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COMMENTARY

Regulation of Ryanodine Receptors by Reactive Nitrogen Species

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ABSTRACT. The ryanodine receptors (RyRs) are large intracellular calcium release channels that play an important role in the control of the calcium levels in excitable and non-excitable cells. Many endogenous modulators such as Mg^{2+}, ATP, or calmodulin can affect the channel activities of the three known mammalian RyR isoforms. RyRs also are known to be redox-responsive. However, the molecular basis and the physiological relevance of redox modulation of RyRs are unclear. Recent evidence suggests that nitric oxide (NO) and related molecules may be endogenous regulators of the skeletal and cardiac muscle RyRs. The two tissues express nitric oxide synthases (NOSs), and NO or NO-related species have been shown to affect Ca^{2+} release channel activities directly via covalent modifications of thiol groups. Both an oxidative and a nitrosative modification of RyRs have been described, leading to either a reversible or irreversible alteration of RyR ion channel activity. Additional mechanisms of regulation may include cyclic GMP-dependent signaling pathways and NO modification of RyR regulatory proteins such as the surface membrane L-type Ca^{2+} channel. Modification of RyRs by NO may influence a variety of physiological functions such as insulin release, vasomotor control, and muscle contraction. BIOCHEM PHARMACOL **57**;10:1079–1084, 1999. © 1999 Elsevier Science Inc.

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NO§ fulfills many of the criteria of a physiological modulator of skeletal and cardiac muscle excitation–contraction coupling [1–3]. Muscle cells express three NO synthase isoforms (eNOS, nNOS, iNOS), are lined by an endothelium, and are exposed to several circulating cell types, all of which liberate NO or related molecules. In cardiac muscle, the result is a frequency-dependent production of NO and modulation of contractile function [4]. However, the exact role of NO in regulating muscle contraction is less clear. NO binds to and activates guanylate cyclase, which accounts for some of its physiological effects; however, NO may also affect cellular functions by non-cGMP-mediated mechanisms primarily involving *S*-nitrosylation and oxidation of free thiols [5]. Below we will briefly review the RyRs and NOSs. We then will focus on recent studies on the regulation of the mammalian skeletal muscle and cardiac muscle RyRs by NO and related species.

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§*Abbreviations*: NO, nitric oxide; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; RyR, ryanodine receptor; RyR1, skeletal muscle RyR; RyR2, cardiac muscle RyR; RyR3, brain RyR; NOS, nitric oxide synthase; SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; SIN-1, 3-morpholinosydnomine; GSNO, *S*-nitrosoglutathione; CysNO, *S*-nitrosocysteine; SNAP, *S*-nitrosyl-*N*-acetylpenicillamine; and cGMP and cAMP, cyclic GMP and cyclic AMP, respectively.

RyRs

RyRs are calcium channels that control the levels of intracellular Ca^{2+} by releasing Ca^{2+} from intracellular calcium-storing organelles [6–8]. They were named RyRs because of the specific binding of the plant alkaloid ryanodine, which has facilitated their purification and characterization. Mammalian tissues express three structurally and functionally related RyRs (RyR1, RyR2, and RyR3) that are encoded by three different genes. RyR1, RyR2, and RyR3 are also known as skeletal, cardiac, and brain RyRs because they were first identified in and isolated from the three respective tissues. They share evolutionary origin, sequence homology, and structural similarities with the other major family of intracellular calcium channels, the inositol 1,4,5-trisphosphate receptors [9]. In striated muscle, the RyRs play a central role in excitation–contraction coupling by releasing Ca^{2+} required for muscle to contract. The skeletal and the cardiac RyRs are located in the junctional SR membrane near transverse (T-) tubule, voltage-sensitive L-type calcium channels (DHPRs). A muscle action potential initiates DHPR conformational changes, which either alter the conformation of the RyRs

by a direct physical interaction (in skeletal muscle) or permit an influx of extracellular Ca^{2+} (in cardiac muscle), with both mechanisms leading to the release of Ca^{2+} from the SR and subsequent muscle contraction [6–8]. RyRs are found in many mammalian tissues, and more than one isoform may be expressed in one tissue. For example, in rabbit slow twitch and diaphragm skeletal muscles, RyR1 and RyR3 coexist in the SR [10].

The RyRs have been isolated as large protein complexes composed of four 560-kDa (RyR polypeptide) and four 12-kDa (FK506 binding protein) subunits [6–8]. cDNA sequencing has indicated that the three mammalian RyR isoforms share 66–70% amino acid homology. The C-terminus represents the most hydrophobic region of the RyRs and accordingly has been proposed to form the Ca^{2+} channel pore. The remaining amino acid sequences are highly hydrophilic and form the "foot structure" that projects into the cytosol toward the T-tubule DHPRs. Various regulatory sites are localized on the large cytosolic foot structure such as those for calcium, magnesium, ATP, and calmodulin. Other proteins physically interact with RyRs and may also modulate the channel activities, such as FK506 binding protein [11], or triadin, an SR junctional protein [6–8]. As discussed below, RyRs also contain reactive thiols, which suggests that reactive oxygen and nitrogen species may have a role in the *in vivo* regulation of channel activity [7, 12–18].

NOSs

NO is derived from one of the chemically equivalent guanidino nitrogens of L-arginine in a reaction catalyzed by one of three NOSs [19]. nNOS (NOS-1), first identified in neurons, and eNOS (NOS-3), first identified in endothelial cells, are most often constitutively expressed [2, 3]. They are activated by extracellular signals that increase intracellular $[Ca^{2+}]$ and thereby facilitate the interaction of the two enzymes with calmodulin. More recent evidence suggests that they can be also activated by Ca^{2+}-independent mechanisms, albeit the putative importance of post-translational modifications in such regulation remains to be proven [20, 21]. The third isoform, iNOS (NOS-2), is transcribed in inflammatory cells and muscles in response to cytokines and bacterial endotoxins [2, 3]. iNOS tightly binds calmodulin in the absence and presence of Ca^{2+} and synthesizes NO and related molecules in a Ca^{2+}-independent manner.

NO is a small chemically reactive molecule that can act as a paracrine autocoid or autocrine second messenger and can exert its cellular effects via cGMP-dependent or -independent pathways [1–3]. The first pathway involves the binding of NO to the heme group of guanylate cyclase, the subsequent enhanced production of intracellular cGMP, and activation of the cGMP-dependent kinase signaling pathway. Alternatively, cGMP can exert control of phosphodiesterases that influence cAMP levels or it can directly gate ion channels. The cGMP-independent pathways usually involve the covalent modification or oxidation of critical thiols or transition metals. NO may directly react with redox centers in proteins, or it may first react with molecular oxygen or superoxide anion (O_2^-) to form reactive NO-related species (NO_x) that are responsible for the post-translational modifications of proteins [5]. For example, the reaction of NO with superoxide yields peroxynitrite, a highly reactive free-radical species that extensively oxidizes [18] and irreversibly activates [16, 18] the RyR. Peroxynitrite has been implicated in the postischemic heart in cellular injuries [22]. In addition to RyRs, a growing list of ion channels are modulated by NO and related molecules including the related inositol 1,4,5-trisphosphate receptor and the cardiac L-type Ca^{2+} channel [23–26].

LOCALIZATION OF NOSs AND RyRs IN STRIATED MUSCLE

Regulation of RyRs by NO (or related molecules) does not require that NOS and RyR co-localize, nor must they be expressed in the same cell. After all, NO, initially identified as the endothelial derived relaxation factor, has been shown to diffuse from endothelial cells to vascular smooth muscle cells to cause vasorelaxation [27]. Nevertheless, a close proximity of the two proteins could be advantageous in that it should restrict NO signaling to specific targets within a limited microenvironment while minimizing aberrant toxic pathways [28]. In striated muscles, all three isoforms of NOS have been identified, and NO has been implicated in the regulation of contractile force [1–3].

In skeletal muscle, nNOS is the predominant isoform. The enzyme is restricted to type II (fast-twitch) muscle fibers of the rat [1]. In some studies nNOS staining has been observed in both type I (slow) and type II muscle fibers [29–31]. In skeletal muscle fibers, nNOS is targeted to neuronal postsynaptic densities by interacting with postsynaptic proteins [32], and to specialized invaginations of the sarcolemma, called caveolae, by binding to caveolin 3 [33] and dystrophin-associated proteins [28]. Caveolin also binds other signaling proteins such as c-src, Ha-ras and $G_{s\alpha}$, which suggests that nNOS may function in caveolae as part of a signaling complex [34]. nNOS is developmentally regulated, being expressed at high levels in rat embryonic and neonatal diaphragms, with a gradual reduction in the expression levels during late postnatal development [35]. Skeletal muscle fibers also express iNOS and eNOS isoforms to varying extents. In rat skeletal muscle fibers, eNOS showed a patchy sarcolemmal and weak sarcoplasmic immunolabeling pattern [30] and co-localized with mitochondrial markers [36], while no expression of iNOS was seen [30]. In guinea pig skeletal muscle, an anti-iNOS antibody weakly stained intracellular structures in "pathogen-free" animals [37]. The staining was intensified by pretreatment with bacterial lipopolysaccharides. Significant eNOS staining of muscle was absent but could be detected in vascular endothelium.

In cardiac muscle, eNOS is the predominant NOS isoform. The enzyme is expressed primarily in the coronary and endocardial endothelia but also has been localized to cardiomyocytes [38]. In canine cardiomyocytes, the enzyme is targeted to caveolae by binding to caveolin 3 [39]. eNOS enzyme activity was modulated in endothelial cells by reversible protein–protein interactions that are controlled by Ca^{2+}/calmodulin and enzyme palmitoylation [40], and it is therefore possible that a similar regulation occurs in cardiomyocytes. A co-purification of eNOS and cardiac RyR has been reported [14]; however, it is not clear whether the two proteins resided in the same membranes, considering the difficulties of preparing pure SR membranes from cardiac homogenates. Much like skeletal muscle, cardiomyocytes express iNOS after endotoxin administration [41].

A physiologically relevant functional coupling of NOS and RyRs has been described in non-muscle cells. Specifically, in canine colon interstitial cells, an activation of RyRs by NOS resulted in a rise of $[Ca]_i$ leading to an increased synthesis and release of NO from the interstitial cells and relaxation of nearby smooth muscle cells [42]. NO-induced, RyR-mediated elevation in $[Ca^{2+}]_i$ has also been suggested to play an important role in the secretory activity of rat pancreatic beta islet cells [43]. Other tissues such as neurons or endothelial cells express RyRs and NOSs; however, whether NO regulates intracellular Ca^{2+} release in these cells and if so, via covalent modification(s) of RyRs or via a cGMP-dependent pathway, or both, remains to be determined.

REGULATION OF RyR BY NO VIA cGMP-DEPENDENT PATHWAYS

NO increases cGMP levels in muscle, and such increases may alter RyR activities [1–3]. One possible mechanism may involve phosphorylation of RyRs by cGMP-dependent protein kinase [44]. cGMP also may indirectly control RyRs by changing the cytosolic levels of RyR effectors such as cADP-ribose. In sea urchin eggs, NO increased the levels of the RyR activator cADP-ribose via a cGMP-dependent mechanism [45]. Treatment of a pheochromocytoma cell line, PC12, with NO donors led to a modest increase in intracellular cADP-ribose levels and release of Ca^{2+} from intracellular stores. The latter was attributed to enhanced opening of RyR2s by a cGMP-dependent pathway [46]. A NO/cGMP/cADP-ribose mediated pathway may also exist in cardiac muscle because cADP-ribose has been reported to modulate the mammalian cardiac RyR [47, 48 but see also 49]. Taken together, the data raise the possibility of NO/cGMP/cADP-ribose regulation of mammalian RyRs, but the importance of this pathway is still unclear.

S-Nitrosylation and Oxidation of RyRs

Contracting muscle produces reactive nitrogen and oxygen intermediates. A functional role of these compounds is supported by the finding that redox-active compounds and antioxidant enzymes modulate excitation–contraction coupling and force production [1–3]. Specifically, both the L-type Ca^{2+} channel [24–26] and RyR [12–18, 50–56] contain sulfhydryls whose oxidation modulates function. Heavy metals, alkylating agents such as *N*-ethylmaleimide, and oxidants such as diamide and H_2O_2 all have been shown to modulate the activity of RyRs. Some oxidants may lead to intersubunit disulfide bond formation and cross-links of RyR subunits [17] or cross-links of RyR with other polypeptides [51].

Tentative early models of the gating mechanism of the skeletal muscle RyR have invoked a complex redox site comprised of several SH groups or two redox sites, one stimulatory and one inhibitory [54]. The model relies on rapid thiol-disulfide exchange reactions driven by changes in membrane potential. These ideas have been extended to the cardiac RyR, which showed a similar redox sensitivity [55]. Currently, no published data are available on the redox responsiveness of RyR3.

RyRs are potential targets for NO and related compounds because they contain a large number of sulfhydryls. The tetrameric mammalian skeletal and cardiac RyRs have a total of 404 and 364 cysteines, respectively (100 and 89 cysteines per 560-kDa subunit [57, 58], and 1 and 2 per FK506 binding protein [59, 60], respectively). In the purified tetrameric cardiac RyR, ~80 cysteines or ~20 per subunit are free, as determined by monobimane reactivity [18]. A direct modulation of RyR1 and RyR2 activities by NO or NO-related species has been demonstrated in vesicle-Ca^{2+} flux, single channel, and [^{3}H]ryanodine binding measurements. NO or NO-related species interact with the skeletal and cardiac RyRs in a complex way because both activation [16–18] and inhibition [13, 14] have been observed. The NO donor SNAP as well as NO generated *in situ* from arginine by endogenous NOS both reduced the rate of Ca^{2+} release from isolated skeletal muscle SR vesicles and the open probability of single skeletal RyR channels incorporated in lipid bilayers [13]. Similarly, an inactivation of single cardiac RyR ion channels by *in situ* generated NO has been described [14]. The effects of NO-related species could be reversed by 2-mercaptoethanol and were considered to be endogenously produced because they were prevented by NOS inhibitors and the NO quencher hemoglobin. On the other hand, Stoyanovsky *et al.* [16] found that NO, delivered in the form of NO gas, and NO donors (NONOates, *S*-nitrosothiols) activated single channels and Ca^{2+} release from skeletal and cardiac SR. Sulfhydryl reducing agents reversed the activating effects of NO and the NO donors but not those of SIN-1, which generates the strongly oxidizing peroxynitrite species. Aghdasi and colleagues [17] reported that NO donors (SNAP and two different NONOates) affected the skeletal muscle RyR in a concentration-dependent manner. Low concentrations had no detectable effect on RyR1 channel activity, as determined in single channel measurements, but were able to block intersubunit cross-links and prevented

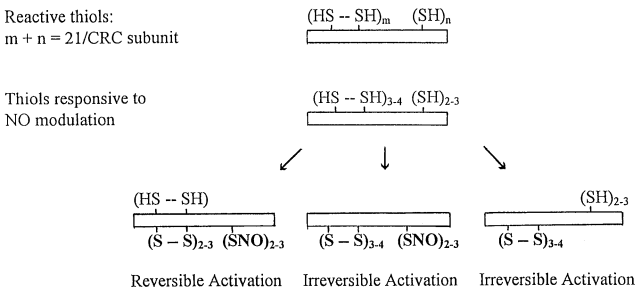

FIG. 1. Schematic diagram of nitrosative and oxidative mechanisms of cardiac RyR activation. The tetrameric receptor has a total of 364 cysteines, of which there are ~21 free cysteines per subunit (84 total are free and reactive [18]). Some of these (vicinal thiols, m) have a tendency to form intramolecular disulfides (dashed lines), whereas others (n) do not. Subsets of both classes of thiols are responsive to NO modulation. Reversible activation of the receptor results from poly-*S*-nitrosylation; concomitant oxidation of up to 2–3 disulfides per subunit does not modify this response (lower left). Oxidation of two extra thiols per subunit—whether associated with poly-*S*-nitrosylation (lower middle) or not (lower right)—leads to irreversible activation of the receptor. Covalent modifications such as poly-*S*-nitrosylation are more likely to be regulatory; oxidation to disulfide (or higher oxides) is more likely to have deleterious effects.

activation of the skeletal RyR channel by the disulfide inducing agent diamide. At higher concentrations, the NO donors activated the RyR1, which was attributed to the formation of intersubunit cross-links. Xu *et al.* [18] found that three NO-related species (GSNO, CysNO, and SIN-1) activated the cardiac RyR in a concentration-dependent manner by poly-*S*-nitrosylation and/or oxidation. Under conditions resulting in maximal channel activation, up to 12 sites per RyR subunit were *S*-nitrosylated and/or oxidized. The level of *S*-nitrosylation appeared to be dependent on channel conformation because it was reduced by the RyR inhibitor Mg^{2+}. Addition of 10 mM dithiothreitol (DTT) resulted in denitrosylation and returned GSNO- but not CysNO-activated channel activities close to control levels. SIN-1, which produces peroxynitrite, oxidized but did not *S*-nitrosylate the cardiac RyR. The post-translational modifications by SIN-1 were associated with channel activation, which was not reversed by DTT. The smaller and more reactive NO-related species CysNO and SIN-1 oxidized a larger number of thiols than did GSNO (~10 by 0.1 mM CysNO and 0.2 mM SIN-1 vs 7–8 by 1 mM GSNO). Thus, the oxidation of additional thiols appeared to be associated with irreversible channel activation (Fig. 1). Taken together, results reported thus far suggest that the effects of NO and NO-related species are concentration-dependent and critically dependent on the experimental conditions such as the redox state of RyRs and the identity of NO-related species. Furthermore, the redox-modifying effects of NO and related molecules appear to be isoform-specific and to be influenced by allosteric effectors of RyRs. Importantly, the ability of RyR to be poly-*S*-nitrosylated represents a novel mechanism of protein regulation reminiscent of polyphosphorylation.

REGULATION OF L-TYPE Ca^{2+} CHANNELS BY NO

In striated muscle, RyRs are regulated by L-type Ca^{2+} channels via either a direct physical interaction (in skeletal muscle) or an influx of extracellular Ca^{2+} (in cardiac muscle) [6–8]. NO therefore also may modulate the release of Ca^{2+} from the SR by interacting with L-type Ca^{2+} channels via cGMP-dependent and independent pathways. Wang *et al.* [26] have suggested a cGMP-mediated regulation of L-type Ca^{2+} channels in cat atrial myocytes. Campbell *et al.* [24] studied the effects of NO-related species (GSNO, CysNO, SIN-1), thiol-oxidizing and reducing agents, and 8-Br-cGMP on basal L-type currents in ferret right ventricular myocytes using the patch clamp technique. Their results indicated that NO-mediated increases in cGMP inhibited the L-type Ca^{2+} channel, whereas *S*-nitrosylation and/or oxidation of the channel stimulated channel activity. An inhibition was observed when the effects of three NO donors (SNAP, CysNO, GSNO) on cardiac L-type Ca^{2+} channels expressed in HEK 293 cells were tested [25]. The inhibitory effects of SNAP were not affected by 8-Br-cGMP, which suggests that NO donors inhibited the recombinant L-type Ca^{2+} channels by a cGMP-independent mechanism. The reasons for the differing effects of NO donors on native [24, 26] and recombinant [25] L-type Ca^{2+} channel activities are not clear but may be due to some variations in the experimental conditions such as the use of different cell lines. Notwithstanding these alternative results, NOS has not been shown to regulate the L-type Ca^{2+} channel independently of cGMP.

CONCLUDING REMARKS

Although recent progress has led to an improved understanding of the interaction of NO and related species with RyRs, several major questions regarding their action on SR Ca^{2+} release remain to be resolved:

1. Is NO a direct physiological modulator of RyRs in striated muscle? The cardiac RyR is endogenously *S*-nitrosylated [18]; however, the extent of *S*-nitrosylation was low and the physiological significance of this reaction (inhibitory or stimulatory) remains to be better established.
2. Do RyRs and NOSs colocalize? A close proximity of the two proteins is not essential but could be advantageous by minimizing aberrant toxic pathways.
3. In which ways are the RyR activities modified by the reactive nitrogen and oxygen intermediates that are formed during muscle activity under normal conditions as well as in fatigued or ischemic tissue? Does NO have a protective or deleterious effect on RyRs [5]? Recently described NOS-specific knockout mouse models [61, 62] might help in clarifying the role of NO in normal as well as fatigued and ischemic muscles.
4. RyRs contain a large number of free thiols, and their

activity is regulated *in vitro* by direct covalent modifications involving the *S*-nitrosylation and oxidation of several classes of sulfhydryls. What is the identity and function of the reactive thiols? Do they form disulfides or higher levels of sulfhydryl oxidation? Does the modification of thiols have deleterious effects or do some thiols serve as buffers and protect from oxidation of critical thiols?

5. Are the interactions of NO with RyRs tissue/isoform specific, and what is the molecular basis of this specificity? Do other proteins such as L-type Ca^{2+} channels (in skeletal muscle) and allosteric effectors such as Mg^{2+}, ATP, or calmodulin influence the interaction of NO with RyRs?

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