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Inhibition of skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase by nitric oxide

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Abstract The effects of nitric oxide on the activities of thapsigargin-sensitive sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Ca^{2+} uptake by sarcoplasmic reticulum (SR) membranes prepared from white skeletal muscle of rabbit femoral muscle were studied. Pretreatment of the SR preparations with nitric oxide at concentrations of up to 250 μM for 1 min decreased the SERCA activity concentration dependently, and also decreased their Ca^{2+} uptake. Both these effects of nitric oxide were reversible. Inhibitors of guanylyl cyclase and protein kinase G (PKG) had no significant effect on the nitric oxide-induced inhibitions of SERCA and Ca^{2+} uptake. Moreover, dithiothreitol did not reverse the inhibitory effects of nitric oxide on SERCA and Ca^{2+} uptake. These findings suggest that nitric oxide inhibits SERCA, mainly SERCA 1, of rabbit femoral skeletal muscle by an action independent of the cyclic GMP-PKG system or oxidation of thiols, and probably by a direct action on SERCA protein.

Key words: Sarcoplasmic reticulum Ca^{2+}-ATPase; Nitric oxide; Sarcoplasmic reticulum; Ca^{2+} uptake; Protein kinase G

1. Introduction

Nitric oxide is generated in the conversion of L-arginine to L-citrulline catalyzed by nitric oxide synthase. Three isoforms of nitric oxide synthase are known: a neural type designated as nNOS (NOS-1), an inducible type iNOS (NOS-2) and an endothelial cell type eNOS (NOS-3). nNOS and eNOS are called constitutive nitric oxide synthase (cNOS) because of their constitutive expressions (for reviews, see [1,2]). cNOS activity is dependent on Ca^{2+}/calmodulin [3] and requires tetrahydrobiopterin [4], whereas iNOS activity is regulated by gene expression inducible by immunological stimuli [1,2]. nNOS has been purified from rat cerebellum [5] and is located immunohistochemically in the brain, retina, myenteric plexus of the intestine, adrenal glands and vascular endothelial cells [3]. In subsequent studies it was also found in the epithelium of the bronchi and trachea [6], and also in rat skeletal muscle, type II (fast) fiber [7] and type I (slow) fiber [8–10] cell membranes. The physiological significance of nitric oxide in skeletal muscle was suggested from the finding that contractile function was augmented by inhibitors of nitric oxide synthase and depressed by nitric oxide donors [7].

Increase in the cytoplasmic concentration of Ca^{2+} ions released from the sarcoplasmic reticulum (SR) results in muscle contraction. Activation of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) decreases the Ca^{2+} concentration by uptake of cytoplasmic Ca^{2+} ions into the SR and results in muscle relaxation [11]. One isoform of SERCA, SERCA 1, is present in the sarcolemma of type II fibers [12,13] and another isoform, SERCA 2, in the cardiac muscle, slow fibers of skeletal muscle and smooth muscle [14]. The mechanism of regulation of cardiac SERCA 2 activity is known: a regulatory protein, phospholamban, inhibits its activity by binding to its catalytic subunit and phosphorylation of phospholamban induced by cyclic AMP-dependent protein kinase reverses this inhibition [15,16]. However, the mechanism regulating SERCA 1 activity has not been studied. Here we report studies on the effect of nitric oxide on the activity of SERCA 1 to determine the mechanism of the effect of nitric oxide on fast fibers of skeletal muscle.

2. Materials and methods

2.1. Preparation of sarcoplasmic reticulum (SR) membranes from rabbit skeletal muscle

SR membranes were prepared as described by Eletr and Inesi [17]. Soon after excision of white skeletal muscle from rabbit hind legs, the tissue was washed and cooled in 0.1 mM EDTA, pH 7.0. Samples of 150 g of trimmed muscle were then homogenized in 450 ml of Medium 1 (10 mM histidine, 10% sucrose, 0.1 mM EDTA, pH 7.0) for 15-s periods every 5 min, for 1 h. During this time, the pH was adjusted with a few drops of 5% NaOH when necessary. The homogenate was centrifuged at $15\,000\times g$ for 20 min. The supernatant was collected and filtered through several layers of washed gauze (1-inch thick filter) to eliminate low-density lipid aggregates. The filtered suspension was then centrifuged at $40\,000\times g$ for 90 min and the resulting precipitate was resuspended in 55 ml of Medium 2 (10 mM histidine, 0.6 M KCl, pH 7.0).

After incubation for 40 min at 2–4°C, the suspension was centrifuged at $15\,000\times g$ for 20 min. Then the top layer (approx. 10% of the total volume) was carefully discarded, to remove low-density lipid aggregates that could still be associated with the preparaton. The remaining supernatant was then collected and recentrifuged at $40\,000\times g$ for 90 min. This resulting precipitate was resuspended in 20 ml of Medium 3 (0.88 M sucrose, 10 mM MOPS, pH 7.0). The entire procedure was carried out at 2–4°C.

2.2. Measurement of thapsigargin-sensitive Ca^{2+}-ATPase (SERCA) activity

SERCA activity in SR membrane preparations was measured by monitoring the release of ^{32}Pi from [γ-^{32}P]ATP (Amersham, PB218). The basic assay mixture consisted of membranes (10 μg of protein),

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Abbreviations: SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; PKG, cyclic GMP-dependent protein kinase; DTT, dithiothreitol

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100 mM KCl, 50 mM Tris-HCl (pH 7.4), 2 mM ATP (containing 100 μM KOH and [γ-^{32}P]ATP), 3 mM $MgCl_2$, 2 μM A23187, 1 mM NaN_3, and appropriate amounts of $CaCl_2$ and EGTA (adjusted to pH 7.4 with up to 29 μM KOH) to produce the required free Ca^{2+} concentration calculated with the software developed by Horiuchi [18]. Assay of SERCA activity was started by addition of 100 μl of the SR preparation to 400 μl of the basic assay mixture and carried out for various incubation periods at 37°C. Thapsigargin-sensitive SERCA activity was defined as the difference between the SERCA activities measured in the presence and absence of 100 nM thapsigargin, as previously described [19]. All assays were started after 5 min preincubation at 37°C with or without thapsigargin.

2.3. Measurement of thapsigargin-sensitive Ca^{2+} uptake activity

Uptake of ^{45}Ca was measured at 25°C by the microfiltration method described by Zhang et al. [20]. The basic reaction mixture contained 20 mM MOPS (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 3 mM ATP, membrane vesicles (up to 10 μg/ml), and appropriate amounts of $CaCl_2$ and EGTA to produce the required free Ca^{2+} concentration [18]. Thapsigargin-sensitive Ca^{2+} uptake activity was defined as the difference between the Ca^{2+} uptake activities measured in the presence and absence of 500 nM thapsigargin. The reaction was started by addition of oxalate and ATP after 5 min of preincubation at 37°C with or without thapsigargin and was terminated at intervals by filtration through Millipore filters (0.45 μm). The filters were washed twice with 2 ml of 2 mM ice cold $LaCl_3$ and 10 mM MOPS (pH 7.0), and the remaining radioactivity was measured in a scintillation counter.

2.4. Drugs

Rp-isomer (Rp-8 bromo cyclic GMPS) was purchased from Biolog Life Sci., Bremen, Germany. 6-(Phenylamino)-5,8-quinolinedione (LY83583) was from Res. Biochem., MA, USA. Inositol 1,4,5-trisphosphate (IP_3) and ryanodine were from Funakoshi, Tokyo. Caffeine was from Wako Pure Chemicals, Osaka, Japan. Heparin was from Sigma, St. Louis, MO, USA. (DL)-(*E*)-Ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexeneamine (NOR3) was from Dojindo Laboratories, Kumamoto, Japan. Gaseous nitric oxide was dissolved in the incubation medium just before experiments, as described previously [21].

3. Results

3.1. Effect of nitric oxide on thapsigargin-sensitive Ca^{2+}-ATPase (SERCA) activity in rabbit skeletal muscle

Sarcoplasmic reticulum (SR) preparations obtained from rabbit femoral muscle exhibited thapsigargin-sensitive SERCA activity: the activity evaluated as Pi liberation from ATP was 3.2 ± 0.3 μmol/min/g protein ($n = 6$) and was constant during a 30-min incubation period under the present experimental conditions (Fig. 1). Pretreatment of the SR preparations with 250 μM nitric oxide for 1 min inhibited the SERCA activity, the extent and duration of inhibition depending on the nitric oxide concentration used in the pretreatment period (Fig. 1). After inhibition, the activity was gradually but completely restored to the control level (Fig. 1). Thus, the inhibitory effect of nitric oxide was reversible under the present conditions. Since nitric oxide is readily oxidized under the present experimental conditions, we next examined the effect of NOR3 which liberates nitric oxide continuously from its molecule. NOR3 also inhibited the activity in a concentration dependent manner, but its inhibitory effect was constant during a 25-min incubation period (Fig. 2).

In another series of experiments, we examined the effects of nitric oxide on Ca^{2+} uptake by SR preparations to confirm its inhibitory effect on SERCA activity. The SR preparations exhibited thapsigargin-sensitive, ATP-dependent uptake of $^{45}Ca^{2+}$ ions and the uptake reached a maximun within about 5 min (Fig. 3A). Pretreatment of the SR preparations with 200 μM nitric oxide inhibited the Ca^{2+} uptake activity. The amount of Ca^{2+} taken up gradually returned to the control level about 15 min after the preincubation period (Fig. 3A). The Ca^{2+} uptake activity was completely restored to the control level about 15 min after pretreatment of the SR preparations with 200 μM nitric oxide (Fig. 4). Thus, Ca^{2+} uptake was inhibited reversibly by nitric oxide. Pretreatment of the preparations with nitric oxide in the presence of 1 μM hemoglobin did not affect the Ca^{2+} uptake activity (data not shown).

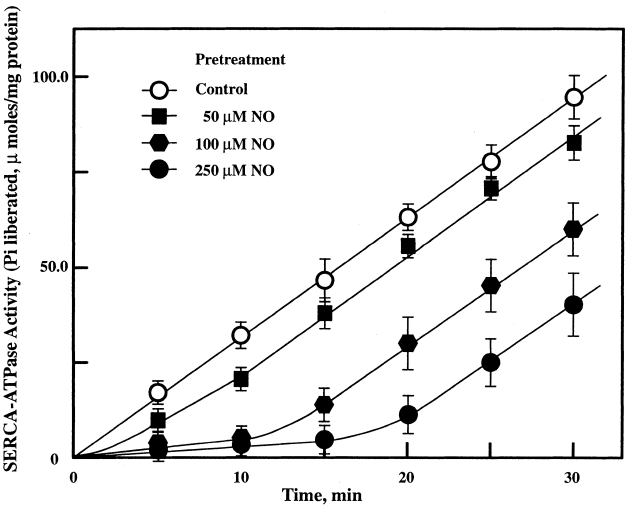

Fig. 1. Effects of nitric oxide on activity of thapsigargin-sensitive Ca^{2+}-ATPase (SERCA) of rabbit SR preparations. Preparations were preincubated without (control) or with various concentrations of nitric oxide (NO) for 1 min. Then, 100-μl volumes of preparations were incubated in 400 μl of assay mixture in the presence of 5 μM free Ca^{2+} ions and 2 μM A23187 to measure SERCA activity. Nitric oxide added during pretreatment was diluted 5-fold with the incubation mixture. SERCA activity was measured over a period of 30 min. Points and bars represent means and standard deviations of values in four to six separate preparations.

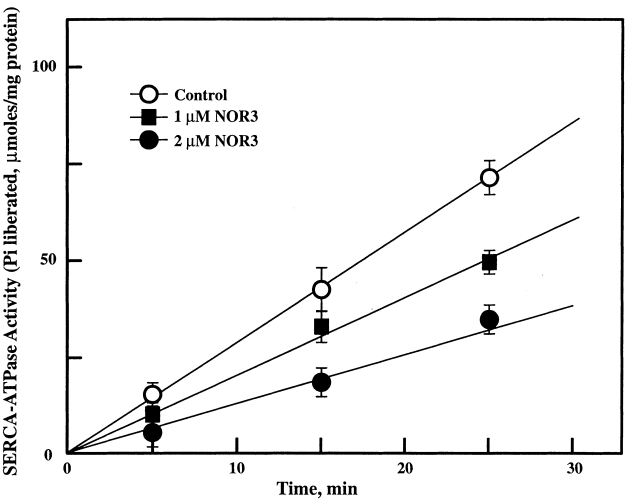

Fig. 2. Effect of NOR3 on SERCA activity of rabbit SR preparations. The SERCA activity of SR preparations was measured in the absence (control) or presence of 1 or 2 μM NOR3 over a period of 25 min. For further details, see Section 2. Points and bars represent means and standard deviations of values in four to six separate experiments.

3.2. Effects of inhibitors of guanylyl cyclase and cyclic GMP-dependent protein kinase (PKG) on nitric oxide-induced inhibition of SERCA activity

Since nitric oxide is known to induce various physiological and pathophysiological events via a cyclic GMP-PKG pathway, we studied the effects of an inhibitor (LY 83583) of guanylyl cyclase and an inhibitor (Rp-8 bromo cyclic GMPS) of PKG on nitric oxide-induced inhibition of SERCA activity. LY 83583 at 1 μM almost completely inhibited increase by dimethylphenylpiperazinium-induced cyclic GMP in rat proximal colon [22], and Rp-8 bromo cyclic GMPS at 10 μM completely inhibited the activity of PKG purified from porcine aorta [23], but had no significant effects on the activity of SERCA in SR preparations with or without pre-

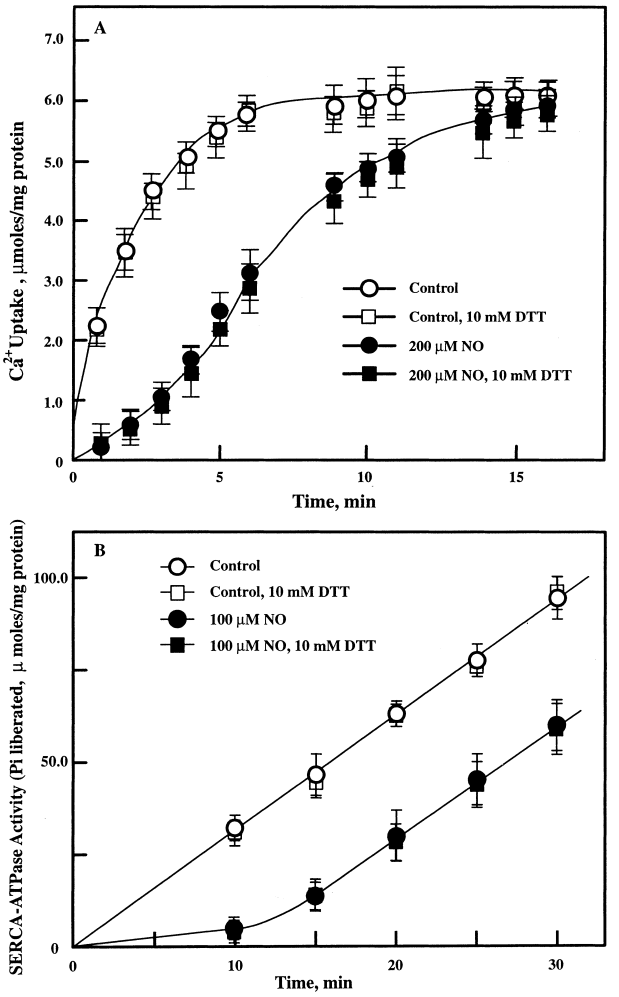

Fig. 3. Effects of nitric oxide and dithiothreitol (DTT) on Ca^{2+} uptake and SERCA activity of rabbit SR preparations. A: Ca^{2+} uptake activities of SR preparations preincubated without (control) or with 200 μM nitric oxide were measured in the absence or presence of 10 mM DTT. The amounts of Ca^{2+} taken up by the SR preparations during the indicated incubation periods were expressed in mmol Ca^{2+}/mg protein. Points and bars represent means and standard deviations for four to six separate measurements. B: Activities of SERCA of SR preparations preincubated without (control) or with 100 μM nitric oxide for 1 min were measured in the absence or presence of 10 mM DTT. For further details, see legend of Fig. 1. Note that 10 mM DTT did not influence the SERCA or Ca^{2+} uptake activity.

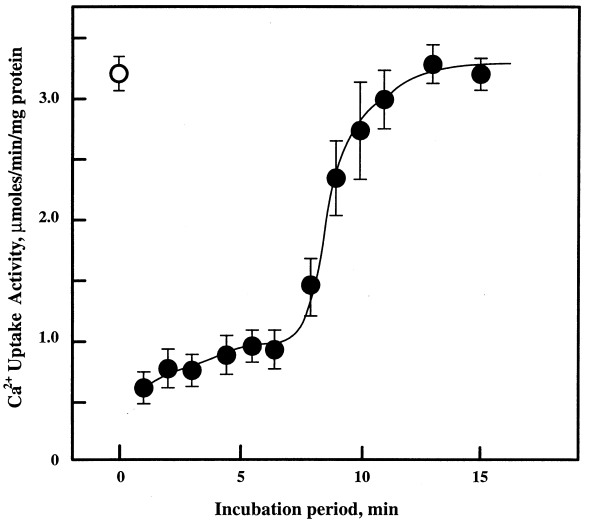

Fig. 4. Recovery of Ca^{2+} uptake activity of rabbit SR preparations from nitric oxide-induced inhibition. The SR preparations were preincubated without (○) or with (●) 200 μM nitric oxide for 1 min. Then they were incubated in assay mixture in the presence of 5 μM Ca^{2+} ions for the indicated times. Ca^{2+} uptake activity was determined by measuring the amount of Ca^{2+} taken up into the SR preparations during a further 45-s incubation period and is expressed as mmol Ca^{2+} taken up per min per mg protein. Points and bars show means and standard deviations for four to six separate measurements. Note that nitric oxide-induced inhibition of Ca^{2+} uptake activity was completely reversed after incubation for 13 min.

treatment with nitric oxide (Fig. 5). They also had no significant effect on the Ca^{2+} uptake activity of untreated or nitric oxide-pretreated SR preparations (data not shown).

3.3. Effects of reductants on nitric oxide-induced inhibition of SERCA activity

Nitric oxide is known to generate reactive oxidants such as NO_2 and NO_3 by oxidation [24]. Active oxygens inhibit many thiol enzymes by oxidizing their thiols and reductants protect these enzymes from oxidation [25–27]. Therefore, we studied whether dithiothreitol (DTT) reverses the inhibition of SERCA activity by nitric oxide. DTT at 10 mM did not affect the SERCA activity of untreated or nitric oxide-pretreated SR preparations (Fig. 3B). It also did not affect the $^{45}Ca^{2+}$ uptake activity of untreated or nitric oxide-pretreated SR preparations (Fig. 3A).

3.4. Effects of agonists and antagonists of SR Ca^{2+} channels on nitric oxide-induced inhibition of SERCA activity

The above findings suggest that nitric oxide inhibits Ca^{2+} uptake into the SR by inhibiting SERCA activity. However, it is also possible that it activates Ca^{2+} channels present in the SR membrane, and so increases Ca^{2+} release from the SR with resultant decreases of Ca^{2+} uptake into the SR. In fact, agonists of SR Ca^{2+} channels, caffeine at 20 mM and IP_3 at 10 μM, decreased $^{45}Ca^{2+}$ uptake into SR preparations by their effects in activating Ca^{2+} releasing channels (Fig. 6A). If the inhibitory effect of nitric oxide on Ca^{2+} uptake involves activation of SR Ca^{2+} channels, antagonists of these SR Ca^{2+} channels should counteract the nitric oxide-induced inhibition of $^{45}Ca^{2+}$ uptake. However, neither an antagonist of IP_3-sensitive Ca^{2+} channels (heparin) nor one of caffeine-sensitive Ca^{2+} channels (ryanodine) had any significant effect on the

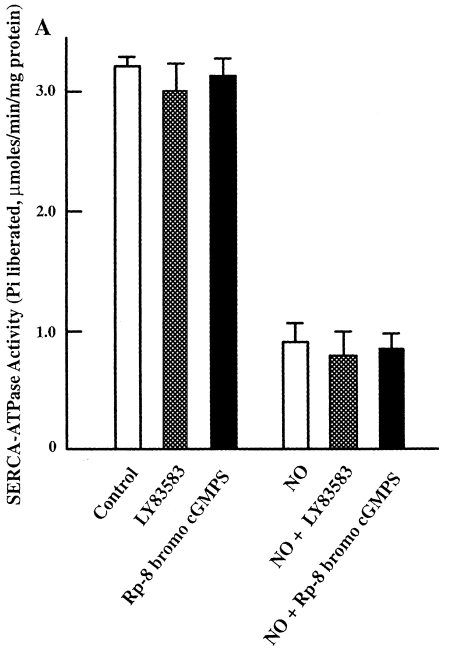

Fig. 5. Effects of LY 83583 and Rp-8 Br cGMPS on nitric oxide-induced inhibition of SERCA activity. Rabbit SR preparations were preincubated without (control) or with 100 μM nitric oxide, 1 μM LY 83583, 10 μM Rp-8 Br cyclic GMPS, nitric oxide plus LY 83583 or nitric oxide plus Rp-8 Br cyclic GMPS for 1 min. Then preparations were incubated for 30 min to measure SERCA activity. The drugs were diluted 5-fold in the incubation mixture.

inhibitory effect of nitric oxide (Fig. 6B). Moreover, these agonists and antagonists had no significant effect on the SERCA activity of SR preparations in the absence or presence of nitric oxide (data not shown).

3.5. *Effects of nitric oxide on ouabain-sensitive Na^+,K^+-ATPase activity of a chicken kidney homogenate*

The effect of nitric oxide on another P type ATPase, Na^+,K^+-ATPase, was also examined. Nitric oxide did not affect the activity of ouabain-sensitive Na^+,K^+-ATPase of a chicken kidney homogenate (data not shown).

4. Discussion

A role of nitric oxide in skeletal muscle contraction was first suggested by studies on rat diaphragm [5], namely, electrical stimulation-induced contraction of the muscle was significantly augmented by inhibitors of nitric oxide synthase and inhibited by exogenous nitric oxide donors. Immunohistochemical studies showed prominent staining of fast fibers of the diaphragm with antibodies against nNOS. The intracellular mechanism of the inhibitory action of nitric oxide on skeletal muscle is unknown. In the present study, we examined the effect of nitric oxide on the SERCA activity of SR preparations from fast fibers of rabbit femoral muscle which express SERCA, mainly the SERCA 1 isoform. The results clearly showed that nitric oxide inhibited the SERCA activity in SR preparations. Results on nitric oxide-induced inhibition of the Ca^{2+} uptake activity of the SR preparations supported this idea. We suppose that depression of SERCA activity resulted in decrease or depletion of Ca^{2+} stores in the SR and consequent inhibition of muscle contraction.

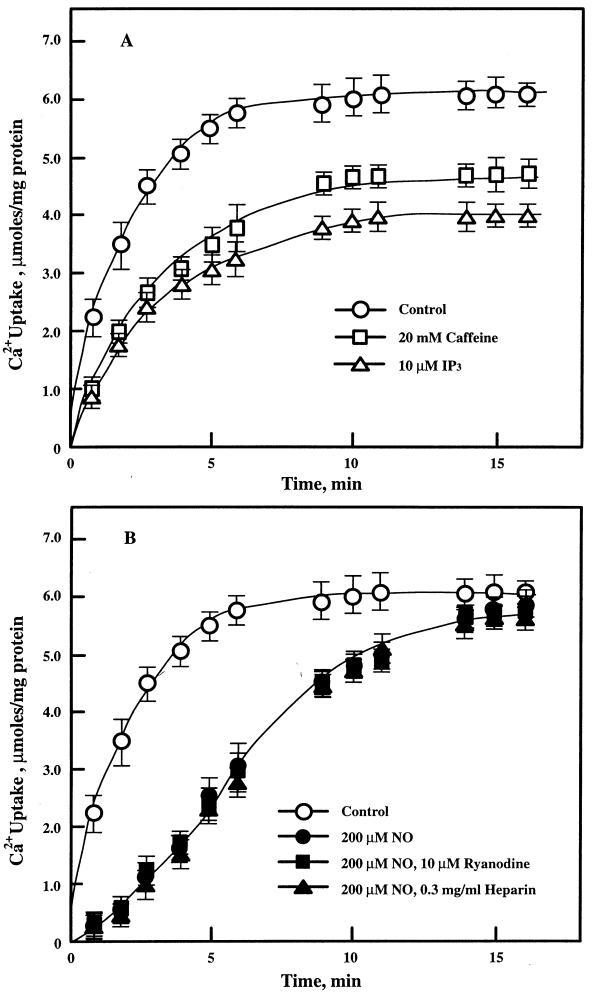

Fig. 6. Effects of caffeine, IP_3, ryanodine and heparin on Ca^{2+} uptake activity and reduction of Ca^{2+} uptake activity by nitric oxide in rabbit SR preparations. A: The Ca^{2+} uptake activity of SR preparations was measured in the absence or presence of 20 mM caffeine or 10 μM IP_3. B: The Ca^{2+} uptake activity of SR preparations preincubated without (control) or with 200 μM nitric oxide for 1 min was measured in the absence or presence of 10 μM ryanodine or 0.3 mg/ml heparin. Ca^{2+} taken up into the SR preparations during various incubation periods is shown as mol Ca^{2+}/mg protein. Points and bars show means and standard deviations for four to six separate measurements.

In the present study, the effects of nitric oxide at concentrations of up to 250 μM on SERCA activity, evaluated by hydrolysis of ATP and $^{45}Ca^{2+}$ uptake, were reversible, but the effect of the nitric oxide persistently releasing compound NOR3 was sustained. The reversible nature of nitric oxide-induced inhibition seems to be due to loss of nitric oxide from the incubation medium by its oxidation. However, although nitric oxide is oxidized rapidly (within several seconds), its effect on SERCA activity persists for some time. The time required for recovery from the inhibitory effects of nitric oxide treatment was related with the concentration of nitric oxide. There are reports that thiol residues such as glutathione and cysteine are dehydrated by *O*-iodide benzoic acid and peroxide, thereby resulting in formation of inter-molecular disulfide bonds [28,29]. Another modification of thiol residues by nitric oxide is *S*-nitrosylation. *S*-Nitrosylation of thiol residues of ryanodine receptors purified from canine heart caused pro-

gressive channel activation [30]. The nitrosylation of GAPDH by nitric oxide was closely related to inhibition of its activity [31]. However, the inhibitory effect of nitric oxide on SERCA does not seem to be related to modifications of thiols, since DTT did not reverse the reduced activities of SERCA and Ca^{2+} uptake induced by nitric oxide. Furthermore, ryanodine did not affect the inhibitory effect of nitric oxide on Ca^{2+} uptake. It is reported that Ca^{2+} release from skeletal muscle SR via ryanodine receptors is activated by caffeine, and reduced by nitric oxide donors [32]. Therefore, nitric oxide may inhibit Ca^{2+} release in the absence of caffeine. However, in the present study, nitric oxide apparently inhibited rather than increased Ca^{2+} uptake, and ryanodine and heparin did not affect Ca^{2+} uptake in the presence of nitric oxide. Thus, these inhibitory effects of nitric oxide also seem to be independent of activation or inhibition of SR Ca^{2+} channels. Since nitric oxide did not affect Na^+,K^+-ATPase in a chicken kidney homogenate, its inhibitory effect on SERCA activity does not seem to be common to P type ATPases.

Interestingly, in rat diaphragm fast fibers, the stimulatory effect of an inhibitor of nNOS on contraction induced by electrical stimulation was partially counteracted by a cyclic GMP analogue, 8-bromo cyclic GMP, and a phosphodiesterase inhibitor, dipyridamole, and the guanylyl cyclase inhibitor LY 83583 increased the contraction induced by electrical stimulation [5]. These results suggest that the cyclic GMP-PKG pathway is involved in the intracellular mechanism of the inhibitory action of nitric oxide. However, our finding of the inhibitory effect of nitric oxide on SERCA activity is incompatible with such a pathway, since LY 83583 and a PKG inhibitor, Rp-8 bromo cyclic GMPS, did not affect the inhibitory effect of nitric oxide on SERCA activity. Nitric oxide may affect SERCA protein directly. Thus, although inhibition by nitric oxide of SERCA, mainly SERCA-1, seems to be one possible mechanism for its inhibition of skeletal muscle contraction, further studies are needed on the relationship between inhibition of SERCA activity and muscle contraction.

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