

MOLECULAR CHARACTERIZATION OF THE CYCLIC NUCLEOTIDE-GATED
CATION CHANNEL OF BOVINE ROD OUTER SEGMENTS

by

ANDRÉA CHRISTINA DOSÉ

B.Sc., San Francisco State University, 1986

M.Sc., San Francisco State University, 1990

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Department of BIOCHEMISTRY AND MOLECULAR BIOLOGY

The University of British Columbia
Vancouver, Canada

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ABSTRACT

The cyclic nucleotide-gated (CNG) cation channel of rod photoreceptors plays an important role in the perception of light. The channel gating is the final step in the visual transduction cascade. The cloning (Kaupp *et al.*, 1989) of what is now known to be the CNG channel α subunit facilitated its molecular characterization. A heterologous expression system was developed to examine molecular aspects of the α and β subunits of the CNG channel. Expression in mammalian cells helped resolve confusion regarding the M_r of the rod CNG channel α subunit. Although the cDNA codes for an 80 kDa protein, the α subunit exists in the rod outer segment as a 63 kDa protein suggesting the existence of a processing mechanism. PCR was used to construct cDNA clones which code for both the 63 and 80 kDa forms of the α subunit of the rod CNG channel. A polyclonal antibody, generated to specifically label the 80 kDa α subunit, was used in conjunction with a monoclonal antibody specific for the 63 kDa α subunit in labeling studies. These antibodies were shown to be mono-specific, capable of differentiating between the two size forms of the α subunit. Immunohistochemical studies demonstrated that the 63 kDa α subunit is the predominant species in the rod outer segment while the 80 kDa form is present in very low quantities. Rod outer segment (ROS) purification on sucrose gradients also showed that the 80 kDa form co-sediments with the 63 kDa α subunit indicating that the two size forms coexist in the outer segment. Examining the α subunits of the photoreceptors of another species demonstrated similar processing of the CNG α subunit polypeptides, however, an olfactory α subunit did not appear to undergo similar processing.

The cloned CNG channel β subunit cDNA codes for a protein with a predicted molecular mass of 155 kDa (Körschen *et al.*, 1995). Heterologous expression of the β subunit cDNA yielded a 240 kDa protein, positively identifying the 240 kDa protein in ROS which co-purifies with the α subunit as the CNG β subunit. Construction and

expression of a truncated form of the β subunit demonstrated that it is the glutamic acid-rich N-terminal portion of the β subunit that is responsible for its anomalous migration on an SDS gel. Co-expression of both subunits in mammalian cells indicated that the β subunit was not responsible for the processing of the α subunit. As seen for the subunits of the native CNG channel the heterologously expressed α and β subunits co-immunoprecipitated. The subunit interaction was not dependent on the 92 N terminal amino acids of the 80 kDa α subunit or on the glutamic acid-rich portion of the β subunit. A method for reconstitution of the heterologously expressed CNG channel was also developed. The expressed α subunit reconstituted alone did not generate functional cGMP-gated channels. Reconstitution of the heterologously expressed channel complex comprising the 80 kDa α and complete β subunits generated functional channels. Compared to the native CNG channel, 10% of the heterologously expressed channel complex exhibited cGMP-gated channel activity. This is the first example of reconstitution of a heterologously expressed cation channel into lipid vesicles for Ca^{2+} efflux measurements. The system presented here will be useful to further define CNG α and β subunit interactions and to carry out structure-function studies on the channel using a biochemical efflux assay.

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LIST OF ABBREVIATIONS

ATP.....	adenosine 5'-triphosphate
BCA.....	bicinchoninic acid
BSA.....	bovine serum albumin
cAMP.....	adenosine 3',5'-cyclic monophosphate
cGMP.....	guanosine 3',5'-cyclic monophosphate
CHAPS....	(3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
CMV.....	cytomegalovirus
CNG.....	cyclic nucleotide-gated
DAB.....	3',3'-diaminobenzidine
DEAE.....	diethylaminoethyl
DMEM.....	Dulbecco's modified Eagle medium
DTT.....	dithiothreitol
ECL.....	enhanced chemiluminescence
EDTA.....	ethylenediamine tetraacetic acid
FBS.....	fetal bovine serum
GABA.....	γ -amino-butyric acid
GARP.....	glutamic acid-rich protein
GTP.....	guanosine 3'-triphosphate
GDP.....	guanosine 3'-diphosphate
HBS.....	Hepes buffered saline
HBSG.....	glucose supplemented Hepes buffered saline
h/e.....	heterologously expressed
Ig.....	immunoglobulin
kb.....	kilobase
kDa.....	kilodalton
LB.....	Luria broth

M _r	relative molecular weight
MEM.....	minimum essential medium
PAGE.....	poly acrylamide gel electrophoresis
PBS.....	phosphate buffered saline
PCR.....	polymerase chain reaction
PDE.....	phosphodiesterase
PMSF.....	phenyl methyl sulfonyl fluoride
RIS.....	rod inner segment
ROS.....	rod outer segment
rpm.....	rotation per minute
SDS.....	sodium dodecyl sulfate
RPE.....	retinal pigment epithelium
Tris.....	tris [hydroxymethyl]aminomethane

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who believed in me even when I didn't.*

INTRODUCTION

1.1 The retina

Light entering the eye must pass through the cornea, the aqueous humor, the lens, and the vitreous chamber before it reaches the retina (**fig 1**). It is the retina that effects the conversion of light into a neurological signal. The retina is the innermost of three tissue layers forming the rear shell of the eyeball. The tough outermost coat is the sclera which protects the delicate inner structures of the eye. The layer just inside the sclera is the choroid, a vascular layer which provides nutrients to ocular tissues. Next is the retinal pigment epithelium and finally the retina which is composed of a highly specialized group of seven different neural cell types. These are the rod photoreceptors, the cone photoreceptors, the bipolar cells, the horizontal cells, the amacrine cells, the ganglion cells, and the interplexiform cells (**fig 2**) (Adler and Farber, 1986).

The bipolar cells synapse with the rods and cones as do the horizontal cells. The bipolar cells play a major role in vertical transmission of photosignals, and horizontal cells mediate lateral interactions between these photoreceptor-bipolar synapses. The ganglion cells synapse with the bipolar cells, and their axons make up the optic nerve. The amacrine cells mediate lateral interactions at the level of bipolar-ganglion synapses, and the interplexiform cells which are morphologically similar to amacrine cells can be presynaptic to both bipolar and horizontal cells and postsynaptic to amacrine cells. Among these cells are found the Müller (or glial) cells which are not considered to be neurons. They receive nutrients from the retinal blood supply and provide them to the retinal neurons. Light passes through these inner retinal layers before reaching the photoreceptors (Adler and Farber, 1986).

The rod and cone cells are the photon receptors, which lie adjacent to the retinal pigment epithelium (RPE), a cellular lining which separates the retina from the blood capillaries of the choroid. Rods and cones are long slender cells. Mammalian rods are

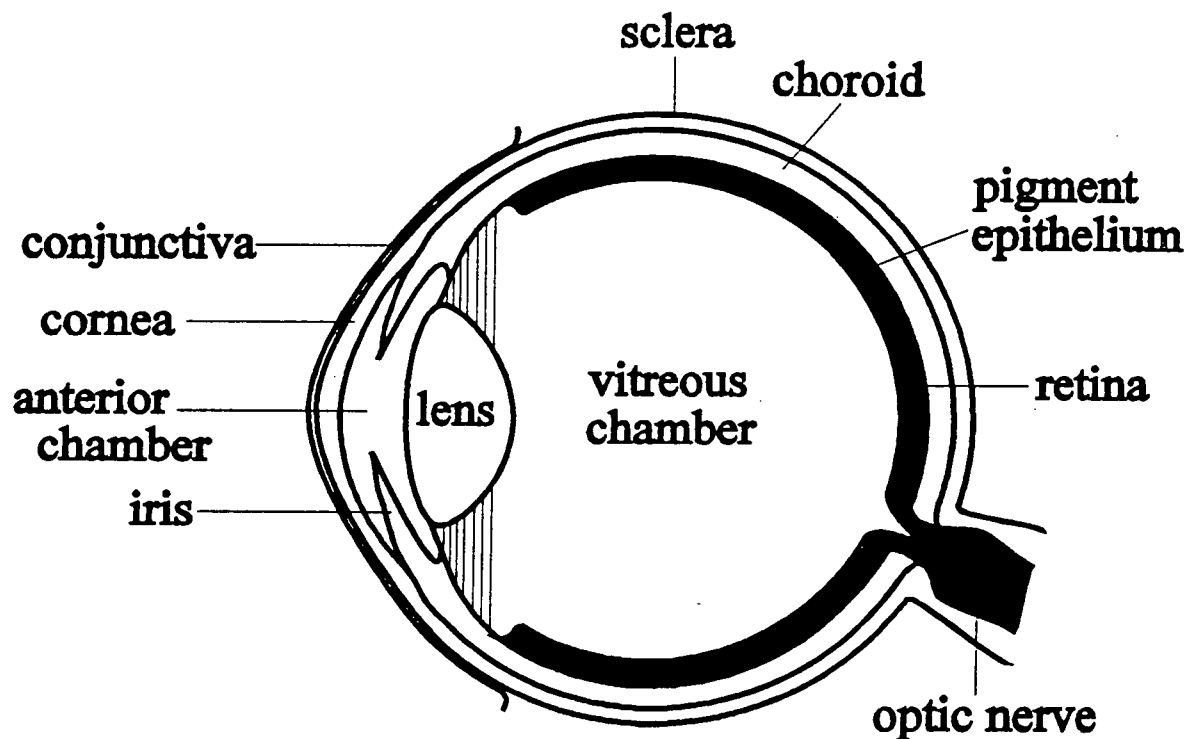


FIG. 1. Diagram of eye in cross section. The front of the eyeball is surrounded by the conjunctiva and the cornea. The cornea is contiguous with the sclera, which surrounds the back of the eyeball. Interior to the sclera at the rear portion of the eye is the choroid layer and the retina. The choroid is a vascular layer which provides nutrients to the retina and the retinal pigment epithelium (RPE). The RPE lies between the choroid and the retina. The retina is the innermost layer and is composed of the photoreceptor cells and other nerve cells.

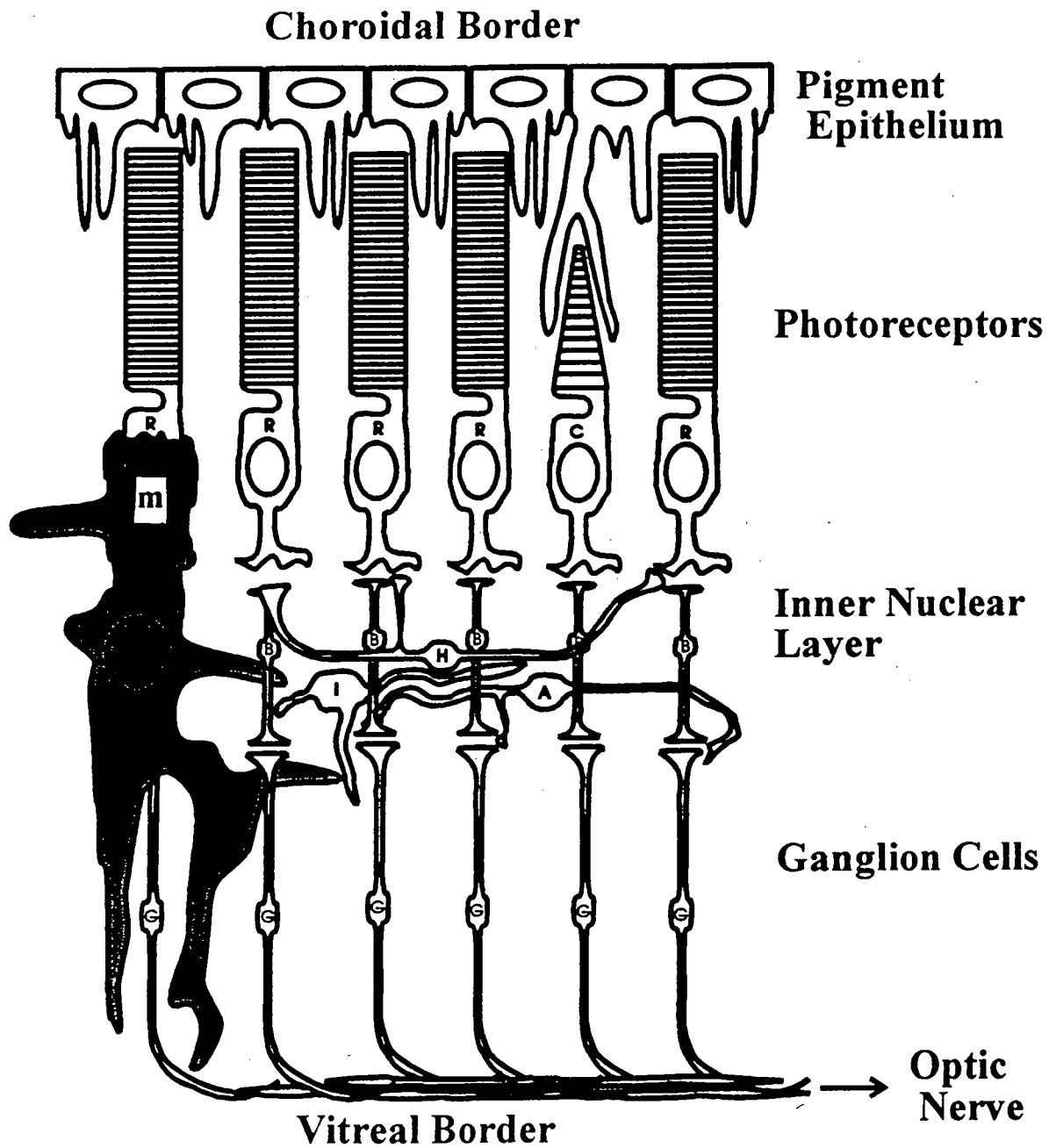


FIG. 2 Diagram of the neuroretinal cells and their synaptic relationships. Cell types are designated as R, rods; C cones; H, horizontal cells; I interplexiform cells; A amacrine cells; B, bipolar cells; G, ganglion cells; M, Müller cells.

1-3 μm in diameter and 40-60 μm in length and cones are 1-1.5 μm wide and 75 μm long. Rods function primarily as photoreceptors for dim light or night vision, and are more predominant towards the periphery of the retina. Cones serve as the photoreceptors for color vision, and are concentrated in the central region of the retina. The human eye contains ~120 million rods, and 6.5 million cones. A similar ratio exists in retinas of cows. Therefore, rod cells have been more extensively studied. It is this cell system that is the focus of this research (Shichi, 1983).

The rod cell can be divided into three regions, the synaptic terminus, the inner segment and the outer segment (**fig 3**). The synaptic terminus which synapses with the bipolar and horizontal cells is the site of release of the neurotransmitter glutamate. Glutamate is continually released until the cell is stimulated by light. Stimulation causes a decrease in neurotransmitter release which initiates the neural signal. The inner segment contains the nucleus, mitochondria, and other subcellular organelles. Outer segments are connected to the inner segments by a narrow cilium ~0.25 μm in diameter. Metabolic fuels and newly synthesized proteins are transported through the connecting cilium to the outer segment. The outer segment is highly specialized for its particular function. It is composed of a stack of thousands of membranous disks surrounded by a plasma membrane. The outer segment contains all the proteins responsible for the visual transduction cascade (Shichi, 1983).

1.2 Visual transduction cascade

A schematic diagram of the visual transduction cascade is presented in figure 4. The photopigment in rods is rhodopsin which constitutes more than 90% of the total protein in each disk. Rhodopsin is an integral membrane protein with 7 transmembrane helices and is chemically linked to the chromophore 11-*cis*-retinal at lysine 296. The primary photochemical event in visual transduction is a light-induced isomerization of the rhodopsin chromophore. After photoisomerization, rhodopsin undergoes a series of

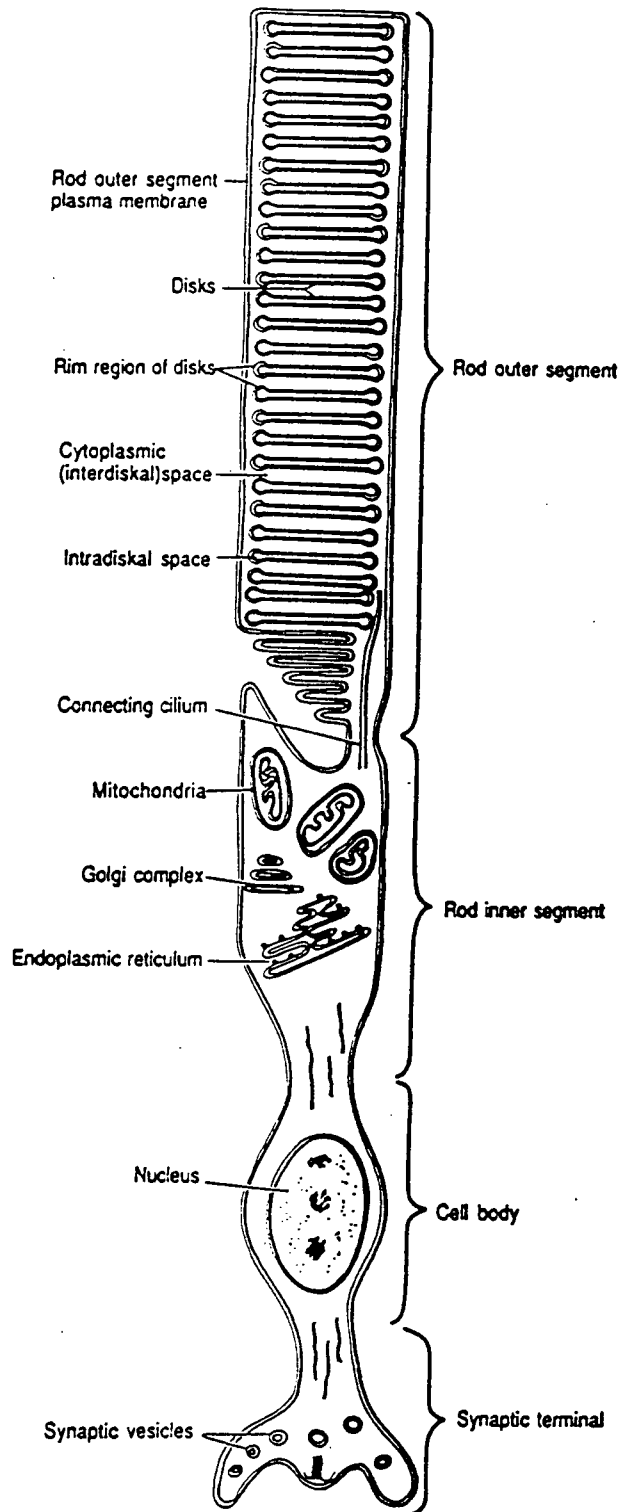


FIG. 3 The rod cell. The rod cell is a highly specialized nerve cell. The outer segment is composed of stacks of membranous disks surrounded by a plasma membrane. The inner segment contains the cellular organelles, and the synaptic terminus synapses with the bipolar and horizontal cells of the retina.

FIG. 4. The molecular mechanism of the phototransduction cascade. STEP 1: A photon of light is captured by the chromophore in rhodopsin which initiates rearrangement through several intermediates to metarhodopsin (M_I) and metarhodopsin II (M_{II}). The inactivation of rhodopsin is believed to involve its phosphorylation by rhodopsin kinase (K_{RH}) followed by capping by 48 kDa protein (alias arrestin, 48K) STEP 2: Metarhodopsin II binds to transducin ($T_\alpha \cdot GDP$), catalyzes the exchange of GTP for GDP on the α subunit of transducin and the transducin subunits dissociate. STEP 3: Two α subunits of transducin ($T_\alpha^* \cdot GTP$) bind to phosphodiesterase (PDE) relieving an inhibitory constraint imposed by PDE_γ resulting in the activation of PDE . Inactivation of PDE is thought to involve the hydrolysis of the terminal phosphate of $T_\alpha \cdot GTP$ which then dissociates from PDE and reassociates with $T_{\beta\gamma}$. STEP 4: Activated PDE hydrolyses cGMP, changes in cGMP concentration (buffered by non-catalytic binding sites) directly regulate the CNG channel in the plasma membrane. The closure of the CNG channel and the continued extrusion of Ca^{2+} through the $Na^+/Ca^{2+}, K^+$ exchanger leads to a decrease in the intracellular Ca^{2+} concentration and subsequently, activation of guanylate cyclase (G.C.) The guanylate cyclase activity is regulated by a calcium-binding protein (M^*).

rapid transitions and is converted to metarhodopsin II (Hargrave and McDowell, 1992). Metarhodopsin II is a relatively stable intermediate which activates transducin, the G protein in the cascade, thereby initiating the phototransduction cascade. Transducin is a heterotrimeric membrane bound protein complex (abbreviated as $T_{\alpha}T_{\beta\gamma}$) (Hargrave *et al.*, 1991; Kahlert and Hofmann, 1991). It contains an α subunit (T_{α} , 39 kDa), that is weakly associated with a $\beta\gamma$ heterodimer ($T_{\beta\gamma}$, 36 and 8 kDa respectively). Guanine nucleotides bind to the α subunit of transducin (Fung *et al.*, 1981). When metarhodopsin II binds to transducin-GDP, GDP is replaced by GTP. Metarhodopsin II is then released, followed by the dissociation of transducin into its subunits, T_{α} -GTP and $T_{\beta\gamma}$ (Pugh and Lamb, 1993). The signal is amplified at this step because each metarhodopsin II can catalyze the binding of GTP to several hundred T_{α} subunits during its active lifetime (Fung *et al.*, 1981).

The next step in the cascade involves the activation of phosphodiesterase (PDE) by T_{α} -GTP. PDE is a peripheral membrane protein consisting of a large heterodimer complex ($PDE_{\alpha\beta}$, 99.2 and 98.3 kDa) which is bound tightly by two identical inhibitory low molecular weight subunits (PDE_{γ} , 9.7 kDa) (Baehr *et al.*, 1979; Hurley and Stryer, 1982; Deterre *et al.*, 1988). T_{α} -GTP and PDE contact each other at the disk membrane surface and two T_{α} -GTP molecules bind tightly to PDE_{γ} , relieving the inhibitory constraint that the PDE_{γ} subunits impose on the α and β catalytic subunits.

The acceleration of PDE activity results in a drop in the cytoplasmic concentration of cGMP which results in the closure of the cGMP-gated cation channel in the plasma membrane. The channel is a heteromultimer composed of a 63 kDa α subunit and a 240 kDa β subunit (Kaupp *et al.*, 1989; Chen *et al.*, 1993; Illing *et al.*, 1994). In the dark, when the channel is open, the current through the channel has a Na^{+} component (about 80%), a Ca^{2+} component (about 15%), and a Mg^{2+} component (about 5%). Based on extracellular concentrations, the relative permeabilities are: $Na^{+}:Ca^{2+}:Mg^{2+} \sim 1:12:2.5$ (Nakatani and Yau, 1988). Before light activation there exists a balance (Miller and

Korenbrod, 1993) between the Ca^{2+} influx through the cGMP-gated channel and the efflux through the $\text{Na}^+/\text{Ca}^{2+}\text{-K}^+$ exchanger, a 230 kDa membrane bound exchanger that also resides in the plasma membrane (fig 5). When the cGMP concentration drops, the channel closes preventing the entry of Ca^{2+} and other cations. The continued activity of the exchanger lowers the concentration of Ca^{2+} in the outer segment (Yau and Nakatani, 1985; Nakatani and Yau, 1988). Free cytosolic Ca^{2+} in dark adapted rods is estimated at ~ 300 nM and upon closure is reduced to ~ 70 nM (Rayer *et al.*, 1990). An inactivation mechanism may prevent the exchanger from lowering cytosolic free Ca^{2+} much below 100 nM (Schnetkamp, 1991).

The light response is followed by inactivation of proteins stimulated in the cascade. Metarhodopsin II initiates its own phosphorylation by stimulating rhodopsin kinase (Palczewski *et al.*, 1991b). Phosphorylated metarhodopsin II is a less potent stimulator of transducin, and is also bound by arrestin (Palczewski *et al.*, 1991a). An important inactivation mechanism is the lowering of free Ca^{2+} in the rod outer segment. Low Ca^{2+} concentrations play a role in deactivating the light-stimulated PDE by relieving the inhibitory effects of S-modulin/recoverin on metarhodopsin II phosphorylation. Low Ca^{2+} concentrations also stimulate the synthesis of cGMP by guanylate cyclase. In the dark, Ca^{2+} /calmodulin is bound to the CNG channel. Upon illumination, the decrease in Ca^{2+} results in the dissociation of calmodulin from the channel. Without Ca^{2+} /calmodulin bound, the channel affinity for cGMP increases. Thus when the cGMP concentration is low, the channel is more sensitive to changes in the amount of cGMP present and capable of detecting the small changes in free cGMP concentration (Hsu and Molday, 1993).

The olfactory signal transduction cascade strongly parallels the cascade in retinal rod cells (fig 6). Molecules, as opposed to photons, are perceived by a large family of odorant receptors in the cilia of the olfactory neuroepithelium (Buck and Axel, 1991).

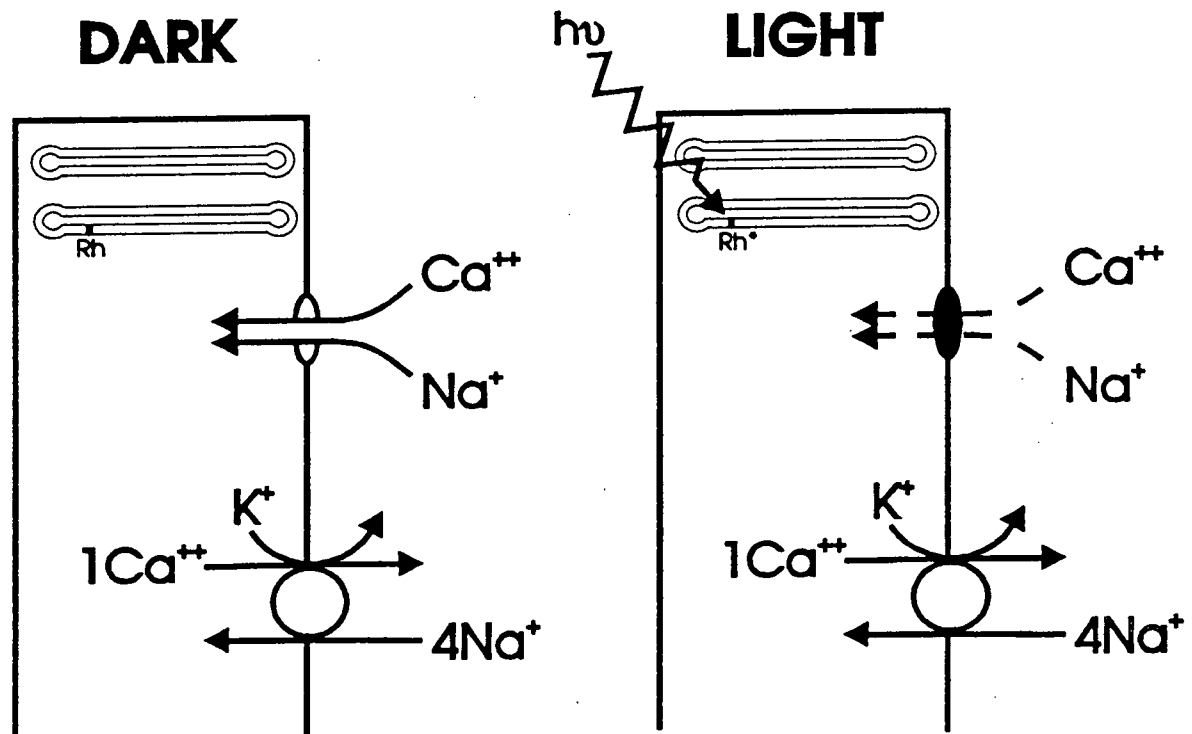


FIG. 5. Diagram depicting the behavior of Ca^{2+} fluxes across the outer segment plasma membrane in darkness and light. In the dark, the Ca^{2+} influx through the CNG channel is balanced by a Ca^{2+} efflux through the Na^+ , Ca^{2+} , K^+ exchanger. In light, upon closure of the CNG channel, the influx of Ca^{2+} is prevented and the result is a decrease in the intracellular Ca^{2+} concentration. Rh, rhodopsin molecule; Rh^* , photoisomerized rhodopsin molecule; $h\nu$, photon. Dashed lines represent terminated ion flux.

The receptors are members of the seven transmembrane helix superfamily which includes rhodopsin. These receptors couple to a G protein, G_{olf} , the α subunit of which has been cloned (Jones and Reed, 1989). Odorant binding stimulates G_{olf} which in turn stimulates adenylate cyclase (Pace *et al.*, 1985). The elevation of the cAMP concentration opens a membrane conductance in olfactory cilia (Nakamura and Gold, 1987). The olfactory cyclic nucleotide-gated channel is a heteromultimer of two known subunits, an α and a β subunit (Dhallan *et al.*, 1990; Liman and Buck, 1994). These subunits show significant sequence similarity to the α and β subunits of rod CNG channels and are considered to be homologous proteins in the cascade (Liman and Buck, 1994).

1.3 Brief overview of selected ion channels

In the late 1980's there existed two general categories of membrane channels, namely, voltage-gated and ligand-gated channels. Voltage-gated channels open in response to voltage differences across a membrane, and ligand-gated channels open as a result of the binding of a ligand. The voltage-gated channel family consists of K^+ , Na^+ , Ca^{2+} , and Cl^- channels. The ligand-gated channel family includes the nicotinic acetylcholine receptor, glycine receptors, GABA receptors, and glutamate receptors. The cyclic nucleotide-gated channels are gated by a ligand, but have structurally they been shown to resemble voltage-gated channels, specifically the K^+ channels (Jan and Jan, 1990, Heginbotham *et al.*, 1992; Goulding *et al.*, 1992). A brief overview of voltage-gated cation channels will be discussed here focusing mainly on their quaternary and tertiary structure.

1.3.1 Sodium channels

The sodium channel field is extensive with biochemical characterizations and cloning studies being carried out in organisms from *D. melanogaster* and *E. electricus* to rats and humans, and in a variety of different tissues. Only a few representative channels will be discussed here.

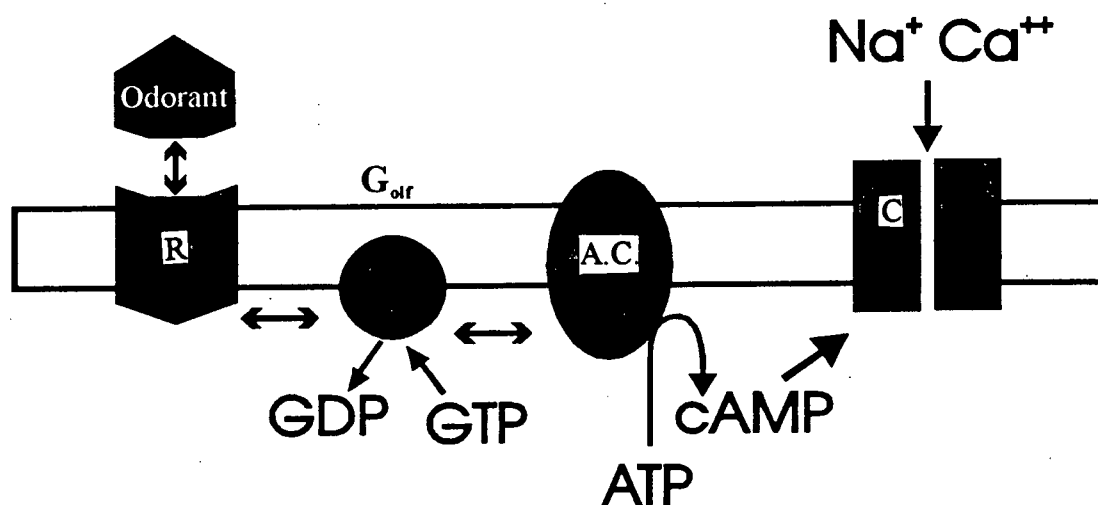


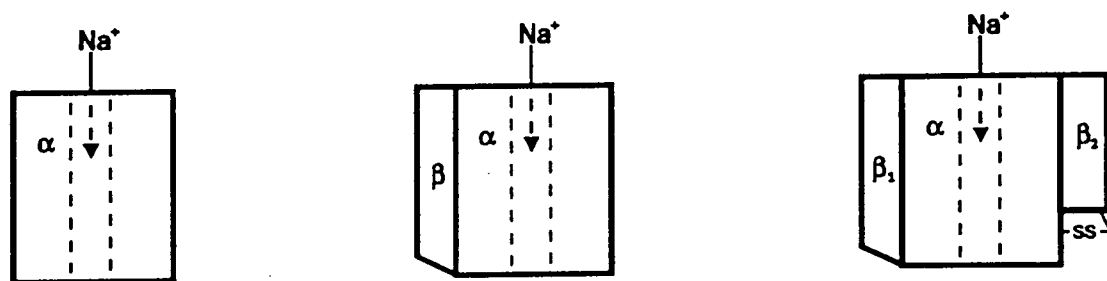
FIG. 6. A schematic model for the mechanism of odorant signal transduction. Receptors (R) in the olfactory neuroepithelium bind odorants. These receptors couple to a G protein (G_{olf}), which stimulates adenylate cyclase (A.C.). The resultant elevation in cAMP concentration opens a CNG channel (C) in the olfactory epithelium.

Sodium channels are a heterogeneous class consisting of a variable number of subunits (**fig 7A**). The Na⁺ channel from the electric eel *Electrophorus electricus* consists of a large single polypeptide of 230-270 kDa (Agnew *et al.*, 1980; Miller *et al.*, 1983). A Na⁺ channel from rat skeletal muscle was found to consist of two subunits, alpha (α , 260 kDa) and beta (β , 38 kDa) (Kraner *et al.*, 1985). A third Na⁺ channel from mammalian brain was shown to consist of a heteromultimeric complex of three subunits, α (260 kDa), β_1 (36 kDa), β_2 (33 kDa) (Hartshorne *et al.*, 1982), β_2 being covalently linked to the α subunit by disulfide bonds (Messner and Catterall, 1985). Using antibodies and protein sequence information, the α subunits have been cloned and sequenced from eel electroplax membranes (Noda *et al.*, 1984), rat skeletal muscle (Trimmer *et al.*, 1989), and rat brain (Noda *et al.* 1986; Kayano *et al.*, 1988) just to name a few.

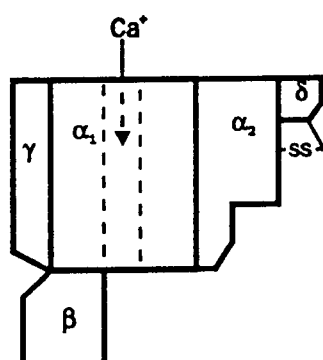
Reconstitution of partially purified voltage-sensitive sodium channels from rat brain, rat skeletal muscle and eel electroplax organ incorporated into phospholipid vesicles resulted in recovery of ion conductance activity (Tamkun *et al.*, 1984; Kraner *et al.*, 1985, Duch and Levinson, 1987). The existence of only the α subunit in the electroplax organ indicated that the β subunit is not necessary for functional activity. Indeed, expression of the α subunit alone in *Xenopus* oocytes is sufficient for Na⁺ channel activity (Goldin *et al.*, 1986; Noda *et al.*, 1986). Biochemical studies have indicated that the β_1 subunit helps to stabilize the α subunit during purification and reconstitution. High ionic strength or reducing agents can selectively dissociate the β_1 and β_2 subunits respectively from the α subunit of the rat brain Na⁺ channel (Messner and Catterall, 1985). Solubilization and reconstitution of purified rat brain channel, after removal of the β_1 subunit, yields a non-functional channel (Messner *et al.*, 1986). In contrast, removal of the β_2 subunit yields a fully functional channel.

All α subunits of Na⁺ channels appear to have similar topological profiles (**fig 8**). They are large polypeptides of 1800-2000 amino acids which contain 4 repeat domains

A) Na^+ CHANNEL SUBUNIT CONFIGURATIONS:



B) Ca^{2+} CHANNEL SUBUNIT CONFIGURATION



**C) K^+ CHANNEL CONFIGURATION:
channel is made up of 4 subunits**

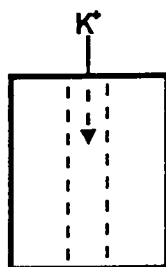


FIG. 7. Subunit configurations for voltage-gated cation channels. The sodium channels exist with one, two or three different subunits. The calcium channels are thought to consist of five different subunits. The potassium channels consist of four similar or identical subunits which make up a core that is similar to the sodium or calcium channel α subunits.

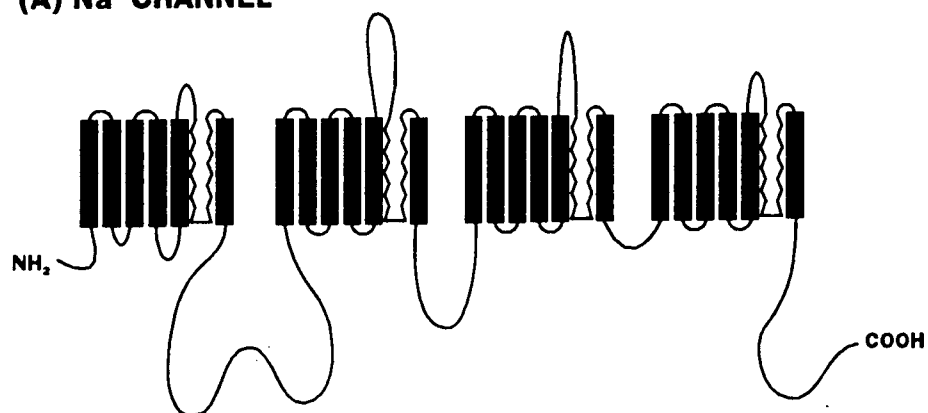
of 300-400 amino acids. Each domain is thought to contain 6 transmembrane helices labeled S1-S6 (Catterall, 1988). Between S5 and S6 is a unique loop that dips into the membrane. This region is thought to be a part of the pore, based on results obtained with the potassium channel (Yool and Schwartz, 1991). The fourth transmembrane segment (S4) of each repeat region has a unique sequence pattern. It is a stretch of predominantly hydrophobic amino acids punctuated every third (or fourth) residue with a positively charged amino acid (either arginine or lysine). First noted in the electroplax channel (Noda *et al.*, 1984), this segment has been proposed to be the voltage sensor of the Na⁺ channel.

1.3.2 Calcium channels

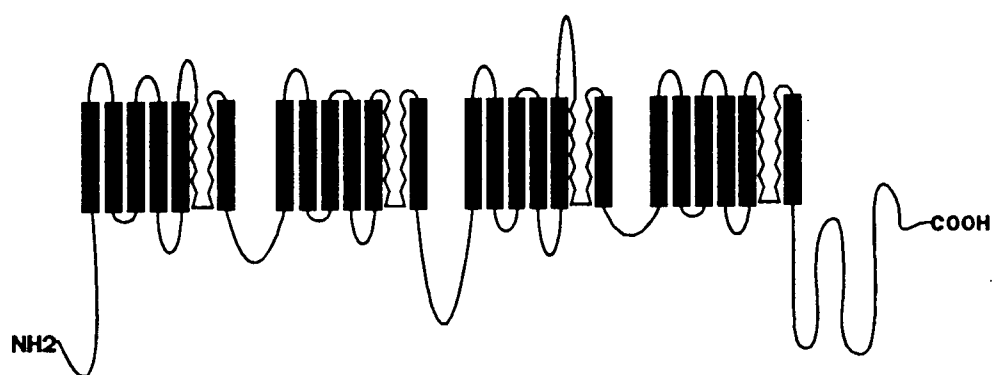
The voltage-dependent calcium channel complex is typically composed of five subunits (**fig 7B**), α_1 (170 kDa), α_2 (140 kDa), β (55 kDa), γ (33 kDa), and δ (24-33 kDa) (Mori, 1994), which exist in 1:1 ratios (Takahashi *et al.*, 1987; Leung, *et al.*, 1988). The α_1 subunit is the major subunit, and contains the channel activity (Noda, 1986). It was first cloned and sequenced in rabbit skeletal muscle (Tanabe *et al.*, 1987). All Ca²⁺ channel α_1 subunits share general structural features with the α subunit of voltage dependent sodium channels. The α_2 subunit was cloned by Ellis and co-workers (1988) and exhibits no similarity to other known protein sequences (Ellis *et al.*, 1988). The α_2 subunit also has the δ subunit linked to it by disulfide bonds (Takahashi *et al.*, 1987). Sequence analysis has indicated that the α_2 subunit and the δ subunit are encoded by the same gene (De Jongh, *et al.*, 1990). The β and γ subunits were also first cloned and sequenced from rabbit skeletal muscle and show no similarity to other known protein sequences (Ruth *et al.*, 1989; Bosse *et al.*, 1990; Jay *et al.*, 1990).

Functional expression in *Xenopus* oocytes has been performed with a number of calcium channels, including the cardiac DHP (dihydropyridine) receptor, smooth muscle

(A) Na⁺ CHANNEL



(B) Ca²⁺ CHANNEL



(C) K⁺ CHANNEL

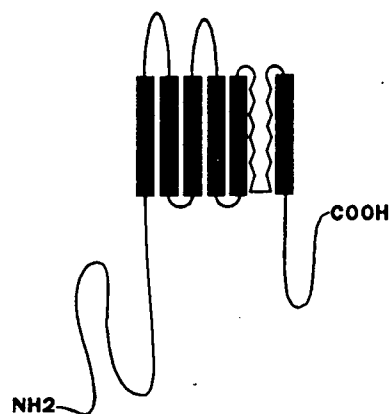


FIG. 8 Predicted topological profiles for voltage-gated cation channels. The Na⁺ and Ca²⁺ channels contain four repeat regions which exhibit sequence similarity. The K⁺ channel sequence is similar to the repeat regions of the Na⁺ and Ca²⁺ channels and is proposed to exist as a tetramer.

L-type calcium channel, and brain non-L-type, non-N-type calcium channel (Mikami *et al.*, 1989; Biel *et al.*, 1990; Mori *et al.*, 1991). The skeletal muscle calcium channel is expressed poorly in *Xenopus* (Nargeot *et al.*, 1992), but microinjection of cDNA into cultured muscle cells from mice with muscular dysgenesis yielded functional calcium channel activity (Tanabe *et al.*, 1988). When skeletal muscle calcium channels from transverse tubules were reconstituted, less than 5% of the channels were active (Curtis and Catterall, 1986)

As mentioned earlier the α_1 subunit of calcium channels exhibits strong sequence similarity to the α subunit of sodium channels and, therefore, have similar predicted membrane topologies (**fig 8**). Ca^{2+} channels contain four repeating units of homology, containing six putative transmembrane segments, the hydrophobic S1, S2, S3, S5, and S6, and a positively charged S4 segment, most likely the voltage sensor. They also contain a putative pore region with highly conserved glutamic acid residues which may be critical for calcium ion selectivity (Heinemann *et al.*, 1992). The α_2/δ subunit complex contains three hydrophobic segments that may constitute transmembrane segments (Ellis *et al.*, 1988). The β subunit is hydrophilic and lacks any typical membrane spanning or membrane associated sequences. The γ subunit has four putative transmembrane domains (Bosse *et al.*, 1990; Jay *et al.*, 1990). The α_2/δ subunits are linked by a disulfide bond, but the points of interaction among the other subunits are not known. Their existence as a complex is supported by co-purification (Curtis and Catterall, 1984; Takahashi *et al.*, 1987) and by co-immunoprecipitation with antibodies directed against the α_1 , α_2 , β , and γ subunits (Leung *et al.*, 1988; Ahljianian *et al.*, 1990).

1.3.3 Potassium channels

The first potassium channel to be cloned was the K^+ channel *Shaker* from *Drosophila* (Papazian *et al.*, 1987; Kamb *et al.*, 1987; Tempel *et al.*, 1987). After the cloning of *Shaker*, other K^+ channels were cloned and sequenced at an impressive rate, and it

became clear that the *Shaker* gene was evolutionarily conserved from *Aplysia* to humans. The *Shaker* gene product is a polypeptide of 616 amino acids. It contains a segment that is 27% identical to a 121-amino acid segment of domain IV of the electroplax Na⁺ channel (Tempel *et al.*, 1987). Indeed, the hydropathy plot revealed six putative transmembrane regions; both Na⁺ and Ca²⁺ channels contain four repeats with the same hydropathy pattern, suggesting that the K⁺ channel may exist as a tetramer. In support of this hypothesis, the K⁺ channel has been shown to assemble as a multimer, most likely a tetramer (MacKinnon, 1991; Pfaffinger and Chen, 1992). No other subunits have been reported (fig 7C).

Expression of K⁺ channels in *Xenopus* oocytes yields functional channels confirmed by patch clamping (Timpe *et al.*, 1988). Several experiments suggest that the assembly of the K⁺ channel tetramer is driven and controlled by sequences near the N-terminus called the T1 domain. The T1 domain is capable of binding to full length channel polypeptide immobilized on a filter, and deletion of T1 creates non-functional channels, in an expression assay. (Li *et al.*, 1992; Shen *et al.*, 1993). The hydrophobic core also appears to stabilize subunit interaction to some extent (Li *et al.*, 1992).

As mentioned earlier, the potassium channels show significant sequence and structural similarity to Na⁺ and Ca⁺⁺ channels (fig 8). They have six transmembrane segments S1-S6. S4 is an amphipathic helix in which typically every third (or fourth) residue is positively charged. Studies with the K⁺ channel mutated in the S4 region (Papazian *et al.*, 1991) support the hypothesis of this being the voltage sensor. The K⁺ channels also have a pore region where the S5-S6 loop dips into the membrane (now called the H5 region). This is the most highly conserved region among all K⁺ clones (Begenisch, 1994). Lopez and co-workers (1994) have presented evidence that the S6 segment of the *Shaker* voltage-gated channel also contributes to the pore. The N-terminus has been shown to play a role in channel inactivation depicted by a "ball and chain" model (Hoshi *et al.*, 1990; Rettig *et al.*, 1992).

1.3.4 Cyclic nucleotide-gated channels

With the cloning of the first cyclic nucleotide-gated channel from bovine retina (Kaupp *et al.*, 1989), the classic definitions of voltage-gated and ligand-gated channels were challenged. Here was a channel gated by a ligand, namely cGMP, without voltage dependence, which apparently demonstrated no sequence homology to any known ligand-gated channel. A report by Jan and Jan (1990) was the first to point out sequence similarities between the bovine cGMP-gated channel and the voltage-gated cation channels (fig 9). Most notable was the alignment of the S4 region where four arginine residues in the CNG channel lined up perfectly with arginines in the voltage sensor motif of the voltage-gated channels (Numa, 1989). The H5 region (or pore) also showed a marked similarity to the pore of the voltage-gated cation channel family (Goulding *et al.*, 1992). Experiments using chimeric retinal-olfactory channels have shown that the S5-S6 linker affects pore diameter and ion permeation indicating that this region does indeed contribute to the lining of the pore (Goulding *et al.*, 1993). Site-directed mutagenesis also showed that a single negative charge within this region controls Ca²⁺ blockage and ionic selectivity (Root and MacKinnon, 1993; Eismann *et al.*, 1994). These two regions and the hydropathy plot undeniably put the CNG channel into the "S4-containing superfamily".

After publication of the bovine rod cGMP-gated channel sequence, a great number of cyclic nucleotide-gated channels were cloned from different tissues. The first to follow was the nucleotide-gated channel from rat and bovine olfactory neurons (Dhallan *et al.*, 1990; Ludwig *et al.*, 1990) which will be described in more detail in a later section. An olfactory channel was also cloned from catfish olfactory neurons (Goulding, *et al.*, 1992). All the olfactory channels are activated more potently by cGMP than cAMP, however, cAMP is the physiological ligand (Dhallan *et al.*, 1990; Ludwig *et al.*, 1990; Goulding, *et al.*, 1992). The rod and cone CNG channel was cloned from mouse, rat, human, and chicken (Pittler *et al.*, 1992; Dhallan *et al.*,

S4 (VOLTAGE SENSOR)

CNG channel		Y P E I R L N R L L R I S R M F E F F Q R T E T R T
		+ + + + - +
Shaker		L A I L R V I R L V R V F R I F K L S R H S K G L Q
Na ⁺ channel	1st domain	V S A L R T F R V L R A L K T I T I F P G L K T I V
	2nd domain	M S V L R S L R L L R I F K L A K S W P T L N I L I
	3rd domain	L G A I K N L R T I R A L R P L R A L S R F E G M K
	4th domain	P T L F R V I R L A R I A R V L R L I R A A K G I R
Ca ²⁺ channel	1st domain	V K A L R A F R V L R P L R L V S G V P S L Q V V L
	2nd domain	I S V R L C I R L L R L F K I T K Y W T S L S N L V
	3rd domain	I S V V K I L R V L R V L R P L R A I N R A K G L K
	4th domain	E S A R I S S A F F R L F R V M R L I K L L S R A E

H5 (PORE)

CNG channel	Y S L Y W S T L T L T T I G -- E T P P P V R D S
	* . * . * . * . *
Shaker	D A F W W A V V T M T T V G Y G D M T P V G F W G
Shab	E A F W W A G I T M T T V G I R D I C P T T A L G
Shaw	L G L W W A L V T M T T V G Y G D M A P K T Y I G

FIG. 9. Alignment of the CNG channel α subunit with voltage-gated cation channels. The S4 region (voltage sensor) and H5 (pore region) are aligned. Charged amino acids are indicated (+ or -) in the CNG channel S4 region. Identical amino acids are indicated by an asterisk (*) in the H5 alignment and conserved amino acids are indicated by a point (.).

1992; Bönigk *et al.*, 1993). Somewhat unexpectedly, CNG channels have also been cloned from kidney (mouse partial sequence, and bovine), heart (rabbit partial sequence), aorta (rabbit) and testis (bovine) (Ahmad *et al.*, 1992; Biel *et al.*, 1993; Hundal *et al.*, 1993; Biel *et al.*, 1994; Weynand *et al.*, 1994). Figure 10 shows a dendrogram of the alignment of the family of CNG channels reported to date, and in appendix I, is an alignment of their predicted amino acid sequences. With the cloning of a second subunit, it became clear that the previously mentioned channels are the α subunits of heteromeric channels. The β subunit of the rod CNG channel was first cloned from human retina (Chen *et al.*, 1993), and subsequently the β subunit of the olfactory CNG channel was cloned from rat olfactory epithelium (Liman and Buck, 1994; Bradley *et al.*, 1994).

1.4 The bovine rod CNG channel

The cyclic GMP-gated channel of rod photoreceptors was the first ion channel found whose gating is controlled by a cyclic nucleotide. Most of what is known about the cyclic nucleotide-gated channel has been observed in the past 10 years. Early reports demonstrated that light causes the membranes of rods and cones to hyperpolarize (Tomita, 1965) implying the existence of a channel. The results of Penn and Haggins, (1969) suggested that the hyperpolarization of the rod outer segment produces a photocurrent which transmits information to the synaptic ends of the cells. Baylor and Fuortes (1970) suggested that there must exist an intermediate substance coupling the absorption of light to a decrease in membrane conductance. The identity of this diffusible signal or messenger was crucial to the understanding of the channel and its gating mechanism. Two conflicting ideas existed; these were the calcium-hypothesis, and the cGMP-hypothesis. The calcium-hypothesis proposed that upon excitation Ca^{2+} is released from an internal reservoir or pool in the ROS and binds to and blocks the membrane conductance. The cGMP-hypothesis proposed that closure of the light regulated conductance is triggered by a drop in intracellular cGMP (Yau and Baylor, 1989).

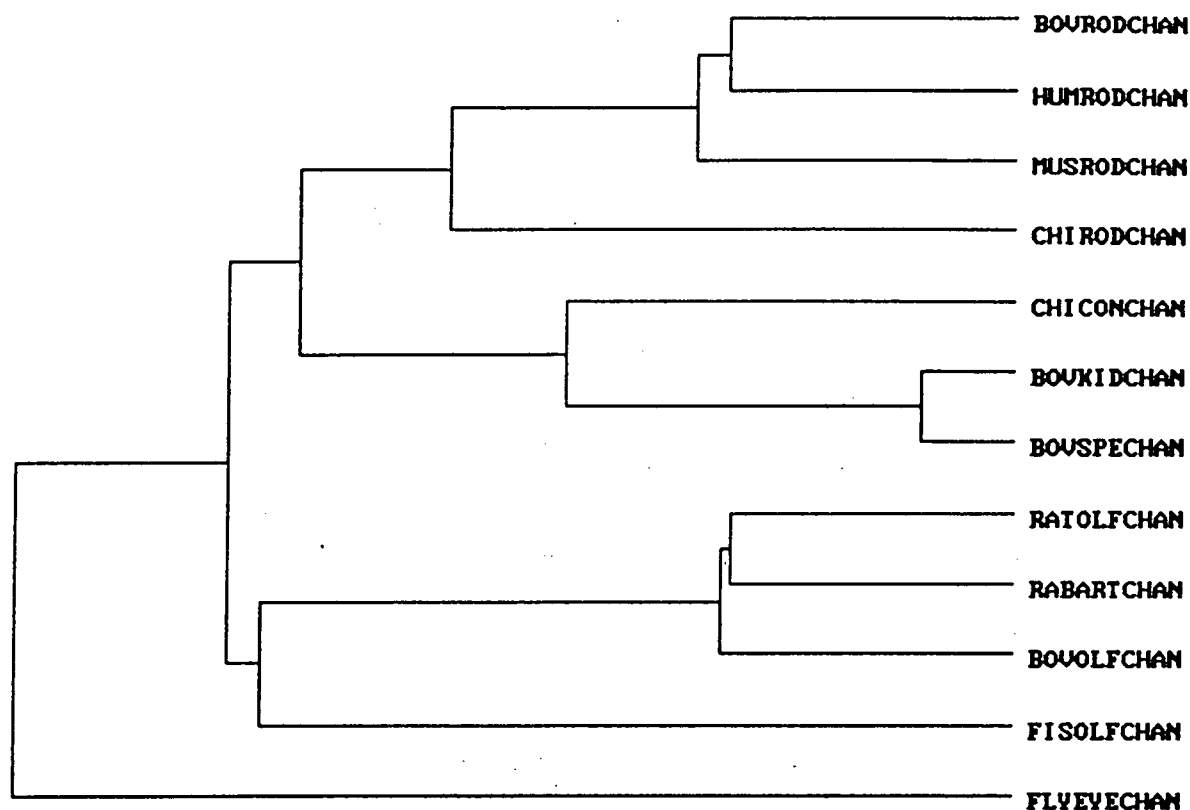


FIG. 10. Dendrogram of the predicted amino acid sequences of cloned CNG channels. Generated using the CLUSTAL program in PCGENE from IntelliGenetics.
 BOURODCHAN-bovine rod CNG channel (Kaupp *et al.*, 1989)
 HUMRODCHAN-human rod CNG channel (Dhallan *et al.*, 1992; Pittler *et al.*, 1992)
 MUSRODCHAN-mouse rod CNG channel (Pittler *et al.*, 1992)
 CHIRODCHAN-chicken rodCNG channel (Bönigk *et al.*, 1993)
 CHICONCHAN-chicken cone CNG channel (Bönigk *et al.*, 1993)
 BOVKIDCHAN-bovine kidney CNG channel (Biel *et al.*, 1994)
 BOVSPECHAN-bovine sperm CNG channel (Weynand *et al.*, 1994)
 RATOLFCHAN-rat olfactory CNG channel (Dhallan *et al.*, 1990)
 RABARTCHAN-rabbit aorta CNG channel (Biel *et al.*, 1993)
 BOVOLFCHAN-bovine olfactory CNG channel (Ludwig *et al.*, 1990)
 FISOLFCHAN-catfish olfactory CNG channel (Goulding *et al.*, 1992)
 FLYEYECHAN-drosophila eye CNG channel (Baumann *et al.*, 1994)

1.4.1 Electrophysiological characterization

In 1985 Fesenko and co-workers presented conclusive evidence that cGMP acts directly on a cation channel in the plasma membrane of vertebrate rod outer segments. Using excised patches of frog rod outer segments, they were able to demonstrate that the addition of cGMP caused an increase in the conductance across the membrane. This conductance was insensitive to the presence of Ca^{2+} . The channel had a half maximum of activation (EC_{50}) of 30 μM and a Hill coefficient (n) of 1.8. The increase in the membrane conductance occurred in the absence of nucleoside triphosphates, ATP and GTP (Cavaggioni and Sorbi, 1981; Fesenko *et al.*, 1985), indicating that phosphorylation was not involved in opening the channel. The response was shown to be specific for 3',5'-cGMP, as addition of 3',5'-cAMP, IMP and 2',3'-cGMP or 5'-GMP showed no effect on the channel activity (Caretta and Cavaggioni, 1983; Fesenko *et al.*, 1985). Analogs of cGMP which are modified at the 8-carbon position of the imidazole ring and 7-deaza-cGMP were still able to induce ion conductance (Caretta *et al.*, 1985; Koch and Kaupp, 1985). The channel was shown to be moderately selective for monovalent cations in the sequence $\text{Na}^+ > \text{Li}^+$, $\text{K}^+ > \text{Rb}^+$, $\text{Cs}^+ > \text{Cl}^-$ (Fesenko *et al.*, 1985).

The results of Fesenko and co-workers (1985) placed the channel in the plasma membrane, but experimental evidence from other labs suggested the presence of a cGMP-gated Ca^{2+} efflux from the disc membranes (Koch and Kaupp, 1985, Caretta *et al.*, 1985, Puckett and Goldin, 1986, and Schnetkamp, 1987). It was not until the work of Cook *et al.* (1989) that the cGMP-gated channel was shown to exist exclusively in the plasma membrane. Using an anti-channel antibody and a technique in which ricin-gold was employed to separate disks and plasma membranes by density gradient centrifugation (Molday and Molday, 1987), it was demonstrated that channel labeling and activity resided in the plasma membrane fraction (Cook *et al.*, 1989).

The most potent blocker of the cGMP-dependent channel is the *L*-stereoisomer of *cis*-diltiazem (Koch and Kaupp, 1985). Work with ROS membrane preparations (Koch and

Kaupp, 1985) and excised patches (Stern *et al.*, 1986) indicated that it has a cytoplasmic cGMP binding site and that it blocks conductance in the micromolar range (2.5-10 μ M). However, in intact rod cells, the light response of a dark-adapted cell was not blocked when a 2.3 mM solution of *l-cis*-diltiazem was dialyzed into the outer segment, and the response was only partially blocked by an extracellular application of 20 μ M solution (Stern *et al.*, 1986).

1.4.2 Biochemical characterization

The original biochemical characterization of the cGMP-gated cation channel activity was carried out by Cook and Kaupp (1986). ROS stripped of peripheral proteins were solubilized in the zwitterionic detergent, CHAPS, and reconstituted into Ca²⁺ loaded liposomes made from soybean phospholipids. Using Arsenazo III as a calcium indicator dye (Caretta and Cavaggioni, 1983; Koch and Kaupp, 1985), the cGMP-stimulated calcium efflux was measured as an increase in absorbance. The subsequent addition of the ionophore A23187 released the remaining Ca²⁺ from the vesicles. Using both these measurements, the "cGMP-dependent channel activity" is defined as:

$$\frac{\text{mol Ca}^{2+} \text{ released by cGMP} \times 100}{(\text{mol Ca}^{2+} \text{ released by cGMP} + \text{mol Ca}^{2+} \text{ released by A23187})}$$

i.e. the percentage of total releasable calcium that is released by cGMP. The value reported for channel activity measured from solubilized ROS membranes stripped of peripheral proteins was typically ~7% (Cook and Kaupp, 1986). In lipid vesicles the channels demonstrated characteristics very similar to those measured for the *in situ* channel (Koch and Kaupp, 1985; Fesenko *et al.*, 1985). The reconstituted channel had an EC₅₀ of 19 μ M cGMP and a Hill coefficient (n) of 2.7. However, it was insensitive to *l-cis*-diltiazem (Cook and Kaupp, 1986).

In 1987, Cook and co-workers reported the purification and identification of the cGMP-gated channel from rod photoreceptors. The solubilized ROS were subjected to ion exchange followed by red-dye affinity chromatography. Purified channels were reconstituted into liposomes and assayed for cGMP-stimulated activity. The EC_{50} of the purified channel for cGMP was 11 μ M with a Hill coefficient (n) of 3.1. When the purified channel was incorporated into planar lipid membranes (Hanke *et al.*, 1988), it exhibited an EC_{50} of 31 μ M and a Hill coefficient (n) of 2.3. The purified channel when reconstituted into liposomes or incorporated into planar lipid bilayers was also unaffected by *L-cis*-diltiazem (Cook *et al.*, 1987).

SDS-PAGE revealed that >90% of the final purified protein consisted of a single band with an apparent molecular mass of 63 kDa. Two conflicting papers published concurrently, also reported the partial purification and identification of cGMP dependent cation channels. Matesic and Liebman (1987) reported the partial purification of a 39 kDa protein from bovine ROS, that could be photoaffinity labeled by 8-azido cAMP. When incorporated into a lipid bilayer, it mediated cGMP-dependent cation fluxes with similar properties to those previously reported (Fesenko *et al.*, 1985; Haynes *et al.*, 1986). In a second paper, Shinozawa and co-workers (1987) reported the detection of a 250 kDa cGMP binding protein in an NH_4Cl -washed frog ROS membrane suspension which when incorporated into a planar lipid bilayer produced a conductance regulated by cGMP. No subsequent papers were published in support of either of these findings.

In the studies of Cook and co-workers (1987) a second band with an apparent molecular mass of 240 kDa was seen co-purifying with the 63 kDa protein. Purification of the cGMP-gated channel on a cAMP-Sepharose affinity column (Gill *et al.*, 1977) also yielded these two polypeptides (Cook and Kaupp, 1986). The 240 kDa protein also co-purified with the 63 kDa protein on a concanavalin A column (Wohlfahrt *et al.*, 1989). Using immunoprecipitation techniques, Molday *et al.*, (1990) showed that the 63 kDa and the 240 kDa proteins were associated and that the 240 kDa protein was the same

protein previously shown to be immunochemically related to red blood cell spectrin (Wong and Molday, 1986). It was, therefore, suggested to be part of a cytoskeletal system within rod outer segments.

A cyclic nucleotide-gated channel was also found in olfactory cilia (Nakamura and Gold, 1987). Early reports that olfactory cilia contain high amounts of an odorant-stimulated adenylate cyclase activity (Pace *et al.*, 1985), suggested that cAMP might serve as an intracellular messenger for olfactory signal transduction. Nakamura and Gold (1987) demonstrated that excised patches of ciliary plasma membrane contain a cAMP-gated conductance with an EC_{50} of $\sim 3 \mu\text{M}$ or $37 \mu\text{M}$ and a Hill coefficient (n) of ~ 1.5 . The channel was activated by both cAMP and cGMP, but cAMP is the physiologically important messenger in vertebrate olfactory transduction (Breer *et al.*, 1990). The reason for this lack of nucleotide selectivity is not clear.

1.4.3 Cloning of the first (α) channel subunit

In 1989 Kaupp and co-workers reported the cloning and functional heterologous expression of the bovine rod cyclic GMP-gated cation channel. Having a means of purifying the channel to near homogeneity (Cook *et al.*, 1987) facilitated the isolation of tryptic peptides for protein sequencing. Degenerate oligonucleotide probes based on these sequences were used to screen a cDNA library. A cDNA clone was isolated which contained an open reading frame encoding 690 amino acids (see appendix). The start codon was assigned to the first ATG triplet found downstream of nonsense codons appearing in frame, and the sequence TAACC preceding the start codon agrees reasonably well with the consensus Kozak sequence (Kozak, 1984). The poly-adenylation signal AATAAA was found 15 bases upstream of the poly d(A) tract. The first ~ 60 amino acids are comprised primarily of the charged amino acids Glu, Asp, and Lys. No extensive sequence identity to other channels was reported. However, a stretch of 80 amino acids from 498-577 was found to show significant similarity to the two tandem cGMP-binding domains of cGMP-dependent protein kinase (Takio *et al.*, 1984).

These regions are reported to be similar in sequence to the regulatory subunits of cAMP dependent protein kinase and the *E. coli* catabolite activator protein (Takio *et al.*, 1984; Weber *et al.*, 1987). Thus, the authors suggested that these amino acids help form a cGMP binding pocket and they have called this region the putative cGMP binding domain (Kaupp *et al.*, 1989).

A hydropathy plot identified six hydrophobic regions, H1-H6 (Kaupp *et al.*, 1989) which are now typically called S1-S6. Only H4 and H5 fulfill the criteria of membrane spanning regions according to Kyte and Doolittle, (1982). Placing the putative cGMP binding region (Fesenko *et al.*, 1985) and the N-terminal (Kaupp *et al.*, 1989) on the cytoplasmic side of the membrane, two hypothetical topological models were proposed. One model depicted the channel with six transmembrane spanning segments and the other with four transmembrane segments. As discussed earlier the current working model (fig 11) resembles the model of the potassium channel.

Proof that the cloned cDNA encoded a functional cGMP-gated channel was obtained by heterologous expression. *In vitro* transcription of the channel cDNA produced mRNA which was injected into *Xenopus* oocytes. Current measurements on excised inside out membrane patches from the oocytes showed a cGMP-gated conductance. The EC_{50} for cGMP was 52.3 μ M and the Hill coefficient (n) was 1.75 (Kaupp *et al.*, 1989), values which are similar to figures reported *in situ* in amphibian (Fesenko *et al.*, 1985) and mammalian rod photoreceptors (Lühning and Kaupp, 1989). cAMP did not activate the channel, and the cGMP-activated current was reduced to one half in the presence of ~40 μ M *l-cis* diltiazem (Kaupp *et al.*, 1989).

Most notable is the fact that the predicted molecular mass of the cGMP gated channel from the cDNA sequence is 79,601 daltons, whereas by SDS-PAGE the channel has an apparent mass of 63,000 daltons (Cook *et al.*, 1987; Wohlfahrt *et al.*, 1989; Cook *et al.*, 1989; Molday *et al.*, 1990; Molday *et al.*, 1991). Although the cDNA sequence does

α Subunit

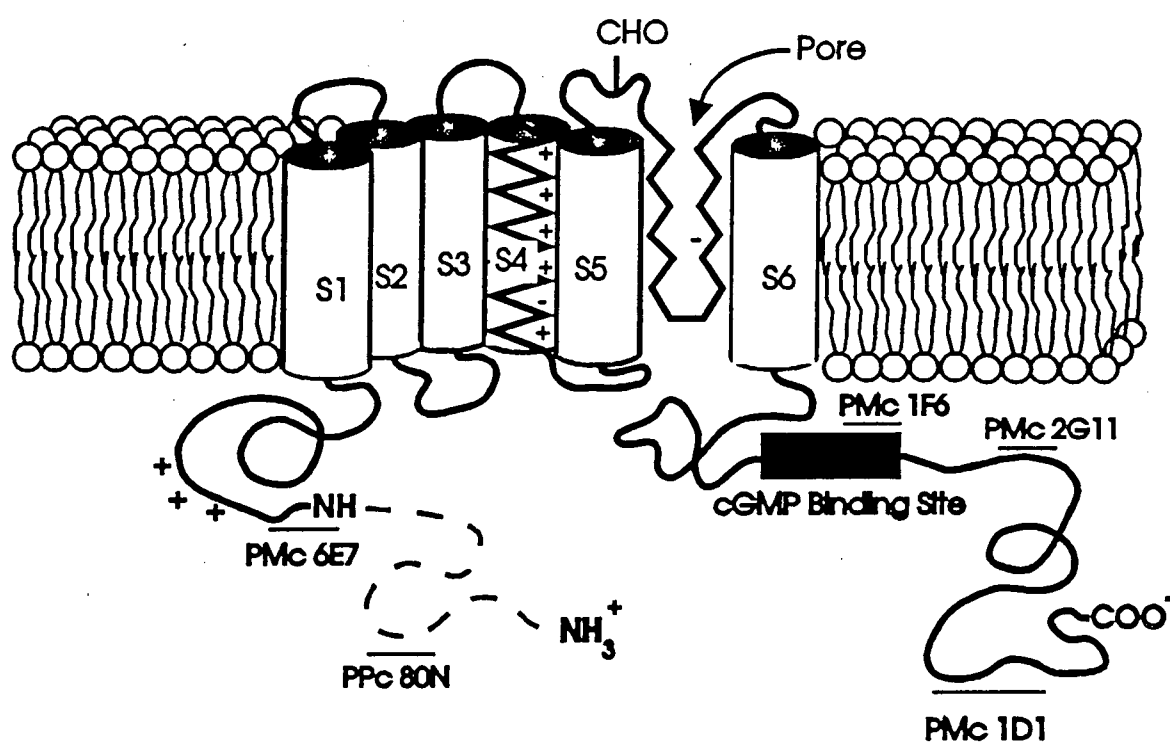


FIG. 11. **Current model of the bovine rod CNG channel α subunit.** Topological profile includes the six putative transmembrane regions, and the proposed pore. Antibody binding sites are indicated, as is the cGMP binding site. The dashed line at the N-terminus represents the 92 amino acids cleaved from the N-terminus of the 80 kDa α subunit to produce the 63 kDa form.

not predict a hydrophobic N-terminal sequence indicative of a typical signal sequence, it appears that the first 92 amino acids are lost from the N-terminus (see below). In some ROS preparations, antibodies that label the 63 kDa channel also label a minor band at 78 kDa, which is thought to be the full length polypeptide predicted by the cDNA sequence (Molday *et al.*, 1992). In one report researchers claimed to affinity purify a 78 kDa protein that exhibited cGMP-gated channel activity upon reconstitution (Hurwitz and Holcombe, 1991).

Compelling evidence, however, supports the hypothesis that the 63 kDa protein is in fact the functional form of the channel. When ROS are prepared from freshly dissected and frozen bovine retinas, in the presence of excess protease inhibitors, and are subjected to electrophoresis and western blotting, the α subunit still exists as a 63 kDa protein (Molday *et al.*, 1991). Western blots of the channel α subunit in three different species also detected a protein with a molecular weight of 63 kDa. N-terminal sequence analysis indicates that the 63 kDa protein purified from bovine ROS lacks the first 92 amino acids of the full length polypeptide predicted by the cGMP-gated channel cDNA sequence (Molday *et al.*, 1991, 1992; Kaupp *et al.*, 1989) (**fig 12**). A monoclonal antibody PMc 6E7, shown to recognize the N-terminus of the 63 kDa form of the channel, densely labeled the cytoplasmic surface of ROS plasma membranes (Molday *et al.*, 1991). Reconstitution of PMc 6E7 immunoaffinity-purified 63 kDa channel yielded cGMP-gated channel activity (Molday *et al.*, 1992).

In 1990, after the cloning of the bovine rod CNG channel, Dhallan and co-workers reported the cloning of a rat olfactory cyclic nucleotide-gated channel. Using the coding region of the bovine rod channel, they screened a rat olfactory cDNA library. The isolated clone encoded 664 amino acids which were 57% identical to the bovine rod sequence. The hydropathy profiles were nearly identical (Dhallan *et al.*, 1990) for the predicted amino acid sequences. The olfactory channel was expressed in human embryonic kidney cells and inside out membrane patches were tested for cyclic

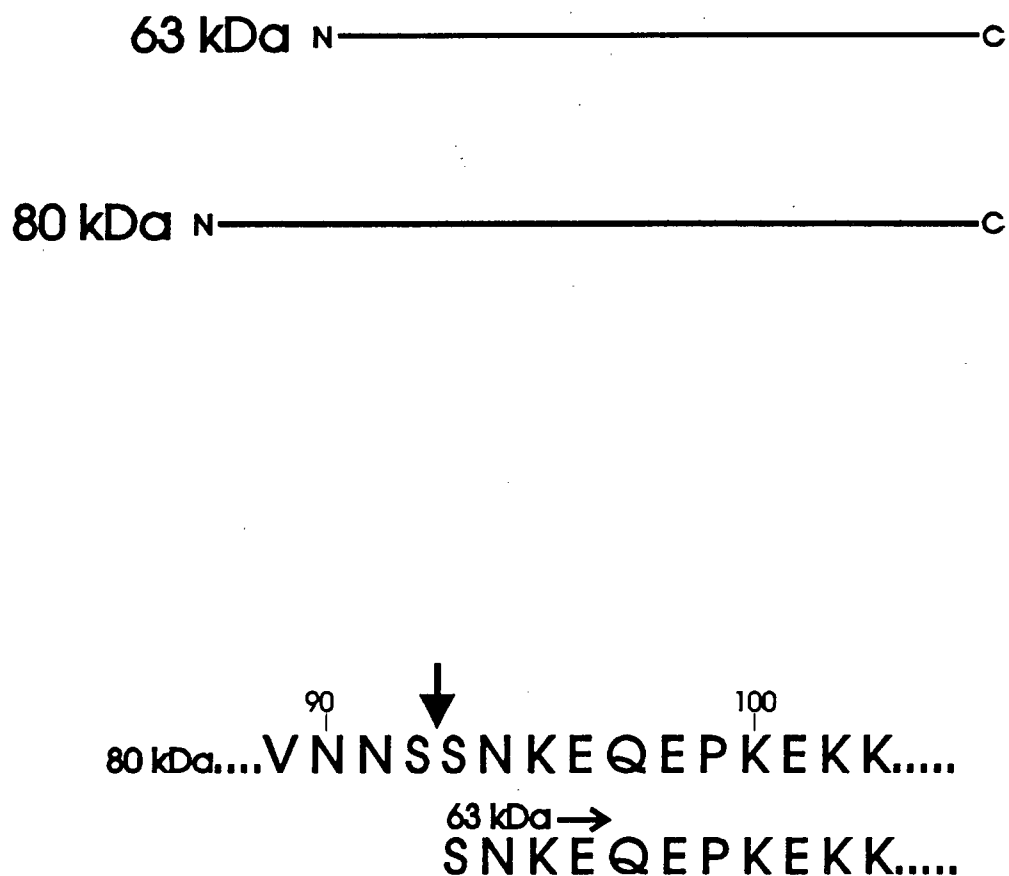


FIG. 12. The N-terminal sequence of the bovine rod CNG channel 63 kDa α subunit. N-terminal sequencing indicated that the 63 kDa α subunit purified from ROS began 92 amino acids downstream from the initiating methionine coded for by the 80 kDa α subunit coding sequence.

nucleotide-gated channel activity. The EC_{50} for cAMP was 38-68 μ M with a Hill coefficient (n) of ~2.0. Shortly thereafter, the cloning of the bovine olfactory CNG channel was reported (Ludwig *et al.*, 1990).

1.4.4 Cloning of the second (β) channel subunit

In 1993, Chen and co-workers reported the presence of a second subunit (now called the β subunit) of the CNG channel in human retinal rod cells which, when co-expressed with the first (α) subunit, confers functional characteristics seen thus far only in the native channel complex. They found two classes of β subunit clones, referred to as hRCNC2a and hRCNC2b, which differ only at their 5' ends and concluded they were alternative splice products. The proteins encoded by the two messages were 623 and 909 amino acids long respectively, and had calculated molecular weights of 70,843 and 102,330. They show an overall nucleotide identity to the human retinal rod α subunit (Dhallan *et al.*, 1992) of 30% in regions of overlap, except for the cyclic nucleotide binding regions which are 50% identical. Immunocytochemistry indicated that the β subunit is localized predominantly in rod outer segments (Chen *et al.*, 1993 & 1994).

The α and β subunits were co-expressed in human embryonic kidney cells (HEK 293) and characterized by patch clamping. The presence of the β subunit restored the flickery activity seen previously in the traces of native channel recordings from photoreceptor outer segments (Haynes *et al.*, 1986, Zimmerman and Baylor, 1986, Matthews and Watanabe, 1988, Quandt *et al.*, 1991), but not in recordings from the α subunit expressed by itself in HEK 293 cells (Chen *et al.* 1993), or in *Xenopus* oocytes (Kaupp *et al.*, 1989). The β subunit co-expressed with the α subunit also reinstated sensitivity to the *l-cis*-diltiazem, which was not observed for the α subunit expressed by itself (Chen *et al.*, 1993, Kaupp *et al.*, 1989).

In a subsequent study, Chen and co-workers (1994) presented results that indicated that the cloned β subunit (Chen *et al.* 1993) was part of the 240 kDa protein that co-purifies with the 63 kDa protein (Wong *et al.*, 1986, Cook *et al.*, 1987, Molday *et al.*,

1990). Furthermore it mediates the Ca^{2+} -calmodulin modulation of the channel (Hsu *et al.*, 1993; Chen *et al.*, 1994). Microsequencing of peptides obtained by digesting the 240 kDa protein from bovine ROS membrane produced peptide sequences that were 77% identical to the human β subunit sequence. Homooligomeric channels formed by the α subunit expressed alone in HEK 293 cells showed no sensitivity to Ca^{2+} -calmodulin, and neither did heterooligomeric channels formed by the α subunit and the shorter alternatively spliced β subunit (2a). However, heterooligomeric channels formed by the α subunit and the longer alternatively spliced β subunit (2b), showed a 1.5- to 2-fold increase in the cGMP EC_{50} in the presence of Ca^{2+} -calmodulin.

In 1994, Illing and co-workers reported that enzymatic cleavage of the 240 kDa protein followed by protein sequencing yielded a sequence which aligned with the β subunit reported by Chen and co-workers (1993), but also a sequence from a seemingly totally unrelated protein. The second set of sequences aligned perfectly with the glutamic-acid rich protein (GARP) cloned from bovine retina by Sugimoto and co-workers (1991). It was thought initially that this was a third subunit, however it has now been shown that the two 'subunits' are in fact two halves of the same protein (Körtschen *et al.*, 1995). The current model for the β subunit is presented in figure 13.

Parallel studies have been carried out with rat olfactory epithelial CNG channel. The cloning of the olfactory β subunit was simultaneously reported by two groups (Bradley *et al.*, 1994; Liman and Buck, 1994). It is 575 amino residues long, and 51% identical to the α subunit, except in the nucleotide binding region where it is 77% identical. Cell-specific expression patterns were examined using *in situ* hybridization, and both the α and β subunits were localized to the neural layer of the olfactory epithelium. The α and β subunits were expressed together and separately in HEK 293 cells, and conductance recordings were obtained from inside-out membrane patches. As in the rod CNG channel, the presence of the β subunit conferred certain functional characteristics not seen when the α subunit is expressed alone.

β - Subunit

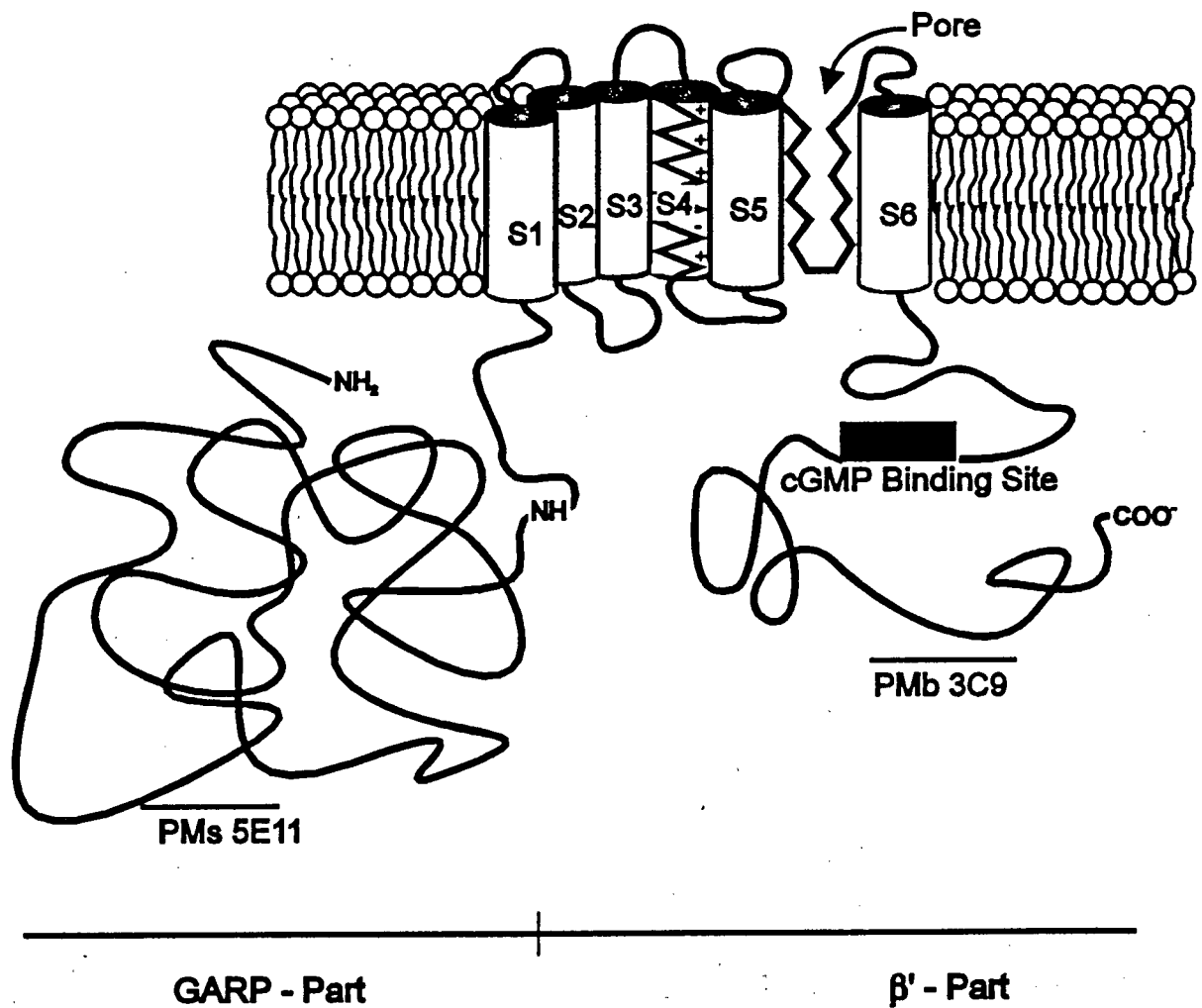


FIG. 13. Current model of the bovine rod CNG channel β subunit. Topological profile includes the six putative transmembrane regions, and the proposed pore. Antibody binding sites are indicated, as is the cGMP binding site.

1.5 Cyclic nucleotide-gated channel antibodies

Several antibodies were used throughout this study. Here they are presented with explanations of their antigens and binding epitopes, if known. Their binding sites are also illustrated in figures 11 and 13.

PMc 1D1 (Cook *et al.*, 1989): A monoclonal antibody raised against purified bovine ROS membranes. It labels the 63 kDa α subunit of the bovine CNG channel in both ROS membranes and purified channel preparations. It crossreacts with proteins in the retinas of human, pig, mouse, rat and guinea pig. The PMc 1D1 epitope was localized close to the C-terminus (Molday *et al.*, 1992), and has been subsequently mapped to amino acids 650-673 using synthetic peptides (M. Illing, R.S. Molday, unpublished results).

PMc 2G11 (Molday *et al.*, 1992): A monoclonal antibody raised to the purified bovine retinal CNG channel. It labels the 63 kDa α subunit in both ROS membranes and purified channel preparations. It crossreacts with proteins in the retinas of human, pig, mouse and rat. The PMc 2G11 epitope has been localized to amino acids 602-615, determined by sequencing of immunopositive clones derived from a λ gt-11 library (Molday *et al.*, 1992), and by immunochemical binding studies using synthetic peptides (M. Illing, R.S. Molday, unpublished results).

PMc 6E7 (Molday *et al.*, 1992): A monoclonal antibody raised to a synthetic peptide corresponding to the first 23 amino acids of the 63 kDa α subunit. It labels the 63 kDa α subunit in both ROS membranes and purified channel preparations. It also crossreacts with proteins in the retinas of pig and mouse. Immunochemical assays with synthetic peptides has mapped the PMc 6E7 epitope to the first 11 amino acids of the 63 kDa α subunit (SNKEQEPKEKK). A similar study included a peptide with a second N-terminal serine preceding serine-93, as predicted by the amino acid sequence of the 80 kDa α subunit (Kaupp *et al.*, 1989). In a competition assay, it was shown that PMc 6E7

recognizes the single serine peptide approximately 1000 times stronger than the double serine peptide (M. Illing, R.S. Molday, unpublished results).

PMc 1F6 (Bönigk *et al.*, 1993): A monoclonal antibody raised to the purified bovine retinal CNG channel. It labels the 63 kDa α subunit in both ROS membranes and purified channel preparations. It crossreacts with the CNG α subunit from chicken retinas and bovine olfactory epithelium. The PMc 1F6 epitope has been mapped to amino acids 576-591 using synthetic peptides (M. Illing, R.S. Molday, unpublished results).

PMs 5E11 (Molday *et al.* 1990): A monoclonal antibody raised against a purified channel preparation. It labels the 240 kDa β subunit of the bovine retinal CNG channel in ROS and purified channel preparations. The epitope is within the GARP portion of the β subunit (Körschen *et al.*, 1995).

PMb 3C9 (Körschen *et al.*, 1995): A monoclonal antibody raised against purified rod CNG channel. It labels the 240 kDa β subunit of the bovine retinal CNG channel in ROS and purified channel preparations. The epitope is within the β' portion of the β subunit.

PPc 80N (present work): A polyclonal antibody raised against a synthetic peptide corresponding to amino acids 41-69 of the 80 kDa α subunit of the bovine rod CNG channel. It labels the 80 kDa α subunit in ROS and heterologously expressing COS-1 cells. The epitope is within the region that is not present in the 63 kDa α subunit.

PPc CC1 (Bönigk *et al.*, 1993): A polyclonal antibody raised against a synthetic peptide corresponding to amino acids 720-735 of the α subunit of the chicken cone CNG channel. It labels cone cells, but not rod cells in the chicken retina. It also labels the 66 kDa cone α subunit in chicken retinas, and the heterologously expressed chicken cone α subunit.

1.6 Thesis investigation

At the outset of this project the cDNA sequence of the CNG cation channel α subunit had just been published. It was not known at the time that it was the α subunit since the

existence of the β subunit had not been reported. The native channel had been studied using electrophysiological and biochemical techniques, but little was known of its molecular properties.

The original activity measurements on the CNG channel were carried out by electrophysiology (Fesenko *et al.*, 1985). These measurements required small quantities of a channel and were carried out within the native membrane. Additionally, biochemical studies provided a means of assaying for cGMP-gated channel activity in ROS (Koch and Kaupp, 1985). Reconstitution and the Ca^{2+} efflux assay facilitated purification, identification, and ultimately the cloning of the α subunit, initially thought to be the only subunit of a homooligomeric channel (Cook *et al.*, 1986, 1987; Kaupp *et al.*, 1989).

Following the cloning of the α subunit, new questions and new experimental possibilities arose. It was observed that the cDNA coded for a polypeptide that was larger than that seen in ROS. Although gated by a ligand, it bore no sequence similarity to other ligand-gated channels. The availability of the cDNA sequence made heterologous expression possible, and in the initial cloning paper (Kaupp *et al.*, 1989) electrophysiological measurements were carried out on *Xenopus* oocytes injected with channel (α subunit) cRNA. It was our goal to take these studies a step further and generate a heterologous system in which molecular properties of the channel could be studied. Also, in order to ascertain the effectiveness of this heterologous expression system for production of large quantities of the channel and to facilitate future structure function studies in our laboratory, a means to reconstitute the heterologously expressed channel was required. As with many projects, what is not known at the beginning, can have great bearing on the outcome of the project.

The original objectives of this thesis were to: 1) subclone the channel, 2) investigate and characterize its molecular properties, 3) examine evidence of channel processing, 4) develop a reconstitution assay for the heterologously expressed channel, and 5) carry out structure-function studies.

Clones which coded for both the 63 and 80 kDa forms of the CNG α subunit were generated by PCR-mutagenesis. Heterologous expression of these clones and determination of their M_r contributed to the positive identification of the 63 kDa polypeptide as the CNG channel of ROS. The characterization of two antibodies enabled the identification of the 80 kDa α subunit in ROS and facilitated differentiation and localization of the two size forms of the α subunit. Immunochemistry also provided evidence of processing of photoreceptor CNG channels in other species.

With the discovery of the β subunit, new questions arose regarding its molecular properties and the interaction of the channel subunits, and these became a part of the thesis. The unusual migration of the β subunit by SDS-PAGE was investigated and coexpression of the α and β subunits indicated that the β subunit was not responsible for the cleavage of the α subunit. Heterologous coexpression also enabled investigation into the coprecipitation observed for the native α and β subunits.

In the early days of this project, the reconstitution and biochemical characterization of a heterologously expressed channel had not been reported. At the completion of this study, reconstitutions of heterologously expressed exchangers and transporters had begun to surface (Reiländer *et al.*, 1992; Shapiro and Ling, 1995). Significant effort was spent in an attempt to generate a reconstitution assay for the heterologously expressed channel. Only when the existence of a β subunit was reported and the β subunit could be coexpressed with the α subunit was success obtained.

MATERIALS AND METHODS

2.1 Bacterial Strains: The following strains of *Escherichia coli* were used for these experiments.

- JM105 *supE endA sbcB15 hsdR4 rspL thiΔ(lac-proAB) F'[traD36 proAB +lacIq lacZΔM15]* (Yannisch-Perron et.al., 1985)
- XL1-Blue *supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac- F'[proAB +lacIq lacZΔM15]Tn10(tet^r)* (Bullock et.al., 1987)
- DH5α/p3 *supE44ΔlacU169 (80lacZΔM15) hsdR17 recA1 endA1 gyrA46 thi-1 relA1 {p3: amber amp^r, amber tet^r, Km^r}* (Sambrook et al., 1989)
- Top10F' *mcrA, Δ(mrr-hsdRMS-mcrBC), φ80ΔlacΔM15,ΔlacX74, deoR recA1, araD139, Δ(ara,leu), 7697, galU, galK, λ⁻, rpsL, endA1, nupG, F'{tet^r}* (Sambrook et al., 1989)

2.2 Vectors: The following expression and cloning vectors were used in these experiments.

- pAX111 A mammalian expression vector constructed by Dr. Rob Kay at Terry Fox Laboratories, Vancouver. It contains a CMV promoter, and SV40 origin of replication. The cloning sites (a SmaI site bracketed by two BamHI sites) are followed by a β-globulin intron and RNA processing signals. It also contains ColE1, the E. coli origin of replication, and a *supF* gene for selection in E.coli strains carrying the p3 episome. The plasmid was a gift from Dr. Rob Kay.

- pAX112 A slightly modified version of pAX111, created by the insertion of the pUC1813 polycloning cartridge into the pAX111 BamHI site.
- pcDNA1 A mammalian expression vector containing the CMV promoter, SV40 transcription termination and RNA processing signals, SV40 and polyoma origins of replication, ColE1 origin and the *supF* gene. Purchased from Invitrogen Corporation.
- pcDNA1
/Amp A modified version of pcDNA1 with the ampicillin resistance gene replacing the *supF* gene. Purchased from Invitrogen Corporation.
- M13mp18 A commonly used cloning vector 7250 bases in length, which is a modified version of M13, a filamentous E.coli bacteriophage (Messing, et.al., 1977). It allows rapid purification of single stranded DNA for sequencing. Purchased from Pharmacia.
- pUC19 A 2686 bp, high copy number, general cloning vector, which contains an ampicillin resistance gene for selection. Purchased from Pharmacia.
- pUC1813 A 2727 bp cloning vector similar to pUC19, but with a modified poly cloning cartridge having symmetrical restriction enzyme sites (Kay and McPherson, 1987). A gift from the laboratory of Caroline Astell, UBC, Vancouver.
- pBluescript A 2960 bp colony producing, high copy number, ColE1 based phagemid. Purchased from Stratagene.

2.3 Constructed Plasmids: The following recombinant plasmids were constructed in the process of this work or were obtained from another laboratory for use in this work.

- pAX80 A 2073 bp fragment containing the entire coding sequence of the α subunit of the bovine rod cyclic nucleotide-gated (CNG) cation channel reported by Kaupp and coworkers (1989), except for three silent mutations engineered to introduce three unique internal restriction enzyme sites, subcloned into the EcoRI site of pAX112.
- pAX63 A 1797 bp fragment containing the coding sequence for the truncated version of the α subunit of the bovine rod CNG cation channel, subcloned into the EcoRI site of pAX112.
- pAX76-HX A 2108 bp fragment obtained from the lab of U.B. Kaupp. containing the entire coding sequence of the α subunit of the bovine olfactory CNG channel, subcloned into Klenow filled XmaI sites of pAX111.
- pCD β A 4179 bp fragment containing the entire coding sequence of the β subunit of the bovine rod CNG channel cloned into the EcoRI site of pcDNA1/Amp.
- pCD β' A 2468 bp fragment containing the truncated version of the β subunit of the bovine rod CNG channel cloned into the EcoRI site of pcDNA1/Amp.
- pCHIROPD A 1935 bp fragment containing the entire coding sequence of the chicken rod CNG channel reported by Bönigk et.al. (1993), subcloned into the EcoRV/XmaI sites of pcDNA1. Obtained from the lab of U.B. Kaupp.

- pCHICON** A 2205 bp fragment containing the entire coding sequence of the chicken cone CNG channel reported by Bönigk and colleagues (1993), subcloned into the HindIII/BamHI sites of pcDNA1. Obtained from the lab of U.B. Kaupp.
- pCGARP** A 2260 bp fragment containing the entire coding sequence of the bovine glutaminc acid rich protein (GARP), subcloned into the EcoRI/XhoI site of pcDNA1/Amp. (Körschen *et al.*, 1995).

2.4 Mammalian Cell lines: Two mammalian cell lines were used for heterologous expression experiments.

- COS-1** African green monkey kidney cells, SV40 transformed, ATCC CRL 1650.
- HEK 293** Human primary embryonic kidney cells; adenovirus-transformed, ATCC CRL 1573.

2.5 Antibodies: The following antibodies were used throughout this work.

Monoclonal-

<u>Name</u>	<u>Specificity</u>	<u>Epitope</u>	<u>Ref</u>
PMc 6E7	bov α subunit	first 11 amino acids of N-terminus of 63 kDa	Molday <i>et al.</i> , 1992
PMc 2G11	bov α subunit	C-terminal amino acids 568-630	Molday <i>et al.</i> , 1992
PMc 1D1	bov α subunit	C-terminal amino acids 645-673	Cook <i>et al.</i> , 1989
PMc 1F6	bov α subunit	C-terminal amino acids 577-591	Bönigk <i>et al.</i> , 1993
PMs 5E11	bov β subunit	GARP portion of β subunit	Molday <i>et al.</i> , 1990
PMb 3C9	bov β subunit	β' portion of β subunit	Körschen <i>et al.</i> , 1995

Polyclonal-

PPc CC1	chi α subunit	C-terminal amino acids 720-735 of chicken cone α	Bönigk <i>et al.</i> , 1993
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PPc 80N bov α subunit N-terminal amino acids 41-69 present work
of 80 kDa

2.6 Peptides: The following peptides were used throughout this work.

<u>Name</u>	<u>Amino Acid. Sequence</u>	<u>Sequence Context</u>
Ser1:	SNKEQEPKEKK	N-terminus of the 63 kDa CNG channel of ROS.
Ser2:	SSNKEQEPKEKK	63 kDa N-terminus (with an additional serine) of the 63 kDa CNG channel of ROS.
peptide 7:	DDDDSASMFEES-ETEN- PHARDSFRSNTHGC	corresponds to amino acids 41-69 of the 80 kDa bovine CNG channel with an additional cysteine at the C-terminal end.
rho 1D4:	TETSQVAPA	corresponds to the 9 C-terminal amino acids of bovine rhodopsin

2.7 Buffers and Reagents: Several buffers were used consistently throughout these experiments.

PBS: 8g NaCl, 0.4g KCl, 0.4g KH_2PO_4 , 2.3g Na_2HPO_4 in 1 liter H_2O .

HBS: 5.94g HEPES, 8.18g NaCl, 2.0g Na_2HPO_4 in 1 liter H_2O .

HBSG: 5.0g HEPES, 8.0g NaCl, 0.4g KCl, 0.99g Na_2HPO_4 , 1.1g glucose in 1 liter H_2O .

NaCl, KH_2PO_4 , Na_2HPO_4 , and glucose were purchased from BDH Inc., Hepes was purchased from Sigma.

2.8 Bacterial Culture: Cells were typically grown shaking at 37°C in Luria Broth (LB, 10% tryptone, 5% yeast extract, 10% NaCl) supplemented with the appropriate antibiotic or on LB agar plates (15 mg/ml agar). Tryptone, yeast extract and agar were

from Difco, NaCl was from Fisher. All pAX111 and pcDNA1 derivatives were grown in DH5 α /p3 cells, in the presence of 40 μ g/ml ampicillin, 8 μ g/ml tetracycline, and 25 μ g/ml kanamycin. The pcDNA1/Amp derivatives were grown in Top10F' cells in the presence of 50 μ g/ml ampicillin. M13mp18 was grown in JM105 cells and pUC vectors and pBluescript^R were grown in XL1-Blue cells (Stratagene). Ampicillin, tetracycline, and kanamycin were purchased from Sigma.

2.9 Mammalian Cell Culture: Mammalian cells were grown at 37°C in a 5% CO₂ atmosphere. COS-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) and HEK 293 cells were grown in Minimal Essential Medium with Earles salts (MEM) or in DMEM. Media were supplemented with 100 units/ml penicillin, 0.1mg/ml streptomycin, and 10% fetal bovine serum (FBS). All cell culture media and antibiotics were purchased from Gibco/BRL. Prior to division, COS-1 cells were trypsinized in 0.05% trypsin in HBS or PBS for three minutes, after which trypsin was quenched by the addition of medium with FBS. HEK 293 cells were trypsinized in 0.05% trypsin, 0.05mM EDTA. Cells were counted on a Bright-Line^R hemocytometer from American Optical.

Trypsin was from Difco and Trypsin/EDTA was purchased from Gibco/BRL.

2.10 Molecular Biology Techniques: The following are routine molecular biology techniques used throughout this work. Most of the procedures can be found in Sambrook *et al.* (1989). When commercially available kits were used, kit protocols were followed, and enzymes and buffers purchased often came with suggested protocols.

Restriction enzyme digestion: The appropriate enzyme was added to a 10 μ l reaction volume containing the DNA to be digested in One-Phor-All buffer (Pharmacia) or a specific buffer supplied with the enzyme. Incubations were typically carried out for 1 hour at 37°C.

Ligations: DNA fragments to be ligated were incubated with T4 DNA ligase (Pharmacia) also in One-Phor-All buffer, supplemented with 1 mM ATP. Ligations were carried out at room temperature for an hour, at 14°C for 4-12 hours, or at 4°C overnight for blunt-ended ligations.

Transformations: E.coli cells were transformed by incubating transformation competent cells with DNA (plasmid or ligation mixtures) for 45 minutes on ice. The DNA/cell mixture was then subjected to a heat shock at 42°C for 90 seconds. Cells were then added to 1ml of prewarmed LB, incubated with shaking at 37°C for up to one hour, and plated out on the appropriate selective media.

Competent cells were prepared from cell cultures grown to an OD₆₀₀ of 0.6. The culture was cooled rapidly on ice and pelleted at 4000 rpm for 10 minutes at 4°C. The cells were resuspended in ice cold 0.1 M CaCl₂ (half the original culture volume) and incubated on ice for 20 minutes. After repelleting, the cells were resuspended in 0.1 M ice cold CaCl₂ (1/20th the original culture volume) and incubated overnight on ice. Glycerol was added to 10%, and cells were stored aliquotted at -70°C. Calcium chloride was purchased from BDH Inc. and glycerol was purchased from Fisher.

Plasmid purifications: For small scale plasmid isolations, Wizard™ Minipreps purchased from Promega were used. For large scale plasmid preparations, QIAGEN Plasmid Kits purchased from Qiagen were used.

DNA fragment purification: Digested fragments were separated by agarose (Gibco/BRL) electrophoresis and isolated using a Bio 101 GENECLAN[®] Kit (Bio/Can Scientific) (for fragments larger than 0.3 kb), or QIAEX Gel Extraction Kit (Qiagen) (for fragments under 300 bases long).

DNA sequencing: DNA was sequenced by the method of Sanger *et al.* (1977) using the Sequenase[®] Kit from Amersham. Protocols provided with the kit were followed for both single- and double-stranded sequencing.

Polymerase Chain Reaction: Reactions were carried out in an ERICOMP™ twin block thermocycler. The reaction volume was 50 µl, containing 16 pmol of each primer, 6 µmoles of each dNTP, and 1.25 units of Taq DNA polymerase (Pharmacia) in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 2 mM MgCl₂. The typical program is listed below:

1 cycle	94°C for 10 seconds
30 cycles	94°C for 10 seconds
	59°C for 30 seconds
	72°C for 1 minute
1 cycle	72°C for 5 minutes
1 cycle	17°C until tubes were retrieved

2.11 Channel Construction and Subcloning: The polymerase chain reaction (PCR) was employed to amplify the cGMP-gated α subunit sequence from bovine retinal cDNA in order to subclone it into expression vectors. The channel sequence was amplified in four fragments or "cassettes". The PCR products were electrophoresed, isolated, digested and subcloned into M13mp18 for sequence confirmation. PCR primers were designed to introduce three unique restriction enzyme cleavage sites within the channel sequence, PstI, SmaI and HindIII. Two different channel cDNAs were constructed, one which encoded a 79.6 kDa polypeptide as dictated by the original cDNA sequence cloned by Kaupp and co-workers (1989), and one which encoded a 69.4 kDa polypeptide beginning at SER-93 (Molday et.al., 1991). Primers were synthesized by the NAPS Unit at UBC. The bases in boldface type are those homologous to the channel sequence. The double underlined base is the silent mutation created by the primer in order to introduce the indicated restriction site. Next to the restriction enzyme site are four random bases added in order to facilitate binding by the restriction enzyme.

PRIMERS P1 and P2 to generate cassette #1.

P1: 5'-GATCGAATTCTCAGTCCTGTGTAGAGTCTGTG-3'
EcoRI

P2: 5'-GATCAAGCTTTAACTGAGTACCCAGATGCCA-3'
HindIII

PRIMERS P3 and P4 to generate cassette #2.

P3: 5'-GATCAAGCTTCCATGAGGTCATCTTTTGAGA-3'
HindIII

P4: 5'-GATCCCCGGGCAGAATTTCAAGCAAGAATT-3'
XmaI

PRIMERS P5 and P6 to generate cassette #3.

P5: 5'-GATCCCCGGGCCGCATTCATGTTGGAAATC-3'
XmaI

P6: 5'-GATCCTGCAGTCTGATTACCTAGAATACTGGC-3'
PstI

PRIMERS P7, P8 and P9: P7 and P8 generate cassette #4A (80 kDa N-terminal), and P7 and P9 generate cassette #4B (63 kDa N-terminal).

P7: 5'-GTACCTGCAGTTCATCAAAACATGCTCTTGC-3'
PstI

P8: 5'-GTCAGAATTCACCATGAAAGAAAGTGATTATC-3'
EcoRI START

P9: 5'-GTCAGAATTCACCATGAGCAATAAGGAGGAACAACC-3'
EcoRI START

The reconstruction of the complete sequence was carried out in two steps. Initially cassettes #1 and #2 were ligated together, as were cassettes #3 and #4A and cassettes #3 and #4B, in XmaI/EcoRI digested pUC18. The ligated cassette pairs were then removed as XmaI/EcoRI fragments. Cassettes #1-2 was then combined with both #3-4A and #3-4B in dephosphorylated EcoRI digested pAX112. Insert orientation was confirmed by restriction enzyme digestion. The resultant expression vectors were called pAX63 and pAX80 (see figure).

2.12 Transfection of Mammalian Cells: Four different methods were employed to transfect COS-1 and HEK 293 cells; DEAE-Dextran, electroporation, BES-buffered calcium phosphate precipitation and HBS-buffered calcium phosphate precipitation.

DEAE-Dextran: (carried out on COS-1 cells) The procedure used was a modification of the method described by Hammarskjöld et.al, (1986). Cells were divided one day prior to transfection and plated at a density of approximately 6×10^5 cells per plate (day 1). On day 2, the monolayer was washed once with TS (140 mM NaCl, 5mM KCl, 1 mM $MgCl_2$, 25 mM Tris-HCL, pH 7.5), once with TD(TS without Mg^{2+} or Ca^{2+}) and then incubated with 7.5 μ g of DNA in 4 ml TS with 1 mg/ml DEAE-dextran for 10 minutes at room temperature and then 40 minutes at 37°C. The DNA/TS solution was then replaced with 4 ml of 20% glycerol in TS and swirled intermittently for 2 minutes. The glycerol solution was removed and the cells were washed once with TS and once with DMEM. The cells were then incubated with DMEM/FBS supplemented with 100 μ M chloroquine (Luthman and Magnusson, 1983) for 5 hours at 37°C, after which the medium was replaced with DMEM/FBS. Cells were harvested 24-60 hours later. DEAE-dextran was purchased from Pharmacia, chloroquine and Tris were purchased from Sigma, $MgCl_2$ was purchased from Fisher.

Electroporation: (carried out on COS-1 cells or HEK 293 cells grown in DMEM) The following procedure is based on a protocol given in the experimental data addendum to the BRL Cell-Porator™ Electroporation System Handbook. Cells were washed in HBSG (HBS supplemented with 6mM glucose) and trypsinized in 0.01% trypsin/HBS for three minutes and diluted to $1.2-2.5 \times 10^5$ cells/ml in 12 ml of HBS. The cell suspension was pelleted and resuspended in 0.5 ml HBSG with 10 μ g plasmid DNA. The cell/DNA mixture was then placed in an Invitrogen 4mm electroporation cuvette, and subjected to an electric pulse using an Invitrogen Cell-Porator™ hooked up to a Pharmacia ECPS 3000/150. The applied voltage and the capacitance were varied from

160-500V and 71-500 μ F, respectively, and the resistance was set at infinite. After exactly two minutes, "zapped" cells were added to fresh prewarmed media and plated out in 10 ml plates.

BES-Buffered Calcium Phosphate Precipitation: (carried out on HEK 293 cells)

The procedure used was a modification of the method described by Chen and Okayama, (1987). On day 1, cells were trypsinized in trypsin/PBS and plated out at a density of 6×10^5 cells/10 ml plate. On day 2, a 240 mM CaCl_2 solution containing 10-25 μ g plasmid DNA was mixed one to one with 2x concentrated BBS buffer (50 mM BES, 280 mM NaCl, 1.4 mM Na_2HPO_4 , pH 6.95). The precipitate was allowed to form for 20 minutes, and the suspension was then added to the media over cells. The plates of cells with the precipitate were placed in a 3% CO_2 incubator overnight. The following morning (day 3) the precipitate was removed, and the cells were washed with DMEM. Fresh DMEM/FBS was added, and plates of cells were returned to 5% CO_2 . After 24 hours (day 4) the cells were ready for harvest. BES was purchased from Calbiochem.

Hepes-Buffered Calcium Phosphate Precipitation: (carried out on COS-1 cells) The

procedure used was a modification of the method described by Sambrook *et al.* (1989). On day 1 cells were trypsinized in trypsin/PBS and plated out at 6×10^5 cells/10 ml plate. In the afternoon of day 2, medium was replaced with calcium-free medium (Gibco/BRL). Three hours later, 10 μ g of plasmid DNA in 500 μ l of 0.248 M CaCl_2 was added slowly to 500 μ l of HBS (pH 7.12), and the precipitate was added immediately to the cells. After incubating for 3.5 hours or overnight (day 3), the precipitate was removed, cells were washed with DMEM, and fresh DMEM/FBS was added. The following day (day 4) the cells were ready for harvest.

2.13 Co-Expressions: For co-expressions, 10 μ g of each plasmid construct was used. Transfections were carried out by either BES or Hepes-buffered calcium phosphate precipitations as described above.

2.14 Solubilization of Whole Cells: The procedure used to solubilize mammalian cultured cells was a modification of the technique reported by Oprian and co-workers (1987). The monolayer of cells was washed three times with PBS or HBS (alone or supplemented with 150 mM KCL and 10 mM CaCl_2) and solubilized in 0.5-1.0 ml solubilization buffer (either 2% CHAPS in PBS, or 180 mM CHAPS, 150 mM KCl, 10 mM CaCl_2 , 10 mM Hepes, pH 7.4). Cells were removed from the plate using a rubber policeman and transferred to a microcentrifuge tube. Solubilization was carried out in the presence of 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF, purchased from Sigma). The solubilized cells were centrifuged for 15 minutes at 4°C to separate the nuclear pellet from the post nuclear extract which could then be examined by SDS-PAGE and western blotting. Protein concentrations were determined using the BCA Protein Assay Reagent Kit from Pierce. CHAPS, (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was purchased from Sigma.

2.15 SDS PAGE and Western Blotting: Electrophoresis of proteins was performed by the standard Laemmli procedure, on a Bio-Rad Protean dual vertical slab gel electrophoresis cell, using 8% gels (Laemmli, 1970). Western blotting was performed on a Hoefer T22 mini-blot transfer apparatus, in 20 mM Tris, pH 7.4, 2 mM EDTA, .01% SDS at 30 mA for 1 hour. After proteins were transferred to ImmobilonTM-P (Millipore), the blot was fixed in methanol and blocked in 0.5% milk/PBS. After blocking, blots were incubated in primary antibody, secondary antibody, and then developed. Washes between incubations were done in PBS. All incubations were carried out for 1/2 hour and washes for about 15 minutes. The primary antibodies were diluted 20 times in 0.1% milk/PBS, and the secondary antibodies (sheep anti-mouse or anti-rabbit Ig, horse radish peroxidase from Amersham) were diluted 5000 fold in 0.1% milk/PBS. Western blots were visualized using the enhanced chemiluminescence

(ECL™) kit also from Amersham. Acrylamide and BIS were purchased from Bio-Rad; SDS was from Fisher.

2.16 Fluorescent Labeling of COS-1 and HEK 293 Cells: The procedure used to fluorescently label mammalian cultured cells was a modification of the technique reported by (Harlow and Lane, 1988). Immunofluorescent labeling of the transfected cells was performed on sterile glass discs which were laid down prior to resettleing of trypsinized cells. After transfection, discs were removed and cells were fixed and permeabilized in ethanol, or fixed in 4% paraformaldehyde and permeabilized in 0.25% saponin, 2% BSA in PBS. All incubations were carried out for 1/2 to 1 hour, and washes between incubations were performed by rinsing discs consecutively in three 100ml beakers of PBS. Blocking was done in 2% BSA in PBS, or 2% goat serum in PBS. Primary antibody labeling was done using a 20-fold dilution of culture fluid containing 1% BSA or goat serum in PBS. The fluorescently labeled secondary antibody was diluted 500-fold in 2% BSA or goat serum/PBS. The disks were mounted on a microscope slide with a drop of 10% Mowiol (Calbiochem) in 25% Glycerol/0.1 M Tris pH 8.5. The paraformaldehyde was purchased from J.B. EM. Services Inc., the saponin was purchased from Fisher.

2.17 Channel Tagging: In order to immunopurify the α subunit from transfected COS cells, the antigenic site of the characterized antibody rho 1D4 was engineered onto the 3' end of the channel α subunit. A PCR primer which was homologous to the 3' end of the α subunit was used (in the place of P1, described earlier) in conjunction with primer P2 (described earlier) to generate a modified cassette #1 with an altered C-terminus. The original stop codon was eliminated, and the 3' end was extended by 27 bases which coded for 9 amino acids from the C-terminal of rhodopsin previously shown to be the

epitope for the rho 1D4 monoclonal antibody (MacKenzie *et al.*, 1984; Hodges *et al.*, 1988). Below is the PCR primer, the annealing region is in boldface.

1D4TAIL downstream primer:

5'-CCACAGACTCTACACAGGACACAGAGCAAAGCCAAGTGGCGCCTGCC
T E T S Q V A P A
TGAGAATTCGATC-3'
STOP EcoRI

The "tagged" cassette #1 was then substituted into the α subunit cDNAs, and the resultant vectors were called pAX631D4 and pAX801D4.

2.18 Monoclonal Antibody Purification: (Goding *et al.*, 1986). Mice were injected with pristane, followed by a peritoneal injection with the desired hybridoma cells. The hybridoma cells were allowed to grow within the mouse as a fluid tumor for several weeks. The mouse was then sacrificed and ascites fluid was harvested. Ascites fluid was mixed with an equal volume of saturated ammonium sulfate and stirred for 35 minutes at 4°C. The precipitated protein was collected by centrifugation at 10,000 rpm for 10 minutes at 4°C and resuspended in 5 ml of 40 mM NaCl, 20 mM Tris, pH 7.4. The protein solution was then dialyzed over a period of 24 hours at 4°C against 4 changes of 20 mM NaCl, 20 mM Tris, pH 7.4, the final change being at room temperature. After centrifugation at 10,000 rpm for 10 minutes at 4°C, the supernatant was collected, and filtered, and loaded onto a DEAE Sephacel column (Pharmacia). The antibody was eluted using a gradient from 20-400 mM NaCl in 20 mM Tris, pH 7.4. Fractions were collected, dialyzed against 10mM Borate, pH 8.4, at 4°C overnight, and examined by SDS PAGE. Tri-sodium-tetraborate was purchased from BDH.

2.19 Polyclonal Antibody Production: A peptide encompassing amino acids 41-69 (plus a cysteine added at the C-terminal) of the 80 kD bovine α subunit, was synthesized

by Ian Clark-Lewis and conjugated to both keyhole limpet hemocyanin and soybean trypsin inhibitor by Michelle Illing. Approximately 150 µg of conjugated peptide was injected intra-muscularly into a New Zealand White rabbit at three week intervals. The first injection was done with complete Freund's adjuvant (Gibco/BRL) and all subsequent injections were done with incomplete Freund's adjuvant (Gibco/BRL).

2.20 Conjugation of Antibody to Sepharose Beads: Monoclonal antibodies were coupled to Sepharose CL-2B with a CNBr activation method (Cuatrecasas, 1970). Typically, 8 ml of Sepharose 2B beads were activated in 50 ml of H₂O adjusted to pH 10-11 with NaOH. Cyanogen bromide (0.15 g) was added to the gently stirring solution, and pH was maintained between 10 and 11 with further addition of 1M NaOH. After about 30 minutes, 20 ml of ice-cold 10 mM borate, pH 8.4 was added. Beads were then washed 4 times in 50ml borate buffer to remove excess CNBr and collected by centrifugation in a clinical table-top centrifuge. Two milligrams of purified antibody was added to 1 ml of activated beads and the suspension was rocked overnight at 4°C. The A₂₈₀ of the supernatant was measured to ascertain the extent of conjugation which was typically 70-90%. Sepharose beads were purchased from Pharmacia, CNBr was purchased from Aldrich Chemical Company Inc., and NaOH from Fisher.

2.21 Immunopurification: Solubilized COS-1 cells were incubated with the appropriate antibody conjugated to Sepharose beads at 4°C for 30 minutes to 4 hours. Typically 500 µl of a ~1 mg/ml solution was incubated with a 50 µl bead volume. The beads were washed five times in PBS, and bound protein was eluted either with 0.1 mg/ml peptide corresponding to the antibody recognition site, or with 5% SDS, 40% sucrose, 10 mM Tris, pH 7.4. These steps were carried out in ULTRAFREE[®]MC 0.45 µm filter units from Millipore.

2.22 Isolation of Bovine Rod Outer Segments: ROS preparations were carried out under dim red light. Typically, 100 freshly dissected or previously frozen retinas were homogenized by gently shaking for 1 minute in homogenization buffer (20% sucrose, 20 mM Tris, pH 7.4, 0.25 μ M $MgCl_2$, 10 mM taurine, 10 mM glucose), and then strained through a fine Teflon screen. The crude ROS suspension was then layered onto six 20ml continuous sucrose gradients (27-60% sucrose, 20 mM Tris, pH 7.4) and centrifuged at 25,000 rpm for 1 hour at 4°C in a Beckman SW-27 rotor. The major pink bands containing both sealed and unsealed ROS were collected and mixed with homogenization buffer to yield a final volume of approximately 120 ml. The ROS were then collected by centrifugation at 8000 rpm for 10 minutes at 4°C in a Sorvall SS-34 rotor, resuspended in approximately 8ml homogenization buffer at approximately 10 mg/ml, and frozen in 0.5 ml aliquots.

2.23 Purification of L- α -Phosphatidylcholine (Asolectin): 5 g of soybean phosphatidylcholine Type IV-S (partially purified asolectin, Sigma) was dissolved in 30ml of chloroform. Distilled acetone (180 ml) containing butylated hydroxytoluene (Sigma) was added slowly, causing aggregation of lipids. The slurry was centrifuged at 10,000 rpm for 20 minutes at -20°C in a SS-34 rotor. The pellets were redissolved in 30ml of chloroform and the precipitation steps repeated. After the second centrifugation step the pellets were dissolved in 50 ml of diethylether containing 0.2% α -tocopherol (vitamin E, Sigma). The solution was centrifuged again at 10,000 rpm for 20 minutes at -20°C to remove insoluble matter. The ether extract was dried in a rotary evaporator, and stored frozen.

2.24 Functional Reconstitution of the Native Channel Complex:

Washing and Solubilizing: Initially, 0.5ml ROS (at a concentration of ~10-15mg/ml) were washed three times in 13ml of hypotonic buffer (10mM Hepes, pH 7.4). ROS were

collected after each wash by centrifuging at 15K for 10 minutes at 4°C in a Sorvall SS-34 rotor, and finally resuspended in approximately 0.4ml of 10mM Hepes. This suspension was added dropwise to rapidly stirring solubilization buffer (10 mM Hepes, pH 7.4, 18 mM CHAPS, 0.15 M KCl, 10 mM CaCl₂, 1 mM DTT, 0.18% asolectin) to a final protein concentration of ~2 mg/ml. Insoluble material was removed by centrifugation at 15K for 10 minutes at 4°C.

Reconstitution: Solubilized ROS were combined 1:1 with an asolectin solution to give a final reconstitution mixture in 10 mM CHAPS, 0.1 M KCl, 10 mM CaCl₂, 10 mg/ml asolectin, 10 mM Hepes, pH 7.4. This mixture was then dialyzed at 4°C for 36-48 hours with three changes of 0.1 M KCl, 10 mM CaCl₂, 1 mM DTT, 5 mM Hepes, pH 7.4, followed by 6 hours with one change in the same buffer lacking calcium.

2.25 Reconstitution of Heterologously Expressed Channel Complex:

Washing and Solubilizing: Transfected cells were removed from plates using a rubber policeman and then washed in HBS and collected by centrifugation. The cells were then resuspended in 10 mM Hepes pH 7.4 with leupeptin (1 µg/ml), aprotinin (4 µg/ml), and PMSF (0.1 mg/ml), and added dropwise to the same solubilization buffer used for ROS for a final concentration of 1 to 2 mg/ml. The solubilized cells were stirred for three minutes, and then centrifuged at 10,000 rpm for 10 minutes at 4°C to remove the nuclear pellet. The supernatant was then run through a TSK-GEL TOYOPEARL DEAE-650 S column (Supelco, Inc.) with a bed volume of approximately 0.5ml. The column was washed with 10 volumes of 15 mM CHAPS, 10 mM Hepes, pH 7.4, 10 mM CaCl₂, 0.1M KCl, 1 mM DTT, 0.18% asolectin, and the bound fraction was eluted with 600 µl aliquots of the same buffer supplemented with 0.7 M KCl.

Reconstitution: 500 µl of column eluted fractions were reconstituted and dialyzed as described for ROS and the rest was used for westerns and concentration assays.

2.26 Channel Activity Assay: Cyclic GMP-induced release of calcium from preloaded vesicles or microsomes was carried out using a SIMAMINCO®DW-2000™ UV-Vis spectrophotometer, in dual wavelength mode. The difference between the absorbance at 630nm and that at 750nm was recorded. In a typical experiment 0.5ml of a liposome or microsome suspension was added to 1.5ml of a 75 μ M solution of the calcium-indicator dye Arsenazo III in dialysis buffer (no Ca^{2+}) described earlier. The cGMP was added as 4 μ l aliquots of 7.5 mM cGMP to give a final concentration of 150 μ M and the efflux of calcium was monitored as an increase in absorbance. Addition of cGMP and other solutes necessitated opening the port causing a dip in the absorbance trace. Samples were stirred with a stirbar during additions and absorbance readings. After each injection of cGMP, the absorbance changes were calibrated by adding 1nmol of Ca^{2+} . To confirm the presence of intact Ca^{2+} -loaded vesicles or microsomes, 1 μ l of a 0.5 mg/ml solution of the ionophore A23187 dissolved in ethanol was added, and Ca^{2+} efflux was measured.

2.27 Subcloning the Second Subunit of the CNG Channel: A clone of the second bovine rod CNG channel subunit (or β subunit) in pCDNA1 (Invitrogen) was obtained from Benjamin Kaupp's lab. A ~4.3 kb EcoRI fragment containing the entire coding sequence was restricted, gel purified and subcloned into pCDNA1/Amp, and the resultant vector was called pCD β . An abbreviated beta clone was also constructed that lacked the GARP (Sugimoto et.al., 1991) portion of the coding sequence. An upstream PCR primer (BETA P1) was designed to introduce a new EcoRI site, a Kozak sequence and a start codon at position 1714 of the β where the sequence first diverges from that of Sugimoto's GARP (see figure). The downstream primer (BETA M2) was on the 3' side of a unique XmnI site at position 2058 of the sequence. The resultant PCR product was subcloned into the vector pCRII (Invitrogen), sequenced, digested out and purified. This EcoRI/ XmnI fragment, containing the first 331 bases of the β sequence (plus a

methionine), was then substituted for the GARP sequence and 331 bases of the β sequence in the original pCD β . The resultant construct was called pCD β' . Below are the two PCR primers; annealing regions are in boldface.

BETA P1:

5'- GATCGAATTCACCATGGTACCTGCCACAGAAGAGCAC-3'
 EcoRI START

BETA M2 :

5'-CTCTTCAAGGAGCGGACGGAG-3'

2.28 Olfactory Channel Subcloning: The α subunit of the CNG channel from bovine olfactory epithelium was obtained from the laboratory of Benjamin Kaupp as a pBluescript clone. A Kozak sequence had been created in front of the start codon. The entire coding sequence was cut out as a HindIII/EcoR1 fragment, blunt-ended by filling in with Klenow fragment, and subcloned into Klenow-filled XmaI sites of pAX111. The resultant expression plasmid was called pAX76-HX.

2.29 Chicken Rod and Cone Channels: The cDNAs for the rod and cone channel α subunits from chicken were obtained from the laboratory of Benjamin Kaupp. The cDNAs had been cloned into the EcoRV/XmaI (rod) and the HindIII/BamHI (cone) sites of pCDNA1 (Invitrogen) and were called pCHIROPD and pCHICON.

2.30 Isolation of Chicken Photoreceptor Cells: Twenty eyes were obtained from the chicken slaughterhouse. After the eyes were enucleated in the head, the retinas were scraped off and added to 4ml of 10% homogenization buffer. The mixture was pipetted repeatedly until homogeneous. After straining, the homogenized retinas were loaded onto sucrose gradients (28-60%) and centrifuged at 26,000 rpm for 1 hour at 4°C. The

red band was collected, brought up to 1ml in homogenization buffer, and frozen in small aliquots.

RESULTS

3.1 *The alpha subunit of the bovine rod CNG channel*

The cloning of the CNG channel of bovine rods (now known to be the α subunit) was reported just prior to the outset of these studies (Kaupp *et al.*, 1989). In order to identify the clone as the CNG channel, Kaupp and co-workers carried out electrophysiological studies on *Xenopus* oocytes injected with α subunit mRNA. The cDNA sequence encoded a protein with a calculated mass of 80 kDa. The purified protein identified as the CNG channel in ROS, however, had a reported mass of 63 kDa (Cook *et al.*, 1987). The molecular mass of the α subunit expressed in *Xenopus* was not ascertained and the relationship between the predicted size of the protein encoded by the cDNA and the size of the α subunit in ROS was not resolved. The N-terminal sequence of the 63 kDa subunit was known although not published at the time. From the published cDNA sequence it could be seen that the sequence of the 63 kDa α subunit started 93 amino acids in from the beginning of the 80 kDa coding sequence. The 63 kDa α subunit, therefore, appeared to be a truncated form of the 80 kDa α subunit.

3.1.1 *Subcloning the α subunit*

The publication of the bovine retinal CNG channel α subunit sequence (Kaupp *et al.*, 1989) opened up the possibility for heterologous expression studies. The CNG channel α subunit clone was not yet available, so it was necessary to generate a clone using PCR. Primers for PCR reactions were designed in order to subclone the channel in four cassettes (**fig 14**). Subcloning by cassettes enabled the introduction of unique restriction enzyme sites within the cDNA sequence which could be useful in future engineering or mutagenesis of the α subunit. The cassettes were numbered #1-#4, cassette #1 being at the 3' end of the cDNA sequence, and #4 at the 5' end. In order to synthesize sequences which code for both the 80 kDa form of the α subunit (Kaupp *et al.*, 1989) and the 63 kDa α subunit (Molday *et al.*, 1991), two different versions of cassette #4 were synthesized. Cassette #4A encoded the entire 5' end of the 80 kDa cDNA sequence.

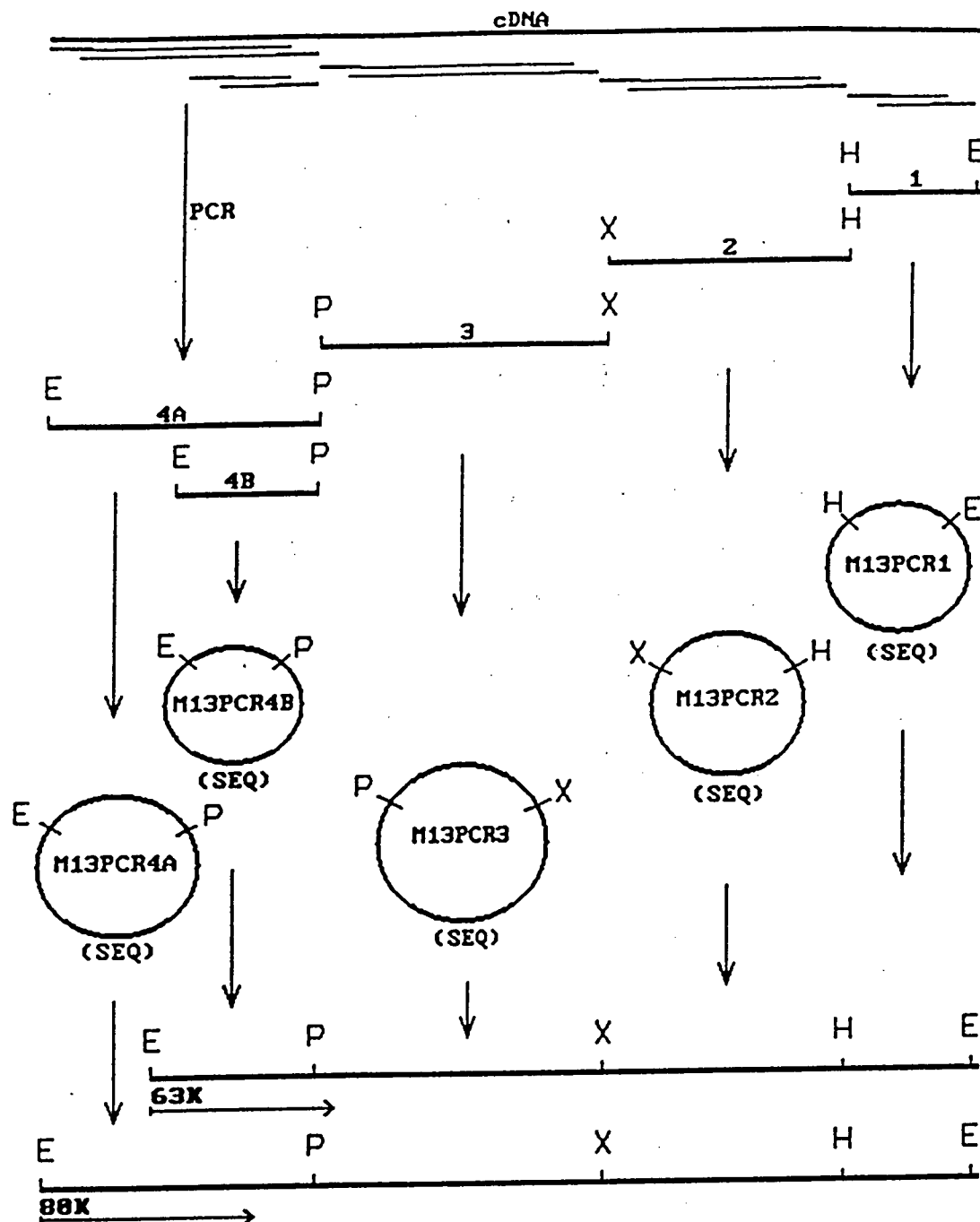


FIG. 14. A schematic diagram depicting the construction of cDNA's coding for the 63kDa and 80kDa α subunits. Polymerase chain reaction (PCR) was used to amplify the α subunit in four cassettes. PCR primers were designed to introduce unique restriction enzyme recognition sites within and at the end of the α subunit sequence. Two different cassettes were amplified at the 5' end (#4A&B). The larger cassette (#4A) coded for the N-terminus of the 80 kDa α subunit, and included the native Kozak sequence and start codon. The smaller cassette coded for the N-terminus of the 63 kDa α subunit and contained a synthetic Kozak sequence and start codon, engineered by PCR extension and mutagenesis. The cassettes were assembled to yield cDNA sequences for the 63 kDa and the 80 kDa α subunits. P, PstI; X, XmaI; H, HindIII; E, EcoRI.

Cassette #4B was missing coding sequence for 92 amino acids at the 5' end, and had a new start codon and Kozak sequence (Kozak, 1984) engineered in its place. The PCR primers were designed such that they directed silent mutations at their priming sites, and thereby introduced three unique restriction enzyme sites into the α subunit sequences: PstI, XmaI and HindIII. PCR fragments #1, #2, #3, and #4A were ligated together to generate cDNA sequence which encoded the entire 80 kDa α subunit of the CNG channel from bovine rods (Kaupp *et al.*, 1989). PCR fragments #1, #2, #3, and #4B were ligated together to generate cDNA sequence which encoded a 63 kDa α subunit with the N-terminus corresponding to the N-terminal sequence of the native 63 kDa CNG channel α subunit (Molday *et al.*, 1991). These α subunit cDNA's were then ligated into the expression vector pAX112, to generate two CNG channel α subunit expression vectors, called pAX80 and pAX63 (fig 15).

3.1.2 Heterologous expression of the α subunit in mammalian cells

The cDNA sequence predicted the mass of the α subunit to be 79.6 kDa (Kaupp *et al.*, 1989). Starting at the experimentally determined N-terminus of the α subunit, the predicted mass of the α subunit is 69.4 kDa (Molday *et al.*, 1991). Interestingly the mass of the ROS α subunit measured experimentally by SDS-PAGE is 63 kDa (Cook *et al.*, 1987). In order to untangle the question of the size of the photoreceptor CNG α subunit, the channel constructs which coded for the 63 and 80 kDa α subunits were heterologously expressed and their apparent molecular masses were determined by western blotting.

The CNG channel α subunit expression vectors were used to transfect COS-1 cells. The post nuclear extracts of cells transfected with either pAX63 or pAX80 were solubilized, electrophoresed and examined on coomassie blue stained gels and by western blotting (fig 16). The blots were labeled with the monoclonal antibody PMc 2G11 which recognizes the 63 kDa native CNG channel α subunit in whole ROS (fig 16, lane a). Antibody labeling revealed bands at 63 kDa in extracts of pAX63 transfected cells

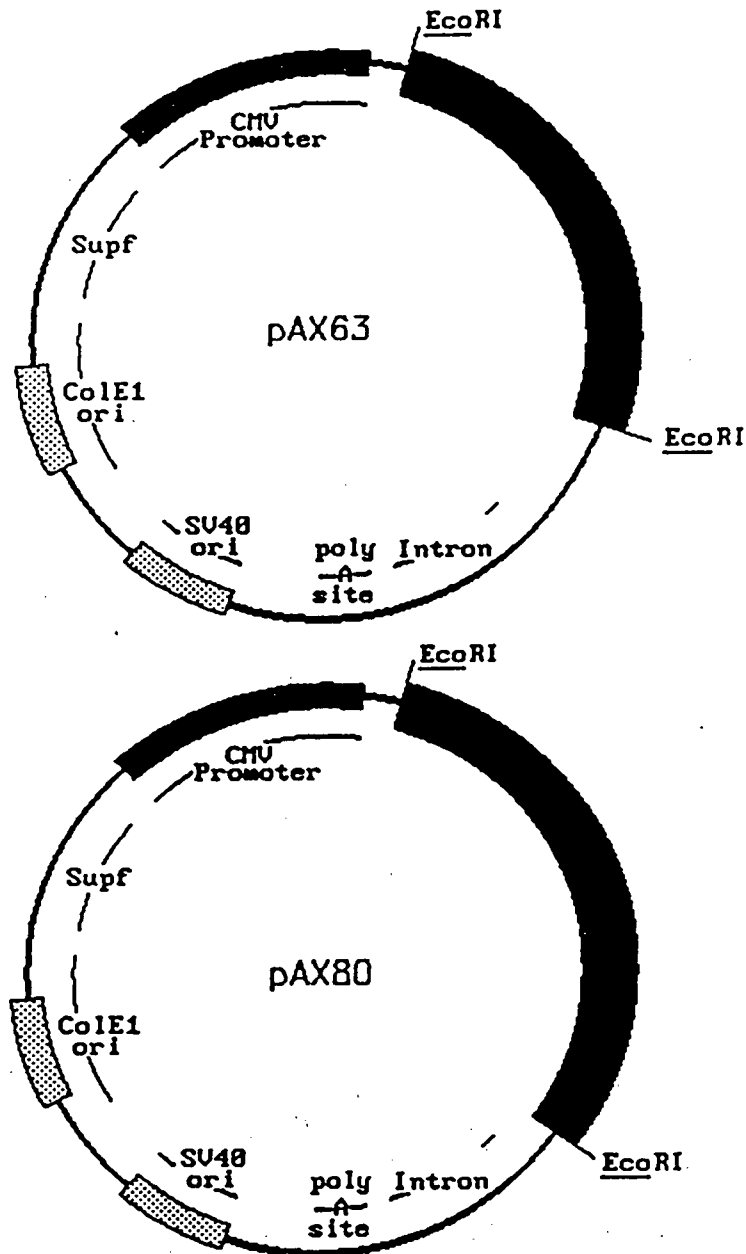


FIG. 15. Maps of pAX63 and pAX80 expression plasmids. The cDNA sequences for the 63 and 80 kDa α subunits were cloned into the EcoRI sites of pAX112. The main features of the expression vector are diagramed. CMV promoter, cytomegalovirus early promoter; *supf*, gene carrying mutations to suppress amber mutations allowing for selection in a suitable *E. coli* host; ColE1 ori, *E. coli* origin of replication; SV40 ori, simian virus 40 origin of replication; β -globulin intron and poly-A site, an intron and polyadenylation signal from the β -globulin gene.

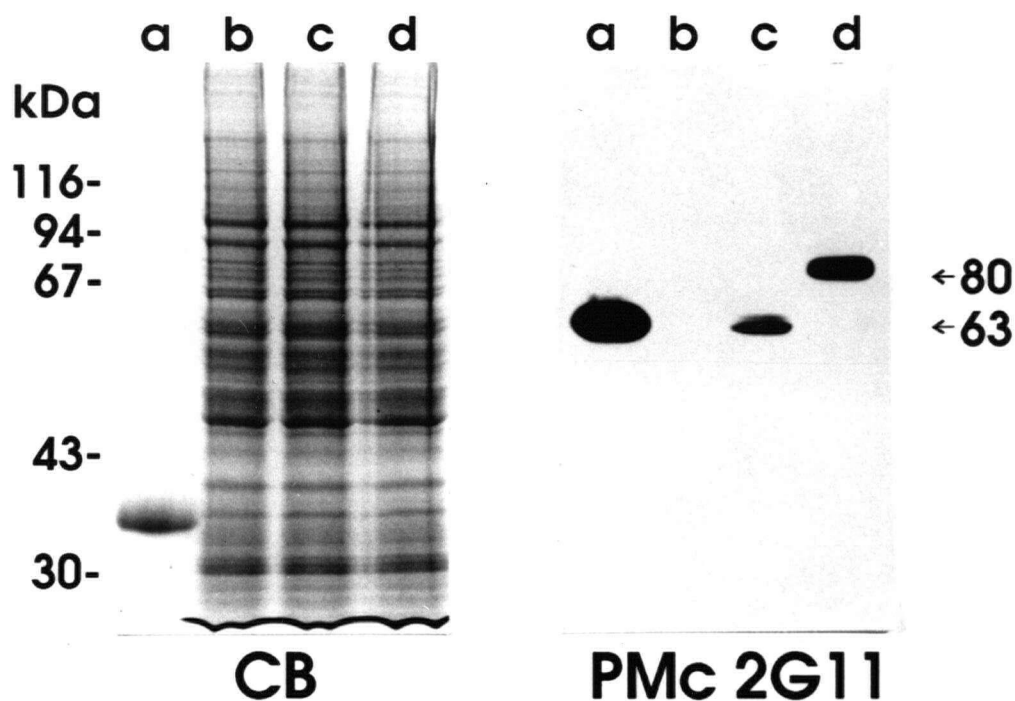


FIG. 16. Western blots of the native rod α subunit, and 63 and 80 kDa α subunits heterologously expressed in COS-1 cells. *Lane a*, isolated ROS (10 μ g); *lane b*, membrane extract from COS-1 cells transfected with pAX112 (control) vector alone (20 μ g); *lane c*, membrane extract from COS-1 cells transfected with pAX63 expression plasmid (20 μ g); *lane d*, membrane extract from COS-1 cells transfected with pAX80 expression plasmid (20 μ g). SDS gels were stained with coomassie blue (CB) and western blots were labeled with anti α subunit monoclonal antibody PMc 2G11 followed by ECL. PMc 2G11 labeled a 63 kDa polypeptide in both the native ROS and the extract containing the heterologously expressed 63 kDa α subunit; a 78 kDa polypeptide was labeled in the extract containing the heterologously expressed 80 kDa α subunit.

(**fig 16, lane c**) and 80 kDa in extracts of pAX80 transfected cells (**fig 16, lane d**). COS-1 cells transfected with expression plasmid alone exhibited no labeling with PMc 2G11 (**fig 16, lane b**).

The heterologously expressed (h/e) 80 kDa α subunit migrates close to the predicted $M_r \sim 78,000$. It is not processed to a 63 kDa protein in COS-1 cells as appears to be the case in rod cells. The truncated h/e α subunit with a predicted molecular weight of 69,400 migrates at approximately 63 kDa. This is the same anomalous migration as that seen for the α subunit in ROS. This heterologously expressed truncated α subunit is therefore referred to as the 63 kDa α subunit or the h/e 63 kDa α subunit. The h/e 63 kDa α subunit typically appeared as a doublet on western blots, the upper band being significantly fainter. This may be due to differential glycosylation. The 63 kDa α subunit was also typically expressed at a lower level than the 80 kDa α subunit, indicating that it may not be as stable as the 80 kDa α subunit in this heterologous expression system. Comparing the band intensities on western blots, an estimate of the number of α subunits per transfected cell can be made. This figure was found to be on the order of 10^6 molecules per cell for the 80 kDa α subunit. The 63 kDa α subunit was typically expressed at less than half of that level.

COS-1 cells transfected with pAX63 or pAX80 were also examined by immunocytochemistry. Cells fixed on glass disks were subjected to immunofluorescent labeling with the monoclonal antibody PMc 2G11 (**fig 17**). Not all cells are transfected, hence, some cells remain unlabeled. Labeling was observed throughout the cells as well as on the plasma membrane (**fig 17 b&c**). The nuclei appear as dark ovals. The mottled labeling is characteristic for the golgi apparatus and endoplasmic reticulum (Munro and Pelham, 1987). COS-1 cells transfected with the expression vector only were not labeled (**fig 17 a**). Phase contrast micrographs of the same cells are included (**fig 17 d,e&f**).

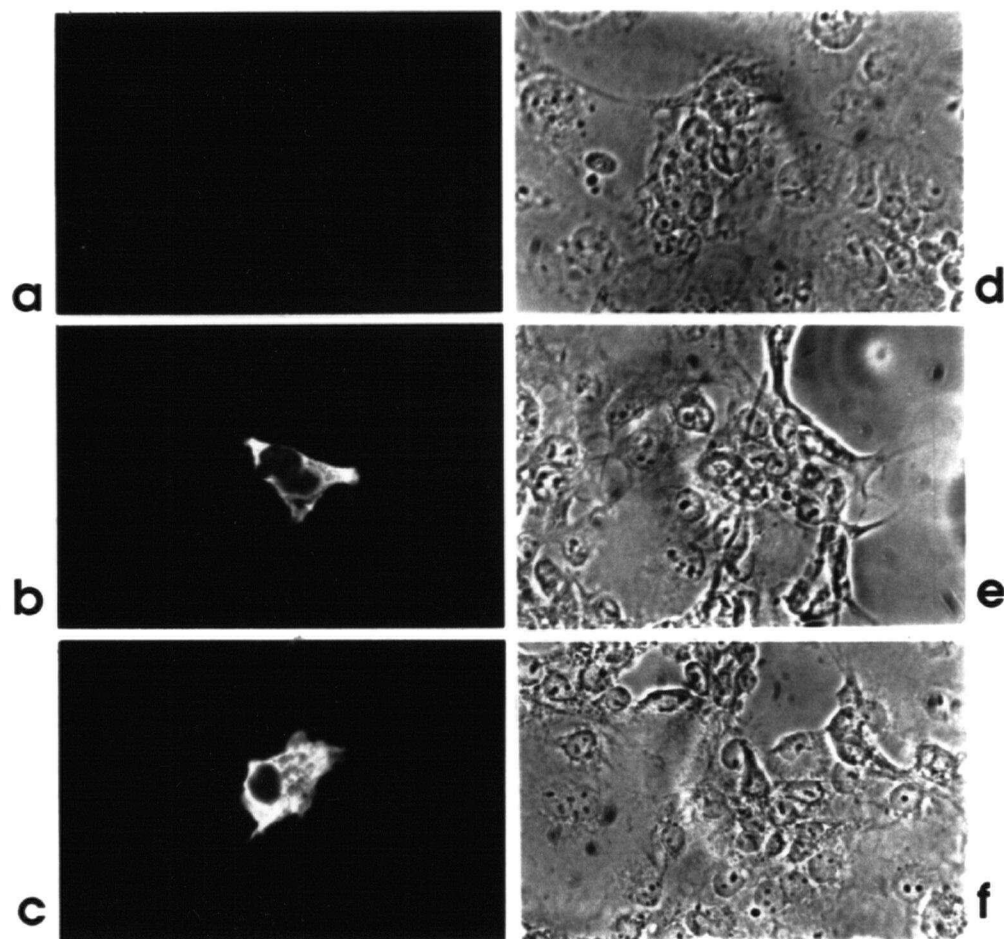


FIG. 17. Immunofluorescent labeling of COS-1 cells expressing the 63 and 80 kDa α subunits of the bovine rod CNG channel. (*a,b,c*) fluorescence micrographs of COS-1 cells transfected with pAX112 (*a*), pAX63 (*b*) and pAX80 (*c*); and (*d,e,f*) phase contrast micrographs of the same cells. COS-1 cells were fixed and permeabilized with methanol on glass discs prior to labeling with PMc 2G11 and fluorescein isothiocyanate-goat anti-mouse Ig.

3.2 Expression patterns of the α subunit

In order to determine which form of the CNG α subunit is present in rod photoreceptors, antibodies specific for each subunit were characterized. As described below, monoclonal antibody PMc 6E7 was shown to be specific for the 63 kDa form and polyclonal antibody PPc 80N was specific for the 80 kDa form of the α subunit.

3.2.1 Monoclonal antibody PMc 6E7

The monoclonal antibody PMc 6E7 (Molday *et al.*, 1992) was raised to a peptide corresponding to the first 23 amino acids of the retinal 63 kDa CNG channel α subunit (section 2.6, Materials and Methods). The reactivity of PMc 6E7 to native 63 kDa α subunit in ROS and the heterologously expressed 63 and 80 kDa α subunits was investigated by gels and western blotting. As shown in figure 18, PMc 6E7 labels a band at 63 kDa in ROS and in extracts of COS-1 cells transfected with pAX63 (fig 18, lane a&c). The extracts of COS-1 cells transfected with pAX80 were not labeled by PMc 6E7 (fig 18, lane b). An identical blot labeled with PMc 2G11 shows labeling of the 63 kDa α subunits in ROS and in pAX63 transfected COS-1 cells and the 80 kDa α subunit in pAX80 transfected COS-1 cells (fig 18, lanes a,c&b).

If the 63 kDa α subunit is the predominant form of the α subunit in ROS, then it should be readily detectable in retinal tissue sections labeled with PMc 6E7. Its presence in ROS *in situ* would further support the view that the 63 kDa form is not due to nonspecific proteolytic degradation during purification. As seen in figure 19a, PMc 6E7 strongly labels the rod outer segments in immunofluorescence labeling studies performed on bovine retinal sections. The specificity of PMc 6E7 labeling was investigated using two peptides Ser-1 and Ser-2 as competing inhibitors. Ser-1 corresponds to the 11 N-terminal amino acids of the 63 kDa α subunit, and Ser-2 is identical to Ser-1 but with an additional serine on the N-terminus (fig 20). The presence of Ser-1 during labeling abolished essentially all PMc 6E7 binding (fig 19b). However, the presence of Ser-2 did

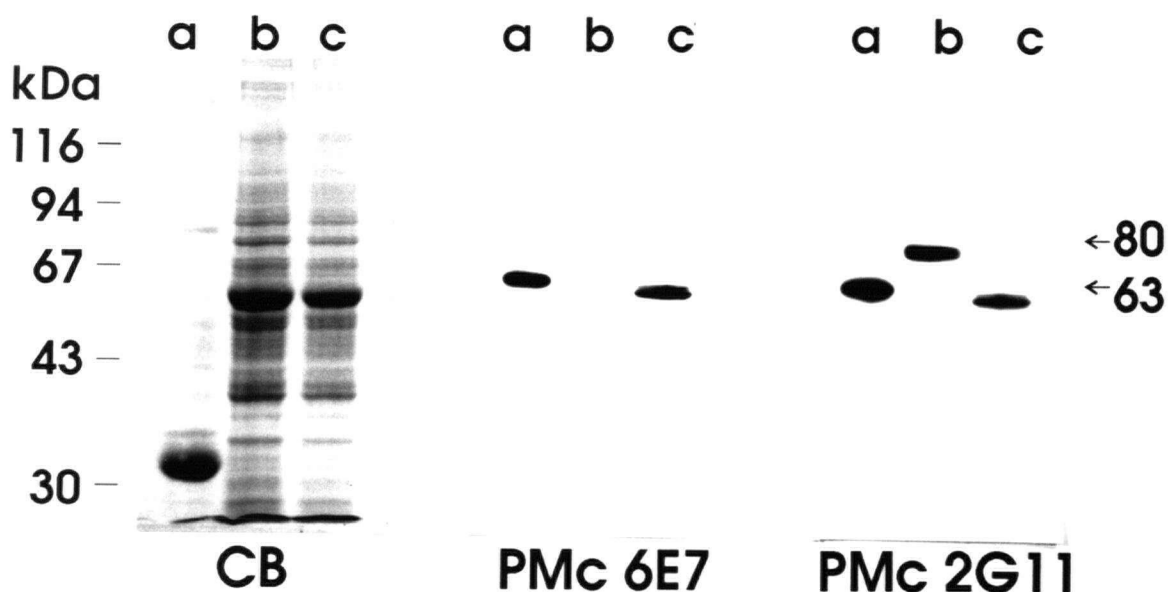


FIG. 18. Western blots of the rod α subunit, and 63 and 80 kDa α subunits heterologously expressed in COS-1 cells and labeled with anti- α subunit monoclonal antibodies PMc 6E7 (63 kDa-specific) and PMc 2G11. SDS gels were stained with coomassie blue (*CB*, *left panel*) or transferred to immobilon and labeled either with PMc 6E7 (against the N-terminus of the 63 kDa α subunit, *middle panel*), or PMc 2G11 (against the C-terminal region of the α subunit, *right panel*). *Lanes a*, isolated whole ROS (10 μ g); *lanes b*, membrane extract from COS-1 cells transfected with pAX80 expression plasmid (20 μ g); *lanes c*, membrane extract from COS-1 cells transfected with pAX63 expression plasmid (20 μ g). PMc 6E7 labeled a 63 kDa polypeptide in both the native ROS and the extract containing the heterologously expressed 63 kDa α subunit, but did not label the extract containing the heterologously expressed 80 kDa α subunit. PMc 2G11 (control) labeled all three α subunits: the rod 63 kDa, the expressed 80 kDa, and the expressed 63 kDa. Primary antibodies were labeled with sheep anti-mouse Ig peroxidase for ECL.

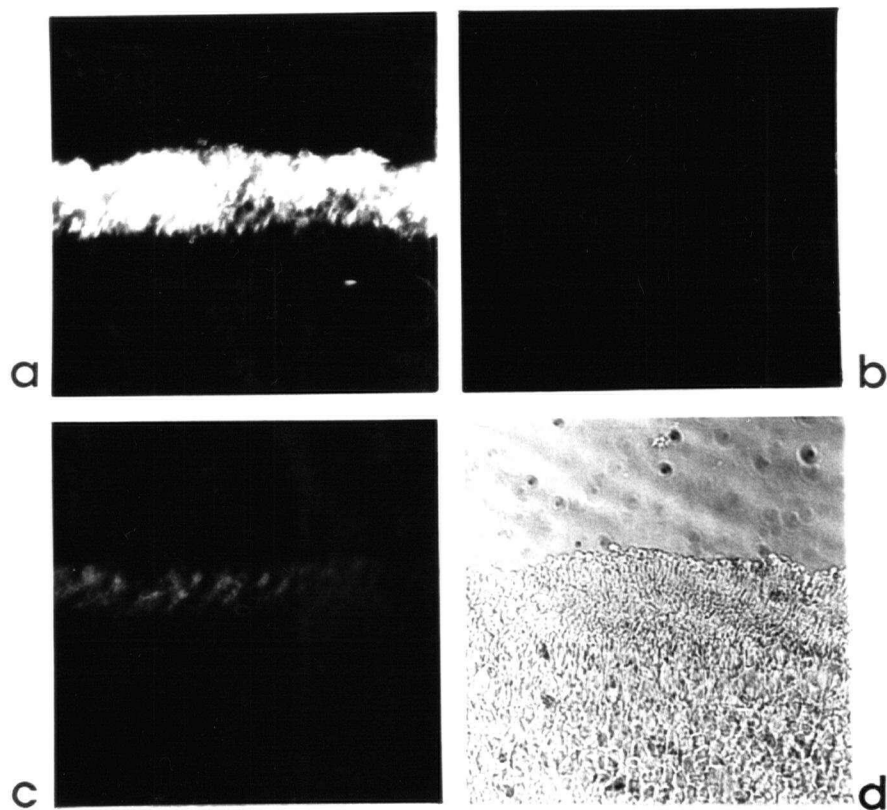


FIG. 19. **Immunofluorescent labeling of bovine retina with 63 kDa-specific anti α subunit monoclonal antibody PMc 6E7.** Cryostat sections of paraformaldehyde-fixed bovine retina were labeled with PMc 6E7 and fluorescein isothiocyanate-goat anti-mouse Ig in the absence (*a*) or in the presence of synthetic peptides Ser-1 (*b*) or Ser-2 (*c*). A phase contrast micrograph is included (*d*). The outer segments are labeled intensely with PMc 6E7. Labeling is partially inhibited in the presence of Ser-2, and is completely inhibited in the presence of Ser-1.

not eliminate all binding (**fig 19c**). This result indicates the presence of the 63 kDa form of the CNG channel α subunit in native rod cells. The results further show that PMc 6E7 has a stronger affinity for the single serine peptide, but that it can bind the double serine peptide with lower affinity.

Peptide inhibition studies were carried out to further explore the reactivity of PMc 6E7 to the various forms of the CNG α subunit. Two peptides Ser-1 and Ser-2, which differ only by an additional serine residue at the N-terminus of Ser-2 (**fig 20**) were used as inhibitors of PMc 6E7 labeling. As shown in figure **21**, Ser-1 was more effective than Ser-2 in inhibiting the binding of PMc 6E7 to the native and heterologously expressed 63 kDa α subunit. Ser-1 peptide completely inhibited labeling of both the native and expressed channel α subunit (**fig 21, lanes a&c**). Ser-2 peptide inhibited most of the PMc 6E7 labeling of the expressed channel, but only showed limited inhibition of labeling of the native channel α subunit (**fig 21, lanes a&c**). Since the native and the expressed 63 kDa α subunit differ only by a methionine at the N-terminus, these results indicate that PMc 6E7 has the highest affinity for a sequence corresponding to Ser-1 or the native 63 kDa α subunit with a free amino group at the N-terminus. Addition of a second serine as for Ser-2 peptide, or a methionine as for the expressed 63 kDa to the recognition site reduces the affinity of the PMc 6E7 antibody for these peptides. Apparently, further extension of the sequence, as found for the 80 kDa expressed channel completely abolished binding of the PMc 6E7 antibody.

In order to examine the heterologously expressed α subunit *in situ*, immunoperoxidase labeling studies were carried out on whole COS-1 cells expressing the α subunits. COS-1 cells transfected with pAX63 and pAX80 were labeled with PMc 6E7. Cells expressing the 63 kDa α subunit were recognized by PMc 6E7 (**fig 22b**), however, those expressing the 80 kDa α subunit were not (**fig 22a**). Synthetic peptides Ser-1 and Ser-2 were used in experiments which examined the ability of PMc 6E7 to

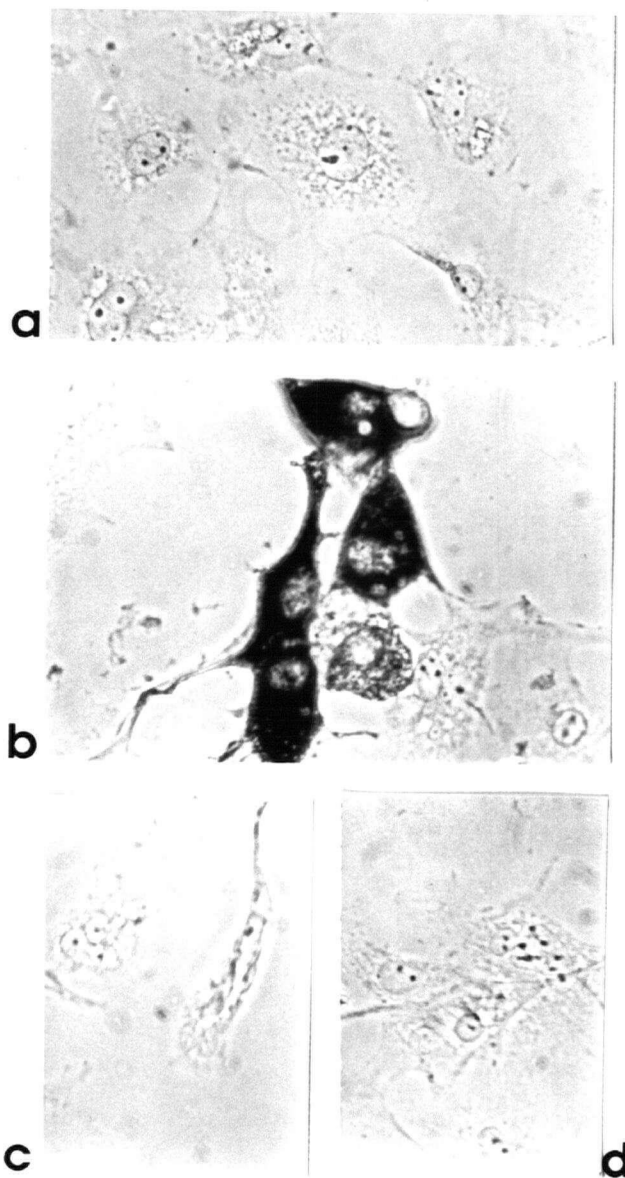


FIG. 22. Immunoperoxidase staining of COS-1 cells expressing the 63 and 80 kDa α subunits of the bovine rod CNG channel and labeled with monoclonal antibody PMc 6E7. COS-1 cells fixed and permeabilized with methanol on glass discs were labeled with PMc 6E7 and horseradish peroxidase-sheep anti-mouse Ig. Immunoreactivity appeared as dark reaction product. (a) COS-1 cells transfected with pAX80 (control) and labeled with PMc 6E7; (b,c,d), COS-1 cells transfected with pAX63 and labeled with PMc 6E7 in the absence of peptide (b); in the presence of Ser-1 (c); or Ser-2 (d). PMc 6E7 did not label COS-1 cells expressing the 80 kDa α subunit. COS-1 cells expressing the 63 kDa α subunit were labeled with PMc 6E7; labeling was inhibited by both Ser-1 and Ser-2.

bind to the heterologously expressed 63 kDa α subunit in the presence of competing peptides. The presence of Ser-1 and Ser-2 competes for the binding of PMc 6E7 (**fig 22c&d**) which supports the blot competition assays. The fact that residual labeling is not seen in the presence of Ser-2 is probably due to the limited sensitivity of DAB visualization.

These results taken together confirm that PMc 6E7 recognizes the native and heterologously expressed 63 kDa α subunit on blots and *in situ*, but the antibody binds the native N-terminus with higher affinity. The labeling is specific and it can be prevented or reduced by the presence of peptides which correspond to the PMc 6E7 recognition site. Most importantly, PMc 6E7 does not recognize the 80 kDa α subunit with its extended N-terminus and provides an effective means for detecting solely the 63 kDa α subunit in cell and membrane preparations.

3.2.2 PPc 80N labeling studies

In order to specifically detect the 80 kDa α subunit in the presence of the 63 kDa α subunit, an antibody was required that would label the 80 kDa α subunit only. It would need to recognize a region in the 80 kDa α subunit that did not exist in the 63 kDa α subunit. The polyclonal antibody PPc 80N was raised against a KLH and ABTI-linked peptide (peptide 7) corresponding to 28 amino acids near the N-terminus of the 80 kDa α subunit (**fig 23**). Coomassie blue stained gels and western blots of solubilized COS-1 cells transfected with pAX63 and pAX80 are shown in figure 24. The western blot was labeled with the polyclonal antibody PPc 80N which recognized the heterologously expressed 80 kDa α subunit, but not the 63 kDa α subunit in COS-1 cells (**fig 24, lane b&c**). In ROS membranes, PPc 80N often labeled a faint band at 80 kDa (**fig 24, lane a**). This same band was also faintly labeled with other α subunit antibodies. In order to demonstrate that the PPc 80N binding was specific, peptide 7 was used in competition studies (**fig 25**). Peptide 7 (amino acids 41-69 of the 80 kDa channel) was a potent inhibitor of PPc 80N binding. The presence of the peptide abolished PPc 80N binding of

80 kDa

MKKVIINTWHSFVNIPNVVGPDVEKEITRM
1
ENGACSSFSGFSGDDDDDSASMFEETEN
41
PHARDSFRDNTHGSGQPSQREQYLPGA
69
ALFNVNNSNKEQEPKEKKKKKKEKKSKP...
| | | | | | | | | | | | | | | | | | | | |
SNKEQEPKEKKKKKKKEKKSKP...
93

63 kDa

FIG. 23. **Peptide 7 binding region near the N-terminus of the 80 kDa α subunit.** The boxed sequence represents the peptide used to generate the polyclonal antibody PPc 80N. The numbering corresponds to that of the 80 kDa α subunit. The N-terminus of the 63 kDa α subunit is included for perspective.

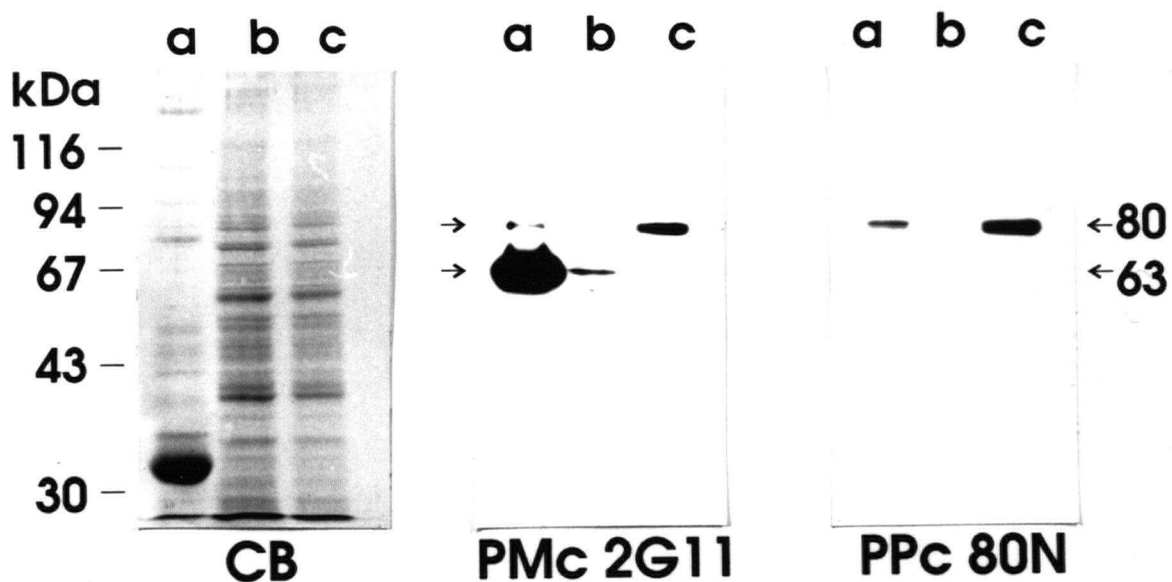


FIG. 24. Western blots of the native rod α subunit and 63 and 80 kDa α subunits heterologously expressed in COS-1 cells and labeled with the 80 kDa-specific polyclonal antibody PPc 80N. SDS gels were stained with coomassie blue (CB, left panel) and western blots were labeled with anti α subunit antibodies PMc 2G11 (monoclonal) and PPc 80N (polyclonal) followed by ECL. Lane a, isolated whole ROS (20 μ g); lane b, membrane extract from COS-1 cells transfected with pAX63 expression plasmid (10 μ g); lane c, membrane extract from COS-1 cells transfected with pAX80 expression plasmid (10 μ g). PPc 80N labeled a 78 kDa polypeptide in both the native ROS and the extract containing the heterologously expressed 80 kDa α subunit, but did not label any polypeptides in the extract containing the heterologously expressed 63 kDa α subunit (right panel). PMc 2G11 (control, middle panel) labeled a 63 kDa polypeptide in both the native ROS and the extract containing the heterologously expressed 63 kDa α subunit. It also labeled a 78 kDa polypeptide in extract containing the heterologously expressed 80 kDa α subunit and faintly labeled a 78 kDa polypeptide in ROS.

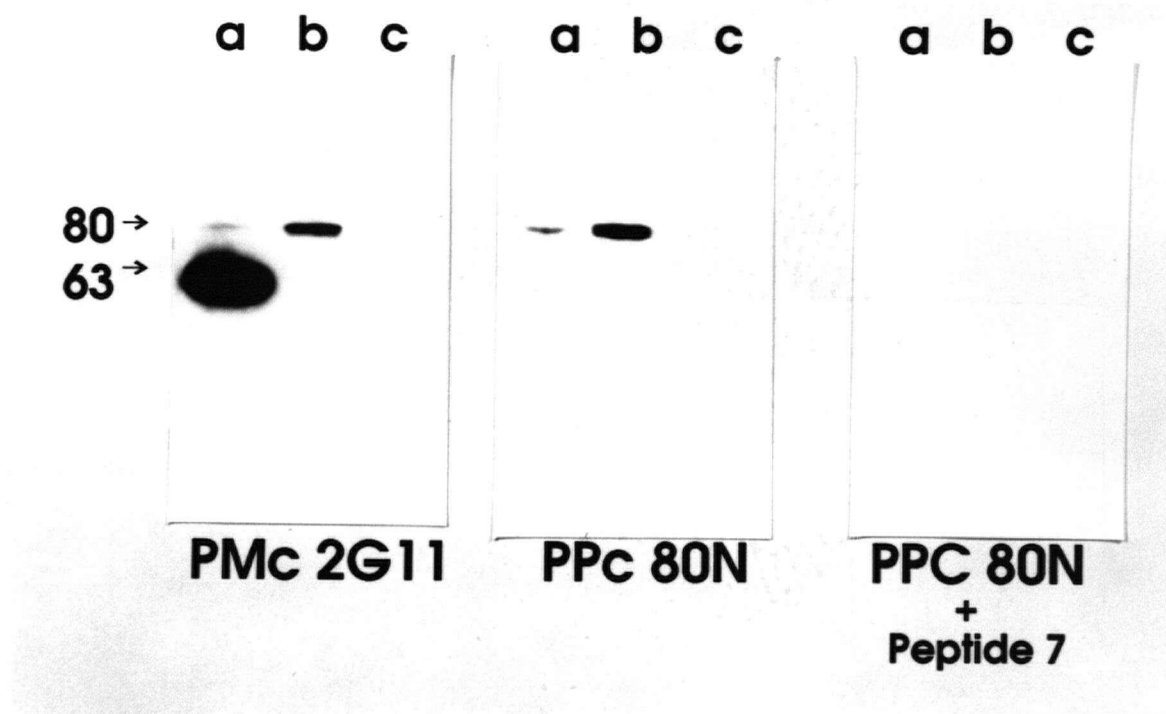


FIG. 25. Western blots of the native rod α subunit and 80 kDa α subunit heterologously expressed in COS-1 cells labeled with 80 kDa specific anti- α subunit polyclonal antibody PPc 80N. Lane a, isolated ROS (20 μ g); lane b, membrane extract from COS-1 cells transfected with pAX80 expression plasmid (10 μ g); lane c, membrane extract from COS-1 cells transfected with pAX112 (control) expression vector (10 μ g). PMc 2G11 labels the 63 kDa α subunit as well as a faint band at 78 kDa in ROS and it labels the 80 kDa α subunit in COS-1 cell extracts. PPc 80N labels the 80 kDa α subunit faintly in native ROS and the 80 kDa α subunit in COS-1 extracts (*middle panel*). Peptide 7 (*right panel*) inhibits PPc 80N binding to both the native and h/e 80 kDa α subunits. Western blots were labeled with anti- α subunit polyclonal antibody PPc 80N and labeled with sheep anti-mouse Ig peroxidase for ECL.

the heterologously expressed 80 kDa α subunit, as well as the faint band in native ROS membranes.

Immunocytochemistry was used to determine if PPc 80N recognizes the heterologously expressed 80 kDa α subunit *in situ*. COS-1 cells transfected with pAX80 were indeed recognized by PPc 80N (**fig 26b**). Cells expressing the 63 kDa α subunit were not labeled (**fig 26a**). Peptide 7 was also a potent competitor for the binding of PPc 80N indicating that the labeling is specific (**fig 26b**). These results together confirm that PPc 80N can be used to identify the 80 kDa α subunit on blots and *in situ*. Most importantly, PPc 80N does not recognize the native or the heterologously expressed 63 kDa α subunit, and along with PMc 6E7 it is possible to differentiate between the two alternate forms of the α subunits.

3.2.3 Localization of the 80 kDa α subunit expressed in rod cells

In order to detect and localize the 80 kDa form of the α subunit in the rod cells, bovine retinal sections were labeled with the PPc 80N antibody for analysis by immunofluorescence. Faint labeling was observed in the rod outer segment layer. (**fig 27a**). No labeling above background was seen in the rod inner segments. The faint labeling of the ROS could be reduced by the presence of peptide 7 (**fig 27b**), indicating that the labeling is real and specific for the 80 kDa α subunit. The secondary antibody, however, exhibited some non-specific labeling, resulting in a high fluorescence background (**fig 27, compare c and d**). A phase contrast micrograph of the retinal section is also included (**fig 27e**). These results suggest that the 80 kDa α subunit may exist in the outer segments of rod cells but at levels that are barely detectable, and that it is not present in significant amounts in the inner segments.

Another biochemical approach was also used to localize the 80 kDa α subunit and to compare the ratio of 63:80 kDa polypeptides within the rod cell. Homogenized retinas were fractionated on a sucrose density gradient. Fractions from the gradient were subjected to electrophoresis and western blots were labeled with both PMc 2G11 and

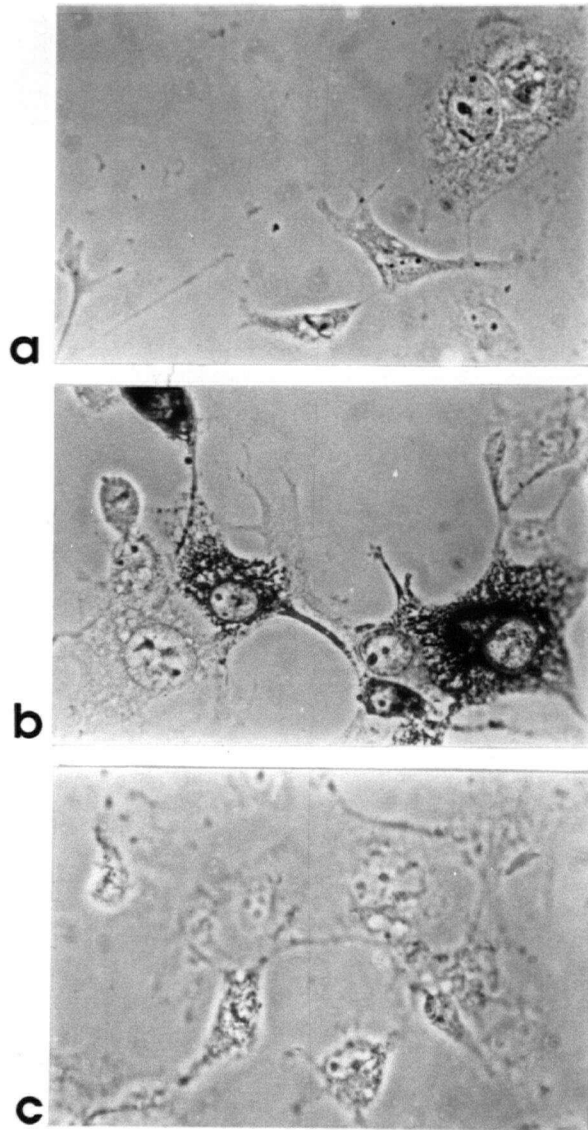


FIG. 26. Immunoperoxidase staining of COS-1 cells expressing the 63 and 80 kDa α subunits of the bovine rod CNG channel. COS-1 cells were fixed and permeabilized with methanol on glass discs and labeled with PPc 80N followed by horse radish peroxidase-sheep anti-mouse Ig. Immunoreactivity appeared as dark reaction product. COS-1 cells transfected with pAX63 (*a*) or pAX80 in the absence (*b*) or presence (*c*) of Peptide 7. PPc 80N did not label COS-1 cells expressing the 63 kDa α subunit. COS-1 cells expressing the 80 kDa α subunit were labeled with PPc 80N; labeling was inhibited with Peptide 7.

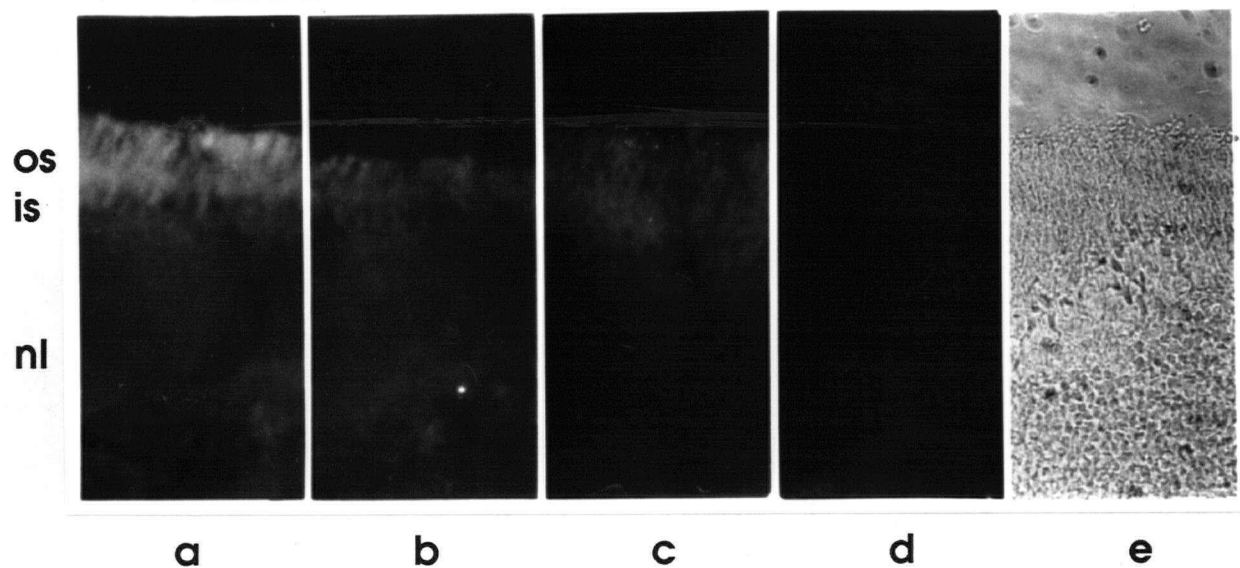


FIG. 27. Immunofluorescent micrographs of bovine retina labeled with 80 kDa-specific anti α subunit polyclonal antibody PPc 80N. Cryostat sections of paraformaldehyde-fixed bovine retina were labeled with PPc 80N (primary antibody) and fluorescein isothiocyanate-goat anti-mouse Ig (secondary antibody). Labeling was carried out in the absence (a) or in the presence of synthetic Peptide 7 (b). Also included are sections labeled with secondary antibody only (c) and primary antibody only (d). A phase contrast micrograph is included (e). The outer segments are labeled lightly with PPc 80N and labeling is reduced in the presence of Peptide 7. The secondary antibody alone causes a low fluorescence background which is eliminated in the absence of secondary antibody. os, outer segment; is, inner segment; nl, nuclear layer.

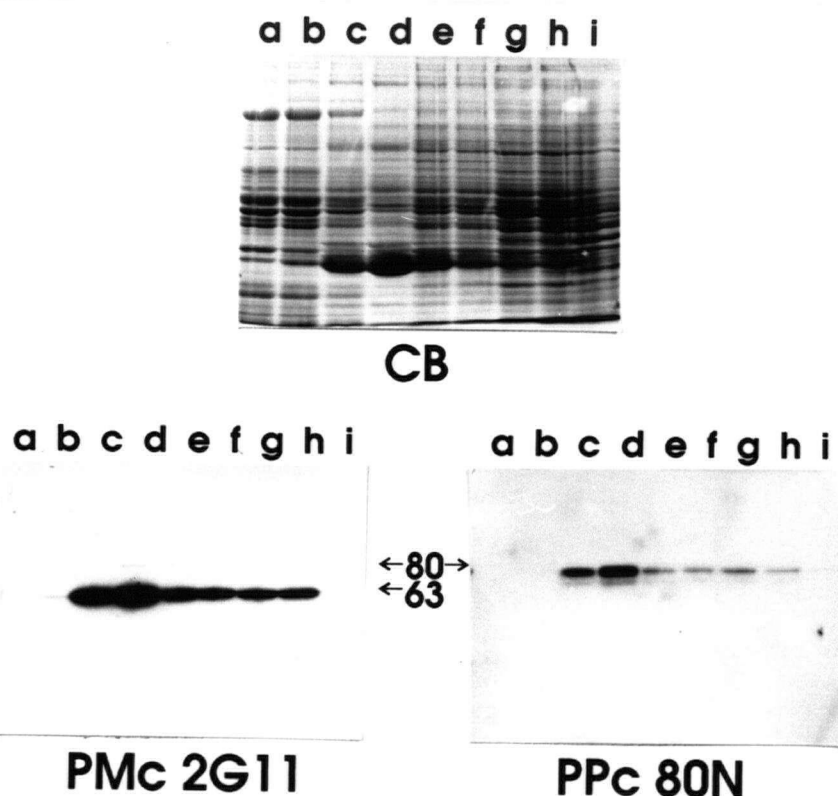


FIG. 28. SDS gels and Western blots of homogenized bovine retinas fractionated on a sucrose gradient. SDS gels were stained with coomassie blue (CB, upper gel) and western blots were labeled either with anti α subunit monoclonal antibody PMc 2G11 (lower left) or with 80 kDa specific anti α subunit polyclonal antibody PPc 80N (lower right). Primary antibodies were labeled with sheep anti-mouse or sheep anti-rabbit Ig peroxidase for ECL. Lane a through i, aliquots taken from gradient beginning at the top (a) and proceeding to the pellet at the bottom of the gradient (i); (lanes c and d represent the pink band harvested during a ROS prep). PMc 2G11 labels the 63 kDa α subunit throughout the gradient. PPc 80N labels the 80 kDa α subunit throughout the gradient with a similar concentration profile to that of the 63 kDa α subunit. The exposure time for the PPc 80N labeled blot was five times as long as that for the PMc 2G11 labeled blot, in order to visualize the 80 kDa α subunit signal in all the lanes.

PPc 80N antibodies (**fig 28**). PMc 2G11 exhibited faint labeling of the 80 kDa polypeptide above the 63 kDa α subunit. By examining a longer exposure of this blot, scanning densitometry showed that the ratio of 63:80 kDa α subunits was the same throughout all the samples. This expression pattern indicates that where the 63 kDa α subunit occurs, a small fraction (less than 0.5%) of the 80 kDa form co-exists. Other parts of the gradient are enriched in inner segments and the retinal pigment epithelium, and apparently they do not express high levels of the 80 kDa α subunit.

3.3 Evidence for truncated forms of the CNG α subunit in other species

Studies outlined above, indicated that the bovine CNG channel α subunit primarily exists as the truncated 63 kDa polypeptide. It was of interest to determine if similar modifications of CNG channel α subunits occur in other species.

3.3.1 Chicken rod and cone α subunit

A study of the CNG channels of chicken retinas (which are now known to be the α subunits) was carried out to ascertain whether the chicken rod and cone photoreceptor α subunits are processed in a similar manner as the bovine photoreceptor α subunit. For these studies, chicken retinas were isolated and subjected to electrophoresis for analysis by western blotting (**fig 29**). The PMc 1F6 monoclonal antibody was used to label the α subunit of both the chicken rod and cone channel. It was raised against the bovine retinal CNG channel α subunit and has been shown to bind to an epitope (Bönigk *et al.*, 1993) which is highly conserved between bovine and chicken photoreceptor and bovine olfactory CNG α subunits (**fig 30**). The PPc CC1 polyclonal antibody was used to label the chicken cone α subunit. This antibody was raised against a peptide specific for the chicken cone channel α subunit sequence and has been shown by immunofluorescence to specifically label cone outer segments in chicken retinas (Bönigk *et al.*, 1993).

Western blots of extracts from COS-1 cells transfected with the chicken rod (pCHIROPD) and cone (pCHICON) α subunits labeled with PMc 1F6 and PPc CC1 are

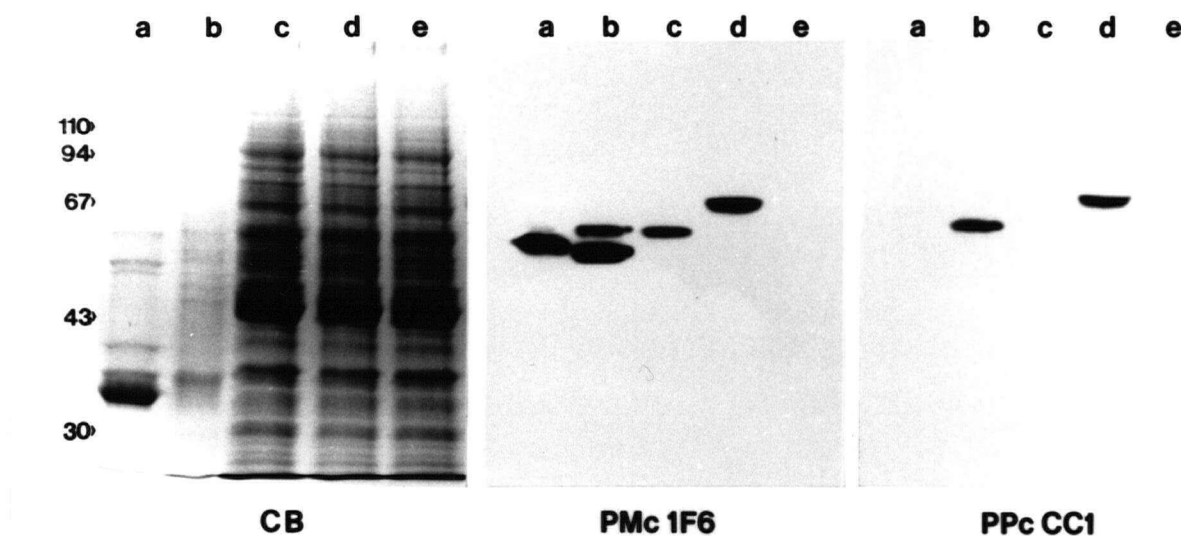


FIG. 29. **SDS gels and Western blots of the native and expressed chicken rod and cone α subunits.** SDS gels were stained with coomassie blue (*CB*, *left panel*) or transferred to immobilon and labeled either with PMc 1F6 (against a highly conserved region near the C-terminus of CNG α subunits, *middle panel*) or PPc CC1 (against the C-terminus of the chicken cone α subunit, *right panel*). *Lane a*, isolated whole ROS (bovine, 10 μ g); *lane b*, chicken retinal membranes (10 μ g); *lane c*, membrane extract from COS-1 cells transfected with pCHIROP expression plasmid (20 μ g); *lane d*, membrane extract from COS-1 cells transfected with pCHICON expression plasmid (20 μ g); *lane e*, membrane extract from COS-1 cells transfected with pAX112 (control) expression vector (20 μ g). PMc 1F6 labeled the rod α subunit in bovine ROS, the rod and cone α subunits in chicken retinal membranes, and both the h/e chicken rod and cone α subunits. PPc CC1 labeled the cone α subunit in chicken retinal membranes and the h/e cone α subunit. Primary antibodies were labeled with sheep anti-mouse Ig peroxidase for ECL.

PMc 1F6 Antibody
Binding Region

BOVRODCHAN	AGNRRTANIKSIGYSDFCLSKDDLMEALTEYPDAKGMLEE	595
CHIRODCHAN	AGNRRTANIRSIGYSDFCLSKDDLMEALTEYPDAKAMLEE	549
CHICONCHAN	SGNRRTANIRSIGYSDFCLSKDDLMEALTEYPEAKKALEE	642
BOVOLEFCHAN	MGNRRTANIRSLGYSDLFCLSKDDLMEAVTEYPDAKRVLEE	572
	*****.******.******.******.*	

FIG. 30. Alignment of the bovine rod, bovine olfactory, and the chicken rod and cone CNG α subunits in the region of the PMc 1F6 binding site. The boxed region represents the synthetic peptide shown to be recognized by the monoclonal antibody PMc 1F6.

shown in figure 29. PMc 1F6 labels a band at ~66 kDa in COS-1 cells expressing the rod α subunit (**fig 29, lane c**) and a band at ~75 kDa in COS-1 cells expressing the cone α subunit (**fig 29, lane d**). PPc CC1 labels the same band as PMc 1F6 in COS-1 cells expressing the cone α subunit (**fig 29, lane d**), but does not label any bands in COS-1 cells expressing the rod α subunit (**fig 29, lane c**). PMc 1F6 therefore has a broader specificity, capable of recognizing both the rod and cone α subunits from chicken retina, whereas PPc CC1 recognizes specifically the chicken cone α subunit.

The apparent molecular weight of the rod and cone α subunit in the chicken retinal tissue was also examined on gels and western blots. Blots were labeled with PMc 1F6 and PPc CC1 in order to determine if the M_r of the native photoreceptor CNG α subunits corresponds to the M_r of the heterologously expressed channel (**fig 29, lanes b**). PMc 1F6 labels two polypeptides in chicken retinas, one with an M_r of ~61,000 and the other with an M_r of ~66,000. PPc CC1, however, only labels the polypeptide at 66,000 daltons in the chicken retina (**fig 29, lane b**). These results indicate that chicken retinas contain detectable levels of both the rod and cone α subunits and that the larger of the two is the cone α subunit. PMc 1F6 labels the bovine rod 63 kDa α subunit (**fig 29, lane a**), but the chicken cone specific antibody PPc CC1 does not label any bands in bovine ROS (**fig 29, lane a**).

Comparing the sizes of the native and the expressed rod and cone α subunits it is evident that the rod and cone α subunits expressed in COS-1 cells are significantly larger than those seen in the chicken retinas. The heterologously expressed rod α subunit has a molecular mass of 66 kDa, but in its native form in the chicken retina it exists as a 61 kDa polypeptide. The heterologously expressed cone α subunit has a mass of 75 kDa, but in its native form it exists as a 66 kDa polypeptide. Like the bovine photoreceptor α subunit, the chicken photoreceptor CNG α subunits exhibit reduced M_r 's, apparently due to post-translational cleavage reactions. These findings suggest that the processing plays a role in the biology or physiology of these channel subunits.

3.3.2 Bovine olfactory CNG α subunit

It is of interest to know whether the same processing seen in retinal CNG α subunits exists for CNG α subunits in other tissues. The bovine olfactory CNG α subunit was analyzed by western blotting both in its native tissue and heterologously expressed in COS-1 cells using the PMc 1F6 antibody. Extracts of COS-1 cells transfected with pAX76-HX expressed a polypeptide with an M_r of ~67,000 that was labeled by PMc 1F6 (**fig 31, lane c**). A polypeptide of the same M_r in olfactory epithelium was also recognized by PMc 1F6 (**fig 31, lane b**). ROS were included for comparison and PMc 1F6 labeling of the 63 kDa α subunit is visible (**fig 31, lane a**). These results indicate that the CNG α subunit in the olfactory epithelium does not undergo the same processing seen for α subunits in photoreceptor cells.

The native olfactory α subunit is a very faint band. In order to confirm that the labeling is specific and not just background, the 1F6 peptide was used in a competition study (**fig 31, right panel**). The presence of the 1F6 peptide abolished PMc 1F6 binding to the ROS, olfactory epithelial native α subunit and the expressed olfactory α subunit. This successful competition confirms that the PMc 1F6 binding was specific. The same band labeled on blot in which ROS were not run directly next to olfactory epithelium.

3.4 Reconstitution of the bovine rod CNG channel α subunit

Electrophysiological studies in several laboratories have shown that the α subunit (an 80 kDa polypeptide) can assemble into functional cGMP-gated channels when expressed in oocytes (Kaupp *et al.*, 1989) and HEK 293 cells (Dhallan *et al.*, 1990). It was therefore concluded that the CNG channel is a homooligomer. To facilitate biochemical characterizations, attempts were made to reconstitute the α subunit into liposomes for analysis of functional properties as had been carried out for the native channel (Cook *et al.*, 1987).

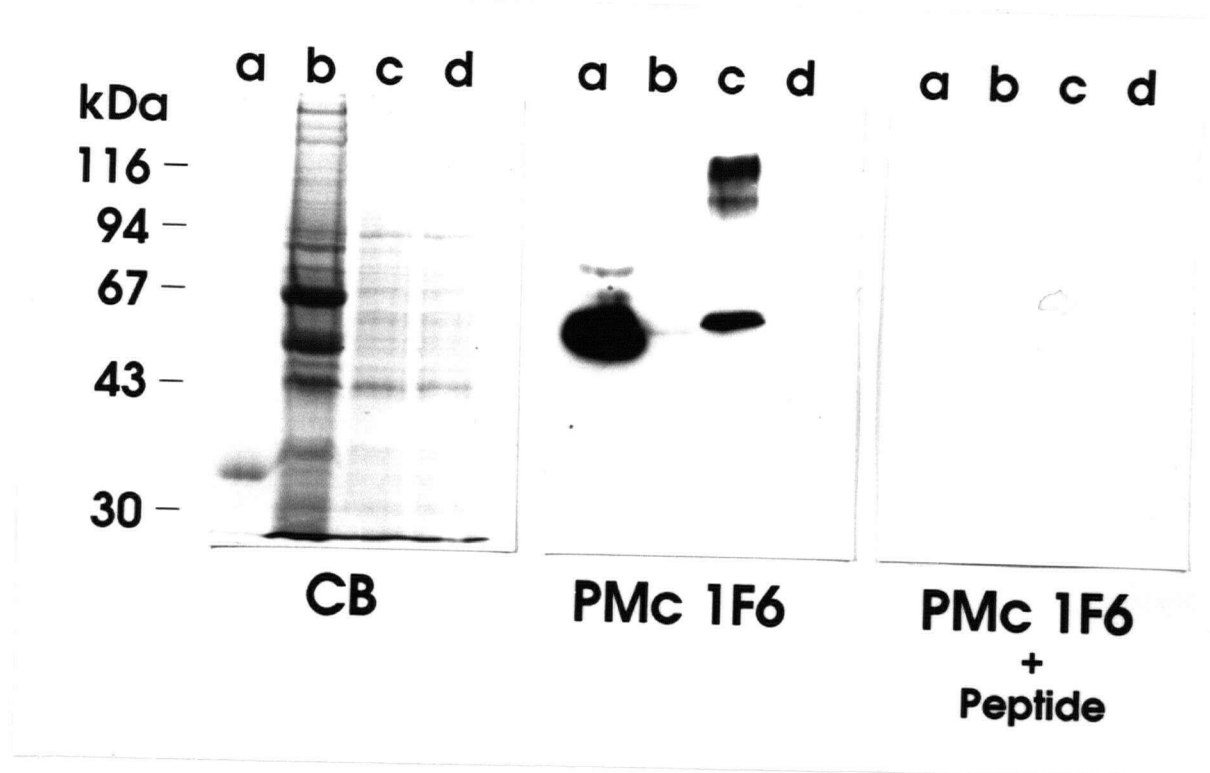


FIG. 31. SDS gels and Western blots of the native and heterologously expressed olfactory α subunits labeled with monoclonal antibody PMc 1F6. Blots were labeled with PMc 1F6 (against a highly conserved region near the C-terminus of CNG α subunits) in the absence (*middle panel*) and presence (*right panel*) of the 1F6 peptide. *Lane a*, isolated whole ROS (bovine, 5 μ g); *lane b*, bovine olfactory epithelium (40 μ g); *lane c*, membrane extract from COS-1 cells transfected with pAX76-HX expression plasmid (7 μ g); *lane d*, membrane extract from COS-1 cells transfected with pAX112 (control) expression vector (7 μ g). PMc 1F6 labeled the rod α subunit in bovine ROS, the olfactory α subunits in bovine olfactory epithelium, and the h/e bovine olfactory α subunit. The presence of the 1F6 peptide inhibited all PMc 1F6 binding indicating specific recognition. SDS gels were stained with coomassie blue (CB, *left panel*) or transferred to immobilon and labeled with sheep anti-mouse Ig peroxidase for ECL.

3.4.1 Direct reconstitution of the α subunit

Initial attempts were made to reconstitute the solubilized COS-1 cell extracts directly into liposomes. COS-1 cells transfected with pAX63 and pAX80 were solubilized and the unpurified post nuclear extract was collected. These extracts were combined with purified lipids in the presence of Ca^{2+} . Dialysis to remove the solubilizing detergent effectively reconstituted the h/e α subunit into Ca^{2+} loaded lipid vesicles. These liposomes were then tested in a Ca^{2+} efflux assay. Upon the addition of cGMP to vesicles containing the heterologously expressed α subunits, no Ca^{2+} efflux was detected. As a positive control, the same experiment was carried out with ROS membranes. The reconstituted ROS membranes exhibited Ca^{2+} efflux upon addition of cGMP (**fig 32**). Both the ROS membranes and COS-1 cells were solubilized at similar protein concentrations (~ 1.7 mg/ml) and equal aliquots of reconstituted vesicles were analyzed on western blots (**fig 32A**). This was the most direct way of quantitatively comparing the amounts of reconstituted h/e α subunit with the amount of the native 63 kDa α subunit reconstituted from ROS. Approximately 20 μg and 2 μg of both COS-1 vesicles and ROS vesicles were examined side by side. The COS-1 vesicles sample with 20 μg of protein (**fig 32, lane d**) appeared to contain less α subunit than 2 μg of ROS vesicles (**fig 32, lane a**). The conclusion was that the amount of heterologously expressed α subunit incorporated into lipid vesicles by this method was at least 20-40 times lower than the amount incorporated from ROS membranes. It was clear that this was not enough heterologously expressed protein for activity measurements and that it would be necessary to purify the α subunit in order to reconstitute it at higher concentrations.

3.4.2 Addition of the rho 1D4 epitope to the α subunit C-terminus

Immunopurification columns are an efficient method to specifically purify and concentrate proteins. An antibody with a characterized binding epitope is required so that a synthetic peptide corresponding to the binding site can be used to elute the protein from the antibody. This also requires that the antibody recognizes the protein and the

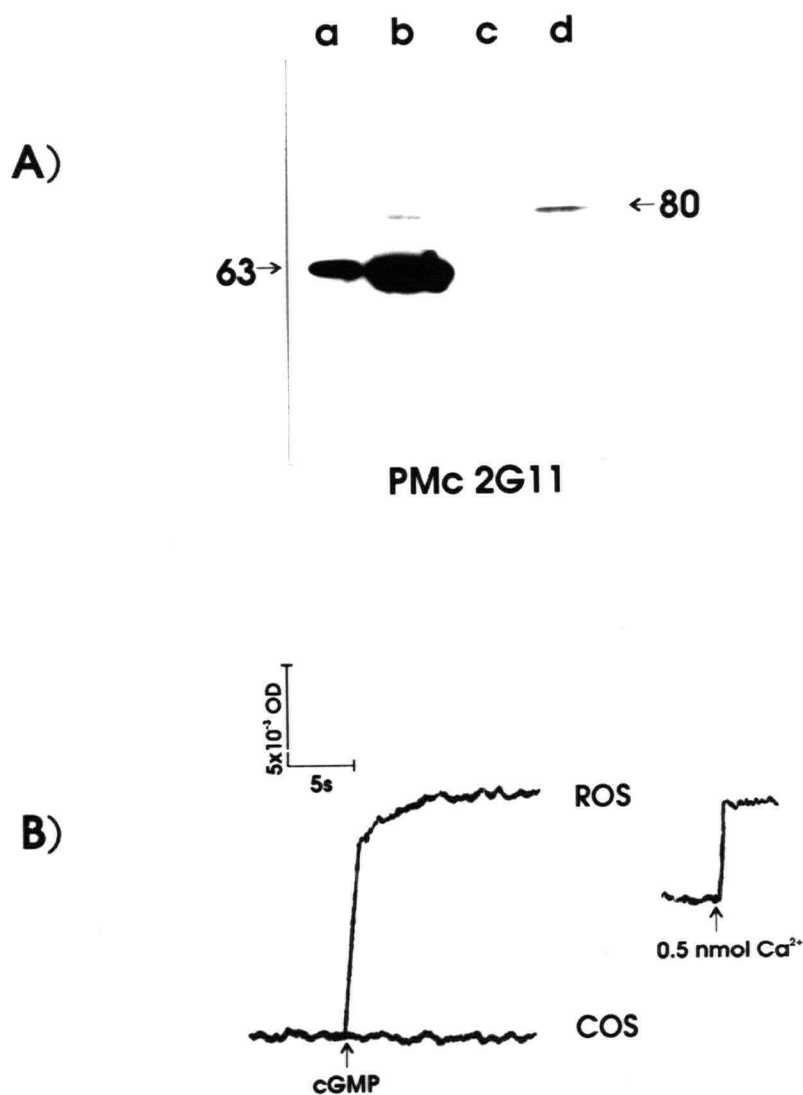


FIG. 32. Reconstitution of ROS membrane proteins and COS-1 cell membrane proteins for analysis of cGMP dependent channel activity. *A)* Western blot labeled with PMc 2G11: *Lane a*, ROS proteins reconstituted into lipid vesicles (2 μ g); *lane b*, ROS proteins reconstituted in to lipid vesicles (20 μ g); *lane c*, pAX80 transfected COS-1 cell membrane proteins reconstituted into lipid vesicles (2 μ g); *lane d*, pAX80 transfected COS-1 cell membrane proteins reconstituted into lipid vesicles (20 μ g). *B)* Activity traces: cGMP-dependent Ca²⁺ efflux activity from reconstituted ROS proteins (*upper trace*) and from reconstituted pAX80 transfected COS-1 cell proteins (*lower trace*). 20 μ g of reconstituted COS-1 cell extracts has less α subunit than 2 μ g of reconstituted ROS membranes. Traces show significant channel activity (Ca²⁺ efflux) upon addition of cGMP (*arrow*) to reconstituted ROS ; no activity is seen for the reconstituted COS-1 cell extracts.

peptide with comparable affinities so that the peptide can successfully compete with the protein for the antibody binding site, thereby eluting the protein. No antibodies meeting these criteria were available for either the 63 kDa α subunit or 80 kDa α subunit. In order to develop a means of immunopurifying of the 63 and 80 kDa α subunits from transfected COS-1 cells, the nine C-terminal amino acids of rhodopsin were engineered onto their C-termini. The C-terminus of rhodopsin is recognized by the well characterized antibody rho 1D4 (MacKenzie *et al.*, 1984, Hodges *et al.*, 1988), and a rho 1D4 immunoaffinity column has been used successfully to purify rhodopsin from COS-1 cells (Oprian *et al.*, 1987). The resultant expression plasmids which coded for 1D4-tagged α subunits were called pAX801D4 and pAX631D4 (**fig 33**).

PCR was used to generate a modified C-terminal cassette #1 which was the cassette at the 5' end of the coding sequence (see original channel construction, **fig 14**). The downstream primer (at the 3' end) was a synthetic oligonucleotide which directed the addition of the coding sequence for 9 rhodopsin C-terminal amino acids onto the C-terminus of the α subunit cDNA. This 1D4TAIL primer (section 2.17) was used in conjunction with primer P2 (section 2.11) as the upstream primer. The resultant PCR product was the recombinant cassette #1 with additional bases coding for 9 extra amino acids added onto the 5' end. This modified cassette was combined with cassettes #2-4A and #2-4B to reconstruct the 80 kDa and 63 kDa 1D4-tagged α subunit sequences (**fig 33**).

Transfection of COS-1 cells with the modified expression vectors followed by solubilization, electrophoresis and western blotting showed that the tagged α subunits were now recognized by the rhodopsin antibody rho 1D4 (**fig 34**). Rho 1D4 labels rhodopsin in ROS membranes at 39 kDa (**fig 34, lane a**) as well as both the 1D4 tagged 63 and 80 kDa heterologously expressed CNG α subunits (**fig 34, lane c&d**). The doublet of the h/e 63 kDa α subunit is again visible. Extracts of cells expressing the 80 kDa channel without the 1D4 tail are not recognized by rho 1D4 (**fig 34, lane b**).

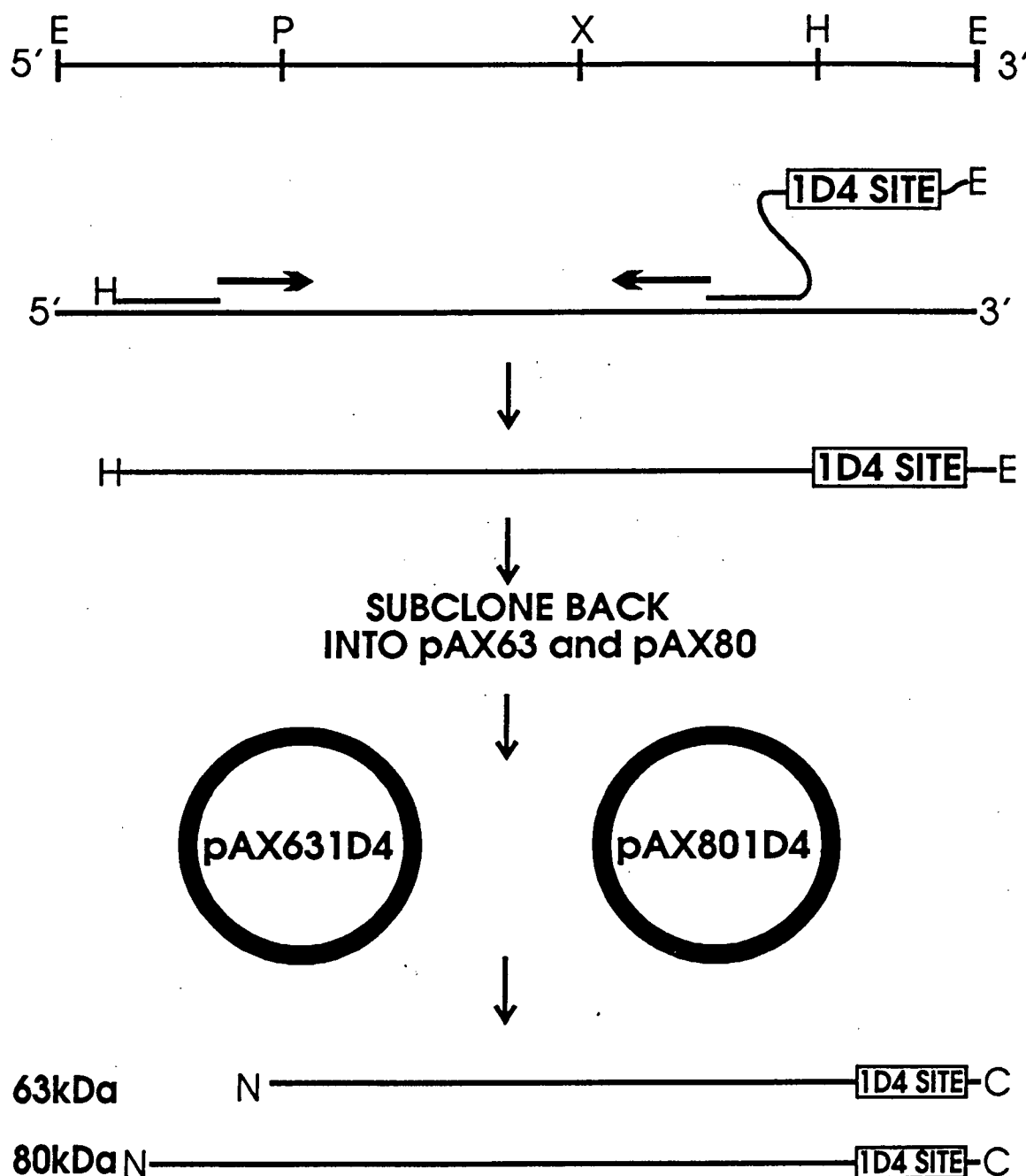


FIG. 33. Schematic diagram of the addition of the rho 1D4 epitope to the C termini of the 63 and 80 kDa α subunits. Cassette #1 (see figure 16) was modified by PCR extension. Twenty-seven bases followed by a stop codon were added to the 3' end. These bases coded for the last nine amino acids of rhodopsin which are recognized by the anti-rhodopsin monoclonal antibody rho 1D4. The extended cassettes were substituted back into pAX63 and pAX80 to generate the expression plasmids pAX631D4 and pAX801D4. The α subunits expressed by these plasmids carried the rho 1D4 epitope at their C-termini.

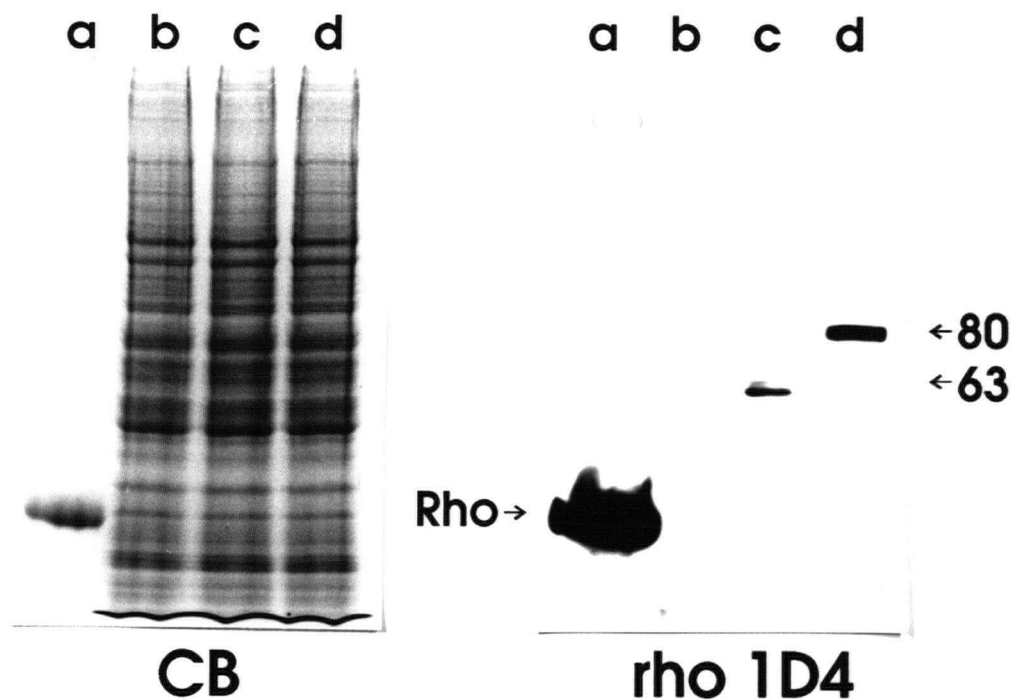


FIG. 34. Western blots of heterologously expressed rho 1D4-tagged 63 and 80 kDa α subunits labeled with anti-rhodopsin monoclonal antibody rho 1D4. SDS gels were stained with coomassie blue (CB) and western blots were labeled with anti rhodopsin monoclonal antibody rho 1D4 and labeled with sheep anti-mouse Ig peroxidase for ECL. *Lane a*, isolated whole ROS (10 μ g); *lane b*, membrane extract from COS-1 cells transfected with pAX80 (negative control) expression plasmid (20 μ g); *lane c*, membrane extract from COS-1 cells transfected with pAX631D4 expression plasmid (20 μ g); *lane d*, membrane extract from COS-1 cells transfected with pAX801D4 expression plasmid (20 μ g). Rho 1D4 labeled the 39 kDa rhodopsin in native ROS as well as 63 and 78 kDa polypeptides in the extracts containing the heterologously expressed rho 1D4-tagged 63 kDa α subunit and 80 kDa α subunit. Rho 1D4 did not recognize the heterologously expressed α subunit lacking the rho 1D4 amino acid tag.

Comparing band intensities on western blots, the addition of the 1D4 epitope to the α subunits appears to have no adverse affect on the expression level.

In order to confirm that the addition of the 1D4 epitope to the C-termini of the heterologously expressed α subunits had no apparent deleterious effects, their expression pattern was examined in COS-1 cells. Immunofluorescence studies carried out on cells transfected with pAX631D4 and pAX801D4 are shown in figure 35. COS-1 cells transfected with pAX801D4 (fig 35b) and with pAX631D4 (fig 35c) are labeled with the anti-rhodopsin monoclonal antibody rho 1D4. Cells transfected with pAX80 (lacking the 1D4 tail) are not labeled by rho 1D4 (fig 35a). The labeling pattern is the same for COS-1 cells expressing α subunits with or without the rho 1D4 tag. Labeling is seen throughout the cell, and on the plasma membrane. Phase contrast micrographs of the same cells are included (fig 35 d,e,f)

3.4.3 Immunopurification of rho 1D4-tagged α subunit

The effectiveness of the rho 1D4 tag for immunopurification of the channel was then ascertained. It was shown that the addition of the rho 1D4 epitope to the 63 and 80 kDa α subunits facilitated their immunopurification (fig 36A&B). For these studies monoclonal antibody rho 1D4 was conjugated to Sepharose beads to generate an immunoaffinity column. The solubilized COS-1 cell extracts were loaded onto the 1D4-Sepharose column, and after washing the column to remove unbound proteins, the 1D4-tagged α subunit was eluted with a peptide corresponding to the Rho 1D4 recognition site (MacKenzie *et al.*, 1984, Hodges *et al.*, 1988). Western blots were used to monitor the immunopurification process. For comparison, ROS and the original COS-1 cell extracts were included on the blot (fig 36, lanes a&b). The unbound and wash fractions contain no signal, indicating that the column removes all the rho 1D4-tagged α subunit (fig 36, lanes c&d). The peptide eluted fraction contains abundant amounts of purified α subunits (fig 36, lanes e). The large smears of labeling seems to be a symptom of over loading, since dilutions of the same samples label as discreet bands (fig 36, lanes f).

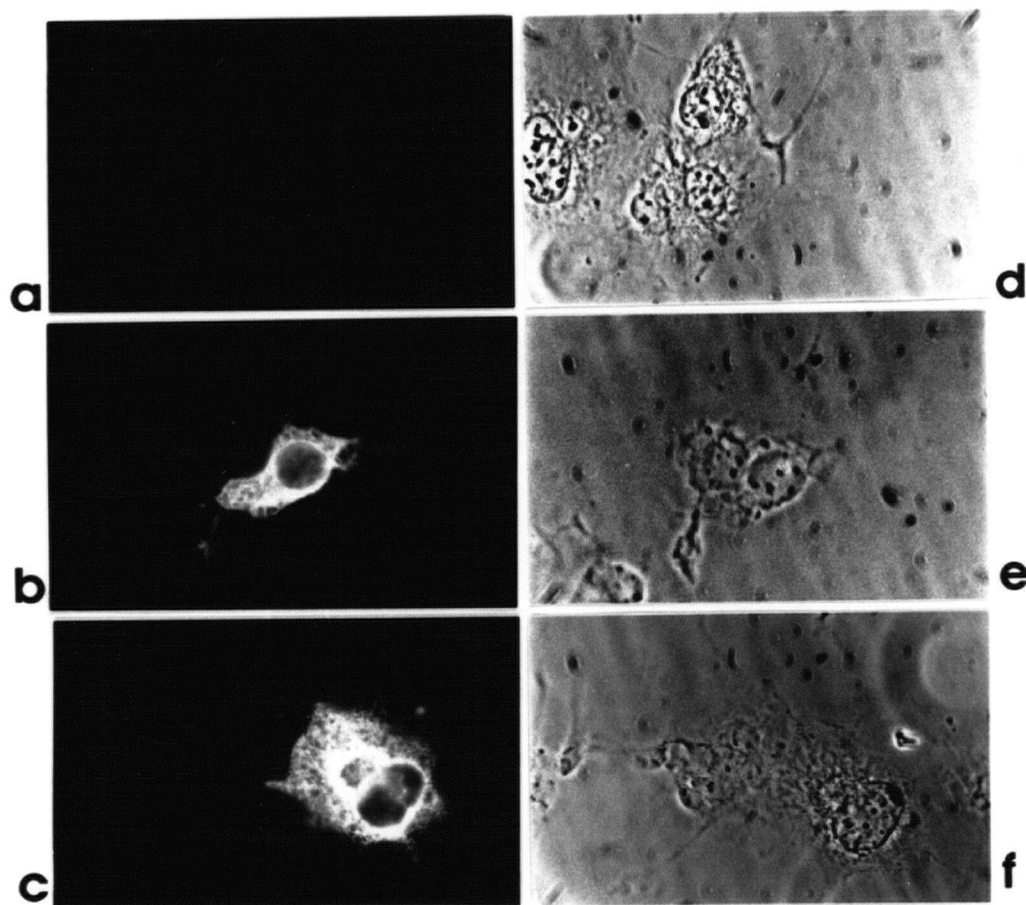


FIG. 35. Immunofluorescent labeling of COS-1 cells expressing the rho 1D4-tagged 63 and 80 kDa α subunits of the bovine rod CNG channel. (*a,b,c*) fluorescence micrographs of COS-1 cells transfected with pAX80 (*a*, control), pAX631D4 (*b*) and pAX801D4 (*c*); (*d,e,f*) phase contrast micrographs of the same COS-1 cells. COS-1 cells were fixed and permeabilized with methanol on glass discs and labeled with anti-rhodopsin antibody rho 1D4 and fluorescein isothiocyanate-goat anti-mouse Ig. COS-1 cells expressing the rho 1D4-tagged 63 kDa α subunit and rho 1D4-tagged 80 kDa α subunit were labeled with rho 1D4. COS-1 cells expressing the α subunit lacking a rho 1D4 tag were not labeled.

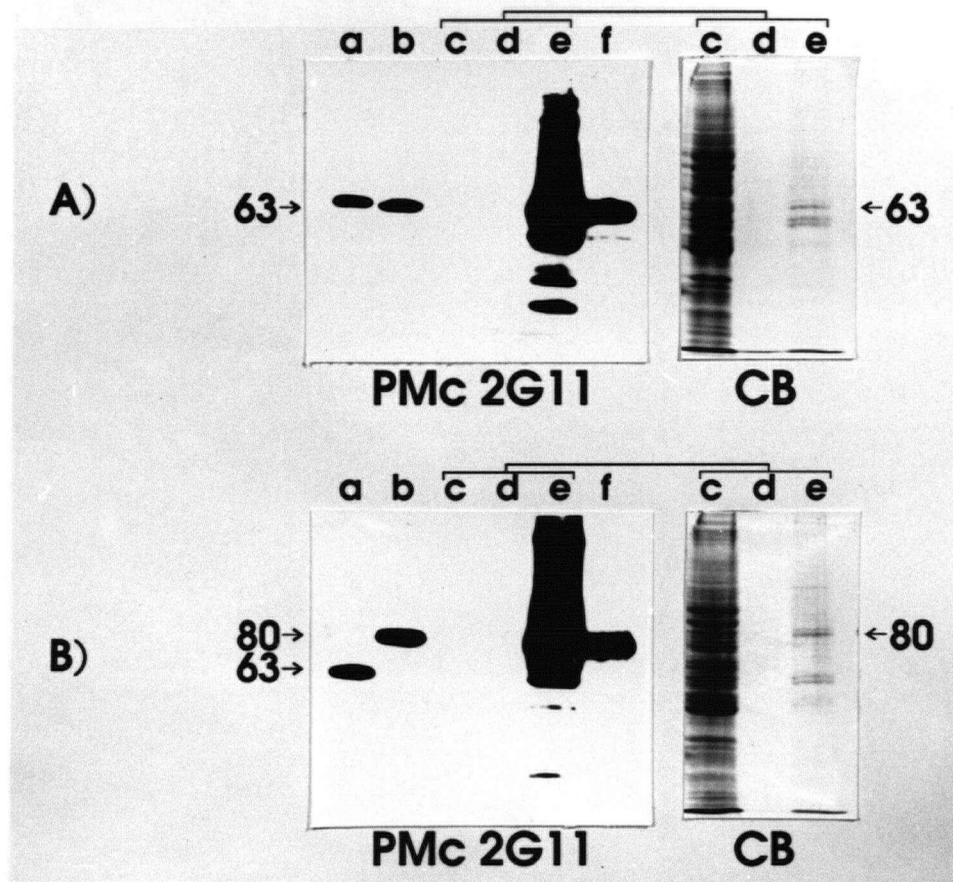


FIG. 36. SDS Gels and western blots of the heterologously expressed rho 1D4-tagged 63 and 80 kDa α subunits purified on a rho 1D4-Sepharose column. SDS gels were stained with coomassie blue (CB, right panels) and western blots were labeled with anti α subunit monoclonal antibody PMc 2G11 and labeled with sheep anti-mouse Ig peroxidase for ECL. *Western blot: lanes a*, isolated whole ROS (10 μ g); *lanes b*, (precolumn sample) membrane extract from COS-1 cells transfected with pAX631D4 (upper left) or pAX801D4 (lower left) expression plasmid (20 μ g); *lanes c*, (unbound) proteins that did not bind to the antibody column; *lanes d*, column wash solution; *lanes e*, (peptide eluted fraction) immunopurified 1D4-tagged 63 kDa (upper) and 80 kDa (lower) α subunits; *lanes f*, 10-fold dilutions of samples in lanes e. *Coomassie blue stained gel: lanes c*, (unbound) proteins that did not bind to the antibody column; *lanes d*, column wash solution; *lane e*, (peptide eluted fraction) immunopurified 1D4-tagged 63 kDa (upper) and 80 kDa (lower) α subunits (10 μ l). Arrows on the right indicate the eluted 1D4-tagged 63(A) and 80 (B) kDa α subunits.

Coomassie blue stained gels of the purified 1D4-tagged α subunits are in the right hand panels (**fig 36, lanes c,d&e**). Faint bands in the peptide eluted fraction as indicated by arrows appear to represent the channel α subunits (**fig 36, lanes e**). A few contaminating proteins are also present in the immunopurified fraction. These may be sticking non-specifically to the affinity column or to unfolded α subunit.

3.4.4 Reconstitution of heterologously expressed 1D4-tagged α subunit

Since it was clear that a higher concentration of the heterologously expressed channel would be required in order to measure activity, immunopurification was employed in order to purify large amounts of the α subunits. COS-1 cells transfected with pAX801D4 were solubilized and purified on a rho 1D4-Sepharose bead column. The peptide eluted fraction was reconstituted into Ca^{2+} loaded liposomes and tested for cGMP-gated Ca^{2+} efflux. The activity measured for the immunopurified 1D4-tagged 80 kDa α subunit was very low, barely above background.

As part of the control, ROS were solubilized in parallel with COS-1 cells, and serial dilutions were performed on the solubilized ROS before reconstitution (**fig 37A**). In this way the lower limit of detectable channel activity was determined. Figure **37B** shows the traces of cGMP-gated Ca^{2+} efflux for dilutions of ROS membranes beginning with 0.34 mg which gave a strong signal, down to the efflux measurement of channel contained in 42.5 μg solubilized ROS membrane protein. The solubilized ROS membranes were diluted in solubilization buffer and the diluted membranes were mixed with consistent portions of reconstitution mixture. The amount of vesicles therefore remained constant while the ROS membrane content (and hence the number of CNG channels) decreased. Consequently, the channel activity (defined as the percentage of total releasable calcium, see section 1.4.2 in Introduction) decreases throughout the series of dilutions, while the Ca^{2+} efflux per milligram of ROS membranes (nmol/mg) remains approximately the same (**fig 37C**).

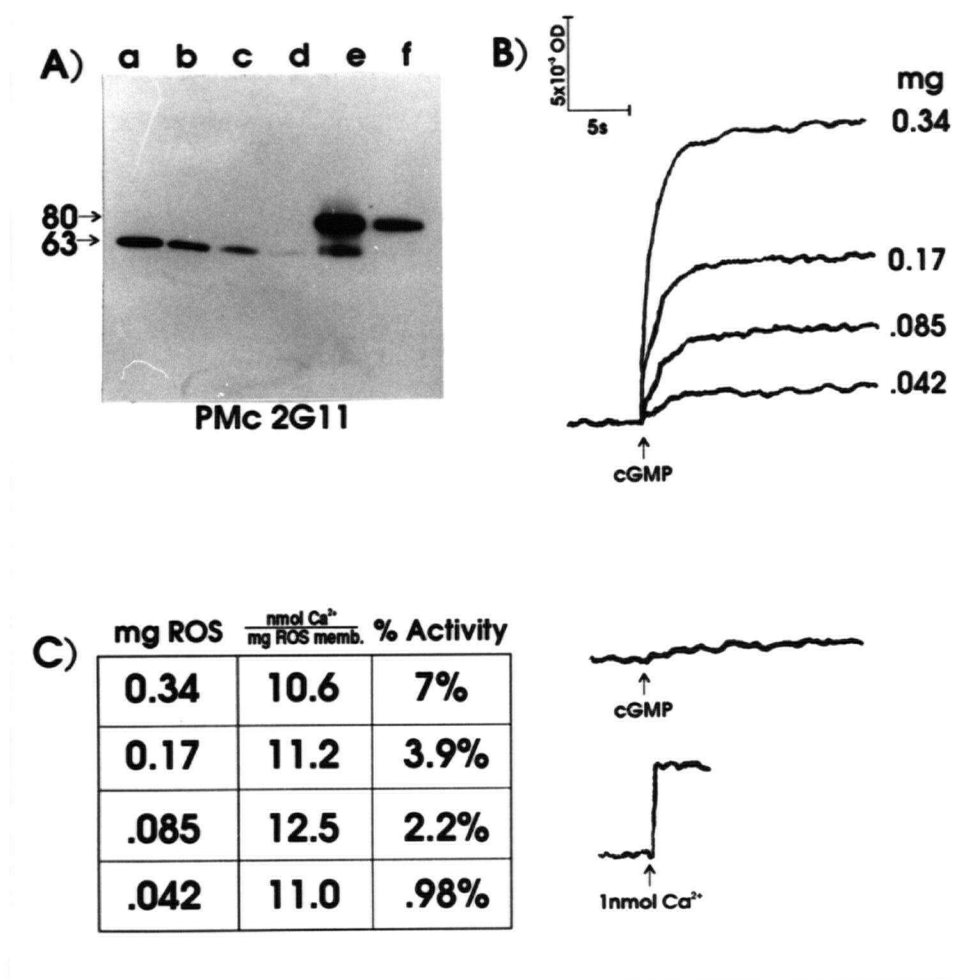


FIG. 37. Reconstitution of serially diluted solubilized ROS membranes and heterologously expressed 1D4-tagged 80 kDa α subunit *A*) Western blot (labeled with PMc 2G11): *Lane a* through *d*, serial dilutions of ROS membranes reconstituted into lipid vesicles: 0.34 mg (*a*), 0.17 mg (*b*), 0.085 mg (*c*), 0.042 mg (*d*); *lane e*, immunopurified 1D4-tagged 80 kDa α subunit reconstituted into lipid vesicles (first peptide eluted fraction); *lane f*, immunopurified 1D4-tagged 80 kDa α subunit reconstituted into lipid vesicles (second peptide eluted fraction). *B*. Activity traces: *upper panel*, Ca^{2+} efflux traces from serially diluted ROS membranes reconstituted into lipid vesicles; *lower panel*, Ca^{2+} efflux traces from immunopurified 1D4-tagged 80 kDa α subunit reconstituted into lipid vesicles. Ca^{2+} efflux from the most diluted solubilized ROS membrane sample is at least twice that seen for the reconstituted immunopurified 1D4-tagged α subunit. *C*) Table presenting calculated values for the serially diluted reconstituted ROS.

In order to qualitatively compare the amount of h/e α subunit reconstituted with the amount of native channel reconstituted, samples of the Ca^{2+} loaded vesicles were electrophoresed and examined by western blot. The immunopurified heterologously expressed channel α subunit was present in the vesicles in greater quantity than that present in the vesicles made with the greatest amount of solubilized ROS (0.34 mg) (**fig 37A, compare lanes e&a**). The amount of Ca^{2+} efflux seen through the heterologous channel, however, was approximately half of that seen for the vesicles made with the least amount of solubilized ROS (42.5 μg) (**fig 37B, compare lanes e&d**). In other words, comparing band intensities on a western blot, the immunopurified heterologously expressed 1D4-tagged 80 kDa α subunit exhibits less than 1% of the activity exhibited by an equal amount of native CNG channel present in solubilized ROS membranes.

3.5 The beta subunit of the bovine rod CNG channel

The cloning of the human rod CNG channel β subunit was reported by Chen and co-workers (1993). It was clear that in order to properly characterize the molecular properties of the CNG channel, the inclusion of the β subunit was mandatory. The cloning of the bovine β subunit was carried out as a collaboration between the laboratories of R.S. Molday and U.B. Kaupp. It was hoped that the β subunit co-expressed with the α subunit would stabilize the heterologously expressed channel during solubilization and reconstitution resulting in a functionally active CNG channel.

3.5.1 Construction of β and β' expression vectors

The latter 3/5 of the bovine β subunit (Körschen *et al.*, 1995) was ~80% identical to the human β subunit (Chen *et al.*, 1993). Figure 38 delineates the regions of overlap between the bovine β subunit and soluble GARP and the human β subunit clones. The first 2/5 of the sequence coded for a protein component which was practically identical to a seemingly unrelated soluble protein cloned from bovine rod cells which was missing in the published human β sequence. The first 571 amino acids of the bovine β subunit

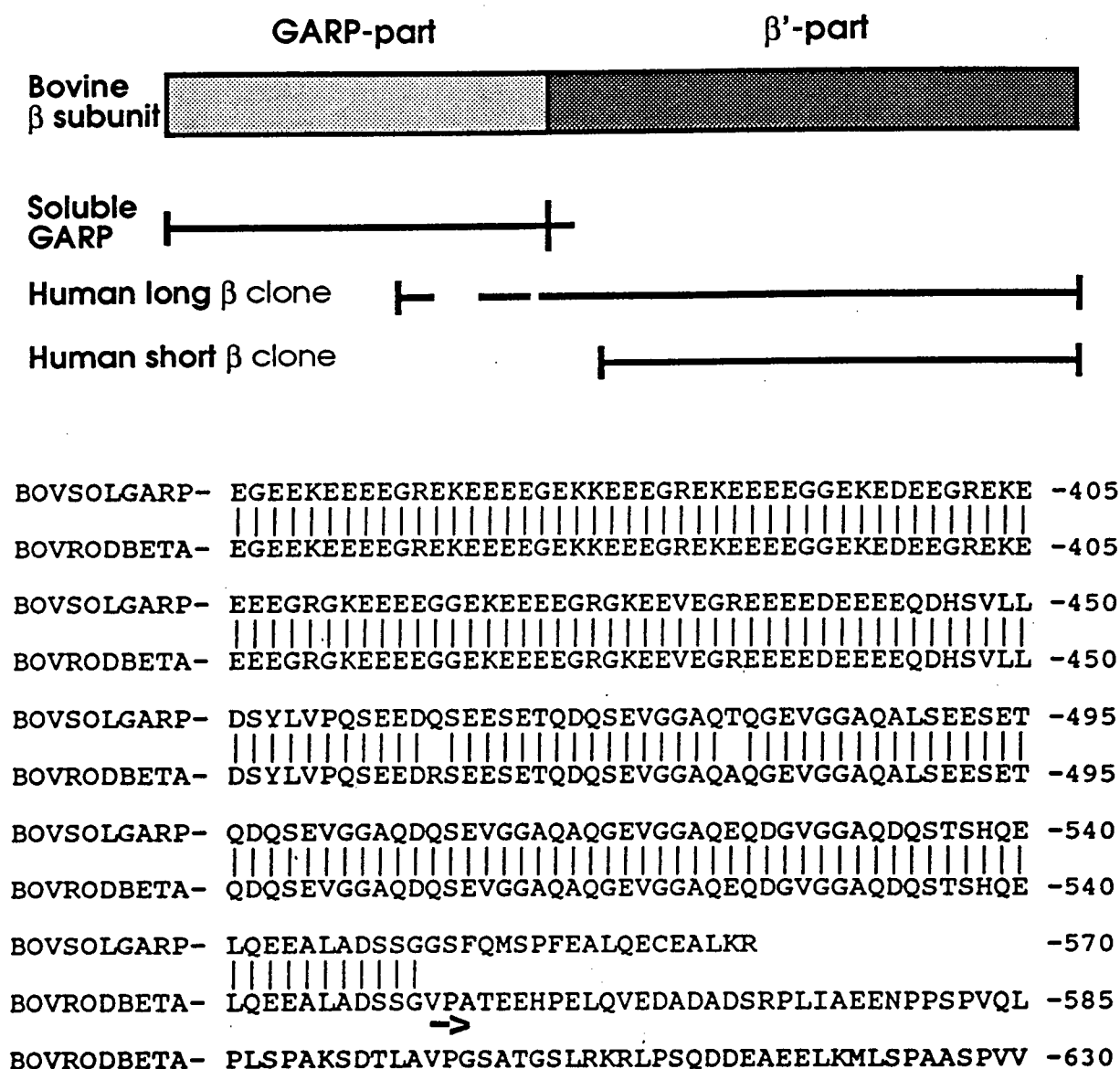


FIG. 38. Region of sequence divergence between the predicted amino acid sequences of the bovine soluble GARP and the complete bovine β subunit. The beginning of the bovine β subunit lines up essentially perfectly (only 3 mismatches) with the bovine retinal glutamic acid-rich protein (GARP) reported by Sugimoto and co-workers, (1991). At amino acid 552 however, the two sequences diverge completely, and the last 19 amino acids of GARP are not present in the β subunit. The remaining bovine β subunit sequence lines up with the longer human β subunit clone reported by Chen and co-workers (1993). The arrow indicates the beginning of the sequence following the initiating methionine of the expressed β' polypeptide.

line up essentially perfectly with bovine retinal glutamic acid-rich protein (GARP) reported by Sugimoto and co-workers, (1991). Of the first 571 amino acids only three mismatches were present, but at amino acid 572 the two sequences diverge completely (fig 38). This divergence coincides with the point at which the bovine β subunit sequence lines up contiguously with the longer human β clone reported by Chen and co-workers (1993).

When the native CNG α subunit is solubilized and reconstituted from ROS, the β subunit is present as a part of the channel complex. It is possible that the complex of the α and β subunits is more stable during solubilization than the α subunit alone. In order to include the β subunit in reconstitution assays, it was necessary to express the bovine rod β subunit heterologously in mammalian cells. The β subunit was subcloned into pCDNA1/Amp and transfected into COS-1 cells or HEK 293 cells.

To help investigate the role of the glutamic acid-rich portion of the bovine β subunit, (Chen *et al.*, 1993), a truncated β subunit (β') was constructed starting at the point where the β subunit sequence diverges from that of GARP (fig 38). The construction is diagrammed in figure 39. A PCR primer was designed to introduce an EcoRI site, followed by a Kozak sequence (Kozak, 1984) and a start codon at the 5' end of the constructed β' sequence. This primer was used in conjunction with a primer (BETA M2, section 2.29), which annealed just downstream from a unique XmnI site. The resultant PCR fragment which corresponded to the N-terminal fragment of the β' -portion of the β subunit was then digested with EcoRI and XmnI. This modified 1600 bp 5' fragment bordered by EcoRI and XmnI was ligated with the XmnI/EcoRI digested 3' portion of the β' sequence into the EcoRI site of pCDNA1/Amp, to generate the expression vector pCD β' (fig 39).

3.5.2 Expression of β and β' subunits in COS-1 cells

As mentioned earlier, the CNG channel β subunit cloned from the human retina was reported to be 100 kDa (Chen, *et al.*, 1993, longer clone). Protein sequencing and

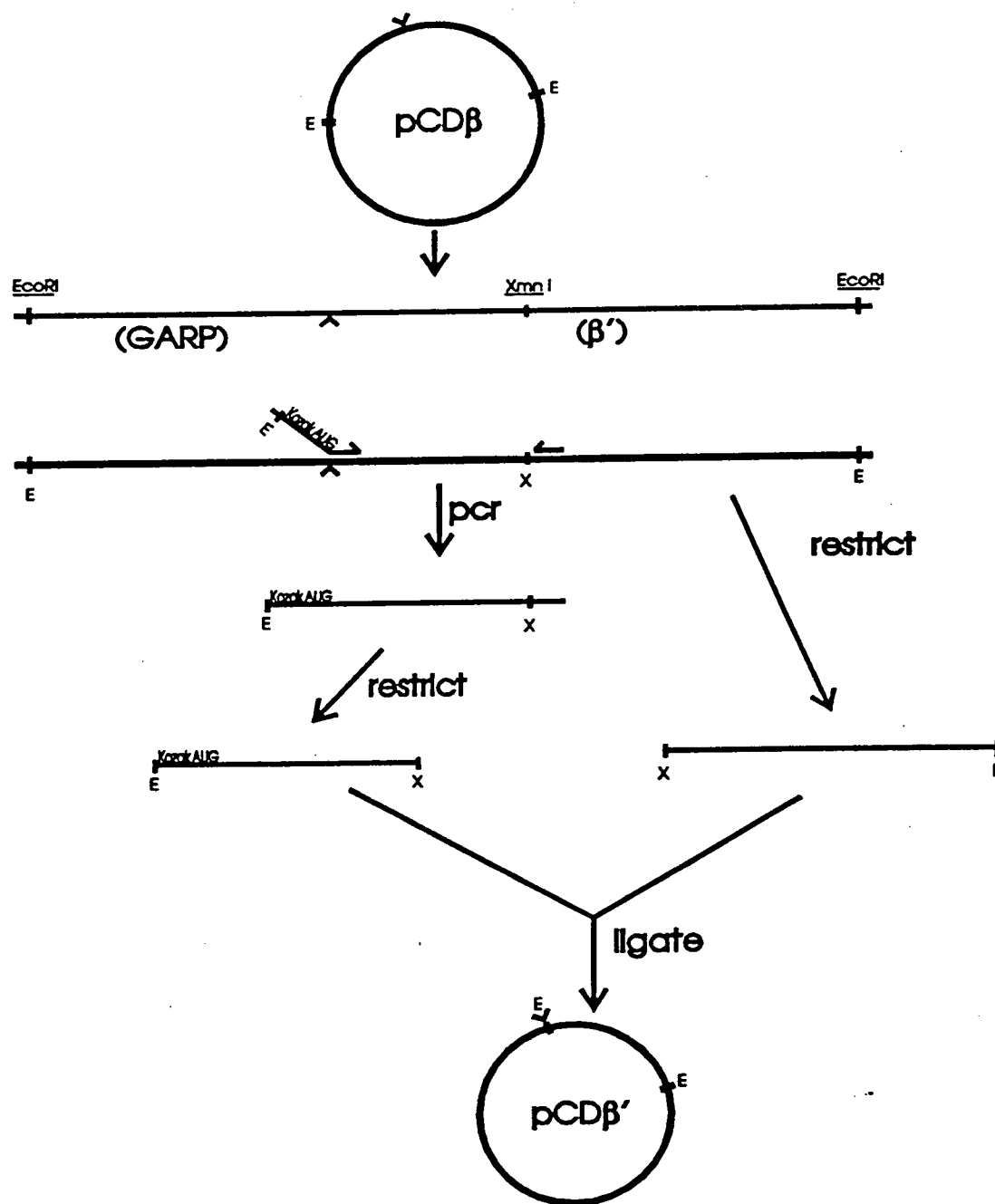


FIG. 39. A schematic diagram of the construction of the truncated β subunit (β') and the construction of the $pCD\beta'$ expression vector. A PCR primer was designed to introduce a new start codon preceded by a synthetic Kozak sequence (Kozak, 1984) and an $EcoRI$ site. This 3' primer was used in conjunction with the 5' primer M2 which annealed just downstream from a unique $XmnI$ site of the β sequence. The resultant PCR product was digested with $EcoRI$ and $XmnI$, and ligated with the $XmnI/EcoRI$ 5' β fragment into $pCDNAI/Amp$. The expression plasmid was called $pCD\beta'$.

antibody studies however, indicated that in bovine and human, the β subunit of ROS has an apparent M_r of 240 kDa by SDS-PAGE (Chen *et al.*, 1994, Molday *et al.*, 1990). Even with the GARP portion at the N-terminus, the predicted molecular mass of the complete β subunit is 155 kDa. In order to resolve the basis for these differences in M_r , the complete β subunit, the C-terminal portion of the β designated β' and GARP were expressed in COS-1 cells and their M_r were compared with the β subunit of the native ROS by SDS-PAGE (fig 40).

The expression vectors pCD β and pCD β' were used to transfect HEK 293 cells, and the solubilized extracts were examined on western blots (fig 40). Also included was the heterologously expressed GARP, the 80 kDa α subunit, and ROS membranes for comparison. The monoclonal antibody PMs 5E11 (Molday *et al.*, 1990) (fig 40, middle panel), which labels the ROS 240 kDa protein (fig 40, lane a), recognized a 240 kDa polypeptide in HEK 293 cells transfected with pCD β (fig 40, lane b). PMs 5E11 also recognized the heterologously expressed GARP (fig 40, lane c), but not the β' polypeptide (lane d), indicating that it recognizes the GARP portion of the β subunit.

The monoclonal antibody PMb 3C9 (fig 40, left panel) which labels the ROS 240 kDa protein (fig 40, lane a) recognized a 240 kDa protein in COS-1 cells transfected with pCD β (fig 40, lane b) as well as a band at 110 kDa in cells transfected with pCD β' (fig 40, lane b). PMb 3C9, however, did not label anything in COS-1 cells expressing GARP indicating that it recognizes the β' portion of the β subunit. PMc 1D1, included as a control can be seen to label the 63 kDa α subunit in the purified native CNG channel (fig 40, lane a) and the heterologously expressed 80 kDa α subunit, but it does not label any part of the CNG channel β subunit (fig 40, lanes b,c&d).

The fact that the expressed β subunit runs at 240 kDa suggests that pCD β contains the entire coding sequence of the bovine CNG β subunit. These results also show that the anomalous migration of the β subunit can most likely be attributed to the GARP

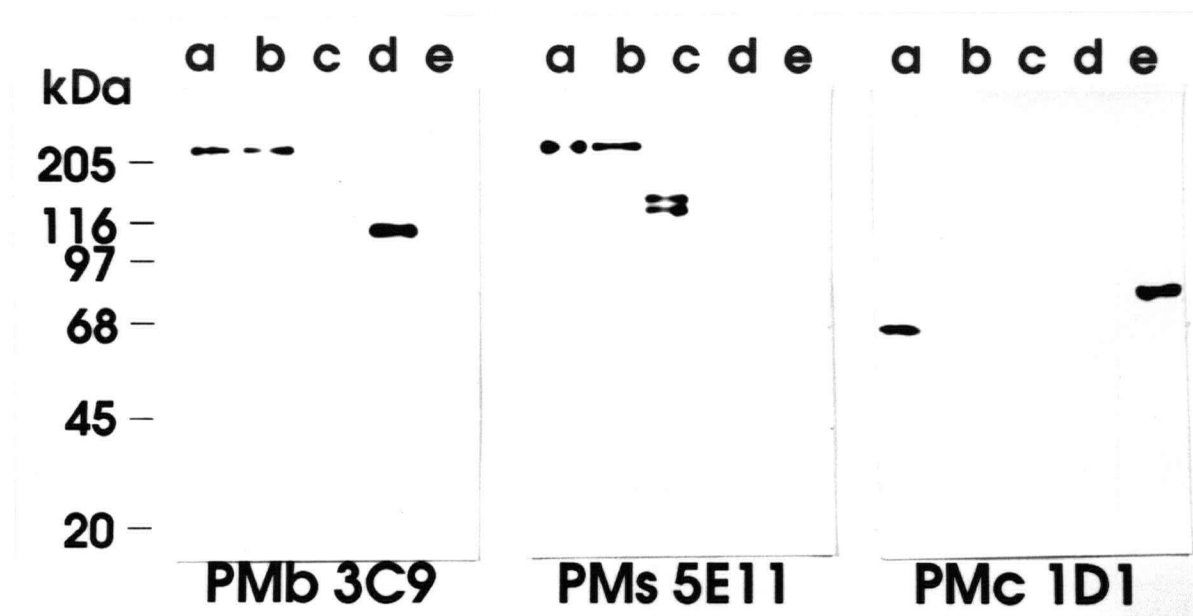


FIG. 40. Western blots of native rod channel, and heterologously expressed complete β subunit, β' -portion, soluble GARP, and α subunit. SDS gels were transferred to immobilon and labeled either with PMb 3C9 (against the C-terminal region of the β subunit, *left panel*), PMs 5E11 (against the GARP-part of the β subunit, *middle panel*) or PMc 1D1 (against the α subunit, *right panel*). Primary antibodies were labeled with sheep anti-mouse Ig peroxidase for ECL. *Lane a*, PMc 1D1-Sepharose immunopurified rod ROS CNG channel; *lane b*, PMb 3C9-Sepharose immunoprecipitated h/e β subunit; *lane c*, PMs 5E11-Sepharose immunopurified h/e GARP; *lane d*, PMb 3C9-Sepharose immunoprecipitated h/e β' polypeptide; *lane e*, PMc 1D1-Sepharose immunopurified h/e 80 kDa α subunit. PMb 3C9 labeled a 240 kDa polypeptide in the native rod channel and the h/e β subunit as well as a 110 kDa polypeptide in the h/e β' polypeptide. PMs 5E11 labeled a 240 kDa polypeptide in the native channel and extracts expressing the β subunit as well as a 130-140 kDa doublet in extracts expressing GARP. PMc 1D1 labeled the 63 kDa α subunit in the native channel and extracts expressing the 80 kDa α subunit.

portion of the β subunit. GARP (calculated M_r of 64.4 kDa) runs as a doublet of M_r ~130-140,000, almost twice its predicted M_r . It is also clear from these blots that the antibody PMs 5E11 recognizes the GARP portion, and the antibody PMb 3C9 recognizes the β' portion of the 240 kDa protein.

In order to examine the expression patterns of the β and β' subunits in COS-1 cells, transfected cells were examined by immunocytochemistry. The h/e soluble GARP was also included as an antibody labeling control. COS-1 cells transfected with pCD β , pCD β' and pCDGARP were fixed on glass disks and labeled with the monoclonal antibodies PMs 5E11 and PMb 3C9 (fig 41). The labeling pattern was similar to that seen in cells expressing the α subunit (compare fig 17). Labeling was observed as a dark reaction product throughout the cells as well as on the plasma membrane. The mottled labeling is characteristic for the golgi apparatus and endoplasmic reticulum (Munro and Pelham, 1987). COS-1 cells expressing the β subunit were recognized by both PMs 5E11 and PMb 3C9 (fig 41 a&d), but cells expressing the β' portion of the β subunit were only recognized by PMb 3C9 (fig 41 b&e). GARP expressing cells (control) were recognized by PMs 5E11 but not PMb 3C9 (fig 41 c&f). These results confirm the labeling pattern of PMs 5E11 and PMb 3C9 obtained by western blotting, and also indicate that the lack of the GARP portion of the β subunit has no obvious deleterious effects on its expression pattern in COS-1 cells.

3.6 Co-expression of α and β CNG channel subunits

It was now possible to co-transfect the α and β subunits into COS-1 cells thereby heterologously expressing the channel complex as it appears to exist in the rod outer segment. This provided a means for addressing several questions. Since the 80 kDa α subunit is processed to 63 kDa in the outer segment, and is tightly associated with the β subunit, could the β subunit be responsible for the proteolytic processing of the α subunit? If the α subunit was not folding properly in the absence of the β subunit, could

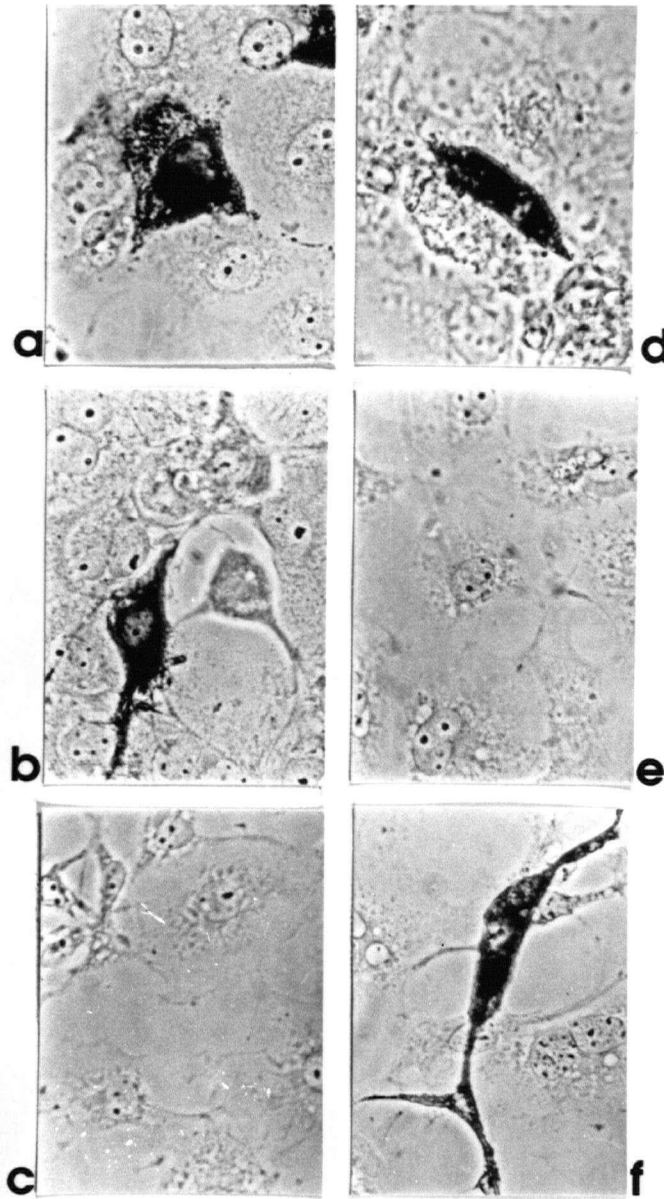


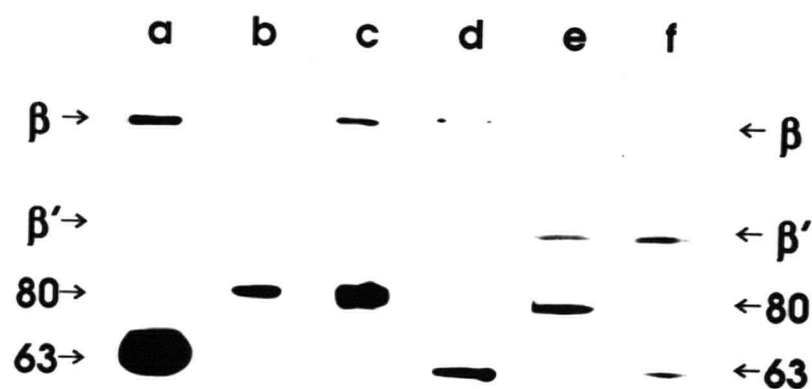
FIG. 41. Immunoperoxidase staining of COS-1 cells expressing the CNG channel β subunit, the β' -portion, and soluble GARP. COS-1 cells were fixed and permeabilized with methanol on glass discs and labeled with PMb 3C9 (against the C-terminal region of the β subunit) or PMs 5E11 (against the GARP-part of the β subunit) followed by horseradish peroxidase-sheep anti-mouse Ig. Immunoreactivity appeared as a dark reaction product. (a,b,c), COS-1 cells transfected with pCD β (a); pCD β' (b) and pCGARP (c) and labeled with PMb 3C9; (d,e,f), COS-1 cells transfected with pCD β (d); pCD β' (e) and pCGARP (f), and labeled with PMs 5E11.

that be corrected by co-expression with the β subunit potentially resulting in an altered expression pattern? Do the heterologously expressed subunits co-purify like the α and β subunits in the native ROS indicating that the expressed subunits are forming a complex like the native CNG channel?

3.6.1 Co-expression of the α and β channel subunits in COS-1 cells

In order to heterologously express the channel complex, COS-1 cells were co-transfected with α and β subunit expression vectors. If the β subunit possessed the ability to proteolytically cleave the α subunit, then co-expression of the 80 kDa α subunit with the β subunit might result in its conversion into the 63 kDa form. As shown by western blotting (**fig 42**), the 80 kDa α subunit co-expressed with the β subunit appeared to be unaffected (**fig 42, lane c**). It ran with the same M_r as the 80 kDa α subunit expressed on its own (**fig 42, lane b**) or when co-expressed with the β' polypeptide (**fig 42, lane e**). Likewise, the 63 kDa α subunit co-expressed with the β (**fig 42, lane d**) and β' (**fig 42, lane f**) subunits was also unaffected. These results indicate that the β subunit itself is not responsible for the removal of the 92 amino acids from the N-terminus of the 80 kDa α subunit.

In order to examine whether the presence of the β subunits had any effect on the expression patterns of the α subunits in COS-1 cells, or vice-versa, co-transfected cells were examined by immunocytochemistry. The expression patterns of the co-expressing α and β subunits were compared using differential fluorescent labeling of two antibodies (**fig 43**). Co-transfected COS-1 cells were labeled with the anti- α subunit polyclonal antibody PPc 80N (rabbit) and the anti- β subunit monoclonal antibody PMs 5E11 (mouse). In order to differentiate between the two antibodies, fluorescein-conjugated anti-mouse Ig (green) and rhodamine-conjugated anti-rabbit Ig (red) were used as secondary antibodies. When fluorescent micrographs were taken and superimposed by double exposure, successfully co-transfected cells appeared yellow. Comparing singly and doubly transfected cells, the presence of either the β subunit (red label, **fig 43a**) or



PMc 2G11/PMb 3C9

FIG. 42. Western blots of native rod channel, and COS-1 cell extracts co-expressing CNG channel α and β subunits. SDS gels were transferred to Immobilon and labeled with both PMc 2G11 (against the α subunit) and PMb 3C9 (against the C-terminal region of the β subunit). Primary antibodies were labeled with sheep anti-mouse Ig peroxidase for ECL. *Lane a*, PMc 1D1-Sepharose immunopurified CNG channel; *lane b*, post-nuclear extracts from COS-1 cells expressing the 80 kDa α subunit; *lane c*, post-nuclear extracts from COS-1 cells expressing the 80 kDa α subunit with the complete β subunit; *lane d*, post-nuclear extracts from COS-1 cells expressing the 63 kDa α subunit with the complete β subunit; *lane e*, post-nuclear extracts from COS-1 cells expressing the 80 kDa α subunit with the β' polypeptide; *lane f*, post-nuclear extracts from COS-1 cells expressing the 63 kDa α subunit with the β' polypeptide. PMb 3C9 labels the 240 kDa complete β subunit and the 110 kDa β' -portion of the β subunit. PMc 2G11 labels the native 63 kDa α subunit and the h/e 80 kDa α subunit.

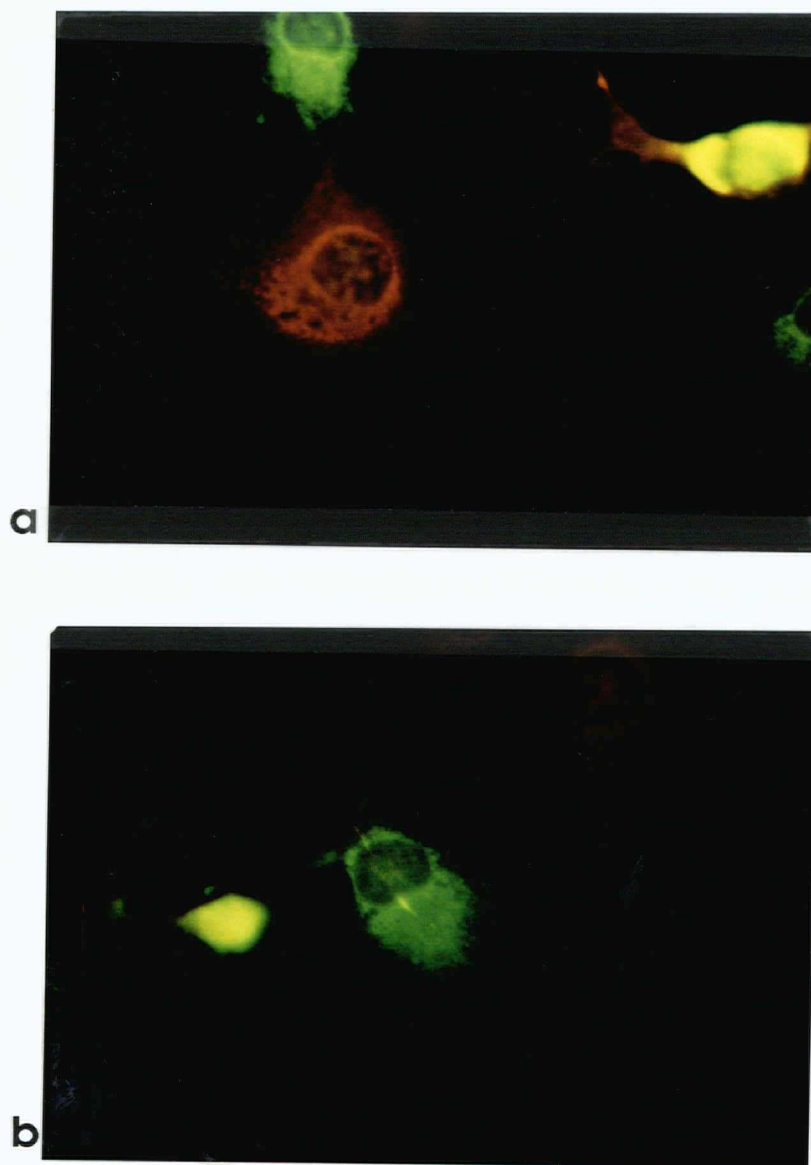


FIG. 43. **Differential immunofluorescent labeling of COS-1 cells co-expressing the α and β subunits of the CNG channel labeled with polyclonal antibody PPc 80N and monoclonal antibody PMb 3C9.** COS-1 cells were co-transfected with (a) pAX80 and pCD β ; (b) pAX80 and pCD β' . Expressing cells were fixed and permeabilized with methanol on glass discs and labeled with PPc 80N (*rabbit*, against the N-terminal region of the 80 kDa α subunit,) and PMb 3C9 (*mouse*, against the C-terminal region of the β subunit). Secondary labeling was carried out with rhodamine conjugated-sheep anti-mouse Ig (red) and fluorescein conjugated-sheep anti-rabbit Ig (green). Cells expressing the α subunit appear green, cells expressing the β subunit or the β' -part appear red, and cells co-expressing the α and β subunits appear yellow.

the β' subunit (red label, **fig 43b**) seem to have no obvious effect on the expression patterns of the 80 kDa α subunit (green), and vice-versa. The same was true for the 63 kDa α subunit co-expressed with the β and β' subunits (data not shown). The proportion of doubly-transfected cells and singly-transfected cells varied from transfection to transfection, but of the total number of transfected cells, typically 30 to 40% were successfully co-transfected.

3.6.2 Co-precipitation of α and β CNG channel subunits

The native α and β subunits from ROS are directly associated and have been shown to co-purify on immunoaffinity columns (Molday *et al.*, 1990). In order to ascertain whether the heterologously co-expressed α and β subunits are associated with each other like the native channel complex from ROS, immunoprecipitation studies were carried out. COS-1 cells were co-transfected with α and β subunits. The co-transfected COS-1 cells were solubilized and their post nuclear extracts were immunoprecipitated with PMc 1D1-Sepharose beads. After immunoabsorption of the co-transfected extracts to the PMc 1D1 beads, the beads were incubated in SDS to elute any bound proteins. These eluted fractions as well as the unbound protein fractions were then subjected to electrophoresis and western blotting (**fig 44**).

The first experiments were carried out with the 80 kDa α subunit and the complete β subunit. The 80 kDa α subunit expressed alone in COS-1 cells can be successfully immunoprecipitated on a PMc 1D1-Sepharose column (**fig 44A, lane e**). There was essentially no α subunit in the unbound fraction (**fig 44A, lane c**) or the wash fraction (**fig 44A, lane d**). Similar immunoprecipitation studies were also carried out on detergent solubilized extracts of COS-1 cells co-transfected with the 80 kDa α and β subunits. As shown in figure **44A (lane f)**, the β subunit co-precipitated with the α subunit. This result indicated that the β subunit is indeed associated with the α subunit in this heterologous expression system.

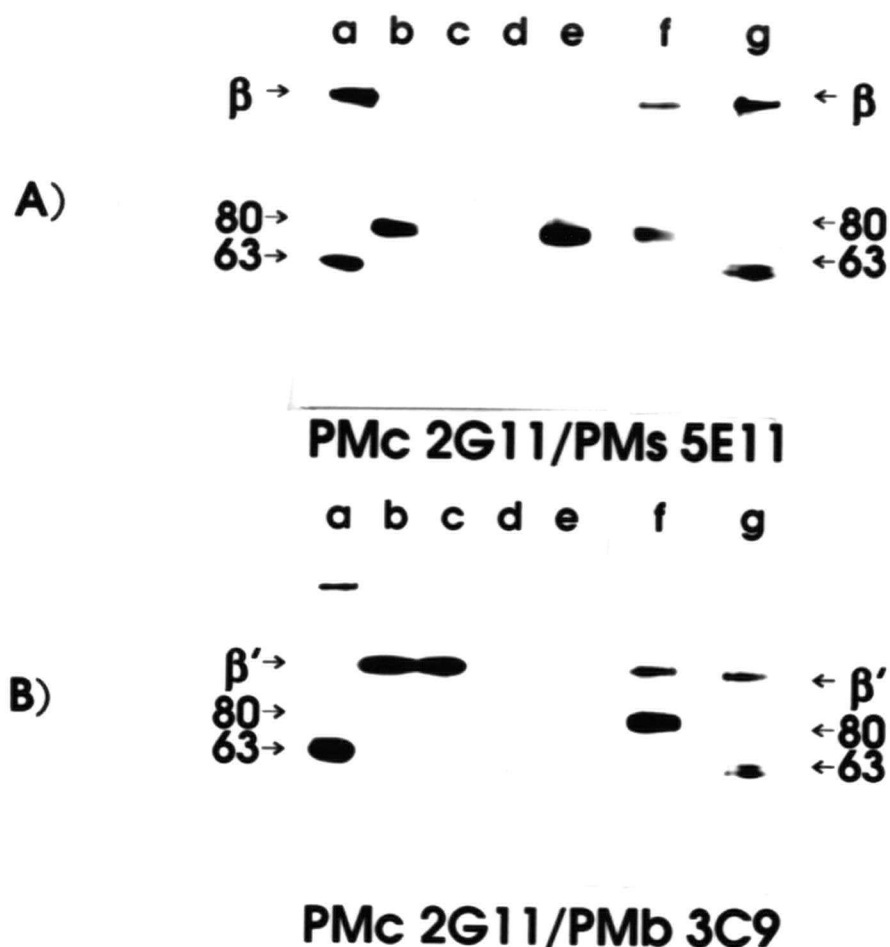


FIG. 44. Western blots COS-1 cell extracts singly or co-transfected with CNG channel α and β subunits purified on a PMc 1D1-Sepharose column. A) demonstrates co-precipitation of the α subunits with the β subunit and **B)** demonstrates co-precipitation of the α subunits with the β' -part of the β subunit. **A.)** *lane a*, PMc 1D1-Sepharose purified native CNG channel; *lane b*, extract from COS-1 cells expressing the 80 kDa α subunit, (*precolumn*); *lane c*, proteins not bound to column; *lane d*, column wash solution; *lane e*, immunoprecipitated protein (*SDS solubilized beads*); *lane f*, β subunit co-precipitated with 80 kDa α subunit; *lane g*, β subunit co-precipitated with 63 kDa α subunit. **B.)** *lane a*, PMc 1D1-Sepharose purified native CNG channel; *lane b*, extract from COS-1 cells expressing the β' polypeptide, (*precolumn*); *lane c*, proteins not bound to column; *lane d*, column wash solution; *lane e*, immunoprecipitated protein (*SDS solubilized beads*); *lane f*, β' polypeptide co-precipitated with 80 kDa α subunit; *lane g*, β' polypeptide co-precipitated with 63 kDa α subunit. Both the 63 kDa and the 80 kDa α subunits were capable of co-precipitating the complete β subunit as well as the β' -part of the β subunit. Blots were double-labeled with PMc 2G11 (against the α subunit) and either PMb 3C9 (against the C-terminal region of the β subunit) or PMs 5E11 (against the N-terminal region of the β subunit).

Immunoprecipitation of membrane extracts from COS-1 cells co-expressing the 63 kDa α and β subunits was carried out to determine if these subunits also assemble into a complex. As shown in figure 44A, (lane g), the β subunit co-precipitated with the 63 kDa α subunit. This result indicates that N-terminal amino acids present in the 80 kDa α subunit are not required for subunit association.

At present it is not clear what function the GARP portion of the β subunit has. It does not seem to influence major electrophysiological properties of the CNG channel complex (Körschen *et al.*, 1995), and does not appear to possess proteolytic activity for the processing of the α subunit. In order to ascertain whether the GARP portion of the β subunit is responsible for subunit association of the α and β complex, the β' polypeptide (lacking GARP) was used in co-precipitation studies. The co-precipitations were carried out as before on PMc 1D1-Sepharose beads. The β' subunit alone is not immunoprecipitated by PMc 1D1-Sepharose beads (fig 44B, lane e). All the expressed β' seen in the precolumn sample of β' expressing COS-1 cell extracts (fig 44B, lane b) is present in the unbound fraction (fig 44B, lane c). The solubilized extracts of COS-1 cells co-transfected with the β' subunit and the 80 kDa or the 63 kDa α subunits were subjected to immunoprecipitation with PMc 1D1-Sepharose beads. The β' subunit co-precipitated with the 80 kDa α subunit (fig 44B, lane f) and with the 63 kDa α subunit (fig 44B, lane g), indicating that the GARP portion of the β subunit is not required for subunit association.

These results together suggest that the heterologously expressed channel complex made up of the α and β subunits associate and co-purify in what appears to be the same manner as the native CNG channel complex from ROS. The cleaved 92 amino acids at the N-terminus of the 80 kDa α subunit is not responsible for the subunit association. Also, the GARP portion of the β subunit is not required for the interaction between the α and β subunits. The regions responsible for the association of the α and β subunits must fall within the region where structurally the two subunits seem to be most similar.

This region includes the transmembrane domains including the S4 region and the pore region, and the cGMP binding site.

3.7 Functional activity of heterologously expressed CNG channel

The previous results have suggested that the α and β subunits were associated with each other upon heterologous expression in COS-1 cells. It remained to be seen whether functional activity could be obtained by solubilizing and reconstituting the heterologously expressed CNG channel complex. Early reconstitution experiments were performed on the heterologously expressed α subunit alone and activity was barely detectable if at all. It was hoped that having the β subunit present during solubilization and reconstitution would somehow restore cGMP-gated channel activity.

3.7.1 Reconstitution of DEAE purified heterologously expressed channel complex

The native CNG channel complex was originally purified on a DEAE column (Cook *et al.*, 1987). In early experiments it was observed that the heterologously expressed α subunit was not efficiently purified on a DEAE column (data not shown). After the β subunit was cloned and co-expressed with the α subunit, it was found that a DEAE column could be used to efficiently purify the heterologously expressed channel complex, from COS-1 cells or HEK 293 cells co-transfected with pAX80 and pCD β . The majority of the h/e channel complex was eluted from the column in a single fraction (**fig. 45A, lane e**). This fraction (500 μ l) was reconstituted into Ca²⁺ loaded vesicles and assayed for cGMP-gated channel activity. Upon addition of cGMP, a Ca²⁺ efflux was measured, indicating the presence of active h/e channel complex (**fig 45B**). As a control, solubilized ROS membranes were reconstituted as well, and assayed for cGMP-gated Ca²⁺ efflux. A qualitative comparison between the amount of native channel complex incorporated into liposomes reconstituted with solubilized ROS membrane proteins and the amount of h/e channel complex incorporated into liposomes, can be made by western blot (**fig 45A**). DEAE-purified h/e channel complex appears to contain even higher

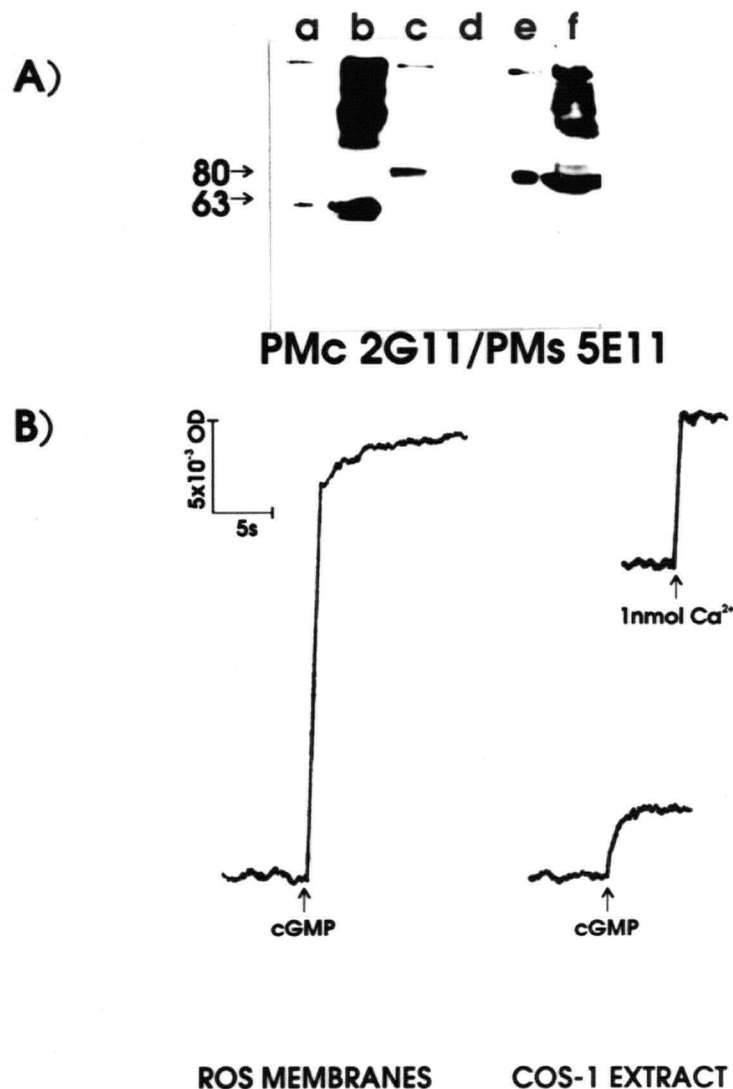


FIG. 45. Reconstitution of the CNG channel from ROS membranes and from COS-1 cell extracts co-expressing the α and β subunits. *A)* Western blot labeled with PMc 2G11 and PMs 5E11: *lane a&b*, 2 and 20 μ l of solubilized ROS membranes reconstituted into lipid vesicles; *lane c*, solubilized extract of COS-1 cells co-expressing 80 kDa α and complete β subunit (*pre-DEAE column*); *lane d*, empty; *lane e&f*, 2 and 20 μ l DEAE purified solubilized extract of COS-1 cells co-expressing 80 kDa α and complete β subunit. *B)* Activity traces: *left trace*, Ca^{2+} efflux from solubilized ROS membranes; *right lower trace*, Ca^{2+} efflux from DEAE purified solubilized extract of COS-1 cells co-expressing 80 kDa α and complete β subunit. Heterologously expressed channel complex exhibited about 1/10th the cGMP stimulated Ca^{2+} efflux activity as the equivalent amount of native channel complex. For calibration 1 nmol of Ca^{2+} is added to Arsenazo III, *right upper trace*.

amounts of channel complex than that seen in solubilized native ROS membranes (**fig 45A, compare lanes f&b**). This is evident when comparing a 10-fold dilution of each (**fig 45A, lane e&a**).

The cGMP induced calcium efflux signal from reconstituted h/e channel complex, however, is significantly lower than the signal obtained from a comparable amount of native channel complex in ROS membranes (**fig 45B**). The percent activity measured for the native complex was approximately 8% (ranging from 7-10%), and the percent activity of the h/e channel complex was approximately 0.8% (ranging from 0.6-0.9%). Comparing the amount of Ca^{2+} released per milligram of reconstituted protein, there is also a 10-fold difference. The average value calculated for ROS membranes was 5.84 nmol/mg and the value calculated for h/e channel complex was 0.53 nmol/mg. It is important to note here that the comparison is being made between ROS membranes and DEAE purified h/e channel complex in which the h/e complex is enriched. By western blot, it appears that similar quantities of solubilized ROS membranes and solubilized DEAE purified COS-1 cells contain comparable amounts of channel complex. So by DEAE purifying the COS-1 extracts, the concentration of h/e channel complex is now comparable to the concentration of channel complex in native ROS membranes. It therefore seems reasonable to compare channel activity and Ca^{2+} efflux efficiency between reconstituted ROS membranes and DEAE purified h/e channel complex.

These results indicate that, with the β subunit present along with the α subunit, the heterologously expressed channel complex can be successfully solubilized and reconstituted into liposomes, to obtain cGMP-gated Ca^{2+} efflux activity. However, it appears that only 1/10th of the h/e channel complex is functionally active. The inactive fraction is most likely made up of subunits from singly transfected cells, or the portion which do not assemble into a functional complex, perhaps due to improper folding.

In order to investigate whether the GARP portion of the β subunit played a role in the formation of functional h/e channel complex, activity measurements were also

performed on reconstituted h/e channel complex comprised of the 80 kDa α subunit and the β' subunit. The Ca^{2+} efflux activity upon the addition of cGMP was only slightly above detectable limits for the h/e complex formed from the 80 kDa α subunit and β' polypeptide. No detectable activity was obtained with h/e complexes formed with the 63 kDa α subunit and either the β subunit or the β' portion. It is not clear whether the lack of activity seen for the 80/ β' , 63/ β and 63/ β' h/e channel complexes is due to these subunit combinations being inactive or perhaps just less likely to form as many functional channel complexes in this heterologous expression system.

DISCUSSION

4.1 *Heterologous expression*

Heterologous expression provides a means for expressing a protein at high levels and out of the context of its native environment. Several eukaryotic expression systems are currently available. Namely, heterologous expressions can be carried out in *Xenopus* oocytes, mammalian cells, or insect cells. Insect cells typically give the highest levels of heterologous protein expression and oocytes typically have the lowest expression levels (Levinson, 1990). Mammalian and insect cells can be cultured in large quantities and are therefore more desirable when the goal is purification of heterologously expressed membrane proteins in significant amounts.

Although large quantities of heterologously expressed proteins are desirable, it is also necessary that the heterologously expressed protein be in a functional state. On initial consideration it seems that mammalian cells would be more likely than insect cells to correctly process a mammalian protein. Although many proteins have been expressed in a functional state in insect cells, Sun *et al.* (1994) have reported that a K⁺ channel expressed in insect cells is found primarily in nonfunctional, insoluble aggregates. In another report Santacruz-Toloza and co-workers (1994) have shown that although patch clamp analyses demonstrate K⁺ channels on the cell surface are active, a large fraction of the expressed protein remains in intracellular organelles where its functional competence is unknown. Problems with K⁺ channels in insect cells are particularly relevant here, since the CNG channels appear to be structurally related to K⁺ channels (Jan and Jan, 1990). For the reasons explained above, COS-1 cells were chosen for heterologous expression of the bovine rod CNG channel. Later, HEK 293 cells were also used. Both are kidney cell lines and since their expression levels are comparable, they were used interchangeably throughout this work.

4.1.1 *Heterologously expressed protein yield*

Two parameters must be optimized in a chosen expression system. One is the expression level (the amount of protein translated within each cell), and the other is the transfection efficiency (the number of cells that are successfully transfected).

The expression level can be affected by the expression vector. The original experiments utilized the expression vector pAX112, a modification of pAX111 which was constructed by Dr Rob Kay, Terry Fox Laboratories, Vancouver. Since pAX111 was never documented in the literature, the commercially available plasmid pCDNA1/Amp was also used for expression. For the α subunit however, it was observed that expression levels were significantly lower in pCDNA1/Amp than pAX112. Therefore the α subunits were expressed in pAX112 and the β subunits were expressed in pCDNA1/Amp. The expression level of the α subunit in COS-1 or HEK 293 cells was estimated to be on the order of 10^6 copies per cell. Reports in the literature on heterologous expression in mammalian cells have ranged from 500-1000 copies per cell for a Ca^{2+} channel (Perez-Reyes *et al.*, 1989) to reports where 10^7 K^+ channels per transfected cell were expressed on the plasma membrane (Sun *et al.*, 1994) or 10^7 copies of rhodopsin were expressed per cell (Oprian *et al.*, 1987).

The transfection efficiency is determined in part by the method of transfection used to introduce DNA into the cells. Early experiments in this work, were performed using the DEAE/Dextran transfection technique (Hammar skjöld *et al.*, 1986). This is a rather laborious method, and also inappropriate for HEK 293 cells since these cells do not adhere strongly to petri dishes. The transfection efficiency is also very low. Typically, in this study less than 10% of COS-1 cells were successfully transfected, but the efficiency was sometimes as low as 1% or less. One of the earmarks of heterologous expression appears to be variability.

The next technique used for transfection was electroporation. An electric pulse was first used to transfer DNA into mammalian cells by Neumann and co-workers (1982).

The technique described in the Materials and Methods section successfully transfected 50% of COS-1 cells; however each electroporation experiment was typically accompanied by 80% cell death. Large scale transfections therefore required so many cells to start that the method became cumbersome.

The calcium phosphate precipitation techniques were the most conducive to large scale transfections. For logistical reasons the method of Chen and Okayama (1987) was preferable for HEK 293 cells and the method of Sambrook and co-workers (1989) was used on COS-1 cells. The transfection efficiency for both methods was typically around 40%. The techniques proved to be quite sensitive to conditions, and results were somewhat variable.

4.1.2 Expression patterns

Once the cells have been successfully transfected, the expression pattern of the heterologously expressed protein can be examined by immunocytochemistry. The presence of a defined outline on an immunolabeled cell is an indication of expression at the plasma membrane. This distribution would be expected for CNG channels since they are localized in the plasma membrane of rod cells (Cook *et al.*, 1989). The patch-clamp data of Dhallan and co-workers (1992) indicates that the photoreceptor α subunit exists as a functional channel in the plasma membranes of HEK 293 cells. The expression patterns of CNG channel subunits as determined by immunofluorescence in both COS-1 and HEK 293 cells also suggests that a significant amount of the expressed protein exists in the endoplasmic reticulum (ER). Proteins labeled in the ER exhibit a characteristic mottled pattern in COS-1 cells (Munro and Pelham, 1987). The CNG channels in the ER are either newly synthesized proteins en route to the plasma membrane, or they are retained in the ER due to incorrect folding. It is not clear whether this portion of the CNG channel is functional or not. In the instance of a plasma membrane Ca^{2+} pump, researchers found that the portion of the expressed protein remaining in the ER was active when tested in microsomes (Enyedi *et al.*, 1993).

4.2 The α subunit

One of the goals of this thesis was to explore the question revolving around the existence of the 63 kDa and the 80 kDa α subunits. The work presented here provides evidence in support of the assertion that the 63 kDa form of the α subunit is not a result of non-specific proteolysis during ROS purification but is the product of a specific cleavage reaction that occurs in the outer segment.

The heterologous expression of the 63 and 80 kDa α subunits provided a means for ascertaining the relative mobilities of the polypeptides coded for by the cloned α subunit cDNA's on SDS gels. Cells transfected with pAX80 expressed a protein recognized by PMc 2G11 which ran close to the predicted molecular mass (~78 kDa) indicating that autoproteolysis to the 63 kDa form did not occur. It also suggested that the cleavage was not the result of a general mammalian cell post-translational modification reaction. Cells transfected with pAX63 expressed a protein which ran at ~63 kDa as found for the α subunit in ROS membranes.

It should be mentioned that the predicted molecular weight for the 63 kDa α subunit minus the 92 amino acids is 69,400. The difference between this value and the observed M_r of 63,000 is most likely due to the anomalous migration of membrane glycoproteins by SDS-PAGE. Examples of inaccurate molecular weight determinations have also been documented for rhodopsin and the peripherin/rds protein of ROS. Both proteins migrate on this electrophoresis system with M_r 's about 10% lower than those predicted by sequence analysis (Molday and Molday, 1987; Connell and Molday, 1990). The possibility of proteolytic degradation at the C-terminus of the α subunit of the channel is ruled out by the fact that the 63 kDa α subunit is labeled by PMc 1D1, which recognizes an epitope 27 amino acids in from the C-terminus (Molday *et al.*, 1992; Illing, M and Molday, R.S., unpublished results).

The 92 N-terminal amino acids do not appear to be a cleaved signal sequence, at least not in the traditional sense. The 92 amino acid stretch is rather large for a signal peptide

and the N-terminus of the channel does not contain a consensus sequence. Channels reported to possess cleaved signal sequences include the ligand-gated channels which typically contain signal peptides of 20-30 amino acids that are cleaved post-translationally (Noda *et al.*, 1983; Shofield *et al.*, 1987; Ellis *et al.*, 1988; Hollman, *et al.*, 1989). A signal sequence is also seen on the ROS $\text{Na}^+/\text{Ca}^{2+}/\text{K}^+$ exchanger (Reiländer, *et al.*, 1992) which like the channel is also localized specifically to the plasma membrane (Reid *et al.*, 1990). The membrane proteins containing signal sequences mentioned above, all have extracellular N-termini.

Many proteins like the channel α and β subunits which are lacking conventional signal sequences are concluded to have an intracellular N-terminus (Wessels and Spiess, 1988) although there are exceptions such as G-protein-coupled receptors like rhodopsin. The first hydrophobic segment therefore acts as a signal-anchor and initiates the translocation process of the following transmembrane segments. In support of this hypothesis is a human α subunit splice variant reported by Dhallan and coworkers (1992). This α subunit, which lacks 36 codons encompassing the first putative transmembrane segment did not produce a functional cGMP channel when transfected into HEK 293 cells. Also in agreement are studies carried out on K^+ channels. The S1 region of a K^+ channel (corresponding to the first putative transmembrane region of the CNG channel subunits) was shown to serve as a signal for incorporation into the membrane (Shen *et al.*, 1993).

Further evidence against the 92 amino acid N-terminus containing some sort of signal sequence was provided by immunofluorescence on whole COS-1 or HEK 293 cells expressing the CNG α subunit. The expression patterns of the 63 and 80 kDa α subunits in COS-1 were the same. If the lack of 92 amino acids were required for insertion into the membrane, then one would expect that most of the 63 kDa polypeptide would be degraded and therefore undetectable. It is true that the 63 kDa α subunit is expressed at a lower level than the 80 kDa α subunit; however, the reason for this is not known.

The absence of a conventional signal sequence does not rule out the possibility that the N-terminus contains some kind of sorting or targeting information. The fact that the channel is preferentially localized to the plasma membrane of ROS, indicates that a specific sorting mechanism may exist directing the channel as well as other membrane proteins specifically to the plasma membrane and not the disk membranes. It is not yet clear what the mechanism for this sorting is, but it could be that specific sequences direct plasma membrane proteins to their destinations.

Functional implications for the two size forms of the channel have not been ruled out. There were early reports of two functionally distinct forms of CNG channel activity in ROS membrane vesicles. They differed in cGMP binding constants and in sensitivity to *l-cis* diltiazem. Along the same line, the α subunit of the rabbit skeletal muscle Ca^{2+} channel has been proposed to undergo similar posttranslational proteolytic cleavage with functional implications. In this case, 320 amino acids are removed from the C-terminus of the Ca^{2+} α subunit (DeJongh *et al.*, 1989). The 212 kDa precursor and the cleaved 175 kDa polypeptides are detected in both cultured muscle cells and freshly isolated muscle membrane preparations in similar proportions. The C-terminal segment contains several potential phosphorylation sites and its absence could affect channel activity (DeJongh *et al.*, 1989).

4.3 Monoclonal antibodies PMc 6E7 and PPc 80N

These studies also report the characterization of two antibodies: monoclonal antibody PMc 6E7 and polyclonal antibody PPc 80N. PMc 6E7 is shown to be specific for the 63 kDa α subunit and PPc 80N is shown to be specific for the 80 kDa α subunit. The use of these two antibodies enabled the differentiation between the two size forms of the α subunits and facilitated their localization. PMc 6E7 and PPc 80N will be discussed in turn.

The characterization of PMc 6E7 involved demonstrating that it labeled the 63 kDa α subunit but not the 80 kDa α subunit on western blots and in COS-1 cells. The fact that

this same antibody labels ROS *in situ*, strongly indicates the presence of the 63 kDa form of the α subunit in the rod outer segment. If the existence of the 63 kDa channel was simply due to non-specific proteolysis during purification, the 63 kDa α subunit should not be detectable in the rod cell particularly not in a fixed frozen retinal section. The addition of paraformaldehyde to the retina occurs only minutes after the removal of the eye from the cow.

The labeling of ROS in whole retinal sections by PMc 6E7 was shown to be specific by the ability of the synthetic peptide Ser-1 to compete for binding. A peptide with an additional serine at the N-terminus was not as efficient at competing for PMc 6E7 binding, and therefore did not abolish all PMc 6E7 labeling of ROS. The heterologously expressed 63 kDa α subunit presumably has a methionine before the first serine, and therefore, more closely resembles the double serine peptide (Ser-2). Peptide competition assays indicated that PMc 6E7 had a higher affinity for the single serine N-terminus of the 63 kDa α subunit than for the double serine (M. Illing, unpublished results). Thus, in whole COS-1 cells, which are expressing an α subunit with a methionine-serine N-terminus, the Ser-2 peptide was also capable of abolishing PMc 6E7 binding as was the Ser-1 peptide. By western blots it is also apparent that the Ser-2 is a more efficient competitor for the h/e α subunit with a met-ser at its N-terminus than for the native α subunit. This provides more evidence for the processing of the N-terminal sequence of the native α subunit, since an exposed serine without an initiating methionine is indicative of proteolytic cleavage.

The characterization of PPc 80N involved demonstrating that it labeled the 80 kDa α subunit but not the 63 kDa α subunit on western blots and in COS-1 cells. Being able to detect the 80 kDa α subunit specifically and distinguish it from the 63 kDa α subunit was very informative. The faint band often seen above the 63 kDa α subunit was now shown to indeed represent the unprocessed 80 kDa α subunit. Both forms of the channel appear to coexist albeit in very different amounts. Interestingly, a band often appears

below the 80 kDa α subunit on a western blots of pAX80 transfected COS-1 cells. The band migrates around 63 kDa and is presumably a degradation product of the 80 kDa α subunit. It is labeled by PMc 2G11, but it is not labeled by PMc 6E7 indicating that it is not the specifically processed 63 kDa α subunit. If the 80 kDa α subunit is processed in the rod cell, the N-terminus could be cleaved in the inner segment, post- or co-translationally, or it could occur in the outer segment. The results presented here argue in favor of the processing occurring in the outer segment.

The labeling of the ROS by PPc 80N in whole retinal sections indicates that the 80 kDa α subunit exists in the outer segment. The fact that the labeling was so faint could be explained in two ways. Either the protein is present in such low amounts that it is barely detectable, or the antibody labels the protein *in situ* with low affinity.

Western blots indicate that the 80 kDa α subunit is present in significantly lower quantities than the 63 kDa in ROS. The 80 kDa band is barely detectable when the 63 kDa band is strongly labeled with PMc 2G11. Considering the ratio of 80:63 kDa α subunits, it is not surprising to see the labeling level of the 80 kDa α subunit so low in ROS. This indicates that the 80 kDa "precursor" is transported to the outer segment prior to removal of the N-terminal amino acids. If the processing occurs in the outer segments, it would be an ongoing process, and some of the uncleaved precursor would be present at all times. It is also possible, however, that the low level of labeling seen with PPc 80N is due to the 80 kDa α subunit epitope being inaccessible to PPc 80N. This possibility seems less likely since PPc 80N efficiently labeled the 80 kDa α subunit in COS-1 cells when examined by both immunofluorescence and western blotting.

Another attractive hypothesis is that the 80 kDa α subunit exists in the inner segment, either briefly as the precursor to the 63 kDa α subunit during protein biosynthesis or as a resident population of CNG channels. It has in fact been shown that in toads there exists a low amount of cGMP-gated activity in the inner segments (Matthews and Watanabe, 1988). However, both the immunofluorescent labeling studies and the subcellular

fractionation studies suggest that this is not the case. Immunofluorescence of sectioned retinas labeled with anti- α subunit antibodies indicated no labeling of the inner segments. Furthermore, subcellular fractionation of homogenized retinas did not identify separate populations of the 63 and 80 kDa α subunits. Were the 80 kDa form to exist only in the inner segment, it would be expected to migrate with the rod inner segments (RIS) in a part of the gradient distinct from that of containing ROS. Instead, the 80 kDa α subunit cosediments with the 63 kDa α subunit. This suggests that the 80 kDa and the 63 kDa are present in the same fractions, i.e. ROS.

Besides posttranslational modification, there are other possible reasons for a protein to exist as two different sizes in a particular tissue. Two genes differing only at their 5' end could be translated to yield two proteins that are labeled by the same antibodies at their C-termini and different antibodies at their N-termini. Alternative splicing can also produce two proteins differing only at their N-terminus.

Considering the extensive screening for CNG channels that has been carried out in many laboratories, the existence of a second gene coding for the 63 kDa α subunit seems unlikely. Data generated from northern blots is not definitive, but the general opinion is that there exists one species of mRNA for photoreceptor α subunits. Kaupp and co-workers (1989) reported a single mRNA estimated to be about 3.2 kilobases long. Human and mouse photoreceptor CNG channel transcripts were reported to be single bands at 3.2-3.5 kb (Dhallan *et al.*, 1992; Pittler *et al.*, 1992). In other laboratories, two additional transcripts were detected in bovine retinal mRNA at 4.6 and 5.2 kb which were attributed to alternate poly (A) site usage as found for opsin mRNA in several species (Pittler *et al.*, 1992; Al-Ubaidi *et al.*, 1990). Northern blots of chicken retinal mRNA yielded one transcript at 3.5 kb for the cone α subunit. The chicken rod α subunit appeared to have two transcripts, one at 2.8 kb and a weaker one at 3.5 kb (Bönigk *et al.*, 1993). The CNG channel cloned from bovine sperm had transcripts at

2.8 and 4.1. Here the authors attributed the larger transcript to an incompletely or alternately spliced mRNA species (Weynand *et al.*, 1994).

Alternative splicing could explain the existence of the 63 kDa and the 80 kDa α subunits, particularly in view of the extensive alternative splicing seen for K⁺ channels (Schwartz *et al.*, 1988). The gene structure of only the human CNG α subunit has been analyzed (Dhallan *et al.*, 1992). In this study the authors specifically looked for differentially spliced variants in the 5' coding region of the mRNA. Only one variant was reported, which contained an in-frame deletion of 36 codons. This variant was inactive when expressed in HEK 293 cells (Dhallan *et al.*, 1992).

It should be mentioned again that Hurwitz and co-workers (1991) reported the purification of an 80 kDa polypeptide which exhibited cGMP-gated Ca²⁺ efflux activity when reconstituted into liposomes. The buffers and conditions are practically identical to those used for the purification of the 63 kDa (Cook *et al.*, 1987), however, the purification was carried out on an 8-Br-cGMP column. The purified polypeptide was reported to run at 78 kDa by SDS-PAGE. It is possible that the authors purified only the 80 kDa α subunit precursor. If such is the case, then it is puzzling why the 63 kDa did not co-purify with the 80 kDa.

4.4 Truncated forms of the CNG α subunit in other species

The finding that the α subunits in both rod and cones from the chicken retina are also processed is important. Both the rod and cone α subunits become processed *in vivo* by proteolytic removal of 60-100 amino acid residues from the N-terminus. This is well within the range of the 92 amino acids removed from the bovine photoreceptor CNG α subunit (Molday *et al.*, 1991). This suggests that the processing of CNG α subunits is general for photoreceptor cells and likely plays an as yet unknown physiological role. Similar processing of the α subunits has also been observed in human and mouse ROS (Molday *et al.*, 1991). The cDNA sequences which code for the human and mouse α subunits predict molecular weights of 79,100 and 79,300 respectively (Dhallan *et al.*,

1992; Pittler *et al.*, 1992), whereas by SDS-PAGE the α subunits migrate with an apparent M_r of 63,000.

Examining the sequences of various photoreceptor CNG α subunits, the bovine, human and mouse sequences share an 11 amino acid sequence surrounding the double serine cleavage site (see appendix boxed region). This would explain the cross reactivity of PMc 6E7 among the three species and also indicates that they are all cleaved at the same site. The chicken rod α subunit shares only 7 amino acids of this sequence most notably missing the first of the two serines. The cone α subunit does not closely resemble the rod α subunits in this region, however it does have two sets of double serines which occur before (amino acids 43&44) and after (amino acids 112&113) the putative cleavage site of the bovine human and mouse α subunit sequences (see appendix).

4.5 Reconstitution of the α subunit

Initial attempts to confirm that the heterologously expressed α subunit was functionally active, indicated that it was necessary to concentrate the channel before reconstitution. Immunopurification requires the availability of characterized antibodies to generate immunoaffinity columns. Available channel antibodies which recognized both the 63 and 80 kDa α subunits could not be successfully used for this purpose. Either the binding site was not known or the antibody recognized the channel protein with a higher affinity than the peptide such that bound protein could not be eluted from a column. Hence, the C-terminal amino acids which corresponded to the rho 1D4 epitope were engineered onto the C-terminus of the α subunit. This same technique has been successfully used for the immunopurification of the cone opsins (Oprian *et al.*, 1991).

The 1D4-tagged α subunit could be efficiently immunopurified. However, upon reconstitution, cGMP-gated channel activity was barely detectable. It is possible that the addition of the 1D4 tail affected channel activity, although not likely.

4.6 The β subunit

The anomalous migration of the β subunit (calculated M_r of 155,000) at 240 kDa is attributed to the GARP portion of the protein. GARP expressed alone was observed to run at twice its predicted molecular mass, most likely due to the large number of negatively charged glutamic acid residues. The β' portion of the β subunit ran with an M_r of 110,000 closer to its predicted M_r of 93,000.

With the discovery of the β subunit it was clear that relevant studies must include both channel subunits. Other channels such as Na^+ and Ca^{2+} channels have been shown to contain subunits in addition to their α subunits. Electrophysiological measurements have indicated that the Na^+ and Ca^{2+} α subunits were functionally expressed on their own in *Xenopus* oocytes. The functional roles of β_1 and β_2 of Na^+ channels are not clear (Goldin, 1994), although β_2 has been implicated in membrane insertion and functional conformation. The various Ca^{2+} channel auxiliary subunits have been suggested to aid in correct assembly of the channel in *Xenopus* oocytes (Schmidt *et al.*, 1986), and in mammalian cells they either increase expression or affect kinetics (Lacerada *et al.*, 1991; Varadi *et al.*, 1991)

Unlike the voltage-gated channels where the various subunits do not demonstrate sequence similarity, the CNG α and membrane bound portion of the β subunit have a 30% overall identity (Chen *et al.*, 1993). This is vaguely reminiscent of ligand gated channels where there exists 18% identity and 54% similarity among the four subunits.

4.7 Co-expression of α and β subunits

Since the presence of the β or β' subunits in co-expression experiments with the 80 kDa α subunit had no effect on its relative molecular weight, it would appear that the β subunit is not responsible for proteolytic cleavage of the α subunit. Thus, for a specific protease one would have to look to the rod cell. Preliminary experiments incubating the h/e 80 kDa α subunit with ROS membranes and ROS soluble proteins gave no hint of specific proteolysis (data not presented).

The native channel purifies as a complex of two subunits, α and β and they have been shown to be directly associated (Cook and Kaupp, 1986; Molday *et al.*, 1990). Using the COS-1 expression system presented here, it could be shown that the two heterologously expressed subunits associate directly as has been observed for the native channel complex. Co-precipitation experiments indicate that the association of CNG channel subunits is not dependent on the N-terminal portion which is cleaved from the 80 kDa α subunit to produce the 63 kDa channel subunit, nor does it require the GARP portion of the β subunit. In the presence of CHAPS, (immunoprecipitation conditions) the subunits were associated, but on SDS gels in the absence of reducing agents the monomers could be separated (data not presented). This is evidence that the assembly is held together by non-covalent interactions (Shen *et al.*, 1993)

Pertinent studies have been carried out for voltage-gated K^+ channels. It has been shown that K^+ channel subunit assembly is directed by a conserved hydrophilic region in the cytoplasmic N-terminus, called the tetramerization 1 (T1) domain (Li *et al.*, 1992; Shen *et al.*, 1993). Such a domain could potentially exist in CNG channels, although the N-terminal regions do not appear to be as well conserved. Photoreceptor CNG α subunits do, however, share one feature in the N-terminal region. They possess regions extremely rich in lysine following the putative cleavage site for the removal of the N-terminus (see appendix).

The *in situ* labeling of the co-expressed channel complex in COS-1 cells indicated that the expression patterns of the α and β subunits were unaffected by each others presence. The labeling pattern of cells expressing only the α subunit was indistinguishable from the pattern seen for the α subunit when co-expressed with the β subunit. Also the fact that PPc 80N antibody recognized the 80 kDa subunit when it was co-expressed with the β subunit, indicated that the presence of the β subunit did not render the α subunit inaccessible to labeling. This is further evidence that the low level

labeling seen by the PPc 80N antibody in the outer segment was a result of low levels of the 80 kDa α subunit.

4.8 Functional activity of the expressed channel complex

Cyclic-GMP gated activity could be convincingly demonstrated only for the heterologously expressed channel complex consisting of the 80 kDa α subunit and the complete β subunit. As estimated by western blots, 10% of heterologously expressed channel complex was active. The inactive population probably represents either singly expressed subunit or non-functional complex. Immunoprecipitation of heterologously expressed channel complex with an antibody against the α subunit never quantitatively precipitates the β subunit and vice versa.

Lack of functional activity does not necessarily indicate that h/e complexes containing the 63 kDa α subunit or the β' subunit are inactive. The Ca^{2+} efflux activity of these combinations may still be below the detection levels of this assay. Indeed, very low levels of activity were observed for h/e channel complex comprising the 80 kDa α subunit and the β' subunit. The implications here are that there is a potential to form functionally active channels.

The first aspect to consider in heterologous expression is levels of protein expression. As mentioned earlier, the 63 kDa α subunit is expressed at a lower level than the 80 kDa in COS-1 cells. It is possible that this lowered expression presents less of the 63 kDa α subunit for assembly with the β subunit for the formation of functional channel complexes. For Ca^{2+} channels the presence of auxiliary subunits increased the expression of the α subunits (Varadi *et al.*, 1991), although this does not appear to be the case for the CNG channel. The presence of the β subunit appears to have no effect on the expression levels of the α subunits and vice versa.

Another aspect that governs function is correct folding of the heterologously expressed protein within the membrane. It is possible for example that the 63 kDa α subunit lacking the 92 N-terminal amino acids has a lower probability of forming a

channel in the membrane. The mechanism is still not fully understood whereby proteins lacking consensus signal sequences are inserted into the membrane. Although the cleaved N-terminus of the 80 kDa α subunit does not appear to be a signal sequence, we cannot rule out a role for it in assisting membrane insertion and folding.

The next aspect to consider is correct subunit-subunit interactions. Of course, subunit interaction cannot always be dissected from folding. The presence of the β subunit may be necessary for the correct folding of the α subunit. The β' portion may play that role but not as efficiently as the complete β subunit, hence, the minimal activity observed for the 80/ β' channel complexes. It has been shown that the β subunit is important for conferring certain native functional characteristics on the h/e channel (Chen *et al.*, 1993), and the GARP portion does not appear to affect the functional activity (Chen *et al.*, 1994).

The native configurations that these subunits are expressed in, namely, the 80 kDa α subunit and the full length β subunit, seem to be the most likely subunit combination to express functional channels. These are the polypeptides as they are synthesized and assembled in the ER. As these studies have indicated, once transported to the outer segment, the 80 kDa α subunit can be processed to the 63 kDa form.

Probably the most important feature determining functional reconstitution is the ability of a channel to retain activity throughout solubilization and reconstitution. Since on its own, the α subunit was marginally active, it is likely that the presence of the β subunit stabilizes the α subunit during solubilization. In support of this observation are biochemical studies on Na^+ channels which have shown that purification and reconstitution of the α subunit in the absence of the β_1 subunit yields a non-functional channel (Messner *et al.*, 1986).

Activity measurements performed on heterologously expressed channels in the past decade were typically carried out as whole cell current recordings in either oocytes or insect cells (Noda *et al.*, 1986; Timpe *et al.*, 1988; Klaiber *et al.*, 1990; Kartner *et al.*,

1991). Patch clamp measurements were also performed on heterologously expressed channels in mammalian cells (for example: Dhallan *et al.*, 1990; Varadi *et al.*, 1991).

The only examples of functional reconstitutions of heterologously expressed channels were measured in lipid bilayers. Channels were expressed in insect or mammalian cells, solubilized and incorporated into lipid bilayers (Bear *et al.*, 1992; Chen, S.R.W. *et al.*, 1993; Sun *et al.*, 1994). This work, therefore, presents the first example of functional reconstitution of a heterologously expressed cation channel into lipid vesicles measured by a biochemical efflux assay.

Reconstitutions of other heterologously expressed membrane proteins into liposomes are beginning to appear in the literature, particularly for transporters (Shapiro and Ling, 1995) and exchangers (Reiländer *et al.*, 1992). Structural information is vital to our understanding of functional mechanisms and at this point heterologous expression is a potential means to obtain large amounts of a protein. Whereas patch clamp studies provide functional confirmation of the proteins expressed on the plasma membrane, reconstitution of the solubilized expressed protein will provide information on the fraction that is in a functional state.

CONCLUDING REMARKS

To fully characterize a channel, at a structural and functional level, it must be available for study in an isolated controlled environment. In order to understand the functional characteristics of a channel, its structural properties must be elucidated. This way three-dimensional structure can be related to function, and in many instances information can be extrapolated to other polypeptides in order to broaden our overall understanding of proteins in general.

Functional studies have been carried out on the bovine rod CNG channel, and molecular cloning (Kaupp *et al.*, 1989) provides the first step in the characterization of its biochemical properties. The present work has sought to contribute to the understanding of this channel at the molecular level. The findings suggest that the channel α subunit, although its cDNA sequence codes for an 80 kDa polypeptide, exists predominantly as a 63 kDa polypeptide in ROS. Heterologous expression contributed to the positive identification of the 63 kDa polypeptide found in ROS as the truncated form of the 80 kDa α subunit. This same 63 kDa form of the α subunit, missing 92 N-terminal amino acids coded for by the cDNA, is the predominant form in the rod outer segment. A faint band seen on western blots above the 63 kDa α subunit was shown to be the 80 kDa α subunit, presumably the uncleaved precursor.

It would seem that the α subunit is synthesized as an 80 kDa polypeptide which associates with the β subunit, is transported to the outer segment and is processed to its 63 kDa form by a specific protease potentially residing in the outer segment. Similar processing mechanisms were shown to occur in the photoreceptor cells of other species.

The β subunit discovered during the course of these studies (Chen *et al.*, 1993) which exhibits 30% homology to the α subunit does not appear to be cleaved. The β subunit N-terminus is radically different from that of the α subunit. The β subunit has a unique bipartite structure. Its N-terminal portion consists of a sequence essentially identical to a glutamic acid rich polypeptide. This protein also exists independently as a soluble

protein (GARP) of unknown function in the ROS (Sugimoto *et al.*, 1991). The GARP portion of the β subunit appears to be responsible for its anomalous migration.

The α and β subunits purified from ROS are directly associated in a noncovalent manner (Molday *et al.*, 1990). The heterologously expressed α and β subunits are also directly associated as demonstrated by co-precipitation with mono-specific antibodies. This association does not depend on the first 92 amino acid N-terminus removed from the 80 kDa protein, nor does it depend on the GARP portion of the β subunit. The β subunit also appeared to stabilize the heterologously expressed channel complex during solubilization.

Successful reconstitution of the CNG 80 kDa α and the β subunit was demonstrated. This lays the ground work for future structure function studies to be carried out in our laboratory. The system presented here will also enable further characterization of the CNG channel subunit interactions. Ultimately, large amounts of correctly folded protein are desirable for crystallization studies to obtain concrete structural information. Since it appears that only 10% of the heterologously expressed channel is functionally active, the expression system needs refinement, and the work here is a first step.

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APPENDIX I

Protein sequence comparison between cloned CNG channels. Identical amino acids marked with an asterisk (*), conserved amino acids marked with a point (.).

BOVRODCHAN	M-----KKVIINTWHSFVNIPNVVGPDVEKEITRMENGACSSF	38
HUMRODCHAN	M-----KNNIINTQQSFVTMPNVIVPDIEKEIRRMENGACSSF	38
MUSRODCHAN	M-----KTNIINTWHSFVNIPNVIVPAIEKEIRRMENGACSSF	38
CHIRODCHAN	M-----KVGVIETHHSHPIIPSVVVQ-----	21
CHICONCHAN	M-----AK--INTQHSYPGMHGLSVRTTDEDIERIENGFI	36
BOVKIDCHAN	M-----AK--ISTQYSHPTRTTHPSVRTMDRDLDCIENGLSR	36
RATOLFCHAN	M-----	1
RABARTCHAN	MSSWRSCARAPLSGSAWRRSAATRRSRRCLKTKRKRWSSGKG	45
BOVOLFCHAN	M-----	1
FISOLFCHAN	MTG-----QAAL-----RSVSSHR-----LSVRSR-----L	22
FLYEYECHAN	MRHFKV-----	6
	*	
BOVRODCHAN	SGDD-DDSASMFESEETENPHARDSFRSNTHGSGQPSQREQYLPG	82
HUMRODCHAN	S-ED-DDSASTSESEENENPHARGSFYSKSLRKGGPSQREQYLPG	81
MUSRODCHAN	S--D-DDNGSLSESENEEDSF----FRSNSYKRRGPSQREQHLPG	76
CHIRODCHAN	--DT-SEDPGLIEKGEN-----RF-----ARQWYLP	45
CHICONCHAN	SLCE-DTSELQRVISMGRHLSGS-QTSPFTGRGAMARLSRFV	79
BOVKIDCHAN	LPCE-ETSELQEGIAMETRGLAES-RQSSFTSQGP-TRLSRLII	78
RATOLFCHAN	-----MTEKSNGVKSSPANNNHNNHPP	23
RABARTCHAN	QSTQCETRRRAQTPCESTGHTWRMTEKSNGVKSSPANNNHNNH	90
BOVOLFCHAN	-----TEKANGVKSSPANNNHNNHAPP	22
FISOLFCHAN	EG-----	24
FLYEYECHAN	-----KAMVQ	11
BOVRODCHAN	AIALFNVNNSNKEQEPKE-----	101
HUMRODCHAN	AIALFNVNNSNNDQEPKE-----	100
MUSRODCHAN	TMALFNVNNSNNDQEPKE-----	95
CHIRODCHAN	AFAQYNINNSNNDKEE-----	61
CHICONCHAN	SLRSWATRHLHHEDQRPDSFLERIRGPELVEVSSRQSNIRSF	124
BOVKIDCHAN	SLRAWSARHLHQEDQRPDSFLERFRGAELQEVSSRESHVQF	123
RATOLFCHAN	SIKANGKDDHRAGSRPQSAADDDTSPELQRLAEMDTPRR----	63
RABARTCHAN	TIKANGKDESRTSRPQS-AADDDTSSELQRLAEMDAPQQ----	129
BOVOLFCHAN	AIKASGKDDHRASSRPQSAADDDTSSELQQLAEMDAPQQ----	61
FISOLFCHAN	-----EAERAESAISRTDGDGDDTCSELQRVTALELPSAEM	62
FLYEYECHAN	SLDISAITGQQTDAEPSK----RSKPSALRR-----	38
BOVRODCHAN	-----KKKKKKEKKS KPDDKNENKKD	122
HUMRODCHAN	-----KKKKKKEKKS KSDNKNENKND	121
MUSRODCHAN	-----KKKKKKEKKS KADDKNEIK-D	115
CHIRODCHAN	-----KKKKKKEKKS KS-----ENKKD	77
CHICONCHAN	REQPGGVNGPWPLARFNVNFSNNTNEDKKEEKKEVKEEKKEE	169
BOVKIDCHAN	QEPDRGRSAWPLARNNTNTCNNSEKDDKA-----	153
RATOLFCHAN	--GRGGFQ---RIVRL-VGVIRDWANKNFRREEPRPDSFLER	102
RABARTCHAN	--RRGGFR---RIVRL-VGVIRQWANRNFRREEPRPDSFLER	168
BOVOLFCHAN	--RRGGFR---RIARL-VGVLEWAYRNFRREEPRPDSFLER	100
FISOLFCHAN	FTQRRPLA---RLVNL-VLSLREWAHKS LVETEQRPD	103
FLYEYECHAN	-----TLQALRQRLTKRNRPK--PPDWFLEKFSN	65

BOVRODCHAN	PEKKKKKEKD-KDKKKKEEKGKDKKEEEKKEVVVIDPSGNTYYNW	166
HUMRODCHAN	PEKKKKK-KD-KEKKKKEEKSKDKKEEEKKEVVVIDPSGNTYYNW	164
MUSRODCHAN	PEKKKKKEKE-KEKKKKEEKTKEKKEEEKKEVVVIDPSGNTYYNW	159
CHIRODCHAN	GERQKNKEK--KEKHKNKDKKKGKEEEKKKDIFIIDPAGNMYIYW	120
CHICONCHAN	EKKEEKKDDK-KDDKKDDKDDKKKEEQKKEVFVIDPSSNMYYNW	213
BOVKIDCHAN	-KKEEK-----EKKEEKKENPKKEEKKKDSVMDPSSNMYYHW	190
RATOLFCHAN	PE-LQTVTTH-QGDDKGGKDGEKGTKKKFELFVLDPAWDYWRW	145
RABARTCHAN	PE-LQTVTTQ-QGDGKGDKDGDGKGTKKKFELFVLDPAWDYWRW	211
BOVOLFCHAN	PE-LHTVTTQ-QGDGKGDKDGEKGTKKKFELFVLDPAWDYWRW	143
FISOLFCHAN	PQ-----AA-NDQSAAPADAPKTKFERWEGFVVSQSDDIYYW	141
FLYEYECHAN	TTNTDKIRKGC PAMEDAALSSEIRGSSVLCNRLSVDPTLHSHYRW	110

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BOVRODCHAN	LFCITLPVMYNWTMIIARACFDELQSDYLEYWLAFDYLSDVVYLL	211
HUMRODCHAN	LFCITLPVMYNWTMVIARACFDELQSDYLEYWLILDYVSDIVYLI	209
MUSRODCHAN	LFCITLPVMYNWTMIIARACFDELQSDYLEYWLIFDYVSNVYLA	204
CHIRODCHAN	LFCITMPVMYNWTMIIARACFDELQNDYLA VWFIVDYVSDVIYIA	165
CHICONCHAN	LTIIAAPVFYNWCM LICRACFDELQIDHIKLWLF LDYCSDIIVVF	258
BOVKIDCHAN	LTVIAVPVFYNWCLLVCRA CFDELQSEHMLWLVLVDYSADILYGM	235
RATOLFCHAN	LFVIAMPVLYNWCLLVARACFSDLQ RNYFVWLVLDYFSDTVYIA	190
RABARTCHAN	LFVIAMPVLYNWCLLVARACFSDLQ RGYFLVWLVLVDYFSDVYIA	256
BOVOLFCHAN	LFLIALPVLYNWCLLVARACFSDLQ KGYIYVWLVLVDYVSDVYIA	188
FISOLFCHAN	LFFIALASLYNWIMLVARACFDQLQ DENFFLWVGLDYLC DVIYIL	186
FLYEYECHAN	LAIVSLAVLYNII FVVGRA VFWEINKSAPAFWYTL DYLCDFIYLL	155

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BOVRODCHAN	DMFVRTRTGYLEQGLLVKEERKLIDKYKSTFQFKLDVLSVIPTDL	256
HUMRODCHAN	DMFVRTRTGYLEQGLLVKEELKLINKYKSNLQFKLDVLSLIPTDL	254
MUSRODCHAN	DMFVRTRTGYLEQGLLVKDRMKLIEKYKANLQFKLDVLSVIPTDL	249
CHIRODCHAN	DMFVRTRTGYLEQGLLVKEEQKLKEKYKSSLQFKLDFLSIIPTDL	210
CHICONCHAN	DMFVRFRGTGFLEQGLLVKDEKKLRDHYTQT VQFKLDVLSLLPTDL	303
BOVKIDCHAN	DMLVRARTGFLEQGLMVMDASRLWKHYTQTL HFKLDVLSLVPTDL	280
RATOLFCHAN	DLIIRLRTGFLEQGLLVKDPKKLRDNYIHTL QFKLDVASIIPPTDL	235
RABARTCHAN	DLFIRLRTGFLEQGLLVKDPKKLRD TYIHTLQFKLDVASIIPPTDL	301
BOVOLFCHAN	DLFIRLRTGFLEQGLLVKDTKKLRDNYIHTM QFKLDVASIIPPTDL	233
FISOLFCHAN	DTCIRLRTGYLEQGLLVKDLAKLRDNYIRT LQFKLDFLSILPTDL	231
FLYEYECHAN	DTLVHMHEGF LDQGLLV RDAFRLRRHYFHTKGWYLDVLSMLPTDL	200

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BOVRODCHAN	LYI-----KFGWNYPEI-RLNRLLRISRMFEFFQRTETRTNY	292
HUMRODCHAN	LYF-----KLGWNYPEI-RLNRLLRFSRMFEFFQRTETRTNY	290
MUSRODCHAN	LYI-----KFGWNYPEI-RLNRLLRISRMFEFFQRTETRTNY	285
CHIRODCHAN	LYF-----KLGLNYPEL-RINRLLRVARMF EFFQRTETRTNY	246
CHICONCHAN	AYL-----KLGLNYPEL-RFNRLLR IARLFEFFDRTE TRTNY	339
BOVKIDCHAN	AYF-----KLGMNYPEL-RFNRLLLKLARLFEFFDRTE TRTNY	316
RATOLFCHAN	IYF-----AVGIHSPEV-RFNRLLLHFARMF EFFDRTE TRTSY	271
RABARTCHAN	IYF-----AVGIHNPEL-RFNRLLLHFARMF EFFDRTE TRTSY	337
BOVOLFCHAN	IYF-----AVGIHNPEV-RFNRLLLHFARMF EFFDRTE TRTSY	269
FISOLFCHAN	LFF-----VTG-YVPQL-RFNRLLLRFSRMFEFFDRTE TRTNY	266
FLYEYECHAN	AYIWWPPETCSSLYLPCPVIVRLNRLLRINRLWEWFDRTE TATGY	245

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BOVRODCHAN	PNIFRISNLVMIIIIIHWNACVYFSISKAIGFGNDTWVYPDVND	337
HUMRODCHAN	PNIFRISNLVMIIVIIHWNACVFYSISKAIGFGNDTWVYPDIND	335
MUSRODCHAN	PNIFRISNLVMIYIVIIHWNACVYYSISKAIGFGNDTWVYPDVND	330
CHIRODCHAN	PNIFRISNLVMIYIVIIHWNACVYYSISKAIGFGADTWVYPNTSH	291
CHICONCHAN	PNMFRIGNLVLYILIIHWNACIYFAISKVIGFGTDSWVYPNVSI	384
BOVKIDCHAN	PNMFRIGNLVLYILIIHWNACIYFAISKFIGFGTDSWVYPNVSN	361
RATOLFCHAN	PNIFRISNLVLYILVIIHWNACIYYVISKSIGFGVDTWVYPNITD	316
RABARTCHAN	PNIFRISNLVLYILVIIHWNACIYYAISKSIGFGVDTWVYPNITD	382
BOVOLFCHAN	PNIFRISNLVLYILIIHWNACIYYAISKSIGFGVDTWVYPNITD	314
FISOLFCHAN	PNAFRICNLILYILVIIHWNACIYYAISKALGLSSDTWVYSGQNK	311
FLYEYECHAN	PNAFRICKVVLAILVLIHWNACMYFAISYEIGFSSDSWVY-NLNG	289

BOVRODCHAN	PDFGRLARKYVYSLYWSTLTLTITIGETPPPVRDSEYFFVVDFLI	382
HUMRODCHAN	PEFGRLARKYVYSLYWSTLTLTITIGETPPPVRDSEYVFFVVDFLI	380
MUSRODCHAN	PEFGRLARKYVYSLYWSTLTLTITIGETPPPVLDSYIFVVDFLI	375
CHIRODCHAN	PEFARLTRKYVYSLYWSTLTLTITIGETPPPVRDSEYFFVVDFLV	336
CHICONCHAN	PEYGRLSRKYIYSLYWSTLTLTITIGETPPPVKDEEYLFVVIDFLV	429
BOVKIDCHAN	PEYGRLSRKYIYSLYWSTLTLTITIGETPPPVKDEEYLFVVIDFLV	406
RATOLFCHAN	PEYGYLAREYIYCLYWSTLTLTITIGETPPPVKDEEYLFVIFDFLI	361
RABARTCHAN	PEYGYLAREYIYCLYWSTLTLTITIGETPPPVKDEEYLFVIFDFLI	427
BOVOLFCHAN	PEYGYLSREYIYCLYWSTLTLTITIGETPPPVKDEEYLFVIFDFLI	359
FISOLFCHAN	T----LSFCYVYCFYWSTLTLTITIGEMPPPVKDEEYVFFVDFLV	352
FLYEYECHAN	TRNNTLORQYIYSFYWSTLTLTITIGETPTPENDVEYLFVVADFLA	334

BOVRODCHAN	GVLIFATIVGNIGSMISNMNAARAEFQARIDAIKQYMHFRNVSKD	427
HUMRODCHAN	GVLIFATIVGNIGSMISNMNAARAEFQARIDAIKQYMHFRNVSKD	425
MUSRODCHAN	GVLIFATIVGNIGSMISNMNAARAEFQSRVDAIKQYMNFRNVSKD	420
CHIRODCHAN	GVLIFATIVGNVGSMSISNMNAARAEFQAKIDAIKQYMHFRNVSKD	381
CHICONCHAN	GVLIFATIVGNVGSMSISNMNASRAEFQAKVDSIKQYMHFRKVTKD	474
BOVKIDCHAN	GVLIFATIVGNVGSMSISNMNASRAEFQAKIDSIKQYMQFRKVTKD	451
RATOLFCHAN	GVLIFATIVGNVGSMSISNMNATRAEFQAKIDAVKHVMQFRKVSKE	406
RABARTCHAN	GVLIFATIVGNVGSMSISNMNATRAEFQAKIDAVKHVMQFRKVSKE	472
BOVOLFCHAN	GVLIFATIVGNVGSMSISNMNATRAEFQAKIDAVKHVMQFRKVSKE	404
FISOLFCHAN	GVLIFATIVGNVGSMIANMNATRAEFQTRIDAIKHVMHFRKVNRT	397
FLYEYECHAN	GVLIFATIVGNIGSMISNMNVARVEFQNRMDGVKQYMAFRRVGHE	379

BOVRODCHAN	MEKRVIKWFDYLTWNKKTVDEREVLKYLDPDKLRAEIAINVHLDTL	472
HUMRODCHAN	MEKRVIKWFDYLTWNKKTVDEKEVLKYLDPDKLRAEIAINVHLDTL	470
MUSRODCHAN	MEKRVIKWFDYLTWNKKTVDEREVLRYLPDKLRAEIAINVHLDTL	465
CHIRODCHAN	MEKRVIKWFDYLTWNKKAVDEREVLKYLDPDKLRAEIAINVHLETL	426
CHICONCHAN	LEARVIKWFDYLTWNKKTVDKEEVLKLNLPDKLKAIEIAINVHLDTL	519
BOVKIDCHAN	LETRVIRWFDYLTWANKKTVDKEEVLKSLPDKLKAEIAINVHLDTL	496
RATOLFCHAN	MEAKVIKWFDYLTWNKKTVDEREVLKLNLPAKLRAEIAINVHLSTL	451
RABARTCHAN	MEAKVIKWFDYLTWNKKTVDEREVLKLNLPAKLRAEIAINVHLSTL	517
BOVOLFCHAN	MEAKVIRWFDYLTWNKKSVDEREVLKLNLPAKLRAEIAINVHLSTL	449
FISOLFCHAN	LETRVIKWFDYLTWNKKTVDQEVLKLNLPDKLRAEIAINVHLDTL	442
FLYEYECHAN	LEARVIRWFAYTWSOGALDEERVLAALPDKLKAEIAIQVHMDTL	424

BOVRODCHAN	KKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYII	517
HUMRODCHAN	KKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYII	515
MUSRODCHAN	KKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYII	510
CHIRODCHAN	KKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYII	471
CHICONCHAN	KKVRIFQDCEAGLLIELVLKLKPTVFS PGDYICKKGDIGREMYII	564
BOVKIDCHAN	RKVRIFQDCEAGLLVELVLKLRPVFS PGDYICKKGDIGREMYII	541
RATOLFCHAN	KKVRIFQDCEAGLLVELVLKLRPVFS PGDYICKKGDIGREMYII	496
RABARTCHAN	KKVRIFQDCEAGLLVELVLKLRPVFS PGDYICKKGDIGREMYII	562
BOVOLFCHAN	KKVRIFQDCEAGLLVELVLKLRPVFS PGDYICKKGDIGREMYII	494
FISOLFCHAN	KKVRIFQDCEAGLLVELVLKLRPVFS PGDYICKKGDIGREMYII	487
FLYEYECHAN	KQVRIFHDTEPGLLEALVLKLQVFS PGDYICKKGDVGKALYIV	469
	..***** * *.*** .*****. *.*****.***.*.....*	
BOVRODCHAN	KEGKLAVVADDGITQFVVLSDGSYFGEISILNIKSGKAGNRRTAN	562
HUMRODCHAN	KEGKLAVVADDGVTQFVVLSDGSYFGEISILNIKSGKAGNRRTAN	560
MUSRODCHAN	KEGKLAVVADDGITQFVVLSDGSYFGEISILNIKSGKAGNRRTAN	555
CHIRODCHAN	KEGKLAVVADDGVTQFVVLSDGSYFGEISILNIKSGKAGNRRTAN	516
CHICONCHAN	KEGKLAVVADDGITQFVVLSDGSYFGEISILNIKSGKSGNRRTAN	609
BOVKIDCHAN	KEGKLAVVAEDGITQFVVLGDGSYFGEISILNIKSGKSGNRRTAN	586
RATOLFCHAN	KEGKLAVVADDGVTQYALLSAGSCFGEISILNIKSGKMGNRRTAN	541
RABARTCHAN	KEGKLAVVADDGVTQYALLSAGSCFGEISILNIKSGKMGNRRTAN	607
BOVOLFCHAN	KEGKLAVVADDGVTQYALLSAGSCFGEISILNIKSGKMGNRRTAN	539
FISOLFCHAN	KEGQLAVVADDGVTQFALLTAGGCFGEISILNIQSGKMGNRRTAN	532
FLYEYECHAN	KRGKLSVVGDDGITVLTATLGAGSVFGEVSVLEIAGNRRTGNRRTAN	514
	* *.***.***.* .*.***.***.*.***.*..*****	
BOVRODCHAN	IKSIGYSDFCLSKDDLMEALTEY PDAKGMLEEK GKQILMKDGLL	607
HUMRODCHAN	IKSIGYSDFCLSKDDLMEALTEY PDAKTMLEEK GKQILMKDGLL	605
MUSRODCHAN	IKSIGYSDFCLSKDDLMEVLTEY PDAKTMLEEK GRQILMKDGLL	600
CHIRODCHAN	IRSIGYSDFCLSKDDLMEALTEY PDAKAMLEEK GKQILMKDGLL	561
CHICONCHAN	IRSIGYSDFCLSKDDLMEALTEY PEAKKALEEK GRQILMKDNLI	654
BOVKIDCHAN	IRSIGYSDFCLSKDDLMEALTEY PEAKKALEEK GRQILMKDNLI	631
RATOLFCHAN	IRSLGYSDLFCLSKDDLMEAVTEY PDAKKVLEER GREILMKEGLL	586
RABARTCHAN	IRSLGYSDLFCLSKDDLMEAVTEY PDAKKVLEER GREILMKEGLL	652
BOVOLFCHAN	IRSLGYSDLFCLSKDDLMEAVTEY PDAKRVLEER GREILMKEGLL	584
FISOLFCHAN	IRSIGYSDFCLSKDDLMEAVA EY PDAQVLEER GREILRKQGLL	577
FLYEYECHAN	VRS LGYSDFCLAKRDLWETLS DYPEARSTLTQRGCQLLRKDGLL	559
	..*.*****.* ** *.....*. *...* ..*.*...*	
BOVRODCHAN	DINIANAGSDPKD-LEEKVTRMESSVDLLQTRFARILAEYESMQQ	651
HUMRODCHAN	DLNIANAGSDPKD-LEEKVTRMEGSVDLLQTRFARILAEYESMQQ	649
MUSRODCHAN	DINIANMGSDPKD-LEEKVTRMEGSVDLLQTRFARILAEYESMQQ	644
CHIRODCHAN	DIEVANLGSDPKD-LEEKVAYMEGSMDRLQTKFARLLAEYDAAQQ	605
CHICONCHAN	DEEAAKAGADPKD-LEEKIDRLETALDTLQTRFARLLAEYSSSQ	698
BOVKIDCHAN	DEELAKAGADPKD-IEEKVEHLETSLDSLQTRFARLLAEYNATQM	675
RATOLFCHAN	DENEVAASMEV-D-VQEKLEQLETNMDTLYTRFARLLAEYTGAQQ	629
RABARTCHAN	DENEVAASMEV-D-VQEKLEQLETNMDTLYTRFGRLLAEYTGAQQ	695
BOVOLFCHAN	DENEVAASMEV-D-VQEKLEQLETNMDTLYTRFARLLAEYTGAQQ	627
FISOLFCHAN	DESVAAGGLGVID-TEEKVERLDASLDILQTRFARLLGEFTSTQR	621
FLYEYECHAN	DEQIFADSQRVHDSIEGGIEKLELSVENLNMRLARLLAEYTASQA	604
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APPENDIX II

Protein sequences for the 63 kDa α subunit, the 80 kDa α subunit, the β' polypeptide and the β subunit.

63 kDa α subunit: 599 amino acids

		5	10	15	20	25	30																							
1	M	S	N	K	E	Q	E	P	K	E	K	K	K	K	K	E	K	K	S	K	P	D	D	K	N	E	N	K	K	
31	D	P	E	K	K	K	K	E	K	D	K	D	K	K	K	E	E	K	G	K	D	K	K	E	E	E	K	K		
61	E	V	V	V	I	D	P	S	G	N	T	Y	Y	N	W	L	F	C	I	T	L	P	V	M	Y	N	W	T	M	I
91	I	A	R	A	C	F	D	E	L	Q	S	D	Y	L	E	Y	W	L	A	F	D	Y	L	S	D	V	V	Y	L	L
121	D	M	F	V	R	T	R	T	G	Y	L	E	Q	G	L	L	V	K	E	E	R	K	L	I	D	K	Y	K	S	T
151	F	Q	F	K	L	D	V	L	S	V	I	P	T	D	L	L	Y	I	K	F	G	W	N	Y	P	E	I	R	L	N
181	R	L	L	R	I	S	R	M	F	E	F	F	Q	R	T	E	T	R	T	N	Y	P	N	I	F	R	I	S	N	L
211	V	M	Y	I	I	I	I	I	H	W	N	A	C	V	Y	F	S	I	S	K	A	I	G	F	G	N	D	T	W	V
241	Y	P	D	V	N	D	P	D	F	G	R	L	A	R	K	Y	V	Y	S	L	Y	W	S	T	L	T	L	T	T	I
271	G	E	T	P	P	P	V	R	D	S	E	Y	F	F	V	V	A	D	F	L	I	G	V	L	I	F	A	T	I	V
301	G	N	I	G	S	M	I	S	N	M	N	A	A	R	A	E	F	Q	A	R	I	D	A	I	K	Q	Y	M	H	F
331	R	N	V	S	K	D	M	E	K	R	V	I	K	W	F	D	Y	L	W	T	N	K	K	T	V	D	E	R	E	V
361	L	K	Y	L	P	D	K	L	R	A	E	I	A	I	N	V	H	L	D	T	L	K	K	V	R	I	F	A	D	C
391	E	A	G	L	L	V	E	L	V	L	K	L	Q	P	Q	V	Y	S	P	G	D	Y	I	C	K	K	G	D	I	G
421	R	E	M	Y	I	I	K	E	G	K	L	A	V	V	A	D	D	G	I	T	Q	F	V	V	L	S	D	G	S	Y
451	F	G	E	I	S	I	L	N	I	K	G	S	K	A	G	N	R	R	T	A	N	I	K	S	I	G	Y	S	D	L
481	F	C	L	S	K	D	D	L	M	E	A	L	T	E	Y	P	D	A	K	G	M	L	E	E	K	G	K	Q	I	L
511	M	K	D	G	L	L	D	I	N	I	A	N	A	G	S	D	P	K	D	L	E	E	K	V	T	R	M	E	S	S
541	V	D	L	L	Q	T	R	F	A	R	I	L	A	E	Y	E	S	M	Q	Q	K	L	K	Q	R	L	T	K	V	E
571	K	F	L	K	P	L	I	D	T	E	F	S	A	I	E	G	S	G	T	E	S	G	P	T	D	S	T	Q	D	

80 kDa α subunit: 691 amino acids

		5		10		15		20		25		30																		
1	M	K	K	V	I	I	N	T	W	H	S	F	V	N	I	P	N	V	V	G	P	D	V	E	K	E	I	T	R	M
31	E	N	G	A	C	S	S	F	S	G	D	D	D	D	S	A	S	M	F	E	E	S	E	T	E	N	P	H	A	R
61	D	S	F	R	S	N	T	H	G	S	G	Q	P	S	Q	R	E	Q	Y	L	P	G	A	I	A	L	F	N	V	N
91	N	S	S	N	K	E	Q	E	P	K	E	K	K	K	K	K	K	E	K	K	S	K	P	D	D	K	N	E	N	K
121	K	D	P	E	K	K	K	K	K	E	K	D	K	D	K	K	K	K	E	E	K	G	K	D	K	K	E	E	E	K
151	K	E	V	V	V	I	D	P	S	G	N	T	Y	Y	N	W	L	F	C	I	T	L	P	V	M	Y	N	W	T	M
181	I	I	A	R	A	C	F	D	E	L	Q	S	D	Y	L	E	Y	W	L	A	F	D	Y	L	S	D	V	V	Y	L
211	L	D	M	F	V	R	T	R	T	G	Y	L	E	Q	G	L	L	V	K	E	E	R	K	L	I	D	K	Y	K	S
241	T	F	Q	F	K	L	D	V	L	S	V	I	P	T	D	L	L	Y	I	K	F	G	W	N	Y	P	E	I	R	L
271	N	R	L	L	R	I	S	R	M	F	E	F	F	Q	R	T	E	T	R	T	N	Y	P	N	I	F	R	I	S	N
301	L	V	M	Y	I	I	I	I	I	H	W	N	A	C	V	Y	F	S	I	S	K	A	I	G	F	G	N	D	T	W
331	V	Y	P	D	V	N	D	P	D	F	G	R	L	A	R	K	Y	V	Y	S	L	Y	W	S	T	L	T	T	T	T
361	I	G	E	T	P	P	P	V	R	D	S	E	Y	F	F	V	V	A	D	F	L	I	G	V	L	I	F	A	T	I
391	V	G	N	I	G	S	M	I	S	N	M	N	A	A	R	A	E	F	Q	A	R	I	D	A	I	K	Q	Y	M	H
421	F	R	N	V	S	K	D	M	E	K	R	V	I	K	W	F	D	Y	L	W	T	N	K	K	T	V	D	E	R	E
451	V	L	K	Y	L	P	D	K	L	R	A	E	I	A	I	N	V	H	L	D	T	L	K	K	V	R	I	F	A	D
481	C	E	A	G	L	L	V	E	L	V	L	K	L	Q	P	Q	V	Y	S	P	G	D	Y	I	C	K	K	G	D	I
511	G	R	E	M	Y	I	I	K	E	G	K	L	A	V	V	A	D	D	G	I	T	Q	F	V	V	L	S	D	G	S
541	Y	F	G	E	I	S	I	L	N	I	K	G	S	K	A	G	N	R	R	T	A	N	I	K	S	I	G	Y	S	D
571	L	F	C	L	S	K	D	D	L	M	E	A	L	T	E	Y	P	D	A	K	G	M	L	E	E	K	G	K	Q	I
601	L	M	K	D	G	L	L	D	I	N	I	A	N	A	G	S	D	P	K	D	L	E	E	K	V	T	R	M	E	S
631	S	V	D	L	L	Q	T	R	F	A	R	I	L	A	E	Y	E	S	M	Q	Q	K	L	K	Q	R	L	T	K	V
661	E	K	F	L	K	P	L	I	D	T	E	F	S	A	I	E	G	S	G	T	E	S	G	P	T	D	S	T	Q	D

β' polypeptide: 823 amino acids

				5					10					15					20						25					30	
1	M	V	P	A	T	E	E	H	P	E	L	Q	V	E	D	A	D	A	D	S	R	P	L	I	A	E	E	N	P	P	
31	S	P	V	Q	L	P	L	S	P	A	K	S	D	T	L	A	V	P	G	S	A	T	G	S	L	R	K	R	L	P	
61	S	Q	D	D	E	A	E	E	L	K	M	L	S	P	A	A	S	P	V	V	A	W	S	D	P	T	S	P	Q	G	
91	T	D	D	Q	D	R	A	T	S	T	A	S	Q	N	S	A	I	I	N	D	R	L	Q	E	L	V	K	L	F	K	
121	E	R	T	E	K	V	K	E	K	L	I	D	P	D	V	T	S	D	E	E	S	P	K	P	S	P	A	K	K	A	
151	P	E	P	A	P	E	V	K	P	A	E	A	G	Q	V	E	E	E	H	Y	C	E	M	L	C	C	K	F	K	R	
181	R	P	W	K	K	Y	Q	F	P	Q	S	I	D	P	L	T	N	L	M	Y	I	L	W	L	F	F	V	V	L	A	
211	W	N	W	N	C	W	L	I	P	V	R	W	A	F	P	Y	Q	T	P	D	N	I	H	L	W	L	L	M	D	Y	
241	L	C	D	L	I	Y	L	L	D	I	T	V	F	Q	M	R	L	Q	F	V	R	G	G	D	I	I	T	D	K	K	
271	E	M	R	N	N	Y	V	K	S	Q	R	F	K	M	D	M	L	C	L	L	P	L	D	L	L	Y	L	K	F	G	
301	V	N	P	L	L	R	L	P	R	C	L	K	Y	M	A	F	F	E	F	N	N	R	L	E	S	I	L	S	K	A	
331	Y	V	Y	R	V	I	R	T	T	A	Y	L	L	Y	S	L	H	L	N	S	C	L	Y	Y	W	A	S	A	Y	E	
361	G	L	G	S	T	H	W	V	Y	D	G	V	G	N	S	Y	I	R	C	Y	Y	W	A	V	K	T	L	I	T	I	
391	G	G	L	P	D	P	R	T	L	F	E	I	V	F	Q	G	L	N	Y	F	T	G	V	F	A	F	S	V	M	I	
421	G	Q	M	R	D	V	V	G	A	A	T	A	G	Q	T	Y	Y	R	S	C	M	D	S	T	V	K	Y	M	N	F	
451	Y	K	I	P	R	S	V	Q	N	R	V	K	T	W	Y	E	Y	T	W	H	S	Q	G	M	L	D	E	S	E	L	
481	M	V	Q	L	P	D	K	M	R	L	D	L	A	I	D	V	N	Y	S	I	V	S	K	V	A	L	F	Q	G	C	
511	D	R	Q	M	I	F	D	M	L	K	R	L	R	S	V	V	Y	L	P	N	D	Y	V	C	K	K	G	E	I	G	
541	R	E	M	Y	I	I	Q	A	G	Q	V	Q	V	L	G	G	P	D	G	K	S	V	L	V	T	L	K	A	G	S	
571	V	F	G	E	I	S	L	L	A	V	G	G	G	N	R	R	T	A	N	V	V	A	H	G	F	T	N	L	F	I	
601	L	D	K	K	D	L	N	E	I	L	V	H	Y	P	E	S	Q	K	F	V	R	K	K	A	R	R	M	L	R	N	
631	N	N	K	P	K	E	K	S	V	L	I	L	P	P	R	A	G	T	P	K	L	F	N	A	A	L	A	A	A	G	
661	K	M	G	A	K	G	G	R	G	G	R	L	A	L	L	R	A	R	L	K	E	L	A	A	L	E	G	R	R	Q	
691	Q	Q	L	L	E	Q	A	K	S	S	E	D	A	A	V	G	E	E	G	S	A	S	P	E	Q	P	P	R	P	E	
721	P	P	A	P	E	A	P	A	P	E	P	T	A	P	E	P	L	A	P	E	A	P	A	P	E	A	P	A	P	S	
751	S	P	P	P	A	S	Q	E	R	P	E	G	D	K	E	A	A	A	P	E	E	H	P	V	R	I	H	V	T	L	
781	G	P	D	P	S	E	Q	I	L	L	V	E	V	P	E	K	Q	E	E	K	E	K	K	E	E	E	E	T	E	E	K
811	E	E	G	E	E	A	R	K	E	K	E	E	E																		

β subunit: 1393 amino acids

	5	10	15	20	25	30																											
1	M	L	G	W	V	Q	R	V	L	P	Q	P	P	G	T	P	Q	K	T	K	Q	E	E	E	E	G	T	E	P	E	P		
31	E	L	E	P	K	P	E	T	A	P	E	E	T	E	L	E	E	V	S	L	P	P	E	E	E	P	C	V	G	K	E	P	
61	V	A	A	V	T	L	G	P	Q	G	T	Q	E	T	A	L	T	P	P	T	S	L	Q	Q	A	Q	V	S	V	A	P	P	
91	E	A	H	S	S	P	R	G	W	V	L	T	W	L	R	K	G	V	E	K	V	V	P	Q	P	A	H	S	S	R	D	P	
121	P	S	Q	N	I	A	A	G	L	E	S	P	D	Q	Q	A	G	A	Q	I	L	G	Q	C	G	T	G	G	S	D	P	P	
151	E	P	S	E	P	S	R	A	E	D	P	G	P	G	P	W	L	L	R	W	F	E	Q	N	L	E	K	M	L	P	P	P	
181	Q	P	P	K	I	S	E	G	W	R	D	E	P	T	D	A	A	L	G	P	E	P	P	G	P	A	L	E	I	K	P	P	
211	P	M	L	Q	A	Q	E	S	P	S	L	P	A	P	G	P	P	E	P	E	E	E	P	I	P	E	P	Q	P	T	P	P	
241	I	Q	A	S	S	L	P	P	P	Q	D	S	A	R	L	M	A	W	I	L	H	R	L	E	M	A	L	P	Q	P	P	P	
271	V	I	R	G	K	G	G	E	Q	E	S	D	A	P	V	T	C	D	V	Q	T	I	S	I	L	P	G	E	Q	E	S	P	
301	E	S	H	L	I	L	E	E	V	D	P	H	W	E	E	D	E	H	Q	E	G	S	T	S	T	S	P	R	T	S	P	P	
331	E	A	A	P	A	D	E	E	K	G	K	V	V	E	Q	T	P	R	E	L	P	R	I	Q	E	E	K	E	D	E	P	P	
361	E	E	E	K	E	D	G	E	E	E	E	E	E	E	G	R	E	K	E	E	E	E	E	G	E	E	K	E	E	E	P	P	
391	R	E	K	E	E	E	E	G	E	K	K	E	E	E	E	G	R	E	K	E	E	E	E	E	G	E	K	E	D	E	P	P	
421	G	R	E	K	E	E	E	E	G	R	G	K	E	E	E	E	G	G	E	K	E	E	E	E	E	G	R	G	K	E	P	P	
451	V	E	G	R	E	E	E	E	D	E	E	E	E	E	Q	D	H	S	V	L	L	D	S	Y	L	V	P	Q	S	E	P	P	
481	D	R	S	E	E	S	E	T	Q	D	Q	S	E	V	G	G	A	Q	Q	S	E	V	G	G	A	Q	G	A	Q	A	L	S	P
511	E	E	S	E	T	Q	D	Q	S	E	V	G	G	A	Q	S	D	S	H	Q	E	L	Q	E	A	A	L	A	D	S	P	P	
541	G	A	Q	E	Q	D	G	V	G	G	A	Q	D	Q	S	D	S	H	Q	E	L	Q	E	A	A	L	A	D	S	P	P	P	
571	G	V	P	A	T	E	E	H	P	E	L	Q	V	E	D	A	D	A	D	S	R	P	L	I	A	E	E	N	P	P	P	P	
601	S	P	V	Q	L	P	L	S	P	A	K	S	D	T	L	A	V	P	G	S	A	T	G	S	L	R	K	R	L	P	P	P	
631	S	Q	D	D	E	A	E	E	L	K	M	L	S	P	A	A	S	P	V	V	A	W	S	D	P	T	S	P	Q	G	P	P	
661	T	D	D	Q	D	R	A	T	S	T	A	S	Q	N	S	A	I	I	N	D	R	L	Q	E	L	V	K	L	F	K	P	P	
691	E	R	T	E	K	V	K	E	K	L	I	D	P	D	V	T	S	D	E	E	S	P	K	P	S	P	A	K	K	A	P	P	
721	P	E	P	A	P	E	V	K	P	A	E	A	G	Q	V	E	E	H	Y	C	E	M	L	C	C	K	F	K	R	P	P	P	
751	R	P	W	K	K	Y	Q	F	P	Q	S	I	D	P	L	T	N	L	M	Y	I	L	W	L	F	F	V	V	L	A	P	P	
781	W	N	W	N	C	W	L	I	P	V	R	W	A	F	P	Y	Q	T	P	D	N	I	H	L	W	L	L	M	D	Y	P	P	
811	L	C	D	L	I	Y	L	L	D	I	T	V	F	Q	M	R	L	Q	F	V	R	G	G	D	I	I	T	D	K	K	P	P	
841	E	M	R	N	N	Y	V	K	S	Q	R	F	K	M	D	M	L	C	L	L	P	L	D	L	L	Y	L	K	F	G	P	P	
871	V	N	P	L	L	R	L	P	R	C	L	K	Y	M	A	F	F	E	F	N	N	R	L	E	S	I	L	S	K	A	P	P	
901	Y	V	Y	R	V	I	R	T	T	A	Y	L	L	Y	S	L	H	L	N	S	C	L	Y	Y	W	A	S	A	Y	E	P	P	
931	G	L	G	S	T	H	W	V	Y	D	G	V	G	N	S	Y	I	R	C	Y	Y	W	A	V	K	T	L	I	T	I	P	P	
961	G	G	L	P	D	P	R	T	L	F	E	I	V	F	Q	G	L	N	Y	F	T	G	V	F	A	F	S	V	M	I	P	P	
991	G	Q	M	R	D	V	V	G	A	A	T	A	G	Q	T	Y	Y	R	S	C	M	D	S	T	V	K	Y	M	N	F	P	P	
1021	Y	K	I	P	R	S	V	Q	N	R	V	K	T	W	Y	E	Y	T	W	H	S	Q	G	M	L	D	E	S	E	L	P	P	
1051	M	V	Q	L	P	D	K	M	R	L	D	L	A	I	D	V	N	Y	S	I	V	S	K	V	A	L	F	Q	G	C	P	P	
1081	D	R	Q	M	I	F	D	M	L	K	R	L	R	S	V	V	Y	L	P	N	D	Y	V	C	K	K	G	E	I	G	P	P	
1111	R	E	M	Y	I	I	Q	A	G	Q	V	Q	V	L	G	G	P	D	G	K	S	V	L	V	T	L	K	A	G	S	P	P	
1141	V	F	G	E	I	S	L	L	A	V	G	G	G	N	R	R	T	A	N	V	V	A	H	G	F	T	N	L	F	I	P	P	
1171	L	D	K	K	D	L	N	E	I	L	V	H	Y	P	E	S	Q	K	F	V	R	K	K	A	R	R	M	L	R	N	P	P	
1201	N	N	K	P	K	E	K	S	V	L	I	L	P	P	R	A	G	T	P	K	L	F	N	A	A	L	A	A	A	G	P	P	
1231	K	M	G	A	K	G	G	R	G	G	R	L	A	L	L	R	A	R	L	K	E	L	A	A	L	E	G	R	R	Q	P	P	
1261	Q	Q	L	L	E	Q	A	K	S	S	E	D	A	A	V	G	E	E	G	S	A	S	P	E	Q	P	P	R	P	E	P	P	
1291	P	P	A	P	E	A	P	A	P	E	P	T	A	P	E	P	L	A	P	E	A	P	A	P	E	A	P	A	P	S	P	P	
1321	S	P	P	P	A	S	Q	E	R	P	E	G	D	K	E	A	A	A	P	E	E	H	P	V	R	I	H	V	T	L	P	P	
1351	G	P	D	P	S	E	Q	I	L	L	V	E	V	P	E	K	Q	E	E	K	E	K	K	E	E	E	T	E	E	K	P	P	
1381	E	E	G	E	E	A	R	K	E	K	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	P	P	

APPENDIX III

Protein sequences for the 63 kDa α subunit, the 80 kDa α subunit, the β' polypeptide and the β subunit.

63 kDa α subunit: 1801 bases

	10	20	30	40	50	60
1	ATGAGCAATAAGGAGCAAGAACCAAGGAAAAGAAGAAAAAGAAAAAGAAAAGAAGAGC					
61	AAGCCAGATGATAAAAATGAAAATAAAAAGGACCCAGAAAAGAAAAGAAGAAAAG					
121	GACAAAGACAAGAAAAAGAAAGAGGAGAAAGGCCAAAGATAAGAAAGAGGAAGAGAAGAAG					
181	GAAGTCGTGGTTATTGATCCCTCAGGAAATACGTATTACAACCTGGCTGTTCTGCATCACC					
241	TTACCTGTTATGTATAACTGGACCATGATTATTGCAAGAGCATGTTTTGATGAACTTCAG					
301	TCTGATTACCTAGATACTGGCTTGCTTTTGATTACTTATCAGATGTAGTCTATCTTCTT					
361	GATATGTTTGTACGAACAAGGACAGGTTACCTAGAACAAGGACTACTGGTGAAGGAAGAG					
421	CGTAAACTGATAGACAAGTATAAATCAACCTTTCAATTTAAACTTGATGTTCTATCAGTG					
481	ATCCCAACTGATCTGCTGTATATTAAGTTTGGCTGGAATTATCCAGAAATTAGATTAAAC					
541	AGATTGTTAAGGATCTCTCGAATGTTTGAGTTCTTCCAGAGAACAGAAACACGGACAAAC					
601	TACCCAAACATCTTCAGGATCTCTAACCTTGTCTATGTACATCATCATCATCCACTGG					
661	AATGCGTGTGTGTAATCTCTATTTCCAAAGCTATTGGGTTTGGAAATGACACATGGGTC					
721	TATCCTGATGTTAATGATCCTGATTTTGGCCGTTTGGCTAGAAAATATGTGTACAGTCTT					
781	TACTGGTCTACACTGACTTTGACCCTATTGGCGAAACACCACCTCCTGTGAGGGATTCT					
841	GAGTATTTCTTTGTGGTGGCTGATTTCTCATTGGAGTGTTAATTTTTGCCACCATTGTC					
901	GGTAACATAGGTTCTATGATTTCCAACATGAATGCGGCCAGGGCAGAATTTCAAGCAAGA					
961	ATTGATGCAATCAAGCAATACATGCATTTTCGAAATGTAAGCAAAGATATGGAGAAGCGA					
1021	GTCATTAAATGGTTTGACTATCTATGGACCAACAAAAAACAGTGGATGAGAGAGAAGTC					
1081	TTGAAGTATCTACCTGATAAACTAAGAGCAGAGATCGCCATCAATGTTTATTAGACACA					
1141	TTAAAAAAGGTGCGCATTTTTTGCAAGCTGTGAAGCTGGTCTGTTGGTGGAGTTGGTCTTG					
1201	AAATTACAACCCCAAGTCTACAGTCCTGGAGATTACATTTGCAAGAAAGGGGACATTGGC					
1261	CGAGAGATGTACATCATCAAGGAAGGCAAACTCGCCGTGGTGGCTGATGACGGGATCACT					
1321	CAGTTTGTAGTATTGAGTGATGGCAGCTACTTTGGTGAATCAGTATCCTTAATATTAAA					
1381	GGTAGCAAAGCTGGCAATCGAAGAACAGCCAATATTAAAAGCATTGGCTACTCAGATCTA					
1441	TTCTGTCTCTCAAAGATGACCTCATGGAAGCTCTAACTGAGTACCCAGATGCCAAAGGT					
1501	ATGCTAGAAGAGAAAGGGAAGCAAATTTTGATGAAAGATGGTCTATTGGACATAAACATT					
1561	GCAAATGCTGGAAGTGATCCTAAAGATCTTGAAGAGAAGGTCACCCGAATGGAGAGCTCA					
1621	GTAGACCTCCTGCAAACAAGGTTTGCTCGGATCCTGGCTGAGTATGAGTCAATGCAGCAG					
1681	AAACTGAAGCAGAGGCTAACCAAGGTTGAGAAATTCCTGAAACCACTTATTGACACAGAA					
1741	TTTTTCAGCTATTGAAGGATCTGGAAGTGAAGTGGGCCCACAGACTCTACACAGGACTGA					

80 kDa α subunit: 2073 bases

	10	20	30	40	50	60
1	ATGAAGAAAGTGATTATCAATACATGGCACTCTTTTGTAAATATTCCCAATGTGGTTGGA					
61	CCAGATGTTGAAAAGGAAATAACAAGGATGGAAAATGGAGCATGCAGCTCCTTTTCTGGT					
121	GATGATGACGACAGTGCCTCTATGTTTGAAGAATCAGAGACTGAGAACCCCCACGCAAGG					
181	GATTCCTTTAGAAAGTAATACACACGGAAGCGGACAACCATCACAGAGGGAGCAATACCTG					
241	CCTGGAGCCATTGCACTTTTAAATGTTAACAACAGCAGCAATAAGGAGCAAGAACCAAAG					
301	GAAAAGAAGAAAAAGAAAAAGAAAAGAAGAGCAAGCCAGATGATAAAAATGAAAATAAA					
361	AAGGACCCAGAAAAGAAAAAGAAGAAAAGAAAAGGACAAAGACAAGAAAAAGAAAGAGGAG					
421	AAAGGCAAAGATAAGAAAGAGGAAGAGAAGAAGGAAGTCGTGGTTATTGATCCCTCAGGA					
481	AATACGTATTACAACTGGCTGTTCTGCATCACCTTACCTGTTATGTATAACTGGACCATG					
541	ATTATTGCAAGAGCATGTTTTGATGAACTTCAGTCTGATTACCTAGAATACTGGCTTGCT					
601	TTTGATTACTTATCAGATGTAGTCTATCTTCTTGATATGTTTGTACGAACAAGGACAGGT					
661	TACCTAGAACAAGGACTACTGGTGAAGGAAGAGCGTAAACTGATAGACAAGTATAAATCA					
721	ACCTTTC AATTTAACTTGATGTTCTATCAGTGATCCCAACTGATCTGCTGTATATTAAG					
781	TTTGGCTGGAATTATCCAGAAATTAGATTAAACAGATTGTTAAGGATCTCTCGAATGTTT					
841	GAGTTCCTCCAGAGAACAGAAACACGGACAAACTACCCAAACATCTTCAGGATCTCTAAC					
901	CTTGTCATGTACATCATCATCATCCACTGGAATGCGTGTGTGTACTTCTCTATTTC					
961	AAAGCTATTGGGTTTGGAAATGACACATGGGCTCTATCCTGATGTTAATGATCCTGATTTT					
1021	GGCCGTTTGGCTAGAAAATATGTGTACAGTCTTTACTGGTCTACACTGACTTTGACCCT					
1081	ATTGGCGAAACACCACCTCCTGTGAGGGATTCTGAGTATTTCTTTGTGGTGGCTGATTTT					
1141	CTCATTGGAGTGTTAATTTTTTGCCACCATTGTGCGTAACATAGGTTCTATGATTTCCAAC					
1201	ATGAATGCGGCCAGGGCAGAATTTCAAGCAAGAATTGATGCAATCAAGCAATACATGCAT					
1261	TTTCGAAATGTAAGCAAAGATATGGAGAAGCGAGTCATTAAATGGTTTGACTATCTATGG					
1321	ACCAACAAAAAACAGTGGATGAGAGAGAAGTCTTGAAGTATCTACCTGATAAACTAAGA					
1381	GCAGAGATCGCCATCAATGTTTCAATTTAGACACATTAAAAAAGGTGCGCATTTTTGCAGAC					
1441	TGTGAAGCTGGTCTGTTGGTGGAGTTGGTCTTGAAATTACAACCCCAAGTCTACAGTCCT					
1501	GGAGATTACATTTGCAAGAAAGGGGACATTGGCCGAGAGATGTACATCATCAAGGAAGGC					
1561	AAACTCGCCGTGGTGGCTGATGACGGGATCACTCAGTTTGTAGTATTGAGTGATGGCAGC					
1621	TACTTTGGTGAAATCAGTATCCTTAATATTAAAGGTAGCAAAGCTGGCAATCGAAGAACA					
1681	GCCAATATTAAAAGCATTGGCTACTCAGATCTATTCTGTCTCTCAAAGATGACCTCATG					
1741	GAAGCTCTAACTGAGTACCCAGATGCCAAAGGTATGCTAGAAGAGAAAGGGAAGCAAATT					
1801	TTGATGAAAGATGGTCTATTGGACATAAACATTGCAAATGCTGGAAGTGATCCTAAAGAT					
1861	CTTGAAGAGAAGGTCACCCGAATGGAGAGCTCAGTAGACCTCCTGCAAACAAGGTTTGCT					
1921	CGGATCCTGGCTGAGTATGAGTCAATGCAGCAGAACTGAAGCAGAGGCTAACCAAGGTT					
1981	GAGAAATTCCTGAAACCACTTATTGACACAGAATTTTCAGCTATTGAAGGATCTGGAAC					
2041	GAAAGTGGGCCCCACAGACTCTACACAGGACTGA					

β' polypeptide: 3041 bases

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      10      20      30      40      50      60
1  GAATTCACCATGGTACCTGCCACAGAAGAGCACCCCGAACTGCAGGTGGAAGACGCTGAT
61 GCTGACAGTCGCCCCCTCATCGCGGAGGAGAACCCACCCTCTCCCGTGCAGCTGCCCCCTG
121 TCTCCTGCCAAATCCGACACCCTTGCAGTCCCAGGATCAGCCACAGGGAGCCTCAGGAAG
181 AGGCTGCCTTCCCAAGATGATGAGGCTGAAGAGCTCAAGATGCTGTCACCGGCTGCATCC
241 CCCGTGGTTGCCTGGTCGGACCCCAACAGCCCAAGGGCACTGATGACCAGGACCGTGCA
301 ACCTCGACAGCCAGCCAGAACAGTGCCATCATCAATGACCGGCTCCAGGAGCTGGTGAAG
361 CTCTTCAAGGAGCGGACGGAGAAGGTGAAGGAGAAGCTCATCGACCCTGACGTCACTCC
421 GATGAGGAGAGCCCCAAGCCCTCCCCAGCCAAAAAAGCCCCAGAGCCGGCCCCAGAAAGTG
481 AAGCCAGCTGAAGCGGGGACGGTGAAGAGGAACACTACTGTGAGATGCTCTGCTGCAAG
541 TTCAAACGCCGCCCTGGAAGAAGTACCAGTTTCCCCAGAGCATCGACCCGCTGACCAAC
601 CTGATGTACATCTTGTGGCTGTTCTTCGTGGTGTGCTGGCCTGGAAGTGAAGTGTGGCTG
661 ATTCCCGTGGCTGGGCTTCCCTATCAGACGCCAGACAAACATCCACCTCTGGCTGCTG
721 ATGGATTACCTGTGTGACCTCATCTACCTCCTGGACATCACCGTGTTCAGATGCGCCTG
781 CAGTTTGTGAGAGCGGGGACATCATTACGGACAAAAAGGAGATGCGCAACAATTACGTG
841 AAATCTCAGCGCTTTAAGATGGACATGCTCTGCCTCTTGCCCTTGGACTTACTCTACTTG
901 AAATTCGGTGTGAATCCCCCTCCTGCGCTTGCCCCGCTGTTTGAAGTATATGGCCTTCTTT
961 GAGTTTAAACAACCGCCTGGAATCCATCCTCAGCAAAGCCTACGTTTACAGGGTTATCAGG
1021 ACCACAGCCTACCTGCTCTACAGCTTACATCTGAACTCATGTCTCTATTACTGGGCATCG
1081 GCCTATGAGGGCCTCGGCTCCACTCACTGGGTTTATGATGGCGTGGGAAACAGTTACATT
1141 CGCTGTACTACTGGGCTGTGAAGACCCTCATCACCATCGGCGGCCTGCCCCGACCCCAAG
1201 ACGCTCTTTGAAATTGTCTTCCAGGGTCTAAACTATTTACGGGCGTCTTCGCTTTCTCC
1261 GTGATGATCGGACAGATGAGAGACGTAGTGGGGGCGCCACCGCGGGACAGACCTACTAC
1321 CGCAGCTGCATGGACAGCACCCTGAAGTATATGAACTTCTACAAGATCCCCAGGTCCGTG
1381 CAGAACCGGGTCAAGACCTGGTACGAATACACCTGGCACTCCCAAGGCATGCTGGATGAG
1441 TCAGAGCTGATGGTGCAGCTTCCGGACAAGATGCGGCTGGACCTCGCCATTGACGTGAAC
1501 TATTCCATCGTCAGCAAAGTGGCACTCTTCCAGGGCTGTGACCGGCTGATGATCTTTGAC
1561 ATGCTGAAGAGGCTGCGCTCTGTAGTCTACCTGCCCAATGATTACGTGTGCAAGAAGGGG
1621 GAGATAGGCCCGGAAATGTACATCATCCAGGCGGGGACAGGTGCAGGTCTTGGGTGGCCCCG
1681 GATGGGAAATCCGTGCTGGTGACGCTGAAGGCTGGATCTGTGTTTCGGAGAAATAAGCTTG
1741 CTGGCTGTAGGGGGCGGGAACCGGCGCACAGCTAACGTGGTGGCCCATGGGTTTACCAAC
1801 CTCTTCATTCTGGATAAGAAGGACCTGAATGAAATTCTGGTGCATTATCCCGAGTCTCAG
1861 AAGTTCGTCCGCAAGAAGGCCAGGCGAATGCTGAGAAATAACAACAAGCCCAAGGAGAAG
1921 AGCGTGCTTATTCTCCCTCCGCGGGCAGGCACCCCCAAGCTCTTCAATGCCGCCCTGGCC
1981 GCAGCAGGAAAGATGGGTGCCAAGGGAGGCAGAGGTGGCAGGCTTGCCCTCCTCAGGGCT
2041 CGACTCAAGGAGCTGGCTGCGCTGGAGGGCCGCAGACAGCAGCAGTTGCTGGAACAGGCC
2101 AAGAGCTCGGAAGACGCCGCCGTGGGAGAGGAGGGATCGGCCTCCCCAGAACAGCCTCCG
2161 CGTCCAGAGCCGCCGCCGCCAGAGGCCCGCCGACCAAGACCGGCCCCAGAGCCCCTG
2221 GCCCCAGAGGCCCGGCCGCCAGAGGCCCGGCCCAAGCTCTCCACCACCTGCCTCCAG
2281 GAGAGGCCGGAGGGAGACAAGGAGGCCGCGGCCAGAAAGAGCACCCGGTGCGGATCCAC
2341 GTGACCCTGGGCCCCGATCCCAGCGAACAGATCCTGTTGGTGGAGGTCCCTGAGAAGCAG
2401 GAGGAGAAGGAGAAAAAGGAGGAGGAGACAGAGGAAAAGGAGGAAGGGGAGGAGGCGAGG
2461 AAGGAGAAAAGGAGGAGTAAGGCGGACGTTAGTGCCTACCCGCTCCCCCAGCCCCCGC
2521 AGGTGCCCCGCTCCCCACCACCCGGCACCCACCTCGAG
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β subunit: 4456 bases

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      10      20      30      40      50      60
1  GGCAAGTCCTGGCTGGTGGACGGCATCCGAGCAGGTGTCAGGATGCTGGGCTGGGTCCAG
61 AGGGTGCTGCCTCAGCCCCCAGGGACCCCTCAGAAGACCAAGCAAGAGGAGGAGGGGACA
121 GAACCAGAGCCAGAGTTGGAGCCGAAGCCTGAAACAGCTCCCGAGGAGACTGAGCTAGAG
181 GAAGTGTCCCTGCCACCAGAGGAGCCCTGCGTGGGGAAGGAAGTGGCTGCGGTTACTCTG
241 GGTCTCAGGGAACCCAGGAGACTGCTCTCACTCCGCCACATCCCTCCAGGCTCAGGTC
301 TCTGTGGCTCCCGAAGCTCACAGCAGTCCCAGAGGCTGGGTGCTGACCTGGCTCAGGAAG
361 GGTGTGGAGAAGGTTGTCCCGCAGCCCGCCACAGCAGCAGGCCCTCCCAGAACATTGCT
421 GCAGGCCTGGAGAGTCCAGATCAGCAGGCAGGAGCACAGATCCTTGGGCAGTGCGGCACT
481 GGGGGCTCAGATGAACCCAGTGAACCCTCCAGGGCCGAAGACCCCGGGCCTGGGCCCTGG
541 CTCCTCCGGTGGTTCTGAACAGAATCTGGAGAAAATGCTGCCTCAGCCCCCGAAAATCTCT
601 GAGGGCTGGAGAGATGAGCCTACAGATGCTGCCTTGGGTCCAGAGCCCCCAGGACCCGCC
661 TTGGAGATCAAGCCCATGCTGCAGGCCAGGAGAGCCCCCTCCCTGCCAGCTCCTGGCCCC
721 CCGGAGCCTGAGGAGGAACCGATCCCAGAGCCCCAGCCCCACTATTCAAGCCTCCTCCCTG
781 CCGCCCCCGCAGGACTCTGCCAGGTTGATGGCATGGATCCTGCACAGGCTGGAGATGGCC
841 CTGCCACAGCCAGTGATCCGTGGGAAGGGTGGGGAGCAGGAGTCTGATGCCCCAGTGACA
901 TGTGACGTGCAGACCATCAGCATCCTCCCTGGAGAGCAAGAGGAGTCGCATCTCATCTTA
961 GAAGAGGTTGACCCTCACTGGGAGGAGGATGAACACCAGGAGGGCAGCACCAGCACCAGC
1021 CCGCGGACCTCAGAGGCAGCTCCAGCTGATGAAGAGAAGGGCAAGGTGGTGGAGCAGACG
1081 CCCAGGGAGCTGCCTCGGATTCAAGAGGAGAAAGAAGATGAGGAGGAGGAGAAGGAAGAC
1141 GGAGAGGAGGAAGAAGAGGAAGGCAGAGAGAAAGAGGAGGAAGAAGGTGAAGAAAAGAG
1201 GAGGAGGAAGGCAGAGAGAAAGAGGAGGAAGAAGGCGAAAAAAAAGAGGAAGAAGGCAGA
1261 GAGAAAGAGGAGGAAGAAGGTGGAGAGAAAGAAGATGAAGAAGGCAGAGAGAAAGAGGAG
1321 GAAGAAGGCAGAGGGAAAGAGGAAGAAGAAGGTGGAGAGAAAGAGGAAGAAGAAGGCAGA
1381 GGGAAAGAGGAGGTGGAAGGCAGAGAGGAGGAGGAAGACGAAGAGGAGGAGCAGGATCAC
1441 AGTGTCCTGCTGGATAGTTATCTGGTGCCACAGAGCGAGGAGGACCGGAGTGAAGAAAGT
1501 GAAACCCAGGACCAGAGTGAAGTGGGCGGAGCCCAGGCCAGGGCGAAGTGGGCGGCGCC
1561 CAGGCCCTGAGTGAAGAAAGTGAACCCAGGACCAGAGTGAAGTAGGCGGAGCCCAGGAC
1621 CAGAGTGAAGTGGGCGGAGCCCAGGCCAGGGCGAAGTGGGTGGAGCCCAGGAGCAGGAC
1681 GGAGTAGGCGGAGCCCAGGACCAGAGCACCTCACACCAGGAGCTGCAAGAAGAGGCCTTG
1741 GCCGACAGCTCAGGAGTACCTGCCACAGAAGAGCACCCCGAACTGCAGGTGGAAGACGCT
1801 GATGCTGACAGTCGCCCCCTCATCGCGGAGGAGAACCCACCCTCTCCCGTGCAGCTGCCC
1861 CTGTCTCCTGCCAAATCCGACACCCTTGCAGTCCCAGGATCAGCCACAGGGAGCCTCAGG
1921 AAGAGGCTGCCTTCCCAAGATGATGAGGCTGAAGAGCTCAAGATGCTGTCACCGGCTGCA
1981 TCCCCCGTGGTTGCCTGGTTCGGACCCACAGCCCACAGGGCACTGATGACCAGGACCGT
2041 GCAACCTCGACAGCCAGCCAGAACAGTGCCATCATCAATGACCGGCTCCAGGAGCTGGTG
2101 AAGCTCTTCAAGGAGCGGACGGAGAAGGTGAAGGAGAAGCTCATCGACCCTGACGTCACC
2161 TCCGATGAGGAGAGCCCCAAGCCCTCCCCAGCCAAAAAAGCCCCAGAGCCGGCCCCAGAA
2221 GTGAAGCCAGCTGAAGCGGGGCGAGTGAAGAGGAACACTACTGTGAGATGCTCTGCTGC
2281 AAGTTCAAACGCCGCCCTGGAAGAAGTACCAGTTTCCCCAGAGCATCGACCCGCTGACC
2341 AACCTGATGTACATCTTGTGGCTGTTCTTCGTGGTGTGCTGGCCTGGAAGTGAAGTGTGG
2401 CTGATTCCCGTGCCTGCGCTGGGCCTTCCCCTATCAGACGCCAGACAACATCCACCTCTGGCTG
2461 CTGATGGATTACCTGTGTGACCTCATCTACCTCCTGGACATCACCGTGTTCAGATGCGC
2521 CTGCAGTTTGTGAGAGGCGGGGACATCATTACGGACAAAAAGGAGATGCGCAACAATTAC
2581 GTGAAATCTCAGCGCTTTAAGATGGACATGCTCTGCCTCTTGCCCTTGGACTTACTCTAC
2641 TTGAAATTCGGTGTGAATCCCCTCCTGCGCTTGCCCCGCTGTTTGAAGTATATGGCCTTC
2701 TTTGAGTTTAAACAACCGCCTGGAATCCATCCTCAGCAAAGCCTACGTTTACAGGGTTATC
2761 AGGACCACAGCCTACCTGCTCTACAGCTTACATCTGAACTCATGTCTCTATTACTGGGCA
2821 TCGGCCTATGAGGGCCTCGGCTCCACTCACTGGGTTTATGATGGCGTGGGAAACAGTTAC
2881 ATTCGCTGTTACTACTGGGCTGTGAAGACCCTCATCACCATCGGCGGCCTGCCCCAGCCCC

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