cGMP-dependent protein kinase protects cGMP from hydrolysis by phosphodiesterase-5

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The physiological effects of cGMP are largely determined by the activities of intracellular receptors, including cGMP-dependent protein kinase (PKG) and cGMP-binding cyclic nucleotide phosphodiesterases (PDEs), and the distribution of cGMP among these receptors dictates activity of the signalling pathway. In the present study, the effects of PKG-I α or PKG-I β on the rate of cGMP hydrolysis by the isolated PDE5 catalytic domain were examined. PKG-I α strongly inhibited cGMP hydrolysis with an IC₅₀ value of 217 nM, which is similar to the physiological concentration of PKG in pig coronary artery reported previously. By contrast, PKG-I β , which has lower affinity for cGMP than does PKG-I α , inhibited cGMP hydrolysis with an IC₅₀ of approx. 1 μ M. Inhibition by PKG-I α was more effective than that by PKG-I β , consistent with their relative affinities for cGMP. Autophosphorylation of PKGs increased their cGMP-binding

affinities and their inhibitory effects on PDE5 hydrolysis of cGMP. Autophosphorylation of PKG-I β increased its inhibitory potency on PDE5 hydrolysis of cGMP by 10-fold compared with a 2-fold increase upon autophosphorylation of PKG-I α . The results indicate that cGMP bound to allosteric cGMP-binding sites of PKG is protected from hydrolysis by PDE5 and that persistent protection of cGMP by either non-phosphorylated or autophosphorylated PKGs may be a positive-feedback control to sustain cGMP signalling.

Key words: cGMP, cGMP-dependent protein kinase (PKG), phosphodiesterase-5, phosphorylation, positive-feedback control, vascular tissue.

INTRODUCTION

cGMP-dependent protein kinase (PKG) plays a central role in effecting many physiological functions following cGMP elevation [1–3]. PKG is activated by elevated cGMP in response to extracellular signals such as NO, natriuretic peptides and guanylin, which activate guanylate cyclases. Activated PKG phosphorylates many intracellular proteins and leads to physiological effects such as relaxation of vascular smooth muscle, airway distension, inhibition of cell proliferation, inhibition of platelet aggregation and apoptosis [1]. On the other hand, cGMP produced in cells is also hydrolysed by cyclic nucleotide phosphodiesterases (PDEs) [4] and, in some instances, it is also bound to PDEs [5]. Extrusion of intracellular cyclic nucleotides by leakage and transporters has been demonstrated [6,7], but the effect of these processes to lower cyclic nucleotides is minor compared with hydrolytic action of PDEs. Total cellular cGMP is mainly determined by the balance between activities of guanylate cyclases and PDEs. To activate signalling through the cGMP pathway, cellular cGMP must reach levels at which PKG binds the nucleotide, but while cGMP is bound to PKG, the nucleotide should be temporally protected from hydrolysis by PDEs. In vascular smooth muscle, PKG-I α and PKG-I β , but not PKG-II, are abundantly expressed, and activation of PKG-I is strongly correlated with decreased tension in vascular and airway smooth muscle [8–10]. Activated PKG phosphorylates myosin light-chain kinase ('MLCK') phosphatase, Ca²⁺-activated K⁺ ('BK_{Ca}') channels, phospholamban and an Ins(1,4,5)P₃-receptor-associated PKG substrate in vascular tissues [12–15] to cause

vascular relaxation. PKG-I-deficient mice fail to relax penile corpus cavernosum in response to NO [16], and aortic rings from these animals do not relax in response to acetylcholine or NO [17]. Thus the cGMP/PKG cascade is established as an important modulator of vascular smooth muscle contraction, and PDE5 is one of the major cGMP-hydrolysing PDEs in vascular tissues [5,11,18,19].

PKG has two allosteric cGMP-binding sites in the N-terminal regulatory region in addition to a more C-terminal catalytic domain [1]. PDE5 has two GAF domains (where GAF is cGMP-specific and -stimulated PDEs, *Anabaena* adenylate cyclase and *Escherichia coli* FhlA) that provide cGMP binding to the regulatory domain of this protein and which are evolutionarily distinct from the cGMP-binding sites of PKG [20]. Intracellular concentrations of cGMP, PKG and PDE5 have been determined in several vascular tissues. Concentrations of cGMP-binding sites of PKG in pig coronary arteries and rabbit corpus cavernosum were estimated to be 600 nM and 58 nM respectively [9,11]. These values are 6- and 3-fold higher than those of cGMP in unstimulated tissue respectively. Moreover, the concentration of allosteric cGMP-binding sites of PDE5 in rabbit corpus cavernosum was 10-fold higher than that of basal cGMP [11]. Considering the *K_D* values of PKG and PDE5 for cGMP, it is likely that a significant portion of cGMP in cells would be bound to PKG and PDE5. cGMP that is active in the PKG-mediated signalling cascade in cells includes free cGMP and PKG-bound cGMP, whereas that which is bound to PDE5 should be temporally unavailable. cGMP bound to either PDE5 or PKG allosteric sites is likely to be unavailable for hydrolysis by PDE. If so, the efficacy of

Abbreviations used: PDE, cyclic nucleotide phosphodiesterase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase.

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cGMP signalling will be significantly influenced by the proportion of cGMP distributed into each of these classes of cGMP-binding sites.

In members of the catabolite gene activator protein-related cyclic nucleotide-binding proteins, including cAMP-dependent protein kinase (PKA) and PKG, the cyclic phosphate moiety is buried deep in the cyclic nucleotide site [21]. The allosteric cAMP-binding sites of the isolated regulatory subunit of PKA protected cAMP from hydrolysis by an unidentified PDE [22] but, when the catalytic subunit of PKA is present, the cAMP-binding affinity is greatly decreased. This would be predicted to increase availability of cAMP for hydrolysis by PDEs, but this has not been studied. In PKGs, the regulatory and catalytic domains reside on the same polypeptide, so the influence of the catalytic domain to decrease cGMP-binding affinity is always present [1]. However, autophosphorylated PKG has increased cGMP-binding affinity. Given these considerations, it is unclear as to whether PKG could significantly protect cGMP from hydrolysis by PDEs. However, it is possible that PKG could protect cGMP from hydrolysis by cGMP-hydrolysing PDEs and thereby sustain signalling through the cGMP cascade. This concept has not been previously tested experimentally. We have hypothesized that, at physiological concentrations of cGMP and its target proteins, cGMP binding to allosteric sites of PKG and PDE5 can alter cGMP availability for hydrolysis by PDE5 and that changes in cGMP-binding affinities of these proteins resulting from phosphorylation or dephosphorylation will markedly impact cGMP signalling. In the present study, we focus on the role of type I PKGs as major intracellular cGMP-binding proteins and the role of these PKGs as the major effector of cGMP action in tissues. As intracellular cGMP increases, it quickly binds to and activates PKG thereby increasing both heterophosphorylation and autophosphorylation activities. When autophosphorylated, PKG has higher cGMP-binding affinity that enhances further cGMP occupancy of its allosteric sites and would be predicted to more potently protect cGMP from hydrolysis by cGMP-hydrolysing PDEs. Protection of cGMP by PKG from hydrolysis by cGMP-hydrolysing PDEs would be a stimulatory aspect of cGMP-dependent signal transduction in cells. In the present study, we demonstrate persistent protection of cGMP by PKG-I α and PKG-I β from hydrolysis by PDE5, and this effect is modulated by the autophosphorylation state of these enzymes.

MATERIALS AND METHODS

Materials

[8-³H]cGMP was purchased from Amersham Biosciences. cGMP, cAMP, ATP and *Crotalus atrox* 5'-nucleotidase were from Sigma. [γ -³²P]ATP was obtained from NEN-DuPont.

Preparation of PKGs

Dimeric native bovine PKG-I α and an N-terminally truncated monomer, PKG-I β (Δ 1–52), were used to test the hypothesis that these proteins could protect cGMP from hydrolysis by PDE catalytic activity. PKG-I α and PKG-I β have very different affinities for cGMP and autophosphorylation increases the cGMP-binding affinity in each. This disparity in cGMP-binding affinity and the ability to modulate affinity through autophosphorylation make this an excellent model to test the hypothesis. PKG-I α was purified to apparent homogeneity from bovine lung as described previously [23]. During the DEAE-Sephadex chromatography in the final purification step, the column was washed exhaustively

with KPME [10 mM potassium phosphate buffer (pH 6.8), 2 mM EDTA and 25 mM 2-mercaptoethanol] containing 0.05 M NaCl and pooled to select for a fraction of PKG-I α that was essentially free of cyclic nucleotide. Purity was analysed using SDS/PAGE followed by Coomassie Brilliant Blue stain. The kinase activity was stimulated approx. 5-fold by the addition of cGMP.

PKG-I β (Δ 1–52) lacking the N-terminal 52 amino acids of human PKG-I β was purified to apparent homogeneity from transfected Sf9 cells as described previously [24]. Purity of the PKG-I β (Δ 1–52) was evaluated by determining the specific catalytic activity compared with that of native PKG-I β and by using SDS/PAGE [10% (w/v) gels] stained with Coomassie Brilliant Blue and silver stain. By this latter analysis, PKG-I β (Δ 1–52) from multiple preparations was >98% pure. cAMP or cGMP content of purified PKG-I β (Δ 1–52) was determined as described previously [24] and was <1% the concentration of total cyclic nucleotide-binding sites. The enzyme was stored at 4 °C in KPME containing 0.15 M NaCl.

The N-terminal sequence of the PKG-I β (Δ 1–52) derived from three preparations was determined to be Arg-Pro-Ala-Thr-Gln-Xaa-Ala-Gln-, which agrees with the known sequence of human PKG-I β beginning at Arg⁵³ (Arg-Pro-Ala-Thr-Gln-Gln-Ala-Gln-) [25].

Characterization of PKG-I β (Δ 1–52)

Specific catalytic activity of PKG-I β (Δ 1–52) was determined to be 2.72 ± 0.15 μ mol/min per mg of protein, which was similar to the value of approx. 2.5 μ mol/min per mg of protein for type I PKGs [8]. The kinase activity was stimulated >10-fold by addition of cGMP. The K_a value for activation of PKG-I β (Δ 1–52) at 30 °C was 2.1 ± 0.53 μ M, which was similar to the value of 0.7 ± 0.15 μ M for wild-type PKG-I β . The K_a was determined using standard protein kinase assay conditions, and the concentration of cGMP in the assay varied from 0.016 to 15.57 μ M. The K_D value of PKG-I β (Δ 1–52) for cGMP shown in the present study was also similar to the value of wild-type PKG-I β . Therefore, in the present study, PKG-I β (Δ 1–52) was used as PKG-I β . Since the cGMP-binding properties of PKG-I β (Δ 1–52) are comparable with those of full-length PKG-I β and because this protein can be produced in abundant quantities in Sf9 cells, we elected to use it as the model for PKG-I β .

Preparation of the PDE5 catalytic domain

Recombinant catalytic domain of bovine PDE5 was partially purified from transfected Sf9 cells by chromatography on DEAE-Sephacel and hydroxyapatite as described previously [26]. The PDE5 catalytic domain exhibited a K_m for cGMP of approx. 5.8 μ M, which is comparable with previous reports [27].

PDE assay

PDE activity was determined by slight modification of the method described previously [11]. The reaction mixture contained 40 mM Mops (pH 7.5), 0.55 mM EGTA, 15 mM magnesium acetate, 0.17 mg/ml BSA, 4 nM PDE5 catalytic domain and 200 nM [³H]cGMP (specific radioactivity of approx. 10 000 c.p.m./pmol). Where indicated, 100 μ M ATP and various concentrations of PKG-I were added to the reaction mixture. The indicated values are concentrations of the reagents in the final assay volume of 125 μ l. Reactions were started by addition of [³H]cGMP after preincubation at 30 °C for 5 min, incubated at 30 °C for the indicated times, and then terminated by addition of 10 μ l of

a stop mix containing 100 mM EDTA, 66 mM theophylline, 21 mM cAMP, 24 mM cGMP and 200 mM Tris/HCl (pH 7.5). *Crotalus atrox* 5'-nucleotidase (200 µg) was added, followed by incubation at 30 °C for 10 min, before termination of the reaction by addition of 1 ml of an ice-cold dilution solution containing 0.1 mM adenosine, 0.1 mM guanosine and 15 mM EDTA. Contents of each assay tube were then filtered on QAE-Sephadex columns as described previously [11]. IC₅₀ values, which represent 50 % inhibition of cGMP hydrolysis by PKG, were calculated using GraphPad Prism 3.0.

cGMP-binding assay

cGMP binding to PKG-I was performed in a reaction mixture containing 40 mM Mops (pH 7.5), 0.55 mM EGTA, 15 mM magnesium acetate, 0.17 mg/ml BSA and [³H]cGMP. The binding reaction was initiated by addition of enzyme. After incubation for indicated periods at 30 °C, 2 ml of ice-cold saturated ammonium sulphate was added to each tube, and the contents were immediately filtered on to 0.45 µm Millipore membranes as described previously [8]. Non-specific binding was determined by incubation of enzyme in the presence of 1 mM unlabelled cGMP.

Preparation of autophosphorylated PKG-Iα and PKG-Iβ

Purified PKG-Iα (0.6 nmol) and PKG-Iβ(Δ1–52) (3 nmol) in KPME were incubated with 200 µM [³²P]ATP (specific radioactivity approx. 30 000 c.p.m./nmol) and 10 mM magnesium acetate in the presence of approx. 50 µM cAMP for 3 h at 30 °C; 100 mM EDTA was added to stop the reaction. The phosphorylated sample was chromatographed on Sephacryl S-200 (0.9 cm × 50 cm) (Amersham Biosciences) equilibrated in KPME containing 0.15 M NaCl to remove free [³²P]ATP. The phosphorylated PKG was concentrated to approx. 500 µl using an Amicon filtration cell equipped with a PM-30 membrane, and the radioactivities and protein concentrations of the samples were measured to determine ³²P incorporation. Non-phosphorylated PKG-Iα and PKG-Iβ were treated similarly, except that ATP and magnesium acetate were omitted in the incubation.

Determination of protein concentration

Protein concentrations were measured by the method of Bradford [28] using BSA as a standard. The values for PKG that were determined by the Bradford method were multiplied by the 0.63 correction factor as described previously [8]. Molar concentrations of PKG were PKG subunit concentrations.

RESULTS

Effects of PKG-Iα and PKG-Iβ on cGMP hydrolysis by the PDE5 catalytic domain

Before testing the effects of purified PKG-Iα and PKG-Iβ [PKG-Iβ(Δ1–52)] on hydrolysis of cGMP by PDE5, *K_D* values of cGMP binding to these two kinases were determined in the absence of ATP. As expected, Scatchard analysis revealed that PKG-Iα and PKG-Iβ each had one high-affinity and one low-affinity binding site for cGMP. *K_D* values of the two cGMP-binding sites of PKG-Iα at 30 °C were 3.7 nM and 38 nM (Table 1). The *K_D* values of PKG-Iβ for cGMP were 54 nM and 750 nM, approx. 10-fold higher *K_D* values for cGMP than PKG-Iα. These *K_D* values of PKG-I were very close to those in previous reports [8,28]. Compared with the *K_D* values of PKG-Iα and PKG-Iβ, the *K_m*

Table 1 *K_D* values of PKG-Iα and PKG-Iβ for cGMP

cGMP-binding assays were performed using 0.5–1.0 nM PKG-Iα or 20–40 nM PKG-Iβ in the absence of ATP for 15 min using concentrations of 3–300 nM cGMP for PKG-Iα and 10–3000 nM cGMP for PKG-Iβ as described in the Materials and methods section. The incubation period was sufficient for these concentrations of PKG-Iα and PKG-Iβ at the lowest concentrations of cGMP to attain equilibration. *K_D* values were calculated by Scatchard plots. Values are the means ± S.E.M. of three independent determinations.

Enzyme	cGMP-binding sites (<i>K_D</i> , nM)	
	High-affinity	Low-affinity
PKG-Iα	3.7 ± 0.57	38 ± 9.8
PKG-Iβ	54.0 ± 7.0	750 ± 96

Figure 1 Effects of PKG-Iα and PKG-Iβ on cGMP hydrolysis by the PDE5 catalytic domain

Solutions containing 4 nM PDE5 catalytic domain and various concentrations of PKG-Iα or PKG-Iβ were preincubated for 5 min at 30 °C, and then the reaction was started by addition of [³H]cGMP at a final concentration of 200 nM. After incubation for 10 min, the reaction was stopped by addition of an excess of cGMP, cAMP and theophylline, and hydrolysed [³H]cGMP was measured after incubation with 5'-nucleotidase. Values are means ± S.E.M. of three independent experiments.

value for cGMP of the partially purified catalytic domain of PDE5 used in the present study was relatively high (*K_m* = 5.6 µM), but agrees with values determined previously for PDE5 holoenzymes [1,26,27].

To examine the effects of PKG-Iα and PKG-Iβ on cGMP hydrolysis by the PDE5 catalytic domain, various concentrations of PKG-Iα or PKG-Iβ were added to the PDE assay containing 4 nM PDE5 catalytic domain and 200 nM [³H]cGMP. The reaction was started by addition of 200 nM [³H]cGMP and, after 10 min, the amount of [³H]cGMP that had been hydrolysed was measured. Under these conditions, 30–40 % of the cGMP in the mixture was hydrolysed by the PDE5 catalytic domain in the absence of PKG. The intracellular concentration of cGMP under basal conditions in pig coronary artery smooth muscle was determined previously to be 90 nM [9]. In a number of models, it is estimated that a 2- to 3-fold increase of cGMP in cells is likely to be sufficient for a maximum physiological response [9,29]. Therefore 200 nM cGMP used in the present study would approach that predicted following physiological stimulation of arterial smooth muscle by NO or natriuretic peptides. PKG-Iα or PKG-Iβ inhibited cGMP hydrolysis by PDE5 (30 % hydrolysis under these conditions) in a concentration-dependent manner (Figure 1). IC₅₀ values of

Figure 2 Effect of ATP on PKG-I α and PKG-I β inhibition of cGMP hydrolysis by the PDE5 catalytic domain

The solution containing the PDE5 catalytic domain and various concentrations of PKG-I α (A) or PKG-I β (B) was preincubated in the absence (–ATP) and presence (+ATP) of ATP for 5 min at 30 °C, and then the reaction was started by addition of [^{3} H]cGMP. After the reaction mixture was incubated at 30 °C for 10 min, hydrolysed [^{3} H]cGMP was measured. The concentrations of the PDE5 catalytic domain, [^{3} H]cGMP and ATP were 4 nM, 200 nM and 100 μ M respectively. Values are means $\pm$ S.E.M. of three independent experiments.

PKG-I α and PKG-I β for cGMP hydrolysis by PDE5 were 31 ± 0.80 nM and 1000 ± 210 nM respectively. These findings strongly indicated that PKG can protect cGMP from hydrolysis.

Effect of ATP on cGMP binding by PKG-I α and PKG-I β and on PKG inhibition of cGMP hydrolysis by the PDE5 catalytic domain

A previous report [30] demonstrated that binding of ATP to the ATP-binding site of PKG-I α decreased its cGMP-binding affinity. We investigated effects of ATP on cGMP binding to PKG-I α and PKG-I β under the conditions used for the current studies, since ATP would always be present in the cellular milieu. Concentrations of PKG-I α (20 nM) and PKG-I β (200 nM) were used that showed approx. 20% inhibition of cGMP hydrolysis by the PDE5 catalytic domain in Figure 1. ATP decreased cGMP-binding affinity for both PKG-I α and PKG-I β in a concentration-dependent manner, but the effect of ATP on cGMP binding to PKG-I β was far less than that on PKG-I α (results not shown). For example, 100 μ M ATP inhibited cGMP binding to PKG-I α and PKG-I β by 57% and 16% respectively. Physiological concentrations of ATP in cells are known to be more than 1 mM. Since at higher concentrations of ATP, cGMP binding to PKG-I α and PKG-I β was similar, 100 μ M ATP was used to examine effects of PKG-I α and PKG-I β on cGMP hydrolysis by the PDE5 catalytic domain. There was no effect of ATP alone on hydrolytic activity of PDE5 (results not shown). The effect of PKG-I α in the presence of ATP was decreased compared with that in the absence of ATP, and an IC₅₀ value of PKG-I α for cGMP hydrolysis by the

Figure 3 Time course of cGMP binding to phosphorylated and non-phosphorylated PKG-I α and PKG-I β

cGMP-binding assays were performed using 25 nM PKG-I α (A) or 100 nM PKG-I β (B) in the presence of 100 μ M ATP for indicated periods, as described in Materials and methods section. Phosphorylated and non-phosphorylated PKGs were prepared in the absence and presence of ATP and magnesium as described in the Materials and methods section. The concentration of [^{3} H]cGMP was 200 nM. Values are means $\pm$ range of two independent experiments.

PDE5 catalytic domain in the presence of ATP was 217 ± 16 nM (Figure 2A). By contrast, inhibition of cGMP hydrolysis by PKG-I β was not strongly affected by ATP. IC₅₀ value of PKG-I β for cGMP hydrolysis in the presence of ATP was more than 1 μ M (Figure 2B). Thus PKG-I α would exhibit more than 5-fold stronger effect on inhibition of cGMP hydrolysis by PDE5 than would PKG-I β in presence of ATP, compared with a 30-fold stronger effect in the absence of ATP.

Inhibition of cGMP hydrolysis by autophosphorylated PKG-I α and PKG-I β

Both PKG-I α and PKG-I β undergo autophosphorylation, and autophosphorylation increases both cGMP-binding affinities and basal kinase activities [31–33]. Thus the autophosphorylated PKGs were used to test the effect of increased cGMP-binding affinity on availability of cGMP for hydrolysis. Time courses of cGMP binding to phosphorylated and non-phosphorylated forms of PKG-I α and PKG-I β , using 200 nM [^{3} H]cGMP in the presence of ATP, are shown in Figure 3. These experiments utilized PKG-I α (25 nM) and PKG-I β (100 nM), which showed 5–10% inhibition of cGMP hydrolysis in the presence of ATP (Figure 3). Bovine PKG-I α can be autophosphorylated at five serines (Ser¹, Ser⁵⁰, Ser⁵⁸, Ser⁶⁴ and Ser⁷²) and two threonines (Thr⁵⁸ and Thr⁸⁴) *in vitro* [34]. After autophosphorylation for 3 h, approx. 4.9 mol phosphate was incorporated/mol of PKG-I α . Phosphorylated and non-phosphorylated PKG-I α were equilibrated with subsaturating levels of [^{3} H]cGMP (200 nM) for 20 min. After equilibration of cGMP binding, approx. 0.8 mol of

Figure 4 Comparison of effects of phosphorylated and non-phosphorylated PKGs on cGMP hydrolysis by the PDE5 catalytic domain

The solution containing the PDE5 catalytic domain and various concentrations of PKG-Iα (A) and PKG-Iβ (B) in the presence of ATP was preincubated at 30 °C for 5 min, and then the reaction was started by addition of cGMP. After the reaction mixture was incubated at 30 °C for 10 min, hydrolysed [^{3} H]cGMP was measured after incubation with 5'-nucleotidase. The concentrations of the PDE5 catalytic domain, [^{3} H]cGMP and ATP were 4 nM, 200 nM and 100 μ M respectively. Values are means $\pm$ S.E.M. of three independent experiments.

cGMP was bound per PKG-Iα subunit, indicating that 40% of the cGMP-binding sites of PKG-Iα were occupied at this concentration of cGMP (Figure 3A). There was no significant difference in the extent of cGMP binding between phosphorylated and non-phosphorylated PKG-Iα. However, the velocity of cGMP binding to phosphorylated PKG-Iα was slightly higher than to non-phosphorylated PKG-Iα. PKG-Iβ is autophosphorylated at Ser⁶³ and Ser⁷⁹ *in vitro* [33,35]. Phosphorylated PKG-Iβ prepared in the present study incorporated 2.08 mol of phosphate/subunit. This suggested that PKG-Iβ was completely autophosphorylated. cGMP binding to phosphorylated and non-phosphorylated PKG-Iβ was very fast and the reactions were equilibrated within 1 min (Figure 3B). cGMP binding to phosphorylated PKG-Iβ at subsaturating cGMP (200 nM) was approx. 3-fold higher than to non-phosphorylated PKG-Iβ. Under these conditions of subsaturating cGMP, binding to phosphorylated and non-phosphorylated PKG-Iβ was 32% and 12% of saturated binding respectively. Affinity of cGMP-binding to phosphorylated PKG-Iβ was approximately twice that of non-phosphorylated PKG-Iβ (results not shown).

Effects of phosphorylated PKG-Iα, phosphorylated PKG-Iβ and the respective non-phosphorylated PKGs on cGMP hydrolysis by the PDE5 catalytic domain in the presence of ATP were compared. Phosphorylated PKG-Iα showed only a 2-fold stronger effect than non-phosphorylated PKG-Iα (Figure 4A). By contrast,

Figure 5 Time course of inhibition of cGMP hydrolysis by the PDE5 catalytic domain in the presence of PKG-Iα or PKG-Iβ

The solution containing the PDE5 catalytic domain and 120 nM PKG-Iα or 1000 nM PKG-Iβ in the presence of ATP was preincubated at 30 °C for 5 min, and then the reaction was started by addition of [^{3} H]cGMP. After incubation for various periods, hydrolysed [^{3} H]cGMP was measured (A). Hydrolysed cGMP and cGMP bound to PKG-Iα were measured under the same conditions as (A) (B). The concentrations of the PDE5 catalytic domain, [^{3} H]cGMP and ATP were 4 nM, 200 nM and 100 μ M respectively. Typical plots from at least two independent experiments are shown.

phosphorylated PKG-Iβ exhibited much stronger inhibition than non-phosphorylated PKG-Iβ. The IC₅₀ value of phosphorylated PKG-Iβ was 166 $\pm$ 4.7 nM, which was approx. 10-fold lower than that of non-phosphorylated PKG-Iβ (Figure 4B).

Time course of cGMP hydrolysis by the PDE5 catalytic domain in the absence and presence of PKG-Iα or PKG-Iβ

Concentrations of PKG-Iα and PKG-Iβ (120 and 1000 nM respectively), which showed 30% inhibition of cGMP hydrolysis (see Figure 2), were used to assess the effectiveness with which PKGs can protect cGMP from hydrolysis by PDE5. A time course of effects of PKG-Iα and PKG-Iβ on inhibition of cGMP hydrolysis by the PDE5 catalytic domain is shown in Figure 5(A). In the absence of PKG, cGMP was almost completely hydrolysed by PDE5 in the absence of PKG after incubation for 2–3 h but, in the presence of PKGs, a significant amount of cGMP was protected against hydrolysis by PDE5 during this prolonged incubation. PDE5 catalytic activity did not decline significantly during this time period. Consistent with the higher affinity of PKG-Iα for cGMP, the inhibition of cGMP hydrolysis by PKG-Iα

was more effective than that by PKG-I β . cGMP binding to PKG-I α was also measured under the same conditions (Figure 5B). cGMP binding to PKG-I α reached a maximum within 10 min, and then the cGMP bound to PKG-I α slowly declined in response to the loss of free cGMP, due to degradation by the PDE5 catalytic domain. Free cGMP was also calculated based on the total bound cGMP and the cGMP that had been hydrolysed. Although free cGMP essentially disappeared during the 3 h incubation in the absence of PKG-I α , when PKG-I α was included, there was still a portion of the cGMP that remained bound to, and protected by, PKG even after the 3 h incubation. These findings suggest that PKG-I α could remain partially activated by cGMP even 3 h after an initial increase in cGMP. Alternatively, PKG may continue to be partially saturated with cGMP and therefore primed for increased ease of activation by subsequent increases in cGMP.

DISCUSSION

We establish in the present study potentially critical roles of allosteric cGMP-binding sites of PKG-I α and PKG-I β in regulating hydrolysis of cGMP by PDE5. In the absence of ATP, PKG-I α has 10-fold higher affinity for cGMP than does PKG-I β . Therefore lower concentrations of cGMP can bind to PKG-I α and activate this kinase. PKG-I α protects cGMP from hydrolysis more strongly than does PKG-I β . Using 200 nM cGMP, both cGMP-binding sites of PKG-I α and the high-affinity site of PKG-I β protect cGMP from hydrolysis in the absence of ATP *in vitro*. Physiologically, more than 1 mM ATP is present in cells. It is known that ATP binding to the PKG-I α kinase domain decreases cGMP-binding to the high-affinity site of PKG-I α [30]. In the present study, we confirmed that ATP decreases binding of cGMP to PKG-I α , but the effect of ATP to decrease cGMP binding to PKG-I β is slight. Considering the K_D value (10–20 μ M) of the ATP-binding site of PKG-I α [30] and the ATP concentration in cells, it would be predicted that PKG is saturated with ATP under physiological conditions. Although ATP weakens inhibition of cGMP hydrolysis by PKG, the results of the present study indicate that physiological concentrations of PKG-I α can still significantly protect cGMP from hydrolysis.

Autophosphorylation of PKGs increases cGMP-binding affinity [32,33]. Both phosphorylated PKG-I α and phosphorylated PKG-I β inhibit cGMP hydrolysis more potently when compared with the non-phosphorylated PKGs. In particular, phosphorylated PKG-I β was 10-fold more effective in protecting cGMP from hydrolysis than non-phosphorylated PKG-I β . Considering physiological concentrations of PKG in vascular tissues, such as pig coronary arteries and rabbit corpus cavernosum, these studies indicate that phosphorylated PKG-I α , phosphorylated PKG-I β and non-phosphorylated PKG-I α each have the potential to significantly protect cGMP from hydrolysis.

PKG-I α bound to cGMP more tightly and protected cGMP from hydrolysis more effectively than PKG-I β . In cultured vascular smooth muscle cells, NO and natriuretic peptides increase intracellular cGMP rapidly, and then cGMP returns nearly to the basal level within 60 min via degradation and efflux into extracellular space [6,36–38]. Our experimental conditions for the rate of degradation of cGMP by hydrolysis should be very close to those in intact cells. The basal level of cGMP in pig coronary artery is approx. 90 nM and stimulated levels are likely to reach approx. 300 nM [9]. In the present study, after incubation for 1–2 h, the sum of free cGMP and bound cGMP returns to near basal level of cGMP estimated for unstimulated vascular smooth

Figure 6 Roles of allosteric cGMP-binding sites of PKG in regulating cGMP hydrolysis

Protection of cGMP by PKG allosteric sites from hydrolysis can be regulated by autophosphorylation and dephosphorylation and thereby this regulation could modulate cGMP signalling.

muscle [9]. However, approx. 50 % of total cGMP remains bound to PKG-I α after total cGMP concentration returns to the basal level. The sum of bound and free cGMP concentrations after 1 h, 2 h and 3 h incubation in the presence of the PDE5 catalytic domain and 120 nM PKG-I α are 94 nM, 59 nM and 37 nM respectively. Using these concentrations of cGMP and 120 nM PKG-I α without the PDE5 catalytic domain, the amount of cGMP bound to PKG-I α was almost equal to that of free cGMP under equilibrated conditions (results not shown). Since $K_D = [\text{free cGMP}][\text{free PKG}]/[\text{bound cGMP}]$, an average of actual K_D values of 120 nM PKG-I α during incubation is 182 ± 13 nM (range 131–206 nM). These findings suggest that cGMP bound to PKG dissociates relatively rapidly from the enzyme in response to the loss of free cGMP, due to degradation by PDE5, and that free cGMP and PKG-I α -bound cGMP are likely to be almost equilibrated during incubation for 3 h.

In earlier studies, we [11,37,39] provided evidence supporting the likelihood that cGMP signalling is regulated by negative feedback via PDE5. Phosphorylation of PDE5 increases cGMP hydrolytic activity 5-fold in intact cells [37,38,40] and 1.5- to 1.7-fold *in vitro* [39,40]. In our more recent report [39], binding of cGMP by allosteric cGMP-binding sites of PDE5 is increased by phosphorylation of PDE5, and this increased binding affinity could provide for sequestration of cGMP by PDE5. Thus regulation of cGMP-hydrolysing and cGMP-binding activities of PDE5 by phosphorylation might be also involved in modulation of intracellular cGMP concentrations and cGMP signalling. Our recent report [39] demonstrated that phosphorylation of purified recombinant PDE5 causes a modest decrease in the cGMP K_m value (from 0.96 to 0.58 μ M). Even the K_m value of

phosphorylated PDE5 is considerably higher than the K_D values of PKG- $I\alpha$ and phosphorylated PKGs for cGMP. Therefore it is predicted that PKG can still protect cGMP from hydrolysis in the presence of phosphorylated PDE5. This is also true for other cGMP-hydrolysing PDEs (PDE1 and PDE2), whose K_m values for cGMP are much lower affinity than the K_D value for cGMP binding by PKG [4].

We have demonstrated in the present study that PKG can sequester cGMP and protect it against hydrolysis by PDEs. In this case, cGMP sequestration by PKG would help to maintain activity of cGMP signalling and persistence of the physiological effects of cGMP. By analogy with PDE5, autophosphorylation of PKG increases both its kinase activity and its cGMP-binding affinity as well. This increases protection of cGMP from hydrolysis by PDE5 at concentrations that approach those in intact cells. It is proposed that autophosphorylation of PKG represents a positive-feedback regulation of cGMP signalling by the cGMP/PKG cascade (Figure 6). Although a typical environment of cGMP protection in vascular tissues has been demonstrated in the present study using PKG and the PDE5 catalytic domain, other cGMP-binding proteins and cGMP-hydrolysing PDEs would play a similar role in regulating cGMP signalling by providing a balance between protection and hydrolysis of cGMP in many other tissues.

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