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Molecular Mechanisms of Cyclic Nucleotide-Gated Channels

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Cyclic nucleotide-gated (CNG) channels are highly specialized to carry out their unique role in cell signaling. Significant progress has been made in the last several years determining the molecular mechanisms for these specializations. The activation of the channels begins with the binding of cyclic nucleotide to a domain in the carboxyl terminal region. This binding, in turn, produces an induced fit of the protein that involves a movement of the C-helix portion of the binding domain. The induced fit of the binding domain is coupled to an allosteric conformational change that opens the channel pore. The pore is formed primarily from the sequence between the S5 and S6 segments. A single glutamic acid in the pore represents the binding site for multiple monovalent cations, the blocking site for external divalent cations, and the site for the effect of protons on permeation.

KEY WORDS: Phototransduction; olfaction; ion channels; cGMP; cAMP.

INTRODUCTION

Ion channels play a fundamental role in the generation of an electrical response to light in photoreceptor cells of the vertebrate retina. The closing of a cyclic nucleotide-gated (CNG) channel in the outer segment of these photoreceptors represents the final step in the enzymatic cascade that begins with the absorption of a photon of light by rhodopsin (see reviews by Stryer, 1986; Yau and Baylor, 1989). The photo-activated rhodopsin catalyzes the exchange of GTP for GDP bound to transducin, which, in turn, activates a phosphodiesterase. The phosphodiesterase catalyzes the hydrolysis of guanosine 3′,5′-cyclic monophosphate (cGMP), lowering the cytosolic concentration of cGMP and closing a cGMP-activated channel in the membrane of the outer segment (Fesenko *et al.*, 1985). The closing of this cation-selective channel causes a hyperpolarization of the photoreceptor outer segment, which is transmitted to the inner segment where it modulates transmitter release. Clearly the cGMP-activated channel plays a central role in visual transduction.

Ion channels that are directly activated by cyclic nucleotides play a role in a number of other cellular processes. The transduction of odorant signals by the olfactory epithelium occurs via a CNG channel (Lancet, 1986; Nakamura and Gold, 1987). In this case the odorant receptors activate a cyclase, which generates adenosine 3′,5′-cyclic monophosphate (cAMP) and opens a cAMP-activated channel. The inhibitory response to glutamate of the on-bipolar cells of the retina has been proposed to be mediated through a cGMP-activated channel (Nawy and Jahr, 1990; Shiells and Falk, 1990). CNG channels have also been demonstrated in a variety of other nonsensory tissues such as the heart, testis, and kidney (Ahmad *et al.*, 1990; Biel *et al.*, 1993, 1994; DiFrancesco and Tortora, 1991; Distler *et al.*, 1994; Marunaka *et al.*, 1991; Weyand *et al.*, 1994). Therefore, a detailed understanding of the molecular mechanisms of these channels' behavior will provide insight into electrical signaling in a number of sensory and physiological processes.

The CNG channels are important regulators of cell function. The channel is permeable to both mono-

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valent and divalent cations. The Ca^{2+} permeability of these channels elevates the level of internal Ca^{2+}, which has been shown to be important in light adaptation in photoreceptors (Matthews *et al.*, 1988; Nakatani and Yau, 1988), activation of a Ca^{2+}-activated Cl^- channel in olfactory receptors (Kleene, 1993; Kurahashi and Kaneko, 1993), transmitter release in cone photoreceptors (Rieke and Schwartz, 1994), and possibly in sperm chemotaxis (Biel *et al.*, 1994; Weyand *et al.*, 1994). Because the permeation of Ca^{2+} and Mg^{2+} is so slow, these divalent cations effectively block the channels, making the single-channel conductance quite small under physiological conditions (see reviews by Yau and Baylor, 1989; Zufall *et al.*, 1994). The CNG channels exhibit varying selectivities for cGMP and cAMP depending on their physiological role. By cooperatively binding multiple cyclic nucleotide molecules, these channels become exquisitely sensitive to changes in the levels of cytosolic cyclic nucleotide (Haynes *et al.*, 1986; Zimmerman and Baylor, 1986), allowing, for example, the rod CNG channel to faithfully detect and signal the drop in cGMP concentration resulting from the absorption of a single photon (Baylor *et al.*, 1979). Whereas CNG channels were originally thought to be static sensors of cyclic nucleotide concentration, it has recently been found that the concentration of nucleotide at which the channels open can be tuned by physiological stimuli. The apparent ligand affinity of the olfactory and rod channels can be modulated by Ca^{2+}/calmodulin (Chen *et al.*, 1994; Chen and Yau, 1994; Gordon *et al.*, 1995b; Hsu and Molday, 1993, 1994; Liu *et al.*, 1994), phosphorylation (Gordon *et al.*, 1992), diacylglycerol analogs (Gordon *et al.*, 1995a), and transition metal divalent cations (Gordon and Zagotta, 1995a,b; Ildefonse and Bennett, 1991; Karpen *et al.*, 1993).

CLONING AND EXPRESSION OF CNG CHANNELS

Kaupp and coworkers first isolated a cDNA clone for a subunit of the bovine rod cGMP-activated channel (Kaupp *et al.*, 1989). In addition, based on sequence homology to the rod CNG channel, clones for other CNG channels have been isolated from a variety of different tissues and species (Baumann *et al.*, 1994; Biel *et al.*, 1993, 1994; Bonigk *et al.*, 1993; Bradley *et al.*, 1994; Chen *et al.*, 1993; Dhallan *et al.*, 1990, 1992; Goulding *et al.*, 1992; Liman and Buck, 1994; Ludwig *et al.*, 1990; Weyand *et al.*, 1994). These channel clones appear to come from a set of distinct but structurally related genes with tissue and species-specific expression (Distler *et al.*, 1994). The rod CNG channel appears to be primarily expressed in the rod photoreceptors. A distinct CNG channel gene was first cloned from olfactory epithelium (Dhallan *et al.*, 1990; Goulding *et al.*, 1992; Ludwig *et al.*, 1990) and subsequently from rabbit aorta (Biel *et al.*, 1993). A third gene for CNG channels has been separately cloned from the cone photoreceptors (Bonigk *et al.*, 1993), the testis (Weyand *et al.*, 1994), and the kidney (Biel *et al.*, 1994). These cDNA clones encode a subunit of CNG channels, referred to as the α subunit or subunit 1, and can, by themselves, produce functional channels when exogenously expressed in either *Xenopus* oocytes or a human embryonic kidney cell line (HEK293). In addition, clones for a separate β subunit, or subunit 2, have been isolated for both the rod (Chen *et al.*, 1993) and olfactory (Bradley *et al.*, 1994; Liman and Buck, 1994) CNG channels. These β subunits do not produce functional channels by themselves, but, when coexpressed with their corresponding α subunits, produce channels with altered permeation, pharmacology, and/or cyclic nucleotide selectivity.

The primary structure and proposed transmembrane topology of the CNG channel polypeptide is diagrammed in Fig. 1. Like the voltage-dependent family of ion channels, CNG channels contain six hydrophobic regions thought to represent transmembrane segments. In addition, the CNG channels exhibit sequence similarity to the voltage-dependent channels, particularly in two regions: the S4 segment, thought to represent the voltage sensor for the voltage-dependent transitions in these channels, and the P-region, thought to comprise part of the channel pore (Jan and Jan, 1990). On the basis of this sequence comparison, it appears that the CNG channels are more closely related to the family of voltage-dependent channels than to the family of ligand-gated channels. Consistent with this transmembrane topology, immunocytochemistry studies have shown that the amino terminal domain is located on the cytoplasmic side of the membrane (Molday *et al.*, 1991), and the beginning of the P-region is located on the extracellular side of the membrane (Wohlfart *et al.*, 1992). The subunit composition of the functional channel is unknown. However, on the basis of their homology to the voltage-dependent K^+ channels and a Hill coefficient for ligand binding that is occasionally greater than 3, the CNG channels may exist as tetramers. When expressed exogenously from only a single subunit, the channel is homo-

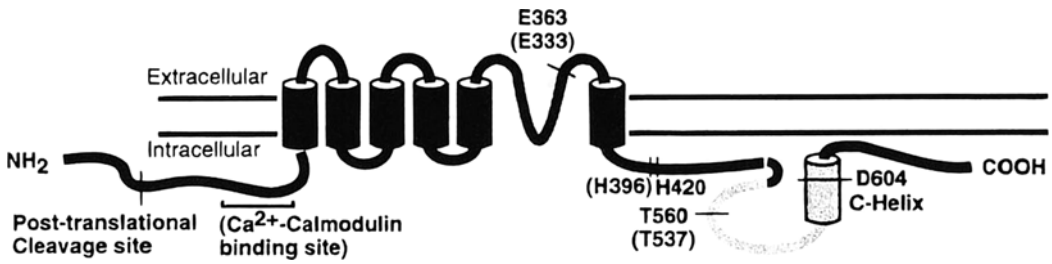


Fig. 1. Diagram of the primary structure and transmembrane topology of the CNG channels. The positions of several important functional regions are labeled. The amino acid positions in the olfactory channel are shown in parentheses.

multimeric. However, the native channel is heteromultimeric, containing some combination of α and β subunits.

The cDNA of the bovine rod cGMP-activated channel predicts a sequence in the amino-terminal domain that is very highly charged, due to the presence of many lysine and glutamic acid residues. It has been suggested that the channel is post-translationally modified by specific cleavage which removes the first 92 amino acids in the amino-terminal domain (Molday *et al.*, 1991). This processing of the channel polypeptide would account for the difference in molecular mass between the protein from the cDNA clone (80 kDa) and the protein isolated from bovine retina (63 kDa). Consistent with this hypothesis, immunofluorescence and immunogold labeling have confirmed that the 63-kDa form of the channel is present in rod outer segments (Molday *et al.*, 1991).

The CNG channels have been studied extensively by expressing the channels from the cDNA clones exogenously in *Xenopus* oocytes or a human embryonic kidney cell line (HEK293). The rod and olfactory CNG channels differ markedly in their apparent affinity for cGMP and cAMP and in the potency of cAMP as an agonist. Both the expressed rod channels and expressed mammalian olfactory channels exhibit a 25- to 40-fold higher apparent affinity for cGMP over cAMP, as determined by the concentration of agonist required to produce half-maximal activation ($K_{1/2}$) (Altenhofen *et al.*, 1991; Dhallan *et al.*, 1990; Kaupp *et al.*, 1989). However, apparent affinity for each of these cyclic nucleotides is about 20-fold higher in the mammalian olfactory channels than in the rod channel. Interestingly, the catfish olfactory channel exhibits a similar apparent affinity for both cyclic nucleotides (Goulding *et al.*, 1992). In addition to apparent affinity, these CNG channels also differ in the potency of cAMP as an agonist. For the expressed rod channel, cAMP is a very poor agonist, activating about 1% of the current activated by cGMP at saturating concentrations (Kaupp *et al.*, 1989). In this respect cAMP appears to be a typical partial agonist that is not as potent as cGMP at promoting the allosteric conformational change upon binding. However, in the expressed olfactory channel, cAMP is a full agonist, activating current to the same level as cGMP at saturating concentrations (Dhallan *et al.*, 1990; Goulding *et al.*, 1992). As indicated below, these differences between the rod and olfactory channels in the ability of agonists to activate the channel result from either differences in the free energy of binding of the cyclic nucleotides or differences in the free energy of the opening allosteric conformational change induced upon binding.

MOLECULAR MECHANISM FOR BINDING OF CYCLIC NUCLEOTIDES

The sequences of the CNG channels also contain, in their intracellular carboxyl terminal region, a domain with significant sequence similarity to the cyclic nucleotide-binding domain of a number of other cyclic nucleotide-binding proteins. The sequence of the cyclic nucleotide-binding domain of the channel is aligned with that of other cyclic nucleotide-binding proteins in Fig. 2A. In addition to cGMP- and cAMP-dependent protein kinases, the cyclic nucleotide-binding domain of the channels have significant sequence similarity to *E. coli* catabolite gene activator protein (CAP). CAP is a cAMP-activated transcription factor whose structure, while bound to cAMP, has been determined by x-ray crystallography to 2.5 Å resolution (McKay and Steitz, 1981; Weber and Steitz, 1987). The cAMP-binding domain of CAP is more than 100 amino acids long and contains many residues, shown in solid boxes, that interact with the bound cAMP. Notice that, while the overall sequence similarity in the binding domains of the channel and CAP is rather

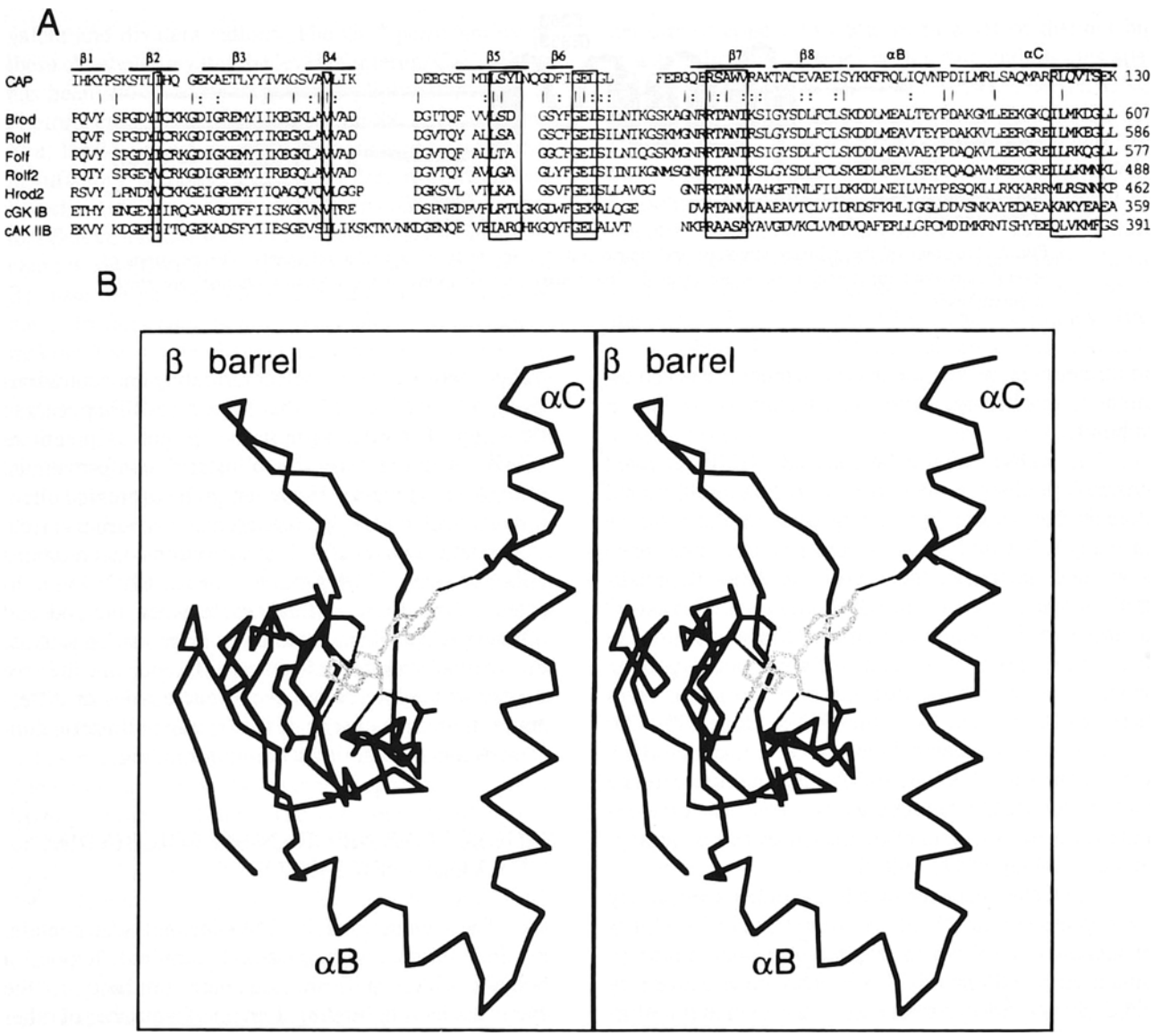


Fig. 2. Summary of the structure of the cyclic nucleotide-binding domain. (A) Amino acid sequence comparison of the cyclic nucleotide binding domains of the catabolite gene activator protein of *E. coli* (CAP), representative CNG channels, and representative cGMP and cAMP dependent protein kinases. Brod, bovine rod CNG channel (Kaupp *et al.*, 1989); Rolf, rat olfactory channel (Dhallan *et al.*, 1990); Folf, fish olfactory channel (Goulding *et al.*, 1992); Rolf2, rat olfactory channel subunit 2 (Bradley *et al.*, 1994); Liman and Buck, 1994); Hrod2, human rod channel subunit 2 (Chen *et al.*, 1994); cGK IB, bovine cGMP-dependent kinase domain B (Takio *et al.*, 1984); cAK IIB, bovine cAMP-dependent kinase IIα domain B (Takio *et al.*, 1984). Lines above the sequence indicate secondary structure motifs present in the crystal structure of CAP; residues in boxes are those that line the nucleotide binding pocket of CAP (Weber and Steitz, 1987). (B) Stereo pair of the backbone structure of the cyclic nucleotide-binding domain of CAP with bound cAMP.

sparse, about 20%, many of the residues that interact with the cAMP are conserved in the CNG channels. Since these residues are spread throughout about 100 amino acids of primary sequence, it seems likely that virtually the whole tertiary structure will be conserved. This structure therefore represents a very good candidate structure for the binding of cyclic nucleotide to the CNG channels.

The structure of CAP, shown in Fig. 2B, comprises eight beta strands, designated β1 to β8, that form a β barrel structure, followed by two α helices, designated αB and αC. The cAMP binds in the *anti*

configuration inside the β barrel with the adenine group interacting with the C-helix. The amino acids interacting with the adenine group are very poorly conserved in the channels and the kinases. To account for some of the specificity for cGMP over cAMP in the channel and kinases, the binding of cGMP has been proposed to be in the *syn* configuration where the 2-amino moiety of cGMP can hydrogen bond to a threonine in β7 found only in the cGMP-binding proteins (Fig. 3A) (Altenhofen *et al.*, 1991; Kumar and Weber, 1992; Shabb *et al.*, 1991; Steinberg *et al.*, 1991; Weber *et al.*, 1987, 1989). While this configuration is very different from the crystal structure of cAMP bound to CAP, it is supported by some experimental evidence. cGMP analogs that contain bulky groups in the 8 position of the guanine ring bind with much higher affinity, maybe because they force the free ligand to adopt the favored *syn* configuration (Koch and Kaupp, 1985; Zimmerman *et al.*, 1985). Interestingly, the chemistry of the substituent at the 8 position appears to be more important than its size (Brown *et al.*, 1993). In addition, mutation of the threonine to an alanine in β7 of the cGMP-activated channel decreases the apparent binding affinity for cGMP more than the apparent affinity for cAMP (Altenhofen *et al.*, 1991).

A different mechanism for cyclic nucleotide selectivity, involving the C-helix, has been suggested by two sets of recent experiments (Goulding *et al.*, 1994; Varnum *et al.*, 1995). In CAP, the purine ring of cAMP points toward the C-helix and makes important hydrogen bonds with it. Since the only differences between cAMP and cGMP lie in their purine ring substitutions, the C-helix was reasoned to be a likely site for selectivity. The importance of this region was confirmed in experiments using chimeric channels where the C-helices of the bovine rod and catfish olfactory channels were exchanged (Goulding *et al.*, 1994). Replacing the C-helix of the rod channel with that of the olfactory channel resulted in a chimera that was activated equally well by cAMP and cGMP, similar to the olfactory channel. Conversely, replacing the C-helix of the olfactory channel with that of the rod channel generated a chimera which was selectively activated by cGMP (the $K_{1/2}$ for cGMP was 100-fold lower than that for cAMP).

The molecular mechanism for the cyclic nucleotide selectivity was recently suggested by Varnum *et al.* (1995). These investigators have shown that aspartate residue 604 (D604) in the bovine rod channel C-helix appears to play a critical role in the selective activation by cGMP. In CAP, the homologous residue is a threonine (T127), which makes an important hydrogen bond with the N6 amino group of cAMP. In the rod channel, the rank order of potency for cyclic nucleotides at saturating concentrations is cGMP > cIMP > cAMP. Upon mutation of D604 to a neutral polar residue, such as glutamine, which is present in the catfish olfactory channel, the order changes to cGMP ≥ cAMP > cIMP. Upon substitution of a nonpolar residue, such as methionine, which is present in the β subunit of the rat olfactory channel, the order becomes inverted relative to the rod channel: cAMP > cIMP > cGMP. These

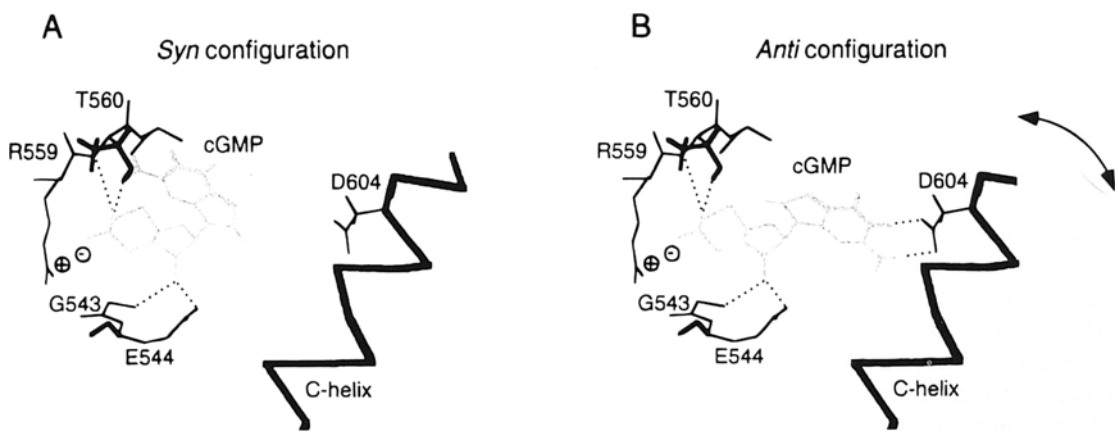


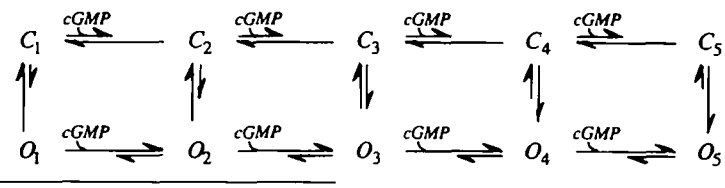
Fig. 3. Model of the cyclic nucleotide binding site of the CNG channel based on analogy with the CAP structure. Key amino acid residues from the rod CNG channel were substituted into a molecular representation of the three-dimensional structure of CAP (Weber and Steitz, 1987): T560 for CAP S83, D604 for CAP T127 (arrows in Fig. 1A). cGMP was substituted for cAMP, and is shown in the *syn* configuration (A) and the *anti* configuration (B) around the N-glycosidic bond. Slight rotation of the torsion angles of N109 in CAP (ϕ, 182–166°; ψ, 267.4° to 255°) at the junction between the B and C α-helices was sufficient to permit H-bonding between D604 and the N1 and N2 hydrogens of (*anti*) cGMP.

results suggest that differences in this residue alone can largely account for the differences in cyclic nucleotide selectivity between the rod channel, the catfish olfactory channel, and channels containing β subunits. Varnum *et al.* proposed that the negatively charged carboxylic acid side chain of D604 forms a pair of hydrogen bonds with the N1 and N2 atoms on the guanine ring (Fig. 3B). For this interaction to occur, the cGMP was proposed to bind in the *anti* configuration, like cAMP in CAP (Fig. 3B). This type of interaction has been shown to occur in high-affinity GTP-binding proteins such as the α subunit of transducin (Noel *et al.*, 1993), EF-Tu (Jurnak, 1985), and H-Ras (Pai *et al.*, 1989). An unfavorable electrostatic interaction between D604 and a pair of unshared electrons at the N1 position of cAMP is suggested to contribute to why cAMP is such a poor agonist on the rod channel. These mutations of D604 affect the free energy of the opening allosteric conformational change much more than the free energy of binding of the cyclic nucleotides, suggesting that this residue may not play a role in the initial contact with cGMP, but may instead contribute to the allosteric stabilization of the nucleotide in the open state of the channel (see below).

MOLECULAR MECHANISM FOR ACTIVATION BY CYCLIC NUCLEOTIDES

The mechanism of activation of CNG channels by the binding of cyclic nucleotides has been studied by several investigators. The dose–response curves of many CNG channels have a Hill coefficient ranging from 2 to 3, indicating that opening of the channel generally requires the binding of at least three molecules of cyclic nucleotide (Yau and Baylor, 1989; Zufall *et al.*, 1994). In addition, the binding of cyclic nucleotides appears to be highly cooperative (Zimmerman and Baylor, 1986). Experiments employing fast jumps in cGMP concentration or voltage have shown that the channels exhibit a rapid gating transition after ligand binding (Karpen *et al.*, 1988a,b). This rapid transition is weakly voltage dependent (roughly an e-fold increase for a 120 mV depolarization) and can account for the voltage dependence in the apparent affinity of the channels for cyclic nucleotide. Studies on the behavior of single channels have demonstrated the occurrence of multiple single-channel conductances (Haynes *et al.*, 1986; Ildefonse and Bennett, 1991; Taylor and Baylor, 1995; Zimmerman and Baylor, 1986). Recently Taylor and Baylor have shown that a major subconductance level is prominent at low cGMP concentrations but not at high cGMP concentrations, suggesting that the subconductance state may represent a partially liganded form of the channel (Taylor and Baylor, 1995). Ildefonse and Bennett have reported the occurrence of as many as four different conductance levels in rod channels reconstituted into planar lipid bilayers, possibly corresponding to channels containing one to four bound ligands (Ildefonse and Bennett, 1991).

These observations are perhaps best explained by the following model (Stryer, 1987):



For this scheme, the channel is considered to exist in two quaternary structures, closed (C) and open (O). The concerted allosteric conformational change between these structures is promoted by the binding of cGMP to each subunit. The greater the number of cGMP molecules that bind, the more stable the concerted conformational change. Within each structure, the binding of cGMP to each subunit is considered to be independent of the number of other bound subunits. This model is derived from a more general model for allosteric transitions in proteins proposed by Monod, Wyman, and Changeux (Monod *et al.*, 1965) for the binding of oxygen to hemoglobin. It is intriguing because it provides a simple molecular explanation for the observed Hill coefficient for cyclic nucleotides, binding cooperativity, and the multiple open states. In addition, the model contains four cyclic nucleotide-binding sites, consistent with a tetrameric structure of the channel. Recently this model has been shown to be consistent with the steady-state behavior of rod and olfactory channels (Goulding *et al.*, 1994; Ildefonse and Bennett, 1991).

With this gating mechanism in hand, experiments have begun to try and determine some of the molecular events that underlie the opening allosteric conformational change. As indicated earlier, the x-ray crystal structure of CAP provides a good candidate structure for cyclic nucleotide binding in the CNG channels. However, as in any allosteric enzyme, the CNG channels must contain at least two different conformations for binding its agonists, a conformation that binds cyclic nucleotide relatively weakly when the channel is closed and a conformation that binds it more tightly when the channel is open. A molecular mechanism for the conformational change in the binding site during the opening allosteric transition in rod CNG channels was suggested by Varnum *et al.* (1995). In this mechanism, the cyclic nucleotide binds to the closed channel primarily through interactions between the β-roll structure and the ribose and exo-cyclic phosphate of the cyclic nucleotide. The opening allosteric conformational change is then thought to involve a movement of the β-roll relative to the C-helix, permitting the formation of a pair of hydrogen bonds between the carboxylate of D604 and the N1 and N2 of the guanine ring (see Fig. 3B). This mechanism is supported by evidence that the C-helix region confers cyclic nucleotide specificity in bovine rod and catfish olfactory CNG channels (Goulding *et al.*, 1994), and that mutations of D604 in the C-helix produce a dramatic effect on the stability of the allosteric transition in a cyclic nucleotide-dependent way (Varnum *et al.*, 1995). A similar mechanism has been proposed for the conformational change in CAP that permits binding to DNA (Weber and Steitz, 1987).

The mechanism for how the conformational change in the cyclic nucleotide binding site is coupled to channel opening is unknown. However, several regions of the channel have been suggested to be involved. Alterations in the amino terminal region are particularly influential on the allosteric transition (Chen and Yau, 1994; Gordon and Zagotta, 1995b; Goulding *et al.*, 1994). The rod and olfactory CNG channels differ markedly in the free energy of their allosteric opening transition; opening in the olfactory channels is much more energetically favorable than opening in the rod channels. However, olfactory channels with a rod amino terminal domain exhibit much less favorable opening, and conversely, rod channels with an olfactory amino terminal domain open much more favorably (Gordon and Zagotta, 1995b; Goulding *et al.*, 1994). Furthermore, substitution into the rod channel of a region from the olfactory channel beginning midway along the amino terminal region and extending through the S2–S3 linker (N-S2 domain) produced even a larger stabilization of the open state (Goulding *et al.*, 1994). The amino terminal domain and S1 segments have been implicated in the process of subunit assembly in voltage-gated K^+ channels (Babila *et al.*, 1994; Li *et al.*, 1992; Shen *et al.*, 1993). These results led Goulding *et al.*, (1994) to suggest that channel opening may involve a change in the strength of intersubunit bonds between N-S2 domains associated with an alteration in subunit orientation, similar to hemoglobin (Perutz, 1990) and gap junction channels (Unwin and Ennis, 1984).

MODULATION OF CHANNEL ACTIVATION

Recent studies have shown that the amino terminal region is involved in the modulation of olfactory CNG channels by Ca^{2+}-calmodulin (Liu *et al.*, 1994). Ca^{2+}-calmodulin causes a decrease in the apparent cyclic nucleotide affinity of both rod (Chen *et al.*, 1994; Gordon *et al.*, 1995b; Hsu and Molday, 1993, 1994) and olfactory (Chen and Yau, 1994; Liu *et al.*, 1994) channels. This modulation occurs by the direct binding of Ca^{2+}-calmodulin to the channels and does not involve the action of a kinase. Liu *et al.* (1994) have shown that the Ca^{2+}-calmodulin binds to a specific domain on the amino terminal region of the olfactory channel (see Fig. 1). Furthermore, they have shown that the decrease in apparent cyclic nucleotide affinity arises from a decrease in the stability of the allosteric opening transition, consistent with the effect of mutations in the amino terminal region discussed above. The modulation of the rod channel involves Ca^{2+}-calmodulin binding not to the α subunit but to the β subunit instead (Chen *et al.*, 1994).

The gating properties of the CNG channels can also be modulated by various transition metal divalent cations including Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Mn^{2+} (Gordon and Zagotta, 1995a,b; Ildefonse and Bennett, 1991; Karpen *et al.*, 1993). For example, low concentrations of intracellular Ni^{2+} (<1 μM) potentiate the response of rod CNG channels. This potentiation is manifested as a dramatic increase in the apparent affinity for cGMP and cAMP and an almost 50-fold increase in the maximal current observed with cAMP. All of these effects of Ni^{2+} can be explained by a mechanism in which Ni^{2+} binds to the channel primarily when the channel is open, promoting the allosteric conformational change induced by cyclic nucleotide binding (Gordon and Zagotta, 1995a). Ni^{2+} produces the opposite effect, an inhibition, in the olfactory channel that

can be explained by a mechanism in which Ni^{2+} binds to the channel primarily when it is closed (Gordon and Zagotta, 1995a). Using chimeras between the rod and olfactory channels, the binding site for Ni^{2+} potentiation was localized to a single histidine residue (H420) in the putative intracellular mouth of the rod channel. The binding site for Ni^{2+} inhibition of the olfactory channel was localized to a single histidine residue (H396) just three amino acids away from the potentiation site. The finding of two residues located only three amino acids apart in the primary sequence, with opposite state dependence to their binding, suggested that this region of the channel undergoes a large movement during the opening transition (Gordon and Zagotta, 1995a,b).

MOLECULAR MECHANISM OF ION PERMEATION

Our understanding of ion permeation through the CNG channels has been greatly aided by structural insights provided by the molecular cloning of these channels. One of the most surprising results to emerge from inspection of their deduced amino acid sequences was the presence of a region that was homologous to the P region (or H5 or SS1–SS2) of voltage-gated K^+ channels (Fig. 1), which links the extracellular ends of the S5 and S6 transmembrane regions (Goulding *et al.*, 1992; Guy *et al.*, 1991). In K^+ channels, the P region has been shown to be the major determinant of ion selectivity and was initially proposed to dip into and out of the membrane, crossing it twice as a β hairpin (see review by MacKinnon, 1995). Subsequent results have shown that the neighboring S5 and S6 segments can also affect ion permeation and, in particular, participate in the binding of intracellular channel blockers. This has led to the view that the S5 and S6 segments form the internal vestibule of the channel whereas the P region may form the narrowest region of the channel which serves as the ion selectivity filter.

The similarity of the amino acid sequence between K^+ channels and CNG channels in the P region is quite surprising since the CNG channels are nonselective cation channels. Although they are good at discriminating cations from anions, they show little or no discrimination between Na^+ and K^+ (Yau and Baylor, 1989; Zufall *et al.*, 1994). Close inspection of the amino acid sequences of the P regions from K^+ channels and CNG channels revealed a deletion of two amino acids, tyrosine and glycine, in the CNG channels. Heginbotham *et al.* (1992) deleted these two residues in Shaker K^+ channels. Remarkably, the mutant channels now displayed many of the properties associated with the pore of the CNG channels. These deletion mutants lost their selectivity for K^+ and became sensitive to block by external Ca^{2+} and Mg^{2+} with high affinity.

Direct evidence that the P region forms the CNG channel pore was provided by Goulding *et al.* (1993) who exchanged the P regions between a bovine rod and catfish olfactory channel. Compared with the olfactory channel, the rod channel has a smaller single-channel conductance (20 vs. 55 pS), a higher degree of selectivity among monovalent cations, and a smaller apparent pore diameter (5.8 vs. 6.3 Å; determined from organic cation permeability; see also Picco and Menini, 1993). A chimeric rod channel containing the olfactory P region displayed the permeation properties appropriate to the olfactory channel. The difference in single-channel conductance could be accounted for quantitatively on the basis of the difference in pore diameter. The fact that the P region determined apparent pore diameter supports the view that it forms the ion selectivity filter at the narrowest part of the pore.

Although the pore of the CNG channels resembles voltage-gated K^+ channels most closely in terms of its amino acid sequence, the CNG channel permeation properties are more like those of voltage-gated L-type Ca^{2+} channels. Similar to Ca^{2+} channels, the CNG channels are permeable to both Ca^{2+} and to monovalent cations (Yau and Baylor, 1989). In addition to permeating the CNG channels, Ca^{2+} also profoundly blocks the flow of current carried by monovalent cations through these channels (Yau and Baylor, 1989; Zufall *et al.*, 1994), similar to the behavior of the voltage-gated Ca^{2+} channels (Almers and McCleskey, 1984; Hess and Tsien, 1984). In Ca^{2+} channels, this behavior is thought to be due to multi-ion occupancy in the pore. Although many of the permeability properties of CNG channels can be explained by a single ion binding site within the pore that binds divalent cations with a higher affinity than monovalent cations (Zimmerman and Baylor, 1992), other evidence favors multi-ion occupancy (Furman and Tanaka, 1990; Sesti *et al.*, 1995). In particular, Sesti *et al.* (1995) report an anomalous mole fraction effect for the rod channel in which the conductance of the channel in a mixture of Li^+ and Cs^+ was greater than the conductance in either Cs^+ or Li^+ alone. Such effects can best be explained by multi-ion channels.

CNG channels also resemble Ca^{2+} channels in that they display a distinct subconductance state that is mediated by a rapid partial blocking effect of external protons

(Goulding *et al.*, 1992; Root and MacKinnon, 1994; Zufall and Firestein, 1993). This block, like that in Ca^{2+} channels, was relieved by raising the external pH and by membrane depolarization. This rapid, short-lived subconductance state is independent of cGMP concentration and is distinct from a longer-lived subconductance state observed at low concentrations of cGMP in photoreceptor channels which is thought to be due to the opening of partially liganded channels (Ildefonse and Bennett, 1991; Taylor and Baylor, 1995).

Where are the divalent cation blocking sites in the pore? Where are the multiple sites that bind permeant ions, allowing the pore to contain multiple ions? Where is the proton binding site? Remarkably, the answer to many of these seemingly distinct questions appears to involve a single residue, a glutamate residue in the P region (rod channel: E363; catfish olfactory channel: E333). Root and MacKinnon (1993) and Eismann *et al.* (1994) found that mutation of this residue to neutral residues nearly abolished block by external Mg^{2+}. Both groups report relatively little effect on block by internal Mg^{2+}, suggesting the presence of a distinct divalent cation binding site near the internal mouth of the channel. The mutations also altered the open channel current–voltage relation, causing a pronounced outward rectification, consistent with an electrostatic role of E333 in increasing the local external cation concentration. Sesti *et al.* (1995) further showed that such mutations converted the behavior of the channel from that of a multi-ion pore to that of a single ion pore because the anomalous mole fraction effect was no longer observed.

Root and MacKinnon (1994) demonstrated that this same glutamate residue (E333) was also responsible for external proton block. In a careful analysis, they were able to distinguish three open conductance states in the catfish CNG channel, a fully open state and two subconductance states, corresponding to channels with zero, one, or two protons bound. To explain the presence of only two apparent proton binding sites in a putatively tetrameric channel, Root and MacKinnon proposed that each binding site consisted of two glutamates forming a carboxyl–carboxylate pair that share a single proton. The influence of neighboring negative charges explains why the pK_a of the site (7.6) is much higher than that of a free glutamate carboxyl side chain (4.3). Given the proximity of the two binding sites, one might have expected that the binding of the first proton would inhibit the binding of the second due to electrostatic effects. However, the binding events were independent, suggesting that a carboxyl–carboxylate was never free but was occupied by a monovalent alkali cation when deprotonated. The homologous glutamate residues in calcium channels have been shown to be important for calcium selectivity (Heinemann *et al.*, 1992; Yang *et al.*, 1993) and may function as the site of multi-ion occupancy.

CONCLUSIONS

Significant progress has been made in elucidating the molecular mechanism for the behavior of CNG channels. However, even with this recent progress, at least two fundamental questions remain: What is the molecular mechanism of the allosteric conformational change that opens the pore? And what is the molecular mechanism by which the channel permeates cations?

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