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

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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 Ca2+ permeability were made using both isotopes and by spectrophotometric techniques using the Ca2+ 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 Ca2+ 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 across the sarcolemma, or surface membrane of the cell. The electrical impulse is transmitted from the surface membrane to the transverse tubule. Depolarization of the transverse tubule causes the release of Ca2+ stored in the SR (2, 3). The Ca2+ that is released into the myoplasm binds to troponin and initiates contraction of the muscle. On repolarization of the surface membrane, the Ca2+ is actively reaccumulated by the SR. Ca2+ 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 transverse tubule leads to release of Ca2+ from the SR. Ca2+-induced Ca2+ release appears to play an important role in cardiac SR, but it is not yet clear whether or not Ca2+-induced Ca2+ release plays a corresponding part in skeletal SR (4). Depolarization-induced Ca2+ release has been studied in several laboratories (5, 6). There is evidence to support the hypothesis that depolarization is the physiological trigger for Ca2+ 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. Ca2+ 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 Ca2+ from SR vesicles. This increase in Ca2+ permeability of the SR causes a stimulation of Ca2+-Mg2+-ATPase activity and an inhibition of active Ca2+ uptake. We also show that the heavy metal tested appears to be binding to a sulfhydryl group on the protein in the SR. Our preliminary results suggest that this protein is not Ca2+-Mg2+-ATPase. In addition to triggering release by heavy metal binding to a sulfhydryl group, we show that oxidation of a sulfhydryl-

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Abbreviation: SR, sarcoplasmic reticulum.

METHODS

Materials. Octyl β-D-glucopyranoside and ATP were purchased from Calbiochem. [γ-32P]ATP and 45CaCl2 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). Ca2+-Mg2+-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 Ca2+ uptake and Ca2+-Mg2+-ATPase activity were made at 37°C in 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM MgCl2/30 μM CaCl2. Uptake and ATPase activity were initiated by the addition of 1 mM Na2ATP to a solution containing SR at 0.2 mg/ml. Uptake was measured with 45CaCl2 (=106 cpm/nmol of Ca2+). 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 CaCl2 and [γ-32P]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 [32P]phosphomolybdate were assayed in a liquid scintillation counter.

Isotopic Measurements of Ca2+ Release. SR vesicles at 5 mg/ml were passively loaded with 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM MgCl2/5 mM 45CaCl2 on ice for 16–24 hr. These samples were then diluted (1:25) into a 100 mM KCl/50 mM Hepes/KOH, pH 7.0/5 mM MgCl2. 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 Ca2+ Efflux. Extravesicular Ca2+ 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 K2SO4/5 mM MgSO4/20 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 Ca2+ were added to calibrate the arsenazo-
Ca²⁺ signal. Uptake was initiated by addition of 0.4 mM ATP. Release was initiated by addition of AgNO₃ or HgCl₂. Neither Ag⁺ or Hg²⁺ interfered with the arsenazo–Ca²⁺ calibration.

RESULTS

As shown in Fig. 1, mercury causes a stimulation of Ca²⁺-Mg²⁺-ATPase activity and a decrease in the total amount of Ca²⁺ accumulated. We found a 4-fold increase in ATPase activity at a Hg²⁺/SR ratio of 5 mol/100 kg. Further increase in the mercury concentration inhibits both ATPase activity and active Ca²⁺ uptake. Shamoo and MacLennan (18, 19) have looked at the effects of HgCl₂ and CH₃HgCl on ATPase activity and active Ca²⁺ uptake in SR vesicles in the presence of potassium oxalate. At approximately the same Hg²⁺/SR ratio, they found an inhibition of both ATPase activity and active Ca²⁺ uptake. Ca²⁺ transport, however, decreases more quickly with increasing Hg²⁺ concentration than does ATPase activity. The experiments shown in Fig. 1 were done in the absence of the Ca²⁺ precipitable anion oxalate. Not only did we find that Hg⁡²⁺ inhibits Ca²⁺ uptake more potently than it inhibits ATPase activity, we also observed that, in the absence of oxalate, Hg²⁺ stimulates ATPase activity. We have also found that AgNO₃ stimulates ATPase activity and inhibits Ca²⁺ uptake into SR vesicles (data not shown). Ag⁺ is not quite as potent in stimulating ATPase activity as Hg²⁺.

One possible explanation for these data is that, in the presence of Hg²⁺, the energy derived from the hydrolysis of ATP is no longer efficiently coupled into the movement of Ca²⁺ across the membrane. As a result of this, the activity of the pump is stimulated. To test for this, we isolated Ca²⁺-Mg²⁺-ATPase by using the method of Banerjee et al. (13) and measured the ATPase activity of the isolated enzyme versus the mercury concentration. Hg²⁺ does not stimulate the ATPase activity of the isolated Ca²⁺-Mg²⁺-ATPase molecule. It does not appear that Hg²⁺ causes an uncoupling of ATPase activity and active Ca²⁺ uptake by acting directly on the Ca²⁺-Mg²⁺-ATPase molecule.

Another possible explanation is that mercury causes the SR to become more leaky to Ca²⁺. The Ca²⁺ that is pumped into the SR leaks out more quickly. The net effect is that less Ca²⁺ is accumulated by the SR and the ATPase activity is stimulated, because there is less of a Ca²⁺ gradient to pump against.

In Fig. 2, we show the results of an experiment designed to measure the Ca²⁺ permeability of the SR in the presence and absence of Hg²⁺. SR vesicles were passively loaded with 5 mM ⁴⁰CaCl₂, and, on equilibration, the sample was diluted 1:25 with a buffer containing no Ca²⁺. If all the Ca²⁺ were to equilibrate, 4% of the initial Ca²⁺ in the vesicle would remain in the vesicles. Hg²⁺ 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 Hg²⁺ present per 100 kg of SR. This corresponds to the concentration of Hg²⁺ that causes maximum stimulation of ATPase activity (Fig. 1). Under these conditions, almost all of the Ca²⁺ present in the SR was released within 30 sec.

To determine whether heavy metal-induced Ca²⁺ 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 HgCl₂/5 mM ⁴⁰CaCl₂/100 mM KCl/50 mM Heps/KOH, pH 7.0/5 mM MgCl₂ 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 Heps, pH 7.0/5 mM MgCl₂. When the SR was incubated in the absence of 2-mercaptoethanol, we obtained a curve almost identical to the 10 μM Hg²⁺ curve shown in Fig. 2. In the presence of a 10-fold excess of 2-mercaptoethanol over Hg²⁺, Ca²⁺ 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 later time points paralleled the "no Hg²⁺" curve in Fig. 2; i.e., they were all lower by 40–50%. It appears that some, but not all, of the Hg²⁺ or Ag⁺ can be displaced by a large excess of reducing agent. Those vesicles in which the Hg²⁺ was removed by 2-mercaptoethanol appear to be as impermeable to Ca²⁺ as normal untreated SR vesicles.

To increase our time resolution, the flux of Ca²⁺ ions across the SR was followed by measuring differential absorption changes of arsenazo III as an indicator of extravascular Ca²⁺ concentration. Fig. 3 shows the time course of Ca²⁺ uptake initiated by addition of ATP followed by Ca²⁺ release initiated by addition of AgNO₃. The Ca²⁺ ionophore A23187 was added on
was added and the final 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$^{2+}$ efflux 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.

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$^+$ ≥ Zn$^{2+}$ > CH$_3$Hg$^+$ > N-ethylmaleimide > Ba$^{2+}$—is almost identical to the binding sequence of heavy metals to such sulphydryl-containing reagents as S-methylcysteine and penicillamine (20). Methylmercuric chloride is less potent than the more hydrophilic mercuric chloride. N-Ethylmaleimide, a relatively specific sulphydryl 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 sulphydryl containing ligands to form a complex. The question arose as to whether or not the oxidation of two neighboring sulphydryl groups to form a disulfide would lead to Ca$^{2+}$ release.

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

| Concentration, $\mu$m |%
|----------------------|------------------|
| Cu$^{2+}$           | 2
| Hg$^{2+}$           | 4
| Ag$^+$              | 10
| Cd$^{2+}$           | 15
| Zn$^{2+}$           | 20
| CH$_3$Hg$^+$        | 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 sulphydryl 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.
approximately the same Hg²⁺/SR ratio (mol/wt), instead of the stimulation of ATPase activity and an inhibition of active Ca²⁺ uptake found in this study, they found only an inhibition of Ca²⁺ uptake. The oxalate present in their experiments prevents the development of a large Ca²⁺ 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 Ca²⁺ gradient by increasing the permeability of the SR to Ca²⁺. The action of the heavy metal is similar to the effect of various detergents on the SR, which increase Ca²⁺ permeability (12, 26) and consequently stimulate ATPase activity.

Shamoo and MacLennan (18, 19) explain their observation that Hg²⁺ is more potent in inhibiting Ca²⁺ uptake than in inhibiting Ca²⁺-ATPase activity in terms of Hg²⁺ binding to the transport or ionophoric site on the Ca²⁺, Mg²⁺-ATPase molecule. They further support this statement by their observation that Hg²⁺ is a potent inhibitor of the ionophoric activity of Ca²⁺, Mg²⁺-ATPase when assayed in a black lipid membrane. Our observations do not indicate that Ca²⁺ uptake in the SR is being inhibited because Hg²⁺ is binding to the ionophoric site on the Ca²⁺, Mg²⁺-ATPase molecule. Our experiments indicate that Ca²⁺ uptake decreases with increased Hg²⁺ concentration because the SR is becoming more leaky to Ca²⁺. Hg²⁺ and the other heavy metals tested are binding to a sulphydryl group on a protein in the SR, which causes a dramatic increase in the Ca²⁺ permeability of the SR.

We have evidence that the release protein is not Ca²⁺, Mg²⁺-ATPase. We have tested SR preparations that have lost their ATPase activity and hence can no longer take up Ca²⁺. These preparations still respond to the addition of heavy metals by increased Ca²⁺ permeability of the SR. Thus, heavy metal stimulation of Ca²⁺ release in SR vesicles is not dependent on a functioning ATPase molecule. Although this does not prove that the release protein is not Ca²⁺, Mg²⁺-ATPase, it does indicate that some other protein in the SR is probably the heavy metal-induced Ca²⁺ release protein.

As measured using arsenazo III as an indicator for extravesicular Ca²⁺, Ag⁺ and Hg²⁺ are very potent in increasing the Ca²⁺ permeability of the SR membrane. Ag⁺ and Hg²⁺ increase the Ca²⁺ permeability of the SR by at least a factor of 1,000. The Ca²⁺ efflux rate is greater than 35 nmol/mg/sec. Rapid mixing should allow determination of how close the rate of Ca²⁺ efflux is to the rate of physiological Ca²⁺ release (4–5 (mol/mg)/sec (9)).

To determine the nature of the binding site, we examined Ca²⁺ release triggered by various heavy metals. Those heavy metals that were most effective in causing Ca²⁺ release react most strongly with sulphydryl groups. It appears that the heavy metals bind to a sulphydryl group on a protein in the SR that triggers Ca²⁺ release. This sulphydryl group is located in a relatively hydrophobic environment. HgCl₂ is a more potent reagent in causing Ca²⁺ release than the more hydrophobic CH₃HgCl.

Ca²⁺ release can also be triggered by oxidation of a sulphydryl group to a disulfide group. Incubation with cupric phenanthroline, which catalyzes air oxidation of sulphydryl groups to disulfides, leads to a large increase in the Ca²⁺ 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 Ca²⁺ from skeletal muscle SR vesicles. The rate and extent of Ca²⁺ release are dramatic compared with those of Ca²⁺ release in-

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**DISCUSSION**

In the present study, we have shown that heavy metals stimulate ATPase activity and inhibit total Ca²⁺ actively accumulated by SR vesicles derived from fast skeletal muscle. This effect is due to a large increase in the Ca²⁺ 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 sulphydryl group on a protein present in the SR. Ca²⁺ release is initiated either by binding of a heavy metal to this sulphydryl group or by oxidation of this sulphydryl group to form a disulfide.

Previously, it has been shown, on a time scale of minutes, that Salyrgan, N-ethylmaleimide, and ZnCl₂ cause release of Ca²⁺ from fragmented SR vesicles (15, 22–24). More recently, it has been shown that HgCl₂ at high concentrations, which totally inhibits ATPase activity, causes a fast release of accumulated Ca²⁺ (25).

The experiments described in this paper were done in the absence of the Ca²⁺ 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
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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