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Original Research Communication

Intracellular Effects of Nitric Oxide on Force Production and Ca^{2+} Sensitivity of Cardiac Myofilaments

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ABSTRACT

The gaseous free radical nitric oxide ($NO^{\cdot}$) has been implicated in a wide range of physiological functions and also has a role in the pathogenesis of cellular injury. It has been suggested that $NO^{\cdot}$ and its congeners may exert effects on actin-myosin crossbridge cycling by modulating critical thiols on the myosin head. To understand the mode and site of actin of $NO^{\cdot}$ in myofibrils, the effects of the $NO^{\cdot}$ donor 3-(2-hydroxy-1-methyl-2-nitrosohydrazine)-*N*-methyl-1-propanamine (NOC-7) have been studied in Triton X-100-treated rabbit cardiac fibers, in which isometric force was measured at controlled degrees of activation. Experiments were undertaken after previous exposure of the preparations to NOC-7 (for 30 min). We found that $NO^{\cdot}$ induced several alterations of myofibrillar function, *i.e.*, decrease in Ca^{2+} sensitivity and Hill coefficient and potentiation of rigor contracture. We attributed the effect on rigor contracture to strong inhibition of myofibrillar creatine kinase (CK) activity, because it could be prevented by exogeneously added CK; such CK inactivation afforded by $NO^{\cdot}$ may result in the myofibrillar ATP-to-ADP ratio. In further experiments, concentration of $NO^{\cdot}$ released from NOC-7 was determined by the electron spin resonance spin-trapping technique; *N*-(dithiocarboxy)sarcosine-Fe^{2+} complex was used as the spin-trap. $NO^{\cdot}$ at cumulative concentration of 0.69 μM was effective in producing both enhancement of rigor contracture and decrease of myofibrillar-bound CK activity; however, Ca^{2+}-sensitivity (pCa_{50}) was significantly decreased at >5.6 μM of $NO^{\cdot}$, suggesting a result from different mechanisms. Thus, the observed decrease in Ca^{2+} sensitivity seems to be associated with direct modification of the regulatory proteins by a relatively higher concentration of $NO^{\cdot}$, and possibly not via inhibition of myofibrillar CK activity. The data reported here indicate that CK may be a pathophysiologically main target for increased $NO^{\cdot}$ formation at low molecular range in the disease state in cardiac muscle. Antiox. Redox Signal. 1, 509–521.

INTRODUCTION

THE GASEOUS FREE RADICAL NITRIC OXIDE ($NO^{\bullet}$) has been implicated in a wide range of physiological functions, including endothelium-dependent relaxation of blood vessels, chemical communication between peripheral nervous and smooth muscle, and long-term modifications of the efficacy of central synapses (Garthwaite, 1991; Moncada *et al.*, 1991). In most $NO^{\bullet}$-regulated tissues, the enzyme responsible for $NO^{\bullet}$ synthesis is present in the tissue itself or in a proximate tissue, and there are $NO^{\bullet}$-responsive targets present in the tissue. One such target is soluble guanylate cyclase, which is activated by $NO^{\bullet}$ (Arnold *et al.*, 1977) to increase production of guanosine 3′,5′-cyclic monophosphate (cGMP). Cardiac muscle expresses a constitutive $NO^{\bullet}$ synthase enzyme, and exogenously produced $NO^{\bullet}$ was shown to reduce developed force through a cGMP-dependent pathway (Finkel *et al.*, 1992; Kobzik *et al.*, 1994). In cardiac muscle, exogenously added $NO^{\bullet}$ produced negative inotropic effects (Finkel *et al.*, 1992; Balligand *et al.*, 1993) but recent re-examination of this issue produced op-

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posite results (Mohan *et al.*, 1996). When the endothelial effects of NO• were inhibited, low NO• (*i.e.*, physiological) concentrations produced a positive inotropic effect whereas negative inotropy could only be attained at concentrations of cGMP or NO•, too high to be considered of physiological significance (Mohan *et al.*, 1996). Thus, the mechanisms underlying NO•'s actions appear to be multifaceted and cannot always be uniquely attributed to cGMP-dependent mechanisms. NO• may directly modulate cardiac function by protein modification, via transnitrosylation or oxidation of cysteine thiol residues (Stamler, 1994). Similarly, many of the NO• effects on cardiac muscle, especially when NO• is generated from NO• donors, are not always related to altered cGMP levels (see Kelly *et al.*, 1996, and references therein).

The isolated heart displays remarkable ATP homeostasis. Cardiac performance and oxygen consumption can vary by nearly an order of magnitude, yet myocardial ATP content remains virtually constant. Myocardial ATP is maintained at a constant level primarily by two chemical reactions. Mitochondrial oxidative phosphorylation synthesizes most of the ATP in heart (Brdiczka and Wallimann, 1994; Saks *et al.*, 1996), and the creatine kinase (CK) reaction (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) resynthesizes the ATP that breaks down during cardiac contraction:

$$\text{Phosphocreatine (PCr)} + \text{ADP} \rightleftarrows \text{creatine} + \text{ATP} \quad [1]$$

Proposed functions of the cellular CK systems include temporal energy buffering, regulation of oxidative phosphorylation, and the transport of chemical potential, in the form of PCr, between sites of ATP production and energy utilization (Saks *et al.*, 1996). Inhibition of myocardial CK compromises contractile function (Tian and Ingwall, 1996); decreased CK activity in the heart has been also implicated in the pathogenesis of cardiomyopathies and heart failure (Saks *et al.*, 1991).

The active site of CK contains a cysteinyl residue that is essential for substrate binding. Thus, CK is susceptible to inactivation by oxidative reactions (Mekhfi *et al.*, 1996). Gross *et al.* (1996) have also shown that an exogenous NO• donor, *S*-nitrosoacetylcysteine, could markedly suppress the ability of isolated rat hearts to recruit contractile reserve in response to a high Ca^{2+} challenge. This was accompanied by a fall in ATP and the unexpected maintenance of PCr content at control levels, suggesting that phosphoryl transfer between PCr and ATP by CK had been inhibited. It remains debatable, however, whether increased NO• concentration at low micromolor range (*i.e.*, ~5 μM) in disease states (when inducible NO• synthase is activated; Kelm and Schrader, 1990) actually participates in myocardial dysfunction.

There are numerous sites within the myocardium that are susceptible to oxidative damage, many of which are intimately involved with the processes of excitation-contraction coupling (*e.g.*, sarcolemmal ion channels and pumps, the sarcoplasmic reticulum, and the contractile proteins). The presence of diffusion barriers, endogenous generators, and scavengers will complicate study of NO• in the intact heart. The skinned fiber technique leaves the contractile structures of myofilaments intact and exposed to the bathing medium. This allows for controlled exposure of the myofilaments to NO• and was used to determine the effects of NO• on maximum Ca^{2+}-activated force ($F_{ca,max}$), resting tension, the Ca^{2+} sensitivity of the contractile proteins and crossbridges in rigor, and on the ability of endogenous myofibrillar-bound CK to rephosphorylate ADP In addition to the evaluation of the functional parameters, electron spin resonance (ESR) spectroscopy was used to monitor the concentration of NO• released from the NO• donor 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC-7) under the experimental system used.

In view of the possibility that pathological concentration of NO• might be involved in myocardial dysfunction, we have now investigated the actual concentration-dependent effect of NO• on the organelles of contraction in myocardium. Here we report that NO• at low micromolar range can modulate myocardial contractile function through modification of CK activity. On the basis of this finding, we

propose that a direct effect of NO• on the myofilaments is likely involved in eliciting the NO•-induced myocardial contractility changes.

MATERIALS AND METHODS

The investigations described here conform with the guide for care and use of laboratory animals published by the U.S. National Institute of Health (NIH Publication NO. 85-23, revised 1985).

The rationale for solution composition, the method of calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, precautions for ethyleneglycol *bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) purity, calcium contamination determination, the measurement of pH, and other details of our experimental protocol have been described elsewhere (Miller and Smith, 1984; Harrison *et al.*, 1988). Experiments were carried out at room temperature (20–22°C).

ESR analysis

Release of NO• from NOC-7 (Dojindo Laboratories, Kumamoto, Japan) was verified by ESR spectroscopy. ESR detection of spin adducts was performed using a JES-FR 100, X-band spectrometer (JEOL, Tokyo) at the following instrument settings: microwave power, 4 mW; magnetic field, 329.990 ± 10 mT; modulation amplitude, 0.2 mT; time constant, 0.03 sec; sweep time, 1 min; and receiver gain, 3.2 × 100. *N*-(dithiocarboxy)sarcosine (DTCS)-Fe^{2+} was used as the spin trap. The disodium salt of DTCS was synthesized through a direct reaction of sarcosine with carbon disulfide, according to a method for the synthesis of the *N*-methyl-D-glucamine dithiocarbamate-sodium salt (Shinobu *et al.*, 1984). DTCS-Fe^{2+} complex was prepared by anaerobically dissolving DTCS disodium salt together with $FeSO_4 \cdot 7H_2O$ in N_2-bubbled saline solution (Fe concentration [Fe] = 50 m*M*; [DTCS]/[Fe] = 3). Desired reaction mixtures (1 ml) were prepared in glass tubes and transferred to a quartz ESR flat cell (0.2 ml), which was in turn placed in the cavity of the ESR spectrometer. Sequential ESR scans were then started 60 sec after the addition of DTCS-Fe^{2+} to the reaction mixture. NO• concentration detected as NO•-DTCS-Fe^{2+} was determined by a double-integration of ESR spectrum, in which 1.0 μ*M* of a 4-hydroxyl-2,2,6,6,-tetramethylpiperidinyloxy (TEMPOL; Aldrich Chem. Co., Milwaukee, WI) solution was used for a primary standard of ESR absorption.

Isolation of ventricular trabeculae

Male New Zealand White rabbits (2.0–2.5 kg) were anesthetized with pentobarbital sodium (50 mg/kg, i.v.); the heart was excised rapidly and flushed with Ringer's solution (in m*M*: 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.0) at room temperature (~22°C); then a trabecula was isolated from the right ventricle. Trabeculae of appropriate size (<200 μm diameter, 2–3 mm long) were commonly situated close to the tricuspid valve annulus or occasionally at the right ventricular apex.

Mounting for isometric force measurement

The preparation was mounted for isometric force measurement by snaring at either end (using nylon monofilaments) between two stainless steel tubes (200 μm o.d. and 100 μm i.d.). The tubes were fixed to a force transducer (Minebea, UL-2GR, Tokyo, Japan) at one end and a fixed point on the other. Sarcomere length was measured by modified uplight microscope and set to 2.1–2.2 μm.

Chemical skinning procedure and tissue activation

Once the muscle had been mounted, it was exposed to a "relaxing" solution (solution A, Table 1) containing nonionic detergent Triton X-100 (1% [vol/vol]) for 20–30 min. This regimen is sufficient to ensure the complete destruction of surface and intracellular membrane diffusion barriers. The chemical skinning agent was then removed by washing in fresh "relaxing" solution. The preparation was acti-

TABLE 1. COMPOSITION OF SOLUTIONS (IN mM EXCEPT WHERE STATED; pH 7.0)

Solutions	*K*[a]	*Mg*[b]	Ca_{total}	*pCa*	*ATP*	*PCr*	*Na*[c]	*EGTA*	*HEPES*
A	140	7	0.02	9.03	5	15	40	10	25
B	140	7	10	4.25[d]	5	15	40	10	25
C	140	7	0.02	7.29	5	15	40	0.2[e]	25
D	140	2.2	0.02	9.03	5	—	40	10	25
E	140	2.2	0.02	9.03	—	—	40	10	25
F	140	2.2	0.02	9.03	—[f]	15	40	10	25

[a]Potassium ions added as KCl and KOH.
[b]Magnesium added as 1 *M* $MgCl_2$, free Mg^{2+} = 2.1–2.5 m*M* in solutions A, B, and mixture of these solutions.
[c]Sodium ions from the salts Na_2ATP and Na_2PCr.
[d]An additional 0.1 m*M* $CaCl_2$ was added to aliquots of this solution to yield "full activating" solution (pCa 4.0). Total chloride concentrations varied from about 110 to 120 m*M*.
[e]1,6-Diaminohexene *N,N,N',N'*-tetraacetic acid (9.8 m*M*) was added to this solution to maintain equivalent ionic strength.
[f]5 m*M* ADP was substituted for ATP in this solution.

vated to produce tension by increasing the free $[Ca^{2+}]$ in the solution. Myofilament Ca^{2+} sensitivity was assessed by altering the $[Ca^{2+}]$ over the appropriate range (test solutions were made by mixing solutions A and B, Table 1). Each test $[Ca^{2+}]$ was applied after prior equilibration of the preparation in solution A to activate the muscle as rapidly and uniformly as possible. We also studied the prior exposed preparation to NO• released from NOC-7 in the rigor state (*i.e.*, during the very strong interaction between actin and myosin filaments that develops in the absence of ATP even at very low calcium concentration). In these experiments, the preparation was initially relaxed in a solution without PCr (solution D, Table 1) to prevent creatine phosphate contamination of the "rigor" solutions (which would otherwise continue to phosphorylate ADP generated by crossbridges from the residual ATP carried over in the preparation when the solution change was made). The rigor contracture was subsequently induced by exposing the preparation to a similar solution without ATP (solution E, Table 1). Each rigor-inducing solution was only applied once to avoid any risk of cumulative ATP contamination. The $MgCl_2$ was reduced in the "rigor" solution to ensure the same free magnesium concentration as the relaxing solution. In some experiments, the ability of endogenous myofibrillar-bound creatine kinase to rephosphorylate ADP was assessed by substituting ADP for ATP (solution F, Table 1).

Preparation and application of NO•

In the present experiments, NO• was only applied once to the preparation. Application of NO• was made to trabeculae during maximal Ca^{2+}-activation for 30 min before assessing myofilament Ca^{2+}-sensitivity and the rigor state. NOC-7 (dissolved in 2 μM NaOH) solution was prepared immediately prior to use.

Data collection and analysis

The output signal of the tension transducer was digitized at appropriate rates using a commercial data acquisition system (MacLab, AD Instruments Pty, Ltd., New South Wales, Australia) and commercially available Chart software. These data can be transferred to Macintosh data handling and graphics programs such as Igor, Excel, and MacDraw II. The relationship between $[Ca^{2+}]$ and tension in our experiments is well described by the Hill equation. Curves were fitted by a least-squares fitting procedure after Levenberg and Marquardt (Brown and Dennis, 1972). This yields values for $K_{1/2}$, the reciprocal of the $[Ca^{2+}]$ producing half-maximum F_{Ca}, and h, the Hill coefficient. To avoid the distortions produced by pooling experimental data, curves were fitted on an individual basis so that the $K_{1/2}$, and any shift, could be determined for each preparation. The Hill coefficients were treated in the same way. $F_{Ca,max}$ is the tension response to pCa 4.0 (which would normally elicit a maximum tension response), and was measured us-

ing solution B (Table 1) with excess calcium added (providing a final pCa of 4.0). Tension responses are shown calibrated in either absolute or relative force. The passive tension induced by stretching the preparation to obtain a sarcomere length of 2.1–2.2 μm was taken as initial resting tension. Absolute force is expressed as the tension generated by the preparation from this initial baseline (independent of an experimental intervention). Active force is taken as the tension generated by the preparation from the resting tension subsequent to an experimental intervention (*i.e.*, absolute tension minus any increase in resting tension). Absolute and active tensions were normalized by being expressed relative to the tension initially obtained at $F_{Ca,max}$. This was determined from five to eight independent control activations made prior to the exposure of the preparation to NO•. Statistical analysis was performed using paired and nonpaired *t*-tests. Significance was taken at the 5% level.

RESULTS

Figure 1 shows experimental protocol for assessing the effects of incubation with NOC-7 for 30 min at full activation state in response to pCa 4.0 on resting tension, the Ca^{2+} sensitivity of the contractile proteins and crossbridge in rigor of the preparations.

Concentration of NO• released from NOC-7

Released NO• concentration from NOC-7 under the same reaction conditions (pCa 4.0 solution, see Fig. 1B and legend for Table 1) as those of isometric force measurement for the ventricular trabeculae (except that the preparation was omitted) was determined by using highly sensitive ESR spectroscopy and spin-trap DTCS-Fe^{2+}. Iron complexes with dithiocarbamate derivatives are commonly used as spin-trapping reagents for NO• because NO• has a high affinity for the iron complexes. The resultant nitrosyl iron complexes exhibit an intense three-line ESR signal. DTCS is fairly stable in air and aqueous media (Yoshimura *et al.*, 1996); thus the water-soluble iron complex is a biologically benign, effective spin-trapping reagent for ESR analysis.

Figure 2B shows the time-dependent ESR spectral change in response to the addition of NOC-7 (1 m*M*). The NOC-7 gradually released NO• when it was added to the bathing medium (pCa 4.0, pH 7.0); the triplet ESR signal (g = 2.040), which is identified as the NO•-DTCS-

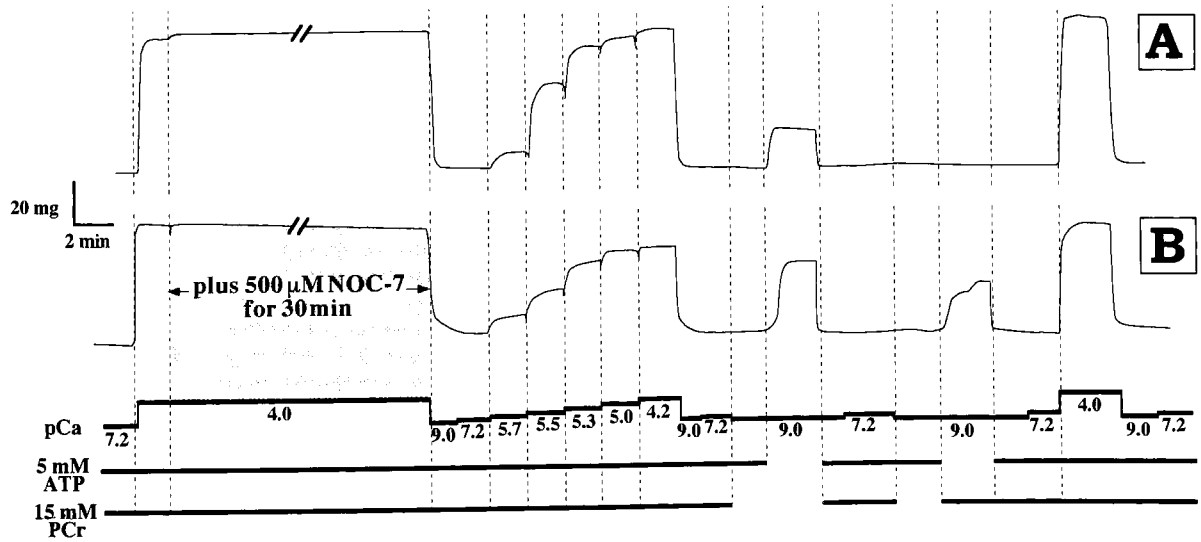

FIG. 1. Experimental traces illustrating the effect of previous exposure to NOC-7 (B, 500 μM for 30 min) on Ca^{2+}-sensitivity (to pCa 5.7–4.2, and to pCa 4.0 to obtain $F_{ca,max}$), rigor contracture (induced by removing ATP from the bathing medium) and on the ability of endogenous myofibrillar-bound CK to rephosphorylate ADP (assessed by substituting ADP for ATP, see Materials and Methods for experimental detail). A time-matched control study without NOC-7 exposure **(A)** was also performed. Two Triton X-100-treated skinned preparations (for time-matched control and NOC-7 exposure) obtained from the same cardiac muscle were studied in parallel. Tracings are representative of five experiments. Gaps in the trace indicate 15-min period in pCa 4.0 activating solution.

Fe^{2+} complex, was detected and its intensity (Fig. 2B) and concentration (Fig. 2A) linearly increased in a time-dependent manner. The spin concentration (determined by a double-integration of the ESR spectrum, in which 1.0 μM of TEMPOL solution was used for a primary standard of ESR absorption) of the formed NO•-DTCS-Fe^{2+} was also increased in a NOC-7 concentration-dependent fashion (Fig. 3). The cumulative NO• concentration up to 30 min was 119.0, 36.1, 5.6, 0.69, and 0.44 μM at 1,000, 500, 50, 5 and 3 μM of NOC-7 concentrations, respectively (Fig. 3).

To assess the accuracy of our method, purified NO• gas was bubbled into the solution containing DTCS-Fe^{2+} complex for 10 min (excess NO• was removed by bubbling pure N_2 gas through the solution), and used for the ESR detection. Detected NO•-DTCS-Fe^{2+} triplet ESR signal was similar to that produced by NOC-7; NO• concentration (determined as spin concentration of NO•-DTCS-Fe^{2+}) was fit well with the authentic NO• saturation-temperature curve ranging from 5 to 37°C (data not shown).

$F_{Ca,max}$ *and resting tension*

Exposure of Triton-skinned trabeculae to NOC-7 (1, 3, 5, 50, 500, and 1,000 μM) for 30 min had no effect on $F_{Ca,max}$ and resting tension (for the effect of 500 μM NOC-7, see Fig. 1B). Even at a concentration of 1,000 μM, there was no effect of NOC-7 exposure for 30 min (gives 119.0 μM of cumulative NO• concentration; $n = 5$ preparations, data not shown).

Ca^{2+}*-sensitivity*

We tested the possibility that NO• alters Ca^{2+} sensitivity by studying submaximal Ca^{2+} activation in the Triton-skinned preparations. Figure 4 illustrates the effect of NO• released from NOC-7 (1–1,000 μM) on myofilament

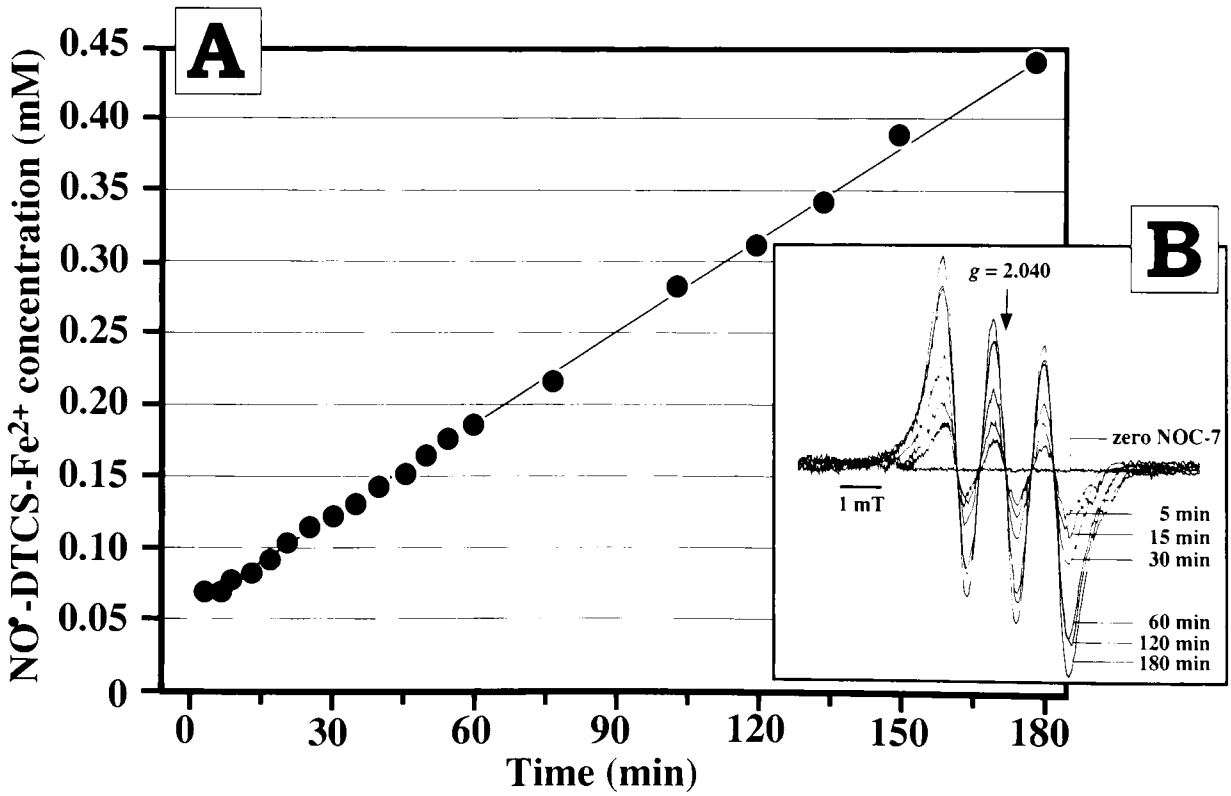

FIG. 2. Time-dependent increase in NO•-DTCS-Fe^{2+} spin concentration (A) and ESR spectral change (B) in response to the addition of NOC-7 (1 m*M*). ESR spectra were recorded in the same reaction medium as that of pCa 4.0 solution for the isometric force measurement experiment described in Fig. 1, except that the Triton-skinned preparations were omitted. The concentration of NO•-DTCS-Fe^{2+} was determined as described in the text. The DTCS-Fe^{2+} complex reacted with NO• released from NOC-7 gives a triplet ESR signal of the NO•-DTCS-Fe^{2+} complex. The signal is increased in a time-dependent manner after the addition of NOC-7.

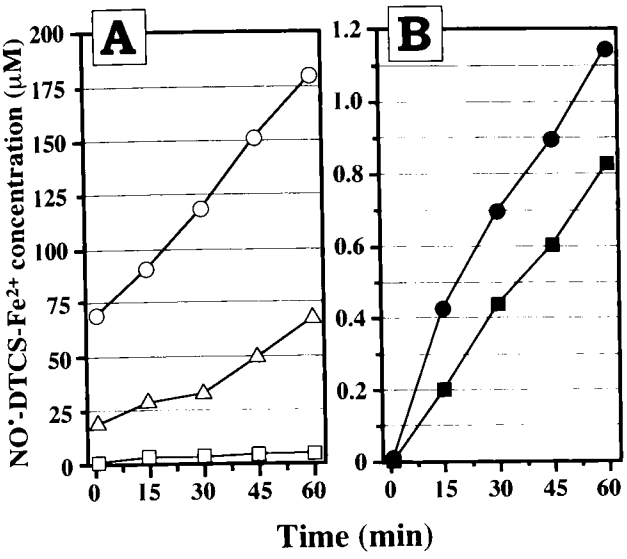

FIG. 3. Concentration-dependent increase in NO•-DTCS-Fe^{2+} spin concentration for 60 min in response to the addition of NOC-7. A. (○) 1 m*M*; (△) 500 μ*M*; (□) 50 μ*M*. **B.** (●) 5 μ*M*; (■) 3 μ*M*. Experimental conditions were identical with those described in the legend of Fig. 2 except 3–1,000 μ*M* NOC-7 was used and the NO•-DTCS-Fe^{2+} concentration was followed for 60 min.

Ca^{2+} sensitivity (for experimental protocol, see Fig. 1). The best-fit curves obtained in the presence of 2 μ*M* NaOH, a vehicle for NOC-7 (1–1,000 μ*M*), were superimposable (Fig. 4A), indicating that NaOH itself tested produced no significant alteration in myofilament Ca^{2+} sensitivity and little change in $F_{Ca,max}$. Exposure of the preparations to NOC-7 elicited a shift of the Ca^{2+}-tension curve to the right in a concentration-dependent fashion (Fig. 4B); pCa_{50} was also decreased by NOC-7 in a concentration-dependent manner (Fig. 5A). Furthermore, NOC-7 produced a concentration-dependent decrease in *h*, binding cooperativity between troponin C and Ca^{2+} (Fig. 5B).

Crossbridges in rigor and endogenous CK activity

Having identified the ability of NO• to elicit inhibition of Ca^{2+} sensitivity of the myofilament with little effect on $F_{Ca,max}$ and resting tension, we next tested NO• on preparations in rigor to reveal whether NO• affects active or nonactive crossbridges, the thin filament site of actin–myosin interaction, or provokes a non-

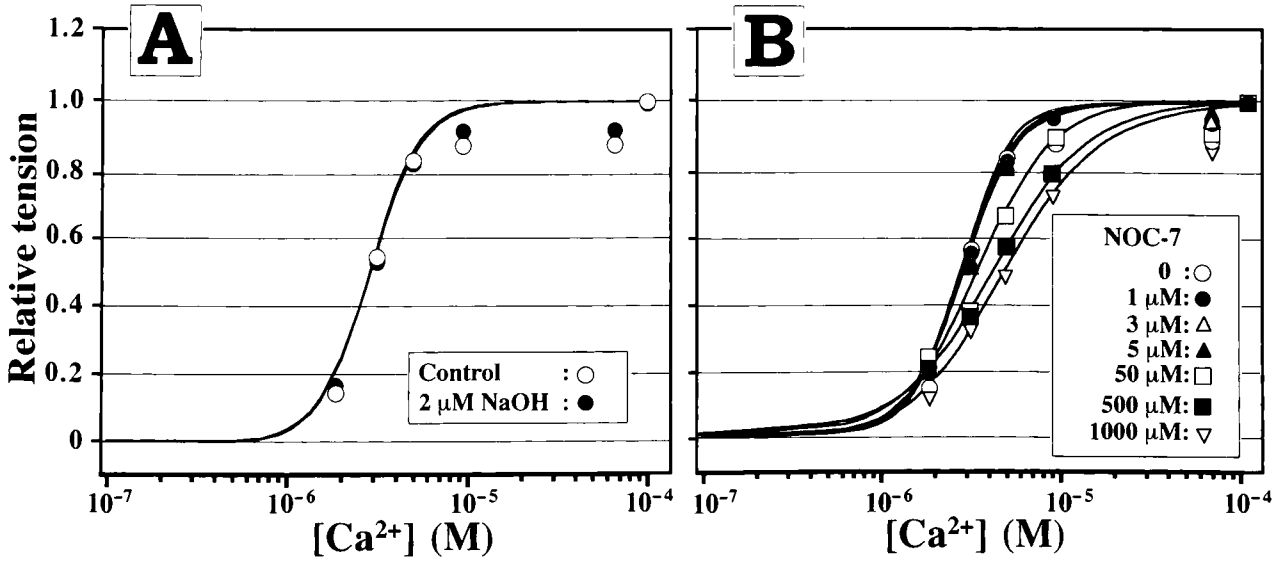

FIG. 4. Graphs demonstrating the effects of vehicle (2 μ*M* NaOH) for NOC-7 (A) and NO• released from NOC-7 (B, 1–1,000 μ*M*) on myofilament Ca^{2+} sensitivity of Triton X-100-treated skinned preparations. The ordinate shows tension, normalized for that series of activations, at different [Ca^{2+}] (abscissa). Experimental conditions were identical with those of Fig. 1. The curves are fitted according to the Hill equation with the mean [Ca^{2+}] for half-maximum response and the Hill coefficient separately determined for each concentration of NOC-7 applied. A time-matched control study without NOC-7 exposure was also performed. Eight Triton X-100-treated skinned preparations (for time-matched control and NaOH exposure, **A**; and for 1 to 1,000 μ*M* NOC-7 exposure for 30 min, **B**) obtained from the same cardiac muscle were studied in parallel. The data are expressed as the mean (n = 5).

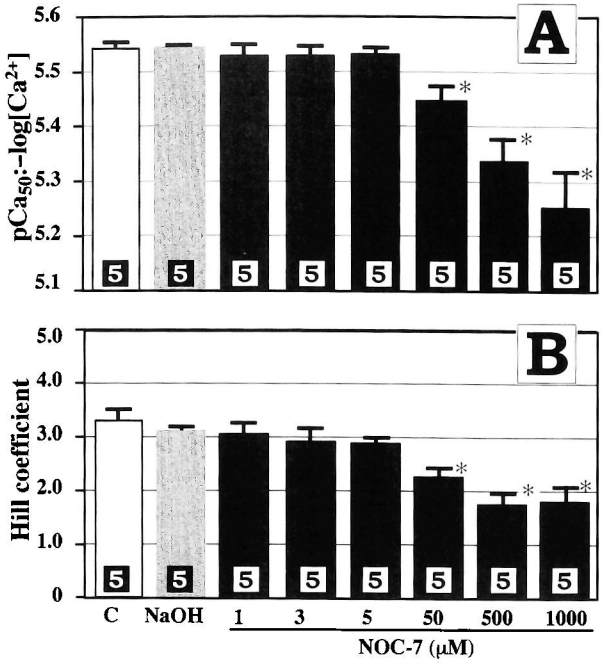

FIG. 5. The effect of NO• released from NOC-7 (1–1,000 μM) on the [Ca^{2+}] required for half-maximum activation (**A**, pCa_{50}) and Hill coefficient (**B**). The values were calculated from the data presented in Fig. 4. Bar heights are means, brackets are SEM, and numbers at the bottom of the bars indicate the number of preparations studied. pCa_{50} and Hill coefficient measured under conditions in time-matched control study are indicated as C (control). *$p < 0.05$ vs. corresponding control.

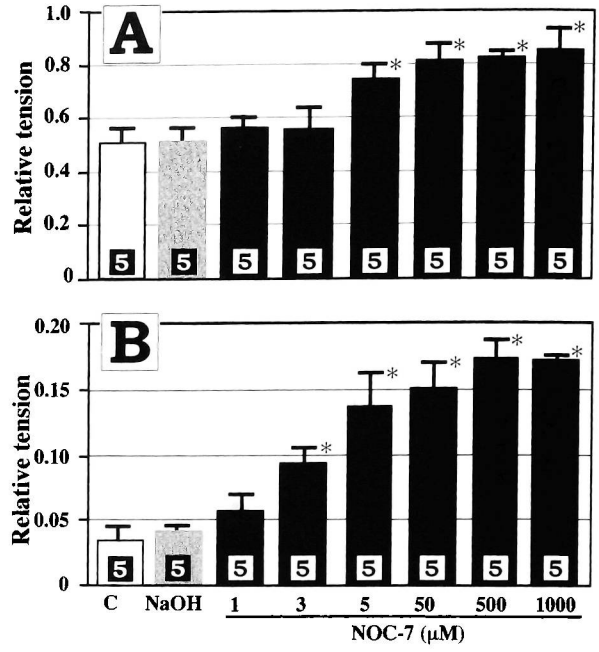

FIG. 6. The effects of NO• released from NOC-7 (1–1,000 μM) on rigor contracture **(A)** and functional assessment of CK activity **(B)**. Experimental conditions were identical with those of Fig. 1. Rigor contracture and functional assessment of CK activity were scaled to the control maximum Ca^{2+}-activated force (in response to pCa 4.0 solution); increased relative tension means decreased CK activity **(B)**. A time-matched control study without NOC-7 exposure was also performed. Eight Triton X-100-treated skinned preparations (for time-matched control, NaOH exposure, and 1–1,000 μM NOC-7 exposure for 30 min) obtained from the same cardiac muscle were studied in parallel. Bar heights are means, brackets are SEM, and numbers at the bottom of the bars indicate the number of preparations studied. Rigor contracture **(A)** and CK activity **(B)** measured under conditions in time-matched control study are indicated as C (control). *$p < 0.05$ vs. corresponding control.

specific disruption. Figure 6A illustrates the results of this series of experiments (for experimental protocol, see Fig. 1). NO• released from NOC-7 exerted concentration-dependent enhancement of rigor contracture; this effect was significant at >5 μM NOC-7 concentrations.

Myofibrillar-bound CK optimizes the local ATP-to-ADP ratio within the myofilaments. We have used this fact to make functional assessment of CK activity by substituting ADP for ATP in our "mock intracellular" solutions. The myofibrillar-bound CK activity, as was relative rigor contracture to rephosphorylated ADP, was decreased (instead, relative tension was increased; Fig. 6B) by NO•; significant decrease occurred at >3 μM NOC-7 concentrations (gives >0.44 μM of cumulative NO• concentration for 30 min; see Fig. 3), suggesting that low concentration of NO• can inhibit CK activity, thereby enhancing rigor state.

Effect of exogenous CK on NOC-7 induced modifications of Ca^{2+} sensitivity and crossbridges in rigor

There is a high activity of myofibrillar CK (~7 IU/mg of noncollagen protein; Saks *et al.*, 1975; Wallimann and Eppenberger, 1985). If the view that the mechanism of the potentiation of the rigor contracture induced by NO• involves decreased endogenous CK activity is correct, it should be possible to inhibit the observed effect of NO• by exogenously added CK. We tested this hypothesis by application of nearly physiological concentration (~20 units/ml) of exogenous CK (EC 2.7.3.2., Type VI-S, from

rabbit heart; Sigma, St. Louis, MO) to our experimental system. In these experiments, 500 μM NOC-7 was used because at this concentration there were nearly maximum effects obtained on Ca^{2+}-sensitivity, rigor contracture, and endogenous CK activity (Figs. 5 and 6). Exogenous CK when added to the system in the concomitant presence of NOC-7 slightly protected against the decreased Ca^{2+} sensitivity induced by NOC-7; this effect, however, was not significant except at highest concentration of CK (20 units/ml) used on h (Fig. 7).

The enhanced crossbridges in rigor afforded by NOC-7 was protected by 20 units/ml CK (Fig. 8A). The observed decrease in myofibrillar-bound CK activity induced by NOC-7 was restored concentration dependently by the addition of exogenous CK to normal (Fig. 8B).

DISCUSSION

The discovery that NO• exhibits many of the physiologic properties associated with endothelial-derived relaxing factor (Furchgott and Zawadzki, 1980; Palmer *et al.*, 1987) has

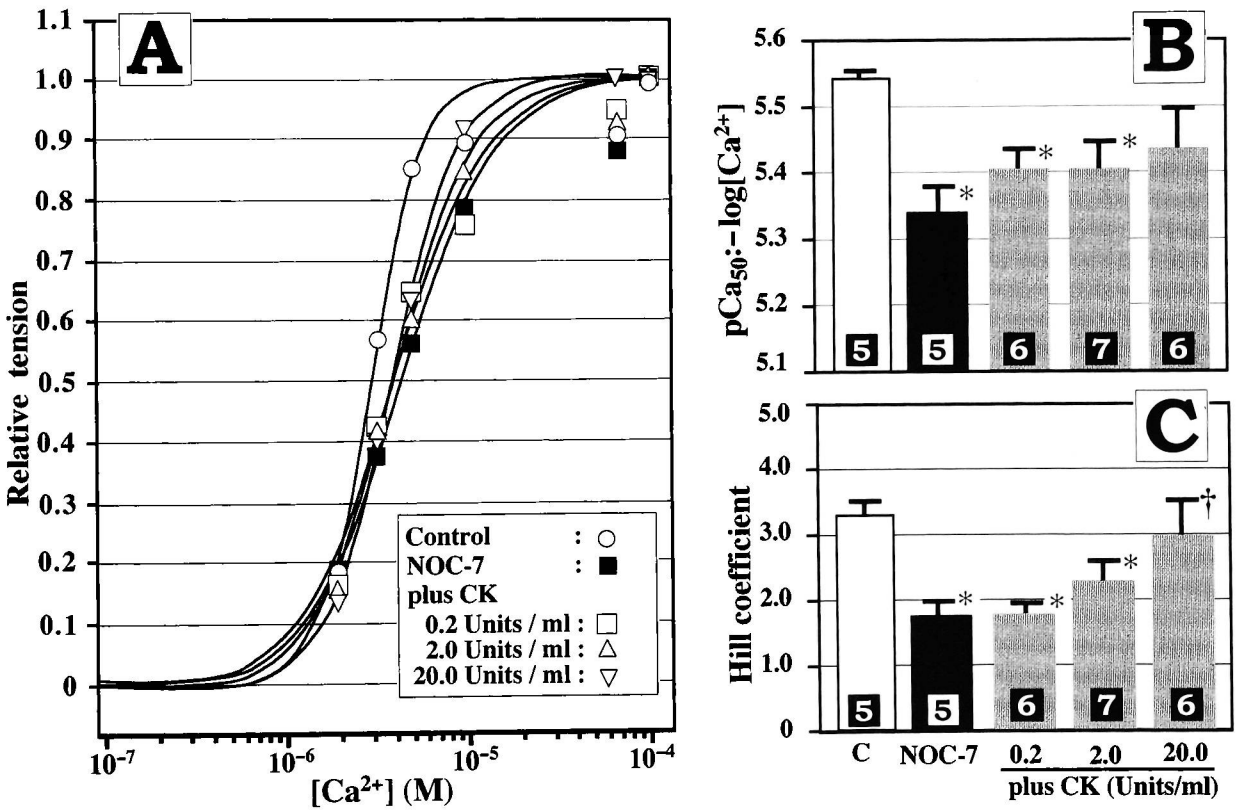

FIG. 7. Effects of exogenous CK on NO•-induced modification of myofilament Ca^{2+} sensitivity. A. Graph showing the effect on Ca^{2+} sensitivity of Triton X-100-treated skinned preparations. The ordinate shows tension, normalized for that series of activations, at different [Ca^{2+}] (abscissa). Experimental conditions were similar to those of Fig. 1 except that exogenous CK (0.2–20 units/ml) was added simultaneously with NOC-7 (500 μM). The curves are fitted according to the Hill equation with the mean [Ca^{2+}] for half-maximum response and the Hill coefficient separately determined for each concentration of NOC-7 applied. A time-matched control study without NOC-7 and exogenous CK was also performed. The data are expressed as the mean (n = 5–7). **B.** The effect on the [Ca^{2+}] required for half-maximum activation (pCa_{50}). The values were calculated from the data presented in **A.** Bar heights are means, brackets are SEM, and numbers at the bottom of the bars indicate the number of preparations studied. pCa_{50} measured under conditions without NOC-7 and exogenous CK in time-matched control study is indicated as C, control. *$p < 0.05$ vs. control. **C.** The effect on Hill coefficient. The values were calculated from the data presented in **A.** Bar heights are means, brackets are SEM, and numbers at the bottom of the bars indicate the number of preparations studied. Hill coefficient measured under conditions without NOC-7 and exogenous CK in time-matched control study is indicated as C (control). *$p < 0.05$ vs. control; †$p < 0.05$ vs. NOC-7 alone.

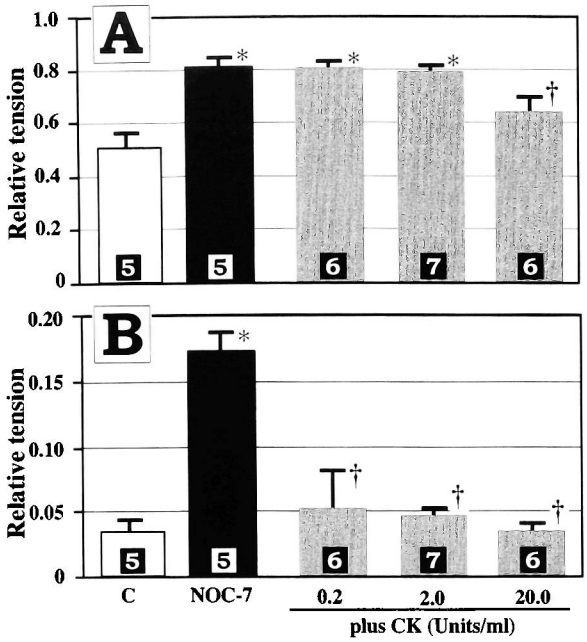

FIG. 8. The effects of exogenous CK on NO•-induced modifications of rigor contracture (A) and functional assessment of CK activity (B). Experimental conditions were similar to those of Fig. 1 except that exogenous CK (0.2–20 units/ml) was added simultaneously with NOC-7 (500 μM). Rigor contracture and the CK activity were scaled to the control maximum Ca^{2+}-activated force (in response to pCa 4.0 solution); increased relative tension means decreased CK activity **(B)**. Bar heights are means, brackets are SEM, and numbers at the bottom of the bars indicate the number of preparations studied. Rigor contracture **(A)** and CK activity **(B)** measured under conditions without NOC-7 and exogenous CK in time-matched control study are indicated as C (control). $*p < 0.05$ vs. corresponding control; $\dagger p < 0.05$ vs. corresponding NOC-7 alone.

opened a new chapter in the biological role of free radicals. There is wide interest in the possibility that oxygen-derived free radicals and nonradical reactive oxygen species, produced during myocardial ischemia and reperfusion (pathological or iatrogenic in origin), can prejudice myocardial function (Okabe *et al.*, 1988, 1991, 1993; Kawakami and Okabe, 1998; Kumasaka *et al.*, 1999).

Zweier *et al.* (1995) have recently demonstrated that NO• formation is increased in the heart under conditions of ischemia, where there is a lack of flow. This increased NO• results in the loss of contractile function after reperfusion. They (Zweier *et al.*, 1995) have also applied ESR spectroscopy in the presence or absence of the exogenous NO• spin trap, *N*-methyl-D-glucamine-dithiocarbamate (MGD)-Fe^{2+}, to detect NO• generation in the ischemic heart. In spite of its importance, until recently, there were few studies investigating the actual concentration-related effect of NO• on the contractile proteins, the subject of the present study.

Numerous possible sites of action have been reported, and among them myofibrillar proteins have been involved. The results of the present study show the following: (1) NO• induced several alterations of myofibrillar function, *i.e.*, decreases in Ca^{2+} sensitivity and Hill coefficient, and potentiation of rigor contracture; and (2) the main effect was a strong inhibition of myofibrillar CK activity. Our studies do not test the direct interaction of NO• and myofibrillar function, because the experiments were undertaken after the NO• donor, NOC-7, was removed from the system. Therefore, the results suggest that the main effect of NO• is due to its ability to inactivate CK during previous exposure, thus inducing a decrease in the local ATP-to-ADP ratio.

CK is an important cellular enzyme involved in energy transduction in muscle cells. In addition to the traditional role of CK as a spatiotemporal ATP/ADP buffer, it has been proposed that the location of the CK isoenzymes in cardiac muscle facilitates the transduction of high-energy phosphates throughout the cell and acts to fine-tune the regulation of energy utilization and production (Wallimann *et al.*, 1992). In myofibrils, CK is an integral part of the M line (for review, see Wallimann and Eppenberger, 1985) and is also distributed across the entire filament (Wegmann *et al.*, 1992). The myofibrillar-bound CK can induce a specific kinetic enhancement of the myofibrillar ATPase (Krause and Jacobus, 1992) and rephosphorylated enough Mg^{2+}-ATP produced by the myosin ATPase reaction to ensure optimal contractile capacities as well as normal Ca^{2+}-sensitivity and crossbridge cycling rate in the absence of Mg^{2+}-ATP. Thus, it can meet the contractile protein energy requirements (Wallimann and Eppenberger, 1985). On the other hand, deprivation of PCr, even in the presence of ATP, decreases the rate of cycling of crossbridges, increases the Ca^{2+} sensitivity, and increases the susceptibility of rigor tension development to a decrease in Mg^{2+}-ATP. The CK,

which exists as functional dimers of M-CK and/or B-CK isoform subunits, contains eight-SH groups, of which two are believed to be at or near the catalytic or substrate binding sites, and appear to be essential for enzyme activity (Watts, 1973; Wallimann *et al.*, 1992). CK activity in solution is inhibited by low micromolar concentrations of *N*-ethylmaleimide, and by a dithiothreitol-reversible oxidation reaction catalyzed by iron, both of which are presumed to be mediated by direct covalent modification of -SH groups on the enzyme (Hamman *et al.*, 1995). NO• is known to bind covalently and to alter the activity of a number of enzymes and transcriptional regulatory factors by direct *S*-nitrosylation and by the intermediate formation of metal–NO• adducts (Stamler, 1994). In the present experiments, NO• can decrease myofibrillar-bound CK activity; exogenous CK protects against the observed decrease in CK afforded by NO•. Whereas it is possible that NO• could have modified CK activity indirectly through some intermediate signaling pathway, a direct reversible modification of the enzyme such as *S*-nitrosylation seems more likely. Taken together, alteration of myofibrillar CK by NO• may be one of the mechanisms involved in the rigor tension development. Indeed, the enhancement of rigor contracture was shown upon previous exposure of Triton-skinned trabeculae to NO•; the demonstrated effect of NO• was inhibited by exogenous CK.

In our experiments, NO• at cumulative concentration of 0.69 μM (for 30 min) was effective on both rigor contracture and myofibrillar-bound CK activity; however, Ca^{2+} sensitivity (pCa_{50}) was significantly modified at >5.6 μM of NO•. These observations are consistent with the view that myofibrillar-bound CK is more sensitive to NO• (even in the low micromolar range, ~5 μM) than Ca^{2+}-sensitivity-influencing factors, suggesting a result of different mechanisms. The cellular mechanisms by which Ca^{2+} sensitivity of the contractile proteins is decreased by NO• and not via decreased CK activity are not known at present. Ca^{2+} sensitivity of tension development may be influenced by a number of factors. Among them, the decreased rate of crossbridge cycling due to substrate deficiency may induce a shift toward lower Ca^{2+} concentrations of the pCa-tension relationship, together with a decrease in cooperativity (Brandt *et al.*, 1982; Ventura-Clapier *et al.*, 1987). This effect can be observed when comparing the pCa-tension relationships obtained with or without PCr (Ventura-Clapier *et al.*, 1987) and can be explained by the efficacy of bound CK to maintain a high local ATP-to-ADP ratio.

Under conditions of restricted exchange of adenine nucleotides between the cytosol and the myofibrillar compartment, bound CK can provide Mg^{2+}-ATP, remove Mg^{2+}-ADP, and buffer proton production in the immediate environment of myosin ATPase (Ventura-Clapier *et al.*, 1994). Inhibition of the CK function leads to a slowing of the off rate of crossbridges and a prolonged duration of the duty cycle of crossbridges. These observations give the explanation that the myofibrillar function is not altered directly through modifications of the regulatory proteins or myosin ATPase but indirectly through an inhibition of myofibrillar CK-inducing substrate deprivation of myosin ATPase and secondary slowing down of crossbridge kinetics. Under our experimental conditions, although NO• in the low micromolar range was able to decrease myofibrillar CK activity dramatically, neither Ca^{2+} sensitivity nor cooperativity was affected. At a higher concentration (>5.6 μM), NO• induced a shift toward higher Ca^{2+} concentrations of the pCa-tension relationship, together with a decrease in cooperativity as well as decrease in CK activity. Thus, the decreased Ca^{2+} sensitivity seems to be associated with direct modification of the regulatory proteins by a relatively higher concentration of NO•, possibly not via inhibition of myofibrillar CK activity. The specific mechanism may involve reduced affinity of troponin C (the Ca^{2+}-binding subunit of the thin-filament regulatory protein troponin) for Ca^{2+}. This could be the result of a direct effect on troponin C or an indirect result of an effect on other regulatory proteins within the thin filament, such as possible alterations in phosphorylation of either myosin light chain-2, troponin I, or troponin T. The precise effect of NO• on the contribution of the phosphorylation of regulatory proteins within the thin filament merits further study.

The increase in resting tension often results

from either cycling crossbridges or from permanently attached crossbridges. Since previous exposure of the preparations to NO• (at any of the concentration tested) was without effect upon the resting tension, we can exclude actively cycling crossbridges (whether Ca^{2+}-dependent or not) from causing the resting tension rise.

It is possible, perhaps likely, that the actions of NO• or its congeners on the regulation of high-energy phosphate metabolism differ as a function of their concentration within the cell. The data reported here indicate that CK may be a pathophysiologically main target for increased NO• formation at low-micromolar range in disease states in cardiac muscle. The recognition of this is of great importance in understanding the pathogenesis, treatment, and development of pharmacological approaches to prevent tissue injury that occurs in disease processes secondary to increased formation of NO•. Especially reversible inhibition of CK during increased NO• formation deserves further study as a potential mechanism underlying cardiac energetic and functional abnormalities in clinically important settings such as myocardial stunning, systemic sepsis, and myocardial inflammatory disease (Ungureanu-Longrois *et al.*, 1995).

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ABBREVIATIONS

ATPase, adenosine triphosphatase; cGMP, guanosine 3′,5′-cyclic monophosphate; CK, creatine kinase; DTCS, *N*-(dithiocarboxy)sarcosine; EGTA, ethyleneglycol *bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; ESR, electron spin resonance; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MGD, *N*-methyl-D-glucamine-dithiocarbamate; NO•, nitric oxide; NOC-7, 3-(2-hydroxy-1-methyl-2-nitrosohydrazine)-*N*-methyl-1-propanamine; PCr, phosphocreatine; TEMPOL, 4-hydroxyl-2,2,6,6-tetramethylpiperidinyloxy.

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