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REDOX SENSING PROPERTIES OF THE RYANODINE RECEPTOR COMPLEX

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1. ABSTRACT

The release mechanism regulating SR Ca^{2+} homeostasis is significantly more sensitive than the uptake mechanisms. The exquisite sensitivity exhibited by ryanodine-sensitive Ca^{2+} channel complexes (*i.e.,* ryanodine receptors, RyRs) to functional perturbation by chemically diverse sulfhydryl-modifying compounds can include phases of activation and inhibition that are dependent on the concentration of the reagent used, the length of exposure, and the nature of the chemical reaction the reagent undertakes with sulfhydryl groups. However the exquisite sensitivity of RyR function to sulfhydryl modification has been generally viewed as significant only in pathophysiological processes. The present paper addresses possible physiological importance of the redox sensing properties of the ryanodine receptor complexes (RyRs) and proposes an underlying mechanism. New data is presented that directly measure the pKa of hyperreactive thiols that occur when the closed conformation of the RyR channel complex is assumed, and that appear to be an integral component of the redox sensor.

2. INTRODUCTION

The Ca^{2+} release mechanism of sarcoplasmic reticulum (SR) was first shown to be sensitive to reagents that interact with protein thiols by exposing SR vesicles loaded with Ca^{2+} to heavy metals (1, 2). Even in these early experiments there were clear indications that the release mechanisms regulating SR Ca^{2+} homeostasis were significantly more sensitive than the uptake mechanisms. Since the initial observations were reported, several laboratories have provided evidence of the exquisite sensitivity exhibited by ryanodine-sensitive Ca^{2+} channel complexes (*i.e.,* ryanodine receptors; RyR) to functional perturbation by chemically diverse sulfhydryl-modifying compounds (recently reviewed in 3, 4). Several classes of non-physiologic organic compounds capable of oxidizing or arylating protein sulfhydryl groups have been utilized to modify the Ca^{2+} release properties of skeletal or cardiac SR by selective modification of RyR1 or RyR2 function, respectively. The functional consequence of sulfhydryl modification of RyRs can include phases of activation and inhibition that are dependent on the concentration of the reagent used, the length of exposure, and the nature of the chemical reaction the reagent undertakes with sulfhydryl groups. These observations have made important contributions toward understanding mechanisms by which oxidizing, reducing, and arylating reactions modify RyR function and its resulting influence on Ca^{2+} transport across SR. However the exquisite sensitivity of RyR function to sulfhydryl modification has generally been generally viewed as significant only in pathophysiological processes. The physiological importance of the redox-sensing property of RyR complexes in healthy cells remains unclear.

2.1. Redox Model of Channel Gating

An original hypothesis proposed by Abramson and Salama suggested that the oxidation of critical sulfhydryl moieties within the RyR complex is a necessary requisite for opening of the channel pore (5). In such a model, oxidation

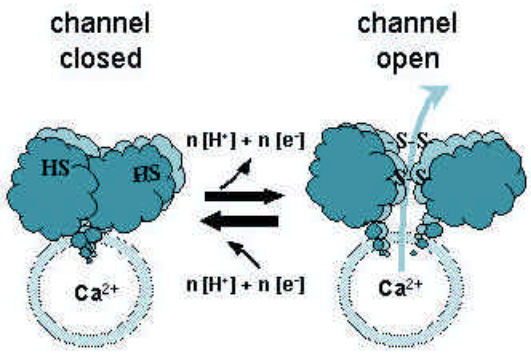

Figure 1. A model proposed by Abramson and Salama (5, 6) relating the oxidation state of critical cysteine thiol moieties to disulfides within RyR primary sequence and channel gating. Disulfides could form either within or between protomers.

and reduction of the critical sulfhyrdyl moieties coincide with gating transitions between conducting and non-conducting states (Figure 1).

Reagents that undergo redox cycling, such as the anthraquinones doxorubicin and daunorubicin, enhance RyR channel open probability (6, 7). In this model, the presence of a redox cycler would either enhance the normal rate of oxidation of critical sulfhydryls to disulfides, or delay the rate of reduction once the disulfides regulating channel open transitions are oxidized. Likewise aryldisulfides such as DTNB or DTDP readily form mixed disulfides with critical thiols within the RyR complex and mimic the "endogenous" protein disulfides that are proposed to be essential for channel opening (8, 9). One major difficulty in reconciling a mechanism of redox mediated gating of the RyR channel complex with physiological activation and inactivation of Ca^{2+} release is the large discrepancy in kinetics with which the two processes proceed. RyR channel gating transitions are extremely rapid with the open state typically lasting no longer than a few milliseconds. Even in the presence of allosteric activators such as bastadin 10 that stabilize long open states, it is unlikely sulfhydryl oxidation is involved given the very rapid reversibility of the compound's effect (10). Quinones that both undergo redox cycling and arylation reactions such as naphthoquinones (11) and benzo[a]pyrene-7,8-dione (12) exhibit complex actions on RyR channel activity that largely depend on concentration and length of exposure, similar to those observed with maleimides under conditions that arylate several classes of RyR1 Cys residues (13). Therefore, sulfhydryl chemistry involving the formation and breaking of disulfide bonds within the RyR complex in the absence of catalysis is extremely slow and leads to long lived irreversible modification of function. In order for oxidation and reduction of critical Cys residues to coincide with each channel gating transition, an efficient means of catalytically transferring electrons from a primary donor to the reactive cysteine(s) within the RyR complex, and subsequently to a terminal acceptor, would be a necessary requisite to support gating. In this regard, a 23 kDa NADH oxidase appears to be closely associated with and modulates the function of RyR2 (14). However NADH oxidoreductase activity is not essential for supporting RyR2 channel gating. Moreover solubilized and purified RyR1 and RyR2 oligomers reconstituted in artificial bilayer lipid membranes (BLM) gate with similar properties to those observed with channels reconstituted directly from junctional SR fused with BLM in the absence of cofactors to support catalytic transfer of electrons (15). Taken together these observations indicate a modulatory role for sulfhdryl chemistry within the RyR complex.

2.2. Redox Control of Calmodulin Binding and RyR Nitrosylation

Reactive sulfhydryl groups within RyR1 have been shown to contribute to the calmodulin-binding (16), and the topic is covered in detail elsewhere in this volume. Of particular interest to the issue of redox control of RyR, is the observation that arylation of Cys 3635 with N-ethylmaleimide resulted in loss of high affinity calmodulin binding and associated channel modulation (17). This finding raised the possibility that functional responses of RyR1 to calmodulin may be related to redox regulation observed with xenobiotic and endogenous sulfhydryl reagents such as glutathione, NADH, and nitric oxide radical ($NO^{\bullet}$), and could at least in part be mediated through hyperreactive Cys3635. Recently Cys3635 has been shown to be the target of selective nitrosylation by $NO^{\bullet}$ (18). Nitrosylation of RyRs has been shown to allosterically modulate channel gating kinetics and ryanodine-binding in a complex manner (covered in detail elsewhere in this volume). However Cys3635 does not appear to be related to redox sensing of GSH and GSSG since expression of site directed mutation C3635A in RyR1 maintained responses to redox environment but lost responses to $NO^{\bullet}$. It is likely that more than one class of sulfhydryl residing within the RyR channel complex is subject to chemical modification, each contributing to specific aspects of function. The major challenge will be to understand how specific sulfhydryl moieties ascribe specific aspects of channel function. Considering the structural complexity of RyR and its associated proteins, this task is likely to be a formidable one.

2.3.Transmembrane Redox Potential of SR

The cytosol of most healthy mammalian cells possesses a redox potential of approximately –230 mV (19). The major cytosolic redox buffers within muscle and non-muscle cells are based on the relative concentration of reduced glutathione (GSH) and oxidized glutathione (GSSG) or NADH and NAD^{+} (20, 21). In the typical mammalian cell, the ratio of [GSH]/[GSSG] in the cytosol is $\geq$60:1 thereby maintaining very reduced redox potential of approximately –230 mV. By contrast, the redox potential of ER lumen is significantly more oxidized (approximately –180 mV); maintained with a 3:1 to 1:1 ratio of [GSH]/[GSSG] (19, 22). It is reasonable to predict that the typical microsomal membrane within which RyRs reside is normally subject to a large transmembrane redox potential difference of 40 to 50 mV with the lumen much more oxidized than the cytosol (Figure 2A). An important question is whether junctional SR of muscle possesses machinery needed to form a transmembrane redox gradient.

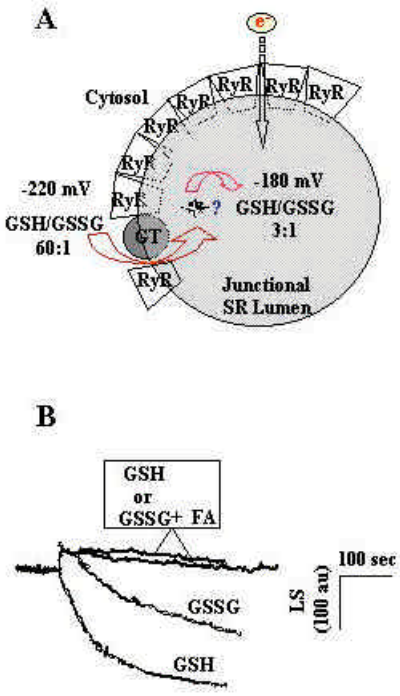

Figure 2. (A) Conceptual model highlighting recent evidence for co-existence within junctional SR of RyR and a transmembrane glutathione transporter (GT). The transporter produces a partition of redox potential between cytoplasmic and lumenal compartments. (B) Light scattering measured spectrally shows that transport of GSH or GSSG (10 mM) from the extravesicular space into the vesicle lumen. The rate of decline in fluorescence (arbitrary units; au) represents the uptake of glutathione into the SR vesicles (thereby decreasing light scattering). Flufenamic acid (FA) blocks all glutathione transport.

In non-muscle cells, one or more transporters have been found to facilitate diffusion of GSH and GSSG across the ER membrane (19, 22, 23). These transporters are thought to be essential for establishing and maintaining the redox potential gradient across the microsomal membrane. If junctional SR is subject to the same large redox gradient observed across ER, then isolated vesicles enriched in RyR should also contain transporters for glutathione. Like ER, junctional SR isolated from skeletal muscle has recently been shown to transport both oxidized and reduced glutathione in two independent studies (24, 25). Initial rates of transport of GSH were 5-times faster than those of GSSG, and were blocked by flufenamic acid (22, 24), suggesting a common pharmacology for SR and ER transporters (Figure 2B). *o*-Phthaldialdehyde has been used to quantitatively measure lumenal glutathione (GSH + GSSG) and the ratio of lumenal GSH/GSSG can be measured in the presence of varying extravesicular redox potential (26, 27). A significant observation was that regardless of the extravesicular redox potential set experimentally, the lumenal ratio of [GSH]/[GSSG] was maintained within narrow limits approximating 3:1, consistent with a significantly more oxidized microsomal lumen, as previously reported for non-muscle ER (19, 22, 23, 26, 28). A most intriguing recent observation is that glutathione transport across SR/ER membranes correlates with the abundance of RyR1, with the fastest transport in terminal cisternae (25). Moreover glutathione transport activity seems to be closely associated with RyR1 in SR.

In summary, junctional SR membranes, much like ER membranes from non-muscle origin, 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), the ER/SR lumen appears to favor a 3:1 ratio of GSH/GSSG at steady state, even if the cytosolic redox potential is highly reduced. A 3:1 GSH/GSSG is consistent with the observation that healthy cells maintain an oxidized lumenal potential (-160 to -180mV) relative to the cytosol (-220 to -230mV). 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. In support of this hypothesis, there is evidence that GSSG can be converted from GSH locally within the ER lumen, although the mechanism(s) remain obscure (22).

2.4.Transmembrane Redox Sensor of RyR Complex

Redox potential can dramatically influence RyR1 function measured with isolated SR and intact muscle fibers. For example Koshita and coworkers found that Ca^{2+} release from SR could be induced by oxidizing compounds such as alcian blue and plumbagin and was partially blocked in the presence of GSH (29). Abramson and coworkers have reported that GSH reduces whereas GSSG enhances the activity of RyR1 (30). In this regard, Marengo and co-workers contributed an insightful observation that RyR1 and RyR2 channels reconstituted in BLM display different patterns of Ca^{2+} dependencies regardless of their origin and that the patterns could be deliberately altered with thiol oxidizing reagents (31). Their results imply that in the reducing environment of the muscle cell, Cys residues critical to function are maintained in the reduced state and may account for low open probability (Po) and low sensitivity to Ca^{2+} activation. However the typical solutions using in the preparation of SR for *in vitro* study does not contain a redox buffer. It is likely that functionally important Cys within a large fraction of RyR complexes auto-oxidize resulting in channels that have high Po and heightened sensitivity to Ca^{2+} activation. One possible physiologic role for hyperreactive sulfhydryl chemistry (see section 1.6, below) within the RyR complex may be to respond to physiologically important changes in localized redox potential in response to redox-active signaling molecules such as nitric oxide (32-34) in the presence of redox buffers.

To study redox regulation of RyR channel activity, Feng et al (24) utilized the bilayer lipid membrane (BLM) preparation to precisely control the redox state on both the cytoplasmic and lumenal faces of the reconstituted channel by adjusting the [GSH]/[GSSG] ratio to form varied redox potentials. Since GSH and GSSG by themselves in excess of 5 mM are highly reducing and oxidizing, respectively, they are likely to influence not only

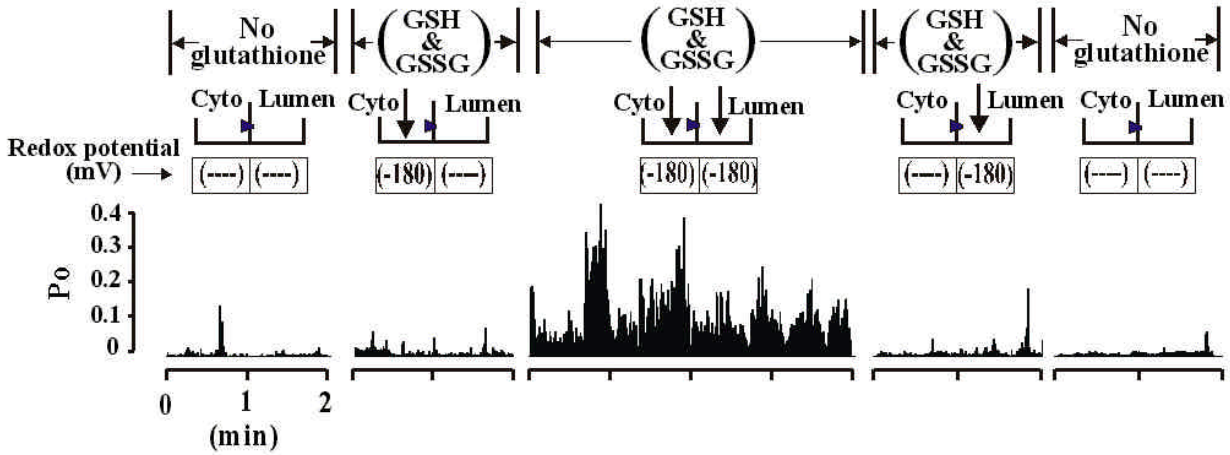

Figure 3. RyR1 responds to transmembrane redox potential in a reversible manner. Long channel records were obtained in the presence or absence of GSH/GSSG on the cytosolic (cyto) and lumenal (lumen) side of the channel as indicated to give –180 mV.

components of the redox sensor but also change the redox state of multiple classes of Cys unrelated to the redox sensor, thereby obfuscating conclusions. Great care was taken to tightly adjust the redox potential with GSH/GSSG ratios whose total concentration did not exceed the physiologically relevant range (1-5 mM) (35). For example, RyR1 channels failed to respond to an cytoplasmic redox potential (RP) of -180 mV (generated by addition 3:1 of [GSH]/[GSSG]; total [glutathione] = 4 mM). Physiologically a cytoplasmic RP of –180 mV would be considered highly oxidizing, yet RyR1 channel function remained unaltered (Figure 3). However, immediately after the addition of 3:1 [GSH]/[GSSG] to generate the same redox potential of -180 mV on the lumenal side of the channel, channel P_o increased 13-fold. RyR1 responded to small (5-10 mV) changes in cytoplasmic redox potential when the lumenal side of RyR1 was fixed at –180 mV. Chemical labeling studies with fluorogenic maleimide CPM (see section 1 and with RyR1 complexes in the closed conformation to promote forming thioether adducts with the most reactive (hyperreactive) thiols (36, 37) resulted in selective loss of redox sensing properties (24).

Kinetic analysis of [^{3}H]ryanodine binding to skeletal SR also revealed tight regulation of RyR1 conformation by redox potential using GSH/GSSG redox buffers that could be eliminated upon labeling of reactive thiols with CPM (38). Transmembrane redox sensing may represent a fundamental mechanism by which ER/SR Ca^{2+} channels respond to localized changes in transmembrane glutathione redox potential produced by physiologic and pathophysiologic modulators of Ca^{2+} release from stores. Moreover many endogenous cellular substrates and xenobiotic compounds of environmental concern can be metabolized *via* electrophilic or redox–active intermediates such as quinones. If hyperreactive thiols within the RyR complex constitute an important biochemical component of a redox sensor, they may convey information about localized changes in redox potential to the Ca^{2+} release process across the microsomal membrane. Ryanodine receptors represent a key Ca^{2+} regulatory channel widely expressed within microsomal membrane of a wide variety of cells where many xenobiotic molecules are metabolized to electrophilic intermediates by the cytochrome P450 system. Co-localization of ryanodine-sensitive Ca^{2+} channels and cytochrome P450 enzyme that catalyze formation of quinone-containing compounds could provide a fundamental mechanism by which localized redox potential is "sensed" by the major intracellular Ca^{2+} store. This mechanism may have both physiological and toxicological significance.

2.5. Hyperreactive Sulfhydryls of RyR Complex: A Key Component of a Transmembrane Redox Sensor

As mentioned above RyR complexes were found to possess a class of Cys moieties that could be distinguished based on their chemical reactivity (36, 37). The rationale for these hinged on the fact that the pKa of the typical protein thiol is above pH 8 and would be expected to be largely protonated (reduced) and have slow chemical reactivity at physiologic cellular pH, especially toward maleimides (39, 40). In fact, disulfide bond-formation (sulfhydryl oxidation), so critical to protein folding, typically takes place within the oxidizing environment of the SR/ER lumen, not in the reducing environment of the cytosol. Only when Cys residues reside within special microenvironments will protein thiol moieties be reactive enough to actively take part in physiologic redox reactions. Examples come from studies of enzyme active sites such as glyceraldehyde-3-phosphate dehydrogenase (41), glutamine-dependent amidotransferases (42), and glutathione reductases (43).

The assay used to probe for hyperreactive thiols within the RyR1 complex utilized the nonfluorescent maleimide, 7-diethyl amino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), which undergoes Michael addition with protein thiols to produce a thioether adduct with high fluorescent yield. Utilizing CPM at very low concentrations (1-50 nM, *i.e.*, 0.02-1.0 pmol/μg SR protein) the kinetics of labeling junctional and longitudinal sarcoplasmic reticulum protein from rabbit skeletal and rat

cardiac muscle was examined in the presence of physiologically and pharmacologically relevant channel activators or inhibitors. Under these conditions, where SR protein thiols greatly exceed the maleimide, the hypothesis that the RyR channel complex possesses a very small number of highly reactive (hyperreactive) thiol groups was tested. If this hypothesis proved incorrect, the anticipated (yet trivial) result would have been relatively slow formation of thioether adducts with the most abundant SERCA pump thiols, since SERCA pump protein accounts for 60-70% of the total SR protein. However a very different result was observed. The CPM assay quantitatively revealed the existence of a very small number ($\leq$1pmol/μg SR) of highly reactive cysteine residues within junctional SR (JSR) membranes enriched in RyR1, traidin and calsequestrin. Formation of CPM thioether adducts proceeded >6-fold faster in the presence of physiologic (*e.g.*, Mg^{2+}) or pharmacologic channel inhibitors when compared to rates in the presence of channel activators *(e.g.* optimal Ca^{2+}). Furthermore longitudinal SR membranes (LSR) lacking the channel complex only displayed slow labeling kinetics regardless of which channel modulator was present. Fluorograms of JSR protein labeled for 45 sec with 10 nM CPM and then size separated by SDS-PAGE revealed that in the presence channel inhibitors CPM formed thioether adducts selectively with the RyR protomer and triadin, whereas CPM labeling in the presence of channel activators form adducts with the abundant SERCA thiols. These results were intriguing because channel associated proteins account for <5% of the total protein of junctional SR preparations and attests to the hyperreactive nature of these thiol groups.

2.6. Direct Measurement of pKa of Hyperreactive Thiols of RyR1 Complex

Underlying the hypothesis that hyperreactive thiols exist within the RyR1 complex and are an essential component of the redox sensor, it is reasonable to predict that a fraction of these thiols may exist in the deprotonated state at physiological pH. In this section we present new data that directly measure the pKa of hyperreactive thiols of the RyR1 complex.

3. EXPERIMENTAL PROCEDURES

3.1. Preparation of SR Membranes

Sarcoplasmic reticulum membrane vesicles enriched in biochemical markers of the terminal cisternae were prepared from back and hind limb skeletal muscles of New Zealand White rabbits according to the method of Saito (24,44). The preparations were stored in 10% sucrose, 5 mM imidazole, pH 7.4 at -80 °C until needed.

3.2. Kinetic Fluorescence Measurement of CPM-Thioether Adducts

Junctional SR (50 μg/ml) was equilibrated in a solution containing 100 mM KCl and an appropriate buffer to obtain pH ranging from 6.5 to 8.5 at 37°C. CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; Molecular Probes, OR) was added at a final concentration of 10 nM to initiate the formation of fluorescent thioether adduct as previously described (36). The kinetics of forming CPM-thioether adducts were continuously measured fluorometrically (SLM 8000, SML Instruments Inc., Urbana, IL). Excitation and emission were set at 397 nm and 465 nm (width of slit = 4 nm), respectively. The buffers used were: 20 mM Mops for pH 6.5- 7.8; 20 mM Tris for pH 8.0 to 8.5; The kinetics of the reaction was measured at each experimental pH under two conditions; one aimed at activating (100 μM Ca^{2+} + 10 mM caffeine) RyR1; the other aimed at inhibiting channel activity (10 mM Mg^{2+}). The rates of increasing fluorescence were sampled at 1 Hz and analyzed by nonlinear regression analysis with Origin© software. The measurements were replicated 4 times and the mean rate plotted as a function of pH. Rates measured in channel-activating buffer were subtracted from the corresponding rates measured in channel-inhibiting buffer. The difference was analyzed by logit-log analysis to calculate the pKa of hyperreactive thiols in the junctional SR preparation.

4. RESULTS

Figure 4 shows the rate curves obtained from reacting 10 nM CPM with 50 μg junctional SR under buffer conditions that either promoted the closed state of the channel (Figure 4A: Mg^{2+}=10 mM Ca^{2+}= 40 nM) or promoted channel opening (Fig. 4B: Mg^{2+} = 0 mM Ca^{2+} = 100 μM caffeine = 10 mM). Formation of CPM-thioether adducts in buffer promoting channel closure proceeded $\geq$6-fold faster than in buffer promoting channel opening 0.0645 sec^{-1} *vs.* 0.0103 sec^{-1} at pH 7.4). The rate of the reaction was highly dependent on the pH, increasing with pH regardless of the constituents of the buffer. Kinetic analysis revealed that rates were significantly greater in buffer containing channel inhibitor compared to those measured in the presence of channel activators at all pH values examined (Figure 4C). The *Open* curve titrated CPM with SR thiols that in general appeared to exhibit low reactivity toward arylation at physiological pH, but became more reactive at basic pH. The heightened reactivity observed with basic pH was likely the consequence of deprotonation of protein thiols. Interestingly under the *Closed* buffer condition, appreciable reaction rates were measured below pH 7 and the rate increased linearly above pH 8. The *Open* curve was subtracted from the *Closed* curve to yield a sigmoidal relationship which discriminated the protonation state of the most reactive thiols (hyperreactive thiols) within the junctional SR preparation (Figure 4D). Logit-log analysis of the hyperreactive thiols revealed a pKa value of 7.39 (Figure 4E). These results reveal that junctional SR contains a unique class of Cys residues, of which half exist in the deprotonated state at physiological intracellular pH (pH = 7.4). Moreover very small changes in cellular pH would be expected to have a pronounced influence of the fraction of hyperreactive Cys in the deprotonated state.

5. DISCUSSION

The pKa of hyperreactive Cys moieties was determined using a fluorometric assay that measures the rate of forming CPM-thioether adducts as a function of pH. Under conditions in the assay buffer that favor the open state of the RyR1 channel complex, the calculated pKa was

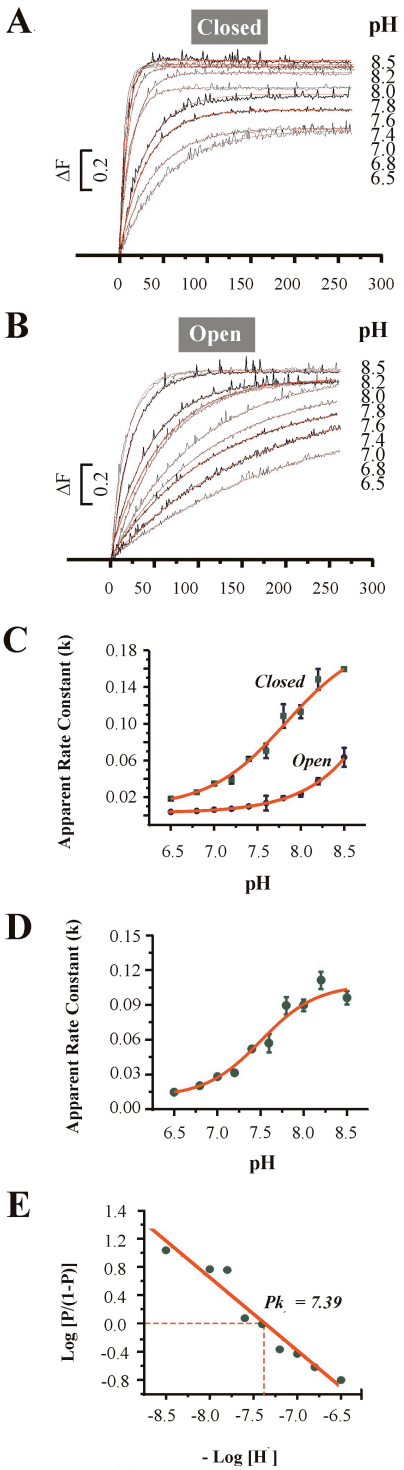

Figure 4. The rate of forming CPM-thioether adducts with junctional SR is highly dependent on pH and the constituents in the buffer that influence conformational state of the RyR1 complex: (A) closed conformation achieved with 10 mM Mg^{2+}; (B) open conformation achieved with 100 μM Ca^{2+} and 10 mM caffeine. (C): Analysis of initial rate constants based on data from (A) and (B). (D): Initial rate constants obtained in the presence of channel activators were subtracted from the corresponding rate constants in the presence of channel inhibitor. The data were best fit by a sigmoidal equation. (E): Logit-log plot from which pKa was calculated.

>8. This was not surprising since protein thiols are generally protonated at physiological pH with the typical Cys thiol exhibiting a pKa of >8 (39). A combination of highly reducing environment, low pO_2, and a pH that favors protonation that are normally found in cell cytosol under physiological conditions, assure low chemical reactivity toward protein thiols. An interesting observation was that the measured pKa of hyperreactive protein thiols associated with RyR1 complexes in buffer conditions promoting channel closure, possessed a pKa of 7.4. Therefore within the intracellular milleaux of a typical muscle cell half of these Cys moieties will exist as a highly reactive deprotonated species (Figure 4).

Taken together the findings indicated that RyR1 channel gating transitions between open and closed conformations were accompanied with changes in the microenvironment of hyperreactive Cys residues. One possible mechanism would invoke the formation of a highly nucleophillic domain that would promote the deprotonation of critical Cys and possible delocalization of their unpaired electrons. In such a scheme, the localized redox potential immediately adjacent to the redox sensing domain would have a dramatic influence on the electron distribution within the redox sensing domain which might in turn influence the stability of the closed state. This mechanism would predict that the closed conformation of the RyR1 complex is the redox sensing conformation, whereas the open conformation would reduce the nucleophilicity of the domain and the reactivity of critical Cys residues would revert to those more typically seen for protein thiols. Thus rapid gating transitions would not coincide with oxidation and reduction of disulfide bonds, but rather the associated conformational transitions would create a reactive domain highly sensitive to local redox environment. Since one state of the channel, the closed state, appears to preferentially assemble the reactive domain with its integral hyperreactive Cys, local redox environment could influence the overall sensitivity of the channel (and SR Ca^{2+} release) to allosteric modulators of physiological and toxicological importance.

6. CONCLUSIONS

Functional modification of enzymes, receptors, and ion channels with reagents that modify protein sulfhydryl groups has been extensively documented. Likewise the RyR complex can be subject to functional modification by sulfhydryl reagents in a complex manner depending on the nature of the chemical reaction taking place and the exact cysteines or disulfides modified. In this respect, both the length of time and conditions of exposing RyR to a given reagent will influence the functional outcome. However, RyR complexes appear to possess a class of highly reactive cysteine moieties whose pKa is near the physiological pH of the typical cytoplasm. The functional role of these hyperreactive cysteines does not mediate channel-gating transitions between open and closed states. Rather, hyperreactive Cys moieties are an essential component of an integral redox sensing mechanism within the RyR complex. The redox sensor does not appear to directly undergo oxidation-reduction

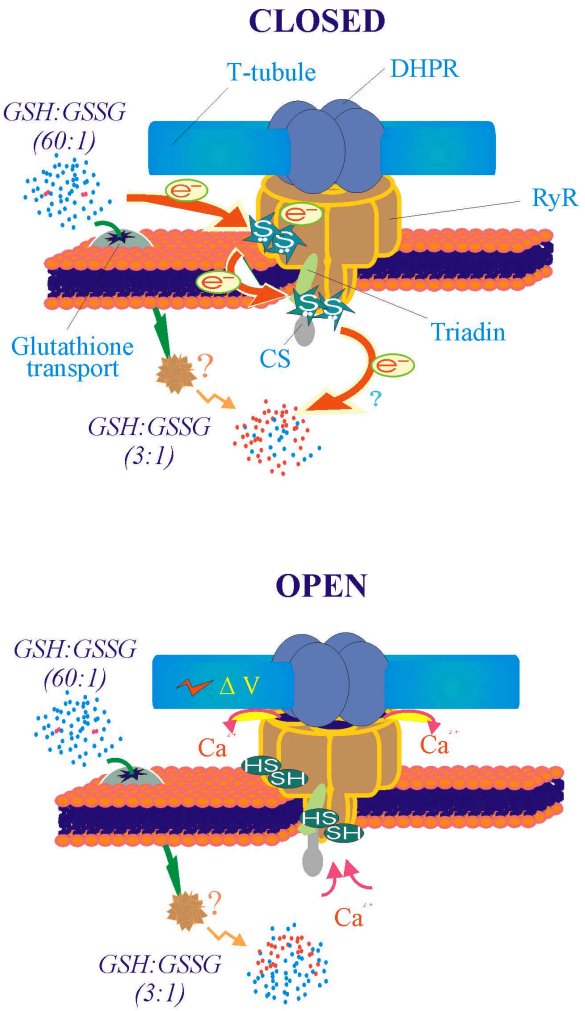

Figure 5. Possible mechanism by which the RyR1 complex senses changes to localized redox environment. In the closed channel conformation, Cys within a nucleophilic domain possesses deprotonated thio-anions that are hyperreactive and have mobile electrons (e^-) that can delocalize. The local redox potential can donate electrons to this "redox sensing" domain. Conformational transitions that coincide with channel opening rearranges the redox sensing domain which looses its nucleophilicity and the critical Cys revert to typical reactivity of other protein thiols.

cycles with channel gating transitions as previously suggested. It is also unlikely that the redox sensor contributes an obligatory mechanistic step in either e-c coupling (skeletal muscle) or CICR (other tissues). Rather the redox sensing properties of RyR complexes are likely to convey important information about localized changes in redox potential that are likely to occur across the SR/ER membrane in physiological and pathophysiologic states. One possible molecular mechanism underlying redox sensing may involve delocalization of mobile electrons within the RyR complex and the local cellular redox buffer (scheme in Figure 5).

7. ACKNOWLEDGEMENT

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Abbreviations: BLM, bilayer lipid membrane; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; DTDP, 2,2'-dithiodipyridine; DTNB, 5,5'-dithiobis(2-nitro)benzoate; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

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