Effects of Potentiators of Muscular Contraction on Binding of Cations by Sarcoplasmic Reticulum

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ABSTRACT Anionic (NO_3^-, Br^-, I^-, and SCN^-) and cationic (Zn^{++} and Cd^{++}) potentiators of the twitch output of skeletal muscle depress the active binding of Ca by sarcoplasmic reticulum isolated from rabbit skeletal muscle. Zinc and Cd exchange for Ca and Mg at the binding sites of the reticular membranes, whereas the anions effectively induce a replacement by Mg of Ca bound actively in the presence of ATP. In the absence of ATP, the passive binding of both Ca and Mg is increased by the anions tested. Furthermore, the anions increase the total capacity of the membrane fragments for passive cation binding. The Ca-stimulated ATPase activity of the membranes is inhibited by Zn and Cd, but not by the anions. Shifts in cations bound to muscle membrane systems caused by agents that increase the force of contraction developed during the twitch are considered to be the primary event modifying excitation-contraction coupling, and thus leading to potentiation.

Several substances differing widely in chemical properties increase the force of contraction developed during twitch of skeletal muscles and muscle fibers (1–4). This increase in tension, or potentiation, sets in very soon after adding the potentiating agents to solutions bathing the muscle or muscle cells. On the basis of results of kinetic studies of the development and reversal of twitch potentiation by two groups of these agents, namely, inorganic anions (i.e., NO_3^-, Br^-, I^-, and SCN^-) and heavy metal cations (e.g., Zn^{++}, Cd^{++}, and UO_2^{++}), the primary site(s) of action of potentiators has been located tentatively on the plasma membrane or the membranes of the transverse tubular system, or both (1–6). However, the nature of the interaction of the potentiating agents with isolated membrane systems from muscle cells has not been investigated probably because of the difficulty in obtaining pure membrane preparations representative of any one cellular membranous system. The microsomal fraction, for example, contains mostly fragmented sarcoplasmic reticulum, but is normally contaminated with transverse tubular membranes

and probably also includes fragments of the plasma membrane (7, 8). The gross microscopic structure and chemical composition of the plasma and sarcoplasmic reticular membranes of skeletal muscle are rather similar, and both membrane systems exhibit a cation exchange type of binding with respect to Ca^{++}, K^+, Na^+, and probably other cations (9–14). Since the excitability and electrical properties of the plasma membrane of the muscle cell are profoundly influenced by exchange of one cation bound to the membrane for another (15), the binding to the membrane of anionic and cationic potentiators and their influence on the binding of physiologically important cations (i.e., Ca^{++}, Mg^{++}, K^+, etc.) probably represent the primary mode of action of the potentiators.

In the present studies I used the membranous materials constituting the microsomal fraction of rabbit skeletal muscle to test the effect of anionic and cationic potentiators on the cation content of the membranes of the muscle cell. Substitution of one of various potentiating anions (i.e., NO_3^-, Br^-, I^-, and SCN^-) for Cl^-, normally present in the medium, interferes with the ATP-activated uptake of Ca by the microsomal fraction, but increases the Mg content of the fraction. The test anions also increase the passive Ca and Mg binding capacities of the isolated membrane fragments. Zinc and Cd, which strongly potentiate twitch tension at a concentration of about 0.1 mM, exchange for Ca and Mg at the binding sites of the membranes, and both cations depress the active binding of Ca. These shifts in cationic content of the reticular membranes and of other membrane systems of the muscle cell caused by the two groups of potentiating agents studied are probably responsible for the prolonged active state of the contractile elements which thus develop a peak tension higher than normal.

METHODS

Preparation of Biological Material The microsomal fraction of rabbit skeletal muscle was isolated by differential centrifugation as described previously (12). Those preparations used in the ATPase activity studies were washed once with 0.6 M KCl to remove adhering myosin and actomyosin (16).

Binding of Cations by Microsomes The binding of Ca, Mg, and K by the microsomal fraction was determined in the presence and absence of ATP. Samples of the microsomal fraction containing 10–15 mg of protein were transferred to polypropylene tubes of the rotor 40 of the Spinco centrifuge, model L, containing the cations and buffers at 23°C as indicated in the legends of the figures. The volume in the tubes was brought to 10 ml, and after 10 min of equilibration the tubes were centrifuged for 30 min at 0° to 5°C at 105,300 *g*. In those studies in which the binding of cations was investigated in the presence of ATP, the nucleotide was added to the polypropylene tubes just before centrifugation. The supernatant solutions were collected after centrifugation, and the pellets and internal walls of the tubes were washed twice with deionized water. Cations trapped in the pellets with supernatant solution were re-

moved by resuspending the pellets in 9 ml of 0.25 M sucrose and centrifuging the suspensions as before. The cations in the washing solutions were measured, and corrections were made for cations trapped in the pellets with washing fluid. The pellets collected were suspended in 7.5 ml of deionized water, and samples of these suspensions were taken for analyses of Ca, Mg, K, and protein.

Analyses 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, as described previously (12).

Analyses of Protein These were performed by the biuret method (17) standardized by Kjeldahl analysis of nitrogen.

Adenosine Triphosphatase Activity (ATPase) The rate of hydrolysis of ATP by samples of the microsomal fraction was determined from the inorganic phosphate liberated during a predetermined period of incubation (1–3 min) under the conditions specified in the legends of Figs. 5 and 6. The amount of ATP hydrolyzed was less than 20% of the original ATP in the assay media. Under these conditions the relationship between the amount of ATP hydrolyzed and the time of the reaction is rectilinear. The reaction was carried out in a total volume of 5.0 ml and initiated by adding 1.0 ml of 5 mM ATP.

Reagents The ATP was obtained from Sigma Chemical Company in the disodium form. All ATP solutions used were adjusted to pH 7.0 with 1 M KOH. Contaminating Ca in ATP was not removed, but the final Ca concentrations were measured in each experiment by absorption spectroscopy and all calculations of pCa were made from the measured values for Ca. All other compounds used were also of reagent grade.

RESULTS

Effect of Anions on the Binding of Ca, Mg, and K by the Microsomal Fraction Selective binding of Ca in the presence of 1.0 mM ATP by microsomes isolated from rabbit skeletal muscle is decreased when 100 mM Cl^- in the medium is replaced by an equal concentration of one of the following anions: SCN^-, I^-, NO_3^-, and Br^- (Fig. 1 *a*). The effect of the substitution of anions was studied in the presence of 4.7 mM Mg and 110 mM K at pCa values ranging from about 9 to 4. The binding of Ca was decreased significantly by SCN^- and I^- at all pCa values studied, but Br^- and NO_3^- were effective only at pCa values below 6.3. Substitution of Cl^- (100 mM) for SCN^-, I^-, Br^-, or NO_3^- at a pCa value of 5 depresses the binding of Ca by 36, 30, 10, and 10%, respectively. At pCa values above 8, microsomes were essentially saturated with respect to Mg and K, and only 20 to 30 μeq of Ca/g of protein were bound, but at higher concentrations of Ca there was a preferential binding of Ca in exchange for Mg and K irrespective of the anion present (Fig. 1 *a* and *b*). This cation exchange of Ca for Mg and K has already been reported by us (12). The extent of displacement of bound Mg and K by Ca depends on the anions in the

medium since the amount of Ca bound varies for different anions. Fig. 1 *b* shows the displacement of Mg and K by Ca at pCa values from about 9 to 4; it is apparent that when one of the anions SCN^-, I^-, NO_3^-, or Br^- substitutes for Cl^-, the effectiveness of Ca in displacing Mg and K from the microsomal binding sites is reduced. The amount of Mg plus K (μequivalents per gram of protein) displaced is approximately equal to the amount of Ca bound in the

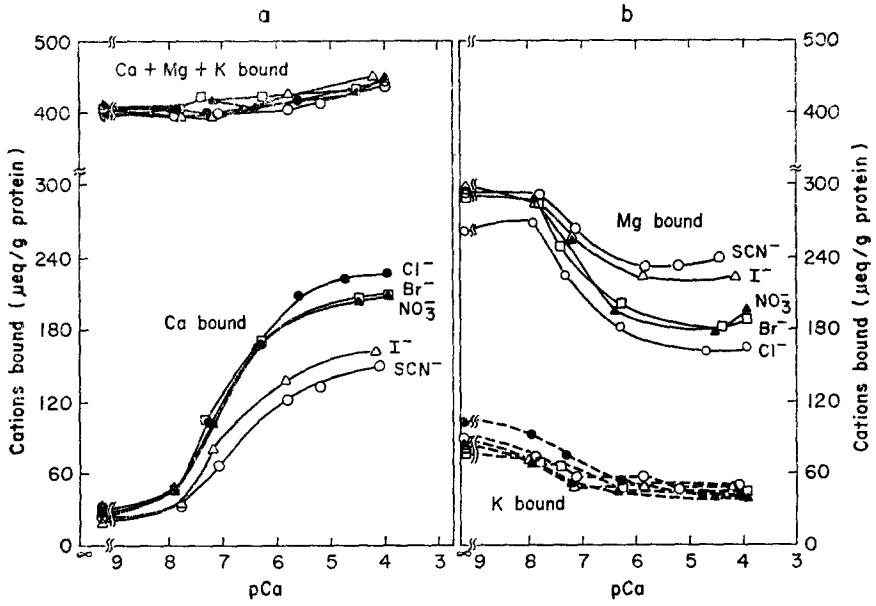

FIGURE 1. Effect of Br^-, NO_3^-, I^-, and SCN^- on the binding of Ca, Mg, and K by sarcoplasmic reticulum isolated from rabbit skeletal muscle. Samples containing 12 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 4.7 mM $MgCl_2$, 10 mM KCl, 20 mM imidazole at pH 7.4, 100 mM of one of the test anions in the K form, 1 mM EGTA, and Ca to give the pCa values designated on the abscissa. Before centrifuging the suspensions for 30 min at 105,400 *g*, 1 ml of 10 mM ATP was added to each tube to give a final volume of 10 ml. The pellets obtained were washed in 0.25 M sucrose, as described in the text, before being analyzed for the cations bound.

case of all anions. Fig. 1 *a* shows that the total Mg plus Ca plus K bound at all pCa values studied in the presence of the various anions remains approximately constant at 400–425 μeq/g of protein, but the maximal binding capacity of the microsomes for Ca appears to be reduced by the presence of all test anions as is indicated by the persistently lower Ca binding in their presence even at pCa values of 4 to 5. On the other hand, the pCa value for 50% saturation with respect to Ca remains approximately constant at 6.8 in the case of all anions, which indicates that the affinity of the binding sites for Ca is not changed by the anion substitutions.

Effects of Various Concentrations of SCN^- and I^- on the Active and Passive Binding of Ca, Mg, and K The effect on the binding of Ca, Mg, and K of substituting SCN^- or I^- for Cl^- at concentrations from 10–210 mM was investigated under conditions for selective ("active") binding of Ca (i.e., 1.0 mM ATP, 4.7 mM Mg, pCa = 5, pH = 7.0, and varying concentrations of K as indicated in the legend of Fig. 2). Furthermore, studies were also carried out on the effect of the anion substitutions on the passive binding of cations in the absence of ATP. Ionic conditions were chosen for the experiments on passive

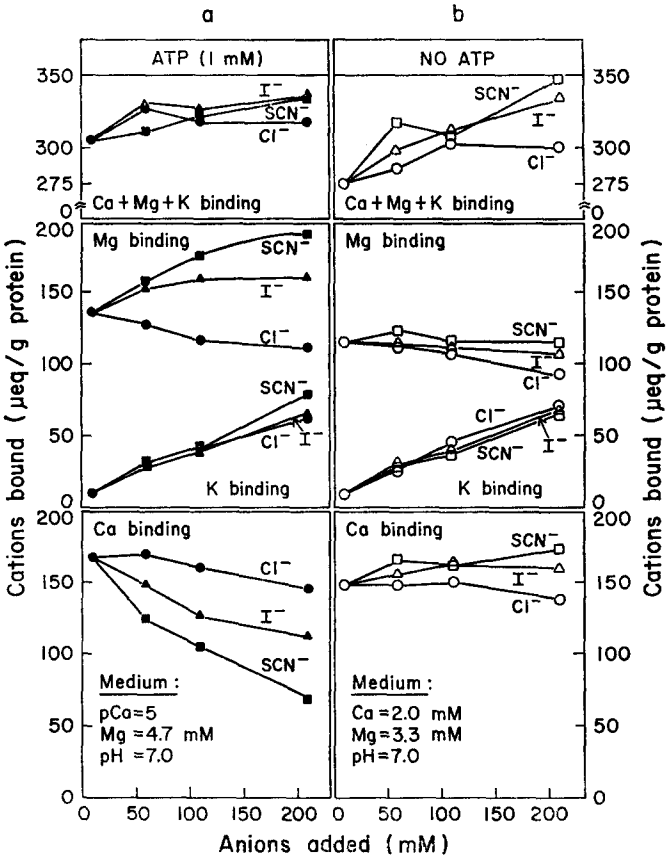

FIGURE 2. Comparison of the effects of anions on the binding of cations bound by isolated sarcoplasmic reticulum in the absence and in the presence of ATP. Samples containing 12 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 10 mM KCl, 20 mM imidazole buffer at pH 7.0, and Ca^{++} and Mg^{++} as indicated. The test anions were added in the K form to give the concentrations indicated. When ATP was also present, it was added just before centrifugation and the free Ca^{++} concentration was adjusted to a pCa value of 5 with a CaEGTA buffer containing 1 mM of total EGTA. The pellets were then washed as described in the text before analyses of cations bound were carried out.

binding of Ca so that originally about 50% of the total binding sites of the microsomal membranes were saturated with respect to Ca, i.e. about the level of Ca binding observed in the presence of ATP, but at a much lower concentration of free Ca^{++} (pCa = 5). This was achieved by raising the concentration of Ca in the medium to 2.0 mM at Mg and K concentrations of 3.3 and 10 mM, respectively (Fig. 2 *b*).

The active uptake of Ca by fragmented sarcoplasmic reticulum is decreased by SCN^- and I^- below that observed in the presence of Cl^- at all concentrations of the test anions studied (Fig. 2 *a*). Increasing the concentration of KCl from 10 to 210 mM decreases the Ca uptake from 170 to 145 μmoles of Ca/g of protein, whereas replacing 210 mM Cl^- by SCN^- or I^- decreases the Ca binding to 70 and 112 μeq of Ca/g of protein, respectively. Intermediate concentrations of SCN^- or I^- also depress the binding of Ca (Fig. 2 *a*). Despite the decrease in Ca binding caused by SCN^- and I^-, total Ca plus Mg plus K retained by the microsomes at the higher concentrations of SCN^- or I^- is slightly higher than total cations bound in the presence of Cl^- (Fig. 2 *a*). Binding of Mg in the presence of Cl^- is lower than that in the presence of equal concentrations of SCN^- or I^-. Higher binding of Mg in the presence of SCN^- or I^- reflects a lower displacement of this cation by Ca in the presence of SCN^- or I^- than that observed in the presence of Cl^-. Thus, the inhibition of Ca uptake induced by SCN^- or I^- permits more Mg to remain bound than is observed in the presence of Cl^-. Superimposed on this indirect effect of the anions on the Mg binding is the direct effect that the test anions have on the passive binding of Mg; this will be discussed later in connection with observations on the effect of anion substitution on the passive binding of divalent cations by the sarcoplasmic reticulum.

The increase in the binding of K as the concentration of anions is increased from 10 to 210 mM probably reflects simply that the binding sites of the microsomes are in equilibrium with increasingly higher concentrations of K added to the medium together with the anions, and, therefore, more binding sites are occupied by K at the higher than at the lower concentrations of K in the medium. The displacement of Ca at the higher concentrations of SCN^- and I^- does not occur simply as a consequence of a competition of K and Ca for the same binding sites because the effect on the binding of Ca is much more prominent in the presence of SCN^- or I^- than in the presence of Cl^- (at the same K concentrations), and, furthermore, the same amount of K is bound irrespective of the anion present (Fig. 2 *a*). Although the concentration of Mg in the medium remained constant at 4.7 mM, the binding of Mg by the microsomes was influenced greatly by the anion present. An increase in the concentration of KCl in the medium from 10 to 210 mM decreases the Mg binding from 135 to 112 μeq/g of protein, but when KSCN or KI replaces KCl, Mg binding increases as the concentration of SCN^- or I^- in the medium is raised.

At concentrations of SCN^- or I^- of 210 mM, the Mg binding is 190 and 160 μeq/g of protein, respectively, as compared to the 112 μeq of Mg/g of protein bound at the same concentration of Cl^- in the medium (Fig. 2 *a*).

Results of the effect of replacing Cl^- by SCN^- or I^- on the binding of Ca, Mg, and K in the absence of ATP (passive binding) are summarized in Fig. 2 *b*. Both anions increase the binding of Ca and Mg above that observed in the presence of Cl^-, and also increase the total cation-binding capacity of the fragmented sarcoplasmic reticulum. The effect is more pronounced at the highest concentration of anions studied (210 mM), but it is consistently observed also at concentrations of the anions of 60 and 110 mM. The binding of K in the presence of Cl^-, or either SCN^- or I^-, does not differ significantly at

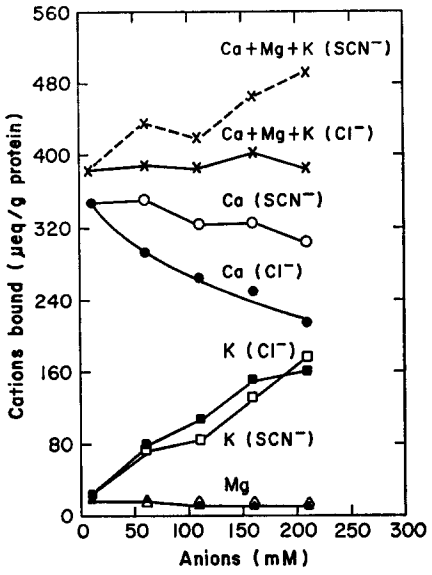

FIGURE 3. Effect of SCN^- on the passive binding of Ca by sarcoplasmic reticulum. Samples containing 10 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 20 mM imidazole at pH 7.0, 2.5 mM $CaCl_2$, 10 mM KCl, and SCN^- or Cl^- as indicated. Both SCN^- and Cl^- were added as the K salt. The suspensions were then centrifuged and washed as described in the text before analyses of cations bound were carried out.

any of the concentrations of the cations studied as was also observed in the presence of ATP.

The total binding of Ca plus Mg plus K in the presence of KCl is about 275 μeq of cations/g of protein at 10 mM KCl, but increases to about 300 μeq/g of protein when the concentration of KCl is 110 mM and remains at this value at higher concentrations. This suggests that at the lower concentrations of K the microsomes are not completely saturated with respect to the cations studied. At saturation in the presence of ATP, the binding capacity of the microsomes is also about 300 μeq of Ca plus Mg plus K per g of protein, which indicates that the same binding sites are involved in passive and active binding of Ca.

The effect of SCN^- on the passive binding of Ca is observed more clearly if the microsomes are essentially saturated with respect to Ca in the absence of Mg. Fig. 3 shows that the binding of Ca and the maximal capacity of the

microsomes for binding cations are both increased when Cl^- is substituted for SCN^-. At the highest concentration of SCN^- studied (210 mM) the binding of Ca was 40% higher than that observed in the presence of Cl^-, and the maximal binding capacity was similarly augmented. The binding of K was not affected by substitution of the anions. Although no Mg was added to the medium, 15–20 μeq of Mg/g of protein remained bound to the fragmented membranes under all conditions of the experiment, and this amount of Mg bound was not affected by the anions studied (Fig. 3).

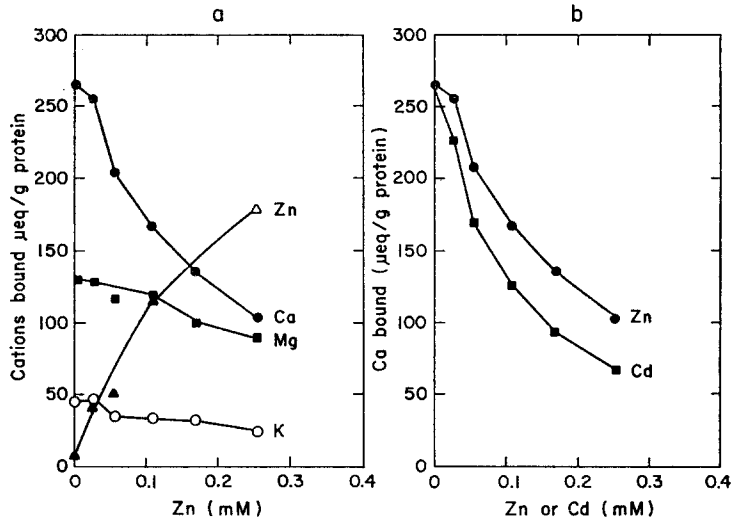

FIGURE 4. Effect of Zn and Cd on the binding of Ca, Mg, and K by the sarcoplasmic reticulum isolated from rabbit skeletal muscle. Samples containing 10 mg of microsomal protein were equilibrated for 10 min at 23°C in solutions containing 20 mM imidazole at pH 7.3, 100 mM KCl, 4.7 mM $MgCl_2$, 0.15 mM $CaCl_2$, and various concentrations of $ZnCl_2$ or $CdCl_2$. Before centrifugation, 1 ml of 10 mM ATP was added to each tube. The pellets were then washed and the cations bound were measured as described in the text. The concentrations of Zn and Cd given on the abscissa are the total concentrations measured in the supernatant solution after centrifugation.

Competition of Zn and Cd with Ca, Mg, and K for the Binding Sites of the Microsomes Previous studies on the passive binding of Zn by skeletal muscle microsomes showed that Zn competes with Ca and Mg for the same binding sites (13). The present study indicates that Ca bound actively in the presence of ATP (1.0 mM) and Mg (4.7 mM) also exchanges for Zn. In addition, Zn displaces Mg and K originally bound by the microsomes, but the total cations bound to the membranes remain approximately constant before and after adding Zn. The amounts of Ca, Mg, and K bound are 260, 135, and 45 μeq/g of protein, respectively, before Zn is added to the medium, and at a Zn concentration in the medium of 0.25 mM, about 40% of the binding sites are in the

Zn form. Under these conditions, Ca and Mg binding decreases to 100 and 90 μeq/g of protein, respectively, whereas 180 μmoles of Zn/g of protein are bound. The amount of K bound remains low at 30–40 μeq/g of protein since most of the binding sites are associated with divalent cations (Fig. 4 *a*).

In another series of experiments, the effectiveness of Cd to depress the active binding of Ca was also tested. As in the case of Zn, Cd decreases the binding of Ca by muscle microsomes, and it appears to be even more potent an inhibitor than Zn (Fig. 4 *b*).

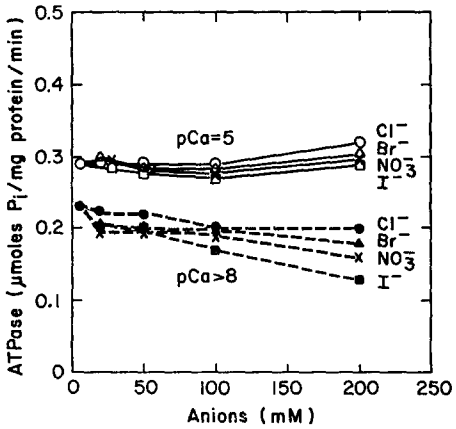

FIGURE 5. Effect of anions on the adenosine triphosphatase activity of sarcoplasmic reticulum isolated from rabbit skeletal muscle. Samples containing 0.6 mg of microsomal protein washed once in 0.6 M KCl were added to tubes containing 4.7 mM $MgCl_2$, 20 mM imidazole at pH 7.0, and the test anions in the K form as indicated. The free Ca^{++} in the medium was adjusted to a pCa value of 5 (solid lines) with a CaEGTA buffer containing 1 mM of total EGTA, or only EGTA (1 mM) was added to reduce the free Ca^{++} to a value of less than 8 (dashed lines). The reaction was initiated with the addition of 1.0 ml of 5 mM ATP to a final volume of 5 ml. ATPase measurements were carried out as described in the text.

Inhibition of ATPase of Sarcoplasmic Reticulum by Potentiators The anionic potentiators tested (i.e., SCN^-, I^-, NO_3^-, and Br^-) do not influence significantly the Ca-stimulated ATPase activity of sarcoplasmic reticulum, but in the absence of Ca (1 mM EGTA added), the anions inhibit the rate of ATP hydrolysis which is Ca-independent. As shown in Fig. 5, I^- is more effective in this respect than NO_3^- and Br^-. Thiocyanate was not tested because it interferes with the analysis of phosphate. These experiments were carried out under conditions identical with those employed in the cation-binding studies, except that the sarcoplasmic reticulum was washed with 0.6 M KCl to remove any myosin and actomyosin that may have been present (16). The concentration of Mg in the medium was 4.7 times that of ATP (i.e., 4.7 and 1.0 mM, respectively). At this high ratio of Mg to ATP, K does not affect the activity

of the enzyme significantly up to concentrations of 200 mM, but at equimolar concentrations of Mg and ATP, K further activates the enzyme (18). A pCa value of 5, employed in these studies, is somewhat inhibitory to the enzymatic activity (18), but it was desirable to compare the effects of the anions on the ATPase activity under suboptimal conditions because the studies on Ca binding indicated maximal binding of Ca at pCa values of 5–6 (Fig. 1 *a*). Weber, Herz, and Reiss (19) have reported maximal uptake of Ca by a similar fraction at pCa values of 6–7. The discrepancy between the values reported here and

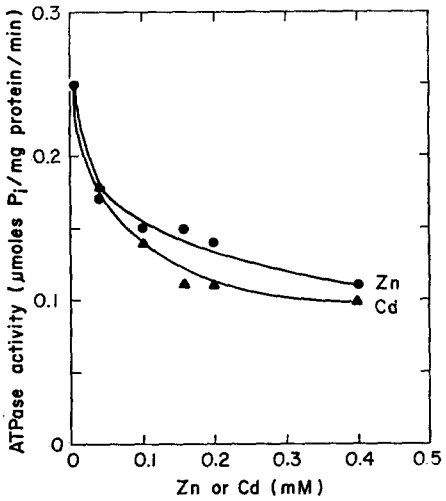

FIGURE 6. Effect of Zn and Cd on the adenosine triphosphatase activity of sarcoplasmic reticulum isolated from rabbit skeletal muscle. Samples containing 0.6 mg of microsomal protein washed once in 0.6 M KCl were added to tubes containing 100 mM KCl, 4.7 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mM imidazole at pH 7.0, and Zn or Cd as indicated. The reaction was started with the addition of 1.0 ml of 5 mM ATP to a final volume of 5 ml. ATPase measurements were carried out as described in the text.

those of Weber et al. probably arises because the pCa values for the present experiments were calculated from the concentration of free Ca^{++} in the media at equilibrium (i.e., after the sarcoplasmic reticulum had been centrifuged and Ca uptake had ceased) whereas the values reported by Weber et al. reflect the concentration of free Ca^{++} at the steady state while the sarcoplasmic reticulum was in suspension and presumably retained maximal values of Ca.

Zinc and Cd both inhibit the ATPase of sarcoplasmic reticulum activated by Mg plus Ca (Fig. 6). A concentration of Zn or Cd of 5×10^{-5} M causes a 35% inhibition of the enzyme activity. The binding of Ca is about equally susceptible to the heavy metal cations (Fig. 4).

All studies of the effect of Zn and Cd on the ATPase activity of sarcoplasmic reticulum were carried out in the presence of Ca, because it is not feasible to

eliminate traces of free Ca^{++} from the assay media with EGTA since this compound also chelates Zn and Cd. Various investigators (21, 33) have shown a correlation between the Ca-activated ATPase of the sarcoplasmic reticulum and the ability of this fraction to accumulate Ca. It appears from the present studies that, although Zn and Cd conceivably may impede Ca uptake by inhibiting the ATPase activity of the reticular membranes, the anionic potentiators presumably modify the cation-binding properties of the membranes by another mechanism.

DISCUSSION

The results show that anionic potentiators of muscular contraction (i.e., SCN^-, I^-, NO_3^-, and Br^-) inhibit the active uptake and increase the passive binding of Ca by isolated sarcoplasmic reticulum. Only a small amount of Ca is bound passively by the sarcoplasmic reticulum membranes (20–30 mg/g of protein) at concentrations of free Ca^{++} of the order of those estimated to exist intracellularly (10^{-6}–10^{-7} M) and, accumulation of the bulk of the Ca by these structures, amounting to 200–250 μeq/g of protein, depends on an active uptake of the ion which requires ATP and presumably is responsible for regulating the concentration of intracellular free Ca^{++} (19–21). The anionic potentiators would be expected, therefore, to interfere with this intracellular regulation of Ca under the control of the sarcoplasmic reticulum if they penetrated the muscle cell into the region of the reticular membranes. Zinc and Cd have an effect on the active binding of Ca by isolated sarcoplasmic reticulum similar to that of the anionic potentiators (Fig. 4), and, if these cations came in contact with the sarcoplasmic reticulum within the cell, the concentration of free myoplasmic Ca^{++} would increase since less Ca would be retained by the reticular membranes in the presence of these cations. This interference of the potentiators (anionic and cationic) with the binding of Ca by sarcoplasmic reticulum could be the basis for the increase in duration of the active state induced in the muscle cell by the presence of the potentiating agents during stimulation (3, 4, 22); i.e., a concentration of free intracellular Ca^{++} beyond the level required for activating the myofibrils would persist for a longer time in the presence than in the absence of the potentiators (22). Detailed studies of the mechanism by which the heavy metal cations and the anions prolong the active state suggest differences between the mode of action of the two groups of potentiators. Thus, the anionic potentiators lower the mechanical threshold and do not affect the action potential, whereas the heavy metals prolong the repolarization phase of the action potential, but do not affect the mechanical threshold (3, 4). Sandow and coworkers (3–5, 22) have suggested that either lowering of the mechanical threshold or prolongation of the repolarization phase increases the effectiveness of membrane depolarization causing release of Ca from the sarcoplasmic reticulum. A lowering of the threshold by anions

may reflect the fact that this type of potentiators sensitizes the release of Ca by sarcoplasmic reticulum to changes in membrane potential so that Ca begins to be released at a membrane potential closer to the resting potential in the presence than in the absence of the potentiators. In the case of the heavy metals, which prolong repolarization, Ca continues to be released until the falling phase of the action potential returns to subthreshold values (3, 4, 22). It should be noted that only in the case of the anions has it been assumed that the potentiating action might be regulated by a direct action of the potentiating agents on the reticulum. The effect of the heavy metals, as discussed above, would be mediated by their effect on the duration of the action potential which would then regulate the release and uptake of Ca by the sarcoplasmic reticulum.

The question arises whether the present findings on the direct effect of the potentiators on the active binding of Ca by isolated sarcoplasmic reticulum and those reported by other workers (23) apply also to the intact muscle cell bathed in solutions containing the potentiating substances. Several observations suggest that neither anionic nor cationic potentiators penetrate the cell to the level of the sarcoplasmic reticulum to exert their potentiating effect. Briefly, the evidence is as follows: Anionic and cationic potentiators exert their effects quickly after they have been added to the exterior of the muscle cell, and these effects are also reversed rapidly (3, 4). Hodgkin and Horowicz (2) found a time constant of 1–3 sec for the effect of adding the potentiating anions to or removing them from the solution bathing a single muscle fiber from the semitendinosus muscle of the frog. Although this time constant is longer than would be expected if the anions acted on the plasma membrane, it is not sufficiently long for their penetration into the myoplasma. The potentiating action of Zn and Cd on frog's sartorius muscle is readily reversed by CaEDTA, a substance which does not penetrate the muscle cell; therefore, Sandow and Isaacson (5) concluded that the primary site of action of Zn and other heavy metal cations probably is the plasma membrane or the transverse tubules or both.

The nature of the interaction of the anionic and cationic potentiators with isolated plasma membrane and transverse tubular membranes has not been studied because of the difficulty in obtaining pure fractions of these membrane components. However, all cellular membranous materials studied have proven to be remarkably similar in their structure, gross chemical composition, and ability to bind cations passively (9–14, 24–27) so that it may be valid to extend the findings on the passive binding properties of one type of membrane to another. The passive binding of cations, particularly Ca, by the plasma membrane has been recognized as an essential feature controlling the permeability properties of the membrane (15, 28). Furthermore, recent studies in our laboratory and those of other workers (29, 30) showed that removal of the

Ca passively bound to the membranes of sarcoplasmic reticulum of rabbit skeletal muscle destroys the integrity of the membrane. The present studies show that the passive binding of divalent cations (Ca and Mg) by the sarcoplasmic reticular membranes is enhanced by SCN^-, I^-, NO_3^-, and Br^- when these anions substitute for Cl^-. This effect of anions on the binding of divalent cations appears to be a general one observed in various systems of biological macromolecules (e.g., plasma proteins) in equilibrium with divalent cations (15). The amounts of Ca and Mg associated with the plasma membrane of the muscle cell probably depend on the equilibrium concentrations of the cations in solutions bathing these membrane systems, and the effect of SCN^-, I^-, NO_3^-, and Br^- would then be to increase the amount of divalent cations associated with these membranes. The increase in divalent cation content of the membrane would be expected to raise the membrane resistance as has been observed for phospholipid membrane models (31, 32), and probably is the basis for the increase in resistance of the muscle plasma membrane which occurs when the above anions replace Cl^- in the bathing solution of the muscle cell (34, 35).

Zinc and Cd, which bind to membrane structures more tightly than do Ca and Mg, substitute for these cations at the binding sites of isolated sarcoplasmic reticulum (Fig. 4) (13) and probably also at the binding sites of the plasma membrane and transverse tubules of the intact cell, since they are expected to have easy access to these structures (36). This substitution of Zn and Cd for other divalent and monovalent cations, which have lower affinity for the ligands of the membranes, has the effect of tightening the molecular structure of the membranes by rigidly cross-linking the lipoprotein complex of the membrane and probably accounts for the observed increase in resistance of the plasma membrane of skeletal muscle of frog caused by Zn (35). Thus, the increase in resistance of the muscle cell membrane caused by anionic and cationic potentiators may originate in both cases from an increase in divalent cation content of the membrane which would tend to decrease its permeability (32).

Zinc and Cd also prolong the repolarization phase of the action potential and thus prolong the active state, presumably by interfering with the diffusion of K out of the cell (4, 5). There is now evidence that the process of repolarization of the muscle cell depends on effluxes of cellular K into the transverse tubules and subsequently from these structures to the outside of the muscle fiber in addition to the outflux of the ion through the plasma membrane (37), but it is not known which of these K fluxes is affected by Zn or Cd. Since the effect is restricted to the movement of K [the rising phase of the action potential spike is normal in the presence of the potentiators (38)], Zn and Cd probably prolong repolarization by interacting at sites not involved in the development of the spike, conceivably at the level of the transverse tubular system.

It is, of course, not improbable that Zn and Cd selectively decrease the permeability of both plasma and T-system membranes to K. Qualitatively the end result is the same as if the heavy metals decreased either the permeability of the membranes of the transverse tubules to K or the rate of free diffusion of K from within the T-tubules to the outside of the muscle fiber (see Adrian and Freygang (39), Peachey (37), and Sandow and Isaacson (5) for discussion of the role of T-tubules in excitation-contraction coupling).

Interesting as these concepts may be, it is assumed that the cation-binding properties for membrane systems isolated from skeletal muscles of frog and rabbit are similar; furthermore, there is no direct information on the effect of potentiators on the cation-binding properties of the outer membranes of any of these muscle cells. Presently we are extending the scope of our investigation to encompass studies on the interaction of potentiators of muscle contraction with isolated sarcolemma. These studies should be particularly elucidative since the evidence obtained with intact muscle cells suggests that the external membranes (physiologically probably including also the transverse tubular membranes) are the site of action of the potentiators (1–5). The results reported here show, however, that if the potentiators penetrate into the myoplasm, they can be expected to increase the release of Ca by the sarcoplasmic reticulum and thus increase the force of contraction developed during the twitch. However, superimposed and preceding this effect is the effect of the potentiators on the passive binding of divalent cations (i.e., Ca and Mg) by the plasma and transverse tubular membranes, as surmised in our studies, which may, indirectly, also affect the release and uptake of Ca by the sarcoplasmic reticulum by modifying the permeability and electrical properties of these membranes with which the potentiators must interact before reaching the sarcoplasmic reticulum in the interior of the cell.

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