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*Am J Physiol Heart Circ Physiol* 275:1002-1010, 1998.

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# Regulation of $K_{Ca}$ -channel activity by cyclic ADP-ribose and ADP-ribose in coronary arterial smooth muscle

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**Li, Pin-Lan, Ai-Ping Zou, and William B. Campbell.** Regulation of  $K_{Ca}$ -channel activity by cyclic ADP-ribose and ADP-ribose in coronary arterial smooth muscle. *Am. J. Physiol.* 275 (*Heart Circ. Physiol.* 44): H1002–H1010, 1998.— The enzymatic pathway responsible for the production and metabolism of cyclic ADP-ribose (cADP-R) in small bovine coronary arteries was characterized, and the role of cADP-R and ADP-ribose (ADP-R) in the regulation of the activity of large-conductance  $Ca^{2+}$ -activated  $K^+$  ( $K_{Ca}$ ) channels was determined in vascular smooth muscle cells (SMC) prepared from these vessels. We found that cADP-R and ADP-R were produced when the coronary arterial homogenates were incubated with 1 mM  $\beta$ -NAD. The time course of the enzyme reactions showed that the maximal conversion rate ( $1.37 \pm 0.03$  nmol·min<sup>-1</sup>·mg protein<sup>-1</sup>) of  $\beta$ -NAD to cADP-R was reached after 3 min of incubation. As incubation time was prolonged, the production of ADP-R was increased to a maximal rate of  $3.66 \pm 0.03$  nmol·min<sup>-1</sup>·mg protein<sup>-1</sup>, whereas cADP-R production decreased. Incubation of the homogenate with cADP-R produced a time-dependent increase in the synthesis of ADP-R. Comparison of coronary arterial microsomes with cytosols shows that the production of both cADP-R and ADP-R in microsomes was significantly greater. In excised inside-out membrane patches of single coronary SMC, the  $K_{Ca}$  channels were activated when  $\beta$ -NAD, the precursor for both cADP-R and ADP-R, was applied to the internal surface. This effect of  $\beta$ -NAD may be associated with the production of ADP-R, because the  $K_{Ca}$ -channel activity was increased by ADP-R in a concentration-dependent manner. The open-state probability of the  $K_{Ca}$  channels increased from a control level of  $0.08 \pm 0.03$  to  $0.17 \pm 0.05$  even at the lowest ADP-R concentration (0.1  $\mu$ M) studied. However, cADP-R reduced the  $K_{Ca}$ -channel activity, and the threshold concentration of cADP-R that decreased the average channel activity of the  $K_{Ca}$  channels was 1  $\mu$ M. These results provide evidence that cADP-R is produced and metabolized in the coronary arterial smooth muscle and that a cADP-R/ADP-R pathway participates in the control of the  $K_{Ca}$ -channel activity in vascular SMC.

adenosine diphosphate-ribose; cyclic adenosine diphosphate-ribose; potassium channel; coronary artery; vascular smooth muscle

CYCLIC ADENOSINE DIPHOSPHATE-RIBOSE (cADP-R) and adenosine diphosphate-ribose (ADP-R) are endogenous metabolites of NAD. cADP-R is formed by cyclizing NAD via ADP-ribosylcyclase, and ADP-R is produced by hydrolysis of NAD by NAD<sup>+</sup> glycohydrolase or hydrolysis of cADP-R by cADP-R hydrolase (7, 15, 18,

19, 22–25, 28, 29, 31, 32). cADP-R and ADP-R have been detected in a variety of tissues such as heart, liver, spleen, and brain and in red blood cells, lymphocytes, pituitary cells, and cultured renal epithelial cells (1, 20, 25, 39, 41, 43). Recent studies indicated that cADP-R causes the mobilization of intracellular  $Ca^{2+}$  by a mechanism that is completely independent of D-myoinositol 1,4,5-trisphosphate (7, 21–28, 30, 32). cADP-R-mediated mobilization of intracellular  $Ca^{2+}$  participates in the regulation of the secretion of insulin, the fertilization of eggs, and the effect of nitric oxide (NO) in nonmuscle tissue (5, 6, 8–10, 23, 41). However, the metabolism and actions of cADP-R and ADP-R in vascular tissue are poorly understood. Because NAD has been reported to modulate the activity of the  $Ca^{2+}$ -activated  $K^+$  ( $K_{Ca}$ ) channels in pulmonary arterial smooth muscle cells (SMC) and to alter vascular tone (33), it is possible that the NAD metabolites cADP-R and ADP-R may serve as intracellular second messengers to gate the  $K^+$  channels in vascular SMC and to regulate vascular tone in the coronary circulation. The purpose of the present study is to determine the production of cADP-R and ADP-R in coronary arteries and the role of these nucleotides in the regulation of the  $K^+$ -channel activity in coronary arterial SMC.

## MATERIALS AND METHODS

*Assay of cADP-R and ADP-R in coronary arterial muscle.* Bovine hearts were obtained from a local slaughterhouse. A branch of the coronary artery was cannulated and filled with 10–20 ml of ice-cold 3% Evans blue in 50 mM sodium phosphate buffer (PBS) containing 0.9% sodium chloride (pH 7.4). The heart was then dissected into  $2 \times 3 \times 1$ -cm pieces and sliced into 300- $\mu$ m-thick tissue sections. Small coronary arteries stained with Evans blue were identified under a dissecting stereomicroscope. These arteries were microdissected, pooled, and stored in ice-cold PBS.

The dissected coronary arteries were cut into very small pieces and homogenized with a glass homogenizer in ice-cold HEPES buffer containing (in mM) 25 Na-HEPES, 1 EDTA, and 0.1 phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 6,000 *g* for 5 min at 4°C, the supernatant containing the membrane protein and cytosolic components was aliquoted and frozen in liquid N<sub>2</sub> and termed the homogenate. Microsome and cytosol were prepared by a differential centrifugation of the homogenate at 10,000 *g* for 20 min and at 100,000 *g* for 90 min. The pellet was the microsomal fraction, whereas the supernatant was the cytosolic fraction. To determine the production of cADP-R and ADP-R, the homogenates (50  $\mu$ g) were incubated for 10 min with 1 mM  $\beta$ -NAD at 37°C in an assay buffer containing (in mM) 250 potassium gluconate, 250 *N*-methylglucamine, 20 HEPES, and 1 MgCl<sub>2</sub> (pH 7.2). The total reaction volume was 0.3 ml. To determine the time course of the production of

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cADP-R and ADP-R, the homogenates were incubated with 1 mM NAD for 1, 2, 3, 4, 5, 10, 30, 60, and 120 min. The reaction mixture was then rapidly frozen in liquid N<sub>2</sub> to terminate the reaction. Before HPLC analysis, the reaction mixtures were centrifuged at 4°C with the use of an Amicon microultrafilter at 3,000 rpm to remove the proteins. The reaction products in the ultrafiltrate were analyzed by an HPLC system with the use of a Hewlett-Packard 1090L solvent delivery system and a 1040A photodiode array detector with a 20- $\mu$ l flow cell (Hewlett-Packard, Avondale, PA). Data were collected and analyzed with a Hewlett-Packard Chemstation. Nucleotides were analyzed on a Supelcosil LC-18 (3  $\mu$ m, 4.6  $\times$  150 mm) with a Supelcosil LC-18 guard column (5  $\mu$ m, 4.6  $\times$  20 mm; Supelco, Bellefonte, PA). The injection volume was 20  $\mu$ l. 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 nucleotides were separated in *solvent A* with a gradient of 5% *solvent B* to 30% *solvent B* over 1 min, held for 25 min, and then increased to 50% *solvent B* over 1 min. The flow rate was 1 ml/min. The column eluate was monitored at 254 nm. Peak identities were confirmed by comigration with known standards and absorbance spectra compared with the known standards. Quantitative measurements were performed by comparison of known concentrations of standards.

To test the applicability of this HPLC analysis for determination of the enzyme activity, purified ADP-ribosylcyclase and NAD<sup>+</sup> glycohydrolase were used to produce cADP-R or ADP-R, which was chromatographed and detected by HPLC as described earlier. Purified ADP-ribosylcyclase and NAD<sup>+</sup> glycohydrolase were purchased from Sigma Chemical. ADP-ribosylcyclase was purified from *Aplysia* and NAD<sup>+</sup> glycohydrolase from bovine spleen. The products of ADP-ribosylcyclase had retention times of 3.0 and 4.1 min, which coeluted with synthetic cADP-R and nicotinamide. The products of NAD<sup>+</sup> glycohydrolase had retention times of 4.1 and 14.6 min, which coeluted with synthetic nicotinamide and ADP-R. In the presence of 1 mM NAD, the maximal conversion rates of cADP-R and ADP-R were 25,897  $\pm$  218 nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg purified ADP-ribosylcyclase protein<sup>-1</sup> and 580.4  $\pm$  22 nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg glycohydrolase protein<sup>-1</sup>, respectively. These results indicate that this HPLC analysis is suitable for determination of the enzyme activity responsible for the production of cADP-R and ADP-R.

*Culture of coronary arterial endothelial and smooth muscle cells.* The bovine coronary arterial endothelial cells (EC) and SMC were cultured as described previously (2, 3, 38). Briefly, the vessels were first rinsed with 5% FCS in medium 199 containing 25 mM HEPES with 1% penicillin, 0.3% gentamycin, and 0.3% nystatin and then cut into segments, and the lumen was filled with 0.4% collagenase in medium 199. After 30 min of incubation at 37°C, the vessels were flushed with medium 199. The detached EC were collected and cultured in RPMI 1640 with 25% FCS, 1% antibiotic solution, 0.3% nystatin, 0.3% gentamycin, 1% glutamine, and 0.1% tylosin. The cells were maintained in an incubator with 5% CO<sub>2</sub> in air at 37°C. EC grew in a typical cobblestone array. The presence of EC markers was used to confirm the purity of the cells. Experiments were performed when cells reached  $\sim$ 95% confluence at either the second or third passage. After EC were collected, strips of denuded vessels were placed into gelatin-coated flasks with medium 199 containing 10% FCS with L-glutamine (1%), tylosin (0.1%), and antibiotics (1%). SMC migrated to the flasks within 3–5 days. Once growth was established, the vessels were removed and cells were grown in medium 199 containing 20% FCS. The identification of the vascular SMC was based on positive staining by an anti-

actin antibody. The cells were grown for 14–16 days to reach confluence. The homogenates of EC and SMC were made as described in *Assay of cADP-R and ADP-R in coronary arterial muscle*. The homogenates were used to study the enzyme activity for the production of cADP-R and ADP-R. All studies were performed using the cells of four passages or less.

*Patch-clamp study.* Small bovine coronary arteries were dissected as described in *Assay of cADP-R and ADP-R in coronary arterial muscle*. The dissected small coronary arteries were first incubated for 30 min at 37°C with collagenase type II (340 U/ml; Worthington), elastase (15 U/ml; Worthington), dithiothreitol (1 mg/ml), and soybean trypsin inhibitor (1 mg/ml) in HEPES buffer consisting of (in mM) 119 NaCl, 4.7 KCl, 0.05 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 glucose, and 10 HEPES (pH 7.4). The digested tissue was then agitated with a glass pipette to free the vascular SMC, 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 then stored at 4°C until used.

Single-channel K<sup>+</sup> currents were recorded using the patch-clamp technique (13). Inside-out patches were used to identify the K<sub>Ca</sub> channels and to determine the effect of cADP-R and ADP-R on K<sup>+</sup> currents in vascular SMC. Patch pipettes were made from borosilicate glass capillaries pulled with the use of a two-stage micropipette puller (PC-87, Sutter) and were heat-polished with a microforge (MF-90, Narishige). The pipettes had tip resistances of 8–10 M $\Omega$  for single-channel recording when filled with 145 mM KCl solution. SMC were placed in a 1-ml perfusion chamber mounted on the stage of a Nikon inverted microscope. After the tip of the pipette was positioned on a cell, a high-resistance seal (5–15 G $\Omega$ ) was formed between the pipette tip and the cell membrane by applying a light suction. Inside-out membrane patches were excised by lifting the pipette membrane complex to the air-solution interface.

A List EPC-7 patch-clamp amplifier (List Biological Laboratories, Campbell, CA) was used to record single-channel currents. The amplifier output signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Currents were digitized at a sampling rate of 3 kHz and stored on the hard disk of a Gateway 486 DS66 computer for off-line analysis. Data acquisition and analysis were performed with pCLAMP software (version 5.7.1, Axon Instruments, Burlingame, CA). Average channel activity ( $NP_o$ ) in patches was determined from recordings of several minutes as

$$NP_o = \left( \sum_{j=1}^N t_j \right) / T$$

where  $N$  is the maximal number of channels observed in conditions of high levels of  $P_o$ ,  $P_o$  is the open-state probability,  $T$  is the duration of the recording, and  $t_j$  is the time with  $j = 1, 2, \dots, N$  channels opening.

The bath solution contained (in mM) 145 KCl, 1.1 MgCl<sub>2</sub>, 10 HEPES, and 2 EGTA, and 300 nM Ca<sup>2+</sup> (pH 7.2) was used for single-channel recordings at the inside-out excised membrane patch. The pipette solution contained (in mM) 145 KCl, 1.8 CaCl<sub>2</sub>, 1.1 MgCl<sub>2</sub>, and 10 HEPES (pH 7.4). All patch-clamp experiments were performed at room temperature ( $\sim$ 20°C). The effects of cADP-R and ADP-R on the K<sup>+</sup>-channel activity were examined in the inside-out patch mode. After inside-out patches were established, a 3-min control recording was obtained at a membrane potential of +40 mV. The solution in the bath was then exchanged with the same bath

solution containing different concentrations of NAD, cADP-R, ADP-R, and their analogs including nicotinamide guanine dinucleotide (NGD), cyclic guanosine diphosphate-ribose (cGDP-R), cyclic inosine diphosphate-ribose (cIDP-R), and 8-bromo-cADP-R (0.1–10  $\mu\text{M}$ ), and a second, successive 3-min recording was obtained. To determine the time course of NAD, cADP-R, and ADP-R on the  $K_{\text{Ca}}$ -channel activity, the  $K_{\text{Ca}}$  current recordings were performed immediately after addition of these drugs and continued for 10 min. In some experiments, the channel currents were recorded when the membrane was depolarized from 0 to 60 mV in steps of 10 mV in the absence and presence of NAD, cADP-R, and ADP-R. These experiments were performed to test the effect of NAD and its metabolites on the voltage dependence of the  $K_{\text{Ca}}$  channels.

To determine the sensitivity of the channels to cytosolic  $\text{Ca}^{2+}$ , the concentration of  $\text{Ca}^{2+}$  in the bath solution was varied from 0.1 to 1  $\mu\text{M}$  and then to 10  $\mu\text{M}$ .  $\text{Ca}^{2+}$  concentration was estimated by a computer program (12) and was confirmed by measuring the free  $\text{Ca}^{2+}$  concentration in the solution using fura 2 (Molecular Probes) with a dual-wavelength spectrofluorometer (Perkin-Elmer). NAD, cADP-R, or ADP-R (10  $\mu\text{M}$ ) was added to examine the effects on the sensitivity of the  $K_{\text{Ca}}$  channels to cytosolic  $\text{Ca}^{2+}$ .

**Statistical analysis.** Data were presented as means  $\pm$  SE. Significance of differences in mean values within and between multiple groups was examined using an ANOVA for repeated measures followed by Duncan's multiple-range test.  $P < 0.05$  is considered statistically significant.

## RESULTS

**Production of cADP-R and ADP-R in coronary arteries.** A representative reverse-phase HPLC chromatogram depicting the profiles of NAD metabolites produced by coronary arterial homogenates is presented in Fig. 1. When the homogenate was incubated with NAD, the products had retention times of 3.0, 4.1 and 14.6 min (Fig. 1B) and coeluted with synthetic cADP-R, nicotinamide, and ADP-R standards, respectively (Fig. 1A). The peak with a retention time of 5.4 min was unreacted NAD. Figure 1C presents a time course of the conversion of NAD to cADP-R and ADP-R by arterial homogenates. The increase in cADP-R was rapid and reached a plateau in 3 min (Fig. 1, inset). A detectable increase was observed within 1 min of incubation, and the maximal conversion rate for cADP-R was  $1.37 \pm 0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ . As incubation time was prolonged, the conversion of cADP-R decreased, whereas the production of ADP-R increased. The maximal ADP-R conversion rate of  $3.66 \pm 0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  was reached within 1 h.

Compared with the activity of purified ADP-ribosylcyclase and NAD<sup>+</sup> glycohydrolase, the amount of cADP-R and ADP-R produced by 1 mg of coronary arterial homogenates, as demonstrated by a quantitative analysis, was equal to that produced by 5.29 ng of ADP-ribosylcyclase and 485 ng of NAD<sup>+</sup> glycohydrolase, respectively. A high concentration of enzymes responsible for the production of ADP-R is present in bovine coronary arteries.

The homogenates were incubated with cADP-R to detect ADP-R production. As shown in Fig. 2A, the product of cADP-R metabolism in the homogenates had

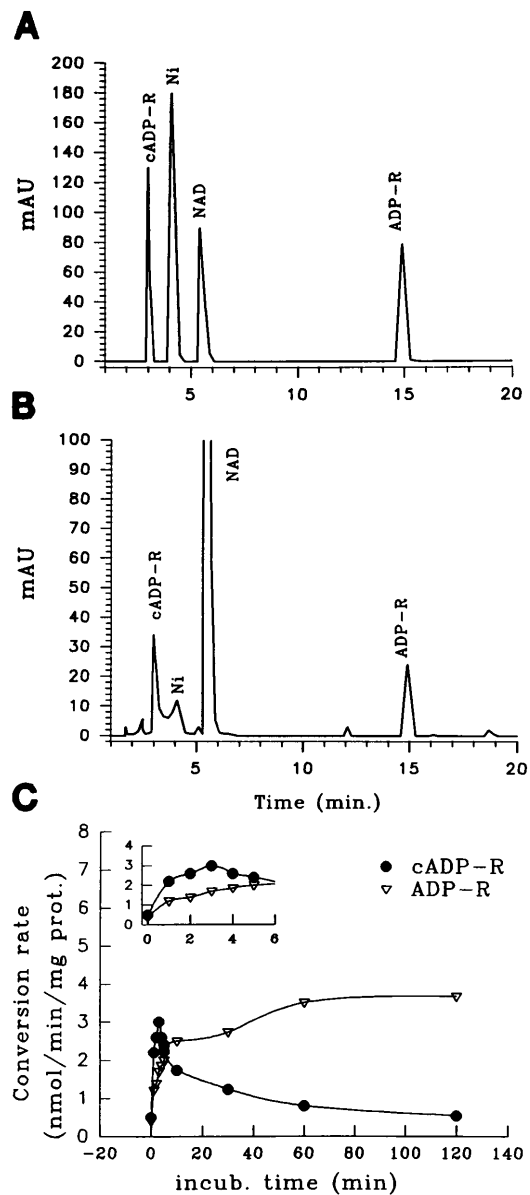


Fig. 1. *B*: reverse-phase HPLC chromatograms depicting production of cyclic ADP-ribose (cADP-R) and ADP-ribose (ADP-R) [in milli arbitrary units (mAU)] in coronary arterial homogenates. Homogenates were prepared from dissected small bovine coronary arteries and incubated with 1 mM NAD. Products were separated and analyzed by reverse-phase HPLC. Metabolites were monitored with an ultraviolet detector at 254 nm. Ni, nicotinamide. Migration times of known standards are shown in chromatogram of *A*. *C*: time course of conversion of NAD into cADP-R and ADP-R in coronary arterial homogenates. *Inset* shows shorter time points from 0 to 5 min, whereas main course indicates longer time points from 0 to 120 min. Conversion rate was calculated as  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .

a retention time of 14.6 min and coeluted with synthetic ADP-R. The peak with a retention time of 3.1 min was unreacted cADP-R. Figure 2B presents a time course of the conversion of cADP-R into ADP-R. A time-dependent increase in the conversion rate of ADP-R was observed. The maximal conversion rate was  $2.2 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .

Table 1 summarizes the maximal conversion rates of NAD into cADP-R and ADP-R in the homogenates,

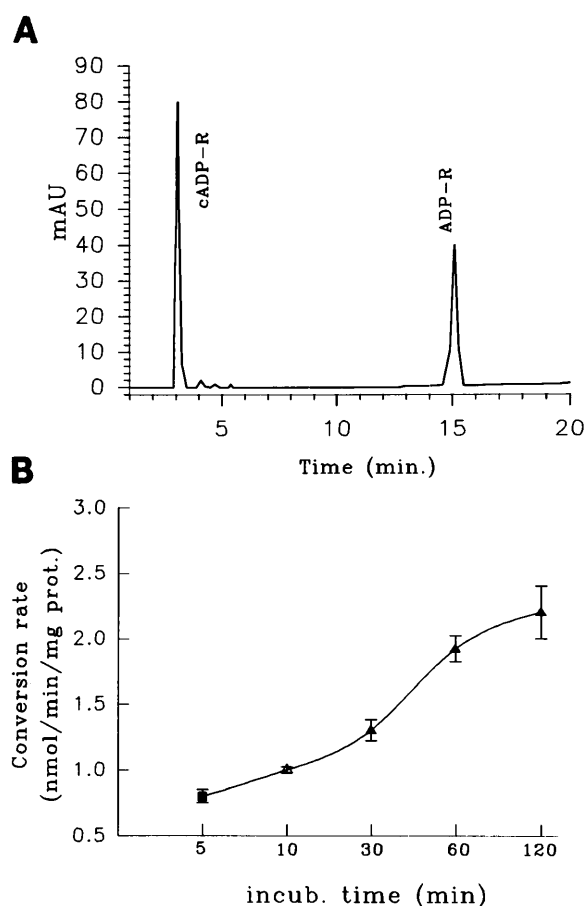


Fig. 2. *A*: reverse-phase HPLC chromatograms depicting conversion of cADP-R into ADP-R in coronary arterial homogenates. Homogenates were prepared from dissected small bovine coronary arteries and incubated with 1 mM cADP-R. *B*: time course of conversion of cADP-R into ADP-R in coronary arterial homogenates.

microsomes, and cytosols prepared from coronary arteries and in the homogenates prepared from cultured coronary SMC and EC. The greater conversion rate of both cADP-R and ADP-R was detected in the microsomes rather than in the cytosols and homogenates. In the homogenate prepared from cultured bovine coronary arterial SMC and EC, both cADP-R and ADP-R were synthesized. In the presence of 1 mM NAD, the

Table 1. *Production of cADP-R and ADP-R in homogenates, microsomes, and cytosols prepared from bovine coronary arteries and in homogenates prepared from cultured coronary SMC and EC*

	Conversion Rate, nmol·min <sup>-1</sup> ·mg protein <sup>-1</sup>	
	cADP-R	ADP-R
Arterial tissue		
Homogenate	1.37 ± 0.03	3.66 ± 0.03
Microsome	2.83 ± 0.15	5.05 ± 0.23
Cytosol	0.13 ± 0.02	1.80 ± 0.03
SMC homogenate	0.54 ± 0.15	2.40 ± 0.20
EC homogenate	0.12 ± 0.03	0.80 ± 0.09

Values represent means ± SE for  $n = 5$  experiments. cADP-R, cyclic ADP-ribose; ADP-R, ADP-ribose; SMC, smooth muscle cells; EC, endothelial cells.

conversion rates of cADP-R and ADP-R were much greater in SMC than in EC.

*Effect of ADP-R on activity of  $K_{Ca}$  channels.* Figure 3*A* depicts the unitary  $K^+$  currents at a membrane potential of +40 mV in an inside-out patch from coronary arterial SMC. The unitary  $K^+$  current has a mean slope conductance of  $256.3 \pm 5$  pS and is consistent with a  $K_{Ca}$  channel (34, 35). In previous studies, we have shown that this  $K^+$  current is activated by increase in intracellular  $Ca^{2+}$  and inhibited by tetraethylammonium and iberiotoxin (34, 35). ADP-R produced a concentration-dependent increase in the activity of the  $K_{Ca}$  channels when added to bath solution in the inside-out patch mode. The  $NP_o$  of the  $K_{Ca}$  channels was increased fivefold at an ADP-R concentration of 10  $\mu$ M. A significant effect was observed at the lowest concentration of ADP-R studied (0.1  $\mu$ M) (Fig. 3*B*). ADP-R had no effect on the single current amplitude of the  $K_{Ca}$  channels (Fig. 3*C*). In the time-course experiments, the  $K_{Ca}$  activity was increased eightfold 1 min after ADP-R (10  $\mu$ M) was added into the bath solution, and activation of the  $K_{Ca}$  channels was maintained during a 10-min recording.

*Effect of cADP-R on activity of  $K_{Ca}$  channels.* In contrast to the effect of ADP-R to increase the activity of the  $K_{Ca}$  channels, cADP-R reduced the  $NP_o$  of the  $K_{Ca}$  channels when applied to the internal surface of inside-out excised patches (Fig. 4*A*). A significant inhibition occurred at a concentration of 1  $\mu$ M, and the  $NP_o$  of the  $K_{Ca}$  channels was decreased by 75% at the highest concentration studied (10  $\mu$ M) (Fig. 4*B*). The amplitude of the  $K_{Ca}$  channels was unaltered by addition of cADP-R (Fig. 4*C*). A significant decrease in the  $K_{Ca}$ -channel activity was only observed 5 min after cADP-R was added into the bath.

*Effect of NAD on activity of  $K_{Ca}$  channels.* NAD, the precursor of cADP-R and ADP-R, produced a concentration-dependent increase in the activity of the  $K_{Ca}$  channels when applied to the internal surface of inside-out patches (Fig. 5*A*). The  $NP_o$  of the  $K_{Ca}$  channels was increased threefold at a concentration of 10  $\mu$ M NAD (Fig. 5*B*). NAD had no effect on the amplitude of the  $K_{Ca}$  channels (Fig. 5*C*). In the time-course experiments, NAD at a concentration of 10  $\mu$ M significantly increased the  $K_{Ca}$ -channel activity only 5 min after being added into the bath solution. In comparison with the effects of ADP-R, a 2-min time delay was observed. NAD-induced increase in the  $K_{Ca}$ -channel activity was spontaneously attenuated during a 10-min recording.

*Effect of analogs of NAD or ADP-riboses on activity of  $K_{Ca}$  channels.* Table 2 summarizes the effects of NAD, cADP-R, ADP-R, and their analogs on the  $K_{Ca}$ -channel activity. NAD and ADP-R at a concentration of 10  $\mu$ M significantly increased the  $NP_o$  of the  $K_{Ca}$  channels, whereas cADP-R decreased the  $NP_o$  of these channels. When cADP-R and ADP-R were simultaneously added into the bath solution, the  $K_{Ca}$ -channel activity was not altered. NGD, cGDP-R, cIDP-R, and even 8-bromo-cADP-R, an inhibitor of cADP-R-induced  $Ca^{2+}$  release, had no significant effect on the  $K_{Ca}$ -channel activity.

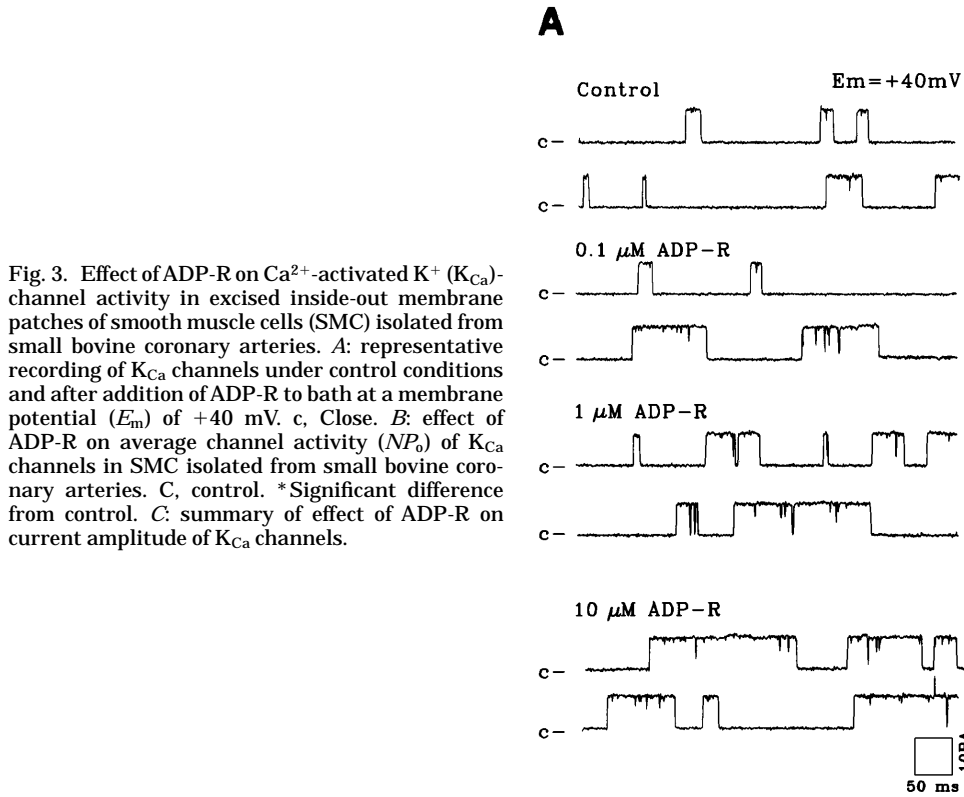
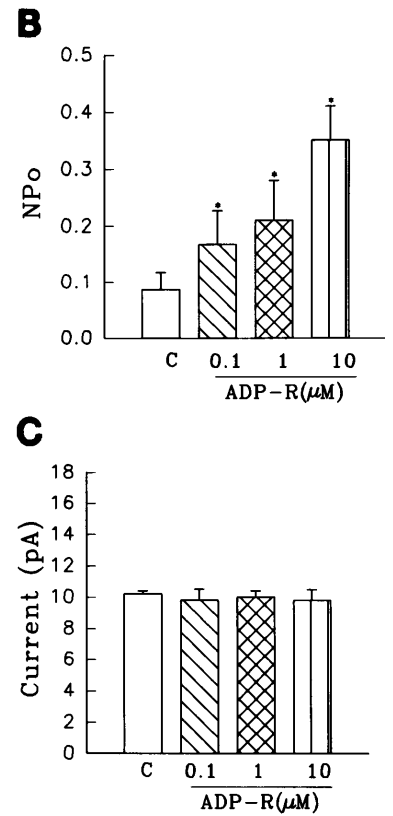


Fig. 3. Effect of ADP-R on  $Ca^{2+}$ -activated  $K^+$  ( $K_{Ca}$ )-channel activity in excised inside-out membrane patches of smooth muscle cells (SMC) isolated from small bovine coronary arteries. *A*: representative recording of  $K_{Ca}$  channels under control conditions and after addition of ADP-R to bath at a membrane potential ( $E_m$ ) of +40 mV. *c*, Close. *B*: effect of ADP-R on average channel activity ( $NP_o$ ) of  $K_{Ca}$  channels in SMC isolated from small bovine coronary arteries. *C*, control. \*Significant difference from control. *C*: summary of effect of ADP-R on current amplitude of  $K_{Ca}$  channels.



*Effect of cADP-R or ADP-R on voltage dependence and  $Ca^{2+}$  sensitivity of  $K_{Ca}$  channels.* In the inside-out patch mode, the  $K_{Ca}$  channels exhibited a voltage-dependent change in the activity. When the membrane

potential was changed from +20 to -60 mV,  $NP_o$  of the  $K_{Ca}$  channels was increased from  $0.015 \pm 0.001$  to  $0.1033 \pm 0.02$ . A calculated voltage ( $pV_{50}$ ) producing a 50% increase in  $NP_o$  of the  $K_{Ca}$  channels was -44 mV.

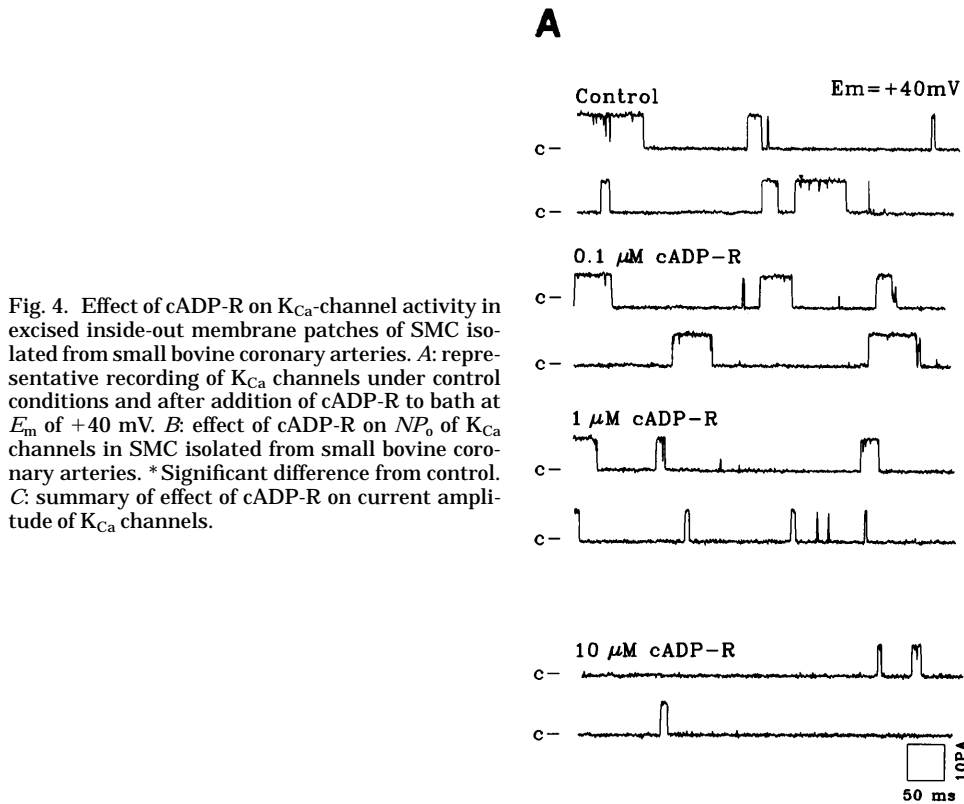
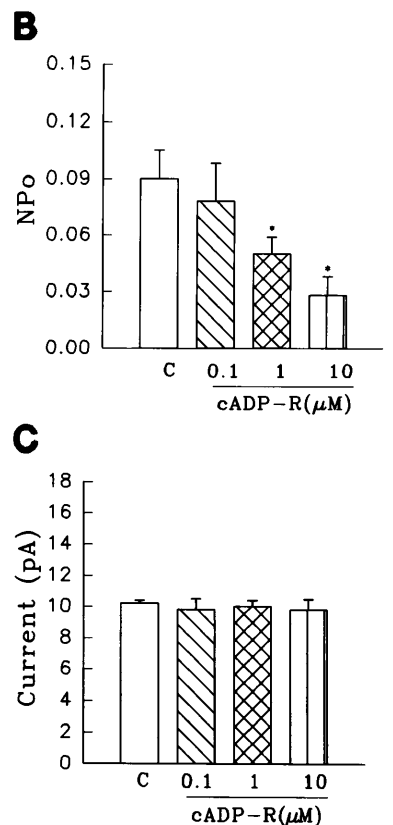


Fig. 4. Effect of cADP-R on  $K_{Ca}$ -channel activity in excised inside-out membrane patches of SMC isolated from small bovine coronary arteries. *A*: representative recording of  $K_{Ca}$  channels under control conditions and after addition of cADP-R to bath at  $E_m$  of +40 mV. *B*: effect of cADP-R on  $NP_o$  of  $K_{Ca}$  channels in SMC isolated from small bovine coronary arteries. \*Significant difference from control. *C*: summary of effect of cADP-R on current amplitude of  $K_{Ca}$  channels.



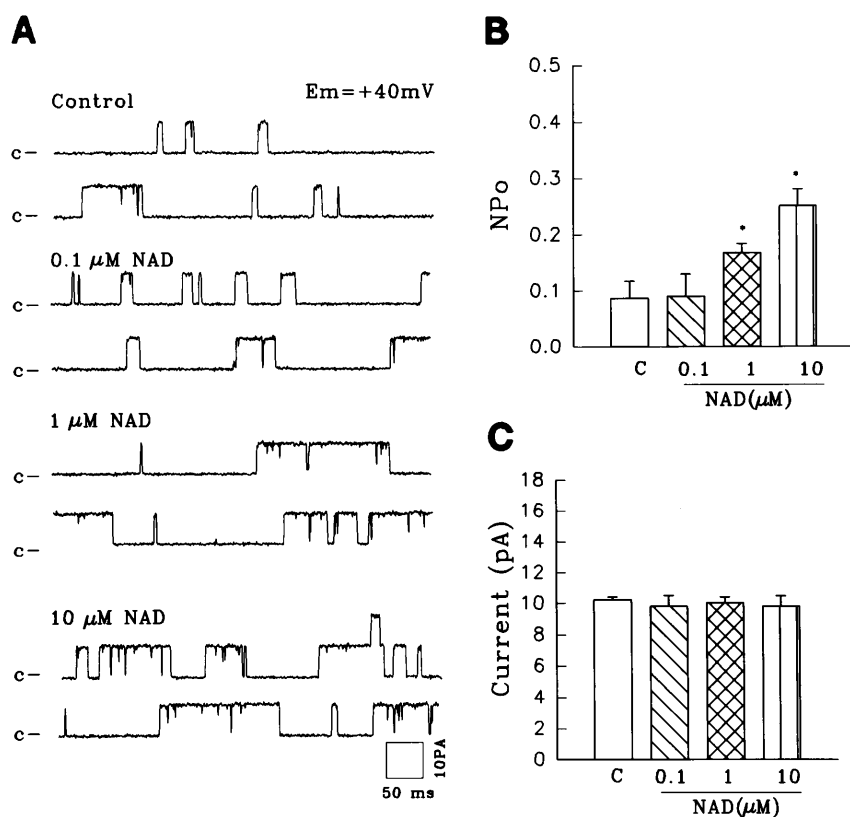


Fig. 5. Effect of NAD on  $K_{Ca}$ -channel activity in excised inside-out membrane patches of SMC isolated from small bovine coronary arteries. *A*: representative recording of  $K_{Ca}$  channels under control conditions and after addition of NAD to bath at  $E_m$  of +40 mV. *B*: effect of NAD on  $NP_0$  of  $K_{Ca}$  channels in SMC isolated from small bovine coronary arteries. \*Significant difference from control. *C*: summary of effect of NAD on current amplitude of  $K_{Ca}$  channels.

ADP-R or cADP-R produced parallel upward or downward shifts of the  $NP_0$  and membrane potential relationship, respectively. The  $pV_{50}$  was  $-43$  mV in the presence of ADP-R and  $-45$  mV in the presence of cADP-R.

The activity of the  $K_{Ca}$  channels was increased in response to an increase in intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ).  $NP_0$  of the  $K_{Ca}$  channels were  $0.06 \pm 0.01$ ,  $0.26 \pm 0.07$ , and  $0.55 \pm 0.06$  at  $[Ca^{2+}]_i$  of 0.1, 1, and 10  $\mu M$ , respectively. Calculated concentration of  $Ca^{2+}$  producing a 50% increase ( $pCa_{50}$ ) in  $NP_0$  was 1.8  $\mu M$ . In the presence of ADP-R and cADP-R,  $Ca^{2+}$ -induced increase in  $NP_0$  of the  $K_{Ca}$  channels was not altered. The calculated  $pCa_{50}$  averaged 1.8 and 2.5  $\mu M$  in the

presence of ADP-R and cADP-R, respectively, which were not significantly different from control.

## DISCUSSION

In the present study, we found that the homogenates prepared from dissected small bovine coronary arteries and cultured SMC produced cADP-R and ADP-R when incubated with NAD. The conversion rate for ADP-R was higher than that for cADP-R. This data provides the first evidence that NAD is metabolized to cADP-R and ADP-R in the vasculature. Recent studies indicated that NAD is converted into cADP-R by cyclization via ADP-ribosylcyclase and into ADP-R by hydrolysis via  $NAD^+$  glycohydrolase (7, 15, 18, 19). In addition, cADP-R can also be hydrolyzed to ADP-R (21, 23–25, 28, 30, 32). These membrane-bound hydrolases and the cyclase have been characterized and purified (14, 18, 19, 32, 36). Using purified ADP-ribosylcyclase and  $NAD^+$  glycohydrolase, we confirmed that the NAD metabolites produced from coronary arterial homogenates and cultured SMC are the same as those produced by corresponding purified enzymes. These findings suggest that coronary arteries contain both ADP-ribosylcyclase and  $NAD^+$  glycohydrolase activity. By determining the time course of the production of ADP-R and cADP-R, we found that the increase in the synthesis of ADP-R was accompanied by a decrease in cADP-R. This suggests that cADP-R may be converted into ADP-R and that cADP-R hydrolase may also participate in the synthesis of ADP-R in coronary arteries. To determine the activity of cADP-R hydrolase, coronary arterial homogenate was incubated with cADP-R and HPLC analysis

Table 2. Effect of NAD, ADP-riboses, and their analogs (10  $\mu M$ ) on  $K_{Ca}$ -channel activity in bovine coronary SMC

	n	$NP_0$	
		Control	After drugs
NAD	5	$0.087 \pm 0.030$	$0.250 \pm 0.03^*$
cADP-R	5	$0.090 \pm 0.015$	$0.028 \pm 0.01^*$
ADP-R	5	$0.087 \pm 0.030$	$0.350 \pm 0.06^*$
ADP-R + cADP-R	7	$0.044 \pm 0.008$	$0.044 \pm 0.01$
NGD	7	$0.044 \pm 0.011$	$0.040 \pm 0.01$
cGDP-R	5	$0.067 \pm 0.023$	$0.056 \pm 0.03$
cIDP-R	4	$0.077 \pm 0.035$	$0.076 \pm 0.03$
8-Br-cADP-R	7	$0.050 \pm 0.010$	$0.046 \pm 0.01$

Values represent means  $\pm$  SE.  $NP_0$ , average channel activity; NGD, nicotinamide guanine dinucleotide; cGDP-R, cyclic guanosine diphosphate-ribose; cIDP-R, cyclic inosine diphosphate-ribose; 8-Br-cADP-R, 8-bromo-cADP-R. \*Significant difference from control at  $P < 0.05$ .

was performed. Interestingly, cADP-R hydrolase activity was also expressed in coronary arteries. On the basis of these results, we conclude that an enzymatic pathway responsible for the formation and metabolism of cADP-R is present in the coronary vasculature. cADP-R is a metabolite of NAD by ADP-ribosylcyclase, and the production of ADP-R may be associated with both NAD<sup>+</sup> glycohydrolase and cADP-R hydrolase.

To define the cell type that possesses the enzymatic pathway for the formation and metabolism of cADP-R, we determined the production of cADP-R and ADP-R in the homogenates prepared from cultured coronary EC and SMC. In the presence of NAD, both EC and SMC produced cADP-R and ADP-R. The conversion rate of cADP-R and ADP-R was greater in SMC than in EC. The presence of the metabolic pathway for the cADP-R formation and hydrolysis in the endothelium and vascular smooth muscle may be of importance in the regulation of coronary vascular tone.

To further determine the localization of the enzyme activity responsible for the production of cADP-R and ADP-R, we examined the metabolism of NAD in the microsomes and cytosols prepared from the coronary arteries. HPLC analysis demonstrated that the productions of both cADP-R and ADP-R were greater in the microsomal fraction compared with those in the cytosolic fraction, suggesting that the enzymes responsible for the production and metabolism of cADP-R are primarily present on the cell membrane. These results are consistent with previous evidence that ADP-ribosylcyclase, NAD<sup>+</sup> glycohydrolase, and cADP-R hydrolase are membrane-bound enzymes in a wide range of mammalian tissues (11, 14, 24, 28, 32, 39). It has been reported that a membrane-bound enzyme complex has high homology with human lymphocyte differentiate antigen CD38, and CD38 has multiple enzyme activities including ADP-ribosylcyclase, cADP-R hydrolase, and NAD<sup>+</sup> glycohydrolase (7, 15, 18, 32, 36, 45). Although we can detect a CD38 protein in coronary arteries by Western blot analysis with the use of a monoclonal anti-human CD38 antibody, the quantitative assay of enzyme activity after immunoprecipitation of homogenates or microsomes with this antibody only partially blocked the production of cADP-R and ADP-R (data not shown). It seems that CD38 is not the only enzyme responsible for the production and metabolism of cADP-R in coronary arteries. The identity of these enzymes remains to be further defined.

The present study also attempted to determine the physiological relevance of cADP-R signaling pathway in coronary arterial smooth muscle. Recent studies indicated that cADP-R may activate the ryanodine receptor to mobilize intracellular Ca<sup>2+</sup> (23, 26, 28). Although vascular SMC possess ryanodine receptors (16, 40), so far there is no evidence suggesting that cADP-R-mediated Ca<sup>2+</sup> mobilization participates in the regulation of vascular tone. In this regard, NO has been reported to increase the production of cADP-R and induce intracellular Ca<sup>2+</sup> mobilization in nonvascular tissue (28, 42). However, this cADP-R-mediated Ca<sup>2+</sup> mobilization is unlikely to mediate the vasodilator

effect of NO, because a rise in intracellular Ca<sup>2+</sup> causes vasoconstriction. Thus it seems that the effect of NO on the production of cADP-R is tissue specific. In a recent review, Lee (23) indicated that NO may activate cADP-R hydrolase, reduce intracellular cADP-R in the vascular smooth muscle, and subsequently lead to lowering of cytosolic Ca<sup>2+</sup>, causing vasodilation. However, this hypothesis has never been confirmed experimentally.

Interestingly, the present study demonstrated that cADP-R and ADP-R alter the activity of the K<sub>Ca</sub> channels in coronary arterial smooth muscle. ADP-R markedly activated the K<sub>Ca</sub> channels, whereas cADP-R inhibited this channel. The activation of the K<sub>Ca</sub> channels may lead to the hyperpolarization of vascular smooth muscle, reduction in intracellular Ca<sup>2+</sup>, and, hence, vasodilation. In contrast, inhibition of the K<sub>Ca</sub>-channel activity has the opposite effect, producing depolarization, increase in intracellular Ca<sup>2+</sup>, and vasoconstriction. It is possible that cADP-R and ADP-R participate in the control of vascular tone by regulating the K<sub>Ca</sub>-channel activity in vascular SMC. It remains to be determined whether cADP-R and ADP-R mediate the effect of vasoactive agonists that serve as the K<sup>+</sup>-channel activator or inhibitor.

NAD also increased the activity of the K<sub>Ca</sub> channels when applied to the internal surface of excised inside-out membrane patches from coronary arterial SMC. This is consistent with recent findings obtained from pulmonary arterial smooth muscle (33). The mechanism by which NAD activates the K<sub>Ca</sub> channels is unknown. The present study suggests that an increase in the production of ADP-R via membrane-bound NAD<sup>+</sup> glycohydrolase or cADP-R hydrolase may be an important mechanism for NAD-induced activation of the K<sub>Ca</sub> channels. A 2-min time delay in the NAD effect on the K<sub>Ca</sub>-channel activity compared with the effects of ADP-R supports the view that the effect of NAD is associated with the production of ADP-R. This production of ADP-R via either NAD<sup>+</sup> glycohydrolase or cADP-R hydrolase, or both, may override the inhibitory effect of cADP-R on the K<sub>Ca</sub>-channel activity, and hence NAD produces activation of the K<sub>Ca</sub> channels. However, in contrast to a sustained increase in the K<sub>Ca</sub>-channel activity produced by ADP-R, NAD-induced activation of the K<sub>Ca</sub> channels ran down spontaneously during a 10-min experimental period. Because cADP-R decreased the K<sub>Ca</sub>-channel activity in this membrane preparation, it is possible that the accumulation of cADP-R during the experimental period contributes to the attenuation of activation of the K<sub>Ca</sub> channels by NAD. This antagonistic effect was confirmed by a simultaneous addition of cADP-R and ADP-R.

The effect of cADP-R and ADP-R on the K<sub>Ca</sub>-channel activity was observed when these nucleotides were added to the internal surface of excised inside-out membrane patches. This indicates that no intracellular soluble cofactors or second messengers are required for their effect. These nucleotides may act directly on the K<sub>Ca</sub>-channel protein or some regulatory protein of this channel in the cell membrane. It seems that ADP-R and cADP-R act on the same site or target, because they



exhibited an antagonistic effect on the  $K_{Ca}$ -channel activity when added simultaneously. However, it remains unknown how ADP-R and cADP-R act on the  $K_{Ca}$  channels. The present study demonstrated that the analogs of ADP-R, cADP-R, and NAD did not alter the  $K_{Ca}$ -channel activity, indicating that ADP-R and cADP-R have a specific site of action on the  $K_{Ca}$  channel or its regulatory elements. This specific mechanism is not dependent on alteration of voltage dependence or  $Ca^{2+}$  sensitivity of the  $K_{Ca}$  channels, because voltage- or  $Ca^{2+}$ -induced activation of the  $K_{Ca}$  channels was not altered by either ADP-R or cADP-R. Given that ADP ribosylation may alter the function of the membrane proteins of cells (4, 37), an enzymatic or nonenzymatic ADP ribosylation of the  $K_{Ca}$ -channel protein or related regulatory protein may contribute to ADP-R-induced activation of the  $K_{Ca}$  channels, and blockade of this ADP ribosylation by cADP-R may be associated with its inhibitory effect on the  $K_{Ca}$ -channel activity. This mechanism will be further explored by determining the ADP ribosylation or ADP-R radiolabeling of the cell membrane proteins from coronary SMC.

Recent studies in our laboratory and by others have demonstrated that cADP-R induced  $Ca^{2+}$  release from sarcoplasmic reticulum in  $\alpha$ -toxin- or  $\beta$ -escin-permeabilized coronary SMC (17, 44). This mobilization of intracellular  $Ca^{2+}$  has been recognized as an important function of cADP-R in nonvascular tissue and cells (28–30). Because the primary purpose of the present study was to characterize the metabolism and actions of NAD and its metabolites, cADP-R and ADP-R, in coronary arteries, we did not directly address the integrative role of endogenously produced cADP-R and ADP-R in the control of cell function of coronary smooth muscle. Considering the effect of cADP-R in mediating the mobilization of intracellular  $Ca^{2+}$  and on the basis of our results in this study, we propose that NAD is converted into cADP-R in the coronary arterial SMC to mobilize intracellular  $Ca^{2+}$  and increase vascular tone, and it is also converted into ADP-R to function as a  $K_{Ca}$ -channel activator in concert with  $Ca^{2+}$ -dependent activation of the  $K_{Ca}$  channel to protect the cell from  $Ca^{2+}$  overloading. Alteration in the production and metabolism of cADP-R or ADP-R in SMC would change vascular tone in coronary circulation.

In summary, the present study characterized the metabolism of cADP-R and ADP-R in small bovine coronary arteries and demonstrated that these intracellular nucleotides alter the activity of the  $K_{Ca}$  channels in coronary vascular SMC. Our results indicate that cADP-R and ADP-R may serve as intracellular signaling molecules that gate the  $K_{Ca}$  channels and control coronary vascular tone.

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## REFERENCES

1. **Beers, K., E. N. Chini, H. C. Lee, and T. P. Dousa.** Metabolism of cyclic ADP-ribose in opossum kidney renal epithelial cells. *Am. J. Physiol.* 268 (*Cell Physiol.* 37): C741–C746, 1995.
2. **Bevyak, G. E., M. J. Hughes, A. R. Johnson, and W. B. Campbell.** Histamine stimulation of prostaglandin and HETE synthesis in human endothelial cells. *Am. J. Physiol.* 255 (*Cell Physiol.* 24): C214–C225, 1988.
3. **Buzzard, C. J., S. L. Pfister, and W. B. Campbell.** Endothelium dependent contractions in rabbit pulmonary artery are mediated by thromboxane  $A_2$ . *Circ. Res.* 72: 1023–1034, 1993.
4. **Duman, R. S., R. Z. Terwilliger, and E. J. Nestler.** Endogenous ADP-ribosylation in brain: initial characterization of substrate protein. *J. Neurochem.* 57: 2124–2132, 1991.
5. **Galione, A.**  $Ca^{2+}$ -induced  $Ca^{2+}$  release and its modulation by cyclic ADP-ribose. *Trends Pharmacol. Sci.* 13: 304–306, 1992.
6. **Galione, A.** Cyclic ADP-ribose: a new way to control calcium. *Science* 259: 325–326, 1993.
7. **Galione, A.** Cyclic ADP-ribose, the ADP-ribosyl cyclase pathway and calcium signalling. *Mol. Cell. Endocrinol.* 98: 125–131, 1994.
8. **Galione, A., H. C. Lee, and W. B. Busa.**  $Ca^{2+}$ -induced  $Ca^{2+}$  release in sea urchin egg homogenates and its modulation by cyclic ADP-ribose. *Science* 253: 1143–1146, 1991.
9. **Galione, A., A. McDougall, W. B. Busa, N. Willmott, I. Gillot, and M. J. Whitaker.** Redundant mechanism of calcium-induced calcium release underlying calcium waves during fertilization of sea urchin eggs. *Science* 261: 348–352, 1993.
10. **Galione, A., H. White, N. Willmott, M. Turner, B. V. L. Potter, and S. P. Watson.** cGMP mobilizes intracellular  $Ca^{2+}$  in sea urchin eggs by stimulating cyclic ADP-ribose synthesis. *Nature* 365: 456–459, 1993.
11. **Glick, D. L., M. R. Hellmich, S. Beushausen, P. J. Tempst, H. Bayley, and F. Strumwasser.** Primary structure of a molluscan egg-specific NADase, a second messenger enzyme. *Cell Regul.* 2: 211–218, 1991.
12. **Godt, R. E.** Calcium-activated tension of skinned muscle fibers of the frog. *J. Gen. Physiol.* 63: 722–739, 1974.
13. **Hamill, O. P., M. R. Neher, and B. Sakmann.** Improved patch clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Arch.* 391: 85–100, 1981.
14. **Hellmich, M. R., and F. Strumwasser.** Purification and characterization of a molluscan egg-specific NADase, a second messenger enzyme. *Cell Regul.* 2: 193–202, 1991.
15. **Howard, M., J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, R. M. E. Parkhouse, T. F. Walseth, and H. C. Lee.** Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* 262: 1056–1059, 1993.
16. **Ito, K., T. Ikemoto, and S. Takakura.** Involvement of  $Ca^{2+}$  influx-induced  $Ca^{2+}$  release in contractions of intact vascular smooth muscle. *Am. J. Physiol.* 261 (*Heart Circ. Physiol.* 30): H1464–H1470, 1991.
17. **Kannan, M. S., A. M. Fenton, Y. S. Prakash, and G. C. Sieck.** Cyclic ADP-ribose stimulates sarcoplasmic reticulum calcium release in porcine coronary artery smooth muscle. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H801–H806, 1996.
18. **Kim, H., E. L. Jacobson, and M. K. Jacobson.** Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. *Science* 261: 1330–1333, 1993.
19. **Kim, U.-H., M.-K. Kim, J.-S. Kim, M.-K. Han, B.-H. Park, and H.-R. Kim.** Purification and characterization of glycohydrolase from rabbit erythrocytes. *Arch. Biochem. Biophys.* 305: 147–152, 1993.
20. **Koshiyama, H. H., H. C. Lee, and A. H. Tashjian.** Novel mechanism of intracellular calcium release in pituitary cell. *J. Biol. Chem.* 266: 16985–16988, 1991.
21. **Lee, H. C.** Specific binding of cyclic ADP-ribose to calcium-storing microsomes from sea urchin eggs. *J. Biol. Chem.* 266: 2276–2281, 1991.
22. **Lee, H. C.** Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. *J. Biol. Chem.* 268: 293–299, 1993.
23. **Lee, H. C.** A signaling pathway involving cyclic ADP-ribose, cGMP, and nitric oxide. *News Physiol. Sci.* 9: 134–137, 1994.

24. **Lee, H. C., and R. Aarhus.** ADP-ribosyl cyclase: an enzyme that cyclizes NAD<sup>+</sup> into a calcium-mobilizing metabolite. *Cell Regul.* 2: 203–209, 1991.
25. **Lee, H. C., and R. Aarhus.** Wide distribution of an enzyme that catalyzes the hydrolysis of cyclic ADP-ribose. *Biochim. Biophys. Acta* 1164: 68–74, 1993.
26. **Lee, H. C., R. Aarhus, M. E. Gurnack, and T. F. Walseth.** Cyclic ADP-ribose activation of the ryanodine receptor is mediated by calmodulin. *Nature* 370: 307–309, 1994.
27. **Lee, H. C., R. Aarhus, and T. F. Walseth.** Calcium mobilization by dual receptors during fertilization of sea urchin eggs. *Science* 261: 352–355, 1993.
28. **Lee, H. C., A. Galione, and T. F. Walseth.** Cyclic ADP-ribose: metabolism and calcium mobilizing function. In: *Vitamins and Hormones*, edited by G. Litwack. Orlando, FL: Academic, 1994, vol. 48, chapt. 5, p. 199–254.
29. **Lee, H. C., R. Graeff, and T. F. Walseth.** Cyclic ADP-ribose and its metabolic enzymes. *Biochimie* 77: 345–355, 1995.
30. **Lee, H. C., T. F. Walseth, R. Aarhus, and D. Levitt.** Cyclic ADP-ribose as a second messenger for mobilizing intracellular calcium stores. *J. Reprod. Devel.* 39: 64–69, 1993.
31. **Lee, H. C., T. F. Walseth, G. T. Bratt, R. N. Hayes, and D. L. Clapper.** Structural determination of a cyclic metabolite of NAD<sup>+</sup> with intracellular Ca<sup>2+</sup> mobilizing activity. *J. Biol. Chem.* 264: 1608–1615, 1989.
32. **Lee, H. C., E. Zocchi, L. Guida, L. Franco, U. Benatti, and A. De Flora.** Production and hydrolysis of cyclic ADP-ribose at the outer surface of human erythrocytes. *Biochem. Biophys. Res. Commun.* 191: 639–645, 1993.
33. **Lee, S., M. Park, I. So, and Y. E. Earm.** NADH and NAD modulates Ca<sup>2+</sup>-activated K<sup>+</sup> channels in small pulmonary arterial smooth muscle cells of the rabbit. *Pflügers Arch.* 427: 378–380, 1994.
34. **Li, P.-L., and W. B. Campbell.** Epoxyeicosatrienoic acids activate potassium channels in coronary smooth muscle through nucleotide binding protein. *Circ. Res.* 80: 877–884, 1997.
35. **Li, P.-L., A. P. Zou, and W. B. Campbell.** Regulation of potassium channel in coronary arterial smooth muscle by endothelium-dependent vasodilators. *Hypertension* 29: 262–267, 1997.
36. **Muller-Steffner, H., M. Muzard, N. Oppenheimer, and F. Schuber.** Mechanistic implications of cyclic ADP-ribose hydrolysis and methanolysis catalyzed by calf spleen NAD<sup>+</sup> glycohydrolase. *Biochem. Biophys. Res. Commun.* 204: 1279–1285, 1994.
37. **Okazaki, I. J., and Moss J.** Mono-ADP-ribosylation: a reversible posttranslational modification of proteins. *Adv. Pharmacol.* 35: 247–280, 1996.
38. **Rosolowsky, M., and W. B. Campbell.** Synthesis of hydroxy-eicosatetraenoic (HETE) and epoxyeicosatrienoic acid (EETs) by cultured bovine coronary artery endothelial cells. *Biochim. Biophys. Acta* 1299: 267–277, 1996.
39. **Ruskino, N., and H. C. Lee.** Widespread occurrence in animal tissues of an enzyme catalyzing the conversion of NAD into a cyclic metabolite with intracellular Ca<sup>2+</sup>-mobilizing activity. *J. Biol. Chem.* 264: 11725–11731, 1989.
40. **Sorrentino, V., and P. Volpe.** Ryanodine receptors: how many, where and why? *Trends Pharmacol. Sci.* 14: 98–103, 1993.
41. **Takesawa, S., K. Nata, H. Yonekura, and H. Okamoto.** Cyclic ADP-ribose in insulin secretion from pancreatic cells. *Science* 259: 370–373, 1993.
42. **Whalley, T., A. McDougall, L. Crossley, K. Swann, and M. Whitaker.** Internal calcium release and activation of sea urchin eggs by cGMP are independent of the phosphoinositide signaling pathway. *Mol. Biol. Cell* 3: 373–383, 1992.
43. **White, A. M., S. P. Watson, and A. Galione.** Cyclic ADP-ribose-induced Ca<sup>2+</sup> release from rat brain microsomes. *FEBS Lett.* 318: 259–263, 1993.
44. **Yu, J.-Z., A.-P. Zou, W. B. Campbell, and P.-L. Li.** Role of cADP-ribose in NO-mediated decrease in [Ca<sup>2+</sup>]<sub>i</sub> in bovine coronary arterial smooth muscle cells (Abstract). *FASEB J.* 12: A406, 1998.
45. **Zocchi, E., L. Franco, L. Guida, U. Benatti, A. Bragellesi, F. Malavasi, H. C. Lee, and A. De Flora.** A single protein immunologically identified as CD38 displays NAD<sup>+</sup> glycohydrolase, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities at the out surface of human erythrocytes. *Biochem. Biophys. Res. Commun.* 196: 1459–1465, 1993.