ALLOSTERIC SITES OF PHOSPHODIESTERASE-5 SEQUESTER CYCLIC GMP

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1. ABSTRACT

Phosphodiesterase-5 (PDE5) and cGMP-dependent protein kinase (PKG) play key roles in cGMP signaling. PDE5 has a catalytic domain (C domain) that hydrolyzes cGMP and a regulatory domain (R domain) that binds cGMP at allosteric sites. We recently demonstrated that in corpus cavernosum, PDE5 concentration exceeds basal cGMP by ~5-fold making it possible that its allosteric sites could bind a significant fraction of the total cellular cGMP. It is hypothesized that the allosteric sites regulate cGMP signaling by sequestering cGMP. At 60 nM cGMP *in vitro*, which approaches a stimulated concentration of cGMP in rabbit corpus cavernosum, isolated R domain inhibits both cGMP hydrolysis by C domain and activation of PKG (IC$_{50}$ values of 388 and 100 nM, respectively). Prior phosphorylation of R domain by cyclic nucleotide-dependent protein kinases, which increases its cGMP-binding affinity, also increases its potency for inhibiting both cGMP hydrolysis by C domain and cGMP activation of PKG (IC$_{50}$ values of 58 and 38 nM, respectively). In rabbit corpus cavernosum, PDE5 concentration (94 nM) exceeds these values. These findings support our hypothesis that physiological concentrations of R domain regulate cGMP signaling by sequestering this nucleotide and that phosphorylation of R domain modulates this effect. This could provide for negative feedback control of cGMP-signaling.

2. INTRODUCTION

Cyclic nucleotides regulate many cellular functions in various tissues. Cyclic GMP and cAMP are produced via activation of guanylyl and adenylyl cyclases by extracellular signals, and then hydrolyzed by phosphodiesterases (PDEs), which returns cyclic nucleotides to basal levels (1-4). Cellular cyclic nucleotide levels are modulated by the relative activities of guanylyl cyclases, adenylyl cyclases, and PDEs. A number of proteins have been suggested to participate in the extrusion of intracellular cAMP and cGMP as a means of modulation of cellular concentrations (5-10). Since these proteins transport other anions, cyclic nucleotides must compete with these anions in order to be extruded. Where it has been studied, it appears that the extrusion of cyclic nucleotides into the extracellular space is quantitatively minor compared to the amount hydrolyzed by PDEs (11). Another possible means of modulation of active cGMP levels in cells is sequestration of this nucleotide by the cGMP-binding sites of intracellular cGMP receptor proteins.

Cyclic GMP-dependent protein kinase (PKG), cGMP-binding PDEs such as PDE2, PDE5 and PDE6, and cGMP-gated ion channels act as intracellular receptors for cGMP (1, 4, 12). In vascular smooth muscle, PDE5, PKG-Iα, and PKG-Iβ are known to modulate cGMP-mediated

relaxation of the muscle. Activation of PKG by cGMP results in phosphorylation of a number of proteins to cause reduction in intracellular calcium (1-3,13), and PDE5 hydrolyzes and binds cGMP specifically (14). Intracellular concentrations of cGMP, PKG, and PDE5 have been determined in several vascular tissues. Concentrations of cGMP-binding sites of PKG and cGMP in pig coronary arteries were estimated to be ~600 nM and 90 nM, respectively (15). In rabbit corpus cavernosum, the concentrations of cGMP-binding sites of PKG and cGMP were estimated to be 58 nM and 18 nM, respectively (16). Moreover, 188 nM of cGMP-binding sites of PDE5 exist in rabbit corpus cavernosum. Thus, concentrations of the cGMP-binding sites of these proteins were much higher than that of cGMP in unstimulated vascular tissues. Considering the cellular concentrations of PKG, PDE5, and cGMP, and the cGMP-binding affinities of these proteins, more than 50% of total cGMP in unstimulated cells could bind to these proteins, and free cGMP could be less than 50% of total cellular cGMP. Therefore, the respective cGMP-binding sites of PDE5 and PKG could compete in binding a significant portion of cGMP in these tissues and may thereby regulate both the hydrolysis of cGMP by PDEs and the concentration of free intracellular cGMP. We have recently demonstrated that PKGIα and PKGIβ protect cGMP from hydrolysis by PDE5 catalytic domain and that autophosphorylated forms of PKGI which have higher affinity for cGMP are more effective in sequestering the nucleotide (17).

Phosphorylation of bovine PDE5 at Ser[92] by PKG or cAMP-dependent protein kinase (PKA) increases cGMP hydrolytic activity (18,19). We found that phosphorylation of PDE5 occurred within 1 min after stimulation by atrial natriuretic peptide in cultured vascular smooth muscle cells, and led to activation of PDE5 hydrolytic activity (20). It was shown more recently that PKG activated by stimulation of 8-bromo-cGMP in human uterine smooth muscle cells led to phosphorylation of PDE5 at Ser[102] (corresponds to Ser[92] of bovine PDE5) and increased PDE5 activity (21). Therefore, activation of PDE5 by phosphorylation via PKG represents at least one form of negative feedback control of cGMP signaling. Cyclic GMP binding to the R domain of PDE5 alone also increases the catalytic activity of PDE5 (22).

We hypothesize that cGMP bound to allosteric sites of PDE5 is temporally unavailable for hydrolysis by PDE, i.e., allosteric sites of PDE5 could sequester cGMP from hydrolysis by cGMP-PDEs. Furthermore, when cGMP is bound to allosteric sites of PDE5, it cannot contribute to PKG activation. Therefore, sequestration of cGMP by allosteric cGMP-binding sites of PDE5 from hydrolysis by PDE and from activation of PKG could significantly regulate cGMP-dependent signal transduction. Since phosphorylation of PDE5 by either PKG or PKA increases its cGMP-binding affinity (18, 23), phosphorylation is expected to strengthen sequestration of cGMP from hydrolysis by cGMP-PDE and from the process of PKG activation. Regulation of this sequestration would provide not only another form of modulation of active cGMP level in cells, but also another route of

negative feedback control of cGMP signaling. In order to test our hypothesis, we have devised an *in vitro* approach that utilizes approximately physiological concentrations of PDE5, PKG, and cGMP. This approach is used to examine effects of allosteric cGMP-binding sites of PDE5 on cGMP hydrolysis by PDE and on activation of PKG. Moreover, effects of phosphorylation of PDE5 on sequestration of cGMP are reported.

3. MATERIALS AND METHODS

3.1. Materials

[8-^{3}H]cGMP was purchased from Amersham Pharmacia Biotech. Cyclic GMP, cAMP, ATP, Crotalus atrox 5' nucleotidase, and protein phosphatase-1 (PP-1) were from Sigma. [γ-^{32}P]ATP was obtained from NEN-DuPont. T-0156, a potent and selective PDE5 inhibitor, was kindly provided by Tanabe Seiyaku Co., Ltd (23).

3.2. Preparation of PKG-Iα and PKG-Iβ

PKG-Iα and PKG-Iβ were purified to apparent homogeneity from bovine lung and aorta, respectively, as described previously (25). Purity of these proteins was analyzed using SDS-PAGE followed by Coomassie Brilliant stain.

3.3. Preparation of PDE5 catalytic domain

Catalytic domain (C domain) of bovine PDE5 was expressed in Sf9 cells as described previously (26) and partially purified by chromatography as follows. Transfected Sf9 cells were disrupted in10 mM K_2HPO_4/KH_2PO_4, pH 6.8, 2 mM EDTA, and 25 mM 2-mercaptoethanol (KPEM) containing protease inhibitor mix (Complete^{TM}, Roche Molecular Biochemicals) by a polytron homogenizer and then centrifuged at 12,000 × g for 30 min at 4° C. The supernatant was loaded onto a hydroxyapatite (Bio-Rad) column (5 × 17 cm) equilibrated in 10 mM sodium phosphate, pH 7.2. After washing the column with 30 mM potassium phosphate, pH 6.8, C domain was eluted from the column using a linear gradient of potassium phosphate (70-350 mM). All purification steps were performed at 4° C. Fractions containing PDE5 activity were concentrated using an Amicon filtration cell equipped with a PM-30 membrane, and then stored in small aliquots in 10% sucrose and 0.15 M NaCl at –70° C.

3.4. Preparation of PDE5 holoenzyme

His-PDE5 holoenzyme was also produced in transfected Sf9 cells as described previously, and then purified to homogeneity using Ni-NTA agarose according to manufacturer's instructions (27).

3.5. Preparation of PDE5 regulatory domain

Regulatory domain (R domain) of PDE5 was produced in E. coli by slight modification of the method reported previously (28). The cDNA fragment coding for the R domain of PDE5 (Val[46]-Glu[539] of human PDE5A1) was amplified by PCR from human fetal lung cDNA (Clontech) using specific primers 5'-AAAAGAATTCTGTTAGAAAAGCCACCAGAGAAATG -3' and 5'-AAAACTCGAGCTCTCTTGTTTCTTCCTCTGCTG-3'. Amplified fragment was subcloned into the EcoRI and

XhoI sites of pGEM-5X3, resulting in the expression plasmid (pGEX-cGB-II) coding for glutathione-S transferase (GST)-fused human PDE5 fragment containing a phosphorylation site and both GAF domains which provide for potential allosteric cGMP-binding. This R domain was phosphorylated by PKA mainly at Ser[102], but there was some phosphorylation of Thr[50]. In contrast, native bovine PDE5 is phosphorylated by PKG or PKA only at Ser[92], which corresponds to Ser[102] of human PDE5. Therefore, the Thr[50] of this R domain was mutated from Arg-Lys-Ala-<u>Thr</u>[50] to Arg-Lys-Ala-<u>Ala</u>[50] using QuickChange site-directed mutagenesis kit (Stratagene) and primer sets: 5'-GAAAAGCCGCCAGAGAAATGGTCAATG -3' plus 5'-CATTGACCATTTCTCTGGCGGCTTTTC-3', producing pGEX-cGB-II-T50A. The pGEX-cGB-II-T50A plasmid was transfected into E. coli (BL21 DE3 strain), and GST fused R domain, which is encoded by the pGEX-cGB-II-T50A plasmid, was produced in E. coli by induction with 100 μM isopropyl-β-D-thiogalactopyranoside at 22° C for 18 hrs. Cells were disrupted in phosphate buffered saline (pH 7.4) by a sonicator and then centrifuged at $48,000 \times g$ for 30 min. The supernatant was loaded on a glutathione Sepharose 4B affinity column, and the R domain was eluted with 10 mM reduced glutathione in Tris-HCl, pH 8.0. The eluate was stored in this solution containing 10 % sucrose and 0.15 M NaCl at -70° C. The R domain was incubated with 5 mM dithiothreitol (DTT) overnight at 4° C before use.

3.6. Preparation of phosphorylated R domain

Purified R domain (2 nmol, 7.5 μM) was incubated with 200 μM ATP and 2 mM magnesium acetate in the presence of 10 μM [3H]cGMP and 3 μM purified PKA. After incubation at 30° C for 2 hr, cIMP was added at a final concentration of 3.3 mM to exchange cGMP from R domain in an additional 1 hr incubation on ice. The R domain was concentrated using ULTRAFREE-0.5 (Millipore) to reduce free cIMP and cGMP concentrations. The concentrated sample was loaded onto Sephacryl S-200 (0.9 × 50 cm) equilibrated with KPED (10 mM K_2HPO_4/ KH_2PO_4, pH 6.8, 2 mM EDTA, 5 mM DTT, and 0.15 M NaCl) to remove free ATP, cGMP, and cIMP. Eluted samples were concentrated to ~500 μl using an Amicon filtration cell equipped with a PM-30 membrane. The stoichiometry of phosphate incorporation into phosphorylated R domain was determined by the previous method using native polyacrylamide gel (23). According to [3H]cGMP content of the phosphorylated sample, phosphorylated R domain containing bound cGMP was 1% of total protein. In certain instances, determination of [32P]protein was used to quantitate phosphate incorporation. Non-phosphorylated R domain was prepared using the same conditions but in the absence of ATP, magnesium acetate, and PKA. Phosphorylated R domain did not contain significant contaminating kinase activity (data not shown).

3.7. Dephosphorylation of phosphorylated R domain

Phosphorylated R domain was incubated at 30° C with PP-1 in 40 mM MOPS, pH 7.5, 0.55 mM EGTA, 15 mM magnesium acetate, 0.168 mg/ml bovine serum albumin, and 20 μM $MnCl_2$. Reaction was terminated by trichloroacetic acid precipitation in the presence of 0.1%

bovine serum albumin, and following centrifugation, supernatants were subjected to Cerenkov counting. In certain instances, reactions were mixed with Laemmli buffer and boiled (29) for 8% SDS-PAGE. Gels were then immunoblotted with anti-PDE5 antibody as described previously (20). After immunoblotting, membranes were exposed to autoradiography.

3.8. PDE assay

PDE activity was determined by slight modification of the method previously described (16). The reaction mixture contained 40 mM MOPS, pH 7.5, 0.55 mM EGTA, 15 mM magnesium acetate, 0.168 mg/ml BSA, 4 nM PDE5 C domain, and indicated concentrations of [3H]cGMP at a specific radioactivity of ~10,000 cpm/pmol. In some experiments, R domain was added to the reaction mixture. The indicated concentrations were those in the final assay volume of 125 μl. After preincubation at 30° C for 5 min, reactions were started by addition of [3H]cGMP and incubated at 30° C for indicated times, and then terminated by addition of 10 μl of a stop mix containing 200 mM Tris-HCl, pH 7.5, 100 mM EDTA, 66 mM theophylline, 21 mM cAMP, and 24 mM cGMP. 10 μl of 20 mg/ml Crotalus atrox 5'-nucleotidase was added to the mixture, which was then incubated 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. The resulting solution was applied to a QAE-Sephadex column (1.5 ml) equilibrated in 0.02 M ammonium formate, pH 7.5. After washing the column with 3 ml of this buffer, aqueous scintillant was added to the eluate, and the radioactivity was then counted. IC_{50} values, which represented 50% inhibition of cGMP hydrolysis by R domain, were calculated using GraphPad Prism 3.0.

3.9. Cyclic GMP-binding assay

Cyclic GMP binding was performed in a reaction mixture containing 40 mM MOPS, pH 7.5, 0.55 mM EGTA, 15 mM magnesium acetate, 0.168 mg/ml BSA, and [3H]cGMP. Addition of enzyme initiated the binding reaction. After incubation for indicated times at 30° C, 1.5 ml cold 10 mM K_2HPO_4 / KH_2PO_4, pH 6.8 (KP buffer), was added to each tube, and the contents were immediately filtered onto a 0.45-μm Millipore membrane that had been pre-wetted with an aliquot of KP buffer. Another 1.5 ml of KP buffer was added to the tube, and this rinse was filtered onto the membrane. The membrane was then washed with 3 × 1.5 ml of cold KP buffer. Membranes were air dried and placed in a counting vial. Nonaqueous scintillant (5 ml) was added to the vial, and radioactivity was counted. Nonspecific binding was determined by incubation of enzyme in the presence of 2 mM unlabeled cGMP. For measuring cGMP binding to PDE5 holoenzyme, 100 μM T-0156, which is a potent PDE5 inhibitor, was added to the reaction mixture to completely inhibit cGMP hydrolysis during incubation. K_D values, which represented 50% of the maximum [3H]cGMP binding, were calculated using GraphPad Prism 3.0.

3.10. PKG kinase assay
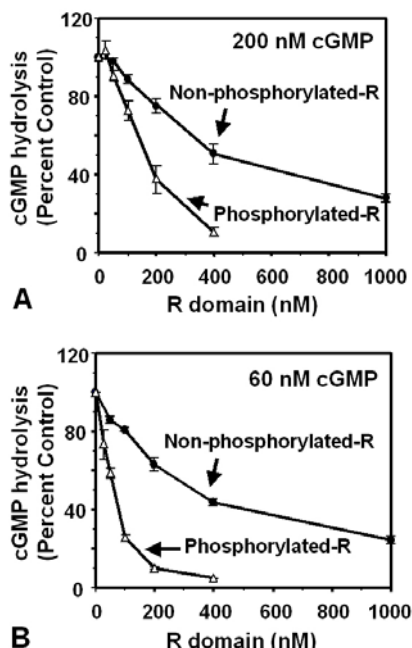
Activity of PKG was measured using a

Figure 1. Effects of phosphorylated and non-phosphorylated R domain on cGMP hydrolysis by isolated PDE5 C domain. The solution containing 4 nM PDE5 C domain and various concentrations of either phosphorylated or non-phosphorylated R domain was preincubated for 5 min at 30º C, and then the reaction was started by addition of [^{3}H]cGMP at final concentrations of 200 nM (A) and 60 nM (B). After incubation for 10 min, hydrolyzed [^{3}H]cGMP was measured. Data are means of three independent determinations ± S.E. In the absence of additions, 25-30% of the [^{3}H]cGMP was hydrolyzed, and this value was taken as the 100% value.

synthetic heptapeptide (RKRSRAE, Peninsula Laboratories Inc.) as a substrate as described previously (30). The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 1 µM synthetic peptide PKA inhibitor (Peninsula Laboratories Inc), 116 µM [^{32}P]ATP (~10,000 cpm/pmol), 17 mM magnesium chloride, and 30 nM PKG. After preincubating with indicated concentrations of cGMP and R domain at 30° C for 30 min, the kinase activity was measured by adding heptapeptide at a final concentration of 108 µM at 30° C for 5 min. The reaction was terminated by spotting the reaction mixture on P 81 phosphocellulose paper (Whatman). IC$_{50}$ values, which represented 50% inhibition of PKG activation by R domain, were calculated using GraphPad Prism 3.0.

3.11. Determination of protein concentrations

Protein concentration was measured by the method of Bradford (31) 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 (30). Protein concentration of R domain was also determined by amino acid analysis and shown to be equal to that determined by the Bradford method. Concentration of C domain was determined by assuming a specific enzyme activity of 10 µmol/min/mg (32). Concentrations of PDE5 and PKG described here represent those of monomers.

4. RESULTS

4.1. Cyclic GMP binding to PDE5 holoenzyme and PDE5 R domain

In order to characterize purified R domain, we compared cGMP-binding affinity of PDE5 holoenzyme and the isolated R domain. [^{3}H]cGMP binding was performed at 30° C for 45 min to equilibrate [^{3}H]cGMP binding to these proteins. K$_D$ values of this PDE5 holoenzyme and R domain were 106 ± 13 nM and 98 ± 15 nM, respectively, which agreed well with values previously reported for wild type and native PDE5 (130~300 nM) (18, 33). Similarity of the K$_D$ value of the isolated R domain to PDE5 holoenzyme validated its usefulness for the current studies. The affinity of the C domain of PDE5 for cGMP was relatively low (Km value=5.6 µM) (26) compared to that of the R domain.

4.2. Characterization of phosphorylated R domain

Phosphorylation of bovine PDE5 holoenzyme at Ser^{92}and the isolated R domain at the corresponding serine by either PKG or PKA is known to increase their cGMP-binding affinities (18, 23). It has been reported that migration of phosphorylated R domain in native gel electrophoresis is slower than that of non-phosphorylated form (23). The non-phosphorylated R domain used in these studies migrated as a single protein band on native gels, and after phosphorylation, the R domain was completely shifted to a slower mobility band (not shown). This indicated that the unphosphorylated R domain did not contain endogenous phosphate at Ser^{102} and that subsequent phosphorylation was complete. The stoichiometry of ^{32}P incorporation was calculated to be 1.2~1.5 mol/mol subunit of R domain. Using the proteins prepared here, we compared cGMP binding to phosphorylated R domain with that to the non-phosphorylated form. Phosphorylation of the isolated R domain markedly increased cGMP-binding affinity from K$_D$ of 98 ± 15 nM to 10 ± 0.39 nM, respectively. Phosphorylation did not affect maximum cGMP binding significantly.

4.3. Effects of phosphorylated and non-phosphorylated R domain on cGMP hydrolysis by C domain

To examine effects of isolated R domain on cGMP hydrolysis by C domain, we measured the hydrolysis of either 200 nM or 60 nM [^{3}H]cGMP by 4 nM C domain in the presence and absence of various concentrations of the R domain. Concentration of cGMP under basal intracellular conditions in pig coronary artery and rabbit corpus cavernosum is 90 nM and 18 nM, respectively (15, 16). It was reported that 2- to 3-fold increase in cGMP produced by stimulation with extracellular signals is sufficient to maximally relax vascular vessels (15). Therefore, 200 nM and 60 nM cGMP used as substrate here would be expected to approach concentrations achieved by physiological stimulation in coronary artery and rabbit corpus cavernosum, respectively. After incubation of the reaction mixture for 10 min, approximately 30-40% of total cGMP in the reaction mixture was hydrolyzed by C domain in the absence of R domain. Addition of the R domain inhibited cGMP hydrolysis in a concentration-dependent manner (Figures 1A and 1B). IC$_{50}$ values of R domain for inhibition of cGMP hydrolysis using 200 nM and 60 nM cGMP differed only slightly (434 ± 61 nM and 338 ± 35 nM, respectively).
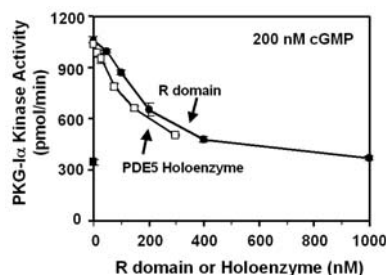
Figure 2. Effects of isolated R domain or PDE5 holoenzyme on PKG-Iα activation by cGMP. 30 nM purified bovine PKG-Iα and various concentrations of isolated R domain or PDE5 holoenzyme were preincubated with 200 nM cGMP for 30 min at 30º C, and then the kinase catalytic activity was measured by addition of substrate and incubation at 30º C for another 5 min. A closed square shows the basal kinase activity of PKG in absence of cGMP. Data are means of four independent experiments of isolated R domain and two independent experiments of PDE5 holoenzyme ± S.E. and range, respectively.
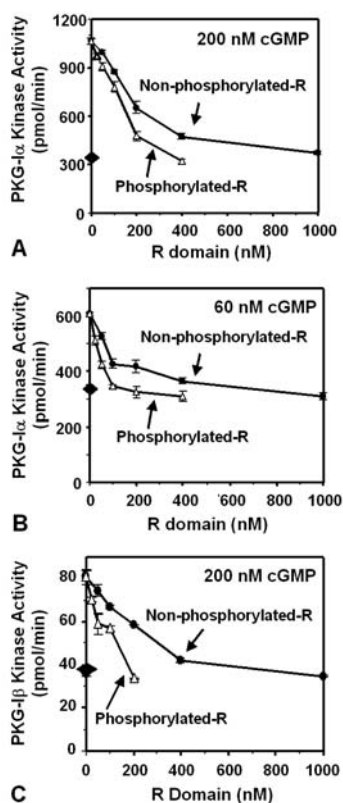

Figure 3. Effects of phosphorylated and non-phosphorylated R domain on activation of PKG-Iα or PKG-Iβ by cGMP. 30 nM PKG-Iα (A and B) or 30 nM PKG-Iβ (C) were preincubated for 30 min at 30º C in the absence and presence of various concentrations of isolated R domain in mixtures containing either 200 nM (A and C) or 60 nM (B) cGMP. Protein kinase catalytic activity was then measured by addition of heptapeptide substrate and incubation at 30º C for an additional 5 min. Closed diamond on the ordinate shows the basal kinase activity of PKG in absence of added cGMP. Data are means of four and two independent experiments for PKG-Iα and PKG-Iβ ± S.E. and range, respectively.

The effect of the phosphorylated R domain on cGMP hydrolysis by C domain was then compared with that of the non-phosphorylated form. Phosphorylated R domain exhibited significantly stronger inhibition of cGMP hydrolysis (Figure 1A and 1B). When 200 nM cGMP was used, the IC_{50} value of the phosphorylated R domain was 144 ± 10 nM, which was approximately 3 times lower than that of the non-phosphorylated R domain. Effects of the phosphorylated and non-phosphorylated R domains using 60 nM cGMP were even stronger (~6 fold) than those observed when 200 nM cGMP was used as substrate. IC_{50} values of the phosphorylated and non-phosphorylated R domains using 60 nM cGMP were 58 ± 2.9 nM and 338 ± 35 nM, respectively. Since phosphorylation increases the cGMP-binding affinity of the R domain ~10 fold, it is reasonable to expect a greater effect of phosphorylated R domain on the inhibition of cGMP hydrolysis at the lower concentration of cGMP.

4.4. Inhibition of PKG-Iα activation by R domain or PDE5 holoenzyme

Effects of the R domain and the PDE5 holoenzyme on cGMP activation of PKG were examined. PKG-Iα (30 nM) was incubated with 200 nM cGMP in the presence and absence of various concentrations of R domain or PDE5 holoenzyme for 30 min to equilibrate cGMP binding to the respective proteins, and then the kinase activity of PKG was measured. In assays containing PDE5 holoenzyme, 100 μM T-0156, a specific PDE5 inhibitor, was included to block breakdown of cGMP by PDE5 catalytic site. This concentration of PKG-Iα is that found in rabbit penile corpus cavernosum (16). Compared with the basal activity of PKG-Iα (~350 pmol/min), 200 nM and 60 nM cGMP activated PKG-Iα kinase activity approximately 3-fold (~1050 pmol/min) and 2-fold (600 pmol/min), respectively. The R domain and the PDE5 holoenzyme inhibited activation of PKG-Iα by cGMP in a concentration–dependent manner with IC_{50} values of 182 ± 19 nM and 128 ± 4.5 nM, respectively (Figure 2). Kinase activity of PKG-Iα in the presence of 1 μM R domain returned to the basal level. High concentrations of R domain did not change basal activity of PKG significantly. These results establish that the cGMP-binding sites on R domain or PDE5 holoenzyme inhibit PKG activity by sequestering cGMP. The R domain and holoenzyme have similar inhibitory potencies.

4.5. Inhibition of activation of PKG-Iα or PKG-Iβ by phosphorylated R domain

Effects of phosphorylated R domain on activation of PKG-Iα by 200 nM and 60 nM cGMP were examined (Figures 3A and 3B). Compared with the effect of non-phosphorylated R domain, the phosphorylated R domain inhibited activation of PKG-Iα with 2-fold greater potency (IC_{50}=182 ± 19 nM and 100 ± 15 nM, respectively). The effect was similar at 60 nM cGMP where phosphorylated and non-phosphorylated R domain inhibited PKG activation with IC_{50} values of 100 ± 11 and 38 ± 1.7 nM, respectively. We also examined effects of phosphorylated and non-phosphorylated R domain on activation of PKG-Iβ, the other PKG-I isoform, by 200 nM cGMP (Figure 3C). This concentration of GMP activated
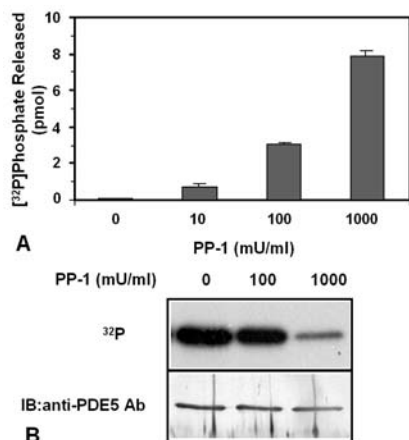
Figure 4. Dephosphorylation of phosphorylated R domain by PP-1. [^{32}P]phosphorylated R domain was prepared as described under "Materials and Methods." 100 nM of the [^{32}P]phosphorylated R domain was incubated for 60 min at 30º C in the absence and presence of various concentrations of PP-1. Reaction was terminated by addition of trichloroacetic acid to precipitate proteins in the mixture. After centrifugation, ^{32}P$_i$ released from [^{32}P]phosphorylated R domain into the supernatant was measured (A). Data are means of two independent experiments ± range. The same reaction mixtures were separated by SDS-PAGE and blotted onto the membrane. The membrane was analyzed by Western blotting with anti-PDE5 antibody, and then exposed to autoradiography (B).

the PKG-Iβ kinase activity approximately 2-fold. IC$_{50}$ values of the phosphorylated and non-phosphorylated R domain for inhibition of PKG-Iβ activation were 73 ± 0.35 nM and 197 ± 21 nM, respectively. The K$_D$ value of PKG-Iβ for activation of kinase activity was reported to be more than 2 to 3 times higher than that of PKG-Iα (1). Compared with the effect on PKG-Iα, inhibition of cGMP activation of PKG-Iβ by phosphorylated R domain was stronger.

4.6. Effects of PP-1 on inhibition of cGMP hydrolysis by phosphorylated R domain

In order to examine reversibility of the phosphorylated R domain effect on the C domain, 100 nM [^{32}P]phosphorylated R domain and various concentrations of phosphoprotein phosphatase 1 (PP-1) were incubated at 30° C for 60 min and then free ^{32}P$_i$ released by protein phosphatase action was determined. ^{32}P$_i$ from the phosphorylated R domain was released by PP-1 in a concentration-dependent manner (Figure 4A). Immunoblot using anti-PDE5 antibody and autoradiography of the same samples are shown in Figure 4B. These analyses demonstrated that PP-1 dephosphorylated phosphorylated R domain and that no proteolytic degradation of the proteins occurred during incubation.

100 nM phosphorylated R domain, which is the physiological concentration of PDE5 in rabbit corpus cavernosum, was used as starting material for examination of effects of dephosphorylation of this protein by PP1 on rate of cGMP hydrolysis by C domain. Phosphorylated R domain showed approximately 80% inhibition of cGMP

hydrolysis in a 10 min incubation (Figure 5A). Although cGMP was almost completely hydrolyzed by C domain in the absence of phosphorylated R domain after a 1 to 3 hr-incubation, phosphorylated R domain persistently inhibited cGMP hydrolysis by C domain during this long incubation. To test whether dephosphorylation of R domain would cause release of this inhibition, PP-1 was added to a reaction that had proceeded for 1 hr. Introduction of PP-1 brought about a significant increase in rate of cGMP hydrolysis, and a phosphoprotein phosphatase inhibitor, microcystin, blocked this increase (Figure 5A). These effects of PP-1 and microcystin on cGMP hydrolysis correlated well with the effects of these agents on ^{32}P content of R domain (Figure 5B).

Cyclic GMP binding to phosphorylated R domain was also measured in the same experiments (Figure 5C). Cyclic GMP binding to phosphorylated R domain reached a maximum within 10 min and then declined in response to the decline in free cGMP due to degradation of the nucleotide by C domain. PP-1 accelerated the decline of cGMP binding to phosphorylated R domain, and microcystin inhibited this effect. Free cGMP was also calculated based on the total bound cGMP and the cGMP that was hydrolyzed (Figure 5D). The amount of free cGMP in the presence of phosphorylated R domain did not change rapidly in both presence and absence of PP-1 during incubation, compared with the changes of bound and hydrolyzed cGMP. These results indicated that in response to dephosphorylation of phosphorylated R domain, cGMP bound to the R domain was relatively rapidly released and no longer protected from hydrolysis by C domain. Moreover, these findings strongly support the possibility that sequestration of cGMP by PDE5 can be regulated by the reversible phosphorylation-dephosphorylation of the enzyme.

5. DISCUSSION

Allosteric cGMP-binding sites of PDE5 and PKG have similar affinities for cGMP, and at physiological concentrations of the enzymes and the nucleotide, competition for cGMP is likely to significantly alter the efficacy of the cGMP signaling pathway. The role of cGMP bound to allosteric sites of PDE5 is a component of cellular cGMP that has not been fully considered previously, while cGMP/PKG cascade and cGMP hydrolysis have been well investigated. We have recently demonstrated that cGMP-binding sites on PKGs protect cGMP from hydrolysis by PDE5 and that this occurs at physiological concentrations of cGMP and PKG. (17). Furthermore, autophosphorylation of PKGs modulates the efficacy of cGMP protection. The present study has demonstrated that allosteric cGMP-binding sites of PDE5 could regulate cGMP signaling by sequestering cGMP from PKG and perhaps from the PDE5 catalytic site as well. The cGMP sequestered by binding of cGMP to PDE5 R domain significantly dampens PKG activation and hydrolysis of cGMP by PDE5 C domain.

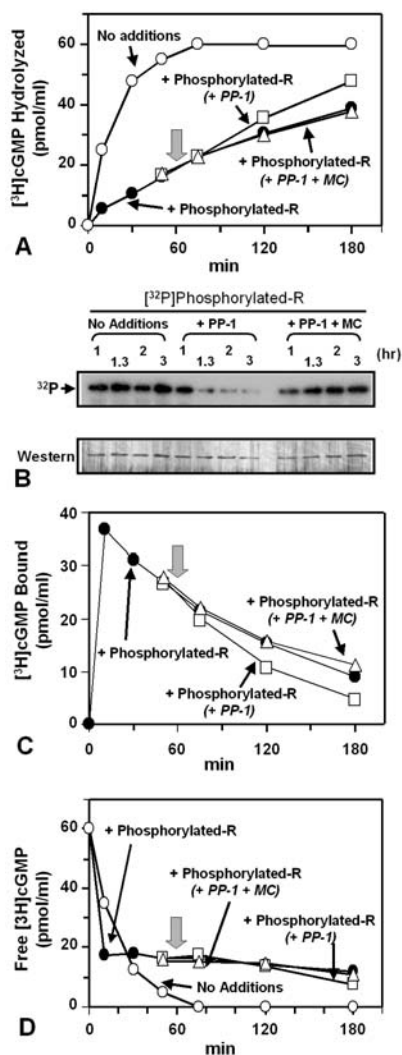The R domain of PDE5 includes the allosteric cGMP-binding sites, and this region of the protein can be

Figure 5. Effects of dephosphorylation of phosphorylated R domain on cGMP hydrolysis by PDE5 C domain. (A) [³H]cGMP and PDE5 C domain were incubated at 30° C for various periods in the presence and absence of phosphorylated R domain (100 nM), and then hydrolyzed [³H]cGMP was measured. After one hour of incubation, 1000 mU/ml PP-1 either with or without 1 μM microcystin (MC) was added to tubes that had already been incubated for 1 hr with phosphorylated R domain (shaded arrow), and then the incubation continued for an additional 2 hrs. Additions made at the 1 hr time point are shown in brackets and italics. (B) Autoradiography and immunoblot of the reaction mixtures by anti-PDE5 antibody are shown using [³²P]phosphorylated R domain under the same conditions as in panel A. (C) The amount of [³H]cGMP bound to R domain in the same experiment as panel A was measured. After incubation for indicated times, aliquots from the reaction mixtures were diluted with KP buffer, and immediately filtered onto a Millipore membrane. (D) Free cGMP was calculated based on the total bound cGMP and the cGMP that had been hydrolyzed in the same experiment as in panel A. The concentration of [³H]cGMP was 60 nM. Data show typical plots from at least three independent experiments.

phosphorylated at a single serine. Our previous report demonstrated that PDE5 holoenzyme or the isolated PDE5 R domain phosphorylated by either PKG or PKA showed 10 times lower K_D value of its allosteric binding sites for cGMP than the corresponding non-phosphorylated form (18, 23). It has been demonstrated that a 2- to 3-fold increase of cGMP by nitric oxide or natriuretic peptides can produce maximum relaxation of vascular smooth muscle (15). Considering the basal and stimulated levels of cGMP, in rabbit corpus cavernosum and pig coronary artery, cGMP concentrations would be expected to range from 20 to 60 nM and 90 to 270 nM, respectively. K_D values of phosphorylated and non-phosphorylated PDE5 holoenzyme for cGMP are 30 nM and 130 nM, respectively, which are very similar to the K_D values of phosphorylated and non-phosphorylated R domain shown here. Therefore, the amount of cGMP bound to PDE5 could be dramatically changed by the extent of PDE5 phosphorylation within the physiological range of cGMP concentrations in vascular tissues, especially in corpus cavernosum.

As reported here, cGMP bound to allosteric cGMP-binding sites of PDE5 is unavailable for cGMP signaling. Sequestration of cGMP could be regulated by phosphorylation of PDE5 under conditions that approach physiological concentrations of cGMP, PDE5, and PKG in intact cells. Phosphorylation of R domain increases affinity of the protein for cGMP. As a result, cGMP binding to phosphorylated R domain is increased 1.5-fold and 2.3-fold at 200 nM and 60 nM cGMP, respectively, and at both concentrations of cGMP, the phosphorylated R domain inhibition of PKG-Iα activation is approximately twice as potent as the non-phosphorylated form. This is in accordance with the ratio of cGMP binding to phosphorylated and non-phosphorylated R domain at each concentration of cGMP. By contrast, IC_{50} value of phosphorylated R domain for inhibition of cGMP hydrolysis is approximately 3-4 times more potent than that of non-phosphorylated R domain at 200 nM and 60 nM cGMP, respectively. These effects of phosphorylation of R domain on rate of hydrolysis of cGMP are in keeping with the increased cGMP binding affinity of the phosphorylated R domain versus the non-phosphorylated form.

It has been reported that PP-1 dephosphorylates phosphorylated PDE5 holoenzyme in intact cells (21). In order to establish that sequestration of cGMP by PDE5 allosteric sites can be regulated by dephosphorylation of PDE5, effect of PP-1 on inhibition of hydrolysis of cGMP by phosphorylated R domain has been demonstrated in the present study. Cyclic GMP bound to the R domain declines in response to dephosphorylation of the protein, and the cGMP released from the allosteric sites is rapidly hydrolyzed by C domain. Thus, phosphorylation and dephosphorylation of PDE5 could reversibly regulate sequestration of cGMP via modulation of cGMP-binding affinity of PDE5.

Regulation of cGMP signaling by PDE5 and PKG are summarized in Figure 6. A portion of cellular cGMP binds to allosteric cGMP-binding sites of PKG to activate the enzyme and cause many physiological effects
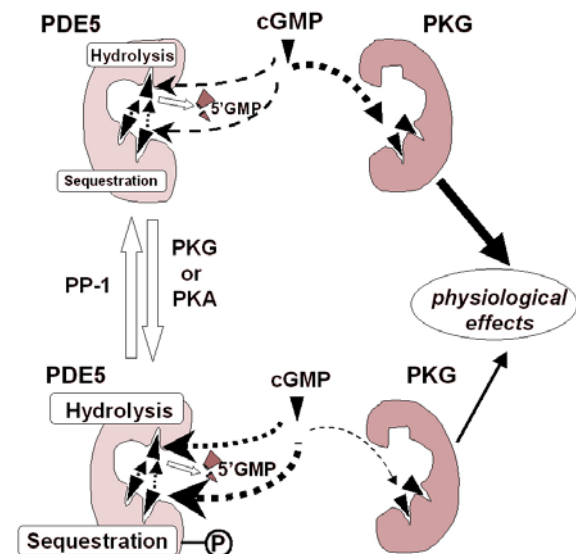
Figure 6. A proposed novel role of allosteric cGMP-binding sites of PDE5 in regulating cGMP-signaling. Sequestration of cGMP by PDE5 allosteric sites is regulated by phosphorylation and dephosphorylation *in vitro* and thereby could modulate physiological effects via the cGMP/PKG cascade.

via protein phosphorylation. A portion of cGMP also binds to allosteric sites of PDE5, and some cGMP is hydrolyzed by the PDE5 catalytic site. Two functions already established for cGMP binding to the PDE5 allosteric sites are 1) substrate-directed stimulation of phosphorylation of the enzyme by PKG or PKA (18, 20), and 2) stimulation of the catalytic site of the enzyme (22). We now propose that allosteric cGMP binding sequesters cGMP from its target receptors such as PKG. Cyclic GMP sequestered by PDE5 allosteric binding sites is temporally unavailable for PKG activation, for hydrolysis by PDE, or perhaps for other proteins such as cGMP-gated ion channels. Activated PKG phosphorylates the R domain of PDE5, which increases cGMP-binding affinity of PDE5 and increases cGMP sequestration by cGMP binding to PDE5. The increased sequestration of cGMP lowers free cGMP thereby reducing that which is available for PKG activation. Upon dissociation of cGMP from activated PKG, it would be less likely to be replaced by another cGMP, and the activation state of PKG would return to basal level.

We have reported that phosphorylation of PDE5 also increases cGMP hydrolytic activity (18, 20). Therefore, within the molecular environment of a single PDE5 molecule, phosphorylation would increase the cGMP occupancy of the allosteric sites as well as the catalytic activity. Upon cGMP dissociation from PDE5 allosteric sites, the nucleotide would be in close proximity to the catalytic site and may be more efficiently hydrolyzed. Increased sequestration and degradation of cGMP caused by phosphorylation of PDE5 would provide negative feedback control of stimulatory processes of cGMP signaling. It should be emphasized that in contrast to degradation and efflux of cGMP, the process of sequestration of cGMP would be largely reversible in response to change of intracellular free cGMP levels and phosphorylation and dephosphorylation of PDE5. If the bound cGMP escapes hydrolysis, it might also act as a reservoir of cGMP or for compartmentalization of cGMP signaling.

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