

INSIGHTS ON THE REGULATION OF THE cGMP-GATED CHANNEL BY
Ca⁺⁺/CALMODULIN AND ON THE PHOSPHORYLATION OF THE β -SUBUNIT BY A
CKII-LIKE PROTEIN KINASE

by

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ABSTRACT

In vertebrate rod photoreceptors, the cGMP-gated channel plays a crucial role by transducing light-induced changes in cGMP into electrical impulses. Regulation of rod cGMP-gated channels by phosphorylation and Ca^{2+} /calmodulin has been examined here.

The activity of a protein kinase responsible for the phosphorylation of the β - but not the α -subunit was found in the cytosol fraction of purified rod outer segment (ROS) preparations. Phosphorylation occurs mainly on the C-terminal portion of the β -subunit and only on serine residues. cGMP-gated channels purified from ROS, phosphorylated by endogenous ROS kinase(s) and reconstituted into phospholipid vesicles did not exhibit a differential cGMP sensitivity behavior when compared to an unphosphorylated control. In addition, no common protein kinase regulators, second messengers or physiological conditions tested altered the phosphate incorporation into the β -subunit. Taken together with inhibitor mapping experiments and phosphorylation assays using GTP as phosphate donor, these results revealed that the endogenous ROS protein kinase responsible for the β -subunit phosphorylation exhibits properties in common with casein kinase II (CK2). Immunofluorescence microscopy of bovine and rat retina sections labeled with either monoclonal or polyclonal anti-CK2 antibodies have confirmed the presence of a CK2-like protein kinase in ROS. In SDS-polyacrylamide gels, the ROS CK2 migrates slightly slower than its human recombinant homolog and may therefore represent a novel CK2 isoform or a CK2 α variant of the enzyme. Further phosphorylation studies using the human recombinant CK2 demonstrates that the rod cGMP-gated channel is effectively a target for CK2 *in vitro*.

The involvement of ROS calmodulin (CaM) in phototransduction was also studied. ROS calmodulin was found associated with the native channel complex under similar calcium conditions to those occurring in dark-adapted ROS. Furthermore, calmodulin did not co-purify with the native channel when ROS membranes were washed in the absence of calcium but in the presence of EDTA. cGMP-dependent Ca^{2+} -efflux assays and CaM-Sepharose chromatography also confirmed that CaM is the authentic ligand for these CaM-binding sites, since no other calcium binding proteins beside calmodulin have been detected in ROS using these techniques. These observations support a model in which calmodulin is a physiological

modulator of the cGMP-gated channel and this regulation may be important for the adaptation and recovery of the photoreceptors by facilitating the reopening of the channels.

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid assay
BSA	bovine serum albumin
CaM	calmodulin
CBP	calcium binding protein
CHAPS	3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulphonate
CKI	casein kinase I
CK2/CKII	casein kinase II
CNG	cyclic nucleotide-gated
cGMP	guanosine 3',5'-cyclic monophosphate
Br-cGMP	8-bromo guanosine 3',5'-cyclic monophosphate
GTP	guanosine 5'-triphosphate
DTT	dithiothreitol
DRB	5,6-dichloro-1-(β -D-ribofuranosyl)benzimidazole
°C	degree Celcius
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethylether) N,N,N',N'-tetraacetic acid
GARP	glutamic acid-rich protein
GC	guanylate cyclase
GCAP	guanylate cyclase activator protein
HEPES	N-2-Hydroxyethylpiperazine- <i>N</i> -2-ethanesulphonic acid
IC ₅₀	50% inhibitory concentration
Ig	immunoglobulin
K _m	Michaelis constant
M _r	molecular weight

MLCK	myosin light chain kinase
n	Hill coefficient
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PP1	protein phosphatase 1
PP2	protein phosphatase 2
PS	phospho serine
PT	phospho threonine
PY	phospho tyrosine
ROS	rod outer segments
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulfate
Ser	serine
TBS	Tris-buffered saline
TCA	trichloroacetic acid
Thr	threonine
Tris	Tris(hydroxymethyl)aminomethane
Tyr	tyrosine
μ l	microliter
μ g	microgram
V _i	initial velocity
V _{max}	maximum velocity
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 VERTEBRATE EYE

The eye is the organ of photoreception, the process by which light energy from the environment is captured by specialized terminated cells of the retina, the rods and cones. The nerve action potentials resulting from light excitation are subsequently relayed to the optic nerve and then to the cerebral cortex in the brain where the information is finally processed to produce an image. Other structures of the eye such as the cornea, the lens, and the iris are secondary to the process of photoreception, although they are essential components of the system necessary for focusing and forwarding the light onto the retina (Fig. 1). The choroid, the aqueous outflow system and the lacrimal apparatus also play alternate roles by nourishing and supporting various tissues of the eye (Forrester *et al.*, 1996).

1.2 RETINA

Visual perception is initiated by absorption of photons by the retina, a highly specialized extension of the central nervous system. The neural retina consists of six different types of neurons: rod and cone photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells (Fig. 2). After light passes through the lens system of the eye and then through the vitreous humor, it enters the retina from the inside. Photons of light will penetrate various cell layers of the retina before reaching the photoreceptor cells. The phototransduction process, or the conversion of light energy to an electric potential, takes place in the photoreceptor cells where photons are first absorbed. The photoreceptor cells transmit the visual information to the ganglion cells in the form of an electric impulse that eventually enter the visual cortex in the brain.

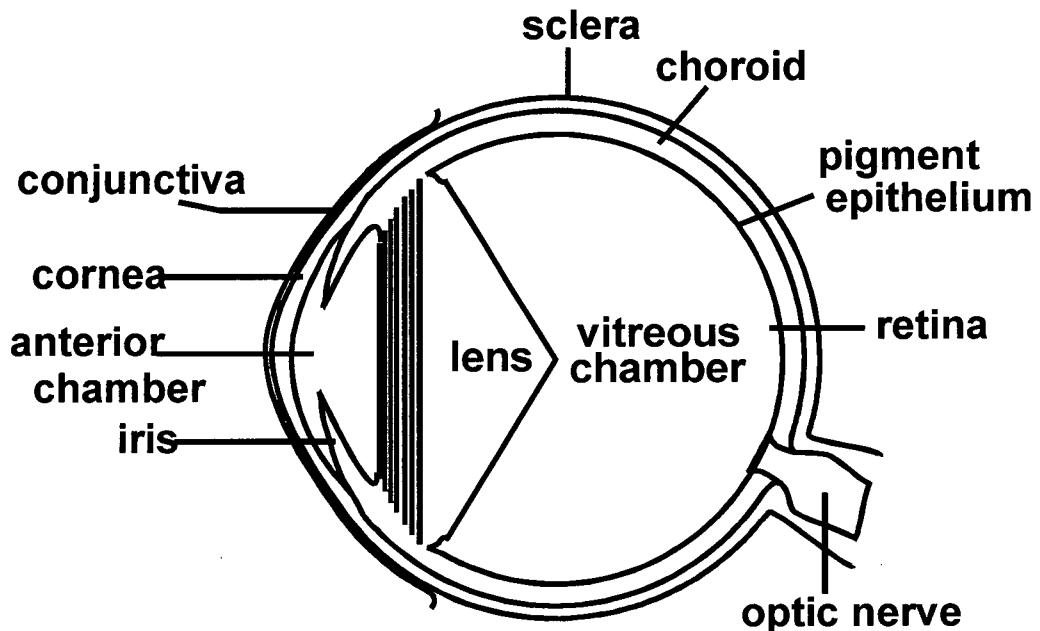


Fig. 1. **Cross-section of the vertebrate eye.** The eyeball is surrounded by the sclera, a cartilaginous tissue contiguous with the cornea. In the frontal portion of the eye, above the cornea, lies the conjunctiva. Beyond the cornea, light passes through the anterior chamber of the eye to the lens. The lens is responsible for focusing images onto the retina, where light is first absorbed. The iris regulates the amount of light entering the eye. The vitreous and anterior chamber contain the vitreous humor, important for maintaining the shape of the eye and for transporting nutrients, oxygen and cellular waste to neighbouring tissues. Interior to the sclera at the rear portion of the eye is the choroid layer and the retina. The choroid provides nutrients to the retina and retinal pigment epithelium (RPE). The retina contains the photoreceptor cells and other neurons (diagram adapted from Dosé, 1995).

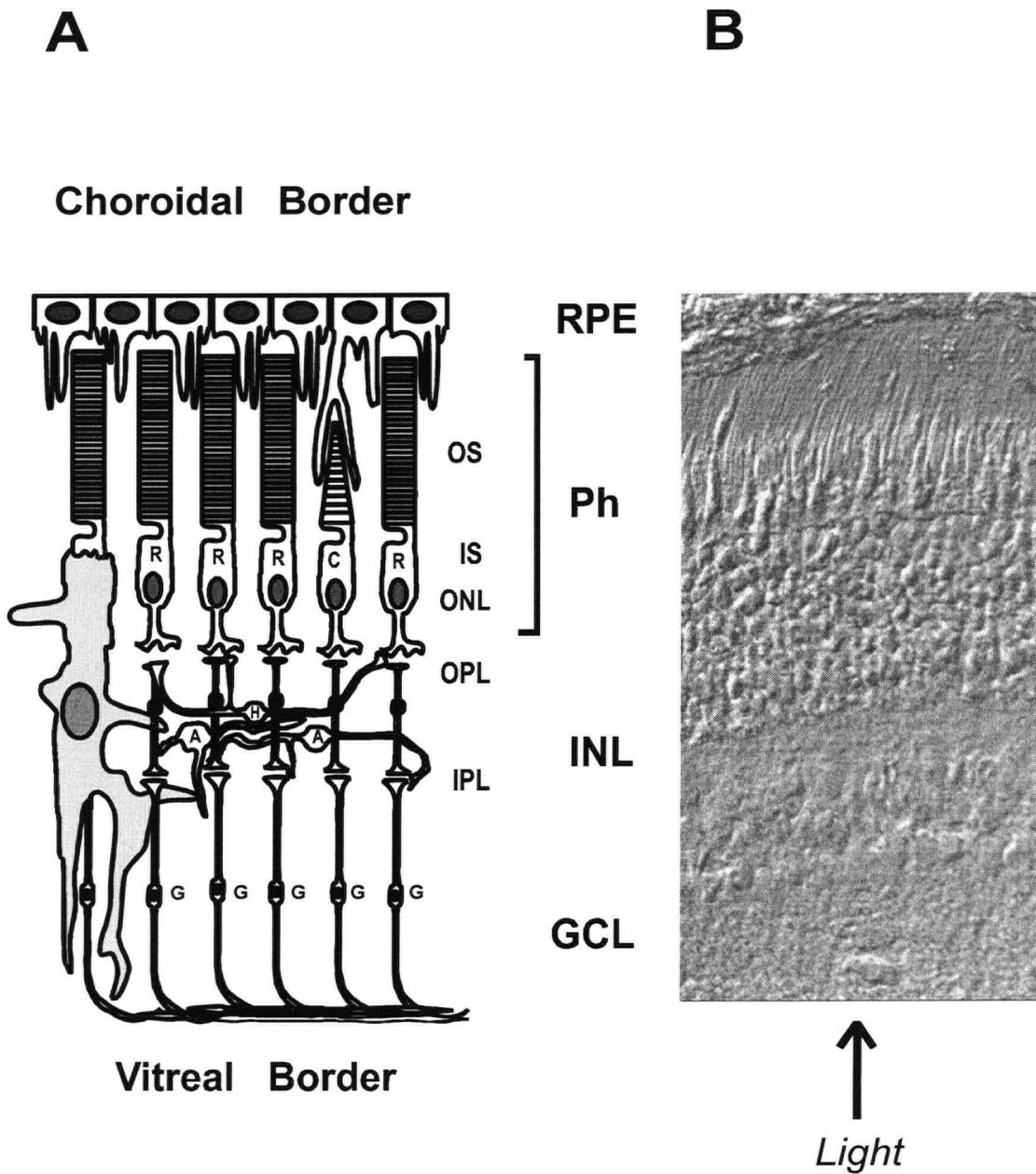


Fig. 2. Organization of the neural retina. Schematic diagram (*A*) and differential interference contrast micrograph (*B*) showing layers of a vertebrate retina. **RPE**, retinal pigment epithelium; **Ph**, photoreceptor cells; **OS**, outer segments of cone (C) and rod (R) photoreceptor cells; **IS**, photoreceptor inner segment; **ONL**, outer nuclear layer containing the cell bodies of photoreceptor cells; **INL**, inner nuclear layer containing cell bodies of bipolar, horizontal (H) and amacrine cells (A); **OPL**, outer plexiform layer containing the neural connections between photoreceptors and bipolar and horizontal cells; **IPL**, inner plexiform layer containing synaptic connections among bipolar, amacrine and ganglion cells (G). **GCL**, ganglion cell layer. The arrow indicates the direction of light entering the eye (diagram adapted from Dosé, 1995 and Hsu, 1993).

1.3 PHOTORECEPTOR CELLS

Rods and cones are the photoreceptor cells that carry out phototransduction in vertebrates. Rods are 2-5 μm in diameter, 100-120 μm in length and are primarily distributed in the peripheral region of the retina. They are responsible for black-and-white vision under dim light and account for the majority of the photoreceptor cells (95%) in the retina of most vertebrates. Cones are less numerous and are often concentrated in the central (macular) region of the retina where they serve as photoreceptors for color vision. Primate cone photoreceptors are slender in the macula with diameter of only 1-1.5 μm ; their diameter reaches 5-8 μm in the peripheral regions of the retina. The cones are generally shorter than the rods in the peripheral part of the retina but can reach up to 60-75 μm in the macular region (Forrester *et al.*, 1996; Shichi, 1983).

Rods and cones have four major functional segments: the outer segment, the inner segment, the cell body and the synaptic terminus. The outer segment of rods and cones is a specialized organelle that carries out phototransduction. The outer segment is physically attached to the rest of the cell at the inner segment by a thin cilium junction. Various metabolites, proteins and lipids generated in the inner segment, are transported to the outer segment through this connecting cilium. The inner segment contains subcellular organelles (endoplasmic reticulum, mitochondria, Golgi apparatus, ribosomes and nucleus) essential for the function of the photoreceptors. The synaptic terminal functions in the transmission of electrical signals from the photoreceptors to the horizontal and bipolar cells via the release of neurotransmitters (Fig. 3).

1.4 ROD PHOTORECEPTOR OUTER SEGMENTS

Photoreceptor outer segments are composed of a highly ordered arrangement of hundreds of flattened disks (up to 1000 per cell). Although cone outer segment disks are a continuation of the plasma membrane, rod outer segment disks are detached from the plasma membrane with the exception of the disks at the basal region. Here new disks are formed from evagination of the plasma membrane (Forrester *et al.*, 1996). Newly formed disks move to the

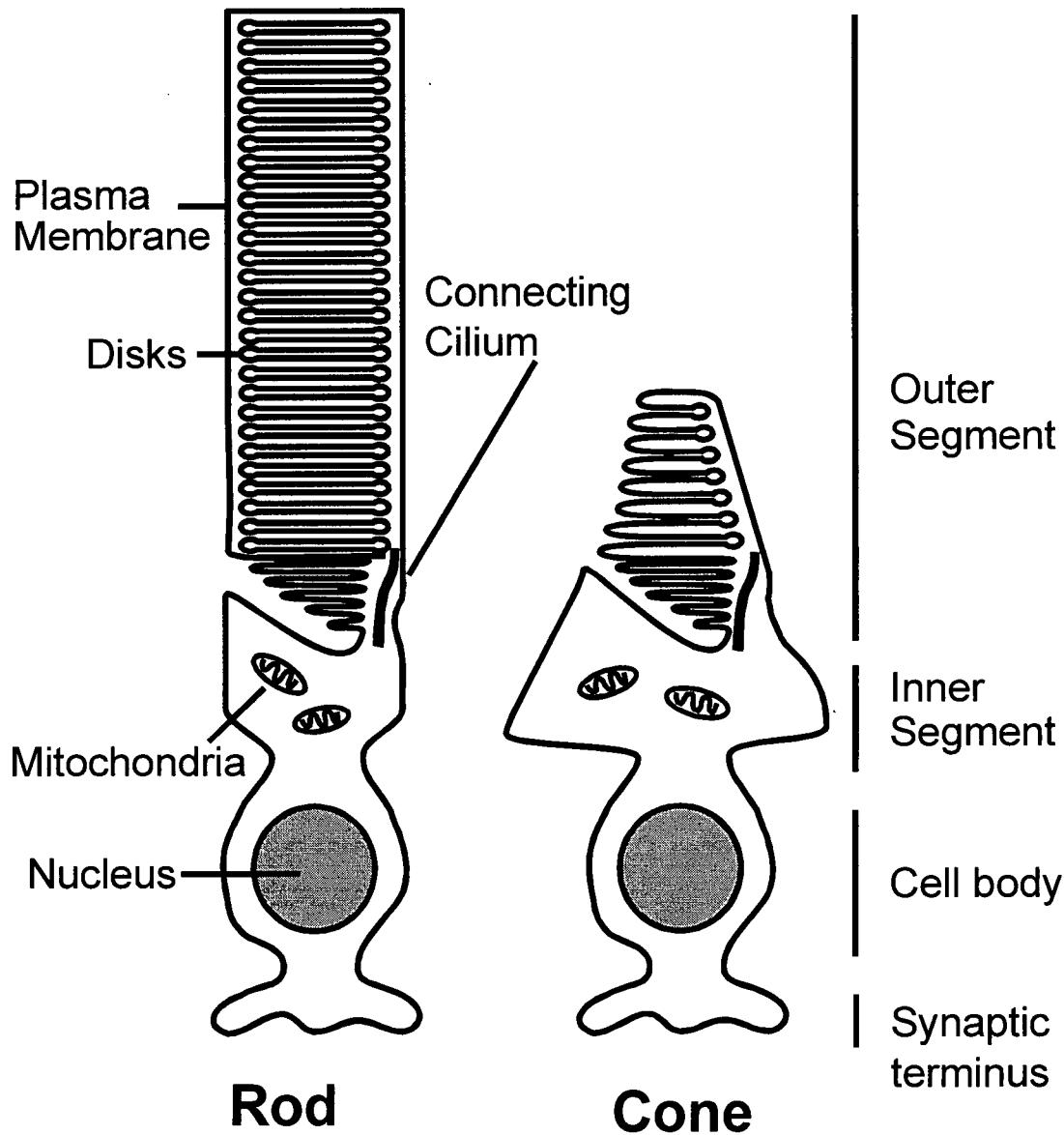


Fig. 3. Schematic diagram of rod and cone photoreceptor cells. Rods and cones are composed of four major functional segments. The outer segment, the inner segment, the cell body and the synaptic terminus. The outer segment of rods and cones is a specialized organelle that carries out phototransduction. The outer segment is physically attached to the rest of the cell at the inner segment by a connecting cilium (diagram adapted from Dosé, 1995).

tips of the outer segment over the course of ten days before being shed and phagocytosed by the RPE cells. In rods, the protein composition of the disk membrane is quite different than that of the plasma membrane. With the exception of the photoreceptor rhodopsin, ROS membrane proteins are sorted to either the disks or the plasma membrane and this polarization is of crucial importance for the proper function of the photoreceptor cells. A method for separating the disk membrane and plasma membrane using Ricin-Gold-Dextran affinity density perturbation has been used to compare the protein composition between these two membranes (Molday and Molday, 1987). A brief description of major ROS membrane proteins (disks and plasma membrane) and soluble proteins are listed in the table I.

1.5 PHOTOTRANSDUCTION

Phototransduction, the process by which light energy is transformed into an electrical impulse, occurs in rod and cone outer segments. The energy stored in a single photon of light, the smallest possible quantal unit of light energy, can be detected by the photoreceptors and cause a measurable receptor potential in a rod of nearly 1 millivolt (Guyton and Hall, 1996). This remarkable sensitivity is due to the extensive amplification (of about a million fold) of the molecular cascade leading to the closure of cGMP-gated cation channels and hyperpolarization of the cells. In rod photoreceptors, three different phases constitute the phototransduction process: the dark current, the visual excitation, and the visual recovery (Reviews: Lagnado and Baylor, 1992; Pugh and Lamb, 1993; Koutalos and Yau, 1996; Yau and Baylor, 1989; Kaupp, 1995).

1.5.1 Dark current

The resting potential of most neurons and sensory receptors remains constant at -60 to -70 mV and mainly dominated by the K^+ distribution across the plasma membrane. In rod photoreceptors, the resting potential in the dark is kept steady around -30 to -40 mV. The difference in the conductivity observed in these two populations of neurons is explained by constant leakage of Na^+ in dark-adapted ROS (Fig.4). Under the same conditions, Na ions

Table I. Localization and function of the principal proteins found in ROS^a

Protein	M _r (kDa)	Function
Disk		
Rhodopsin	38	Photoreceptor protein
ABCR	220	Transport of retinal ^b
Peripherin/rds	35 (39)	Structure and maintenance of disk rims ^c
ROM-1	33 (37)	Structure and maintenance of disk rims
Retinol dehydrogenase	37	Conversion of all- <i>trans</i> retinal to retinol
Plasma membrane		
cGMP-gated channel		Non-selective cation channel allowing the entry of Na ⁺ / Ca ²⁺ in ROS upon cGMP binding
α-subunit	63 (79.6)	
β-subunit	240	Part of the oligomeric channel. Contains several regulatory components
Na ⁺ /K ⁺ -Ca ²⁺ exchanger	230 (130)	Exchange Ca ²⁺ /K ⁺ for extracellular Na ⁺
Rhodopsin	38	Photoreceptor protein
GLUT-1	50	Facilitative glucose transport in ROS
Soluble and membrane-associated		
Phosphodiesterase α, β & γ	11, 84, 88	Hydrolyses cGMP to 5'-GMP
Arrestin	48	Binds phosphorylated rhodopsin
Transducin α, β & γ	39, 37, 8	G-protein. Activates PDE
Phosducin	33	Binds and regulate βγ-transducin
Calmodulin	20 (16.5)	CBP. Regulates cGMP-gated channels
Phosphatase 2A	38	Removes phosphate groups on rhodopsin
Protein kinase C	85	Phosphorylates rhodopsin
Rhodopsin kinase	68	Phosphorylates rhodopsin
Recoverin	23.3	Inhibition of rhodopsin phosphorylation
Guanylate cyclase	112	Synthesize cGMP from GTP

^aTable adapted from R.S. Molday (Molday and Molday, 1993)

^bHypothesized function (Sun *et al.*, 1999)

^cHypothesized function (Connell and Molday, 1990; Goldberg *et al.*, 1998)

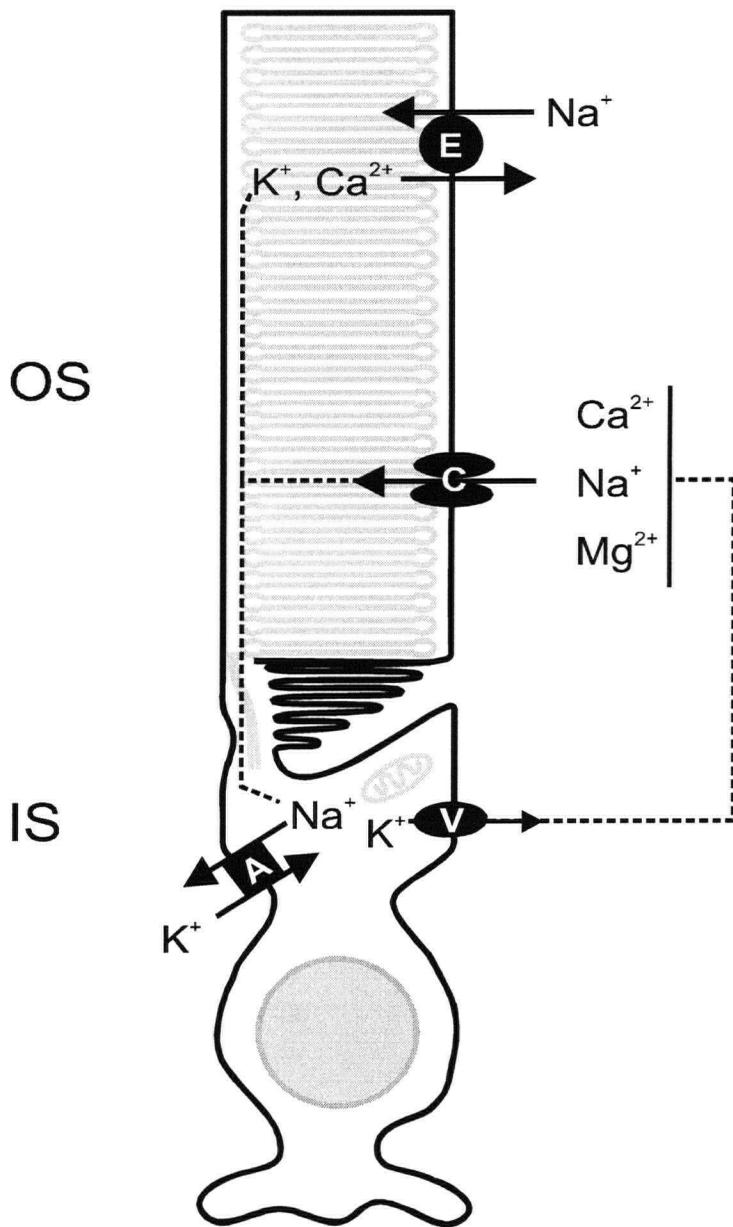


Fig. 4. Dark current in rod photoreceptors. The dark current consist of an inward current carried by Na^+ , Ca^{2+} and Mg^{2+} and an outward current carried by K^+ from a voltage-gated K^+ channel (V). Na^+ , Ca^{2+} and Mg^{2+} enter the outer segment (OS) through a cGMP-gated cation channel (C). Ca^{2+} entering the outer segment is extruded by a $\text{Na}^+/\text{Ca}^{2+}$ - K^+ exchanger (E) and Na^+ is pumped out of the photoreceptor by a Na^+/K^+ ATPase (A) in the inner segment (IS). Potassium ions entering the photoreceptor cell at the inner segment are required to maintain high intracellular K^+ levels and perpetuation of the dark current in rods. The extrusion mechanism of Mg^{2+} is still unknown (Diagram adapted from Hsu, 1993).

entering the outer segment are extruded from the inner segment, creating a steady-state current (dark current) of 25-71 pA (Baylor *et al.*, 1979a, b; Hagins *et al.*, 1970, and Stryer, 1986). The cGMP-gated channel is responsible for the entry of calcium, sodium and magnesium, creating an inward current in ROS (Cook *et al*, 1987, 1989; Nakatani and Yau, 1988a; Hagins *et al.*, 1970; Yau and Nakatani, 1985). The Ca^{2+} concentration in the outer segment is kept constant in the dark by the Na^+/K^+ - Ca^{2+} exchanger (Yau and Nakatani, 1984; Schnetkamp, 1986; Cook and Kaupp, 1988). This protein catalyzes the transport of one calcium ion against its electrochemical gradient by coupling it to the efflux of one K^+ and the influx of four Na^+ (Schnetkamp *et al.*, 1989; Cervetto *et al.*, 1989). The balancing outward current is carried by K^+ from the inner segment. Na^+ and K^+ gradients across the plasma membrane are maintained by an active Na^+/K^+ ATPase (Sillmann *et al.*, 1969; Hagins *et al.*, 1970; Stirling and Lee, 1980). Potassium ions are then extruded by voltage-gated K^+ channels (Fig 4.).

1.5.2 Visual excitation

During the process of visual excitation, light modulates the membrane potential by affecting the movement of ions across the plasma membrane. The molecular pathway allowing the energy of one single photon of light to be amplified and transformed into an electrical impulse is a G protein-mediated event similar to signal transduction processes that occur in cells responding to hormones such as glucagon and adrenaline. Like other G protein-mediated pathways, the signal coming from the photon is relayed by multiple proteins acting in a cascade and causing rapid and astronomical amplification of the original signal (Yau, 1994; Pugh and Lamb, 1993). All the steps, from photon absorption to hyperpolarization, as well as the proteins involved in the molecular amplification cascade and their function are described below (Fig. 5).

Absorption of photons by rhodopsin

In rods, the first event of the visual excitation is the absorption of a photon by the photoreceptor rhodopsin. Rhodopsin consists of the membrane protein opsin and a covalently bound prosthetic group, the carotenoid pigment 11-*cis*-retinal. Upon light absorption, the

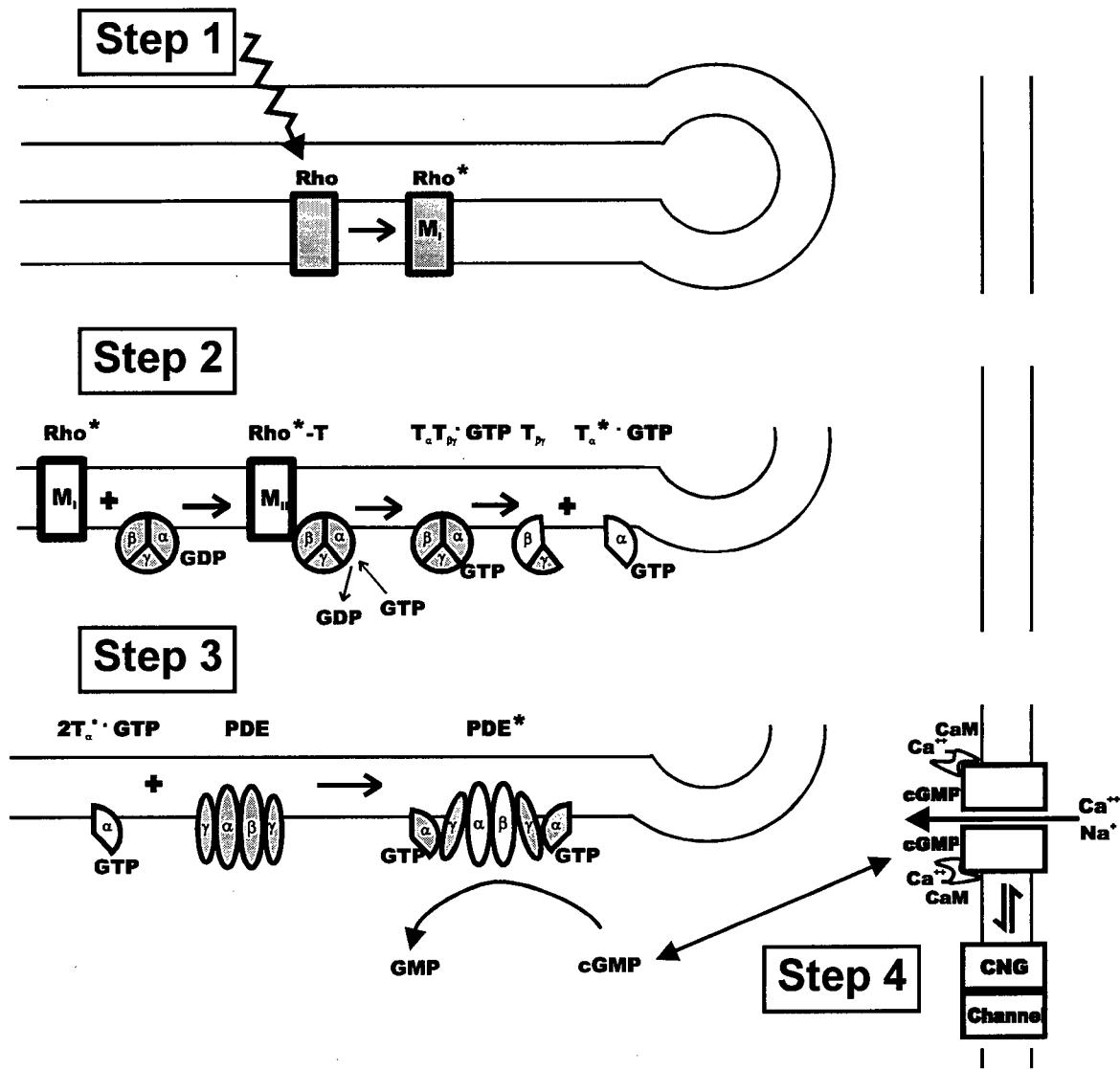


Fig. 5. Visual excitation in rods. This diagram illustrates the molecular mechanism of phototransduction. Step 1, light activates rhodopsin (rho); Step 2, activated rhodopsin (rho* or M_{II}) activates transducin (T) by catalyzing the exchange of GDP for GTP on the α-subunit of transducin, causing this subunit to dissociate from the βγ subunits; Step 3, the α-subunit of activated transducin then activates the cGMP-phosphodiesterase (PDE); Step 4, hydrolysis of cGMP to GMP by PDE results in the closure of the cGMP-gated channel, preventing cations from entering the outer segment and causing the cell to hyperpolarize. For more details, see section 1.5.2 (Diagram adapted from Kim, 1998).

excited retinal molecule isomerizes rapidly to an all-*trans* form through a series of unstable intermediates as the molecule readjusts itself within the opsin (Birge, 1990). Photobleaching of rhodopsin to metarhodopsin II occurs in 1 msec, causing the cytoplasmic surface of the protein to be catalytically active (Baylor, 1996). In this state, rhodopsin activates the GTP-binding protein transducin (Emeis *et al.*, 1982; Bennett *et al.*, 1982). The retinal moiety is then released from the opsin in the last step.

Activation of transducin by metarhodopsin II

The next step in the visual cascade is the activation of the G protein transducin by activated rhodopsin (metarhodopsin II). Transducin is an oligomeric protein composed of three subunits termed α , β and γ (Table I). Upon light absorption, metarhodopsin II catalyses the exchange of bound GDP for GTP on the α -subunit of transducin in the first amplification step of phototransduction (Fung and Stryer, 1980; Kühn *et al.*, 1981; Fung, 1983). Within a fraction of a second, one molecule of activated rhodopsin can activate nearly 500 transducin molecules (Fung *et al.*, 1981). The exchange of GDP for GTP on the α -subunit of transducin causes the α -subunit to dissociate from the $\beta\gamma$ subunits. The α -subunit interacts with cGMP phosphodiesterase (PDE) in the next step of phototransduction.

Activation of PDE by the α -subunit of transducin

The second amplification step in phototransduction is carried out by PDE, a multimeric soluble protein responsible for the hydrolysis of cGMP to 5'-GMP. The inactive form of PDE is composed of four subunits; two catalytic (α and β) and two regulatory (γ) subunits (Table I). The γ -subunit is inhibitory and its association to the catalytic $\alpha\beta$ -subunits inactivates the catalytic function of PDE (Fung and Griswold-Prenner, 1989; Wensel and Stryer, 1990; Yamazaki *et al.*, 1990). The dissociated α -subunit of transducin activates PDE by binding the γ -subunit, thus changing the interaction between this subunit and the $\alpha\beta$ -subunits (Arshavsky and Bownds, 1992; Artemyev *et al.*, 1992; Antonny *et al.*, 1993). One activated molecule of PDE can hydrolyse around 1,000 cGMP molecules per second (Wheeler and Bitensky, 1977; Lieberman and Pugh, 1980).

Closure of cGMP-gated channels and hyperpolarization or rods

A relative high concentration of cyclic GMP maintains a significant number of cGMP-gated channels open in the dark. Under these conditions, the cGMP concentration inside ROS remains stable ~60 μ M. However, more than 85% of the total ROS cGMP is bound to the non-catalytic cGMP-binding site of PDE, leaving 4-10 μ M free cGMP available to bind cGMP-gated channels in the dark (Yamazaki *et al.*, 1980; Charbonneau *et al.*, 1990). It is estimated that at this cGMP concentration, only 1-5% of the total ROS cGMP-gated channels are kept open in the dark (Nakatani and Yau, 1988b). Upon light absorption, decreased levels in cGMP due to hydrolysis by PDE will result in the closing of the cation channels (Koch and Kaupp, 1985; Fesenko *et al.*, 1985). Closure of the channels will stop the influx of Na^+ , Ca^{2+} and Mg^{2+} , decreasing the cation permeability of the ROS plasma membrane and subsequently lowering the inward current. The constant extrusion of potassium in the inner segment by the voltage-gated K^+ channels will cause the rod photoreceptor cell to hyperpolarize. The greater the amount of light energy striking the rods, the greater the degree of hyperpolarization. This characteristic is extremely important since it allows the eye to discriminate between a wide range of light intensities. At maximum light intensity, when approximately ten rhodopsin molecules are activated per rod cell, the membrane potential approaches -70 to -80 mV (Penn and Hagins, 1972; Baylor *et al.*, 1979a,b; Lamb *et al.*, 1981)

Release of neurotransmitters

Dark-adapted rods constantly release neurotransmitters at the synaptic terminus. Hyperpolarization of rods decreases the amount of neurotransmitters released by a yet unknown mechanism. In cone photoreceptors, a potential mechanism of neurotransmitter release has been linked to the calcium-entry pathway. In photoreceptor inner segments, Ca^{2+} /calmodulin induces the production of nitric oxide (NO) by activating a nitric oxide synthase (Koch, 1994). The NO produced can activate a soluble guanylate cyclase, leading to the production of cGMP in the inner segment. The influx of calcium resulting from the subsequent activation of cGMP-gated channels found in the inner segment has been proposed to modulate exocytosis of neurotransmitters vesicles (Kurennny *et al.*, 1994; Rieke and Schwartz, 1994). The neurotransmitters used for synaptic transmission in the retina have not

all been identified yet. However, it is known that cones and rods release the inhibitory neurotransmitter glutamate at their synapse. A decrease in the release of inhibitory neurotransmitters evokes an excitatory response in bipolar cells. The response is then transmitted to ganglion cells and eventually to the brain (Fig. 2. refs: Guyton and Hall, 1996).

1.5.3 Visual recovery

The mechanisms of termination of the visual excitation cascade and the restoration of the dark current are currently not as well understood as the mechanisms of transmission of the visual signals in rods. However, considerable effort has been made during the last decade toward understanding how bleached ROS return to their resting state (Lagnado and Baylor, 1992; Palczewski, 1994). Some of the steps required to terminate the phototransduction process and to restore the resting potential are described below (Fig. 6).

QUENCHING OF THE PHOTOTRANSDUCTION CASCADE

Rhodopsin inactivation and regeneration

The quenching of rhodopsin catalytic activity constitutes the first step of the visual recovery, leading to the restoration of the dark state. Although the quenching of the visual cascade is a rapid and precise process, the decay of metarhodopsin II is quite slow and occurs in several minutes. The inactivation of rhodopsin, therefore, is essential to terminate the activation of transducin by preventing metarhodopsin II from reinitiating the signal transduction cascade. Rhodopsin is inactivated by rhodopsin kinase-mediated phosphorylation (Kühn and Dreyer, 1972; Miller *et al.*, 1986) and by subsequent binding of the soluble protein arrestin to phosphorylated metarhodopsin II. This prevents further activation of transducin (Wilden and Kühn, 1982; Kühn *et al.*, 1984; Zuckerman *et al.*, 1985). In the next step of the quenching process, all-*trans* retinal is released from the opsin and reduced to all-trans retinol by the enzyme retinol dehydrogenase (Wald, 1968; Lion *et al.*, 1975; Ishiguro *et al.*, 1991). Retinol can no longer bind to opsin, but is transported to the retinal pigment epithelium (RPE) by an interphotoreceptor retinoid binding protein (Bok and Heller, 1976; Saari and Bredberg, 1988; Deigner *et al.*, 1989). Opsin is dephosphorylated by protein phosphatase 2 (PP2) and is re-used to regenerate rhodopsin (Fowles *et al.*, 1989; Palczewski *et al.*, 1989). RPE cells

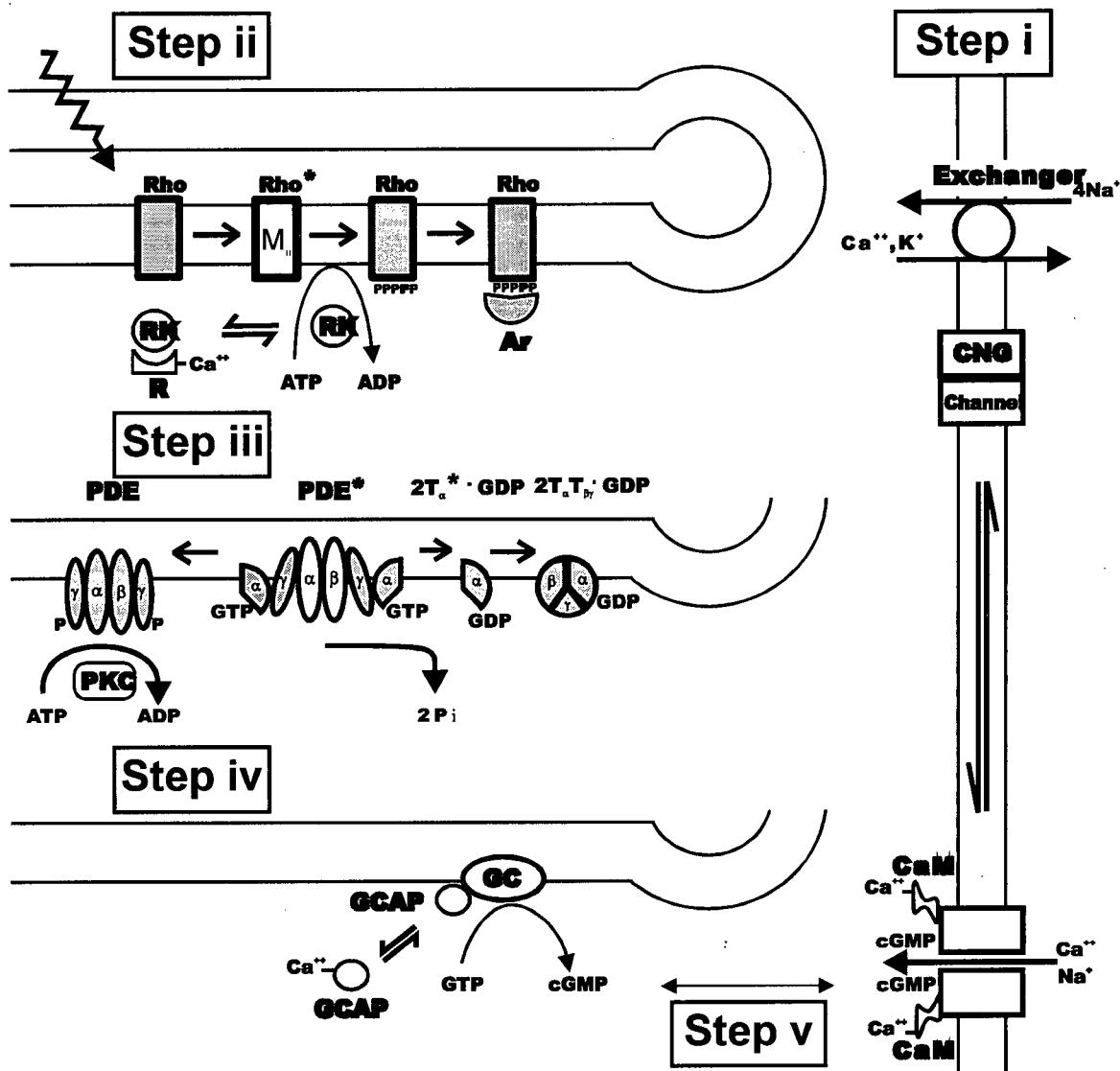


Fig. 6. Visual recovery in rods. This diagram illustrates the visual recovery pathway. Step i, a decrease in the intracellular Ca^{2+} concentration due to a constant extrusion by the $\text{Na}^+/\text{Ca}^{2+}-\text{K}^+$ exchanger results in the dissociation of recoverin from rhodopsin kinase and dissociation of calmodulin from the cGMP-gated channel. This causes the channel to respond to smaller levels of cGMP than those required to keep the channel open in the dark; Step ii, rhodopsin is inactivated by rhodopsin kinase (RK) and arrestin (Ar); Step iii, transducin α -subunit is inactivated by GTP hydrolysis and reassociates with transducin $\beta\gamma$ subunits. PDE is inactivated after dissociation from transducin; Step iv, cyclic GMP is resynthesized from GMP by monophosphate kinase, nucleoside diphosphate kinase and guanylate cyclase (GC); Step v, newly synthesized cGMP causes the cGMP-gated channel to re-open, allowing the influx of cations in the outer segments. Calcium subsequently inactivates guanylate cyclase through a guanylate cyclase-activating protein (GCAP) and allows calmodulin to reassociate with the channel. For more details, see section 1.5.3 (Diagram adapted from Kim, 1998).

contain all the enzymes necessary for the conversion of all-*trans* retinol to 11-*cis* retinal. The 11-*cis* retinal is then transported back to the rod photoreceptors by the interphotoreceptor retinoid binding protein where it binds to opsin to regenerate rhodopsin.

Inhibition of rhodopsin phosphorylation by recoverin

A mechanism involving the Ca^{2+} -entry pathway and the calcium-binding protein (CBP) recoverin has been recently proposed for a more efficient termination of the visual cascade at low calcium levels (Müller and Koch, 1998). Pioneer studies by Kawamura (1993) indicated that rhodopsin kinase activity is altered by recoverin. Recoverin is a soluble and/or membrane associated CBP found in ROS, RIS and even in bipolar cells (Euler and Wässle, 1995; Ames *et al.*, 1996). In dark-adapted ROS (high Ca^{2+} concentration), recoverin forms a complex with rhodopsin kinase, preventing further phosphorylation of rhodopsin. The calcium levels in ROS decrease during visual excitation, leading to the dissociation of recoverin from rhodopsin kinase. This will cause an increase in the phosphorylation of rhodopsin and consequently, a more efficient shut down of the visual excitation cascade (Gorodovikova *et al.*, 1994; Calvert *et al.*, 1995; Chen *et al.*, 1995). In a more recent study based on the results of *in vitro* experiments, Otto-Bruc and co-workers (1998) observed, however, that phosphorylation of rhodopsin is insensitive to regulation by Ca^{2+} and recoverin.

Transducin inactivation

The α -subunit of transducin possess a slow intrinsic GTPase activity (Kühn, 1980; Kühn *et al.*, 1981) that is accelerated by the binding to the γ -subunit of PDE and the membrane-bound protein RGS-9 (Angleson *et al.*, 1993; He *et al.*, 1998; Makino *et al.*, 1999). The catalytic activity of transducin is responsible for the hydrolysis of the bound GTP to GDP and the subsequent inactivation of the α -subunit of transducin. This increase in transducin GTPase activity by the PDE γ -subunits can be partially suppressed by cGMP, thus regulating with precision the concentration of this second messenger inside the ROS (Arshavsky and Bownds, 1992). The inactivated α -subunit of transducin now bound to GDP can reassociate with the $\beta\gamma$ -subunits.

Deactivation of PDE

Inhibition of the catalytic activity of PDE is thought to be coupled with the inactivation of transducin since this last event allows the γ -subunits to inhibit the catalytic $\alpha\beta$ -subunits of PDE (Sitaramayya and Lieberman, 1983).

cGMP-GATED CHANNELS REOPENING AND Ca^{2+} FEEDBACK PATHWAY

In dark-adapted rods, the open configuration of cGMP-gated channels is maintained by cGMP. During photoexcitation, reduction of the pool of cGMP caused by cGMP hydrolysis will result in dissociation of cGMP from the channel. This induces a conformational change leading to closure of the cation channel. The reopening of the cGMP-gated channels in the dark is highly dependent on the inhibition of PDE and the catalytic activity of guanylate cyclase. Channel reopening is also dependent on the calcium entry pathway. Ca^{2+} acts on the guanylate cyclase activator proteins (GCAPs) to modulate the activity of guanylate cyclase (Palczewski *et al.*, 1994; Dizhoor *et al.*, 1995; Frins *et al.*, 1996) and calmodulin, a ubiquitous Ca^{2+} binding protein that has been shown to bind and alter the sensitivity of the channel for cGMP (Hsu and Molday, 1993; Hsu and Molday, 1994; Chen *et al.*, 1994).

cGMP resynthesis and facilitative channel reopening by GC

Cyclic GMP metabolism requires the action of three enzymes in ROS. After the hydrolysis of cGMP to 5'-GMP by PDE, GDP is synthesized from GMP by guanosine monophosphate kinase. GDP is subsequently converted to GTP by a nucleoside diphosphate kinase. GTP is the substrate of guanylate cyclase (GC) for cGMP resynthesis. In dark-adapted ROS, the concentration of free cGMP is approximately 4-10 μM and the calcium concentration is around 500 nM. Under these conditions, the activity of guanylate cyclase is low. It is currently known that light regulates the activity of GC through the calcium entry pathway and the action of two calcium-binding proteins, the guanylate cyclase activator proteins GCAP-1 and GCAP-2. It has been suggested that one or both GCAPs stimulate guanylate cyclase at low calcium concentration (50-400 nM) (Gorczyca *et al.*, 1995; Palczewski *et al.*, 1994; Dizhoor *et al.*, 1995; Frins *et al.*, 1996; Olshevskaya *et al.*, 1997; Müller and Koch, 1998).

Channel reopening by Ca^{2+} /calmodulin

Ca^{2+} /calmodulin binds the β -subunit of the rod cGMP-gated channel *in vitro* and causes a 1.5-2 fold decrease in the ligand sensitivity (Hsu and Molday, 1993). It has been proposed that the binding of calmodulin plays a role in the visual recovery by increasing the cation influx at low cGMP levels up to 6-fold. This allows the cGMP-gated channels to respond to low level of cGMP, similar to those occurring after the visual excitation. During photoactivation the calcium concentration decreases rapidly below 50 nM, causing a negative feedback mainly responsible for the initiation of light adaptation in ROS. Below this Ca^{2+} concentration, calmodulin dissociates from the cGMP-gated channel. Inhibition by calmodulin is now relieved and the channel can respond to lower levels of cGMP than that required to keep the channel open in the dark. Reopening of the channel will restore Ca^{2+} levels and allow Ca^{2+} /calmodulin to reassociate with the cGMP-gated channel (Molday, 1996). The olfactory cyclic nucleotide gated channel has also been shown to interact with $\text{Ca}^{2+}/\text{CaM}$ (Chen and Yau, 1994). In this case, the interaction results in a 20-fold decrease in ligand sensitivity, supporting a model in which calmodulin is a major contributor of adaptation in olfactory neurons. Because the effect of $\text{Ca}^{2+}/\text{CaM}$ on the ligand sensitivity of the rod cyclic nucleotide gated (CNG) channel is so low, it is still unresolved whether calmodulin modulation of the channels contributes to the visual recovery under physiological conditions (see section 1.6.3).

1.6 MOLECULAR PROPERTIES OF CNG CHANNELS

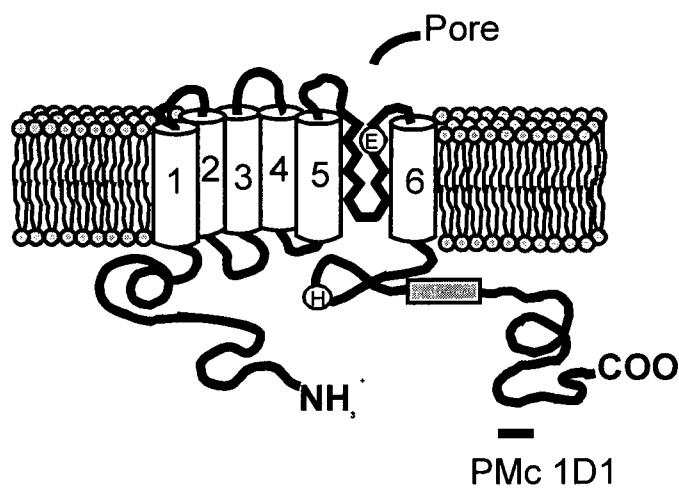
Cyclic nucleotide gated channels are non-selective cation channels that can be directly activated by cyclic nucleotides cGMP and/or cAMP. CNG channels were first discovered in photoreceptor cells (Fesenko *et al.*, 1985; Haynes and Yau, 1985) and olfactory sensory neurons (Nakamura and Gold, 1987), but are now found in a wide variety of tissues and cell types among vertebrates and invertebrates. During the last decade, more than a dozen genes encoding different CNG channels and channel subtypes have been identified and cloned. CNG channels have been detected in the central nervous system (ganglion cells, vomeronasal receptor neurons (Leinders-Zufall *et al.*, 1995; Bradley *et al.*, 1997; Taniguchi *et al.*, 1996)) and in non-neuronal tissue (heart, testis, kidney, keratinocytes, and muscle (Hundal *et al.*,

1993; Biel *et al.*, 1993, 1994; Ahmad *et al.*, 1992; Weyand *et al.*, 1994; Oda *et al.*, 1996; Santy and Guidotti, 1996). The exact function of CNG channels in most of these cell types is still under investigation, but it is believed that they play a central role by producing a Ca^{2+} signal that links cyclic nucleotide metabolism to Ca^{2+} -regulated pathways in the cell. Taking into account the broad variety of tissues expressing these channels and the different cell requirements dictated by the environment, it is not surprising to observe striking differences in ion selectivity and sensitivity as well as ligand specificity among channels expressed in different cells. The rod cGMP-gated channel, for example, is less sensitive to cyclic nucleotides than the olfactory channel and has a different ligand specificity with a preference for cGMP over cAMP (25-40 higher affinity) (Kaupp, 1995). Although CNG channels belong to the superfamily of ligand-gated ion channels on the basis of their ability to bind cyclic nucleotides, they are structurally more related to the family of voltage-gated channels. The structure, molecular function and regulation of the rod cGMP-gated channel will be discussed in detail in the following sections.

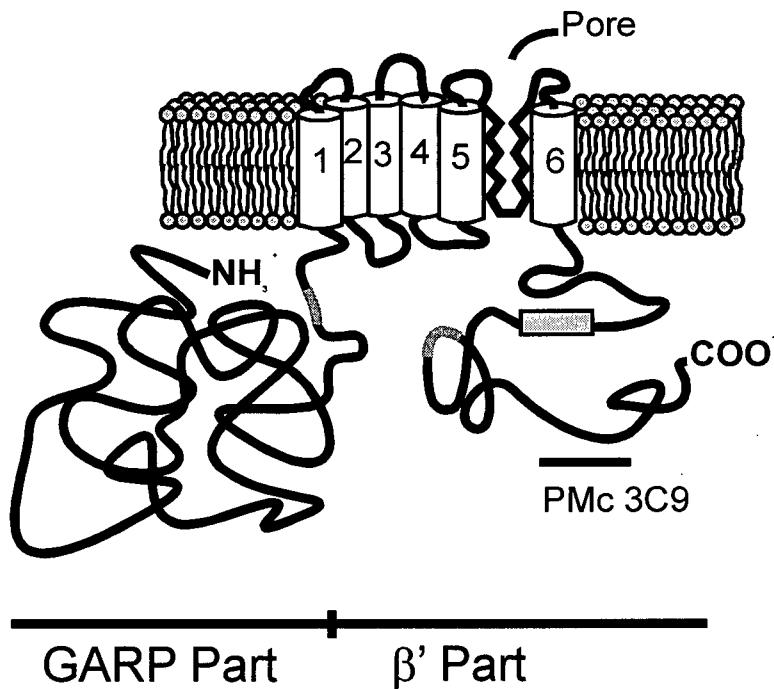
1.6.1 Structure of rod cGMP-gated channels

The rod cGMP-gated channel is a hetero-oligomer composed of α - (Cook *et al.*, 1987; Kaupp *et al.*, 1989) and β -subunits (Chen *et al.*, 1993) assembled together in a yet unknown stoichiometry. It is however suggested that the subunits form a heterotetrameric structure, similar to K^+ voltage-gated channels (Liu *et al.*, 1996; Varnum and Zagotta, 1996). The α -subunit has six membrane-spanning segments (S1-S6) and a pore-forming region between S5 and S6 (Fig. 7.). The cGMP-binding domain is located between the S6 segment and the carboxyl terminus on the cytoplasmic side of the membrane. The α -subunit of the cGMP-gated channel complex also harbors a glutamate residue in the pore region that is responsible for divalent cation binding (Ca^{2+} and Mg^{2+}). Divalent cation binding to the pore reduces the permeability of the channel for monovalent ions and influences the ion selectivity and gating (Root and MacKinnon, 1993; Eismann *et al.*, 1994; Sesti *et al.*, 1995). A histidine residue (H420) in the C-terminal portion of the rod CNG channel α -subunit has been shown to play a major role in the activation by divalent transition metal cations (Ni^{2+} , Zn^{2+} , Cd^{2+} , Co^{2+} and Mn^{2+}) and the subsequent increase in cGMP sensitivity of the rod cGMP-gated channel

A



B



GARP Part β' Part

Fig. 7. Structural model for the rod cGMP-gated channel. The rod cGMP-gated channel is a hetero-oligomer composed of α - (A) and β -subunits (B) assembled together in an unknown stoichiometry. 1-6 denotes six putative transmembrane α -helices. H denotes a histidine residue (H420) that can modulate the gating properties of the channel through the interaction with transition metal divalent cation. E shows the presence of a glutamic acid residue (glu363) in the pore-forming region. Two shaded areas on the β -subunit structure indicate the position of two calmodulin-binding sites. The shaded rectangle denotes the cGMP-binding site. The positions of epitopes recognized by the monoclonal antibodies PMc 1D1 or PMc 3C9 are also indicated on respective subunits (Diagram adapted from Dr. R.S. Molday).

(Ildefonse and Bennett, 1991; Karpen *et al.*, 1993; Gordon and Zagotta, 1995a,b). Although the bovine β -subunit shows only 30% sequence similarity with the α -subunit, the topology of the β -subunit is nearly identical to that of the α -subunit. The β -subunit also possess a cGMP binding domain near the C-terminus, but the negatively charged glutamate residue in the pore-forming region is replaced by a glycine residue. A unique feature of the β -subunit is the presence of a large N-terminal domain that is rich in glutamate, aspartic and proline residues and essentially identical to a bovine glutamic acid-rich protein (GARP) (Körschen *et al.*, 1995). The glutamic acid and proline residues are responsible for the anomalous migration of the β -subunit by SDS-gel electrophoresis. GARP is also found in human, bovine and rat ROS cytosol as splice variants of the β -subunits (Körschen *et al.*, 1995; Colville and Molday, 1996). A full-length version, called f-GARP and a truncated version (t-GARP) migrate in SDS-polyacrylamide gels as 120-140 kDa and 55-62 kDa polypeptides, respectively (Colville and Molday, 1996). The function of soluble GARPs as well as the GARP-part expressed as an integral part of the β -subunit is unknown, although possible interactions with other ROS proteins have been observed (Loewen and Molday, unpublished results).

The rod cGMP-gated channel is regulated *in vitro* by Ca^{2+} /calmodulin (Hsu and Molday, 1993). Recent studies have identified two unconventional and distinct calmodulin-binding sites on the β -subunit of the channel. The first calmodulin-binding site is located in the N-terminus before the first transmembrane domain. Deletion of this site totally abolishes Ca^{2+} /calmodulin modulation and binding to the channel complex. The other site, located downstream of the cGMP-binding site in the C-terminal region binds strongly Ca^{2+} /calmodulin but is, by itself, insufficient to confer the modulatory properties of calmodulin on the cGMP-gated channel (Grunwald *et al.*, 1998; Weitz *et al.*, 1998). Regulation of the channel complex by Ca^{2+} /calmodulin as well as a more exhaustive description of the implication of those sites is explained further in the section 1.6.3

1.6.2 Activation and molecular function of rod cGMP-gated channels

Channel activation occurs by direct binding of cyclic nucleotides to a region highly conserved in the C-terminus of all CNG channels. This sequence, composed of a stretch of nearly 130 amino acids, is homologous to cyclic nucleotide binding domains of cyclic

nucleotide-dependent protein kinases (PKA, PKG) and the catabolic gene-activator protein of *E. coli* for which the 3D-structure has been determined (McKay and Steitz, 1981; Weber and Steitz, 1987). The rod cGMP-gated channel can be activated by direct binding of cGMP, and to a lesser degree by cAMP. The channel sensitivity for cyclic nucleotides not only depends on the structure of the cGMP-binding site itself, but also on structural elements distributed in the N- and C-terminal portions of the channel (Goulding *et al.*, 1994; Gordon and Zagotta, 1995a). Binding of cyclic nucleotides to CNG channels induce conformational changes in the protein that cause the pore to open. Although the β -subunits of the cGMP-gated channel bind cyclic nucleotides, they fail to produce a functional channel when exogenously expressed in cultured *Xenopus* oocytes or human epithelial kidney cells (HEK 293). In contrast, the α -subunits expressed in these cells can assemble by themselves to form a functional homomeric channel that lacks some of the electrophysiological, molecular and pharmacological properties of the native channel such as the flickering nature of the channels opening and closing, the Ca^{2+} /calmodulin sensitivity and the inhibition by l-cis-diltiazem (Kaupp *et al.*, 1989; Dhallan *et al.*, 1992; Chen *et al.*, 1993; Nizzari *et al.*, 1993). Coexpression of the β -subunit with the α -subunit produces a channel complex with electrophysiological and pharmacological behaviors similar to those observed for the native channel (Chen *et al.*, 1993, 1994). The physiological function of the rod cGMP-gated channel has been extensively reviewed (Zagotta, 1996; Zagotta and Siegelbaum, 1996; Wei *et al.*, 1998) and is described in previous sections (1.5.2 and 1.5.3).

1.6.3 Regulation of rod cGMP-gated channels

To date, several factors affecting the activity of the rod cGMP-gated channel have been identified. These regulatory factors alter the influx of cations inside the outer segments by different mechanisms. Known and suggested regulators of the channel are listed in Table II and their involvement and mechanisms of action are described below.

Light

As a result of photoexcitation, the intracellular pool of cGMP decreases due to its hydrolysis by light-activated PDE. Reduced levels of cGMP will cause the cGMP-gated

Table II. Known regulators of the rod cGMP-gated cation channel

Regulator	Effect	Function	Reference
Accepted			
Light	Indirect	closes channel by reducing [cGMP]	Baylor and Fuortes, 1970; Koch and Kaupp, 1985
cGMP	Direct	keeps channel open	Koch and Kaupp, 1985
Ca ²⁺ /calmodulin	Direct	reduces cGMP sensitivity	Hsu and Molday, 1993
Transition metal cations	Direct	block channel pore	Gordon and Zagotta, 1995a,b; Karpen <i>et al.</i> , 1993
Ca ²⁺	Direct	blocks channel pore	Root and MacKinnon, 1993; Menini <i>et al.</i> , 1988
Suggested			
Phosphorylation	Direct/Indirect	Affects cGMP sensitivity	Gordon <i>et al.</i> , 1992 Molokanova <i>et al.</i> , 1997
Endogenous inh.factor	Direct	reduces cGMP sensitivity	Gordon <i>et al.</i> , 1995

channels to close and block the influx of cations into the ROS (Hagins *et al.*, 1970; Yee and Liebman, 1978; Koch and Kaupp, 1985).

Ca²⁺ and Calcium-binding proteins

A tight control of the rod CNG channel by calcium is not so surprising, taking into account that the entry of Ca²⁺ in ROS is linked to several feedback mechanisms crucial for light adaptation. The interaction of calcium with the channel is both direct, involving the binding to specific residues and indirect via the binding of calmodulin.

The direct binding of extracellular calcium and magnesium to a glutamate residue located at position 363 of the pore-forming region leads to a blockage of the channels (Root and MacKinnon, 1993; Eismann *et al.*, 1994). The high sensitivity of the channel for external Ca²⁺ and Mg²⁺ can be reduced dramatically by the mutant E363Q, suggesting that carboxyl groups form a cation-binding site in the pore region. The β-subunit lacks this glutamate residue and for this reason, the relative ion permeability of the heterotetrameric channel (native) is different from the homotetrameric channel composed exclusively of α-subunits. Another cation-binding site with lower affinity for Ca²⁺ and Mg²⁺ (Ki ~ 1 mM) has been found located on the intracellular side of the channel. Binding of internal Ca²⁺ and Mg²⁺ to this site can also block the channel (Menini, *et al.*, 1988; Colamartino *et al.*, 1991; Zimmerman and Baylor, 1992; Picones and Korenbrot, 1995).

It has been shown previously that Ca²⁺/calmodulin interacts with the β-subunit of the rod cGMP-gated channel *in vitro* at relatively high [Ca²⁺], leading to a two-fold decrease in the apparent affinity of the complex for cGMP (Hsu and Molday, 1993). A similar observation has been made in olfactory sensory neurons, where Ca²⁺/calmodulin binds to the α-subunit of the olfactory channel and decreases the ligand sensitivity around 20-fold (Chen and Yau, 1994). Half a decade ago, Liu and collaborators (1994) reported a mechanism by which Ca²⁺/calmodulin decreases the affinity of the olfactory CNG channel for cyclic nucleotides. From this study, and more recent work (Varnum and Zagotta, 1997), it appears that an interaction between the N- and C-terminal domains of the α-subunit are responsible for the high ligand affinity of the olfactory channel and that binding of Ca²⁺/calmodulin disrupts this interaction, thereby decreasing the ligand sensitivity of the channel for cyclic nucleotides. It is

not clear yet if the rod cGMP-gated channel responds to Ca^{2+} /calmodulin in the same way, but it is strongly suggested that Ca^{2+} /calmodulin affects the gating properties of the rod channel by interacting with a high affinity N-terminal CaM-binding site. In their model, Wei and co-workers (1998) suggested that a network of interactions linking N-terminal domains on the β -subunit to possibly the α -subunit would be disrupted upon CaM binding, thus causing allosteric changes and a decrease in the ligand sensitivity of rod channels. Regulation of the rod cGMP-gated channel by Ca^{2+} /calmodulin has been proposed to be part of the negative feedback that restores the dark state by allowing the channel to respond to smaller changes in cGMP. However, some experiments performed by Gray-Keller and collaborators (1993) have shown no effect of CaM or CaM inhibitors on the dark current and on the light response in intact rods, leading to the possibility that regulation of the rod cGMP-gated channel by CaM may not constitute a relevant physiological process. Electrophysiological studies using truncated frog ROS, however, have later shown that Ca^{2+} modulates the activity of the cGMP-gated channel in a similar manner to what has been observed *in vitro*, suggesting that the small change in ligand sensitivity observed upon Ca^{2+} /calmodulin binding may play only a minor role in rod adaptation. At present it is still controversial whether Ca^{2+} /calmodulin participates in the light adaptation and recovery of rod photoreceptor cells.

The existence of an endogenous calcium-binding protein, with a different calcium requirement, but the same modulatory properties as calmodulin has been suggested in frog rods (Gordon *et al.*, 1995). In this study an endogenous inhibitory factor was found to modulate the activity of the cGMP-gated channel over a broader range of calcium concentrations. The inhibitory factor was irreversibly inactivated (or removed) from the channel at low Ca^{2+} concentration (tens of nanomolar), whereas calmodulin was removed reversibly only below 1 μM Ca^{2+} concentration. Because the inhibitory factor and calmodulin show equivalent channel inhibition, it has been postulated that these two calcium-binding proteins bind to the same site. Similar findings, based on patch-clamp experiments, have been reported for the fish cone photoreceptor (Rebrik and Korenbrot, 1998) and the mammalian olfactory receptor neuron (Balasubramanian *et al.*, 1996). There is however no biochemical evidence to date for the existence of such endogenous factors.

Protein phosphorylation

Protein phosphorylation and its effect on the activity of the rod cGMP-gated channel has been investigated previously. There is, however, no known direct biochemical studies reporting phosphate incorporation into the rod cGMP-gated channel in a kinase-catalyzed reaction. More than ten years ago, Szuts (1985) observed that two large ROS membrane proteins (220- and 240-kDa) are phosphorylated *in vitro* in a light-dependent manner. The identity and the role of these proteins, however was not known at that time and no further biochemical studies have been conducted since then. It was not until the work of Gordon and collaborators (1992) that phosphorylation of the rod cGMP-gated channel was investigated. In this study, the authors observed that Ser/Thr phosphatase inhibitors (okadaic acid, microcystin-LR, and calyculin-A) increase up to three-fold the apparent affinity of the endogenous cGMP-gated channel for its ligand in inside-out patches from frog and salamander rod outer segments. It was also observed that addition of exogenous Ser/Thr phosphatases, PP1 and PP2A modulate the cGMP sensitivity differently, suggesting the existence of at least two phosphorylation sites that regulate the channel in a reciprocal manner (Gordon *et al.*, 1992). It is not clear from this study whether the endogenous protein phosphatases, the PP1 and PP2, act directly on the rod CNG channel.

Patch-clamp experiments further demonstrated that the recombinant rod cGMP-gated channel α -subunit expressed in *Xenopus* oocytes can be modulated by tyrosine, but not serine or threonine phosphorylation (Molokanova *et al.*, 1997). Inside-out patches from oocytes membrane were used to test the effect of Ser/Thr phosphatase inhibitors (okadaic acid, calyculin A and microcystin LR), tyrosine phosphatase inhibitors (orthovanadate and pervanadate) and protein tyrosine kinase inhibitors (lavendustin A and erbstatin) on the activity of the rod cGMP-gated channel. It was observed that only the tyrosine phosphatase inhibitors, but not the Ser/Thr phosphatase inhibitors, decrease the sensitivity of the recombinant channel for cGMP. Also, the addition of protein tyrosine kinase inhibitors caused an increase in the apparent affinity of the channel for cGMP. Taken together these studies suggested that phosphorylation of the channel decreases the cGMP sensitivity of the rod CNG channel. A similar observation has been made using inside-out patches from salamander rod outer segments containing the native channel (Molokanova *et al.*, 1997).

PKC-mediated phosphorylation of the olfactory CNG channel α -subunit has been recently reported (Müller *et al.*, 1998) using rat and bovine recombinant channels heterogenously expressed in human embryonic kidney cells (HEK 293). Excised patches treated with phorbol ester (PMA), a specific PKC activator, increased the cGMP sensitivity of the α -homomeric olfactory CNG channel approximately 5-fold. Mutation of a single serine residue, located in proximity of the CaM-binding site on the α -subunit, was sufficient to abolish the modulation by PMA/PKC. Although phosphorylation of this site does not affect the calmodulin-induced modulation of the olfactory channel, the results reported in this study suggest that the PKC-mediated phosphorylation increases the ligand sensitivity of the channel when the CaM-binding site is not occupied (Müller *et al.*, 1998).

1.7 PHOSPHORYLATION OF ION CHANNELS

Protein phosphorylation and its effect on ion channel activity has been extensively studied in the past and is one of the most widespread mechanism of ion channel modulation (Review: Levitan, 1985, 1988, 1994). In the process of protein phosphorylation, a γ -phosphate group of ATP (or GTP) is transferred to serine, threonine, or tyrosine residues in a protein kinase-catalyzed reaction. The covalently linked phosphate group can affect the catalytic or structural properties of the protein in several ways. These include conformational change, protein recruitment or inhibition of protein binding. There are several protein kinases identified and characterized to date, most of them having specific substrate requirements and unique regulatory properties (section 1.8). Protein phosphorylation is not limited to certain categories of ion channels. It is not uncommon to observe a given ion channel serving as a substrate for more than one protein kinase and modulating the channel in a cooperative or reciprocal manner (Levitan, 1988, 1994). A few examples of regulation by phosphorylation are discussed below.

Modulation of ion channels by phosphorylation

It is believed that phosphorylation of ion channels in the nervous system, skeletal muscle, heart, kidney and many other tissues can influence the electric properties of the cells

by modulating the ion fluxes across the plasma membrane. For example, the nicotinic acetylcholine receptor, a heteropentameric ligand-gated ion channel found in neurons, skeletal muscle and in the electric organ of *Torpedo*, is a substrate for PKA, PKC and tyrosine kinases (Huganir and Greengard, 1983; Huganir *et al.*, 1984; Safran *et al.*, 1987). Phosphorylation of the acetylcholine receptor complex reduces the ligand sensitivity of the channel and increases the rate of receptor desensitization, thus affecting the information transport at synapses (Downing and Role, 1987; Eusebi *et al.*, 1985; Huganir *et al.*, 1986). PKA phosphorylation of GABA_A receptor, a ligand-gated Cl⁻-selective channel, can cause a rapid desensitization of the GABA-induced current in neurons and other cells expressing this channel (Browning *et al.*, 1990). Phosphorylation of GABA_A by PKC and CaM-kinase II is also thought to play a role in the receptor sensitivity to ethanol (Wafford *et al.*, 1991; Wafford and Whiting, 1992). The Na⁺ channel, a member of the super-family of voltage-gated ion channels found in various tissues, is phosphorylated by both PKA and PKC (Costa *et al.*, 1982; Rossie and Catterall, 1987, 1989; West *et al.*, 1991). The effect of PKC and PKA phosphorylation are similar, resulting in a decrease in the activity of the sodium channel and reduction in ion flux (Rossie *et al.*, 1987; Numann *et al.*, 1991).

Other effects of phosphorylation on ion channel and Ca²⁺ ATPase modulation

Although direct changes in the catalytic properties of ion channels are commonly seen upon protein phosphorylation, it is not the only effect of protein phosphorylation. Observations made on the nicotinic acetylcholine receptor show that PKA phosphorylation stabilizes the receptor subunits and promotes their assembly and the aggregation of receptors at the synaptic terminus of neurons (Ross *et al.*, 1987; Green *et al.*, 1991). Aggregation of acetylcholine receptor at nerve-muscle synapses may also be triggered by tyrosine phosphorylation (McMahan and Wallace, 1989). In a more recent study, Enyedi and co-workers (1996) reported the activation of a Ca²⁺ pump isoform (4b) by PKC phosphorylation in an inhibitory region, downstream of a calmodulin-binding domain. Although no cross-talk has been observed between the calmodulin activation pathway, and the PKC phosphorylation pathway of the Ca²⁺ ATPase pump isoform 4b, a subsequent study showed that PKC

phosphorylation of the "a" forms of the Ca^{2+} pump isoform 2 and 3 prevents the binding and the stimulation of the pump by calmodulin (Enyedi *et al.*, 1997).

1.8 PROTEIN KINASES

Protein kinases play crucial roles in numerous signal transduction cascades and other vital biochemical pathways such as cell cycle regulation, glycolysis, protein metabolism and degradation. The central role of protein kinases in regulating cellular function is emphasized by the observation that many kinases are encoded by proto-oncogenes. To date, more than a hundred different protein kinases have been identified, representing a structurally and functionally diverse group of enzymes. Although they all have evolutionary related catalytic domains, protein kinases differ greatly in their substrate specificity and regulatory properties (Taylor, 1987). Regulatory properties are usually provided by non-catalytic regions present on the catalytic subunit or on regulatory subunits. In many cases, these regulatory domains act as a pseudosubstrates, inhibiting the catalytic functions of the protein kinase by binding to the active site (Soderling, 1990). Protein kinases can be classified into two categories, based on their ability to phosphorylate Tyr or Ser/Thr residues on proteins. For any proteins, the ability of a given kinase to phosphorylate these residues depends on the primary structure (consensus sequence), the accessibility and the specificity of the protein kinase. It is now known that most protein kinases have a preference for Ser/Thr and Tyr residues located in the vicinity of one or more basic residues, but this is not true for all kinases as we shall see later (section 1.8.3).

1.8.1 Protein kinase inhibitors

Protein kinase inhibitors are important to study the role of phosphorylation on signal transduction cascades as well as other cellular processes and to help identify the kinases implicated in these pathways. Most protein kinase inhibitors act by binding to the active site or regulatory site crucial for the function of the enzyme. Other inhibitors consist of peptides containing consensus sequences recognized specifically by certain protein kinases. Protein kinases and their inhibitors used in the present study are listed in Table III.

Table III. Inhibitors used in this study and their protein kinase targets

Inhibitor	Target kinase	IC_{50}^1
Bisindolylmaleimide I	PKC (α , β , δ , ϵ)	PKC (α , β , δ , ϵ): 8.4; 18; 210; 132 nM; PKA: 2 μ M
Calphostin C	PKC	PKC: 50 nM; MLCK: >5 μ M; PKA: >50 μ M;PKG: >20 μ M; $p60^c\text{-}SRC$: >25 μ M
H-89, dihydrochloride	PKA	PKA: 50 nM; MLCK: 30 μ M; CaM kinase II: 30 μ M; PKC: 30 μ M
KN-93	CaM kinase II	CaM kinase II: 370 nM
ML-7	MLCK	MLCK: 300 nM; PKA: 21 μ M; PKC: 42 μ M
Protein kinase G inhibitor	PKG	PKG: 86 μ M; PKA: 550 μ M
Lavendustin A	EGF receptor tyr kinase $p60^c\text{-}SRC$	EGF receptor tyr kinase: 11 nM; $p60^c\text{-}SRC$: 500 nM
5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole (DRB)	CKII	CKII (6 μ M)
Heparin	CKII and other kinases ¹	CKII: 1.4 nM
Staurosporine	Broad Range Ser/Thr	PKA: 15 nM; PKC: 0.7-7 nM; PKG: 8.5 nM; MLCK: 1.3 nM; CaM kin.II: 20 nM

¹See Zollner, 1993.

1.8.2 Casein kinase II

Casein kinase II (CK2) is a ubiquitous cyclic nucleotide-independent Ser/Thr protein kinase found in all eukaryotic cells and tissues examined to date, including gray matter and cortical growth cones (Tuazon and Traugh, 1991; Allende and Allende, 1995; Mitev *et al.*, 1994; Edgar *et al.*, 1997). CK2 has been found primarily in the cytosol of these cells, but it is also associated with the plasma membrane, nucleus and mitochondria (Hathaway and Traugh, 1982). An astonishing number of substrates differing greatly in function and localization have been reported to be phosphorylated by CK2 over the past 40 years. Evidence accumulated so far indicates that casein kinase II may play a central role in the control and division of cells by phosphorylating and regulating a wide variety of oncoproteins, transcription factors, and enzymes involved in nucleic acid synthesis (Litchfield and Lüscher, 1993). CK2 has also been reported to phosphorylate substrates involved in the regulation of signal transduction pathways. CK2 is usually found as a heterotetramer composed of two catalytic α -subunits (and/or α') and two regulatory β -subunits, although there are observations that the kinase is also active as a monomer in human spleen (Gounaris *et al.*, 1987) and a dimer in rat and porcine liver (Delpech *et al.*, 1986; Baydoun *et al.*, 1981).

A variety of different casein kinase II isoforms are found in different species and in different cells (Tuazon and Traugh, 1991). In mammals and birds, the catalytic α -subunits and the α' isoform are products of two distinct genes, but are expressed at similar levels in most cells (Litchfield *et al.*, 1990). The α - and α' -subunits are catalytically active by themselves and have molecular masses ranging from 41- to 44-kDa and 37- to 42-kDa, respectively. The β -subunits (24- to 26-kDa) play regulatory roles and are responsible for stimulating the protein kinase activity of the holoenzyme up to ten-fold (Cochet and Chambaz, 1983). It has also been suggested that the β -subunits are involved in the stabilization of the α -subunit against proteolysis and heat denaturation and contribute in the modulation of substrate specificity through interaction of the holoenzyme with specific substrates and inhibitors (Meggio *et al.*, 1992). To date, no other physiological regulator of casein kinase II has been identified, although several activators and inhibitors have been identified and used to characterize and regulate the enzyme *in vitro*.

Casein kinase II phosphorylates Ser/Thr residues located within acidic sequences composed of glutamic and aspartic residues (S/T X₊₁X₊₂D/E₊₃; X denotes any nonbasic and non-bulky amino acids). It is also possible that additional acidic residues, located at position -2 to +7 from the Ser/Thr residue act as positive determinants for the enzyme (Meggio *et al.*, 1994). CK2 is very unusual among protein kinases in that it can transfer phosphate groups from GTP ($K_m \sim 20 \mu M$) as well as ATP ($K_m \sim 10 \mu M$). ATP is used preferentially in the presence of Mg²⁺, but in the presence of Mn²⁺, GTP is the main phosphate donor (Gatica *et al.*, 1993). The ability of CK2 to transfer phosphate from both ATP and GTP has been used in the past along with the substrate casein and the inhibitor heparin to identify and characterize CK2 in a variety of cells (Tuazon and Traugh, 1991). Inhibition of CK2 by heparin is very effective ($IC_{50} = 1.4 \text{ nM}$), although the effect of heparin inhibition varies with different substrates and in some cases can be totally abolished (Hattaway *et al.*, 1980; Feige *et al.*, 1980; Taylor *et al.*, 1987). CK2 is not inhibited by staurosporine, a broad-range Ser/Thr kinase inhibitor, but it is inhibited by 2,3-bisphosphoglycerate, benzimidazole derivatives (DRB), and polyanionic compounds such as heparan sulfate, inositol hexasulfate and peptides rich in glutamic and aspartic acid residues (Zollner, 1993; Tuazon and Traugh, 1991). Physiological inhibitors of CK2 have not yet been identified, although small levels of heparin in the liver and 2,3-bisphosphoglyceric acid in red blood cells may be sufficient to regulate casein kinase II *in vivo* (Tuazon and Traugh, 1991).

1.9 CALMODULIN

Calmodulin is a ubiquitous calcium-binding protein that acts as a calcium sensor and transducer in a wide variety of cellular regulatory pathways. X-ray crystallography studies of Ca²⁺/calmodulin have revealed an unusual dumbbell-shape structure composed of two globular domains joined by a seven-turn α -helix (Babu *et al.*, 1985; Chattopadhyaya *et al.*, 1992). Each globular domain is composed of two paired helix-turn-helix motifs, also known as EF hands (Kretsinger, 1976). The oxygen atoms present in the backbone and side chains of the loop coordinate Ca²⁺ ions at these sites. With the exception of annexins, most CBPs contain similar EF hand motifs, but their number and coordination geometry may differ, allowing different

CBPs to bind calcium over a wide range of affinity and selectivity (Van Eldik and Watterson, 1998). Upon calcium binding to any of these sites, calmodulin undergoes major conformational rearrangements that expose a hidden non-polar surface. Hydrophobic interactions between calmodulin and its target protein mediate the activation or inhibition of the protein through a Ca^{2+} -dependent regulatory pathway. Calmodulin targets are extremely varied and include protein kinases, phosphatases, adenylate cyclases, PDE, ion channels, and Ca^{2+} ATPases (Van Eldik and Watterson, 1998). Ca^{2+} /calmodulin is known to activate proteins by binding near an inhibitory domain, causing the autoinhibitory domain to dissociate from the active site (e.g. Ca^{2+} ATPase pump isoform 4b). It is believed that protein inhibition by calmodulin follows a similar mechanism, disrupting the stable conformation of a protein through its association with an autoactivation domain (Van Eldik and Watterson, 1998).

Calmodulin has been identified in ROS where it is found both in the cytosol and associated with the plasma membrane (Kohnken *et al.*, 1981; Nagao *et al.*, 1987). To date, few calmodulin targets have been identified in ROS. Besides its interaction with the rod cGMP-gated channel (Hsu and Molday, 1993), a possible correlation between Ca^{2+} /calmodulin, adenylate cyclase and phosducin has been suggested (Willardson *et al.*, 1996). Calmodulin overlay experiments however have shown that several ROS proteins, ranging from 29-kDa to 240-kDa, bind Ca^{2+} /calmodulin (Nagao *et al.*, 1987; Morelli *et al.*, 1989; Hsu and Molday, 1993, 1994). The identity of most of these proteins is still unknown and it is possible that some of these polypeptides are proteolytic fragments of the cGMP-gated channel β -subunit (240-kDa). In addition, calmodulin has not yet been linked to the phosphorylation pathway in ROS despite the apparent abundance of phosphoproteins (Szuts, 1985; Binder *et al.*, 1989; Warren and Molday, unpublished results) and the identification and localization of the calmodulin-binding protein calcineurin in ROS preparations (Dickinson and Molday, unpublished results).

1.10 THESIS INVESTIGATION

The main objective of the present study was to further our knowledge on the regulation of the rod cGMP-gated channel by protein phosphorylation and Ca^{2+} /calmodulin interaction.

The first aim of this study was to investigate whether or not the rod cGMP-gated channel is a target for endogenous ROS protein kinases. This was accomplished using phosphorylation assays and affinity chromatography. A biochemical characterization of the phosphorylation reaction was also performed to identify which amino acids are being phosphorylated, in which part of the channel and at what frequency. Phosphoamino acid analysis, limited proteolysis experiments and phosphorylation assays were carried out here to accomplish these tasks. Cyclic GMP-dependent Ca^{2+} efflux assays were used in conjunction with phosphorylation assays to determine the role of phosphorylation in the regulation of the cGMP-gated channel.

In a second study, the identity of the endogenous ROS protein kinase involved in this reaction was investigated using inhibitor mapping experiment, Western blotting and immunofluorescence.

In the final area of study, Western blotting and immunoprecipitation experiments were used to clarify the role of Ca^{2+} /calmodulin in phototransduction. The interaction of ROS calmodulin with the native rod cGMP-gated channel was investigated under similar calcium concentration to those prevailing in dark-adapted or bleached ROS. The potential existence of other channel modulators was also investigated using the cGMP-dependent efflux assay and the approaches mentioned above.

CHAPTER 2

PHOSPHORYLATION OF THE cGMP-GATED CHANNEL β -SUBUNIT

2.1 MATERIALS

2.1.1 Animal tissues

Frozen bovine retinas were obtained from Schenk Packing Co (Stanwood, WA).

2.1.2 Chemicals

$[\gamma^{32}\text{P}]$ ATP and $[\alpha^{32}\text{P}]$ ATP were purchased from New England Nuclear (Boston, MA). Immobilon-P and nitrocellulose membranes were purchased from the Millipore Corporation (Bedford, MA) and reagents used for enhanced chemiluminescence (ECL) were obtained from Amersham Corporation. Calmodulin-Sepharose affinity chromatography support was obtained from Pharmacia Biotech (Uppsala, Sweden). Kallikrein was a product of Sigma and the protease inhibitor Pefabloc was from Boehringer Mannheim. Phosphocellulose plates used for phosphoamino acid analysis, Biomax films for autoradiography, and X-Omat Blue XB-1 films for western blotting were obtained from Eastman Kodak Co (Rochester, NY). The Chelex-100 column was from Bio-Rad Laboratories and the bicinchoninic acid (BCA) assay kit was obtained from Pierce Chemicals (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co (St-Louis, MO) or Fisher Scientific (Fair Lawn, NJ).

2.1.3 Immunoreagents

All the monoclonal antibodies against the cGMP-gated channel (Fig. 6) used in this study were previously generated in the laboratory by fusion of mouse myeloma cells with spleen cells from BALB/c mouse immunized with affinity-purified ROS proteins or recombinant polypeptides (Lane *et al.*, 1986). Affinity chromatography support PMc 1D1-was made by coupling purified PMc 1D1 to Sepharose 2B using the cyanogen bromide method (March *et al.*, 1974).

2.2 METHODS

2.2.1 Bovine rod outer segment preparations

Bovine ROS were prepared under dim red light as described previously (Molday and Molday, 1987). Briefly, hundred retinas were gently agitated in approximately 50 ml of homogenization buffer containing 20% (w/v) sucrose, 10 mM β -D-glucose, 10 mM taurine, 0.25 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4. Outer segments were collected by filtration of the homogenate through a Teflon filter (300 μ m mesh) and approximately 8-10 ml of the suspension was layered on each of six 28-60% (w/v) sucrose gradients. Following centrifugation in a SW28 rotor (Beckman Instruments) at 24,000 rpm for 1 hr at 4°C, the band containing intact outer segments was gently removed from the tube and the ROS were washed once with 20 ml of homogenization buffer by centrifugation in a Sorvall SS-34 rotor (Dupont Co.) at 12,000 rpm for 15 min. The washed ROS were resuspended in homogenization buffer containing 0.7 mM Pefabloc and stored at -70°C until further use. The protein concentration was determined by bicinchoninic acid (BCA) assay and was approximately 9-12 mg/ml. Soluble proteins were washed away or collected directly after hypotonic lysis of bleached ROS under dim red light (Molday and Molday, 1987, 1999). Briefly, ROS were resuspended in 5 to 10 volumes of hypotonic buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM dithiothreitol (DTT), and 2 mM EDTA. After 5 min incubation on ice, the samples were centrifuged at 30,000 rpm for 10 min in a Beckman Optima ultracentrifuge. The membrane pellet was resuspended in 5 to 10 volumes of hypotonic buffer and the procedure repeated two more times. The pellet was finally resuspended in 10 mM HEPES-KOH, 1 mM DTT (DTT was omitted when the sample was used for affinity chromatography) to give a final protein concentration of 7-9 mg/ml by the BCA assay.

2.2.2 Purification of the cGMP-gated channel

ROS membrane proteins were solubilized in the dark in 10 mM HEPES-KOH, pH 7.4, 18 mM CHAPS, 150 mM KCl, 2 mM CaCl₂ and the cGMP-gated channel complex was purified by affinity chromatography using PMc 1D1-Sepharose (anti α -subunit) or CaM-Sepharose. In most cases, 100 μ l of beads (bed volume) was incubated with 500 μ l of solubilized ROS membrane proteins (~ 2 mg/ml proteins) at 4°C for 90 min. The unbound

fraction was removed by centrifugation and the beads washed ten times in 1 ml of solubilization buffer (described above). The proteins were normally eluted from the column using the electrophoresis sample buffer without BME except in the case of calmodulin-Sepharose affinity chromatography where 2 mM EGTA was used to elute the proteins off the column.

2.2.3 Phosphorylation of the rod cGMP-gated channel

ROS membranes were resuspended in ROS cytosol isolated after hypotonic lysis of the outer segments (2 mg/ml proteins). The 200 μ l reaction mixture contained 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂, 1 mM DTT and the phosphorylation reaction was generally initiated by the addition of 100 μ M cold ATP, 3 mM MgCl₂ and 5 μ Ci [γ -³²P]ATP (or [α -³²P]ATP for control). All the steps were done in parallel under either normal room lighting or complete darkness. The effect of different regulators including cAMP, cGMP, Br-cGMP, Ca²⁺, phorbol ester, Ca²⁺/calmodulin and EDTA was investigated by pre-incubating various levels of these substances for 5 min in the reaction mixture (Table IV). After 15 min incubation at 30°C, the reaction was stopped either by addition of the electrophoresis sample buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 100 mM β -Mercaptoethanol, 40% sucrose) or by washing the membrane 5 times in 10 mM HEPES-KOH, pH 7.4, 2 mM EDTA and 1mM DTT. Samples used for affinity chromatography were solubilized as described above. Purified or unpurified samples were subjected to electrophoresis and the proteins were stained with coomassie blue before autoradiography. Relative band intensities were compared by scanning the autoradiograms using a LKB laser densitometer.

2.2.4 Phosphate quantification

The cGMP-gated channel was phosphorylated and purified by PMc 1D1-Sepharose affinity chromatography as described above. The concentration of channel was quantified by the method of Kaplan and Pedersen (1985) using BSA as a standard. Briefly, purified and SDS-denatured channel samples were precipitated in 20% trichloroacetic acid (TCA) in a buffer containing 10 mM Tris-Cl, pH 7.5, 1% SDS and spotted onto nitrocellulose filters. The filters were washed with 6% TCA and stained using an amido black solution (0.5% amido

black, 45% ethanol, 10% acetic acid). The background was removed using a 90% methanol, 2% acetic acid solution and the filters were dried before isolation of the spots in Eppendorf tubes. Amido black labeling was removed by incubation in a 25 mM NaOH, 50 µM EDTA and 50% ethanol solution for 20 min under constant mixing. Filters were discarded and the absorption was measured at 630 nm. A filter paper-based assay was used to measure phosphate incorporation in TCA-precipitated samples (Bray, 1960; Witt and Roskoski, 1975).

2.2.5 Phosphoamino acid analysis

After phosphorylation of ROS proteins *in vitro*, the cGMP-gated channel was purified by PMc 1D1-Sepharose affinity chromatography and subjected to electrophoresis on a 8.5% SDS-PAGE gel. The proteins were transferred onto Immobilon-P membranes and the membranes cut into 1 mm² pieces. The pieces containing ³²P-labeled cGMP-gated channel β-subunits were incubated with (50 µg/ml) tosyl-phenylalanine chloromethyl ketone-treated trypsin (Sigma) for 4 hrs in 50 mM NH₄HCO₃ at 37°C and subsequently washed 4 times with distilled water. Supernatants were pooled, lyophilized, and resuspended in water. Phosphopeptides were washed three more times and then dissolved in 6N HCl and hydrolyzed under N₂ for 1hr at 110°C. Acid-hydrolyzed peptides were washed thrice with 200 µl dH₂O and spotted on 20 X 20-cm thin-layer cellulose plates along with 1 µg of phosphoserine, phosphothreonine and phosphotyrosine. Electrophoresis was carried out for 70 min at 750 V in 1% pyridine, 10% (v/v) acetic acid buffer. Samples were detected by autoradiography.

2.2.6 Limited proteolysis

Proteolysis was carried out as described by Kim and co-workers (1998). Briefly, washed ROS membranes were phosphorylated (see above), washed to remove ATP, resuspended in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂ and 1 mM DTT (1 mg/ml proteins) and digested with porcine pancreatic kallikrein at final concentration of 0.25, 0.5 and 1 U/ml for 25min at 25°C. ROS membranes were washed thrice in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂ and 0.7 mM Pefabloc. Membrane proteins were solubilized and the cGMP-gated channel purified by affinity chromatography using PMc 1D1-Sepharose columns. Phosphopeptides were separated by SDS-PAGE, detected by coomassie

blue and identified by western blotting using PMb 3C9 monoclonal antibody and autoradiography.

2.2.7 Reconstitution of membrane proteins into lipid vesicles

ROS were washed three times under dim red light in a hypotonic buffer containing 5 mM HEPES-KOH, pH 7.4, 150 mM KCl, 1 mM DTT and 4 mM EDTA to lyse the outer segments. The membrane proteins were solubilized at 4°C for 15 min in 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 2 mM CaCl₂ and 150 mM KCl and were spun at 40,000 rpm for 10 min in a TLA100.4 rotor (Beckman) to remove residual aggregated material. The cGMP-gated channel was purified by affinity chromatography using CaM-Sepharose and eluted using a buffer containing 18 mM CHAPS, 5 mM HEPES-KOH, pH 7.4, 2 mg/ml asolectin, 150 mM KCl and 2 mM EGTA. The purified cGMP-gated channel was added to an equal volume of soybean L- α -phosphatidylcholine (Sigma type IV) to give a final concentration of 10 mg/ml lipid, 5 mM HEPES-KOH buffer, pH 7.4, 10 mM CHAPS. The detergent was dialyzed out for 48 hrs in a dialysis buffer containing 5 mM HEPES-KOH, pH 7.4, 5 mM CaCl₂ and 150 mM KCl. The excess calcium outside the lipid vesicles was removed by dialyzing the samples for 2 hrs in dialysis buffer without calcium (5 mM HEPES-KOH, pH 7.4 and 150 mM KCl). Phosphorylation of the membrane protein was carried out before solubilization, unless otherwise specified. The modified Bradford assay was used to determine the protein concentration of solubilized ROS and purified channel before reconstitution (Read and Northcote, 1981).

2.2.8 cGMP-dependent Ca²⁺ efflux assay

Activity measurements were determined as described by Cook *et al.* (1986, 1987). Lipid vesicles containing the purified cGMP-gated channel were passed through a Chelex column to remove the calcium outside the vesicles. A solution containing 0.3 ml of the reconstituted channel in lipid vesicles was added to 1.7 ml of Arsenazo III, a calcium indicating dye that absorbs at 650 nm upon calcium binding. The calcium released from the vesicles was triggered by the addition of cGMP and monitored using a dual wavelength spectrophotometer (SLM Aminco DW2000). To test the effect of calmodulin on the

phosphorylated channel, 7.5 nM calmodulin was added to the vesicle solution prior to the activity measurements. The data obtained for the cGMP-dependent Ca^{2+} efflux assay was analyzed using the curve fit program of Sigma Plot (Jandel Scientific). The Michaelis constant (K_m) and the Hill coefficient (n) were calculated by plotting the initial velocity over the maximal velocity at saturating cGMP concentration as a function of cGMP concentrations.

2.2.9 Binding of the phosphorylated β -subunit to calmodulin-Sepharose

Briefly, the channel was phosphorylated as described above and the reaction was terminated by washing the membranes in a ice-cold buffer containing 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM EDTA. Membrane proteins were solubilized as above and incubated with 100 μl of calmodulin-Sepharose beads for 90 min at 4°C. Unbound proteins were removed by washing the beads 10 times in 1 ml of solubilization buffer. Bound proteins were released by replacing Ca^{2+} with EGTA in the solubilization buffer. The samples were applied to an SDS-polyacrylamide gel and visualized by autoradiography.

2.2.10 Polyacrylamide gel electrophoresis and western blotting

Protein samples were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis using the buffer system of Laemmli (1970). Protein samples contained in 8%-12% SDS-polyacrylamide gel slices were either stained by coomassie blue or electroblotted onto Immobilon-P membranes in 25 mM Tris-HCl, pH 8.4, 190 mM glycine and 5-10% methanol. For western blotting, the membranes were blocked in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.5, 140 mM NaCl, and 2 mM KCl) containing 2% milk for 20 min at 25°C and then incubated for 45 min at 25°C with the mAb hybridoma culture fluid (PMb 3C9) diluted 10 fold in PBS containing 0.02% milk. The membranes were washed thoroughly with PBS and incubated with sheep anti-mouse immunoglobulin (Ig) conjugated to horseradish peroxidase at 1/6000 dilution. Proteins of interest were detected by enhanced chemiluminescence (ECL).

2.3 RESULTS

2.3.1 The rod CNG channel β -subunit is phosphorylated *in vitro* by an endogenous kinase

The phosphorylation state of the cGMP-gated channel was investigated using a fraction of ROS cytosol containing endogenous protein kinases. After incubation of ROS membrane proteins with [γ -³²P]ATP and ROS cytosol, the channel was solubilized, purified by PMc 1D1-Sepharose affinity chromatography and submitted to gel electrophoresis and autoradiography. Several membrane proteins were phosphorylated in ROS, the most predominant phosphoprotein being rhodopsin and its multimers (Fig. 8, *autoradiogram lane a* and *b*). The β -subunit, but not the α -subunit of CNG, was also phosphorylated in the presence of magnesium (Fig. 8, *lane c*). To test whether protein labeling was caused by nucleotide binding, ROS membrane proteins were treated with [α -³²P]ATP. Under these conditions, no labeling of the β -subunit or other ROS membrane proteins was observed, indicating that the γ -phosphate group of ATP is transferred to the β -subunit of the channel in a protein kinase-catalyzed reaction (not shown). The amount of phosphate groups incorporated on the β -subunit was calculated by scintillation counting and protein quantification using the Pedersen assay (Kaplan and Pedersen, 1985). After phosphorylation of ROS membrane proteins, the complex was purified on a PMc 1D1-Sepharose affinity column. Both the α - and the β -subunits of the rod CNG channel were eluted in a buffer containing 3% SDS and the protein concentration and the ³²P counts were determined. Based on the premise that the native channel is a heterotetramer composed of two α - and two β -subunits (Liu *et al.*, 1996), an average incorporation of one to two phosphate groups per five β -subunits was determined.

A phosphoprotein migrating as a 105-kDa protein was observed in the purified channel preparation. The protein was most evident in channel preparations in which no protease inhibitors were added. Proteolysis experiments using kallikrein followed by western blotting using PMb 3C9 revealed that this 105-kDa phosphoprotein is indeed the β' -part of the cGMP-gated channel, suggesting that the C-terminal portion of the β -subunit is a target for ROS endogenous protein kinases (Fig. 9, *lane a, b, c, and d*). This experiment also shows that GARP comprised in the N-terminal domain of the β -subunit is very sensitive to proteolysis, whereas the β' -part (105-kDa) and the α -subunit of the cGMP-gated channel remain relatively

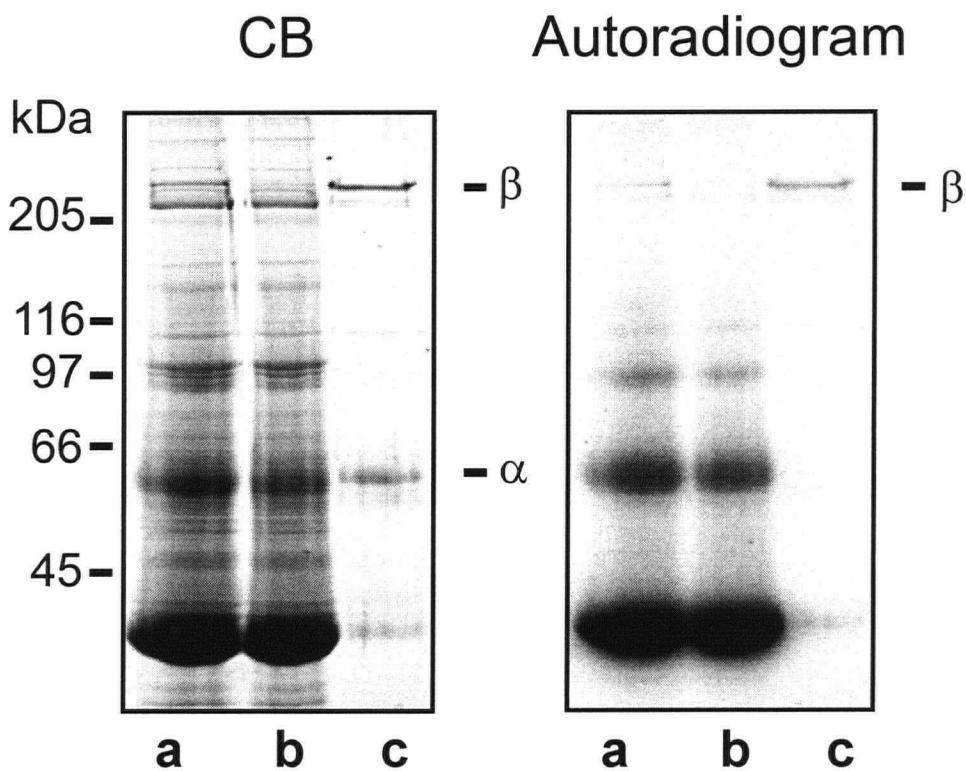


Fig. 8. *In vitro* phosphorylation of the rod cGMP-gated channel β -subunit by endogenous ROS protein kinase(s). ROS membranes were incubated for 15 min at 30°C in a solution containing [γ - 32 P]ATP, MgCl₂ and a lysate containing ROS soluble protein kinases. The membrane proteins were subsequently solubilized in CHAPS and the cGMP-gated channel purified by affinity chromatography using PMc 1D1-Sepharose. Samples were subjected to SDS-polyacrylamide gel electrophoresis on a 9% slab gel, stained with Coomassie Blue (CB) and the phosphoproteins detected by autoradiography. *lane a*, ROS membranes solubilized in CHAPS; *lane b*, unbound fraction and *lane c*, bound fraction from a PMc 1D1-Sepharose column. Lanes contained either 40 μ g of ROS or 3 μ g of purified channel. α and β indicate the position of the α - and β -subunit of the cGMP-gated channel, respectively.

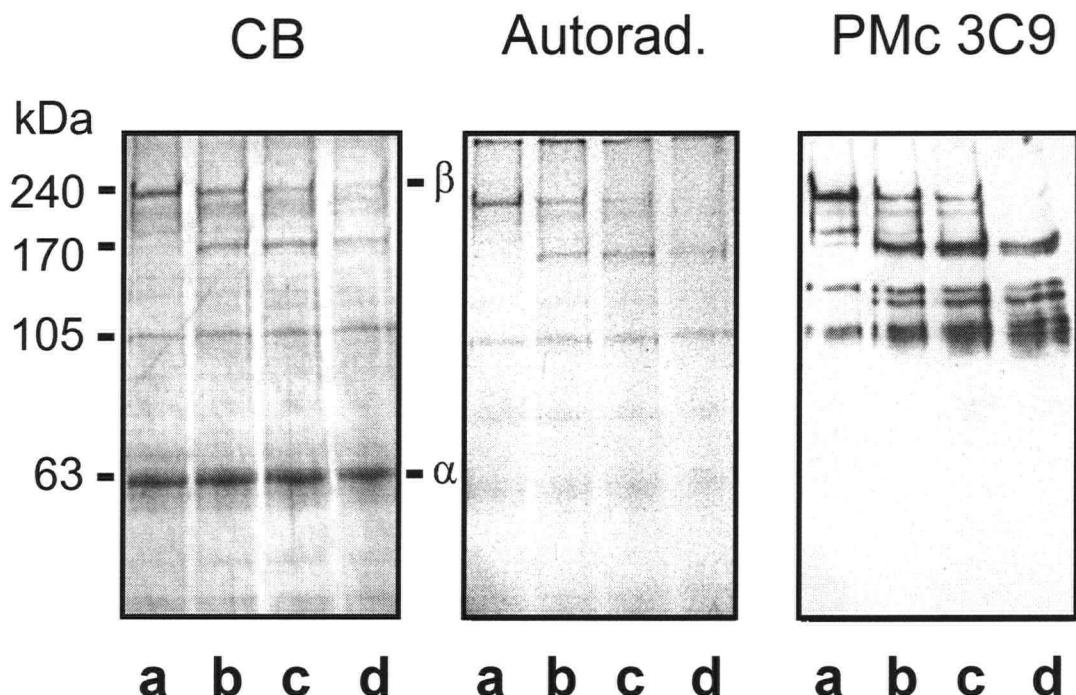


Fig. 9. Proteolysis of the phosphorylated β -subunit of the cGMP-gated channel. ROS membranes were phosphorylated *in vitro* by endogenous ROS protein kinases and treated with either 0, 0.3, 0.5, or 1.0 μ g/ml kallikrein (*lane a, b, c, and d*, respectively). The channel complex was purified by PMc 1D1-Sepharose and the samples (3 μ g) were separated on SDS-polyacrylamide gels and then analyzed by either Coomassie Blue staining (CB), autoradiography or Western blotting with PMb 3C9 antibody.

resistant to degradation by kallikrein at 1 µg/ml. On the basis of these studies it appears that most degradation products observed on the coomassie blue-stained gel are proteolytic fragments of the β -subunit and these peptides are phosphorylated (Fig. 9).

To further characterize the phosphorylation reaction, the phosphoamino acids present within the β -subunit were determined. For these studies, the β -subunit and the β' -part of the channel were isolated on SDS-PAGE gels after phosphorylation and transferred onto Immobilon-P membranes. The proteins were digested with trypsin, and the residual polypeptides were hydrolyzed by HCl. The resulting 32 P-labeled amino acids were spotted on phosphocellulose plates along with phosphoamino acid standards and subjected to electrophoresis. As shown in Fig. 10, only phosphoserine residues were observed. The relatively weak signal observed may be due to low phosphate incorporation in the β -subunit.

2.3.2 Regulation of the phosphorylation reaction

The effect of various experimental conditions on the phosphorylation of the β -subunit *in vitro* were investigated. As previously shown, phosphorylation of rhodopsin was light-dependent (Fig. 11, *lane a, b, c, and d*). In contrast, the level of phosphate incorporation into the β -subunit was independent of light. Cyclic GMP is the second messenger in phototransduction and for this reason, its effect on phosphorylation was also investigated. Addition of cGMP and the non-hydrolyzable analogue Br-cGMP to the phosphorylation reaction (up to 200 µM) did not have any additional effect on phosphate incorporation by the β -subunit (Table IV). Likewise, no effects were observed with the addition of cAMP, Ca²⁺, Ca²⁺/calmodulin, EDTA, and the PKC activator phorbol ester (Table IV).

2.3.3 Effect of phosphorylation on the activity of the rod cGMP-gated channel β -subunit

The activity of many ion channels is now known to be regulated by protein phosphorylation (Levitan, 1988, 1994). However, the exact mechanisms by which phosphate groups alter the activity of these channels are not well understood. In order to study the role of β -subunit phosphorylation on the cGMP-gated channel, ROS were treated with endogenous protein kinases in the presence and absence of ATP. After phosphorylation, the channel was isolated by calmodulin-Sepharose affinity chromatography and reconstituted into calcium-

Autoradiogram

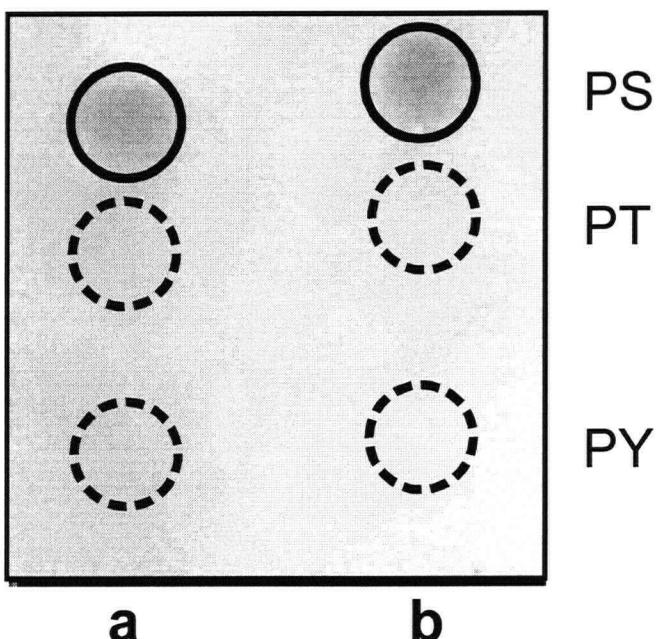


Fig. 10. Phosphoamino acid analysis of phosphorylated cGMP-gated channel β -subunits. The native cGMP-gated channel β -subunit (*b*) and the β' -part of the channel β -subunit (*a*) were phosphorylated *in vitro*, purified by PMc 1D1-Sepharose, processed for amino acid hydrolysis, analyzed by electrophoresis on thin layer cellulose plates, stained with ninhydrin, and subjected to autoradiography as described in "methods". Positions of standards phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY) are indicated.

Autoradiogram

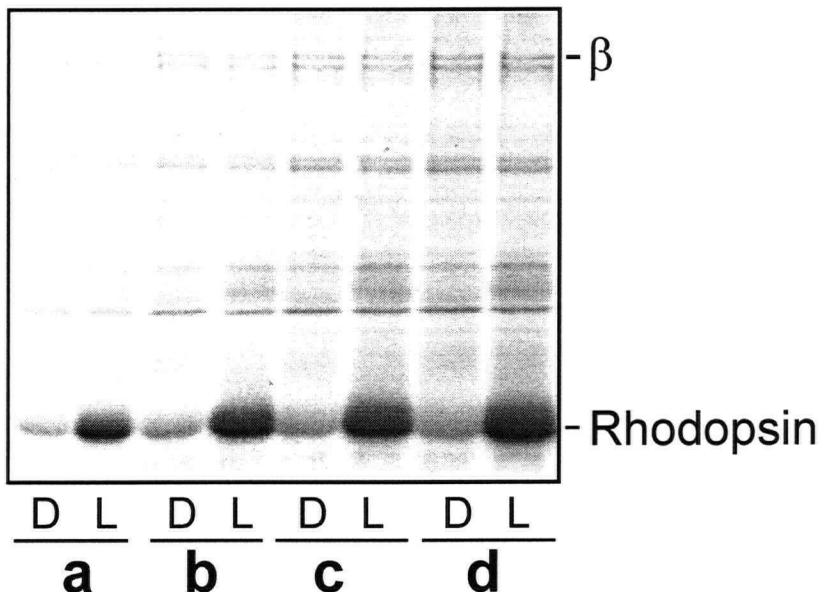


Fig. 11. Effect of light and dark on the phosphorylation of the cGMP-gated channel β -subunit. Washed ROS membranes resuspended in a ROS lysate were phosphorylated *in vitro* under dark (D) or light (L) conditions. The latter was exposed to normal room lighting. Both samples (50 μ l) were incubated for 15 min at 30°C and the reaction was stopped by the addition of one volume of electrophoresis sample buffer. Phosphorylated ROS membranes were subjected to electrophoresis and the phosphoproteins identified by autoradiography. *lane a*, 10 μ g ROS; *lane b*, 20 μ g ROS; *lane c*, 30 μ g ROS; *lane d*, 40 μ g ROS.

Table IV. Factors that did not influence the *in vitro* phosphorylation of the cGMP-gated channel β-subunit.

Regulator	Concentration	Effect
Light/Dark		-
Ca ²⁺	0.05, 0.2, 0.5, 1 and 2 mM	-
EDTA	0.5, 1 and 2 mM	-
Phorbol ester	10 and 100 μM	-
Ca ²⁺ /phorbol ester	See above	-
Ca ²⁺ /calmodulin	0.5, 1 and 5 μM	-
cGMP	20, 50 and 200 μM	-
Br-cGMP	20, 50 and 200 μM	-
cAMP	50 and 100 μM	-

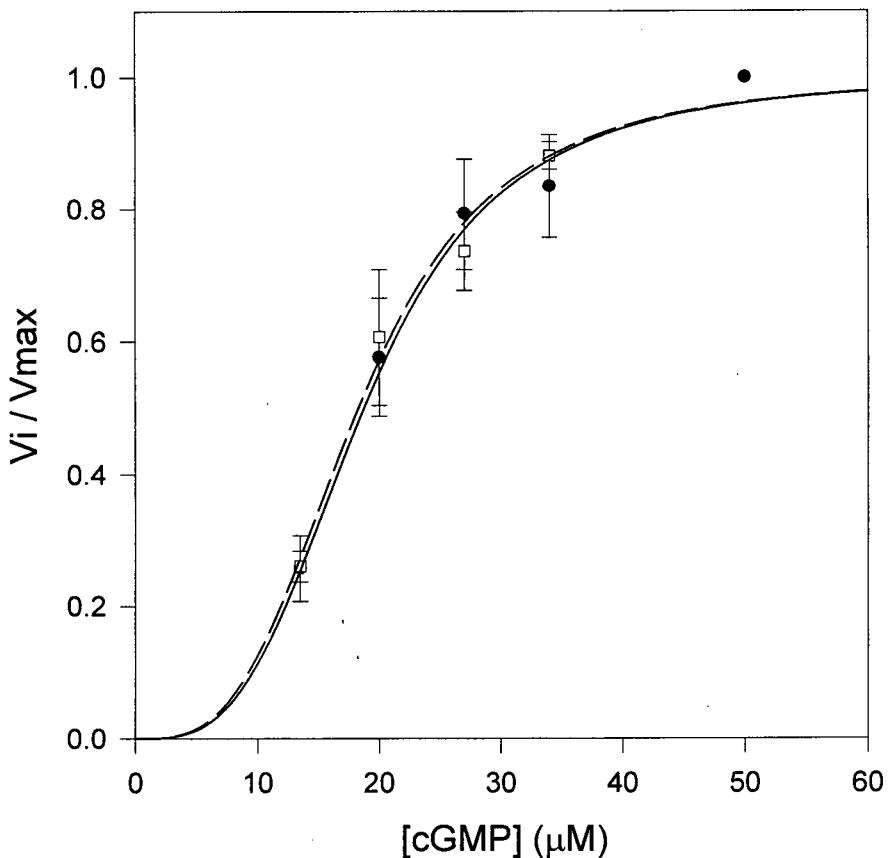


Fig. 12. **Effect of the phosphorylation of the channel on its activity.** Bovine ROS membranes were phosphorylated and solubilized in CHAPS. The phosphorylated and unphosphorylated cGMP-gated channels were subsequently purified by calmodulin-Sepharose chromatography and reconstituted into Ca^{2+} -containing vesicles for cGMP-dependent Ca^{2+} efflux assay. The initial velocities (V_i) were normalized to the velocity at high concentration (V_{max}) of cGMP (50 μM) and the data representing the mean from three experiments was analyzed by the curve fit program of Sigma Plot using the Hill equation $V/V_{\text{max}} = [\text{cGMP}]^n / ([\text{cGMP}]^n + K_m^n)$ where K_m and n are the Michaelis constant and the Hill coefficient, respectively. The nonphosphorylated cGMP-gated channel (●) had a $K_m = 18.7 \pm 0.5 \mu\text{M}$ and $n = 3.3 \pm 0.3$ and the phosphorylated channel (□) had a $K_m = 18.3 \pm 0.6 \mu\text{M}$ and $n = 3.2 \pm 0.3$.

containing lipid vesicles for analysis of cGMP-dependent calcium efflux from the vesicles. As shown in Fig. 12, no significant difference in the dose-response curves are obtained for the phosphorylated and nonphosphorylated channel. In three different experiments, the K_m calculated for the phosphorylated samples ($K_m = 18.3 \pm 0.6 \mu\text{M}$) was comparable to K_m of the nonphosphorylated control ($K_m = 18.7 \pm 0.5 \mu\text{M}$). The cyclic GMP binding cooperativity (n) was also similar for both samples ($n_{\text{control}} = 3.3 \pm 0.3$; $n_{\text{phospho}} = 3.2 \pm 0.3$), and close to the previously reported value of 3.8. (Hsu and Molday, 1993). To test whether or not the control was already phosphorylated, we performed additional experiments in which the same control was compared to channels treated with alkaline phosphatase, previously shown to remove the covalently bound phosphate groups on the β -subunit (not shown). No effect on the K_m or n values was observed by this treatment.

2.3.4 Cross-talk between channel phosphorylation and calmodulin inhibition

Calmodulin affects the activity of the Ca^{2+} ATPase isoforms 2a and 3a, by increasing the activity of the protein (Verma *et al.*, 1994). It is now known that Ca^{2+} /calmodulin binds an autoinhibitory region and releases the inhibition, thereby activating the pump. Recent experiments showed that phosphorylation by PKC is sufficient to prevent subsequent Ca^{2+} /calmodulin binding and activation of the Ca^{2+} pump isoform 2a and 3a (Enyedi *et al.*, 1997). Since Ca^{2+} /calmodulin has been shown to modulate the cGMP sensitivity of the rod CNG channels (Hsu and Molday, 1993), the effect of β -subunit phosphorylation on calmodulin binding and inhibition was investigated. Phosphorylated ROS membranes were solubilized and the β -subunit was purified by calmodulin-Sepharose affinity chromatography in presence of calcium. As shown in Fig. 13, the phosphorylated and unphosphorylated β -subunits were purified in equal amounts by calmodulin-Sepharose chromatography (Fig. 13 CB and Autoradiogram, *lane c* and *d*). On the basis of this experiment, it appears that phosphorylation of the β -subunit does not inhibit or enhance the binding of Ca^{2+} /calmodulin to the channel.

The phosphorylated and unphosphorylated channels reconstituted in calcium-containing lipid vesicles were also tested for their ability to respond to Ca^{2+} /calmodulin inhibition. The sensitivity of the unphosphorylated cGMP-gated channel for its ligand was considerably

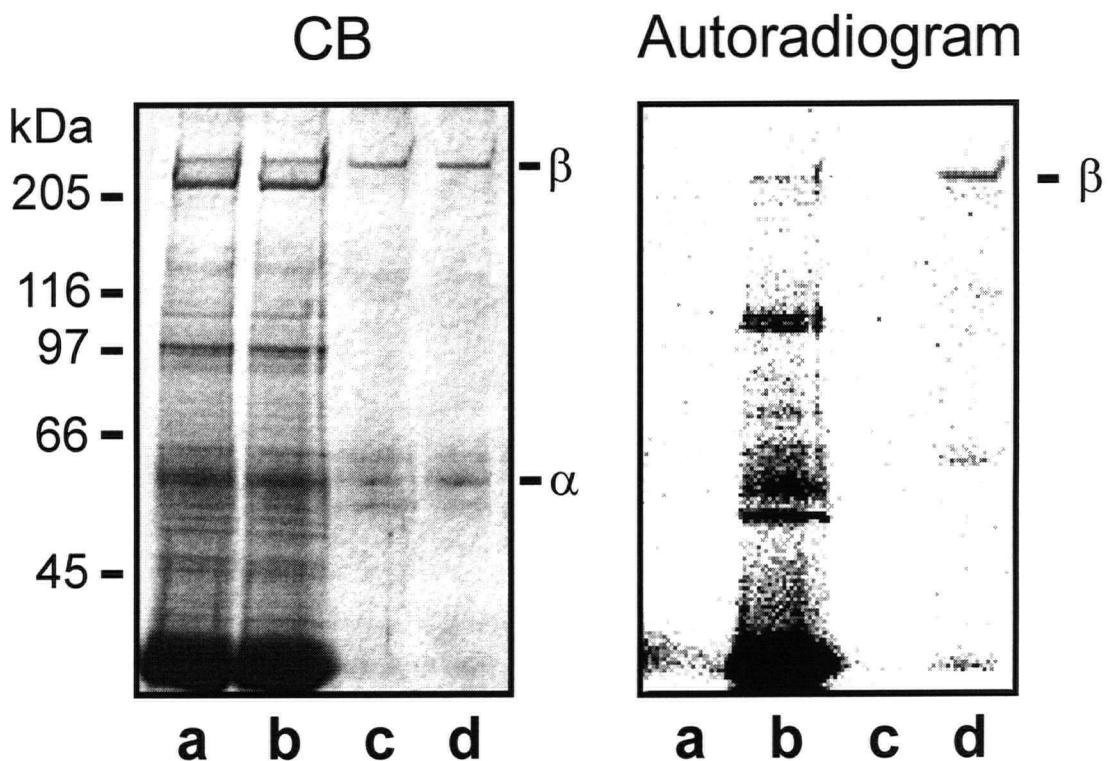


Fig. 13. Cross-talk between the phosphorylation pathway and the binding of Ca^{2+} /calmodulin to the channel β -subunit. Washed ROS membranes were phosphorylated and solubilized in CHAPS. Phosphorylated and nonphosphorylated samples were purified by calmodulin-Sepharose chromatography, loaded onto a 9% SDS-polyacrylamide gel and subjected to electrophoresis. The presence of the cGMP-gated channel complex in the purified samples was detected by Coomassie Blue staining (CB) and phosphorylation was confirmed by autoradiography. *lane a*, unbound fraction from unphosphorylated ROS sample purified by CaM-Sepharose; *lane b*, unbound fraction from phosphorylated ROS sample purified by CaM-Sepharose; *lane c*, bound fraction from unphosphorylated ROS sample purified by CaM-Sepharose; *lane d*, bound fraction from phosphorylated ROS sample purified by CaM-Sepharose.

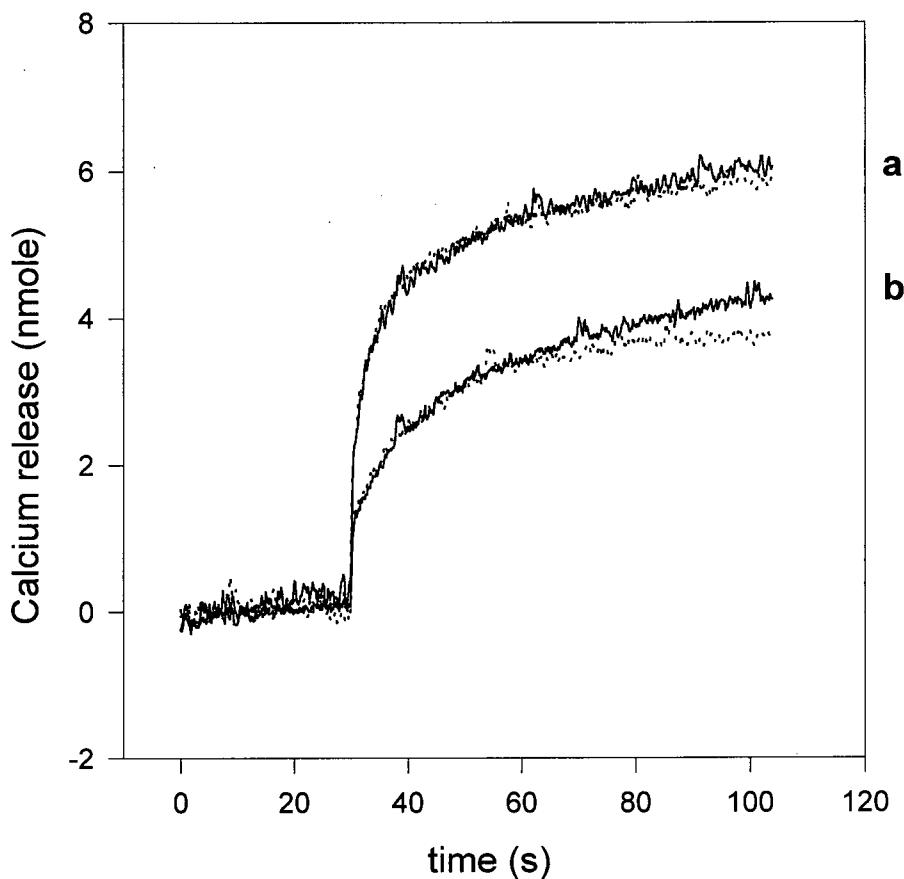


Fig. 14. **Effect of phosphorylation on the calmodulin regulation of the cGMP-gated channel.** Phosphorylated (dashed line) and unphosphorylated (solid line) channels were purified by CaM-Sepharose chromatography and reconstituted into Ca^{2+} -containing lipid vesicle for cGMP-dependent Ca^{2+} efflux assay. A cGMP concentration of 27 μM was used to initiate the efflux of Ca^{2+} either in the presence (*b*) or absence (*a*) of 7.5 nM calmodulin.

decreased by the addition of 7.5 nM calmodulin as previously shown. A similar effect was observed for the phosphorylated channel (Fig. 14, *curve a* and *b*).

2.4 DISCUSSION

In this study the rod cGMP-gated channel has been shown to be phosphorylated *in vitro* by an uncommon protein kinase endogenous to ROS. To date, several phosphoproteins have been reported in ROS (Kapoor and Chader, 1984; Szuts, 1985; Binder *et al.*, 1989; Farber *et al.*, 1979). Although, the identity of most of these proteins is unknown, in some instances efforts have been made to link their phosphorylation to the phototransduction process. The phosphorylation of some proteins like rhodopsin (Kühn and Dreyer, 1972; Miller *et al.*, 1986) and phosducin (Lee *et al.*, 1990; Yoshida *et al.*, 1994) has been successfully shown to be an integral part of the ROS phototransduction pathway, whereas the phosphorylation of others such as the cGMP-gated channel and GC has not (Wolbring and Schnetkamp, 1995; Gordon *et al.*, 1992). In a preliminary study, Szuts reported (1985) the phosphorylation of a 220- and 240-kDa membrane proteins of frog ROS in a cGMP- and light-dependent manner. Although it was suggested that the 220-kDa protein was a rim protein (now known as the ABCR), the identity of the 240-kDa protein was unknown at that time. Experiments carried out here show that the 240-kDa protein in bovine ROS, now known as the cGMP-gated channel β -subunit, is also phosphorylated *in vitro* but this reaction occurs independently of light and cGMP. Similar results obtained from the light-dependent phosphorylation experiments were observed before and it was suggested that the low phosphate incorporation by the 240-kDa protein was responsible for this observation (Szuts, 1985). Consistent with previous reports (Kühn and Dreyer, 1972; Bownds *et al.*, 1972), the results presented here show that rhodopsin incorporates a significantly lower amount of phosphate groups under dark condition (between 12-15 times less as determined by laser densitometry) than under light conditions. In contrast, light-dependent phosphorylation of the β -subunit was not observed suggesting either that this reaction is not light-dependent or there is a loss or deactivation of the light-dependent kinase during the preparation and the hypotonic lysis of ROS membranes.

The second messengers cGMP and cAMP were also used to determine if these nucleotides alter phosphorylation of the channel (Binder *et al.*, 1989; Farber *et al.*, 1979; Szuts,

1985). In this study, the addition of cGMP or Br-cGMP to a mixture of endogenous protein kinases failed to alter the level of phosphorylation of the β -subunit.

These results prompted a search for physiological protein kinase regulators that may link channel phosphorylation to visual transduction or related processes in ROS. The use of various protein kinase regulators such as Ca^{2+} /calmodulin (CaM kinase), cAMP (PKA), Ca^{2+} , EDTA, and phorbol ester (PKC) did not alter the phosphorylation of the β -subunit. The lack of protein kinase regulation is quite surprising and raises some doubts about the physiological involvement of the phosphorylation of the β -subunit in ROS. There are a few examples of protein kinases characterized for their apparent lack of regulation *in vivo* (Allende and Allende, 1995). The ubiquitous protein kinase CK2 is one such enzyme. In addition to its puzzling lack of regulation, the unusual preference of this kinase for Ser/Thr surrounded by clusters of acidic residues makes it a good candidate for the phosphorylation of the channel β -subunit, especially in the N-terminus where many regions rich in glutamic acid residues are found. To date it is unclear whether or not the GARP-part of the β -subunit is phosphorylated. Results obtained from limited proteolysis experiments and presented here show undoubtedly that the β' -part of the channel β -subunit is phosphorylated. More experiments are needed to localize the phosphorylation site(s) in this subunit.

The function of the various splice variants of GARP found in ROS is unknown. Although the presence of the GARP part on the β -subunit does not affect the electrophysiological properties of the channel (Körshen *et al.*, 1995), it has been suggested that GARP may play important physiological roles by maintaining the spatial distribution of the channel in the membranes and/or maintaining the structure of the outer segments through its interaction with cytoskeletal proteins (Colville and Molday, 1996). A possible connection between the phosphorylation of the β -subunit and the maintenance of ROS structure through GARP may explain partially the apparent lack of regulation observed in this study.

To examine whether phosphorylation of the β -subunit modulates cGMP-gated channels, the activity of phosphorylated and unphosphorylated channels was measured using the cGMP-dependent Ca^{2+} efflux assay. Using this assay, previous studies have established that the half-maximal velocity of the native cGMP-gated channel reconstituted in lipid vesicles is reached at cGMP concentration ranging from 18 to 23 μM (Cook *et al.*, 1986; Koch *et al.*, 1987). In our

assay, we repeatedly observed that both the phosphorylated and unphosphorylated samples have similar kinetic behaviors, with K_m values of 18.3 ± 0.6 and $18.7 \pm 0.5 \mu\text{M}$, respectively. In all the samples tested, the cooperativity for cGMP was equivalent to the values reported in the literature ($n \sim 3.8$) and similar between the phosphorylated and unphosphorylated channel. The little variation in K_m values calculated from our experiments is not significant enough to justify the involvement of phosphorylation in regulating the Ca^{2+} influx. The low phosphate incorporation on the β -subunit calculated suggests that the phosphorylation reaction is far from optimum (one to two phosphates per five β -subunit) and may explain why no major changes in cGMP sensitivity is observed upon phosphorylation.

In opposition to our findings, Gordon and co-workers (1992) suggested that two or more phosphorylation sites affect the cGMP sensitivity of the channel in a reciprocal manner. Phosphorylation of the channel was not directly shown, but rather it was observed that the use of Ser/Thr protein phosphatase inhibitors alter the ligand affinity of the frog rod cGMP-gated channel. From this observation, they drew the conclusion that the activity of the channel is modulated directly, or indirectly through a channel-associated regulatory protein by interplay of phosphorylation and dephosphorylation. Contradictory results obtained from patch-clamp experiments performed on *Xenopus* oocytes expressing the recombinant α -subunit suggest that tyrosine, but not Ser/Thr phosphorylation affects the cGMP sensitivity of the channel (Molokanova *et al.*, 1997). This was observed by testing the effect of tyrosine kinase and tyrosine phosphatase inhibitors along with Ser/Thr phosphatase inhibitors on the cGMP-induced current. The authors associated this divergence to the different electrophysiological properties exhibited by the recombinant homomeric channel α -subunit versus those of the native channel. Under all the conditions tested here, the channel β -subunit was invariably phosphorylated where the α -subunit was not. In addition, phosphate groups were only found on serine residues. It is likely that the effect observed by Molokanova and co-workers is due to endogenous *Xenopus* oocyte protein tyrosine kinase and phosphatases not found in ROS. A similar observation might explain the results obtained by Gordon, since frog ROS exhibit some differences in protein properties and physiology, attributable to evolutionary divergence between species (Koutalos, 1992; Whalen *et al.*, 1990; Hayashi and Yamazaki, 1991; Fong *et al.*, 1985).

Enyedi and co-workers (1997) showed that phosphorylation of the Ca^{2+} pump isoforms 2a and 3a prevents the binding and modulation of Ca^{2+} /calmodulin. Inspired by these results, the effect of calmodulin on the phosphorylation of the channel β -subunit was studied. Phosphorylation of the β -subunit was found to have no effect on the binding of calmodulin to the channel complex. Activity measurements also indicated that the phosphorylation of the β -subunit and calmodulin modulation of the channel constitute two independent mechanisms in ROS.

CHAPTER 3

DETECTION OF A CK2-LIKE PROTEIN KINASE IN THE OUTER SEGMENT OF ROD PHOTORECEPTORS

3.1 MATERIALS

3.1.1 Animal tissues

Fresh bovine eyes were obtained from J and L Beef Ltd (Surrey, BC) and frozen bovine retinas were purchased from Schenk Packing Co (Stanwood, WA).

3.1.2 Chemicals

[γ -³²P]GTP was purchased from New England Nuclear. The Ser/Thr kinase inhibitors bisindolylmaleimide I, calphostin C, H-89, KN-93, ML-7, PKG inhibitor peptide, DRB, staurosporine and the tyrosine kinase inhibitor lavendustin A were obtained from Calbiochem (La Jolla, CA). Heparin and dephosphorylated bovine milk casein were purchased from Sigma. Protein G-agarose was from Genex Corporation (Gaithersburg, MD). The recombinant, fully active human casein kinase II was purchased from Upstate Biotechnology (Lake Placid, NY). All other chemicals were obtained from Sigma or Fisher Scientific or from distributors as listed in Chapter 2 (section 2.2.1).

3.1.3 Immunoreagents

The monoclonal and polyclonal antibodies against the α -subunit of CK2 were purchased from Calbiochem (La Jolla, CA).

3.2 METHODS

3.2.1 Preparation of bovine ROS, ROS membranes and ROS lysates

Bovine ROS were prepared under dim red light as described previously (Molday and Molday, 1987, 1999). ROS membranes and ROS lysates (cytosol fraction) were prepared as follows: 10 mg (protein) of isolated ROS in 20% w/v sucrose, 10 mM β -D-glucose, 10 mM

taurine, 0.25 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4 were centrifuged in a TL100.4 rotor (Beckman instruments) at 40,000 rpm for 10 min and lysed in 5 to 10 volumes of hypotonic buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM DTT, and 2 mM EDTA. After 5 min incubation on ice, the samples were centrifuged at 30,000 rpm for 10 min in a TLA45 rotor using a Beckman Optima ultracentrifuge. The membrane pellet was resuspended in 5 to 10 volumes of hypotonic buffer and the procedure was repeated two more times. The pellet was finally resuspended in 10 mM HEPES-KOH, 1 mM DTT (DTT was omitted when the sample was used for affinity chromatography) to give a final protein concentration of 7-9 mg/ml determined by the BCA assay. The lysates from two hypotonic washes were pooled and used for protein kinase assays and western blot analyses. The KCl concentration of ROS lysate was adjusted to 150 mM.

3.2.2 Phosphorylation of rod cGMP-gated channels

The experimental procedure was carried out as described in Chapter 2 (section 2.2.3).

3.2.3 Purification of cGMP-gated channels

The procedure was carried out as described in Chapter 2 (section 2.2.2) with the following modification. The cGMP-gated channel complex was purified using PMc 1D1-Sepharose, exclusively.

3.2.4 Inhibitor mapping

Washed ROS membranes resuspended in 50 µl of ROS lysate containing 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂, 1 mM DTT were pre-incubated in presence of various serine, threonine and tyrosine kinase inhibitors for 10 min at 30°C (Table III). The phosphorylation reaction was initiated as mentioned previously and the samples incubated for 10 more minutes. The reaction was stopped by the addition of an equal volume of electrophoresis sample buffer. Samples (40 µl) were subjected to electrophoresis on 8.5% SDS-polyacrylamide gels. The gels were stained with coomassie blue and the radioactivity was detected by autoradiography.

3.2.5 Immunofluorescence microscopy

Immunofluorescence microscopy was carried out using the method of Johnson and Blanks (1984) with some modifications. Fresh bovine eyes were dissected and fixed by immersion in 4% (w/v) paraformaldehyde-PBS, pH 7.4 at 25°C for 5 h. Retina pieces were cut (~0.5 cm²) and the paraformaldehyde gradually replaced with a solution containing 10% (w/v) sucrose in PBS, pH 7.4. Fixed retina pieces were frozen in Tissue Tek over the surface of liquid nitrogen. The embedded retinas were sectioned and used for immunolabeling within 72 h. Ten-μm cryosections of bovine and rat retinas were first blocked with 10% goat serum in PBS containing 0.2% Triton X-100 for 20 min at 25°C. The sections were then incubated overnight at 25°C with either anti-CK2α polyclonal antiserum or an anti-CK2α monoclonal antibody diluted 5 and 10 times, respectively, in PBS containing 0.1% Triton X-100 and 2% goat serum. As a control, the anti-CK2 rabbit polyclonal antiserum was pre-absorbed with a 20-amino acid residue synthetic peptide which had been used as the immunogen for the production of antibody. The labeled sections were washed four times with PBS and then incubated 1 h at 25°C with either goat anti-rabbit (1:1000) or goat anti-mouse (1:800) Ig conjugated with Cy3 fluorescent dye. The cryosections were subsequently washed four more times with PBS. Immunofluorescence microscopy was carried out using a Zeiss Axioplan fluorescent microscope. Photographs were taken using Kodak Tri-X pan 400 films.

3.2.6 Phosphorylation of the cGMP-gated channel β-subunit by exogenous CK2

The cGMP-gated channel complex was bound to the PMc 1D1-Sepharose affinity matrix (described above) and phosphorylated in detergent by the addition of 0.5 μg of exogenous casein kinase II (2.3 U). The 100 μl reaction mixture contained 50 μl of the channel bound to the PMc 1D1-Sepharose matrix in 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂, 3 mM MgCl₂, 100 μM ATP and 5 μCi [γ -³²P]ATP. The phosphorylation reaction was initiated by adding casein kinase II and the samples were incubated for 10 min at 30°C. The reaction was stopped by washing away the excess ATP in ice-cold solubilization buffer. The channel was eluted using the electrophoresis sample buffer and loaded onto 9% SDS-polyacrylamide gels. Phosphoproteins were detected by autoradiography.

3.2.7 Phosphorylation of casein by endogenous ROS kinases

ROS (8 mg protein) were hypotonically lysed, washed thrice in 1-2 ml of a 10 mM HEPES-KOH, pH 7.4, 1 mM DTT solution and spun at 40,000 rpm for 10 min. The ROS lysate (soluble fraction) was collected after the second wash and kept at -70°C until further use. A 100 µl aliquot of the ROS lysate was diluted 1:3 in 10 mM HEPES-KOH, pH 7.4 and incubated for 4 h with the monoclonal antibody anti-CK2α at 4°C. The immunocomplex was removed by immunoprecipitation using protein G-Sepharose and the ROS lysate depleted of CK2 was utilized in the casein phosphorylation experiments.

The 50 µl phosphorylation reaction mixture contained 1 µg casein dissolved in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂, 3 mM MgCl₂, 100 µM ATP and 5 µCi [γ -³²P]ATP. The phosphorylation reaction was initiated by adding the ROS lysate CK2 depleted or undepleted to the reaction mixture (diluted 1:30) and the samples were incubated for 10 min at 30°C. The reaction was stopped by adding one volume of electrophoresis sample buffer. The samples were applied onto a 10% SDS-polyacrylamide gel and the phosphorylation state of casein was observed by autoradiography.

3.2.8 Reconstitution of the CK2-phosphorylated channel in lipid vesicles and cGMP-dependent Ca²⁺ efflux assay

ROS were washed thrice in a hypotonic buffer containing 5 mM HEPES-KOH, pH 7.4, 150 mM KCl, 1 mM DTT and 4 mM EDTA and the membrane proteins were solubilized at 4°C for 15 min in 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 2 mM CaCl₂ and 150 mM KCl. The cGMP-gated channel was purified by CaM-Sepharose affinity chromatography and phosphorylated for 20 min at 30°C by the addition of 0.5 µg (2.3 U) recombinant human CK2 in a 200 µl reaction mixture containing 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂, 5 µCi [γ -³²P]ATP, 100 µM ATP, 3 mM MgCl₂, 100 µl CaM-Sepharose beads (bed volume). The cation channel complex was eluted after phosphorylation using a buffer containing 18 mM CHAPS, 5 mM HEPES-KOH, pH 7.4, 2 mg/ml asolectin, 150 mM KCl and 2 mM EGTA. The purified cGMP-gated channel was subsequently reconstituted in lipid vesicles as described above (section 2.2.7). Activity measurements were carried out as

described by Cook *et al.* (1986, 1987). Lipid vesicles containing the CK2-phosphorylated and unphosphorylated purified cGMP-gated channel were passed through a Chelex column to remove the excess calcium outside the vesicles. The reconstituted channel (0.3 ml) was added to 1.7 ml of Arsenazo III. The calcium released from the vesicles was triggered by the addition of cGMP and monitored using a dual wavelength spectrophotometer (SLM Aminco DW2000). The data obtained for the cGMP-dependent Ca^{2+} efflux assay was analyzed using the curve fit program of Sigma Plot (Jandel Scientific). The Michaelis constant (K_m) and the Hill coefficient (n) were calculated by plotting the initial velocity over the maximal velocity at saturating cGMP concentration as a function of cGMP concentration.

3.2.9 Polyacrylamide gel electrophoresis and western blotting

Protein samples were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis using the buffer system of Laemmli (1970). Protein samples contained in 8%-12% SDS-polyacrylamide gel slices were either stained by coomassie blue or electroblotted onto Immobilon-P membranes in 25 mM Tris-HCl pH 8.4, 190 mM glycine and 5-10% methanol. For western blotting, the membranes were blocked in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.5, 140 mM NaCl, and 2 mM KCl) containing 2% milk for 20 min at 25°C and then incubated for 45 min at 25°C in a PBS solution containing 0.02% milk and 2 $\mu\text{g}/\text{ml}$ of anti-casein kinase II α -subunit antibody. The membranes were washed thoroughly with PBS and incubated with sheep anti-mouse immunoglobulin (Ig) conjugated to horseradish peroxidase at 1/6000 dilution. CK2 was detected by enhanced chemiluminescence.

3.3 RESULTS

3.3.1 The cGMP-gated channel is phosphorylated by soluble and membrane-associated protein kinases

To examine whether phosphorylation of the channel β -subunit is due to a ROS soluble or membrane-associated protein kinase, membrane proteins were incubated either with or without ROS lysate in the presence of [γ -³²P]ATP. As shown in Fig. 15 (CB, *lane c* and *d*), the amount of β -subunit purified by affinity chromatography was roughly the same in both

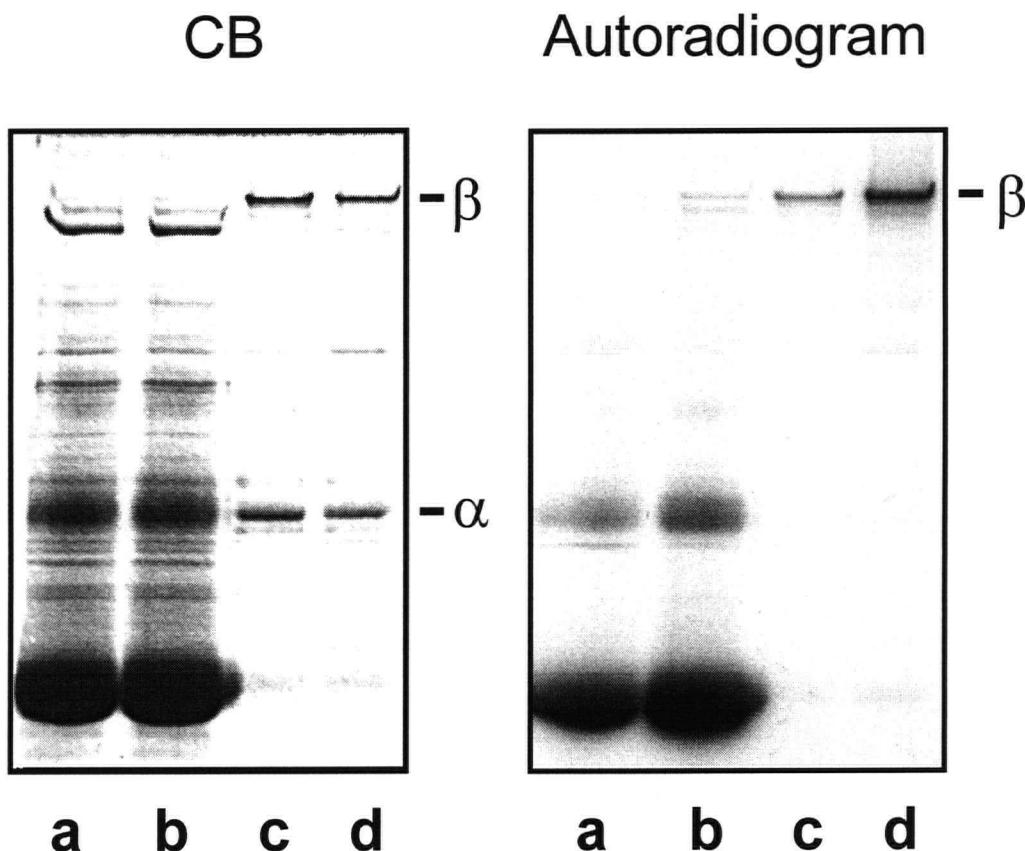


Fig. 15. Effect of ROS cytosol on the phosphorylation of the cGMP-gated channel β -subunit. ROS membranes were hypotonically lysed, washed by centrifugation and resuspended in either HEPES buffer or ROS lysate containing endogenous ROS soluble proteins. The phosphorylation reaction was initiated by addition of $[\gamma^{32}\text{P}]$ ATP and the samples were incubated for 15 min at 30°C. The phosphorylated membranes were washed in HEPES and solubilized in CHAPS and the cGMP-gated channel complex purified by PMc 1D1-Sepharose chromatography. The samples were loaded onto a 9% SDS-polyacrylamide gel and subjected to electrophoresis. ROS membrane proteins were detected by Coomassie Blue (CB) staining and phosphorylation determined by autoradiography. *lane a*, unbound fraction from sample phosphorylated in HEPES; *lane b*, unbound fraction from sample phosphorylated using a ROS lysate; *lane c*, bound fraction from sample phosphorylated in HEPES; *lane d*, bound fraction from sample phosphorylated using a ROS lysate.

samples, but the labeling was nearly two-fold greater in the sample incubated with a lysate containing ROS soluble proteins (Fig. 15. autoradiogram, *lane c* and *d*). At this stage it is not clear whether the β -subunit is the substrate for multiple kinases or a soluble protein kinase that also associates with ROS membranes.

3.3.2 Inhibitor mapping

Various Ser/Thr and Tyr kinase inhibitors were used to identify the kinase(s) responsible for the phosphorylation of the cGMP-gated channel β -subunit (Table III and V). ROS membranes were pre-incubated with different protein kinase inhibitors and the phosphorylation reaction was initiated by addition of [γ -³²P]ATP. As shown on the left autoradiogram in Fig. 16, the relative labeling intensities of the channel β -subunit were not decreased by the addition of bisindolylmaleimide I (*lane b*), calphostin C (*lane c*), H-89 (*lane d*), KN-93 (*lane e*), ML-7 (*lane f*), the PKG inhibitor (*lane g*), and even staurosporine (*lane h*) when compared to a positive control in which no protein kinase inhibitors were added (*lane a*). Rhodopsin provides a good control to evaluate the potency of some inhibitors since the protein kinases responsible for its phosphorylation have been purified and characterized (Lee *et al.*, 1981; Palczewski *et al.*, 1988; Williams *et al.*, 1997; Udovichenko *et al.*, 1997). Addition of bisindolylmaleimide I, a potent and specific PKC inhibitor, significantly reduces the phosphorylation of rhodopsin as well as most phosphoproteins found in ROS (Fig. 16, *lane b*) whereas phosphorylation of the β -subunit of the channel remains the same. Staurosporine, a broad-range Ser/Thr kinase inhibitor also reduces significantly the rhodopsin phosphorylation by inhibiting PKC and rhodopsin kinase, but has no effect on the phosphorylation of the channel β -subunit (Fig. 16, *lane h*). However DRB, a potent and specific inhibitor of casein kinase II completely abolished the phosphorylation of the β -subunit when used at concentration equivalent to ten times the IC₅₀ (*lane j*). Except for rhodopsin, the labeling of other ROS phosphoproteins was unchanged by the addition of DRB when compared to a positive control in which no protein kinase inhibitors were added (*lane i*). Although no inhibition of rhodopsin phosphorylation by the enantiomer 5,6-dichloro-1-(β -D-ribofuranosyl) benzimidazole

Table V. Inhibitor mapping

Inhibitor	Target kinase	Concentration used	Effect
Bisindolylmaleimide I	PKC (α , β , δ , ε)	200 μ M	-
Calphostin C	PKC	5 μ M	-
H-89	PKA	0.8 mM	-
KN-93	CaM kinase II	7.4 μ M	-
ML-7	MLCK	0.84 mM	-
Protein kinase G inhibitor	PKG	5.5 mM	-
Lavendustin A	EGF receptor tyr kinase pp60 ^{c-SRC}	10 μ M	-
DRB	CK2	60 μ M	+
Heparin	CK2 and other kinases	140 nM	+/-
Staurosporine	Broad-range Ser/Thr protein kinases	2 μ M	-

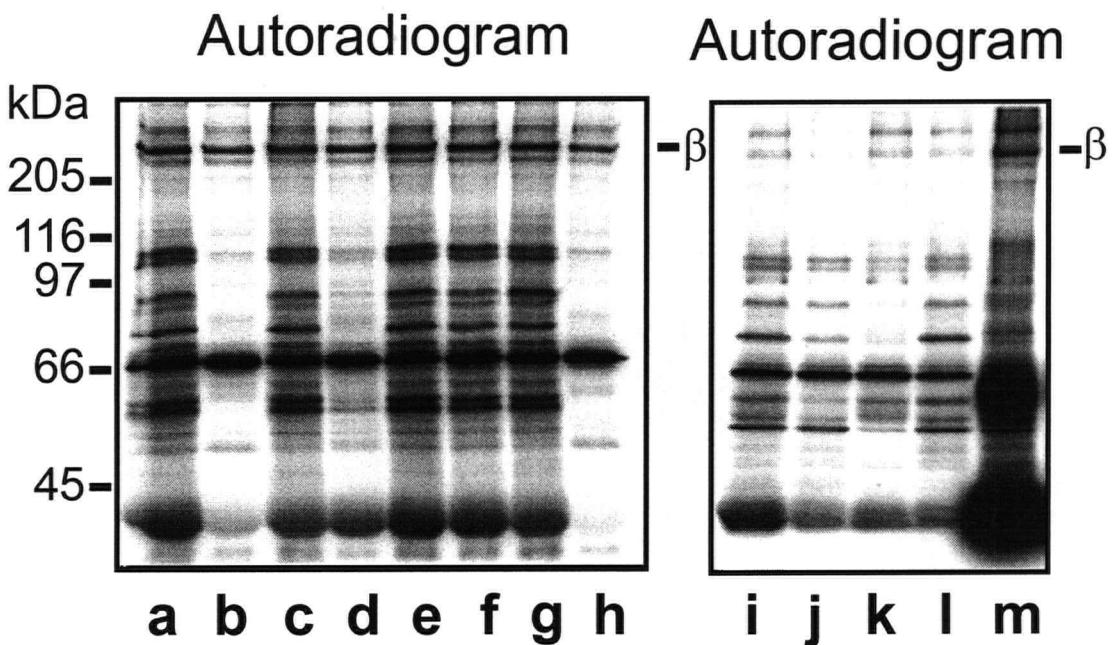


Fig. 16. Screening of endogenous ROS protein kinase using Ser/Thr protein kinase inhibitors. Washed ROS membranes were resuspended in ROS lysate containing endogenous ROS protein kinases and pre-incubated in absence or presence of several Ser/Thr protein kinase inhibitors for 10 min at 30°C. The phosphorylation reaction was initiated by the addition of [γ -³²P]ATP or [γ -³²P]GTP in the presence of 3 mM MgCl₂ or MnCl₂, respectively and the samples were incubated for 10 min. The reaction was stopped by the addition of electrophoresis sample buffer and the samples loaded onto 9% SDS-polyacrylamide gels. After electrophoresis, the gels were dried and the phosphoproteins identified by autoradiography. *lane a* and *i*, uninhibited ROS; *lane b*, 200 μ M bisindolylmaleimide I; *lane c*, 5 μ M calphostin C; *lane d*, 0.8 mM H-89; *lane e*, 7.4 μ M KN-93; *lane f*, 0.84 mM ML-7; *lane g*, 5.5 mM PKG inhibitor; *lane h* and *k*, 2 μ M Staurosporine; *lane j*, 60 μ M 5,6-dichloro-1-(β -D-ribofuranosyl) benzimidazole; *lane l*, 140 nM Heparin; *lane m*, phosphorylation of ROS membranes using [γ -³²P]GTP.

has been reported, the inhibitor 5,6-dichloro-1-(β -ribofuranosyl) benzimidazole is known to inhibit effectively rhodopsin kinase ($K_i \sim 4 \mu\text{M}$; Zollner, 1993). Since the inhibition of rhodopsin by the D enantiomer was fairly weak, we can assume that the solution was fairly pure. Surprisingly, the addition of heparin did not have significant effect on the kinase(s) involved in the channel phosphorylation, even when used one hundred time above the IC_{50} for CK2 (*lane l*). When compared to the positive control (*lane i*), half the radioactivity was still detected on the β -subunit after phosphorylation in the presence of heparin (*lane l*). Besides its extreme sensitivity to heparin inhibition ($IC_{50}=1.4 \text{ nM}$), CK2 can be distinguish from other protein kinases by its ability to use both ATP and GTP as phosphate donors (Gatica *et al.*, 1993). As shown in Fig. 16, the β -subunit of the cGMP-gated channel, rhodopsin and other ROS phosphoproteins incorporate γ -phosphate groups of GTP in the presence of Mn^{2+} (*lane m*). In the presence of Mg^{2+} , no labeling was seen on the channel β -subunit or most ROS proteins, suggesting that the presence of manganese is crucial for the transfer of γ -phosphate groups from GTP (not shown). This agrees with previous findings that GTP is the preferred substrate for casein kinase II with Mn^{2+} and ATP is the preferred substrate with Mg^{2+} (Gatica *et al.*, 1993).

3.3.3 Casein kinase II in photoreceptor rod outer segments

Identification of a CK2 isoform in bovine ROS

An anti-casein kinase II monoclonal antibody that cross-reacts with human, bovine and rat CK2 α -subunit was used to investigate the presence of casein kinase II in bovine ROS. The reactivity of the antibody is shown by western blot analysis in Fig. 17. When compared with a human recombinant CK2 (*lane a*), a higher molecular weight protein recognized by the monoclonal antibody was detected in bovine ROS (*lane b*). The protein is found predominantly in a lysate containing ROS soluble proteins (*lane c*) ; very little is found associated with ROS membranes (*lane d* and *e*). Our results also indicate that this isoform of CK2 does not co-precipitate with the cGMP-gated channel (*lane f*).

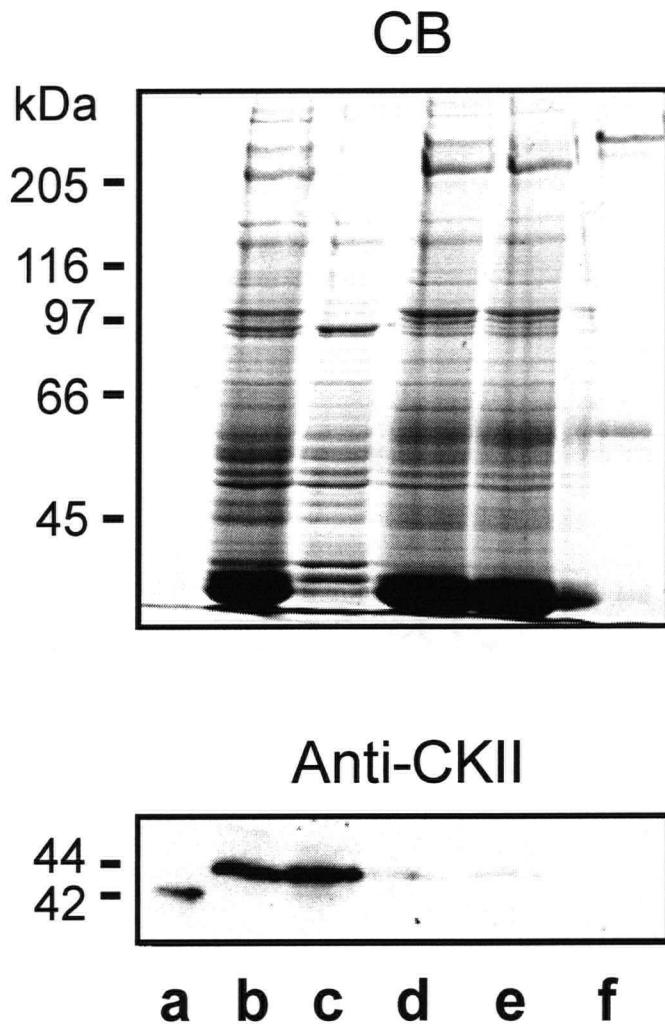


Fig. 17. Western blot of ROS, ROS cytosol, solubilized ROS membrane and purified cGMP-gated channel labeled with a monoclonal antibody against CK2 α -subunit. Human recombinant casein kinase II (lane *a*), bovine ROS (lane *b*), ROS cytosol (lane *c*), solubilized ROS membranes (lane *d*), unbound (lane *e*) and bound (lane *f*) fraction from PMc 1D1-Sepharose were subjected to SDS gel electrophoresis and either stained with Coomassie Blue (CB) or transferred to Immobilon-P membranes and labeled with an anti-CK2 α monoclonal antibody. Lanes contained either 40 μ g of ROS or 3 μ g of purified channel.

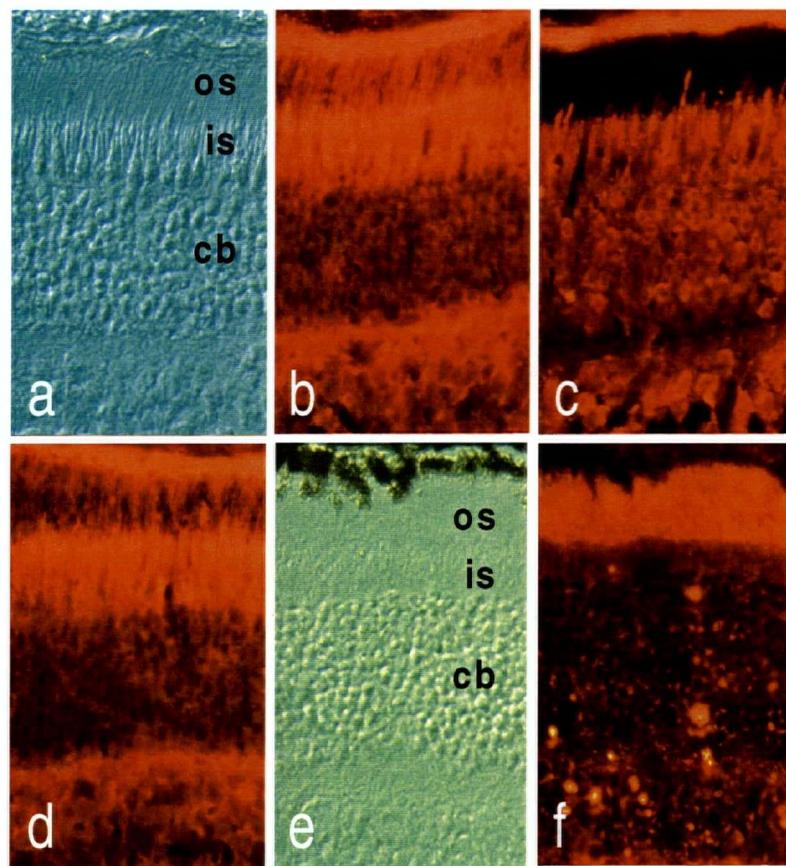


Fig. 18. Localization of CK2 α -subunit in the photoreceptor layer of bovine and rat retinas by immunofluorescence microscopy. Cryosection of bovine and rat retinas were labeled with either anti-CK2 α monoclonal or polyclonal antibodies and Cy3-labeled goat anti-mouse or anti-rabbit Ig, respectively. Fluorescent micrographs of bovine retina labeled with (b) anti-CK2 α polyclonal antibody; (c) the same antibody pre-absorbed with a 20-amino acid residue CK2 α blocking peptide; (d) anti-CK2 α monoclonal antibody. Fluorescent micrograph of rat retina labeled with (f) anti-CK2 α monoclonal antibody. Differential interference contrast micrographs of bovine (a) and rat (e) photoreceptor layer showing the outer segment (os), the inner segment (is) and the cell body (cb).

Localization of casein kinase II in ROS by immunofluorescence microscopy

In order to verify that the CK2 isoform is a true ROS component, rat and bovine retinas cryosections were labeled with anti-CK2 α monoclonal antibody for immunofluorescence microscopy. As shown in Fig. 18, casein kinase II was observed in ROS from both species, but the labeling was more intense in rat rod outer segments (*d* and *f*). Labeling was also observed in many other retinal cell layers and for this reason, we examined the distribution of CK2 using another CK2 α antibody for which a control peptide was available. The use of a polyclonal anti-CK2 α antibody confirmed that casein kinase II or a CK2 isoform is found in ROS (*b*). Labeling of the ROS was essentially inhibited by the addition of a blocking peptide corresponding to the residues 70-89 of CK2 α -subunit. However, the labeling of the inner segment and cell body layers as well as other retina cell layers was still observed (*c*). Western blot analyses revealed that the anti-CK2 α antiserum labels other proteins in a retina mock sample, suggesting the presence of at least another antibody in the polyclonal antiserum. Since the labeling of these retinal cells can not be inhibited by the addition of a CK2 blocking peptide, it is likely that this other antibody recognize a more common antigen, different from CK2 and present in other retinal cells, but absent from ROS (not shown).

3.3.4 The rod cGMP-gated channel is phosphorylated *in vitro* by casein kinase II

To examine whether or not the cGMP-gated channel is a potential substrate for casein kinase II, the affinity-purified channel was incubated with human recombinant CK2 in the presence of [γ -³²P]ATP. Figure 19A shows that the β -subunit, and possibly the α -subunit of the cGMP-gated channel, is indeed a target for casein kinase II (*lane a*).

With the exception of heparin inhibition, all the characteristic features of casein kinase II including the inhibition by DRB, the ability to use both GTP and ATP as phosphate donor and the lack of inhibition by staurosporine (Tuazon and Traugh, 1991; Zollner, 1993) have been observed in ROS using the channel β -subunit as substrate.

To further characterize a ROS protein kinase that seems to possess most CK2 properties, the ability of ROS endogenous protein kinase to phosphorylate bovine milk casein was examined. As shown in Fig. 19B, casein was a target for endogenous ROS protein kinases (Autoradiogram, *lane b* and *c*). In addition, a ROS lysate sample depleted of the endogenous

A
Autoradiogram

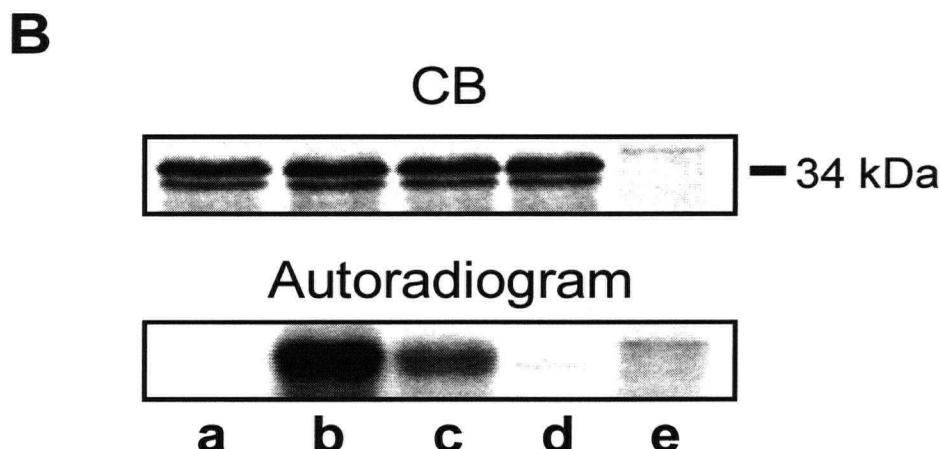
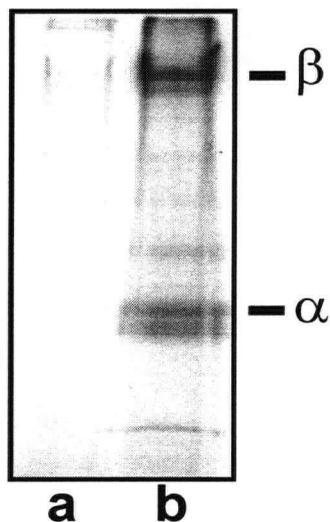


Fig. 19. Phosphorylation of the cGMP-gated channel by a human recombinant CK2 and phosphorylation of casein using a ROS lysate. *A*, Washed ROS membranes were solubilized in CHAPS and the cGMP-gated channel purified by PMc 1D1-Sepharose. Subsequently, the native channel complex bound to the PMc 1D1-Sepharose matrix was phosphorylated in detergent by the addition of exogenous casein kinase II (2.3 U), eluted from the column and subjected to SDS gel electrophoresis. Phosphorylation of the channel was detected by autoradiography. *lane a*, purified channel incubated with [γ -³²P]ATP; *lane b*, purified channel incubated with exogenous CK2 and [γ -³²P]ATP. *B*, the ability of a lysate containing endogenous ROS protein kinases to phosphorylate bovine milk casein *in vitro* was tested. After phosphorylation, the samples (1 μ g casein) were applied to a 10% SDS-polyacrylamide gel and subjected to electrophoresis. The phosphorylation state of casein was observed by autoradiography. *lane a*, casein; *lane b*, casein phosphorylated using a ROS lysate; *lane c*, casein phosphorylated using a ROS lysate diluted (1:30); *lane d*, casein phosphorylated using a CK2-depleted ROS lysate; *lane e*, phosphorylated ROS lysate.

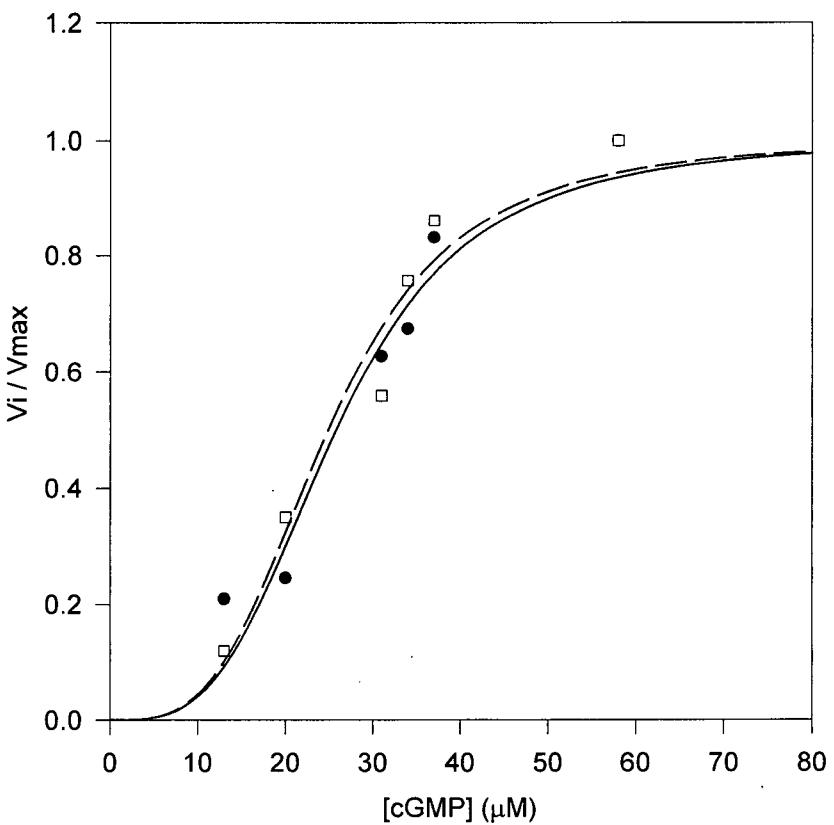


Fig. 20. Effect of CK2 phosphorylation on the cGMP-gated channel activity. Washed ROS membranes were solubilized in CHAPS and the cGMP-gated channel was purified by calmodulin-Sepharose. The native channel complex was subsequently phosphorylated by the recombinant human CK2 and reconstituted into Ca^{2+} -containing vesicles for cGMP-dependent Ca^{2+} efflux assay. The initial velocities (V_i) were normalized to the velocity at saturating concentration (V_{max}) of cGMP (58 μM) and the data was analyzed by the curve fit program of Sigma Plot using the Hill equation $V/V_{\text{max}} = [\text{cGMP}]^n / ([\text{cGMP}]^n + K_m^n)$ where K_m and n are the Michaelis constant and the Hill coefficient, respectively. The nonphosphorylated cGMP-gated channel (●) had a $K_m = 25.8 \mu\text{M}$ and $n = 3.3$ and the CK2-phosphorylated channel (□) had a $K_m = 24.9 \mu\text{M}$ and $n = 3.4$.

CK2-like protein failed to phosphorylate casein *in vitro* (Fig. 19B, *lane d*), suggesting that the protein previously identified by western blot and immunofluorescence microscopy is most likely a casein kinase II isoform or a CK2 α -subunit variant.

3.3.5 Effect of CK2 phosphorylation on the activity of the cGMP-gated channel

The activity of the CK2-phosphorylated cGMP-gated channel was compared to that of the unphosphorylated channel. As shown in Fig. 20, phosphorylation of the channel α - and β -subunit by exogenous CK2 did not have significant effect on its activity. The cGMP sensitivity of the CK2-phosphorylated and unphosphorylated channel was comparable with K_m values of 24.9 and 25.8 μM , respectively. The cyclic GMP gating cooperativity was basically identical for both samples with $n=3.3$ and 3.4, respectively.

3.4 DISCUSSION

The cGMP-gated channel β -subunit is a target for a soluble ROS protein kinase

Phosphorylation assays indicate that isolated bovine rod outer segments contain an unusual soluble protein kinase responsible for the phosphorylation of the channel β -subunit. Predominant labeling of the channel by a ROS lysate indicated that a protein kinase present in the ROS cytosol is the prime enzyme responsible for the phosphorylation of the β -subunit. Approximately half the phosphorylation intensity was still observed when the purified cGMP-gated channel was incubated with [γ - ^{32}P]ATP alone. A similar phosphate transfer behavior has been observed on rhodopsin from a sample incubated in either HEPES buffer or a ROS lysate fraction.

Two possibilities can account for these observations. Rhodopsin kinase is a soluble protein also found associated with ROS membranes (Kühn, 1978; Anant and Fung, 1992; Palczewski *et al.*, 1988; Bentrop *et al.*, 1993). Being located in the vicinity of rhodopsin, rhodopsin kinase can respond quickly to rapid turnovers in the visual excitation cascade by phosphorylating rhodopsin and quenching its activity (Kühn and Dreyer, 1972; Miller *et al.*, 1986). It has been shown previously that rhodopsin kinase can be released from the membrane or inactivated upon treatment with urea (Bentrop *et al.*, 1993). This observation explains why the phosphorylation of rhodopsin from a ROS membrane sample is still observed after

incubation without ROS soluble proteins. Besides rhodopsin kinase, a myriad of protein kinases have been found associated with biological membranes (Cooper, 1994; Nelsetuen and Bazzi, 1991). Several studies report the association of casein kinase II with cell membranes and in rare cases, with some of its protein substrates (Hathaway and Traugh, 1982). This observation could explain the less intense labeling of the β -subunit after phosphorylation in the absence of ROS soluble proteins. One point however remains unclear; assuming that the incubation time is sufficient to allow complete phosphorylation of ROS proteins, the labeling intensities should be roughly the same on both samples no matter the amount of protein kinase present. For this reason it can not be excluded that the β -subunit may be a target for more than one protein kinase. According to our observation, the soluble protein kinase would phosphorylate the channel predominantly and at different sites than the membrane-associated kinase. However, our experimental results do not favor this alternative, unless the membrane-associated kinase is also characterized by its lack of regulation and its sensitivity to DRB inhibition.

Identification of a CK2-like protein kinase in ROS

Only a few ROS protein kinases have been purified and characterized to date. These include rhodopsin kinase, protein kinase C and nucleotide-dependent protein kinases (Palczewski *et al.*, 1988; Wolbring and Cook, 1991; Williams *et al.*, 1997; Farber *et al.*, 1979). Studies on the regulation of the β -subunit phosphorylation using physiological characteristics of these kinases such as light-activation, Ca^{2+} /phorbol ester stimulation and cAMP-dependence failed to link the activity of these enzymes to the phosphorylation of the channel β -subunit *in vitro*. Most characteristic features of casein kinase II such as the atypical lack of regulation by known physiological regulators, the ability to transfer γ -phosphate groups from both ATP and GTP, the sensitivity to DRB inhibition, the lack of inhibition by staurosporine and the preference for acidic proteins like casein have been observed in this study. However, one of the hallmarks of CK2, the inhibition by nanomolar quantities of heparin, has not yet been observed here. There are at least two potential explanations for this observation: i) Phosphorylation of the channel β -subunit is catalyzed by an additional or a different protein kinase that is more resistant to heparin inhibition than CK2; ii) Phosphorylation of the β -

subunit by a CK2-like protein kinase is resistant to heparin inhibition. Using the specific CK2 inhibitor DRB favors the second explanation, since the labeling on the cGMP-gated channel β -subunit can be totally abolished by this inhibitor in the range of concentration expected for CK2 inhibition. The second possibility is also supported by the observation that a casein kinase II isoform or a CK2 α -subunit variant has been identified in ROS. The ROS CK2-like protein identified by western blotting and immunofluorescence microscopy migrates higher in SDS-polyacrylamide gels than the human recombinant CK2 and for this reason could represent a novel isoform that lacks some characteristic properties of the original enzyme. A novel isoform with altered properties is however unlikely since heparin inhibits CK2 by competing with polyanionic substrates at the active site. A sequence potentially responsible for conferring the substrate specificity of CK2 for acidic substrates has been identified within the active site of the enzyme and shows total conservation among all CK2 α variants and α' isoforms inter- and intra-species identified and characterized to date (Tuazon and Traugh, 1991).

The resistance of casein kinase II to heparin inhibition has been reported previously (Taylor *et al.*, 1987). In this study, Taylor and co-workers observed that phosphorylation of the nuclear protein nucleoplasmin by purified CK2 kinases from either *Xenopus* oocytes or calf thymus is insensitive to heparin. It was suggested that the unusual long stretch of polyglutamic acid in nucleoplasmin may be responsible for this effect by competing advantageously with anionic inhibitors such as heparin and thereby raising the concentration of heparin needed for inhibition. This is in agreement with the work of Meggio and co-workers (1983) from which it was shown that CK2 has a greater affinity for long (~70 residues) rather than short (~10 residues) polyglutamic acid peptides. Considering that the GARP-part of the channel is highly rich in glutamic acid residues (Colville and Molday, 1996), this could explain why the phosphorylation of the cGMP-gated channel β -subunit is insensitive to heparin inhibition.

Western blotting and immunofluorescence microscopy confirms the presence of a casein kinase II-like protein in ROS. As discussed previously, the ROS homolog has a different mobility than the human recombinant CK2 enzyme on SDS-polyacrylamide gels and may either represent a novel isoform of the enzyme, or be highly similar to previously reported

α -subunit variants and CK2 α' isoforms identified from various species and tissues (Tuazon and Traugh, 1991). Immunofluorescence labeling using two distinct anti-CK2 α antibodies confirms that the labeling observed in ROS from western blot analyses is not due to contamination by other retinal cells. The labeling of both rat and bovine ROS suggests that the presence of CK2 is conserved among various species and that its presence may be physiologically significant.

Although the CK2-like protein has not yet been purified from ROS, it has been shown here that the bovine rod cGMP-gated channel is a substrate for casein kinase II *in vitro*. However, phosphorylation of both the β - and the α -subunits by the human recombinant CK2 raises important questions about the exact identity and properties of the ROS endogenous CK2-like protein, since phosphorylation of the α -subunit by this protein has not been observed with ROS lysates. The presence of two soluble GARP variants in bovine ROS may account for this observation (Colville and Molday, 1996). Although there are 16 potent CK2 phosphorylation sites on the channel α -subunit, the absence of long stretches of glutamic acid residues makes this protein a less preferred substrate over the β -subunit and the two other soluble GARP variants. In a similar manner to what was observed with the heparin inhibition and taking into account the unusual acidic nature as well as the abundance of these polyanionic peptides in ROS, it is not surprising to observe the absence of labeling on the α -subunits in the presence of various forms of GARP. It is possible that during the phosphorylation of the affinity-purified cGMP-gated channel by exogenous CK2, the GARP-part on the β -subunit was insufficient to saturate all the kinases, causing some enzymes to phosphorylate other low-affinity substrates, including the channel α -subunit.

Effect of CK2 phosphorylation on the cGMP-gated channel activity

A possible role for the CK2 phosphorylation of the channel was investigated. Although the cGMP-gated channel α - and β -subunits were both phosphorylated *in vitro* by casein kinase II, no effect on the activity of the channel was observed. Taking into account the fundamental role of the cGMP-gated channel in phototransduction, it is likely that the phosphorylation of the channel β -subunit is of physiological importance. However, as demonstrated by the milk

phosphoprotein casein, not all phosphorylation is functionally important. It is believed that CK2 phosphorylation of caseins in mammary glands is primarily of nutritional importance, regulating the process of micelle formation and secretion (Bingham, 1979; Fiat and Jolles, 1989; Sood and Slattery, 1997).

CHAPTER 4

CALMODULIN INTERACTION WITH THE cGMP-GATED CHANNEL

4.1 MATERIALS

4.1.1 Animal tissues

Frozen bovine retinas were purchased from Schenk Packing Co (Stanwood, WA).

4.1.2 Chemicals

All chemicals were purchased from Sigma (St-Louis, MO) or Fisher Scientific (Fair Lawn, NJ) or obtained from distributors as listed in chapter 2 and 3.

4.1.3 Immunoreagents

The monoclonal antibody PMb 3C9 raised against the C-terminus of the cGMP-gated channel was generated in the laboratory as described before (section 2.1.3). The calmodulin monoclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

4.2 METHODS

4.2.1 Preparation of bovine ROS, ROS membranes and ROS lysates

Bovine ROS were prepared under dim red light as described previously (Molday and Molday, 1987, 1999). ROS membranes and ROS lysates for depletion experiments were prepared as follow: 10 mg (protein) of isolated ROS in 1 ml of a solution containing 20% (w/v) sucrose, 10 mM β -D-glucose, 10 mM taurine, 0.25 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4 was centrifuged in a TL100.4 rotor (Beckman Instruments) at 40,000 rpm for 10 min and lysed in 5 to 10 volumes of hypotonic buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM DTT, 2 mM EDTA. After 5 min incubation on ice, the samples were centrifuged at 30,000 rpm for 10 min in a TLA45 rotor using a Beckman Optima ultracentrifuge. The membrane pellet was resuspended in 5 to 10 volumes of hypotonic buffer and the procedure was repeated two more times. The pellet was finally resuspended in 10 mM HEPES-KOH, pH 7.4, 1 mM DTT (DTT was omitted when the sample was used for affinity chromatography) to give a final

protein concentration of 7-9 mg/ml by BCA assay. The lysates from two hypotonic washes were pooled and either used directly or diluted 1:3 in 10 mM HEPES-KOH, pH 7.4, and the endogenous ROS calmodulin was depleted using an anti-calmodulin antibody and protein G-agarose (see below).

4.2.2 Co-immunopurification of calmodulin with the channel

Ten mg (protein) of isolated ROS in 20% w/v sucrose, 10 mM β-D-glucose, 10 mM taurine, 0.25 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4, was centrifuged in a TL100.4 rotor (Beckman Instruments) at 40,000 rpm for 10 min, separated into two samples (~5 mg proteins) and washed thrice under dim red light in a buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM DTT with either 1 μM CaCl₂ or 2 mM EDTA. ROS membrane proteins were solubilized in 10 mM HEPES-KOH, pH 7.4, 18 mM CHAPS, 150 mM KCl, 1 μM CaCl₂ and the cGMP-gated channel complex from both samples was purified by PMc 1D1-Sepharose affinity chromatography. In most cases, 100 μl of beads (bed volume) was used and incubated with 500 μl of solubilized ROS membrane proteins (~ 2 mg/ml proteins) at 4°C for 90 min. The unbound fraction was removed by centrifugation and the beads were washed ten times in 1 ml of solubilization buffer. The samples were eluted using the electrophoresis sample buffer (without BME) and the proteins were loaded onto a 8% and a 12% SDS-polyacrylamide gel. The proteins were stained by coomassie blue and calmodulin was detected by western blotting.

4.2.3 Quantification of the amount of calmodulin bound to the channel

Two aliquots of ROS (5 mg proteins per sample) were hypotonically lysed and washed separately under dim red light in hypotonic buffer containing either 1 μM CaCl₂ or 2 mM EDTA. ROS membrane proteins were solubilized in 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 1 μM CaCl₂ and the cGMP-gated channel was purified by PMc 1D1-Sepharose affinity chromatography. The purified channel sample was applied to a 12% SDS-polyacrylamide gel along with known amount of exogenous bovine brain calmodulin standard. Proteins were electroblotted for 12 min and the amount of calmodulin that co-precipitated with the channel was compared to calmodulin standards by western blotting. Microgram quantities

of cGMP-gated channel α - and β -subunits were obtained as determined by protein assay of Kaplan and Pedersen (1985) using BSA as a standard.

4.2.4 Calmodulin-Sepharose affinity chromatography

The following procedure was carried out as described above. Hypotonically lysed ROS in either 1 μ M CaCl₂ or 2 mM EDTA were solubilized as above and incubated with 100 μ l of calmodulin-Sepharose beads for 90 min at 4°C. Unbound proteins were removed by washing the beads 10 times in 1 ml of solubilization buffer. Bound proteins were released by replacing Ca²⁺ by 2 mM EGTA in the solubilization buffer. The samples were applied to a SDS-polyacrylamide gel and visualized by coomassie blue staining and western blotting.

4.2.5 Calmodulin depletion from ROS cytosol

ROS (10 mg proteins) were hypotonically lysed and washed three times under dim light in 1-2 ml of 10 mM HEPES-KOH, pH 7.4, 1 mM DTT and 2 mM EDTA and spun at 40,000 rpm for 10 min. The ROS lysate containing calmodulin, other calcium binding proteins and ROS soluble proteins was collected after the second wash and dialyzed for 12 hrs against a buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM DTT, 150 mM KCl, 0.5 mM CaCl₂. ROS lysate fractions were subsequently diluted 1:3 in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂ and incubated with 5 μ g of anti-calmodulin IgG monoclonal antibody for 6 hrs at 4°C. The immunocomplex was captured by incubation with protein G-agarose (75 μ l bead volume) overnight at 4°C under mild agitation. The ROS cytosol depleted of calmodulin was separated from the column by spinning the samples at 2000 rpm and used immediately or kept no longer than one week at -70°C. An aliquot of the ROS lysate depleted of calmodulin along with a sample of undepleted ROS lysate and the eluant from protein G-agarose were loaded onto a 12% SDS-polyacrylamide gel. Removal of calmodulin from the ROS lysate was confirmed by western blot.

4.2.6 Depletion experiments

Ten mg (protein) of isolated ROS in 20% (w/v) sucrose, 10 mM β -D-glucose, 10 mM taurine, 0.25 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4, were centrifuged at 40,000 rpm for 10

min and washed three times in a buffer containing 10 mM HEPES-KOH, pH 7.4, 1 mM DTT, 2 mM EDTA. ROS membrane proteins were solubilized as follow: Washed ROS were separated in three aliquots and resuspended in 200 μ l of either ROS lysate depleted of calmodulin, ROS lysate containing calmodulin, or the hypotonic buffer in which EDTA has been replaced by 2 mM CaCl₂. Resuspended ROS membranes from three samples were solubilized for 15 min at 4°C in 900 μ l of a solution containing 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 2 mM CaCl₂. The cGMP-gated channel complex from these samples was purified by using calmodulin-Sepharose affinity chromatography. The purified protein samples were applied onto a 9% SDS-polyacrylamide gel and the cGMP-gated channel β -subunit detected by coomassie-blue staining and western blotting.

4.2.7 Reconstitution of ROS membrane proteins in lipid vesicles and cGMP-dependent Ca²⁺ efflux assay

ROS were washed thrice in a hypotonic buffer containing 5 mM HEPES-KOH, pH 7.4, 150 mM KCl, 1 mM DTT and 4 mM EDTA and the membrane proteins were solubilized at 4°C for 15 min in 18 mM CHAPS, 10 mM HEPES-KOH, pH 7.4, 2 mM CaCl₂ and 150 mM KCl. ROS membrane proteins were subsequently reconstituted in lipid vesicles as described in a previous section (2.2.6). Activity measurements were done as described by Cook *et al.* (1986, 1987). Lipid vesicles containing ROS membrane proteins were passed through a Chelex column to remove the excess calcium outside the vesicles. A solution (0.3 ml) containing the reconstituted channel was added to 1.7 ml of Arsenazo III. The presence of other CBPs was investigated by adding 100 μ l of either 10 mM HEPES-KOH buffer, calmodulin depleted ROS lysate or calmodulin containing ROS lysate. The calcium released from the vesicles was triggered by the addition of cGMP and monitored using a dual wavelength spectrophotometer (SLM Aminco DW2000). The data obtained for the cGMP-dependent Ca²⁺ efflux assay was analyzed using the curve fit program of Sigma Plot (Jandel Scientific). The Michaelis constant (K_m) and the Hill coefficient (n) were calculated by plotting the initial velocity over the maximal velocity at saturating cGMP concentration as a function of the cGMP concentration.

4.2.8 Polyacrylamide gel electrophoresis and western blotting

Protein samples were separated according to their molecular weight by SDS-polyacrylamide gel electrophoresis using the buffer system of Laemmli (1970). SDS-polyacrylamide gels were either stained by coomassie blue or electroblotted onto Immobilon-P membranes in 25 mM Tris-HCl pH 8.4, 190 mM glycine and 5-10% methanol. For western blotting, the membranes were blocked in phosphate-buffered saline (PBS, 0.01 M phosphate buffer, pH 7.5, 140 mM NaCl, and 2 mM KCl) containing 2% milk for 20 min at 25°C and then incubated 45 min at 25°C in a PBS solution containing 0.02% milk and either 2 µg/ml of anti-calmodulin antibody or the mAb hybridoma culture fluid PMb 3C9 diluted 10 fold in PBS. The membranes were washed thoroughly with PBS and incubated with sheep anti-mouse immunoglobulin (Ig) conjugated to horseradish peroxidase at 1/6000 dilution. Calmodulin and the cGMP-gated channel β-subunit were detected by enhanced chemiluminescence.

4.3 RESULTS

4.3.1 Co-precipitation of ROS calmodulin with the cation channel complex in the presence, but not in absence, of Ca²⁺

In dark-adapted ROS, the Ca²⁺ concentration ranges between 220 and 554 nM (McNaughton *et al.*, 1986; Ratto *et al.*, 1988; Korenbrodt and Miller, 1989; Lagnado *et al.*, 1992). Although *in vitro* experiments have established that ROS calmodulin modulates the ligand sensitivity of the cGMP-gated channel under these physiological conditions (Hsu and Molday, 1993, 1994), the participation of calmodulin in rod adaptation and recovery is not well accepted. In an attempt to clarify the role of calmodulin in phototransduction, a variety of experiments reproducing the calcium concentration that prevail in dark and bleached ROS were designed. In a first experiment, immunoprecipitation assays were used to examine the interaction between endogenous ROS calmodulin and the native channel under physiological calcium concentration. As shown in Fig. 21, calmodulin is found in the ROS cytosol (*lane c* and *d*), but also associated with ROS membranes (*lane e* and *f*). Membranes washed in a hypotonic buffer containing 4 mM EDTA still contained bound calmodulin, although no calcium was present in the solution (*lane f*). Finally, calmodulin is bound to the cation channel

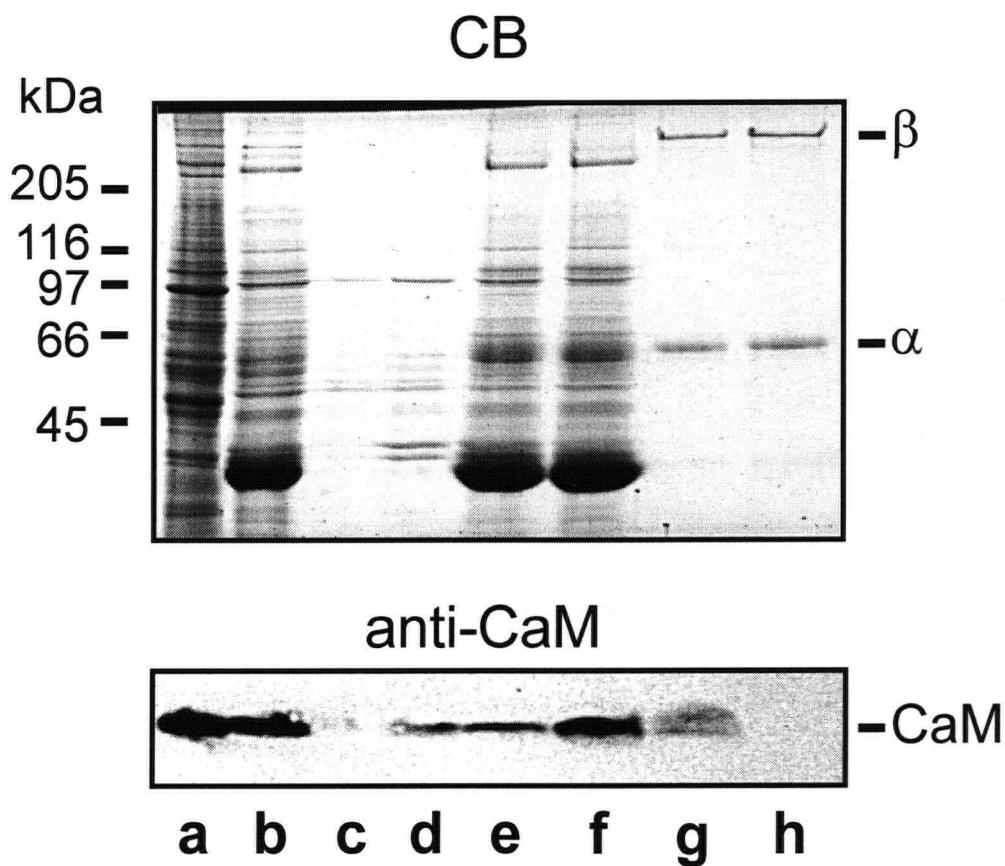


Fig. 21. Co-purification of ROS calmodulin with the rod cGMP-gated channel. ROS membranes were washed in HEPES buffer containing either 1 μ M CaCl_2 or 2 mM EDTA. ROS membranes were solubilized in CHAPS and the cGMP-gated channel from both samples was purified by PMc 1D1-Sepharose. The samples were subjected to SDS gel electrophoresis and the proteins were detected by Coomassie Blue (CB) staining. Calmodulin was identified by Western blotting using an anti-calmodulin monoclonal antibody. *lane a*, 15 μ g human carcinoma cell lysate; *lane b*, ROS; ROS lysate from membranes lysed in presence (*lane c*) or absence (*lane d*) of Ca^{2+} ; Unbound fraction from solubilized ROS membranes washed in presence (*lane e*) or absence (*lane f*) of Ca^{2+} ; Bound fraction to PMc 1D1 from ROS membranes washed in presence (*lane g*) or absence (*lane h*) of Ca^{2+} . Lanes contained either 40 μ g of ROS or 1.5 μ g of purified channel.

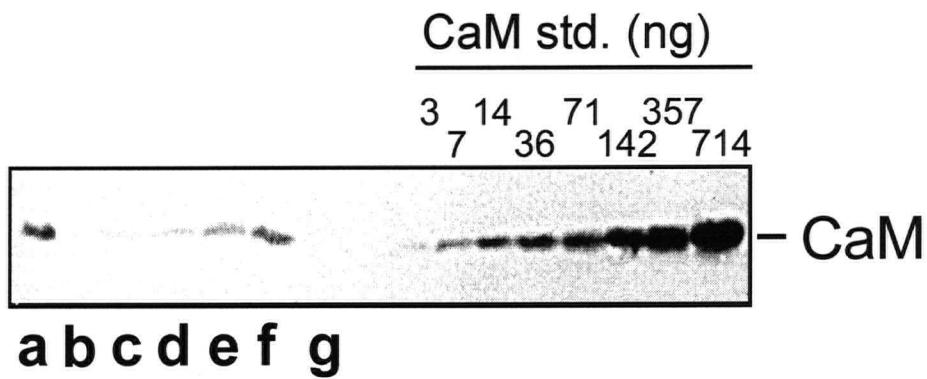


Fig. 22. Quantification of the amount of calmodulin co-purified with the cGMP-gated channel β -subunit. Co-purification of calmodulin with the native channel complex was carried out as described previously (Fig. 21). ROS (*lane a*), ROS lysate from membranes lysed in presence (*lane b*) or absence (*lane c*) of Ca^{2+} , the unbound fractions to PMc 1D1 from solubilized ROS membranes washed in presence (*lane d*) or absence (*lane e*) of Ca^{2+} and the bound fractions to PMc 1D1 from ROS membranes washed in presence (*lane f*) or absence (*lane g*) of Ca^{2+} were applied to a 12% SDS-polyacrylamide gel along with calmodulin standard. The proteins were subjected to electrophoresis, transferred onto Immobilon-P and labeled with an anti-calmodulin monoclonal antibody.

complex in presence, but not in absence of 1 μ M Ca^{2+} (Western blot, *lane g* and *h*, respectively).

The amount of calmodulin that co-precipitated with the channel was calculated by western blotting and protein quantification using the Pedersen assay (Kaplan and Pedersen, 1985). In this study, the endogenous ROS calmodulin that co-purified with the cGMP-gated channel was compared to a ladder of calmodulin standards (Fig. 22, *lane d*). Taking in account that the native channel is a heterotetramer most likely composed of two α - and two β -subunits (Liu *et al.*, 1996) and that only the β -subunit binds Ca^{2+} /calmodulin (Hsu and Molday, 1993, 1994; Chen *et al.*, 1994; Körschen *et al.*, 1995), calmodulin binds to the β -subunit of the cGMP-gated channel in an approximate ratio of 1:3.5. This result suggests that in the presence of calcium, most CaM-binding sites on the cGMP-gated channel β -subunits are occupied by the endogenous ROS calmodulin.

4.3.2 The rod cGMP-gated channel is purified by CaM-Sepharose affinity chromatography in the presence, but not in absence, of Ca^{2+}

Calmodulin-Sepharose affinity chromatography was used to characterize further the association of calmodulin with the channel under conditions that predominate in dark-adapted and bleached ROS. As shown in Fig. 23, the cGMP-gated channel from ROS membranes washed in the presence of calcium was not purified by calmodulin-Sepharose, suggesting that calmodulin or another calcium-binding protein was already bound to the β -subunit (CB and 3C9 western blot, *lane h*). In contrast, the rod cGMP-gated channel from ROS membranes washed in the absence of Ca^{2+} was purified by CaM-Sepharose affinity chromatography. This indicates that the CaM-binding sites on the rod cGMP-gated channel were unoccupied (CB and 3C9 western blot, *lane i*). Exogenous addition of bovine brain calmodulin to this sample inhibits the binding of the channel to calmodulin-Sepharose by competing for the CaM-binding sites on the β -subunit (CB, 3C9 and CaM western blot, *lane g* and *j*).

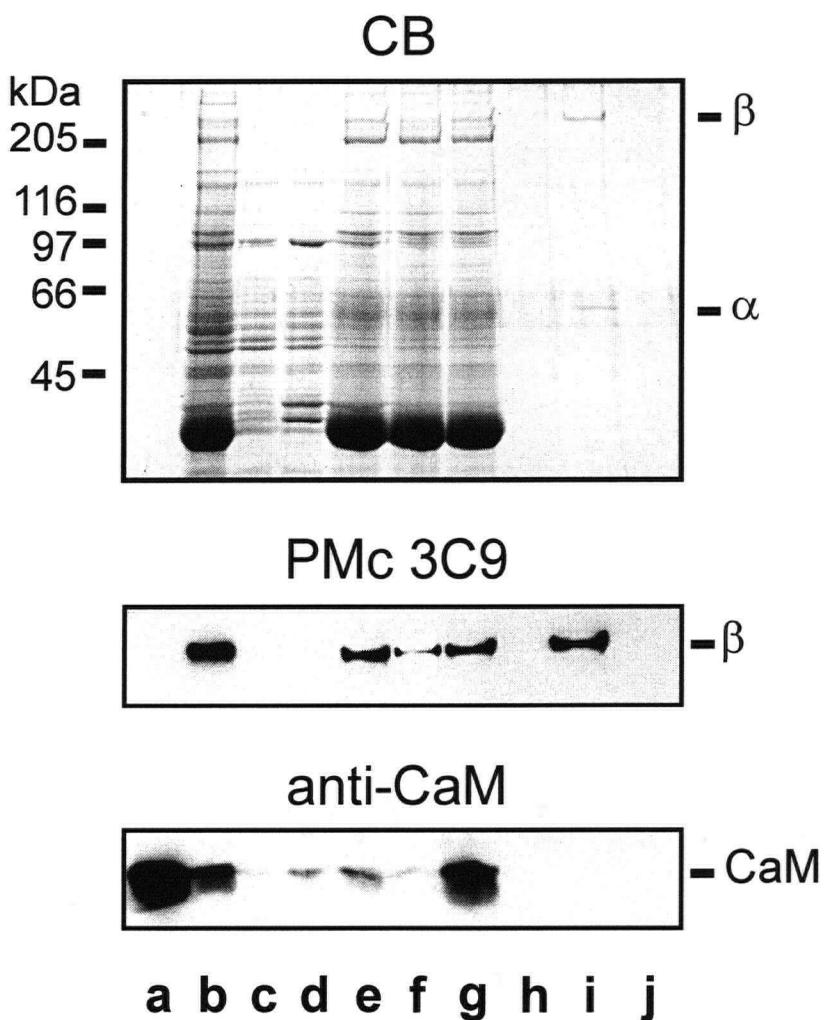


Fig. 23. CaM-Sepharose purification of the rod cGMP-gated channel in the presence, but not in absence, of Ca^{2+} . ROS membranes were washed in HEPES buffer containing either 1 μM CaCl_2 or 2 mM EDTA. ROS membranes were solubilized in CHAPS and the cGMP-gated channel from both samples was purified by calmodulin-Sepharose. The samples were subjected to SDS gel electrophoresis and the proteins were either stained by Coomassie Blue (CB) or transferred to Immobilon-P membranes for Western blotting. The cGMP-gated channel β -subunit was labeled with PMb 3C9 monoclonal antibody and calmodulin was identified using an anti-calmodulin monoclonal antibody. *lane a*, 7.5 μg bovine brain calmodulin; *lane b*, ROS; ROS lysate from membranes lysed in presence (*lane c*) or absence (*lane d*) of Ca^{2+} ; Unbound fractions to CaM-Sepharose from solubilized ROS membranes washed in presence (*lane e*) or absence (*lane f*) of Ca^{2+} ; The latter was incubated with 2 μg bovine brain calmodulin during chromatography (*lane g*); Bound fractions to CaM-Sepharose from ROS membranes washed in presence (*lane h*), absence (*lane i*) of Ca^{2+} or absence of Ca^{2+} and incubated with exogenous CaM during chromatography (*lane j*). Lanes contained either 40 μg of ROS or 2 μg of purified channel.

4.3.3 Only calmodulin binds to the CaM-binding sites on the channel β -subunit

The binding and regulation of the cGMP-gated channel by an endogenous inhibitory factor with different calcium requirements than calmodulin has been previously suggested by Gordon and her co-workers (1995). Based on equivalent channel inhibition, it was suggested that the novel CBP and calmodulin bind the same site. To investigate a potential interaction between other ROS proteins and the β -subunit at the CaM-binding sites, calmodulin was depleted from ROS lysates and the capacity of the cGMP-gated channel complex to bind to CaM-Sepharose in the presence of other ROS soluble proteins was examined.

As shown in Fig. 24, the channel can only be purified by calmodulin-Sepharose if the solubilized sample was incubated in the presence of HEPES buffer or ROS lysate depleted of calmodulin (Fig. 24A CB and 3C9 western blot, *lane e* and *g*). Incubation of the rod cGMP-gated channel with a ROS lysate containing endogenous calmodulin prevented the purification of the channel complex by CaM-Sepharose chromatography. This suggests that the unconventional CaM-binding sites on the β -subunit are specific for the ROS calmodulin and that other ROS proteins do not interact with the channel at these sites (Fig. 24A CB and 3C9 western blot, *lane f*). As shown in Fig 24B, calmodulin was successfully removed from a ROS lysate containing calmodulin and other soluble proteins (Fig. 24B, *lane c* and *d*).

4.3.4 The ligand sensitivity of cGMP-gated channels is modulated preferentially by calmodulin in ROS

To determine if other channel modulators are present in ROS, the effect of ROS soluble proteins on the activity of the cGMP-gated channel was tested. ROS lysates depleted and undepleted of calmodulin were incubated with ROS membrane proteins reconstituted in calcium-containing lipid vesicles and the activity of the rod cGMP-gated channel was measured. When compared to a positive control, the sensitivity of the rod cGMP-gated channel for cGMP was decreased approximately 1.3-fold in the presence of a ROS lysate containing calmodulin (Table VI; Fig. 25B, ▲). However, the addition of a ROS lysate depleted of calmodulin only had minor effects on the affinity of the cGMP-gated channel for its ligand when compared to the same control in which the ROS lysate was replaced by HEPES buffer (Fig 25A and B, ● and ■). The K_m values were essentially identical for both samples

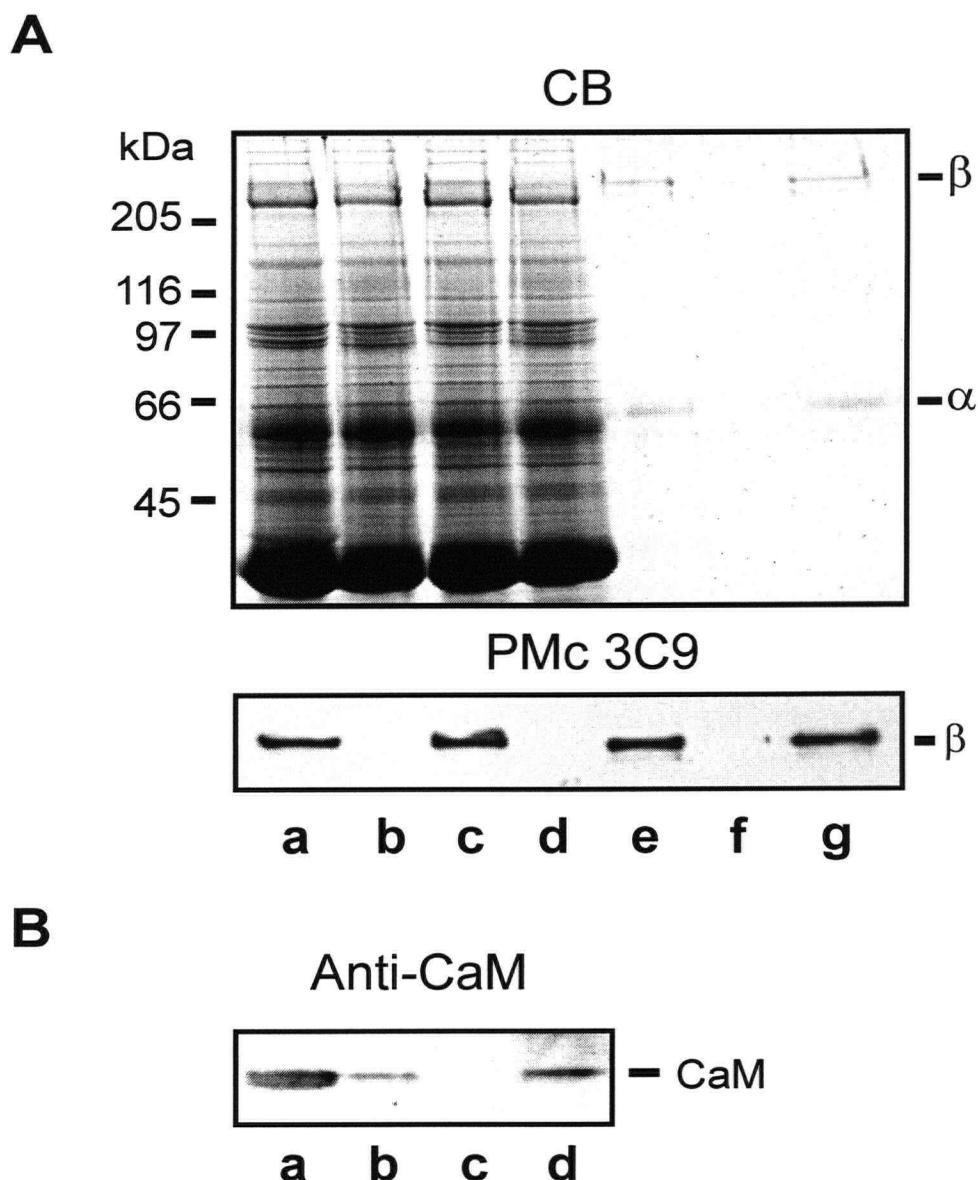


Fig. 24. Investigation of other ligands for the CaM-binding sites on the channel β -subunit. *A*, ROS membranes were washed in a hypotonic buffer containing 2 mM EDTA and solubilized in CHAPS. ROS lysates depleted or undepleted of calmodulin were added to the solubilized membranes before purification of the channel by calmodulin-Sepharose. Bovine ROS (*lane a*), unbound fractions from CaM-Sepharose column incubated with solubilized ROS containing HEPES (*lane b*), ROS lysate (*lane c*) and ROS lysate depleted of calmodulin (*lane d*) and bound fraction of CaM-Sepharose from solubilized ROS samples containing HEPES (*lane e*), ROS lysate (*lane f*) and ROS lysate depleted of calmodulin (*lane g*). The samples were subjected to SDS gel electrophoresis, transferred to Immobilon-P membranes and labeled with β -subunit-specific antibody PMb 3C9. *B*, Western blot of ROS lysate (*lane a*), ROS lysate diluted 1:10 (*lane b*), ROS lysate depleted of calmodulin (*lane c*) and bound fraction to Protein G-agarose (*lane d*) labeled with anti-calmodulin monoclonal antibody.

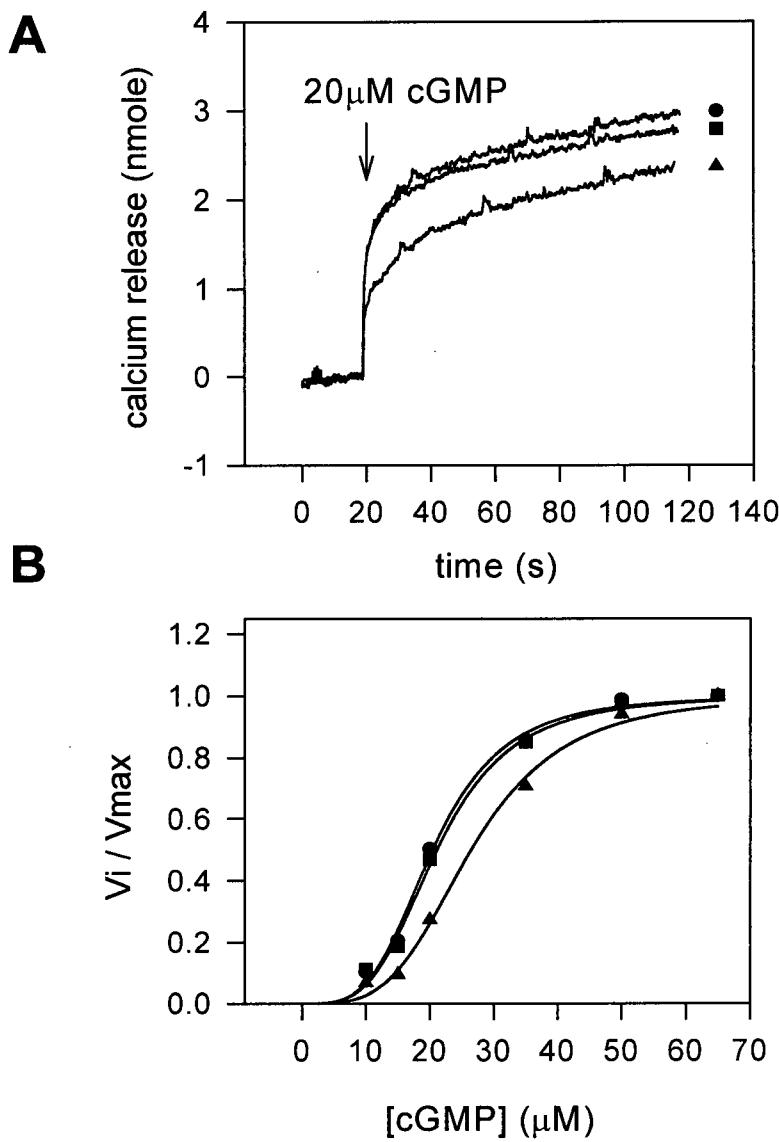


Fig. 25. Investigation of other cGMP-gated channel modulators in ROS. *A*, the native cGMP-gated channel was reconstituted into Ca^{2+} -containing lipid vesicle for cGMP-dependent Ca^{2+} efflux assay. The presence of other potential channel modulators was investigated by adding 100 μl of either HEPES buffer (●), ROS lysate depleted (■) or ROS lysate undepleted (▲) of calmodulin. A cGMP concentration of 20 μM was used to initiate the efflux of Ca^{2+} . *B*, initial velocities (V_i) obtained for cGMP concentration ranging from 10 to 65 μM were normalized to the velocity at saturating concentration (V_{\max}) of cGMP (65 μM) and the data was analyzed by the curve fit program of Sigma Plot using the Hill equation $V/V_{\max} = [c\text{GMP}]^n / ([c\text{GMP}]^n + K_m^n)$ where K_m and n are the Michaelis constant and the Hill coefficient, respectively. The cGMP-gated channel incubated with HEPES (●) had a $K_m = 20.5 \mu\text{M}$ and $n = 3.7$, the channel incubated with a ROS lysate undepleted of calmodulin (▲) had a $K_m = 26.5 \mu\text{M}$ and $n = 3.7$ and the channel incubated with a ROS lysate depleted of calmodulin (■) had a $K_m = 21.1 \mu\text{M}$ and $n = 3.6$.

($K_m=21.1 \mu M$ and $K_m=20.5 \mu M$, respectively) and close to the value reported previously for the native channel (Hsu and Molday, 1993). The abolition of the shift in the dose-response curve (Fig 25A and B, ▲ and ■) coincides with the total removal of calmodulin from the ROS lysate as monitored by western blot (Fig 24B), suggesting that calmodulin is the sole cGMP-gated channel protein modulator in ROS. In a similar manner to what has been observed in the past for calmodulin, addition of the ROS lysate shifted the apparent cGMP affinity without affecting the gating kinetics of the channel for its ligand (Table VI; Hsu and Molday, 1993).

4.4 DISCUSSION

To date, there is no direct evidence linking the presence of Ca^{2+} /calmodulin in ROS to the regulation of the rod cGMP-gated channel *in vivo* (Gray-Keller *et al.*, 1993; Nakatani *et al.*, 1995). However, Ca^{2+} /calmodulin binding to the channel β -subunit as well as its role in the modulation of the native cGMP-gated channel has been well characterized *in vitro* (Weitz *et al.*, 1998; Grunwald *et al.*, 1998; Hsu and Molday, 1993, 1994). In the present study, the endogenous ROS calmodulin co-purifies with the native rod cGMP-gated channel from ROS membranes washed in presence, but not in absence of calcium. This suggests that calmodulin is bound to the channel under similar conditions to those occurring in dark-adapted ROS, when the calcium concentration is believed to be around 500 nM (Gray-Keller and Detwiler, 1994). On the other hand, under the calcium concentration that reproduces roughly the conditions prevailing in bleached ROS ($[\text{Ca}^{2+}]<50 \text{ nM}$), endogenous calmodulin does not co-purify with the channel. As determined by co-immunoprecipitation studies, the ratio of calmodulin bound per β -subunit (1:3.5) indicates that most channels bind calmodulin in the presence of calcium. This is also supported by the observation that the cGMP-gated channel from ROS membranes washed in absence of calcium binds to CaM-Sepharose. In contrast, the channel from ROS membranes washed in presence of calcium does not bind to CaM-Sepharose, since the CaM-binding sites are already occupied by endogenous CaM. Taken together, these results indicate that Ca^{2+} /calmodulin binds to and modulates the activity of the channel in a Ca^{2+} -dependent

Table VI. Kinetic values obtained for the cGMP-gated channel incubated with ROS cytosol depleted or undepleted of calmodulin

Conditions	Km (μM)	n
Literature¹		
Control	19	3.8
+Calmodulin (235 nM)	33	3.8
Experimental		
Control	20.5	3.7
+ROS lysate undepleted	26.5	3.7
+ROS lysate depleted	21.1	3.6

¹Hsu and Molday, 1993

manner and support the role of this channel regulation in the recovery of the rod photoreceptors.

Immunoprecipitation studies and CaM depletion experiments carried out in this study indicate that calmodulin is the main calcium-binding protein that binds and regulates the cGMP-gated channel in ROS. Based on the relatively small effect of CaM on channel activity and the suggestion that another frog ROS CBP regulates the channel, it has been proposed that the unconventional CaM-binding sites on the β -subunit may in fact represent the real target for a ROS inhibitory factor (Gordon *et al.*, 1995; Weitz *et al.*, 1998). To test this possibility, the availability of these sites for other ROS soluble proteins was examined. In contrast to the findings of Gordon and her co-workers (1995), no other ROS proteins were observed to bind the CaM-binding sites on the channel β -subunit. Effectively, the cGMP-gated channel was purified by CaM-Sepharose chromatography when incubated with a ROS lysate depleted of calmodulin, but not when incubated with a ROS lysate containing calmodulin. In addition, co-immunoprecipitation studies carried out here indicate that the CaM-binding sites are saturated with endogenous ROS calmodulin under Ca^{2+} conditions similar to those occurring in dark-adapted ROS.

Moreover, ion-flux measurements using ROS lysates from which calmodulin had been removed showed that no other ROS soluble proteins modulate the native rod channel in reconstituted systems. The activity of the cGMP-gated channel under these conditions was comparable to a positive control with K_m values of 21.1 and 20.5 μM , respectively. These values showed similar kinetic properties to those reported previously for the native channel (Table VI). Addition of ROS lysate containing calmodulin to a preparation of ROS vesicles was found to shift the dose-response curve for cGMP-dependent activity to higher cGMP concentration, in a similar manner to what has been observed using purified CaM from ROS or brain sources (Hsu and Molday, 1994). The cGMP sensitivity of the channel was decreased nearly 1.3-fold in the presence of ROS lysate, a value equivalent to the small changes observed previously for ROS (1.7-fold decrease) and purified channel (1.3-fold decrease) vesicle preparations incubated with calmodulin. In accordance with these observations, it appears likely that calmodulin is the sole protein modulator of the cGMP-gated channel in ROS and the authentic ligand of the unusual CaM-binding sites on the β -subunit. A question however

remains: Is the small contribution of Ca^{2+} /calmodulin in the modulation of the rod cGMP-gated channels physiologically relevant? As previously suggested, it is perhaps possible that the related CaM-binding sites present on channel subunits expressed in different tissues arose differently over time, becoming stronger or weaker depending on their evolutionary path (Grunwald *et al.*, 1998). On this basis, calmodulin would contribute only minimally to photoreceptor adaptation and recovery.

SUMMARY

The results of this study suggest that the rod cGMP-gated channel is indeed a target for ROS protein kinase(s). Unlike the results of previous studies carried out on the recombinant α -subunit heterologously expressed in *Xenopus* oocytes, results presented here show directly that the β -subunit, but not the α -subunit, of the native rod channel complex is phosphorylated in an ATP-dependent manner (Gordon *et al.*, 1992). However, although the channel is phosphorylated, no significant change in the cGMP sensitivity of the native channel has been observed. This suggests that phosphorylation of the cGMP-gated channel may play other roles or simply that the experimental conditions were not adequate enough to allow detection of phosphorylation-induced changes in channel activity. Biochemical characterization of this reaction shows that the phosphate transfer was not optimum and this may explain why no change in activity was detected.

Further characterization of the phosphorylation reaction shows that phosphorylation occurs exclusively on serine residues. In addition, our data indicates that the C-terminal portion of the β -subunit is the main target for kinase(s)-catalyzed phosphorylation. However, it is possible that the GARP-part of the channel may also incorporate γ -phosphate(s) from ATP. Future work will be needed to localize more precisely the sites of phosphorylation within the β -subunit and obtain information about the role of this modification in rod cell physiology.

Surprisingly, none of the physiological conditions including addition of protein kinase regulators and second messengers modified the catalytic properties of the kinase(s) involved in the phosphorylation of the β -subunit. Casein kinase II, a well-characterized Ser/Thr protein kinase, is well-known for its unusual properties. In addition to its lack of regulation *in vivo*, this protein kinase has an unusual preference for acidic substrates, making it a potential candidate for the phosphorylation of the channel β -subunit.

Inhibitor mapping experiments and phosphorylation assays carried out here indicate that ROS contain a kinase that exhibits most of the casein kinase II properties, with the exception of heparin inhibition. As discussed previously, the acidic nature of the GARP-part of the channel β -subunit may account for these observations, by competing advantageously with heparin at

the CK2 catalytic sites. The presence of a CK2-like protein kinase has been confirmed in ROS by western blotting and immunofluorescence microscopy. The novel ROS CK2 has a different mobility than the human recombinant homolog on SDS-polyacrylamide gels and may therefore represent a new isoform or a CK2 α variant of the enzyme. At this stage, the involvement of the CK2-like protein kinase in the phosphorylation of the β -subunit is not certain. However, phosphorylation assays show that the cGMP-gated channel is a substrate for the human recombinant casein kinase II *in vitro*.

Regulation of the rod cGMP-gated channel by Ca^{2+} /calmodulin constitutes another controversial modulatory mechanism in ROS. Experiments carried out here help support a model in which calmodulin is an integral part of phototransduction in rods. Immunoprecipitation studies confirm that endogenous ROS calmodulin associates with the native cGMP-gated channel under similar calcium conditions to those occurring in dark-adapted ROS. Furthermore, CaM-Sepharose chromatography and western blotting studies show that most endogenous ROS calmodulin molecules are bound to the channel under the same conditions. These observations represent a first and important step towards clarifying and defining the physiological role of calmodulin in phototransduction.

Besides the regulation of cGMP-gated channels by calmodulin, it has been suggested that other CBPs may regulate CNG channels (Gordon *et al.*, 1995; Balasubramanian *et al.*, 1996). To clarify this issue, a couple of experiments were designed to directly determine if other calcium-binding proteins are present in ROS and alter the cGMP-gated channel activity. Purification of the cGMP-gated channel by CaM-Sepharose chromatography in the presence of ROS lysate depleted of calmodulin indicates that no other proteins bind the CaM-binding site on the β -subunit. Activity measurements revealed that the dose-response curve was shifted to the right upon addition of ROS lysate undepleted of calmodulin to the calcium-containing vesicles. This effect was abolished when a ROS lysate depleted of calmodulin was used, suggesting that calmodulin is the sole Ca^{2+} -dependent modulator of the rod cGMP-gated channel.

REFERENCES

- Ahmad, I., Korbmacher, C., Segal, A.S., Cheung, P., Boulpaep, E.L., and Barnstable, C.J. 1992. Mouse cortical collecting duct cells show non-selective cation channel activity and express a gene related to the cGMP-gated rod photoreceptor channel. *Proc. Natl. Acad. Sci. U.S.A.* **89**:10262-10266.
- Allende, J.E., and Allende, C.C. 1995. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* **9**:313-323.
- Ames, J.B., Tanaka, T., Stryer, L., and Ikura, M. 1996. Portrait of a myristoyl switch protein. *Curr. Opin. Struct. Biol.* **6**:432-438.
- Anant, J.S., and Fung, B.K. 1992. In vivo farnesylation of rat rhodopsin kinase. *Biochem. Biophys. Res. Commun.* **183**:468-473.
- Angleson, J.K., and Wensel, T.G. 1993. A GTPase-accelerating factor for transducin, distinct from its effector cGMP phosphodiesterase, in rod outer segment membranes. *Neuron.* **11**:939-949.
- Antonny, B., Otto-Bruc, A., Chabre, M., and Vuong, T.M. 1993. GTP hydrolysis by purified α -subunit of transducin and its complex with the cyclic GMP phosphodiesterase inhibitor. *Biochemistry.* **32**:8646-8653.
- Arshavsky, V.Y., and Bownds, M.D. 1992. Regulation of deactivation of photoreceptor G protein by its target enzyme and cGMP. *Nature.* **357**:416-417.
- Artemyev, N.O., Rarick, H.M., Mills, J.S., Skiba, N.P., and Hamm, H.E. 1992. Sites of interaction between rod G-protein α -subunit and cGMP-phosphodiesterase γ -subunit: Implications for the phosphodiesterase activation mechanism. *J. Biol. Chem.* **267**:25067-25072.
- Babu, Y.S., Sack., J.S., Greenough, T.J., Bugg, C.E., Means, A.R., and Cook, W.J. 1985. Three-dimensional structure of calmodulin. *Nature.* **315**:37-40.
- Balasubramanian, S., Lynch, J.W., and Barry, P.H. 1996. Calcium-dependent modulation of the agonist affinity of the mammalian olfactory cyclic nucleotide-gated channel by calmodulin and a novel endogenous factor. *J. Membrane Biol.* **152**:13-23.
- Baydoun, H., Hoppe, J., Freist, W., and Wagner, K.G. 1981. The ATP substrate site of a cyclic-nucleotide-independent protein kinase from porcine liver nuclei. *Eur. J. Biochem.* **115**:385-389.
- Baylor, D.A. 1996. How photons start vision. *Proc. Natl. Acad. Sci. U.S.A.* **93**:560-565.

- Baylor, D.A., and Fuortes, M.G. 1970. Electrical responses of single cones in the retina of the turtle. *J. Physiol.* (London). **207**:77-92.
- Baylor, D.A., Lamb, T.D., and Yau, K.-W. 1979a. The membrane current of single rod outer segments. *J. Physiol.* **288**:589-611.
- Baylor, D.A., Lamb, T.D., and Yau, K.-W. 1979b. Response of retinal rods to single photons. *J. Physiol.* **288**:613-634.
- Bennett, N., Michel-Villaz, M., and Kühn, H. 1982. Light-induced interactions between rhodopsin and the GTP-binding protein. *Eur. J. Biochem.* **127**:97-103.
- Bentrop, J., Planger, A., and Paulsen, R. 1993. An arrestin homolog of blowfly photoreceptors stimulates visual-pigment phosphorylation by activating a membrane-associated protein kinase. *Eur. J. Biochem.* **216**:67-73.
- Biel, M., Altenhofen, W., Hulin, R., Ludwig, J., Freichel, M., Flockerzi, V., Dascal, N., Kaupp, U.B., and Hofmann, F. 1993. Primary structure and functional expression of a cyclic nucleotide-gated channel from rabbit aorta. *FEBS Lett.* **329**:134-138.
- Biel, M., Zong, X., Distler, M., Bosse, E., Klugbauer, N., Murakami, M., Flockerzi, V., and Hofmann, F. 1994. Another member of the cyclic nucleotide-gated channels family expressed in testis, kidney, and heart. *Proc. Natl. Acad. Sci. U.S.A.* **91**:3505-3509.
- Binder, B.M., Brewer, E. and Bownds, M.D. 1989. Stimulation of protein phosphorylation in frog Rod Outer Segments by protein kinase activators. Suppression of light-induced changes in membrane current and cGMP by protein kinase C activators. *J. Biol. Chem.* **264**:8857-8864.
- Bingham, E.W. 1979. Role of mammary casein kinase in the phosphorylation of milk proteins. *J. Dairy Res.* **46**:181-185.
- Birge, R.R. 1990. Photophysics and molecular electronic applications of the rhodopsins. *Annu. Rev. Phys. Chem.* **41**:683-733.
- Bok, D., and Heller, J. 1976. Transport of retinol from the blood to the retina: Autoradiographic study of the pigment epithelial cell surface receptor for plasma retinol-binding protein. *Exp. Eye Res.* **22**:395-402.
- Bownds, D., Dawes, J., Miller, J., and Stahlman, M. 1972. Phosphorylation of frog photoreceptor membranes induced by light. *Nature New Biol* (London). **237**:125-127.

- Bradley, J., Zhang, Y., Bakin, R., Lester, H.A., Ronnett, G.V., and Zinn, K. 1997. Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: A potential effector of synaptic plasticity in brain neurons. *J. Neurosci.* **17**:1993-2005.
- Bray, G.A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochemistry*. **1**:279-285.
- Browning, M.D., Bureau, M., Dudek, E.M., and Olsen, R.W. 1990. Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ -aminobutyric acid A receptor. *Proc. Natl. Acad. Sci. U.S.A.* **87**:1315-1318.
- Calvert, P.D., Klenchin, V.A., and Bownds, M.D. 1995. Rhodopsin kinase inhibition by recoverin. *J. Biol. Chem.* **270**:24127-24129.
- Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W., and McNaughton, P.A. 1989. Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature (London)*. **337**:740-743.
- Charbonneau, H., Prusti, R.K., LeTrong, H., Sonnenberg, W.K., Mullaney, P.J., Walsh, K.A., and Beavo, J.A. 1990. Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. *Proc. Natl. Acad. Sci. U.S.A.* **87**:288-292.
- Chatopadhyaya, R., Meador, W., Means, A., and Quiocho, F. 1992. Calmodulin structure refined at 1.7 Å resolution. *J. Mol. Biol.* **228**:1177-1192.
- Chen, C.-K., Inglese, J., Lefkowitz, R.J., and Hurley, J.B. 1995. Ca^{2+} -dependent interaction of recoverin with rhodopsin kinase. *J. Biol. Chem.* **270**:18060-18066.
- Chen, T.-Y., Illing, M., Molday, L.L., Hsu, Y.-T. Yau, K.-W., and Molday, R.S. 1994. Subunit 2 (or β) of retinal rod cGMP-gated cation channel is a component of the 240-kDa channel-associated protein and mediates Ca^{2+} -calmodulin modulation. *Proc. Natl. Acad. Sci. USA*. **91**:11757-11761.
- Chen, T.-Y., Peng, Y.-W., Dhallan, R.S., Ahamed, B., Reed, R.R., and Yau, K.-W. 1993. A new subunit of the cyclic-gated cation channel in retinal rods. *Nature*. **362**:764-767.
- Chen, T.-Y., and Yau, K.-W. 1994. Direct modulation by Ca^{2+} -calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. *Nature*. **368**:545-548.
- Cochet, C., and Chambaz, E.M. 1983. Oligomeric structure and catalytic activity of G type casein kinase. *J. Biol. Chem.* **258**:1403-1406.

- Colamartino, G., Menini, A., and Torre, V. 1991. Blockage and permeation of divalent cations through the cyclic GMP-activated channel from tiger salamander retinal rods. *J. Physiol. (London)*. **440**:189-206.
- Colville, C.A., and Molday, R.S. 1996. Primary structure and expression of the human β -subunit and related proteins of the rod photoreceptor cGMP-gated channel. *J. Biol. Chem.* **271**:32968-32974.
- Connell, G.J., and Molday, R.S. 1990. Molecular cloning, primary structure, and orientation of the vertebrate photoreceptor cell protein peripherin in the rod outer segment disk membrane. *Biochemistry*. **29**:4691-4698.
- Cook, N.J., Hanke, W., and Kaupp, U.B. 1987. Identification, purification and functional reconstitution of the cyclic GMP-dependent channel from rod photoreceptors. *Proc. Natl. Acad. Sci. U.S.A.* **84**:584-589.
- Cook, N.J., and Kaupp, U.B. 1988. Solubilization, purification and reconstitution of the sodium-calcium exchanger from bovine retinal rod outer segments. *J. Biol. Chem.* **263**:11382-11388.
- Cook, N.J., Molday, L.L., Reid, D., Kaupp, U.B., and Molday, R.S. 1989. The cGMP-gated channel of bovine rod photoreceptors is localized exclusively in the plasma membrane. *J. Biol. Chem.* **264**:6996-6999.
- Cook, N.S., Zeilinger, C., Koch K.-W., and Kaupp, U.B. 1986. Solubilization and functional reconstitution of the cGMP-dependent cation channel from bovine rod outer segment. *J. Biol. Chem.* **261**:17033-17039.
- Cooper, J.A. 1994. Membrane-associated tyrosine kinases as molecular switches. *Semin. Cell Biol.* **5**:377-387.
- Costa, M.R., Casnelli, J.E., and Catteral, W.A. 1982. Selective phosphorylation of the α subunit of the sodium channel by cAMP-dependant protein kinase. *J. Biol. Chem.* **257**:7918-7921.
- Deigner, P.S., Law, W.C., Canada, F.J., and Randon, R.R. 1989. Membranes as the energy source in the endergonic transformation of vitamin A to 11-cis-retinol. *Science*. **244**:968-971.
- Delpech, M., Levy-Favatier, F., Moisand, F., and Kruh, J. 1986. Rat liver nuclear protein kinase NI and NII. *Eur. J. Biochem.* **160**:333-341.
- Dhallan, R.S., Macke, J.P., Eddy, R.L., Shows, T.B., Reed, R. R., Yau, K.-W., and Nathans, J. 1992. Human rod photoreceptor cGMP-gated: amino acid sequence, gene structure, and functional expression. *J. Neurosci.* **12**:3248-3256.

- Dizhoor, A.M., Olshevskaya, E.V., Henzel, W.J., Wong, S.C., Stults, J.T., Ankoudinova, I., and Hurley, J.B. 1995. Cloning, sequencing, and expression of a 24-kDa Ca^{2+} -binding protein activating photoreceptor guanylyl cyclase. *J Biol Chem.* **270**:25200-25206.
- Dosé, A. 1995. Molecular characterization of the cyclic nucleotide-gated cation channel of bovine rod outer segments. Ph.D. Thesis. University of British Columbia.
- Downing, J.E.G., and Role, L.W. 1987. Activators of protein kinase C enhance acetylcholine receptor desensitization in sympathetic ganglion neurons. *Proc. Natl. Acad. Sci. U.S.A.* **84**:7739-7743.
- Edgar, M.A., Pasinelli, P., DeWit, M., Anton, B., Dokas, L.A., Pastorino, L., DiLuca, M., Cattabeni, F., Gispen, W.H., and De Graan, P.N. 1997. Phosphorylation of the casein kinase II domain of B-50 (GAP-43) in rat cortical growth cones. *J. Neurochem.* **69**:2206-2215.
- Eismann, E., Müller, F., Heinemann, S.H., and Kaupp, U.B. 1994. A single negative charge within the pore region of a cGMP-gated channel controls rectification, Ca^{2+} blockage and ionic selectivity. *Proc. Natl. Acad. Sci. U.S.A.* **91**:1109-1113.
- Emeis, D., Kuhn, H., Reichert, J., and Hofmann, K.P. 1982. Complex formation between metharhodopsin II and GTP-binding protein in bovine photoreceptor membranes leads to a shift of the photoproduct equilibrium. *FEBS Lett.* **143**:29-34.
- Enyedi, A., Elwess, N.L., Filoteo, A.G., Verma, A.K., Paszty, K., and Penniston, J.T. 1997. Protein kinase C phosphorylates the "a" forms of plasma membrane Ca^{2+} pump isoforms 2 and 3 and prevents binding of calmodulin. *J. Biol. Chem.* **272**:27525-27528.
- Enyedi, A., Verma, A.K., Filoteo, A.G., and Penniston, J.T. 1996. Protein kinase C activates the plasma membrane Ca^{2+} pump isoform 4b by phosphorylation of an inhibitory region downstream of the calmodulin-binding domain. *J. Biol. Chem.* **271**:32461-32467.
- Euler, T., and Wässle, H. 1995. Immunocytochemical identification of cone bipolar cells in the rat retina. *J. Comp. Neurol.* **361**:461-478.
- Eusebi, F., Molinaro, M., and Zani, B.M. 1985. Agents that activate protein kinase C reduce acetylcholine sensitivity in cultured myotubes. *J. Cell Biol.* **100**:1339-1342.
- Farber, D.B., Brown, B.M., and Lolley, R.N. 1979. Cyclic nucleotide dependent protein kinase and the phosphorylation of endogenous proteins of retinal rod outer segments. *Biochemistry.* **18**:370-378.
- Feige, J.J., Pirollet, F., Cochet, C., and Chambaz, E.M. 1980. Selective inhibition of a cyclic nucleotide-independent protein kinase (G type casein kinase) by naturally occurring glycosaminoglycans. *FEBS Lett.* **121**:139-142.

- Fesenko, E.E., Kolesnikov, S.S., and Lyubarsky, A.L. 1985. Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature*. **313**:310-313.
- Fiat, A.M., and Jolles, P. 1989. Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. *Mol. Cell Biochem.* **87**:5-30.
- Fong, S.L., Landers, R.A., and Bridges, C.D. 1985. Varieties of rhodopsinin frog rod outer segment membranes: analysis by isoelectric focusing. *Vision Res.* **25**:1387-1397.
- Forrester, J., Dick, A., McMenamin, P., and Lee, W. 1996. The eye: Basic sciences in practice. Saunders, London.
- Fowles, C., Akhtar, M., and Cohen, P. 1989. Interplay of phosphorylation and dephosphorylation in vision: protein phosphatases of bovine rod outer segments. *Biochemistry*. **28**:9385-9391.
- Frins, S., Bönigk, W., Müller, F., Kellner, R., and Koch, K.-W. 1996. Functional characterization of a guanylyl cyclase-activating protein from vertebrate rods. *J. Biol. Chem.* **271**:8022-8027.
- Fung, B.K.-K. 1983. Characterization of transducin from bovine retinal rod outer segments. Separation and reconstitution of the subunits. *J. Biol. Chem.* **258**:10495-10502.
- Fung, B.K.-K., and Griswold-Prenner, I. 1989. G protein-effector coupling: Binding of rod phosphodiesterase inhibitory subunit to transducin. *Biochemistry*. **28**:3133-3137.
- Fung, B.K.-K., Hurley, J.B., and Stryer, L. 1981. Flow of information in the light-triggered cyclic nucleotide cascade of vision. *Proc. Natl. Acad. Sci. U.S.A.* **78**:152-156.
- Fung, B.K.-K., and Stryer, L. 1980. Photolyzed rhodopsin catalyzes the exchange of GTP for GDP in retinal rod outer segment membranes. *Proc. Natl. Acad. Sci. U.S.A.* **77**:2500-2504.
- Gatica, M., Hinrichs, M.V., Jedlicki, A., Allende, C.C., and Allende, J.E. 1993. Effect of metal ions on the activity of casein kinase II from *Xenopus laevis*. *FEBS Lett.* **315**:173-177.
- Goldberg, A.F.-X., Loewen, C.J., and Molday, R.S. 1998. Cysteine residues of photoreceptor peripherin/rds: role in subunit assembly and autosomal dominant retinitis pigmentosa. *Biochemistry*. **37**:680-685.
- Gorczyca, W.A., Polans, A.S., Surgucheva, I.G., Subbaraya, I., Baehr, W., and Palczewski, K. 1995. Guanylyl cyclase activating protein. *J. Biol. Chem.* **270**:22029-22036.

- Gordon, S.E., Brautigan, D.L., and Zimmerman, A.L. 1992. Protein phosphatases modulate the apparent agonist affinity of the light-regulated ion channel in retinal rods. *Neuron*. **9**:739-748.
- Gordon, S.E., Downing-Park, J., and Zimmerman, A.L. 1995. Modulation of the cGMP-gated ion channel in frog rods by calmodulin and an endogenous inhibitory factor. *J. Physiol.* **3**:533-546.
- Gordon, S.E., and Zagotta, W.N. 1995a. Localization of regions affecting an allosteric transition in cyclic nucleotide-activated channel. *Neuron*. **14**:857-864.
- Gordon, S.E., and Zagotta, W.N. 1995b. A histidine residue associated with the gate of the cyclic nucleotide-activated channels in rod photoreceptors. *Neuron*. **14**:177-183.
- Gorodovikova, E.N., Gimelbrant, A.A., Senin, I.I., and Philippov, P.P. 1994. Recoverin mediates the calcium effect upon rhodopsin phosphorylation and cGMP hydrolysis in bovine retina rod cells. *FEBS Lett.* **349**:187-190.
- Goulding, E.H., Tibbs, G.R., and Siegelbaum, S.A. 1994. Molecular mechanism of cyclic nucleotide-gated channel activation. *Nature*. **372**:369-374.
- Gounaris, A., Trangas, T.T., and Tsipalis, C.M. 1987. Soluble cAMP-independent protein kinase from human spleen. *Arch. Biochem. Biophys.* **259**:473-480.
- Gray-Keller, M.P., and Detwiler, P.B. 1994. The calcium feedback signal in the phototransduction cascade of vertebrate rods. *Neuron*. **13**:849-861.
- Gray-Keller, M.P., Polans, A.S., Palczewski, K., and Detwiler, P.B. 1993. The effect of recoverin-like calcium-binding proteins on the photoresponse of retinal rods. *Neuron*. **10**:523-531.
- Green, W.N., Ross, A.F., and Claudio, T. 1991. cAMP stimulation of acetylcholine receptor expression is mediated through posttranslational mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* **88**:854-858.
- Grunwald, M.E., Yu, W.-P., Yu, H.-H., and Yau, K.-W. 1998. Identification of a domain on the β -subunit of the rod cGMP-gated cation channel that mediates inhibition by calcium-calmodulin. *J. Biol. Chem.* **273**:9148-9157.
- Guyton, A.C., and Hall, J.E. 1996. Textbook of medical physiology. 9th edition. W.B. Saunders. 637-650.
- Hagins, W.A., Penn, R.D., and Yoshikami, S. 1970. Dark current and photocurrent in retinal rods. *Biophys. J.* **10**:380-412.

- Hathaway, G.M., Lubben, T.H., and Traugh, J.A. 1980. Inhibition of casein kinase II by heparin. *J. Biol. Chem.* **255**:8038-8041.
- Hathaway, G.M., and Traugh, J.A. 1982. Casein kinases-multipotential protein kinases. In: *Current topics in cellular regulation*. edited by E. Stadtman and B. Horecker, pp. 101-127. Academic Press. New-York.
- Hayashi, F., and Yamazaki, A. 1991. Polymorphism in purified guanylate cyclase from vertebrate rod photoreceptors. *Proc. Natl. Acad. Sci. U.S.A.* **88**:4746-4750.
- Haynes, L.W., and Yau K.-W. 1985. Cyclic GMP-sensitive conductance in outer segment membrane of catfish cones. *Nature*. **317**:61-64.
- He, W., Cowan, C.W., and Wensel, T.G. 1998. RGS9, a GTPase accelerator for phototransduction. *Neuron*. **20**:95-102.
- Hsu, S.-C. 1993. Glucose metabolism in phototransduction. Ph.D. Thesis. University of British Columbia.
- Hsu, Y.-T., and Molday, R.S. 1993. Modulation of the cGMP-gated channel of rod photoreceptor cell by calmodulin. *Nature*. **361**:76-79.
- Hsu, Y.-T., and Molday, R.S. 1994. Interaction of calmodulin with the cyclic GMP-gated channel of rod photoreceptor cells. *J. Biol. Chem.* **269**:29765-29770.
- Huganir, R.L., Delcour, A.H., Greengard, P., and Hess, G.P. 1986. Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. *Nature*. **321**:774-776.
- Huganir, R.L., and Greengard, P. 1983. cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **80**:1130-1134.
- Huganir, R.L., Miles, K., and Greengard, P. 1984. Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **81**:6968-6972.
- Hundal, S.P., DiFrancesco, D., Mangoni, M., Brammar, W.J., and Conley, E.C. 1993. An isoform of the cGMP-gated retinal photoreceptor channel gene expressed in the sinoatrial node (pacemaker) region of rabbit heart. *Biochem. Soc. Trans.* **21**:119S.
- Ildefonse, M., and Bennett, N. 1991. Single-channel study of the cGMP-dependent conductance of retinal rods from incorporation of native vesicles into planar lipid bilayers. *J. Membr. Biol.* **123**:133-147.
- Ishiguro, S.-I., Suzuki, Y., Tamai, M., and Mizuno, K. 1991. Purification of retinol dehydrogenase from bovine retinal rod outer segments. *J. Biol. Chem.* **266**:15520-15524.

- Johnson, L.V., and Blanks, J.C. 1984. Application of acrylamide as an embedding medium in studies of lectin and antibody binding in the vertebrate retina. *Current Eye Res.* **3**:969-973.
- Kaplan, R.S., and Pedersen, P.L. 1985. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal. Biochem.* **150**:97-104.
- Kapoor, C.L., and Chader, G.J. 1984. Endogenous phosphorylation of retinal photoreceptor outer segment protein by calcium phospholipid-dependent protein kinase. *Biochem. & Biophys. comm.* **122**:1397-1403.
- Karpen, J.W., Brown, R.L., Stryer, L., and Baylor, D.A. 1993. Interaction between divalent cations and the gating machinery of cyclic GMP-activated channels in salamander rods. *J. Gen. Physiol.* **101**:1-25.
- Kaupp, U.B. 1995. Family of cyclic nucleotide gated ion channels. *Curr. opin. Neurobiol.* **5**:434-442.
- Kaupp, U.B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N.J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T., and Numa, S. 1989. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature.* **342**:762-766.
- Kawamura, S. 1993. Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. *Nature.* **362**:855-857.
- Kim, T.S.Y. 1998. Topological organization, functional characterization and localization of the bovine rod photoreceptor Na/Ca-K exchanger. Ph.D. Thesis. University of British Columbia.
- Kim, T.S.Y., Reid, D.M., and Molday, R.S. 1998. Structure-function relationships and localization of the Na/Ca-K exchanger in rod photoreceptors. *J. Biol. Chem.* **273**:16561-16567.
- Koch, K.-W. 1994. Calcium as modulator of phototransduction in vertebrate photoreceptor cells. *Rev. Physiol. Biochem. Pharmacol.* **125**:149-192.
- Koch, K.-W., Cook, N.J., and Kaupp, U.B. 1987. The cGMP-dependent channel of vertebrate rod photoreceptors exists in two forms of different cGMP sensitivity and pharmacological behavior. *J. Biol. Chem.* **262**:14415-14421.
- Koch, K.-W., and Kaupp, U.B. 1985. Cyclic GMP directly regulates a cation conductance in membranes of bovine rods by a cooperative mechanism. *J. Biol. Chem.* **260**:6788-6800.

- Kohnken, R.E., Chafouleas, J.G., Eadie, D.M., Means, A.R., and McConnell, D.G.J. 1981. Calmodulin in bovine rod outer segments. *J. Biol. Chem.* **256**:12517-12522.
- Korenbrot, J.I., and Miller, D.L. 1989. Cytoplasmic free calcium concentration in dark-adapted retinal rod outer segments. *Vision Res.* **29**:939-948.
- Körschen, H.G., Illing, M., Seifert, R., Sesti, F., Williams, A., Gotzes, S., Colville, C., Müller, F., Dosé, A., Godde, M., Molday, L., Kaupp, U.B., and Molday, R.S. 1995. A 240 kDa protein represents the complete β subunit of the cyclic nucleotide-gated channel from rod photoreceptor. *Neuron*. **15**:627-636.
- Koutalos, Y. 1992. High-pH form of bovine rhodopsin. *Biophys. J.* **61**:272-275.
- Koutalos, Y., and Yau, K.-W. 1996. Regulation of sensitivity in vertebrate rod photoreceptors by calcium. *Trends Neurosci.* **19**:73-81.
- Kretsinger, R.H. 1976. Calcium-binding proteins. *Annu. Rev. Biochem.* **45**:239-266.
- Kühn, H. 1978. Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. *Biochemistry*. **17**:4389-4395.
- Kühn, H. 1980. Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. *Nature*. **283**:587-589.
- Kühn, H., Bennett, N., Michel-Villaz, M., and Chabre, M. 1981. Interactions between photoexcited rhodopsin and GTP-binding protein: Kinetics and stoichiometric analyses from light-scattering changes. *Proc. Natl. Acad. Sci. U.S.A.* **78**:6873-6877.
- Kühn, H., and Dreyer, W.J. 1972. Light-dependent phosphorylation of rhodopsin by ATP. *FEBS Lett.* **20**:1-6.
- Kühn, H., Hall, S.W., and Wilden, U. 1984. Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. *FEBS Lett.* **176**:473-478.
- Kurenni, D.E., Moroz, L.L., Turner, D.W., Sharkey, K.A., and Barnes, S. 1994. Modulation of ion channels in rod photoreceptors by nitric oxide. *Neuron*. **13**:315-324.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**:680-685.
- Lagnado, L., and Baylor, D.A. 1992. Signal flow in visual transduction. *Neuron*. **8**:995-1002.

- Lagnado, L., Cervetto, L., and McNaughton, P.A. 1992. Calcium homeostasis in the outer segments of retinal rods from the tiger salamander. *J. Physiol.* **455**:111-142.
- Lamb, T.D., McNaughton, P.A., and Yau, K.-W. 1981. Spatial spread of activation and background desensitization in toad rod outer segments. *J. Physiol.* **319**:463-496.
- Lane, R.D., Crissman, R.S., and Ginn, S. 1986. High efficiency fusion procedure for producing monoclonal antibody against weak immunogens. *Methods Enzymol.* **121**:183-192.
- Lee, R.H., Brown, B.M., and Lolley, R.N. 1981. Protein kinases of retinal rod outer segments: identification and partial characterization of cyclic nucleotide dependent protein kinase and rhodopsin kinase. *Biochemistry*. **20**:7532-7538.
- Lee, R.H., Brown, B.M., and Lolley, R.N. 1990. Protein kinase A phosphorylates retinal phosphducin on serine 73 in situ. *J. Biol. Chem.* **265**:15860-15866.
- Leinders-Zufall, T., Rosenboom, H., Barnstable, C.J., Shepherd, G.M., and Zufall, F. 1995. A calcium-permeable cGMP-activated cation conductance in hippocampal neurons. *Neuroreport*. **6**:1761-1765.
- Levitan, I.B. 1985. Phosphorylation of ion channels. *J. Memb. Biol.* **87**:177-190.
- Levitan, I.B. 1988. Modulation of ion channels in neurons and other cells. *Ann. Rev. Neurosci.* **11**:119-136.
- Levitan, I.B. 1994. Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* **56**:193-212.
- Liebman, P.A., and Pugh, E.N.Jr. 1980. Phosphodiesterase activation in visual receptor membranes. *Nature*. **287**:734-736.
- Lion, F., Rotmans, J.P., Daemen, F.J.M., and Bonting, S.L. 1975. Biochemical aspects of the visual process: XXVII. Stereospecificity of ocular dehydrogenases and the visual cycle. *Biochim. Biophys. Acta*. **384**:283-292.
- Litchfield, D.W., Lozemann, F.J., Piening, C., Sommercorn, J., Takio, K., Walsch, K.A., and Krebs, E.G. 1990. Subunit structure of casein kinase II from bovine testis. *J. Biol. Chem.* **265**:7638-7644.
- Litchfield, D.W., and Lüscher, B. 1993. Casein kinase II in signal transduction and cell cycle regulation. *Mol. Cell. Biochem.* **127/128**:187-199.
- Liu, D.T., Tibbs, G.R., and Siegelbaum, S.A. 1996. Subunit stoichiometry of cyclic nucleotide-gated channels and effects of subunit order on channel function. *Neuron*. **16**:983-990.

- Liu, M., Chen, T.-Y., Ahamed, B., Li, J., and Yau, K.-W. 1994. Calcium-calmodulin modulation of the olfactory cyclic nucleotide-gated cation channel. *Science*. **266**:1348-1354.
- Makino, E.R., Handy, J.W., Li, T., and Arshavsky, V.Y. 1999. The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5 G protein beta subunit. *Proc. Natl. Acad. Sci. U.S.A.* **96**:1947-1952.
- March, S.C., Parikh, I., and Cuatrecasas, P. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* **60**:149-152.
- McKay, D.B., and Steitz, T.A. 1981. Structure of catabolite gene activator protein at 2.9-Å resolution suggests binding to left-handed B-DNA. *Nature*. **290**:744-749.
- McMahan, U.J., and Wallace, B.G. 1989. Molecules in basal lamina that direct formation of synaptic specializations at neuromuscular junctions. *Dev. Neurosci.* **11**:227-247.
- McNaughton, P.A., Cervetto, L., and Nunn, B.J. 1986. Measurement of the intracellular free calcium concentration in salamander rods. *Nature*. **322**:261-263.
- Meggio, F., Boldyreff, B., Marin, O., Pinna, L.A., and Issinger, O.-G. 1992. Role of β subunit of casein kinase-2 on the stability and specificity of the recombinant reconstituted holoenzyme. *Eur. J. Biochem.* **204**:293-297.
- Meggio, F., Marin, O., and Pinna, L.A. 1994. Substrate specificity of protein kinase CK2. *Cell. Molec. Biol. Res.* **40**:401-409.
- Meggio, F., Pinna, L.A., Marchiori, F., and Borin, G. 1983. Polyglutamyl peptides: a new class of inhibitors of type-2 casein kinases. *FEBS Lett.* **162**:235-238.
- Menini, A., Rispoli, G., and Torre, V. 1988. The ionic selectivity of the light-sensitive current in isolated rods of the tiger salamander. *J. Physiol. (London)*. **402**:279-300.
- Miller, J.L., Fox, D.A., and Litman, B.J. 1986. Amplification of phosphodiesterase activation is greatly reduced by rhodopsin phosphorylation. *Biochemistry*. **25**:4983-4988.
- Mitev, V., Pauloin, A., and Houdebine, L.M. 1994. Purification and characterization of two casein kinase type II isozymes from bovine brain gray matter. *J. Neurochem.* **63**:717-726.
- Molday, R.S. 1996. Calmodulin regulation of cyclic-nucleotide-gated channels. *Curr. Opin. Neurobiol.* **6**:445-452.
- Molday, R.S., and Molday, L.L. 1987. Differences in the protein composition of bovine retinal rod outer segment disk and plasma membranes isolated by a ricin-gold-dextran density perturbation method. *J. Cell Biol.* **105**:2589-2601.

- Molday, R.S., and Molday, L.L. 1993. Isolation and characterization of rod outer segment disk and plasma membranes. *Methods in Neurosciences*. **15**:131-150.
- Molday, R.S., and Molday, L.L. 1999. Purification, characterization and reconstitution of cyclic nucleotide-gated channels. *Methods Enzymol.* **294**:246-260.
- Molokanova, E., Trivedi, B., Savchenko, A., and Kramer, R.H. 1997. Modulation of rod photoreceptor cyclic nucleotide-gated channels by tyrosine phosphorylation. *J. Neurosci.* **17**:9068-9076.
- Morelli, A., Damonte, G., Panfoli, I., and Pepe, I. 1989. Proteins of rod outer segments of toad retina: binding with calmodulin and with GTP. *Biochem. Biophys. Res. Comm.* **163**:363-369.
- Müller, F., Bonigk, W., Sesti, F., and Frings, S. 1998. Phosphorylation of mammalian olfactory cyclic nucleotide-gated channels increases ligand sensitivity. *J. Neurosci.* **18**:164-173.
- Müller, F., and Koch, K.-W. 1998. Calcium-binding proteins and nitric oxide in retinal function and disease. *Acta Anat.* **162**:142-150.
- Nagao, S., Yamazaki, A., and Bitensky, M.W. 1987. Calmodulin and calmodulin binding proteins in amphibian rod outer segments. *Biochemistry*. **26**:1659-1665.
- Nakamura, T., and Gold, G.H. 1987. A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature*. **325**:442-444.
- Nakatani, K., Koutalos, Y., and Yau, K.-W. 1995. Ca^{2+} modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors. *J. Physiol. (London)*. **484**:69-76.
- Nakatani, K., and Yau, K.-W. 1988a. Calcium and magnesium fluxes across the plasma membrane of the toad rod outer segment. *J. Physiol.* **395**:695-729.
- Nakatani, K., and Yau, K.-W. 1988b. Guanosine 3'-5'-cyclic monophosphate-activated conductance studied in a truncated rod outer segment of the toad. *J. Physiol. (London)*. **395**:731-753.
- Nelsestuen, G.L., and Bazzi, M.D. 1991. Activation and regulation of protein kinase C enzymes. *J. Bioenerg. Biomembr.* **23**:43-61.
- Nizzari, M., Sesti, F., Giraudo, M.T., Virginio, C., Cattaneo, A, and Torre, V. 1993. Single-channel properties of cloned cGMP-activated channels from retinal rods. *Proc. R. Soc. London [biol].* **254**:69-73.

- Numann, R., Catterall, W.A., and Scheuer, T. 1991. Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science*. **254**:115-118.
- Oda, Y., Hackos, D., Korenbrot, J.I., Timpe, L., Largman, C., and Mauro, T. 1996. Keratinocyte differentiation induces alternatively spliced forms of a cGMP-gated channel. *Molec. Biol. Cell*. **7**:251a.
- Olshevskaya, E.V., Hughes, R.E., Hurley, J.B., and Dizhoor, A.M. 1997. Calcium binding, but not a calcium-myristoyl switch, controls the ability of guanylyl cyclase-activating protein GCAP-2 to regulate photoreceptor guanylyl cyclase. *J. Biol. Chem.* **272**:14327-14333.
- Otto-Bruc, A.E., Fariss, R.N., Van Hooser, J.P., and Palczewski, K. 1998. Phosphorylation of photolyzed rhodopsin is calcium-insensitive in retina permeabilized by alpha-toxin. *Proc. Natl. Acad. Sci. U.S.A.* **95**:15014-15019.
- Palczewski, K. 1994. Is vertebrate phototransduction solved? New insights into the molecular mechanism of phototransduction. *Invest. Ophthalmol. Vis. Sci.* **35**:3577-3581.
- Palczewski, K., Hargrave, P.A., McDowell, J.H., and Ingebritsen, T.S. 1989. The catalytic subunit of phosphatase 2A dephosphorylates phosphoopsin. *Biochemistry*. **28**:415-419.
- Palczewski, K., McDowell J.H., and Hargrave, P.A. 1988. Purification and characterization of rhodopsin kinase. *J. Biol. Chem.* **263**:14067-14073.
- Palczewski, K., Subbaraya, I., Gorczyca, W.A., Helekar, B.S., Ruiz, C.C., Ohguro, H., Huang, J., Zhao, X., Crabb, J.W., Johnson, R.S., Walsh, K.A., Gray-Keller, M.P., Detwiler, P.B., and Baehr, W. 1994. Molecular cloning and characterization of retinal photoreceptor guanylyl cyclase-activating protein. *Neuron*. **13**:395-404.
- Penn, R.D., and Hagins, W.A. 1972. Kinetics of the photocurrent of retinal rods. *Biophys. J.* **12**:1073-1094.
- Picones, A., and Korenbrot, J.I. 1995. Permeability and interaction of Ca^{2+} with cGMP-gated ion channels differ in retinal rod and cone photoreceptors. *Biophys. J.* **69**:120-127.
- Pugh, E.N., Jr., and Lamb, T.D. 1993. Amplification and kinetics of the visual steps in phototransduction. *Biochim. Biophys. Acta*. **1141**:111-149.
- Ratto, G.M., Payne, R., Owen, W.G., and Tsien, R.Y. 1988. The concentration of cytosolic free calcium in vertebrate rod outer segments measured with Fura-2. *J. Neurosci.* **8**:3240-3246.
- Read, S.M., and Northcote, D.H. 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.* **116**:53-64.

- Rebrik, T.I., and Korenbrot, J.I. 1998. In intact photoreceptors, a Ca^{2+} -dependent, diffusible factor modulates the cGMP-gated ion channels differently than in rods. *J. Gen. Physiol.* **112**:537-548.
- Rieke, F., and Schwartz, E.A. 1994. A cGMP-gated current can control exocytosis at cone synapses. *Neuron*. **13**:863-873.
- Root, M.J., and MacKinnon, R. 1993. Identification of an external divalent cation-binding site in the pore of a cGMP-activated channel. *Neuron*. **11**:459-466.
- Ross, A.F., Rapuano, M., Schmidt, J.H., and Prives, J.M. 1987. Phosphorylation and assembly of nicotinic acetylcholine receptor subunits in cultured chick muscle cells. *J. Biol. Chem.* **262**:14640-16647.
- Rossie, S., and Catterall, W.A. 1987. Cyclic AMP-dependent phosphorylation of voltage-sensitive sodium channels in primary cultures of rat brain neurons. *J. Biol. Chem.* **262**:12735-12744.
- Rossie S., and Catterall, W.A. 1989. Phosphorylation of the alpha subunit of rat brain sodium channels by cAMP-dependent protein kinase at a new site containing Ser686 and Ser687. *J. Biol. Chem.* **264**:14220-14224.
- Rossie, S., Gordon, D., and Catterall, W.A. 1987. Identification of an intracellular domain of the sodium channel having multiple cAMP-dependent phosphorylation sites. *J. Biol. Chem.* **262**:17530-17535.
- Saari, J.C., and Bredberg, L. 1988. CoA- and Non-CoA-dependent retinol esterification in retinal pigment epithelium. *J. Biol. Chem.* **263**:8084-8090.
- Safran, A., Sagi-Eisenberg, R., Neumann, D., and Fuchs, S. 1987. Phosphorylation of the acetylcholine receptor by protein kinase C and identification of the phosphorylation site within the receptor δ subunit. *J. Biol. Chem.* **262**:10506-10510.
- Santy, L.C., and Guidotti, G. 1996. Reconstitution and characterization of two forms of cyclic nucleotide-gated channel from skeletal muscle. *Am. J. Physiol.* **271**:E1051-1060.
- Schnetkamp, P.P.M. 1986. Sodium-calcium exchange in the outer segments of bovine rod photoreceptors. *J. Physiol* (London). **373**:25-45.
- Schnetkamp, P.P.M., Basu, D.K., and Szerencsei, R.T. 1989. $\text{Na}^+-\text{Ca}^{2+}$ exchange in bovine rod outer segments requires and transports K^+ . *Am. J. Physiol.* **257**:C153-C157.
- Sesti, F., Eismann, E., Kaupp, U.B., Nizzari, M., and Torre, V. 1995. The multi-ion nature of the cGMP-gated channel from vertebrate rods. *J. Physiol.* (London). **487**:17-36.

- Shichi, H. 1983. Biochemistry of vision. Academic Press, New-York.
- Sillman, A.J., Ito, H., and Tomita, T. 1969. Studies on the mass receptor potential of the isolated frog retina: On the basis of the ionic mechanism. *Vision Res.* **9**:1443-1451.
- Sitaramayya, A., and Liebman, P.A. 1983. Mechanism of ATP quench of phosphodiesterase activation in rod disk membranes. *J. Biol. Chem.* **258**:1205-1209.
- Soderling, T.R. 1990. Protein kinases. Regulation by autoinhibitory domains. *J. Biol. Chem.* **265**:1823-1826.
- Sood, S.M., and Slattery, C.N. 1997. Monomer characterization and studies of self-association of the major beta-casein of human milk. *J. Dairy Sci.* **80**:1554-1560.
- Stirling, C.E., and Lee, A. 1980. [3H] Oubain autoradiography of frog retina. *J. Cell. Biol.* **85**:313-324.
- Stryer, L. 1986. Cyclic GMP cascade of vision. *Annu. Rev. Neurosci.* **9**:87-119.
- Sun, H., Molday, R.S., and Nathans, J. 1999. Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease. *J. Biol. Chem.* **274**:8269-8281.
- Szuts, E.Z. 1985. Light stimulates phosphorylation of two large membrane proteins in frog photoreceptors. *Biochemistry*. **24**:4176-4184.
- Taniguchi, M., Kashiwayanagi, M., and Kurihara, K. 1996. Intracellular dialysis of cyclic nucleotides induces inward currents in turtle vomeronasal receptor neurons. *J. Neurosci.* **16**:1239-1246.
- Taylor, A., Allende, C.C., Weinmann, R., and Allende, J.E. 1987. The phosphorylation of nucleoplasmin by casein kinase-2 is resistant to heparin inhibition. *FEBS Lett.* **226**:109-114.
- Taylor, S.S. 1987. Protein kinases: a diverse family of related proteins. *BioEssays*. **7**:24-29.
- Tuazon, P.T., and Traugh, J.A. 1991. Casein kinase I and II-multipotential serine protein kinases: structure, function and regulation. *Adv. Second Messenger Phosphoprot. Res.* **23**:123-164.
- Udovichenko, I.P., Newton, A.C., and Williams, D.S. 1997. Contribution of protein kinase C to the phosphorylation of rhodopsin in intact retinas. *J. Biol. Chem.* **272**:7952-7959.
- Van Eldik, L., and Watterson, M.D. 1998. Calmodulin and signal transduction. *Academic Press (London)*.

- Varnum, M.D., and Zagotta, W.N. 1996. Subunit interactions in the activation of cyclic nucleotide-gated ion channels. *Biophys. J.* **70**:2667-2679.
- Varnum, M.D., and Zagotta, W.N. 1997. Interdomain interactions underlying activation of cyclic nucleotide-gated channels. *Science*. **278**:110-113.
- Verma, A.K., Enyedi, A., Filoteo, A.G., and Penniston, J.T. 1994. Regulatory region of plasma membrane Ca²⁺ pump. 28 residues suffice to bind calmodulin but more are needed for full auto-inhibition of the activity. *J. Biol. Chem.* **269**:1687-1691.
- Wafford, K.A., Burnett, D.M., Leidenheimer, N.J., Burt, D.R., and Wang, J.B. 1991. Ethanol sensitivity of the GABA_A receptor expressed in Xenopus oocytes requires 8 amino acids contained in the gamma 2L subunit. *Neuron*. **7**:27-33.
- Wafford, K.A., and Whiting, P.J. 1992. Ethanol potentiation of GABA_A receptors requires phosphorylation of the alternatively spliced variant of the $\gamma 2$ subunit. *FEBS Lett.* **313**:113-117.
- Wald, G. 1968. Molecular basis of visual excitation. *Science*. **162**:230-239.
- Weber, I.T., and Steitz, T.A. 1987. Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5-Å resolution. *J. Molec. Biol.* **198**:311-326.
- Wei, J.-Y., Roy, D.S., Leconte, L., and Barnstable, C.J. 1998. Molecular and pharmacological analysis of cyclic nucleotide-gated channel function in the central nervous system. *Prog. Neurobiol.* **56**:37-64.
- Weitz, D., Martin, Z., Müller, F., Beyermann, M., Körschen, H.G., Kaupp, U.B., and Koch, K.-W. 1998. Calmodulin controls the rod photoreceptor CNG channel through an unconventional binding site in the N-terminus of the β -subunit. *EMBO J.* **17**:2273-2284.
- Wensel, T.G., and Stryer, L. 1990. Activation mechanism of retinal rod GMP phosphodiesterase probed by fluorescein-labeled inhibitory subunit. *Biochemistry*. **29**:2155-2161.
- West, J.W., Numann, R., Murphy, B.J., Scheuer, T., and Catterall, W.A. 1991. A phosphorylation site in the Na⁺ channel required for modulation by protein kinase C. *Science*. **254**:866-868.
- Weyand, I., Godde, M., Frings, S., Weiner, J., Müller, F., Altenhofen, W., Hatt, H., and Kaupp U.B. 1994. Cloning and functional expression of a cyclic nucleotide-gated channel from mammalian sperm. *Nature*. **368**:859-863.

Whalen, M.M., Bitensky, M.W., and Takemoto, D.J. 1990. The effect of the gamma-subunit of the cyclic GMP phosphodiesterase of bovine and frog (*Rana catesbeiana*) retinal rod outer segments on the kinetic parameters of the enzyme. *Biochem. J.* **265**:655-658.

Wheeler, G.L., and Bitensky, M.W. 1977. A light-activated cyclic GMP phosphodiesterase. *Proc. Natl. Acad. Sci. U.S.A.* **74**:4238-4242.

Wilden, U., and Kühn, H. 1982. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Biochemistry*. **21**:3014-3022.

Williams, D.S., Liu, X., Schlamp, C.L., Ondek, B., Jaken, S., and Newton, A.C. 1997. Characterization of protein kinase C in photoreceptor outer segment. *J. Neurochem.* **69**:1693-1702.

Williardson, B.M., Wilkins, J.F., Yoshida, T., and Bitensky, M.W. 1996. Regulation of phosducin phosphorylation in retinal rods by Ca^{2+} /calmodulin-dependent adenylyl-cyclase. *Proc. atl. Acad. Sci. U.S.A.* **93**:1475-1479.

Witt, J.J., and Roskoski, R. 1975. Rapid protein kinase assay using phosphocellulose-paper absorption. *Anal. Biochemistry*. **66**:253-258.

Wolbring, G., and Cook, N.J. 1991. Rapid purification of protein kinase C from bovine retinal rod outer segments. *Euro. J. Biochem.* **201**:601-606.

Wolbring, G., and Schnetkamp, P.P.M. 1995. Activation by PKC of the Ca^{2+} -sensitive guanylyl cyclase in bovine retinal rod outer segments measured with an optical assay. *Biochemistry*. **34**:4689-4695.

Yamazaki, A., Hayashi, F., Tatsumi, M., Bitensky, M.W., and George, J.S. 1990. Interactions between the subunits of cyclic GMP phosphodiesterase in *Rana Catesbeiana* rod photoreceptors. *J. Biol. Chem.* **265**:11539-11548.

Yamazaki, A., Sen, I., Bitensky, M., Casnellie, J.E., and Greengard, P. 1980. Cyclic GMP-specific high affinity, noncatalytic binding sites on light-activated phosphodiesterase. *J. Biol. Chem.* **255**:11619-11624.

Yau, K.-W. 1994. Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. *Invest. Ophtalmol. Visual Sci.* **35**:9-32.

Yau, K.-W., and Baylor, D.A. 1989. Cyclic GMP-activated conductance of retinal photoreceptor cells. *Annu. Rev. Neurosci.* **12**:289-327.

Yau, K.-W., and Nakatani, K. 1984. Electrogenic Na-Ca exchange in retinal rod outer segment. *Nature*. **311**:661-663.

- Yau, K.-W., and Nakatani, K. 1985. Light-induced reduction of cytoplasmic free calcium in retinal rod outer segment. *Nature*. **313**:252-255.
- Yee, R., and Liebman, P.A. 1978. Light-activated phosphodiesterase of the rod outer segment. Kinetics and parameters of activation and deactivation. *J. Biol. Chem.* **253**:8902-8909.
- Yoshida, T., Willardson, B.M., Wilkins, J.F., Jensen, G.J., Thornton, B.D., and Bitensky, M.W. 1994. The phosphorylation state of phosducin determines its ability to block transducin subunit interactions and inhibit transducin binding to activated rhodopsin. *J. Biol. Chem.* **269**:24050-24057.
- Zagotta, W.N. 1996. Molecular mechanisms of cyclic-nucleotide gated channels. *J. Bioenergetics Biomembranes*. **28**:269-278.
- Zagotta, W.N., and Siegelbaum, S.A. 1996. Structure and function of cyclic nucleotide-gated channels. *A. Rev. Neurosci.* **19**:235-263.
- Zimmerman, A.L., and Baylor, D.A. 1992. Cation interactions within the cyclic GMP-activated channel of retinal rods from the tiger salamander. *J. Physiol.* **449**:759-783.
- Zuckerman, R., Buzdygon, B., Philip, N., Liebman, P., and Sitaramayya, A. 1985. Arrestin: An ATP/ADP exchange protein that regulates cGMP phosphodiesterase activity in retinal rod disk membranes (RDM). *Biophys. J.* **47**:37a.
- Zollner, H. 1993. Handbook of enzyme inhibitors. VCH publications. 2nd edition. Weinheim.