Micromolar concentrations of cupric ion (Cu\textsuperscript{2+}) and mercaptans such as cysteine, cysteamine, and homocysteine trigger large and rapid Ca\textsuperscript{2+} release from skeletal muscle sarcoplasmic reticulum (SR) vesicles. At the concentrations used, Cu\textsuperscript{2+} alone does not induce Ca\textsuperscript{2+} release nor does cysteine alone; both are required to induce Ca\textsuperscript{2+} release from SR. Cu\textsuperscript{2+} is known to catalyze the autooxidation of cysteine to its disulfide form cystine; Cu\textsuperscript{2+}/mercaptan-induced Ca\textsuperscript{2+} release appears to be caused by Cu\textsuperscript{2+}-catalyzed formation of a mixed disulfide between the exogenous mercaptan and a critical sulfhydryl on a transmembrane protein. In the oxidized state the SR is highly permeable to Ca\textsuperscript{2+}. Supporting evidence for this interpretation is as follows. 1) The order of Ca\textsuperscript{2+}-releasing reactivity of the mercaptans is the same as the order in which these compounds undergo oxidation to disulfide forms in the presence of Cu\textsuperscript{2+}. 2) Ca\textsuperscript{2+} efflux induced by cysteine and Cu\textsuperscript{2+} can be reversed by the addition of the disulfide reducing agent dithiothreitol. 3) Hypochlorous acid and plumbagin, both potential sulfhydryl oxidants, induce rapid Ca\textsuperscript{2+} efflux from SR vesicles; in addition, Cu\textsuperscript{2+}, which catalyzes H\textsubscript{2}O\textsubscript{2} oxidation of cysteine, enhances H\textsubscript{2}O\textsubscript{2}-induced release. Oxidation-induced Ca\textsuperscript{2+} release from SR can be partially reversed or blocked by ruthenium red or the local anesthetics procaine and tetracaine. The Ca\textsuperscript{2+} efflux rates are strongly Mg\textsuperscript{2+} dependent and are significantly higher in heavy SR than in light SR. These data suggest that the Ca\textsuperscript{2+} efflux thus induced is via the "Ca\textsuperscript{2+}-release channel" and that the oxidation state of a critical sulfhydryl group on this protein may be the principal means by which the Ca\textsuperscript{2+} permeability of the SR is regulated in vivo.

The sarcoplasmic reticulum (SR) is known to play a major role in the regulation of intracellular Ca\textsuperscript{2+} concentration in muscle and thereby in the generation of force. In skeletal, and in some striated muscles, the rapid movement of Ca\textsuperscript{2+} into the cytosol has been attributed mainly to efflux of Ca\textsuperscript{2+} from the SR triggered by an action potential across the T-tubule. The removal of Ca\textsuperscript{2+} from the cytosol, leading to relaxation, is via a Ca\textsuperscript{2+} pump which is integral to the SR membrane. However, despite extensive studies both in vitro and in vivo, the underlying mechanism responsible for Ca\textsuperscript{2+} release from the SR in skeletal and striated muscle is largely unknown. Several hypotheses have been put forth in attempts to link the action potential's arrival at the T-tubule to Ca\textsuperscript{2+} release from the SR. 1) Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (2-5); 2) depolarization of the SR membrane (6, 7); 3) changes in pH (8); 4) voltage-dependent charge displacement within the T-tubular membrane (9), which may be responsible for the movement of feet (10) or bridges (11) at the triadic junction spanning the T-tubule/SR gap; and 5) Ca\textsuperscript{2+} release induced by inositol 1,4,5-triphosphate (12, 13). The issue is not resolved and the physiological significance of the methods used to trigger Ca\textsuperscript{2+} release from SR remains controversial. The proposed mechanisms have been reviewed recently by A. Martonosi (14).

The theories mentioned above have one thing in common. Efflux of Ca\textsuperscript{2+} from the SR is presumed to be mediated by a Ca\textsuperscript{2+}-channel distinct from the Ca\textsuperscript{2+}-pump (Ca\textsuperscript{2+},Mg\textsuperscript{2+}-ATPase). In most studies, Ca\textsuperscript{2+} release has been described phenomenologically in terms of the agents which induce the changes in Ca\textsuperscript{2+} permeability, with little or no discussion on a molecular level as to the mechanisms by which these agents regulate the opening and closing of the "Ca\textsuperscript{2+}-release channel."

We have previously reported that micromolar concentrations of heavy metal ions (i.e. Cu\textsuperscript{2+}, Hg\textsuperscript{2+}, Ag\textsuperscript{+}, Cd\textsuperscript{2+}, or Zn\textsuperscript{2+}) trigger the rapid release of Ca\textsuperscript{2+} by apparently binding to a sulfhydryl group on an integral membrane protein of SR vesicles (15); the potency of these heavy metals to induce Ca\textsuperscript{2+} release from SR was found to be similar to their relative binding affinities to sulfhydryl groups. Upon actively accumulating Ca\textsuperscript{2+} in the presence of ATP, Ag\textsuperscript{+} (5-10 \mu M) induces rapid Ca\textsuperscript{2+} release (~58 nmol of Ca\textsuperscript{2+}/mg of protein/s) from heavy SR vesicles (16). Ag\textsuperscript{+}-induced release was examined as a function of pH, Mg\textsuperscript{2+}, and ionic strength; in light, heavy, and intermediate SR; and in the presence of known blockers of Ca\textsuperscript{2+} efflux. The data strongly suggest that Ag\textsuperscript{+} acts at the physiological site of Ca\textsuperscript{2+} release (16). Other investigators have reported that less reactive sulfhydryl reagents like organic mercurials (50-100 \mu M) also induce Ca\textsuperscript{2+} release from SR (17, 18).

Also, we (15) and other investigators (19) have shown that cupric phenanthroline, which is known to catalyze the air oxidation of sulfhydryls to disulfides (20), can induce Ca\textsuperscript{2+} release from the SR, though it has been suggested (19) that
the mode of action of cupric phenanthroline may involve cross-linking of the Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-ATPase and not the oxidation of sulfhydryls on a separate Ca\textsuperscript{2+} channel, as we have suggested (15).

Clearly, heavy metals do not play a role in physiological Ca\textsuperscript{2+} release from SR. However, given that they seem to strongly interact with the Ca\textsuperscript{2+} release channel, they are useful as probes of the functions of this channel and especially the role, if any, of this critical sulfhydryl in the regulation of the Ca\textsuperscript{2+} permeability of the SR in vivo. In the present report we show that cysteine and other biologically common mercaptans, in the presence of Cu\textsuperscript{2+}, induce large and rapid increases in the Ca\textsuperscript{2+} permeability of the SR. Cu\textsuperscript{2+} is known to catalyze the oxidation of cysteine to its disulfide form, cystine (21, 22).

We have found that the relative potency of the mercaptans to cause release (in the presence of Cu\textsuperscript{2+}) parallels the rate at which these compounds undergo Cu\textsuperscript{2+}-catalyzed oxidation to disulfides. Hypochlorous acid (23) and plubamin (24), which can act as sulfhydryl oxidants in the absence of Cu\textsuperscript{2+}, induce Ca\textsuperscript{2+} release from SR vesicles, though at somewhat lower rates. Also, Cu\textsuperscript{2+} enhances release induced by H\textsubscript{2}O\textsubscript{2} as well as sulfhydryl oxidation by H\textsubscript{2}O\textsubscript{2} (34). The rate of Ca\textsuperscript{2+} efflux induced by Cu\textsuperscript{2+} and cysteine is strongly dependent on free [Mg\textsuperscript{2+}] and is optimal at physiological pH and ionic strength. Ca\textsuperscript{2+} efflux induced by Cu\textsuperscript{2+}/cysteine is also much faster in heavy SR vesicles than in light and can be partially blocked or reversed by known inhibitors of Ca\textsuperscript{2+} release, namely ruthenium red (25, 26), procaine, and tetracaine (26). These properties of Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release from SR are very similar to Ag\textsuperscript{+} and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (16), suggesting a common site of action. The rapid increase in Ca\textsuperscript{2+} permeability of the SR induced by sulfhydryl oxidation may be 1) coincidental with no physiological significance or 2) an indication that sulfhydryl oxidation is the chemical reaction which links the depolarization of the T-tubule membrane with Ca\textsuperscript{2+} release from the SR or 3) an indication that this critical sulfhydryl is involved in regulation of the Ca\textsuperscript{2+} channel, though its normal function does not involve undergoing oxidation. A preliminary report on the work presented here has appeared in abstract form (27).

### Materials and Methods

Preparation of SR Vesicles—Rabbit skeletal sarcoplasmic reticulum vesicles were prepared according to the method of MacLennan (28). Light and heavy SR (LSR and HSR) vesicles were prepared by further fractionation of the crude SR preparation on a sucrose step gradient as described previously (16). SR, LSR, and HSR vesicles were suspended at approximately 30 mg of protein/ml in 100 mM KCl plus 20 mM HEPES at pH 7.4 and frozen in liquid nitrogen until used. Protein concentrations were determined by the method of Lowry et al. (29).

Ca\textsuperscript{2+} Efflux Experiments—Ca\textsuperscript{2+} efflux experiments were performed with SR vesicles which were loaded with Ca\textsuperscript{2+} either passively or actively in the presence of ATP. Extravesicular Ca\textsuperscript{2+} concentrations were measured through the differential absorption changes of arsenazo III at 675-685 nm with a time-sharing dual wavelength spectrophotometer (30). The use of this dye and the standard controls demonstrating its specificity for Ca\textsuperscript{2+} in similar SR experiments have been previously reported (31). The reagents used to induce Ca\textsuperscript{2+} release from SR vesicles produced negligible changes in the absorption of the Ca\textsuperscript{2+} indicator and did not appear to interfere with the specific and quantitative measurements of free Ca\textsuperscript{2+} concentrations. Ca\textsuperscript{2+} efflux measurements from actively loaded SR vesicles were performed as follows. SR vesicles were suspended at a protein concentration of 0.25 mg/ml in 100 mM KCl, 20 mM HEPES, pH 7.4, 0.1 mM arsenazo III, and various concentrations of MgCl\textsubscript{2} at room temperature. Two additions of Ca\textsuperscript{2+} were made to verify the linearity of the dye's response to Ca\textsuperscript{2+} (final [Ca\textsuperscript{2+}] = 15 or 20 mM) as indicated, and the differential absorption changes of the dye were continuously monitored. ATP (0.5 mM) was added to initiate active Ca\textsuperscript{2+} uptake, and the time course of Ca\textsuperscript{2+} uptake was recorded until Ca\textsuperscript{2+} loading was completed (as judged by a leveling-off of the dye signal). Ca\textsuperscript{2+} release was induced by sequentially adding various concentrations of cysteine (or other mercaptans) then Cu\textsuperscript{2+} or by adding a sulfhydryl oxidizing agent. The maximum efflux rates were calculated by determining the slope of the efflux curve at its steepest phase. The Ca\textsuperscript{2+} efflux rate is expressed in units of nmol of Ca\textsuperscript{2+} effluxed per mg of SR protein or as a rate constant in s\textsuperscript{-1}.

Effects of pH and Ionic Strength—Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release was measured as a function of pH and ionic strength using passively loaded SR vesicles. For pH-dependent Ca\textsuperscript{2+} efflux, SR vesicles were Ca\textsuperscript{2+} loaded by incubating SR vesicles (10 mg of protein/ml) containing 100 mM MgCl\textsubscript{2}, 0.5 mM CaCl\textsubscript{2}, 10 HEPES, and 16 PIPES at pH values ranging from 6.75 to 8.0. For measurements of Ca\textsuperscript{2+} efflux as a function of ionic strength the vesicles were suspended at 10 mg of protein/ml in solutions containing (in mM): 20 HEPES, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, pH 7.25, and various concentrations of KCl and sucrose to obtain a final osmolarity of approximately 225 mosM. In both studies, the vesicles were incubated on ice for 12-18 h to allow complete equilibration of free Ca\textsuperscript{2+} concentration across the SR membrane. The passively loaded SR vesicles were rapidly diluted by a factor of 40 in similar but Ca\textsuperscript{2+}-free media containing 0.1 mM arsenazo III at room temperature. The differential absorption of the Ca\textsuperscript{2+} indicator was continuously monitored to follow the passive efflux of Ca\textsuperscript{2+} from the lumen of the SR (at 1 mM Ca\textsuperscript{2+}) to the extravesicular medium (at 25-35 mM Cu\textsuperscript{2+}). Sequential additions of ATP were made to determine a rate constant for Ca\textsuperscript{2+} efflux (in nmol of Ca\textsuperscript{2+}/mg of protein/s) divided by the total internal Ca\textsuperscript{2+} loading of the vesicles (in nmol of Ca\textsuperscript{2+}/mg of protein); percentage of Ca\textsuperscript{2+} effluxed was calculated by dividing the amount of Ca\textsuperscript{2+} effluxed due to Cu\textsuperscript{2+} to cysteine by the total internal Ca\textsuperscript{2+} loading of the vesicles. Points plotted are the mean ± SE of four runs.

Measurement of Sulfhydryl Oxidation Rates with DTNB—The reagent DTNB (or Ellman's reagent) has been used extensively to quantitatively determine sulfhydryl concentration at pH above 8.0 (22). DTNB provides a convenient optical assay for studying sulfhydryl group oxidation since it does not interact with disulfides and because it exhibits a large change in absorption at 410 nm upon reacting with sulfhydryls. Rates of sulfhydryl oxidation of cysteine, cysteine, cysteamine, and homocysteine were determined in the presence of Cu\textsuperscript{2+} using DTNB with the following protocol. An aliquot of a solution (0.9 ml) containing 100 mM KCl, 5 mM HEPES, and either 1 or 10 mM MgCl\textsubscript{2}, pH 7.0 (Solution A) was mixed with 0.1 ml of a solution B containing 1 mM DTNB dissolved in 120 mM Tris-HCl, pH 8.5. The final pH of the mixture (A + B) was 8.1. The absorbance at 410 nm of this mixture provided the "reference" baseline absorption against which other mixtures were compared. The sulfhydryl compound (30 mM) to be tested was added to 0.9 ml of solution A plus 2, 4, or 6 mM Cu\textsuperscript{2+}. At chosen time intervals, 0.1 ml of the mixture was removed and the absorbance at 410 nm was recorded. The concentration of nonoxidized sulfhydryl groups left in the medium was computed by comparing the absorption of the reaction mixture with that of a control reaction mixture which did not contain Cu\textsuperscript{2+}. The resulting plot of remaining free —SH groups versus time was linear, as expected (this reaction is zero order with respect to [Cu\textsuperscript{2+}] (22); the rate of sulfhydryl oxidation was computed from the slope of this line.

Reagents—The buffers HEPES and PIPES and the sulfhydryl reducing agent dithiothreitol (DTT) were purchased from Research Organics (Cincinnati, OH). Imidazole was purchased from Aldrich. Hepes and sodium-mercaptoethanesulfonic acid (H\textsubscript{2}S\textsubscript{2}O\textsubscript{3}), MgCl\textsubscript{2}, and CaCl\textsubscript{2} were purchased from J. T. Baker Chemical Co. ATP and the Ca\textsuperscript{2+} ionophore A23187 were purchased from Behring Diagnostics. Vacuum-distilled hypochlorous acid (HOC1) was a gift of Dr. J. Michael Albrich of the Oregon Graduate Center. The enzymes superoxide dismutase and catalase and all other reagents were purchased from Sigma.
RESULTS
Sulfhydryl Oxidation with Mercaptans Induces Ca\textsuperscript{2+} Release from SR Vesicles—In Fig. 1, the differential absorption changes (ΔA = A\textsubscript{t} - A\textsubscript{0}) of arsenazo III were used to monitor ATP-dependent Ca\textsuperscript{2+} uptake followed by Cu\textsuperscript{2+}/cysteine-induced Ca\textsuperscript{2+} release from SR vesicles. Two equal additions of Cu\textsuperscript{2+} to the SR reaction mixture produced equal changes in ΔA verifying the linearity of the dye response in the medium. An addition of ATP initiated active Ca\textsuperscript{2+} uptake by SR vesicles as inferred by the decrease in ΔA. Sequential additions of Cu\textsuperscript{2+} (2 μM) and then cysteine (10 μM) induced a partial but rapid release of the accumulated Ca\textsuperscript{2+}, recorded by a rapid rise in ΔA. Reversing the order of cysteine and Cu\textsuperscript{2+}-additions did not alter the rate or extent of Ca\textsuperscript{2+} release. However, the addition of either reagent alone or of a premixed solution of Cu\textsuperscript{2+} and cysteine to Ca\textsuperscript{2+}-loaded SR vesicles did not induce Ca\textsuperscript{2+} release. Indeed, after a few minutes a mixture of Cu\textsuperscript{2+} and cysteine in these proportions contains little or no reduced cysteine because of the Cu\textsuperscript{2+}-catalyzed oxidation of cysteine to form cystine (21, 22). Addition of oxidized cysteine (cystine) did not induce Cu\textsuperscript{2+} release from SR (data not shown). Upon completion of Ca\textsuperscript{2+} efflux (indicated by a leveling-off of the absorption changes of the Ca\textsuperscript{2+} indicator), subsequent addition of 2 μM ruthenium red resulted in the active reuptake of the released Ca\textsuperscript{2+}. Alternatively, ruthenium red added at the beginning of the experiment or just after ATP-dependent Ca\textsuperscript{2+} uptake blocked cysteine-induced Ca\textsuperscript{2+} release (not shown). Similarly, other inhibitors of Ca\textsuperscript{2+} release like procaine (10 mM) or tetracaine (1 mM) blocked or reversed cysteine-induced Ca\textsuperscript{2+} release (not shown). At the end of each experimental run, the Ca\textsuperscript{2+} ionophore A23187 was added to release all the Ca\textsuperscript{2+} stored in the lumen of the SR and to verify that the total Ca\textsuperscript{2+} released (after A23187) was equal to the total Ca\textsuperscript{2+} taken up by the vesicles. Under the present experimental conditions, the total Ca\textsuperscript{2+} actively sequestered by the SR vesicles was consistently in the range of 100 ± 10 nmol of Ca\textsuperscript{2+}/mg of protein.

The experiment shown in Fig. 1 was repeated with cysteine and the structurally related mercaptans cysteamine and homocysteine. In Fig. 2, the Cu\textsuperscript{2+} concentration was kept constant at 2 μM while the concentrations of cysteine, cysteamine, and homocysteine were varied over a wide range of values. For each experimental run, the rate of Ca\textsuperscript{2+} efflux induced by the addition of Cu\textsuperscript{2+} and the mercaptan was calculated from the maximum slope of the efflux trace, and the rates of Ca\textsuperscript{2+} efflux were plotted as a function of mercaptan concentration. The relationship between rate of efflux and mercaptan concentration exhibits several interesting features. 1) At its optimal concentration, cysteine is more reactive than cysteamine, and both are much more effective in triggering release than homocysteine. (Note the change in scale between Fig. 2, A and B.) 2) Ca\textsuperscript{2+} efflux rates first increase with increasing mercaptan concentration, reaching a maximum rate, then decrease with further increase in mercaptan concentration. 3) Cu\textsuperscript{2+}/mercaptan-induced Ca\textsuperscript{2+} release is inhibited by excess mercaptan (−10-fold or more over [Cu\textsuperscript{2+}]). Thus, the mercaptans act as both agonists (at low concentrations) and as inhibitors (at high concentrations) of Ca\textsuperscript{2+} release. These features are consistent with Cu\textsuperscript{2+}-catalyzed formation of mixed disulfide bonds between the thiol group on the added cysteine and a critical thiol group on the Ca\textsuperscript{2+} channel. For fixed protein and Cu\textsuperscript{2+} concentrations, at low cysteine concentrations the ratio of cysteine:Cu\textsuperscript{2+}:channel appears to favor formation of mixed disulfides, while at higher cysteine concentrations the relatively small amount of Cu\textsuperscript{2+} spends more of its time catalyzing the formation of cysteine and less time catalyzing mixed disulfide formation. On the other hand, the Cu\textsuperscript{2+}-catalyzed oxidation of cysteine is known to be approximately first order with respect to Cu\textsuperscript{2+}. Assuming that the mechanism of mixed disulfide formation is the same as that of cystine formation, a plot of Ca\textsuperscript{2+} efflux rate versus [Cu\textsuperscript{2+}] should be roughly linear. In Fig. 3 this prediction is borne out; Ca\textsuperscript{2+} efflux rates versus [Cu\textsuperscript{2+}] are plotted at two fixed concentrations of cysteine, 5 and 20 μM; the protein concentration was 0.25 mg/ml in each case. The curve is linear over a range of Cu\textsuperscript{2+} concentration from zero up to equimolarity with the added cysteine.

The rate at which mercaptans autoxidize in the presence of Cu\textsuperscript{2+} parallels their ability to induce Ca\textsuperscript{2+} release from SR, as shown in Tables I and II. This is a noteworthy observation, but we emphasize that in the first case (autoxidation of mercaptans, Table I), the reaction is between two identical molecules, while in the second (Cu\textsuperscript{2+} efflux induced by Cu\textsuperscript{2+} and mercaptan) Ca\textsuperscript{2+} efflux rates are used to assess relative rates of reaction between dissimilar molecules. For Table I, the ratio of sulfhydryl oxidation of cysteine, cysteamine and homocysteine was measured with DTNB as described above. Cysteine and cysteamine self-oxidized rapidly compared to homocysteine, while glutathione oxidation (not shown) was too slow to detect over the time scale of these experiments. As shown in Table II, glutathione is also ineffective at inducing Ca\textsuperscript{2+} release when in the presence of Cu\textsuperscript{2+}, while cysteine, cysteamine, and homocysteine are effective and to degrees comparable to their autoxidation reactivity. The efflux experiments summarized in Table II were performed essentially in the same manner as those of Figs. 1 and 2, the exception being that mercaptans other than cysteine were added at the concentrations indicated. The chemical reactivity of sulfhydryl groups is known to be enhanced by the close proximity of an amine; thus, it is not surprising that cysteine and cysteamine are more reactive than homocysteine (homocysteine’s hydrocarbon chain is one carbon longer than those of cysteine and cysteamine). Penicillamine’s sulfhydryl is attached to a tertiary carbon, which is also known to greatly

\begin{align*}
\Delta A &= A_t - A_0 \\
0.5 \text{ mM ATP} &
\end{align*}

\begin{align*}
2 \mu M \text{ RR} &
\end{align*}

\begin{align*}
10 \mu M \text{ Ca}^{2+} &
\end{align*}

\begin{align*}
10 \mu M \text{ Cys} &
\end{align*}

\begin{align*}
2 \mu M \text{ Cu}^{2+} &
\end{align*}

\begin{align*}
A23187 &
\end{align*}

\begin{align*}
1 \text{ min} &
\end{align*}

FIG. 1. Ca\textsuperscript{2+} uptake and release from sarcoplasmic reticulum vesicles. SR vesicles were suspended at a protein concentration of 0.25 mg/ml in 100 mM KCl, 20 mM HEPES, 1 mM MgCl\textsubscript{2}, and 0.1 mM arsenazo III. Two aliquots of 10 μM CaCl\textsubscript{2} were then added, and the difference in absorbance between 675 and 685 nm was monitored as a measure of external [Ca\textsuperscript{2+}]. Uptake was initiated by addition of 0.5 mM ATP (free [Mg\textsuperscript{2+}] ~0.6 mM). When uptake was judged to be complete, small amounts of Cu\textsuperscript{2+} and cysteine were added sequentially, triggering Ca\textsuperscript{2+} release. Ca\textsuperscript{2+} release rates were calculated from the steepest slope of the release curve. Addition of 2 μM ruthenium red (RR) caused immediate reuptake of 90% of the released Ca\textsuperscript{2+}. Addition of the Ca\textsuperscript{2+} ionophore A23187 caused complete release of the remaining internal Ca\textsuperscript{2+}.
reduce reactivity (33). Consistent with sulfhydryl oxidation-induced Ca\textsuperscript{2+} release, the oxidized forms of cysteine and cysteamine were not effective at inducing Cu\textsuperscript{2+}-catalyzed Ca\textsuperscript{2+} release either in the presence or absence of Cu\textsuperscript{2+}, nor was methionine, the S-methyl derivative of homocysteine. The sulfur atom of methionine can form strong complexes with heavy metal ions but this configuration does not generally lead to oxidation (33).

In addition to Cu\textsuperscript{2+}/mercaptan-induced Ca\textsuperscript{2+} release from SR, we have observed Ca\textsuperscript{2+} release induced by sulfhydryl oxidants, summarized in Table III. Plumbagin most likely acts by oxidizing neighboring sulfhydryls to disulfides (24) and although less certain, it is probable that hypochlorous acid (HOCI) acts in the same way. Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is a well-known thiol oxidant whose activity as a thiol oxidant is greatly stimulated by the presence of Cu\textsuperscript{2+} ion (34); thus, the stimulation of H\textsubscript{2}O\textsubscript{2}-induced release with Cu\textsuperscript{2+} again implies sulfhydryl oxidation.

If Ca\textsuperscript{2+} release is caused by the oxidation of a critical sulfhydryl to a disulfide, then a disulfide reducing agent should in principle reverse the effect of Cu\textsuperscript{2+} and cysteine. In Fig. 4, SR vesicles were suspended in a standard KCl buffer with 1 mM MgCl\textsubscript{2}, loaded with 20 mM Ca\textsuperscript{2+} using 1 mM Mg\textsuperscript{2+}-ATP, and then Ca\textsuperscript{2+} efflux was induced by addition of 10 mM cysteine and 2 mM CuCl\textsubscript{2} (free [Mg\textsuperscript{2+}] ~0.6 mM) and Ca\textsuperscript{2+} efflux was initiated by adding the indicated amounts of mercaptan followed by addition of 5 mM CuCl\textsubscript{2}. Ca\textsuperscript{2+} efflux rates were calculated from the steepest portion of the efflux curve. (Note the difference in scale between the two figures.)

Table I

| Reagent       | [Cu\textsuperscript{2+}] | [Mg\textsuperscript{2+}] | Oxidation rate μM/s |
|---------------|--------------------------|--------------------------|---------------------|
| Cysteine      | 2                        | 1                        | 0.060               |
|               | 4                        | 1                        | 0.108               |
|               | 6                        | 1                        | 0.162               |
|               | 4                        | 10                       | 0.133               |
| Cysteamine    | 4                        | 1                        | 0.070               |
| Homocysteine  | 4                        | 1                        | 0.004               |

The mercaptans listed were suspended at 30 μM in 100 mM KCl, 5 mM HEPES, and either 1 or 10 mM MgCl\textsubscript{2}, pH 7.0 (0.9-ml total volume). 2 mM CuCl\textsubscript{2} was added and then at the times indicated, 0.1 ml of 120 mM Tris-HCl, 1 mM DTNB, pH 8.5, was added (final pH, 8.1). The concentration of unoxidized —SH groups in solution is proportional to the absorbance of light by DTNB at 410 nm; absorbance at 410 nm was monitored in a Beckman model DU-7 spectrophotometer. The rate of oxidation of —SH groups was calculated from the slope of the resulting absorbance versus time plot.
taken together strongly support the concept that the Ca$^{2+}$ releasing activity of Cu$^{2+}$ and cysteine involves the formation of a mixed disulfide between a sulfhydryl on the Ca$^{2+}$ channel and the sulfhydryl on the added cysteine.

**Properties of Cu$^{2+}$/Cysteine-induced Ca$^{2+}$ Release—Ag$^+$ and Ca$^{2+}$-induced Ca$^{2+}$ release rates have been reported to be strongly affected by Mg$^{2+}$, pH, and ionic strength; also, the rates of Ca$^{2+}$ release induced by these two methods are greatly dependent upon where the SR vesicles were derived from (i.e., from longitudinal SR (LSR) or from terminal cisternae (HSR)). We measured Ca$^{2+}$ release rates induced by Cu$^{2+}$/cysteine as a function of these parameters and found strong similarities between Cu$^{2+}$/cysteine-induced Ca$^{2+}$ release and Ca$^{2+}$ release induced by these other methods. The rate of Ca$^{2+}$ efflux induced by Cu$^{2+}$/cysteine is plotted as a function of total Mg$^{2+}$ in the medium in Fig. 5; SR vesicles (0.25 mg of protein/ml) were suspended in 100 mM KCl, 20 mM HEPES, 0.1 mM arsenazo III, 15 μM CaCl$_2$, and 0.5, 1, 3, 5, 7.5, or 10 μM CuCl$_2$.
vesicles (26) and in skeletal muscle fibers (36). We also measured the rate of Cu²⁺-catalyzed cysteine oxidation at high (10 mM) and low (1 mM) Mg²⁺ concentration, and we found a slight stimulation of the oxidation rate at the higher Mg²⁺ concentration (Table I). The lower rates of efflux at higher [Mg²⁺] thus seem to be due to inhibition of Ca²⁺ transport across the membrane and not inhibition of sulfhydryl oxidation.

To examine Ca²⁺ release from SR as a function of pH and ionic strength, SR vesicles (10 mg of protein/ml) were passively loaded with Ca²⁺ by incubation overnight in 0.8 mM Ca²⁺ and various concentrations of sucrose, KCl, buffers, and pH (see figure captions). The vesicles were diluted by a factor of 40 (final [protein] = 0.25 mg/ml) into Ca²⁺ free but otherwise identical media containing 0.1 mM arsenazo III. Ca²⁺ efflux was initiated by addition of 10 μM cysteine and 2 μM Cu²⁺ and monitored spectrophotometrically as described previously. The rate constants (efflux rate in nmol of Ca²⁺/s/mg of protein divided by Ca²⁺ loading in nmol of Ca²⁺/mg of protein) and extent (%) of Cu²⁺/cysteine-induced Ca²⁺ release as functions of pH and ionic strength are plotted in Figs. 6 and 7. The rate of Ca²⁺ release has a broad maximum at around pH 7−7.25 and decreases with decreasing ionic strength; both characteristics are similar to Ca²⁺ release triggered by Ag⁺ (16). The rate constant reported at pH 7.25, 100 mM KCl, 0.8 mM MgCl₂, corresponds to an efflux rate of ~1.6 nmol of Ca²⁺/s/mg of protein.

The rate of Ca²⁺ release induced by Cu²⁺/cysteine is several times (~4−5 times) faster from HSR than LSR. SR vesicles were suspended at 0.25 mg of protein/ml in 100 mM KCl, 20 mM HEPES, 1 mM MgCl₂, pH 7.0, with 0.1 mM arsenazo III and loaded with 20 μM CaCl₂ by addition of 0.5 mM Mg²⁺-ATP (free [Mg²⁺] = 1 mM) as described in the legend to Fig. 1; Ca²⁺ release was initiated by addition of 10 μM cysteine and 2 μM CuCl₂. Under these conditions, the Ca²⁺ efflux rate from HSR was 9.8 nmol of Ca²⁺/mg of protein/s while the rate of efflux from LSR was about 2.3 nmol of Ca²⁺/mg of protein/s.

**DISCUSSION**

With the aid of the metallochromic indicator arsenazo III, we have shown that various mercaptans in the presence of Cu²⁺ induce rapid Ca²⁺ release from SR. We have confirmed these results with Millipore filtration techniques using "Ca²⁺" as a tracer, with the metallochromic indicator antipyrylazo III, and with a Ca²⁺-sensitive electrode (data not shown). Ca²⁺ release from SR induced by Cu²⁺/cysteine shares many similarities with Ag⁺- and Ca²⁺-induced Ca²⁺ release; all three methods of inducing Ca²⁺ release are inhibited by procaine, tetracaine, and ruthenium red. Also all three show similar responses to free Mg²⁺, pH, and ionic strength. In addition, Cu²⁺/cysteine-induced Ca²⁺ release is significantly faster in HSR than LSR, another common characteristic of Ag⁺- and Ca²⁺-induced Ca²⁺ release.

Ag⁺ and the other heavy metals appear to induce Ca²⁺ release from SR by binding to a critical sulfhydryl group which is somehow closely linked to the site of Ca²⁺ release in vivo, but the Cu²⁺/mercaptan effect seems to be due to Cu²⁺-catalyzed cross-linking of the mercaptan’s sulfhydryl group with this critical protein sulfhydryl. Arguments supporting this oxidative mechanism of Ca²⁺ release are as follows. 1) Ca²⁺ release induced by Cu²⁺ and cysteine is reversed by addition of an excess of the disulfide reducing agent DTT (Fig. 4). 2) The rates of Cu²⁺-catalyzed oxidation of mercaptans (Table I) parallel the rates of Ca²⁺ release induced by these mercaptans (Table II). 3) The Ca²⁺ release rate induced by Cu²⁺ and cysteine appears to be first order with respect to Cu²⁺ (Fig. 3), as expected for Cu²⁺-catalyzed oxidation (21, 22). 4) Hypochlorous acid (23) and plumbagin (24), both potential sulfhydryl oxidants, induce Ca²⁺ release from SR in the absence of Cu²⁺, while Cu²⁺, which enhances the rate of
H₂O₂ oxidation of cysteine stimulates H₂O₂-induced release (Table III).

An issue of interest is the role of redox reactions in excitation-contraction coupling in skeletal muscle. We have shown that formation of a mixed disulfide induces Ca²⁺ release from SR. However, the means we have used to reduce this disulfide, thus closing the channel (i.e. with DTT), is too slow to be of physiological significance. If sulfhydryl redox reactions control the opening and closing of the Ca²⁺ channel, these reactions would almost certainly be enzymatically catalyzed. The activities of several nontransport proteins are known to be regulated by the oxidation states of sulfhydryl pairs which can switch from the dithiol to the disulfide state and back on a time scale of a few milliseconds. For example, lipoamide dehydrogenase is regulated by intracellular NAD(H) (37) and glutathione reductase is regulated by intracellular NADP(H) (37). Alternatively, Robillard and Kones (38) describe a dithiol/disulfide exchange reaction which may be the means of regulating the activities of a number of microbial membrane transport systems. In view of the parallels between Ca²⁺- and oxidation-induced Ca²⁺ release and the fact that the molecular mechanism underlying Ca²⁺-induced Ca²⁺ release is unknown, we suggest that the underlying mechanism of Ca²⁺-induced Ca²⁺ release may involve oxidation or some other perturbation of this critical sulfhydryl. For example, Ca²⁺ may regulate an enzyme responsible for catalyzing sulfhydryl redox reactions linked to Ca²⁺ release or, if Ca²⁺-induced Ca²⁺ release involves direct binding of Ca²⁺ to the Ca²⁺ channel, this critical sulfhydryl may be one of the functional groups involved in gating the channel, whether it undergoes oxidation or not.

We have emphasized an interpretation of our results in which Cu²⁺ acts as a catalyst in the formation of a mixed disulfide between the exogenous mercaptan and an essential sulfhydryl on the Ca²⁺ channel. We note, however, that the Cu²⁺-catalyzed oxidation of cysteine does transiently produce Cu⁺. Under aerobic conditions, however, Cavallini et al. (21) place an upper limit on the number of copper atoms in the +1 state at 20% while Zwart et al. (22) estimate this number to be more like 2%. The +1 oxidation state of copper is reportedly more stable in an aerobic environment when the Cu⁺ ion is at the center of a coordination complex between two or more high affinity ligands (such as sulfhydryl groups) (39). However, given the low initial concentration of Cu²⁺ and the even lower Cu⁺ concentration, Cu⁺ binding or complex formation would seem an unlikely mechanism even without the data supporting the oxidation hypothesis (i.e. the cysteine dependence in Fig. 2, the reversal with DTT, and plumbagin, HOCI, and Cu²⁺/H₂O₂-induced release). Another possible explanation for the effects of Cu²⁺ and cysteine would be that Cu²⁺ catalyzes a thiol/disulfide exchange reaction between the cysteine thiol and a protein disulfide (40), though the oxidative mechanism again seems more likely.

Whether or not sulfhydryl oxidation is engaged in the in vivo gating of the Ca²⁺ channel of the SR, it is interesting to note that the mercaptan penicillamine (which is either a sulfhydryl oxidant or reductant, depending on conditions) has been used to slow down the onset of muscular dystrophy in chickens (41). Also, elevated levels of the Ca²⁺ have been implicated in the onset of this disease (42). We suggest that, in the clinical context with the absence of Cu²⁺, penicillamine may protect the same critical sulfhydryl involved in Cu²⁺/cysteine-induced release from pathological oxidation; the result of this protection would be maintenance of normal resting-state Ca²⁺ permeability levels for the membrane and thus lower cytosolic Ca²⁺ concentrations. Seen in this light, oxidation-induced Ca²⁺ release from SR may hold important clues for understanding muscular dystrophy in addition to understanding the functions of normal muscle.

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