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Nitric Oxide Activates or Inhibits Skeletal Muscle Ryanodine Receptors Depending on Its Concentration, Membrane Potential and Ligand Binding

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Abstract. We show that rabbit skeletal RyR channels in lipid bilayers can be activated or inhibited by NO, in a manner that depends on donor concentration, membrane potential and the presence of channel agonists. 10 μM *S*-nitroso-N-acetyl-penicillamine (SNAP) increased RyR activity at −40 mV within 15 sec of addition to the *cis* chamber, with a 2-fold increase in frequency of channel opening (F_o). 10 μM SNAP did not alter activity at +40 mV and did not further activate RyRs previously activated by 2 mM *cis* ATP at +40 or −40 mV. In contrast to the increase in F_o with 10 μM SNAP, 1 mM SNAP caused a 2-fold reduction in F_o but a 1.5-fold increase in mean open time (T_o) at −40 mV in the absence of ATP. 1 mM SNAP or 0.5 mM sodium nitroprusside (SNP) induced ~3-fold reductions in F_o and T_o at +40 or −40 mV when channels were activated by 2 mM *cis* ATP or in channels activated by 6.5 μM peptide A at −40 mV (peptide A corresponds to part of the II–III loop of the skeletal dihydropyridine receptor). Both SNAP-induced activation and SNAP/SNP-induced inhibition were reversed by 2 mM dithiothreitol. The results suggest that *S*-Nitrosylation or oxidation of at least three classes of protein thiols by NO each produced characteristic changes in RyR activity. We propose that, in vivo, initial release of NO activates RyRs, but stronger release increases [NO] and inhibits RyR activity and contraction.

Key words: Ryanodine receptors — Nitric oxide — Regulatory thiols — Oxidation — Skeletal Muscle — Calcium channels

Introduction

The ubiquitous molecular species nitric oxide (NO) has many functions including modulation of muscle contraction, signaling in endothelial and nerve cells and immunological mechanisms. One of the first physiological roles described for NO was relaxation of vascular smooth muscle (Moncada & Higgs, 1993) and it has since been found to induce relaxation in many visceral smooth muscles. All 3 endogenous NO synthase (NOS) enzymes, which produce NO from L-arginine, have also been found in skeletal muscle, with neuronal NOS (nNOS) expressed in concentrations in excess of those in brain (Kobzik et al., 1994). The high concentrations of NOS suggest a role for NO in skeletal muscle function and, indeed, NO inhibits skeletal muscle contraction (Kobzik et al., 1994; El Dwairi et al., 1998) and actomyosin ATPase activity (Perkins, Han & Sieck, 1997). However, activation of cGMP-dependent pathways account for only a small part of the NO-evoked tension depression in skeletal muscle (Kobzik et al., 1994; Reid, 1998). It is likely that NO elicits a major effect on contraction of skeletal muscle by *S*-Nitrosylation or oxidation of sulfhydryl (−SH) groups on proteins that regulate excitation–contraction coupling (ECC) and cytoplasmic [Ca^{2+}] in skeletal muscle (Reid, 1998).

ECC in skeletal muscle leads to Ca^{2+} release from SR through ryanodine receptor (RyR) calcium release channels which form the junctional foot between the T-tubule and SR membranes (Franzini-Armstrong, 1970). The RyR is a homotetramer and each subunit has a molecular mass of 560 kD (Otsu et al., 1990). The cytoplasmic domain of the RyR interacts with the surface membrane dihydropyridine receptor (DHPR) L-type Ca^{2+} channels. Ca^{2+} release occurs in response to depolarization of the plasma membrane, which is detected by a voltage sensor in the DHPR. Upon depolarization, DHPRs undergo a conformational change—associated with membrane-bound charge movements—which is transmitted to the RyR via the cytoplasmic loop between domains II and III in the DHPR (Tanabe et al., 1990).

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The RyR channel opens in response to the signal transmission and cytoplasmic [Ca^{2+}] increases. A 20 amino acid peptide (peptide A), corresponding to the N-terminal part of the II–III loop, activates Ca^{2+} release from the SR and activates RyR channels in bilayers (El-Hayek et al., 1995; Dulhunty et al., 1999; Gurrola et al., 1999). The peptide A region is thought to facilitate DHPR II–III loop binding to the RyR. Agents that interact with the RyR, or proteins that modulate RyR channel gating, can effect Ca^{2+} release and contraction.

SH reagents modulate current flow through RyR channels by covalent modification associated with oxidation of cysteine residues (Homlberg et al., 1991; Boraso & Williams, 1994; Favero, Zable & Abramson, 1995; Eager, Roden & Dulhunty, 1997). Small nitrosothiol (SNO) molecules increase cardiac RyR activity by *S*-Nitrosylation of several cysteine residues on each monomer of the channel protein and by oxidation of several other cysteine residues to form disulfide bridges within the RyR protein complex (Xu et al., 1998). NO may *S*-Nitrosylate or oxidize the same cysteine residues that are modified by other SH reagents, or they may modify separate residues. There have been conflicting reports that NO-donors either activate or inhibit RyRs from both skeletal and cardiac muscle (Mészáros, Minarovic & Zahradníková, 1996; Stoyanovsky et al., 1997; Zahradníková et al., 1997; Xu, Meissner & Stamler, 1998). The different results obtained in different laboratories may depend on the concentration of NO donors used and/or the potential at which activity is recorded. The effects of NO may also depend on interactions between NO and other modulators of RyR activity.

The aim of the present study was to examine the voltage-dependence, ATP-dependence and peptide A-dependence of NO-donors added at low (10 μM) or high (1 mM) concentrations to the cytoplasmic side of the RyR channel. Novel observations are that NO can either activate or inhibit skeletal RyRs, depending on concentration, membrane potential and the presence or absence of ATP or peptide A. Low concentrations of *S*-nitroso-N-acetyl-penicillamine (SNAP) activated RyRs at −40 mV in the absence of ATP. Higher concentrations of SNAP or sodium nitroprusside (SNP) inhibited RyRs in a potential-independent manner, with strongest inhibition seen in the presence of the physiological agonists, ATP and peptide A. The rapid reversal of all effects of the NO donors by 2 mM dithiothreitol (DTT) indicates that NO acted by *S*-Nitrosylation or oxidation of protein thiol groups. The results suggest (i) that *S*-Nitrosylation or oxidation of RyRs by NO may strongly influence RyR channel gating under physiological conditions and (ii) that this modulation of RyR activity could contribute to the in vivo modulation of contraction by NO under normal or pathological conditions.

Materials and Methods

Preparation of SR Vesicles

Preparation of SR vesicles was based on Saito et al. (1984) and has been described in Laver et al. (1995). Rabbit back and hindlimb muscle was dissected, minced and centrifuged to yield a crude microsomal fraction that was then run on a sucrose gradient. Heavy SR vesicles were collected and the crude fraction and heavy SR were stored at −70°C.

Lipid Bilayers and Voltage Clamp

Lipid bilayers were formed from palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2 by weight, Avanti Polar Lipids) in *n*-decane, painted across an aperture, with a diameter of 180 μM, in a Delrin cup. Bilayer potential was controlled using an Axopatch 200-A amplifier (Axon Instruments, Foster City, CA) and is given relative to the *cis* chamber. Channel activity was recorded with bilayer potential voltage clamped at either +40 or −40 mV. Potentials were changed every ~30 sec.

Solutions for Single Channel Recording

The normal *cis* solution contained (in mM): 230 cesium methane sulfonate ($CsCH_3SO_3$), 20 CsCl, 10 *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and 1 $CaCl_2$ and was adjusted to a pH of 7.4 with CsOH. The *trans* solution contained (in mM): 30 $CsCH_3SO_3$, 20 CsCl, 0.1 $CaCl_2$ and 10 TES and was adjusted to a pH of 7.4 with CsOH. 250/50 mM Cs^+ *cis/trans* provided an osmotic gradient to facilitate incorporation of SR vesicles (Miller & Racker, 1976). SR vesicles were added to the *cis* chamber to a final concentration of 2–10 μg/ml. Following vesicle incorporation (and observation of channel activity), the *cis* chamber was perfused to remove extra vesicles and thus prevent multiple RyR incorporation. Cs^+ was used as the conducting ion because it has a high conductance through RyRs and a low conductance through SR K^+ channels. The cytoplasmic side of the SR membrane faced the *cis* solution after fusion with the bilayer (Kourie, Foster & Dulhunty, 1997). *Trans* Cs^+ was increased to 250 mM, by addition of 200 mM $CsCH_3SO_3$, for channel recording under symmetrical conditions. Experiments were carried out at room temperature (20–25°C).

Recording and Analysis of Single Channel Activity

Single channel currents were displayed on an oscilloscope, recorded on videocassette and stored on optical disc. Current was recorded at 1 kHz (−3dB, 4-pole Bessel) and digitized at 2 kHz with a TL-1 DMA interface (Labmaster, Axon Instruments). Mean current (I', the average of all data points during a period of recording), open probability (P_o), open time (T_o) and open frequency (F_o) were determined from 120 sec records using an in-house channel analysis program, Channel 2 (developed by P.W. Gage and M. Smith). The threshold discriminator for channel opening was set just above the baseline noise, rather than at 50% of the maximum current, to include openings to submaximal conductance levels at <50% of the maximum conductance. “Average

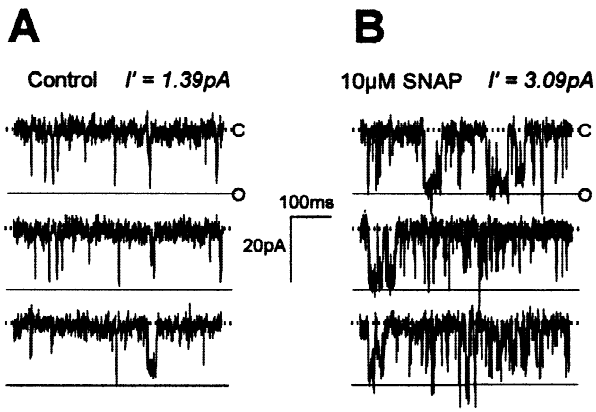

Fig. 1. Activation of a single skeletal muscle RyR by 10 μM SNAP. The *cis* Ca^{2+} concentration for data in this and subsequent figures was 1 mM. The records show single channel recording from one RyR at −40 mV under control conditions (*A*) and following application of 10 μM SNAP. (*B*). Openings of the channel are downward current steps to the maximum open channel conductance (continuous line labeled O) from the closed current level, shown by the dashed line (*C*). The records show 1500 msec of continuous activity under control conditions and during exposure to SNAP. Mean current (in pA) for the 1500 msec of activity is shown above each set of recordings.

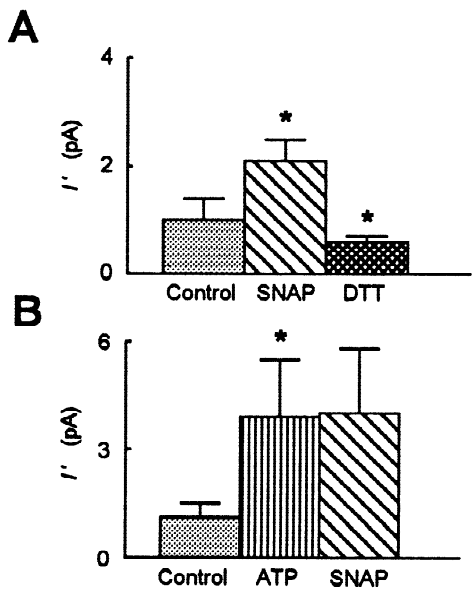

Fig. 2. The increase in mean current through RyRs induced by 10 μM *cis* SNAP at −40 mV is reversed by 2 mM *cis* DTT and is not seen in the presence of 2 mM *cis* ATP. Average data is shown for the effect of 10 μM SNAP on mean current (I') for six experiments at −40 mV. (*A*) 10 μM SNAP caused an increase in average mean current (cross-hatched bin) from the control level (stippled bin) and then DTT reversed the increase in mean current (double cross-hatched bin). (*B*) 2 mM ATP induced an increase in the average mean current (vertically striped bar) and the subsequent addition of SNAP had no additional effect (cross-hatched bar). Asterisks, in this and subsequent figures, indicate that the average data was significantly different from the preceding data set ($P < 0.05$). The significant SNAP-induced increase in the average mean current was reversed by DTT (*A*). ATP also significantly increased the average mean current (*B*). The vertical capped bars show ±1 SEM.

mean current" is the sum of mean current values in *n* channels divided by *n*.

Statistics

The Student's two-tailed paired *t*-test was used to test for significance of difference between data. Values are expressed as means ± 1 SEM. Differences were considered significant when $P < 0.05$.

Drugs

All Solutions were prepared using MilliQ deionized water. SNAP (Sigma) was dissolved in ethanol (EtOH). Stock solutions of SNAP were made at concentrations of 1 mM or 100 mM. A 1/100 dilution of these stock solutions was made to give final bath concentrations of 10 μM and 1 mM SNAP, respectively. The final bath concentration of EtOH was 1% in each instance. ATP (Sigma), dithiothreitol (DTT) (Sigma) and sodium nitroprusside (SNP) (Sigma) were dissolved in deionized water.

Results

10 μM *cis* SNAP Activates RyRs at −40 mV

Control RyR activity at −40 mV, with 100 μM *cis* Ca^{2+}, consisted of brief openings to the maximum conductance and to submaximal conductance levels (Fig. 1*A*). Activity at −40 mV increased when 10 μM SNAP was added to the *cis* chamber, with a noticeable increase in the number of brief channel openings and a second channel sometimes became active in the bilayer (Fig. 1*B*). There was no change in the maximum single channel conductance at −40 mV, which was 421 ± 22 pS before, and 429 ± 33 pS after, activation by 10 μM SNAP (Fig. 1). The mean current I' through RyRs increased in 6 of 6 experiments within 11 ± 5 sec of adding 10 μM SNAP, with an average increase in I' from 1.0 ± 0.4 pA to 2.1 ± 0.4 pA (Fig. 2*A*). The average I' of all channels under control conditions at −40 mV was 1.1 ± 0.2 pA ($n = 40$).

The possibility that 10 μM SNAP increased RyR activity by *S*-Nitrosylation or oxidation of regulatory thiols associated with the channel protein was examined by adding the reducing agent, DTT, to SNAP-activated channels. DTT is capable of denitrosylating nitrosothiol groups as well as reducing disulfide bridges (Xu et al., 1998). The activation with 10 μM SNAP was reversed by 2 mM DTT, with average I' in the 6 experiments falling to 0.6 ± 0.1 pA within 14 ± 4 sec of application. 2 mM DTT added alone to the *cis* chamber does not activate skeletal (Haarmann, Fink & Dulhunty, 1999) or

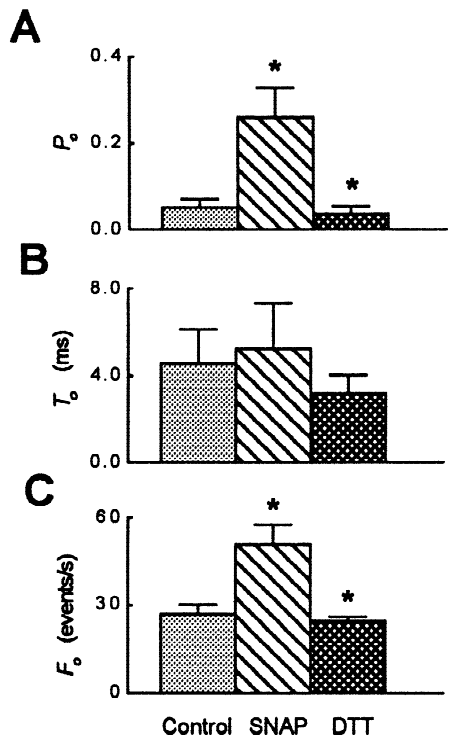

Fig. 3. 10 μM SNAP reversibly increased open probability of RyRs at −40 mV, by increasing the average frequency of opening, but not the average mean open time of 5 single RyR channels. (*A*) Average open probability (P_o). (*B*) Average mean open time, (T_o). (*C*) Average open frequency, (F_o) at −40 mV. SNAP (cross-hatched bars) increased P_o (*A*) and F_o (*C*) compared with control (stippled bars), but had no significant effect on the average T_o (*B*). DTT reversed the increases in P_o and F_o (double cross-hatched bars). The average P_o and F_o were significantly increased by SNAP and then significantly reduced by DTT (*A, C*). The vertical capped bars show ±1 SEM.

cardiac RyRs (Eager et al., 1997). Since SNAP has no oxidative capacity on its own, the result suggests that the NO released by 10 μM SNAP increased activity by *S*-Nitrosylation or oxidation of −SH groups on the RyR protein or on associated regulatory proteins.

In marked contrast to the increase in activity at −40 mV, RyR activity at +40 mV was unchanged after addition of 10 μM *cis* SNAP. I' for all channels at +40 mV under control conditions was 1.5 ± 0.2 pA ($n = 54$) and similar to the 1.1 pA at −40 mV (above). Average control I' in 13 bilayers at +40 mV was 0.9 ± 0.2 pA and 1.2 ± 0.3 pA after addition of 10 μM SNAP.

10 μM *cis* SNAP Does Not Activate RyRs at −40 mV if 2 mM ATP is Present in the *cis* Chamber

SNAP-induced activation was examined in the presence of *cis* ATP. I' at −40 mV increased from 1.3 ± 0.4 pA to 4.3 ± 0.9 pA ($n = 14$) with 2 mM ATP. Application of 10 μM *cis* SNAP to the ATP-activated channels did not cause any additional activation, and average I' (between 100 sec and 220 sec after SNAP addition) was 4.0 ± 1.8 pA ($n = 4$) (Fig. 2*B*). As at −40 mV, addition of ATP increased activity at +40 mV in 6 out of 6 bilayers, but none of the channels were further activated by 10 μM SNAP. Average I' at +40 mV was 1.1 ± 0.4 pA under control conditions, 4.4 ± 0.6 pA in the presence of ATP, and 4.2 ± 0.5 pA after adding the NO donor.

10 μM SNAP Activates RyRs at −40 mV by Increasing the Frequency of Channel Opening

The effect of 10 μM SNAP on the single channel parameters of RyRs at −40 mV, in the absence of ATP, was examined in 5 experiments in which one channel only was active in the bilayer. P_o and F_o increased in all 5 channels, but T_o increased in only 1 channel. Average P_o and F_o increased significantly (~5-fold or ~3-fold, respectively) with 10 μM SNAP, but average T_o did not change (Fig. 3). The increase in both P_o and F_o was reversed by DTT. Both P_o and F_o fell to control levels after addition of 2 mM *cis* DTT in each of the 5 channels and average P_o and F_o were significantly reduced to levels not significantly different from control (Fig. 3). The reversal of changes in single channel parameters by DTT provides further evidence that activation with 10 μM SNAP was due to *S*-Nitrosylation or oxidation of cysteine residues associated with RyRs, by NO released from the donor.

Weak Inhibition of RyR Activity by 1 mM SNAP in the Absence of ATP

SNAP added to the *cis* chamber at 1 mM did not increase RyR activity at −40 or +40 mV. Instead, I' at −40 mV fell in 5 out of 6 channels, with average I' being 1.8 ± 0.6 pA under control conditions and 0.9 ± 0.2 pA after adding 1 mM SNAP (Fig. 4*A*). P_o and F_o fell in 5 of the 6 channels, while T_o surprisingly increased in all 6 channels. Thus there was no significant effect on average I' and P_o, because the average ~1.5-fold increase in T_o was counterbalanced by an average ~2-fold decrease in F_o (Fig. 4). In contrast to −40 mV, there was no consistent change in any parameters of channel activity at +40 mV in the 6 channels exposed to 1 mM SNAP and average I' was 0.8 ± 0.5 pA under control conditions or 0.8 ± 0.2 pA after adding 1 mM SNAP. The significant increase in T_o at −40 mV suggests that *S*-Nitrosylation or oxidation of a second class of lower affinity activating thiol groups with 1 mM SNAP led to an increase in T_o. On the other hand, the reduction in F_o (as well as the fall in I' and P_o in most channels) with 1 mM SNAP, in contrast to the increase in F_o with 10 μM SNAP, suggests that the higher concentration of SNAP exerted an additional inhibitory action on the channels—possibly by modification of a separate class of inhibitory thiol groups.

Strong Inhibition of RyR Channels by 1 mM SNAP in the Presence of ATP

The inhibitory effect of 1 mM *cis* SNAP was substantially greater when the NO donor was added in the presence of 2 mM *cis* ATP, and was independent of bilayer potential (inhibition was seen +40 and −40 mV). Application of ATP produced the expected increase in RyR activity, with an increase in channel open dwell time (Fig. 5). When 1 mM SNAP was added to the ATP-activated RyRs, channel openings became brief and infrequent within 10–18 sec of application and activity was reduced to less than that recorded before ATP addition (Fig. 5). A fall in activity at +40 mV was seen in 7 of 7 bilayers with a significant average decrease in I' from 3.7 ± 0.3 pA in ATP-activated channels to 1.0 ± 0.3 pA within 15 ± 3 sec of adding 1 mM SNAP (Fig. 6*A*). Similarly, I' at −40 mV fell significantly from an ATP-activated value of 3.2 ± 1 pA to 1.0 ± 0.4 pA after adding 1 mM SNAP ($n = 6$, Fig. 6*B*). One channel did not open at −40 mV and was not included in the average data at that potential.

1 mM SNAP-induced inhibition was reversed by 2 mM *cis* DTT in 6 of 7 channels. Average I' at +40 or −40 mV increased from 1.0 ± 0.3 pA or 1.0 ± 0.4 pA, respectively with SNAP, to 2.6 ± 0.9 pA or 3.8 ± 1.1 pA after adding DTT. I' with DTT was similar to I' with ATP alone (Fig. 6).

Inhibition of ATP-Activated RyRs by 1 mM SNAP is Due to a Reduction in F_o and T_o

Single channel properties were measured in 5 experiments in which bilayers contained only one channel. Addition of ATP rapidly activated RyRs, increasing P_o, T_o and F_o in all 5 channels at +40 and −40 mV and significantly increasing average P_o and T_o by 4- to 5-fold, but without significantly increasing average F_o (Fig. 7). The subsequent addition of 1 mM *cis* SNAP was followed by a fall in P_o, T_o and F_o in all 5 channels with significant 3- to 4-fold reductions in the average parameters. The magnitude of the fall in T_o with 1 mM SNAP was less than the reduction in the other parameters and some channel openings with SNAP plus ATP remained longer than openings under control conditions before activation by ATP.

The effect of 1 mM SNAP on ATP-activated channels was reversed by DTT. P_o increased in all 5 channels following addition of 2 mM DTT, due to increases in T_o and F_o to levels that were not significantly different from those seen with ATP alone (Fig. 7). Therefore the inhibitory action of 1 mM SNAP was due to *S*-Nitrosylation or oxidation of cysteine residues which must have differed from the residues modified by NO to activate the channels, since the membrane potential-dependence and ATP-dependence of activation and inhibition differed.

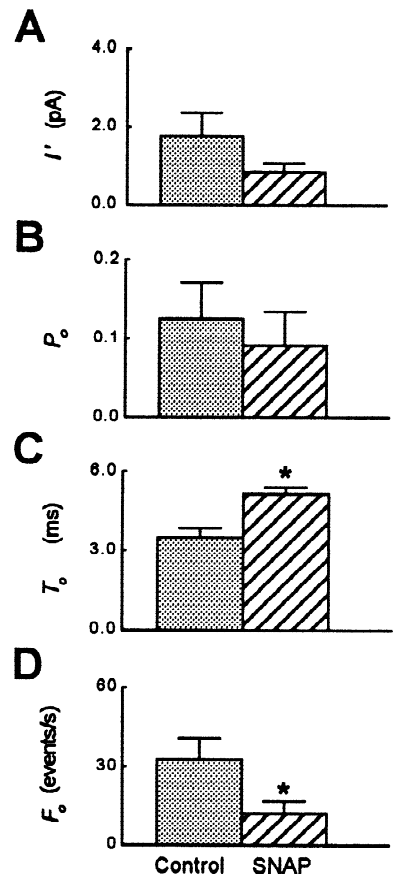

Fig. 4. Addition of 1 mM SNAP increased the mean open time, but decreased the frequency of opening of RyR channels at −40 mV in the absence of ATP. The effect of 1 mM SNAP on average I' (*A*), P_o (*B*), T_o (*C*), and F_o (*D*) of RyRs is shown at −40 mV. In *A* 1 mM SNAP had no significant effect on the average I', although I' fell in 5 out of the 6 channels. (*B*) SNAP did not significantly alter average P_o, despite a decrease in P_o in 5 out of 6 channels. The lack of effects of 1 mM SNAP on I' and P_o was due to opposing effects of a significant increase in average T_o (*C*) and a significant decrease in average F_o (*D*). The vertical capped bars show ±1 SEM.

Temporary Reversal of SNAP-induced Inhibition by a Change in Membrane Potential

The inhibition of skeletal RyR activity in the presence of 1 mM SNAP was different from the irreversible loss of cardiac RyR activity induced by reactive disulfides (Eager et al., 1997), since inhibition could be reversed by DTT (Fig. 7) and high activity was temporarily restored when the bilayer potential was returned to +40 mV after a 10–30 sec voltage pulse to −40 mV (Fig. 8). Average I' in 6 channels, at +40 mV (between 100 sec and 220 sec after addition of 1 mM SNAP to *cis* solution containing 2 mM ATP), was 1.7 ± 0.4 pA and increased to 3.2 ± 0.6 pA immediately after a voltage pulse to −40 mV. In-

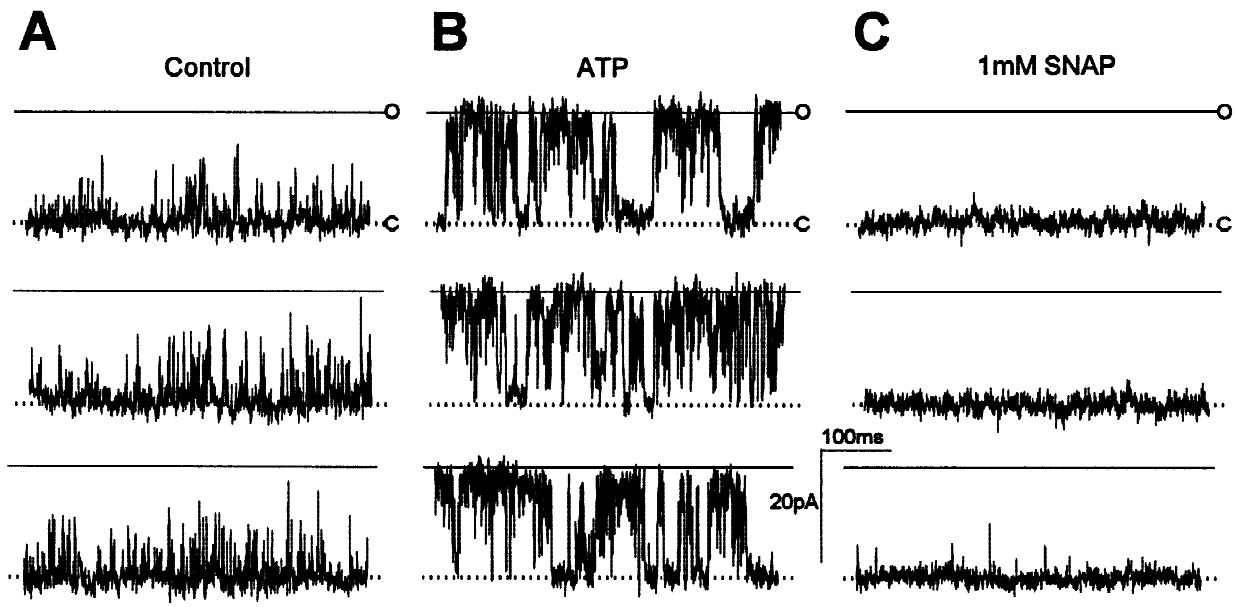

Fig. 5. Addition of 1 mM SNAP reduced the activity of ATP-activated RyR channels. A single channel recording from one RyR under control conditions (*A*) and following cumulative additions of 2 mM ATP (*B*) and then 1 mM SNAP (*C*) at a bilayer potential of +40 mV. Channel openings are upward current deflections from the closed current level (dashed line labeled *C*) to submaximal conductance levels and the maximum single channel conductance (continuous line labeled O). The records show 1500 msec of continuous activity under each condition. ATP elicited a rapid increase in RyR activity and SNAP then suppressed this increased activity within 15 sec of application.

creased activity remained for ~30 sec following the change in potential and then channel activity subsided to the SNAP-inhibited level ($I' = 1.4 \pm 0.4$ pA) (Fig. 8). Similarly, inhibition at −40 mV was temporarily relieved when potential was returned to −40 mV after a period at +40 mV ($n = 6$).

Inhibition of RyR Activity by 0.5 mM SNP

To confirm that the inhibition induced by 1 mM SNAP was due to NO, not to an interaction between SNAP and the RyR, the effect of a chemically unrelated NO donor, SNP, was examined. Following the usual ~5-fold increase in average I' of RyRs with ATP at +40 mV, addition of 0.5 mM SNP significantly reduced I' within 16 ± 5 sec of its addition to 4 bilayers, to an average value that was not significantly different from control (Fig. 9*A*). The 76% reduction in I' with 0.5 mM SNP was similar to the 73% reduction in I' observed with 1 mM SNAP. Significant inhibition of ATP-activated channels by 0.5 mM SNP was also seen at −40 mV ($n = 4$). These results provide further evidence that the SNAP-induced reduction in channel activity was due to an effect of the NO released from the donor.

Inhibition of RyRs by 1 mM SNAP Following Activation by a DHPR II–III Loop Peptide

To determine whether strong inhibition by NO depended specifically on the presence of ATP, channels were activated by a different physiological agonist, peptide A, which corresponds to Thr^{671}-Leu^{690} of the skeletal DHPR (El-Hayek et al., 1995). As expected (Dulhunty et al., 1999), peptide A added to the *cis* chamber at 6.3 μM blocked 5 out of 5 RyR channels at +40 mV, with a significant ~5-fold decrease in average I' (Fig. 9*C*). In contrast, at −40 mV, the peptide caused an increase in activity in all 5 channels with an increase open dwell time (Fig. 9*B*) and a significant ~2-fold increase in I' (Fig. 9*D*). Subsequent addition of 1 mM *cis* SNAP did not alter the peptide A-inhibited activity at +40 mV, but caused an 81 ± 5% reduction in RyR activity at −40 mV. The loss of activity after addition of SNAP to peptide A-activated channels shows that NO-induced inhibition of RyR activity is not specifically dependent on ATP, and proceeds in the presence of the DHPR II–III loop which either bound to the RyR in vivo, or binds during ECC (Dulhunty et al., 1999).

Discussion

Several effects of SNAP on single RyR channel activity were observed and attributed to *S*-Nitrosylation or oxidation of thiol groups by NO, since the effects were reversed by DTT and were reproduced by the chemically unrelated NO donor, SNP. The results show that (i) NO has differential effects on RyR activity depending on its concentration, membrane potential and activating ligands and (ii) at least three classes of functionally active thiols

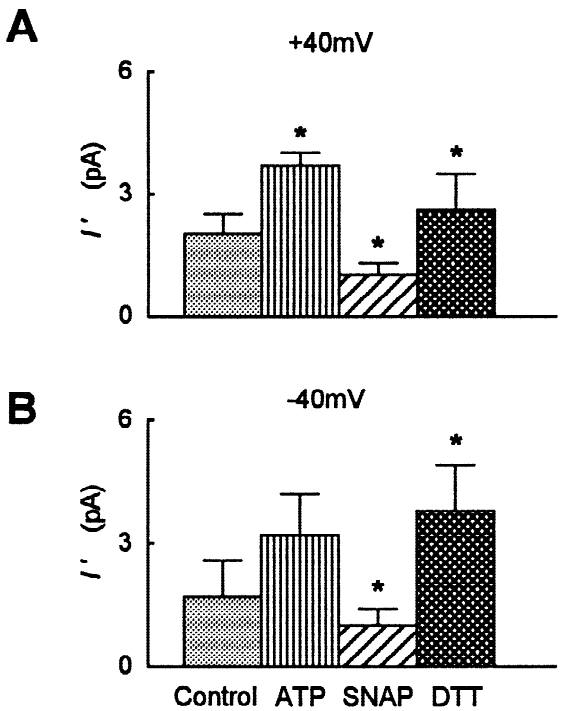

Fig. 6. 1 mM *cis* SNAP caused a voltage-independent reduction in average mean current of ATP-activated RyRs, which was reversed after addition of 2 mM *cis* DTT. Average I' is shown at +40 mV (*A*, $n = 7$) and −40 mV (*B*, $n = 6$). In *A* and *B*, average control I' is shown first (stippled bins), followed by I' after addition of 2 mM ATP (vertically striped bins), then 1 mM SNAP (diagonal bins) and finally 2 mM DTT (double cross-hatched bins). Addition of ATP increased I', the subsequent addition of SNAP then reduced I', and activity was then restored to ATP-activated levels after adding DTT. The increase in I' with ATP was significant at +40 mV. Average I' after addition of SNAP was significantly less than the average ATP-activated I' at both potentials, and average I' after addition of DTT was significantly greater than that in the presence of ATP and SNAP. The vertical capped bars show ±1 SEM.

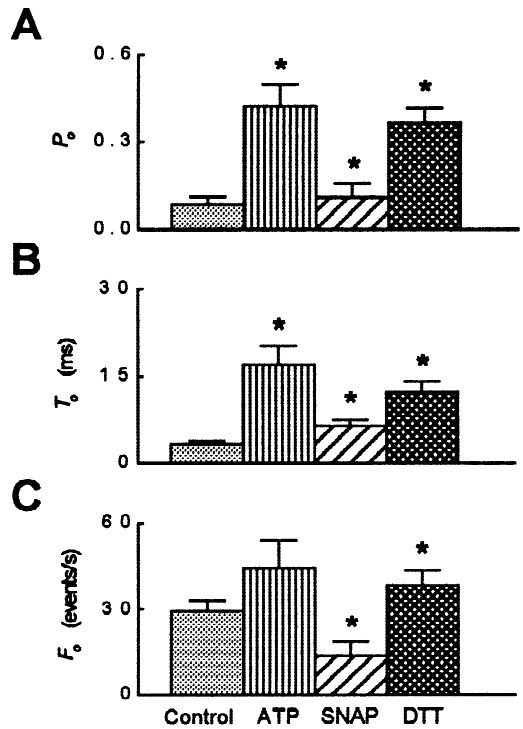

Fig. 7. Reversible reductions in both the duration and frequency of channel opening contribute to the fall in I' and P_o during RyR inhibition after adding 1 mM SNAP to ATP-activated channels. Average data are shown for the effect of 1 mM SNAP on P_o (*A*), T_o (*B*) and F_o (*C*) of 5 single RyRs at +40 mV. ATP (2 mM) caused an increase in average P_o and T_o. F_o was greater with ATP than under control conditions in all 5 channels, although the average increase was not statistically significant using the students *t*-test. SNAP then induced a significant reduction in average P_o, T_o and F_o and DTT finally reversed the SNAP-induced decline with significant increases in P_o, T_o and F_o after its addition. The vertical capped bars show ±1 SEM.

on the RyR, or associated regulatory proteins, are modified by NO.

S-Nitrosylation of Protein Thiols

S-Nitrosylation allows NO to covalently modify proteins and alter their function. An *S*-Nitrosothiol (RSNO) formed with a protein thiol can oxidize a neighboring protein thiol (Eq. 1) or another RSNO (Eq. 2) to form a protein disulfide (R-S-S-R).

$$\mathrm{RSNO + RSH \rightarrow R{-}S{-}S{-}R + NO^{\cdot}} \quad (1)$$

$$\mathrm{RSNO + RSNO \rightarrow R{-}S{-}S{-}R + 2NO^{\cdot}} \quad (2)$$

RSNOs are stable in vivo and in vitro in many protein species (Simon et al., 1996) and in RyRs during purification (Xu et al., 1998). Since DTT both reduces disulfides and denitrosylates RSNO (Xu et al., 1998), it is not clear whether the reactions in our study stopped with the formation of stable RSNOs, or whether oxidation proceeded to R-S-S-R.

The concentration of NO produced by 10 μM SNAP is of the order of 40 nM (Simonsen et al., 1999) and is within measured physiological concentrations of NO (*see* Discussion below). Thus the NO-affinity of activating thiol groups must be several orders of magnitude higher than their affinity for H_2O_2, or reactive disulfides which activate RyRs at 0.1–5 mM (Boraso & Williams, 1994; Eager et al., 1997; Marengo, Hidalgo & Bull, 1998).

Classes of SH Groups that are S-Nitrosylated or Oxidized by NO

At least 3 classes of thiols were modified by NO in the present study. 10 μM SNAP increased F_o at −40 mV in the absence of ATP, suggesting *S*-Nitrosylation or oxi-

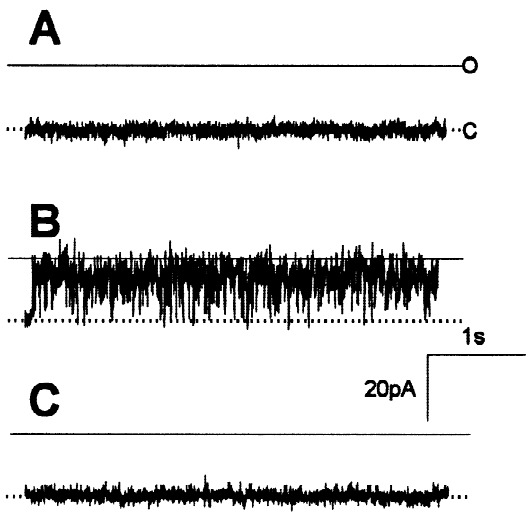

Fig. 8. The inhibition of RyR activity after adding 1 mM SNAP to the *cis* chamber can be temporarily relieved by an 80 mV voltage pulse. Single channel activity recorded at +40 mV is shown before (*A*), immediately after (*B*) and >30 sec after (*C*) a voltage pulse to −40 mV. Channel openings are upward current steps from the closed current level (dashed line, labeled C) to the maximum single channel conductance (continuous line, labeled O). Three records of continuous activity lasting 4 sec are shown for the same channel under each condition. (*A*) Channel activity is suppressed by 1 mM SNAP. (*B*) Channel activity was restored immediately after a 30 sec voltage pulse to −40 mV, and the increased activity lasted for ~30 sec. (*C*) At >30 sec after the voltage step, RyR activity returned to the SNAP-inhibited level.

dation of one class of high affinity −SH groups, located in the membrane field. The additive effect of modification of a second class of lower affinity, voltage-dependent, activating, thiol by 1 mM SNAP increased T_o at −40 mV. Finally, modification of a third class of low affinity, voltage-independent thiol groups by 1 mM SNAP inhibited channels by decreasing F_o and T_o at −40 and +40 mV. This inhibition was most prominent after channels were activated with ATP or peptide A. Different changes seen in channel activity as the concentration of SNAP increased are presumably the cumulative effects of oxidation of higher and lower affinity −SH groups. In addition, each of the effects could be due to the cumulative modification of more than one thiol, so that each class of thiols we describe may contain several −SH groups or indeed several subclasses.

If modification of one class of thiols has a "fingerprint" action on RyR gating mechanisms, we can compare classes of thiols modified by NO and by other oxidants. Activation with 10 μM SNAP was characterized by increased F_o, in contrast to increased T_o in cardiac or skeletal RyRs activated by dithiodipyridines (DTDPs) or thimerosal (TMS) (Eager & Dulhunty, 1999; Haarmann et al., 1999). Thus different classes of activating thiols are targeted by NO or DTDPs/TMS. Both 10 μM SNAP and the reactive disulfide, gliotoxin, increase F_o (D.

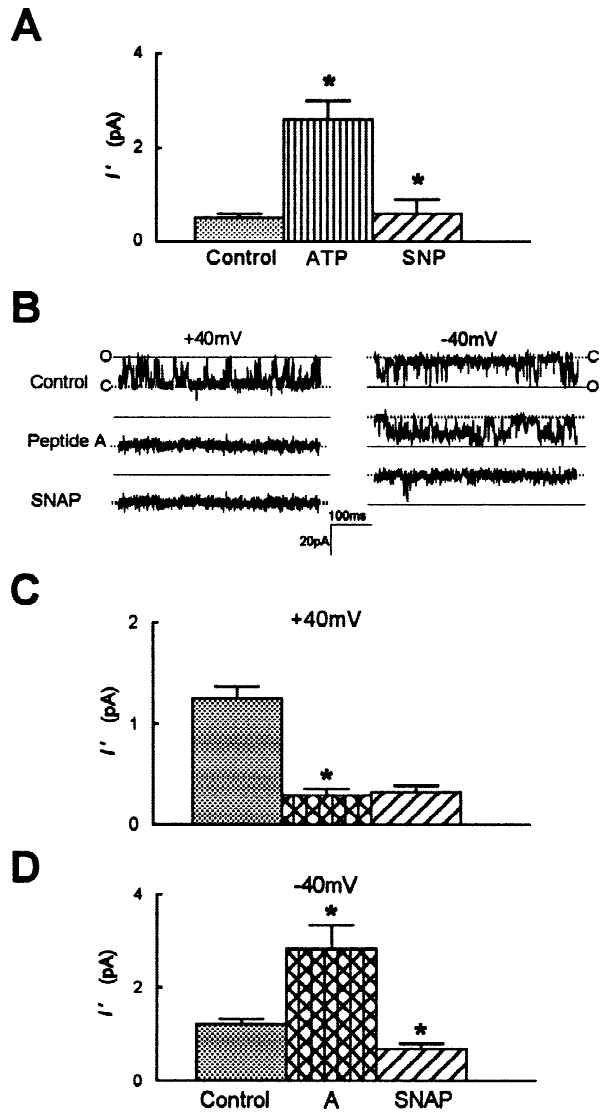

Fig. 9. Inhibition of ATP-activated RyRs by 0.5 mM SNP was similar to inhibition by 1 mM SNAP (Fig. 7), and peptide A-activated channels were inhibited by 1 mM SNAP in the same way as ATP-activated channels (Fig. 7). (*A*) Average changes in I' after adding 0.5 mM *cis* SNP at +40 mV. The histogram shows control activity (stippled bin), activation by 2 mM *cis* ATP (vertical striped bin) and inhibition of ATP-activated channels after adding 0.5 mM SNP (diagonal bars) (n = 4). (*B*) Effects of 6.3 μM *cis* peptide A on activity of a single RyR at +40 and −40 mV, and effects of subsequent addition of 1 mM *cis* SNAP. Channel openings are upward at +40 mV or downward at −40 mV, from a closed current level (dotted line C) to the maximum single channel conductance (continuous line, O). Peptide A decreased channel activity at +40 mV, but increased activity at −40 mV. 1 mM SNAP inhibited peptide A-activated openings at −40 mV. (*C* and *D*) Average I' at +40 mV (*C*) or −40 mV (*D*), under control conditions (stippled bin), after addition of peptide A (double cross-hatched bins) and finally after adding 1 mM *cis* SNAP. Addition of peptide A caused a significant decrease in I' at +40 mV, the subsequent addition of SNAP had no further effect. At −40 mV, peptide A significantly increased I', and subsequent addition of 1 mM SNAP caused a significant reduction in I'. Vertical capped bars show ±1 SEM.

Green, S.M. Pace, P. Waring, A. Hurne, J.D.E. Hart and A.F. Dulhunty, *unpublished results*) and may modify the same class of thiols. The increase in T_o with 1 mM SNAP or 1 mM DTDPs/TMS could depend on modification of the same class of low affinity thiols. Finally, inhibition with 1 mM SNAP differed from loss of activity with DTDPs/TMS, which is not alleviated by changing bilayer potential (Eager et al., 1997; Haarmann et al., 1999), indicating that two distinct classes of low affinity inhibiting thiols are available for modification. Thus, there are at least 4 classes of thiols associated with skeletal RyR channel gating.

Each monomer of the RyR contains at 89 (cardiac) or 101 (skeletal) cysteine residues (Otsu et al., 1990). Some residues are involved in structural disulfides, while others present free thiols. There are 21 free −SH groups per monomer in cardiac RyRs (Xu et al., 1998). *S*-Nitrosylation of up to 3 of these groups by small SNO compounds results in channel activation that is reversed by denitrosylation (Xu et al., 1998). Oxidation (in contrast to *S*-Nitrosylation) of 5 to 6 other −SHs per subunit by SNOs (forming R-S-S-R) had no effect on channel activity, but oxidation of an additional 3 to 5 thiols produced activation that could not be reversed by 20 mM DTT. Xu et al. (1998) concluded that the RyR differentiates physiologically between nitrosative and oxidative signals. The failure of 20 mM DTT to reverse SNO-induced oxidation, contrasts with reversal of DTDP/TMS-induced oxidation by 2 mM DTT (Eager & Dulhunty, 1998). Either the cysteines oxidized by DTDP/TMS differ from those oxidized by the SNOs, or mixed disulfides with DTDP/TMS are accessible to DTT, while the R-S-S-Rs formed by SNO modification are inaccessible. Since both activation and inhibition in the present study were reversed by 2 mM DTT, the NO reaction in our hands may have stopped at *S*-Nitrosylation and not continued to the formation of R-S-S-R.

Production of NO in Skeletal Muscle

Neuronal NOS (nNOS) is expressed in fast twitch (type II) fibers and is concentrated near the sarcolemma. Endothelial NOS (eNOS) is heterogenously expressed in muscle fibers and is colocalized with mitochondrial succinate dehydrogenase (Kobzik et al., 1994, 1995), while inducible NOS (iNOS) is cytosolic (Williams et al., 1994). NO produced by nNOS and iNOS in cell monolayers is ~100–1300 nM (Laurent, Lepoivre & Tenu, 1996). The concentrations of NO produced by 10 μM and 1 mM SNAP were 40 nM to 4 μM according to Simonsen et al. (1999) which is close to physiological range of 100 nM to 1.3 μM. The differential effects of NO on RyRs suggest a feedback loop (Mészáros et al., 1996), in which the initial or resting release of NO activates RyRs, elevating cytoplasmic [Ca^{2+}], further activating nNOS and increasing [NO]. When [NO] becomes high enough to inhibit RyRs, cytoplasmic [Ca^{2}] falls and NO production falls.

S-Nitrosylation of RyRs in vivo

S-Nitrosylation and oxidation of proteins in vivo proceed in the presence of the reducing agent, glutathione (GSH), if the affinity for the *S*-Nitrosylating or oxidizing reagents is greater than that for GSH (e.g., Koshita, Miwa & Oba, 1993). GSH is present at 0.5 to 10 mM in mammalian skeletal muscle fibers (Meister & Anderson, 1983) and the normal cytoplasmic ratio of GSH:GSSG is ~45 (Sies et al., 1972; Meister & Anderson, 1983; Curello et al., 1985). The GSH:GSSG in heart falls to 8 after 90 min of ischemia followed by reperfusion—due to a decline in [GSH] and increase in [GSSG] as GSH is oxidized by GSH peroxidase, reactive oxygen species and NO (Curello et al., 1985). The reduced GSH:GSSG leaves muscle proteins vulnerable to reactive oxygen species, and to oxidative damage (Curello et al., 1985; Steare & Yellon, 1995; Sirsjö et al., 1996).

Physiological Effects of *S*-Nitrosylation of RyRs

Activation of RyRs by low [NO] should increase cytoplasmic [Ca^{2+}] and enhance contraction. Although we can find no reports of NO increasing twitch tension, 100 μM SNAP slows the decline in force that occurs during repetitive isometric contraction of soleus or EDL fibers (Murrant, Woodley & Barclay, 1994; Murrant & Barclay, 1995). A positive effect of increased Ca^{2+} release on contraction might be negated by the inhibitory effect of NO on the contractile proteins (Perkins et al., 1997). Higher [NO]s do depress contraction (Kobzik et al., 1994), which could be a combined effect of reduced [Ca^{2+}] and reduced contractile function. Since RyRs must be active in order to be depressed by NO, it is likely that NO exerts its greatest inhibitory effect on contraction during sustained activity, and may well contribute to high frequency fatigue in skeletal muscle.

In conclusion, the results show that NO modulates RyR activity by *S*-Nitrosylation or oxidation of several classes of cysteine residues associated with the protein. The modifications of protein thiols can increase or decrease channel activity. The effects of NO depend on its concentration, the membrane potential and the degree of channel activation. The effects of NO on the RyR are likely to contribute to changes in skeletal muscle contractile force induced by NO in normal muscle cells and to oxidative damage under physiological conditions.

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