Skeletal Muscle Ryanodine Receptor Is a Redox Sensor with a Well Defined Redox Potential That Is Sensitive to Channel Modulators

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The sarcoplasmic reticulum (SR) is a subcellular organelle that controls the contractile state of muscle by regulating the Ca²⁺ concentration in the cytosol. With hydrolysis of ATP, the SR actively accumulates Ca²⁺ into its lumen leading to muscle relaxation. Depolarization of the transverse tubule membrane results in the release of Ca²⁺ from the SR and muscle contraction. The Ca²⁺ release protein is pharmacologically characterized by its ability to bind the plant alkaloid ryanodine with high affinity and high specificity, and hence this protein is now known as the ryanodine receptor (RyR). [³H]ryanodine has been used to identify the Ca²⁺ release protein and is important in characterizing this receptor (1, 2). It has been repeatedly demonstrated that reagents that open the Ca²⁺ release channel, increase equilibrium binding of ryanodine. The binding of ryanodine has become a functional probe to characterize the redox state of the receptor. For the first time, hyperreactive sulfhydryl groups associated with the Ca²⁺ release mechanism have been observed at the level of skinned fibers in Ca²⁺ flux measurements in single channel measurements and at the level of high affinity ryanodine binding measurements (4–9). Moreover, it has been observed that reactive oxygen species activate Ca²⁺ release from SR (10–13) and may act as redox active signaling molecules to activate Ca²⁺ transport (14). It is clear from the above studies that redox reactions may play a critical role in controlling the kinetics of the Ca²⁺ release mechanism. Furthermore, from experiments carried out with a fluorescent maleimide, it has been shown that the reactivity of these hyperreactive thiols is very sensitive to the concentration of Ca²⁺ channel modulators such as Ca²⁺, Mg²⁺, and caffeine (15).

The redox potential within the cell is controlled by the concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), NAD⁺, and NADH. Zable et al. (16) have shown that GSH inhibits Ca²⁺ channel activity and equilibrium ryanodine binding, whereas GSSG stimulates the activity of the receptor. This suggests that changes in the cellular redox potential may influence the degree of activation of the Ca²⁺ release mechanism and effect the myoplasmic Ca²⁺ concentration and the contractile state of muscle.

Instead of using the more traditional method of measuring equilibrium ryanodine binding, in this study a model was developed in which the rate of ryanodine binding was related to the redox state of the receptor. For the first time, hyperreactive sulfhydryl groups associated with the Ca²⁺ release mechanism from skeletal muscle SR were shown to have a well defined redox potential, and this redox potential was controlled by physiologically relevant Ca²⁺ channel activators and inhibitors. Although it is unlikely that the redox state of these thiols controls excitation-contraction coupling, our results suggest that during oxidative stress, these hyperreactive thiols oxidize and activate the SR Ca²⁺ release mechanism and alter the Ca²⁺ sensitivity of the release channel. Under mild oxidative stress, relatively small changes in the cellular redox potential can contribute to significant stimulation of the ryanodine receptor.

EXPERIMENTAL PROCEDURES

SR vesicles were isolated from rabbit fast twitch skeletal muscle by the method of MacLennan (17) with small modifications. 50 μM dithiothreitol and 0.2 μg/ml leupeptin were included in all buffers except for the final SR resuspension buffer. Samples were stored in liquid N₂.

The initial rate of [³H]ryanodine binding was determined from time-dependent measurements at 3, 6, 9, and 12 min at 37 °C. SR was pretreated with 0.1–0.3 mM GSH for 10 min at room temperature in binding buffer containing 250 mM KCl, 15 mM NaCl, 20 mM Pipes, pH 7.1. The time-dependent reaction was initiated by addition of 4 nM [³H]ryanodine, Ca²⁺ (buffered with EGTA), and various concentrations of GSSG and GSH to yield the desired solution redox potential calculated from Equation 7. For Ca²⁺-dependent measurements, Ca²⁺ was buffered with 50 μM EGTA to a free Ca²⁺ concentration as calculated by WinMaxx (18). The binding reaction was quenched by rapid filtration
through Whatman GF/B filters mounted on a 48-well Brandel Cell Harvester. Filters were rinsed twice with binding buffer containing 50 μM Ca^{2+}. Scintillation vials were filled with scintillation fluid, shaken overnight, and counted the following day. The initial binding rate was calculated from a linear regression fit of 4 time-dependent measurements of bound ryanodine. The derived slope is the initial rate of ryanodine binding. Subtraction of non-specific binding does not affect the rate of binding, and therefore no subtraction was made.

SR was labeled with 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM) using a method similar to that of Liu et al. (15). SR at 0.1 mg/ml was incubated in standard ryanodine binding buffer (without ryanodine) containing 1.0 mM CaCl2 and 20 mM CPM at room temperature with rigorous stirring for 3 min. The reaction was quenched by addition of EGTA and 0.1–0.3 mM GSH, and samples were then incubated for 10 min at room temperature. Ca^{2+}-dependent binding of ryanodine was fit to a Hill equation of the form,

\[ B' = \frac{B'_{\text{max}}[Ca]^{n_B}}{K_r + [Ca]^{n_B}} \] (Eq. 1)

where \( B' \) is the initial rate of ryanodine binding (pmol/mg/min), \( B'_{\text{max}} \) is the maximum rate of binding, \( K_r \) is the apparent affinity for Ca^{2+} of the receptor, and \( n_B \) is the Hill coefficient, a measure of the degree of cooperativity for Ca^{2+} activation of receptor binding. Data were fit using a nonlinear regression curve fitting routine (Sigma Plot for Windows version 5.0). EC_{50} = (K_p)^{1/3}. CPM was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma.

RESULTS

The redox potential of the SR sulfhydryl groups that control ryanodine binding was determined by measuring the rate of [3H]ryanodine binding versus the redox potential of the aqueous environment. As shown in Fig. 1A, the amount of binding increased linearly with time over the first 12 min of exposure to [3H]ryanodine. Also, the initial rate of ryanodine binding increased as the solution redox potential became more positive (more oxidizing). Although only four traces are shown in Fig. 1A, in a typical redox titration the initial rate of ryanodine binding was measured at twelve different redox potentials.

To determine the redox potential of receptor thiols, the rate of ryanodine binding was measured as a function of the solution redox potential. To fit these data, a simple model is proposed in which two rate constants, \( k_+ \) and \( k_- \), describe the rate of ryanodine binding to the reduced receptor, \( R_{\text{red}} \) and the oxidized receptor, \( R_{\text{ox}} \) respectively. As the solution redox potential becomes more positive, the fraction of receptors in the oxidized state increases and the initial rate of ryanodine binding \( B' \) increases according to Equation 2.

\[ B' = \frac{dB/dt_{\text{initial}} = k_+(Ry)(R_{\text{red}}) + k_-(Ry)(R_{\text{ox}})}{R_{\text{ox}}} \] (Eq. 2)

The maximum rate of binding, \( B'_{\text{max}} = k_+(Ry)(R_{\text{red}}) \), occurs when all of the receptors are oxidized (\( R_{\text{ox}} = R_{\text{red}} \)), and the minimum rate of binding, \( B'_{\text{min}} = k_-(Ry)(R_{\text{ox}}) \), occurs when all of the receptors are reduced (\( R_{\text{red}} = R_{\text{ox}} \)). Combining these terms yields the following equations.

\[ (B' - B'_{\text{min}}) / (B'_{\text{max}} - B') = \frac{R_{\text{ox}}}{R_{\text{red}}} \] (Eq. 3)

\[ B'_{\text{max}} / B'_{\text{min}} = k_+ / k_- \] (Eq. 4)

In a typical redox titration, as shown in Fig. 1B, \( k_+ / k_- = 5.2 \pm 1.4 \) (based on 21 independent measurements at different Ca^{2+} concentrations). Interestingly, the ratio of \( k_+ / k_- \), was found to be independent of the experimental conditions. Although \( B'_{\text{min}} \) varied slightly with Ca^{2+}, caffeine, and Mg^{2+} concentration, the ratio of \( B'_{\text{max}} / B'_{\text{min}} \) was always approximately equal to 5.2.

If two protein sulfhydryl groups are oxidized to a disulfide, upon the addition of oxidized glutathione, the oxidation reaction is described by Equation 5.

\[ P - (SH)_2 + GSSG \rightleftharpoons P - S-S + 2 GSH \] (Eq. 5)

At equilibrium, \( K_{\text{ox}} = (P - S-S)(GSH^2)/((P - (SH)_2)(GSSG)) \), and therefore is as follows.

\[ (P - S-S)/(P - (SH)_2) = \frac{K_{\text{ox}}}{R_{\text{ox}}/R_{\text{red}} + K_{\text{ox}}/GSSG/GSH^2} = \frac{(B' - B'_{\text{min}})(B'_{\text{max}} - B')}{(B'_{\text{max}} - B')} \] (Eq. 6)

The binding of ryanodine (Equation 2) is significantly slower than the oxidation reaction (Equation 5). Reconstitution data with an artificial bilayer lipid membrane has shown that the Ca^{2+} release protein rapidly responds (less than 1 min) to changes in the local redox potential (19). It is therefore valid to assume that the oxidation reaction has reached equilibrium before the earliest time point at which ryanodine binding is measured (3 min).

Using GSH and GSSG as a redox buffer, the redox potential of the solution is defined by,

\[ E_{\text{redox}} = E^0 + 2.3 R T / (n F) \log_{10}(GSSG/GSH^2) \] (Eq. 7)

and the redox potential of the receptor is equal to the following,

\[ E_{\text{redox, R}} = E^0 + 2.3 R T / (n F) \log_{10}(I/K_{\text{ox}}) \] (Eq. 8)

where the redox potential is referenced to a normal hydrogen electrode. \( E^0 \) is the standard potential of glutathione, \(-0.24 \) V, \( R \) is the gas constant \( 8.31 \text{ deg}^{-1} \text{ mol}^{-1} \text{ K}^{-1} \), \( T \) is the absolute temperature \( K \), \( n \) is the number of electrons transferred \( n = 2 \), and \( F \) is the Faraday constant \( 96,406 \text{ J} \text{ V}^{-1} \text{ mol}^{-1} \) (20). Thiols that are more easily oxidized have larger values of \( K_{\text{ox}} \) and more negative redox potentials.

Combining Equations 6–8 yields

\[ 2.303 R T / n F \log_{10}((B' - B'_{\text{min}})(B'_{\text{max}} - B')) = E_{\text{redox}} - E_{\text{redox, R}} \] (Eq. 9)

where the minimum and maximum initial rates of binding \( B'_{\text{min}} \) and \( B'_{\text{max}} \) are obtained from the highly reduced and oxidized data shown at the limits of the redox titration (Fig. 1B). By plotting the left side of Equation 9 versus \( E_{\text{redox}} \), the redox potential of the receptor, \( E_{\text{redox, R}} \), is obtained from the x-intercept of Fig. 1C. Moreover, the fact that Fig. 1C is linear with a slope of 1 indicates that Equations 3 and 6 are valid. That is \( (B' - B'_{\text{min}})(B'_{\text{max}} - B') \) is proportional to GSSG and is inversely proportional to GSH^2, as is expected for the formation of a pure disulfide. It is not proportional to 1/GSH, which is what would be expected if oxidation to a mixed disulfide had been responsible for activation of the receptor. In Fig. 1B, the initial rate of ryanodine binding, \( B' \), is plotted versus the solution redox potential, \( E_{\text{redox}} \). The solid line shown in Fig. 1B is obtained from solving Equation 9 for \( B' \).

\[ B' = (r B'_{\text{max}} + B'_{\text{min}})(1 + r) \] (Eq. 10)

where \( r = 10^{(E_{\text{red}} - E_{\text{redox}})/2.303 R T} \). As shown in Fig. 1B, the redox potential of thiols associated with activation of ryanodine binding was dependent on the experimental conditions. The trace on the left was derived from SR that is incubated in 0.1–0.3 mM GSH for 10 min at room temperature prior to the measurement of the initial rate of binding at the indicated solution redox potential \( E_{\text{redox}} \). The trace on the right was derived from a redox titration carried out on SR vesicles without GSH pretreatment. Preincubation of the SR with GSH caused a 65 mV shift in the redox potential of the receptor to more negative values. In both the control and the pre-reduced samples, the maximum and minimum initial rates of binding \( (B'_{\text{max}} \) and \( B'_{\text{min}} \) were identical, indicating that the same...
A group of thiols were being oxidized in the two redox titrations shown in Fig. 1B. Along with this shift in redox potential of the receptor, it was observed that the initial rates of ryanodine binding were highly reproducible only if the SR was first pre-reduced with GSH. As shown in Fig. 1D, going back and forth between -140 and -180 mV or between -105 and -198 mV (data not shown) does not alter time-dependent ryanodine binding as long as the protein is pretreated with GSH. Samples of SR vesicles that were not pre-reduced did not show reproducible binding characteristics at more positive redox potentials (data not shown). All data presented in subsequent figures are from samples that are pre-reduced at room temperature for 10 min with GSH as indicated. Furthermore, it should be noted that the initial rate of ryanodine binding is controlled by the solution redox potential, as defined by Equation 7, not by the absolute amount of GSH or GSSG in the buffer.

The Ca^{2+} release channel is activated by Ca^{2+}, sensitized to activation by Ca^{2+} in the presence of caffeine and is inhibited by Mg^{2+}. Redox titrations were carried out at various free Ca^{2+} concentrations, and the calculated redox potential of RyR1 thiols was plotted versus Ca^{2+}, in the presence and absence of caffeine (Fig. 2A) or Mg^{2+} concentration (Fig. 2B).

Not only does the Ca^{2+} concentration affect the redox poten-
tial of sulfhydryl groups involved in activation of the ryanodine receptor (Fig. 2A) but also the solution redox potential effects the Ca\(^{2+}\) dependence of the initial rate of ryanodine binding (Fig. 3). As is evident in Table I, at highly oxidizing redox potentials (more positive than 2140 mV), the receptor becomes more sensitive to activation by Ca\(^{2+}\) (K\(_{d}\)Ca\(^{2+}\) and the EC\(_{50}\) decreases). The Hill coefficient \(n_H\) is unaffected by the redox potential of the solution. The data indicate a strong coupling between the Ca\(^{2+}\) binding site and the redox-sensitive thiols associated with RyR1.

The hyperreactive thiols on the Ca\(^{2+}\) release mechanism of SR sense the state of the release channel (15). They are specifically labeled by nanomolar concentrations of the alkylating reagent CPM, when the channel is in a closed configuration. In Fig. 4A, a redox titration is shown following pretreatment of the SR with 20 nM CPM, under conditions in which the Ca\(^{2+}\) release channel is in its closed state (1.0 mM CaCl\(_2\)). Under these conditions, no increase in the rate of ryanodine binding is observed as the solution redox potential increases. The receptor no longer demonstrates redox sensitivity. In control experiments in which the Ca\(^{2+}\) release channel is in its open state during CPM pretreatment (50 \(\mu\)M Ca\(^{2+}\)), a subsequent redox titration was identical to that shown in Fig. 1B (data not shown). A normal redox titration is observed when CPM binds to non-hyperreactive thiols. Although the ryanodine receptor still shows a biphasic Ca\(^{2+}\) concentration dependence, following CPM treatment the enhanced rate of ryanodine binding observed at more positive redox potential (Fig. 3) is not present (Fig. 4). CPM eliminates redox control of the ryanodine receptor by blocking hyperreactive thiols.

**DISCUSSION**

In this paper, we demonstrate for the first time that hyperreactive thiols on the skeletal muscle sarcoplasmic reticulum ryanodine receptor (RyR1) have a well defined redox potential that is sensitive to the concentrations of channel modulators such as Ca\(^{2+}\), Mg\(^{2+}\), and caffeine. Conditions that favored the opening of the Ca\(^{2+}\) release channel (micromolar Ca\(^{2+}\) and millimolar caffeine) caused the redox potential of the receptor to become more negative, which favored the oxidation of critical thiols to a disulfide, and an enhancement of the rate of ryanodine binding (Fig. 1B). Conditions that close down the Ca\(^{2+}\) release channel (submicromolar Ca\(^{2+}\), millimolar Ca\(^{2+}\) or Mg\(^{2+}\) (Fig. 2B)), increase the receptor redox potential, which favors the reduced form of critical sulfhydryls and a decrease in the rate of ryanodine binding. Just as caffeine sensitizes the ryanodine receptor to activation by Ca\(^{2+}\), caffeine also main-
tained the redox potential of the receptor at more negative values, even at low Ca\(^{2+}\) concentrations.

This control of the redox potential of hyperreactive thiols by physiologically relevant channel modulators suggests that these thiols play a role in controlling channel gating during excitation-contraction coupling. However, even under conditions which strongly favor the activation of the Ca\(^{2+}\) release protein, 50 \(\mu\)M Ca\(^{2+}\) and 10 mM caffeine, the redox potential does not get more negative than –175 mV. The normal cytoplasmic redox potential is approximately –230 mV (22), as measured in cultured pancreatic cells. This is 55 mV more negative than the redox potential of the receptor under maximum activating conditions. Under these conditions, only 1.6% of the total receptors are oxidized and activated by thiol oxidation (calculated from Equations 6 and 9). Under less optimal conditions for channel activation, the receptor redox potential is more positive, and the open probability of the channel should be extremely small. The only condition in which a significant fraction of these thiols are oxidized is when the cell is oxidatively stressed and the cellular redox potential increases, or if the redox potential in the myoplasm is more positive than the estimated value of –230 mV (22). Under these conditions, one would expect that small changes in the cellular redox potential to have a major influence on excitation-contraction coupling.

In perhaps a related mode of operations, the OxyR transcription factor of Escherichia coli activates antioxidant genes in response to elevated H\(_2\)O\(_2\) levels (23). The redox potential of OxyR is –185 mV, which is significantly higher than that of the cellular redox potential under normal non-oxidatively stressed conditions (~270 mV). However, an oxidatively induced drop in the GSH/GSSG ratio leads to an increase in the cellular redox potential and an activation of OxyR. In a similar manner, an increase in the cellular redox potential during oxidative stress (induced by fatigue, aging, or ischemia) should result in oxidation of the ryanodine receptor/Ca\(^{2+}\) release protein and the opening of the Ca\(^{2+}\) release pathway.

Our previous studies have shown that oxidation induced by quinones, porphyrins and H\(_2\)O\(_2\) is strongly Cu\(^{2+}\) dependent (8, 10, 24). A likely explanation for the Ca\(^{2+}\) dependence of oxidation-induced Ca\(^{2+}\) release from SR vesicles is directly related to the measurements of redox potential described in this manuscript. Ca\(^{2+}\) lowers the redox potential of the ryanodine receptor, which then allows these thiols to be oxidized more easily. We propose that conformational changes induced by channel activators stabilize one or both members of a redox pair, which in an appropriate redox environment results in thiol oxidation. It is well known that the SR Ca\(^{2+}\) release channel can be activated by non-thiol reagents in the absence of GSH and GSSG. An oxidation reaction is not required to open the Ca\(^{2+}\) release channel in vitro. However, as shown in Fig. 3, the degree of activation of the receptor is strongly dependent on the solution redox potential. In a more oxidized environment, there is a large stimulation of the receptor. Whereas in a more reduced environment, there is a relatively small degree of channel activation by Ca\(^{2+}\). The gain of the system appears to be set by the cellular redox potential. The labeling of hyperreactive thiols by CPM eliminates redox potential control of 

| \(E_{\text{so}}\) (mV) | Control | –60 | –90 | –120 | –140 | –160 | –220 | –230 |
|-----------------|---------|-----|-----|------|------|------|------|------|
| \(n^a\)         | 0.77    | 0.78| 0.82| 0.71 | 0.68 | 0.69 | 0.72 | 0.77 |
| \(K^a\)         | 2.06    | 0.50| 0.69| 1.49 | 1.97 | 2.00 | 2.06 | 2.01 |
| \(EC_{50}^a\)   | 2.50    | 0.41| 0.63| 1.76 | 2.70 | 2.71 | 2.72 | 2.48 |

\(^a\) \(\mu\)M

The Hill coefficient \(n_H,K_d\), and \(EC_{50}\) for Ca\(^{2+}\)-dependent activation of the rate of ryanodine binding as a function of the solution redox potential. All values listed are derived from the data shown in Fig. 3.
RyR1. It does not eliminate Ca\(^{2+}\)-dependent activation and inhibition of RyR1. Studies that ignore the cellular redox environment are ignoring an important effector of Ca\(^{2+}\) channel function.

A number of recent studies have shown that some thiol oxidizing reagents induce a biphasic concentration- and time-dependent activation and subsequent inactivation of RyR1 (10, 25). At low concentrations, the receptor is activated in a time-dependent manner. At higher concentrations, a time-dependent inactivation of ryanodine binding is observed after a delay of 30–50 min. The inactivation is likely to be caused by an oxidation of a second set of less reactive thiols. In this study, time-dependent inactivation was not observed. Experiments were designed to look at relatively short exposures to less oxidizing environments. The sulphydryls examined are hyper-reactive thiols whose oxidation is responsible for activation of the ryanodine receptor, not those responsible for subsequent inactivation.

As shown in Fig. 1B, pre-incubation of the SR with GSH caused a 65 mV shift in the redox potential of the receptor to more negative values. Two possible explanations explain this large shift in the redox potential of RyR1 upon pre-reduction with GSH come to mind. Despite the fact that most of the SR preparation was isolated in the presence of 50 μM dithiothreitol, oxidation during the last stages of the isolation procedure may have caused a shift in the redox potential of RyR1 to a more positive value. The redox potential of those hyper-reactive thiols that control ryanodine binding may be altered by the oxidation of a second class of “regulatory thiols.” Aghdasi et al. (9) has previously shown that SR vesicle preparations are sensitive to oxidation by dissolved oxygen. Alternatively, the 10-min incubation with GSH may be required to transport GSH across the SR membrane and generate a suitable redox environment on the luminal side of the SR (21). In other words, the luminal redox potential may influence the redox potential of hyper-reactive thiols on the cytoplasmic face of the SR.

The carboxyl-terminal domain of the IP\(_3\) receptor from the endoplasmic reticulum has a similar amino acid sequence to that of the ryanodine receptor. It has been shown that increased ratios of GS/GSH activate IP\(_3\) binding (26), and it has been suggested that there are conserved sequences, which are strong candidates for sites of redox reactions common to both the IP\(_3\) and RyR (27). The RyR1, RyR2, and the IP\(_3\) receptors may contain similar or related molecular mechanisms for dealing with oxidative stress.

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