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The Effect of Oxygen Free Radicals on Calcium Permeability and Calcium Loading at Steady State in Cardiac Sarcoplasmic Reticulum

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SUMMARY

It has been proposed that oxygen free radical production is an important mediator of the myocardial dysfunction during the course of acute ischemia. We tested this hypothesis by characterizing the pathway of calcium efflux across sarcoplasmic reticulum (SR) membranes affected by oxygen free radicals. The effect of oxygen free radicals on the steady state calcium load, calcium permeability, and Ca,Mg-ATPase activity of isolated canine cardiac SR vesicles was investigated at pH 7.0. *In vitro* generation of oxygen free radicals by xanthine oxidase (0.09 units/ml), acting on xanthine in doses up to 50 μM as a substrate, increased the permeability of the SR vesicles to calcium, determined by measuring net efflux of calcium after stopping pump-mediated fluxes, and decreased total intravesicular calcium and free intravesicular calcium with no effect on Ca,Mg-ATPase activity. The effect of oxygen free radicals on calcium permeability was calcium gradient-dependent. Xanthine alone or xanthine plus denatured xanthine oxidase had no effect on this system. Superoxide dismutase (SOD, 56 units/ml), but not denatured SOD, significantly inhibited the effect of xanthine-xanthine oxidase reaction. The calcium permeability of the SR membrane decreased with decreasing calcium load. In addition, inasmuch as extravesicular calcium exerts only a slight effect on calcium permeability, the decrease in the permeability with calcium load is specifically related to the calcium load. Oxygen free radical-induced increase in calcium permeability was unaffected by Mg concentration between 2.1 and 21 mM. In summary, our data reveal that $\cdot O_2^-$ can produce a diminished level of accumulated calcium, which is reflected by the decreased calcium load and an increase in passive calcium permeability, and that the decreased calcium accumulation in the presence of the xanthine-xanthine oxidase system may not be mainly due to an inhibited calcium pump but due to an increased calcium permeability. Our results also suggest that increased SR membrane passive calcium permeability induced by oxygen free radicals is not carrier mediated. It is postulated that, with the oxygen free radical-mediated progressive increase in calcium permeability, free cytosolic calcium concentrations would increase in ischemic myocardium.

Myocardial ischemia results in a series of metabolic events that includes the autooxidation of catecholamines, a reduction in intracellular pH, the breakdown of ATP to hypoxanthine and xanthine, an increase in reducing equivalents, and activation of the cyclooxygenase system (1–4). All of these reactions favor the univalent reduction of molecular oxygen to oxygen free radicals and their metabolites (superoxide anion, $\cdot O_2^-$, hydrogen peroxide, H_2O_2, and the hydroxyl radical, $\cdot OH$) (5). These metabolites of molecular oxygen are highly toxic and are capable of extensive tissue damage. With reperfusion and reintroduction of molecular oxygen into this previously ischemic vascular bed, the myocardium is then "primed" for the production of a "burst" of oxygen intermediates and further extensive tissue damage (3). However, the sources and sinks of these oxygen free radicals produced as a result of the ischemic/reperfusion process have not been adequately identified.

Our laboratory has provided evidence that a major target organelle attacked by the ischemic process is the portion of excitation-contraction coupling system that regulates calcium delivery (the sarcolemma and SR) to the contractile proteins and not the contractile proteins *per se* (6–9). Further, using the xanthine-xanthine oxidase system, it has been shown that $\cdot O_2^-$ and its metabolites, H_2O_2 and $\cdot OH$, depress calcium transport by cardiac SR (10, 11) and can attack sarcolemmal muscarinic receptors, resulting in depression of muscarinic binding (12).

One of the consequences of oxidation can be impairment of membrane permeability (13–15). In particular, the inability of

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ABBREVIATIONS: SR, sarcoplasmic reticulum; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SOD, superoxide dismutase; PLA_2, phospholipase A_2.

cells to regulate intracellular calcium levels has been proposed as a source of oxidative toxicity (16–18). Hypothetically, oxygen free radicals could increase calcium flux through the lipid bilayer or a protein pore. To our knowledge, only two studies have examined the effect of oxygen free radicals on the calcium permeability of cardiac (10) or skeletal (19) SR. Both studies assumed that oxygen free radical-mediated oxidation of membrane lipids was responsible for the increase in calcium permeability.

It is recognized that cardiac SR vesicles actively take up calcium into an enclosed vesicular space because this uptake is reduced to an extravesicular binding when ATP is replaced with nonhydrolyzable analogues and because the accumulated calcium is readily released by calcium ionophores (20). The level of steady state calcium uptake varies with temperature, pH, and Mg^{2+}, nucleotide, and extravesicular calcium concentrations (20–22). These variables presumably alter steady state uptake by affecting one or more of the various routes of calcium flux.

The passive efflux pathways in cardiac SR include the efflux through a calcium channel and a passive leak parallel to the channel and the pump (20). In the previous study (10) we have shown that oxygen free radicals produce an increase in passive calcium permeability of cardiac SR vesicles. To gain further insight into the effect of oxygen free radicals on calcium flux behavior of canine cardiac SR vesicles, the present study focuses on the interaction of steady state calcium accumulation and passive calcium permeability in the presence of oxygen free radicals generated by the xanthine oxidase reaction, acting on xanthine as a substrate. We report that oxygen free radicals can reduce intravesicular bound and free calcium of SR and seem to be responsible for the increase in passive calcium efflux.

Materials and Methods

Isolation of cardiac SR. SR vesicles were isolated from the left ventricle of healthy, adult, filaria-free dogs as follows. Dogs were anesthetized with sodium pentobarbital (25 mg/kg) and the heart was rapidly excised and placed in ice-cold 0.9% NaCl. The free wall of the left ventricle was cleaned of connective tissue, minced, and then homogenized (1 g of muscle/3 volumes of 10 mM imidazole buffer, pH 7.0) for 1 min at 4° in a Sorvall Omnimixer. The homogenate was centrifuged at 4000 × *g* for 20 min. The pellet was rehomogenized in 3 volumes of 10 mM imidazole and centrifuged again at 4000 × *g* for 20 min. The supernatant from this and the previous centrifugation were combined, poured through four layers of cheesecloth, and centrifuged at 10,000 × *g* for 15 min. The supernatant was filtered through eight layers of cheesecloth and centrifuged at 31,000 × *g* for 1 hr. The pellets were rehomogenized in 1 M KCl, 10 mM imidazole buffer by use of a Teflon pestle and then centrifuged at 145,000 × *g* for 1 hr. The final pellet was resuspended in 30% sucrose, 10 mM imidazole, pH 7.0, to a final concentration of about 7 mg of SR protein/ml. Protein concentration was determined by the method of Lowry *et al.* (23). The isolated SR was kept at −40° for 1 to 4 days.

Calcium loading. Calcium uptake was measured in the absence of calcium-precipitating anions at 27° by filtration, through 0.45-μm Millipore filters, of 1-ml aliquots from a 10-ml bath containing 100 mM KCl, 20 mM imidazole, pH 7.0, 10 mM NaN_3, 100 μM ATP, 2.1 mM $MgCl_2$, 0.1 μCi of ^{45}Ca/ml, and 4 μM added calcium.

It is assumed in all experiments that the total calcium in the reaction bath is distributed among four compartments. These are as follows: Ca_o, the calcium in solution outside the vesicles; Ca_{bo}, the calcium bound to the outside of the vesicles; Ca_{bi}, calcium bound to intravesicular binding sites; and Ca_i, calcium free within the intravesicular space. Thus the total calcium in the bath is

$$Ca_T = Ca_o + Ca_{bo} + Ca_{bi} + Ca_i$$

and the calcium associated with the SR is

$$Ca_t = Ca_{bo} + Ca_{bi} + Ca_i$$

The total calcium in the reaction bath was measured by atomic absorption spectrophotometry after wet ashing of the reaction bath including SR. The total calcium associated with the SR was obtained by Millipore filtration and was calculated from the total ^{45}Ca in the reaction bath and the ^{45}Ca in the filtrates of the reaction bath. The uptake reaction was begun by addition of ATP, Ca, and Mg to an otherwise complete reaction bath.

Calcium efflux. Calcium efflux was measured, after steady state calcium uptake was reached, by quenching pump-mediated calcium fluxes and observing net release of calcium by filtering aliquots of the reaction bath at various times after addition of the quench reagents. The initial apparent first order rate constant was obtained by linear regression of the natural logarithm of the calcium uptake determined by Millipore filtration at various times after quenching the pump-mediated calcium fluxes. Quenching of the pump-mediated fluxes was produced in two ways, by addition of EGTA to a final concentration of 2.5 mM or by addition of glucose (12.5 mM final concentration). The initial value ($Ca_t - Ca_{bo}$) was determined by extrapolating the first order efflux curve to the time of addition of EGTA quench. The free calcium was calculated according to the method of Fabiato and Fabiato (24).

Ca,Mg-ATPase activity. The ATPase activity was determined from the rate of $^{32}P_i$ release from [γ-^{32}P]ATP by the method of Feher and Briggs (25).

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 (0.09 units/ml, from cow milk, 1.0 unit/mg of protein; Boehringer Mannheim, Indianapolis, IN) and xanthine (Sigma Chemical Co., St. Louis, MO) as a substrate was used at pH 7.0. The oxidation of xanthine by xanthine oxidase yields $\cdot O_2^-$ that is disproportionated in H_2O_2 and O_2. This reaction can proceed spontaneously or it can be catalyzed by SOD.

The *in vitro* rate of $\cdot O_2^-$ generation from xanthine (25 μM)-xanthine oxidase (0.09 units/ml) system was performed by spectrophotometrically following the conversion of xanthine to urate at 37°, pH 7.0 (26). Superoxide anion production measured by monitoring the SOD-inhibitable reduction of cytochrome *c* at 550 nm was 2.8×10^{-7} nmol of $\cdot O_2^-$/liter/sec, which compares favorably with that reported by Brawn and Fridovich (27). We used SOD (56 units/ml, from bovine blood, 2800 units/mg of protein; Sigma) to scavenge $\cdot O_2^-$. For control studies, SOD and xanthine oxidase were denatured by boiling at 100° for 20 min. The timed sequence of reagent addition is described in Results. All materials were reagent grade unless otherwise indicated.

Statistical analyses. The statistical tests of significance used were one-way analysis of variance and the Dunnett's multiple range test (28). Statistical software was obtained from Ricoh (Tokyo, Japan). A significance level of $p > 0.05$ was used to reject the null hypothesis.

Results

Fig. 1 shows the effect of exogenously generated oxygen radicals from the xanthine-xanthine oxidase system on steady state calcium load, calcium permeability, and Ca,Mg-ATPase activity of SR vesicles. Xanthine alone in concentrations ranging from 5 to 80 μM (Fig. 1, A and B) or 5 to 150 μM (Fig. 1C) had no effect on steady state calcium load (Fig. 1A), calcium efflux (Fig. 1B), and ATPase activity (Fig. 1C). In the presence of xanthine oxidase (0.09 units/ml), a significant decrease in

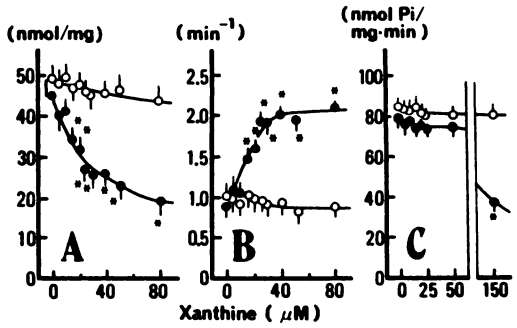

Fig. 1. Effect of xanthine on SR steady state calcium load ($Ca_t - Ca_{bo}$) (A), passive calcium permeability (B), and Ca,Mg-ATPase activity (C), in the presence (●) or absence (○) of xanthine oxidase (0.09 units/ml). The reaction bath, sequence of addition of xanthine and xanthine oxidase, calculation of initial first order rate constant, *K/v*, and measurement of net passive efflux were similar to those described in Table 1. ATPase was measured by assaying the rate of $^{32}P_i$ released from [γ-^{32}P]ATP in the calcium uptake reaction bath without ^{45}Ca. Calcium-independent ATPase was measured by including 2 mM EGTA. Each *point* represents the mean ± standard error of four or five experiments. *Significantly ($p < 0.01$) different from control (xanthine only).

steady state calcium load and an increase in initial first order rate constant, with xanthine concentrations ranging from 20 to 40 μM, was observed. The data obtained between 40 and 80 μM indicated no further changes in the initial first order rate constant even when there was an additional decrease of steady state calcium load. The calcium load and the initial first order rate constant, therefore, did not correlate well over the whole range of concentrations used. However, xanthine concentration up to 50 μM in the presence of xanthine oxidase had no effect on the ATPase activity. A relatively higher xanthine concentration (150 μM) depressed ATPase activity.

To characterize the oxygen free radical-sensitive pathway of calcium permeability across SR membrane in our experimental system, we first investigated the effect of calcium load on the initial first order rate constant under control conditions in the absence of an oxygen free radical-generating system. If the initial efflux obtained after quenching the pump is a diffusional flux, then it should not depend on the quench method. We have used two quench methods. Addition of EGTA quenches the pump by lowering activator calcium, and glucose plus hexokinase quenches the pump by converting substrate ATP to ADP and glucose 6-phosphate. The initial first order rate constant was nearly identical when efflux was initiated by EGTA or by glucose plus hexokinase (Table 1). The quench reagents stopped the calcium-dependent liberation of P_i associated with the forward operation of the pump, and the P_i levels did not increase with time (data not shown). This evidence, coupled with other observations (20), suggests that calcium efflux after quenching of ATPase activity is not associated with turnover of the Ca,Mg-ATPase and can be considered to be passive efflux.

The validity of using EGTA or glucose plus hexokinase to measure passive calcium efflux requires that no portion of this efflux is due to nucleotide-dependent carrier action. If nucleotide-independent carrier action is involved, we might expect effects of internal or external calcium on the initial calcium efflux. To examine these effects, the extent of calcium loading was varied by adding varying amounts of EGTA to the incubation solution before beginning the uptake reaction. As can be seen in Table 1, the initial first order rate constants were nearly identical when EGTA or glucose plus hexokinase was used. Further, the rate constants decreased with decreasing calcium load.

We next examined the effect of oxygen radicals on calcium load and passive calcium efflux. The results of the experiments are shown in Table 1. Xanthine or xanthine oxidase alone and xanthine plus denatured xanthine oxidase had no significant ($p > 0.01$) effect on calcium load and initial first order rate constant (not shown). The combination of xanthine plus xanthine oxidase that elicited no effect on the ATPase activity induced a significant decrease in the steady state calcium load and increase in the rate constant. These effects of oxygen free radicals were significantly inhibited by SOD (Table 1) but not by denatured SOD (not shown). The calcium load, varied by adding 0–20 μM EGTA to the reaction bath, exerted only a slight effect on any of the changes mediated by the generation of oxygen free radicals. However, steady state outside calcium concentration was not identical for all of these experiments. To test for effects of Ca_o on calcium efflux, calcium or EGTA was added during calcium efflux initiated by glucose plus hexokinase. In these experiments (Table 2), Ca_o had a slight but nonsignificant effect on the first order rate constants under control conditions. In the presence of the xanthine-xanthine oxidase system, there was a significant increase in the initial first order rate constant. This effect of oxygen free radicals was inhibited by SOD; Ca_o between 0.001 and 33.9 μM had no effect on this system, suggesting that the decrease in the calcium permeability with calcium load is specifically related to the calcium load and that oxygen free radicals can produce an increase in passive calcium efflux.

To further describe the effect of oxygen free radicals on the calcium efflux pathway, the initial efflux of calcium from SR was measured at various Mg concentrations in the presence of xanthine and xanthine oxidase. If nucleotide-dependent carrier action were involved in the passive calcium flux, then it should be altered by Mg. We determined the first order rate constant for calcium efflux for SR when Mg was varied from 2.1 to 21 mM. Xanthine, xanthine oxidase alone (Fig. 2A), and xanthine plus denatured xanthine oxidase (Fig. 2B) had no effect on the first order rate constant. A marked increase in the first order rate constant was observed in the presence of xanthine plus xanthine oxidase (Fig. 2B). SOD, but not denatured SOD, completely inhibited the effect of the xanthine-xanthine oxidase reaction (Fig. 2C). Mg showed no apparent effect on this system (Fig. 2).

The above evidence suggests that the inhibition of steady state calcium uptake is due to alteration in the passive calcium permeability of the SR membrane. If the view that oxygen free radical-induced decrease in steady state calcium load results in a "leaking out" of transported calcium before binding within the SR vesicle is correct, oxygen free radicals would decrease Ca_{bi} and Ca_i, and this effect would be inhibited by SOD. We tested this hypothesis by determination of the passive calcium efflux, J_p. Passive calcium efflux, J_p, is driven by the intravesicular free calcium. Ca_i is not directly measured in our experiments. However, the total internal calcium can be calculated according to

$$Ca_i + Ca_{bi} = Ca_t - Ca_{bo}$$

provided Ca_{bo} is known. The passive efflux of calcium, J_p, from SR vesicles was measured at various calcium loads obtained by

TABLE 1

Effect of free radicals produced from the xanthine-xanthine oxidase system on SR steady state calcium load and passive calcium permeability

Steady state calcium uptake was attained in 10-ml reaction bath with 20 mM imidazole buffer, pH 7.0, containing 100 mM KCl, 10 mM NaN_3, 100 μM ATP, 2.1 mM $MgCl_2$, 11 μM total Ca, 0.080–0.092 mg/ml SR protein, 0–20 μM EGTA, and, when added, 25 μM xanthine (X), 0.09 units/ml xanthine oxidase (XO), and 56 units/ml SOD. The time sequence of additions was designed to ensure exposure of SR to oxygen radicals for 1 min and SOD for 0.5 min before initiation of calcium uptake by substrates (Ca^{2+} + Mg^{2+} + ATP). The initial first order rate constant K/v was calculated as described under Materials and Methods. Net passive efflux was begun by the addition of EGTA (2.5 mM) or glucose (12.5 mM) plus hexokinase (0.06 mg/ml) 1.0 min after beginning uptake. The values are means ± standard errors (eight experiments).

Experimental addition	Agent used	Steady state $Ca_t - Ca_{bo}$	Ca_{bo}	EGTA quench		Glucose + hexokinase	
				Ca_o	K/v	Ca_o	K/v
		nmol/mg	nmol/mg	μM	min^{-1}	μM	min^{-1}
None	Control	48 ± 4	8.6 ± 0.4	<0.001	0.98 ± 0.03	3.5	0.92 ± 0.05
	X + XO[a]	28 ± 2[a]	8.4 ± 0.6		1.81 ± 0.06[a]		1.93 ± 0.08[a]
	X + XO + SOD	39 ± 4[b]	9.0 ± 0.5		1.12 ± 0.07[b]		0.98 ± 0.07[b]
4 μM EGTA	Control	40 ± 2	6.1 ± 0.3	<0.001	0.86 ± 0.06	2.3	0.91 ± 0.03
	X + XO	21 ± 3[a]	6.0 ± 0.5		1.33 ± 0.08[a]		1.43 ± 0.09[a]
	X + XO + SOD	36 ± 2[b]	6.3 ± 0.8		0.92 ± 0.06[b]		0.99 ± 0.03[b]
8 μM EGTA	Control	22 ± 3	5.9 ± 0.4	<0.001	0.65 ± 0.03	1.3	0.70 ± 0.02
	X + XO	12 ± 2[a]	5.6 ± 0.7		1.25 ± 0.04		1.32 ± 0.06[a]
	X + XO + SOD	20 ± 1[b]	5.7 ± 0.7		0.72 ± 0.01[b]		0.82 ± 0.07[b]
12 μM EGTA	Control	16 ± 3	4.8 ± 0.2	<0.001	0.62 ± 0.04	0.7	0.68 ± 0.05
	X + XO	9 ± 2[a]	4.0 ± 0.7		0.89 ± 0.03[a]		0.94 ± 0.02[a]
	X + XO + SOD	17 ± 2[b]	4.6 ± 0.4		0.63 ± 0.08[b]		0.73 ± 0.06[b]
20 μM EGTA	Control	14 ± 2	3.2 ± 0.1	<0.001	0.39 ± 0.03	0.3	0.43 ± 0.05
	X + XO	7 ± 2[a]	3.0 ± 0.8		0.78 ± 0.07[a]		0.75 ± 0.06[a]
	X + XO + SOD	12 ± 1[b]	3.0 ± 0.7		0.45 ± 0.02[b]		0.52 ± 0.03[b]

[a] Significantly different from control ($p < 0.01$).
[b] Significantly different from X + XO group.

TABLE 2

Effect of outside calcium concentration on free radical-induced change in SR passive calcium permeability

The reaction bath and the sequence of additions for xanthine (X), xanthine oxidase (XO), or SOD were essentially the same as described for Table 1. Net calcium efflux was initiated by addition of 12.5 mM glucose plus 0.06 mg/ml hexokinase 1.0 min after calcium uptake was begun. At 1.2 min, Ca_o was changed by addition of 2.5 mM, 0.5 mM, or 0.1 M EGTA or 10 and 25 μM calcium. All values are means ± standard errors (six experiments).

Experimental Addition	Agent Used	Ca_o	K/v
		μM	min^{-1}
2.5 mM EGTA	Control	0.001	1.09 ± 0.10
	X + XO		1.84 ± 0.08[a]
	X + XO + SOD		0.96 ± 0.12[b]
0.5 mM EGTA	Control	0.008	0.92 ± 0.05
	X + XO		1.76 ± 0.10[a]
	X + XO + SOD		1.11 ± 0.04[b]
0.1 mM EGTA	Control	0.084	0.96 ± 0.08
	X + XO		1.67 ± 0.03[a]
	X + XO + SOD		1.02 ± 0.09[b]
None	Control	8.9	0.89 ± 0.06
	X + XO		1.72 ± 0.08[a]
	X + XO + SOD		0.98 ± 0.04[b]
10 μM Ca	Control	18.9	0.91 ± 0.09
	X + XO		1.50 ± 0.06[a]
	X + XO + SOD		1.12 ± 0.03[b]
25 μM Ca	Control	33.9	0.84 ± 0.11
	X + XO		1.42 ± 0.03[a]
	X + XO + SOD		0.93 ± 0.05[b]

[a] Significantly different from control ($p < 0.01$).
[b] Significantly different from X + XO group.

actively loading the vesicles in the presence of varying low concentrations of EGTA. By plotting the passive efflux J_p against the calcium load ($Ca_t - Ca_{bo} = Ca_i + Ca_{bi}$, the sum of free and bound intravesicular calcium, in nmol mg^{-1}), Ca_{bi} can be determined from the extrapolated intercept of the line onto the abscissa (20). Fig. 3 shows the measured and calculated data that determine the passive calcium efflux J_p after the attainment of steady state in actively loaded populations. As

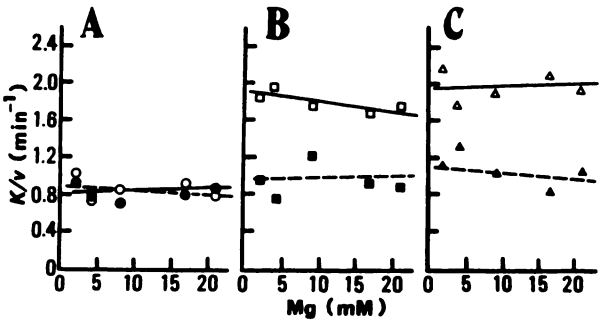

Fig. 2. Effect of Mg concentration on apparent first order rate constant for passive efflux from actively loaded SR. Reaction conditions were identical to those of Table 2 except the Mg concentration was 2.1–21 mM, protein concentration was 0.062–0.081 mg/ml, and total calcium was 8.5–11.2 μM. The sequence of addition of reagents is as described in Table 1. Agents used are: A, xanthine (○) and xanthine oxidase (●); B, xanthine plus xanthine oxidase (□) and xanthine plus denatured xanthine oxidase (■); C, xanthine plus xanthine oxidase plus SOD (▲) and xanthine plus xanthine oxidase plus denatured SOD (△). Each *point* represents the mean from two separate studies performed in duplicate.

expected, Ca_{bi} and Ca_i were unchanged by the addition of either xanthine or xanthine oxidase alone and xanthine plus denatured xanthine oxidase, but xanthine plus xanthine oxidase produced SOD-inhibitable decrease in Ca_{bi} and Ca_i.

Discussion

The SR of cardiac muscle is a potential mediator of the early responses to ischemia and hypoxia and could contribute to the intracellular calcium overload seen in the final contracture phase. Depression of SR function during ischemia would result in the loss of regulation of intracellular calcium (29). Schwartz *et al.* (30) found a defect in SR calcium release after 12–60 min of ischemia, whereas, with 30 min of ischemia, Hess *et al.* (31) found calcium uptake to be depressed. These studies indicate that the time course for damage to the SR is more rapid than

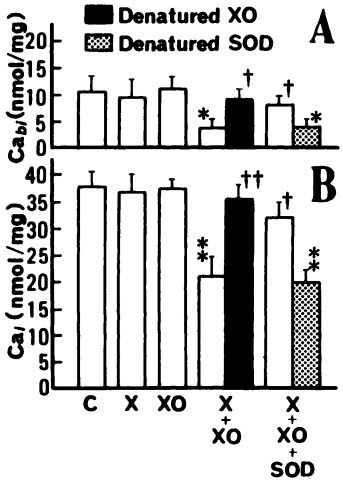

Fig. 3. Effect of oxygen free radicals produced from xanthine (*X*) – xanthine oxidase (*XO*) system on intravesicular bound calcium (A, CA_{bi}) and intravesicular free calcium (B, Ca_i) for SR. Steady state calcium uptake was attained at 27° in a bath containing 100 mM KCl, 20 mM imidazole, pH 7.0, 10 mM NaN_3, 100 μM ATP, 2.1 mM $MgCl_2$, 11.0 μM total Ca, 0.080–0.092 mg of SR protein per ml, and, when added, 25 μM xanthine, 0.09 units/ml xanthine oxidase or denatured xanthine oxidase, and 56 units/ml SOD or denatured SOD. The sequence of addition of reagents is as described in Table 1. Seady state calcium uptake was varied by including 0–25 μM EGTA in the reaction bath. Net passive efflux was begun by adding 2.5 mM EGTA. The initial passive efflux was calculated as $J_p = K/v(Ca_t - Ca_{bo})$. The intravesicular bound calcium, Ca_{bi}, was taken as the extrapolated intercept of the line of the plots of each obtained J_p onto the *abscissa*, calcium load. Ca_i was calculated according to $Ca_i = Ca_t - Ca_{bo} - Ca_{bi}$. Ca_{bi} and Ca_i measured under standard conditions are indicated as *C*, control. Each column represents the mean (five to seven experiments) and the error bar, ± 1 SE. *, **Significantly (*$p < 0.05$; **$p < 0.01$) different from XO; †, ††significantly (†$p < 0.05$; ††$p < 0.01$) different from X + XO.

previously appreciated and indicate that SR dysfunction may be related to the loss of tension development during the course of ischemia. The generation of oxygen free radicals in the ischemic heart would appear to be a good candidate for the mediator of this potential SR dysfunction (32–34).

The major findings of this study are as follows. First, oxygen free radicals from the reaction of xanthine, in doses up to 50 μM, plus xanthine oxidase (0.09 units/ml) produced a diminished level of accumulated calcium, which was reflected by the decreased calcium load ($Ca_i + Ca_{bi}$), and produced an increase in passive calcium permeability with no effect on Ca,Mg-ATPase activity. This is contradictory to our earlier report (10) in which a significant inhibition in the Ca,Mg-ATPase activity was observed. In those studies, we used Sigma xanthine oxidase, which is heavily contaminated with PLA_1. In the present study we used Boeringer-Mannheim xanthine oxidase, which is absolutely free of PLA_2 contamination (35). The PLA_2 can adversely affect the ATPase activity, independent of free radical effect. These results led us to the conclusion that oxygen free radical-mediated decrease in SR steady state calcium load may not mainly be due to an inhibited calcium pump but due to an increased calcium permeability. Further observations suggesting that Ca,Mg-ATPase is not involved as a carrier of the oxygen free radical-induced increase in the efflux through a passive leak included: (a) initial flux is unaffected by conversion of ATP to ADP by glucose plus hexokinase; and (b) permeability is unaffected by Mg. It has been known for some time that calcium efflux can be mediated by the Ca,Mg-ATPase in the reversal of the ATPase-dependent calcium uptake reaction with cardiac SR (36). The reversal of the ATPase requires addition of EGTA to preloaded vesicles, a situation similar to that used by us to study passive calcium efflux. Our experimental conditions, however, are not optimal for calcium efflux mediated by the reversal of the ATPase (20, 37). Furthermore, outside calcium concentration, Ca_o, only minimally affects the first order rate constant between 0.001 and 33.9 μM. Thus, it is suggested that net reversal of the ATPase is not an important component of the oxygen free radical-induced increase in passive calcium permeability and this passive route is not regulated by Ca_o under the conditions used.

Second, oxygen free radicals produced a decrease in calcium bound to intravesicular binding sites, Ca_{bi}, and calcium free within the intravesicular space, Ca_i. The calcium gradient bears on the importance of the passive pathway with respect to calcium accumulation and release. The gradient can be calculated from Ca_i and Ca_o. The intravesicular calcium concentration can be calculated from the free intravesicular calcium load and the intravesicular volume. The enclosed SR volume has been estimated by a number of investigators (38–40), and values range from 2 to 5 μl/mg (38–40). When Ca_o was near the physiological range, 0.15–0.4 μM, and intravesicular volume, v, was assumed to be 5 μl/mg, the steady state gradient was about 18.3×10^3 for xanthine control and about 11.8×10^3 for xanthine plus xanthine oxidase (Fig. 3). Assuming $v = 2$ μl/mg, then the gradient was about 122.0×10^3 at 0.15 μM calcium concentration for xanthine control and about 79.3×10^3 for xanthine plus xanthine oxidase (Fig. 3). From these results and calculations, it appears that SR can attain gradient and that the effect of oxygen free radicals on calcium permeability is calcium gradient dependent.

It has been suggested that the chemical reactivity of $\cdot O_2^-$ is quite low (41, 42) but others (43) argue that reaction

$$\cdot O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$$

is too slow to compete with the dismutation reaction

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

and that $\cdot O_2^-$ can initiate phospholipid autoxidation provided conjugated diene hydroperoxides are already present. Lynch and Fridovich (44) also showed that lipid hydroperoxides sensitize membranes to $\cdot O_2^-$. Further, lipid peroxidation results in increases in both membrane fluidity and membrane permeability (45). These observations and our results suggest that $\cdot O_2^-$ is involved in a mechanism that may cause increased passive calcium permeability, inasmuch as all the effects of oxygen free radicals generated from the xanthine-xanthine oxidase reaction are inhibited by SOD, which is a very specific enzyme (46). Also, a strong possibility exists for the formation of $\cdot OH$ radical via Fenton and Haber-Weiss reactions, due to contaminant iron salts as shown below:

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

The protective effect of SOD may be due to prevention of $\cdot O_2^-$-dependent reduction of Fe^{3+} to Fe^{2+} and thus inhibition of $\cdot OH$ radical formation. In this regard, SOD has been shown to depress $\cdot OH$ radical formation (47–49). In the present investigation, we did not confirm the fractional contribution of contaminant reactive iron in the formation of $\cdot OH$ radical by

the iron chelator desferrioxamine. It is clear, however, that oxygen free radicals increase the passive permeability of the SR vesicles to calcium and produce a diminished level of accumulated calcium. This is reflected by the decreased calcium load that is not caused by inhibition of the calcium pump; therefore, the increased SR membrane passive calcium permeability is not carrier mediated. The oxygen free radical-mediated increase in passive calcium permeability may contribute, at least in part, to the decrease in intravesicular calcium.

Xanthine oxidase has been proposed to be the source of injurious O_2 free radicals in the reperfused ischemic heart (32, 50). The normally perfused rat and dog heart contain almost all of this enzyme in the dehydrogenase form. The dehydrogenase form hydroxylates purines and by hydration/dehydrogenation transfers electrons to NAD^+. During a period of ischemia, xanthine dehydrogenase is rapidly converted to an oxidase form (50–52), which uses molecular oxygen as the electron acceptor producing superoxide anion and H_2O_2. The breakdown of ATP in the ischemic heart causes accumulation of hypoxanthine, the substrate for the enzyme, while the reperfusion provides the O_2. In the ischemic dog myocardium, 0.07 units of xanthine oxidase/g of protein is reported to be present (50), which corresponds to 1.4 units/100 g of cardiac tissue, and is estimated to produce 0.28 μmol of $\cdot O_2^-$ anion/min. This amount of $\cdot O_2^-$ anion is more than enough to significantly contribute to cellular damage. In the present investigation, we used 0.09 units/ml xanthine oxidase, which induced significant increases in calcium permeability and decreases in intravesicular calcium.

Having identified the *in vitro* effect of xanthine plus xanthine oxidase calcium loading at steady state in SR, one can now ask what the consequences of SR injury would be to cardiac function. The final phase of hypoxic ischemic injury is characterized by a progressive increase in resting tension with the formation of rigor complexes that herald the irreversible phase of myocardial ischemia. The formation of rigor complexes most probably involves a significant increase in intracellular calcium concentration. The increase in intracellular calcium would appear to be the final common pathway for ischemic contracture.

It has long been assumed that the source for this intracellular calcium overload was the extracellular space, owing to the concentration gradient across the sarcolemmal membrane. However, this concept has been challenged by Bourdillion and Poole-Wilson (53), analyzing calcium influx and efflux in the ischemic rabbit intraventricular septum. These investigators demonstrated no net increase in tissue uptake of calcium during the ischemic phase and no change in net efflux. It is only after reperfusion and reoxygenation that an increase in total tissue calcium is observed that can be explained by the influx of calcium from the extracellular to the intracellular compartment. These data strongly support a primary intracellular source of free calcium as the initiating event for the final irreversible phase of myocardial ischemia. Inasmuch as it is now recognized that the SR of cardiac muscle serves as the major source and sink of coupling calcium in the excitation-contraction coupling sequence (54), with the oxygen free radical-induced progressive increase in calcium permeability, free cytosolic calcium concentrations would increase in ischemic myocardium.

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