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Phosphorylation of phosphodiesterase-5 is promoted by a conformational change induced by sildenafil, vardenafil, or tadalafil

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1. ABSTRACT

Phosphodiesterase-5 (PDE5) inhibitors (sildenafil, vardenafil, or tadalafil) or phosphorylation by cyclic nucleotide-dependent protein kinase causes an apparent conformational change in PDE5, as indicated by a shift in migration on non-denaturing PAGE gels and an altered pattern of tryptic digestion. Combination of cGMP and a PDE5 inhibitor or phosphorylation does not cause a further gel shift or change in tryptic digest. Phosphorylation of PDE5 is stimulated by inhibitors, and combination of cGMP and inhibitor does not cause further phosphorylation. Dephosphorylation of PDE5 by either purified phosphoprotein phosphatase-1 or -2A catalytic subunit or by a crude phosphatase mixture is not affected by cGMP or inhibitors, suggesting that phosphorylation itself maintains conformational exposure of the phosphorylation site. The combined results imply that cGMP binding to the catalytic site initiates negative feedback control of many cellular cGMP signaling pathways by directly stimulating phosphorylation and activation of PDE5; by exploiting this molecular mechanism, PDE5 inhibitors stimulate their own potencies.

2. INTRODUCTION

Mammalian cyclic nucleotide phosphodiesterases (PDEs) are a superfamily of eleven enzymes that catalyze the hydrolysis of the cyclic nucleotides, adenosine or guanosine 3',5'-monophosphate (cAMP or cGMP, respectively) (1-6). Cyclic AMP and cGMP mediate myriad cellular processes (7, 8). PDEs play a critical role in attenuating and terminating cyclic nucleotide signaling events by blunting or lowering the intracellular concentration of these nucleotides. All mammalian PDEs contain a conserved catalytic domain of ~270 amino acids in the carboxyl-terminal portion of the protein and a less well conserved amino terminus that in many instances serves as a regulatory domain (R domain) (1, 4).

The cGMP-binding, cGMP-specific PDE, also known as phosphodiesterase-5 (PDE5), can be phosphorylated *in vitro* at a single serine (Ser-102 of human PDE5A1) or in intact cells by cGMP-dependent protein kinase (PKG) (9-12) or cAMP-dependent protein kinase (PKA) (13-15). Phosphorylation increases affinity of the catalytic site for cGMP, thereby activating PDE5

catalytic activity (9, 11, 13, 15-17). Recently, we showed that phosphorylation or cGMP binding slows mobility of the isolated R domain of PDE5 on non-denaturing PAGE (native-PAGE), consistent with an apparent conformational change in this region of the protein when it is phosphorylated or saturated with cGMP (18). While a number of studies on apparent conformational change induced by cGMP on PDE5 holoenzyme or isolated R domain have been performed (18-20), none has examined the effect of catalytic site-specific ligands on the conformation of intact PDE5. Apparent change in conformation of PDE5 and increased exposure of the phosphorylation site have been thought to be elicited exclusively by cGMP binding to the allosteric site (14, 15, 18). In the present study, we tested if: 1) PDE5 catalytic site occupation by substrate analogs such as PDE5 inhibitors causes an apparent conformational change in PDE5 that is similar to that produced by cGMP interaction with the enzyme or by phosphorylation, 2) an associated conformational change exposes the enzyme to cyclic nucleotide-dependent protein kinases for increased phosphorylation, and 3) the same ligand-binding reactions that promote phosphorylation of PDE5 also regulate dephosphorylation.

3. MATERIALS AND METHODS

3.1. Materials

[gamma-^{32}P]ATP was purchased from PerkinElmer Life Sciences (Boston, MA). Sildenafil was purified from Viagra® tablets using the method established in this laboratory (21). Tadalafil was synthesized based on the procedure of Daugan *et al*. (22). Vardenafil was provided by Bayer AG (Wuppertal, Germany). Cyclic GMP, cAMP, ATP, milrinone, rolipram, IBMX, okadaic acid, trichloroacetic acid, BSA, trypsin, PP1c and PP inhibitor-2 were from Sigma (St. Louis, MO). PP2A was purchased from Calbiochem (San Diego, CA). Microcystin-LR was from Alexis (San Diego, CA). Preformed native gels, native gel sample buffer and prestained low molecular weight SDS-PAGE standards were from Bio-Rad (Hercules, CA). Sypro Ruby protein gel stain was from Molecular Probes (Carlsbad, CA). S-200 HR resins were purchased from Amersham (Piscataway, NJ).

3.2. Expression and Purification of PDE5

His-tagged constructs of full-length bovine and human PDE5A1, and the isolated R domain fragment (residues 1-540) of human PDE5A1 were expressed and purified as previously reported (23). Sf9 cell pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl) containing protease inhibitor mixture (Complete™, EDTA-free, Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Cell suspension was kept on ice for 90 min, followed by homogenization of 20-ml aliquots on ice by 2 x 6-s bursts in an Ultra Turrex microhomogenizer (Tekmar, Mason, OH) with a 20-s recovery between bursts. Lysates were centrifuged (10,000 rpm) in a Beckman JA-20 rotor for 30 min at 4º C. The supernatant was diluted 3X with lysis buffer and then loaded onto a nickel-nitrolotriacetic acid agarose (Qiagen, Valencia, CA) column (0.9 x 2.5 cm) equilibrated with lysis buffer. The column was washed first with 20 mL lysis buffer, twice with 20 mL-aliquots of wash buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole), and then elution buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 150 mM imidazole) was soaked into the resin and stopped for 2 h at 4º C. Four eluants of 2.5 mL each were collected. Fractions containing PDE5 protein were pooled, and dialyzed against 200 vol dialysis buffer (50 mM Tris-HCl, pH 7.5, 25 mM 2-mercaptoethanol). Proteins were concentrated by dialysis against 50 mM Tris-HCl, pH 7.5, 25 mM 2-mercaptoethanol, 20 % v/v glycerol, flash-frozen in liquid nitrogen and stored at -70º C. The enzymes were more than 90 % homogeneous by 12 % SDS-PAGE followed by Coomassie Blue staining. Protein concentration was determined by the Bradford method (24) using bovine serum albumin (BSA) as standard.

3.3. Preparation of Canine Lung Homogenate

Fresh canine lung was minced and homogenized in 4X volume of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 25 mM 2-mercaptoethanol, 100 mM NaCl, 1 % v/v Triton X-100 with a Cuisinart apparatus (high speed, 3 bursts of 30 s each at 4° C). Supernatant fraction used for assays was obtained by centrifugation in a Beckman JA-20 rotor for 30 min (10,000 X g*)*.

3.4. Gel Electrophoresis

For SDS-PAGE, samples were boiled in 30 mM Tris-HCl, pH 6.8, 12.5 % v/v glycerol, 17 mM SDS, 160 mM 2-mercaptoethanol, 0.00125 % w/v bromophenol blue for 5 min and subjected to 12 % SDS-polyacrylamide gel electrophoresis. For native-PAGE, samples were combined with native-PAGE sample buffer (63 mM Tris-HCl, pH 6.8, 40 % v/v glycerol, 0.01 % Bromophenol blue Bio-Rad, Hercules, CA) and loaded into 50 microliter-wells of precast Tris-HCl acrylamide (7.5 or 10 %) gels (Bio-Rad). Electrophoresis was conducted at room temperature in native-PAGE running buffer (25 mM Tris, 192 mM glycine, pH 8.3, Bio-Rad) for 40 min at 200 V, according to the manufacturer's instructions. For studies of effects of ligands on PDE5 conformation, PDE5 holoenzyme (1 micromolar) was incubated in 50 mM Tris-HCl, pH 7.5, 5 mM Mg^{2+} acetate and 1.4 micrograms BSA in the absence or presence of cGMP (10 mM), inhibitor (25 micromolar), or a combination of cGMP (10 mM) and inhibitor (25 micromolar), in a final volume of 10 – 15 microliters for 30 min on ice before subjecting the proteins to electrophoresis. High concentrations of cGMP or inhibitors were necessary to achieve saturating levels over the 1 micromolar PDE5 that was needed for detection of the band on the gels. High levels of cGMP were also needed because of ongoing hydrolysis of cGMP by the PDE5 catalytic site. For studies of the effect of phosphorylation on PDE5 mobility, the enzyme was first phosphorylated (as described below in section entitled *Phosphorylation of Proteins*) before subjecting it to native-PAGE. Proteins were visualized by either Coomassie Blue or Sypro® Ruby staining. For Sypro Ruby staining, gels were incubated twice for 30 min each in 50 % v/v methanol, 7 % v/v acetic acid, stained overnight in Sypro Ruby protein gel stain (Molecular

Probes, Carlsbad, CA) and de-stained in 10 % v/v methanol, 7 % v/v acetic acid for 30 min. Gels were rinsed twice for 5 min each in water before visualizing under UV light using Molecular Imager FX (Bio-Rad).

3.5. Limited Tryptic Digest

PDE5 holoenzyme (1.2 micrograms) was incubated in 50 mM Tris-HCl, pH 7.5, and 5 mM Mg^{2+} acetate in the absence or presence of cGMP (10 mM) or inhibitor (25 micromolar), or a combination of cGMP (10 mM) and inhibitor (25 micromolar), in a final volume of 10 – 15 microliters for 30 min on ice. Trypsin [0.012 microgram, 1:100 w/w, trypsin to PDE5] was added and the reaction was incubated at 30º C for 10 min. The reaction was terminated by addition of SDS-PAGE sample buffer followed immediately by boiling for 5 min. The mixture was then subjected to 12 % SDS-PAGE, and the peptides were visualized by Coomassie Blue or Sypro Ruby staining.

3.6. Phosphorylation of Proteins

PDE5 holoenzyme and R domain were phosphorylated using purified bovine catalytic subunit (C subunit) of PKA (25). To test effects of cGMP and/or PDE5 inhibitor on phosphorylation, 1 micromolar PDE5 was added to PKA C subunit (0.2 micromolar) in a phosphorylation mixture [0.2 mM ATP (with trace [^{32}P]ATP), 50 mM Tris-HCl, pH 7.5, 5 mM Mg^{2+} acetate] in the presence of cGMP (10 mM), inhibitor (10 micromolar), or a combination of cGMP (10 mM) and inhibitor (10 micromolar) at 30º C. At indicated times, aliquots of the mixture were spotted onto phosphocellulose paper (P81), washed four times in 0.5 % phosphoric acid, dried, and counted. Concomitantly, aliquots were used for either SDS- or native-PAGE followed by autoradiography.

To prepare quantitatively phosphorylated PDE5 for phosphoprotein phosphatase studies, PDE5 holoenzyme (~6 micromolar) was phosphorylated at 30º C in a mixture using C subunit of PKA (~0.9 micromolar), in a final volume of 0.6 mL in the absence of cGMP. This design avoided potential carry-over of cGMP into the subsequent dephosphorylation reaction. After 1.5 to 2 h, aliquots of the reaction mixture were spotted on P81 paper, washed four times in 0.5 % phosphoric acid, dried, and counted to determine the incorporation of phosphate into PDE5. The rest of the reaction mixture was applied to a Sephacryl S-200 high resolution (HR) column (0.9 x 54 cm) equilibrated in 50 mM Tris-HCl, pH 7.5 and 100 mM NaCl; phospho-PDE5 was eluted using the same buffer. Fractions containing PDE5 were pooled or used separately for dephosphorylation experiments. Native RII alpha of PKA, which was used as a control substrate for the phosphoprotein phosphatase reaction, was phosphorylated under similar conditions, except that cAMP (1 mM) was added to the incubation.

3.7. Dephosphorylation of Phospho-PDE5

[^{32}P]Phospho-PDE5 was dephosphorylated using either purified catalytic subunit of phosphoprotein phosphatase-1 (PP1c), purified catalytic subunit of phosphoprotein phosphatase-2A (PP2A), or crude homogenate in buffer containing 0.5 mg/ml BSA, 1 mM Mn^{2+} chloride, 1 mM Mg^{2+} acetate, 1 mM dithiothreitol and 50 mM Tris-HCl, pH 7.5, in a total volume of 30 microliters. Dephosphorylation was conducted in the presence or absence of various ligands. RII alpha was used as control substrate for the phosphatase activity. At indicated times, the reaction was terminated by addition of trichloroacetic acid (15 % v/v final concentration), briefly vortexed, and kept at –20º C for 15 min, or at 4º C for at least 1 h. The reaction was then centrifuged at maximum speed in a bench-top microcentrifuge for 10 min at 4º C; an aliquot of the supernatant was removed and counted in aqueous scintillant to determine the ^{32}Pi released. Phosphatase activity was also measured in the presence of phosphoprotein phosphatase inhibitor-2, or a combination of microcystin and okadaic acid.

3.8. Statistical Analyses

All values are given as mean ± standard error of mean (S.E.M.) as determined by GraphPad Prism graphics software (GraphPad Software Inc., San Diego, CA). The software uses the following equation: S.E.M. = standard deviation/$n^{1/2}$, where standard deviation is determined as $[\Sigma(y_i - y_{mean})^2/(n - 1)]^{1/2}$. All S.E.M. values reported fit within a 95 % confidence interval, which quantifies the precision of the mean. The software was also used to generate curves and nonlinear regression lines.

4. RESULTS

4.1. Effects of Catalytic Site-Specific Ligands and Phosphorylation on Migration of PDE5 on Native-PAGE

Cyclic GMP binding to PDE5 has been shown to induce an apparent conformational change in the enzyme (19, 20, 26), as shown by slower migration of the protein on native-PAGE. Recently, we showed that cGMP binding to the isolated R domain of PDE5 also causes a similar change (18). To test whether binding of ligands to PDE5 catalytic site alone also affects the conformation of the enzyme, purified recombinant His-tagged PDE5 was pre-incubated on ice for 30 min in the absence or presence of catalytic site-specific inhibitors (sildenafil, vardenafil or tadalafil, 25 micromolar each) to saturate the PDE5 catalytic site, or sufficient cGMP (10 mM) to saturate both the allosteric and catalytic sites. Excess unbound ligands were then removed by subjecting the mixtures to native-PAGE as described under *Materials and Methods.* Figure 1 shows that in the absence of effectors, PDE5 migrated mainly as a single band, although a minor, more slowly migrating band was also present. Preincubation with cGMP shifted most of the PDE5 to a more slowly migrating band whereas migration of the BSA marker did not change. A similar position-shift of PDE5 was obtained by preincubation of PDE5 with 25 micromolar of either sildenafil, vardenafil or tadalafil (catalytic site-specific ligands). Combination of either inhibitor with cGMP (10 mM) did not cause a greater shift in mobility, although the proportion of the slowly migrating band increased. Milrinone (25 micromolar), a PDE3-selective inhibitor, or rolipram, a PDE4-selective inhibitor (25 micromolar), did not affect mobility. Despite the effect of catalytic site-

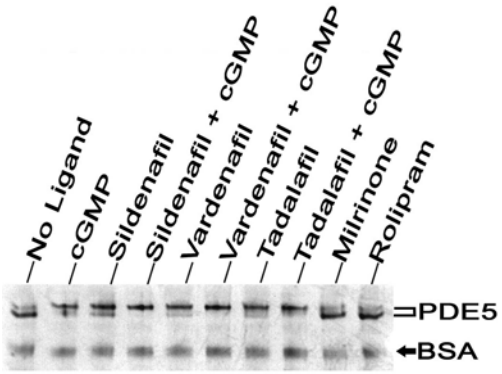

Figure 1. Effects of ligands on PDE5 migration on native-PAGE. Purified His-tagged PDE5 (1 micromolar) was incubated in the absence (no ligand) or presence of various ligands (cGMP, 10 mM, sildenafil, 25 micromolar; vardenafil, 25 micromolar; tadalafil, 25 micromolar; milrinone, 25 micromolar; rolipram, 25 micromolar) on ice in a total volume of 10 – 15 microliters as described under "Materials and Methods." After 30 minutes, 2X volume of sample buffer was added to the incubation and samples (1 microgram PDE5) were immediately subjected to native PAGE as described under "Materials and Methods" and stained with Sypro Ruby. BSA (1.4 micrograms) served as an internal control for gel loading and to show specificity of the ligand effect. The gel shown above is representative of many experiments, with two different protein preparations.

pecific ligands on mobility of PDE5, zaprinast, a PDE5 catalytic site-specific ligand, did not show a similar effect in an earlier report (18). The affinity of zaprinast for PDE5 is at least 100 times less than that of any of the PDE5 inhibitors used here; thus a concentration of zaprinast greater than 2.5 mM (more than the 1 mM in the report by (18) would be required to elicit an effect similar to that of sildenafil. Consistent with the effects of potent inhibitors (Figure 1), zaprinast (7.5 mM) caused a greater than 90 % shift of PDE5 from the lower to the upper band, but at less than 2 mM it produced a barely detectable shift (data not shown).

Migration of PDE5 that had been phosphorylated in the absence of any ligand using [gamma-^{32}P]ATP and a high concentration of PKA C subunit was also retarded on native-PAGE in a manner similar to that observed following preincubation with cGMP and sildenafil (Figure 2, top panel); autoradiography (Figure 2, botton panel) of the Coomassie blue stained gel (Figure 2, top panel) showed that radioactivity was associated only with the more slowly migrating protein band (Figure 2). Even when the stoichiometry of phosphorylation ranged from 0.1-1 mol per subunit, the radioactive band was associated only with the upper position shown in Figure 2 (results not shown). This suggested that either phosphorylation of a single subunit within the dimer is sufficient for the full mobility shift (conformational change), or that both subunits of the dimer are phosphorylated virtually simultaneously. A similar effect of phosphorylation on native gel migration of the isolated R domain of PDE5 was shown earlier (18).

4.2. Effects of Catalytic Site-Specific Ligands on Limited Tryptic Digestion of PDE5

To confirm that ligands were inducing a conformational change in PDE5, limited tryptic digests were employed. PDE5 was preincubated with cGMP (10 mM), various inhibitors (25 micromolar each), or a combination of each inhibitor and cGMP and subjected to limited trypsin digestion as described under *Materials and Methods*. As shown in Figure 3, the pattern of digestion was similar when proteolysis was performed in the presence of cGMP (lane 3) or PDE5-selective inhibitors (lanes 4-6), but the pattern differed from that produced by digestion of PDE5 in the absence of ligands (lanes 2 and 9). The peptide pattern generated in the presence of milrinone (lane 7), a PDE3-selective inhibitor, or rolipram (lane 8), a PDE4-selective inhibitor, was similar to that produced in the absence of ligand (lanes 2 and 9). Trypsin treatment in the presence of both cGMP and a PDE5 inhibitor (lanes 10-12) produced a pattern that was similar to that with either cGMP or inhibitor alone. Differences are clearly shown at the bands indicated by arrows ***a***, ***b***, ***c***, ***d*** and ***e***. In the presence of ligands that bind to PDE5, prominent peptide bands indicated by arrows ***a*** (~ 94 kDa) and ***d*** (~34 kDa) were observed. In the absence of any ligand, or in the presence of non-PDE5-selective inhibitors, peptide bands indicated by arrows ***b*** (~ 45 kDa), arrow ***c*** (40 kDa) and arrow ***e*** (32 kDa) were observed. These ligands did not affect the tryptic digest pattern of PKA C subunit, catalase, ovalbumin or thyroglobulin (data not shown).

4.3. Stimulation of PDE5 Phosphorylation by cGMP or PDE5 Catalytic Site-Specific Ligands

The apparent change in conformation of PDE5 induced by cGMP has been shown to provide for efficient phosphorylation of a serine in the R domain of PDE5 (Ser-92 of bovine PDE5 and Ser-102 of human PDE5) (14, 15, 18). It was thought that this cGMP effect was due to cGMP binding to the PDE5 allosteric site. Although in previous studies occupation of the catalytic site by IBMX under the conditions used did not promote phosphorylation (14), this possibility was re-evaluated since we observed effects of PDE5-selective inhibitors on PDE5 conformation similar to that induced by cGMP. Phosphorylation of PDE5 by PKA C subunit was stimulated in the presence of sildenafil in a time-dependent fashion in a manner similar to the stimulation observed with cGMP (Figure 4). Maximum phosphate incorporation into PDE5 in the presence of cGMP (10 mM) or sildenafil (10 micromolar) was ~1 mol phosphate per PDE5 monomer (Figure 4A). This incorporation was similar to values previously reported (14, 18, 19). Combination of cGMP and sildenafil, or extending the time of phosphorylation to 5 h did not increase phosphate incorporation further compared to that obtained in the presence of cGMP (data not shown). Aliquots of the reaction taken at various time points were subjected to SDS-PAGE, stained with Coomassie blue (Figure 4B), and then subjected to autoradiography (Figure 4C). The stimulation of phosphorylation shown in Figure 4A is also seen in the autoradiograph (Figure 4C) of the gel

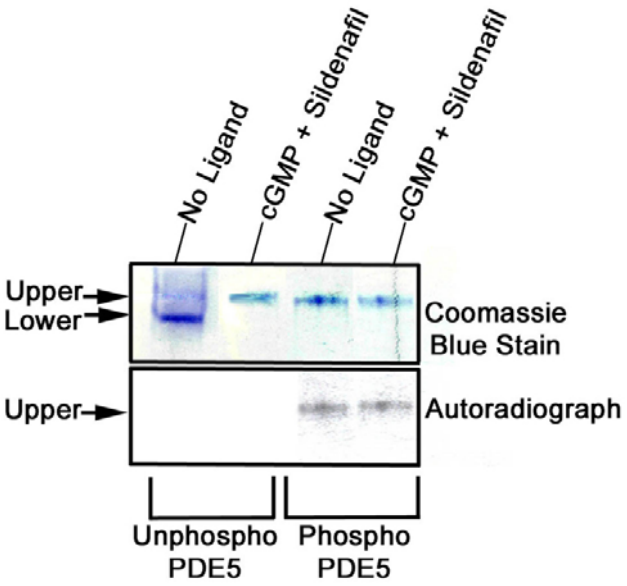

Figure 2. Effect of phosphorylation on PDE5 mobility on native-PAGE**.** PDE5 in the absence (no ligand) or presence of 10 mM cGMP and 10 micromolar sildenafil was incubated in the presence of C subunit (0.8 micromolar) and [gamma-^{32}P]ATP (0.5 mM) for 1 hour at 30º C, then subjected to native-PAGE as described under "Materials and Methods." *Top panel*: Coomassie blue stained gel. *Bottom panel:* Autoradiograph of gel shown in top panel. The gel and autoradiograph shown above are representative of many experiments, with two different protein preparations.

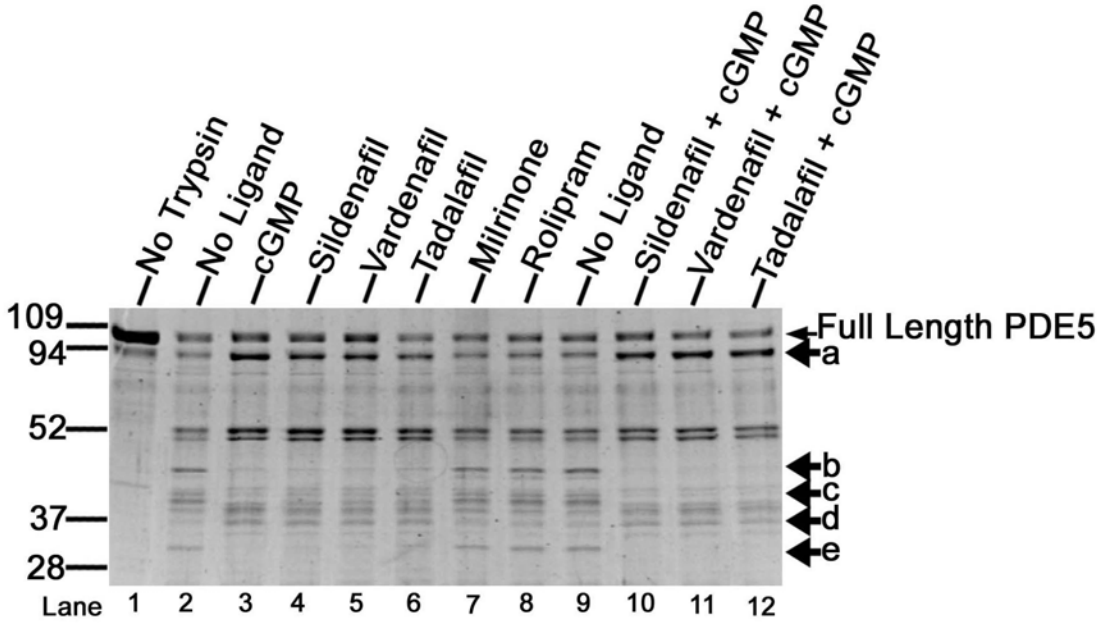

Figure 3. Effects of ligands on limited tryptic map of PDE5. PDE5 was prepared as described under "Materials and Methods." Aliquots of the reaction equivalent to 1.2 micrograms of PDE5 were then pre-incubated in the absence (no ligand) or presence of various ligands (cGMP, 10 mM, sildenafil, 25 micromolar; vardenafil, 25 micromolar; tadalafil, 25 micromolar; milrinone, 25 micromolar; rolipram, 25 micromolar) on ice. Trypsin (0.012 microgram) was added after 30 minutes pre-incubation, and the mixture was incubated at 30º C. After 10 minutes, SDS-PAGE sample buffer was added, and the samples immediately boiled for 5 minutes. The samples were then subjected to 12 % SDS-PAGE and stained with Sypro Ruby. Peptide fragments indicated by arrows ***a***, ***b***, ***c***, ***d*** and ***e*** clearly show differences in proteolytic digest in the absence or presence of PDE5-specific ligands. The gel shown above is representative of at least five experiments.

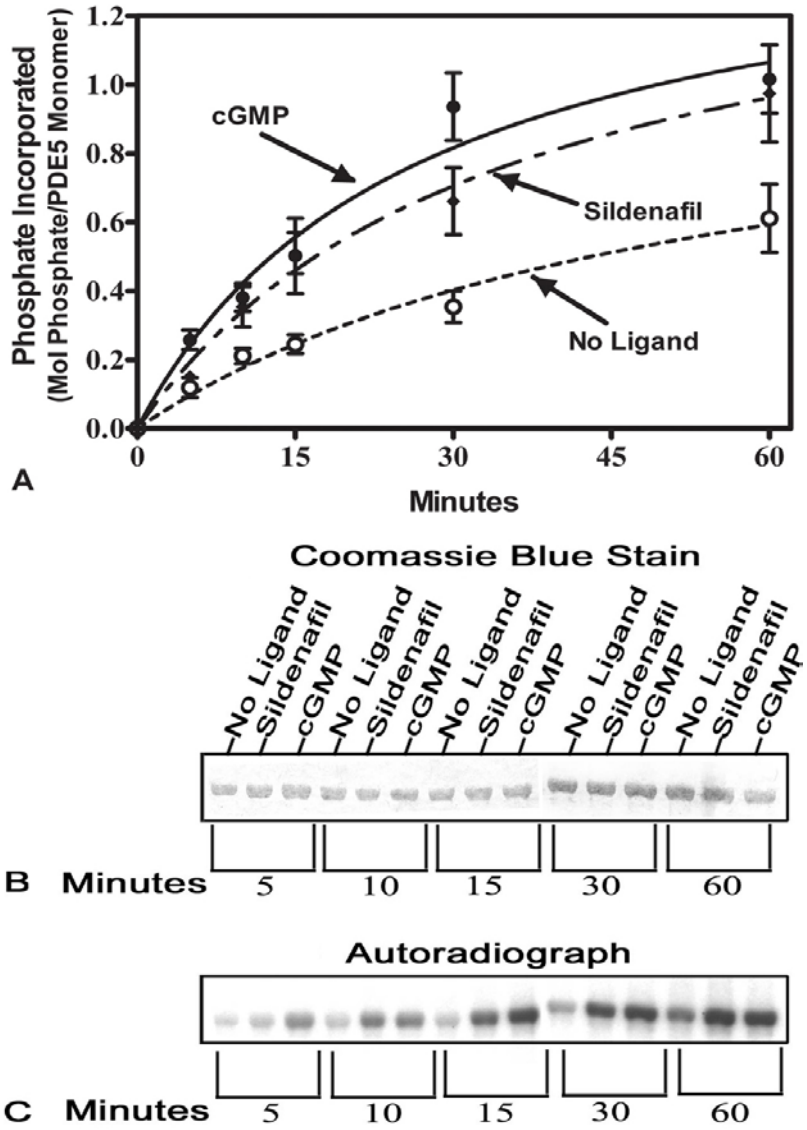

Figure 4. Effects of cGMP and sildenafil on PDE5 phosphorylation. Purified PDE5 (1 micromolar) was phosphorylated in the absence (no ligand) or presence of cGMP (10 mM) or sildenafil (10 micromolar) using purified C subunit (0.2 micromolar) and [^{32}P]ATP (0.2 mM). At the indicated times, aliquots of the reaction were either (***A***) spotted on phospho-cellulose paper, washed 4 times in phosphoric acid, dried and counted by Cerenkov radiation, as described under "Materials and Methods," or (***B***) subjected to SDS-PAGE, Coomassie blue stained, and (***C***) autoradiography. Results in ***A*** are the mean of four experiments, with each point performed in triplicate (no ligand, open circles; cGMP, filled circles; sildenafil, filled diamonds). Image in ***B*** is representative of four experiments. Autoradiograph in ***C*** is derived from gel shown in ***B***.

shown in Figure 4B. Cyclic GMP or sildenafil stimulation of PDE5 phosphorylation by PKA C subunit was also concentration-dependent (data not shown).

To ascertain the specificity of sildenafil stimulation of PDE5 phosphorylation, PDE5 (holoenzyme or R domain) was phosphorylated in the absence or presence of cGMP and various PDE inhibitors. As shown in Figure 5A, phosphorylation of PDE5 holoenzyme by PKA C subunit was stimulated more than 2-fold in the presence of PDE5 catalytic site-specific inhibitors in a manner similar to the stimulation in the presence of cGMP. Milrinone or rolipram, PDE3- and PDE4-selective inhibitors, respectively, did not affect PDE5 phosphorylation. Under the conditions used for the experiment shown in Figure 5, IBMX, a weak and nonspecific PDE inhibitor, also stimulated PDE5 phosphorylation at a concentration near its IC_{50} value (data not shown). In contrast, phosphorylation of R domain was not stimulated by catalytic site-specific ligands (Figure 5B), confirming that stimulation of holoenzyme phosphorylation by these ligands was due to interaction with the PDE5 catalytic site, and not to interaction with the kinase.

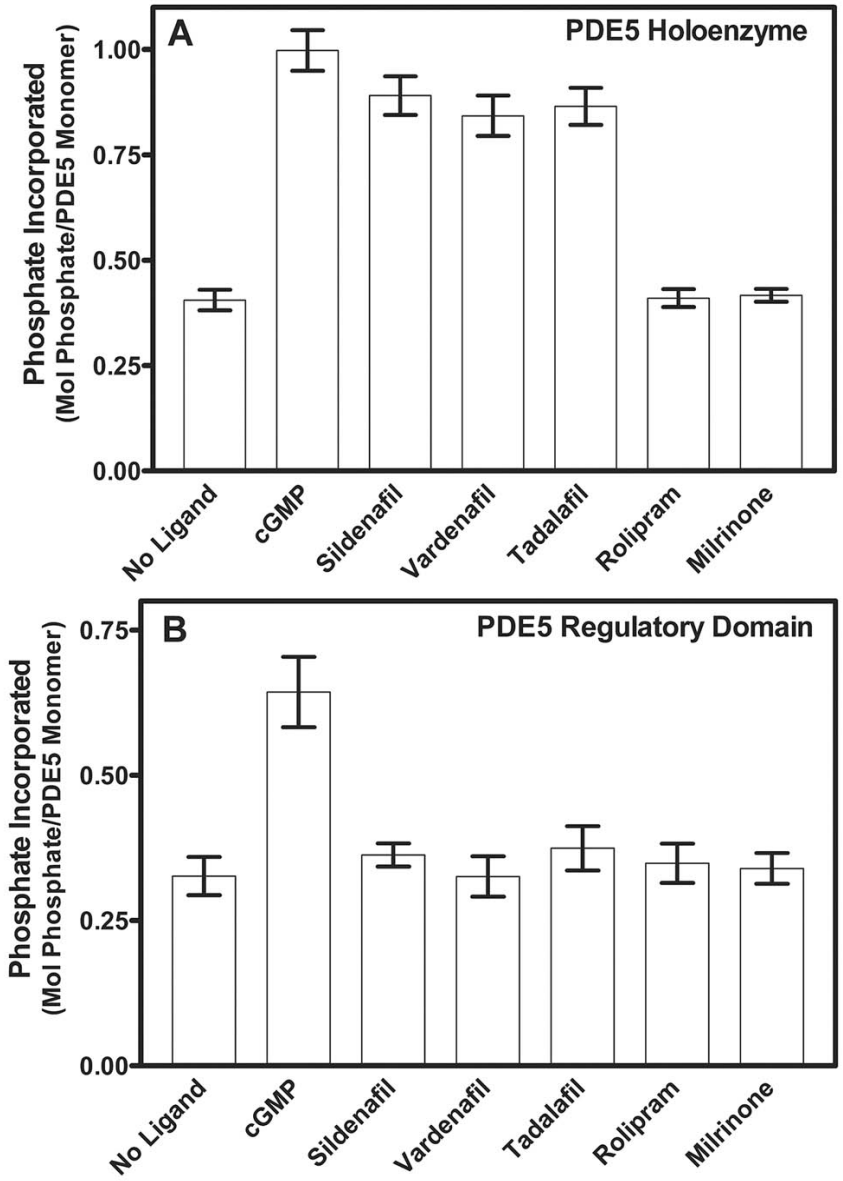

Figure 5. Effect of various ligands on PDE5 phosphorylation. Purified PDE5 (1 micromolar) was phosphorylated in the absence (no ligand) or presence of cGMP (10 mM) or various PDE inhibitors (10 micromolar each) using purified C subunit (0.2 micromolar) and [^{32}P]ATP (0.2 mM). After 30 minutes, the reaction was stopped by addition of EDTA (87.5 mM final concentration), aliquots from each incubation were spotted on phospho-cellulose paper, washed 4 times in phosphoric acid, dried, and counted by Cerenkov radiation, as described under "Materials and Methods." Panel ***A*** shows ligand effects on phosphorylation of PDE5 holoenzyme (n = 4, ± S.E.M.), and panel ***B*** shows lack of effects of ligands other than cGMP on phosphorylation of the isolated regulatory domain (n = 3, ± S.E.M.).

However, phosphorylation of R domain was stimulated in the presence of cGMP, as shown earlier (18).

4.4. Ligands Do Not Regulate Dephosphorylation of PDE5

Since substrate-directed factors can either enhance or impede dephosphorylation of a protein (27, 28), we tested whether dephosphorylation of PDE5 by phosphoprotein phosphatases is affected by a ligand-induced change in conformation of the enzyme. PDE5 was phosphorylated quantitatively (~0.7 - 1 mol phosphate per PDE5 monomer) in the absence of cGMP as described under *Materials and Methods*. Using PP1c, which has been shown to dephosphorylate PDE5 both *in vitro* and in intact cells (10), addition of cGMP (0.001 – 10 mM) or preincubation with cGMP (10 mM) did not affect the rate of dephosphorylation of PDE5 under conditions in which dephosphorylation was linear with time, and less than 30 %

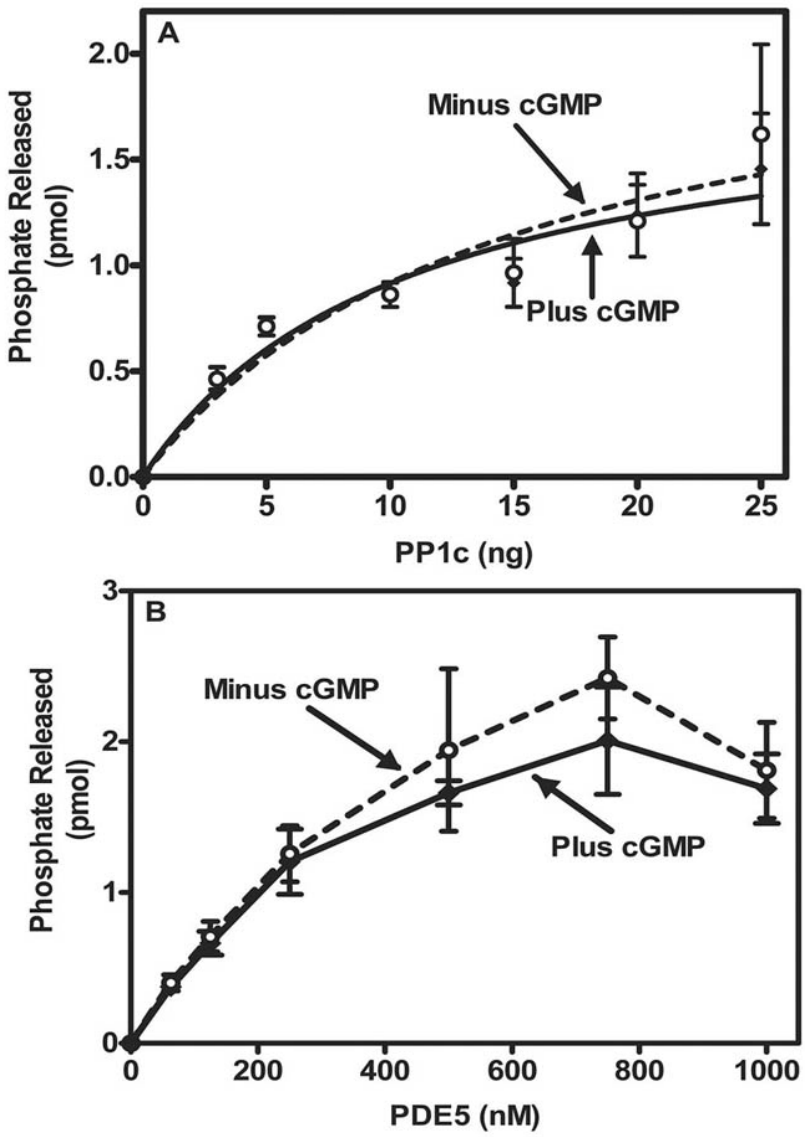

Figure 6. Effect of cGMP on PDE5 dephosphorylation by PP1c. [^{32}P]Phospho-PDE5 was incubated in the absence (open circles) or presence of cGMP (100 micromolar, filled diamonds). The dephosphorylation reaction was initiated by addition of purified PP1c catalytic subunit and the reaction was allowed to proceed for 15 minutes. After 15 minutes, the reaction was terminated by addition of trichloroacetic acid (15 % w/v final concentration). The reaction mixture was treated as described under "Materials and Methods," and aliquots were counted in aqueous scintillant. ***A***, PP1c concentration-dependence of dephosphorylation of PDE5 (267 nM). ***B***, PDE5 concentration on dephosphorylation, using 3 nM PP1c. Results are the mean of three experiments using two different protein preparations, with each point performed in triplicate.

of the total substrate was dephosphorylated (data not shown). There was also no effect of cGMP on dephosphorylation using various concentrations of PP1c (Figure 6A) or PDE5 (Figure 6B). Sildenafil (10 micromolar), 5'-GMP (0.001 – 10 mM) or cAMP (1 mM) did not appreciably affect PDE5 dephosphorylation by PP1c in a 15-min incubation (Figure 7). Cyclic GMP also did not affect the rate of dephosphorylation when PDE5 phosphorylated in the presence of cGMP was used as substrate for PP1c (data not shown). Dephosphorylation of PDE5 by PP2A was also unaffected by these ligands (data not shown). As shown in Figure 8, dephosphorylation of PDE5 by different dilutions of crude canine lung homogenate, which would contain phosphoprotein phosphatases and their regulatory proteins, was not affected by the absence or presence of cGMP, sildenafil, or a combination of cGMP and sildenafil. The initial rates of dephosphorylation of PDE5 by equimolar concentrations of purified catalytic subunits of PP1c and PP2A were similar (data not shown). Release of phosphate by these purified

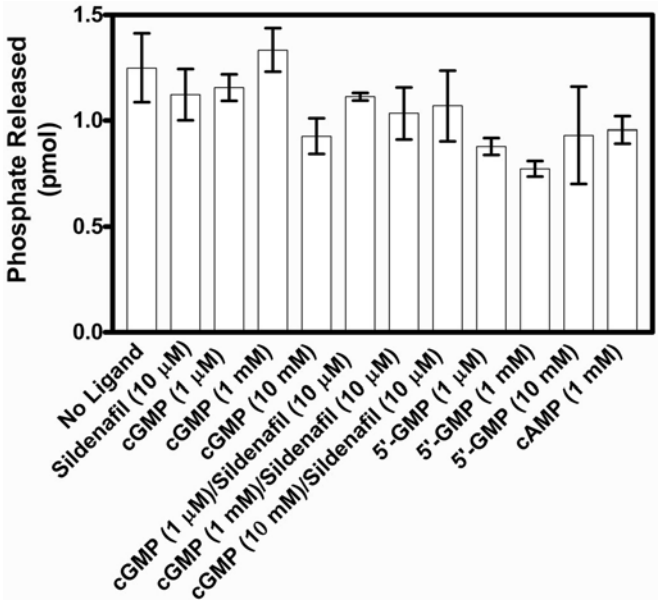

Figure 7. Effect of various ligands on PDE5 dephosphorylation by PP1c. Phospho-PDE5 (250 nM) was incubated in the absence or presence of various ligands as indicated. Dephosphorylation and subsequent treatment of the reaction was done as in Figure 6. Results are the mean of six experiments using two different protein preparations, with each point performed in triplicate.

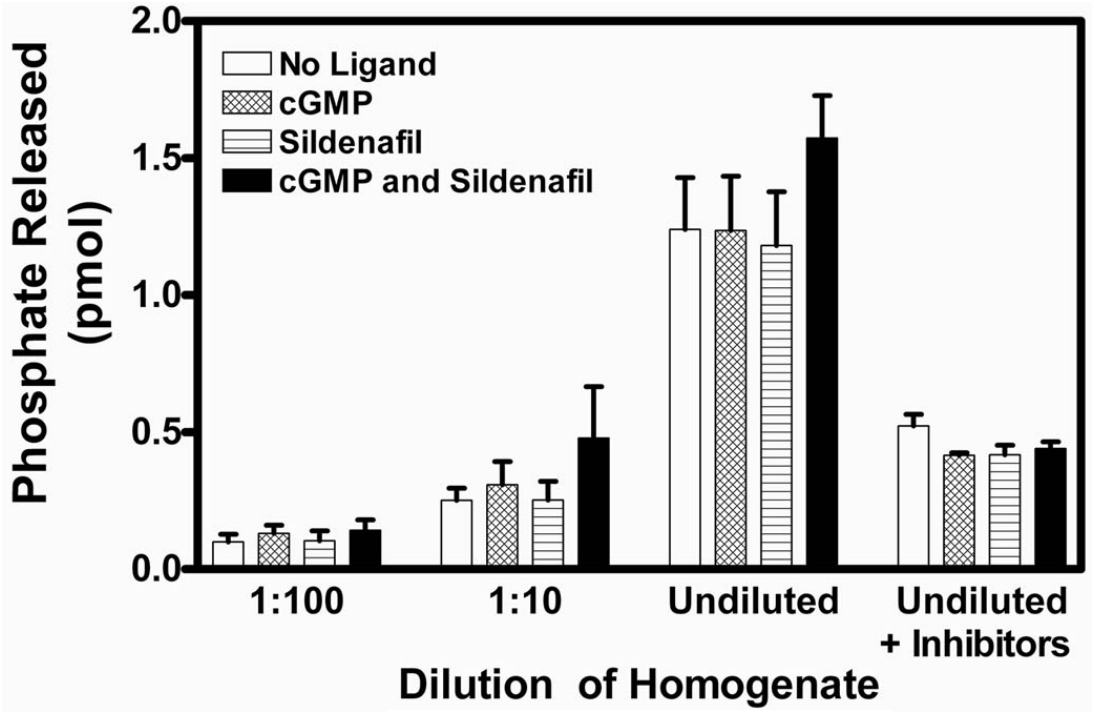

Figure 8. Dephosphorylation of PDE5 by phosphatases in crude canine lung homogenate. Phospho-PDE5 (500 – 600 nM) was incubated in the absence or presence of cGMP (10 mM), sildenafil (10 micromolar) or a combination of cGMP (10 mM) and sildenafil (10 micromolar). Dephosphorylation reaction was initiated by addition of 5 microliters of homogenate (of the indicated dilutions) and the reaction was allowed to proceed for 15 minutes. As a control experiment, a mixture of the phosphatase inhibitors, microcystin (187.5 nM) and okadaic acid (37.5 nM), was added to separate reactions. After 15 minutes, the reaction was terminated by addition of trichloroacetic acid (15 % w/v). The reaction was treated as described under *"Materials and Methods*," and aliquots counted in aqueous scintillant. Results are the mean of four experiments using two different protein preparations, with each point performed in triplicate.

phosphatases or phosphoprotein phosphatases in crude homogenate was inhibited potently (> 90 %) by PP inhibitor-2 (0.9 micromolar), or a combination of microcystin (0.05 micromolar) and okadaic acid (0.25 micromolar), indicating specificity of the assay for phosphoprotein phosphatases as opposed to proteases or some other degradative enzymes.

In the dephosphorylation studies, phospho-RII alpha of PKA was used as a control (results not shown) since it is an excellent substrate for PP1c and other phosphoprotein phosphatases (29, 30). Under our assay conditions (0.5 micromolar phospho-RII alpha or phospho-PDE5), PDE5 and RII alpha were dephosphorylated at a similar rate by PP1c; K_m values for dephosphorylation of phospho-PDE5 by PP1c in the absence or presence of cGMP were 514 or 409 nM, respectively, while the K_m values for phospho-RII alpha in the absence or presence of cGMP were 290 or 273 nM, respectively. These ligands had no effect on the rate of RII alpha dephosphorylation (data not shown).

5. DISCUSSION

Ligand-induced change in conformation occurs in at least two PDEs (PDE2 and PDE5) (18, 26, 31, 32) and is associated with changes in activity of these enzymes (15, 33). The present results using native gel electrophoresis and tryptic digests show for the first time that PDE5 inhibitors induce an apparent conformational change in PDE5. This change induced by inhibitors alone appears to be similar, if not identical, to that induced by high cGMP, conditions under which the catalytic site, as well as the allosteric site of the enzyme, is occupied, and the enzyme is in the activated state (9, 16). A combination of cGMP and inhibitor does not cause a further detectable change in electrophoretic migration of the protein. The apparent conformational change produced by inhibitors may underlie, at least in part, the stimulation of allosteric cGMP binding following inhibitor interaction with the catalytic site (34).

The results of native gel mobility shifts alone elicited by the inhibitors are consistent with increased elongation or surface electropositivity of the enzyme (35, 36). If an increased surface electronegativity occurs (26), it is apparently overridden by a more dominant change in elongation (35, 36). The two bands on native gels, which represent two apparent conformations of PDE5, are present in some protein preparations even without treatment with ligands or phosphorylation, suggesting the existence of a natural or intrinsic equilibrium that accounts for varying levels of the presumed active conformer in the basal state (16). This supports the interpretation that in the absence of either ligand binding to the catalytic site or phosphorylation of the R domain, the enzyme is primarily in a less active conformation, which is represented by the more rapidly migrating band. Addition of cGMP and/or phosphorylation converts the more rapidly migrating band (less active conformer) to a more slowly migrating band (more active conformer) of PDE5. Addition of PDE5 inhibitor also converts the more rapidly migrating band (less active conformer) to a more slowly migrating band (which exhibits the conformation of the more active conformer but is kinetically inactive because of the presence of the inhibitor). Occupation of the catalytic site, occupation of both allosteric and catalytic sites, or phosphorylation elicits a similar shift on native gels, but we cannot be certain that the conformers represented in each of these bands are identical. In fact, migration of unphospho- and phospho-isolated R domain in the presence of cGMP were both slowed on native gels, but migration of the bands was not identical, therefore suggesting some differences (18).

Phosphorylation alone increases affinity for ligands at the PDE5 catalytic site (9, 13, 17). Thus, the "principle of reciprocity" (37) or "linkage theory" (38) predicts that binding of ligands to the catalytic site would promote phosphorylation. The present results show that catalytic site-specific PDE5 inhibitors cause a conformational change that stimulates phosphorylation, which is the first demonstration of such a process for any PDE. This confirms the original prediction and supports the interpretation that optimum conformation of PDE5 for phosphorylation can be achieved by different molecular routes. The previous observation of substrate-directed (cGMP) regulation of phosphorylation of PDE5 (14, 15, 18) could be explained in part not only by cGMP binding to the allosteric site, which was first suggested, but also by cGMP binding to the catalytic site of the enzyme. Since cGMP binding to the allosteric and catalytic sites are mutually stimulatory, it is currently not possible to assess whether either one of these is quantitatively more stimulatory for phosphorylation in intact cells. Although there are numerous ligands that are specific for the PDE5 catalytic site, there are no compounds that exclusively occupy the allosteric cGMP-binding sites.

This is the first demonstration that occupation of the catalytic site of a PDE regulates phosphorylation of the enzyme. Based on this effect and the principle of reciprocity (37), it is predicted that phospho-PDE5 has increased affinity for inhibitor, thereby providing a feed-forward effect that increases the potency of these medications. Once PDE5 is phosphorylated, the phosphate could then maintain the enzyme in the more active conformation. This is supported by the studies of Mullershausen *et al*. (39). It is not likely that catalytic site occupancy exposes a site other than Ser-102 for phosphorylation since we previously showed that the homologous bovine Ser-92 was the only residue phosphorylated in the presence of cGMP and the non-specific PDE inhibitor, IBMX (14, 15), and in the present study a combination of cGMP and sildenafil did not increase phosphate incorporation into PDE5.

PDE5 phosphorylation was shown to be robustly stimulated in brain slices by PDE5 inhibitors (40). In some tissues, cGMP elevation would result from the presence of PDE5 inhibitors, which should stimulate phosphorylation due to both allosteric and catalytic site cGMP binding, but the inhibitors could also directly induce a conformational change in PDE5 that promotes efficient phosphorylation of the enzyme by activated PKG or PKA. Without this effect,

in some tissues such as penile corpus cavernosum, cGMP may not be significantly increased in the absence of sexual arousal induction of a robust agonist drive via nitric oxide. Moreover, while PDE5 phosphorylation would be relatively slow at basal cGMP since this nucleotide is required for activation of PKG (10, 11, 14, 40), activated PKA in the absence of PKG activation could cause some phosphorylation in the presence of the inhibitor-induced conformation of PDE5, thereby shifting the enzyme to a more activated state. This scenario is supported by the fact that PDE5 is slowly phosphorylated *in vivo* in PKG-deficient cells (with normal PKA levels) stimulated with a combination of 8-Br-cAMP and 8-Br-cGMP (10) or nitric oxide (9).

Even though regulation of phosphorylation of PDE5 by cGMP or inhibitors is substrate-directed (14, 15), dephosphorylation of the enzyme by a variety of phosphoprotein phosphatases is not regulated by ligands tested under the conditions used herein. That cGMP or PDE5 inhibitors have no effect on dephosphorylation of PDE5 suggests that phosphorylation maintains the enzyme in an optimum conformation for activity and for ready dephosphorylation. Together, our results show that ligand interaction with the catalytic domain of PDE5 induces an apparent conformation that is similar to that induced by phosphorylation and, in fact, exposes Ser-102 for phosphorylation; therefore, in this activated conformation binding of these ligands does not affect dephosphorylation of PDE5. Additionally, cGMP binding and/or phosphorylation maintains PDE5 in a conformation that promotes cGMP degradation through negative feedback regulation and inhibitor potency through a feed-forward process that exploits the mechanism of negative feedback regulation. Therefore, both cGMP-binding and phosphorylation processes represent potential negative feedback mechanisms for cGMP signaling in cells. Our report represents the first comprehensive attempt at deciphering the mechanism of regulation of dephosphorylation of any PDE and the findings herein significantly extend our understanding of the complex pharmacological action of PDE5-selective inhibitors that are currently in clinical use.

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Abbreviations: PDE: phosphodiesterase, GAF: mammalian cGMP-binding phosphodiesterase; Anabaena adenylyl cyclases; Escherichia coli FhlA, PKA: cAMP-dependent protein kinase, PKG: cGMP-dependent protein kinase, IBMX: 3-isobutyl-1-methylxanthine, PP1: phosphoprotein phosphatase-1, PP2A: phosphoprotein phosphatase-2A, BSA: bovine serum albumin, C subunit: catalytic subunit, R domain: regulatory domain

Key Words: Phosphodiesterase-5, Protein Kinase A, Protein Kinase G, cGMP, Phosphoprotein Phosphatase, PP1, PP2A, Phosphorylation, Dephosphorylation, Conformation, PDE5 Inhibitors, Sildenafil, Vardenafil, Tadalafil

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