Downregulation of cGMP-dependent protein kinase-1 activity in the corpus cavernosum smooth muscle of diabetic rabbits

Shaohua Chang, Joseph A. Hypolite, Marielena Velez, Arun Changolkar, Alan J. Wein, Samuel Chacko, and Michael E. DiSanto

1Division of Urology, 2Department of Pathobiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Submitted 31 October 2003; accepted in final form 10 June 2004

Downregulation of cGMP-dependent protein kinase-1 activity in the corpus cavernosum smooth muscle of diabetic rabbits.

First published June 17, 2004; 10.1152/ajpregu.00639.2003.—Increased guanosine 3',5'-cyclic monophosphate (cGMP), induced by nitric oxide release, is crucial for corpus cavernosum smooth muscle (CCSM) relaxation within the penis. This CCSM relaxation (necessary for penile erection) is impaired in men with erectile dysfunction (ED), especially those men with diabetes. One of the effector proteins for cGMP is cGMP-dependent protein kinase-1 (PKG-1). PKG-1 knockout mice exhibit detrusor overactivity (Am J Physiol Regul Integr Comp Physiol 279: R1112–R1120, 2000) and, more relevant to this study, ED (Proc Natl Acad Sci USA 97: 2349–2354, 2000), suggesting an in vivo role for PKG-1 in urogenital smooth muscle relaxation. In the current study, using normal rabbit CCSM, Western blot analysis revealed high expression of PKG-1 at levels almost equivalent to aorta (previously shown to have high PKG-1 expression) and that the two known alternatively spliced isoforms of PKG-1 (α and β) are expressed in nearly equal amounts in the CCSM. However, in response to alloxan-induced diabetes, there was a decrease in expression of both PKG-1 isoforms at the mRNA and protein levels as determined by real-time RT-PCR and Western blotting, respectively, but with the PKG-1α isoform expression decreased to a greater extent. Moreover, diabetes was associated with significantly decreased PKG-1 activity of CCSM in vitro, correlating with decreased CCSM relaxation. Immunofluorescence microscopy revealed a diabetes-associated decrease in PKG-1 in the CCSM cells. In conclusion, our results demonstrate for the first time a significant downregulation of PKG-1 expression associated with decreased PKG-1 activity in the CCSM in response to diabetes. Furthermore, these results suggest a mechanistic basis for the decreased efficacy of phosphodiesterase V inhibitors in treating diabetic patients with ED.

Penile tumescence (erection) and detumescence (return to flaccid state) are regulated by a complex neurophysiological process of relaxation and contraction, respectively, of corpus cavernosum smooth muscle (CCSM) (for a review, see Ref. 15). Failure of the CCSM to relax properly results in the inability of men to obtain an erection sufficient for sexual satisfaction and has been termed erectile dysfunction (ED). The incidence of ED throughout the world is expected to reach 322 million worldwide by the year 2025 (41).

Guanosine 3',5'-cyclic monophosphate (cGMP) is the direct intracellular mediator of the CCSM relaxation (60). In response to sexual stimuli, release of nitric oxide (NO) causes elevation of cGMP levels via stimulation of guanylate cyclase activity (34), which leads to relaxation of the CCSM and ultimately to penile erection (4). However, the exact molecular mechanism by which cGMP causes smooth muscle (SM) relaxation is not clear.

The recently developed orally active phosphodiesterase V (PDE5) inhibitors, which include sildenafil, vardenafil, and tadalafil, have exploited the fact that the cGMP produced in the CCSM can be broken down to GMP and that by blocking this degradation one can increase bioavailable cGMP and hence improve CCSM relaxation (necessary for erection). However, this class of drug is not effective for all men with ED with one of the largest groups of nonresponders being diabetic men. For example, Vickers and Satyanarayana (63) reported (based on a global efficacy question) a 56 vs. 84% efficacy of the PDE5 inhibitor sildenafil in diabetic compared with nondiabetic men with ED. Thus, in a significant portion of the male diabetic population with ED, maintaining cGMP levels is apparently not sufficient to improve CCSM relaxation, suggesting that some other cGMP-mediating mechanism may have been altered in the pathogenetic process.

One of the effector proteins that has been identified for cGMP is cGMP-dependent protein kinase-1 (PKG-1) (19). There are two PKG-1 isoforms, known as PKG-1α (76 kDa) and PKG-1β (78 kDa), and they are generated via alternative splicing of a single gene (20, 64). The genomic structure of human PKG-1 consists of 19 exons: the first two 5′-exons known as 1α and 1β are specific, respectively, for PKG-1α and PKG-1β, whereas the remaining 17 exons are common between the two isoforms (44). Unlike cAMP-dependent protein kinase (PKA) which exists as a tetramer, the PKG-1 holoenzyme exists as a dimer (56). Although the cGMP-binding sites and catalytic domains are very similar, some differences do exist between these two isoforms in their NH2-terminal autoinhibitory domains. In addition, PKG-1α has been shown to have a higher affinity for cGMP than the PKG-1β isoform (48). PKG-1α is highly expressed in the Purkinje cell of the cerebellum, in platelets, lung, and in SM cells, whereas the PKG-1β isoform is primarily expressed in SM cells (33).

Studies have shown that PKGs and cAMP-dependent kinases (PKAs) are highly homologous protein kinase families with similar substrate specificities, and phosphorylation of cellular proteins by both families of kinases leads to alterations in calcium mobilization, protein phosphatase activity, ion channel function, gene transcription, SM contractility, and platelet aggregation (for a review, see Ref. 19). However,
recent studies using recombinant overexpression (12) or knockout mice deficient in PKGs (46) have shown that cGMP kinases regulate very specifically distinct pathways that are separate from those regulated by PKAs.

The PKG-1 knockout mouse has also demonstrated the physiological significance of this enzyme in the lower urinary tract. These PKG-1-deficient mice completely lack NO-cGMP-dependent SM relaxation (46) and exhibit hyperactive voiding patterns (45) and, more relevant to this paper in particular, PKG-1 knockout mice show physiological signs of ED (23). More recently, Kotera et al. (37) reported data consistent with PKG-1 protecting cGMP from hydrolysis by PDE5, further demonstrating the importance of this enzyme to penile erection. Thus a decrease in the expression and/or activity of PKG-1 in response to diabetes could diminish the biological activity of cGMP making CCSM relaxation (necessary for erection) more difficult to achieve. The goals of the current study were to examine the expression, localization, and activity of PKG-1 in the CCSM from normal rabbits, determine whether any alterations in PKG-1 regulation occur in response to diabetes, and finally whether these changes correlate with alterations in the contractility of CCSM isolated from diabetic rabbits.

METHODS

Diabetic rabbit model. All studies involving animals were approved by the University of Pennsylvania Animal Use and Care Committee. Diabetes was induced by injection of alloxan (100 mg/kg body wt) into the ear vein of 12-wk-old male New Zealand White rabbits weighing ~6 lbs. The blood glucose levels were checked 1 wk after the alloxan injection (using a glucometer) and again before euthanasia after 6 mo. Only those rabbits that maintained blood glucose levels of 300 mg/dl or higher over the 6-mo period were used for this study. Age-matched normal rabbits served as controls.

Isolation of rabbit CC. The two CC were removed from both normal and diabetic rabbits and cleaned of the adjacent tissue as euthanasia after 6 mo. Only those rabbits that maintained blood body wt) into the ear vein of 12-wk-old male New Zealand White rabbits were equilibrated at Lo in Tyrode's buffer at 37 °C for 15 min to allow stabilization of the muscle at the resting level, and the optimal force development (Lo) was determined by increasing the concentration of phenylephrine (1.02 μM) to 125 mM KCl was achieved. After being washed three times, the force produced by CCSM at the basal level in physiological studies or immediately snap-frozen in liquid nitrogen and stored at -70 °C for subsequent mRNA and protein analyses. For in vitro enzymatic analyses, only corpora frozen for less than 3 days were used.

Force measurements. Strips of CC (~50 mg and 3 × 10 mm) were suspended longitudinally in 15 ml of Tyrode's buffer at 37 °C as previously described (7) and placed in organ baths containing Tyrode's buffer (124.9 mM NaCl, 2.5 mM KCl, 23.8 mM NaHCO3, 6 mM H2O 0.5 mM MgCl2, 0.4 mM NaH2PO4, 1.8 mM CaCl2, and 5.5 mM dextrose) equilibrated with 95% O2-5% CO2 at 37 °C for physiological studies or immediately snap-frozen in liquid nitrogen and stored at -70 °C for subsequent mRNA and protein analyses. For in vitro enzymatic analyses, only corpora frozen for less than 3 days were used.

Real-time PCR. Real-time PCR was performed using the Light Cycler (Roche, IN). Basically, a mastermix of the following reaction components was prepared to the indicated end-concentration: 1 μl of forward primer (0.4 μM), 1 μl of reverse primer (0.4 μM), 4 μl × 5 PCR buffer (BD Biosciences Clontech, Palo Alto, CA), 2 μl of dNTP (100 μM), 0.4 μl of DMSO, 2 μl of SYBR Green I (Sigma), 11 μl of water, and 0.6 μl of Titanium Taq DNA polymerase (BD Biosciences Clontech). Then, 19 μl of the mastermix were filled into the Light Cycler glass capillaries and 1 μl of cDNA (produced as described above) was added as the PCR template. Capillaries were closed, centrifuged, and placed into the Light Cycler rotor. The following experimental protocol was used: denaturation (95 °C for 30 s) followed by an amplification program repeated 20–35 cycles (95 °C for 5 s and then 68 °C for 20 s) using a single fluorescence measurement. Also, a melting curve program (60–95 °C with a heating rate of 0.1 °C/s with continuous fluorescence measurement) was run and finally a cooling step to 40 °C. The specificity of each PCR product was verified by the melting curve analysis and gel electrophoresis.

The expression of PKG-1 isoform was determined by constructing standard amplification curves using known amounts of purified PCR product.

Protein extraction and Western blot analysis. Frozen pieces of CC (~20–30 mg) were ground to a powder as described above. Next, total extractable protein was isolated and protein concentration was determined as previously described (7). Then, 20 μg of the extractable proteins contained in the supernatant fraction of each sample were separated by 12% SDS-PAGE on large-format (16 × 16 cm) gels and transferred to an Immobilon-P membrane (Millipore, Bedford, MA) overnight at 30 V (Bio-Rad mini-transfer unit) in buffer (25 mM Tris, 192 mM glycine, 2% methanol). After being blocked with 5% nonfat milk for 1 h in PBS containing 0.1% Tween 20 (PBST), the membrane was incubated with rabbit anti-cGMP-dependent protein kinase antibody (KAP-PK005: Stressgen Biotechnologies, Victoria, BC Canada) at 1:4,000 dilution in PBS for 2 h at room temperature. The membrane was then washed two times with PBST and further incu-
bated with secondary horseradish peroxidase-linked antibody (1:5,000 dilution of donkey anti-rabbit, NA934; Amersham Biosciences, Bucking-

hamshire, UK) for 1 h at room temperature. Membranes were then

washed five times with PBST and PKG-1 protein expression was
detected using an enhanced chemiluminescence kit (ECL) from Am-
ersham Biosciences. The amount of total PKG-1 was determined by

reflectance scanning densitometry as described under RNA extraction

and semiquantitative RT-PCR and expressed as optical density per

milligram of total extractable protein. All reactions were kept in the

linear range. Identical gels were run for all samples and stained with

Coomassie blue to confirm that the samples were not subject to

proteolytic breakdown and to confirm equal protein loading of gels.

cGMP-dependent protein kinase assay. In vitro activity of PKG

was measured using a modification of a previously described method

(25). Briefly, frozen tissue was ground to a powder as described above

and then homogenized at 50 mg tissue/ml of extraction buffer con-
taining 10 mM HEPES, 1 mM EDTA, 10 mM dithiothreitol, 1 mM

IBMX, 125 mM KCl, 0.8 mM PMSF, 1 mM antipain, 1 mM E64, and 0.1 mg/ml trypsin inhibitor (all from Sigma). The homogenate was then centrifuged at 30,000 g for 5 min and the supernatant was assayed for soluble PKG activity. Briefly, PKG activity was determined by measuring the transfer of the [γ-32P]ATP to the relatively specific substrate for PKG (ALISASGPAAPLA) from Bachem (Torrance, CA). The assay was carried out in a total volume of 70 μl containing 150 μM substrate, 10 mM HEPES, 35 mM β-glycerophosphate, 4 mM magnesium acetate, 200 μM [γ-32P]ATP (2.5 Ci/tube), 5 μM synthetic protein kinase A inhibitor, and 0.5 mM EDTA (all from Sigma except hot ATP from Amersham Biosciences, Piscataway, NJ). The reaction was stopped by spotting 50 μl of the reaction mixture onto phosphocellulose paper (Whatman P81, Whatman Biosciences, Clifton, NJ). The paper was then washed four times in 5 ml of 0.5% o-phosphoric acid for 10 min each and then transferred to a scintillation vial containing 10 ml of Cytoscint scintillation fluid (ICN, Irvine, CA). The protein concentra-
tion of each extract was determined using the Bradford protein

assay (3) and then PKG-specific activity was expressed as picomoles

de phosphate incorporated per minute per milligram of total protein.

The assay was carried out both in the presence and absence of 5 μM

exogenous cGMP (Sigma).

Effect of diabetes on CCSM contractility. The two CC were

isolated from both 6-mo diabetic and age-matched male New Zealand

White rabbits as described in METHODS. Strips of CC were

attached to a force transducer and the length of optimal force develop-

ment (Lₒ) was set as described in METHODS. In response to increasing concentrations of phenylephrine, CCSM from diabetic rabbits developed more force per gram of tissue compared to normal rabbits (Fig. 1). As can be seen in Fig. 2A, on average the CCSM from diabetic rabbits generated more force to each

<table>
<thead>
<tr>
<th>A: Normal</th>
<th>B: Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>EFS</td>
</tr>
<tr>
<td>SNP</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Force generation and relaxation of corpus cavernosum smooth muscle (CCSM) from normal and diabetic rabbits. CC strips from age-matched normal (A) and diabetic (B) rabbits were equilibrated with 95% O₂-5% CO₂ in Tyrode’s buffer at 37°C for 30 min and then length of optimal force development (Lₒ) was determined. After equilibration at Lₒ for an additional 15 min, the responses to increasing amounts of phenylephrine (PE; 1.02, 3.08, 9.25, 27.7, and 83.3 μM) were recorded. Next, pulses of electrical field stimulation (EFS; at frequencies of 1, 2, 4, 8, 16, 32, and 64 Hz) were applied and the relaxation responses were recorded. Finally, the precontracted CCSM was relaxed by the addition of increasing amounts of the nitric oxide donor sodium nitroprusside (SNP; 2.46, 7.40, 22.2, 66.6, and 200 μM). Top: traces are representative of 3–4 separate determinations performed on different pairs of normal and diabetic rabbits and the average results are shown in Fig. 2. One can see that the CCSM from the diabetic rabbit generated more force in response to all concentrations of PE and was more difficult to relax by both EFS as well as SNP. Weights and dimensions of the normal and diabetic CC strips shown at top were approximately the same.
concentration of phenylephrine with approximately twice as much force produced on average to the maximum 250-μM dose.

After reaching maximum force, pulses of EFS were applied to the precontracted CCSM. As can be seen in Fig. 1 and summarized in Fig. 2B, the CCSM from normal rabbits relaxed more efficiently to EFS than the CCSM from the diabetic rabbits, and at the highest frequency administered (64 Hz), the CCSM from normal rabbits relaxed on average by ~50%, whereas the CCSM from the diabetic rabbits only achieved ~15% relaxation. Similarly, the phenylephrine-precontracted CCSM from normal rabbits was relaxed more efficiently by the NO donor SNP than CCSM from the diabetic rabbits (Fig. 1). At the highest concentration of SNP (200 μM), the CCSM from the age-matched normal rabbits was relaxed from the phenylephrine-precontracted state on average by ~70%, whereas the CCSM from the 6-mo diabetic rabbits was relaxed by only ~40% (Fig. 2C). The CCSM from the diabetic rabbits at the resting state also produced more force in response to increasing concentrations of SNP.

Expression levels of PKG-1 in rabbit CCSM. To determine the relative expression of PKG-1 in rabbit CC, protein was extracted from rabbit CC, urinary bladder, aorta, and heart as well as from the NIH 3T3 cell fibroblast cell line, and 20 μg of total extractable protein were applied to a large-format SDS-PAGE gel and Western blot analysis for PKG-1 expression performed as described in Methods. As can be seen in Fig. 3, the normal CC expressed significant amounts of PKG-1 protein at levels almost equivalent to the rabbit aorta (n = 3). Also, the expression of PKG-1 in rabbit urinary bladder was almost as high as in CC. By comparison, it can be seen that the cardiac muscle in the heart expressed much lower amounts of PKG-1, whereas the NIH 3T3 cells expressed only trace amounts of PKG-1. Furthermore, because large-format SDS-PAGE gels were run, one can see in Fig. 3 that the PKG-1α and PKG-1β isoforms appear to be separable under these conditions. Thus it can be seen that the CC expressed both the larger PKG-1β isoform (~78 kDa) and that these two isoforms are expressed in near equal amounts in the urinary bladder and the aorta as well.

Effect of diabetes on PKG-1 mRNA expression. To determine the expression of PKG-1 at the mRNA level, three PCR primers were prepared as described in Methods. The upstream primers were designed to sequences specific to either PKG-1α or PKG-1β (exons 1α and 1β, respectively), whereas a common downstream primer was designed to a region identical between the two PKG-1 alternatively spliced isoforms. Semi-quantitative RT-PCR revealed that the expression of the higher cGMP affinity PKG-1α isoform was decreased while the expression of the internal control actin remained constant in both the CC and in the bladder (Fig. 4A). Four more pairs of CC from normal and 6-mo diabetic rabbits are shown in Fig. 4B confirming a selective reduction in PKG-1α mRNA expression. The decreased expression was further confirmed and quantitated using the same primers to perform real-time RT-PCR as described in Methods. As can be seen in Fig. 5A, the...
semiquantitative RT-PCR was performed in which expression of PKG-1 was signiﬁcantly decreased in the CCSM from diabetic rabbits compared with normal rabbits by RT-PCR. A: using a PCR primer pair in which the upstream primer was anchored in the PKG-1a-speciﬁc exon 1a as described in METHODS, semiquantitative RT-PCR was performed in which α-actin was ampliﬁed as an internal control. It can be seen in this gel that the expression of the higher internal α-actin control remained constant for both the CC and bladder. B: using the same strategy employed in A, 4 more pairs of normal and diabetic CC were analyzed by semiquantitative RT-PCR. Lanes N1–N4 are from normal CC and lanes D1–D4 are from diabetic CC. The α-actin cDNA was again ampliﬁed as an internal control.

expression of PKG-1α was signiﬁcantly lower in the CCSM from diabetic rabbits compared with the CCSM from normal rabbits as indicated by the rightward shift in the ﬂuorescence plot (indicating that it takes more cycles for the message to be ampliﬁed to similar levels as in the control). After determining the PCR efﬁciency for this product, it was determined that the CCSM from the diabetic rabbit expressed ~8.6-fold less PKG-1α mRNA than CCSM from age-matched normal rabbits. Similarly, real-time RT-PCR performed for the expression of PKG-1β (Fig. 5B) revealed that the expression of this isoform was also decreased but to a lesser extent (~2.5-fold) than the decrease in PKG-1α mRNA expression. Both the PKG-1α and PKG-1β PCR products were sequenced to conﬁrm their identity. Because the rabbit PKG-1β sequence had not been previously reported, the results from our sequencing of this cDNA will be deposited in the GenBank.

Effect of diabetes on PKG-1 protein expression. Western blot analysis using antibody speciﬁc to PKG-1 was performed to determine whether the decrease in PKG-1 expression observed at the mRNA level in the CCSM in response to diabetes is also observed at the protein level as well. As can be seen in Fig. 6A, the overall expression of PKG-1 was decreased on average by 24.5 ± 0.4% (n = 4 pairs). However, it was the α-isoform that was decreased more signiﬁcantly on average (~46.3 ± 4.6%) than the β-isoform (decreased by 7.4 ± 0.7%). A similar selective PKG-1α decrease (44 ± 7.2%) compared with PKG-1β (9.5 ± 2.1%) was also found to occur in the urinary bladder in response to 6 mo of diabetes (Fig. 6B; n = 4 pairs). Identical gels stained with Coomassie blue conﬁrmed equal protein loading and no signiﬁcant difference in the expression of SM myosin or actin (Fig. 6, C and D).

In vitro determination of CCSM PKG-1 activity. With the use of a synthetic peptide, the sequence of which (ALISASG-
PAAPLAO) was based on an in vivo target sequence for PKG-1, we determined the activity of PKG-1 in extracts of rabbit CCSM using an in vitro assay as described in METHODS. Under basal conditions (no exogenous cGMP added to the assay), the activity of PKG-1 was ~37% lower in the extracts prepared from the CCSM of diabetic rabbits (3.27 ± 0.82) compared with the activity in extracts from the CCSM of control rabbits (5.28 ± 0.61 pmol·min⁻¹·mg protein⁻¹; Fig. 7A). Similarly, in the presence of 5 μM exogenous cGMP, the activity of PKG-1 was ~42% lower in extracts prepared from the CCSM of diabetic rabbits (48.82 ± 12.6) compared with CCSM from control rabbits (82.53 ± 8.2 pmol·min⁻¹·mg protein⁻¹; Fig. 7B). It can also be seen in Fig. 7 that the activity of PKG-1 in the CCSM extracts (both from normal and diabetic rabbits) is increased ~14-fold in the presence of exogenous cGMP revealing a similar activity ratio in both animals. We also noted a similar increase in PKG-1 activity in response to exogenous cGMP in extracts from rabbit aorta, but the increase in extracts from urinary bladder smooth was only about twofold (data not shown).

**Immunohistochemical staining of PKG-1 in CC.** Because PKG-1 has been shown to exist in many different cell types, we performed double-labeling immunofluorescent microscopy to determine the cellular localization of PKG-1 in CC tissue sections from diabetic and age-matched normal rabbits as described in METHODS. As can be seen in Fig. 8, A and B, the CC sections from the diabetic rabbits stained much less intensely than those from the normal rabbits correlating with our RT-PCR and Western blotting results described above. Furthermore, one can see that decreased PKG-1 staining was evident in the CCSM cells (compare Fig. 8, A and B, stained for PKG-1 with Fig. 8, C and D, stained for α-actin). One can see in the merged images in Fig. 8, E and F, that the SM cells appear yellow reflecting the merged staining of the green α-actin and the red PKG-1 staining. Interestingly, in the merged images, one can also see that PKG-1 is expressed in the nuclear region of some SM cells as well (reflecting its known role, at least of PKG-1β, in modulating gene transcription), but because as expected the α-actin antibody does not stain the nucleus region, the nucleus appears red in the merged image. Immunofluorescence microscopy performed on CCSM sections but in which the second antibody was omitted showed that the second antibody (directed against rabbit IgG) does not cross-react directly with the rabbit tissue itself (data not shown).

**DISCUSSION**

The results of this study reveal a significant decrease in PKG-1 in the corpora cavernosa (the two long cylindrical rods of SM in the penis critical for erection) in response to 6 mo of...
alloxan-induced diabetes. Because cGMP is a direct intracellular mediator of CCSM relaxation (necessary for erection) and PKG-1 is considered one of the most important downstream targets and effector proteins for cGMP, this decreased PKG-1 expression may be expected to make erection more difficult to achieve. Indeed, the decreased PKG-1 expression we observed in diabetic male rabbits correlates with the decreased relaxation exhibited by CCSM from alloxan-induced diabetic rabbits reported in this study (Figs. 1 and 2) and by others (1, 59). Furthermore, the CCSM from these diabetic rabbits generated significantly more force in response to EFS (Fig. 2D), suggesting that the decreased PKG-1 expression may heighten the basal tone and sensitivity of the CCSM to contractile agents in diabetic rabbits.

Although Sullivan et al. (54) recently reported a trend toward increased NO-induced relaxation in 6-mo alloxan-induced diabetic rabbits, this contrasts with an earlier finding of impaired SNP-induced relaxation in 3- and 6-mo alloxan-induced diabetic rabbits by the same group in Thompson et al. (59). This difference may reflect the fact that gaseous NO was added directly in the former study as opposed to NO being generated via SNP along with the fact that the optimal length of the muscle (L.o) was not set. The approximate sixfold increase in IC50 for SNP-induced relaxation in diabetic rabbits reported in the latter study is similar to the decreased SNP-induced relaxation reported in our current study. The results of these two above studies are consistent in the fact that they both suggest diabetes-induced changes occurring above the level of cGMP. However, they do not rule out downstream changes. Although the authors suggest that guanylate cyclase expression is decreased in the diabetic rabbits (59), this hypothesis is only based on the observation that cGMP formation is decreased in diabetic corpus cavernosum, but yet in diabetic rats cGMP formation was actually found to be enhanced (42) and also a paper by Sullivan et al. (53) showed that nitroprusside-stimulated cGMP was unaffected in the cavernosal tissue of 3- and 6-mo diabetic rabbits. Thus the changes in PKG-1 activity reported in the current study could be involved in the mechanism of decreased contractility in the CCSM from diabetic rabbits.

Western blot analysis of various tissues and cells revealed expression of PKG-1 in the rabbit CCSM and urinary bladder at levels almost as high as in the aorta (Fig. 3). Because the aorta has been previously reported to have one of the highest PKG-1 expression levels compared with other tissues (33, 57), a physiological role for PKG-1 in the lower urogenital tract is suggested as has also been suggested by PKG-1 knockout mice studies (23, 45, 46). In contrast, the expression of PKG-1 in cardiac muscle was much lower as has been previously reported (33, 37). Expression of PKG-1 was barely detectable in the nonmuscle NIH 3T3 cell line correlating with its reported higher expression in contractile cell types (2, 28).

Our large-format SDS-PAGE gel system used in our Western blot analyses was able to separate the PKG-1α from the slightly larger alternatively spliced PKG-1β isoform as shown in Fig. 3. Performing Western blot analysis of these large gels,
using an antibody that recognizes a common region in the two PKG-1 isoforms (located in the COOH-terminal region) as described in METHODS, allowed us to make direct measurements of the PKG-1 isoform composition in particular tissues. Interestingly, as can be seen in Fig. 3, our Western analysis showed near equal expression of the two PKG-1 isoforms at the protein level for normal rabbit CCSM, urinary bladder, and aorta, although conflicting data exist in the literature. For example, using Northern blot analysis, the mRNA expression of PKG-1α has been shown to be much greater in human aorta (57) but much less in bovine aorta (33) than the alternatively spliced PKG-1β isoform. Also, with the use of Northern analysis, a separate study suggested a much higher expression of PKG-1β relative to PKG-1α in urinary bladder (44). However, these previous studies employed separate probes or antibodies to measure the individual isoform expression and thus, due to documented differences in antibody sensitivities or probe affinities (33), may not allow for direct comparisons in isoform expression as does our Western blot system. Biochemical purification of PKG-1 from bovine aorta has, however, suggested that the expression of PKG-1α and PKG-1β is nearly equivalent (Fig. 3) for rabbit aorta (64).

In response to 6 μmol of alloxan-induced diabetes, those rabbits that reached and maintained blood glucose levels greater than 300 mg/dl exhibited a decrease in the expression of PKG-1α at the mRNA level as determined by semi-quantitative RT-PCR (using α-actin as an internal control) and by real-time RT-PCR (Figs. 4 and 5). Although the PKG-1β mRNA expression (Fig. 5B) was also reduced in the diabetic rabbits, the reduction was much less than for PKG-1α. This selective reduction of PKG-1α was also evident at the protein level as well based on Western blotting (Fig. 6A). The fact that PKG-1α protein expression was also selectively reduced compared with PKG-1β in urinary bladder in response to diabetes (Fig. 6B) suggests a similar PKG-1 response to diabetes in these two urogenital SMs. Although there is a commercially available PKG-1α-specific antibody, it did not cross-react with our rabbit PKG-1α. However, the PKG-1β antibody (KAP-PK005: Stressgen Biotechnologies) did cross-react with our rabbit PKG-1β and showed only a slightly reduced (6.75 ± 1.21%) expression of PKG-1β in the CC from the diabetic compared with normal rabbits (supplemental data for this article may be found at http://ajpregu.physiology.org/cgi/content/full/00639.2003/DC1). Thus these results further support the observation made using the common PKG-1 antibody that the PKG-1α isoform is reduced most significantly.

A reduction in PKG-1α expression would be expected to have a greater physiological effect on the CCSM than a reduction in PKG-1β because PKG-1α has been shown to have a higher affinity constant \(K_\text{d} \approx 9.1 \text{ vs. } 20 \mu M \text{ (38)}\) for cGMP (33) as well as a lower half-maximal activation value \(K_\text{a} \text{ reported in bovine as } 0.1 \text{ vs. } 1.3 \mu M \text{ (48) and } 0.20 \text{ vs. } 0.44 \mu M \text{ (64) in 2 separate studies}.\) Also, COS-7 cell clones in which recombinant PKG-1α was overexpressed phosphorylated vasodilator-stimulated protein, which is a downstream target of PKG-1, to a higher level than recombinant COS-7 cells overexpressing PKG-1β (40). Moreover, PKG-1 cell-permeable activators that preferentially activate PKG-1α over PKG-1β have suggested that PKG-1α is the main mediator of SM relaxation in rat aorta (58, 61) and in pig coronary arteries (52).

Our in vitro determination of PKG-1 activity confirmed our hypothesis that the decreased PKG-1 expression (in particular, PKG-1α) would alter the enzymatic activity of PKG-1. Under basal conditions (with no addition of exogenous cGMP), PKG-1 enzymatic activity was decreased by –37% in response to diabetes (Fig. 7A). The roughly 5-pmol min⁻¹ mg protein⁻¹ specific activity in normal rabbit CCSM is slightly higher than the 4-pmol min⁻¹ mg protein⁻¹ specific activity previously reported for the rat aorta (25) and 3.9-pmol min⁻¹ mg protein⁻¹ specific activity reported for rat myometrium (24) using this same assay. Likewise, the ~14-fold increase in normal CCSM PKG-1 activity in response to 5 μM exogenous cGMP is similar to the roughly 10-fold increases reported for the rat aorta (25) and rat myometrium (24). However, the PKG activity in normal bladder extracts, although similar to basal activity in aorta and CCSM, was only increased by about twofold in response to 5 μM exogenous cGMP (data not shown), suggesting an altered PKG regulation compared with the more tonic-type CCSM and aortic SM. In the presence of exogenous cGMP, PKG-1 activity was again lower in CCSM from diabetic rabbits (~42%) compared with CCSM from normal rabbits. The substrate used for these studies was based on the sequence surrounding the phosphorylation site in bovine lung cGMP-binding cGMP-specific phosphodiesterase (13), which is a potent and relatively specific substrate for PKG compared with PKA (11). Nonetheless, a PKA-specific inhibitor was also included in the assay to prevent cross-phosphorylation by PKA. Thus diabetes is associated with a decrease in PKG-specific activity in the CCSM of diabetic rabbits compared with normal rabbits both in the presence and absence of exogenous cGMP, although the activity ratio remains constant.

Finally, although our results demonstrated that PKG-1 expression was decreased at the level of mRNA, protein, and functional in vitro activity, we sought to determine whether PKG-1 expression was decreased in the SM cells. Using an antibody that recognizes both PKG-1 isoforms (made to the common COOH-terminal region shared by the two PKG-1 alternatively spliced isoforms), we showed, using immunofluorescent microscopy, that the expression of PKG-1 was decreased in the SM bundles of the CCSM in response to diabetes (Fig. 8, A and B) while expression of α-actin in the CCSM cells remained constant (Fig. 8, C and D).

A decrease of PKG-1 in the CCSM would be predicted to modulate CCSM contractility through several different molecular mechanisms. First, PKG-1 has been shown to phosphorylate and inactivate RhoA (the activator of ROK) (50, 51). Thus decreased PKG-1 expression would be expected to increase ROK activity and thereby increase the phosphorylation of SM myosin phosphatase (SMMP) making it less active and favoring CCSM contraction. Second, the NH₂-terminal region of PKG-1 has been shown to bind to the COOH-terminal region of SMMP and stabilize it (55). Again, decreased PKG-1 expression would make the SMMP less active and favor CCSM contraction. PKG-1 has also been demonstrated to activate Ca-activated K channels in SM cells (47) that have been shown to cause hyperpolarization and inactivate voltage-gated Ca²⁺ channels and lead to decreased calcium levels and vasorelaxation. Thus decreased PKG-1 expression would again lead to increased tone and/or contractility through this pathway as well. PKG-1 can also phosphorylate and activate a number of other ion channels, pumps, and receptors and in doing so
regulate SM contractility (for a review, see Ref. 19). Finally, PKG-1 has been recently suggested to be involved in suppressing extracellular matrix protein expression (14). A decreased PKG-1 expression attenuating this effect would be expected to decrease CCSM contractility as well.

Interestingly, as can be seen in the merged double-stained sections in Fig. 8, E and F, although the PKG-1 and the α-actin colocalized in the cytoplasm of the SM cells, one can see that there is also a unique expression of PKG-1 (red) in the endothelial cells surrounding the CCSM bundles and in some of the other interstitial cell types. Also, it can be seen that there is a unique expression of PKG-1 in the nuclear region of some of the CCSM cells. This is not surprising because it has been reported that PKG-1β has been shown to exhibit effects on transcriptional activity (5, 21).

Our in vitro physiological determinations correlate well with our molecular findings of a decreased PKG-1 expression in response to diabetes. We observed decreased relaxation of phenylephrine-precontracted CCSM from diabetic rabbits in response to both EFS as well as SNP (Figs. 1 and 2). A decreased CCSM relaxation in response to diabetes induced by different mechanisms has been reported in a number of different species. In addition to the alloxan-induced diabetes studies described above, decreased CCSM relaxation has also been reported in streptozotocin-induced diabetic rats (27, 29, 30, 32) and recently in streptozotocin-induced diabetic mice (43). Although some studies reported no effect of diabetes on SNP-induced CCSM relaxation (22, 30, 65), the duration of diabetes in these studies was generally less than 8 wk. The duration of diabetes in our study was 6 mo and we found that shorter periods of diabetes have less of an effect on CCSM contractility (unpublished observation by DiSanto and Chacko). Also, unlike other shorter-term diabetes studies that did not find a change in the EFS-induced contraction of resting CCSM from diabetic compared with normal animals (31, 32), our study did find increased contractility (Fig. 2D).

An altered CCSM relaxation in humans with diabetes has also been reported as well. A decrease in autonomously mediated relaxation to EFS (36, 49) and endothelin-dependent relaxation (49) has been reported for CCSM from diabetic vs. nondiabetic men. Also, it has been shown that the adenosine triphosphate-sensitive K channel-subtype modulators pinacidil and levromakalim were less effective in relaxing CCSM from men with diabetes than CCSM from nondiabetic men (62). Similarly, increases in human CCSM contraction in response to diabetes have also been reported. Insulin-dependent diabetic men were shown to have a higher maximum rate constant for the onset of contraction to phenylephrine than nondiabetic men (10). However, two studies failed to find a difference in relaxation of CCSM from diabetic and nondiabetic men. In one study, SNP relaxation of norepinephrine-precontracted CCSM was not significantly different between diabetic and nondiabetic men, although there were only nine patients in each group and there was a trend toward the CCSM from diabetics being more difficult to relax (49). In a second study, it was found that there was no significant difference in the rate constant for relaxation of phenylephrine-precontracted CCSM between men with diabetes and nondiabetic men in response to the NO donor nitroglycerin (9). However, a recent study showed that the PDE5 inhibitor sildenafil augmented SNP-induced hypotension but not nitroglycerin-induced hypertension, suggesting a different mechanism of action of these two drugs in vascular SM.

To our knowledge, the only study that examined whether there was an alteration in PKG-1 expression in the CC of humans with ED was an immunohistochemical study performed by Klotz et al. (35) that examined a small group of 23 patients. The results of this study revealed no distinct differences in the distribution of PKG-1 between potent and impotent patients correlating with our alloxan-induced diabetic rabbit results presented in Fig. 8. Although the authors of this study note that there appeared to be no difference in PKG-1 immunoreactivity between potent and impotent patients, they also point out that their studies were not quantitative and suggest that more quantitative methods, as used in our current study, may reveal differences. Also, the above study only included two diabetic patients and the type, severity, or duration of the diabetes was not noted. Finally, the results of the above study differ sharply from our data and others (18, 26) in that PKG-1 immunoreactivity was found only in the CCSM cells.

Using the same 6-mo alloxan-induced rabbit model of diabetes described in this study, we previously found that diabetes is associated with an increase in both the CCSM sensitivity and force generation in response to endothelin-1 (ET-1), the mechanism of which involves an overexpression of both the ETα and ETβ receptors (6). In this same study, we further demonstrated an overexpression of the Rho-kinase-α (ROKα) isoform. Taken together, the increased sensitivity to ET and the overexpression of ROKα would be expected to increase the tone of the CCSM. Coupled with a decrease in the NO/cGMP-mediated pathway of CCSM relaxation reported in this paper, these molecular changes in the contractile pathways of CCSM may drive the CCSM into a more tightly contracted state, making it more difficult for PDE5 inhibitors to cause CCSM relaxation. Moreover, these molecular changes may at least partly explain the decreased efficacy of PDE5 inhibitors in diabetic men with impotence. Also, as it has been previously reported that the ability of cGMP to activate PKG-1 seems to decrease with the age of the CCSM cell (39), further studies need to be performed to determine if decreased PKG-1 expression/activity may be a common mechanism involved in the pathogenesis of organic ED. Finally, pharmacological or transgenic activation of PKG-1, alone or in combination with other treatments, may prove clinically useful in the treatment of ED.

**REFERENCES**


DECREASED PKG-1 ACTIVITY IN CCSM OF DIABETIC RABBITS


