ANTIOXIDANTS & REDOX SIGNALING
Volume 1, Number 1, 1999
Mary Ann Liebert, Inc.

Novel Mechanisms Involved in Superoxide Anion Radical-Triggered Ca^{2+} Release from Cardiac Sarcoplasmic Reticulum Linked to Cyclic ADP-Ribose Stimulation

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ABSTRACT

It has been suggested that cyclic adenosine 5′-diphosphoribose (cADPR) directly activates the cardiac isoform of the ryanodine receptor (RyR)/Ca^{2+} release channel. We have previously shown that selective activation of RyR/Ca^{2+} release channel by superoxide anion radical ($O_2^{\cdot -}$) is dependent of the presence of calmodulin and identified calmodulin as a functional mediator of $O_2^{\cdot -}$-triggered Ca^{2+} release through the RyR/Ca^{2+} release channel of cardiac sarcoplasmic reticulum (SR). We now demonstrate that although the effect of $O_2^{\cdot -}$ on Ca^{2+} efflux from RyR/Ca^{2+} release channel at higher concentrations ($>5\ \mu M$) is due to its ability to produce a loss in function of calmodulin thereby decreasing calmodulin inhibition, $O_2^{\cdot -}$ radicals at lower concentrations ($<5\ \mu M$) may be able to stimulate Ca^{2+} release only in the presence of calmodulin from the SR via increased cADPR synthesis; it is also shown that cADPR is a modulator that can activate the Ca^{2+}-release mechanism when it is in a sensitized state by the presence of calmodulin, possibly, at physiological concentration. In addition, the SR vesicles immediately upon addition of cADPR, but not NAD^+, did exhibit Ca^{2+} efflux stimulation. When heart homogenate was incubated with $O_2^{\cdot -}$, conversion of NAD^+ into cADPR was stimulated; the reduction of homogenate Ca^{2+} uptake (by increasing Ca^{2+} efflux through RyR/Ca^{2+} release channel) occurred. Thus $O_2^{\cdot -}$ radical is responsible for cADPR formation from NAD^+ in the cellular environment outside of the SR of heart muscle. The results presented here provide the first evidence of a messenger role for $O_2^{\cdot -}$ radical in cADPR-mediated Ca^{2+} mobilization in myocardium. Antiox. Redox Signal. 1, 55–69.

INTRODUCTION

THE RISE IN CYTOPLASMIC CA^{2+}, identified first as the trigger of muscle contraction (Heilbrunn and Wiercinski, 1947), is now recognized as a universal signal that controls numerous processes in all eukaryotic cells. The similar recognition that Ca^{2+} is stored in and released from both the sarcoplasmic reticulum (SR) of muscle (Ebashi and Endo, 1968) and the endoplasmic reticulum (ER) of nonmuscle cells (Somlyo, 1984; Pozzan *et al.*, 1994) led to major questions still being asked about the identity of the molecular mechanisms of Ca^{2+} release from the SR/ER (Somlyo and Somlyo, 1994; Berridge, 1997). Release of Ca^{2+} from SR/ER

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can be triggered by the binding of the second messenger inositol 1,4,5-trisphosphate (IP_3) to the IP_3 receptor/Ca^{2+} release channel (for review, see Berridge, 1993). It also can be mediated by the ryanodine receptor (RyR)/Ca^{2+} release channel in response to a surface membrane action potential and/or a change in the concentration of a second messenger by a mechanism referred to in muscle as excitation-contraction coupling.

Depending upon the experimental concentration of ryanodine used, it was found to either stimulate or inhibit Ca^{2+} efflux from isolated cardiac (Okabe *et al.*, 1991) and skeletal (Meissner, 1986) SR membrane vesicles. These effects are consistent with single channel experiments where micromolar concentrations of ryanodine "lock" the channel into a reduced-conductance, high-open probability (*Po*) state, and millimolar concentrations lead to irreversible channel closure (Lai *et al.*, 1989; Kawakami and Okabe, 1998).

Recent study (Mészáros *et al.*, 1993) suggests that cyclic adenosine 5'-diphosphoribose (cADPR) can release intracellular Ca^{2+} in a variety of mammalian cells including cardiac myocytes. An isoform of ADP-ribosyl cyclase, the enzyme responsible for cADPR synthesis, has been purified from the ovotestis of the marine mollusc, *Aplysia californica* (Lee and Aarhus, 1991), and subsequently cloned (Glick *et al.*, 1991). A homologous enzyme with dual ADP-ribosyl cyclase and cADPR hydrolase activities is widely expressed in the plasma membrane of mammalian cells (Rusinko and Lee, 1989) and is similar or identical to the human leukocyte antigen CD38 (States *et al.*, 1992). CD38 has been cloned from human insulinoma (Takasawa *et al.*, 1993) and rat pancreatic islets (Koguma *et al.*, 1994); its expression in CDS1 cells leads to cADPR-sensitive Ca^{2+} release (Summerhill *et al.*, 1993).

At present, there is much interest in the possibility that cADPR may be an endogenous regulator of one or more isoforms of the RyR/Ca^{2+} release channel. However, cADPR may not bind directly to RyR but to accessory proteins, probably calmodulin, that may couple cADPR to channel activation (Lee *et al.*, 1994). Consistent with this notion, 8-amino-cADPR, which acts as a selective cADPR antagonist, does not block caffeine- or ryanodine-induced Ca^{2+} release (Walseth and Lee, 1993). Previously, we have shown that selective activation of RyR/Ca^{2+} release channel by superoxide anion radical ($O_2^{\cdot -}$) is dependent of the presence of calmodulin and identified calmodulin as a functional mediator of $O_2^{\cdot -}$-triggered Ca^{2+} release through the RyR/Ca^{2+} release channel (Kawakami and Okabe, 1998). In the present study, we have explored the possibility that cADPR is synthesized in response to exposure of cardiac cells to low concentrations of $O_2^{\cdot -}$ and acts as a modulator of Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. $O_2^{\cdot -}$ anion radicals at relatively low concentrations (<5 μM) can stimulate Ca^{2+} release from heavy fraction of cardiac SR vesicles due to increased cADPR synthesis, and present results point to the importance of calmodulin in modulating the effect of $O_2^{\cdot -}$. The results also provide the first evidence of a messenger role for $O_2^{\cdot -}$ in cADPR-mediated Ca^{2+} mobilization in myocardium.

MATERIALS AND METHODS

Heavy SR vesicles preparation and $^{45}Ca^{2+}$ efflux measurements

Canine cardiac heavy SR was isolated by discontinuous sucrose gradient centrifugation by a modified method as described (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 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.2, and protease inhibitors (1 μg/ml of pepstatine and leupeptin, 1 m*M* iodoacetamide, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.1 m*M* 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 centrifuging, the supernatant was discarded. The pellet was fractioned overnight on a discontinuous sucrose gradient (10%, 31%, 40%, and 50%) in a solution of 400 m*M* KCl, 20 m*M* MOPS pH 6.8, and 100 μM of $MgCl_2$, $CaCl_2$, and ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in a Beck-

man SW27 rotor at 25,000 rpm. The final pellets were resuspended in 0.3 *M* sucrose, 20 m*M* MOPS pH 6.8, also containing the above mixture of protease inhibitors. Protein concentration was determined by the method of Lowry *et al.* (1951). The resulting heavy SR vesicles were preincubated overnight on ice in 2 m*M* $^{45}CaCl_2$ (New England Nuclear, Boston, MA), 150 m*M* KCl, and 20 m*M* MOPS pH 6.8. They were then diluted 20-fold into a Ca^{2+}-releasing medium containing 0.5 m*M* disodium ATP, 150 m*M* KCl, 20 m*M* MOPS pH 6.8, and 1 m*M* EGTA/Ca buffer to adjust the pCa to 5. $^{45}Ca^{2+}$ efflux was quenched with ice-cold quench solution containing 1 m*M* $LaCl_3$, 10 m*M* $MgCl_2$, 150 m*M* KCl, and 20 m*M* 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 m*M* 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (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 m*M* HEPES, and 4 m*M* EGTA pH 7.4. The hypertonic suspension was centrifuged for 30 min at 150,000 × *g* and the pellet was washed twice with 20 m*M* HEPES pH 7.4. The final pellet was resuspended in 0.3 *M* sucrose and 20 m*M* 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 μ*M* calmodulin (from bovine brain, Fluka AG, Buchs, Switzerland) and then subjected to preparative sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The washing and the boiled extracts of SR stimulated the incorporation of ^{32}P from 10 μ*M* [γ-^{32}P]ATP into EGTA-washed SR proteins in the presence of 0.5 m*M* $CaCl_2$. 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 m*M* EGTA and by several hypotonic washes in the absence of the chelator, resulted in the depletion of calmodulin (Carafoli *et al.*, 1980).

Whole-heart homogenate $^{45}Ca^{2+}$ uptake

The minced canine cardiac left ventricle was homogenized in 5 volumes of ice-cold 10 m*M* imidazole buffer pH 7.0 with a Polytron homogenizer (Kinematica, Luzern, Switzerland) with PT 20 probe, setting 6, for two 10-sec periods. Oxalate-supported $^{45}Ca^{2+}$ uptake rates of the SR in the whole-heart homogenate were modified from the previously described methods (Okabe *et al.*, 1991). The uptake conditions were maintained at 37°C and included 34.7 m*M* KCl, 6 m*M* imidazole buffer pH 7.0, 3.3 m*M* potassium oxalate, 5 m*M* disodium ATP, 5 m*M* $MgCl_2$, 0.18 m*M* $CaCl_2$ with 0.05 μCi of ^{45}Ca/ml and saponin (100 μg/ml in final concentration, Sigma). The reaction was stopped by filtration through a set of prefilters (Millipore, Bedford, MA) and 0.45-μm Millipore filters, and the filtrate was counted in a liquid scintillation spectrometer. The rate of uptake was calculated from the linear regression of $^{45}Ca^{2+}$ uptake determined at 0.5, 1.0, 1.5, and 2.0 min after initiation of uptake; the specific activity of the filtrate $^{45}Ca^{2+}$ is unchanged throughout this assay (Feher *et al.*, 1988). In the presence of hypoxanthine (80 μ*M*, Sigma Chemical, St. Louis, MO)-xanthine oxidase (0.1 U/ml; activity, 35.8 μ*M*/min; Boehringer-Mannheim Biochemical, Indianapolis, IN), the $^{45}Ca^{2+}$ uptake (in nanomoles per milligram) was linear with time and the $^{45}Ca^{2+}$ uptake rate (in nanomoles per milligram per minute) was independent of the volume of the homogenate taken for the assay. The uptake assays were completed within 20 min after homogenization.

Electron spin resonance analysis

The spin-trapping studies were performed with the desired mixture containing 5,5-di-

methyl-1-pyrroline-*N*-oxide (DMPO; Labotec, Tokyo, Japan; 99–100% pure, gas chromatographic assay by Dojindo Laboratories, Kumamoto, Japan). Electron spin resonance (ESR) detection of the spin adduct was carried out at room temperature with a JEOL (model JES-RE3X) X-band spectrometer connected with the JEOL computer systems 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 ± 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 the MnO. An absolute concentration of DMPO—OOH was determined by a double integration of the ESR spectrum, in which 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 protein concentration of 1 mg/ml were incubated for 10 min at 22°C or heated for 10 min at 95°C 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 hypoxanthine. Next, vesicles underwent sedimentation for 30 min at 100,000 × g in a Beckman airfuge. Xanthine oxidase was added 2.5 min before the sedimentation and superoxide dismutase (SOD, 10 μg/ml; 3,000 units/ml Sigma Chemical) was added 30 sec before the addition of xanthine oxidase. The supernatants of samples not heated at 95°C were incubated for 10 min at 95°C. 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).

Intravesicular free Ca^{2+} (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°C 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 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 net release in Ca^{2+} by Millipore filtration. Quenching of the pump-mediated fluxes was produced by 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 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 by the method of Feher and Briggs (1980).

Synthesis of 8-amino-cADPR

8-Amino-cADPR was synthesized from commercially available precursor (8-azido-AMP, Sigma) essentially as described by Walseth and Lee (1993), using a two-step procedure consisting of the synthesis of 8-amino-NAD^+ by chemically coupling 8-amino-AMP (prepared from 8-azido-AMP by treatment with dithiothreitol) to β-nicotinamide mononucleotide (β-NMN) followed by enzymatic conversion to 8-amino-cADPR. The chemical coupling of 8-amino-AMP to β-NNM to form 8-amino-NAD^+ was performed by carbodiimide coupling. 8-Amino-AMP (0.1 μmol), β-NMN (1 μmol) and $MgCl_2$ (2 μmol) were combined in a microfuge tube and evaporated to dryness using a SpeedVac concentrator. The coupling reaction was initiated by adding 20 μl of 1.5 *M* HEPES-NaOH pH 6.8 and 20 μl of 1.5 *M* 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide-HCI (EDC) and incubated at 37°C for 12–20 hr. The yield of 8-amino-NAD^+ was between 45 and 53%. The reaction products were diluted to 1 ml with water and injected onto an AG MP-1 column (0.6 × 15 cm) which was eluted at a flow rate of 1 ml/min using a trifluoroacetic acid gradient from 1.5 to 150 m*M* over 30 min. The 8-amino-NAD^+ peak was collected and evaporated to dryness on a SpeedVac concentrator. 8-Amino-NAD^+ was converted to 8-amino-cADPR using ADP-ribosyl cyclase purified from *Aplysia* ovotestis. The 8-amino-NAD^+ was reconstituted with 1 ml of 25 m*M* HEPES-NaOH pH 6.8 and incubated for 2–4 hr at room temperature with ADP-ribosyl cyclase. The resulting 8-amino-cADPR was purified by AG MP-1 chromatography. The purified 8-amino-cADPR was evaporated to dryness on a SpeedVac concentrator and stored at −80°C. The molar extinction coefficient of 8-amino-cADPR used was 16,000 $M^{-1}\cdot cm^{-1}$ (274 nm) (Walseth and Lee, 1993). ADP-ribosyl cyclase was purified from ovotestis of *Aplysia kurodai*, a species common around the Japanese coast, by the method described previously (Lee and Aarhus, 1991) with a slight modification. The cytoplasmic fraction obtained from the *Aplysia* ovotestis was applied to sequential column chromatography with CM-Toyopearl 650 M (Tosoh), SP-Toyopearl 650S (Tosoh), and Mono S HR 5/5 (Pharmacia-LKB). Usually, 4–6 mg of the homogeneous enzyme could be purified from 40–50 grams of ovotestis with a recovery of 12%. The purified cyclase dissolved in 20 m*M* HEPES-NaOH pH 7.4, 1 m*M* EDTA, and 250 m*M* NaCl at a protein concentration of more than 250 μg/ml was quite stable and could be stored for several months at 4°C without loss of enzyme activity. The protein concentration was determined by the DC protein assay (Bio Rad) using bovine serum albumin as standard.

NAD^+ conversion reactions

The minced canine cardiac left ventricle was homogenized in 5 volumes of ice-cold 10 m*M* Tris-maleate pH 6.8, also containing 0.1 m*M* phenylmethylsulfonyl fluoride and 0.1 m*M* benzamidine, with a Polytron homogenizer with PT 20 probe, setting 6, for two 10-sec periods. The supernatant obtained after centrifugation (2,000 × *g*, 5 min) was dialyzed overnight against 100 volumes of 0.3 *M* sucrose containing 10 m*M* Tris-maleate pH 6.8. NAD^+ conversion reactions were initiated by adding 100 μM [*adenine*-^{14}C] NAD^+ (New England Nuclear) or cold NAD^+ to the homogenate di-

luted to 0.8–1.0 mg/ml with 150 m*M* KCl, 5 m*M* $MgCl_2$, and 20 m*M* MOPS pH 7.2 at 37°C. The reactions were quenched with 0.5 *M* ice-cold perchloric acid, followed by centrifugation (Tomy Seiko LC06-SP, Tokyo, Japan; 2,000 rpm, 5 min); supernatants, whose pH was readjusted to 6.5 with 0.1 *M* NaOH, were analyzed by high-performance liquid chromatography (HPLC). NAD^+ and cADPR were quantified by either their radioactivity or 254-nm ultraviolet absorption and identified by HPLC analysis of appropriate cold standards run under identical conditions. HPLC was performed using AG MP-1 (Bio-Rad) resin and a nonlinear gradient of trifluoroacetic acid (Lee *et al.*, 1989). Deproteinized samples (50 μl) were injected and eluted with 0.25 *M* NH_4-formate pH 4.0. Radioactivity was determined from 150-μl samples collected after ultraviolet detection.

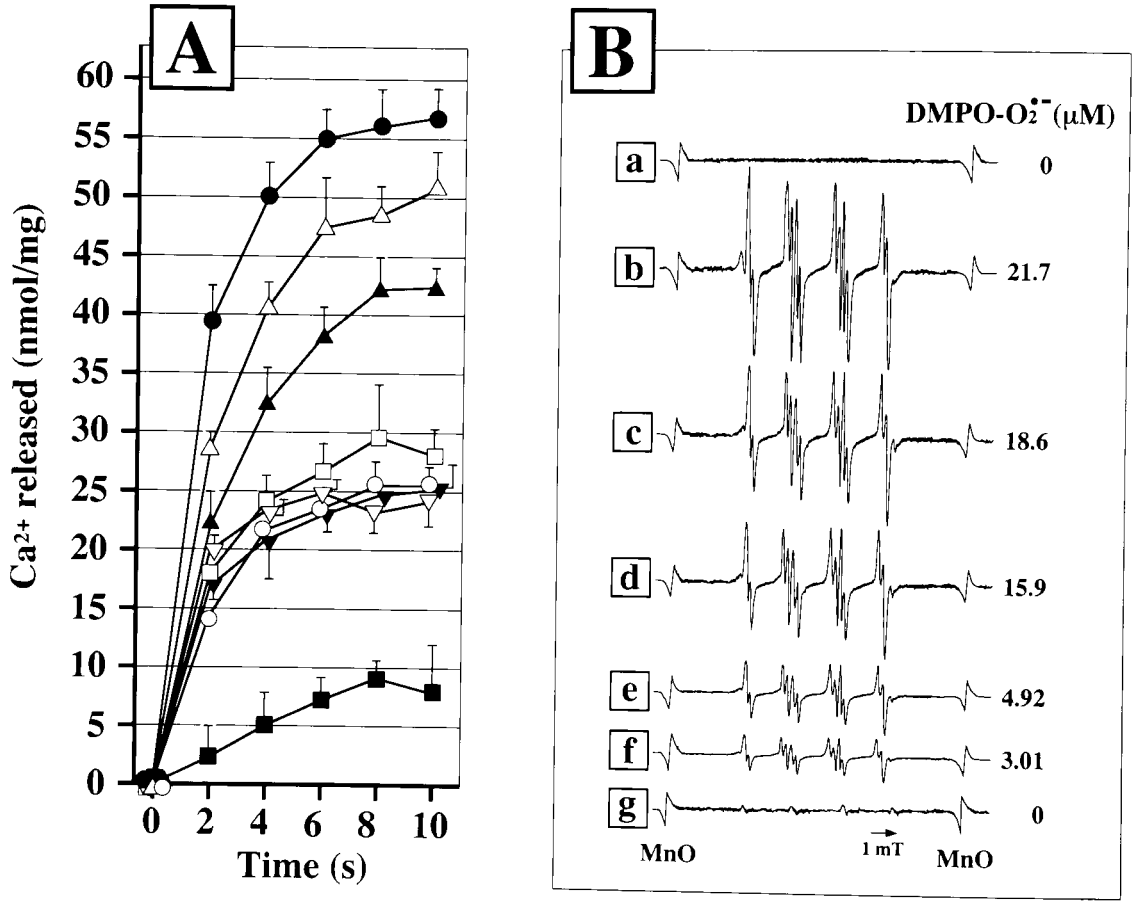

FIG. 1. Effect of $O_2^{\cdot-}$ radicals generated from hypoxanthine (2.5–50 μ*M*)-xanthine oxidase (0.2 U/ml) reaction on $^{45}Ca^{2+}$ efflux from isolated heavy SR vesicles. A. Inhibitions by SOD (□, 10 μg/ml in the presence of 50 μ*M* hypoxanthine and 0.2 U/ml xanthine oxidase) and ryanodine (■, 300 μ*M* in the presence of 50 μ*M* hypoxanthine and 0.2 U/ml xanthine oxidase) of the effect of hypoxanthine (○, 0 μ*M*; ▼, 2.5 μ*M*; ▽, 5 μ*M*; ▲, 10 μ*M*; △, 30 μ*M*; and ●, 50 μ*M*)-xanthine oxidase reaction. Time sequence additions were designed to ensure exposure of the SR to the oxygen-derived free radical-generating system for 2.5 min before initiation of the reaction. SOD was added 30 sec before the free radical exposure. The SR vesicles were preincubated with or without ryanodine for 10 min; then the reaction was initiated. Data represent the average of measurements carried out with five independent cardiac SR preparations; five SR preparations isolated from different cardiac muscles were studied in parallel. Error bars are standard errors. **B.** ESR spectra of spin adducts produced by the hypoxanthine (a, 0 μ*M*; b, 50 μ*M*; c, 30 μ*M*; d, 10 μ*M*; e, 5 μ*M*; f, 2.5 μ*M*; in the presence of 88 m*M* DMPO)-xanthine oxidase (0.2 U/ml) reaction, and effect of SOD (g, 10 μg/ml in the presence of 50 μ*M* hypoxanthine and 0.2 U/ml xanthine oxidase). 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. SOD was added 30 sec before the addition of hypoxanthine/xanthine oxidase to the reaction medium containing 88 m*M* DMPO. At 2.5 min after the addition of hypoxanthine/xanthine oxidase, the 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.

RESULTS

Increase in $^{45}Ca^{2+}$ efflux induced by $O_2^{\cdot -}$

In preliminary experiments, 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; Wako Chemicals, Osaka, Japan; HPLC assay, 98%). $^{45}Ca^{2+}$-loaded SR vesicles were diluted into Ca^{2+}-releasing medium containing 10 μM free Ca^{2+} to induced Ca^{2+}-induced Ca^{2+} release. As seen in Fig. 1A, in the presence of fixed concentration of xanthine oxidase (0.2 U/ml), hypoxanthine enhanced the $^{45}Ca^{2+}$ efflux from SR in a concentration-dependent fashion; the $^{45}Ca^{2+}$ efflux from the vesicles produced by the highest concentration of hypoxanthine (50 μM) used was drastically inhibited by SOD and 300 μM ryanodine, which at this high concentration of ryanodine blocks the RyR/Ca^{2+} release channel (Kawakami and Okabe, 1998), indicating that the effect afforded by hypoxanthine-xanthine oxidase on $^{45}Ca^{2+}$ efflux may be due to $O_2^{\cdot -}$ generated and stems from its interaction with the RyR/Ca^{2+} release channels.

The participation of $O_2^{\cdot -}$ in the observed effect elicited by hypoxanthine-xanthine oxidase reaction was further verified by ESR spectroscopy with DMPO as the spin trap under the same reaction conditions as those of Fig. 1A (except that SR vesicles and $^{45}Ca^{2+}$ were omitted). Hyperfine coupling constants (hfcc's) of the spin adducts observed (Fig. 1B) were analyzed as follows: one nitrogen, a_N = 1.41 mT, one hydrogen in β-position, $a_{H\beta}$ = 1.41 mT, and one hydrogen in γ-position, $a_{H\gamma}$ = 0.13 mT. The component of the spectrum was assigned to DMPO-$O_2^{\cdot -}$. The concentration of DMPO-$O_2^{\cdot -}$ adduct was dependent on the concentration of hypoxanthine (in the presence of 0.2 U/ml xanthine oxidase), suggesting that the increase in $^{45}Ca^{2+}$ efflux was $O_2^{\cdot -}$ concentration dependent (Fig. 1). cADPR (1.0 μM), 8-amino-cADPR (1.0 μM), NAD^+ (1.0 μM), and calmodulin (2 μM) had no effect on ESR spectra of spin adducts observed using DMPO on hypoxanthine (2.5–50 μM)-xanthine oxidase (0.2 U/ml) reaction (data not shown).

The modulation of the activity of the RyR/Ca^{2+} release channel by calmodulin (Meissner and Henderson, 1987) has prompted the hypothesis that $O_2^{\cdot -}$ anion radical produces a loss in function of calmodulin in heavy SR vesicle, thereby increasing the release of Ca^{2+} through RyR/Ca^{2+} release channel. Indeed in our system, $O_2^{\cdot -}$ (generated from hypoxanthine-xanthine oxidase reaction) reduced the calmodulin content of SR vesicles, in a concentration-dependent manner; the loss afforded by hypoxanthine-xanthine oxidase was blunted by SOD (Fig. 2).

Calmodulin and cADPR interaction on $^{45}Ca^{2+}$ efflux

As can be seen in Fig. 3A, cADPR (1.0 μM; >99% purity, Amersham International) caused an increase in $^{45}Ca^{2+}$ efflux; the $^{45}Ca^{2+}$ efflux activated by cADPR was virtually abolished by 8-amino-cADPR (1.0 μM), a potent antagonist of cADPR. Calmodulin (2 μM) also diminished the effect of cADPR. A calmodulin concentration of 2 μM was used because this concentra-

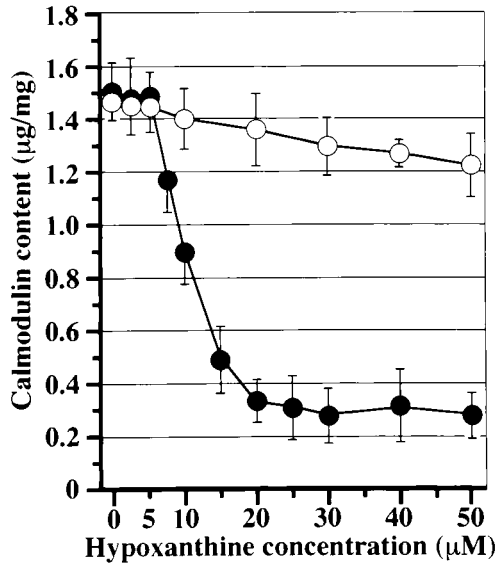

FIG. 2. Effect of $O_2^{\cdot -}$ radicals generated from hypoxanthine (●, 0 and 2.5–50 μM)-xanthine oxidase (0.2 U/ml) reaction on calmodulin content of the SR vesicles, and effect of SOD (○, 10 μg/ml). Time sequence additions were designed to ensure exposure of the SR to the $O_2^{\cdot -}$ radical-generating system for 2.5 min, and SOD was added before $O_2^{\cdot -}$ radical exposure as described in the text. Data represent the average ± standard error of measurements carried out with three cardiac SR preparations; three SR preparations isolated from different cardiac muscles were studied in parallel.

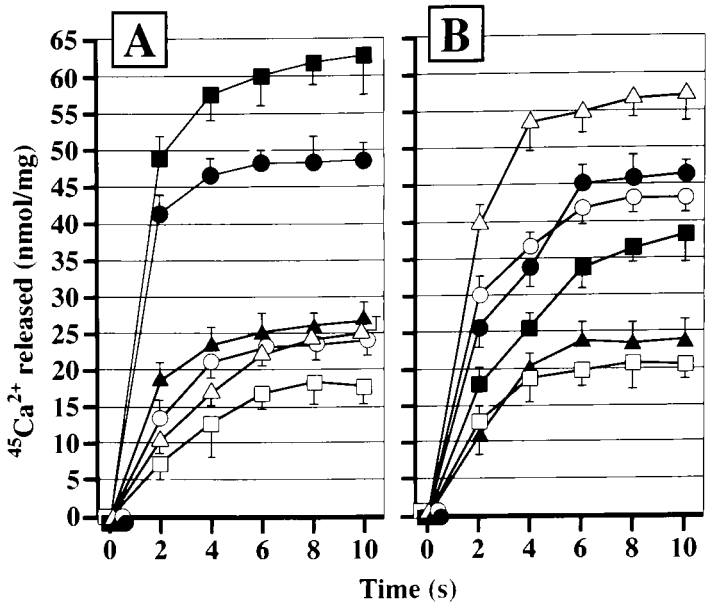

FIG. 3. Effect of cADPR responsible for calmodulin on $^{45}Ca^{2+}$ efflux from isolated heavy SR vesicles. A. Inhibitions by 8-amino-cADPR (▲, 1.0 μM) and high concentration of calmodulin (□, 2 μM) and stimulation by low concentration of calmodulin (■, 0.25 μM) of the effect of cADPR (●, 1.0 μM), and effect of NAD^+ (△, 1.0 μM) in native SR vesicles. cADPR was added simultaneously with initiation of the reaction, NAD^+ was added 2.5 min before initiation of the reaction, and 8-amino-cADPR or calmodulin (0.25 and 2 μM) was added before the addition of cADPR. None (controls) (○) samples were also run in a time-matched manner. Data represent the average of measurements carried out with four cardiac SR preparations; four SR preparations isolated from different cardiac muscles were studied in parallel. Error bars are standard errors. **B.** Effects of cADPR and exogenous calmodulin in EGTA-washed calmodulin-depleted SR vesicles. cADPR (●, 1.0 μM; ■, in the presence of 0.5 μM calmodulin; and △, in the presence of 0.75 μM calmodulin) was added simultaneously with initiation of the reaction; 8-amino-cADPR (▲, 1.0 μM in the concomitant presence of 0.5 μM calmodulin and cADPR) was added before the addition of cADPR; and calmodulin (□, 0.5 μM alone; ■, 0.5 μM plus cADPR; and △, 0.75 μM plus cADPR) was added before the addition of 8-amino-cADPR. None (controls) (○) samples were also run simultaneously. Data represent the average of measurements carried out with four cardiac SR preparations; four SR preparations isolated from different cardiac muscles were studied in parallel. Error bars are standard errors.

tion was found to have a maximally inhibitory effect on Ca^{2+} efflux from cardiac Ca^{2+}-release vesicles (Meissner and Henderson, 1987). However, 0.25 μM calmodulin enhanced the effect of cADPR. The precursor of cADPR, NAD^+ (1.0 μM; >99% purity, Sigma) had no effect on this system. Thus, the SR vesicles by itself have no ability (possibly no ADP-ribosyl cyclase activity) to form cAMPR from NAD^+

The removal of endogenous calmodulin from SR vesicles by a hypotonic treatment, immediately followed by a hypertonic wash in the presence of 4 mM EGTA and by several hypotonic washes in the absence of the chelator, produced a marked increase in $^{45}Ca^{2+}$ efflux (control in Fig. 3B). The demonstrated increase was restored by the addition of exogenous calmodulin (0.5 μM) to nearly normal, indicating that our prepared calmodulin-depleted SR is valid for assessing the effect of endogenous calmodulin. With the addition of cADPR in EGTA-washed calmodulin-depleted SR vesicles, cADPR lost its ability to cause $^{45}Ca^{2+}$ efflux; the addition of exogenous calmodulin (0.5 μM) to the system reproduced 8-amino-cADPR-inhibitable effect of cADPR (Fig. 3B). Furthermore, the reproduced effect of cADPR was enhanced when EGTA-SR was treated with 0.75 μM instead of 0.5 μM calmodulin (Fig. 3B). Table 1 shows the data confirming Ca_i and Ca^{2+}-ATPase activity in parallel reactions that were identical in all respects to $^{45}Ca^{2+}$ efflux experiments (Fig. 3). cADPR produced 8-amino-cADPR-inhibitable decrease in Ca_i. The effect of cADPR was calmodulin-inhibitable at higher concentration (2 μM); instead, the decreased Ca_i elicited by cADPR was enhanced by 0.25 μM calmodulin. NAD^+ had no effect

on Ca_i. In EGTA-washed calmodulin-depleted SR vesicles, decreased Ca_i (produced by calmodulin depletion) was significantly protected by exogenously added calmodulin (0.5 μM); under this conditions, cADPR produced 8-amino-cADPR-inhibitable decrease in Ca_i (compared with 0.5 μM calmodulin alone). When EGTA-SR was treated with 0.75 μM calmodulin, the effect of cADPR was enhanced significantly compared with the value with 0.5 μM calmodulin (in the presence of cADPR). cADPR-sensitive $^{45}Ca^{2+}$ efflux thus requires $<\mu M$ of calmodulin, but not calmodulin concentration (2 μM), which shows direct inhibitory effect on the RyR/Ca^{2+} release channel (Meissner and Henderson, 1987; Kawakami and Okabe, 1998). 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 cADPR and calmodulin are due to their direct effects rather than to altered catalytic activity of the Ca^{2+} pump.

Effect of $O_2^{\cdot -}$ responsible for calmodulin and cADPR interaction

To assure the influence of the cellular environment outside of the SR of heart muscle on the effect of $O_2^{\cdot -}$ responsible for calmodulin and cADPR interaction, additional experiments were carried out on ventricular homogenate Ca^{2+} uptake as an indicator of SR function. We chose to use the homogenate oxalate-supported Ca^{2+} uptake activity for the following reasons: (i) this activity is a rapid, reliable, and accurate assessment of SR function (Solaro and Briggs, 1974); (ii) uptake rates can be determined within a few minutes of homogenization so that degradation of SR activity is minimal (Rapundalo *et al.*, 1986); and (iii) although the oxalate-supported Ca^{2+} uptake has the disadvantage of being somewhat ambiguous because of the several pathways for Ca^{2+} (Feher and Lipford, 1985), it has the advantage of being completely representative of the tissue SR. None of the SR has been dis-

TABLE 1. EFFECT OF cADPR RESPONSIBLE FOR CALMODULIN ON Ca_i AND Ca^{2+}-ATPASE ACTIVITY FOR SR VESICLES

	Native SR		*EGTA-washed SR*	
Experimental addition	*Ca_i (nmol/mg)*	*Ca^{2+}-ATPase activity (nmol Pi/mg-min)*	*Ca_i (nmol/mg)*	*Ca^{2+}-ATPase activity (nmol Pi/mg-min)*
None (control)	30.1 ± 3.2	90.6 ± 5.2	12.6 ± 0.3[a]	88.9 ± 7.3
cADPR	11.8 ± 1.1[b]	85.4 ± 7.3	14.0 ± 1.8	71.3 ± 6.2
8-Amino-cADPR	27.8 ± 2.9	91.3 ± 4.6		
8-Amino-cADPR plus cADPR	24.5 ± 2.6[c]	87.9 ± 6.7		
CaM (2 μM) plus cADPR	36.0 ± 3.0[c]	88.4 ± 8.1		
CaM (0.25 μM) plus cADPR	4.5 ± 2.8[c]	93.8 ± 6.4		
NAD^+	32.6 ± 5.3	92.4 ± 5.5		
CaM (0.5 μM)			25.8 ± 0.4[b]	70.9 ± 11.0
CaM (0.75 μM)			28.3 ± 0.9[b]	74.6 ± 8.3
CaM (0.5 μM) plus cADPR			17.4 ± 1.2[d]	80.6 ± 10.9
CaM (0.75 μM) plus cADPR			12.1 ± 0.4[e]	83.7 ± 6.5
CaM (0.5 μM) plus 8-amino-cADPR plus cADPR			23.9 ± 0.5[f]	84.5 ± 4.9

cADPR (1.0 μM) was added simultaneously with initiation of the reaction; NAD^+ (1.0 μM) was added 2.5 min before initiation of the reaction; 8-amino-cADPR (1.0 μM) was added before the addition of cADPR; and calmodulin (0.25, 0.5, 0.75, or 2.0 μM) was added before the addition of 8-amino-cADPR or cADPR. None (control) and controls with 8-amino-cADPR alone or calmodulin alone were also run in a time-matched manner. Values are mean ± standard error (four to five experiments). p values are the result of analysis of variance and Dunnett's multiple-range test.

[a] $p < 0.01$ vs. corresponding Ca_i in native-SR (none-control).
[b] $p < 0.01$ vs. corresponding none (control).
[c] $p < 0.01$ vs. corresponding cADPR alone.
[d] $p < 0.01$ vs. corresponding calmodulin (0.5 μM) alone.
[e] $p < 0.05$ vs. corresponding calmodulin (0.5 μM) plus cADPR.
[f] $p < 0.01$ vs. corresponding calmodulin (0.5 μM) plus cADPR.

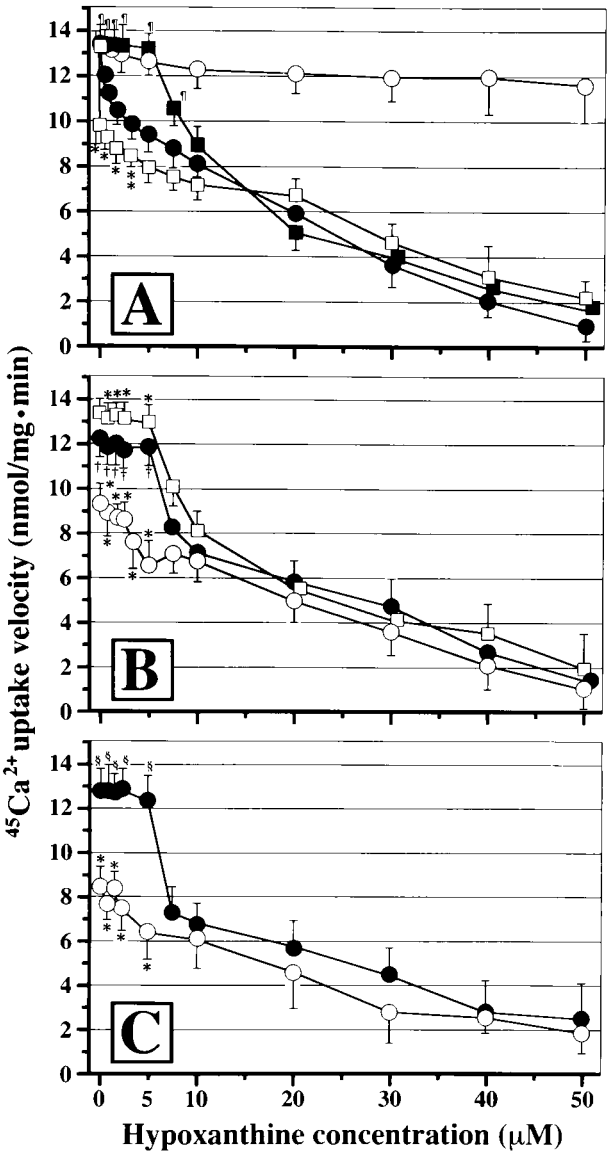

FIG. 4. Modifications afforded by SOD (10 μg/ml), exogenous calmodulin (0.25 μM), NAD^+ (1.0 μM), cADPR (1.0 μM), and 8-amino-cADPR (1.0 μM) of the effect of $O_2^{\cdot -}$ radical generated from hypoxanthine (0.2–50 μM)-xanthine oxidase (0.2 U/ml) reaction on whole heart homogenate $^{45}Ca^{2+}$ uptake rate. A. Inhibition by SOD (○) of the effect of $O_2^{\cdot -}$ radicals (●), and stimulation by exogenous calmodulin (□) of the effect exerted by lower concentrations (<5 μM) of hypoxanthine (in the presence of xanthine oxidase), and effect of 8-amino-cADPR (■) on the calmodulin stimulation. The time sequence additions were designed to ensure exposure of the homogenate to the $O_2^{\cdot -}$ radical generating system for 2.5 min before initiation of the reaction. SOD or 8-amino-cADPR was added to the reaction bath (in the presence of xanthine oxidase alone) before the $O_2^{\cdot -}$ radical exposure; calmodulin was added before the addition of 8-amino-cADPR or $O_2^{\cdot -}$ radical exposure. Data represent the average of measurements carried out with five heart homogenates; five homogenates prepared from different cardiac muscles were studied in parallel. Error bars are standard errors. $^*p < 0.01$ and $^{**}p < 0.05$ vs. corresponding hypoxanthine alone; and $^¶p < 0.01$ vs. corresponding calmodulin (in the presence of hypoxanthine) (analysis of variance followed by paired two-tailed Welch *t*-test if analysis of variance was significant). **B.** Stimulation by NAD^+ (○) and inhibition by 8-amino-cADPR (□) of the effect exerted by lower concentrations (<5 μM) of hypoxanthine (in the presence of xanthine oxidase), and effect of 8-amino-cADPR (●) on the NAD^+ stimulation. Experimental conditions were identical to those in A, except that NAD^+ was added simultaneously with the $O_2^{\cdot -}$ radical exposure and 8-amino-cADPR was added before the addition of NAD^+ or $O_2^{\cdot -}$ radical exposure. Data represent the average of measurements carried out with five heart homogenates; five homogenates prepared from different cardiac muscles were studied in parallel. Error bars are standard errors. $^*p < 0.01$ vs. corresponding hypoxanthine alone in **A**; and $^¶p < 0.01$ vs. corresponding NAD^+ (in the presence of hypoxanthine) (analysis of variance followed by paired two-tail Welch *t*-test if analysis of variance was significant). **C.** Stimulation by cADPR (○) of the effect exerted by lower concentrations (<5 μM) of hypoxanthine (in the presence of xanthine oxidase), and effect of 8-amino-cADPR (●) on the cADPR stimulation. Experimental conditions were identical to those in **A**, except that cADPR was added simultaneously with initiation of the reaction (2.5 min after the start of $O_2^{\cdot -}$ radical exposure) and 8-amino-cADPR was added before the addition of cADPR. Data represent the average of measurements carried with five heart homogenates; five homogenates prepared from different cardiac muscles were studied in parallel. Error bars are standard errors. $^*p < 0.01$ vs. corresponding hypoxyanthine alone in **A**; and $^§p < 0.01$ vs. corresponding cADPR (in the presence of hypoxanthine) (analysis of variance followed by paired two-tail Welch *t*-test if analysis of variance was significant).

carded during the isolation of purified SR (Feher *et al.*, 1980).

Previously, it has been shown that homogenate oxalate-supported Ca^{2+} uptake measured in the presence of azide is not due to mitochondria (Solaro and Briggs, 1974) or sarcolemma (Trumble *et al.*, 1980) and is thus restricted to SR. The effect of the hypoxanthine-xanthine oxidase reaction on homogenate oxalate-supported $^{45}Ca^{2+}$ uptake rate in the presence of saponin, a steroid glycoside that permeabilizes cells by interacting with cholesterol in the cell membrane (Burgess *et al.*, 1983), is shown in Fig. 4A. The uptake rate was decreased in a hypoxanthine concentration-dependent manner; the observed effect was blunted by SOD. At hypoxanthine concentrations (<5 μM) that were incapable of reducing

calmodulin content of SR vesicles (Fig. 2), there was a decrease in $^{45}Ca^{2+}$ uptake rate and the observed decrease was enhanced by calmodulin (0.25 μM); when 8-amino-cADPR was added, there was a sift of the concentration–response curve (in the presence of calmodulin) up and to the right (Fig. 4A). NAD^+ elicited further decrease in $^{45}Ca^{2+}$ uptake rate (Fig. 4B) compared with hypoxanthine alone (<5 μM). The decreases in $^{45}Ca^{2+}$ uptake rate induced by <5 μM hypoxanthine itself and additional NAD^+ were totally abolished by 8-amino-cADPR (a sift of the concentration-response curves up and to the right was also observed in the presence or absence of NAD^+). The addition of cADPR to the system decreased $^{45}Ca^{2+}$ uptake rate at hypoxanthine concentrations ranging from 0.2 to 5 μM (Fig. 4C) that permitted NAD^+ to induced $^{45}Ca^{2+}$ uptake reduction; the observed effect of cADPR was also virtually abolished by 8-amino-cADPR (Fig. 4C).

Conversion of NAD^+ into cADPR induced by $O_2^{\cdot -}$

Figure 5 shows that heart muscle can convert NAD^+ into cADPR. When NAD^+ was added to cardiac ventricular homogenates, formation of cADPR occurred, indicating that cardiac muscle (but not SR vesicles themselves, see Fig. 3) possesses ADP-ribosyl cyclase activity (Lee and Aarhus, 1991). The NAD^+ consumption was enhanced in the presence of hypoxanthine (2.5 μM)-xanthine oxidase (0.2 U/ml) (Fig. 5A) and cADPR formation was enhanced also by hypoxanthine-xanthine oxidase reaction (Fig. 5B). The stimulatory effect by hypoxanthine-xanthine oxidase on cADPR formation was SOD inhibitable.

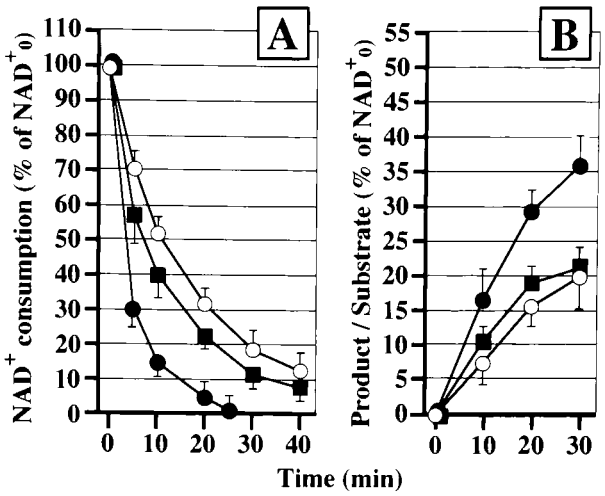

FIG. 5. Stimulation by $O_2^{\cdot -}$ radicals generated from hypoxanthine (2.5 μM)-xanthine oxidase (0.2 U/ml) reaction of conversion of NAD^+ into cADPR in cardiac muscle homogenate. NAD^+ consumption (A) and formation of cADPR (B) were measured. Time sequence additions was ensure exposure of the homogenates to the complete $O_2^{\cdot -}$ radical generating system (●) for 2.5 min before initiation of NAD^+-conversion reactions. SOD (■, 10 μg/ml) was added before $O_2^{\cdot -}$ radical exposure. Controls with xanthine oxidase alone (○) were run simultaneously. The reaction was quenched at the indicated time points. Data represent the average of measurements carried out with six heart preparations. Error bars are standard errors.

DISCUSSION

This study provides evidence for $O_2^{\cdot -}$ anion radical-mediated stimulation of cADPR synthesis and for a functional role of cADPR as a direct activator of the SR RyR/Ca^{2+} release channels in myocardium. The evidence is based the abilities of $O_2^{\cdot -}$ to convert NAD^+, a precursor of cADPR, into cADPR, in cardiac homogenates, and of authentic cADPR to activate RyR/Ca^{2+} release channels in isolated heavy SR vesicles.

During the initial investigations on the Ca^{2+}-releasing activity of $O_2^{\cdot -}$, we noted the requirement of calmodulin (Okabe *et al.*, 1987, 1989, 1991; Kawakami and Okabe, 1998), and suggested that $O_2^{\cdot -}$ can lead to RyR/Ca^{2+} release channel activation by displacement of calmodulin from SR vesicles; a plausible site of attack by $O_2^{\cdot -}$ may be calmodulin-dependent inhibitory mechanism or mechanisms of RyR/Ca^{2+} release channels. Although the effect of $O_2^{\cdot -}$ on $^{45}Ca^{2+}$ efflux at higher concentrations (>5 μM generated from >5 μM hypoxanthine in the presence of 0.2 U/ml xanthine oxidase) is due to its ability to produce a loss in function of calmodulin (Figs. 1 and 2), thereby decreasing calmodulin inhibition (Kawakami and Okabe, 1998), the results of the current study demonstrate that $O_2^{\cdot -}$ anion radicals at lower concentrations (<5 μM) may be able to stimulate Ca^{2+} release only in

the presence of calmodulin from the SR *via* increased cADPR synthesis (Figs. 3–5 and Table 1); thus, $O_2^{\cdot -}$ radicals have dual effects.

The validity of using the homogenate oxalate-supported Ca^{2+} uptake rate as an index of SR function has been previously documented (Okabe *et al.*, 1991), and it is now established that part of the Ca^{2+} uptake activity resides in vesicles containing RyR/Ca^{2+} release channels that prevent Ca^{2+} accumulation under the usual assay conditions (Okabe *et al.*, 1991). The major efflux pathway for Ca^{2+} is through RyR/Ca^{2+} release channels (Lai *et al.*, 1988). Thus, Ca^{2+} uptake rate by SR (in the homogenates) could be depressed by either reducing the Ca^{2+} influx by reducing Ca^{2+}-ATPase activity or increasing Ca^{2+} efflux by opening the RyR/Ca^{2+} release channels. Previously, we have shown that under the conditions used in the present study hypoxanthine-xanthine oxidase reaction depressed oxalate-supported Ca^{2+} uptake and steady-state Ca^{2+} accumulation with no effect on Ca^{2+}-ATPase activity in isolated SR vesicles (Okabe *et al.*, 1991). In the present study, hypoxanthine at <5 μM in the presence of 0.2 U/ml xanthine oxidase was shown to stimulate calmodulin-, NAD^+-, and cADPR-sensitive and 8-amino-cADPR-inhibitable decreases in SR $^{45}Ca^{2+}$ uptake rate in homogenates (Fig. 4). In addition to this, when the homogenate was incubated with $O_2^{\cdot -}$ (generated from 2.5 μM hypoxanthine in the presence of 0.2 U/ml xanthine oxidase), conversion of NAD^+ into cADPR was also stimulated (Fig. 5). Ryanodine (300 μM to close the RyR/Ca^{2+} release channel in heart homogenate) (Feher *et al.*, 1988) inhibited all the effects elicited by hypoxanthine (0.2–50 μM)-xanthine oxidase on the homogenate oxalate-supported $^{45}Ca^{2+}$ uptake (data not shown). These results led us to the conclusion that cardiac muscle homogenates possess ADP-ribosyl cyclase activity and $O_2^{\cdot -}$ can stimulate Ca^{2+} release from SR due to increased cADPR synthesis. The cardiac SR immediately upon addition of cADPR, but not NAD^+, did exhibit $^{45}Ca^{2+}$ efflux stimulation (Fig. 3A), implying that the SR by itself does not possess ADP-ribosyl cyclase activity. Furthermore, the stimulated $^{45}Ca^{2+}$ efflux (exerted by cADPR) was abolished by ryanodine (at a concentration that blocks the RyR/Ca^{2+} release channel) (data not shown). The properties of cADPR in the SR are thus consistent with its ability to activate RyR/Ca^{2+} release channels as well as enhance Ca^{2+}-induced Ca^{2+} release by these channels. It is reported that both NADase and ADP-ribosyl cyclase activities were detected in cardiac muscle homogenates (Mészáros *et al.*, 1995), and when compared to either nicotinamide or even to ADPR formation, cADPR is only produced in minute amounts (Mészáros *et al.*, 1995). We did not observe any quantitative difference in cADPR and other reaction products of NAD^+, nicotinamide, and ADPR, in the present study.

The ability of cADPR to stimulate $^{45}Ca^{2+}$ efflux from the SR vesicles appeared to depend on calmodulin, because the effect of cADPR was enhanced by calmodulin in native SR (Fig. 3A); cADPR lost its ability to stimulate $^{45}Ca^{2+}$ efflux in EGTA-washed calmodulin-depeleted SR (Fig. 3B). Additional calmodulin (0.5 μM) to the EGTA-washed SR vesicles reproduced the effect of cADPR; treatment of EGTA-washed SR with 0.75 μM instead of 0.5 μM calmodulin caused more potent $^{45}Ca^{2+}$ efflux than that caused by cADPR in the presence of 0.5 μM calmodulin (Fig. 3B). cADPR sensitivity thus requires calmodulin. The effect of calmodulin does not appear to be mediated through enzymatic reactions because neither an inhibitor of calcineurin, cypermethrin (Enan and Matsumura, 1992), nor inhibitors of Ca^{2+}-calmodulin-dependent protein kinase II, the antagonistic peptide, fragment 290–309 (Hashimoto *et al.*, 1987), or the substrate peptide syntide-2 (Payne *et al.*, 1988) blocked the cADPR-sensitivity-conferring activity. Furthermore, calmodulin has been 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). Therefore, calmodulin may interact directly with and sensitize the Ca^{2+}-release mechanism of cADPR on RyR/Ca^{2+} release channel in cardiac SR.

The cardiac RyR/Ca^{2+} release channel is activated by cADPR (Mészáros *et al.*, 1993) but inhibited by calmodulin in the absence of ATP (Smith *et al.*, 1989). However, in our system, 2 μM calmodulin inhibited cADPR-induced

$^{45}Ca^{2+}$ efflux even in the presence of 0.5 m*M* ATP; instead, 0.25 μM calmodulin enhanced both the effect (that was 8-amino-cADPR-inhibitable) of $O_2^{\cdot -}$ radicals in homogenate experiments (Fig. 4A) and the effect exerted by cADPR on $^{45}Ca^{2+}$ efflux from isolated SR vesicles (Fig. 3A). Therefore, it is likely that calmodulin at low concentration may be not sufficient to inhibit Ca^{2+} release from SR through RyR/Ca^{2+} release channels but sufficient to confer cADPR sensitivity to the channels, suggesting cADPR is a modulator that can activate the Ca^{2+}-release mechanism when it is in a sensitized state (by the presence of a low concentration of calmodulin, possibly a physiological concentration).

In the current experiments on Ca_i, we also evaluated the ability of cADPR to stimulate Ca^{2+} release. Data (Table 1) showing that cADPR can decrease Ca_i only under the conditions of calmodulin stimulation without changing Ca^{2+}-ATPase activity and that mild stimulation (by 0.25 μM calmodulin) enhances the observed effect of cADPR and that further calmodulin stimulation (2 μM) inhibits it are entirely compatible with a hypothesis that cADPR-induced decrease in the Ca^{2+} accumulation results in a "releasing out" of transported Ca^{2+} through cADPR-sensitive RyR/Ca^{2+} release channels before binding within the SR vesicles. Moreover, the effect of cADPR is dependent on the presence of calmodulin.

The mechanism(s) by which $O_2^{\cdot -}$ anion radical stimulates cADPR synthesis in cardiac muscle has not been completely settled in the current study. For $O_2^{\cdot -}$ to play a role in enhancing cADPR production, a marked change in its enzyme property, such as conversion from NADase to cyclase, would be required. It is tempting to speculate that $O_2^{\cdot -}$ may inhibit the hydrolysis of cADPR (yielding ADPR) catalyzed by NADase or alter cyclase, and is noteworthy that $O_2^{\cdot -}$ is responsible for cADPR formation from NAD^+ in the cellular environment outside of the SR of heart muscle. The results presented here suggest that $O_2^{\cdot -}$-induced Ca^{2+} release from SR vesicles is at least a two-step process: $O_2^{\cdot -}$ radical at low concentrations (<5 μM) induces Ca^{2+} release by stimulating synthesis of cADPR whose action requires calmodulin; at higher concentrations (>5 μM), $O_2^{\cdot -}$ produces a loss in function of calmodulin thereby stimulating Ca^{2+} release. The precise effective concentrations of $O_2^{\cdot -}$ on a two-step process were indistinguishable from those generated from hypoxanthine-xanthine oxidase reaction, but the data presented here provide the most direct proof of its identity. The $O_2^{\cdot -}$ concentration-related effects within physiological range on Ca^{2+} mobilization in myocardium and the role played by $O_2^{\cdot -}$ as the physiological trigger in cADPR-mediated Ca^{2+} signaling merit further study.

ACKNOWLEDGMENTS

This work was supported by Grants 09877356 (E.O.) and 10557167 (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.

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