experiment, 35% of *dcp-1* GLC stage 14 egg chambers had a severe dumpless phenotype, and 63% of these were TUNEL-positive (also see Table 1). With either TUNEL or AO, a delay in apoptosis was observed in the *dcp-1* GLCs. This delay, however, may not be directly due to loss of *dcp-1* function because a delay in apoptosis is observed in other dumpless mutants (*15*). These authors have suggested that an inhibitor of apoptosis may be present in the nurse cells and that removal of the inhibitor by dumping is required for the completion of apoptosis.

17. Ultrastructural analysis has revealed large (0.5 μm) gaps in the nuclear envelope of nurse cells before the stage of nurse cell dumping [E. Okada and C. H. Waddington, *J. Embryol. Exp. Morphol.* **7**, 583 (1959); A. P. Mahowald and M. P. Kambysellis, in *The Genetics and Biology of* Drosophila, M. Ashburner and T. R. F. Wright, Eds. (Academic Press, New York, 1980), vol. 2d, pp. 141–224]. With a nuclear β-Gal marker, wild-type nurse cell nuclei become permeable during stage 10B, with β-Gal leakage into the cytoplasm (*8*). Both *dcp-1* alleles are caused by insertion of P elements that carry a nuclear β-Gal marker (*12*). We examined either heterozygous *dcp-1/CyO* or *dcp-1* GLC ovaries for β-Gal activity using X-gal as a substrate. We followed the protocol in (*31*), except that ovaries were fixed in 1% glutaraldehyde [in 0.1 M phosphate buffer (pH 6.9)] for 7 min.
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Activation of the Cardiac Calcium Release Channel (Ryanodine Receptor) by Poly-S-Nitrosylation

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Several ion channels are reportedly redox responsive, but the molecular basis for the changes in activity is not known. The mechanism of nitric oxide action on the cardiac calcium release channel (ryanodine receptor) (CRC) in canines was explored. This tetrameric channel contains ~84 free thiols and is S-nitrosylated in vivo. S-Nitrosylation of up to 12 sites (3 per CRC subunit) led to progressive channel activation that was reversed by denitrosylation. In contrast, oxidation of 20 to 24 thiols per CRC (5 or 6 per subunit) had no effect on channel function. Oxidation of additional thiols (or of another class of thiols) produced irreversible activation. The CRC thus appears to be regulated by poly-S-nitrosylation (multiple covalent attachments), whereas oxidation can lead to loss of control. These results reveal that ion channels can differentiate nitrosative from oxidative signals and indicate that the CRC is regulated by posttranslational chemical modification(s) of sulfurs.

Mammalian tissues express three major isoforms of nitric oxide synthase (NOS) (*1*). All three NOSs have been identified in cardiac or skeletal muscle in close association with the sarcolemma and have been implicated in the regulation of force production (*1–3*). Cytosolic Ca^{2+}, the primary determinant of force, is released from the sarcoplasmic reticulum (SR) by a ryanodine-sensitive CRC in response to a muscle action potential. In cardiac muscle, the CRC is opened by Ca^{2+} ions that enter the myocyte through a voltage-sensitive dihydropyridine receptor or L-type Ca^{2+} channel in a process known as Ca^{2+}-induced Ca^{2+} release (*4*). Ca^{2+} ions can also increase the activity of NOS 1 and NOS 3 (*1*, *3*). Nitric oxide (NO) then exerts its effects by covalently modifying or oxidizing critical thiols or transition metals in proteins (*5*).

NO may be a physiological modulator of excitation-contraction (E-C) coupling. It is produced at the sarcolemma, it cycles in the beating heart on millisecond time scales, and it modulates contractility (*3*). Both the L-type Ca^{2+} channel (*6*, *7*) and CRC (*8*) are potential targets of NO or related molecules because they contain sulfhydryls whose oxidation modulates their function and influences E-C coupling (*9*). Indeed, intramolecular disulfide formation is thought to be the molecular correlate of NO-mediated changes in channel activity (*7*, *10*). In this model, channels cannot distinguish NO signals from other redox active species—that is, they are thought to sense changes in oxidation state rather than the individual species responsible for such change. Our results indicate that poly-S-nitrosylation reversibly activates the CRC, whereas comparable degrees of thiol oxidation do not. We further identify at least one other redox site whose oxidation irreversibly activates the release channel and could impair muscle function.

CRC purified from canine hearts contained *S*-nitrosothiol (SNO) groups (74 ± 35 pmol of SNO per milligram of CRC; $n = 6$) if dithiothreitol (DTT) was eliminated from the standard buffers used in purification (*11*, *12*), whereas inclusion of DTT resulted in almost complete loss of SNO (0.5 pmol of SNO per milligram of CRC; $n = 2$). In other words, the CRC is endogenously S-nitrosylated and this posttranslational modification is reversible. Low-mass SNOs are one class of endogenous compounds capable of protein S-nitrosylation (*13*). To examine their effects on single cardiac CRCs, we incorporated proteoliposomes containing purified channels into planar lipid bilayers (*11*, *14*). Channel activity can be reliably monitored in a medium containing monovalent cations because the cardiac CRC does not conduct

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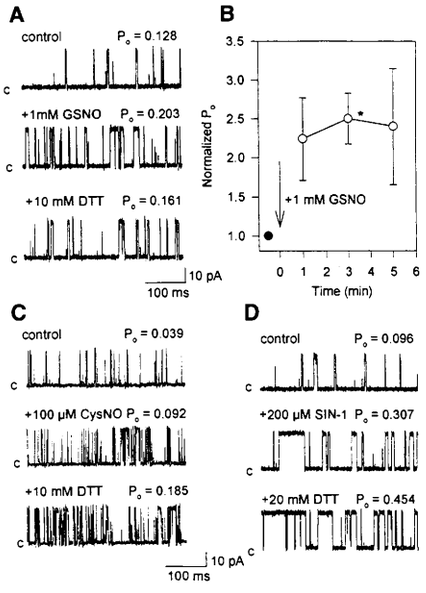

Fig. 1. Effects of GSNO (**A** and **B**), CysNO (**C**), and SIN-1 (**D**) on cardiac CRC activity. (A) Effect of GSNO. Proteoliposomes containing the purified CRC were fused with a planar lipid bilayer. Single channel currents, shown as upward deflections from the closed levels (c on left), were recorded in symmetric medium of 0.25 M KCl and 20 mM potassium Hepes (pH 7.4) containing 2 μM free Ca^{2+} before (top trace) and 1 min after successive addition of 1 mM GSNO (middle trace) and 10 mM DTT (bottom trace) to the cis (SR cytosolic) side of the bilayer chamber. Holding potential was +35 mV. (B) Time course of GSNO effects. P_o values were obtained from recordings as in (A) from 1-min data files before (solid circle) and after (open circles) addition of 1 mM cytosolic GSNO and are means ± SE of seven experiments. *Significantly different ($P < 0.05$) from P_o before addition of GSNO. (C) Effect of CysNO. A single CRC was recorded as in (A) before (top trace) and after successive addition of 100 μM CysNO (middle trace) and 10 mM DTT (bottom trace) to the cis chamber. (D) Effect of SIN-1. A single CRC was recorded as in (A) before and after successive addition of 200 μM SIN-1 (middle trace) and 20 mM DTT (bottom trace) to the cis chamber.

Table 1. Cardiac CRC: Channel activation and S-nitrosylation. Data are means ± SE of the number of experiments indicated in parentheses. The control P_o (without sulfhydryl reactive compound) was 0.14 ± 0.02 (n = 69).

Compound	mM	P_o (% of control)		SNO/CRC subunit (mol/mol)	
		−DTT	+DTT	−DTT	+DTT
GSNO	0.1	108 ± 32 (4)		0.4 ± 0.2 (6)	
	0.5	148 ± 20 (15)*		1.1 ± 0.1 (4)	0.2 ± 0.1 (4)
	1.0	236 ± 37 (7)*	144 ± 29 (7)†	2.7 ± 0.5 (12)	0.7 ± 0.3 (5)
	2.0	223 ± 58 (8)			
CysNO	0.01	116 ± 20 (8)	105 ± 9 (8)	0.3 ± 0.2 (3)	
	0.03	156 ± 20 (6)*	499 ± 211 (6)		
	0.10	339 ± 77 (9)*	527 ± 107 (9)*	1.6 ± 0.3 (15)	0.001 ± 0.002 (4)
SIN-1	0.1	173 ± 15 (3)*		0 ± 0 (3)	
	0.2	220 ± 24 (7)*	262 ± 43 (7)*	0.06 ± 0.06 (3)	
	0.5	173 ± 51 (5)			
	1.0	158 ± 66 (5)			
N-Ethylmaleimide	2.0	273 ± 76 (6)*			

*Significantly different from control (before addition of SNO or SIN-1) ($P < 0.05$ as determined by Student's unpaired t test). †Significantly different ($P < 0.05$) from samples treated with 1 mM GSNO.

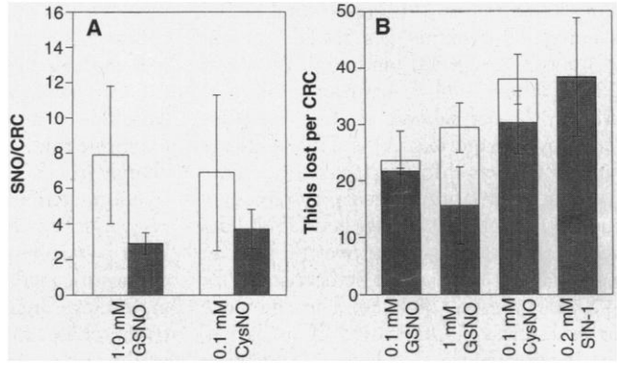

Fig. 2. S-Nitrosylation and oxidation of the CRC. (**A**) Control of S-nitrosylation by endogenous effectors. Proteoliposomes containing the purified cardiac CRC reacted with GSNO or CysNO in the presence of 2 μM or 10 μM free Ca^{2+} (open bars) (which had the same effect) or 5 mM Mg^{2+} (shaded bars) and the extent of S-nitrosylation was determined as described (*12*). Data are means ± SE of three determinations. (**B**) Redox-related modifications by NO donors. Proteoliposomes containing the purified CRC reacted with monobromobimane, which detects free but not S-nitrosylated or oxidized thiols before and after treatment with GSNO, CysNO, or SIN-1 as described in (*18*). The number of thiols lost to S-nitrosylation (open bars) or oxidation (shaded bars) by NO donors is shown. Data are means ± SE of five to eight determinations. Before treatment with the NO donors, the number of free cysteines was 84 ± 4 (n = 13) (21 per CRC subunit), as determined by bimane reactivity.

anions such as Cl^- and it conducts monovalent cations more efficiently than Ca^{2+} (*15*). With K^+ as the current carrier, single channel conductance is 770 pS (*16*). In preliminary experiments, SNOs activated the CRC, so recordings were made in the presence of a submaximally activating concentration of free Ca^{2+} (2 μM) in the cis (SR cytosolic) chamber. Addition of 1 mM S-nitrosoglutathione (GSNO) to the cis chamber resulted in increased CRC activity without an apparent change in single channel conductance (Fig. 1A). Channel open probability (P_o) increased from 0.128 to 0.203 within 1 min after addition of the nitrosothiol. A similar 2- to 2.5-fold activation was observed 1 to 5 min after addition of GSNO in seven separate recordings (Fig. 1B). Addition of 10 mM DTT to the cis chamber returned channel activity close to that of untreated channels (Fig. 1A; Table 1), whereas DTT by itself had no effect (n = 9). DTT could have reversed either S-nitrosylation or thiol oxidation to disulfide. The SH-alkylating reagent *N*-ethylmaleimide activated the channel to a similar extent as GSNO (Table 1), demonstrating that direct covalent modification of thiols (that is, by alkylation or nitrosylation) can affect channel activation.

S-Nitrosylation of the CRC was directly measured (*12*) in proteoliposomes after reaction with GSNO under conditions that led to activation of single channels (*14*) (Fig. 1A). Channel activation required the nitrosylation of many thiols. Modification of ~2 sites per CRC was associated with minimal activation, modification of ~4 sites per CRC was associated with modest (~50%) activation, and modification of ~11 sites per CRC (~3 per subunit) was associated with two- to threefold activation (Table 1). Addition of a concentration of DTT that resulted in loss of most NO groups reversed the activation. Thus, the ryanodine receptor appears to be progressively activated as up to ~3 SH groups per CRC subunit are S-nitrosylated.

The structure of the target protein and nature of the NO donor and milieu determine the efficiency of thiol modification by either nitrosylation or oxidation (*5*, *13*, *17*). In particular, the extent of S-nitrosylation was about twofold greater in the active channel conformation (induced by 10 μM Ca^{2+}) than in the inactive state (induced by 5 mM Mg^{2+}) (Fig. 2A). The specificity of the nitrosylating compound was further illustrated by the effects of S-nitrosocysteine (CysNO), a smaller (less restricted) and more potent NO donor than GSNO. Cytosolic CysNO (0.1 mM) increased P_o from 0.039 to 0.092,

a condition under which 6.4 thiols per CRC (~2 per subunit) were nitrosylated (Table 1). CysNO concentrations as low as 30 μM produced a significant increase in P_o. A lower concentration (10 μM) that did not activate the channel resulted in only one SNO per CRC (Table 1). Addition of 10 mM DTT resulted in NO group removal. However, whereas DTT returned GSNO-activated channel activity close to that of controls (Fig. 1A and Table 1), it did not reverse the effects of CysNO (Fig. 1C and Table 1). These results are best rationalized by CysNO oxidizing channel sites that are not accessible to GSNO or DTT (*13*).

To test the possibility that oxidation of the CRC can lead to irreversible activation, we quantified the number of free thiols on the purified receptor with a lipophilic fluorescent monobromobimane before and after exposure to GSNO and CysNO (*18*). The purified, tetrameric CRC has a total of 364 cysteines [89 cysteines per 560-kD subunit (*19*) and 2 per FK506 binding protein 12.6 (*20*)] of which ~84 ± 4 (mean ± SE; n = 13) or ~21 per subunit are free, as determined by bimane reactivity. This is a large number of free thiols; typically, no more than one or two intramolecular cysteines are reactive in proteins. CysNO and GSNO decreased the number of reactive thiols to an extent greater than could be accounted for by S-nitrosylation (Fig. 2B). The difference (or excess loss) is indicative of oxidation. The inverse relationship between oxidation and nitrosylation by GSNO is consistent with a report that NO prevents disulfide-mediated activation of the ryanodine receptor (*21*)—that is, S-nitrosylation may block formation of some disulfides. GSNO oxidized 5.5 thiols per CRC subunit and CysNO oxidized 7.3 thiols per CRC (two extra thiols per subunit) (Fig. 2B). SNOs are not known to oxidize thiols beyond disulfide (*7, 13, 17*) (although we cannot definitively exclude radical-based higher oxidation of sulfur mediated by CysNO homolysis). Thus, CysNO led to apparent formation of one more disulfide per CRC subunit.

We also tested the effects of 3-morpholinosydnonimine (SIN-1), which generates the NO-related species peroxynitrite ($OONO^-$) (*17*). This molecule shares with SNOs a reactive predilection for thiols, but it is a stronger oxidant (*17*). Like CysNO, SIN-1 produced severalfold activation of the channel, which could not be reversed by reducing conditions (Fig. 1D and Table 1) and caused the (same) loss of ~10 thiols per CRC subunit (Fig. 2B). However, the functional effects of SIN-1 were associated with thiol oxidation in the absence of S-nitrosylation (Fig. 2B). Taken together, our results suggest that (i) oxidation of 5.5 thiols per CRC subunit (presumably forming two or three disulfides) occurs readily but without affecting CRC function (0.1 mM GSNO in Fig. 2B; Table 1); and (ii) oxidation of seven or more thiols per subunit (or more than three disulfides) is associated with irreversible activation (SIN-1 and CysNO in Fig. 2B and Table 1). Thus, apparent formation of one extra disulfide per subunit may be responsible for the irreversibility. Alternatively, oxidation of a distinct class of thiols exhibiting differential reactivity toward these compounds could account for the results. Although we are entirely open to the possibility that formation of sulfinic or sulfonic acids contributes to irreversible channel activation, it is not unusual to find intramolecular disulfides resistant to DTT reduction, particularly when conformational changes are produced in proteins (*22*).

Intramolecular disulfide formation is typically favored as the mechanism of redox regulation in proteins in general and in ion channels in particular (*7, 10, 23*). However, it is difficult to reconcile NO activation of the CRC with oxidation or to argue for a "disulfide switch" in the regulation. Quite extensive thiol oxidation occurred without a change in CRC function, and oxidative activation, attributed to loss of two extra thiols, was irreversible, making it more likely to be of pathophysiological than of physiological significance. Endogenous CRC effector molecules including Mg^{2+}, adenosine triphosphate, and calmodulin (*15*) may naturally protect from oxidation by changing protein conformation; the structure dictates the geometry and proximity of dithiols and thus their propensity for forming disulfides. On the other hand, reversible dose-dependent activation is well explained by multiple covalent modifications of the CRC. Specifically, the extent of S-nitrosylation (poly-S-nitrosylation) correlated with the degree of activation; S-alkylation likewise activated the CRC; and denitrosylation reversed the activity. This modus operandi includes the possibility that low-level CRC oxidation facilitates activation by S-nitrosylation; indeed, oxidation and nitrosylation occurred simultaneously (Fig. 2B). Other covalent modifications or reversible chemical additions to sulfurs, such as S-thiolation by reactive disulfides and sulfenic acid derivatization by peroxides, may have comparable effects on the CRC (*24*).

Our findings further illustrate that thiols in proteins can recognize both nitrosative and oxidative events and, moreover, can distinguish between them. Thus, either particular thiols in the CRC (monothiols versus dithiols) subserve different sensory and regulatory functions, or particular chemical modifications of thiols (nitrosylation versus oxidation) elicit distinct functional changes, or both. Recent identification of polynitrosylated proteins in vivo (*25*) strengthens the case for such chemical modifications having physiological relevance. The use of multiple covalent attachments as a means to modulate protein function is reminiscent of regulation by phosphorylation.

The CRC contains a large number of thiols and is thus poised for regulation by redox events. We offer the following model. In the resting state, cardiac muscle may produce small amounts of (S)NO that are sufficient to down-regulate the L-type Ca^{2+} channel by a guanosine 3′,5′-monophosphate (cGMP)-dependent mechanism (possibly a tonic effect) (*3, 7, 26*). The CRC is maintained in a low activity state by low [Ca^{2+}] and effectors such as Mg^{2+}, which prevent S-nitrosylation. With muscle activation, the influx of Ca^{2+} activates the CRC and NOS. (S)NO production overrides cGMP inhibition of the L-type Ca^{2+} channel (*7*) and leads to concerted activation of the CRC (now in a permissive conformation) by poly-S-nitrosylation. Auxiliary chemical additions at sulfurs, mediated by reactive oxygen or nitrogen species, may also affect CRC activity (*24*). The subsequent decrease in Ca^{2+} will inactivate NOS and switch the channel conformation to favor denitrosylation (*1, 3*). The allosteric mechanism would resemble that in hemoglobin, where S-nitrosylation occurs in the R structure and denitrosylation occurs in the T structure (*27*). By contrast, oxidative or nitrosative stress may have deleterious consequences because of excessive modification of the receptor. Indeed, some thiols may serve to buffer responses and protect from excessive oxidation. We suggest that NO and related molecules may regulate E-C coupling through discrete mechanisms. On the one hand, they can inhibit the L-type channel via cGMP; on the other hand, they sensitize the muscle to Ca^{2+}-induced Ca^{2+} release by chemical modifications of thiols.

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11. Canine cardiac SR vesicle fractions enriched in CRC activities were prepared in the presence of protease inhibitors [L. Xu, G. Mann, G. Meissner, *Circ. Res.* **79**, 1100 (1996)], The CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}-solubilized canine heart 30 S CRC complex was isolated by rate-density centrifugation. Gradient fractions were either concentrated by Centricon 30 (Amicon, Danvers, MA) centrifugation (to determine endogenous SNO levels) or reconstituted into proteoliposomes by removal of CHAPS by dialysis as described [H. B. Lee, L. Xu, G. Meissner, *J. Biol. Chem.* **269**, 13305 (1994)], except that the 20-hour dialysis buffer contained 10 mM potassium Pipes (pH 7.4), 0.25 M KCl, 10 μM EGTA, 20 μM Ca^{2+}, and 0.1 mM phenylmethylsulfonyl fluoride, and DTT was excluded from the buffers in some experiments. Protein concentrations were determined by using Amido Black [R. A. Kaplan and P. L. Pederson, *Anal. Biochem.* **150**, 97 (1985)].
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Functional Expression of a Mammalian Odorant Receptor

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Candidate mammalian odorant receptors were first cloned some 6 years ago. The physiological function of these receptors in initiating transduction in olfactory receptor neurons remains to be established. Here, a recombinant adenovirus was used to drive expression of a particular receptor gene in an increased number of sensory neurons in the rat olfactory epithelium. Electrophysiological recording showed that increased expression of a single gene led to greater sensitivity to a small subset of odorants.

Olfactory transduction begins with the binding of an odorant ligand to a protein receptor on the olfactory neuron cell surface, initiating a cascade of enzymatic reactions that results in the production of a second messenger and the eventual depolarization of the cell membrane (*1*). This relatively straightforward and common signaling motif is complicated by the existence of several thousand odorants, mostly low-molecular-weight organic molecules, and nearly a thousand different putative receptors (*2, 3*). The receptors are believed to be members of the superfamily of G protein–coupled receptors (GPCRs) that recognize diverse ligands, including the biogenic amine neurotransmitters. Although the putative odorant receptors constitute the largest subfamily of GPCRs, in some ways they remain the most enigmatic, because no particular mammalian receptor has been definitively paired with any ligand. Functional expression of cloned odorant receptors would allow the characterization of the chemical receptive fields that provide the basis for coding and organization in the olfactory system.

A functional expression system for odorant receptors requires both that the receptors are properly targeted to the plasma membrane, and that they couple efficiently with a second messenger system that produces a measurable response to ligand stimulation. On the simple assumption that olfactory neurons themselves would be the most capable cells for expressing, targeting, and coupling odorant receptors, we have endeavored to use the rat nasal epithelium as an expression system, driving the expression of a particular receptor by including it in a recombinant adenovirus and infecting rat nasal epithelia in vivo. Here, we relied on the large number of putative odorant receptors, and their approximately equal expression among the 6 million neurons of the rat olfactory epithelium, to identify the average increase in response in an epithelium in which one of these receptors is overexpressed (*4*). This can be measured extracel-

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