Distinguishing the Roles of the Two Different cGMP-binding Sites for Modulating Phosphorylation of Exogenous Substrate (Heterophosphorylation) and Autophosphorylation of cGMP-dependent Protein Kinase*

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The role of each of the two different cGMP-binding sites (referred to as slow and fast sites) of type I cGMP-dependent protein kinase (PKG) in altering the rate of catalysis of phosphorylation of exogenous substrates (heterophosphorylation) or the rate of autophosphorylation has not been resolved. In the present study, the cGMP concentration required for half-maximal activation ($A_{50}$) of wild-type PKG type Iβ (WT) was 5-fold higher for heterophosphorylation than for autophosphorylation. cGMP occupation of the slow site was associated with an increase in the autophosphorylation rate, whereas occupation of the fast and slow site together was associated with a decrease in the autophosphorylation rate compared with the rate observed with occupation of the slow site alone. The contributions of each cGMP-binding site were investigated using PKG mutants containing substitutions of an invariant threonine residue that is critical for high affinity cGMP-binding in each site. Site-directed mutagenesis of Thr-317 of the fast site (T317A) increased the cGMP $A_{50}$ for heterophosphorylation 4-fold at 30 °C, with nominal effect on cGMP $A_{50}$ for autophosphorylation compared with WT. The analogous slow site mutation (T193A) increased the cGMP $A_{50}$ for heterophosphorylation and autophosphorylation 32- and 64-fold, respectively. Compared with WT, the cGMP $A_{50}$ of the double mutant (T193A/T317A) for heterophosphorylation was increased 300-fold, whereas the cGMP $A_{50}$ for autophosphorylation was similar to that of T193A. Thus, occupation of both cGMP-binding sites of PKG is required for maximal stimulation of heterophosphorylation, whereas occupation of the slow site alone is sufficient for stimulation of the rate of autophosphorylation, and additional occupation of the fast site reduces this rate.

Several regulatory roles have been ascribed to cGMP-dependent protein kinase (PKG) in mammalian cells (1–5).

Three mammalian isozymic forms of PKG have been reported: type Iα, type Iβ, and type II; each of these exists as a homodimer of subunits that contain a regulatory domain and a catalytic domain in a single polypeptide sequence. The regulatory portions of the type I enzymes comprise the amino-terminal ~50% of the polypeptide and contain an autoinhibitory domain, autophosphorylation sites, and two cyclic nucleotide-binding sites (Fig. 1A). The primary sequences of the types Iα and Iβ PKG isoforms differ only in their amino-terminal segment of ~100 residues, sharing 36% identity in this region (6), which contains the dimerization domain, autophosphorylation sites, and autoinhibitory domain. The cGMP-binding sites in types Iα and Iβ PKG have identical amino acid sequences but exhibit different kinetic properties and cyclic nucleotide analog specificities (7). These intramolecular binding sites are distinguished from each other by their relative affinities for cGMP, and they are described as the low affinity cGMP-binding site (fast dissociating site or fast site) and the high affinity cGMP-binding site (slow dissociating site or slow site) (8).

In the absence of cyclic nucleotide, PKG is maintained in a catalytically inactive state for phosphorylation of exogenous substrate (heterophosphorylation) by an autoinhibitory domain that includes a pseudosubstrate site (9). When intracellular cyclic nucleotide levels increase, heterophosphorylation is activated, but the mechanism by which cyclic nucleotide binding stimulates this activity is unknown. Evidence suggests that activation of the PKG either by cyclic nucleotide association with the two allosteric cyclic nucleotide-binding sites or by autophosphorylation is associated with a conformational change (9–13) that removes the influence of the autoinhibitory domain on the catalytic site, thus reducing the efficiency of pseudosubstrate site competition for substrate binding. The $K_a$ of cGMP for the stimulation of heterophosphorylation most closely relates to the average affinity constants of the nucleotide for both sites. However, the utility of two cyclic nucleotide-binding sites in PKG (or PKA) in enzyme function is unknown.

Cyclic nucleotide binding also stimulates the intramolecular autophosphorylation of type I PKG, resulting in altered functional characteristics of the enzyme (14–16). Autophosphorylation of bovine type Iβ PKG in the presence of cGMP or cAMP modifies Ser-63 and Ser-79 (Fig. 1A), and autophosphorylation of Ser-79 is associated with a 4-fold increase above the basal
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Experimental Procedures

Purification of Iβ PKG—Recombinant human type Iβ PKG was purified to homogeneity from Spodoptera frugiperda (Sf9) cells as previously described by (19). Bovine type Iβ PKG was purified to homogeneity from aorta according to Francis et al. (20). Purity was determined by SDS-polyacrylamide gel electrophoresis. Autophosphorylation—10 nm type Iβ PKG was incubated in the presence of 100 μM ATP (∼2,000,000 cpm/μl [γ-32P]ATP), 5 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, and the presence or absence of the indicated cGMP concentration. At 30 °C, the incubation period was either 10 min or 1 h, and the incubation at 4 °C proceeded for 9 h. To assess [32P] incorporation, aliquots of the reaction mixture were added to SDS-2-mercaptoethanol reducing buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis. Type Iβ PKG bands were excised and counted.

PKG Kinase Assay (Heterophosphorylation)—The catalytic activity of PKG was determined as described previously (20) by measuring the incorporation of [32P] into a synthetic heptapeptide (RRRSRAE, Peninsula Laboratories, Inc.) (21). The reaction mixtures contained 10 nM type Iβ PKG, 20 mM Tris, pH 7.4, 200 μM ATP, 136 μg/ml of the heptapeptide, 20 mM magnesium acetate, 100 mM 3-isobutyl-1-methylxanthine, 20 μM synthetic peptide inhibitor of PKA (22), and −50,000 cpm/μl [γ-32P]ATP, with the indicated concentrations of cGMP or dilution buffer. The incubation period was 15 min at 30 °C or 1 h at 4 °C. The reaction was terminated by spotting 85% of the reaction mixtures onto phosphocellulose papers (Whatman P-81, 2 × 2 cm), which were then washed in 75 mM phosphoric acid and counted as described previously (23).

It should be noted that because the cGMP A50 values for phosphorylation of exogenous substrate are obtained using conditions of initial rate, these values represent Ks. The cGMP A50 values for autophosphorylation do not represent Ks because the substrate concentration is depleted progressively during the reaction, and the initial rate of autophosphorylation is reduced at relatively high concentrations of cGMP.

Determination of Protein Concentration—Protein was determined by the method of Bradford (24) using bovine serum albumin as the standard protein or by total [14C]serum binding. Results of Picotag analysis and total [14C]serum binding indicated that protein determination by the Bradford method using bovine serum albumin as the standard overestimated the PKG concentration by 33% (7). Therefore, this correction factor was used when the Bradford method was employed for protein determination.

Materials—[γ-32P]ATP was purchased from NEN Life Science Products. DEAR-Sephsach, Sephacryl S-200, and low molecular weight SDS-polyacrylamide gel electrophoresis standards were purchased from Amersham Pharmacia Biotech. Heptapeptide substrate (RRRSRAE) was obtained from Peninsula Laboratories, Inc. P-81 phosphocellulose paper was purchased from Whatman. Tosylphenylalanyl chloromethyl ketone-treated trypsin was from Roche Molecular Biochemicals. cGMP, cAMP, ATP, magnesium acetate, bovine serum albumin, 2-mercaptoethanol and Bradford reagent were from Sigma.

Results

Comparison of the Kinetic Features of cGMP-stimulated Autophosphorylation and Heterophosphorylation of Native and WT PKG—Recombinant human type Iβ PKG (WT PKG) has been shown previously to exhibit activities comparable to those of bovine type Iβ PKG (native) in all respects (19, 25). Fig. 2 demonstrates that cGMP stimulation of the rate of autophosphorylation and cGMP stimulated the rate of heterophosphorylation were also comparable between native and WT PKG. There was no detectable difference between native and WT PKG with respect to the rate and stoichiometry of autophosphorylation or the rate of heterophosphorylation observed in the presence of saturating cGMP. The stoichiometry of autophosphorylation approached 2 mol of phosphate/mol of enzyme subunit for both native and WT PKG (not shown), and one-dimensional phosphopeptide maps of trypsin digests of autophosphorylated native and WT PKG were indistinguishable (not shown). In vitro autophosphorylation of Ser-63 and Ser-79 of native Iβ PKG occurs at different rates (16). At 30 °C
in the presence of saturating cGMP concentrations, phosphorylation of Ser-63 is complete in ~7 min, whereas phosphorylation of Ser-79 saturates in ~2 h. Unless otherwise indicated, the autophosphorylation reactions for each of the PKGs in the present study were terminated at a time point that was experimentally determined to fall in the linear portion of the Ser-79 modification curve generated in the presence of saturating cGMP concentrations.

The cGMP $A_{50}$ for autophosphorylation was lower than that for heterophosphorylation by 12- and 5-fold at 30 °C for native and WT PKGs, respectively (Fig. 2). When examined at 4 °C, the cGMP $A_{50}$ for autophosphorylation of WT PKG was 2-fold lower than that for heterophosphorylation (Fig. 2). Reactions were performed at 30 °C so that results would be comparable to previous studies (19) and at 4 °C to approximate conditions of cGMP dissociation assays. These data indicated that the rates of autophosphorylation and heterophosphorylation were regulated differentially by cGMP-binding.

Comparison of Kinetic Features of cGMP-stimulated Autophosphorylation and Heterophosphorylation of WT PKG and cGMP Binding Site Mutant PKGs—Both of the cGMP-binding sites of PKG and cGMP-gated ion channel contain a critical Thr that is proposed to form a hydrogen bond interaction with the 2-amino group of cGMP (Fig. 1B) (17, 18). cGMP-stimulated autophosphorylation and heterophosphorylation of recombinant h-Iβ PKG containing point mutations in the fast cyclic nucleotide dissociation site (T317A), in the slow cyclic nucleotide dissociation site (T193A), or in both cyclic nucleotide-binding sites (T193A/T317A) was examined (Fig. 3). Total phosphate incorporation into the mutant enzymes in the presence of saturating cGMP concentrations was comparable to that of WT PKG, with the exception of the double mutant (T193A/T317A), which had 60% phosphate incorporation compared with WT PKG (not shown). Therefore, heterophosphorylation (19) and autophosphorylation activity of recombinant proteins could be compared directly. Maximum activity (as seen in Figs. 2 and 3) refers to the maximal phosphotransferase activity (heterophosphorylation or autophosphorylation) of the individual recombinant enzymes measured in the presence of saturating cGMP concentrations. Mutation of the fast site (T317A) had little effect on the cGMP $A_{50}$ for autophosphorylation (increased 1.7-fold); however, the cGMP $A_{50}$ for heterophosphorylation was increased 5-fold (Table I). These data suggested that cGMP-binding to the fast site does not significantly stimulate autophosphorylation under these conditions but does contribute appreciably to maximal cGMP stimulation of heterophosphorylation.

When the autophosphorylation reaction was examined under conditions that measure predominantly modification of Ser-63 (10 min at 30 °C) (Fig. 4), the rate of autophosphorylation was stimulated maximally at low concentrations of cGMP, which occupy predominantly the slow site, whereas high concentrations of cGMP, which also occupy the fast site, decreased the autophosphorylation rate as compared with the rate of lower cGMP concentrations (Fig. 4). These data supported the proposal that slow site binding is the major component of cGMP-stimulated autophosphorylation.

This interpretation was also supported by comparison of the cGMP $A_{50}$ of T193A with that of T193A/T317A. The cGMP $A_{50}$ for autophosphorylation of T193A/T317A was only 2.6-fold greater than that of T193A, but the cGMP $A_{50}$ for heterophosphorylation of T193A/T317A was 28-fold greater than that of T193A (Table I). Mutation of the slow site increased the cGMP $A_{50}$ for autophosphorylation and heterophosphorylation 34- and 20-fold, respectively, compared with that of WT PKG. These data demonstrate directly that cGMP-binding to the slow site is a significant component of the stimulation of catalytic activity for heterophosphorylation as well as the major component of stimulation of autophosphorylation.

The results reported here provide the first evidence for positive cooperativity in the cGMP-stimulated catalytic activity of type I PKG toward the heptapeptide substrate (Hill coefficient of 1.6 for WT PKG measured at 4 °C) (Table I). The PKG containing the fast site mutation (T317A) had reduced positive cooperativity with a Hill coefficient of 0.8 at 4 °C. A Hill coefficient of <1 could indicate that the effect of cGMP-binding to the two sites regulates catalytic activity more independently, i.e. by a less coupled mechanism. Therefore, fast site mutation not only increased the concentration of cGMP required for half-maximal stimulation of heterophosphorylation but also reduced the positive cooperativity observed with cGMP-stimulated heterophosphorylation. These data supported the conten-
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Table I
Kinetic features of cGMP stimulation of PKG Activity

| PKG          | Temperature °C | cGMP A50 Hetero-p | cGMP A50 Auto-p | cGMP A50 fold effect | Hill coefficient |
|--------------|----------------|-------------------|----------------|----------------------|-----------------|
| Wild type    | 4              | 0.034 ± 0.004     | 0.016 ± 0.003  | 1                    | 1.65 ± 0.27     |
|              | 30             | 0.57 ± 0.07       | 0.12 ± 0.03    | 1.41 ± 0.24          |                 |
| T317A        | 4              | 0.17 ± 0.02       | 0.027 ± 0.005  | 5                    | 20              |
| T193A        | 4              | 0.67 ± 0.06       | 0.54 ± 0.08    | 20                   | 1.42 ± 0.16     |
| T193A/T317A  | 4              | 19 ± 2            | 1.40 ± 0.52    | 560                  | 1.1 ± 0.1       |

* Hetero-p, catalytic activity toward substrate (heterophosphorylation).

** Auto-p, autophosphorylation.

![Graph](image)

Fig. 4. Effects of cGMP concentration on initial rate of autophosphorylation of bovine and human Iβ PKG. cGMP-stimulated autophosphorylation of 10 nM bovine Iβ PKG (native) or WT h-Iβ PKG or fast site mutant T317A was measured at 30 °C as described under “Experimental Procedures”; reactions were terminated at 10 min. Results are the mean of two experiments.

Discussion

It was established earlier that occupation of the slow cGMP-binding site of PKG occurs at relatively low cGMP concentrations, whereas at higher cGMP concentrations the fast site is also occupied, and both binding sites are thought to be involved in the activation of PKG for heterophosphorylation (8, 26). Whether activation of autophosphorylation follows the same pattern in response to cGMP-binding could have important physiological implications. The results of the present study suggest that cGMP-binding to the slow site provides a significant component of cGMP-stimulated heterophosphorylation and that cGMP-stimulated autophosphorylation is caused primarily by cGMP-binding to the slow cyclic nucleotide-binding site. However, occupation of the fast site by cGMP is stimulatory for heterophosphorylation. Therefore, as cellular cGMP levels progressively increase, it would be predicted that initially the rate of autophosphorylation would accelerate because of cGMP-binding to the high affinity “slow” site. However, further elevation in cGMP would lead to cGMP-binding to the low affinity “fast” site as well and thereby decrease the rate of autophosphorylation even though the rate is still faster than that with no cGMP.

The interpretation of the current results are at variance with those of studies using slow site and fast site selective cGMP analogs to activate type Iα PKG (27). From the latter studies, it was concluded that binding of cGMP to either the slow or fast site is stimulatory for autophosphorylation, whereas simultaneous binding to both sites is inhibitory for autophosphorylation (27). These conclusions relied in large part on the observation that fast site binding, after occupation of the slow site, has a direct stimulatory effect on the activity of the enzyme for heterophosphorylation (27). However, studies using single cyclic nucleotide analogs should be interpreted with caution. Even though N2-monobutyryl-cGMP binds with somewhat higher affinity to the fast site than to the slow site (26), the results were interpreted with the assumption that fast site binding has significant influence on the expression of heterophosphorylation.

The conformational change that occurs with slow site binding is that under subsaturating conditions the phosphorylation of PKG compared with cGMP, 2-monobutyryl-cGMP binds with somewhat higher affinity to the fast site than to the slow site (26), and the results were interpreted with the assumption that only the fast site would be occupied under these conditions. However, studies using single cyclic nucleotide analogs should be interpreted with caution. Even though N2-monobutyryl-cGMP is 10-fold selective (compared with cGMP) for the fast site (26), this level of relative selectivity is probably not sufficient to produce an absolute site selectivity for this analog because of the very high innate cyclic nucleotide affinity of the slow site compared with the fast site. From our results using type Iα PKG reported here, it is suggested that cGMP-binding to the slow site alone accounts for stimulation of autophosphorylation. The effect of cyclic nucleotide binding to the fast site is to diminish the rate of autophosphorylation that has been promoted by cGMP-binding to the slow binding site.

When cGMP binds to PKG, the enzyme undergoes a progressive conformational change that is associated with activation of catalysis (5, 11–13). This induced conformational change disrupts interactions between the catalytic and autoinhibitory domains (1, 5, 9). Results suggest that occupation of the slow site alone causes a substantial conformational change in PKG (7, 13). Results in the present study demonstrate directly that cGMP-binding to the slow site alone stimulates autophosphorylation and has a critical role in stimulating heterophosphorylation. The conformational change that occurs with slow site binding alone apparently allows for increased exposure of Ser-63 and Ser-79 to the catalytic site. cGMP-binding to the fast site is important for full expression of cGMP-stimulated heterophosphorylation (8). This effect could be direct, or it could be brought about by a more indirect role of this component, such as stimulation of slow site binding.

Fast site binding decreases cGMP dissociation from the slow site in in vitro and phosphotransferase activity significantly. It is proposed that fast site binding, after occupation of the slow site, has a direct stimulatory effect on the activity of the enzyme for heterophosphorylation in addition to its stimulatory effect on...
cGMP-binding to the slow site. These two effects presumably act in concert to stimulate heterophosphorylation.

Based on the present studies using a model heptapeptide substrate, autophosphorylation of PKG is likely to have physiological relevance and may play a “feed-forward” role in cGMP signaling PKG activation considering the following observations: 1) autoactivation by autophosphorylation of both type I PKG isoforms occurs in the respective autoinhibitory domains even though the amino acid sequences of the autophosphorylated regions for the two isozymes are very different, and most of these autophosphorylated sites do not conform to consensus phosphorylation sequences for heterophosphorylation in PKGs (16); 2) autophosphorylation reduces the cAMP A50 for both isozymes for heterophosphorylation, thus facilitating cross-activation by cAMP (16); 3) autophosphorylation of type Iα (29) and Iβ PKGs (30) is affected minimally by the presence of high concentrations of substrate, suggesting that autophosphorylation/autoactivation occurs in preference to heterophosphorylation (16); 4) autoactivation of type I PKG by autophosphorylation occurs at physiological concentrations of enzyme and cyclic nucleotides (16).

From results presented here, the cGMP concentration required to achieve A50 of autophosphorylation of native and wild type Iβ PKG at 30 °C is 100–120 nM, which approximates the concentration of cGMP in vascular smooth muscle cells under basal conditions (31). Therefore, even basal intracellular conditions would be predicted to foster a relatively high level of autophosphorylation of PKG, particularly because a significant level of cGMP is maintained in the cells. At 10−7 M cGMP the stimulation of autophosphorylation is six times that for heterophosphorylation. Therefore, PKG autophosphorylation could prove to be a significant determinant of PKG activity for heterophosphorylation (16) because the activity of non-autophosphorylated type Iβ PKG varies from 16 to 30% of maximum at the lower and upper ends of the physiological range of cGMP concentration, respectively, an increase in activity of 1.9-fold (16). In contrast, the activity of autophosphorylated type Iβ PKG is 56% of maximum at the upper end of the physiological range of cGMP, a 3.5-fold increase over that of non-autophosphorylated enzyme at the basal concentration of cGMP (56 versus 16%). Therefore, changes in the state of autophosphorylation caused by an alteration in the balance between either the rate of self-modification or phosphoprotein phosphatase activity could have profound effects on PKG for heterophosphorylation under basal conditions and in response to changes in cyclic nucleotide levels; this effect would be particularly evident at low levels of cyclic nucleotides as in the initial phase of increasing cyclic nucleotide levels, i.e. a feed-forward mechanism. At high concentrations of cGMP, the diminished rate of autophosphorylation may contribute to a negative feedback regulatory mechanism. Thus, it seems likely that the cellular state of PKG activity for heterophosphorylation is determined by the concerted actions of cyclic nucleotide binding and autophosphorylation events.

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