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Department of Biochemistry and Molecular Biology
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Vancouver, Canada
Date June 30, 1994
ABSTRACT

During the past decade, great advances have been made towards deciphering the molecular mechanism of vision. Findings from various laboratories have led to the establishment of the visual cascade and the identification of the main members of this pathway. However, little is known so far about the intricate regulation of the various enzymes involved, in particular, the roles of Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-binding proteins. This thesis describes the finding of a novel regulation of the visual transduction process through the Ca\textsuperscript{2+}-calmodulin dependent modulation of the cGMP-gated channels.

An unknown 17/20 kDa protein found in the EDTA extract of bovine rod outer segments (ROS) was purified and identified as calmodulin by a variety of biochemical and immunochemical studies. The cGMP-gated channel complex, which plays an important role in phototransduction, was found to be the major calmodulin binding protein in ROS membranes by calmodulin affinity chromatography and Western blotting analysis with iodinated calmodulin. This association between calmodulin and the channel complex, which is mediated through a high molecular weight channel associated protein, was found to be conserved among various species.

The effect of calmodulin on the ROS cGMP-gated channel activity was investigated by a Ca\textsuperscript{2+} influx assay using ROS membrane vesicles pre-loaded with Arsenazo III dye. In the absence of calmodulin, the channel displayed an apparent Km of 19 ± 0.4 µM for cGMP, while in the presence of calmodulin, its apparent Km increased to 33 ± 2 µM. Similar shifts in the Km of the channel for cGMP were observed using extruded ROS membrane vesicles containing either dichlorophosphonazo III or neutral red. This effect is specific for calmodulin since other Ca\textsuperscript{2+}-binding proteins such as bovine recoverin and brain S-100 caused no change on channel activity. The calmodulin-mediated shift in the affinity of the channel for cGMP could be reversed by mastoparan, a peptide inhibitor of calmodulin. In addition to Ca\textsuperscript{2+}, calmodulin also appeared to affect the translocation of various monovalent and divalent cations across the ROS membrane.
Calmodulin modulation of the channel was observed to occur within a physiological Ca$^{2+}$ range of 20-300 nM.

The calmodulin effect was shown to be mediated through the 240 kDa channel associated protein as determined by a Ca$^{2+}$ efflux assay using the immunoaffinity purified channel complex reconstituted into lipid vesicles. In the absence of calmodulin, the reconstituted channel complex displayed a $K_m$ of 33 $\mu$M for cGMP, while in the presence of calmodulin, the $K_m$ increased to 44 $\mu$M. N-terminal sequence analysis of a 105 kDa calmodulin-binding fragment of the 240 kDa protein revealed a sequence that matched with the recently cloned $\beta$-subunit of the human rod cGMP-gated channel.

In summary, the presence of calmodulin in ROS has been detected. This protein binds preferentially to the $\beta$-subunit of the cGMP-gated channel complex and can modulate the affinity of the channel for cGMP. A model of the calmodulin binding to the channel complex is presented and the importance of the calmodulin mediated regulation of the channel activity is discussed.
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LIST OF ABBREVIATIONS

ATP .................. adenosine 5'-triphosphate
BCA .................. bicinehoninic acid
BSA .................. bovine serum albumin
cAMP .................. adenosine 3', 5'-cyclic monophosphate
cGMP .................. guanosine 3', 5'-cyclic monophosphate
CHAPS ............... (3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
DEAE .................. diethylaminoethyl
DFP .................. diisopropylfluorophosphate
DTT .................. dithiothreitol
ECL .................. enhanced chemiluminescence
EDTA .................. ethylenediamine tetraacetic acid
EGTA .................. ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCCP .................. carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone
FITC .................. fluorescein isothiocyanate
GDP .................. guanosine diphosphate
GMP .................. guanosine monophosphate
GTP .................. guanosine triphosphate
h .................. hour
HEPES ............... (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
Ig .................. immunoglobulin
kDa .................. kilodalton
min .................. minute
Mr .................. relative molecular weight
PBS .................. phosphate buffered saline
PDE .................. phosphodiesterase
ROS .................. rod outer segments
SDS................. sodium dodecyl sulphate
Tris................ (Tris[hydroxymethyl]aminomethane)
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CHAPTER 1

INTRODUCTION

1.1. PHOTORECEPTOR ROD CELL

1.1.1. Structure

The photoreceptor rod cell is a highly specialized neuron found in the peripheral region of the retina. It functions to mediate vision under dim light conditions. In the human retina, the rod cell is generally 1-3 μm in diameter and 40-60 μm in length (Shichi, 1983). It is divided into 4 major compartments: the outer segment, the inner segment, the cell body, and the synaptic terminal (Fig. 1). The outer segment is the primary site for visual transduction. This compartment is joined to the inner segment via a nonmotile cilium containing microtubule doublets and actin and myosin filaments. This contractile system is believed to mediate the formation of new disks at the base of the outer segment (Williams, 1991). The inner segment contains the metabolic machinery of the cell including the endoplasmic reticulum, golgi apparatus, and mitochondria. Newly synthesized ROS proteins are believed to be transported from Golgi apparatus in the inner segment via vesicles (Bird et al., 1988). These vesicles fuse with the ciliary plasma membrane and the proteins are sorted to either disk or plasma membranes by an unknown mechanism. Mitochondria supply the majority of the energy, in the form of ATP, required by the various metabolic processes of the rod cell. The cell body is the compartment that encloses the nucleus. Messenger RNAs from the nucleus direct the synthesis of various rod cell proteins. The synaptic terminal of the rod photoreceptor cell contains numerous neurotransmitter vesicles. In the dark, neurotransmitters are continuously released to activate the connecting bipolar and horizontal cells. The release of these neurotransmitters are inhibited in response to light.
Fig. 1: Schematic representation of a vertebrate photoreceptor rod cell.

The photoreceptor rod cell is divided into four major compartments: the outer segment, the inner segment, the cell body, and the synaptic terminal (diagram provided by Dr. R. S. Molday).
1.1.2. Rod outer segments

Each outer segment is made up of a stack of hundreds of disks surrounded by a plasma membrane. At the base of the outer segment, evagination of the ciliary plasma membrane produces continuously folded nascent disk membranes from which mature disks and the plasma membrane form (Steinberg et al., 1980). A disk consists of two closely spaced lamellar membranes joined together by a hairpin loop structure known as the rim region. The disks have been shown to interact with the plasma membrane at these rim regions through filamentous structures (Roof and Heuser, 1982). In the outer segment, approximately 95% of the membranes are disk membranes while only 5% of the membranes are plasma membranes (Molday and Molday, 1987).

Intact rod outer segments can be prepared free of other retinal cells and organelles by gently shaking the retinas in a sucrose solution followed by density gradient centrifugation (Godchaux and Zimmerman, 1979; Molday and Molday, 1987). The protein components of the outer segments can be separated into soluble and membrane fractions (Godchaux and Zimmerman, 1979; Kühn, 1980). Using a ricin-gold-dextran membrane perturbation method, Molday and Molday (1987) have shown that the membrane fraction can be further separated into disk and plasma membranes. Many of the proteins found in the soluble and membrane fractions as listed in Table I (Molday and Molday, 1993), have been identified and characterized by various laboratories. The soluble fraction includes enzymes required for the visual cascade, glucose metabolism, and calcium-dependent pathways. As for the membrane fraction, studies have shown that the disk and plasma membranes, with the exception of rhodopsin, have quite distinct protein compositions.

In terms of lipids, the two membrane systems also appear to have distinct compositions. Plasma membranes have been reported to have a high content of cholesterol, unsaturated C\textsubscript{18} (18:2; 18:3) fatty acids and the saturated C\textsubscript{14} fatty acids, whereas the disk membranes are high in the saturated C\textsubscript{18} fatty acid and unsaturated C\textsubscript{22}
Table I

Rod outer segment proteins

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td><strong>Soluble and membrane-associated proteins</strong></td>
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<tr>
<td>Phosphodiesterase</td>
<td></td>
</tr>
<tr>
<td>α-subunit</td>
<td>88,000</td>
</tr>
<tr>
<td>β-subunit</td>
<td>84,000</td>
</tr>
<tr>
<td>γ-subunit</td>
<td>11,000</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>85,000</td>
</tr>
<tr>
<td>Rhodopsin kinase</td>
<td>68,000</td>
</tr>
<tr>
<td>Arrestin</td>
<td>48,000</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>43,000</td>
</tr>
<tr>
<td>Transducin</td>
<td></td>
</tr>
<tr>
<td>α-subunit</td>
<td>39,000</td>
</tr>
<tr>
<td>β-subunit</td>
<td>37,000</td>
</tr>
<tr>
<td>γ-subunit</td>
<td>8,000</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td>44,000</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>38,000</td>
</tr>
<tr>
<td>Phosducin</td>
<td>33,000</td>
</tr>
<tr>
<td>Recoverin</td>
<td>26,000</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>16,700</td>
</tr>
<tr>
<td>Phosphatase 2A</td>
<td>38,000</td>
</tr>
<tr>
<td><strong>Other proteins</strong></td>
<td></td>
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<tr>
<td>Glycolytic enzymes</td>
<td></td>
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<td>Nucleotide diphosphokinase</td>
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<td>Pentose shunt enzymes</td>
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<td>Guanylate kinase</td>
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<td>Tubulin</td>
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<td><strong>Disk membrane proteins</strong></td>
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<td>Rim protein</td>
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<td>Rhodopsin</td>
<td>38,000</td>
</tr>
<tr>
<td>Peripherin/rds</td>
<td>35,000</td>
</tr>
<tr>
<td>ROM-1</td>
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<tr>
<td>Retinol dehydrogenase</td>
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<tr>
<td><strong>Plasma membrane proteins</strong></td>
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</tr>
<tr>
<td>cGMP-gated channel-associated protein</td>
<td>240,000</td>
</tr>
<tr>
<td>Na+/K+-Ca2+ exchanger</td>
<td>230,000</td>
</tr>
<tr>
<td>cGMP-gated channel</td>
<td>63,000</td>
</tr>
<tr>
<td>Glut-1 glucose transporter</td>
<td>50,000</td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>38,000</td>
</tr>
<tr>
<td>Guanylate cyclase?</td>
<td>112,000</td>
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(22:6) docosahexaenoic acid (Boesze-Battaglia and Albert, 1989). Thus, specific sorting mechanisms are likely to exist in the rod cell to direct the lipids and proteins to their respective membrane systems.

1.2. PHOTOTRANSDUCTION

Phototransduction is the process by which the photoreceptors convert absorbed light into electrical signals. In the dark, there is a continuous flow of current across the photoreceptor plasma membrane. The direction of flow is inward at the outer segment and outward at the inner segment (Fig. 2). By suction electrode recording studies, it was shown that this circulating dark current reaches a value of 20-50 pA per vertebrate rod cell (Baylor et al., 1979a, b). The inward current, mainly in the form of Na\(^+\) ions, is electrically balanced by an outward K\(^+\) current at the inner segment. This is brought about by the maintenance of Na\(^+\) and K\(^+\) gradients across the inner segment plasma membrane by the electrogenic action of Na\(^+\)/K\(^+\) ATPase. Sodium ions from the inward current are pumped out of the rod inner segment into the extracellular medium where the \([\text{Na}^+]\) is much higher, in exchange for the translocation of K\(^+\) ions into the inner segment from the extracellular medium where the \([\text{K}^+]\) is lower. Potassium ions can then exit the inner segment down their concentration gradient via voltage sensitive K\(^+\)-channels in the plasma membrane of the inner segment to complete the circuit.

The Na\(^+\) current entering the outer segment is suppressible by light. This suppression is referred to as the photocurrent. Generally, it has been found that the isomerization of a single rhodopsin will give rise to a peak photocurrent of 0.2-1.5 pA. In other words, the dark current will be suppressed by 0.2-1.5 pA. This photocurrent has three main features. First, the response of photocurrent is linear for 5-10 isomerization of rhodopsin per rod cell (Penn and Hagins, 1972; Baylor et al., 1979 a, b). Second, individual photons will cause the suppression of the dark current only over a restricted length of the outer segment due to compartmentalization of the photoresponse (Lamb and
Fig. 2: **Hyperpolarization of the rod cell in response to light.**

In the dark, Na$^+$ enters the ROS through the plasma membrane specific cation channels. Na$^+$ then flows into the inner segment and is continuously extruded by Na$^+$/K$^+$ ATPase in exchange for the influx of K$^+$ (3 Na$^+$ extruded for the influx of 2 K$^+$). The K$^+$ translocated into the inner segment by Na$^+$/K$^+$ ATPase then exits the inner segment through the voltage-gated potassium channels. The continuous flow of this dark current enables the release of neurotransmitter vesicles from the synaptic terminal. In the light, the photoactivated process causes the closure of the ROS cation channels. The continuous action of Na$^+$/K$^+$ ATPase to pump out Na$^+$ from the inner segment will then result in the hyperpolarization of the ROS plasma membrane. This hyperpolarization diminishes the release of neurotransmitter vesicles from the synaptic terminal.
Yau, 1981). Thirdly, the single photon response is multistage in nature due the underlying complex pathways of the visual cascade system (Fuortes and Hodgkin, 1964).

1.2.1. Ca\textsuperscript{2+} vs cGMP as the primary messenger for photoexcitation

In the early 1970s, various laboratories directed much of their efforts toward identifying the primary messenger which relays the photoabsorption event on the disk membrane to a change in plasma membrane conductance. Two distinct hypotheses were proposed at the time. The first hypothesis, proposed by Yoshikami and Hagins (1970), suggested that the divalent cation Ca\textsuperscript{2+}, is the primary messenger of photoexcitation. In their model (Fig. 3), it was suggested that in the dark, intracellular Ca\textsuperscript{2+} concentration is relatively low and there is a high accumulation of Ca\textsuperscript{2+} within the disks. The light activated process causes the release of Ca\textsuperscript{2+} from the disks and an increase in the intracellular Ca\textsuperscript{2+} level. This increase in Ca\textsuperscript{2+} suppresses the dark current by directly blocking the cation channels in the plasma membrane. The second hypothesis, also known as the cGMP hypothesis, stemmed from the findings of several laboratories (Bitensky et al., 1971; Cohen et al., 1978; Kilbride and Ebrey, 1979; Woodruff and Bownds, 1979). This hypothesis suggests that the light activated process induces a change in the intracellular cGMP concentration which ultimately affects the cation conductance of the plasma membrane (Fig. 4). The first hypothesis was eventually disproved by a number of experimental results. First, it was shown that Ca\textsuperscript{2+} closed the channels more slowly than did light (Yau and Nakatani, 1985). Second, ROS depleted of Ca\textsuperscript{2+} could still respond to light (Matthews et al., 1985; Cote et al., 1985). Third, the cation channels on excised ROS plasma membrane patches are opened by the addition of cGMP, but not by the addition of Ca\textsuperscript{2+} (Fesenko et al., 1985). The cGMP hypothesis, together with the discovery of the various visual cascade enzymes, formulated the present model of visual transduction.
Fig. 3. Calcium hypothesis for visual excitation.

In the dark, calcium ions are believed to be sequestered within the ROS disks and this maintains a relatively low level of Ca\(^{2+}\) within the cytoplasm. Under this condition, Na\(^{+}\) flows into the outer segment unhindered through the plasma membrane specific cation channels. In the light, the light activated process will cause the release of Ca\(^{2+}\) from the disks. The elevated level of cytoplasmic Ca\(^{2+}\) will then prevent further entry of Na\(^{+}\) by directly blocking the cation channels.
Fig. 4. cGMP hypothesis for visual excitation.

In the dark, the elevated level of cGMP maintains a significant number of cation channels in their open state and this allows the influx of Na\(^+\) into the outer segment. In the light, the photobleaching of rhodopsin activates the visual cascade resulting in a decrease in the level of cGMP. This decrease in the level of cGMP will then cause the closure of the cation channels and prevent further influx of Na\(^+\) into the outer segment.
1.3. VISUAL TRANSDUCTION CASCADE

The visual transduction process is divided into two major stages: the initial light stimulated excitation step and the subsequent recovery to the dark resting state. Briefly, the visual excitation cascade (Fig. 5) is initiated when light induces the photoisomerization of the 11-*cis*-retinal chromophore of rhodopsin to its all trans-form. This photoexcited form of rhodopsin then activates transducin by catalyzing the exchange of GTP for GDP on the α-subunit of transducin. Activated transducin turns on the cGMP-dependent phosphodiesterase which catalyzes the hydrolysis of cGMP to 5'-GMP. In the dark, an elevated level of cGMP maintains the cGMP-gated channels in their open state and allows the influx of Na⁺ and Ca²⁺ into the outer segment. The light-stimulated decrease in the cGMP level causes the closure of these cGMP-gated cation channels. Closure of these cation channels results in the hyperpolarization of the rod outer segment plasma membrane and initiates the transmission of electrical signals. Recovery of the rod cell to its dark resting state involves the deactivation of rhodopsin, transducin, phosphodiesterase, and the stimulation of guanylate cyclase to resynthesize cGMP required for the reopening of the cGMP-gated channels.

1.3.1. Photoactivation of rhodopsin

Vertebrate rhodopsin, a membrane protein of 38 kDa, is present in high abundance in ROS disk membranes. In the dark, it is conjugated to an 11-*cis*-retinal chromophore via a Schiff base to a specific lysine residue. In the case of bovine rhodopsin, the linkage is at lysine 296, in the middle of the seventh transmembrane helix (Thomas and Stryer, 1982). Upon absorption of a photon, 11-*cis*-retinal isomerizes to its all trans isomer within 200 femtoseconds (Schoenlein et al., 1991). This isomerization of retinal leads to the formation of various rhodopsin photolysis intermediates beginning with bathorhodopsin and proceeding on to form lumirhodopsin, metarhodopsin I and finally to metarhodopsin II (Lamola et al., 1974). Formation of metarhodopsin II then leads to changes in the
Fig. 5: A diagram showing the basic reactions of the visual transduction pathway.

In the dark, elevated cGMP concentrations maintain a number of cGMP-gated channels in their open conformation. This allows the influx of Na\(^+\) and Ca\(^{2+}\) into the outer segment and maintains the outer segment in a partially depolarized state. Photobleaching of rhodopsin, involving the isomerization of 11-cis retinal to its all-trans isomer, results in the formation of activated (meta II) rhodopsin which catalyzes the exchange of bound GDP for GTP on transducin. Transducin will then activate phosphodiesterase which catalyzes the hydrolysis of cGMP to GMP. The decrease in free cGMP concentration within the outer segment will cause a closure of the cGMP-gated channels to the influx of Na\(^+\) and Ca\(^{2+}\) and a transient hyperpolarization of the outer segment. The recovery of the ROS to its dark resting state occurs through 1) the inactivation of rhodopsin by a rhodopsin kinase (RK) catalyzed phosphorylation reaction and the binding of arrestin (Ar); 2) inactivation of transducin by hydrolysis of bound GTP to GDP; 3) inhibition of phosphodiesterase by rebinding of the inhibitory subunits to the catalytic subunits; 4) resynthesis of cGMP from GTP by guanylate cyclase (GC); and 5) the reopening of the cGMP-gated channel as cGMP levels increase.
environment of various rhodopsin amino acids (Chahre, 1985; Chen and Hubbell, 1978) and the dissociation of retinal from the opsin (Bownds, 1967; Cooper et al., 1987).

1.3.2. Activation of transducin

Photoreceptor ROS transducin is a member of the heterotrimeric GTP binding protein family. Each transducin consists of an α-, β-, and γ-subunit. In its inactive form, transducin is peripherally associated with the ROS disk membrane and its α-subunit contains a bound GDP molecule. Upon photoactivation, metarhodopsin II associates with the α-subunit of transducin via rhodopsin's cytoplasmic loops (Kühn and Hargrave, 1981; König et al., 1989). Metarhodopsin II catalyzes the exchange of GTP for GDP on the α-subunit of transducin (Kühn, 1980). The binding of GTP to transducin results in a conformational change which causes the α-subunit of transducin to dissociate from both the β, γ-subunits and metarhodopsin II (Kühn, 1980). The free metarhodopsin II can then interact with another transducin complex. A single metarhodopsin II can activate several hundred transducin molecules. This represents the first step of signal amplification in visual activation.

1.3.3. Hydrolysis of cGMP by PDE

PDE in ROS is composed of one α-, one β-, and two γ-subunits. The cGMP hydrolysis function of the enzyme resides in the α- and β-subunits while the γ-subunits act as inhibitory subunits (Hurley and Stryer, 1982). In addition to its catalytic site, PDE also has two additional high affinity noncatalytic cGMP binding sites (Yamazaki et al., 1980). The phosphodiesterase complex becomes activated when the two γ inhibitory subunits are inactivated by binding to two GTP-γs subunits (Wensel and Stryer, 1990). Removal of the inhibitory restraint of these subunits increases the phosphodiesterase activity by 50 to 100 fold (Baehr et al., 1979; Hurley and Stryer, 1982). Approximately, one hundred
phosphodiesterase molecules are activated from a single photon absorption. Each PDE molecule can lead to the hydrolysis of up to $10^3$ cGMP molecules.

1.3.4. Closure of the cGMP-gated channel

The free cGMP concentration in the ROS is approximately 4-10 µM. This maintains a significant number of the cGMP-gated channels in their open state (Nakatani and Yau, 1988a). The opening of these channels allows the influx of Na$^+$ and Ca$^{2+}$ into the outer segment. The activation of phosphodiesterase leads to a rapid hydrolysis of cGMP to 5'-GMP. This results in a local depletion of cGMP and causes the closure of the nearby plasma membrane cGMP-gated channels.

1.3.5. Deactivation and regeneration of rhodopsin

To turn off the visual activation cascade, rhodopsin, transducin, and phosphodiesterase have to be inactivated and cGMP has to be regenerated. Inactivation of photoactivated rhodopsin involves the phosphorylation of rhodopsin followed by the binding of arrestin to phosphorylated rhodopsin. Phosphorylation of rhodopsin has been shown to be a light-dependent process (Kühn and Dreyer, 1972; Bownds et al., 1972). This phosphorylation reaction occurs at serine and threonine residues and is universal among various species. More than 85% of phosphate incorporation is found at the C-terminal region of rhodopsin (Hargrave et al., 1980). Under conditions of high level of rhodopsin bleaching, the extent of phosphorylation is proportional to the fraction of rhodopsin being bleached with a maximum of 9 moles of phosphate incorporated into each mole of rhodopsin (Wilden and Kühn, 1982).

Rhodopsin undergoes a conformational change upon photobleaching. However, rhodopsin cannot be phosphorylated until the formation of metarhodopsin II (Yamamoto and Shichi, 1983; Seckler and Rando, 1989). This conformational change is necessary to allow the cytoplasmic surface of the opsin to interact with rhodopsin kinase. Rhodopsin
kinase is a serine and threonine kinase that is dependent on Mg$^{2+}$. It is a member of a new class of receptor kinases that phosphorylate the G-protein coupled receptors (Palczewski and Benovic, 1991).

Phosphorylation of rhodopsin is insufficient to completely stop the activation of transducin by metarhodopsin II (Wilden et al., 1986), although a graded decrease in transducin activation is associated with an increasing level of rhodopsin phosphorylation (Miller et al., 1986). A complete inhibition of metarhodopsin's ability to activate transducin requires the further binding of arrestin to the phosphorylated form of metarhodopsin II (Kühn et al., 1984). Arrestin dissociates from metarhodopsin II when the all-trans-retinal chromophore is reduced to all-trans-retinol by retinol dehydrogenase (Hofmann et al., 1992, Ishiguro et al., 1991). Upon arrestin dissociation, metarhodopsin II is dephosphorylated by phosphatase 2A (Palczewski et al., 1989). All-trans-retinol is transported to the retinal pigment epithelial cells and converted back to 11-cis-retinal (Bok and Heller, 1976). The newly synthesized 11-cis-retinal is then transported back into the ROS where it reassociates with opsin to regenerate rhodopsin.

1.3.6. Deactivation of transducin and phosphodiesterase

The α-subunit of transducin has intrinsic GTPase activity. It is believed that GTP hydrolysis deactivates GTP-Τα (Dratz et al., 1987; Vuong and Chabre, 1990). The resulting inactive GDP-Τα complex dissociates from the γ-subunit of phosphodiesterase. This free GDP-Τα subunit can then reassociate with the β- and γ- heterodimers of transducin. However, some studies have suggested that the rate of GTP hydrolysis is too slow and that Τα inactivation may require an alternate pathway (Erickson et al., 1992). The catalytic activity of phosphodiesterase is inactivated when the γ inhibitory dimers reassociates with the α- and β- catalytic subunits of phosphodiesterase.

1.3.7. Resynthesis of cGMP
Guanylate cyclase is a key enzyme responsible for visual recovery. This enzyme catalyzes the formation of cGMP from GTP. The activity of this enzyme is inhibited by pyrophosphate, a by-product of the cGMP synthesis (Hakki and Sitaramayya, 1990), and is dependent on the level of intracellular Ca\(^{2+}\) (Koch and Stryer, 1988). The resynthesis of cGMP by guanylate cyclase results in an increase in the level of cGMP. This elevated level of cGMP reopens the cGMP-gated channels to allow the influx of Na\(^{+}\) and Ca\(^{2+}\) into the outer segment.

1.3.8. Roles of calcium in visual transduction

In the dark, cytoplasmic Ca\(^{2+}\) in the photoreceptor ROS is kept at a constant level by two transporting systems. Influx of calcium ions entering the outer segment via the cGMP-gated channels are counterbalanced by their constant extrusion through the plasma membrane specific Na\(^{+}\)/Ca\(^{2+}\)-K\(^{+}\) exchanger. This maintains the cytoplasmic Ca\(^{2+}\) at a concentration of between 0.2-0.6 μM (McNaughton et al., 1986; Ratto et al., 1988). When light triggers the visual activation cascade, a decrease in cGMP concentration results in the closure of the cGMP-gated channels. The closure of the cGMP-gated channels prevents the influx of Ca\(^{2+}\) into the outer segment. Since the activity of the Na\(^{+}\)/Ca\(^{2+}\)-K\(^{+}\) exchanger is not affected by the light activated process, Ca\(^{2+}\) continues to be extruded and the Ca\(^{2+}\) level within the outer segment decreases to below 0.1 μM.

Koch and Stryer (1988) proposed that the main target of this Ca\(^{2+}\) flux between light and dark conditions is guanylate cyclase. The activity of this enzyme was shown to increase 5-20 fold when Ca\(^{2+}\) is lowered from 300 nM to 20 nM. This effect appeared to be mediated through a soluble factor. It was proposed that in the dark when the Ca\(^{2+}\) level is high, this factor binds to Ca\(^{2+}\) and is maintained in its inactive state. Guanylate cyclase is in turn maintained at its basal level of activity (Fig. 6). When the photobleaching of rhodopsin results in a decrease in the level of Ca\(^{2+}\) through the closure of the cGMP-gated channels, Ca\(^{2+}\) dissociates from this soluble factor. This activated
Fig. 6: Calcium-dependent modulation of guanylate cyclase.

In the dark, elevated levels of cGMP maintain a relatively high concentration of Ca\(^{2+}\) within the outer segment through the opening of the cGMP-gated channels. The modulator (M) of guanylate cyclase binds Ca\(^{2+}\) and is in its inactive state. Under these conditions, guanylate cyclase is kept at its basal level of activity. When the light activated process causes a decrease in the level of cGMP ① and the closure of the cGMP-gated channels, the continuous action of Na\(^{+}/Ca^{2+}-K^{+}\) exchanger will then result in a decrease in the level of Ca\(^{2+}\) within the outer segment ②. The dissociation of Ca\(^{2+}\) from this modulator will activate it and this protein will then stimulate the resynthesis of cGMP from GTP ③. As the level of cGMP rises within the outer segment, the cGMP-gated channels will reopen and replenish the level of Ca\(^{2+}\) within the outer segment to its dark resting state ④. The rebinding of Ca\(^{2+}\) to the modulator will once again deactivate it and minimize the stimulation of guanylate cyclase activity ⑤.
factor then stimulates guanylate cyclase to resynthesize cGMP. Once the cGMP-gated channels reopen in response to an elevated level of cGMP, the Ca^{2+} level within the outer segment rises. This allows Ca^{2+} to rebind to the guanylate cyclase activating factor and restores the guanylate cyclase to its basal level of activity.

A 26 kDa Ca^{2+} binding protein, recoverin, was originally believed to be this Ca^{2+}-dependent modulator of guanylate cyclase (Dizhoor et al., 1991; Lambrecht and Koch, 1991). However, subsequent studies indicated that recoverin is unlikely to be the modulator of guanylate cyclase (Hurley et al., 1993). Recombinant recoverin, expressed in *E. coli.*, failed to stimulate rod guanylate cyclase. In addition, at the low Ca^{2+} concentrations required to stimulate guanylate cyclase, recoverin dissociates from the ROS membranes. More recently, it has been shown that recoverin affects instead, phosphodiesterase activation in a Ca^{2+}-dependent manner, through regulating rhodopsin phosphorylation (Kawamura, 1993). With increasing Ca^{2+} concentrations, this protein was found to decrease the extent of rhodopsin phosphorylation and thus have a direct effect on the rate of phosphodiesterase activation. This modulation is thought to play a role in visual adaptation, a phenomenon characterized by a decrease in sensitivity of the photoreceptor cell in the presence of background illumination (Fain and Matthews, 1990; Pugh Jr. and Lamb, 1990; Shapley and Enroth-Cugell, 1984).

1.4. THE cGMP-GATED CHANNEL COMPLEX

1.4.1. Properties of the cGMP-gated channel

The cGMP-gated channel of ROS is a cation selective channel that does not discriminate well between monovalent cations (Fesenko et al., 1985; Hodgkin et al., 1985). The relative permeabilities of various monovalent cations have been measured and are shown in the decreasing order: NH_{4}^{+} > Li^{+} > Na^{+} > K^{+} > Rb^{+} > Cs^{+}. The cGMP-gated channel is also permeable to divalent cations such as Ca^{2+} and Mg^{2+}. In the dark, the inward current is mainly carried by Na^{+} and to a lesser degree by Ca^{2+} and Mg^{2+}.
(Hodgkin et al., 1985; Nakatani and Yau, 1988a; Cervetto et al., 1988). Suppression of the Na⁺ component of this current results in the hyperpolarization of the rod cell. The Ca²⁺ component plays a role in visual recovery, while the Mg²⁺ component has no known function in phototransduction so far.

Voltage clamping analyses of isolated ROS indicate that the current-voltage relation shows an outward rectification (Bader et al., 1979; Bodoia and Detwiler, 1985; Baylor and Nunn, 1986). At negative voltages, the current-voltage relation is flat, implying that the rod dark current is insensitive to physiological changes in membrane potential (between -40 mV in dark to -80 mV in light, Cervetto and Fuortes, 1978). This rectification of the cGMP-gated channels together with the absence of leakage channels allows the effective propagation of the light-induced hyperpolarization through the outer segment (Yau and Baylor, 1989).

Single-channel current recordings of the rod plasma membrane patches at low cGMP concentrations (Haynes et al., 1986; Zimmerman and Baylor, 1986) indicate that the channel has a maximum single channel conductance of 25 pS in the absence of divalent cations. The channel openings last for a few milliseconds and display a characteristic flickery nature. This suggests that the cGMP molecules may be loosely bound to the channel. This fast gating kinetics of the channel improves the signal to noise ratio for photon detection and also allows the rapid propagation of the photoresponse. Single channel activity is suppressed when a low concentration of Ca²⁺ or Mg²⁺ is added. Bursts of high-frequency flickering events are observed in place of the single channel opening events, suggestive of the intermittent blocking of the open channel by divalent cations. At physiological potentials, the unit conductance is lowered from 25 to 0.1 pS and this translates into a decrease of unitary current from a maximum of 1 pA to 4 fA. Thus, a large number of channels must be open to maintain the dark current at a fixed size. This effectively reduces the level of background noise.
Several pharmacological agents have been shown to block the cGMP-gated channel conductance. One of these inhibitors is the Ca$^{2+}$ channel antagonist l-cis-diltiazem which blocks the conductance at micromolar concentrations in both the ROS membrane preparations and excised ROS plasma membrane patches (Koch and Kaupp, 1985; Stern et al., 1986). However, it does not inhibit the activity of the purified channels that have been reconstituted into lipid vesicles (Cook et al., 1987). The compound, 3',4'-dichlorobenzamil, which is a derivative of the Na$^+$ channel blocker amiloride has also been reported to block channel activity at micromolar range (Nicol et al., 1987). More recently, Nicol (1993) has shown that pimozide, a Ca$^{2+}$ channel and calmodulin antagonist, is also an effective blocker of the rod cGMP-gated channel in the micomolar range.

1.4.2. Purification and localization of the cGMP-gated channel

The rod cGMP-gated channel was first purified by Cook et al. (1987) from CHAPS solubilized ROS membranes by DEAE anion exchange and TSK AF-red affinity chromatography. The purified channel preparation, as visualized on SDS polyacrylamide gels, consisted of a prominent 63 kDa and a fainter 240 kDa band. This preparation exhibited cGMP-dependent calcium flux activity when reconstituted into lipid vesicles. Further studies by Molday et al. (1990) revealed that these two proteins are tightly associated to one another. The channel complex, consisting of the 63 kDa and the 240 kDa proteins, could be copurified by immunoaffinity chromatography using either an anti-63 kDa cGMP-gated channel monoclonal antibody PMc 1D1 or an anti-240 kDa protein monoclonal antibody PMs 4B2. More recently, Brown et al. (1993) demonstrated that both the 63 kDa and the 240 kDa proteins can be photoaffinity labelled by 8-p-azidophenacylthio-cGMP, an analog of cGMP. This suggests that the cGMP-gated channel complex may consist of at least two distinct subunits, each with the capability to bind cGMP.
Various biochemical and immunocytochemical analyses using the anti-63 kDa cGMP-gated channel monoclonal antibody PMc 1D1 and the anti-240 kDa protein monoclonal antibody PMs 4B2 have been carried out to study the distribution of the channel in rod outer segments. The results indicate that the cGMP-gated channel complex is exclusively localized to the ROS plasma membrane and its density is approximately 300 μm⁻² (Cook et al., 1989; Molday et al., 1990).

1.4.3. Molecular cloning of the cGMP-gated channel complex

The sequence of the 63 kDa channel subunit was determined by Kaupp et al. (1989) by screening a retinal cDNA library using degenerate oligonucleotide probes generated from the partial amino acid sequences of the tryptic channel fragments. The full length cDNA encodes a membrane protein of 690 amino acids (Mr. 79,601). Homologues of this channel subunit have been cloned and sequenced from several other species. The mouse and human rod channel sequences are 85% identical to the bovine sequence (Pittler et al., 1992; Dhallan et al., 1992) while the chicken rod and cone subunits share 76% and 65% identity with the bovine sequence, respectively. When the full length cDNA was transcribed into mRNA and microinjected into Xenopus oocytes, the expressed channel displayed many of the electrophysiological properties of the ROS channel (Kaupp et al., 1989). It was observed to be activated by cGMP with a K₁/₂ of 52 μM and a cooperativity of 1.75. However, it was less sensitive to the channel blocker, L-cis-diltiazem, and it did not display rapid flickering of the channel conductance.

Until recently, it was believed that the channel was composed of a homooligomeric complex. Chen et al. (1993), however, demonstrated the presence of a second subunit (designated as the β-subunit) of the channel by homology cloning from a human retinal library. The human β-subunit shares 30% homology with the original bovine channel (now designated as the α-subunit). The full length cDNA of the β-subunit encodes a membrane protein of 909 amino acids (Mr 102,330). Alternate splicing
produces a shorter protein product that has its first 286 amino acids at its N-terminus missing. The relative distribution of these two forms of the β-subunit in ROS, however, is not known. When this β-subunit was transiently expressed in human kidney 293 cells, the expressed channel was inactive as determined by patch clamping study. But, when this β-subunit was coexpressed with the α-subunit, the resulting complex displayed ion conductance properties that were more similar to the native ROS channel. In particular, the coexpressed α- and β-subunits displayed rapid flickering of the channel conductance and increased sensitivity to inhibition by L-cis-diltiazem.

1.4.4. Structural analysis and topography of the cGMP-gated channel subunits

Sequencing analysis of the α- and β-subunits of the cGMP-gated channels of various species revealed several interesting features. Stretches of 80-100 amino acids close to the C-termini of the channels have been identified as the cGMP binding sites (Kaupp et al., 1989; Chen et al., 1993; also see Fig. 7), on the basis of their similarity to the putative cGMP binding domain of the bovine lung cGMP-dependent protein kinase (Takio et al., 1984). Hydropathy plots of the channel sequences suggest the presence of 5 hydrophobic segments, designated as H1 - H5, which are of sufficient length to span the lipid bilayer. In the bovine α-subunit, there are 5 consensus sequences for N-linked glycosylation at Asn residues 90, 91, 177, 327, and 423. However, by lectin binding, endoglycosidase treatment, and immunochemical studies, the channel α-subunit is likely to be N-linked glycosylated only at Asn position 327 (Wohlfart et al., 1989, 1992). In contrast, there is no consensus sequence for a N-linked glycosylation site in the human β-subunit.

The full length cDNA of the bovine α-subunit encodes an 80 kDa protein. However, the purified ROS membrane channel subunit generally displays a molecular weight of 63 kDa on the SDS polyacrylamide gels. N-terminal sequence analysis of the purified α-subunit revealed the absence of the first 92 amino acids from the N-terminus of
Fig. 7: Sequence alignment of the cyclic nucleotide binding domains of channels from different species.

Alignment of the potential cyclic nucleotide binding sites of rod and cone α subunits, the rod β subunit, and the olfactory subunit. Identical amino acids are indicated by ( : ). A high degree of sequence identity within the nucleotide binding domain is observed.
the channel. The α-subunits of other mammalian species also display an apparent Mr. 63 K on the SDS polyacrylamide gels, thus suggesting that this type of N-terminal truncation may be universal for mammalian photoreceptor channels (Molday et al., 1991). Analysis of the chicken rod and cone α-subunits also indicate the occurrence of some type of cleavage reactions (Bönigk et al., 1993). The cleavage site between Ser 92 and 93 of the bovine sequence, N-N-S-S-N-K-E, is conserved in other mammals (Fig. 8). Related but nonidentical sequences are also found in the α-subunits of chicken photoreceptors.

A modified voltage sensor motif S4, as found in the voltage-gated shaker K⁺ channel, is present in both the photoreceptor and olfactory nucleotide gated channel subunits (Jan and Jan, 1990; Kaupp et al., 1989). In voltage gated channels, this motif generally consists of up to 7 repeats of a positively charged arginine or lysine residue separated by two hydrophobic amino acids. This motif is thought to span the lipid bilayer. For the photoreceptor and olfactory channel subunits, there are only four of these repeats and the motif also contains several negatively charged residues (Fig. 9). It is likely that this divergence of the S4 motifs of the photoreceptor and olfactory channels from its voltage gated channel counterpart is responsible for their insensitivity to physiological changes in membrane potential (Kaupp, 1991).

In addition, the photoreceptor and olfactory channel subunits also contain a segment (Fig. 9) which shows considerable similarity to the pore region of the voltage gated K⁺ channel (Goulding et al., 1992; Heginbotham et al., 1992; Bönigk et al., 1993). The pore region has been suggested to consist of two antiparallel β strands connected by a hairpin turn (Yellen et al., 1991; Durell and Guy, 1992). The pore regions of the individual subunits are likely to line the internal cavity of the channel complex in order to translocate ions across the membrane.

Based on immunocytochemical labelling, biochemical, and sequence analysis, a model showing the structural features of the α-subunit of the cGMP-gated channel has been proposed (Bönigk et al., 1993) as shown in Figure 10. The H1-H5 hydrophobic
### N-TERMINAL CLEAVAGE SITE

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<th>Sequence</th>
<th>Range</th>
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<td>N N S S N K E</td>
<td>(90-96)</td>
</tr>
<tr>
<td>Human Rod (α)</td>
<td>N N N S N K D</td>
<td>(89-95)</td>
</tr>
<tr>
<td>Mouse Rod (α)</td>
<td>N N N S N K D</td>
<td>(84-90)</td>
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<tr>
<td>Chicken Rod (α)</td>
<td>N N N S N K D</td>
<td>(53-59)</td>
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<tr>
<td>Chicken Cone (α)</td>
<td>S N N T N E D</td>
<td>(145-151)</td>
</tr>
</tbody>
</table>

Fig. 8: Sequence alignment of the N-terminal cleavage sites of the cGMP-gated channels from different species.

Alignment of the N-terminal cleavage sites from bovine, human, mouse, and chicken rod and cone cGMP-gated channels.
**VOLTAGE SENSOR MOTIF (S4)**

<table>
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<th>Species</th>
<th>Motif Alignment</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
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<td>(360-380)</td>
</tr>
<tr>
<td>Bovine Rod (α)</td>
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<tr>
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**PUTATIVE PORE**

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<tr>
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Fig. 9: Sequence alignment of the putative voltage sensor motifs (S4) and pore regions of the cyclic nucleotide gated channels.

The voltage sensor motifs (S4) and pore regions of the bovine rod channel were compared with those of the voltage-gated Shaker K+ channel and other cyclic nucleotide gated channels.
segments and the S4 motif are likely to span the membrane while the putative pore is viewed as extending into the membrane in an extended β conformation. The N-terminal and C-terminal segments of the channel are both oriented to the cytoplasmic side. The Asn 327 glycosylation site is exposed to extracellular environment. The cGMP binding domain of the channel, located near the C-terminus, is likely to interact with the pore to control cation translocation. The β-subunit, which displays sequence homology with the α-subunit, probably has similar structural features.

1.5. CALMODULIN

Calmodulin was first discovered as a component required to activate cAMP-phosphodiesterase from bovine brain (Cheung, 1970). Subsequently, it was shown by Teo and Wang (1973) to be a Ca$^{2+}$-binding protein that confers Ca$^{2+}$ sensitivity to this enzyme. Calmodulin has been purified from bovine brain by either the conventional chromatographic methods such as DEAE anion exchange, hydroxyapatite, and gel filtration chromatography or by more specific affinity chromatographic methods using ligands such as troponin I and melittin (Grand et al., 1979; Kincaid, 1987) or antipsychotic drugs such as phenothiazine, trifluoroperazine, and chlorpromazine (Charbonneau and Cormier, 1979; Jamieson and Vanaman, 1979) immobilized onto agarose.

1.5.1. Molecular cloning of calmodulin

The first calmodulin cDNA sequence was obtained from the electroplax tissue of the electric eel (Munjaal et al., 1981). Subsequently, the calmodulin cDNA sequences were determined from a variety of species such as chicken (Putkey et al., 1983), toad (Chien and Dawid, 1984), rat (Nojima and Sokabe, 1987), human (Wawrzynczak and Perham, 1984), fruit fly (Smith et al., 1987), yeast (Davis et al., 1986), slime mold (Goldhagen and Clarke, 1986), and trypanosome (Tschudi et al., 1985). The amino acid sequences of these calmodulins show a high degree of homology with one another.
Fig. 10: Working model for the organization of the α-subunit of bovine rod cGMP-gated channel within the lipid bilayer.

The first 92 amino acids (dashed line) as predicted from cDNA sequence analysis are absent in the channel in ROS membranes possibly by a posttranslational cleavage reaction. Segments labeled H1 - H5 and the S4 voltage sensor motif are possible transmembrane α-helix segments and the segment between H4 and H5 is the putative pore region. Immunogold labelling studies have established the N and C terminus on the cytoplasmic side and Asn-327 containing an N-linked oligosaccharide on the extracellular side. The cGMP binding domain is located near the C-terminus.
1.5.2. Structure of calmodulin

Calmodulin is a protein of 148 amino acids. It is dumbbell shaped with two of its globular lobes joined by an 8-turn central $\alpha$-helix. It has 4 structurally and functionally similar domains (Fig. 11) which are related to the Ca$^{2+}$-binding motif of parvalbumin (Vanaman et al., 1977). Each Ca$^{2+}$-binding domain, also known as an EF-hand, consists of a loop of 12 amino acids flanked by two $\alpha$-helices that are oriented at approximately 90° to one another (Kretsinger and Nockolds, 1973). Each lobe has two EF-hands and within each EF-hand, the negatively charged residues and a main-chain carbonyl oxygen of the loop form a pentagonal bipyramid with six coordinating residues to facilitate Ca$^{2+}$ binding. Five of these residues coordinate a Ca$^{2+}$ or Mg$^{2+}$ ion while the sixth coordinates a water molecule (Kretsinger et al., 1988).

Calmodulin has 7 $\alpha$-helices spanning residues 7-19, 29-40, 46-55, 65-92, 102-112, 119-128, and 138-148. The helices are separated by non helical stretches containing 6-9 amino acids. These helices are involved in the Ca$^{2+}$-binding helix-loop-helix structure. The central $\alpha$-helix is shared by domains II and III (a.a. 41-64 and 93-118 respectively). Beta sheet structures occur between the pair of domains in each lobe. In addition, calmodulin has four reverse turns within each Ca$^{2+}$-binding loop (Babu et al., 1985).

Within each half of calmodulin, there is a hydrophobic cleft. The aromatic rings of Phe 19 and 68 are situated at the surface of the cleft in the N-terminal half while Phe 92 and 114 are found at the cleft surface in the C-terminal half. The entrance leading to these clefts are blocked from the solvent by lysine residues. It is believed that the binding of Ca$^{2+}$ by calmodulin exposes these hydrophobic clefts to allow them to interact with the target sites (Babu et al., 1985).

1.5.3. Post-translational modification

Calmodulin undergoes various post-translational modifications. The N-terminus of calmodulin has been shown to be acetylated (Watterson et al., 1980). In addition, most
Fig. 11: Structure and sequence of calmodulin.

a. A schematic diagram of calmodulin. Calmodulin is dumbbell shaped with 2 globular lobes, each containing two Ca$^{2+}$-binding sites (Babu et al., 1985). b. Bovine calmodulin is a protein of 148 amino acids. The Ca$^{2+}$-binding loops of the 4 EF-hands (amino acids 20-31, 56-67, 93-104, and 129-140) are underlined. The first amino acid, alanine, is acetylated and the protein is trimethylated at lys-115 (*).
calmodulins are trimethylated at lysine position 115. This N-methylation reaction is catalyzed by S-adenosylmethionine:calmodulin N-methyltransferase (Sitaramayya et al., 1980). Although this modification does not affect its modulation of the cAMP phosphodiesterase activity, this methylation has been implicated in its modulation of NAD kinase activity (Rowe et al., 1986; Roberts et al., 1986). Calmodulin is also a substrate for protein carboxymethyltransferase at either aspartic or glutamic acid residues (Freitag and Clarke, 1981). Finally, the carboxyl terminal lysine of calmodulin can be cleaved by a calmodulin converting enzyme, resulting in a change in mobility on SDS polyacrylamide gels (Murtaugh et al., 1983).

1.5.4. Calcium binding to calmodulin

Calcium binding to calmodulin has been examined by a variety of methods including equilibrium and flow dialysis and spectroscopic analysis (Yoshida et al., 1983; Minowa and Yagi, 1984; Burger et al., 1984; Iida and Potter, 1986; Ogawa and Tanokura, 1984). At physiological concentration of salts, the Ca$^{2+}$-binding curves indicate that there are four apparent binding sites with $K_d$ in the range of $5 \times 10^{-6}$ to $10^{-5}$ M. The binding of Ca$^{2+}$ to calmodulin appears to be a sequential event leading to a stepwise Ca$^{2+}$-induced conformational transition of calmodulin (Klee and Vanaman, 1982). The apo form of calmodulin, Ca$_0$CaM, is believed to form a Ca$_2$CaM intermediate first upon Ca$^{2+}$ addition. This form then goes on to become Ca$_4$CaM upon the binding of two additional Ca$^{2+}$ ions.

In addition to Ca$^{2+}$, calmodulin can also bind many other divalent cations and trivalent lanthanides. Some of these cations (i.e. Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, and Tb$^{3+}$), like Ca$^{2+}$, will induce a conformational change leading to an activation of calmodulin dependent enzymes. Others, such as Mg$^{2+}$, Be$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ will bind to calmodulin and cause a different conformational change which will not activate the target enzymes (Klee, 1988).
1.5.5. Calmodulin binding sequences

The mechanisms of calmodulin interaction with its target sites were studied by the utilization of various calmodulin binding peptide hormones and toxins (DeGrado, 1988; Anderson and Malencik, 1986). The Ca$^{2+}$-dependent association of these peptides to calmodulin appears to correlate with their abilities to form positively charged amphiphilic helices (Giedroc et al., 1983; McDowell et al., 1985; DeGrado et al., 1985; Cox et al., 1985) which are characterized by clusters of hydrophobic residues adjacent to clusters of basic residues (Fig. 12).

The primary sequences of a number of calmodulin binding proteins have been determined and their precise calmodulin binding domains have been mapped (Fig. 13). The calmodulin binding sequences are generally 25 residues or less. These sequences contain a large number of positively charged residues and little or no negatively charged residues. Hydrophobic residues generally repeat with a 3 to 4 residue period to match the $\alpha$-helix.

Studies of calmodulin binding peptides with circular dichroism, proton NMR, and fluorescence emission indicate that the peptide binding to calmodulin leads to an increase in helicity of the complex. The hydrophobic helical faces of the peptides comes in direct contact with calmodulin while their hydrophilic faces are exposed to the surrounding environment (O'Neil and DeGrado, 1990). Photoaffinity labelling analyses indicate that the hydrophobic patches on the two halves of calmodulin must come together to form a single binding site for either the peptide or the target enzyme binding (Kauer et al., 1986; O'Neil et al., 1989).

1.6. FUNCTIONAL ROLES OF CALMODULIN

Calmodulin is present in virtually all eukaryotic cells (Klee and Vanaman, 1982). It acts as a regulatory protein to modulate the activities of a large number of proteins involved in cellular Ca$^{2+}$ signalling pathways. These include cAMP metabolism, protein
Fig. 12: **Helical wheel plot of a model amphiphilic calmodulin binding peptide.**

Calmodulin binds with high affinity to the basic amphiphilic α-helical peptide (Ac-WKKLLKLLLLKL-CONH₂) in which one face of the helix is hydrophobic while the other face is positively charged (O’Neil and DeGrado, 1990).
**Fig. 13:** Amino acid sequences of the calmodulin binding domains of various target proteins.

The amino acid sequences of the calmodulin binding sites of various known proteins and their dissociation constants for calmodulin are listed (O'Neil and DeGrado, 1990). These sequences generally form amphiphilic α-helices.
phosphorylation and dephosphorylation, cation transport, cytoskeletal organization, and cell cycle control.

1.6.1. Cyclic nucleotide metabolism

Cyclic AMP acts as a second messenger in the action of various hormones. It is synthesized by adenylate cyclase from ATP and is degraded by a phosphodiesterase to 5'-AMP. Calmodulin regulates both the synthesis and hydrolysis of cAMP through modulating the activities of adenylate cyclase and phosphodiesterase. Several forms of both enzymes have been shown to be activated by calmodulin (Brostrom et al., 1975; Cheung et al., 1975; Klee and Vanaman, 1982). Thus, calmodulin has the effect of causing a transient change in cAMP level in response to an increase in free cytoplasmic Ca\(^{2+}\).

Cyclic GMP is a key messenger in the visual cascade and in the cGMP-dependent protein kinase activation. This compound is synthesized by guanylate cyclase from GTP and hydrolyzed by cGMP phosphodiesterase. In the protozoan *Tetrahymena pyriformis*, guanylate cyclase has been reported to be activated by calmodulin (Nagao et al., 1979). However, in photoreceptor rod outer segments, the activities of both cGMP-phosphodiesterase and guanylate cyclase have been shown to be unaffected by calmodulin (Del Priore and Lewis, 1983; Koch and Stryer, 1988).

1.6.2. Protein phosphorylation

Calmodulin is involved in the phosphorylation of a large number of proteins through the activation of various kinases. In smooth muscle, it stimulates the myosin light chain kinase to phosphorylate myosin light chain which activates the actomyosin ATPase. This subsequently results in ATP hydrolysis and the contraction of myosin, leading to the contraction of smooth muscle (Hoar et al., 1979; Sherry et al., 1978). Calmodulin is also
involved in glycogen metabolism by stimulating the activity of phosphorylase kinase, leading to glycogen breakdown (Cohen et al., 1978; Shenolikar, et al., 1979).

Calmodulin dependent multiprotein kinase, also known as CaM-PK II, is a kinase with a broad substrate specificity (Kennedy et al., 1983a, b; Yamauchi and Fujisawa, 1983). In brain, this kinase phosphorylates synapsin-1 to control neurotransmitter release (Llinas et al., 1985). It also phosphorylates tyrosine and tryptophan hydroxylases to regulate catecholamine and serotonin synthesis (Vulliet et al., 1984; Yamauchi et al., 1981). In skeletal muscle, CaM-PK II inhibits glycogen synthesis by the phosphorylation of glycogen synthase (Cohen, 1986). In cardiac muscle, CaM-PK II phosphorylates phospholamban and increases the Ca$^{2+}$-ATPase activity to accumulate Ca$^{2+}$ within the sarcoplasmic reticulum (Simmerman et al., 1986). In liver, in addition to modulating glycogen synthase, this kinase is also involved in the α-adrenergic agonist response by modulating the activity of pyruvate kinase and phenylalanine hydroxylase (Schworer et al., 1985a, b; Doskeland et al., 1984). Phosphorylation of pyruvate kinase leads to a decrease in its affinity for its substrate while phosphorylation of phenylalanine hydroxylase leads to the activation of this enzyme.

1.6.3. Protein dephosphorylation

Calcineurin, also known as protein phosphatase 2B, is present in various tissues (Ingebritsen et al., 1983). This serine and threonine specific phosphatase is a heterodimer composed of a 61 kDa and a 19 kDa subunit and is stimulated by Ca$^{2+}$ and calmodulin (Stewart et al., 1983). The 61 kDa catalytic subunit binds calmodulin while the 19 kDa subunit binds Ca$^{2+}$ (Merat et al., 1985; Klee et al., 1979; Aitken et al., 1984). Calcineurin can be found in both the soluble and membrane fractions, with the membrane bound species likely to be associated with cytoskeletal elements (Pallen et al., 1985; Tallant and Cheung, 1983).
Calcineurin plays an important role in the regulation of glycogen metabolism. It dephosphorylates the α-subunit of phosphorylase kinase (Antoniw and Cohen, 1976) to prevent glycogen breakdown. It is also involved in the cAMP-dependent pathway by dephosphorylating the regulatory subunit of cAMP-dependent protein kinase, leading to the deactivation of the enzyme. Calcineurin stimulates phosphatase-1 activity by dephosphorylating inhibitor-1 (Cohen, 1982; Hemmings et al., 1984). In addition, this enzyme dephosphorylates various microtubule associated proteins including Tau factor and microtubule associated protein-2 (MAP-2). This promotes microtubule assembly (Goto et al., 1985).

1.6.4. Cation transport

Intracellular cation levels are regulated by a variety of pumps, exchangers, and channels. Many of these ion translocators are regulated by calmodulin. The red blood cell Ca\(^{2+}\)-ATPase pump, which extrudes Ca\(^{2+}\) from the cytoplasm, is activated by calmodulin (Carafoli and Zurini, 1982; Penniston, 1982; Gopinath and Vincenzi, 1977). In heart myocyte plasma membranes, calmodulin has been suggested to play a role in modulating the Na\(^+\)/Ca\(^{2+}\) exchanger activity through phosphorylation by calmodulin dependent kinase (Caroni and Carafoli, 1983). In addition, calmodulin has been shown to increase the activity of a Ca\(^{2+}\)-dependent Na\(^+\) channel of Paramecium (Saimi and Ling, 1990) and to decrease the activity of the Ca\(^{2+}\)-release channels of the sarcoplasmic reticulum in cardiac and skeletal muscle (Smith et al., 1989) and the unglycanated MIP26 gap junction channels in lens (Swamy and Abraham, 1992).

1.6.5. Cytoskeletal organization

Calmodulin interacts with various cytoskeletal structures. Calmodulin has been shown to regulate the microtubule assembly and disassembly process. In the presence of Ca\(^{2+}\)-calmodulin, brain microtubules were shown to undergo dissociation with a
concurrent inhibition of its assembly process (Nishida and Sakai, 1980; Keller et al., 1982; Job et al., 1981). Similarly, calmodulin has been shown to stimulate the polymerization of G-actin (Piazza and Wallace, 1985) and the dissociation of caldesmon from F-actin (Sobue et al., 1981). In red blood cells, calmodulin binds to spectrin and other cytoskeletal elements (Berglund et al., 1984; Burns and Gratzer, 1985). In neurons and other cell types, calmodulin associates with various structural proteins including fodrin, a homologue of red blood cell spectrin (Glenney Jr. et al., 1982).

1.6.6. Control of cell proliferation

Ca\(^{2+}\)-calmodulin is believed to play a role in mitosis by regulating the progression of cells from the G1 phase into the S phase (Hazelton et al., 1979). Both Ca\(^{2+}\) and calmodulin are found in abundance in the centrosomal region of the mitotic spindle (Welsh et al., 1978; Wolniak et al., 1980). Transient increases in Ca\(^{2+}\) have been associated with the breakdown of the nuclear envelope, chromatin condensation, and chromosomal movement during anaphase (Keith et al., 1985; Poenie et al., 1985; Baitinger et al., 1990).
1.7. THESIS INVESTIGATION

In photoreceptor cells, intracellular calcium plays important roles in modulating the visual recovery process. These modulations are mediated through various Ca$^{2+}$-binding proteins. Prior to the initiation of this thesis work, little was known about the identities of the Ca$^{2+}$-binding proteins in ROS and their involvement in the visual pathway. In an attempt to learn more about the biochemical mechanisms underlying Ca$^{2+}$ feedback in ROS, functional characterization of an unknown 17/20 kDa Ca$^{2+}$-binding protein, extracted from the bovine ROS membranes by EDTA, was carried out in this thesis. This characterization was accomplished through identification of this Ca$^{2+}$-binding protein and its main target in ROS membranes and elucidation of the modulatory role of this Ca$^{2+}$-binding protein on its target.

This thesis investigation is divided into four major chapters. Chapter 2 describes the extraction, purification, identification, and localization of a 17/20 kDa protein from bovine ROS membranes. The identification of this Ca$^{2+}$-binding protein as calmodulin led to the studies in Chapter 3 which describe the purification and identification of calmodulin binding proteins on ROS membranes. Using calmodulin affinity chromatography and Western blotting analysis with radioiodinated calmodulin and specific monoclonal antibodies, a 240 kDa protein which forms a complex with the $\alpha$-subunit of the cGMP-gated channel was found to be the major calmodulin binding protein on the ROS plasma membranes. This association between calmodulin and the channel complex was also examined among photoreceptor ROS of various vertebrates.

Chapter 4 describes studies which investigate the potential modulation of the cGMP-gated channel by calmodulin. A series of cation influx assays carried out on extruded ROS membrane vesicles either in the presence or absence of calmodulin revealed that calmodulin regulates the channel activity by changing its affinity for cGMP. The specificity of this effect was examined in terms of its cation specificity, Ca$^{2+}$-dependency, and inhibition by calmodulin antagonists.
Chapter 5 looks into the characterization of the 240 kDa channel associated protein. The importance of this 240 kDa protein in mediating the calmodulin effect was studied by reconstitution assays and proteolytic analysis. In addition, a 105 kDa fragment of the 240 kDa protein was found to contain the calmodulin binding site.
CHAPTER 2
IDENTIFICATION AND CHARACTERIZATION OF A 20 kDa PROTEIN, CALMODULIN, FROM THE EDTA EXTRACT OF PHOTORECEPTOR ROD OUTER SEGMENTS

2.1. MATERIALS
Bovine eyes were obtained from J & L Meats (Surrey, British Columbia). Murine anti-calmodulin monoclonal antibody was from Upstate Biotechnology Incorporated. DEAE Sephacel and Ultrogel AcA-54 were purchased from Pharmacia. $^{45}$CaCl$_2$ and $[^{125}]$ Bolton-Hunter reagent were bought from New England Nuclear. Arthrobacter ureafaciens neuraminidase was acquired from Boehringer-Mannheim. Paraformaldehyde and Tissue-Tek were bought from J. B. EM Services and Miles, respectively. Horseradish peroxidase conjugated sheep anti-mouse F'ab fragment and the ECL kit were purchased from Amersham. The BCA protein assay kit was from Pierce and nitrocellulose membranes were from Schleicher & Schuell. All other chemicals were obtained from Sigma, Fisher Scientific, or Calbiochem.

2.2. METHODS
2.2.1. Preparation of ROS membranes
ROS membranes were prepared according to the method described by Molday and Molday (1987). Briefly, retinas dissected from 100 bovine eyes were gently shaken in homogenizing solution (20 mM Tris-HCl pH 7.2, 20 % sucrose, 10 mM β-D-glucose, 10 mM taurine, and 0.25 mM MgCl$_2$). The retinal mixture was filtered through a Teflon screen and the filtrate was loaded onto six 20 ml 30% - 50% w/v continuous sucrose gradients (same composition as the homogenizing solution with the exception of the sucrose concentration). The gradients were then placed in a SW28 rotor and centrifuged at 25,000 rpm for 1 h at 4°C using a Beckman ultracentrifuge. The ROS band was
collected and diluted 1:5 with the homogenizing solution. The collected ROS were pelleted by centrifuging at 12,000 rpm using a Sorvall SS-34 rotor. The pellet was then resuspended in 8 ml of homogenizing solution. Typically, 70-80 mg of ROS proteins were obtained from 100 bovine retinas.

2.2.2. Purification of the 20 kDa protein from ROS membranes

Eighty milligrams of ROS proteins were washed twice in 10 mM Tris-HCl pH 7.2 and 0.5 mM GTP and the soluble proteins were removed by pelleting the membranes at 15,000 rpm using a Sorvall SS-34 rotor. The ROS membrane pellet was washed once in 10 mM Tris-HCl pH 7.2 and 150 mM NaCl. EDTA extraction was then carried out by washing the membranes twice with 10 mM Tris-HCl pH 7.2 containing 1 mM EDTA. This pooled EDTA extract was loaded onto a DEAE Sephacel column (1.5 ml bed volume) equilibrated at 4°C in 10 mM Tris-HCl pH 7.2, 1 mM EDTA, and 0.2 M NaCl. The 20 kDa protein was eluted off the column using a linear NaCl gradient from 0.2-0.7 M. Fractions containing the 20 kDa protein as analyzed by SDS polyacrylamide gel electrophoresis were dialyzed against 10 mM Tris-HCl pH 7.2 and concentrated to 0.6 ml using either a Savant Speed Vac or Aquacide II-A. The sample was then loaded onto a 1.5 X 42 cm Ultrogel AcA-54 gel filtration column equilibrated in 10 mM Tris-HCl pH 7.2, 0.1 M NaCl, and 1 mM EDTA. Fractions containing the 20 kDa protein were dialyzed against distilled water and concentrated by lyophilization.

2.2.3. Gel filtration chromatography

The relative molecular weights of the 20 kDa protein and calmodulin were compared by gel filtration chromatography. Half a ml of the purified 20 kDa protein or bovine brain calmodulin (0.5 mg/ml) was loaded onto an 1.5 X 48 cm of Ultrogel AcA-54 gel filtration column. The column was equilibrated at 4°C in the buffer consisting of either 10 mM HEPES pH 7.4, 100 mM NaCl, and 1 mM CaCl₂ or 10 mM HEPES pH 7.4, 100
mM NaCl, and 1 mM EGTA. One ml fractions were collected and analyzed by SDS polyacrylamide gel electrophoresis. The peak fractions of the 20 kDa protein and calmodulin were assigned as the elution volumes (Ve) of these two proteins for the purpose of calculating their apparent molecular weights. Blue dextran (~2 x 10^3 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) were run separately as molecular weight standards.

2.2.4. Preparation of ROS disk and plasma membranes

A nontrypsinized preparation of ROS disk and plasma membranes was carried out according to the method of Molday and Molday (1987). Briefly, unbleached ROS (80 mg of proteins) in 12 ml of homogenizing solution were treated with 0.1 unit of *Arthrobacter ureafaciens* neuraminidase for 2 h at 4 °C. The neuraminidase treated ROS were washed with homogenizing solution by centrifugation at 10,000 rpm for 15 min using a Sorvall SS-34 rotor. The ROS pellet was gently resuspended in 6 ml of homogenizing solution, mixed with 1.5 ml of ricin-gold-dextran (A_520 ~ 4), and allowed to incubate for 14 h at 4 °C. The ricin-gold-dextran labelled ROS were washed once in homogenizing solution and 3 times in 10 mM Tris-HCl pH 7.2. The ROS pellet was resuspended in 1 mM Tris-HCl pH 7.2 and incubated for 2 h at 4 °C. The hypotonically lysed ROS membranes were repeatedly extruded through a syringe needle (gauge 14) for 20 min to separate the disks from the plasma membranes. The membrane mixture was pelleted by centrifugation at 17,000 rpm for 30 min. The membrane pellet was then resuspended in 8 ml of 10 mM Tris-HCl pH 7.2 and loaded onto six continuous gradients consisting of 9 ml of 25% - 50% w/v sucrose (containing 10 mM Tris-HCl pH 7.2) overlaying a 1 ml of 60 % w/v sucrose. The gradients were centrifuged at 37,000 rpm for 3 h in a SW41 rotor. The band containing the disk membranes was collected, diluted with 5 volumes of 10 mM Tris-HCl pH 7.2, and spun at 17,000 rpm for 30 min. The disk membrane pellet was resuspended in 3 ml of 10 mM Tris-HCl pH 7.2. The ROS plasma membranes, which
pellet to the bottom of the gradients, were collected, washed with 10 mM Tris-HCl pH 7.2, and resuspended in 0.5 ml of 10 mM Tris-HCl pH 7.2.

2.2.5. Extraction of calmodulin from ROS disk and plasma membranes

ROS disk and plasma membranes were prepared as described above. One mg of disk membrane proteins and 0.6 mg of plasma membrane proteins were washed with 10 mM Tris-HCl pH 7.2 containing 0.15 M NaCl to remove glyceraldehyde-3-phosphate dehydrogenase. The pelleted membrane samples were then incubated with 100 µl of the EDTA buffer to extract calmodulin. The extracted samples were then analyzed by SDS polyacrylamide gel electrophoresis.

2.2.6. Immunofluorescence localization of calmodulin

A bovine retina was fixed in 3 % paraformaldehyde in phosphate buffered saline solution for 3 h, embedded in acrylamide, and frozen in Tissue-Tek according to the method described by Johnson and Blanks (1984). Retinal cryostat sections, 10 µm in thickness, were cut and blocked in 3 % bovine serum albumin (BSA) and 1.5 % Triton X-100 in PBS for 2 h. Anti-calmodulin monoclonal antibody diluted 10 X with 3 % BSA in PBS was added to the blocked retinal sections and allowed to incubate at 4 °C overnight. The sections were washed to remove unbound primary antibody and incubated with fluorescein isothiocyanate conjugated goat anti-mouse Ig for 1 h at 4 °C. Immunofluorescence microscopy was carried out using a Zeiss Axiophot photomicroscope.

2.2.7. Radioiodination of calmodulin

Bovine brain calmodulin (40 µg) was radioiodinated by [125I] Bolton Hunter reagent (Bolton and Hunter, 1973). The iodinated calmodulin was isolated by gel
filtration. Typically, the radioiodinated calmodulin had a specific activity of 4 μCi / μg of protein.

2.2.8. SDS polyacrylamide gel electrophoresis, Western blotting analysis, and protein concentration determination

SDS polyacrylamide gel electrophoresis was carried out using the buffer system of Laemmli (1970). For the Ca$^{2+}$-dependent mobility shift analysis, 1 mM CaCl$_2$ or 1 mM EGTA was added to the running gel buffer. For Western blotting analysis with the anti-calmodulin monoclonal antibody, protein samples were run on a 12 % SDS polyacrylamide gel. The proteins were electrophoretically transferred at 0.2 A for 20 min from the SDS polyacrylamide gel onto an Immobilon membrane in the presence of the transfer buffer (12.5 mM Tris-HCl, 96 mM glycine, and 10 % methanol). The blot was blocked in 0.5 % Tween in PBS for 1 h at room temperature, incubated in anti-calmodulin antibody (1 μg/ml) for 1 h, washed with 0.05 % Tween in PBS, and then incubated in horseradish peroxidase conjugated goat anti-mouse Ig for 1 h. Visualization of the antibody labelling was carried out by ECL according to the manufacturer's direction. For the $^{45}$Ca$^{2+}$ labelling study, the purified 20 kDa protein and calmodulin were run on a 12 % SDS polyacrylamide gel and transferred onto nitrocellulose membrane. The blot was equilibrated in 10 mM imidazole pH 6.8, 60 mM KCl, and 5 mM MgCl$_2$ for 1 h at room temperature. The blot was then labelled with 1 μCi/ml of $^{45}$CaCl$_2$ for 10 min, washed with the equilibration buffer, air dried, and visualized by autoradiography. For the iodinated calmodulin labelling study, the labelling was carried out according to the method of Flanagan and Yost (1984). Protein samples were run on an 8 % SDS polyacrylamide gel and transferred onto an Immobilon membrane. The blot was blocked in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1mM CaCl$_2$, and 0.05% Tween 20 for 1 h and labelled with iodinated calmodulin (2 μCi/ml) in the blocking buffer for 90 min at room
temperature. The blot was washed with the blocking buffer and air dried. Labelled protein bands were visualized by autoradiography.

The protein content of ROS membranes was determined by the BCA protein assay kit using bovine serum albumin as a standard.

2.3. RESULTS

2.3.1. Extraction and purification of a 20 kDa protein from ROS

Selective extractions of soluble proteins from ROS were carried out by first washing the intact ROS with a hypotonic solution (10 mM Tris-HCl pH 7.2 and 0.5 mM GTP), following by an isotonic solution (10 mM Tris-HCl pH 7.2 and 150 mM NaCl) prior to treatment with EDTA. The initial hypotonic wash lysed the ROS and removed majority of the ROS soluble proteins, including phosphodiesterase, transducin, and S-antigen (data not shown). The subsequent isotonic wash removed glyceraldehyde-3-phosphate dehydrogenase from ROS membranes (data not shown) as reported by Hsu and Molday (1990). A 20 kDa protein (Fig. 14, lane e) was eluted by EDTA from the ROS membranes together with several other proteins with molecular weights ranging from 26 kDa to 160 kDa (Fig. 14, lane b). The 20 kDa protein which comigrated with bovine brain calmodulin by SDS polyacrylamide gel electrophoresis was subsequently purified by DEAE anion exchange and gel filtration chromatography (Fig. 14, lanes c and d). The gel filtration step was utilized to remove a 90 kDa protein which sometimes cofractionated with the 20 kDa protein. A 26 kDa protein which was coeluted with the 20 kDa protein by EDTA was later identified as recoverin.

2.3.2. Identification of the 20 kDa protein as calmodulin

The purified 20 kDa protein was compared with bovine brain calmodulin by gel filtration molecular weight sizing, $^{45}$Ca labelling, Ca$^{2+}$-dependent mobility shift by SDS polyacrylamide gel electrophoresis, Western blotting with anti-calmodulin monoclonal
Fig. 14: Extraction and purification of calmodulin from bovine ROS membranes.

Bovine ROS calmodulin was selectively extracted with an EDTA solution after the ROS membranes had been washed with hypotonic and isotonic solutions. Calmodulin was purified from the EDTA extract by DEAE anion exchange and gel filtration chromatography. The protein samples were analyzed on a 12% SDS polyacrylamide gel by staining with Coomassie blue. Lane a, bovine ROS membranes (30 μg); lane b, EDTA extract of ROS membranes; lane c, DEAE column purified calmodulin; lane d, gel filtration column purified calmodulin; lane e, Sigma bovine brain calmodulin (1 μg).
antibody, and amino acid composition analysis. When the 20 kDa protein and calmodulin were chromatographed on an Ultrogel AcA-54 gel filtration column either in the presence of Ca\(^{2+}\) or EGTA, they displayed apparent molecular weights in the range of 35 to 37 kDa (Table II). The higher than expected values for the molecular weights of both proteins suggest that the 20 kDa protein is probably axially asymmetric, a recognized characteristic of calmodulin.

The potential Ca\(^{2+}\)-binding property of the 20 kDa protein was investigated by \(^{45}\)Ca\(^{2+}\) labelling. Figure 15 shows that the 20 kDa protein (lane a), like calmodulin (lane b), can bind Ca\(^{2+}\). Another unique property exhibited by calmodulin is its ability to undergo a Ca\(^{2+}\)-dependent mobility shift when electrophoresed on a SDS polyacrylamide gel. In the absence of Ca\(^{2+}\) (+EGTA), the 20 kDa protein and calmodulin displayed an apparent molecular weight of 20 kDa. In the presence of Ca\(^{2+}\), both proteins migrated with an apparent molecular weight of 17 kDa (Fig. 16). Because of this ambiguity in the molecular weight assignment, the 20 kDa protein is referred to as the 17/20 kDa protein.

Western blotting analysis with an anti-calmodulin monoclonal antibody showed that the antibody labelled the 17/20 kDa protein in intact ROS (Fig. 17, lane a) and in the ROS EDTA extract (Fig. 17, lane b), as well as the purified bovine brain calmodulin (Fig. 17, lane c). The amino acid analysis of the purified 17/20 kDa protein (Table III) indicated that the amino acid composition of the 17/20 kDa protein is very similar to that of calmodulin but not to other Ca\(^{2+}\)-binding proteins of similar molecular weights, such as troponin C, calcineurin B, S-100 α, and S-100 β.

2.3.3. Immunofluorescence labelling

Immunofluorescence labelling of retinal sections by the anti-calmodulin monoclonal antibody suggested that calmodulin is ubiquitously distributed among all retinal cell layers, including the outer segments of the photoreceptor rod cells (Fig. 18). In
**Table II**

*Gel filtration molecular weight sizing of the 17/20 kDa protein*

<table>
<thead>
<tr>
<th></th>
<th>17/20 kDa protein</th>
<th>Calmodulin</th>
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</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
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<td>36 kDa</td>
</tr>
<tr>
<td>EGTA</td>
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</table>
Fig. 15: Calcium-45 binding analysis of the 20 kDa protein.

The purified 20 kDa protein (lane a, 0.5 μg) and bovine brain calmodulin (lane b, 0.5 μg) were run on a 12% SDS polyacrylamide gel and the proteins were transferred onto a nitrocellulose membrane. The blot was then incubated in a $^{45}\text{CaCl}_2$ solution. The $^{45}\text{Ca}^{2+}$ binding was visualized by autoradiography.
Fig. 16: Calcium-dependent mobility shift analysis of the 17/20 kDa protein by SDS polyacrylamide gel electrophoresis.

The purified 20 kDa protein (lanes a, 1 µg) and bovine brain calmodulin (lanes b, 1 µg) were run on a 12% SDS polyacrylamide gel either in the presence of Ca$^{2+}$ or EGTA. The two proteins displayed an apparent Mr of 20 K in the presence of EGTA and an apparent Mr of 17 K in the presence of Ca$^{2+}$. 
Fig. 17. Western blotting analysis with an anti-calmodulin monoclonal antibody.

Whole ROS proteins (lanes a, 30 μg), EDTA extract of ROS membranes (lanes b), and bovine brain calmodulin (lanes c, 0.5 μg) were run on a 12 % SDS polyacrylamide gel. The gel slices were either stained with Coomassie blue (CB) or transferred onto an Immobilon membrane and subjected to Western blotting analysis with an anti-calmodulin monoclonal antibody (CaM).
Table III

Comparison of the amino acid composition of the 17/20 kDa protein with those of other Ca\(^{2+}\)-binding proteins

<table>
<thead>
<tr>
<th>Residue</th>
<th>17/20 kDa protein</th>
<th>CaM</th>
<th>Troponin C</th>
<th>Calcineurin B</th>
<th>S-100 α</th>
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<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) The content of cysteine and tryptophan residues cannot be determined by amino acid composition analysis.
a control study, preadsorption of the anti-calmodulin antibody by Protein-G beads greatly diminished antibody labelling.

2.3.4. Extraction of calmodulin from ROS disk and plasma membranes

In order to determine whether calmodulin binding proteins in ROS are localized to the disk or plasma membrane, ROS disk and plasma membranes were separated by the ricin-gold-dextran affinity perturbation method under nontrypsinizing conditions (Fig. 19, lanes a and c). A subsequent extraction of these two membrane preparations by EDTA indicated that calmodulin binds preferentially to the ROS plasma membranes (Fig. 19, lanes b and d). When ROS membranes were lightly trypsinized to allow a better disk and plasma membrane separation, similar results were also obtained (data not shown).

2.3.5. Calmodulin binding proteins in ROS

Calmodulin binding proteins in ROS were determined by labelling with iodinated calmodulin. ROS proteins were first separated into soluble and membrane protein fractions (Fig. 20 (left), lanes b and c) and the membrane fraction was further divided into disk and plasma membranes (Fig. 20 (left), lanes d and e). These protein samples were then run on a SDS polyacrylamide gel and transferred onto an Immobilon PVDF membrane. The membrane was subjected to Western blotting analysis with iodinated calmodulin. Calmodulin binding components in the ROS soluble protein fraction consist mainly of a protein of 67 kDa (Fig. 20 (right), lane b). The membrane associated calmodulin binding proteins have molecular weight ranging from 35 kDa to 240 kDa. These proteins are preferentially localized to the ROS plasma membranes (Fig. 20 (right), lanes c, d, and e).

2.4. DISCUSSION
Fig. 18: **Immunofluorescence localization of calmodulin.**

Fluorescent micrographs of retinal sections labelled with either (b) anti-rhodopsin monoclonal antibody rho 1D4, (c) anti-calmodulin monoclonal antibody, or (d) flowthrough from a Protein G column used to adsorb out the anti-calmodulin antibody. The photoreceptor layers, including the outer segment (OS), inner segment (IS), and cell body (CB), visualized under phase contrast microscopy are shown on (a).
Fig. 19. Extraction of calmodulin from ROS disk and plasma membranes.

ROS disk and plasma membranes were prepared by the ricin-gold-dextran perturbation method. The isolated disk (lane a, 30 μg) and plasma membranes (lane c, 40 μg) were then subjected to an EDTA wash, and their respective supernatants after centrifugation of the membranes are shown in lanes b and d. Lane e is bovine brain calmodulin (1 μg). The protein samples were analyzed on a 12% SDS polyacrylamide gel and stained with Coomassie blue.
Fig. 20: **Labelling of the ROS proteins with iodinated calmodulin.**

ROS proteins were divided into soluble and membrane fractions. The membrane proteins were further separated into disk and plasma membranes. The protein samples were run on an 8% SDS polyacrylamide gel. The gel slices were either stained with Coomassie blue or transferred onto an Immobilon membrane and subjected to iodinated calmodulin labelling. Lanes a, whole ROS proteins (40 µg); lanes b, soluble ROS extract (7 µg); lanes c, stripped ROS membrane proteins (30 µg); lanes d, ROS disk membranes (25 µg); lanes e, ROS plasma membranes (30 µg).
Selective extraction of ROS membranes under various conditions led to the detection of several proteins that appeared to interact with the ROS membranes in a divalent cation dependent manner. A 20 kDa protein found in the EDTA extract of ROS membranes became the focus of this study. This protein was purified by DEAE anion exchange and gel filtration chromatography. Subsequent studies by gel filtration molecular weight sizing, $^{45}$Ca labelling, Ca$^{2+}$-dependent mobility shift on SDS polyacrylamide gel, amino acid composition analysis, and Western blotting analysis with anti-calmodulin monoclonal antibody identified this protein as calmodulin. Immunofluorescence labelling of retinal sections with an anti-calmodulin monoclonal antibody showed that calmodulin is found in rod photoreceptor ROS as well as other retinal layers and is not a contaminant from other retinal layers.

Recoverin, a 26 kDa protein originally hypothesized to modulate ROS guanylate cyclase activity (Lambrecht and Koch, 1991; Dizhoor et al., 1991) but more recently found to regulate rhodopsin phosphorylation in ROS (Kawamura, 1993), was also present in this EDTA extract. However, this protein does not appear to interact with the membranes as tightly as calmodulin, since only small amounts of this protein remained bound to the ROS membranes after the initial hypotonic and isotonic washes. Unlike iodinated calmodulin which readily bound to distinct bands on the Western blots, iodinated recoverin failed to label any ROS proteins. In addition, a recoverin affinity column also failed to extract proteins from the solubilized ROS membranes in a Ca$^{2+}$-dependent manner (data not shown).

Calmodulin has also been detected in photoreceptor outer segments from various sources, including bovine, frog, squid, and teleost (Kohnken et al., 1981; Morelli et al., 1989; Nagao et al., 1987; de Couet et al., 1986; Asai et al., 1989; Nagle and Burnside, 1983). This suggests that calmodulin may play an important functional role in the photoreceptor rod cell. No definitive identification of the calmodulin binding proteins in ROS, however, had been made. As a first step in the characterization of the calmodulin
binding proteins in ROS, the ROS proteins were separated into soluble and membrane fractions, and the membrane fraction was further divided into disk and plasma membranes. Analysis of the ROS proteins by either selective EDTA extraction or iodinated calmodulin labelling on Western blots suggests that the calmodulin binding proteins are preferentially localized to ROS plasma membranes.

ROS disk and plasma membranes have been previously shown to have quite distinct protein compositions (Molday and Molday, 1987). Proteins such as the cGMP-gated channel complex and the Na⁺/Ca²⁺-K⁺ exchanger are found exclusively in the plasma membranes (Cook et al., 1989; Molday et al., 1990; Reid et al., 1990). The preferential association of calmodulin to ROS plasma membranes raises the possibility that calmodulin may be interacting with one of these plasma membrane specific proteins involved in phototransduction. Thus, it may have a role in regulating the visual transduction process. Identification of a major calmodulin binding protein in ROS plasma membranes is described in Chapter 3.
CHAPTER 3
IDENTIFICATION OF THE cGMP-GATED CHANNEL COMPLEX
AS THE MAJOR CALMODULIN BINDING PROTEIN IN BOVINE
ROS MEMBRANES

3.1. MATERIALS

Calmodulin Sepharose, DEAE Sephadex and Sepharose 2B beads were obtained from Pharmacia. DEAE-650 S gel was purchased from Supelco and cyanogen bromide was bought from Fisher. $^{[125]}$ Bolton-Hunter reagent was from New England Nuclear. Immobilon membranes were acquired from Millipore and the protein assay kit was obtained from Bio-Rad. Arsenazo III, asolectin, proteolytic inhibitors, cGMP, and all other chemicals were purchased from Sigma.

3.2. METHODS

3.2.1. Calmodulin affinity chromatography

ROS membranes were prepared from freshly dissected bovine retinas as previously described (Molday et al., 1987). Proteolytic inhibitors: 0.1 mM DFP, 5 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin were present in all subsequent chromatographic steps. The ROS membranes were washed three times in 10 mM HEPES pH 7.4 containing 1 mM EDTA and solubilized in solubilization buffer (10 mM HEPES pH 7.4, 150 mM KCl, 10 mM CaCl₂, 1 mM DTT, 18 mM CHAPS, and 0.2 % asolectin) at a protein concentration of 1.5-2 mg/ml. Forty three mg of solubilized ROS proteins were loaded onto 2 calmodulin columns (2.5 ml each) equilibrated in the solubilization buffer at 4 °C. After washing the columns with 10 column volumes of washing buffer (10 mM HEPES pH 7.4, 15 mM CHAPS, 150 mM KCl, 1 mM CaCl₂, 1 mM DTT, and 0.18 % asolectin), the channel complex was eluted off the columns with washing buffer in which 1 mM CaCl₂ was replaced by 1.5 mM EDTA. Fractions (0.75 ml) were collected and their
absorbance was measured at 280 nm. Peak fractions containing the channel complex were adjusted to 2 mM CaCl₂ and mixed 1:1 with 18 mg/ml of asolectin and 2 mM CHAPS in the dialyzing buffer (10 mM HEPES pH 7.4, 0.1 M KCl, 0.1 mM DTT, and 2 mM CaCl₂). The sample was first dialyzed against 5 changes of the dialyzing buffer for 2 days and then dialyzed against 3 changes of the same buffer without Ca²⁺ over a period of 8 h. The reconstituted channel was used for Ca²⁺ efflux assays (Cook et al., 1987).

For the purification of channel complex from various species, 0.5-0.7 mg of bovine, pig, and rat and 3 mg of frog ROS membranes were first washed with the EDTA solution to remove endogenous calmodulin, and then solubilized and mixed with 100 µl of calmodulin-Sepharose beads. After washing the beads with washing buffer, the respective channel complexes were eluted off the beads with elution buffer containing 2 mM EDTA. Asolectin was included with CHAPS in order to maximize the interaction between calmodulin and the channel complex. In the absence of asolectin, the CHAPS concentration had to be decreased below 15 mM. Other detergents (in the absence of asolectin) such as 1 % Triton X-100 can be used to substitute CHAPS while 1 % sodium cholate cannot be used. This detergent appeared to interfere with the interaction between calmodulin and the channel complex.

3.2.2. PMc 6E7 antibody affinity chromatography

PMc 6E7 monoclonal antibody was purified by ammonium sulphate precipitation and DEAE anion exchange chromatography. The antibody was precipitated from mouse ascites fluid with 50 % ammonium sulphate at 4°C. The precipitate was pelleted at 10,000 rpm using a Sorvall SS-34 rotor. The pellet was resuspended in 10 mM Tris·HCl pH 7.2 containing 20 mM NaCl and dialyzed against 3 changes of the same buffer. The antibody solution was then loaded onto a DEAE Sephadex column equilibrated at room temperature in 10 mM Tris·HCl pH 7.2. The column was washed with 5 column volumes of the equilibration buffer, and the antibody was eluted with a 0 - 0.3 M NaCl gradient. One ml
fractions were collected and analyzed by both A₂₈₀ measurements and SDS polyacrylamide gel electrophoresis. The purified antibody was dialyzed against 10 mM borate pH 8.4 and coupled to CNBr activated Sepharose 2B beads (Molday et al., 1990).

Purification of the cGMP-gated channel complex using the PMc 6E7 antibody column was carried out essentially by the same procedure used for calmodulin affinity chromatography except DTT was omitted and 0.9 mg/ml of synthetic peptide corresponding to the N-terminal of the 63 kDa ROS channel (Ser-Asn-Lys-Glu-Gln-Glu-Pro-Lys-Glu-Lys-Lys-Lys-Lys-Lys-Lys) was used to elute the channel complex (Molday et al., 1991).

3.2.3. DEAE purification of the cGMP-gated channel complex

ROS were washed three times with 10 mM HEPES pH 7.4, 1 mM DTT, and 1 mM EDTA. The ROS membranes were solubilized at a final protein concentration of 1.6 mg/ml in 10 mM HEPES pH 7.4, 150 mM KCl, 18 mM CHAPS, 0.2 % asolectin, 2 mM CaCl₂, and 0.1 mM DFP. The DEAE fractionation of ROS proteins was carried out according to the method of Cook et al. (1987). Briefly, the solubilized ROS membrane proteins were loaded onto 4 ml of DEAE-650 S column equilibrated at 4 °C in the solubilization buffer. The column was washed with 10 column volumes of the washing buffer (10 mM HEPES pH 7.4, 150 mM KCl, 15 mM CHAPS, 0.18 % asolectin, 2 mM CaCl₂, and 0.1 mM DFP). The bound proteins were eluted off the column with 0.75 M KCl in the washing buffer, and 1.5 ml fractions were collected. Fractions containing the channel complex were pooled and reconstituted into lipid vesicles according to the method of Cook et al. (1987) as described above.

3.2.4. Calcium efflux assay on reconstituted channels

The dialyzed lipid vesicles containing the reconstituted channel complex (0.3 ml) were mixed with 1.7 ml of 55 μM Arsenazo III dye in the vesicle dialysis buffer. The
calcium efflux assays were induced by the addition of 4 μl of varying concentrations of cGMP. The absorbance change of the Arsenazo III dye induced by Ca\(^{2+}\) efflux from the vesicles was monitored spectrophotometrically at 650-730 nm using a SLM Aminco DW 2000 dual wavelength spectrophotometer.

3.2.5. Ca\(^{2+}\)-dependent association of calmodulin with the channel complex

Anti-channel α-subunit monoclonal antibody PMc 1D1 was purified from mouse ascites fluid and coupled to Sepharose 2B beads as described above. The channel complex was immobilized onto the antibody column using the method described above except: 1 % Triton X-100 was used in place of CHAPS and NaCl was used in place of KCl. One hundred μl of the antibody beads containing the immobilized channel complex was washed with 2 column volumes of the washing buffer containing 0.1 % Triton X-100. Three hundred μl of bovine brain calmodulin (50 μg) were adjusted to 1 mM CaCl\(_2\) and 0.1 % Triton X-100 and allowed to incubate with the beads for 2 h at 4 °C. The beads were then washed with 20 column volumes of the washing buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.1 % Tx-100, and 1 mM CaCl\(_2\)). The bound proteins were eluted off the beads with the washing buffer containing 2 mM EDTA in place of 1 mM CaCl\(_2\).

3.2.6. SDS polyacrylamide gel electrophoresis, Western blotting analysis, and protein concentration determination

SDS polyacrylamide gel electrophoresis was carried out as previously described. Calmodulin was iodinated by the Bolton-Hunter reagent and \(^{125}\)I-calmodulin labelling was carried out as described in section 2.2.8.. For immunoblotting analysis, the blot was first labelled with PMc 6E7 and PMs 5E11 and then relabelled with \(^{125}\)I-goat antimouse Ig for visualization by autoradiography. The protein concentration of various solubilized samples were determined by the Bio-Rad protein assay kit using γ-globulin as a standard.
3.3. RESULTS

3.3.1. Identification of the cGMP-gated channel complex as a major calmodulin binding protein

In order to identify the calmodulin binding proteins, ROS membranes were solubilized in detergent and passed through a calmodulin column. Two polypeptides of apparent Mr 63 K and 240 K were isolated as the major constituents (Fig. 21 (left), lane b). These polypeptides comigrated with the 63 kDa α-subunit of the channel and the 240 kDa channel associated polypeptide of the cGMP-gated channel complex isolated on an anti-channel immunoaffinity column (Molday et al., 1990; Molday et al., 1991; Fig. 21 (left), lane c). Western blots labelled with antibodies PMc 1D1 and PMs 5E11 to the channel α-subunit and to the 240 kDa protein, respectively, confirmed that the calmodulin affinity column extracted the 63 kDa/240 kDa cGMP-gated channel complex from ROS membranes (Fig. 21 (middle)).

A Western blot labelled with $^{125}$I-calmodulin in the presence of Ca$^{2+}$ was used to identify the calmodulin binding proteins. Whereas calmodulin labelled several polypeptides of apparent Mr 240 K, 140 K, 70 K, 67 K, and 35 K in both the ROS membranes and the calmodulin affinity purified fraction (Fig. 21 (right), lanes a and b), only the 240 kDa polypeptide was labelled with calmodulin in the immunoaffinity purified channel complex (Fig. 21 (right), lane c). This labelling is specific since in the presence of EGTA, no labelling by iodinated calmodulin was observed. The intense labelling of the 67 kDa and 70 kDa proteins in the calmodulin column eluant as compared to the 240 kDa protein is probably due to the low transfer efficiency of the 240 kDa protein.

3.3.2. Calmodulin affinity chromatography

Purification of the channel complex from ROS membranes was carried out using calmodulin affinity chromatography. Figure 22a shows the SDS polyacrylamide gel
Fig. 21: Purification of the cGMP-gated channel complex by calmodulin and immunoaffinity chromatography and identification of the 240 kDa channel-associated protein as a major calmodulin binding polypeptide of ROS membranes.

ROS were subjected to an EDTA wash to remove soluble proteins. The membranes were then solubilized and passed through either a calmodulin or antibody affinity column. Lanes a, ROS membranes after the extraction of soluble proteins (30 μg); lanes b, EDTA eluant from a calmodulin affinity column; and lanes c, N-terminal peptide eluant from a N-terminal specific anti-channel α-subunit monoclonal (PMc 6E7) column. Left: SDS polyacrylamide gel stained with Coomassie Blue (CB); Middle: Western blot labelled with both an anti-63 kDa channel α-subunit monoclonal antibody (PMc 1D1) and an anti-240 kDa monoclonal antibody (PMs 5E11). Right: Western blot labelled with 125I-calmodulin (CaM).
Fig. 22: Purification of the bovine ROS channel complex by calmodulin affinity chromatography.

a. SDS polyacrylamide gel electrophoretic analysis of the calmodulin column fractions. Fifteen μl of sample from each column fraction was run on an 8 % SDS polyacrylamide gel and the gel was stained with Coomassie blue (CB). The results indicated that the 63/240 kDa channel complex is the predominant component eluted off the calmodulin column. b. Elution profile of the channel complex from a calmodulin affinity column. The 63/240 kDa channel complex was eluted off the calmodulin column by EDTA and the absorbance at 280 nm of each fraction (0.75 ml) was monitored. Peak fractions (3-7) were pooled and reconstituted into lipid vesicles.
electrophoretic analysis of the column fractions and Figure 22b shows the chromatographic profile. The pooled peak fractions from the calmodulin columns were reconstituted into lipid vesicles for calcium efflux assays (Cook et al., 1987). Figure 23a is a plot of the channel activation by varying concentrations of cGMP. The channel displayed a Km of 30 μM for cGMP and a Hill coefficient (n) of 3.9 (Fig. 23b). These values are comparable to those of the channel purified by DEAE anion exchange and TSK AF-red affinity chromatography (Km = 11 μM and n = 3.1; Cook et al., 1987).

Purification of the channel complex by calmodulin affinity chromatography recovered approximately 33% of the net activity from the pooled fractions (3-7). The purified channel displayed a specific activity of 163 units/mg protein, representing a 36 fold purification. For the purpose of comparison, purification of the channel complex by DEAE anion exchange column (Cook et al., 1987) was carried out as well. The DEAE chromatography method recovered 50% of the net activity. The fractionated channel complex was purified 14 fold and had a specific activity of 65.7 units/mg protein (Table IV).

Calmodulin affinity chromatography, thus, not only gave a channel complex of comparable yield as the DEAE column but also of higher purity and specific activity. The 36 fold purification of the channel complex obtained by calmodulin affinity chromatography is lower than the 110 fold purification reported by Cook et al. (1987) using the combined DEAE anion exchange and the TSK AF-red affinity chromatography. However, the 240 kDa protein was reported to be either absent or present in minute quantity in their channel preparations.

SDS polyacrylamide gel electrophoretic analysis was also carried out to compare the purity of the channel complex obtained from these two chromatographic procedures. Both columns extracted the majority of the channel complex from the solubilized ROS as evident from Western blotting analysis with anti-channel complex monoclonal antibodies PMs 5E11 and PMc 1D1 (Fig. 24 (right), lanes b and e). However, whereas the DEAE
Fig. 23: Activation of the calmodulin affinity purified channel complex by cGMP.

The channel complex purified by calmodulin affinity chromatography was reconstituted into lipid vesicles. 0.3 ml of vesicles containing 8 μg of the channel complex were assayed at varying concentrations of cGMP. a. The relative initial velocities of the channel complex were plotted as a function of cGMP concentration. The solid curved line was drawn from the sigmoidal isotherm \( \frac{V_o}{V_{\text{max}}} = 1 - (1 + 10^m(pK_m-pC_{\text{cGMP}}))^{-1} \) using a Km of 30 μM and a Hill coefficient \((n)\) of 3.9. b. Hill plot analysis of the channel activation as a function of cGMP concentration. The slope of the straight line is 3.9.
### Table IV

**Comparative purification of the channel complex by DEAE anion exchange and calmodulin affinity chromatography**

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<th>Channel activity</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Units&lt;sup&gt;a&lt;/sup&gt; /ml</th>
<th>Total units</th>
<th>Units/mg of protein</th>
<th>Recovery %</th>
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<td>DEAE pool</td>
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<td>0.17</td>
<td>11.3</td>
<td>102</td>
<td>65.7</td>
<td>49.6</td>
<td>14</td>
</tr>
<tr>
<td>Solubilized ROS</td>
<td>28</td>
<td>1.52</td>
<td>6.8</td>
<td>190</td>
<td>4.47</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>CaM (unbound)</td>
<td>28</td>
<td>1.20</td>
<td>0.6</td>
<td>16.8</td>
<td>0.50</td>
<td>8.8</td>
<td>---</td>
</tr>
<tr>
<td>CaM Pool</td>
<td>7.5</td>
<td>0.05</td>
<td>8.5</td>
<td>63.8</td>
<td>163</td>
<td>33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36</td>
</tr>
</tbody>
</table>

<sup>a</sup> One unit of channel activity is defined as the amount of Ca<sup>2+</sup> released by saturating concentration of cGMP that is equivalent to 1% of one ml of lipid vesicles.

<sup>b</sup> Determined by pooling fractions 3-7.
fractionated channel complex was contaminated with \( \text{Na}^+ / \text{Ca}^{2+} - \text{K}^+ \) exchanger (Mr = 230 K), guanylate cyclase (Mr = 112 K), and rhodopsin (Mr = 38 K), the calmodulin column eluant consisted of mainly the 240 kDa and 63 kDa components of the channel complex (Fig. 24 (left), lanes c and f).

### 3.3.3. Channel complexes from ROS of other species

In addition to bovine ROS membranes, calmodulin affinity chromatography was used to purify the channel complexes from ROS membranes of various mammals. Figure 25 (left) shows a Coomassie blue stained SDS polyacrylamide gel of the channel complexes purified from bovine, pig, and rat ROS membranes. Identification of the 63 kDa polypeptides as the \( \alpha \)-subunit of the cGMP-gated channels was confirmed by Western blotting analysis with the anti-\( \alpha \)-subunit monoclonal antibody PMc 1D1 (Fig. 25, right).

In frog ROS membranes, 3 bands corresponding to proteins of apparent Mr 240 K, 112 K, and 74 K were observed in the EDTA eluted fractions from a calmodulin affinity column (Fig. 26 (left), lane d). The 74 kDa protein stained only faintly with Coomassie blue. Anti-\( \alpha \)-subunit monoclonal antibody PMc 1F6, which binds to a highly conserved segment of this subunit in various species (Bönigk et al., 1993), was used to identify the frog \( \alpha \)-subunit of the cGMP-gated channel. As shown in Figure 26 (right), PMc 1F6 labelled the 63 kDa channel \( \alpha \)-subunit in bovine preparations and the 74 kDa polypeptide in frog preparations. The anti-240 kDa channel associated protein monoclonal antibody PMs 4B2 (Molday et al., 1990) did not cross-react with any protein in frog ROS membrane preparations. A Western blot labelled with iodinated calmodulin revealed that calmodulin intensely labelled the 112 kDa protein in addition to a 63 kDa protein (Fig. 26 (middle), lanes c and d). The 63 kDa protein is likely a homolog of the bovine 67 kDa protein which has been shown not to be a component of the cGMP-gated channel complex.
Fig. 24: Comparative purification of the channel complex by DEAE and calmodulin affinity chromatography.

Solubilized ROS membrane proteins (lanes a and d) were loaded onto either a DEAE or a calmodulin column. Lanes b and e are the flowthrough from the columns, respectively. Lanes c and f are the respective eluants from the two columns. The protein samples were analyzed on an 8 % SDS polyacrylamide gel. The gel slices were either stained with Coomassie blue (left) or transferred onto an Immobilon membrane and subjected to Western blotting analysis with both the anti-cGMP-gated channel α-subunit monoclonal antibody PMc 1D1 and the anti-240 kDa protein monoclonal antibody PMs 5E11 (right).
Fig. 25: Purification of the channel complex from ROS of various mammals.

Calmodulin affinity chromatography was used to purify the cGMP-gated channel complexes (lanes b, d, and f) from bovine (lane a), pig (lane c), and rat (lane e) ROS membranes. The protein samples were analyzed on an 8% SDS polyacrylamide gel and stained with Coomassie blue (left). The presence of the cGMP-gated channel α-subunit from each species was confirmed by Western blotting analysis with the anti-cGMP-gated channel α-subunit monoclonal antibody PMc 1D1 (right). The relative positions of the α-subunit of the cGMP-gated channel and the high molecular weight channel associated protein are marked with arrows.
Fig. 26: CaM affinity purification and Western blot analysis of the cGMP-gated channel complexes from bovine and frog ROS membranes.

Solubilized bovine and frog ROS membranes (lanes a and c, 30 μg) were loaded onto calmodulin-Sepharose columns in the presence of Ca\(^{2+}\) and bound proteins were eluted with EDTA (lanes b and d). SDS polyacrylamide gels were either stained with Coomassie blue (CB) or transferred onto Immobilon membranes and labelled with either radiiodinated CaM or anti-channel α-subunit monoclonal antibody PMc 1F6 and anti-240 kDa monoclonal antibody PMs 4B2. The relative positions of the α-subunit of frog cGMP-gated channel as detected with PMc 1F6 monoclonal antibody are marked with arrows.
3.3.4. Extraction of calmodulin by immobilized channel complex

In order to further demonstrate the Ca$^{2+}$-dependent interaction of calmodulin with the cGMP-gated channel complex, this complex was immobilized onto the anti-α-subunit monoclonal antibody PMc 1D1 Sepharose beads. SDS polyacrylamide gel electrophoretic analysis confirmed the binding of the channel complex to the antibody beads (Fig. 27, lane c). Bovine brain calmodulin was then incubated with the antibody beads containing immobilized channel complex in the presence of Ca$^{2+}$. The bound proteins were eluted off the beads by EDTA. As shown in Figure 27 (lane e), bovine brain calmodulin was extracted by the channel complex. When a similar binding experiment was carried out with the EDTA extract of ROS membranes, a 90 kDa protein, in addition to calmodulin, was found to bind to the immobilized channel complex in a Ca$^{2+}$-dependent manner (data not shown). This suggests that another Ca$^{2+}$ binding protein can also interact with the channel complex.

3.4. DISCUSSION

In order to determine the role of calmodulin in the Ca$^{2+}$-dependent regulation of ROS processes, identification of the calmodulin binding proteins was first carried out. Using calmodulin affinity chromatography, the cGMP-gated channel complex consisting of the 63 kDa α-subunit and the 240 kDa channel associated polypeptide, was found to be the major calmodulin binding protein on ROS membranes. By Western blotting analysis using iodinated calmodulin, this association between calmodulin and the channel complex was found to be mediated through the 240 kDa protein. Identification of the plasma membrane specific cGMP-gated channel complex as the major calmodulin binding protein in ROS membranes is in agreement with the findings in Chapter 2 in which calmodulin was found to be preferentially associated with the ROS plasma membranes.

Calmodulin affinity chromatography is a useful method for purification of the channel complex. In a single step, the channel complex can be isolated in high yield and
Fig. 27: Extraction of calmodulin by immobilized cGMP-gated channel complex.

The bovine cGMP-gated channel complex consisting of the 63 kDa α-subunit and the 240 kDa channel associated protein was immobilized onto anti-α-subunit PMc 1D1 monoclonal antibody Sepharose beads. The association of the channel complex to the antibody beads was confirmed on a 10% SDS polyacrylamide gel. Lane a is the ROS membranes (30 μg). Lane b is the PMc 1D1 beads boiled in the gel loading buffer and lane c is the PMc 1D1 antibody beads with immobilized channel complex. The positions of the channel complex components and antibody heavy and light chains are marked by arrows. Bovine brain calmodulin (lane d, 0.5 μg) was adjusted to 1 mM CaCl₂ and incubated with the PMc 1D1 antibody beads immobilized with the channel complex. The beads were washed and the bound calmodulin was eluted by EDTA (lane e).
with a greater than 90 % purity as assessed by SDS polyacrylamide gel electrophoresis. The channel complex purified by calmodulin affinity chromatography is functionally active as determined by the reconstitution assays. The $K_m$ and Hill coefficient of the reconstituted channel complex are comparable to those of the channel complex prepared by DEAE anion exchange and TSK AF-red affinity chromatography as described by Cook et al. (1987). Calmodulin affinity chromatography is also a valuable method for the purification of the channel complexes from various species. This channel complex can also be purified from frog ROS membranes, suggesting that the association between the channel $\alpha$-subunit and a calmodulin binding subunit may be universal among vertebrates. In frog ROS membranes, the 74 kDa protein is most likely the $\alpha$-subunit of the channel since it binds an anti-$\alpha$-subunit antibody. The 112 kDa calmodulin binding protein may itself constitute the calmodulin binding component of the channel complex, or alternatively, it may be a proteolytic fragment of the 240 kDa protein which is more efficiently transferred and renatured than the 240 kDa protein.

The association between calmodulin and the channel complex was also shown by the $Ca^{2+}$-dependent binding of calmodulin to the channel complex immobilized onto an anti-channel $\alpha$-subunit antibody-Sepharose support. The 90 kDa protein which was found to co-elute with calmodulin from the column by EDTA may be another $Ca^{2+}$-binding protein that associates with the channel. It will be interesting to determine whether this protein binds to the $\alpha$-subunit or to the 240 kDa channel associated protein. Based on the above studies, this interaction between calmodulin and the channel complex may play a role in regulating the function of the channel. Investigation of the effect of calmodulin on the channel activity is described in Chapter 4.
CHAPTER 4
MODULATION OF THE cGMP-GATED CHANNEL OF
PHOTORECEPTOR ROS BY CALMODULIN

4.1. MATERIALS
Polycarbonate membranes were obtained from Nuclepore. Dichlorophosphonazo III was purchased from Fluka. DEAE Sephacel and Ultrogel AcA 54 were from Pharmacia. Centricon-10 concentrator was acquired from Amicon. Bovine brain S-100 protein, bovine brain calmodulin, Arsenazo III, Sephadex G50, phenyl Sepharose, neutral red, FCCP, and all other chemicals were obtained from Sigma.

4.2. METHODS
4.2.1. Calcium influx assay using Arsenazo III and dichlorophosphonazo III dyes
ROS membranes were prepared as previously described. ROS membrane vesicles were loaded with Arsenazo III dye as follows: ROS membranes were washed twice in buffer A (10 mM HEPES pH 7.4, 1 mM DTT, 1 mM EDTA, and 0.1 mM DFP) and once in buffer B (2 mM HEPES pH 7.4, 0.2 mM DTT, and 0.02 mM DFP) to remove soluble proteins. The membrane pellet was resuspended in buffer B at a protein concentration of 12 mg/ml and bleached by continuous white light. After one cycle of freezing and thawing, the membranes were suspended in 10 mM HEPES pH 7.4, 100 mM KCl, 1 mM DTT, and 2 mM Arsenazo III and sonicated for 1 min in a beaker of water using a broad tip sonicator probe (Heat Systems-Ultrasonics, Inc.) at setting 7. The sonicated ROS membranes were then extruded 3 times using a Lipid Extruder (Lipex Biomembranes, Vancouver, B.C.) through two layers of 800 nm pore size Nuclepore polycarbonate filters (Hope et al., 1985). The extrusion of ROS membranes was repeated using 400 nm and 200 nm pore size polycarbonate filters. The membrane vesicles were passed through a Sephadex G50 column (1.5 X 28 cm) equilibrated at 4 °C in buffer C (10 mM HEPES pH
7.4, 0.1 mM DTT, and 100 mM KCl) to remove untrapped dye. The eluted vesicles were further dialyzed against buffer C for 3 h at 4 °C. ROS membrane vesicles containing trapped Arsenazo III dye were then added to buffer C in a cuvette to a final volume of 2 ml and at a final protein concentration of 0.29 mg/ml either in the absence or presence of 5 μg/ml (235 nM) calmodulin. An aliquot (4 μl) of stock CaCl₂ was then added to give a final concentration of 100 μM CaCl₂. One min after the addition of calcium, the calcium influx assay was initiated by the injection of 4 μl of varying concentrations of cGMP. The change in Arsenazo III dye absorbance upon binding to Ca²⁺ was monitored at 650-730 nm using a SLM Aminco DW 2000 dual wavelength spectrophotometer.

For dichlorophosphonazo III (Wohlfart et al., 1990), a similar dye trapping procedure was employed. The assays were carried out in the presence of CaCl₂ or MgCl₂ (200 μM). For assays using Mg²⁺ as the main influx ion, 1 μM CaCl₂ was also included. The divalent cation influx assays were carried out either in the presence or absence of 235 nM bovine brain calmodulin and the assays were initiated by the addition of varying concentrations of cGMP. The change in dye absorbance was monitored at 600-672 nm using the SLM Aminco DW 2000 dual wavelength spectrophotometer.

4.2.2. Inhibition of the calmodulin effect by mastoparan

ROS membrane vesicles containing Arsenazo III dye were prepared as described above. ROS membrane vesicles were added to buffer C (10 mM HEPES pH 7.4, 0.1 mM DTT, and 100 mM KCl) to a final volume of 2 ml at a final protein concentration of 0.3 mg/ml. Varying concentrations of calmodulin (0.8-235 nM) either in the presence or absence of 430 nM mastoparan were added. The calcium influx assays were carried out by the addition of CaCl₂ (100 μM) followed by the injection of 12.5 μM cGMP 1 min later. The change in dye absorbance was monitored as described above.

4.2.3. Cation influx assay using neutral red dye
ROS were washed once in buffer D (10 mM HEPES/arginine pH 7.4, 0.5 mM DTT, and 1 mM EDTA) and once in buffer E (10 mM HEPES/arginine pH 7.4 and 0.5 mM DTT) to remove soluble proteins. The membrane pellet was then resuspended in buffer E at a protein concentration of 14 mg/ml and subjected to one cycle of freezing and thawing. The membranes were mixed with an equal volume of the resuspension buffer (20 mM HEPES/arginine pH 7.4, 10 % sucrose, 2.5 % Ficoll, and 0.5 mM DTT) and extruded 4 times through one layer of 800 nm pore size Nuclepore polycarbonate filter using the Lipid Extruder. The extrusion of ROS membranes was repeated using the 400 nm pore size polycarbonate filter. The neutral red assay was carried out according to a modified method of Schnetkamp (1990) as follows: The membrane vesicles were added to the assay buffer containing 20 mM HEPES/arginine pH 7.4, 5 % sucrose, 0.5 mM DTT, 30 μM neutral red, 1 μM FCCP, and with or without 400 nM calmodulin. The final assay volume was 2 ml and with a protein concentration of 0.5 mg/ml. The assay was initiated by the addition of 50 μl of 0.5 M CaCl₂, MgCl₂, BaCl₂, MnCl₂, NaCl, KCl, LiCl, or CsCl (12.2 mM final concentration) and 0.4 mM CaCl₂ (10 μM final concentration). Four μl of 10 mM cGMP (20 μM final concentration) was added 1 min later. The change in absorbance was monitored at 540-650 nm using a SLM Aminco DW 2000 dual wavelength spectrophotometer. For measuring the Ca²⁺ dependence of calmodulin effect, Na⁺ (12.2 mM) was used as the main influx ion and the assay mixture contained varying concentrations of Ca²⁺ ranging from 10 nM to 1 μM. Buffers of varying Ca²⁺ concentrations were prepared according to the program of Fabiato (1988). The following ratios of Ca²⁺ to 1 mM EGTA were used (estimated free Ca²⁺ in parentheses): 0.134 (10 nM), 0.279 (25 nM), 0.436 (50 nM), 0.607 (100 nM), 0.755 (200 nM), 0.830 (316 nM), 0.860 (398 nM), 0.908 (631 nM), and 0.940 (1000 nM).

4.2.4. Purification of calmodulin and recoverin from bovine photoreceptor ROS
Bovine ROS calmodulin was purified as described in section 2.2. For the purification of recoverin, ROS membranes from 200 bovine retinas were washed with 100 ml of 10 mM HEPES pH 7.4 and 1 mM EDTA. The supernatant was loaded onto a 4 ml DEAE Sephadex column equilibrated at 4 °C in 10 mM HEPES pH 7.4, 0.1 mM DTT, and 1 mM EDTA. The column was washed with 5 column volumes of equilibration buffer and recoverin was eluted off the column with 0.125 M NaCl in the same buffer. The DEAE fractions were dialyzed at 4 °C against 10 mM HEPES pH 7.4, 0.5 M NaCl, 0.1 mM DTT, and 0.1 mM CaCl2 and then with the same buffer without the NaCl. Recoverin was eluted with 10 mM HEPES pH 7.4, 0.1 mM DTT, and 5 mM EGTA. The phenyl Sepharose column eluant was concentrated down to 1 ml with a Centricon-10 concentrator and loaded onto an 1.5 x 54 cm Ultrogel AcA-54 gel filtration column equilibrated in 10 mM HEPES pH 7.4 and 100 mM NaCl. One ml fractions were collected and analyzed by SDS polyacrylamide gel electrophoresis. Fractions containing recoverin were dialyzed against 10 mM HEPES pH 7.4 and lyophilized down to 0.25 ml.

4.3. RESULTS

4.3.1. Effect of calmodulin on the affinity of the channel for cGMP

The effect of calmodulin on the cGMP-gated channel activity was investigated by Ca2+ influx assays using ROS membrane vesicles loaded with the divalent cation sensitive dye, Arsenazo III. The rate of Ca2+ influx was measured spectrophotometrically (Koch and Kaupp, 1985) as a function of cGMP concentration in the presence and absence of calmodulin. As shown in Figure 28a and b, the apparent Km for cGMP increased from 19 ± 0.4 μM in the absence of calmodulin to 33 ± 2 μM in the presence of Ca2+-calmodulin. This change in Km translates into a relatively large change in the rate of Ca2+ influx (6-
Fig. 28: Effect of calmodulin on the activation of the cGMP-gated channel.

a. The relative initial velocity of channel activation by cGMP was plotted against the concentration of cGMP either in the presence (■) or absence (●) of calmodulin. In the absence of calmodulin, the channel had an apparent Km of 19 μM and a Hill coefficient of 3.8 for cGMP. In the presence of calmodulin, its Km increased to 33 μM, but its Hill coefficient remained the same at 3.8. These assays were carried out in three separate experiments using different ROS membrane vesicle preparations. In all cases, an increase in Km (from 19 ± 0.4 μM to 33 ± 2 μM) in the presence of calmodulin (235 nM) without significant change in either the Vmax or the Hill coefficient (n= 3.7 ± 0.1 and n= 3.5 ± 0.6 in the absence and presence of calmodulin, respectively) was observed. The solid curved lines are calculated from a sigmoidal relationship using the indicated Km and Hill coefficients.

b. A typical trace for cGMP dependent influx of Ca²⁺ into ROS vesicles containing trapped Arsenazo III. A cGMP concentration of 12.5 μM was used to initiate the influx of Ca²⁺ either in the presence (+) or absence (-) of calmodulin.
Dichlorophosphonazo III, a divalent cation sensitive dye, that had been previously used by Wohlfart et al. (1990) to measure Mg$^{2+}$ translocation through the reconstituted cGMP-gated channel complex, was also used in this study to further verify the calmodulin effect. This dye was trapped within ROS membrane vesicles and assays were carried out using either Ca$^{2+}$ or Mg$^{2+}$ as the main influx ion. When Ca$^{2+}$ was used as the main influx ion, a similar shift in the affinity of the channel for cGMP was observed, as had been observed with Arsenazo III loaded ROS membrane vesicles (Fig. 29a). The Km of the channel for cGMP increased from 22 μM to 33 μM while its Hill coefficient remained relatively unchanged (n= 3.0). When Mg$^{2+}$ was used as the influx ion (Fig. 29b), the channel displayed a Km of 37 μM with a Hill coefficient of 2.4. In the presence of calmodulin, the Km of the channel for cGMP increased to 45 μM without significantly changing its Hill coefficient (n= 2.1). The higher Km and lower Hill coefficient observed with Mg$^{2+}$ translocation may be due to the differential gating of this cation by the channel complex.

Neutral red, a dye that is sensitive to changes in surface electrostatic potential induced by cation influx (Schnetkamp, 1990), was also used to examined the calmodulin effect. For Ca$^{2+}$ influx rates using the neutral red assay (Fig. 30), Ca$^{2+}$-calmodulin was observed to increase the Km for cGMP from 34 μM to 51 μM without significantly affecting either the Vmax or the Hill coefficient (n= 2.4 and 2.2 respectively). The higher Km and lower Hill coefficient obtained with this assay may be due to the hydrophobic nature of this dye and its interaction with the phospholipids and/or the channel.

### 4.3.2. Specificity of the calmodulin effect

In order to assess whether the observed calmodulin-induced change in the affinity of the cGMP-gated channel for cGMP is a unique phenomenon or is a universal property
Fig. 29: Effect of calmodulin on the activation of the rod channel by cGMP as determined by the dichlorophenazo III assay system.

a. Calcium influx assays were carried out using ROS membrane vesicles containing dichlorophosphonazo III dye either in the absence (●) or presence (■) of 235 nM calmodulin. Calmodulin was shown to increase the Km of the channel for cGMP from 22 μM to 34 μM without affecting either its maximum velocity or cooperativity for cGMP (n= 3.0). The solid curved lines were drawn from a sigmoidal isotherm using the indicated Km and Hill coefficients. b. Magnesium influx assays were carried out as above either in the absence (●) or presence (■) of calmodulin. The Km of the channel was shown to increase from 37 μM in the absence of calmodulin to 45 μM in the presence of calmodulin. Their respective Hill coefficients, however, remained relatively unchanged (n= 2.4 and 2.1 respectively).
Fig. 30: Effect of calmodulin on the activation of the rod channel by cGMP as determined by the neutral red assay system.

Calcium influx assays were carried out using extruded ROS membrane vesicles in the presence of neutral red. The assays were carried out either in the absence (●) or presence (■) of calmodulin. The initial velocities of the channel were plotted as a function of cGMP concentration. The Km of the channel was observed to increase from 34 μM to 51 μM in the presence of calmodulin while its Hill coefficient remained relatively unchanged (n= 2.4 and 2.2 respectively). The solid curved lines were drawn using the indicated Km and Hill coefficient values.
shared by various Ca\textsuperscript{2+}-binding proteins, bovine ROS recoverin and brain S-100 proteins were added to the Arsenazo III loaded vesicles in place of calmodulin. In addition, calmodulin isolated from bovine ROS was compared with bovine brain calmodulin for its effect on channel activity. Recoverin was purified from the EDTA extract of ROS by DEAE anion exchange, phenyl Sepharose, and gel filtration chromatography and the purified recoverin was analyzed by SDS polyacrylamide gel electrophoresis as shown in Figure 31. Figure 32 shows the cGMP-dependent influx of Ca\textsuperscript{2+} into ROS membrane vesicles in the presence of various Ca\textsuperscript{2+}-binding proteins. Whereas bovine brain calmodulin and ROS calmodulin decreased the channel activity equally well (Fig. 32b), bovine ROS recoverin and brain S-100 protein had no effect on channel activity under similar conditions (Fig. 32a).

4.3.3. Inhibition of the calmodulin effect by mastoparan

In order to reverse the calmodulin effect, two anti-calmodulin agents, mastoparan and calmidazolium, were used. In the absence of mastoparan, the half maximal inhibition (IC\textsubscript{50}) of the channel by calmodulin in the presence 12.5 \mu M cGMP was found to be 1.85 ± 0.25 nM (averaged from 3 trials; Fig. 33). In the presence of 430 nM mastoparan, higher calmodulin concentrations were required to modulate the channel activity, and the half maximal inhibition (IC\textsubscript{50}) of the channel was found to increase over ten fold to 20.3 ± 3.8 nM (averaged from 3 trials). Higher mastoparan concentration was not used since this peptide was found to affect the channel activity at concentrations above 800 nM. Calmidazolium, a potent hydrophobic calmodulin inhibitor, was found to inhibit the channel activity at concentrations above 560 nM. However, at or below this concentration, it was ineffective in reversing the calmodulin effect to any significant extent. It has been reported that the hydrophobic nature of the calmodulin inhibitors can result in their incorporation into lipid bilayers (Tanimura et al., 1991). As a result, these inhibitors can become unavailable for binding to calmodulin. This may explain the
Fig. 31: Purification of recoverin from bovine ROS.

Bovine ROS were washed with EDTA to extract soluble proteins. Recoverin was purified from this EDTA extract by DEAE anion exchange, phenyl Sepharose, and gel filtration chromatography. The protein samples were run on a 9% SDS polyacrylamide gel and the gel was stained with Coomassie blue. Lane a, intact ROS (30 μg); lane b, EDTA extract of ROS (5 μg); lane c, pooled recoverin from the anion exchange column; lane d, pooled recoverin from the phenyl Sepharose column; lane e, gel filtration column purified recoverin.
Fig. 32: Effect of various Ca$^{2+}$-binding proteins on the cGMP-gated channel activity.

a. ROS membrane vesicles were trapped with Arsenazo III dye. The Ca$^{2+}$ influx assays were carried out at 12.5 μM cGMP in the absence (-CaM) or in the presence of 200 nM recoverin, S-100, and calmodulin. b. Ca$^{2+}$ influx was measured in the absence or presence of 20 nM bovine brain calmodulin or bovine photoreceptor calmodulin.
Fig. 33: Inhibition of the calmodulin effect by mastoparan.

The calcium influx assays were carried out using extruded vesicles containing Arsenazo III dye. The assays were carried out using varying concentrations of calmodulin (0.8-235 nM) either in the presence (■) or absence (○) of 430 nM mastoparan. A cGMP concentration of 12.5 μM was used to initiate the assay and the relative initial velocity of Ca²⁺ influx in the absence of calmodulin was designated as 100 % activity.
inhibition of the channel activity by mastoparan and calmidazolium at high concentrations and the failure of calmidazolium to inhibit the calmodulin effect.

4.3.4. Cation selectivity and calcium dependence of the calmodulin effect

The cGMP-gated channel is permeable to a variety of monovalent and divalent cations. A neutral red dye assay was used to determine the effect of Ca\textsuperscript{2+}-calmodulin on the cGMP-dependent influx rate of different cations into ROS membrane vesicles. At 20 \mu M cGMP, Ca\textsuperscript{2+}-calmodulin decreased the initial rate of ion influx by 2-3 fold for all cations except Mg\textsuperscript{2+} (Fig. 34). The smaller decrease for Mg\textsuperscript{2+} (1.4 fold) may be due to the interference of high Mg\textsuperscript{2+} concentration on the interaction of Ca\textsuperscript{2+} with calmodulin (Ogawa and Tanokura, 1984).

The calmodulin modulation of the channel activity was shown to be a Ca\textsuperscript{2+}-dependent process. In a similar neutral red dye assay system, Na\textsuperscript{+} was used as the main influx ion and the assay was carried out in the presence of varying concentrations of Ca\textsuperscript{2+} ranging from 10 nM to 1 \mu M. At a constant cGMP concentration of 20 \mu M, the calmodulin effect was observed to occur within the range of 20 to 300 nM Ca\textsuperscript{2+} (Fig. 35). In the control study in which calmodulin was omitted from the assay mixture, the channel activity was relatively unaffected by Ca\textsuperscript{2+}.

4.4. DISCUSSION

The role of calmodulin in modulating the activity of the cGMP-gated channel was investigated using the extruded ROS membrane vesicle system containing various divalent cation sensitive dyes. In all cases, it was found that the presence of calmodulin resulted in a decrease in the affinity of the channel (ie. increase in Km) for cGMP without affecting the number of cGMP molecules required to open the channel (ie. Hill coefficients remain constant). This effect was shown to be specific for calmodulin since two other calcium
Fig. 34: Effect of calmodulin on the influx of various cations.

Cation influx assays were carried out using extruded ROS membrane vesicles in the presence of neutral red. The initial rates of cGMP-dependent influx of different monovalent (Na+, K+, Li+, and Cs+) and divalent (Mg2+, Ba2+, and Mn2+) cations into ROS membrane vesicles were measured in the presence (solid) and absence (cross-hatched) of calmodulin. A cGMP concentration of 20 µM was used to initiate cation influx. The initial velocity of Ca2+ flux in the absence of calmodulin was set at 100%. All ions except Mg2+ showed a 2-3 fold decrease in initial velocities in the presence of Ca2+-calmodulin.
Fig. 35: Calcium dependence of calmodulin effect on the cGMP-gated channel activity.

The Na⁺ influx assays were carried out using extruded ROS membrane vesicles in the presence of neutral red and at varying concentrations of Ca²⁺ (10-1000 nM). The assays were carried out either in the presence (■) or absence of calmodulin (●). The cGMP-dependent (20 µM) Na⁺ influx in the presence of 10 nM Ca²⁺, but in the absence of calmodulin, was designated as 100 % activity. Results of three sets of experiments carried out in the presence of calmodulin are shown with standard deviations marked by error bars.
binding proteins, bovine brain S-100 and retinal recoverin, had no effect on the channel activity.

The half maximal inhibition value of $1.85 \pm 0.25$ nM obtained from the dose-response curve of the calmodulin inhibition of channel activity indicates that the association between calmodulin and the channel complex is quite strong. Other calmodulin binding proteins have binding constants for calmodulin in the range of 1-600 nM (O'Neil and DeGrado, 1990). Inhibition of the calmodulin effect was found to be dependent on the type of inhibitors used. Only mastoparan, the least hydrophobic of the compounds used, was capable of substantially reversing the calmodulin effect in the lipid-rich assay system employed here.

The binding of calmodulin to the channel complex affected the $K_m$ but not the $V_{max}$ or cooperativity of the channel for cGMP. These parameters were found to be consistent by a variety of assay methods. This suggests that calmodulin affects the binding affinity of the channel for cGMP but not the ion translocation properties of the channel.

The calmodulin effect on channel activity was observed over a $Ca^{2+}$ concentration of 50-300 nM. This is within the range of the $Ca^{2+}$-dependent activation of guanylate cyclase (Koch and Stryer, 1988) and physiological $Ca^{2+}$ concentration in photoreceptor outer segments (McNaughton et al., 1986; Ratto et al., 1988; Korenbrot and Miller, 1989; Kaupp and Koch, 1992). Earlier, Caretta et al. (1988) observed that $Ca^{2+}$ decreased the binding affinity of a cGMP analogue to ROS membranes. It is likely that this observed $Ca^{2+}$ effect on cGMP binding is related to the effect of $Ca^{2+}$ on calmodulin modulation of the cGMP-gated channel as reported here.

The above studies suggest that changes in cytoplasmic $Ca^{2+}$ concentration which are known to occur during the photoresponse can affect the activity of the cGMP-gated channel. The channel is opened in response to the cooperative binding of cGMP to the channel subunits. The affinity of the channel for cGMP is further modulated by the level of cytoplasmic $Ca^{2+}$ through the binding of $Ca^{2+}$-calmodulin to the channel complex.
This Ca\(^{2+}\)-calmodulin effect on the channel is most pronounced at low cGMP concentrations as found under physiological conditions (Nakatani and Yau, 1988a). Regulation of the channel by cGMP and Ca\(^{2+}\)-calmodulin is in some ways analogous to the regulation of hemoglobin by oxygen and pH (Bohr effect) in which small changes in pH alter the affinity of hemoglobin for oxygen.

The key question that one could raise regarding calmodulin modulation of the channel is "Does this effect occur in ROS under physiological conditions?" To address this question, experimental designs must involve the use of whole ROS. This system, however, can cause potential problems due to the complexities associated with intact systems. Earlier studies dealing with the incorporation of Ca\(^{2+}\) buffer into detached ROS indicated that intracellular Ca\(^{2+}\) dictates the rate of photorecovery (Torre et al., 1986; Nakatani and Yau, 1988b). Although these observations are in general agreement with the idea of a Ca\(^{2+}\)-calmodulin mediated shift in the affinity of the channel for cGMP, it is not possible to distinguish the role played by calmodulin with those of other Ca\(^{2+}\)-regulated processes, such as those of guanylate cyclase and phosphodiesterase. Gray-Keller et al. (1993) recently described experiments involving measurements of the kinetics of light response following the dialysis of various Ca\(^{2+}\)-binding proteins into a detached gecko rod outer segment. Addition of exogenous recoverin was found to prolong the photorecovery phase possibly due to its potential inhibition on rhodopsin phosphorylation while calmodulin was found to have little or no effect. Results in this chapter indicate that calmodulin binds strongly to the channel complex (half maximal inhibition or IC\(_{50}\) = 2 nM). With an estimated calmodulin concentration of 4 \(\mu\)M in the ROS (Kaupp and Koch, 1992), the addition of exogenous calmodulin would unlikely elicit an additional effect, particularly if calmodulin remains bound to the channel complex in high Ca\(^{2+}\). Another approach to study calmodulin modulation of the channel in intact ROS is to measure the effect of calmodulin inhibitors on the phototransduction process. However, the application of these calmodulin inhibitors in membrane systems is not straightforward.
Several studies including studies on the ROS membrane vesicle system, as described above, indicate that the calmodulin inhibitors can directly inhibit the conductance of not only the cGMP-gated channel but also a variety of other types of channels in a Ca\(^{2+}\)-independent manner (Nicol, 1993; Kihira et al., 1990; Klockner and Isenberg, 1987). This suggests that these inhibitors are not acting through calmodulin. The hydrophobic nature of the inhibitors could result in their incorporation into the lipid bilayers, thereby decreasing their overall effective concentration. In a highly membranous system such as the intact rod outer segment, one would have to determine the actual target site of these inhibitors as well as their effective concentrations in the cytoplasm. Without this information, interpretation of inhibition results would be ambiguous. The physiological effect of calmodulin on modulation of the cGMP-gated channel thus remains an open question at the present time.
CHAPTER 5
CHARACTERIZATION OF THE 240 kDa CHANNEL ASSOCIATED PROTEIN

5.1. MATERIALS

Bovine pancreatic trypsin and chymotrypsin, porcine pancreatic kallikrein, soybean trypsin inhibitor, CHAPS, asolectin, Arsenazo III, bovine brain calmodulin, and other chemicals were purchased from Sigma. \(^{[125]}\) Bolton-Hunter reagent was obtained from New England Nuclear and horseradish peroxidase conjugated sheep anti-mouse F'ab fragment and the ECL kit were acquired from Amersham. Centricon-30 concentrator was bought from Amicon, and Immobilon membrane was obtained from Millipore.

5.2. METHODS
5.2.1. Purification of the channel complex by PMc 6E7 antibody affinity and DEAE anion exchange chromatography

ROS membranes (40 mg protein) were solubilized in 30 ml of 10 mM HEPES pH 7.4, 150 mM KCl, 18 mM CHAPS, 0.2 % asolectin, 2 mM CaCl\(_2\), and 0.1 mM DFP. The solubilized sample was loaded onto two antibody columns (3 ml bed volume each) that had been equilibrated at 4 °C in the same buffer. After washing the columns with 10 column volumes of the washing buffer (10 mM HEPES pH 7.4, 150 mM KCl, 15 mM CHAPS, 0.18 % asolectin, 1 mM CaCl\(_2\), and 0.1 mM DFP), the channel complex was eluted off the columns with washing buffer containing the synthetic peptide (0.9 mg/ml) corresponding to the N-terminus of the cGMP-gated channel (Molday et al., 1991). Fractions (1 ml) collected were then loaded onto a 1.5 ml DEAE anion exchange column. After washing the column with 5 column volumes of washing buffer in the presence of 1 mM DTT, the channel complex was eluted from the column using 0.45 M KCl in the
washing buffer. One ml fractions were collected, pooled, and reconstituted into lipid vesicles according to the method described by Cook et al. (1987).

5.2.2. Calcium efflux assay of the immunoaffinity purified channel complex reconstituted into lipid vesicles

The calcium efflux assay was carried out according to the method described by Cook et al. (1987). The assay mixture consisted of 0.3 ml vesicles (6.5 µg protein) and 1.7 ml of 55 µM Arsenazo III dye in dialysis buffer containing 1 µM CaCl₂. The assay was carried out either in the presence or absence of 120 nM bovine brain calmodulin. The Ca²⁺ efflux assay was initiated by the addition of varying concentrations of cGMP. Changes in dye absorbance were monitored at 650-730 nm using a SLM Aminco DW 2000 dual wavelength spectrophotometer.

5.2.3. Mild proteolytic digestion of ROS membranes with trypsin, chymotrypsin, and kallikrein

ROS membranes were washed twice with 10 mM HEPES pH 7.4, 1 mM DTT, and 1 mM EDTA. The membrane pellet was resuspended in 10 mM HEPES pH 7.4 at a protein concentration of 10 mg/ml for tryptic and chymotryptic digestion and 5 mg/ml for kallikrein digestion. Solutions containing trypsin (0.8 µg/ml), chymotrypsin (1.6 µg/ml), and kallikrein (1.3 units/ml) were added to an equal volume of the resuspended ROS membrane mixture and digestions were allowed to proceed for 30 min at room temperature. The digestions were terminated by washing the membranes twice with 10 mM HEPES pH 7.4 containing 50 µg/ml of soybean trypsin inhibitor for trypsin and 0.5 mM DFP for chymotrypsin and kallikrein. The digested membranes were then solubilized in CHAPS, reconstituted into lipid vesicles, and subjected to Ca²⁺ efflux assays as described earlier.
5.2.4. Purification of the kallikrein-treated channel complex by PMc 6E7 antibody affinity chromatography

ROS membranes which had been treated with kallikrein (10 mg) were solubilized in 10 mM HEPES pH 7.4, 150 mM NaCl, 1 mM DTT, 0.1 mM DFP, and 2 mM CaCl\(_2\). The solubilized sample was loaded onto a 1 ml PMc 6E7 antibody column. The column washing and elution procedures were carried out as previously described in section 3.2.2.

5.2.5. Protein sample preparation for N-terminal sequence analysis

For N-terminal sequence analysis, ROS membrane proteins (120 mg) were subjected to kallikrein treatment and then passed through three 5 ml calmodulin Sepharose columns. The EDTA eluants from the calmodulin affinity columns were concentrated with a Centricon-30 concentrator. Polypeptides were separated on an 8% SDS polyacrylamide gel and electrotransferred onto an Immobilon membrane. The protein bands were visualized by staining with Ponceau S. The desired protein bands were excised from the membrane, destained in distilled water, and sent to the protein microsequencing service facility at the University of Victoria for N-terminal sequence analysis.

5.2.6. SDS polyacrylamide gel electrophoresis and Western blotting analysis

SDS polyacrylamide gel electrophoresis and electrotransfer of ROS membrane proteins onto Immobilon membranes were carried out as previously described. For the inhibition of \(^{125}\)I-calmodulin binding to the 240 kDa protein by anti-240 kDa monoclonal antibodies PMs 3C9, PMs 5E11, and PMs 4B2, the blots were first blocked for 1 h with 50 mM Tris pH 7.4, 100 mM NaCl, 0.05 % Tween 20, and 1 mM CaCl\(_2\). The blots were then incubated in the same buffer containing 10 times diluted hybridoma culture fluid for another hour. Iodinated calmodulin (2 \(\mu\)Ci/ml) was then added and allowed to incubate with blots for 1 h. After washing away the unbound calmodulin, the labelled bands on the blots were visualized by autoradiography. For blots labelled with the anti-240 kDa protein
and anti-63 kDa channel α-subunit monoclonal antibodies only, horseradish peroxidase conjugated sheep anti-mouse Ig was added instead of $^{125}$I-calmodulin. Labelled protein bands were visualized by ECL.

5.3. RESULTS

5.3.1. Mediation of the calmodulin effect through the 240 kDa channel associated protein

Calmodulin-induced shift in the affinity of the channel for cGMP has been demonstrated, in the previous chapter, using ROS membrane vesicles trapped with divalent cation sensitive dyes. In order to verify that this effect is mediated through the calmodulin-binding 240 kDa protein, $\text{Ca}^{2+}$-efflux assays were carried out using PMc 6E7 antibody column purified channel complex which had been reconstituted into lipid vesicles (Fig. 36). In the absence of calmodulin, the channel displayed a $K_m$ of 33 $\mu$M and a Hill coefficient of 3.3. In the presence of calmodulin, the $K_m$ of the channel increased to 44 $\mu$M while its Hill coefficient remained relatively unchanged ($n= 3.3$ and $3.1$, respectively). The shift in the affinity of the channel for cGMP in the reconstituted system is not as large as that observed in the intact membrane system. This difference could be attributed to potential changes in channel conformation induced by solubilization and reconstitution.

The antibody column purified channel complex was also analysed by SDS polyacrylamide gel electrophoresis and Western blotting with $^{125}$I-calmodulin. The purified channel complex was shown to consist of the characteristic 240 kDa and 63 kDa protein bands. The 240 kDa protein was the only protein band labeled by iodinated calmodulin on the Western blot (data not shown). Thus, in the absence of other calmodulin binding proteins, the observed shift in the affinity of the channel for cGMP is most likely the result of calmodulin interaction with the 240 kDa channel associated protein.
Fig. 36: Effect of calmodulin on the cGMP-dependent activation of the reconstituted channel complex.

The PMc 6E7 antibody column purified channel complex was concentrated by DEAE anion exchange chromatography and reconstituted into lipid vesicles. Calcium efflux assays were carried out either in the absence or presence of 120 nM calmodulin. The relative initial velocities of the channel were plotted as a function of varying concentrations of cGMP. The solid curved lines represent a sigmoidal isotherm using Km values of 33 and 44 μM and Hill coefficients of 3.3 and 3.1 in the absence (■) and presence (■) of calmodulin, respectively. The purified channel complex had a specific activity of 187 units/mg protein.
5.3.2. Effectiveness of anti-240 kDa protein monoclonal antibodies in inhibiting calmodulin binding to the channel complex

Several monoclonal antibodies have been generated against the 240 kDa channel associated protein. These antibodies, PMs 3C9, PMs 5E11, and PMs 4B2, were used on Western blots in an attempt to inhibit iodinated calmodulin binding to the 240 kDa protein. The blots were incubated in these antibody solutions prior to calmodulin labelling. As shown in Figure 37, none of the antibodies inhibited calmodulin binding to the 240 kDa protein, implying that the epitopes of these antibodies on the 240 kDa protein are distant from the calmodulin binding site.

5.3.3. Effect of proteolysis on the cGMP-gated channel complex

Proteolytic digestions of ROS membranes were carried out using various enzymes in an attempt to destroy the calmodulin binding site on the 240 kDa protein, and hence, the sensitivity of the channel complex to calmodulin. The enzyme digestions were carried out under conditions which minimized cleavage of the 63 kDa channel \( \alpha \)-subunit. The proteolyzed ROS membrane proteins were reconstituted into lipid vesicles for \( \text{Ca}^{2+} \) efflux assays. Monoclonal antibody PMc 6E7 (Molday et al., 1991) directed against the N-terminus of the 63 kDa channel \( \alpha \)-subunit and monoclonal antibodies PMc 1D1 and PMc 2G11 directed against the C-terminal regions of the \( \alpha \)-subunit were used to label Western blots to examine the intactness of the \( \alpha \)-subunit.

Chymotrypsin, an enzyme that preferentially cleaves peptide bonds on the carboxyl side of aromatic amino acids, was found to cleave a variety of ROS membrane proteins including the 240 kDa protein (Fig. 38). Western blots of the chymotrypsinized ROS membrane proteins (Fig. 38) gave 2 closely spaced bands with PMc 1D1 labelling and 3 closely spaced bands with PMc 6E7 and PMc 2G11 labelling. This implied that cleavages had occurred at two different regions of the C-terminus of the \( \alpha \)-subunit. Reconstitution
Fig. 37: Inability of anti-240 kDa protein monoclonal antibodies to inhibit calmodulin binding to the 240 kDa protein.

Various anti-240 kDa protein monoclonal antibodies were used in an attempt to inhibit iodinated calmodulin binding to the 240 kDa protein by Western blot analysis. Left, Coomassie blue stained SDS polyacrylamide gel of the stripped ROS membranes (30 μg). Right, lane a, iodinated calmodulin labelling of ROS membrane proteins without prior incubation with anti-240 kDa protein monoclonal antibodies; lanes b, c, and d are the iodinated calmodulin labelling of the ROS membrane proteins in the presence of anti-240 kDa protein monoclonal antibodies PMs 3C9, 4B2, and 5E11 respectively.
Fig. 38: Chymotrypsin digestion of ROS membranes.

ROS membranes were subjected to mild chymotrypsin digestion. The digested membranes were run on an 8% SDS polyacrylamide gel. The intactness of the channel α-subunit was monitored by Western blotting analysis with anti-α-subunit monoclonal antibodies PMc 1D1, 6E7, and 2G11. Lanes a are the undigested ROS membranes (30 μg) and lanes b are the chymotrypsinized ROS membranes (30 μg).
of the chymotrysinized ROS membrane proteins into lipid vesicles gave no channel activity as determined by the Ca\(^{2+}\) efflux assay.

Treatment of ROS membranes with trypsin also resulted in the cleavage of various ROS membrane proteins (Fig. 39). Western blotting analysis with monoclonal antibodies PMc 6E7 and PMc 1D1 indicated that trypsin cleaved at the N-terminal region of the α-subunit and removed the PMc 6E7 binding site. Analysis with PMs 4B2 indicated that the 240 kDa protein was broken down to various smaller fragments. The trypsinized ROS membrane proteins were also reconstituted into lipid vesicles. Calcium efflux assays of the reconstituted vesicles indicated that trypsinization of the channel complex appeared to slightly shift the Km of the channel for cGMP to the left (Km shifted from 32 μM to 28 μM; Fig. 40a and b). Addition of calmodulin to the assay mixture decreased the channel activity for both samples (Fig. 41). However, lowering calmodulin concentration from 200 to 25 nM resulted in a much more rapid reversal of the calmodulin effect for the trypsinized ROS membrane vesicles than for the untrypsinized ROS membrane vesicles. This suggests that the calmodulin binding site was still intact but its ability to bind calmodulin was diminished by the proteolytic cleavage. Calmodulin affinity chromatography of the trypsinized ROS membrane proteins yielded a 59 kDa trypsinized form of the channel α-subunit and a 70 kDa fragment of the 240 kDa protein (Fig. 42, left). However, this 70 kDa protein failed to be labelled by iodinated calmodulin on the Western blot (Fig. 42, right).

A third proteolytic enzyme, kallikrein, was also used to digest ROS membranes. As shown in the Coomassie blue stained SDS polyacrylamide gel (Fig. 43), the 240 kDa protein appeared to be the only protein cleaved by this enzyme. Western blotting analysis with PMc 1D1 and PMc 6E7 monoclonal antibodies indicated that the α-subunit of the channel remained relatively intact. Analysis with PMs 4B2 monoclonal antibody indicated that the 240 kDa protein was cleaved into smaller fragments. The reconstituted vesicles of the kallikrein treated ROS membrane proteins gave a Km of 29 μM and a Hill coefficient
Fig. 39: Trypsin digestion of ROS membranes.

ROS membranes were subjected to mild trypsin digestion. The digested membranes were run on an 8% SDS polyacrylamide gel and the intactness of the channel α-subunit and the 240 kDa protein was monitored by Western blotting analysis with anti-α-subunit monoclonal antibodies PMc 1D1 and PMc 6E7 and anti-240 kDa protein monoclonal antibody PMs 4B2. Lanes a are the undigested ROS membranes (30 μg) and lanes b are the trypsinized ROS membranes (30 μg).
Fig. 40: Reconstitution of trypsinized ROS membrane proteins.

\[ \frac{V_0}{V_{\text{max}}} \]

Ca\textsuperscript{2+} efflux assays were carried out to compare the effect of trypsinization on channel activity. Intact or trypsinized ROS membrane proteins were solubilized and reconstituted into lipid vesicles. Aliquots of 0.3 ml of vesicles were used for the Ca\textsuperscript{2+} efflux assays at varying concentrations of cGMP. The relative initial velocities of the channel were plotted as a function of the cGMP concentration. a. The intact channel displayed a Km of 32 \( \mu \)M and a Hill coefficient of 3.5. b. The trypsinized channel displayed a Km of 28 \( \mu \)M and a Hill coefficient of 3.3. The solid curved lines in a and b were drawn using their respective Km and Hill coefficients.
Fig. 41: Effect of trypsin treatment of ROS membranes on the calmodulin modulation of the channel activity.

Untrypsinized (a) and trypsinized (b) ROS membranes were reconstituted into lipid vesicles. The reconstituted vesicles were subjected to the Ca^{2+} efflux assays at 17.5 μM cGMP in the presence of 0, 25, and 200 nM calmodulin.
Fig. 42: Purification of the trypsinized channel complex by calmodulin affinity chromatography.

Trypsinized ROS membranes were solubilized and passed through a calmodulin column. The bound proteins were then eluted with EDTA. The protein samples were analyzed on an 8% SDS polyacrylamide gel (left). Western blotting analysis with iodinated calmodulin was carried out to detect calmodulin binding proteins (right). Lanes a, stripped ROS membranes (30 μg); lanes b, calmodulin column eluant from the ROS membranes; lanes c, trypsinized ROS membranes (30 μg); lanes d, calmodulin column eluant from the trypsinized ROS membranes. The position of the 70 kDa fragment of the 240 kDa protein is marked by the arrow.
Fig. 43: Digestion of ROS membranes with kallikrein.

ROS membranes were digested with kallikrein. The intactness of the channel complex was monitored by SDS polyacrylamide gel electrophoresis and Western blotting analysis with anti-α-subunit monoclonal antibodies PMc 1D1 and PMc 6E7. Lanes a are the ROS membranes (30 μg) and lanes b are the kallikrein-treated ROS membranes (30 μg).
of 3.1 for cGMP (Fig. 44). No Attempts to determine whether the kallikrein treatment of ROS membranes would reverse the calmodulin effect were made since the calmodulin binding site was still intact after this treatment as described below.

5.3.4. Identification of a calmodulin binding fragment of the 240 kDa protein

When ROS membranes were digested with kallikrein as described above, the 240 kDa protein was degraded while the 63 kDa α-subunit remained relatively intact. The proteolyzed ROS membranes were solubilized in CHAPS and passed through a PMc 6E7 antibody column. The bound proteins were then eluted off the column by the competing synthetic peptide. A 105 kDa protein was found to coelute with the 63 kDa channel α-subunit as shown on the Coomassie blue stained SDS gel (Fig. 45 (left), lane d). This 105 kDa fragment was shown to be labelled on Western blots by both iodinated calmodulin and monoclonal antibody PMs 3C9 but not by monoclonal antibodies PMs 5E11 and PMs 4B2 (Fig. 45).

In order to isolate this 105 kDa fragment in large quantity, calmodulin affinity chromatography was employed. The 105 kDa protein was transferred onto an Immobilon membrane and the protein band was subjected to N-terminal sequence analysis. The amino acid sequence was found to have a high degree of homology with the N-terminal region of the recently cloned human cGMP-gated channel β-subunit (Chen et al., 1993). The sequence alignment is shown in Figure 46. The N-terminal sequence of the 105 kDa fragment is upstream of the initiation site for the shorter form of the human β-subunit (Chen et al., 1993). This suggests that the longer form of the β-subunit is the predominant species expressed in bovine ROS.

5.4. DISCUSSION

Calmodulin modulates the activity of the cGMP-gated channel by changing its affinity for cGMP. This modulation is mediated through the 240 kDa channel associated
Fig. 44: Reconstitution of the kallikrein-treated ROS membrane proteins.

Kallikrein-treated ROS membranes were solubilized and reconstituted into lipid vesicles. The Ca$^{2+}$-efflux assays were carried out at varying concentrations of cGMP using 0.3 ml of reconstituted lipid vesicles per assay. The relative initial velocities of the reconstituted channels were plotted as a function of cGMP concentration. The solid curved lines were drawn using a Km of 29 µM and a Hill coefficient of 3.1.
Fig. 45: Purification of the kallikrein-treated cGMP-gated channel complex by the anti-α-subunit PMc 6E7 monoclonal antibody column.

ROS membranes treated with kallikrein were solubilized and passed through an anti-α-subunit PMc 6E7 antibody column. The bound proteins were eluted off the column by synthetic peptides. The purified channel complex was analysed by SDS polyacrylamide gel electrophoresis and Western blotting analysis with either iodinated calmodulin or anti-240 kDa protein monoclonal antibodies PMs 3C9, 4B2, and 5E11. Lanes a, stripped ROS membranes (30 µg); lanes b, intact channel complex purified by PMc 6E7 column; lanes c, kallikrein-treated ROS membranes (30 µg); lanes d, kallikrein-treated channel complex purified by PMc 6E7 column. The positions of the 105 kDa calmodulin binding fragment of the 240 kDa protein are indicated by arrows.
Fig. 46: N-terminal sequence alignment of the 105 kDa fragment with the cGMP-gated channel β-subunit.

The N-terminal sequence of the 105 kDa kallikrein fragment of the 240 kDa protein was aligned with the human rod cGMP-gated channel β-subunit (HRCN2 B). The initiation site of the shorter transcript of the human β-subunit is marker by an asterisk.
protein. This was verified by Ca\(^{2+}\) efflux assays using reconstituted channels that were purified by immunoaffinity chromatography. The purified channel complex which consists of the 63 kDa \(\alpha\)-subunit and the 240 kDa channel associated protein is devoid of other contaminating calmodulin binding proteins as analyzed by iodinated calmodulin binding on Western blots. The reconstituted channel displayed a similar decrease in the affinity of the channel for cGMP in the presence of calmodulin.

Attempts were made to inhibit the interaction between calmodulin and the channel complex by either direct competition with anti-240 kDa protein monoclonal antibodies or by mild proteolytic cleavages to remove the calmodulin binding site. In Western blotting experiments, none of the available anti-240 kDa protein monoclonal antibodies were able to inhibit calmodulin binding to the 240 kDa protein. This suggests that the epitopes for these antibodies are distant from the calmodulin binding site. Digestion of the ROS membranes with chymotrypsin not only cleaved the 240 kDa protein, but it also removed the C-terminal end of the \(\alpha\)-subunit, rendering the channel inactive. This suggests that the C-terminal segment of the channel may be important for the channel activity. Digestion with trypsin, on the other hand, cleaved the 240 kDa protein and the N-terminal end of the channel. The trypsinized channel complex was functionally active and showed increased affinity for cGMP. However, it is not known whether this increased affinity is the result of the cleavage of the 240 kDa protein or the N-terminus of the channel \(\alpha\)-subunit. In addition, the trypsinized channel complex still displayed calmodulin sensitivity but with a decreased affinity for calmodulin. Calmodulin affinity chromatography of the trypsinized ROS proteins yielded a 70 kDa fragment of the 240 kDa protein and the degraded 59 kDa \(\alpha\)-subunit. The 70 kDa fragment, however, was not labelled by iodinated calmodulin on the Western blot. This is likely the result of the decreased affinity of the proteolyzed 70 kDa fragment for calmodulin. It will be interesting in the future to determine the N-terminal sequence of this 70 kDa fragment. Digestion of the ROS membranes with kallikrein appeared to cleave only the 240 kDa protein. The digested channel complex
after reconstitution, was still functionally active. Purification of the kallikrein-treated channel complex by an anti-channel antibody column yielded a polypeptide of 105 kDa that binds calmodulin and the intact 63 kDa channel α-subunit.

Amino terminal sequence analysis of the 105 kDa protein gave a sequence that showed a high degree of homology with the recently cloned human β-subunit of the cGMP-gated channel (Chen et al., 1993). Sequences corresponding to the human β-subunit of the cGMP-gated channel have also been detected earlier from the peptide mapping analysis of the bovine 240 kDa protein (Illing and Molday, unpublished data). Detection of the β-subunit within the 240 kDa protein is in agreement with the finding of Brown et al. (1993), indicating that the 240 kDa protein as well as the 63 kDa α-subunit is labelled by a cGMP photoaffinity derivative.

The longer transcript of the β-subunit is likely to be the predominant form expressed in bovine ROS as suggested by the N-terminal sequence analysis of the 105 kDa fragment of the 240 kDa protein. The full length cDNA of the human β-subunit encodes a protein of 102 kDa. Thus, it is possible that the β-subunit is only a component of the 240 kDa protein. Analysis of the human β-subunit revealed a stretch of 20 amino acids close to N-terminus of the channel (a.a. 283-302) as a potential calmodulin binding site. This stretch contains a large number of positively charged residues. The hydrophobic residues within this stretch repeat with a 3 to 4 residue period to form an amphiphilic helix.

This β-subunit can be regarded as the regulatory component of the channel complex. Based on the observation that the 63 kDa α-subunit of the channel is present in more or less similar amounts as the 240 kDa protein (Molday et al., 1990), each channel complex potentially consists of a 240 kDa polypeptide (or one β-subunit) associated with 3 or more α-subunits. Upon binding to Ca$^{2+}$-calmodulin, it is possible that the β-subunit may undergo a conformational change, leading to a decrease in the overall affinity of the channel complex for cGMP. Under conditions of low intracellular Ca$^{2+}$, the dissociation of calmodulin from the β-subunit will then result in an increase in the affinity of the
channel complex for cGMP. Thus, the Ca$^{2+}$-dependent interaction of calmodulin with the β-subunit has the effect of increasing the sensitivity of the channel to cGMP and allows it to readily respond to fluctuations in cGMP concentration between dark and light conditions.
SUMMARY

Photobleaching of rhodopsin in rod photoreceptors activates the visual cascade system leading to a decrease in the cGMP concentration and the closure of cGMP-gated channels in the rod outer segment plasma membrane. Calcium and Ca\(^{2+}\)-binding proteins play important roles in the recovery of the rod cell to its dark resting state by regulating the activation of phosphodiesterase and the resynthesis of cGMP by guanylate cyclase (Kawamura and Murakami, 1991; Kawamura, 1993; Koch and Stryer, 1988). The regulation of phosphodiesterase is mediated through a 26 kDa protein, recoverin/S-modulin, which modulates the level of rhodopsin phosphorylation and ultimately affects the rate of phosphodiesterase activation (Kawamura and Murakami, 1991; Kawamura, 1993). Another Ca\(^{2+}\)-binding protein, originally thought to be recoverin, is responsible for modulating the guanylate cyclase activity in an as yet undefined manner (Koch and Stryer, 1988; Hurley et al., 1993).

This thesis describes the detection and purification of calmodulin from bovine photoreceptor ROS and the identification of the cGMP-gated channel complex as the major calmodulin binding protein in ROS membranes. In addition, by a variety of assay methods using extruded ROS membrane vesicles and reconstituted cGMP-gated channel complex, calmodulin was shown to increase the apparent Km of the channel for cGMP (Table V). This effect has been shown to be mediated through the 240 kDa channel associated protein. The effect was observed over a physiological Ca\(^{2+}\) concentration range of 20-300 nM. This suggests that changes in cytoplasmic Ca\(^{2+}\) concentration during the photoresponse can affect the activity of the cGMP-gated channel. The channel is opened in response to the cooperative binding of cGMP to the channel subunits. The affinity of the channel for cGMP can be further modulated by the level of cytoplasmic Ca\(^{2+}\) through the binding of Ca\(^{2+}\)-calmodulin to the channel complex.
<table>
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<th>Hill Coeff.</th>
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<th>Hill Coeff.</th>
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Table V

Summary of the calmodulin modulation of the cGMP-gated channel complex as determined by various assay systems.
The Ca\textsuperscript{2+}-calmodulin effect on the cGMP-gated channel can be incorporated into the current model for photoexcitation and photorecovery (Stryer, 1986; Chabre and Deterre, 1989; Pugh Jr. and Lamb, 1990; Kaupp and Koch, 1992). In the dark, the free cGMP level in ROS, estimated to be about 4-10 \( \mu \text{M} \), maintains a small, but significant number of channels in their open state (Nakatani and Yau, 1988a), allowing for the influx of Na\textsuperscript{+} and Ca\textsuperscript{2+} (Fig. 47a). Under these conditions, cytoplasmic Ca\textsuperscript{2+} is maintained at a relatively high concentration (approximately 0.3 \( \mu \text{M} \)) by balancing the influx of Ca\textsuperscript{2+} through the channel with the efflux of Ca\textsuperscript{2+} through the Na\textsuperscript{+}/Ca\textsuperscript{2+}-K\textsuperscript{+} exchanger. Under these conditions, guanylate cyclase is maintained at its basal level of activity and the cGMP-gated channel is in its low affinity (high Km) state for cGMP through Ca\textsuperscript{2+}-calmodulin binding to the 240 kDa channel-associated protein. Photoexcitation leads to the activation of phosphodiesterase and a decrease in cGMP levels (Fig. 47b). The decrease in total cGMP has been reported to be only 10-15\% (Cote et al., 1986), but the decrease in free cGMP has not yet been measured. Since the channel is in its low affinity state, it may be positioned on the cGMP dose-response curve to be sensitive to a small decrease in free cGMP concentration, thus, facilitating its closure. A decrease in cytoplasmic Ca\textsuperscript{2+} levels (estimated to be below 100 nM) will occur as a result of the closure of the channel and the continuous extrusion of Ca\textsuperscript{2+} through the Na\textsuperscript{+}/Ca\textsuperscript{2+}-K\textsuperscript{+} exchanger. This decrease in Ca\textsuperscript{2+} will activate guanylate cyclase (Koch and Stryer, 1988) and limit PDE activation (Kawamura and Murakami, 1991). Under these conditions, calmodulin will also dissociate from the channel complex and cause the channel to switch to its high affinity state for cGMP. The channel will now reopen at a lower cGMP level thus facilitating the recovery of the ROS to its dark level as cGMP synthesis proceeds. The opening of the channel will in turn restore the Ca\textsuperscript{2+} level to its dark level, leading to the inactivation of guanylate cyclase and the return of the channel to its low affinity state (Fig. 47c). Thus, reversible binding of calmodulin to the channel complex in response to
a
1 ↑ cGMP
2
3 ↑ Ca²⁺
4 ↓ G.C.

GTP → cGMP

High Km

b
1 ↑ cGMP
2
3 ↑ Ca²⁺
4 ↓ G.C.

GTP → cGMP

High Km → Low Km

c
1 ↑ cGMP
2
3 ↑ Ca²⁺
4 ↓ G.C.

GTP → cGMP

Low Km → High Km

cGMP-gated channel complex
Na/Ca-K exchanger
Calmodulin
Fig. 47: Possible role for calmodulin modulation of the channel during the visual transduction process.

a. In the dark, an elevated level of cGMP ① maintains a significant number of cGMP-gated channels in their open state and allows the influx of Na⁺ and Ca²⁺ into the outer segment ②. The balanced influx of Ca²⁺ through the channel with the efflux of Ca²⁺ through the Na⁺/Ca²⁺-K⁺ exchanger results in a relatively high level of Ca²⁺ within the outer segment ③. The high level of Ca²⁺ results in the association of calmodulin to the channel complex and maintains the channel in its low affinity state for cGMP ④. The channel, in its low affinity state, will respond to a decrease in the level of cGMP during photoexcitation of the outer segment. This high level of Ca²⁺ will also deactivate the modulator of guanylate cyclase and minimize the stimulation of guanylate cyclase activity ④.

b. Photobleaching of rhodopsin results in the activation of phosphodiesterase and a decrease in the level of cGMP ①. This causes the closure of the cGMP-gated channel ② and a decrease in intracellular Ca²⁺ ③ due to the continuous extrusion of Ca²⁺ by Na⁺/Ca²⁺-K⁺ exchanger. This drop in Ca²⁺ will cause calmodulin to dissociate from the channel complex and shift the channel from its low affinity state to its high affinity state for cGMP ④. The low level of Ca²⁺ will also activate guanylate cyclase through a Ca²⁺ sensitive modulator ④. The combined effect of resynthesizing cGMP and making the channel more sensitive to cGMP levels would facilitate the recovery of the outer segment to its dark level.

c. As the channels reopen due to a rise in cGMP level ①②, the Ca²⁺ level in the outer segment is restored ③. This results in the rebinding of calmodulin to the channel and conversion of the channel to its low affinity state ④. Guanylate cyclase is also restored to its basal level of activity ④.
changes in the level of Ca\(^{2+}\) has the effect of increasing the sensitivity of the channel to small changes in cGMP which may occur during the photoresponse.

Light adaptation characterized by a decrease in rod sensitivity in the presence of background illumination has been shown to be mediated by Ca\(^{2+}\) (Torre et al., 1986; Nakatani and Yau, 1988b). Reduced Ca\(^{2+}\) induced under conditions of light adaptation will result not only in an increase in cGMP (Koch and Stryer, 1988), but also in a higher affinity of the channel for cGMP, thus resulting in the observed reduction in the flash sensitivity characteristic of light adaptation. On this basis, Ca\(^{2+}\) may regulate photorecovery and light adaptation not only by regulating guanylate cyclase activity and PDE activation (Koch and Stryer, 1988; Kawamura and Murakami, 1991), but also by modulating the affinity of the channel for cGMP. In addition to the visual system, a similar calcium mediated regulation of the cAMP-gated channels has been reported in the olfactory system (Kramer and Siegelbaum, 1992). It is possible that this calcium mediated modulation of the cyclic nucleotide gated channels is not limited to the visual and olfactory signal transduction systems. This regulation may potentially be an important component in the auditory and gustatory signal transduction pathways as well.

The identity of the 240 kDa channel associated protein has been under study. In addition to the \(\beta\)-subunit recently identified by Chen et al. (1993), the 240 kDa protein also contains a glutamic acid rich protein (Illing, Williams, Colville, and Molday, unpublished data) that had been cloned and sequenced earlier by Sugimoto et al. (1991). This glutamic acid rich protein (GARP) is potentially covalently linked to the \(\beta\)-subunit to give an apparent Mr 240 K by SDS polyacrylamide gel electrophoresis. Since the N-terminal sequence analysis of the 105 kDa calmodulin binding fragment of the 240 kDa protein does not contain the sequence of this glutamic acid rich protein, this protein is likely to be binding to an N-terminal stretch of the \(\beta\)-subunit upstream of the kallikrein cleavage site. Thus, the cGMP-gated channel complex can be envisioned as composed of three \(\alpha\)-subunits associated tightly with one or more \(\beta\)-subunits (Fig. 48). This \(\beta\)-subunit,
Fig. 48: Schematic model of the cGMP-gated channel complex.

The cGMP-gated channel complex can be envisioned as a pentameric complex consisting of 3 α-subunits, one β-subunit, and one glutamic acid rich protein. The glutamic acid rich protein is associated with the β-subunit, possibly by covalent linkage. Calmodulin binds to the cGMP-gated channel complex in a Ca\textsuperscript{2+}-dependent manner through the β-subunit.
which has the glutamic acid rich protein attached to it, interacts with calmodulin in a Ca\(^{2+}\)-dependent manner. It will be interesting to determine the type of interaction between the \(\beta\)-subunit and the glutamic acid rich protein as well as the stoichiometric ratios of individual components of the cGMP-gated channel complex.
REFERENCES


