

Conversion of phosphodiesterase-5 (PDE5) catalytic site to higher affinity by PDE5
inhibitors

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The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; PKG, cGMP-dependent protein kinase; GAF, mammalian cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA; IBMX, 3-isobutyl-1-methylxanthine; KPM, 10 mM potassium phosphate (pH 6.8) containing 15 mM β -mercaptoethanol; KP, 10 mM potassium phosphate (pH 6.8); C domain, catalytic domain; R domain, regulatory domain; GAF, cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA.

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Abstract

Phosphodiesterase-5 (PDE5) specifically hydrolyzes cGMP, thereby contributing to modulation of intracellular levels of this nucleotide. In the present study, pre-incubation with cGMP increased PDE5 catalytic activity for cGMP degradation and converted the PDE5 catalytic site to a form that was more potently inhibited by each of the three PDE5 catalytic site-specific inhibitors; sildenafil, vardenafil, and tadalafil. These results implied that elevated cGMP initiates a physiological negative feedback on the cGMP pathway by increasing the affinity of the PDE5 catalytic site for cGMP. This increase in catalytic site activity or affinity for inhibitors could be caused by binding of cGMP to either the PDE5 allosteric sites, catalytic site, or both. Whether occupation of the catalytic site alone could mediate the effect was examined utilizing radiolabeled PDE5 inhibitors in the absence of cGMP. Exchange-dissociation of [³H]sildenafil (Viagra®), [³H]vardenafil (Levitra®), or [³H]tadalafil (Cialis®) from full-length PDE5 or isolated catalytic domain revealed two kinetic components (slow and fast). Extended pre-incubation of full-length PDE5, but not isolated catalytic domain, with ³H-inhibitors converted the biphasic pattern to a single slow (high-affinity) component. Studies of amino-terminally truncated PDE5 established that full-length GAF-B subdomain conjoined with the catalytic domain was sufficient for this conversion. In conclusion, binding of substrate or substrate analogs such as tadalafil and vardenafil to the catalytic site converts a fast (low-affinity) inhibitor dissociation component of the PDE5 catalytic site to a slow (high-affinity) inhibitor dissociation component. This effect is predicted to improve the substrate affinity or inhibitory potencies of these compounds in intact cells.

Introduction

Many proteins are postulated to be in dynamic equilibrium between two states, T and R [taut (T):less active with low affinity for substrate or ligand; relaxed (R): more active with higher affinity for substrate or ligand] (Monod et al., 1965). Interaction of such proteins with one or more ligands often perturb this equilibrium to favor one state over another. Many cyclic nucleotide phosphodiesterases (PDEs) exhibit less active and more active forms (Rocque et al., 1997; Soderling and Beavo, 2000; Francis et al., 2005; Conti and Beavo, 2007). Interconversion of R and T states could underlie these alternative activity states.

Mammalian PDEs are subdivided into 11 families based on elution position from ion-exchange columns, order of discovery, amino acid sequence, and specific catalytic and regulatory characteristics (Conti and Jin, 1999; Soderling and Beavo, 2000; Francis et al., 2001). The catalytic domain (C domain) of PDEs is highly conserved among PDE families. Amino- and extreme carboxyl-terminal sequences are divergent among PDE families and vary in length within families. In some PDEs, these sequences are responsible for selective regulatory characteristics such as binding of allosteric ligands or other small molecules and/or phosphorylation, which can influence the equilibrium of PDEs between less and more active states.

PDE5 is a homodimer; each monomer contains a regulatory (R) domain and a C domain that catalyzes breakdown of cGMP to 5'-GMP (Corbin and Francis, 1999). The R domain contains several functional subdomains including a phosphorylation site (Ser-102

in hPDE5), allosteric cGMP-binding sites, and dimerization contacts (Thomas et al., 1992; McAllister-Lucas et al., 1995; Zoraghi et al., 2005; Blount et al., 2006). Binding of inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) or sildenafil to the PDE5 catalytic site increases affinity of the allosteric site in the R domain for cGMP (Francis et al., 1980; Corbin and Francis, 1999). Allosteric binding of cGMP provided by GAF-A in the PDE5 R domain is kinetically heterogeneous, regulates cGMP-dependent exposure of the phosphorylation site, and increases affinity of the catalytic site for cGMP, thereby stimulating cGMP hydrolysis at sub-saturating levels of cGMP (Thomas et al., 1990b; Corbin and Francis, 1999; Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003; Blount et al., 2004; Zoraghi et al., 2005). Cyclic GMP binding to GAF-B has not been demonstrated but GAF-B is important in enzyme functions (Zoraghi et al., 2005). Stimulation of cGMP hydrolysis by PDE5 following cGMP elevation in intact cells has been demonstrated (Wyatt et al., 1998; Mullershausen et al., 2003; Rybalkin et al., 2003). Stimulation is likely due to cGMP binding to the high-affinity GAF-A site however cGMP binding to the low-affinity GAF-B site may also contribute to increased hydrolysis.

Conformational change induced by ligand binding occurs in several PDEs including PDE2, PDE4, and PDE6 (Stroop and Beavo, 1991; Artemyev and Hamm, 1992; Francis et al., 1998; Laliberte et al., 2000; Francis et al., 2003; Terry et al., 2003). Similarly, PDE5 undergoes ligand-dependent conversion from one physical state to another. Previous work in this laboratory indicates that allosteric cGMP-binding exists in more than one kinetic and physical form. Cyclic GMP exchange-dissociation kinetics for allosteric sites of PDE5 holoenzyme or isolated R domain are curvilinear (Thomas et al.,

1992; Francis et al., 2003; Zoraghi et al., 2005). Cyclic GMP binding to PDE5 stimulates phosphorylation, (Thomas et al., 1990b; Wyatt et al., 1998; Mullershausen et al., 2001; Francis et al., 2002) which activates catalysis (Corbin et al., 2000), and converts the biphasic kinetic profile of allosteric cGMP-binding to a single high-affinity species (Francis et al., 2002).

Using tritiated PDE5-catalytic site specific inhibitors (sildenafil, vardenafil, tadalafil) we found that PDE5 exhibits a fast component and a slow component of ^3H -inhibitor exchange-dissociation, but proportions of the components differ for each inhibitor (Corbin et al., 2003; Blount et al., 2004). Inhibitor binding to the catalytic site of PDE5 has recently been shown to stimulate phosphorylation consistent with the interpretation that inhibitors induce a perturbation in PDE5 structure (Bessay et al., 2007). Due to these findings, we hypothesize that the two kinetic components represent different states of PDE5 that are inter-convertible through a C domain-mediated process, although the R domain is likely to influence this conversion. This report demonstrates that occupation of the catalytic site of full-length PDE5 causes a time-dependent conversion of the enzyme to a form exhibiting a single slow-dissociation kinetic pattern; this implies conversion of PDE5 to a higher affinity form for these inhibitors. In addition, the presence of GAF-B in the R domain conjoined to the C domain is required for this process.

Materials and Methods:

Materials

[³H]cGMP and DEAE-Sephacel were purchased from Amersham Biosciences Inc. (Piscataway, NJ). 3-Isobutyl-1-methylxanthine (IBMX), histone type II-AS, *Crotalus atrox* snake venom, 5'-GMP, and cGMP were obtained from Sigma Chemical Co. (St. Louis, MO). Full-length recombinant bovine PDE5 was isolated from infected Sf9 cells using Ni/NTA agarose (QIAGEN) as described previously (Blount et al., 2004). Sildenafil was purified from tablets of Viagra™ following the method previously established in this laboratory (Corbin et al., 2003; Francis et al., 2003). Purified sildenafil was radiolabeled with tritium by Amersham Biosciences Inc. Tadalafil was synthesized according to US Patent Number 6,140,329 (Inventor Alain Claude-Marie Daugan: ICOS Corporation, Bothell, Washington). After confirming the compound structure by mass spectrometry, tadalafil was submitted to Amersham Biosciences Inc. for radiolabeling with tritium. HPLC results from Amersham indicated that [³H]sildenafil was > 98% pure while the [³H]tadalafil preparation was > 99% pure. Vardenafil and [³H]vardenafil were provided by Bayer HealthCare A.G., Wuppertal, Germany. All three ³H-inhibitors that had been stored for over a year were subjected to Sephadex G-25 chromatography, which tightly adsorbs PDE inhibitors and provides high resolution (Corbin et al., 2003; Francis et al., 2003). All three ³H-inhibitors were resolved in single peaks, co-eluted with purified unlabeled inhibitors and had IC₅₀ values similar to those of unlabeled inhibitors, suggesting that the ³H-inhibitors were unaltered after storage for one year.

Isolated C Domain

The C domain was constructed from bovine PDE5 cDNA to contain amino acids E535-Q860. Primers introduced *NdeI* and *XhoI* restriction sites to the 5' and 3' end of the fragment, respectively. After amplification, the DNA fragment was subcloned into the His-tagged expression vector pET15b (Novagen, Inc.). Next, four residues were mutated to change the DNA fragment from a bovine to human PDE5 sequence. The resulting plasmid was sequenced, determined to be correct, and then transformed into *E. coli* strain BL21 (Codonplus) for overexpression.

E. coli that contained the isolated catalytic (C) domain was grown in enriched LB medium (10 g bacto-tryptone, 5 g bact-yeast extract, 10 g NaCl per liter with 0.4% glucose, and 100 mg AMP added after autoclaving) at 37 °C to A600 = 0.7, and then 0.1 mM isopropyl β -D-thiogalactopyranoside was added for further growth at 15 °C overnight. Cells were centrifuged at 4000 rpm in a Beckman JA-10 rotor (Beckman Coulter, Inc., Fullerton, CA) for 10 min at 4 °C. The resulting pellet was resuspended in 4 ml extraction buffer [20 mM Tris base (pH 8), 0.3 M NaCl, 15 mM imidazole, 1 mM β -mercaptoethanol, and 1 EDTA-free Complete[®] protease inhibitor cocktail tablet per 50 ml solution (Roche Applied Sciences, Indianapolis, IN)] by pipetting up and down. Cells were homogenized by sonication using a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT) by pulsing four times at 1 min each. After centrifugation at 9000 rpm in a Beckman JA-20 rotor for 20 min, the supernatant was collected and loaded onto a 0.9 x 3-cm (~ 2 ml) Ni-NTA agarose column (QIAGEN) equilibrated with extraction buffer. The column was washed with 150 ml of a buffer containing 20 mM

Tris base (pH 8), 0.3 M NaCl, 15 mM imidazole, and 1 mM β -mercaptoethanol followed by a 50 ml wash with buffer that contained 20 mM Tris base (pH 8), 50 mM NaCl, 15 mM imidazole, and 1 mM β -mercaptoethanol. Isolated C domain was eluted from the Ni-NTA column with elution buffer [20 mM Tris base (pH 8), 0.3 M NaCl, 150 mM imidazole, and 1 mM β -mercaptoethanol] and collected in 1 ml fractions. Fractions were subjected to SDS-PAGE, protein assay (Bradford, 1976), and PDE activity analysis. Fractions that contained the C domain were pooled and flash frozen after adding glycerol to a final concentration of 20%.

PDE5 Truncation Constructs

Full-length human PDE5A1 cDNA (Tanabe-Seiyaku Pharmaceutical Co. Ltd., Saitama, Japan) was the template used to generate His-tagged PDE5A1 truncation mutants. All constructs were created by introduction of start and stop codons at the appropriate loci. Primers introduced *EcoRI* and *NotI* sites to the start and stop codons, respectively, for cloning into the vector. The resulting PCR fragment was inserted into pAcHLT-A vector (BD Pharmingen) that was linearized by digestion with *EcoRI* and *NotI*. Boundaries for GAF-A and GAF-B were selected based on homology with other GAF-containing PDEs (PDE2 and PDE6), because the precise boundaries of these functional modules are not known. The first construct (E420-N875) contained the full-sequence for GAF-B and extended through the C-terminus of PDE5 including the entire C domain. The second truncation construct (G466-N875) contained the C-terminal half of GAF-B and extended through the C terminus of PDE5 including the entire C domain. All constructs were confirmed by DNA sequencing before co-transfection with BaculoGold linear

baculovirus DNA (BD Pharmingen) according to the protocol from BD Pharmingen. Proteins were expressed in Sf9 cells, purified to near homogeneity using NTA-Ni resin, and characterized as described previously (Blount et al., 2006). Constructs were flash frozen in Tris buffer [20 mM Tris (pH 8), 50 mM NaCl] containing a final concentration of 20 % glycerol.

³H-Inhibitor Membrane Filtration Binding

Full-length bovine His-tagged PDE5 (80 μ l; 0.035 nM final) was added to 1 ml binding reaction mixture that contained 0.2 mg/ml histone IIA-S, various concentrations of ³H-inhibitor, and buffer that consisted of 10 mM potassium phosphate (pH 6.8) and 25 mM β -mercaptoethanol (KPM). ³H-inhibitor adhered to the test tube when added in the absence of or prior to addition of histone. Histone also increased retention of PDE5 on the Millipore membranes. Reaction mixture containing the enzyme was incubated on ice or at 30 °C for varying lengths of time as indicated. Millipore nitrocellulose membranes (0.45 μ m) were placed under house vacuum and prewetted with 1 ml cold 10 mM potassium phosphate (KP), pH 6.8, that contained 0.1% Triton X-100. Next, a 200 μ l aliquot of 25% Triton X-100 in KPM (room temperature) was added to the reaction tube. The entire contents of the tube were immediately applied to the pre-wetted filter. The reaction tube was then washed once with 3 ml cold 0.1% Triton X-100 in KP, and the wash was also applied to the filter. Filter membranes were removed, dried, and transferred to 6-ml scintillation vials. Five ml of non-aqueous scintillant were added to the tubes which were then placed in a scintillation counter.

PDE Activity

PDE activity was determined as previously described (Blount et al., 2006) with 0.4 μ M [3 H]cGMP as substrate.

Statistical Analyses

All values are given as mean \pm standard error of mean (SEM) as determined by GraphPad Prism graphics software. The software uses the following equation: SEM= standard deviation/ $n^{1/2}$, where standard deviation is determined as $[\sum(y_i - y_{\text{mean}})^2 / (n-1)]^{1/2}$. All SEMs reported fit within a 95% confidence interval, which quantifies the precision of the mean.

Results

Cyclic GMP pre-incubation stably stimulates PDE5 catalytic site activity

PDE5 catalytic activity was maximally stimulated after pre-incubation with 0.63 mM cGMP (Fig. 1). Higher cGMP concentrations caused no further increase. Stimulation by cGMP reached a maximum following 10 min pre-incubation and was stably maintained at 30 °C for 2 h after dilution (data not shown). Following pre-incubation, the extent of stimulation by cGMP was not affected by dilutions ranging from 300- to 96,000-fold followed by overnight incubation at 30 °C to foster cGMP dissociation. At 96,000-fold dilution the cGMP concentration was 0.025 μ M before the PDE assay. If the cGMP affinity for the allosteric sites was increased by cGMP pre-incubation, it is conceivable that the K_D approached or surpassed this value. Sildenafil (0.5-500 nM) or vardenafil (0.03 -30 nM) added to the pre-incubation under the same conditions had no significant effect.

Addition of cGMP increases inhibitor potency

Since PDE5 inhibitors are substrate analogs of cGMP, the results of Fig. 1 predicted that pre-incubation of PDE5 with cGMP would also increase affinity for PDE inhibitors. To test this, PDE5 was incubated in the absence or presence of cGMP (2.5 mM) at 4 °C for 0 min, 15 min or 5 h. The incubation mixture was then diluted 500-fold to lower cGMP to at least 5 μ M assuming that there was no cGMP hydrolysis during the incubation. PDE assays were performed on enzyme that was not pre-incubated with cGMP head-to-head with enzyme that had been incubated with cGMP followed by 500-fold dilution. The

activity of cGMP-treated and -untreated enzyme was the same indicating that cGMP had been significantly diluted to a level that did not have significant carry-over in the assay and therefore further cGMP stimulation of PDE5 catalytic activity was prevented (data not shown). The diluted enzyme was then added to a PDE assay reaction mixture that contained increasing concentrations of sildenafil, tadalafil, or vardenafil to measure PDE5 catalytic activity and determine IC_{50} values for each inhibitor.

Pre-incubation with cGMP had a dramatic effect on sildenafil potency for PDE5 (Fig. 2). Full-length PDE5 that was not pre-treated with cGMP and diluted 500-fold in buffer yielded an IC_{50} for sildenafil of 4.1 ± 0.6 nM, a value that was consistent with those from previous reports (Corbin et al., 2003; Blount et al., 2004). Full-length PDE5 pre-incubated for 15 min with cGMP was ~4-fold more potent for sildenafil (0.9 ± 0.2 nM). After 5 h of pre-incubation, PDE5 was ~8-fold more potent for sildenafil (0.5 ± 0.1 nM).

Cyclic GMP pre-incubations also increased the potency of tadalafil for the PDE5 catalytic site (Fig. 2). The IC_{50} for tadalafil without cGMP pre-incubation was 3.4 ± 0.7 nM. The IC_{50} decreased ~2-fold to 2.1 ± 0.4 nM after 15-min pre-incubation with cGMP. After 5 h of pre-incubation with cGMP, potency for tadalafil increased ~3-fold to 1.2 ± 0.2 nM. Vardenafil potency was also affected by cGMP (data not shown). Similar to tadalafil, potency was increased ~2-fold after a 15-min pre-incubation with cGMP and ~3-fold after 5 h incubation with cGMP.

Time-dependent conversion of PDE5 from a two-component to a one-component ³H-inhibitor exchange-dissociation pattern by pre-incubation with a catalytic site-specific ³H-inhibitor

Cyclic GMP increased the potency of the PDE5 catalytic site for PDE5-selective inhibitors in a time-dependent manner; however it was unclear if the effect was due to cGMP binding to the catalytic site or to the R domain. In the absence of cGMP, and using PDE5-specific inhibitors tadalafil and vardenafil as catalytic-site specific analogs of cGMP, the pattern of exchange-dissociation kinetics for each was employed to determine if the time-dependent increase in affinity of the catalytic site could be produced by ligand binding only to the catalytic site.

PDE5 was pre-incubated with saturating concentrations (30 nM) of ³H-inhibitor for either 15 min or 12 h at 4 °C. An aliquot was then removed to determine ³H-inhibitor binding at 0 time (B_0). Unlabeled inhibitor (~100-fold excess) was then added to the reaction mixture and aliquots were removed and filtered at various times (B_t) to follow the dissociation (exchange) of radiolabeled inhibitor from the enzyme. As seen in Fig. 3 (top panel), the [³H]tadalafil exchange-dissociation pattern after 15 min of pre-incubation was biphasic with approximately 50% of the binding associated with a slow-dissociation component and 50% being associated with a fast-dissociation component. When the resulting curve was fitted for one-site binding kinetics, the R^2 value was 0.2, indicating that the subsequent dissociation of tadalafil after 15 min pre-incubation was not linear. Pre-incubation under the same conditions for 12 h prior to initiating the exchange-dissociation yielded slow dissociation of the ligand and the pattern was linear via R^2

value of 0.8 when fitted to one-site binding kinetics. Total ^3H -inhibitor binding did not change by varying the pre-incubation time (data not shown). PDE5 was also pre-incubated with [^3H]tadalafil for 5 h prior to initiating exchange-dissociation, and the pattern was intermediate to those initiated after 15 min and 12 h pre-incubations. Approximately 70% of the dissociation existed in the high-affinity component and 30% existed in the low-affinity component (data not shown).

The same experiments were performed for [^3H]vardenafil exchange-dissociation (Fig. 3, bottom panel). After 15-min pre-incubation, [^3H]vardenafil dissociation exhibited two components; 70% of the dissociation was high-affinity and 30% displayed low-affinity. The goodness of fit for one-site binding kinetics gave an R^2 value of 0.3. A shift to essentially 100% higher-affinity component was observed after 12 h pre-incubation with [^3H]vardenafil. The R^2 value for this curve was 0.8, again supporting linearity and thus confirming the shift of the low-affinity component to a high-affinity component that was similar to the rate for the slow component for [^3H]tadalafil exchange-dissociation. Pre-incubation with [^3H]vardenafil for 5 h at 4 °C caused an intermediate conversion of the enzyme [high-affinity component (85%) and a low-affinity (15%), (data not shown)].

^3H -Inhibitor exchange-dissociation behavior reveals that the PDE5 isolated C domain also exhibits two components

Exchange-dissociation was performed for both [^3H]tadalafil and [^3H]vardenafil using isolated C domain (E535-Q860) in head-to-head experiments with full-length PDE5. The C domain and the full-length PDE5 were first saturated with ^3H -inhibitor (30 nM) for 30

min at 4 °C. Exchange-dissociation of the respective radiolabeled inhibitor from the enzyme after addition of excess unlabeled inhibitor was studied as described above. Tadalafil dissociation from full-length PDE5 showed a 70% high-affinity component and a 30% low-affinity component (Fig. 4, top panel). [³H]tadalafil dissociation from the isolated C domain exhibited a curve that was not significantly different from that of full-length PDE5. The data demonstrated that the isolated C domain, which is monomeric, also displays at least two exchange-dissociation components. The pattern of [³H]vardenafil dissociation from full-length PDE5 was identical to that for isolated C domain (Fig. 4, bottom panel). In this case the same proportion of the two components was observed for each protein (~ 80% in the high-affinity component and ~20% in the low affinity component). The combined results suggested that both full-length PDE5 and isolated C domain exist in at least two forms that have different catalytic site affinities for ligands. The relative amount of these forms varies depending on the ligand used. Full-length PDE5 is dimeric and isolated C domain is monomeric; therefore, the presence of two exchange-dissociation components requires neither dimerization nor the PDE5 R domain.

The low-affinity exchange-dissociation component of isolated PDE5 C domain is not converted by pre-incubation with inhibitors

After it was established that the isolated monomeric C domain of PDE5 was composed of two kinetically distinct inhibitor-dissociation components, an attempt was made to alter the proportion of each component by pre-incubation with [³H]inhibitors as was observed for full-length PDE5. Isolated C domain was pre-incubated at 4 °C for 15 min, 5 h, or 12

h with saturating concentrations of either [³H]tadalafil or [³H]vardenafil as described above. However, in contrast to the time-dependent shift in the pattern observed for full-length PDE5, pre-incubation of the isolated C domain with [³H]tadalafil did not significantly affect the biphasic dissociation kinetics (Fig. 5), i.e., the low-affinity component was not converted to the high-affinity component. The same experiment performed with [³H]vardenafil yielded similar results (data not shown), i.e., neither the 15-min, 5-h, or 12-h pre-incubation with [³H]vardenafil altered the exchange-dissociation profile. As noted above, for each inhibitor, pre-incubation altered the relative amounts of the two components, but total binding and the rate of each dissociation component did not change.

Truncated forms of PDE5 exhibit different patterns of conversion of exchange-dissociation components

Results described above suggested that the R domain contributes to the stable shift of the catalytic site to the high-affinity form. Previous studies indicated that specific regions of the R domain affect potency of certain classes of inhibitors (Blount et al., 2006). To determine the region of the R domain required for this shift, a series of amino-terminal truncations of PDE5 were made: PDE5 Δ 1-419 (E420-N875) contained GAF-B and C domain, and PDE5 Δ 1-465 (G466-N875) contained the carboxyl-terminal portion of the GAF-B sequence and the C domain. These two truncation constructs, full-length PDE5, or isolated C domain were assayed in a head-to-head manner following pre-incubation with [³H]tadalafil at 4 °C for 15 min, 5 h, or 12 h before measuring exchange-dissociation rates as described above (Fig. 6). Full-length PDE5 exhibited the same time-dependent

shift to one high-affinity component as previously observed (Fig. 3). Exchange-dissociation for PDE5 Δ 1-419, like full-length PDE5, revealed that pre-incubation with saturating [³H]tadalafil converts the enzyme into a form with a single, high-affinity component in a time-dependent manner (Fig. 6, top panel). After 15 min pre-incubation, 70% of the dissociation was associated with the high-affinity component while 30% was low-affinity. After 5-h pre-incubation, this mutant was converted into 80% high-affinity and 20% low-affinity components (data not shown). Similar to full-length PDE5, PDE5 Δ 1-419 exhibited only a single high-affinity component after 12 h of pre-incubation with [³H]tadalafil. Prolonged pre-incubation of radiolabeled tadalafil and the isolated C domain of PDE5 did not affect the biphasic dissociation kinetics of the inhibitor (Fig. 5). Like the isolated C domain of PDE5, PDE5 Δ 1-465 exhibited a stable biphasic dissociation pattern with 30% of the enzyme remaining in the low-affinity form and 70% remaining in the high-affinity form regardless of the time of pre-incubation (Fig. 6, bottom panel).

Discussion

Several reports have demonstrated that cGMP stimulates PDE5 activity (Corbin et al., 2003; Mullershausen et al., 2003; Rybalkin et al., 2003) but the mechanism that mediates this stimulation is unknown. A previous report by Rybalkin et al. demonstrated that PDE5 in freshly prepared crude extracts could be activated ~10-fold by cGMP with an EC₅₀ of ~0.1 μM; this activation was lost following storage of the enzyme. Cyclic GMP activated the purified PDE5 described herein by 2- to 3-fold with maximum effect at 630 μM cGMP. The lower degree of activation in the current study could relate to the differences in the enzyme preparations; the enzyme in the Rybalkin study was freshly prepared and not pure whereas PDE5 in this study was purified but had been stored for some time. If the enzyme is progressively activated with storage, this could account for the lower degree of stimulation by cGMP. Likewise, the difference in concentration of cGMP required for maximal stimulation may also relate to the differences in the enzyme preparations and might infer a role for cGMP binding to GAF-B in the context of the holoenzyme. It is established that PDE5 has at least two distinct binding sites for cGMP, represented by the catalytic site and the high-affinity GAF-A site, but whether GAF-B binds cGMP and thereby affects catalytic function is not known. Pre-incubation of PDE5 with saturating levels of cGMP for increasing periods of time rapidly converts the enzyme into a high-affinity state for catalytic-site specific compounds, but the mechanisms responsible for the increased potency of PDE5 have been elusive. GAF-B has been shown to impact affinity of GAF-A for cGMP (Zoraghi et al., 2005), therefore there is potential for alteration in the affinity for cGMP activation of the enzyme for

cGMP due to a change in the interaction between GAF-A and GAF-B in PDE5

holoenzyme

Studies have shown that full-length PDE5 may exist in at least two physical states (Thomas et al., 1990a; Blount et al., 2004). The present report is the first to demonstrate that ligand binding to the C domain in intact PDE5 can convert the lower affinity kinetic population of PDE5 to a higher affinity population. Moreover, we show here that the isolated monomeric C domain, like the isolated R domain, can also exist in at least two forms. It is presumed that the multiple states of full-length PDE5 can result from modifications of either the R domain, C domain, or both. When compared in a head-to-head manner with full-length PDE5, the isolated C domain displays similar biphasic kinetics of inhibitor exchange-dissociation. Therefore, the biphasic characteristics of ^3H -inhibitor dissociation from the C domain do not require influence from a kinetically heterogeneous R domain. However, unlike the behavior of full-length PDE5, the low-affinity kinetic component in the isolated C domain is not stably converted to the high-affinity component under the conditions used here. These observations suggest that portions of the R domain are required for stable conversion of a low-affinity state into a one high-affinity state. The fact that conversion of PDE5 to a higher affinity state requires considerably longer for inhibitors than for cGMP suggests either that inhibitors bind to the catalytic site by a different mechanism than does cGMP or that the cGMP effect is due to binding of this ligand to both catalytic and allosteric sites simultaneously.

These combined data show that PDE5 exists in at least two states that can be altered by ligand binding to the catalytic site. While this is a novel finding for PDE5, different kinetic forms of the catalytic site have previously been reported in other members of the PDE superfamily. Similar to PDE5, multiple forms of PDE4 have been characterized by different binding kinetics of the PDE4-specific inhibitor rolipram. PDE4 exhibits two kinetically distinct components of rolipram-binding; a low-affinity rolipram-binding component (LARBS) and a high-affinity rolipram-binding component (HARBS) (Schneider et al., 1986; Jacobitz et al., 1996). HARBS and the LARBS are more accurately described as two distinct binding-affinity states of the same catalytic site rather than separate sites (Jacobitz et al., 1996). Brain is rich in HARBS, with the percentage varying from 47 to 66% (Zhao et al., 2003). By contrast, only LARBS is detected in peripheral tissues. The identification of distinct pharmacological PDE4 forms may have therapeutic consequences since it has been hypothesized that inhibitors selective for LARBS and with low affinity for HARBS may have fewer side effects than those that preferentially interact with HARBS.

The PDE5 R domain contains several features that impact the catalytic site including: 1) a phosphorylation site (Ser-102 in hPDE5A) that, once phosphorylated, increases the affinity of the catalytic site for substrate, 2) a GAF-A domain that binds cGMP with high affinity ($K_D = 0.2 \mu\text{M}$), which then increases affinity of the catalytic site for substrate, and 3) a GAF-B domain which has recently been shown to contribute to potency of certain inhibitors (Blount et al., 2006). Results reported herein suggest that an intact GAF-B is also required for the shift of full-length PDE5 into a form with one high-affinity

component by pre-incubating with inhibitor. This effect of GAF-B on the catalytic site is a hitherto unknown function for PDE5. It has been previously reported that the high-affinity binding state of PDE4 for rolipram requires both the amino-terminal domain and the catalytic domain, while the low-affinity binding state requires only the catalytic domain (Rocque et al., 1997). Given these findings in conjunction with the results reported in this manuscript, it is suggestive that many PDEs may require some portion of the R domain for conversion to a high-affinity binding state.

Amino-terminal truncation constructs of hPDE4A revealed that amino acids 265-331 have no effect on catalytic activity or rolipram inhibition of the PDE4 catalytic site, but this deletion causes loss of high-affinity rolipram binding. This 66 amino-acid segment contains the C-terminal portion of the UCR-2 domain as well as the sequence between this domain and the PDE4 catalytic site (Jacobitz et al., 1996). In addition, the sequence containing GAF-B in PDE5 has recently been shown to influence affinity of the catalytic site for vardenafil-based inhibitors (Blount et al., 2006). These studies as well as the data reported herein demonstrate that amino acids adjacent to the catalytic site contribute importantly to high-affinity binding of PDE4- or PDE5-selective inhibitors.

Given the similarities between the characteristics of PDE4 and PDE5 conformers, it is possible that the ratio of the high-affinity ligand binding form to the low-affinity form will vary from tissue to tissue. Further experimentation using extracts of various tissues is needed to answer this question. The relative abundance of the two kinetic forms of PDE5 might differ in different intracellular compartments as well. This poses a

potentially interesting feature that will now have to be considered when examining the effects of cGMP hydrolysis and development of PDE5 inhibitors for clinical treatment. As demonstrated in the present report, the ratio of high-affinity and low-affinity forms of PDE5 in a given tissue could vary dramatically depending on the levels of natural ligands and exposure to drugs.

Several negative feedback mechanisms for the cGMP pathway involving PDE5 have been described (Corbin et al., 2003). One of these occurs by cGMP binding to the allosteric site of PDE5, which activates the catalytic site directly as well as by promoting phosphorylation of the enzyme. The present findings suggest an additional mechanism by which PDE5 is involved in negative feedback regulation of the cGMP pathway. The PDE5 inhibitors are considered to be substrate analogs that can be used to probe for actions of cGMP at the catalytic site. Results using these inhibitors imply that when cGMP increases, PDE5 should bind the ligand at the catalytic site and cause conversion to the high-affinity form, thus promoting more cGMP breakdown. This mechanism also has important pharmacological implications. The PDE5 inhibitors not only directly convert the catalytic site of the enzyme into a higher affinity state, but they also block cGMP hydrolysis, thereby enhancing elevation of cGMP. Both effects would convert the enzyme into the high-affinity state, thus accentuating the potency of each inhibitor. Sexual arousal is necessary for any of the PDE5-selective inhibitors to be effective in promoting increased tumescence of penile tissue. After the inhibitor is administered to a patient, it may be hours after absorption before sexual arousal occurs. In this scenario, there would be sufficient time for the inhibitor to bind to the catalytic site of PDE5 and

convert it into a high-affinity state. Therefore, after arousal the drug should be more effective, i.e., more potent, due to shift of the low-affinity form of PDE5 to the high-affinity form and, in turn, reversal of inhibition may be slowed. This could explain in part why many men taking PDE5 inhibitor-medications report that the drugs are still effective well after the reported pharmacokinetic plasma clearance ($t_{1/2}$) values for these inhibitors (Moncada et al., 2004; Gupta et al., 2005; Valiquette et al., 2005).

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Footnotes

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The abbreviations used are: PDE, cyclic nucleotide phosphodiesterase; PKG, cGMP-dependent protein kinase; GAF, mammalian cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA; IBMX, 3-isobutyl-1-methylxanthine; KPM, 10 mM potassium phosphate (pH 6.8) containing 15 mM β -mercaptoethanol; KP, 10 mM potassium phosphate (pH 6.8); C domain, catalytic domain; R domain, regulatory domain; GAF, cGMP-binding phosphodiesterase, *Anabaena* adenylyl cyclases, *Escherichia coli* FhlA.

Legends for Figures

Fig 1. Pre-incubation with cGMP increases PDE5 catalytic activity. 20 μ l (0.092 mg/ml) recombinant bovine PDE5 was added to a 5 μ l solution of KPM, bovine serum albumin (3mg/ml), and 10 μ l 0-10 mM cGMP. After 15 min at 30 °C, each reaction mixture was diluted 300-fold in KPM-1 mg/ml bovine serum albumin, and 100 μ l was added to 150 μ l PDE assay mixture for determination of PDE catalytic activity in a 5-min incubation at 30 °C as described in *Materials and Methods*. The final [3 H]cGMP substrate concentration was 0.36 μ M. The highest concentration of unlabeled cGMP that would have carried over from the pre-incubation into the PDE assay was 2.2 μ M; however, it was determined that 20 % of the cGMP was degraded in the pre-incubation. Therefore, the highest cGMP carry-over was 1.8 μ M. This could account for the slight inhibition seen at the highest cGMP concentration (2.4 mM) used in the pre-incubation. Data are derived from three experiments performed in triplicate. Carry-over of unlabeled cGMP into the PDE assay was not corrected for specific radioactivity change. Therefore, the cGMP stimulation observed could be underestimated.

Fig 2. Pre-incubation with cGMP increases potency of inhibition of PDE catalytic activity by PDE5 inhibitors. PDE5 (6 μ M) was pre-incubated for 0 min, 15 min, or 5 h at 4 °C in the absence and presence of cGMP (2.5 mM). After a 500-fold dilution, the enzyme was added to the PDE assay reaction mixture as described in Fig. 1 containing increasing concentrations of sildenafil (top panel) or tadalafil (bottom panel). PDE activity was determined in a 15-min incubation as described in *Materials and Methods*

using 0.4 μ M (final concentration) [3 H]cGMP as substrate. Data are derived from three experiments performed in triplicate.

Fig 3. Pre-incubation of PDE5 with 3 H-inhibitors for 12 h slows the exchange-dissociation rate. PDE5 (0.35 nM final concentration in assay) was added to 4.5 ml of binding reaction mixture containing either [3 H]tadalafil (top panel) or [3 H]vardenafil (bottom panel) at 30 nM (final concentration). To determine the zero time point, a 520- μ l aliquot of this mixture was filtered as described in *Materials and Methods*. Following 15-min or 12-h preincubation of the enzyme with either [3 H]tadalafil or [3 H]vardenafil, the stoichiometry of inhibitor binding (B_o) was not significantly different. 100-fold excess of corresponding unlabeled inhibitor was added to the remaining incubating binding reaction mixture at 4 $^{\circ}$ C after various pre-incubation times (15 min or 12 h). Aliquots were removed and filtered by the same procedure at the indicated time points. [3 H]tadalafil dissociation after 15 min, $n=9$; after 12 h, $n=5$. [3 H]vardenafil dissociation after 15 min, $n=6$; after 12 h, $n=10$.

Fig 4. The pattern of exchange-dissociation rates of 3 H-inhibitor from full-length PDE5 and isolated catalytic domain is similar. PDE full-length or isolated C domain (0.035 nM final) was added to 4.5 ml of binding reaction mixture containing either [3 H]tadalafil (top panel) or [3 H]vardenafil (bottom panel) (30 nM final concentration) and incubated for 1 h. The zero time point was determined as described in Fig 3. Following 15-min or 12-h preincubation of enzymes with either [3 H]tadalafil or [3 H]vardenafil, the stoichiometry of inhibitor binding (B_o) was not significantly different. 30 μ l of a 1 mM

solution of corresponding unlabeled inhibitor was added to the remaining incubating binding reaction mixture at 4 °C. Aliquots were removed and filtered by the same procedure at the indicated time points. [³H]tadalafil dissociation for full-length PDE5, $n=6$; for isolated C domain, $n=12$. [³H]vardenafil dissociation for full-length PDE5, $n=5$; for isolated C domain, $n=8$.

Fig 5. Exchange-dissociation of [³H]tadalafil from isolated catalytic domain is not altered by pre-incubation with inhibitor. PDE5 holoenzyme or isolated C domain (80 μM; 0.035 nM final) was added to 4.5 ml of binding reaction mixture containing either [³H]tadalafil (30 nM final concentration) and pre-incubated for either 15 min, 5 h, or 12 h at 4 °C. The zero time point was determined as described in Fig 3. Following 15-min, 5-h, or 12-h preincubation of PDE5 isolated C domain with [³H]tadalafil, the stoichiometry of inhibitor binding (B_0) was not significantly different. 30 μl of a 1 mM solution of corresponding inhibitor was added to the remaining incubating binding reaction mixture at 4 °C. Aliquots were removed and filtered by the same procedure at the indicated time points. [³H]tadalafil dissociation after 15 min, $n=13$; after 5 h, $n=5$; after 12 h, $n=5$.

Fig 6. Exchange-dissociation of [³H]tadalafil from PDE5Δ1-419 but not PDE5Δ1-465 is affected by pre-incubation. PDE5Δ1-419 (top panel) or PDE5Δ1-465 (bottom panel) (80 μl; 0.035 nM final) was added to 4.5 ml of binding reaction mixture containing [³H]tadalafil (30 nM final concentration) after being pre-incubated for either 15 min or 12 h at 4 °C. The zero time point was determined as described in Fig 3. Following 15-min or 12-h preincubation of the respective constructs with [³H]tadalafil, the stoichiometry of

inhibitor binding (B_0) was not significantly different. 30 μ l of a 1 mM tadalafil solution was added to the remaining incubating binding reaction mixture at 4 °C. Aliquots were removed and filtered by the same procedure at the indicated time points. [3 H]tadalafil dissociation for PDE5 Δ 1-419 after 15 min, $n=6$; after 12 h, $n=6$. [3 H]tadalafil dissociation for PDE5 Δ 1-465 after 15 min, $n=5$; after 12 h, $n=5$.

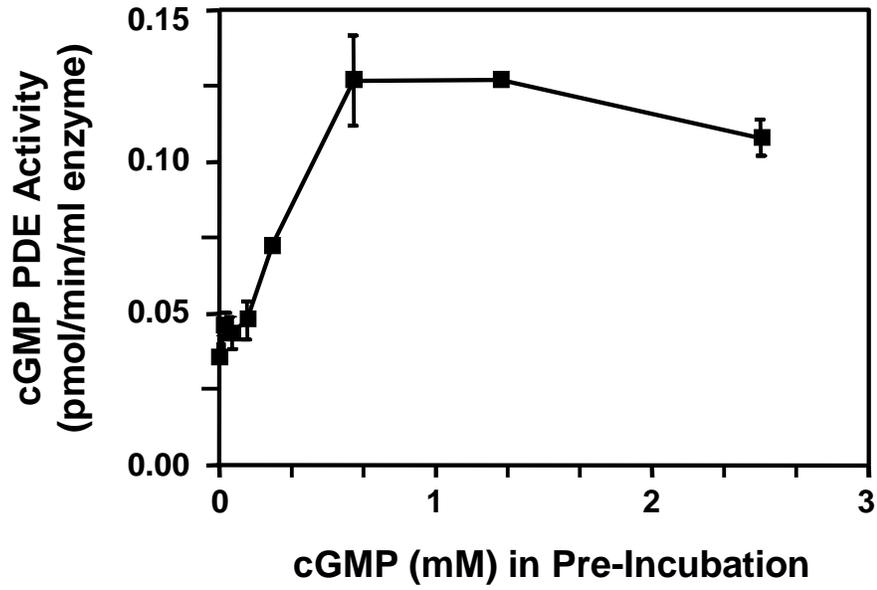


Figure 1

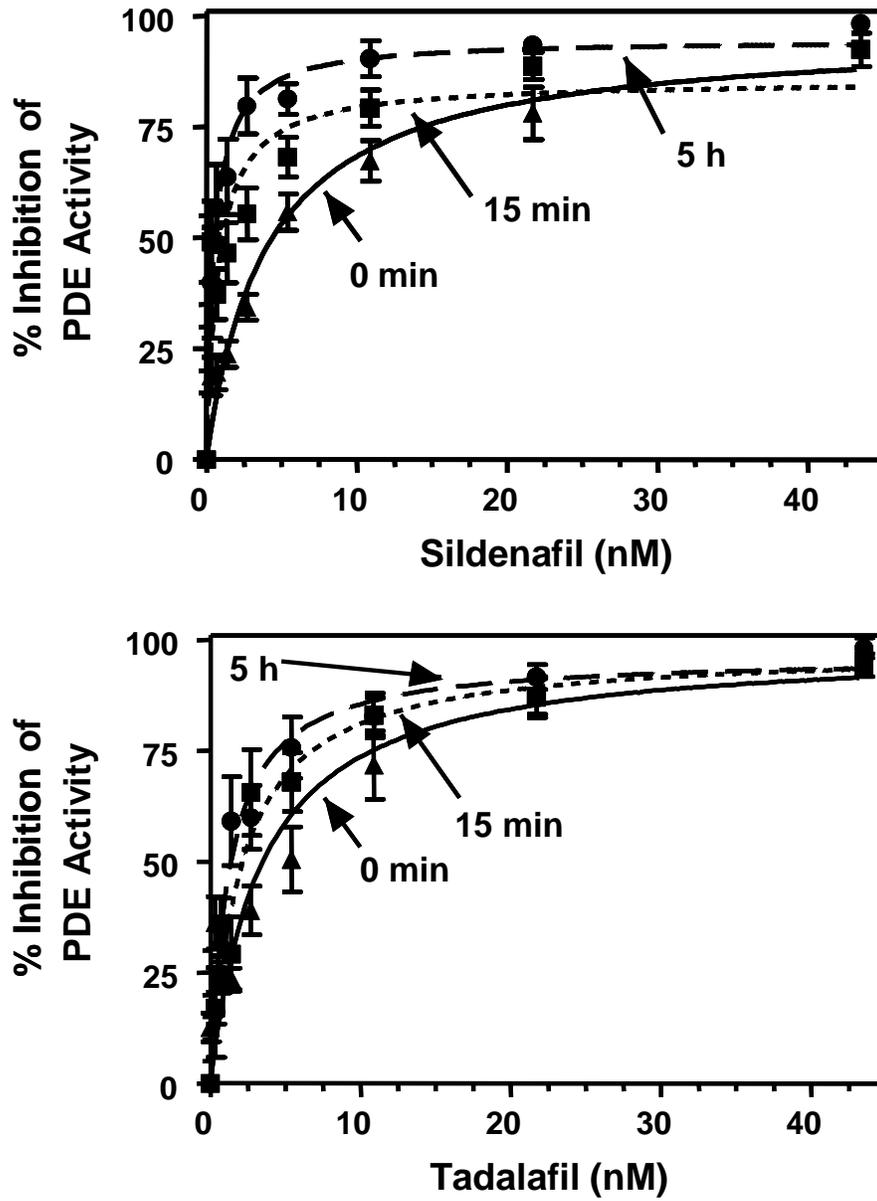


Figure 2

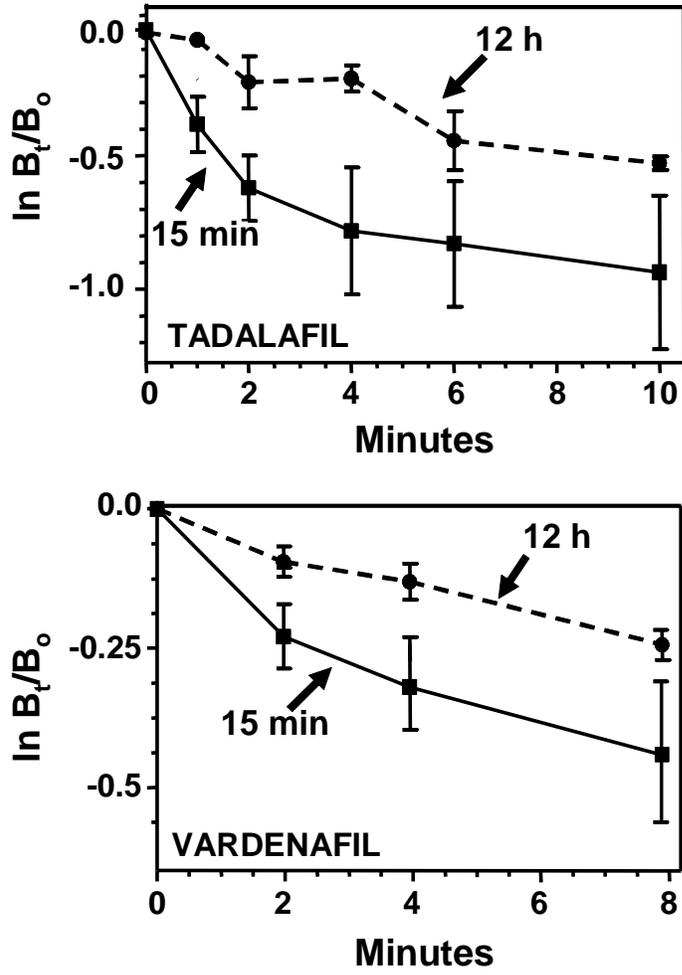


Figure 3

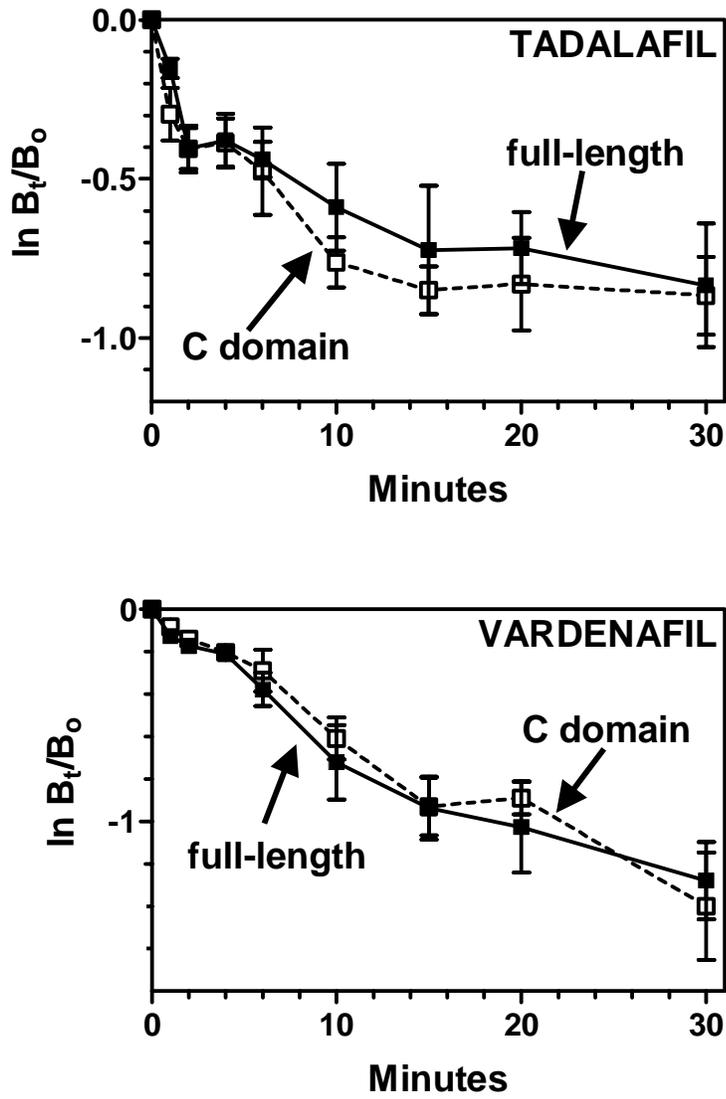


Figure 4

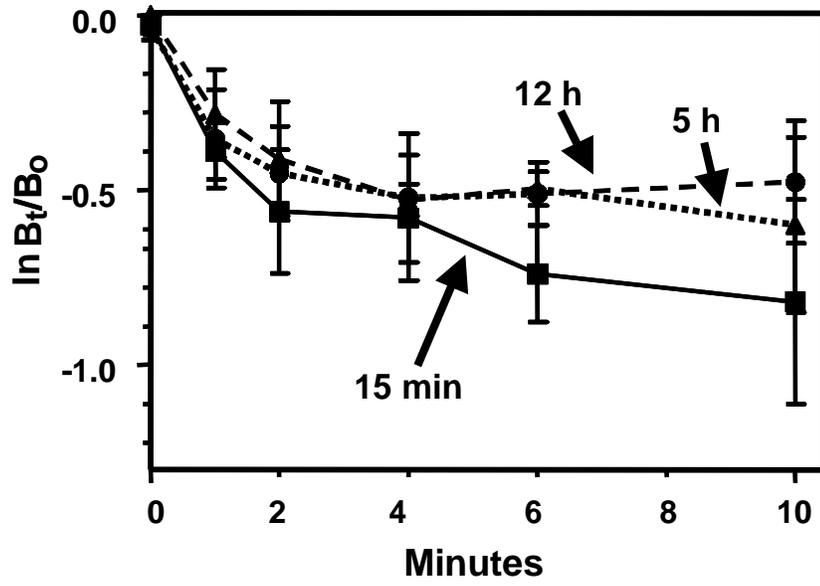


Figure 5

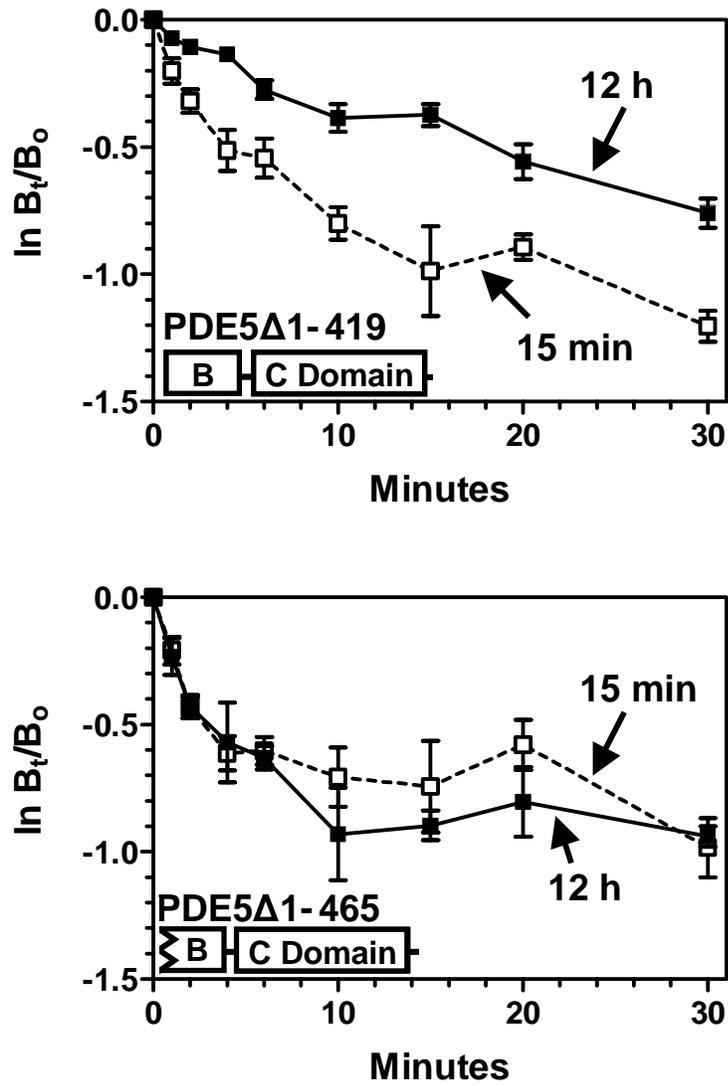


Figure 6