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Cyclic ADP-Ribose Contributes to Contraction and Ca^{2+} Release by M_1 Muscarinic Receptor Activation in Coronary Arterial Smooth Muscle

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Key Words
Cyclic adenosine diphosphate-ribose · Muscarinic receptors · Calcium · Coronary artery · Vascular smooth muscle cells

Abstract
The present study determined the role of cyclic ADP-ribose (cADPR) in mediating vasoconstriction and Ca^{2+} release in response to the activation of muscarinic receptors. Endothelium-denuded small bovine coronary arteries were microperfused under transmural pressure of 60 mm Hg. Both acetylcholine (ACh; 1 nmol/L to 1 μmol/L) and oxotremorine (OXO; 2.5–80 μmol/L) produced a concentration-dependent contraction. The vasoconstrictor responses to both ACh and OXO were significantly attenuated by nicotinamide (Nicot; an ADP-ribosyl cyclase inhibitor), 8-bromo-cADPR (8-Br-cADPR; a cADPR antagonist) or ryanodine (Ry; an Ry receptor antagonist). Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was determined by fluorescence spectrometry using fura-2 as a fluorescence indicator. OXO produced a rapid increase in $[Ca^{2+}]_i$ in freshly isolated single coronary arterial smooth muscle cells (CASMCs) bathed with Ca^{2+}-free Hanks' solution. This OXO-induced rise in $[Ca^{2+}]_i$ was significantly reduced by pirenzepine (PIR; an M_1 receptor-specific blocker), Nicot, 8-Br-cADPR or Ry. The effects of OXO on the activity of ADP-ribosyl cyclase (cADPR synthase) were examined in cultured CASMCs by measuring the rate of cyclic GDP-ribose (cGDPR) formation from β-nicotinamide guanine dinucleotide. It was found that OXO produced a concentration-dependent increase in the production of cGDPR. The stimulatory effect of OXO on ADP-ribosyl cyclase was inhibited by both PIR and Nicot. These results suggest that the cADPR signaling pathway participates in the contraction of small coronary arterial smooth muscle and Ca^{2+} release induced by activation of M_1 muscarinic receptors.

Introduction

Cyclic ADP-ribose (cADPR), an endogenous metabolite of nicotinamide adenine dinucleotide, has been identified as an intracellular second messenger in a variety of mammalian cells. This signaling nucleotide initiates Ca^{2+}-induced Ca^{2+} release through activation of ryanodine (Ry) receptors (RyRs), which is completely independent of the inositol 1,4,5-triphosphate (IP_3) signaling pathway [1–10]. cADPR-mediated signaling has been indicated to participate in the regulation of many cell functions or physiological processes, including insulin secretion, egg fertilization, cell proliferation and nitric oxide-induced Ca^{2+} movement [3, 5, 9, 11–14]. Recent studies in our laboratory have demonstrated that inhibition of cADPR formation produces coronary vasodilatation, and nitric oxide inhibits Ca^{2+} mobilization through the cADPR sig-

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naling pathway in coronary arterial smooth muscle cells (CASMCs) [11, 14, 15]. However, it remains unclear whether the cADPR signaling pathway is linked to any agonist receptors on the cellular membrane surface. Also, little is known regarding the vasoactive agonists that exert their effects on coronary arteries through this signaling pathway.

Previous studies have shown that the neurotransmitter acetylcholine (ACh) contracts the vascular smooth muscle without the endothelium [16, 17]. Its direct vasoconstrictor effect, in combination with its stimulatory action on endothelial nitric oxide, may play an important role in the regulation of vascular tone. However, the mechanisms by which activation of muscarinic ACh receptors (mAChRs) directly contracts vascular smooth muscle are not fully understood. In NG108-15 neuronal cells, activation of M_1 mAChRs has been reported to increase the production of cADPR [18]. Given that ACh was reported to contract nonvascular smooth muscle through RyR-mediated Ca^{2+} release [19, 20], we hypothesized that cADPR may serve as an intracellular second messenger mediating mAChR agonist-induced Ca^{2+} mobilization via RyR activation and vasoconstriction in small bovine coronary arteries. The present study was designed to test this hypothesis. Using endothelium-denuded, pressurized small bovine coronary arteries, we first examined the effects of cADPR antagonist and RyR blocker on ACh- and oxotremorine (OXO; an M_1 receptor agonist)-induced vasoconstriction. By measuring the intracellular Ca^{2+} ($[Ca^{2+}]_i$) release response in single CASMCs, we then determined the contribution of the cADPR-signaling pathway to Ca^{2+} release induced by M_1 receptor activation. Also, to directly measure the effects of M_1 receptor activation on cADPR production, we examined the effects of M_1 receptor activation and blockade on the activity of ADP-ribosyl cyclase in bovine CASMCs using high-performance liquid chromatography (HPLC) analysis.

Materials and Methods

Isolation of Small Coronary Arteries

Fresh bovine hearts were obtained from a local abattoir. The left ventricular wall was rapidly dissected and immersed in ice-cold phosphate-buffered saline (PBS; Sigma; pH 7.4). The myocardial sections were transported immediately to the laboratory and used to dissect intramyocardial coronary arteries from the branches of the left anterior descending artery. The artery segments (5–8 mm in length) were cleaned of fat and connective tissues under a dissection microscope and placed in cold PBS. A segment of hair was passed through the rings of the lumen of small coronary arteries. The endothelium was removed by gently rotating the hair [21].

Vascular Reactivity in in vitro Perfused Small Coronary Arteries

Vascular reactivity in isolated perfused and pressurized small bovine coronary artery was measured as we described previously [22]. Dissected small coronary arteries were transferred to a water-jacketed perfusion chamber and cannulated with two glass micropipettes. The outflow cannula was clamped, and the arteries were bathed under the transmural pressure of 60 mm Hg in physiological saline solution containing (in mmol/L): NaCl, 119; KCl, 4.7; $CaCl_2$, 1.6; $MgSO_4$, 1.17; NaH_2PO_4, 1.18; $NaHCO_3$, 24; EDTA, 0.026, and glucose, 5.5 (pH 7.4). The internal diameter of the arteries was measured with a video system composed of a stereomicroscope (Leica MZ8), a charge-coupled device camera (KP-MI AU, Hitachi), a video monitor (VM-1220U, Hitachi), a video measuring apparatus (VIA-170, Boeckeler Instrument) and a video printer (UP890 MD, Sony). The arterial images were recorded continuously with a videocassette recorder (M-674, Toshiba). Only arterial segments with an internal diameter of 0.1–0.2 mm at a pressure of 60 mm Hg were used in the present study.

After a 60-min equilibration period under the transmural pressure of 60 mm Hg, ACh (1 nmol/L to 1 μmol/L) or OXO (2.5–80 μmol/L) was cumulatively added to the bath solution to induce vasoconstriction. When ACh- or OXO-induced contraction reached a stable plateau at each concentration, the internal diameter of the vessel was measured and recorded. To examine the contribution of the cADPR or IP_3 signaling pathway to OXO- and ACh-induced vasoconstriction, the arteries were pretreated for 30 min with one of the following compounds: nicotinamide (Nicot; an inhibitor of ADP-ribosyl cyclase) (6 mmol/L) [23], 8-bromo-cADPR (8-Br-cADPR; a cADPR antagonist) (30 μmol/L) [3], Ry (an antagonist of RyRs) (30 μmol/L) [3, 24] or 2-aminoethoxydiphenyborate [2-APB; a cell membrane-permeable inhibitor of IP_3 receptors (IP_3R)] (30 μmol/L) [25]. Then, ACh or OXO was added, and the vasoconstrictor response was observed as described above. In additional groups of arteries, analogues of Nicot and 8-Br-cADPR, niacin (6 mmol/L) and 8-Br-cAMP (30 μmol/L), respectively, were tested as negative controls. The contractile responses to ACh or OXO were expressed as the percentage reduction in vascular internal diameters. Physiological saline solution in the bath was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 and maintained at 37 ± 0.1°C throughout the experiment.

Preparation of Single CASMCs

Single smooth muscle cells from small bovine coronary arteries were prepared by enzymatic dissociation as we described previously [14]. Briefly, dissected small coronary arteries were first incubated for 25 min at 37°C with collagenase type II (340 units/ml; Worthington), elastase (15 units/ml; Worthington), dithiothreitol (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml) in HEPES buffer consisting of (in mmol/L): NaCl, 119; KCl, 4.7; $CaCl_2$, 0.05; $MgCl_2$, 1; glucose, 5, and HEPES, 10 (pH 7.4). The digested arterial tissue was then agitated with a glass pipette to free vascular smooth muscle cells, and the supernatant was collected. Remaining tissue was further digested with fresh enzyme solution, and the supernatant was collected at 5-min intervals for an additional 15 min. The supernatants were pooled and diluted 1:10 with HEPES buffer and stored at 4°C until used.

Measurement of $[Ca^{2+}]_i$ in Single CASMCs

Determination of $[Ca^{2+}]_i$ in single CASMCs was performed using fura-2 as an indicator. The cells were first loaded with fura-2 acetoxymethyl ester (fura-2-Am 5 μmol/L; Molecular Probes, Eugene, Oreg.,

USA) at 37°C for 45 min. A polylysine-coated glass coverslip was mounted at the bottom of a chamber, which was mounted horizontally on a Nikon Diaphoto TMD inverted microscope (Nikon, Japan). The cells on the coverslip were incubated with Ca^{2+}-free Hanks' buffer for 20 min to allow the complete hydrolysis of intracellular fura-2-Am ester to fura-2. OXO (80 μmol/L) was applied to the bath solution to induce Ca^{2+} release. To examine the specificity of OXO on mAChRs and the role of the cADPR signaling pathway in OXO-induced Ca^{2+} release, CASMCs were pretreated for 15 min with pirenzepine (PIR; a selective inhibitor of M_1 mAChRs) (0.1 μmol/L), Nicot (6 μmol/L), Ry (30 μmol/L) or 8-Br-cADPR (30 μmol/L). The concentrations of these inhibitors were based on the results obtained from in vitro microperfusion of coronary arteries and their ability to inhibit OXO-induced vasoconstriction. In another group of CASMCs, 2-APB alone or in combination with 8-Br-cADPR was added to the bath solution, and then the OXO-induced Ca^{2+} release response was observed. The ratio of fura-2 emissions, when excited at wavelengths of 340 and 380 nm, was monitored using a fluorescent ratio spectrofluorometric microscope (PTI) [11].

Culture of CASMCs

To determine the effects of OXO on ADP-ribosyl cyclase activity, cultured bovine CASMCs were used. As we described previously [26], endothelium-denuded coronary arteries were rinsed with medium 199 containing 5% FCS, a 2% solution of antibiotics (penicillin-streptomycin-amphotericin B), 0.3% gentamicin and 0.3% nystatin, and cleaned of connective tissues. After being cut into small pieces, the arteries were placed into dishes with the lumen side down and incubated in medium 199 containing 10% FCS, 1% glutamine, 1% antibiotic solution, 0.3% gentamycin, 0.3% nystatin and 0.1% tylosin for 3–5 days until vascular smooth muscle cells migrated to the dishes. Once smooth muscle cell growth had been established, the vessels were removed, and the cells were grown in medium 199 containing 20% FCS. All cells were maintained in an incubator with 5% CO_2 in air at 37°C. The smooth muscle cells were identified by positive staining with an anti-α-actin antibody. All studies were performed using the cells of 2–4 passages.

HPLC Assay of ADP-Ribosyl Cyclase Activity

Cultured CASMCs at confluence were rinsed 3 times with 10 ml of chilled PBS and collected using a cell scraper at 4°C. The cells were divided into 4 different Eppendorf tubes (1 ml each) and preincubated at 37°C for 10 min. To examine the effects of OXO, PIR and Nicot on ADP-ribosyl cyclase activity, CASMCs were treated with OXO (80 μmol/L) in the presence of PIR (0.1 μmol/L), Nicot (6 μmol/L) or PBS (control), respectively. After incubation at 37°C for 15 min, the cells were washed with Hanks' solution. In another group of experiments, CASMCs were incubated with different concentrations of OXO at 37°C for 1, 3, 5 and 15 min to stimulate cADPR production and to determine the time course of OXO-induced activation of ADP-ribosyl cyclase, respectively. The pellets of cells were suspended in HEPES buffer (pH 7.0) containing (in mmol/L): HEPES, 10; NaCl, 148; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 0.3, and glucose, 5.5. They were then sonicated 6 times (each time for 20 s) with a sonifier cell disrupter (Branson, Model 185) at 4°C. After centrifugation at 3,500 *g* for 10 min, the supernatant was collected. To determine ADP-ribosyl cyclase activity, the supernatant of 100 μg of protein was incubated with 100 μmol of β-nicotinamide guanine dinucleotide (β-NGD^+) at 37°C for 60 min. The reaction mixtures were centrifuged at 4°C through an Amicon microultrafilter at 13,800 *g* to remove proteins, and then analyzed by HPLC with a fluorescence detector (Hewlett-Packard 1090 HPLC system and 1046A spectrofluorometer). The excitation wavelength of 300 nm and the emission wavelength of 410 nm were used to detect the fluorescent products. All HPLC data were collected and analyzed by a Hewlett-Packard Chemstation [11].

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, Bellefonte, Pa., USA). The injection volume was 20 μl. The mobile phase consisted of 150 m*M* ammonium acetate (pH 5.5) containing 5% methanol (solvent A) and 50% methanol (solvent B). The solvent system was a linear gradient of 5% solvent B in A to 30% solvent B in A over 1 min, held for 25 min, and then 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. Quantitative measurements were performed by comparison of known concentrations of standards [11].

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM). The significance of the differences in mean values between and within multiple groups was examined using analysis of variance for repeated measures followed by Duncan's multiple range test. Student's t test was used to evaluate the statistical significance of differences between two paired observations. $p < 0.05$ was considered statistically significant.

Results

ACh-Induced Contraction in Endothelium-Denuded Small Coronary Arteries

During a 60-min equilibration period, the internal diameters of small coronary arteries were not significantly changed. There were no significant differences in baseline internal diameters between groups of vessels in each experiment. Cumulative addition of ACh (1 nmol/L to 1 μmol/L) induced a concentration-dependent vasoconstriction. After washing and equilibration, repeated addition of ACh produced similar contraction to that in the first experiment, indicating that ACh-induced coronary vasoconstriction is repeatable in this preparation.

To determine the contribution of the cADPR signaling pathway to ACh-induced vasoconstriction, the vessels were treated for 30 min with Nicot (6 μmol/L), Ry (30 μmol/L) or 8-Br-cADPR (30 μmol/L), respectively. In the presence of Nicot, ACh-induced contraction was significantly attenuated. The maximal contraction was changed from 49.8 ± 4.0 to 15.8 ± 2.6% (n = 5) (fig. 1A). Similarly, in the presence of Ry or 8-Br-cADPR, ACh-induced concentration-dependent contraction was significantly decreased (fig. 1B, C). Ry decreased the maximal contraction induced by ACh from 46.2 ± 4.1 to 32.8 ± 3.5% (n = 6), while 8-Br-cADPR decreased it from 43.0 ± 3.7 to 22.5 ± 5.3% (n = 5).

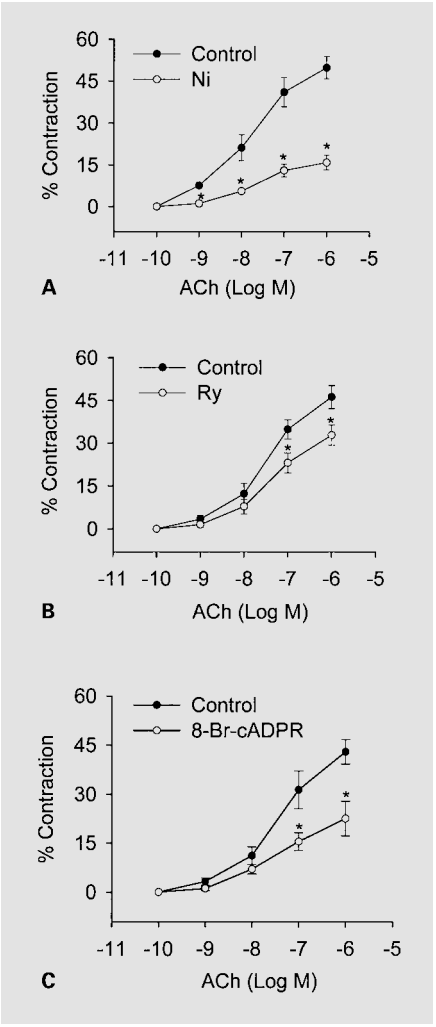

Fig. 1. ACh-induced contraction of endothelium-denuded small coronary arteries. A Concentration-dependent vasoconstrictor response to ACh in the absence or presence of the ADP-ribosyl cyclase inhibitor Nicot (Ni; 6 mmol/L). B Concentration-dependent vasoconstrictor response to ACh in the absence or presence of the RyR blocker Ry (30 μmol/L). C Concentration-dependent vasoconstrictor response to ACh in the absence or presence of a cell-permeable cADPR antagonist, 8-Br-cADPR (30 μmol/L). * $p < 0.05$ compared to control (n = 5–6).

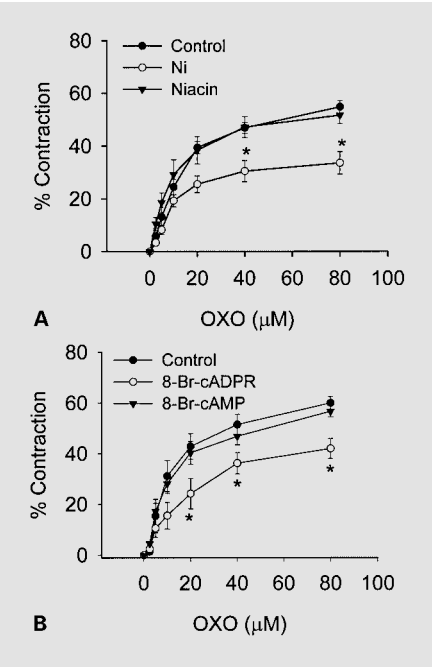

Fig. 2. Concentration-contraction curves in response to OXO in endothelium-denuded small coronary arteries. A OXO-induced concentration-dependent vasoconstriction in the absence or presence of Nicot (Ni; 6 mmol/L). B OXO-induced concentration-dependent vasoconstriction in the absence or presence of 8-Br-cADPR (30 μmol/L). * $p < 0.05$ compared to control (n = 5–6).

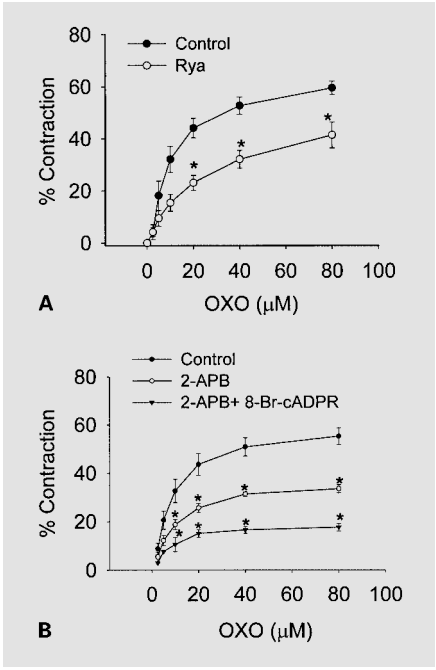

Fig. 3. Effects of RyR or IP_3R blockade on OXO-induced vasocontraction. A OXO-induced concentration-dependent vasoconstriction in the absence or presence of Ry (30 μmol/L). B OXO-induced concentration-dependent vasoconstriction in the absence or presence of the cell-permeable IP_3R antagonist 2-APB (30 μmol/L) alone or in combination with 8-Br-cADPR (30 μmol/L). * $p < 0.05$ compared to control (n = 6).

OXO-Induced Contraction in Endothelium-Denuded Small Coronary Arteries

In endothelium-denuded bovine small coronary arteries, cumulative addition of OXO (2.5–80 μmol/L) also produced a concentration-dependent contraction (fig. 2). OXO-induced contraction usually began 30 s to 1 min after application of OXO to the bath solution and lasted for more than 20 min. OXO at a concentration of 80 μmol/L caused the maximal contraction of 59.3 ± 1.4% (n = 18).

To determine whether OXO-induced vasoconstriction involves the cADPR signaling pathway, the arteries were pretreated with the compounds which block cADPR production or action. In the presence of Nicot (6 mmol/L), OXO-induced contraction at concentrations of 20–80 μmol/L was significantly reduced (fig. 2A). The maximal contraction was decreased from 54.8 ± 2.4 to 33.6 ± 4.3% (n = 6). Moreover, blockade of the cADPR signaling pathway by a specific antagonist, 8-Br-cADPR (30 μmol/L), significantly attenuated OXO-induced coronary vasoconstriction (fig. 2B). However, neither niacin nor 8-Br-cAMP, the analogues of Nicot and 8-Br-cADPR, respectively, had an effect on OXO-induced coronary vasoconstriction.

Table 1. Effects of an M_1 mAChR antagonist and cADPR-RyR inhibitors on basal $[Ca^{2+}]_i$ in smooth muscle cells from bovine coronary arteries

Group	$[Ca^{2+}]_i$, n*M*	n	p
Control	123.8 ± 3.4	46	
PIR	117.5 ± 4.8	15	0.3
Nicot	139.4 ± 13.0	17	0.1
8-Br-cADPR	116.0 ± 5.3	14	0.3
Ry	137.1 ± 12.3	16	0.1

Each value represents the mean ± SEM for the number indicated.

Effects of RyR and IP_3R Antagonists on OXO-Induced Vasoconstriction

It has been reported that cADPR-mediated Ca^{2+} release is associated with the activation of RyR on the sarcoplasmic reticulum (SR) of muscle cells. The present study also examined the effect of RyR blocker on OXO-induced coronary vasoconstriction. It was found that the contraction induced by OXO at concentrations of 10–80 μmol/L was also significantly decreased by Ry (30 μmol/L). The maximal contraction was reduced from 59.5 ± 2.6 to 34.9 ± 5.0% (n = 6) (fig. 3A).

Since OXO-induced coronary vasoconstriction was not completely blocked by the Ry receptor or cADPR antagonist, we examined the effect of IP_3R antagonist on OXO-induced contraction. In the presence of 2-APB (30 μmol/L), OXO-induced vasocontraction was significantly reduced. The maximal contraction was attenuated from 55.2 ± 3.5 to 33.5 ± 1.7% (n = 6). In the presence of both 8-Br-cADPR and 2-APB, however, OXO only produced a 17.6 ± 1.6% contraction, representing a 68% decrease in OXO-induced vasoconstriction (n = 6; fig. 3B).

OXO-Induced Ca^{2+} Release

The basal $[Ca^{2+}]_i$ in bovine CASMCs was 123.8 ± 3.4 n*M* (n = 46 cells). After CASMCs were pretreated with PIR (0.1 μmol/L), Nicot (6 mmol/L), 8-Br-cADPR (30 μmol/L) and Ry (30 μmol/L) for 15 min, the basal $[Ca^{2+}]_i$ was not significantly altered. The results from these experiments are presented in table 1.

As shown in figure 4A, OXO (80 μmol/L) evoked a rapid Ca^{2+} transient in CASMCs exposed to Ca^{2+}-free Hanks' solution with 1 m*M* EGTA. Peak $[Ca^{2+}]_i$ increased from 122.1 ± 5.9 to 343.1 ± 14.4 n*M* (n = 22). When the cells were pretreated with the M_1 mAChR blocker PIR

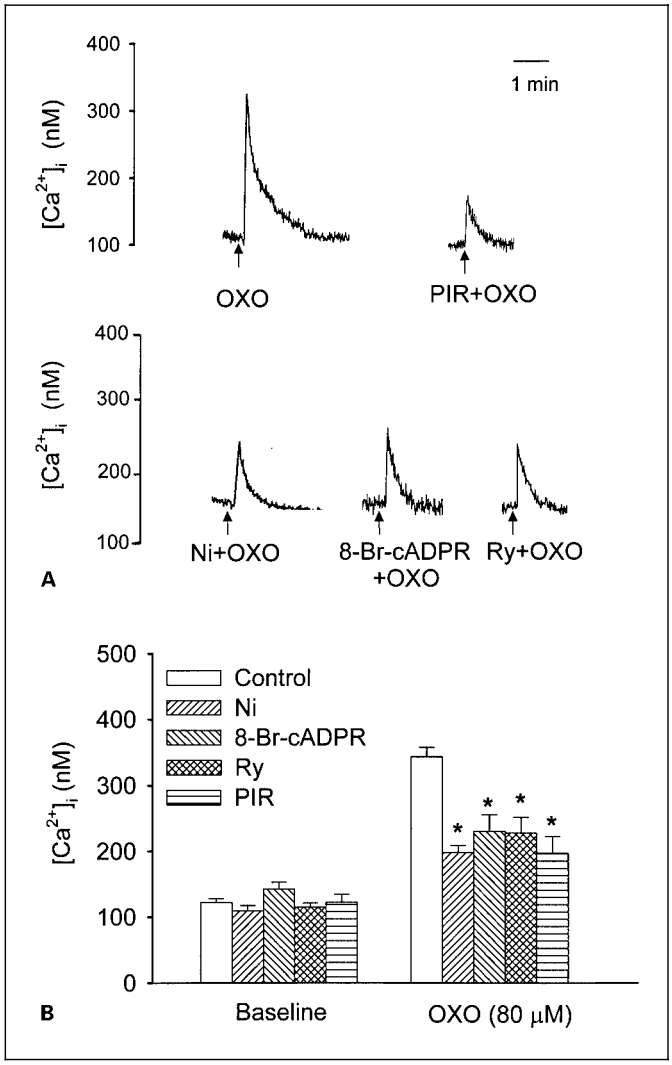

Fig. 4. Inhibition of OXO-induced Ca^{2+} release by PIR, Nicot (Ni), 8-Br-cADPR and Ry in smooth muscle cells from small bovine coronary arteries. A Representative recordings of the ratio of fura-2 fluorescence. B Summarized data showing the changes from baseline $[Ca^{2+}]_i$ to the integrated peak transient $[Ca^{2+}]_i$ response to OXO in the presence of PIR (0.1 μmol/L, n = 15), Nicot (6 mmol/L, n = 17), 8-Br-cADPR (30 μmol/L, n = 14) or Ry (30 μmol/L, n = 16). * $p < 0.05$ compared to control.

(0.1 μmol/L) for 15 min, the OXO (80 μmol/L)-induced maximal increase in $[Ca^{2+}]_i$ was significantly attenuated to 197.8 ± 10.8 n*M*, a reduction of 42.3% (n = 15). Inhibition of cADPR production in CASMCs with Nicot (6 mmol/L) also significantly reduced the OXO-induced Ca^{2+} release to 229.9 ± 25.3 n*M*, a 33% reduction (n = 17). When the cADPR antagonist 8-Br-cADPR was used to treat CASMCs, OXO-induced Ca^{2+} release was mark-

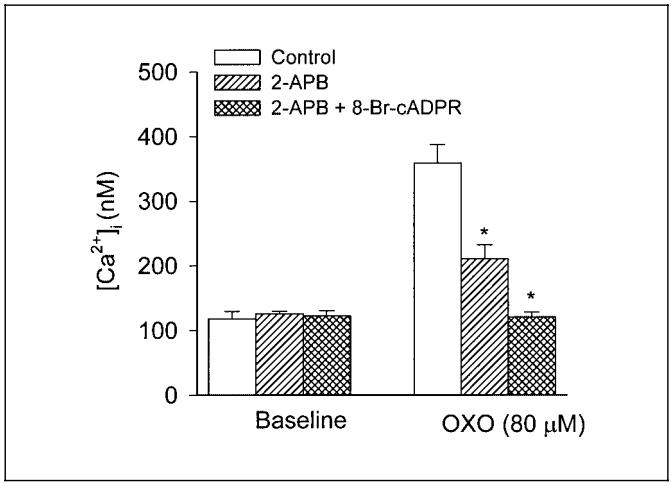

Fig. 5. OXO-induced Ca^{2+} release in the absence or presence of the IP_3R antagonist 2-APB (30 μmol/L) alone or in combination with 8-Br-cADPR (30 μmol/L). * $p < 0.05$ compared to control.

edly inhibited by 34%. Similarly, in the presence of Ry (30 μmol/L), OXO-stimulated Ca^{2+} release was significantly attenuated by 35% (n = 16). The results are summarized in figure 4B.

Consistent with the results obtained from arterial preparations, OXO-induced Ca^{2+} release was also reduced by the IP_3 antagonist 2-APB. In the presence of 2-APB, the maximal Ca^{2+} release response was blocked by 56.2%. A combination of 2-APB and 8-Br-cADPR substantially inhibited the Ca^{2+} release response to OXO by 94.5% (n = 6) (fig. 5).

ADP-Ribosyl Cyclase Activity in Cultured CASMCs

Figure 6A presents a typical fluorescence HPLC chromatograph showing cyclic GDP-ribose (cGDPR) production from β-NGD^+ by cultured CASMCs. This product coeluted with the cGDPR standard at a retention time of 2.2 min. Under control conditions, the conversion rate of β-NGD^+ to cGDPR was 23.5 ± 0.96 pmol/min/mg protein (n = 5). When CASMCs were incubated with OXO, a time- and concentration-dependent increase in cGDPR production was observed. At concentrations of 40–80 μmol/L, OXO significantly stimulated the conversion of β-NGD^+ to cGDPR, even with 1-min incubation. The maximal conversion rate of β-NGD^+ to cGDPR was 57.5 ± 3.3 pmol/min/mg protein (n = 5), which was observed at 80 μmol/L OXO with 15 min of incubation with CASMCs (fig. 6B). In the presence of PIR (0.1 μmol/L), even the highest concentration of OXO (80 μmol/L) could not increase the activity of ADP-ribosyl cyclase. Similarly, pretreatment of CASMCs with the ADP-ribosyl cyclase inhibitor Nicot (6 mmol/L) also significantly inhibited OXO-stimulated conversion of β-NGD^+ to cGDPR (n = 5) (fig. 6C).

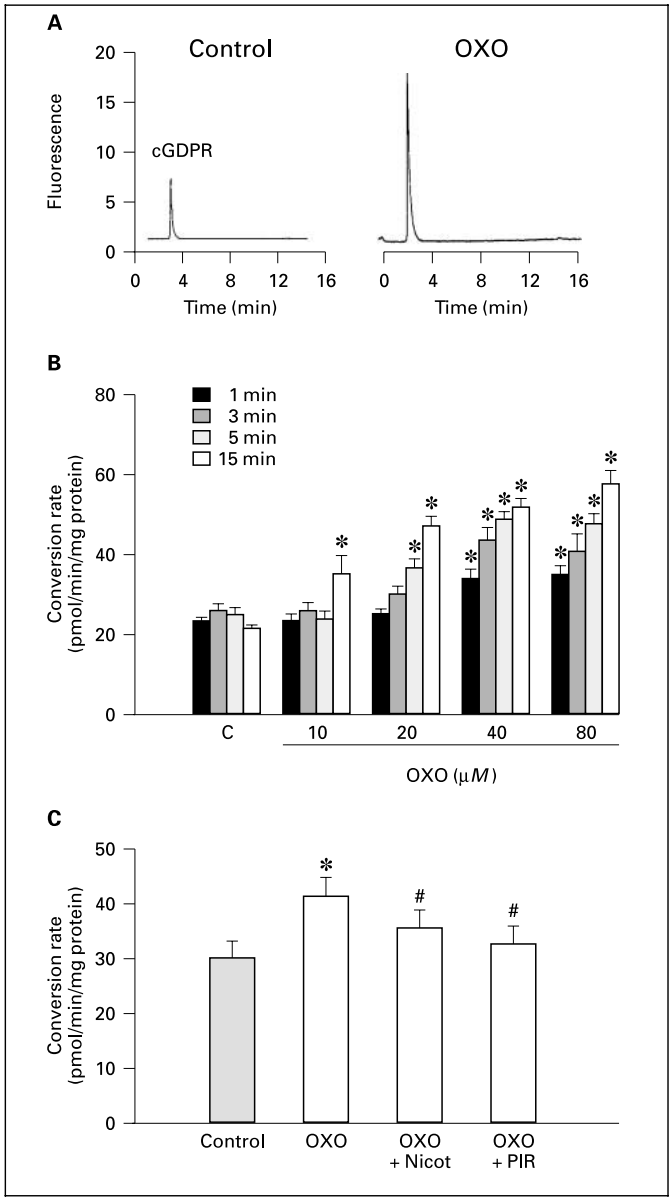

Fig. 6. Concentration-dependent activation by OXO of ADP-ribosyl cyclase. A Typical fluorescence chromatograms depicting the effect of OXO on cGDPR production in homogenates of cultured CASMCs. B Summarized data showing a time course of OXO-induced concentration-dependent conversion of β-NGD^+ to cGDPR by ADP-ribosyl cyclase. C Summarized data showing the conversion rate of β-NGD^+ to cGDPR by ADP-ribosyl cyclase in the presence of OXO, OXO + PIR or OXO + Nicot (n = 5). * $p < 0.05$ compared to control (n = 5).

Discussion

The present study demonstrated that (1) both ACh and OXO induced contraction in endothelium-denuded small coronary arteries, (2) inhibition of cADPR production or action attenuated ACh- and OXO-induced coronary vasoconstriction, (3) stimulation of M_1 mAChRs by OXO evoked Ca^{2+} release from RyR-sensitive stores in single CASMCs, and (4) OXO-induced M_1 mAChR activation increased cADPR production. These results suggest that cADPR contributes to the contraction of small coronary arterial smooth muscle and Ca^{2+} release induced by M_1 mAChR activation.

ACh produces vasodilatation in most vascular beds when the endothelium is functioning normally [16, 27, 28]. ACh-induced vasodilation is linked with the release of endothelium-derived nitric oxide, prostaglandin I_2 (PGI_2) and epoxyeicosatrienoic acids [27, 29]. When the endothelium is removed or dysfunctional, ACh produces vasoconstriction in many vasculatures, including the coronary, cerebral, mesentery and renal circulation [30, 31]. However, the mechanism by which ACh directly contracts vascular smooth muscle is poorly understood. In the present study, we first explored the role of the cADPR signaling pathway in mediating ACh-induced vasoconstriction in endothelium-denuded small coronary arteries. Using a selective ADP-ribosyl cyclase inhibitor, Nicot, and a specific antagonist of cADPR action, 8-Br-cADPR, we found that blockade of cADPR production or action significantly attenuated ACh-induced vasoconstriction. These results provide direct evidence that the cADPR signaling pathway contributes to the vasoconstrictor response to ACh in the coronary circulation.

Previous studies have indicated that the effect of cADPR is dependent on RyR-mediated Ca^{2+} mobilization from Ca^{2+}-ATPase-dependent stores in the sarcoplasmic reticulum [24, 25]. In the present study, exogenous application of Ry, a RyR blocker, significantly attenuated the vasoconstrictor response to ACh. This result is consistent with the findings that RyR blockade inhibited Ca^{2+} mobilization, Ca^{2+} oscillations and vasoconstriction in porcine coronary arterial smooth muscle [32], gallbladder smooth muscle [33], smooth muscle of the gastric fundus [34] and trachea [19]. Taken together, these results support the view that the cADPR signaling pathway participates in ACh-induced vasoconstriction through RyR activation in arterial smooth muscle cells.

There are 4 pharmacologically defined subtypes of mAChRs, namely M_1, M_2, M_3 and M_4. By gene cloning, however, 5 subtypes of mAChRs, m_1 to m_5, were found in different tissues or cells [35]. In bovine coronary arteries, M_1, M_2 and M_3 mAChRs have been reported [36, 37], and they may stimulate different signaling pathways to produce vasodilation or vasoconstriction. Since a recent study has reported that M_1 mAChRs increase the activity of ADP-ribosyl cyclase in NG108-15 neural cells [18], we hypothesized that cADPR may serve as a second messenger mediating the vasoconstriction induced by the activation of M_1 mAChRs in CASMCs. To test this hypothesis, we examined the effect of blockade of cADPR production and actions on coronary vasoconstriction induced by the M_1 mAChR agonist OXO. Consistent with the effects of ACh, OXO contracted endothelium-denuded small coronary arteries. The inhibition of cADPR production and blockade of cADPR actions significantly blunted OXO-induced coronary vasoconstriction. Although OXO-induced vasoconstriction in these endothelium-denuded coronary arteries was not completely blocked by cADPR antagonist and RyR blocker, it is believed that the cADPR signaling pathway participates, at least in part, in OXO-induced coronary vasoconstriction. This cADPR-mediated mechanism produces a vasoconstrictive effect to a similar extent to that of the IP_3 signaling pathway (fig. 3). It is likely that both the cADPR and IP_3 signaling pathways are involved in vasoconstriction induced by M_1 mAChR activation.

To determine whether OXO-induced vasoconstriction is directly associated with cADPR-mediated Ca^{2+} release from sarcoplasmic reticulum in CASMCs, we examined the effects of inhibition of cADPR production and action on OXO-induced Ca^{2+} release in single CASMCs. OXO was found to produce a rapid and transient increase in Ca^{2+}, which was inhibited by a specific M_1 AChR antagonist, PIR. Since the cells were bathed with Ca^{2+}-free solution, this Ca^{2+} increase represented a Ca^{2+} release from the sarcoplasmic reticulum. Consistent with the results obtained from perfused arterial preparation, pretreatment of CASMCs with Nicot, 8-Br-cADPR and Ry blocked OXO-induced Ca^{2+} release. Although we did not detect OXO-induced Ca^{2+} oscillations in CASMCs, the rapid Ca^{2+} release response observed in the present study may lead to Ca^{2+} activation of coronary arterial smooth muscle resulting in vasoconstriction. In the present study, we also observed the inhibitory effect of IP_3R blockade on the OXO-induced Ca^{2+} release response, which may contribute to the IP_3R-mediated vasoconstriction induced by OXO. When both RyR and IP_3R were blocked by a combination of 8-Br-cADPR and 2-APB, the OXO-induced Ca^{2+} release response was substantially blocked. It should be noted that 2-APB may also affect store-operated Ca^{2+}

entry in addition to its inhibition of IP_3-induced Ca^{2+} release [38, 39]. However, since Ca^{2+} release responses were measured in Ca^{2+}-free solution in the present studies, it is impossible that Ca^{2+} entry participates in the Ca^{2+} response to OXO. Taken together, these results further indicate that activation of M_1 receptors produces Ca^{2+} release and vasoconstriction through both cADPR- and IP_3-mediated pathways.

By HPLC analysis, we found that incubation of CASMCs with OXO produced a time- and concentration-dependent activation of ADP-ribosyl cyclase, which was blocked by its inhibitor, Nicot, and a specific M_1 mAChR antagonist, PIR. This activation of ADP-ribosyl cyclase occurred rapidly even in the first minute of incubation of CASMCs with OXO. These results provide biochemical evidence that activation of M_1 mAChRs increased the production of cADPR in CASMCs, which is consistent with previous studies showing that the activation of mAChRs increased the production of cADPR in adrenal chromaffin cells and NG108-15 neuronal cells [18, 40]. In neuronal cells, ADP-ribosyl cyclase is directly coupled to M_1 mAChRs through G proteins. When these receptors are activated, the production of cADPR increases, resulting in Ca^{2+} mobilization from the endoplasmic reticulum through RyR activation [18, 41]. In these cells, cADPR serves as a second messenger to couple M_1 mAChRs to RyRs, thereby producing the effect of cADPR on neuronal activity. Taking these results together, we conclude that cADPR may play a role as a second messenger to couple activation of M_1 mAChRs and RyR-mediated Ca^{2+} release from the sarcoplasmic reticulum of vascular smooth muscle cells, thereby contributing in part to M_1 mAChR-mediated vasoconstriction in the coronary circulation.

In summary, the present study demonstrated that blockade of cADPR production and action significantly attenuated ACh- and OXO-induced vasoconstriction in small coronary arteries. In isolated single CASMCs, inhibition of both cADPR production and action substantially decreased OXO-induced Ca^{2+} release from the sarcoplasmic reticulum. OXO activated ADP-ribosyl cyclase, thereby increasing the production of cADPR. Our results suggest that cADPR-mediated Ca^{2+} mobilization represents a novel signaling pathway participating in the vasoconstrictor response to M_1 AChR activation in coronary arteries.

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