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MECHANISMS OF AUTOINHIBITION IN CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

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1. ABSTRACT

Cyclic AMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) are autoinhibited through multiple interactions between their respective regulatory and catalytic domains. A large portion of this autoinhibition occurs through interactions between residues within the catalytic domain and those within either a substrate-like sequence (-RRXSX-) or pseudosubstrate sequence (-RRXAX-) in the regulatory domains. These contacts effectively inhibit catalysis by blocking substrate binding. Particularly important contacts involve the P^{-2}, P^{-3}, and P^{+1} residues where either serine, which is potentially autophosphorylated, or alanine occupies the P^{0} position. The primary sequence is apparently less important for autoinhibition in PKGs than in PKAs, and a conserved serine at P^{+2} in PKGs is important for autoinhibitory contacts. Elements outside the substrate-related sequences also contribute to autoinhibition in both PKA and PKG. For example, synthetic peptides with relatively short pseudosubstrate sequences are weak inhibitors; while heat-denatured RII subunit does not inhibit catalytic subunit, it is still rapidly autophosphorylated; and truncated PKGs lacking the substrate-like sequence are still partially autoinhibited. Thus, capacity for autoinhibition of PKA or PKG is provided by contacts involving direct interactions with the catalytic site and by contacts that stabilize an inactive conformation.

2. INTRODUCTION

Autoinhibition (self-inhibition) is a key biochemical regulatory mechanism that contributes importantly to controlling enzyme catalytic activities and other protein functions. Relief of autoinhibition results in activation of the respective protein and is critical in regulation of all physiological pathways. In the broadest sense, self-regulation through autoinhibition can occur in any protein that exists in active and inactive conformers. Reversible conversion between inactive and active conformers requires existence of a molecular switch that is controlled by alterations in the energy of contacts inherent in protein-protein interactions within that molecule. Quantitative alterations in the energy of these contacts can be initiated by binding allosteric regulators, association/dissociation with other proteins, or covalent modifications. In all instances, conversion between inactive and active conformations alters contacts between structural elements within a single protein chain, between subunits of the same protein, or between separate proteins. In some instances, autoinhibition is provided by a very limited segment of protein sequence. In other instances, it is conferred by diverse contacts distributed across the protein surface, thereby maintaining the protein in a less active conformation. In many proteins, both schemes are employed; each conforms to the general description of intrasteric autoinhibition (1-14).

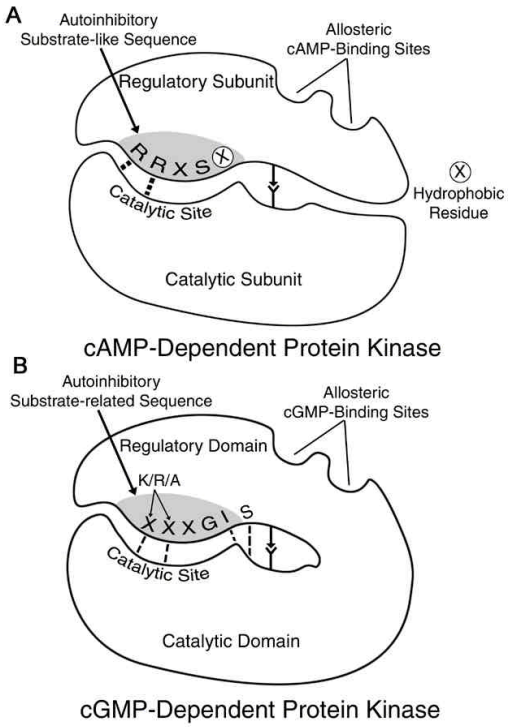

Figure 1. Schematic depiction of contacts that contribute to autoinhibition in PKAs and PKGs. PKA is depicted as a single R subunit and a single C subunit although the native enzyme is a heterotetramer of dimerized R subunits and two C subunits. PKG is depicted as a monomer although all known PKGs exist as homodimers. Autoinhibition in PKA can occur between a single R subunit and a single C subunit. In PKGs autoinhibition occurs through interactions within a single monomer. The gray stippled area depicts the substrate-related sequence in each enzyme. The interaction depicted by the arrow and Y interface reflects features within each of the proteins other than the substrate-related sequence that are required for effective autoinhibition. 1A. For PKA autoinhibition, a substrate-like sequence (-RRX**S**X-) containing either serine (RII subunit) or glycine/alanine (RI subunit) at P^0 interacts with the catalytic site of C subunit to competitively block protein/peptide substrate binding. The arginines at P^{-2} and P^{-3} are particularly important for PKA autoinhibition. The heavy lines extending from the arginines (R) in R subunit to the catalytic subunit depict the important contribution that this duo of basic residues makes to PKA autoinhibition. 1B. For PKG autoinhibition, the homologous substrate-related sequence is shown as –XXXGIS- because the P^{-2} and P^{-3} positions are occupied by either arginine, lysine or alanine in known PKGs (Table 1). Only three of the six known PKGs contain two basic residues at these positions. Other contacts important in PKG autoinhibition include P^{+1} isoleucine/valine and P^{+2} serine. This serine is juxtaposed, but technically outside, a classical definition of a PKG substrate-like sequence. It is depicted as forming an autoinhibitory contact with PKG catalytic domain, but whether this contact involves the catalytic domain or sites in the regulatory domain is not known.

Reversible autoinhibition/activation in protein kinases is a pivotally important regulatory mechanism that has been evolutionarily conserved despite marked divergence in protein structures, substrate specificities, and regulatory features of these enzymes (11; 15-22). Cyclic AMP-dependent protein kinase (PKA) was the first protein kinase shown to be autoinhibited, and it was the first protein kinase shown to be inhibited by a substrate-like inhibitory domain (4; 23-25). Reversible processes such as ligand binding, phosphorylation, oxidation, and nitrosylation can introduce sufficient energy to shift the equilibrium of a protein between the autoinhibited and activated state. In some instances, proteins self-modify to modulate and/or reverse autoinhibition. Such is the case for autophosphorylation which occurs in type II PKA, PKGs, calmodulin-dependent protein kinase, myosin light chain kinase, and others (15; 26-33). In PKA and PKGs, self-incorporation of phosphate(s) into the autoinhibitory region of the protein increases its ease of activation by cyclic nucleotides, and, in the case of PKGs, autophosphorylation increases kinase activity in absence of cyclic nucleotide (31-38). This autoactivation could be considered to be the counter process to autoinhibition.

3. GENERAL MECHANISMS OF AUTOINHIBITION OF CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

There are two major mechanisms involved in effecting autoinhibition in cyclic nucleotide-dependent protein kinases. One involves a short segment of sequence in the regulatory domains of these proteins which mimics the substrate consensus sequence and locks into the catalytic site . The second involves other contacts between the regulatory domain and regions outside the catalytic site; these contribute to autoinhibition by either stabilizing the substrate consensus sequence interactions with the catalytic site or by maintaining the kinase in a less active conformation (4; 12; 17; 37-41). Both processes contribute importantly to inactivating the kinases. The regulatory and catalytic components of cAMP-dependent protein kinases (PKAs) reside on separate subunits, i.e., the regulatory subunit (R subunits) and the catalytic subunit (C subunit) (23; 24). Although the inactive form of PKA is a tetramer consisting of two R subunits and two C subunits, autoinhibition occurs in the complex of a single R subunit with a single C subunit as depicted in Figure 1A (42-44). Most cGMP-dependent protein kinases (PKGs) are homodimers, and the regulatory and catalytic domains are contained on a single polypeptide chain (37-39; 41; 45-49). Autoinhibition occurs within a single monomer as shown in Figure 1B (50). Notably, monomeric forms of PKA and PKG occur naturally in some lower forms (51-53).

3.1. Autoinhibition through substrate-like sequences

The autoinhibitory domains of many protein kinases include a short segment of sequence that mimics the consensus substrate sequence for that particular kinase. In some instances, this sequence can be autophosphorylated, in which case it is a true substrate sequence. If not, it is termed a pseudosubstrate sequence. The similarity of such a sequence to substrates allows it to

Table 1. Inhibitory Sequences in PKAs, PKGs, and PKA-Specific Protein Kinase Inhibitor Resemble PKA and PKG Substrate Consensus Sequence

Substrate Consensus Sequence for PKA and PKG	-**RRX**S/T**X**-
cAMP-Dependent Protein Kinases (R subunits):	
RIα (human, bovine, porcine, rat, mouse)	-GRR**RRGA**ISAEV-
RIβ (mouse)	-ARR**RRGG**VSAEV-
RIIα (bovine, porcine, at, mouse)	-K/RFT**RRVS**VCAET-
RIIβ (human, rat)	-RFT**RRAS**VCAEA-
Drosophila melanogaster R	-PVR**RRGG**ISAEP-
Caenorhabditis elegans R	-SGG**RRTG**ISAEP-
Dictyostelium discoideum R	-TRK**RRGA**ISSEP-
Saccharomyces cerevisiae R	-MAQ**RRTS**VGE-
cGMP-Dependent Protein Kinases:	
PKGIα (human and bovine)	-TRK**RRGA**ISSEP-
PKGIβ (human and bovine	-PRT**KRQA**ISAEP-
PKGII	-RRG**AKAG**VSAEP-
Caenorhabditis elegans PKG	-QRA**KKIA**VSAEP-
Drosophila melanogaster G1 gene PKG	XXX**KKQG**VSAES-
Drosophila melanogaster G2-T1 gene PKG	-QRQ**RALG**ISAEP-
PKA-Specific Protein Kinase Inhibitor	**RRNA**IHDIL-

PKAs and PKGs preferentially phosphorylate proteins at serine or threonine in a consensus substrate sequence of –Arg-Arg-X-Ser/Thr-X-. Autoinhibitory domains of all known R subunits and PKGs contain a related sequence as does PKI. Residues in bold denote the sequence homologous to the consensus substrate sequence. Underlined amino acids occupy the P^0 position which can be phosphorylated when this residue is serine or threonine. When P^0 is occupied by glycine or alanine as occurs in RI subunits and PKGs, the sequence is denoted as a pseudosubstrate site.

bind directly into the kinase catalytic cleft to competitively block protein/peptide substrate binding and catalysis. The pioneering concept of this mechanism of autoinhibition for protein kinases was first recognized in the PKAs (3). For PKAs and PKGs, the preferred consensus sequence for phosphorylation is RRXS/TX (Table 1) (8; 9; 54-58). While some variation within this motif is tolerated, e.g., lysine can replace one or both arginines, basic residues located at P^{-2} and P^{-3} (phosphorylatable serine or threonine=P^0) are critical in defining a good substrate for either PKA or PKG. In addition, while not required, a hydrophobic residue at P^{+1} is an important positive factor in substrate efficacy for both kinases. For the remainder of this document, residues amino- or carboxyl-terminal to the (pseudo)phosphorylation site (P^0 residue) are designated as minus or plus residues, respectively.

Complete amino acid sequences are now available for R subunits from many species (Table 1) (59-61). These confirm that autoinhibitory domains of all known R subunits contain a sequence that closely mimics the classical PKA consensus substrate sequence. There is overwhelming support for the importance of the substrate-like sequence in PKA for the efficacy with which R subunit inhibits C subunit. RII subunits contain a substrate-like sequence (-RRX*S*X-) that can be autophosphorylated by PKA catalytic subunit (C subunit) (26). In all known cases, RI subunits contain a pseudosubstrate sequence (-RRX*A*X- where alanine or glycine occupies the position of the phosphorylatable serine or threonine). When the P^0 alanine or glycine in RIα subunit is mutated to serine, this position can also be phosphorylated by C subunit, which occurs in RII subunit. In all known R subunits either isoleucine or valine occurs at P^{+1}, and a hydrophobic residue in this position is favored in PKA substrates. For PKA, the veracity with which the autoinhibitory sequence mimics that of an optimal substrate consensus sequence has been established as critical to high-affinity association between R and C subunits and, therefore, effective autoinhibition. Since R and C subunits can dissociate upon activation, a more precise substrate-like sequence may facilitate a timely reassociation.

The case for a dominant role for the pseudosubstrate mechanism of autoinhibition of PKGs is less compelling. Only three of the six known PKGs (mammalian PKGIβ, Drosophila DG1 gene product, and *C. elegans* PKG) contain prototypical substrate-like sequences in the homologous autoinhibitory domain (–KRQ*A*I-, -KKQ*G*V-, and -KKI*A*V-, respectively). Other PKGs have only a single basic residue (lysine or arginine) at either P^{-2} or P^{-3} (62-66) . These latter sequences are not good mimics of PKG substrates (Table 1), and even the presence of lysine rather than arginine at P^{-2} or P^{-3} makes these less than optimal substrate-like sequences (57; 67). An excellent substrate consensus sequence (-^{81}RKFTK^{85}-) located near the autoinhibitory sequence (-^{59}RAQGI^{63}-) in PKGIα does not provide for autoinhibition following removal of the more amino-terminal sequence by partial proteolysis (68). This example demonstrates that a less than ideal substrate-like sequence can fulfill the requirements for autoinhibition if it is appropriately

Table 2. Contribution of Residues in RI Subunit Pseudosubstrate Sequences To Autoinhibition

Regulatory Subunit	Sequence Surrounding Pseudosubstrate Sequence in RI Subunit								
	-5	-4	-3	-2	-1	P^0	+1	+2	
	92	93	94	95					
Wild Type	. .R	R	R	R	G	A	I	S.	.
Δ1-91	M R	R	R	R	G	A	I	S.	.
Δ1-94			M	R	G	A	I	S.	.
Δ1-94*				R	G	A	I	S.	.
Δ1-93.R95A		M	R	**A**	G	A	I	S.	.
Δ1-95					G	A	I	S.	.
Δ1-92/R95A	M	R	R	**A**	G	A	I	S.	.
RI I98A	. . R	R	R	R	G	A	**A**	S.	.

The residue number in bovine RI is indicated above the sequences. Each sequence begins with the amino-terminal residue in that particular truncated RI subunit, and mutated residues are in bold. Sequences preceded by dots are contained within full length RI. Alanine that occupies P^0 in the RI pseudosubstrate sequence is underlined. IC_{50} is defined as concentration of RI subunit required to inhibit 50% of Kemptide phosphorylation by PKA C subunit. C subunit concentration was 0.02 nM.

positioned in relationship to the catalytic site. Since autoinhibitory and catalytic domains of PKGs are contained within the same polypeptide, it may be less crucial that the pseudosubstrate sequence of PKG closely emulates a substrate sequence.

3.2. Autoinhibition through non-substrate-like sequences

In addition to the requirement for a substrate-related sequence for productive autoinhibition of PKA and PKG catalysis, other regions of both proteins contribute importantly to maintaining the inactive conformations. Myriad studies using limited proteolysis, site-directed mutagenesis, truncation mutagenesis, scanning mutagenesis, and synthetic peptides have attempted to define regions of R subunits and PKGs that contribute to autoinhibition. Significant progress has been made in determining the general location of segments of sequence that contribute to autoinhibiton, but there is still a paucity of information defining specific interactions. The absence of x-ray crystallographic structures for either PKA holoenzyme or PKG in the inactive state continues to stall advances in our understanding of this important aspect of cyclic nucleotide signaling.

4. AUTOINHIBITION OF cAMP-DEPENDENT PROTEIN KINASES (PKAs)

Association of R subunit and C subunit to form PKA holoenzyme is an extremely high-affinity interaction. In absence of cAMP, either RI or RII subunit homodimer combines with any of the C subunit isozymes with an apparent affinity of ~0.2-0.4 nM (41; 69-73). While much is known about requirements for PKA autoinhibition, the mechanisms involved are not yet fully understood. Information on this topic derives largely from a multitude of studies utilizing truncated forms of R subunits, synthetic peptides that mimic the region surrounding the substrate or pseudosubstrate sequence in R subunits, and the x-ray crystallographic structure of C subunit co-crystallized with the high-affinity, heat-stable PKA inhibitor (PKI) (71; 74-79). Much of the R subunit autoinhibition of C subunit is effected by a substrate-like sequence located in the amino-terminal portions of either RI or RII subunits (-RRX*A*X- and -RRX*S*X-, respectively) (Figure 1A). These prototypical sequences directly interact with the catalytic site in C subunit, but the consensus substrate sequence alone is insufficient to sustain potent autoinhibition. Heat-denatured RII is still rapidly phosphorylated by C subunit in the autoinhibitory substrate site (-RRX*S*X-), but it does not inhibit catalysis (4). PKI specifically inhibits C subunit in part by mimicking interactions that occur between C subunit catalytic site and the substrate-like sequence in R subunit, but the potency of PKI inhibition is dramatically enhanced by interactions that are unique to that complex (80-82). Peptides that duplicate the substrate-like sequence in R subunit or PKI are weak inhibitors of catalysis (see below).

4.1. Studies of autoinhibition of PKA using modified forms of R subunits

It is well established that arginines within the substrate-like sequences of RI and RII subunits are important in interaction of either R subunit with C subunit. Removal of either of the two arginines in the pseudosubstrate sequence (-RRX*A*X-) of RI subunit (Δ1-94, Δ1-94*, Δ1-93.R95A, Δ1-92.R95A in Table 2) or the substrate-like sequence of RII subunit (-RRX*S*X-) causes a profound loss in R subunit inhibitory potency toward C subunit (73; 77).

Because the RII subunit autophosphorylation site is such a good substrate for PKA, and because arginine is required for autophosphorylation, Corbin et al. proposed that the autophosphorylation sequence contains basic residues and serves as a recognition sequence for C subunit, such that it competitively inhibits substrate phosphorylation (3). RI subunit is not autophosphorylated because alanine replaces the phosphorylatable serine, i.e., it is a true pseudosubstrate sequence (Table 1). The fact that the

Table 3. Effect of Substitutions and Length on Inhibitory Potencies of Synthetic RI Subunit Peptides Toward PKA Catalytic Subunit

Peptide Number	Peptide Sequence	IC_{50} (μM)
	P^0	
1	^{82}PPPPNPVVKGRRRRG<u>A</u>IS^{99}	12
2	^{82}PPPPNPVVKGRR**A**RG<u>A</u>IS^{99}	6230
3	^{82}PPPPNPVVKGRRR**A**G<u>A</u>IS^{99}	517
4	^{82}PPPPNPVVKGRRRRG<u>A</u>**A**S^{99}	350
5	^{92}RRRRG<u>A</u>IS^{99}	18
6	**A**RRRG<u>A</u>IS^{99}	48
7	**AA**RRG<u>A</u>IS^{99}	203
8	^{94}RRG<u>A</u>IS^{99}	810
9	^{94}RRG<u>A</u>IS-AEVYTEEDAASYVRKVIPKDYKT^{122}	14

The parent peptide is RItide (82-99) (peptide 1). Substituted amino-acids are in bold. The Ala at the P^0 position is underlined.

proteolytically-sensitive autoinhibitory region of R subunit is protected in PKA holoenzyme and autophosphorylation of RII subunit decreases its affinity for C subunit, provides further support for the pseudosubstrate hypothesis. These results along with those from the co-crystal of PKI-C subunit have established that substrate-like sequences in either R subunit or PKI occupy the substrate-binding site in C subunit, thereby preventing substrate binding and maintaining PKA in an inhibited state.

Autophosphorylation of RII subunit at Ser-95 (or of RI subunit mutated to replace the homologous P^0 alanine with serine) by C subunit occurs in the absence of cAMP. This suggests that the P^0 alanine in RI subunit pseudosubstrate sequence is closely approximated to the γ-phosphate of MgATP. Replacement of alanine at P^0 in RI subunit by bulky amino acids results in reduced affinity for C subunit, and MgATP further reduces C subunit affinity for each of these mutant RI subunits. This suggests that a bulky group at P^0 of RI subunits may spatially interfere with MgATP bound at the active site of C subunit.

The impact of P^{-2} and P^{-3} arginines (-RRX*S/A*X-) on inhibitory potencies of R subunits has been studied using site-directed and truncation mutagenesis. Substitution of alanine for both P^{-2} and P^{-3} arginines in RI subunit causes loss of inhibition of C subunit (73). Mutation of either P^{-2} or P^{-3} arginine of RI subunit to alanine, or deletion of these residues, increases IC_{50} values substantially (Table 2), demonstrating the importance of each in C subunit inhibition. Deletion of both P^{-2} and P^{-3} arginines in RI subunit through truncation mutagenesis (Δ?95 in Table 3) causes a synergistic increase in the IC_{50} value compared to a truncated RI subunit retaining the complete RI subunit pseudosubstrate sequence (Δ1-91 in Table 3). Retention of either P^{-2} or P^{-3} arginine in an amino-terminally truncated RI subunit blunts the loss in inhibitory potency.

In RI subunit, two additional arginines are located immediately amino-terminal to the pseudosubstrate site (-RR<u>RRX*A*X</u>-), but there is no indication that these residues contribute significantly to autoinhibition. When alanine replaces arginine at either P^{-2} or P^{-3}, the remaining arginines at P^{-3} and P^{-4} cannot fulfill the role of the P^{-2}-P^{-3} arginine duo within the RI pseudosubstrate site (73). This indicates that interactions between the arginines in the pseudosubstrate site and acidic residues in the catalytic site cannot spatially shift to substitute the two required arginines at P^{-2}-P^{-3} with those in P^{-3}-P^{-4}.

A hydrophobic residue at P^{+1} and the length of the aliphatic side chain at this position are also important for potent R subunit interaction with C subunit. Isoleucine or valine at P^{+1} is conserved in autoinhibitory domains of all known species of PKA, PKG and PKI. Substitution of alanine for the P^{+1} isoleucine in RI subunit autoinhibitory peptide sequence diminishes inhibitory potency ~20-fold. Substituting glutamine for this isoleucine increases the length of the side chain and hydrophilicity at this position, and the efficacy with which this mutant RI subunit inhibits C subunit is also reduced. Steric constraints at P^{+1} are also supported by peptide substrate studies.

Contacts other than those involving the substrate-like autoinhibitory sequence also contribute to autoinhibition of PKA. As noted above, the P^0 serine in heat-denatured RII is still efficiently phosphorylated by C subunit, but there is no inhibition of catalytic activity presumably due to loss of critical structure in RII (4; 77). Site-directed mutagenesis of C subunit has established that it contains a high-affinity R subunit-binding domain involving Trp-196, Leu-198, His-87, Lys-189, Lys-213, and Lys-217 (83-85). Conversion of His-87 to Gln and Trp-196 to either Ala or Arg has little effect on catalysis or inhibition of C subunit by PKI, but interaction with R subunit is lost, as also happens when Lys-189, Lys-213, and Lys–217 in C subunit are altered. This emphasizes the importance of autoinhibitory interactions outside the substrate-related sequence described above. These and other observations indicate that many structural elements of C subunit that interact with R subunit and PKI are shared, but other contacts important for inhibition are novel.

4.2. Studies of autoinhibition of PKA using synthetic peptides

A collection of synthetic RI subunit peptides has been used to assess the comparative and qualitative contributions of specific residues to autoinhibition of C subunit (Table 3) (73). Peptides with the same substrate-like sequence (peptides 1, 5, and 9) as found in R subunit are very weak inhibitors compared to R subunit. Pepides with lengthy amino-terminal (peptide 1) or carboxyl-terminal extensions (peptide 9) around the pseudosubstrate sequence (peptide 5) are essentially equipotent with the latter (IC_{50} values of 12-18 μM), i.e., ~ 60,000-fold weaker than RI subunit. These peptides lack component(s) that provide for the remarkable potencies of these same sequences in R subunits. The substrate-like sequence in R subunits may provide for a novel conformation that, in combination with the direct contacts made by the substrate-like sequence itself, is particularly potent for inhibiting C subunit. In this scenario, when key components in the substrate-like sequence of R subunit are removed, substituted or otherwise modified, both components of inhibition are lost simultaneously.

The principal RI peptides shown in Table 3, peptides 1 (RI 82-99), 5 (RI 92-99), and 9 (RI 94-122) are specific inhibitors of C subunit. In contrast, synthetic peptides based on sequences found in protein kinase C (RFARKGALRQKNVHEVKN) and Ca^{2+}/calmodulin-dependent protein kinase (MHRQETVDCLKKFNARRKLKGAILTTMLA) and which contain sequences similar to RI subunit pseudosubstrate sequence (underlined) do not inhibit C subunit even at very high concentrations.

4.3. Inhibition of PKA by the heat stable PKA inhibitor, PKI

The PKI family of proteins inhibits PKA C subunit isoforms Cα and Cβ, but not Cγ, with high specificity and potency (86-88). Like R subunit, PKI contains a pseudosubstrate sequence (Table 1) that interacts with the catalytic site in C subunit. Studies using PKI peptide analogs have also demonstrated the importance of the P^{-3} arginine, P^{-2} arginine, and P^{+1} isoleucine in the pseudosubstrate sequence, as well as that of residues amino-terminal to this sequence, for potent PKI inhibition of C subunit (89). Details of the interaction of residues within the PKI pseudosubstrate site with those in C subunit catalytic site have been revealed in the x-ray crystal structure of the co-crystal of a PKI peptide (residues 5-24) and C subunit (78; 79). This structure provides enormous insight into some of the most crucial interactions that account for inhibition of C subunit by the substrate-like sequence in PKI and, by extrapolation, in R subunits.

C subunit is an asymmetric bilobate protein in which the catalytic site lies in a deep cleft bisecting the lobes. The more carboxyl-terminal lobe of C subunit provides most of the interaction with the substrate-like consensus sequence to precisely position it in the catalytic cleft (78; 90). In the co-crystal of C subunit complexed with a peptide derived from the inhibitory segment of PKI (residues 5-24), PKI pseudosubstrate P^{-3} and P^{-2} arginines form hydrogen bonds with four glutamic acids in C subunit. P^{-3} arginine interacts with Glu-127, and perhaps also with Glu-331, which is located in a cluster of six acidic residues near the carboxyl terminus. Glu-170 and Glu-230 ion pair with the P^{-2} arginine. The P^{+1} isoleucine of PKI pseudosubstrate site sits in a hydrophobic pocket near the enzyme surface. These interactions effectively lock the PKI pseudosubstrate sequence into the catalytic site of C subunit. Results derived from the co-crystal of C subunit and PKI reveal the specific interactions which have been described in biochemical studies of the pseudosubstrate P^{-3} and P^{-2} arginines and the P^{+1} hydrophobic residue in both R subunit and PKI.

While PKI and R subunit make common contacts with C subunit to effect inhibition, each forms novel contacts with C subunit as well. The PKI P^{-6} arginine hydrogen bonds with a glutamic acid in C subunit and orients the amphipathic helix at the amino-terminal end of PKI (78; 90). This amphipathic helix of PKI interacts with a hydrophobic groove on the surface of C subunit and is critical in conferring high-affinity binding of PKI to the kinase. However, R subunit lacks a structural counterpart, and sequence amino-terminal to the pseudosubstrate sequence in R subunits is apparently not required for potent inhibition of C subunit.

5. AUTOINHIBITION OF cGMP-DEPENDENT PROTEIN KINASES (PKGs)

Autoinhibition in PKGs is similar to PKA in many respects, but there are clear distinctions between the two. This most likely results from differences in overall structures of the kinases as well as to very specific differences within the catalytic site. For example, PKI is an extremely weak inhibitor of PKGs in contrast to being a potent inhibitor of PKA (87; 91). As in PKA, interactions between PKG catalytic site and a substrate-related sequence in its regulatory domain contribute importantly to effect autoinhibition, but evidence suggests that these contacts differ somewhat from those occurring in PKA (40). In addition, contacts outside the substrate-related sequence participate in autoinhibition as well. Regions of PKG that contribute to autoinhibition have been determined by a number of techniques including limited proteolysis, site-directed and truncation mutagenesis, and synthetic peptides based on PKG sequences.

In mammalian tissues, three major forms of PKGs have been identified. These include two type I PKGs (PKGIα and PKGIβ), which are products of alternative splicing in the amino-terminal ~100 residues, and one type II PKG (PKGII) (46; 48; 62; 63; 92-95). Unlike PKA, regulatory and catalytic domains of PKGs reside within a single polypeptide, and autoinhibition of catalysis and relief of that inhibition by cGMP binding occurs within each monomer as depicted in Figure 1B (50). The autoinhibitory and autophosphorylation domains of PKGs are complex, overlapping, and located in the amino-terminal portion of the proteins, i.e., the approximately first 100 residues (Figure 1B). However, the exact boundaries of these overlapping domains are not known.

Table 4. Summary of Characteristics of PKG Fragments Produced by Partial Proteolysis

Enzyme	Apparent Subunit Molecular Mass kDa	Kinase Activity Ratio -/+ cGMP	K_a for cGMP μM
Native PKGIα	76	0.2	0.2
Trypsinized PKGIα (^{78}QAFRKFKT-)	65	0.7	ND
Native PKGIβ	78	0.07	0.4
Proteolytic Fragments of PKGIβ			
EndoK PKGIβ (^{75}RQAISAEP-)	70	0.09	ND
Trypsin PKGIβ (^{76}QAISAEP-)	70	0.51	0.2
Chymotrypsin PKGIβ (^{86}DIQDLS-)	67	0.71	ND

Purified bovine PKGs were subjected to partial proteolysis using endoproteinase Lys-C (EndoK), trypsin or chymotrypsin. Following proteolysis, each proteolyzed PKG was purified to apparent homogeneity. Amino-terminal sequence of each was determined using sequential Edman degradation.

PKGs contain a substrate-related sequence that is homologous to the classical autoinhibitory sequence found in R subunits of PKA. However, few of these sequences in PKGs have the two arginines that are so crucial for effective PKA autoinhibition (Table 1). Furthermore, whereas autophosphorylation of RII subunit by C subunit occurs at a single residue within the autoinhibitory substrate consensus sequence, autophosphorylation of PKGs occurs at multiple sites distributed throughout the amino-terminal region of the proteins (29; 31; 33). Research into autoinhibition of PKGs has largely focused on the sequence that is homologous to the autoinhibitory sequence in R subunits, and the effects of autophosphorylation on autoinhibition have also been studied (33; 96).

5.1. Studies of PKG autoinhibition using modified PKGs

Since regulatory and catalytic domains of PKG are contained within a single polypeptide, it is difficult to determine the affinity with which autoinhibition occurs in these kinases. Among PKGs, potencies of the autoinhibitory domains may differ since "basal" activity measured in the absence of cyclic nucleotide varies in the purified PKGs. Furthermore, K_a values for cGMP activation of PKGIα and PKGIβ differ, although the sequences of their cyclic nucleotide-binding sites are identical (48; 97). PKGIα and PKGIβ isoforms are derived from alternative splicing of mRNA and differ only in their amino-terminal 100 amino acids.

Limited proteolysis of PKGIα and PKGIβ generates a collection of amino-terminally truncated PKGs with varying degrees of autoinhibition (40; 68). For PKGIα, trypsin treatment produces a 65-kD protein that is largely cGMP-independent (-cGMP/+cGMP activity ratio of 0.75) (Table 4), and whose amino-terminal sequence begins at Gln-78 (Gln-Ala-Phe-Arg-Lys-Phe-Thr-Arg-), thereby excluding the pseudosubstrate sequence located between Arg-59 and Ile-63 (-^{59}Arg-Ala-Gln-*Gly*-Ile^{63}-). This clearly establishes the overall importance of this latter region of sequence in PKG to autoinhibition. However, retention of partial cGMP-dependence of the catalytic activity of the fragment indicates that contacts remain which prevent full expression of PKGIα catalytic activity in the absence of cGMP. Furthermore, a substrate sequence (-^{81}Arg-Lys-Phe-Thr-Arg^{85}-) that is retained in this PKGIα fragment and which is actually a site for autophosphorylation is insufficient to effect potent autoinhibition. This emphasizes that the precise location of the pseudosubstrate sequence within the sequence/structure of PKGs is necessary for effective autoinhibition.

Partial proteolysis of PKGIβ also emphasizes the importance of the substrate-related sequence for autoinhibition (Table 4). A fragment whose amino-terminus begins at Arg-75 (^{75}Arg–Gln-*Ala*-Ile-Ser-Ala^{80}-) retains a single basic residue at P^{-2} in its pseudosubstrate sequence, but catalytic activity is almost fully dependent on cGMP (activity ratio –cGMP/+cGMP <0.1) similar to native PKG. A second PKG fragment that lacks basic residues at both P^{-2} and P^{-3} is approximately half-active in absence of cGMP (activity ratio –cGMP/+cGMP ~0.5). This result advances understanding of PKGIβ autoinhibition in several ways. First, it documents the powerful influence of a basic residue at P^{-2} and the lack of an absolute requirement for one at P^{-3}. In this regard, it differs significantly from requirements for effective PKA autoinhibition. Second, it demonstrates that a significant amount of autoinhibition (~50%) persists even in absence of the two basic residues that are the key elements of a consensus sequence in a true pseudosubstrate site. Third, it establishes that PKGIβ autoinhibition results from multiple contacts within and carboxyl-terminal to the pseudosubstrate sequence. While this may also be true for PKA autoinhibition, it has been difficult to demonstrate experimentally.

An Arg75Ala mutant of PKGIβ has an activity ratio of ~0.5, which is almost identical to that of the PKGIβ proteolytically truncated through this residue (40; 98). This suggests that the lysine at P^{-3} in intact PKGIβ does not contribute significantly to potency with which this region interacts with the catalytic site. Pseudosubstrate sequences of mammalian PKGIα and PKGII and that predicted for the *Drosophila* G2 PKG gene product have a single basic

Table 5. Effect of Substitutions and Length on the Inhibitory Potencies of PKGIβ Peptides Toward PKGIβ

Peptide Number	Peptide Sequence	IC_{50} (mM)
10	^{71}ProArgThrLysArgGlnAlaIleSerAlaGluPro^{82}	0.5
11	^{71}Pro**Ala**ThrLysArgGlnAlaIleSerAlaGluPro^{82}	2.7
12	^{71}ProArgThr**Ala**ArgGlnAlaIleSerAlaGluPro^{82}	1.4
13	^{71}ProArgThr**Arg**ArgGlnAlaIleSerAlaGluPro^{82}	0.04
14	^{71}ProArgThrLys**Ala**GlnAlaIleSerAlaGluPro^{82}	2.1
15	^{71}ProArgThrLysArgGlnAla**Ala**SerAlaGluPro^{82}	0.8
16	^{74}LysArgGlnAlaIleSerAlaGluPro^{82}	2.7
17	^{58}GlnAlaGlnLysGlnSerAlaSerThrLeuGlnGlyGlu-ProArgThrLysArgGlnAlaIleSerAlaGluPro^{82}	1.0
18	^{71}ProArgThrLysArgGlnAlaIleSerAlaGluPro-ThrAlaPheAspIleGlnAspLeuSerHis^{92}	0.3

The parent synthetic peptide is PKGIβ (71-82) (peptide 10). Substituted amino acids are highlighted and underlined.

residue (arginine) located at either P^{-2} or P^{-3} (Table 1). These enzymes are efficiently autoinhibited which suggests that an arginine at either P^{-2} or P^{-3} along with other interactions provides contacts sufficient for maintaining PKGs in the inactive state. It also seems likely that either a duo of lysines as occurs in the PKGs from *C. elegans* and *Drosophila G1* or a single lysine at P^{-2} as occurs in PKGII is sufficient for autoinhibition. However, the results of Collins and Uhler suggest that a single lysine at P^{-3} cannot fulfill the autoinhibitory role (98).

As in R subunits, hydrophobicity in the residue located at P^{+1} is preserved in the pseudosubstrate sites of all known PKGs. Isoleucine or valine occupies this position in all known PKGs (Table 1). When this isoleucine in either PKGIα or PKGIβ is replaced with a hydrophilic residue, autoinhibition is greatly diminished (99). A similar conclusion regarding P^{+1} is drawn from studies with synthetic peptides as well (Table 5) (100).

Another residue that contributes to autoinhibition in PKGs is the conserved serine at P^{+2}. A point mutation to convert this serine to alanine in either PKGIα or PKGIβ compromises the potency of autoinhibition and elevates basal kinase activity significantly (J. Busch, unpublished results). This residue can be autophosphorylated in PKGIβ despite being "out of the P^0 position," and substitution of aspartate for this serine in PKGIβ produces a constitutively active kinase (33; 98). The PKG-catalyzed transfer of the γ-phosphate from ATP to a P^{+2} serine demonstrates a clear difference in the catalytic sites of PKGs and C subunit. In combination, these results suggest that serine at P^{+2} in the autoinhibitory domain of PKGs may form contacts within the PKG catalytic site and thereby contribute to potency of autoinhibition or activation following autophosphorylation.

It has long been known that PKG readily phosphorylates serine in the P^{+2} position in RI subunit (Table 1) (101). Serine also occurs at P^{+2} in a number of PKG substrates where phosphate is transferred to a serine in the P^0 position, e.g., PDE5, histone 2B, phosphorylase kinase α subunit, and hormone-sensitive lipase (102). Therefore, interaction between PKG catalytic site residues and a serine at P^{+2} may not be restricted to autoinhibitory contacts and raises the possibility that contact at this point may have relevance to substrate interactions. However, despite the presence of serine at P^{+2} in these PKG substrates, this site is apparently not phosphorylated when serine occupies the P^0 position (57; 103). Substitution of alanine for the P^{+2} serine in the synthetic PDE5 peptide does not significantly alter the kinetic properites of PKG for phosphorylation of the P^0 serine. Despite the evidence that contacts within and carboxyl-terminal to the PKG pseudosubstrate site contribute importantly to interaction between its regulatory and catalytic domains, it is difficult to quantitate relative contributions of specific residues in this region to autoinhibition.

5.2. Effect of autophosphorylation on PKG autoinhibition

PKGs autophosphorylate multiple sites in their amino-terminal ~100 amino acids. In PKGIα?at least six sites can be autophosphorylated (31; 33). Four of these are modified within a segment of 30 amino acids, whereas in PKGIβ two sites are modified within a distance of 17 amino acids. Most of these autophosphorylations occur at sites that do not mimic a typical consensus substrate sequence. For PKGIβ?introduction of phosphate at the conserved P^{+2} serine (-KRQAI**S**AE-) through autophosphorylation is important for autoactivation, perhaps by weakening interaction between the substrate-like sequence and the catalytic site. In addition, both affinity for cyclic nucleotide and PKG basal activity increase. Therefore, autophosphorylation at certain sites can effectively relieve autoinhibition. This could result from electrostatic repulsion between the negatively charged phosphate and similarly charged residues within the catalytic domain, steric distortion of the topography of the autoinhibitory region to block inhibitory contacts, disruption of interactions involving the P^{+2} serine, or a combination of these processes.

5.3. Inhibition of PKG by RI subunit

PKG and PKA are homologous kinases. RI subunit competitively inhibits PKGIα catalytic activity *in vitro*, including autophosphorylation (101). It has a K_i of

~0.2 μM when either histone H2B or a heptapeptide substrate is used. This indicates that RI subunit is ~ 50-200 fold less potent for inhibiting PKG catalytic activity than that of C subunit. As noted above, PKG also phosphorylates RI subunit *in vitro* at the P^{+2} serine adjacent to the pseudosubstrate region (-RRG*A*I**S**-). This serine is homologous to a PKG autophosphorylation site in its own autoinhibitory domain and does not conform to a substrate consensus sequence. The K_m for PKG phosphorylation of RI subunit is ~2 μM, a concentration that is approached in some tissues. C subunit does not phosphorylate the same site in RI, whereas the P^0 serine in RII subunit is located in an ideal substrate consensus sequence (-RRXSV-) and is readily phosphorylated by both C subunit and PKG. Several tissues appear to contain excess free RI subunit. Whether RI subunit inhibits PKG in intact tissues is not known, and whether this demonstrated interaction and phosphorylation *in vitro* contributes to physiological cross-talk for regulation of cyclic nucleotide signaling remains questionable.

5.4. Studies of PKG autoinhibition using synthetic peptides

A collection of synthetic peptides based on the PKGIββpseudosubstrate sequence has been used to assess contribution of residues within and adjacent to the pseudosubstrate sequence (^{74}Lys-Arg-Gln-*Ala*-Ile) to inhibition of PKG kinase activity (Table 5) (100). The parent peptide (peptide 10, Iβ (71-82)) inhibits activated PKGIββwith an IC_{50} of 0.5 mM. This value is 50-fold greater than those for inhibition of PKA C subunit by peptides that mimic RI subunit pseudosubstrate region (peptides 1 (12 uM), 5 (18 uM), and 9 (14 μM) in Table 3). Substitution of the basic residues in PKGIβ parent peptide (peptide 10), i.e., P^{-5} arginine, P^{-3} lysine, or P^{-2} arginine with alanine (peptides 11, 12, and 14) decreases their inhibitory potencies 3- to 5-fold. Replacement of lysine at P^{-3} by arginine (peptide 13) increases the inhibitory potency (IC_{50} ~35 μM) for PKG, and this compares well with the inhibitory potency of RI-subunit parent peptide (peptide 1) toward C subunit (12 μM) (Table 3). This suggests that in all known instances, the autoinhibitory pseudosubstrate sequences within PKGs are not designed for optimum potency as occurs and is required for effective autoinhibition in PKA.

Interaction of PKG pseudosubstrate sequence and the catalytic site may be innately weak compared with the analogous interaction on PKA. Since regulatory and catalytic domains of PKG are juxtaposed within the same polypeptide chain, an optimized substrate-related sequence could impede activation by cGMP. A weaker interaction of the substrate-related sequence with the active site may be favored in the process of natural selection of PKG regulation. This may also apply in other kinases whose regulatory and catalytic activities are co-localized in the same polypeptide.

Peptide 16, which lacks the first three residues of the amino-terminus of peptide 10 (Table 5), has decreased inhibitory potency (6-fold). Inhibitory potency of peptide 17 (which includes PKGIβ residues 58-82) is somewhat less than that of peptide 10 and provides no evidence that residues amino-terminal to P^{-6} might contribute importantly to potent inhibition of PKGIβ. Ser-79 is autophosphorylated in intact PKGIβ, which causes partial activation of this enzyme. However, although peptides 10, 16, and 17 contain site(s) that are autophosphorylated in intact PKGIβ (Ser-63 and/or Ser-79), none of these is phosphorylated detectably by PKGIβ? Extension of the carboxyl-terminal sequence of peptide 10 by ten residues beyond the substrate-like sequence (peptide 18) has little effect on inhibitory potency.

Based on peptide studies, P^{-5} arginine, P^{-3} lysine, and P^{-2} arginine could contribute moderately to potency of PKGIβ autoinhibition. However, results with peptide analogs of autoinhibitory domains of kinases must be judged reservedly. Despite a small effect of substitutions in the parent PKGIβ peptide on PKG inhibition, several of these same changes in PKG holoenzyme significantly alter extent of autoinhibition. Notably, substitutions in the PKGIβ peptides have a less profound effect on inhibitory potencies than similar substitutions in RI subunit pseudosubstrate peptides.

As for PKA, potent PKGIβ autoinhibition clearly involves interactions outside the substrate-like sequence. As a consequence of the physical association of regulatory and catalytic domains in PKG, it is suggested that there are numerous low-affinity interactions between PKGIβ autoinhibitory domain and the active site. This is supported by the observation that each of the P^{-5}, P^{-3}, and P^{-2}, and P^{+1} residues in PKGIβ peptides contributes to a similar extent, albeit weakly, to inhibition. Addition of ten PKGIβ residues (83-92) to the carboxyl terminus of peptide 10 improves inhibitory potency of the resulting peptide (peptide 18) slightly, suggesting that residue(s) in this region may also interact with the active site region.

6. SUMMARY

PKG and PKA are homologous enzymes that exhibit similar, but not identical, substrate specificities and have similar substrate-like autoinhibitory sequences. Therefore, it is likely that there will be many similarities in the residues involved in substrate binding and autoinhibiton for these kinases. The importance of PKI pseudosubstrate P^{-3} and P^{-2} arginines and the P^{+1} hydrophobic residue to potent inhibition of C subunit is clearly established by x-ray crystallographic structure of the co-crystal. PKI P^{-3} arginine forms hydrogen bonds with Glu127 and Glu331, and P^{-2} pseudosubstrate arginine hydrogen bonds with Glu230 and Glu170, essentially interlocking the pseudosubstrate sequence and the active site. PKI P^{+1} isoleucine lies in a hydrophobic pocket comprised of Leu198, Pro202, and Leu205 near the surface of C subunit and is believed to participate in properly orientating the P site residue. With the exception of Glu331, which is outside the conserved catalytic core, each of the glutamates involved in forming hydrogen bonds with PKI P^{-6}, P^{-3}, and P^{-2} arginines is invariant in PKGIα and PKGIβ. C subunit

hydrophobic residues that interact with the PKI P^{+1} hydrophobic residue are also highly conserved as phenylalanine, proline, and valine in PKGIα?PKGIβ?PKGII, and Drosophila G2 gene product. Since PKI lacks P^{-4} or P^{-5} basic residues, the crystal structure gives no insight into how these residues in R subunit or PKGs might interact with the active site. In addition, recent work supports a potential role for the conserved serine at P^{+2} in PKGs. In R subunits P^{+2} is occupied by either cysteine or serine. Whether this position participates in interactions between R subunits and the catalytic domain of PKA is not known.

Results of peptide studies suggests that the P^{-5}, P^{-3}, P^{-2}, and P^{+1} pseudosubstrate sequence residues may contribute to autoinhibition of both type ? α PKA and PKGIβ. The peptide studies suggest potentially important contacts involving RI-subunit residues P^{-5} and P^{-4} arginines that are not detected using truncated RI subunits. This assumes of course that conformation of the synthetic peptides mimics the conformation of the autoinhibitory region in the intact protein. The parent peptide of the PKGIβ substrate-related sequence is profoundly less potent than is the substrate-like sequence of RI subunit, primarily because the PKGIβ P^{-3} site contains lysine instead of arginine. The overall impotence of PKA RI subunit or PKGIβ pseudosubstrate sequence peptides, the results with PKGs, RI subunit, or RII subunit derived from proteolysis or truncation mutagenesis, and heat-denaturation studies of RII subunit imply that there must be other interactions between autoinhibitory and catalytic domains of these respective enzymes.

PKA is somewhat unique among protein kinases that are regulated by pseudosubstrate sequences by the fact that the pseudosubstrate sequence and catalytic domain are located on separate subunits, whereas these domains are contiguous in many protein kinases including PKG, myosin light-chain kinase, protein kinase C, and calmodulin-dependent protein kinase. Compared to PKA, the structural arrangement of PKG is more typical of members of the protein kinase family; consequently, consideration of autoinhibitory mechanisms in PKG may be a more suitable prototype for studying overall mechanisms of autoinhibition.

7. ACKNOWLEDGEMENT

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