Hydrogen Peroxide Stimulates the Ca\(^{2+}\) Release Channel from Skeletal Muscle Sarcoplasmic Reticulum*

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Terence G. Favero‡§, Anthony C. Zable§, and Jonathan J. Abramson§

From the ‡Department of Biology, University of Portland, Portland, Oregon 97203 and the §Department of Physics, Portland State University, Portland, Oregon 97207

Hydrogen peroxide (H\(_2\)O\(_2\)) at millimolar concentrations induces Ca\(^{2+}\) release from actively loaded sarcoplasmic reticulum vesicles and induces biphasic \([^{3}\text{H}]\)ryanodine binding behavior. High affinity \([^{3}\text{H}]\)ryanodine binding is enhanced at concentrations from 100 \(\mu\text{M}\) to 10 \(\text{mM}\) (3-4 fold). At H\(_2\)O\(_2\) concentrations greater than 10 \(\text{mM}\), equilibrium binding is inhibited. H\(_2\)O\(_2\) decreased the \(K_d\) for \([^{3}\text{H}]\)ryanodine binding by increasing its association rate, while having no effect on the rate of dissociation of \([^{3}\text{H}]\)ryanodine from its receptor. H\(_2\)O\(_2\) (1 \(\text{mM}\)) also reduced the EC\(_{50}\) for Ca\(^{2+}\) activation from 632 \(\text{nM}\) to 335 \(\text{nM}\). These effects were completely abolished in the presence of catalase, ruthenium red, and/or Mg\(^{2+}\) (mm). H\(_2\)O\(_2\)-stimulated \([^{3}\text{H}]\)ryanodine binding is not further enhanced by either doxorubicin or caffeine. The direct interaction between H\(_2\)O\(_2\) and the Ca\(^{2+}\) release mechanism was further demonstrated in single-channel reconstitution experiments. Peroxide, at submillimolar concentrations, activated the Ca\(^{2+}\) release channel following fusion of a sarcoplasmic reticulum vesicle to a bilayer lipid membrane. At millimolar concentrations of peroxide, Ca\(^{2+}\) channel activity was inhibited. Peroxide stimulation of Ca\(^{2+}\) channel activity was reversed by the thiol reducing agent dithiothreitol. Parallelizing peroxide induced activation of ryanodine binding, Ca\(^{2+}\) transport, and single Ca\(^{2+}\) channel activity, it was observed that the ryanodine receptor formed large disulfide-linked protein complexes that dissociated upon addition of dithiothreitol.

Reactive oxygen species (ROS) have been shown to mediate various pathological conditions in a variety of tissues (1). These molecular oxygen-derived intermediates may be generated either by electron reduction or energy activation (i.e. light). Among these ROS include hydrogen peroxide (H\(_2\)O\(_2\)), singlet oxygen (O\(_2\)), hypochlorous acid (HOCl), superoxide radical (O\(_2^-\)), and the hydroxyl radical (OH\(^-\)).

In various muscle types, ROS have been implicated in alteration of normal Ca\(^{2+}\) homeostasis via disruption of normal sarcoplasmic reticulum (SR) function. This may be accomplished by inhibiting the Ca\(^{2+}\) ATPase pump and/or by activating the Ca\(^{2+}\) release channel. A number of studies have detailed the effects of one or more ROS on whole muscle tissue or on isolated sarcoplasmic reticulum (SR) derived from smooth, cardiac, and/or skeletal muscles (2-10). Treatment of these tissues with ROS attempt to induce oxidative stress similar to that experienced during ischemia and/or reperfusion. In smooth muscle, O\(_2^-\) has been shown to inhibit both Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake into the SR while stimulating inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release (3, 4). In cardiac muscle, HOCl reduced contractile function (7) and inhibited SR Ca\(^{2+}\) uptake (11). On a more microscopic level, H\(_2\)O\(_2\) activated cardiac SR Ca\(^{2+}\) channel gating activity at \(\mu\text{molar}\) concentrations (9). Superoxide also decreased Ca\(^{2+}\) uptake into cardiac SR vesicles by increasing the Ca\(^{2+}\) permeability of the SR via opening the SR Ca\(^{2+}\) release channel (2). These studies indicate that, while it is not the only intracellular organelle affected, the SR is a likely target for ROS in these tissues.

Reduced oxygen species may also interfere with skeletal muscle function. Reperfusion of ischemic muscle, vitamin E deficiency, and muscular dystrophy are important pathological conditions where ROS have been implicated in cytotoxic damage (12). Reactive oxygen intermediates generated during repetitive exercise have also been shown to promote muscle fatigue (13). Despite this evidence, only a few studies have examined the direct effect of ROS on skeletal muscle and SR function. The oxidant peroxysulfate and the OH radical both inhibited Ca\(^{2+}\) uptake into isolated SR vesicles, while peroxysulfate also increased the Ca\(^{2+}\) permeability of the SR membrane (8). In other work describing the effect of sulfhydryl oxidizing reagents on SR Ca\(^{2+}\) release, HOCl, and the highly reactive O\(_2^-\), were shown to stimulate Ca\(^{2+}\) release from actively loaded SR vesicles (10, 14). Calcium release induced by ROS was shown to be blocked by SH reducing agents and ruthenium red, indicating the SR Ca\(^{2+}\) release channel was a primary target for their interaction.

Although the mechanism underlying excitation-contraction coupling in skeletal muscle is poorly understood, oxidation of thiols has been shown to regulate the SR Ca\(^{2+}\) release channel, which mediates Ca\(^{2+}\) release preceding muscle contraction (14). Oxidation-induced opening of the SR Ca\(^{2+}\) channel has also been confirmed at the single-channel level following the fusion of SR vesicles to planar lipid bilayer membranes (BLM) (15). In this report, we show that the cellular oxidant, H\(_2\)O\(_2\), modifies the SR Ca\(^{2+}\) release channel. Moreover, activation of the Ca\(^{2+}\) release channel by H\(_2\)O\(_2\) appears to result from an oxidation of key thiol groups to a disulfide. These results may be significant in understanding muscle cell dysfunction under oxidative stress and may provide insight into the molecular regulation of the Ca\(^{2+}\) release channel.
MATERIALS AND METHODS

Preparation of SR Vesicles—For all studies, sarcoplasmic reticulum vesicles were prepared from rabbit hind leg and back white skeletal muscle according to the method of MacLennan (16). The protein concentration was determined by absorption spectroscopy (17).

Measurement of Ca$^{2+}$ Fluxes—Ca$^{2+}$ fluxes across SR vesicles were measured using a dual wavelength spectrophotometer (14, 18), by measuring the differential absorption changes of arsenazo III (ASIII) at 675-605 nm. The vesicle suspension was incubated in a buffer containing 100 mM KCl, 20 mM Hepes, 1 mM MgCl$_2$, 20 mM free Ca$^{2+}$, and 200 mM ASIII. Uptake was initiated by the addition of 0.5 mM Mg$^{2+}$-ATP. Upon achieving steady state Ca$^{2+}$ uptake, release was initiated by the addition of H$_2$O$_2$, and the free extravesicular Ca$^{2+}$ concentration was recorded as a function of time. The Ca$^{2+}$-ASIII signal was calibrated with the addition of 4–8 mM Ca$^{2+}$. Ca$^{2+}$ uptake and efflux rates were determined from the maximal slope of the extravesicular Ca$^{2+}$ concentration versus time.

Ca$^{2+}$-stimulated ATPase Activity—Ca$^{2+}$-independent, Ca$^{2+}$-dependent, and A23187-stimulated Ca$^{2+}$ ATPase activities were determined spectrophotometrically for untreated and peroxide-treated SR vesicle preparations. The standard assay buffer contained 100 mM KCl, 20 mM Hepes, 1 mM MgCl$_2$, 1 mM EGTA, 3 mM NADH, 1 mM phosphonooxy pyruvate, 5 units of lactate dehydrogenase, 5 units of pyruvate kinase, and 0.5 mM Mg-ATP at pH 7.0 in a 1-ml volume. This assay was carried out in a stepwise fashion. Ca$^{2+}$-independent (Mg$^{2+}$-dependent) ATPase activity was initiated by the addition of SR (0.1 mg) to the cuvette, and the absorbance changes at 340 nm were recorded for 2 min (22°C). To measure Ca$^{2+}$-stimulated activity, 8 mM Ca$^{2+}$ was added, and again the absorbance changes were monitored for 3 min. The Ca$^{2+}$ ionophore, A23187, was added and the reaction run to completion. SR (1 mg/ml) was incubated at several different H$_2$O$_2$ concentrations for 1 min prior to conducting the various assays. Calcium uptake and Ca$^{2+}$ ATPase assays were carried out in identical buffers. The data are the average of three independent experiments and are expressed as a percentage of the control.

$[^{3}H]$Ryanodine Binding—Ryanodine binding measurements were conducted according to the methods of Pessah et al. (19). Briefly, SR membranes (200 µg/ml) were incubated at 37°C for 3 h (unless otherwise noted) in a medium containing 250 mM KCl, 15 mM NaCl, 15 mM [H]$^{3}$ryanodine, and 20 mM Hepes at pH 7.1. Depending on the conditions of the assay, Ca$^{2+}$ (100 µM EGTA + various concentrations of Ca$^{2+}$), and H$_2$O$_2$, were present during the incubation procedure. The binding reaction was quenched by rapid filtration through Whatman GF/B glass fiber filters, which were then rinsed with 5 ml of ice-cold buffer containing 50 mM Ca$^{2+}$. The filters were placed in polytubes (Fisher), filled with 3 ml of scintillation mixture (Beckman, ReadySafe), shaken overnight, and counted the following day. The experiments were repeated at least twice on two different SR preparations to yield statistically identical results. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled ryanodine. For details of individual experiments refer to figure captions.

Measurement of Association/ Dissociation Kinetics—The association rate of [H]$^{3}$ryanodine was measured by quenching the binding reaction by filtration at times ranging from 1 to 480 min after the addition of SR vesicles to the [H]ryanodine reaction medium. Dissociation of [H]$^{3}$ryanodine from the equilibrium complex followed equilibration of 1 min [H]$^{3}$ryanodine with SR membranes for 2 h at 37°C. The SR was then diluted into a 100-fold excess of the assay medium, or the assay medium containing 1 or 10 mM H$_2$O$_2$. Determinations of residual specific binding were made at times ranging from 5 to 160 min.

Equilibrium Binding Analysis—Equilibrium binding data from saturation analysis were fit to a one-site model. The dissociation constant, Kd, and the maximal binding capacity, Bmax, were determined using the Enzfitter computer program.

Single Ca$^{2+}$ Channel Analysis—Ca$^{2+}$ release channel reconstitution into a bilayer membrane was carried out as described elsewhere (21). Briefly, isolated SR vesicles, suspended in 0.3 M sucrose, were added to the cis side of a dual chamber set-up, separated by a planar bilayer lipid membrane (BLM) composed of a 5:3 mixture of phosphatidylethanolamine and phosphatidylserine at 50 mg/ml suspended in decane. The cis chamber contained 500 mM CsCl, 200 µM CaCl$_2$, 10 mM Hepes, pH 7.2, while the trans side contained 100 mM CsCl, 10 mM Hepes, pH 7.2. Following the fusion of a single vesicle, 200 µM EGTA was added to the cis chamber to stop further fusions and the chamber was immediately rinsed with identical buffer containing no added Ca$^{2+}$ or EGTA. Subsequent channel activity was measured at a holding potential of +25 mV with respect to the trans (ground) side. In bilayer

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Fig. 1. The Effect of H$_2$O$_2$ on Ca$^{2+}$-dependent ATPase activity, A23187-stimulated ATPase activity, the rate of Ca$^{2+}$ uptake, and the amount of Ca$^{2+}$ uptake. SR (1 mg/ml) was incubated at different H$_2$O$_2$ concentrations for 1 min prior to conducting the various assays. The final assay conditions for uptake experiments were: SR (0.2 mg/ml); buffer, 100 mM KCl and 20 mM Hepes at pH 7.0, 1 mM Mg$^{2+}$, 0.5 mM Mg-ATP. For ATPase experiments, all concentrations were similar except SR concentrations (0.1 mg/ml). The data are the average of three independent experiments and are expressed as a percentage of control. In the absence of H$_2$O$_2$, Ca$^{2+}$-dependent ATPase activity was 0.45 ± 0.04 µmol/mg/min; A23187-stimulated ATPase activity = 1.20 ± 0.02 µmol/mg/min; total amount of Ca$^{2+}$ uptake = 111 ± 12 nmol/mg; and the maximal rate of Ca$^{2+}$ uptake = 12.98 ± 1.11 nmol/mg.

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RESULTS

Ca$^{2+}$ Transport Measurements—Steady-state Ca$^{2+}$-dependent ATPase activity of intact SR vesicles was observed to be stimulated biphasically following exposure to H$_2$O$_2$ for 1 min. The stimulation of ATPase activity induced by H$_2$O$_2$ (1–20 mM) parallels a reduction in both the rate and amount of Ca$^{2+}$ uptake when the SR membrane was pretreated with H$_2$O$_2$ (Fig. 1). However, in the presence of the Ca$^{2+}$ ionophore A23187, ATPase activity was not modified by H$_2$O$_2$ concentrations up to 80 mM. It appeared that, in the absence of the Ca$^{2+}$ ionophore, modification of ATPase activity and Ca$^{2+}$ uptake were due to alterations in the Ca$^{2+}$ permeability of the SR induced by H$_2$O$_2$.

In Fig. 2, it was demonstrated that H$_2$O$_2$ directly stimulated Ca$^{2+}$ release from actively loaded SR vesicles. Calcium was released at concentrations of H$_2$O$_2$ as low as 1 mM, while the release rates appeared to saturate between 50 and 80 mM H$_2$O$_2$. The maximal release rate derived from the kinetic eval-
Hydrogen Peroxide and SR Ca\textsuperscript{2+} Channel

During experiments, the program ENZFITTER (Elsevier-Biosoft) was used to determine the influence of H\textsubscript{2}O\textsubscript{2} on the dissociation of bound [\textsuperscript{3}H]ryanodine from its receptor (Fig. 5 and Table I). A linear transformation of the dissociation constant, \( K_d \), was observed with up to 80 \textmu M H\textsubscript{2}O\textsubscript{2}. Maximal receptor occupancy was observed at <10 \textmu M. At concentrations greater than 10 \textmu M, binding of [\textsuperscript{3}H]ryanodine to its receptor was inhibited. No binding was detected at 100 \textmu M H\textsubscript{2}O\textsubscript{2}.

When Ca\textsuperscript{2+} release was measured in the presence of the Ca\textsuperscript{2+} channel inhibitor, ruthenium red (10 \textmu M), no release of Ca\textsuperscript{2+} was observed with up to 80 \textmu M H\textsubscript{2}O\textsubscript{2}. The ability to block Ca\textsuperscript{2+} release with a specific channel inhibitor suggests that H\textsubscript{2}O\textsubscript{2} was affecting the Ca\textsuperscript{2+} release mechanism of SR, and was not causing an increase in the SR Ca\textsuperscript{2+} permeability by non-specific effects such as lipid peroxidation.

\textsuperscript{3}H]Ryanodine Binding Studies—A more direct assay to monitor the interaction between the Ca\textsuperscript{2+} release protein and H\textsubscript{2}O\textsubscript{2} was obtained through the use of the highly specific Ca\textsuperscript{2+} channel probe, ryanodine. H\textsubscript{2}O\textsubscript{2} caused a biphasic interaction between [\textsuperscript{3}H]ryanodine and its receptor (Fig. 3). [\textsuperscript{3}H]Ryanodine binding was stimulated above 100 \textmu M H\textsubscript{2}O\textsubscript{2}. Maximal receptor occupancy was observed at <10 \textmu M. At concentrations greater than 10 \textmu M, binding of [\textsuperscript{3}H]ryanodine to its receptor was inhibited. No binding was detected at 100 \textmu M H\textsubscript{2}O\textsubscript{2}.

When catalase, an enzyme that detoxifies H\textsubscript{2}O\textsubscript{2} by converting it to H\textsubscript{2}O and O\textsubscript{2}, was present at 5 units/ml in the incubation medium, no stimulation or reduction in binding was observed (Fig. 3). In addition, when assayed in the presence of 5 \textmu M ruthenium red, H\textsubscript{2}O\textsubscript{2} failed to enhance [\textsuperscript{3}H]ryanodine binding (Table I). These data further suggest that H\textsubscript{2}O\textsubscript{2} interacts directly with the SR Ca\textsuperscript{2+} release channel.

In order to more fully describe the interaction between H\textsubscript{2}O\textsubscript{2} and the ryanodine receptor, time-dependent association and dissociation experiments were conducted. Increasing H\textsubscript{2}O\textsubscript{2} concentrations stimulated the rate of association of [\textsuperscript{3}H]ryanodine to its receptor (Fig. 4a and Table I). A linear transformation of the association data highlighted this more clearly (Fig. 4b). Interestingly, at 100 \textmu M H\textsubscript{2}O\textsubscript{2}, when equilibrium measurements (3-h incubation) indicated only a small amount of ryanodine binding, time-dependent measurements show a rapid acceleration in the rate of ligand binding followed by a rapid dissociation.

Experiments (at 1 and 10 \textmu M H\textsubscript{2}O\textsubscript{2}) were also carried out to determine the influence of H\textsubscript{2}O\textsubscript{2} on the dissociation of bound [\textsuperscript{3}H]ryanodine from its receptor (Fig. 5 and Table I). SR vesicles were labeled with [\textsuperscript{3}H]ryanodine in the absence of H\textsubscript{2}O\textsubscript{2} and allowed to equilibrate for 2 h at 37 °C. Aliquots were subsequently diluted 100-fold into a buffer that contained 0, 1, or 10 \textmu M H\textsubscript{2}O\textsubscript{2} without ryanodine. Peroxide in the dissociation buffer had no effect on the dissociation of [\textsuperscript{3}H]ryanodine from its receptor (Fig. 5 and Table I). These data coupled with the H\textsubscript{2}O\textsubscript{2}-dependent increase in the rate of association suggested that the increase in [\textsuperscript{3}H]ryanodine binding caused by H\textsubscript{2}O\textsubscript{2} (Fig. 3) was due solely to an enhancement of the ryanodine association kinetics.

An increase in association rate with little or no change in the dissociation rate should result in a decrease in the equilibrium dissociation constant, \( K_d \). In equilibrium binding studies (Fig. 6), we observed that 1 \textmu M H\textsubscript{2}O\textsubscript{2} reduced the \( K_d \), from 10.19 to 7.17 \textmu M, a result in agreement with the \( K_d \) calculated from the ratio of \( \frac{K_a}{K_r} \) (Table I). With either method of calculating the \( K_d \), there is a small increase in the affinity of the receptor for binding ryanodine in the presence of peroxide (1 \textmu M). As shown in Fig. 6, there is also an increase in the number of high affinity ryanodine binding sites (\( B_{\text{max}} \), control = 4.2; 1 \textmu M peroxide, 5.4 pmol/mg).

Several activators, including caffeine, have been shown to alter the Ca\textsuperscript{2+}-dependent binding of ryanodine to its receptor. In Fig. 7, it is demonstrated that H\textsubscript{2}O\textsubscript{2} sensitized the ryanodine receptor to activation by Ca\textsuperscript{2+}. In the absence of H\textsubscript{2}O\textsubscript{2}, the EC\textsubscript{50} for Ca\textsuperscript{2+} binding was determined to be 632 nm, while in the presence of 1 \textmu M H\textsubscript{2}O\textsubscript{2}, the EC\textsubscript{50} was 335 nm. It was also observed that known activators and inhibitors of Ca\textsuperscript{2+} release similarly affect peroxide’s ability to stimulate high affinity [\textsuperscript{3}H]ryanodine binding to SR (Table II). Activation of ryanodine binding by H\textsubscript{2}O\textsubscript{2} was considerably decreased by two Ca\textsuperscript{2+} channel inhibitors, Mg\textsuperscript{2+} and ruthenium red. H\textsubscript{2}O\textsubscript{2} had no effect on the ability of doxorubicin to stimulate binding, since binding had already been maximally activated by doxorubicin. However, H\textsubscript{2}O\textsubscript{2} stimulated binding well beyond that induced by 5 \textmu M caffeine.

Peroxide’s interaction with the Ca\textsuperscript{2+} release mechanism from SR could result from an oxidation of critical thiols on the receptor, which might be expected to be reversible upon addition of reducing agents. To determine if the stimulatory effect...
of H_2O_2 on [^3]Hryanodine binding required H_2O_2 to be continuously present, time-dependent association experiments were conducted following H_2O_2 catalase, and DTT treatment (Fig. 8). SR vesicles were incubated with 1 mM H_2O_2 in the [^3]Hryanodine binding medium for 30 min preceding the addition of either DTT (0.5 mM) or catalase or both. We observed that the addition of DTT to the binding medium inhibited the H_2O_2-induced stimulation of ryanodine binding whether H_2O_2 was present or had been converted to H_2O and O_2 by catalase (Fig. 8). This suggested that H_2O_2-stimulated binding was due to an oxidation of sulfhydryl groups, associated with the ryanodine receptor, to disulfides. When catalase alone was added following the initial 30-min incubation period, the stimulatory effect of H_2O_2 persisted. The oxidation-induced stimulatory effect of H_2O_2 on [^3]Hryanodine binding occurred during the initial incubation period and continued throughout the course of the binding experiment. This effect was persistent unless DTT reduced the oxidized SH groups to their native state.

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**DISCUSSION**

Recently, ROS have drawn attention for their potential to disrupt normal muscle function by targeting specific proteins for modification. H_2O_2 is reportedly produced physiologically in muscle cells undergoing oxidative stress (ischemia, reperfusion, exercise, etc.). Modification of the Ca^{2+} release mechanism of sarcoplasmic reticulum by H_2O_2 may promote muscle dysfunction during such pathophysiological conditions. We have examined the interaction between hydrogen peroxide and the Ca^{2+} transport mechanisms from skeletal muscle SR.

When ATPase activity and Ca^{2+} uptake were assayed as a function of H_2O_2 concentration with intact SR vesicles, a biphasic concentration dependence was observed. Peroxide concentrations up to 20 mM increased the permeability of intact SR vesicles, which resulted in an increased Ca^{2+}-dependent ATPase activity and a decreased net accumulation of Ca^{2+}. This was not due to an interaction with the Ca^{2+}-pump, since peroxide did not affect the ability of the Ca^{2+}-ATPase to hydrolyze ATP when analyzed in the presence of the Ca^{2+} ionophore, A23187. Exposure of the SR to H_2O_2 concentrations greater than 20 mM apparently decreased the Ca^{2+} permeability of the SR, which stimulated Ca^{2+} uptake into SR vesicles and inhibited the steady-state ATPase activity.

An apparent discrepancy between the biphasic effect of H_2O_2 on ATPase activity and active Ca^{2+} uptake (Fig. 1) and the monophasic stimulation of Ca^{2+} release (Fig. 2) can be explained by differences in the manner in which these assays were carried out. In Fig. 2, the SR vesicles were first actively loaded with Ca^{2+}, and then exposed to H_2O_2. The initial Ca^{2+} release rate is a measure of the initial rate at which H_2O_2 reacts with the Ca^{2+} release mechanism and opens the Ca^{2+} release channel. In contrast, the data presented in Fig. 1 were

| Treatment          | \( K_{diss} \) (min\(^{-1}\)) | \( K_{-1} \) (min\(^{-1}\)) | \( K_{1} \) (min\(^{-1}\)mM\(^{-1}\)) | \( K_{diss}(K_{-1}K_{1}) \) (nM) | \( K_{diss} \) (Scatchard) |
|--------------------|-------------------------------|-------------------------------|-------------------------------------|---------------------------------|---------------------------|
| Control            | 0.0065                        | 0.00322                       | 0.00022                             | 14.64                           | 10.19                     |
| 1 mM H_2O_2        | 0.0090                        | 0.00315                       | 0.00039                             | 8.07                            | 7.17                      |

**TABLE I**

The observed association rate for untreated SR and SR treated with 1 mM H_2O_2. The \( K_{diss} \) and \( K_{-1} \) were determined from nonlinear regression of the data shown in Fig. 4 using the program ENZFITTER. \( K_{diss} = (K_{diss} - K_{-1})[L] \) where [L] is the ligand concentration. The \( K_{diss} \) (Scatchard) was derived from the plot in Fig. 6.
taken over a period of several minutes and represent measurements of ATPase activity and net Ca\(^{2+}\) uptake following exposure of SR vesicles to H\(_2\)O\(_2\). As shown in Fig. 4a, high concentrations of H\(_2\)O\(_2\) (10–100 mM) first activated the release mechanism and then inhibited the Ca\(^{2+}\) channel as a function of time. Thus, the effect of high concentrations of H\(_2\)O\(_2\) was to stimulate rapid initial release of Ca\(^{2+}\) from SR vesicles. However, the Ca\(^{2+}\) ATPase activity and Ca\(^{2+}\) uptake, which was measured on the time scale of several minutes, displayed a biphasic H\(_2\)O\(_2\) concentration dependence (Fig. 1).

While activation of Ca\(^{2+}\) release by H\(_2\)O\(_2\) (EC\(_{50}\) ≈ 13 mM) and modification of Ca\(^{2+}\)-dependent ATPase activity occurs at [H\(_2\)O\(_2\)] of approximately 15 mM, submillimolar concentrations of H\(_2\)O\(_2\) were observed to activate high affinity ryanodine binding (EC\(_{50}\) ≈ 0.65 mM). A higher concentration of H\(_2\)O\(_2\) is required to modify Ca\(^{2+}\) fluxes across SR vesicles, than to alter ryanodine binding. A likely explanation for this discrepancy is that the ryanodine binding measurements, which were carried out over a time scale of hours, represent equilibrium measurements. SR thiols were oxidized in less than 30 min (Fig. 8). The transport assays, however, do not reflect equilibrium measurements. A relatively large excess of H\(_2\)O\(_2\) was required to oxidize thiols and modify transport on the time scale of the assays shown in Figs. 1 and 2.

The mechanism underlying H\(_2\)O\(_2\)-induced Ca\(^{2+}\) release appears to involve a direct interaction with the ryanodine receptor from the terminal cisternae. Peroxide had a biphasic effect on high affinity [\(^3\)H]ryanodine binding to the receptor (Fig. 3).
slightly decreased the As an activator, H2O2 increased the apparent affinity of the bilayer experiments using two separate SR preparations.

| A | B | C | D | E |
|---|---|---|---|---|

Fig. 9. Polyacrylamide gel electrophoresis of SR membranes following treatment with H2O2 and reducing agents as described in the methods. H2O2 treatment: lanes 1 and 3, no H2O2; lanes 2 and 6, 1 mM H2O2; lanes 3 and 7, 10 mM H2O2; lanes 4 and 8, 100 mM H2O2; lanes 1–4, solubilized with Laemmli buffer without DTT; lanes 5–8, solubilized with Laemmli buffer + 2 mM DTT.

sulfhydryl interactions on the Ca2+ release protein. In Figs. 8 and 11, it is demonstrated on the level of ryanodine binding and single-channel activity that the stimulatory effect of H2O2 was reversible upon addition of thiol reducing agents. Following incubation with H2O2, the oxidant may be removed (by the addition of catalase) without altering its stimulatory effect (Fig. 8). Only when DTT was added did the stimulation of [3H]ryanodine binding return to normal levels. It appears that activation of the Ca2+ release channel via peroxide was mediated by a direct SH oxidation, and that reduction of this disulfide restores the receptor to its native state. This was also supported by the data derived from the electrophoresis experiments (Fig. 9).

As an activator, H2O2 increased the apparent affinity of the Ca2+ binding site responsible for activating [3H]ryanodine binding (Fig. 6) and induced rapid Ca2+ release (Fig. 2). H2O2 slightly decreased the Kd for [3H]ryanodine binding (Fig. 6) by accelerating the rate at which ryanodine binds to its receptor (Fig. 4, a and b). The failure of H2O2 to stimulate [3H]ryanodine binding in the presence of the Ca2+ channel inhibitors ruthenium red and Mg2+ (Fig. 3 and Table II) further supports the hypothesis that H2O2 directly interacts with the Ca2+ release protein from SR.

At high concentrations, H2O2 demonstrated a time-dependent activation and inactivation of ryanodine binding (Fig. 4). This time-dependent loss of ryanodine binding led to complete inactivation of the receptor, which suggests that the receptor was damaged via intermolecular cross-linking, extensive protein oxidation, or destruction of its supporting membrane by lipid peroxidation.

Peroxide-induced stimulation of Ca2+ release, [3H]ryanodine binding, and single-channel activity appears to be mediated by

Fig. 10. Peroxide modification of Ca2+ release channel activity. Following fusion of an SR vesicle to a BLM, the cis chamber was perfused with standard buffer (500 mM CsCl, no added Ca2+) and current was recorded as a function of time (A). B, 0.1 mM H2O2 was added to the cis side; C, H2O2 was raised to 1.0 mM; D, H2O2 raised to 10 mM; E, 100 μM Ca2+ cis added. The open state probabilities (P_o) for traces A–E are as follows: A, P_o = 0.05; B, P_o = 0.85; C, P_o = 0.10; D, P_o = 0.01. The time for each trace was 500 ms/trace. Current traces were recorded at a holding potential of +25 mV with respect to the trans side (ground) of the bilayer. These observations were made in five independent bilayer experiments using two separate SR preparations.

Analysis of SR proteins by SDS-PAGE strongly suggests that activation of the release mechanism by H2O2 induced the formation of disulfide-linked high molecular mass aggregates or multi-protein complexes. As the concentration of H2O2 was increased from 1 to 100 mM, we observed a progressive decrease in the density of protein bands at 450 kDa (ryanodine receptor) and 170 kDa. At 100 mM H2O2, both bands completely disappeared. These data parallel the concentration-dependent increase in association of [3H]ryanodine to its receptor (Fig. 4, a and b), peroxide-stimulated Ca2+ release rate (Fig. 2), and activation of Ca2+ channel activity by peroxide (Fig. 10). These data directly demonstrate that during activation of the SR Ca2+ release channel by H2O2, several proteins form a complex via oxidation of protein thiols localized on the SR Ca2+ release channel.

It has been previously demonstrated that oxidation of SH groups to disulfides activates the Ca2+ release mechanism of SR, while reduction of the disulfides inhibits Ca2+ release (14). As shown in Fig. 8, addition of the reducing agent DTT to peroxide-treated SR reduced ryanodine binding to a level equal to that observed under control conditions. The addition of DTT also restored the high molecular mass complex containing the 450-kDa junctional foot protein to its native state (Fig. 9). Recently, it has been shown that during activation of Ca2+ release by a number of known channel activators (Ca2+, caffeine, ATP, and ryanodine), hyperreactive thiols associated with the ryanodine receptor and triadin no longer interact with nanomolar concentrations of the fluorescent maleimide, 7-di-
ethylamino-3(4'-maleimidyl phenyl)-4-methycoumarin (21).
Moreover, activation leads to the formation of a high molecular weight complex linked via disulfide bridges (22). These results strongly suggest that during physiological activation of the Ca\(^{2+}\) release process, the Ca\(^{2+}\) release channel is disulfide-linked to other key SR proteins, and that reduction of these disulfide linkages results in channel closure. The formation of a disulfide-linked protein complex induced by the addition of H\(_2\)O\(_2\) may reflect a normal protein-protein interaction that occurs during Ca\(^{2+}\) release channel activation.

Lower concentrations of H\(_2\)O\(_2\) were required to activate and inhibit Ca\(^{2+}\) channel activity in single-channel measurements (Figs. 10 and 11) than were needed to modify \[^3\]H\(\text{ryanodine}\) binding (Fig. 3) and Ca\(^{2+}\) release from SR vesicles (Fig. 2). Although the reasons for this higher sensitivity of the reconstituted channel are not well understood, this phenomenon has been previously observed with other agonists in this and other laboratories (23). Interestingly, the concentrations of peroxide required to activate single-channel gating in skeletal SR are substantially lower than those required to activate Ca\(^{2+}\) channel gating in single cardiac Ca\(^{2+}\) channels. At micromolar Ca\(^{2+}\) concentrations, 3–5 mM peroxide was required to activate single-channel activity (9). Consistent with our results, cardiac SR Ca\(^{2+}\) channel activation was also inhibited by the addition of the disulfide reducing agent DTT.

Oxidation of protein thiolis by ROS may be relevant in various pathophysiological conditions. Harmful oxygen species have been shown to disrupt normal cell Ca\(^{2+}\) concentrations during reperfusion of cardiac (24) and skeletal muscle tissue (25) following an extended period of ischemia (24). In heart muscle, this may also be accompanied by a modification of the Ca\(^{2+}\) transient (26). The increase in resting Ca\(^{2+}\) is most likely mediated by Ca\(^{2+}\) entry via a damaged sarcolemma and/or by the disruption of the internal SR membrane systems.

While it has not been conclusively established that endogenous sulfhydryl reactivity regulates the skeletal muscle Ca\(^{2+}\) release channel in vivo, our current understanding of excitation-contraction coupling from experiments performed in vitro strongly suggest that modification of SH groups can control the gating pattern of the SR Ca\(^{2+}\) release channel. The continued use of SH probes may provide us with more detailed information regarding SR Ca\(^{2+}\) channel function.

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