

Redox Modulation of L-Type Calcium Channels in Ferret Ventricular Myocytes

Dual Mechanism Regulation by Nitric Oxide and S-Nitrosothiols

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ABSTRACT The effects of NO-related activity and cellular thiol redox state on basal L-type calcium current, $I_{Ca,L}$, in ferret right ventricular myocytes were studied using the patch clamp technique. SIN-1, which generates both $NO\cdot$ and O_2^- , either inhibited or stimulated $I_{Ca,L}$. In the presence of superoxide dismutase only inhibition was seen. 8-Br-cGMP also inhibited $I_{Ca,L}$, suggesting that the NO inhibition is cGMP-dependent. On the other hand, S-nitrosothiols (RSNOs), which donate NO^+ , stimulated $I_{Ca,L}$. RSNO effects were not dependent upon cell permeability, modulation of SR Ca^{2+} release, activation of kinases, inhibition of phosphatases, or alterations in cGMP levels. Similar activation of $I_{Ca,L}$ by thiol oxidants, and reversal by thiol reductants, identifies an allosteric thiol-containing "redox switch" on the L-type calcium channel subunit complex by which $NO\cdot/O_2^-$ and NO^+ transfer can exert effects opposite to those produced by $NO\cdot$. In sum, our results suggest that: (a) both indirect (cGMP-dependent) and direct (S-nitrosylation/oxidation) regulation of ventricular $I_{Ca,L}$, and (b) sarcolemma thiol redox state may be an important determinant of $I_{Ca,L}$ activity.

KEY WORDS: cardiac electrophysiology • calcium homeostasis • ionic channels • N-oxides • S-nitrosylation

INTRODUCTION

Nitric oxide (NO-related activity) plays a key role in many important biological responses, including vasodilation, platelet inhibition, immune responses, cell adhesion, and neurotransmission (Ignarro, 1990; Marletta et al., 1990; Moncada et al., 1991; Stamler et al., 1992*b*; Snyder, 1992; Schmidt et al., 1993; Michel and Smith, 1993; Dinerman et al., 1993; Shah and Lewis, 1993; Lincoln, 1994; Schmidt and Walter, 1994; Stamler, 1995; Feelisch and Stamler, 1996). It is believed that the free radical $NO\cdot$ exerts many of its effects through an indirect pathway involving activation of guanylate cyclase and increased levels of cyclic guanosine monophosphate (cGMP). However, NO signaling also encompasses the actions of other naturally occurring N-oxides, such as S-nitrosothiols (RSNOs)¹ and peroxynitrite ($OONO^-$) which display reactivity pro-

files that are different from $NO\cdot$ itself (Stamler et al., 1992*b*). In particular, the NO group of RSNOs has NO^+ properties that enable S-nitrosylation of proteins, while peroxynitrite oxidizes protein thiols (Feelisch and Stamler, 1996). As a result, cellular redox state can be an important factor in determining NO-related responses (Stamler et al., 1992*b*).

Posttranslational modification of protein thiols can regulate protein function. Regulation by redox state and NO/RSNOs has been described for numerous intra- and extracellular proteins (e.g., Stamler et al., 1992*a, b*; Gopalakrishna et al., 1993; Lander et al., 1993; Duche et al., 1994; Stamler, 1994, 1995). Membrane bound ion channels have received particular attention (e.g., Ruppertsberg et al., 1991; Lipton et al., 1993; Bolotina et al., 1994; Stamler, 1994; Gozlan and Ben-Ari, 1995; Stamler, 1995; Lipton et al., 1996). Direct effects of N-oxides appear to derive from reactions of vicinal thiols that serve as allosteric regulators of protein and channel function (Lipton et al., 1993; Stamler, 1994; Stamler, 1995). In addition to direct redox regulation, NO can modify ion channel function by cGMP (e.g., Schmidt et al., 1993; Lincoln, 1994). Thus, a picture has emerged wherein NO-regulation of cellular functions occurs via either cGMP or S-nitrosylation/oxidation of metal- and/or thiol-containing proteins. Nonetheless, it is still widely believed that different NO-related species exert similar or identical effects on cellu-

Preliminary accounts of this work have appeared previously in abstract form (Campbell et al. 1995. *Circulation*. 92:I433; Campbell et al. 1995. *Endothelium*. 3[Suppl.]:S36; Campbell et al. 1996. *Biophys. J.* 70:A314).

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¹Abbreviations used in this paper: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; GNSO, S-nitrosoglutathione; $I_{Ca,L}$, L-type calcium current; NEM, N-ethylmaleimide, RSNO, S-nitrosothiols; SOD, superoxide dismutase; TTX, tetrodotoxin.

lar protein and channel function through common signalling mechanisms. Furthermore, dual mechanisms of ion channel modulation have not been described for NO.

In cardiac muscle, the L-type calcium current, $I_{Ca,L}$, triggers the excitation-contraction coupling process through Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum (Bers, 1991; Wier, 1992). Mechanisms of indirect modulation of $I_{Ca,L}$ by intracellular second messenger pathways have attracted intense experimental attention (Hartzell, 1988; McDonald et al., 1994; Campbell and Strauss, 1995). With regard to NO and cardiac function, it has been demonstrated that: (a) NO-related activity can modulate cardiac contractility both in vivo and in vitro (Brady et al., 1993; Shah and Lewis, 1993; Balligand et al., 1995; Hare et al., 1995; Mohan et al., 1996); (b) NO may be indirectly involved in muscarinic inhibition of β -adrenergically stimulated $I_{Ca,L}$, either through activation of cGMP-dependent phosphodiesterases (Fischmeister and Shrier, 1989; Mery et al., 1993; Han et al., 1994, 1995) or cGMP-dependent protein kinase and/or phosphatases (Levi et al., 1989; Mery et al., 1991; Mubagwa et al., 1993; Herzig et al., 1993; Levi et al., 1994; Summii and Sperelakis, 1995; Wahler and Dollinger, 1995); and (c) at least some working cardiac myocyte types possess a constitutive nitric oxide synthase (NOS; Balligand et al., 1995; Kanai et al., 1995) and a Ca^{2+} -independent NOS (iNOS) which can be upregulated upon exposure to cytokines (Brady et al., 1992; Finkel et al., 1992; Balligand et al., 1994; Haywood et al., 1996). However, in all of these previous studies the molecular identity of the NO-related effector species was assumed to be equivalent to free radical NO \cdot , and neither cellular redox state or possible direct effects were considered.

In this paper we demonstrate for the first time that NO-related modulation of the sarcolemmal L-type calcium channel of mammalian cardiac muscle can involve both indirect control by cGMP and direct control by channel subunit S-nitrosylation/oxidation. We also demonstrate that these two signalling pathways have opposite effects upon L-type calcium channel function. Finally, we demonstrate that sarcolemmal thiol redox state can directly and selectively modulate ventricular $I_{Ca,L}$.

METHODS

Myocyte Isolation, Electrophysiological Techniques, and Data Analysis

Single myocytes were enzymatically isolated (collagenase, protease, elastase) from the right ventricles of 10–20 wk-old male ferrets via the Langendorff perfusion method as previously described (Campbell et al., 1993; Qu et al., 1993a, b). After isolation, myocytes were stored in normal (1.8 mM $CaCl_2$, 5.4 mM KCl) control saline (described below) at room temperature (22–24°C) until used. All experiments were conducted within 6–12 h after initial myocyte isolation.

Myocytes were voltage clamped using the single-microelectrode “gigaseal” patch clamp technique in the whole cell recording configuration (Hamill et al., 1981; Marty and Neher, 1995). The amplifier was an Axopatch 1-C (Axon Instruments, Inc., Foster City, CA). All other recording and stimulating apparatus, as well as recording chamber, simple gravity feed perfusion system, inverted microscope, and modified stage, were as previously described in detail (Campbell et al., 1993; Qu et al., 1993a, b). Patch pipettes were fabricated from 1.5-mm O.D. borosilicate tubing (TW 150F-4; WP Instruments) and gently heat polished to a final approximate diameter of 1 μ m (MF-83 microforge; Narishige Scientific Instruments, Tokyo, Japan). When filled with intracellular recording solution (described below) pipette resistances were 2–5 M Ω . After zeroing current flow across the pipette tip (junction potentials were 5 mV or less and have not been corrected for in the data presented), gigaseals were initially formed in normal Na^+ solution (described below). After obtaining the whole cell configuration, at least 10 min were allowed to pass to allow adequate internal perfusion and stabilization of current gating parameters (Marty and Neher, 1995).

Current traces and voltage clamp pulses were recorded on videotape using a 4-channel A/D VCR adaptor (PCM 4; Medical Systems Corp., Greenvale, NY). Currents were filtered (8 pole Bessel) at 1–2 kHz and were subsequently digitized offline using a 125 kHz A/D board under pCLAMP software control (Axon Instruments). All analysis was conducted on $I_{Ca,L}$ recordings that were not “leakage corrected” using commercially available software packages (pCLAMP, Lotus, FigP). Measurements of both the mean peak and isochronal 500 ms I-V relationship or extrapolation of “leak currents” from linear regression of data obtained from –70 to –40 mV (no net inward current activated; see Fig. 4A) indicated that the mean peak leak current under both control and S-nitrosoglutathione conditions was only 1–2.4% of the mean peak $I_{Ca,L}$ at 0 mV. Therefore, the residual nonsubtracted leakage current was not a significant contributing factor in the results presented. When applicable, statistical significance was tested using a paired *t* test at *P* < 0.05. Data are presented as mean \pm SEM.

All reported measurements were on the basal $I_{Ca,L}$, i.e., $I_{Ca,L}$ recorded without any previous β -adrenergic stimulation or muscarinic inhibition. All experiments were conducted at room temperature (22–24°C). In all experiments myocytes were held at a holding potential of HP = –70 mV. The data points in all figures correspond to the net peak inward $I_{Ca,L}$ elicited in response to a 500 ms voltage clamp pulse to 0 mV applied at a frequency of 0.167 Hz, except for the results presented in Fig. 4 (I-V curve, inactivation kinetics). Due to the slow and variable (\sim 1–2 ml/min) perfusion times of the recording chamber used (vol \sim 2 ml; Campbell et al., 1993; Qu et al., 1993a, b), no quantitative conclusions on the kinetics of onset of compound effects can be reached from the data presented; hence, effects are given as mean values after steady-state conditions had been obtained. These factors, in combination with possible differences in redox state of individual myocytes, account for the variable time-course of effects illustrated in some of the figures. In an attempt to account for cellular variability, possible differences in internal perfusion, and other experimental variables, mean results from at least *n* \geq 3 myocytes were obtained for each specific experimental manipulation.

Solutions and Compounds

Previous studies from our laboratory (Qu et al., 1993a, b) have demonstrated that ferret right ventricular myocytes do not appear to possess a macroscopically measurable T-type calcium current. Hence, the calcium currents analyzed in this study are purely L-type. Gigaseals were formed and the whole cell configu-

ration obtained in control extracellular solution (mM): 144 NaCl, 5.4 KCl, 1 MgSO₄, 1.8 CaCl₂, 10 HEPES, pH = 7.40. Myocytes were then subsequently perfused with an Na⁺- and K⁺-free extracellular I_{Ca,L} solution (mM): 144 N-methyl-D-glucamine-Cl (NMDG-Cl), 5.4 CsCl, 1 MgSO₄, 1.8 CaCl₂, 10 HEPES, pH = 7.40. The patch pipette contained K⁺-free intracellular solution (mM): 120 CsCl, 20 TEA-Cl, 1 MgSO₄, 5 EGTA, 5 Mg-ATP, 5 Tris creatine phosphate, 0.2 GTP, 10 HEPES, pH = 7.40. As has been previously demonstrated (Qu et al., 1993a, b), by using these solutions a pure I_{Ca,L} can be recorded from these myocytes over periods of up to 45–60 min without any significant “run down” (e.g., Figs. 5, 6 B, and 9 A and D). Whether this represents appropriate properties of the recording solutions used or instead is a reflection of different intracellular regulatory properties that minimize run down of ferret right ventricular basal I_{Ca,L} is presently unclear. In any event, in nearly all circumstances an experiment was terminated due to factors other than run down (typically loss of seal resistance). As a result, the “perforated patch” technique (e.g., Horn and Marty, 1988; Han et al., 1994, 1995) was not used.

Stock solutions (50 mM) of S-nitrosothiols were freshly synthesized by mixing equal volumes of 100 mM reduced glutathione or L-cysteine in 250 mM HCl, 0.1 mM EDTA and 100 mM NaNO₂ in distilled water at room temperature (Arnelle and Stamler, 1995) and stored in the dark on ice until used. Stocks were diluted (with readjustment of pH back to 7.40 with CsOH) in the extracellular I_{Ca,L} recording solution to a final concentration of 1 mM immediately before use. Staurosporine was dissolved in DMSO to give stock solutions that were refrigerated until used. These stocks were then diluted in extracellular I_{Ca,L} solution to a final staurosporine concentration of 200 nM. While the final level of DMSO in the I_{Ca,L} solution ($\leq 0.1\%$) had no measurable effect upon I_{Ca,L}, in experiments involving staurosporine an equal amount of DMSO was added to the control I_{Ca,L} solution to serve as an internal control for possible solvent effects. Aliquots of stock solutions of H-7 (1-[5-isoquinolinesulfonyl]-2-methylpiperazine-HCl) were made in extracellular I_{Ca,L} solution, stored in the refrigerator, and diluted immediately before use to a final concentration of 50 μ M. SIN-1 (3-morpholiniosydnonimine-HCl) solutions were made fresh for each individual experiment and were used within 5–10 min of preparation. Reduced glutathione, cysteine, superoxide dismutase, dithiothreitol (DTT), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), and all compounds used to make both control and I_{Ca,L} extracellular and intracellular solutions were obtained from Sigma. High purity ryanodine, 8-bromo-cyclic GMP, staurosporine, H-7, and tetrodotoxin (TTX) were obtained from Calbiochem Corp. (La Jolla, CA). SIN-1 was obtained from Alexis Chemical Corp. (San Diego, CA).

Conventions and Definitions

For ease of presentation the following conventions and definitions have been used: (a) Unless otherwise indicated, “I_{Ca,L}” will refer to the peak inward basal I_{Ca,L} recorded at 0 mV. At 0 mV I_{Ca,L} is an inward current; accordingly, data points in all figures have been plotted on a negative axis. In all appropriate figures the numbers by any given data point correspond to the indicated current recordings shown in the inset, while the solid lines above the data points correspond to the period of indicated compound application; (b) “indirect” will refer to modulatory effects on I_{Ca,L} due to the involvement or activation of intracellular second messenger systems (e.g., guanylate cyclase and cGMP); and (c) “direct” will refer to modulatory effects on I_{Ca,L} due to redox, S-nitrosylation, and/or oxidation reactions of sarcolemmal thiol groups which are accessible to the extracellular environment (Stamler, 1994, 1995).

RESULTS

Indirect Inhibitory Effects of Nitric Oxide (NO[•]) on I_{Ca,L}

SIN-1 has been widely used in cardiac electrophysiological studies as a specific “NO-donor” compound. During its spontaneous breakdown sequence SIN-1 generates both NO[•] and O₂⁻ (superoxide) free radicals, which can then react at nearly diffusion-limited rates ($k \approx 3.7 \times 10^7$ /M/s) to produce peroxynitrite: SIN-1 \rightarrow O₂⁻ + NO[•] \rightarrow OONO⁻ (Feelisch, 1991; Saran et al., 1990; Stamler et al., 1992b; Feelisch and Stamler, 1996). The effects of 1 mM SIN-1 on ferret right ventricular I_{Ca,L} were not straightforward, in that it could either reversibly decrease ($-26 \pm 5\%$; 5/13 myocytes; Fig. 1A) or increase I_{Ca,L} ($+35 \pm 8\%$; 5/13 myocytes;

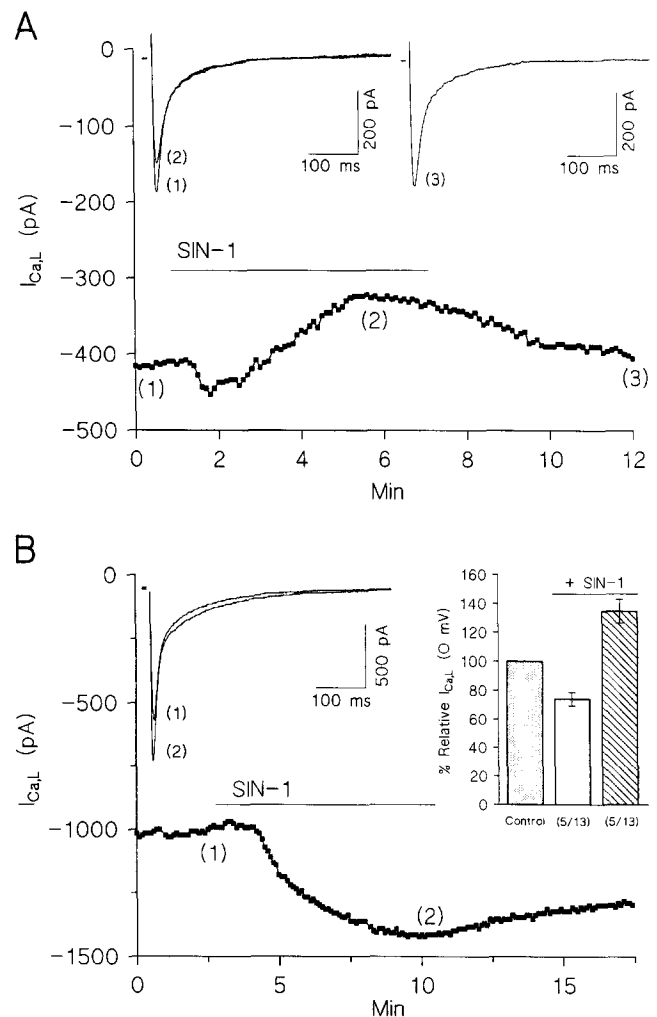


FIGURE 1. Effects of extracellularly applied 1 mM SIN-1 on basal I_{Ca,L}. (A) Representative example of reversible inhibition of I_{Ca,L} by SIN-1. (B) Representative example of reversible stimulation of I_{Ca,L} by SIN-1. (Inset) Summary of 1 mM SIN-1 effects. In 5/13 myocytes SIN-1 inhibited I_{Ca,L} by $-26 \pm 5\%$, while in 5/13 myocytes it stimulated I_{Ca,L} by $+35 \pm 8\%$.

Fig. 1B). In three additional myocytes, SIN-1 produced biphasic effects on $I_{Ca,L}$.

We hypothesized that in myocytes in which SIN-1 inhibited $I_{Ca,L}$, the cellular redox conditions were such that the effects of $NO\cdot$ were dominant, leading to inhibition of $I_{Ca,L}$ through cGMP. On the other hand, in those myocytes in which SIN-1 stimulated $I_{Ca,L}$, we hypothesized that S-nitrosylation- and/or oxidation-mediated signals lead to channel activation. To test for cGMP-dependent mechanisms we reevaluated the effects of 1 mM SIN-1 in the presence of 750–3,000 U/ml superoxide dismutase (SOD), so as to minimize production of $OONO^-$. Under these conditions 1 mM SIN-1 produced a consistent and reversible inhibition of $I_{Ca,L}$ of $-17 \pm 4\%$ ($n = 3$ myocytes; Fig. 2A). Moreover, extracellular application of 10–100 μ M 8-Br-cGMP consistently inhibited $I_{Ca,L}$ (Fig. 2B). In five myocytes where steady-state effects were obtained, 100 μ M 8-Br-cGMP inhibited $I_{Ca,L}$ by $-30 \pm 9\%$. Compared to SIN-1 + SOD, the inhibition produced by 8-Br-cGMP

was greater (Fig. 2B, inset), possibly due to the fact that 8-Br-cGMP is relatively phosphodiesterase resistant. Finally, myocytes were preincubated for at least ≥ 8 min in 100 μ M 8-Br-cGMP (to allow steady-state inhibition of $I_{Ca,L}$ to be obtained) and then exposed to 1 mM SIN-1. In the continual presence of 8-Br-cGMP, SIN-1 was either without effect (2/7 myocytes) or significantly increased $I_{Ca,L}$ by $+105 \pm 17\%$ (5/7 myocytes; Fig. 2C). In no instance in the presence of 8-Br-cGMP was a further inhibition of $I_{Ca,L}$ by SIN-1 observed. Taken in aggregate, these results indicate that: (a) $NO\cdot$ can inhibit ferret right ventricular $I_{Ca,L}$ through an indirect cGMP-dependent pathway, and (b) $OONO^-$ may be capable of stimulating $I_{Ca,L}$, either through redox and/or S-nitrosylation reactions.

Stimulatory Effects of S-Nitrosothiols (NO^+ Transfer) on $I_{Ca,L}$

To test the hypothesis that the stimulatory effects produced by SIN-1 in some myocytes may have been due to

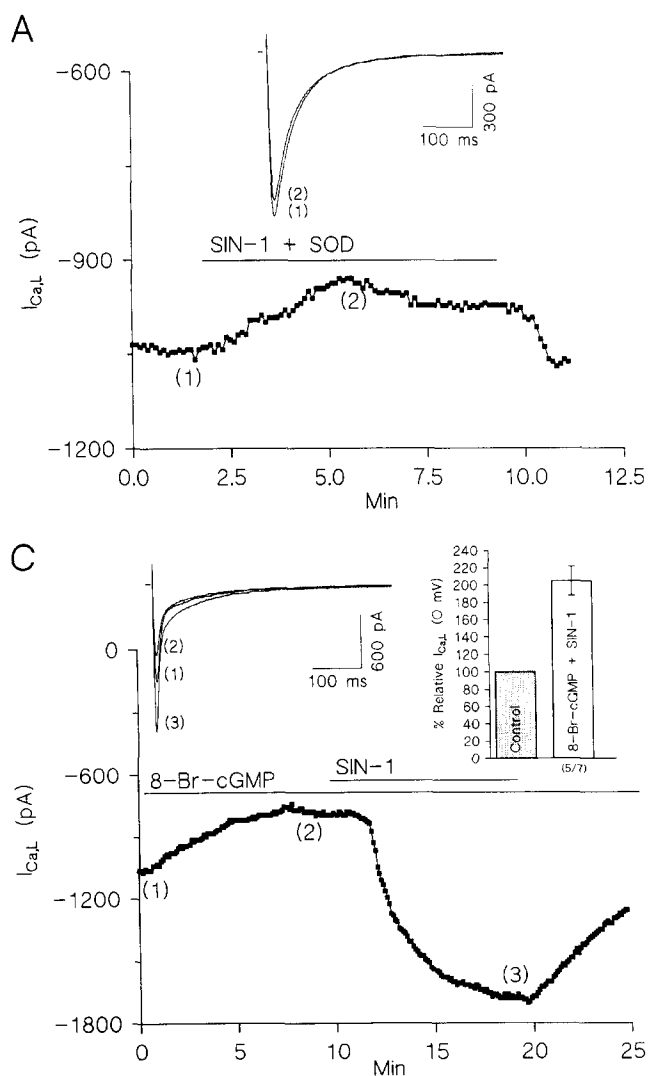


FIGURE 2. (A) SIN-1 and SOD. In the presence of superoxide dismutase (SOD; which would promote $NO\cdot$ production and prevent $OONO^-$ formation) SIN-1 consistently inhibited $I_{Ca,L}$. Representative result from one myocyte exposed to 1 mM SIN-1 + 1500 U/ml SOD. $I_{Ca,L}$ was reversibly inhibited by $\sim 18\%$. (B) Representative results from one myocyte on extracellular application of 100 μ M 8-Br-cGMP. 100 μ M 8-Br-cGMP consistently inhibited $I_{Ca,L}$. (Inset) Summary and comparison of mean inhibitory effects produced by 100 μ M 8-Br-cGMP ($-30 \pm 9\%$ inhibition, $n = 5$ myocytes) and 1 mM SIN-1 + SOD ($-17 \pm 4\%$ inhibition, $n = 3$ myocytes). (C) Representative results from one myocyte on the effects of 1 mM SIN-1 after inhibitory steady-state effects of 100 μ M 8-Br-cGMP had been obtained. (Inset) Summary of effects of 1 mM SIN-1 in the presence of 100 μ M 8-Br-cGMP. In the presence of 8-Br-cGMP, in 2/7 myocytes SIN-1 had no effect on $I_{Ca,L}$, while in 5/7 myocytes it significantly stimulated $I_{Ca,L}$ by $+105 \pm 17\%$. In the presence of 8-Br-cGMP in no instance was an inhibition of $I_{Ca,L}$ by SIN-1 observed.

direct channel modification, we studied the effects of two naturally occurring S-nitrosothiols that support NO⁺ transfer chemistry (i.e., S-nitrosylation), S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CySNO). Extracellular application of 1 mM GSNO consistently produced a significant increase in I_{Ca,L} (+61 ± 11%, n = 7 myocytes) which was slowly reversible upon wash-out (Fig. 3 A). The stimulation of I_{Ca,L} could be repeated upon a second application of GSNO, and in no instance was inhibition of I_{Ca,L} observed. Thus, the effects of GSNO (NO⁺) on I_{Ca,L} were diametrically oppo-

site to those produced by NO⁻. Since when perfusing GSNO 1 mM Na⁺ was also reintroduced into the extracellular solution (see METHODS) control measurements were conducted in the presence of 50 μM tetrodotoxin (TTX). In the presence of TTX, 1 mM GSNO increased I_{Ca,L} by +63 ± 18% (n = 6 myocytes; Fig. 3 B), a mean value of stimulation very similar to that observed under control conditions. The results obtained with TTX indicated that the 1 mM Na⁺ reintroduced into the extracellular recording solution was not a significant factor in mediating the effects of GSNO upon I_{Ca,L}.

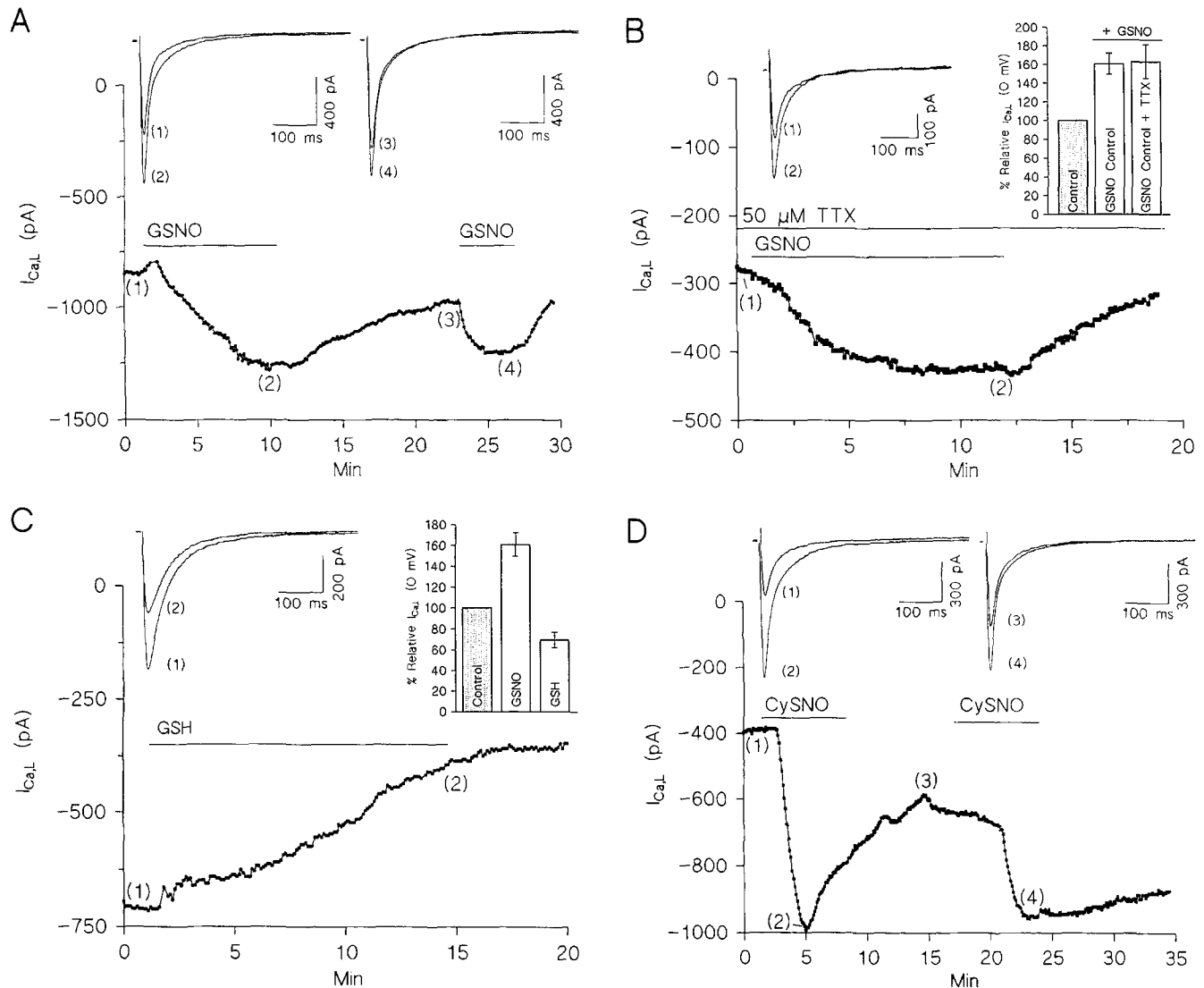


FIGURE 3. Effects of extracellular RSNOs (S-nitrosylation) and RSH (reduction) on I_{Ca,L}. (A) 1 mM GSNO consistently stimulated I_{Ca,L}. Representative result from one myocyte. The effects were both reversible and repeatable. (B) Representative result from one myocyte on the effects of 1 mM GSNO on I_{Ca,L} in the presence of 50 μM TTX. (Inset) Summary and comparison of stimulatory GSNO effects under control conditions and in the presence of 50 μM TTX. Mean stimulation values: GSNO control, +61 ± 11% (n = 7 myocytes); GSNO control + TTX, +63 ± 18% (n = 6 myocytes). (C) 1 mM reduced glutathione (GSH) consistently inhibited I_{Ca,L}. Results in this myocyte are representative of 6 similar experiments. (Inset) Summary and comparison of GSNO and GSH results. Mean values of stimulation/inhibition as follows: 1 mM GSNO, +61 ± 11% (n = 7 myocytes); 1 mM GSH, -31 ± 7% (n = 6 myocytes). (D) 1 mM S-nitrosocysteine (CySNO) consistently stimulated I_{Ca,L}. Representative result from one myocyte, where CySNO initially transiently stimulated I_{Ca,L} by nearly 150% (see text for details).

Finally, in a separate series of control experiments the effects of extracellular reduced glutathione (GSH, 1 mM) were determined. In contrast to GSNO, 1 mM GSH consistently inhibited $I_{Ca,L}$ ($-31 \pm 7\%$; $n = 6$ myocytes; Fig. 3 C). Therefore, the stimulatory effects of GSNO were specifically related to the NO group.

Like GSNO, 1 mM S-nitrosocysteine (CySNO) also

consistently and reversibly increased $I_{Ca,L}$ (Fig. 3 D). However, by comparison to GSNO, the initial stimulatory effect was more transient ($n = 4$ myocytes). This was probably related to the short half-life of CySNO (Feelisch and Stamler, 1996). Because GSNO is both stable and cell impermeable, we used it to probe for potential extracellular redox sites (see below).

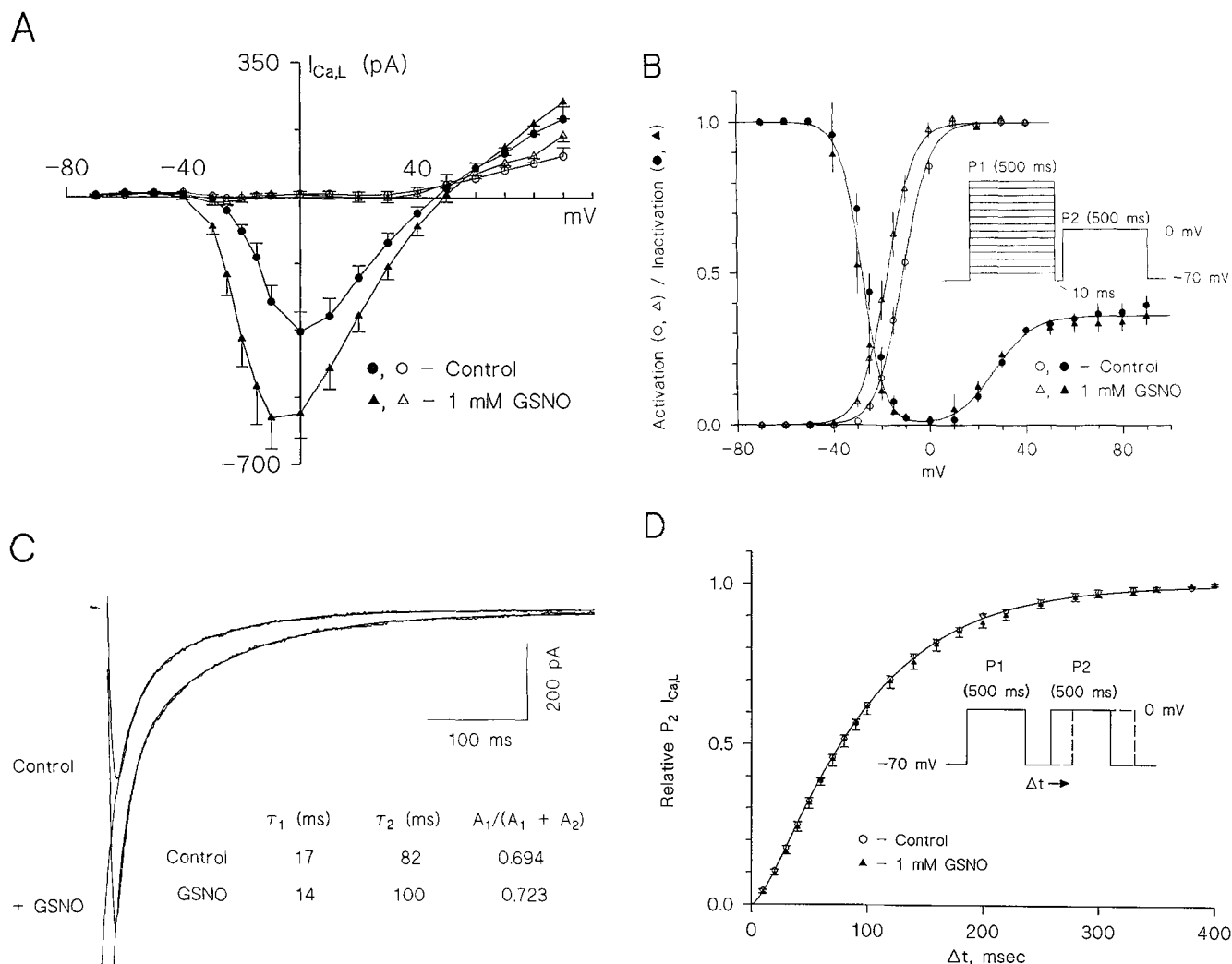


FIGURE 4. Effects of 1 mM extracellular GSNO on $I_{Ca,L}$ macroscopic gating characteristics. Mean data for all panels obtained from a total of 7 myocytes. (A) Peak (solid symbols) and isochromal (500 ms; open symbols) current-voltage (I-V) relationships obtained in control and after GSNO application. (B) Estimated steady-state activation curves (open symbols) and measured steady-state inactivation curves (solid symbols). Activation curves were constructed from the peak I-V relationships of (A) (Qu et al., 1993a, b) and could be fit to a single Boltzmann relationship with the following parameters: control, $V_{1/2} = -11$ mV, $k = 5.5$ mV; GSNO, $V_{1/2} = -17.5$ mV, $k = 5.5$ mV. At 0 mV, the mean activation variables in control and GSNO were 0.88 and 0.96, respectively; this would produce an estimated mean increase of $I_{Ca,L}$ at 0 mV of only 9%. The mean steady-state inactivation relationships (measured using the protocol in schematic inset) for both control and GSNO could be fit with the sum of two Boltzmann relationships "f" and "r" (Qu et al., 1993a, b; values in mV): $f = 1/(1 + \exp[(V + 28)/4.5])$, $r = 0.31/(1 + \exp[22 - V]/8)$. (C) Kinetics of $I_{Ca,L}$ macroscopic inactivation at 0 mV. Representative double exponential fits with indicated parameters obtained from one myocyte in control and GSNO ($A_1/[A_1 + A_2]$ = relative initial amplitude of fast component τ_1). Mean fit parameters as follows: control, $\tau_1 = 17.5 \pm 2.1$ ms, $\tau_2 = 79.3 \pm 2.4$ ms, $A_1/(A_1 + A_2) = 0.636 \pm 0.044$; 1 mM GSNO, $\tau_1 = 13.9 \pm 1.3$ ms, $\tau_2 = 89.8 \pm 4.5$ ms, $A_1/(A_1 + A_2) = 0.694 \pm 0.033$. Statistical significance levels: τ_1 , $0.01 < P < 0.02$; τ_2 , $0.02 < P < 0.05$; $A_1/(A_1 + A_2)$, $0.02 < P < 0.05$. (D) Mean kinetics of recovery from inactivation at HP = -70 mV (protocol shown in schematic inset). Mean recovery data for both control and GSNO could be fit with the same sigmoid recovery curve (Qu et al., 1993a, b): $I_{Ca,L} = I_{Ca,L,max} (1 - \exp[-t/\tau_{rec}])^n$, with $\tau_{rec} = 75$ ms and $n = 1.55$.

Effects of GSNO on $I_{Ca,L}$ macroscopic gating characteristics. GSNO increased the amplitude of the peak current-voltage (I-V) relationship ($+61 \pm 11\%$ increase at 0 mV) without significantly affecting either the apparent reversal potential or the isochronal (500 ms) I-V relationship (Fig. 4A). The $V_{1/2}$ of the mean estimated steady-state activation curve (Fig. 4B) was slightly shifted by -6.5 mV in the hyperpolarized direction by GSNO, without any significant alteration in other estimated activation parameters (see Fig. 4B, legend). Similarly, GSNO had no significant effect on either the mean "U-shaped" steady-state inactivation relationship (Fig. 4B) or the kinetics of recovery from inactivation measured at $HP = -70$ mV (Fig. 4D; see legend). While the parameters of $I_{Ca,L}$ macroscopic inactivation in GSNO were calculated to be significantly different from control conditions (Fig. 4C; mean values to double exponential fits given in Fig. 4C legend), the overall alterations in inactivation kinetics were nonetheless minor. It should be noted that a -6.5 mV shift in the estimated activation curves would increase peak $I_{Ca,L}$ at 0 mV by only 9%. Hence, additional factors (i.e., increases in Np_o and/or i) must also be operative to account for the stimulatory effects of GSNO observed.

Is the Stimulatory Effect of GSNO on $I_{Ca,L}$ due to Indirect or Direct Mechanisms? Tests of Potential Indirect Mechanisms

Inhibition of SR Ca^{2+} release. GSNO could potentially stimulate $I_{Ca,L}$ through reduction or inhibition of Ca^{2+} -induced Ca^{2+} release from the sarcoplasmic reticulum, which in turn would reduce a significant component of Ca^{2+} -induced inactivation (Mitchell et al., 1984; Balke and Wier, 1991; Imredy and Yue, 1994; Campbell and Strauss, 1995; Y. Qu and D.L. Campbell, unpublished observations). To test this SR Ca^{2+} hypothesis, myocytes were first preincubated for >60 min in $20 \mu\text{M}$ ryanodine to achieve complete steady-state block of the RY_2 -type SR Ca^{2+} release channel (Meissner, 1995). In addition, $20 \mu\text{M}$ ryanodine was also included in the patch pipette solution. However, after pretreatment with ryanodine 1 mM GSNO was still able to consistently and significantly increase $I_{Ca,L}$ by $+65 \pm 26\%$ ($n = 3$ myocyte; Fig. 5), a value comparable to that observed under control conditions (see Fig. 8). These results indicate that the effects GSNO are not obligatorily dependent upon modulation of SR Ca^{2+} release.

Kinase activation. GSNO could indirectly stimulate $I_{Ca,L}$ through activation of a protein kinase or kinases (e.g., cAMP-dependent PKA, PKC, or some other presently unknown kinase "PKX" [Hartzell et al., 1995]). To test this indirect kinase hypothesis two different nonspecific kinase inhibition protocols were applied. Both protocols involved simultaneous extracellular application of two kinase inhibitor compounds, 200 nM staurosporine and $50 \mu\text{M}$ H-7. At these high concentra-

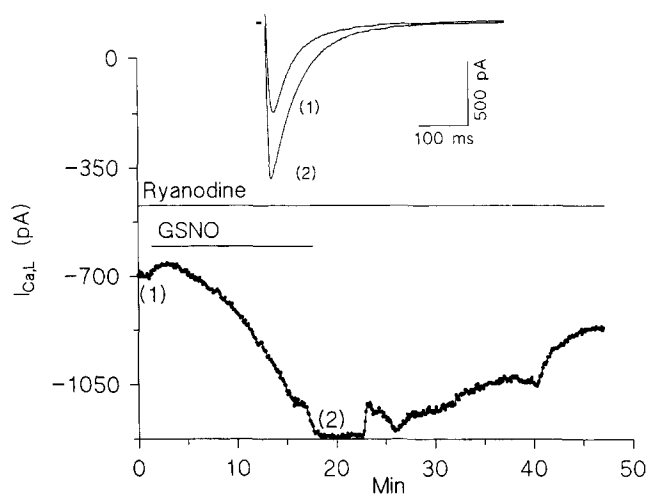


FIGURE 5. RSNO and SR regulation. Reduction or inhibition of SR Ca^{2+} release. Representative results from one myocyte after SR Ca^{2+} release had been inhibited with $20 \mu\text{M}$ ryanodine (see text for details). 1 mM GSNO increased $I_{Ca,L}$ to an extent similar to that observed under control conditions (see Fig. 8).

tions, the combination of staurosporine + H-7 should have inhibited all presently known kinases in cardiac myocytes (Hartzell, 1988; McDonald et al., 1994; Campbell and Strauss, 1995).

In the first protocol a series of control $I_{Ca,L}$ recordings were obtained using the standard control patch pipette solution (5 mM ATP, 5 mM creatine phosphate, and $200 \mu\text{M}$ GTP present; see METHODS). Staurosporine + H-7 were then extracellularly applied for 8–20 min, whereupon 1 mM GSNO was applied. Initial application of staurosporine + H-7 consistently produced a significant inhibition of $I_{Ca,L}$ of $-69 \pm 6\%$ (Fig. 6A and inset). These results indicate that, under the recording conditions employed, a significant component of the basal $I_{Ca,L}$ in ferret right ventricular myocytes is maintained by the activity of a (presently unidentified) kinase or kinases (Tsien et al., 1986; Hartzell, 1988; Ono and Fozzard, 1992, Herzig et al., 1993; Ono and Fozzard, 1993; McDonald et al., 1994; Hartzell et al., 1995; Campbell and Strauss, 1995). However, 1 mM GSNO was still able to increase the $I_{Ca,L}$ remaining after significant nonspecific kinase inhibition (Fig. 6, B and C). In a total of 6 myocytes in which this first protocol was applied, 1 mM GSNO increased the basal $I_{Ca,L}$ remaining after 8–20 min exposure to staurosporine + H-7 by $+54 \pm 10\%$ (see Fig. 8).

To further test the indirect kinase hypothesis, a more stringent second kinase inhibition protocol was also applied. Myocytes were first preincubated for >1 h (and typically >2 –3 h) in staurosporine + H-7, plus staurosporine + H-7 were continually perfused under all recording conditions. In an attempt to eliminate or mini-

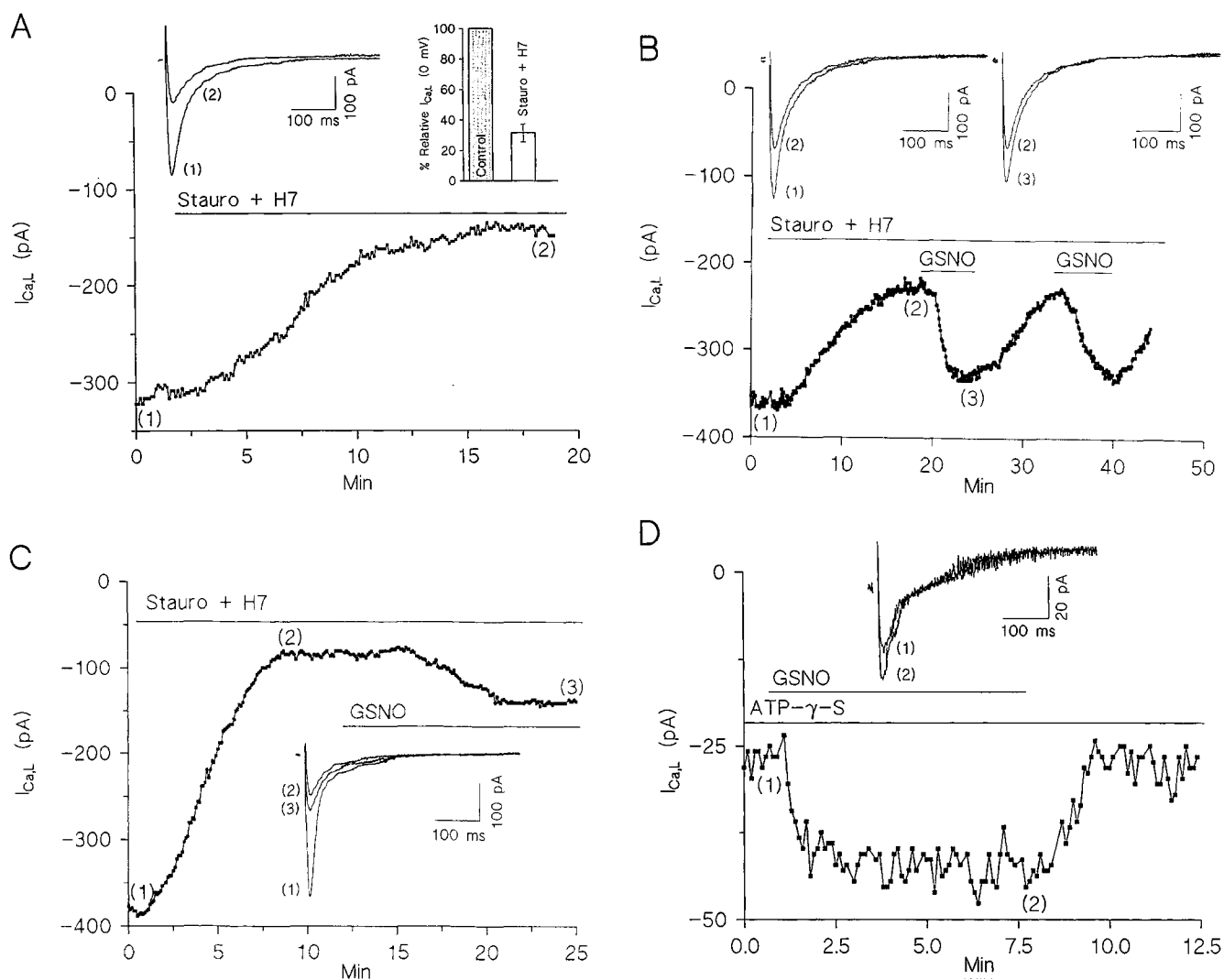


FIGURE 6. RSNO and kinase regulation. (A, B, and C) Representative results of first kinase inhibition protocol (see text for details). (A) Extracellular application of 200 nM staurosporine + 50 μ M H-7 consistently inhibited basal $I_{Ca,L}$. Representative results from one myocyte. (*Inset*) Under these conditions, staurosporine + H-7 (8–20 min exposures) produced a mean inhibition of $I_{Ca,L}$ of $-69 \pm 6\%$ ($n = 8$ myocytes). (B and C). After “steady-state” staurosporine + H-7 inhibition had been achieved, 1 mM GSNO increased $I_{Ca,L}$ to an extent similar to that observed under control conditions (see Fig. 8). Results from two different myocytes. (D) Representative results of second kinase inhibition protocol (patch pipette ATP replaced with ATP- γ -S; see text for details) from one myocyte. 1 mM GSNO reversibly increased residual basal $I_{Ca,L}$ to an extent similar to that observed under control conditions (see Fig. 8). Similar results observed in a total of 9 myocytes.

mize all intracellular phosphorylation reactions, the patch pipette solution was modified by including 50 μ M H-7 and replacing control 5 mM ATP with 5 mM ATP- γ -S (a nonhydrolyzable ATP-analogue) and completely removing creatine phosphate and GTP. Finally, myocytes were perfused for at least 15–20 min before GSNO application. Under these conditions the peak “basal” $I_{Ca,L}$ was quite small (typically under 100 pA). Nonetheless, 1 mM GSNO still reversibly increased the residual basal $I_{Ca,L}$ by $+65 \pm 16\%$ ($n = 9$ myocytes; Fig. 6 D).

Phosphatase inhibition. GSNO could potentially stimulate $I_{Ca,L}$ through inhibition of basally active phosphatase activity (e.g., Ono and Fozzard, 1993; Imredy

and Yue, 1994). To nonspecifically inhibit or attenuate phosphatase activity 120 mM KCl in the patch pipette was replaced with 120 mM CsF (Shenolikar and Nairn, 1991; Neumann et al., 1993; Imredy and Yue, 1994). Under these conditions, in many myocytes ($n = 8$) peak $I_{Ca,L}$ continued to progressively grow in amplitude even after 20 min of CsF perfusion. Such behavior would be consistent with inhibition of (presently unidentified) basally active phosphatases, resulting in “unopposed” basal kinase activity. Due to this complication, recordings were made from relatively small myocytes, so as to achieve both adequate internal perfusion of CsF and steady-state effects within 20 min. Under

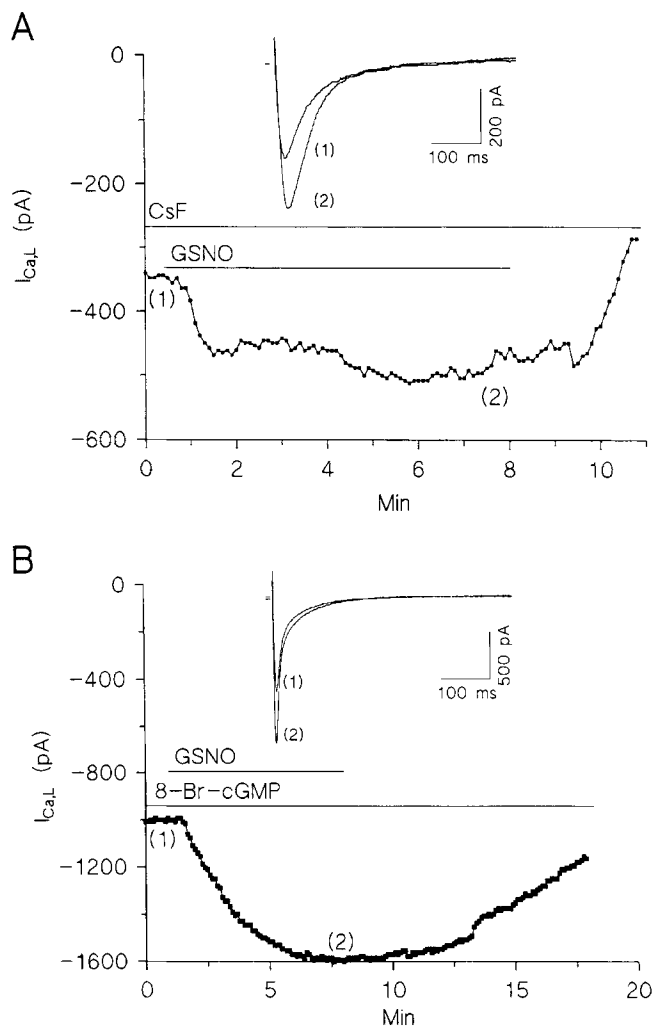


FIGURE 7. (A) GSNO and phosphatase inhibition. Representative results from one myocyte after intracellular perfusion of 120 mM CsF for 20 min (see text for details). 1 mM GSNO reversibly increased $I_{Ca,L}$ (see Fig. 8). (B) GSNO and alterations in cGMP levels. Representative results from one myocyte which had been preincubated in 100 μ M 8-Br-cGMP for \sim 10 min. 1 mM GSNO reversibly increased $I_{Ca,L}$ (see Fig. 8).

such conditions, 1 mM GSNO was still able to significantly and reversibly increase basal $I_{Ca,L}$ by $+48 \pm 7\%$ ($n = 3$ myocytes; Fig. 7 A).

Alterations in intracellular cGMP levels. GSNO could potentially stimulate $I_{Ca,L}$ by lowering intracellular cGMP levels. To test this possibility, myocytes were preincubated for ≥ 10 minutes in 100 μ M 8-Br-cGMP to allow steady-state inhibitory effects to be obtained. Since 8-Br-cGMP is relatively phosphodiesterase resistant, this maneuver should have effectively "clamped" (or at least minimized any changes in) the intracellular level of cGMP. In the continual presence of 8-Br-cGMP, 1 mM GSNO was still able to significantly and reversibility stimulate $I_{Ca,L}$ by $+73 \pm 31\%$ ($n = 5$ myocytes; Fig. 7

B). Hence, the stimulatory effects of GSNO on $I_{Ca,L}$ are not obligatorily dependent upon alterations in intracellular cGMP levels.

The mean results from all of the tests for possible indirect mechanisms (SR Ca^{2+} release, kinases, phosphatases, cGMP) are summarized in Fig. 8. The stimulatory effects of GSNO on $I_{Ca,L}$ under all of the conditions tested were very similar to those obtained under control conditions.

Possible Direct Mechanisms: Tests for an Extracellular "Redox Switch"

The opposing effects on $I_{Ca,L}$ of reduced glutathione (reducing agent) versus GSNO and $OONO^-$ (S-nitrosylating and oxidizing agents) raised the possibility that the modulatory effects of these compounds were mediated through direct redox and/or S-nitrosylation reactions at an extracellular "redox switch(es)" containing critical thiol groups (e.g., Lipton et al., 1993; Stamler, 1994; Gozlan and Ben-Ari, 1995; Lipton et al., 1996). To test this hypothesis we examined the effects of extracellular application of three thiol-specific reagents: (a) DTT (dithiothreitol), a thiol-specific reducing agent; (b) DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]), a thiol-specific oxidizing agent; and (c) NEM (*N*-ethylmaleimide), an irreversible thiol alkylating agent.

Effects of DTT and DTNB. Fig. 9, A and B show representative results obtained from two different myocytes on the effects on $I_{Ca,L}$ of 2 mM DTT and 200 μ M DTNB. Extracellular application of DTT produced a significant inhibition of $I_{Ca,L}$. Furthermore, the inhibi-

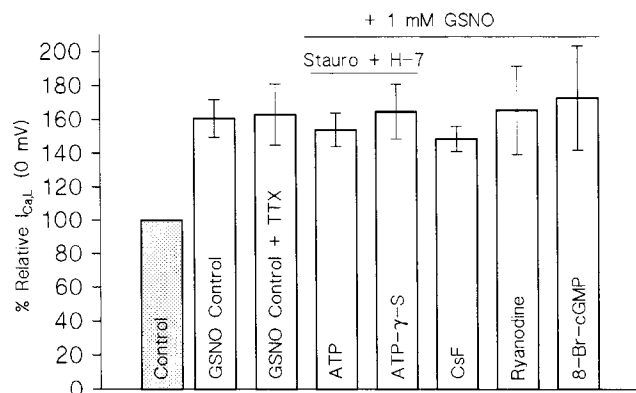


FIGURE 8. Mechanism of GSNO stimulation: summary. The stimulatory effects of GSNO on $I_{Ca,L}$ under all conditions tested were very similar to those produced under control conditions. Mean % stimulation values as follows: GSNO control, $+61 \pm 11\%$ ($n = 7$); GSNO control + TTX, $+63 \pm 18\%$ ($n = 6$); 20 μ M ryanodine, $+65 \pm 26\%$ ($n = 3$); 120 mM CsF, $+48 \pm 7\%$ ($n = 3$); staurosporine + H-7: first kinase inhibition protocol (ATP), $+54 \pm 10\%$ ($n = 6$), second kinase inhibition protocol (ATP- γ -S), $+65 \pm 16\%$ ($n = 9$); and 8-Br-cGMP, $+73 \pm 31\%$ ($n = 5$).

tory effects of DTT could be reversed by subsequent application of DTNB. In addition, DTNB also produced a significant stimulation of $I_{Ca,L}$ (Fig. 9B). Similar results (1–5 mM DTT, inhibition; 200 μ M DTNB, stimulation) were obtained in 5 myocytes. DTNB is relatively membrane impermeant (Aizenmann et al., 1989; Lei et al., 1992; Tang and Aizenmann, 1993). Therefore, its effects on $I_{Ca,L}$ may be attributed to redox reactions with sarcolemmal thiol groups. These effects therefore indicate that alteration of the redox state of sarcolemmal thiol groups can significantly modulate $I_{Ca,L}$, with thiol reduction producing down regulation and oxidation up regulation of $I_{Ca,L}$.

Effects of DTT on GSNO response. GSNO is cell impermeant. Thus its effects on $I_{Ca,L}$ more than likely occur

through action at the cell surface. To determine if the effects of GSNO could be reversed by reduction of extracellular thiol groups, the effects of DTT were explored. Fig. 9, C and D show that GSNO-mediated increases in $I_{Ca,L}$ could be partially reversed in a concentration-dependent manner by simultaneous application of 1–5 mM DTT ($n = 4$ myocytes). While these results do not demonstrate that DTT and GSNO are exerting their opposing effects through interactions at the same extracellular redox site(s), they do establish that GSNO-mediated stimulation of $I_{Ca,L}$ is reversible and mediated by interactions with extracellular sarcolemmal thiol groups. Almost certainly, GSNO catalyzes S-nitrosylation-mediated disulfide formation (Arnelle and Stamler, 1995).

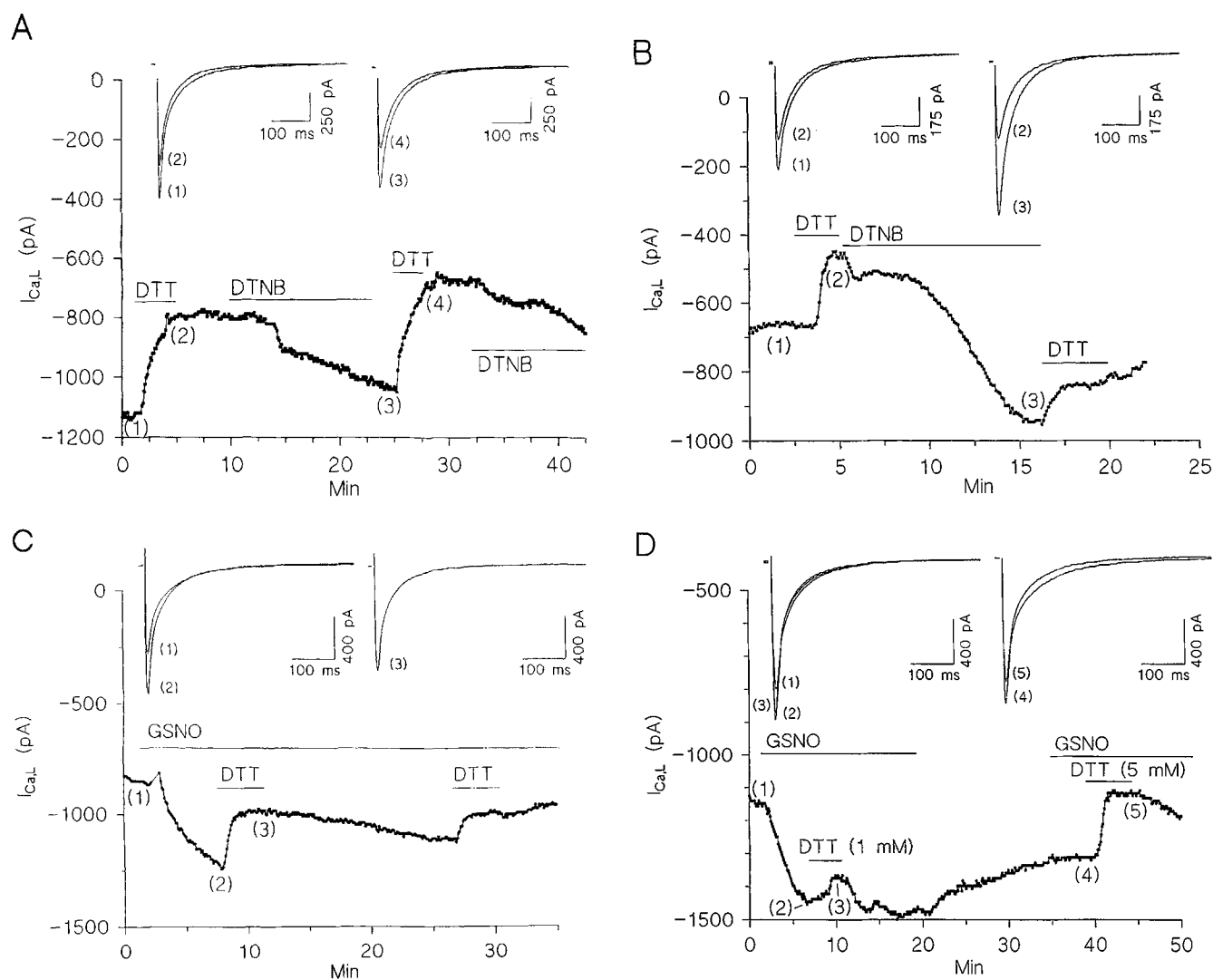


FIGURE 9. The extracellular redox site: DTT/DTNB. Effects of alterations in redox state of extracellular thiol groups by DTT (reducing agent) and DTNB (oxidizing agent; see text for details). (A and B) Effects of extracellular application of 2 mM DTT and 200 μ M DTNB. Results from two different myocytes. Sarcolemmal thiol reduction directly inhibited while thiol oxidation stimulated $I_{Ca,L}$. (C and D) Simultaneous application of 1–5 mM DTT (2 mM in C, 1 and 5 mM in D) was able to partially reverse previous stimulation of $I_{Ca,L}$ by 1 mM GSNO. Results from two different myocytes.

Effects of NEM. *N*-ethylmaleimide has been demonstrated to covalently modify protein thiol groups, thereby preventing subsequent S-nitrosylation reactions (Stamler et al. 1992a; Bolotina et al., 1994; Lipton et al., 1996). If the direct extracellular redox switch hypothesis is correct, then it should have been possible to eliminate or greatly attenuate the effects of GSNO by prior extracellular application of 1 mM NEM. The effects of NEM alone on $I_{Ca,L}$ were complicated, in that it initially significantly stimulated and then subsequently inhibited $I_{Ca,L}$ (Fig. 10 A; $n = 5$ myocytes). The early stimulation is consistent with modification of extracellular thiols, whereas later inhibition may derive from nonspecific alkylation of other (intracellular) thiol containing proteins (e.g., kinases or phosphatases; Figs. 6, 7, and 8).

Due to these complicated biphasic effects, attempts were made to obtain steady-state effects by first incubating myocytes for 20–30 min in 0.1–1 mM NEM. However, these attempts were thwarted by the fact that

NEM exposure times of >20 min resulted in significant damage to or killing of all the exposed myocytes. As a result, the following alternative protocol was applied: (a) in any given myocyte, 1 mM GSNO was first applied for ~3–5 min to obtain an estimate of control stimulation; (b) 1 mM NEM was then applied for 10–15 min to obtain approximate steady-state effects; and (c) 1 mM GSNO was then reapplied. Fig. 10, B and C show representative results obtained from two different myocytes where this GSNO-NEM-GSNO protocol was applied (a) GSNO initially stimulated $I_{Ca,L}$; (b) 1 mM NEM ultimately reversed the GSNO-mediated stimulation back to or below initial control levels; and (c) the second application of GSNO either failed to produce any effect (Fig. 10 B) or was significantly attenuated compared to the initial control stimulation (Fig. 10 C; compare to Figs. 3 A and 6 B). Similar results were observed in a total of 7 myocytes. These results strongly suggest that the stimulatory effects of GSNO on $I_{Ca,L}$ are being me-

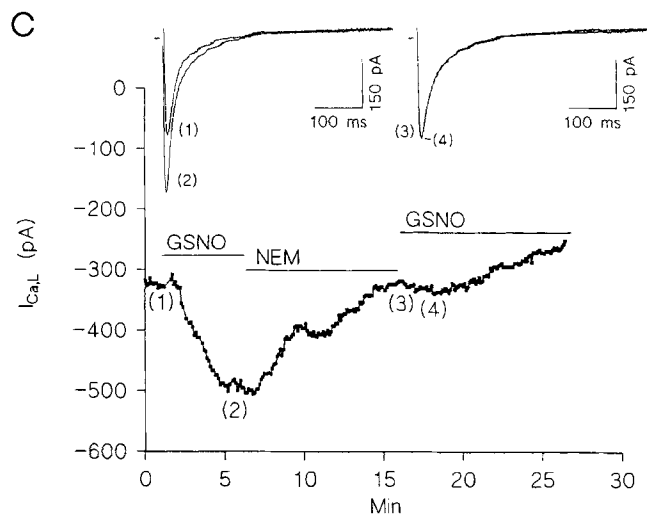
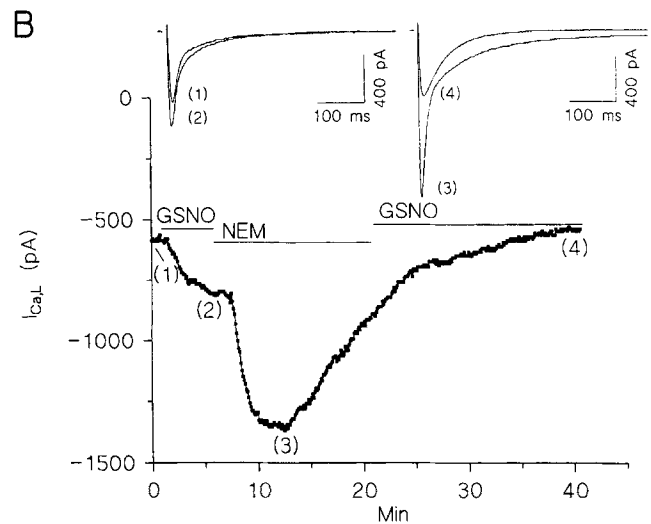
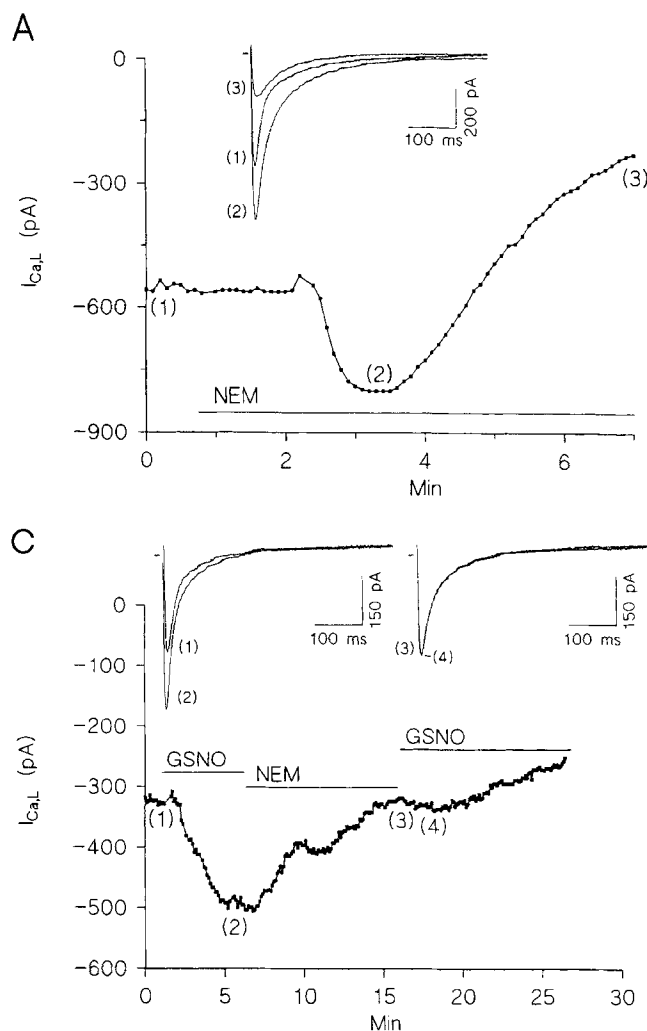


FIGURE 10. The extracellular redox site: NEM/GSNO. Effects of NEM, an irreversible thiol alkylating agent. (A) Extracellular 1 mM NEM consistently produced biphasic effects upon $I_{Ca,L}$, i.e., an initial stimulation followed by a maintained inhibition. Representative results from one myocyte. (B and C). NEM abolishes or inhibits GSNO-mediated stimulation. Results from two different myocytes using the GSNO-NEM-GSNO protocol described in the text. In both myocytes illustrated, an approximate 10–15-min exposure to 1 mM NEM was either able to completely abolish (B) or significantly attenuate (C) the stimulatory effects of a second application of 1 mM GSNO compared to the stimulation produced under initial control conditions (compare to Figs. 3 A and 6 B).

diated through interactions with an extracellular redox switch, i.e., through direct S-nitrosylation and/or disulfide reactions.

DISCUSSION

Our results indicate that there are at least two opposing signal transduction pathways by which NO-related activity ($\text{NO}\cdot$, OONO^- , RSNOs) can modulate ferret right ventricular basal $I_{\text{Ca,L}}$ (Fig. 11). Increases in cGMP inhibit $I_{\text{Ca,L}}$, whereas S-nitrosylation and/or oxidation of the channel subunit complex stimulate $I_{\text{Ca,L}}$. Direct redox modulation is not dependent upon changes in $[\text{Ca}^{2+}]_i$, kinase activation, phosphatase inhibition, or alterations in cGMP levels (although these indirect intracellular processes may be capable of modulating or altering direct effects; see below). Rather, reduction of allosteric-site thiols leads to direct down regulation of $I_{\text{Ca,L}}$, while thiol oxidation leads to direct up regulation.

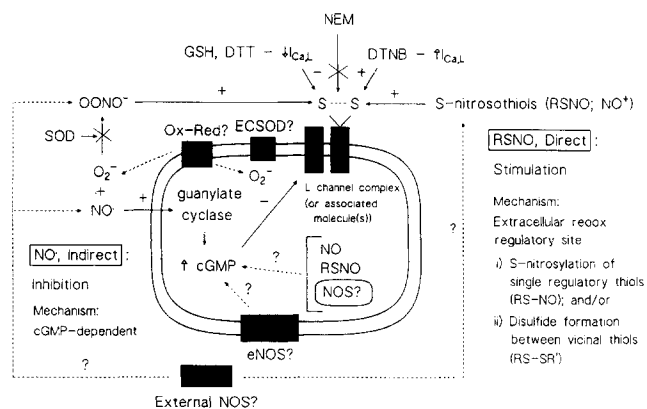


FIGURE 11. Hypothesized model for direct and indirect modulation of ventricular $I_{\text{Ca,L}}$ by NO-related activity (NO^+). +, denotes stimulation, -, inhibition, solid arrows designate established pathways, dashed arrows potential physiological pathways. NOS, nitric oxide synthase; *Ox-Red*, NAD(P)H oxidoreductases; and *ECSOD*, extracellular superoxide dismutase (Stamler, 1996). For illustrative purposes the redox site (represented schematically by the extracellular S-S domain) is shown associated with the L-channel subunit complex or another closely associated molecule(s). However, neither the exact location nor the total number of direct modulatory redox sites is presently known. This redox site(s) is accessible to extracellular reducing (GSH, DTT) and oxidizing (OONO^- , DTNB) agents, with reduction causing direct inhibition and oxidation direct stimulation of $I_{\text{Ca,L}}$. The effects of this site(s) can be abolished by alkylation of critical thiols within it by NEM. Within this framework, NO-related activity can inhibit $I_{\text{Ca,L}}$ through an indirect intracellular mechanism involving activation of guanylate cyclase, or stimulate $I_{\text{Ca,L}}$ via oxidation of the redox site(s). RSNOs promote disulfide formation by S-nitrosylation (Arnelo and Stamler, 1995). Therefore, dual site regulation by S-nitrosylation (single thiols) or oxidation (vicinal thiols) is possible. NOS activity generates NO, OONO^- , and RSNO; thus, both cellular redox state and location (i.e., cellular source) of NO synthases or NO-donors could be important factors in determining the response of cardiac myocyte L-type calcium channels to N-oxides.

The actions of endogenous NO and RSNOs will therefore be critically dependent upon both the site of synthesis and the local redox milieu (see below). To the best of our knowledge, this is the first demonstration of redox modulation of cardiac L-type calcium channels, and that NO and RSNOs can exert functionally opposite effects upon L-type (or, for that matter, any) channel function.

The exact location of this redox site(s) is at present undetermined. One obvious candidate location is the L-channel subunit complex, either the pore forming α_1 subunit, an associated auxiliary channel subunit, or a presently unrecognized auxiliary subunit (e.g., Isom et al., 1994; Hofmann et al., 1994; Mori, 1994; Chiamvimonvat et al., 1995; Campbell and Strauss, 1995; Stea et al., 1995). While the ferret ventricular α_1 subunit has yet to be cloned, the sequences of other α_{1C} subunits indicate ~ 10 – 15 cysteine residues located at numerous putative extracellular sites (e.g., Stea et al., 1995), raising the distinct possibility of multiple redox sites (see below). Regarding auxiliary subunits, the present model of the calcium channel $\alpha_2\text{-}\delta$ subunit proposes a disulfide group connecting the two subunits (Jay et al., 1991). Redox alterations at such a site could also be possible, since it has been demonstrated that coexpression of various auxiliary subunits can alter the amplitude of heterologously expressed L-channel α_1 subunits (Wei et al., 1991; Wellig et al., 1993; Pérez-García et al., 1995). Alternatively, the redox site(s) may be located on another adjacent membrane-bound molecule(s) which can directly interact with the L-channel complex upon redox activation/inactivation (Lander et al., 1993; Brown, 1993; Clapham, 1994). Furthermore, the possibility of multiple redox sites is not excluded (see below). The biphasic effects of NEM on $I_{\text{Ca,L}}$ could be interpreted as evidence for dual-site allosteric regulation.

Comparison to Previous Results

Cardiac myocytes. Our data indicate that ferret ventricular basal $I_{\text{Ca,L}}$ is directly dependent upon sarcolemmal thiol redox state, an unrecognized factor in all previous myocyte studies. Cellular redox state may therefore be an important unrecognized factor in determining the effects of many indirect, second messenger-mediated neuromodulatory compounds on cardiac myocytes, and may account, at least in part, for many of the contradictory results reported in the cardiac neuromodulatory literature (Hartzell, 1988; McDonald et al., 1994; Campbell and Strauss, 1995).

The effects of N-oxides on $I_{\text{Ca,L}}$ in isolated cardiac myocytes have been conducted almost exclusively under conditions of previous β -adrenergic stimulation or M_2 -muscarinic inhibition, and have focused exclusively upon the possible indirect effects of $\text{NO}\cdot$. Since our mea-

measurements were conducted on basal $I_{Ca,L}$, it is somewhat difficult to directly compare them with previous studies. For example, SIN-1 indirectly inhibits the β -adrenergically stimulated $I_{Ca,L}$ of bullfrog ventricular (Mery et al., 1993), rabbit SA nodal (Han et al., 1994, 1995), and guinea pig ventricular myocytes (Wahler and Dollinger, 1995). However, SIN-1 has been reported to stimulate $I_{Ca,L}$, at least under certain experimental conditions. In frog ventricular myocytes 0.1–10 nM SIN-1 was reported to have no significant effect on basal $I_{Ca,L}$ but did increase (by 40%) $I_{Ca,L}$ stimulated by a nonmaximal concentration (0.3 μ M) of forskolin (Mery et al., 1993). In guinea pig ventricular myocytes 10–100 μ M SIN-1 was reported to increase basal $I_{Ca,L}$ by 8–24%, while in the presence of 10 nM isoproterenol the effects of 10 μ M SIN-1 were complicated in that it could either stimulate or inhibit $I_{Ca,L}$ depending upon the magnitude of the isoproterenol-induced stimulation (Wahler and Dollinger, 1995). None of these previous studies considered direct effects, and most did not appreciate that SIN-1 produces mainly OONO⁻ (not NO[•]); nonetheless, they emphasize that the effects of SIN-1 are likely to be redox state dependent, and raise the possibility that NO-mediated effects on $I_{Ca,L}$ may also be modulated or altered by second messenger-mediated and/or phosphorylation reactions.

Our finding that NO-related activity can inhibit $I_{Ca,L}$ through an indirect cGMP-dependent mechanism is in agreement with numerous previous reports. However, the effects of RSNOs or cellular redox state on $I_{Ca,L}$ (either basal, β -adrenergically stimulated, or M_2 -muscarinic inhibited) have not been adequately studied in other isolated myocyte systems. For example, Mohan et al. (1996) report both stimulatory and inhibitory effects of NO on contractility of feline right ventricular papillary muscles. The applicability of our results to $I_{Ca,L}$ in other cardiac myocyte types therefore awaits future determination. Nonetheless, our results clearly indicate that under basal conditions NO[•] and RSNOs can exert discrete and opposite effects upon $I_{Ca,L}$. Therefore, the assumption that NO only exerts physiologically relevant indirect effects on cardiac $I_{Ca,L}$ through activation of guanylate cyclase clearly needs to be revised.

Other cell types and ion channels. The effects of extracellularly accessible sulfhydryl modification on rabbit lung smooth muscle L-type Ca^{2+} channel α_1 subunits expressed alone appear to be significantly different from those exerted on native right ventricular L-type channel subunit complexes. Specifically, sulfhydryl reduction (DTT) had no effect on currents generated by α_1 subunits; however, sulfhydryl oxidation significantly inhibited currents, and this inhibition could then be reversed by DTT (Chiamvimonvat et al., 1995). These differences may arise from either additional redox-sensitive

interactions with auxiliary β and/or $\alpha_2\text{-}\delta$ subunits in native myocytes, posttranslational modifications, and/or $\beta/\alpha_2\text{-}\delta$ subunit interactions that induce conformational changes in the α_1 subunit (and vice versa) which alter redox responses. While these results indicate the presence of redox-sensitive modulatory sites on the L-type Ca^{2+} channel α_1 subunit (Chiamvimonvat et al., 1995), they also emphasize the difficulties associated with extrapolating physiological function from simplified expression systems in which only individual channel subunits are expressed. It is interesting to note that another recent study has demonstrated significant differential oxygen sensitivity of native L-type calcium channels in rabbit smooth muscle cells isolated from pulmonary arterial conduit and resistance vessels (Franco-Obregón and López-Barneo, 1996). Specifically, hypoxia inhibited $I_{Ca,L}$ in proximal (conduit) smooth muscle myocytes but stimulated $I_{Ca,L}$ in distal (resistance) smooth muscle myocytes. The molecular mechanisms underlying this differential oxygen sensitivity are presently unknown (e.g., different channel isoforms and/or different auxiliary subunits?). Nonetheless, these results demonstrate that vascular smooth muscle $I_{Ca,L}$ can be selectively modulated by redox state and raise the distinct possibility of significant regional and/or tissue differences in redox-sensitive modulatory properties of L-type calcium channels in the cardiovascular system.

A direct, cyclic-GMP-independent modulation by "NO" of the charybdotoxin-sensitive Ca^{2+} activated K^+ channel of rabbit aortic smooth muscle has been described (Bolotina et al., 1994). Similar to our results, this direct NO-mediated inhibition could also be blocked by NEM, suggesting involvement of an extracellular thiol-containing redox switch. It has been hypothesized that direct activation of this K_{Ca} channel derives from the ability of protein RSNO to promote disulfide formation between vicinal thiols (Stamler, 1994; Arnelle and Stamler, 1995). A similar mechanism has also been hypothesized for NO-mediated direct regulation of type I adenylyl cyclase (Duhe et al., 1994), protein kinase C (Gopalakrishna et al., 1993), and the NMDA receptor (Lipton et al., 1993).

Redox modulation of native and cloned neuronal NMDA receptors has been extensively studied. DTT stimulates, while both DTNB and S-nitrosylation inhibit the NMDA receptor (Aizenmann et al., 1989; Kuryatov et al., 1994; Pan et al., 1995). Therefore, under normal oxygenated conditions, NMDA receptor-mediated Ca^{2+} influx into neurons would be attenuated, while $I_{Ca,L}$ -mediated Ca^{2+} influx into ventricular muscle would be enhanced. Recent mutagenesis studies have also identified at least three separate NMDA receptor subunit sites with different reactivity profiles to DTT, DTNB, and NEM (Köhr et al., 1994; Sullivan et al., 1994;

Omerovic et al., 1994, 1995), suggesting multiple processes involving several pairs of cysteine residues. This raises the possibility that redox modulation of ventricular $I_{Ca,L}$ may involve several distinct redox sites, possibly located on different molecules. This question can, in principle, be addressed in future studies combining cellular, biophysical, and molecular biological techniques.

Possible Pharmacological and Physiological Implications

“NO Donor” compounds. The identification of a putative extracellular redox site can explain the variable effects of SIN-1 on $I_{Ca,L}$ observed in previous studies. Depending upon the redox conditions of any given myocyte, the effects of SIN-1 could vary from inhibition ($NO\cdot$) to stimulation ($OONO^-/RSNO$). We also studied the effects of diethylamine-NO (DEA-NO) and spermine-NO, which produce $NO\cdot$ (Feelisch and Stamler, 1996). DEA-NO consistently stimulated while spermine-NO consistently inhibited $I_{Ca,L}$ ($n = 5$ myocytes each), effects which were duplicated by the carrier molecules alone (DEA, spermine, respectively). Thus, it would appear that the cardiac L-type calcium channel may have a modulatory polyamine site(s), similar to that described for the NMDA receptor (Johnson, 1996). In summary, the effects of many NO-donor compounds reported in the cardiac literature need to be carefully reevaluated.

S-nitrosothiols. The effects of endogenous RSNOs have been almost exclusively attributed to assumed homolytic (free radical) cleavage mechanisms: $RSNO \rightarrow RS\cdot + NO\cdot$. However, the pharmacological and physiological profiles of numerous biologically relevant RSNOs do not correlate with charge, lipophilicity, stability in solution, or rates of spontaneous $NO\cdot$ release (Stamler, 1994, 1995). These results suggest that in biological systems the effects of RSNOs will be dependent upon site(s) of production and are exerted in significant part through heterolytic cleavage reactions: $RSNO \rightarrow RS^- + NO^+$. We have used RSNOs (GSNO, CySNO) as exemplary NO^+ transferring compounds to demonstrate that S-nitrosylation can produce direct stimulatory effects on cardiac $I_{Ca,L}$ distinct from inhibitory effects mediated by cGMP. Our results highlight that direct stimulation of cardiac $I_{Ca,L}$ is likely to be produced by other endogenous nitrosating species.

Physiological implications. Both the atria and ventricles possess an extensive endocardial endothelium which produces NO, possibly on a beat-to-beat basis (Schulz et al., 1991; Shah and Lewis, 1993; Siney and Lewis, 1993; Disriol et al., 1995). It has been recently reported that in feline right ventricular papillary muscle the effects of both cGMP and various NO-related agents (S-nitroso-N-acetyl-penicillamine, SIN-1, sodium nitroprusside) could be modulated by the status of the endocardial endothelium, and that these NO-related agents could produce

in a concentration-dependent manner both positive and negative inotropic effects (Mohan et al., 1996). While endocardial endothelium removal has been reported not to alter ferret papillary muscle action potential duration (Shah et al., 1992), and its effects have been mainly attributed to (presently unidentified) diffusible substances which appear to increase myofilament Ca^{2+} sensitivity (Wang and Morgan, 1992; Shah and Lewis, 1993), our results nonetheless raise the distinct possibility that the effects of the endocardial endothelium on cardiac function may also be mediated, at least in part, through endogenous RSNOs or NO-related oxidizing agents acting on $I_{Ca,L}$.

Myocardial cells can also be exposed to N-oxides produced by intracardiac nerve terminals, smooth muscle, coronary endothelium, and various circulating cell types (Ignarro, 1990; Moncada et al., 1991; Klimaschewski et al., 1992; Dinerman et al., 1993; Michel and Smith, 1993; Schmidt and Walter, 1994; Tanaka and Chiba, 1995). Some cardiac myocytes also possess an endogenous constitutive NO synthase (e.g., rabbit SA-node cells: Han et al., 1994, 1995; rat ventricular myocytes: Balligand et al., 1995; Kanai et al., 1995) and a cytokine inducible NOS (Brady et al., 1992; Finkel et al., 1992; Balligand et al., 1994; Haywood et al., 1996). The physiological significance of many of these findings is presently unclear. However, our data suggest that the both the location (i.e., cellular source) from which N-oxides and RSNOs are generated and the local redox environment could be important factors in determining myocyte response to N-oxides. For example, if NO/RSNOs are produced intracellularly within cardiac myocytes, the inhibitory effects of cGMP may predominate. Alternatively, if NO/O_2^- are generated either at the sarcolemmal surface or from other cellular sources (e.g., endothelial cells, neurons, circulating cells) stimulatory NO^+ transfer and/or oxidation reactions are likely to dominate. As a result, the activity of both membrane bound NAD(P)H oxidoreductases (which generate O_2^-) and extracellular superoxide dismutases (ECSODs; Stamler, 1996) could also be important factors (schematically depicted in Fig. 11). The possible roles of these enzyme systems in cardiac NO-related responses is presently unknown.

Finally, a redox site directly modulating $I_{Ca,L}$ could also be of immediate relevance under pathological conditions. Ischemic conditions would produce a more reducing extracellular environment, which would lead to direct inhibition of $I_{Ca,L}$. Such an effect could be cardioprotective by reducing the energy requirements of ischemic myocytes, as well as reducing the harmful effects of released catecholamines. Our results also raise the interesting possibility of physiologically relevant $NO\cdot/O_2^-$ effects during periods of increased cardiac activity resulting from contraction-associated localized

production of O_2^- (Reid et al., 1992; Consentino et al., 1994) or reperfusion injury. In particular, the opposite effects of NO and $OONO^-$ on $I_{Ca,L}$ suggest that under certain conditions NO may actually increase contractility if synthesized concomitantly with O_2^- . However, such effects could also become potentially arrhythmogenic.

Summary

We find that: (a) both cGMP-dependent and redox (S-nitrosylation and/or thiol oxidation) dependent mecha-

nisms are involved in modulation of ferret right ventricular $I_{Ca,L}$; (b) NO-related activity can exert both inhibitory and stimulatory effects upon $I_{Ca,L}$; and (c) Ca^{2+} homeostasis in ventricular myocytes is directly and selectively regulated by cellular redox state. These findings strongly suggest that direct redox modulation of sarcolemmal L-type calcium channels is an important and physiologically relevant mechanism for modulation of mammalian cardiac function.

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