Retinal Bipolar Neurons Express the Cyclic Nucleotide-Gated Channel of Cone Photoreceptors

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Submitted 6 September 2002; accepted in final form 22 October 2002

INTRODUCTION

Cyclic nucleotide-gated (CNG) channels are ligand-gated nonspecific cation channels that open when cyclic nucleotides [guanosine 3′,5′-cyclic monophosphate (cGMP) or cAMP] bind to specific regions on the cytoplasmic face of the channel. The central role of these channels in visual and olfactory transduction is well established, but in addition to their role in primary sensory transduction, CNG channels are thought to be important in a variety of other physiological processes. In the retina, for example, CNG channels have been implicated in nitric oxide signaling (Ahmad et al. 1994). Also, CNG channels may mediate the sign-inverting synapse in the ON visual pathway, where glutamate released from photoreceptor terminals in darkness hyperpolarizes ON bipolar cells through a metabotropic glutamate receptor (mGluR6) (Nakajima et al. 1993). In this pathway, the proposed mechanism is that mGluR6 cascade is analogous to phototransduction, i.e., the existence of CNG channels in ON bipolar cells has yet been forthcoming, and the molecular identity of any CNG channels expressed in these cells remains unknown.

METHODS

Cloning a CNG channel from goldfish retina using RT-PCR

All animal procedures were carried out in accordance with National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee. Goldfish and rat retinas were separated from hemisected eyes and rapidly frozen. Total RNA was extracted by the method of Cathala et al. (1983) and stored at −80°C under ethanol. Alternatively, poly(A)⁺ RNA was extracted using the micro Poly A⁺ Pure kit (Ambion). For Northern blots, samples of total RNA or poly(A)⁺-selected RNA were used. Reverse transcription using Superscript II reverse transcriptase (Life Technologies) was carried out according to the manufacturer’s protocol. In brief, 1–5 µg RNA was added to diethyl pyrocarbonate (DEPC)-treated water to a final volume of 11 µl. The solution was heated to 95°C for 5 min and then placed on ice. Random hexamer primers (3 µg) were added, and the mixture was incubated at 70°C for 10 min and then cooled on ice. After addition of 4 µl 5× first-strand synthesis buffer, 250 U of reverse transcriptase were added and the mixture was incubated at 42°C for 60 min.

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buffer, 2 μl 0.1 M DTT, 1 μl dNTPs (10 mM each), 10 units RNasin, and 200 units Superscript II, the synthesis reaction was carried out at 42°C for 1 h. The RNA was then digested with RNase H (1.5 units) for 20 min at 37°C, followed by heating to 95°C for 5 min. RNasin was obtained from Eppendorf, and all other reagents were obtained from Life Technologies.

Conventional PCR was then performed using Platinum Taq DNA polymerase (Life Technologies) and 2 μl reverse-transcribed cDNA in 50 μl PCR buffer and reactants. The standard amplification protocol consisted of 95°C for 5 min, followed by 45 cycles of 95, 55, and 72°C for 1 min each, ending with 72°C for 4 min. Primers were designed that are predicted to amplify cDNA for α subunits of CNG channels from a wide variety of vertebrate species. The forward primer was 5'-GGCGGTTCTTCGAT-3' and the reverse primer was 5'-CAGAAGAGCTTGAGTA-3'. These primers embrace the region from the extracellular loop between S1 and S2 to the cyclic nucleotide-binding domain of CNG channel α subunits and produce an amplified product of approximately 1160 bp. cDNA products of the correct size were gel purified and subcloned into pGEM-T Easy cloning vector (Promega). Selected clones were sequenced on an automated DNA sequencer.

To extend the cDNA to the 3' and 5' ends of the mRNA, we used 3' rapid amplification of cDNA ends (RACE) (3' RACE System; Life Technologies) and 5' RACE (5' RACE System; Life Technologies), following the manufacturer’s protocols, except that Thermoscript reverse transcriptase (Life Technologies) was used instead of Super- script II for 5' RACE. Because the predicted N-terminus of the protein turned out to be shorter than many other known CNG channels, we pursued an alternative strategy to confirm that the end of the cDNA obtained by 5' RACE was complete and correct. For this purpose, we used oligo-capping RACE (GeneRacer kit; Invitrogen), in which RNA was first treated with calf intestinal phosphatase to remove 5' phosphate group that allows subsequent ligation of an RNA adaptor sequence prior to reverse transcription. This strategy produced a longer stretch of 5' untranslated region (UTR) than conventional 5' RACE, but the predicted start of the coding sequence and the proximal part of the UTR were identical to that obtained with 5' RACE. The accession number of the full cDNA is AY167423.

The expected size of the full-length mRNA was determined from Northern blots of total RNA or poly(A)+ RNA. Blots were hybridized with a radiolabeled, 1100-bp probe synthesized from a PCR fragment of the cloned CNG channel. After prehybridization incubation for 4 h at 68°C, hybridization was carried out overnight at 68°C in a solution containing 5× standard saline citrate (SSC), 1× Denhardt’s solution, 20 mM NaH2PO4/NaHPO4 (pH 6.7), 50% formamide, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. Blots were washed at 68°C for 15 min in 2× SSC, 0.1% SDS, followed by two 15-min washes in 0.2× SSC, 0.1% SDS. Images were acquired on a phosphorImager.

**Single-cell RT-PCR**

For single-cell RT-PCR, cell contents were aspirated into a whole-cell patch pipette containing pipette solution made with RNase-free water (Ambion). To prevent extraneous material from entering the patch pipette, a small amount of the cell was left outside the pipette to plug the tip. Also, only cells that were free of attached cellular debris and well separated from surrounding material were collected. The pipette contents (approximately 1 μl) were then expelled into a siliconized 0.5-ml microfuge tube containing 10.5 μl RNase-free water. We then added 4 μl 5× first-strand synthesis buffer, 0.5 μl 0.1 M DTT, 2 μl RNasin (2 units/μl), 1 μl d-nucleoside triphosphate (dNTPs) (10 mM each), and 1 μl of random hexamer primers (3 μg/μl). After incubation at room temperature for 10 min, 1 μl (200 units) of Superscript II reverse transcriptase was added, and the cDNA synthesis was carried out for 1 h at 42°C. The RNase H treatment was omitted. All components were obtained from Life Technologies except RNasin, which was obtained from Eppendorf. Two rounds of PCR amplification were then performed using the thermocycler protocol described above for analysis of CNG channels in whole retina. The first-round primers were the same as those used for whole-retina PCR analysis (see METHODS). Both specific and degenerate second-round PCR primers were designed based on the sequence of the CNG channel cDNA obtained from goldfish retinal RNA. For the second round, the specific forward primer was 5'-GGTCTCTCATTGACGACCGTTG-3' and the specific reverse primer was 5'-GATGTTGGGCGTCTTCGAT-3'. The degenerate forward primer was 5'-GGACGGGTWTCCTNGARC-3' and the degenerate reverse primer was 5'-TTGTCASAGRTAATCRAACC-3'. The expected size of the amplified cDNA from the specific second-round primers is 1.135 bp, whereas the degenerate primers produce an expected fragment of 655 bp. Amplified products of the correct size were gel-purified, subcloned in pGEM-T Easy, and sequenced.

Three control experiments were performed in parallel with each single-cell RT-PCR experiment. First, cell contents were aspirated and all experimental steps were performed, except the reverse transcriptase enzyme was omitted (− RT control). Second, a sham cell collection was carried out, in which a patch pipette was placed in the bath and a small amount of external fluid was aspirated instead of a cell (no cell control). The pipette contents were then expelled and all experimental steps were carried out as for collected cells. Third, the two rounds of PCR were performed, but water was substituted for reverse-transcribed cDNA in the first round (no DNA control). In the experiments reported here, all three control experiments were negative for amplified PCR products of the expected size.

**In situ hybridization**

Isolated cells were obtained from goldfish retina by mechanical trituration after papain digestion, as detailed previously (Heidelberger and Matthews 1992). Cells were plated directly onto microscope slides, allowed to attach for 25 min, and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h at 4°C. Cells were washed, cryoprotected in sucrose, frozen, and stored at −80°C.

Specific RNA probes for cone CNG channels of goldfish retina were synthesized in sense and antisense directions from cDNA obtained by RT-PCR. The probe length was 1,100 bp. Synthesis, hybridization, and detection of digoxigenin-labeled probes were carried out according to the manufacturer’s protocol (Roche), using anti-digoxigenin antibody conjugated to alkaline phosphatase for detection.

**Immunocytochemistry**

Immunostaining for protein kinase C (PKC) was used to mark ON bipolar cells (Suzuki and Kaneko 1990). Goldfish retinas were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, frozen, and sectioned at 12 μm. Alternatively, enzymatically dissociated cells were fixed for 40 min in 4% paraformaldehyde in PBS. Anti-PKC antibody (NOVUS Biologicals; clone MCS, ab31) was diluted 1:300 in PBS containing 4% goat serum and 0.1% Tween 20. Signals were detected in retina sections using Alexa-488-conjugated secondary antibody and confocal microscopy (Olympus FV-300). In isolated cells, signals were detected using the Vectastain ABC-peroxidase kit (Vector Laboratories), according to the manufacturer’s directions.

**RESULTS**

**Molecular identification of a CNG channel α subunit expressed in goldfish retina**

The large size and distinctive morphology of goldfish bipolar neurons facilitate physiological studies and allow ready
identification of the cells in dissociated cell preparations (see following text). Because of these advantages, we chose to study the expression of CNG channels in os bipolar cells of goldfish retina. No CNG channels had yet been molecularly characterized in goldfish, so we first needed to identify transcripts for α subunits of CNG channels expressed in goldfish retina. For this purpose, we carried out RT-PCR using degenerate PCR primers that target conserved regions in the nucleic acid sequences of known CNG channel α subunits from a variety of species and cell types. To minimize potential problems arising from amplification of any contaminating genomic DNA, we selected primers that were predicted to span introns, based on the organization of the genes encoding the CNG channel of human rods (Dhallan et al. 1992) and chick cones (Bönigk et al. 1996).

Primers were tested using total RNA isolated from goldfish and rat retinas, with the latter serving as a positive control. To estimate the sensitivity of each primer set, total RNA was serially diluted, and primers were chosen that produced detectable products of the appropriate size from 1 to 10 pg of goldfish RNA. The primer pair selected for further study (see METHODS) yielded an amplified product approximately 1,100 bp in length, embracing the region of the encoded protein from the extracellular loop between S1 and S2 to the cyclic nucleotide-binding domain. In positive-control experiments using RNA from rat retina, the amplified cDNA produced by these primers corresponded to the expected portion of the α subunit of CNG channels of rat rod photoreceptors (data not shown). Additional control experiments confirmed that the primers did not produce an ampiclon of the correct size from goldfish genomic DNA, except when RNA was also added to the genomic DNA and a reverse transcriptase reaction was carried out. In other words, the primers detect only reverse-transcribed cDNA derived from expressed mRNA and are not sensitive to contaminating genomic DNA.

RT-PCR was performed on goldfish retinal RNA, and cDNA of the expected size was gel isolated, subcloned, and sequenced. A total of 17 clones was sequenced, all of which were identical. Database searches revealed that the goldfish cDNA is approximately 70% identical to nucleic acid sequences of CNG channel α subunits from rods and cones of mammals and chick, indicating that the PCR product represents part of the coding sequence for the α subunit of a CNG channel expressed in goldfish retina. To confirm that the amplified cDNA stems from a bona fide mRNA present in goldfish retina, we hybridized Northern blots of total RNA with antisense probe synthesized from the goldfish PCR fragment. Figure 1A shows that the probe revealed a single band of 2,500–3,000 bp, which is similar in size to known mRNAs of CNG channels in other species.

To obtain the full coding sequence of the cloned channel, we used 3′ RACE to extend the cDNA through the 3′ UTR to the poly(A)− tail of the mRNA. In the 5′ direction, the cDNA was extended by 5′ RACE, and the resulting sequence was further confirmed by oligo-capping (Maruyama and Sugano 1994; Volloch et al. 1994). Including 5′ and 3′ UTRs, the resulting full-length mRNA is 2,654 bp in length, which agrees with the results of the Northern blot. The predicted amino acid translation from the coding region of the mRNA is shown in Fig. 1B, compared with the peptide sequence of the CNG channel of chick cone photoreceptors (Bönigk et al. 1993). Approximately 66% (418 of 637) of the amino acid residues are identical in the chick cone channel and the goldfish retinal CNG channel. An additional 16 nonidentical residues conserve the charge at the corresponding position (K/R or D/E substitutions). The highest similarity is found in the cyclic nucleotide-binding domain and in the pore region, with moderate similarity in the...
transmembrane segments. The N- and C-termini are most divergent.

Localization of the CNG channel transcript to goldfish cone photoreceptors

To establish which cells in goldfish retina express the CNG channel transcript identified by RT-PCR, we synthesized antisense and sense RNA probes from the amplified cDNA and carried out in situ hybridization in isolated cells, which were obtained by dissociation of papain-digested retina (Heidelberger and Matthews 1992). All isolated cones in the dissociated preparation were labeled with the antisense probe, but not the sense probe, as illustrated Fig. 2. The region between the nucleus and the ellipsoid was intensely labeled, whereas the ellipsoid, outer segment, connecting axon, and synaptic terminal exhibited less reaction product. This result demonstrates that the CNG channel α subunit cloned from goldfish retina is equivalent to the cone-specific CNG channel of mammalian and chick retina, CNG3 (Bönigk et al. 1993; Hirano et al. 2000).

We next turned to RT-PCR from single cones to determine whether the CNG channel detected by in situ hybridization in isolated goldfish cones is in fact the same as the CNG channel we identified by RT-PCR from goldfish retina. Individual cones were aspirated into patch pipettes and reverse transcription was carried out as described in METHODS. Two rounds of PCR were performed, employing the primers used previously for whole-retina PCR for the first round, followed by nested second-round primers based on the sequence of the cloned CNG channel. Figure 3A shows that amplicons of the correct size were observed in three of five individual cones (lanes 1, 2, 3, 5, and 6 contain cDNA from single cones; lanes 4 and 7 are control lanes). After gel isolation, the cDNAs of the expected size were subcloned and sequenced. All positive bands were identical to the CNG channel α subunit identified previously by RT-PCR from intact retina. Figure 3B shows results obtained from the same set of first-round PCR reactions but using a different set of degenerate second-round primers, which are expected to produce an amplified product of approximately 650 bp. With this second set of primers, the expected product was observed in two cells, one of which had been negative with the first set of second-round primers. Sequencing again confirmed that the amplified cDNA was identical to the CNG channel cloned previously from goldfish retina. Thus four of five isolated cones examined with single-cell PCR were found to express the CNG channel transcript we characterized earlier by RT-PCR from goldfish retinal RNA. We assume that the failure to detect the transcript in one cone represents a limitation of the single-cell PCR technique, rather than heterogeneity among cones. The result from single-cell RT-PCR confirms the conclusion from in situ hybridization that the CNG channel isoform we have identified in goldfish retina is the α subunit of the cone photoreceptor CNG channel. We therefore refer to this α subunit as gfCNG3, because it is apparently the homologue of the mammalian cone-specific subtype, CNG3.

The cone CNG channel, gfCNG3, is expressed in goldfish bipolar neurons

To examine the expression of CNG channels in bipolar cells, we exploited the morphological uniqueness of different classes of bipolar cells in goldfish (Sherry and Yazulla 1993). For example, one important class of ON bipolar cells, which depolarize in response to illumination, can be distinguished based on the large size of their synaptic terminals and their large, flask-shaped somata (Saito and Kujiraoka 1982; Sherry and Yazulla 1993). As illustrated in Fig. 4A, these cells can be readily recognized in retina sections by virtue of their intense labeling with antibodies against PKC, which selectively labels ON bipolar cells in teleost retina (Suzuki and Kaneko 1990). Cells of this type have large cell bodies (arrowhead, Fig. 4A) located near the middle of the inner nuclear layer of the retina (INL), and especially large, bulbous synaptic terminals (white asterisk, Fig. 4A) in the inner portion of the inner plexiform layer (IPL). Figure 4A also shows that PKC immunoreactivity marks cone-driven ON bipolar cells, which have smaller somata (arrow, Fig. 4A) and synaptic terminals (black asterisk, Fig. 4A). Bipolar cells largely retain their distinctive morphology after enzymatic dissociation of the retina, and ON cells can again be distinguished from OFF cells based on PKC immunoreactivity, as shown in Fig. 4, B and C. As expected, isolated bipolar cells with large synaptic terminals were uniformly positive for PKC (Fig. 4B), whereas PKC-negative OFF bipolar cells had smaller terminals (Fig. 4C). In preparations of dissociated cells, then, we assume that large-terminal bipolar cells with the morphology illustrated in Fig. 4B are ON cells. Small-
terminal bipolar cells, on the other hand, represent a mixture of off and on types.

In addition to labeling in cones, in situ hybridization in dissociated goldfish retinal neurons also revealed gfCNG3 expression in single bipolar cells. Figure 5 (top) shows that the antisense probe directed against the goldfish cone CNG channel consistently labeled bipolar cells that have large, bulbous synaptic terminals (101 of 102 large-terminal bipolar cells were stained by the antisense probe). By contrast, no labeling was observed in large-terminal bipolar cells incubated with the sense probe (Fig. 5, bottom). Retinal horizontal cells, which were also found in the isolated cell preparations, did not express gfCNG3: hybridization of the antisense probe was detected in only 1 of 60 horizontal cells examined. The labeling in bipolar cells was predominantly localized to the perinuclear region of the soma and was less intense in the dendrites, axon, and synaptic terminal. Because the large-terminal bipolar neurons are on bipolar cells (see Fig. 4), the results with single-cell in situ hybridization demonstrate the expression of the cone CNG channel in at least a subset of on bipolar cells.

Besides the large-terminal bipolar cells, other bipolar cells with small terminals and isolated bipolar cell somata without axons or terminals were also observed in the dissociated retina preparation. These cells represent a mixture of on and off bipolar cells. Of this latter group, 66% (185 of 282) were labeled by the antisense probe, and the remainder were unlabeled (Fig. 5, middle). A simple explanation is that the unlabeled cells represent off bipolar cells, whereas the labeled cells represent on bipolar cells (as the large-terminal cells certainly do). Thus the cone CNG channel of goldfish retina is expressed in a subset of bipolar cells, which includes on bipolar cells.

FIG. 3. Single-cell RT-PCR identifies the CNG channel isoform expressed in cone photoreceptors from goldfish retina. Cytoplasm was collected from single, isolated cones using a patch pipette. After 2 rounds of RT-PCR (see methods), the resulting cDNA was electrophoresed in an agarose gel. Lanes 1–3 and 5–7 show results from individual cone photoreceptors, and lanes 4 and 7 show control experiments in which the reverse transcriptase was omitted. A: specific second-round primers were used, based on the known sequence of the CNG channel α subunit identified with RT-PCR from whole-retinal RNA. The expected size of the product is 1,135 bp (arrows). B: degenerate second-round primers were used, which are expected to amplify an approximately 655-bp product from transcripts of known CNG channel α subunits. In both A and B, positive bands were subcloned and sequenced to identify the expressed CNG 3 and 5.
To confirm the identity of the CNG channel expressed in bipolar cells, we carried out single-cell RT-PCR on individual, isolated bipolar cells. Experiments were restricted to large-terminal, ON bipolar cells, which were almost uniformly positive (101 of 102 cells) in the in situ hybridization experiments. Figure 6A illustrates an isolated, living bipolar of the type used for single-cell PCR. The procedure was similar to that described above for cone photoreceptors. Figure 6B shows an example of results from one such experiment. In this instance, amplified cDNA of the expected size was observed in three of five large-terminal bipolar cells, whereas control experiments in which the reverse transcriptase enzyme was omitted (−RT control) or in which no reverse-transcribed DNA was added to the PCR reactions (no DNA control) were negative. In three experiments, no bands of the expected size were observed in five −RT controls from bipolar cells, three −RT controls from cones, three no DNA controls, or in five control collections in which the pipette was lowered to a cell but extracellular fluid was collected instead of the cell. By contrast, positive bands were observed in 8 of 10 bona fide PCR experiments. [This 80% success rate is comparable to that obtained in RT-PCR experiments from single cones (see RESULTS), and thus, likely represents the level of detectability achieved under our experimental conditions.] In eight bipolar cells with positive results, sequencing confirmed that the detected cDNA was identical to the goldfish cone CNG channel. Thus single-cell PCR directly confirms that ON bipolar cells in goldfish retina express mRNA for the CNG channel of cone photoreceptors, gfCNG3.

**DISCUSSION**

Taking the first in-frame methionine downstream from a 5′ end-stop codon to represent the translation initiation site (Kozak 1996), we predict the goldfish cone CNG channel to consist of 637 amino acids, which is similar in length to the
mouse cone CNG channel (631 amino acids) (Hirano et al.
2000) and the short form of the rat cone CNG channel
expressed in olfactory epithelia (632 amino acids) (Meyer et al.
2000). As demonstrated in Fig. 1B by the alignment with the
longer α subunit of chick cone photoreceptors (735 amino
acids) (Bönigk et al. 1993), the shorter length of the goldfish
channel reflects truncation of the N-terminus, which is a known
site of alternative splicing in cone channels (Bönigk et al.
1996; Meyer et al. 2000). Despite extensive testing of the 5’
end of the cDNA, we never detected longer variants in gold-
fish, which suggests that the short form is at least the dominant
form expressed in fish retina, if not the only form.

The binding specificity of CNG channels for different cyclic
nucleotide agonists is strongly influenced by three amino acids
in the cyclic-nucleotide binding region whose side chains are
thought to interact with the purine base of the bound nucleotide
(Scott and Tanaka 1998; Scott et al. 2000). CNG channels
of photoreceptors preferentially bind cGMP over cAMP and have
phenylalanine, lysine, and aspartate residues at the three im-
portant positions (F580, K643, and D651 in the chick cone
channel shown in Fig. 1B). By contrast, the corresponding
amino acids are tyrosine, arginine, and glutamate in mamma-
lian olfactory CNG channels, which are approximately equally
sensitive to cAMP and cGMP. At the homologous positions,
the goldfish cone CNG channel is identical to other photo-
ceptor channels (F481, K544, and D552) and is thus expected
to be preferentially activated by cGMP. This in turn implies
that the relevant internal messenger controlling the channel in
bipolar cells is cGMP instead of cAMP.

What signal pathways might alter cGMP levels and hence
affect cGMP-gated channels in bipolar cells? gfCNG3 is ex-
pressed in ON bipolar cells in goldfish retina, which suggests
that this channel isoform could mediate the synaptic action of
glutamate in ON cells (Nawy and Jahr 1990, 1991; Shiells and
Falk 1990). In this pathway, the metabotropic glutamate recep-
tor, mGluR6, may be coupled via a G protein to phosphodies-
erase, which lowers cGMP concentration in the bipolar cell
soma, mGluR6, may be coupled via a G protein to phosphodies-
erase, which lowers cGMP concentration in the bipolar cell
tor, mGluR6, may be coupled via a G protein to phosphodies-
erase, which lowers cGMP concentration in the bipolar cell
x. This work was supported by National Eye Institute Grant EY-13251.

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J Neurophysiol • VOL 89 • FEBRUARY 2003 • www.jn.org