Cyclic Nucleotide-Gated Ion Channels

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Kaupp, U. Benjamin, and Reinhard Seifert. Cyclic Nucleotide-Gated Ion Channels. Physiol Rev 82: 769–824, 2002; 10.1152/physrev.00008.2002.—Cyclic nucleotide-gated (CNG) channels are nonselective cation channels first identified in retinal photoreceptors and olfactory sensory neurons (OSNs). They are opened by the direct binding of cyclic nucleotides, cAMP and cGMP. Although their activity shows very little voltage dependence, CNG channels belong to the superfamily of voltage-gated ion channels. Like their cousins the voltage-gated K⁺ channels, CNG channels form heterotetrameric complexes consisting of two or three different types of subunits. Six different genes encoding CNG channels, four A subunits (A1 to A4) and two B subunits (B1 and B3), give rise to three different channels in rod and cone photoreceptors and in OSNs. Important functional features of these channels, i.e., ligand sensitivity and selectivity, ion permeation, and gating, are determined by the subunit composition of the respective channel complex. The function of CNG channels has been firmly established in retinal photoreceptors and in OSNs. Studies on their presence in other sensory and nonsensory cells have produced mixed results, and their purported...
roles in neuronal pathfinding or synaptic plasticity are not as well understood as their role in sensory neurons. Similarly, the function of invertebrate homologs found in Caenorhabditis elegans, Drosophila, and Limulus is largely unknown, except for two subunits of C. elegans that play a role in chemosensation. CNG channels are nonselective cation channels that do not discriminate well between alkali ions and even pass divalent cations, in particular Ca\(^{2+}\). Ca\(^{2+}\) entry through CNG channels is important for both excitation and adaptation of sensory cells. CNG channel activity is modulated by Ca\(^{2+}\)/calmodulin and by phosphorylation. Other factors may also be involved in channel regulation. Mutations in CNG channel genes give rise to retinal degeneration and color blindness. In particular, mutations in the A and B subunits of the CNG channel expressed in human cones cause various forms of complete and incomplete achromatopsia.

I. INTRODUCTION

Ion channels that are directly activated by cyclic nucleotides [cyclic nucleotide-gated (CNG) channels] are relatively recent arrivals in the world of ion channels. Their discovery was intimately tied with the quest for the intracellular messenger that mediates the photoresponse. Their discovery was intimately tied with the quest for the intracellular messenger that mediates the photoresponse. Their discovery was intimately tied with the quest for the intracellular messenger that mediates the photoresponse.

Within a relatively short time, similar channels were identified in cone photoreceptors (153), chemosensitive cilia of olfactory sensory neurons (OSNs) (293), and the pineal gland (94). Molecular cloning of CNG channels became possible when the channel protein was purified and unequivocally identified by functional reconstitution into artificial liposomes and lipid bilayers (79, 151). Partial amino acid information derived from the purified protein allowed the successful cloning and functional expression of the first CNG channel gene (188). The molecular identification of a CNG channel has sparked much progress over the past several years. It is now obvious that CNG channels are not unique to photoreceptors and OSNs, but are expressed in other neurons and nonneuronal tissues alike. CNG channels belong to a heterogeneous gene superfamily of ion channels that share a common transmembrane topology and pore structure and that harbor in their COOH-terminal region a binding domain for nucleoside 3',5'-cyclic monophosphates (cNMPs). Other members of this superfamily are the so-called hyperpolarization-activated and cyclic nucleotide-gated (HCN) pacemaker channels (for review, Ref. 189), the ether-a-gogo (EAG) and human eag-related gene (HERG) family of voltage-activated K\(^{+}\) channels (for review, Ref. 117), and several plant K\(^{+}\) channels commonly referred to as KAT, AKT, and KST channels (for review, see Ref. 359). The focus of this review is on CNG channels.
binding protein calmodulin and by phosphorylation. The potential mechanisms of this modulation and its physiological significance are presented in section IX.

III. EXCURSION ON CYCLIC NUCLEOTIDES

Studies on cellular cAMP and cGMP signaling frequently relied on the use of chemical derivatives of cAMP and cGMP. Ever since the first substances have been deployed for studies of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) and phosphodiesterases (PDE), the list of new derivatives has grown long. More importantly for the subject of this review, derivatives of cyclic nucleotides were also instrumental for functional studies of CNG channels in various cellular systems. cAMP and cGMP have been modified at the cyclic phosphodiester group, the 2′- and 3′-hydroxyls of the ribofuranose moiety, and at the adenine and guanine ring systems (see Fig. 12). Phosphorothioate derivatives of cAMP and cGMP have been employed to study the activation properties of native CNG channels in photoreceptors (217, 442) and olfactory neurons (217) and the heterologously expressed A1 and A2 channels (217; see sect. vii). Cyclic nucleotides substituted at the C-8 position have proven particularly valuable for cellular studies. For one reason, a number of substituents at C-8 render these molecules more membrane permeant than cAMP and cGMP itself. For example, 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP) and 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP) are 6- and 90-fold, respectively, more lipophilic than cGMP (63). Therefore, these compounds readily penetrate membranes providing a simple route for delivery to the intracellular binding sites. Another favorable feature of most but not all C-8-substituted derivatives is their high potency in activating CNG channels (54, 66, 203, 390, 442). For example, in rod photoreceptors and OSNs, 8-BrcGMP and 8-pCPT-cGMP activate CNG channels at ~10- and 80-fold, respectively, lower concentrations than cGMP (112, 411, 442). Finally, 8-BrcGMP and 8-pCPT-cGMP are poor substrates for several PDE isoforms and resist hydrolysis. 8-pCPT-cGMP is not measurably hydrolyzed by three different PDEs, and the rate of 8-BrcGMP hydrolysis is 4- to 40-fold lower than that for cGMP (63). The PDE6 of rods and cones hydrolyzes 8-BrcGMP 170- to 500-fold more slowly than cGMP (19, 442). Thus the triad of lipophilicity, potency of activation, and resistance to hydrolysis makes these C-8-substituted cyclic nucleotides attractive agents for the study of CNG channels.

Owing to the multiple actions of cyclic nucleotides inside intact cells, however, results obtained with these derivatives may sometimes be difficult to interpret. Delivering, for example, a relatively high concentration of cyclic nucleotides to intact cells will eventually activate all cAMP- and cGMP-dependent processes including phosphorylation by PKA and PKG, activation or inhibition of some PDE isoforms, and eventually cAMP-dependent regulation of gene expression. In addition, hydrolysis-resistant analogs may behave as competitive antagonists that bind to the catalytic site of PDE and thereby hinder hydrolysis of both cAMP and cGMP. As a result, endogenous cyclic nucleotides may accumulate during the course of the experiment. Finally, the infusion of cells with cyclic nucleotides either from a pipette in the whole cell configuration or by bathing in a medium containing membrane-permeable analogs is inherently slow in relation to the speed of action on kinases, PDEs, and CNG channels. Therefore, it will be exceedingly difficult to experimentally dissect the action of cyclic nucleotides on CNG channels from other cellular effects.

This kind of experiment can be ameliorated using chemical derivatives “dubbed” caged cyclic nucleotides. Caged compounds are molecules whose biological activity has been disabled by chemical modification. Photoysis cleaves the modifying group (“uncaging”), thereby rapidly releasing the active molecule. (For a collection of reviews on caged compounds, see Reference 261.) Four different classes of caging groups have been used: 4,5-dimethoxy-2-nitrobenzyl (DMNB), 1-(2-nitrophenyl)ethyl (NPE), desoxybenzoinyl (Desyl), and derivatives of (7-methoxy-coumarin-4-yl)methyl (MCM) (Fig. 1). Synthesis of caged cyclic nucleotides by esterification of the phosphodiester group produces mixtures of axial and equatorial forms. The diastereomers differ significantly in solubility and solvolytic stability (150). We recommend that the pure isomeric forms are used for experiments. To be useful, caged cyclic nucleotides must meet specific requirements.

First, they should dissolve well in aqueous solution (~100 μM-10 mM). The higher the concentration of the caged compound inside the cell, the more cAMP or cGMP is released per flash. Second, caged compounds must be resistant toward solvolysis (the caging group is attached to the cyclic nucleotide through an ester group that can undergo hydrolysis in an aqueous medium). Otherwise, the free cyclic nucleotide is produced during the course of an experiment. Third, caged cyclic nucleotides should display high photoefficiencies, i.e., high molar absorbivities and high quantum yields. Finally, the photochemical reaction that releases the cyclic nucleotide should be fast (~1 ms) compared with the physiological reaction under study.

The NPE- and DMNB-caged compounds either photolyze relatively slowly (NPE) or display rather low photoefficiencies (DMNB) (Table 1). Desyl-caged cAMP is very sensitive to solvolysis in aqueous buffer solution (Table 1), and the MCM-caged cyclic nucleotides are poorly soluble (148). However, caged derivatives of MCM combine a set of favorable properties rendering them...
ideal tools for intracellular studies (Table 1). For example, [6,7-bis(carboxymethoxy)coumarin-4-yl)methyl (BCMCM) esters of cAMP and cGMP are highly soluble (1 mM) (147) and display a high quantum yield (0.1–0.15, Ref. 147; Table 1). The (7-diethylaminocoumarin-4-yl)-methyl (DEACM) derivatives have an even higher quantum yield and absorptivity than the BCMCM-caged congeners. Moreover, MCM-based caged cyclic nucleotides are extremely stable in aqueous solution and react quickly within a few nanoseconds. The CMCM and BCMCM compounds are negatively charged, therefore, their high solubility; consequently, they are poorly membrane permeable and must be introduced into the cell by means of the recording pipette. Recently, bismethoxy and bisethoxy esters of BCMCM have been synthesized. These compounds are neutral, penetrate cell membranes, and accumulate inside the cell due to hydrolysis of the ester groups at the coumaryl moiety (V. Hagen and U. B. Kaupp, unpublished data). They are compounds of choice for studies on cell suspensions.

Caged cyclic nucleotides have been used to study 1) the rate of activation of the rod CNG channel in excised patches (185) and in the whole cell configuration (150, 339); 2) the Ca\(^{2+}\) permeability of CNG channels in intact rods and cones, OSNs, and cell lines (97, 311); 3) the desensitization of the olfactory CNG channel by Ca\(^{2+}\)/calmodulin (CaM) (45); and 4) cyclic nucleotide-stimulated Ca\(^{2+}\) entry in mammalian spermatozoa (417).

IV. CELLULAR FUNCTION

The function of CNG channels has been firmly established in rod and cone photoreceptors, in extraretinal photoreceptors, and in sensory neurons of the olfactory epithelium. Electrophysiological studies of CNG channels in these sensory neurons provided a wealth of information regarding their ligand sensitivity, mechanism(s) of activation, modulation, and ion selectivity. The underlying channel polypeptides have been identified by molecular cloning; coexpression of the respective subunits in cell lines produces channels that recapitulate many, if not all, properties of the CNG channels in their native membranes. Although CNG channels also exist in other neurons and nonneuronal tissues, their specific functions are yet to be determined rigorously. Several other ion channels, whose molecular identity is uncertain or not known, have been speculated to be directly controlled by cyclic nucleotides.

A. CNG Channels in Vertebrate and Invertebrate Photoreceptors

Photoreceptors in invertebrates fall into two morphologically distinct subtypes: rhabdomeric photoreceptors with a microvilli-derived photosensitive structure and ciliary photoreceptors, whereas vertebrate photoreceptors are of the ciliary type. The ciliary-type photoreceptors, whether vertebrate or invertebrate, respond to light either with a depolarization or hyperpolarization, whereas the rhabdomere-type invertebrate photoreceptors respond exclusively with a depolarization. Ciliary photoreceptors, whether depolarizing or hyperpolarizing, vertebrate or invertebrate, use cGMP-signaling pathways. The targets of these pathways are CNG channels that either open or close in response to light. Although the phototransduction mechanism in depolarizing rhabdomeric photoreceptors is not entirely clear, the light-dependent channels belong to the family of trp/trpl channels (365). Notwithstanding this well-established pathway, the presence of CNG channels in rhabdomeric photoreceptors has been considered. We will discuss the function of CNG channels in 1) the hyperpolarizing rods and cones, 2) the pinealocytes, 3) the depolarizing photoreceptor in the parietal eye of some lizards, 4) the hyperpolarizing ciliary photoreceptor of an invertebrate, and 5) rhabdomeric photoreceptors of invertebrates.
Ca$^{2+}$CNG channels close but the exchanger continues to clear Ca$^{2+}$ (reviewed in Refs. 268, 329; Fig. 2). In light, when Ca$^{2+}$ cause it provides the only source for Ca$^{2+}$ to be similar to DMNB-cGMP. J. E. Corrie and D. R. Trentham.

1. CNG channels in the outer segment of rod and cone photoreceptors

Rods respond to a light stimulus with a brief hyperpolarization by closing CNG channels in the surface membrane of the outer segment (for review, see Ref. 431). In the dark, channels are activated by the binding of cGMP, allowing a steady cation current ("dark current") to flow into the outer segment. Light triggers a sequence of enzymatic reactions that leads to the hydrolysis of cGMP. When CNG channels close, the inward current ceases and the cell hyperpolarizes. The enzyme cascade comprises the photopigment rhodopsin (R), the G protein transducin (T), and a PDE. Light stimulation decreases the cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]i) and increases the activity of the guanylyl cyclase (GC) that synthesizes cGMP. The resulting decline in [Ca$^{2+}$]i provides a negative feedback mechanism that controls at least three biochemical processes. First, the activity of the guanylyl cyclase (GC) that synthesizes cGMP is stimulated as Ca$^{2+}$ levels decrease. The Ca$^{2+}$ sensitivity of the GC is relayed by two small Ca$^{2+}$-binding proteins, designated GC-activating proteins (GCAP1 and GCAP2). At rest, when [Ca$^{2+}$]i is lowered to 50-100 nM, the GCAPs prevail in the inactive form with Ca$^{2+}$ bound. In light, when [Ca$^{2+}$]i is lowered to 50-100 nM, Ca$^{2+}$ dissociates from GCAPs; the Ca$^{2+}$-free form then stimulates GC activity (for review, see Refs. 202, 313). Second, the lifetime of active PDE is shortened through the phosphorylation of light-activated rhodopsin (R*) by the rhodopsin kinase. This reaction is mediated by another small Ca$^{2+}$-binding protein, CaM (169, 280). All three reactions by various degrees help to restore the dark state and to adjust the light sensitivity of the cell (reviewed in Ref. 330).

A similar transduction scheme exists in cones, the photoreceptors responsible for vision in bright light. The fundamentally same events underlie phototransduction in rods and cones, and the two photoreceptor types utilize similar protein isoforms of the enzyme cascade. However, the light sensitivity of cones is 30- to 100-fold lower than that of rods, and cones adapt over a wider range of light conditions.

### Table 1. Properties of caged cyclic nucleotides

<table>
<thead>
<tr>
<th>Solubility, μM</th>
<th>λ$_{max}$ nm</th>
<th>Absorbivity, ε[M$^{-1}$ cm$^{-1}$]</th>
<th>φ</th>
<th>Half-life, h</th>
<th>τ</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPE-cGMP</td>
<td>225</td>
<td>255</td>
<td>17,000</td>
<td>0.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NPE-8-BrcGMP$^*$</td>
<td>35</td>
<td>204</td>
<td>19,400</td>
<td>0.33</td>
<td>300</td>
<td>ND</td>
</tr>
<tr>
<td>DMNB-cGMP</td>
<td>125$^*$</td>
<td>347</td>
<td>5,500</td>
<td>0.004</td>
<td>ND</td>
<td>300</td>
</tr>
<tr>
<td>DMNB-8-BrcGMP$^*$</td>
<td>100</td>
<td>346</td>
<td>5,800</td>
<td>0.005</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>MCM-cGMP$^*$</td>
<td>15$^*$</td>
<td>327</td>
<td>13,300</td>
<td>0.21</td>
<td>&gt;500$^*$</td>
<td>Few ns</td>
</tr>
<tr>
<td>DEACM-cGMP$^*$</td>
<td>120</td>
<td>403</td>
<td>19,300</td>
<td>0.25</td>
<td>&gt;1,000</td>
<td>Few ns</td>
</tr>
<tr>
<td>CMC-cGMP$^*$</td>
<td>&gt;1,000</td>
<td>325</td>
<td>11,200</td>
<td>0.1</td>
<td>&gt;1,000</td>
<td>Few ns</td>
</tr>
<tr>
<td>BCMCM-cGMP$^*$</td>
<td>&gt;1,000</td>
<td>347</td>
<td>11,200</td>
<td>0.1</td>
<td>&gt;1,000</td>
<td>Few ns</td>
</tr>
</tbody>
</table>

intensities than rods (reviewed in Ref. 330). It has been suggested that differences in the Ca\(^{2+}\) homeostasis underlie the distinct light sensitivity and adaptation range of the two photoreceptor types. Important elements that control the dynamics and size of the changes in \([\text{Ca}^{2+}]_i\) are cell volume, the rate of Ca\(^{2+}\) clearance by the Na\(^+/\text{Ca}^{2+}\)-K\(^+\) exchanger, the Ca\(^{2+}\)-buffering capacity of the cytoplasm, and Ca\(^{2+}\) entry through CNG channels (see Ref. 274 for a thorough discussion). Several observations demonstrate that the CNG channels in rods and cones differ in ion permeation, ligand sensitivity, and modulation by Ca\(^{2+}\). The relative ion permeability \(P_{\text{Ca}}/P_{\text{Na}}\) of CNG channels is more than three times larger in cones than in rods (21.7 and 6.5, respectively; Refs. 322, 414), and under physiological ionic conditions, the fraction of the dark current carried by Ca\(^{2+}\) is about twofold larger in cones than in rods (311, 318). The Na\(^+/\text{Ca}^{2+}\)-K\(^+\) exchange current in cones is at least one order of magnitude larger than that in rods. From these observations it has been inferred that the light-stimulated changes in \([\text{Ca}^{2+}]_i\) are far larger and faster in cones compared with rods.

The cGMP sensitivity of the CNG channel and its modulation by Ca\(^{2+}\) is also different in intact outer segments of rods and cones. At elevated \([\text{Ca}^{2+}]_i\) (i.e., in the dark state), the \(K_{1/2}\) for cGMP can be as large as 550 \(\mu\text{M}\) in cones (mean \(K_{1/2} = 335.5 \, \mu\text{M}\); Ref. 333) compared with rods (37.8–40 \(\mu\text{M}\); Refs. 295, 352). In truncated or electropermeabilized rods, the Ca\(^{2+}\)-dependent modulation of the ligand sensitivity is only 1.5- to 2-fold, similar to the effect of Ca\(^{2+}\)/CaM on detached membrane patches (295, 333, 352). In contrast, the range of the CNG channel modulation in intact cones is much wider than in rods and is not well mimicked by Ca\(^{2+}\)/CaM in detached patches from the outer segment (145). This has led to the hypothesis that an unknown factor, which is lost upon patch excision, is responsible for the larger range of modulation of the ligand sensitivity in cones (333). In summary, the cGMP sensitivity, its modulation by \([\text{Ca}^{2+}]_i\), and the Ca\(^{2+}\) permeation are profoundly different in CNG channels of rods and cones, supporting the notion that the CNG channel is a pivotal determinant of the dynamics of Ca\(^{2+}\) homeostasis in vertebrate photoreceptor cells.

CNG channels in cones serve a second function that is absent in rods. Light produces a graded hyperpolarization in rods and cones that is up to 35 mV in amplitude. Not all of this response range is effectively transmitted to the postsynaptic bipolar and horizontal cells. The highly nonlinear input-output relation of the rod synapse is largely accounted for by the voltage dependence of presynaptic Ca\(^{2+}\) channels. At the dark resting voltage of −35 mV, a fraction of the Ca\(^{2+}\) channel is open, and the continuous Ca\(^{2+}\) entry sustains a tonic release of the neurotransmitter glutamate from the synaptic terminal. The Ca\(^{2+}\) channels are characterized by an activation threshold of approximately −45 mV (14). Therefore, when a rod is hyperpolarized to values more negative than approximately −45 mV, the Ca\(^{2+}\) channels close and synaptic transmission ceases (12, 27). In contrast to rods, synaptic transmission in cone photoreceptors continues as the light-induced voltage response grows to −70 mV.

FIG. 2. Ca\(^{2+}\) feedback mechanism in rod and cone photoreceptors involving cyclic nucleotide-gated channels. PDE, phosphodiesterase; GCAP, guanylyl cyclase-activating protein.
Cyclic nucleotide–gated ion channels

(23, 114, 308). Whereas the small overlap of the voltage range of \( \text{Ca}^{2+} \) channel activation and the voltage range produced by light can explain signal clipping at the rod synapse, it fails to explain the broader voltage range over which synaptic transmission operates in cones. This conundrum has been partially solved by the discovery of CNG channels in the inner segment and synaptic terminal of cones (341, 358). The density of CNG channels in the inner segment is low, whereas in the cone terminal these channels appear to come in clusters (358). If the clusters were located near release sites, CNG channels would be ideally suited to control the \( \text{Ca}^{2+} \)-dependent release of glutamate. In fact, experimental maneuvers that activate CNG channels also trigger exocytotic events and release glutamate from the cone terminal (341, 358). The cGMP sensitivities measured in patches of membrane excised either from the outer segment or the axon terminal are indistinguishable, suggesting that CNG channels from both locales are built from identical or similar subunits. The cGMP sensitivity of the CNG channels in the synapse is as unusually low as that of CNG channels in the cone outer segment of the fish retina (\( K_{1/2} = 206 \) and 305.5 \( \mu \text{M} \), respectively; Refs. 333, 358). We note, however, that the high \( K_{1/2} \) value in fish cones required an intact cone photoreceptor, whereas the \( K_{1/2} \) of synaptic channels was determined in excised patches.

CNG channels could serve two different functions in the cone synapse. First, these channels might extend the voltage range over which synaptic transmission operates by providing a sustained \( \text{Ca}^{2+} \) influx even at very negative voltages. Second, nitric oxide (NO) is a good candidate to serve as retrograde neurotransmitter that is released onto cone terminals from other retinal cells (358). An NO synthase (NOS) is predominantly found in the inner segment of rods and cones and in processes of bipolar cells in the outer plexiform layer of the retina (204, 227, 240). Furthermore, a soluble form of guanylate cyclase (sGC) is found in the inner segment of cones and is stimulated by NO (204). Thus CNG channels may play an important role in the modulation of synaptic transmission by NO in the axon terminals of cones.

2. CNG channels in pinealocyte photoreceptors

The pineal regulates various physiological functions by nocturnal secretion of the hormone melatonin. Light sensitivity of the pineal has been retained in most vertebrates, except mammals. Pinealocytes, the light-sensitive cells, display hyperpolarizing responses to brief pulses of light (328, 398) and express several retinal proteins including arrestin, recoverin, rhodopsin kinase, phosducin, GC, and a cGMP-specific PDE (for review, see Refs. 212, 250). Dryer and Henderson (94) recorded CNG channel activity from excised inside-out patches of dissociated photoreceptors from the chick pineal. These CNG channels in extraretinal photoreceptors feature all the hallmarks of CNG channels in retinal photoreceptors (94, 95). Activation is half-maximal between 10 and 50 \( \mu \text{M} \) cGMP. Even fully activated channels display frequent brief transitions to the closed state. For this reason, the open probability (\( P_o \)) becomes not unity at saturating cGMP concentrations. The brief closing events are more frequent at negative than at positive membrane potentials. Similar properties have been reported for CNG channels from retinal photoreceptors (158, 262, 305). Moreover, expression of several CNG channel subunits in the pineal has been confirmed by in situ hybridization and immunohistochemistry (see sect. vi). These results collectively show that the light response in pinealocytes of lower vertebrates is produced by activation of a cGMP-signaling pathway, which leads to the closure of cGMP-selective ion channels. Chick pineal cells display a circadian rhythm in cGMP concentration (152, 388). It is therefore conceivable that CNG channels are involved in regulating the output of the intrinsic circadian oscillator.

3. CNG channels in parietal-eye photoreceptors

Some lizards do have a parietal-eye, or third eye, on top of their head. The parietal eye seems likely to convey information about changes in light intensity and spectral composition during dusk and dawn. The parietal-eye photoreceptors resemble in their morphology rod and cones of the vertebrate retina, yet they depolarize in response to a flash of light (377). This suggested that parietal-eye photoreceptors, like rhodometric photoreceptors of the invertebrate eye, might utilize a phosphoinositide-signaling cascade rather than the cGMP-signaling pathway of retinal rods and cones. It came as a surprise when Finn et al. (105) convincingly demonstrated that the outer segment membrane of the parietal-eye photoreceptors harbors a high density of CNG channels with all the hallmarks of CNG channels from rods and cones: the channels are selectively activated by cGMP, cAMP is much less effective, the channels are nonselective among monovalent cations and are permeable to \( \text{Ca}^{2+} \) ions, channels are blocked by \( \text{l-cis-diltiazem} \), and \( \text{Ca}^{2+}/\text{CaM} \) reduces the cGMP-activated current by reducing the ligand sensitivity.

What type of CNG channel is expressed in the parietal-eye? CNG channels of retinal cones are significantly more \( \text{Ca}^{2+} \) permeable than those of rods (113, 146, 155, 322). The relative selectivity for \( \text{Ca}^{2+} \) over alkali cations has been determined from reversal potentials (\( V_{rev} \)) under well-defined ionic conditions in excised patches from rod and cone (322) and parietal-eye photoreceptors (105). In cones of striped bass, \( P_{\text{Ca}}/P_{\text{Na}} = 21.7 \); in rods of tiger salamander, \( P_{\text{Ca}}/P_{\text{Na}} = 5.9 \) (146); and in the parietal-eye, \( P_{\text{Ca}}/P_{\text{Na}} = 8.1-10.3 \) (105). Thus, at least with respect to the \( \text{Ca}^{2+} \) permeability, the CNG channel in parietal-eye pho-
oreceptors behaves more like the CNG channel of rods than that of cones. However, permeability ratios of native CNG channels are not invariant but depend on the cGMP concentrations (146; see sect. viii). When comparing relative ion permeabilities, this complication must be kept in mind.

The depolarizing light response is produced by an increase in the cytosolic cGMP concentration that is controlled by an unusual cGMP-signaling pathway (426). In the dark, cGMP is synthesized continuously by GC activity and rapidly degraded by PDE activity. The elevated PDE activity in the dark seems to rest on a constitutively active G protein, whereas the mechanism that keeps GC active in the dark is not known. Light acts by inhibiting the PDE through another G protein, permitting the cGMP concentration to rise and CNG channels to open.

4. CNG channels in hyperpolarizing photoreceptors of invertebrates

The retina of some molluscan eyes is composed of two layers of photoreceptors: depolarizing rhabdomeric-type cells, similar to those found in most other invertebrates, and ciliary photoreceptors that hyperpolarize in light (269). The mechanism underlying the hyperpolarizing light responses has been studied in two scallop species, Pecten and Lima. Light stimulation under voltage clamp activates an outward current that is accompanied by a decrease in the cellular input resistance. The V_{rev} of the light-stimulated current lies near the equilibrium potential for K^+ (E_K) (126), demonstrating that the light-dependent channel is highly K^+ selective and that the hyperpolarizing light response is brought about by opening K^+ channels rather than by closing nonselective cation channels, as in retinal rods and cones. In a series of incisive experiments, Gomez and Nasi (85) convincingly demonstrated that 1) the inositol trisphosphate (IP_3)/Ca^{2+}-signaling pathway is not crucial for phototransduction, 2) the photoreceptors rely on cGMP as the internal messenger of the transduction cascade, and 3) the light-dependent channel is opened by cGMP. The latter two observations imply that light elevates cGMP, although it is unknown whether this involves the inhibition of a PDE or the stimulation of a GC.

In contrast to the Ca^{2+}-permeable CNG channels of retinal photoreceptors and OSNs, the Pecten channel is virtually impermeable to Ca^{2+}, and the K_v/2 values for blockage by extracellular Ca^{2+} and Mg^{2+} are 1–2 orders of magnitude higher (299). The significant K^+ selectivity, the lack of Ca^{2+} permeability, and the weak divalent block suggest that the pore architecture is more like that of K^- channels than that of CNG channels. It is interesting to note that a cGMP-sensitive K^+ channel also seems to underlie the light response of a photosensitive neuron in the abdominal ganglion of a marine mollusc (136). This cell generates slow, depolarizing light responses due to the closure of K^- channels that are kept open in the dark by cGMP. The protein(s) forming the cGMP-dependent K^- channel is unknown. Its molecular identification is eagerly awaited, as it will certainly further our understanding of the molecular mechanisms that govern ion selectivity in CNG channels.

5. CNG channels in rhabdomeric photoreceptors of invertebrates?

During the 1970s and 1980s, when the cGMP-signaling pathway in vertebrate photoreceptors was elucidated, several groups examined the possibility that light also regulates the cGMP (or cAMP) concentration in depolarizing rhabdomeric photoreceptors of invertebrates and that cGMP mediates the light response by opening ion channels in the microvilli membrane. Injection of cGMP into Limulus ventral photoreceptors produced a depolarization that mimics the receptor potential (176). Superfusion with cGMP of membrane patches excised from the light-sensitive lobe of the ventral photoreceptor activated channels that closely resembled the channels activated by light in cell-attached patches (13). In contrast, perfusion of photoreceptors from Drosophila eyes with various cGMP analogs was without effect (73). The cDNAs of CNG channel subunits have been cloned from Drosophila melanogaster and Limulus polyphemus (22, 69, 278). The precise sites of expression of the two channel subunits are not known. Further advances with respect to the function of CNG channels in invertebrate brain will require precise cellular and subcellular localization of the channel proteins.

B. CNG Channels in Chemosensory Cells

Chemosensory cells of vertebrates can be subdivided in three major subgroups: olfactory sensory neurons, neurons of the vomeronasal organ, and taste receptor cells. For each of these different senses, the involvement of CNG channels in signal transduction has been proposed. Much less is known about chemosensory transduction in invertebrates. Recent studies with the nematode C. elegans, however, suggest that CNG channels may play an important role in chemotaxis to odorants and salt in this model organism. In this section, we examine the evidence for the involvement of CNG channels in vertebrate and invertebrate chemosensation.

1. OSNs

OSNs are embedded in the olfactory epithelium lining the cavity of the nose. OSNs are bipolar neurons; a single dendrite extends to the apical surface of the neuroepithelium, and a single axon projects to the olfactory
bulb. From the tip of the dendrite ~20–50 thin cilia extend into the layer of mucus that covers the epithelium. The cilium is the site where the chemoelectrical transduction takes place. It is the functional equivalent of the outer segment of retinal photoreceptors. It houses all the molecular components to register odorants, to amplify the signal by a series of enzymatic reactions, and to generate the electrical response. Like rods, OSNs are exquisitely sensitive; they can respond to stimulation by a few odorant molecules. This high sensitivity is accompanied by a rich selectivity. Humans are probably able to discriminate between more than 10,000 or so different odorous compounds. This enormous achievement is endowed by an array of several hundreds up to a thousand odorant receptors with overlapping specificity for a few odorants (for review, see Ref. 327).

Quite remarkably, a cousin of the retinal CNG channels takes center stage in odorant signaling; the vast majority of OSNs respond to brief pulses of odorants with a transient receptor current by opening cAMP-gated channels in the ciliary membrane (107, 293). In contrast to the outer segment of photoreceptors, where the light-sensitive current is solely carried by CNG channels, the receptor current originating in chemosensitive cilia has two ionic components: an inward cationic component mediated by CNG channels, followed by an inward anionic component mediated by Ca$^{2+}$-activated Cl$^{-}$ channels. In addition to cAMP, other signaling molecules have been implicated in odorant transduction, especially the two gaseous messengers NO and carbon monoxide (CO). We will critically examine the experimental foundation for these hypotheses.

Finally, a cGMP-signaling pathway that is targeting a highly cGMP-selective CNG channel has been identified in a small subset of OSNs, whereas components that furnish the prototypical CAMP-signaling pathway are absent in those cells. These observations provide compelling evidence that cGMP serves as the principal messenger for chemosensory signaling in some OSNs.

**A) THE PROTOTYPICAL CAMP-SIGNALING PATHWAY.** A milestone in olfactory research was the cloning of a family of odorant receptors by Buck and Axel (61). Since then a vast number of putative odorant receptors have been cloned. Although functional expression of these receptors in heterologous systems has been accomplished in only a few cases (219, 415), it is taken for granted that these receptors feed into a common CAMP-signaling pathway. The principal players of this signaling pathway are shown in Figure 3, top. The binding of odorants to their cognate receptors in the membrane of chemosensitive cilia first activates a G protein (Golf) and then an adenylyl cyclase (ACIII). The ensuing rise in the concentration of cAMP opens CNG channels and thereby produces a depolarization of the cell membrane. Like its retinal cousins, the CNG channel in OSNs is highly Ca$^{2+}$-permeable (97, 113, 294), and channel activation causes a rapid increase of [Ca$^{2+}$]$_i$ (230, 232). The odorant-stimulated rise of [Ca$^{2+}$]$_i$ plays an important role in both excitation and odor adaptation. It serves as a feedforward signal that enhances the depolarizing response by activating Ca$^{2+}$-dependent Cl$^{-}$ channels, which, in fact, carry a large fraction of the receptor current (197, 220). In rat OSNs, as much as 85% of the olfactory response can be mediated by Cl$^{-}$ channels (251). The increase of [Ca$^{2+}$]$_i$ serves also as a delayed negative feedback signal that reduces the cAMP sensitivity of the CNG channels and stimulates cAMP hydrolysis by a Ca$^{2+}$-dependent ciliary form of PDE (PDE1C2) (42). Both processes, channel desensitization and PDE activation, are controlled by Ca$^{2+}$/CaM (42, 72, 248).

In a series of elegant double-pulse experiments using short puffs of odorants or flashes of ultraviolet light that rapidly release cAMP from a caged compound, Kurahashi and Menini (224) show that the principal mechanism underlying odorant adaptation acts at the CNG channel and that regulatory mechanisms upstream of the channel contribute little to the odorant-induced reduction of the cell’s sensitivity, at least on a time scale of several tens of seconds. Additional mechanisms of adaptation that appear to operate on a longer time regime include phosphorylation of odorant receptors (38) and adenylyl cyclase (412).

**B) NO AND CO: GASEOUS MESSENGERS INVOLVED IN ODORANT SIGNALING?** NO is a short-lived molecule capable of diffusing across membranes and reacting with a variety of targets. It is produced by various isoforms of NOS, some of which are activated by Ca$^{2+}$/CaM. The physiological concentrations of NO are in the picomolar range (8). The most common action of NO involves the activation of sGC, i.e., NO stimulates the synthesis of cGMP. However, NO can also regulate the activity of various proteins by reacting with cysteine sulfhydryls, a covalent modification known as S-nitrosylation (for review, see Ref. 50). The direct activation by NO was also reported for the olfactory CNG channel (52). Perfusion of membrane patches excised from the soma or dendrite of OSNs of tiger salamander with NO donors like S-nitrosocysteine (SNC) produced single-channel events similar to those observed in the presence of cAMP. NO-stimulated channel activity persisted for up to 30 min in the absence of NO donors and was mimicked by sulfhydryl-modifying reagents like N-ethylmaleimide (NEM), iodoacetamide (IAA), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in a reversible manner. SNC was also a potent agonist of the homomeric channel by NO (412).
sensitive (51). These observations collectively support the notion that NO works through S-nitrosylation. However, it is noted that CNG channels in rod and cone photoreceptors and from C. elegans do not become activated by covalent modification with NO (209, 358, 400), although their respective A subunits carry a cysteine residue at a homologous position.

Stimulation of OSNs with a short pulse of 500 μM SNC in the whole cell recording configuration elicits a sizable inward current that, in contrast to the current in excised patches, returns to baseline within 1.5 s (52). The authors reasoned that this current was due to covalent modification by NO, because the recording pipette contained no GTP, which is required for cGMP synthesis. This observation then indicates that the S-nitrosylation product inside the cell is 100–1,000 times less stable than in the excised membrane patch. Although redox reactions inside the cell might rapidly reverse the S-nitrosylation, it is conceivable that some endogenous GTP was still available to support cGMP synthesis either because equilibration between the pipette and the cell interior was not complete and/or because GTP was generated from ATP by transphosphorylation reactions involving guanylate kinase and nucleotide diphosphate kinase activity (see Ref. 422 and references therein). The chemical nature of the modification in native channels from OSNs and heterologously expressed A2 subunits seems to be different as well. The NO-induced channel activity persisted for many minutes in excised patches of OSNs, but rapidly faded with a rate constant of 3.8 s⁻¹ (τ = 260 ms) in patches with the A2 homomeric channel (51). Such a rapid decay would seem incompatible with the formation of high-molecular-weight nitrosothiol species, which are stable on a time scale of several minutes to hours (378). This raises the intriguing possibility that reversible binding of either SNC or NO itself gates the channel open.

Lynch (256), working with rat OSNs, was unable to reproduce the actions of NO donors and sulfhydryl-modifying reagents on the native olfactory CNG channel. In fact, the NO donors SIN-1 and SNC at moderate concentrations inhibit the cAMP-stimulated currents rather than activating the unliganded channels. Moreover, the oxidizing agent DTNB caused a permanent inhibition. Inhibition brought about by either agent was reversed by a 2-min wash with dithiothreitol. The similar inhibitory effects of NO and DTNB suggest that two neighboring S-nitrosothiols combine to form a relatively stable disulfide bond. Because NEM displays no inhibitory action, it can be
concluded that alkylation of either one or both of the paired cysteines does not replicate the effect of the desulfide formation.

Future work must establish the physiological function of covalent NO modification of the olfactory CNG channel. Two questions are particularly pertinent. The concentration of NO in the olfactory epithelium has not been determined; therefore, it is not known whether the concentrations of NO in the ciliary layer reach the high levels of NO needed to activate the olfactory channel (125). Second, NO potently activates sGC in cells (EC$_{50}$ ~20–250 nM; Refs. 28, 380). The high sensitivity raises the question what fraction of CNG channels become activated by S-nitrosylation at NO concentrations that fully activate sGC and thereby cGMP synthesis.

An alternative mechanism of the action of NO in odorant signaling involving sGC and cGMP was proposed by other groups. These investigations were stimulated by the fact that the CNG channel of chemosensitive cilia is exquisitely sensitive to both cAMP and cGMP. In fact, the $K_{1/2}$ of half-maximal activation is twofold lower for cGMP than for cAMP (1.0–2.4 μM and 2–4 μM, respectively; Refs. 40, 58, 72, 112, 208, 293). The slightly higher sensitivity for cGMP spurred several authors to explore a potential physiological role for channel regulation by cGMP.

Breer and Shepherd (49) proposed that the NO/cGMP system is involved in both excitation and some form of olfactory adaptation. What is the experimental evidence for these hypotheses? Hefty doses of odorant stimulate a retracted elevation of cGMP concentration in isolated olfactory cilia or cultured OSNs (48, 220, 404). The response is abolished by $N^\omega$-nitro-L-arginine, a selective inhibitor of NO formation by NOS. These observations have been interpreted to indicate that an odorant-induced Ca$^{2+}$ influx activates NOS via Ca$^{2+}$/CaM and, thereby, initiates NO production. The highly membrane-permeable and diffusible NO might activate sGC in this and in neighboring cells. Specifically, Breer and Shepherd (49) proposed that recruitment of adjacent neurons via the NO/cGMP system could serve as a mechanism to encode very intense stimuli. The NO/cGMP system, however, is unlikely to play such a role in the adult epithelium, because developing and regenerating OSNs, but not mature OSNs, contain NOS activity (47, 196, 221, 345).

A variation of this theme has been proposed by Zufall and collaborators (234, 235, 444). These authors provide evidence that the CO/cGMP system might be responsible for a long-lasting form of odor adaptation. Variants of this hypothesis can be traced to previous reports showing that pretreatment of olfactory preparations with membrane-permeable cGMP derivatives attenuates the second messenger response to odorant stimuli in rat cilia and the receptor current in the olfactory epithelium of the bullfrog (319). CO, like NO, stimulates the synthesis of cGMP by sGC (59, 115, 193); therefore, CO has been proposed to serve as an endogenous gaseous messenger in the nervous system (260). Indeed, OSNs contain high levels of the CO-producing enzyme heme oxygenase-2 and produce CO upon stimulation with odorants (173, 174, 404).

How might CO engender odor adaptation? Odor stimulation elicits two kinetically distinct inward currents: a large and fast transient current mediated by the cAMP-signaling system, followed by a small and persistent current of only a few picamperes in amplitude (444). It is this “background” current that appears to depend on cGMP and is underlying long-term adaptation. The authors specifically proposed that the small background current is flowing through CNG channels and that cGMP-dependent Ca$^{2+}$ entry is a crucial step in the development of long-lasting adaptation. The results are based on an experimental protocol involving successive puffs of odorant. The first conditioning pulse of odorant provides the reference cellular response and sets into motion the adapting processes. The second test pulse probes the change in odorant sensitivity caused by the first pulse. Using this experimental design, Kurashashi and Menini (224) and Reisert and Matthews (335) identified a form of odorant adaptation that is virtually instantaneous and operates on a time scale of a few seconds. The amplitude of the adapted response depends on the time of the delivery of the test pulse. For short interpulse times (~1–5 s), the response amplitude is significantly reduced; amplitudes gradually increase with the interpulse time span (224, 230, 335). The time constant for complete recovery of the sensitivity is of the order of 3–10 s and depends on the odorant concentration of the conditioning pulse (224); intense stimuli require longer times for complete recovery than weaker stimuli. The recovery time critically depends on the rate of Ca$^{2+}$ clearance from the cell by a Na$^+$/Ca$^{2+}$ exchange mechanism (230, 335), underpinning the idea that Ca$^{2+}$/CaM modulation of the CNG channel accounts for most odorant adaptation.

The understanding of long-term adaptation is incomplete. For example, does a stimulated cell enter a state of short-term adaptation first, then recover from stimulation, and finally transit to a state of long-lasting adaptation? For obvious reasons, the paired-pulse protocol (pulse every 30 s) designed to study long-term adaptation does not cover the time regime of short-term adaptation (<10 s) (444). Therefore, it is not clear whether the lower sensitivity revealed by the second pulse reflects a delayed or incomplete recovery from the first pulse rather than a slowly progressing adaptation. How does long-term adaptation relate to short-term adaptation if a Ca$^{2+}$-dependent shift of CNG channel sensitivity is underlying both forms of adaptation? It is intriguing that cells recover from the massive Ca$^{2+}$ influx during the cAMP-mediated odorant response within 5–10 s or so (224, 230, 335), whereas it takes several minutes to recover from the much smaller cGMP-mediated response. Does this finding imply that the
feedback mechanisms, i.e., hydrolysis of the cyclic nucleotide and extrusion of Ca$^{2+}$, are different for the cAMP and cGMP response? Because the rate of clearance from the Ca$^{2+}$ load by the Na$^+/Ca^{2+}$ exchanger sets the recovery time (335), the long-term effects in some cells might result from an altered Ca$^{2+}$ homeostasis that is characterized by a slower rate of Ca$^{2+}$ clearance. This interpretation is supported by the observation that only a subpopulation of cells displays long-term adaptation.

C) A cGMP-selective CNG channel in mammalian chemosensory neurons. A small population of OSNs that project to a group of atypical glomeruli in the main olfactory bulb, the so-called necklace glomeruli, houses a different repertoire of signaling molecules (178, 273). This subgroup of OSNs expresses an olfactory-specific guanylyl cyclase (GC-D), a cGMP-stimulated isof orm of PDE (PDE2), and a cGMP-selective CNG channel (splice variant of the cone A3). These three proteins are highly enriched in the chemosensitive cilia. Most intriguingly, the characteristic markers for the enzymatic makeup of the prototypical cAMP pathway in OSNs, i.e., $G_{s}$, ACIII, PDE1C2, and three distinct CNG channel subunits (A2, A4, and B1b), are absent from this subset of neurons (see Fig. 3, bottom). These findings rule out the coexistence of the known cAMP-signaling pathway and this novel cGMP-dependent pathway and argue for cGMP as the principal messenger in this subset of OSNs. GC-D is a member of the family of receptor-type GCs that become activated by binding of peptide hormones to the extracellular domain (111). Although no ligand has yet been identified for GC-D, this subgroup of OSNs may not respond to normal volatile odorants, but, possibly, to ligands that control some aspects of reproductive behavior. Because very few OSNs use this cGMP-signaling pathway, it has not been feasible to electrically record from these cells. It is, however, anticipated that stimulation of this unique cell type with the cognate ligand of GC-D will produce a depolarizing receptor current and an increase in [Ca$^{2+}$], by opening cGMP-selective channels.

2. The vomeronasal organ

The vomeronasal organ (VNO) or Jacobson’s organ is a chemosensitive organ present in most vertebrates. It is important for the detection of pheromones that convey information between individuals of the same species. The sensory neurons of the VNO have a bipolar organization, and their axons terminate in a specialized brain region, the accessory olfactory bulb. In situ hybridization studies revealed that a modulatory CNG channel subunit that is also found in OSNs (A4, see nomenclature in sect. v) is reportedly expressed in these neurons, whereas the principal A2 subunit of OSNs is lacking (30). The A4 subunit itself does not form functional CNG channels (44, 242), raising the intriguing possibility that it might coassemble with members of other channel families.

Alternatively, the A4 subunit might become activated by another ligand of unknown nature. Indeed, Broillet and Firestein (53) reported that the A4 subunit, when heterologously expressed, produced NO-activated Ca$^{2+}$-selective channels and that single NO-activated channels from VNO neurons have some properties in common with the heterologously expressed channels. The significance of these findings is unclear, because recent evidence suggests that signaling in the VNO is mediated by the phospholipase C/IP$_3$ pathway and TRP channels (231, 243, 270, 445).

3. Taste receptor cells

A cyclic nucleotide-sensitive conductance with quite unique properties has been described in a subset of taste cells in the frog. Superfusion of excised inside-out patches from the apical end of frog taste receptor cells suppressed a current (207) that reversed at about ~50 mV under symmetrical biionic conditions (110 mM intracellular K$^+$/110 mM extracellular Na$^+$), arguing that the channel is K$^+$ selective. Maneuvers intended to raise the intracellular cGMP concentration (perfusion with IBMX or 8-Br-cGMP) reduced whole cell inward currents that reverse at ~0 mV. The discrepancy between results acquired in the excised-patch and whole cell configuration raises questions as to the nature of the ionic conductance. The underlying channels appear to have a ligand sensitivity and selectivity that are both distinctively different from those of other CNG channels. The action of cAMP and cGMP on excised patches was exquisitely sensitive ($K_{1/2} = 77–160$ nM and $K_{1/2} = 16–36$ nM, respectively). Moreover, cAMP and cGMP are significantly more potent than their 8-bromo-substituted analogs, whereas for the photoreceptor and olfactory CNG channels the opposite is true. The dependence of current suppression on the concentration of cAMP, cGMP, and 8-bromo-substituted congeners is described by a simple binding isotherm (Hill coefficient $n$ is unity). From the indirect modulation by cyclic nucleotides of Ca$^{2+}$-activated K$^+$ channels in the same patch, it has been inferred that the cNMP-suppressible conductance is also Ca$^{2+}$ permeable, although this interpretation has not been substantiated by direct demonstration of Ca$^{2+}$ permeation. The authors propose that tastants, by binding to G protein-coupled receptors, activate transducin, which is also present in taste cells (351), which in turn activates a CAMP-specific PDE. The ensuing drop in cAMP concentration activates the CAMP-suppressible inward current, leading to membrane depolarization and a rise of [Ca$^{2+}$]. The molecular identity of the CNG channel isom is
activated rather than inactivated by cyclic nucleotides and, therefore, unlikely to form the cNMP-suppressible conductance alone.

4. Chemosensation in invertebrates

A precedent for chemosensory signaling using cGMP-selective CNG channels exists in the nematode C. elegans (74, 210). The tax-2 and tax-4 genes of C. elegans encode two distinct CNG channel subunits required for chemosensation and thermosensation. Tax-2 and tax-4 mutants display defects in the chemotaxis to volatile compounds and salts, as well as in thermotaxis. Mutations in either gene affect similar behavioral responses, and tax-2 and tax-4 are expressed in the same olfactory, gustatory, and thermosensory neurons, suggesting that the native channel is composed of these two subunits.

The ceB (Tax-2) and ceA (Tax-4) proteins have been localized to sensory neurons that also express some of the 29 different receptor GC genes of C. elegans. The homomeric ceA channel and the heteromeric ceA/ceB channel highly prefer cGMP over cAMP. Both observations suggest that the CNG channels mediate an electrical response that relies on a cGMP-rather than a cAMP-signaling pathway.

In addition to their function in sensory transduction, both the tax-2 and tax-4 genes play a role in the guidance of sensory axon outgrowth during development and the maintenance of normal axon morphology throughout the adult stage (74, 75). In mammals, CNG channels may serve a similar function because CNG channel activity is present in growth cones of cultured OSNs (181), and the A4 subunit was immunohistochemically localized to growing fibers of cultured hippocampal neurons (46).

The congruence of developmental and behavioral defects in C. elegans mutants might be explained by either a primary defect in neuronal connectivity that perturbs normal behavior or by the fact that the correlated neuronal activity is required to refine synaptic connections during development like in sensory systems of vertebrates (75). This latter hypothesis was specifically tested in mice lacking a functional cAMP-sensitive CNG channel due to targeted disruption of the A2 subunit gene. Two groups reported that the peripheral olfactory projections are in part influenced by neuronal activity (438, 439), whereas another group concluded that the olfactory CNG channel, and by inference chemosensory activity, is not required for generating synaptic specificity in the olfactory bulb (244).

C. CNG Channels in the Brain

1. cGMP-sensitive currents in retinal neurons and glia cells

Bipolar cells are retinal interneurons that receive synaptic input from photoreceptors. Glutamate, released from the synaptic terminal in the dark, hyperpolarizes ON-bipolar cells (301, 375) through activation of a metabotropic glutamate receptor (mGluR6; Ref. 292) and subsequent suppression of a nonselective cation current. When glutamate release ceases in the light, the bipolar cells depolarize (ON-response). The nonselective cation current is enhanced when cGMP was introduced into the cell through a patch pipette, indicating that the current might flow through CNG channels. The mGluR6 receptor is thought to signal through a G protein to a PDE. Activation of the PDE would stimulate cGMP hydrolysis, thereby closing the CNG channel. This led to the concept that a cGMP-signaling pathway similar to that in the photoreceptor outer segment operates in ON-bipolar cells. However, subsequent experiments did not support this concept (300). When intracellularly perfused with the hydrolysis-resistant analogs 8-pCPT-cGMP and 8-BrcGMP or the PDE inhibitor IBMX, bipolar cells continue to respond to glutamate, and no difference was observed in the kinetics and the amplitude of the response (300), whereas similar treatments of rods result in profoundly altered kinetics and size of light responses (355, 442). These results argue against regulation of the glutamate-sensitive current by PDE activity. Moreover, the properties of the presumptive cGMP-sensitive channel do not match those of known CNG channels. For example, the cGMP-sensitive current in bipolar cells is not blocked by extracellular Ca$^{2+}$ (376). Finally, several different antibodies against known subunits of retinal CNG channels (A1, A2, A3, and B1a, see sect. v) do not label ON-bipolar cells (408; F. Müller, unpublished observations). These results argue against the presence of a photoreceptor-type CNG channel in ON-bipolar cells.

There is also some suggestion of a rodlike CNG channel in retinal ganglion cells (2, 190). About one-half of the ganglion cells in the rat retina respond to stimulation by NO with an inward current (190). This current is mimicked by either intracellular perfusion with cGMP or extracellular application of the membrane-permeable analogs 8-BrcGMP and 8-pCPT-cGMP, suggesting that the cGMP-sensitive current flows through CNG channels. This conclusion appears to be supported by in situ hybridization and PCR that detect transcripts coding for an A1 subunit in ganglion cells (2).

However, an electrophysiological study in salamander (164) found no evidence that treatment with membrane-permeant cGMP analogs, IBMX, or NO donors led to the activation of CNG channels. Moreover, the cGMP-sensitive current displays properties that do not match the properties of known CNG channels. First, the blockage of the current by divalent cations is weak and does not result in a strong outward rectification, i.e., the voltage dependence of blockage is weak. Second, the current in ganglion cells reverses at roughly 0 mV under bionic conditions (Cs$^+$ inside, Na$^+$ outside), whereas CNG channels would display a distinc-
tively more positive $V_{\text{rev}}$ under similar ionic conditions (see sect. viii on ion selectivity). Third, under these ionic conditions, the current ($I$)-voltage ($V$) relations of CNG channels display a distinctive inward rectification, due to the smaller conductance of CNG channels for $\text{Cs}^+$ compared with $\text{Na}^+$ (see, for example, Refs. 112, 255, 271, 309, 416), whereas the cGMP-sensitive current in ganglion cells is rectifying in the outward direction (2). Moreover, antibodies against known CNG channel subunits do not label ganglion cells in the mammalian retina (408; Müller, unpublished observations). With the proviso that CNG channels in photoreceptors and ganglion cells are antigenically similar, a low channel density cannot explain this failure. As an example, in the rod outer segment, 1–2% of open CNG channels sustain a current of 30 pA (297, 434), whereas the cGMP-sensitive conductance in ganglion cells carries currents up to 500 pA.

8-BrcGMP and NO donors also enhance whole cell currents in acutely dissociated or cultured retinal Müller cells and stimulate $\text{Ca}^{2+}$ influx (228), whereas 8-BrcAMP has no effect on whole cell currents. A fraction of the CGMP-sensitive current seemed to be carried by a $\text{Ca}^{2+}$-activated $\text{K}^+$ channel and another fraction by a nonselective cation channel. Transcripts encoding a CNG channel subunit have been amplified from cultured human Müller cells using primers specific for the A1 subunit of the cGMP-gated channel of rod photoreceptors. These pieces of evidence have been interpreted to indicate that Müller cells express a rodlike CNG channel. Again, immunohistochemical studies do not support the presence of a rodlike CNG channel in retinal glia cells (408; Müller, unpublished observations).

2. Cyclic nucleotide-sensitive currents in hippocampal neurons

A variety of different transcripts encoding CNG channel subunits have been detected in several brain areas by in situ hybridization, cloning of cDNA, and PCR (see sect. vi); however, studies on the functional characterization of neuronal CNG channels in situ are sparse. Two laudable examples are the work by Leinders-Zufall et al. (233) and Bradley et al. (46). At rest ($\approx 80$ mV), a whole cell inward current is evoked in hippocampal neurons upon superfusion with 8-BrcGMP. Although this basic observation is shared by both reports, the underlying currents appear to be different.

In one study, the $I-V_m$ relation of the 8-BrcGMP-activated current was almost linear in the presence of 2.5 mM $\text{Ca}^{2+}$ and 1 mM $\text{Mg}^{2+}$ in the bath (233). The current was 1.5- to 2-fold larger in the absence of divalent cations, but the shape of the $I-V_m$ relation was largely unchanged. This result contrasts with the strong blockage of known CNG channels by extracellular divalent cations, whether $\text{Ca}^{2+}$ or $\text{Mg}^{2+}$, which produces a pronounced outward rectification of currents in photoreceptors and OSNs. Furthermore, the $I-V_m$ relation has been recorded with 140 mM $\text{Cs}^+$ in the pipette and 140 mM $\text{Na}^+$ in the bath. Under these biionic conditions, known CNG channels show a distinctively positive $V_{\text{rev}}$ ($\approx 20$ mV), and the $I-V_m$ relation is slightly inwardly rectifying (112, 255, 271, 309, 416); in contrast, the currents in hippocampal neurons reverse either at $\approx 0$ mV or at $\approx 40$ mV (Figs. 2C and 3C, respectively, of Ref. 233) and rectify slightly in the outward direction.

Leinders-Zufall et al. (233) also attempted to study $\text{Ca}^{2+}$ permeability by estimating the $\text{Ca}^{2+}$ current flowing through this channel relative to the $\text{Na}^+$ current. To this end, the authors compared the current amplitudes in $\text{Na}^+$ and in various choline/\text{Ca}^{2+} mixtures. At 30, 100, and 1,000 $\mu$M $\text{Ca}^{2+}$, the current ratio $I_{\text{Ca}}/I_{\text{Na}}$ was roughly 0.25, 0.5, and 0.7, respectively, implying that $\text{Ca}^{2+}$ carries almost as much current as $\text{Na}^+$ at more than 100-fold lower concentrations. Similar experiments with CNG channels in rods and heterologously expressed subunits yielded entirely different results. At 70–100 mM external $\text{Ca}^{2+}$, $I_{\text{Ca}}$ amounts to only 1–2% of $I_{\text{Na}}$ in the native rod CNG channel, to roughly 10% in the A2 homomeric channel, and to 14% in the Drosophila channel (22, 65, 97). In conclusion, the currents recorded from hippocampal neurons (233) do not match the properties of currents flowing through known CNG channels.

The $I-V_m$ relation reported by Bradley et al. (46) is slightly outwardly rectifying in the absence of external divalent cations, and 1 mM $\text{Mg}^{2+}$ blocked the inward current at $\approx 80$ mV almost completely. Although the currents have not been further characterized, this piece of evidence is consistent with the idea that the current is carried by CNG channels.

3. cAMP-sensitive currents in invertebrate neurons

A cAMP-activated cation current is widely distributed among central molluscan neurons (for references, see Refs. 191, 192, 382). In neurons of Helix, Aplysia, and Pleurobranchaea, the current persists in the presence of inhibitors of PKA (165, 192, 382), arguing that the cAMP action on channels is direct and does not involve phosphorylation. In excised membrane patches, perfusion with 1 mM cAMP produces single-channel events of $\approx 40$ pS (382). These two observations have been taken as evidence that the underlying channels belong to the class of CNG channels. The unit conductance of 40 pS was compared with Na$^+$ conductance by estimating the $\text{Ca}^{2+}$ permeability of CNG channels. The unit conductance of 40 pS was compared with Na$^+$ conductance by estimating the $\text{Ca}^{2+}$ permeability of CNG channels.
D. CNG Channels in Spermatozoa

Cyclic nucleotides are key elements of cellular signaling in sperm of both vertebrates and invertebrates (for reviews, see Refs. 118, 406). cAMP and cGMP mediate several cellular responses, including acrosomal exocytosis, swimming behavior, and chemotraction. The swimming behavior of sperm is controlled by factors, usually short peptides, that are secreted by the egg or cellular structures of the oviduct. In a few species, primarily marine invertebrates with external fertilization, the amino acid sequence of the peptides and their cognate membrane receptors on the surface of the sperm have been identified (119, 275, 406). For example, speract, a short peptide from the sea urchin Strongylocentrotus purpuratus, activates a receptor-type GC and thereby stimulates a rise of the intracellular cGMP concentration (118, 406). Speract also gives rise to an increase of \([Ca^{2+}]_i\). These observations suggest that cGMP activates a \(Ca^{2+}\) conductance, although a direct causal relationship has not been established rigorously. Owing to their high \(Ca^{2+}\) permeability, CNG channels are among the prime candidates for the \(Ca^{2+}\)-entry pathway. Unfortunately, in sea urchin, where their potential function would have been so obvious, no CNG channels have been detected by homology screening (R. Gauss and U. B. Kaupp, unpublished observations). Instead, Gauss et al. (120) identified the first member of the family of pacemaker channels (or HCN channels), which are controlled by both cyclic nucleotides and voltage. The HCN channel in \(S.\ purpuratus\) is highly selective for cAMP and is not \(Ca^{2+}\) permeable. Its function in spermatozoa is not known.

The search for CNG channels was more successful in mammalian sperm. The testicular expression of several CNG channel subunits (A3, B1, and B3) has been suggested by cloning of cDNA from testis libraries or by Northern analysis (34, 35, 122, 416, 417). Antibodies specific for the A3 and B1 subunits labeled the flagellum of mature sperm and precursor cells in cross-sections of seminiferous tubules (417). Heterologous expression of the A3 subunit cloned from testis produces channels that are cGMP sensitive and cAMP selective: the \(K_{1/2}\) for cAMP is \(~200\)-fold higher than the \(K_{1/2}\) for cGMP (8.3 and 1,720 \(\mu M\), respectively; Ref. 416). Therefore, these channels might be involved in a cGMP-stimulated \(Ca^{2+}\) influx into intact sperm (417). cGMP-stimulated channel activity was detected in small vesicles that might have been derived from cytoplasmic droplets and in patches excised from osmotically swollen sperm. The low success rate of establishing a giga-seal resistance and of detecting channel activity prevented a more thorough characterization of the native channel in situ. Cyclic nucleotide-mediated \(Ca^{2+}\) influx into sperm was studied by confocal laser scanning microscopy (417). The 8-bromo- and 8-pCPT-analogs of cGMP were delivered from caged compounds by brief flashes of ultraviolet light. Photolysis of both caged compounds evokes a \(Ca^{2+}\) influx into sperm; the respective derivatives of cAMP are much less effective. The \(Ca^{2+}\) influx depends on the presence of extracellular \(Ca^{2+}\) and was greatly reduced at high extracellular Mg\(^{2+}\) concentrations.

Because knock-out mice lacking the A3 subunit are fertile (33), at this point we can only speculate about a functional role of cGMP-selective CNG channels in sperm. CNG channels might be involved in some aspect of motility control or, more specifically, in chemotactic swimming behavior or in the process of capacitation or acrosomal exocytosis. Another concern is the failure so far to identify a receptor-type GC in mammalian sperm, leaving alone its cognate ligand. There is also no evidence in sperm for the presence of the \(Ca^{2+}\)-regulated GCs (GC-E and GC-F) known from retinal photoreceptors.

Mouse sperm express two other channels (CatSper1 and CatSper2) that bear similarity with a single repeat of the four-repeat structure of voltage-activated \(Ca^{2+}\) channels (332, 336). Targeted disruption of the CatSper1 gene results in male sterility; moreover, the cAMP-induced \(Ca^{2+}\) influx is abolished in mutant mice. The CatSper channels are unrelated to CNG or HCN channels; in particular, they are lacking in a cAMP/cGMP-binding domain. The cloned CatSper channel genes so far have resisted functional expression, suggesting that they might require additional subunits to become functional. An intriguing possibility is that CNG and CatSper subunits coassemble to form \(Ca^{2+}\)-permeable and cyclic nucleotide-sensitive ion channels.

E. Miscellaneous Nonneuronal Tissues

Cyclic nucleotide-sensitive channels have been reported to exist in several nonneuronal cells. In this section we discuss some of the available evidence.

1. Airway epithelial cells

Xu et al. (427) report the expression of transcripts coding for the CNG channel from rod photoreceptors (A1 and B1 subunits) in a human alveolar cell line (A549). Furthermore, the authors compared whole cell currents recorded from the alveolar cell line to whole cell currents from A1-transfected HEK293 cells and from untransfected controls in the presence and absence of 8-BrcGMP. The averaged current amplitude recorded from one set of alveolar cells in the presence of 8-BrcGMP was about twofold larger than the averaged amplitudes recorded from another set of alveolar cells without 8-BrcGMP. A problem with this approach is that it does not allow correcting for leak currents in one and the same cell. Moreover, when working with transfected cell lines, the fluorescence of the cotransfected green fluorescent pro-
tein (GFP) does not always correlate with channel activity. Therefore, the contribution of the cGMP-activated current to the total whole cell current is not known. Moreover, the blockage of CNG channels by \( \text{L-cis-diltiazem} \) in rods and cones and OSNs is strongly voltage dependent (112, 154, 266), whereas the blockage in A549 cells is not. This study would have been more convincing had the cGMP-sensitive currents been unequivocally identified in excised membrane patches or in the whole cell configuration by infusion of cGMP from the recording pipette. CNG channels have also been involved in liquid homeostasis of lung in 6-mo-old sheep but not in 6-wk-old sheep on the basis of a pharmacological study using dichlorobenzil, amiloride, and pimozide, which, among other channels, also block CNG channels (179, 180).

2. Gonadotropin-releasing hormone-secreting neuronal cell line

Vitalis et al. (405) report the identification by PCR of transcripts for the CNG channel subunits A2, A4, and B1 in a neuronal cell line (GT1) secreting the gonadotropin-releasing hormone (GnRH). These three subunits make up the native CNG channels in chemosensitive cilia of OSNs (40, 357). Perfusion of inside-out patches excised from GT1 neurons with 200 \( \mu \text{M cAMP} \) produced single-channel events with a unit conductance of \( \sim 60 \text{pS} \) in 140 mM symmetrical \( \text{Na}^+ \) and in the presence of 4 mM divalent cations on both sides of the membrane. Under these conditions, all known CNG channels are severely blocked, and single-channel events cannot be resolved. For example, in high extracellular \( \text{Ca}^{2+} \), the unit conductance of CNG channels in rods and OSNs has been estimated from noise analysis to be significantly smaller than 1 pS (37, 87, 140, 199). It, therefore, seems unlikely that the 60-pS channel is produced by the CNG channel subunits detected by PCR.

3. Endothelial cells of pulmonary artery

Wu et al. (425) sought to identify CNG channels in endothelial cells by recording \( I-V_m \) relations in the whole cell configuration under various conditions. The ionic selectivity, the \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) dependence of blockage, and the rectification behavior of the endothelial cell currents are incompatible with the properties of known CNG channels. Therefore, the authors’ claim is equivocal that the currents are carried by a CNG channel comprising the A2 subunit.

4. Kidney

cGMP-sensitive channels have been identified in cells of the renal inner medullary collecting duct (IMCD cells; Ref. 241). These channels exhibit a complex pattern of regulatory mechanisms. The constitutively active channels appear to be under the dual control of cGMP and PKG. In excised patches, cGMP reduced the \( P_e \) directly by 39% through a phosphorylation-independent mechanism. cGMP also inhibited the channel by 96.1% through a phosphorylation-dependent mechanism involving PKG. When guanosine 5’-O-(3-thiotriphosphate), an activator of G proteins, was present, PKG had no effect. The authors suggested that channel activity is controlled by the complex interaction between PKG and a \( G_i \) protein. In a subsequent report from the same laboratory (401), a seemingly related channel was studied in a cell line derived from the inner medullary collecting duct. The authors argue that this channel exhibits a cation selectivity, unit conductance, \( \text{Ca}^{2+} \) permeability, and pharmacology similar to the classic CNG channels and support their notion by cloning of cDNA (182) that displays 99% nucleotide sequence identity to the respective cDNA of the A1 subunit cloned from the mouse retina (324). The authors also suggest that the differences between the retinal and renal cDNA sequence may account for the functional differences. We take issue with this view.

First, the 20- and 28-pS channel activity in IMCD cells was recorded in the presence of millimolar concentrations of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) in the extracellular medium. Under these ionic conditions, classic CNG channels are severely blocked and display a strong outward rectification, and single-channel recordings are not feasible (see above). Second, CNG channels are not constitutively active in the absence of cGMP or cAMP (see, however, sect. vii). Third, CNG channels are neither inhibited by cGMP nor downregulated by PKG activity or activated by \( G_i \) proteins. Fourth, the diltiazem sensitivity of the renal channel is almost two orders of magnitude lower than that of retinal CNG channels. The authors probably used the racemate of diltiazem for their blocking experiments. However, only the \( \text{L-cis} \)-form of the diastereomeric diltiazem blocks CNG channels at micromolar concentrations (203).

F. Conclusions

The function of CNG channels in photoreceptors and OSNs has been established beyond reasonable doubt, whereas for other cell types either their expression is uncertain or their function is not fully understood. Most needed is a convincing demonstration of cyclic nucleotide-sensitive currents in excised patches and quantitative comparison with the properties of known CNG channels. A panel of caged cyclic nucleotides now provides powerful tools to record CNG channel activity in the whole cell configuration or by \( \text{Ca}^{2+} \) imaging. We trust that these techniques will clarify some of the issues addressed in this section.

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V. MOLECULAR DIVERSITY OF CYCLIC NUCLEOTIDE-GATED CHANNEL SUBUNITS

A. Diversity of Subunit Genes

The advent of molecular cloning has uncovered a bewildering number of genes from various species encoding different CNG channel subunits. The chase for new CNG channel genes has come to a preliminary end by the recent release of the genomic sequences of several species, including human, D. melanogaster, C. elegans, and Arabidopsis thaliana. The CNG channel genes and subunits have received a bewildering variety of different names, which inevitably caused some confusion. Subunits that have initially been cloned from either rod, cone, or OSN have been named according to their cellular origin. When it was realized that CNG channels form heteromultimers, the previously cloned subunits have been designated “first” or α-subunit and the novel subunits have been designated “second” or β-subunit. However, subunits are not specific for rod, cone, and OSN but are expressed in other cells as well. Therefore, classification of channel subunits based on the tissue of original identification became inappropriate. Moreover, it was recognized that the so-called α-subunits form functional channels on their own and, therefore, have been also referred to as “principal” subunits, whereas the β-subunits are unable to form functional channels and, therefore, have been designated “modulatory” subunits. A comprehensive list of the various designations across subunits, tissues, and species is given by Richards and Gordon (340).

Throughout this review, for the mammalian genes we use the new nomenclature that most laboratories working with CNG channels have agreed on (Table 2; Ref. 43). It follows two guidelines. First, based on sequence comparison, CNG channel genes fall into different subfamilies. In mammals, two gene subfamilies can be distinguished. Homologous members of these two subfamilies are found in the genomes of species as distant as human, C. elegans, and Drosophila. The encoded polypeptides of the two subfamilies are referred to as A and B subunits. Roman letters have been previously used to classify the subunits of several families of ligand-gated channels. Members of a subfamily are numbered (A1, A2, etc.). Second, a designation used in the past for one gene cannot be used for another gene even if the former usage has been discontinued. This rule prevented the numbering of A and B subunits based on functional relatedness or functional context rather than on the order of molecular cloning or baptizing. Moreover, this rule created gaps in the numbering system. For example, one subunit of olfactory CNG channels has been previously thought to be a B subunit (formerly CNGB2). This subunit is now designated CNGB4. As a consequence, the designation CNGB2 is excluded from future use, and one of the other B subunits cannot be renamed but must keep its original designation (CNGB3).

The NCBI database contains the sequences of six different human genes encoding CNG channels. These genes have been known already from either cloning of human cDNA or cloning of orthologs from other vertebrates including bovine, mouse, rabbit, rat, and chick. The Drosophila and C. elegans genomes harbor, respectively, four and six different CNG channel genes. The mammalian CNG channel genes fall into two different gene subfamilies (see phylogenetic tree in Fig. 4). Members of one subfamily represent the principal subunits that, except one (CNGA4), form functional channels on their own and are responsible for several key properties of CNG channels. This subfamily consists of four members, designated here CNGA1, CNGA2, CNGA3, and CNGA4. For brevity, we refer to these subunits as A1 to A4. The A1 subunit is the founding member, first identified in retinal rods (188). The principal subunit identified in OSNs (90, 254) is designated A2, and the principal subunit identified in cones (39) is designated A3 (see Table 2). The sequence similarity among A1 to A3 is high, ranging from 75.1 to 78.9% (Table 3).

The A4 subunit, first identified in rat OSNs (44, 242), is set apart from A1 to A3. It seems to fall into a subfamily of its own, except it would represent the only member of that subfamily. Only one other ortholog in humans is known. At the time when this subunit was cloned, it was believed that the olfactory CNG channel, like the channel of retinal rods, is built from two different subunits. Moreover, like the CNGB1 and CNGB3 subunits described below, A4 does not form functional channels on its own. Therefore, A4 has been frequently referred to as the “β subunit” or second subunit of the olfactory CNG channel. However, based on the overall sequence similarity and

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**Table 2. Nomenclature of CNG channel subunits**

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Reported Names</th>
<th>Original Cloning</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNGA1</td>
<td>Rod CNG channel, CNGB1a, RCNC1</td>
<td>188</td>
</tr>
<tr>
<td>CNGA2</td>
<td>Olfactory CNG channel, CNGB2a, CCNC1</td>
<td>90, 254</td>
</tr>
<tr>
<td>CNGA3</td>
<td>Cone CNG channel, CNGB3a, OCNC1</td>
<td>30, 416</td>
</tr>
<tr>
<td>CNGA4</td>
<td>Second/modulatory subunit of olfactory CNG channel, CNGB4a, CCNC2</td>
<td>44, 242</td>
</tr>
<tr>
<td>CNGB1</td>
<td>Second/modulatory subunit of rod CNG channel, CNGB1β</td>
<td>214</td>
</tr>
<tr>
<td>CNGB3</td>
<td>Second/modulatory subunit of cone CNG channel, CNGB3β</td>
<td>122</td>
</tr>
</tbody>
</table>

CNG, cyclic nucleotide gated.
functional sequence motifs, it is more akin to A1 to A3 than to the two B subunits (186). For example, A4 shares 49.2–51.7% sequence identity with A1 to A3, but only 24.9 and 16.3% identity with B1 and B3, respectively. Therefore, it seems appropriate to classify this subunit as an A subunit.

The second subfamily comprises two members, designated CNGB1 and CNGB3, which have been first identified in rod and cone photoreceptors, respectively (71, 122, 214). These subunits are often referred to as modulatory subunits, because, unlike the A1 to A3 subunits, they do not form functional channels on their own. However, when coexpressed with A subunits in a heterologous cell system, the resulting heteromeric channels display properties that are characteristically different from the homomeric A channels with respect to ligand sensitivity and selectivity, gating, pharmacology, ion selectivity, and modulation by Ca\(^{2+}\)/CaM. The B1 and B3 subunits share 48.1% sequence identity with each other, but only between 22.4 and 31.8% with A1 to A3 (Table 3).

Genes encoding CNG channel subunits have been also identified from invertebrates, notably *C. elegans*, *D. melanogaster*, and *L. polyphemus*. The cDNAs of two different genes have been cloned from *C. elegans*, tax-2 and tax-4 (74, 210), and an additional four genes have been identified by the *C. elegans* Genome Project. How do these subunits relate to their mammalian cousins? Tax-4 is closely related to the mammalian A subunits and produces functional channels when heterologously expressed. Tax-2 is phylogenetically related to the mammalian B subunits and does not form functional channels on its own. We will refer to these subunits as ceCNGA (Tax-4) and ceCNGB (Tax-2). The other four *C. elegans* genes seem to fall into a subfamily on its own. For the time being, we will refer to these subunits as ceCNG3 to ceCNG6.

Two genes encoding CNG channel subunits have been cloned from *D. melanogaster* (22, 278). Furthermore, the sequences of two other subunits have been determined by the *Drosophila* Genome Project. One subunit displays significant sequence similarity with the

![Figure 4](image.png)
mammalian A1 to A3 subunits and produces functional channels in a heterologous cell system (22). We will refer to this subunit as dmCNGA. Another subunit (accession number AAF46757) clearly falls into the subfamily of mammalian B subunits; we will refer to this subunit as dmCNGB. The other two subunits of Drosophila, like four of the C. elegans genes, are not as easily categorized as the others and will be provisionally designated dmCNG3 and dmCNG4.

The subunit cloned from L. polyphemus is significantly longer than its mammalian counterparts and may represent an A subunit (69).

The subdivision into A and B subunits is also supported by a comparison of the genomic structure of CNG channel genes. The NH$_2$-terminal region up to S2 of the mammalian A1 to A3 subunits is encoded by several small exons, whereas the region from S2 to the COOH terminus is encoded by a single large exon. This exon structure is conserved among A1 to A3 and from chick to human (41, 89, 419) (Fig. 4). The exon structure between B1 and B3 is also conserved, but entirely different from that of A1 to A3. The human B1 gene is composed of 33 small exons (9, 10); there appears to be no conservation of splice sites between A and B subunit genes. Most notably, the region from S2 up to the COOH terminus is encoded by 12 exons in the B subunits, but only by a single exon in A1 to A3. The exon structure of the NH$_2$-terminal region up to S2 is largely conserved between A4 and A1 to A3, whereas that of the COOH-terminal region is not conserved, arguing that the A4 gene falls into a subfamily of its own.

Several splice variants have been identified of the A3 and the B1 subunits (36, 40, 41, 71, 214, 273, 276, 395, 417). The various splice forms of A3 differ in their NH$_2$-terminal region (Fig. 5). Why cone photoreceptors, pinealocytes, sperm, and a subpopulation of OSNs express different splice forms of the A3 gene is not known, and the electrophysiological properties of the various A3 forms have not been systematically compared.

The two most important splice forms of the B1 gene are B1a and B1b, expressed in rods and OSNs, respectively. The B1b subunit is a short form of B1a, which is lacking the glutamic acid-rich part (see sect. v.B). Several B1 splice forms that differ only in their very NH$_2$-terminal region are expressed in testis (36, 417).

B. Functional Domains

The major structural sequence motifs and functional domains of CNG channels are depicted in Figure 6 and are discussed below.

1. Transmembrane topology

The current model for the membrane topology of the A and B subunits of CNG channels is illustrated in Figure 6. The core structural unit consists of six membrane-spanning segments, designated S1-S6, followed by a cNMP binding domain near the COOH terminus. A pore region of ~20–30 amino acids is located between S5 and S6. The S4 segment in CNG channels resembles the voltage-sensor motif found in the S4 segment of voltage-gated K$^+$, Na$^+$, and Ca$^{2+}$ channels. Because the voltage-sensor motif, the six transmembrane segments, and the pore region are also characteristic features of voltage-gated channels, it has been suggested that CNG channels and voltage-gated channels are members of a gene superfamily of cation channels, which evolved from a common primordial channel (160, 175).

Experimental evidence in support of the topological model was first obtained from immunogold labeling for electron microscopy of the CNG channel from rod photoreceptors. In these studies, both the NH$_2$ and COOH termini were localized to the cytoplasmic side of the ROS plasma membrane (282), and a glycosylated segment connecting S5 to the pore region was localized to the extracellular side (420). The topological model for this and other A subunits received further support from a gene fusion approach using enzyme reporters (161). Although similar studies are lacking for the B subunits, because of the significant sequence similarity and similar structural features, most likely A and B subunits share a common transmembrane topology.

2. Voltage-sensor motif

The S4 segment of all CNG channels comprises a charged sequence motif that is reminiscent of the “voltage-sensing” S4 segment of voltage-gated channels. This segment, in voltage-gated channels, has been proposed to serve as sensor of membrane voltage that upon depolarization moves toward the extracellular surface and thus opens the channel. The S4 segment of K$^+$ channels is characterized by five to seven positively charged residues (Arg or Lys) at every third position interspersed with predominantly hydrophobic residues (Fig. 7A). It is sufficiently long to be able to span the membrane as an amphipathic α-helix. Studies using site-directed mutagenesis and state-dependent labeling of residues with fluorescent dyes or with antibodies supported the voltage-sensor hypothesis (218, 259, 353). Indeed “gating currents” were observed directly demonstrating that charges of S4 were transferred during voltage changes (11). Recent evidence suggests that the S4 helix, upon depolarization, undergoes a 180° rotation with a slight tilt (68, 124); this movement is sufficiently large to transfer positive charges from an intracellular to an extracellular environment.

The core of the S4 segment in CNG channels displays significant sequence similarity to the respective region of K$^+$ channels, although it includes only three or four regularly spaced Arg or Lys residues (Fig. 7A). The functional
significance of the S4 segment is, however, still elusive, as CNG channels show very little voltage dependence. A few studies have been carried out to address this question. Transfer of the S4 segment from the rat A2 subunit to the voltage-gated dmEAG channel produced a chimeric channel that is activated by depolarization, suggesting that a

![Genomic structure of cyclic nucleotide-gated (CNG) channel genes.](image)

**A**
- A1
- A2
- A3
- A4

**B**
- NH₂
- COOH
- rat A3b
- rat A3a
- chick pineal A3a
- chick pineal A3b
- chick retina A3
- bov. retina A3
- hum. retina A3

**FIG. 5.** Genomic structure of cyclic nucleotide-gated (CNG) channel genes. A: exon structure of the six human CNG channel genes. Red lines demarcate exon boundaries. B: splice variants of the A3 gene cloned from various tissues and species. The numbering of exons is according to Reference 41.
CNG channel S4 segment is capable of serving as a voltage sensor (391). However, when the extracellular S3-S4 loop was transferred from A2 to dmEAG, the channel chimera apparently became constitutively active. The authors went on to show that the midpoint potential of activation ($V_{1/2}$) of this chimera was shifted to such large negative values that, within the physiological range of membrane voltage, channels are fully activated and thereby became voltage insensitive. A mechanism that might explain this result is that hydrophilic amino acids within the S3-S4 loop of CNG channels interfere with the gating movements by “locking” the S4 segment and thereby the channel in the open state. A Glu residue present in the S3-S4 loop (E246) appears to be important; exchange of the respective position of dmEAG (A345E) shifted the $V_{1/2}$ by 50 mV to more negative values. In addition, it was shown that the sign of the charge is not important; Arg at this position was as effective as Glu in shifting $V_{1/2}$ (391).

A similar mechanism is conceivable for CNG chan-
nels. The S4 charges might be “locked” in a fixed conformation by hydrophilic residues in the S3-S4 loop, and gating of these channels, despite the S4 motif, is solely promoted by the binding of cyclic nucleotides. Unfortunately, to date, it has not been possible to test this hypothesis by “unlocking” the S4 segment to produce a voltage-activated CNG channel mutant. Moreover, sequence alignment of the S3-S4 loop reveals only little...
similarity among CNG channels (Fig. 7A). Most importantly, E246 is only found in A1 to A3 subunits. The S3-S4 loop of other CNG channels, for example, dmA, is less hydrophilic than that of rat A2, yet the voltage dependence of dmA channels is not enhanced compared with A2. Clearly, more work is needed to eventually understand the role of the S4 segment of CNG channels.

3. P region

For K⁺ channels, the view of the P region and the question of how selectivity is produced was taken into a new era when the crystal structure of the bacterial KcsA K⁺ channel was solved (93). The pore region of KcsA comprises roughly 25 amino acids that form an inwardly directed helix and the selectivity filter, which consists of the GYG signature sequence of K⁺ channels (Fig. 7B). The Tyr residue of the GYG motif interacts specifically with two Trp residues of the pore helix to form a sheet of aromatic amino acids (a total of 12 in a tetramer) that is positioned like a cuff around the selectivity filter and is thought to behave like a layer of strings stretched radially outward to hold the pore open at its proper diameter (93).

Can we envision a similar P region architecture for CNG channels? Sequence conservation among K⁺ channels and CNG channels in the P region is quite remarkable (Fig. 7B). Within the pore helix, many residues are identical or at least conserved, arguing for a common pore architecture of K⁺ channels and CNG channels. However, the sequence similarity diverges exactly at the selectivity filter. At the respective position of the Tyr residue in the K⁺ channel, a negatively charged (Glu, Asp) or at least a functionally charged residue exists in most CNG channels (Fig. 7B). The structural features of a selectivity filter made of up to four negative charges in tetrameric channels are difficult to predict because the electrostatic interaction of the carboxylic side chains strongly depends on their mean charge and distance. To date, we rely on several indirect methods that have been employed to study pore structures. For example, several studies employed the substituted cysteine accessibility method (SCAM) (5): each amino acid within the P region is consecutively replaced by a cysteine, and the accessibility of the reactive thiol group is tested from either side of the membrane with thiol-specific reagents [i.e., (2-sulfonatoethyl) methane thiosulfonate (MTSES), [2-(trimethylammonium)ethyl]-methane thiosulfonate (MTSET), or (2-aminoethyl)-methane thiosulfonate (MTSEA)]. From these results an accessibility map of the pore region can be constructed. Differences in the pattern of reactivity of cysteine-substituted residues between the open and closed states of the channel indicate that a conformational change takes place in or around the pore loop. Previous studies have also concluded that the pore region at or near the negatively charged residue E363 is involved in channel gating (24, 62, 121).

4. Cyclic nucleotide-binding domain

All CNG channel subunits contain a COOH-terminal domain (comprising –80–100 amino acid residues) that exhibits significant sequence similarity to the cNMP-binding domains of PKA and PKG and the catabolite activator protein of Escherichia coli (CAP). The three-dimensional structure of CAP has been determined by X-ray crystallography (265). The cAMP-binding site of CAP, comprising three α-helices (A, B, and C) and eight β-strands (β1 to β8), served as template for cNMP-binding domains of other proteins (Fig. 8). The β-strands form a flattened barrel consisting of two antiparallel β-sheets, each with four strands, connected in a jelly-roll topography. The three helices are connected to the ends of the β-barrel. The A helix is NH₂ terminal of the β-barrel, whereas the B helix,
immediately followed by the C helix, is COOH terminal of the β-barrel. The cyclic nucleotide is bound within the binding site through a network of polar and nonpolar interactions. The phosphate and the ribose ring of cAMP interact with the protein through several hydrogen bonds and through electrostatic contacts. These interactions involve residues in the loop linking α6 and α7. In contrast, the adenine ring interacts primarily through hydrophobic and stacking interactions with residues in and near the C helix. The location of β-strands and α-helices in the cNMP-binding domain of CNG channels, HCN channels, protein kinases, EPAC, and CAP is shown in Figure 9.

5. The glutamic acid-rich part of the B1a subunit

The B1a subunit of the CNG channel of rod photoreceptors features a unique bipartite structure (214). The membrane-spanning so-called β’ part is homologous to A subunits, whereas the large cytoplasmic NH2-terminal domain is lacking in all other CNG channel subunits. Even more intriguing, except for a few amino acid residues, this NH2-terminal domain is identical with two soluble glutamic acid-rich proteins (GARPs) (78, 213, 383), which seem to be specifically expressed in rod photoreceptors. We refer to the soluble proteins as GARP1 and GARP2 and to the respective channel domain as GARP-part (213). GARP1 is twice as large as GARP2 and, therefore, these two proteins have also been designated full GARP and truncated GARP (f-GARP and t-GARP, respectively; Ref. 78). The B1a subunit and GARP1/GARP2 are derived from two different genes. The two soluble GARP1/GARP2 probably represent alternatively spliced forms (9, 10). Splice variants of the rod B1a lacking the GARP-part are found in OSNs (40, 357) and testis (417). The presumptive B3 subunit expressed in cone photoreceptors is much shorter than B1a (122) and has no GARP-related sequences, suggesting that GARPs serve a rod-specific function. In fact, specific anti-GARP antibodies stain the outer segment of rods but not of cones (213).

The common NH2-terminal region of GARPs carries four short proline-rich repeats R1 to R4 (~15 amino acids) that are highly conserved among each other and that represent the most conserved structural elements between GARPs from different species (see dot blot in Fig. 6).

Peptide affinity chromatography suggested that these repeats are involved in protein-protein interaction between GARP and the PDE, the GC, and the retina-specific ATP-binding cassette (ABCR) transporter (213). The ABCR transporter (6, 172) probably translocates all-trans...
retinal complexed together with phosphatidylethanolamine as a Schiff's base (384). The ABCR is better known as “rim” protein because it is confined to the rim of the disk membrane (172, 315). Reportedly, the GC is also located near the disk margin, although the electron microscopic evidence is less compelling than for ABCR (249). The soluble GARPs are tightly associated with the margin of the disk, probably by binding to one or several of the rim-confined proteins. The interaction between GARPs and ABCR and GC has recently been called into

![Alignment of cNMP-binding sites of vertebrate and invertebrate CNG channels, HCN channels, cyclic nucleotide-dependent kinases, EPAC, and the catabolite gene activator protein CAP.](http://physrev.physiology.org/)

**FIG. 9.** Alignment of cNMP-binding sites of vertebrate and invertebrate CNG channels, HCN channels, cyclic nucleotide-dependent kinases, EPAC, and the catabolite gene activator protein CAP. The ratA1 subunit was taken as a reference. Residues identical or similar to the respective amino acid in the ratA1 subunit are shown with a black or gray background, respectively. RatA1 (91), ratA2 (90), ratA3 (356), ratA4 (44, 242), dmA (22), ceA (210), ratB1 (356), mouseB3 (122), ceB (74), dmB (accession AAF46757), dm3 (278), dm4 (accession BAA89278), ce3 (accession T20936), ce5 (accession AAF36062), ce6 (accession T21969), HCN1 (354), PKA1 (cAMP-binding site 1 of PKA) (396), PKG1 (cGMP binding site 1 of PKG) (389), EPAC (86), and CAP (4) are shown.
question (325). Using immunoprecipitation techniques, Poetsch et al. (325) showed that both ABCR and GC cannot be pulled down with GARP-specific antibodies, no matter whether the potential binding partners have been covalently attached to each other by cross-linking reagents. Instead, peripherin, another protein located at the disK rim, seems to interact with GARP2 and the GARP/H11032 part of the B1a channel subunit. The interaction between B1b subunit and proteins at the disK margin is expected to force the CNG channels to align in circles that are stacked along the length of the outer segment (Fig. 10).

Such a nonuniform distribution might account for the high variability of channel density in the rod outer segments determined by patch-clamp recording (184), although the authors do not favor this explanation.

GARP2, the most abundant GARP species, has been proposed to interact with the PDE (213). In fact, recombinant GARP2 inhibits light-activated PDE with a $K_{1/2}$ of $10 \text{nM}$. We have reexamined the control of PDE activity by using purified native GARP2 and observed only a weak inhibition of PDE activity, whereas recombinant GARP2 was a potent inhibitor (H. G. Körshen, unpublished results). The difference between native and recombinant GARP2 suggests that the small fusion part of the recombinant GARP2 (57 amino acid residues) might be responsible for the inhibitory action.

The CNG channel also undergoes an interaction with the Na+/Ca2+-K+ exchanger that involves the A rather than the B subunit (21, 281, 325, 364). The molar ratio of exchanger to channel is about two (21, 334), implying that two exchanger molecules are bound per channel. The juxtaposition of the channel, which promotes Ca2+ influx and the Na+/Ca2+-K+ exchanger, which promotes Ca2+ efflux, suggests that Ca2+ dynamics inside the cell are localized to microdomains in the vicinity of the channel. The high density of negatively charged Glu residues may serve as a low-affinity Ca2+ buffer that kinetically impedes the diffusion of Ca2+ away from the site of entry.

C. Posttranslational Modifications

1. Proteolytic processing

Molecular characterization of CNG channel proteins was initiated when Kaupp et al. (188) cloned the cDNA for the A subunit of the CNG channel from bovine rods.

---

**Fig. 10. Interaction of the rod CNG channel with other proteins.** Left: interaction with peripherin and the Na+/Ca2+-K+ exchanger. Right: predicted ringlike distribution of CNG channels in the outer segment membrane of rods.
full-length cDNA codes for a polypeptide of 690 amino acids with a predicted molecular mass of 79.6 kDa. This size is in good agreement with the molecular mass (~78 kDa) of the heterologously expressed polypeptide measured by SDS gel electrophoresis, but considerably larger than the apparent molecular mass of 63 kDa observed in bovine ROS and purified channel preparations (382). Immunocytochemical labeling studies of retinal tissue has firmly established that the 63-kDa subunit is the predominant form of the A subunit in rods. The discrepancy in size is primarily due to the absence of the NH$_2$-terminal 92 amino acid residues. The truncated A subunit has also been detected in chicken retinal ex-pig retinas (283). Shortened forms of the rod and cone A subunits have also been detected in chicken retinal extracts (39). These results led to the view that the NH$_2$ terminus of the A subunits undergoes photoreceptor-specific posttranslational cleavage. The role for this proteolytic processing is not known at the present time, but it may be required for targeting of the channel to the plasma membrane of the outer segment or for interaction with other photoreceptor proteins.

Proteolytic processing seems to be absent in the A2 subunit of the rat olfactory CNG channel, because the heterologously expressed and the deglycosylated native subunit display a similar molecular mass of ~75 kDa, which reasonably agrees with the predicted molecular mass of 76 kDa for the cloned subunit (40). For the same reasons, the B1a and B1b subunits of the rod and olfactory CNG channels, respectively, do not seem to undergo posttranslational processing (40, 214).

2. Glycosylation

Enzymatic deglycosylation indicated that the 63-kDa A1 subunit but not the 240-kDa B1a subunit contains an N-linked oligosaccharide chain (421). The A1 subunit of the bovine rod CNG channel is N-glycosylated at residue N327 (420). The glycosylated and deglycosylated forms of A1 differ in molecular mass by 2 kDa. Glycosylation has not been studied in the A3 subunit.

The olfactory A2 subunit is heavily glycosylated. In Western blots of isolated cilia, an A2-specific antibody recognizes two bands: a sharp but faint ~75-kDa band and a prominent diffuse band at 110–145 kDa (40). Treatment of cilia preparations with glycosidase abolished the diffuse band entirely and at the same time strongly enhanced the 75-kDa signal, demonstrating that the vast majority of the A2 subunit exists in a highly glycosylated form. It seems likely that the glycosylated 110- to 145-kDa form is specifically targeted to cilia, whereas the non- or low glycosylated 75-kDa form might be expressed elsewhere in OSNs, for example, the soma or dendrite. The A4 and B1b subunits of the olfactory CNG channel are not glycosylated (40).

The roles for proteolytic processing and N-glycosylation are not known at the present time, but both processes may be required for targeting of channels to the outer segment and the chemosensitive cilia.

VI. MOLECULAR COMPOSITION AND TISSUE DISTRIBUTION

The list of tissues in which CNG channel subunits are reportedly expressed has been rapidly expanding. It includes nonneuronal tissue such as heart, lung, kidney, pancreas, liver, spleen, testis (Table 5), and various brain areas such as cortex, cerebellum, hippocampus, thalamus, hypothalamus, retinal olfactory bulb, gustatory and olfactory epithelium, VNO, pineal gland, and pituitary (Table 4). Although high expression levels of CNG channels allowed a comprehensive characterization in photoreceptors and OSNs, their study in other tissues, because of the much lower abundance, is technically demanding and hence much less advanced. A comprehensive list of the expression pattern of CNG channel subunits in various species is given by Richards and Gordon (340).

A. Molecular Composition of CNG Channels in Photoreceptors and Olfactory Neurons

The first indication that CNG channels are built from several distinct subunits came from the functional characterization of the heterologously expressed A1 subunit of rod photoreceptors. The recombinant A1 channel recapitulates several of the key properties of the CNG channel in intact rods, but also deviated in some aspects (188). Furthermore, the channel protein purified by either immunoadfinity or CaM-affinity chromatography consists of two prominent polypeptides that migrate on SDS-polyacrylamide gels with apparent molecular masses of 63 and 240 kDa, respectively. Initially, it was thought that only the 63-kDa polypeptide comprised the channel itself and the 240-kDa component represented a spectrinlike cytoskeletal protein that was tightly associated with the CNG channel (79, 279). Subsequent studies have revealed that the 240-kDa polypeptide is the second subunit of the channel (214). It is now generally believed that CNG channels in various tissues form heteromeric complexes consisting of distinct subunits. Because A and B subunits from nematode to human functionally coassemble with each other (104), a large number of distinct channels can, in principle, be combinatorially generated from the six subunit types. Whether the promiscuous assembly of diverse subunits is a general strategy to generate CNG channels that suit a particular cellular function is not known and deserves further examination. It is also not known whether in some tissues CNG channels exist as a homomeric complex.
The native CNG channel in rods consists of two types of subunits, A1 and B1a (Fig. 11). Purified channel preparations do only contain these two polypeptides, and coexpression of the A1 and B1a subunits produces channels that recapitulate most, if not all, of the key properties of the native channel from rods (70, 71, 214). Notably, the properties of native channels and various combinations of heterologously expressed subunits were compared using five functional criteria: 1) the sensitivity for activation by cAMP, 2) the discrimination between Na\(^+\) and K\(^+\), 3) the single-channel conductance, 4) the kinetics of open-closed transitions, and 5) the ability to adopt a subconductance state. The A2A4B1b channel resembles the native channel in each of the five criteria, whereas other combinations (e.g., A2A4 or A2B1b) or homomeric A2 channels display clear differences in all of the criteria with respect to the native channel (40, 44, 242, 357). However, subtle differences between native and A2A4B1b channels were noted that may either result from different glycosylation patterns of the native and heterologously expressed subunits or from the expression system. In fact, two studies have shown that the kinetic properties of A2A4 channels depend on the expression system. The channels partially desensitize when ex-

The native CNG channel in rods consists of two types of subunits, A1 and B1a (Fig. 11). Purified channel preparations do only contain these two polypeptides, and coexpression of the A1 and B1a subunits produces channels that recapitulate most, if not all, of the key properties of the native channel from rods (70, 71, 214). Notably, the rapid flickery gating that is so characteristic of the native channel (156, 392, 399, 441) and the high sensitivity to blockage by \(\text{L-cis-diltiazem}\) (203, 379) require the coexpression of the A1 and B1a subunits (71, 214).

Although the CNG channel from cones has not been as extensively studied as that in rods, it likely is also composed of A and B subunits (A3 and B3) (39, 122, 416). Similar to the rod CNG channel, the channel from cones displays flickery activity and is blocked by micromolar concentrations of \(\text{L-cis-diltiazem}\) in a voltage-dependent manner (154, 158). Coexpression of A3 and B3 produces channels that display flickery gating behavior and are blocked by \(\text{L-cis-diltiazem}\) (122).

The CNG channels in the chemosensitive cilia of OSNs unlike those in photoreceptors consists of three different subunits: A2, A4, and B1b (40). The mRNAs of all three subunits are expressed in OSNs, and the three subunit polypeptides are colocalized in the cilia. Furthermore, the properties of native channels and various combinations of heterologously expressed subunits were compared using five functional criteria: 1) the sensitivity for activation by cAMP, 2) the discrimination between Na\(^+\) and K\(^+\), 3) the single-channel conductance, 4) the kinetics of open-closed transitions, and 5) the ability to adopt a subconductance state. The A2A4B1b channel resembles the native channel in each of the five criteria, whereas other combinations (e.g., A2A4 or A2B1b) or homomeric A2 channels display clear differences in all of the criteria with respect to the native channel (40, 44, 242, 357). However, subtle differences between native and A2A4B1b channels were noted that may either result from different glycosylation patterns of the native and heterologously expressed subunits or from the expression system. In fact, two studies have shown that the kinetic properties of A2A4 channels depend on the expression system. The channels partially desensitize when ex-

### Table 4. Subunit distribution in brain

<table>
<thead>
<tr>
<th>Subunit Region</th>
<th>RT-PCR</th>
<th>Northern/RPA</th>
<th>In Situ</th>
<th>Western</th>
<th>Immunohistochemistry</th>
<th>Reference Nos.</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>2, 39</td>
</tr>
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<td>ND</td>
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<td>+</td>
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<td>±</td>
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<td></td>
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<td>+</td>
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<td>B1</td>
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<td>Rod photoreceptors or retina</td>
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<tr>
<td>Retina</td>
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<td>ND</td>
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<td>–</td>
<td>ND</td>
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<td>ND</td>
<td>122</td>
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+, Positive results; -, negative results; ±, positive and negative results; ND, not determined; NA, not applicable.
pressed in *Xenopus* oocytes (242), but do not do so in HEK293 cells (44).

The strategy taken to examine the stoichiometry and arrangement of subunits has been to express wild-type and mutant subunits. The mutants are expected to change the channel properties in characteristic ways and thereby "report" the number and arrangement of individual subunits in the channel complex. For example, Liu et al. (246) coexpressed the wild-type A1 subunit with a chimeric A1 subunit in which the pore region is replaced with that of the A2 subunit. The wild-type and chimeric subunits, when expressed alone, display a single-channel conductance of 30 and 85 pS, respectively. Upon coexpression, four additional intermediate conductances are observed that, in principle, could arise from the four possible combinations of two subunits forming a pentameric channel. With the use of homo- and heterodimer constructs, some evidence was provided that two of the intermediate conductances might arise from different arrangements of wild-type and chimeric constructs in a tetrmeric channel, i.e., the same subunits can be adjacent or opposite to each other (246). Experimental evidence in favor of the tetrmeric complex was also provided by Ni$^{2+}$ potentiation studies (135).

More recently, the Ni$^{2+}$ technique has been extended to heteromeric rod CNG channels (A1B1b) (159, 372), unfortunately with conflicting results. Shammat and Gordon (372) coexpressed A and B subunits with either constructed or mutated Ni$^{2+}$ potentiation sites and concluded that the native rod channel exists in an AABB arrangement. These authors assumed that a channel with engineered C-linker histidines in the B subunit (N546H) but mutated C-linker histidines in the A subunit (H420Q) can only be potentiated by Ni$^{2+}$ when two B subunits are localized adjacent to each other. This view was challenged by He et al. (159), who could show that Ni$^{2+}$ potentiation is mediated by the wild-type B subunit in the absence of A subunit histidines. Potentiation even persisted when residues in the C-linker of the B subunit that possibly bound Ni$^{2+}$ were mutated, suggesting that regions other than the C-linker might be responsible for Ni$^{2+}$ potentiation in the heteromeric channel. In contrast to Shammat and Gordon (372), He et al. (159) proposed a diagonal ABAB stoichiometry. However, their experiments using the blockage by L-cis-diltiazem and the cAMP activation properties of coexpressed tandem constructs provided no evidence against an AAAB or BBBA arrangement, which they explicitly stated in their discussion.

Therefore, it seems important to rigorously determine the subunit stoichiometry and arrangement of native channels by other methods. Indeed, a biochemical approach based on cross-linking experiments of native rod channels favors an AAAB stoichiometry (Weitz, D., N. Ficek, E. Kremmer, and U. B. Kaupp, unpublished data). Nevertheless, direct structural techniques such as single-particle electron microscopy or biophysical methods able to detect each subunit in the oligomeric channel complex would be highly desirable.

**B. Subunit Distribution in the Brain and Nonneuronal Tissues**

The distribution of CNG channel subunits in the brain has been examined by various techniques (Table 4). These studies have produced conflicting results. The need for extensive PCR amplification and long development times for the in situ hybridization suggests that the levels of mRNAs encoding channel subunits are very low and, in combination with the use of different primers or cDNA probes, amplification protocols, and hybridization conditions, may account for the variable results. Finally, expression of the A1, A2, and A3 subunits in the cortex is each developmentally regulated in a specific temporospatial pattern (346), which further enhances the experimental complexity. For example, the level of the A1 transcript in rat cortical areas is high at birth (postnatal day 0) and gradually decays with time until it becomes practically nondetectable at postnatal day 55, whereas a similar developmental regulation was not observed in the hippocampal region (346).
The expression of CNG channel subunits in nonneuronal tissue has been studied by various techniques (Table 5). Except for testis and spermatozoa, the experimental evidence for expression of any of the known six CNG channel genes in nonneuronal tissue is weak. In testis, the expression of mRNA encoding A3, B1, and B3 has been shown by cloning of cDNAs and Northern analysis (34, 122, 416, 417). With the use of several polyclonal antibodies, the A3 and B1 subunits have been localized immunohistochemically to the flagellum of ejaculated sperm and precursor cells in cross-sections of seminiferous tubules. Cloning identified one short and three long transcripts of the B1 subunit (417). The highly abundant short transcript would give rise to a polypeptide that begins shortly before S1 (molecular mass 74.3 kDa). The three longer transcripts differ in their 5'-terminal region, are much less abundant, and would give rise to polypeptides with a molecular mass of 104–106 kDa. Western analysis of cauda epididymal and ejaculated sperm detected only a 80-kDa polypeptide but provided no evidence for polypeptides of higher molecular mass (417). Therefore, the long B1 subunits must be expressed at rather low levels, if at all.

The presence of CNG channel transcripts in nonneuronal tissues other than testis has been studied by Northern analysis. Biel et al. (34) demonstrated expression of A3 transcripts in renal cortex and renal medulla and a weak signal in cardiac atrium and ventricle. Ahmad et al. (3) detected the presence of A1 in heart and kidney of rat, whereas in canine, Zhang et al. (437) were unable to detect A1 transcripts in liver, spleen, lung, heart, and kidney (437). No signals using probes for A1-A3 were detected in liver of rabbit; adrenal gland and diencephalon of bovine; and brain, kidney, and pancreas of rat (92). The A1 transcript was also detected in keratinocytes (310). Ruiz et al. (350) could not detect by Northern analysis the A2 message in heart using several specific probes; however, using RNase protection

<table>
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<th>Subunit</th>
<th>Region</th>
<th>RT-PCR</th>
<th>Northern</th>
<th>In Situ</th>
<th>Western</th>
<th>Immunohistochemistry</th>
<th>Reference Nos.</th>
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<td>Heart</td>
<td>±</td>
<td>±</td>
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+, Positive results; –, negative results; ±, positive and negative results; ND, not determined; NA, not applicable.
assay, they were able to detect the A2 transcript in mouse heart and brain, but not in liver.

Ding et al. (91) studied the expression pattern of A1 in rat by four different techniques. These authors detected transcripts in total RNA or poly(A) + RNA from pineal and pituitary gland, retina, adrenal gland, and in testis with a size of 3.1–3.5 kb, consistent with the size of cloned cDNA. Transcripts of 1.8 kb and of ~6 kb were detected in kidney and spleen, respectively. No signals were detected in total RNA or poly(A) + RNA of heart, brain, lung, liver, skeletal muscle, and kidney. The RNase protection assay suggested the presence of A1 transcripts in retina, brain, lung, and spleen, but not in liver, heart, testis, and kidney. A third detection method, in situ hybridization, suggested the presence of A1 transcripts in alveolar tissue of lung, the endothelial layer of aorta (but not in smooth muscle layers), thymus, and spleen. These authors also reported intense staining in hippocampus and cerebellum and weaker staining in the cortex. Finally, using RT-PCR, A1 fragments could be amplified from all tissues tested.

The expression pattern has been also studied by RT-PCR, again with mixed results (Table 5). For example, A2 seems to be expressed in heart of rabbit, but not in rat and bovine, whereas A3 was detected in bovine heart, but not in rat and rabbit (92).

In summary, although many of these studies are suggestive, none of them conclusively demonstrates the presence of A1 transcripts in alveolar tissue of lung, the endothelial layer of aorta (but not in smooth muscle layers), thymus, and spleen. These studies also reported intense staining in hippocampus and cerebellum and weaker staining in the cortex. Finally, using RT-PCR, A1 fragments could be amplified from all tissues tested.

VII. LIGAND SENSITIVITY AND SELECTIVITY

The ligand sensitivity and selectivity of native and exogenously expressed CNG channels have been studied by measuring the currents activated in excised inside-out membrane patches upon superfusion with various concentrations of ligand. All known native CNG channels respond to both cAMP and cGMP, but lower concentrations of cGMP than cAMP are required to open the channels. In rods and cones, CNG channels sharply discriminate between cGMP and cAMP, whereas channels in OSNs respond equally well to both ligands, i.e., are much less selective (Fig. 12). Moreover, CNG channels from OSNs have a much higher sensitivity for both cAMP and cGMP than photoreceptor CNG channels. Figure 12 illustrates yet another important difference between CNG channels from photoreceptors and OSNs: cAMP is a full agonist of the olfactory channel but only a partial agonist of the rod channel.

The analysis of dose-response relations showed that channel activation is steeply dependent on cGMP concentration. In double-logarithmic plots (log $I_{\text{max}}$ vs. log [cGMP]), the limiting slopes ranged between ~1.5 and ~3.5. The slope of the dose-response relation and the limiting slope was interpreted to indicate that several cGMP molecules bind to the channel in a cooperative manner. Because each subunit harbors a single cNMP-binding site (188) and because homo- and heteromeric channels most likely form tetrameric complexes (246), maximally four ligand molecules bind to the channel.

Numerous studies examined the molecular basis of ligand sensitivity and selectivity and cooperativity. Selectivity can be achieved either by differential control of the affinity for binding of the ligand, efficacy of gating, or a combination of both. Binding affinity is a measure of how tightly cyclic nucleotides bind to the channel; it has not been determined yet for any CNG channel by a direct binding assay but has been inferred from models that describe the activation of the channel (see below). Efficacy refers to the ability of the ligand to gate the channel open once it has been seated in the binding cavity. Although the distinction between binding and gating is a useful concept, in practice it has been proven difficult to
experimentally dissect binding from gating, because both processes are inextricably coupled to each other (for a brilliant account, see Ref. 77). However, in some instances, efficacy can be gleaned from the relative maximal open probability \( \frac{P_{\text{max}}}{P_{\text{max}}(\text{cAMP})/P_{\text{max}}(\text{cGMP})} \) obtained at saturating ligand concentrations. For example, cAMP is a poor agonist for the native CNG channel from rods (390) (\( P_{\text{max}} \) in the presence of saturating cAMP concentrations is \( \sim 20\% \) of that in the presence of cGMP) or for the homomeric A1 channels, where relative \( P_{\text{max}} \) is only a few percent (7, 134), whereas for A2 channels, cAMP is able to fully open the channel (relative \( P_{\text{max}} \) reaches nearly unity).

Several important questions were addressed by a multitude of studies. Which amino acid residues interact with the ligand, and what is the molecular nature of these interactions? Which of these interactions are state dependent, i.e., occur either in the open or the closed state, and which are state independent? What is the molecular basis for ligand sensitivity and discrimination? Which part of the channel is involved in gating, i.e., the transition from the closed to the open state of the channel and which part is involved in binding?

The search for key residues involved in binding and gating have been guided by previous work on ligand binding and activation mechanism in PKA and PKG and the CAP protein. Several regions determine the binding and the activation process, including the cNMP-binding domain itself, the linker region connecting S6 and the cNMP-binding domain (“C-linker”), the NH2-terminal domain, and the pore region.

A. cNMP-Binding Site

Molecular modeling and mutagenesis studies predicted 10 different interactions between cGMP and the binding pocket involving 8 amino acid residues, 5 with the purine ring and 5 with the ribofuranose (7, 366, 368, 369, 394, 402) (Fig. 13). It is tacitly assumed that interactions with the common ribofuranose moiety are largely similar for all cyclic nucleotides, whereas differential interaction with the purine ring controls ligand selectivity. The location of these residues in the cNMP-binding fold is shown in Figure 9. The five interactions with the ribofuranose include hydrogen bonding between T560 (numbers refer to bovine A1) and O6’ of the diester group, between G543 and O2’ of the ribose moiety, and between I545 and O2’ or O3’. A salt bridge is formed between R559 and the negatively charged phosphate group. Finally, an ionic hydrogen bond is established between E544 and O2’. The five interactions with the purine ring include a hydrogen bond between the hydroxyl group of T560 and N2C. This interaction has been proposed to be mediated by an intercalated water molecule (367). Residue F533 is predicted to undergo an aromatic-aromatic interaction with the purine ring. Finally, residue D604 either shares a single hydrogen bond with N1 (367) or two hydrogen bonds with N1 and N2C of cGMP (402). cGMP and cAMP differ in their structure only at C6-N1-C2. cAMP cannot form a hydrogen bond with either N1 or N2C, resulting in an unfavorable electrostatic interaction between the negatively charged carboxylate side chain of D604 and the free electron pair at N1 of cAMP (402).

Differential interaction of cAMP and cGMP with D604 in the C helix of the cNMP-binding domain has been proposed to underlie ligand discrimination in CNG channels (402). At the equivalent position in CAP, residue T127 in one subunit and S128 from the opposite subunit of the CAP dimer form hydrogen bonds with the N2C amino group of adenine (409) (Fig. 8). In agreement with this prediction, the efficacy of gating by cAMP is enhanced 1) in A1 mutants with a neutral residue at position 604 (D604Q and D604M) (402); 2) at low pH, when D604 is presumably protonated (131); and 3) in channels that include A4, B1, and B3, i.e., subunits, which carry a neutral or positively charged amino acid at the respective position (44, 122, 131, 242).

![Differential interaction of cAMP and cGMP with D604 in the C helix of the cNMP-binding domain](http://physrev.physiology.org/10.220.33.1/2017/6/13/interaction-amino-acid-residues-cnmp-binding-site-cgmp.png)
One would have hoped that this model explains the higher gating efficacy by cAMP in olfactory (A2) channels compared with rod (A1) channels. Unfortunately, several lines of evidence suggest that various CNG channel subtypes may employ distinct amino acid residues and mechanisms to achieve ligand selectivity. For example, A2 orthologs from mammals and catfish (90, 137, 254) have a glutamate or glutamine, respectively, at this position, yet these subunits form channels that are almost indistinguishable with respect to the efficacy of gating by cAMP. Furthermore, A1 and A3 from bovine, mouse, and rat all carry an aspartate residue at the equivalent position, yet the gating efficacy by cAMP relative to that by cGMP varies from \(-0.02\) (bovine A1; Ref. 133) to 0.14 (mouse A3; Ref. 443) to almost 1.0 (rat A3; Ref. 273). Another caveat of these mutagenesis studies is that contacts formed by residues F533, K596, D604, and T560 with the purine ring are not independent from each other (369, 402) and, consequently, it is difficult to assign the selectivity switch to any one of these residues alone. Finally, the model requires cGMP to bind to the anti conformation, whereas other models propose the syn conformation (369).

The interaction of the cyclic nucleotide with the \(β\)-roll of the cNMP-binding domain was probed with phosphorothioate derivatives of cAMP and cGMP (Rp-cAMP, Rp-cGMP, Sp-cAMP, and Sp-cGMP; Refs. 217, 442). Although these compounds bind to the different CNG channels, their mode of action is quite diverse. Both Rp- and Sp-derivatives of cGMP are full agonists of native rod (442) and homomeric A1 channels (217), but only Sp-cGMP is an agonist of homomeric A2 channels, whereas Rp-cGMP is a competitive antagonist (217). Rp-cAMP is an antagonist of the rod CNG channel, but a partial agonist of the olfactory CNG channel. In contrast, Sp-cAMP fully activates the olfactory CNG channel (217). Similarly, Rp- and Sp-cAMP are both antagonists of the homomeric A1 channel, whereas A2 channels discriminate between the two analogs: Sp-cAMP is an agonist, whereas Rp-cAMP is an antagonist. These results indicate that the interaction of \(β\)-roll residues of the cNMP-binding domain with the cyclic nucleotide is different for various channels and that one has to be cautious to generalize results obtained from one channel type.

Recently, a chimera between the cyclic nucleotide-binding domain of A1 and the DNA-binding domain of CAP has been crystallized (370). At low resolution (7 Å), the overall structure of this chimera is the same as that of CAP. This seems to be a promising avenue toward a three-dimensional structure at high resolution, and it might be a unique opportunity to compare the cNMP-binding domains of different A and B subunits. However, some caution must be taken in the interpretation of the results. In fact, the A1-CAP chimera binds both cAMP and cGMP with equal affinity, whereas DNA binding is promoted by cAMP only and not by cGMP, like in the wild-type CAP protein. However, within the context of the CNG channel, this binding domain mediates full channel activation by cGMP (full agonist), but only poor activation by cAMP (partial agonist), illustrating the profound impact of protein environment on the properties of a functional domain in an allosteric protein.

B. C-Linker Region

Low concentrations of transition metal ions, including Ni\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\), potentiate the response of the rod CNG channel to subsaturating cGMP concentrations but have little effect on the maximal response at saturating cGMP concentrations (171, 183). Ni\(^{2+}\) also increased the potency of cAMP to activate homomeric A1 channels by almost 50-fold (133). Subsequent studies identified H420 in A1 as part of the Ni\(^{2+}\)-binding site responsible for the potentiation (133). Nickel potentiation in homomeric A1 channels involves the interaction of at least two H420 residues localized on adjacent subunits (135), which presumably coordinate the transition metal ion. However, a H420Q mutant of A1 when coexpressed with wild-type B1 forms heteromeric channels, whose activity can be potentiated by Ni\(^{2+}\), suggesting that potentiation of the native channel involves residues from both A1 and B1 subunits (159). In contrast, Ni\(^{2+}\) inhibited the activation of the olfactory A2 channel. This action is mediated by another histidine residue (E396) (134). The two His residues are each located in the C-linker region of the respective channel protein, suggesting that this region participates in the conformational changes leading to channel opening (134).

Swapping experiments with the C-linker region between A1 and ceA (Tax-4) channels (314) and between A2 and A3 channels (443) also suggest that this domain somehow relays the binding event to the gating machinery near or inside the pore. Three amino acid residues in the C-linker seem to account for most of the difference in efficacy of activation by cAMP between mouse A3 and rabbit A2 (443). When the residues I439, D481, and D494 of rat A3 are replaced by the respective amino acids found in rabbit A2 (I439V, D481A, and D494S), the efficacy of activation by cAMP is significantly enhanced. Quite sadly, the A2 ortholog from catfish features the identical residues at these positions as bovine A3, yet with respect to efficacy of gating, this subunit behaves like all other A2 orthologs and not like an A3 subunit (137). Furthermore, three other residues were identified in the C linker of A1 that when changed to the respective residues in ceA (Tax-4) improved the efficacy of gating (314).

C. NH\(_2\)-Terminal Region

The involvement of the NH\(_2\)-terminal region in channel activation was suggested by studies using chimeras...
between A1 and A2 subunits (139). The NH₂ terminus in A2 interacts by means of a CaM-binding site (248) with the cNMP-binding domain in the COOH-terminal region (403). This interaction lowers the change in free energy between the unliganded open and closed states. Binding of CaM interferes with this interaction and thereby increases the \( K_{1/2} \) of activation. This allosteric control seems to be less important for the A1 channel.

D. Pore

Many pore mutants show alterations in channel gating (24, 25, 62, 121, 343). Because of the principle of reciprocity, changes in gating are likely to affect binding as well. In fact, several mutations at or near E363 in A1 and the corresponding position E340 in A2 demonstrated that gating defects in the mutant channel are associated with an increase in the \( K_{1/2} \) of activation (62, 121). Likewise, an A3 pore mutant (T368S) found in patients with deficient color vision shows dramatically altered gating kinetics, a lower maximal \( P_o \), and a 10-fold increase of the \( K_{1/2} \) of activation (Tränkner, D., H. Jägle, S. Kohl, R. Seifert, L. T. Sharpe, U. B. Kaupp, and B. Wissinger, unpublished data).

E. Conformational Changes of Protein Domains During Activation

Inspired by the crystal structures of KcsA and CAP, recent studies focused on the nature of the conformational rearrangements that led to CNG channel opening. Important insight into the secondary structure of subdomains as well as into their movement was obtained by applying SCAM (5) to residues of the cNMP-binding site (263), the putative pore helix (247), and S6 (108), but also by applying a histidine scan to residues in the C linker (177).

The ligand initially binds to the closed channel involving interactions between the \( \beta \)-roll structure of the cNMP-binding site and the ribofuranose moiety of the cyclic nucleotide. Subsequently, a movement of the C helix relative to the \( \beta \)-roll structure occurs (263). This movement is somehow transduced into a rotation of the \( \alpha \)-helical C linker (177), which connects the transmembrane segment S6 with the C-NMP-binding site. Finally, the rotation around the central axis of the C-linker helix is likely to become transmitted to the S6 region. This step seems to be important for channel activation. The region located COOH terminally of S6 seems to serve different functions in Shaker \( K^+ \) channels and CNG channels. In Shaker \( K^+ \) channels, this region mechanically occludes the channel entrance (84) and harbors the activation gate (168). In CNG channels, ions can enter the inner vestibule in both the open and closed state, arguing against an intracellular location of the activation gate (108).

Instead, some experiments suggest that the selectivity filter itself is the location of the gate. SCAM studies of the state dependence of the accessibility of pore residues of A1 indicate that the pore helix undergoes a 100–180° rotation around its long axis during channel activation. Possibly, this conformational change either occurs in parallel with or precedes the movement of the gate. Future work needs to unravel at higher structural resolution how movements of the cNMP-binding domain, the C linker, and the pore helix are interconnected.

F. Kinetic Models

It is useful to discuss channel activation within the framework of linear state or sequential models and cyclic allosteric models (for an insightful discussion of different models, see Ref. 239). The sequential model involves binding of \( n \) molecules of cGMP to the closed channel, followed by a gating conformational change of the fully liganded channel to the open configuration (see Fig. 14). The binding of ligand to each subunit is described by the binding constant \( K_T \) and the open-closed transition by the equilibrium constant \( L \).

The four-site sequential model would be most appropriate for CNG channels because of their tetrameric structure. This model predicts a limiting slope of the dose-response relation of four; however, in most studies slopes between two and three were obtained. A neat explanation for this discrepancy is provided by Ruiz et al. (347). They found that the slope of dose-response relation constructed from single-channel recordings is consistently steeper (Hill coefficient 3.0) than the slope of the dose-response relation deduced from macroscopic currents (Hill coefficient <2.0). However, large variations in \( K_{1/2} \) were observed in multichannel patches. The authors suggest that the sum of channels with high Hill coefficients yet variable \( K_{1/2} \) values will give rise to artifically shallow dose-response relations. Notwithstanding this complication, Karpen et al. (185) used a three-site sequential model to describe the kinetics of channel activation using jumps in cGMP concentration (caged cGMP) and voltage. A two-site sequential model has been used to describe some of the differences in ligand sensitivity and selectivity between CNG channel subfamilies (134, 402). The analysis of dose-response relations suggested that the allosteric transition from the closed to the open configuration was energetically more favorable when cGMP was bound to homomeric A1 and A2 channels than when cAMP was bound, i.e., the difference in the activation by cGMP and cAMP was primarily attributed to differences in the free energy of the allosteric transition (\( \Delta G_{1/2} \)), whereas changes in \( K_T \) alone could not account for the behavior of the cAMP as partial agonist of the rod channel (134). Similarly, the difference between activation of A2 channels compared with A1 channels could be accounted for by the principle of reciprocity.
for by varying only the equilibrium constant $L$, implying that the initial binding of cAMP and cGMP, characterized by the binding constant $K_T$, is similar (134). However, in another study by Zagotta and co-workers group (402), a 10-fold lower affinity was obtained for binding to the rod channel of cAMP compared with cGMP $[K_T(cAMP) = 2,020 \mu M; K_T(cGMP) = 226 \mu M]$. The problem is this: “high efficacy” ligands, characterized by $L$ values $\geq 20$ are indistinguishable from each other, because they yield maximal $P_o = 0.95$ at saturating ligand concentrations (see Ref. 77). This problem was overcome by Li and Lester (238). Using single-channel kinetics of the olfactory A2 subunit, these authors showed that cGMP binds with much higher affinity than cAMP (30-fold difference in $K_T$) and that the equilibrium constant $L$ that governs the open-closed transitions is largely similar for cAMP and cGMP.

In the linear state models, the unliganded and partially liganded states do not lead to the open configuration. The cyclic allosteric model proposed by Monod et al. (288) is a more general form of sequential models that permits this transition (Fig. 14). The allosteric model assumes that the binding sites are equivalent but that the ligand affinity is higher in the open than in the closed states. The opening of the unliganded channel, characterized by the equilibrium constant $L_0$ is usually low ($L_0 < 1$). Because the open channels bind ligands more tightly than the closed channels, the stability of the open configuration is enhanced by a factor $f$ each time an additional ligand molecule binds to the channel.

Three predictions can be derived from the model of Monod et al. (288): 1) channels should show openings independent from the ligand, 2) each liganded state should be associated with a unique open state, and 3) the equilibrium constant $L$ and, therefore, the $P_o$ should increase by a constant factor $f$ for each ligand that binds. One of the three predictions of the model of Monod et al. (288) is satisfied by experiment, and two are not. In fact, CNG channels show ligand-independent openings (200, 323, 348, 349, 393). The spontaneous open probability ($P_{sp}$) is significantly lower for A1 than for A2 channels $[P_{sp} = 1.25 \times 10^{-4}$ for a chimeric channel comprising the A1 sequence and an olfactory A2 pore, $P_{sp} = 1.5 \times 10^{-5}$ for the A1 subunit (348), and $P_{sp} = 2.25 \times 10^{-3}$ for A2 (393)], indicating that the free energy of gating is smaller in olfactory channels than that in rod channels. Transplantation of the NH$_2$-terminal domain from A2 subunit to A1 produces chimeric channels whose $P_{sp}$ is nearly identical to that of A2 (393). These and previous results show that the NH$_2$-terminal domain determines differences in both ligand gating and spontaneous openings between rod and olfactory CNG channels and that channel activation occurs by a cyclic allosteric mechanism. The analysis of the allosteric mechanism was taken one significant step further by Ruiz and Karpen (349). They succeeded to

![FIG. 14. Three different models of channel activation. Left: sequential model (red) and cyclic allosteric model (red and blue). Right: coupled-dimer model. Subunits can exist in the inactive (“tense”) conformation (T, squares) or the active (“relaxed”) conformation (R, circles). $K_T$ and $K_R$ are the equilibrium constants for binding of the agonist A to the T and R states, respectively; $L_0$, equilibrium gating constant between the unliganded T and R states; $L$, equilibrium gating constant between $T_{A\alpha}$ and $R_{A\alpha}$ in the sequential model (red); $f$ is a multiplicative factor, $f = K_R/K_T$. [Modified from Li et al. (239) and Richards and Gordon (340).]
covalently tether a cGMP photoaffinity analog to the A1 channel and thereby permanently activate the channel (55). This technique allows to lock exactly one, two, three, or four cGMP molecules onto their binding sites and, in combination with single-channel recordings, to observe the gating of single channels with fixed ligand occupancies (348). Channels occupied by four ligands stay open most of the time; unliganded channels and channels with one ligand open with a $P_o$ of $10^{-5}$. When two and three ligands were locked in place, $P_o$ is 0.01 and 0.33, respectively. These $P_o$ values are not in the ratios predicted by the model of Monod et al. (288). More interestingly, when less than four ligands are bound, the opening events show frequently smaller subconductance states, as if the flipping of each subunit opens the channel wider, and the probability distribution of subconducting states depends on the number of ligands bound. These observations are inconsistent with the simple model of Monod et al. (288) as outlined in Figure 14.

As elegantly as these experiments are conceived, they are not entirely unequivocal. The issue of multiple conducting states is unresolved. Several authors do not find subconductance states or as many as four (29, 170, 245, 348, 349, 386). An alternative route to test the model of Monod et al. (288) has been taken by Liu et al. (245). To dissect the impact of each individual binding event on channel activation, these authors constructed channels with a constrained number of functional cNMP-binding sites. This goal was achieved by coexpressing subunits with wild-type binding sites and mutant subunits with disabled binding sites. The binding of the ligand was abolished by mutations of R559 and T560; these residues have previously been shown to decrease ligand sensitivity by several orders of magnitude (7, 394). The results of Liu et al. (245) and Ruiz and Karpen (348, 349) qualitatively agree with each other in as much as both show a progressive increase in $P_o$ with increasing numbers of bound ligands. However, the two studies significantly differ in the factor by which each bound ligand increases $P_o$. For example, binding of one ligand does not change $P_o$ in the tethered ligand approach, whereas $P_o$ was 0.017 in the disabled binding-site approach. Whatever mechanism might explain this discrepancy, both studies show that the conventional model of Monod et al. (288) is inadequate to fully comprehend the complexity of CNG channel activation. Liu et al. (245) therefore proposed an interesting variation of the model of Monod et al. (288) in which the channel is composed of a pair of dimers [coupled-dimer (CD) model, see Fig. 14]. In the CD model, each subunit in a dimer binds the ligand independently, yet the two subunits undergo a concerted allosteric conformational change to open. Moreover, each of the two dimers makes this transition independently.

VIII. ION SELECTIVITY

CNG channels strongly select cations over anions, discriminate poorly among monovalent cations, and are also permeable to divalent cations, in particular Ca$^{2+}$. In physiological ionic solutions, therefore, both monovalent and divalent cations permeate CNG channels, and any one ion species carries only a fraction of the total current. The Ca$^{2+}$ permeability of CNG channels is a crucially important part of their cellular function. Changes in CNG channel activity are inevitably associated with changes in [Ca$^{2+}$], that initiate various excitatory or adapting cellular processes.

A. Selectivity for Alkali Ions

The selectivity of CNG channels for monovalent cations was determined from relative ion permeabilities recorded under biionic conditions in excised patches. Ion selectivity of native CNG channels has been first examined in intact cells by changing ionic solutions at the extracellular side of the cell (67, 166, 167, 222, 223, 272, 297, 432). Although these early studies were complicated by the lack of control over the intracellular concentrations of ions, they revealed that CNG channels from rod and cone photoreceptors and OSNs do not select strongly among alkali cations.

With the use of the excised inside-out patch configuration, the following sequence of permeability ratios was obtained for the channel from salamander rods: Li$^+$ > Na$^+$ > K$^+$ > Rb$^+$ > Cs$^+$ = 1.14:1:0.98:0.84:0.58 (271). Similar permeability sequences have been reported for CNG channels from frog rods (103), mammalian rods (255), and depolarizing photoreceptors of the lizard parietal eye (105, 106). The permeability of the CNG channel from salamander rods was also probed with several large organic cations (320). From space-filling models of the tested cations, the narrowest part of the pore is estimated to be at least a 3.8 Å $\times$ 5 Å rectangle.

The sequence of ion selectivities and permeability ratios in native CNG channels of rods and recombinant A1 subunits are rather similar, except for Li$^+$, which is more permeant than Na$^+$ in rod CNG channels, but less permeant in A1 homomeric channels (188, 255). Coexpression of A1 and B1a subunits yielded channels with permeability ratios similar if not identical to native channels (214).

The ion selectivity of the CNG channel of cones is similar to that of rods (321): K$^+$ > Na$^+$ > Li$^+$ > Rb$^+$ > Cs$^+$ = 1.11:1:0.99:0.96:0.8.

A small difference, however, is noticeable. In cones, Li$^+$ is equally or less permeable than Na$^+$, whereas in rods it is more permeable. The ion selectivities of recombinant A1 and A3 homomeric channels from chick are by and large identical (39).
The selectivity sequence of the native CNG channel from OSNs is qualitatively similar to that of rods and cones (112): Na\(^+\) > K\(^+\) > Li\(^+\) > Rb\(^+\) > Ca\(^{2+}\) = 1.0:0.81:0.74:0.60:0.52 and similar to that of A2 homomorphic channels of catfish OSNs (138). The diameter of the channel pore probed by the permeability of large organic cations was 5.8–5.9 Å for the A1 homomeric channel and 6.3–6.4 Å for the A3 the homomeric channel (138). Similar experiments with the native CNG channel from rat OSNs yielded slightly larger pore dimensions (6.5 Å × 6.5 Å) (16).

In conclusion, selectivity for alkali ions is similar in various isoforms of CNG channels and across species.

**B. Blockage by Divalent Cations**

To permeate, Ca\(^{2+}\) must bind to a site inside the pore and thereby block the current carried by monovalent cations. The characteristic features of the blocking action of extracellular Ca\(^{2+}\) are summarized in Figure 15. The current recorded from an excised patch in the outside-out configuration is progressively suppressed by increasing concentrations of Ca\(^{2+}\) (Fig. 15A). The blockage is strongly voltage dependent: inward currents are more effectively suppressed than outward currents (Fig. 15A, red trace). The voltage dependence of Ca\(^{2+}\) blockage is characteristic of a positively charged and permeable blocking molecule. This is illustrated by plotting the fraction of unblocked current against \(V_m\) (Fig. 15B). Starting from +80 mV, blocking efficacy increased continuously when \(V_m\) is made less positive. At around −40 mV, blockage is maximal, although not complete, and is relieved again at more negative values of \(V_m\). The relief of blockage at negative \(V_m\) has been interpreted as facilitation of Ca\(^{2+}\) permeation (76). The voltage dependence of the Ca\(^{2+}\) blockage has been analyzed (371) using the Woodhull model that originally has been used to describe the proton block of Na\(^+\) channels (423). One parameter in the Woodhull model, the “effective valence” \(z\) represents the number of blocking charges. In the three channel types tested, \(z\) adopts values of −2, supporting the notion that a single Ca\(^{2+}\) blocks the ionic pathway. Another parameter that characterizes the fraction of the membrane voltage acting at the blocking site, which is often equalled to the position of the ion binding site, adopts values of −0.4, indicating that the site is located in the membrane approximately one-third of its thickness (98).

The Ca\(^{2+}\) affinity of the intrapore binding site has been determined by measuring the Ca\(^{2+}\) (and Mg\(^{2+}\)) dependence of the blockage of monovalent currents (113, 371). The dose-response relation between normalized current and extracellular Ca\(^{2+}\) (Fig. 15C) over the entire voltage range is well described by a simple binding isotherm, supporting the notion that CNG channels carry a single binding site inside the pore and that the occupancy by a single Ca\(^{2+}\) is sufficient to occlude the channel (371).

A glutamate (Glu) residue in the P loop of the CNG channel A1 to A3 subunits has been identified as an important structural element of Ca\(^{2+}\) binding. When this Glu residue is replaced by a neutral amino acid, Ca\(^{2+}\) blockage is almost entirely abolished (98, 121, 317, 343) (Fig. 15E). Because a Glu residue is provided by each A subunit, in homotetrameric channels, a set of four glutamate residues is expected to form the high-affinity intrapore Ca\(^{2+}\)-binding site. The net negative charge of the Glu residues appears to be an important determinant of the Ca\(^{2+}\) affinity. Experimental maneuvers that either enhance or diminish the negative charge at this site also decrease or increase, respectively, the \(K_{1/2}\) of blockage. First, when the Glu residue is replaced by various neutral residues, in each case, the high-affinity Ca\(^{2+}\)-binding site is abolished, whereas substitution of Glu with Asp, another negatively charged amino acid, enhances rather than impairs the Ca\(^{2+}\)-binding affinity (121, 317, 371). Second, protonation of the Glu residues also weakens the Ca\(^{2+}\) affinity. At low external pH, a higher extracellular Ca\(^{2+}\) is required to produce the same fraction of blockage than at high pH (338, 371) (Fig. 15F). The four Glu carboxylates form two identical noninteracting protonatable sites (\(pK_a\) of 7.6) in channels composed of the catfish A2 subunit (344). Protonation of these sites produces two substates of smaller conductance. Third, reduction of the negative charge in heteroligomeric channels consisting of A and B subunits (with a Gly residue at the respective position in the pore loop) also results in a lower Ca\(^{2+}\) affinity (214) (Fig. 15C). Thus experimental measures expected to either reduce or eliminate the negative charge at the Ca\(^{2+}\)-binding site by three independent mechanisms also reduce or eliminate Ca\(^{2+}\) binding. These results underscore the importance of negative charges for binding of Ca\(^{2+}\) inside the pore vestibule.

Various homomeric CNG channels formed by either A1, A2, A3, or dmA distinctively differ in their sensitivity to blockage by extracellular Ca\(^{2+}\) (113, 371), implying that the sites inside the pore bind Ca\(^{2+}\) with different affinity (Fig. 15D). However, the pore region itself, including the key Glu residue, is highly conserved among A1-A3 subunits and dmA (Fig. 15G), suggesting that residues outside the pore region either participate directly in the binding of Ca\(^{2+}\) or modify the geometric arrangement of glutamates. By an extensive mutagenesis study, Seifert et al. (371) showed that all aspects of Ca\(^{2+}\) blockage are transferred from one channel type to another when the S5-P-S6 region is exchanged between different channels, whereas swapping of domains outside the S5-P-S6 region has no or only small effects. The S5-P-S6 region serves as a basic pore module that governs both blockage and ion permeation in CNG channels. These authors also showed that several amino acid residues at the extracellular ends of the S5 and S6
segments located near the membrane water interface, as well as the linkers that connect these segments with the pore loop, determine how strongly Ca\textsuperscript{2+} interacts with the pore.

CNG channels not only differ in their interaction with Ca\textsuperscript{2+}, but also in their pH sensitivity. The reduction of monovalent currents by external protons in the A1 and A2

**FIG. 15.** Ca\textsuperscript{2+} blockage of CNG channels. A: current-voltage relations of heterologously expressed homomeric A1 channels in the presence of various concentrations (see labels in micromolar) of extracellular Ca\textsuperscript{2+}. Currents carried by potassium are strongly blocked by Ca\textsuperscript{2+} in a dose-dependent fashion. [Data from Seifert et al. (371).] B: voltage dependence of the fraction of unblocked currents (see text). [Data from Seifert et al. (371).] C: dose-response relation of Ca\textsuperscript{2+} blockage at ~60 mV of the heterologously expressed A1 channel (black circle) compared with the coexpressed A1B1 channel (blue circle). Coexpression of the A1 and B1 subunits leads to a reduction in the apparent affinity of the intrapore binding site for Ca\textsuperscript{2+}. [Data from Körschen et al. (214).] D: dose-response relation of Ca\textsuperscript{2+} blockage for different heterologously expressed A subunits. The apparent affinity of the intrapore binding site differs among channels. [Data from Baumann et al. (22) and Frings et al. (113).] E: dose-response relation of Ca\textsuperscript{2+} blockage of heterologously expressed A1 channels (solid circles) and the E363Q mutant channel (open squares). The apparent affinity of the intrapore binding site in the mutant is reduced by about three orders of magnitude. [Data from Eismann et al. (98).] F: dose-response relation of Ca\textsuperscript{2+} blockage of heterologously expressed A channels at different pH values. The apparent affinity of the intrapore binding site is reduced by protonation of pore glutamates. [Calculated from data in Seifert et al. (371).] G: alignment of the pore region of A1, A2, A3, dmA, and B1.
channels seems to be controlled by acidic groups with a pK$_a$ of ~6.5 and 7.6, respectively (344, 371). As transplantation of the S5-P-S6 region from one channel type to another transfers both the extracellular pH sensitivity and the sensitivity to Ca$^{2+}$ blockage, it has been suggested that different protonation patterns of the Glu residues underlie the different Ca$^{2+}$ sensitivities (371).

### C. Ca$^{2+}$ Permeation

Ca$^{2+}$ selectivity of CNG channels has been probed by two independent experimental measures. First, the permeability for Ca$^{2+}$ relative to that for monovalent cations, $P_{Ca}/P_M$, has been determined from $V_{rev}$ measured under various biionic conditions. Second, the fraction of the total ionic current carried by Ca$^{2+}$ was determined under physiological conditions.

#### 1. Relative permeabilities $P_{Ca}/P_M$

All CNG channels are more permeable to Ca$^{2+}$ than to Na$^+$ (Table 6). Relative ion permeabilities $P_{Ca}/P_{Na}$ are 6.5 and 10.3 in salamander rods and lizard parietal eye, respectively, but 21.7 in cones of striped bass (105, 146, 322). The difference in $P_{Ca}/P_{Na}$ between CNG channels of rods and cones is also observed in recombinant channels formed from the respective A1 and A3 subunits alone (113).

The interaction between photoreceptor CNG channels and divalent cations depends on the cGMP concentration. Torre and co-workers (64, 67) were the first to notice that elevating the level of cGMP modifies the ionic selectivity for divalent cations of the rod CNG channel. For example, Mn$^{2+}$, which under normal conditions does not appreciably permeate the channel, becomes permeable at high cGMP concentrations. This effect has now been examined in more detail by measuring the dependence of $P_{Ca}/P_{Na}$ on the cGMP concentration (146).

#### 2. Fractional Ca$^{2+}$ currents

An important measure of Ca$^{2+}$ permeability is the fraction of the total current ($I_t$) that is carried by Ca$^{2+}$ under physiological conditions. It is also referred to as fractional Ca$^{2+}$ permeability ($P_f$) (361). $P_f$ adopts values between 0 and 1. The fraction of the dark current carried by Ca$^{2+}$ in intact rods has originally been estimated using an indirect method. This method relies on the measurement of the current generated by the electrogenic transport of cations by the Na$^+/Ca^{2+}$-K$^+$ exchanger and comparison with the changing current through the light-dependent CNG channel. This method consistently provided estimates that 10–18% of the dark current is carried by Ca$^{2+}$ (141, 229, 297, 435). The electrogenic Na$^+/Ca^{2+}$-K$^+$ exchange current in cones is at least 10-fold larger than in rods (163, 298, 318), and hence, separating the electrogenic from the photocurrent is fraught with error (J. Korenbrot, unpublished observations). Therefore, the analysis of the fractional Ca$^{2+}$ current in cones is

**Table 6. Relative ion permeabilities for divalent cations**

<table>
<thead>
<tr>
<th></th>
<th>$P_{Ca}/P_{Na}$</th>
<th>$P_{Na}/P_{Na}$</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rod</td>
<td>6.5†</td>
<td>ND</td>
<td>146, 322</td>
</tr>
<tr>
<td>Parietal eye</td>
<td>10.3</td>
<td>ND</td>
<td>105</td>
</tr>
<tr>
<td>A1 homomer</td>
<td>3.1†</td>
<td>3.3†</td>
<td>113</td>
</tr>
<tr>
<td>Cone</td>
<td>21.7†</td>
<td>ND</td>
<td>146</td>
</tr>
<tr>
<td>A3 homomer</td>
<td>10.9</td>
<td>9.3†</td>
<td>113</td>
</tr>
<tr>
<td>OSNs</td>
<td>6.5</td>
<td>ND</td>
<td>225</td>
</tr>
<tr>
<td>A2 homomer</td>
<td>6.8†</td>
<td>11.9†</td>
<td>113</td>
</tr>
<tr>
<td>dmA1 homomer</td>
<td>34.9†</td>
<td>11.2†</td>
<td>22</td>
</tr>
</tbody>
</table>

* Some authors used the Fatt and Ginsborg (101) approximation to calculate $P_{Ca}/P_{Na}$; others used the equation derived by Lewis (237). Some authors used ion concentrations, instead of activities. To make figures comparable all data have been calculated using the equation given by Lewis and using ion activities.
less reliable than that in rods. Notwithstanding these experimental difficulties, Perry and McNaughton (318) reported a roughly twofold larger Ca$^{2+}$ current in salamander cones than in rods (21 and 10%, respectively).

Neher and collaborators (361, 440) developed a method to measure $P_f$ values in glutamate and acetylcholine receptor channels. The method requires the simultaneous measurement of total membrane current $I_t$ by means of the patch-clamp technique and Ca$^{2+}$ flux through spectroscopic techniques using fluorescent Ca$^{2+}$ indicators (reviewed in Ref. 302). Frings and Korenbrot and their collaborators applied this method to study fractional Ca$^{2+}$ currents in recombinant CNG channels (97, 113) and native CNG channels of intact rods and cones (311). An important prerequisite of the accurate determination of $P_f$ is the fast and synchronous activation of CNG channels. This goal was achieved by using caged cyclic nucleotides. These photolabile substances release the ligand upon irradiation with a flash of ultraviolet light, thereby activating CNG channels within a few milliseconds (150, 185, 187).

With the use of this direct method, $P_f$ values of 14 and 21% were determined for the percentage of current carried by Ca$^{2+}$ in rods of catfish and salamander (311), respectively, at 1 mM extracellular Ca$^{2+}$. These results are similar to those that are based on the analysis of the Na$^+$/Ca$^{2+}$/K$^+$ exchange current. In cones of striped bass and catfish, the fractional Ca$^{2+}$ currents are more than twofold larger than in rods (33 and 34%, respectively) (311). These results also demonstrate that the differences in fractional Ca$^{2+}$ current are maintained across several species. The differences in $P_f$ between CNG channels of rods and cones in combination with the known difference in the clearance rate of Ca$^{2+}$ from the outer segment suggests that changes in [Ca$^{2+}$]o will be larger and faster in cones than in rods. A difference in Ca$^{2+}$ balance may underlie the dissimilarity in the light sensitivity, the kinetics of the light response, and characteristics of the light and dark adaptation of the two types of photoreceptors.

The small size of the chemosensitive cilia makes measurements of $P_f$ in intact OSNs inpracticable. A value of $P_f$ has been estimated though in HEK293 cells by coexpression of the A2, A4, and B1b subunits that produce channels with properties that are largely identical to those of native channels (40). At 2 mM external Ca$^{2+}$, 41% of the current are carried by Ca$^{2+}$ (97). Given the high [Ca$^{2+}$] of 3–7 mM (83, 337) in the olfactory mucus that covers the cilia, we expect that most, if not all, of the current passing the olfactory CNG channel is carried by Ca$^{2+}$.

The relationship between Ca$^{2+}$ concentration and $P_f$ has been systematically analyzed in recombinant channels formed from A subunits alone (97, 113). While all A isoforms carry a pure Ca$^{2+}$ current at [Ca$^{2+}$] ≥ 10 mM ($P_f$ ~ 1), they are set apart from each other by the Ca$^{2+}$ dependence of $P_f$. The difference has been characterized by the constant $K_{Pr}$, i.e., the extracellular Ca$^{2+}$ concentration at which one-half of the current is carried by Ca$^{2+}$ ($P_f = 0.5$) (97). $K_{Pr}$ has a characteristic value for each A isoform. The rod A1 channels display the lowest $K_{Pr}$ (0.4 mM), and $P_f$ is essentially unity when Ca$^{2+}$ is above 1 mM, whereas $K_{Pr}$ = 4.2 mM for the dmA subunit and extracellular Ca$^{2+}$ values >10 mM are required to achieve a $P_f$ = 1. The $K_{Pr}$ value for the A2 and A3 channels are similar to each other and intermediate between A1 and dmA.

Two striking differences exist between the native CNG channels from rod and cone and the corresponding A homomeric channels. First, the $K_{Pr}$ of the recombinant channels is lower in rods than in cones, whereas in the native channels this is reversed. As a consequence, the $P_f$ value at 1 mM Ca$^{2+}$ in cone channels is about twofold higher than that in rod channels, whereas for recombinant channels it is about three times lower. Second, the Ca$^{2+}$ dependence of $P_f$ is extremely steep in the recombinant A1 channel but remarkably shallow in the native rod channel; even when extracellular Ca$^{2+}$ is 10 mM, $P_f$ is still only 0.5. The mechanisms responsible for these functional differences are not known. One possibility is that the B subunits, which carry a Gly instead of a Glu residue in the pore loop, create pores that are distinctively different from those in A homomeric channels. A second possibility is that the arrangement and dielectric environment of the Ca$^{2+}$-coordinating residues are changed by Mg$^{2+}$. The $P_f$ values in the native CNG channels were all measured in the presence of external Mg$^{2+}$, whereas the studies in A homomeric channels were conducted without Mg$^{2+}$. Because Mg$^{2+}$ binds to (113) and permeates (433) CNG channels, the presence of Mg$^{2+}$ might interfere with the interaction between Ca$^{2+}$ and the pore.

D. Comparison of CNG Channels and Voltage-Gated Ca$^{2+}$ Channels

Comparison of similarities and differences between CNG channels and voltage-gated Ca$^{2+}$ channels can help to understand the permeation mechanism in CNG channels. In homomeric CNG channels and in voltage-gated Ca$^{2+}$ channels, a set of four glutamate residues forms the cation-binding site in the pore (100, 428). Both channels conduct monovalent cations in the absence of Ca$^{2+}$, showing values of single-channel conductance in the range of 20–80 pS in Ca$^{2+}$-free solution. Micromolar concentrations of extracellular Ca$^{2+}$ block monovalent currents in both channel types, but voltage-gated Ca$^{2+}$ channels show higher Ca$^{2+}$ affinity ($K_i$ ~ 1 μM) compared with CNG channels ($K_i$ between 3 and 500 μM). Finally, current recordings at high Ca$^{2+}$ concentrations have revealed a similar conductance of Ca$^{2+}$ relative to that of Na$^+$. The single-channel conductance of voltage-gated
Ca\(^{2+}\) channels from ventricular heart cells is 85 pS with 150 mM Na\(^+\) and 9 pS with 110 mM Ca\(^{2+}\) (162). A relative Ca\(^{2+}\)/Na\(^+\) conductance of ~0.1 is also observed for native olfactory CNG channels and homomeric dmA channels (22, 97), although the absolute single-channel conductance for Ca\(^{2+}\) is likely to be at least 10-fold smaller in CNG channels than in Ca\(^{2+}\) channels. While these similarities in pore structure and conducting properties point to a common mechanism for ion permeation, some dissimilarities illustrate the different tasks the two channel types fulfill under physiological conditions. The higher Ca\(^{2+}\) affinity of voltage-gated Ca\(^{2+}\) channels causes a virtually complete suppression of monovalent currents and produces pure Ca\(^{2+}\) currents at 1–2 mM extracellular Ca\(^{2+}\), whereas the known native CNG channels conduct mixed cation currents at this extracellular Ca\(^{2+}\) concentration (297, 318). Despite their high Ca\(^{2+}\) affinity, voltage-gated Ca\(^{2+}\) channels conduct substantial Ca\(^{2+}\) currents, possibly sustained by electrostatic repulsion of two Ca\(^{2+}\) that can occupy the pore at the same time. Such a mechanism appears to be much less effective in CNG channels, although double occupancy may also play a role. Indeed, the Ca\(^{2+}\) dependence of \(P_T\) for various A subunits is significantly steeper than predicted from the Goldman-Hodgkin-Katz equation (97). This result was interpreted to indicate that more than one Ca\(^{2+}\) can occupy the CNG channel pore. Nevertheless, high Ca\(^{2+}\) affinity in CNG channels is clearly correlated with a low Ca\(^{2+}\) conductance, and the most substantial Ca\(^{2+}\) influx is mediated by low-affinity channels. In conclusion, several structural features common to both channel types give rise to pure Ca\(^{2+}\) currents in voltage-gated Ca\(^{2+}\) channels and to mixed cation currents with a high Ca\(^{2+}\) fraction in CNG channels.

IX. CHANNEL MODULATION

CNG channels, unlike other ligand-gated channels, do not desensitize in the continued presence of the ligand. However, the activity of CNG channels appears to be modulated by Ca\(^{2+}\)-binding proteins and phosphorylation/dephosphorylation. In this section we discuss the underlying mechanisms and the physiological significance of this modulation.

A. Phosphorylation/Dephosphorylation

Repeated measurement of the dose-response curve of channel activation in excised patches of ROS membranes disclosed a slow increase in cGMP sensitivity over time (128). The decrease in \(K_{1/2}\) was usually 2- to 3-fold but could be as large as 10-fold. The enhancement of ligand sensitivity was slowed down by both ATP and inhibitors of Ser/Thr phosphatases and was accelerated by purified type 1 phosphatase, suggesting that phosphorylation might control the conversion of channels between states of high and low ligand sensitivity. When the membrane patch was excised into a Ca\(^{2+}\)-free medium (containing 1–2 mM EGTA), the time-dependent decrease of \(K_{1/2}\) was irreversibly abolished. This observation suggests that an unknown factor, possibly a Ca\(^{2+}\)-dependent phosphatase tightly adhering to the membrane patch, is permanently inactivated or lost in Ca\(^{2+}\)-free medium.

The A1 subunit of the rod CNG channel, when heterologously expressed in Xenopus oocytes, displays a seemingly similar decrease of \(K_{1/2}\) after patch excision (287). Unlike in the native rod CNG channel, inhibitors of Ser/Thr phosphatases are without effect, whereas orthovanadate and pervanadate, inhibitors of phosphotyrosine phosphatases (PTPs), slow down the progressive sensitivity enhancement (285, 287). The effect is reduced but not entirely abolished by the mutation Y498F in the \(\beta_1\)-strand of the cNMP-binding site, consistent with the idea that this residue in oocytes is phosphorylated by a protein tyrosine kinase (PTK). In Ca\(^{2+}\)-free medium, the time-dependent effect of PTPs on A1 subunits in oocyte membranes persists, whereas in native ROS membranes no change in ligand sensitivity occurs (128, 287). Genistein, a PTK inhibitor, slows dramatically channel activation and reduces maximal currents by twofold (284, 286). These effects occur in the absence of ATP and were taken as evidence that genistein, in addition to inhibiting tyrosine phosphorylation, also promotes an allosteric inhibitory interaction between the PTK and the channel that does not involve phosphorylation (284, 286). The action of genistein was also observed in native channels of rods, cones, and OSNs, raising the possibility that CNG channels are part of a regulatory complex that contains PTKs (286).

The \(K_{1/2}\) value of the A3 subunit of the cone CNG channel in HEK293 cells shifts from 19 to 56 \mu M upon treatment with phorbol esters (290). The change in ligand sensitivity involves phosphorylation of two serine residues (S577 and S579) in the cNMP-binding domain. The \(\delta\)-isoform of PKC is specifically expressed in cone outer segments and might mediate the phosphorylation. Phosphorylation and its effect on ligand sensitivity have not been studied in the native CNG channel of cone photoreceptors.

In the chick, the cGMP sensitivity of the cone CNG channel is under the control of a circadian rhythm (201). During the subjective night, the sensitivity is approximately twofold higher than during the subjective day. Circadian modulation of ligand sensitivity is driven, at least in part, by rhythms in the activities of two protein kinases: the Erk form of mitogen-activated protein kinase and the Ca\(^{2+}/CaM\)-dependent protein kinase II (CaMKII). Perturbation of these signaling pathways causes phase-dependent changes in the cGMP sensitivity of the cone CNG channel. The mechanism of the sensitivity regulation...
has not been determined. It may involve phosphorylation of A or B subunits by Erk or CaMKII or phosphorylation of other signaling molecules that interact with the channels and lead to modulation of their sensitivity.

The cGMP sensitivity of the A2 subunit of the olfactory CNG channel is greatly enhanced (10-fold decrease of $K_{1/2}$) by PKC-mediated phosphorylation of a serine residue (S93) adjacent to a CaM-binding site. However, the shift of $K_{1/2}$ is not observed in native CNG channels from phorbol ester-treated OSNs (289).

In conclusion, the physiological implications of phosphorylation of CNG channels, whether in rods, cones, or OSNs or whether by PKC, PTK, Erk, or CaMKII, are not completely understood and deserve further study in intact OSNs or whether by PKC, PTK, Erk, or CaMKII, are not completely understood and deserve further study in intact cells. It seems necessary 1) to show that CNG channels in fact become phosphorylated in situ, 2) to identify the respective protein kinases and phosphatases along with their regulatory mechanisms, 3) to identify the channel subunits and amino acid residues involved, and 4) to characterize the cellular function of this modulation.

B. Ca$^{2+}$-Binding Proteins

A variety of ion channels, notably Ca$^{2+}$-permeable channels, are modulated by Ca$^{2+}$/CaM (236). Often Ca$^{2+}$/CaM downregulates channel activity and, thereby, provides a mechanism that interrupts Ca$^{2+}$ entry and terminates the cellular response. CNG channels are no exception to this rule. The ligand sensitivity of CNG channels is modulated by binding of CaM or by as yet unidentified Ca$^{2+}$-binding proteins (for review, see Ref. 280). CaM lowers the ligand sensitivity of CNG channels from rod, cone, and OSN to different extents and by way of different subunits.

1. Olfactory channel

Ca$^{2+}$/CaM increases the $K_{1/2}$ (i.e., lowers the ligand sensitivity) of the native olfactory CNG channel for cAMP up to 50-fold (17, 72). The change in $K_{1/2}$ is thought to be the main adaptive feedback mechanism in OSNs (224) (see sect. vB). CaM binds to a short segment in the NH$_2$-terminal region of the A2 subunit in a Ca$^{2+}$-dependent fashion (248, 413). The $K_{1/2}$ of CaM binding to the target peptide (4–12 nM; Refs. 248, 413) and the EC$_{50}$ of the Ca$^{2+}$/CaM action on channel activity (21 nM; Ref. 72) are of similar magnitude, suggesting that this target sequence is a major determinant of the CaM action. The amino acid sequence of the target peptide belongs to a common form of CaM-binding sites. These sites are characterized by aromatic residues at positions 1 and 14 of the motif and additional hydrophobic residues at positions 5 and 8. Several positively charged residues are interspersed between the hydrophobic residues. In the $\alpha$-helical conformation, basic and hydrophobic residues become located on opposite sides of an amphipathic helix.

2. Rod channel

The native CNG channel of rod photoreceptors is exquisitely sensitive to regulation by Ca$^{2+}$/CaM ($K_{1/2}$ (CaM) of 1–2 nM; Refs. 20, 169, 413), yet the decrease of cGMP sensitivity, by comparison with the olfactory channel, is modest (maximal 2-fold increase of $K_{1/2}$ for cGMP activation; Refs. 20, 130, 169, 295, 413). The Ca$^{2+}$ dependence of the modulation of the native channel [$K_{1/2}$(Ca$^{2+}$) = 48 nM; Ref. 295] and the binding of CaM to the target peptide [$K_{1/2}$(Ca$^{2+}$) = 117 nM; Ref. 413] are similar and well within the range of Ca$^{2+}$ concentrations during the light response (141, 211, 229, 264). In contrast to the olfactory A2 subunit, the A1 subunit of rod CNG channels does not bind CaM, and homomeric channels composed of A1 subunits are not modulated by CaM (70, 169, 214). CaM sensitivity is conferred to heteromeric rod channels by an unconventional CaM-binding site in the NH$_2$-terminal region of the B1a subunit (143, 413).

Although CaM modulation of the rod CNG channel is well established in vitro, its significance as an adaptive mechanism has been questioned on various grounds (141, 215, 295, 352; for review, see Ref. 280). Two of the most nagging problems have been that the less than twofold decrease in cGMP sensitivity of the native rod channel by CaM seems small compared with the large changes in the cell’s sensitivity during light adaptation, and efforts to demonstrate in vivo that a living rod is using that mechanism yielded mixed results (88, 142, 216, 280, 295, 352). Even an unidentified endogenous factor has been implied because exogenous CaM does not always fully recapitulate the change in the Ca$^{2+}$ dependence of the cGMP sensitivity that is observed after patch excision or after truncation of the ROS (130, 352). It is unlikely that this factor is another Ca$^{2+}$-binding protein. Removal of endogenous CaM from the cytosol of ROS by immunoprecipitation also removes all Ca$^{2+}$-dependent modulatory activity (R. S. Molday, personal communication).

3. Cone channel

Although cone CNG channels in excised patches from some species are weakly modulated by CaM [heterologously expressed chicken A3 (41), native cone CNG channels of striped bass (145)], cones from other species were found to be insensitive to CaM (native catfish (157), human and bovine A3 (41, 144, 436)]. These observations would not support a role of CaM in sensitivity regulation of the cone’s light response, but matters seem to be more complex. Surprisingly, the A3 subunit of the cone CNG channel, like the A2 subunit of OSNs, comprises in its NH$_2$-terminal region a fairly conserved CaM target motif that, in various binding assays, shows CaM binding (41,
These seemingly paradoxical findings might be reconciled by the idea that the cGMP sensitivity of the CNG channel of cones is controlled by another Ca$^{2+}$-binding protein and that CaM can act as a weak partial agonist in some but not all species. Experimental support for this hypothesis has been provided by Rebrik and Korenbrod (333). In electropermeabilized cones, the $K_{1/2}$ (cGMP) increased from 84.3 $\mu$M in the absence of Ca$^{2+}$ to 335 $\mu$M in the presence of 20 $\mu$M Ca$^{2+}$ (the range is 67–550 $\mu$M). The Ca$^{2+}$-dependent modulation of $K_{1/2}$ progressively and irreversibly vanished during recording of ligand sensitivity in low Ca$^{2+}$ medium. In membrane patches detached from cone outer segments, the $K_{1/2}$ is reduced only $\sim$1.5-fold in a solution free of Ca$^{2+}$, and the modulation is quickly lost after exposure of the patch to the Ca$^{2+}$-free solution (145). Finally, CaM does not mimic in detached membrane patches the effect of the diffusible factor. These results argue that a soluble factor, which is lost during perfusion, reversibly interacts with the CNG channel in a Ca$^{2+}$-dependent manner. In electropermeabilized and truncated rods, the extent of Ca$^{2+}$-dependent modulation of $K_{1/2}$ is significantly smaller than in cones, and it is essentially recapitulated by CaM in excised patches (295, 333, 352).

How does binding of CaM bring about a decrease in apparent ligand affinity? The mechanism(s) has mostly been studied using the olfactory A2 subunit. The NH$_2$- and COOH-terminal regions of A2 interact with each other (403). The CaM target motif participates in this interdomain interaction; deletion of this segment disrupts the CaM sensitivity of the channel (248) and the interdomain binding (403) and increases the $K_{1/2}$ value of channel activation. The interaction between NH$_2$ and COOH terminus leads to an energetically more favorable conformational stabilization the open state of the channel. This interaction is antagonized by binding of CaM, which results in an increase in $K_{1/2}$ of channel activation. In this respect, CaM affects gating rather than the strength of ligand binding (248, 403).

How general is this domain interaction among CNG channel subunits and does this mechanism also hold for the native channel? Interaction between NH$_2$- and COOH-terminal regions of the A1 subunit of the rod channel has also been proposed (132), although this subunit does not carry a CaM-binding site. The evidence was mostly based on overlay assays of Western blots. However, using a different binding assay (pull-down of NH$_2$- and COOH-terminal fusion constructs), Varnum and Zagotta (403) reported no interaction between NH$_2$- and COOH-terminal regions of A1. In fact, the NH$_2$- and COOH-terminal constructs of A1 were used as negative controls to emphasize the specificity of the interaction in A2, begging the question of how specific these assays are. We suggest that this interaction in the rod CNG channel is either weak and functionally less important or is nonexistent (see also Ref. 57 for discussion).

The dissociation of CaM from the native rod channel is distinctively faster at high (large $P_o$) than at low cGMP concentration (low $P_o$) (130). This observation suggests that Ca$^{2+}$/CaM binds more tightly to the closed than to the open state of the channel, i.e., binding is state dependent. For the rod channel, which spends most of the time in the closed state (i.e., low $P_o$) (430), such a mechanism is deemed suggestive. Gordon (127) proposed that Ca$^{2+}$/CaM also preferentially binds to the closed state of the olfactory CNG channel, which is closed at rest and opens upon stimulation of OSNs with odors. However, for the negative-feedback modulation to produce rapid adaptation, Ca$^{2+}$/CaM must bind effectively and rapidly to the open state. In a series of elegant experiments, Bradley et al. (45) addressed this issue. Using caged cyclic nucleotides, they measured the decline of the cAMP- or cGMP-induced currents due to the action of Ca$^{2+}$/CaM. In native channels from OSNs, the current decline was complete within 0.5 s, whereas for heterologously expressed A2, the decline proceeded $\sim$100 times more slowly. Coexpression of combinations of A2, A4, and B1b subunits demonstrated that only the A2A4B1b channel species matched the rapid kinetics of the native channel, whereas A2A4 and A2B1b displayed distinctively slower current declines. The slower time course resulted from a 200-fold smaller rate constant $k_{on}$ of Ca$^{2+}$/CaM binding to the A2 channel compared with the A2A4B1b or native channel. More importantly, the $k_{on}$ for A2 channels decreased by 10-fold going from high to low values of $P_o$, suggesting that binding of Ca$^{2+}$/CaM in fact is state dependent. In contrast, $k_{on}$ for the binding of Ca$^{2+}$/CaM to the native or A2A4B1b channel was independent of $P_o$. The rate of dissociation of Ca$^{2+}$/CaM from the channel was too slow to be measured and, therefore, $k_{off}$ could not be determined. The dissociation constant $K_d$ depends on the ratio $k_{off}/k_{on}$ of the respective rate constants. Thus these results do not allow a conclusion as to which channel state, open or closed, Ca$^{2+}$/CaM preferentially binds to. In line with these in vitro studies, CNGA4 null mice displayed a pronounced defect in odor adaptation (291). In a paired-pulse paradigm, the amplitude of the electro-olfactogram (EOG) response to the second pulse was about one-half that of the first EOG response in wild-type mice exposed to cineole, whereas in CNGA4 mice, the first and second peak responses were nearly identical.

Many mechanisms of CNG channel function have been studied in homomeric channels (A1 to A3). The new works by Bradley and Munger and collaborators (45, 291) and previous works by Bönigk, Dzeja, Körschen, Chen, and collaborators (40, 71, 97, 214) give a reminder that all aspects of channel activation, gating, ion selectivity, and modulation are finely tuned by the subunit composition of CNG channels. This work also cautions against rash gen-
eralizations; a mechanism established for homomeric A subunits may be entirely different in another setting of A and B subunits.

C. Miscellaneous

Short-chain analogs of diacylglycerol (DAG) reduced the cGMP-induced current in native CNG channels from rods (81, 129). Because full-length DAG has a stimulatory rather than inhibitory effect (422), the functional implications are unclear.

The rod CNG channel is inhibited by phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (422). The inhibitory action of PIP$_2$ is strongest in heteromeric A1B1 channels, less strong in A1 monomers, and intermediate in membrane patches from rod outer segments. The olfactory CNG channel is not inhibited. The functional significance of a regulatory role of PIP$_2$ for CNG channel activity in rod photoreceptors at present is not known.

X. PHARMACOLOGY

The pharmacology of CNG channels is poorly understood, partly for lack of suitable compounds with which to probe CNG-mediated currents. A number of organic compounds have been reported to block current through CNG channels. The list of blockers includes L-cis-diltiazem (71, 106, 112, 122, 154, 203, 214, 266, 267, 331, 342, 379); pimozide (306); amiloride and its derivative (112, 307); tetracaine (109, 110, 362); polyamines (253, 257, 304); W-7, a calmodulin inhibitor (198, 444); H-8, a PKA/PKG inhibitor (410); and ruthenium red and neomycin (258). Except for pseudechotoxin, a peptide venom of the Australian King Brown snake (56), most inhibitors block the channel at micromolar concentrations.

L-Cis-diltiazem has been studied most extensively. It blocks the CNG channels of rods and cones and OSNs in a voltage-dependent fashion (154, 266, 331). The drug exerts its effect from the cytoplasmic face of the channel; extracellular application is much less effective (342, 379). In the fish, L-cis-diltiazem blocks the rod CNG channel at 10-fold lower concentrations than the cone CNG channel. The Michaelis constants of inhibition were 0.9 and 8.7 μM at +30 mV for rod and cone, respectively (154). For both rod and cone CNG channels, the sensitivity to L-cis-diltiazem is conferred upon the heteromeric channels by the B subunits (71, 122). Intriguingly, the L-cis-diltiazem sensitivity is lost upon solubilization and subsequent functional reconstitution of the rod CNG channel, although A and B subunits are still present (79, 80).

The most potent blocking agent for CNG channels is pseudechotoxin (56). It inhibits the homomeric A2 channel with a $K_i$ of 5 nM and the homomeric A1 channel with a $K_i$ of 100 nM. The peptide is several orders of magnitude less effective in blocking the heteromeric channels.

XI. CHANNELOPATHIES AND KNOCK-OUT MODELS

Mutations in the genes encoding the A1 subunit cause a rare autosomal recessive form of retinitis pigmentosa (RP) (96), a genetically heterogeneous group of diseases that are characterized by a progressive degeneration of the rod and cone photoreceptors ultimately leading to blindness. Three of the five subunit alleles are null mutants because they would encode proteins that are lacking in most or all of the functional domains (Fig. 16). The other two alleles (S316F and R654 1bp deletion) encode channels that, while functional, mostly fail to reach the plasma membrane when heterologously expressed in HEK293 cells, suggesting that photoreceptor degeneration is due to the paucity or entire lack of the rod CNG channel. Another form of autosomal recessive RP is caused by mutation of the CNGB1 gene encoding the B1 subunit of the rod CNG channel (18).

Mutations in either the CNAG3 or the CNGB3 genes, encoding, respectively, the A3 and B3 subunits of the cone photoreceptors, cause achromatopsia (or total color blindness) (205, 206, 387), a rare autosomal recessive disorder characterized by the total loss of color discrimination, by photophobia, nystagmus, and severely reduced visual acuity (for review, see Refs. 373, 374). Kohl and Wissinger and collaborators (206, 418) identified 46 mutations in the CNAG3 gene of families originating from Germany, Norway, and the United States of America. Most mutations (39 of 46) represent amino acid substitutions. Four mutations (R277C, R283W, R436W, and 304); W-7, a calmodulin inhibitor (198, 444); H-8, a PKA/PKG inhibitor (410); and ruthenium red and neomycin (258). Except for pseudechotoxin, a peptide venom of the Australian King Brown snake (56), most inhibitors block the channel at micromolar concentrations.

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FIG. 16. Mutations in the human subunits A1, A2, and B1a causing achromatopsia or retinal degeneration. X, stop codon mutation; fs, frame-shift mutation.
hibit no detectable responses to odorants, i.e., suffer from general anosmia (60). This result argues that cAMP is the major second messenger mediating olfactory signal transduction. The disruption of the CNGA2 gene also had biochemical and morphological consequences (15). The olfactory epithelium in A2−/− compared with wild-type mice was thinner and showed lower expression of an olfactory marker protein. Moreover, the olfactory bulb was smaller, and tyrosine hydroxylase expression was reduced in the majority of periglomerular neurons but was retained in atypical or “necklace” glomeruli, suggesting that the OSNs that project to this subset of glomeruli use an alternate signaling pathway. In fact, Juilfs and Meyer and collaborators (178, 273) show that a small subpopulation of OSNs, which projects to these atypical glomeruli, uses a cGMP-signaling pathway and that the signaling components that furnish the two populations of OSNs are entirely different.

Recording from hippocampal slices of A2−/− mice, Parent et al. (316) showed that several measures of basal synaptic activity seem to be unaltered, and high-frequency stimulation used to induce long-term potentiation (LTP) produced no significant differences between wild-type and knock-out mice. However, on weaker more physiological theta-burst stimulation, the initial amplitude of the response decayed faster in knock-out mice compared with wild-type mice (316). The authors interpret these results to indicate that CNG channels play a significant role in LTP in the hippocampus.

Deletion of the A3 subunit produced mice lacking any cone-mediated photoresponses, whereas the rod pathway was completely intact (33). The A3−/− mice were also fertile.

Given the reportedly widespread expression of various CNG channel subunits in neuronal and nonneuronal tissue, it is surprising that in A2- and A3-deficient mice no phenotypic alterations were reported other than loss of smell and vision, respectively, and that patients suffering from either RP or achromatopsia associated with defects in the respective A1, B1, or A3 CNG channel subunits display no other phenotypic abnormalities.

XII. OUTLOOK

Ion channels that are directly gated by cyclic nucleotides play important roles in vision and olfaction. Although many important questions regarding CNG channel function have been adequately answered, future studies need to address the following issues.

1) The stoichiometry and arrangement of subunits in the native channel complex are either not known or controversially discussed. Most of the elegant biophysical studies on channel gating and modulation have been using homomeric A subunits. Whether the models and mechanisms derived from these studies also hold for the heteromeric channels deserves further work.

2) As with other ion channels, the structure at low and high resolution is not known. Low-resolution images could shed light on the quaternary structure and arrangement of subunits. A high-resolution structure could provide information on the molecular mechanism of ion selectivity, permeation, channel gating, and ligand selectivity.

3) The physiological function of CNG channels in cells other than sensory neurons is ill-defined. The available tools now allow us to address this issue more rigorously than has been possible in the past.

4) More than 40 mutations in CNG channel genes have been identified that give rise to various forms of achromatopsia. The mutant channels provide a rich source for the study of the functional significance of individual amino acid residues.

5) CNG channels carry up to three identified CaM-binding sites (e.g., the olfactory channel). The function of each of these sites and their interaction is not completely understood. Moreover, other as yet unidentified Ca2+-binding sites or other factors are probably involved in the regulation of CNG channels.

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