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INHIBITION BY FREE RADICAL SCAVENGERS AND BY CYCLOOXYGENASE INHIBITORS OF THE EFFECT OF ACIDOSIS ON CALCIUM TRANSPORT BY MASSETER MUSCLE SARCOPLASMIC RETICULUM*

EIICHIRO OKABE,† YASUSHI KATO, HARUO KOHNO, MICHAEL L. HESS‡ and HARUO ITO

Department of Pharmacology, Kanagawa Dental College, 82 Inaoka-Cho, Yokosuka, 238 Japan; and ‡Department of Medicine (Cardiology), Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

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Abstract—*In vitro*, arachidonic acid depressed calcium transport by sarcoplasmic reticulum (SR) in the homogenate of canine masseter muscle. This effect was inhibited by superoxide dismutase (SOD), a scavenger of the superoxide anion radial ($\cdot O_2^-$), at pH 7.0, and by SOD plus *d*-mannitol, a scavenger of hydroxyl free radical ($\cdot OH$), at pH 5.5. Indomethacin and 2-aminomethyl-4-*tert*-butyl-6-propionyl phenol (ONO-3144), a compound known to accelerate the conversion of prostaglandin G_2 (PGG_2) to PGH_2 and scavenge free radicals, inhibited the effect of arachidonic acid at both pH 7.0 and pH 5.5. PGG_2, but not PGH_2, duplicated the effect of arachidonic acid. The effect of PGG_2 on SR function was similar to that of exogenous free radicals generated from the xanthine–xanthine oxidase system. Incubation at pH 5.5, in the absence of an exogenous free-radical generating system, depressed SR calcium transport in the homogenate and in isolated SR. This effect in the homogenate was inhibited by indomethacin or by ONO-3144. At 10-min incubation at pH 5.5, SOD partially and temporarily reversed the depressant effect of acidosis. The addition of SOD plus *d*-mannitol completely reversed the system. *d*-Mannitol alone was ineffective. Arachidonic acid was able to mimic these effects of acidosis, except that arachidonic acid further depressed isolated SR calcium transport. These results demonstrate that acidosis can depress SR calcium transport in the homogenate of masseter muscle by an oxygen-free radical mechanism by the generation of $\cdot O_2^-$ and $\cdot OH$. Our results also demonstrate that significant oxygen radical generation can occur through the cyclooxygenase pathway of arachidonic acid metabolism at an acidotic pH in the cellular environment outside of the SR of the muscle cell, and seems to be responsible for the generation of the $\cdot OH$ derived from $\cdot O_2^-$.

It has been shown previously that the generation of free radicals from xanthine–xanthine oxidase system uncouples oxalate-supported calcium uptake from the Ca^{2+}-dependent ATPase activity in cardiac sarcoplasmic reticulum (SR)§ [1–3]. We interpreted this finding as a defect in the phospholipid permeability barrier of the SR to calcium [4]. An uncoupling of SR calcium transport from ATP hydrolysis has been observed during the course of skeletal muscle ischemia [5]. In skeletal muscle, Arkhipenko *et al.* [5, 6] found an increase in lipid peroxidation products and disturbed calcium transport during limb tourniquet ischemia.

There is abundant evidence that, during ischemia, there is a switch from aerobic to anaerobic metabolism with increased rates of glycolysis and, therefore, the generation of protons at an increased rate [7]. The accumulation of these hydrogen ions leads to a decrease in intramuscular pH, a finding now documented by direct interstitial pH measurements [8] and nuclear magnetic resonance studies [9]. Further, under ischemic conditions, with the decreased tissue oxygen concentration, there is an increase in reducing equivalents [10]. Under these conditions, the univalent reduction of oxygen is more likely and, therefore, the production of superoxide anion and other free radicals is favored. Any free radical generated during ischemia could readily interact with the unsaturated fatty acids of phospholipid membranes [11].

Prostaglandins are synthesized *de novo* from membrane phospholipids or fatty acids in response to a variety of stimuli including trauma, nerve stimulation, hypoxia, and ischemia [12–14]. Further, it is known that one of the intermediate steps in the biosynthesis of prostaglandins, the enzymatic conversion of the endoperoxide G_2 to the endoperoxide H_2, is accompanied by the release of a free oxygen radical [15]. In a recent study [16], we postulated that dysfunction of masseter muscle SR calcium transport induced by arachidonic acid and prostaglandin G_2 (PGG_2) could be attributed to free radical production with resultant lipid peroxidation. Lipid per-

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† Address all correspondence to: Eiichiro Okabe, D.D.S., Ph.D., Department of Pharmacology, Kanagawa Dental College, 82 Inaoka-Cho, Yokosuka, 238 Japan.

§ Abbreviations: SR, sarcoplasmic reticulum; SOD, superoxide dismutase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E_2; and ONO-3144, 2-aminomethyl-4-*tert*-butyl-6-propionyl phenol hydrochloride.

oxidation, in turn, leads to membrane alterations from phospholipid depletion and also leads to the inhibition of membrane associated enzymes [17].

In this study, we have hypothesized that acidosis and free oxygen radicals or the processes that lead to their production may interact in a way such that the damage induced by free oxygen radicals may be enhanced by the presence of acidosis. With the implication of both free radical production and a decrease in pH, the present study was designed to investigate the possibility that the effect of acidosis on SR calcium transport may be mediated by production of free oxygen radicals via intermediate steps in the synthesis of prostaglandins in canine masseter muscle.

MATERIALS AND METHODS

Sarcoplasmic reticulum preparation. SR vesicles were prepared from minced canine masseter muscle by a previous method [16], as follows. Healthy, adult dogs were anesthetized with sodium pentobarbital (25 mg/kg, i.v.), and the masseter muscle was rapidly removed and placed in ice-cold 0.9% NaCl. The muscle was cleaned of fat and connective tissue, minced and then homogenized twice (1 g muscle/4 vol. of 10 mM imidazole buffer, pH 7.0) for 1 min at 4° in a Sorvall Omnimixer. The homogenate was centrifuged at 10,000 *g* for 20 min. The pellet was rehomogenized in 4 vol. of 10 mM imidazole and centrifuged at 10,000 *g* for 20 min. The supernatant fractions from this and the previous centrifugation were combined, poured through four layers of cheesecloth, and then centrifuged at 12,000 *g* for 15 min. The supernatant fraction was filtered through eight layers of cheesecloth and centrifuged at 31,000 *g* for 90 min. The pellets from this spin were rehomogenized, by use of a Potter–Elvehjem homogenizer with a Teflon pestle, in 1 M KCl, 10 mM imidazole buffer, and then centrifuged at 145,000 *g* for 60 min. The SR pellet was rehomogenized in 30% sucrose, 20 mM Tris–HCl, pH 7.0, and stored at −40° for 1–4 days. Protein concentration of this SR preparation and the unfractionated homogenate was determined by the method of Lowry *et al.* [18].

Calcium uptake velocity. Oxalate-supported calcium uptake rate of the SR was assayed as described previously [16]. Calcium uptake rates of the unfractionated homogenate were modified from a previously described method [19]. The validity and reproducibility of this method have been confirmed by Feher *et al.* [20] and by Newman [21]. The reaction bath (5 ml) contained 34.7 mM KCl, 6 mM imidazole, 3.3 mM NaN_3, 3.3 mM potassium-oxalate, 5 mM Na_2 ATP, 5 mM $MgCl_2$, 0.18 mM $CaCl_2$ with 0.05 μCi $^{45}Ca^{2+}$/ml. The reaction was stopped by filtration through a set of prefilters and Millipore filters (0.45 micropore diameter), and the filtrate was counted in a liquid scintillation spectrometer. All studies were performed at 27°.

Preincubation procedures. The SR or the unfractionated homogenate was preincubated at pH 5.5 for various times in 3.0 ml of incubation mixture (SR: 43.2 mM KCl, 7.5 mM imidazole, 4.2 mM potassium-oxalate and 4.2 mM NaN_3; homogenate: 14.4 mM KCl, 2.5 mM imidazole, 1.4 mM potassium-oxalate and 1.4 mM NaN_3), pH 5.5. After preincubation, the pH was increased by adding 1.0 ml of solution (SR: 389.3 mM KCl, 67.5 mM imidazole, 38.2 mM potassium-oxalate and 38.2 mM NaN_3; homogenate: 129.8 mM KCl, 22.5 mM imidazole, 12.6 mM potassium-oxalate and 12.6 mM NaN_3), pH 8.5. The pH of the final incubation mixtures were 7.01 ± 0.03 (N = 32) in the SR studies and 7.03 ± 0.02 (N = 41) in the homogenates.

The pH of each individual reaction was adjusted with the imidazole buffer following the method of Fabiato and Fabiato [22] and then re-checked prior to the initiation of the reaction sequence. The cyclooxygenase inhibitor indomethacin (Sigma Chemical, Co., St. Louis, MO; 5 mg/kg, i.v.) was injected 30 min before the removal of masseter muscle and isolation of SR. Indomethacin stock solution was prepared immediately prior to the administration by dissolving three parts indomethacin and one part sodium carbonate in distilled water. From this stock solution, indomethacin was dissolved in 10 ml of 0.9% NaCl solution, and administered by slow i.v. infusion. The concentrations of reagents used varied with the purpose of the experiment and are described more fully in Results.

All solutions were prepared with deionized, distilled water using analytical grade reagents. The results are presented as specific activity (μmoles Ca^{2+}/mg protein·min).

Generation of free radicals and use of free radical scavengers. To produce a flux of oxygen-derived free radicals, an enzyme–substrate system consisting of xanthine oxidase (Sigma) and xanthine (Sigma) as a substrate was used at pH 7.0 or 5.5. The oxidation of xanthine by xanthine oxidase yields superoxide anion radical ($\cdot O_2^-$) primarily (equation 1), that is disproportionated into hydrogen peroxide (H_2O_2) and oxygen (O_2) (equation 2). This reaction can proceed spontaneously or it can be catalyzed by superoxide dismutase (SOD). Hydrogen peroxide will then react with $\cdot O_2^-$ to form hydroxyl free radical ($\cdot OH$) or perhaps singlet oxygen ($^1O_2$) (equation 3) [23, 24].

$$\text{xanthine} \xrightarrow{\text{xanthine oxidase}} \text{urate} + \cdot O_2^- \quad (1)$$

$$2\cdot O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (2)$$

$$\cdot O_2^- + H_2O_2 \longrightarrow OH^- + \cdot OH + {}^1O_2 \quad (3)$$

Singlet oxygen, however, may be readily scavenged either by hypoxanthine or by xanthine and urate which are the products of this enzymic reaction [23].

The generation of $\cdot O_2^-$ from our xanthine–xanthine oxidase system was confirmed spectrophotometrically following the conversion of xanthine to urate [24]. Superoxide anion production measured by monitoring the reduction of cytochrome c^{3+} at 550 nm was 6.0×10^{-7} nmoles $\cdot O_2^-$ per liter per sec at 27°, pH 7.0, and 3.0×10^{-7} nmoles $\cdot O_2^-$ per liter per sec at pH 5.5. This compares favorably with that reported by Brawn and Fridovich [25], indicating that our xanthine–xanthine oxidase system is a valid means for assessing the effect of scavengers.

We used SOD (Sigma) to scavenge $\cdot O_2^-$ and *d*-mannitol (Sigma) to scavenge $\cdot OH$ [26]. For control

studies, SOD and xanthine oxidase were denatured by boiling at 100° for 20 min. The timed sequence and concentration of reagent addition are described in Results.

Use of prostaglandins and fatty acids. One of the following agents was incubated at pH 7.0 or 5.5 with masseter muscle homogenate from non-treated animals or indomethacin-treated animals:

(1) 11,14,17-Eicosatrienoic acid (P-L Biochemicals, Milwaukee, WI; 200 μg/ml) incubated with non-treated homogenate. This unsaturated fatty acid is not a substrate for cyclooxygenase and, therefore, does not stimulate prostaglandin synthesis [27]. It was used to determine whether or not 200 μg/ml of a fatty acid produces non-specific effects.

(2) Arachidonic acid (P-L Biochemicals; 200 μg/ml) incubated with non-treated homogenate.

(3) Arachidonic acid (200 μg/ml) incubated with indomethacin-treated homogenate.

(4) PGG_2, 10 μg/ml.

(5) PGH_2, 10 μg/ml.

(6) PGE_2 (Ono Chemical Co., Osaka, Japan), 10 μg/ml.

Note that, because their molecular weights are similar, PGG_2, PGH_2, and PGE_2 at 10 μg/ml provide similar molar concentrations. PGG_2, PGH_2 or PGE_2 was incubated with non-treated homogenate.

(7) PGG_2 (10 μg/ml) incubated with indomethacin-treated homogenate.

(8) PGG_2 (10 μg/ml) plus SOD (10 μg/ml) or denatured SOD (10 μg/ml) incubated with non-treated homogenate.

(9) PGG_2 (10 μg/ml) plus 20 mM *d*-mannitol incubated with non-treated homogenate.

(10) PGG_2 (10 μg/ml) plus SOD (10 μg/ml) or denatured SOD (10 μg/ml) plus 20 mM *d*-mannitol incubated with non-treated homogenate.

(11) Arachidonic acid (200 μg/ml) plus SOD (10 μg/ml) or denatured SOD (10 μg/ml) incubated with non-treated homogenate.

(12) Arachidonic acid (200 μg/ml) plus 20 mM *d*-mannitol incubated with non-treated homogenate.

(13) Arachidonic acid (200 μg/ml) plus SOD (10 μg/ml) or denatured SOD (10 μg/ml) plus 20 mM *d*-mannitol incubated with non-treated homogenate.

(14) Arachidonic acid (200 μg/ml) plus 2-aminomethyl-4-*tert*-butyl-6-propionyl phenol hydrochloride (ONO-3144, 0.4 mM) incubated with non-treated homogenate. Aishita *et al.* [28] have shown that ONO-3144 inhibits thromboxane A_2 synthetase and does not inhibit cyclooxygenase activity. Moreover, ONO-3144 is an effective reducing agent and is a particularly good scavenger of oxidants [28, 29] formed by prostaglandin endoperoxide metabolism (e.g. peroxide-induced conversion of PGG_2 to PGH_2). ONO-3144 (Ono Chemical Co.) was dissolved immediately prior to the experiment in distilled water.

(15) PGG_2 (10 μg/ml) plus ONO-3144 (0.4 mM) incubated with non-treated homogenate.

The prostaglandin cyclic endoperoxides PGG_2 and PGH_2 were biosynthesized from radiolabeled arachidonic acid by using the cyclooxygenase from sheep seminal vesicle microsomes. After biosynthesis and isolation, PGG_2 and PGH_2 were dissolved in acetone and stored at −80°. Confirmation of the purity of endoperoxides was obtained by the method of Hamberg *et al.* [30]. Additional confirmation of the authenticity of the endoperoxides was obtained by showing that they induced platelet aggregation *in vitro*. A stock solution of PGE_2 was prepared by dissolving PGE_2 in 100% ethanol.

Statistical significance tests were performed using Student's *t*-test or an analysis of variance. A value of $P < 0.05$ was accepted as indicating a significant difference.

RESULTS

Effect of free radicals generated from a xanthine–xanthine oxidase system on homogenate calcium

Table 1. Effect of free radicals produced from the xanthine–xanthine oxidase system and free radical scavengers on homogenate calcium uptake velocity at pH 7.0 and 5.5*

Treatment	N	Calcium uptake velocity (1×10^{-3} μmoles Ca^{2+}/mg·min) pH 7.0	pH 5.5
Control	5	16.9 ± 0.3	8.1 ± 0.2†
Xanthine	6	17.2 ± 0.5	7.7 ± 0.4
Xanthine plus xanthine oxidase	6	9.3 ± 0.2‡	2.5 ± 0.7‡
Xanthine plus xanthine oxidase plus SOD	6	14.2 ± 0.7§	2.9 ± 0.9
Xanthine plus xanthine oxidase plus SOD plus mannitol	6	14.3 ± 0.6	7.0 ± 0.8§
Xanthine plus denatured xanthine oxidase	4	16.2 ± 0.8§	7.2 ± 0.5§
Xanthine plus xanthine oxidase plus denatured SOD	4	8.6 ± 0.4‖	2.8 ± 0.8
Xanthine plus xanthine oxidase plus denatured SOD plus mannitol	4	9.0 ± 0.5¶	2.4 ± 0.7¶

* Reaction conditions: 34.7 mM KCl, 6 mM imidazole, 3.3 mM NaN_3, 3.3 mM potassium-oxalate, 5 mM Na_2 ATP, 5 mM $MgCl_2$, 0.18 mM $CaCl_2$, and, when added, 0.1 mM xanthine, 0.1 mg/ml xanthine oxidase, 10 μg/ml SOD, and 20 mM *d*-mannitol at 27°. The sequence of addition of reagents is as described in the text. All values are means ± S.E.M.

† Significantly ($P < 0.01$) different from pH 7.0 control.

‡ Significantly ($P < 0.01$) different from xanthine alone.

§ Significantly ($P < 0.01$) different from xanthine plus xanthine oxidase.

‖ Significantly ($P < 0.01$) different from xanthine plus xanthine oxidase plus SOD.

¶ Significantly ($P < 0.01$) different from xanthine plus xanthine oxidase plus SOD plus mannitol.

uptake velocity at pH 7.0 and pH 5.5. This experiment required the sequential addition of homogenate, xanthine oxidase, SOD, and *d*-mannitol. The reaction bath was as described in Materials and Methods, and the homogenate was preequilibrated to the desired pH for 2.5 min prior to addition of Mg^{2+}, Ca^{2+}, and ATP (also preequilibrated to 27°) to an otherwise complete reaction. Xanthine was added before the addition of homogenate, and xanthine oxidase was added 1.0 min before either SOD or substrates (Ca^{2+} + Mg^{2+} + ATP). SOD was added 0.5 min before *d*-mannitol or substrates. *d*-Mannitol was added 0.5 min before substrates. This sequence assured that the homogenate was exposed to the free-radical generating system for 1.0 min, to SOD for 0.5 min, and to SOD plus *d*-mannitol for 0.5 min prior to the start of calcium uptake. Table 1 shows the results of this study. Control studies in the absence of free-radical generating system at pH 5.5 produced a significant depression of calcium uptake velocity when compared to pH 7.0. The addition of xanthine alone had no effect on calcium uptake velocity at either pH 7.0 or pH 5.5. Xanthine oxidase together with xanthine depressed calcium uptake velocity at both pH 7.0 and 5.5. In the presence of xanthine, denatured xanthine oxidase had no effect at either pH 7.0 or 5.5. SOD inhibited the depressed calcium uptake velocity produced by xanthine oxidase at pH 7.0 but not at pH 5.5. At pH 7.0, denatured SOD had no effect on the depression of calcium transport induced by the xanthine–xanthine oxidase system. In the presence of SOD, but not denatured SOD, at pH 5.5, *d*-mannitol inhibited the depression of calcium uptake velocity induced by the generation of free radicals from the xanthine–xanthine oxidase system.

Effect of SOD, d-*mannitol and normalization of the pH to 7.0 following preincubation at pH 5.5 on homogenate and isolated SR calcium uptake velocity.*

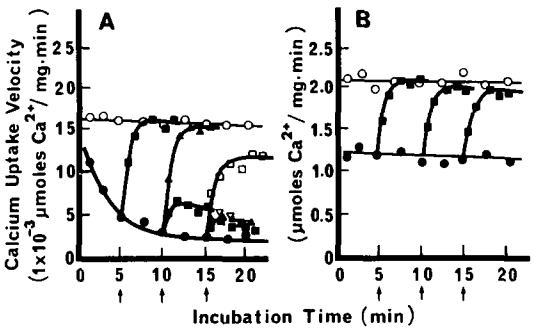

Fig. 1. Time-course of change of calcium transport by the unfractionated homogenate (A) and isolated sarcoplasmic reticulum (B) at pH 7.0 (○) and pH 5.5 (●), and the effect of changing pH to 7.0 (■) in the presence of superoxide dismutase (10 µg/ml; ▲), *d*-mannitol (20 mM; ▽), or superoxide dismutase plus *d*-mannitol (□) after 5, 10 and 15 min of preincubation at pH 5.5. The arrows indicate the time of changing pH to 7.0 from 5.5 and addition to the pH 5.5 reaction bath of superoxide dismutase, *d*-mannitol or superoxide dismutase plus *d*-mannitol. Each point represents the mean from three separate studies performed in duplicate.

Having established an additive effect of both hydrogen ions and free radicals, we next turned our attention to the effects of acidosis in the absence of an exogenous free-radical generating system and the effects of free radical scavengers on this system.

In the homogenate studies (Fig. 1A), preincubation at pH 5.5 caused a 50% decrease in homogenate calcium uptake velocity within 5 min and this depression of homogenate calcium transport was reversed with normalization of the pH to 7.0. However, at 10-min preincubation at pH 5.5, there was minimal reversibility with normalization of the pH to 7.0. By 15 min preincubation at pH 5.5, the ability to reverse the system by normalizing the pH was lost. Also shown is the effect on calcium uptake velocity of the addition of SOD, *d*-mannitol, or SOD plus *d*-mannitol simultaneously with normalization of the pH to 7.0 at various times following preincubation at pH 5.5, Following a 10-min preincubation at pH 5.5, the addition of SOD to this system was capable of restoring calcium uptake velocity to pH 7.0 controls. At 15-min preincubation at pH 5.5, this effect was negligible since the recovery in calcium uptake velocity was the same as with the restoring of the pH alone. Simultaneously with the restoration of the pH to 7.0, the addition of *d*-mannitol alone had no effect on the depression of homogenate calcium uptake velocity induced by 15-min preincubation at pH 5.5, while SOD plus *d*-mannitol partially reversed the effects of preincubation at pH 5.5 for 15 min.

Preincubation of isolated SR at pH 5.5 depressed SR calcium uptake velocity (Fig. 1B). Following normalization of the pH to 7.0 after the isolated SR had been preincubated for 5, 10 or 15 min at pH 5.5, calcium uptake velocity was restored to that seen without reduction in pH.

Arachidonic acid-induced change of calcium transport and effect of indomethacin, ONO-3144 and free radical scavengers at pH 7.0 and 5.5. It appeared from the results cited above that acidosis depressed SR calcium transport in the homogenate in part by a mechanism dependent on free radicals that are generated from the cellular environment of muscle cells. To determine the relationship between acidosis and unsaturated fatty acids, we investigated the effect of arachidonic acid on calcium transport at pH 7.0 and 5.5. The results of these experiments are shown in Figs. 2 and 3. At pH 7.0 (Fig. 2), the depression of homogenate calcium uptake velocity induced by arachidonic acid was dependent on the time of incubation, and both indomethacin and ONO-3144 inhibited the depression of calcium uptake velocity induced by arachidonic acid (Fig. 2A). As shown in Fig. 2B, the addition of SOD brought about a significant and almost complete reversal of the reduced calcium uptake induced by arachidonic acid after 5, 10 and 15 min of incubation. In the isolated SR (Fig. 2, panels C and D), there was a significant depression of calcium uptake velocity 10–20 min after the addition of arachidonic acid. This depression of calcium uptake velocity induced by arachidonic acid was inhibited significantly by indomethacin or ONO-3144 (Fig. 2C). The addition of SOD at 10 and 15 min restored

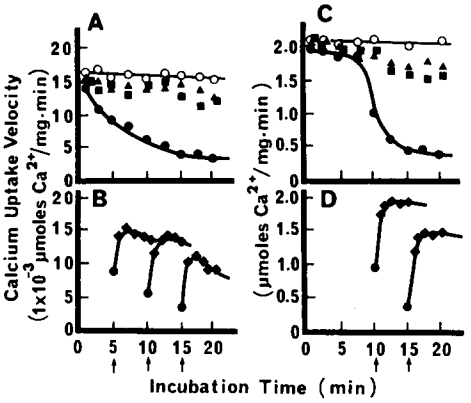

Fig. 2. Time-course of effect of indomethacin (▲), ONO-3144 (0.4 mM; ■) and superoxide dismutase (10 µg/ml; ◆) on arachidonic acid (100 µg/ml; ●) -induced change of calcium transport by the unfractionated homogenate (A, B) and isolated sarcoplasmic reticulum (C, D) at pH 7.0. Arachidonic acid, 11,14,17-eicosatrienoic acid (100 µg/ml; ○) or ONO-3144 was added at time zero. Indomethacin (5 mg/kg, i.v.) was administered 30 min before the removal of masseter muscle and isolation of sarcoplasmic reticulum. The arrows indicate the time points for the addition of superoxide dismutase. Key: (●, ▲, ■, ◆) with arachidonic acid; and (○) without arachidonic acid. Each point represents the mean from three separate studies performed in duplicate.

calcium uptake velocity towards the value seen in 11,14,17-eicosatrienoic acid control (Fig. 2D). 11,14,17-Eicosatrienoic acid had no effect on the time-course of homogenate and isolated SR calcium uptake velocity.

Figure 3 shows the comparable experiment to Fig. 2 at pH 5.5. The effect of 11,14,17-eicosatrienoic acid was negligible since the time-course of homogenate and isolated SR calcium uptake velocity at pH 5.5 incubated with 11,14,17-eicosatrienoic acid was the same as the controls at pH 5.5 (Fig. 1).

In the homogenate studies (Fig. 3, panels A and B), the depression of calcium uptake velocity induced by incubation at pH 5.5 was inhibited completely by indomethacin or ONO-3144. Further, it is seen that following 10 min of incubation at pH 5.5, the addition of *d*-mannitol alone to this system had no effect on the depression of calcium uptake velocity. SOD added at 10 min partially reversed the effect of incubation at pH 5.5 for 10 min. The addition of SOD plus *d*-mannitol completely reversed the system. When arachidonic acid was added instead of 11,14,17-eicosatrienoic acid, there was a further significant depression of calcium uptake velocity. This depression induced by arachidonic acid was inhibited by indomethacin or by ONO-3144. Following 5 min of incubation with arachidonic acid at pH 5.5, the addition of *d*-mannitol alone had no effect. In the presence of SOD, however, *d*-mannitol effectively reversed the system.

The time-course of the changes in isolated SR calcium uptake velocity induced by arachidonic acid at pH 5.5 is shown in Fig. 3, panels C and D. Arachidonic acid caused an immediate 65% decrease in calcium uptake velocity, followed by a more gradual decline to less than 20% of the 11,14,17-eicosatrienoic acid control value within 10–20 min. This depression of isolated SR calcium uptake velocity was inhibited by indomethacin or by ONO-3144 (Fig. 3C). Also shown is the effect on calcium uptake velocity of the addition of SOD, *d*-mannitol, or SOD plus *d*-mannitol at 5 and 10 min after incubation with arachidonic acid at pH 5.5 (Fig. 3D). The addition of SOD alone or *d*-mannitol alone at 5 or 10 min after the incubation had no effect on the time-course of calcium uptake velocity. However, the addition of SOD plus *d*-mannitol at 5 min restored calcium uptake velocity towards the 11,14,17-eicosatrienoic acid control. At 10 min the addition of SOD plus *d*-mannitol also reversed the calcium uptake velocity depression induced by arachidonic acid.

Effects of prostaglandins and fatty acids on homogenate calcium uptake velocity at pH 7.0 *and pH* 5.5. These results suggest that the acidosis-induced depression of SR calcium transport in the homogenate is the result of free radicals generated by the metabolism of arachidonic acid through the cyclooxygenase pathway. Therefore, we hypothesized that the metabolism of arachidonic acid would duplicate the effect of free radicals generated from a xanthine–xanthine oxidase system on calcium transport by SR in the homogenate at pH 7.0 and pH 5.5. A test of this hypothesis is presented in Table 2. The reaction bath was as described in Materials and Methods, and the homogenate prepared from normal or indomethacin-treated animals was pre-equilibrated to the desired pH for 2.5 min prior to the addition of substrates. The conditions of the sequential addition of SOD and *d*-mannitol were the same as those of Table 1, except that xanthine was omitted and arachidonic acid, 11,14,17-eicosatri-

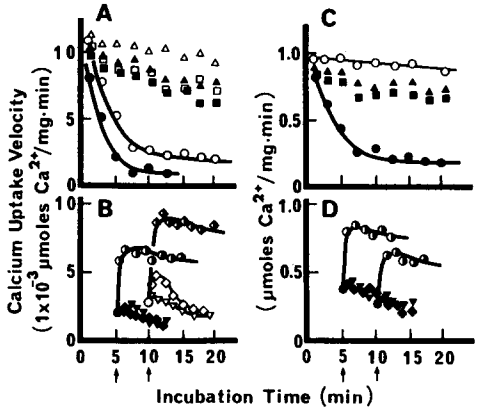

Fig. 3. Time-course of effect of indomethacin (△, ▲), ONO-3144 (0.4 mM; □, ■), superoxide dismutase (10 µg/ml; ◇, ◆), *d*-mannitol (20 mM; ▽, ▼) and superoxide dismutase plus *d*-mannitol (◈, ◑) on arachidonic acid (100 µg/ml; ●) -induced change of calcium transport by the unfractionated homogenate (A, B) and isolated sarcoplasmic reticulum (C, D) at pH 5.5. Arachidonic acid, 11,14,17-eicosatrienoic acid (100 µg/ml; ○) or ONO-3144 was added at time zero. The arrows indicate the time points for the addition of superoxide dismutase, *d*-mannitol or superoxide dismutase plus *d*-mannitol. Key: (●, ▲, ■, ◆, ▼, ◑) with arachidonic acid; and (○, △, □, ◇, ▽, ◈) with 11,14,17-eicosatrienoic acid. Each point represents the mean from three separate studies performend in duplicate.

Table 2. Effect of superoxide dismutase, mannitol and ONO-3144 on prostaglandin- and fatty acid-induced decrease in homogenate calcium uptake velocity*

Treatment	N	Calcium uptake velocity (1×10^{-3} μmoles Ca^{2+}/mg·min) pH 7.0	pH 5.5
Control	5	17.2 ± 0.1	8.5 ± 0.3
11,14,17-Eicosatrienoic acid	5	16.4 ± 0.4	8.0 ± 0.5
Arachidonic acid	8	8.6 ± 0.5†	3.8 ± 0.2†
Indomethacin plus arachidonic acid	8	15.9 ± 0.4‡	7.6 ± 0.5‡
PGG_2	7	7.2 ± 0.8§	3.2 ± 0.7§
Indomethacin plus PGG_2	6	8.2 ± 0.5†	3.0 ± 0.6†
PGH_2	6	16.8 ± 0.3‖	8.5 ± 0.1‖
PGE_2	5	16.2 ± 0.7‖	8.2 ± 0.5‖
Denatured SOD	5	16.0 ± 0.2	7.9 ± 0.3
PGG_2 plus SOD	8	14.9 ± 0.6‖	3.5 ± 0.6
PGG_2 plus denatured SOD	6	7.6 ± 0.7¶	3.0 ± 0.8
PGG_2 plus mannitol	6	7.0 ± 0.8	3.2 ± 0.9
PGG_2 plus SOD plus mannitol	8	16.2 ± 0.7	7.9 ± 0.5‖
PGG_2 plus denatured SOD plus mannitol	6	7.2 ± 0.4**	3.5 ± 0.7**
Arachidonic acid plus SOD	8	15.4 ± 0.2‡	3.9 ± 0.3
Arachidonic acid plus denatured SOD	6	7.1 ± 0.5††	3.6 ± 0.5
Arachidonic acid plus mannitol	6	6.9 ± 0.6	3.5 ± 0.4
Arachidonic acid plus SOD plus mannitol	8	16.7 ± 0.2	8.3 ± 0.6‡
Arachidonic acid plus denatured SOD plus mannitol	7	8.1 ± 0.4‡‡	4.0 ± 0.4‡‡
Arachidonic acid plus ONO-3144	5	16.2 ± 0.7‡	7.6 ± 0.5‡
PGG_2 plus ONO-3144	6	16.5 ± 0.8‖	7.8 ± 0.3‖

* The sequence of addition and final concentration of reagents, and reaction conditions are as described in the text. All values are mean ± S.E.M.
† Significantly ($P < 0.01$) different from 11,14,17-eicosatrienoic acid.
‡ Significantly ($P < 0.01$) different from arachidonic acid.
§ Significantly ($P < 0.01$) different from acetone vehicle and control.
‖ Significantly ($P < 0.01$) different from PGG_2 alone.
¶ Significantly ($P < 0.01$) different from PGG_2 plus SOD.
** Significantly ($P < 0.01$) different from PGG_2 plus SOD plus mannitol.
†† Significantly ($P < 0.01$) different from arachidonic acid plus SOD.
‡‡ Significantly ($P < 0.01$) different from arachidonic acid plus SOD plus mannitol.

enoic acid, PGG_2, PGH_2, PGE_2, acetone or ethanol vehicle was added instead of xanthine oxidase. ONO-3144 was added 1.0 min after arachidonic acid or PGG_2, or 0.5 min before the substrates.

The acetone or ethanol vehicles, in the same concentrations as in the various solutions, had no effect on calcium uptake velocity at either pH 7.0 or pH 5.5. Similarly, indomethacin, SOD, *d*-mannitol, SOD plus *d*-mannitol, or ONO-3144 had no effect when compared to the controls. Arachidonic acid and PGG_2 produced statistically significant depression of calcium uptake velocity at both pH 7.0 and pH 5.5. The depression of calcium uptake velocity caused by arachidonic acid or PGG_2 was inhibited by ONO-3144. The depression of calcium uptake induced by arachidonic acid but not by PGG_2 was inhibited by the treatment with indomethacin at either pH 7.0 or pH 5.5.

At pH 7.0, SOD but not denatured SOD inhibited the depressed calcium uptake velocity caused by arachidonic acid or PGG_2. *d*-Mannitol was, by itself, incapable of inhibiting the depressed calcium uptake velocity induced by arachidonic acid or PGG_2. At pH 5.5, SOD had no effect on the depression of calcium uptake velocity caused by arachidonic acid or PGG_2. *d*-Mannitol, together with SOD but not with denatured SOD, was capable of inhibiting the system.

The addition of 11,14,17-eicosatrienoic acid, PGH_2 or PGE_2 caused no significant reduction in calcium uptake velocity at either pH 7.0 or pH 5.5.

DISCUSSION

Our study demonstrates that acidosis can depress calcium transport by sarcoplasmic reticulum via the generation of free radicals associated with the synthesis of prostaglandins within the cellular environment of the muscle cell. The use of the muscle homogenate model permits one to study SR function as measured by oxalate supported, unidirectional calcium flux in the presence of the normal cellular environment and in the absence of any potential artifact that may occur with ultracentrifugation [20]. This technique permits exposure of the intracellular space to biochemical manipulation. In our study, the reduced calcium uptake velocity induced by free oxygen radicals generated from the xanthine–xanthine oxidase system was inhibited by SOD at pH 7.0. This is consistent with the known production of superoxide anion radical ($\cdot O_2^-$) by the xanthine oxidase reaction. We have shown previously that a decrease in pH 7.0 to 5.5 uncouples SR calcium transport from ATP hydrolysis [16]. When the experiment was carried out at pH 5.5, SOD was unable to inhibit the effects of the xanthine oxidase

system on SR calcium transport. This suggests that in addition to $\cdot O_2^-$ another free radical was generated under the circumstances of the low pH. At pH 5.5 both SOD and *d*-mannitol are needed to inhibit the effects of the xanthine–xanthine oxidase on SR calcium transport. This could possibly incriminate the hydroxyl radical ($\cdot OH$) or more likely an hydroxyl radical intermediate such as an alkoxy radical as the destructive species of the xanthine–xanthine oxidase reaction at pH 5.5.

The time sequence studies of the effects of acidosis on isolated SR calcium transport and on SR calcium transport in the homogenate result in the following significant observations. (1) There is an immediate depression of homogenate calcium transport due to increased hydrogen ion concentration which is independent of free radicals and is capable of being reversed by the normalization of the pH. This reversibility is no longer present if the acidosis is prolonged beyond 5 min. (2) Between 5- and 10-min preincubation at pH 5.5, it would appear that there is a generation of $\cdot O_2^-$. This is inferred by the reversibility of the depression in calcium transport with the addition of SOD and decreasing the hydrogen ion concentration (normalization of the pH to 7.0). At 10- and 15-min preincubation, SOD had little effect other than that observed by decreasing hydrogen ion concentration. (3) Between 10- and 15-min preincubation at pH 5.5, it would appear that the more lethal $\cdot OH$ is generated. (4) However, acidosis had no effect on isolated SR calcium transport. These results led us to the conclusion that acidosis-induced depression of SR calcium transport in the homogenate is due to free radicals generated from the cellular environment of the muscle cell.

The mechanism, by which hydrogen ions appear to facilitate some reactions, which generates free radicals is not known. It is well established that $\cdot O^-$ is normally produced in small amounts, and there are several potential sources for its production. These include a number of autooxidation reactions such as hydroxyquinones, leukoflavins, catecholamines and thiols [31, 32]. Oxygenated hemoglobin and myoglobin liberate $\cdot O^-$ [33]. A number of oxidative enzymes, such as xanthine oxidase, produce $\cdot O_2^-$ [34]. Free radicals are also produced in the course of arachidonic acid and other unsaturated fatty acid metabolism by the cyclooxygenase and lipoxygenase pathways [35]. A plausible but hypothetical mechanism by which acidosis could induce generation of free radicals is the disruption of lysosomes. For example, lysosomal enzymes include phospholipases [36] which may release unsaturated fatty acids, including arachidonic acid from phospholipids. Free radicals may then be generated from arachidonic acid metabolism. In the present experiments, arachidonic acid but not 11,14,17-eicosatrienoic acid (an unsaturated fatty acid which is not a substrate of cyclooxygenase) produced depression of SR calcium transport in both the isolated SR and in the homogenate at pH 7.0 and pH 5.5. These effects of arachidonic acid were inhibited by indomethacin or by ONO-3144, a compound known to accelerate the conversion of PGG_2 to PGH_2. Furthermore, in our system, arachidonic acid-induced depression of SR calcium transport was inhibited by SOD at pH 7.0 or by the combination of SOD and *d*-mannitol at pH 5.5, suggesting that the effect of arachidonic acid is dependent on the generation of free radicals. In addition, acidosis enhanced the depressive effect of arachidonic acid on SR calcium transport in the homogenate. These results show that cyclooxygenase system is an active participant in the mechanism by which low pH generates free oxygen radicals in the homogenate.

If the view that the mechanism of the depression of SR calcium transport in the homogenate induced by acidosis involves increased production of prostaglandins and generation of free radicals is correct, it should be possible to reproduce the depression of SR calcium transport in homogenate by exogenously administered prostaglandins. We tested this hypothesis by application of various prostaglandins or related compounds to our experimental system. Arachidonic acid produced the depression of SR calcium transport in the homogenate at both pH 7.0 and pH 5.5. This effect was inhibited by the inhibition of the increased prostaglandin synthesis by indomethacin, and by ONO-3144 that scavenges free radicals and accelerates the conversion of PGG_2 to PGH_2 resulting in a marked decrease in PGG_2 [28]. PGG_2, but not PGH_2, duplicated the effect of arachidonic acid. The effect of PGG_2 at pH 7.0 was inhibited by SOD, a scavenger of $\cdot O_2^-$. *d*-Mannitol, a known scavenger of $\cdot OH$, together with SOD, was capable of inhibiting the effect of PGG_2 at pH 5.5. 11,14,17-Eicosatrienoic acid, PGE_2 and PGH_2 did not produce any of the changes in SR calcium transport in the homogenate. These results are consistent with the view that a marked increase in prostaglandin synthesis can produce the depression of SR calcium transport. Furthermore, the findings point out that the important compound in the production of this depression is PGG_2. This provides additional direct evidence for the participation of free radicals, since the effect of PGG_2 is inhibited by free radical scavengers.

It is known that the conversion of PGG_2 to PGH_2 releases free radicals [35, 37]. The exact nature of these radicals is not known. The identification of the free radicals which are responsible for the altered SR function in both isolated SR and in the homogenate induced by acidosis, arachidonic acid, or PGG_2 rests at the present entirely on the use of scavengers. In the case of SOD, which is a very specific enzyme [34], the conclusion seems safe that reversal of the observed effects can be interpreted as indicative that the $\cdot O_2^-$ is involved. In the case of *d*-mannitol, this conclusion must, at the present, remain tentative. The results in both the homogenate and in the isolated SR suggest that, at pH 7.0, the radical responsible for the effects on calcium uptake is the $\cdot O_2^-$, since all these effects are inhibited by SOD. When the pH was reduced to 5.5, it appeared that another radical was involved in addition to $\cdot O_2^-$. We suggest that this second radical is the $\cdot OH$. As pointed out by Beauchamp and Fridovich [38], at the lower pH, the concentration of $\cdot O_2^-$ would be reduced and the concentration of the perhydroxyl radical ($\cdot HO_2$) would be correspondingly increased. Furthermore, the spontaneous dismutation of these radicals to form H_2O_2 would be accelerated markedly at the low

pH. These conditions would favor the accelerated formation of ·OH [39]. Clearly, further work is required to elucidate the exact nature and role of the radicals generated by the conversion of PGG_2 to PGH_2 in the complex biochemical mechanisms that lead to alterations in the SR calcium transport system of the muscle cell. It is already clear, however, that a burst of prostaglandin synthesis initiated by acidosis leads to the generation of free radicals that are immediately responsible for altered SR function and that ·OH or a closely related species of free radicals is involved in the depression of SR calcium transport in the homogenate at an acidotic pH.

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