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Inhibition of cGMP-Dependent Protein Kinase by the Cell-Permeable Peptide DT-2 Reveals a Novel Mechanism of Vasoregulation

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ABSTRACT

Cyclic GMP-dependent protein kinase (PKG) serves as an important physiological regulator of vascular reactivity and tone. However, available inhibitors of PKG have exhibited variable effects in intact tissue, hindering the elucidation of the functional role of PKG in blood vessels. In this study, we have determined the effects of our previously engineered potent and selective PKG Iα inhibitor DT-2 on basal and cGMP-stimulated purified recombinant PKG, and compared DT-2 with commonly used PKG inhibitors (8*R*,9*S*,11*S*)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7*b*,11*a*-trizadibenzo-(*a*,*g*)-cycloocta-(*c*,*d*,*e*)-trinden-1-one (KT-5823), Rp-8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS), and (β-phenyl-1,*N*2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-PET-cGMPS). As expected, all inhibitors reduced cGMP-stimulated PKG activity. However, only DT-2 decreased cGMP-independent or basal PKG activity, whereas KT5823 showed no effect and the Rp-compounds actually had partial agonist activity. To evaluate the potential functional impact of this unique inhibition by DT-2 under physiologically relevant conditions, we analyzed the inhibitors in isolated pressurized cerebral arteries. KT-5823 and Rp-8-pCPT-cGMPS demonstrated marginal reversal of vasodilation induced by 8-Br-cGMP. By comparison, DT-2 completely reversed 8-Br-cGMP induced dilations with comparable potency to Rp-8-Br-PET-cGMPS. In fact, DT-2 constricted arteries beyond their starting (pre-8-Br-cGMP) diameters and caused constriction even in the absence of exogenous 8-Br-cGMP, an effect that was not observed with any other inhibitor. The direct constricting effect of DT-2 was essentially abolished in cultured arteries, where PKG expression was reduced by approximately 90%. These findings indicate that DT-2 not only effectively inhibits cGMP-stimulated PKG activity but also reduces basal PKG activity both in vitro and in vivo. Moreover, these distinctive inhibitory properties of DT-2 suggest an important role for constitutive PKG activity in the continuous regulation of cerebral artery tone.

Type I cyclic GMP-dependent protein kinase (PKG I) mediates fundamental physiological functions of cGMP in vascular smooth muscle (for review, see Pfeifer et al., 1999; Lincoln et al., 2001; Schlossmann et al., 2003). Relaxation of smooth muscle induced by important endogenous vasodilators such as nitric oxide and natriuretic peptides is dependent on the activation of soluble and particulate guanylyl cyclases, respectively. The subsequent increase in intracellular cGMP leads to an activation of PKG by occupation of cGMP-binding sites on the enzyme. PKG modulates excitation-contraction coupling in smooth muscle by reducing intracellular Ca^{2+} levels (Wellman et al., 1996; Carrier et al., 1997; Feil et al., 2002) and promoting dephosphorylation of regulatory myosin light chains (Kawada et al., 1997; Wu et al., 1998). The physiological targets of the two splice variants PKG Iα and PKG Iβ, believed to be critical for smooth muscle relaxation, include large-conductance potassium channels (Carrier et al., 1997), the GTPase RhoA (Sauzeau et al., 2000), phospholipase C (Xia et al., 2001), inositol 1,4,5-

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ABBREVIATIONS: PKG, cGMP-dependent protein kinase; Rp-cGMPS, guanosine 3′,5′-monophosphorothioate, Rp isomer; DTT, DL-1,4-dithiothreitol; TLCK, L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone; TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone; SBTI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; CAPS, 3-[cyclohexylamino]-L-propanesulfonic acid; MHC, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PSS, physiological saline solution; 8-Br-cGMP, 8-bromo-cGMP; Rp-8-pCPT-cGMPS, 8-(*p*-chlorophenylthio)-guanosine 3′,5′-monophosphorothioate, Rp isomer; Rp-8-Br-PET-cGMPS, β-phenyl-1,N^2-etheno–guanosine 3′,5′-monophosphorothioate, Rp isomer.

trisphosphate receptor (Komalavilas and Lincoln, 1994), phospholamban (Koller et al., 2003), Inositol trisphosphate receptor-associated cGMP kinase substrate (IRAG; Schlossmann et al., 2000), and the myosin binding subunit of myosin phosphatase (Surks et al., 1999; MacDonald et al., 2000). PKG may also play a pivotal role in determining contractile and proliferative smooth muscle cell phenotypes (Boerth et al., 1997; Lincoln et al., 1998; Brophy et al., 2002).

The exact molecular mechanisms leading to PKG activation are not yet fully understood. The central dogma of PKG activation involves a cGMP-induced conformational change, unhinging of its auto-inhibitory site, and subsequent exposure of the catalytic domain (Francis and Corbin, 1994; Pfeifer et al., 1999). However, autophosphorylation of N-terminal serine and threonine residues also seems to modulate kinase activity in all three isozymes, types Iα, Iβ, and II (Hofmann and Flockerzi, 1983; Smith et al., 1996; Vaandrager et al., 2003). Thus, it seems that PKG might maintain some intrinsic basal or constitutive level of activity even in the absence of cGMP. However, potential physiological roles of this constitutive PKG activity are completely unknown. The present study provides the first evidence for a possible functional role of basal PKG activity.

For the elucidation of the functional roles of PKG in smooth muscle cells, selective and, in particular, cell-permeable inhibitors of this enzyme would be of great value. Unfortunately, available inhibitors of PKG have exhibited considerable variability in terms of selectivity, potency, efficacy, and permeability. A number of PKG inhibitors have been developed to aid in the dissection of PKG-dependent cellular pathways. cGMP analogs derived from the diastereomer Rp-cGMPS, intended to compete directly with cGMP at the regulatory domain of PKG (Schwede et al., 2000), are in wide use. Various modifications have been made to Rp-cGMPS in recent years to increase inhibitory potency, membrane permeability, resistance to degradation by cellular phosphodiesterases, and selectivity for PKG over other cellular kinases such as PKA (Butt et al., 1994, 1995). On the other hand, KT-5823, a staurosporine analog, inhibits PKG by competing directly with ATP at the catalytic domain. Unfortunately, inconsistent and disparate experimental results with Rp-cGMPS derivatives and KT-5823 have questioned the utility and reliability of these agents. These problems are often even more apparent in studies of living tissues or cells compared with in vitro analyses of kinase activity (Wyatt et al., 1991; Komalavilas and Lincoln, 1996; Burkhardt et al., 2000) and may complicate interpretation of functional studies. In recognition of these issues, we have recently devised approaches and strategies aimed at the development of new PKG inhibitors having higher potency and efficacy in vivo compared with existing compounds.

In previous studies, we described DT-2, a novel and competitive peptide inhibitor of PKG Iα that consists of two parts: 1) a substrate-competitive sequence identified for its tight binding of PKG and 2) a membrane translocation signal from the HIV-tat protein (Dostmann et al., 2000, 2002). DT-2 proved to be highly potent (K_i = 12.5 nM) against PKG-dependent phosphorylation and exhibited substantial selectivity (~1000-old) for PKG over PKA. In isolated blood vessels, DT-2 exhibited potent and selective inhibition of type I PKG, was internalized into vascular smooth muscle cells, and antagonized nitric oxide-mediated vasorelaxation. Inhibitory properties of DT-2 are distinct from other agents such as cGMP-competitive (Rp-cGMP enantiomers) and ATP-competitive (KT-5823) inhibitors in that DT-2 interferes directly with protein substrate-PKG interaction. This ability to directly block PKG binding and phosphorylation of cellular target proteins provides a comprehensive type of inhibition unique in the field of PKG inhibitors. Here we characterize the functional utility of this peptide, comparing its effects both in vitro and in intact arteries with those of the most potent and selective PKG inhibitors currently available. DT-2 not only demonstrates a very favorable profile of potency and efficacy versus other PKG inhibitors; also, because of its unique mechanism of kinase inhibition, it has revealed a key role for basal PKG activity in regulation of vascular tone.

Materials and Methods

Animal and Tissue Preparation. Male rats were euthanized with an injection of sodium pentobarbital (120 mg/kg, i.p.) and subsequent decapitation, in accordance with the University of Vermont Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Brains were removed and placed in cold (4°C) physiological saline solution (PSS) containing 119 mM NaCl, 4.7 mM KCl, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 0.03 mM EDTA, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$, and 10.6 mM glucose, pH 7.4. Cerebral arteries were dissected, gently stripped of connective tissue, and treated as indicated below.

Expression and Purification of Recombinant PKG Iα. PKG Iα was prepared as described previously (Dostmann et al., 1999) with the following modifications. Sf9 insect cells were grown in SF-900 II SFM medium containing 50 μg/μl gentamicin, 2% pluronic acid, 2% fetal bovine serum, and maintained in suspension at a replication rate of 18 to 24 h (110 rpm at 28°C). Maximal viability and optimal protein expression performance was achieved between passages 5 and 12. In general, 98% viability was confirmed using flow cytometry (Epics XL-MCL; Beckman Coulter, Fullerton, CA) and incubating cells in the presence of 2.5 μg/ml propidium iodide. In preparation for baculovirus infection, cells were diluted to a density of 8×10^5 cells/ml and grown overnight to a density of 1.2 to 1.5×10^6 cells/ml in SF-900 II SFM media containing 5% fetal bovine serum. Baculovirus stocks (Bac-to-Bac-system; Invitrogen, Carlsbad, CA) carrying the PKG Iα gene with typical titers of approximately 1.7×10^9 plaque-forming units/ml were obtained after three exhaustive 5-day amplifications. Cells were infected with virus stock to a final 1:20 ratio and incubated for 72 h. After centrifugation at 4000*g* for 10 min, the pellet was resuspended in 1 ml of cold buffer A (50 mM phosphate, pH 6.5, 10 mM DL-1,4-dithiothreitol (DTT), 5 mM EDTA, 5 mM EGTA, and 10 mM benzamidine) plus protease inhibitors [50 μg/ml L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone (TLCK), 100 μg/ml tosyl phenylalanyl chlormethyl ketone, 100 μg/ml soybean trypsin inhibitor (SBTI), 50 μg/ml antipain, and 170 μg/ml PMSF] per gram of wet cell pellets. The cells were lysed using a French Pressure cell at 1200 psi, diluted with ice-cold buffer A plus 50 μg/ml TLCK, 100 μg/ml L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone (TPCK), 100 μg/ml SBTI, 50 μg/ml antipain, and 170 μg/ml PMSF to 2 ml/g wet pellet and centrifuged at 25,000 rpm at 4°C for 30 min. The clear supernatant was loaded on a 2.5 ml of 8-(2-aminoethyl-)amino-cAMP-agarose column at 4°C and washed with 20 volumes buffer A plus 50 μg/ml TLCK, 100 μg/ml TPCK, 100 μg/ml SBTI, 170 μg/ml PMSF, followed by 20 volumes of buffer A plus 500 mM NaCl and 500 volumes of buffer A. PKG Iα was recovered from the column using a discontinuous and isocratic elution profile (2.5 ml per fraction) at room temperature using buffer A plus 50 μg/ml TLCK, 100 μg/ml TPCK, 100 μg/ml SBTI, 170 μg/ml PMSF, and 1 mM cAMP.

Peak fractions were pooled and dialyzed against a total volume of 10 liters of buffer B (50 mM phosphate, pH 6.8, 2 mM benzamidine, 1 mM EDTA, and 100 mM β-mercaptoethanol) changing the buffer 4 to 5 times over a period of 48 h. Additional dialysis for up to 5 days was performed to assure the complete removal of cAMP. SDS-PAGE, immunoblot analysis, and mass spectrometric analysis (MicroMass LCT/z-spray nanoflow) confirmed that the enzyme preparations were not degraded and virtually cyclic-nucleotide free. The typical yield for PKG preparations was 5 to 10 mg/l Sf9 cell suspension.

Inhibition of PKG by DT-2, KT-5823, and Rp-cGMPS analogs. We tested the ability of PKG inhibitors to suppress kinase activity of basal and cGMP-stimulated enzyme using our standard phosphotransferyl assay protocol (Dostmann et al., 1999) with the following modifications. A constant concentration of the PKG specific substrate W15 (TQAKRKKSLAMA; 16 μM) was incubated with buffer A (25 mM MES, pH 6.9, 5 mM NaCl, 0.5 mM magnesium acetate, 0.2 mM EDTA, 1 mg/ml bovine serum albumin, 10 mM dithiothreitol, 100 μM ATP, 50 to 60 μCi of [γ-^{32}P]ATP, specific activity 250–350 cpm/pmol) at 30°C. The phosphorylation reaction was started by adding PKG to a final concentration of 2 nM. Basal and maximal PKG-mediated reaction (2 min) velocities were determined with buffer A and 500 nM cGMP, respectively. The inhibitors were tested on basal and fully active PKG at concentrations 10-fold above their published K_i/IC_{50} values. Autophosphorylation was induced by incubating the enzyme for 30 min in the presence of 0.1 μM Mg-ATP and 1 μM cGMP or 100 μM cAMP at 30°C according to Chu et al. (1998). After extensive dialysis against buffer B plus 50 mM β-mercaptoethanol, kinase activity was analyzed as described above.

Western Analysis and Immunofluorescence. For Western blots, cerebral arteries were harvested from rat brains, frozen in liquid nitrogen and homogenized in lysis buffer containing 150 mM NaCl, 10 mM EDTA, 15 mM $MgCl_2$, 40 mM CAPS, 1 mM $NaVO_3$, 1 mM NaF, 2.5 mM urea, 1 mM DTT, 15 mM PMSF, and 0.2% deoxycholate (w/v), 0.2% SDS (w/v), 1% Nonidet P-40 (w/v), and 10% (w/v) glycerol. Lysis buffer also contained mammalian (1:100; Sigma) and bacterial (1:1000; Sigma) protease inhibitor cocktail. Samples were centrifuged, added to sample buffer containing 0.1 mM DTT, and heated (95°C) for 2 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked by 30 min of exposure to Tris-buffered saline containing 0.2% Tween 20 and 5% dry milk. Membranes were exposed to the following primary antibodies overnight at 4°C: polyclonal rabbit anti-PKGI (Calbiochem, San Diego, CA), monoclonal mouse anti-smooth muscle myosin heavy chain (MHC II) (Abcam Limited, Cambridgeshire, UK), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon Labs, Temecula, CA). Membranes were then exposed to the following fluorescence-labeled secondary antibodies in the dark at room temperature for 40 min: anti-rabbit Alexa Fluor 680 (Molecular Probes, Eugene, OR) for PKG and anti-mouse Alexa Fluor 800 (Rockland Immunochemicals, Gilbertsville, PA) for MHC II and GAPDH. Protein detection was carried out using an Odyssey Infrared Imaging System (LI-CORE Inc. Biosciences, Lincoln, NE) and associated software (excitation at 680 nm and emission at 750 nm for PKG; excitation at 780 nm and emission at 810 nm for MHC II and GAPDH).

For immunofluorescence staining, cerebral artery segments were fixed with 4% formaldehyde PBS for 15 min. Segments were then washed with fresh PBS, transferred to glass slides, permeabilized with 0.2% Triton X, and blocked with 1% bovine serum albumin PBS for 1 h. Arteries were then exposed to rabbit-anti PKGI (primary; see above) antibody overnight at 4°C and subsequently treated with fluorescent-labeled (Cy5) anti-rabbit (Molecular Probes, Eugene, OR) antibody for 1 h at room temperature. Arteries were washed with PBS and topped with coverslips. Immunofluorescence was detected with a Bio-Rad 2000 laser scanning confocal microscope (excitation at 650 nm, emission at 670 nm for Cy5). Identical settings (laser intensity, iris, and signal gain) were applied for assessment of fresh and cultured arteries.

Arterial Diameter Measurements. Posterior cerebral arteries (internal diameter, 100–200 μm) were isolated in ice-cold PSS and cannulated on glass pipettes within a glass-bottomed pressure myograph chamber. The chamber bath was continuously superfused with warmed (37°C), gassed (95% O_2/5% CO_2) PSS. Pipettes were connected to a PSS-containing reservoir, such that intravascular pressure could be increased and decreased by raising and lowering the reservoir. An in-line manometer allowed for continuous monitoring of pressure. Arterial diameters were measured using a video dimension analyzer (Living Systems, Inc., Burlington, VT). Arteries were tested for viability with 60 mM KCl (those attaining less than 30% constriction were discarded). For concentration-effect curves, vessels were pressurized to 80 mm Hg and allowed to develop myogenic tone. Arteries were dilated by approximately 75% with 8-Br-cGMP (50 μM) and subsequently exposed to various PKG inhibitors. Maximal (passive) arterial diameters were obtained via treatment with Ca^{2+}-free PSS containing EGTA (2 mM) and the Ca^{2+} channel blocker nisoldipine (10 μM).

Arterial Culture. Posterior cerebral arteries dissected from rat brains were placed in serum-free Dulbecco's modified Eagle's media with Ham's F-12 nutrient supplement containing penicillin (100 units/ml) and placed in an incubator at 37°C and 5% CO_2. Arteries were removed from media after 2 to 5 days and subjected to molecular and/or functional assessments as indicated.

Materials. [γ-^{32}P]ATP (4500 Ci/mmol) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Insect Sf9 cells, SF-900 II SFM media, gentamycin, pluronic acid, and the Bac-to-Bac expression system was from Invitrogen. 8-(2-Aminoethyl)amino-cAMP columns, cAMP, cGMP, and cGMP analogs were purchased from BioLog (Bremen, Germany). KT-5823 was obtained from Calbiochem (San Diego, CA). Propidium iodide was purchased from Serologicals Corp. (Norcross, GA). Phosphocellulose filters were from Whatman (Clifton, NJ). TLCK, TPCK, SBTI, PMSF, benzamidine, DTT, 1,4-bis[2-(5-phenyloxazolyl)]-benzyl, 2,5-diphenyloxazol, and all other chemicals were purchased from Sigma (St. Louis, MO).

Data Analysis. Data are given as mean ± S.E.M. For functional data, nonlinear regression curves were calculated using Prism software (GraphPad, San Diego, CA) and used to determine IC_{50} values. For group comparisons, data were subjected to one-way analysis of variance and, where appropriate, individual comparisons were made with a Newman-Keuls post test. Statistical significance is indicated where $p < 0.05$.

Results

Inhibition of basal and stimulated PKG activity. In our earlier work, we demonstrated that the novel peptide inhibitors DT-2 and DT-3 are potent and highly selective inhibitors of PKG type I (Dostmann et al., 2000). In addition, their cell-penetrating properties as fusion constructs with fragments derived from the *Drosophila melanogaster* Antennapedia protein (DT-3) and the HIV transactivator of transcription (tat) protein (DT-2), made them ideal tools to study the physiological roles of PKG in vivo (Dostmann et al., 2002). We now provide a direct comparative study of these inhibitors against other well-known and widely used PKG inhibitors. For kinase assays, recombinant PKG Iα was purified from insect Sf9 cells using the baculovirus expression system (Feil et al., 1993; Dostmann et al., 1999). Special care was taken to prevent the formation of the tryptic fragment Δ1–77, which exhibits constitutive kinase activity (Heil et al., 1987). In addition, all protein preparations were subjected to an extensive dialysis protocol to obtain approximately 90% cyclic nucleotide free enzymes (only cAMP was detected; see *Materials and Methods*). Nanoflow-electrospray-ionization mass spectrometry confirmed purity, low N-terminal auto-

phosphorylation, and minor cyclic nucleotide levels (data not shown). Figure 1 compares the reaction velocities of basal and fully activated PKG Iα using the substrate TQAKRKK-SLAMA (Dostmann et al., 1999). Responses were obtained in the presence and absence of PKG inhibitors. In the presence of saturating levels of cGMP (500 nM), all inhibitors (at concentrations 10-fold above their reported IC_{50}/K_i values) reduced PKG Iα activity, albeit to very different levels. KT-5823 (5 μM) had only a marginal inhibitory effect, whereas the Rp-cGMPS analogs inhibited the kinase significantly although not completely. 5 μM Rp-8-pCPT-cGMPS and 350 nM Rp-8-Br-PET-cGMPS caused reductions in V_{max} for the active kinase from 9.52 $\pm$ 0.48 to 4.66 $\pm$ 0.16 μmol/min/mg ($p < 0.001$) and 3.79 $\pm$ 0.27 μmol/min/mg ($p < 0.001$), respectively. In contrast, DT-2 had a much greater inhibitory effect, reducing cGMP-activated kinase activity below basal levels to 0.66 $\pm$ 0.06 μmol/min/mg ($p < 0.001$). In the absence of cGMP, PKG Iα showed an intrinsic kinase activity of 1.23 $\pm$ 0.09 μmol/min/mg, which again was significantly reduced by DT-2 to 0.32 $\pm$ 0.03 μmol/min/mg ($p < 0.01$). It should be noted that basal activity could be raised to 2.5 $\pm$ 0.2 μmol/min/mg, and again inhibited with DT-2, when the enzyme was subjected to autophosphorylation in the presence of cGMP/cAMP and Mg/ATP to mimic in vivo conditions (data not shown) (Chu et al., 1998). In separate experiments, KT5823 showed no effect on basal PKG activity, whereas the cyclic nucleotide antagonist Rp-8-pCPT-cGMPS actually activated the kinase with V_{max} values of 2.47 $\pm$ 0.39 μmol/min/mg ($p < 0.05$). Rp-8-Br-PET-cGMPS showed a similar tendency to increase PKG activity to 2.16 $\pm$ 0.41 μmol/min/mg although this trend did not reach statistical significance. The results from Fig. 1 demonstrate that only DT-2 has the capacity to effectively inhibit PKG Iα to levels well below the enzyme's resting activity.

Reversal of 8-Br-cGMP dependent vasodilations in cerebral arteries. To extend our evaluation to the whole-tissue level, we compared the effects of DT-2 with other PKG inhibitors in intact resistance arteries (Fig. 2). Posterior cerebral artery segments (internal diameter, 150–200 μm) were constricted by increasing intravascular pressure to 80 mm Hg (inducing myogenic tone; Davis et al., 1999) and were subsequently dilated by approximately 75% with the cGMP analog 8-Br-cGMP. These experiments were conducted in the presence of 10 μM 1H-[1,2,4]oxadiazolo[4,3a]-quinoxaline-1-one to prevent endogenous cGMP production. The ATP-competitive inhibitor KT-5823 (Fig. 2A) had a modest effect on 8-Br-cGMP–dilated vessels, producing a maximal reversal of 24 $\pm$ 11% and an IC_{50} of 7.2 $\pm$ 2.5 μM, $n = 5$. The cGMP-analog inhibitor Rp-8-pCPT-cGMPS (Fig. 2B) elicited moderate reversal of 8-Br-cGMP dilation (maximum, 54 $\pm$ 14%, $n = 5$) at higher concentrations (>30 μM) but exhibited relatively poor inhibition overall (IC_{50} = 27.4 $\pm$ 2.2 μM). In fact, Rp-8-pCPT-cGMPS often enhanced vasodilation at concentrations less than 50 μM. We employed another cGMP-competitive inhibitor, Rp-8-Br-PET-cGMPS (Fig. 2C), which is currently considered the most permeable, selective, and po-

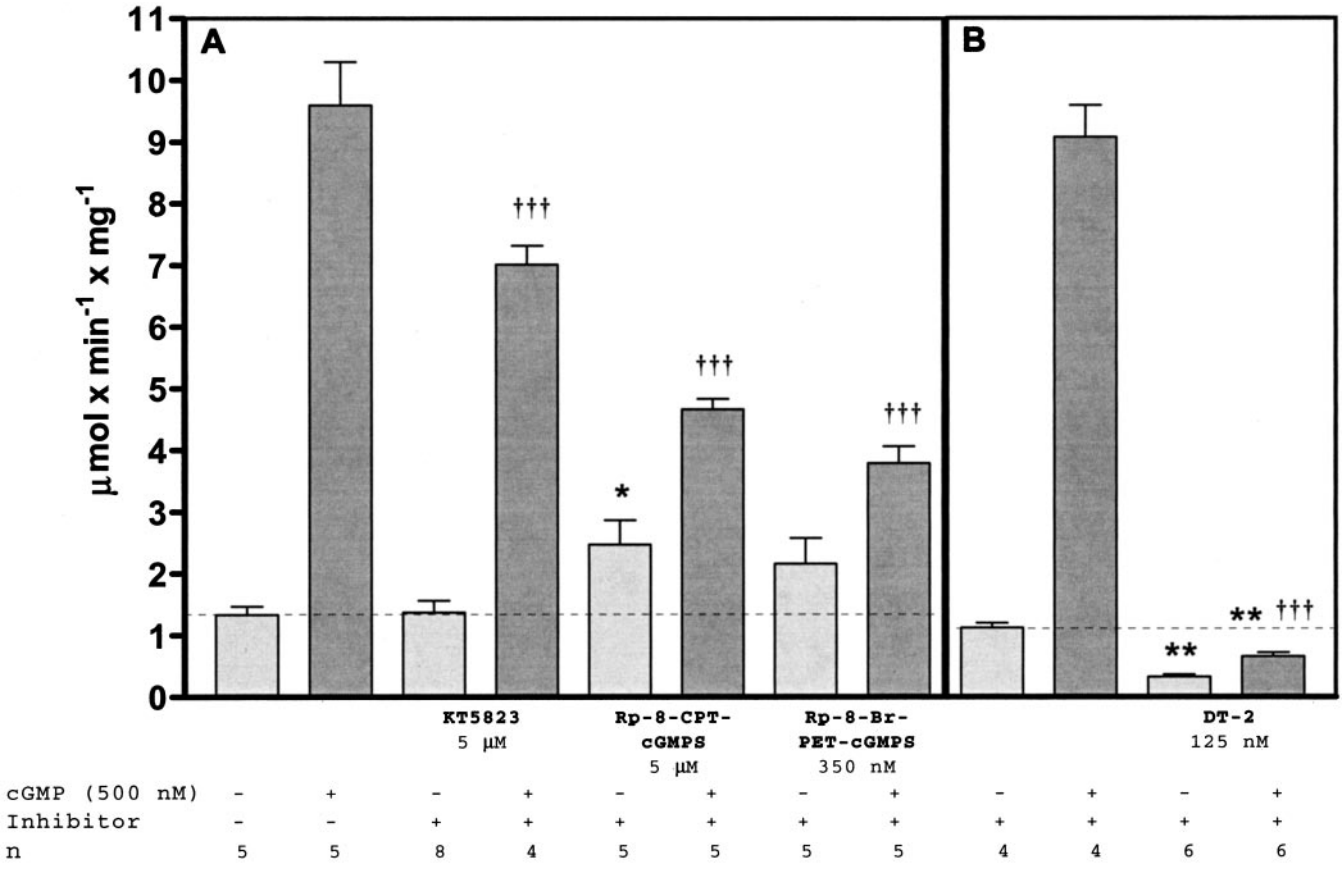

Fig. 1. Basal and stimulated PKG activity. Inhibition of PKG Iα by KT-5823, Rp-8-pCPT-cGMPS, and Rp-8-Br-PET-cGMPS (A) and DT-2 (B) was assayed in the absence and presence of cGMP as described under *Materials and Methods*. Dashed lines indicate basal levels of PKG activity (activity in the absence of cGMP) for control experiments. *, $P < 0.05$; **, $P < 0.01$ versus (−cGMP) control; †††, $P < 0.001$ versus (+cGMP) control.

tent inhibitor available. Rp-8-Br-PET-cGMPS substantially and potently (IC_{50} = 1.3 ± 0.2 μM, n = 6) reversed 8-Br-cGMP dilations, although the net recovery of tone was incomplete (maximal of 72 ± 3%). DT-2, on the other hand, completely reversed 8-Br-cGMP dilations with considerable potency (IC_{50} = 3.7 ± 0.4 μM, n = 15; Fig. 2D). In fact, at concentrations above 3 μM, DT-2 consistently decreased arterial diameter beyond its starting (pre-8-Br-cGMP) point (maximum, 152 ± 13% reversal), indicating a recovery of tone in excess of the cGMP-mediated dilation. Arteries were similarly treated with either the cell penetrating peptide (DT-6; YGRKKRRQRRRPP) or the nonpermeable PKG inhibitor (W45; LRK_5H) to serve as controls for DT-2. Figure 3 shows concentration-effect curves for all the inhibitors and peptides tested. Maximal reversal and IC_{50} values are summarized in Table 1. Overall, Rp-8-Br-PET-cGMPS was slightly but significantly more potent than DT-2 against 8-Br-cGMP dilations whereas DT-2 elicited significantly greater net recovery of tone (152 ± 13% versus 72 ± 3%, $p <$ 0.01). Importantly, the control peptide W-45 (10 μM) had no effect and DT-6 (10 μM) had only a modest effect on 8-Br-cGMP-dilated arteries (Fig. 3 and Table 1).

PKG Expression and DT-2–Induced Constriction. Based on our initial in vitro observation of cGMP-independent PKG activity (Fig. 1), we hypothesized that basal PKG activity may contribute to continuous modulation of vascular tone. Consequently, short-term DT-2–induced PKG inhibition might favor constriction. Therefore, we tested the direct effects of DT-2 on arterial tone in the absence of cGMP and the presence of the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3*a*]-quinoxaline-1-one to minimize the influence of endogenous cGMP. In fresh pressurized cerebral arteries, 10 μM DT-2 caused substantial constriction, suggesting a possible contribution of constitutive PKG activity to the regulation of myogenic tone (Fig. 4A). This constriction was 148 ± 13% (n = 5) of the constriction elicited by direct smooth muscle depolarization using 60 mM KCl. We next hypothesized that if the constrictor effect of DT-2 were in fact dependent on inhibition of basal PKG activity, then its effect would be markedly decreased with reduced PKG expression. Detection of PKG by Western analysis using a PKG Iα/Iβ antibody (Keilbach et al., 1991) (Fig. 4B) revealed that arteries cultured in serum-free media for 5 days reduced PKG expression by 87 ± 3%. Expression of neither GAPDH nor smooth muscle myosin heavy chain type II was changed during the culture period. Immunostaining confirmed considerable down-regulation of PKG in smooth muscle cells of cultured arteries (Fig. 4B). Although the contractile responses to increasing pressure and to KCl were preserved in cultured arteries, 10 μM DT-2 elicited little or no constriction (3 ± 1% of 60 mM KCl, n = 5; Fig. 4C). This finding is summarized in Fig. 4D and clearly indicates a direct dependence of DT-2 constrictor effects on PKG.

Discussion

The actions of PKG may be extremely important for the regulation of vascular smooth muscle contractility and hence maintenance of blood pressure and flow. Although various inhibitors have been employed over the past 2 decades to specifically study PKG, a number of problems associated with stability, cell permeability, potency, efficacy, and selectivity have limited their use as discriminating tools. Nevertheless, much of our current understanding of the role of

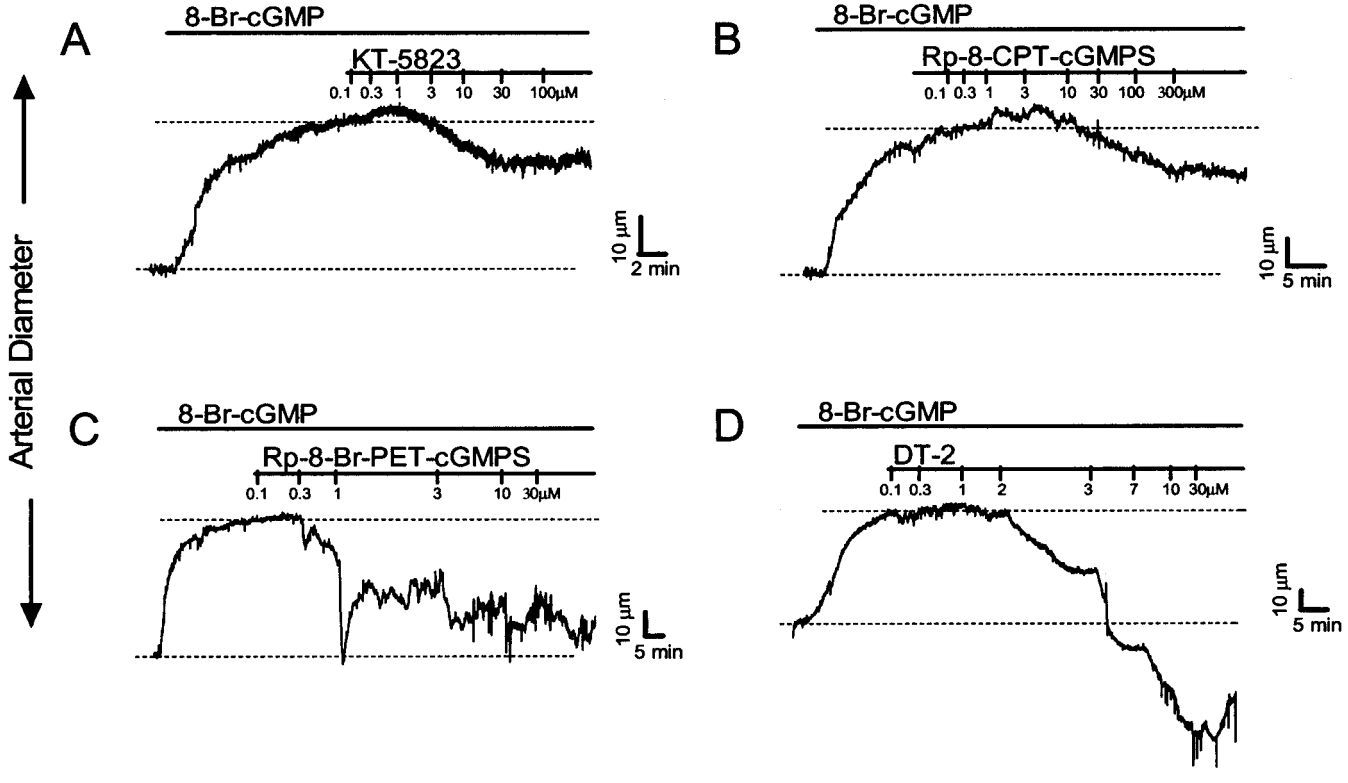

Fig. 2. Effects of PKG inhibitors on 8-Br-cGMP–induced dilations in pressurized cerebral arteries. Arteries were pressurized to 80 mm Hg, dilated with 50 μM 8-Br-cGMP, and exposed to increasing concentrations of KT-5823 (A), Rp-8-pCPT-cGMPS (B), Rp-8-Br-PET-cGMPS (C), and DT-2 (D). For each trace, dashed lines indicate resting diameters at 80 mm Hg pressure (bottom) and diameters after 8-Br-cGMP exposure (top).

PKG in vascular smooth muscle signaling has been inferred from the effects of these agents. We recently described DT-2, a novel competitive inhibitor of PKG, consisting of a library-derived PKG Iα inhibitor peptide (W45) coupled to the tat(43–58) membrane translocation peptide (Dostmann et al., 2000). Although the mechanism of translocation is not entirely clear, we showed that DT-2 readily internalized into intact cerebral artery smooth muscle cells. Moreover, DT-2 antagonized NO-mediated vasodilations but did not inhibit PKA-dependent responses (Dostmann et al., 2002).

In the present study, we compared DT-2 with other commonly used compounds that are considered the most potent and effective PKG inhibitors currently available. In establishing a reliable protein kinase activity assay system for this comparative analysis, we found that purified recombinant PKG exhibited significant activity even in the absence of cGMP. As expected, addition of cGMP considerably enhanced PKG activity well above this basal level. The ATP-competitive inhibitor KT-5823 had no effect on basal kinase activity and only marginally reduced cGMP-stimulated activity. These limited effects on PKG activity were reflected in the minimal influence of KT-5823 on 8-Br-cGMP dilations in functional experiments. This ineffectiveness of KT-5823 may reflect inconsistent cell permeability and/or stability and suggests that negative results with KT-5823 in intact tissues may reflect poor inhibition rather than noninvolvement of PKG (Fouty et al., 1998; Shimoda et al., 2002). The Rp-cGMPS derivatives Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS demonstrated partial inhibition of cGMP-stimulated PKG activity, with Rp-8-Br-PET-cGMPS proving to be more effective. These effects were likewise mimicked by the functional data, in which Rp-8-Br-PET-cGMPS more potently and effectively reversed 8-Br-cGMP mediated vasodilations. Interestingly, the Rp-cGMPS compounds, particularly Rp-8-pCPT-cGMPS, actually tended to increase basal PKG activity (see Fig. 1). This finding was unexpected and suggests a dual role for Rp-cGMPS derivatives as partial agonists. Indeed, in functional experiments, Rp-8-pCPT-cGMPS augmented vasodilation at concentrations less than 50 μM, and similar small dilations to Rp-8-Br-PET-cGMPS were observed in the absence of 8-Br-cGMP (data not shown). Concentration dependent partial agonistic properties of the related Rp-cAMPS analogs have recently been reported (Gjertsen et al., 1995). Such partial activation of PKG by Rp-cGMP analogs implies that complete inhibition of PKG is probably unattainable with these agents. Certainly, caution should be used when interpreting effects of KT-5823 and Rp-cGMPS inhibitors, especially because application of these compounds is commonly used as the sole method for discerning PKG-dependent mechanisms in cells and intact tissues.

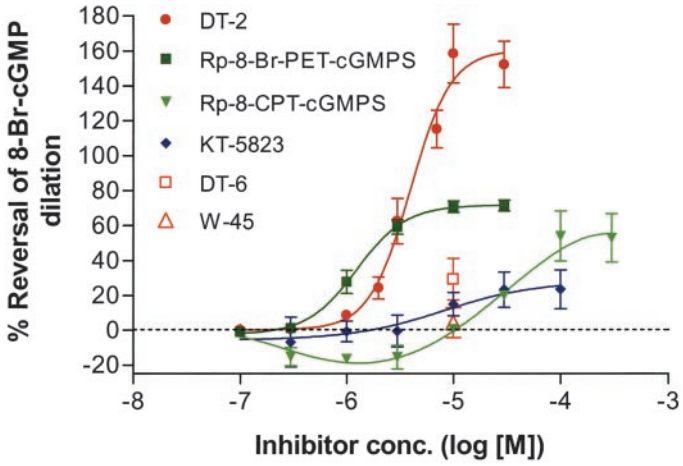

Fig. 3. Concentration-effect curves for PKG inhibitors against 8-Br-cGMP–induced dilations. Plot shows percentage reversal of 8-Br-cGMP dilations in cerebral arteries for all inhibitors tested. Values below the dashed line indicate additional dilation. Means ± S.E.M. and nonlinear regression curves are shown for each inhibitor. Open symbols indicate mean responses to control peptides W45 and DT-6.

TABLE 1

Relative potencies and efficacies of various PKG inhibitors on 8-Br-cGMP–induced vasodilation

Inhibitor	IC_{50}	Maximum Reversal
	μM	%
KT-5823	7.2 ± 2.5	24 ± 11 (n= 5)
Rp-8-CPT-cGMPS	27.4 ± 2.2	54 ± 14 (n= 5)
Rp-8-Br-PET-cGMPS	1.3 ± 0.2	72 ± 3 (n= 6)
DT-2 (YGRKKRRQRRRPP-LRK_5H)	3.7 ± 0.4	152 ± 13 (n=15)
DT-6 (YGRKKRRQRRRPP)		29 ± 12 (n= 7)
W45 (LRK_5H)		4 ± 8 (n= 4)

In our kinase assay, DT-2 exhibited powerful inhibition of PKG. This inhibition was distinct from other inhibitors in that it included reductions in basal as well as cGMP-stimulated kinase activity. Unlike other inhibitors tested, DT-2 always depressed PKG activity significantly below basal levels, regardless of whether the kinase was exposed to cGMP. Correspondingly, in functional experiments, DT-2 completely reversed vasodilations to 8-Br-cGMP, actually constricting arteries beyond their starting (pre-8-Br-cGMP) diameters. Importantly, the combinatorial library derived PKG inhibitory sequence of DT-2, W-45, alone had no significant influence on cerebral artery tone, whereas the membrane translocation signal part of DT-2, DT-6, alone had only modest effects (approximately 25% reversal) on 8-Br-cGMP–mediated dilations. This is consistent with our previous findings (Dostmann et al., 2000) showing that W45 alone is not internalized into cerebral artery smooth muscle cells and that DT-6 alone, although readily internalized, exhibits relatively weak PKG inhibition. Together, these data indicate that the merging of the two sequences is necessary to provide intracellular delivery and effective inhibition of PKG. Even in the absence of exogenous cGMP, DT-2 caused a significant increase in resting tone, consistent with reductions in basal or constitutive PKG activity in arterial myocytes. Overall, our results indicate that DT-2 elicits vasoconstriction through comprehensive inhibition of PKG activity (i.e., both basal and stimulated), and suggest a key role for constitutive PKG activity in continuous regulation of vascular tone (Fig. 5). Indeed, this proposal is consistent with the recently observed hypertensive effect of PKG I ablation in mice (Pfeifer et al., 1998).

In culture, arterial myocytes lose PKG expression (Boerth et al., 1997). Similarly, in our study, culture of intact cerebral arteries for 5 days decreased PKG expression by approximately 90%. Loss of PKG expression in cultured myocytes has been linked to down-regulation of contractile proteins and the transition of these cells to a noncontractile phenotype (Boerth et al., 1997; Brophy et al., 2002). Although arterial tone was somewhat reduced in our 5-day cultured arteries compared with fresh arteries, contractility was essentially preserved (as indicated by responses to KCl and

increasing pressure), suggesting that at least in intact vessels, this period of culture does not significantly alter phenotype. Indeed, we observed only very slight reductions in MHCII expression (see Fig. 4) after 5 days in culture. Certainly, the expression of contractile proteins other than MHC may in fact decline after 5 days in culture, contributing to reduced contractility. However, down-regulation of PKG would presumably concomitantly reduce the modulation of tone afforded by constitutive activity of the kinase, making the net effect of culture rather difficult to predict. In any case, given

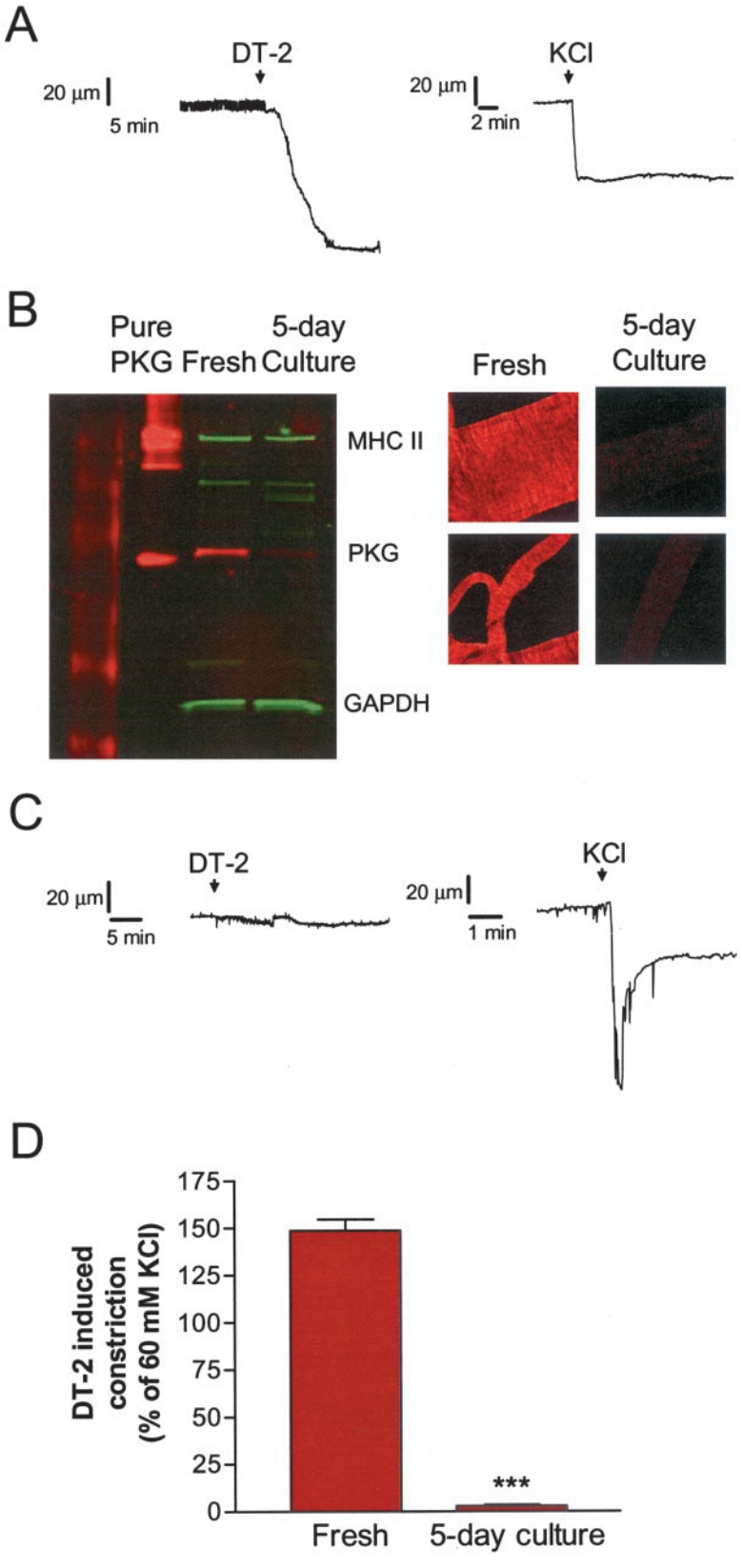

Fig. 4. Dependence of DT-2–induced constriction on PKG expression. A, fresh cerebral arteries were pressurized to 80 mm Hg and exposed to 7 μM DT-2. Responses to 60 mM KCl were recorded in the same arteries. B. Western blots (left) show substantial down-regulation of PKG I protein expression in cerebral artery lysates after 5 days in serum-free culture compared with fresh arteries. Expression of the housekeeping protein GAPDH and the contractile protein myosin heavy chain II (MHC II) were also monitored. Immunohistochemistry (right) reveals PKGI-positive staining in smooth muscle of intact cerebral arteries, which is considerably decreased after 5 days in culture. C, constrictor responses to DT-2 were greatly reduced in 5-day cultured arteries although responses to KCl were preserved. D, the bar graph summarizes DT-2 mediated constrictions relative to 60 mM KCl constrictions in fresh and cultured arteries (***. $p < 0.001$ versus fresh).

that cultured arteries exhibited down-regulation of PKG, with stable tone, this approach allowed us to assess whether the constrictor effect of DT-2 was related to PKG or to some other mechanism. Under conditions of low smooth muscle PKG expression, DT-2–induced constriction was essentially abolished, indicating that the constrictor effect of DT-2 is largely if not entirely dependent on PKG inhibition.

In our study, we have detected PKG activity in the absence of cGMP. The molecular nature of intrinsic PKG activity is still poorly understood, partly because in the absence of a crystal structure, molecular models fail to describe the interplay of N-terminal autophosphorylation and cGMP-binding in modulating kinase activity. Recent studies have shown that autophosphorylation and cGMP-binding mediate PKG stimulation and that both events induce similar conformational changes necessary for kinase activity (Chu et al., 1998; Busch et al., 2002; Wall et al., 2003). Our N-terminal phosphate-free and 90% cyclic nucleotide-free PKG Iα preparations have never been exposed to cGMP and Mg/ATP; thus, the enzyme was kept in the lowest possible basal state. We determined basal activities of approximately 1.2 and 2.5 μmol/min/mg for nonautophosphorylated and autophosphorylated kinase preparations, respectively. Given the maximal reaction velocity of approximately 10 μmol/min/mg, the high degree of relative cGMP stimulation of PKG activity (4 to 8 fold) usually measured from mammalian and recombinant sources, may reflect different autophosphorylation levels. Irrespective of the state of autophosphorylation, the conditions used in our experiments shown in Fig. 1 represent the most stringent conditions possible to assess a possible functional role of basal PKG activity. In future studies, we aim to investigate the degree of PKG autophosphorylation in smooth muscle of intact blood vessels as well as its contribution to vascular function.

Basal or constitutive PKG activity may be particularly critical in vascular smooth muscle, where multiple cellular targets that regulate excitation-contraction coupling are known to be modulated by PKG dependent phosphorylations. Short-term regulation of these proteins is known to decrease cellular Ca^{2+} concentration and reduce the Ca^{2+} sensitivity of the contractile apparatus, leading to depressed contractility. Our findings suggest that even without short-term stimulation by cGMP, a permissive level of PKG-I dependent phosphorylation of one or more of these targets is sufficient to provide a constant brake on contraction, opposing vascular tone.

To our knowledge, a vasoregulatory role for basal PKG activity has not been previously reported. Schubert et al. (1999) have suggested that tonic activity of PKG does not contribute to continuous regulation of rat cerebral artery tone based on the lack of effect of Rp-8-Br-PET-cGMPS on arterial tone under resting conditions. However, as our data indicate, Rp-8-Br-PET-cGMPS inhibits only cGMP-stimulated activity, not basal PKG activity, and is therefore not an effective tool for discerning the vascular influence of PKG at very low cellular cGMP levels. The current study demonstrates that the novel competitive peptide inhibitor DT-2 is a unique and comprehensive tool for the study of PKG in intact tissues and suggests a potentially important role for basal PKG activity in the regulation vascular contractility. Furthermore, our findings indicate that development and refinement of this new class of peptide inhibitors is warranted.

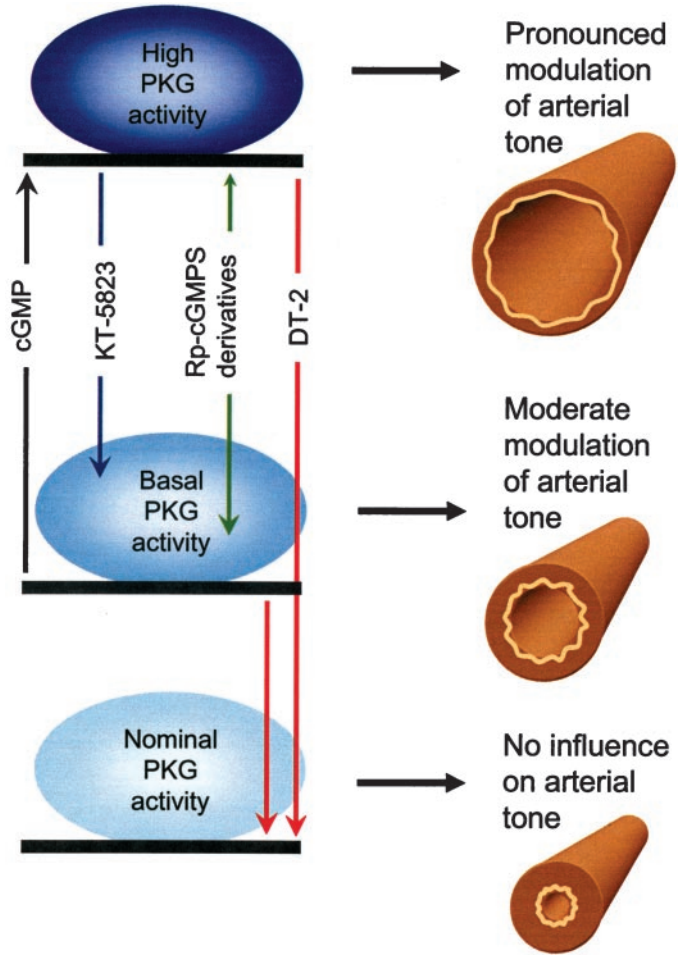

Fig. 5. Model of vascular smooth muscle PKG activity and its impact on vascular tone. We propose that constitutive activity of PKG (Basal PKG Activity) exerts continuous modulation of vascular tone even at minimal cGMP levels. Increased cGMP further stimulates the kinase (High PKG Activity), enhancing its modulatory influence. Available inhibitors, including KT-5823 (blue arrow) and the Rp-cGMPS derivatives (green arrow), such as Rp-8-pCPT-cGMPS and Rp-8-Br-PET-cGMPS, reverse cGMP-stimulated PKG activity to varying degrees, but only toward basal levels. Rp-cGMPS derivatives may also yield partial kinase stimulation as indicated by the double-headed arrow. DT-2 (red arrow), on the other hand, inhibits not only the cGMP-stimulated PKG activity but also a substantial portion of the basal PKG activity, essentially abolishing vasoregulation by the kinase.

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