0026-895X/98/030497-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**497–503 (1998).

Superoxide Anion Radical-Triggered Ca^{2+} Release from Cardiac Sarcoplasmic Reticulum through Ryanodine Receptor Ca^{2+} Channel

MIDORI KAWAKAMI and EIICHIRO OKABE

Department of Pharmacology and ESR Laboratory, Kanagawa Dental College, Yokosuka, Kanagawa 238, Japan

Received July 14, 1997; Accepted October 31, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

The ryanodine receptor Ca^{2+} channel (RyRC) constitutes the Ca^{2+}-release pathway in sarcoplasmic reticulum (SR) of cardiac muscle. A direct mechanical and a Ca^{2+}-triggered mechanism (Ca^{2+}-induced Ca^{2+} release) have been proposed to explain the *in situ* activation of Ca^{2+} release in cardiac muscle. A variety of chemical oxidants have been shown to activate RyRC; however, the role of modification induced by oxygen-derived free radicals in pathological states of the muscle remains to be elucidated. It has been hypothesized that oxygen-derived free radicals initiate Ca^{2+}-mediated functional changes in or damage to cardiac muscle by acting on the SR and promoting an increase in Ca^{2+} release. We confirmed that superoxide anion radical ($O_2^{\cdot-}$) generated from hypoxanthine-xanthine oxidase reaction decreases calmodulin content and increases $^{45}Ca^{2+}$ efflux from the heavy fraction of canine cardiac SR vesicles; hypoxanthine-xanthine oxidase also decreases Ca^{2+} free within the intravesicular space of the SR with no effect on Ca^{2+}-ATPase activity. Current fluctuations through single Ca^{2+}-release channels have been monitored after incorporation into planar phospholipid bilayers. We demonstrate that activation of the channel by $O_2^{\cdot-}$ is dependent of the presence of calmodulin and identified calmodulin as a functional mediator of $O_2^{\cdot-}$-triggered Ca^{2+} release through the RyRC. For the first time, we show that $O_2^{\cdot-}$ stimulates Ca^{2+} release from heavy SR vesicles and suggest the importance of accessory proteins such as calmodulin in modulating the effect of $O_2^{\cdot-}$. The decreased calmodulin content induced by oxygen-derived free radicals, especially $O_2^{\cdot-}$, is a likely mechanism of accumulation of cytosolic Ca^{2+} (due to increased Ca^{2+} release from SR) after reperfusion of the ischemic heart.

Several attempts have been made to integrate the two most popular hypotheses of myocardial stunning and reperfusion injury: (1) accumulation of cytosolic Ca^{2+} and (2) increase in oxygen-derived free radical production (Kukreja and Hess, 1992; Opie, 1992). According to this unifying hypothesis, oxygen-derived free radicals initiate Ca^{2+}-mediated functional changes or damage by acting on the SR and promoting Ca^{2+} entry into the cytosol. In support of this is the finding that oxygen-derived free radicals, generated by the xanthine-xanthine oxidase system, depress SR Ca^{2+} uptake in canine heart homogenates (Hess *et al.*, 1984) and isolated SR vesicles (Okabe *et al.*, 1983). Because the net Ca^{2+} uptake in the SR is a result of the activity of Ca^{2+}-ATPase and of the SR Ca^{2+}-release channel, an abnormal Ca^{2+} uptake may be the result of the dysfunction of either or both structures. The site or sites of action for oxygen-derived free radicals damage are unknown, although previous studies on the SR have focused on damage to the Ca^{2+} pump. Direct effects of oxygen-derived free radicals on SR Ca^{2+}-release channels may be important in understanding their potential contribution to ischemia/reperfusion injury and developing strategies to protect against such injury. Previously, we found that decreased SR Ca^{2+} uptake in response to oxygen-derived free radicals is associated with an enhanced Ca^{2+} loss by the Ca^{2+}-release process (Okabe *et al.*, 1988, 1991). We now provide evidence that $O_2^{\cdot-}$ dramatically alters the gating characteristics of the reconstituted RyRC from the heavy fraction of cardiac SR vesicles due to decreased calmodulin content; this is a novel mechanism for SR Ca^{2+} release by oxygen-derived free radicals in cardiac muscle. These findings indicate that an elevation in cytosolic Ca^{2+} due to abnormal Ca^{2+} handling,

This work was supported by Grants 09877356 (E.O.) and 07557119 (E.O.) from Scientific Research Fund of The Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Research Fund of JEOL Ltd., Tokyo, Japan.

ABBREVIATIONS: SR, sarcoplasmic reticulum; Ca_i, intravesicular free Ca^{2+}; $O_2^{\cdot-}$, superoxide anion radical; RyRC, ryanodine receptor Ca^{2+}-release channel(s); P_o, open probability; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMPO, 5,5-dimethyl-1-ryrroline-*N*-oxide; MnO, manganese oxide marker; SOD, superoxide dismutase; DMSO, dimethylsulfoxide; $HO^{\cdot}$, hydroxyl radical; Ca^{2+}-ATPase, Ca^{2+}-stimulated, Mg^{2+}- dependent ATPase; SH, sulfhydryl.

such as the increase in P_o of RyRC, by the SR may contribute to *in vivo* reperfusion injury.

Materials and Methods

Heavy SR vesicles preparation and $^{45}Ca^{2+}$ efflux measurements. Canine cardiac heavy SR was isolated by discontinuous sucrose gradient centrifugation according to a modified method described previously (Valdivia *et al.*, 1991). Briefly, canine ventricular muscles were minced in a food processor and homogenized for 60 sec in buffer containing 0.3 M sucrose, 20 mM MOPS, pH 7.2, and protease inhibitors (1 μg/ml pepstatin and leupeptin, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 10 μg/ml aprotinin). The homogenate was centrifuged for 20 min at 11,000 $\times$ *g*. The supernatant was centrifuged for 60 min at 119,000 $\times$ *g*. After centrifugation, the supernatant was discarded. The pellet underwent fractionization overnight on a discontinuous sucrose gradient (10%, 31%, 40%, and 50%) in a solution of 400 mM KCl, 20 mM MOPS, pH 6.8, and 100 μM $MgCl_2$, $CaCl_2$, and EGTA in a Beckman Instruments (Columbia, MD) SW27 rotor at 25,000 rpm. The final pellets were resuspended in 0.3 M sucrose and 20 mM MOPS, pH 6.8, in addition to the previously mentioned mixture of protease inhibitors. Protein concentration was determined according to the method of Lowry *et al.* (1951). The resulting heavy SR vesicles were preincubated overnight on ice in 2 mM $^{45}CaCl_2$ (New England Nuclear Research Products, Boston, MA), 150 mM KCl, and 20 mM MOPS, pH 6.8. They then were diluted 20-fold into a Ca^{2+}-releasing medium containing 150 mM KCl, 20 mM MOPS, pH 6.8, and 1 mM EGTA/Ca^{2+} buffer to adjust the pCa to 5. $^{45}Ca^{2+}$ efflux was quenched with ice-cold quench solution containing 1 mM $LaCl_3$, 10 mM $MgCl_2$, 150 mM KCl, and 20 mM MOPS, pH 6.8. After filtration through Millipore (Bedford, MA) filters (0.45 μm) and washing of the filters with the quenching solution, the radioactivity retained by the filter was determined by liquid scintillation counting.

EGTA washing of heavy SR vesicles. To remove endogenous calmodulin from SR, the vesicle suspension was diluted 1:100 in 20 mM HEPES, pH 7.4, kept 20 min on ice and made hypertonic by the addition of the same volume of 1.2 M KCl, 20 mM HEPES, and 4 mM EGTA, pH 7.4. The hypertonic suspension was centrifuged for 30 min at 150,000 $\times$ *g*, and the pellet was washed twice with 20 mM HEPES, pH 7.4. The final pellet was resuspended in 0.3 M sucrose and 20 mM MOPS, pH 6.8, containing the mixture of protease inhibitors and used immediately. Confirmation of the depletion of endogenous calmodulin was obtained according to the method of Schulman and Greengard (1978), in which EGTA-washed SR vesicles were phosphorylated with [γ-^{32}P]ATP (New England Nuclear Research Products) in the presence of 30 μg of washing extract or 0.6 μM calmodulin (from bovine brain; Fluka AG, Buchs, Switzerland) and then subjected to preparative sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The washing and boiled extracts of SR stimulated the incorporation of ^{32}P from 10 μM [γ-^{32}P]ATP into EGTA-washed SR proteins in the presence of 0.5 mM $CaCl_2$. With the assumption that kinase is activated only by calmodulin, this result demonstrates the presence of calmodulin in the extracts. Moreover, it was found that a hypotonic treatment, followed immediately by a hypertonic wash in the presence of 4 mM EGTA and by several hypotonic washes in the absence of the chelator, resulted in the depletion of calmodulin (Carafoli *et al.*, 1980).

ESR analysis. The spin-trapping studies were performed with the desired mixture containing DMPO (Labotec, Tokyo, Japan; 99–100% pure, gas chromatographic assay by Dojindo Laboratories, Kumamoto, Japan). ESR detection of the spin adduct was carried out at room temperature with a JEOL (model JES-RE3X) X-band spectrometer connected with the JOEL computer system Esprit (Tokyo, Japan). Hyperfine coupling constants were calculated based on the resonance frequency measured with a microwave frequency counter and the resonance field measured with the JEOL field measurement unit model ES-FC5. ESR spectra were recorded at the instrument settings of 0.05-mT (100 kHz modulation amplitude), 10-mT recording range, 2-min recording time, 0.1-sec time constant, 8-mW (9.414-GHz microwave power), and 335.6 $\pm$ 5-mT magnetic field.

A quantitative analysis of the spin adducts of $O_2^{\cdot-}$ was performed as described previously (Mitsuta *et al.*, 1990). After recording of the ESR spectra, the signal intensity of DMPO—$O_2^{\cdot-}$(—OOH) was normalized as a relative height against the standard signal intensity of MnO. An absolute concentration of DMPO—OOH was determined by a double integration of the ESR spectrum, in which a 1.0 μM concentration of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl solution was used as a primary standard of ESR absorption.

Calmodulin content of heavy SR vesicles. Heavy SR vesicle fractions at a protein concentration of 1 mg/ml were incubated for 10 min at 22° or heated for 10 min at 95° in media containing either 20 mM K-piperazinediethanesulfonic acid, pH 7.0, 0.1 M KCl, 100 μM EGTA, and 106 μM Ca^{2+} (10 μM free Ca^{2+}) or 0.125 M borate, pH 8.4, 0.075 M NaCl, 0.2% bovine serum albumin, and 1 mM EGTA ($<10^{-10}$ M free Ca^{2+}) in the presence or absence of 20 μM hypoxanthine (Sigma Chemical, St. Louis, MO). Next, vesicles underwent sedimentation for 30 min at 100,000 $\times$ *g* in a Beckman airfuge. Xanthine oxidase (0.1 unit/ml; activity, 35.8 μM/min; Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added 2.0 min before the sedimentation, and SOD (10 μg/ml; 3000 units/ml; Sigma Chemical) was added 30 sec before the addition of xanthine oxidase. The supernatants of samples not heated at 95° were incubated for 10 min at 95°. The calmodulin content of the supernatant fractions were measured with the use of an ^{125}I-calmodulin radioimmunoassay kit from New England Nuclear Research Products).

Ca_i. Ca_i was calculated after passive Ca^{2+} efflux, J_p, from heavy SR vesicles was measured as described previously (Okabe *et al.*, 1988). Briefly, steady state Ca^{2+} uptake was measured in the absence of Ca^{2+}-precipitating anions at 27° by filtration through 0.45-μm Millipore filters of 1.0-ml aliquots from a 10-ml bath containing 100 mM KCl, 20 mM imidazole, pH 7.0, 10 mM NaN_3, 100 μM disodium ATP, 2.1 mM $MgCl_2$, 0.1 μCi of $^{45}Ca^{2+}$/ml, and 4 μM added Ca^{2+}. Total Ca^{2+} in the reaction bath was determined by atomic absorption spectrophotometry after wet ashing of the reaction bath including SR. The total Ca^{2+} associated with the SR was obtained by Millipore filtration and was calculated on the basis of the total $^{45}Ca^{2+}$ in the reaction bath and the $^{45}Ca^{2+}$ in the filtrates of the reaction bath. The uptake reaction was begun by the addition of ATP, Ca^{2+}, and Mg^{2+} to an otherwise complete reaction bath.

Passive Ca^{2+} efflux was measured after steady state Ca^{2+} uptake was reached through quenching of pump-mediated Ca^{2+} fluxes and observation of the net release in Ca^{2+} by Millipore filtration. Quenching of the pump-mediated fluxes was produced by the addition of EGTA to a final concentration of 2.5 mM. The initial apparent first-order rate constant, K/v, was obtained by linear regression of the natural logarithm of the Ca^{2+} uptake determined through Millipore filtration at various times after the addition of EGTA. The initial passive Ca^{2+} efflux, J_p, was obtained from the product of the first order-rate constant and the initial Ca^{2+} load.

It is assumed in all experiments that total Ca^{2+} in the reaction bath is distributed among four compartments; these are Ca_o, the Ca^{2+} in solution outside the vesicles; Ca_{bo}, the Ca^{2+} bound to the outside of the vesicles; Ca_{bi}, the Ca^{2+} bound to intravesicular binding site; and Ca_i, Ca^{2+} free within the intravesicular space. The initial value ($Ca_t - Ca_{bo}$) was determined by extrapolating the first-order efflux curve to the time of the addition of EGTA quench. The initial passive Ca^{2+} efflux, calculated as $J_p = K/v\ (Ca_t - Ca_{bo})$, is driven by Ca_i. Ca_i is not directly measured in the current experimental system. However, the total internal Ca^{2+} can be calculated as $Ca_i + Ca_{bi} = Ca_t - Ca_{bo}$, provided Ca_{bo} is known. The J_p value from SR vesicles was measured at various loads obtained by actively loading the vesicles in the presence of 0–25 μM EGTA. By plotting each obtained J_p value against the Ca^{2+} load ($Ca_t - Ca_{bo} = Ca_i + Ca_{bi}$, the sum of free and bound intravesicular Ca^{2+}), Ca_{bi} can be determined from

the extrapolated intercept of the line onto the abscissa. Ca_i was calculated according to $Ca_i = Ca_t - Ca_{bo} - Ca_{bi}$.

Ca^{2+}-ATPase activity. The ATPase activity was determined from the rate of $^{32}P_i$ release from [γ-^{32}P]ATP according to the method of Feher and Briggs (1980).

Planar phospholipid bilayer experiments. Single-channel recordings were carried out by incorporating the native or EGTA-washed calmodulin-depleted heavy SR vesicles into planar phospholipid bilayers according to a previous method (Smith *et al.*, 1985). The planar phospholipid bilayers, composed of phosphatidylethanolamine (bovine heart; Avanti Polar Lipids, Birmingham, AL) dispersed in decane at a concentration of 25 mg/ml, were painted across a 200-μm-diameter hole in the styrene copolymer septum between the two experimental chambers containing 5 mM $CaCl_2$, 50 mM choline chloride, and 10 mM HEPES/Tris, pH 7.2. Heavy SR vesicles (10 μg/ml) then were added to the designated *cis* chamber, and the solution was fortified with choline chloride to produce a 7:1 gradient across the membrane. Vesicle fusion was monitored as steplike conductance increases that resulted in a Cl^--specific macroscope current. After fusion, the *cis* chamber was perfused with 1 mM Ca-EGTA (10 μM free Ca^{2+}), and 250 mM HEPES/125 mM Tris, pH 7.4, and the *trans* chamber was perfused with 250 mM glutamic acid, and 10 mM HEPES, adjusted to pH 7.4 with $Ca(OH)_2$, to give a solution with $\approx$67 mM free Ca^{2+}. Channel opening results in a flow of ions across the bilayer, which was amplified by a patch-clamp amplifier (Axopatch; Axon Instruments, Foster, CA), and stored on a videocassette tape recorder through a PCM converter system (RP-880; NF Instruments, Yokohama, Japan) filtered at 1 kHz and digitized at 2 kHz. All experiments were recorded at room temperature (22°). All recordings were made with the *cis* chamber voltage-clamped at 0 mV relative to ground. P_o of channels, and the lifetimes of open and closed events were identified by 50% threshold analysis. P_o values were calculated from 3-min records of steady state recordings. Channel openings are presented as upward deflections.

Results

The rapid progress made in recent years on the mechanism of the channel pathway is partly attributable to the use of ryanodine as a tool to probe this mechanism. Ryanodine has a biphasic effect: at low concentration, it locks the channel in a low-conductivity configuration; and at higher concentration, it determines channel blockade (Meissner, 1986). This biphasic action has been related to the presence of multiple ryanodine binding sites. Interaction with the high affinity site induces the formation of the low-conductivity state; interaction with the low affinity site or sites inactivates the channel (Pessah and Zimanyi, 1991). We described previously the optimal conditions for specific closure of the heavy SR Ca^{2+}-release channel by ryanodine (Okabe *et al.*, 1991). Briefly, short periods (0.5 min) of incubation with ryanodine concentrations that saturated the high affinity ryanodine-binding site decreased the steady state Ca^{2+} load of the SR vesicles; the increase in steady state Ca^{2+} load by a longer (10-min) incubation with high concentrations (250–750 μM) of ryanodine occurs through closure of Ca^{2+}-release channels. We assessed the effect of hypoxanthine-xanthine oxidase reaction on Ca^{2+} release from SR vesicles loaded passively with $^{45}Ca^{2+}$ in the presence of ryanodine (300 μM at 10 min of incubation). $^{45}Ca^{2+}$-loaded SR vesicles were diluted into Ca^{2+}-releasing medium containing 10 μM free Ca^{2+} to induce Ca^{2+}-induced Ca^{2+} release (Fig. 1). As seen in Fig. 1A, hypoxanthine-xanthine oxidase reaction drastically enhanced the $^{45}Ca^{2+}$ efflux from SR; the $^{45}Ca^{2+}$ efflux from the vesicles in the presence of hypoxanthine-xanthine oxidase

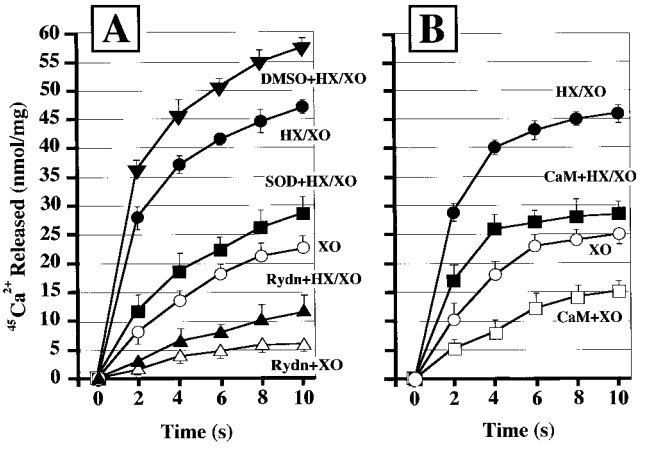

Fig. 1. Inhibition by SOD, ryanodine (A), and calmodulin (B) of the effect of oxygen-derived free radicals generated by hypoxanthine (*HX*, 20 μM)-xanthine oxidase (*XO*, 0.1 unit/ml) reaction on $^{45}Ca^{2+}$ efflux from isolated heavy SR vesicles. The time sequence additions were designed to ensure exposure of the SR to the oxygen-derived free radical-generating system for 2.0 min before initiation of the reaction. SOD (10 μg/ml), DMSO (150 mM), or calmodulin (*CaM*, 2.0 μM) was added before the free radical exposure. The SR vesicles were preincubated with or without ryanodine (*Rydn*, 300 μM; Wako Chemicals, Osaka, Japan; high performance liquid chromatography assay, 98%) for 10 min; then, the reaction was initiated. Data represent the average of measurements carried out with six (A) or seven (B) cardiac SR preparations. *Error bars*, standard errors.

reaction was inhibited by 300 μM ryanodine, which at this high concentration blocks the RyRC, indicating that the effect of hypoxanthine-xanthine oxidase on Ca^{2+} efflux stems from its interaction with the RyRC. The observed effect of hypoxanthine-xanthine oxidase was potentiated by DMSO and inhibited by SOD. Furthermore, the stimulatory effect by hypoxanthine-xanthine oxidase reaction on the $^{45}Ca^{2+}$ efflux was diminished by exogenously added calmodulin (Fig. 1B).

The radical species responsible for the effect elicited by hypoxanthine-xanthine oxidase reaction was verified by ESR spectroscopy with DMPO as the spin trap. Fig. 2 shows the ESR spectra of spin adducts observed using DMPO on hypoxanthine-xanthine oxidase reaction system. The spectrum in the presence of hypoxanthine-xanthine oxidase was constructed from the spectra of two types of spin adducts. Hyperfine coupling constants for one of the spin adducts were analyzed as follows: one nitrogen, a_N = 1.41 mT; one hydrogen of β position, $a_{H\beta}$ = 1.41 mT; and one hydrogen of γ position, $a_{H\gamma}$ = 0.13 mT (Buettner, 1987). The hyperfine coupling constants of the other spin adduct are a_N = 1.49 mT and $a_{H\beta}$ = 1.49 mT. Each component of the spectrum was assigned to DMPO—$O_2^{\cdot-}$(—OOH) and HO—DMPO spin adduct (DMPO—OH). When SOD (5 and 10 μg/ml) was added to the system, this signal was blunted effectively in a concentration-dependent fashion, suggesting that the reaction between $O_2^{\cdot-}$ and DMPO is inhibited by SOD due to a competition reaction between DMPO and SOD for $O_2^{\cdot-}$ (Finkelstein *et al.*, 1982). On the addition of DMSO to the system, which should eliminate $HO^{\cdot}$, another spectrum appeared. This signal increased with dependence on the decrease of that of DMPO—OH. The hyperfine coupling constants of new signal are a_N = 1.64 mT and $a_{H\beta}$ = 2.24 mT. These values coincide with the values reported for 5,5,2-trimethyl-1-pyrrolidinyl-oxyl (DMPO—CH_3) (Buettner, 1987). It is known

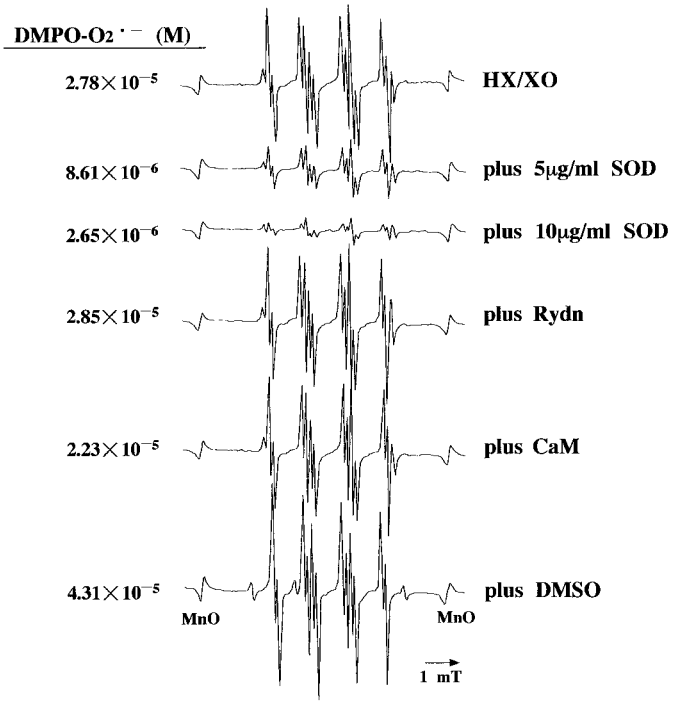

Fig. 2. ESR spectra of spin adducts produced by the hypoxanthine (*HX*)-xanthine oxidase (*XO*) reaction. ESR spectra were recorded in the same reaction medium as that of Ca^{2+} release assay except that SR vesicles and $^{45}Ca^{2+}$ were omitted and, when added, the medium contained 20 μM hypoxanthine, 0.1 unit/ml xanthine oxidase, 5 and 10 μg/ml SOD, 300 μM ryanodine (*Rydn*), 2.0 μM calmodulin (*CaM*), and 150 mM DMSO. SOD, ryanodine, calmodulin, or DMSO was added 30 sec before the addition of hypoxanthine/xanthine oxidase to the reaction medium containing 100 mM DMPO. At 2 min after the addition of hypoxanthine/xanthine oxidase, ESR spectrum was recorded as described in Materials and Methods. Signals appearing at both sides of the ESR spectra correspond to Mn^{2+} (MnO) installed in the ESR cavity as a reference. The concentration of DMPO—$O_2^{\cdot-}$(—OOH) was determined as described in the text.

that DMSO reacts selectively with the HO$^{\cdot}$ and the equivalent amount of methyl radical ($^{\cdot}CH_3$) is generated and that the short half-life of DMPO—$O_2^{\cdot-}$ that has been reported is attributable to the partial reaction of HO$^{\cdot}$ with DMPO—$O_2^{\cdot-}$ (Kohno *et al.*, 1991); the stability of DMPO—$O_2^{\cdot-}$ thus is dependent on the concentration of HO$^{\cdot}$. Therefore, when HO$^{\cdot}$ had been eliminated by DMSO, the intensity of DMPO—$O_2^{\cdot-}$ is increased. Indeed, DMSO significantly enhanced the effect of hypoxanthine-xanthine oxidase on $^{45}Ca^{2+}$ efflux from SR vesicles (see also Fig. 1A) due to the ability of DMSO to stabilize $O_2^{\cdot-}$ anion radicals; furthermore, the increase in $^{45}Ca^{2+}$ efflux was $O_2^{\cdot-}$ concentration dependent (Figs. 1A and 2). Ryanodine and calmodulin had no effect on ESR spectra of spin adducts observed using DMPO on hypoxanthine-xanthine oxidase reaction (Fig. 2). These results strongly suggest that $O_2^{\cdot-}$ activates RyRC and calmodulin may be a functional modulator of $O_2^{\cdot-}$-induced increase in Ca^{2+} release through the RyRC.

The modulation of the activity of the RyRC by calmodulin (Meissner and Henderson, 1987), coupled with evidence supporting site (SR)-specific loss of calmodulin during myocardial ischemia (Turla *et al.*, 1985), has prompted the hypothesis that $O_2^{\cdot-}$ anion radicals (generated from hypoxanthine-xanthine oxidase) produce a loss in function of calmodulin in heavy SR vesicles, thereby increasing the release of Ca^{2+} through RyRC. To test this hypothesis further, we examined the influence of hypoxanthine-xanthine oxidase reaction on endogenous calmodulin content of heavy SR vesicles.

Hypoxanthine-xanthine oxidase reduced the calmodulin content of SR vesicles; the loss afforded by hypoxanthine-xanthine oxidase was blunted by SOD (Fig. 3A). The loss of calmodulin seems to be the proximal cause of the effect of $O_2^{\cdot-}$ because removal of endogenous calmodulin from heavy SR vesicles can mimic the effect of hypoxanthine-xanthine oxidase reaction on $^{45}Ca^{2+}$ efflux (Figs. 3, B and C). With the generation of $O_2^{\cdot-}$ from hypoxanthine-xanthine oxidase reaction in EGTA-washed calmodulin-depleted SR vesicles, there was no increase in $^{45}Ca^{2+}$ efflux (Fig. 3B). Inasmuch as it seems that rather drastic experimental conditions used in the calmodulin extraction experiments cause some of the damage to the SR vesicles, it was investigated whether the addition of exogenous calmodulin reverses the effect of the depletion of calmodulin. A calmodulin concentration of 2 μM was used because this concentration was found to have a maximally inhibitory effect on Ca^{2+} efflux from cardiac Ca^{2+}-release vesicles (Meissner and Henderson, 1987). The addition of exogenous calmodulin (2 μM) to the experimental system brought about an effective reversal of the stimulated $^{45}Ca^{2+}$ efflux induced by the removal of endogenous calmodulin from SR vesicles to normal; the effect of $O_2^{\cdot-}$, which was not observed in calmodulin-depleted SR vesicles on $^{45}Ca^{2+}$ efflux, was reproduced. SOD also was effective on this system

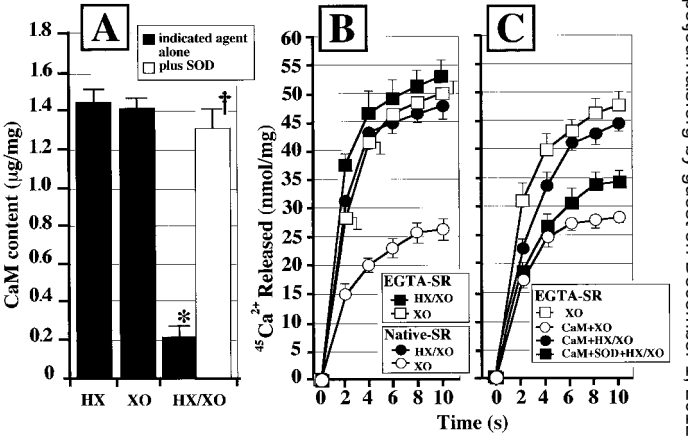

Fig. 3. Effect of oxygen-derived free radicals generated by hypoxanthine-xanthine oxidase reaction responsible for calmodulin in heavy SR vesicles. A, Calmodulin (*CaM*) content of the SR vesicles. Time sequence additions were designed to ensure exposure of the SR to hypoxanthine (*HX*, 20 μM)-xanthine oxidase (*XO*, 0.1 unit/ml) reaction for 2.0 min, and SOD (10 μg/ml) was added before the free radical exposure. Data represent the average ± standard error of measurements carried out with seven cardiac SR preparations. *, $p < 0.01$, significantly different from hypoxanthine. †, $p < 0.01$, significantly different from corresponding value for hypoxanthine/xanthine oxidase (analysis of variance followed by unpaired two-tailed Welch t test if analysis of variance was significant). B, $^{45}Ca^{2+}$ efflux from native and EGTA-washed calmodulin (*CaM*)-depleted SR vesicles in the presence or absence of hypoxanthine (*HX*, 20 μM)-xanthine oxidase (*XO*, 0.1 unit/ml) reaction. Time sequence additions were designed to ensure exposure of the SR vesicles to the oxygen-derived free radical-generating system for 2.0 min before initiation of the reaction. Data represent the average of measurements carried out with five cardiac SR preparations. *Error bars*, standard errors. C, Effect of exogenous calmodulin (*CaM*, 2.0 μM) on $^{45}Ca^{2+}$ efflux from EGTA-washed calmodulin-depleted SR vesicles in the presence or absence of hypoxanthine (*HX*, 20 μM)-xanthine oxidase (*XO*, 0.1 unit/ml). The sequence of addition of hypoxanthine and xanthine oxidase was similar to that described in B. SOD (10 μg/ml) or calmodulin (2.0 μM) was added before free radical exposure. Data represent the average of measurements carried out with six cardiac SR preparations. *Error bars*, standard errors.

(Fig. 3C). This set of observations suggests that the treatment involved in the calmodulin-extraction procedure has only a negligible effect and the RyRC is quite sensitive to $O_2^{\cdot-}$ only in the presence of calmodulin.

Finally, we examined the direct influence of $O_2^{\cdot-}$ responsible for calmodulin interaction on single-channel gating behavior of RyRC using the planar lipid bilayer/heavy SR vesicle fusion technique while confirmation of Ca_i and Ca^{2+}-ATPase activity was followed simultaneously in parallel reactions that were identical in all respects (Fig. 4 and Table 1). All single-channel recordings of Ca^{2+}-release channel were made with the *cis* chamber containing 0.1 unit/ml xanthine oxidase. Hypoxanthine-xanthine oxidase reaction produced an increase in P_o from 0.028 to 0.462 and significantly decreased Ca_i with no effect on Ca^{2+}-ATPase activity; the observed effect was blunted effectively by SOD (Fig. 4A and Table 1). Fig. 4D demonstrates the closure of the channels by 300 μM ryanodine even in the presence of hypoxanthine-xanthine oxidase, indicating that $O_2^{\cdot-}$ is activating the RyRC. The results for the effect of ryanodine on Ca_i are shown in Table 1. In the experiments with 300 μM ryanodine preincubation for 10 min to block the RyRC, Ca_i of native SR was increased significantly compared with the value without ryanodine preincubation; the observed effect of hypoxanthine-xanthine oxidase in the experiments without ryanodine preincubation was nearly completely abolished by ryanodine. When the Ca^{2+}-release channel from EGTA-washed calmodulin-depleted SR vesicles has been incorporated into the lipid bilayers, the P_o was 0.509 in the presence of xanthine oxidase alone *cis*; in the presence of hypoxanthine-xanthine oxidase reaction, the effect of $O_2^{\cdot-}$ was resolved poorly (Fig. 4B). The addition of exogenous calmodulin (2 μM) to the system *cis* reproduced the effect of $O_2^{\cdot-}$ (Fig. 4C); there was SOD-inhibitable reduction in Ca_i (Table 1). The Ca^{2+}-ATPase activity was not changed in any of the experimental conditions tested (Table 1); therefore, it is likely that all the effects exerted by $O_2^{\cdot-}$ generated from hypoxanthine-xanthine oxidase reaction are due to its direct effects rather than to altered catalytic activity of the Ca^{2+} pump.

The effects of exogenous calmodulin and ryanodine on Ca_i of native- and EGTA-washed calmodulin-depleted SR vesicles were investigated further (Table 1). We find that exogenously added calmodulin increases Ca_i of native SR and significantly protects against the decreased Ca_i induced by hypoxanthine-xanthine oxidase reaction. Furthermore, Ca_i of native SR treated with 10 μM instead of 2.0 μM calmodulin was nearly identical to the value of control (2.0 μM calmodulin plus xanthine oxidase alone) when the SR vesicles were exposed to hypoxanthine-xanthine oxidase reaction. Similarly, 10 μM calmodulin protected Ca_i of EGTA-washed SR vesicles from the reproduced effect of hypoxanthine-xanthine oxidase observed in the presence of 0.2 μM calmodulin. Ryanodine was able to mimic the effects of a high concentration (10 μM) of calmodulin (in native and EGTA SR) and SOD (in EGTA SR) on Ca_i. These findings strongly suggest that the calmodulin-dependent inhibitory mechanism of RyRC is the site at which $O_2^{\cdot-}$ radicals exert the observed effect.

Discussion

Postischemic reperfusion may lead to a progressive normalization of intracellular Ca^{2+} homeostasis, which is asso-

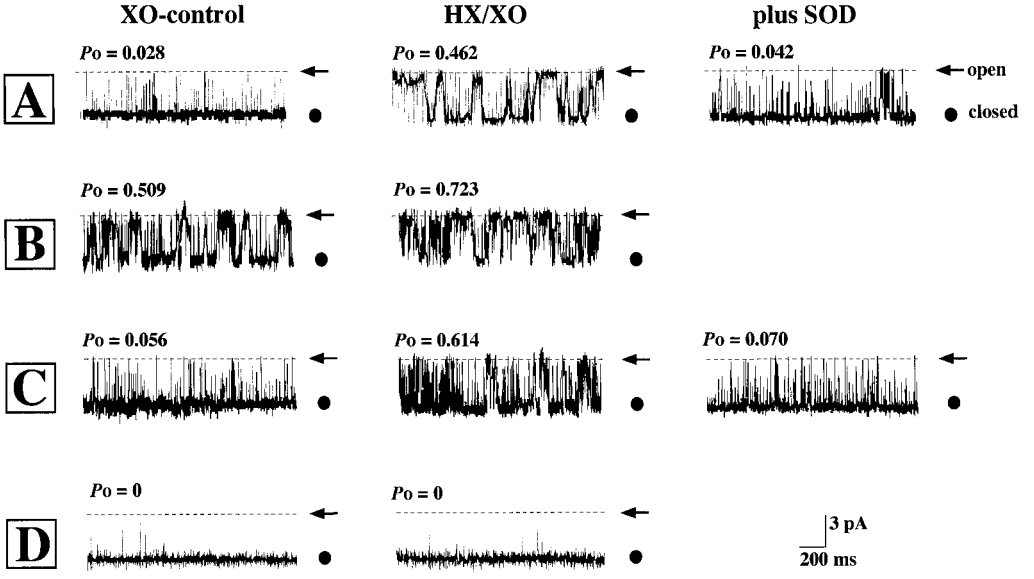

Fig. 4. Effect of oxygen-derived free radicals generated by hypoxanthine-xanthine oxidase reaction responsible for calmodulin on native and EGTA SR Ca^{2+}-release channels and the effect of ryanodine. *Dotted lines*, open-channel level. *Current traces*, recorded at a holding potential of 0 mV with respect to the *trans* side (ground) of the bilayer. A, Single-channel current fluctuations at 2.0 min after the addition of hypoxanthine (*HX*, 20 μM) to the *cis* chamber containing xanthine oxidase (*XO*, 0.1 unit/ml) and effect of SOD (10 μg/ml) in native SR. SOD was added to the *cis* chamber before the addition of hypoxanthine. B, Single-channel current fluctuations at 2.0 min after the addition of hypoxanthine to the *cis* chamber containing xanthine oxidase in EGTA-washed calmodulin-depleted SR. Reaction conditions were similar to those described in A except that EGTA-washed SR vesicles were used. C, Effects of exogenous calmodulin on single-channel current fluctuations in EGTA-washed calmodulin-depleted SR. Experimental conditions were similar to those described in A except that EGTA-washed SR vesicles were used in the presence of exogenous calmodulin. Calmodulin (2.0 μM) was added to the *cis* chamber before the addition of hypoxanthine. Exogenous calmodulin reverses the effect of the depletion of endogenous calmodulin; under this condition, the effect elicited by hypoxanthine/xanthine oxidase [observed in native SR (A)] is reproduced. D, Closure of the channels by 300 μM ryanodine (for 10 min of preincubation before the addition of hypoxanthine) in native SR. Experimental conditions were similar to those described in A except that ryanodine was added to the *cis* chamber.

TABLE 1

Effect of oxygen-derived free radicals generated by hypoxanthine-xanthine oxidase reaction responsible for calmodulin on Ca_i and Ca^{2+}-ATPase activity for SR

For determination of Ca_i or Ca^{2+}-ATPase activity, time sequence addition was designed to ensure exposure of the native and EGTA-washed SR vesicles to the oxygen-derived free radical generating system [20 μM hypoxanthine (HX) plus 0.1 unit/ml xanthine oxidase (XO)] for 2.0 min before initiation of the reaction. SOD (10 μg/ml) or calmodulin (CaM) (2.0 μM or 10 μM*) was added before the free radical exposure. The SR vesicles were preincubated with or without ryanodine (300 μM) for 10 min; then, the reaction was initiated. Values are mean ± standard error (five to seven experiments). *p*-values are the result of analysis of variance and Dunnett's multiple-range test.

Experimental addition	Native SR		EGTA SR	
	Ca_i	Ca^{2+}-ATPase activity	Ca_i	Ca^{2+}-ATPase activity
	nmol/mg	*nmol P_i/mg/min*	*nmol/mg*	*nmol P_i/mg/min*
XO	32.8 ± 4.6	86.6 ± 5.6	14.3 ± 0.5[e]	92.4 ± 6.8
HX/XO	16.1 ± 1.2[a]	70.9 ± 9.6	13.8 ± 1.6	82.6 ± 8.3
SOD plus HX/XO	26.4 ± 1.6[b]	72.8 ± 5.3		
CaM plus XO	48.3 ± 4.8[a]	91.8 ± 10.1	29.4 ± 2.4[a]	74.8 ± 11.8
CaM plus HX/XO	30.4 ± 2.7[bc]	80.7 ± 7.9	12.4 ± 1.9[c]	81.9 ± 9.9
CaM plus HX/XO*	42.9 ± 3.8[d]	82.6 ± 4.8	23.8 ± 1.6[d]	94.6 ± 16.5
CaM plus SOD plus HX/XO			25.0 ± 2.8[d]	76.3 ± 6.6
Ryanodine plus XO	52.6 ± 5.9[a]	88.4 ± 4.6	33.2 ± 2.5[a]	82.9 ± 7.4
Ryanodine plus HX/XO	46.2 ± 3.8[b]	84.9 ± 3.4	28.8 ± 3.8	78.8 ± 10.2
Ryanodine plus CaM plus HX/XO			31.6 ± 3.1[d]	83.2 ± 5.9

[a] $p < 0.01$ versus corresponding XO alone.
[b] $p < 0.01$ versus corresponding HX/XO.
[c] $p < 0.01$ versus corresponding XO alone in the presence of 2.0 μM CaM.
[d] $p < 0.01$ versus corresponding HX/XO in the presence of 2.0 μM CaM.
[e] $p < 0.01$ versus corresponding XO alone in native SR.

ciated with functional recovery, or an exacerbation of Ca^{2+} overload, which is associated with the development of irreversible cellular injury. Due to the importance of Ca^{2+} in the pathophysiology of ischemic injury, the effects of ischemia and reperfusion on the systems involved in Ca^{2+} homeostasis have raised special interest.

Myocardial ischemia results in a series of metabolic events that include the autoxidation 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. All of these reactions favor the univalent reduction of molecular oxygen to reactive oxygen species (Tompson and Hess, 1986; Okabe *et al.*, 1987). These metabolites of molecular oxygen are highly toxic and capable of extensive tissue damage. With reperfusion and reintroduction of molecular oxygen into this previously ischemic vascular bed, the myocardium is "primed" for production of a "burst" of reactive oxygen intermediates and further extensive tissue damage (Hess and Manson, 1984). The sources and sinks of the reactive oxygen species produced as a result of the ischemia/reperfusion process have not been identified adequately. However, workers at our laboratory have provided evidence that during the course of short term, normothermic ischemia, the SR of cardiac muscle is one of the first major intracellular organelles injured by the ischemia/reperfusion (Hess *et al.*, 1981; Krause and Hess, 1984). The cytosolic Ca^{2+} overload produced during ischemia/reperfusion in large part represents a redistribution of intracellular Ca^{2+}. On the basis of these findings, it has been speculated that reactive oxygen species-induced increase of Ca^{2+} release from SR through RyRC might be involved in the Ca^{2+} overload observed after reperfusion of the ischemic heart.

The results of the current study demonstrate that $O_2^{\cdot-}$ is involved in a mechanism that stimulates Ca^{2+} release from SR through RyRC and that the effect of $O_2^{\cdot-}$ is dependent on the presence of calmodulin. This postulate is inferred from significant observations: (1) hypoxanthine-xanthine oxidase reaction stimulates ryanodine-inhibitable $^{45}Ca^{2+}$ efflux from SR (at a concentration that blocks the RyRC) (Fig. 1A), (2) the observed effect of hypoxanthine-xanthine oxidase also was inhibited by SOD (Fig. 1A) and exogenously added calmodulin (Fig. 1B), (3) oxygen-derived free radical species generated from hypoxanthine-xanthine oxidase reaction is confirmed to be $O_2^{\cdot-}$ by ESR study (Fig. 2) and the increase in $^{45}Ca^{2+}$ efflux is $O_2^{\cdot-}$ concentration dependent (Figs. 1A and 2), (4) the increased $^{45}Ca^{2+}$ efflux induced by hypoxanthine-xanthine oxidase reaction is observed only in the presence of calmodulin (Fig. 3, B and C), and (5) hypoxanthine-xanthine oxidase reduces the calmodulin content of SR vesicles (which is SOD inhibitable) (Fig. 3A).

The ubiquitous Ca^{2+}-binding protein calmodulin has been implicated as a regulator of Ca^{2+} release. Seiler *et al.* (1984) first observed that calmodulin was associated with high-molecular-weight proteins in the SR, later identified as the RyRC (Lai *et al.*, 1988), and is shown to cause an inhibition of Ca^{2+} release from cardiac SR vesicles in the absence of hydrolyzable nucleotide substrate (Meissner and Henderson, 1987). The effects of calmodulin are reversed by mastoparan, a calmodulin-binding peptide (Smith *et al.*, 1989). These observations suggest that calmodulin is interacting directly with RyRC and not through the regulation of protein phosphorylation; therefore, if $O_2^{\cdot-}$ anion radicals attack the calmodulin-dependent inhibitory mechanism (which is not involved in calmodulin-dependent kinase) of RyRC, the channel should be activated.

In the current experiments, we also evaluated the effect of $O_2^{\cdot-}$ (generated from hypoxanthine-xanthine oxidase reaction) on Ca_i of SR. Data (Table 1) showing that $O_2^{\cdot-}$ can decrease Ca_i only under the conditions of calmodulin stimulation without changing Ca^{2+}-ATPase activity and that further calmodulin stimulation inhibits the demonstrated ryanodine-sensitive effect of $O_2^{\cdot-}$ are entirely compatible with a hypothesis that $O_2^{\cdot-}$-induced decrease in the Ca^{2+} accumulation results in a "releasing out" of transported Ca^{2+} through RyRC before binding within the SR vesicles; moreover, the effect of $O_2^{\cdot-}$ is dependent on the presence of calmodulin. The observed preventive effect of ryanodine on $O_2^{\cdot-}$-induced

change in Ca_i thus is due to the ability of this agent to close RyRC. These suggestions are strengthened by the fact that hypoxanthine-xanthine oxidase reaction can increase Ca^{2+}-release channel P_o, and the modifications afforded by SOD, endogenous and exogenous calmodulin, and ryanodine (Fig. 4) were similar to those on $^{45}Ca^{2+}$ efflux and Ca_i (Ca_i is decreased by increasing P_o), indicating the results of similar mechanisms. Calmodulin seems to be an inhibitory species of RyRC and is an important accessory protein in modulation of the effect of $O_2^{\cdot-}$.

In summary, the results of the current study clearly show that $O_2^{\cdot-}$ can lead to Ca^{2+}-release channel activation by displacement of calmodulin from heavy SR vesicles and suggest that a plausible site of attack by $O_2^{\cdot-}$ may be calmodulin-dependent inhibitory mechanism or mechanisms of RyRC. In addition to the large number of agents that impart pharmacological effects at SR Ca^{2+}-release channels, a variety of chemical oxidants and, in particular, SH-oxidizing reagents have been shown to activate Ca^{2+}-release. The SH-oxidizing reagents that have been tested compete with ryanodine binding to SR vesicles, which indicates these agents act at or near the ryanodine binding site (Zaidi *et al.*, 1989). The activation or opening of Ca^{2+}-release channels by SH-oxidizing reagents could be reversed by SH-reducing agents (e.g., cysteine, dithiothreitol) (Hilkert *et al.*, 1992). However, cysteine and dithiothreitol had no effect on the observed effects elicited by hypoxanthine-xanthine oxidase reaction on $^{45}Ca^{2+}$ efflux and single-channel gating behavior (data not shown). Therefore, SH oxidation/reduction, if any, seems unlikely to have induced the activation of Ca^{2+} release in our system. Taken together, it is highly likely that $O_2^{\cdot-}$ acts at a specific site on the RyRC, possibly calmodulin-modulated reaction step or steps, although no details were provided concerning this mechanism in the current experiments. The increased opening of the RyRC induced by $O_2^{\cdot-}$ would result in the elevation of the cytosolic concentration of Ca^{2+} and contribute to *in vivo* reperfusion injury.

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Send reprint requests to: Dr. Eiichiro Okabe, D.D.S., Ph.D., Professor & Chairman, Department of Pharmacology and ESR Laboratory, Kanagawa Dental College, 82 Inaoka-Cho, Yokosuka, Kanagawa 238, Japan. E-mail: okabe@kdcnet.ac.jp