Autophosphorylation of Type I\(\beta\) cGMP-dependent Protein Kinase Increases Basal Catalytic Activity and Enhances Allosteric Activation by cGMP or cAMP*

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Autophosphorylation of purified bovine I\(\beta\) isozone of cGMP-dependent protein kinase (I\(\beta\) cGK) in the presence of cGMP or cAMP increased basal kinase activity (−cGMP) as much as 4-fold and reduced the \(K_a\) for both cGMP and cAMP; maximum catalytic activity (+cGMP) was not altered. Autophosphorylation proceeded with at least two rate components. The faster rate correlated with phosphorylation of Ser-63. The slower rate, as well as the increase in basal kinase activity and decrease in \(K_a\) for cyclic nucleotides, correlated with phosphorylation of Ser-79. Autophosphorylation of either residue was an intramolecular reaction. Autophosphorylation of a proteolytically generated I\(\beta\) cGK monomer lacking amino-terminal residues 1–64 increased basal activity (3-fold) and decreased \(K_a\) for cAMP (15-fold). This indicates that autophosphorylation of Ser-79 did not require dimeric cGK and that the phosphorylation of Ser-79 in the monomer was sufficient to alter enzymatic characteristics of I\(\beta\) cGK. These studies suggested that increases in intracellular cGMP or cAMP could result in autophosphorylation of I\(\beta\) cGK, which would increase basal kinase activity as well as the sensitivity of cGK to activation by cGMP or to cross-activation by cAMP. Autophosphorylation could also prolong the increased kinase activity after decline of the second messenger.

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§ The abbreviations used are: cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis.
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Basal kinase activity of non-autophosphorylated and autophosphorylated Iβ cGK. 1.0 µM Iβ cGK was autophosphorylated as described under “Experimental Procedures” in the absence (no cyclic nucleotide) or presence of 5 µM cGMP, or 10 µM cAMP, or in the presence of 5 µM cGMP alone (Non-Autophosphorylated). These reaction mixtures were then diluted 500-fold in cold buffer A containing 0.5 mg/ml bovine serum albumin and assayed for protein kinase activity in the presence and absence of 10 µM cGMP. Basal kinase activity is the percent of maximum kinase activity measured in the presence of cGMP. Error bars = S.D. (n); n = 6 except for autophosphorylation in absence of cyclic nucleotide for which n = 2.

Phosphorylation of Iα cGK in the presence of cAMP was reported to increase basal kinase activity and decrease the $K_a$. The increased basal kinase activity elicited by autophosphorylation was stable for at least 90 min when Iβ cGK was stored at 30 °C prior to the kinase assay (not shown) and was linear for at least 2 h at 30 °C during the kinase assay (Fig. 3). This activation was not reversed when >99.5% of cyclic nucleotides were removed by gel filtration chromatography (not shown). These data indicated that the increased basal activity was due to a stable modification of cGK.

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Fig. 3. Stability of autophosphorylation-stimulated activity. 0.7 μM Iβ cGK was autophosphorylated in the presence of 10 μM cAMP for 2.25 h as described under “Experimental Procedures.” Reaction mixtures were then diluted 200-fold in cold buffer A containing 0.2 mg/ml bovine serum albumin and assayed for protein kinase activity in the absence and presence of 10 μM cGMP. The phosphorylated enzyme, which had a basal activity 4-fold greater than control, was then subjected to a 2-h protein kinase assay at 30°C for various times in the presence and absence of 0.5 μM cGMP.

for cAMP by ~6-fold (21). Our results confirmed the increase in basal activity of Iα cGK by cAMP and indicated that cGMP-stimulated autophosphorylation also increased the basal activity of Iα cGK (not shown). In addition to the effect on basal activity, cAMP- or cGMP-stimulated autophosphorylation of Iβ cGK decreased the $K_a$ for either cyclic nucleotide—2-fold (0.83–0.48 μM cGMP, 7.6–2.8 μM cAMP) (Fig. 4).

Fig. 4 illustrates the impact of autophosphorylation on the sensitivity of Iβ cGK to physiological concentrations of cGMP or cAMP (shown in dashed boxes). Porcine coronary artery smooth muscle is rich in cGK, and intracellular concentrations of cGMP (0.09–0.27 μM) and cAMP (0.42–1.26 μM) have been determined in that tissue (31, 32). As seen in Fig. 4, kinase activity of non-autophosphorylated Iβ cGK varied from 16 to 30% of maximum at the lower and upper end of the physiological range of cyclic nucleotides (0.09–0.27 μM), respectively, an increase of 1.9-fold. In contrast, the activity of autophosphorylated Iβ cGK was 56% of maximum at 0.27 μM cGMP and represented a 3.5-fold increase over that of non-autophosphorylated enzyme at 0.09 μM cGMP (56 versus 16%). Similarly, the activity of non-autophosphorylated Iβ cGK at 1.26 μM cAMP, which is at the upper end of the physiological range, was 1.5-fold greater than that exhibited at 0.42 μM cAMP. The activity of autophosphorylated Iβ cGK was 3.3-fold greater at 1.26 μM cAMP compared with that of non-autophosphorylated enzyme at 0.42 μM cAMP. These data demonstrated that the phosphorylation state of Iβ cGK, as well as the level of cyclic nucleotides, is a significant determinant of Iβ cGK activity.

Autophosphorylation of Iβ cGK was also studied at concentrations of enzyme as well as concentrations of cyclic nucleotides that approximate intracellular levels in porcine coronary artery smooth muscle (31, 32). Treatment of this tissue with either 10 μM sodium nitroprusside or 100 μM isoproterenol causes a ~3-fold elevation of cGMP and cAMP, respectively (see above) (32). Incubation of Iβ cGK in the presence of Mg/ATP and either cGMP (0.3 μM), cAMP (1.2 μM), or a combination of both cyclic nucleotides reported for the agonist-treated arteries resulted in stoichiometric phosphate incorporation (1.1–1.4 [32P] mol/mol Iβ cGK subunit). Basal activity was 2.5-fold greater than the basal activity of cGK that had been incubated with the same concentrations of cyclic nucleotide alone (no Mg/ATP) (not shown).

The effect of autophosphorylation on the $K_a$ for cGMP could be due to changes in cGMP dissociation characteristics. Iβ cGK was autophosphorylated in the presence of Mg/ATP and 20 μM [3H]cGMP or with [3H]cGMP alone. The reaction was halted by addition of excess EDTA and chilling the samples to 4°C. The dissociation of [3H]cGMP in the presence of 100-fold excess unlabeled cGMP was measured as described under “Experimental Procedures.” Dissociation was rapid under these conditions (~80% dissociated in 4 s), and no difference was observed between autophosphorylated and non-autophosphorylated Iβ cGK (not shown). The presence of histone VIII-S (0.5 mg/ml, final concentration) in the incubation significantly decreased the rate of dissociation, but no difference in dissociation rates between autophosphorylated and non-autophosphorylated Iβ cGK could be detected (Fig. 5).

[3H]cGMP dissociated from the fast and slow sites of Iβ cGK with a $t_{1/2}$ of 3.7 s and 8.1 min, respectively (not shown). Under the same conditions, dissociation rates from the fast and slow sites of Iα cGK yielded a $t_{1/2}$ of 6.2 s and 18.2 min, respectively (not shown). These rates are in accordance with published values (15, 22).

Comparison of Effect of cGMP on Autophosphorylation of Iα and Iβ cGK—The stoichiometry of phosphate incorporation into purified Iα cGK has been reported to be higher in the presence of cAMP than in the presence of cGMP (19). This was
also generally observed with Iβ cGK in the present studies and was probably due to the slower rate of phosphate incorporation in the presence of cGMP compared with that in the presence of cAMP. However, there was a difference in the pattern of cGMP-stimulated autophosphorylation of Iα and Iβ cGK (Fig. 6). With Iα cGK, autophosphorylation was stimulated (33) by cGMP concentrations that saturate only one cyclic nucleotide-binding site (15). At cGMP concentrations sufficient to partially saturate the second cyclic nucleotide-binding site, autophosphorylation was inhibited (33) (Fig. 6). Cyclic nucleotide analog studies (not shown) suggested that the extent of autophosphorylation of Iα cGK was inversely related to the affinity of the cyclic nucleotide for both binding sites. This supports the original observation that autophosphorylation was stimulated by cyclic nucleotide bound in the slow site alone, or perhaps in the fast site alone, but was inhibited by saturation of both cyclic nucleotide-binding sites (33). However, defining the contribution of the binding sites based on the use of cyclic nucleotide analogs is problematic since those that are relatively fast site-selective are still likely to bind to the high affinity site at lower concentrations than is required for their association with the fast site. Therefore, partial saturation of only the fast site without prior saturation of the slow site is unlikely. Thus, fast site-selective cyclic nucleotide analogs may stimulate autophosphorylation by binding to the slow site. This introduces the possibility that cyclic nucleotide binding to the slow site stimulates autophosphorylation of Iα cGK, whereas binding to the fast site inhibits the reaction.

Under the same conditions, autophosphorylation of Iβ cGK was not inhibited by saturating cGMP concentrations (Fig. 6). This lack of inhibition was apparently not due to the lower cGMP affinity of Iβ cGK relative to Iα cGK, since autophosphorylation of Iβ cGK was also stimulated at low concentrations and not inhibited at high concentrations of the cyclic nucleotide analog 8-Br-β-phenyl-1,3′-etheno-cGMP (not shown), which has a ~28-fold higher affinity for Iβ cGK than does cGMP (34).

Identification of Phosphoacceptor Sites—The two apparent time components of autophosphorylation (Fig. 2) were examined for multiple-site autophosphorylation. Iβ cGK was autophosphorylated in the presence of cAMP and [γ-32P]ATP for 7 min or 2 h and then proteolyzed with trypsin. The resulting 32P-peptides were resolved by high-performance liquid chromatography C8 reverse phase chromatography (Fig. 7). When Iβ cGK was incubated for 7 min (0.8 mol [32P]/mol Iβ cGK subunit), the only significant 32P-peptide peak eluted at ~15% acetonitrile (Fig. 7A), confirming the primary phosphoacceptor site as Ser-63 (56). The basal kinase activity and Kₐ for cAMP of cGK autophosphorylated for 7 min was unchanged compared with that of the non-autophosphorylated cGK (not shown). The profile derived from Iβ cGK autophosphorylated for 2 h (1.8 mol [32P]/mol Iβ cGK subunit) in the presence of cAMP also had a major radiolabeled peak that eluted with ~15% acetonitrile, as well as three other peaks that eluted with ~30% acetonitrile (Fig. 7B). The basal activity and Kₐ for cAMP of cGK autophosphorylated for 2 h was 5-fold greater and 2.6-fold lower, respectively, than that of the non-autophosphorylated enzyme (not shown). All three peaks eluting with ~30% acetonitrile were determined to be derived from the same phosphopeptide, TQAI5'AEPTAFD1ODLSVTLTFYPK... , but slight differences in the peptides accounted for the different elution positions. Peak 1 contained the peptide as written above. The amino terminus of peak 2 was Gln-76 rather than Arg-75. Upon sequential Edman degradation of these peptides, the 32P was released at Ser-79. The amino terminus of peak 3 was blocked; however, tandem mass spectrometric analysis determined that the mass of the peak 3 peptide was consistent with the peak 2 peptide containing one phosphoserine and having Gln-76 cyclized. The slower rate of autophosphorylation correlated with modification of Ser-79 since phosphorylation of Ser-63 was essentially complete within 7 min (Fig. 7).

Studies of the Requirement of Quaternary Structure for Autophosphorylation of Iβ cGK—The order of reaction of Iβ cGK autophosphorylation was examined. The initial rate (Ser-63) of phosphate incorporation into Iβ cGK was constant over a 40-fold range of enzyme concentration (13–513 nM). A Van't Hoff plot would yield a slope of 1 for an intramolecular reaction or a slope of 2 for an intermolecular reaction (35). As seen in Fig. 8, autophosphorylation of Ser-63 was first-order (slope = 0.98) and, therefore, independent of enzyme concentration, which represents an intramolecular reaction. The reaction was carried out for only 2 min due to the lability of Iβ cGK at low concentrations. Because of this lability, examination of the low-rate component of autophosphorylation (Ser-79) in native cGK proved difficult. This problem was circumvented by utilizing a monomeric form of Iβ cGK. During extended storage, purified dimeric Iβ cGK is proteolyzed carboxyl-terminal to the dimerization domain, thus producing cGMP-dependent monomer (22, 36). An aged Iβ cGK preparation was determined to contain approximately 20% full-length and 80% monomeric...
enzyme. SDS-PAGE revealed that proteolysis generated two different monomers with molecular masses of ~70 and ~68 kDa. Sequential Edman degradation of the monomers determined that the amino terminus of the 70-kDa monomer was Ser-65 and that of the 68-kDa monomer was Ser-79. Thus, neither contained Ser-63, the primary phosphoacceptor. This preparation was used to examine the order of reaction for Ser-79 modification.

Aged Iβ cGK, containing a mixture of full-length and monomeric enzyme, was diluted over a 64-fold range (20–1300 nM) and autophosphorylated in the presence of cAMP. The reaction was halted at 7 min by adding SDS-reducing buffer and boiling. Samples were subjected to SDS-PAGE to resolve the full-length protein and the two monomers. Monomer bands were excised, and the radioactivity was measured. The 70-kDa Iβ cGK monomer provided an opportunity to examine the effects of Ser-79 autophosphorylation in the absence of Ser-63. For Iβ cGK, the reaction was terminated after 2 min by spotting aliquots onto phosphocellulose papers (Whatman P81), and [32P] incorporation was determined (slope = 0.98). For the Iγ cGK monomer, the reaction was terminated by addition of SDS-reducing buffer and boiling. [32P] incorporation was determined by measuring the radioactivity associated with the monomer band isolated from SDS-PAGE gels stained with Coomassie Blue. (Slope = 1.1.)

**DISCUSSION**

Kinase activity of Iβ cGK is increased by cGMP or cAMP in three ways. First, cyclic nucleotide association with the binding sites of the enzyme relieves autoinhibition of the catalytic site. Second, cyclic nucleotide-stimulated autophosphorylation of the autoinhibitory domain impedes interaction of the pseudosubstrate site with the catalytic center. Finally, autophosphorylation increases sensitivity of the enzyme to activation by cyclic nucleotides. At subsaturating levels of cyclic nucleotide, these effects in combination would produce a significantly greater stimulation of kinase activity than would occur by either process alone. Thus, cyclic nucleotides cause the enzyme to autophosphorylate and concomitantly autoactivate.

For several reasons, the increased basal activity of both Iα and Iβ cGK following autophosphorylation cannot be explained by trace amounts of cyclic nucleotides carried over from the autophosphorylation incubation. First, dilution of non-autophosphorylated Iβ cGK that had been incubated in the absence or presence of cGMP or cAMP yields identical activities. Second, the characteristics of cGMP dissociation from autophosphorylated Iα (19, 20, 37) or Iβ cGK do not differ from those of the unmodified enzymes. Third, the increased basal activity is terminal to this residue. It also suggested that there are no major phosphoacceptors carboxyl-terminal to Ser-79. The availability of the 70-kDa Iβ cGK monomer provided an opportunity to examine the effects of Ser-79 autophosphorylation in the absence of Ser-63. The 70-kDa Iβ cGK monomer was separated from both the full-length enzyme and the 68-kDa Iβ cGK monomer by DEAE-Sephacel chromatography as described previously (25). The 70-kDa Iβ cGK monomer was then subjected to autophosphorylation. Basal kinase activity and cAMP $K_p$ for the autophosphorylated 70-kDa Iβ cGK monomer was compared with that of the non-autophosphorylated 70-kDa Iβ cGK monomer (Fig. 9). Autophosphorylation increased basal kinase activity from 17 to 52% of maximum and decreased the $K_p$ of cAMP 15-fold. These results confirmed that modification of Ser-79 is sufficient to produce the functional effects observed with autophosphorylation of full-length Iβ cGK. In addition, the rate of Ser-79 modification in the absence of Ser-63 was similar (~2-fold greater) to that of the native dimer (not shown). This suggested that in the native dimeric cGK, autophosphorylation of these sites in Iβ cGK is not hierarchical.
not reversed upon removal of substrates and ligands by gel filtration chromatography. Thus, the increase in basal kinase activity and the increase in affinity for cyclic nucleotides result from a stable modification of the enzyme that closely correlates with autophosphorylation.

Autophosphorylation of Iβ cGK under these conditions modifies two residues, Ser-63 and Ser-79. Phosphorylation of Ser-63 is rapid and saturates in ~7 min at 30°C but has minimal, if any, effect on kinase activity or cyclic nucleotide sensitivity. Phosphorylation of Ser-79 proceeds at a rate ~10-fold slower than that for phosphorylation of Ser-63 and correlates with an increased basal kinase activity and decreased $K_a$ for cyclic nucleotides. It is not certain if autophosphorylation of Ser-79 in dimeric Iβ cGK is sufficient to increase basal activity or if modification of both Ser-63 and Ser-79 is required. However, modification of Ser-79 in a monomeric Iβ cGK lacking Ser-63 is sufficient to elicit these changes in activity. It is possible that Ser-63 alters an unknown activity of the enzyme, such as stability to proteolysis, intracellular localization, or substrate recognition.

Van't Hoff analysis reveals that the autophosphorylation of Iβ cGK is intramolecular. This is confirmed by the recent observation in this laboratory that the rate of Iβ cGK autophosphorylation remains constant in the presence or absence of a 5-fold excess of pre-autophosphorylated Iβ cGK (Francis et al. (56)).

$\alpha$ and Iβ cGK contain substrate sites (autophosphorylation sites) and putative pseudosubstrate sites within their autoinhibitory domains. The sequence of a pseudosubstrate site resembles that of a substrate and is consequently thought to confer inhibition of catalysis by competition with the active site of the enzyme (17, 38–40). However, by definition the pseudosubstrate site lacks a phosphoacceptor site. The pseudosubstrate sites of Iβ cGK (56) and of Iα cGK (11, 12, 41) are $^{74}$KROQ$^{15}$SAEP and $^{95}$RAOG$^{14}$SAEP, respectively. The residue preceding the asterisk (*) is thought to occupy the phosphate acceptor site (P0) because it corresponds to RRXS$^X$ of the consensus phosphorylation sequence in type II regulatory subunit of cAK. The major sites of autophosphorylation in Iα cGK (Ser-50, Thr-58, Ser-72, Thr-84) (42) and Iβ cGK (Ser-63 and Ser-79) flank the pseudosubstrate site. In the case of Iβ cGK, Ser-79 is located in the P$^{12}$ position. Phosphorylation of this residue is proposed to cause the activity change in Iβ cGK, but phosphorylation of the homologous residue in Iα cGK (Ser-64) presumably cannot explain the activity change in this enzyme since this site is reported to be only a minor phosphoacceptor (18).

The mechanism by which autophosphorylation or cyclic nucleotide binding activates cGK is unknown; however, these processes could be quite similar. Because of the presence of autophosphorylation sites in the vicinity of the autoinhibitory domain, activation is likely due to displacement of the pseudosubstrate site from the catalytic site. In order for phospho-transfer to either exogenous substrates or autophosphorylation sites to occur, the tight interaction between the catalytic domain and the autoinhibitory domain must be disrupted. Such a disruption could be brought about by a conformational change due to electrostatic repulsion between the incorporated phosphate, which carries a negative charge, and a negative charge(s) in the catalytic domain. The structure of the cAK catalytic subunit (C-subunit) co-crystallized with a peptide derived from the heat-stable protein kinase inhibitor (43) confirms results generated from biochemical studies (38, 44, 45) indicating that electrostatic interactions are crucial to C-subunit-substrate association. Two Arg residues in the consensus substrate recognition sequence (RRXS$^X$) are found in the protein kinase inhibitor as well as in the autoinhibitory region of the cAK regulatory subunits and are thought to interact with four Glu residues of the C-subunit (43). Incorporation of an electronegative phosphate group near the two Arg residues in the consensus recognition sequence might repel the Glu residues in the active site of the C-subunit. Indeed, autophosphorylation of the Ser residue in the autoinhibitory site (RRVS$^V$) of the type II regulatory subunit reduces its affinity for C-subunit by 10-fold (46), and phosphorylation of the Ser-99 residue in the pseudosubstrate site (P$^{99}$RRGAI$^S$) of type I regulatory subunit by cGK greatly reduces its affinity for C-subunit (47, 48). The position of Ser-99 in type I regulatory subunit is homologous to that of the phosphoacceptor Ser-79 in Iβ cGK (P$^{79}$). The catalytic domain of type I cGK shares 41% identity to the C-subunit (determined by using Ref. 49) and includes the four Glu residues discussed above. Therefore, it is feasible that electrostatic repulsion between the phosphate group of the autophosphorylated Ser-79 (Iβ cGK) and the Glu residues of the catalytic domain could elicit a conformational change that activates the enzyme. However, evidence indicates that the catalytic and regulatory domains of cAK and cGK interact at positions outside the pseudosubstrate site (50–52, 56). Thus, autophosphorylation must also overcome these additional sites of interaction. Using synthetic peptides, it was recently demonstrated that phosphorylation alters the charge of basic residues that flank the phosphoacceptor (53). This effect, in combination with repulsion of the catalytic domain or other components of the regulatory domain, and a reduction in the hydrophobicity of the pseudosubstrate region could provide a potent force to induce conformational changes resulting in activation of the enzyme.

Purified bovine Iα cGK contains endogenous covalent phosphate, indicating that phosphorylation of this isoform occurs in vivo (19), although the sites of in vivo modification have not been determined. The following additional observations suggest that autophosphorylation of both Iα and Iβ cGK has physiological relevance and plays a role in activation. 1) Autoactivation by autophosphorylation of sites in the autoinhibitory domain occurs with both isoforms even though the amino acid sequences of the autophosphorylated regions for the two isoforms are very different. 2) Autophosphorylation reduces the $K_a$ for cAMP in both isoforms, thus facilitating cross-
activation by cAMP. 3) Autoactivation of Iβ cGK by autophosphorylation occurs at presumed physiological concentrations of enzyme and cyclic nucleotides. 4) Autophosphorylation of Iα (54) or Iβ cGK (24) is unaffected by the presence of substrate, suggesting that autophosphorylation/autoactivation occurs in preference to phosphorylation of exogenous substrates. Furthermore, in vitro autophosphorylation of Iβ cGK proceeds as rapidly as phosphorylation of exogenous substrates such as the heptapeptide RLRSRAE and the in vivo substrate vimentin (24). The in vitro autophosphorylation rate of the secondary site is relatively slow, but cGK could be maintained in a partially autophosphorylated state in vivo due to the presence of significant steady-state levels of cyclic nucleotides even in the presence of low levels of stimulants in most tissues (55). Again, this would depend on the relative rates of autophosphorylation and dephosphorylation.

In vitro autophosphorylation of Iα or Iβ cGK is an intramolecular reaction that activates these enzymes and increases the sensitivities to activation by cGMP or cAMP. When cGMP is elevated, autophosphorylation may account for as much of the increased cGK activity as that contributed by cGMP binding to the enzyme. Finally, autophosphorylation could protract the effects of extracellular signals that induce transient elevations of cyclic nucleotide levels.

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