Allosteric Activation of cGMP-Specific, cGMP-Binding Phosphodiesterase (PDE5) by cGMP†

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ABSTRACT: The effects of cGMP binding on the catalytic activity of cGMP-specific, cGMP-binding phosphodiesterase (PDE5) are unclear because cGMP interacts with both allosteric and catalytic sites specifically. We studied the effects of cGMP on the hydrolysis of a fluorescent substrate analogue, 2′-*O*-anthraniloyl cGMP, by PDE5 partially purified from rat cerebella. The preparation contained PDE5 as the major cGMP-PDE activity and was not contaminated with cAMP- or cGMP-dependent protein kinases. The Hill coefficients for hydrolysis of the analogue substrate were around 1.0 in the presence of cGMP at concentrations <0.3 μM, while they increased to 1.5 at cGMP concentrations >1 μM, suggesting allosteric activation by cGMP at concentrations close to the bulk binding constant of the enzyme. Consistent with an allosteric activation, increasing concentrations of cGMP enhanced the hydrolysis rate of fixed concentrations of 2′-*O*-anthraniloyl cGMP, which overcame competition between the two substrates. Such activation was not observed with cAMP, cyclic inosine 3′,5′-monophosphate, or 2′-*O*-monobutyl cGMP, indicating specificity of cGMP. These results demonstrate that cGMP is a specific and allosteric activator of PDE5, and suggest that in cells containing PDE5, such as cerebellar Purkinje cells, intracellular cGMP concentrations may be regulated autonomously through effects of cGMP on PDE5.

cGMP functions as an intracellular messenger in numerous cell types and physiological systems (*1*). In the central nervous system, cGMP plays a key role in synaptic plasticity in various brain regions including the cerebellum (*2*), the striatum (*3*), and the hippocampus (*4*). In the cerebellum, long-term depression, a change of synaptic transmission strength between parallel fibers and Purkinje cells that is thought to underlie cerebellar motor learning (*5*), involves the nitric oxide−cGMP pathway (*2*). Depolarization of Purkinje cells combined with photolysis of a caged-cGMP compound can induce long-term depression in Purkinje cells, but only when these two signals are paired within a time window of 300 ms (*6*), suggesting that temporal regulation of intracellular cGMP dynamics is important for this form of plasticity. Soluble guanylyl cyclase produces cGMP in Purkinje cells in response to nitric oxide derived from surrounding neurons (*7*). The degradation of cGMP is controlled by phosphodiesterases (PDEs),[1] and Purkinje cells have been shown to express at least three different isoforms of PDEs. These are calmodulin-dependent PDE (PDE1) (*8*), cGMP-specific, cGMP-binding PDE (PDE5) (*9*), and cGMP-specific, 3-isobutyl-1-methylxanthine (IBMX)-insensitive PDE (PDE9) (*10*). Compounds that inhibit PDE5 activity, such as zaprinast or dipyridamole, facilitated cGMP-dependent long-term depression (*11*). IBMX or zaprinast, but not vinpocetine, a PDE1 inhibitor, inhibited cGMP breakdown following exogenous nitric oxide-dependent cGMP production in Purkinje cells (*7*). These pharmacological studies suggest that PDE5 plays a major role in shaping cGMP dynamics in Purkinje cells.

PDE5 binds cGMP with high specificity at two allosteric cGMP-binding sites (*12*) within the GAF (cGMP-specific cGMP-stimulated PDE, Adenylate cyclase, and FhlA) domains (*13*). The bulk binding constant of cGMP is estimated to be less than 1 μM at neutral pH (*14*). PDE5 hydrolyzes cGMP specifically (*15*). Mutant proteins deficient in cGMP-binding sites showed similar catalytic activity to wild type (*16*), indicating that the basal activity (without bound cGMP) of PDE5 is independent of these sites. Although PDE inhibitors such as IBMX or zaprinast are competitive with substrate, they enhanced the cGMP binding (*15*), indicating an interaction between catalytic and binding sites. According to "the reciprocity theory", it is suggested that cGMP binding to the allosteric site may affect the catalytic activity (*17*).

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[1] Abbreviations: PDE, phosphodiesterase; GAF, cGMP-specific cGMP-stimulated PDE, adenylate cyclase, and FhlA; IBMX, 3-isobutyl-1-methylxanthine; cIMP, cyclic inosine 3′,5′-monophosphate; Bu-cGMP, 2′-*O*-monobutyl cGMP; Ant-cGMP, 2′-*O*-anthraniloyl cGMP; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.

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This idea is supported by the fact that GAF domains of cGMP-stimulated PDE (PDE2) and PDE5 are homologous to each other (*17*). These various observations suggest that cGMP may be an allosteric activator of PDE5; however, this hypothesis has not yet been demonstrated. Furthermore, although it is known that phosphorylation by cAMP-dependent protein kinase (PKA) (*18*) or cGMP-dependent protein kinase (PKG) (*19*) regulates the catalytic activity of PDE5, cGMP binding to PDE5 is prerequisite for PKG-dependent phosphorylation (*16*). Therefore, it is unclear whether cGMP binding alone activates the catalytic activity of PDE5.

The present study was conducted to clarify whether cGMP acts as an allosteric activator of PDE5 or not. Since both binding and catalytic sites may interact with each other, we did not use mutant PDE5 proteins deficient in cGMP binding (*16*) for this study. Although some cGMP analogues are reported to displace [^{3}H]-cGMP bound to the allosteric site (*15*, *20*), the effect of analogue binding on the catalytic activity of PDE5 was unclear. Cyclic inosine 3′,5′-monophosphate (cIMP) and 2′-*O*-monobutyl cGMP (Bu-cGMP) can be hydrolyzed by PDE5 to some extent, but they are by no means good substrates for routine assay (*15*). We previously reported that 2′-*O*-anthraniloyl cGMP (Ant-cGMP) (*21*) is a good substrate of PDE5 (*7*). Ant-cGMP does not activate PDE5 through binding to the allosteric site, because it did not alter the Hill coefficient of PDE5. Using this hydrolyzable analogue, we demonstrate that PDE5 activity is specifically activated by cGMP in a direct, allosteric manner.

EXPERIMENTAL PROCEDURES

Partial Purification of PDE5. The method of PDE5 purification used was based upon that previously described for purification from lung tissue (*22*). However, since PDE5 immunoreactivity was identified only in Purkinje cells in the central nervous system (*23*), we paid attention to the removal of the major PDE activity in the cerebellum, PDE1 (*8*), and enzymes that are known to affect PDE5 activity, including PDE6 γ-subunit-like protein (*24*), PKG (*19*), and PKA (*18*), rather than to obtaining highly pure preparations.

All procedures were performed at 4 °C unless otherwise stated. Ten adult male Wistar rats were decapitated under ether anaesthesia to collect cerebella. The pia mater including superficial blood vessels was carefully removed with fine forceps. Cerebella were homogenized with a glass−Teflon homogenizer in 10 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 2 mM $MgSO_4$, 1 mM dithiothreitol, 60 μM leupeptin hemisulfate, 10 μg/mL aprotinin, 1.4 μM pepstatin, 10 μg/mL soybean trypsin inhibitor, 0.2 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine. The homogenate was centrifuged (Beckman XL90, type 70Ti rotor) at 10^5g for 30 min.

The supernatant was loaded onto a DEAE Sepharose-FF (Pharmacia) column (15 mm i.d. × 55 mm) preequilibrated with 20 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl and 10 mM $MgSO_4$. Proteins were eluted by a linear gradient of NaCl concentrations (50−250 mM) at a rate of 0.6 mL/min (60 mL), and the elution pattern monitored by the absorbance at 280 nm (AC5100L UV monitor, Atto Co., Ltd.) showed two peaks as previously reported (*9*). PDE5 immunoreactivity was found in the first peak; however, separation from the second peak (major peak containing PDE1) was not complete.

The contents of the entire first peak including the overlap with the second peak were added to 4 volumes of 20 mM Tris-HCl buffer, pH 7.0, containing 1 mM $MgSO_4$, 1 mM imidazole, 10 mM β-mercaptoethanol, and 0.1 mM EGTA. The mixture was loaded onto a Blue Sepharose affinity column (HiTrap Blue, Pharmacia, 5 mL) preequilibrated with the same buffer at 1 mL/min using a syringe pump (WPI, SP100i). The column was washed with the same buffer containing 0.8 M NaCl until the absorbance of the washouts returned to baseline, and then with 10 mL of the same buffer alone. PDE5 activity was eluted with 15 mL of the same buffer containing 0.5 M KSCN (*22*). The HiTrap Blue column choromatography removed calcineurin, a major calmodulin-binding protein that competes with PDE1 on the calmodulin-conjugated column (*25*) (data not shown).

The elutant buffer was immediately exchanged by filtration (VivaSpin, M_r >50 000) with 20 mM Tris-HCl buffer, pH 7.0, containing 1 mM $MgSO_4$, 1 mM imidazole, 10 mM β-mercaptoethanol, and 1 mM $CaCl_2$. Proteins were then passed through a calmodulin-conjugated Sepharose 4B (Pharmacia) column (7 mm i.d. × 20 mm) preequilibrated with the same buffer (*25*). The flow-through fraction was concentrated by filtration as above in 20 mM sodium phosphate buffer, pH 7.2. To elute PDE1, the column was washed with the same buffer containing 500 mM NaCl and with the buffer alone, and then PDE1 immunoreactivity was eluted with the same buffer containing 5 mM EGTA.

The flow-through fraction containing PDE5 was loaded onto a HiTrap-chelating column (1 mL, Pharmacia) that was preloaded with 0.1 mM $ZnCl_2$ and preequilibrated with 20 mM sodium phosphate buffer, pH 7.2. The column was washed with 10 mL of the same buffer containing 200 mM NaCl and then 10 mL of the same buffer alone. PDE5 was eluted with 10 mL of the same buffer containing 20 mM EDTA at 0.5 mL/min after incubation for 1 h at room temperature. The elutant was concentrated by filtration as above. In some experiments, a PD10 desalting column (Pharmacia) and a small DEAE Sepharose FF column (14 mm i.d. × 30 mm) were used instead of filtration, and results were similar. Proteins were stored on ice and used within 24 h. Protein concentrations were determined using a Protein Assay kit (BioRad).

PDE Assay. A fluorescent substrate analogue, Ant-cGMP (Figure 1A) (*21*), is commercially available (Calbiochem), and often used to assay PDE activity (*26*). The hydrolytic product of Ant-cGMP, 2′-*O*-anthraniloyl GMP, was slowly (approximately minutes) and nonenzymatically converted into 3′-*O*-anthraniloyl GMP (*21*). Therefore, we analyzed the formation of these two compounds using an HPLC system (Gilson) as a measure of PDE activity.

Partially purified PDE5 (15 μL) was incubated with 150 μL of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM Ant-cGMP, 0−100 μM cGMP, 1 mM 5′-AMP, 30 mM $MgSO_4$, and 4 mM β-mercaptoethanol at 37 °C for 30 min. As a method for termination of the reaction, the boiling method is not suitable, because anthraniloyl compounds are readily degraded by heat (*21*) (data not shown). The reaction was terminated by addition of 30 μL of cold 30% trichlo-

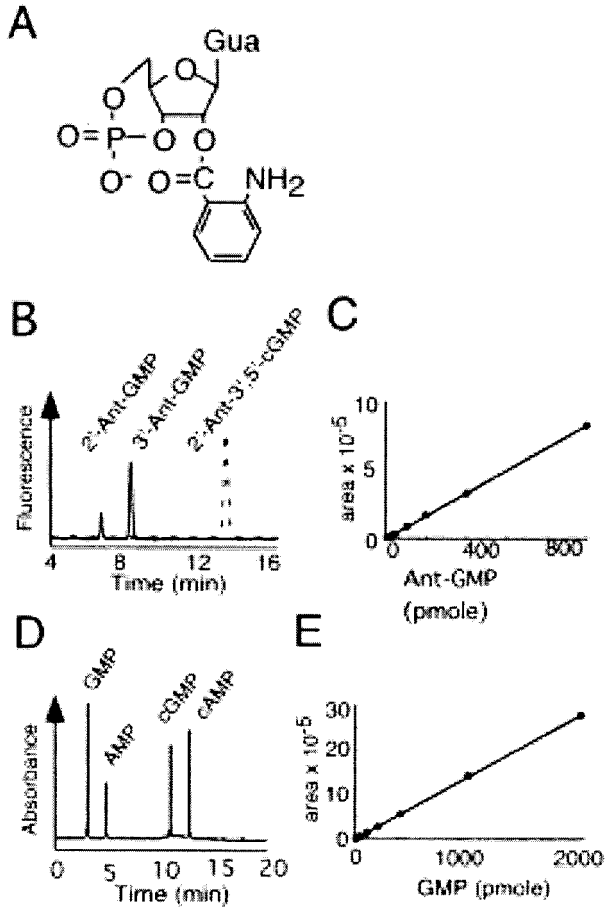

FIGURE 1: Detection of Ant-cGMP and cGMP. (A) Chemical formula of Ant-cGMP. (B) Chromatogram of Ant-cGMP, 2′Ant-GMP, and 3′Ant-GMP on HPLC. (C) Standard curve for Ant-GMPs. (D) Chromatogram of cGMP and 5′-GMP. (E) Standard curve for 5′-GMP.

roacetate, followed by washing with water-saturated ether 4 times. Aliquots were analyzed with a C18 column (YMC-pack Pro 4.6 × 150 mm, YMC Co., Ltd.). Fluorescence was monitored (excitation 350 nm, emission 430 nm) in 84 mM sodium phosphate buffer, pH 4.0, containing 12.8% acetonitrile. The retention times of 2′-Ant-GMP, 3′-Ant-GMP, and Ant-cGMP were 7.1, 9.0, and 13.8 min, respectively, as shown in Figure 1B. Ant-cGMP hydrolysis was calculated from the sum of areas for the two Ant-GMP isomers using the standard curve obtained by synthetic Ant-GMP (0.2−2000 pmoles, $r = 0.9999$, Figure 1C). The peak area of the products increased in proportion to the reaction time (up to 1 h). Usually less than 1% of Ant-cGMP was hydrolyzed under our reaction condition. GMP (2.9 min) and cGMP (10.7 min) were separated by the same column in 80 mM sodium phosphate buffer, pH 4.0, with a 2−80% acetonitrile gradient (19 min) at 0.8 mL/min, and detected by absorbance at 255 nm (Figure 1D). GMP production was calculated using a standard curve obtained by authentic 5′-GMP (1−2000 pmol, $r = 0.9999$, Figure 1E). The peak area of GMP increased in proportion to the reaction time up to 1 h. The retention time of 5′-AMP, which was included in the reaction to suppress nonselective esterase activities in crude extracts, was 4.8 min in the gradient elution condition and did not interfere with the separation and quantitative analysis of GMP. To subtract PDE-independent hydrolysis, Ant-cGMP hydrolysis in the presence of 50 μM zaprinast was also measured, since the major cGMP-hydrolyzing PDE activities are considered to be PDE1, PDE5, and PDE9, all of which are sensitive to zaprinast. Zaprinast-insensitive hydrolysis was less than 10% of the total activity. This portion of breakdown of Ant-cGMP and cGMP was insensitive to IBMX and partially nonenzymatic, suggesting a contribution from nonspecific esterase activity and thermal degradation.

Synthesis of Anthraniloyl GMP. Ant-GMP, as the standard, was synthesized under dim light from 1.5 mmol of isatoic anhydride and 5′-GMP (1 mmol/15 mL of water), and purified by the use of Sephadex LH-20 and TSK-gel DEAE-SW (Tosoh) column chromatography (*27*). Ant-GMP was identified by a C18 column as a single peak, or by silica thin-layer chromatography (1-propanol/aqueous ammonia/water containing 0.5 g/L EDTA = 6:3:1, volume ratio) as the only spot with brilliant blue fluorescence ($\lambda_{ex} = 365$ nm).

Kinetic Model for Detection of Allosterism. Our model for allosteric kinetics is based on the following assumptions that are predicted from our experimental results. First, Ant-cGMP does not interact with the allosteric site. This was suggested by the observation that the Hill coefficient of Ant-cGMP hydrolysis without cGMP was 1.0 (Figure 4B). Second, binding of cGMP to the allosteric site activated the enzyme. This is predicted since the Hill coefficients changed at cGMP concentrations near the reported bulk K_d value for cGMP (*14*) (Figure 4B). A steady-state approximation method showed the generalized formula for total Ant-cGMP hydrolysis rate (υ) catalyzed by PDE5 molecular species bound to 0, 1, and 2 cGMPs (eq 1):

$$\upsilon = \frac{AP\left(\frac{\kappa_2}{K_{A1}} + \frac{\kappa_4 B_1}{K_{A2}}G + \frac{\kappa_6 B_1 B_2}{K_{A3}}G^2\right)}{1 + \frac{A}{K_{A1}} + \left(B_1 + \frac{1}{K_{G1}} + \frac{AB_1}{K_{A2}}\right)G + B_1\left(B_2 + \frac{1}{K_{G2}} + \frac{AB_2}{K_{A3}}\right)G^2 + \frac{B_1 B_2}{K_{G3}}G^3} \quad (1)$$

where P, A, and G are the concentrations of total PDE5, Ant-cGMP, and cGMP, respectively. B_1 and B_2 are binding constants of the first and second cGMP-binding sites, respectively. Reaction rate constants of Ant-cGMP hydrolysis by PDE5 with 0, 1, and 2 bound cGMPs are denoted as κ_2, κ_4, and κ_6, respectively. Michaelis constants for Ant-cGMP and cGMP of each state of PDE5 are expressed as K_{A1}, K_{A2}, K_{A3}, K_{G1}, K_{G2}, and K_{G3}, respectively.

When all of the PDE5 species display identical hydrolytic rates, i.e., when there is no activation, eq 1 expresses competitive inhibition of Ant-cGMP hydrolysis by cGMP as shown in eq 2, which then allows us to estimate υ using the observed V_{max}, K_A, and K_G.

$$\upsilon = \frac{V_{max}A}{K_A + \frac{K_A}{K_G}G + A} \quad (2)$$

To consider a typical case of allosteric activation, we assumed that the rate constant of PDE5 bound to two molecules of cGMPs (κ_6) is k-fold larger than the others (κ_2 and κ_4). The apparent K_M for each substrate was assumed to

Table 1: Summary of Purification Procedures

procedures	volume (mL)	protein (mg)	protein recovery (%)	total activity[a] (nmol of GMP/min)	specific activity [nmol of GMP min^{-1} mg^{-1}]	purification (*x*-fold)
homogenate	10.0	90.9	100.0	17.0	0.2	1.0
supernatant	10.0	45.4	49.9	40.9	0.9	4.7
DEAE Sepharose gradient elutant	25.0	26.0	28.6	101.4	3.9	20.8
Blue Sepharose KSCN elutant	12.5	19.3	21.2	77.2	4.0	21.3
calmodulin-conjugated Sepharose flow-through	25.1	9.3	10.2	80.0	8.6	45.6
chelating column EDTA elutant	6.0	1.8	2.0	21.2	11.8	62.9

[a] The cGMP hydrolysis activities in the materials indicated were measured with 100 μM cGMP.

be independent of cGMP binding for simplicity. Then, eq 1 gives eq 3.

$$v = \frac{V_{max}A}{K_A + \frac{K_A}{K_G}G + A} + \frac{V_{max}A}{K_A + \frac{K_A}{K_G}G + A} \times \frac{(k-1)B_1B_2G^2}{1 + B_1G + B_1B_2G^2} \quad (3)$$

Although we cannot estimate the rate from this equation since values for k, B_1, and B_2 are not known, eq 3 tells us the essential feature of allosteric activation of PDE5. Namely, the rate of Ant-cGMP hydrolysis should be a summation of competitive inhibition (the first term), that reduces the rate in a hyperbolic manner, and the second term, whose change is bell-shaped; i.e., the second term increases at first until it reaches a peak and then decreases as cGMP concentrations increase. Mathematical analysis revealed that eq 3 also has a peak as a local maximum. These considerations predicted that Ant-cGMP hydrolysis in the presence of varied concentrations of cGMP should have a peak that is indicative of allosteric activation, while the peak should not be found with nonallosteric kinetics.

We estimated the Hill coefficient for Ant-cGMP hydrolysis, n, from the Hill equation:

$$\log (V_{max}/v - 1) = \log K - n \log S \quad (4)$$

where

$$S = \frac{K_A}{K_G}G + A \quad (5)$$

V_{max} was estimated from the bi-reciprocal plot. K is the apparent Michaelis constant. All numerical calculation was done using Microsoft Excel software. Results of linear regression were accepted only if regression coefficients were >0.99.

Phosphorylation of PDE5. PDE5 was concentrated with a spin-column (VivaSpin, M_r >50 000) in 50 mM Tris-HCl, pH 7.4 (0.45 mL, 1.8 mg/mL), and then incubated with 12.5 mM β-mercaptoethanol, 2 mM $MgSO_4$, 0.2 mM ATP, 1000 cpm/pmol of [γ-^{32}P]-ATP (5×10^8cpm), 50 μM cGMP, and 0.25 mM IBMX, in the presence or absence of 60 000 units of PKG-1 (Calbiochem) for 1 h at 30 °C (*19*). The PKG reaction was terminated by addition of 300 nM KT5823, a specific PKG inhibitor. Phosphorylation of PDE5 was analyzed by SDS−PAGE followed by autoradiography (BAS5000 image analyzer, Fuji Film). After exposure for 30 min, the digitized image was analyzed on a Power Macintosh 7600 personal computer (Apple) equipped with a cpu-accelerator board (466 MHz, Advantec), using NIH image software.

Antibodies and Western Blotting. A specific antibody against rat PDE5 was a gift from Drs. K. Omori and J. Kotera (*23*). Specific antibodies against PKG-1 (Calbiochem), the catalytic subunit of PKA (Transduction Laboratories), PDE1 (Chemicon), and PDE6 γ-subunit (CytoSignal Inc., USA) were purchased. For Western blotting, horseradish peroxidase-conjugated anti-rabbit IgG (Vector Laboratories, USA) was used as the second antibody. Chemiluminescence was detected by an ECL kit (Amersham-Pharmacia). The exposure time required for detection of PDE5 was usually 30 min, while those for other proteins were 5−10 min.

RESULTS

Characterization of Partially Purified Cerebellar PDE5. Previous studies showed that cGMP activated Ant-cGMP hydrolysis by PDE5 in living Purkinje cells (*7*). To characterize the direct action of cGMP on PDE5, we partially purified PDE5 from rat cerebella by the use of four chromatographic procedures as described under Experimental Procedures. An example of the purification experiments is summarized in Table 1. Although cGMP hydrolysis was assayed at each step after dialysis, total activities in the supernatant and homogenate were lower than those of the DEAE-cellulose elutant, suggesting the existence of endogenous PDE inhibitors in the cerebellum. The cGMP hydrolytic activity was purified about 63-fold. The final preparation contained 131 ± 17 μg/mL (mean ± SEM, $n = 28$) of total protein in ~3 mL of 20 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgSO_4$ and 250 mM NaCl. Silver staining of SDS−PAGE gels detected a 98 kDa protein as expected (*15*) whose content was about 1% of the total staining. V_{max} for cGMP hydrolysis of our preparation (26.4 ± 1.7 nmol min^{-1} mg^{-1}, $n = 8$) was also 1% of the reported value for highly purified PDE5 (2599 nmol min^{-1} mg^{-1} after the Zn-chelating column procedure) (*22*). Immunoreactivity for PDE5 was found in the final preparation, but those for PKG, PKA, PDE6 γ-subunit, and PDE1 were not recognized (Figure 2). The preparation hydrolyzed cGMP with a K_M = 1.51 ± 0.54 μM ($n = 8$, data not shown), which is in good agreement with the K_M of full-length wild-type bovine PDE5 (2.0 ± 0.4 μM) (*16*). Zaprinast dose-dependently suppressed

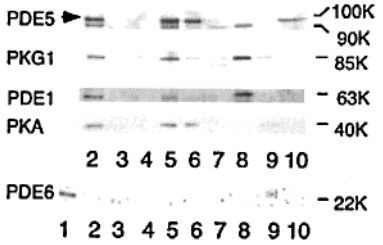

FIGURE 2: Western blotting analysis of purification steps using specific antibodies against PDE5 (98 kDa, indicated by an arrow), PKG1 (85 kDa), PDE1 (63 kDa), PKA catalytic subunit (PKA, 40 kDa), and PDE6 γ-subunit (PDE6, 22 kDa). These blots represent a single purification out of 2−5 experiments. Each lane contained 3 μg of total proteins. Lane 1, DEAE-Sepharose flow-through (shown for PDE6γ only); 2, DEAE-Sepharose NaCl gradient elutant; 3, Blue Sepharose flow-through; 4, Blue Sepharose NaCl washout; 5, Blue Sepharose KSCN elutant; 6, calmodulin column flow-through; 7, NaCl washouts of calmodulin column; 8, calmodulin column EGTA elutant; 9, chelating column flow-through; 10, chelating column EDTA elutant. Approximate molecular weights estimated from authentic enzymes (PKG-1, PDE-1, and PKA) and standard proteins (GibcoBRL, BenchMark Protein Ladder) are indicated on the right (Da).

PDE activity in the preparation with an $IC_{50} = 0.45 \pm 0.01$ μM ($n = 3$, Figure 3A), which is consistent with the reported IC_{50} for PDE5 (*9*, *16*), but not that for PDE1 (*9*) or PDE9 (*28*, *29*). An activator (calcium-calmodulin) and an inhibitor (vinpocetin) (*30*) of PDE1 did not affect Ant-cGMP hydrolysis (Figure 3B). PDE2 was not identified in rat cerebellum (*31*), and its specific inhibitor (EHNA) (*32*) did not affect Ant-cGMP hydrolysis (Figure 3B). These results confirmed that the major cGMP-hydrolyzing activity in the preparation was mediated by PDE5. Incubation of the preparation with Mg-ATP, IBMX, and cGMP did not cause phosphorylation of PDE5, excluding contamination with PKG activity (Figure 3C). Further addition of exogenous PKG did cause phosphorylation of PDE5 (Figure 3C).

Thus, our preparation contained PDE5 as the major cGMP-PDE activity, and enzymes that are known to affect PDE5 activity through phosphorylation, PKA, PKG, and PDE6 γ-subunit-like protein, were excluded, although other unknown influencing factors are not excluded. During PDE5 purification, the major part of PKG-1 immunoreactivity was unexpectedly found to bind to the calmodulin-affinity column and was eluted with EGTA (Figure 2). This suggests a specific interaction of PKG-1 with calmodulin, or other calmodulin-binding proteins. The rest of the PKG and PKA immunoreactivities were separated by a zinc-chelating column. An antibody against PDE6 γ-subunit recognized only a 22 kDa protein in the cerebellar cytosol, which was separated by DEAE chromatography. We did not find a 14 kDa protein as reported in dog airway (*24*). Although the antibody against PDE5 recognized a 90 K protein, this was not an isoform of PDE5, because it was separated by calmodulin-conjugated Sepharose column chromatography.

Hill Coefficient Analysis. PDE5 in the final fraction also hydrolyzed Ant-cGMP (K_M for Ant-cGMP $= 11.8 \pm 0.6$ μM, $V_{max} = 0.35 \pm 0.08$ μmol min^{-1} mg^{-1}, $n = 6$). In the absence of cGMP, Ant-cGMP hydrolysis followed ordinary Michaelis−Menten kinetics and displayed a Hill coefficient of 1.02 ± 0.02 ($n = 7$, Figure 4A), indicating that Ant-cGMP did not modulate catalytic activity in an allosteric manner. This result also suggested that trace cGMP binding to PDE5

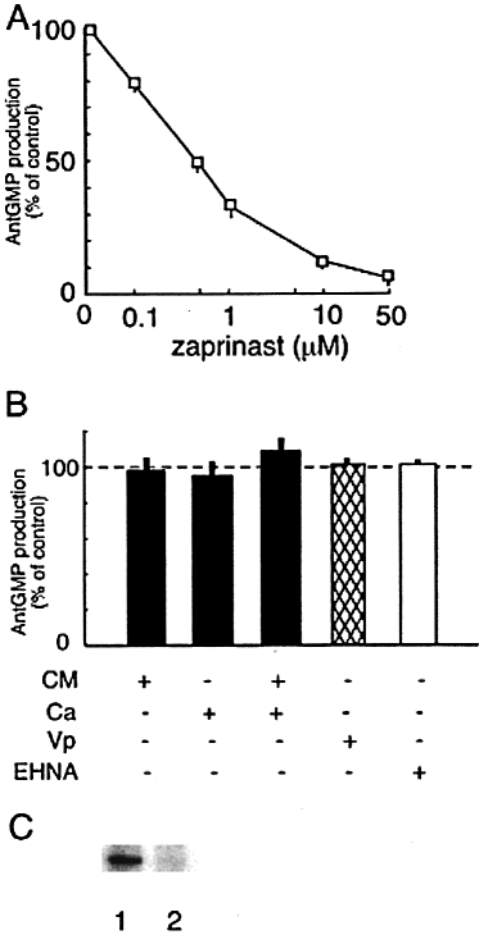

FIGURE 3: Characterization of the partially purified PDE5. (A) Zaprinast dose-dependently inhibited hydrolysis of 10 μM Ant-cGMP, as determined by measurement of product (Ant-GMP). Hydrolysis without zaprinast (277 nmol min^{-1} mg^{-1}) was taken as 100%. Average and SEM (vertical bars) of 4 experiments are shown. (B) Effects on Ant-cGMP hydrolysis (1 mM Ant-cGMP + 10 μM cGMP) of PDE1 activators (100 nM calmodulin, CM, 0.2 mM $CaCl_2$, Ca), a PDE1 inhibitor (0.1 mM vinpocetine, Vp), and a PDE2 inhibitor (20 μM EHNA). Averages of 3 separate experiments are shown as percent of control without drugs (344 nmol min^{-1} mg^{-1}). Vertical bars indicate SEM. (C) Autoradiograms showing addition of exogenous PKG phosphorylated PDE5 (lane 1), while phosphorylation was not observed in the absence of exogenous PKG (lane 2). All lanes contained Mg-ATP, cGMP, IBMX, and 3 μg of total proteins.

in the preparation, if any, was not significant and did not cause allosteric modification of the catalytic activity. When Ant-cGMP hydrolysis was measured in the presence of cGMP at a 1:10 molar ratio, the apparent Hill coefficient was significantly increased to 1.47 ± 0.22 ($n = 4$, $p < 0.001$, *t*-test, Figure 4A). Although precise Hill coefficients cannot be calculated under these conditions, the results are consistent with an allosteric activation of PDE5 by cGMP.

We next measured Ant-cGMP hydrolysis in the presence of constant concentrations of cGMP to obtain precise Hill coefficients as a function of cGMP concentrations. As shown in Figure 4B, Hill coefficients near 1 were obtained when cGMP concentrations were at 0−0.3 μM. With cGMP concentrations higher than 1 μM, Hill coefficients were significantly increased up to around 1.5. The change in Hill coefficients occurred at a cGMP concentration consistent with the reported bulk K_d value for cGMP (about 1 μM at

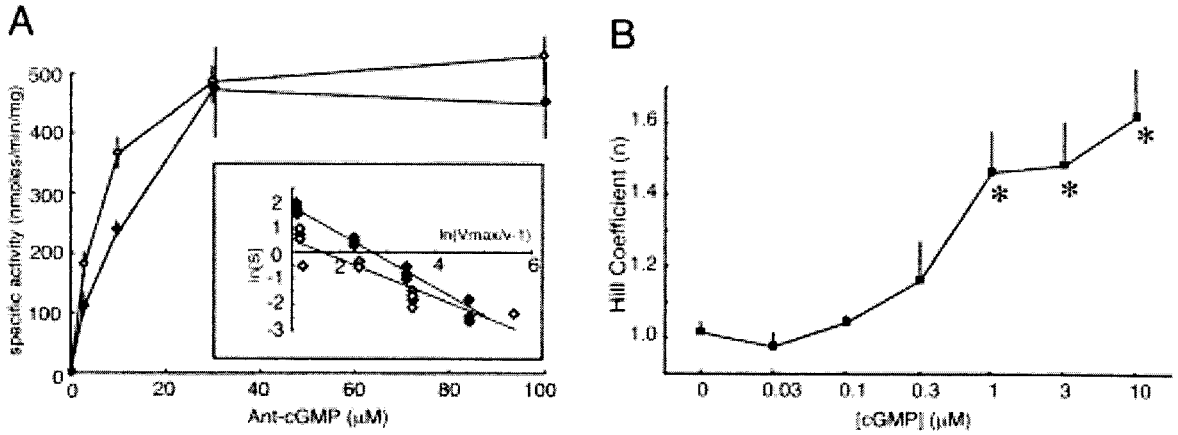

FIGURE 4: (A) Hydrolysis rate of 4 concentrations of Ant-cGMP was assayed in the absence (open symbols) or presence (closed symbols, Ant-cGMP/cGMP = 10) of cGMP. Mean and SEM of 4 independent results are shown. The inset shows the Hill plot of the same set of data. (B) Hill coefficients of Ant-cGMP hydrolysis (1, 3, 10, 30, and 100 μM) in the presence of cGMP at the concentrations indicated. Results from 3−7 independent experiments are shown. Asterisks indicate a significant difference from 0 μM cGMP ($p < 0.001$, *t*-test).

neutral pH) (*14*). These results further indicate that cGMP is an allosteric activator of PDE5.

Detection of Allosteric Activation. Although the increase in the Hill coefficients suggested allosteric activation of the catalytic activity of PDE5, the results shown in Figure 4A did not reveal any significant increase in the V_{max} of Ant-cGMP hydrolysis (0.39 ± 0.01 μmol min^{-1} mg^{-1}, $n = 4$). Under these conditions of 1:10 concentration of cGMP to Ant-cGMP, the apparent K_M for Ant-cGMP hydrolysis was slightly, but significantly increased (13.5 ± 0.3 μM, $n = 4$, $p < 0.001$). Thus, cGMP seemed to lower the affinity of Ant-cGMP as the substrate, indicating that competition between the two substrates masked allosteric activation. To unveil this activation, we measured the effect of 0−100 μM cGMP on the hydrolysis of constant concentrations of Ant-cGMP (0.1 or 1 mM). Under these experimental conditions, Ant-cGMP hydrolysis is also expected to be reduced due to competitive inhibition by cGMP, and, indeed, Ant-cGMP hydrolysis was reduced at high cGMP concentrations as shown in Figure 5A. However, a consideration of the kinetics of PDE activity (Experimental Procedures) predicted that Ant-cGMP hydrolysis should reach a peak if cGMP activates PDE5 allosterically. Figure 5A indeed demonstrated that hydrolysis of 1 mM Ant-cGMP shows a transient increase with a peak at 10−30 μM cGMP. When the Ant-cGMP concentration was 0.1 mM, only a little peak appeared in the same range of cGMP concentrations (Figure 5A), suggesting that competition was predominant. The differences between the two curves were significant at cGMP = 10 ($p < 0.05$, *t*-test) and 30 ($p < 0.001$, *t*-test) μM. When the rate of Ant-cGMP hydrolysis was calculated according to eq 3, these results were best simulated with parameters whereby $B_1 = 3$ nM, $B_2 = 1$ μM, and $k = 1.3$ (Figure 5B). The calculated rate first decreased slightly, then increased, reaching a peak at around 10−30 μM cGMP, and then decreased. This behavior is qualitatively identical to the observed data in Figure 5A. Simulation by eq 2 did not give a peak at any cGMP concentrations (Figure 5B).

Such a peak was not observed when Bu-cGMP, cIMP, or cAMP was used instead of cGMP (Figure 5C). Bu-cGMP displayed only competitive inhibition of Ant-cGMP hydrolysis, consistent with a previous report that Bu-cGMP is a substrate of PDE5, but does not interact with its allosteric site (*15*). Cyclic IMP has been reported to compete with cGMP weakly at both catalytic and binding sites (*15*, *20*); however, no significant peak was observed, suggesting that cIMP is not an activator of PDE5. Cyclic AMP showed neither competition nor activation, signifying that our preparation contained PDE activities specific to cGMP. Thus, the peak was generated by cGMP specifically. Combined with the result that Ant-cGMP did not alter the Hill coefficient, these results indicate that the action of cGMP on the cGMP-binding site activates PDE5. The peak was not observed when cIMP or Bu-cGMP was used instead of Ant-cGMP, probably due to their low hydrolytic rates (data not shown). The effect of cGMP was evident in Tris-HCl or HEPES−NaOH buffers, but somewhat weak in sodium phosphate buffer. We tested the effects of 2′,3′-cGMP, pyrophosphate, glycerophosphate, mercaptoethanol, EDTA, zinc chloride, triethylamine, L-lysine, L-arginine, and ammonium acetate, but none of these compounds affected the cGMP-dependent activation of PDE5 (data not shown).

DISCUSSION

The present study revealed that cGMP specifically activates partially purified PDE5 in an allosteric manner. We previously reported that the hydrolytic rate of Ant-cGMP infused into Purkinje cells was accelerated by nitric oxide in a manner dependent on soluble guanylyl cyclase and PDE5 activities (*7*). The present results support the idea that the observed fluorescence change was due to cGMP-dependent activation of PDE5 within living Purkinje cells. Together, we suggest that the cGMP concentrations in cerebellar Purkinje cells are autonomously regulated through its action on PDE5, the specific sensor and hydrolyzer of cGMP.

Basal cGMP concentration in corpus cavernosum is estimated to be lower (18 nM) than cGMP-binding protein concentrations in the tissue, suggesting a role of PDE5 as a cGMP buffer and a feedback regulator of intracellular cGMP concentration (*33*). On the other hand, the cerebellum contains extraordinary high cGMP concentration. The basal concentration of cGMP (free plus bound) in Purkinje cells is estimated to be 0.8 μM, based on the basal cGMP concentration measured in cerebellar slices (1.6 pmol/mg of protein) (*34*) and an estimation of the volume occupied by Purkinje cells in the slice (*35*). Purkinje cells also express

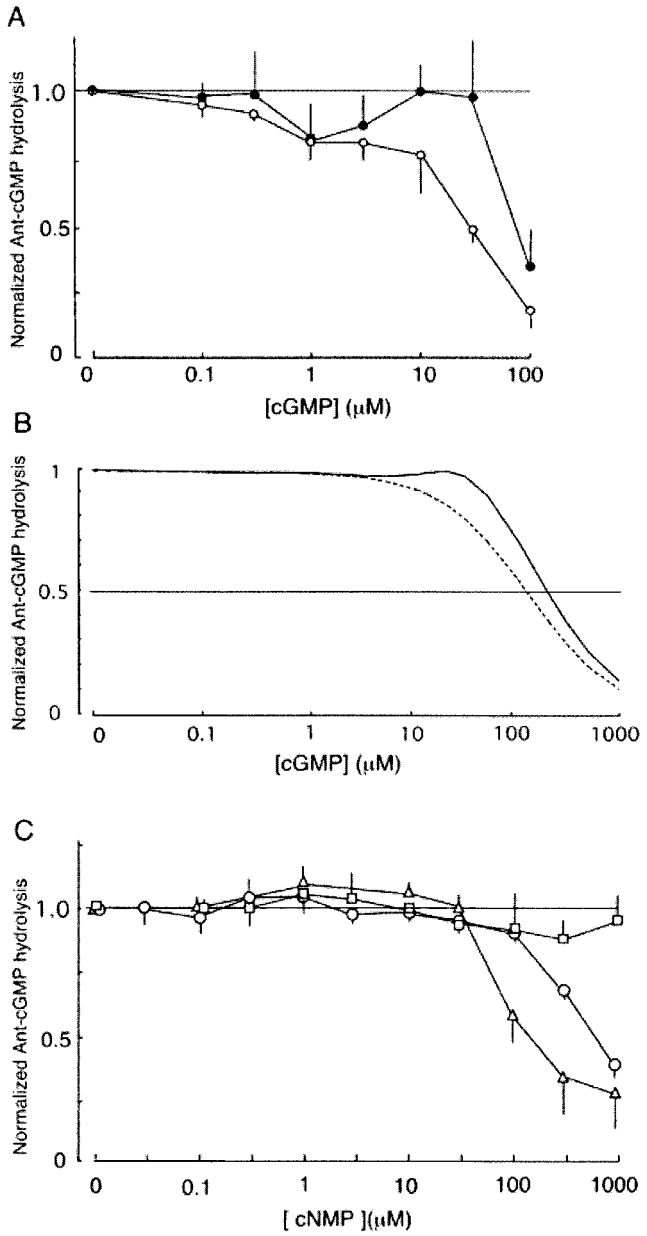

FIGURE 5: Detection of allosteric activation. (A) cGMP dependency of Ant-cGMP (1 mM, closed circles; 0.1 mM, open circles) hydrolysis by partially purified PDE5. Mean and SEM of 3−8 independent results are shown. Normalized Ant-cGMP hydrolysis indicates that Ant-GMP production at the indicated cGMP concentrations was divided by that at cGMP =0. Note that the normalized Ant-cGMP hydrolysis = 1 does not necessarily means 'no activation', as cGMP competes with Ant-cGMP. (B) Simulation of cGMP dependency of 1 mM Ant-cGMP hydrolysis according to eqs 2 (dotted line) and 3 (solid line). Parameters used are A = 1000 μM, K_A = 12 μM, K_G = 1.5 μM, B_1 = 5 nM, B_2 = 1 μM, and k = 1.3. (C) Ant-cGMP (1 mM) hydrolysis in the presence of cAMP (squares), Bu-cGMP (circles), or cIMP (triangles). Results from 3−8 independent experiments are shown.

cGMP-binding proteins other than PDE5 such as PKG-1β and olfactory-type cGMP-dependent cation channels (*1*), but the intracellular concentrations of cGMP-binding sites are not known. We speculate that the allosteric activation of PDE5 will keep the basal cGMP concentrations in Purkinje cells below those required for activation of potential cGMP targets. The K_a for cGMP of PKG-1β is 1.2 μM (*17*), which is consistent with the cGMP concentration required to increase the Hill coefficient of PDE5.

In addition to having the highest basal cGMP concentration in the brain, the cerebellum shows a robust increase in cGMP concentration by application of moderate concentrations of glutamate receptor agonists such as 3 μM AMPA (*36*), or by glutamatergic transmission by parallel fiber activity (*7*, *37*). This cGMP rise can evoke long-term depression (*2*) provided it coincides with concomitant depolarization of the Purkinje cell within a narrow time window of around 300 ms (*6*). Therefore, intracellular cGMP concentrations must be rapidly controlled to enable normal neuronal function, and cGMP-dependent activation of PDE5 may play a pivotal role in this regulation. In addition to direct activation, cGMP binding to the allosteric sites increases the susceptibility of PDE5 to phosphorylation that increases the catalytic activity of PDE5 (*16*). Thus, in cells where PDE5 and PKG are coexpressed, phosphorylation-dependent activation of PDE5 will follow allosteric activation. Although allosterism and phosphorylation are independent mechanisms, we speculate that in concert they will limit the lifetime of cGMP-mediated signals (*38*). Cerebellar Purkinje cells express protein phosphatase-1, protein phosphatase-2A, and calcineurin (*39*, *40*) as well as PKG-1 (*41*). Phosphorylation of G-substrate, another substrate of PKG, inhibits protein phosphatases-1 and -2A (*42*). Thus, when PKG is activated, the major dephosphorylating activity for PDE5 in Purkinje cells may be calcineurin, suggesting that the lifetime of cGMP may also depend on intracellular calcium concentrations. When both cGMP and calcium concentrations are raised, a condition that induces long-term synaptic depression (*6*), activation of calcineurin will reduce the content of phosphorylated PDE5 by dephosphorylation of PDE5 or G-substrate as reported for inhibitor-1 (*43*). Under such conditions, sustained increases in cGMP concentration are expected. Our preliminary observations suggested that PDE5 phosphorylated by PKG can be dephosphorylated by calcineurin and protein phosphatase-1 (data not shown).

We showed that cGMP at concentrations greater than 1 μM increased the Hill coefficient for Ant-cGMP hydrolysis. Previous studies could not detect such changes in the Hill coefficient (equivalent to nonlinear bi-reciprocal plot), because they used [^{3}H]-cGMP as the reporter substrate (*18*). The change in the Hill coefficient takes place at cGMP concentrations near the K_M for hydrolysis, which may also make detection of the allosterism difficult. We developed a kinetic model for competition between Ant-cGMP and cGMP on the catalytic site to support further the idea of allosteric activation. An assumption of our model that Ant-cGMP does not interact with the cGMP-binding site was substantiated by the finding that the Hill coefficient for Ant-cGMP hydrolysis without cGMP was just 1. Furthermore, the cGMP-activation site of PDE2, which is considered as homologous to the cGMP-binding site of PDE5 (*17*), did not interact with cGMP analogues with modification at the 2′ position (*44*), supporting the idea that Ant-cGMP does not interact with the cGMP-binding site of PDE5. Our kinetic model best simulated the observed data with an activation factor k = 1.3, suggesting that the magnitude of activation is not large, at least in an isolated enzyme preparation. This may account for the difficulty in detecting the allosteric effect of cGMP on the catalytic activity in previous studies. Binding constants for each cGMP-binding sites (B_1 and B_2) of PDE5 are not known. Results of the present simulation suggested

that the two sites have different affinities for cGMP as already reported (*12*). We speculate that the allosteric activation suggests a positive cooperativity between these cGMP-binding sites. Estimation of B_2 according to eq 3 suggested a value consistent with previous reports on the bulk binding constant (*14*). Although mathematical analysis mimicked the data qualitatively, this simulation addresses only one simple case of the possible mechanisms derived from eq 1. Therefore, refinement of the model will be necessary, as well as further characterization of the binding sites.

PDEs are involved in many aspects of diseases such as asthma (*45*). PDE inhibitors have a wide-ranging potential therapeutic benefit, recently exemplified by the specific inhibitor sildenafil (*46*). The effects of competitive PDE inhibitors are influenced by the concentrations of the substrate cyclic nucleotides. Since the generation of the substrate (such as cGMP) is regulated by different signal transduction systems (such as nitric oxide) from degrading systems, it is potentially difficult to control (*47*). Our preliminary experiments did not find any compound that mimicked or inhibited the allosteric activation by cGMP, with the exception of phosphate buffer, which sometimes, but not reproducibly, inhibited activation. However, we believe that this cGMP-dependent activation via the allosteric site represents a new potential target of PDE5 inhibition.

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