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Abstract—The role of endogenous ADP-ribosylation in mediating the activation of the Ca^{2+}-activated K^+ channels was determined in bovine coronary arteries. Endogenous ADP-ribosylation was examined by incubating coronary arterial homogenates or lysates of cultured coronary arterial smooth muscle cells with [adenylate-^{32}P]NAD. Four ^{32}P-labeled proteins were observed at 51, 52, 80, and 124 kDa in the homogenates and lysates. This reaction was enhanced by the addition of 11,12-epoxyeicosatrienoic acid (11,12-EET), a cytochrome P450–derived eicosanoid, and GTP to the incubation. By Western blot analysis, 42- and 70-kDa proteins were recognized by specific antibodies against ADP-ribosyltransferase in the coronary arterial homogenates and smooth muscle cell lysate but not in the lysate of endothelial cells. The 52-kDa acceptor protein of endogenous ADP-ribosylation comigrated with a protein ADP-ribosylated by cholera toxin and was recognized and immunoprecipitated by an anti–$G_S\alpha$ antibody. These results suggest that $G_S\alpha$ is one of several acceptors of the ADP-ribose moiety. As shown by the patch-clamp technique, 11,12-EET stimulated the activation of the K^+ channels in the smooth muscle cells, and this activation was completely blocked by novobiocin, vitamin K_1, 3-aminobenzamide, and *m*-iodobenzylguanidine, inhibitors of endogenous mono-ADP-ribosyltransferases. We conclude that endogenous mono-ADP-ribosyltransferases are present in smooth muscle from bovine coronary arteries. These enzymes transfer ADP-ribose to the cellular proteins such as $G_S\alpha$ and may mediate intracellular signal transduction in coronary vascular smooth muscle. In the coronary circulation, the ADP-ribosylation signaling pathway may play an important role in mediating the activation of the K^+ channels induced by 11,12-EET. (*Circ Res*. 1999;85:349-356.)

Key Words: mono-ADP-ribosyltransferase ■ K^+ channel ■ coronary artery ■ eicosanoid ■ epoxyeicosatrienoic acid

W e have recently reported that epoxyeicosatrienoic acids (EETs), a family of cytochrome P450–derived eicosanoids, serve as endothelium-derived hyperpolarizing factors in the coronary circulation. The EETs activate the Ca^{2+}-activated K^+ (K_{Ca}) channels, hyperpolarize vascular smooth muscle, and dilate vessels.[1,2] Similar results have been reported in other vascular beds.[3–5] The mechanism by which EETs activate the K_{Ca} channels remains unknown. More recently, we demonstrated that EETs activate the K_{Ca} channels in coronary smooth muscle through a guanine nucleotide binding protein, G_S.[6] Like EETs, cholera toxin also activated this K_{Ca} channel. Given that cholera toxin is an ADP-ribosyltransferase, we wondered whether EETs act similarly. In fact, a recent study indicated that EETs stimulated endogenous ADP-ribosylation in liver cells.[7] It remains to be determined whether endogenous mono-ADP-ribosyltransferase is activated by EETs in coronary arterial smooth muscle and whether ADP-ribosylation is involved in the EET-induced activation of the K_{Ca} channels.

Mono-ADP-ribosyltransferases are present in many animal tissues.[8–11] Although there are different types of mono-ADP-ribosyltransferases in various tissues and they differ in their physiochemical properties, substrate specificity, and localization, all mono-ADP-ribosyltransferases catalyze the formation of *N*-glycosidic bonds between ADP-ribose and several specific amino acid residues.[11,12] These enzymes include (1) NAD:arginine ADP-ribosyltransferase, which catalyzes the transfer of the ADP-ribose moiety of NAD to arginine, arginine methyl ester, or histones, (2) NAD:diphthamide ADP-ribosyltransferase, which transfers ADP-riboses to a modified histidine residue, and (3) NAD:cysteine ADP-ribosyltransferase, which catalyzes the transfer of the ADP-riboses to cysteine.[9,13,14]

The role of ADP-ribosylation in intracellular signal transduction has been extensively studied. Bioactive hormones or second messenger systems regulate ADP-ribosylation of cellular proteins, particularly guanine nucleotide binding pro-

teins or G proteins. Endogenous ADP-ribosylation of $G_S\alpha$ occurs in adipocytes in response to activation of adenosine receptors[15] and in platelets by activation of prostacyclin or thrombin receptors[16,17] and release of nitric oxide.[17–19] The stimulation of β-adrenergic receptors increased ADP-ribosylation of $G_S\alpha$ in hepatocytes.[20] ADP-ribosylation of $G_S\alpha$ results in its activation and a persistent increase in adenylate cyclase activity. Moreover, ADP-ribosylation of $G_i\alpha$ leads to its inhibition and also produces activation of adenylate cyclase and alteration in ion channel activity.[14,21] In addition to G proteins, ADP-ribosylation of other proteins such as protein kinases may regulate intracellular signal transduction processes. ADP-ribosylation of effector molecules such as actin[22,23] and ion channels may also regulate cell function. Previous studies have indicated that a number of cellular proteins can be ADP-ribosylated.[9,12] However, little is known regarding mono-ADP-ribosyltransferase and endogenous mono-ADP-ribosylation in vascular tissues. It has yet to be determined whether endogenous ADP-ribosylation occurs in the vascular smooth muscle and whether it plays a role in signal transduction of vascular smooth muscle cells.

The purpose of the present study was to determine whether coronary arterial smooth muscle cells and endothelial cells express mono-ADP-ribosyltransferases and to address the role of endogenous mono-ADP-ribosylation in mediating the effect of EETs on the K^+ channel activity in coronary arteries. We examined the expression of mono-ADP-ribosyltransferase with the use of autoradiography and Western blot analysis in combination with immunoprecipitation and determined the role of ADP-ribosylation in the control of the K_{Ca} channel activity using the patch-clamp technique.

Materials and Methods

Preparation of Homogenate of Small Bovine Coronary Arteries

Bovine hearts were obtained from a local slaughterhouse. A branch of the coronary artery was cannulated and filled with 10 to 20 mL of ice-cold 3% Evan's blue in 50 mmol/L sodium phosphate containing 0.9% sodium chloride (pH 7.4) (physiological salt solution [PSS]) and 6% albumin. The heart was then dissected into $2\times3\times1$-cm pieces and sliced into 300-μm-thick tissue sections. Small coronary arteries (200 to 300 μm) stained with Evan's blue were identified under a dissecting stereomicroscope. These arteries were microdissected, pooled, and stored in ice-cold PSS. The dissected coronary arteries were cut into very small pieces and homogenized with a glass homogenator in ice-cold HEPES buffer containing (in mmol/L) Na-HEPES 25, EDTA 1, and PMSF 0.1. After centrifugation of the homogenate at 6000g for 5 minutes at 4°C, the supernatant containing membrane and cytosolic components, termed homogenate, was aliquoted and frozen in liquid N_2 and stored at -80°C until used. Microsomal and cytosolic fractions were prepared by a differential centrifugation of the homogenates at 2000g for 10 minutes, 10 000g for 20 minutes, and 100 000g for 90 minutes. The 100 000g-pellet was the microsomal fraction whereas supernatant was the cytosolic fraction.

Assay of Endogenous ADP-Ribosylation

ADP-ribosylation assay was carried out as described by Seki et al.[7] Homogenate (140 μg) was incubated in 200 μL of reaction mixture containing (in mmol/L) Tris-HCl (pH 7.5) 200, EDTA 2, MgCl$_2$ 10, NAD$^+$ 0.002, and 5 μCi of [^{32}P]NAD$^+$ with or without 1 mmol/L ATP and/or 0.5 mmol/L GTP. 11,12-Epoxyeicosatrienoic acid (11,12-EET), which was synthesized and purified as we described

previously,[2] was added to the reaction mixture at a concentration of 1 or 10 μmol/L. The reaction was stopped 30 minutes later by the addition of 0.8 mL of 10% (wt/vol) trichloroacetic acid (TCA) and 10 μL of 0.1% BSA. After being kept on ice for 30 minutes, the sample was centrifuged at 12 000g for 10 minutes. The precipitates were dissolved in SDS-PAGE gel sample buffer and heated at 100°C for 3 minutes. Protein was separated by SDS-PAGE according to the method of Laemmli.[24] The gel was dried, and radioactivity was detected by autoradiography on Kodak Omat film. Cholera toxin–stimulated ADP-ribosylation was determined by the same method as described above, except for the addition of 5 μg of cholera toxin to the mixture after a 5-minute preincubation of the homogenate with different concentrations of 11,12-EET (10^{-9} to 10^{-5} mol/L) or its vehicle. In some experiments, proteins separated by SDS-PAGE were transferred to nitrocellulose and analyzed by immunoblotting using a specific antibody against $G_S\alpha$ to localize ADP-ribosylated acceptor protein, as described below.

Immunoprecipitation

Immunoprecipitation was performed as described previously.[25] Briefly, coronary arterial homogenate (140 μg) was incubated with [^{32}P]NAD$^+$ as described above, and then the rabbit polyclonal antibody against the α subunits of G_S (10 to 30 μL) was added to the reaction mixtures. Samples were incubated for 2 hours at 4°C under constant rotation, and then protein A–Sepharose beads (60 μL) of 12.5% (wt/vol) were added. Before the addition, the Sepharose beads were allowed to swell for 30 minutes in precipitation buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, and 1% Triton X-100) and washed 3 times with 1 mL of precipitation buffer. After the reaction mixtures were incubated overnight, the Sepharose beads were pelleted (12 000g for 5 minutes) and washed twice with 1 mL of washing buffer A containing 1% (wt/vol) Nonidet P-40, 0.5% (wt/vol) SDS, 600 mmol/L NaCl, and 50 mmol/L Tris-HCl (pH 7.4) and once with 1 mL of washing buffer B containing 300 mmol/L NaCl, 10 mmol/L EDTA, and 100 mmol/L Tris-HCl (pH 7.4). The beads were reconstituted with 50 μL of buffer B and heated at 100°C for 3 minutes and then centrifuged at 3000 rpm for 5 minutes. The supernatants were dissolved in SDS-PAGE gel sample buffer and proteins were separated by SDS-PAGE as described above. The gel was dried, and radioactivity was detected by autoradiography on Kodak Omat film.

Preparation of Antibody Against a Synthetic Peptide Derived From the Amino Acid Sequence of Human Mono-ADP-Ribosyltransferase

According to the cDNA sequence of human mono-ADP-ribosyltransferase gene,[26] an amino acid sequence was deduced with the aid of the Translate program in GCG computer software (Madison, Wis). A unique 14-residue peptide (NH$_2$-Cys-Ala-Gly-Gln-Ser-Arg-Glu-Asp-Tyr-Ile-Tyr-Gly-Phe-Spc-Gln-COOH) (M_r=1636.8) with high predicted antigenicity was synthesized at the Protein and Nucleic Acid Facility (Medical College of Wisconsin, Milwaukee). The synthesized peptide was purified by reverse-phase HPLC and conjugated to keyhole-limpet hemocyanin by the EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) method (Imject Immunogen EDC conjugation kit, Pierce). The conjugate was dialyzed for 18 hours against PBS buffer, and 250 μg was mixed 1:1 with Freund's complete adjuvant and then injected intradermally into 20 sites on the shaved back of adult New Zealand White rabbits. The animals were given booster injections at 30-day intervals with a similar mixture but using Freund's incomplete adjuvant. Preimmune serum was obtained before the initial immunization, and subsequent blood collection was 5 days after the booster injection. Blood was withdrawn from the ear vein, and the serum was separated, aliquoted, and stored frozen at -80°C. The serum was evaluated for antibody titer by using a standard ELISA assay.[27]

Western Blot Analysis

Forty micrograms of protein of the homogenates, microsomes, or cytosols was subjected to SDS-PAGE (12% running gel) after heating at 100°C for 3 minutes. The protein was electrophoretically transferred at 30 V overnight onto a nitrocellulose membrane in transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, 20% (vol/vol) methanol, and 0.0025% SDS. Nonspecific binding sites were blocked by immersing the membrane in Tris-Tween buffer solution (TBS-T) containing 50 mmol/L Tris-HCl (pH 7.5), 0.2 mmol/L NaCl, 0.05% Tween 20, and 5% blocker (Amersham) with continuous shaking. The membrane was washed once for 15 minutes and twice for 5 minutes in fresh TBS-T buffer and then incubated with 1:1000 polyclonal antibody against the synthetic peptide of human mono-ADP-ribosyltransferase or a specific monoclonal antibody against rat ADP-ribosyltransferase (RT6.2)[28] for 1 hour at room temperature. The membrane was washed 3 times with TBS-T and then incubated for 1 hour with 1:1000 HRP-labeled goat anti-rabbit or donkey anti-mouse IgG. After washing the membrane 5 times for 10 minutes to remove the unbound secondary antibody, 10 mL of detection solution 1 and 2 (1:1) (Amersham) was added directly to the blots on the surface carrying the protein. After incubation for 1 minute at room temperature, the membrane was wrapped in Saran wrap and then exposed to Kodak Omat film.[29]

Culture of Coronary Arterial Endothelial Cells and Smooth Muscle Cells

Culture of coronary arterial endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) was performed as described previously.[30] Briefly, after separate enzymatic dispersion of arterial ECs and VSMCs, cells were washed 3 times each with PSS. Then, cells were pelleted by centrifugation and plated in 25-cm^2 flasks. Medium 199 was used for VSMCs and RPMI 1640 was used for ECs. Media contained 20% FBS and antibiotics (ampicillin and gentamicin). The flasks were placed in a 5% CO_2 in an air incubator at 37°C, and the cells were grown for 14 to 16 days to confluence. Cells were used for Western blot analysis for mono-ADP-ribosyltransferase before 5 passages.

Patch-Clamp Study

Small bovine coronary arteries were dissected as described above. Small bovine coronary arterial smooth muscle cells were enzymatically dissociated, and K$^+$ channels were characterized as we described previously.[6,31] Briefly, the dissected small coronary arteries were digested with collagenase and elastase to dissociate VSMCs, and a List EPC-7 patch-clamp amplifier (List Biological Laboratories, Inc) was used to record single-channel currents.

The effects of specific mono-ADP-ribosyltransferase inhibitors, novobiocin, vitamin K$_1$, 3-aminobenzamide, and *m*-iodobenzylguanidine were first tested on the K$_{Ca}$ channel activity in cell-attached and inside-out patch modes. After a cell-attached patch or an inside-out patch was established, a 3-minute control recording was obtained at a membrane potential of +40 mV. Then, the bath solution was changed to include *m*-iodobenzylguanidine (1 to 100 μmol/L), vitamin K$_1$ (1 to 100 μmol/L), novobiocin (10 to 1000 μmol/L), or 3-aminobenzamide (10 to 1000 μmol/L), and a second successive 3-minute recording at each concentration was obtained. To determine the effects of these inhibitors on EET-induced activation of the K$^+$ channels, patch-clamp recordings were performed in the cell-attached patch mode. A 3-minute control recording was obtained at a membrane potential of +40 mV. Then, the bath solution was exchanged with the solution containing 11,12-EET (100 nmol/L) (n=7), and a second successive 3-minute recording was obtained. In another group of cells, *m*-iodobenzylguanidine (100 μmol/L), vitamin K$_1$ (100 μmol/L), novobiocin (1000 μmol/L), or 3-aminobenzamide (1000 μmol/L) was added to the bath solution. A 3-minute control recording was obtained at a membrane potential of +40 mV. Then, the solution in the bath was exchanged with the solution containing 11,12-EET (100 nmol/L), and a second successive 3-minute recording was obtained (n=8).

Statistical Analysis

Data were presented as mean±SEM. 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. A Student's *t* test was used to examine significance of difference in 2 groups. $P < 0.05$ was considered statistically significant.

Results

Identification of Endogenous ADP-Ribosylation in Coronary Arterial Homogenates

Figure 1 presents a typical autoradiography of endogenous ADP-ribosylation in the homogenates prepared from small bovine coronary arteries. Proteins with molecular sizes of 51, 52, 80, and 124 kDa were ADP-ribosylated under control conditions in the presence or absence of GTP (1 mmol/L) (Figure 1A). When coronary homogenates were treated with 11,12-EET, ADP-ribosylation was significantly increased in the presence of GTP (Figure 1A). Figure 1B summarizes the effects of 11,12-EET on the ADP-ribosylation in coronary arterial homogenates. The density of the 52-kDa autoradiographic band was markedly increased by 11,12-EET in the presence of GTP. This effect was specific for GTP, because ATP did not affect basal or EET-induced ADP-ribosylation (data not shown). Cholera toxin stimulated the ADP-ribosylation of the 45-, 52-, and 80-kDa proteins. Pretreatment of the homogenate with 11,12-EET significantly inhibited the ADP-ribosylation of these proteins by cholera toxin. The inhibition was concentration related (Figure 1C and 1D), suggesting that 11,12-EET–induced endogenous ADP-ribosylation may competitively inhibit cholera toxin–induced ADP-ribosylation. To exclude the possibility of nonenzymatic ADP-ribosylation, free, unlabeled ADP-ribose (0.1 mmol/L) was added to the reaction mixtures for assay of endogenous ADP-ribosylation in the presence of ^{32}P-NAD. Free ADP-ribose had no effect on the ADP-ribosylation reaction (Figure 2A).

ADP-Ribosylation of G$_S$

To confirm the ADP-ribosylation of G$_S$, the proteins were electrophoretically transferred onto nitrocellulose membranes. After autoradiography, Western blot analysis was performed on the same membrane using an anti–G$_S$α antibody. As shown in Figure 2B, a 52-kDa immunoreactive band was detected, which comigrated with the ^{32}P-labeled band on the gel. This suggests that the ADP-ribosylated 52-kDa protein was a G$_S$α. To further determine the endogenous ADP-ribosylation of G$_S$, the purified G$_S$α subunit was added into the reaction mixture, and immunoprecipitation was performed (Figure 3). When a purified G$_S$α subunit was added to the reaction mixtures, the 52-kDa ^{32}P-labeled protein was markedly increased (lane +G$_S$α). When the reaction mixtures were immunoprecipitated using an anti–G$_S$α subunit antibody, only one band with a molecular size of 52 kDa was detected (lane beads+anti-G$_S$α).

Western Blot Analysis of Mono-ADP-Ribosyltransferase in Bovine Coronary Arteries

Figure 4 presents a typical Western blot by using mono-ADP-ribosyltransferase antibodies. Two proteins, 42 and 70 kDa,
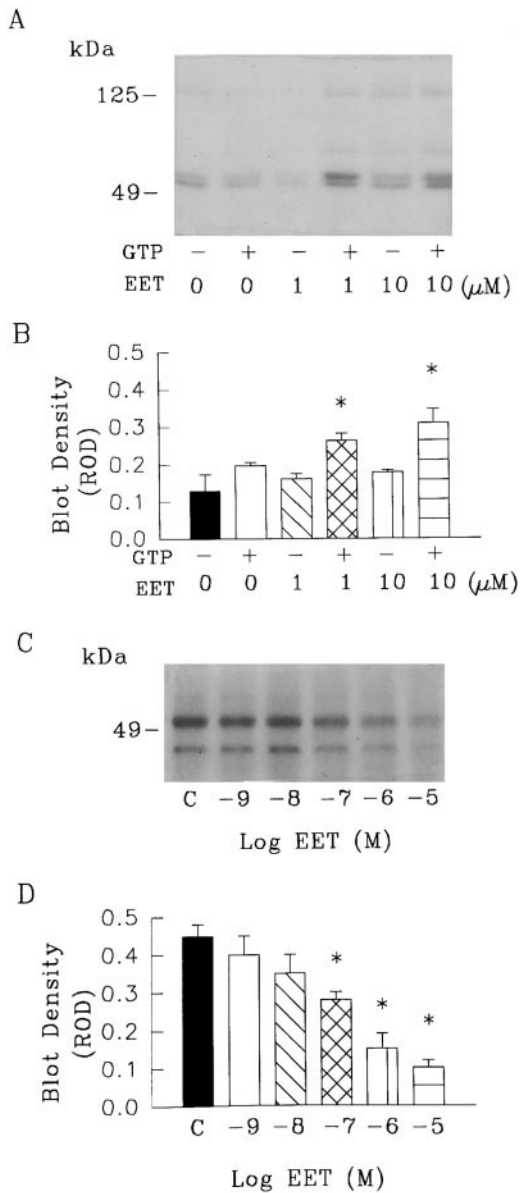
Figure 1. Autoradiographs of ADP-ribosylation in coronary arterial homogenates. A, Endogenous ADP-ribosyltransferase activity in the presence of GTP and 11,12-EET. Homogenate (140 μg) was incubated in 200 μL of reaction mixture with 5 μCi of [^{32}P]NAD$^+$. The ^{32}P-ADP–ribosylated proteins were separated in SDS-PAGE, and the radioactivity was detected by autoradiography. B, Summary of the density of the autoradiographic 52-kDa band in the presence and absence of 11,12-EET (n=4). C, Cholera toxin–induced ADP-ribosylation with or without 11,12-EET pretreatment. ADP-ribosylation reaction was performed as described above, and cholera toxin (5 μg) was added to the reaction mixture. D, Summary of the density of the autoradiographic 52-kDa band produced by cholera toxin after 11,12-EET pretreatment. *Significant difference from controls (in panel B, EET=0 μmol/L; in panel D, bar C).

Figure 2. ADP-ribosyltransferase activity and Western blot of $G_S\alpha$ in the absence and presence of free ADP-ribose. Free ADP-ribose (0.1 mmol/L) was added to the reaction mixture. A, Typical autoradiogram of ADP-ribosylation in coronary arterial homogenates. B, Western blot of the same gel in panel A, using anti–$G_S\alpha$ antibody.

were recognized by an antibody against human mono-ADP-ribosyltransferase (Figure 4A) and also by an anti–RT6.2 ADP-ribosyltransferase antibody (Figure 4B). The proteins were detected in the coronary arterial homogenates, microsomes, and cytosols. More of the proteins were found in the cytosolic fraction than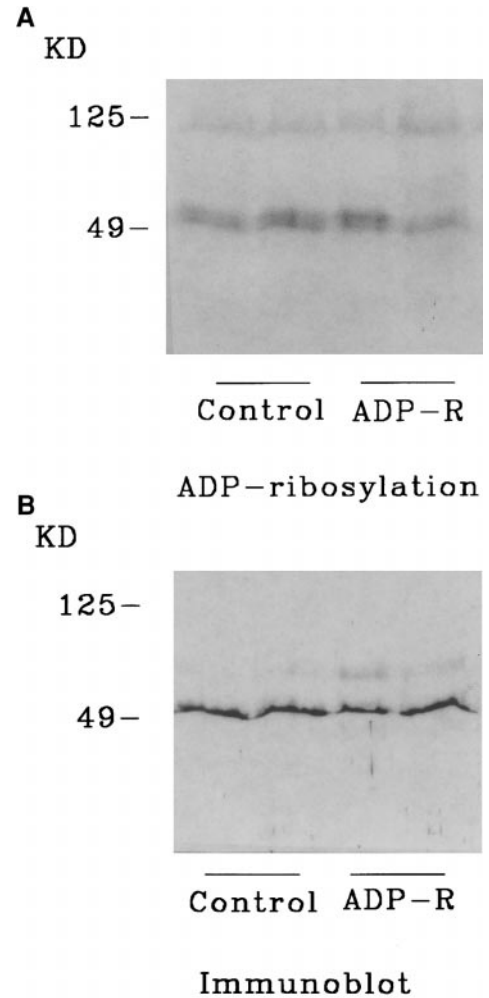 the microsomal fraction. These ADP-ribosyltransferases were also detected in lysates of cultured bovine coronary arterial VSMCs but not in ECs.

Effect of Inhibition of Endogenous ADP-Ribosylation on the K_{Ca} Channel Activity

Table 1 summarizes the effects of the inhibitors of mono-ADP-ribosyltransferases on the open probability (NP_O) of the K_{Ca} channels. In the cell-attached patch mode, 100 μmol/L *m*-iodobenzylguanidine and vitamin K_1 and 1000 μmol/L novobiocin and 3-aminobenzamide significantly reduced the NP_O of the K_{Ca} channels. However, in the inside-out patch mode, none of these inhibitors had effects on the NP_O of K_{Ca} channels, suggesting that these inhibitors of mono-ADP-ribosyltransferases act on the enzyme and do not have a direct effect on the membrane channel.

Effect of Inhibition of Endogenous ADP-Ribosylation on 11,12-EET–Induced Activation of the K_{Ca} Channels

Figure 5A presents typical recordings of single K^+ currents in cell-attached patches, depicting the effects of 11,12-EET (100
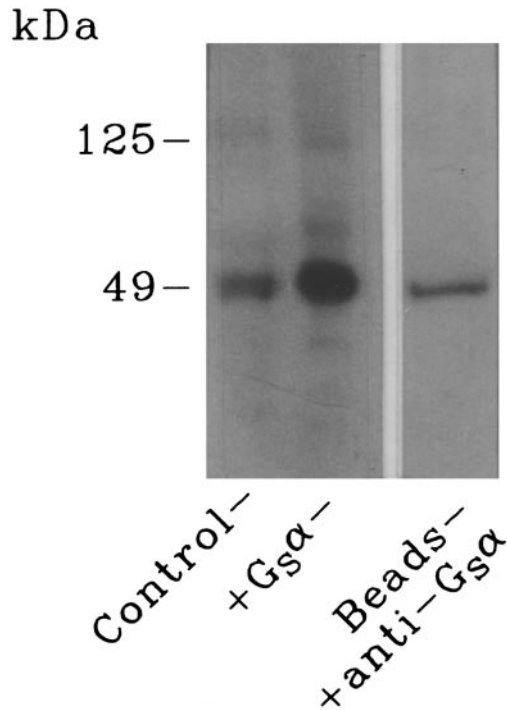
Figure 3. Typical autoradiogram depicting the ADP-ribosylation of coronary homogenates after the addition of purified $G_S\alpha$ (lane $+G_S\alpha$) and immunoprecipitation of ADP-ribosylated $G_S\alpha$ by anti–$G_S\alpha$ antibody (lane beads+anti-$G_S\alpha$).
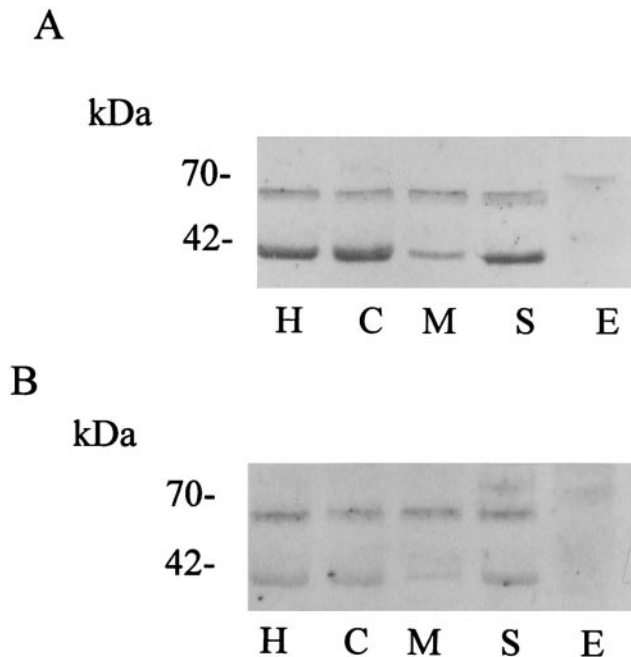

Figure 4. Western blot analysis of ADP-ribosyltransferases in homogenates, microsomes, and cytosols prepared from small bovine coronary arteries. A, An antibody against a synthetic peptide derived from human ADP-ribosyltransferase. B, An antibody against rat lymphocyte RT6.2 ADP-ribosyltransferase activity. H, C, and M indicate homogenates, cytosols, and microsomes prepared from bovine coronary arteries, respectively. S and E indicate the homogenates prepared from cultured coronary smooth muscle cells and endothelial cells, respectively.

nmol/L) and mono-ADP-ribosyltransferase inhibitor, *m*-iodo-benzylguanidine (M-IG) (100 μmol/L). Previous studies have documented this channel as a K_{Ca} channel[6] and that 11,12-EET increases this channel activity. In the presence of M-IG, the effects of 11,12-EET to increase opening of K_{Ca} channels were substantially blocked (M-IG+11,12-EET). Figure 5B summarizes the effects of 11,12-EET and M-IG on the NP_O of the K_{Ca} channels. The amplitude of the K_{Ca} channels was not affected (Figure 5C). Table 2 summarizes the effects of 11,12-EET on the NP_O of the K_{Ca} channels in the absence and presence of the inhibitors of mono-ADP-ribosyltransferase. In the absence of the inhibitors, 100 nmol/L 11,12-EET produced a 5-fold increase in the NP_O of the K_{Ca} channels. In the presence of any one of 4 inhibitors of mono-ADP-ribosyltransferases, the effects of 11,12-EET on the NP_O of the K_{Ca} channels were blocked.

Discussion

Endogenous ADP-ribosylation has been reported in a variety of animal tissues,[8–11] and the genes for several mono-ADP-ribosyltransferases have been cloned.[26,28,32–36] The present study provides the first evidence for the presence of mono-ADP-ribosyltransferases in smooth muscle of bovine coronary arteries. These enzymes catalyze the endogenous ADP-ribosylation of cellular proteins in the presence of NAD. Four smooth muscle cell proteins with molecular sizes of 51, 52, 80, and 124 kDa were ADP-ribosylated. By use of Western blot analysis with antibodies against either human or rat mono-ADP-ribosyltransferases, 42- and 70-kDa mono-ADP-ribosyltransferases were detected in coronary arterial smooth muscle. These results indicate that coronary arterial smooth muscle expresses mono-ADP-ribosyltransferases, which catalyze the ADP-ribosylation of cellular proteins.

To further determine the localization of the mono-ADP-ribosyltransferases in coronary arteries, we first examined the protein expression of the enzymes in cultured coronary ECs and smooth muscle cells using Western blot analysis. Clearly, smooth muscle cells expressed the 42- and 70-kDa ADP-ribosyltransferases like the arterial homogenate. In contrast, ECs did not express these proteins. Thus, it appears that endogenous ADP-ribosylation in coronary arteries occurs only in smooth muscle cells. Moreover, Western blot analysis indicated that the mono-ADP-ribosyltransferases in coronary arterial smooth muscle were primarily present in the cytosolic fraction. This is consistent with previous findings indicating that endogenous ADP-ribosylation occurred in the cytosol of rat liver cells[7] and human platelets.[13,16–18] However, several studies demonstrated that some arginine-specific mono-ADP-ribosyltransferases in rabbit or human skeletal and cardiac muscle,[34–38] rodent lymphocytes,[28] and human testis[26] are GPI-anchored proteins and present on the surface of cells. The present study did not attempt to define which isoforms of the mono-ADP-ribosyltransferases are present in bovine coronary arterial smooth muscle.

The acceptor proteins for mono-ADP-ribosyltransferases possess a wide spectrum of molecular weights from 20 to 120 kDa and varied activities. These acceptor proteins include G proteins, actin, Ca^{2+}-dependent ATPase, cAMP-independent protein kinase, GAPDH, elongation factor-2, and some un-

TABLE 1. Effect of Mono-ADP-Ribosyltransferase Inhibitors on the K_{Ca} Channel Activity (NP_0) in Cell-Attached and Inside-Out Patches of Coronary Smooth Muscle Cells

			Inhibitor Concentration, μmol/L			
Inhibitor	n	Control	1	10	100	1000
Cell-attached patches						
M-IG	6	0.054 ± 0.01	0.039 ± 0.01	0.04 ± 0.01	$0.038\pm0.01^*$	ND
Vit K	7	0.075 ± 0.03	0.044 ± 0.01	0.037 ± 0.01	$0.021\pm0.02^*$	ND
Novo	6	0.065 ± 0.02	ND	0.052 ± 0.01	0.033 ± 0.07	$0.036\pm0.01^*$
3-AB	5	0.05 ± 0.01	ND	0.04 ± 0.01	$0.03\pm0.01^*$	$0.014\pm0.01^*$
Inside-out patches						
M-IG	6	0.056 ± 0.02	0.056 ± 0.01	0.053 ± 0.01	0.052 ± 0.01	ND
Vit K	5	0.073 ± 0.02	0.069 ± 0.02	0.053 ± 0.01	0.05 ± 0.02	ND
Novo	5	0.075 ± 0.02	ND	0.081 ± 0.03	0.068 ± 0.02	0.077 ± 0.03
3-AB	5	0.083 ± 0.02	ND	0.089 ± 0.02	0.087 ± 0.03	0.076 ± 0.02

Vit K indicates vitamin K_1; Novo, novobiocin; 3-AB, 3-aminobenzamide; and ND, not determined.
*Significant difference from control.
Values are mean$\pm$SE.

known cellular proteins.[14–23] In the present study, we found that a major ADP-ribosylated protein (52 kDa) was the same molecular size as a protein that was ADP-ribosylated by cholera toxin. This 52-kDa protein was recognized by an antibody against $G_S\alpha$. The addition of purified $G_S\alpha$ into the reaction mixtures markedly increased ADP-ribosylation of a 52-kDa protein, and immunoprecipitation of the reaction mixtures by an anti–$G_S\alpha$ antibody also demonstrated a 52-kDa protein that was ADP-ribosylated. Taken together, these data suggest that endogenous mono-ADP-ribosyltransferases may catalyze ADP-ribosylation of $G_S\alpha$ in coronary arterial smooth muscle. In addition, endogenous

ADP-ribosylation also occurs on proteins with molecular weights of 51, 80, and 124 kDa. The identity of these proteins remains to be determined.

Previous studies have indicated that endogenous ADP-ribosylation of cellular proteins may play an important role in the transmembrane and intracellular signal transduction. A number of hormones or bioactive substances such as nitric oxide, prostacyclin, adenosine, and isoproterenol activated or modulated the activity of mono-ADP-ribosyltransferases in various animal tissues.[15–20] However, little is known about the role of mono-ADP-ribosyltransferases in the regulation of vascular function. Recent studies in our laboratory demon-
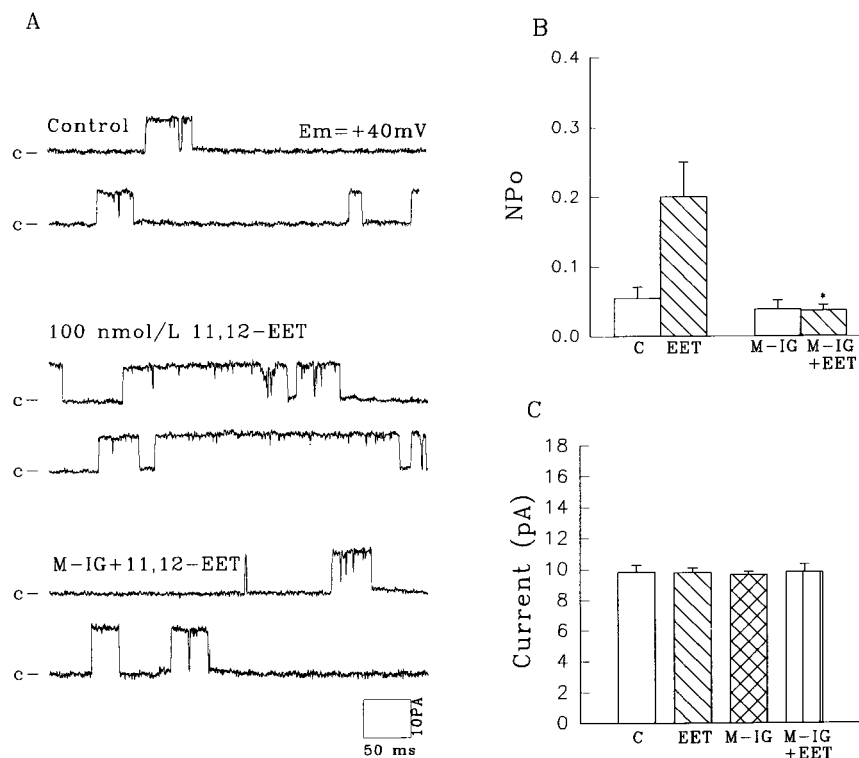

Figure 5. A, Representative recordings depicting the effects of 11,12-EET and M-IG on the K_{Ca} channel activity in the cell-attached patches. B and C, Effects of 11,12-EET (100 nmol/L) and M-IG (100 μmol/L) on the NP_O (B) and current amplitude (C) of the K_{Ca} channels. *Significant difference compared with the value obtained before administration of M-IG.

TABLE 2.   Effect of Mono-ADP-Ribosyltransferase Inhibitors on 11,12-EET–Induced Increase in the K_{Ca} Channel NP_0 in Cell-Attached Patches of Coronary Smooth Muscle Cells

Protocol	Vehicle	11,12-EET (100 nmol/L)
Control	0.068 ± 0.012	0.33 ± 0.13*
M-IG (100 μmol/L)	0.054 ± 0.016	0.038 ± 0.01
Vit K (100 μmol/L)	0.054 ± 0.03	0.045 ± 0.03
Novo (1000 μmol/L)	0.035 ± 0.01	0.039 ± 0.01
3-AB (1000 μmol/L)	0.04 ± 0.02	0.02 ± 0.01

*Significant difference from vehicle control. Values are mean$\pm$SE.

strated that EETs, a family of cytochrome P450–derived eicosanoids, activate K_{Ca} channels, hyperpolarize vascular smooth muscle, and dilate coronary arteries. These EETs represent endothelium-derived hyperpolarizing factors in the coronary circulation.[1,2,6] They contribute to the endothelium-dependent relaxation and the control of coronary vascular tone. Regarding the mechanism of EET activation of the K_{Ca} channel, we reported that EETs, particularly 11,12-EET, require GTP to activate the K_{Ca} channels in coronary smooth muscle and that blockade of $G_S\alpha$ activity by anti–$G_S\alpha$ antibody abolished 11,12-EET–induced activation of the K_{Ca} channels, suggesting an involvement of $G_S\alpha$ in the effect of EETs.[6] The mechanism by which EETs activate $G_S\alpha$ remains unknown. A recent study indicated that EETs, particularly 14,15-EET, produced a concentration-dependent stimulation of ADP-ribosylation through an endogenous mono-ADP-ribosyltransferase in the liver cell cytosol.[7] We showed that, like the EETs, cholera toxin, an exogenous ADP-ribosyltransferase, also activated K_{Ca} channels in coronary smooth muscle cells.[6] Therefore, we hypothesize that EETs may also stimulate the activity of a mono-ADP-ribosyltransferase and subsequently activate $G_S\alpha$ and K_{Ca} channels. To test this hypothesis, we examined the effect of 11,12-EET on the ADP-ribosylation in coronary arterial homogenates. The addition of 11,12-EET to the reaction mixtures of coronary arterial homogenate and [^{32}P]-NAD markedly enhanced the ADP-ribosylation of all 4 acceptor proteins in the presence of GTP, suggesting that the mono-ADP-ribosyltransferase activity was stimulated by 11,12-EET. Using the patch-clamp technique, we examined the role of mono-ADP-ribosylation in mediating the effects of 11,12-EET on the K_{Ca} channel activity in bovine coronary arterial smooth muscle. 11,12-EET significantly enhanced the activity of K_{Ca} channels. In the presence of 4 chemically distinct inhibitors of mono-ADP-ribosyltransferases, 3-aminobenzamide, *m*-iodobenzylguanidine, novobiocin, and vitamin K_1, the 11,12-EET–induced increase in the K_{Ca} channel activity was substantially blocked. These results suggest that mono-ADP-ribosyltransferases may play an important role in mediating the effects of EETs on K_{Ca} channel activity.

The mechanism by which activation of mono-ADP-ribosyltransferases alters the activity of the K_{Ca} channels remains unknown. It is possible that ADP-ribosylation of $G_S\alpha$ is critical, given that activation of G_S increases the activity of the K_{Ca} channels.[39,40] We also found that cholera toxin induced ADP-ribosylation of $G_S\alpha$ in bovine coronary arterial smooth muscle and markedly increased the activity of the K_{Ca}

channels.[6] The activation of K_{Ca} channels by the combination of cholera toxin and 11,12-EET was the same as either alone, suggesting that they may act by a common mechanism.[6] Along these lines, stimulation of endogenous ADP-ribosylation by 11,12-EET inhibited cholera toxin–stimulated ADP-ribosylation of $G_S\alpha$. Therefore, stimulation of endogenous ADP-ribosylation of $G_S\alpha$ by 11,12-EET may result in its activation and subsequently increases the activity of the K_{Ca} channels.

In summary, the present study has demonstrated that mono-ADP-ribosyltransferases are present in bovine coronary arterial smooth muscle. The mono-ADP-ribosyltransferases transfer ADP-ribose moiety from NAD to 51, 52, 80, and 124 kDa proteins. The 52-kDa protein is a major acceptor protein and appears to represent $G_S\alpha$. 11,12-EET stimulates endogenous ADP-ribosylation of $G_S\alpha$ in coronary arterial homogenate. Specific inhibitors of endogenous mono-ADP-ribosyltransferase decreased the activity of K_{Ca} channels and blocked 11,12-EET–induced activation of K_{Ca} channels. These results suggest that mono-ADP-ribosyltransferases participate in the regulation of the activity of K_{Ca} channels in vascular smooth muscle and may play an important role in the control of coronary vascular tone. The mono-ADP-ribosyltransferase–mediated signaling pathway contributes to EET-induced vasodilation and hence to the actions of these endothelium-derived hyperpolarization factors.

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