the other hand, our results

indicate that approximately 80 % of the maximal Ca bound actively (i.e., in the presence of ATP) by the microsomal fraction is associated with binding sites of the lipoprotein membranes and therefore does not contribute to the intravesicular Ca activity.

The results of our studies, which indicate that ATP induces an increased association of Ca with binding sites of the microsomal membrane fragments, are summarized in Figs. 1–3. In the three types of experiments, binding of a given quantity of Ca results in displacement of an approximately equal quantity of Mg or Mg plus K previously bound by the membranes. The retention of Mg and K is determined by a mass law type of interaction between the cations and anionic groups in the membranes whose average pK value is 6.6 (18). These groups were previously shown to bind Ca also (and H, Zn, and Na) (18). Since Ca, Mg, and K compete for the same sites (18), the binding sites in the membranes are essentially saturated with respect to Mg or Mg and K before adding ATP at the ionic composition of the assay medium used to study Ca binding; i.e., 10^{-8} M to 10^{-4} M Ca, 3.8 mM Mg, and 10 mM or 110 mM K (Figs. 1 and 2). The exchange of bound Mg and K for Ca induced by ATP suggests, a priori, that the nucleotide selectively increases the apparent affinity of the binding sites of the membranes for Ca and, consequently, this cation, even at relatively low concentrations in the assay medium, competes effectively with Mg and K for the binding sites of the membranes.

This interpretation assumes that the original retention of Mg and K in the membrane fraction occurs because of an electrostatic interaction between anionic sites of the membranes and the cations and that no activity gradients of these cations are maintained across the vesicular membranes.[2] Movement of Ca from the suspending medium into the membrane phase could occur

[2] Distribution of the cations between the equilibrating medium and the membranous fraction according to Gibbs-Donnan equilibrium would in fact tend to produce a higher activity of free Ca^{++}, Mg^{++}, and K^{+} in the membranous phase than in the equilibrating medium. However, this effect does not influence our results since we washed the microsomal fraction with a nonionic solution (0.25 M sucrose) to remove ions not adsorbed to the membranes before measuring the cations retained by the microsomes. This washing procedure effectively "fixes" the adsorbed cations while removing Ca, Mg, and K that had moved to the membranous phase (in association with diffusible anions, e.g., Cl^{-}) in accordance with the Gibbs-Donnan distribution of ions. It is irrelevant in this respect whether we consider the membrane material as existing in the form of vesicles with semipermeable membranes or merely as fragmented open pieces of membrane. In both instances, the Gibbs-Donnan effects are operative and in both cases the binding sites act as the fixed charges which determine the Gibbs-Donnan distribution of diffusible ions (45). The effectiveness of the washes with sucrose solution in removing free ions from the membranous fraction is also independent of the structure assumed by the fragmented membranes and of whether or not sucrose permeates the membranes of the vesicles. If the membrane fragments are in the form of vesicles, we need to assume only that free ions in the vesicles are in equilibrium with free ions outside. For ions which are not transported actively such equilibrium must in fact exist whether ATP is present or not. For those ions which are transported, the steady state maintained in the presence of ATP will revert to an equilibrium state once ATP is removed. Thus, depleting the external medium of the vesicles of free cations leads to depletion of free cations in the vesicles. Those cations adsorbed to binding sites will remain to maintain electrical neutrality in the membrane fraction.

either in conjunction with an equivalent quantity of anions (Cl^-, inorganic phosphate (Pi), etc.) or in exchange for cations which would be bound to the membranes under the experimental conditions. If an equal number of equivalents of Cl^- or Pi and Ca^{++} were taken up by the membranes, without release of the cations (Mg and K) bound before the uptake of Ca, the total cations bound would be higher in the presence than in the absence of ATP. The observation that the total Ca plus Mg plus K bound at pH values above 6.2 remains approximately constant before and after adding ATP (Fig. 3) excludes the possibility that the fraction of Ca retained by the microsomes exists in the vesicles either as Ca + 2 Cl^- or as $Ca_3(PO_4)_2$. We also showed more directly that phosphate does not accumulate in the membrane fraction in studies in which we established that essentially no inorganic phosphate was retained by the membranes under conditions for maximal uptake of Ca.

An alternate possibility is that the concentration of free Ca^{++} in some compartment(s) of the membranes containing also the binding sites is in fact increased by ATP to the extent that the Ca^{++} accumulated competes effectively with Mg^{++} and K^+ for the binding sites. It can be estimated from the data in Fig. 1 that the concentration of free Ca^{++} in such compartment(s) needs to rise only to about 2 mM to induce the same binding of Ca and displacement of Mg and K at the binding sites as that caused by ATP. We calculated that at this concentration of free Ca^{++} in the hypothetical compartment(s) the total concentration of Ca (Ca bound plus Ca free) in the microsomal fraction would be about 10 mM and that about 50 % of the total binding sites in the membranes would be associated with Ca and 50 % with Mg, or Mg and K. We assume in these calculations that once Ca is transported into the compartments it is bound passively to the membranes as determined by the affinity constant for the interaction between the binding sites of the membranes and Ca in the absence of ATP (Fig. 1) (18).

Whether the effect of ATP in promoting exchange of Ca for bound Mg and K is induced directly by selectively increasing the affinity of the binding sites for Ca or indirectly by transporting the Ca across the vesicular membranes into a compartment where the cation will then be in equilibrium with the binding sites, a large fraction of the Ca accumulated by relaxing factor in the presence of ATP is adsorbed to binding sites. This conclusion is inescapable, given the passive affinity of the microsomal membranes for Ca (11, 18). If the Ca retained by relaxing factor in the presence of ATP remained in the free form, the concentration of free Ca^{++} in the vesicles would be of the order of 10–15 mM, and, unless the interior of the vesicular membranes did not contain binding sites, Ca^{++} at this concentration would compete effectively with Mg^{++} and K^+ for the binding sites (Fig. 1). The total concentration of Ca in the microsomal fraction is probably considerably higher than the 10–15 mM estimated from the wet weight of the pellets and their Ca content since we did not

correct for the intervesicular space. However, the Ca retained by the microsomal membranes in the presence of ATP never exceeds the concentration of the binding sites in the membranes (350 μeq/g of protein at pH 6.7) and usually corresponds to only 50–60 % of the total binding sites available at neutral pH for the binding of Ca (Figs. 1 and 2). We do not know whether the binding sites are distributed equally in the external and internal surfaces of the vesicles. Ohnishi and Ebashi (42) have suggested that the rapidity of the Ca uptake by the vesicles (3×10^{-9} moles of Ca^{++} per cm^2 of surface area per sec) probably indicates an absorption of the cation to the outer surface of the vesicles. If a distinction can be made between external and internal binding sites in terms of the assessibility of cations to these sites, we should be able to detect it by further fragmenting the vesicular membranes to expose more of the internal binding sites. The results of our experiments in this direction are as yet inconclusive.

The number of binding sites available for binding of Ca, Mg, and K depends on the concentration of H^+ in the medium since this cation competes effectively with the other cations for the binding sites in the membranes (Fig. 3) (18). Thus, the amount of Ca, Mg, and K associated with the membranes increases with increasing pH in the absence of ATP. The same is true for Mg and K in the presence of ATP, but the binding of Ca has a pH optimum of 6.4–6.6 under the latter conditions. These results were discussed amply in a previous section and we might just bring to focus here that at pH 4.9 ATP promotes adsorption of Ca almost exclusively at the expense of H which at this pH value displaced Mg and K from the binding sites before the adsorption of Ca (Fig. 3).

The role of Mg in the binding of Ca promoted by ATP is an intriguing one. The cation is required for maximal selective uptake of Ca in the presence of ATP (6, 7, 38) and, in concentrations up to 15 mM, does not compete with Ca bound actively at the adsorption sites of the membranes (Fig. 5). Potassium, on the other hand, is not required for active binding of Ca and at 110 mM depresses the binding of this cation significantly (Fig. 4). Weber et al. (11) also reported recently the apparent noncompetition of Mg with Ca in active binding of Ca by relaxing factor. It has also been observed by other workers that K and other monovalent cations decrease the active binding of Ca (38, 39).

The relationship between active binding of Ca and ATP hydrolysis cannot be determined accurately by centrifugation. It appears, however, that hydrolysis of ATP is required for binding of Ca, although various workers disagree on the relationship existing between the hydrolysis of ATP and the binding of Ca (7, 11, 40, 41). The work of Hasselbach and Makinose (8) and that of Weber et al. (11) indicate that 1–2 moles of Ca are bound by relaxing factor for each mole of "extra splitting" of ATP, but Ebashi and Yamanouchi

(41) showed that at low concentrations of ATP this value may be as high as 10 moles of Ca bound per mole of ATP hydrolyzed. The work of Martonosi and Feretos (40) also shows that it is possible to dissociate the binding of Ca from the ATPase activity of the microsomal fraction of skeletal muscle of rabbit; i.e., by simply adjusting the experimental conditions they demonstrated Ca uptake without an increase, or even a decrease, in the ATPase activity. These findings of Ebashi and Martonosi are difficult to reconcile with the hypothesis that an active transport mechanism, employing ATP as the immediate energy donor, is operative in promoting transmembrane transport of Ca into the microsomal vesicles. If increased binding of Ca by the membranes under the influence of ATP reflects an increased affinity of the binding sites for Ca under these conditions (rather than equilibration of the sites with relatively high concentrations of compartmentalized free Ca in the vesicles), we should visualize the role of ATP as one in which the nucleotide acts to modify the binding sites retaining the Ca in the membranes rather than to activate a hypothetical carrier which transports the cations across the vesicular membranes (8, 11).

The fraction of Ca bound by isolated sarcoplasmic membranes is not influenced by caffeine in concentrations up to 10 mM (Fig. 8). However, the studies of Herz and Weber (37) showed that at maximal active uptake of Ca by relaxing factor isolated from skeletal muscle of rabbit and frog, 8 mM caffeine releases 20–40 % of the total bound Ca. We could also induce superprecipitation of actomyosin previously "relaxed" by relaxing factor by adding 5 mM caffeine after the system contained an amount of Ca higher than that adsorbed to the binding sites of the membranes of the relaxing factor in the actomyosin suspension (Fig. 9). These results suggest that a fraction of Ca more labile than the adsorbed Ca which we detect in our studies is concentrated by the relaxing factor under optimal conditions. In vivo, the Ca accumulated in the membrane system in excess of the adsorbed Ca may be the Ca which is easily translocated within the muscle. However, since this fraction of Ca accounts for only about 20 % of the total Ca that can be concentrated in the relaxing factor, we should like to entertain the possibility that the adsorbed Ca is normally in a very dynamic state and that physiological stimulation of the muscle cell causes a rapid desorption-adsorption cycle of Ca by the membranes of the sarcoplasmic reticulum.

We should like to refer to experiments showing accumulation of calcium oxalate within the microsomal vesicles of rabbit skeletal muscle (7, 8) and in the terminal cisternae of striated muscle fibers of frog (44). Whereas the oxalate experiments with the isolated vesicles suggest that at high pCa values Ca moves into the vesicles against an activity gradient (7, 8, 11), they have limited application to the question of whether the bulk of the Ca accumulated by the fragmented microsomal membranes is retained as ionized Ca or is

associated with the binding sites of the membranes. A concentration of 5 mM oxalate used in these experiments (7) restricts the Ca activity in the vesicles (and in the external medium) to 4×10^{-7} M as determined from the solubility product of calcium oxalate. If we assume the same activity coefficient for inside and outside the vesicles, the concentration of internal Ca at the point where calcium oxalate precipitates in the vesicles is about 100 times higher than the Ca activity (7); i.e., 4×10^{-5} M. Since the concentration of Ca accumulated in the vesicles in the absence of oxalate is at least 10^{-2} M, the concentration of free Ca permitted in the vesicles by 5 mM oxalate is only 0.4 % of the Ca taken up by the microsomal fraction. It may be argued that in the absence of oxalate the concentration of internal free Ca is in fact higher than that permitted by 5 mM oxalate, but Fig. 1 shows that if the free Ca in equilibrium with the binding sites of the membrane rises to 2 mM, about 80 % of the total Ca (Ca bound plus Ca free) retained by the vesicles (about 10 mM) will be bound. Thus, it appears that the Ca bound by the membranes must represent a large fraction of the total Ca accumulated. Weber et al. (11) have recently arrived at a similar conclusion. They observed that the solubility product for calcium oxalate in the vesicles calculated from the product of the Ca accumulated in excess of calcium oxalate and the free oxalate is invariably higher than the true solubility product and that the calculated value varies from one oxalate concentration to another in a manner suggesting that some of the Ca accumulated is bound to the membranes.

We show above that the observation that calcium oxalate precipitates within the reticular vesicles provides only limited information concerning the state of the Ca in these structures. A similar argument reconciles the apparent discrepancy between our contention that in vitro a large fraction of the Ca taken up by sarcoplasmic reticulum is adsorbed to binding sites of the membranes and the observation that calcium oxalate accumulates in the terminal cisternae of muscle fibers (44).

Between 80 and 100 μmoles of Ca per g of microsomal protein were invariably found associated with binding sites of isolated sarcoplasmic reticulum under the described experimental conditions. We could not establish precisely what fraction this Ca represents of the maximal uptake capacity of the reticulum membranes under steady-state conditions because the Ca binding is measured under equilibrium conditions, whereas the maximal Ca uptake is observed at steady state. However, the value for maximal uptake of Ca at steady state as measured by the Millipore filtration technique (38) and by the titration procedure described in connection with Fig. 9 is about 20 % higher than that observed for the binding of the divalent cation. We could obtain these maximal values only if an ATP-regenerating system (i.e., phosphocreatine–creatine phosphokinase) was also present. In the presence of ATP, without a regenerating system, the value for the maximal Ca uptake,

as determined by the Millipore filtration procedure, was similar to that for the Ca which we report to be bound even when the measurement was made before all ATP was hydrolyzed. Thus, we consider the value of 80 % for the fraction of total Ca accumulated, which is bound, a conservative figure. It is probable that the amount of Ca bound under conditions for maximal Ca uptake (i.e., at steady state and in the presence of the ATP-regenerating system) is higher than that reported here since our studies show that the Ca capacity of the binding sites of the reticulum membranes at neutral pH is about 350 μeq/g of protein.

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