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Catalytic site amino acids of PKGI-alpha influence allosteric cGMP binding

Jennifer L. Busch[1], Thomas M. Bridges[2], Robyn Richie-Jannetta[3], Brian P. Hollett[1], Sharron H. Francis[2], Jackie D. Corbin[2]

[1]Department of Biology, Wheaton College, Wheaton, IL 60187; [2]Department of Molecular Physiology and Biophysics and [3]Department of Biochemistry, Vanderbilt University, Nashville, TN 37232-0615

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1. ABSTRACT

Ser-64, an autophosphorylation site in the autoinhibitory subdomain of cGMP-dependent protein kinase type I-alpha (PKGI-alpha), lowers affinity for cGMP and suppresses catalytic activity (1). Using the structure of homologous cAMP-dependent protein kinase as a model, three conserved residues (Gln-401, His-404, Cys-518) in the PKGI-alpha catalytic site are predicted to be juxtaposed to Ser-64 (2). Individual point mutants (Q401A, H404A and C518A) and a double mutant (S64A/H404A) have been generated. cGMP or cAMP affinities (K_a) of each mutant protein for phosphotransferase activation and allosteric [^{3}H]cGMP-binding affinity (K_D) of each mutant protein are significantly improved over those of wild-type (WT) PKGI-alpha. However, affinities (K_m) of the mutant PKGs for peptide substrates or ATP are unaltered. Kinase activity ratio (-GMP/+cGMP) of H404A is greater than that for WT, Q401A, or C518A, and similar to that for S64A and S64A/H404A. These results reveal a unique mechanism whereby catalytic domain residues predicted to be spatially close to Ser-64 of the regulatory domain weaken the intrinsically high affinity of PKGI-alpha for cGMP and provide for autoinhibition of catalytic activity.

2. INTRODUCTION

Cyclic GMP-dependent protein kinase type Iα (PKGIα) is a homodimer, and each PKG monomer contains a regulatory domain (R domain) and a catalytic domain (C domain); salient functions and regulation of kinase activities are retained within a single monomer (3-5). Each C domain has a Mg/ATP-binding site and a protein/peptide substrate-binding site. Each R domain has an extended leucine zipper dimerization subdomain near the amino terminus, two allosteric cGMP-binding sites, and an autoinhibitory subdomain that includes several autophosphorylation sites (including Ser-64) and a pseudosubstrate sequence (4; 6). The pseudosubstrate sequence (-^{59}RAQ**G**I^{63}-) mimics a protein substrate sequence but lacks a phosphorylatable residue; in place of the phosphorylatable serine or threonine found in substrates, Gly-62 (underlined and bolded) in PKGIα occupies the phosphorylation position (P^0). In the inactive state of PKGIα, the protein/peptide substrate-binding site in the C domain has been thought to interact with the pseudosubstrate site and other nearby features, thereby blocking substrate access to the catalytic site (7; 8). Cyclic GMP binding to the allosteric sites and/or

autophosphorylation in the R domain disrupts this interaction and activates the enzyme (6; 9-14). Autophosphorylation of PKGIα has also been shown to cause a 10-fold increase in affinity for cAMP, thus increasing the likelihood of "cross-activation" of PKG by cAMP under physiological conditions (15; 16). Thus, definition of the elements within PKG that contribute to regulation of its activation by cGMP and/or cAMP and autoinhibition of its phosphotransferase activity addresses biological questions that are increasingly important in understanding the role of PKG in cyclic nucleotide signaling in many tissues (5; 17-24).

Cyclic AMP-dependent protein kinase (PKA), which is homologous to PKG, has a dimeric R subunit and two C subunits that interact with the dimeric R subunit. When cAMP binds to the R subunits, the C subunits are activated and dissociate as monomers. In contrast, PKG is comprised of two subunits in which the R and C domains are conjoined on a single polypeptide; upon activation by cGMP binding, the PKG monomers undergo a marked conformational elongation that disrupts autoinhibition (11; 25). The R subunit of PKA or R domain of PKG contains an autoinhibitory subdomain that includes a pseudosubstrate sequence and that suppresses phosphotransferase activity of the respective catalytic sites. The presence of a basic amino acid in the P^{-3} position [three amino acids amino-terminal to the phosphorylation position (P^0)] of the pseudosubstrate sequence in PKGIα is a characteristic feature of most cyclic nucleotide protein kinase substrates. A serine (italicized and bolded in the PKGIα sequence) or cysteine in the P^{+2} position (-^{59}RAQGI***S***^{64}-), which has previously been considered to be outside the pseudosubstrate sequence, is conserved in cAMP- and cGMP-dependent protein kinases. RIα and RIβ of PKA have a serine at the P^{+2} position (Ser-99 and Ser-100, respectively), and PKA RIIα and RIIβ have a cysteine (Cys-97 and Cys-98, respectively) at this position. All three PKG enzymes have a serine at P^{+2}, i.e., Ser-64, Ser-79, and Ser-125 in PKGIα, PKGIβ, and PKGII, respectively. The side chain of either serine or cysteine has the capacity to interact with other amino acids through hydrogen bonding.

Work in this laboratory and in that of others has shown that the P^{+2} serine in the pseudosubstrate sequence of either WT PKGIα or WT PKGIβ weakens the intrinsic affinity of the allosteric binding sites for cGMP; substitution of alanine for these respective serines (S64A in PKGIα or S79A in PKGIβ) significantly increases cGMP-binding affinity (1; 26). This reveals that Ser-64 or Ser-79 maintains the respective PKG in a state of lowered sensitivity to the physiologically active ligand, cGMP, and explains, at least in part, the mechanism whereby autophosphorylation of these residues increases sensitivity of PKGs to activation by low levels of cGMP. The respective serines in these pseudosubstrate sequences have also been shown to increase effectiveness of autoinhibition (1, 26). Replacement of these serines by alanine in PKGIα or PKGIβ substantially weakens autoinhibition, and replacement by bulkier amino acids, e.g., aspartic acid or asparagine, nearly abolishes autoinhibition. Thus, contacts involving the Ser at P^{+2} in the pseudosubstrate sites of PKGI isoenzymes contribute to maintenance of a low activity state of the enzymes in at least two ways: a) by suppressing the intrinsically high affinity of the allosteric cGMP-binding sites for cGMP and b) by fostering autoinhibition and decreasing access of substrate to the catalytic site. The suppression of sensitivity to cGMP activation by Ser-64 provides a mechanism whereby autophosphorylation of Ser-64 disrupts interaction with other elements within the protein and rapidly relieves the constraint on cGMP sensitivity. This vaults the enzyme into a full-blown expression of its intrinsic sensitivity to cGMP activation. Thus, this change in affinity is not a gain due to improved function but rather a gain in function due to relief of an autoinhibitory braking action on sensitivity to cGMP, the activating second messenger.

Autoinhibition of PKGIα is accomplished through interaction between the R and C domains within a single monomer, and evidence suggests that amino acids both in and near the pseudosubstrate sequence contribute importantly to this interaction (1; 7; 27). Therefore, the goal of the present study has been to identify amino acids within the C domain that interact with this region to change the intrinsic cGMP-binding affinity and enzyme activity. Since the x-ray crystal structure of the closely related PKA holoenzyme has been determined, amino acid contacts between the pseudosubstrate sequence of PKA RIα and C subunits have been used as a model for identifying potentially important contacts in the homologous PKG (2). Gln-401, His-404 and Cys-518 of PKGIα have been selected based on their conservation with and correspondence to PKA C subunit amino acids (Gln-84, His-87 and Cys-199, respectively) that make contacts with the pseudosubstrate region of RIα.

According to the crystal structure of PKA holoenzyme, the hydroxyl of the Ser-99 (P^{+2} position) side chain in the RIα subunit forms a hydrogen bond with the ε-amino group of the side chain of Gln-84 in the C subunit; Gln-84, in turn, forms a hydrogen bond with the nearby His-87 of the C subunit (2). An important role for His-87 in interaction between PKA R and C subunits is supported by the fact that point mutation of His-87 substantially weakens C subunit affinity for the R subunit, consistent with weakened autoinhibitory interactions (28-30). Moreover, His-87 appears to be involved in contacts made with P^{+2} amino acids of several peptide substrates (31). The PKA crystal structure shows that Cys-199 in the C subunit is also near Ser-99 in RIα (2). A role of Cys-199 in R-C subunit interaction is supported by the finding that it can be covalently modified by iodoacetic acid in the absence, but not in the presence, of PKA R subunit (32).

Both R and C domain interactions in PKGI can impact cGMP-binding affinity. In the presence of Mg/ATP, cGMP-binding affinity (K_D) is weakened (15; 33; 34). The P^{+2} Ser-64 and Ser-79 in the regulatory domains of PKGIα and PKGIβ, respectively, affect cGMP-binding affinity (1). In PKA, Gln-84, His-87 and Cys-199 make contacts with the PKA R subunit, including the P^{+2} amino acid. Gln-84, His-87 and Cys-199 of PKA C subunit are conserved as Gln-401, His-404 and Cys-518 in PKGIα. Therefore, it is hypothesized that these residues in PKGI contribute importantly to affinity for cGMP binding and autoinhibition of kinase activity.

3. MATERIALS AND METHODS

3.1. Site-directed mutagenesis of PKGI~

PKGIα Gln-401 was mutated to Ala (Q401A) and His-404 was mutated to Ala (H404A) using long-range amplification PCR site-directed mutagenesis. pBluescript KS^+ + PKGIβ was the template: the sequences of PKGIα and PKGIβ are identical except for the first ~100 amino acids. Oligonucleotide primers (Operon or Alpha DNA) with the following sequences, shown 5' to 3', and oligonucleotides with complementary sequences (not shown) were constructed to be identical to wild-type (WT) PKGIα cDNA sequence except for the underlined portions, which serve to mutate the amino acids: Q401A, GAGCGCAGGAGCACATCCGCTC; and H404A, CAAGACAGCAGGAGGCCATCCGCTCAGAGAAGC. After amplifying the cDNA and verifying incorporation of the mutation by sequencing, mutant PKGIs were digested with NcoI and SacI. The resulting 860-base pair fragment was subcloned into a pVL1392 + hcGKIα vector (35).

PKGIα Cys-518 was mutated to Ala (C518A) using two PCR steps. First, two mutant fragments were generated using template pVL1392 + PKGIα and a pair of oligonucleotide primers, each of which contained a flanking primer and a primer containing the mutation. Flanking and mutated primer pair (shown 5' to 3', with mutations designated by underlining) GCTGTAACCTGCCTTGTGATTG and GGGGCTACATACTCTGGAGTCCCAGCAAAAGTCCAT GTTTTC generated a 652-base pair fragment with the mutation near its 3' end. Flanking and mutated primer pair GCAACACTTGGTATTATAGGAGG and GAAAACATGGACTTTTGCTGGGACTCCAGAGTATGT AGCCCC produced a 383-base pair fragment with the mutation near its 5' end. Second, these two fragments were used as templates with the two flanking primers for another round of PCR to generate a ~1000-base pair fragment with the mutation near the center of the sequence. This fragment was ligated into a pCR2.1-TOPO vector (Invitrogen), transformed, and amplified. Following purification and verification of the mutation through sequencing, pCR2.1-TOPO-C518A was digested with SacI and AflII, and the resulting 545-base pair fragment was subcloned into a pVL1392 + PKGIα vector.

Double mutant S64A/H404A was constructed by ligating the relevant mutant fragments from the PKGIα single mutant plasmids pVL1392 + S64A and pVL1392 + H404A (1).

All vectors were propagated in *Escherichia coli*, and plasmids were sequenced by the dideoxy chain termination method (36).

3.2. Expression of PKGI~ protein

All tissue culture procedures were performed in Sf9 insect cells (*Spodoptera frugiperda;* Pharmingen) maintained at 27 ^oC in Grace's cell medium supplemented with 10% fetal bovine serum. pVL1392 + hcGKIα transfer vectors (2-4 μg) containing the mutant sequences and BaculoGold DNA (0.5 μg; Pharmingen) were co-transfected into Sf9 cells. Recombinant baculoviruses were harvested, and viral titer was amplified as described previously (1). Recombinant baculoviruses containing the WT PKGIα sequence and the S64A PKGIα sequence had been made previously (1).

1-2 x 10^7 cells were infected with recombinant baculovirus in T-175 tissue culture flasks. At 72 h post-infection, the cells were scraped from the plates and harvested by centrifugation (4000 x g for 10 min at 4 ^oC). Cell pellets containing PKGIα were resuspended in 20 mL cold KPEM (10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 25 mM 2-mercaptoethanol) plus a protease inhibitor mixture (Complete; Roche Molecular Biochemicals) used at the recommended concentration, and homogenized on ice by 2 4-sec bursts in an Ultra-Turrax microhomogenizer. Homogenates were centrifuged at 4 ^oC for 20 min at 12,000 x g.

3.3. Purification of recombinant PKGI~

Crude extracts from homogenizations were loaded onto columns of 0.6 mL cAMP-Agarose (Sigma; 11-atom spacer) or 1 mL 8-aminoethylamino-cAMP-Agarose (Biolog), each equilibrated in KPEM + protease inhibitor. After extracts were loaded, columns were washed sequentially with 5-10 mL KPEM + protease inhibitor, ~10 mL KPEM + protease inhibitor + 1 M NaCl, and 10 mL KPEM + protease inhibitor. Recombinant protein was eluted from the column using ~15 mL KPEM + protease inhibitor + 1 mM cAMP over a 24-h time period. Eluted fractions were concentrated in Centriplus YM-30 centrifugal filter devices (Millipore; Amicon Bioseparations) by centrifugation three times at 4 ^oC for 60 min at 1100 x g. Before each centrifugation, 4 mL of KPEM was added to the sample to lower the final cAMP concentration ~10-fold as determined by spectrophotometry (extinction coefficient for cAMP = 14650 M^{-1} cm^{-1}; wavelength = 259 nm). PKGIα purity in these samples was analyzed by 10% SDS-PAGE and Coomassie Blue staining. Protein concentration was determined with the Bradford method (37).

3.4. Determination of PKGI~ catalytic activity

WT and mutant PKGIα catalytic activities were determined by the method of Wolfe *et al* using a synthetic heptapeptide (RKRSRAE) as substrate (38; 39). 5 μL PKGIα (0.2-1 nM dimeric enzyme) was added to 40 μL kinase reaction mixture [20 mM Tris, pH 7.4, 20 mM magnesium acetate, 200 μM ATP, 100 μM IBMX, 170 μM RKRSRAE, 0.9 μM PKA peptide inhibitor (5-24), and ~15,000 cpm/μL [^{32}P]ATP)] in the absence and presence of 10 μM cGMP. Assays were conducted at 30 ^oC for 10-20 min, and aliquots were spotted onto Whatman P-81 cation exchange paper to determine the amount of $^{32}P_i$ transferred to peptide substrate. The P-81 papers were washed four times in 75 mM phosphoric acid, washed once in ethanol, dried, and counted by the Cerenkov method.

3.5. Determination of K_a (cGMP and cAMP) values

Protein kinase activation constants (K_a) for cGMP and cAMP for WT and mutant PKGIα enzymes were determined by incubating 10 μL dimeric PKGIα (0.2-5 nM final concentration) with 40 μL kinase reaction

mixture in the absence and presence of increasing concentrations of cyclic nucleotide. These reactions were incubated at 30 ºC for 11-30 min and stopped using Whatman P-81 cation exchange paper and 75 mM phosphoric acid as described above.

3.6. Determination of K_D for cGMP

WT and mutant PKGIα were diluted to 62.5 nM dimeric PKGIα in KPEM containing 1 mg/ml BSA and then further diluted to a final concentration of 0.15 nM dimeric PKGIα (0.6 nM cGMP-binding sites) in cGMP-binding assay mixture (2 M NaCl, 50 mM potassium phosphate, 1 mM EDTA, 0.5 mg/ml histone IIAS and 200 μM IBMX). Diluted PKGIα was incubated with increasing concentrations of [^{3}H]cGMP at 30 ºC for 1 h. Reactions were stopped by adding 2 ml ice-cold saturated ammonium sulfate and were filtered on PVDF paper pre-wet with 1 ml of ice-cold ammonium sulfate. The filter papers were washed three times each with 2 ml ice-cold ammonium sulfate and dried. Papers were then suspended in 1.5 ml 2% SDS, shaken, and 10 ml aqueous scintillant was added prior to counting.

3.7. Determination of K_m and V_{max} for heptapeptide substrates and K_m for ATP

0.2 nM dimeric purified native bovine lung PKGIα, recombinant WT PKGIα, or mutant PKGIα was incubated with 10 μM cGMP, kinase reaction mixture minus peptide substrate, and increasing concentrations of one of two heptapeptide substrates (RKISASE or RKRSRAE). After 10 min at 30 ^{o}C, incubation reactions were stopped and processed by the Whatman P-81 cation exchange paper and phosphoric acid wash technique described above. P-81 papers were counted by the Cerenkov method.

To determine K_m for ATP of PKGIα proteins, ~2 nM dimeric PKGIα was incubated with 10 μM cGMP and kinase reaction mixture including 83 nM [^{32}P]ATP, in the presence of increasing concentrations of unlabeled ATP. Reactions proceeded at 30 ^{o}C for 13 min and were stopped by P-81 paper application and phosphoric acid washing as described above. Enzyme activity in the presence of specific ATP concentrations was calculated from the amount of $^{32}P_i$ transferred to the heptapeptide substrate RKRSRAE. Final ATP concentrations were calculated by adding [^{32}P]ATP and unlabeled ATP concentrations.

3.8. Data analysis

Data are reported as mean ± standard error of the mean (S.E.M.). The number of independent experiments (n) performed are indicated, and each independent experiment was done in duplicate. Statistical analyses to determine whether values of the PKGIα mutants are significantly different from those of WT PKGIα are performed using one-way ANOVA tests (Prism software). $P < 0.05$ is considered statistically significant.

3.9. Materials

cAMP and cGMP were purchased from Sigma. Cyclic AMP-affinity resin was purchased from Sigma and BioLog. Protease inhibitor tablets were purchased from Roche Molecular Biochemicals. Heptapeptides RKRSRAE and RKISASE were obtained from Bachem and Multiple Peptides Systems, respectively. [^{32}P]ATP was purchased from NEN (Perkin-Elmer), PKA inhibitor peptide (5-24) was purchased from Peninsula Labs, and [^{3}H]cGMP was purchased from Amersham Biosciences. P-81 cation exchange chromatography paper was purchased from Whatman, and nitrocellulose 0.45 μm filter paper was purchased from Millipore. Pre-cast SDS polyacrylamide gels were obtained from BioRad Laboratories.

4. RESULTS

4.1. Cyclic nucleotide-binding affinities of PKGI~ catalytic domain mutants are increased

Whether Gln-401, His-404, or Cys-518 could directly contact Ser-64 or play a similar role to that of Ser-64 in regulating binding affinities for cGMP or cAMP has been assessed by generating single point mutations of each of these residues, as well as a double mutant involving Ser-64 and His-404 (S64A/H404A), and determining their respective kinetic properties. The respective mutant enzymes have been expressed as described in Materials and Methods and purified to >90% purity as shown on 10% SDS-PAGE gels (Figures 1A and 1B). Each of these recombinant proteins migrates as ~78 kDa proteins, which is indistinguishable from the migration of native PKGIα purified from bovine lung.

Affinity for cyclic nucleotide binding has been determined in two ways. First, the cyclic nucleotide-binding affinities for PKGIα mutants Q401A, H404A, C518A and the double mutant S64A/H404A have been determined indirectly by measuring the concentration of cyclic nucleotide required to half-activate kinase phosphotransferase activity (K_a). As shown in Figure 2A and Table 1, the potency of cGMP for activation of phosphotransferase activity is increased in the mutant proteins; the affinities of Q401A, H404A and C518A for cGMP are increased 7-, 33- and 6-fold, respectively, which are reflected in lower K_a values. Among these three residues, the 33-fold increase in affinity of the H404A mutation for cGMP is similar to the 29-fold improvement in affinity determined for the S64A mutant (Table 1). The similarity of the effects of these two individual mutations and their proposed proximity in the model prompted us to determine if the effects on cGMP binding in a double mutant (S64A/H404A) are additive. As shown in Table 1, the affinities of the respective mutant PKGs (S64A, H404A and S64A/H404A) are the same (3.6±0.7 nM, 3±1 nM, and 3.0±0.2 nM, respectively), suggesting that the effects of Ser-64 and His-404 could be interrelated.

In addition to cGMP, cAMP can also activate PKG phosphotransferase activity, and autophosphorylation of PKGIα (most likely including Ser-64) is reported to increase affinity for cAMP by 10-fold with little effect on affinity for cGMP (15). This improved cAMP affinity has implication for conferring an increased physiological role for this nucleotide in PKGIα-mediated effects. The potencies with which cAMP activates phosphotransferase

Table 1. Kinase activation (K_a cGMP and K_a cAMP) and binding affinity (K_D cGMP) values for purified recombinant WT and mutant PKGIα

PKGI~	K_a cGMP (nM)	K_a cAMP (nM)	K_D cGMP (nM)
WT	103 ± 9	7676 ± 1257	88 ± 19
Q401A	15 ± 2**	1529 ± 467**	17 ± 12**
C518A	17 ± 4**	4525 ± 507**	33 ± 9*
H404A	3 ± 1**	227 ± 66**	30 ± 8*
S64A	3.6 ± 0.7**	ND[1]	8 ± 1[2]
S64A/H404A	3.0 ± 0.2**	ND	ND

[1]ND, not determined, [2]Reported in Busch *et al* (1). Cyclic nucleotide binding to WT or catalytic domain mutant PKGIα is determined by one of two methods. The K_a cGMP and K_a cAMP (data also shown graphically in Figures 2A and 2B) are determined as described in the Figure 2 legend. The K_D values are determined by incubating 0.15 nM dimeric enzyme (0.6 nM binding sites) in the presence of increasing concentrations of [^{3}H]cGMP at 30 ^{o}C for 1 h as described under Materials and Methods. n>3; *p<0.05 compared to the WT value, **p<0.001 compared to WT value.

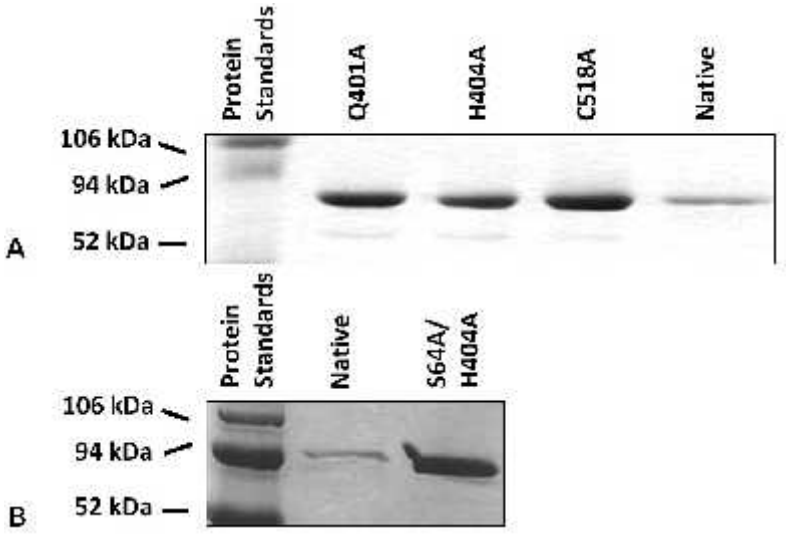

Figure 1. SDS-PAGE analysis of purified native WT and mutant PKGIα proteins. 1A. PKGIα (native, 1 μg) is purified from bovine lung according to the procedure outlined in Francis *et al*, and recombinant single mutant PKGIα (1.4-2 μg) is purified from Sf9 cells (48). The protein is boiled for 4 minutes in the presence of 10% SDS, 2 mM 2-mercaptoethanol, and 1 mg/mL bromophenol blue dye and electrophoresed on a 10% SDS-PAGE gel (BioRad). The gel is stained with Coomassie Brilliant Blue dye. 1B. S64A/H404A purified from Sf9 cells is boiled for 4 minutes in the presence of 10% SDS, 2 mM 2-mercaptoethanol, and 1 mg/mL bromophenol blue dye and electrophoresed on a 10% SDS-PAGE gel. 0.75 μg PKGIα purified from bovine lung is electrophoresed as a control (48). The gel is stained with Coomassie Brilliant Blue dye.

activities of the Q401A, H404A, or C518A mutant PKG proteins are also increased; the fold increases in affinity for cAMP for the respective proteins are 5-, 34- and 1.5-fold, respectively (Figure 2B, Table 1). Interestingly, the magnitude of the increase in affinity of the H404A mutant for cAMP (34-fold) is like that for cGMP. However, unlike the effects of autophosphorylation of PKGIα, the ratio of affinity for cAMP versus cGMP among the WT, Q401A, H404A and C518A PKGs does not significantly change (75- to 270-fold preference for cGMP).

Affinity (K_D) for cGMP has been directly determined using the [^{3}H]cGMP-binding assay in the absence of substrates as described in Materials and Methods. As found by K_a measurement, the K_D values also reveal improved cGMP affinity for each mutant (Table 1). Cyclic GMP affinities obtained using the two methods are similar except for that of H404A. Cyclic GMP affinity of H404A determined by the [^{3}H]cGMP binding assay is 10-fold weaker than that determined by activation of phosphotransferase activity (Table 1) and may relate to the different conditions employed in the assays. Cyclic GMP dissociation studies show that the high affinity cGMP-binding site is not altered in the mutant PKGs (data not shown).

4.2. Effect of PKGI~ catalytic domain mutations on autoinhibition

A cyclic nucleotide-dependent protein kinase activity ratio is determined by dividing phosphotransferase activity in the absence of cyclic nucleotide by that in the presence of a fully-activating concentration of cyclic nucleotide. This is an accepted technique for testing the extent of autoinhibition of cyclic nucleotide-dependent protein kinases. Higher ratios signify less autoinhibition. Mutation of Ser-64 to either Ala or Thr in the R domain of

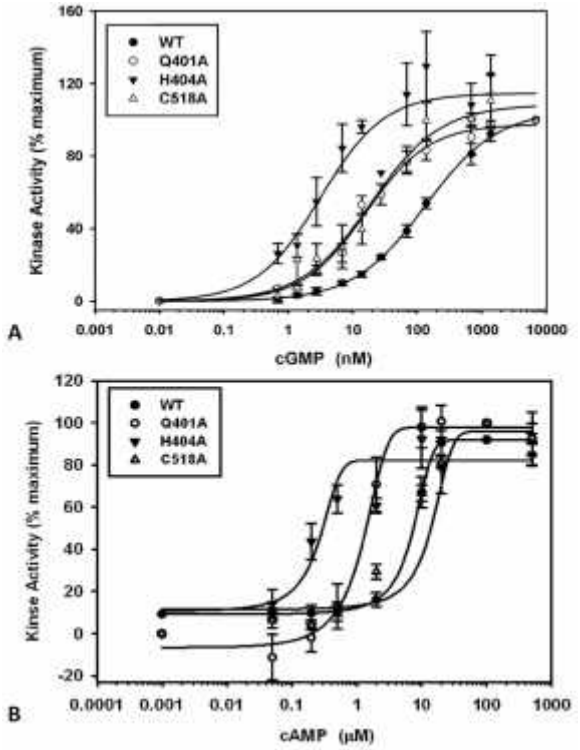

Figure 2. PKGIα catalytic domain mutations improve cGMP and cAMP affinity. 2A. Protein kinase activities for WT and mutant PKGIα are determined in the absence and presence of increasing concentrations of cGMP using 0.5 nM dimeric PKGIα and the RKRSRAE heptapeptide as substrate for 11 min at 30 ^{o}C as described in Materials and Methods. 100% maximal kinase activity is defined as the activity at 6800 nM cGMP (WT, n=7; H404A, n=4; Q401A and C518A, n=3; each done in duplicate). 2B. Protein kinase activities are determined in the absence and presence of increasing concentrations of cAMP using 5 nM dimeric PKGIα and the RKRSRAE heptapeptide as substrate for 11-30 min at 30 ^{o}C as described in Materials and Methods. 100% maximal kinase activity is defined as the activity at ~100 mM cAMP.

PKGIα not only increases affinity of the enzyme for cGMP but also increases the kinase activity ratio of the enzyme by 2- to 3-fold. Moreover, mutation of this residue to larger residues (Asp or Asn) also increases affinity for cGMP and generates a nearly constitutively active kinase (activity ratios of 0.83 ± 0.03 and 0.78 ± 0.04, respectively) (1). These results indicate that Ser-64 plays an important role in autoinhibition and that introduction of bulk either through a phosphate group or a larger amino acid results in significant disruption of autoinhibition (1). Given that cGMP-binding affinity is also substantially increased by mutation of Gln-401, His-404, or Cys-518 in the C domain, we hypothesize that these amino acids may also contribute to interactions by which Ser-64 holds the enzyme in an inactive conformation.

To examine the role of Gln-401, His-404 and C518A in the phosphotransferase functions of PKGIα, the kinetic characteristics of the PKGIα mutants (Q401A, H404A, C518A and S64A/H404A) have been studied and compared with WT and/or native PKGIα. Using WT PKGIα as a control, kinase activities of the mutant proteins have been determined in the absence and presence of 10 μM cGMP in order to calculate the activity ratios, which reflect the degree of autoinhibition. Activity ratios of the Q401A and C518A mutant proteins are not significantly different ($p > 0.05$) from that of WT PKGIα (Figure 3A). However, the activity ratio of H404A (0.45 ± 0.06) is increased ~2-fold over that of WT PKGIα (0.24 ± 0.03; $p < 0.05$) (Figure 3A), indicating that His-404 is partially responsible for holding PKGIα in a more inactive conformation, i.e., it is a significant contributor to autoinhibition. Effects of the individual mutations of S64A and H404A on the activity ratios are not statistically different from those in the double mutation (S64A/H404A) (Figure 3B). This indicates that the effects of these two residues on PKGIα autoinhibition are not additive. The PKG proteins have been purified using cAMP-affinity columns, but residual cAMP in the protein preparations has no effect on cyclic nucleotide-activation as indicated by the absence of a change in the activity ratio following extensive sequential dilution up to 100,000-fold (data not shown).

4.3. Effects of PKGI~ catalytic domain mutations on phosphotransferase activity

When activated by cyclic nucleotides, PKGIα catalyzes transfer of the gamma-phosphate of ATP to a protein or peptide substrate. Since Gln-401 and His-404 are located in the predicted Mg/ATP-binding region of PKGIα and Cys-518 is located in its substrate-binding region, effects of an alanine replacement of these residues on specific features of the catalytic process have been more thoroughly studied.

The PKGIα catalytic rate as measured experimentally depends on several parameters, including maximum velocity (V_{max}), affinity for peptide substrates (K_m peptide), and affinity for ATP (K_m ATP). In order to avoid potential unknown caveats among substrates, the maximum catalytic activities (V_{max}) of the PKGIα C domain mutants [in the presence of saturating cGMP (10 μM)] have been determined using two heptapeptide substrates, RKISASE and RKRSRAE. The RKISASE peptide mimics the phosphorylation sequence for PKGI in PDE5, and the RKRSRAE peptide is slightly modified from a sequence based on the phosphorylation sequence for PKGI in histone H2B (39; 40). As shown in Table 2, V_{max} values for phosphorylation of RKISASE and RKRSRAE by recombinant WT PKGIα are comparable (3.38 ± 0.59 and 3.46 ± 0.44 μmol/min/mg, respectively), and a similar rate (3.08 ± 0.88 μmol/min/mg) has been obtained for native PKGIα phosphorylation of RKISASE. These values agree with the previously published V_{max} value of 5.1 ± 0.4 μmol/min/mg (41). The V_{max} values of Q401A, H404A and C518A for both peptides are decreased 3.5- to 7-fold compared with that of WT PKGIα (Table 2).

Compared with the WT PKGIα, mutations of Gln-401, His-404, or Cys-518 do not produce significant

Table 2. Specific enzyme activities (V_{max}) for purified WT and mutant PKGIα in the presence of two different substrates

PKGI~	RKISASE; V_{max} («mol/min/mg)	RKISASE; Fold-effect	RKRSRAE; V_{max} («mol/min/mg)	RKRSRAE; Fold-effect
Native	3.08 ± 0.88	1.1		
WT	3.38 ± 0.59	1.0	3.46 ± 0.44	1.0
Q401A	0.77 ± 0.13**	4.4	0.69 ± 0.08**	5.0
H404A	0.51 ± 0.07**	6.6	0.98 ± 0.12**	3.5
C518A	0.71 ± 0.09**	4.8	0.51 ± 0.06**	6.8

Specific enzyme activities are determined by dividing the catalytic activity [μmol $^{32}P_i$ transferred from [^{32}P]ATP to the peptide substrate per minute of incubation time per volume of enzyme (μmol/min/mL]) by the protein concentration (mg/mL) as determined by the Bradford method (37). Catalytic activity is determined as specified in Materials and Methods: 2 nM dimeric PKGIα, 10 μM cGMP, and either 170 μM RKRSRAE or 1 mM RKISASE is used as substrate. Reactions are incubated at 30 ^{o}C for 10-11 minutes and stopped as described. n=4; **p<0.001 compared to WT values.

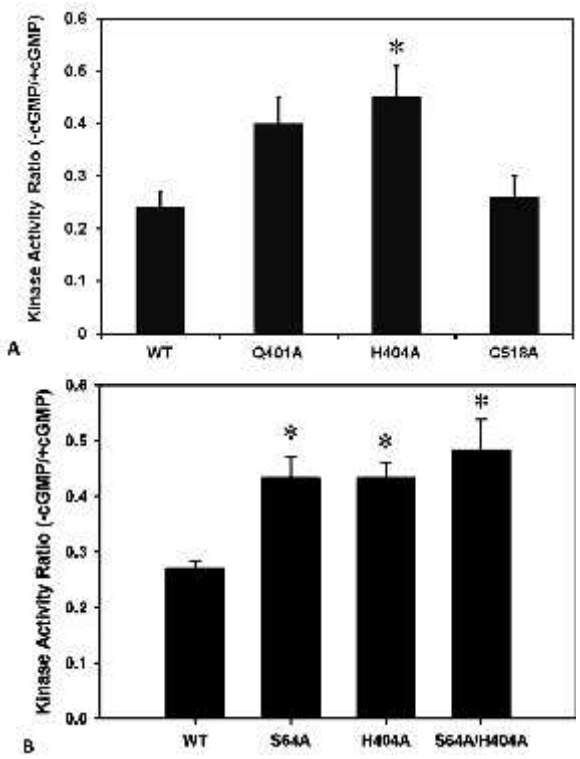

Figure 3. Protein kinase activity ratios of WT and mutant PKGIα enzymes. 3A. Using 25 ng purified PKGIα (~0.2 nM), kinase assays are performed in the absence and presence of 10 μM cGMP as described in Materials and Methods. The protein kinase activity ratio is defined as the PKG catalytic activity in the absence of cGMP divided by the PKG kinase activity in the presence of a saturating concentration of cGMP (n = 7, each in duplicate; *p<0.05 compared with WT value). 3B. Kinase assays are performed in the absence and presence of 10 μM cGMP as described Materials and Methods. (WT, n = 7; S64A, n=4; H404A, n=3; S64A/H404A, n=3; each in duplicate; *p<0.05 compared with WT value).

changes in K_m for the respective heptapeptide substrates (Table 3). The WT PKGIα K_m values (K_m RKISASE = 233 ± 101 μM and K_m RKRSRAE = 140 ± 29 μM) that have been determined in this study are somewhat higher than those previously published (K_m RKISASE = 80 μM (41); K_m RKRSRAE = 28.8 μM (39), 38 μM (41)). These differences are most likely explained by variations in assay conditions rather than by enzyme modification, since in the present study the K_m for RKISASE of the purified recombinant WT PKGIα (233 ± 101 μM) is similar to the K_m for RKISASE of PKGIα purified from bovine lung (249 ± 72 μM). The K_m values of the three PKGIα mutants (Q401A, H404A, and C518A) for ATP (22.7 ± 2.9 μM, 27.6 ± 3.2 μM and 29.7 ± 3.6 μM, respectively) are similar to that of WT (25.8 ± 1.7 μM) when RKRSRAE is used as substrate (Table 3). This calculated K_m value of ATP for WT PKGIα is similar to a previously published value of 37.2 ± 7.3 μM (42).

5. DISCUSSION

In an attempt to identify amino acids in the C domain of PKGIα that contribute to regulation of cyclic nucleotide-binding affinity and which are important for regulating PKGIα autoinhibition mediated through Ser-64, three C domain single mutants (Q401A, H404A, C518A) and one double mutant (S64A/H404A) have been studied and compared with the S64A mutant. Like mutation of Ser-64 (S64A), each of the C domain single mutations as well as the double mutation significantly increases affinity for cGMP and cAMP, although these alterations do not modify the cyclic nucleotide specificity of the enzyme. Results presented herein suggest that His-404 could interact with Ser-64 to mediate these effects. Indeed, the cGMP-binding affinities are similarly increased when either His-404 or Ser-64 is mutated to alanine, and the effects of a double mutation have no greater effect. Both the S64A and the H404A mutant proteins have a ~30-fold higher affinity (K_a) for cGMP (3.6 ± 0.7 nM and 3 ± 1 nM, respectively) than does the WT PKGIα (103 ± 9 nM). When both of these sites are simultaneously mutated (S64A/H404A), the cGMP-binding affinity (K_a = 3.0 ± 0.2 nM) is similar to that for the single mutants. Since the relative cAMP/cGMP affinities do not increase in the mutant proteins, the effects are different from the effect of autophosphorylation, which increases cAMP affinity ~10-fold but has little effect on PKG affinity for cGMP (15).

Analysis of the changes in the energy of binding suggests that the potential contact between Ser-64 and His-404 could be a direct hydrogen bond. Hydrogen bonds display Gibbs free energies of 2.5 – 4 kcal/mol (43). The difference between the Gibbs free energy [Δ G = -RT (ln K_a)] of WT PKGIα and that of mutant PKGIα is termed

Table 3. Binding affinities (K_m) of substrates RKISASE and RKRSRAE and of ATP for purified WT and mutant PKGIα

PKGI~	RKISASE; K_m («M)	RKISASE; Fold-effect	RKRSRAE; K_m («M)	RKRSRAE; Fold-effect	ATP; K_m («M)	ATP; Fold-effect
Native	249 ± 72	1.1				
WT	233 ± 101	1.0	140 ± 29	1.0	25.8 ± 1.7	1.0
Q401A	487 ± 222	2.1	125 ± 49	0.9	22.7 ± 2.9	0.9
H404A	475 ± 233	2.0	199 ± 67	1.4	27.6 ± 3.2	1.1
C518A	370 ± 131	1.6	215 ± 91	1.5	29.7 ± 3.6	1.2

K_m for substrates is determined by incubating 0.2 nM dimeric PKGIα for 10 minutes at 30 °C in the presence of increasing heptapeptide concentrations as explained in Materials and Methods. K_m for ATP is determined by incubating 2 nM dimeric PKGIα (final concentration) in the presence of increasing concentrations of [^{32}P]ATP for 13 minutes at 30 °C as described in Materials and Methods. n=3; no statistical differences between WT and any mutants.

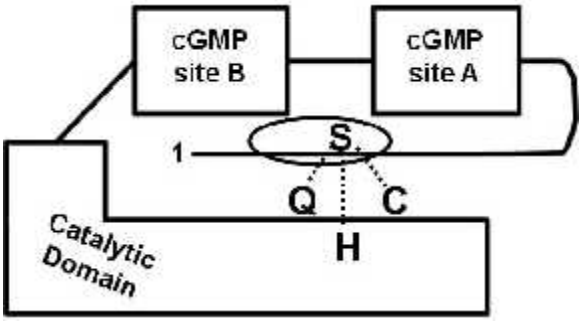

Figure 4. Proposed model of the interactions between Ser-64, Gln-401, His-404, and Cys-518. A PKGIα monomer containing a regulatory domain and a catalytic domain is depicted. The regulatory domain begins with the first amino acid (designated by '1') and continues through the autoinhibitory subdomain (oval) and the two cGMP-binding sites (designated cGMP site A and cGMP site B). This model suggests that Ser-64 makes direct contacts (shown in the dotted lines) with catalytic domain amino acids Gln-401 (Q), His-404 (H), and Cys-518 (C). Alternatively, Ser-64 may interact indirectly with any of these residues via another amino acid (not shown). Mutation of any of these residues would affect the interaction between the catalytic domain and the autoinhibitory site of the regulatory domain. This model, influenced partially by Osborne *et al* (47), also suggests that Ser-64 lies between the catalytic domain and the two cGMP-binding sites. Localized conformational changes to Ser-64 could affect the cGMP-binding sites and increase affinity for cyclic nucleotides.

&Φιγυρε Δ(ΔG). Calculated Δ(ΔG) values are negative for all mutants (S64A, H404A, S64A/H404A, Q401A, and C518A), but the highest negative values are obtained with the S64A, H404A, and S64A/H404A mutants (-2.01 kcal/mol, -2.12 kcal/mol, and -2.12 kcal/mol, respectively). When correcting for changes in hydrophobicity, these values may increase 2-fold (44), equal to a free energy change consistent with losing one hydrogen bond. Since negative Δ(ΔG) values signify that protein stability and/or binding energy between a ligand and mutant is weakened by mutation, Ser-64 and His-404 may form part of a unique and important protein substructure that, when disrupted, alters the C domain and the entire protein to cause an increase in cyclic nucleotide-binding affinity and a decrease in the potency of autoinhibition. Gln-401 and Cys-518 also act to restrict cyclic nucleotide-binding affinity, although to a lesser extent. It is unknown whether these residues mediate their effect by directly interacting with residues within the R domain or by serving a broader structural role.

The His-404 mutant (H404A) has a significantly increased PKGIα basal activity (0.45 ± 0.06), i.e. in the absence of cGMP, as compared to WT PKGIα (0.24 ± 0.03), suggesting that His-404 is involved in holding the enzyme in a more inactive conformation. Ser-64 in the R domain has already been shown to contribute to maintaining the inactive state (1). Indeed, the S64A phosphotransferase activity (activity ratio = 0.43 ± 0.04) in the absence of cGMP is similar to that of H404A. To gain insight into whether the conserved Ser-64 within the R domain and the His-404 in the C domain act independently or are inter-dependent, a S64A/H404A double mutant has been created. The activity ratio of the S64A/H404A mutant (0.48 ± 0.06) is not statistically different from that of the proteins containing a single mutation at either Ser-64 or His-404. Since the activity ratio of the S64A/H404A mutant is not increased compared to the activity ratio of either of the single mutants, Ser-64 and His-404 may directly impact and/or contact each other. This result corroborates the conclusion of the kinase activity study, which implies that Ser-64 and His-404 form important contacts involved in PKGIα functions. However, other amino acids are most likely involved, since cGMP-dependence is not totally obliterated by these mutations.

Several conclusions can be drawn from these results. First, like Ser-64 in the autoinhibitory domain, Gln-401, His-404 and Cys-518 in the catalytic domain restrict the intrinsically strong cGMP-binding affinity provided by the allosteric cGMP-binding sites in PKGIα; regulation of this affinity is critical for regulation of PKGI catalytic activity and myriad physiological processes by cellular levels of cGMP. Cyclic GMP-binding affinity is increased when any one of these amino acids is mutated. This result is intriguing when compared to those in a previous study which found that the entire C domain promotes stronger cGMP-binding affinity; a PKGIα mutant lacking amino acids 352-670 has weaker cGMP-binding affinity at each of the two cGMP-binding sites (45). Second, of the three C domain amino acids analyzed herein, His-404 is the strongest determinant (by 3- to 5-fold) of cyclic nucleotide-binding affinity. The finding that the K_D of H404A for [^{3}H]cGMP binding is 10-fold higher than its K_a for activation of the kinase suggests that His-404 preferentially affects kinase activation over cGMP

binding. To our knowledge, these findings are the first implication of single amino acids in the C domain of a cyclic nucleotide-dependent kinase having a role in cyclic nucleotide-binding affinity of the R domain.

The targeted amino acids in this study are in the C domain of PKGIα; therefore, possible repercussions of the mutations on catalytic activities have been tested. Typically, PKGIα catalysis is determined by phosphorylation of peptide substrates, which depends on several kinetic parameters including peptide substrate placement in the active site, peptide-binding affinity, and affinity for the Mg/ATP complex. Two commonly used heptapeptides for studies of PKGI catalytic function are RKISASE and RKRSRAE. Sequences of these differ at the P^{-1}, P^{+1} and P^{+2} positions; RKISASE has small neutral amino acids (Ile and Ala) at P^{-1} and P^{+1}, whereas RKRSRAE has an arginine in each of these positions. At the P^{+2} position, RKISASE contains a Ser, and RKRSRAE contains an Ala. It has been determined that the V_{max} values of Q401A, H404A and C518A with RKISASE as substrate are reduced 4- to 6-fold and with RKRSRAE as substrate are reduced 3- to 7-fold. Affinities for heptapeptide substrate or ATP (K_m RKISASE, K_m RKRSRAE, or K_m ATP) for Q401A, H404A and C518A are not significantly different from that of WT PKGIα. These results contrast with work done by Gibbs *et al* using yeast PKA C-subunit in which dual mutation of His-131 (the yeast C-subunit homologue of PKGIα His-404) and Glu-130 by alanine substitutions causes a decrease in affinity for both heptapeptide substrate (Kemptide, LRRASLG) and MgATP (46). Although this difference may be explained by the simultaneous mutation of these two residues, which may have had more effect on affinity than did His-131, it does point out an interesting distinction between PKG and PKA. The current results provide unique insight into an unidentified mechanism of PKGIα by which Glu-401, His-404 and Cys-518 affect V_{max}. By interactions involving either the Mg/ATP binding region or substrate binding region, these amino acids may participate in optimally positioning amino acids directly involved in phosphate transfer and in fostering potent autoinhibition.

The results in this study demonstrate that three C domain residues (Gln-401, His-404 and Cys-518) significantly impact interactions of the C domain of PKGIα with the autoinhibitory subdomain and/or cyclic nucleotide-binding sites of the R domain. These interactions are explained by a model (Figure 4), part of which is influenced by a recently-published crystal structure of the cGMP-binding sites within the PKGIα regulatory domain (47). First, Gln-401, His-404 and Cys-518 are situated close to each other in the catalytic domain and interact directly (or indirectly) with Ser-64 of the regulatory domain. Cellular mutation of any of these four PKGIα residues interferes with autoinhibition and causes the enzyme to assume a more active conformation. Second, Ser-64 is spatially located near the cGMP-binding sites. This positioning reflects crystal structure information showing that an amino acid just carboxy-terminal to the autoinhibitory subdomain is located between the two cGMP-binding sites (47). When key interactions are lost through mutations of any of these four residues to alanine, resultant localized conformational changes alter the structure of the nearby cGMP-binding sites and increase the binding affinity for cyclic nucleotides. Ser-64, Gln-401, His-404 and Cys-518, as well as residues outside the substrate-binding region of PKGIα, can selectively affect cGMP binding, autoinhibition, and phosphotransferase activity. This would influence stimulation of PKG downstream events such as lowered blood pressure, decreased blood clotting, enhanced penile erection, and opening of airways.

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Send correspondence to: Jennifer L. Busch, Biology Department, Wheaton College, 501 College Avenue, Wheaton, IL 60187, Tel: 630-752-5645, Fax: 630-752-7278, E-mail: jennifer.busch@wheaton.edu