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MOLECULAR PROPERTIES OF MAMMALIAN PROTEINS THAT INTERACT WITH cGMP: PROTEIN KINASES, CATION CHANNELS, PHOSPHODIESTERASES, AND MULTI-DRUG ANION TRANSPORTERS

Sharron H. Francis, Mitsi A. Blount, Roya Zoraghi, and Jackie D. Corbin

Department of Molecular Physiology & Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615

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1. ABSTRACT

Cyclic GMP is a critical second messenger signaling molecule in many mammalian cell types. It is synthesized by a family of guanylyl cyclases that is activated in response to stimuli from hormones such as natriuretic peptides, members of the guanylin family, and chemical stimuli including nitric oxide and carbon monoxide. The resulting elevation of cGMP modulates myriad physiological processes. Three major groups of cellular proteins bind cGMP specifically at allosteric sites; interaction of cGMP with these sites modulates the activities and functions of other domains within these protein groups to bring about physiological effects. These proteins include the cyclic nucleotide (cN)-dependent protein kinases, cN-gated cation channels, and cGMP-binding phosphodiesterases (PDE). Cyclic GMP also interacts with the catalytic sites of many cN PDEs and with some members of the multi-drug anion transporter family (MRPs) which can extrude nucleotides from cells. The allosteric cN-binding sites in the kinases and the cN-gated channels are evolutionarily and biochemically related, whereas the allosteric cGMP-binding sites in PDEs (also known as GAF domains), the catalytic sites of PDEs , and the ligand-binding sites in the MRPs are evolutionarily and biochemically distinct from each other and from those in the kinase and channel families. The sites that interact with cGMP within each of these groups of proteins have unique properties that provide for cGMP binding. Within a given cell, cGMP can potentially interact with members of all these groups of proteins if they are present. The relative abundance and affinities of these various cGMP-binding sites in conjunction with their subcellular compartmentation, proximity to cyclases and PDEs, and post-translational modification contribute importantly in determining the impact of these respective proteins to cGMP signaling within a particular cell.

2. INTRODUCTION

Cyclic GMP and cAMP are important second messengers in mammalian cells (1-16). In many cells cAMP and cGMP are both present, but with a few exceptions, the cAMP concentration is typically much higher than that of cGMP (3- to 1000-fold). The two cNs primarily target a limited number of proteins that preferentially interact with one or the other nucleotide, the degree of discrimination of the numerous cN-binding sites

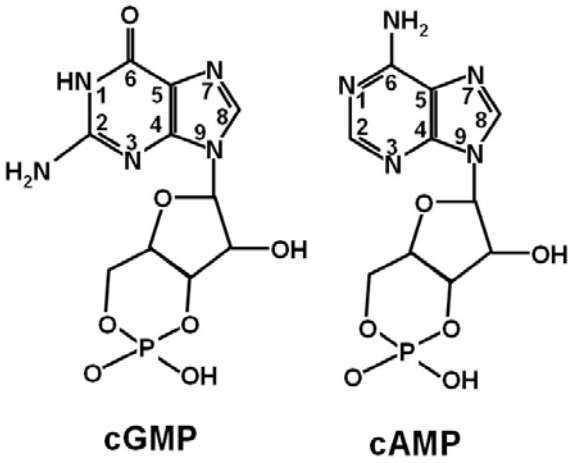

Figure 1. Molecular structures of 3',5'-guanosine cyclic monophosphate (cGMP) and 3',5'-adenosine cyclic monophosphate (cAMP). Structures of the two molecules differ only in the pyrimidine rings and the positions of the ring are numbered 1-6.

for either cGMP or cAMP is quite varied and is certainly far from absolute specificity. As a result, considerable caution must be exercised when discussing "cGMP-specific" and "cAMP-specific" binding sites in proteins; use of the terms "cGMP-selective" or "cAMP-selective" is more accurate. Since intracellular cAMP concentration in most cells markedly exceeds that of cGMP, cAMP can potentially interact with cGMP-selective sites such as those in the cGMP-dependent protein kinases (PKG) that actually have higher affinity for cGMP. Conversely, if the concentration of cGMP rises to high enough levels, it can bind to cAMP-dependent protein kinase (PKA) and activate the kinase function; this process is known as "cross-activation." Most of the proteins discussed in this chapter have affinities for cGMP or cAMP in the submicromolar to micromolar range, which is well within the range of estimated physiological concentrations. Cross-activation is a significant concern in interpreting studies in which the experimental conditions produce excessively high levels of either cAMP or cGMP. Selective compartmentation of certain cN-binding proteins in proximity to the cyclases which produce either cGMP or cAMP can further confound the degree of functional selectivity since local concentrations of one or the other nucleotide may be quite high (17-28).

In aqueous solution, cNs are in a dynamic equilibrium between *syn* and *anti* conformations based on the orientation around the bond between the ribose and N-9 of the purine (29). The spatial topography of the various cN-binding sites described herein preferentially accommodates one or the other conformation. In addition, the sites may be highly selective for cGMP, or alternatively, they may be relatively non-selective and interact well with both cGMP and cAMP. Many of the differences in the cN-binding sites have been spatially mapped and functionally defined through the use of a battery of cN analogs. The selectivity of these analogs for particular targets has been important in dissecting mechanisms of cN signaling in intact cells (30-45). The determinants of guanine versus adenine selectivity are provided through distinctive features in each of the respective cN-binding sites. Cyclic GMP and cAMP differ only in the pyrimidine portion (at N-1, C-2, and C-6) of the guanine or adenine rings (Figure 1). Cyclic GMP is protonated at N-1, contains an amino group at C-2, and an oxygen at C-6. Cyclic AMP has a single substitution on the purine, i.e., an amino group at C-6, and the N-1 position is not protonated and is therefore more electronegative (29). The cyclic phosphate moiety is a key component in the binding of nucleotides to the allosteric cN-binding sites on the cN-dependent protein kinases, cN-gated channels, as well as both allosteric and catalytic cN-binding sites on cN-PDEs, since the respective 5'-nucleotide monophosphates do not bind well. In many of the sites the equatorial and axial oxygens in that ring are important determinants of the effectiveness of the bound nucleotide to elicit changes such as activation of function. Substitution of sulfur for one of the oxygens in the cyclic phosphate moiety typically has marked effects on the efficacy of action of cN analogs. Among the various groups of cN-binding sites in mammalian proteins, contact with the ribose moiety is important in some sites, but not in others.

For investigators working in the field of cN signaling, it is imperative that all cN-binding proteins in a particular tissue be considered as targets when either the cellular cGMP or cAMP level is altered. Not only do cNs interact with and activate signaling proteins such as the cN-dependent protein kinases and the cN-gated channels, but when cN are bound to these various sites, e.g., the sites in PKA, PKG, or GAF domains of PDEs, they are temporally sequestered from the cellular milieu and unavailable to either activate the signaling pathway or to be hydrolyzed by PDEs (46-48). When released from the site of sequestration, this same cN pool could become progressively available to activate target proteins or to be hydrolyzed by PDE catalytic sites. Cyclic GMP, the focus of this chapter, may remain bound to high affinity cN-binding sites on PKGs, PDEs, or cN-gated channels thereby sensitizing these proteins to rapid activation by small increases in cellular cGMP (2, 49). Thus, it is critical to consider the population of potential cGMP-binding proteins in a cell, the relative amounts of these respective proteins, and the dynamic equilibrium of cGMP association/dissociation from the various cN-binding sites. All of these factors are involved in determining the balance between the level of the input signal, i.e., cGMP, and the output through the activated signaling pathways. This chapter will provide a general overview of the physical and functional features of each of the sites in mammalian proteins that are known to interact with cGMP. For more detailed information the reader is referred to comprehensive reviews devoted to each particular group of proteins.

3. STRUCTURAL AND FUNCTIONAL CHARACTERISTICS THAT DISTINGUISH FAMILES OF MAMMALIAN cGMP-BINDING PROTEINS

A key aspect of the ongoing research into cN-signaling pathways has been the effort to identify the cellular proteins that bind cNs and to characterize the

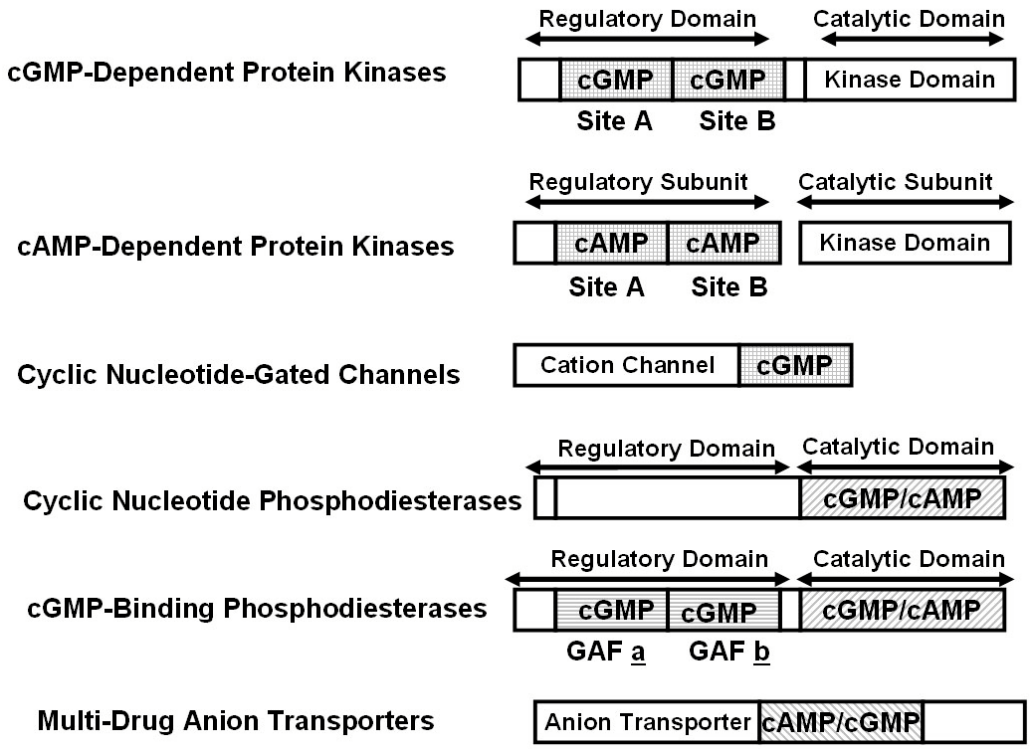

Figure 2. Cartoon depiction of modular structures of cellular proteins that interact with cGMP. Cyclic GMP binding domains are found in many chimeric proteins that contain other functional domains as well; these include domains such as protein kinase domains, phosphodiesterase catalytic domains, and channels.

properties of functional domains within these proteins. This approach is critical in developing a fuller understanding of the biochemical properties of these proteins and in designing selective pharmacological agents to specifically intervene at selective cN-binding sites. Most mammalian proteins that interact with cGMP are chimeric proteins containing allosteric cN-binding sites and other functional modules such as protein kinase domains or ion-transporting channel domains. The group of cN-binding sites that was the first to be well characterized belongs to the bacterial catabolite gene activator protein (CAP)-related family of cN-binding sites (50-52). More recently, it has been demonstrated that allosteric sites (also known as GAF domains) in some cN PDEs bind cGMP with high affinity, and these sites are entirely distinct in structure from the sites in the CAP-related proteins (53-57). The third group of sites that interacts with cGMP is the catalytic site of PDEs, which is also evolutionarily and biochemically distinct from both the CAP-related sites and the allosteric sites in PDEs (53, 58-62). Lastly, some members of the MRP family can extrude cN from the cell, but these proteins generally interact with a variety of heterocyclic compounds, and the physical features that define the ligand-binding site are poorly understood (63, 64).

3.1. Proteins containing cGMP-binding sites that are structurally homologous to the cN-binding site in the bacterial catabolite gene activator protein

Based on the genomic sequence, CAP-related cN-binding sites occur in a number of protein families, but the function for many of these is not known. All CAP-related binding sites are similar in overall structure, but they differ in cGMP/cAMP selectivity and affinities, cN-analog specificity, and modulation of function by structural features outside the binding site (2, 39, 40, 42, 50, 65-69). Differences in the CAP-related cN-binding sites in the various protein families are evident based on selective interaction of cN analogs with particular sites, results of site-directed mutagenesis, and comparison of x-ray crystal structures. The exact dimensions that provide for a stable cN-binding site in CAP-related sites are not specifically known, but a single isolated cN-binding site derived from type I regulatory subunit of PKA, consisting of ~128 AA, binds cAMP with high affinity (70). It seems likely that these general dimensions will also apply for the homologous cN-binding sites found in PKG, cN-regulated guanine nucleotide-exchange factors (GEFs), and cN-gated channels. The GEFs identified thus far appear to be highly selective for cAMP and will not be discussed further (40, 71-73).

3.1.1. Cyclic nucleotide-dependent protein kinases

The PKG and PKA families are homologous proteins that have evolved from a common ancestor (Figure 2) (74). PKG and PKA have a 50- to 200-fold selectivity for cGMP and cAMP, respectively, and most commonly the cellular effect of cGMP or cAMP is thought to be mediated by the respective kinase. However, in several systems, a physiological level of cGMP or cAMP can cross-activate the other kinase (75-80).

In mammalian tissues, PKAs are ubiquitously expressed, but PKGs are less abundant. Evidence for PKGs has been found in smooth muscle, cerebellum, bone, platelets, heart, and the gastrointestinal epithelial cell. Mammalian tissues contain three forms of PKG, i.e., PKGIalpha, PKGIbeta, and PKGII (65, 81-88). PKGIalpha and PKGIbeta are alternative splice variants of the same gene while PKGII is derived from a separate gene. All three PKGs are prototypical ligand-activated serine/threonine protein kinases, all are homodimers, and they share a similar arrangement of functional modules within a single polypeptide chain. Each is a chimeric protein with an N-terminal regulatory (R) domain and a more C-terminal protein kinase catalytic domain (74, 85, 87). The peptide substrate specificities of PKGIalpha and PKGIbeta are essentially identical, but that of PKGII differs (65, 89). For both PKGI isoforms and PKGII, phosphorylation of consensus sequences in some proteins may require selective anchoring of the particular PKG with that protein (90-92). Two homologous cN-binding sites of ~110 AA each are arranged in tandem in the R domain; binding of either cGMP or cAMP to these sites relieves autoinhibition and fully activates kinase catalytic activity (2, 36, 93, 94). The mechanistic importance of two cN-binding sites is unclear for either PKG or PKA, but the two sites within a given subunit of PKGIalpha or a PKA regulatory subunit exhibit positive cooperativity, and full activation requires occupation of both sites. However, cooperativity is more pronounced in PKA than in PKGIalpha, and there is no evidence for cooperative cGMP binding by PKGIbeta or PKGII.

The duo of cN-binding sites in PKGs and PKA are products of an ancient gene duplication that occurred prior to divergence of PKG and PKA so that the more N-terminal sites in the two groups of kinases are more homologous to one another than to the more C-terminal site within the same group (74, 95). The more N-terminal cN-binding site in PKG and PKA is commonly called site A and the more C-terminal site is called site B. In both enzymes, the two sites are kinetically distinct and show diverse cN analog-binding specificities (36). In PKGIalpha and PKGIbeta, the more N-terminal cGMP-binding site is the higher affinity site (96), but in PKGII, the more N-terminal site is the lower affinity site as occurs in the regulatory subunit of PKA (97). In both PKGs and PKAs, the two intra-subunit sites differ approximately 10-fold in affinity for cN binding.

The characteristics of the cN-binding sites in PKGs and PKAs for binding cNs are modulated by influences outside the actual binding sites. PKGIalpha and PKGIbeta, differ only in the N-terminal ~100 AA; this segment precedes the sequence encompassing the cGMP-binding sites (85, 98). Despite the fact that the AA sequences of the cGMP-binding sites in these PKG isoforms are identical, the cN-binding affinities of these isoforms differ by ~10-fold, and the cN-analog specificities of the isoforms also differ substantially (65, 99). These differences in cGMP-binding properties are dictated by the differences in AA sequence in the N-terminal 100 AA including the leucine zipper dimerization subdomain and the autoinhibitory subdomain (100, 101). In addition, point mutation of certain AA in the autoinhibitory domains of the respective PKGs dramatically increases the cN-binding affinity of the proteins (102, 103). For example, substitution of a neutral AA for a single Ser in the autoinhibitory domain of PKGIalpha or Ibeta increases cGMP-binding affinity ~10- to 20-fold. In addition, the K_D values for cGMP-binding by PKGs are in the range of 0.1-1 μM, and autophosphorylation of sites within the N-terminal 100 AA of the PKGI isoforms significantly increases cN-binding affinity (103-105). These examples demonstrate clearly that the binding properties of the cN-binding sites in PKGs are the product of the intrinsic features of the binding sites as well as influences imposed by elements of the structure elsewhere in the protein.

Currently, an x-ray crystal structure of PKG cGMP-binding sites is lacking, but a number of x-ray crystallographic structures have been determined for the closely related cN-binding sites in the RI and RII domains of PKA and Epac2(exchange protein directly activated by cAMP-2) (67, 106, 107). The sites in PKG with cGMP bound have been modeled based on the x-ray crystallographic structure of CAP (52). Basic features of the cN-binding sites in these proteins include three α-helices, i.e., the N-terminal A helix and the more C-terminal B and C helices, and an eight-stranded (β1-β8), anti-parallel flattened β-barrel structure. In the x-ray crystallographic structures of the PKA regulatory subunit, cAMP is bound in a deep pocket, and three AA (Arg, Glu, and a backbone contact) form direct bonds with the cN. The Glu interacts with the ribose 2'-OH, and the Arg and an Ala interact with the equatorial oxygen of the cyclic phosphate ring. Three glycines are located at critical turns in the structure, thereby contributing to the correct folding of the binding pocket. Eukaryotic CAP-related cN-binding sites that prefer cGMP utilize a hydrogen bond between either a Thr or Ser and the C-2 amino group in the guanine; in many, but not all of these proteins, this contact provides a substantial component of cGMP selectivity (95). In sites that are cAMP-selective, Ala typically occupies the position of this Thr, but the structural basis for preference of adenine over guanine is not well established. Hydrophobic AA in each of the cN-binding sites in PKA contribute importantly to the affinity with which cN is bound; a Trp and a Tyr in the binding sites in PKA form stacking interactions with the adenine moiety in cAMP. It is likely that the cN-binding sites in PKG also utilize hydrophobic stacking since in both PKG and PKA, cN binds with higher affinity at reduced temperatures consistent with hydrophobic interactions within the respective binding pockets. The cyclic phosphate ring of cAMP or cGMP is buried in the cN-binding pocket, and while bound, the cyclic phosphate ring is protected from the hydrolytic action of cN PDEs (46, 47).

Studies using a large number of cN analogs and the structures revealed by x-ray crystallographic indicate that PKGs and PKAs bind the cN in the syn conformation, although in the evolutionarily-related bacterial CAP, cAMP binds in the anti conformation (50). Analogs with bulky substitutions at C-8 favor the syn conformation of the cN

and commonly have higher binding affinity for PKG than cGMP (33, 36, 65, 99, 108). Studies of the effects of a collection of cN analogs on functions of the cN-dependent protein kinases also confirm the importance of contacts with both the 2'-OH and the cyclic phosphate, and in both proteins, the specific substitution at C-6 provides a critical determinant. For high-affinity binding of cGMP to PKG, the C-2 amino group of guanine is critical. Cyclic nucleotide analogs such as cIMP that lack an amino group at C-2 bind weakly to PKG, whereas analogs with increased hydrogen bonding potential at C-2 bind with higher affinity than does cGMP. Bulky substitutions at N-1, C-2, and the C-8 are well tolerated by both PKG and PKA. Analysis of an extensive collection of analogs with modifications on the purine portion of the nucleotides has also determined interactions that select for preferential binding of the analogs to either site A or site B in PKG or PKA, but almost all cN analogs will interact to some extent with both sites in these kinases (33, 36, 109). For this reason, cN analogs that preferentially bind to either site A or site B should be described as site A- or site B-selective NOT as site A- or site B-specific despite. Likewise, to date there are no cN analogs that have been determined to be specific for PKGIalpha or PKGIbeta despite the fact that some commercial groups persist in marketing analogs with these descriptions.. While a given cN analog may show higher potency of activation of either PKGIalpha or PKGIbeta, typically that analog can activate both PKGs (2, 32, 65, 99, 108, 110).

The concentration of cGMP that is required to activate each of the PKGs differs significantly. While the AA sequences of the cN-binding sites of PKGIalpha and PKGIbeta are identical, the K_a for stimulation of kinase catalytic activity differs approximately 5-10 fold (65); for PKGIalpha the K_a is ~0.1 μM whereas for PKGIbeta the K_a is 0.5-1.0 μM. For PKGII, the K_a for activation is ~1 μM, similar to the value for PKGIbeta, but the AA sequences of the cN-binding sites in PKGII differ substantially from those in PKGI (89). PKGIalpha and PKGIbeta are commonly co-expressed, and with some exceptions, both appear to be largely cytosolic. However, a small portion of these enzymes are compartmentalized due to selective interaction with other proteins within the cell (91, 92, 111). This suggests that PKGIalpha and PKGIbeta may differentially respond to increases in cGMP (16). Variations in the cGMP within different subcellular compartments could selectively modulate PKG activity irrespective of the differences in cGMP-binding affinity. To date, PKGI and PKGII have not been shown to be co-expressed. Unlike the PKGI isoforms, PKGII is found primarily in the particulate portion of the cell, and this localization is apparently required for its effective phosphorylation of membrane-associated substrates (90, 112, 113). Compartmentation to the membrane may provide not only for co-localization with substrates but also for adequate exposure to cGMP produced by ligand-activated receptor guanylyl cyclases that are membrane-bound. This compartmentation could facilitate activation of PKGII by even slight activation of the cyclases since the resulting local concentration of cGMP could be quite high. Co-localization of the kinases with certain PDEs may also be advantageous in dampening cGMP signaling in some cases.

3.1.2. Cyclic nucleotide-gated cation channels

The ion-transporting functions of two major groups of channels are directly modulated by cN binding (6, 11-15, 69, 114-117). Both groups of channels belong to the superfamily of voltage-gated cation channels. In both groups, four subunits assemble to form the channel pore; each subunit in the channel contains a more N-terminal transmembrane domain and a more C-terminal allosteric CAP-related cN-binding site that extends into the cytoplasm. The selectivity and affinity of the cN-binding site on the different channels in these two groups for either cGMP or cAMP varies markedly, as does the degree of activation of ion flux by either cN.

One group of channels includes a family of proteins dubbed the cN-gated cation channels (CNG) (11, 12, 14, 114-116, 118, 119). These channels conduct Ca^{2+} and Na^+, but Ca^{2+} is preferred under physiological conditions. The subunit composition and regulatory features of each set of CNG channels can impact the relative Ca^{2+}: Na^+ selectivity. In the absence of cN, the pore is closed due to autoinhibitory influences. When either cAMP or cGMP binds to the allosteric cN-binding sites on the channel subunits, autoinhibition is relieved, and the channel pore adopts a more open state thereby allowing for increased ion flux across the membrane (12, 14, 118-122).

The other group of cN-regulated channels includes a family of proteins described as the hyperpolarization-activated and cyclic nucleotide-regulated cation–nonselective channels (HCN); these account for the I_h current measured in many excitable cells (13, 15, 117, 123, 124). HCN channels transmit both K^+ and Na^+ but are impermeable to Ca^{2+}; K^+ is preferred by 4-7 fold. Opening of the HCN channels is dually regulated by the plasma membrane potential and by cN binding. With increasing hyperpolarization of the plasma membrane potential, the probability that these channels will adopt the open state increases. However, upon association of cN with these channels, activation of the channel function is shifted to more electropositive membrane potentials, and the rates of channel opening and firing are increased (13, 15, 69, 117, 124, 125).

Cyclic nucleotide binding or dissociation from the allosteric cN-binding site on either CNG or HCN subunits induces a reversible conformational change that is communicated to the rest of the protein, thereby altering ion flux through the pore. In both CNG and HCN channels, the cN-binding domain retains the salient features of the sites previously described for CAP, PKG, and PKA including the six critical AA (three Gly residues, Arg, Glu, and a backbone contact). The AA sequences of the cN-binding sites in the HCN subunits share more identity with the cN-binding sites in the cN-dependent protein kinases than do the cN-binding sites in the CNG subunits.

CNG channels occur in many tissues throughout the body including rod and cone photoreceptor cells,

neuronal tissues including olfactory neurons, retinal ganglion cells and sympathetic neurons, testis, aorta, kidney, pinealocytes, sperm, and heart. The density of these channels within tissues varies greatly. In rod photoreceptors, CNG channel density is quite high (~1 μM), but in most tissues, the abundance of these channels in tissues is quantitatively low compared to the cN-dependent protein kinases. The CNG channels are activated (opened) by cN binding, not by changes in voltage. In photoreceptor cells or olfactory neurons, the change in pore activity elicited by cN binding produces the electrical response to either light or odorants. CNG subunits have been divided into two general classes, alpha and beta, that are evolutionarily related (11, 12, 14). In addition, there are numerous splice variants of the various subunits. The class containing the alpha-subunits includes CNGA1-4, each of which can form functional cN-gated homomultimeric channels when individually expressed in heterologous systems. However, the characteristics of the channels formed only by alpha-subunits differ from those of native CNG channels. The beta-subunit proteins (CNGB1 and 3) do not form cN-gated channels when expressed in heterologous systems. However, when they are co-expressed with one of the alpha-subunits, heteromultimeric channels with pore properties resembling those of native channels are produced. On this basis, the beta-subunits are presumed to be modulatory components of the CNG channels. Both types of subunits contain an N-terminus that extends into the cytoplasm, a long region comprised of six transmembrane segments, an ion-conducting pore, and a C-terminal cytoplasmic ~120 AA cN-binding domain. Native CNG channels are comprised of both alpha- and beta-subunits, but the subunit composition of channels differs in various cell types. For instance, three different CNG subunits comprise the channel in cilia of olfactory neurons (CNGA2, CNGA4, and an isoform of CNGB1), two different CNG subunits comprise the cone photoreceptor channel (CNGA3 and CNGB3), and the rod photoreceptor channel involves three CNGA1 subunits and one CNGB1 subunit (126-129). Variations in subunit composition can confer distinct regulatory and functional features to that particular population of pores.

The mechanism by which cN binding translates into CNG channel opening is currently unknown, but cN binding to these channels is known to be highly cooperative (Hill coefficient ~2-4) (12, 122, 130-132). This cooperativity has the potential to sensitize channel responsiveness to small changes in intracellular cN concentration. Furthermore, the channel does not desensitize in the continued presence of cN. The cN-binding site in the CNG may exist in equilibrium between two conformations, (either closed or open). When the site is in the closed state, it is proposed that the cN is bound with low affinity, and when the channel is in the open state, the cN is bound more tightly (69, 122, 130). When cN occupies the cN-binding site, structural changes occurring in the cN-binding domain are functionally coupled to the rest of the channel to apparently foster the open conformation. Full occupation of the cN-binding domains on a given channel is required for full activation.

The similarity of AA sequences of cN–binding domains among members of the family of CNG channels is high (~80%), but similarity with related cN-binding domains in PKA, PKG, and CAP is low (~20%). Members of the CNG channel family typically have high affinity for cGMP, but the degree of cN-binding selectivity, the affinity of the channel for these nucleotides, and the efficacy with which cGMP or cAMP activates a particular channel varies considerably. The CNG from the rod photoreceptor cells of the retina has a 20- to 50-fold higher affinity for cGMP than for cAMP, but even at saturating levels of cAMP, the channel is only partially activated. In contrast, the olfactory CNG has similar affinity for the two cNs and pore permeability is fully activated by both. Furthermore, the olfactory CNG channels have ~40-fold higher affinity for cGMP than that found for the photoreceptor channels, and the affinity for cAMP is ~1000-fold greater than that of the photoreceptor. Affinities of CNGs for the cNs is in the range of 0.5-80 μM (12, 133-136)

Cyclic nucleotide selectivity in CNG channels is determined by multiple elements in the channel structure, and some of these are not in the CAP-related cN-binding site *per se* (12, 136-140) The conserved Thr found in other CAP-related cGMP-preferring sites such as those in PKG is suggested to interact directly with the C-2 amino group of guanine; this conserved Thr is present in all CNG cN-binding sites irrespective of their degree of cGMP-selectivity. The Thr at this position in the cN-binding sites of CNG channels is commonly described as a co-determinant of cN specificity since its mere presence does not always translate into a higher affinity for cGMP than for cAMP (12). This is illustrated by the rod photoreceptor and olfactory CNG channels. The subunits in both channels have a Thr at this position yet the rod channel is highly cGMP-selective and the olfactory channel has similar affinities for cGMP and cAMP. Contacts derived from the C-helix of the cN-binding pocket are also extremely important in determining cN selectivity (12, 116, 120, 140). An Asp in this helix is apparently another co-determinant that contributes a significant portion of the cGMP-selectivity profile of the CNG channels. The negatively charged carboxyl group of the Asp side chain is predicted to form a bidentate bond with the N-1 hydrogen and the C-2 amine of the guanine ring of cGMP (141); this interpretation is supported by a recent model of the cN-binding domain based on the structure of CAP(142). Replacement of the Asp with a non-charged AA increases cAMP-binding affinity suggesting that the electronegativity of the side chain may further contribute to cGMP/cAMP discrimination by impairing interaction with cAMP; this may occur through electrostatic repulsion involving the non-protonated N-1 in cAMP. Indeed, in channel cN-binding sites that favor cAMP, an uncharged AA occupies the position of the Asp. The importance of the C-helix in determining cN-selectivity has been demonstrated by the exchange of the C-helices between the cGMP-selective rod CNG channel and the cGMP/cAMP nonselective olfactory CNG channel. When the C-helix of the olfactory channel is used to create a chimera with the rod CNG channel, the result is a cGMP/cAMP non-selective channel. Conversely, when the C-helix of the rod channel replaces the C-helix of the olfactory channel, the resulting protein is selectively activated by cGMP with >100-fold affinity for cGMP over cAMP (143).

Regions outside the pocket also affect cN-binding affinity (12, 138, 140). Both the N-terminal region of the CNG channels and the C-linker sequence that connects the cN-binding domain to the channel contribute importantly to cN-binding affinity and cN-gated channel opening. Three AA in the C-linker sequences of CNGA2 and CNGA3 strongly affect cN-selectivity. When these residues in the cGMP-selective cone photoreceptor channel are replaced by the corresponding AA from the cN-nonselective olfactory channel, cAMP-binding affinity increases (136).

The various CNG channels share many regulatory mechanisms. These influences include: a) the subunit composition of the channel, b) phosphorylation of serines and/or tyrosines in the channel proteins, c) effects of Ca^{2+}, d) oxidation status of selected cysteines, e) effects of various metabolites such as diacylglycerol, and f) properties of the linker sequence that connects the cN-binding domain to the rest of the channel. However, specific details of the regulatory influences vary substantially. Phosphorylation of specific Tyr residues in the subunits of the rod photoreceptor CNG channel subunits decreases the sensitivity of pore opening in response to cN (144, 145) .

The Ca^{2+}/calmodulin regulation of CNG channels, including the number of Ca^{2+}/calmodulin molecules bound per channel and the particular subunit to which the Ca^{2+}/calmodulin complex is bound is quite varied. In general, elevation of intracellular Ca^{2+} decreases the cN-binding affinity of the pore. In many instances, the regulation appears to involve binding of the Ca^{2+}/calmodulin complex, but other Ca^{2+}-binding proteins may also be involved (146-151). For rat olfactory CNG channel, apocalmodulin is reported to bind to both the CNGB1b and CNGA4 subunits; the apocalmodulin-channel complex is then presumably poised for rapid modulation by increases in Ca^{2+} (152). In both photoreceptor and olfactory channels, direct binding of the Ca^{2+}/calmodulin complex decreases channel sensitivity to cN binding and activation of the channel. The effect of Ca^{2+}/calmodulin to decrease cN binding affinity is greater in magnitude in the olfactory channel (cGMP-binding affinity is decreased > 50-fold) compared to the effect in the rod photoreceptor channel where cGMP-binding affinity is decreased 2-3 fold. Binding of Ca^{2+}/calmodulin may involve either the alpha or beta-subunits in the channels. Ca^{2+}/calmodulin effect on the rod photoreceptor channel is conferred through the beta-subunit of the channel. Two binding sites for the Ca^{2+}/calmodulin complexes have been identified in the CNGB1 subunit; one is located in the N-terminal region of the channel, and the other is located C-terminal to the cN-binding domain (153). Only one Ca^{2+}/calmodulin complex is bound to the olfactory channel, and this interaction apparently occurs in the N-terminal segment of the CNGA2 subunit. Binding of Ca^{2+}/calmodulin to a specific region in the N-terminal segment of the CNGA2 subunit of the olfactory channel reduces sensitivity of the channel to cN activation, and tyrosine phosphorylation of the CNGB1 subunits blunts the inhibitory effect of Ca^{2+}/calmodulin (144, 145, 154).

The cN analog-binding specificity of CNGs has not been exhaustively studied (43-45, 135, 155). However, it is clear that the pattern of analog effects on channel function, i.e., acting as either an agonist or an antagonist, does not exactly mimic the effects of these analogs on the cGMP-binding sites in PKGs (45). Analogs with substitutions at C-8 (particularly hydrophobic substitutions) typically bind to the channels (and to PKGs) with higher affinity than does cGMP. Likewise, the cyclic phosphate moiety is critical to cN binding and channel activation. For the rod cGMP-gated CNG channel α-subunit, Rp-8-Br-cGMPS, Sp-8-Br-cGMPS, and 8-pCPT-cGMPS act as agonists to activate the channel; for PKGs the Rp-8-pCPT-cGMPS is an an antagonist and can be used to discriminate between actions of these two cGMP-binding proteins (45

The second group of cN-regulated channels is the HCN channels, and these account for cellular currents known as I_h currents (9, 15, 123, 124, 156-158). These channels are comprised of four very similar members (HCN1-HCN4) that share ~60% AA sequence identity and exhibit similar functional properties; AA sequence identity among the cN-binding sites in these proteins is ~90%. HCN channels play a particularly important role in the function of excitable tissues such as the rhythmic cardiac and neuronal pacemaker activities, but they are expressed in other tissues. HCN1 channels are particularly abundant in both pre- and post-synaptic regions of neurons. Functional characteristics of the native I_h currents that are provided by the HCN channels include: 1) pore permeability to both K^+ and Na^+ with K^+:Na^+ ranging between 3-4, 2) activation of the channel current by hyperpolarizing membrane potential, 3) shift of pore activation to less polarized membrane potential by direct binding of cN to the channel subunits, and 4) Cs^+ blockade of the pore (13, 159).

Like the CNG channels, HCN channels are comprised of four subunits to create a central aqueous pore. Each subunit within the multimeric HCNs contains a voltage-sensor domain, a transmembrane domain, and a C-terminally located cN-binding domain that is connected to the rest of the protein by a linker sequence. The x-ray crystallographic structure for a cN-binding site derived from a HCN-channel has been determined and shown to resemble the structure found in other CAP-related sites (69). Whether the channels are comprised of a single type of subunit, i.e., a homomultimeric channel, or by a combination of subunits from different HCNs, i.e., a heteromultimeric channel, is not clear. However, when each subunit is individually expressed in heterologous systems, a channel activity with characteristics that resemble native I_h channel currents can be measured. For instance, cells expressing HCN2 constructs had an I_h current with K_a values for cAMP and cGMP of 0.5 and 6 μM, respectively (15). This closely approximates values determined for native I_h channels in sinoatrial node channels from heart (0.2 and 8 μM for cAMP and cGMP, respectively).

Occupation of the cN-binding domain by either cGMP or cAMP induces a conformational change that

promotes opening of the pore. In contrast to the CNG channel subunits, the sites in the HCN channels are typically cAMP-selective despite having the Thr residue that enhances cGMP selectivity in PKGs and certain CNGs. As in the CNGs, contacts derived from the C-helix contribute to cN selectivity. At the position occupied by the Asp-604 in the rod photoreceptor cGMP-preferring CNG channel, there is an Ile in HCN channel subunits. Based on mutagenesis studies in rod CNG channels, it is predicted that this switch from an acidic to a neutral AA may account for the cAMP preference of these channels. The x-ray crystal structure of a cN-binding fragment of HCN2 has provided insight into the features that contribute to determining cAMP/cGMP selectivity and the conformational rearrangements associated with cN binding and activation (69). As in CNGs and cN-dependent protein kinases, the ion-transporting function of the HCN channels is blocked by autoinhibitory influences. In the case of the HCN channels, this autoinhibition can be overcome by either a sufficient hyperpolarization of the plasma membrane potential or by cN binding to sensitize the pore to less electronegative potentials. Binding properties of these cN may also be affected by novel features in the site since cAMP is bound in the anti conformation and cGMP is bound in the syn conformation (69). Results from the crystal structure of HCN2 channels suggest that cAMP selectivity may be enhanced through hydrophobic interactions between the adenine ring and side chains of Val, Met, Leu, and Arg residues, as well as hydrogen-bonding interactions between the C-6 amide of cAMP and the backbone carbonyl oxygen of a nearby Arg.

HCN channels play a particularly important role in cardiac pacemaker tissues and in neuronal cells that exhibit cyclical patterns of depolarization (9, 156, 157, 160-162). HCN channels have a wide tissue distribution; HCN1 channels are particularly abundant in brain and in some synapses, whereas HCN2 and HCN4 are abundant in heart. The HCN channels are also found in non-excitable tissues, but the functional characteristics or the role of these channels in these tissues is not understood. In cardiac tissue, the HCN pacemaker channels play a pivotal role in the response to cAMP elevation following sympathetic stimulation. Upon elevation of intracellular cAMP in response to activation of adenylyl cyclases by beta-adrenergic agonists, cAMP binds to the cN-binding site on the HCN channels to make these channels more excitable and increase heart rate.

The respective HCN channels exhibit different activation kinetics and different degree of cN regulation (69, 125, 163-165). For example, opening of the pores formed by the HCN1 channel displays the fastest kinetics, but the membrane voltage shift that is induced by cAMP binding is minor (2-5 mV) . The kinetics of the opening of the HCN2 are somewhat slower than that for HCN1, and those for the HCN4 channels are even slower (124, 159, 166-168). However, in the HCN2 and HCN4 channels, cAMP binding causes a dramatic rightward shift (15-25 mV) in the membrane potential at which the pores open. Differences in cN-induced gating properties may relate to inherent differences in the cN-binding sites, differences in the communication between functional domains in the various subunits within the channels, variations in the subunit composition of the particular channels, and the presence of splice variants and regulatory influences. Evidence suggests that HCN channels can also be reversibly modulated by phosphorylation/dephosphorylation events, and by changes in intracellular Ca^{2+} levels (117, 169-171).

3.2. Cyclic GMP binding to cyclic nucleotide phosphodiesterases

3.2.1. Catalytic site

The balance between synthesis of cNs by adenylyl or guanylyl cyclases and breakdown by PDEs largely determines cellular cN levels. PDEs serve as key regulators of cAMP- and cGMP-signaling pathways by controlling the spatial and temporal components of cN signals as well as the steady-state levels of intracellular cAMP and cGMP. Hydrolysis of cAMP and cGMP occurs at a single site in the catalytic domain (C domain) of the PDEs; some PDE catalytic sites are highly specific for either cAMP or cGMP, whereas others hydrolyze both nucleotides and are known as dual-specificity PDEs (59, 61, 62, 172-178). Among the dual-specificity PDEs, the relative affinities (K_m values) of the catalytic sites for cAMP or cGMP and the efficiency (k_{cat}) with which each cN is hydrolyzed vary widely. The catalytic sites of PDEs are not homologous to the CAP family of cN-binding sites that include the PKGs and cN-gated channels or to the allosteric cGMP-binding GAF domains found in some PDEs.

PDEs have been subdivided into three evolutionarily distinct classes, classes I, II, and III. All known mammalian PDEs are class I PDEs, and to date only this class contains the predicted allosteric cGMP-binding GAF domains discussed below (176). There are 11 known families of mammalian PDEs that are products of 20 or more genes; they have been classified by DNA sequence analysis and by biochemical and pharmacological characteristics. Almost all class I PDEs are dimeric, but the contribution of oligomerization to function is poorly understood (179). Each PDE monomer is a chimeric protein that contains a conserved C domain of ~270 AAs (58, 62, 174, 176, 177). The C domain of each class I PDE is complexed with a more N-terminal domain that may commonly regulates catalytic function, provides for subcellular location, promotes dimerization, or mediates other functions, such as protein-protein interactions (56, 57, 180-182). By convention, the N-terminal region is described as the regulatory (R) domain although regulatory processes have not been established for several PDE families.

The catalytic sites among the eleven families of class I PDEs share many common features that provide for interaction with cNs, but the PDEs differ widely in affinity and specificity for cNs. Aside from the issue of varying degrees of cN specificity, the K_m values for hydrolysis of cNs by PDE catalytic sites range from 0.05->100 μM, and the affinities of these sites can be modulated by regulatory elements outside the catalytic site; these include processes

such as phosphorylation/dephosphorylation and ligand binding to allosteric sites (183-188). Even within a group of closely related PDEs such as members of the PDE1 family, the affinity for each of the nucleotides varies remarkably (189). For example, the K_m values for cAMP vs cGMP for three isoforms are as follows: PDE1A2 (113 and 5 μM, respectively; 23-fold cGMP selective), PDE1B1 (24 vs 3 μM, respectively; 8-fold cGMP selective), PDE1C2 (1 and 1 μM, respectively; no cN selectivity. These differences are likely to be due to specific structural variations within the cN-binding pocket of the respective C domains. However, among these same PDE1 isoforms, the variations in affinity for the substrates do not translate into significant differences in the rates of hydrolysis since the k_{cat} values for cAMP and cGMP are quite similar for each of the three.

Class I PDEs share a high degree of AA conservation in the C domains. Seventeen AA in the C domain are invariant among mammalian PDEs and twelve of the invariant AA are in the catalytic pocket (58, 60, 176, 190-193). This conservation of primary sequence suggests that all PDE C domains share a similar overall three-dimensional structure. This prediction has been borne out thus far in the overall topology of the isolated C domains revealed by the x-ray crystal structures of PDEs 1B, 3B, 4B, 4D, 5, and 9 (60, 192, 194-197). Based on these x-ray crystal structures, the conserved PDE C domains are comprised of 16 α-helices within an overall compact structure. The structure can be subdivided into three subdomains, and the catalytic site is located in a hydrophobic pocket formed by the juxtaposition of these regions.

The x-ray crystal structures of the mammalian PDE catalytic sites reveal common elements that provide for specific and high affinity interaction with cN and various competitive inhibitors (62, 193-195, 198-200). These sites in PDEs are entirely different in structure from allosteric cN-binding sites, and as revealed by comparison of structures of cGMP-specific vs cAMP-specific PDEs, the cN selectivity of these respective catalytic sites cannot be accounted for by overall structural features (197). The catalytic sites in different PDEs exhibit specific structural and spatial features that provide for the selectivity of that particular PDE for cGMP vs cAMP. Furthermore, many PDE inhibitors that are competitive with cN are highly specific for the catalytic sites in one PDE family (190, 201-206). The specificity of these inhibitors is largely provided by the defining features of a given catalytic site. The catalytic sites of the PDEs with known structures contain two metal-binding sites; one site is located at the bottom of the active site. In most PDEs studied thus far, this is occupied by a tightly-bound Zn^{2+} (60, 191, 192, 197, 199, 207, 208). A closely apposed second site contains a more loosely bound second metal, perhaps Mg^{2+} or Mn^{2+}.. The metal components of the catalytic site are primarily involved in catalysis, but the coordination provided by one or both of these metals is also likely to be important for proper formation of the catalytic site pocket and for anchoring of the cyclic phosphate moiety.

The continued discovery of the many novel characteristics of the various PDE catalytic sites is helping to refine our understanding of the mechanisms that determine cN specificity and the structural features that either optimize or discriminate among structurally related PDE inhibitors. Analysis of the individual structures of two cAMP-specific PDEs with 5'-AMP in the site compared with that of one cGMP-specific PDE with 5'-GMP reveals that the overall positioning of the products (and presumably the substrate) is quite similar despite the difference in specificity (190, 191). Although these structures reveal the positioning of the low affinity product, investigators have used these structures to predict important interactions of the high affinity substrates in these sites. Three groups of interactions within the catalytic site have been established to be important for cN-binding affinity and catalytic function. The importance of many of these AA in providing for PDE catalytic function and cN specificity has been predicted based on AA sequence homology or is supported by the results of site-directed mutagenesis. However, these approaches are limited. Predictions based on homology require the identification of a sufficient number of AA that are conserved among all class I PDEs or that are selectively conserved among either cAMP-specific or cGMP-specific PDEs. When considering individual PDE families, additional contacts contribute importantly to the particular characteristics, e.g., different affinities, with which these catalytic sites interact with cN substrate, and these are more difficult to identify based on homology comparisons.

The three shared structural features critical for cN binding to the catalytic sites of all class I PDEs that have been studied include 1) a hydrophobic pocket that includes four AA at its core, 2) an invariant Gln that directly contacts the purine, and 3) coordination to the cyclic phosphate moiety (190-193, 197, 209). First, a collection of four conserved hydrophobic AAs are approximated in three-dimensional space in the catalytic site to form a hydrophobic pocket containing a double-sided "hydrophobic clamp" around the space occupied by the heterocyclic rings of the cN substrates or those of competitive inhibitors such as 3-isobutyl-1-methylxanthine, sildenafil, tadalafil, vardenafil, and zardaverine. These four hydrophobic AA are not invariant among mammalian PDEs, but the two of the positions that form either side of the "hydrophobic clamp" are always occupied by aromatic AAs such as Phe, Tyr, or Trp. This "clamp" is critical in providing high-affinity interaction with cNs and many inhibitors. In co-crystals formed between the isolated PDE C domains and either PDE inhibitors or adenine-containing or guanine-containing molecules, the cyclic ring structure of the purines or purine analogs is stacked in the pocket that contains these two aromatic AAs as well as other hydrophobic AAs. One of these aromatic AA is close enough to form pi-pi electron interactions with the purine ring of the cNs.

Second, the γ-amide and the carbonyl-oxygen from the side chain of an invariant Gln form bidentate contacts with either cAMP or cGMP, and also form contact some inhibitors; this interaction provides for high-affinity recognition of both cGMP and cAMP. In addition to providing for this high-affinity interaction, evidence is

mounting that restriction of the rotational flexibility and therefore the orientation of the γ-amide group of the Gln side chain provides for the high degree of cGMP- or cAMP-selectivity seen in some PDE families. For this reason, it has been dubbed the "glutamine switch" (191). In both cGMP-specific and cAMP-specific PDEs, the orientation of the γ-amide group in the Gln side chain is frequently fixed by an extended network of hydrogen bonds described as a hydrogen-bond relay, and the side arm has little if any rotational flexibility. However, the orientation of the fixed γ-amide group differs by ~180^o in the two types of PDEs. In the orientation favoring cGMP-selective binding, the γ-amide of Gln is oriented such that the amide group can form a hydrogen bond with the C-6 oxygen and the carbonyl oxygen of the Gln can form a hydrogen bond with the N-1 hydrogen. In the orientation favoring cAMP-selective binding, the Gln side chain is oriented so that the carbonyl oxygen can form a hydrogen bond with the C-6 amide, and the γ-amide can form a hydrogen bond with the N-7 of the adenine ring. However, in PDEs that have dual specificity for cGMP and cAMP, the stabilizing hydrogen-bond relay for this invariant Gln is purportedly absent or weak, and free rotation of the side chain allows it to assume either orientation and high-affinity interaction with either cN. The universality of this mechanism for cN selectivity in all PDEs is yet to be demonstrated.

Third, the cyclic phosphate moiety of the cNs is critically important for high-affinity interaction of these nucleotides with all known PDE catalytic sites. Although no co-crystal of a PDE with the intact cN substrate has been obtained, modeling based on the bound state of the 5'-monophosphate product has allowed investigators to infer important contacts with the cyclic phosphate ring, particularly in the region of the catalytic site containing the bound metals. Occupation of the metal sites most likely serves several important functions in the PDE catalytic site; these include a) formation of the proper tertiary structure of the catalytic site, b) anchoring the substrate to the site through interactions with the cyclic phosphate moiety, and c) contributing to polarization of water and generation of a reactive hydroxyl nucleophile for hydrolysis of the cyclic phosphate bond (193, 207, 210).

In contrast to the CAP-related cN-binding site, PDE catalytic sites do not form important contacts with the ribose moiety, and large substitutions at the 2'-OH are well tolerated. This structural pattern includes the catalytic sites of the dual-specificity PDEs 1 and 2, and the cGMP-specific PDEs 5 and 6 (34, 35, 37, 211-213). Substitutions at the N-1/C-2 positions are also typically well tolerated, including substitutions such as N^2-hexyl- and 1-N^2-phenyletheno- groups on the pyrimidine portion of the molecule. Some of these analogs are hydrolyzed by PDEs, but others are not well tolerated. In contrast, cN analogs containing substitutions at C-8 or replacement of the oxygens within the cyclic phosphate group are poorly tolerated. A number of heterocyclic inhibitors that lack the cyclic phosphate moiety or even the purine structure have now been shown to interact with the PDE catalytic sites with 1000- to 10,000-fold higher affinity than does the natural substrate (214, 215) . So, it is clear that with proper chemical design, additional high affinity contacts within and surrounding the catalytic site can be exploited to provide for potent and selective contact with a particular PDE catalytic site. It should be emphasized that a wide variety of pharmacological agents that interact with the PDE catalytic sites (sildenafil, tadalafil, vardenafil, zaprinast, IBMX, caffeine, papaverine) do not significantly bind to either the allosteric cGMP-binding GAF domains (see below) or the cGMP-binding sites in the CAP-related proteins. This further emphasizes the structural and biochemical distinctions among these families of cGMP-binding sites.

3.2.2. Allosteric regulatory sites on phosphodiesterases

The domains in PDEs that provide for allosteric cGMP binding belong to a widespread family of domains known as GAF domains (55, 57, 216). The AA sequences, tertiary structures, and signature sequences for these cGMP-binding domains are novel and are not related to any other known family of cN-binding proteins. The GAF acronym is derived from the names of the first three classes of proteins recognized to contain this domain: mammalian cGMP-binding PDEs, Anabaena adenylyl cyclases, and Escherichia coli FhlA. GAF domains are commonly described as one of the largest families of small molecule-binding domains, but direct evidence of ligand binding has only been demonstrated for only a few. Increasingly, roles other than ligand binding are being documented.

In mammalian tissues, only three families of proteins are known to contain GAF domains. Of the eleven known families (PDEs 1-11), five (PDEs 2, 5, 6, 10, and 11) contain GAF domains in the N-terminal portion. A duo of GAFs, dubbed a and b, arranged in tandem is most common. PDEs 2, 5, 6, 10, and some isoforms of PDE11 have two GAFs aligned in this way (217, 218). In PDEs 2, 5, and 6, allosteric cGMP binding is provided by one or more of these GAFs, and cGMP-binding stoichiometry for these three PDEs is one cGMP bound per PDE monomer, i.e., two cGMP molecules bound per dimer of PDEs 2, 5, or 6 (56, 219-222). In PDEs 5 and 6, the GAF domain binding shows more than 100-fold selectivity for cGMP versus cAMP (182, 219). The cN-binding specificity of the R domain in PDE2 favors cGMP by a 10-20 fold preference, but it also interacts with cAMP (223). The K_D values for cGMP binding by GAFs in these PDEs are in the range of 0.01-0.4 μM. No functional role such as cGMP binding has been determined for the GAFs in PDEs 10 and 11.

The x-ray crystal structure of only one cGMP-binding GAF domain has been determined, i.e., GAF b in the truncated dimeric R domain of PDE2 (56). This is the first structure of a GAF containing bound cGMP and as such has advanced insight into structure/function relationships in this particular group of cGMP-binding sites. There is little conservation of AA sequence among the few PDE GAFs that are known to bind cGMP. In the absence of significant conservation of primary sequence, it is almost impossible to predict critical cGMP-binding contacts. The truncated PDE2 R domain structure that has been determined contains both GAFs a and b. Despite

overall structural similarity between these GAFs, the GAF b domains bind cGMP and are spatially well separated, but the GAF a domains do not bind cGMP and are dimerized (56).

The GAF domains in PDE2 are rich in beta-sheets (beta1-beta6). These beta-sheets are packed on the back side with 2-4 α-helices and on the other side with a mixture of short α-helices and loops, which in GAF b forms the sides of the cGMP-binding pocket. In the crystal structure of the PDE2 R domain, cGMP is bound only in GAF b and is in the anti conformation. This result is in agreement with the stoichiometry of one cGMP bound per PDE2 monomer and with predictions based on cN analog–binding studies. Cyclic nucleotide analog studies have established that the allosteric sites in PDEs 2, 5, and 6 are remarkably specific for cGMP. Substitutions at any position on the nucleotide have a major impact on binding affinity; even cIMP, which differs only in lacking the C-2 amide, binds with >10-fold lower affinity (37, 176).

In the x-ray crystal structure of PDE2 R domain, the cGMP molecule along with three water molecules is almost entirely buried in a deep pocket in GAF b where it is coordinated by 11 contacts. This large number of contacts may stabilize the anti conformation of cGMP and the energetically strained boat conformation of the cyclic phosphate ring (56). The pattern of contacts revealed in this x-ray crystal structure demonstrates the difficulty in identifying a general cN-binding signature sequence in GAF domains; some contacts involve the protein backbone, and the binding motif is not completely conserved in other cGMP-binding PDE GAF domains, e.g., GAF a in PDE5. However, based on the crystal structure of the PDE2A GAF domains and sequence similarities among other PDE GAFs that are likely to bind cGMP, Beavo and coworkers have proposed a fingerprint sequence comprised of 11-residues [$SX_{(13\text{-}18)}FDX_{(18\text{-}22)}IAX_{(21)}$ $Y/NX_{(2)}VDX_{(2)}TX_{(3)}TX_{(19)}E/Q$] for cGMP binding (56). Not all of the AA listed directly contact the cGMP and therefore are not likely to be conserved among other cGMP-binding GAFs. Furthermore, the K_D values for cGMP binding to PDE GAFs vary considerably indicating that there must be novel contacts for cGMP in different GAFs.

The guanine and ribose of cGMP make a total of six polar and two hydrophobic contacts with AAs of PDE2 GAF b (56). This is consistent with results of cN analog studies that infer the importance of the ribose in cGMP binding to PDE GAFs. Using the x-ray crystal structure as a guide, Wu et al. modified the PDE2 R domain using site-directed mutagenesis to establish that three residues, the Phe-Asp dyad and one of the Thr residues in the fingerprint sequence, are required for determining a significant portion of cGMP/cAMP selectivity (223). The carboxyl group of Asp in the Phe-Asp dyad interacts with the N-1 hydrogen of guanine, the aromatic ring of Phe stacks with the guanine ring, and Thr interacts with the C-2 amide group via a water molecule. Although the protein with wild type PDE2 sequence exhibits about a 20-fold preference for cGMP, individual substitution of each of these three AA significantly increases the cAMP-binding affinity and slightly decreases cGMP-binding affinity. As a result, the site still binds cGMP with high affinity, but the marked selectivity for cGMP is lost because the site no longer efficiently discriminates against cAMP. The C-6 carbonyl group of guanine interacts with the main-chain amide group of Asp in the Phe-Asp duo. Cyclic AMP has an amino group at C-6, which is incompatible with contact to the backbone amide bond at the above mentioned Asp. In addition, N-1 in cAMP is not protonated and is electronegative in comparison with cGMP; therefore this position cannot form a hydrogen-bond with the Asp side chain. Substitution of Asn for this Asp does not significantly impair cGMP-binding affinity, indicating that the negative charge is not required for high-affinity cGMP binding, but cAMP-binding affinity is improved 2-fold. Substitution of Ala for Asp impairs cGMP-binding affinity and increases cAMP-binding affinity, suggesting that the longer side chain of the Asp or Asn may sterically interfere with cAMP positioning in the site. In the x-ray crystal structure, Phe stacks with the guanine ring of cGMP. Results of these studies using site-directed mutagenesis also suggest that Phe in the Phe-Asp dyad is primarily a negative determinant for cAMP binding rather than being particularly important for cGMP binding.

As mentioned before, the cyclic phosphate moiety of the cNs is critically important for binding to any known PDE allosteric site, but this portion of cGMP makes no direct contacts with side chains of AA in the binding pocket of the PDE2 GAF b (56). The exocyclic oxygens of the cyclic phosphate group form contacts through two hydrogen bonds with backbone amides and a water molecule; the negatively charged phosphate group of cGMP is stabilized by a positive charge from an adjacent α-helix. Point mutation of each of the AAs that are involved in contacts with the phosphate or ribose of cGMP essentially abolishes cN binding (223). On this basis, it has been suggested that the major portion of binding energy for cGMP in the GAFs of PDEs derives from these critical contacts with the cyclic phosphate and the ribose. This interpretation is also consistent with the fact that related ligands such as IBMX, caffeine, guanine, or 5'-GMP do not detectably interact with these cGMP-binding GAFs in PDE2, PDE5, or PDE6. Although analog studies indicate that the 2'-OH is an important contact, no contact of this position with AA in the binding pocket was revealed in the x-ray crystallographic structure of PDE2 GAF b (56).

In addition to the AA that form direct contacts with cGMP in the binding pocket of the PDE2 R domain, other AA also contribute importantly to determining the characteristics of these GAFs for cN binding. Notably, the conserved NKFD motif that lies outside the cGMP-binding pocket contributes to allosteric cGMP binding in PDE5 and is structurally important in maintaining cGMP binding in the PDE2 R domain (223-225). As in other cGMP-binding sites discussed above, the intrinsic cGMP-binding affinity of these GAFs is also subject to reversible regulation by regions outside the binding site itself, and these sites most likely exist in an active state and an autoinhibited state. Phosphorylation of a single Ser (Ser-102) in human PDE5 increases the allosteric cGMP-binding affinity of the GAF

domain by ~10-fold, and the cGMP-binding affinity is also reversibly sensitive to the oxidation/reduction modifications within the R domain (187, 226). In addition, in both PDE2 and PDE5, the cGMP-binding affinity of the isolated R domain is greater than that of the holoenzyme, and the isolated cGMP-binding GAFs (GAF b in PDE2 and GAF a in PDE5) have significantly higher binding affinity than that found for the intact R domain (182, 223). Thus, sequences external to the actual GAF domains significantly impact the intrinsic cGMP-binding affinity of these sites.

3.3. Multi-drug anion transporters

Energy-dependent extrusion of cNs from cells is a well established phenomenon. Plasma levels of cNs fluctuate in a number of physiological and pathophysiological situations and have been suggested to be a bioindicator in a number of conditions. However, the physiological or pathophysiological relevance of the extrusion of cNs from the intracellular space is still debatable. A possible role has been advanced for extracellular/intercellular cGMP in modulating cellular processes (227) , but no extracellular receptors for cGMP have yet been identified, and this possiblity is still in question. Cyclic GMP extrusion from cells can occur against a concentration gradient, requires hydrolysis of ATP, and in some instances is saturable (228, 229). Cyclic GMP stimulates this cGMP-transporter ATPase. The majority of studies of cGMP transport have been performed in inside-out erythrocyte membrane vesicles, but more recently studies have been carried out in a variety of intact cells transfected with proteins that are conjectured to be "cGMP-transporters." Overall, results suggest that at low cGMP concentrations the cN is primarily transported by a multispecific efflux pump with the characteristics of an organic anion transport system. Most experimental evidence indicates that the rapid catalytic action of PDEs to hydrolyze cGMP and/or cAMP is by far the most important determinant in reducing intracellular cN content (230-232). The consensus in the field is that extrusion of cN from the cell may in some rare instances contribute slightly to this process, but it is not a major factor in dampening cellular cN levels. Irrespective of these considerations, it is clear that cNs are present in plasma from healthy individuals and these levels change in many conditions. The tissue source of these plasma cNs and the mechanism(s) responsible for moving cNs from intracellular spaces into the extracellular space and the plasma are not known.

In recent years, several members of the multidrug resistance protein (MRP) family have been demonstrated to transport cNs across cell membranes in an ATP-dependent manner, yet other members of this family do not transport cNs. MRPs are widely distributed in the body, and it is clear that when certain members of this family of proteins are present in a cell, they have the potential to bind and extrude cGMP. Cyclic nucleotide-transport activity has been reported for MRP4, MRP5, and MRP8 (63, 64, 233-239). MRP5 has been detected in three different cell types in the human heart including vascular smooth muscle cells, cardiomyocytes, and vascular endothelial cells (240). It is also found in smooth muscle cells in the ureter, urethra, bladder, blood vessels, and penile corpus cavernosum, and in the glandular epithelium of the prostate (241, 242). Overexpression of MRP5 was associated with high affinity (K_m~2 μM) cGMP transport. MRP4 is also implicated in cGMP transport and is found in vascular smooth muscle and in the human kidney proximal tubules (236, 243). In cells overexpressing MRP4, cGMP is transported (K_m~10 μM) in an ATP-dependent manner, and PDE5 inhibitors such as sildenafil and zaprinast block the transport. However, MRP4 transports other small molecules including glucuronide and sulfate conjugates of steroids, so that cNs would most likely compete with these molecules for access to the transporter. There is no evidence that the MRP proteins are related in structure to any of the other known cGMP-binding proteins..

The field of MRP-mediated cN transport is still in its infancy. Cyclic GMP is actively transported across human erythrocyte membranes using either intact cells or inside-out membrane vesicles. While it is commonly agreed that MRPs 4, 5, and 8 can transport cNs across membranes, the specific properties that have been reported for this transport do not always agree. Early studies using vesicular uptake indicate that MRPs 4 and 5 are high-affinity cN transporters. In those studies, MRP4 exhibit K_m values of 10 μM for cGMP and 45 μM for cAMP compared to the K_m values of MRP5 of 2 μM for cGMP and 380 μM for cAMP. More recently, in studies of cells over-expressing MRP4 or MRP5, cNs were transported with low affinity, and the transport was not saturable even at 600 μM cN (237). In addition, in these latter studies, decline in intracellular cN levels was modest at best. Lastly, some reports suggest that another transporter, an organic anion transporter (OAT1), can mediate cGMP efflux from cells overexpressing this protein, but this possibility has not been investigated thoroughly.

PDE inhibitors such as IBMX, trequensin, zaprinast, dipyridamole, and sildenafil also inhibit cN transport activity by some members of the MRP family (244, 245), but in other reports this effect is absent. The reason for this discrepancy is not clear. If these PDE inhibitors are potent inhibitors of the MRPs, this would argue that PDE inhibitors currently in clinical use (e.g., dipyridamole and sildenafil) may act through two mechanisms, i.e., blocking PDE catalysis and blocking cN extrusion from the cell. Both processes could thus contribute to cN accumulation in cells. However, if extracellular cGMP acts as a primary intercellular signaling molecule, a decline in the local cGMP concentration could have important effects on cellular functions (227).

4. CROSS-ACTIVATION AND CROSS-TALK IN SIGNALING PATHWAYS THAT ARE ALTERED BY CHANGES IN cGMP

The complex array of cellular proteins that interact with cGMP poses a challenge to investigators studying cN-signaling processes. Some of the cN-binding sites in these proteins are highly selective for cGMP, while others interact with cAMP as well. As emphasized in the Introduction, the specificity of many (perhaps most) of these cN-binding sites is best described as cGMP-selective

rather than cGMP-specific. Depending on the population of cN-binding proteins within a given cell type, elevation of cellular cGMP can lead to increased signaling through cGMP-activated pathways, increased signaling through cAMP-activated pathways, or decreased signaling through cAMP-activated pathways. The outcome of such a scenario depends not only on the population of cN-binding proteins within a given cell, but also on considerations such as compartmentation of the proteins and the cN signal (25, 26, 246). Direct cross-activation of "cAMP-specific" sites in PKA by cGMP has been documented to occur in numerous instances (75). Likewise, cGMP competition with cAMP at the catalytic sites of dual-specific PDEs such as PDEs 1, 2, and 3 can produce an elevation of cAMP and increased signaling through cAMP pathways simply as a result of the competition of cGMP as substrate resulting in a lower rate of cAMP breakdown . In contrast, cGMP binding to GAFs in PDE2 can increase hydrolysis of both cGMP and cAMP at the catalytic site . In adrenal medullary cells, elevation of cGMP by natriuretic peptides activates PDE2 which in turn increases cAMP hydrolysis and thereby lowers cAMP levels and cAMP-induced aldosterone production. Colocalization/compartmentation of cGMP-binding proteins is also likely to be a factor. The CNG and HCN channels, the MRP transporters, and PKGII are all membrane-associated proteins. A significant portion of the guanylyl cyclase activity (both ligand-activated and nitric oxide-stimulated) in many cells is also membrane-bound so that cGMP generated within the microenvironment of the plasma membrane is likely to a) have immediate access to these cN-binding sites and b) the local cGMP concentration may be quite high. Cyclic GMP-hydrolyzing PDEs are also found in association with the cell membrane and undoubtedly contribute to rapid dampening of cGMP signaling through these various protein targets. Therefore, it should be clear that considerable caution must be employed when interpreting the results of studies in which either cGMP or cAMP is increased.

5. SUMMARY AND PERSPECTIVE

Multiple types of sites in mammalian proteins interact with cGMP. These include the CAP-related sites in PKG and PKA, the CAP-related sites in the CNG and HCN families of cN-gated channels, the GAF domain sites in certain cN PDEs, the catalytic sites of both cGMP-specific and dual-specific PDEs, and the site(s) in MRPs. The tissue distribution, subcellular compartmentation, cN-selectivity, and cN-binding affinity and regulation of these proteins dictate the response of the signaling pathways to increases in cGMP resulting from agonist stimuli and pharmacological intervention. The differences in the evolutionary origins and the novel biochemical properties of these sites are critical in distinguishing among these protein targets; development of pharmacological agents that selectively target one of these groups of cGMP-binding sites is also immeasurably aided by these differences.

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Send correspondence to: Dr Sharron H. Francis, Dept. Molecular Physiology & Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, Tel: 615-322-4383, Fax: 615-343-3794, E-mail: Sharron.francis@vanderbilt.edu

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