Cilostazol improves endothelium-derived hyperpolarizing factor-type relaxation in mesenteric arteries from diabetic rats

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Matsumoto, Takayuki, Tsuneo Kobayashi, Kentaro Wakabayashi, and Katsuo Kamata. Cilostazol improves endothelium-derived hyperpolarizing factor-type relaxation in mesenteric arteries from diabetic rats. Am J Physiol Heart Circ Physiol 289: H283–H291, 2005.—We previously reported that in mesenteric arteries from streptozotocin (STZ)-induced diabetic rats that 1) endothelium-derived hyperpolarizing factor (EDHF)-type relaxation is impaired, possibly due to a reduced activity of cyclic monophosphate (cAMP) via increased phosphodiesterase 3 (PDE3) activity (Matsumoto T, Kobayashi T, and Kamata K. Am J Physiol Heart Circ Physiol 285: H283–H291, 2003) and that 2) PKA activity is decreased (Matsumoto T, Wakabayashi K, Kobayashi T, and Kamata K. Am J Physiol Heart Circ Physiol 287: H1064–H1071, 2004). Here we investigated whether chronic treatment with cilostazol, a PDE3 inhibitor, improves EDHF-type relaxation in mesenteric arteries isolated from STZ rats. We found that in such arteries 1) cilostazol treatment (2 wk) improved ACh-, A-23187-, and cyclopiazonic acid-induced EDHF-type relaxations; 2) the ACh-induced cAMP accumulation was transient and sustained in arteries from cilostazol-treated STZ rats; 3) the EDHF-type relaxation was significantly decreased by a PKA inhibitor in the cilostazol-treated group, but not in the cilostazol-untreated group; 4) cilostazol treatment improved both the relaxations induced by cAMP analogs and the PKA activity level; and 5) PKA catalytic subunit (Cat-α) protein was significantly decreased, but the regulatory subunit RII-β was increased (and the latter effect was significantly decreased by cilostazol treatment). These results strongly suggest that cilostazol improves EDHF-type relaxations in STZ rats via an increase in cAMP and PKA signaling.

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VASCULAR COMPLICATIONS are the main causes of morbidity and mortality in patients with diabetes. Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro- and microangiopathy in humans and in animal models of the disease (11, 42, 43, 47). Endothelial cells relax the tone of the underlying vascular smooth muscle cells by releasing a number of vasoconstrictor substances, including nitric oxide (NO), prostacyclin, and an as-yet-elusive endothelium-derived hyperpolarizing factor (EDHF) (7). Although NO has generally been considered to be the principal mediator of endothelium-dependent relaxations, it has become increasingly clear that NO-independent endothelium-derived relaxing factors might play an important role in local vasomotor control. The contribution made by EDHF to relaxation is dependent on vessel size, it being more prominent in the smaller, physiologically more important arteries than in larger ones (5, 12, 14). Impaired endothelium-dependent relaxations have been reported in various types of blood vessels in several animal models of diabetes (11). Although there is an accumulating body of evidence to show that in large conduit arteries, such as the aorta, the impairment of NO signaling seen in diabetes is attributable to endothelial dysfunction (11, 16, 18, 22–25), there is limited information about endothelial dysfunction in the diabetic microvasculature (10, 13, 30, 51). In particular, little attention has been paid to the potential role of an impaired release or impaired action of EDHF in diabetes. Because small-vessel dysfunction (such as in retinopathy, nephropathy, and neuropathy) is one of the major complications seen in diabetes (27, 50), an impairment of EDHF-mediated responses may have important implications for the mechanisms by which diabetes leads to vascular dysfunction. Thus an improvement in EDHF signaling could be an interesting therapeutic target in cases involving diabetic vasculopathy.

Although the identity of EDHF is still controversial (5, 12, 14), there is evidence that EDHF-type relaxation involves the transfer of a mediator from the endothelium to the smooth muscle via myoendothelial gap junctions (44). Interestingly, it was recently reported that cAMP facilitates EDHF-type relaxation in conduit arteries by enhancing electrotonic conduction via gap junctions (6, 15). Endogenous formation of cAMP may therefore play an important role in the EDHF phenomenon, because agonists such as ACh are capable of promoting the endothelial synthesis of cAMP through a mechanism that is independent of the formation of prostanoids (19, 48). Furthermore, elevations in smooth muscle cAMP levels have been shown to facilitate electrotonic signaling within the vascular media and thereby to amplify and prolong the transmission of ACh-induced hyperpolarizations to smooth muscle cells remote from the endothelium (15). The intracellular level of cAMP is tightly regulated by both control of its rate of synthesis [by adenyly cyclases (ACs)] (9) in response to extracellular signals and control of its rate of hydrolysis [by phosphodiesterases (PDEs)] (4, 32, 35). After an intracellular elevation of cAMP, reversible phosphorylation of several protein substrates, including the gap-junction component connexin, by cAMP-dependent PKA exerts influences over many physiological processes (26, 36, 49). It is known that PKA is activated by cAMP and comprises two regulatory (R) and two catalytic (C) subunits (26), and several pieces of evidence indicate that PKA activity is determined by the expression balance between the C and R subunits (8, 45). In recent studies, we have demonstrated 1) that alterations in EDHF-type relaxation in mesenteric arteries from streptozotocin (STZ)-induced

diabetic rats may be attributable to an increase in PDE3 activity, leading to a reduction in the action of cAMP (31), and 2) that both cAMP analog-activated vasodilation and PKA activation are impaired in STZ mesenteric arteries, possibly due to an imbalance between PKA C and R subunit expressions (34). Taken together, these results suggest that the cAMP and PKA signaling pathway could be intimately involved in the specific alteration of EDHF-mediated responses seen in the mesenteric artery in STZ rats.

Cilostazol {6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)-butoxy]-3, 4-dihydro-2(1H)-quinolinone} is a specific inhibitor of PDE3. Its major effects are prevention of platelet aggregation and a dilation of blood vessels via an increase in tissue cAMP levels (20, 29). Although there is an accumulating body of evidence to show that cilostazol has beneficial effects on diabetic complications in both patients and animal models (21, 39), no study has yet investigated the effect of cilostazol on EDHF-type relaxation. In this study, we carried out just such an investigation, while also trying to determine the underlying mechanism using mesenteric arteries isolated from STZ-induced diabetic rats.

MATERIALS AND METHODS

Reagents. A-23187; apamin; cyclopiazonic acid (CPA); charybdotoxin; SQ-22536; STZ; phenylephrine; indomethacin; N\textsuperscript{3}-nitro-L-arginine (l-NNA); IBMX; 8-bromo-cAMP (8-BrCAMP); N\textsuperscript{6}, O\textsuperscript{2}-dibutylryl-cAMP (DBcAMP); EDTA; EGTA; PMSF; leupeptin; apro tinin; and monoclonal β-actin antibody were all purchased from Sigma Chemical (St. Louis, MO). PKA inhibitor (PKI) 14–22 amide, cell-permeable, myristoylated was from Calbiochem-Novabiochem (La Jolla, CA). All drugs were dissolved in saline, except where otherwise noted (e.g., A-23187, CPA, and IBMX were dissolved in DMSO). Horseradish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI), whereas antibodies for PKA C and R subunit isoforms (Cat-α, RI-α, RI-β, and RR-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 STZ (65 mg/kg) 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 Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accredited by the Ministry of Education, Culture, Sports, Science, and Technology). Rats were considered diabetic if their blood glucose exceeded 400 mg/dl at 7 days after the injection of STZ (519.2 ± 14.0 mg/dl; n = 30). To examine the therapeutic, not preventive, effect of cilostazol, some STZ-induced diabetic rats were given cilostazol (100 mg/kg po, daily) for 2 wk starting 10 wk after the STZ injection (i.e., in our established diabetic model). Twelve weeks after the STZ injection, the rats were euthanized by decapitation while under diethyl ether anesthesia. Control rats were euthanized in the same way 12 wk after receiving their buffer injection. Thus we studied three groups: controls, cilostazol-untreated, and cilostazol-treated diabetic groups.

Measurement of plasma glucose, cholesterol, triglyceride, insulin, and blood pressure. Plasma parameters and blood pressure were measured as described previously (25, 33).

Measurement of isometric force. Vascular isometric force was recorded as in our previous paper (31, 34). Rats were anesthetized with diethyl ether and euthanized by decapitation 12 wk after treatment with STZ, STZ plus cilostazol, or buffer. 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 NaHCO\textsubscript{3}, 1.8 CaCl\textsubscript{2}, 1.2 NaHPO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 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% O\textsubscript{2}, 5% CO\textsubscript{2}) 10-ml bath of 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-NNA and 10 μM indomethacin (to block NO synthase and cyclooxygenase, respectively) before administration of phenylephrine (1 μM). Once the phenylephrine-induced contraction had stabilized, vasodilator responses were elicited either in a cumulative manner (ACh) or in a single concentration-effect manner in the case of A-23187 (calcium ionophore), CPA [sarco(endo)plasmic reticulum Ca\textsuperscript{2+} ATPase (SERCA) inhibitor], 8-BrCAMP (a cell-permeable cAMP analog rated as having greater resistance to hydrolysis by PDE), and DBcAMP (a cell-permeable cAMP analog), the last-named agent being applied in the presence of 100 μM IBMX, a nonselective inhibitor of PDE. Such relaxation responses were also generated in the combined presence of l-NNA (100 μM), indomethacin (10 μM), and PKI (5 μM) or of charybdotoxin (100 nM) plus apamin (100 nM). In the experiments involving the use of IBMX, an equieffective concentration of phenylephrine was used (from 1 to 10 μM).

Enzyme immunoassay for cAMP. Mesenteric rings from control rats, STZ rats, and STZ rats treated with cilostazol were incubated for 40 min at 37°C in oxygenated KHS containing 100 μM l-NNA plus 10 μM indomethacin with, in some experiments, 100 μM SQ-22536, a specific AC inhibitor. Phenylephrine (1 μM) was added 5 min before ACh (3 μM) stimulation. Rings were frozen in liquid N\textsubscript{2} after the addition of ACh and stored at −80°C. The mesenteric tissues were homogenized in 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. PKA activity in lysates was measured as described previously (34). The homogenates were centrifuged, and the supernatant (10 μg of protein) was used in a nonradioactive assay for cAMP-dependent protein kinase (PKA assay system; Promega). For the positive control (10 ng of 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 was quantitated by spectrophotometry.

Western blotting. The protein levels of the various PKA subunits were quantified by using immunoblotting procedures, essentially as described before (34). Mesenteric arterial tissues (two pooled vessels per 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. The lysate was cleared by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected, and the proteins were solubilized in Laemmli buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, IL). 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 (40 kDa; 1:1,000), anti-PKA Cat-β (42 kDa; 1:500), anti-PKA RI-α (49 kDa;
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1.250), anti-PKA RI-α (51 kDa; 1:1,000), or anti-PKA RI-β (53 kDa; 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, IL). To normalize the data, we used β-actin as a housekeeping protein. The β-actin protein levels were determined after stripping the membrane and probing with β-actin monoclonal primary antibody (42 kDa; 1:5,000) with HRP-conjugated anti-mouse IgG as the secondary antibody. The optical densities of the bands on the film were quantified by using densitometry with correction for the optical density of the corresponding β-actin band.

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

RESULTS

General parameters. As shown in Table 1, the plasma glucose, cholesterol, and triglyceride levels were significantly higher in STZ rats than in control rats. Body weight and the plasma insulin level were significantly lower in STZ rats than in control rats. Plasma HDL cholesterol levels were similar between STZ rats and control rats. Treatment with cilostazol did not alter plasma parameters or systolic blood pressure in our established diabetic rats.

EDHF-type relaxation. To investigate the EDHF-type relaxation evoked by ACh in the rat mesenteric artery, the vasorelaxation response to ACh in the presence of 100 μM l-NNA plus 10 μM indomethacin was examined in the following three groups of rats: a control group, a cilostazol-untreated STZ group, and a cilostazol-treated STZ group. The tension, developed in response to 1 μM phenylephrine, was not significantly different among the three groups (2.34 ± 0.03 g for control, \( n = 14; \) 2.26 ± 0.06 g for STZ, \( n = 14; \) and 2.3 ± 0.05 g for cilostazol-treated STZ, \( n = 12 \)). The maximum relaxation responses shown by mesenteric arteries were significantly smaller in the untreated STZ group than in the control group (52.1 ± 5.4% in the untreated STZ group vs. 78.8 ± 2.2% in the control group, \( P < 0.001 \)) (Fig. 1A). This attenuated maximal response was significantly improved by chronic cilostazol treatment (67.7 ± 2.8% in the cilostazol-treated STZ group; \( P < 0.05 \) vs. the untreated STZ group) (Fig. 1A). The concentration of ACh inducing a 50% relaxation (EC50) was significantly lower in the control group than in the untreated STZ group (control, 77.8 ± 7.4 nM vs. untreated STZ, 207.4 ± 28.8 nM, \( P < 0.001 \); Fig. 1A). This decreased sensitivity was significantly improved by cilostazol treatment (cilostazol

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**Table 1. Values of various parameters in control rats and cilostazol-treated and -untreated rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>STZ</th>
<th>Cilostazol-treated STZ</th>
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<tr>
<td>Body weight, g</td>
<td>578.0±17.7 (15)</td>
<td>260.0±7.4* (15)</td>
<td>274.0±8.7* (15)</td>
</tr>
<tr>
<td>Plasma glucose, mg/dl</td>
<td>143.7±2.4 (15)</td>
<td>575.8±13.9* (15)</td>
<td>610.0±10.5* (15)</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>96.5±2.2 (15)</td>
<td>163.3±7.0* (15)</td>
<td>157.1±4.2* (15)</td>
</tr>
<tr>
<td>Plasma HDL, mg/dl</td>
<td>53.8±1.9 (15)</td>
<td>56.0±1.7 (15)</td>
<td>58.0±1.7 (15)</td>
</tr>
<tr>
<td>Plasma triglyceride, mg/dl</td>
<td>144.0±11.6 (15)</td>
<td>648.7±30.0* (15)</td>
<td>571.0±35.6* (15)</td>
</tr>
<tr>
<td>Plasma insulin, pg/ml</td>
<td>1,590.0±70.9 (15)</td>
<td>311.9±44.6* (15)</td>
<td>492.1±77.5* (15)</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>103.1±1.8 (15)</td>
<td>101.7±2.7 (15)</td>
<td>108.7±3.2 (15)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of determinations is shown within parentheses. STZ, streptozotocin. *\( P < 0.001 \) vs. controls.
group, 84.8 ± 9.9 nM; P < 0.001 vs. STZ; Fig. 1A). Preparations incubated with 30 mM K\(^+\), charybdotoxin (100 nM) plus apamin (100 nM), or denuded of their endothelium were essentially devoid of vasorelaxant activity in all groups (data not shown). We then examined the EDHF-type relaxation induced by the Ca\(^{2+}\) ionophore A-23187 and the SERCA inhibitor CPA. In these experiments, the tension, developed in response to 1 \(\mu\)M phenylephrine, was not significantly different among the three groups (2.43 ± 0.05 g for control, \(n = 15\); 2.42 ± 0.06 g for STZ, \(n = 15\); and 2.45 ± 0.06 g for cilostazol-treated STZ, \(n = 14\)), as in the ACh-application study. The EDHF-type relaxation responses induced by A-23187 (0.3, 1 \(\mu\)M) and CPA (10 \(\mu\)M) were each significantly weaker in the STZ group than in the controls, and these impaired relaxations were significantly improved by cilostazol treatment (Fig. 1, B and C). These results indicate that treatment with cilostazol improved the EDHF-type relaxation response in our STZ rats.

\textit{cAMP accumulation.} cAMP levels were measured in rat mesenteric arterial rings treated with 100 \(\mu\)M L-\(\text{N}{\text{G}}\)-nitro-L-arginine (L-NNA) plus 10 \(\mu\)M indomethacin for 40 min before 1 \(\mu\)M phenylephrine application (Fig. 2). Under our conditions, cAMP levels in the absence of 3 \(\mu\)M ACh stimulation were not significantly different among the three groups. An ACh-induced cAMP accumulation was evident in all groups at 15 s, but the elevated cAMP level declined rapidly, being significantly reduced at 60 s (vs. 15 s) in the control and untreated STZ groups. This decline between 15 and 60 s after ACh stimulation was greater in the untreated STZ group than in the controls (\(P < 0.01\), Fig. 2B). In rings from cilostazol-treated STZ rats, however, a sustained accumulation in cAMP level was seen after ACh stimulation. These results suggest that PDE activity was increased in STZ rats and that this increase was effectively inhibited by cilostazol treatment.

\textit{Effects of a PKA inhibitor on EDHF-type relaxation.} PKA contributes to cAMP signaling and to its impact on vascular function (17, 36). To examine the part played by PKA in the present EDHF-type relaxation, rings were incubated with PKI (as well as with L-NNA plus indomethacin) for 40 min before administration of phenylephrine. As shown in Fig. 3, under these conditions the ACh-induced EDHF-type relaxation was attenuated by PKI in the control group (Fig. 3A), and for that group the EC\(_{50}\) value was significantly greater after PKI treatment than after vehicle treatment (\(P < 0.001\), Fig. 3A). Surprisingly, an inhibitory effect of PKI was not evident in the STZ group (Fig. 3B). When rings from cilostazol-treated STZ rats were incubated with PKI, however, the ACh-induced EDHF-type relaxation and the sensitivity to ACh were significantly attenuated (Fig. 3C), as they were in the control group.

\textit{Effects of cAMP analogs on relaxation responses.} The data in Fig. 3 and those in a previous report from Kamata’s laboratory (31) demonstrate that cAMP and PKA signaling plays an important role in EDHF-type relaxation. To clarify the nature of the cAMP-mediated vasodilation, we examined the relaxing effects of two cAMP analogs in rings from the three groups (in the presence of 100 \(\mu\)M L-NNA plus 10 \(\mu\)M indomethacin). As reported previously (34), the relaxation responses induced by 8-BrcAMP (a cell-permeant cAMP analog that is rated as more resistant to PDE) or another cAMP analog (DBcAMP, applied in the presence of IBMX) were each significantly weaker in the STZ group than in the control group (Fig. 4). These impaired relaxations were significantly enhanced by cilostazol treatment of STZ rats (Fig. 4).

\textit{PKA activity.} In our previous report (34), PKA activity was found to be decreased in the STZ-diabetic mesenteric artery. Furthermore, the data in Fig. 4 demonstrate that cilostazol treatment enhances the impaired cAMP-mediated responses seen in the STZ-diabetic mesenteric artery. To investigate whether PKA activity was improved by cilostazol treatment, we measured this activity in mesenteric arteries from all three groups. As reported previously (31), PDE activity is increased in the STZ-diabetic mesenteric artery. We therefore examined the effects of DBcAMP treatment on PKA activity in mesenteric artery rings after pretreatment with the PDE inhibitor.
IBMX (20 μM). As shown in Fig. 5, there was no significant difference in PKA activity among vehicle-treated rings from the three groups. On stimulation with 100 μM DBCAMP, PKA activity was significantly increased in the control group but not in the STZ group. The DBCAMP-stimulated level of PKA activity was significantly greater in rings from cilostazol-treated STZ rats than in those from STZ rats not treated with cilostazol (P < 0.05).

Expressions of the proteins for PKA subunits. To investigate the possible mechanisms underlying the impairment of cAMP analog-induced PKA activity seen in mesenteric arteries from STZ-induced diabetic rats and the improvement in this activity after cilostazol treatment, we examined whether the expressions of the proteins for the various PKA subunits might be altered in mesenteric arteries from the three groups. Immunoblot analysis of mesenteric arteries from control, STZ, and cilostazol-treated STZ rats (using anti-PKA subunit antibodies) allowed detection of immunoreactive proteins (Fig. 6). The PKA C subunit Cat-α protein level was significantly lower in the diabetic group than in the controls, and cilostazol treatment had no influence on this decreased expression. Furthermore, the expressions of the proteins for the PKA Cat-β, RI-α, and RII-α subunits showed no differences among the three groups.
the role of cAMP and PKA signaling in these arteries. Indeed, chronic treatment of STZ rats with cilostazol enhanced cAMP and PKA signaling in the mesenteric artery. This stimulation of the vascular cAMP and PKA pathway by cilostazol is supported by several lines of evidence. First, Kamata’s laboratory (31) found that chronic treatment with cilostazol increased ACh-induced cAMP accumulation in the STZ rat mesenteric artery, and acute treatment with cilostamide, a specific PDE3 inhibitor, also increases cAMP accumulation in such rats. Second, chronic treatment with cilostazol increased the relaxations induced by cAMP analogs in our STZ-diabetic rats. Third, the DBcAMP-stimulated level of PKA activity, as well as the control level, was enhanced in mesenteric arteries from cilostazol-treated STZ rats. Fourth, PKA activity is known to be regulated by the expression levels of PKA subunits in several cells (8, 26, 45). This is supported, for instance, by evidence that PKA activity is decreased both by induction of the RI-α subunit and by the degradation of the C subunit in several cells (26), as well as by evidence that in RII-β gene-knockout mice, an increased level of RII-α compensates for the loss of RII-β in brown fat cells (8). This switching of the PKA isoform from type II to type I results in an increased basal level of PKA activity and an increased energy expenditure (8). Such RII-β knockout mice have been noted to be leaner and also protected against diet-induced obesity, insulin resistance, and dyslipidemia (8, 45). In our diabetic model, a decrease in the level of the PKA Cat-α subunit and an increase in the PKA RII-β subunit have been observed in the mesenteric artery (34 and the present Fig. 6). In the present study, this increased level of the RII-β subunit was greatly decreased by cilostazol treatment. It was noteworthy that the EDFH-type relaxation was inhibited by PKI in mesenteric arteries from cilostazol-treated diabetic rats, as well as in those from the nondiabetic controls. On the other hand, such an inhibition by PKI was not seen in the cilostazol-untreated STZ mesenteric artery. To judge from these results, the impaired EDFH-type relaxation seen in STZ rats is attributable to decreased cAMP and PKA signaling. In fact, in the diabetic state an abnormality in the cAMP signaling pathway has been observed in several tissues (1, 37, 38). Moreover, EDFH-type relaxations are potentiated by an increase in the intracellular cAMP level (6, 14, 15, 31, 48).

Several studies have suggested that impairment of EDFH-mediated responses is present in disease states, indicating the potential for therapeutic interventions (12). For example, chronic treatment with an angiotensin-converting enzyme inhibitor, with an angiotensin-receptor antagonist, or with a diuretic normalizes EDFH-mediated responses in spontaneously hypertensive rats (12, 40). Moreover, treatment with folate, with an aldose reductase inhibitor, or with calcium dobesilate (an angioprotective agent) restores impaired EDFH-mediated responses in diabetes (2, 10, 12). In addition, dietary supplements and exercise have beneficial effects on EDFH responses (12). However, the mechanisms underlying these drug-induced and adjuvant-induced improvements in EDFH responses remain poorly understood. EDFH-mediated responses are clearly affected in a variety of pathological conditions, and the fact that the above therapeutic or adjuvant interventions can restore these responses suggests that an improvement in these EDFH-mediated responses contributes to the observed beneficial effect. Especially interesting is the possibility that an enhancement of EDFH-mediated responses

**Fig. 6.** Analysis of protein expressions for PKA catalytic and regulatory subunits in mesenteric arteries from control, STZ, and cilostazol-treated STZ rats. Top: representative Western blots of PKA subunits. Bottom: bands were quantified as described in MATERIALS AND METHODS. Data are means ± SE of six determinations (subunits/β-actin). Number of determinations is shown within parentheses. **P < 0.01 vs. control. †P < 0.05 vs. STZ.

(Fig. 6). On the other hand, the expression of the protein for PKA R subunit RII-β was significantly greater in the STZ group than in the control group. Surprisingly, cilostazol treatment greatly attenuated this increased expression.

**DISCUSSION**

In the present study, we examined whether the impaired EDFH-type relaxation seen in mesenteric arteries isolated from established diabetic rats is improved by chronic cilostazol treatment. To address specifically the possible association between changes in EDFH-type relaxation and changes in the cAMP and PKA signaling pathway in this artery, we chose the established STZ rat as our model. We did this because in this chronic diabetic model we and others have demonstrated 1) an impairment of ACh-induced, EDFH-type relaxation (13, 31) and 2) evidence of a reduction in the action of cAMP, such as increased PDE3 activity and decreased PKA activity (31, 34). In this chronic diabetic model, the plasma lipid and glucose concentrations are both increased (23, 30, 31, 34). When we administered cilostazol for 2 wk to such established STZ rats, the cilostazol did not improve the blood lipid or glucose levels; thus its beneficial effects are clearly unrelated to a correction of the hyperlipemia or hyperglycemia.

A novel, intriguing, and potentially important finding made in this study was that the modest increase in EDFH-type relaxation seen in mesenteric arteries from cilostazol-treated STZ rats was associated with evidence of a marked change in

<table>
<thead>
<tr>
<th>Subunits/β-actin</th>
<th>Control</th>
<th>STZ</th>
<th>Cilostazol-treated</th>
</tr>
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<tr>
<td><strong>Catα</strong></td>
<td>1.00</td>
<td>0.75</td>
<td>1.25</td>
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<tr>
<td><strong>Catβ</strong></td>
<td>0.80</td>
<td>0.65</td>
<td>1.10</td>
</tr>
<tr>
<td><strong>RIα</strong></td>
<td>0.90</td>
<td>0.75</td>
<td>1.15</td>
</tr>
<tr>
<td><strong>RIβ</strong></td>
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<td>0.55</td>
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may contribute to improvements in diabetic microvascular complications such as retinopathy, nephropathy, and neuropathy (because EDHF plays important roles in the microvasculature). Although cilostazol is used clinically in several diseases, such as peripheral artery disease (20), the present study 1) provides the first evidence of its potential to be a therapeutic drug for the improvement of EDHF-mediated responses in diabetic states and 2) strongly suggests that the reduction of EDHF-mediated responses in diabetes may be improved by manipulation of the cAMP and PKA pathway.

Several recent studies (3, 20, 28, 41, 46) have reported that cilostazol has beneficial effects on the cardiovascular system through several cellular pathways. For instance, cilostazol possesses the ability to inhibit not only PDE3 activity but also adenosine uptake (20, 29). Cilostazol has also been reported to inhibit superoxide generation and tumor necrosis factor-α formation and to suppress NF-κB activation and transcription (28, 41). As yet, it remains unclear whether there is a direct relationship between the effects of cilostazol on EDHF responses and its effects on these cellular pathways. Further investigation is required on this point.

In conclusion, our study demonstrates that chronic cilostazol treatment of STZ-diabetic rats improves EDHF-type relaxation in the mesenteric artery via increased cAMP and PKA signaling. We believe that our findings should stimulate further interest in cilostazol as a potential therapeutic drug for use against diabetes-associated vascular disease, especially microvascular disease.

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REFERENCES

29. Makino A, Ohuchi K, and Kamata K. Mechanisms underlying the attenuation of endothelium-dependent vasodilatation in the mesenteric