Neuron, Vol. 15, 627–636, September, 1995, Copyright © 1995 by Cell Press

A 240 kDa Protein Represents the Complete β Subunit of the Cyclic Nucleotide-Gated Channel from Rod Photoreceptor

Heinz Gerd Körschen,* Michelle Illing,† Reinhard Seifert,* Federico Sesti,* Andrew Williams,† Sebastian Gotzes,* Caroline Colville,† Frank Müller,* Andrea Dosé,† Matthias Godde,* Laurie Molday,† U. Benjamin Kaupp,* and Robert S. Molday†
*Institut für Biologische Informationsverarbeitung
Forschungszentrum Jülich
Postfach 1913
52425 Jülich
Germany
†Department of Biochemistry and Molecular Biology
University of British Columbia
Vancouver, British Columbia V6T 1Z3
Canada

Summary

The cyclic nucleotide-gated channel from rod photoreceptors is composed of two distinct subunits (α and β). The properties of the α subunit, which can form functional channels by itself, are modified by coexpression with a homologous polypeptide, designated the β subunit. However, the α subunit from rod photoreceptor membranes copurifies with a 240 kDa protein that is significantly larger than this putative β subunit. We now demonstrate by peptide sequencing and by cloning and functional expression of cDNA that the 240 kDa protein represents the complete β subunit with an unusual bipartite structure. The N-terminal part is essentially identical to a glutamic acid-rich protein (GARP), whereas the C-terminal part is highly homologous to the previously cloned human "β subunit." Expression of the complete β subunit in HEK 293 cells results in a polypeptide with the same apparent molecular weight as the 240 kDa protein of the native rod channel. Coexpression of the α subunit with the full-length β subunit yields hetero-oligomeric channels with properties characteristic of the native channel.

Introduction

Cyclic nucleotide–gated (CNG) channels of rod and cone photoreceptor cells play a central role in vertebrate phototransduction by controlling the flow of cations across the outer segment plasma membrane in response to light-induced changes in cGMP (for reviews, see Yau and Baylor, 1989; Miller et al., 1994; Kaupp, 1995). Molecular cloning studies indicate that the CNG channel of rod photoreceptors consists of two distinct subunits, designated α and β subunits (Kaupp et al., 1989; Chen et al., 1993). The α subunit has been most extensively characterized. It contains a cGMP-binding domain, a voltage sensor motif, a pore region, and six membrane-spanning segments (Heginbotham et al., 1992; Bönigk et al., 1993; Henn et al., 1995). Expression of the α subunit by itself results in functional channels that have most but not all of the electrophysiological properties of the native rod channel (Kaupp et al., 1989; Dhallan et al., 1992; Chen et al., 1993; Nizzari et al., 1993). More recently, a second subunit, designated the β subunit, has been cloned from a human retinal cDNA library (Chen et al., 1993). This β subunit is ~30% identical to the α subunit in amino acid sequence and contains structural features similar to those found in the α subunit. Expression of the β subunit alone does not form functional channels; however, coexpression of the β subunit with the α subunit in HEK 293 cells results in channels that have electrophysiological properties found in the native rod channel, in addition to those found with expression of the α subunit alone. These include the flickering nature of the channels opening and closing, micromolar sensitivity to l-cis-diltiazem, and Ca^{2+}/calmodulin sensitivity (Chen et al., 1993, 1994).

Cloning studies suggest that there are two alternatively spliced forms of the β subunit, which differ only in length. The shorter form has a predicted molecular mass of ~70 kDa; the longer form contains an additional segment at the N-terminus and has a molecular mass of ~102 kDa. However, β subunit polypeptides in the 70–102 kDa range have not been detected in purified rod channel preparations (Cook et al., 1987; Molday et al., 1990; Chen et. al., 1994). Instead, the CNG channel isolated from bovine rod outer segment membranes contains two distinct polypeptides that migrate by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with apparent molecular masses of 63 and 240 kDa. The 63 kDa protein corresponds to the α subunit (Kaupp et al., 1989; Molday et al., 1991), whereas the 240 kDa protein has been shown to bind calmodulin and to contain partial amino acid sequences of the cloned human "β subunit" (Hsu and Molday, 1993; Chen et al., 1994).

To begin to study the structural and functional properties of the 240 kDa protein of the rod CNG channel and to define its relationship to the "β subunit" of the channel, we have determined the primary structure of the 240 kDa protein of the CNG channel from bovine rod photoreceptors. Our results indicate that the 240 kDa protein represents the complete β subunit with a unique bipartite structure. Coexpression of the complete β subunit with the α subunit results in a functional channel that exhibits molecular and electrophysiological properties similar to those of the native rod photoreceptor channel.

Results

The Primary Structure of the 240 kDa Protein Corresponds to the Complete β Subunit of the Rod Photoreceptor CNG Channel

Initially, amino acid sequence information was obtained from N-terminal microsequence analysis of cyanogen bromide (CNBr) and proteolytic peptides derived from the 240

kDa protein of the affinity-purified bovine rod channel. Thereby, peptides sequences were obtained that are either highly homologous to sequences of the human "β subunit" (Chen et al., 1994) (Figure 1, PEP3–PEP7) or identical to sequences contained in a bovine retina-specific glutamic acid–rich protein (GARP) earlier cloned by Sugimoto et al. (1991) (Figure 1, PEP1 and PEP2).

To define the relationship of GARP and the "β component" of the 240 kDa protein, bovine retinal cDNA libraries were initially screened with a PCR fragment amplified from bovine retinal cDNA. Several overlapping cDNA clones were isolated and used to generate the final recombinant clone (pbRCNGCβ). As shown in Figure 1, this cDNA codes for a protein of 1,394 amino acid residues with a calculated molecular mass (M_w) of 155,064 Da. The deduced polypeptide sequence contains amino acid sequences identical to those obtained from peptide microsequencing. It embodies two regions that show a high degree of sequence similarity with two entirely different, previously cloned proteins. The hydrophilic N-terminal part of bRCNGCβ (amino acids 1–571; GARP-part) is essentially identical to the sequence of bovine GARP, but lacks the C-terminal 19 amino acids. The translational initiation site of pbRCNGCβ was assigned to the same ATG triplet found in cDNA encoding GARP (Sugimoto et al., 1991). The sequence from residues 572–1394 (designated here as the β′-part) is highly homologous to the sequence of the proposed "β subunit" of the human rod CNG channel (hRCNC2; Chen et al., 1993) and comprises the predicted hydrophobic membrane-spanning segments (S1–S6), the cGMP-binding site, and the pore region.

Northern Blot Analysis of Retinal mRNA Transcripts

The size of messenger RNA of the 240 kDa protein was analyzed by Northern blotting. A cDNA probe that specifically hybridized to the β′-part of pbRCNGCβ recognized a single transcript of ~7.4 kb in poly(A)$^+$ RNA from bovine retina (Figure 2, lane 1). A second probe, specific for the GARP-part of pbRCNGCβ, also hybridized to the ~7.4 kb transcript, but it also recognized two smaller transcripts of ~4.2 and ~1.6 kb (Figure 2, lane 2). These results suggest that the 7.4 kb transcript codes for the 240 kDa protein (i.e., the complete β subunit), whereas the smaller transcripts code for GARP and possibly a shorter form of GARP. Alternatively spliced transcripts that code for the β′-part alone must be rare, if they exist at all.

Western Blot Analysis of the β Subunit Expressed in HEK 293 Cells

The difference between the calculated M_w (~155 kDa) of bRCNGCβ and the M_w of the 240 kDa protein of the native rod channel as estimated by SDS–PAGE may be due to anomalous electrophoretic mobility of this protein caused by the large number of negatively charged glutamic acid residues. We examined this possibility by separately expressing the cDNAs for the full-length β subunit, the β′-part, and GARP in HEK 293 cells and comparing the mobilities of the expressed proteins by SDS–PAGE with that of the 240 kDa protein from the rod channel purified from

```
MLGWVQRVLPQPPGTPQKTKQEEEGTEPEPELEPKPETAPEETELEEVSLPPEEPCVGKE 60

VAAVTLGPQGTQETALTPPTSLQAQVSVAPEAHSSPRGWVLTWLRKGVEKVVPQPAHSSR 120

PSQNIAAGLESPDQQAGAQILGQCGTGGSDEPSEPSRAEDPGPGPWLLRWFEQNLEKMLP 180
------PEP1----------
QPPKISEGWRDEPTDAALGPEPPGPALEIKPMLQAQESPSLPAPGPPEPEEEPIPEPQPT 240
                         ---PEP2---
IQASSLPPPQDSARLMAWILHRLEMALPQPVIRGKGGEQESDAPVTCDVQTISILPGEQE 300

ESHLILEEVDPHWEEDEHQEGSTSTSPRTSEAAPADEEKGKVVEQTPRELPRIQEEKEDE 360

EEEKEDGEEEEEEGREKEEEEGEEKEEEEGREKEEEEGEKKEEEGREKEEEEGGEKEDEE 420

GREKEEEEGRGKEEEEGGEKEEEEGRGKEEVEGREEEEDEEEEQDHSVLLDSYLVPQSEE 480

DRSEESETQDQSEVGGAQAQGEVGGAQALSEESETQDQSEVGGAQDQSEVGGAQAQGEVG 540
                                ▼
GAQEQDGVGGAQDQSTSHQELQEEALADSSGVPATEEHPELQVEDADADSRPLIAEENPP 600
              --------PEP4-------
------PEP3------           -PEP5-
SPVQLPLSPAKSDTLAVPGSATGSLRKRLPSQDDEAEELKMLSPAASPVVAWSDPTSPQG 660

TDDQDRATSTASQNSAIINDRLQELVKLFKERTEKVKEKLIDPDVTSDEESPKPSPAKKA 720
                                                  ------ S1 -
PEPAPEVKPAEAGQVEEEHYCEMLCCKFKRRPWKKYQFPQSIDPLTNLMYILWLFFVVLA 780
--------          -------- S2 --------
WNWNCWLIPVRWAFPYQTPDNIHLWLLMDYLCDLIYLLDITVFQMRLQFVRGGDIITDKK 840
      -------- S3 -------     --------- S4 ---------
EMRNNYVKSQRFKMDMLCLLPLDLLYLKFGVNPLLRLPRCLKYMAFFEFNNRLESILSKA 900
    -------- S5 ---------                        ----- pore -
YVYRVIRTTAYLLYSLHLNSCLYYWASAYEGLGSTHWVYDGVGNSYIRCYYWAVKTLITI 960
-----    -------- S6 -------
GGLPDPRTLFEIVFQGLNYFTGVFAFSVMIGQMRDVVGAATAGQTYYRSCMDSTVKYMNF 1020

YKIPRSVQNRVKTWYEYTWHSQGMLDESELMVQLPDKMRLDLAIDVNYSIVSKVALFQGC 1080
               ------------------------------ cGMP-binding site -
DRQMIFDMLKRLRSVVYLPNDYVCKKGEIGREMYIIQAGQVQVLGGPDGKSVLVTLKAGS 1140
--------------------------------                          ---
VFGEISLLAVGGGNRRTANVVAHGFTNLFILDKKDLNEILVHYPESQKLLRKKARRMLRN 1200
------PEP6------              -------------PEP7-------------
NNKPKEKSVLILPPRAGTPKLFNAALAAAGKMGAKGGRGGRLALLRARLKELAALEAAAR 1260

QQQLLEQAKSSEDAAVGEEGSASPEQPPRPEPPAPEAPAPEPTAPEPLAPEAPAPEAPAP 1320

SSPPPASQERPEGDKDAARPEEHPVRIHVTLGPDPSEQILLVEVPEKQEEKEKKEEETEE 1380

KEEGEEARKEKEEE----------------------------------------------- 1394
```

Figure 1. Deduced Amino Acid Sequence of the β Subunit of the CNG Channel of Bovine Rod Photoreceptors

Numbers of amino acid residues are given at the right end of each line. Peptide sequences PEP1–PEP7 (line above sequence) were determined by microsequencing of fragments of the 240 kDa protein produced by partial proteolysis with CNBr, endoproteinase-Lys-C, or kallikrein and were identical to sequences deduced from cloning studies. Amino acid residues at positions 11 and 12 of PEP1 could not be determined. Structural segments similar to those of the α subunit are represented by lines above the sequence: S1–S6, putative membrane spanning regions; S4, voltage sensor motif; and the pore segment, which lines the cavity of the channel. The arrowhead indicates the end of the GARP-part (residues 1–571) and the start of the β′-part (residues 572–1394). The GARP-part is nearly identical to the bovine glutamic acid–rich protein (GARP; Sugimoto et al., 1991) and a GARP clone obtained by screening a retinal expression library; the exceptions are at the following positions: residues 341 (Lys), 482 (Arg), and 499 (Ala) are replaced by Glu, Gln, Thr, respectively, in GARP (of Sugimoto et al., 1991). A 19 amino acid segment at the C-terminal end of both GARP proteins is missing in bRCNGCβ.

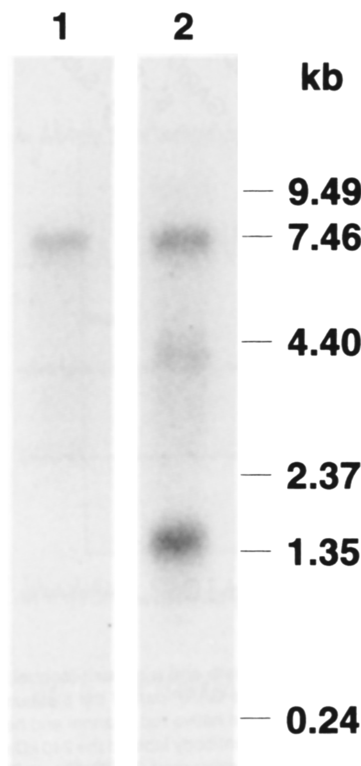

Figure 2. Northern Blot Analysis of Poly(A)$^+$ RNA from Bovine Retina
Lane 1 shows a hybridization carried out with a cDNA probe corresponding to amino acid residues 580–712. Lane 2 represents a hybridization with a cDNA probe containing only GARP-specific sequences. In both lanes, transcripts of ~7.4 kb were detected. Two additional bands of ~4.2 and ~1.6 kb were detected with the GARP-specific probe (lane 2).

outer segment membranes. Two monoclonal antibodies, one raised against the β′-part (PMb 3C9) and one raised against GARP (PMs 5E11), were used to detect the expression of these proteins by Western blotting. As shown in Figure 3, both antibodies labeled a polypeptide of M_w ~240 kDa in purified rod channel preparations and in HEK 293 cells transfected with pbRCNGCβ. In addition, the PMb 3C9 antibody recognized a polypeptide of M_w ~110 kDa in HEK 293 cells transfected with a cDNA containing only the β′-part (calculated M_w 92.7 kDa). The PMs 5E11 antibody recognized a doublet of M_w ~130–140 kDa in HEK 293 cells transfected with the cDNA encoding GARP (calculated M_w 64.4 kDa). The similar M_w values obtained for the native and heterologously expressed β subunits support the view that pbRCNGCβ contains the complete coding region of this subunit. The anomalous migration (apparent M_w) of the expressed GARP indicates that this component of the complete β subunit, with its high content of negatively charged glutamic acid residues, is largely responsible for the anomalous migration and apparent M_w of the β subunit (~240 kDa) by SDS–PAGE. The absence of bands in the purified channel corresponding to shorter forms of the β subunit is in agreement with earlier results indicating that the native human rod channel does not contain significant amounts of truncated β subunits (Chen et al., 1994). In control studies, the monoclonal antibodies raised against the β subunit did not bind to the α subunit (~63 kDa) of the CNG channel (Figure 3).

The Complete β Subunit Is Expressed in the Outer Segments of Rod Photoreceptor Cells

The expression pattern of the β subunit was determined by immunohistochemistry on cryosections of the bovine retina. Monoclonal antibodies PMs 5E11 and PMb 3C9 and polyclonal antibody PPc 32K against an epitope close to the C-terminus of the β subunit all intensely stained the outer segment layer of rod cells (Figure 4, OS). In addition, weak immunoreactivity was detected in the outer plexiform layer (OPL) and the nerve fiber layer (NFL) with antibody PMs 5E11. PPc 32K showed an even weaker staining of the nerve fiber layer. Together, these results demonstrate that the 240 kDa protein, i.e. the complete β subunit, is predominantly found in rod photoreceptor outer segments.

Electrophysiological Properties of the CNG Channel Expressed in HEK 293 Cells

Previous studies on the coexpression of α and β subunits were carried out with an incomplete β subunit lacking the GARP-part (Chen et al., 1993, 1994). Therefore, we examined the properties of hetero-oligomeric channels composed of the α subunit and the complete β subunit (240 kDa protein). Expression of the β subunit alone did not give rise to cGMP-activated currents (~80 patches), similar to the finding of Chen et al. (1993) with the incomplete β subunit. Coexpression of cDNAs encoding bRCNGCβ and the α subunit in HEK 293 cells gave rise to cGMP-activated channel activity in inside-out patches. Figure 5 shows single-channel recordings from excised inside-out patches of HEK 293 cells expressing the α and β subunits (Figure 5A) or the α subunit alone (Figure 5B). The mean single-channel conductance of the hetero-oligomeric (α + β) channel was 23 pS at +50 mV and 15 pS at −50 mV. The respective single-channel conductances for the homo-oligomeric (α) channel were 30 and 25 pS. The noise level of the open state of the hetero-oligomeric channel is significantly larger than that of the homo-oligomeric channel. Finally, the gating of homo- and hetero-oligomeric channels is different. The single-channel events of the hetero-oligomeric channel are not as well resolved as those of the homo-oligomeric channel (compare the single-channel currents activated by 100 μM cGMP in Figures 5A and 5B). l-cis-diltiazem at micromolar concentrations (10 μM) effectively blocked activity of the hetero- but not homo-oligomeric channels (Figures 5A and 5B). This finding verified that hetero-oligomeric channels had been formed because blockage with l-cis-diltiazem requires the association of the β′-part of the second subunit with the α subunit (Chen et al., 1993). The blockage by l-cis-diltiazem was quantitatively examined by measuring macroscopic currents in the presence of different concentrations of l-cis-diltiazem (Figure 6A). Hetero-oligomeric channels had a half-maximal inhibition constant (K_i) for l-cis-diltiazem of

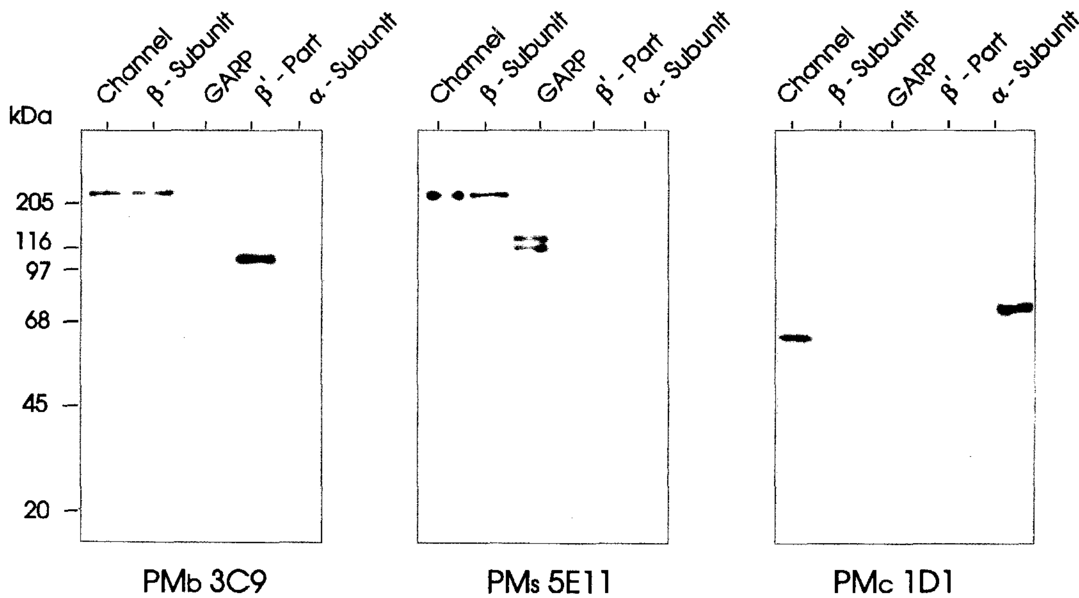

Figure 3. Western Blot Analysis

Western blots of the native rod channel and heterologously expressed complete β subunit, GARP, β′-part, and α subunit (control) probed with monoclonal antibodies PMb 3C9 (against the C-terminal region of the β subunit), PMs 5E11 (against the GARP-part of the β subunit), and PMc 1D1 (against the α subunit). The PMb 3C9 antibody specifically labeled a 240 kDa polypeptide in both the native rod channel and heterologously expressed full-length β subunit, as well as a 110 kDa polypeptide in the expressed β′-part. The PMs 5E11 antibody labeled the 240 kDa polypeptide in both the native channel and expressed full-length β subunit, as well as a 130–140 kDa doublet in the expressed GARP extract. The PMc 1D1 antibody labeled the 63 kDa polypeptide (α subunit) in the rod channel preparation and the 80 kDa polypeptide corresponding to the expressed α subunit; the difference in M_w between these two forms of the α subunit is due to the absence of an N-terminal segment on the α subunit of the native channel (Molday et al., 1991).

3.5 μM (at 0 mV) and an e-fold change in K_i by ~55 mV; both values are essentially identical with those of the native channel from salamander rod photoreceptors (McLatchie and Matthews, 1992). Mean values for half-maximal activation by cyclic nucleotides ($K_{1/2}$ ± SD at V_m = +80 mV) were 40.1 ± 9 μM (n = 10) for cGMP and 2100 μM (n = 1) for cAMP. The mean Hill coefficients for cGMP and cAMP were 2.2 ± 0.5 and 1.2, respectively. These values are not significantly different from those determined for the native bovine channel (Lühring et al., 1990) or the heterologously expressed α subunit (Kaupp et al., 1989; Altenhofen et al., 1991).

We next examined the ionic selectivity and Ca^{2+} blockage of hetero-oligomeric channels. The current–voltage (I–V) relations from an inside-out patch recorded under symmetrical bi-ionic conditions are shown in Figure 6B. Relative ion permeabilities were calculated from the reversal voltage (V_{rev}). Mean values for V_{rev} are as follows:

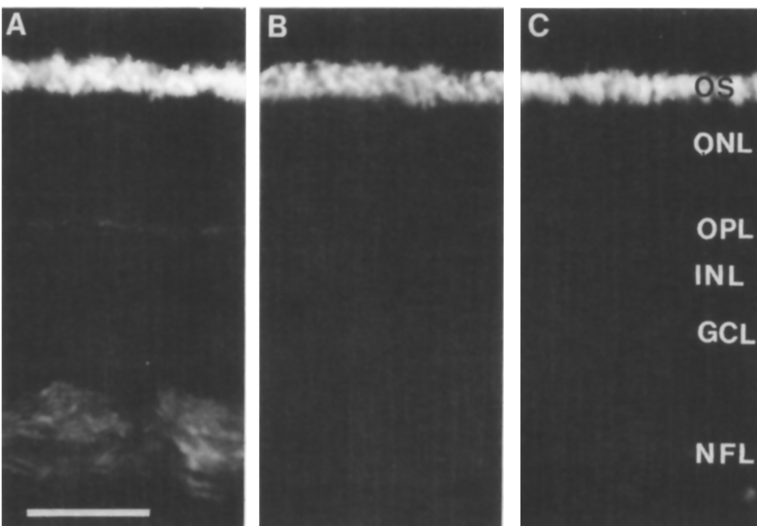

Figure 4. Immunohistochemical Localization

Vertical cryosections of bovine retina stained with antibodies PMs 5E11 (A), PPc 32K (B), and PMb 3C9 (C). Immunofluorescence was visualized with a secondary antibody coupled to the dye Cy3. All three antibodies intensely stained outer segments (OS) of photoreceptors. In addition, PMs 5E11 shows a weak immunofluorescence in the outer plexiform layer (OPL) and the nerve fiber layer (NFL). PPc 32K shows very weak staining in the NFL. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Bar, 50 μm.

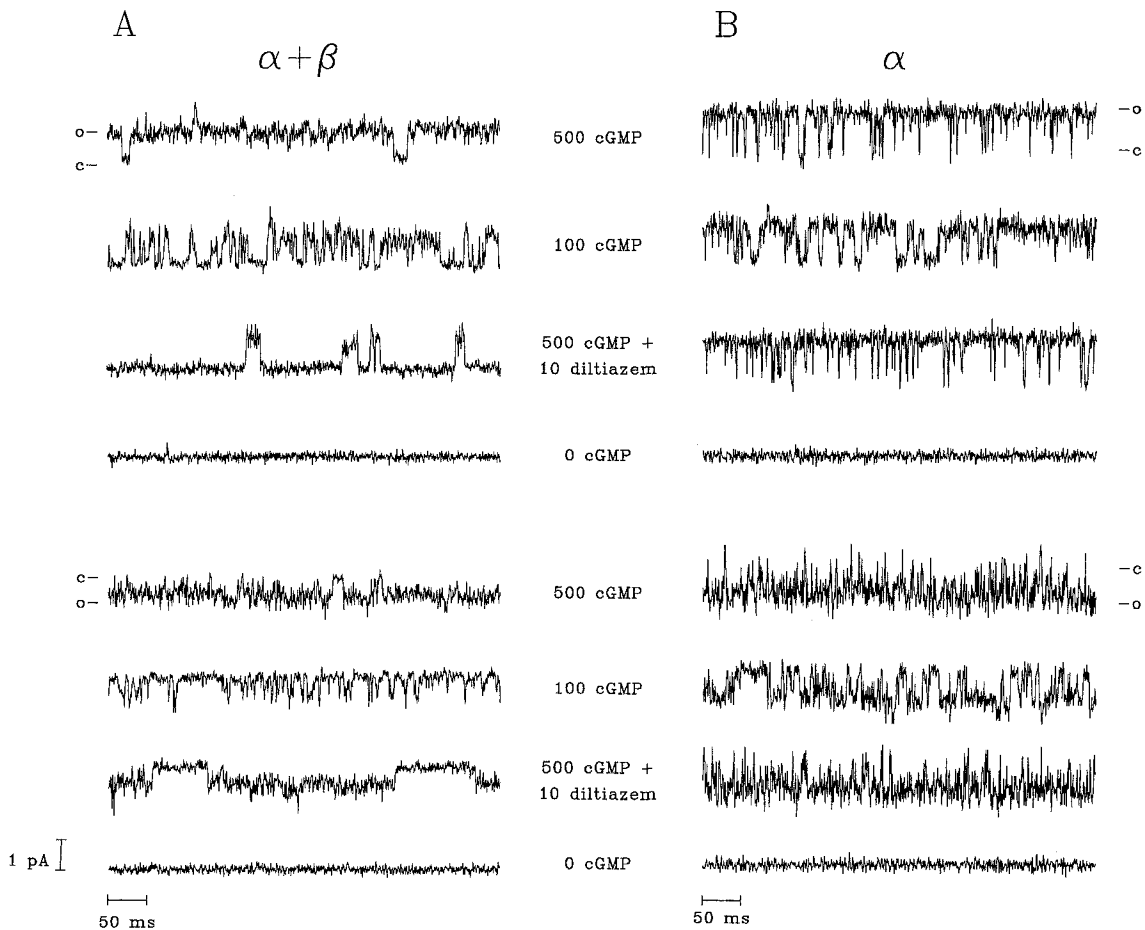

Figure 5. Single-Channel Activity of Hetero-Oligomeric and Homo-Oligomeric Channels

(A) The recordings show single-channel currents from an inside-out patch of HEK 293 cells transfected with plasmids encoding the α and β subunit (hetero-oligomeric channel). The indicated concentrations of cGMP or L-cis-diltiazem are given in micromolars. The holding potential was either +50 mV (upper four traces) or −50 mV (lower four traces). Recordings were filtered at f_c = 1 kHz.
(B) Similar recordings as in (A) with the homo-oligomeric channel.
c, closed channel; o, open channel.

NH_4^+, −21.6 ± 1.3 mV (n = 8); Li^+, −5.8 ± 1.8 mV (n = 7); Na^+, −1.2 ± 0.5 mV (n = 8); Rb^+, +5.7 ± 0.8 mV (n = 7); Cs^+, +13.0 ± 2.7 mV (n = 7). Permeability ratios (P_i/P_{K+}) calculated from V_{rev} yield the following series of ion selectivity: $NH_4^+ > Li^+ > Na^+ \sim K^+ > Rb^+ > Cs^+$ in the proportions 2.3:1.2:1.0:1:0.8:0.6. Relative ion permeabilities of the homo-oligomeric α subunit determined in control experiments were virtually identical to those previously described (Kaupp et al., 1989; Eismann et al., 1994). The permeability series for the hetero-oligomeric channel quantitatively agrees with the selectivity of the native channel (Menini, 1990; Furman and Tanaka, 1990; Lühring et al., 1990) but differs from that of the homo-oligomeric channel. Notably, the higher Li^+ permeability of the native channel compared with the α-homo-oligomeric channel is completely restored by coexpression of both subunits.

Figure 7A shows I–V relations from an outside-out patch at various concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$). As the $[Ca^{2+}]_o$ was increased, the cGMP-activated current was suppressed and the I–V relation became outwardly rectifying. The voltage dependence of Ca^{2+} blockage was analyzed by plotting the ratio of unblocked currents (I/I_{max}) measured in the presence of various Ca^{2+} concentrations (I) and in Ca^{2+}-free solution (I_{max}) against V_m. To illustrate the difference in Ca^{2+} blockage between homo-oligomeric (α) and hetero-oligomeric (α + β) channels, I/I_{max} versus V_m is compared in Figure 7B for a single $[Ca^{2+}]_o$ of 21 μM. Coexpression of subunits changed the Ca^{2+} blockage in a characteristic way. The relief of Ca^{2+} blockage at hyperpolarizing voltages was less pronounced, and the efficiency of blockage was decreased. By analyzing plots similar to that shown in Figure 7B for different $[Ca^{2+}]_o$ values, K_i values were determined. Figure 7C compares the dose–response relations of Ca^{2+} blockage at −60 mV for homo- and hetero-oligomeric channels. Mean values for K_i (V_m = −60 mV) are 4.9 ± 1.9 μM (n = 9) for the homo-oligomeric channels (α) and 47.4 ± 10.2 μM (n = 7) for the hetero-oligomeric channels (α + β). The results on the ion permeability and Ca^{2+} blockage demonstrate that bRCNGCβ takes part in lining the aqueous pore and contributes to the

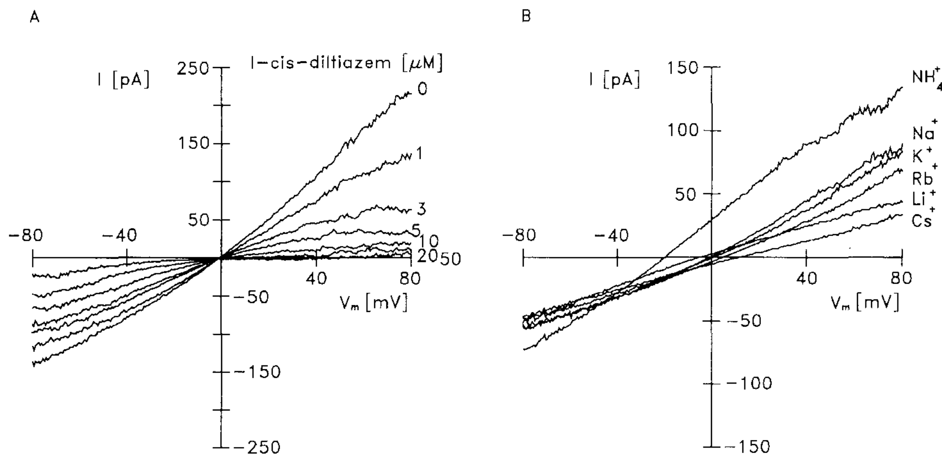

Figure 6. Blockage by L-cis-Diltiazem and Ionic Selectivity of Hetero-Oligomeric Channels

(A) The recordings show current–voltage (I–V) relations of an inside-out patch in the presence of the indicated concentrations of L-cis-diltiazem.

(B) I–V recordings under symmetrical bi-ionic conditions with the indicated cation solution (at 100 mM) in the medium perfusing the cytoplasmic side of the membrane.

Ca^{2+}-binding site of CNG channels. A glutamate residue in the pore-forming (P) region of the α subunit is crucially important for ion selectivity and channel blockage by external Ca^{2+} (Root and MacKinnon, 1993; Eismann et al., 1994; Sesti et al., 1995). This residue is exchanged by a glycine residue in a similar P motif of the β subunit. The glycine residue is probably responsible for the lower sensitivity to Ca^{2+} blockage of the hetero-oligomeric channel.

The currents carried by hetero-oligomeric channels consisting of α and β subunits were also diminished by Ca^{2+}/calmodulin (Figure 7D, compare traces 3 and 4), whereas homo-oligomeric (α) channels did not respond to Ca^{2+}/calmodulin (Figure 7E, compare traces 3 and 4), as previously reported (Chen et al., 1994). When the α subunit was coexpressed with a truncated β subunit that was lacking the GARP-part, channels were sensitive to Ca^{2+}/calmodulin (Figure 7F, compare traces 3 and 4). At saturating cGMP concentrations, Ca^{2+}/calmodulin had no effect (Figures 7D and 7F, compare traces 1 and 2). The formation of hetero-oligomeric channels in these experiments was verified by blockage with 10 μM l-cis-diltiazem (Figures 7D and 7F, trace 5). These results confirm earlier findings (Chen et al., 1994) that the β′-part of the 240 kDa protein contains the Ca^{2+}/calmodulin-binding site and show that the GARP-part of the β subunit does not modify these properties, at least on this level of analysis.

Discussion

In this study, we have identified the 240 kDa protein as the complete β subunit of the CNG channel from bovine rod photoreceptors. This subunit has the unusual structural feature of being composed of two distinct parts. The bovine β′-part comprising the C-terminal 823 amino acids is ~86% identical to an analogous β′-part (amino acids 116–909) of the human "β subunit" (Chen et al., 1993). It contains the cGMP-binding domain, a voltage sensor–like motif, the pore region, and the calmodulin-binding site. Coexpression studies of the complete β subunit or the β′-part with the α subunit indicate that the channel properties conferred by the 240 kDa protein of the native rod channel are mediated by the membrane-spanning β′-part of the subunit. As shown here and by Chen et al. (1993, 1994), the β′-part is responsible for calmodulin modulation of the channel activity, the ion selectivity, micromolar sensitivity to the inhibitor l-cis-diltiazem, and the flickering gating behavior of the channel. The stoichiometric relationship of α and β subunits in forming the native hetero-oligomeric channel is not yet known. The hydrophilic N-terminal GARP-part of the complete β subunit contains many glutamic acid residues and several repeat regions. The high net negative charge resulting from the glutamic acid residues is largely responsible for the anomalously high apparent M_w of both the complete β subunit (~240 kDa) and heterologously expressed GARP (130–140 kDa) measured by SDS–PAGE. The high net negative charge likely causes a significant reduction in the amount of SDS bound to the protein, and thus results in an abnormally slow electrophoretic mobility. The abnormally high apparent M_w of the rod $Na^+/Ca^{2+}–K^+$ exchanger has been attributed to a similar effect (Reiländer et al., 1992). The binding of the native rod channel to anion exchange (diethylaminoethyl) resins (Cook et al., 1987) also appears to be due in part to the high content of glutamic acid residues in the GARP-part of the β subunit.

Although the cDNA of the human rod "β subunit" cloned by Chen et al. (1993) contains the β′-part and a small part of a GARP-like component, it does not appear to code for the complete human β subunit. Comparison of the bovine and human sequences suggests that a large portion of the N-terminal region of the complete human β subunit is missing. The incompleteness of the cloned human "β subunit" is further evident from the finding that the apparent M_w of the expressed human "β subunit" (~130–150 kDa) is considerably lower than the apparent M_w of a band

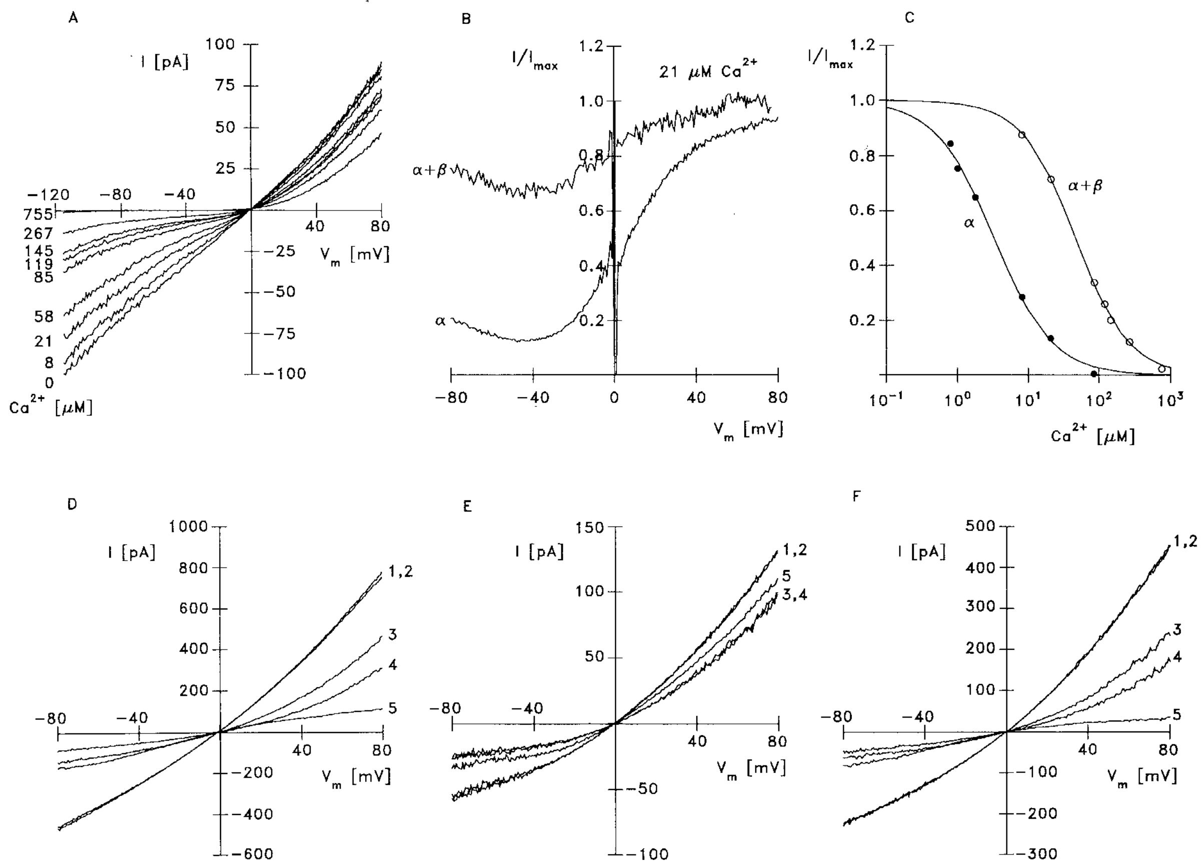

Figure 7. Electrophysiological Properties of Hetero-Oligomeric Channels Expressed in HEK 293 Cells

(A) Blockage of hetero-oligomeric channels by extracellular Ca^{2+}. The recordings show I-V relations of an outside-out patch in the presence of the indicated Ca^{2+} concentrations. The pipette solution contained 1 mM cGMP to activate channels.

(B) Comparison of normalized currents in the presence (α + β) and absence (α) of the β subunit. Currents in the presence of 21 μM Ca^{2+} (I) were normalized to the currents recorded under divalent-free conditions (I_{max}).

(C) Dose-response relation of blockage by external Ca^{2+} at −60 mV in the presence (α + β) and absence (α) of the β subunit. Constants of half-maximal inhibition (K_i) are 3.3 μM (α) and 46.5 μM (α + β). The Hill coefficients were $n \approx 1$.

(D–F) Effect of Ca^{2+}/calmodulin on the cGMP-activated current of hetero-oligomeric (α + β) channels (D), homo-oligomeric (α) channels (E), and hetero-oligomeric channels containing a truncated form of the β subunit that is lacking the GARP-part (F). The bath solutions (traces 1–4) contained 120 mM KCl, 10 mM HEPES, 50 μM Ca^{2+} (2 mM nitrilotriacetic acid, 0.8 mM $CaCl_2$; pH 7.4, adjusted with KOH), and the following additions: 1 mM cGMP (trace 1); 1 mM cGMP and 350 nM calmodulin (trace 2); 60 μM cGMP (trace 3); 60 μM cGMP and 350 nM calmodulin (trace 4). The bath solution for trace 5 contained 100 mM KCl, 10 mM HEPES, 10 mM EGTA, 10 μM l-cis-diltiazem, and 500 μM cGMP (pH 7.4, adjusted with KOH).

(~240 kDa) that is recognized by an antibody directed against the β′-part of the isolated human rod CNG channel (Chen et al., 1994). Probably, the clones encoding the human "β subunit" represent either partial clones or splice variants (Chen et al., 1993) that are not expressed in the rod photoreceptor. Other mammalian rod CNG channels also appear to contain a high molecular weight polypeptide associated with the α subunit (Hsu and Molday, 1994). These high molecular weight polypeptides probably represent the complete β subunit of native rod channels.

The β subunit confers upon the rod CNG channel sensitivity to modulation by Ca^{2+}/calmodulin and blockage by l-cis-diltiazem. These properties are similar in hetero-oligomeric channels containing either the complete β subunit or the form that lacks the GARP-part. Thus, at least at this level of analysis, the GARP-part does not seem to influence major electrophysiological properties of the rod CNG channel, including ligand sensitivity. However, there may be subtle differences (e.g., in channel gating) that need to be addressed by future work.

Ca^{2+} plays an important role in the adaptation of rod and cone photoreceptors by controlling the activity of several key enzymes of the signaling pathways (for reviews, see Pugh and Lamb, 1990; Fain and Matthews, 1990; Yau, 1991; Koch, 1994). CNG channels are crucially important for Ca^{2+} homeostasis because they represent the only pathway for Ca^{2+} entry into the outer segment. The changes in relative ion permeability and in Ca^{2+} blockage upon the addition of the β subunit suggest that Ca^{2+} permeation may also be affected. It will be important for future work to determine the relative contribution of Ca^{2+} current through the hetero-oligomeric channel, as has been recently measured for the homo-oligomeric α subunit (Frings et al., 1995).

The unusual bipartite structure of the β subunit raises several intriguing questions. First, what is the function of

the GARP-part in the 240 kDa protein? It could provide a means for the channel to interact with other proteins, e.g., the $Na^+/Ca^{2+}-K^+$ exchanger or cytoskeletal elements. Recently, it has been reported that the rod CNG channel can associate with the $Na^+/Ca^{2+}-K^+$ exchanger under certain conditions (Bauer and Drechsler, 1992). Alternatively, the GARP-part may be needed for targeting of CNG channels to the rod outer segment, or it may be part of the fibrous elements observed to extend from the plasma membrane to the rim regions of the disc membrane in rod outer segments (Roof and Heuser, 1982).

Photoreceptor CNG channel genes are also expressed in other parts of the retina and in nonneuronal tissues (Ahmad et al., 1992, 1994; Weyand et al., 1994; Biel et al., 1993; for review, see Yau, 1994; Kaupp, 1995). Do cone photoreceptors and nonneuronal cells expressing CNG channels also utilize 240 kDa-like β subunits, or do they express the GARP-part or β′-part components as separate entities? If so, do the GARP-part and the β′-part associate with each other? Our findings and a previous report (Sugimoto et al., 1991) argue that at least the GARP-part is expressed as a separate polypeptide in the retina. It remains to be shown by future work whether β subunits in other cells are built up as a modular system consisting of two distinct parts that can be alternatively expressed as single or separate polypeptides, and thereby increase the functional diversity of the CNG channel family.

Experimental Procedures

Isolation and Microsequence Analysis of Peptides from the 240 kDa Protein

The CNG channel was isolated from bovine rod outer segment membranes on a calmodulin–Sepharose column as previously described (Hsu and Molday, 1994). The 240 kDa protein was separated from the 63 kDa α subunit by SDS–PAGE and digested with CNBr or endoproteinase-Lys-C by standard procedures (Eckerskorn and Lottspeich, 1989). Peptides were isolated by high pressure liquid chromatography using a C18 reverse-phase column and subjected to N-terminal microsequence analysis. Peptide sequences PEP1, PEP2, PEP6, and PEP7 were derived from CNBr peptide fragments; PEP4 and PEP5, from endoproteinase-Lys-C fragments. Sequence PEP3 was obtained by kallikrein digestion of the purified channel and isolated on a calmodulin-Sepharose column (Chen et al., 1994).

Isolation and Characterization of cDNA Clones

Degenerate oligonucleotide primers corresponding to amino acid sequences KYMAFFE (residues 426–432) and QMIFD (residues 627–631) of the human retinal rod "β subunit" hRCNC2 (Chen et al., 1993) were used to amplify a fragment from bovine retina cDNA. The resulting PCR fragment was used to screen an oligo(dT) and a random-primed cDNA library made from poly(A)$^+$ RNA of the bovine retina. Several partial clones were isolated. Clone pbR6RE (~2.6 kb) contains the 5′-terminal region of the β subunit, including the same translation initiation site found in bovine GARP. There was no stop codon found in frame 5′-terminal of this ATG triplet. A second, overlapping clone, pbR13ON (~2.3 kb), encodes the middle part of the β subunit. A third clone, pbR3OE (~1.6 kb), contains sequences extending beyond the 3′ end of the coding region. These three cDNA clones were combined to give pbRCNGCβ using standard procedures. The clone pbRCNGCβ (4282 bp) contains the complete coding region of the β subunit. The β′-part cDNA clone was generated by ligation of a XmnI-digested 3′ fragment of the pbRCNGCβ clone with a PCR fragment containing a ATG start codon at the beginning of the β′-part. The β′-part clone coded for amino acid residues 572–1394.

A full-length GARP clone pSP14GARP was obtained by screening a bovine retinal cDNA expression library with monoclonal antibodies PMs 5E11 and PMs 4B2 directed against the 240 kDa protein. The deduced protein sequence of the pSP14GARP cDNA was identical to that reported by Sugimoto et al. (1991), except for the following three positions: residues 341 (Lys), 482 (Arg), and 499 (Ala) in SP14GARP are substituted with Glu, Gln, and Thr, respectively. DNA sequencing was carried out by the dideoxy chain termination method using [α-^{35}S]dATP.

Northern Blot Analysis

Poly(A)$^+$ RNA from bovine retina was isolated using a FastTrack RNA isolation kit (Invitrogen). For Northern blots, 10 μg of poly(A)$^+$ RNA was hybridized under high stringency conditions with ^{32}P-labeled probes synthesized by random priming (Ambion) as previously described (Bönigk et al., 1993). Probes 1 and 2 were amplified by PCR from pbRCNGCβ.

Monoclonal and Polyclonal Antibodies against the CNG Channel

Monoclonal antibody PMc 1D1 against the α subunit and monoclonal antibodies PMs 5E11 and PMs 4B2 against the GARP-part of the β subunit have been described previously (Molday et al., 1990). Monoclonal antibody PMb 3C9 against the β′-part was generated from a hybridoma cell line obtained by fusion of spleen cells from a mouse immunized with the purified rod CNG channel with NS-1 cells, as previously described (Molday and MacKenzie, 1983). Polyclonal antibody PPc 32K was obtained from a rabbit immunized with a synthetic peptide (amino acids 1292–1334 of the β subunit) coupled to keyhole limpet hemocyanin as previously described (Molday et al., 1991; Bönigk et al., 1993).

Western Blotting of CNG Channel Subunits

The CNG channel of rod photoreceptors was immunoprecipitated from Triton X-100–solubilized bovine rod outer segment membranes using a PMc 1D1–Sepharose immunoaffinity matrix (Molday et al., 1990). The cDNAs for the complete β subunit (pbRCNGCβ), GARP (pSP14GARP), the β′-part, and the α subunit were subcloned into pcDNAI (Invitrogen) and individually expressed in HEK 293 cells using a calcium phosphate transfection procedure (Chen and Okayama, 1987). Cell extracts were solubilized in 1% Triton X-100. The expressed β subunit and β′-part were immunoprecipitated with PMb 3C9–Sepharose; the expressed GARP was immunoprecipitated with PMs 5E11–Sepharose; and the expressed α subunit was immunoprecipitated with PMc 1D1–Sepharose. The samples were run on 10% SDS–PAGE, transferred to Immobilon-P (Millipore), and sequentially labeled with the primary monoclonal antibody and sheep anti-mouse Ig-peroxidase for enhanced chemiluminescence (Amersham) detection.

Immunocytochemistry

Cryosections of bovine retina were sequentially labeled with the primary antibody and goat anti-mouse Ig–Cy3 or goat anti-rabbit Ig–Cy3 secondary antibody as previously described (Bönigk et al., 1993).

Functional Expression

Channel-specific cDNAs were expressed in HEK 293 cells as previously described (Baumann et al., 1994). Macroscopic currents of excised inside-out patches were recorded under voltage-clamp conditions as described (Altenhofen et al., 1991; Bönigk et al., 1993) with solutions as in Baumann et al., 1994. The blockage of cGMP-activated currents by external Ca^{2+} was determined as described (Eismann et al., 1994; Baumann et al., 1994). The solution in the pipette contained 124 mM KCl, 10 mM HEPES, 10 mM EGTA, 26 mM KOH (pH 7.4), and 1 mM cGMP. The bath solution contained 100 mM KCl, 10 mM HEPES, 10 mM EGTA, and 45–50 mM KOH (depending on the amount of Ca^{2+} needed to give the indicated free Ca^{2+} concentrations pH 7.4). Leak currents of patches in the inside-out and outside-out configurations were measured and subtracted from cGMP-activated currents as described in Baumann et al. (1994). Relative ion permeabilities were calculated from V_{rev} measured under symmetrical bi-ionic conditions as described (Baumann et al., 1994).

Acknowledgments

We thank Drs. A. Baumann, E. Eismann, and I. Weyand for critical reading of the manuscript; Dr. W. Bönigk, Dr. W. Baehr, and N. Beyert

for providing the cDNA libraries; Dr. Beyermann (FMP, Berlin) for synthesis of a C-terminal peptide of the β subunit; S. Köppen and M. Bruns for technical assistance; A. Eckert for typing the manuscript; the Nucleic Acid Service Unit (University of British Columbia) for synthesis of oligonucleotide primers; and the Protein Microsequencing Center (University of Victoria, B. C.) for microsequence analysis of peptides. This work was supported by grants from the Deutsche Forschungsgemeinschaft (U. B. K.), the National Institutes of Health, the Medical Research Council of Canada, and the v. Humboldt Stiftung (R. S. M.). R. S. M. is a v. Humboldt Forschungspreis awardee.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received May 17, 1995; revised July 7, 1995.

References

Ahmad, I., Korbmacher, C., Segal, A. S., Cheung, P., Boulpaep, E. L., and Barnstable, C. J. (1992). Mouse cortical collecting duct cells show nonselective cation channel activity and express a gene related to the cGMP-gated rod photoreceptor channel. Proc. Natl. Acad. Sci. USA *89*, 10262–10266.

Ahmad, I., Leinders-Zufall, T., Kocsis, J. D., Shepherd, G. M., Zufall, F., and Barnstable, C. J. (1994). Retinal ganglion cells express a cGMP-gated cation conductance activatable by nitric oxide donors. Neuron *12*, 155–165.

Altenhofen, W., Ludwig, J., Eismann, E., Kraus, W., Bönigk, W., and Kaupp, U. B. (1991). Control of ligand specificity in cyclic nucleotide-gated channels from rod photoreceptors and olfactory epithelium. Proc. Natl. Acad. Sci. USA *88*, 9868–9872.

Bauer, P. J., and Drechsler, M. (1992). Association of cyclic GMP-gated channels and Na^+–Ca^{2+}–K^+ exchangers in bovine retinal rod outer segment plasma membranes. J. Physiol. *451*, 109–131.

Baumann, A., Frings, S., Godde, M., Seifert, R., and Kaupp, U. B. (1994). Primary structure and functional expression of a *Drosophila* cyclic nucleotide-gated channel present in eyes and antennae. EMBO J. *13*, 5040–5050.

Biel, M., Altenhofen, W., Hullin, R., Ludwig, J., Freichel, M., Flockerzi, V., Dascal, N., Kaupp, U. B., and Hofmann, F. (1993). Primary structure and functional expression of a cyclic nucleotide-gated channel from rabbit aorta. FEBS Lett. *329*, 134–138.

Bönigk, W., Altenhofen, W., Müller, F., Dose, A., Illing, M., Molday, R. S., and Kaupp, U. B. (1993). Rod and cone photoreceptor cells express distinct genes for cGMP-gated channels. Neuron *10*, 865–877.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. *7*, 2745–2752.

Chen, T.-Y., Peng, Y.-W., Dhallan, R. S., Ahamed, B., Reed, R. R., and Yau, K.-W. (1993). A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. Nature *362*, 764–767.

Chen, T.-Y., Illing, M., Molday, L. L., Hsu, Y.-T., Yau, K.-W., and Molday, R. S. (1994). Subunit 2 (or β) of retinal rod cGMP-gated cation channel is a component of the 240-kDa channel-associated protein and mediates Ca^{2+}-calmodulin modulation. Proc. Natl. Acad. Sci. USA *91*, 11757–11761.

Cook, N. J., Hanke, W., and Kaupp, U. B. (1987). Identification, purification, and functional reconstitution of the cyclic GMP-dependent channel from rod photoreceptors. Proc. Natl. Acad. Sci. USA *84*, 585–589.

Dhallan, R. S., Macke, J. P., Eddy, R. L., Shows, T. B., Reed, R. R., Yau, K.-W., and Nathans, J. (1992). Human rod photoreceptor cGMP-gated channel: amino acid sequence, gene structure, and functional expression. J. Neurosci. *12*, 3248–3256.

Eckerskorn, C., and Lottspeich, F. (1989). Internal amino acid sequence analysis of proteins separated by gel electrophoresis after tryptic digestion in polyacrylamide matrix. Chromatographia *28*, 92–94.

Eismann, E., Müller, F., Heinemann, S. H., and Kaupp, U. B. (1994). A single negative charge within the pore region of a cGMP-gated channel controls rectification, Ca^{2+} blockage and ionic selectivity. Proc. Natl. Acad. Sci. USA *91*, 1109–1113.

Fain, G. L., and Matthews, H. R. (1990). Calcium and the mechanism of light adaptation in vertebrate photoreceptors. Trends Neurosci. *13*, 378–384.

Frings, S., Seifert, R., Godde, M., and Kaupp, U. B. (1995). Profoundly different calcium permeation and blockage determine the specific function of distinct cyclic nucleotide–gated channel. Neuron *15*, 169–179.

Furman, R. E., and Tanaka, J. C. (1990). Monovalent selectivity of the cyclic guanosine monophosphate-activated ion channel. J. Gen. Physiol. *96*, 57–82.

Heginbotham, L., Abramson, T., and MacKinnon, R. (1992). A functional connection between the pores of distantly related ion channels as revealed by mutant K^+ channels. Science *258*, 1152–1155.

Henn, D. K., Baumann, A., and Kaupp, U. B. (1995). Probing of the transmembrane topology of cyclic nucleotide-gated channels by a gene fusion aproach. Proc. Natl. Acad. Sci. USA *92*, 7425–7429.

Hsu, Y.-T., and Molday, R. S. (1993). Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. Nature *361*, 76–79.

Hsu, Y.-T., and Molday, R. S. (1994). Interaction of calmodulin with the cyclic GMP-gated channel of rod photoreceptor cells. J. Biol. Chem. *269*, 29765–29770.

Kaupp, U. B. (1995). Family of cyclic nucleotide-gated channels. Curr. Opin. Neurobiol., in press.

Kaupp, U. B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N. J., Kangawa, K., Matsuo, H., Hirose, T., et al. (1989). Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature *342*, 762–766.

Koch, K.-W. (1994). Calcium as modulator of phototransduction in vertebrate photoreceptor cells. Rev. Physiol. Biochem. Pharmacol. *125*, 149–192.

Lühring, H., Hanke, W., Simmoteit, R., and Kaupp, U. B. (1990). Cation selectivity of the cGMP-gated channel of mammalian rod photoreceptors. In Sensory Transduction Life Sciences, A. Borsellino, L. Cervetto, and V. Torre, eds. (New York: Plenum Press), pp. 169–173.

McLatchie, L. M., and Matthews, H. R. (1992). Voltage-dependent block by L-*cis*-diltiazem of the cyclic GMP-activated conductance of salamander rods. Proc. R. Soc. Lond. (B) *247*, 113–119.

Menini, A. (1990). Currents carried by monovalent cations through cyclic GMP-activated channels in excised patches from salamander rods. J. Physiol. *424*, 167–185.

Miller, J. L., Picones, A., and Korenbrot, J. I. (1994). Differences in transduction between rod and cone photoreceptors: an exploration of the role of calcium homeostasis. Curr. Opin. Neurobiol. *4*, 488–495.

Molday, L. L., Cook, N. J., Kaupp, U. B., and Molday, R. S. (1990). The cGMP-gated cation channel of bovine rod photoreceptor cells is associated with a 240-kDa protein exhibiting immunochemical cross-reactivity with spectrin. J. Biol. Chem. *265*, 18690–18695.

Molday, R. S., and MacKenzie, D. (1983). Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry *22*, 653–660.

Molday, R. S., Molday, L. L., Dose, A., Clark-Lewis, I., Illing, M., Cook, N. J., Eismann, E., and Kaupp, U. B. (1991). The cGMP-gated channel of the rod photoreceptor cell: characterization and orientation of the amino terminus. J. Biol. Chem. *266*, 21917–21922.

Nizzari, M., Sesti, F., Giraudo, M. T., Virginio, C., Cattaneo, A., and Torre, V. (1993). Single-channel properties of cloned cGMP-activated channels from retinal rods. Proc. Roy. Soc. Lond. (B) *254*, 69–73.

Pugh, E. N., Jr., and Lamb, T. D. (1990). Cyclic GMP and calcium: the internal messengers of excitation and adaptation in vertebrate photoreceptors. Vis. Res. *30*, 1923–1948.

Reiländer, H., Achilles, A., Friedel, U., Maul, G., Lottspeich, F., and Cook, N. J. (1992). Primary structure and functional expression of

the Na/Ca,K-exchanger from bovine rod photoreceptors. EMBO J. *11*, 1689–1695.

Roof, D. J., and Heuser, J. E. (1982). Surfaces of rod photoreceptor disk membranes: integral membrane components. J. Cell Biol. *95*, 487–500.

Root, M. J., and MacKinnon, R. (1993). Identification of an external divalent cation–binding site in the pore of a cGMP-activated channel. Neuron *11*, 459–466.

Sesti, F., Eismann, E., Kaupp, U. B., Nizarri, M., and Torre, V. (1995). The multi-ion nature of cGMP-gated channel from vertebrate rods. J. Physiol., in press.

Sugimoto, Y., Yatsunami, K., Tsujimoto, M., Khorana, H. G., and Ichikawa, A. (1991). The amino acid sequence of a glutamic acid-rich protein from bovine retina as deduced from the cDNA sequence. Proc. Natl. Acad. Sci. USA *88*, 3116–3119.

Weyand, I., Godde, M., Frings, S., Weiner, J., Müller, F., Altenhofen, W., Hatt, H., and Kaupp, U. B. (1994). Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. Nature *368*, 859–863.

Yau, K.-W. (1991). Calcium and light adaptation in retinal photoreceptors. Curr. Opin. Neurobiol. *1*, 252–257.

Yau, K.-W. (1994). Cyclic nucleotide-gated channels: an expanding new family of ion channels. Proc. Natl. Acad. Sci. USA *91*, 3481–3483.

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

GenBank Accession Number

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number X89626.