Phosphorylation Increases Affinity of the Phosphodiesterase-5 Catalytic Site for Tadalafil

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ABSTRACT
Phosphodiesterase-5 (PDE5) is phosphorylated at a single serine residue by cyclic nucleotide-dependent protein kinases. To test for a direct effect of phosphorylation on the PDE5 catalytic site, independent of cGMP binding to the allosteric sites of the enzyme, binding of the catalytic site-specific substrate analog [3H]tadalafil to PDE5 was measured. Phosphorylation increased [3H]tadalafil binding 3-fold, whereas cGMP caused a 1.6-fold increase. Combination of both treatments caused more than 4-fold increase in [3H]tadalafil binding, and effects were additive only at submaximal stimulation. Consistent with the increase in affinity, phosphorylation slowed the [3H]tadalafil exchange-dissociation rate from PDE5 more than 6-fold. Finally, phosphorylation increased affinity for hydrolysis of a catalytic site-specific cGMP analog, 2'-O-antraniloylcGMP, by ~3-fold. The combined results showed that phosphorylation activates PDE5 catalytic site independently of cGMP binding to the allosteric sites. The results suggested that phosphorylation acts in concert with allosteric cGMP binding to stimulate the PDE5 catalytic site, which should promote negative feedback regulation of the cGMP pathway in intact cells. By increasing the affinity of the catalytic site, phosphorylation should also consequently increase the potency and duration of PDE5 inhibitor action.

Mammalian phosphodiesterases (PDEs) are a superfamily of metallophosphohydrolases that catalyze the hydrolysis of cAMP and/or cGMP (Conti and Beavo, 2007). Some of the 11 PDE families are regulated by cGMP binding to allosteric sites (Zoraghi et al., 2004; Beavo et al., 2006) and/or by phosphorylation (Beavo et al., 2006; Conti and Beavo, 2007). One PDE that is phosphorylated both in vitro and in vivo is the cGMP-binding, cGMP-specific phosphodiesterase, also known as PDE5 (Thomas et al., 1990b; Wyatt et al., 1998; Corbin et al., 2000). PDE5 plays a prominent role in cGMP breakdown in many tissues (Francis et al., 2001; Corbin et al., 2005), and it is the therapeutic target in the treatment of erectile dysfunction (Francis and Corbin, 2005) and pulmonary hypertension in humans (Leibovitch et al., 2007). PDE5 is a homodimer of ~100 kDa subunits, and each subunit contains an N-terminal regulatory domain and a conserved C-terminal catalytic domain (Thomas et al., 1990a; McAllister-Lucas et al., 1993). Within the regulatory domain are two tandem GAF (cGMP-stimulated PDE, Anabaena adenyl cyclase, Escherichia coli Fh1A protein) subdomains (Thomas et al., 1990a; McAllister-Lucas et al., 1993; Aravind and Ponting, 1997). Binding of cGMP to this subdomain stimulates phosphorylation of the enzyme by cyclic nucleotide-dependent protein kinase (Thomas et al., 1990b); binding of cGMP also stimulates catalytic activity (Okada and Asakawa, 2002; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003).

Although allosteric cGMP binding stimulates PDE5 catalytic function (Okada and Asakawa, 2002; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003), controversy exists on a direct effect of phosphorylation on the PDE5 catalytic site. Phosphorylation increases the affinity of both the allosteric and catalytic sites for ligand binding (Corbin et al., 2000; Francis et al., 2002). Increased cGMP hydrolytic activity of PDE5 in intact cells and crude lysates correlates with phosphorylation of the enzyme (Wyatt et al., 1998; Murphy, 2001; Rybalkin et al., 2002; Mullershausen et al., 2003; Shimizu-Albergine et al., 2003), but whether this effect of phosphorylation on the PDE5 catalytic site is direct, or whether it is an indirect stimulatory effect of phosphorylation on allosteric cGMP binding that then directly activates the catalytic site is unknown. Catalytic function has usually been assessed by electrophoresis; PKA, cAMP-dependent protein kinase; KP, potassium phosphate; Ant, 2'-O-antraniloyl.

ABBREVIATIONS: PDE, phosphodiesterase; PKG, cGMP-dependent protein kinase; TLC, thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis; PKA, cAMP-dependent protein kinase; KP, potassium phosphate; Ant, 2'-O-antraniloyl.
by measuring cGMP hydrolysis; however, cGMP interacts with both catalytic and allosteric sites, the latter of which has been shown to stimulate the former (Okada and Asakawa, 2002; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003).

Phosphorylation of PDE5 is tightly regulated under physiological conditions, requiring cGMP not only for increasing exposure of the PDE5 phosphorylation site, representing substrate activation, but also for activation of PKG for the phosphorylation of the enzyme (Thomas et al., 1990b; Turko et al., 1998a). Phosphorylation enhances and stabilizes cGMP-induced PDE5 activation, thus compromising increased cGMP production by guanylyl cyclase (Murthy, 2001; Mullershausen et al., 2003). Phosphorylation should also facilitate the persistence of PDE5 activation at low substrate concentrations (Mullershausen et al., 2003). Here, we use substrate analogs that interact only with the catalytic site of PDE5 to show that phosphorylation directly stimulates the PDE5 catalytic site independently of cGMP binding to the allosteric site.

**Materials and Methods**

**Materials.** [γ-32P]ATP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Tadalafil was synthesized based on the procedure of Daugen et al. (2003) and radiolabeled by Amersham Biosciences (Piscataway, NJ). G-25 Sephadex superfine resins and [6-3H]cGMP were purchased from Amersham. Cyclic GMP, isatoic anhydride, LH-20 Sephadex resins, ATP, potassium phosphate, histone IIAS, and bovine serum albumin were from Sigma-Aldrich (St. Louis, MO). Silica gel thin-layer chromatography (TLC) plates were purchased from Macherey-Nagel Inc. (Easton, PA).

**Expression and Purification of PDE5.** His-tagged constructs of full-length bovine and human PDE5A1, and isolated regulatory domain of human PDE5 (residues 1–540), were expressed and purified as described previously (Corbin et al., 2003; Blount et al., 2004; Zoraghi et al., 2005), with slight modification. SF9 cell pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, and 300 mM NaCl) containing protease inhibitor mixture (Complete, EDTA-free; Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Cell suspensions were kept on ice for 90 min, followed by homogenization of 20-ml aliquots on ice by 2 × 6-s bursts in an Ultra Turrex microhomogenizer (Tekmar-Dohrmann, Mason, OH) with a 2-s recovery between bursts. Lysates were centrifuged at 10,000 rpm in a Beckman JA-20 rotor for 30 min at 4°C. The supernatant was diluted three times with lysis buffer, and then it was loaded onto a nickel-nitrilotriacetic acid agarose (QIAGEN, Valencia, CA) column (0.9 × 2.5 cm) equilibrated with lysis buffer. The column was washed first with 20 ml of lysis buffer and then twice with 20 ml aliquots of wash buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 20 mM imidazole). Elution buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 150 mM imidazole) was soaked into the resin, and flow was stopped for 2 h at 4°C. Four eluants of 2.5 ml each were then collected, and elution fractions containing PDE5 protein were pooled and dialyzed against 200 volumes of dialysis buffer (50 mM Tris-HCl, pH 7.5, 25 mM 2-mercaptoethanol) overnight at 4°C. Proteins were then concentrated by dialysis against storage buffer (50 mM Tris-HCl, pH 7.5, 25 mM 2-mercaptoethanol, and 20% (v/v) glycerol), flash-frozen in liquid nitrogen and stored at −70°C. Purity of protein was >95% as assessed by 12% SDS-PAGE followed by Coomassie Blue staining. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

**Phosphorylation of Proteins.** PDE5 holoenzyme was phosphorylated using bovine catalytic subunit (C subunit) of PKA that had been purified to apparent homogeneity as described previously (Flockhart and Corbin, 1984). PDE5 (0.5–2 μM) was added to PKA C subunit (0.2–0.8 μM) in a phosphorylation mixture [0.2 mM ATP (with addition of trace [γ-32P]ATP), 50 mM Tris-HCl, pH 7.5, and 5 mM magnesium acetate] in the absence of cGMP at 30°C. After 1.5 to 2 h, aliquots of the reaction mixture were spotted on Whatman P81 papers, which were washed four times in 0.5% phosphoric acid, dried, and counted to determine the incorporation of phosphate into PDE5. The remaining reaction mixture was used immediately or stored at 4°C for not more than 24 h for use in subsequent experiments.

**[3H]cGMP Binding Assay.** PDE5 cGMP binding activity was measured as described previously using 0.4 μM [3H]cGMP in a total reaction volume of 100 μl (Corbin et al., 2000; Blount et al., 2004). For 2′-O-anthraniloyl cGMP (Ant-cGMP) competition binding studies, PDE5 (100 nM) was added to the reaction mixture in the presence of cGMP (0–100 μM) or Ant-cGMP (0–100 μM) on ice. After 30 min, 1 ml of cold KPM (10 mM potassium phosphate, pH 6.8, and 25 mM 2-mercaptoethanol) was added to the reaction. All binding reactions were vacuum filtered on Millipore filters (pore size, 0.45 μm) premoistened with 1 ml of KPM. The filters were rinsed twice with 1-ml aliquots of cold KDP, dried, and counted in 5 ml of nonaqueous scintillant.

**[3H]Tadalafil Binding Assay.** [3H]Tadalafil binding to PDE5 was determined as described previously (Blount et al., 2004). In brief, unphospho- and phospho-PDE5 (135 pM) were incubated in a 2-ml reaction mixture containing 0 to 6 nM [3H]tadalafil, 0.2 mg/ml type II-AS histone, and KPM in the absence or presence of 10 μM cGMP. After 30 min, 200 μl of 25% Triton X-100 in KP (10 mM potassium phosphate, at room temperature) was added to the reaction mixture, which was then vacuum-filtered on Millipore paper (0.45-μm pore size) premoistened with ice-cold 0.1% Triton X-100 in KP. The filter was then rinsed twice, each time with 1 ml of ice-cold 0.1% Triton X-100 in KP, dried, and counted in 5 ml of nonaqueous scintillant. For [3H]tadalafil dissociation/exchange experiments, PDE5 was incubated with 8 nM [3H]tadalafil; after 30 min, 1 ml of the reaction was added to 100 μl of 25% Triton X-100 in KP, and this mixture was vacuum-filtered on Millipore papers as mentioned above to determine binding time at 0 (B0). An ~15,000-fold excess unlabeled tadalafil was then added to the remaining reaction mixture, and at the indicated times (Bi), 1-ml aliquots were removed and subjected to vacuum filtration on Millipore filters as described above to determine [3H]tadalafil remaining bound. Papers were dried, and radioactivity was determined in 5 ml of nonaqueous scintillant. In these binding experiments, PDE5 was diluted in a solution of 2 mg/ml bovine serum albumin and KPM.

**Synthesis of Ant-cGMP.** Ant-cGMP was synthesized as described previously (Hirotasuka, 1982) with slight modification. In brief, 1 mmol of cGMP was dissolved in 10 ml of water at 38°C with stirring. The pH was adjusted to 9.6 with 2 N NaOH. Isatoic anhydride (1.5 mmol) was slowly added with continuous stirring, maintaining the pH at 9.6 by continuous addition of NaOH. After 2 h, a 6-ml aliquot of the reaction mixture was applied to a Sephadex LH-20 column (1.6 × 88 cm) wrapped in aluminum foil and pre-equilibrated in 30% (v/v) ethanol at room temperature. Components of the reaction were eluted with the same buffer under gravity. Fractions (4 ml) were collected, and fractions containing Ant-cGMP were identified by UV fluorescence and confirmed by TLC using cellulose gel. Pure fractions exhibiting only a single spot on TLC corresponding to Ant-cGMP were pooled, lyophilized, and then dissolved in 50 mM Tris-HCl buffer, pH 8.0. The absorbance and fluorescence profiles of the dissolved samples were then measured (see Spectral Measurements). Stocks of Ant-cGMP were stored in the dark at −20°C until ready for use, and experiments using Ant-cGMP were performed under low light conditions.

**Thin-Layer Chromatography.** TLC was conducted under conditions previously optimized by Hirotasuka (1982) using either silica or cellulose gels. TLC was performed with 1-propanol/NH4OH/water (6:3:1 v/v/v). After 1 to 2 h, Ant-containing compounds (Ant-cGMP
and Ant-cGMP) were detected on the chromatogram using a hand-held Blak Ray UV-lamp (365 nm; UVP, Inc., Upland, CA), and their positions were marked with pencil. cGMP was detected using a hand-held Mineralight UV-lamp (254 nm; Ultra-violet Products Inc., San Gabriel, CA) or by iodine staining in a closed chamber.

**Spectral Measurements.** After Ant-cGMP synthesis, absorption spectra were measured at room temperature with a DU-40 spectrophotometer (Beckman Coulter, Fullerton, CA). Samples were scanned from 200 to 450 nm. Fluorescence emission and excitation spectra were also measured at room temperature with a FlexStation II microplate reader using SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Samples were excited at 330 nm, and emission scans were measured from 360 to 600 nm.

**Ant-cGMP Hydrolysis by PDE5.** Ant-cGMP hydrolysis was conducted as described previously by Hiratsuka (1982), with modification. PDE5 concentrations and reaction time were determined from pilot studies to give kinetically valid data. Purified unphospho- and phospho-PDE5 (40 nM final concentration in reaction) were incubated at 30°C with 50 mM Tris-HCl, pH 7.5, and 0 to 200 μM Ant-cGMP in a total volume of 50 μl. After 10 min, the reaction was terminated by placing in a boiling water bath for 3 min; the reaction mixture was then centrifuged for 10 min in a benchtop microcentrifuge at maximum speed, and a 30-μl aliquot of the supernatant was subjected to TLC on silica gel. Ant-GMP remaining was visualized using a hand-held UV lamp, and then it was scraped from the TLC plate and eluted from the silica gel with 0.5 ml of 0.5 M Tris-HCl, pH 8.0. The mixture was centrifuged at maximum speed in a benchtop microcentrifuge for 10 min. A 150-μl aliquot of the supernatant was dissolved in 1 ml of N,N-dimethyl formamide, and fluorescence was measured by exciting the samples at 330 nm and emission measured at 410 nm. The amount of Ant-GMP was fluorometrically determined based on fluorescence of known amounts of Ant-cGMP.

**Statistical Analyses.** Values given are the mean ± S.E.M. as determined by GraphPad Prism graphics software (GraphPad Software Inc., San Diego, CA). The software uses the following equation: $y = y_0 + A \frac{x-x_0}{b}$. 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 non-linear regression lines. To determine significant difference, data were also analyzed using paired Student’s t test (two-tailed).

**Results**

**Cyclic GMP-Independent Phosphorylation of PDE5.** PDE5 has been shown to be phosphorylated at a faster rate by PKG than by PKA, and the reaction is stimulated by allosteric cGMP binding (Thomas et al., 1990b). However, using a high concentration of C subunit of PKA and a long incubation time, up to 1 mol of phosphate was incorporated per PDE5 monomer in the absence of cGMP (Fig. 1). This incorporation was similar to that obtained in the presence of high cGMP (10 mM), which used similar conditions to those used previously for maximum stimulation of phosphate incorporation (Thomas et al., 1990b; Corbin et al., 2000; Francis et al., 2002). Longer incubations up to 12 h did not further increase phosphate incorporated in the absence or presence of cGMP (data not shown).

**Phosphorylation of PDE5 Increases Its Affinity for [3H]Tadalafil Independently of Allosteric cGMP Binding.** To test for a direct effect of phosphorylation on PDE5 catalytic domain, which does not involve allosteric cGMP binding, we used [3H]tadalafil, a high potency PDE5 inhibitor that interacts as a substrate analog only with the catalytic site of the enzyme. $K_D$ for binding of [3H]tadalafil was determined by incubating unphospho- and phospho-PDE5 in the presence of increasing concentrations of [3H]tadalafil as described under Materials and Methods, and in the presence or absence of 10 μM cGMP. As shown in Fig. 2A, phosphorylation alone significantly increased the affinity for [3H]tadalafil by 3-fold ($K_D = 5.4 ± 1.7$ nM for unphospho-PDE5 to $K_D = 1.8 ± 0.37$ nM for phospho-PDE5; $P = 0.0058$). As shown in Fig. 2B, in the absence of phosphorylation, cGMP modestly but significantly increased the affinity of PDE5 for [3H]tadalafil by 1.7-fold ($K_D = 3.3 ± 0.62$ and 5.4 ± 1.7 nM in the presence and absence of cGMP, respectively; $P = 0.036$). The combination of cGMP and phosphorylation increased the affinity for the ligand by more than 4-fold ($K_D = 1.2 ± 0.32$ nM; Fig. 2B), compared with unphospho-PDE5 with no cGMP. The maximum bound [3H]tadalafil was similar under these different conditions (0.7–0.8 mol tadalafil/PDE5 monomer).

The effect of phosphorylation of PDE5 on the exchange-dissociation kinetics of [3H]tadalafil was also examined. Unphospho- and phospho-PDE5 were saturated with 8 nM [3H]tadalafil and, at time $t = 0$, aliquots were removed and filtered ($B_0$). Unlabeled tadalafil (~15,000-fold excess) was then added to the incubation, and aliquots were removed at various times and filtered as described previously (Blount et al., 2004) to determine [3H]tadalafil remaining bound ($B_t$) at a given time point. As shown in Fig. 3, the exchange-dissociation kinetics of [3H]tadalafil from unphospho-PDE5 displayed a curvilinear pattern, suggesting at least two rate components as was observed previously (Blount et al., 2004). The average half-life ($t_{1/2}$) for dissociation/exchange of [3H]tadalafil was 9.4 min. Phospho-PDE5 displayed linear kinetics for [3H]tadalafil dissociation/exchange, and $t_{1/2} (59$ min) increased more than 6-fold over that of unphospho-enzyme ($P = 0.0024$). This decrease in dissociation/exchange rate implied increased affinity, which was consistent with the results derived from the [3H]tadalafil binding isotherm shown in Fig. 2. The combined results from Figs. 2 and 3 showing effects of phosphorylation on [3H]tadalafil binding to PDE5 are summarized in Table 1.

![Fig. 1. Cyclic GMP-independent phosphorylation of PDE5. PDE5 was incubated with PKA C subunit and [γ-32P]ATP in the absence of cGMP at 30°C for 2 h. At the indicated times, aliquots of the reaction (15–20 pmol of PDE5 monomer, in triplicate) were removed and spotted on Whatman P81 filter paper (Whatman, Clifton, NJ), washed in H$_2$PO$_4$, dried, and counted by Cerenkov radiation as described under Materials and Methods. As control, PDE5 phosphorylation was also carried out in the presence of 10 nM cGMP (bar graph). The result shown is the mean ± S.E.M. of values from at least six experiments, using two different protein preparations, with each point performed in triplicate.](image-url)
Synthesis, Purification, and Characterization of Ant-cGMP. Ant-cGMP is a cGMP analog that has been shown to be hydrolyzed by PDEs in bovine heart (Hiratsuka, 1982) and rat cerebellar extracts (Okada and Asakawa, 2002). Using the method of Hiratsuka (1982), Ant-cGMP was synthesized with yields of approximately 60%. Rf values, absorption, and fluorescence profiles of the product were similar to published values and profiles. Based on kinetic data, Ant-cGMP does not bind to PDE5 allosteric sites (Hiratsuka, 1982; Okada and Asakawa, 2002). To confirm this, we tested whether Ant-cGMP binds to PDE5 allosteric sites by conducting a competition binding study. Initial results surprisingly showed competition of Ant-cGMP for allosteric cGMP binding to PDE5 even though TLC analysis indicated the product to be free of contaminants. Thus, we further purified the Ant-cGMP using a Sephadex G-25 superfine column (0.9 × 14 cm) equilibrated in 10 mM KP buffer, pH 6.8 (Fig. 4). The Sephadex G-25 profile suggested an 5% contaminant present (first peak) in the preparation. This contaminant was tentatively identified as cGMP based on its elution position (Francis et al., 1988) and UV absorption spectrum. This level of cGMP, not detected by UV on TLC, could interfere in reactions requiring significant levels of Ant-cGMP. The presence of a small amount of cGMP in the Ant-cGMP preparation was not surprising considering that cGMP was the starting material for Ant-cGMP synthesis. The more purified compound (second peak of the G-25 Sephadex profile), identified as Ant-cGMP by TLC analysis and fluorescent spectrum, did not compete with [3H]cGMP for binding to PDE5 holoenzyme (Fig. 5A) or isolated regulatory domain (Fig. 5B). Cyclic GMP effectively

Fig. 2. Effect of phosphorylation and cGMP on [3H]tadalafil binding to PDE5. Unphospho- or phospho-PDE5 (135 pM) was incubated in the absence (A, minus cGMP) or presence (B, plus cGMP) of 10 μM cGMP and increasing concentrations of [3H]tadalafil (0–6 nM) for 45 min in 2-ml reaction mixture as described under Materials and Methods. The reaction was then subjected to Millipore filtration, and filter papers were dried and counted in 6 ml of aqueous scintillant (n = 3; ±S.E.M.). PDE5 was phosphorylated as described in Fig. 1. Data were analyzed using paired t test.

Fig. 3. Effect of phosphorylation on [3H]tadalafil dissociation/exchange from PDE5. Unphospho- or phospho-PDE5 (400–800 pM) was incubated with 8 nM [3H]tadalafil for 30 min in 10 ml of reaction mixture as described under Materials and Methods. One-milliliter aliquots of the reaction were then subjected to Millipore filtration, and papers were dried and counted in 6 ml of aqueous scintillant (n = 4; ±S.E.M.). PDE5 was phosphorylated as described in Fig. 1.

Fig. 4. G-25 Sephadex superfine chromatography of Ant-cGMP. Three hundred microliters of 55 mM stock Ant-cGMP was subjected to G-25 Sephadex superfine chromatography (0.9 × 14 cm) in KP buffer (10 mM potassium phosphate; pH 6.8). Ant-cGMP was eluted with the same buffer under gravity, and 1.5-ml fractions were collected. Absorbance of each fraction at 260 nm was then determined. The result shown is representative of three experiments.

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<tr>
<th>[3H]Tadalafil Binding K_{D}</th>
<th>[3H]Tadalafil Dissociation t_{1/2}</th>
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<td>nM</td>
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<tr>
<td>Unphospho-PDE5</td>
<td>5.4 ± 1.7</td>
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<td>Phospho-PDE5</td>
<td>1.8 ± 0.37</td>
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Synthesis, Purification, and Characterization of Ant-cGMP.
competed with $[^3H]cGMP$ for binding to PDE5 holoenzyme and isolated regulatory domain.

**Phosphorylation of PDE5 Increases Ant-cGMP Hydrolysis.** Tadalafil is not degraded by PDE5. To test for an effect of PDE5 phosphorylation on hydrolytic activity independent of cGMP binding to the allosteric sites, we used purified Ant-cGMP, which is hydrolyzed by PDEs, but it does not bind to the allosteric sites (Hiratsuka, 1982; Okada and Asakawa, 2002; Fig. 5). Phosphorylation significantly increased the affinity of the catalytic site for Ant-cGMP hydrolysis by nearly 3-fold ($K_m = 24.7 \pm 6.7$ versus $65.1 \pm 18 \mu M$; $P = 0.001$). Even though analysis indicated that the maximum rate of hydrolysis of Ant-cGMP was not significantly affected by phosphorylation, the rate of hydrolysis at lower Ant-cGMP concentrations was more than 2-fold higher for phospho-PDE5 (Fig. 6). By comparing the results of Figs. 5 and 6, it can be seen that Ant-cGMP did not bind to the PDE5 allosteric sites even at the highest concentration (100 $\mu M$) used for the studies of Ant-cGMP hydrolysis.

**Discussion**

Using PDE5 that had been quantitatively phosphorylated in the absence of cGMP, and ligands that interact only with the catalytic site of PDE5, we demonstrate in this report that phosphorylation directly affects the catalytic site of PDE5 independently of cGMP binding to the allosteric sites. This effect of phosphorylation on the catalytic site affects affinity ($K_m$ or $K_p$) for Ant-cGMP or the inhibitor tadalafil, thus representing two ligands with very different structures. Our analyses suggest that the maximum rate of hydrolysis ($V_{max}$) of the substrate analog or maximum binding ($B_{max}$) of inhibitor is not significantly affected. Phosphorylation seems to affect the overall structure of the catalytic site because inhibitor binding and substrate hydrolysis are similarly affected, despite the fact that these different classes of ligands have different chemical structures and sizes, and that they make novel and distinct contacts within the catalytic pocket (Turko et al., 1998b; Sung et al., 2003; Huai et al., 2004).

Our results represent the first attempt to investigate a direct effect of PDE5 phosphorylation in the absence of cGMP binding to the regulatory domain. PDE5 is not a very good substrate for PKA compared with PKG (Thomas et al., 1990b; Corbin et al., 2000), but in the present study PDE5 has been phosphorylated in the absence of cGMP using high concentrations of C subunit of PKA. It is necessary to perform these experiments in the absence of cGMP because we have shown that phosphorylation increases the affinity of PDE5 allosteric sites for cGMP (Corbin et al., 2000; Francis et al., 2002), which could subsequently stimulate the catalytic site, thus complicating interpretation of results.

We have previously shown that phosphorylation of purified recombinant PDE5 increases hydrolysis of cGMP (Corbin et al., 2000). In addition, we and others have shown that PDE5 phosphorylation correlates with increased cGMP hydrolytic activity measured in extracts of treated cells (Wyatt et al., 1998; Murthy, 2001; Rybalkin et al., 2002; Mullershausen et al., 2003; Shimizu-Albergine et al., 2003). It is proposed that PDE5 phosphorylation provides a mechanism for negative feedback control that counters the intracellular cGMP increase. The allosteric effect of cGMP to increase PDE5 catalyzed breakdown of cGMP is mitigated by an increase in phospho-PDE5.
lytic activity represents one mechanism of negative feedback control of the cGMP pathway that has already been established. In concert, as intracellular cGMP increases and binds to PDE5 to expose its phosphorylation site, and PKG is simultaneously activated, PDE5 would be phosphorylated and activated, thereby increasing cGMP hydrolysis and enhancing the negative feedback effect mediated by allosteric cGMP binding.

This mechanism not only applies to negative feedback of the cGMP pathway but also to the pharmacological actions of PDE5 inhibitors; by increasing affinity of the catalytic site of PDE5 for ligands such as tadalafil, phosphorylation would potentiate the potency and pharmacological effects of PDE5 inhibitors (Corbin et al., 2003). Thus, the negative feedback mechanism for the cGMP pathway becomes a positive feedback mechanism for PDE5 inhibitors. This implies that lower dosages of the PDE5 inhibitors are used than would be needed otherwise for efficacy in treating patients. The effect of phosphorylation to improve inhibitor binding may also contribute to prolonged action (increased $t_{1/2}$) of the drug in vivo: increased smooth muscle cell retention of the inhibitor caused by improved inhibitor binding to PDE5 would render it less available for ultimate degradation by the liver or elimination by excretory processes.

The present results not only reveal another important mechanism for negative feedback regulation of the cGMP pathway (Table 2) but also further define the important role that PDE5 phosphorylation plays in this process. When intracellular cGMP is low, PDE5 would be predicted to exist in a low activity state. As the intracellular concentration of cGMP increases, PDE5 catalytic activity would increase due to increased substrate availability (mass action). Increased intracellular cGMP would not only increase allosteric cGMP binding by mass action, but interaction of this nucleotide with the catalytic site would induce a conformational change that would cause a further increase in allosteric cGMP binding (Francis et al., 1980). As a result, binding of cGMP to the allosteric sites would increase hydrolysis at the catalytic site (Okada and Asakawa, 2002; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003), further increasing the negative feedback effect on the cGMP pathway. Binding of cGMP to the allosteric sites would also contribute to the negative feedback response independently of catalytic activity by sequestering a significant portion of the cellular cGMP, therefore inhibiting effects of cGMP on PKG and other cGMP receptors (Gopal et al., 2001). Increased intracellular cGMP would also activate PKG and foster phosphorylation of PDE5 (Wyatt et al., 1998; Rybalkin et al., 2002; Mullershausen et al., 2003). Phosphorylation would then stimulate the allosteric and catalytic sites, thereby magnifying the catalytic and allosteric cGMP binding activities of PDE5 (Corbin et al., 2000), maximizing the negative feedback response and maintaining the activation state of the enzyme even at low concentrations of cGMP (Mullershausen et al., 2003). Thus, cGMP binding to the allosteric sites and phosphorylation work in concert to regulate PDE5 catalytic activity and to attenuate cellular cGMP signaling. In summary, at the molecular level the reciprocal interactions that occur among the catalytic, allosteric, and phosphorylation sites represent positive feedback control of PDE5 (Fig. 7; Table 2), resulting in negative feedback action on the cGMP pathway and positive feedback control on PDE5 inhibitor binding.

An effect of allosteric ligands or phosphorylation is usually accompanied by changes in structure and/or interdomain interactions of proteins. These modifications alter the equilibrium between at least two conformational states; the allosteric modifier or phosphorylation stabilizes one of these states. These effects have initially been observed in the AMP- and/or phosphorylation-dependent conversion of inactive, low affinity “T state” glycogen phosphorylase b to the active, high affinity “R state” glycogen phosphorylase a (Krebs and Fischer, 1956). In at least one other enzyme, PKG, phosphorylation (autophosphorylation) and/or cGMP binding relieves autoinhibition and increases catalytic and cGMP binding activities of the enzyme, and these effects are accompanied by an increased Stokes radius and slowed migration of PKG on native-PAGE, consistent with a change in conformation of

### Table 2

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<thead>
<tr>
<th>Action</th>
<th>Effect on PDE5</th>
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<tr>
<td>Mass action—increased in intracellular cGMP</td>
<td>Increased cGMP hydrolysis at the catalytic site</td>
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<tr>
<td>Interaction of cGMP or inhibitor with catalytic site</td>
<td>Increased allosteric cGMP binding</td>
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<tr>
<td>Binding of cGMP to allosteric site</td>
<td>Increased cGMP hydrolysis or binding of inhibitors at the catalytic site</td>
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<tr>
<td>Phosphorylation</td>
<td>Increased allosteric cGMP binding</td>
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</table>

![Fig. 7. Hypothetical model of PDE5 showing reciprocal stimulation by modifiers of different functional domains. The indicated reciprocal stimulations result in negative feedback effect on cGMP pathway or positive feedback effect on PDE5 inhibitor action. A and B show the respective GAF subdomains; S is the phosphorylatable serine (Ser-102 in human and Ser-92 in bovine PDE5). Revised figure with permission from Corbin and Francis (1999).](image)
the enzyme (Chu et al., 1998). Our previous report (Francis et al., 2002) showed that phosphorylation increases the affinity of PDE5 R domain for cGMP and affects the mobility of PDE5 R domain on native-PAGE, consistent with a change in conformation of the R domain. Phosphorylation also retards the migration of PDE5 holoenzyme on native-PAGE (Bessay et al., 2007), and the present study shows that phosphorylation increases the catalytic activity of the enzyme. The isolated catalytic domain of PDE5 may also display different conformations (Blount et al., 2004; Wang et al., 2006). Binding of inhibitor to PDE5 catalytic site retards the migration of holoenzyme on native-PAGE, consistent with a change in conformation (Bessay et al., 2007).

Regulation of PDE5 activity involves reciprocal interactions among several components of the enzyme (Fig. 7). Phosphorylation alone increases affinity for ligands at the PDE5 catalytic site; thus, the “principle of reciprocity” (Weber, 1975) would predict that binding of ligands to the catalytic site would promote phosphorylation. Indeed, we observe stimulation of PDE5 phosphorylation by catalytic site-specific ligands (Bessay et al., 2007). By concerted effect of all of these intramolecular processes, PDE5 becomes a better catalyst for dispensing cGMP-mediated signaling, or a higher affinity protein for binding PDE5 inhibitors.

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References


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