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Heavy metals induce rapid calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle

(sulfhydryl reagent/permeability)

JONATHAN J. ABRAMSON*, JONATHAN L. TRIMM*, LYLE WEDEN†, AND GUY SALAMA‡

*Physics Department and †Chemistry Department, Environmental Sciences and Resources Program, Portland State University, Portland, Oregon 97207; and ‡Department of Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

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ABSTRACT **Micromolar concentrations of mercury, silver, and other reagents known to react with sulfhydryl groups are shown to stimulate ATPase activity and inhibit active calcium uptake in sarcoplasmic reticulum vesicles derived from rabbit fast skeletal muscle. These effects are caused by a dramatic increase in the calcium permeability of the sarcoplasmic reticulum. Measurements of Ca^{2+} permeability were made using both isotopes and by spectrophotometric techniques using the Ca^{2+} indicator arsenazo III. Air oxidation of a sulfhydryl group to a disulfide group also leads to a large increase in the calcium permeability of the sarcoplasmic reticulum.**

The sarcoplasmic reticulum (SR) is responsible for the regulation of relaxation and contraction in skeletal muscle. It accomplishes this by controlling the Ca^{2+} concentration in the region of the myofibrils (1). The cycle of contraction and relaxation is initiated by a neural impulse that propagates a wave of depolarization along the sarcolemma, or surface membrane of the muscle cell. The electrical impulse is transmitted from the surface membrane to the transverse tubule. Depolarization of the transverse tubule causes the release of Ca^{2+} stored in the SR (2, 3). The Ca^{2+} that is released into the myoplasm binds to troponin and initiates contraction of the muscle. On repolarization of the surface membrane, the Ca^{2+} is actively reaccumulated by the SR. Ca^{2+} uptake by the SR leads to a decrease in myoplasmic calcium, which leads to relaxation of the muscle cell.

Several hypotheses have been presented to explain how depolarization of the *t* tubule leads to release of Ca^{2+} from the SR. Ca^{2+}-induced Ca^{2+} release appears to play an important role in cardiac SR, but it is not yet clear whether or not Ca^{2+}-induced Ca^{2+} release plays a corresponding part in skeletal SR (4). Depolarization-induced Ca^{2+} release has been studied in several laboratories (5, 6). There is evidence to support the hypothesis that depolarization is the physiological trigger for Ca^{2+} release, but experimental difficulties, primarily osmotic effects (7–9) and the lack of a reliable method for measuring membrane potential, raise doubts regarding this proposal. Ca^{2+} release can also be triggered by increasing the pH of the medium containing the SR (10, 11).

In this paper, we present data showing that various heavy metals cause the fast release of Ca^{2+} from SR vesicles. This increase in Ca^{2+} permeability of the SR causes a stimulation of Ca^{2+},Mg^{2+}-ATPase activity and an inhibition of active Ca^{2+} uptake. We also show that the heavy metals tested appear to be binding to a sulfhydryl group on a protein in the SR. Our preliminary results suggest that this protein is not Ca^{2+},Mg^{2+}-ATPase. In addition to triggering release by heavy metal binding to a sulfhydryl group, we show that oxidation of a sulfhydryl group to a disulfide also causes a very large increase in the Ca^{2+} permeability of SR vesicles.

METHODS

Materials. Octyl β-D-glucopyranoside and ATP were purchased from Calbiochem. [γ-^{32}P]ATP and $^{45}CaCl_2$ were purchased from New England Nuclear. All other chemicals were purchased from Sigma.

Preparations. SR vesicles were prepared from rabbit white skeletal muscle according to the method of MacLennan (12). Ca^{2+},Mg^{2+}-ATPase was isolated by extraction with octyl glucoside (13). Protein concentration was determined by the method of Lowry *et al.* (14).

Assays. Isotopic measurements of Ca^{2+} uptake and Ca^{2+},Mg^{2+}-ATPase activity were made at 37°C in 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$/30 μM $CaCl_2$. Uptake and ATPase activity were initiated by the addition of 1 mM Na_2ATP to a solution containing SR at 0.2 mg/ml. Uptake was measured with $^{45}CaCl_2$ (≈10^5 cpm/nmol of Ca^{2+}). The reaction was stopped by filtration through a Millipore 0.45-μm type HA filter (15). Filters were washed and dried, and radioactivity was determined in Aquasol-2 universal liquid scintillation cocktail (New England Nuclear). ATPase activity was measured using unlabeled $CaCl_2$ and [γ-^{32}P]ATP. The reaction was terminated by the addition of ice-cold trichloroacetic acid. The precipitated protein was pelleted, and the supernatant was added to butyl acetate/ammonium molybdate (16). The labeled phosphomolybdate complex was extracted into the butyl acetate phase by extended Vortex mixing. The phases were separated by centrifugation, and samples from the butyl acetate phase containing [^{32}P]phosphomolybdate were assayed in a liquid scintillation counter.

Isotopic Measurements of Ca^{2+} Release. SR vesicles at 5 mg/ml were passively loaded with 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$/5 mM $^{45}CaCl_2$ on ice for 16–24 hr. The samples were then diluted (1:25) into a 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$. The experiment was carried out at 37°C. Samples were removed at various times and filtered through Millipore 0.45-μm type HA filters. Filters were washed and dried, and radioactivity was determined.

Spectrophotometric Measurements of Ca^{2+} Efflux. Extravesicular Ca^{2+} was measured using arsenazo III as an indicator. Measurements were made using a time-sharing dual-wavelength spectrophotometer at 675–685 nm (17). SR at ≈0.2 mg/ml was incubated in 67 mM K_2SO_4/5 mM $MgSO_4$/20 mM Tris maleate, pH 7.0/100 μM arsenazo III (Sigma, purified grade). The temperature was regulated at either 22°C or 37°C. Two aliquots of 25 μM Ca^{2+} were added to calibrate the arsenazo–

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Abbreviation: SR, sarcoplasmic reticulum.

Ca^{2+} signal. Uptake was initiated by addition of 0.4 mM ATP. Release was initiated by addition of $AgNO_3$ or $HgCl_2$. Neither Ag^+ or Hg^{2+} interfered with the arsenazo–Ca^{2+} calibration.

RESULTS

As shown in Fig. 1, mercury causes a stimulation of Ca^{2+},Mg^{2+}-ATPase activity and a decrease in the total amount of Ca^{2+} accumulated. We found a 4-fold increase in ATPase activity at a Hg^{2+}/SR ratio of 5 mol/100 kg. Further increase in the mercury concentration inhibits both ATPase activity and active Ca^{2+} uptake. Shamoo and MacLennan (18, 19) have looked at the effects of $HgCl_2$ and CH_3HgCl on ATPase activity and active Ca^{2+} uptake in SR vesicles in the presence of potassium oxalate. At approximately the same Hg^{2+}/SR ratio, they found an inhibition of both ATPase activity and active Ca^{2+} uptake. Ca^{2+} transport, however, decreases more quickly with increasing Hg^{2+} concentration than does ATPase activity. The experiments shown in Fig. 1 were done in the absence of the Ca^{2+} precipitable anion oxalate. Not only did we find that Hg^{2+} inhibits Ca^{2+} uptake more potently than it inhibits ATPase activity, we also observed that, in the absence of oxalate, Hg^{2+} stimulates ATPase activity. We have also found that $AgNO_3$ stimulates ATPase activity and inhibits Ca^{2+} uptake into SR vesicles (data not shown). Ag^+ is not quite as potent in stimulating ATPase activity as Hg^{2+}.

One possible explanation for these data is that, in the presence of Hg^{2+}, the energy derived from the hydrolysis of ATP is no longer efficiently coupled into the movement of Ca^+ across the membrane. As a result of this, the activity of the pump is stimulated. To test for this, we isolated Ca^{2+},Mg^{2+}-ATPase by using the method of Banerjee *et al.* (13) and measured the ATPase activity of the isolated enzyme versus the mercury concentration. Hg^{2+} does not stimulate the ATPase activity of the isolated Ca^{2+},Mg^{2+}-ATPase molecule. It does not appear that Hg^{2+} causes an uncoupling of ATPase activity and active Ca^{2+} uptake by acting directly on the Ca^{2+},Mg^{2+}-ATPase molecule.

Another possible explanation is that mercury causes the SR to become more leaky to Ca^{2+}. The Ca^{2+} that is pumped into the SR leaks out more quickly. The net effect is that less Ca^{2+} is accumulated by the SR and the ATPase activity is stimulated, because there is less of a Ca^{2+} gradient to pump against.

In Fig. 2, we show the results of an experiment designed to measure the Ca^{2+} permeability of the SR in the presence and absence of Hg^{2+}. SR vesicles were passively loaded with 5 mM $^{45}CaCl_2$ and, on equilibration, the sample was diluted 1:25 with

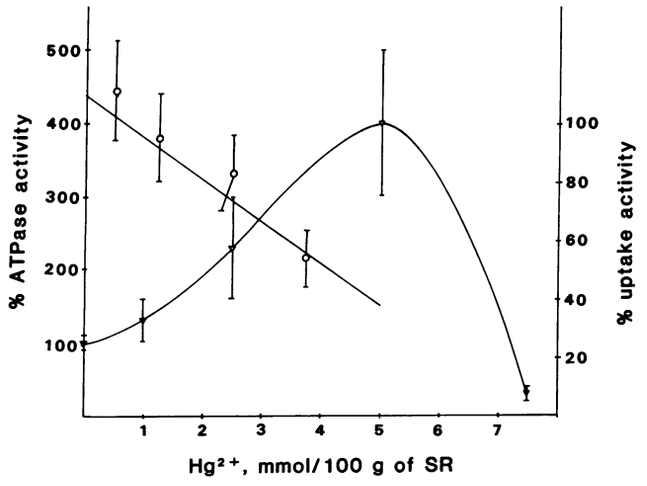

FIG. 1. Calcium uptake (○) and ATPase activity (▽) as a function of $HgCl_2$ concentration in SR vesicles. Values given are mean ± SEM.

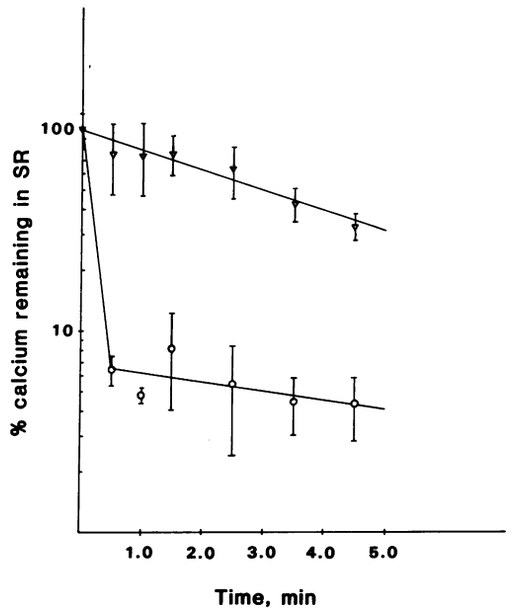

FIG. 2. Calcium efflux in the presence and absence of Hg^{2+}. SR vesicles at 5 mg/ml were incubated overnight at 0°C in 5 mM $^{45}CaCl_2$/100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$ and then diluted 1:25 (t = 0) into 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$ with (○) and without (▽) 10 μM $HgCl_2$. Values given are mean ± SD.

a buffer containing no Ca^{2+}. If all the Ca^{2+} were to equilibrate, 4% of the initial Ca^{2+} in the vesicle would remain in the vesicles. Hg^{2+} was present in the dilution medium at a concentration of 10 μM. On dilution of the SR into this medium, the SR concentration was 0.2 mg/ml. There are 5 mol of Hg^{2+} present per 100 kg of SR. This corresponds to the concentration of Hg^{2+} that causes maximum stimulation of ATPase activity (Fig. 1). Under these conditions, almost all of the Ca^{2+} present in the SR was released within 30 sec.

To determine whether heavy metal-induced Ca^{2+} release is a reversible effect, we modified our experimental procedure slightly and this time added a large excess of reducing agent. SR at 5 mg/ml was incubated with 250 μM $HgCl_2$/5 mM $^{45}CaCl_2$/100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$ at room temperature for 15 min, 2.5 mM 2-mercaptoethanol was added, and this preparation was incubated for 30 min at room temperature and then allowed to equilibrate at 0°C for 16–24 hr. The SR was then diluted 1:25 with 100 mM KCl/50 mM Hepes, pH 7.0/5 mM $MgCl_2$. When the SR was incubated in the absence of 2-mercaptoethanol, we obtained a curve almost identical to the 10 μM Hg^{2+} curve shown in Fig. 2. In the presence of a 10-fold excess of 2-mercaptoethanol over Hg^{2+}, Ca^{2+} remaining in the SR at the first time point (t = 30 sec) was 40–50% of that at t = 0, and the values found at all later time points paralleled the "no Hg^{2+}" curve in Fig. 2; i.e., they were all lower by 40–50%. It appears that some, but not all, of the Hg^{2+} or Ag^+ can be displaced by a large excess of reducing agent. Those vesicles in which the Hg^{2+} was removed by 2-mercaptoethanol appear to be as impermeable to Ca^{2+} as normal untreated SR vesicles.

To increase our time resolution, the flux of Ca^{2+} ions across the SR was followed by measuring differential absorption changes of arsenazo III as an indicator of extravesicular Ca^{2+} concentration. Fig. 3 shows the time course of Ca^{2+} uptake initiated by addition of ATP followed by Ca^{2+} release initiated by addition of $AgNO_3$. The Ca^{2+} ionophore A23187 was added on

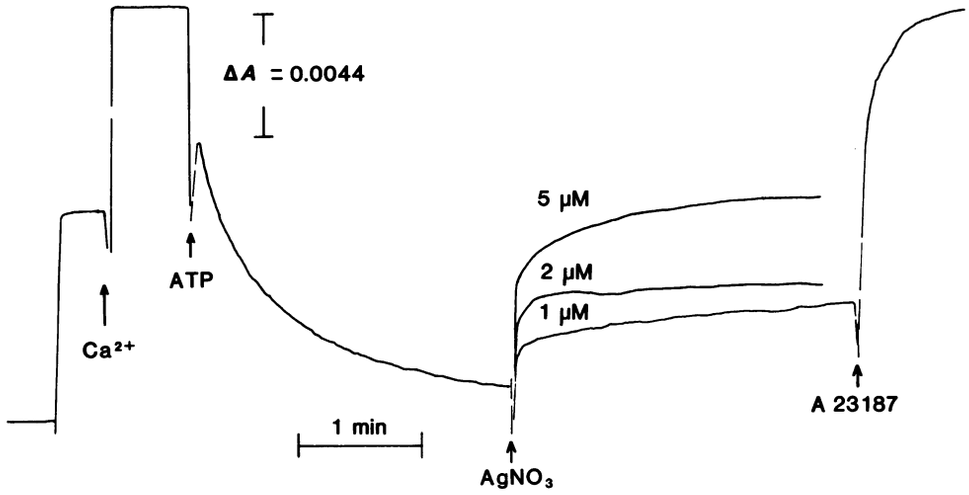

FIG. 3. Calcium uptake of SR vesicles followed by release triggered by addition of $AgNO_3$ at various concentrations. SR vesicles at 0.22 mg/ml were incubated at 25°C in 67 mM K_2SO_4/5 mM $MgSO_4$/20 mM Tris maleate, pH 7.0/100 μM arsenazo III. Two aliquots of Ca^{2+} were added to bring the final Ca^{2+} concentration to 50 μM. Then, 400 μM ATP was added and Ca^{2+} uptake was recorded (absorbance decreased), after which $AgNO_3$ was added and Ca^{2+} release was recorded. Finally, A23187 (1 μg/ml) was added and the remaining Ca^{2+} was released by the SR vesicles (absorbance increased). Extravesicular Ca^{2+} was monitored by a dual-wavelength spectrophotometer measuring changes in the absorption at 675–685 nm.

completion of the experiment to cause the efflux of the remaining Ca^{2+}.

In Fig. 4, the initial rate of Ca^{2+} efflux averaged over the first 6 sec and the total percentage of Ca^{2+} efflux are plotted as functions of Ag^+ and Hg^{2+} concentrations. The initial rate was averaged over the first 6 sec, because it takes 2 to 3 sec to manually add and mix samples (ATP or Ag^+). Neither Ag^+ nor Hg^{2+} interfered with calibration of the arsenazo signal versus the free Ca^{2+} concentration. Increasing the Ag^+ concentration increased both the initial rate and the total percentage effluxed. In the case of Hg^{2+}-induced Ca^{2+} release, both the rate of efflux and the total Ca^{2+} effluxed were less sensitive to increasing concentration. A small amount of Hg^{2+} caused a relatively large amount of total Ca^{2+} to efflux. Efflux rates measured at 37°C were 3 to 4 times higher than efflux rates measured at 25°C.

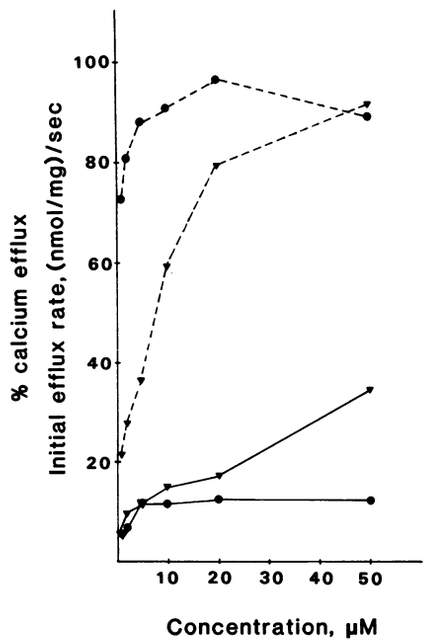

FIG. 4. Percent calcium efflux (---) and initial efflux rate (——) as a function of $HgCl_2$ (●) or $AgNO_3$ (▼) concentration. Initial efflux rates were averaged over the first 6 sec. Experiments were done at 37°C as described in Fig. 3.

To determine the nature of the binding site that initiates Ca^{2+} release in SR vesicles, we examined the relative potency of several heavy metals in causing Ca^{2+} release. In Table 1, we show the concentrations of various agents that cause release of 50% of passively loaded ^{45}Ca in 30 sec. The sequence—$Cu^{2+} > Hg^{2+} > Ag^+ > Cd^+ \geq Zn^{2+} > CH_3Hg^+ >$ *N*-ethylmaleimide > Ba^{2+}—is almost identical to the binding sequence of heavy metals to such sulfhydryl-containing reagents as *S*-methylcysteine and penicillamine (20). Methylmercuric chloride is less potent than the more hydrophilic mercuric cloride. *N*-Ethylmaleimide, a relatively specific sulfhydryl reagent, causes release of Ca^{2+} at a somewhat higher concentration. These experiments can be done by incubating the SR (5 mg/ml) in a solution containing the heavy metal (at a concentration 25-fold that shown in Table 1) or the SR can be diluted 1:25 (final SR concentration = 0.2 mg/ml) into a solution containing the heavy metal at the concentration shown in the table. It is important to maintain the heavy metal/SR ratio (mol/wt) constant.

A reagent such as Hg^{2+} is bifunctional; i.e., it can react with two sulfhydryl containing ligands to form a complex. The question arose as to whether or not the oxidation of two neighboring sulfhydryl groups to form a disulfide would lead to Ca^{2+} release.

Table 1. Relative potency of various reagents in causing Ca^{2+} release

	Concentration, μM
Cu^{2+}	2
Hg^{2+}	4
Ag^+	10
Cd^{2+}	15
Zn^{2+}	20
CH_3Hg^+	30
MalNEt	40
Ba^{2+}	No release

SR vesicles at 5 mg/ml were incubated overnight on ice in 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$/5 mM $^{45}CaCl_2$ and then diluted 1:25 with buffers lacking Ca^{2+} and containing heavy metal or sulfhydryl reagents at various concentrations. The concentration of reagent (after dilution of the SR) that causes 50% of the Ca^{2+} to be released in 30 sec is given. MalNEt, *N*-ethylmaleimide.

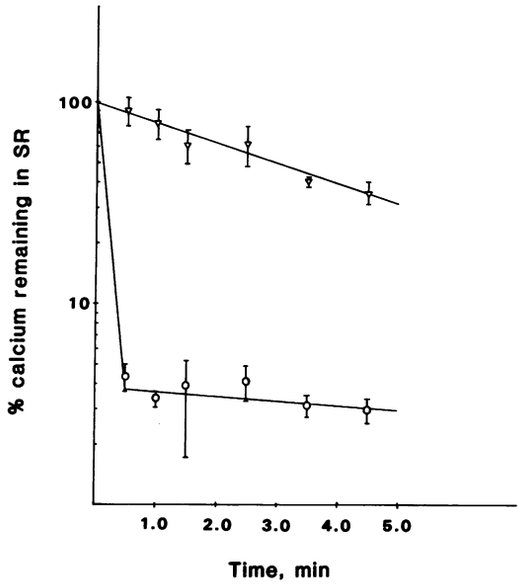

FIG. 5. Calcium release in the presence and absence of cupric phenanthroline. SR vesicles at 5 mg/ml were incubated overnight as described in Fig. 2 with (○) and without (▽) 5 μM $CuCl_2$/10 μM phenanthroline. Controls were run with $CuCl_2$ alone, phenanthroline alone, and buffer alone. At $t = 0$, samples were diluted 1:25 into buffer lacking Ca^{2+}, $CuCl_2$, and phenanthroline. ^{45}Ca remaining in the SR was assayed as a function of time. Values given are mean ± SD.

Cupric phenanthroline is known to catalyze the air oxidation of SH to S—S (21). In Fig. 5, we show the effect of cupric phenanthroline on the permeability of SR vesicles passively loaded with ^{45}Ca. In these experiments, SR vesicles at 5 mg/ml were incubated overnight with 5 μM $CuCl_2$/10 μM *o*-phenanthroline (Sigma) in the presence of 5 mM $^{45}CaCl_2$ and then diluted 1:25 into a buffer without Ca^{2+}. Ca^{2+} efflux was measured as a function of time. Controls with buffer alone, 5 μM $CuCl_2$ alone, and 10 μM *o*-phenanthroline alone were the same as the control shown.

We also attempted to reverse the Cu^{2+}/phenanthroline effect by adding a large excess of 2-mercaptoethanol in a manner similar to that described in the Hg^{2+} and Ag^+ experiments. In these experiments, we found that 2-mercaptoethanol was not at all successful in reversing the effect of cupric phenanthroline.

DISCUSSION

In the present study, we have shown that heavy metals stimulate ATPase activity and inhibit total Ca^{2+} actively accumulated by SR vesicles derived from fast skeletal muscle. This effect is due to a large increase in the Ca^{2+} permeability of the membrane (at least 3 orders of magnitude) that is triggered by the addition of heavy metals. It appears that the heavy metals tested react with a sulfhydryl group on a protein present in the SR. Ca^{2+} release is initiated either by binding of a heavy metal to this sulfhydryl group or by oxidation of this sulfhydryl group to form a disulfide.

Previously, it has been shown, on a time scale of minutes, that Salyrgan, *N*-ethylmaleimide, and $ZnCl_2$ cause release of Ca^{2+} from fragmented SR vesicles (15, 22–24). More recently, it has been shown that Hg^{2+} at high concentrations, which totally inhibits ATPase activity, causes a fast release of accumulated Ca^{2+} (25).

The experiments described in this paper were done in the absence of the Ca^{2+} precipitable anions oxalate and phosphate. Shamoo and MacLennan (18, 19) have done experiments similar to the one described in Fig. 1 in the presence of K^+ oxalate. At approximately the same Hg^{2+}/SR ratio (mol/wt), instead of the stimulation of ATPase activity and an inhibition of active Ca^{2+} uptake found in this study, they found only an inhibition of Ca^{2+} uptake. The oxalate present in their experiments prevents the development of a large Ca^{2+} gradient across the membrane. The stimulation of ATPase activity that we report here is due to the ability of the heavy metals tested to decrease the Ca^{2+} gradient by increasing the permeability of the SR to Ca^{2+}. The action of the heavy metal is similar to the effect of various detergents on the SR, which increase Ca^{2+} permeability (12, 26) and consequently stimulate ATPase activity.

Shamoo and MacLennan (18, 19) explain their observation that Hg^{2+} is more potent in inhibiting Ca^{2+} uptake than in inhibiting Ca^{2+}-ATPase activity in terms of Hg^{2+} binding to the transport or ionophoric site on the Ca^{2+},Mg^{2+}-ATPase molecule. They further support this statement by their observation that Hg^{2+} is a potent inhibitor of the ionophoric activity of Ca^{2+},Mg^{2+}-ATPase when assayed in a black lipid membrane. Our observations do not indicate that Ca^{2+} uptake in the SR is being inhibited because Hg^{2+} is binding to the ionophoric site on the Ca^{2+},Mg^{2+}-ATPase molecule but rather that Ca^{2+} uptake decreases with increased Hg^{2+} concentration because the SR is becoming more leaky to Ca^{2+}. Hg^{2+} and the other heavy metals tested are binding to a sulfhydryl group on a protein in the SR, which causes a dramatic increase in the Ca^{2+} permeability of the SR.

We have evidence that the release protein is not Ca^{2+},Mg^{2+}-ATPase. We have tested SR preparations that have lost their ATPase activity and hence can no longer take up Ca^{2+}. These preparations still respond to the addition of heavy metals by increased Ca^{2+} permeability of the SR. Thus, heavy metal stimulation of Ca^{2+} release in SR vesicles is not dependent on a functioning ATPase molecule. Although this does not prove that the release protein is not Ca^{2+},Mg^{2+}-ATPase, it does indicate that some other protein in the SR is probably the heavy metal-induced Ca^{2+} release protein.

As measured using arsenazo III as an indicator for extravesicular Ca^{2+}, Ag^+ and Hg^{2+} are very potent in increasing the Ca^{2+} permeability of the SR membrane. Ag^+ and Hg^{2+} increase the Ca^{2+} permeability of the SR by at least a factor of 1,000. The Ca^{2+} efflux rate is greater than 35 nmol/mg/sec. Rapid mixing should allow determination of how close the rate of Ca^{2+} efflux is to the rate of physiological Ca^{2+} release [4–8 (μmol/mg)/sec (9)].

To determine the nature of the binding site, we examined Ca^{2+} release triggered by various heavy metals. Those heavy metals that were most effective in causing Ca^{2+} release react most strongly with sulfhydryl groups. It appears that the heavy metals bind to a sulfhydryl group on a protein in the SR that triggers Ca^{2+} release. This sulfhydryl group appears to be located in a relatively hydrophilic environment. $HgCl_2$ is a more potent reagent in causing Ca^{2+} release then the more hydrophobic CH_3HgCl.

Ca^{2+} release can also be triggered by oxidation of a sulfhydryl group to a disulfide group. Incubation with cupric phenanthroline, which catalyzes air oxidation of sulfhydryl groups to disulfides, leads to a large increase in the Ca^{2+} permeability of the SR membrane. This effect is not reversed by addition of a large excess of reducing agent. The inability of reducing agents to reverse the oxidation caused by cupric phenanthroline has also been reported with myosin chymotryptic subfragment one. It appears that cupric phenanthroline treatment may lead to the generation of oxygen free radicals during oxidation (27).

We have shown that heavy metals induce rapid release of Ca^{2+} from skeletal muscle SR vesicles. The rate and extent of Ca^{2+} release are dramatic compared with those of Ca^{2+} release in-

duced by addition of Ca^{2+} (28–30) or by conditions designed to depolarize the SR membrane (8) in vesicle preparations. If the heavy metals are acting by binding to the "physiological release protein," sulfhydryl reagents may prove to be an important tool in characterization and identification of the Ca^{2+} release protein.

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