Diabetes-related changes in cAMP-dependent protein kinase activity and decrease in relaxation response in rat mesenteric artery

Takayuki Matsumoto, Kentaro Wakabayashi, Tsuneo Kobayashi, and Katsuo Kamata.
Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan
Submitted 17 February 2004; accepted in final form 26 April 2004

Matsumoto, Takayuki, Kentaro Wakabayashi, Tsuneo Kobayashi, and Katsuo Kamata. Diabetes-related changes in cAMP-dependent protein kinase activity and decrease in relaxation response in rat mesenteric artery. *Am J Physiol Heart Circ Physiol* 287: H1064–H1071, 2004.—Using superior mesenteric artery rings isolated from age-matched controls and streptozotocin (STZ)-induced diabetic rats, we recently demonstrated that EDHF-type relaxation is impaired in STZ-induced diabetic rats, possibly due to a reduced action of cAMP via increased phosphodiesterase (PDE) activity (Matsumoto T, Kobayashi T, and Kamata K. *Am J Physiol Heart Circ Physiol* 285: H283–H291, 2003). Here, we investigated the activity and expression of cAMP-dependent protein kinase (PKA), an enzyme that is produced by a pleiotropic and plays key roles in the transduction of many external signals through the cAMP second messenger pathway and in cAMP-mediated vasorelaxation. The relaxation induced by cilostamide, a selective PDE3 inhibitor, was significantly weaker in superior mesenteric artery rings from STZ-induced diabetic rats than in those from age-matched controls. The relaxation responses to 8-bromo-cAMP (8Br-cAMP) and N6O2-dibutylryl-adenosine-cAMP (db-cAMP), a cell-permeant cAMP analog, were also impaired in the STZ diabetic group. PKA activity in the db-cAMP-treated mesenteric artery was significantly lower in the STZ diabetic group. The expression levels of the mRNA and protein for PKA catalytic subunit Cat-α were significantly decreased in the STZ diabetic group, but those for PKA regulatory subunit isoform RI-β were increased. We conclude that the abnormal vascular relaxation responsiveness seen in STZ-induced diabetic rats may be attributable not only to increased PDE activity but also to decreased PKA activity. Possibly, the decreased PKA activity may result from an imbalance between PKA catalytic and regulatory subunit expressions.

protein kinase A; streptozotocin

ADENOSINE 3’,5’-CYCLIC MONOPHOSPHATE (cAMP) acts as a second messenger in the intracellular signal transduction of a wide variety of extracellular stimuli in several tissues (3, 39). In the vascular system, cAMP plays important roles in the regulation of vascular tone and in the maintenance of the mature contractile phenotype in smooth muscle cells (31, 41). The intracellular levels of cAMP are tightly regulated, both by control of its rate of synthesis (by adenyl cyclases) in response to extracellular signals and by rate of control of its hydrolysis [by cyclic nucleotide phosphodiesterases (PDEs)] (3, 38, 39). The newly minted cAMP then binds to its receptor, cAMP-dependent protein kinase (PKA), and causes reversible phosphorylation of protein substrates that regulate a vast number of cellular processes, such as metabolism, cell growth and differentiation, apoptosis, gene expression, ion channel conductivity, and vascular tone (41, 53, 55).

There are two types of PKA, type I (PKA-I) and type II (PKA-II), and these share a common catalytic (C) subunit but contain distinct regulatory (R) subunits (RI and RII, respectively) (53, 55, 57). Through biochemical studies and gene cloning, four isoforms of the R subunits (RI-α, RI-β, RII-α, and RII-β) have been identified. Three distinct C subunits (Cat-α, Cat-β, and Cat-γ) have also been identified. In the holoenzyme state, PKA exists in an inactive form. After an increase in intracellular cAMP, the R subunits bind to cAMP, resulting in the dissociation of the holoenzyme into a regulatory dimer and two C monomers. The free C subunits can then phosphorylate various substrates. Thus both C and R subunits are important for PKA-mediated functions (30, 53). In addition to their biochemical differences, the isoforms of PKA may have different effects on a number of physiological and pathophysiological processes and signaling mechanisms in cells, and these may underlie the differential effects of cAMP. This is supported by, for instance, the finding that, although the total R subunits-to-C subunits ratio in normal tissue is relatively constant at around 1:1, the relative amounts of RI and RII vary and depend to a large extent on both physiological conditions and the hormonal status of the tissue (11, 19, 35, 54). One recent study (9) showed that in RII-β gene knockout mice, an increased level of RI-α compensates for the loss of RII-β in brown fat cells. The switching of the PKA isofrom from type II to type I results in an increased basal level of PKA activity and increased energy expenditure. Such RII-β knockout mice have been noted to be leaner and also protected against diet-induced obesity, insulin resistance, and dyslipidemia (2, 9, 51). These results demonstrate clearly that RI and RII are functionally distinct.

Diabetes mellitus is associated with vascular complications, including an impairment of vascular responsiveness to neurotransmitters in the macro- and microvasculature (10). In the macrovasculature, an accumulating body of evidence suggests that the relaxation responses induced in aortic strips by endothelium-dependent agents are weaker in streptozotocin (STZ)-induced diabetic rats than in nondiabetic control rats (8, 23, 26, 28, 45, 47, 49). Moreover, several reports have indicated that diabetic rats show an impairment of ACH-induced, endothelium-dependent hyperpolarization/relaxation in the microvasculature (16, 36, 62). The contribution made by EDHF to relaxation is dependent on vessel size, being more prominent in the smaller, physiologically more important arteries than in larger...
ones (5, 40). Thus an impairment of EDHF-mediated responses may have important implications for the mechanisms by which diabetes leads to vascular dysfunction because small vessel dysfunction (such as in retinopathy, nephropathy, and neuropathy) is one of the major complications seen in diabetes. Recently, we demonstrated that EDHF-type relaxation was impaired in STZ-induced diabetic rats and that this impairment might be attributable to a reduction in the action of cAMP via increased PDE activity (37). There are several reports indicating that cAMP-mediated responses, such as β-receptor-mediated responses, are altered in diabetic states. For example, Type I diabetic patients showed a decreased β-adrenergic responsiveness of the heart beat in isoproterenol infusion experiments (4). In ventricular cardiomyocytes and papillary muscle from STZ diabetic rats, the β-adrenergic stimulatory pathway involves an additional defect upstream of the adenylate cyclase/G protein system (25, 61). Furthermore, in the vascular system, the isoproterenol-induced relaxation response is impaired in the STZ diabetic aorta (24). However, no study has yet investigated the relationship between diabetic vascular pathology and the cAMP downstream (cAMP-PKA) system.

In the present study, we designed experiments to investigate the diabetes-related changes in cAMP-induced vasodilation in the rat superior mesenteric artery. We were especially interested in PKA activity in diabetes. We also asked whether mesenteric arteries from control and established diabetic rats might differ in their PKA subunit expression profiles.

MATERIALS AND METHODS

Reagents. STZ, phenylephrine, indomethacin, N6-nitro-l-arginine (l-NAME), 3-isobutyl-1-methylxanthine (IBMX), 8-bromo-cAMP (8Br-cAMP), N6,O2-dibutyryl-cAMP (db-cAMP), EDTA, EGTA, PMSF, leupeptin, aprotonin, monoclonal β-actin antibody, and probe-inhibitor cocktail were all purchased from Sigma Chemical (St. Louis, MO). Cilostamide was from Calbiochem-Novabiochem (La Jolla, CA). All drugs were dissolved in saline except where otherwise noted (e.g., IBMX and cilostamide were dissolved in DMSO). Horse- 

radish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were antibodies for PKA catalytic and regulatory subunit isoforms (Cat-α, RII-α, and RII-β) were from BD Biosciences (San Jose, CA). PKA Cat-β subunit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals and experimental design. Male Wistar rats (8 wk old and 180–230 g body wt) received a single injection via the tail vein of 65 mg/kg STZ dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan).

Measurement of plasma glucose. Twelve weeks after the injection of STZ or buffer, plasma glucose was determined using a commercially available enzyme kit, which made use of the O-toluidine method (Wako Chemical; Osaka, Japan).

Measurement of isometric force. Vascular isometric force was recorded as in our previous paper (37). Rats were anesthetized with diethyl ether and euthanized by decapitation 12 wk after treatment with STZ. The superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO3, 1.8 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, and 11.0 dextrose. The artery was carefully cleaned of all fat and connective tissue, and ring segments 2 mm in length were suspended by a pair of stainless steel pins in a well-oxygenated (95% O2-5% CO2) bath of 10 ml KHS at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T, Nihon Kohden; Tokyo, Japan). The tissues were equilibrated for 40 min in the presence of 100 μM l-NAME and 10 μM indomethacin (to block nitric oxide synthase and cyclooxygenase, respectively) before the administration of phenylephrine (1 μM). Once the phenylephrine-induced contraction had stabilized, vasodilator responses were elicited either in a cumulative manner (cilostamide, a specific inhibitor of PDE3) or in a single concentration-effect manner [8Br-cAMP (a cell-permeable cAMP analog rated as having greater resistance to hydrolysis by PDE) and db-cAMP (a cell-permeable cAMP analog), the latter in the presence of 10 μM IBMX, a nonselective inhibitor of PDE]. In the experiments with IBMX, an equefficient concentration of phenylephrine was used (1–10 μM).

In vitro kinase assay for PKA activity. Mesenteric arteries were pretreated with 20 μM IBMX for 30 min at 37°C and then treated with 100 μM db-cAMP or vehicle (deionized water) for 30 min at 37°C. They were then rapidly frozen in liquid N2 and stored at −80°C. The mesenteric tissues were homogenized in a homogenization buffer containing (in mM) 25 Tris·HCl (pH 7.4), 150 NaCl, 1 EDTA, 1 EGTA, 0.3 PMSF, 0.04 leupeptin, and 0.02 aprotinin. The homogenates were centrifuged, and the supernatant (10 μg protein) was used in a nonradioactive assay for PKA (PKA assay system, Promega). For the positive control (10 ng PKA; provided with the kit), we used the same assay conditions as for the artery samples. For the negative control (deionized water), we again used the same assay conditions as for the artery samples. Phosphorylated and nonphosphorylated peptide bands were visualized on a 0.8% agarose gel, and the former were quantitated by spectrophotometry.

Enzyme immunoassay for cAMP. Mesenteric rings from diabetic and age-matched control rats were incubated for 30 min at 37°C in oxygenated KHS containing 20 μM IBMX and then incubated for 30 min at 37°C with either 100 μM db-cAMP or vehicle. The stimulated level of cAMP was then determined. To this end, rings were rapidly frozen in liquid N2 and stored at −80°C. cAMP was then extracted in 6% trichloroacetic acid, followed by neutralization with water-saturated diethyl ether, and an enzyme immunoassay (Amersham Biosciences UK; Little Chalfont, UK) was performed.

Measurement of expression of mRNAs for PKA subunit isoforms using RT-PCR. RNA was isolated by the guanidium method (7). Briefly, rat superior mesenteric arteries were carefully isolated and then cleaned of fat and connective tissue. The arteries were homogenized in RNA buffer, and the RNA was quantified by ultraviolet absorbance spectrophotometry. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA using oligo(dT)20 and a ThermoScript RT-PCR System (Invitrogen; Carlsbad, CA). All primers were synthesized by Sigma-Genosys (St. Louis, MO). Individual sequences, PCR conditions, product size, and GenBank accession numbers are shown in Table 1. The PCR products so obtained were analyzed on ethidium bromide-stained agarose (1.5%) gels. The PCR products were quantified by scanning densitometry, with the amount of each product being normalized with respect to the amount of GAPDH product.

Western blot analysis. Mesenteric arterial tissues (three pooled vessels) were homogenized in ice-cold homogenization buffer containing 20 mM Tris·HCl (pH 7.5), 1 mM MgCl2, and protease inhibitor cocktail. Homogenates were centrifuged at 3,000 g for 5 min. The supernatant was collected, and the proteins were solubilized in modified mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). Samples (20 μg/lane) were resolved by electrophoresis on 12% SDS-PAGE gels and then transferred onto
polyvinylidene difluoride membranes. Briefly, after the residual protein sites on the membrane were blocked with Block ace (Dainippon pharm; Osaka, Japan), the membrane was incubated with anti-PKA Cat-α (1:1,000), anti-PKA Cat-β (1:500), anti-PKA RII-α (1:1,000), or anti-PKA RII-β (1:1,000) in blocking solution. HRP-conjugated anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in Tween-PBS, followed by detection using SuperSignal (Pierce). To normalize the data, we used β-actin as a housekeeping protein. The β-actin protein levels were determined after the membrane was stripped and probed with β-actin monoclonal primary antibody (1:5,000), with HRP-conjugated anti-mouse IgG as the secondary antibody. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.

**Statistical analysis.** Data are expressed as means ± SE. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after one-way ANOVA, with a probability level of P < 0.05 being regarded as significant. Statistical comparisons between concentration-response curves were made using a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (P < 0.05 again being considered significant).

**RESULTS**

**Blood glucose levels and animal body weights.** As reported previously (37), at the time of the experiment, all STZ-treated rats exhibited hyperglycemia, their blood glucose concentrations (599.1 ± 26.4 mg/dl, n = 12) being significantly higher than those of the age-matched nondiabetic control rats [99.8 ± 6.4 mg/dl, n = 12 (P < 0.001)]. The body weights of the diabetic rats (240.2 ± 4.3 g, n = 12) were significantly less than those of the age-matched control rats [518.9 ± 21.7 g, n = 12 (P < 0.001)] at the time of the experiment.

**PDE inhibitor-induced relaxation.** cAMP signaling in mammalian cells is terminated by cyclic nucleotide PDEs, a multifamily class of enzymes that catalyze the hydrolysis of cyclic nucleotides to 5'-nucleotide monophosphates (which do not activate cAMP effector proteins; Refs. 3 and 38). Among the various PDEs, the two of greatest interest are PDE3 and PDE4 because they preferentially hydrolyze cAMP (48). To investigate cAMP-mediated relaxation in the rat mesenteric artery, we first tested the effect of cilostamide (10⁻⁹–10⁻⁵ M), a specific PDE3 inhibitor, when it was added cumulatively to rings precontracted by phenylephrine (1 μM) in the presence of 100 μM l-NNa plus 10 μM indomethacin. The tension developed in response to 1 μM phenylephrine was 2.16 ± 0.15 g in diabetic mesenteric rings (n = 7) and 2.34 ± 0.06 g in those from the age-matched controls (n = 7, no significant difference). The concentration-response curves (Fig. 1) showed that the peak relaxation induced by cilostamide was significantly weaker in mesenteric arteries from diabetic rats [63.9 ± 6.9% and 84.6 ± 3.3% of the phenylephrine-induced tone in diabetic (n = 7) and age-matched controls (n = 7), respectively (P < 0.05)]. The EC₅₀ values for the cilostamide-induced relaxations were 232.7 ± 26.9 and 141.6 ± 10.1 nM in diabetic and age-matched controls, respectively (P < 0.01). On the other hand, when Ro 20-1724 (10⁻⁵–10⁻³ M), a PDE4-specific inhibitor, was added cumulatively to rings precontracted by phenylephrine (1 μM) in the presence of 100 μM l-NNa plus 10 μM indomethacin, it induced almost no relaxation in either of the two (data not shown).

**Effects of cAMP analogs on relaxation responses.** To clarify the cAMP-mediated vasodilation, we examined the relaxing effect of a cAMP analog on rings from diabetic and age-matched control rats. In the presence of 100 μM l-NNa plus 10 μM indomethacin, 8Br-cAMP (1 mM), a cell-permeant cAMP analog that is rated as more resistant to PDEs, caused a relaxation of mesenteric artery rings from age-matched controls, the peak being by 23.4 ± 2.2% (n = 8). However, the relaxation response to this dose of 8Br-cAMP was significantly smaller in the diabetic group [11.7 ± 1.8%, n = 6 (P < 0.01); Fig. 2A]. We also examined the effect of another cAMP analog (db-cAMP) in the presence of IBMX, a PDE inhibitor. As

### Table 1. Oligonucleotide primer sequences for PKA subunit isoforms and GAPDH and PCR protocols

<table>
<thead>
<tr>
<th>cDNA (GenBank Accession No.)</th>
<th>PCR Primer Sequences</th>
<th>PCR Protocols</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA Cat-α (X57986)</td>
<td>5'-TCCTTTGGGAGTAGATGC-3'</td>
<td>94°C/60 s, 94°C/60 s, 94°C/60 s</td>
<td>563 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GGGAAGAAAGGGGTAAC-3'</td>
<td>59°C/60 s, 59°C/60 s, 59°C/60 s</td>
<td></td>
</tr>
<tr>
<td>PKA Cat-β (D10770)</td>
<td>5'-CAGATCGTGCTAAGATGAG-3'</td>
<td>94°C/60 s, 94°C/60 s, 94°C/60 s</td>
<td>543 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GTCATGAAAGGTTGCTGATGC-3'</td>
<td>56°C/60 s, 56°C/60 s, 56°C/60 s</td>
<td></td>
</tr>
<tr>
<td>PKA RII-α (NM_013181)</td>
<td>5'-GCAAAGACATGATGACGACG-3'</td>
<td>94°C/60 s, 94°C/60 s, 94°C/60 s</td>
<td>393 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GTTTGCCCATTACATTAC-3'</td>
<td>54°C/60 s, 54°C/60 s, 54°C/60 s</td>
<td></td>
</tr>
<tr>
<td>PKA RII-β (J02934)</td>
<td>5'-AGATGGAATCAGGACTCTGCAG-3'</td>
<td>94°C/60 s, 94°C/60 s, 94°C/60 s</td>
<td>383 bp</td>
</tr>
<tr>
<td></td>
<td>5'-GCGACGTTGCTACATGACC-3'</td>
<td>56°C/60 s, 56°C/60 s, 56°C/60 s</td>
<td></td>
</tr>
<tr>
<td>GAPDH (X02231)</td>
<td>5'-TGCGCTCGAATGTTGCTGAG-3'</td>
<td>94°C/60 s, 94°C/60 s, 94°C/60 s</td>
<td>308 bp</td>
</tr>
<tr>
<td></td>
<td>5'-AGATGGAATCAGGACTCTGCAG-3'</td>
<td>54°C/60 s, 54°C/60 s, 54°C/60 s</td>
<td></td>
</tr>
</tbody>
</table>

Among the PKA family class of enzymes that catalyze the hydrolysis of cyclic nucleotides to 5'-nucleotide monophosphates (which do not activate cAMP effector proteins; Refs. 3 and 38). Among the PKA subunit isoforms and GAPDH and PCR protocols, the two of greatest interest are PDE3 and PDE4 because they preferentially hydrolyze cAMP (48). To investigate cAMP-mediated relaxation in the rat mesenteric artery, we first tested the effect of cilostamide (10⁻⁹–10⁻⁵ M), a specific PDE3 inhibitor, when it was added cumulatively to rings precontracted by phenylephrine (1 μM) in the presence of 100 μM l-NNa plus 10 μM indomethacin. The tension developed in response to 1 μM phenylephrine was 2.16 ± 0.15 g in diabetic mesenteric rings (n = 7) and 2.34 ± 0.06 g in those from the age-matched controls (n = 7, no significant difference). The concentration-response curves (Fig. 1) showed that the peak relaxation induced by cilostamide was significantly weaker in mesenteric arteries from diabetic rats [63.9 ± 6.9% and 84.6 ± 3.3% of the phenylephrine-induced tone in diabetic (n = 7) and age-matched controls (n = 7), respectively (P < 0.05)]. The EC₅₀ values for the cilostamide-induced relaxations were 232.7 ± 26.9 and 141.6 ± 10.1 nM in diabetic and age-matched controls, respectively (P < 0.01). On the other hand, when Ro 20-1724 (10⁻⁵–10⁻³ M), a PDE4-specific inhibitor, was added cumulatively to rings precontracted by phenylephrine (1 μM) in the presence of 100 μM l-NNa plus 10 μM indomethacin, it induced almost no relaxation in either of the two (data not shown).

**Effects of cAMP analogs on relaxation responses.** To clarify the cAMP-mediated vasodilation, we examined the relaxing effect of a cAMP analog on rings from diabetic and age-matched control rats. In the presence of 100 μM l-NNa plus 10 μM indomethacin, 8Br-cAMP (1 mM), a cell-permeant cAMP analog that is rated as more resistant to PDEs, caused a relaxation of mesenteric artery rings from age-matched controls, the peak being by 23.4 ± 2.2% (n = 8). However, the relaxation response to this dose of 8Br-cAMP was significantly smaller in the diabetic group [11.7 ± 1.8%, n = 6 (P < 0.01); Fig. 2A]. We also examined the effect of another cAMP analog (db-cAMP) in the presence of IBMX, a PDE inhibitor. As
IBMX depressed contraction, the concentration of phenylephrine used in experiments involving IBMX was increased to between 1 and 10 μM. The tension developed in response to 1–10 μM phenylephrine in the presence of IBMX was 1.90 ± 0.09 g in diabetic mesenteric rings (n = 7) and 1.95 ± 0.09 g in the age-matched control group (n = 7, no significant difference). The peak amplitude of the relaxation induced by 30 μM db-cAMP in rings pretreated with IBMX was significantly smaller in the diabetic group than in the age-matched control group [79.6 ± 4.96% and 92.7 ± 1.73% of the phenylephrine-induced tone in diabetic (n = 7) and age-matched control (n = 7) groups, respectively (P < 0.05); Fig. 2B].

Effects of cAMP analogs on PKA activity and cAMP accumulation in rat mesenteric artery. PKA has been shown to contribute to cAMP signaling and to its impact on vascular function (41). As reported previously (37), PDE activity is increased in the diabetic mesenteric artery. We therefore examined the effects of db-cAMP treatment on PKA activity, after pretreatment with the PDE inhibitor IBMX (20 μM), in mesenteric artery rings. As shown in Fig. 3, there was no significant difference in vehicle-treated PKA activity between the diabetic and age-matched control groups. After stimulation with 100 μM db-cAMP, PKA activity was significantly increased in the age-matched control group [1.08 ± 0.25 and 1.96 ± 0.17 U/ml in vehicle- and db-cAMP-treated mesenteric arteries, respectively (P < 0.05)] but not in the diabetic group (1.24 ± 0.14 and 1.31 ± 0.23 U/ml in vehicle- and db-cAMP-treated mesenteric arteries, respectively). The db-cAMP-stimulated level of PKA activity in mesenteric arteries was significantly lower in the diabetic group than in the age-matched controls (P < 0.05).

Next, to confirm that PKA received the same degree of stimulation in mesenteric arteries from age-matched controls and STZ-induced diabetic rats, cAMP levels were measured in rat mesenteric arterial rings that had first been treated with 20 μM IBMX for 30 min and then incubated for 30 min with 100 μM db-cAMP or vehicle (the conditions also used for PKA activity measurements). Under our conditions, vehicle-treated cAMP levels were not significantly different between diabetic and age-matched control groups [41.6 ± 7.4 and 47.7 ± 8.0 pmol/mg protein in diabetic (n = 3) and age-matched control (n = 3) rats, respectively]. Moreover, db-cAMP-treated cAMP levels were not significantly different between the two groups [92.0 ± 17.4 and 94.3 ± 10.7 pmol/mg protein in diabetic (n = 4) and age-matched control (n = 4) rats, respectively]. Thus our PKA activity assay system was successful at demonstrating similarities between the diabetic and control groups.

Expression of mRNAs and proteins for PKA subunits. To investigate the possible mechanisms underlying the impaired cAMP analog-induced PKA activity seen in mesenteric arteries from STZ-induced diabetic rats, we examined whether the expressions of mRNAs and proteins for the various PKA subunits (see Introduction) might be altered in the diabetic state. Using RT-PCR analysis on the total RNA isolated from superior mesenteric arteries from age-matched controls and STZ-induced diabetic rats, we found the following. The expression of GAPDH mRNA showed no difference between the diabetic and age-matched control groups, but the expression of mRNA for the PKA C subunit Cat-α was significantly lower in the diabetic rats (compared with the age-matched controls). On the other hand, the expression of mRNA for the PKA R subunit RII-β was significantly increased in the diabetic group (Fig. 4).

A

B

Fig. 1. Concentration-response curves for the relaxations induced by cilostamide (phosphodiesterase 3-specific inhibitor) in isolated rings of superior mesenteric artery obtained from age-matched controls and diabetic rats. Ordinate shows relaxation of the superior mesenteric artery as a percentage of the contraction induced by phenylephrine (1 μM). In each experiment, Nω-nitro-L-arginine (L-NNA; 100 μM) + indomethacin (10 μM) was applied 40 min before the phenylephrine application and was present thereafter. Each data point represents the mean ± SE from 7 experiments; the SE is included only when it exceeds the dimension of the symbol used. *P < 0.05, diabetic vs. control.

Fig. 2. Decrease in cAMP derivative-induced relaxation in diabetic mesenteric arteries. In each experiment, L-NNA (100 μM) plus indomethacin (10 μM) was applied 40 min before the phenylephrine application and was present thereafter. A: 8-bromo-cAMP (8Br-cAMP; 1 mM) was added after the 1 μM phenylephrine-induced contraction had reached a steady-state level. B: Nω-O-dibutyryl-cAMP (db-cAMP; 30 μM) was added after the induction of contraction by 1–10 μM phenylephrine in the presence of 10 μM IBMX. Results are expressed as means ± SE; n = 6–8. *P < 0.05 and **P < 0.01, diabetic vs. control.
The expressions of mRNAs for the PKA Cat-β, RI-α, and RII-α subunits showed no differences between the diabetic and age-matched control groups (Fig. 4).

Immunoblot analysis of mesenteric arteries from diabetic and age-matched control rats (using anti-PKA subunit antibodies) allowed detection of immunoreactive proteins (Fig. 5). In parallel with the mRNA expression profiles, the PKA C subunit Cat-α protein levels were significantly lower in the diabetic group than in the age-matched controls, whereas the expression of the protein for the PKA R subunit RII-β was significantly higher in the former than in the latter group. The expressions of the proteins for the PKA Cat-β, RI-α, and RII-α subunits showed no differences between the diabetic and age-matched control groups (Fig. 5).

DISCUSSION

In the present study, we made two major findings related to 1) the impairment of cAMP-mediated relaxation and PKA activity in the mesenteric artery in STZ-induced diabetic rats, and 2) the abnormal pattern of expression of PKA subunits in that diabetic artery.

To assess cAMP-dependent relaxation in mesenteric arteries obtained from diabetic and age-matched control rats, we used cilostamide, which inhibits PDE3 and increases intracellular cAMP, 8Br-cAMP, and db-cAMP. Because complex interactions between different vasodilator pathways in vascular smooth muscle have been proposed for nitric oxide synthase and cyclooxygenase (32), we investigated vasodilator responses in the presence of inhibitors of these enzymes (l-NNA and indomethacin). In mammalian cells, cAMP signaling is terminated by PDEs (3, 38, 48). Of the PDE families identified in vascular smooth muscle cells, members of the PDE3 (cGMP inhibited) and PDE4 (cAMP specific) families have been shown to contribute to the regulation of cAMP signaling and its impact on vascular smooth muscle cell function (3, 38, 48). More specifically, inhibitors of PDE3 or PDE4 increase vascular smooth muscle cell cAMP levels, and an inhibition of PDE3 activity has marked effects on vascular smooth muscle cell contraction-relaxation coupling (i.e., it induces vasodilation). Several studies have found PDE3 activities to be altered in diabetes (42, 43), and our recent study (37) demonstrated that PDE3 activity in the mesenteric artery was increased in STZ-induced diabetic rats. In the present study, our first finding was that inhibition of PDE3 by cilostamide induced a relaxation response in the mesenteric artery that was attenuated in STZ-induced diabetic rats (Fig. 1). The concentration-response curve for cilostamide showed not only a significant rightward shift but also a significantly decreased maximum response in the diabetic group. Thus our data suggest that the impairment of cilostamide-induced relaxation seen in diabetic rats was not...
entirely attributable to increased PDE activity. On the other hand, an inhibition of PDE4 activity induced almost no vasodilation, as reported by others (34, 48). We speculated that the impairment of vascular responsiveness in the diabetic group might be attributable to an abnormality in the cAMP signaling pathway as well as to an increased PDE activity. To clarify this point, we studied cAMP analog-mediated relaxation. The first analog we tested, 8Br-cAMP, is effective at activating PKA but much less effective at activating PKG, and it is rated as being more resistant to PDE (15, 18). In our study, the 8Br-cAMP-mediated relaxation was significantly impaired in the diabetic group (Fig. 2). Furthermore, when we tested a second analog, we found the db-cAMP-mediated relaxation seen in the presence of the PDE inhibitor IBMX to also be reduced in the diabetic group (Fig. 2). Taken together, the above results suggest that in the diabetic mesenteric artery, relaxation is impaired downstream of cAMP.

PKA represents a key component in the cAMP signaling pathway, because it is responsible for the intracellular transduction of most cAMP-mediated physiological effects in a variety of cells (53, 55, 57). Activation of PKA in vascular smooth muscle cells by an elevated cytoplasmic cAMP concentration results in relaxation as a result of phosphorylations both of effector proteins, including myosin light chain kinase, and of the ion channels that regulate the cytosolic Ca²⁺ concentration and hyperpolarization (e.g., K⁺ channels) (41, 52, 58). Furthermore, PKA-mediated phosphorylations underpin the EDHF phenomenon in several arteries (21, 56). In the present study, cAMP analog-stimulated PKA activity was decreased in the diabetic mesenteric artery (Fig. 3), and under the same conditions as those used for the PKA activity assay, cAMP levels in mesenteric arteries were very similar between control and diabetic rats. Therefore, we directly demonstrated that PKA activity was impaired in the diabetic mesenteric artery. This is consistent with the previous finding that PKA activity is altered in the diabetic state (12, 44). As mentioned above, it has been assumed that activation of PKA may represent one of the mechanisms by which cAMP and cAMP analogs induce vasorelaxation (17). On the other hand, several studies have suggested that PKA is not the sole mediator of cAMP-dependent vasodilation (13, 18, 59, 60). Although the precise mechanism by which cAMP causes relaxation in the rat mesenteric artery is not certain, our data indicate that the impairment of cAMP-mediated relaxation seen in the diabetic state may be, at least in part, due to a relative lack of PKA activity. Future experiments will need to address this question.

In the native state, PKA exists as a tetramer holoenzyme consisting of two R subunits and two C subunits. In the holoenzyme state, PKA is in an inactive form. After an increase in intracellular cAMP, the regulatory subunits bind to cAMP, which results in the dissociation of the holoenzyme into a R dimer and two C subunit monomers. The free catalytic subunits can then phosphorylate various substrates (30, 53, 55, 57). Here, we report, for the first time, decreased Cat-α protein and increased RI-β protein in the diabetic mesenteric artery (as compared with controls) (Fig. 5). Similar changes in expression were seen at the mRNA level (Fig. 4), indicating transcriptional alterations in the diabetic state. These changes imply decreased PKA holoenzyme (R₂/C₂) targeting and therefore decreased local pools of C subunits. These expression abnormalities may contribute to the decreased cAMP-induced PKA activity and vasodilation/relaxation observed in the diabetic mesenteric artery. This conclusion is supported by the findings showing that the inactive holoenzyme can form at saturating cAMP levels, leading to decreased cAMP-responsive transcription, in cells overexpressing R subunits, and 2) maximally cAMP-stimulated cells overexpressing RI-α show less nuclear translocation of a microinjected C subunit (29, 30). Further support for this hypothesis is provided by the previous observations that in hepatocytes and neuroblastoma cells, cAMP signaling is decreased both by induction of the RI-α subunit and by degradation of the C subunit (20, 50).

Although the mechanism underlying such differences in the expressions and activities of PKA subunits between diabetic and control rats remains unclear, metabolic and/or hormonal alterations might be involved. In rats fed a low-protein diet, insulin secretion by the islets was decreased, and this impairment was associated with reduced PKA Cat-α subunit expression (14). Moreover, the expression of the gene that encodes the regulatory subunit of PKA was increased in this model (14). Furthermore, PKA RI-β subunit knockout mice have been noted both to be leaner and to be protected against diet-induced obesity, insulin resistance, and dyslipidemia (2, 9, 51). These results suggest that alleles at loci coding for PKA regulatory subunits should be included in the list of candidate genes determining susceptibility both to obesity and to the diabetes associated with obesity. In our STZ diabetic model, long-term insulin deficiency and hyperglycemia are associated with metabolic abnormalities such as increases in plasma triglyceride, cholesterol, and low-density lipoproteins (27). In addition, the levels of counterregulatory hormones such as catecholamines, adrenocorticotropic hormone, and glucagon...
are markedly higher in the diabetic condition (1, 6). Thus it is possible that the observed abnormalities in the expression levels of PKA subunits may be due to changes in the plasma levels of the above hormones. This speculation is supported by the finding that PKA subunit expression is regulated by several hormones (22, 33, 46). However, to establish causal relationships will require research focusing, for example, on time-course changes both in the expressions of the mRNAs for PKA subunits and in PKA activity in diabetic state.

In conclusion, we found that cAMP-mediated relaxation was impaired in the STZ diabetic mesenteric artery and that this impairment may be attributable to reduced PKA activity, in turn, resulting from an alteration in the pattern of expression of PKA subunits. Future research into vascular signaling mechanisms should continue to focus on physiological and therapeutic means of stimulating the cAMP signal transduction cascade in diabetic vasculopathy.

ACKNOWLEDGMENTS

The authors thank A. Iwasaki, T. Katohno, and N. Hirata for technical help.

GRANTS

This study was supported in part by the Ministry of Education, Science, Sports and Culture, Japan, and by the Promotion and Mutual Aid Cooperation for Private Schools in Japan, and by the Takeda Science Foundation.

REFERENCES