Transmembrane Redox Sensor of Ryanodine Receptor Complex*

Wei Feng‡, Guohua Liu‡, Paul D. Allen§, and Isaac N. Pessah‡¶

From the ‡Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616 and the §Department of Anesthesia, Brigham and Women’s Hospital, Boston, Massachusetts 02114

Inositol 1,4,5-trisphosphate receptors (IP₃R) and ryanodine receptors (RyR) mediate the release of endoplasmic and sarcoplasmic reticulum (ER/SR) Ca²⁺ stores and regulate Ca²⁺ entry through voltage-dependent or ligand-gated channels of the plasma membrane. A prominent property of ER/SR Ca²⁺ channels is exquisite sensitivity to sulfhydryl-modifying reagents. A plausible role for sulfhydryl chemistry in physiologic regulation of Ca²⁺ release channels and the fidelity of Ca²⁺ release from ER/SR is lacking. This study reveals the existence of a transmembrane redox sensor within the RyR1 channel complex that confers tight regulation of channel activity in response to changes in transmembrane redox potential produced by cytoplasmic and luminal glutathione. A transporter selective for glutathione is co-localized with RyR1 within the SR membrane to maintain local redox potential gradients consistent with redox regulation of ER/SR Ca²⁺ release. Hyperreactive sulfhydryl moieties previously shown to reside within the RyR1 complex (Liu, G., and Pessah, I. N. (1994) J. Biol. Chem. 269, 33028–33034) are an essential biochemical component of a transmembrane redox sensor. Transmembrane redox sensing may represent a fundamental mechanism by which ER/SR Ca²⁺ channels respond to localized changes in transmembrane glutathione redox potential produced by physiologic and pathophysiologic modulators of Ca²⁺ release from stores.

A change in cytosolic Ca²⁺ concentration serves as a signal for modulating a wide range of cellular activities (1–3). A major mechanism for increasing cytosolic Ca²⁺ includes release of Ca²⁺ from internal stores (endoplasmic or sarcoplasmic reticulum, ER or SR) via a genetic superfamily of Ca²⁺ release channels including inositol 1,4,5-trisphosphate receptors (IP₃R) and ryanodine receptors (RyR) (4–6). A prominent functional property of all of these channels is exquisite sensitivity to reduction and oxidation by sulfhydryl reagents (7–12). The functional consequences of sulfhydryl modification of RyRs include phases of activation and inhibition, revealing that multiple classes of sulfhydryl groups residing on Cys residues of all three isoforms of RyR channel complexes are important for native functioning and subject to chemical modification (11, 12). However, defining a role for sulfhydryl redox chemistry in RyR function has been controversial since the initial suggestion that sulfhydryl oxidation is a key step in channel activation (13). A plausible physiological role for redox control of ER/SR Ca²⁺ release channels and its attendant mechanism has remained elusive.

It is known that glutathione (GSH) and glutathione disulfide (GSSG) constitute the major redox buffer system of skeletal muscle and many non-muscle cells (14, 15). In the typical mammalian cell, the ratio of [GSH]/[GSSG] in the cytosol is ≈30:1, thereby maintaining very reduced redox potential (RP) of approximately −220 mV (16). By contrast, the RP of the ER lumen is significantly more oxidized (approximately −180 mV) and is maintained with a 3:1 to 1:1 ratio of [GSH]/[GSSG] (16, 17). Thus, the typical microsomal membrane within which the RyR and IP₃R reside is normally subject to a large transmembrane RP difference of 40–50 mV with the lumen much more oxidized than the cytosol (16, 17).

To study redox regulation of RyR channel activity, the bilayer lipid membrane (BLM) preparation affords precise control of the redox state on both the cytoplasmic (cis) and luminal (trans) faces of the reconstituted channel by adjustment of the [GSH]/[GSSG] ratio to form varied redox potentials. In the present work, we provide direct evidence that RyR1 channel activity follows transmembrane redox potential. Chemical labeling studies with CPM indicate previously identified hyperreactive sulfhydryl moieties within the RyR1 complex (18, 19) constitute an essential component of a unique transmembrane redox sensor.

EXPERIMENTAL PROCEDURES

Preparation of SR Membranes—Sarcoplasmic reticulum membrane vesicles were prepared from back and hind limb skeletal muscles of New Zealand White rabbits according to the method of Saito et al. (20) with some modifications. During the SR preparation, GSH and GSSG were included in the homogenization buffer, and the glutathione RP was made to −220 mV, which mimics the typical cytoplasmic RP in vivo. The preparations were stored in 10% sucrose, 10 mM Hepes, pH 7.4, at −80 °C until needed. GSH and GSSG Stock Solutions—GSH was dissolved in degassed Hepes (20 mM) buffer, and the solution was adjusted to pH 7.0. Aliquots (−0.2 ml) were transferred to vials and sealed after blowing with argon. The vials were stored at −20 °C for no longer than 60 days. Once thawed and opened for use, the vial was discarded. GSSG solution was also made and stored in a similar manner except without degassing and argon protection. Samples of GSH and GSSG were taken from both sides of the BLM chamber at the end of channel recordings to verify that the initial redox potential did not change during the course of the experiments. Redox buffers were found to be stable for at least 1 h.

Transport Measurements by Light Scattering Techniques—Osmotically induced changes in microsomal vesicle size and shape (21) were monitored at 400 nm at a right angle to the incoming light beam, using a fluorimeter (F-2000, Hitachi). A decrease in light scattering reflected...
vesicular swelling, a consequence of osmotic changes as GSH and GSSG transported into the vesicle lumen. Briefly, SR vesicles (25 μg/ml) were equilibrated in a hypotonic medium (5 mM K-PIPES, pH 7.0). The osmotically induced changes in light scattering were measured after the addition of a small volume (~10% of the total incubation volume) of concentrated and stock solutions of the compounds to be tested. No changes in light scattering were observed when an identical assay solution was diluted with the same volume of stock buffer lacking GSH or GSSG. Sucrose was used to test the selectivity of GSH- and GSSG-induced changes in light scattering because it was shown to be weakly permeable to SR membranes (21). Fluorogenic acid, a known anion transport inhibitor (22), was used to test the specificity of GSH or GSSG permeation of junctional SR.

Preparation of GSH and GSSG Extracts from SR Lumen—SR vesicles (100 μg) were incubated in 1 ml of solution A (100 mM KCl, 20 mM MOPS, 100 μM CaCl₂), and an indicated combination of [GSH] and [GSSG] at 37 °C for 15 min. The mixtures were then centrifuged at 16,000 × g for 25 min at 4 °C. Each supernatant was carefully decanted from its respective pellet, and the latter was rinsed three times with 1 ml of solution A. The pellets were resuspended and homogenized in 100 μl of buffer containing 1 mM potassium P₃ buffer, 0.5% CHAPS (w/v), and alamethicin (22) (0.1 mg/mg protein) and then incubated for 30 min at 37 °C to permit the release of all vesicular glutathione. Following incubation, 100 μl of 5% trichloroacetic acid in reequilibrated buffer (20 mM KCl, 50 mM K₂HPO₄, 1% diethylstilbestrol, 10 mM acetic acid) was added into the solubilization mixture and the extract centrifuged at 16,000 × g for 25 min. The supernatant was collected, applied to a Microcon YM-3 (3000 molecular weight cut-off, Millipore) and centrifuged at 16,000 × g for 1 h. The filtrate (~200 μl), which represented the total SR luminal glutathione (GSH + GSSG), was analyzed for [GSH]/[GSSG] as described below.

Measurement of GSH and GSSG Content—The GSH and GSSG content of each luminal extract was determined in a manner similar to the method of Senft et al. (23), using the fluorescence probe o-phthalaldehyde (OPA) with a few modifications. To measure total glutathione ([GSH + GSSG], 25 μl of each extract was added into 65 μl of 1 mM potassium P₃ buffer containing 100 μM dithionite to fully reduce GSSG. The reaction medium was incubated for 1 h at room temperature followed by addition of 80 μl of 0.1 mM potassium P₃. To quantify the total GSH product produced from the reduction reaction, 20 μl of OPA (5 mg/ml) was introduced into the mixture and permitted to incubate at room temperature for 90 min. OPA fluorescence was measured with a fluorescence spectrophotometer (model F-2000, Hitachi) at excitation 365 nm/emission 430 nm. The portion of total glutathione attributable to GSSG was analyzed in the manner described for total glutathione without the use of dithionite. To determine the original background content of luminal SR GSH and GSSG before the addition of exogenous glutathione, 100 μg of SR was incubated with alamethicin (0.1 mg/mg SR protein) to release the glutathione to the solution bathing the SR vesicles. These values were used as background correction for determining the net exogenous GSH and GSSG transported into SR vesicles.

Single-channel Kinetics in BLM—Reconstitution of RyR1 and recording of channel activity were performed as previously reported (24) with some modifications. RyR1 channels were reconstituted into planar lipid bilayer (5:2 phosphatidylethanolamine:phosphatidylcholine, Northern Lipids Inc., 50 mg/ml in decane) by introducing SR vesicles to the cis chamber. The cis chamber contained 0.7 ml of 250 or 500 mM CaCl₂, 50–200 μM CaCl₂, and 10 mM Hepes, pH 7.4, whereas the trans side (virtually grounded) contained 50 or 100 mM CaCl₂, 50–100 μM CaCl₂, and 10 mM Hepes, pH 7.4. Upon the fusion of SR vesicle into bilayer, cis chamber was perfused, and the cis/trans CaCl₂ gradient was reversed. Single-channel activity was measured using a patch clamp amplifier (Dagan 3900) at a holding potential as indicated in each figure. The data were filtered at 1 kHz before being acquired at 10 kHz by a DigiData 1200 (Axon Instruments, Foster City, CA). The data were analyzed using pClamp 6 (Axon) and CDA 1.0 (provided by Dr. G. Liu) without additional filtering. The length of representative current traces was ≥1 s. Average Pₒ was calculated from ≥1 min recording.

RESULTS AND DISCUSSION

Junctional SR membranes from rabbit skeletal muscle prepared in the presence of a [GSH]/[GSSG] redox buffer with a potential of −220 mV (calculated from the Nernst relationship (16)) resulted in a high percentage (43%) of reconstituted channels (n = 102) exhibiting a characteristic low open probability (Pₒ) gating mode (Fig. 1A). The remaining channels (57%) exhibited a high Pₒ gating mode. This observation was consistent with those of previous reports (25–27). Although the molecular details underlying low Pₒ and high Pₒ gating modes has remained unclear, the oxidation of one or more classes of sulfhydryl moieties was implicated in stabilizing these experimentally observed gating modes. Low Pₒ and high Pₒ gating modes represent stable RyR1 channel gating behaviors that have been observed in BLM experiments. These gating modes appeared to depend on the overall redox state of the receptor complex because low Pₒ behavior was promoted by treatment with reducing agents, whereas high Pₒ behavior was promoted by oxidizing agents. A characteristic of these gating behaviors was that once they were achieved, the channels did not require the continued presence of reducing or oxidizing equivalents to maintain their gating modes. This observation strongly suggested that low Pₒ and high Pₒ gating behavior may result from the breaking or forming intra- or inter-subunit disulfide bonds (25). This hypothesis was further supported by the observation that gating behaviors can be interconverted by the subsequent addition of reducing or oxidizing reagent. Although channels exhibiting low and high Pₒ gating modes were found to be tightly responsive to transmembrane RP, the present study focuses primarily on channels exhibiting the low Pₒ gating mode. To test the response of the RyR1 channel to cytosolic oxidation, the channel was challenged with a highly oxidized RP of −180 mV generated by adding [GSH]/[GSSG] at a ratio of 3:1 (total [glutathione] = 4 mM) to the cis chamber of the BLM (Fig. 1D). Surprisingly, the channel showed a negligible change in gating activity (Pₒ = 0.011). However, immediately after the addition of 3:1 [GSH]/[GSSG] to generate the same redox potential −180 mV on the luminal (trans) side of the channel, channel Pₒ increased 13-fold. (mean Pₒ rose from 0.011 to 0.138 based on 2–4 minute continuous recordings before and after setting the trans redox potential; Fig. 1C). If channel activation were simply the result of inclusion of an oxidizing potential of −180 mV on the luminal face of the channel, then the higher channel activity should persist after removal cytoplasmic RP. However, removal of the cytoplasmic RP by extensive perfusion of the cis chamber with an identical solution lacking glutathione would not support this hypothesis because the channel activity would not increase after removal of cytoplasmic RP.

FIG. 1. Transmembrane glutathione redox buffer is essential for the transmembrane redox sensing of RyR1. A RyR1 channel from skeletal muscle SR vesicles was incorporated into BLM, as described under “Experimental Procedures.” The channel activity was recorded in the presence of 7 μM free cis Ca²⁺ at a holding potential of −40 mV (A). The introduction of 3 mM GSH and 1 mM GSSG, which corresponded to a RP of approximately −180 mV (16), into the cis side had a negligible influence on channel activity (B). Subsequent inclusion of the same [GSH]/[GSSG], which gave −180 mV in the trans side, significantly enhanced channel open probability (Pₒ; C). Immediately following the removal of GSH and GSSG from the cis side by extensive perfusion of the cis chamber, the channel Pₒ returned to control levels (D). Similar perfusion of the trans chamber to remove the trans redox buffer did not further alter channel activity (E). The figure shows the Pₒ histograms of 2–4 min of continuous record and an expansion of a representative 2 s of current trace for each manipulation of redox conditions. Mean Pₒ was calculated from the entire length of continuous data and denoted in each section of the figure. The current fluctuation is downward; a dashed line is the maximal amplitude; arrows labeled c and o indicate closed and open levels, respectively. This experiment was repeated for a total of 11 separate channels that gave similar results.
Experimental design to obtain transmembrane (cis/trans) redox potential (RP) of -180 & -180 (mV)

| Group | Open probability (Po) |
|-------|-----------------------|
|       | R. P (mV) defined | Control (before | % of control |
|       | (cis/-180 trans/-180) | R.P defined) | |
| a     | 0.153±0.019        | 0.026±0.012     | 797±242 (n=4) |
| c     | 0.146±0.014        | 0.032±0.012     | 666±206 (n=3) |
| b     | 0.171±0.051        | 0.027±0.015     | 866±240 (n=9) |
| d     | 0.235±0.034        | 0.051±0.033     | 748±428 (n=3) |

FIG. 2. RyR1 responds transmembrane redox potential independent of the concentration of glutathione applied in the buffer. The upper panel (conditions a, b, c, d) shows the combination of concentrations of GSH and GSSG in the cis and trans chambers to obtain a symmetric transmembrane RP of -180 mV. The lower panel summarizes data for 19 channels. The average P_o values before and after defining the transmembrane RP are given as absolute values and as percent of activity recorded during the control period. Each P_o was calculated from 1 to 2 min of continuous record before and after setting the symmetrical transmembrane RP to -180 mV.

One resulted in a 13-fold decrease in channel P_o (mean P_o from 0.138 to 0.011; Fig. 1D). Responsiveness to transmembrane redox potential has been observed in 83 of 106 separate reconstitution experiments from junctional SR prepared with or without redox buffering in the initial steps (including channels exhibiting both low and high P_o gating modes) and appears to represent a common feature of RyR1 channel regulation.

RyR1 channels responded to local changes in transmembrane RP irrespective of the absolute concentration of glutathione used in the buffer. Both the cytoplasmic and luminal sides of the membrane were set to -180 mV using a variety of [GSH] and [GSSG] ranging from 0.1 to 4 mM (Fig. 2, top panel, conditions a-d). RyR1 channels exhibited P_o values ranging from 0.014 to 0.084 in the presence of 10 mM cis Ca^{2+} and undefined RP (no GSH/GSSG). RyR1 channels responded with a 7–8-fold enhancement in gating activity once a symmetrical -180 mV transmembrane RP was established (Fig. 2, lower panel). In n = 19 separate reconstitution experiments, channel activity was enhanced to the same degree (7–8-fold of the respective control activity in the absence of a defined transmembrane RP) and was independent of the absolute [GSH] and [GSSG] used to control activity in the absence of a defined transmembrane RP. Thus, one unique physiologic role of redox sensing afforded to ER/SR Ca^{2+} channels is the continuous alignment of Ca^{2+}-induced Ca^{2+} release gain with small changes of transmembrane redox potential. High concentrations of either GSH or GSSG (≥2 mM) added singly to the cytoplasmic side of the RyR1 channel resulted in persistent changes in channel gating behavior (data not shown) as previously reported (28). These effects are likely due to the extreme reduction and oxidation potentials that nonselectively alter protein thiols/disulfides and adversely impact a physiologically relevant transmembrane redox sensor (11, 12, 28).

In healthy non-muscle cells, the ER transmembrane RP gradient is maintained by one or more transporters that facilitate diffusion of either GSH or GSSG and the process is blocked by fluafenamic acid (17). To establish the physiological relevance of a trans SR membrane redox sensor within the RyR1 complex, we determined whether or not junctional SR possesses a transport mechanism necessary to generate a transmembrane redox gradient. First, light scattering measured spectrally shows that transport of GSH or GSSG (10 mM) from the extravesicular space into the vesicle lumen (Fig. 3A). The rate of decline in fluorescence represents the uptake of GSH or GSSG into the SR vesicles (thereby decreasing light scattering). Based on the initial rate, junctional SR vesicles transported GSH -5 times faster than GSSG, consistent with recent measurements performed with liver microsomes (17). Despite the higher initial rates of GSH over GSSG, the steady state capacity of SR vesicles for GSH was only 2-fold higher than GSSG within 10 min (ΔF = 40 versus 80; Fig. 3A). Transport of glutathione is selective because the addition of equimolar sucrose to the transport medium produces only a very small change in light scattering in the same time frame as previously shown (21). Flufenamic acid is a known blocker of glutathione transport across ER membrane vesicles prepared from liver (17). Likewise, movement of GSH and GSSG across junctional SR was fully inhibited by 1 mM fluafenamic acid (Fig. 3A). Second, we measured the transmembrane GSH and GSSG ratio at steady state (10 min) using o-phthalaldehyde as a quantitative indicator (23). This method permits direct quantitative analysis of total luminal glutathione (GSH + GSSG) and GSH. In this manner, the ratio of luminal GSH/GSSG can be measured in the presence of varying extravesicular RP Fig. 3B shows several ratios of [GSH]/[GSSG] added to the SR buffer to give extravesicular RP ranging from -231 mV to -180 mV. After a 10-min equilibration, the vesicles were extracted and the rela-
The transmembrane RP is altered (reduction). Regardless of the extravesicular redox potential set experimentally, the luminal ratio of [GSH]/[GSSG] converged to 3:1 (Fig. 3B, fifth column). These results reveal for the first time the ability of SR lumen to clamp the ratio of its [GSH]/[GSSG] within narrow limits, despite the presence varying cytoplasmic RP. A 3:1 ratio of [GSH]/[GSSG] is consistent with a significantly more oxidized microsomal lumen, as demonstrated previously (16, 17). The ability of the ER/SR membranes, such as ER membranes from non-muscle origin (17), possess a selective transporter for GSH and GSSG. Although a common feature of this microsomal transporter is a preference for GSH over GSSG (based on initial rates), steady state analysis reveals the ability of the ER/SR lumen to favor a 3:1 ratio of GSH/GSSG regardless of the cytosolic RP. A 3:1 GSH/GSSG is consistent with the observation that healthy cells maintain an oxidized luminal potential (−160 to −180 mV) relative to the cytosol (−220 mV) (16). How the ER/SR lumen maintains an oxidized potential despite the preference for transport of GSH is unclear. One possibility is that GSH is oxidized to GSSG within the ER/SR lumen and that the latter is preferentially retained (17). In support of this hypothesis, there is evidence that GSSG can be formed locally within the ER lumen, although the mechanism(s) remain obscure (17, 29, 30). The existence of a GSH/GSSG transporter co-localized with RyR1 within junctional SR membranes would be expected if transmembrane redox sensing is a significant physiologic modulator of RyR1 function.

The ability of the RyR1 complex to respond to transmembrane RP and the apparent ability of SR/ER to create a transmembrane redox gradient raises the possibility that RyR1 channel activity could be actively regulated by subtle localized changes in transmembrane RP. Fig. 4 show the typical response of RyR1 channels to small changes in transmembrane RP. In the absence of a defined RP, low Po channels exhibited infrequent gating activity even when cis Ca2+ was present in an optimal range (30–100 μM) Ca2+ (Fig. 4, A and C; top traces). Once the transmembrane RP was buffered to −220 to −180 mV (cis/trans), the Po increased 2.7-fold from 0.019 to 0.052 (Fig. 4A, middle trace). However, upon adjusting cis to a more oxidized −180 mV, the channel Po immediately increased an additional 2.1-fold (Fig. 4A, bottom trace) for a total enhancement of nearly 6-fold. In this regard, RyR1 channels were under tight control by transmembrane RP, regardless of their gating mode (low versus high Po). Fig. 4B shows an example of a high Po channel closely following as little as +10–20 mV incremental jumps in RP on the cis side relative to a fixed luminal RP of −180 mV.

Modulation of channel Po is independent of the direction in which the transmembrane RP is altered (reduction → oxidation or oxidation → reduction). For example, once the transmembrane RP was set to symmetrical −180 mV, the Po increased 4.2-fold compared with the initial Po under undefined RP (Fig. 4C, compare top and middle traces). Subsequent reduction of cis to −210 mV reduced Po 2-fold. These results reveal that RyR1 channels tightly follow the cis/trans RP in both oxidizing and reducing directions. Similarly, the Po of low Po RyR1 channels closely followed as little as −10 mV incremental jumps in RP on the cis side relative to a fixed trans of −180 mV in transmembrane RP (Fig. 4D). In separate experiments, the cytoplasmic RP was kept fixed at −160 mV and the luminal RP incrementally titrated from −160 to −190 mV in 5 to −10 mV increments (n = 4, data not shown). As the RP difference across the BLM increased, channel Po increased accordingly (227% enhanced Po, at −160 mV cis versus −190 mV trans). These results reveal that once RP is set on both the cis and trans sides, RyR1 channel activity tightly follows incremental changes in RP on either side of the membrane. Right regulation of gating activity by transmembrane RP may be likened to the tight voltage regulation observed with voltage-gated ion channels, therefore representing a fundamentally new mode in channel regulation.

The response of the RyR1 channels to a physiologically relevant redox difference on cytoplasmic and luminal sides of the membrane indicates that an essential component of the redox sensing mechanism may span the ER/SR membrane and may involve the redox state of essential Cys moieties. The redox difference across the ER/SR membrane may therefore provide the driving force for electron transfer reactions that modify the gating kinetics in response to physiological ligands. From what has been known for many redox-sensitive biochemical processes, including targets of transcription factors, antioxidants, cytokines, as well as ion channels/transporters, cell growth-related genes, kinases, phosphatases, etc., electron flow through CH2-SH moieties of conserved Cys residues within proteins account for their redox-sensing properties (14). An essential component of RyR1 redox sensing may involve the transfer of electrons among two or more closely spaced Cys moieties within the channel complex. The RyR1 complex has
been shown to possess a small number of highly reactive sulfhydryl moieties in which chemical reactivity appears to dramatically increase with decreasing $P_c$, by the presence of mm Mg$^{2+}$ and/or nm Ca$^{2+}$ (18, 19, 24). Consistent with previous reports (18, 19), the fast labeling kinetics of these hyperreactive thiols by the fluorescent maleimide CPM (7-diethylamino-5-[4-(maleimidylphenyl)-4-methylcoumarin) took place under conditions that favor RyR1 channel closure (e.g. 10 mm Mg$^{2+}$). The hyperreactive SR thiols rapidly formed thioether adducts with 0.2–1 pmol of CPM/µg of SR protein ($t_{1/2}$ of 11.67–15.37 s; $n = 4$, data not shown). To determine whether chemical modification of hyperreactive Cys moieties after gating behavior, channels were arylated with CPM under conditions known to promote selective labeling of only hyperreactive Cys residues and the functional consequences determined in the BLM. Fig. 5, A and C, reveals that short-term (≤5 min) exposure of a channel to 40 and 50 nm CPM, respectively, caused negligible changes of $P_c$ ($t_{1/2}$, A and C) and mean open dwell time (Fig. 5B) ($n = 8$). Reconstitution of RyR1 channel from such specifically CPM-relabeled SR vesicles also demonstrated unchanged channel gating behavior ($n = 13$, data not shown). However, prolonged incubation of CPM produced additional nonspecific interactions with less reactive Cys residues and thus led to a time-dependent irreversible inactivation of the channel (Fig. 5C; $n = 3$). These results reveal that formation of thioether adducts with the most reactive (hyperreactive) Cys residues within the RyR1 complex does not alter overt aspects of channel gating behavior.

Are the hyperreactive thiols associated with the RyR1 complex an essential component of the transmembrane redox sensor? Fig. 6 addresses this important question by comparing the responses of two separate channels to parallel changes in transmembrane RP without and with chemical modification of the hyperreactive Cys residues with CPM. Both channels responded strongly to the instillation of a symmetric −180 mV transmembrane RP and were negatively regulated by a −220/−180 mV cis/trans gradient (compare Fig. 6, A and B, traces 2–4). The channel in panel B, after exposure to 20 nm CPM for

![Image](https://example.com/image1.png)

**FIG. 5.** Selective labeling of hyperreactive thiols does not alter RyR1 channel gating behavior. In A, a RyR1 channel was pretreated at −30 mV in the presence of varying cis [Ca$^{2+}$] (traces 1 and 2) and [Mg$^{2+}$] (traces 3 and 4). CPM (40 nm) was introduced into the cis chamber for 5 min (traces 5 and 6) and then removed by extensive perfusion of the chamber (traces 7 and 8). $P_c$ was analyzed and is denoted above the representative traces. Mean open time and the curve fit ($\tau_o$ and $\tau_z$, best fit with double exponential) of the channel before and after CPM treatment are displayed in B. In C, channel activity was initially recorded in the presence of 2 nm cis Mg$^{2+}$ before and after a 40-s exposure to 40 nm CPM (+CPM). The cis chamber was extensively perfused with buffer containing 100 µM Ca$^{2+}$ but lacking Mg$^{2+}$ and CPM. Channel gating was continuously followed after a second exposure to 50 nm CPM (+CPM) on the cis side for ~30 min. Prolonged arylation of protein thiols with CPM resulted in an irreversible inhibition of channel activity.

![Image](https://example.com/image2.png)

**FIG. 6.** CPM labeling of hyperreactive thiols does not alter the transmembrane redox sensor. In A, the single channel was incorporated in BLM, and its activity was recorded at −25 mV in 7 and 30 µM [Ca$^{2+}$], respectively (traces 1 and 2). [GSH]/[GSSG] = 3 mM/1 mM (giving −180 mV RP) was symmetrically applied into both the cis and trans chambers (trace 3) resulting in a 17-fold enhancement of $P_c$. The subsequent addition of GSH to 9.72 mM into the cis chamber (−210 mV; trace 4) reduced the $P_c$, 12-fold. Extensive perfusion of both cis and trans was performed to remove transmembrane RP, and the channel activity was followed in the presence of 7 and 30 µM Ca$^{2+}$, respectively (traces 5 and 6). Transmembrane RP was re-established with [GSH]/[GSSG] = 3 mM/1 mM to both cis and trans (trace 7). The experiment was repeated four times. In B, following the incorporation of the channel into BLM at −40 mV in the presence of 7 µM cis Ca$^{2+}$ (trace 1), [GSH]/[GSSG] = 0.95 mM/0.10 mM was introduced into the cis (−180 mV; trace 2). Subsequently, [GSH]/[GSSG] = 4.0 mM/1.79 mM (also −180 mV) was applied in trans (trace 3). A further reduction of cis to −220 mV was made by varying cis [GSH]/[GSSG] to 4.55 mM/0.1 mM (trace 4). Extensive perfusion of both chambers removed transmembrane RP (trace 5). Incubation of CPM (20 nm) in the cis for 120 s was terminated by perfusion (trace 6). After removal of CPM, cis and trans [GSH]/[GSSG] levels were adjusted to 0.95 mM/0.10 mM and 4.0 mM/1.79 mM, respectively (symmetrical −180 mV; trace 7). In the last step, 10 µM ryanodine (RY) was included in cis (short trace). This is a representative of three experiments with similar results.

120 s, failed to respond to a −180/−180 mV symmetric cis/trans redox gradient (Fig. 6B, compare traces 6 and 7), whereas the control channel maintained its redox-sensing properties (Fig. 6A, compare traces 6 and 7). Despite the loss of transmembrane redox sensing, the CPM-modified channel, as predicted, maintained unchanged gating behavior and sensitivity to 10 µM ryanodine (Fig. 6B, short trace, labeled Ry). In separate experiments, RyR1 channels had been reconstituted in BLM after hyperreactive thiols were specifically aryalted by CPM under the conditions described above. It was found that these pretreated channels gated normally but lacked sensitivity to transmembrane RP changes ($n = 3$, data not shown).

Our findings reveal the existence of a transmembrane redox sensor within a microsomal Ca$^{2+}$ channel and represent the first direct evidence linking transmembrane RP with a specific biochemical mechanism regulating microsomal Ca$^{2+}$ transport. Small changes in localized RP appear to dramatically influence the RyR1 activity regardless of cytoplasmic Ca$^{2+}$ levels. Redox sensing is therefore likely to have significant regulatory impact on Ca$^{2+}$-induced IP$_3$ release, reassessing the broad distribution of IP$_3$-Rs and RyR receptors, redox control of microsomal Ca$^{2+}$ release channels may represent a fundamental mechanism by which mammalian cells regulate Ca$^{2+}$ signaling and homeostasis in response to localized changes in redox potential. Hyperreactive thiols within the channel complex are an essential component of a transmembrane redox sensor, which is likely to contribute important regulatory functions during normal intracellular signaling (mediated for example by nitric oxide) (31–
34) and may be involved in mediating changes in Ca\textsuperscript{2+} signaling during oxidative stress (24).

REFERENCES

1. Berridge, M. J. (1993) *Nature* 361, 315–325
2. Clapham, D. E. (1995) *Cell* 80, 259–268
3. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) *Nature* 395, 645–648
4. Nakai, J., Dirksen, R. T., Nguyen, H. T., Pessah, I. N., Beam, K. G., and Allen, P. D. (1998) *Nature* 395, 72–75
5. Kisselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X.; Birnbaumer, L., and Muallem, S. (1998) *Nature* 396, 478–482
6. Ma, H. T. Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) *Science* 287, 1647–1651
7. Missiaen, L., Taylor, C. W., and Berridge, M. J. (1991) *Nature* 352, 241–244
8. Bootman, M. D., Taylor, C. W., and Berridge, M. J. (1992) *J. Biol. Chem.* 267, 25113–25119
9. Joseph, S. K., Ryan, S. V., Pierson, S., Renard-Rooney, D., and Thomas, A. P. (1992) *J. Biol. Chem.* 270, 3588–3593
10. Vanilging, S., Sipta, H., Missiaen, L., De Smedt, H., De Smet, P., Casteels, R., and Parys, J. B. (1999) *Cell Calcium* 25, 107–114
11. Pessah, I. N., and Feng, W. (2000) *Antioxid. Redox Signal.* 2, 17–25
12. Dullunty, A., Haarmann, C., Green, D., and Hart, J. (2000) *Antioxid. Redox Signal.* 2, 27–34
13. Abramson, J. J., and Salama, G. (1989) *J. Bioenerg. Biomembr.* 21, 283–294
14. Sen, C. (1998) *Biochem. Pharmacol.* 55, 1747–1758
15. Sies, H. (1999) *Free Radic. Biol. Med.* 27, 916–921
16. Hwang C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* 257, 1496–1502
17. Bánhegyi, G., Luini, L., Puskás, F., Rossi, R., Fulceri, R., Braun, L., Mile, V., di Simplicio, P., Mandl, J., and Benedetti, A. (1999) *J. Biol. Chem.* 274, 12213–12216
18. Liu, G., Abramson, J. J., Zable, A. C., and Pessah, I. N. (1994) *Mol. Pharmacol.* 45, 189–200
19. Liu, G., and Pessah, I. N. (1994) *J. Biol. Chem.* 269, 33028–33034
20. Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984) *J. Cell Biol.* 99, 875–885
21. Meissner, G. (1988) *Methods Enzymol.* 157, 417–437
22. Bánhegyi, G., Marcelongo, P., Fulceri, R., Hinds, C., Burchell, A., and Benedetti, A. (1997) *J. Biol. Chem.* 272, 13584–13590
23. Senft, A. P., Dalton, T. P., and Shertzer, H. G. (2000) *Anal. Biochem.* 286, 80–86
24. Feng, W., Liu, G., Xia, R., Abramson J. J., and Pessah, I. N. (1999) *Mol. Pharmacol.* 55, 821–831
25. Marengo, J. J., Hidalgo, C., and Bull, R. (1998) *Biophys. J.* 74, 1263–1277
26. Murayama, T., Oba, T., Katayama, E., Oyamada, H., Oguchi, K., Kobayashi, M., Otsuka, K., and Ogawa, Y. (1999) *J. Biol. Chem.* 274, 17297–17308
27. Oba, T., Murayama, T., and Ogawa, Y. (2000) *Biophys. J.* 78, 724
28. Zable, A. C., Favero, T. G., and Abramson, J. J. (1997) *J. Biol. Chem.* 272, 7069–7077
29. Bánhegyi, G., Marcelongo, P., Puskás, F., Fulceri, R., Mandl, J., and Benedetti, A. (1998) *J. Biol. Chem.* 273, 2758–2762
30. Wells, W. W., Xu, D. P., Yang, Y. F., and Rocco, P. A. (1990) *J. Biol. Chem.* 265, 15361–15364
31. Aghdasi, B., Reid, M. B., and Hamilton, S. L. (1997) *J. Biol. Chem.* 272, 25462–25467
32. Xu, L., Eu, J. P., Meissner, G., and Stamler, J. S. (1998) *Science* 280, 234–237
33. Salama, G., Menshikova, E. V., and Abramson, J. J. (2000) *Antioxid. Redox Signal.* 2, 5–16
34. Eu, J. P., Sun, J., Xu, L., Stamler, J. S., and Meissner, G. (2000) *Cell* 102, 499–509