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Susceptibility of caffeine- and Ins(1,4,5)P_3-induced contractions to oxidants in permeabilized vascular smooth muscle

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Abstract

Two principal pathways of Ca^{2+} release from the sarcoplasmic reticulum of excitable and non-excitable cells have been described: one pathway dependent on the second messenger D-*myo*-inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), and a second pathway sensitive to Ca^{2+} and regulated by caffeine and ryanodine. It was found that the Ca^{2+}-pump activity of vascular smooth muscle sarcoplasmic reticulum is inhibited by superoxide anion radicals ($O_2^{\cdot -}$); however, the effects of reactive oxygen intermediates on sarcoplasmic reticulum Ca^{2+} release in vascular muscle cells are not well defined. The purpose of the present study was to evaluate the effects of reactive oxygen intermediates generated from the hypoxanthine/xanthine oxidase reaction system on contractions induced by caffeine, Ins(1,4,5)P_3 and norepinephrine in staphylococcal α-toxin-permeabilized rabbit mesenteric arteries. This system generates $O_2^{\cdot -}$, H_2O_2, and hydroxyl radicals. We wished to identify which class of reactive oxygen intermediates is responsible for the associated loss of vascular smooth muscle contractile function. Caffeine and Ins(1,4,5)P_3 produced a transient contraction when the sarcoplasmic reticulum of the permeabilized preparations was preloaded with pCa 7.0 solution for 5 min before washing with 0.5 mM EGTA solution; norepinephrine also produced a transient contraction. Exposure of the preparations to hypoxanthine/xanthine oxidase (for 30 min) attenuated caffeine-induced contraction, but was without effect on Ins(1,4,5)P_3-induced contraction. The observed effect of hypoxanthine/xanthine oxidase exposure was superoxide dismutase-inhibitable, suggesting $O_2^{\cdot -}$ involvement. Hypoxanthine/xanthine oxidase also inhibited norepinephrine-induced contraction. The effect of hypoxanthine/xanthine oxidase on norepinephrine contraction was protected by catalase, but not by superoxide dismutase and dimethyl sulfoxide; exogenously added H_2O_2 mimicked the effect of hypoxanthine/xanthine oxidase exposure. H_2O_2, added exogenously, was without effect on Ins(1,4,5)P_3-induced contraction. It is suggested that the pathway of Ca^{2+} release from the sarcoplasmic reticulum dependent on Ins(1,4,5)P_3 is insensitive to $O_2^{\cdot -}$. Instead, caffeine-induced Ca^{2+} release mechanisms may be susceptible to $O_2^{\cdot -}$ and H_2O_2, rather than $O_2^{\cdot -}$ and hydroxyl radicals, may be the active agent in the norepinephrine-induced contraction. Our results are also consistent with the view that the attenuation by H_2O_2 of the norepinephrine-induced contraction may be linked to the receptor-associated pathway of Ins(1,4,5)P_3 formation, but not to degradation processes of Ins(1,4,5)P_3.

Keywords: Free radical; Ca^{2+} store; Smooth muscle, vascular

1. Introduction

Reactive oxygen intermediates (superoxide anion radical ($O_2^{\cdot -}$), H_2O_2 and hydroxyl radical ($HO^{\cdot}$)) are produced under various physiological and pathophysiological conditions (see Slater, 1984; Werns et al., 1986). Reactive oxygen intermediates have been implicated in ischemia-reperfusion and other pathophysiological states of the cardiovascular system (Slater, 1984; Okabe et al., 1993). In some of the pathophysiological conditions, the first target of oxygen free radicals is the vascular system (Rubanyi, 1988). Reactive oxygen intermediates may be involved in processes such as the endothelium-dependent relaxation of coronary arteries (Rubanyi and Vanhoute, 1986), but an accumulation of these intermediates such as $HO^{\cdot}$ can inhibit endothelium-dependent coronary artery relaxation (Todoki et al., 1992). Several investigators have also suggested that reactive oxygen intermediates interfere with normal contractile function in vascular smooth muscle. Lamb and Webb (1984) found that free radicals generated by electrolysis inhibit contractile responses of rat tail and dog coronary arteries, in vitro. Xanthine-oxidase-derived radicals were reported by Wolin and Belloni (1985) to decrease tension in norepinephrine-precontracted vessel

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rings. Although these studies have examined the effects of oxidants on the vasculature, the species of reactive oxygen intermediates responsible for the smooth muscle dysfunction and the mechanisms of the effects at the cellular level remain undefined.

Calcium ions play a determining role in several cellular processes, including excitation-contraction coupling. Although the systems responsible for the Ca^{2+} dynamics of excitation-contraction coupling in smooth muscle are not as well defined as those in skeletal and cardiac muscles, sarcoplasmic reticulum is believed to play a major role as both a sink (Ford and Hess, 1975) and source (Somlyo et al., 1985) of activator Ca^{2+}. There have been several reports that oxygen radicals can inhibit or inactivate sarcoplasmic reticulum Ca^{2+} pumps that are responsible for the removal of Ca^{2+} from cytosol. Superoxide radicals have been reported to produce dysfunction of smooth muscle sarcoplasmic reticulum (Suzuki and Ford, 1991) and cardiac sarcoplasmic reticulum (Okabe et al., 1983, 1988). Furthermore, Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine receptor Ca^{2+} channel is stimulated by xanthine oxidase-derived radicals in cardiac muscle (Okabe et al., 1991). Two principal pathways of Ca^{2+} release from the sarcoplasmic reticulum of excitable and non-excitable cells have been described: one pathway dependent on the second messenger, D-*myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) (Berridge, 1987), and a second pathway sensitive to Ca^{2+} and regulated by caffeine and ryanodine (Fleischer and Inui, 1989). In smooth muscle, both $Ins(1,4,5)P_3$ and ryanodine receptor-mediated Ca^{2+} release mechanism have been described previously (for a review, see Somlyo and Somlyo, 1994). However, the effects of reactive oxygen intermediates on sarcoplasmic reticulum Ca^{2+} release in vascular muscle cells are not well defined. Nishimura et al. (1988) and Kitazawa et al. (1989) developed a method of permeabilizing vascular smooth cells with staphylococcal α-toxin. According to their results, the α-toxin-permeabilized vascular smooth muscle retained intact receptors and signal transduction systems. In the present study, we used α-toxin-permeabilized rabbit mesenteric arteries to examine the effects of the hypoxanthine–xanthine oxidase reaction system, which generates $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$, the major aim of the study being a better understanding of which of these species is responsible for the associated loss of the vascular smooth muscle contractions induced by caffeine, $Ins(1,4,45)P_3$ and norepinephrine.

2. Materials and methods

2.1. Animals

Male albino (New Zealand) rabbits (2.0–2.5 kg) were used for all studies.

2.2. Vessel preparation and isometric tension recording

In accordance with our institutional Animal Care Committee guidelines, the animals were killed by bleeding under anesthesia (pentobarbital sodium, 50 mg/kg, i.v.), then small mesenteric arteries were dissected away and cut into rings. A ring of muscle (150–200 μm in diameter, 100–150 μm in length) was prepared in physiologic salt solution of the following composition: 140 mM NaCl, 5.0 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, and 5.0 mM HEPES (brought to pH 7.4 with NaOH).

Force recordings were obtained at room temperature from the rings mounted in a 1.0-ml volume chamber by means of two tungsten wires passed through the lumen of

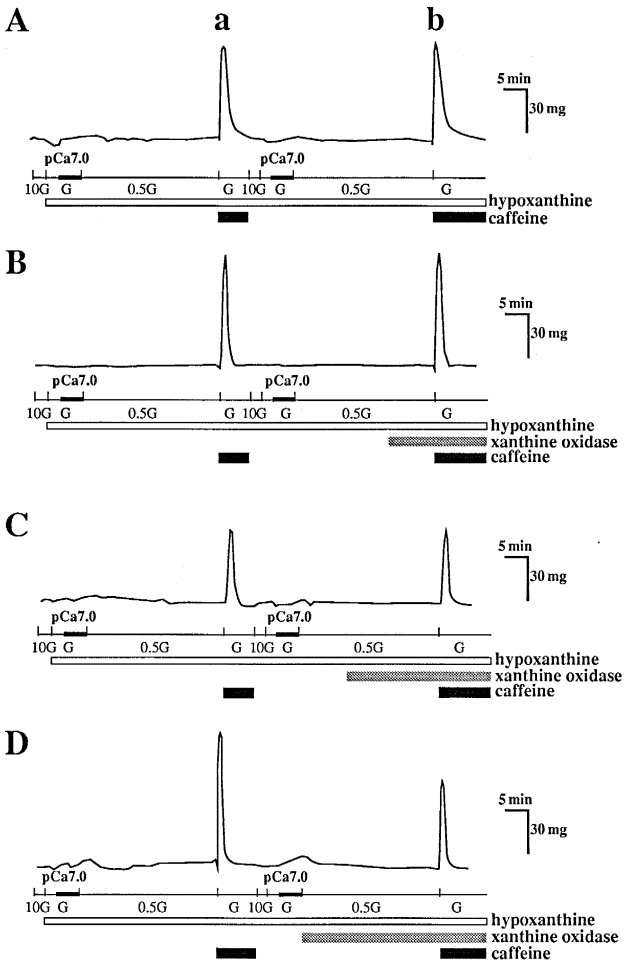

Fig. 1. The effect of hypoxanthine-xanthine oxidase reaction on contractions induced by caffeine in the α-toxin-permeabilized rabbit mesenteric artery. Contractions induced by 30 mM caffeine were studied in the absence (but hypoxanthine alone was present, lane a) or presence (lane b) of hypoxanthine (100 μM)-xanthine oxidase (0.01 U/ml). The permeabilized preparations were exposed to hypoxanthine-xanthine oxidase for 10 (B), 20 (C) and 30 min (D) before the addition of caffeine; a time-matched control study in the presence of hypoxanthine alone was also performed (A). Four α-toxin-permeabilized preparations (for time-matched control, 10, 20 and 30 min of exposure) obtained from the same vessel were studied in parallel. Tracings are representative of four experiments.

Table 1
Effect of hypoxanthine (HX)-xanthine oxidase (XO) reaction on caffeine- and Ins(1,4,5)P_3-induced contraction

HX/XO exposure	Caffeine contraction (%)		Ins(1,4,5)P_3 contraction (%)
	−SOD	+SOD	−SOD
10 min	94.9 ± 12.9		94.3 ± 9.1
20 min	93.2 ± 7.5		81.6 ± 19.5
30 min	56.6 ± 6.6 [a]	104.2 ± 6.6 [b]	97.0 ± 11.6

The experimental conditions were similar to those described in Figs. 1 and 2; the permeabilized artery preparations were exposed to HX/XO for 10, 20 and 30 min before the addition of caffeine or Ins(1,4,5)P_3 (in the presence of HX/XO). Superoxide dismutase (SOD; 100 U/ml) was added concomitantly with HX. In all preparations tested without HX/XO exposure (in the presence of HX alone), the caffeine- or Ins(1,4,5)P_3-induced response was stable (time-matched control, Fig. 2A-a and 2B-a) during the experimental period. Percentages of the responses evoked by caffeine and Ins(1,4,5)P_3 observed 30 min after the addition of XO to pre-HX/XO exposure responses in time-matched controls (caffeine, 95.3 ± 4.3; Ins(1,4,5)P_3, 108.5 ± 5.2) are taken as 100%, and other responses (run simultaneously) are calculated in relation to this. The results are means ± S.E.M. ($n = 4$); n refers to the number of rabbits from which the mesenteric artery was taken. [a] $P < 0.01$ in comparison with 10 min value; [b] $P < 0.01$ in comparison with corresponding value without SOD.

the ring; one wire was attached to the chamber, and the other was attached to a force transducer (Minebea, UL-2GR, Tokyo, Japan) mounted on a micromanipulator (Narishige, MM-113, Tokyo, Japan). The α-toxin-permeabilized smooth muscle was prepared and studied by the methods described by Nishimura et al. (1988). After contractions were measured for the intact preparation, the muscle was treated with α-toxin (50 μg/ml, for 13 min) in a cytoplasmic substitution solution of the following composition: 130 mM K propionate, 4.0 mM $MgCl_2$, 3.75 mM Na_2-ATP, 20 mM Tris/maleate, 10 mM creatine phosphate and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 6.84. After the treatment with α-toxin the skinned preparations were used for the experiments.

2.3. Oxygen radical generating system

To produce a flux of oxygen radicals, an enzyme substrate system was used that consisted of xanthine oxidase (0.01 U/ml), and hypoxanthine (100 μM) as a substrate. The oxidation of hypoxanthine by xanthine oxidase yields $O_2^{\cdot -}$ that is split into H_2O_2 and O_2. This reaction can proceed spontaneously or can be catalyzed by superoxide dismutase. We used superoxide dismutase to scavenge $O_2^{\cdot -}$. Catalase and dimethyl sulfoxide (DMSO) were used to scavenge H_2O_2 and $HO^{\cdot}$, respectively; deferoxamine, a powerful iron chelator, was also used. The timed sequence of reagent addition is described in the Section 3.

2.4. Electron spin resonance (ESR) analysis

The production of $O_2^{\cdot -}$ radicals by the hypoxanthine-xanthine oxidase reaction was verified by ESR spectroscopy. ESR detection of the spin adduct was performed by a JES-FR 100, X-band spectrometer (Jeol, Tokyo, Japan) at the following instrument settings: modulation amplitude, 0.25 mT; recording range, 5 mT; recording

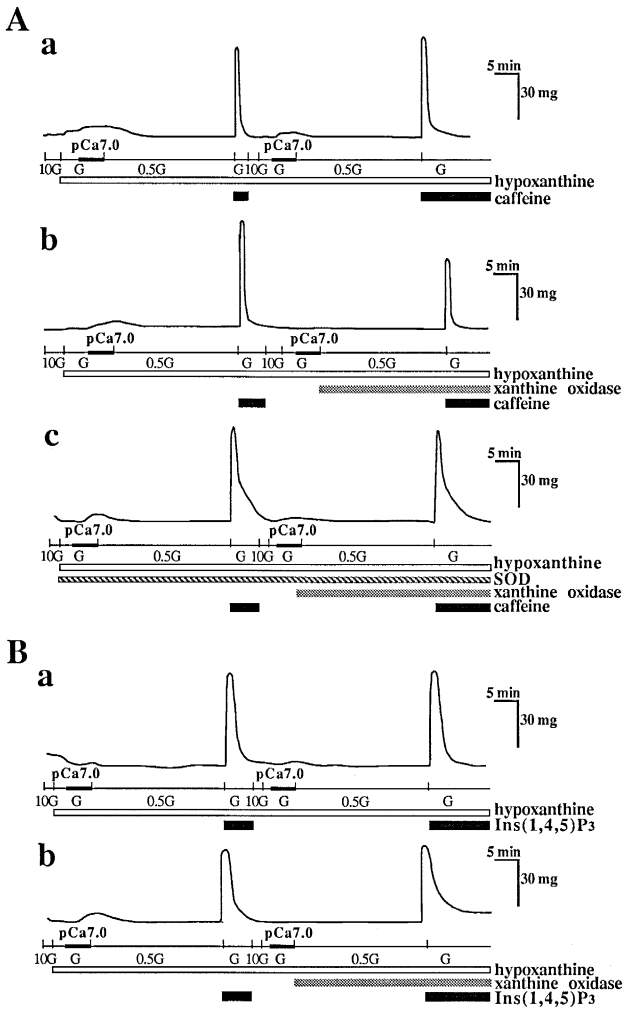

Fig. 2. The effect of hypoxanthine-xanthine oxidase exposure on contractions induced by caffeine (A) and Ins(1,4,5)P_3 (B) in α-toxin-permeabilized rabbit mesenteric artery, and effect of superoxide dismutase (SOD). Reaction conditions were identical to those of Fig. 1D. The permeabilized preparations were exposed to hypoxanthine-xanthine oxidase for 30 min before the addition of 30 mM caffeine (A-b and c) or 250 μM Ins(1,4,5)P_3 (B-b); a time-matched control study for caffeine (A-a) or Ins(1,4,5)P_3 (B-a) in the presence of hypoxanthine alone was also performed. SOD (100 U/ml) was added concomitantly with hypoxanthine (A-c). Five α-toxin-permeabilized preparations (for time-matched controls, caffeine and Ins(1,4,5)P_3; hypoxanthine plus xanthine oxidase, caffeine and Ins(1,4,5)P_3; and SOD plus hypoxanthine plus xanthine oxidase, caffeine) obtained from the same vessel were studied in parallel. Tracings are representative of four experiments.

time, 2 min; time constant, 0.03 s; microwave power, 8 mW; and magnetic field, 335.6 ± 5 mT. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO; 100 mM) was used as the spin trap. The desired reaction mixtures (200 μl) were prepared in the reaction chamber and transferred to a quartz ESR flat cell (130 μl), which, in turn, was placed in the cavity of the ESR spectrometer. Sequential ESR scans were then started 30 s after the addition of DMPO to the reaction mixture. To quantitate the DMPO spin adducts detected, the Mn^{2+} standard ESR spectrum (MnO) was obtained.

2.5. Reagents

The following drugs and chemicals were used: caffeine, catalase, creatine phosphate, EGTA, hypoxanthine, norepinephrine hydrochloride, superoxide dismutase (from bovine blood, 3300 U/mg of protein) (Sigma, St. Louis, MO, USA); Ins(1,4,5)P_3, dimethyl sulfoxide, hydrogen peroxide (Wako, Osaka, Japan; the H_2O_2 concentration was estimated spectrophotometrically by measuring its absorbance at 240 nm with extinction coefficient, $\epsilon = 81$ $l \cdot mol^{-1} \cdot cm^{-1}$, then it was used for experiments); deferoxamine mesylate (Ciba-Geigy, Basel, Switzerland); xanthine oxidase (Boehringer-Mannheim, Mannheim, Germany; activity, 35.8 μM/min); 5,5-dimethyl-1-pyrroline-*N*-oxide (Labotec, Tokyo, Japan); and staphylococcal α-toxin (Gibco BRL Life Technologies, Grand Island, NY, USA). All other reagents were of analytical grade.

2.6. Data presentation

All data were expressed as means ± S.E.M. Two sets of statistical comparisons were made. Student's *t*-test for paired samples was used when two populations were compared with each other. Comparisons of subsequent interventions with the controls were made using a one-way analysis of variance, followed by Duncan's multiple range test. Differences were considered significant at $P < 0.05$.

3. Results

Caffeine increases the Ca^{2+} sensitivity of Ca^{2+}-releasing channels for Ca^{2+}-induced Ca^{2+} release in sarco-

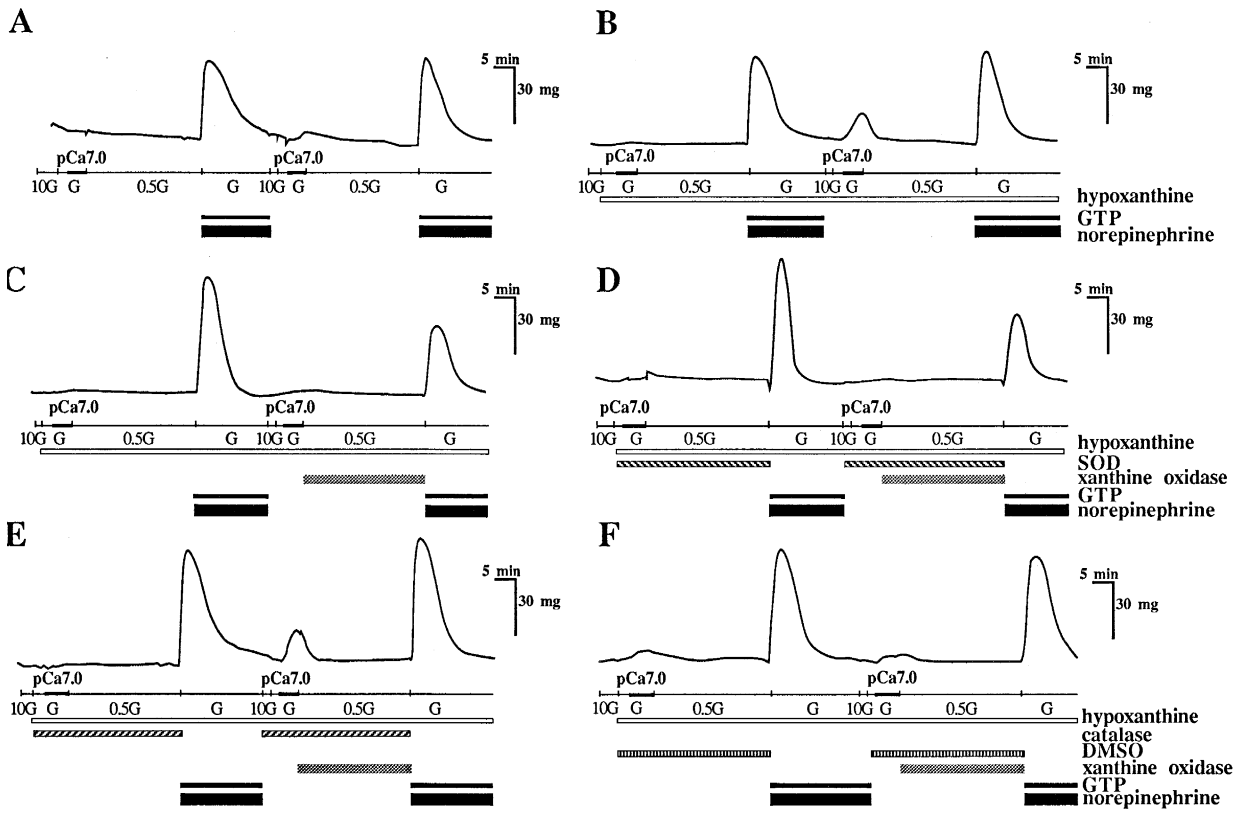

Fig. 3. The effect of previous hypoxanthine-xanthine oxidase exposure on contractions induced by norepinephrine in α-toxin-permeabilized rabbit mesenteric artery, and effects of various reactive oxygen species scavengers. Reaction conditions were identical to those of Fig. 1D except that contractions were generated by 10^{-5} M norepinephrine in the presence of 1 mM GTP. The permeabilized preparations were exposed to hypoxanthine-xanthine oxidase for 30 min and the preparations were washed serially, then responses were generated with norepinephrine (C–F); a time-matched control study in the absence or presence of hypoxanthine alone was also performed (A and B). Superoxide dismutase (SOD; 100 U/ml, D), catalase (10 U/ml, E) or DMSO (100 mM, F) was added 39 min before the addition of norepinephrine and GTP. Six α-toxin-permeabilized preparations (for time-matched controls in the presence and absence of hypoxanthine alone, hypoxanthine plus xanthine oxidase, and hypoxanthine plus xanthine oxidase in the presence of SOD, catalase or DMSO) obtained from the same vessels were studied in parallel. Tracings are representative of five experiments.

plasmic reticulum and, therefore, has been widely used as a convenient pharmacological tool to investigate Ca^{2+}-induced Ca^{2+} release mechanisms and the characteristics of the Ca^{2+} storage sites in smooth muscle cells (Sawynok and Yaksh, 1993). The permeabilized artery bathed in nominally Ca^{2+}-free solution responded reproducibly to caffeine if the sarcoplasmic reticulum was previously loaded at pCa 7.0 (Fig. 1A). The pre-exposure responses (Fig. 1, lane a) and post-exposure responses (Fig. 1, lane b) elicited by caffeine were not significantly different from each other when the permeabilized artery was exposed to hypoxanthine/xanthine oxidase for 10 and 20 min before the addition of caffeine (Fig. 1B and C, and Table 1). However, the caffeine-induced contraction was effectively suppressed by 30 min of exposure (before the addition of caffeine) to hypoxanthine/xanthine oxidase (Fig. 1D and Table 1).

Oxygen free-radical species generated from the hypoxanthine/xanthine oxidase reaction under the same reaction conditions as those of Fig. 1 was verified by using highly sensitive ESR spectroscopy and the spin-trap DMPO in the absence of the artery preparations. Hyperfine coupling constants (hfcc's) of the spin adducts observed (ESR spectra not shown) were analyzed as follows: one nitrogen, $a_N = 1.41$ mT, one hydrogen in β-position, $a_{H\beta} = 1.41$ mT, and one hydrogen in γ-position, $a_{H\gamma} = 0.13$ mT. The component of the spectrum was assigned to DMPO-$O_2^{\cdot -}$ (–OOH). The signal intensity of the second peak of the spectrum (normalized as a relative height against the standard signal intensity of the MnO marker) reached its maximum value (1.03) immediately after the addition of hypoxanthine/xanthine oxidase to the bathing media in the presence of DMPO; the relative signal intensity was decreased, in a time-dependent manner, when DMPO was added 5 (signal intensity, 0.97) to 30 min (signal intensity, 0.62) after initiation of the hypoxanthine/xanthine oxidase reaction. Thus, the oxygen free-radical exposure conditions were chosen based on the results depicted in Fig. 1 and on ESR studies. We used 30 min of exposure before the addition of stimulants.

The observed effect of 30 min of hypoxanthine/xanthine oxidase exposure before the addition of caffeine (Fig. 2A-b) was inhibited by superoxide dismutase (Fig. 2A-c and Table 1). We also studied the effect of hypoxanthine/xanthine oxidase on contraction elicited by Ins(1,4,5)P_3 in α-toxin-permeabilized preparations. The exposure of the preparations to hypoxanthine/xanthine oxidase had no significant effect on the Ins(1,4,5)P_3-induced contraction (Fig. 2B and Table 1), suggesting that the pathway of Ca^{2+} release from the sarcoplasmic reticulum dependent on Ins(1,4,5)P_3 is insensitive to oxygen free radicals; instead, caffeine-induced Ca^{2+} release mechanisms may be susceptible to oxygen free radicals. Because one of the major intracellular effects of α_1-adrenoceptor activation is mediated by the synthesis and release of Ins(1,4,5)P_3, we next examined the ability

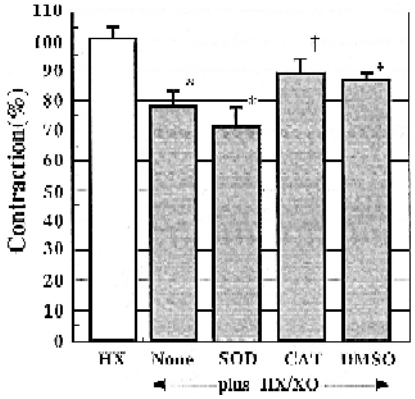

Fig. 4. Bar graph showing the effects of various reactive oxygen species scavengers on contractions induced by norepinephrine with previous hypoxanthine (HX)-xanthine oxidase (XO) exposure. The experiments were performed under the same experimental conditions as those of Fig. 3. Percentages of the responses evoked by norepinephrine observed after early hypoxanthine/xanthine oxidase exposure against pre-exposure responses in time-matched control (in the absence of hypoxanthine/xanthine oxidase, see Fig. 3A; 122.2 ± 10.8) are taken as 100%, and other responses (run simultaneously) are calculated in relation to this. The results are means ± S.E.M. ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken. * $P < 0.01$ in comparison with hypoxanthine alone; † $P < 0.05$ in comparison with hypoxanthine/xanthine oxidase-no drug. SOD indicates superoxide dismutase; and CAT, catalase.

of norepinephrine to generate contraction and the effect of hypoxanthine/xanthine oxidase reaction in the permeabilized artery preparations. The inhibitory effect of several compounds on norepinephrine-induced vascular responses is known to be due to oxidation of the catecholamine (Furchgott, 1956). In the present series of studies, therefore, norepinephrine was added after hypoxanthine/xanthine oxidase was removed from the organ chamber; the artery preparations were exposed to hypoxanthine/xanthine oxidase and the preparations were washed serially, then responses were generated with norepinephrine. Fig. 3 shows representative tracings of an experiment designed to examine the effect of hypoxanthine/xanthine oxidase on the response elicited by norepinephrine. The contraction in response to norepinephrine was significantly attenuated by the hypoxanthine/xanthine oxidase reaction (Fig. 3C); the effect produced by hypoxanthine/xanthine oxidase was slightly enhanced by superoxide dismutase (Fig. 3D) and inhibited by catalase (Fig. 3E) (see summarized data in Fig. 4). DMSO slightly, but not significantly, protected against the observed effect of hypoxanthine/xanthine oxidase (Fig. 3F and Fig. 4). This suggests the possible involvement of H_2O_2 in the inhibitory effect of hypoxanthine/xanthine oxidase on norepinephrine-induced contraction. Experiments similar to that depicted in Fig. 3, except that H_2O_2 was used instead of hypoxanthine/xanthine oxidase, were performed. Previous exposure to H_2O_2 of the permeabilized preparations produced significant inhibition of norepinephrine-induced contraction (Fig. 5B). The effect of H_2O_2 was protected

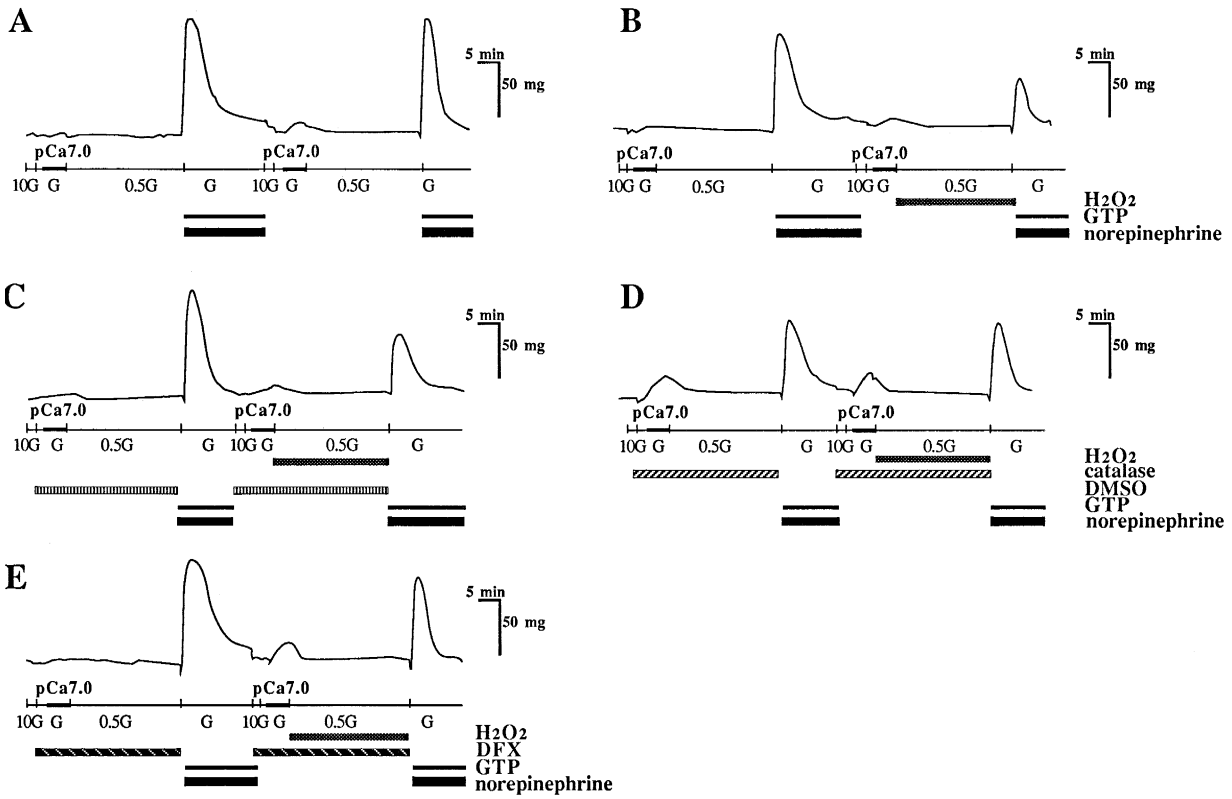

Fig. 5. The effect of previous H_2O_2 exposure on contractions induced by norepinephrine in α-toxin-permeabilized rabbit mesenteric artery, and effects of various reactive oxygen scavengers. Reaction conditions were identical to those of Fig. 3 except that H_2O_2 was used instead of the hypoxanthine-xanthine oxidase reaction. The permeabilized preparations were exposed to H_2O_2 (500 μM) for 30 min and the preparations were washed serially, then responses were generated with 10^{-5} M norepinephrine (B–E); a time-matched control study in the absence of H_2O_2 was also performed (A). DMSO (100 mM, C), catalase (10 U/ml, D) or deferoxamine (DFX, 1 mM; E) was added 39 min before the addition of norepinephrine and GTP. Five α-toxin- permeabilized preparations (for time-matched control, H_2O_2, and H_2O_2 in the presence of DMSO, catalase or deferoxamine) obtained from the same vessels were studied in parallel. Tracings are representative of eight experiments.

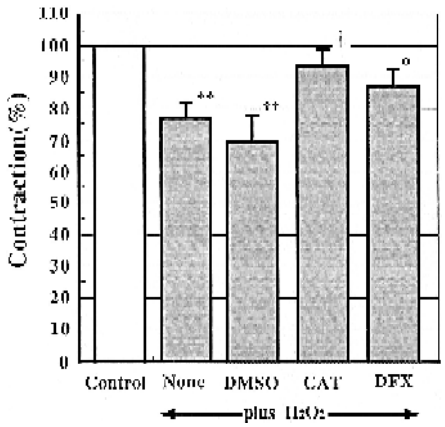

Fig. 6. Bar graph showing the effects of various reactive oxygen species scavengers on contractions induced by norepinephrine with previous H_2O_2 exposure. The experiments were performed under the same experimental conditions as those of Fig. 5. Percentages of the responses evoked by norepinephrine observed after early H_2O_2 exposure against pre-exposure responses in time-matched control (see Fig. 5A; 103.8 ± 5.4) are taken as 100%, and other responses (run simultaneously) are calculated in relation to this. The results are means ± S.E.M. ($n = 8$); n refers to the number of rabbits from which the mesenteric artery was taken. $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with control; $^{\dagger}P < 0.05$ in comparison with H_2O_2-no drug. CAT indicates catalase; and DFX, deferoxamine.

against by catalase (Fig. 5D), but not by DMSO (Fig. 5C) and deferoxamine (Fig. 5E) (see also summarized data in Fig. 6). H_2O_2 (500 μM) exposure (for 30 min) had no significant effect on 250 μM Ins(1,4,5)P_3-induced contraction (data not shown).

4. Discussion

There is substantial evidence to support the role of sarcoplasmic reticulum Ca^{2+} cycling mechanism in the maintenance of tension in vascular muscle. Although tonic tension clearly depends on Ca^{2+} influx (Lodge and Van Breemen, 1985), Ca^{2+} entering the smooth muscle cell may first be accumulated into the peripheral sarcoplasmic reticulum (Van Breemen, 1977). Receptor stimulation, by enhancing Ins(1,4,5)P_3 production, induces a permeable sarcoplasmic reticulum, through which Ca^{2+} could cycle, to be released in the vicinity of the myofilaments. It is known that caffeine activates Ca^{2+} channels in the sarcoplasmic reticulum of vascular muscle, which mediate Ca^{2+}-induced release of Ca^{2+} (Herrmann-Frank et al., 1991). These channels are distinct from the channels that

mediate sarcoplasmic reticulum Ca^{2+} release when the cell is stimulated by agonists such as norepinephrine. By acting on specific membrane receptors, agonists stimulate the formation of Ins(1,4,5)P_3, which binds to and opens specific Ins(1,4,5)P_3 receptor channels in the sarcoplasmic reticulum membrane (Ehrlich and Watras, 1988). In the present study, exposure of α-toxin-permeabilized rabbit mesenteric artery preparations to hypoxanthine/xanthine oxidase attenuated the caffeine-induced contraction, but was without any effect on Ins(1,4,5)P_3-induced contraction (Table 1); we have demonstrated that the attenuation by hypoxanthine/xanthine oxidase exposure is superoxide dismutase-inhibitable. The results are consistent with an inhibitory action of $O_2^{\cdot -}$ on Ca^{2+}-induced release of Ca^{2+}, but not on Ins(1,4,5)P_3-dependent Ca^{2+} release.

The α-toxin-permeabilized smooth muscle preparation was first described by Kerrick and colleagues (Cassidy et al., 1979). More recently, it was found that this preparation retains receptor- and G protein-coupled alterations in myofilament Ca^{2+} sensitivity (Nishimura et al., 1988; Kitazawa et al., 1989, 1991). This may be related to the fact that α-toxin forms small pores approximately 2–3 nm in diameter, which admit small ions and water-soluble molecules but do not allow diffusional removal of essential cytoplasmic proteins (Hohman, 1988). Nishimura et al. (1988) reported that the activation of adrenoceptors by norepinephrine on the permeabilized membrane induces Ca^{2+} release from the sarcoplasmic reticulum and produces contraction via formation of Ins(1,4,5)P_3. If the degradation process but not the formation of Ins(1,4,5)P_3 is sensitive to the hypoxanthine/xanthine oxidase reaction, norepinephrine-induced contraction could not be modified by the hypoxanthine/xanthine oxidase reaction. On repeated norepinephrine stimulation of α-toxin-permeabilized rabbit mesenteric artery preparations in the absence of exogenous GTP, the contraction declines as a function of the number of contraction-relaxation cycles (Nishimura et al., 1992). Therefore, to assess the effect of the hypoxanthine/xanthine oxidase reaction in our system, norepinephrine-induced contraction was evoked in the presence of exogenous GTP. In addition, Ca^{2+} ionophore A23187 and ionomycin were not included in all solutions to ensure that sarcoplasmic reticulum would be involved in any of the responses studied. Under the conditions reported here, the contraction evoked by norepinephrine was significantly attenuated by the hypoxanthine/xanthine oxidase reaction (Fig. 4). Superoxide dismutase induced some enhancement of the effect of hypoxanthine/xanthine oxidase, and catalase significantly protected against the observed effect of the hypoxanthine/xanthine oxidase reaction (Fig. 4), most likely in conjunction with H_2O_2 generation. There are several products of the hypoxanthine/xanthine oxidase reaction that could mediate the attenuation of norepinephrine-induced contraction observed in the present study. The fate of $O_2^{\cdot -}$ generated from hypoxanthine/xanthine oxidase is varied. First, it may react with biological molecules to cause cellular dysfunction. Secondly, it may interact with water to form H_2O_2, a spontaneous reaction whose rate is enhanced by superoxide dismutase (Okabe et al., 1991). Also, formation of $HO^{\cdot}$ radical via Fenton and Haber-Weiss reactions, due to contaminant iron salts as shown below, is well possible:

$$O_2^{\cdot -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^- \quad (2)$$

The data strongly support a role for H_2O_2 as a mediator of the effect of hypoxanthine/xanthine oxidase on norepinephrine-induced contraction because protection against the effect of hypoxanthine/xanthine oxidase could only be obtained with catalase, but not with superoxide dismutase or DMSO. The slight enhancement of the effect of hypoxanthine/xanthine oxidase produced by superoxide dismutase may be due to an anticipated increase in H_2O_2 production via an alternate divalent reduction pathway (Fridovich, 1970). Our studies have shown that H_2O_2 produced a catalase-inhibitable attenuation of the contraction evoked by norepinephrine; $HO^{\cdot}$ scavenger DMSO and deferoxamine had no effect on the attenuated norepinephrine-induced contraction (Fig. 6). Deferoxamine is a powerful iron chelator. It binds tightly to Fe(III) and, therefore, cannot be reduced to Fe(II) by $O_2^{\cdot -}$ for $HO^{\cdot}$ radical production (Eqs. (1) and (2)). These results suggest that it may be H_2O_2 rather than $HO^{\cdot}$ and $O_2^{\cdot -}$ is most likely to be responsible for the demonstrated effect of hypoxanthine/xanthine oxidase on norepinephrine-induced contraction. The conclusion that the degradation processes of Ins(1,4,5)P_3 are not sensitive to H_2O_2 is based, in part, on the finding that the contraction elicited by Ins(1,4,5)P_3 was not affected by H_2O_2 exposure; thus H_2O_2 either produced by hypoxanthine/xanthine oxidase reaction or added exogenously may modify the formation processes of Ins(1,4,5)P_3, thereby inhibiting the Ins(1,4,5)P_3-dependent Ca^{2+} release from sarcoplasmic reticulum linked to generation of the contraction.

Wolin and Belloni (1985) reported that xanthine oxidase-derived radicals decreased contractile tension in vessels that had been tonically contracted by norepinephrine. These investigators suggested that $O_2^{\cdot -}$ may mediate the vessel injury because superoxide dismutase but not catalase prevented the loss of contractile tension. Although the results of this earlier study suggested a role of oxidant-dependent attenuation of vascular contractile function, the experimental preparation did not allow the investigators to determine whether the loss of contractile tension was due to oxidation and inactivation of norepinephrine or to direct damage to the vascular preparation. A major difference between the study of Wolin and Belloni (1985) and the present study is that we now assessed the contractile effectiveness of norepinephrine before and after exposure of the permeabilized preparation to hypoxanthine/xanthine oxidase. As the preparations were thoroughly washed after exposure to hypoxanthine/xanthine oxidase, we believe

that our data provide strong evidence in support of direct smooth muscle damage.

Although our results clearly established a role for reactive oxygen intermediates in hypoxanthine/xanthine oxidase-induced loss of contractile responsiveness in the α-toxin-permeabilized artery preparation, the cellular mechanism involved remains unclear. Potential sites of the attenuation of norepinephrine-induced contraction by hypoxanthine/xanthine oxidase include adrenoceptors and alterations in second messenger function; the degradation processes of Ins(1,4,5)P_3 and disruption of contractile protein function are not the sites of the action, because the contraction evoked by exogenous Ins(1,4,5)P_3 was unaffected by the hypoxanthine/xanthine oxidase reaction (Table 1). Further studies should provide more insight into the cellular mechanism of oxidant-induced vascular smooth muscle dysfunction.

Staphylococcal α-toxin enters the plasma membrane of susceptible cells and forms very stable non-selective pores. These pores permit compounds of a molecular mass of < 1 kDa to diffuse while retaining larger proteins (Ahnert-Hilger et al., 1989), thus the xanthine oxidase molecule (130 kDa) is not permeable. This implies that reactive oxygen species generated from the hypoxanthine/xanthine oxidase reaction may act outside the cells. The superoxide anion radical is generally known as a poor oxidant in aqueous solution (Sawyer and Valentine, 1981), and the stronger oxidant, $HO^{\cdot}$, was felt to be a potentially more toxic agent. The greater reactivity of $HO^{\cdot}$ presents a problem because $HO^{\cdot}$, once formed, can react indiscriminately with many molecules. Thus it is unlikely to reach a critical macromolecular target unless its generation occurs specifically at the target (Fridovich, 1986). Since $O_2^{\cdot -}$ is generally a poor reactant with many molecules, its concentration is more likely to be conserved; thus it is available to react with any susceptible molecule. If any functional molecule is reactive to $O_2^{\cdot -}$, it can be the site of damage caused by reactive oxygen intermediates. Therefore, a possible explanation for the effect of hypoxanthine/xanthine oxidase reported upon here is that $O_2^{\cdot -}$ generated outside of the smooth muscle cells may affect the caffeine-sensitive superficial Ca^{2+} store (Van Breemen and Saida, 1989; Wong and Klassen, 1993), and that Ins(1,4,5)P_3-sensitive deep sarcoplasmic reticulum function may be modified by H_2O_2 because extracellular H_2O_2 quickly crosses the cell membrane. This mechanism would integrate the role of reactive oxygen intermediates described in this study with the vascular pathophysiology linked to alterations in local blood flow, e.g., inflammation and reperfusion of ischemic tissues.

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