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The Pharmacology of Cyclic Nucleotide-Gated Channels: Emerging from the Darkness

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Abstract

Cyclic nucleotide-gated (CNG) ion channels play a central role in vision and olfaction, generating the electrical responses to light in photoreceptors and to odorants in olfactory receptors. These channels have been detected in many other tissues where their functions are largely unclear. The use of gene knockouts and other methods have yielded some information, but there is a pressing need for potent and specific pharmacological agents directed at CNG channels. To date there has been very little systematic effort in this direction - most of what can be termed CNG channel pharmacology arose from testing reagents known to target protein kinases or other ion channels, or by accident when researchers were investigating other intracellular pathways that may regulate the activity of CNG channels. Predictably, these studies have not produced selective agents. However, taking advantage of emerging structural information and the increasing knowledge of the biophysical properties of these channels, some promising compounds and strategies have begun to emerge. In this review we discuss progress on two fronts, cyclic nucleotide analogs as both activators and competitive inhibitors, and inhibitors that target the pore or gating machinery of the channel. We also discuss the potential of these compounds for treating certain forms of retinal degeneration.

INTRODUCTION

CNG channels play a key role in visual and olfactory signal transduction in retinal photoreceptor cells and olfactory receptor neurons. In these sensory neurons, CNG channels generate an electrical signal by responding to light- and odorant-induced changes in intracellular levels of cyclic nucleotides [1-3]. In retinal rod photoreceptors, the level of cGMP is relatively high in the dark, and the sustained entry of Na^+ and Ca^{2+} ions through CNG channels maintains the cell in a partially depolarized state. When the visual pigment rhodopsin absorbs a photon, it becomes enzymatically active and catalyzes the exchange of GTP for bound GDP on many molecules of the G-protein transducin (for reviews of phototransduction see [4-16]). The GTP-bound form of transducin in turn activates a cGMP phosphodiesterase that catalyzes the hydrolysis of cGMP. As a result, CNG channels in the plasma membrane close, causing a membrane hyperpolarization that decreases the release of transmitter onto second order cells of the retina [17,18]. Recovery of the dark state requires both shut-off of the excitation pathway and synthesis of cGMP to reopen channels. The interruption of Ca^{2+} influx through the channels is critical for the timing of recovery. Ca^{2+} continues to be extruded by a light-independent Na^+/Ca^{2+}-K^+ exchanger, which causes a decrease in intracellular Ca^{2+}. This stimulates the activation of guanylyl cyclase to resynthesize cGMP and the deactivation of

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rhodopsin by rhodopsin kinase. A similar pathway operates in cones, but each molecular constituent differs somewhat from its rod counterpart. Cones are much less sensitive to light, give briefer light responses, and adapt over a wider range of light intensities (see reviews cited earlier).

In contrast, the signaling cascade in olfactory sensory neurons generates responses with polarity opposite to those found in rods and cones. Stimulation of a diverse array of odorant receptors triggers an increase in intracellular cAMP *via* activation of G_{olf} and adenylyl cyclase type III (reviewed in [19-23]). This rise in cAMP directly activates CNG channels leading to a depolarizing influx of Na^+ and Ca^{2+} ions. The olfactory response is further shaped by activation of calcium-activated chloride channels. The olfactory response is terminated by receptor phosphorylation, GTP hydrolysis by G_{olf}, and reduction of CNG channel activity by calcium-calmodulin feedback.

Significant activation of CNG channels requires the binding of three molecules of cGMP [24-30]. Thus, these channels behave as molecular amplifiers, with large changes in activity resulting from small changes in cyclic nucleotide concentration. The gating kinetics of the channel are very rapid, and do not limit the response [31,32]. CNG channels have been embraced by biophysicists as a paradigm for the study of ligand gating and protein allostery [33-35]. They are well-suited for this purpose because they can be studied at the level of a single protein molecule, but, unlike many other ion channels, they do not inactivate or desensitize.

Native CNG channels are composed of different combinations and splice variants of six pore-forming subunits, including both α (CNGA1-4) and β (CNGB1 & 3) subunits. Although many of the α subunits can be functionally expressed as homomultimers, co-expression of the β subunits is known to confer distinct functional properties, in terms of ion permeation, ligand sensitivity, gating mechanisms, and regulation. Cloning, functional expression, and biochemical experiments have demonstrated that rod channels are composed of two types of subunits: CNGA1 and CNGB1 [36-45]. Recent evidence suggests that the stoichiometry of the native rod photoreceptor channel is 3 CNGA1 to 1 CNGB1 [46-48]. The CNG channel in cone photoreceptors is composed of CNGA3 and CNGB3; in an intriguing twist compared to rod channels, functional evidence suggests that the stoichiometry of the cone channel is 2:2 [49]. The native olfactory channel contains three subunit types, including CNGA2, CNGA4, and CNGB1.3, in a ratio of 2:1:1 [50-54]. Although they are relatively insensitive to membrane potential, CNG channels belong to the superfamily of voltage-gated channels, and both A and B subunits contain six transmembrane helices (S1-S6) and a pore (P) region between S5 and S6 [55,56]. Each subunit contains a single cGMP-binding site near its cytoplasmic C-terminus [57,58]. Like voltage-gated potassium channels, evidence indicates that CNG channels function as tetramers [27,28,30,59-62].

CNG channels are permeable to monovalent and divalent cations, including Na^+, K^+, Ca^{2+}, and Mg^{2+} [33]. Ca^{2+} and, to a lesser degree, Mg^{2+} occupy the pore a high percentage of the time under physiological conditions, which significantly lowers the single channel conductance [24,25]. The relatively tight binding of Ca^{2+} to the pore results in the channel showing some selectivity for Ca^{2+} over other cations [63], and the permeation mechanism bears a qualitative resemblance to that of voltage-gated Ca^{2+} channels. Homomeric CNG channels are the most similar to Ca^{2+} channels, because they also have a ring of glutamates that coordinate Ca^{2+} at the equivalent pore position [64,65]. At somewhat higher than normal extracellular Ca^{2+} levels, CNG channels become “pure” Ca^{2+} channels, albeit with lower throughput rates for Ca^{2+} than typical voltage-gated Ca^{2+} channels [63,66]. These observations illustrate two quantitative differences: first, the affinity and selectivity of CNG channels for Ca^{2+} is not as high [65,67], and second, the charge repulsion that creates high Ca^{2+} flux in voltage-gated Ca^{2+} channels is

much less prominent (or perhaps nonexistent) in CNG channels. Native, heteromeric channels bind Ca^{2+} less well than homomeric channels [67] because the B subunits contain a glycine at the equivalent position. Although they carry a more mixed cationic current, native channels are still selective for Ca^{2+}: 15% of the dark current in rods is carried by Ca^{2+} [68], even though the concentration of extracellular Ca^{2+} is about 1% of the Na^{+} concentration.

As is true for most eukaryotic ion channels, the three-dimensional structure of CNG channels is not yet known. The pore is thought to be similar in structure to that of other P-loop-containing channels. The seminal work of MacKinnon and colleagues in determining the structure of the bacterial K^{+} channel KcsA [69-71] has given ion channel researchers a template for understanding pores from this large family. On the other hand, there are substantial differences in permeation and gating between CNG channels and K^{+} channels, and thus the analogies go only so far. In addition to the different cations that pass through CNG channels, there is evidence that the main gate for ions is in the outer selectivity filter [72-76], rather than at the intracellular entrance to the pore where the gate in K^{+} channels is found [77-80]. (This region does widen during opening of CNG channels, but the orifice is too large to limit the flow of ions even in the closed channel [76].) Some guidance to the structure of the cyclic nucleotide binding region comes from the recent x-ray crystal structure of the C-terminal fragment of HCN2 [62], a eukaryotic hyperpolarization-activated, cyclic nucleotide-modulated channel. HCN channels are responsible for neuronal and cardiac pacemaker activity. They are primarily voltage-gated, but gating is modulated by the binding of cyclic nucleotides. The C-terminal fragment contains the cytoplasmic cyclic nucleotide binding domain (CNBD) and the C-linker that connects the binding region to the pore. The fragments assemble as tetramers with a four-fold axis of rotational symmetry. The CNBD consists of an eight-stranded antiparallel β roll flanked by α helices. Cyclic nucleotides interact with the β-roll and the C-terminal α-helix. Based on sequence similarity, CNG channels are likely to have the same basic topology in the binding region. However, much remains to be learned, not only about static structure, but also about the sequence of events that underlie channel gating.

CNG channels may be regulated by several physiologically-relevant signaling pathways. Olfactory desensitization is mediated in part by direct binding of calcium-calmodulin to the olfactory CNG channel, which causes a dramatic reduction in the channel's apparent sensitivity to cAMP. Both the rod and cone CNG channel isoforms are also regulated by calcium-calmodulin, although the effect is less dramatic. The effects of calcium-calmodulin on olfactory and retinal CNG channels are reviewed in [81]. Several other modulators have dramatic effects on channel activation, but their relevance *in vivo* has yet to be definitively established. Transition metal divalent cations, such as Zn^{2+} and Ni^{2+}, are known to enhance activation of the rod CNG channel, and inhibit activation of the olfactory isoform [72,82-84]. Phosphorylation at tyrosine and/or serine residues is known to alter the apparent cyclic nucleotide affinity of the rod and olfactory CNG channels [85-87]. In addition, experiments using the tyrosine-kinase inhibitor genistein, suggest that the rod channel may also be regulated by direct interaction with a tyrosine kinase [88]. Recently, lipid modulators have been shown to regulate the activity of CNG channels. Activation of rod CNG channels is dramatically reduced by application of diacylglycerol analogs and *all-trans*-retinal [89,90], while activation of olfactory CNG channels is inhibited by cholesterol depletion [91] and application of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [92]. Finally, modification of C460 by nitric oxide has been reported to activate homomeric channels composed of CNGA2, independently of cyclic nucleotides [93]. Despite the plethora of natural compounds that modulate the activity of CNG channels, this review will focus on modulation of CNG channel function by non-natural pharmacological agents; for consideration of physiological regulation of CNG channel function, the reader is directed to several recent reviews on this topic [33,81,86,94].

CNG channels may have functions in the retina in addition to their key role in phototransduction. CNG channels have been found to modulate transmitter release at the cone-bipolar cell synapse [95]. Perhaps Ca^{2+} entry through these channels can regulate transmitter release in a mostly voltage-independent fashion at other synapses as well. In this vein, CNG channels may be involved in mediating the presynaptic effects of nitric oxide [96]. Elsewhere in the retina, CNG channels have been reported in a subset of bipolar cells and ganglion cells [97-102]. However, the function, even the presence, of CNG channels in these cells has been difficult to firmly establish and remains controversial [103].

Although we know something about the molecular functions of CNG channels, how they participate in the physiology of non-sensory tissues remains almost purely speculative. In the brain, there is evidence that CNG channels are responsible for the prolonged depolarization of hippocampal CA1 neurons following muscarinic receptor activation [104], and the induction of hippocampal LTP following low-frequency, theta-burst stimulation was reduced in mice in which CNGA2 was genetically ablated [105]. CNG channels have been detected in many other tissues, including heart, kidney, testis, liver, lung, skeletal muscle, adrenal gland, pancreas, and colon [33,35]. There is intriguing evidence that CNG channels play a part in resistance to colon cancer mediated by bacterial enterotoxins, suggesting possible treatments that would target CNG channels [106].

Until recently there has been very little systematic effort to develop potent and specific pharmacological agents directed at CNG channels.Most of what can loosely be termed CNG channel pharmacology came from testing reagents known to target protein kinases or other ion channels, or by accident (retrospectively termed serendipity) when researchers were investigating other intracellular pathways that might regulate CNG channels. Predictably, these studies have not produced selective agents. The use of gene knockout technology in mice has helped to define some of the purposes of CNG channels, but the foregoing discussion clearly points to a need for specific pharmacological agents.

CYCLIC NUCLEOTIDE DERIVATIVES – ACTIVATORS AND COMPETITIVE INHIBITORS

Modifications of the cyclic nucleotide can alter affinity, gating efficacy, and membrane permeability. However, achieving selectivity for CNG channels is a major challenge as cyclic nucleotide-dependent protein kinases are sensitive to much lower concentrations of the natural cyclic nucleotides. Moreover, the effects of cyclic nucleotide analogues on other targets such as cyclic nucleotide phosphodiesterases (PDEs), Epac, a cAMP-sensitive guanine nucleotide exchange factor, and hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channels need to be evaluated. The latter two classes are relatively recent discoveries and thus have received considerably less attention as cyclic nucleotide binding proteins. While hundreds of cyclic nucleotide analogues have been synthesized over the past four decades (see [107], where many of the synthetic methods were worked out), few systematic studies of their effects on CNG channels have been carried out; the majority of studies have focused on cAMP- and cGMP-dependent protein kinases and cyclic nucleotide phosphodiesterases (reviewed in [108]). Nonetheless, several analogues have been used in studies of CNG channel function and structure, and new strategies for generating CNG-channel-selective cyclic nucleotide analogues are emerging.

Different classes of CNG channels have distinct preferences for cGMP and cAMP (Fig. 1). As CNG channels were discovered and cloned from various tissues it became clear that different combinations of the pore-forming A and B subunits respond to cAMP and cGMP differently. For instance, cAMP and cGMP are full activators of native olfactory CNG channels with similar potencies, while native rod photoreceptor CNG channels require ~10-fold higher cGMP

and ~500-fold higher cAMP concentrations for half maximal activation [3,109,110]. Furthermore, cAMP is only a partial activator of the rod channel, eliciting 10-40% of the maximal cGMP-activated current at saturating concentrations of cAMP (depending on the species). This nucleotide preference highlights the importance of evaluating more than one class of CNG channel and suggests the additional challenge of producing cyclic nucleotide analogues that are selective for a single type of CNG channel.

An understanding of how cyclic nucleotides bind to and activate CNG channels, as well as the structural features that underlie nucleotide selectivity will be important guides in the development of CNG channel-selective analogues. Although the three-dimensional structures of vertebrate CNG channels are not yet known, the essential structural blueprint for a cyclic nucleotide-binding domain (CNBD) is conserved from bacteria to mammals, as revealed by atomic resolution structures of CNBDs from diverse proteins: a transcription factor, protein kinases, a guanine nucleotide exchange factor, a voltage-sensitive potassium channel and a bacterial potassium channel [62,111-117]. The CNBD consists of an eight-stranded antiparallel β roll with one α helix (A) at the N-terminus of the domain and two (B and C) at the C-terminus. The cyclic nucleotide binds to a cleft on the β roll with the cyclic phosphate making an electrostatic interaction with a conserved arginine residue (R558 in bovine CNGA1). Mutation of this residue in a chimeric CNG channel results in reduced cyclic nucleotide affinity but allows full activation at saturating cyclic nucleotide concentrations [118]. The ribose binds to the β roll *via* hydrogen bonding interactions, while contacts between the purine ring and the β roll and are largely hydrophobic. Additional contacts with the purine ring are made by the C-helix, especially in the open state. Of particular note is aspartate 604 in bovine CNGA1, which is thought to be largely responsible for nucleotide preference by contributing two hydrogen bonds to cGMP (N1 and N2) while presenting an unfavorable interaction with the N1 position of cAMP [119]. CNGA2 and CNGA3 contain glutamate and aspartate at this position, and all three subunits show strong selectivity for cGMP over cAMP when they are expressed on their own. In contrast, CNGA4, CNGB1 and CNGB3 do not contain acidic residues here. This is thought to be the primary reason why these subunits, which do not express on their own, make native heteromeric channels more sensitive to cAMP than homomultimers formed from CNGA1-3 alone. In general, though, how cyclic nucleotide binding triggers channel opening is largely unknown. One indicator of the complexity of this process is that CNGA2 exhibits a higher probability of opening than CNGA1 with either cGMP or cAMP bound, and evidence suggests that regions spread throughout the CNGA2 subunit contribute to this [120]. Finally, there is the surprising observation that the CNBD from HCN2 binds cAMP in the *anti* conformation (about the N-glycosidic bond) and cGMP in the *syn* conformation even though cAMP and cGMP modulate activation of the channel to the same extent [62].

Ribose and Cyclic Phosphate Modifications

CNG channels are very sensitive to alterations of the ribose and the cyclic phosphate: 2'-deoxy-cGMP, 2',3'-cGMP and 5'-GMP are unable to activate rod CNG channels [1,109]. Furthermore, while phosphorothioate derivatives of cGMP, S_P-cGMPS and R_P-cGMPS (Fig. 2), are able to activate the rod CNG channel, 12- and 70-fold higher concentrations are required relative to cGMP [121]. In studies of the native olfactory CNG channel S_P-cAMPS and R_P-cAMPS were 4- and 75-fold less potent than cAMP, and R_P-cAMPS was only a partial activator, eliciting ~35% of the maximal current at saturation [122]. This sensitivity to modification at the cyclic phosphate has been utilized to generate "caged" cyclic nucleotides, analogues with modifications to the cyclic phosphate that prevent channel activation but can be cleaved by photolysis to rapidly release cGMP [123,124]. For example, the 4,5-dimethoxy-2-nitrobenzyl ester of cGMP was used in flash photolysis experiments to measure the gating kinetics of the CNG channel from retinal rods [32].

Purine Ring Modifications

Other than at the C8 position, modifications to the purine ring system have resulted in compounds with decreased apparent affinity for CNG channels (Fig. 2). For example, 2-aminopurine 3',5'-cyclic monophosphate that lacks the C6 oxygen of cGMP is unable to activate CNG channels from retinal rods. Substitution of the 6-oxy group with sulfur is tolerated, albeit with a 3-fold reduction in potency [109]. Modification of both the 2-amino and the nitrogen at position 1 was achieved with the synthesis of β-phenyl-1, N^2-etheno-cGMP (PET-cGMP) [125]. This modification introduces significant nonpolar bulk to the purine ring, and PET-cGMP is unable to activate homomeric rat CNGA1 channels when present at 2.5 mM. However, in conjunction with modifications at other positions that rescue binding affinity, the PET modification may turn out to be useful in creating competitive antagonists (see below). PET-cGMP can potentiate channel activation by subsaturating concentrations of cGMP, an effect that is maximal at ~100 μM PET-cGMP, indicating that this analogue can bind to the channel [126].

A number of cGMP analogues with substitutions at the C8 position have been tested on CNG channels (Fig. 3). In addition to exhibiting an increased apparent affinity, these analogues have the advantage of being generally resistant to degradation by PDE [127-130], and hydrophobic substitutions can increase membrane permeability. Early studies of 8-Br-cGMP demonstrated that it is about 10-fold more potent than cGMP in activating retinal rod CNG channels [121, 131,132]. In addition, 8-N_3-cGMP, 8-(*p*-azidophenacylthio)-cGMP, 8-benzylthio-cGMP, and 8-Fl-cGMP [8-(5-thioacet-amidofluorescein)-cGMP)] were also reported to be potent activators, the latter two more potent than 8-Br-cGMP [57,109,132]. To systematically examine the effects of charge and hydrophobicity at this position on channel activation, Brown *et al*. synthesized a series of 8-amino- and 8-thio-substituted cGMP analogues and tested them on native retinal rod channels [133]. Results from these experiments suggest that amine (*n*-propylamino, carboxyethylamino, and aminoethylamino) substitutions at the C8 position alter the electronic structure of the guanine ring system, leading to lower potency. Positively charged groups several bond lengths from the guanine ring (aminoethylthio and trimethy-laminoethylthio) are also detrimental. Conversely, nonpolar (*n*-propylthio) or negatively charged groups at the same position (sulfoethylthio and carboxyethylthio) yield activators that exhibit similar or slightly greater potency than 8-Br-cGMP. The photoaffinity analogue 8-(*p*-azidophenacylthio)-[^{32}P]cGMP labels a stretch of hydrophobic amino acids within the CNBD. This region may be responsible for the greater potency of cGMP analogues with hydrophobic substituents at the C8 position [58]. One such compound, 8-(*p*-chlorophenylthio)-cGMP (CPT-cGMP), has found use in several studies as a membrane-permeant activator of CNG channels that is highly resistant to hydrolysis by PDEs [91,134]. Finally, it appears that the cyclic nucleotide-binding pocket of CNG channels is capable of accommodating very large substituents at the C8 position. This is illustrated by the ability of 8-Fl-cGMP, more than twice the mass of cGMP, to fully activate the rod photoreceptor channel, with potency greater than 8-Br-cGMP [109,132]. Despite the potency of different C8 substituted derivatives, many of these compounds are also very potent activators of protein kinase G (PKG) [125,135]. Thus, other modifications will likely be required to produce channel-specific agents.

Competitive Antagonists

As protein kinases display greater affinity for cAMP and cGMP relative to CNG channels, it seems unlikely that alterations at a single position will yield a CNG channel-selective modulator. Greater success may be achieved by modifying several positions on the nucleotide. Some progress has been made by combining phosphorothioate modification of the cyclic phosphate (either R_P or S_P isomers) with phenyletheno (PET) derivatization of the 2-amino and N1 positions of the guanine ring in cGMP. Specifically, S_P-8-Br-PET-cGMPS was reported to be a potent activator of PKG, but a competitive inhibitor of the rod CNG channel

[126]. Conversely, R_P-8-Br-cGMPS activates the homomeric rod CNG channel but antagonizes activation of PKG [134]. However, these agents bind quite poorly to CNG channels, and their specificities against different isoforms and other cyclic nucleotide binding proteins remain at issue. Also, it would be desirable to have analogs that act on only one protein at a given concentration. In another study R_P-8-CPT-cGMPS was found to be an effective competitive inhibitor of homomeric CNGA2 channels [122]. Further work will be required to determine if it acts similarly on native olfactory channels.

Multivalent Ligands

A novel approach for producing ligands that bind selectively and with high affinity to proteins containing multiple ligand binding sites has been employed in the synthesis of highly potent CNG channel activators. The concept is to connect two ligands with flexible polymer chains of defined average length, until a compound is found that optimally spans two binding sites on the protein of interest (Fig. 4) [136]. The largest enhancement in affinity occurs when the average length of the polymer chain matches the average distance between binding sites. These multivalent compounds are referred to as polymer-linked ligand dimers (PLDs). The enhancement in apparent affinity is due to a dramatic slowing of the off-rate. When one ligand moiety of the pair dissociates, its tendency to rebind is very high because it has a high effective concentration in a hemisphere swept out by the polymer chain (Fig. 4b). Thus, the effective off-rate of the PLD is given by the normal off-rate at a single binding site multiplied by the probability that the second site is not occupied (which is very low).

A series of polymer-linked cGMP dimers of varying length was generated by reacting 8-SH-cGMP with vinyl sulfone-derivatized polyethylene glycol (Fig. 4a) [136]. The compounds were tested on homomeric rod and olfactory CNG channels expressed in oocytes, and on cGMP-dependent protein kinase (PKG). Fig. (5a) shows that for both channels, there was a gradual increase in apparent affinity with increasing polymer length until an optimal average length was reached. Flexible polymers like PEGs have average lengths that are proportional to the square root of the number of monomers [137]. At the optimal length (30 Å for retinal channels and 39 Å for olfactory channels) the enhancement of apparent affinity (cf. cGMP) was 250-fold for the rod channel and 800-fold for the olfactory channel. Kinetic experiments with the olfactory channel showed that the off-rate for the optimal PLD was slowed by a factor of several thousand. The PLDs were less effective in activating PKG (30-fold enhancement – Fig. 5b), most likely because one of the two cGMP-binding sites in each regulatory subunit does not bind C_8-substituted cGMP derivatives as well as cGMP itself [135]. The optimal PLD was different for each target protein, showing that the PLD strategy can reveal agents with a selectivity for specific proteins, even if their binding sites are conserved. This was best illustrated by the 39 Å cGMP PLD, which was the first cyclic nucleotide derivative to activate a CNG channel (olfactory CNGA2) more potently than PKG, by a factor of about 20 (Fig. 5). There is significant promise for the development of even more potent and selective CNG channel modulators *via* changes to the number of ligands (cGMP trimers and tetramers), refinements to the optimal polymer length, alterations of polymer stiffness, or the incorporation of additional modifications to the cyclic nucleotide.

PORE BLOCKERS AND GATING MODIFIERS

The two CNG channel blockers that are most commonly used are *l-cis*-diltiazem, an optical isomer of the clinically-relevant Cardizem (an antihypertensive drug), and dichloro-benzamil, a derivative of amiloride, the well-known blocker of epithelial sodium channels (Fig. 6). *l-cis*-diltiazem (LCD) was first reported to block CNG channels in 1985 by Koch and Kaupp, who demonstrated inhibition of cGMP-dependent ion flux from bovine rod outer segment vesicles [131]. This compound was subsequently tested on membrane patches excised from the rod photoreceptors of the salamander, *Ambystoma tigrinum*. In these studies, LCD was

found to inhibit the rod photoreceptor CNG channel with a $K_{1/2}$ of ~ 1 μM at +30 mV, whereas the more common isomer, *d-cis*-diltiazem was at least an order of magnitude less effective [138]. LCD has also been shown to block the CNG channels from olfactory receptor neurons and cone photoreceptors, although the affinity for these channels is lower (~ 50 μM) [139-141]. Inhibition of the rod channel by LCD is noncompetitive and voltage-dependent, with the $K_{1/2}$ increasing about 5 – 10-fold at negative voltages [141-143]. High affinity block of the rod CNG channel by LCD requires the presence of the CNGB1 subunit, as channels formed by the CNGA1 subunit are less sensitive by nearly two orders of magnitude [41]. A similar finding has also been reported for the CNG channel from cone photoreceptors [144]. Thus, LCD can be used as a diagnostic tool to assess the presence of CNG channel β subunits. Because it is relatively membrane-permeant, LCD is also effective when applied from the extracellular surface in the whole-cell recording mode [143]. Although the actual binding site for LCD remains unknown, it is thought to be within the transmembrane domain ($z\delta \sim 0.5$ [141,145]), and it has been suggested to inhibit channel current through effects on gating, rather than direct blockage of the pore [141]. Although commonly used as a diagnostic tool to demonstrate the presence of CNG channels, these results must be interpreted with caution, as LCD can by no means be considered a specific blocker. Although the affinity of LCD for L-type calcium channels is ~ 10-fold lower than that of *d-cis*-diltiazem, it is still an effective blocker at low or sub μM concentrations, comparable to those required to inhibit the rod CNG channel, and much lower than the concentrations required to inhibit the olfactory and cone isoforms [146,147]. More recently, LCD has also been shown to have cardioprotective effects that are not related to block of calcium channels [148,149], and it has been shown to block voltage-dependent sodium channels at μM concentrations [150]. Despite these limitations, however, LCD has been proposed for use in clinical treatment of retinal degeneration due to altered cGMP metabolism, which causes calcium overload and apoptosis, and it has been shown to be partially neuroprotective in rodent models of retinal degeneration [151].

Dichlorobenzamil (DCB), an amiloride derivative, was shown to inhibit CNG channel currents in ion-flux experiments and when applied to the cytoplasmic face of inside-out patches excised from frog rod outer segments [152] (Fig. 6). Block was half-maximal at low μM concentrations and was relatively independent of voltage. This compound is equally effective on CNG channels found in the sensory neurons of rat olfactory epithelium [139,153], but, to our knowledge, its effectiveness on the channel isoform found in cone photoreceptors has not been reported. DCB has been used to demonstrate the presence of CNG channels in hippocampal neurons, airway epithelium, and intestine [104,154,155]. Once again, however, DCB cannot be considered a selective antagonist of CNG channels. Although originally developed as an inhibitor of the sodium-calcium exchanger at low μM concentrations [156], it has also been shown to inhibit voltage-gated sodium, calcium, and potassium channels in a similar concentration range [157-159].

Several additional pharmacological agents known to target other ion channels have also been shown to block CNG channels (Fig. 7). Pimozide, a calcium channel blocker used as an antipsychotic drug [160] has been reported to block the CNG channel in frog rod photoreceptors when applied to inside-out patches at low μM concentrations [161], and the related compound, clopimozide, was reported to block the channel from bovine rods in ion flux experiments when applied at low μM concentrations [131]. However, attempts to reproduce the effect of pimozide in ion flux experiments and on excised inside-out patches containing the heterologously-expressed bovine rod channels (CNGA1 + CNGB1) have not been successful ([131] and Fig. 8). Amiloride, typically used at very low μM concentrations to block the epithelial sodium channel [162], has been shown to block rod and olfactory CNG channels [140,143]. The block by amiloride is strongly voltage-dependent, with the $K_{1/2}$ for the olfactory channel increasing from 17 μM at +100 mV to 400 μM at -100 mV [140]. D-600, a derivative of the L-type calcium channel blocker verapamil, has also been shown to block the olfactory CNG channel at

relatively high concentrations (K_i = 200 μM) at -20 mV, but was more effective at depolarized potentials (K_i = 12 μM at +100 mV) [140]. However, other calcium channel blockers, such as nimodipine, nifedipine, and verapamil are ineffective on the rod CNG channel [131]. Nifedipine, however, has been reported to block the frog olfactory CNG channel [163]. More recently, dequalinium, a blocker of small-conductance calcium-activated potassium channels, has been reported to be a high-affinity and voltage-dependent blocker of both homomeric and heteromeric rod CNG channels [164,165].

Finally, tetracaine (compound **1**, Table 1), a local anesthetic that blocks both sodium and calcium channels, has been shown to block rod CNG channels at micromolar concentrations [142,166,167]. (Mutagenesis studies of sodium channels have suggested a bimodal interaction of local anesthetics with the pore; an electrostatic interaction with negatively charged residues in the selectivity filter and a hydrophobic interaction with aromatic residues on the pore-lining helix [168-172].) Further work on homomeric retinal (CNGA1) and olfactory (CNGA2) channels established that block by tetracaine is voltage- and state-dependent. The apparent K_D for block of open channels is 170 μM in contrast to 220 nM for closed channels [173]. Evidence was also presented that tetracaine interacts with the pore glutamate residues [73]. The very strong preference of tetracaine for closed channels may have to do with the precise arrangement of glutamate residues in the closed configuration. For a closed state blocker the efficiency of block increases with the fraction of closed channels. This can be a desirable property in a therapeutic setting for channels like CNG channels that are thought to spend most of their time in the closed state. More recently, a multiply charged tetracaine derivative that blocks CNG channels at subnanomolar concentrations was reported (compound **4**,Table 1) [174]. Like tetracaine this compound binds preferentially to closed channels. The extra charges increase the apparent affinity for the pore, and also confer a steep voltage dependence of block. Compound **4** is selective for CNG channels over brain Na^+ channels. The highly charged moiety likely interacts more strongly with the selectivity filter of rod CNG channels, which contain three (heteromeric channel) to four (homomeric channel) glutamates, than the selectivity filter of Na^+ channels, which contains aspartate, glutamate, and lysine [175]. Several additional tetracaine derivatives were synthesized to examine the effects of increased positive charge and alterations to the hydrophobic character of tetracaine for blockade of homomeric CNGA1 (Table 1) [176]. The results show that the addition of one or two positively charged groups to the tertiary amine end of tetracaine results in dramatic increases in potency and voltage-dependence of block. In contrast, addition of a positively charged amine to the hydrophobic butyl tail of tetracaine is deleterious to block. A derivative with a polar N-acetyl group at the butyl tail or a derivative lacking the butyl tail are both very poor blockers, further reinforcing the importance of hydrophobic character at this end. Among this series of tetracaine derivatives the apparent K_D for block varies over nearly eight orders of magnitude, which provides a fair amount of guidance for how to produce blockers that may be more specific for CNG channels.

A second category of CNG channel blockers includes a number of calmodulin antagonists (Fig. 9). Undoubtedly, many of the CNG channel blockers in this category were discovered during experiments designed to elucidate the mechanism and physiological role of calcium-calmodulin (Ca-CaM) regulation in olfactory transduction. Binding of the Ca-CaM complex is known to reduce the cyclic nucleotide affinity of all three isoforms of CNG channels [177-179]. Although the physiological significance of this regulation is unclear for rod and cone channels [180,181], binding of Ca-CaM to the native olfactory channel causes a twentyfold increase in the $K_{1/2}$ for cAMP, which is thought to underlie olfactory desensitization [81,178]. Kleene [182] reported that application of the calmodulin antagonists, trifluoperazine and W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride), reduced the cAMP-dependent current when applied at low μM concentrations to the cytoplasmic face of membrane patches excised from frog olfactory neurons. Thus, although they can discriminate

between the cAMP-activated current and the Ca^{2+}-activated chloride current in olfactory neurons, these reagents will be of little use for investigating the Ca-CaM regulation of olfactory transduction. Furthermore, trifluoperazine and W-7 have been reported to inhibit other ion channels, including calcium channels [183], sodium channels [184], potassium channels [185], and chloride channels [186], at similar concentrations, so they certainly cannot be regarded as specific blockers of CNG channels.

Pharmacological agents targeting other signaling pathways have also been shown to block CNG channels (Fig. 9). An inhibitor of soluble guanylyl cyclase, LY83,583, has been reported to block olfactory CNG channels in salamander [187], but this compound is ineffective on rat ([188] and Fig. 8) and zebrafish channels [189]. LY83,583 is also ineffective at blocking heteromeric rod channels (Fig. 8), suggesting caution in extrapolating blocking efficacy across both species and channel isoforms. Reagents targeting Ca^{2+}-signaling pathways have also been reported to block CNG channels. Ruthenium red, a well-known blocker of the ryanodine receptor, has been shown to be a high-affinity, but voltage-dependent, blocker of the zebrafish olfactory CNG channel [189]. At -60 mV, ruthenium red was shown to block cAMP-activated currents at sub-μM concentrations, but the efficacy was reduced by over two orders of magnitude at +60 mV. Neomycin, an aminoglycoside antibiotic known to inhibit the activity of phospholipase C, blocks the zebrafish olfactory CNG channel at 100 – 200 μM [189]. Finally, H-8, an inhibitor of the cAMP-dependent protein kinase, has also been shown to directly inhibit the rod CNG channel at mM concentrations [190].

The information above has important consequences for all of ion channel pharmacology. When a blocker that is widely used on a well-known channel is found to block a lesser-known class of channels such as CNG channels, its utility plummets. Thus, many cherished pharmacological agents will have to be reevaluated.

In an attempt to discover more specific, high-affinity blockers for CNG channels, Brown and colleagues screened a variety of snake, spider, and scorpions venoms for peptide toxins that would inhibit CNG channels when applied to the extracellular face of the channel. In 1999, these efforts resulted in the purification of pseudechetoxin (PsTx) from the venom of the Australian King Brown snake (Pseudechis australis) [191]. This 25 kDa toxin is a member of the family of cysteine-rich secretory proteins (CRISP), which are widespread in the venoms of elapid snakes [192]. Other members of this family are thought to inhibit L-type calcium channels [193] and large conductance calcium-activated potassium channels [194]. When applied to the extracellular face of HEK-293 cells expressing rat CNGA2, PsTx blocked currents at low nM concentrations. It was slightly less effective on CNGA1 channels, and almost totally ineffective on the cone channel isoform, CNGA3. PsTx is also largely ineffective on heteromeric CNG channels containing CNGB1 or CNGB3 subunits [195], reducing its utility in native preparations. PsTx has been shown to inhibit channel current by forming high-affinity contacts with the extracellular pore turret, thereby occluding the entrance to the transmembrane pore [195]. A comparision of PsTx with the homolog, pseudecin, which is 97% identical but blocks CNGA2 with a 20-fold lower affinity, led to the suggestion that the cysteine-rich carboxy terminus might be the site of channel interaction [192]. Recently, the crystal structure has been solved for stecrisp, triflin, and natrin, other members of the CRISP family [194,196,197]. The structure reveals that the cysteine-rich carboxy terminus forms a separate domain, which is similar in structure to sea anemone toxins, BgK and ShK, that target potassium channels. If this domain from PsTx can be expressed as a functional inhibitor, it will facilitate further development of PsTx as a tool to study the structure and function of CNG channels.

TARGETING CNG CHANNELS IN RETINAL DISEASE

The involvement of CNG channels in retinal disease can be divided into two categories. In the first category mutations in rod and cone CNG channel subunits impair vision by causing improper functioning or trafficking of the channels. In the second category mutations cause defects in cGMP metabolism, and CNG channels are important in the pathophysiology of the diseases. Regarding the first category, mutations in either the CNGA1 [198-200] or CNGB1 [201] subunits of rods cause autosomal recessive forms of retinitis pigmentosa, a heterogeneous group of diseases that cause progressive degeneration of rod and cone photoreceptors, and ultimately blindness (see also [202]). The known mutants either lack major channel domains, or are functional but mostly fail to reach the cell surface [198,203,204]. Mutations in either the CNGA3 [205-207] or CNGB3 [208-210] subunits of cones cause complete achromatopsia, also known as total colorblindness or rod monochromacy. This is an autosomal recessive disorder characterized by a complete inability to discriminate between colors and a severe loss of visual acuity. Regarding the second category, a number of forms of retinitis pigmentosa affect phototransduction and cGMP metabolism [211,212]. Mutations that result in increased cGMP levels, such as mutations in the cGMP phosphodiesterase of rods or in guanylyl cyclase activating protein 1, cause a massive influx of Na^+ and Ca^{2+} through CNG channels [211, 213,214]. This leads to metabolic overload, as well as direct toxicity and apparent activation of the apoptotic pathway [215]. (Interestingly, some of the disease-associated mutations in the cone channel subunits CNGB3 [216,217] and CNGA3 [218] have been shown recently to cause a gain of channel function, which would result in the same problems as a rise in cGMP.) An important experimental model for the phosphodiesterase defect has been the rd mutation in mice [219,220]. Six years ago it was reported that the Ca^{2+} channel blocker *d-cis*-diltiazem can rescue photoreceptors and preserve visual function in the rd mouse [221]. Unlike its optical isomer, *l-cis*-diltiazem, this compound exhibits little or no interaction with rod CNG channels [138]. The hypothesis for why it was able to rescue photoreceptors is that the high cGMP levels in rd rods and consequent depolarization caused by CNG channels leads to the activation of voltage-gated Ca^{2+} channels and excessive Ca^{2+} entry. However, this conclusion has been called into question in two more recent studies [222,223], where no beneficial effects of *d-cis*-diltiazem were observed. Even if protective effects are seen under some circumstances, Ca^{2+} channel blockers will have many other effects on retinal function. It has been noted in a number of reviews that an attractive treatment for the disorders involving high cGMP levels would be a specific and slowly reversible blocker of CNG channels [224]. Although *l-cis*-diltiazem was found to be partially neuroprotective in mouse studies [151], CNG channel blockers that are more potent, specific, and membrane-permeant would likely be extremely useful for treating these blinding diseases.

HYPERPOLARIZATION-ACTIVATED, CYCLIC NUCLEOTIDE-MODULATED CHANNELS

Another physiologically important class of ion channels that is modulated by the direct binding of cyclic nucleotides is the hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channels [225]. These channels are formed from the products of four known genes termed HCN1-HCN4. Two of these members (HCN2 and HCN4) are exquisitely sensitive to intracellular levels of cyclic nucleotides, which enhance their activity. Structurally and functionally, HCN channels can be described as a chimera between a voltage-activated potassium channel (albeit with a reversed polarity and reduced potassium selectivity) and a cyclic nucleotide-gated ion channel. Each subunit of an HCN channel contains six putative transmembrane helices and a reentrant loop forming a cation-selective pore that permits the flow of both potassium and sodium ions. The carboxy-terminal region of the channel contains a cyclic nucleotide-binding domain, which regulates the voltage-dependence of channel

activation. The four isoforms of HCN channels vary in terms of voltage-dependence, gating kinetics, sensitivity to cAMP, and tissue localization.

HCN channels are activated by membrane hyperpolarization, giving rise to the depolarizing I_h current, which plays a key role in the generation of rhythmic activity in a variety of excitable tissues, including the heart and brain. This current is best known for its role as the "pacemaker" channel in the sinoatrial (SA) node of the heart. In cells of the SA node, HCN channels are activated by the hyperpolarization following the cardiac action potential. The inward mixed-cation current carried by these channels slowly depolarizes the cell until threshold is reached, and another action potential is initiated. These channels are also thought to play a role in generation of rhythmic activity in the brain, taste transduction, and synaptic plasticity. Finally, HCN channels have been implicated in the pathophysiology of neuropathic pain [226].

Further efforts to characterize the functions of HCN channels in general, and individual isoforms in particular, have been hampered by a striking lack of selective pharmacological tools. I_h is often distinguished by substantial block by extracellular Cs^+ in the low mM range, and insensitivity to 2 mM intracellular Ba^{2+}. However, several other K^+ channels are also blocked by Cs^+ in this concentration range. The best-known blocker of HCN channels is the Zeneca compound, ZD7288 [227]. A K_i of 41 μM has been reported for block of mouse HCN1, although this may be an underestimate of the true affinity due to a slow phase of block that did not reach steady state [228]. Block of HCN channels by ZD7288 has a complex dependence on voltage and exhibits an unexpectedly high zδ between 3 and 5. Although block of I_h by ZD7288 was originally reported to not be state-dependent, block is now thought to require prior activation of the channel, and ZD7288 can be trapped in the pore by channel closure [228]. It seems likely that some of the discrepancies in the literature arose from differences in the experimental protocols. Although this blocker is relatively effective, it does not select between HCN isoforms, and it has also been shown to inhibit currents through AMPA and NMDA receptors [229], as well as T-type calcium channels [230], calling its specificity into question. Two other organic blockers, zatebradine (UL-FS49) [231,232] and ivabradine (S-16257) [233] have also been used to block HCN channels.

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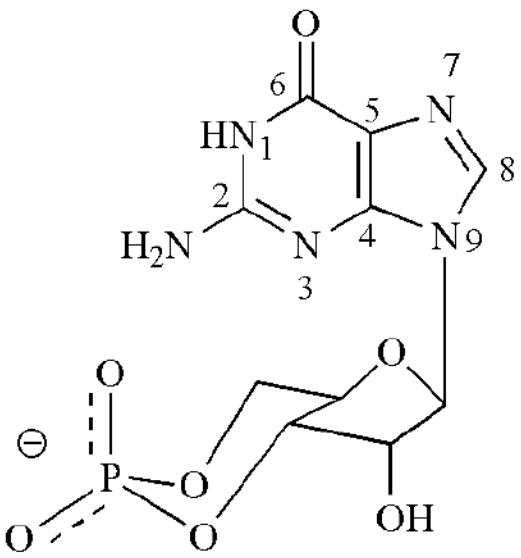
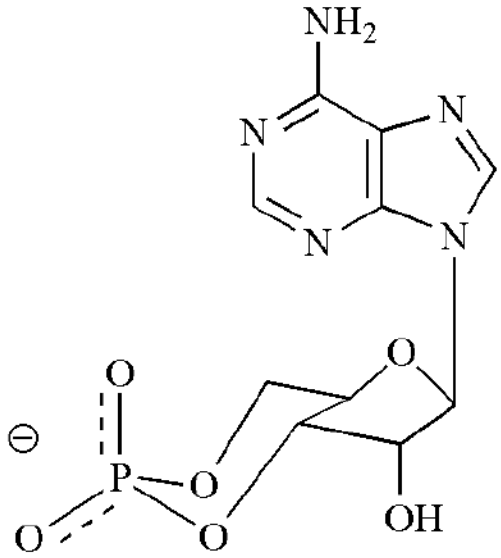


cGMP



cAMP

Fig. (1).
Structures of cGMP and cAMP both drawn in the *syn* conformation with respect to the N-glycosidic bond. Numbering of the purine ring is shown for cGMP.

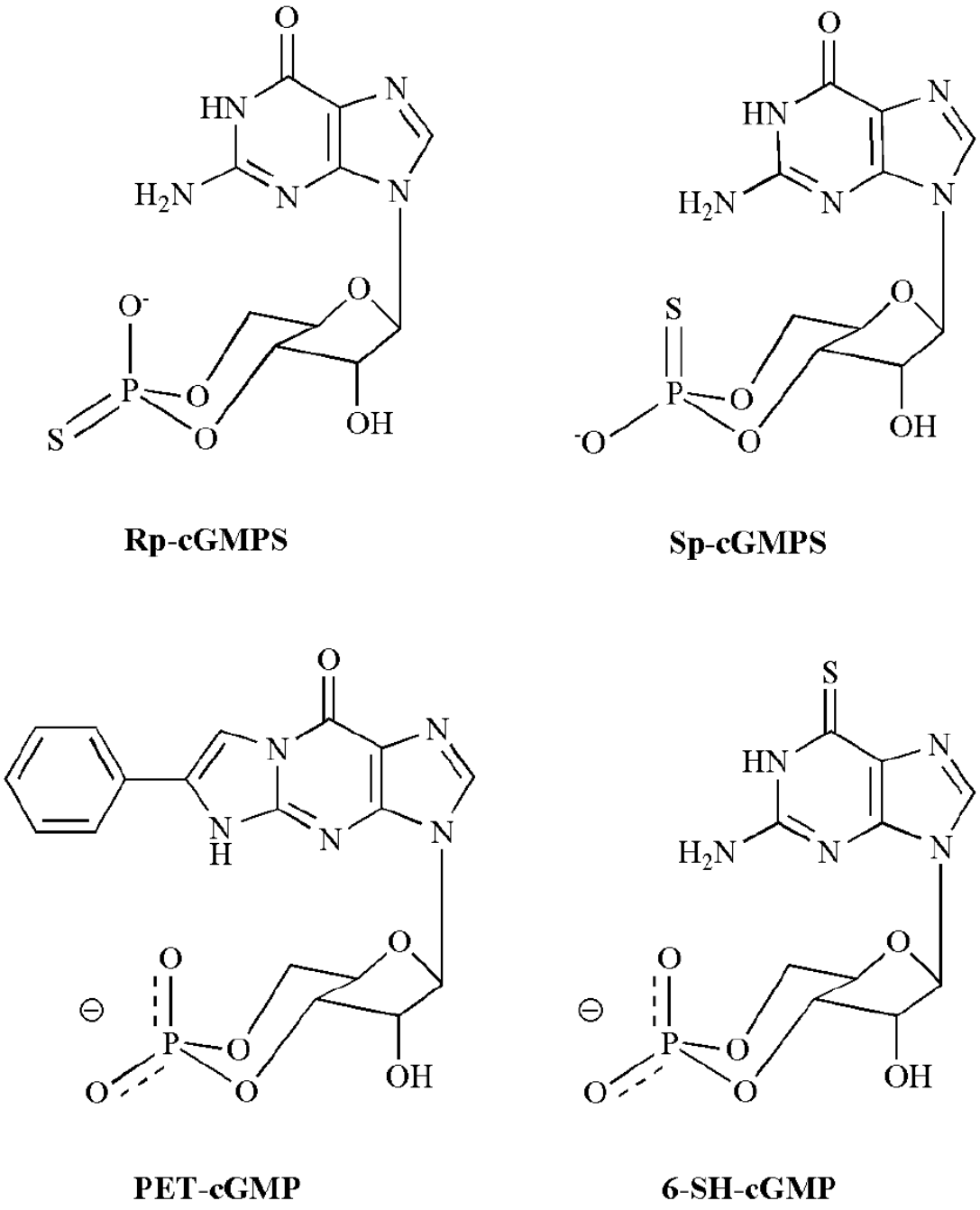


Fig. (2).
Structures of selected analogues of cGMP modified at the cyclic phosphate and purine ring.

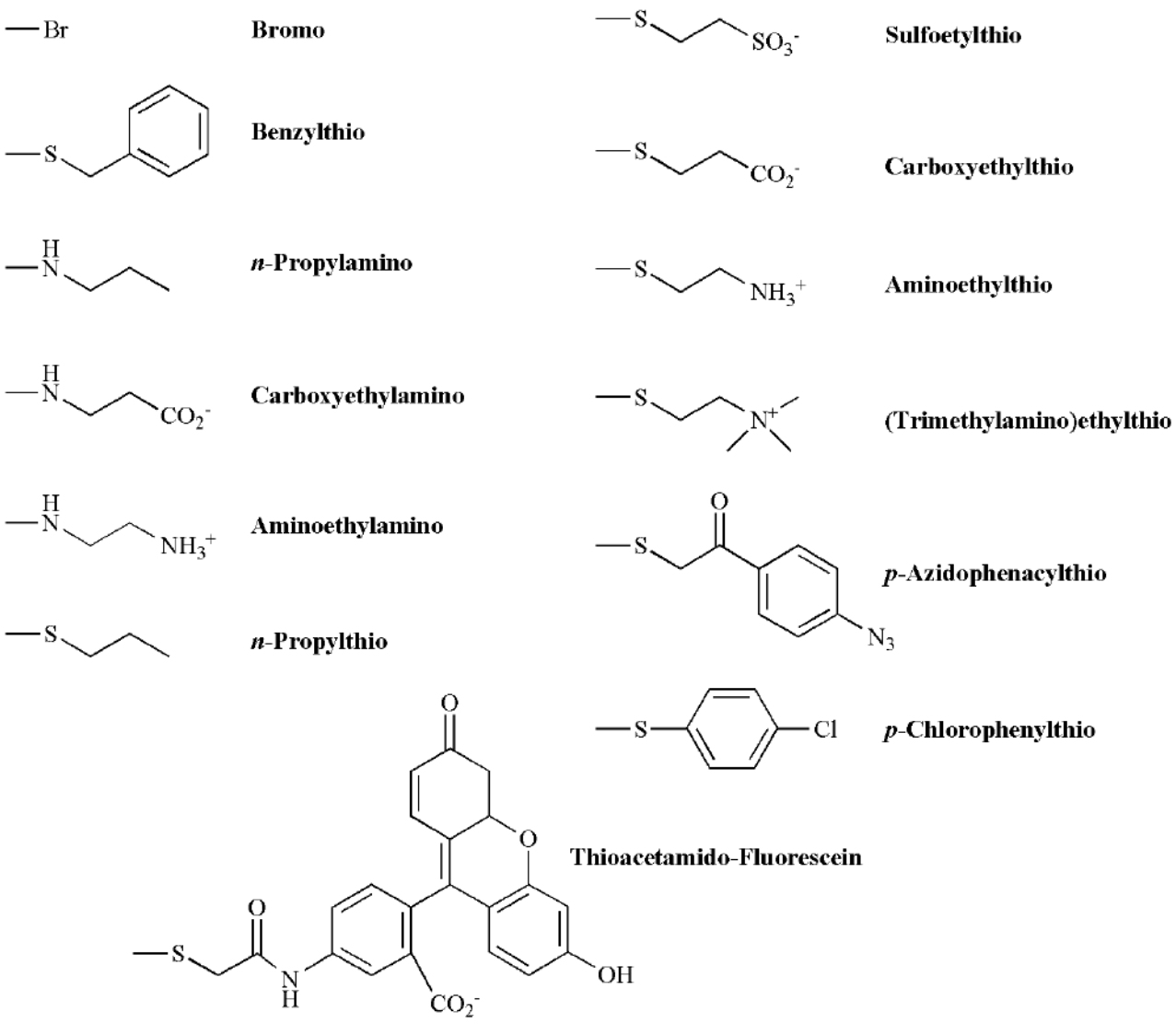


Fig. (3).
Modification of cGMP at the C8 position. Structures of selected C8 substituents.

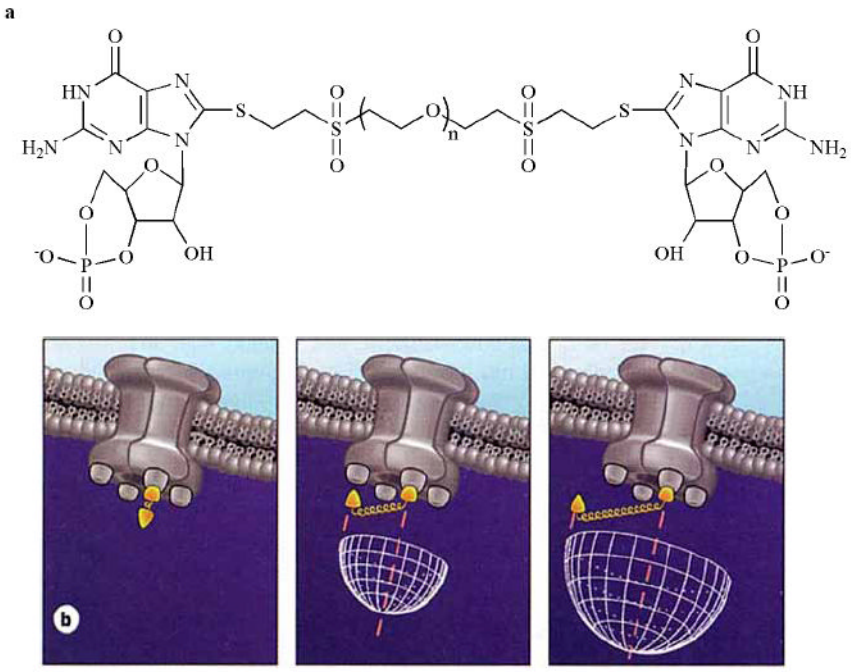


Fig. (4).

Polymer-linked ligand dimers (PLDs). **a**, Structure of a PLD containing two cGMP moieties and a polyethylene glycol (PEG) linker, with *n* ethylene glycol units. **b**, Diagrams of PLDs binding to a channel with four ligand-binding sites: PLDs are shown with polymers whose average length is too short (left), just right (center), or longer (right) than necessary, to allow the ligands to span two binding sites on the channel. Reproduced from ref. [136] by copyright permission of Nature Publishing Group (http://www.nature.com/).

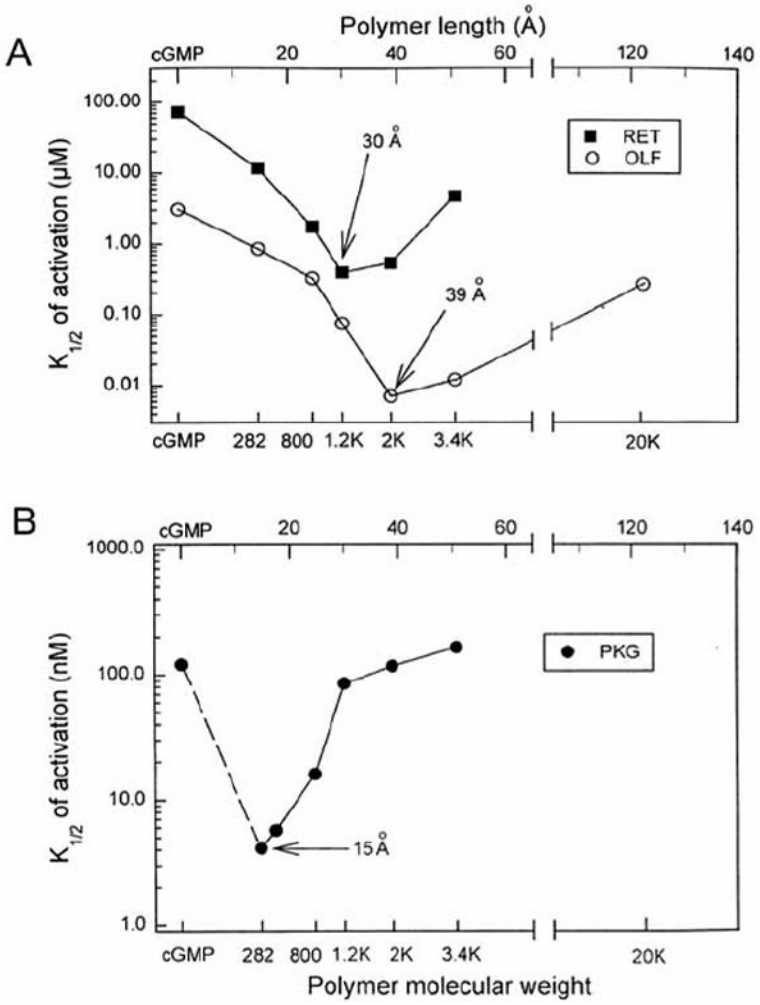


Fig. (5).

Identification of optimal polymer-linked dimers (PLDs) for cGMP-binding proteins. **a**, Activation of CNG channels (RET and OLF, CNGA1 and CNGA2). **b**, Activation of cGMP-dependent protein kinase (PKG). Arrows indicate PLDs optimal for activating each target protein. Polymer lengths were estimated from previous determinations, assuming an increase with the square root of the M_r. Reproduced from ref. [136] by copyright permission of Nature Publishing Group (http://www.nature.com/).

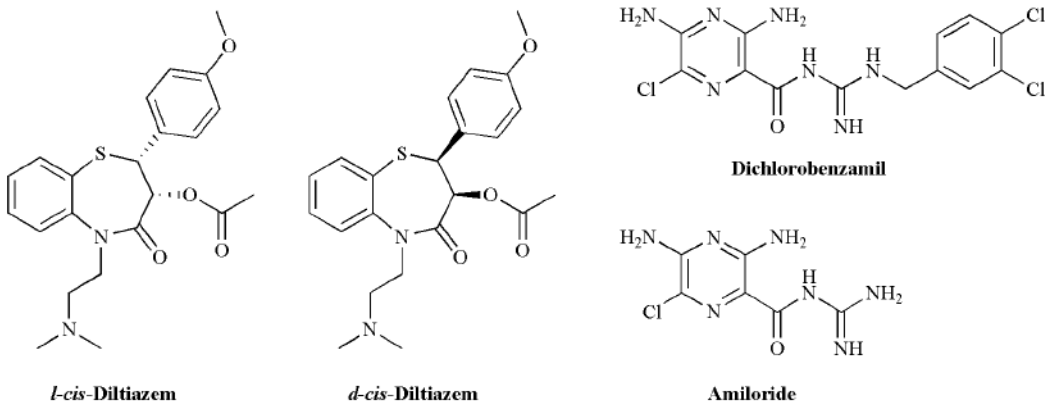


Fig. (6).
Structures of *l-cis*- and *d-cis*- diltiazem, dichlorobenzamil and amiloride.

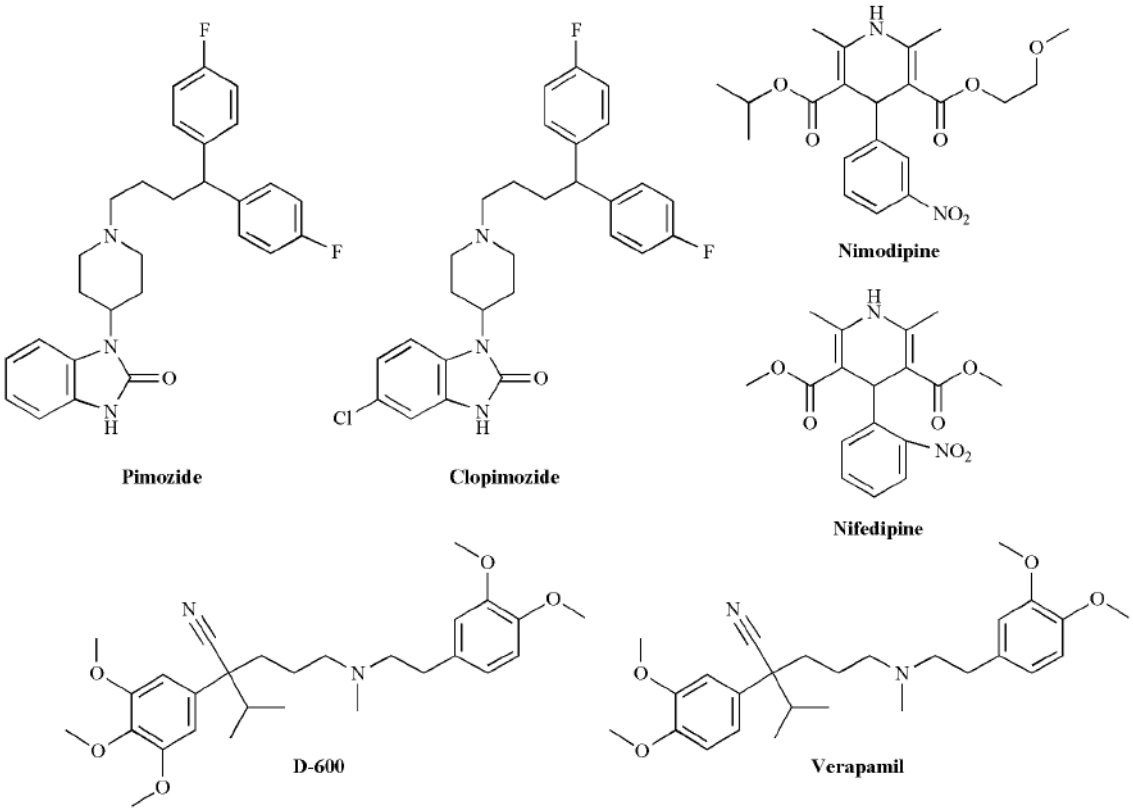


Fig. (7).
Structures of Ca^{2+} channel blockers that have been reported to block CNG channels.

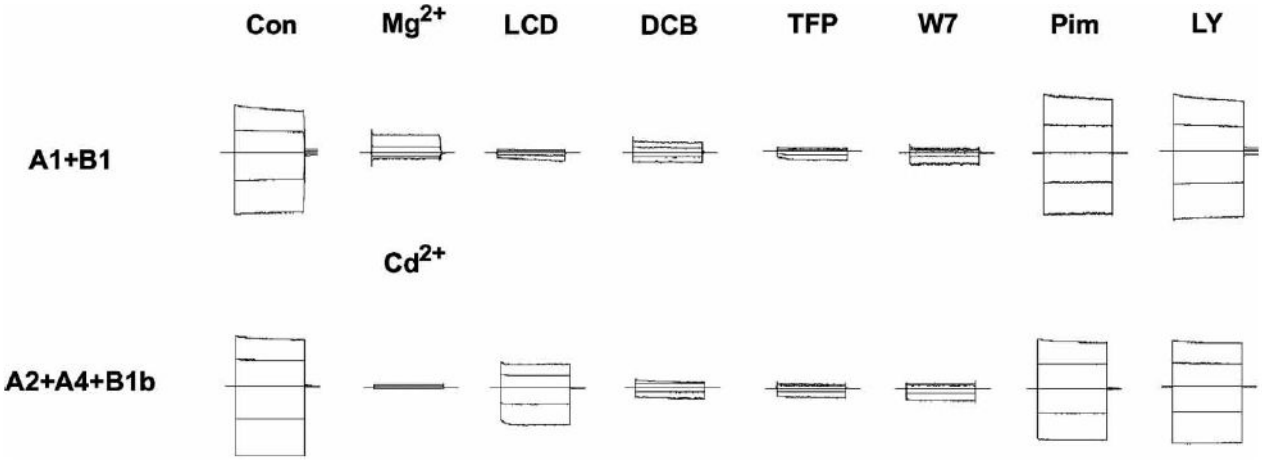


Fig. (8).

Extent of inhibition of heteromeric retinal rod and olfactory CNG channels by various pharmacological agents. Conventional whole-cell currents were recorded from HEK-293 cells expressing the rod (CNGA1 + CNGB1) or olfactory (CNGA2 + CNGA4 + CNGB1b) channels. Expression of all subunits was confirmed by measuring cyclic nucleotide dose-response relations and LCD affinity on excised inside-out patches. Currents were elicited by 250 ms voltage steps from a holding potential of 0 mV to potentials ranging from -50 to +50 mV in 25 mV increments. Control (Con) currents in the absence of inhibitor for both heteromeric channels ranged from 1-5 nA at -50 mV. Because the effects of many of the agents were only partially reversible, each set of traces was obtained from a different cell and normalized to a control trace taken at -50 mV on the same cell. Abbreviations for inhibitors not defined in the text are: TFP, trifluoperazine; Pim, Pimozide; and LY, LY83,583. External Mg^{2+} and Cd^{2+} were applied at 10 mM and 3 mM respectively; all other pharmacological agents were present at 50 μM. The currents shown in the presence of inhibitors were obtained after steady-state block was achieved, usually several minutes after extracellular application. Pimozide and LY83,583 are known to be membrane-permeant on the timescale of this experiment (~10 min), yet no channel block was detected (see text). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 0.5 mM EDTA, and 10 mM Hepes, pH 7.4; the internal solution contained 140 mM KCl, 5 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, and 1 mM cGMP.

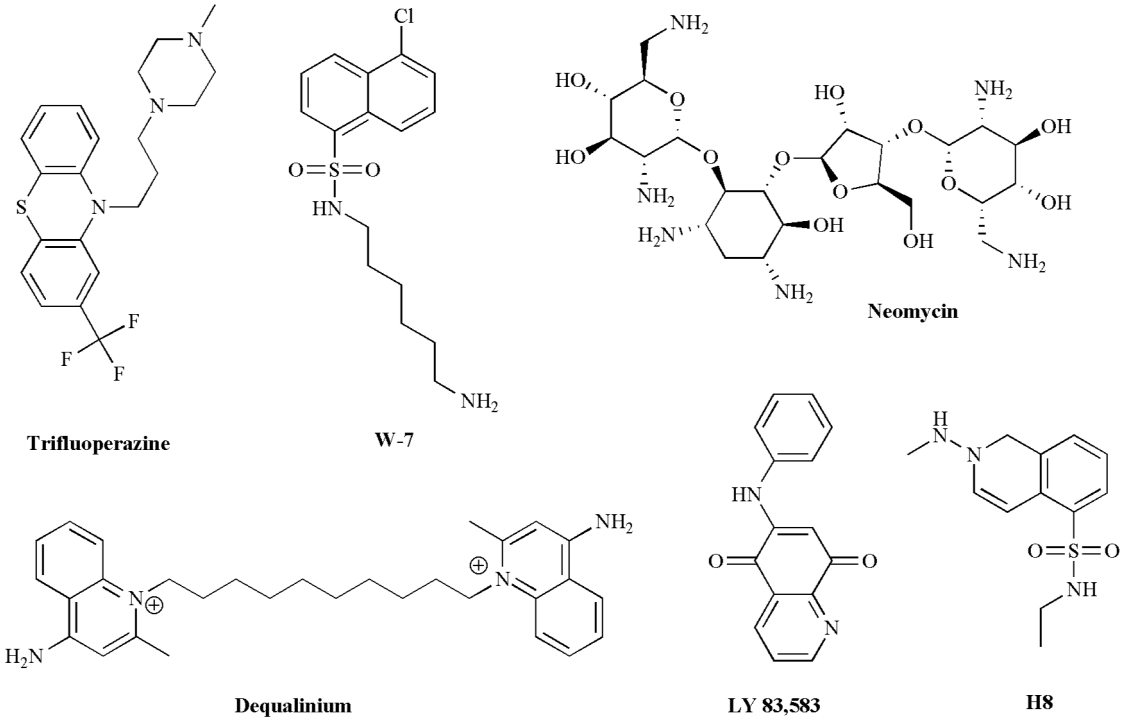
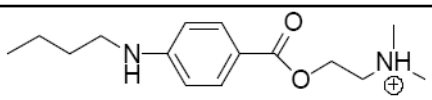
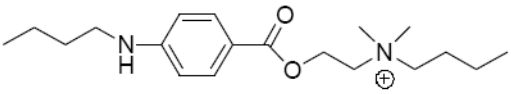
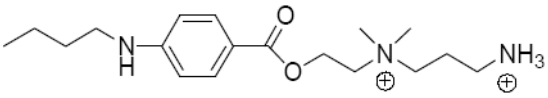
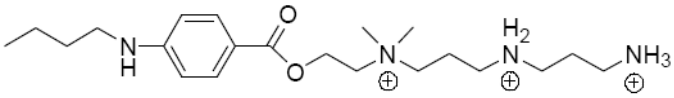
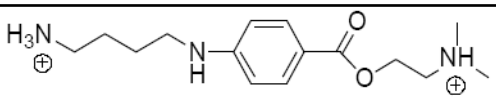
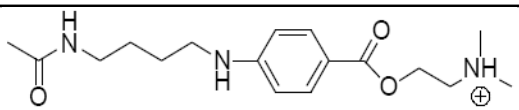
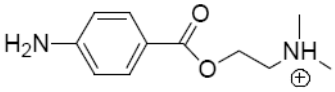


Fig. (9).

Structures of compounds with diverse activities: Ca^{2+}/CaM antagonists, trifluoperazine and W-7; an antibiotic, neomycin; an SK K^+ channel blocker, dequalinium; a guanylyl cyclase inhibitor, LY83,583; a PKA inhibitor, H-8. All of these compounds have been reported to block CNG channels.

Table 1

Tetracaine Analogue Structures and Block of CNGA1 Channels

Compound		$K_{D(40)}$ (μM)	zδ
1		6.8	0.47
2		3.1	0.45
3		0.074	1.8
4		0.00003	2.6
5		210	1.1
6		2500	0.47
7		2300	0.91

$K_{D(40)}$: The apparent dissociation constant at +40 mV. **zδ**: the charge carried by a blocker (z) multiplied by the fraction of the electric field (δ) traversed to reach its binding site. Each structure shown is the predominant form at neutral pH.