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# CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES

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**Abstract**—The actions of several hormones and neurotransmitters evoke signal transduction pathways which rapidly elevate the cytosolic concentrations of the intracellular messengers, cAMP and cGMP. The cyclic-nucleotide dependent protein kinases, cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), are the major intracellular receptors of cAMP and cGMP. These enzymes become active upon binding respective cyclic nucleotides and modulate a diverse array of biochemical events through the phosphorylation of specific substrate proteins. The focus of this review is to describe the progress made in understanding the structure and function of both PKA and PKG.

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## 1. INTRODUCTION

Considerable research has focused on elucidating the action of cyclic AMP (cAMP), since the discovery by Sutherland and colleagues that it is an intracellular second messenger for hormone-mediated events. The major intracellular receptor for cAMP is the cAMP-dependent protein kinase (PKA), which controls many biochemical events through phosphorylation of target proteins (Fig. 1). Cyclic GMP is an independent intracellular messenger, but unlike cAMP, this nucleotide binds to several proteins (Fig. 1), including the cGMP-dependent protein kinase (PKG). It is now known that the action of many neurotransmitters, prostaglandins and hormones proceed through receptor-mediated signaling pathways that activate these cyclic nucleotide-dependent protein kinases. PKA and PKG contain conserved structural regions, in common with all protein kinases, which compose a catalytic core of approximately 250 amino acids. The catalytic core contains the sequences responsible for substrate binding and catalysis of the phosphoryl

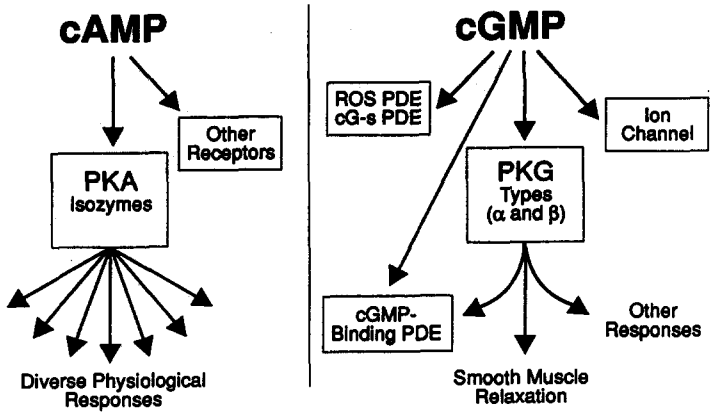


FIG. 1. A comparison of cAMP and cGMP action (adapted with permission from Corbin *et al.*, 1990).

transfer reaction. The overall primary structures of PKA and PKG are quite related, and along with protein kinase C, form the AGC subfamily of intracellular messenger-dependent protein kinases (Fig. 2). All members of the AGC subfamily, have related mechanisms of activation, similar substrate specificities and primary structures which distinguish them from other members of the protein kinase superfamily (Hanks *et al.*, 1988).

Due to the many important cellular processes that PKA and PKG mediate, their activity is tightly controlled. The catalytic core of either kinase is maintained in an inactive state by interaction with specific inhibitory sequences located on regulatory units. The structural organization within the regulatory units of both PKA and PKG are similar and consist of several well defined subdomains which are responsible for inhibition of catalytic activity and other important modulatory functions, namely dimer formation, cyclic-nucleotide binding and subcellular localization. Cyclic-nucleotide binding to the regulatory unit activates the kinase by causing the displacement of the inhibitory sequences from the catalytic core. While the regulatory and catalytic units of both kinases are structurally related, their quaternary structures are distinct (Fig. 3A). The regulatory and catalytic units of PKG are contained within the same polypeptide chain and the holoenzyme is a homodimer of $M_r$ 140,000. In contrast, PKA has a tetrameric holoenzyme complex, $M_r$ 170,000, composed of separate catalytic and regulatory subunits.

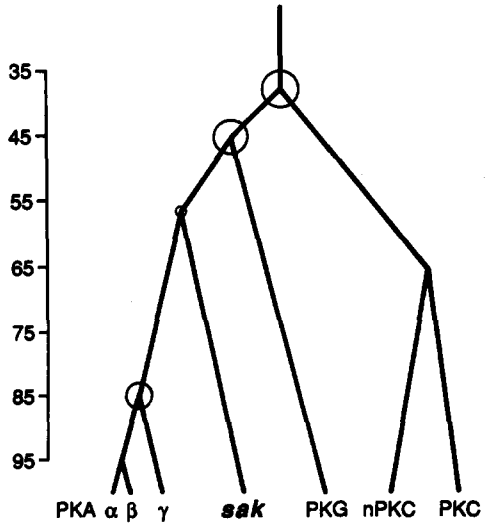


FIG. 2. The divergence of the AGC subfamily of second messenger dependent protein kinases. Reprinted from Beushausen *et al.*, 1988, with permission of the authors and the copyright holder, Cell press, Cambridge.

An array of PKA and PKG isozymes have been identified in many organisms. For example, human genes encoding three catalytic subunits (Fig. 2) and four regulatory subunits (Fig. 4A) for PKA have been identified. So far two PKG isoforms have been identified and may be splice variants of a single gene (Fig. 4A). Each PKA subunit isoform is expressed in a particular group of tissues and as a result selected cell-types express a complexed mixture of multiple isozymes. While the substrate specificity and kinetic rates of each PKA catalytic subunit isoform are identical, the regulatory units are functionally diverse, exhibiting different affinities for cyclic-nucleotide analogs and subcellular localizations. Furthermore, the diverse biochemical effects associated with the different neurotransmitters or hormones that stimulate cAMP production have been proposed to occur through the site-specific activation of particular PKA subtypes. These PKA subtypes are localized at subcellular sites through interactions of the regulatory subunits with anchoring proteins. Subcellular localization and site-specific activation of the kinase may be important mechanisms to insure that individual hormones or neurotransmitters are able to trigger the correct biochemical events associated with their action.

This review focuses particularly on protein chemistry and recombinant approaches which have successfully defined individual amino acids, or short stretches of sequence, that perform, or participate in particular functions of the cAMP- or cGMP-dependent protein kinases. Historically, PKA has been used as a prototype to identify these sites (reviewed by Taylor *et al.*, 1990; Beebe and Corbin, 1986), while later experiments have been repeated on PKG. Since this review is biased toward structural aspects of the cyclic-nucleotide dependent kinases, less emphasis

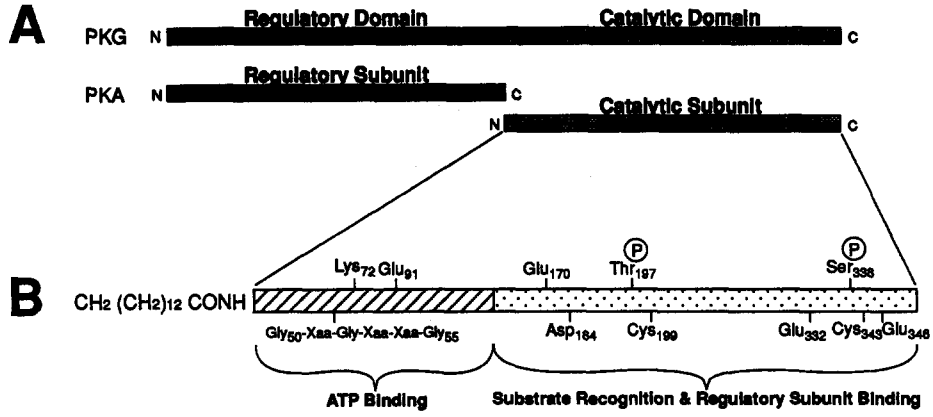


FIG. 3. The catalytic units of PKA and PKG. A: The domain organization of PKA and PKG. B: Essential residues in the catalytic core of PKA.

has been placed upon the cellular processes controlled by kinase phosphorylation. However, the mechanism of two prominent cyclic-nucleotide kinase mediated events which have been recently elucidated will be discussed, namely, the role of PKA in the activation of cAMP-responsive genes and the role of PKG in smooth muscle relaxation. There are several excellent reviews which provide detailed information on the range of cellular events which are controlled by the cyclic-nucleotide kinases (Lincoln and Corbin, 1983; Krebs *et al.*, 1985; Beebe and Corbin, 1986; Corbin *et al.*, 1990).

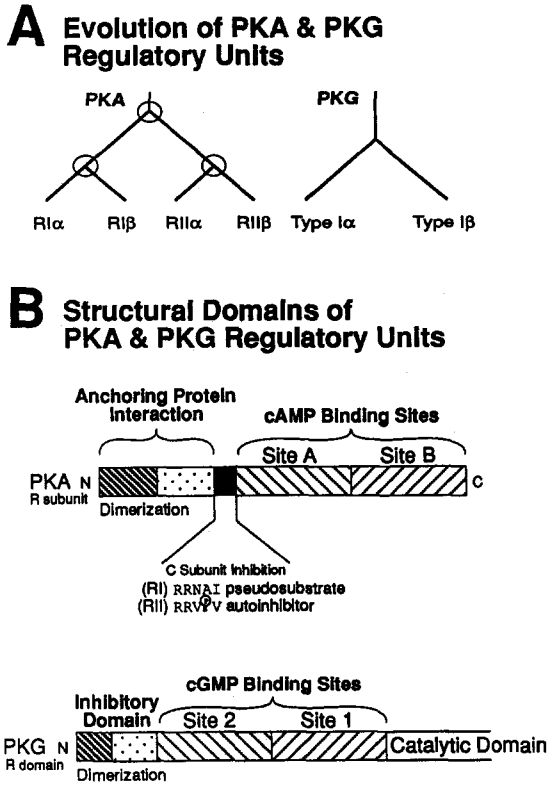


FIG. 4. The regulatory units of PKA and PKG. A: The evolution of PKA and PKG regulatory unit isoforms. B: the structural organization of the PKA regulatory subunits and the PKG regulatory domains.

## 2. THE cAMP AND cGMP SIGNALING PATHWAYS

Sutherland and colleagues were the first to report that levels of urinary cAMP and cGMP were regulated by different hormones (Hardman and Sutherland, 1969). Since then it has been established that each cyclic-nucleotide is synthesized through a distinct signaling pathway and modulates different cellular processes (reviewed by Corbin *et al.*, 1990). The generation of cAMP is catalyzed by adenylyl cyclase. Ligand–receptor interactions, coupled to G proteins, transduce extracellular signals through the membrane stimulating adenylyl cyclase on the inner membrane surface (Krupinski *et al.*, 1989). Several adenylyl cyclase isoforms have been identified and characterized and it appears that all are membrane proteins (Bakalyar and Reed, 1990). Thus, newly synthesized cAMP is released from the membrane and diffuses into the cell to activate PKA. Individual hormones which act through PKA, are somehow able to mediate the trafficking of cAMP, creating compartmentalized pools of cyclic-nucleotide (Barsony and Marks, 1990). For example, forskolin promotes cAMP accumulation in the nucleus while isopreterenol and prostaglandin E2 trigger cytoplasmic accumulation (Barsony and Marks, 1990). Accumulation of compartmentalized cAMP pools results in selective activation of particular localized PKA isoforms.

Cyclic GMP is synthesized from GTP by guanylyl cyclases (Hardman and Sutherland, 1969), but unlike adenylyl cyclase, different enzyme classes are localized to the membrane, cytoskeleton and cytoplasm (reviewed by Shulz *et al.*, 1989). Each guanylyl cyclase class is regulated by different effectors and promotes accumulation of cGMP in different cellular compartments. For example, the cytoplasmic guanylyl cyclases are activated by free radicals and nitrovasodilators, while the membrane bound class is regulated by the peptide hormone atrial naturitic

factor (Mittal and Murad, 1977; Chinkers *et al.*, 1989).

The biochemical actions of cAMP and cGMP are compared and contrasted in Fig. 1. Processes controlled by cAMP are all thought to proceed through a single molecule, PKA, while the activity of several proteins, including PKG, are modulated by cGMP. Cyclic GMP also modulates the gating of certain ion-channels and allosterically controls phosphodiesterase activity (Kaupp *et al.*, 1989; Beavo and Reifsnyder, 1990). The various functions of cGMP are particularly involved in visual transduction where cGMP-gated ion-channels, phosphodiesterases and PKG are all active (Maelicke, 1990). PKA is present at considerably higher levels in most tissues than PKG, which may explain why more events are controlled through the cAMP-signaling pathway.

## 3. THE cAMP-DEPENDENT PROTEIN KINASE

### 3.1. THE CATALYTIC SUBUNIT

Phosphorylation of substrate proteins by the catalytic subunit of PKA triggers the wide variety of physiological responses which are controlled through the cAMP signaling pathway (Fig. 1). With the exception of chemotaxis in *Dictyostelium discoidium* (Klein *et al.*, 1988) and the gating of certain ion-channels (Hockberger and Swandulla, 1987), all cAMP-responsive events require activation of PKA. The catalytic subunit (ATP: protein phosphotransferase, EC 2.7.1.37) is a monomeric enzyme of 349 amino-acids (Peters *et al.*, 1977, Shoji *et al.*, 1981). Three mammalian catalytic subunit isoforms exist, Cα, *Cβ* and Cγ have been identified (Fig. 2). Originally identified as isoelectric variants of purified protein preparations, all apparently have identical substrate specificities (Sugden *et al.*, 1976; Peters *et al.*, 1977; Bechtel *et al.*, 1977). This finding has been confirmed by molecular cloning experiments in which cDNAs encoding the catalytic subunit isoforms from a variety of species have been isolated. The catalytic subunit isoforms, Cα and C*β*, have been cloned from bovine, murine, porcine and human cDNA libraries and are 93% identical in amino-acid sequence (Uhler *et al.*, 1986a,b; Showers and Mauer 1986, Adavani *et al.*, 1987). Both Cα and C*β* are ubiquitously expressed in all tissues, although C*β* is the predominant brain isoform (Uhler *et al.*, 1986b). The third mammalian isoform, Cγ, has been recently cloned, and shown to be a testis-specific isoform. Sequence comparison suggests Cγ has diverged significantly from Cα and C*β* (Fig.2), showing only 79% and 83% identity, respectively (Beebe *et al.*, 1990). Three yeast catalytic subunit isoforms exist, TPK 1, TPK 2, and TPK 3 (Toda *et al.* 1987a). These yeast catalytic subunits apparently have overlapping substrate specificities since gene disruption experiments have shown that expression of only one TPK gene is required for cellular function (Toda *et al.*, 1987b). Catalytic subunit isoforms have also been cloned from *Drosophila, Caenorhabitis elegans* and *Aplysia*, all of which are closely related to their mammalian homologs in both size and primary structure (Foster *et al.*, 1988; Beushausen *et al.*, 1988, Gross *et al.*, 1990). Two splice variants of the *Aplysia* catalytic subunit are found (Beushausen *et al.*, 1988) and recently, a related form, sak, (Fig. 1) which is selectively expressed in the ovotestis, has been identified (Beushausen and Bayley, 1991). Whether sak is a third catalytic subunit isoform or a distinct, but related *Aplysia* kinase which regulates events specifically associated with fertilization, remains to be firmly established.

#### 3.1.1. *Catalytic Subunit Structure*

The catalytic subunit contains a region of approximately 250 amino acids, common to all protein kinases, known as the catalytic core (Hanks *et al.*, 1988). This core, which lies between residues 40 to 285, is composed of several conserved sequence motifs, representing distinct domains involved in catalysis, namely ATP binding, substrate binding and phosphoryltransfer (Fig. 3B). Post-translational modification occurs at conserved sites introducing phosphate and myristate into the catalytic subunit (Shoji *et al.*, 1981; Carr *et al.*, 1982).

#### 3.1.2. *ATP-Binding*

Before catalysis can proceed, each catalytic subunit must bind a peptide substrate and the second substrate, ATP. Both peptide and ATP bind with high affinity at separate sites within the catalytic core of the kinase. The catalytic subunit binds ATP with a $K_m$ of 7 $\mu$M (Whitehouse *et al.*, 1983) at a binding site located in the amino-terminal portion of the catalytic core (Fig. 3B). A combination of chemical modification and mutagenesis experiments, pioneered by the laboratory of Susan Taylor, have identified the ATP binding site (Taylor *et al.*, 1990). Specific labeling of lysine 72 with the ATP analog fluorosulfonylbenzoyl 5′-adenosine (FSBA) irreversibly inhibited kinase activity (Zoller and Taylor, 1979). Differential labelling of lysine residues with acetic-anhydride confirmed that the region flanking Lys 72 was also important for ATP binding (Buechler *et al.*, 1989). The involvement of lysine 72 was also confirmed by others who used a different ATP analog, *lin*-benzo ATP, to perform florescence displacement studies (Bhatangar *et al.*, 1983, 1984). These studies also suggested that cysteines 199 and 343 were also involved in ATP binding (Puri *et al.*, 1985). The catalytic cores of all protein kinases contain an invariant lysine or arginine corresponding to residue 72 and which most likely interacts with the γ-phosphate of ATP (Zoller and Taylor, 1979; Hanks *et al.*, 1988). Accordingly, replacement of these invariant basic residues by site-directed mutagenesis has become a standard strategy used to establish whether cloned proteins have protein kinase activity. For example, replacement of Lysine 295 in pp $60^{v\text{-}src}$ or Lysine 1018 of the insulin receptor with neutral amino acids produces molecules with inactive kinase cores (Snyder *et al.*, 1985; Kamps and Sefton, 1986; Chou *et al.*, 1987; Ebina *et al.*, 1987). Most protein kinases are sensitive to inhibition by certain ATP analogs, which therefore can be used as nonselective membrane-permeable inhibitors of kinase activity. For example, the iosquinolinesulfonamide derivative, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide,

($H_8$), binds to the ATP-binding site with an inhibition constant of 1.2 $\mu$M (Hagiwara *et al.*, 1986).

The catalytic subunit's ATP-binding loop is located twenty-two residues upstream from lysine 72. This region displays the consensus glycine rich sequence *Gly*–Thr–*Gly*–Ser–Phe–*Gly*–Arg–Val, (Fig. 3B) which is conserved in all protein kinases (Hanks *et al.*, 1988). The same positioning of three glycines is present in other classes of nucleotide-binding proteins, namely the dehydrogenases and GTP-binding proteins and their crystal structures provide a structural model for the protein kinase ATP-binding domain (Rossmann *et al.*, 1974; Bourne *et al.*, 1991). Work underway to solve the crystal structure of the catalytic subunit will prove or disprove this prediction (Sowadski *et al.*, 1985).

Carboxyl groups also participate in additional ATP binding. Ion-pairing of water and carboxyl groups bind $Mg^{2+}$, forming a ternary complex with the $Mg^{2+}$ ATP substrate (Granot *et al.*, 1980; Armstrong *et al.*, 1979). Chemical modification of the catalytic subunit with carbodiimides has identified several carboxyl groups involved in catalysis, and in particular ATP binding. The hydrophobic carbodiimide dicyclohexylcarbodiimide (DCCD) inhibits the catalytic subunit and presumably binds to carboxyl groups in the ATP-binding site since $Mg^{2+}$ ATP protects the kinase from DCCD inhibition (Toner-Webb and Taylor, 1987). Two reactive carboxyl groups Asp 184 and Glu 91 have been identified (Buechler and Taylor, 1988). In the absence of $Mg^{2+}$ATP, DCCD mediates the cross-linking of Asp 184 and Lys 72 suggesting both residues are in close proximity to the active site (Buechler and Taylor, 1990).

### 3.1.3. *Substrate Binding and Phosphotransfer*

The catalytic subunit recognizes peptide substrates which contain two basic amino-acids spaced by one or two residues from the target serine or threonine (Kemp *et al.*, 1976). Catalysis proceeds as a transfer of the phosphate from bound ATP, onto the deprotonated hydroxyl side chain of the target serine or threonine (Ho *et al.*, 1988; Taylor *et al.*, 1990). Kinetic analysis on the pH dependency of $V_{max}/K_m$ has suggested that an acidic side-chain with a $pK_a$ of 6.2 may be involved in the deprotonation of the target serine or threonine (Yoon and Cook, 1987). Therefore, carboxyl groups appear to participate in recognition and catalysis of peptide substrates, presumably by bonding with the basic residues of the substrate.

Chemical modifications of carboxyl groups with the hydrophilic carbodiimide, 1-ethyl-3(3-dimethylamino-propyl)-carbodiimide (EDC) has identified certain side-chains important for catalysis. Incubation with EDC irreversibly inactivates the catalytic subunit, however, unlike DCCD, preincubation with $Mg^{2+}$ATP is unable to prevent inhibition (Buechler and Taylor, 1990). Furthermore, the catalytic subunit was protected from EDC-mediated inactivation by pseudosubstrate inhibitor peptide (Buechler and Taylor, 1990). Combined, these findings suggest that EDC modifies carboxyl groups which participate in substrate binding. Two regions of protein were identified from EDC protection studies, Glu 170, and a cluster of six carboxyl groups at the carboxyl terminus (Buechler and Taylor, 1990). Apparently, the role of Glu 170 in catalysis is to interact directly with the basic side-chains of the peptide substrate (Fig. 3B). Other protein kinases which recognize and phosphorylate substrates rich in basic residues, namely PKG, PKC, phosphorylase kinase and myosin light chain kinase also contain a conserved carboxyl group corresponding to Glu 170 (Buechler and Taylor, 1990). A similar role has been proposed for Glu 332, which lies within the C-terminal acidic cluster, from recent studies of Taylor *et al.* (1990) which demonstrated that mutant recombinant catalytic subunit containing alanine in place of Glu 332 has a decreased catalytic activity. Although Glu 332 lies outside the conserved kinase catalytic core, it is thought that this carboxyl group may be important for interacting with one of the two arginines in the peptide substrates.

Additional amino acids involved in substrate recognition have been identified with peptide based affinity labels. Substrate peptides (Leu–*Arg*–*Arg*–Ala–*Ser*–Leu–Gly), where the target serine was replaced with 3-nitro-2-pyridinesulfenyl cysteine, were designed in an attempt to identify which residues on the catalytic subunit interacted with the target serine (Bramson *et al.*, 1982). These peptides irreversibly inhibited the catalytic subunit and identified Cys 199 as a component of the active site. Peptide based affinity labels where either arginine 1 or arginine 2 were replaced with $N^{\delta}$-(bromoacetyl)-ornithine were used to cross-link the catalytic subunit (Mobashery and Kaiser, 1988). Peptides modified at the arginine 1 position identified Thr 197 and Glu 346, while those modified at the arginine 2 position identified Thr 197 and Cys 199 (Mobashery and Kaiser, 1988).

Threonine 197, is an active site residue of particular interest since it is phosphorylated in the catalytic subunit (Fig. 3B) (Shoji *et al.*, 1981). Levin and *et al.* (1988) have shown phosphothreonine as necessary, not only for peptide substrate recognition, but also for interaction with the regulatory subunits of the kinase. A mutant yeast catalytic subunit (TPK 1) that was not inhibited by the regulatory subunit, contained a single nucleotide change, producing a threonine to alanine substitution at the TPK 1 position corresponding to threonine 197 (Levin *et al.*, 1988). Substitution of aspartate or glutamate at this position restored regulatory subunit interaction, suggesting that a negatively charged phosphothreonine is a determinant for binding (Levin and Zoller, 1990). The R subunits contain pseudosubstrate sequences that are competitive inhibitors of the catalytic subunit. It is likely that the R subunits recognize the active site of the kinase in a similar manner to that of substrates.

### 3.1.4. *Post-translational Modification*

The catalytic subunit undergoes two different forms of post-translational modification. The amino-terminal glycine becomes myristylated and phosphate is incorporated at two sites within the kinase. Sequence determination of the catalytic subunit identified two phosphopeptides. The phosphorylated

residues were Thr 197 and Ser 338 (Shoji *et al.*, 1979, 1983). It is still not known if the incorporation of either phosphate is the result of autophosphorylation or the action of some other protein kinase. As described in the previous section, the apparent function of phosphothreonine 197 (Fig. 3B) is as a determinant for regulatory subunit interaction (Levin and Zoller, 1990). The role of phosphoSer 338 is still uncertain. It is located close to an acidic cluster (residues 332–346) proposed to function in the recognition of the peptide substrates (Taylor *et al.*, 1990). It is unlikely that Serine 338 is an essential determinant for substrate recognition because it is not conserved between the yeast and mammalian catalytic subunits, yet both have overlapping substrate specificities (Toda *et al.*, 1987b; Zoller *et al.*, 1988).

The catalytic subunits of mammals, *Aplysia* and *Drosophila* all have a conserved amino-terminal glycine residue which serves as a substrate for *n*-myristyltransferase (Fig. 3B). This enzyme attaches the long-chain fatty acid myristic acid [$CH_3(CH2)_{12}COOH$], via an amide linkage, onto the α-amino group of the catalytic subunit (Carr *et al.*, 1982; Towler, *et al.*, 1988a). The bovine catalytic subunit was the first molecule to be identified as a myristylated protein (Carr *et al.*, 1982). Since then, several proteins involved in signal transduction, including other protein kinases, phosphatases and GTP-binding proteins are known to become myristylated (reviewed by Towler *et al.*, 1988b). Myristylation is commonly thought to promote targeting of proteins to sites on, or near, membranes. A mutant of $pp60^{v\text{-}src}$, which cannot be myristylated, is not targeted to the membrane and, as a result, is no longer oncogenic (Kamps *et al.*, 1985). In contrast, the corresponding mutation in PKA (Gly to Ala) causes no apparent changes in its catalytic activity or subcellular targeting (Clegg *et al.*, 1989). Therefore, the function of myristylation in PKA is nonessential for full biological activity. Furthermore, the recombinant catalytic subunit, expressed in *E. coli*, is active whether it is myristylated or not (Slice and Taylor, 1989; Duronio *et al.*, 1990). Possibly myristylation of the catalytic subunit, in conjunction with anchoring of the regulatory subunits, promotes correct subcellular localization of PKA. As will be discussed later, the kinase is localized to particular subcellular environments through interactions of the regulatory subunits with particular anchoring proteins (Scott *et al.*, 1990; Fraser *et al.*, 1991; Carr *et al.*, 1991).

### 3.2. The Regulatory Subunits

The primary function of the regulatory subunits (R) is to inhibit the catalytic subunit. Two R subunit classes exist, distinguished by their order of elution from DEAE-cellulose, which form the type I and II PKA holoenzymes respectively (Corbin *et al.*, 1975; Hofmann *et al.*, 1975). Detailed analysis of both R subunits, RI and RII, show that they differ in molecular weight, protein sequence, phosphorylation state, tissue distribution and subcellular localization. Clearly, the most striking difference is that RII can be phosphorylated by the catalytic subunit, whereas RI cannot (Erlichman *et al.*, 1974; Rosen and Erlichman, 1975; Hofmann *et al.*, 1975; Rangel-Aldao and Rosen 1976).

Protein sequencing and cloning studies have identified two isoforms of each R subunit class (Titani *et al.*, 1984; Clegg *et al.*, 1988; Takio *et al.*, 1982; Stein *et al.*, 1984). The predominant isoforms, RIα and RIIα (Fig. 4A), are expressed in most tissues (Lee *et al.*, 1983; Scott *et al.*, 1987), while RIβ and RIIβ are selectively expressed, primarily in central nervous system and reproductive tissues (Clegg *et al.*, 1988; Jahnsen *et al.*, 1986; Cadd and McKnight, 1989). The expression of either RII isoform is controlled in a tissue specific manner (Øyen *et al.*, 1988; Cadd and McKnight, 1989). For example, RIIα expression in the testis is developmentally regulated (Øyen *et al.*, 1989), while RIIβ expression in granulosa cells is stimulated by follicle stimulating hormone (Ratoosh *et al.*, 1987). Separation of the regulatory subunits by ion-exchange chromatography has been used by several investigators to show that individual hormones preferentially activate either the type I or the type II PKA holoenzyme (reviewed by Harper *et al.*, 1985). For example, type I PKA is preferentially activated by glucagon in hepatocytes (Schwoch, 1978, Byus *et al.*, 1979) and by corticotropin-releasing factor, isoproterenol, or forskolin in AtT20 cells (Litvin *et al.*, 1984). Type II PKA is activated by human gonadotrophic hormone in ovarian follicles, but the same hormone activates type I PKA in the corpus luteum (Hunzicker-Dunn, 1981). In osteoblasts, parathyroid hormone activates types I PKA whereas prostaglandin E2 ($PGE_2$) activates type II PKA (Livesey *et al.*, 1982).

Homologs of RIα, classified by their lack of an autophosphorylation site, have been identified in lower eukaryotes and cloned from *Drosophila*, *Dictyostelium discoideum* and *Caenorhabiditis elegans*. These molecules contain conserved structural features, when compared to their mammalian counterparts (Mutzel *et al.*, 1987; Lu *et al.*, 1990). Up to five regulatory subunits have been identified in *Aplysia* (Eppler *et al.*, 1986), of which one form, an RI homolog, has been cloned (H. Bayley, personal communication). So far, the only RII homolog found in lower organisms has been cloned from yeast (Toda *et al.*, 1987b). This molecule, termed BCY 1, contains a fifty amino-acid extension at the amino terminus, in comparison to mammalian RIIα, but undergoes autophosphorylation by either the yeast or mammalian catalytic subunits (J. D. Scott, unpublished observations).

The domain structure of the regulatory subunits is summarized in Fig. 4B; they are highly asymmetric proteins (Erlichman *et al.*, 1974) and composed of a linear array of well-defined and functionally distinct subdomains, responsible for dimerization, subcellular localization, inhibition of the catalytic subunit and cAMP binding (Fig. 4B). The amino-terminal third of each regulatory subunit contains sites which participate in protein–protein interactions while the carboxyl-terminal two-thirds represent two tandem repeated sequences, which have arisen as a result of gene-duplication, and fold to form the cAMP binding domains (Weber *et al.*, 1982; Titani *et al.*, 1984).

### 3.2.1. *R Subunit Dimerization*

The PKA holoenzyme is a tetramer consisting of two catalytic subunits and a regulatory subunit dimer (Fig. 3A). The regulatory subunit dimer is a stable complex which remains intact after release of the catalytic subunits. The only regulatory subunit which does not form dimers is that found in *Dictyostelium discoideum* and contains a deletion in the amino-terminus (Mutzel *et al.*, 1987). In RI, both protomers are orientated in an antiparallel manner and covalently linked through interchain disulfide bonds formed between Cys 16 and Cys 37 (Zick and Taylor, 1982; Bubis *et al.*, 1987, 1988). Dimerization of RII does not involve cysteines, but has been shown by limited proteolysis to occur between the first 45 residues of each protomer (Potter *et al.*, 1978; Weber and Hilz, 1979, Reimann, 1986). Expression of recombinant RIIα truncated at the amino-terminus revealed that deletion of the first 14 residues abolishes dimerization (Cupp *et al.*, 1989; Scott *et al.*, 1990). Furthermore, dimerization could be conferred on carrier proteins if expressed as chimeras containing just the first 30 amino acids of murine RIIα (Scott *et al.*, 1990). These results suggest that residues 1–30 represent an independent RIIα dimerization subdomain, functional when removed from the rest of the molecule (Fig. 4B). Secondary structure predictions imply that residues 11–23 of RIIα can form a β-sheet. Crystallographic analysis proposes that β-sheets often participate in protein–protein interactions. Residues 1–30 of are highly conserved in all known RII sequences (Scott *et al.*, 1987; Øyen *et al.*, 1989). Since conserved structure often represents conserved function, it is likely that this region represents almost the complete dimerization domain.

### 3.2.2. *Subcellular Localization*

Subcellular localization of the PKA is directed through the R subunit (Sarkar *et al.*, 1984). Although both RI isoforms are primarily cytoplasmic, certain tissues contain up to 75% of either RII isoform in particulate form, associated with either the plasma membrane, cytoskeletal components, secretory granules, or the nuclear membranes (Rubin *et al.*, 1972; Nigg *et al.*, 1985; Salvatori *et al.*, 1990; Joachim and Schwoch, 1990). Type II kinase localization is dictated through association of RII with specific anchoring-proteins (Rubin *et al.*, 1979; Leiser *et al.*, 1986, Sarker *et al.*, 1984). Tissue-specific patterns of RII-anchoring proteins have been detected by protein-blotting techniques or fractionation with RII-sepharose affinity columns (Lohmann *et al.*, 1984, Carr *et al.*, 1991). Presumably, tissue-specific kinase function is directed through unique complements of RII-anchoring proteins and localization influences which substrates are most accessible to the catalytic subunit upon activation. Consistent with this theory, it has been noted that a significant subset of RII-anchoring proteins are PKA substrates themselves (Theurkauf and Vallee, 1982; Lohmann *et al.*, 1984). Recently two novel RII anchoring proteins have been cloned and are PKA substrates *in vitro*. Both molecules, Ht 21 and Ht 31, contain clusters of consensus phosphorylation sites within a twenty residue region of sequence (Fraser *et al.*, 1991; Fraser and Scott, manuscript in preparation). Phosphorylation may unmask activities residing within each anchoring-protein and may be the mechanism which triggers individual cAMP-mediated responses.

RII-anchoring proteins are likely to play a central role in cAMP-mediated signal transduction. So far little is known about their structure, subcellular location, or tissue distribution and only three RII-anchoring proteins have been characterized in any detail. Cytoskeletal attachment of PKA occurs through interactions between RII and microtubule-associated protein 2 (MAP 2) (Theurkauf and Vallee, 1982). The site on MAP 2 that contacts RII has been localized to a 31-residue peptide in the amino-terminal regions of the molecule (Rubino *et al.*, 1989; Obar *et al.*, 1989). Complementary studies have defined the site on RII responsible for MAP 2-anchoring (Scott *et al.*, 1990; Luo *et al.*, 1990). The MAP 2 binding site is contained within the first 79 residues and requires a dimeric RII protein, since disruption of dimerization destroys anchoring (Cupp *et al.*, 1989; Scott *et al.*, 1990). The topology of the anchored type II kinase (Fig. 5) is such that the catalytic subunits are optimally positioned to phosphorylate the MAP 2. Accordingly, MAP 2 is phosphorylated up to 11 times by PKA and furthermore, most RII-anchoring proteins are substrates for kinase (Theurkauf and Vallee, 1982; Lohmann *et al.*, 1984). Phosphorylation of RII-anchoring proteins by PKA may unmask other biological activities residing within the molecules.

Other RII-anchoring proteins such as the brain protein p75 also bind through the same or overlapping site as MAP 2 (Scott *et al.*, 1990; Luo *et al.*, 1990). Several p75 analogs, ranging in size from $M_r$ 60,000 to 150,000, have been reported in different species and may represent members of a family of structurally related RII-anchoring proteins (Bergman *et al.*, 1989, 1991). Several studies have shown that the p150/75 family is expressed specifically in neural and neuroendocrine tissues and preferentially associates with the RIIβ isoform (Sarkan *et al.*, 1984). RII also associates with the calmodulin-dependent phosphatase calcinurin (Hathaway *et al.*, 1981). Because proteins in the p150/75 family and calcinurin associate with both PKA and calmodulin, these RII-binding proteins appear to function in both cAMP and $Ca^{2+}$-mediated transduction pathways.

### 3.2.3. *Kinase Inhibition (Pseudosubstrate Hypothesis)*

The primary function of the regulatory subunit is inhibition of the catalytic subunit. In the absence of cAMP, the regulatory subunits competitively inhibit the catalytic subunit with nanomolar affinities (Hofman, 1980; Builder *et al.*, 1980). The 'hinge-region' is a proteinase-sensitive region between residues 90 and 100 on the regulatory subunit (Fig. 4B) which is the primary, but not the only site for contact with the catalytic subunit (Takio *et al.*, 1984a; Titani *et al.*, 1984). The involvement of pseudosubstrate sequences within the hinge-region, for inhibition of the catalytic subunit has been established by protein chemistry and molecular biology techniques (Fig. 6). Several other protein kinases including PKG, protein

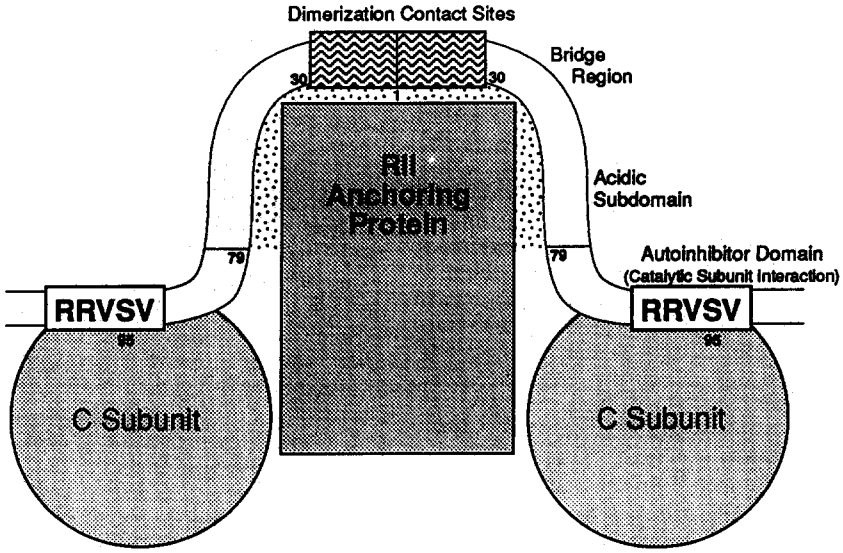


FIG. 5. The topology of the anchored type II PKA holoenzyme. Reprinted from Scott *et al.*, 1990, with permission of the copyright holder, the American Society of Biological Chemists, Inc., Bethesda.

kinase C, the smooth and skeletal muscle myosin light chain kinases and the $Ca^{2+}$/calmodulin kinase II are inhibited by pseudosubstrate sequences (House and Kemp, 1987; Olson *et al.*, 1990; Kennelly *et al.*, 1987; Payne *et al.*, 1988; Colbran *et al.*, 1988). Therefore, pseudosubstrate inhibition is a general mechanism for kinase regulation (reviewed by Soderling, 1990). Furthermore, inhibition by 'substrate-like' sequences may be the mode of regulation for the tyrosine kinase $pp60^{c\text{-}src}$ (Grandori, 1989).

The free regulatory subunit, when saturated with cAMP, is particularly sensitive to trypsin cleavage at the hinge region (Weber and Hilz, 1979; Potter *et al.*, 1978). Corbin *et al.* (1978) destroyed catalytic subunit interaction by modifying RII with the arginine-specific reagent, 2, 3-butanedione and were the first to show that arginine residues were determinants for kinase inhibition. Corbin proposed that arginines were components of 'pseudosubstrate sites' which bound at the active site of the kinase since the regulatory subunits are competitive inhibitors of the catalytic subunit (Corbin *et al.*, 1978). It was later established that hinge-region sequences do resemble consensus substrate sites for the catalytic subunit (Takio *et al.*, 1984a; Titani *et al.*, 1984). The type I R subunit contains a true pseudosubstrate site, *Arg–Arg*–Asn–*Ala/Gly*–Ile where the target serine is replaced with alanine or glycine. RIα and RIβ, contain alanine and glycine as their pseudosubstrate residues respectively (Fig. 6) and differ from each other in the concentrations of cAMP required to distrupt their interaction with the catalytic subunit (Clegg *et al.*, 1987; Cadd *et al.*, 1990). The type II R subunit contains an autophosphorylation site of sequence *Arg–Arg*–Val–*Ser*–Val (Scott *et al.*, 1987; Lee *et al.*, 1983; Clegg *et al.*, 1988). Mutation of the autophosphorylation site, serine 145, in the yeast RII homolog, BCY 1, has marked effects upon catalytic subunit binding affinity (Kuret *et al.*, 1988). Replacement of serine 145 with alanine or glycine, created regulatory subunits with 2–10-fold higher affinities for the catalytic subunit while substitution with glutamic acid or lysine decreased inhibitory potency 2–5-fold (Kuret *et al.*, 1988). Additional regions of the regulatory subunits, other than the pseudosubstrate site, are also required to bind the catalytic subunit. It has been shown recently, that mutation of arginines 92 or 93 abolishes kinase inhibition, but creates a regulatory subunit which is still able to associate with the catalytic subunit in a cAMP-dependent manner (Wang *et al.*, 1991).

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PKI | S | **G** | **R** | T | G | **R** | **R** | N | | **I** | H | D | I | L |
| RIα | K | **G** | **R** | R | - | **R** | **R** | G | | **I** | S(P) | **A** | **E** | V |
| RIβ | K | A | **R** | R | - | **R** | **R** | G | | V | S(P) | **A** | **E** | V |
| RIIα | P | **G** | **R** | F | D | **R** | **R** | V | (P) | V | C | **A** | **E** | T |
| RIIβ | I | N | **R** | F | T | **R** | **R** | A | (P) | V | C | **A** | **E** | A |
| PKGα | G | P | **R** | T | T(P) | **R** | A | Q | | **I** | S | **A** | **E** | P |
| PKGβ | E | P | **R** | - | T | **K** | **R** | Q | | **I** | S | **A** | **E** | P |

FIG. 6. The Pseudosubstrate and Autoinhibitor sequences in PKA and PKG regulatory units. This diagram was composed from published sequences of PKA and PKG regulatory units, PKI (Scott *et al.*, 1985c), RIα (Titani *et al.*, 1984), RIβ (Clegg *et al.*, 1987), RIIα (Takio *et al.*, 1984a), RII β (Stein and Rubin, 1985), PKGα (Takio *et al.*, 1984b) and PKGβ (Wernet *et al.*, 1989). Highly conserved residues essential for kinase inhibition are boxed while the shaded area represents the pseudosubstrate or autoinhibitor molecule.

### 3.2.4. *PKI*

In addition to the regulatory subunits, the catalytic subunit can be inhibited by a heat-stable protein kinase inhibitor (PKI) (Walsh *et al.*, 1971). Multiple forms of PKI exist and at least three isoforms have been identified from rabbit skeletal muscle (Ferraz *et al.*, 1979; McPherson *et al.*, 1979; Whitehouse and Walsh, 1982). The inhibitors from rabbit skeletal muscle are proteins of about 75 residues which irreversibly bind the catalytic subunit with high

affinity ($K_i$ 1–0.2 nM), in the presence of $Mg^{2+}$ATP (Scott *et al.*, 1985a,b; Whitehouse *et al.*, 1983). Sequence analysis and peptide studies have localized the inhibitory site on PKI to the first 24 amino acids (Fig. 6), which includes a pseudosubstrate site (Scott *et al.*, 1985a,b,c, 1986; Cheng *et al.*, 1985, 1986). Recently, a cDNA encoding a 70 residue rat testis PKI isoform has been cloned and characterized (Van Patten *et al.*, 1991). This molecule contains the conserved pseudosubstrate sequence, but the remainder of the molecule is only 38% identical to the rabbit skeletal form (Scott *et al.*, 1985c).

Detailed kinetic analysis of PKI peptide analogs have defined the structural parameters governing kinase inhibition (Scott *et al.*, 1985a, 1986; Cheng *et al.*, 1985, 1986; Glass *et al.*, 1989a,b; Reed *et al.*, 1987, 1989; Katz *et al.*, 1989). The pseudosubstrate site is located between residues 18 and 22 (Fig. 6) and requires arginines 18 and 19 for full activity (Scott *et al.*, 1985a,b,c, 1986; Cheng *et al.*, 1985, 1986; Van Patten *et al.*, 1991). Additional contact with the catalytic subunit is provided by residues 5–12 (Glass *et al.*, 1989a,b). Conformational analysis of PKI peptides by circular dichromism (CD) suggests that residues 5–12 form an α-helix (Reed *et al.*, 1987, 1989). PKI peptides are valuable tools for inhibiting the kinase in crude extracts because of their high specificity for the catalytic subunit. This approach has been extended by the groups of Avruch and Mauer who both have synthesized mini genes encoding PKI, the peptide product of which blocks cAMP-responsive transcription of neuropeptide genes (Grove *et al.*, 1987, 1989; Day *et al.*, 1989). PKI peptides can block PKG activity *in vitro*, although with a potency four orders of magnitude lower than for PKA (Glass *et al.*, 1986).

### 3.2.5. *R Subunit Phosphorylation*

Both classes of regulatory subunit are phosphorylated by protein kinases other than the catalytic subunit, although the functional significance of these events is unclear. In the presence of cAMP, a single molecule of phosphate is incorporated into RIα by PKG (Geahlan and Krebs, 1980a,b). However, in the absence of cAMP, RIα is not phosphorylated, but instead competitively inhibits PKG with a $K_i$ of 250 nM (Geahlan and Krebs, 1980b). Phosphorylation occurs close to the pseudosubstrate site of RIα (Fig. 6), lowering the inhibitory potency for the catalytic subunit and decreasing the number of available cAMP binding sites (Geahlan *et al.*, 1981; Hashimoto *et al.*, 1981). Whether or not any of these effects occur *in vivo* is unclear, since attempts to demonstrate RI phosphorylation by PKG in rat soleus muscle were negative (Geahlan *et al.*, 1981).

Incubation of the type II regulatory subunit with the catalytic subunit promotes phosphorylation of serine 95. This lowers the affinity of RII for the catalytic subunit and decreases the rate of holoenzyme reassociation (Rangel-Aldao and Rosen, 1976). Each RII protomer, isolated in the absence of catalytic subunit, contains up to 2 molecules of phosphate which are incorporated at other sites within the protein (Rymond and Hoffmann, 1982). A detailed study by Hemmings *et al.* (1982), identified four potential phosphorylation sites, located between residues 44 and 76. All four sites can become phosphorylated by the synergistic action of two kinases. Glycogen synthase kinase 3 (GSK 3) can phosphorylate serines 44 and 47, but only after serines 74 and 76 have been phosphorylated by casein kinase II (CKII) (Hemmings *et al.*, 1982; Roach, 1990). The synergistic action of protein kinases, like CK II and GSK 3, permits the rapid introduction of multiple phosphates, clustered at a particular site, into a substrate molecule (reviewed by Roach, 1990).

### 3.2.6. *cAMP Binding and Holoenzyme Activation*

Each regulatory subunit monomer contains two high-affinity binding sites for cAMP which are located downstream from the dimerization and pseudosubstrate regions (Fig. 4B). When both sites are occupied, the regulatory subunit adopts a conformation with lower affinity for the catalytic subunit (Rannels and Corbin, 1979, Titani *et al.*, 1984; Takio *et al.*, 1984a). The cAMP binding sites, termed sites A and B, share considerable sequence identity and have arisen as a result of tandem gene duplication (Rannels and Corbin, 1979; Weber *et al.*, 1982; Titani *et al.*, 1984; Takio *et al.*, 1984a). Both sites have different cAMP exchange rates, specificities for cAMP analogs and sensitivities to affinity labeling with azido-cAMP compounds (reviewed by Weber *et al.*, 1979; Døskeland and Øgreid, 1981; Beebe and Corbin, 1986; Taylor *et al.*, 1990). Site A, the more amino-terminal site, shows relatively fast dissociation of cAMP and a preference for N-6 or C-6-substituted cAMP analogs. In contrast, site B exhibits slower cAMP dissociation kinetics and preference for C-8-substituted analogs (Weber *et al.*, 1979; Rannels and Corbin, 1980a,b; Øgreid *et al.*, 1985; Beebe *et al.*, 1984). Sites A and B act synergistically during PKA activation and exhibit a strong positive cooperativity with Hill coefficients of 1.4–1.6 (Døskeland and Øgeid, 1984). There are differences in the cAMP analog preferences of RI and RII and selective activation of type I kinase is obtained with certain combinations of cAMP analogs, while type II kinase activation is obtained with other combinations (Robinson-Steiner and Corbin, 1982; Øgreid *et al.*, 1985).

The cyclic nucleotide binding domains of all PKA and PKG isoforms show extensive sequence similarity to the cAMP-binding site of catabolite repressor protein (CAP), a DNA-binding protein from *E. coli* (Weber *et al.*, 1982). Weber *et al.* (1987) have built a model for the cAMP-binding sites of the R subunits by fitting their sequences into the crystallographic coordinates of CAP (Fig. 7). The cAMP-binding domain is a β-barrel structure composed of eight β-strands (Fig. 7). Residues conserved between CAP and the regulatory subunits are located at sites involved in the binding of the cyclic phosphate ring and the ribose moiety of cAMP (Fig. 7). Three residues are invariant in all cAMP-binding domains, Gly 323 (numbering is based on RIα) which defines the end of the β-strand, Glu 324 which interacts with the ribose moiety of the nucleotide and Arg 333 which is thought to interact with the cyclic phosphate ring (Fig. 7). The involvement of these residues in cAMP

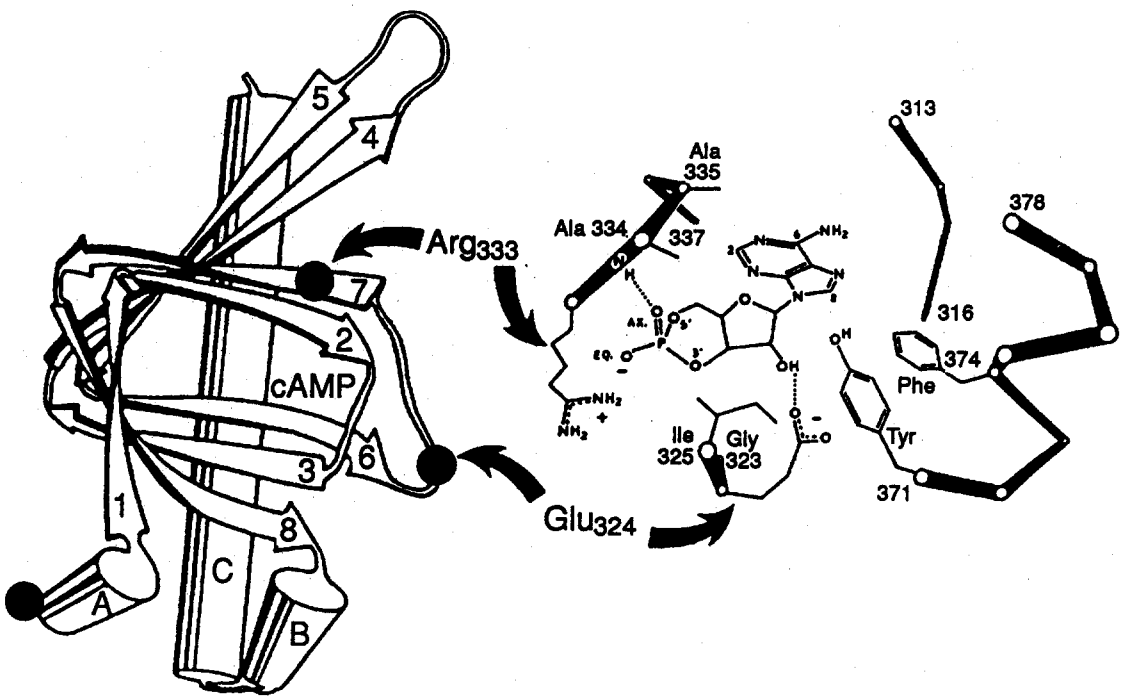


FIG. 7. A model for the cAMP-binding site (Site B) of PKA, built from the crystallographic coordinates of the *E. coli* catabolite repressor protein, CAP (Weber *et al.*, 1982). The invariant residues required for direct interaction with the cyclic nucleotide are indicated.

binding and kinase activation has also been demonstrated through an independent line of investigation, namely, functional and genetic analysis of cAMP-resistant type I regulatory subunits (Murphy and Steinberg, 1985; Steinberg *et al.*, 1987a,b). Sequence analysis of mutants, isolated from mutagenized S49 lymphoma cells, identified single amino acid substitutions in either site A or B which corresponded to the Gly 323, Glu 324 or Arg 333 (Steinberg *et al.*, 1987b; Øgreid *et al.*, 1988; Houge *et al.*, 1990; Clegg *et al.*, 1987). Point mutations in RI, introduced by site-directed mutagenesis, at Gly 200 (Site A), Glu 324 and Arg 333 (Site B) have confirmed that these residues are required for normal cAMP binding (Woodford *et al.*, 1989; Correl *et al.*, 1989; Ringheim and Taylor, 1990; Houge *et al.*, 1990; Steinberg *et al.*, 1991). Mutational inactivation of site B has more effect on kinase activation than mutation of site A, as mutation of Gly 324 completely blocks cAMP binding (Clegg *et al.*, 1987; Correll *et al.*, 1989). Furthermore, deletion of site B abolishes cooperativity, and creates a regulatory subunit which activates the kinase at high concentrations of cAMP (Øgreid and Døskeland, 1981; Bubis *et al.*, 1987).

Although the PKA holoenzyme can bind up to 4 cAMP molecules at saturation, the exact number required for activation of the catalytic subunit is still in question. Upon binding a single cAMP, the regulatory subunits undergo conformational changes which cause 'charge-shifts' in the holoenzyme (Cobb *et al.*, 1987; Wolfe *et al.*, 1990). As a result, the 'charge-shifted' form has a different elution pattern on DEAE resins and has been isolated as a ternary complex where 50% of the cAMP binding sites are saturated (Cobb *et al.*, 1987; Wolfe *et al.*, 1990). The distribution of cAMP is equal between sites A and B (Wolfe *et al.*, 1990). This holoenzyme form has a higher Hill coefficient for cAMP and is now 'primed' for activation (Wolfe *et al.*, 1990). A model for PKA activation is presented in Fig. 8A and is compared with a similar model for PKG. PKA activation occurs in two phases (Fig. 8A). A single molecule of cAMP binds to either site A or B in each regulatory subunit, causing conformational rearrangement of the inactive holoenzyme, priming it for activation. This creates a ternary complex with a heightened sensitivity for cAMP. Upon binding of additional cAMP to the remaining binding sites, other conformational changes occur, which are transduced to the pseudosubstrate site causing a release of the active catalytic subunit (Fig. 8A).

### 3.3. PKA FUNCTION

The sole function of the catalytic subunit is to catalyze the transfer of phosphate from the $\gamma$-position of ATP onto a target serine or threonine recognized in the substrate. Phosphorylation of target proteins alters their activity, which in turn, promotes changes in cellular function and metabolism. Furthermore, protein phosphorylation is a reversible process, with the dephosphorylation step catalyzed by the phosphoprotein phosphatases (reviewed by Fischer, 1983; Cohen and Cohen, 1989). Glycogen phosphorylase was the first enzyme whose activity was shown to be altered as a consequence of reversible phosphorylation (Fischer and Krebs, 1955). Later these investigators showed that this reaction was catalyzed by phosphorylase kinase and required the intracellular messenger cAMP (Krebs *et al.*, 1959). Ten years later,

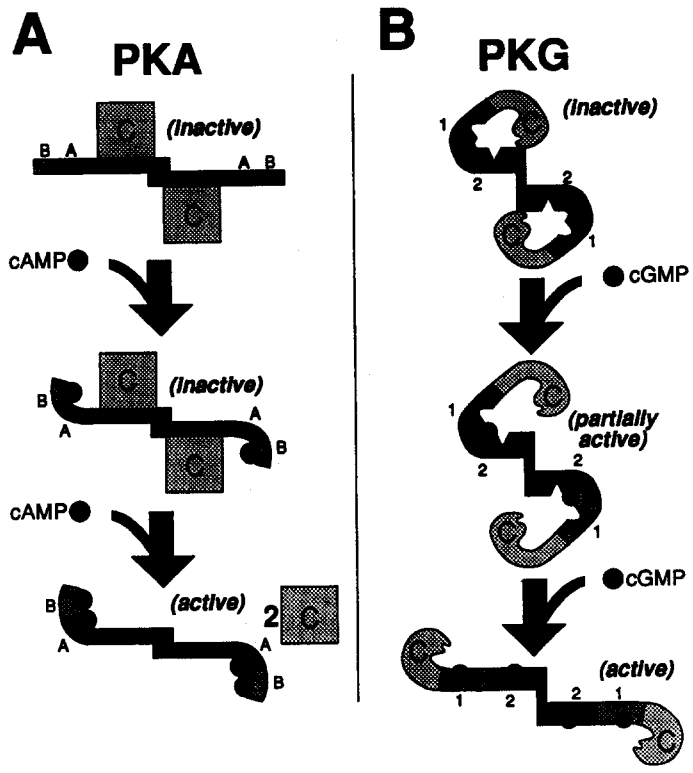


FIG. 8. The mechanisms of PKA and PKG activation. The modes of activation for PKA (A) and PKG (B) are compared. The mechanistic details for activation of either kinase are presented in the text.

the enzyme responsible for the phosphorylation of phosphorylase kinase was purified and shown to be a cAMP-dependent protein kinase (Walsh *et al.*, 1968; Kuo and Greengard, 1969). At this time it was recognized that this cAMP-dependent protein kinase had a relatively broad substrate specificity and was likely the mediator of all the functions performed by cAMP. Since then, the number of molecules identified as undergoing phosphorylation by PKA, has increased dramatically. With the advent of molecular cloning, the number of published protein sequences are increasing, allowing for computer aided searches for consensus 'PKA phosphorylation sites' to be routinely preformed. These strategies have identified many new proteins which might be phosphorylated by PKA *in vitro*, but it should be emphasized that many of them do not satisfy the criteria developed by Krebs and colleagues (Krebs, 1985; Krebs and Beavo, 1979; Krebs *et al.*, 1985) that establish the physiological significance of a given phosphorylation event. These criteria are listed below:

(1) Phosphorylations by PKA occur at substrate sites on the surface of target proteins where a pair of basic residues precede the target serine or threonine by one or two residues (*Lys/Arg–Lys/Arg–Xaa–Xaa–Ser/Thr–Xaa*).
(2) The $K_m$ value for phosphorylation must lie within the range of physiological concentrations reported for the substrate protein.
(3) Measurable changes in the biological activity of the substrate protein must accompany phosphorylation.
(4) Phosphorylation occurs *in vivo*, and in response to agents which elevate intracellular cAMP.
(5) Phosphorylation is reversible and, phosphate can be removed by the phosphoprotein phosphatases.

### 3.3.1. *Substrate Specificity*

By the mid-1970s the number of proteins phosphorylated by pKA was large enough to identify consensus phosphorylation sites. Ljungstrom *et al.* (1974) showed that the purified catalytic subunit phosphorylated native or denatured liver type pyruvate kinase at the sequence Leu–Arg–Arg–Ala–*Ser*–Val–Ala (Humble *et al.*, 1975). Around the same time Bylund and Krebs (1975) showed that denaturation of chicken egg lysozyme exposed two phosphorylation sites. Tryptic digestion of the protein at one site yielded the sequence Arg–Tyr–*Ser*–Leu–Gly. A consensus heptapeptide, Leu–Arg–Arg–Ala–*Ser*–Leu–Gly, derived from both of these sequences was synthesized by Kemp *et al.*, (1976). This peptide, known as Kemptide, now serves as a universal substrate for the catalytic subunit with a $K_m$ of 4.7 $\mu$M and a $V_{max}$ of 15.8 $\mu$ mol/min/mg (Whitehouse *et al.*, 1983). It should be noted that several other protein kinases, including the PKG and histone, myelin basic protein and S6 kinase can also use Kemptide as a substrate (reviewed by Glass *et al.*, 1981; Kemp and Pearson, 1990).

Kemptide analogs have been used to demonstrate that two basic residues, preferably arginine, are

essential determinants for substrate recognition (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1977). Furthermore, a single residue, of any type other than acidic amino acids, is the optimal spacing between the basic subset and the target serine or threonine (Feramisco *et al.*, 1980). A pair of lysine residues can replace arginines as suitable substrate determinants but are often spaced two residues away from the target serine or threonine (Yeaman *et al.*, 1977). A third arginine, six residues upstream of the target serine or threonine is often found in substrate proteins (Zetterqvist and Ragnarsson, 1982). The primary structure of many protein substrates for the catalytic subunit has been analyzed and three consensus substrate sites have been proposed (these are presented below).

(1) *Arg*–*Arg*–Xaa–*Ser*–Xaa

(2) *Lys*–*Arg*–Xaa–Xaa–*Ser*–Xaa

(3) *Arg*–Xaa–Xaa–*Arg*–*Arg*–Xaa–*Ser*–Xaa.

While primary structure is a major determinant for PKA substrates, the conformation of the peptide backbone is also important. Nuclear magnetic resonance (NMR) and kinetic analysis of the catalytic subunit phosphorylation of Kemtide analogs have ruled out the possibility of $\alpha$ helix or $\beta$ sheet and suggest a random coil structure is adopted by the substrate peptide (Granot *et al.*, 1980; reviewed by Kemp *et al.*, 1979; Mildvan *et al.*, 1983; Taylor *et al.*, 1990). Subsequent NMR studies by the same group with Kemptide and *N*-methylated analogs proposed that the peptide substrate binds to the active site with a particular extended coil structure (Rosevear *et al.*, 1984) which facilitates hydrogen bonding between the extended peptide backbone and active site resides in catalytic subunit (Bramson *et al.*, 1987; Thomas *et al.*, 1987). Circular dichromism (CD), studies of phosphoKemptide analogs suggest that the extended coil structure of the substrate is lost upon its phosphorylation (Hider *et al.*, 1985). Substrate binding also causes marked changes in the structure of the catalytic subunit. Near- and far-ultraviolet CD studies show that Kemptide binding promotes a two step change in catalytic subunit structure, causing a net decrease in $\alpha$ helix and an increase $\beta$ sheet (Reed and Kinzel, 1984 a,b, Reed *et al.*, 1985).

### 3.3.2. *cAMP-Responsive Transcriptional Activation*

The number of cellular processes controlled by PKA phosphorylation is many and varied, primarily because of the enzyme's broad substrate specificity. Several excellent reviews deal with specific cAMP-mediated cellular events (Krebs and Beavo, 1979; Mokuno *et al.*, 1988; Bidey *et al.*, 1988). This article will focus only on the recent advances made in understanding the mechanism of cAMP-responsive gene activation (reviewed by Roesler *et al.*, 1988; Goodman, 1990). Early studies on the regulation of neuropeptide gene expression focused on cAMP and showed that somatostatin mRNA levels increased in primary diencephalic cultures after exposure to forskolin, a potent activator of adenylyl cyclase (Montminy *et al.*, 1986a). The DNA element responsible for cAMP-regulated expression was characterized by deletion analysis of a somatostatin-chloramphenocol acetyl transferase (CAT) reporter gene construct (Montminy *et al.*, 1986b). These studies identified a DNA sequence, between 29 and 60 base pairs upstream from the transcriptional start site, which could confer 'cAMP-responsiveness' when ligated onto a heterologous promoter. This sequence was designated the 'cAMP-responsive element' or CRE (Montminy *et al.*, 1986b). The somatostatin CRE contains an 8 base palindrome, 5′-TGACGTCA-3′, and is conserved in many other genes regulated by cAMP (reviewed by Goodman, 1990). Using the somatostatin CRE as an affinity ligand, a binding protein, CRE-binding protein (CREB), was purified and shown to be a substrate for PKA (Montminy and Bilezikjian, 1987). Characterization of cDNA's encoding CREB provided its primary structure and identified consensus phosphorylation sites for PKA, protein kinase C, and casein kinase II (Hoeffler *et al.*, 1988; Gonzalez *et al.*, 1989). Phosphorylation of CREB appears to be important for somatostatin gene regulation *in vitro* as Yamamoto *et al.* (1988) showed the catalytic subunit stimulates the level of transcription up to 20-fold. In related experiments, Mauer (1989) demonstrated that overexpression of either catalytic subunit isoform (C$\alpha$ or C$\beta$) stimulates transcription of the cAMP-responsive prolactin gene, while transfection of a mini gene which encodes a peptide inhibitor of the kinase decreased transcription (Day *et al.*, 1989).

While direct evidence for the phosphorylation of CREB *in vivo* by the catalytic subunit has yet to be established, several lines of evidence suggest the type II PKA is specifically required for activation of somatostatin transcription. A mutant pheochromocytoma, cell line PC 12 IB-126, which expresses diminished amounts of RII, is unable to activate a cAMP-responsive somatostatin reporter gene (Montminy *et al.*, 1986b). Cyclic AMP-responsive transcription of the somatostatin reporter gene is restored, only when RII$\beta$ is introduced into PC 12 IB-126 cells (Tortora and Cho-Chung, 1990). The exact role of type II PKA in transcriptional activation is still unclear. One early postulate is that RII alone regulated CREB by a mechanism independent of kinase activity, however, this has been discounted in the light of conclusive evidence, from several groups, that the active catalytic subunit is absolutely required for cAMP-responsive transcription (Riabowol *et al.* 1988; Buchler *et al.*, 1988; Mauer, 1989; Mellon *et al.*, 1989). Possibly RII, which can compartmentalize the type II PKA through interactions with anchoring proteins could tether the enzyme close to the sites of transcription, i.e. the nucleus (Sarkar *et al.*, 1984; Scott *et al.*, 1990; Luo *et al.*, 1990). Upon the appropriate external signal, elevated, cAMP will release the catalytic subunit from an anchored type II holoenzyme, which then is translocated into the nucleus. Quite recently nuclear translocation of the catalytic subunit has been demonstrated (Meinkoth *et al.*, 1990), in which, a fluorescent catalytic subunit was microinjected into the cytoplasm and translocated to the nucleus within 10 min, while the regulatory subunits did not translocate in similar experiments (Meinkoth *et al.*, 1990).

## 4. cGMP-DEPENDENT PROTEIN KINASE

The structural organization and catalytic properties of the cGMP-dependent protein kinase (PKG) are similar to the cAMP-dependent protein kinase (Lincoln and Corbin, 1977). The level of PKG is relatively low in most tissues with the exception of lung, cerebellum and smooth muscle (Lincoln and Corbin, 1983). Although PKG has a relatively broad substrate specificity (Glass *et al.*, 1981), its restricted tissue distribution suggests it regulates fewer biological processes than PKA. Several lines of evidence suggest that the enzyme mediates the relaxation of smooth muscle, inhibition of platelet aggregation and certain hemodynamic effects (reviewed by Lincoln, 1989, Walter, 1989). These biological effects occur in response to agents that elevate cGMP such as atrial naturietic peptide, nitrovasodilators and endothelial relaxing factor (Gruetter *et al.*, 1979; Francis *et al.*, 1989; Corbin *et al.*, 1990).

The cGMP-dependent protein kinase was originally identified in various species of Arthropods (Kuo and Greenguard, 1970). Since then, homologs have been identified in *Drosophila*, silk worms, the protozoan *Paramecium* and several mammalian species (Inoue *et al.*, 1976; Migiletta and Nelson, 1988; Kalderon and Rubin, 1989). Most of the properties of PKG have been described from studies of the bovine lung enzyme which has been sequenced and is a molecule of 670 amino acids with a subunit molecular weight of 76,331 (Takio *et al.*, 1984b). Recently two isoforms of PKG, designated types Iα and Iβ, have been identified which have different amino-terminal domains (Fig. 4A) and may have arisen as a result of alternate splicing from a single gene (Lincoln *et al.*, 1988; Wolfe *et al.*, 1989a,b; Francis *et al.*, 1989). Two PKG isoforms have also been identified and cloned from *Drosophila* and show considerable sequence homology to the bovine isoforms (Kalderon and Rubin, 1989; Foster *et al.*, 1988). These *Drosophila* cDNAs were used as probes to clone the mammalian PKG isoforms and sequence analysis has shown that the type Iα and type Iβ diverge, primarily in the first 105 aminoacids (Wernet *et al.*, 1989; Sandberg *et al.*, 1989).

The structural organization of PKG is similar, but not identical, to PKA (Fig. 3A and 4B) and contains segments with strong homology to both the catalytic and regulatory subunits of PKA (Hashimoto *et al.*, 1982; Takio *et al.*, 1983, 1984b). The major difference between these kinases is that PKG is a dimer composed of two identical subunits, that remain associated upon activation by cGMP (Gill *et al.*, 1977; Lincoln *et al.*, 1978). One interpretation of these findings is that PKG is a 'chimeric kinase' which has evolved as a result of convergent gene splicing to incorporate both the regulatory and catalytic domains into the same polypeptide chain (Takio *et al.* 1984b). The following sections will discuss PKG structure and function with particular emphasis on the similarities and differences with PKA.

### 4.1. PKG Structure

PKG was first discovered by Kuo and Greenguard (1970) and later shown to be a dimeric cGMP-binding protein of $M_r$ 150,000, consisting of two identical subunits ($M_r$ 75,000), which could undergo autophosphorylation (Lincoln *et al.*, 1976; de Jonge and Rosen, 1977; Gill *et al.*, 1977; Lincoln *et al.*, 1978). The chimeric structure of PKG was initially identified by a combination of functional studies and later confirmed by sequencing of the protein. Limited proteolysis of the silk worm PKG activated the kinase and demonstrated that the enzyme could be fragmented into a $M_r$ 34,000 catalytic unit and a $M_r$ 36,000 cGMP binding domain (Inoue *et al.*, 1976). Concomitantly, it was noted that PKG and PKA could both phosphorylate some of the same substrates, suggesting each kinase had similar substrate specificities and presumably homologous catalytic units (Glass *et al.*, 1981). This was confirmed when the ATP binding site of PKG was labeled with [$C^{14}$] FSBA at the lysine 388, which is homologous to lysine 72 in PKA and conserved in all protein kinase (Fig. 3B). Furthermore, the protein sequence surrounding this region was shown to be highly homologous to the PKA (Hashimoto *et al.*, 1982). In complimentary experiments, sequence analysis of a 91 residue region of the bovine lung PKG, which contained the autophosphorylation site was shown to be homologous to the pseudosubstrate sites in both regulatory subunits of the PKA (Takio *et al.*, 1983).

#### 4.1.1. *Catalytic Core*

By homology to other protein kinases, the catalytic core of PKG is located between residues 356 and 621 (Hanks *et al.*, 1988). Conserved sequences within this region form the ATP binding site, recognize peptide substrates, and interact with the intramolecular pseudosubstrate site located in the regulatory unit (Fig. 3B). The regulatory unit of PKG is located amino-terminal of the catalytic core on the same polypeptide chain and is homologous to the regulatory subunits of the PKA (Takio *et al.*, 1983, 1984b). Distinct functional units within the regulatory section are responsible for dimerization, intramolecular inhibition of the catalytic unit, and cGMP binding (Fig. 4B).

#### 4.1.2. *Dimerization*

Little PKA, the extreme amino-terminus of each PKG protomer participates in dimerization (Fig. 4B). Characterization of a monomeric form of the type Iβ PKG isolated from bovine aorta smooth muscle revealed that removal of the first 63 amino acids by endogenous proteinases disrupted dimerization (Wolfe *et al.*, 1989a; Sandberg *et al.*, 1989). Such PKG monomers retain a dependence on cGMP for activation, thus indicating that the catalytic core interacts with cGMP binding sites and the inhibitory domain within the same polypeptide chain rather than across chains as has been suggested (Wolfe *et al.*, 1989b). The type Iβ PKG is extended by 16 residues at the amino terminus when compared to the type Iα Isoform (Wernet *et al.*, 1989; Sandberg *et al.*, 1989; Francis *et al.*, 1989) Since the amino terminal sequences of both PKG isoforms are distinct, it is likely that the structural information required for

dimerization is contained within the secondary structure motifs.

#### 4.1.3. *Autophosphorylation and Inhibition*

The autophosphorylation sites within the inhibitory domains of PKG types Iα and Iβ are dissimilar (Fig. 6) (Wolfe *et al.*, 1989a; Wernet *et al.*, 1989). Sequences of the bovine smooth muscle isoforms are distinct for the first 105 amino acids, but identical throughout the remainder of the molecule (Wernet *et al.*, 1989). In the presence of cGMP, the type Iα PKG becomes autophosphorylated at threonine 58, but in the presence of cAMP it also phosphorylates Ser-50, Ser-72, and Thr-84 (Aitken *et al.*, 1984; Døskeland *et al.*, 1987). In contrast, the type Iβ isoform becomes autophosphorylated exclusively on serine residues (Wolfe *et al.*, 1989a). The isoform-specific, inhibitory domains associate with identical catalytic units and have distinct activation properties, which is reflected by their different cGMP analog selectivities (Hofmann *et al.*, 1985; Corbin *et al.*, 1990). In common with both regulatory subunits of the PKA, the PKG isoforms contain pseudosubstrate sequences (Fig. 6), which presumably interact at the active site in the kinase domain (Lincoln *et al.*, 1978; Wernet *et al.*, 1989).

#### 4.1.4. *cGMP Binding and Activation*

Immediately preceding the inhibitory domain are two cGMP-binding sites, Sites 1 and 2, which have evolved as a result of gene duplication (Fig. 4B). Sites 1 and 2 act allosterically to activate the kinase domain with a Hill coefficient of 1.6 (Corbin and Døskeland, 1983; Corbin *et al.*, 1986, Døskeland *et al.*, 1987). Interestingly, like the type I PKA isoforms, both PKG types have different activation kinetics, which is in part reflected by their responsiveness to different cGMP analogs. Analogs modified at C-1 or C-2 elicit similar responses from both isozymes, while C-6 or C-8 modified analogs are more effective in activating type Iα (Wolfe *et al.*, 1989a). For example, 8-bromo-cGMP and 8-butyryl-cGMP are highly effective activators of the type Iα kinase but have little or no effect on the type Iβ (Corbin *et al.*, 1986; Francis *et al.*, 1988; Wolfe *et al.*, 1989a). Detailed analysis of type Iα kinase specificity for 46 different cGMP analogs suggested that both intra-subunit binding sites prefer cyclic nucleotides in the *syn* conformation (Corbin *et al.*, 1986). The relative insensitivity of the type Iβ isoform for 8-bromo- or 8-butyryl-cGMP may reflect a preference for the cGMP anticonformer in one or other of the cGMP binding sites (Wolfe *et al.*, 1989a). Presumably, 8-bromo- or 8-butyryl-cGMP analogs can be used as probes on examine type Iα kinase function alone.

The similarity of the cAMP- and cGMP-dependent protein kinases is clearly reflected by the structural conservation exhibited by their cyclic nucleotide-binding sites. Both cyclic nucleotides activate either enzyme, although cAMP has a 200-fold lower affinity for PKG, as does cGMP for the PKA (Døskeland *et al.*, 1983; Corbin *et al.*, 1986). Models of cAMP- and cGMP-binding sites have been presented which are based upon sequence homologies with the cAMP-binding protein CAP (Fig. 7) for which the crystal structure is known (Weber *et al.*, 1989). Molecular modeling and sequence analysis infer that the cAMP- and cGMP-binding site are distinguished by an alanine or threonine, in the β7 sheet which dictates their cyclic nucleotide specificity (Weber *et al.*, 1989; Shabb *et al.*, 1990). The threonine hydroxyl group in the cGMP-binding sites is proposed to form a hydrogen bond with the guanine-2-amino group of cGMP. Furthermore, this threonine is conserved in all known cGMP-binding domains (Kalderon and Rubin, 1989; Foster *et al.*, 1988; Wernet *et al.*, 1989; Sandberg *et al.*, 1989). The importance of this side-chain was demonstrated experimentally by Shabb *et al.* (1990) who converted a cAMP-binding site of the type I regulatory subunit into a cGMP-binding site by replacing Ala 334 with threonine. The resulting mutant type I regulatory subunit had a marked increase in cGMP affinity as measured by protein kinase activation and cGMP binding (Shabb *et al.*, 1990).

Like PKA, PKG undergoes a 'charge shift' upon cyclic nucleotide binding, which can be monitored by an altered elution position during ion-exchange chromatography (Wolfe *et al.*, 1987). This property has been used to examine the mechanism of PKG activation by cGMP. PKG activation is a two stage process (Fig. 8B). Initially, cGMP saturates the site 1, which is closest to the catalytic unit in linear sequence. Two events follow when site 1 is saturated: (1) the kinase becomes 50% active and (2) site 2 is primed for cGMP binding. When cGMP saturates site 2 the enzyme becomes fully active, presumably by fully displacing the amino-terminal pseudosubstrate site from the active site of the enzyme (Fig. 8B).

While the PKA and PKG have similar mechanisms of activation (Fig. 8), there are several important differences. If both kinases are 50% saturated with cyclic nucleotides, cAMP is equally distributed between sites A and B while all of cGMP is found in site 1 (Cobb *et al.*, 1987; Wolfe *et al.*, 1987). Bound cGMP can be readily removed from PKG by various chromatographic procedures, while bound cAMP cannot be removed from PKA. Possibly the most physiologically relevant differences is that half-saturated PKG is 50% active (Fig. 8B) while the PKA (Fig. 8A) is inactive. This could suggest that PKG may be partially active at subthreshold levels of cGMP.

### 4.2. PKG Substrate Specificity and Function

Studies comparing the substrate specificities of the cAMP- and cGMP-dependent protein kinases have shown that both enzymes can phosphorylate the same sites on several target proteins (reviewed by Glass and Krebs, 1980). The determinants for substrate specificity of PKG has been studied using a peptide corresponding to the amino-acid sequence around serine 32 in histone 2B (Glass and Krebs, 1980). This peptide Arg–*Lys*–Arg–*Ser*–*Arg*–Lys–Glu is highly basic and is selectively phosphorylated by PKG with a $K_m$ of 21 μM and a $V_{max}$ 4.4 μmol/min/mg (Glass and Krebs, 1979; Glass *et al.*, 1981). These kinetic values are favorable when compared to phosphorylation by the PKA, which has a $K_m$ of 100 μM and a

$V_{max}$ of 16.5 μmol/min/mg. Comparison of the histone 2B sequence with several PKG substrate sites including and autophosphorylation site in the type Iα isoform (Ile–Gly–Pro–*Arg*–Thr–*Thr*–*Arg*–Ala–Gln–Gly–Ile), have led to the proposal of a consensus substrate site (Takio *et al.*, 1983; Glass and Smith 1983; Thomas *et al.*, 1990; Kemp and Pearson, 1990). The PKG phosphorylation site, Arg–Arg–*Ser*–*Arg*–Xaa, like those for PKA, can contain clusters of basic residues upstream of the target serine of threonine. However, unlike PKA sites, basic residues were also located downstream (Glass *et al.*, 1981; Glass, 1983; Glass and Smith, 1983). Furthermore, PKG catalyzes the phosphorylation of serine or threonine residues equally, while PKA shows a preference for serine (Glass and Smith, 1983).

PKG mediates smooth muscle relaxation, platelet aggregation in response to vasodilators, and participates in a feedback loop which regulates the activity of another cGMP-binding protein, the cGMP-dependent phosphodiesterase (Francis *et al.*, 1988; Corbin *et al.*, 1990; Halbrügge *et al.*, 1990; Thomas *et al.*, 1990a). In all three of these examples, PKA can mimic the effects of PKG but at a much slower rate. In smooth muscle, the major recipient of cGMP appears to be PKG, and C-8 modified cGMP analogs which preferentially activate the type Iα isoform, were able to promote relaxation of pig coronary artery at concentrations 1500-fold lower than dibuteryl cAMP (Francis *et al.*, 1988). Vasodilators also promote platelet aggregation in a process which involves the phosphorylation of a $M_r$ 46,000 protein called vasodilator-stimulated phosphoprotein (VASP). Elevation of cGMP caused a rapid phosphorylation of VASP on 1 or 2 serine residues while elevation of cAMP only caused phosphorylation at one of these sites. (Walter, 1989; Halbrügge *et al.*, 1990).

Perhaps the most intriguing role for PKG may be in the regulation of the cGMP-dependent phosphodiesterase (cG-BPDE) which catalyzes the degradation of cGMP. Like PKG, and cG-BPDE is widely distributed in tissues and present in high levels in lung tissues (Corbin *et al.*, 1990). Structural analysis of the cG-BPDE suggest it contains separate sites for the binding and hydrolysis of cGMP (Thomas *et al.*, 1990b). When the cGMP binding sites are occupied, the cG-BPDE becomes phosphorylated on a serine residue by PKG, at a rate 10-fold greater than PKA (Thomas *et al.*, 1990a). The effect of PKG phosphorylation on cG-BPDE catalysis is as yet unknown but may be part of a negative feedback loop to activate the enzyme and, in the process of decreasing the cGMP levels, turn off the kinase.

## 5. CONCLUSIONS

Structural and functional analysis has shown that the cAMP-dependent and cGMP-dependent protein kinases are related enzymes which respond to different intracellular messengers. The functional similarities between PKA and PKG are reflected by studies comparing their substrate specificities which have shown that both enzymes can phosphorylate the same sites on several target proteins. Therefore, if unregulated, PKA and PKG can often trigger the same biological effects. Future research will undoubtedly focus on elucidating the specific functions of each kinase and the reason for multiple isoforms. Diversity between PKA and PKG is provided by their regulatory units which exhibit different affinities for cyclic-nuclotide analogs and subcellular localizations. Therefore, compartmentalization of PKA and PKG is a potential mechanism to sequester individual kinase isoforms close to specific pools of substrate. Selective activation of compartmentalized kinase pools through trafficking of cyclic nucleotide may explain why individual hormones and neurotransmitters are able to cause specific phosphorylation events while utilizing a seemingly universal pathway.

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