Functional changes in adenylyl cyclases and associated decreases in relaxation responses in mesenteric arteries from diabetic rats

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Submitted 21 September 2004; accepted in final form 4 May 2005

Matsumoto, Takayuki, Kentaro Wakabayashi, Tsuneo Kobayashi, and Katsuo Kamata. Functional changes in adenylyl cyclases and associated decreases in relaxation responses in mesenteric arteries from diabetic rats. Am J Physiol Heart Circ Physiol 289: H2234–H2243, 2005. First published May 13, 2005; doi:10.1152/ajpheart.00971.2004.—To assess the functional change in adenylyl cyclases (AC) associated with the diabetic state, we investigated AC-mediated relaxations and cAMP production in mesenteric arteries from rats with streptozotocin (STZ)-induced diabetes. The relaxations induced by the water-soluble forskolin (FSK) analog NKh477, which is a putative AC activator, were not by the β-adrenoceptor agonist isoproterenol (Iso) and the AC activator FSK, were reduced in intact diabetic mesenteric artery. In diabetic rats, however, Iso-, FSK-, and NKH477-induced relaxations were attenuated in the presence of inhibitors of nitric oxide synthase and cyclooxygenase. To exclude the influence of phosphodiesterase (PDE), we also examined the relaxations induced by several AC activators in the presence of 3-isobutyl-1-methylxanthine (IBMX; a PDE inhibitor). Under these conditions, the relaxation induced by Iso was greatly impaired in STZ-diabetic rats. This Iso-induced relaxation was significantly attenuated by pretreatment with SQ-22536, an AC inhibitor, in mesenteric rings from age-matched controls but not in those from STZ-diabetic rats. Under the same conditions, the relaxations induced by FSK or NKH477 were impaired in STZ-diabetic rats. Neither FSK- or A-23187 (a Ca2+ ionophore)-induced cAMP production was significantly different between diabetics and controls. However, cAMP production induced by Iso or NKH477 was significantly impaired in diabetic mesenteric arteries. Expression of mRNAs and proteins for AC5/6 was lower in diabetic mesenteric arteries than in controls. These results suggest that AC-mediated relaxation is impaired in the STZ-diabetic rat mesenteric artery, perhaps reflecting a reduction in AC5/6 activity.

adenosine; 3′,5′-cyclic monophosphate; forskolin; isoproterenol; mesentery; streptozotocin;

The multiple G protein-coupled receptors (GPCR) expressed in vascular tissue, including β-adrenergic receptors (β-AR), can reduce vascular tone. These GPCRs exert their effects by coupling to the heterotrimetric G protein Gs, thereby stimulating the activities of adenylyl cyclases (ACs). Such AC activity produces the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP), which plays pivotal regulatory roles in a wide variety of signal transduction pathways in various tissues (3). In the vascular system, cAMP plays important roles in both the regulation of vascular tone and the maintenance of the mature contractile phenotype of smooth muscle cells (SMCs). cAMP is produced from ATP by the action of AC in response to a variety of extracellular signals, such as hormones, growth factors, and neurotransmitters (12, 27). cAMP activity is terminated by cyclic nucleotide phosphodiesterases (PDEs), a multifamily class of enzymes that catalyze the hydrolysis of cyclic nucleotides to 5′-nucleotide monophosphates, which do not activate cAMP effector proteins (39, 41). When AC activity in smooth muscle produces cAMP, this, via activation of protein kinase A (PKA), alters intracellular Ca2+ dynamics and contractile function by phosphorylating Ca2+-channels, Ca2+-ATPases, and myosin light chain kinase (44, 59).

At least nine closely related isoforms of AC (AC1 through AC9) have been cloned and characterized in mammals, each encoded by a distinct gene (11, 12, 22, 62). These isoforms are divided into subfamilies on the basis of their regulatory patterns in response to products of other second messenger pathways (11, 12, 22, 62). Group 1 includes AC1, AC3, and AC8 isoforms, which are stimulated by Ca2+/calmodulin (11, 12, 22, 62). Group 2 includes AC2, AC4, and AC7 isoforms, which are regulated by G protein βγ-subunits (11, 12, 22, 62). Group 3 includes AC5 and AC6 isoforms, which are inhibited by micromolar concentrations of Ca2+ (11, 12, 22, 62), regulated by PKA and protein kinase C (PKC), and inhibited by βγ-subunits (11, 12, 22, 62). Group 4 includes the AC9 isoform, which is insensitive to Ca2+ (βγ-subunits, or forskolin (11, 12, 22, 62). There is a significant heterogeneity in the distribution and biochemical properties of the different isoforms, and each tissue or cell type possesses a unique combination of these isoforms. For example, the Ca2+-inhibitable isoforms AC5 and AC6 are the most abundant ones in the heart (11, 12, 22). Although these two isoforms are equally prevalent at birth, AC5 mRNA becomes predominant in the adult rat heart. Furthermore, with aging there is an increase in AC5 and a decrease in AC6, an isoform shift that may influence cardiac function (27). Both of these isoforms can be phosphorylated and inhibited by PKA, which thereby provides feedback regulation within the transduction cascade (11, 12, 22). The various AC isoforms are all activated by Gs; however, different ACs have varying affinities for Gs, a finding that may explain the variety of tissue responses to a given adrenergic receptor agonist. Similarly, both the inhibition of AC by Gi-coupled receptors and its activation by βγ-subunits are isozyme specific (11, 12, 22). There are a number of reports indicating that AC activity is altered in several diseases (15, 23, 29, 54). Moreover, recent data suggest that an increase in the cellular expression of AC has the potential to improve and restore β-AR function in cardiovascular disease (18, 50).

Diabetes mellitus is associated with vascular complications, including impairments of the vascular responsiveness to neurotransmitters in the macro- and microvasculature (10, 13, 48, 49, 68). Several reports have indicated that cAMP-mediated

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responses, such as β-AR-mediated responses, are altered in the diabetic state. For example, type 1 diabetic patients showed a decreased β-adrenergic responsiveness of the heart beat in isoproterenol (Iso) infusion experiments (4). In ventricular cardiomyocytes and papillary muscle isolated from rats with streptozotocin-induced diabetes, the β-adrenergic stimulatory pathway involves an additional defect upstream of the AC/G protein system (32, 67). Furthermore, in the vascular system of such rats, the iso-induced relaxation response is impaired in both the aorta (31) and the basilar artery (42). As mentioned above, the existence of multiple forms of PDE, AC, and PKA allow cells to tailor their responsiveness (25), yet so far no study has investigated the relationship between diabetic vasculopathy and the cAMP signaling system. For example, it was recently reported that cAMP facilitates endothelium-derived hyperpolarizing factor (EDHF)-type relaxation in conduit arteries by enhancing electrotonic conduction via gap junction (8, 21). Endogenous formation of cAMP may therefore play an important role in the EDHF phenomenon, because agonists such as ACh are capable of promoting endothelial synthesis of the cAMP through a mechanism that is independent of the formation of prostanoids (33, 63). We have recently shown that the mesenteric artery from the STZ-diabetic rat exhibits an impaired EDHF-type relaxation and that this impairment might be attributable to a reduced action of cAMP, in turn resulting from increased PDE activity (38). We also recently reported that cAMP-mediated (but not AC mediated) relaxation is impaired in the STZ-diabetic mesenteric artery and that this impairment may be attributable to reduced PKA activity, which in turn results from an alteration in the pattern of expression of PKA subunits (40). Although our previous studies suggested that an abnormality downstream of cAMP signaling was present in the STZ-diabetic mesenteric artery, it remained unclear whether the functions of ACs (components of upstream of cAMP signaling) are altered in the diabetic mesenteric artery.

The present study was undertaken to investigate any diabetes-related changes in AC-mediated relaxation and cAMP production in the rat superior mesenteric artery. Moreover, because cAMP is degraded by PDE, we assessed AC-induced relaxation and cAMP production in the presence of a PDE inhibitor. We also asked whether mesenteric arteries from control and established diabetic rats might differ in the expression profiles of their AC isoforms.

MATERIALS AND METHODS

Reagents. STZ, phenylephrine (PE), indomethacin, Nω-nitro-l-arginine (l-NNA), 3-isobutyl-1-methylxanthine (IBMX), isoprote-nol (Iso), forskolin (FSK), SQ-22536, phenylmethylsulfonyl fluoride (PMSF), and A-23187 were all purchased from Sigma Chemical (St. Louis, MO), and 6-[(3-dimethylamino)propionyl]forskolin (NKH477) was a gift from Nippon Kayaku. All drugs were dissolved in water, except IBMX, FSK, and A-23187, which were dissolved in dimethyl sulfoxide. Horseradish peroxidase (HRP)-linked secondary anti-rabbit antibody was purchased from Promega (Madison, WI), and the antibodies for AC5/6, AC4, and AC8 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals and experimental design. Male Wistar rats (8 wk old, 180- to 230-g body weight) 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, Culture, Sports, Science, and Technology, Japan).

Measurement of plasma glucose and insulin. Twelve weeks after the injection of STZ or buffer, plasma glucose was determined using a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibagay, Gunma, Japan).

Measurement of isometric force. Vascular isometric force was recorded as reported previously (38, 40). Rats were anesthetized with diethyl ether and euthanized by decapitation 12 wk after treatment with STZ or buffer. The superior mesenteric artery was randomly 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, 1.2 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 separately suspended by a pair of stainless steel pins in a well-oxygenated (95% O2-5% CO2) bath of 10 m of KHS at 37°C. The rings were stretched until resting tension was 1.0 g (found to be optimal for inducing maximal contractions in preliminary experiments) 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). Once the PE-induced contraction had stabilized, relaxation responses were elicited in a cumulative manner (Iso, FSK, or the water-soluble FSK analog NKH477, 10^{-5}–10^{-4} M). Complex interactions between different vasodilator pathways in vascular smooth muscle have been proposed for nitric oxide synthase (NOS) and cyclooxygenase (COX) (37, 69). We therefore investigated some relaxation responses after equilibration for 40 min in the combined presence of l-NNA (100 μM) and indomethacin (10 μM), to block NOS and COX, respectively, before administration of PE (1 μM). In a second series of experiments, we examined the effects of cAMP generation (such as that induced by a single application of Iso, FSK, or NKH477) on relaxation responses in the combined presence of l-NNA (100 μM), indomethacin (10 μM), and IBMX (10 μM; a cyclic nucleotide PDE inhibitor). We also examined the effect of a single application of Iso in the presence of the above three inhibitors (l-NNA, indomethacin, and IBMX) plus 100 μM SQ-22536, a cell-permeable AC inhibitor. In the experiments with IBMX, an equieffective concentration of PE was used (1–10 μM).

Enzyme immunoassay for cAMP. Mesenteric rings from diabetic and age-matched control rats were incubated for 1 h at 37°C in oxygenated KHS containing 50 μM IBMX. The rings were then incubated for 15 min with one of the following: 1) FSK, 2) NKH-477, 3) Iso, 4) the Ca^{2+} ionophore A-23187 (all 10 μM), or 5) vehicle. The stimulated level of cAMP was then determined in each case. 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, finally, an enzyme immunoassay (Amersham Biosciences UK) was performed.

Measurement of expression of mRNAs for ACs using RT-PCR. Total RNA was isolated using the guanidinium method (9). Briefly, 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. Individual sequences, PCR conditions, product size, and GenBank accession numbers are shown in Table 1. To ensure that we were within the exponential phase of the semiquantitative PCR reaction, the appropriate number of cycles was newly established for each set of samples. 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
RESULTS

Blood glucose and insulin levels and animal body weights. As in previous reports (38, 40), at the time of the experiment (1) all STZ-treated rats exhibited hyperglycemia, with blood glucose concentrations (632.2 ± 23.7 mg/dl, n = 8, P < 0.001) significantly higher than those of age-matched nondiabetic control rats (101.0 ± 7.5 mg/dl, n = 8), and 2) body weights of diabetic rats (248.0 ± 11.6 g, n = 8, P < 0.001) were significantly lower than those of age-matched controls (572.2 ± 19.5 g, n = 8). Plasma insulin levels were significantly lower in STZ-treated rats (0.28 ± 0.07 ng/ml, n = 6, P < 0.001) than in controls (1.70 ± 0.08 ng/ml, n = 6).

Relaxation responses to Iso, FSK, and NKH477. To investigate AC-mediated relaxation in the rat mesenteric artery, we first tested the effects of Iso (10⁻⁹–10⁻⁵ M), a Gₛα-coupled receptor agonist, FSK (10⁻⁹–10⁻⁵ M), a direct AC activator, and NKH477 (10⁻⁹–10⁻⁵ M), a water-soluble FSK analog, when added cumulatively to rings precontracted with PE (1 µM). The tension developed in response to 1 µM PE was 2.34 ± 0.32 g in diabetic mesenteric rings (19.5 g, n = 19, no significant difference). The body weights (0.07 ± 0.08 ng/ml, n = 6) were 6.41 ± 0.46 g in diabetic and 10.220.32.246 on June 29, 2017 http://ajpheart.physiology.org/ Downloaded from http://ajpheart.physiology.org/ by 10.220.32.246 on June 29, 2017

#### Table 1. Oligonucleotide primer sequences for AC 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, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC3 (M55075)</td>
<td>5’-GAGGCTGACTTCCGAGC-3’</td>
<td>94°C/60 s</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>5’-GCGGCAACGGTGAGGAAGA-3’</td>
<td>58°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>26 cycles</td>
<td></td>
</tr>
<tr>
<td>AC4 (NM_019285)</td>
<td>5’-GATGCGTGGTGGCGCCGATTT-3’</td>
<td>94°C/60 s</td>
<td>516</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 cycles</td>
<td></td>
</tr>
<tr>
<td>AC5 (NM_022600)</td>
<td>5’-AGAGGAGCAACAATCACAG-3’</td>
<td>94°C/60 s</td>
<td>409</td>
</tr>
<tr>
<td></td>
<td></td>
<td>58°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td>AC6 (NM_012821)</td>
<td>5’-CTAAGGCGATCCGAGAAGT-3’</td>
<td>94°C/60 s</td>
<td>293</td>
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<td></td>
<td>55°C/60 s</td>
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<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 cycles</td>
<td></td>
</tr>
<tr>
<td>AC7 (AF542508)</td>
<td>5’-GCCTGACTGAGCCAGCTGTC-3’</td>
<td>94°C/60 s</td>
<td>256</td>
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<td></td>
<td>57°C/60 s</td>
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<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28 cycles</td>
<td></td>
</tr>
<tr>
<td>AC8 (NM_017142)</td>
<td>5’-GATTCACGAACTGGAGC-3’</td>
<td>94°C/60 s</td>
<td>502</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72°C/60 s</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35 cycles</td>
<td></td>
</tr>
<tr>
<td>GAPDH (X02231)</td>
<td>5’-TCGCTCAAGATTTGCAAGCA-3’</td>
<td>94°C/60 s</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td></td>
<td>54°C/60 s</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>72°C/60 s</td>
<td></td>
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</tbody>
</table>

AC, adenyl cyclase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Product normalized with respect to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) product.

Measurement of expression of AC proteins using Western blotting. Mesenteric arteries (3 pooled vessels in each group) were homogenized in ice-cold lysis buffer containing 50 mM Tris·HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS containing 1 mM PMSF. Homogenates were centrifuged at 13,000 g for 10 min. The supernatant was collected, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid (BCA) protein assay reagent kit (Pierce). Samples (40 µg/lane) were resolved by electrophoresis on 7.5% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes. Briefly, after the residual protein sites on the membrane were blocked using Block ace (Dainippon-pharm, Osaka, Japan), the membrane was incubated with anti-AC5/6 (1:200), anti-AC4 (1:200), or anti-AC8 (1:200) in blocking solution. HRP-conjugated anti-rabbit or anti-goat antibody (Vector) was used at a 1:10,000 dilution in Tween-containing PBS, followed by detection using SuperSignal (Pierce).

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

AC isozyme expression in mesenteric arteries (Table 2). To exclude the influence of NOS and COX, we investigated the relaxation induced by Iso, FSK, and NKH477 (10⁻⁴ M), a water-soluble FSK analog, when added cumulatively to rings precontracted with 1 µM PE. The tension developed in response to 1 µM PE was 2.34 ± 0.32 g in diabetic mesenteric rings (19.5 g, n = 19, no significant difference). The body weights (0.07 ± 0.08 ng/ml, n = 6) were 6.41 ± 0.46 g in diabetic and 10.220.32.246 on June 29, 2017
teric arteries from diabetic rats [14.8 ± 1.7 and 33.0 ± 4.0% of the PE-induced tone in diabetic rats (n = 6) and age-matched controls (n = 6), respectively (P < 0.01)]. The EC_{50} values for the Iso-induced relaxations were not significantly different (Table 2). On the other hand, when FSK (10^{-9}–10^{-5} M) or NKH477 (10^{-9}–10^{-5} M) were added cumulatively to rings precontracted by PE (1 μM) in the presence of 100 μM l-NNA plus 10 μM indomethacin, the maximum relaxations were not different between age-matched controls and STZ-diabetic rats (Fig. 2, B and C), although the whole dose-

Table 2. EC_{50} values for Iso-, FSK-, and NKH477-induced relaxation in mesenteric arteries from age-matched control and STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Iso Control, nM</th>
<th>Iso Diabetic, nM</th>
<th>FSK Control, nM</th>
<th>FSK Diabetic, nM</th>
<th>NKH477 Control, nM</th>
<th>NKH477 Diabetic, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>207.9±22.7</td>
<td>218.1±41.0</td>
<td>41.4±5.7</td>
<td>35.2±6.4</td>
<td>14.2±1.5</td>
<td>42.7±7.0*</td>
</tr>
<tr>
<td>l-NNA + Indo</td>
<td>830.0±189.2</td>
<td>640.1±85.9</td>
<td>108.8±8.6</td>
<td>291.2±33.9</td>
<td>41.5±5.2</td>
<td>94.0±20.0†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 to 10 experiments. Iso, isoproterenol; FSK, forskolin; STZ, streptozotocin; l-NNA, N^\text{G}-nitro-l-arginine (l-NNA); Indo, indomethacin (10^{-5} M). *P < 0.01, diabetic vs. control. †P < 0.05; ‡P < 0.001, l-NNA + Indo-treated diabetic vs. l-NNA + Indo-treated control.
**Response curve** showed a significant rightward shift in the diabetic group (Fig. 2, B and C, and Table 2).

**Time course of relaxations induced by Iso, FSK, and NKH477.** In our previous study, PDE activity was found to be increased in the diabetic rat mesenteric artery (38). To exclude the involvement of PDE activity in the Iso- or direct AC activator-induced relaxations in the mesenteric artery, we examined the time course of these response in the presence of 10 μM IBMX, a PDE inhibitor. IBMX itself depressed contraction, the concentration of PE used in experiments involving IBMX was increased to between 1 and 10 μM. The tension developed in response to 1–10 μM PE in the presence of IBMX was 2.03 ± 0.09 g in diabetic mesenteric rings (n = 12) and 1.90 ± 0.06 g in age-matched control mesenteric rings (n = 13, no significant difference). Under these conditions, the Iso (100 nM)-induced relaxation was significantly weaker in mesenteric arteries from diabetic rats (Fig. 3). Additional pretreatment with 100 μM SQ-22536 (an AC inhibitor) in combination with 100 μM l-NNa, 10 μM indomethacin, and 10 μM IBMX markedly reduced the relaxation induced by Iso (100 nM) in mesenteric artery from the controls but not from STZ-diabetic rats (Fig. 3). When one of the direct AC activators, either FSK (10 nM) or NKH477 (3 nM), was applied to rings precontracted by PE (1–10 μM) in the presence of 100 μM l-NNa, 10 μM indomethacin, and 10 μM IBMX, the relaxation induced by each of these AC activators was significantly weaker in the diabetic group than in the controls (Fig. 4).

**Measurement of cAMP production.** As reported previously (38), the impaired EDHF-type mesenteric artery relaxation seen in STZ-induced diabetic rats may be attributable to a reduced action of cAMP, in turn resulting from increased PDE3 activity. For that reason, cAMP levels were measured in rings treated for 1 h with 50 μM IBMX, a PDE inhibitor, followed by stimulation for 15 min with one of several drugs (Fig. 5). Under our conditions, vehicle-treated cAMP levels were not significantly different between the diabetic and age-matched control groups. Although a FSK (10 μM)-induced cAMP production was evident in both groups, the elevated cAMP level was not significantly different between diabetic and control rats (Fig. 5A). Interestingly, the NKH477 (10 μM)-induced cAMP production (Fig. 5B) was significantly lower in diabetic rats than in control rats, as was the Iso (10 μM)-induced cAMP production (Fig. 5C). In contrast, the A-23187 (10 μM)-induced cAMP production was not significantly different between diabetic and control rats (Fig. 5D).

**Expressions of mRNAs and proteins for AC isoforms.** Among the nine mammalian transmembrane AC isoforms, a calcium-inhibitable AC (AC5 or AC6) plays important roles in the cardiovascular system (12, 22, 58). NKH477, a watersoluble FSK derivative, stimulates AC5 more potently than the other AC isoforms (65), and it is therefore used as a selective AC5 activator (30, 58). Having found, as described above, that the NKH477-induced relaxation (Figs. 1, 2, and 4B) and cAMP production (Fig. 5B) were impaired in STZ-diabetic mesenteric arteries, we went on to investigate whether the expressions of
the mRNAs and proteins for AC5/6 might be altered in the diabetic state. Using RT-PCR analysis on the total RNA isolated from mesenteric arteries obtained from age-matched control and STZ-induced diabetic rats (Fig. 6A), we found that whereas there was no difference in the expression of GAPDH mRNA between the two groups of rats, the expression of the mRNA for AC5 was significantly weaker in the diabetic rats than in the age-matched control rats. The expression of the mRNA for AC6, however, was only slightly (not significantly) decreased (Fig. 6A). Immunoblots of mesenteric arteries treated with anti-AC5/6 antibody after their removal from diabetic and age-matched control rats allowed detection of immunoreactive proteins with molecular masses of ~180 kDa (Fig. 6B). The specificity of the AC5/6 antibody was confirmed by preadsorption of the primary antibody with the peptide to which the antibody was raised (data not shown). Isoforms AC5/6 observed by Western blotting in the mesenteric artery are ~40 kDa heavier than expected. From an initial cloning study and sequence analysis, AC5/6 is predicted to have 1,184 (28) or 1,165 amino acids (70), whereas AC6 is anticipated to have 1,166 amino acids (36), which equates to ~139 kDa. The discrepancies between the observed and predicted sizes are probably accounted for by N-linked glycosylation at the consensus sites present in all AC isoforms in the second hydrophobic domain (M2) (56). Thus the ~40-kDa heavier than expected protein bands obtained with AC-selective antibodies are likely to represent glycosylated forms of the protein.

Indeed, Sobolewski et al. (58) reported that deglycosylation of membrane fractions with N-glycosidase F resulted in a downward shift in the bands specific for AC5/6 in rat pulmonary artery smooth muscle cells. In the present study, the AC5/6 protein level was significantly lower in the diabetic rats than in the age-matched controls (Fig. 6B). Furthermore, when we investigated the expression level of other AC isoforms in mesenteric arteries, there was no significant alteration in the mRNA levels for AC3 and AC8 between diabetic rats and controls (Fig. 6C). On the other hand, the mRNA level for AC7 was significantly increased in diabetic rats (Fig. 6C). The mRNA and protein levels for AC4 were also significantly increased in the diabetic rats compared with the age-matched controls (Fig. 6, C and D). The AC8 protein level was similar between diabetic rats and age-matched controls (Fig. 6D).

DISCUSSION

In the present study, we made two major findings, namely, the presence of 1) impairments of AC stimulant-mediated relaxation and AC activity in the mesenteric artery from STZ-induced diabetic rats and 2) a decreased expression of AC5/6 isoforms in that diabetic artery. To access AC-mediated relaxation in mesenteric arteries obtained from diabetic and age-matched control rats, we first used Iso, a β-AR agonist, and FSK, a direct AC activator (Fig. 1). Our first finding was that the Iso- and the FSK-induced relaxations were not impaired in diabetic mesenteric artery. Complex interactions between different vasodilator pathways in vascular smooth muscle have been proposed for NOS and COX (37, 69). Also, some studies suggest that the L-arginine/NO system contributes to vasodilation in response to the β-AR stimulation (with Iso) or cAMP elevation (with FSK) (20). Thus we suggest that these drug-induced vasodilations in mesenteric arteries from diabetic and age-matched controls were comparable because of various endothelium-derived factors. These data are consistent with the reports of several
arteries in STZ-diabetic rats (6). To exclude these endothelial-derived factors, we next investigated the relaxation responses in the presence of inhibitors of NOS and COX (e.g., L-NNA and indomethacin). We found that the Iso-induced relaxation was attenuated in STZ-induced diabetic rats (Fig. 2A). Generally, stimulation of β2-AR leads to vascular relaxation, which involves the following signaling cascade: β2-AR/Gs protein/AC/cAMP/PKA (52, 66). In addition, β2-AR agonists seem to induce relaxation via a cAMP-PKA-independent mechanism in smooth muscle in some tissues (47, 60). Consequently, we cannot be sure whether impairment of the Iso-induced relaxation in the diabetic mesenteric artery was attributable to an impairment of AC activity. When the direct AC activator FSK was cumulatively applied to mesenteric artery rings, the concentration-response curve showed a significant rightward shift in the diabetic group (Fig. 2B), leading us to hypothesize that the impaired relaxation response in that group was dependent on a decreased AC activity.

To test this hypothesis, we examined relaxation responses in the presence of a PDE inhibitor (Figs. 3 and 4) because previous studies have found PDE3 activity to be altered in diabetes (45, 46). Indeed, we recently demonstrated that PDE3 activity in the mesenteric artery was increased in STZ-induced diabetic rats (38). Under the above conditions, the Iso-induced relaxation was 1) greatly impaired in STZ-induced diabetic rats (Fig. 3) and 2) significantly reduced by pretreatment with an AC inhibitor in age-matched control rats but almost unchanged in STZ-induced diabetic rats (Fig. 3). These results strongly suggest that in the diabetic mesenteric artery, AC-mediated effects no longer contributed to the Iso-mediated relaxation. Similarly, the relaxations induced by FSK and the water-soluble FSK derivative NKH477 in the presence of a PDE inhibitor were significantly impaired in the diabetic mesenteric artery (Fig. 4). These results support AC activation-induced relaxation being impaired in the diabetic mesenteric artery. However, we recently reported that cAMP-mediated relaxation is impaired in the diabetic mesenteric artery and that this impairment may be attributable to reduced PKA activity, which in turn results from an alteration in the pattern of expression of PKA subunits (40). It is very difficult to assess AC-mediated cAMP-induced relaxation in the diabetic mesenteric artery because downstream components, such as the activities of PDE (38) and PKA (40), are altered in the diabetic state. To assess whether the impairment of the above relaxation
responses were entirely attributable to changes in the activities of these cAMP downstream components, we examined examples of AC stimulant-induced cAMP production in the presence of a PDE inhibitor. Interestingly, despite the application of a high concentration of Iso (10 μM) to mesenteric artery rings, cAMP production was significantly lower in the diabetic group than in the controls (Fig. 5C), strongly suggesting that β-AR-induced AC activity is impaired in the STZ-induced diabetic mesenteric artery. This interpretation is supported by previous findings that catecholamine-induced AC activation is impaired in several tissues in diabetic states (2, 43, 57, 61). On the other hand, the high-dose FSK (10 μM)-induced cAMP production showed no significant alteration between diabetic and age-matched control rats (Fig. 5A). In contrast, that induced by the water-soluble FSK derivative NKH477 (10 μM) was greatly impaired in the diabetic mesenteric artery (Fig. 5B). FSK, like digitalis, is a natural plant extract that is used in traditional medicine (53). All AC isoforms, with the possible exception of AC9, are activated by FSK (12, 22). This activation mechanism is currently explained as follows. In AC, FSK binds to the catalytic core at the opposite end of the ventral cleft that contains the active site and activates the enzyme by gluing together the two cytoplasmic domains in the core (C1 and C2) with a combination of hydrophobic and hydrogen bond interaction (71). Although the efficacy of FSK has been confirmed in human studies (7, 19), its poor tissue selectivity has hampered its clinical use. Recently, however, the water-soluble FSK derivative NKH477 was introduced for the treatment of human heart failure (51, 55). NKH477 is a derivative in which a 3-(dimethylamino)propionyl group is attached to FSK at the C6 position, and it has been shown to stimulate AC5 more potently than other AC isoforms (65). A recent crystallographic study predicted a relatively large open space between the C6/C7 positions of FSK and its binding site within AC (64, 71). This implies that an FSK derivative modified in these positions might display an altered isoform selectivity without suffering a disruption of its activity; this is consistent with the findings for NKH477 (65). Furthermore, Jourdan et al. (30) demonstrated that AC5 mRNA and protein were expressed in pulmonary artery smooth muscle cells and suggested that the AC5 was functional because NKH477 induced a significantly greater increase in cAMP than did FSK. In the present study, the NKH477-induced cAMP production was greater than that induced by FSK in the control mesenteric artery (Fig. 5A and B). Moreover, NKH477-induced relaxation responses in the absence and presence of l-NNA plus indomethacin in control mesentery were greater than FSK-induced responses (Figs. 1 and 2, Table 2). NKH477-induced relaxation was significantly impaired in diabetic mesenteric artery in the absence and presence of l-NNA plus indomethacin (Figs. 1C and 2C, Table 2). NKH477 (3 mM) induced a greater relaxation than FSK (10 nM) in the control mesenteric artery (Fig. 4A and B). Furthermore, the AC5 mRNA and protein levels were significantly lower in the diabetic mesenteric artery (Fig. 6A and B). These data suggest that both AC5 activity and the relaxation induced by AC5 activation are impaired in the diabetic mesenteric artery, an interpretation consistent with the diabetes-related decrease we observed in the AC5 mRNA and protein expression levels. As mentioned above, the FSK-induced total AC activity showed no apparent change between the diabetic and control groups (Fig. 5A). Hence, we speculate that one (or more) AC isoforms shows a compensatory upregulation in the diabetic mesenteric artery (see below). However, the FSK-induced relaxation was impaired in our diabetic rats (Fig. 4A). It is conceivable that this impaired relaxation is attributable to a decreased PKA activity in diabetic rats or that the FSK concentration employed in this experiment (10 nM) was too low to alter total AC activity. Because relaxation is the final output of complicated cascades, further investigation is required on these points.

In the present study, we demonstrated for the first time that in the mesenteric artery isolated from the STZ-induced diabetic rat, the observed downregulation of AC catalytic activity results, at least in part, from reductions in the levels of AC5/6 mRNA and protein. Although the underlying mechanism remains unclear, metabolic and/or hormonal alterations might be involved. For example, in cells expressing AC5, insulin augments cAMP production through phosphatidylinositol 3,4,5-trisphosphate activation of PKC-ζ (34). Thus we speculate that this insulin effect is not present in our insulinohippocampal diabetic model. In apparent conflict with our results, Hashim et al. (23, 24) demonstrated that the stimulatory effects of Iso, glucagon, NaF, and FSK on AC activity are enhanced in aortas from short-term (5 days) STZ-induced diabetic rats and that these enhancements were attributable to the hyperglycemia. However, differences in the diabetes duration and the blood vessel examined could explain this discrepancy. Indeed, in our STZ diabetic model, the long-term insulin deficiency and hyperglycemia are associated with metabolic abnormalities such as increases in plasma triglyceride, cholesterol, and low-density lipoproteins (35). In addition, the levels of counterregulatory hormones such as catecholamines, adrenocorticotropic hormone, and glucagon are markedly higher in the diabetic condition (1, 26). Thus it is possible that this discrepancy in the expression levels and activities of AC isoforms is due to desensitization of AC functions following chronic exposure to these hormones. This speculation is supported by the previous finding that AC activity is altered by chronic treatment with a number of hormones (5, 14).

It is important to realize that membrane catalytic activity is the sum of the catalytic activities of the various AC isoforms coexisting therein (even within a single cell type). This is important because different isoforms show different sensitivities to various types of stimulation, including those mediated via βγ-subunits (12, 17, 22). Thus the impact of the activities of multiple cell surface receptors, both stimulatory and inhibitory, on the accumulation of intracellular cAMP will depend on the characteristics of the particular AC isoforms that are expressed there. Indeed, in the present study FSK-induced cAMP production in the mesenteric artery was found to be similar between diabetic and age-matched control rats (Fig. 5A), and A-23187-induced cAMP production was not significantly different between these two groups (Fig. 5D). Furthermore, AC4 mRNA and protein level and AC7 mRNA level were significantly increased in diabetic mesenteric artery compared with controls (Fig. 6A and B). On the other hand, AC8 mRNA and protein level and AC3 mRNA level did not change in both groups (Fig. 6C and D). These results suggest that the presence of a Ca2+-stimulated AC isoform in the mesenteric artery, which is likely to be AC8 on the basis of the recognized enhancing effect of Ca2+ on AC8 activity (16), 2) that the Ca2+-stimulated level of this isoform does not differ between
the controls and our diabetic rats, and 3) that the compensatory regulation was in existence in diabetic mesentery between AC isoforms. However, to establish a causal relationship will require research focusing on, for example, the time course of the diabetes-related changes in the expressions of the mRNAs and proteins for AC isoforms and 2) AC activity in the mesenteric artery.

Finally, taking the relevant literature and our evidence together leads us to propose that in the cAMP signaling cascade, several abnormalities in physiological function (relaxation parameters), biochemical activity (decreased AC and PKA activity, increased PDE activity), and mRNA/protein levels may be present in the STZ-induced diabetic mesenteric artery (Refs. 38, 40; present data). Although these data do not necessarily indicate a cause-and-effect relationship, the parallel changes among these activities lend credence to the idea that a loss in cAMP signaling plays a role in the progression of diabetic vasculopathy, which is a prominent clinical feature of diabetes. We believe that our findings should stimulate further interest in cAMP signaling in the progression of diabetic vasculopathy, which is a prominent clinical feature of diabetes.

We thank Nippon Kayaku Co. for the kind gift of NKH477. We also thank A. Iwasaki, T. Katohno, and N. Hirata for technical help.

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