Cellular Signalling 21 (2009) 1768–1774

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Allosteric-site and catalytic-site ligand effects on PDE5 functions are associated with distinct changes in physical form of the enzyme

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ARTICLE INFO

Article history:
Received 8 June 2009
Received in revised form 27 July 2009
Accepted 28 July 2009
Available online 6 August 2009

Keywords:
Phosphodiesterase
PDE
PDE5
PDE5 inhibitor
Sildenafil
Tadalafil
GAF
Cyclic GMP
Negative feedback
Allosteric regulation

ABSTRACT

Native phosphodiesterase-5 (PDE5) homodimer contains distinct non-catalytic cGMP allosteric sites and catalytic sites for cGMP hydrolysis. Purified recombinant PDE5 was activated by pre-incubation with cGMP. Relatively low concentrations of cGMP produced a Native PAGE gel shift of PDE5 from a single band position (lower band) to a band with decreased mobility (upper band); higher concentrations of cGMP produced a band of intermediate mobility (middle band) in addition to the upper band. Two point mutations (G659A and G659P) near the catalytic site that reduced affinity for cGMP substrate retained allosteric cGMP-binding affinity like that of WT PDE5 but displayed cGMP-induced gel shift only to the middle-band position. The upper band could represent a form produced by cGMP binding to the catalytic site, while the middle band could represent a form produced by cGMP binding to the allosteric site. Millimolar cGMP was required for gel shift of PDE5 when added to the pre-incubation before Native PAGE, presumably due to removal of most of the cGMP during electrophoresis, but micromolar cGMP was sufficient for this effect if cGMP was included in the native gel buffer. cGMP-induced gel shift was associated with stimulation of PDE5 catalytic activity, and the rates of onset and reversibility of this effect suggested that it was due to cGMP binding to the allosteric site. Incubation of PDE5 with non-hydrolyzable, catalytic site-specific, substrate analogs such as the inhibitors sildenafil and tadalafil, followed by dilution, did not produce activation of catalytic activity like that obtained with cGMP, although both inhibitors produced a similar gel shift to the upper band as that obtained with cGMP. This implied that occupation of the catalytic site alone can produce a gel shift to the upper band. PDE5 activation or gel shift was reversed by lowering cGMP with dilution followed by at least 1 h of incubation. Such slow reversibility could prolong effects of cGMP on PDE5 in cells after decline of this nucleotide. Reversal was also achieved by Mg^{++} addition to the pre-incubation mixture to promote cGMP degradation, but Mg^{++} addition did not reverse the gel shift caused by sildenafil, which is not hydrolyzed by PDE5. Upon extensive dilution, the effect of tadalafil, a potent PDE5 inhibitor, to enhance catalytic-site affinity for this inhibitor was rapidly reversed. Thus, kinetic effect of binding of a high-affinity PDE5 inhibitor to the catalytic site is more readily reversible than that obtained by cGMP binding to the allosteric site. It is concluded that cGMP or PDE5 inhibitor binding to the catalytic site, or ligand binding to both the catalytic site and allosteric site simultaneously, changes PDE5 to a similar physical form; this form is distinct from that produced by cGMP binding to the allosteric site, which activates the enzyme and reverses more slowly.

1. Introduction

Mammalian cyclic nucleotide PDEs are a superfamily (PDE1–PDE11) of metallo-phosphohydrolases that catalyze hydrolysis of cyclic nucleotides to the respective 5′-nucleotide [1–7]. They contain a conserved catalytic (C) domain that varies in specificity and affinity for cAMP and cGMP and differs in inhibitor sensitivity [3,6,8]. PDEs are regulated by myriad processes, including subcellular localization [9], cGMP or cAMP binding [3,10,11], phosphorylation [3,8,12–18], or Ca^{2+}/calmodulin binding [19,20]. Phosphodiesterase-5 (PDE5) is highly selective for cGMP breakdown, plays a prominent role in determining cGMP levels in many tissues, and is the therapeutic target for treatment of erectile dysfunction (ED) by "PDE5-selective inhibitors" such as sildenafil (Viagra™), vardenafil (Levitra™), tadalafil (Cialis™), and udenafil (Zydena™) [21–27]. Thus far we have identified 10 mechanisms by which PDE5 is activated after cGMP elevation, and we have proposed that this brings about negative feedback control of the cGMP-signaling pathway. PDE5 is a homodimer [28] with separate cGMP sites for catalysis and allosteric regulation at the more carboxyl-terminal C domain and amino-terminal regulatory (R) domain, respectively, of

Abbreviations: PDE, cyclic nucleotide phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; C domain, catalytic domain; R domain, regulatory domain; PAGE, polyacrylamide gel electrophoresis; KP, 10 mM potassium phosphate (pH 6.8); GAF, cGMP-binding PDE, *Anabaena* adenylyl cyclase, *E. coli* FhlA protein.

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doi:10.1016/j.cellsig.2009.07.012

each monomer. Binding of ligand to the catalytic site stimulates cGMP binding to the allosteric site [28], and conversely [29–33]. Within the R domain are two tandem GAF (cGMP-binding PDE, *Anabaena* adenylyl cyclase, *E. coli* Fh1A protein) subdomains [28,34,35]. Binding of cGMP to PDE5 GAF stimulates the catalytic site [30–33]. We have recently shown that this is a direct effect on the catalytic site [30,36]. A concerted effect of catalytic-site and allosteric-site binding of ligand, along with phosphorylation [37–39], could serve for powerful negative feedback control of cGMP signaling, thereby enhancing PDE5-mediated dampening or termination of the signal. This concerted effect would also increase cGMP sequestration by the PDE5 allosteric sites [17,40,41], which would further reduce free cGMP and dampen cGMP signaling. The negative feedback mechanisms should translate into positive feedback for PDE5 inhibitors that are in clinical use since these inhibitors are substrate analogs that are not metabolized in the smooth muscle cells and improve catalytic-site affinity in a time-dependent manner [42].

We have discovered two kinetic species and two physical forms of PDE5 [36,43]. Ligand binding or phosphorylation can cause conversion of one form to another. Whether ligand binding to the allosteric site, catalytic site, or both, causes conversion of these species and forms, and whether the kinetic species represent the physical forms are unknown. The kinetic species are characterized by "high-affinity" or "low-affinity" of the catalytic site for cGMP or PDE5 inhibitors, as well as high-affinity and low-affinity of the allosteric cGMP-binding site for cGMP; the physical forms are characterized by distinct mobilities on Native PAGE. Evidence for a third physical form that is produced by ligand binding is presented herein. Establishment of the existence and mechanisms of interconversion of the kinetic species or physical forms is important in understanding the regulation of cGMP action and pharmacology of inhibitor effects. Results in the present study improve understanding of molecular mechanisms that impact PDE5 inhibitor therapy and allow new directions for medications that affect PDE5 and cGMP signaling.

2. Materials and methods

2.1. Materials

Sildenafil was purified from Viagra® tablets using the method established in this laboratory [30,44]. Purified sildenafil was radiolabeled with tritium by GE Healthcare. Tadalafil was synthesized according to Daugan et al. [45]. After confirming the compound structure by mass spectrometry, tadalafil was submitted to GE Healthcare for radiolabeling with tritium. Vardenafil and [^{3}H]vardenafil were provided by Bayer AG (Wuppertal, Germany). Cyclic GMP, cAMP, 3-isobutyl-1-methylxanthine (IBMX), histone type II-AS, *Crotalus atrox* snake venom, and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Pre-formed native gels and native gel sample buffer were from Bio-Rad (Hercules, CA). Sephacryl S-200 High Resolution gel filtration resin was purchased from Amersham (Piscataway, NJ). [^{3}H]cGMP was purchased from GE Healthcare (Piscataway, NJ).

2.2. Expression and purification of PDE5A1

Human cDNA coding for full-length PDE5A1 (hPDE5A1) was ligated into the baculovirus expression vector pAcHLT-A (BD Biosciences PharMingen). The resulting plasmid pAcA-PDE5 was used to make point mutations (G659A and G659P) with the QuikChange site-directed mutagenesis kit (Stratagene). WT and mutant PDE5 proteins were obtained using the Sf-9 cell-baculovirus system (BD Biosciences PharMingen) as previously described [46,47].

Purified His-tag recombinant bovine PDE5A1 was obtained as described [30].

Native PAGE, SDS-PAGE, enzyme phosphorylation, [^{3}H]cGMP-binding kinetics, and catalytic properties verified structural and functional integrity of the unmutated PDE5 constructs. All recombinant PDE5A1 proteins exhibited a purity of >98% as determined by SDS-PAGE. Bovine PDE5A1 constructs were flash-frozen using liquid N_2 in Tris buffer (20 mM Tris, pH 8, and 50 mM NaCl) containing a final concentration of 20% glycerol. Human PDE5A1 constructs were flash-frozen in 10 mM potassium phosphate, pH 6.8, 25 mM β-mercaptoethanol, and 1 mM EDTA (KPEM) + 150 mM NaCl and 10% sucrose. Recombinant PDE5 preparations were stored at −80 °C.

PDE activity was determined as described previously [46] with 0.4 μM [^{3}H]cGMP as substrate.

2.3. Native polyacrylamide gel electrophoresis (Native PAGE)

20-μl samples in KPM + 100 mM NaCl were combined with 5-μl ligand and 5-μl 7 mg/ml BSA and pre-incubated as indicated. 5-μl 40% v/v glycerol containing 0.01% bromophenol blue (Bio-Rad, Hercules, CA) was added, and the mixture was loaded into 50-μl 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, PDE5 holoenzyme (1 μM) was incubated in 50 mM Tris–HCl, pH 7.5, and 1.75 mg/ml BSA in the absence or presence of ligands in a final volume of 10–30 μl for the time and temperature indicated before subjecting the proteins to electrophoresis. Proteins were visualized by either Coomassie Blue or silver staining.

2.4. [^{3}H]cGMP Millipore membrane filtration protein binding

432-μl recombinant bovine PDE5 (225 nM) that had been diluted in 10 mM KP (pH 6.8) + 1 mg/ml BSA was added to 48 μl of a mixture containing 10 mM KP, 1 mM EDTA, 4 mg/ml histone type II-AS (Sigma), 0.25 mM 3-isobutyl-1-methylxanthine (IBMX), and 4.9 μM [^{3}H]cGMP. The reaction mixture was incubated for the indicated times at 30 °C and an aliquot (72 μl) was removed and pipetted into 2 ml ice-cold KP and immediately filtered under house vacuum through Millipore nitrocellulose membranes (0.45 μm pore size) that had been pre-wetted with 2 ml of ice-cold 10 mM KP. The reaction tube was then washed twice with 2-ml aliquots of ice-cold KP, and the washes were also applied to the filter. Filter membranes were removed, dried, and transferred to 6-ml scintillation vials containing 5 ml of nonaqueous scintillant for counting.

2.5. Statistical analyses

All values are given as 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.s fit within a 95% confidence interval, which quantifies the precision of the mean.

3. Results

3.1. Time-dependent activation of PDE5 by pre-incubation with cGMP

Purified, recombinant bovine PDE5 was activated in a time-dependent manner by pre-incubation with 710 μM cGMP prior to dilution and assay of PDE activity (Fig. 1A). The peak of activation was achieved by 20 min, which correlated with the relatively slow time course of binding of cGMP to the allosteric site of PDE5 (Fig. 1B). A 15-min pre-incubation with 34,000 μM cAMP did not produce significant PDE5 activation (not shown).