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# Forum Minireview

# RyR1 Modulation by Oxidation and Calmodulin

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## ABSTRACT

**Alteration of skeletal muscle function by reactive oxygen species and nitric oxide (NO) may involve regulation of the activity of the skeletal muscle $Ca^{2+}$ release channel (also known as RyR1). We have shown that oxidants can activate RyR1 and produce inter-subunit disulfide bonds. Both effects are prevented by pretreatment with either NO donors or *N*-ethylmaleimide under conditions that modify less than 5% of the total sulfhydryls on RyR1. Oxidation-induced intersubunit crosslinking can also be prevented by the binding of either $Ca^{2+}$calmodulin or apocalmodulin to RyR1. Also, both $Ca^{2+}$calmodulin and apocalmodulin binding are blocked by oxidation of RyR1. In contrast, alkylation with *N*-ethylmaleimide or reaction with NO donors preferentially blocks apocalmodulin binding to RyR1, suggesting the existence of a regulatory cysteine within the apocalmodulin binding site. We have demonstrated that $Ca^{2+}$calmodulin and apocalmodulin bind to overlapping, but nonidentical, sites on RyR1 and that cysteine 3635 is close to or within the apocalmodulin-binding site on RyR1. This cysteine is also one of the cysteines that form the intersubunit disulfide bonds, suggesting that calmodulin binds at an intersubunit contact site. Our findings are consistent with a model in which oxidants regulate the activity of RyR1 directly by altering subunit–subunit interactions and indirectly by preventing the binding of either $Ca^{2+}$-bound calmodulin or apocalmodulin. NO also has both a direct and an indirect effect: it blocks the ability of oxidants to generate intersubunit disulfide bonds and prevents apocalmodulin binding.** Antiox. Redox Signal. 2, 41–45.

## INTRODUCTION

Reactive oxygen species (ROS) and nitric oxide derivatives (NO) are endogenous modulators of skeletal muscle function (Reid *et al.*, 1993; Kobzik *et al.*, 1994). ROS and NO are continually synthesized by muscle fibers and other cell types within muscle tissue (Kobzik *et al.*, 1994; Reid, 1996). These redox-active molecules exert tonic influences on a variety of processes within the myocyte, including excitation-contraction (E-C) coupling. The molecular mechanisms by which ROS and NO modulate E-C coupling are the focus of growing research interest. Among the regulatory proteins that exhibit redox sensitivity, the ryanodine-sensitive $Ca^{2+}$ release channel of the sarcoplasmic reticulum (SR) has been a primary focus of research for several laboratories (Abramson *et al.*, 1983, 1988a,b, 1989, 1995; Zorzato *et al.*, 1986; Zaidi *et al.*, 1989; Boraso and Williams, 1994; Favero *et al.*, 1995; Eager *et al.*, 1997; Eager and Dulhunty, 1998, 1999), including our own (Aghdasi *et al.*, 1997a,b; Wu *et al.*, 1997; Moore *et al.*, 1999; Zhang *et al.*, 1999). The SR $Ca^{2+}$ release channel is a homotetrameric protein composed of four 565-kDa subunits. Opening of the channel releases calcium from intracellular stores and is a critical step in E-C coupling. Channel activity is highly sensitive to redox-active agents, including ROS and NO, and, therefore, represents an attractive regulatory target. This manuscript briefly reviews our findings in this area, examining redox effects on channel function and structure.

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## ALKYLATION AND OXIDATION EFFECTS ON THE CHANNEL

Our initial study (Aghdasi *et al.*, 1997a) examined changes in channel structure and function produced by sulfhydryl reagents. One of our earliest observations was that SR vesicle preparations and isolated single channels exhibit a significant degree of spontaneous oxidation. This reflects autooxidative modification of protein sulfhydryls and is acutely reversible by treating the channels with dithiothreitol (DTT), a thiol-reducing agent. Because redox status of the protein *in vivo* cannot be determined with existing methods, we established the practice of pretreating isolated channels or SR vesicles with DTT prior to mechanistic studies using other sulfhydryl reagents. This strategy enables us to evaluate the full range of redox response that the protein undergoes; it also insures that we obtain data under the reduced conditions that are likely to pertain to the intact cell.

N-Ethylmaleimide (NEM), a sulfhydryl alkylating agent, has complex effects on isolated channels reconstituted into planar lipid bilayers. The channel undergoes time-dependent changes in spontaneous opening activity that has three characteristic phases: NEM first inhibits the channel, then activates the channel, and finally inhibits activity again. NEM shows a similar three-phase effect on the binding of [$^3$H]ryanodine to SR vesicle preparations. NEM initially decreases [$^3$H]ryanodine binding (phase 1), followed by a recovery of binding activity (phase 2), and then a final phase of inhibition (phase 3). We interpret these changes to reflect progressive and/or cumulative alkylation of RyR1 sulfhydryls by NEM.

The oxidizing agent diamide has very different effects on channel activity. We found that diamide activates the channel and enhances [$^3$H]ryanodine binding. Diamide also alters the channel response to NEM. Phase 1 inhibition is abolished; instead, single channels activated by diamide are further activated by the addition of NEM. All functional effects of diamide are reversed by subsequent exposure to the reducing agent DTT. Diamide also has characteristic effects on channel structure, inducing covalent cross-links between subunits of the $Ca^{2+}$ release channel tetramer. Diamide causes intersubunit dimerization of both the full-length 565-kDa subunits and the 400-kDa fragments that are produced by endogenous calpain digestion. This latter finding indicates that the inter-subunit cross-link does not involve sulfhydryls within the amino-terminal 170-kDa fragment of the protein. NEM alkylation of a subset of sulfhydryls (prior to the onset of the phase 2 effects) blocks the formation of intersubunit cross-links by diamide. Integrating these observations, we concluded that diamide either cross-links some of the sulfhydryls alkylated prior to the phase 2 functional effects of NEM or causes a conformational change in the $Ca^{2+}$ release channel that renders these hyperreactive sulfhydryls inaccessible to NEM alkylation. These data laid the groundwork for mapping sites of subunit–subunit contact in the $Ca^{2+}$ release channel tetramer and for identifying the functionally important sulfhydryls.

## HYDROGEN PEROXIDE AND NO AS CHANNEL MODULATORS

In a subsequent study (Aghdasi *et al.*, 1997b), we tested the effects of ROS and NO on channel function. Hydrogen peroxide ($H_2O_2$) was evaluated as a probe for ROS effects; a panel of chemically distinct NO donors was used to assess NO effects. We confirmed that $H_2O_2$ activates the channel, an effect previously reported by Favero *et al.* (1995); $H_2O_2$ increases the probability of channel opening and increases [$^3$H]ryanodine binding. $H_2O_2$ also stimulates intersubunit dimer formation. These effects mimic the action of diamide and, like diamide, the $H_2O_2$ effects are acutely reversed by DTT exposure. Both $H_2O_2$ and NO donors block phase 1 inhibition of the channel by NEM, duplicating diamide effects. At relatively high concentrations, the actions of NO donors are indistinguishable from those of $H_2O_2$ and diamide: NO donors activate the channel and produce intersubunit cross-links, and both effects are reversible by DTT.

At lower concentrations, however, NO donors have a distinct action. Concentrations of NO donors that have no detectable effect

on channel activity, when administered alone, block intersubunit cross-linking and channel activation by diamide. These findings suggest a model in which NO exerts biphasic effects on channel activity that are concentration-dependent. At low levels, NO appears to prevent oxidation of regulatory sulfhydryls and thereby limits channel activation. At higher concentrations, NO stimulates oxidative activation that is identical to the action of $H_2O_2$.

## LOCALIZATION OF REGULATORY SULFHYDRYLS

A companion study (Wu *et al.*, 1997) addressed the location and reactivity of regulatory sulfhydryls in greater detail. We used calpain and tryptic cleavage, two-dimensional SDS-polyacrylamide gel electrophoresis, amino-terminal sequencing, sequence-specific antibody Western blotting, and [$^{14}$C]NEM labeling to identify the domains involved in the functional effects described above. Results of these studies are consistent with a model in which diamide, an oxidizing agent, produces an intermolecular cross-link between adjacent subunits of the calcium channel tetramer. All of the cysteines involved in the crosslink appear to be either in the region between amino acids ~2,100 and 2,843 or the region between amino acids 2,844 and ~4,685. Oxidation exposes a new calpain cleavage site in the central domain of the channel, in the region near amino acid 2,100. Some of the sulfhydryls that react most rapidly with NEM are located in the amino-terminal domain between amino acids 426 and 1,396.

## REDOX MODULATION AND CALMODULIN BINDING

Calmodulin (CaM) has biphasic effects on the channel, increasing activity in low (nanomolar) calcium environments and inhibiting activity at high (micromolar) calcium levels (Tripathy *et al.*, 1995). Our data suggest that both calcium-bound CaM ($Ca^{2+}$ CaM) and $Ca^{2+}$-free CaM (apoCaM) bind to one site per subunit of the RyR1 tetramer (Moore *et al.*, 1999). Recently, we have demonstrated that the capacity of the $Ca^{2+}$ release channel to bind CaM is closely related to the oxidation state of the channel (Zhang *et al.*, 1999). Oxidation blocks the binding of [$^{125}$I]CaM at both micromolar and nanomolar $Ca^{2+}$ concentrations. Conversely, binding of CaM (either form) to the channel inhibits oxidative cross-linking between channel subunits. Under conditions where <5% of the total sulfhydryls on each channel subunit are alkylated by NEM, [$^{125}$I] apoCaM binding and oxidation-induced intersubunit crosslinking are completely blocked. In contrast, binding of $Ca^{2+}$ [$^{125}$I]CaM is unaffected. NO donors have effects similar to NEM (unpublished observations). These studies indicate that the binding sites for apoCaM and $Ca^{2+}$ CaM have distinct features. The data further suggest that oxidation may alter channel activity both directly by producing inter-subunit disulfide bonds and indirectly by altering the binding of CaM. Last, the results suggest that CaM may protect the channel from oxidative modification, *e.g.*, during conditions of oxidative stress.

We have also shown that CaM bound to RyR1, either at n*M* or $\mu M$ $Ca^{2+}$, protects sites after amino acids 3,630 and 3,637 from cleavage by trypsin, suggesting that both the apoCaM and $Ca^{2+}$ CaM binding sites are close to this site (Moore *et al.*, 1999). Although allosteric protection of these sites is possible, it seems unlikely that a partial agonist (apoCaM) and an inhibitor ($Ca^{2+}$ CaM) would allosterically regulate the same region of RyR1 in the same fashion. Our data favor a model in which apoCaM and $Ca^{2+}$ CaM bind close to a site of subunit–subunit contact on RyR1, but the determinants on both RyR1 and CaM important for binding are likely to be different for apoCaM and $Ca^{2+}$ CaM.

## IDENTIFICATION OF A CYSTEINE INVOLVED IN REDOX MODULATION OF RyR1

The sequence between the two sites protected from trypsin by bound CaM is AVVACFR, and we have identified the cysteine within this sequence as one of the cysteines involved in the intersubunit disulfide bond induced by oxidants. We found that both

cysteines needed for the oxidation-induced inter-subunit crosslink could be protected from alkylation with N-ethylmaleimide by bound calmodulin (Moore *et al.*, 1999). We then showed, using amino-terminal amino acid sequencing together with analysis of the distribution of [$^3$H]NEM labeling with each sequencing cycle, that cysteine 3635 of RyR1 was rapidly labeled by NEM and that this labeling is blocked by bound calmodulin. These findings suggested that cysteine 3,635 is located at an inter-subunit contact site that is close to or within the calmodulin-binding site.

## SUMMARY AND CONCLUSIONS

Our research demonstrates that the SR $Ca^{2+}$ release channel undergoes reversible redox modulation and is sensitive to biological mediators, including $H_2O_2$ and NO derivatives. Either ROS or NO can induce oxidative activation of the channel. This process appears to target a small subset of the available sulfhydryls (one of which is cysteine 3,635) and involves the formation of disulfide cross-links between subunits. Also, calmodulin and oxidation appear to modulate RyR1 activity by regulating inter-subunit interactions in a mutually exclusive manner.

Overt activation via channel oxidation is most likely to be important in pathologic processes, e.g., malignant hyperthermia, sepsis, reperfusion injury, *etc.* At lower levels, ROS and NO appear to affect the channel in a more complex manner. Oxidative activation by ROS may be partial and the functional effects more subtle. NO may be capable of inhibiting oxidative activation and could thereby oppose the action of ROS. Such opposition is consistent with ROS and NO effects on unfatigued muscle contraction, in which force is increased by ROS (Reid *et al.*, 1993) and decreased by NO (Kobzik *et al.*, 1994). Thus, it is conceivable that ROS and NO could modulate channel function and E-C coupling *in vivo* via such a mechanism. We do not suggest, however, that redox mechanisms are essential for interaction of the voltage sensor with the calcium release channel.

Finally, our most recent observations indicate that redox mechanisms can strongly influence protein–protein interactions between the $Ca^{2+}$ release channel and CaM. This represents a novel mechanism by which ROS might indirectly affect channel activity. Also, this finding raises the larger issue of secondary regulation as a general mechanism. Calcium channel interaction with other modulatory proteins, *e.g.*, FKBP, may also be influenced by protein oxidation state. Such mechanisms would provide an additional level of regulatory complexity by which ROS and NO might fine-tune channel function.

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## ABBREVIATIONS

apoCaM, Apocalmodulin or $Ca^{2+}$-free calmodulin; CaM, calmodulin; DTT, dithiothreitol; E-C coupling, excitation-contraction coupling; $H_2O_2$, hydrogen peroxide; NEM, *N*-ethylmaleimide; NO, nitric oxide; redox, oxidation-reduction; ROS, reactive oxygen species; RyR1, ryanodine receptor, type 1; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum.

## REFERENCES

ABRAMSON, J.J., TRIMM, J.L., WEDEN, L., and SALAMA, G. (1983). Heavy metals induced rapid calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle. Proc. Natl. Acad. Sci. USA **80,** 1526–1530.

ABRAMSON, J.J., BUCK, E., SALAMA, G., CASIDA, J.E., and PESSAH, I.N. (1988a). Mechanism of anthraquinone-induced calcium release from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **263,** 18750–18758.

ABRAMSON, J.J., CRONIN, J., and SALAMA, G. (1988b). Oxidation induced by phthalocyanine dyes cause rapid calcium release from sarcoplasmic reticulum vesicles. Arch. Biochem. Biophys. **263,** 245–255.

ABRAMSON, J.J., and SALAMA, G. (1989). Critical sulfhydryls regulate calcium release from sarcoplasmic reticulum. J. Bioenerg. & Biomemb. **21,** 283–294.

ABRAMSON, J.J., ZABLE, A.C., FAVERO, T.G., and

SALAMA, G. (1995). Thimerosal interacts with $Ca^{2+}$ release channel ryanodine receptor from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **270,** 29644–29647.

AGHDASI, B., ZHANG, J.Z., WU, Y., REID, M., and HAMILTON, S.L. (1997a). Multiple classes of sulfhydryls modulate the skeletal muscle $Ca^{2+}$ release channel. J. Biol. Chem. **272,** 3739–3748.

AGHDASI, B., REID, M.B., and HAMILTON, S.L. (1997b). Nitric oxide protects the skeletal muscle $Ca^{2+}$ release channel from oxidation induced activation. J. Biol. Chem. **272,** 25462–25467.

BORASO, A., and WILLIAMS, A.J. (1994). Modification of the gating of the cardiac sarcoplasmic reticulum $Ca^{2+}$ release channel by $H_2O_2$ and dithiothreitol. Am. J. Physiol. **267,** H1010–1016.

EAGER, K.R., RODEN, L.D., and DULHUNTY, A.F. (1997). Actions of sulfhydryl reagents on single ryanodine receptor $Ca^{(2+)}$-release channels from sheep myocardium. Am. J. Physiol. **272,** C1908–1918.

EAGER, K.R., and DULHUNTY, A.F. (1998). Activation of the cardiac ryanodine receptor by sulfhydryl oxidation is modified by $Mg^{2+}$ and ATP. J. Membr. Biol. **163,** 9–18.

EAGER, K.R., and DULHUNTY, A.F. (1999). Cardiac ryanodine receptor activity is altered by oxidizing reagents in either the luminal or cytoplasmic solution. J. Membr. Biol. **167,** 205–214.

FAVERO, T.G., ZABLE, A.C., and ABRAMSON, J.J. (1995). Hydrogen peroxide stimulates the $Ca^{2+}$ release channel from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **270,** 25557–25563.

KOBZIK, L., REID, M.B., BREDT, D.S., and STAMLER, J.S. (1994). Nitric oxide in skeletal muscle. Nature **372,** 546–548.

MOORE, C.P., RODNEY, G., ZHANG, J.-Z., SANTACRUZ-TOLOZA, L., STRASBURG, G.M., and HAMILTON, S.L. (1999). ApoCalmodulin and $Ca^{2+}$ calmodulin bind to the same region on the skeletal muscle $Ca^{2+}$ release channel. Biochemistry **38,** 8532–8537.

MOORE, C.P., ZHANG, J.-Z., and HAMILTON, S.L. (1999). A role for cysteine 3635 of RyR1 in redox modulation and calmodulin binding. J. Biol. Chem. **274,** 36831–36834.

REID, M.B. (1996). Reactive oxygen and nitric oxide in skeletal muscle. News Physiol. Sci. **11,** 114–119.

REID, M.B., KHAWLI, F.A., and MOODY, M.R. (1993). Reactive oxygen in skeletal muscle III. Contractility of unfatigued muscle. J. Appl. Physiol. **75,** 1081–1087.

TRIPATHY, A., XU, L., MANN, G., and MEISSNER, G. (1995). Calmodulin activation and inhibition of skeletal muscle $Ca^{2+}$ release channel (ryanodine receptor). Biophys. J. **69,** 106–119.

WU, Y., AGHDASI, B., DOU, S.J., ZHANG, J.Z., LIU, S.Q., and HAMILTON, S.L. (1997). Functional interactions between cytoplasmic domains of the skeletal muscle $Ca^{2+}$ release channel. J. Biol. Chem. **272,** 25051–25061.

ZAIDI, N.F., LAGENAUR, C.F., ABRAMSON, J.J., PESSAH I., and SALAMA, G. (1989). Reactive disulfides trigger $Ca^{2+}$ release from sarcoplasmic reticulum via an oxidation reaction. J. Biol. Chem. **264,** 21725–21736.

ZHANG, J.-Z., WU, Y., WILLIAMS, B.Y., RODNEY, G., MANDEL, F., STRASBURG, G.M., and HAMILTON, S.L. (1999). Oxidation of the skeletal muscle $Ca^{2+}$ release channel alters calmodulin binding. Am. J. Physiol. **276,** (Cell Physiol. 45), C46–C53.

ZORZATO, F., MARGRETH, A., and VOLPE, P. (1986). Direct photoaffinity labeling of junctional sarcoplasmic reticulum with [$^{14}$C] doxorubicin. J. Biol. Chem. **261,** 13252–13257.

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2. Michael Y. Song , Ayako Makino , Jason X.-J. Yuan . 2011. Role of Reactive Oxygen Species and Redox in Regulating the Function of Transient Receptor Potential Channels. *Antioxidants & Redox Signaling* **15**:6, 1549-1565. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
3. Lufang Zhou, Miguel A. Aon, Ting Liu, Brian O'Rourke. 2011. Dynamic modulation of Ca2+ sparks by mitochondrial oscillations in isolated guinea pig cardiomyocytes under oxidative stress. *Journal of Molecular and Cellular Cardiology* . [CrossRef]
4. Barbara W. Brandom, Mihaela VisoiuMalignant Hyperthermia 1682-1692. [CrossRef]
5. Melissa A. Chambers, Jennifer S. Moylan, Michael B. Reid. 2009. Physical inactivity and muscle weakness in the critically ill. *Critical Care Medicine* **37**, S337-S346. [CrossRef]
6. Leigh Ann Callahan, Gerald S. Supinski. 2009. Sepsis-induced myopathy. *Critical Care Medicine* **37**, S354-S367. [CrossRef]
7. Chris van der Poel, Joshua N Edwards, William A Macdonald, D George Stephenson. 2008. EFFECT OF TEMPERATURE-INDUCED REACTIVE OXYGEN SPECIES PRODUCTION ON EXCITATION-CONTRACTION COUPLING IN MAMMALIAN SKELETAL MUSCLE. *Clinical and Experimental Pharmacology and Physiology* . [CrossRef]
8. Cecilia Hidalgo , Paulina Donoso . 2008. Crosstalk Between Calcium and Redox Signaling: From Molecular Mechanisms to Health Implications. *Antioxidants & Redox Signaling* **10**:7, 1275-1312. [Abstract] [Full Text PDF] [Full Text PDF with Links]
9. Michael B. Reid. 2008. Free radicals and muscle fatigue: Of ROS, canaries, and the IOC. *Free Radical Biology and Medicine* **44**:2, 169-179. [CrossRef]
10. Victor S. Sharov, Christian SchöneichChapter 6 Oxidative Modification of Ca2+ Channels, Ryanodine Receptors, and the Sarco/Endoplasmic Reticulum Ca2+-ATPase **61**, 113-130. [CrossRef]
11. Dr. Livia C. Hool , Ben Corry . 2007. Redox Control of Calcium Channels: From Mechanisms to Therapeutic Opportunities. *Antioxidants & Redox Signaling* **9**:4, 409-435. [Abstract] [Full Text PDF] [Full Text PDF with Links]
12. Hong-mei HAN, Ri-sheng WEI, Anthony F LAI, Chang-cheng YIN. 2006. Molecular nature of sulfhydryl modification by hydrogen peroxide on type 1 ryanodine receptor1. *Acta Pharmacologica Sinica* **27**:7, 888-894. [CrossRef]
13. C. Hidalgo. 2005. Cross talk between Ca2+ and redox signalling cascades in muscle and neurons through the combined activation of ryanodine receptors/Ca2+ release channels. *Philosophical Transactions of the Royal Society B: Biological Sciences* **360**:1464, 2237-2246. [CrossRef]
14. Paula Aracena , Wei Tang , Susan L. Hamilton , Cecilia Hidalgo . 2005. Effects of S-Glutathionylation and S-Nitrosylation on Calmodulin Binding to Triads and FKBP12 Binding to Type 1 Calcium Release Channels. *Antioxidants & Redox Signaling* **7**:7-8, 870-881. [Abstract] [Full Text PDF] [Full Text PDF with Links]
15. Ramamurthy Mahalingam, Nina Fedoroff. 2003. Stress response, cell death and signalling: the many faces of reactive oxygen species. *Physiologia Plantarum* **119**:1, 56-68. [CrossRef]
16. Andrei A Kochegarov. 2003. Intracellular calcium channels and their modulators. *Expert Opinion on Therapeutic Patents* **13**:6, 815-850. [CrossRef]
17. M. B. Reid, Y.-P. Li. 2001. Cytokines and oxidative signalling in skeletal muscle. *Acta Physiologica Scandinavica* **171**:3, 225-232. [CrossRef]