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Cyclic ADP ribose-mediated Ca^{2+} signaling in mediating endothelial nitric oxide production in bovine coronary arteries

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Zhang, Guo, Eric G. Teggatz, Andrew Y. Zhang, Matthew J. Koeberl, Fan Yi, Li Chen, and Pin-Lan Li. Cyclic ADP ribose-mediated Ca^{2+} signaling in mediating endothelial nitric oxide production in bovine coronary arteries. *Am J Physiol Heart Circ Physiol* 290: H1172–H1181, 2006. First published October 21, 2005; doi:10.1152/ajpheart.00441.2005.—The present study tested the hypothesis that cyclic ADP ribose (cADPR) serves as a novel second messenger to mediate intracellular Ca^{2+} mobilization in coronary arterial endothelial cells (CAECs) and thereby contributes to endothelium-dependent vasodilation. In isolated and perfused small bovine coronary arteries, bradykinin (BK)-induced concentration-dependent vasodilation was significantly attenuated by 8-bromo-cADPR (a cell-permeable cADPR antagonist), ryanodine (an antagonist of ryanodine receptors), or nicotinamide (an ADP-ribosyl cyclase inhibitor). By in situ simultaneously fluorescent monitoring, Ca^{2+} transient and nitric oxide (NO) levels in the intact coronary arterial endothelium preparation, 8-bromo-cADPR (30 μM), ryanodine (50 μM), and nicotinamide (6 mM) substantially attenuated BK (1 μM)-induced increase in intracellular [Ca^{2+}] by 78%, 80%, and 74%, respectively, whereas these compounds significantly blocked BK-induced NO increase by about 80%, and inositol 1,4,5-trisphosphate receptor blockade with 2-aminethoxydiphenyl borate (50 μM) only blunted BK-induced Ca^{2+}-NO signaling by about 30%. With the use of cADPR-cycling assay, it was found that inhibition of ADP-ribosyl cyclase by nicotinamide substantially blocked BK-induced intracellular cADPR production. Furthermore, HPLC analysis showed that the conversion rate of β-nicotinamide guanine dinucleotide into cyclic GDP ribose dramatically increased by stimulation with BK, which was blockable by nicotinamide. However, U-73122, a phospholipase C inhibitor, had no effect on this BK-induced increase in ADP-ribosyl cyclase activity for cADPR production. In conclusion, these results suggest that cADPR importantly contributes to BK- and A-23187-induced NO production and vasodilator response in coronary arteries through its Ca^{2+} signaling mechanism in CAECs.

nucleotide; vasodilation; endothelium; endothelium-derived relaxing factor

CYCLIC ADP RIBOSE (cADPR), endogenously generated from nicotinamide (Ni) adenine dinucleotide, has emerged as a novel intracellular Ca^{2+}-mobilizing second messenger in a wide variety of mammalian cells. The actions of this signaling nucleotide are completely independent of the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-signaling pathway, but it exerts its action by targeting ryanodine (Ry) receptors (RyRs) (8, 15, 16, 31–33, 44). Elevation of intracellular cADPR levels followed by Ca^{2+} mobilizition and activation of Ca^{2+}-dependent effects within cells has been found to be essential for versatile physiological processes, such as cell cycle regulation, cell proliferation, gene expression, egg fertilization, neurotransmitter release, muscle contraction, and nitric oxide signaling (10, 29, 55). Recent studies (17, 35, 36, 52, 55, 57) in our laboratory and by others have reported that in vascular smooth muscle (VSM), cADPR production participates in its constrictor response to Ins(1,4,5)P_3-independent agonists, such as acetylcholine, oxotremorine, 5-hydroxytryptamine, and endothelin, and these actions of cADPR are associated with activation of RyRs and consequent Ca^{2+} mobilization. However, so far it remains unclear whether cADPR is involved in the regulation of endothelial function, in particular, in participating in endothelial NO-dependent vasodilator response.

It has been reported that Ca^{2+} activation of endothelial cells is one of the most important mechanisms mediating the production of endothelium-derived relaxing factors (EDRFs), such as NO and prostacyclins (17, 50, 54, 56), thereby mediating endothelium-dependent vasodilation (EDVD). Despite extensive studies, it has still been controversial which Ca^{2+} signaling pathway is responsible for the elevation of intracellular Ca^{2+} in endothelial cells in the process of EDVD. In regard to the regulation of Ca^{2+} level in the vascular endothelium, it has been demonstrated that on stimulation with inflammatory agonists, such as thrombin, histamine, bradykinin (BK), and oxidants, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) could increase 5–10 times compared with the basal level. This agonist-induced increase in $[Ca^{2+}]_i$ occurs in two distinct phases, a transient rise due to intracellular Ca^{2+} store depletion, which involves generation of Ins(1,4,5)P_3 and Ins(1,4,5)P_3-induced Ca^{2+} release from the endoplasmic reticulum (ER), and a sustained phase due to Ca^{2+} entry into the cell from extracellular medium (Ca^{2+} influx). Increased intracellular Ca^{2+} levels, either Ca^{2+} influx or release, were found to stimulate the enzymatic binding of Ca^{2+}/calmodulin (CaM) to endothelial NO synthase (eNOS), resulting in rapid conversion of L-arginine into citrulline, producing NO (14, 42, 49). However, there is considerable evidence that blockade of Ins(1,4,5)P_3 signaling could only partially attenuate agonist-induced Ca^{2+} release as well as store-operated calcium influx and partially decreased NO production in endothelial cells (11). Other studies have reported that Ins(1,4,5)P_3 is not

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involved in the regulation of BK-induced Ca^{2+} increase in endothelial cells (21) and that cyclopiazonic acid-enhanced Ca^{2+} increase and EDRF release in bovine pulmonary arterial endothelial cells is not Ins(1,4,5)P_3 dependent (39). In addition, the activation of protein tyrosine kinases and phosphatases is also found to participate in the process of endothelial NO generation independently of the phospholipase C signaling pathway (13). Interestingly, ADP-ribosyl cyclase was recently reported to give rise to BK signal transduction from receptors to its effector enzymes (9, 23, 25). Based on these results, we hypothesized that cADPR may serve as an intracellular second messenger in mediating BK-induced Ca^{2+} mobilization in arterial endothelial cells and thereby stimulates the production of NO and participates in the EDVD.

To test this hypothesis, we first examined the effects of cADPR antagonist, ADP-ribosyl cyclase inhibitor, and RyRs blocker on BK-induced vasodilation by using pressurized intact small bovine coronary arterial preparation. By simultaneously measuring $[Ca^{2+}]_i$ and NO production in the intact endothelium of small bovine coronary arteries using a high-speed wavelength-switching fluorescence imaging system, we then determined the contribution of this cADPR signaling pathway to Ca^{2+} release and NO production induced by BK receptor activation or Ca^{2+} ionophore A-23187. Moreover, to directly measure the effects of BK on cADPR production in isolated coronary arterial endothelial cells (CAECs), we quantified the intracellular cADPR levels using cADPR cycling assay and determined BK-induced changes in intracellular cADPR levels. We then went to analyze the effects of BK on the activity of ADP-ribosyl cyclase to produce cADPR in bovine CAECs using HPLC technique. In addition, the role of Ins(1,4,5)P_3 signaling pathway in the endothelial NO production was also observed. Our results provide strong evidence that cADPR-mediated Ca^{2+} signaling pathway is importantly involved in the production of NO and EDVD in coronary arteries.

MATERIALS AND METHODS

Microdissection and microperfusion of small coronary artery. Fresh bovine hearts were obtained from a local abattoir. The left ventricular wall was rapidly dissected and immersed in ice-cold physiological saline solution (PSS) containing (in 10^{-3} M) 119 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose (pH 7.4). This myocardial section was immediately transported to the laboratory. Small intramural coronary arteries from the left anterior descending artery were carefully dissected and placed in cold PSS until cannulation (up to 4 h). Segments of small arteries (100–200 μm, internal diameter) were transferred to a water-jacketed perfusion chamber and cannulated with two glass micropipettes at their in situ length, as we described previously (59). The outflow cannula was clamped, and the arteries were pressurized to 60 mmHg. The superfusate (PSS) in the bath was bubbled with 95% O_2-5% CO_2 and maintained at pH 7.4 and 37°C. This condition for isolated artery preparation was chosen based on a number of previous studies (3, 34, 43, 47, 59). Because there is no hemoglobin in the superfusate, enough O_2 supply is needed to maintain tissue PO_2 for normal endothelial and VSM function. By adjusting bubbling rate, an appropriate balance of O_2 supply and consumption could be reached, which maintains an appropriate environment for studies of vasomotor responses in this isolated artery preparation (34, 43, 59). The internal diameter of 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 continuously recorded with a videocassette recorder (M-674, Toshiba).

After a 60-min equilibration period, the arteries were precontracted by ~50% of their resting diameter with the thromboxane A_2 analog U-46619. Once steady-state contraction was obtained, cumulative dose-response curves to the endothelium-dependent vasodilator BK (10^{-10}-10^{-6} M) were determined by measuring changes in the internal diameter. To examine the contribution of the cADPR signaling pathway to BK-induced vasodilator response, the arteries were perfused by lumen and pretreated for 30 min with one of the following compounds: Ni (6 mM, an inhibitor of ADP-ribosyl cyclase), 8-bromo-cADPR (30 μM, 8-Br, a cADPR antagonist), Ry (50 μM, an antagonist of RyRs), and dose-response curves to the vasodilator were further established. As done in our previous studies, to prevent the interaction of different drugs, various inhibitors were added into lumen perfusate, but BK was added into the bath solution separately. Between pharmacological interventions, the arteries were washed three times with PSS and allowed to equilibrate in drug-free PSS for 20–30 min. The vasodilator response was expressed as percent relaxation of U-46619-induced precontraction based on changes in the internal diameter of the arteries. The inhibition efficiency was then calculated based on these curves of dilator response to cumulative addition of BK (10^{-10}-10^{-6} M) in the absence and presence of inhibitors, such as Ni, 8-Br, or Ry, at each corresponding dose. PSS in the bath was continuously bubbled with 95% O_2-5% CO_2 and maintained at 37 ± 0.1°C throughout the experiment.

Fluorescent imaging measurement of $[Ca^{2+}]_i$ and NO levels in intact endothelium of coronary arteries. As Li's laboratory (54) described previously, a fluorescence imaging analysis system was set up to simultaneously monitor $[Ca^{2+}]_i$ and intracellular NO levels. In brief, the longitudinally cut-open arterial segment was put into a recording chamber with the vessel lumen side downward facing the objective of an inverted microscope. Care was taken to keep the endothelium intact. The chamber was filled with Hanks' buffered saline solution containing (in mM) 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 3 Na_2HPO_4, 0.4 KH_2PO_4, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, and 10 HEPES (pH 7.4). After a 30-min equilibration, the artery was loaded with fura-2 AM (10 μM) as an indicator for $[Ca^{2+}]_i$ as well as 4,5-diaminofluorescein (DAF-2) diacetate (DAF-2 DA, 10 μM) for NO. After dye loading for 40 min at room temperature was completed, individual endothelial cells on the endothelium of coronary arteries were visualized by an inverted microscope with epifluorescence at attachments. With a digital camera, the fluorescence images were then captured and analyzed as follows. A fura-2 fluorescence ratio of excitation at 340 nm to that at 380 nm (F_{340}/F_{380}) was determined to calculate $[Ca^{2+}]_i$. In contrast, endothelial NO production was first expressed as a relative fluorescence (f), which is the net increment of DAF-2 fluorescence at excitation/emission of 480/535 nm relative to its basal value ($f = \Delta F/F_0 \times 1{,}000$), where F is DAF-2 fluorescence intensity obtained during experiments and F_0 is its basal fluorescence intensity. Then a differential conversion of time-dependent NO-DAF-2 fluorescence curve (df/d*t*) was performed to accurately calculate NO production rate and plotted against the reaction time and presented this curve with $[Ca^{2+}]_i$ changes in parallel. The area under the curve (AUC) of df/d*t* was calculated to represent the cumulative amount of NO in the cells.

BK (10^{-6} M) was added to the bath solution to stimulate NO production. To examine the contribution of the cADPR pathway to BK-induced change of $[Ca^{2+}]_i$ and endothelial [NO], the arteries were respectively preincubated for 30 min with Ni (6 mM), 8-Br (30 μM), or Ry (50 μM), and the response to BK was then determined. Fluorescent image was measured every 10 min in a single area of the endothelial layer. Results were expressed as the integrated fluorescence intensity within the area observed. In addition, the contribution of this cADPR pathway to the effect of A-23187 (2 μM), a receptor-independent activator of NO production, was monitored. To clarify

the possible involvement of Ins(1,4,5)P_3 pathway in BK-induced increase in $[Ca^{2+}]_i$ and endothelial [NO], the arteries were also preincubated for 30 min with 2-aminethoxydophenyl borate (2-APB, 50 μM) or 2-APB together with 8-Br in parallel.

Culture of CAECs. The bovine coronary arterial endothelial cells (BCAECs) were cultured as described previously (58). Briefly, the arteries were rinsed with RPMI 1640 containing 5% FCS, 2% penicillin-streptomycin solution, 0.3% gentamicin, and 0.3% nystatin, as well as cleaned-off connective tissues. The lumen of the arteries was filled with 0.25% collagenase type II (Sigma) in RPMI 1640 and incubated at 37°C for 30 min. The arteries were then flushed with RPMI 1640, and the detached endothelial cells were collected, cultured in RPMI 1640 (containing 25% FCS, 1% glutamine, and 1% penicillin-streptomycin solution), and maintained in an incubator with 5% CO_2-95% room air at 37°C. The endothelial cells were identified by their morphological appearance (i.e., cobblestone array) and by positive staining for von Willebrand factor antigen. All studies were performed by using the cells of 3 to 4 passages.

Cycling assay for intracellular cADPR level. The cADPR basal level in cultured BCAECs was determined as described previously (20). About 6×10^6 cultured CAECs per sample were used. After respectively exposed to Ni (6 mM), U-73122 (100 nM), or vehicle solution, followed by the presence of a differential concentration of BK (0.5, 1, and 2 μM) at 37°C for 5 min, CAECs were scraped by a plastic scraper and spined down by centrifugation. The centrifugal pellets were extracted with 0.5 ml of 0.6 M perchloric acid at 4°C. Perchloric acid was removed by mixing the aqueous sample with a solution containing 3 vol of 1,1,2-trichlorotrifluoroethane to 1 vol of tri-*n*-octylamine. After centrifugation for 10 min at 1,500 *g* was completed, the aqueous layer containing the cADPR was removed and then incubated overnight at 37°C with a enzyme mixture containing 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NADase, 2.5 mM $MgCl_2$, and 20 mM sodium phosphate (pH 8.0). All nucleotides except cADPR in the samples were hydrolyzed.

Reactions were conducted in 96-well plates. Added to 0.1 ml of cADPR or other nucleotide samples were 50 μl of reagent containing 0.3 μg/ml ADP-ribosyl cyclase, 30 mM nicotinamide, and 100 mM sodium phosphate (pH 8). This initiated the conversion of cADPR in the samples to NAD^+. The conversion was allowed to proceed for 15 min at room temperature. The cycling reagent (0.1 ml) was then added, which contained 2% ethanol, 100 μg/ml alcohol dehydrogenase, 20 μM resazurin, 10 μg/ml diaphorase, 10 μM flavin mononucleotide, 10 mM nicotinamide, 0.1 mg/ml BSA, and 100 mM sodium phosphate (pH 8). The cycling reaction was allowed to proceed for 2–4 h, and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically using a fluorescence plate reader. With known concentrations of standards cure, quantitative measurements were performed. The results shown are means ± SE from at least three independent measurements.

HPLC analysis of ADP-ribosyl cyclase activity. Cultured CAECs at confluence were rinsed three times with 10 ml of chilled PBS and collected by using a cell scraper at 4°C. The cells were divided into four different Eppendorf tubes (1 ml each) and preincubated at 37°C for 10 min. To examine the effects of BK, Ni, and U-73122 on ADP-ribosyl cyclase activity, CAECs were treated with BK (1 μM) in the presence of Ni (6 mM), U-73122 (100 nM), or PBS (control), respectively. After incubation at 37°C for 15 min was completed, the cells were washed with Hanks' solution. The pellets of cells were suspended in HEPES buffer containing (in mM) 10 HEPES, 148 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.3 $MgCl_2$, and 5.5 glucose (pH 7.0). They were then sonicated 6 times (each time for 20 s) with a sonifier cell disrupter (model 185, Branson) at 4°C. After centrifugation at 3,500 *g* for 10 min was completed, the supernatant was collected. To determine ADP-ribosyl cyclase activity, the supernatant of 100 μg of protein was incubated with 100 μM 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 1046Å 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.

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). The injection volume was 20 μl. The mobile phase consisted of 150 mM 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 commigration with known standards. Quantitative measurements were performed by comparison of known concentrations of standards (17, 18, 34).

Statistics. Data are expressed as means ± SE. The significance of the differences in mean values between and within multiple groups was examined by using ANOVA 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

Effects of 8-Br, Ry, and Ni on BK-induced vasodilation in intact bovine small coronary arteries. To explore whether cADPR antagonist 8-Br, Ry receptor blocker Ry, and ADP-ribosyl cyclase inhibitor Ni have effects on the EDVD response to BK, the concentration-response curves of BK were first determined in each group as control. The vasodilator response to BK was then redetermined in the presence of various inhibitors, respectively. As shown in Fig. 1*A*, 8-Br (30 μM perfused into the lumen of cannulated coronary arteries for 15 min) had no significant effect on the basal vascular tone, but it markedly impaired BK-induced concentration-dependent vasorelaxation by 36%. Similarly, both RyR blockade with Ry (50 μM perfused into the lumen for 15 min; Fig. 1*B*) and inhibition of ADP-ribosyl cyclase by Ni (6 mM perfused into the lumen; Fig. 1*C*) significantly attenuated the vasodilator responses to BK (1 μM) by 45% and 30%, respectively.

Effects of 8-Br, Ry, Ni, and 2-APB on endothelial $[Ca^{2+}]_i$ and NO level in intact coronary arterial endothelium. Figure 2*A* presents typical fluorescence microscopic images showing Ca^{2+} and NO signals within endothelial cells. Under control condition, BK (1 μM) induced a rapid large increase in fura-2 F_{340}/F_{380} fluorescence ratio (red) accompanied by DAF-2 fluorescence increase within endothelial cells, indicating a parallel increase in $[Ca^{2+}]_i$ and NO level in this intact endothelium. Pretreatment of the endothelium with 8-Br, although it had no effect on the basal F_{340}/F_{380} ratio of fura-2 and DAF-2 fluorescence in the endothelium, significantly attenuated BK-induced changes in fura-2/Ca^{2+} signal and substantially blocked the DAF-2/NO signal. To validate that these signals are from endothelial cells, some arteries were deendothelized and the fluorescent images were acquired again when BK was added. It was demonstrated that neither fura-2 nor DAF-2 signal could be observed in the deendothelized arteries. We also performed experiments to confirm the specificity of these two signals by use of L-NAME to block NOS activity. In these experiments, it was found that N^G-nitro-L-arginine methyl ester (L-NAME)

completely blocked DAF-2 fluorescence-Ca^{2+} response (data not shown). The relationship of BK-induced increases in $[Ca^{2+}]_i$ and NO in the endothelium and their time course were plotted as Fig. 2*B*. BK induced a rapid peak in $[Ca^{2+}]_i$, followed by a sustained plateau, which represents intracellular Ca^{2+} release (peak response) and Ca^{2+} influx (plateau), respectively. The increase in $[Ca^{2+}]_i$ was accompanied by a relatively slow increase in NO production as represented by DAF-2 fluorescence intensity change. The maximal increase in DAF-2/NO signal occurred 2 min after Ca^{2+} peak elevations. After the arteries were incubated with 8-Br (30 μM), Ni (6 mM), or Ry (50 μM) for 15 min, the BK-induced elevation in $[Ca^{2+}]_i$ was significantly decreased and the corresponding

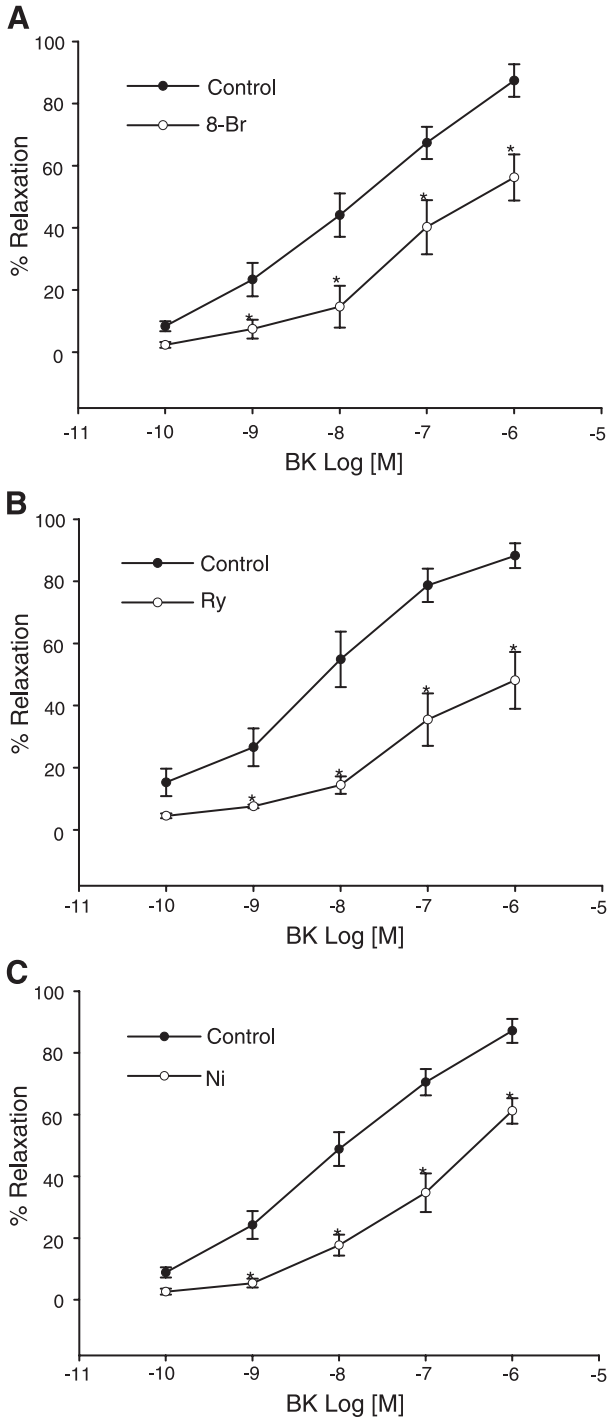

Fig. 1. Effect of inhibition of cyclic ADP ribose (cADPR) on concentration-dependent vasodilator responses to bradykinin (BK, 1 μM) in freshly isolated and pressurized small bovine coronary arteries. *A*: effect of 8-bromo-CADPR (8-Br, 30 μM) for 15 min on vasodilator responses to BK with maximal inhibitions of 36% (n = 6 hearts). *B*: effect of ryanodine (Ry, 50 μM) on vasodilator responses to BK with maximal inhibitions of 45% (n = 6 hearts). *C*: effect of nicotinamide (Ni, 6 mM) on vasodilator responses to BK with maximal inhibitions of 30% (n = 6 hearts). *$P < 0.05$ vs. control.

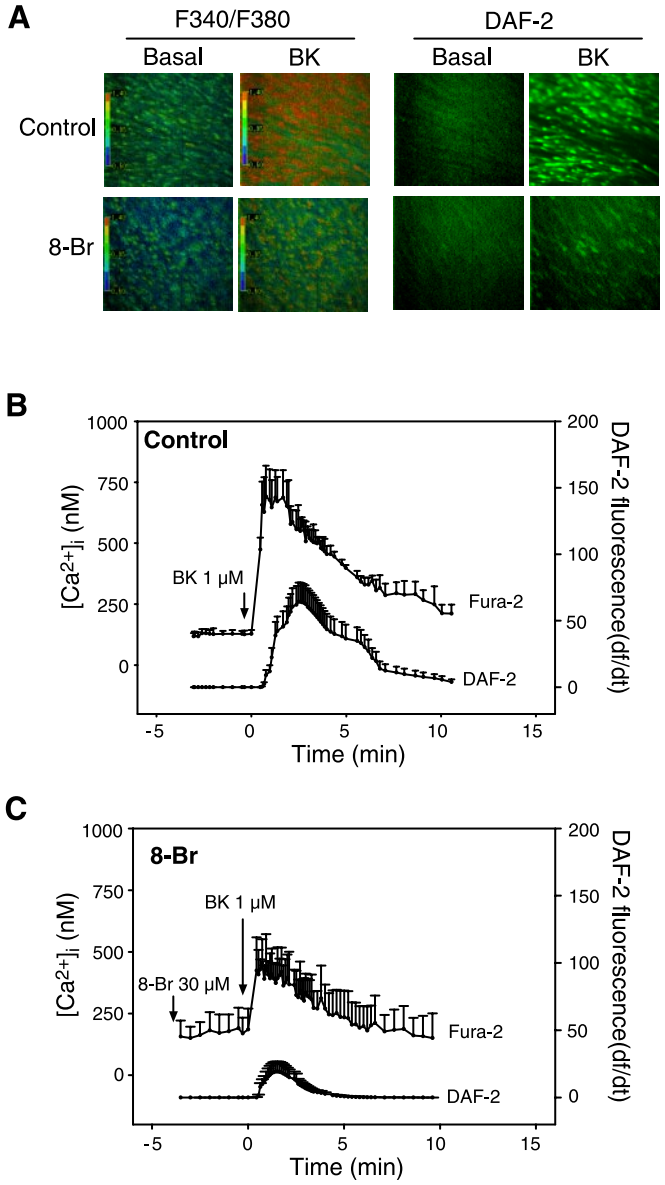

Fig. 2. Effect of inhibition of cADPR production and action on BK-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and nitric oxide (NO) in intact endothelium of small bovine coronary arteries. *A*: representative fura-2 fluorescence ratio (F_{340}/F_{380}) images and representative 4,5-diaminofluorescein (DAF-2) fluorescence images under control conditions or during stimulation with BK (1 μM) and change after pretreatment with or without 8-Br (30 μM). *B*: relationship between BK-induced increase in $[Ca^{2+}]_i$ and endothelial NO generation under control condition (n = 8 hearts). df/d*t*, differential conversion of time-dependent NO-DAF-2 fluorescence curve. *C*: simultaneous recordings of BK-induced increase in $[Ca^{2+}]_i$ and DAF-2 fluorescence in intact endothelium of small coronary artery after pretreatment with 8-Br (n = 8 hearts).

increase in DAF-2/NO fluorescence intensity was dramatically blocked. Figure 2*C* also presents the results obtained from 8-Br-treated arteries. It was clear that in the presence of 8-Br, the peak increase in Ca^{2+} transient in response to BK was significantly attenuated with a $\Delta[Ca^{2+}]_i$ from 599 ± 72 nM to 129 ± 51 nM. Corresponding to this attenuation of Ca^{2+} increase, NO response to BK was dramatically blocked by 8-Br with a net production from 126 ± 31 to 26 ± 4 AUC. Meanwhile, Ca^{2+} ionophore A-23187 was found to cause a similar increase in endothelial $[Ca^{2+}]_i$ and NO levels to that induced by BK. Antagonism of cADPR by 8-Br (30 μM) blunted both Ca^{2+} and NO responses to A-23187 (Fig. 3, *A* and *B*). Moreover, both blockade of RyRs by 50 μM Ry and inhibitor of ADP-ribosyl cyclase by Ni significantly reduced the peak Ca^{2+} response to BK and markedly downregulated BK-induced NO increase in endothelial cells (Fig. 4, *A* and *B*).

To assess the contribution of cADPR and Ins(1,4,5)P_3 signaling pathway to the BK-induced endothelial $[Ca^{2+}]_i$ and NO level in the intact coronary arterial endothelium, blockade of Ins(1,4,5)P_3 signaling by 2-APB (50 μM) and/or inhibition of cADPR signaling by 8-Br (30 μM) were performed in the endothelium preparation before the addition of BK. As shown in Fig. 5*A*, inhibition of Ins(1,4,5)P_3 signaling alone causes a mild decrease in $[Ca^{2+}]_i$ as well as endothelial NO. However, pretreatment with 2-APB and 8-Br together substantially

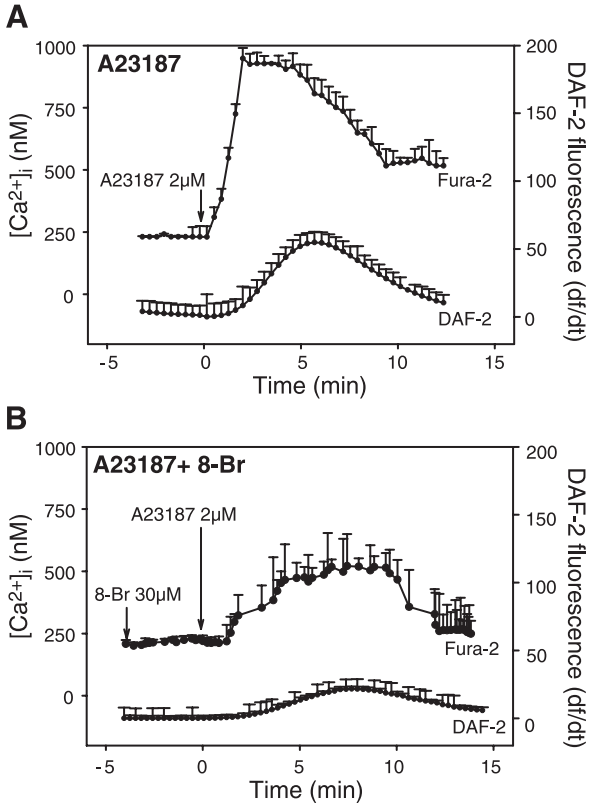

Fig. 3. Effect of inhibition of cADPR production and action on A-23187 (2 μM)-induced increase in $[Ca^{2+}]_i$ and NO in intact endothelium of small bovine coronary arteries. *A*: relationship between A-23187-induced increase in $[Ca^{2+}]_i$ and endothelial NO generation under control condition (n = 8 hearts). *B*: simultaneous recordings of A-23187-induced increase in $[Ca^{2+}]_i$ and DAF-2 fluorescence in intact endothelium of small coronary artery after pretreated with 8-Br (n = 8 hearts).

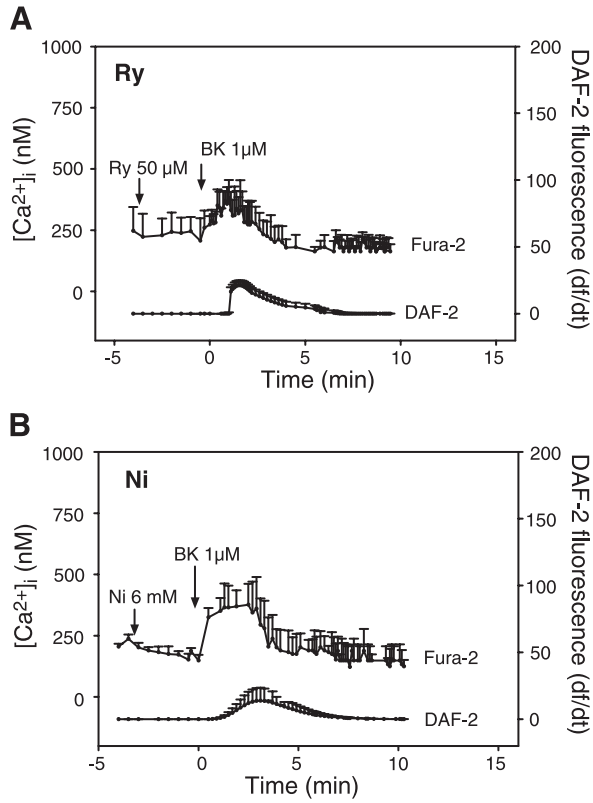

Fig. 4. Simultaneous recordings of BK-induced increase in $[Ca^{2+}]_i$ and DAF-2 fluorescence in intact endothelium of small coronary artery after pretreated with Ry (50 μM, *A*, n = 8 hearts) or Ni (6 mM, *B*, n = 8 hearts).

blocked the peak Ca^{2+} response to BK and completely abolished BK-induced NO increase in endothelial cells (Fig. 5*B*).

Figure 6 summarized these data depicting the maximal increase in Ca^{2+} and NO levels in response to BK under control condition and after treatments of various antagonists or inhibitors. As shown in Fig. 6*A*, BK (1 μM) evoked a rapid Ca^{2+} transient in the arterial endothelium by 599 ± 72 nM (n = 8 arterial preparations). When the arteries were pretreated with the cADPR antagonist 8-Br (30 μM) for 15 min, the BK-induced maximal increase in $[Ca^{2+}]_i$ was significantly attenuated to 129 ± 51 nM, a reduction of 78% (n = 8 arterial preparations). Inhibition of cADPR production with Ni (6 mM) also significantly reduced the BK-induced Ca^{2+} release to 156 ± 45 nM, a 74% reduction (n = 8 arterial preparations). Furthermore, blockade of RyRs by Ry (50 μM) attenuated BK-stimulated Ca^{2+} release to 121 ± 37 nM, an 80% reduction (n = 8 arterial preparations). In contrast to the antagonism of cADPR, addition of 2-APB alone attenuated BK-stimulated Ca^{2+} release to 435 ± 37 nM, an 27% reduction (n = 8 arterial preparations). However, with the use of pretreated vessel with 2-APB and 8-Br together, BK-induced $[Ca^{2+}]_i$ was dramatically blocked with a $\Delta[Ca^{2+}]_i$ by 43 ± 3 nM. As shown in Fig. 6*B*, BK alone produced a marked increase in NO fluorescence (ΔAUC by 126 ± 31) and the addition of 8-Br (30 μM) markedly blocked BK-induced increase in NO fluorescence (ΔAUC 26 ± 4). Similarly, the addition of Ni (6 mM) or Ry (50 μM) also decreased BK-stimulated NO response (ΔAUC by 21 ± 1 and 22 ± 4, respectively, n = 8 arterial preparations). Ry at this concentration even significantly reduced NO

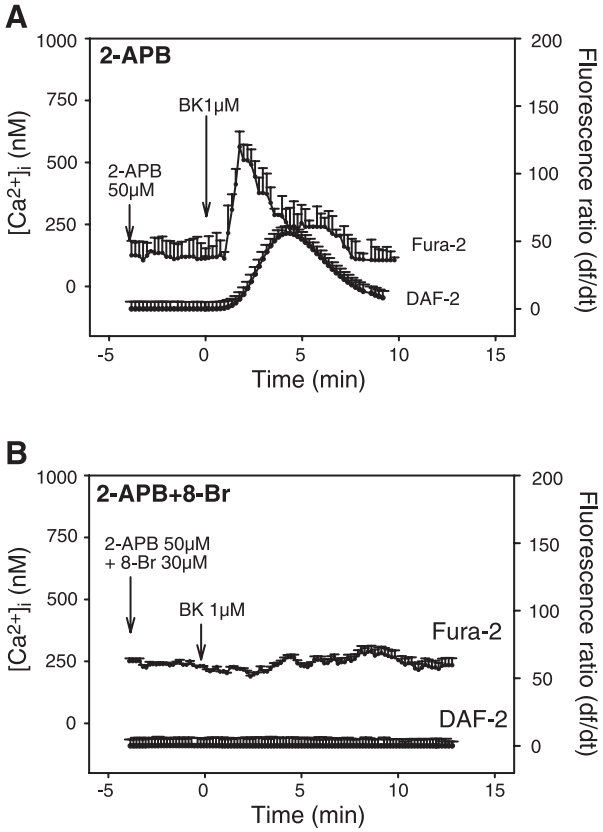

Fig. 5. Simultaneous recordings of BK-induced increase in $[Ca^{2+}]_i$ and DAF-2 fluorescence in intact endothelium of small coronary artery after pretreated with 2-aminethoxydiphenyl borate (2-APB, 50 μM; $n = 8$ hearts) or 2-APB plus 8-Br (30 μM; $n = 8$ hearts).

levels under control condition. However, 2-APB alone partially blocked BK-stimulated NO increase (ΔAUC by 96 ± 5, $n = 8$ arterial preparations), whereas this endothelial NO production was completely blocked in the presence of 2-APB and 8-Br together.

Effects of Ni on BK-induced cADPR increase in CAECs. Using cultured CAECs, we determined cADPR levels in the absence or presence of BK. As summarized in Fig. 7*A*, cADPR was detectable in CAECs with a concentration range at 1–5.5 pmol/mg protein. In the control CAECs, the basal level of cADPR was 2.29 ± 0.16 pmol/mg protein ($n = 8$ experiments). When the CAECs were stimulated with BK for 5 min, the intracellular cADPR level substantially increased. Interestingly, the relative higher response to BK, a 5.82 ± 0.79 pmol/mg protein increase in cADPR concentration, was not found at 2 μM but at a 1 μM BK dose ($n = 8$ experiments, $P < 0.05$). Inhibition of ADP-ribosyl cyclase with Ni (6 mM) significantly reduced both cADPR basal levels and BK-induced cADPR generation by about 40% reduction ($n = 8$ experiments). However, the addition of U-73122 (100 nM) was not found to inhibit BK (1 μM)-induced intracellular cADPR generation (Fig. 7*B*, $n = 8$ experiments).

ADP-ribosyl cyclase activity in CAECs. By fluorescence HPLC analysis, cyclic GDP ribose (cGDPR) production converted from β-NGD^+ by cultured CAECs was measured, which indicated ADP-ribosyl cyclase activity in CAECs. This conversion of β-NGD^+ into cGDPR was used because cGDPR cannot be metabolized by ADP-ribosyl cyclase, leading to more accurate quantities of ADP-ribosyl cyclase. This product coeluted with the cGDPR standard at a retention time of 2.2 min. Under control conditions, the conversion rate of β-NGD^+ into cGDPR was 33.15 ± 2.72 pmol·min^{-1}·mg $protein^{-1}$ ($n = 6$ experiments). When CAECs were incubated with BK, a concentration-dependent increase in cGDPR production was observed. BK significantly stimulated the production of cGDPR, even at 1 min incubation time point (data not shown). The maximal conversion rate of β-NGD^+ to cGDPR was 49.18 ± 0.93 pmol·min^{-1}·mg $protein^{-1}$ ($n = 6$ experiments), which was observed at 1 μM BK with 5 min of incubation with CAECs. In the presence of the ADP-ribosyl cyclase inhibitor Ni (6 mM), BK-induced increase in the conversion of β-NGD^+ to cGDPR was substantially inhibited ($n = 6$ experiments), and even at the highest concentration studied, BK (10 μM) could not increase the activity of ADP-ribosyl cyclase. In contrast, U-73122 had no effect on BK (1 μM)-induced activation of ADP-ribosyl cyclase ($n = 8$ experiments) (shown as Fig. 8).

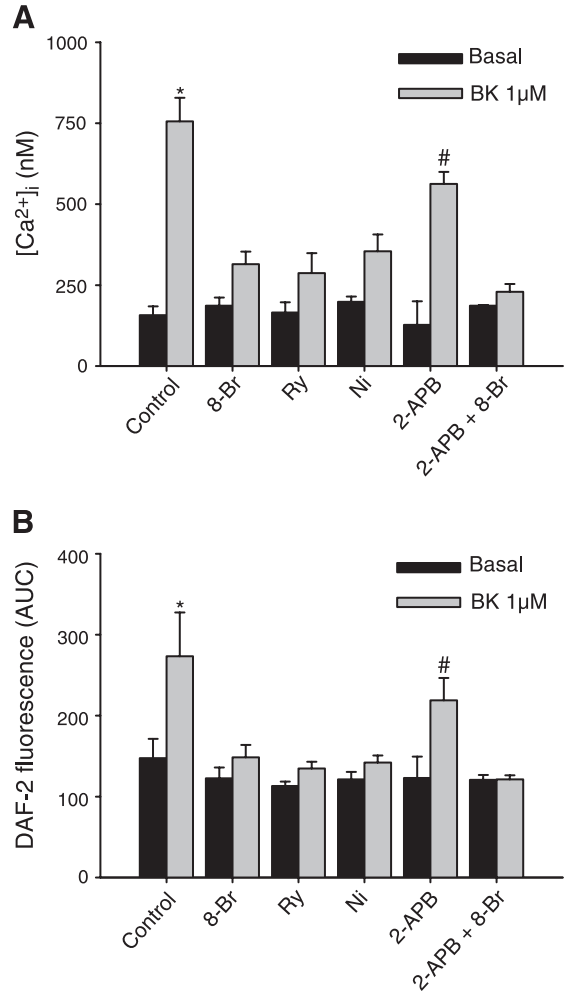

Fig. 6. Summarized data for effect of 8-Br (30 μM), Ry (50 μM), Ni (6 mM), 2-APB (50 μM), or 2-APB plus 8-Br (30 μM) on BK (1 μM)-induced increase in $[Ca^{2+}]_i$ (*A*) and NO (*B*) in intact endothelium of small bovine coronary arteries. AUC, area under curve. Values are means ± SE; $n = 8$ arterial preparations from different hearts. *$P < 0.05$ vs. control; #$P < 0.05$ vs. in presence of 2-APB and 8-Br together.

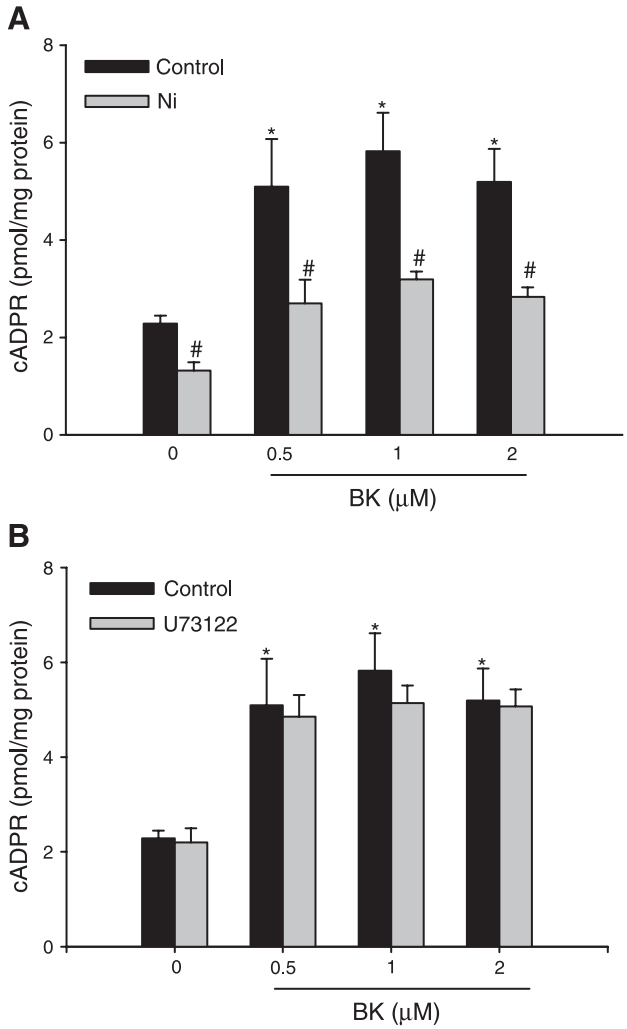

Fig. 7. Effects of Ni and U-73122 on BK-induced increase in cADPR levels in isolated coronary arterial endothelial cells (CAECs). *A*: results when CAECs were exposed to differential concentrations of BK (0, 0.5, 1, and 2 μM) with or without Ni (6 mM) at 37°C for 5 min before cycling assay. *B*: results when CAECs were exposed to differential concentrations of BK (0, 0.5, 1, and 2 μM) with or without U-73122 (100 nM) at 37°C for 5 min before cycling assay. Values are means ± SE; $n = 8$ experiments. *$P < 0.05$ vs. basal control in absence of BK; #$P < 0.05$ vs. control in presence of differential concentration of BK.

DISCUSSION

In the present study, we demonstrated that inhibition of cADPR production significantly attenuated BK-induced concentration-dependent coronary arterial vasodilation. In the intact coronary arterial endothelium, BK evoked Ca^{2+} release from RyRs-sensitive stores in CAECs, which was accompanied by an increase in NO production. Moreover, we confirmed that this BK-induced intracellular Ca^{2+} increase and NO response is not mainly associated with Ins(1,4,5)P_3 signaling but with cADPR levels in these cells. cADPR cyclase activation by BK may contribute to enhanced production of cADPR. We also demonstrated that blockade of cADPR production significantly attenuated endothelial intracellular Ca^{2+} increase and NO generation in response to another stimulator, Ca^{2+} ionophore A-23187, of NO production in the endothelium. These results suggest that cADPR-mediated Ca^{2+} signaling plays an important role in the regulation of endothelial NO production and thereby plays a critical role in EDVD.

It is well known that the EDVD is of importance in the regulation of coronary circulation, which is involved in the actions of increased vasoactive agents such as arachidonic acid (41), ATP (4, 28), acetylcholine (1), A-23187 (46), and BK (7, 40). Both BK- and A-23187-induced vasodilation is linked to the release of endothelium-derived NO (7, 40), prostaglandin I_2 (38), and endothelium-dependent hyperpolarizing factor, such as epoxyeicosatrienoic acids (6), and BK is considered as a more classic Ca^{2+}-dependent activator of eNOS (5, 53). As reported in a previous study from Li's laboratory (54), BK can produce a rapid and simultaneous increase in intracellular Ca^{2+} and endothelial NO. This BK-induced NOS activation is largely dependent on intracellular Ca^{2+} mobilization and subsequent binding of Ca^{2+}/CaM to eNOS and displacement of eNOS from caveolin-1 (12, 19). Moreover, many studies have been reported on the role of Ca^{2+} influx (2, 30), Ca^{2+}-mobilizing second messenger, and relative receptors in the endothelial intracellular Ca^{2+} release (11, 13, 27, 39, 45). Recently, a cellular spatiotemporal organization pattern for Ca^{2+} mobilization under the plasma membrane in endothelial cells has been indicated to be critical for the regulation of NO generation (26). However, the mechanism by which BK leads to such Ca^{2+} increase still remains very controversial and has yet to be clarified.

The present study first explored the role of the cADPR signaling pathway in mediating BK-induced vasodilation in perfused and pressurized small coronary arteries. We found that inhibition of cADPR production or antagonism of its action significantly attenuated BK-induced vasodilation in these coronary arteries. Moreover, consistent with the previous findings that cADPR acts via RyRs-mediated Ca^{2+} mobilization from the sarcoplasmic reticulum, the present study confirmed that exogenous application of a large dose of Ry markedly impaired the vasodilator response of coronary arter-

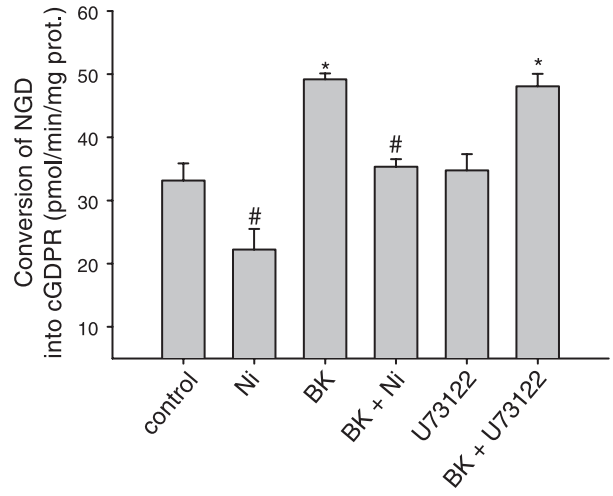

Fig. 8. Effects of Ni and U-73122 on BK-induced enhancement of ADP-ribosyl cyclase activity in isolated CAECs. Summarized data show conversation rate of β-nicotinamide guanine dinucleotide (β-NGD^+) into cyclic GDP ribose (cGDPR) (indicating the activities of ADP-ribosyl cyclase) in homogenates of CAECs in response to BK (1 μM) in the absence or presence of Ni (6 mM) or U-73122 (100 nM). prot, protein. Values are means ± SE; $n = 6$ experiments. *$P < 0.05$ vs. control in absence of BK; #$P < 0.05$ vs. control or in presence of BK only.

ies to BK. To our knowledge, these results provide the first direct evidence that a cADPR signaling pathway is involved in the vasodilator response to BK in coronary circulation.

To determine whether BK-induced response is directly linked to cADPR-mediated Ca^{2+} release from the ER in CAECs, a newly developed fluorescence imaging system was used to simultaneously measure Ca^{2+} transient and NO production in the intact coronary endothelium under control conditions or during blockade of cADPR-RyRs signaling. In these experiments, BK was found to produce a rapid and transient increase in Ca^{2+} through intracellular mobilization and Ca^{2+} influx, which was accompanied by an increase in intracellular NO. However, when the arteries were pretreated with cADPR-RyRs signaling inhibitors or blockers, Ni, 8-Br, or Ry separately, BK-induced Ca^{2+} release was significantly attenuated and NO production was obviously blocked. This supports the view that BK stimulates Ca^{2+} release in CAECs through cADPR-RyRs signaling pathway.

It should be noted that the present findings do not rule out the role of Ins(1,4,5)P_3-mediated Ca^{2+} signaling in BK-induced NO production. It is well known that Ins(1,4,5)P_3-mediated Ca^{2+} signaling is generally present in various mammalian cells and via G protein-coupled B_2 receptor BK may activate Ins(1,4,5)P_3 signaling pathway (11, 27). In the present study, however, blockade of Ins(1,4,5)P_3 pathway by 2-APB was only found to partially inhibit the intracellular Ca^{2+} mobilization and NO production induced by BK. If 8-Br together with 2-APB was used to treat the intact endothelium of coronary arteries, the BK-induced Ca^{2+} release response was blocked more substantially compared with that by using 8-Br alone. These results are consistent with other evidence that the maximal inhibition effect of carboxyamidotriazole on vascular endothelial growth factor A-induced NO production in human umbilical vein endothelial cells was only ~50% depending on Ins(1,4,5)P_3 pathway (11). In addition, ~50% inhibition of BK-induced vasodilation with >80% inhibition of Ca^{2+} and NO increase by inhibition of cADPR-RyRs signaling pathway also indicates that BK-induced vasodilaition is not only associated with Ca^{2+}-NO-mediated mechanism. It is possible that cADPR primarily influences BK-induced coronary vasodilation via its Ca^{2+} release and consequent NO production. There is a concern whether this cADPR-Ca^{2+}-NO regulation is only specific to BK-induced response. However, our results indicate that cADPR signaling seems to be involved in a general process of endothelial Ca^{2+} and NO response because 8-Br, an antagonist of cADPR, also markedly blunted A-23187-induced Ca^{2+} increase and NO production in endothelial cells.

Through biochemical analysis, we further provided evidence that cADPR production was activated by BK. Using a fluorescence cycling assay, we found that stimulation CAECs with BK caused a significant increase in intracellular cADPR levels. Consistent with previous results in sea urchin eggs and other tissues or cells, the concentration of cADPR in CAECs was found at a range of picomole per milligram of tissue protein, which by calculation is relative to a tissue level of nanomoles (37, 48, 51). When these CAECs were challenged by BK, intracellular cADPR levels were significantly increased. Inhibition of ADP-ribosyl cyclase with Ni significantly reduced this BK-induced cADPR generation. However, inhibition of phospholipase C by U-73122 did not influence either basal or BK-induced cADPR levels. These biochemical results further strengthened the conclusion from our functional studies in the arteries or CAECs that BK stimulates cADPR production and thereby activates NO generation, which produces EDVD.

To define the enzymatic pathway for BK-induced elevation of cADPR in CAECs, we performed HPLC analysis to determine the activation of ADP-ribosyl cyclase by BK. It was demonstrated that incubation of CAECs with BK produced a concentration-dependent enhancement of ADP-ribosyl cyclase activity, which could be significantly blocked by its inhibitor Ni and not by phospholipase C inhibitor U-73122. This activation of ADP-ribosyl cyclase occurred rapidly even in the first minute of the incubation of CAECs with BK. In previous studies, the regulation of ADP-ribosyl cyclase has been found to be involved in several membrane or cytosolic receptors, such as muscarinic acetylcholine receptors in NG108–15 neuronal cells (24), angiotensin II or β-adrenergic receptors in ventricular myocytes (22), and M_1 muscaric receptors in VSM cells (17). It has been proposed that these receptors could be directly coupled with ADP-ribosyl cyclase, and, therefore, their activation could result in increased production of cADPR (22, 24). With respect to BK, there is evidence that B_2 receptors are directly coupled to the membrane-bound form of ADP-ribosyl cyclase in human airway smooth muscle cells (9) or NG108–15 neuronal cells (23), suggesting that BK could stimulate cADPR production via this coupling mechanism. The present study did not attempt to explore this detail coupling mechanism; however, our results suggest that BK could potentially stimulate ADP-ribosyl cyclase through this coupling because BK-induced Ca^{2+} response and cADPR production are rather rapid.

In summary, the present study demonstrated the following. First, inhibition of cADPR production and antagonism of its action significantly impaired BK-induced EDVD in the intact bovine small coronary arteries. Second, in the intact endothelium of coronary arteries, inhibition of cADPR-RyRs Ca^{2+} signaling pathway not only substantially decreased BK-induced Ca^{2+} release but also substantially blocked BK-induced NO generation. Third, BK was found to increase intracellular cADPR levels, and inhibition of cADPR cyclase with Ni significantly reduced this BK-induced increase in cADPR levels. Fourth, BK-induced increase in intracellular cADPR levels was associated with activation of ADP-ribosyl cyclase. Fifth, in addition, the contribution of cADPR to Ca^{2+}/NO release was found linked to the response to other agonists, such as A-23187. Therefore, we conclude that cADPR-mediated Ca^{2+} represents an important Ca^{2+} signaling mechanism, which importantly contributes to BK-induced NO production in coronary arteries via its Ca^{2+} mobilization action. Therefore, this signaling nucleotide is of importance in the regulation of EDVD response in coronary circulation.

GRANTS

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