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Review

Modulation of cyclic-nucleotide-gated channels and regulation of vertebrate phototransduction

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Summary

Cyclic-nucleotide-gated (CNG) channels are crucial for sensory transduction in the photoreceptors (rods and cones) of the vertebrate retina. Light triggers a decrease in the cytoplasmic concentration of cyclic GMP in the outer segments of these cells, leading to closure of CNG channels and hyperpolarization of the membrane potential. Hence, CNG channels translate a chemical change in cyclic nucleotide concentration into an electrical signal that can spread through the photoreceptor cell and be transmitted to the rest of the visual system. The sensitivity of phototransduction can be altered by exposing the cells to light, through adaptation processes intrinsic to photoreceptors. Intracellular Ca^{2+} is a major signal in light adaptation and, in conjunction with Ca^{2+}-binding proteins, one of its targets for modulation is the CNG channel itself. However, other intracellular signals may be involved in the fine-tuning of light sensitivity in response to cues internal to organisms. Several intracellular signals are candidates for mediating changes in cyclic GMP sensitivity including transition metals, such as Ni^{2+} and Zn^{2+}, and lipid metabolites, such as diacylglycerol. Moreover, CNG channels are associated with protein kinases and phosphatases that catalyze changes in phosphorylation state and allosterically modulate channel activity. Recent studies suggest that the effects of circadian rhythms and retinal transmitters on CNG channels may be mediated by such changes in phosphorylation. The goal of this paper is to review the molecular mechanisms underlying modulation of CNG channels and to relate these forms of modulation to the regulation of light sensitivity.

Key words: cyclic-nucleotide-gated channel, modulation, phosphorylation, protein–protein interaction, insulin-like growth factor I, calmodulin, photoreceptor, sensory transduction.

Cyclic-nucleotide-gated channels and phototransduction

Rods and cones are the cells of the vertebrate retina that transduce visual information into neural signals. These remarkable cells not only possess all the molecular machinery necessary for generating the light response but they also contain systems for adjusting light sensitivities in accord with the level of background illumination. The only exogenous ingredients needed for signal transduction are the chromophore 11-*cis* retinal, provided by the retinal pigment epithelium (RPE) (Dowling, 1987), and Ca^{2+}, which enters through ion channels and is the crucial signal for adaptation (Fain et al., 2001). Unlike invertebrate photoreceptors (Crow and Bridge, 1985; Renninger et al., 1989), until recently there has been little evidence for extrinsic modulation of the phototransduction cascade by chemical transmitters.

The molecular steps of rod phototransduction are well understood (Fig. 1). Single photons induce isomerization of rhodopsin, leading to activation of the G-protein transducin and of phosphodiesterase, which hydrolyzes cyclic GMP (cGMP). This leads to a decrease in the cytoplasmic concentration of cGMP and closure of cyclic-nucleotide-gated (CNG) channels. The resulting decrease in the steady inward 'dark current' hyperpolarizes the membrane potential, ultimately leading to a decrease in the tonic release of the neurotransmitter glutamate from the presynaptic terminals.

The genes encoding CNG channels have been cloned, and their transmembrane structures have been deduced from the primary amino acid sequence (for a review, see Zagotta and Siegelbaum, 1996). Even though voltage has little effect on channel opening, CNG channel proteins are members of the voltage-gated superfamily of ion channel proteins, having particularly high homology with voltage-gated K^+ channels (Fig. 2) (Jan and Jan, 1990). Like K^+ channels, each subunit of a CNG channel has a cytoplasmic N terminus, six membrane-spanning segments and a cytoplasmic C terminus. In addition, the fourth membrane-spanning segment of CNG channels has several positively charged amino acids spaced three residues apart. In voltage-gated channels, these charges function as the voltage sensor for gating, but even though this

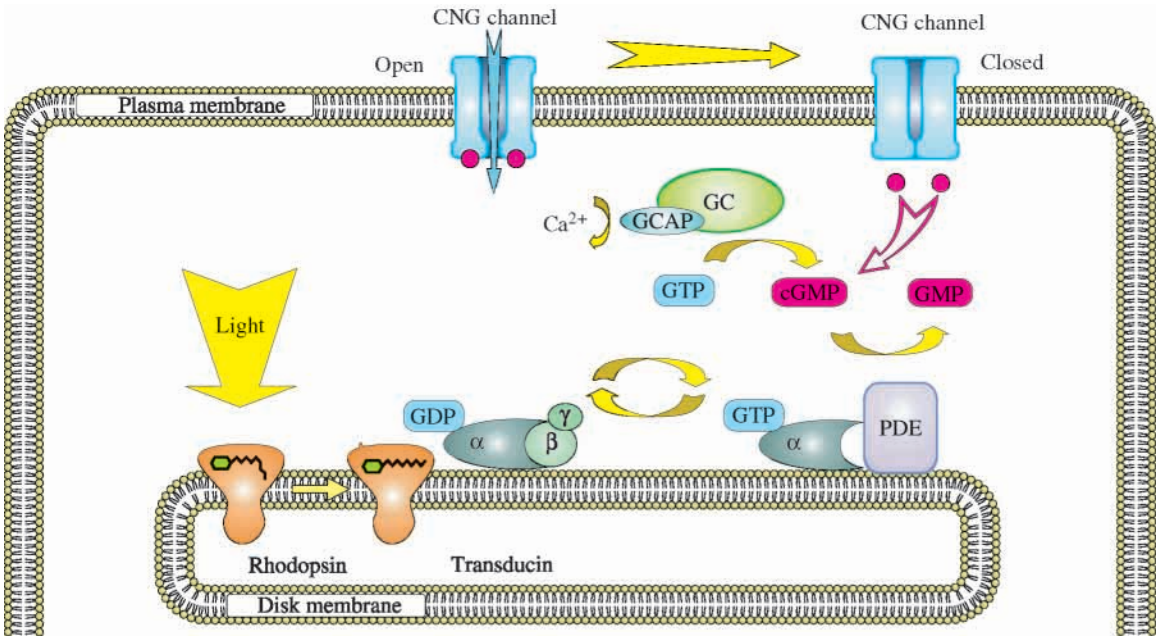

Fig. 1. The phototransduction cascade. Light causes photoisomerization of rhodopsin, activating the heterotrimeric G-protein transducin. The GTP-bound α-subunit activates phosphodiesterase (PDE), which degrades cGMP to GMP. The decrease in cGMP concentration leads to closure of cyclic-nucleotide-gated (CNG) channels, resulting in two effects, a decrease in Ca^{2+} influx and hyperpolarization of the membrane potential. The resulting decrease in intracellular Ca^{2+} concentration is important for adaptation. Lowered intracellular Ca^{2+} concentration disinhibits guanylate-cyclase-activating protein (GCAP), leading to activation of guanylate cyclase (GC) and resynthesis of cGMP.

motif is fairly well conserved in CNG channels, gating of these channels is relatively voltage-insensitive. The region between the fifth and sixth membrane-spanning segment is a re-entrant loop, known as the 'P' domain, because it has proved to be the crucial pore-lining region of the channel that determines the conductance and ion-selectivity properties of the channels (Goulding et al., 1993). CNG channels are thought to be tetramers (Liu et al., 1996), with each of four subunits contributing a pore-lining 'P' domain.

Native CNG channels in photoreceptors and olfactory neurons are heteromultimers, containing homologous α- and β-subunits (Kaupp et al., 1989; Dhallan et al., 1990; Goulding et al., 1992; Liman and Buck, 1994; Korschen et al., 1995; Gerstner et al., 2000). Exogenous expression of rod or olfactory α-subunits in *Xenopus laevis* oocytes results in functional CNG channels with properties that are similar, but not quite identical, to those of their native counterparts. Expression of β-subunits alone fails to produce functional channels; when they are co-expressed with α-subunits, the resulting channels more closely resemble native channels.

Our understanding of photoreceptor CNG channel gating and modulation has been greatly helped by structural and functional comparisons with closely homologous CNG channels from olfactory receptor neurons. The vertebrate olfaction signaling cascade involves odorants binding to G-protein-coupled receptors, activation of adenylate cyclase and synthesis of cyclic AMP, leading to activation of olfactory CNG channels and depolarization of the membrane potential. Like rod CNG channels, olfactory CNG channels are voltage-

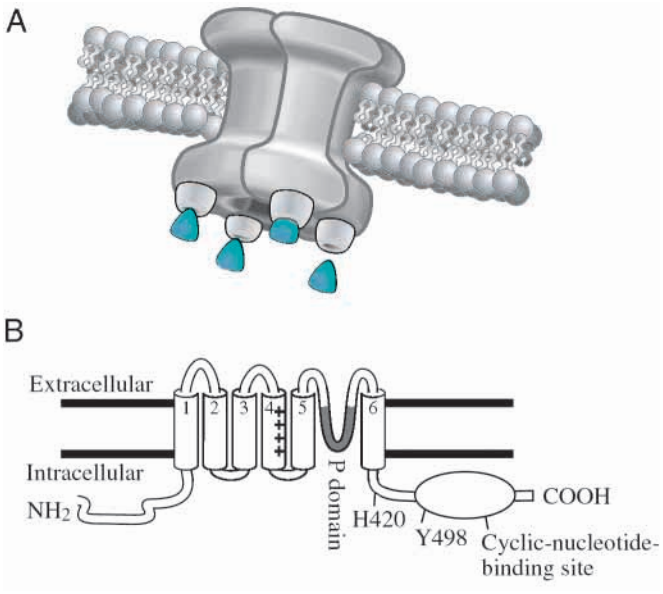

Fig. 2. (A) Three-dimensional structure of a cyclic-nucleotide-gated (CNG) channel. Four subunits are arranged to form a common pore. There is a cyclic-nucleotide-binding site on each subunit. (B) Diagram of the primary structure of the α-subunit. The cylinders indicate hydrophobic segments thought to represent transmembrane domains. The S4 segment is the voltage sensor for voltage-dependent transition in these channels; the region between S5 and S6 is thought to be part of the channel pore (P domain). The cyclic-nucleotide-binding domain is in the C-terminal region of the CNG channel.

insensitive, non-selective in their permeability to monovalent cations and do not desensitize with prolonged exposure to ligand. However, olfactory CNG channels have a larger-diameter pore and can be fully activated by either cyclic AMP or cyclic GMP (Dhallan et al., 1990; Goulding et al., 1992). Analysis of chimeric CNG channels, formed by substituting segments of rod with olfactory CNG channels, has elucidated crucial segments, and in some cases individual amino acids, that mediate the effects of modulators on CNG channel gating (Table 1).

Photoreceptor CNG channels play the central role in phototransduction. Therefore, intracellular or extracellular messengers that alter the behavior of CNG channels could potentially alter vision. For example, changing the sensitivity of CNG channels to cGMP (i.e. changing the $K_{1/2}$ for activation) could result in a change in visual sensitivity, either in response to signals external to an organism, such as light itself during light and dark adaptation, or in response to internal signals, such as circadian or hormonal regulation. Sensory signaling can be altered by affecting upstream steps in the phototransduction cascade, thereby changing the concentration of cyclic nucleotides. These effects are not the subject of this review, even though the changes in electrical signals that result would ultimately be mediated by CNG channels. The goal of this review is to consider the various ways in which photoreceptor CNG channels are modulated in the hope of elucidating the molecular mechanisms of visual modulation.

Modulation of CNG channels by Ca^{2+}/calmodulin

Ca^{2+} plays a central role in photoreceptor adaptation. The closure of CNG channels during illumination leads to a decrease in the influx of Ca^{2+}, which permeates through both rod and cone CNG channels. However, the activity of the $Na^+/Ca^{2+}/K^+$ exchanger is maintained, so the combination of decreased influx through CNG channels and maintained efflux through the exchanger results in a net decrease in the cytoplasmic Ca^{2+} concentration. The decrease in Ca^{2+} concentration, through largely unknown mechanisms, appears to contribute to shaping the kinetics of the rising and falling phases of the light response (Matthews, 1985; Lagnado and Baylor, 1994; Gray-Keller and Detwiler, 1994). However, the fall in Ca^{2+} concentration is more clearly important for mediating adaptation during steady illumination (for a review, see Pugh et al., 1999). In rod outer segments, Ca^{2+} regulates phototransduction *via* several Ca^{2+}-binding proteins. Thus, recoverin regulates rhodopsin phosphorylation and helps terminate the activity of rhodopsin, and guanylate-cyclase-activating protein (GCAP) regulates cGMP synthesis by guanylate cyclase, replenishing cGMP after the peak of the light response. A mathematical model of rod phototransduction suggests that GCAP and recoverin play a major role in mediating the effects of Ca^{2+} on light adaptation, at low and high ambient light levels, respectively (Koutalos et al., 1995; Koutalos and Yau, 1996).

In addition to these effects, Ca^{2+}, in conjunction with calmodulin in rods (Hsu and Molday, 1993) or other Ca^{2+}-binding proteins in cones (Rebrik and Korenbrot, 1998), directly binds to and inhibits CNG channels by reducing their sensitivity to cGMP. In rods, Ca^{2+}/calmodulin binds to the β-subunit of the rod CNG channel protein (Chen et al., 1994; Hsu and Molday, 1993; Weitz et al., 1998; Grunwald et al., 1998). The binding of Ca^{2+}/calmodulin to CNG channels can alter the interaction between the N and C termini of channel subunits, which may be important for gating (Varnum and Zagotta, 1997). According to this scenario, the drop in intracellular Ca^{2+} concentration during the light response should favor the reopening of CNG channels, thus extending the operating range of the rod light response. However, the shift in cGMP sensitivity is rather small (two- to threefold) compared with the large change in rod light sensitivity during light adaptation (100- to 1000-fold), suggesting that this mechanism makes only a minor contribution to adaptation in rods (Koutalos and Yau, 1996), although it makes a larger contribution to adaptation in cones (Rebrik and Korenbrot, 1998).

It should be noted that Ca^{2+}/calmodulin also modulates CNG channels in olfactory neurons, but here the binding site is on the α-subunit rather than other subunits of the channel (Liu et al., 1994). Like the rod CNG channel, the cyclic-nucleotide-sensitivity (to both cGMP and cAMP) of olfactory CNG channels is reduced by Ca^{2+} (Kramer and Siegelbaum, 1992). Odorant responses are mediated by an increase in cyclic AMP concentration, which opens CNG channels, depolarizing the cell and allowing Ca^{2+} influx. The increased Ca^{2+} influx, followed by inhibition of CNG channels by Ca^{2+}/calmodulin, constitutes a negative feedback system that plays a crucial role in olfactory adaptation in these cells (Kurahashi and Menini, 1997).

Regulation by nitric oxide

Nitric oxide (NO) is an important signaling molecule in the retina (for a review, see Cudeiro and Rivadulla, 1999). The major target for NO in most cell types is soluble guanylate cyclase, which, by synthesizing cGMP, can lead to activation of CNG channels. In some olfactory neurons, NO can directly activate CNG channels even in the absence of cyclic nucleotides (Broillet and Firestein, 1996), but there is no evidence that NO has a direct affect on photoreceptor CNG channels (Trivedi and Kramer, 1998). One crucial locus for NO action appears to be a cysteine residue (C460) in the α-subunit of the olfactory channel (Broillet, 2000), a residue that is also conserved in the olfactory β-subunit and in the rod α- and β-subunits. Why the rod channels are unaffected by NO remains a mystery. Moreover, since the physiological relevant dose of NO is unclear, the physiologically significance of CNG channel modulation by NO remains uncertain.

Modulation by transition metals

The sensitivity of CNG channels can also be altered by transition metals, such as Ni^{2+} and Zn^{2+} (Karpen et al., 1993;

Table 1. *Modulators of photoreceptor and olfactory cyclic-nucleotide-gated (CNG) channels*

	Photoreceptor CNG channel			Olfactory CNG channel		
Modulator	Effect	Mechanism and site of action	References	Effect	Mechanism and site of action	References
Ca^{2+} *via* Ca^{2+}-binding proteins	Decrease in cyclic nucleotide sensitivity	Ca^{2+}/CaM binds to N terminus of β-subunit	Chen et al., 1994; Hsu and Molday, 1993; Grunwald et al., 1998; Rebrik and Korenbrot, 1998; Weitz et al., 1998	Decrease in cyclic nucleotide sensitivity	Ca^{2+}/CaM and other Ca^{2+}-binding proteins bind to N terminus of α-subunit	Kramer and Siegelbaum, 1992; Chen and Yau, 1994; Liu et al., 1994
Transition metals (Ni^{2+}, Zn^{2+})	Increase in cyclic nucleotide sensitivity	Coordination of H420 of adjacent subunits and promotion of open state	Karpen et al., 1993; Gordon and Zagotta, 1995a	Decrease in cyclic nucleotide sensitivity	H396	Gordon and Zagotta, 1995b
Nitric oxide	None reported			Activation	S-nitrosylation of α-subunit (C460) and β-subunit	Broillet and Firestein, 1996; Broillet, 2000
Serine/threonine phosphorylation	Decrease/increase in cyclic nucleotide sensitivity	Rod channel S577, S579 of cone α-subunit	Gordon et al., 1992; Muller et al., 2001	Increase in cyclic nucleotide sensitivity	S93	Muller et al., 1998
Tyrosine phosphorylation	Decrease in cyclic nucleotide sensitivity	Y498 of α-subunit	Molokanova et al., 1997; Molokanova et al., 1999a	None reported		
Protein–protein interaction with protein tyrosine kinase	Inhibition of cGMP-elicited current; decrease in cyclic nucleotide sensitivity	S6 transmembrane domain and flanking regions of α-subunit	Molokanova et al., 1999b; Molokanova et al., 2000; Molokanova and Kramer, 2001	None reported		
Phospholipid metabolites	Decrease in cyclic nucleotide sensitivity	Transmembrane domains	Gordon et al., 1995; Crary et al., 2000	Decrease in cyclic nucleotide sensitivity		Crary et al., 2000

CaM, calmodulin.

Gordon and Zagotta, 1995a). Like other divalent cations (notably Ca^{2+} and Mg^{2+}), at sufficiently high concentrations these ions induce a voltage-dependent block by binding to sites within the permeation pathway of the CNG channel. However, Ni^{2+} and Zn^{2+} have an additional effect on channel gating. Experiments on rod and olfactory CNG channel α-subunits have demonstrated that the two channels are affected in remarkably different ways by these metals and have elucidated the structural basis for this difference. Rod α-subunits contain a crucial histidine (H420) necessary for potentiation of channel gating by Ni^{2+}. The olfactory α-subunit lacks a histidine at the position equivalent to H420. However, unlike the rod channel, it has a histidine at position 396, which is involved in the functionally opposite effect. Thus, channels composed of olfactory α-subunits exhibit a depression of cGMP sensitivity in the presence of transition metals. From a functional perspective, there is the intriguing possibility that, in the retina, the free concentration of these ions (especially Zn^{2+}) may be sufficiently high, either at rest and/or in response to activity or neuromodulator actions, to play a physiological role in rod CNG channel regulation. Free or loosely bound Zn^{2+} can be detected in rods and cones (Wu et al., 1993; Kaneda et al., 2000), and Zn^{2+} is tightly bound to rhodopsin (Shuster et al., 1996) and phosphodiesterase, where it is essential for enzymatic function (He et al., 2000). Exposure to light results a dramatic redistribution of chelatable Zn^{2+} in rods (Ugarte and Osborne, 1999), raising the possibility that Zn^{2+} plays a dynamic role in phototransduction, perhaps with CNG channels as an important target.

Regulation by lipid metabolites

Recent studies have shown that certain lipid metabolites, including diacylglycerol (DAG), modulate native and expressed rod CNG channels (Gordon et al., 1995; Crary et al., 2000; Womack et al., 2000). Even though DAG is an activator of protein kinase C (PKC), the effect of DAG on CNG channels does not require the catalytic activity of protein kinases (Gordon et al., 1995). It is unclear whether DAG binds to hydrophobic regions of the CNG channel protein or whether it deforms the bilayer in the vicinity of CNG channels, altering their ability to open. Invertebrate phototransduction is thought to be mediated by phospholipase C (PLC) (Ranganathan et al., 1995), and DAG metabolites have been implicated in activating the light-sensitive ion channels in *Drosophila melanogaster* photoreceptors (Chyb et al., 1999). Hence, there are interesting parallels in the finding that DAG also modulates CNG channels from vertebrate photoreceptors. It has long been known that components of the phosphoinositide signaling pathways exist in rod outer segments. In recent work, the PLC isoforms PLCβ4 (Peng et al., 1997) and PLCγ1 (Ghalayini et al., 1998), as well as a G_q-type G-protein ($G_{\alpha 11}$) capable of activating PLC (Peng et al., 1997), have been immunocytochemically localized to vertebrate rod outer segments. Moreover, PLCγ1 is translocated to membranes in response to light (Ghalayini et al., 1998). Despite these findings, exogenous introduction of PLC, inositol trisphosphate, or PKC fails to alter light responses measured from truncated rod outer segments (Jindrova and Detwiler, 1998). Thus, it remains to be demonstrated that products of lipid metabolism have a physiological role in mediating or modulating the vertebrate light response.

Regulation of CNG channels by phosphorylation

Neurotransmitter modulation of physiological processes, such as neurotransmitter release and muscle contraction, often involves 'cross-talk', in which one biochemical signaling pathway can modulate the activity of another. For years, investigators have searched for cross-talk in vertebrate phototransduction, with mostly negative results. PKC, which is prominent in rod outer segments, appears to have no effect on light sensitivity (Xiong et al., 1997; Jindrova and Detwiler, 1998). Cyclic-AMP-dependent protein kinase (PKA), although capable of phosphorylating phototransduction proteins including rod guanylate cyclase (Wolbring and Schnetkamp, 1996) and phosducin (Willardson et al., 1996), also has no clear role in acute modulation of the light response. Neither PKA nor PKC has any reported effects on rod CNG channel activity, although other protein kinases and phosphatases do appear to modulate rod CNG channels (see below), supporting the idea that the phototransduction cascade might be regulated by neurotransmitter-elicited 'cross-talk'. There is recent evidence for modulation of cone CNG channels by an endogenous Ca^{2+}-independent form of PKC (Muller et al., 2001).

Studies suggest that CNG channels can be modulated by changes in phosphorylation state catalyzed by serine/threonine protein kinases and phosphatases (Gordon et al., 1992) and, more recently, by protein tyrosine kinases (PTKs) and phosphatases (PTPs) (Molokanova et al., 1997). Two approaches have been used to investigate modulation by changes in phosphorylation state. First, researchers have studied changes in activity brought about by unidentified kinases or phosphatases endogenous to cells expressing the channels (either photoreceptors or exogenous expression systems) (Gordon et al., 1992; Molokanova et al., 1997). The involvement of these enzymes has been deduced from the use of specific kinase or phosphatase inhibitors. In the second approach, defined kinases or phosphatases are applied directly to CNG channels in a cell-free system (usually an excised inside-out membrane patch) (Muller et al., 1998). Unfortunately, kinases and phosphatases are often quite 'promiscuous': proteins that are not natural physiological targets may nonetheless still be substrates for the enzymes in a cell-free system. Therefore, conclusions about modulation based solely on exogenous enzyme application should be made with caution.

Rod CNG channels formed by expressing the bovine rod α-subunit gene in *Xenopus laevis* oocytes exhibit a particularly clear type of modulation, attributable to changes in tyrosine phosphorylation state (Molokanova et al., 1997; Molokanova

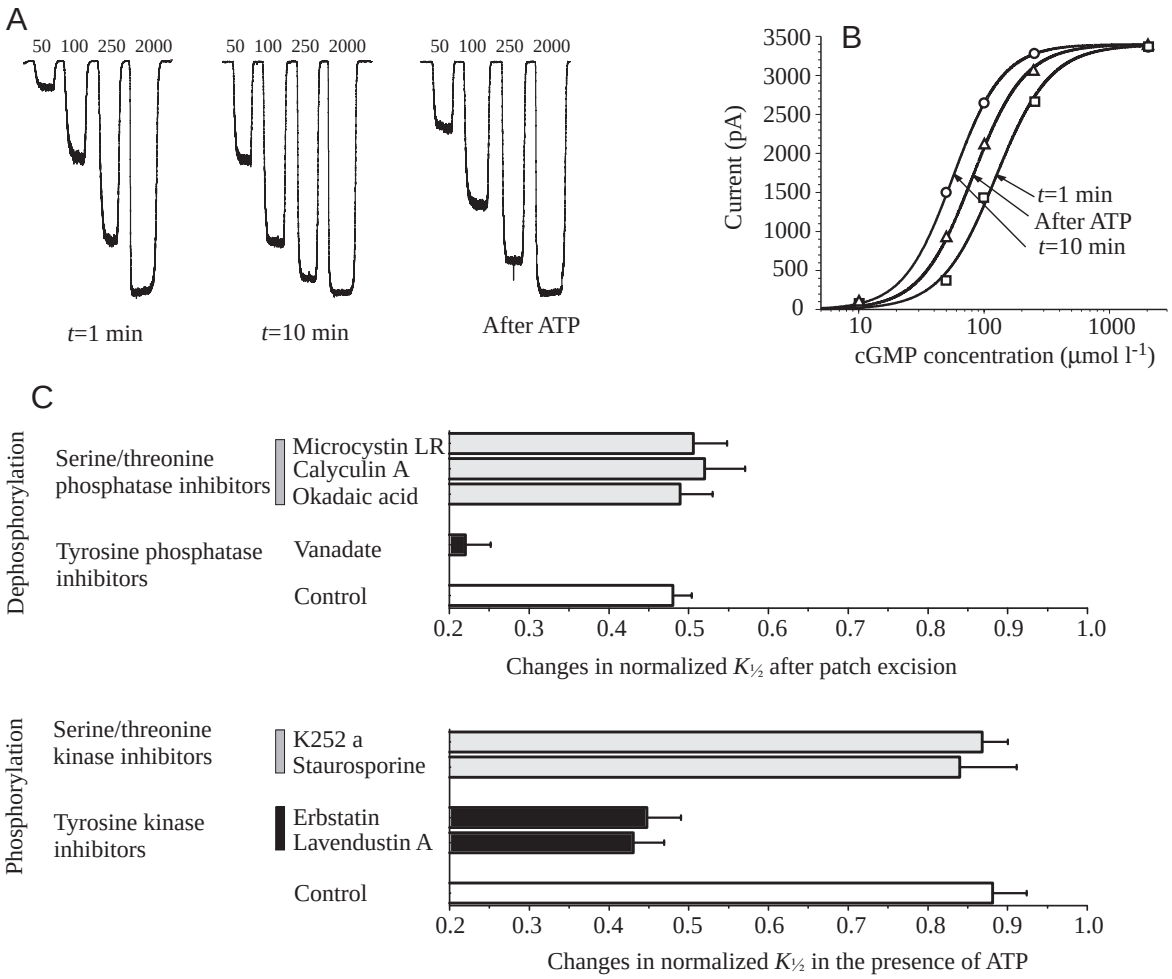

Fig. 3. Modulation of cyclic-nucleotide-gated (CNG) channels by tyrosine phosphorylation. (A) Changes in amplitude of cGMP-activated current through CNG channels in an excised membrane patch. Currents were elicited by application of cGMP at concentrations of 50, 100, 250 and 2000 μmol l^{-1} and recorded 1 and 10 min after patch excision and after a 3 min application of 200 μmol l^{-1} ATP. (B) Changes in cGMP-sensitivity of CNG channels after patch excision and subsequent transient application of ATP. (C) Changes in the cyclic-nucleotide-sensitivity ($K_{1/2}$) exhibited during the first 10 min after excision in the presence of serine/threonine and tyrosine protein kinases and phosphatases inhibitors. Values are means + S.E.M. (N=6–16 for different inhibitors).

et al., 1999a). The key observation is a spontaneous two- to threefold increase in channel cGMP sensitivity, such that CNG currents activated by sub-saturating, but not saturating, cGMP concentrations increase after patch excision (Fig. 3A). On average, the $K_{1/2}$ for activation by cGMP shifts from approximately 120 μmol l^{-1} to approximately 60 μmol l^{-1} within 10 min of patch excision (Fig. 3B). Addition of ATP to the superfusate partly reverses the effect, but the sensitivity once again begins to increase when ATP is removed. ATP-γ-S, which can often support irreversible thio-phosphorylation of proteins, elicits an irreversible effect on cGMP sensitivity. Non-hydrolysable ATP analogs, such as AMP-PNP, which cannot act as substrates in phosphorylation reactions, have no effect. The simplest interpretation of these results is that a protein phosphatase, which dephosphorylates the channel, increases the cGMP sensitivity, while a protein kinase, which phosphorylates the channels in the presence of ATP, makes the channels less sensitive to cGMP. Since the effects occur spontaneously in excised patches, the putative kinases and phosphatases must be constitutively active. Native CNG channels in rod photoreceptors are modulated in a similar manner but, in the absence of Ca^{2+}, an external transmitter is required to trigger changes in phosphorylation state (see below).

Pharmacological experiments support the above hypothesis and focus attention on PTKs and PTPs instead of serine/threonine-specific enzymes. The increase in cGMP sensitivity upon patch excision is greatly reduced by vanadate, a PTP inhibitor, and the effect of ATP is blocked by lavendustin A or erbstatin, selective PTK inhibitors (Fig. 3C). In contrast, specific inhibitors of serine/threonine kinases and phosphatases have no effect. Finally, a specific tyrosine in the cyclic-nucleotide-binding domain of the rod channel α-subunit (Y498) has been identified as a crucial site required for modulation. Substitution of this tyrosine with a phenylalanine eliminates modulation. The olfactory CNG channel α-subunit

lacks a tyrosine at the equivalent position (F477), and it does not exhibit modulation. However, when a tyrosine is substituted into this position (Y477F), modulation is introduced. Taken together, it seems very likely that the oocyte contains constitutively active PTK(s) and PTP(s) that phosphorylate or dephosphorylate Y498 in the rod channel, decreasing and increasing cGMP sensitivity, respectively.

Activity-dependence of modulation by tyrosine phosphorylation

Modulation of the rod CNG channel is influenced in an intriguing manner by its open *versus* closed state. Studies on homomeric rod CNG channels containing α-subunits show that the channel can only be dephosphorylated when it is opened with cGMP and can only be phosphorylated when it is closed by removing cGMP. Application of a saturating concentration of cyclic AMP, a very weak partial agonist of the rod channel, only weakly alters the ability of PTP to modulate the channel, supporting the notion that channel opening, rather than ligand occupancy of the cyclic-nucleotide-binding site, is responsible for the activity-dependent effects of cyclic nucleotides. Molokanova et al. (Molokanova et al., 1999b) have proposed a model in which PTK and PTP compete for the same or overlapping sites on the channel, accounting for the opposing activity-dependent effects on phosphorylation and dephosphorylation (Fig. 4). According to this scenario, the open channel conformation favors binding to PTP and the closed conformation favors binding to PTK. Moreover, the interaction between the channel and either protein is mutually exclusive.

The activity-dependence of modulation introduces an interesting bistability to rod CNG channel behavior. Thus, phosphorylation, which decreases cGMP sensitivity, leads to an increase in the probability of channels being closed, increasing phosphorylation by PTKs, which further decreases cGMP sensitivity. Conversely, dephosphorylation, which increases cGMP sensitivity, leads to an increase in the probability of channels being open, increasing dephosphorylation by PTPs and further increasing cGMP sensitivity. In other words, when the channels are open (e.g. in the dark), they will be drawn into a stable phosphorylated state, and when they are closed (in the light) they will be drawn into a stable dephosphorylated state. The activity-dependence of modulation has been demonstrated for rod channels expressed in oocytes, but has not yet been demonstrated for native CNG channels. If it indeed applies to rods, such activity-dependence might have important functional consequences for modulation of phototransduction by offsetting or counteracting the negative feedback effects of light adaptation.

Regulation by protein–protein interactions

Rather than living as lonely isolated membrane proteins, ion channels are much more gregarious, forming macromolecular complexes with various signaling molecules including

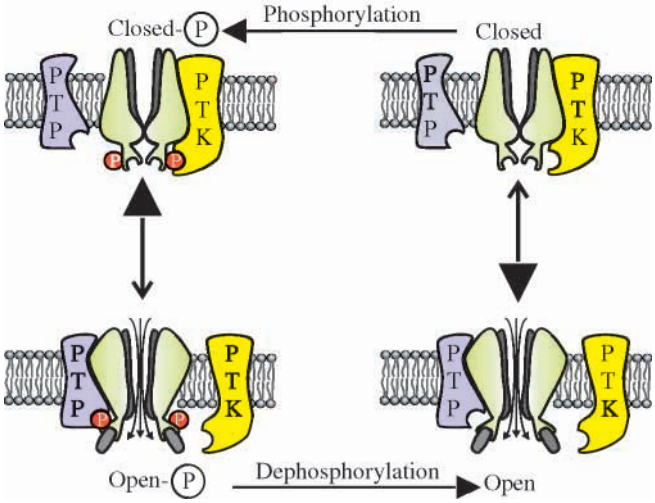

Fig. 4. Schematic model illustrating the activity–dependence of modulation of cyclic-nucleotide-gated (CNG) channels by tyrosine phosphorylation and dephosphorylation. The top and bottom diagrams represent closed and open channels, respectively; the left and right diagrams represent phosphorylated and dephosphorylated channels, respectively. The ovals represent cGMP molecules. The relative thickness of the arrowheads represents changes in the favorability of gating. PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase.

cytoskeletal proteins, G-proteins and protein kinases and phosphatases. Recent biochemical studies have shown that the native rod CNG channel is bound to the $Na^+/Ca^{2+}/K^+$ exchanger (Bauer and Drechsler, 1992; Schwarzer et al., 2000). In addition, the N-terminal domain of the β-subunit of the rod channel is similar to other glutamic-acid-rich proteins found in photoreceptors, which bind to components of the phototransduction cascade, such as phosphodiesterase and guanylate cyclase (Korschen et al., 1999), forming a macromolecular assembly termed a ‘transducisome’.

We have obtained indirect evidence that the rod CNG channel is stably associated with PTK(s). Moreover, at least under some circumstances, the PTK(s) can alter channel function, even in the absence of ATP. Thus, PTKs can modulate CNG channels in two ways: first, by catalyzing phosphorylation and, second, through a non-catalytic allosteric effect that inhibits CNG channel gating.

The non-catalytic inhibition mediated by PTKs can be elicited by applying genistein, a PTK inhibitor that specifically interacts with the ATP-binding site on the enzyme (Molokanova et al., 1999b). Application of genistein dramatically slows the gating of the rod CNG channels and reduces the steady-state current activated by cGMP. Various results, including the observation that agents specific for PTKs prevent genistein inhibition, suggest that genistein inhibition is not mediated by a direct interaction between genistein and the CNG channel, but rather involves an indirect effect mediated by a PTK. The affinity and efficacy of genistein are much higher for closed than for open channels, following the same activity-dependent pattern of phosphorylation of CNG

channels by PTKs. These results and others strongly suggest that genistein inhibition involves genistein binding to the PTK which, through an allosteric interaction with the channel, hinders channel gating (Molokanova and Kramer, 2001).

The effect of genistein is not limited to rod CNG channels expressed in oocytes, but also applies to native CNG channels in rods and cones and, to a lesser extent, to olfactory neurons (Molokanova et al., 2000). Moreover, the same criteria used to suggest that genistein acts indirectly (through a PTK) also apply to inhibition of the native channels. It is unclear whether CNG channels and PTK have an intimate or just a casual relationship with one another. Thus, we do not know whether the PTK responsible for genistein inhibition is part of a stable complex with the CNG channel or whether it normally dissociates and reassociates with the channel. Additional biochemical experiments are needed to identify the PTKs that regulate CNG channels and to understand the nature of their relationship.

Modulation by neurotransmitters

Can phototransduction be modulated by extrinsic neurotransmitters? In photoreceptors from invertebrates, such as *Limulus polyphemus*, efferent neurotransmitters can alter light sensitivity by influencing components of the phototransduction cascade (for a review, see Barlow, 1990). Thus, octopamine changes the frequency of spontaneous and evoked 'quantum bumps' (O'Day and Lisman, 1985), indicating a change in the rate of rhodopsin isomerization. In vertebrate retina, neurotransmitters such as dopamine (Akopian and Witkovski, 1996; Stella and Thoreson, 2000), GABA (Barnes and Hille, 1989) and glutamate (Picaud et al., 1995) can modulate the activity of voltage-gated ion channels in the inner segments or terminals of rods and cones, presumably shaping the light response and altering synaptic transmission. However, until recently, little was known about transmitter actions on the phototransduction cascade itself, which occur in outer rather than inner segments.

Few types of receptors for extrinsic signaling molecules have been found in the plasma membrane of rod outer segments. Those that occur include dopamine receptors (Udovichenko et al., 1998), adenosine A2 receptors (McIntosh and Blazynski, 1994) and receptors for insulin-like growth factor I (IGF-I) (Waldbillig et al., 1991). IGF-I is particularly interesting because it is synthesized and released from retinal pigment epithelial cells (Waldbillig et al., 1991), which lie immediately adjacent to rod outer segments. Moreover, IGF-binding proteins, which may participate in the delivery of IGF-I to receptors, are concentrated in the interphotoreceptor matrix, situated between the RPE and the plasma membrane of outer segments. The RPE plays several crucial roles in supporting photoreceptors, including providing the photopigment 11-*cis* retinal to rod and cone outer segments and

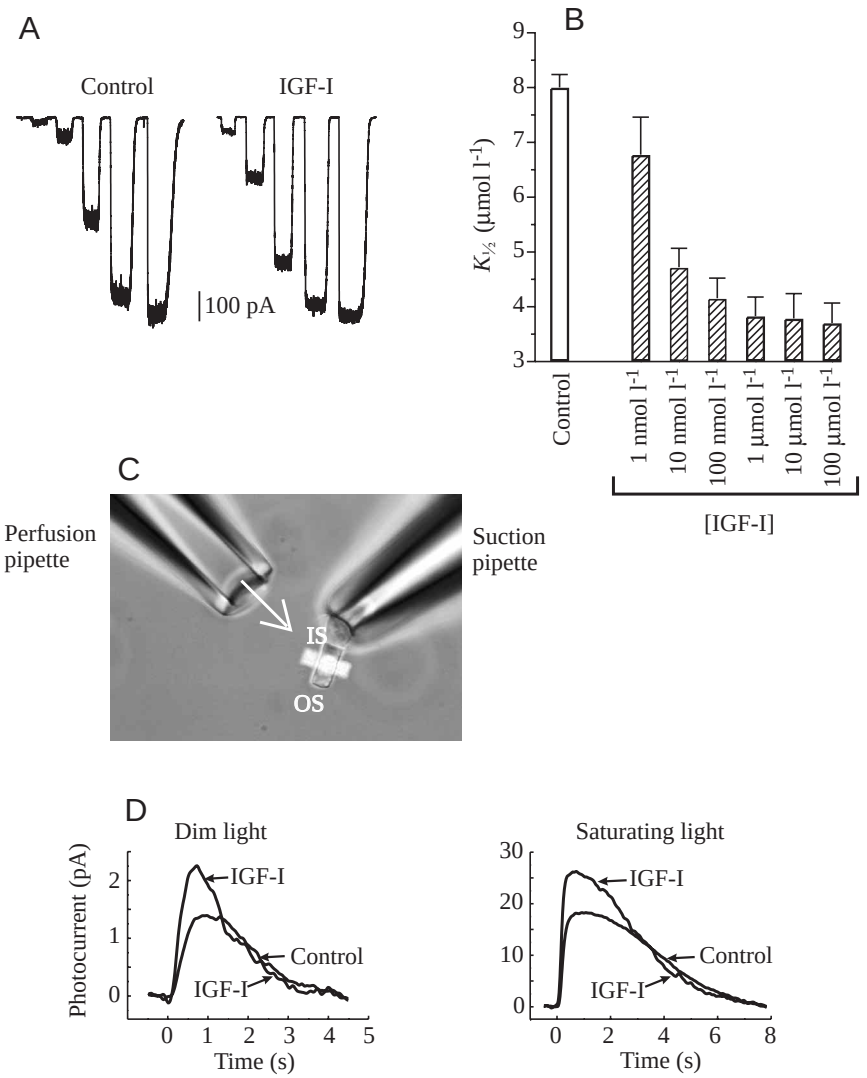

Fig. 5. Insulin-like growth factor I (IGF-I) increases the cyclic-nucleotide-sensitivity of rod cyclic-nucleotide-gated (CNG) channels. (A) An increase in amplitude of the currents activated by application of 8-Br-cGMP (1, 2.5, 10, 25 and 250 μmol l^{-1} from left to right) in excised patches from rods exposed to control saline or to saline containing 10 μmol l^{-1} IGF-I for 10 min prior to patch excision. (B) Effects of a 10 min pretreatment with 1 nmol l^{-1} to 100 μmol l^{-1} IGF-I (values are means + S.E.M., N=7–25) on the apparent affinity of CNG channels. (C) A suction pipette was used to record the light response of the rods *via* the inner segment (IS) while permitting continuous superfusion of control or IGF-I-containing solutions on the outer segment (OS). (D) Average rod photoresponse waveforms in response to dim and saturating 10 ms light flashes (at time zero) recorded in control saline and 4–6 min after the beginning superfusion with 1 μmol l^{-1} IGF-I.

regulating the shedding of discs and from the apical end of outer segments and secretion of several growth factors important for photoreceptor development and long-term survival (Bok, 1993). The finding that one of the growth factors released by the RPE (IGF-I) also acutely regulates the light response (Savchenko et al., 2001) suggests that the RPE also has a more dynamic neuromodulatory function.

Application of IGF-I to rod outer segments leads to a two- to threefold increase in the sensitivity of CNG channels to cGMP (Savchenko et al., 2001) (Fig. 5A,B). The effect of IGF-I occurs within tens of seconds and dissipates with equal rapidity after removal of IGF-I. Various lines of evidence suggest that the effect of IGF-I involves a complex signaling pathway, ending with tyrosine dephosphorylation of the rod CNG channel. Parallel experiments performed on rod CNG channel α-subunits expressed in *Xenopus laevis* oocytes, which have their own IGF-I receptors, show that IGF-I also increases the cGMP sensitivity of the channels, but only if the crucial tyrosine (Y498) is present. Hence, when this tyrosine is substituted with a phenylalanine (mutant Y498F), the channels are unaffected when the oocyte is exposed to IGF-I. These results suggest that the effect of IGF-I not only involves tyrosine dephosphorylation but that the crucial target is the same specific tyrosine residue implicated in spontaneous modulation in oocytes.

Further studies (Savchenko et al., 2001) show that IGF-I alters the light response of rods (Fig. 5C,D). Suction electrode recordings of photocurrents from salamander rods show that IGF-I increases flash responses at both dim and saturating light intensities. Focal electroretinogram recordings from mammalian retina also show that IGF-I increases the population light response from rods. The increase in the saturating light response is consistent with modulation of the CNG channels. The resting concentration of free cGMP in the dark is thought to be approximately 5 μmol l^{-1}, sufficient to open 2–5 % of the CNG channels at any instant in time. By increasing the sensitivity of CNG channels to cGMP, a greater percentage of channels will be opened (e.g. 10 %), increasing the amount of current available to be turned off by saturating light. The increased cGMP sensitivity of the channels will be offset to some extent by the negative feedback systems inherent in rod phototransduction. Thus, the increase in the dark CNG current will result in an increase in Ca^{2+} influx, and the resulting increase in internal Ca^{2+} concentration, in conjunction with GCAP, should inhibit guanylate cyclase, reducing the cytoplasmic concentration of cGMP. At steady state, the size of the IGF-I effect should be inversely dependent on the efficiency of this homeostatic mechanism. For example, IGF-I increases the CNG current elicited by 5 μmol l^{-1} cGMP by approximately 300 %, whereas the saturating light response is increased by only 25 %.

It is possible that IGF-I plays a role in slow forms of light adaptation in rods. Rapidly decaying 'photoreceptor adaptation', lasting seconds to minutes, is intrinsic to rods and cones and can be attributed entirely to modulation of the phototransduction cascade by light-driven changes in intracellular Ca^{2+} concentration (for a review, see Pugh et al., 1999). A much more slowly recovering change in responsiveness, lasting minutes to hours, termed 'photochemical adaptation', requires the RPE and has been attributed to regeneration of bleached photopigment (Dowling, 1987). In addition to this type of slow adaptation, which is only apparent at high light intensities sufficient to bleach most of the rhodopsin, there are more subtle changes in light sensitivity in response to hormones or associated with free-running circadian rhythms (see below). In addition, slow light-driven diurnal changes have been reported (Schaeffel et al., 1991; Birch et al., 1994). The modest effects elicited by IGF-I are more likely to be involved in these more subtle slow changes in light sensitivity.

Circadian regulation of CNG channels

A variety of events in retinal photoreceptors are regulated in a circadian manner. Photoreceptors exhibit circadian rhythms in morphological features including the shedding of discs from outer segments, retinomotor movements and outer segment renewal (for a review, see Cahill and Besharse, 1993). The absolute sensitivity of the light response (Lu et al., 1991; McGoogan et al., 1998) and the relative contribution of rods and cones in driving postsynaptic responses (Manglapus et al., 1998; Manglapus et al., 1999) also vary in a circadian manner. Recent studies have show that the cGMP-sensitivity of cone photoreceptor CNG channels varies with a circadian rhythm (Ko et al., 2001). Isolated chick cones appear to have an endogenous circadian clock resulting in a two- to threefold increase in the cGMP-sensitivity of the CNG channels during the subjective night compared with the subjective day. Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II or the MAP kinase Erk, which exhibit circadian rhythms in their kinases activities, causes phase-dependent changes in the apparent affinities of the CNG channels for cGMP, suggesting that the rhythms in enzyme activity drive the rhythm in channel sensitivity. The biochemical event ultimately responsible for circadian channel modulation has not been determined. It will be interesting to determine whether tyrosine phosphorylation or dephosphorylation of the channel plays a role.

Concluding remarks

The proximal light-elicited signal that opens and closes CNG channels in rods and cones is a change in the cytoplasmic cGMP concentration. However, like virtually all ion channels, CNG channels are subject to modulation by a variety of other intracellular signaling systems. The sensitivity of CNG channels to cyclic nucleotides can be altered by Ca^{2+}/calmodulin, transition metals, phospholipid metabolites, changes in phosphorylation state and interactions with membrane proteins such as protein tyrosine kinases. CNG channels contain specific domains that act as receptor sites for each of these intracellular signals and, at least in some cases, photoreceptor cells possess specialized biochemical cascades

for transmitting these modulatory signals to the channels. In addition, extracellular transmitters, such as IGF-I, can activate signaling cascades, leading to modulation of CNG channels. The presence of specialized transmission and reception machinery suggests that modulation of CNG channel sensitivity plays an important physiological role in rods. However, whereas the signaling systems are becoming well understood, the message that they carry is still not clear. Does modulation of CNG channels contribute to changes in light sensitivity during slow forms of adaptation such as diurnal regulation? What role does modulation of CNG channels play in mediating changes in light sensitivity triggered by signals intrinsic to organisms, such as circadian or hormonal signals? The advent of molecular biological techniques, in conjunction with biochemical and physiological studies, will undoubtedly help to define the role of CNG channel modulation in fine-tuning visual sensitivity.

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