Published in final edited form as:
J Comp Neurol. 2000 May 22; 421(1): 80–94.

Cloning and Immunocytochemical Localization of a Cyclic Nucleotide–Gated Channel α-Subunit to All Cone Photoreceptors in the Mouse Retina

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Abstract

Cyclic nucleotide–gated channels (CNGC) are ligand-gated ion channels that open and close in response to changes in the intracellular concentration of the second messengers, 3′,5′-cyclic adenosine monophosphate and 3′,5′-cyclic guanosine monophosphate. Most notably, they transduce the chemical signal produced by the absorption of light in photoreceptors into a membrane potential change, which is then transmitted to the ascending visual pathway. CNGCs have also been implicated in the signal transduction of other neurons downstream of the photoreceptors, in particular the ON-bipolar cells, as well as in other areas of the central nervous system. We therefore undertook a search for additional cyclic nucleotide–gated channels expressed in the retina. Following a degenerate reverse transcription polymerase chain reaction approach to amplify low-copy number messages, a cDNA encoding a new splice variant of CNGC α-subunit was isolated from mouse retina and classified as mCNG3. An antiserum raised against the carboxy-terminal sequence identified the retinal cell type expressing mCNG3 as cone photoreceptors. Preembedding immunoelectron microscopy demonstrated its membrane localization in the outer segments, consistent with its role in phototransduction. Double-labeling experiments with cone-specific markers indicated that all cone photoreceptors in the murid retina use the same or a highly conserved cyclic nucleotide–gated channel. Therefore, defects in this channel would be predicted to severely impair photopic vision.

Indexing terms

rat; calcium/calmodulin; phototransduction

Cyclic nucleotide–gated channels (CNGC) are nonselective cation channels, constructed out of α- and β-subunits in a likely tetrameric configuration, with intracellular ligand-binding domain(s). When expressed in heterologous systems, the α-subunits can form functional ion channels. With the coexpression of the α- and β-subunits, the channels acquire additional characteristics that reflect those seen in situ (Kaupp, 1995; Molday, 1996; Wei et al., 1998), for example, the ability to be modulated by calcium (Chen et al., 1993; Körschen et al., 1995). These channels are highly regulated (Zimmerman, 1995) and may be the downstream effectors of nitric oxide–signaling mechanisms (Ahmad et al., 1994; Savchenko et al., 1997).

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CNGCs appear to derive from three genes: those encoding channels from rod photoreceptors (CNG1), olfactory epithelia (CNG2), and testis/cone photoreceptors (CNG3).

The role of CNGCs has been best elucidated for phototransduction, which occurs in the outer segments of rod and cone photoreceptors using highly related, but distinct, proteins. Light absorbed by the visual pigments, rod opsin or the cone opsins, respectively, induces a conformational change in these seven transmembrane-domain receptors. This shift, in turn, activates the G-protein, transducin, which regulates the activity of a 3′,5′-cyclic guanosine monophosphate (cGMP)-phosphodiesterase, resulting in a local reduction in the levels of intracellular cGMP. This concentration change is sensed and transduced into an electrical signal by the CNGCs present in the plasma membranes of these cells. This signal is then propagated to and processed by the higher-order neurons of the visual system.

In general, less is known about the components involved in cone function as compared to those in rods. The cones contain visual pigments with different spectral sensitivities that allow the organism to discriminate color during photopic vision. Cones make up an estimated 1% of photoreceptors in rat (Szél and Röhlich, 1992) and 3% in mouse (Carter-Dawson and LaVail, 1979; Jeon et al., 1998); however, the average cone density in the mouse retina (≈12,400) is similar to that found in the periphery of primate retina (Jeon et al., 1998). Although these animals have rod-dominated retinae, they are dichromats, in that they possess *s*hort-wavelength cone (S-cone) and *m*edium-wavelength cone (M-cone) types (Jacobs et al., 1991; Szél et al., 1996; Sun et al., 1997).

Although the central role of CNGCs in sensory transduction is well documented, these channels have also been implicated in the physiology of various cell types within the retina. In particular, a CNGC has been proposed to mediate signaling in the ON-bipolar cells (Nawy and Jahr, 1990; Shiells and Falk, 1990), and the search was undertaken in the present study to molecularly identify low-abundance candidates in the mouse retina using sensitive reverse transcription-polymerase chain reaction (RT-PCR) approaches. The mouse was chosen because it is becoming increasingly prominent in vision research due to the growing availability of transgenic animals and to the power of its genetics.

We report the isolation of a cDNA from mouse retina encoding a new splice variant of a CNGC α-subunit, named mCNG3. Using antiserum generated against a carboxy-terminal peptide, we show that this channel is expressed in cones and that the same or a highly similar CNGC is likely to be used by all cones in the retina. Some of these results have appeared in abstract form (Hirano et al., 1998).

MATERIALS AND METHODS

All experiments were performed in accordance with the guidelines for the welfare of experimental animals issued by the Federal Governments of the United States and of Germany and were approved by the institutional animal-use committees.

Cloning

The complementary DNA (cDNA) of a CNGC was isolated from mouse retina using a combination of RT-PCR and RACE PCR (rapid amplification of cDNA ends PCR) techniques. First-strand cDNA template was reverse transcribed from total RNA isolated from mouse (C57BL/6) retinae with oligo (dT) as primer following standard protocols. The initial fragments were amplified using degenerate primers, designed from the highly conserved sequence of the cGMP binding site of the mouse rod photoreceptor cGMP-gated channel [Pittler et al., 1991; ol-1, amino acid (a.a.) residues SPGDYIC; 5′-(A/T)(G/C)(C/T)CC(A/G/T)-GG(A/G/C)GA(C/T)TA(C/T)AT(A/G/T)TG-3′, sense, and ol-2, a.a. residues KDDLMEA, 5′-AC(C/T)TCCAT

(G/C)A-(A/G)(A/G)TC(A/G)TC(C/T)TT-3′, antisense]. Two rounds of PCR were performed in a 50-μl reaction volume containing first-strand cDNA as template, 200 pmol of each primer, 200-μM deoxynucleotides, 2.5 U Taq polymerase (Boehringer Mannheim, Indianapolis, IN), and 1× supplied buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3 (20°C)] and first-strand cDNA as template, or for the second round, 1 μl (1/50th) of the first-round PCR. The following protocol was used: 5 minutes at 94°C, then 40 seconds at 94°C, 1 minute at 55°C, 1 minute at 74°C for 35 cycles, followed by 5 minutes at 74°C. The expected 250-base pair (bp) PCR product was gel-purified, digested with Sau3AI, which cleaves the mouse rod photoreceptor cGMP-gated channel fragment, the likely major contaminant, and cloned into the Sma I site of pBluescript SK(−) (Stratagene, La Jolla, CA). The inserts of more than 30 successful recombinants were sequenced (Sequenase 2.0 kit, United States Biochemical/Amersham Life Sciences, Cleveland, OH). Sequence analysis revealed that the rod photoreceptor CNG channel (Pittler et al., 1991), a cone photoreceptor CNG-like channel (1–21, Bönigk et al., 1993), and one previously unidentified channel were isolated (1–26). A few sequences that did not appear to encode cyclic nucleotide–gated channels were found and disregarded.

Screening of a mouse retina cDNA library in λZAP (Duvoisin et al., 1995) using the initially obtained 1-26 sequence as probe failed to produce true positives. Therefore, the cDNA was extended another 630 bp by a second round of degenerate PCR, using ol-2 primer and a degenerate primer designed from the fifth putative transmembrane sequence [a.a. residues IIHWNAC, ol-3A, 5′-AT(A/C/T)AT-(A/C/T)CA(C/T)TGGAA(C/T)GC(A/G/C/T)TG-3′, sense]. For a second round of PCR, the downstream primer was replaced by a nested specific primer (cg-3, 5′-TGATGT-ACATCTCTCTTCCA-3′), derived from the initially obtained 1-26 cDNA sequence.

To obtain the full 5′ end of the cDNA, the RACE PCR technique of Frohman et al. (1988) was used (5′RACE PCR kit, GIBCO/Bethesda Research Labs, Gaithersburg, MD), following the manufacturer's instructions. The cg-3 was used as primer for the reverse transcription, and a nested primer (cg-5, 5′-AATGTACTTCCGGGAGAGTC-3′) was used with the anchor primer for two rounds of PCR amplification. The products were cloned and sequenced. The analysis indicated that an incomplete 5′ end had been obtained; therefore, a second round of RACE PCR was done using another primer closer to the 5′ end (cg-13, 5′-AGTCTGGTGGCTTCTGTTCT-3′). The products were cloned, sequenced, and found to contain the putative beginning of the open reading frame (ORF) and 380 bp of 5′-untranslated region (UTR).

The genomic organization of the rod photoreceptor CNG α-subunit indicated that the majority of the coding region is present in one large 3′-exon (Dhallan et al., 1992). On the assumption that this channel shared a similar genomic organization, we screened a mouse genomic DNA library in bacteriophage λ EMBL3 (Clontech, ML1043j) using the conditions described in Duvoisin et al. (1995). A partial clone assembled from PCR fragments above was used as a probe. Two λ recombinant phages giving the strongest signal were pursued. Southern blot analysis allowed us to identify a 2-kb Bam HI-restriction digest product, which was subcloned and sequenced as above. The recombinant clone (7B13) contained the putative 3′ end of the ORF and a 410-bp 3′-UTR.

To confirm that we had the full-length cDNA of a single CNGC, the cDNA was reamplified in two overlapping halves using specific 5′- and 3′-primers on first-strand cDNA synthesized from mouse retina total RNA (Chomczynski and Sacchi, 1987). The PCR products (cg12 & cg-9, 5′-ATCTCAGCCTTCAGCTTGTC-3′ and cg-28, 5′-TCAA-GCTGGACATCCTGTCT-3′ & cg-25, 5′-AGTCTTTGAAG-CATCCTCGG-3′) were cloned and a recombinant plasmid carrying the complete open reading frame for the cone cyclic

nucleotide–gated channel was constructed from the overlapping clones (cg12-9 and 7B13) using SpeI and StuI restriction sites. To verify the construct, the 2.8-kb cDNA insert of the plasmid (NC-2) was sequenced over its full length on both strands. Sequence analysis of the NC-2 insert revealed that the full-length cDNA was that of the putative cone photoreceptor CNGC. The nucleotide sequence data reported in this article will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Database under the accession number AJ243933.

Sequence analysis was carried out using the GCG suite of programs (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI) and the DNAStar (Laser-gene, Madison, WI) programs.

Antibodies

Primary antibodies and their dilutions were as follows: a mouse monoclonal antibody (mAb) against the α-isozyme of protein kinase C (PKC) (clone MC5; Amersham, Arlington Heights, IL, 1:100), a rabbit polyclonal antibody against human S-cone opsin (JH455, kindly provided by J. Nathans, Johns Hopkins School of Medicine, 1:5,000), and affinity-purified polyclonal antiserum raised in rabbits against a carboxy-terminal peptide from the mCNG3 sequence (see below, 1:2,000). The labeling of the primary antibodies was visualized using fluorochrome-conjugated secondary antibodies: Alexa 594 goat anti-rabbit IgG (1:500) and Alexa 488 goat anti-mouse IgG (1:500, both from Molecular Probes, OR). The JH455 antiserum recognizes the S-opsin in a variety of species, including mouse (Wang et al., 1992). We will use the following terminology for the cone types: S-cones for the *s*hort-wavelength, including ultraviolet, cones and M-cones for the *m*edium-wavelength cones.

Characterization of anti-peptide antiserum

Generation of the antiserum—Polyclonal antibodies were raised in rabbits against a 15-a.a. carboxy-terminal peptide (FSPDRENSEDASKTD) along with an amino-terminal cysteine for coupling to keyhole limpet hemocyanin. The antiserum was purified by affinity chromatography using the mAbTrap GII kit (Pharmacia), according to manufacturer's instructions.

Determination of antiserum specificity

Western blot analysis: Western blots were performed on homogenates prepared from mouse retina, cerebellum, liver, and tongue. Mice were deeply anesthetized with halothane, decapitated, and the tissues were dissected and frozen immediately in liquid nitrogen. Tissues were homogenized in buffer containing 0.32 M sucrose, 0.02 M Tris-HCl, pH 7.4, 5 mM EGTA, and protease inhibitor cocktail (Complete mini, Boehringer Mannheim, Mannheim, Germany). After a low-speed spin (1000 *g*, 5 minutes), the supernatant was spun at 16,000 *g* for 20 minutes at 4°C. The pellets were resuspended in homogenization buffer, but the sucrose was replaced by 10% glycerol. Twenty micrograms protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on an 8% gel and was transferred to nitrocellulose using standard techniques. After blocking in 5% (wt/vol) powdered milk in Tris-buffered saline (TBS: 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 0.05% (vol/vol) Tween-20 (TBST) for 1–3 hours at room temperature, the membranes were incubated in the peptide antiserum (1: 2,000) overnight. After five washes for 5 minutes each in TBST, the blots were incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (New England Bio-labs, 1:2,000) in 5% milk in TBST for 0.5–1 hour. Following the same wash protocol, the signal was developed using enhanced chemiluminescence (SuperSignal Substrate, Pierce).

Western blots of retina protein showed that the mCNG3 antiserum recognized a single band with an apparent molecular weight of ≈70 kDa, close to the calculated molecular weight of 73

kDa (Fig. 1). No bands were detected in protein homogenates from cerebellum and liver, whereas there was a band of ≈40 kDa present in tongue homogenate.

Heterologous expression of cDNAs: The cDNA insert from NC-2 was removed using XbaI and BamHI of the polylinker region of pBLSK, and the resulting fragment was subcloned into similarly digested pCMV5 (Chen et al., 1991). Expression plasmids were transfected using Super-Fect (Qiagen, Hilden, Germany) into human embryonic kidney 293 (HEK), which were maintained at 37°C in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO/Life Technologies, Eggenstein, Germany). After allowing 2 days for expression, the HEK cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 minutes, rinsed well with 0.1 M PBS, pH 7.4, and processed using standard indirect immunofluorescence methods.

The antiserum efficiently labeled HEK cells transfected with EC1 (data not shown), but not untransfected cells or mock-transfected cells. HEK cells transiently transfected with other recombinant CNGCs—human rod CNGC α-and β-subunits (Dhallan et al., 1992; Chen et al., 1993), mouse rod CNGC α-subunit (Pittler et al., 1991), rat olfactory channel α-subunit (Dhallan et al., 1990), bovine rod CNGC β-subunit (Körschen et al., 1995), bovine testis β-subunits (4C, 4D, 4E, Biel et al., 1996)—also showed no cross-reactivity with the mCNG3 antiserum. Therefore, the antiserum does not appear to recognize α-subunits from human (or mouse) rod photoreceptor, rat olfactory channel, or the β-subunits from bovine testis and human and bovine retina.

Immunocytochemistry

Light microscopy: Immunocytochemical labeling was carried out using the indirect immunofluorescence method. Retinae of mouse (C57BL), albino rat, chicken (*Gallus gallus*), New Zealand White rabbit, monkey (*Macaca fascicularis*), ox, goldfish (*Carassius auratus*), tree shrew (*Tupaia belangeri*), pig, and guinea pig were investigated. Eyes of ox and pig were obtained from the local slaughterhouse. The other animals were deeply anesthetized before decapitation and/or enucleation, as follows: The mice and rats were anesthetized with 4% halothane, then cervically dislocated or decapitated. The chickens were cervically dislocated. The rabbits were initially administered ketamine and Rompun (xylazine; Bayer) (0.25 mg/kg ketamine, 0.1 mg/kg Rompun, i.m.) followed by an overdose of sodium pentobarbital (100–200 mg/kg Nembutal i.v.; Abbott). The guinea pigs were given ketamine (80 mg/kg) and Rompun (8 mg/kg) intraperitoneally. The monkeys (150 mg/kg, i.v.) and tree shrews (60 mg/kg, i.p.) were given lethal doses of sodium pentobarbital (Nembutal). The eyecups were fixed for 15–30 minutes in 4% paraformaldehyde, cryoprotected in graded sucrose (10, 20, and 30%), and sectioned vertically at 12–14 μm on a cryostat. Details of the double-immunolabeling procedure are provided elsewhere (Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994). In brief, double-immunolabeling experiments were performed by incubating the sections in a mixture of antibodies followed by a cocktail of secondary antibodies. Control sections were processed excluding the primary antibodies, using preimmune serum, or by preincubation of the antiserum with the peptide antigen (1 mg/ml). These negative controls resulted in no specific labeling.

The sections were examined and photographed under epifluorescence on a Zeiss photomicroscope (Axiophot, Oberkochen, Germany) using 40×, 63×, and 100× objectives and the appropriate fluorescence filter sets (Alexa 594: BP546, FT580, LP590; Alexa 488: 450–490, FT510, LP520). For Alexa 488 photomicrographs, an additional green interference filter (515–565) was inserted in the microscope tube. The fluorescence filters were wedge corrected,

such that shifting from one filter to the other did not cause displacement of the image. Black-and-white photomicrographs were taken on Kodak TMY 400 film.

Labeling of all cone photoreceptors with peanut agglutinin (PNA) was done after the antibody labeling process. The tissue sections were blocked in 0.5 mg/ml BSA in PBS for 15 minutes, then incubated in the same solution containing fluorescein isothiocyanate (FITC)-conjugated PNA (0.3 mg/ml, Vector Laboratories, Burlingame, CA) for 30–45 minutes at room temperature. After rinsing for 30 minutes in PBS, the sections were mounted in Mowiol (Höchst, Frankfurt/M., Germany).

Electron microscopy: The labeling was performed on mouse retina using preembedding immunoelectron microscopy techniques, according to a modification of the method of Sassoè-Pognetto et al. (1994) and described in detail in Koulen et al. (1998). In brief, the eyecups were fixed for 50 minutes in 4% paraformaldehyde. The retinae were dissected, cryoprotected in graded sucrose, and frozen and thawed three times to improve antibody penetration. Vibratome sections were cut at 50 μm into cold PBS, pH 7.4. The antibody was diluted in the same media used for light microscopy, except that Triton X-100 was omitted, and the sections were incubated for four days at 4°C. The immunolabeling was visualized by the ABC method (Vectastain Elite ABC kit, Vector Laboratories, Burlin-game, CA) using 3,3′-diaminobenzidine (DAB) as substrate. The immunolabel was silver intensified and gold toned, and the tissue was flat embedded in Glycidether 100-based epoxy resin (Koulen et al., 1998; Serva, Heidelberg, Germany). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The grids were examined and photographed on a Zeiss EM10 electron microscope.

RESULTS

Molecular characterization of a new CNGC α-subunit

To identify additional members of the CNGC family whose expression might be restricted to the retina, mouse retina total RNA was reverse transcribed and the cDNA was amplified by PCR using degenerate oligonucleotide primers, whose sequence was derived from the highly conserved cyclic nucleotide binding domain from the mouse rod photoreceptor CNGC channel (Pittler et al., 1991). The complete 2.8-kb cDNA was obtained by various RT-PCR techniques (degenerate RT-PCR, 5′- and 3′-RACE PCR) and by a screen of a mouse genomic library.

Analysis of the deduced a.a. sequence indicated that the cDNA contained a protein-coding region of 631 a.a. with a calculated molecular mass of 72.6 kDa. From Clustal analysis of CNGC sequences, it was observed that this mouse cDNA showed greatest similarity to the family of CNGC α-subunits and is more distantly related to the β-subunit branch. Within the three principal classes of CNGC α-subunits, the new sequence falls squarely in the group of CNG subunits cloned from cone photoreceptors (Bönigk et al., 1993), testis (Weyand et al., 1994), and kidney (Biel et al., 1994). These subunits correspond to the type CNG3, according to the classification of Biel et al. (1995). Therefore, we have named this *m*ouse *c*yclic *n*ucleotide–*g*ated channel cDNA, mCNG3.

Pairwise sequence analysis indicated that mCNG3 is most closely related (92% identity) to the CNGgust channel, recently isolated from rat taste buds (Misaka et al., 1997). This high degree of similarity indicates that the polypeptide expressed in rat retina is likely to be CNG-gust. The overall a.a. sequence identity between mCNG3 and the next most closely related group of sequences was on the order of 80% (bovine testis/cone channel, 84%; human cone channel, 83%; chicken cone channel, 77%). In contrast, mCNG3 showed only 64% identity to the mouse rod photoreceptor CNG1 α-subunit (Pittler et al., 1991).

Analysis of the primary a.a. sequence indicates that mCNG3 possesses the structural characteristics of a CNGC subunit (Zagotta and Siegelbaum, 1996; Biel et al., 1999b), including six putative transmembrane segments (designated S1–S6), a pore region (P), and a highly conserved cyclic nucleotide binding domain in the carboxy-terminus (Fig. 2). By sequence analogy, both the amino-and the carboxy-termini are predicted to be intracellular. There is a conserved threonine residue in the cyclic nucleotide binding domain found in CNGCs that show preference for cGMP over 3′,5′–cyclic adenosine monophosphate (cAMP) (Varnum et al., 1995); therefore, mCNG3 is predicted to be a cGMP-gated channel. There is a potential N-linked glycosylation site in the extracellular loop between the S5 and the pore, the same domain that has been shown to be glycosylated in the rod CNG1 (Wohlfahrt et al., 1992). In addition, there are several consensus sequences for phosphorylation sites in the amino- and carboxy-terminal domains for cAMP-dependent kinase, PKC, and a protein tyrosine kinase (indicated by asterisks in Fig. 2). These sites may be used to modulate the channel properties, because phosphorylation is known to decrease the channel affinity for cyclic nucleotides (Gordon et al., 1992; Molokanova et al., 1997, 1999).

The sequence alignment of mCNG3 with related CNG channel α-subunits—rat taste buds (Misaka et al., 1997, 1999), bovine testis/cone photoreceptor (Weyand et al., 1994; Biel et al., 1994), human cones (Yu et al., 1996; Wissinger et al., 1997), and chicken cones (Bönigk et al., 1993)—is shown in Figure 2. The central portion of the protein, containing all six transmembrane domains, including the voltage-sensor motif in S4, the pore region, and the cyclic nucleotide binding domain, is the most conserved; indeed, the pore region is completely conserved in all of the species. In the sequence of mCNG3 and CNG-gust, there are two prominent gaps near the amino-terminus when compared to the bovine, human, and chicken sequences, corresponding to the deletion of exon 3 and of exons 5 and 6 (Bönigk et al., 1996), respectively. There is a putative calcium–calmodulin binding domain (Ca^{2+}-CaM, Liu et al., 1994) between Asp26 and Leu50 of mCNG3 that spans the first gap and that is different from the Ca^{2+}-CaM-binding motif previously identified in exon 4 of the full-length chicken cone CNGC α-subunit (Bönigk et al., 1996). A putative PKC phosphorylation site on Ser41 exists in the 12-a.a. stretch between the two characteristic aromatic or long-chain aliphatic residues, Leu32 and Trp45, of the proposed Ca^{2+}-CaM binding domain (O'Neill and DeGrado, 1990; Ikura et al., 1992; Liu et al., 1994). The second gap is characterized by a string of highly charged (E,K) residues, a motif found in some CNGC subunits and thought to influence ligand sensitivity. Despite the high degree of identity between mCNG3 and CNGgust, there are numerous gaps and a.a. substitutions in the amino-terminal sequence. Generally, the regions of greatest sequence divergence are at the amino-and carboxy-termini, reflecting probable differences in channel kinetics, channel regulation, and protein–protein interactions (Zimmerman, 1995). Therefore, to maximize specificity for mCNG3, a carboxy-terminal peptide was chosen to generate polyclonal antiserum in rabbits.

Photoreceptor outer segments express mCNG3 protein

In vertical sections of retinae immunostained with the antiserum against mCNG3 (Fig. 3), immunofluorescence was observed in the photoreceptor layer of retina only. This staining pattern was found in the retinae of rat (Fig. 3A), mouse (Fig. 3B) and chicken (Fig. 3C); the antiserum did not appear to produce labeling in the retina of the other species examined, namely, ox, monkey, rabbit, pig, tree shrew, guinea pig, and goldfish. Within the photoreceptors, the labeling appeared to be confined principally to the outer segment structure, with some faint labeling extending downward into the inner segments. The use of preimmune serum or the preincubation of the antiserum with the peptide abolished specific labeling on retina sections. The sparse distribution of labeled outer segments in the rod-dominated rodent retinae, as well as the labeled double cone in chicken retina (Fig. 3C, arrow), suggested that it

was the cone photoreceptors or a subtype of these photoreceptors that express the mCNG3 protein.

Electron microscopic localization of mCNG3

Preembedding immunoelectron microscopy using mCNG3 antiserum on mouse retina (Fig. 4) revealed intense silver-intensified DAB labeling in conelike photoreceptor outer segments, within a sea of unlabeled rod photoreceptor outer segments. The outer segments of cone photoreceptors lie proximal to those of the rod photoreceptors in the mouse retina (Carter-Dawson and LaVail, 1979). Thus, the relative depth of the labeled structures within the outer segment layer of the retina, i.e., abutting the inner segment layer, suggests that it is the cones that are being selectively labeled. Cones make up 3% of the photoreceptors in mouse retina (Carter-Dawson and La-Vail, 1979;Jeon et al., 1998) and show an overall relatively uniform density (Szél et al., 1992), on the order of ≈12,400 cells/mm^2 (Jeon et al., 1998), throughout the retina, which is consistent with the frequency of labeled outer segment observed with the mCNG3 antiserum. The pointed morphology of the outer segment, particularly the one on the right (Fig. 4), is also suggestive of cone photoreceptors.

Localization of mCNG3 to plasma membranes of outer segments

A high-power electron micrograph (Fig. 5) of a labeled cone photoreceptor outer segment shows the silver-intensified DAB reaction product localized almost exclusively to the layered folds of the plasma membrane that make up the disks of the cone photoreceptor.

Colocalization of mCNG3 with an S-cone marker

It is not possible, however, to determine by shape or relative distributions of the labeled structures whether this labeling occurs in all cone photoreceptors or in just a subtype of cones. To answer this question, we double-labeled retina sections with the mCNG3 antibody and cone subtype-specific markers. Because the S-cone opsin antibody (JH455) and the mCNG3 antibody are both polyclonal antisera raised in rabbit, we were not able to compare the stainings directly. Therefore, we show in Figure 6 that the anti-PKC monoclonal antibody from Amersham (clone MC5) labeled the outer segments of S-cone photoreceptors, in addition to the rod bipolar cells within the inner nuclear layer (Greferath et al., 1990), in mouse retina (Fig. 6A). The same section was labeled for the S-cone opsin with JH455, which labels the whole extent of the S-cones (Fig. 6B). The identification of the PKC-immunoreactive outer segments as belonging to S-cones can be readily made from the one-to-one correspondence observed in the high-power magnification views of the outer segment layers in Figure 6D (PKC) and 6E (JH455). Similar results were obtained in rat retina (data not shown). In this section, likely taken from inferior mouse retina where S-cones predominate (Szél et al., 1992;Wikler et al., 1998), all photoreceptors recognized by the PKC mAb were S-cones. Thus, the anti-PKC mAb can be used as a marker for S-cones in murid retina (Ohki et al., 1994;Wikler et al., 1998).

Figure 7 shows a vertical section of mouse retina double-stained for PKC (Fig. 7A,D) and for mCNG3 (Fig. 7B,E). The S-cones were identified by their PKC immunoreactivity in Figure 7A. The immunofluorescence of the mCNG3 labeling is restricted to the outer segments of photoreceptors (Fig. 7B). The colocalization of the two immunoreactivities can be seen clearly in the high-power magnifications of the outer segment layer in Figure 7D (PKC) and 7E (mCNG3). In this experiment, most, if not all, of the cone photoreceptors appear to be of the S-type. Similar results were obtained using rat retina (data not shown). These double-labeling experiments demonstrate that S-cones express the mCNG3 protein.

Both M-cones and S-cones express mCNG3

Although the cones show an overall uniform distribution throughout the rodent retina (Szél et al., 1992; Wang et al., 1992), the two cone types, the S- and M-cones, are present in the mouse retina in largely complementary gradients (Szél et al., 1992, 1996; Wang et al., 1992). The S-cones predominate in inferior retina, whereas the M-cones are enriched in the superior regions. Double-labeling experiments using the anti-PKC mAb and the mCNG3 antiserum on sections of rat retina, which does not show as stark a difference in their cone fields (Szél and Röhlich, 1992; Szél et al., 1996), are shown in Figure 8. We observed that all PKC-immunoreactive S-cone outer segments showed colocalization with mCNG3 labeling (arrows in Fig. 8B), but there were, in addition, photoreceptor outer segments that did not show double-labeling with the S-cone marker. From the relative density of the labeled outer segments, these non-S-cone photoreceptors are likely to correspond to the M-cones, and not to the rod photoreceptors.

To confirm that all cone photoreceptors express the mCNG3 protein, double-labeling experiments were carried out using FITC-PNA, which labels all cones in the mouse retina (Blanks and Johnson, 1984; Szél et al., 1992), and the mCNG3 antiserum. The FITC-PNA labels the cone photoreceptor and the interphotoreceptor matrix surrounding each cone (Blanks and Johnson, 1984). For each outer segment labeled with the mCNG3 antiserum (Fig. 9A), there was a corresponding sheath of FITC-PNA immunofluorescence (Fig. 9B), indicating that all cones, and both cone types, express the mCNG3 channel.

DISCUSSION

The mCNG3 cDNA encodes a CNGC α-subunit

In this study, we report the molecular identification of a new splice variant of a cone photoreceptor CNGC α-subunit, isolated from mouse retina. The sequence similarity is supported by the immunocytochemical localization to cone photoreceptor outer segments, where it most likely transduces the light signal into a membrane potential change. Its ultrastructural localization to the disk membranes of the cone photoreceptor outer segments places it in the same cellular compartment as the other components of the visual transduction machinery, namely the opsins, transducins and phosphodiesterase (Yau, 1994). In cones, the disks of the outer segment are composed of infoldings of the plasma membrane, where the cGMP-sensitive channels transducing the light response in photoreceptors are found (Cobbs et al., 1985; Haynes and Yau, 1985).

All cones appear to use the same CNGC to mediate phototransduction

From the double-labeling experiments, we conclude that the mCNG3 antiserum labels all cones in the murid retina. The number and pattern of outer segments labeled in the rod-dominated mouse and rat retinae at the light microscopic level, as well as at the ultrastructural level, are consistent with cone labeling; moreover, the double-labeling experiments with cone-specific markers demonstrated clearly that both S-cones and M-cones express the mCNG3 α-subunit. Although S-cones and M-cones use different opsins and therefore have different spectral sensitivities, they appear to use the same CNGC for transducing this light information. Consistent with this finding is the similarity in response kinetics of all three types of cones in the primate retina (Schnapf et al., 1990). However, it seems that there may be subtle differences in signaling between the cone types, because the inhibitory γ-subunit of the cGMP phosphodiesterase of the S- and M-cones appear to derive from different genes (Hamilton and Hurley, 1990). In addition, it is not yet determined whether cone channels contain a unique β-subunit (Biel et al., 1999b) that may confer additional functional differences. It is known that CNG3 (α-subunit) and CNG4 (β-subunit) are both expressed in the bovine testis and can form functional channels when combined in heterologous expression systems (Biel et al., 1996).

There is also evidence that homomeric CNGα channels may be used in some tissues (Torre et al., 1992; Bradley et al., 1994; Biel et al, 1996).

The hypothesis that the same channel is used in all cone photoreceptors is supported by molecular genetic evidence. If all cones use the same polypeptide to mediate their signal transduction, then defects in this channel would be predicted to severely impair photopic vision. Indeed, Biel and colleagues (1999a) very recently described electroretinogram studies on a CNG3 knockout mouse that showed that these animals completely lacked a cone-mediated light response. Furthermore, work by Wissinger and colleagues demonstrated that mutations in the gene encoding the cGMP-gated channel in cone photoreceptors are associated with total color blindness (achromatopsia) in humans (Kohl et al., 1998). Indeed, all of the point mutations identified in the human gene (*CNGA3*) by Kohl et al. (1998) involve residues conserved in the mouse CNG3 sequence, suggesting the importance of these residues in the proper function of the cGMP-gated channel.

Although all cone types appear to use the same channel for their signaling, it is clear that the rods use a different CNGC α-subunit (CNG1; Kaupp et al., 1989; Pittler et al., 1991; Kaupp, 1995) as well as a distinct β-subunit (CNG4; Chen et al., 1993: Körschen et al., 1995; Biel et al., 1996). Indeed, the two classes of photoreceptor appear to use related, but distinct, polypeptides for most, if not all, components of their signal transduction cascade, including the CNGCs (Bönigk et al., 1993), the transducins (Lerea et al., 1986; Peng et al., 1992) and phosphodiesterases (Hurley, 1987). Physiological differences in kinetics and modulation of phototransduction (Yau, 1994) between rods and cones undoubtedly arise from these molecular differences, as well as reflect their use under different light conditions. Recently, Kaupp and colleagues (Körschen et al., 1999) reported the localization of glutamic-acid-rich proteins (GARPs) to rod, but not cone, photoreceptors. They proposed a role for the GARPs in assembling various signaling molecules in the rods and, via an inhibitory effect on the cGMP phosphodiesterase, in dampening of the phototransduction system to prevent the unnecessary cGMP turnover during daylight. The β-subunit of the rod cGMP-gated channel itself contains a large cytosolic amino-terminal GARP region; therefore, the absence of staining in cones suggests that cone channels lack a β-subunit, or that the GARP domain of such a subunit is selectively missing, or perhaps that it is too distantly related to be recognized by the available GARP antibodies. Interestingly, there is a similar, highly charged glutamate- and lysine-rich region of as yet undetermined function found in both chick rod and cone CNGC α-subunits (Bönigk et al., 1993, 1996), as well as in the bovine cone CNGC α-subunit (Fig. 2). This particular domain is missing in mCNG3, because of the splicing out of exon 5.

Splice variants of the full-length cone photoreceptor channel gene

In fact, the mCNG3 cDNA lacks exons 3, 5, and 6 when compared to the full-length chick cone photoreceptor channel α-subunit gene (Bönigk et al., 1996). Because both protein products showed immunocytochemical localization to cone outer segments, it is likely that both channels mediate phototransduction, but it suggests that functional differences exist between the two species. Splice variants of the cone photoreceptor channel have been previously described by Bönigk et al. (1996); however, these splice variants, possessing deletions of exons 5 and/or 6 in the amino-terminus, were not expressed in the retina, but rather in the pineal organ and in testis.

The differentially spliced forms would be expected to have different kinetics, ligand sensitivities, and regulation. For example, the ligand sensitivity of the chick cone photoreceptor channel splice variant lacking exons 5 and 6 is increased compared to that of the full-length protein (Bönigk et al., 1996). Interestingly, we have identified a possible Ca^{2+}-CaM binding domain in the amino-terminus of mCNG3, which arises from the conjunction of exons 2 and 4—a splice that does not occur in the previously characterized chick, bovine, or human cone

channel α-subunits. The basic amphiphilic structure of this domain is highly similar to that reported for the olfactory channel α-subunit (Liu et al., 1994), although the sequence similarity is only 44% (32% identity).

At present, the data on the modulation of cone photoreceptor channels by Ca^{2+}–CaM are conflicting. Yu et al. (1996) reported finding no modulation by Ca^{2+}–CaM for the homomeric human CNG3 channel, whereas Biel et al. (1996) reported significant effects of Ca^{2+}–CaM on ligand affinity and on inhibition by pimozide and L-*cis*-diltiazem on the bovine testis/cone homomeric CNG3 channel. Both the human and bovine sequences contain an insert of 38 a.a. compared to mCNG3, that not only disrupts the proposed Ca^{2+}–CaM binding domain, but also lacks the first of two aromatic or long-chain aliphatic residues, identified as critical by Liu et al. (1994) for Ca^{2+}–CaM regulation of the olfactory channel. This observation may explain why Yu et al. (1996) did not see modulation by calmodulin, as was also the case for the native cone channel from catfish (Haynes and Stotz, 1997). Bönigk and colleagues (1996) identified another Ca^{2+}–CaM site in exon 4 of the full-length chick cone channel and reported a small, but consistent, decrease in sensitivity to cGMP with the application of Ca^{2+}–CaM. Therefore, the detectability of modulation as well as yet unidentified Ca^{2+}–CaM binding domains or associated regulatory factors may account for the discrepancies. It will be interesting to see whether the proposed Ca^{2+}–CaM domain of the mCNG3 channel confers sensitivity to Ca^{2+}–CaM modulation to the mouse cone channel.

Müller et al. (1998) studied the effects of PKC phosphorylation of the olfactory channel at a site flanking the Ca^{2+}–CaM site identified in exon 4 and reported an increase in ligand sensitivity with phosphorylation, without counteracting the modulation by Ca^{2+}–CaM. The corresponding serine residue is present in mCNG3; however, it does not conform to a PKC phosphorylation consensus site. There is instead a potential phosphorylation site directly in the middle of the proposed Ca^{2+}–CaM binding domain of mCNG3. Further characterization will be necessary to determine whether the mCNG3-containing channel shows sensitivity to modulation via PKC-mediated phosphorylation.

Other roles for CNGCs in the retina

Rieke and Schwartz (1994) provided physiological evidence that cGMP-gated channels play a functional role in the cone terminals by permitting these photoreceptors to extend their range of neurotransmitter release over a much broader range of voltage than the rods via their calcium permeability. CNGCs also have been proposed as targets of synaptic regulation through feedback by nitric oxide onto cone terminals (Savchenko et al., 1997). However, the mCNG3 label appeared to be restricted to the outer segments. It may be that the CNG channels in the outer segments involved in phototransduction are different from the channels present in terminals, or that the epitope is masked in the terminal, or that the density of channels is too low to be detected by indirect immunofluorescence. Further work will be required to determine whether novel nonphotoreceptor CNGCs participate in visual information processing in the retina.

Acknowledgments

U.S. National Institutes of Health; Grant numbers: R01 EY09534, F32 EY06488; Grant sponsor: New York Academy of Medicine David F. Warfield Fellowship in Ophthalmology, Research to Prevent Blindness Career Development Award; Grant sponsor: Max Planck Society Fellowship; Grant sponsor: Deutsche Forschungsgemeinschaft; Grant number: SFB 269/B4.

We thank Dr. J. Nathans for his generosity in providing the S-cone opsin antiserum and Drs. M. Biel, U.B. Kaupp, R.S. Molday, R.R. Reed, and K.-W. Yau for sending CNGC plasmids. We acknowledge the skilled technical assistance of A. Hildebrand, W. Hofer, G.S. Nam, and F. Boij, the computer graphics work of D. Benzaid, and the secretarial assistance of I. Odenthal. We thank Drs. J.H. Brandstätter and L. Peichl for critically reading and improving the manuscript, and finally Dr. A. Francesconi for engaging discussions throughout the project. This work was supported

by the U.S. National Institutes of Health (R01 EY09534 to R.M.D.; F32 EY06488 to A.A.H.), New York Academy of Medicine David F. Warfield Fellowship in Ophthalmology (A.A.H.), Research to Prevent Blindness Career Development Award (R.M.D.), Max Planck Society Fellowship (A.A.H.), and the Deutsche Forschungsgemeinschaft (SFB 269/B4, H.W.).

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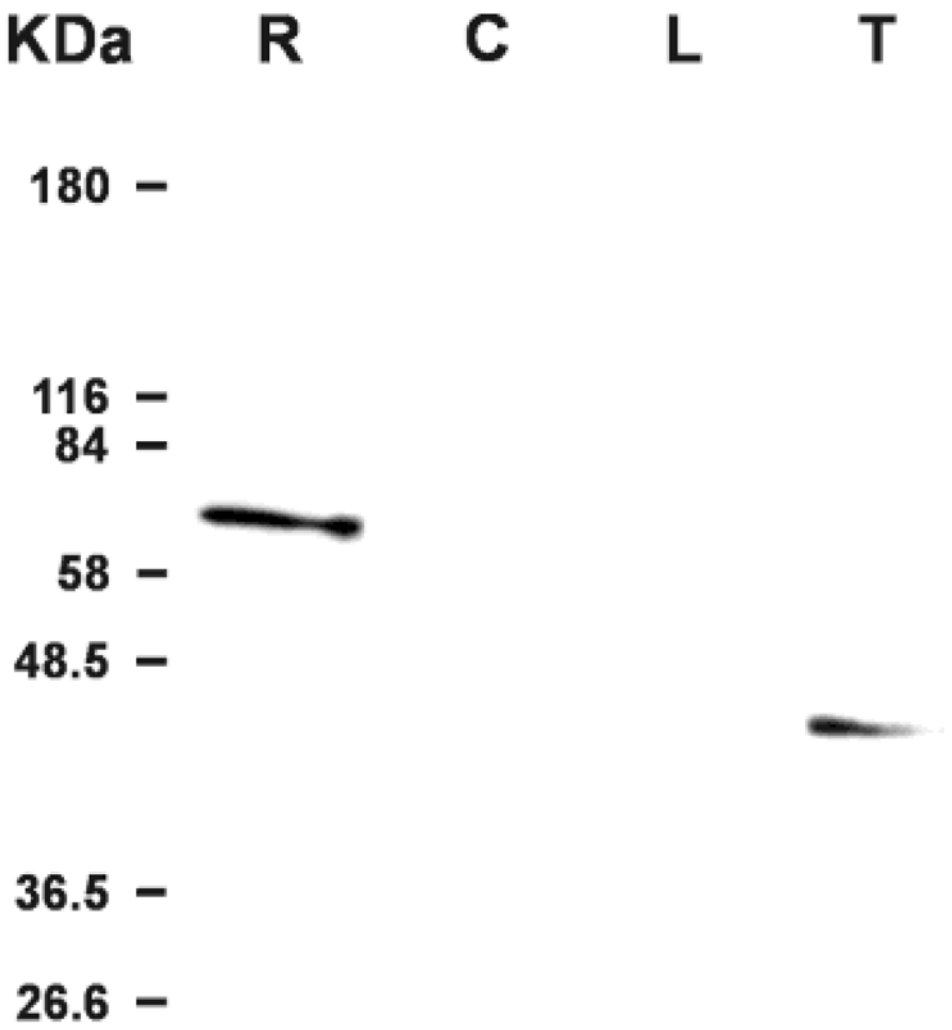

Fig. 1.

Specificity of the antiserum against the mCNG3 protein. Western blot analysis showed a single band in retina labeled with an apparent molecular weight of ≈70 kDa by the mCNG3 antiserum. No bands were detected in cerebellum or in liver; however, in tongue a ≈40 kDa band was observed. The positions of the molecular weight markers are shown to the left in KDa. R, retina; C, cerebellum; L, liver; T, tongue.

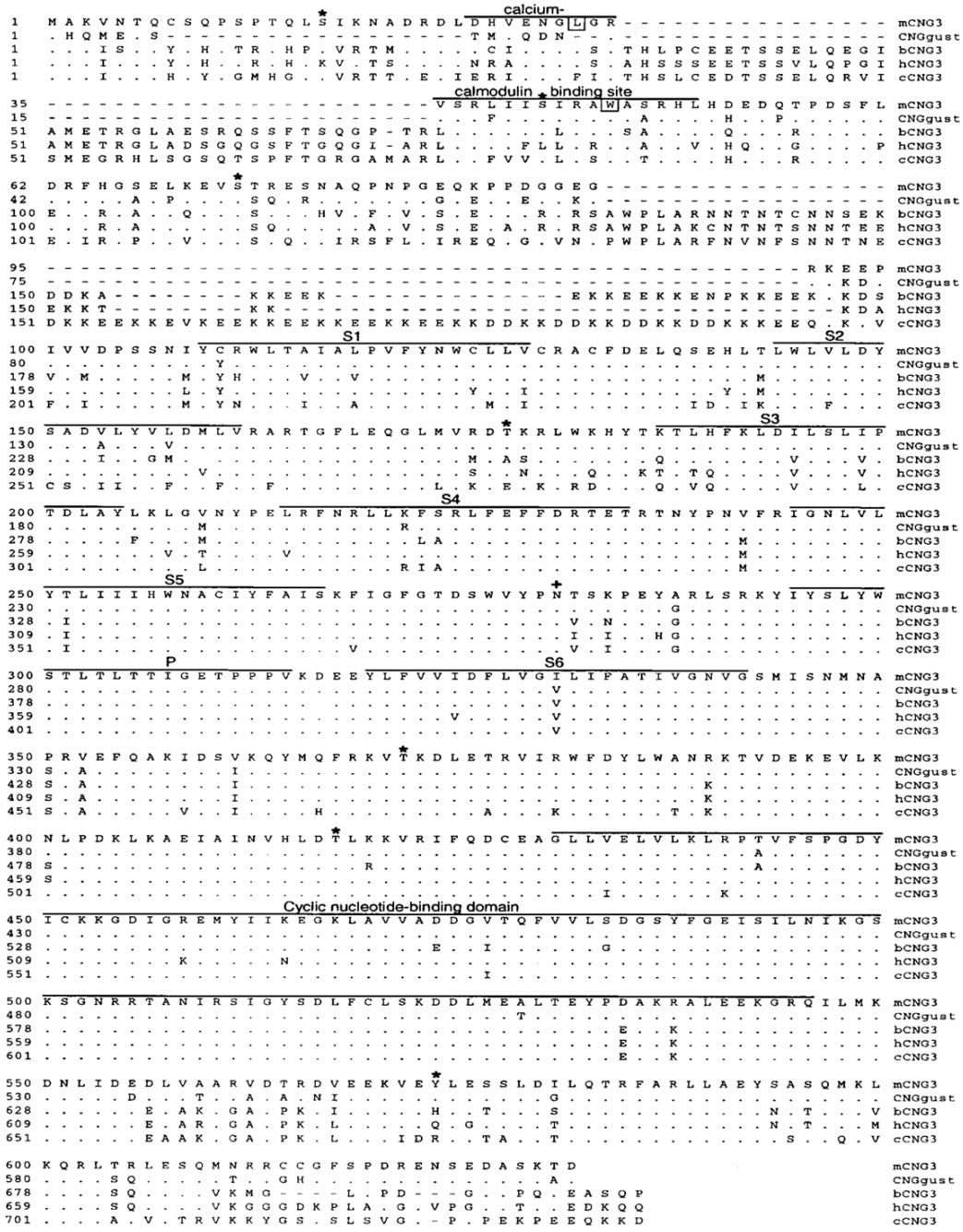

Fig. 2.

Sequence alignment with related cyclic nucleotide–gated channel α-subunits. The mCNG3 cDNA protein-coding sequence is shown on the first line and aligned with it in descending order of sequence similarity are the rat CNGgust (Misaka et al., 1997), bovine cone/testis CNG3 (bCNG3, Biel et al., 1994; Weyand et al., 1994), human cone CNG3 (hCNG3, Yu et al., 1996; Wissinger et al., 1997) and chicken cone CNG3 (cCNG3, Bönigk et al., 1993) cDNA sequences. The following conserved structural characteristics of cyclic nucleotide–gated channels are denoted: The six putative transmembrane domain sequences, S1 through S6, the pore (P) region, and the cyclic nucleotide-binding domain in the carboxy-terminus. A proposed calcium–calmodulin binding site is found in the amino-terminus. Putative posttranslational

modification sites include one for N-glycosylation (+) and several for protein phosphorylation (*). Gaps in the sequence alignment are denoted by hyphens (-) and amino acid identity to mCNG3, by periods (.).

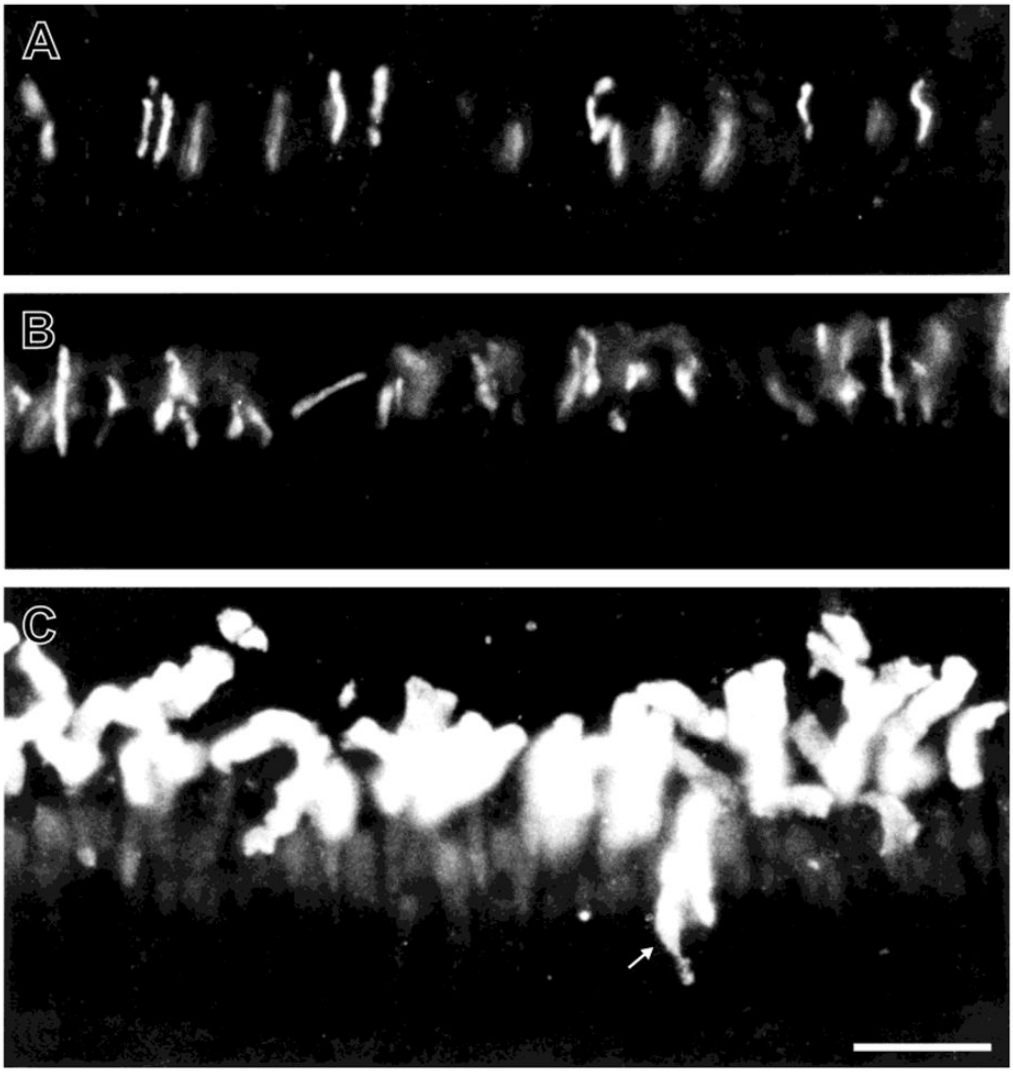

Fig. 3.

Fluorescence micrographs of photoreceptor labeling by antiserum to mCNG3 in (**A**) rat, (**B**) mouse, and (**C**) chicken retinae. Note that the immunoreactivity is concentrated in the outer segments with much fainter labeling extending into the inner segments. The arrow points to a double cone in chicken retina (C). Scale bar = 20 μm.

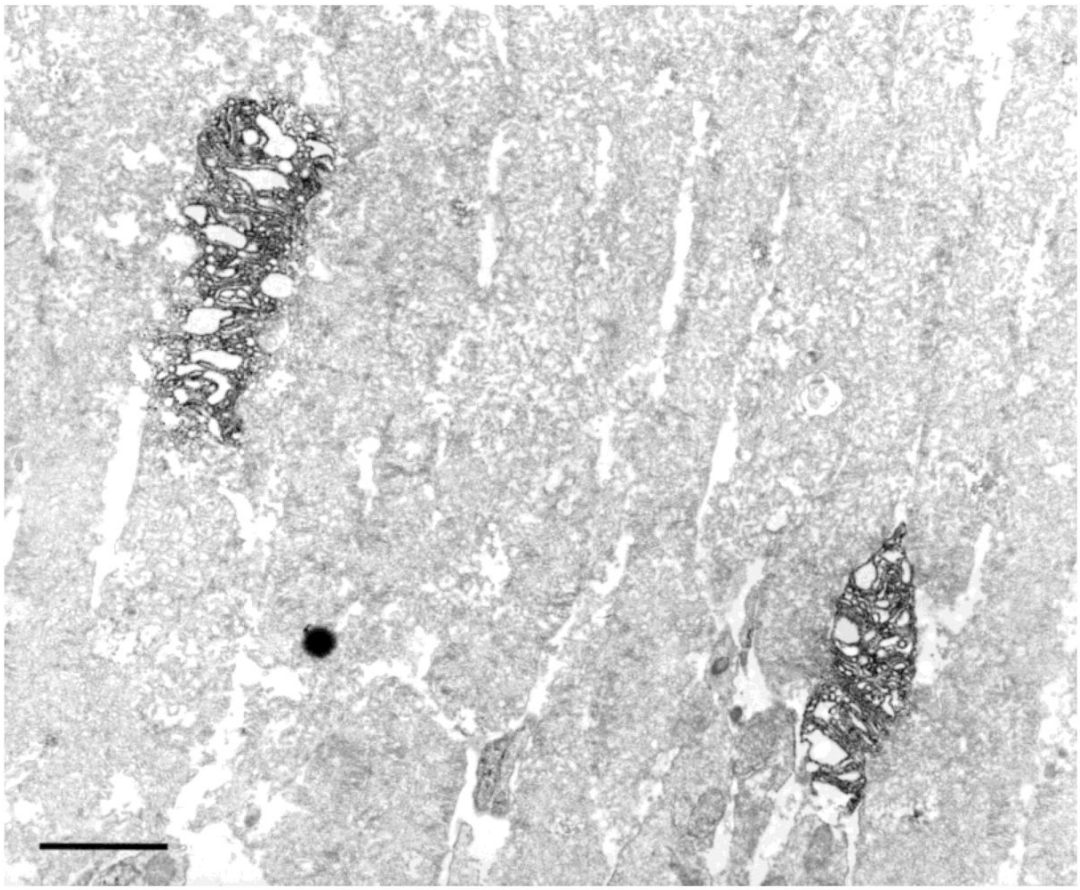

Fig. 4.

Electron micrograph of cone photoreceptor channel immunoreactivity in the photoreceptor layer of mouse retina. The figure shows silver-intensified diaminobenzidine reaction product in the outer segments of regularly spaced photoreceptors, which are likely to be cones from their relative frequency in a rod-dominated retina and their depth within the outer segment layer. Scale bar = 2 μm.

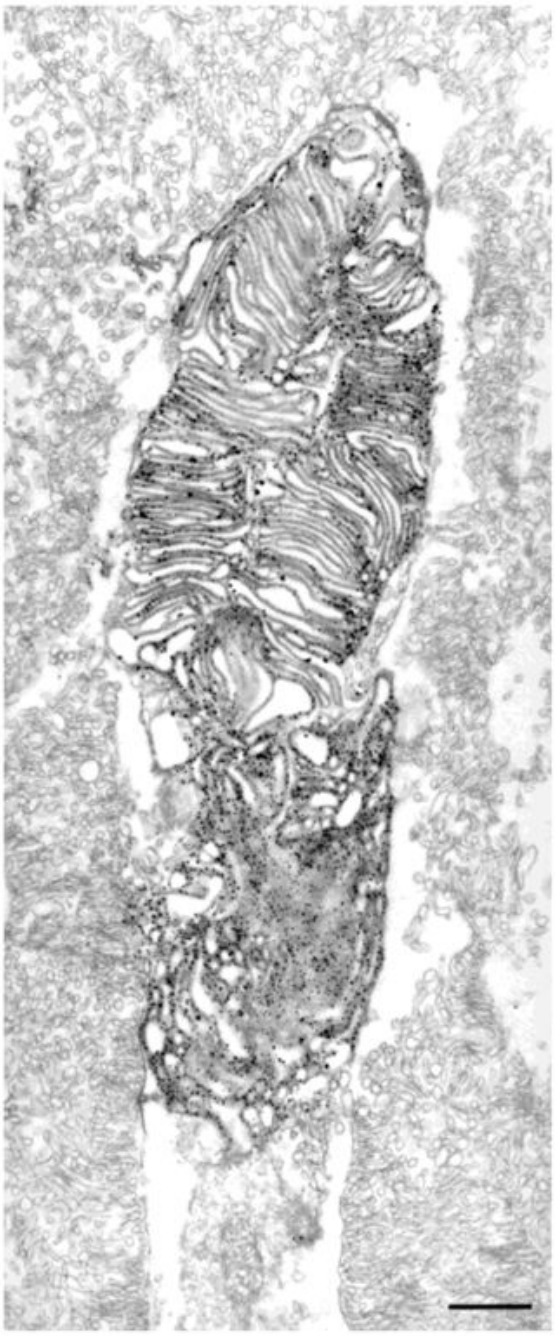

Fig. 5.
High-power electron micrograph shows silver-intensified diaminobenzidine reaction product in the plasma membranes of a labeled cone outer segment. Scale bar = 1 μm.

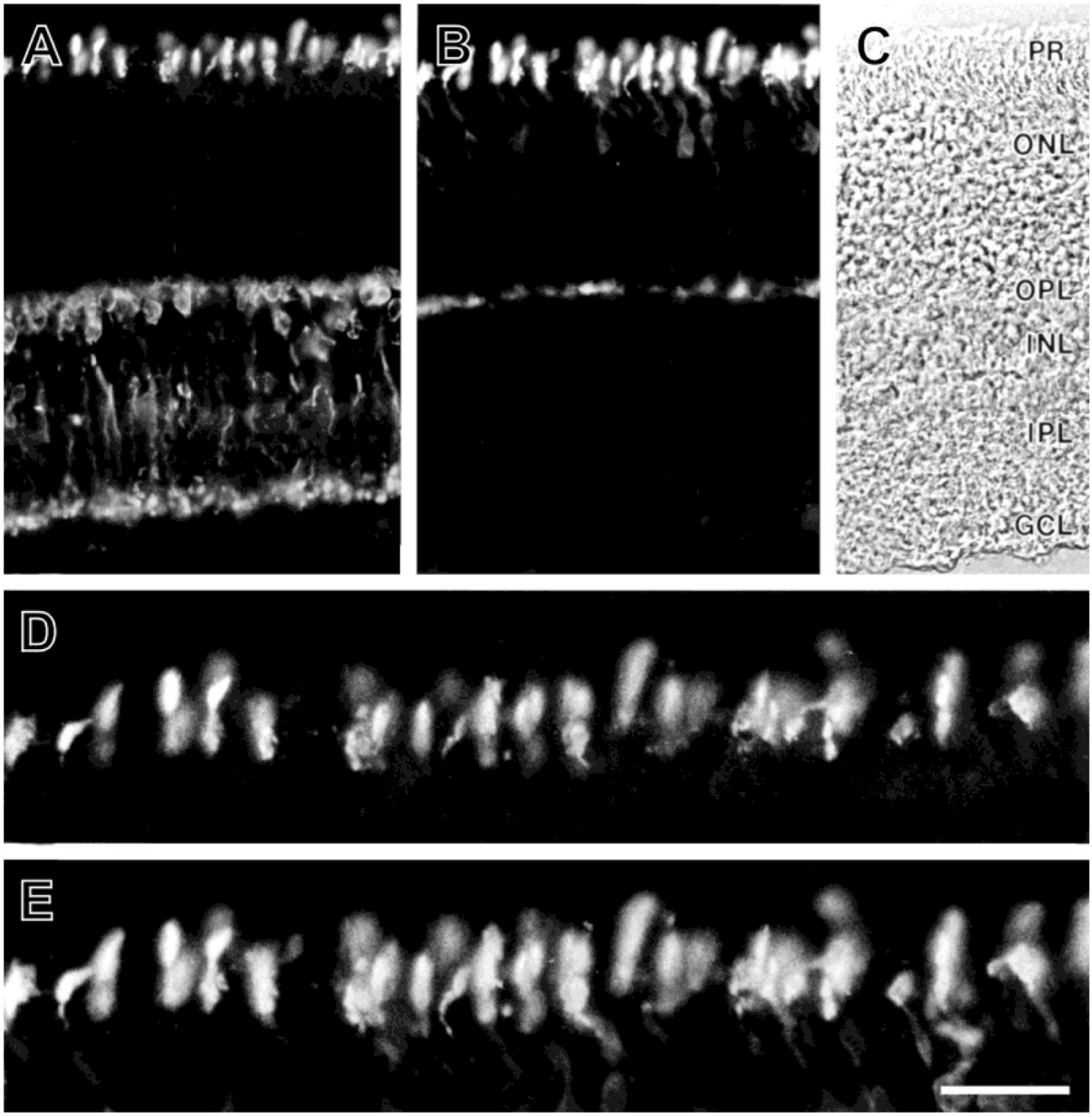

Fig. 6.

Fluorescence micrographs shows that an antibody to protein kinase C (PKC) labels S-cones specifically in the photoreceptor layer of mouse retina. **A:** An anti–PKC antibody (clone MC5) labeled outer segments in the photoreceptor layer (PR) as well as the rod bipolar cells in the inner nuclear layer (INL). **B:** A human S-cone opsin antiserum (JH455) was used to identify the S-cones. The retinal layers are shown using Nomarski optics in (**C**). Higher magnification views of the PR layer show the colocalization of PKC (**D**) and blue cone opsin (**E**) immunoreactivity. ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 42 μm for A–C; 20 μm for D,E.

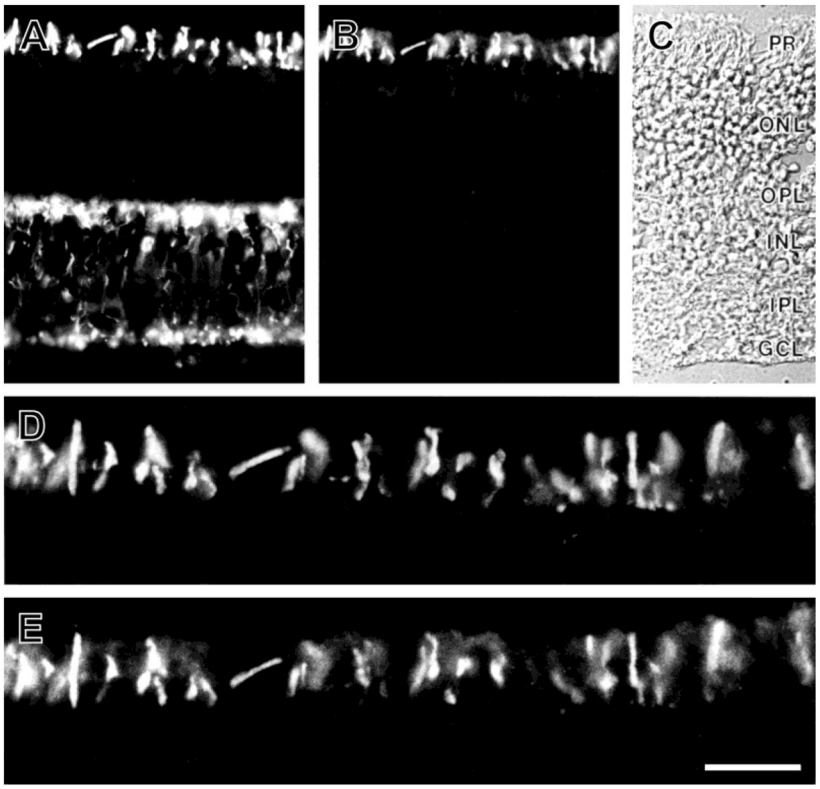

Fig. 7.

Fluorescence micrographs shows that S-cones express the mCNG3 protein in mouse retina. **A:** The anti–protein kinase C (PKC) antibody identified outer segments of S-cones in the photoreceptor layer (PR) and the rod bipolar cells in the inner nuclear layer (INL). **B:** The antiserum to mCNG3 labeled the same outer segments in the PR layer as the anti-PKC antibody. The retinal layers are shown with Nomarski optics in (**C**). Higher magnification views of the PR layer shows the colocalization of anti-PKC (**D**) and mCNG3 (**E**) immunoreactivity, indicating that S-cones express this CNG channel subunit. In this section, taken most likely from inferior retina, all photoreceptors recognized by the mCNG3 antiserum appear to be S-cones. ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 42 μm for A–C; 20 μm for D,E.

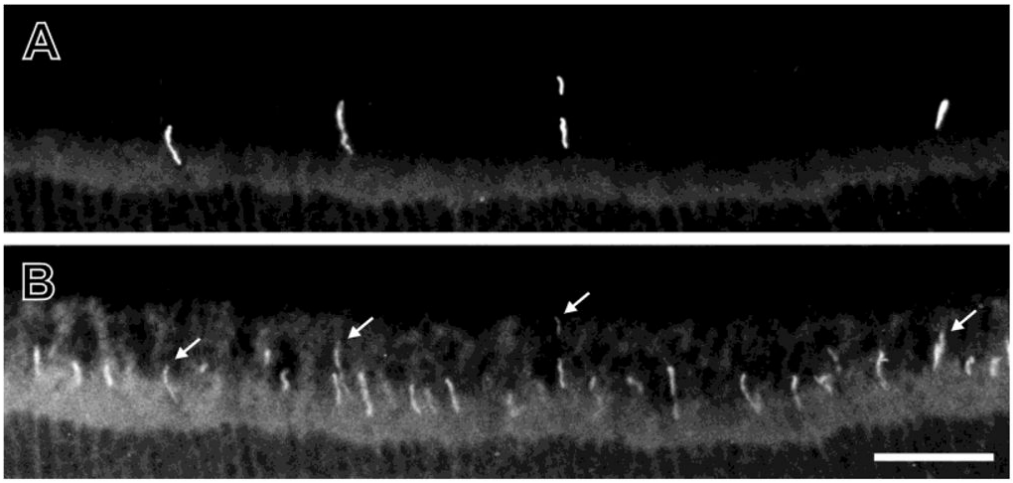

Fig. 8.

Fluorescence micrographs shows that S-cones and likely M-cones express the mCNG3 channel subunit in rat retina. **A:** The anti-protein kinase C (PKC) antibody identified the outer segments of S-cones. **B:** The mCNG3 antiserum labeled the four outer segments recognized by the anti-PKC antibody (arrows) plus additional outer segments, of the presumed M-cones. Scale bar = 20 μm.

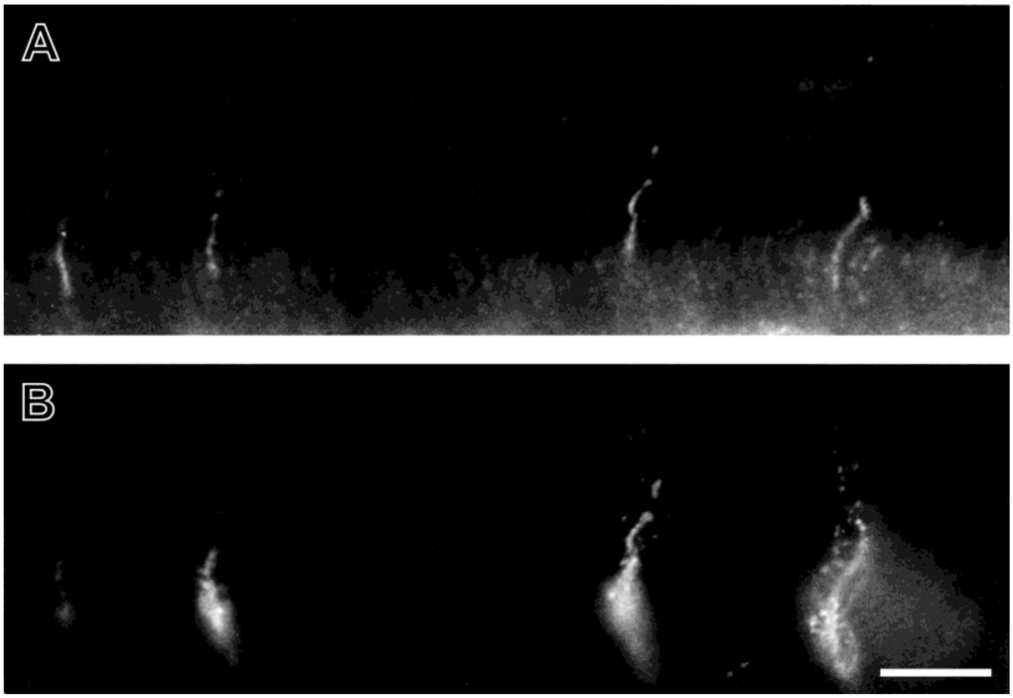

Fig. 9.

Fluorescence micrographs shows the colocalization of a cone photoreceptor marker, peanut agglutinin (PNA), and the mCNG3 immunoreactivity in rat retina. **A:** The interphotoreceptor matrix surrounding each cone is labeled by fluorescein isothiocyanate (FITC)–PNA (Blanks and Johnson, 1984). **B:** The cone photoreceptor outer segments labeled by the mCNG3 antiserum are observed within each shell of FITC–PNA labeling, indicating that all cones express a mCNG3-like protein. Scale bar = 10 μm.