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Nitric oxide inhibits Ca^{2+} mobilization through cADP-ribose signaling in coronary arterial smooth muscle cells

JIANG-ZHOU YU, DAVID X. ZHANG, AI-PING ZOU,
WILLIAM B. CAMPBELL, AND PIN-LAN LI
Departments of Pharmacology and Toxicology and Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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Yu, Jiang-Zhou, David X. Zhang, Ai-Ping Zou, William B. Campbell, and Pin-Lan Li. Nitric oxide inhibits Ca^{2+} mobilization through cADP-ribose signaling in coronary arterial smooth muscle cells. *Am J Physiol Heart Circ Physiol* 279: H873–H881, 2000.—The present study was designed to determine whether the cADP-ribose-mediated Ca^{2+} signaling is involved in the inhibitory effect of nitric oxide (NO) on intracellular Ca^{2+} mobilization. With the use of fluorescent microscopic spectrometry, cADP-ribose-induced Ca^{2+} release from sarcoplasmic reticulum (SR) of bovine coronary arterial smooth muscle cells (CASMCs) was determined. In the α-toxin-permeabilized primary cultures of CASMCs, cADP-ribose (5 μM) produced a rapid Ca^{2+} release, which was completely blocked by pretreatment of cells with the cADP-ribose antagonist 8-bromo-cADP-ribose (8-Br-cADPR). In intact fura 2-loaded CASMCs, 80 mM KCl was added to depolarize the cells and increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Sodium nitroprusside (SNP), an NO donor, produced a concentration-dependent inhibition of the KCl-induced increase in $[Ca^{2+}]_i$, but it had no effect on the U-46619-induced increase in $[Ca^{2+}]_i$. In the presence of 8-Br-cADPR (100 μM) and ryanodine (10 μM), the inhibitory effect of SNP was markedly attenuated. HPLC analyses showed that CASMCs expressed the ADP-ribosyl cyclase activity, and SNP (1–100 μM) significantly reduced the ADP-ribosyl cyclase activity in a concentration-dependent manner. The effect of SNP was completely blocked by addition of 10 μM oxygenated hemoglobin. We conclude that ADP-ribosyl cyclase is present in CASMCs, and NO may decrease $[Ca^{2+}]_i$ by inhibition of cADP-ribose-induced Ca^{2+} mobilization.

adenosine 3′,5′-cyclic diphosphate-ribose; coronary artery; vascular smooth muscle cells

ADENOSINE 5′-CYCLIC DIPHOSPHATE-RIBOSE (cADP-ribose) was first reported to be present in sea urchin eggs and to possess Ca^{2+} mobilizing activity (10, 37). Recent studies indicate that cADP-ribose is produced in a number of mammalian tissues, including heart, liver, spleen, brain and red blood cells, pituitary cells, and cultured renal epithelial cells (5, 29, 46, 51). Basal concentrations of cADP-ribose in cardiac muscle, liver, and brain are estimated to be 100–200 nM (15, 31). Like sea urchin eggs, cADP-ribose also causes Ca^{2+} mobilization in these mammalian tissues and cells. Therefore, cADP-ribose has been proposed as a Ca^{2+}-mobilizing second messenger in mammalian cells. It may mediate the secretion of hormones such as insulin and catecholamines, the fertilization of eggs, the estrogen response in rat uterus, and the effects of nitric oxide (NO) in nonmuscle tissues (9, 14, 18, 33, 37, 42, 46).

cADP-ribose mobilizes intracellular Ca^{2+} by a mechanism completely independent of D-*myo*-inositol 1,4,5-trisphosphate (IP_3), since the IP_3 receptor antagonist heparin cannot block the effect of cADP-ribose (18, 15, 33, 34). Recent studies have indicated that cADP-ribose activates ryanodine receptors by a calmodulin-dependent mechanism (35) and that inhibitors of Ca^{2+}-induced Ca^{2+} release (CICR) such as tetracaine, procaine, and ruthenium red selectively inhibit the cADP-ribose but not IP_3-sensitive Ca^{2+} release. Agonists of CICR such as caffeine and Ca^{2+} potentiate the Ca^{2+}-releasing activity of cADP-ribose (16, 30, 31, 36). However, the role of cADP-ribose in the control of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in vascular smooth muscle cells is poorly understood. Recently, Kannan et al. (25) reported that cADP-ribose stimulates Ca^{2+} release from isolated coronary arterial smooth muscle cells (CASMCs). It remains to be determined whether endogenous cADP-ribose can also act as an intracellular second messenger to mediate agonist responses in coronary vascular smooth muscle cells and to participate in the control of $[Ca^{2+}]_i$ in these cells.

NO activates soluble guanylyl cyclase and the production of cGMP, which results in vasodilation (2, 4, 22). However, there is strong evidence to suggest that other mechanisms independent of the cGMP pathway, such as direct activation of K^+ channels (8, 11), decrease in $[Ca^{2+}]_i$ (7, 19, 26), and reduction of 20-hydroxyeicosatetraenoic acid production (1), may also contribute to the vasodilator effect of NO. The mechanisms responsible for the decrease in $[Ca^{2+}]_i$ by NO are poorly understood. Given that cADP-ribose participates in the control of $[Ca^{2+}]_i$, it is possible that inhibition of cADP-ribose-mediated Ca^{2+} release is in-

Address for reprint requests and other correspondence: P.-L. Li, Dept. of Pharmacology and Toxicology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226 (E-mail: pli@post.its.mcw.edu).

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volved in the NO-induced decrease in $[Ca^{2+}]_i$ in CASMCs. The present study was designed to determine the involvement of the cADP-ribose signaling pathway in the regulation of intracellular Ca^{2+} in CASMCs and to determine whether NO reduces $[Ca^{2+}]_i$ through the cADP-ribose-mediated signaling pathway in these cells.

MATERIALS AND METHODS

Dissection of coronary arteries and culture of smooth muscle cells. Smooth muscle cells were isolated and cultured as described previously (44). Briefly, the coronary arteries were dissected from bovine hearts obtained from the local slaughterhouse. The dissected coronary arteries were incubated for 30 min with 5% fetal bovine serum (FBS) in medium 199 containing 25 mM HEPES with 1% penicillin, 0.3% gentamycin, and 0.3% nystatin. After the endothelial cells were removed, the arteries were cut into very small pieces and digested with 0.2% collagenase at 37°C overnight. CASMCs in the suspension were washed three times with medium 199 by centrifugation (1,000 rpm for 10 min), placed in a six-well plate or petri dish (10^4 cells/ml), and cultured in medium 199 containing 20% FBS and antibiotics in a 5% CO_2 incubator at 37°C. Confluence was reached in 5–6 days. The primary cultures of CASMCs were used to study the activities of both ADP-ribosyl cyclase and cADP-ribose hydrolase and to measure the $[Ca^{2+}]_i$ and Ca^{2+} release response to various stimuli. The identity of CASMCs was demonstrated by a positive staining with an antibody against smooth muscle α-actin.

Measurement of $[Ca^{2+}]_i$ in intact CASMCs. Fura 2-acetoxymethyl ester (AM) (Molecular Probes, Eugene, OR) was used for monitoring $[Ca^{2+}]_i$ (48). Subconfluent CASMCs on glass coverslips were washed with Hanks' buffer containing (in mM) 130 NaCl, 5.4 KCl, 20 HEPES, 5.5 glucose, 1.25 $CaCl_2$, and 1 $MgCl_2$ and were incubated with 5 μM fura 2-AM at 37°C for 30 min. After the free fura 2-AM was washed out, the coverslip was mounted on a chamber and then on the stage of an inverted microscope (Nikon Diaphot). Cells were incubated with Hanks' buffer for 20 min at room temperature to allow for complete hydrolysis of intracellular fura 2-AM to fura 2, and then the ratio of fura 2 emissions, when excited at 340 and 380 nm, was monitored by using a fluorescent microscopic spectrometric system (PTI). $[Ca^{2+}]_i$ were calculated from the ratio of fluorescence at 340 nm vs. 380 nm (F_{340}/F_{380}) using the following equation

$$[Ca^{2+}]_i\ (nM) = K_d(F_0/F_S)(R-R_{min})/(R_{max}-R)$$

where R is the ratio F_{340}/F_{380}, K_d is the dissociation constant of Ca^{2+}, R_{min} and R_{max} are minimal and maximal F_{340}/F_{380} ratios, respectively, and F_0/F_S represents the maximal and minimal signal intensity at 380 nm, respectively. K_d was 224 nM. R_{min}, R_{max}, and F_0/F_S were determined and calculated from in situ calibrations. R_{max} was calculated from the fluorescence intensity after permeabilizing the cells with 5 mM ionomycin and represented maximal $[Ca^{2+}]_i$. R_{min} was obtained by addition of 2 mM EGTA and represented the minimal $[Ca^{2+}]_i$ (12, 48).

To examine the role of endogenous cADP-ribose in the control of $[Ca^{2+}]_i$ in CASMCs, KCl (80 mM) was used to depolarize CASMCs and to produce CICR. Next, 8-bromo-cADP-ribose (100 μM), a cell-permeant cADP-ribose antagonist, ryanodine (10 μM), or SNP (100 μM) was added to the bath, and the ratio F_{340}/F_{380} was recorded. In the presence of 8-bromo-cADP-ribose or sodium nitroprusside (SNP), the KCl-induced Ca^{2+} release response was observed by recording the changes in the ratio F_{340}/F_{380}. U-46619, a thromboxane A_2 mimetic, has been reported to contract coronary vessels through activation of membrane Ca^{2+} channels and IP_3 production (47, 53). To determine the effect of 8-bromo-cADP-ribose and SNP on agonist-induced Ca^{2+} influx and the IP_3-mediated Ca^{2+} signaling pathway, cells were treated with U-46619 (100 nM) in the presence or absence of 8-bromo-cADP-ribose and SNP, and changes in $[Ca^{2+}]_i$ were measured.

Assay of Ca^{2+} release from the sarcoplasmic reticulum of α-toxin-permeabilized CASMCs. To directly determine the effect of cADP-ribose on Ca^{2+} release from the sarcoplasmic reticulum (SR), the permeabilized CASMCs were used. Permeabilization and fura 2-AM loading were performed by a modification of the method described recently by Kannan et al. (25). Briefly, the primary cultures of CASMCs on coverslips were first incubated with 5 μM fura 2-AM at 37°C for 30 min. The coverslips were then washed and mounted on a slide chamber. The slide chamber was then mounted on the stage of Nikon Diaphot inverted microscope. The cells were incubated and permeabilized by addition of 5 μg/ml α-toxin in low-Ca^{2+} Hanks' buffer (pCa 9.0 = 1 nM Ca^{2+} concentration) for 10–15 min. α-Toxin made pores on the cell membrane by binding to the cell surface, forming headers with other molecules and inserting into the plasma membrane. The pores made by α-toxin allowed the molecules with size <4,000 Da to pass through, but α-toxin itself did not enter into the cells. This prevented functional intracellular proteins from leaving from the cells and protected the organelles from damage (43). The permeabilized cells were incubated in pCa 6.0 Hanks' buffer for 10–20 min to load Ca^{2+} in the SR and then were bathed with pCa 9.0. With the use of fluorescent microscopic spectrometry, Ca^{2+} release was monitored when different compounds (5 μM cADP-ribose, 70 μM IP_3, and 100 μM 8-bromo-cADP-ribose) were added to the bath solution. The doses of cADP-ribose, IP_3, and 8-bromo-cADP-ribose were chosen based on previous studies (18, 25). The fluorescence intensity of intracellular fura 2 was determined and recorded when excited at 340 and 380 nm, and Ca^{2+} release was indicated by the ratio F_{340}/F_{380}. Because the cells were permeabilized, $[Ca^{2+}]_i$ could not be calculated. The direct effect of SNP on cADP-ribose- or IP_3-induced Ca^{2+} release was detected by preexposure of the permeabilized cells to SNP (100 μM) before addition of cADP-ribose or IP_3.

HPLC analysis of cADP-ribose and ADP-ribose synthesis. Homogenates were prepared from CASMCs as we described previously (39, 40). Primary cultures of CASMCs at confluence were rinsed with 10 ml of chilled PBS (pH 7.0; Sigma) and were collected using a cell scraper at 4°C. The cells were resuspended and homogenized in HEPES buffer (pH 7.0) containing (in mM) 10 HEPES, 148 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.3 $MgCl_2$, and 5.5 glucose, sonicated three times with a sonifier cell disruptor (model 185; Branson) for 20 s, and then centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was considered as the cell homogenate and was used to determine the activities of ADP-ribosyl cyclase and cADP-ribose hydrolase. To determine the activity of ADP-ribosyl cyclase, the homogenates (100 μg) were incubated with 1 mM nicotinamide guanine dinucleotide (NGD) at 37°C for 30 min. Before HPLC analysis, the reaction mixtures were centrifuged at 4°C through an Amicon microultrafilter at 3,000 rpm to remove the proteins. The reaction products were analyzed by an HPLC system (Hewlett-Packard 1090; Hewlett-Packard, Avondale, PA). A fluorescent product of ADP-ribosyl cyclase, cGDP-ribose, was detected using a Hewlett-Packard 1046A spectrofluorometer. The excitation wavelength was 300 nm, and the emission wavelength was

410 nm. Previous studies have confirmed that ADP-ribosyl cyclase converted both NAD into cADP-ribose and NGD into cGDP-ribose (21). However, unlike cADP-ribose, cGDP-ribose is not hydrolyzed by tissue cADP-ribose hydrolase. Therefore, we used the conversion rate of NGD into cGDP-ribose to represent the ADP-ribosyl cyclase activity, which avoided the influences of cADP-ribose hydrolysis. To determine the activity of cADP-ribose hydrolase, the homogenates were incubated with 1 mM cADP-ribose at 37°C for 30 min, and the reaction products were chromatographed and analyzed using a Hewlett-Packard HPLC system with a 1040A photodiode array detector. The column effluent was monitored at 254 nm. Data were collected and analyzed by a Hewlett-Packard Chemstation.

Nucleotides were resolved on a 3-μm Supelcosil LC-18 column (4.6 × 150 mm) with a 5-μm Supelcosil LC-18 guard column (4.6 × 20 mm; Supelco). The injection volume was 20 μl. For cGDP-ribose, the mobile phase consisted of 150 mM ammonium acetate (pH 5.5) containing 5% methanol (*solvent A*) and 50% methanol (*solvent B*). For ADP-ribose, the mobile phase consisted of 10 mM potassium dihydrogen phosphate (pH 5.5) containing 5 mM tetrabutylammonium dihydrogen sulfate (*solvent A*) and acetonitrile (*solvent B*). The solvent system was a linear gradient that started with 5% *solvent B* then increased to 30% *solvent B* over 1 min, and 25 min later increased to 50% *solvent B* over 1 min. The flow rate was 0.8 ml/min. Peak identities were confirmed by comigration with known standards and ultraviolet absorbance spectra compared with the known standards. Quantitative measurements were performed by comparison of known concentrations of standards (40, 41).

The HPLC analysis was also used to determine the permeability of 8-bromo-cADP-ribose in CASMCs. These CASMCs were incubated with 8-bromo-cADP-ribose (100 μM) for 20 min and then were washed three times with PBS. The cells then were pelletted and homogenized. 8-Bromo-cADP-ribose eluted at 4.58 min was quantitated by HPLC. Detected 8-bromo-cADP-ribose represents the amount of this nucleotide that entered the cells, since it is not produced within the cells. A cell number-dependent accumulation of 8-bromo-cADP-ribose was found within the cells with an average entry rate of 0.5 nmol · mg protein^{-1} · min^{-1}, suggesting that 8-bromo-cADP-ribose enters into the cells without conversion or degradation.

To determine the effect of SNP on the activities of ADP-ribosyl cyclase and cADP-ribose hydrolase, the homogenates were first incubated with different concentrations of SNP (0.1–100 μM) for 3 min, the incubation was continued for 30 min after addition of 1 mM NGD for 30 min at 37°C, and the reaction was terminated by centrifugation at 3,000 rpm at 4°C through an Amicon microultrafilter. The reaction products were separated and analyzed by HPLC. In another experimental group, 10 μM oxygenated hemoglobin (OxyHb), an NO scavenger (13), was added to the reaction mixtures before addition of SNP to confirm that the effect of SNP is due to release of NO. To determine the reversibility of the SNP effect, OxyHb was added 30 min after the incubation of the homogenate with SNP. The effect of cGMP on the activities of ADP-ribosyl cyclase was determined in the presence of 5 mM ATP, since phosphorylation by cGMP-dependent protein kinase may require ATP. The homogenates were incubated with cGMP (0.1–100 μM) for 30 min at 37°C and then with 1 mM NGD for 30 min in the presence of cGMP. The reaction products were separated and analyzed by HPLC. To determine the role of guanylyl cyclase in the NO-induced reduction of the ADP-ribosyl cyclase activity, 1H-(1,2,4)-oxadiazolo[4,2-α]quinoxaline-1-one (ODQ), a soluble guanylyl cyclase inhibitor (38), was added to the reaction mixtures before addition of SNP.

Statistical analysis. Data are presented as means ± SE; *n* indicates the number of bovine hearts. The significance of the differences in mean values between and within multiple groups was examined using an ANOVA for repeated measures followed by a Duncan's multiple range test. A Student's *t*-test was used to evaluate statistical significance of differences between two paired observations. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of cADP-ribose on the SR Ca^{2+} release in α-toxin-treated CASMCs. Figure 1*A* presents a typical recording depicting the Ca^{2+} release response from the SR of permeabilized CASMCs and the effect of the cADP-ribose antagonist 8-bromo-cADP-ribose on the SR Ca^{2+} release induced by cADP-ribose. In these experiments, the cells were permeabilized by α-toxin, which allowed an access of cADP-ribose to the SR. Addition of cADP-ribose (5 μM) produced a rapid Ca^{2+}

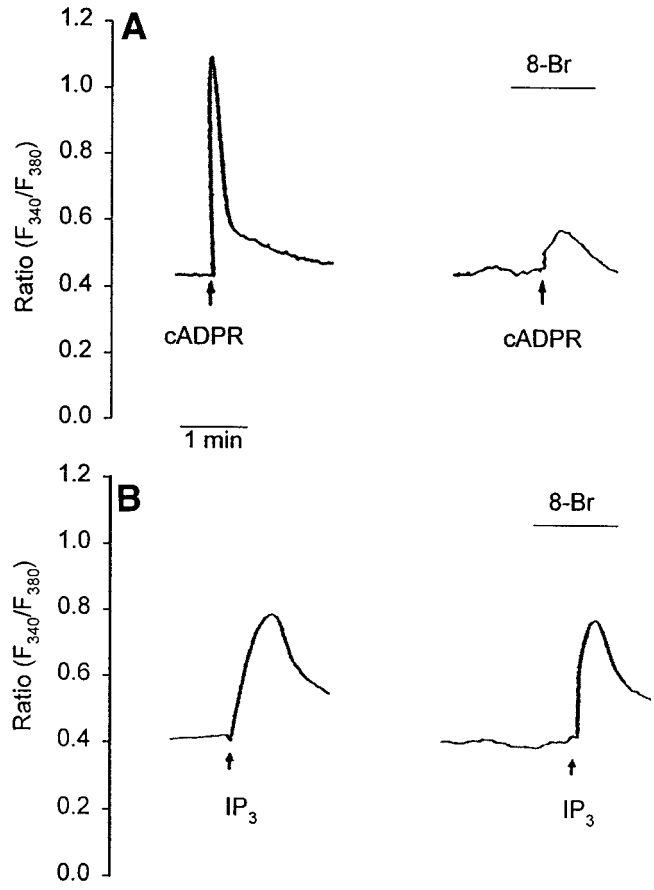

Fig. 1. Ca^{2+} release from sarcoplasmic reticulum (SR) of α-toxin-permeabilized primary cultures of bovine coronary arterial smooth muscle cells. *A*: representative recordings of Ca^{2+} release from the SR in response to cADP-ribose (cADPR; 5 μM) in the absence or presence of 8-bromo-cADP-ribose (8-Br; 100 μM). Ca^{2+} release is indicated by a ratio of fura 2 fluorescence at 340 and 380 nm (F_{340}/F_{380}) using fluorescent microscopic spectrometry. *B*: representative recordings of Ca^{2+} release from the SR in response to D-*myo*-inositol 1,4,5-trisphosphate (IP_3; 70 μM) in the absence or presence of 8-bromo-cADP-ribose.

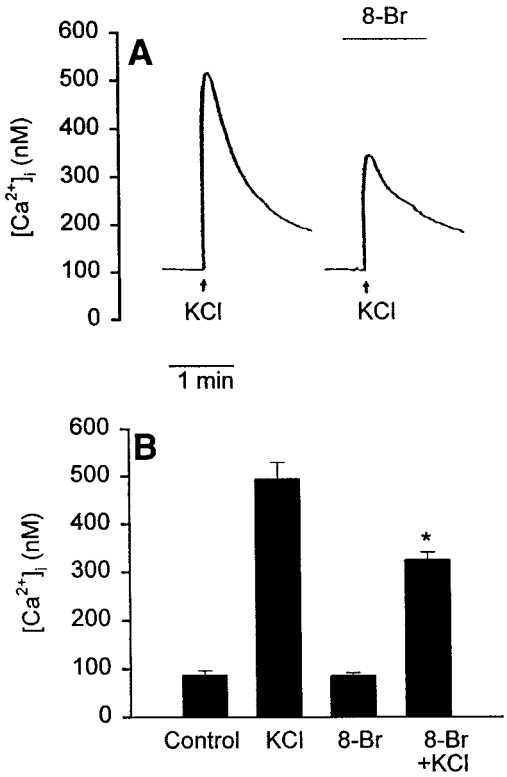

Fig. 2. Effects of 8-bromo-cADP-ribose on KCl-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in intact cultured bovine coronary arterial smooth muscle cells. *A*: representative recordings of F_{340}/F_{380} using a fluorescent microscopic spectrometry. KCl-induced transient increase in $[Ca^{2+}]_i$ was reduced in the presence of 8-bromo-cADP-ribose. *B*: summarized data showing integrated peak transient $[Ca^{2+}]_i$ response to KCl (80 mM) in the absence or presence of 8-bromo-cADP-ribose. *Significant difference from the values obtained during addition of KCl alone ($P < 005$, $n = 8$).

release response. In the presence of the cADP-ribose antagonist 8-bromo-cADP-ribose (100 μM, $n = 6$), the effect of cADP-ribose on the SR Ca^{2+} release was substantially blocked. As shown in Fig. 1*B*, IP_3 at a concentration of 70 μM ($n = 6$) also produced the release of Ca^{2+} in α-toxin-permeabilized cells (Fig. 1*B*). However, 8-bromo-cADP-ribose had no effect on the Ca^{2+} release from the SR induced by IP_3. In these permeabilized cells, SNP at a concentration of 100 μM was without effect on cADP-ribose- or IP_3-induced intracellular Ca^{2+} release (data not shown).

Effect of 8-bromo-cADP-ribose on KCl-induced increase of $[Ca^{2+}]_i$ in intact CASMCs. Because 8-bromo-cADP-ribose is a membrane-permeable antagonist of cADP-ribose, it was used to study the role of endogenous cADP-ribose in the control of $[Ca^{2+}]_i$ in intact fura 2-loaded cells. As shown in Fig. 2*A*, KCl produced a rapid initial increase in $[Ca^{2+}]_i$, and the initial peak of this Ca^{2+} transient represented the Ca^{2+} release response (24, 45). Pretreatment of the cells with 8-bromo-cADP-ribose (100 μM) for 20 min significantly attenuated this increase in $[Ca^{2+}]_i$ induced by KCl (Fig. 2*A*). Figure 2*B* summarizes the effect of 8-bromo-cADP-ribose on the KCl-induced peak increase in $[Ca^{2+}]_i$. 8-Bromo-cADP-ribose had no significant effect on basal $[Ca^{2+}]_i$ in CASMCs. In the presence of 8-bromo-cADP-ribose, however, the KCl-induced peak increase in $[Ca^{2+}]_i$ was decreased from 500.2 ± 31.7 to 346.3 ± 25.1 nM.

Effect of SNP on KCl-induced increase in $[Ca^{2+}]_i$ in the absence or presence of 8-bromo-cADP-ribose and ryanodine. In fura 2-loaded intact CASMCs, SNP (100 μM) significantly inhibited the KCl-induced rapid initial increase in $[Ca^{2+}]_i$ (Fig. 3). In the presence of 8-bromo-cADP-ribose (100 μM), the effect of SNP to decrease the KCl-induced Ca^{2+} response was significantly attenuated. SNP only inhibited the KCl-induced $[Ca^{2+}]_i$ increase by 42%, which was significantly lower than 72% in the absence of 8-bromo-cADP-ribose. Ryanodine at a concentration of 10 μM also significantly decreased the KCl-induced Ca^{2+} response by 70%. In the presence of ryanodine, SNP did not further inhibit the KCl-induced increase in $[Ca^{2+}]_i$.

In additional experiments, we incubated the cells in the Ca^{2+}-free solution for 20 min, added $CaCl_2$ (1 mM) to the bath solution, and monitored the increase in $[Ca^{2+}]_i$. Addition of $CaCl_2$ also produced a rapid increase in $[Ca^{2+}]_i$ by 33 nM, which represented CICR. In the presence of SNP (100 μM), this $CaCl_2$-induced Ca^{2+} release was substantially blocked, suggesting that SNP inhibits CICR. These results indicate that failure of SNP to further reduce KCl-induced Ca^{2+} release in the presence of 8-bromo-cADP-ribose is not due to a failure of NO action at the low levels of intracellular Ca^{2+} rather than due to blockade of the cADP-ribose pathway.

Effect of SNP on U-46619-induced increase in $[Ca^{2+}]_i$ in the absence or presence of 8-bromo-cADP-ribose. The thromboxane A_2 mimetic U-46619 induces Ca^{2+} influx, stimulates IP_3 production, and consequently increases intracellular Ca^{2+}, which results in vasocon-

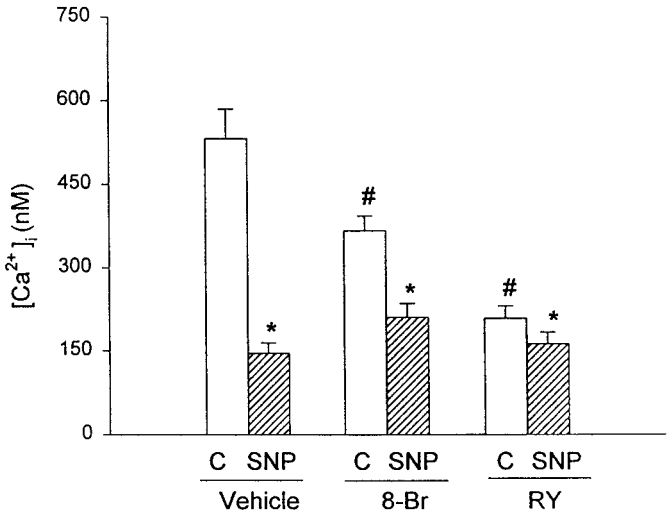

Fig. 3. Effect of sodium nitroprusside (SNP) on KCl-induced increase in $[Ca^{2+}]_i$ in cultured bovine coronary arterial smooth muscle cells in the absence and presence of 8-bromo-cADP-ribose (100 μM; $n = 9$) and ryanodine (Ry; 10 μM; $n = 11$) *Significant difference from control (C, $P < 005$). #Significant difference from control in vehicle group.

striction (47, 53). In the present study, U-46619 (100 nM) increased $[Ca^{2+}]_i$ by 60 nM. Pretreatment of the cells with 8-bromo-cADP-ribose (100 μM) alone or in combination with SNP (100 μM) had no effect on the U-46619-induced increase in $[Ca^{2+}]_i$ (Fig. 4).

Effect of SNP on the activities of ADP-ribosyl cyclase and cADP-ribose hydrolase. In the primary cultures of CASMCs, the conversion rate of NGD to cGDP-ribose was 0.72 ± 0.09 nmol · min^{-1} · mg $protein^{-1}$ (n = 7), and the conversion rate of cADP-ribose to ADP-ribose was 1.07 ± 0.13 nmol · min^{-1} · mg $protein^{-1}$ (n = 7). These results indicate that CASMCs express the activities of ADP-ribosyl cyclase and cADP-ribose hydrolase. To determine the effect of NO on the activity of ADP-ribosyl cyclase and cADP-ribose hydrolase, the homogenates of CASMCs were incubated with SNP at concentrations of 0.1–100 μM. As shown in Fig. 5, SNP produced a concentration-dependent decrease in the conversion rate of NGD to cGDP-ribose, indicating an inhibition of the ADP-ribosyl cyclase activity. SNP at a concentration of 100 μM, a concentration that is predicted to produce 150 nM NO (49), significantly reduced the cADP-ribose cyclase activity by 43%. The conversion rate of NGD to cGDP-ribose was decreased from 0.72 ± 0.09 to 0.40 ± 0.08 nmol · min^{-1} · mg $protein^{-1}$ (n = 7). In contrast, SNP had no effect on the activity of cADP-ribose hydrolase, as measured by the conversion rate of cADP-ribose to ADP-ribose.

Previous studies reported that SNP can release other molecules in addition to NO, such as free radicals and cyanides (3, 20). To rule out the effect of these SNP products, we added OxyHb to the reaction mixture to bind NO and then redetermined the ADP-ribosyl cyclase activity. OxyHb (10 μM) completely prevented the inhibitory effect of SNP on the ADP-ribosyl cyclase activity, suggesting that the inhibitory effect of SNP is due to NO release. To determine whether the inhibitory effect of SNP on the ADP-ribosyl cyclase activity can be reversed by scavenging NO, OxyHb was added to the reaction mixture after SNP was incubated with the homogenate for 30 min. Interestingly, OxyHb completely restored the ADP-ribosyl cyclase activity, even in the presence of SNP, suggesting that the inhibitory effect of NO on ADP-ribosyl cyclase is reversible (Fig. 6*A*).

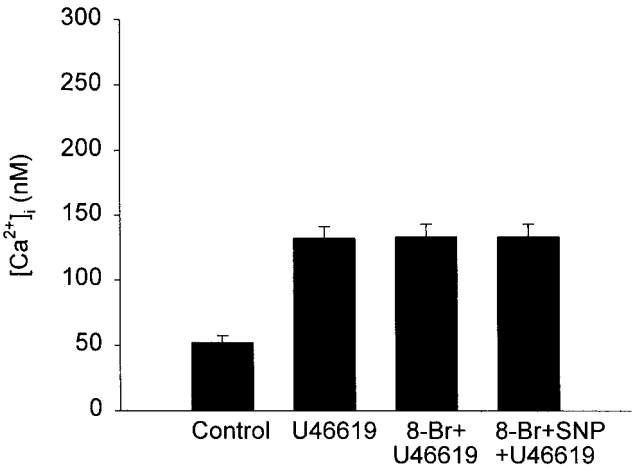

Fig. 4. Effect of 8-bromo-cADP-ribose and SNP on U-46619-induced increase in $[Ca^{2+}]_i$ in cultured bovine coronary arterial smooth muscle cells (n = 8).

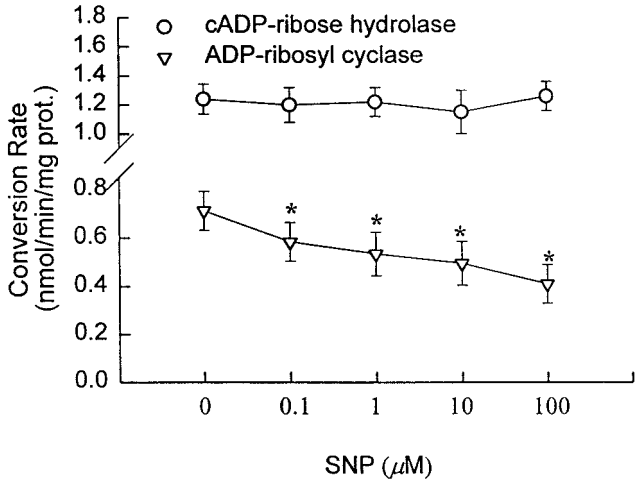

Fig. 5. Effect of SNP on the activities of ADP-ribosyl cyclase (the conversion rate of nicotinamide guanine dinucleotide to cGDP-ribose) and cADP-ribose hydrolase (the conversion rate of cADP-ribose into ADP-ribose) in the homogenate of bovine coronary arterial smooth muscle cells. *Significant difference from control (without SNP; $P < 005$; n = 7).

Effect of guanylyl cyclase inhibition and cGMP on the ADP-ribosyl cyclase activity. It is known that NO induces vascular relaxation through the activation of guanylyl cyclase and an increase in cGMP content of vascular smooth muscle cells (2, 4, 22). Recent studies in nonvascular tissues reported that NO increases intracellular cGMP and consequently stimulated the production of cADP-ribose (18, 31, 50, 52). To determine whether the NO-induced decrease in the production of cADP-ribose in CASMCs is also associated with the activation of guanylyl cyclase, we examined the effects of the guanylyl cyclase inhibitor ODQ and cGMP on the ADP-ribosyl cyclase activity in these cells. SNP (100 μM) significantly decreased the conversion rate of NGD to cGDP-ribose. ODQ (100 μM) had no effect on SNP-induced inhibition of the ADP-ribosyl cyclase activity (Fig. 6*B*). Moreover, direct addition of cGMP (0.1–100 μM) and ATP (5 mM) to the reaction mixtures did not alter the ADP-ribosyl cyclase activity (data not shown), suggesting that cGMP is not an activator or inhibitor of ADP-ribosyl cyclase in CASMCs.

DISCUSSION

In the present study, we demonstrated that cultured bovine CASMCs synthesized and metabolized cADP-ribose and that cADP-ribose mobilized Ca^{2+} from the SR of these cells. The cADP-ribose antagonist 8-bromo-cADP-ribose significantly attenuated the initial peak Ca^{2+} transient induced by KCl, suggesting that blockade of cADP-ribose actions reduces intracellular Ca^{2+} mobilization. These results strongly indicate that cADP-ribose mediates a Ca^{2+} signaling pathway that participates in the control of $[Ca^{2+}]_i$ in CASMCs.

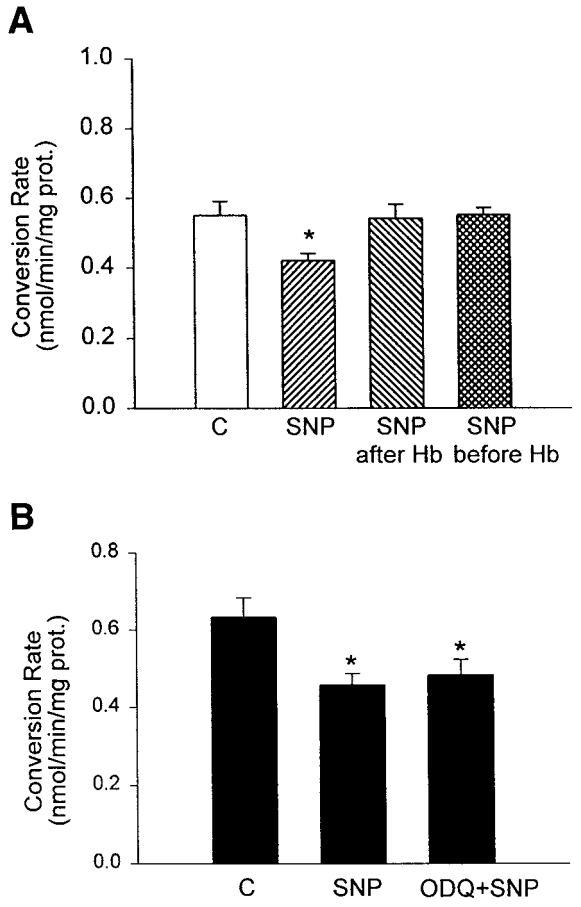

Fig. 6. Effect of oxygenated hemoglobin (OxyHb) and guanylyl cyclase inhibition by 1H-(1,2,4)-oxadiazolo[4,2-α]quinoxaline-1-one (ODQ) on SNP-induced decrease in the activity of ADP-ribosyl cyclase in the homogenate of cultured bovine coronary arterial smooth muscle cells. *A*: effect of OxyHb on SNP-induced inhibition of ADP-ribosyl cyclase activity before Hb and after Hb. *B*: effect of ODQ on SNP-induced decrease in the activity of ADP-ribosyl cyclase in the homogenate of bovine coronary arterial smooth muscle cells. *Significant difference from control ($P < 005$, $n = 4$).

Previous studies have indicated that cADP-ribose produces intracellular Ca^{2+} mobilization to a similar extent to IP_3 in nonvascular cells (15, 18, 30, 32). A recent study by Kannan et al. (25) reported that cADP-ribose induced the SR Ca^{2+} release in β-escin-permeabilized smooth muscle cells freshly isolated from porcine coronary arteries. With the use of α-toxin-permeabilized CASMCs, the present study directly observed the release of Ca^{2+} from the SR in response to cADP-ribose. In the presence of a specific antagonist of cADPR-ribose, 8-bromo-cADP-ribose, the effect of cADP-ribose on the SR Ca^{2+} release was completely blocked. However, 8-bromo-cADP-ribose did not alter the IP_3-induced Ca^{2+} release response in these cells. Taken together, these data confirm that cADP-ribose mobilizes intracellular Ca^{2+} in CASMCs through a mechanism independent of IP_3.

NO produces vasodilation through a decrease in $[Ca^{2+}]_i$ in vascular smooth muscle cells (7, 19, 26). However, the mechanism by which NO reduces $[Ca^{2+}]_i$ in these cells is poorly understood. The present study examined the effect of the NO donor SNP on the cADP-ribose- and IP_3-sensitive Ca^{2+} release in α-toxin-permeabilized CASMCs. SNP was not found to have an effect on either the cADP-ribose- or IP_3-induced Ca^{2+} release response in these cells, suggesting that NO does not directly alter the action of cADP-ribose and IP_3 on the SR. Because cADP-ribose and IP_3 were exogenously administered to induce Ca^{2+} from the SR, these experiments did not address whether endogenously produced cADP-ribose plays a role in the control of $[Ca^{2+}]_i$ and in mediating the effect of NO on $[Ca^{2+}]_i$.

To answer this question, we examined the effects of the cADP-ribose antagonist 8-bromo-cADP-ribose on the KCl-induced increase in $[Ca^{2+}]_i$ in CASMCs. 8-Bromo-cADP-ribose has been reported to be cell membrane permeable in previous studies (31). With the use of HPLC analysis, the present study also demonstrated that 8-bromo-cADP-ribose can enter and accumulate in cultured CASMCs. Therefore, this cell membrane-permeant cADP-ribose antagonist can be used to study the role of endogenous cADP-ribose in the control of $[Ca^{2+}]_i$. In these experiments, KCl was found to increase $[Ca^{2+}]_i$. Pretreatment of CASMCs with 8-bromo-cADP-ribose significantly attenuated the KCl-induced Ca^{2+} release response, as indicated by the decrease in the initial peak of the Ca^{2+} transient. These results suggest that endogenous cADP-ribose-mediated Ca^{2+} release from the SR contributes to $[Ca^{2+}]_i$ in CASMCs. This effect of cADP-ribose may be associated with CICR activation. It is documented that KCl depolarizes the cell membrane of vascular smooth muscle cells and subsequently activates voltage-dependent Ca^{2+} channels, resulting in Ca^{2+} influx. Increased Ca^{2+} influx activates CICR and consequently produces a large global increase in $[Ca^{2+}]_i$ and vasoconstriction (6). There is considerable evidence that cADP-ribose participates in CICR in nonvascular tissues (16, 30, 31, 36). However, the mechanism by which cADP-ribose mediates CICR remains unknown. It is possible that a small increase in $[Ca^{2+}]_i$ by influx results in cADP-ribose production, activation of cADP-ribose-mediated Ca^{2+} mobilization, or augmentation of the sensitivity of the Ca^{2+} pool to cADP-ribose, thereby leading to CICR and a large global increase in $[Ca^{2+}]_i$ throughout the cytoplasma and nucleus.

Importantly, the present study demonstrated that the NO donor SNP significantly inhibited the KCl-induced increase in $[Ca^{2+}]_i$ in CASMCs and that 8-bromo-cADP-ribose attenuated the inhibitory effect of NO. The effect of SNP on the KCl-induced increase in $[Ca^{2+}]_i$ may be associated with inhibition of endogenous cADP-ribose-mediated Ca^{2+} release in these smooth muscle cells. This view is supported by three lines of evidence. First, SNP primarily attenuated the initial Ca^{2+} transient to KCl in CASMCs, and 8-bromo-cADP-ribose abolished the inhibitory effect of SNP. It has been demonstrated that the initial peak of the

Ca^{2+} transient in single cell measurements primarily reflects Ca^{2+} release, and a sustained increase in $[Ca^{2+}]_i$ mainly indicates Ca^{2+} influx (24, 45). The interaction of 8-bromo-cADP-ribose with SNP on the initial peak of the KCl-induced Ca^{2+} transient indicates actions on intracellular Ca^{2+} release. Second, ryanodine was also found to reduce the initial KCl-induced Ca^{2+} transient and to block the effect of SNP. Because ryanodine is known to inhibit Ca^{2+} release from the SR independent of IP_3, the ryanodine-sensitive component of the KCl-induced Ca^{2+} response should represent Ca^{2+} release through SR ryanodine receptors. It seems that SNP primarily inhibits the KCl-induced increase in $[Ca^{2+}]_i$ through ryanodine-sensitive mechanism, a mechanism of the action of cADP-ribose (17, 31, 32).

Finally, we examined the effect of 8-bromo-cADP-ribose and SNP on the U-46619-induced increase in $[Ca^{2+}]_i$. U-46619, a thromboxane A_2 mimetic, produces a rise of $[Ca^{2+}]_i$ through an increase in Ca^{2+} influx and IP_3-mediated Ca^{2+} release (47, 53). In these experiments, U-46619 was found to produce an increase in $[Ca^{2+}]_i$ in CASMCs. Neither blockade of cADPR nor addition of SNP altered the U-46119-induced Ca^{2+} response. This further suggests that SNP at a concentration used in this study does not alter Ca^{2+} influx or IP_3-mediated Ca^{2+} signaling.

However, the view that NO inhibits cADP-ribose-mediated Ca^{2+} release is not in concordance with the findings of previous studies in nonvascular cells. Previous studies have reported that NO increases the production of cADP-ribose and consequently increases Ca^{2+} release from the endoplasmic reticulum in nonvascular cells such as macrophage lines, neurons, pancreatic β-cells, and urchin eggs (18, 31, 50, 52). The mechanism by which NO decreases $[Ca^{2+}]_i$ in vascular smooth muscle cells but increases $[Ca^{2+}]_i$ in some other cells remains unknown. It has been assumed that NO may have different effects on the enzyme activities responsible for the production and degradation of cADP-ribose in vascular smooth muscle cells compared with other cells (31).

To test this hypothesis, we examined the effects of NO on the ADP-ribosyl cyclase and cADP-ribose hydrolase activity in CASMCs. Interestingly, we found that NO decreased the ADP-ribosyl cyclase activity, but it had no effect on the cADP-ribose hydrolase activity. Although these results do not support the view that NO increases the cADP-ribose hydrolase activity (31), the inhibition of the ADP-ribosyl cyclase activity may decrease the concentrations of cADP-ribose and lower $[Ca^{2+}]_i$. Therefore, based on these observations, we conclude that cADP-ribose increases SR Ca^{2+} release in vascular smooth muscle cells and that NO inhibits the ADP-ribosyl cyclase activity, decreases the production of cADP-ribose, and reduces $[Ca^{2+}]_i$ in these cells.

Because NO has been reported to stimulate the ADP-ribosyl cyclase activity through the production of cGMP in nonvascular cells (18, 31, 50), we wondered whether alteration of the production of cGMP is also involved in the inhibitory effect of NO on the ADP-ribosyl cyclase activity in CASMCs. To address this issue, the effect of ODQ, a guanylyl cyclase inhibitor, on the SNP-induced decrease in the ADP-ribosyl cyclase activity was examined in the homogenate of CASMCs. We found that ODQ had no effect on the ADP-ribosyl cyclase activity, suggesting that the activation of guanylyl cyclase does not contribute to the effect of NO on the ADP-ribosyl cyclase activity in CASMCs. Consistent with these results, addition of cGMP even at concentrations much higher than physiological concentrations was without effect on the ADP-ribosyl cyclase activity. Thus the inhibitory effect of NO on the ADP-ribosyl cyclase activity is mediated by mechanisms independent of cGMP that remain to be determined.

The present study did not attempt to determine the role of the cADP-ribose signaling pathway in mediating the vasomotor response to other agonists. However, there is increasing evidence indicating that cADP-ribose serves as a second messenger mediating the effects of a number of agonists that mobilize intracellular Ca^{2+} in nonvascular tissues or cells (9, 42, 46). In longitudinal intestinal muscle and tracheal smooth muscle, cholecystokinin and 5-hydroxytryptamine have been reported to act through the cADP-ribose pathway (17). However, a recent study reported that the cADP-ribose signaling pathway was not involved in the vasoconstrictor response of rabbit airway smooth muscle to carbachol (23). Further studies are needed to define which types of agonists act through the cADP-ribose pathway in vascular smooth muscle cells.

In summary, the present study demonstrated that cADP-ribose produced SR Ca^{2+} release in CASMCs and that SNP inhibited the production of cADP-ribose and consequently decreased intracellular Ca^{2+} mobilization in these cells. These results indicate that endogenous cADP-ribose may play an important role in the control of $[Ca^{2+}]_i$ in vascular smooth muscle cells and in the mediation of the NO-induced decrease in $[Ca^{2+}]_i$ and vasodilation in the coronary circulation.

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