Molecular cloning, functional expression and chromosomal localization of a human homolog of the cyclic nucleotide-gated ion channel of retinal cone photoreceptors

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Abstract **We have cloned from human retina a cyclic nucleotide-gated (CNG) ion channel that is distinct from the one found in rod photoreceptors. This channel protein is highly homologous to the CNG channel that recently has been cloned from bovine testis and kidney and has been shown to be present in retinal cone photoreceptors. When expressed in human embryonic kidney cells, the protein forms functional ion channels with properties broadly similar to those described for the cloned bovine channel. The gene for this channel resides on chromosome 2.**

Key words: Cyclic nucleotide-gated channel; cGMP; Cone photoreceptor; Visual transduction; Human gene

1. Introduction

Cyclic nucleotide-gated (CNG) channels are a recently recognized family of ion channels [1–4]. The first member was identified as a key component of visual transduction in retinal rod photoreceptors [5–7]. Subsequently, similar ion channels were found in retinal cone photoreceptors [8] and olfactory receptor neurons [9]. It now appears that these ion channels are not confined to sensory receptor neurons, but are present in other neural and non-neural tissues as well. At the same time, new members of the family continue to emerge (for review, see [3]).

The cDNAs encoding the CNG channels involved in visual and olfactory transductions have been cloned from several vertebrate species [10–18]. It is now clear that the native channels are composed of at least α- and β-subunits. The α-subunit can form homo-oligomeric channels that are activated by cyclic nucleotides. In contrast, the β-subunit cannot form functional homo-oligomeric channels, but confers specific functional properties to the channel complex when co-expressed with the α-subunit [19–25]; but see [26].

The rod, cone and olfactory channels, which are the most studied members of the CNG channel family, show certain differences in properties. Apart from being different in the half-activation constant and the relative cGMP and cAMP efficacies (mostly between the visual and olfactory channels), the channels also have different Ca^{2+} permeabilities; namely, the cone and olfactory channels are more permeable to Ca^{2+} than the rod channel [27–29]. In addition, the native rod and olfactory channels are inhibited by Ca^{2+}-calmodulin [30–32]. For the olfactory channel, this inhibition involves direct binding of Ca^{2+}-calmodulin to the α-subunit [33] whereas the inhibition of the rod channel depends on Ca^{2+}-calmodulin binding to the β-subunit [34,22]. So far, the effect of Ca^{2+}-calmodulin on the native cone channel has only been studied in fish, and no modulation was found [35]. However, the cloned bovine cone channel has recently been reported to form homo-oligomeric channels that can be modulated by Ca^{2+}-calmodulin [25]. This finding suggests that there may be species variations in the properties of these channels.

In this report, we describe the cloning of a cDNA that appears to encode the human ortholog of the bovine cone channel. The knowledge of the sequence and gene location will provide a useful basis for examining human diseases involving this channel gene. In the retina, mutations in the rod CNG channel have been shown to lead to certain cases of autosomal recessive retinitis pigmentosa [36]. At the same time, we were curious to know whether the human cone channel (α-subunit) is sensitive to Ca^{2+}-calmodulin as reported for the bovine homolog.

2. Materials and methods

2.1. cDNA clone isolation

Poly(A)$^+$-RNA was isolated from human retina with an oligo(dT)-selection method (Micro-FastTrack, Invitrogen). Oligo(dT)-primed cDNA was then synthesized using the SuperScript™ Choice System (Life Technologies). Degenerate primers corresponding to several conserved regions of known CNG channels were used to amplify specific cDNA fragments. Two sets of primers, corresponding to the amino acid sequences IIIHWN and IFQDCEA, and MISNMN and IFQDCEA, respectively, generated products of the appropriate sizes; these were subcloned into pCRII (TA cloning kit, Invitrogen) and sequenced. Two types of sequence were obtained: one was identical to part of the human rod CNG channel α-subunit cDNA [13] and the other was different. The latter was used to screen an adult human retinal cDNA library in λgt10. One positive clone was obtained after screening 2×10^5 recombinants (0.2×SSC, 0.1% SDS, 42°C, 30 min), and was used to rescreen the same library. Five more positive clones were obtained after screening 1×10^6 recombinants. The insert of the longest clone (3.7 kb) was subcloned into pBluescript and sequenced. Its sequence was compared to those of known CNG channels and found to lack the 5′-end. The missing 5′-end was amplified using adaptor primers provided in the Marathon cDNA amplification kit (Clontech) and the gene-specific primers TATAGAAGACAGGCAGGGCGATGG and TGTAATGCTGCCACAGCCTGTTGG (corresponding to amino acids 174–181 and 236–243, respectively, in the final clone). The resulting fragment of 438 bp was ligated to the partial clone using a unique *Bfrl* site. The final clone, with an insert of 4.1 kb, was sequenced on both strands. For eukaryotic expression, a recombinant plasmid was constructed by inserting a *Hin*dIII fragment that contained the full coding region and part of the 5′-untranslated region into the polylinker site of pCIS (Genentech) [11].

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Abbreviations: CNG channel, cyclic nucleotide-gated channel; PCR, polymerase chain reaction; NIGMS, National Institute of General Medical Sciences

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2.2. Chromosomal localization

DNAs from human-rodent somatic cell hybrids (NIGMS human/rodent somatic cell hybrid mapping panel, purchased from a core facility at Johns Hopkins School of Medicine) were analyzed by PCR. DNA (100 ng) was incubated with 5 pmol of the primers CAGACTTACACAAGACAG and GACTCATCTTTGGGTAATTTGC and 1 U of Taq DNA Polymerase (Boehringer Mannheim, Germany) in 25 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. After 35 cycles (60 s at 94°C, 60 s at 55°C and 120 s at 72°C per cycle; GeneAmp PCR 9600 System, Perkin Elmer), the PCR products were analyzed by agarose gel electrophoresis. Human genomic DNA was used as a positive control, and mouse and Chinese-hamster-ovary (CHO) cell genomic DNA were used as negative controls.

2.3. Expression and electrophysiological characterization

Transient expression was performed by transfecting the cloned cDNA into human embryonic kidney (HEK) 293 cells (American Type Culture Collection) using the calcium-phosphate method [37,38]. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a humified atmosphere containing 5.5% CO_2. Recordings from excised, inside-out membrane patches [39] were made with an EPC9 amplifier and the PULSE-/PULSE-FIT software package (both from HEKA, Germany, distributed by Instrutech, New York). Single-channel data were analyzed with the software TAC (SKALAR Instruments, distributed by Instrutech, New York). Both programs were run on a Macintosh Quadra 650 computer. Macroscopic current was filtered at 2.9 kHz (4-pole Bessel filter) and sampled at 3 kHz; single-channel current was filtered at 2.9 kHz and sampled at 25 kHz.

The patch pipettes were fabricated from borosilicate glass capillaries. For mascrocopic currents, the patch pipettes had a tip lumen diameter of 1.5–2 μm and a resistance of 2–4 MΩ. For single-channel recordings, the pipettes were coated with sticky wax (Kerr, Emeryville, CA) and had a tip lumen diameter of less than 1 μm and a resistance of 5–7 MΩ.

For most experiments, the pipette and the bath solutions both contained 140 mM NaCl, 5 mM KCl, 2 mM EGTA (ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid) and 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid])/NaOH, pH 7.4. When calmodulin was tested, the bath solution contained 50 μM buffered free Ca^{2+}, achieved by substituting 2 mM nitrilotriacetic acid and 704 μM $CaCl_2$ for the EGTA. When the effect of extracellular divalent cations was examined, the pipette solution contained one of the following: (i) 2 mM $CaCl_2$/1 mM $MgCl_2$, (ii) 50 μM buffered free Ca^{2+} (see above), or (iii) 1 mM EGTA with zero added $CaCl_2$ and $MgCl_2$. cGMP or cAMP was added to the bath solution as needed.

A solenoid-controlled rotary valve system [40] was used to change solutions; the change was usually complete within 2 s. All experiments were performed at room temperature (20–25°C).

3. Results and discussion

PCR amplification with degenerate primers corresponding to conserved sequences of known CNG channels was performed on human retinal cDNA. Subsequent screening of a human retinal cDNA library yielded a clone distinct from the human rod CNG channel α-subunit cDNA [13]. Its sequence predicts a 695 amino acid polypeptide (Fig. 1) with a calculated molecular weight of 78.9 kDa, and has motifs similar to those of other CNG channels. Its sequence shows 85% identity (91% similarity based on conserved residues) to the recently published sequence for the bovine cone CNG channel [17,18], with the differences mostly in the N- and C-termini. This close homology suggests that our cDNA clone probably represents the human ortholog of the bovine cone channel. Its identities to the chicken cone [16] and human rod [13] CNG channel α-subunit cDNAs are 76% (86% similarity) and 62% (75% similarity), respectively.

To identify the chromosomal localization of the gene, PCR

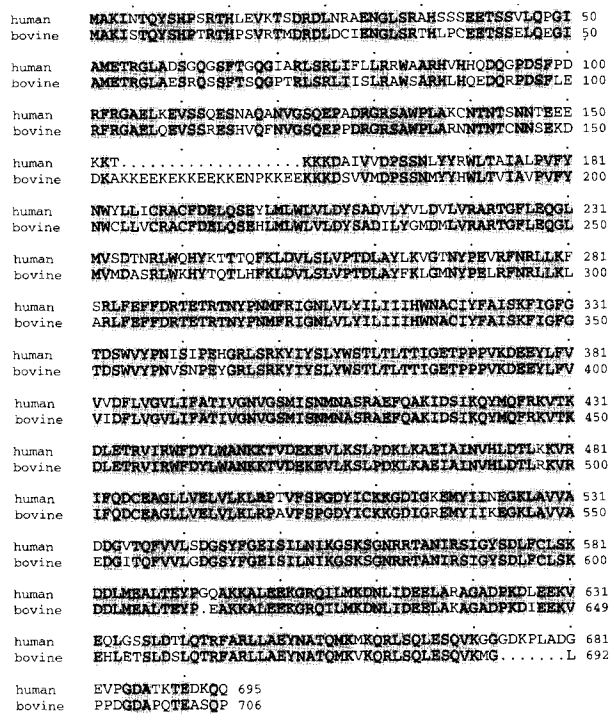

Fig. 1. Amino acid sequence comparison between the bovine cone channel and the human homolog. Identical residues are shaded. Gaps were introduced to produce optimal alignment. Alignment was made using the GCG package (Wisconsin).

was performed on the DNAs from human-rodent somatic cell hybrids. Gene-specific primers amplified fragments of similar sizes from the DNA of a single hybrid and from human genomic DNA, consistent with the gene residing on chromosome 2 (data not shown). No fragments were generated with mouse and CHO cell genomic DNA.

To check functional expression, we transiently transfected HEK 293 cells with the cloned cDNA. Two to 3 days after transfection, inside-out membrane patches were excised from the cells and found to be sensitive to bath-applied cyclic nucleotides. With cGMP as ligand, the current showed a half-activation constant ($K_{1/2}$) of 18.1 ± 3.4 μM cGMP at −60 mV and a Hill coefficient of 2.1 ± 0.2 (mean ± SD, $n = 9$) (Fig. 2a, ■). The current evoked by cAMP was typically about 10-fold smaller than the cGMP-activated current; the corresponding $K_{1/2}$ and Hill coefficient at −60 mV were 1322 ± 54 μM and 1.5 ± 0.3 ($n = 4$), respectively (Fig. 2a, ○ and △). These characteristics are similar to those reported for the cloned bovine cone CNG channel [17,18].

Macroscopic current-voltage (*I-V*) relations were measured in either the absence or the presence of extracellular divalent cations. All recordings were made at a saturating concentration (1 mM) of cGMP. When divalent cations were absent on both sides of the membrane, the *I-V* relation was almost linear (Fig. 2b). In the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+} on the extracellular side, which approximated physiological conditions, the *I-V* relation showed strong outward rectification (Fig. 2c). At a lower concentration of extracellular divalent cations, such as 50 μM Ca^{2+}, some inward rectification was also observed (Fig. 2d). These results are again similar to those for the cloned bovine cone channel [18,29].

The drug L-*cis*-diltiazem [6] blocks the native retinal CNG

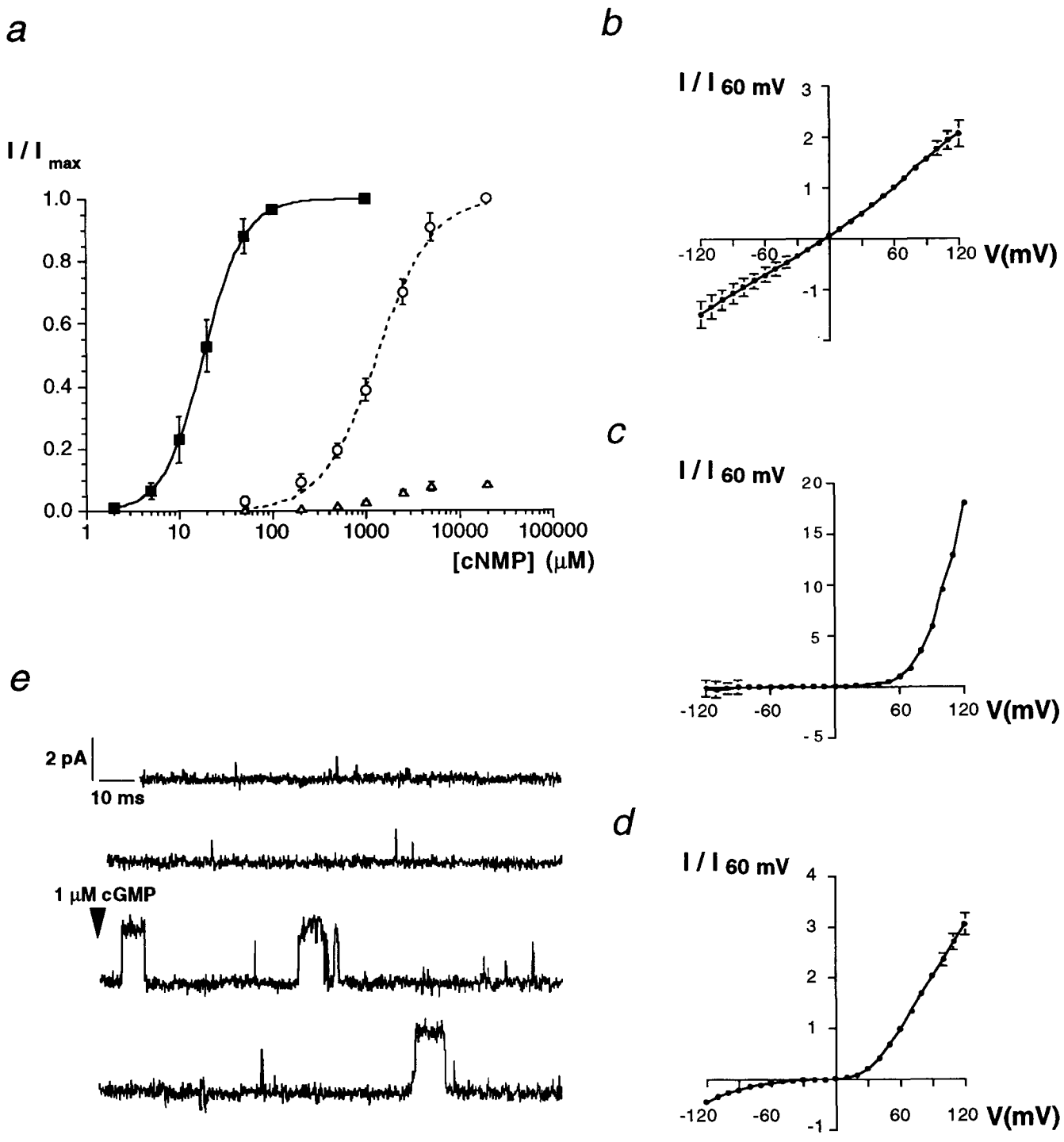

Fig. 2. Electrophysiological properties of homo-oligomeric channels formed by the human protein. (a) Dose-response relations at −60 mV for cGMP (■, $n=9$) and cAMP (△, $n=3$, with current indicated as a fraction of the maximal cGMP-activated current; ○, $n=4$, with maximal current scaled to the same height as the cGMP-activated current). Averaged data are shown. Curve fits were drawn according to $I/I_{max} = C^n/[C^n+K_{1/2}{}^n]$ where I/I_{max} is the normalized current, C the concentration of cyclic nucleotide, $K_{1/2}$ the half-activation constant and n the Hill coefficient. See text for fitted $K_{1/2}$ and n values. (b–d) Macroscopic current-voltage relations measured at a saturating cGMP concentration (1 mM) in the absence of divalent cations (b), or in the presence of extracelluar 50 μM Ca^{2+} (c) or 2 mM Ca^{2+} and 1 mM Mg^{2+} (d). Each point in the *I-V* relation was normalized with respect to the current at +60 mV and is averaged from two to six experiments. Vertical bars indicate SD. The mean value for the current at +60 mV was 650 ± 964 pA for (b) ($n=6$), 717 ± 844 pA for (c) ($n=2$) and 3.4 ± 0.3 pA for (d) ($n=2$). Background currents ranged from 1 to 27 pA (+60 mV) and are already subtracted. Points are connected by straight lines. (e) Single-channel currents induced by 1 μM cGMP at +60 mV in the absence of divalent cations (bottom two traces).

channels. When applied from the cytoplasmic side at positive membrane potentials, half-maximal inhibition occurs at ~1 μM for the native rod channel [41–44] and ~10 μM for the native cone channel [42]. In experiments with our expressed protein, 10 μM L-*cis*-diltiazem had no effect on the cGMP-activated current at +60 mV (3 experiments, data not shown).

Previously, it was found that L-*cis*-diltiazem at as high as 100 μM blocked the cloned bovine cone channel by only 17%, though the applied membrane potential was not reported [18]. The high sensitivity of the native rod channel to L-*cis*-diltiazem is now known to be conferred by a β-subunit coupled to the α-subunit [19,22]. The same may be true for the native cone channel [25].

We have also investigated the effects of the modulatory protein, calmodulin. Ca^{2+}-calmodulin strongly inhibits the native olfactory channel by binding to its α-subunit [32,33], and also inhibits the native rod channel by binding to its β-subunit [30,31,34,22]. On the other hand, the native cone channel, at least from catfish [35], is not modulated by Ca^{2+}-calmodulin. Consistent with this last result, we have found that calmodulin up to 1 μM did not affect the cGMP-activated current of our expressed protein in the presence of 50 μM Ca^{2-}. Surprisingly, the homo-oligomeric channels formed by the cloned bovine homolog apparently show a sensitivity to calmodulin, with the $K_{1/2}$ for cGMP activation increasing by up to 3-fold in the presence of Ca^{2+}-calmodulin [25].

Sample single-channel recordings are shown in Fig. 2e. We observed both brief (≤1 ms) and considerably longer (~10 ms or longer) openings. The brief openings were also detected in the absence of cGMP, but not in mock-transfected cells. These may have resulted from a very low residual cGMP concentration in the bath solution in our experiments, or from spontaneous channel openings which have been described for the native cone channel in fish [45]. At +60 mV, the single-channel conductance is ~38 pS for the long openings. For the bovine homolog, we have calculated a similar single-channel conductance based on the recent expression data of Biel and co-workers [25]; however, Weyand and colleagues [17] have reported a value of 21 pS.

In summary, we have cloned the cDNA encoding a human retinal CNG channel that appears to be the human ortholog of the bovine cone CNG channel. Its gene was found to reside on chromosome 2. The chromosomal localization will be useful in the search of candidate genes for retinal disorders. Mutations in the rod channel α-subunit have been linked to certain cases of autosomal recessive retinitis pigmentosa, characterized by a degeneration of the rod photoreceptors [36]. It may be clinically relevant to know whether mutations in the cone channel gene can lead to cone-specific diseases. Since the cone CNG channel gene is present in sperm, heart, kidney, and other tissues (for review, see [3]), mutations may also be linked to non-retinal diseases. When expressed in HEK 293 cells, the human protein revealed electrophysiological properties that were broadly similar to those described for the bovine homolog [17,18,29]. The only discrepancy is that, whereas a sensitivity to Ca^{2+}-calmodulin was reported for the bovine homolog [25], we did not find this modulation for the human channel. This difference may reflect a variation between species, even among mammals. Finally, both the human and bovine [18,25] channel homologs showed little sensitivity to L-*cis*-diltiazem, unlike the native cone channel in fish [42]. This difference suggests the presence of a β-subunit in the native cone channel complex that is responsible for the high sensitivity to L-*cis*-diltiazem [25], as is the case for the native rod channel [19,22]. Whether a cone β-subunit distinct from that in rods exists remains to be determined.

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