Molecular Cloning and Expression of a Modulatory Subunit of the Cyclic Nucleotide-gated Cation Channel

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The cDNA of three variants of a cyclic nucleotide-gated (CNG) channel modulatory subunit (CNG4c–CNG4e) has been cloned. CNG4c, CNG4d, and CNG4e differ slightly from each other within an amino-terminal sequence that was originally reported as part of the bovine retinal glutamic acid-rich protein (GARP). The core region of CNG4 is homologous to the second subunit of the human rod photoreceptor channel (hRCNC2b), suggesting that both proteins are alternatively spliced products of the bovine and human homologue of the same gene. CNG4 transcripts are present in retina, testis, kidney, heart, and brain. Expression of CNG4 in HEK293 cells did not lead to detectable currents. Coexpression of CNG4 with the principal subunit of the bovine testis CNG channel (CNG3) resulted in currents that differed in several aspects from that induced by CNG3 alone. The heterooligomeric CNG3/CNG4 channel expressed in sensory or nonsensory cells is also coassembled with a modulatory subunit. Here, we report the cloning of three variants of a cDNA encoding an additional CNG channel subunit that modulates the functional properties of the CNG3 channel.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of CNG4c–CNG4e—A 491-bp cDNA fragment corresponding to amino acids Tyr427 through His584 of the modulatory subunit of the human rod photoreceptor channel (19) was used to screen a dilog(dT)-primed bovine testis library constructed in the pDNA2 vector (Invitrogen). Five clones were obtained. The clone containing the biggest insert, pcGT4 (nucleotides (nt) 1215–3253, numbering of nucleotides according to the sequence of CNG4c) was sequenced on both strands using T7 DNA polymerase in the chain termination method. A specifically primed cDNA library was constructed in the pDNA2 vector using 5′-µg of bovine testis poly(A)+ RNA and 0.5 µg of 5-phosphorylated primer cg79r (5′-TATCCACATGCTTGG-3′, nt 1809–1826). Screening with the 254-bp HaelI-(1239)-PflMI-(1493) fragment derived from the 5′ end of pcGT4 resulted in the isolation of 15 additional clones. The three longest clones (pcGT7, pcGT9, and pcGT10) were sequenced on both strands. pcGT7 (nt 131–1823), pcGT9 (nt 79–1824), and pcGT10 (nt 73–1826) were identical over the whole overlapping sequence region with the exception of a 27-bp in-frame deletion present in pcGT10 (nt 249–275) and a 54-bp in-frame deletion present in pcGT9 (nt 229–282). The composed cDNA sequence of pcGT4 and pcGT7, pcGT9, or pcGT10, respectively, contained one large open reading frame starting directly at the 5′ end of the sequence. The missing 5′ terminus of the cDNA was cloned by the 5′ RACE technique (Life Technologies, Inc., 5′ RACE kit), essentially following the instructions of the manufacturer. Briefly, 1 µg of poly(A)+ RNA was reverse-transcribed using primer cg79r. The single-stranded cDNA was tailed using dCTP and terminal transferase and PCR-amplified using the Life Technologies, Inc. anchor primer and a nested primer (cg74r, 5′-GTA-ACTGTCTCCCAGCCTACC-3′, nt 1504–1524). The PCR profile was as follows: initial denaturation for 5 min at 95 °C; 35 cycles of 94 °C 1 min, 52 °C 1 min, 72 °C 1.5 min; and a final extension at 72 °C for 5 min. PCR fragments > 500 bp were cloned into Smal-cut pUC18 vector. About 1000 colonies were hybridized with the 254-bp HaelI-(1239)-PflMI-(1493) fragment derived from the 5′ end of pcGT4. The three longest clones obtained, pRACE2 (nt 39–1524), pRACE4 (nt 10–1524), and pRACE12 (nt 1–1524), were completely sequenced on both strands. All three clones were identical with pcGT10 in the overlapping region and contained an additional 5′-sequence, including a consensus initiation ATG preceded by an in-frame stop-codon. Other five 5′ RACE clones

Cyclic nucleotide-gated (CNG) channels comprise a class of nonselective cation channels that are directly and cooperatively activated by the binding of cGMP or cAMP (1, 2). Originally, CNG channels have been characterized in sensory cells by electrophysiological techniques and molecular cloning (3, 4). In vertebrates, three different genes encode CNG channels. CNG1 is expressed in rod photoreceptors (5–8), CNG3 in cone photoreceptors (9–12), and CNG2 in olfactory neurons (13). There is solid evidence that expression of CNG3 channels is not restricted to sensory cells. Electrophysiologically, CNG3 is expressed both in sensory and nonsensory cells (14). CNG3 channels have been detected in photosensitive cells of pineal glands (14), in retinal bipolar cells (15, 16), and in some retinal ganglion cells (17). PCR amplification has identified partial sequences of CNG1–3 channels expressed in sensory or nonsensory cells (18). In addition, a functional olfactory-like channel (rACNG) has been cloned from rabbit aorta (20). Furthermore, mammalian CNG3 was originally cloned from bovine kidney (21) and testis (9).

Additional subunits (referred to as β subunit or subunit 2) have been cloned from the rod outer segment (hRCNC2a and hRCNC2b, Ref. 22) and from olfactory neurons (rOCNC2, Refs. 23 and 24). These new subunits do not form functional channels by themselves. They coassemble with CNG1 and CNG2 to form heterooligomeric complexes and induce channel properties that are present in the native channels but are absent from the channels formed by the CNG1 and CNG2 homooligomers (22–24). The "new" but physiological properties include activation of the channel by cAMP at physiologically observed concentrations, single-channel flickering, and block of the current by L-cis-diltiazem. It was unknown whether or not the CNG3 channel expressed in sensory or nonsensory cells is also coassembled with a modulatory subunit. Here, we report the cloning of three variants of a cDNA encoding an additional CNG channel subunit that modulates the functional properties of the CNG3 channel.
were analyzed and overlapped completely with the sequence of either pGCT7, pGCT9, or pGCT10.

PCR Analysis—CNG3- and CNG4-specific sequences were amplified in PCR reactions containing 10 ng oligo(dt)-primed cDNA of bovine and rat tissues and primer pairs as follows: primer pair cng208f (5'-ATC- TACTTGCAGCA(G/TAC)TT-3') and cng21r (5'-GACTC-TGGCTTCCTGA(T/A)TT-3') corresponding to the peptides Y124PFAISKET and N129KGSK522y of CNG3 (21), respectively, was used to amplify a 722-bp CNG3-specific fragment. Primer pair cng205f (5'-TCACCTAGGTGTGA(T/G)TA(G/TAC)TT-3') and cng207r (5'-GCTGTGATGAT(G/A)TACAT(T/C)TC-3') corresponding to the peptides T4350SHVYDDEG and E656MYIIQA of CNG4c, respectively, was used to amplify a 554-bp CNG4-specific fragment. PCR products were essentially as described for 5'RACE.

Northern blot Analysis—Poly(A) RNA (10 μg) isolated from each of the tissues indicated was size-fractionated on a 1.5% agarose gel, transferred to Hybond-N membranes (Amersham Corp.) and cross-linked by ultraviolet light. Three 32P-labeled probes were used: a 274-bp fragment derived from CNG4c (probe A; nt 106–379), a 308-bp fragment derived from CNG4c (probe B; nt 431–739), and a 127-bp fragment specific for GARP (probe C; nt 1776–1902 of GARP; Ref. 26). Hybridization was at high stringency starting with probe A. After autoradiographic exposure the probe was stripped off by washing the filter for 30 min in 0.1xSSC, 0.1% SDS at 95 °C. Subsequently the filter was rehybridized with probe B. After exposure, the stripping procedure was repeated and the filter was probed with probe C.

Construction of Expression Vectors CNG4c–CNG4e/CMV and Expression in HEK293 Cells—For transient expression in HEK293 cells the recombinant plasmids pCNG4c-pCNG4e-CMV were constructed by inserting cDNA fragments representing the complete coding region of CNG4c-CNG4e (nt 43–2946) into the polylinker site of the pcDNA3 vector (Invitrogen). pCNG4c-pCNG4e-CMV were used for transient expression using the calcium phosphate method either alone or in combination with equimolar amounts of plasmid pcGK26-CMV (21) encoding the bovine CNG3 channel. When CNG4d was expressed without CNG3 a small amount (0.10 of the molar amount of CNG4) of the green fluorescent protein (GFP, Ref. 26) cloned in the pcDNA3 vector was coexpressed. Only cells showing green fluorescence were used for electrophysiological experiments.

Electrophysiological Recording—Electrophysiological experiments were performed with HEK293 cells 2–4 days after transfection with the respective cDNAs. The current was recorded from inside-out patches using a List EPC 7 amplifier and pclamp software (Axon Instruments) running on an IBM-compatible computer. The bath and pipette contained a Ca2+-free Ringer solution composed of (mM): 140 NaCl, 5 KCl, 10 HEPES, 1 EGTA (pH 7.4). The Ca2+-free bath contained (mM): 140 NaCl, 5 KCl, 10 HEPES, 0.704 CaCl2, 2 sodium nitroprusside (pH 7.4). This buffer yielded a free Ca2+ concentration of 50 μM (27) and was used in combination with calmodulin. cGMP (Sigma) was directly dissolved in the bath solutions before the experiments. A 10 mM stock solution of t-cis-diltiazem was prepared in water. Calmidazolium and pimozide were dissolved at 10 mM in dimethyl sulfoxide (Me2SO). Calmodulin purified from porcine brain (28) was dissolved at a concentration of 2.5 mg/ml in a buffer containing 100 mM Tris and 1 mM CaCl2 (pH 7.4). If not stated otherwise, the inside-out patch was held at 0 mV and stepped to ±105 mV for 150 ms in increments of 15 mV every 5 s. The currents were corrected for leak current and capacitative artifacts by subtraction of the currents recorded in the absence of cGMP. The magnitude of the current was measured 10–20 ms before ending the voltage pulse. A multibarreled pipette, which was placed within 200 μm of the patch, allowed rapid and complete solution changes. Calmodulin was applied for 1 min and the other compounds were perfused for at least 30 s before starting an experiment. Single-channel recordings were made from excised inside-out patches with symmetrical Ca2+-free solution in the bath and pipette were sampled at 10 kHz and filtered at 2 kHz. Experiments were carried out at room temperature (22 °C ± 1 °C). All results are given as mean ± S.E.

RESULTS

Primary Structure of CNG4—To identify modulatory subunits associated with CNG3, we screened cDNA libraries from bovine testis which expresses CNG3 transcripts at a high density (9, 19, 21) with a probe from the β subunit of the human rod photoreceptor channel (hRCNC2b). We identified three splice variants of a common primary transcript designated as CNG4c, CNG4d, and CNG4e. The nucleotide sequence of the three variants, CNG4c, encodes for a protein of 939 amino acid residues with a calculated molecular mass of 104,474 daltons (Fig. 1). The length of the cloned sequence is in good agreement with the size of the mRNA as detected in Northern analysis (Fig. 2B). The cDNAs of CNG4d and CNG4e are identical with CNG4c with the exception of 27- and 54-bp deletions in the amino-terminal region. The deduced proteins of CNG4d (930 residues) and CNG4e (921 residues) have calculated molecular masses of 103.602 and 102,700 daltons, respectively. Analysis of several clones as well as sequencing of PCR fragments obtained with primers flanking the variable sequence (data not shown) revealed that CNG4d is by far the most frequent form (CNG4d:CNG4c:CNG4e = 20:2:1) in bovine testis. No evidence was obtained that testis expresses a CNG4e variant with a truncated amino terminus as reported for the short form of the rod photoreceptor β subunit hRCNC2a (22).

The sequence from residues 116–999 of CNG4c is highly homologous to the sequence of hRCNC2b. By contrast, the N114-terminus of CNG4c shows only minor homology to hRCNC2b. The major part of this divergent sequence (residues 10–115) is present in a 65-kDa glutamic acid-rich protein (GARP) that has been cloned from bovine rod outer segment (25). Within the common region of 320 bp the cDNAs of CNG4c and GARP differ only at three positions. These differences most likely reflect an individual sequence polymorphism since they were also present in a partial GARP sequence amplified from bovine testis mRNA with GARP-specific primers (data not shown). The deletions present in CNG4d and CNG4e are localized within the 320-bp GARP sequence. PCR analysis indicated that at least the 27-bp deletion giving rise to CNG4d is also represented in the GARP-specific cDNA (data not shown).

Tissue Expression of CNG4—Northern blot hybridizations were performed with probes specific for the 320-bp common sequence (Fig. 2A, probe A), for CNG4 (probe B), and GARP (probe C). In bovine testis probes A and B detected two major mRNA species of 3.3 and 3.5 kb (Fig. 2B) corresponding to the length of the cloned sequence. No signal was observed with the GARP-specific probe C, even after long exposure. However, GARP fragments corresponding to the carboxyl-terminal half of the protein could be amplified by PCR from bovine testis cDNA (data not shown), indicating that low amounts of GARP mRNA, which were not detected by Northern analysis, are present in testis. The CNG4-specific probe B detected a single mRNA species of 7.5 kb in bovine retina (Fig. 2B). Probe A hybridized with the same 7.5-kb species and in addition with a 4.4-kb mRNA. The 4.4-kb mRNA was selectively recognized by the GARP-specific probe C, indicating that unlike testis, bovine retina contains significant amounts of GARP-specific transcripts.

Northern analysis with probes A and B identified CNG4 transcripts only in testis and retina. To detect possible low level expression of channel subunits, we performed PCR reactions with degenerate primers deduced from the core regions of CNG3 and CNG4. Specific fragments were obtained from the cDNA of several bovine and rat tissues (Fig. 2C). The sequences of rat CNG3 and CNG4 are highly homologous to their bovine counterparts with nucleotide sequence identities of 89 and 90%, respectively. CNG3 could be detected in bovine retina, kidney, and heart as well as in rat kidney, heart, and brain in line with previous results (21). By contrast, CNG4 was present in all rat tissues tested but was absent from bovine kidney and heart mRNA.

Formation of Functional Heterooligomeric Channels of CNG3 and CNG4—To determine whether CNG4c-CNG4e subunits could form functional CNG channels, we transiently transfected HEK293 cells with expression vectors containing the...
The expressed CNG4d protein was detected by an antibody and migrated on a SDS gel with an apparent molecular mass of 150 kDa (data not shown). This apparent molecular weight is considerably larger than the mass of 104 kDa calculated from the cDNA sequence. A similar discrepancy has been observed with hRCNC2b (29). The cells expressing the CNG4d protein never had any cyclic nucleotide-activated current in excised inside-out patches. However, the three CNG4 variants modified the properties of the current induced by CNG3 alone and restored some of the physiologically observed properties of the mammalian and amphibian rod and cone photoreceptors (1, 30). The currents induced by the 1:1 coexpression of CNG3 with CNG4c or CNG4e showed essentially the same properties as the current induced by CNG3 (data not shown). As discussed above the splice variant CNG4d is the major expressed isoform in testis. Therefore, detailed analysis were only carried out with the CNG4d isoform. The results of most experiments were confirmed with the other two isoforms.

CNG4d weakened the outward rectification observed in the presence of 2 mM extracellular Ca\(^{2+}\) with the homooligomeric CNG3 channel (data not shown) and induced single-channel flickering (Fig. 3D). Furthermore, CNG4d increased the affinity of the channel for cyclic nucleotides (Table I). The increase in affinity was more prominent with cAMP (6-fold decrease of \(K_i\) at +60 mV) than with cGMP (1.5 fold decrease of \(K_i\)). Interestingly, the high degree of cooperativity found for the activation of the CNG4d channel was blocked by \(\text{l-cis-diltiazem}\) with a half-maximal inhibition (IC\(_{50}\)) of 10 \(\mu\)M (Fig. 3A), whereas the homooligomeric CNG3 channel was affected only marginally by 100 \(\mu\)M \(\text{l-cis-diltiazem}\) (Fig. 3B). The IC\(_{50}\) value of the heterooligomeric channel is in the range of IC\(_{50}\) values reported for native cone photoreceptors (30), whereas the native (31) and the heterologously expressed rod channel (22) is about 10 times more sensitive for \(\text{l-cis-diltiazem}\). Like the native rod and cone channels the CNG3/CNG4d channel was blocked by \(\text{l-cis-diltiazem}\) in a time- and voltage-dependent manner (Fig. 3C). The block was more effective at positive than at negative membrane potentials (Fig. 3C).

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![Fig. 1. Primary structure of bovine testis CNG4c–CNG4e. A, scheme of CNG4c. The open box represents the amino acid sequence with the amino terminus on the left and the carboxyl terminus at the right. Putative transmembrane domains (1–6), the pore region (P), and the binding site for cGMP are indicated. The sequence common with GARP is shown as a gray box. B, nucleotide sequence, deduced amino acid sequence of CNG4c, and its alignment with subunit 2 of human retinal rod cGMP-gated cation channel (hRCNC2b). The nucleotide sequence was assembled from clones pRACE12, pcGT7, and pcGT4. Amino acids identical between CNG4c and hRCNC2b are boxed. Gaps in the sequence of hRCNC2b are represented by dashes. At positions 15 and 750 of hRCNC2b stretches of 15 and 2 glutamic acid residues, respectively, are indicated by indices. The putative transmembrane domains, the pore region, and the cGMP binding site are shown by lines below the amino acid sequences. The sequence missing in CNG4d and CNG4e is illustrated by black and gray boxes, respectively. The stop codons preceding the initiation ATG and at the end of the open reading frame of CNG4c, respectively, are double-underlined. The sequence of CNG4c, which is also present in GARP, is marked by arrows. The sequences flanking the 5′ and 3′ end of the common region in GARP are shown in minuscule over the nucleotide sequence of CNG4c. Three nucleotide exchanges with respect to the published sequence of GARP, which were found both in GARP itself and CNG4c, are also shown in minuscule over the sequence.
and 3′-untranslated sequences (5′-UTR and 3′-UTR) are shown as horizontal lines. The sequence common to both proteins is indicated in black. Probes A–C are shown as black lines below the proteins. B, Northern analysis of CNG4 and GARP expression. Ten micrograms of poly(A)+ RNA of bovine testis and retina RNA was used in each lane. The same blot was subsequently hybridized with probes A, B, and C as described under “Experimental Procedures.” Autoradiographic exposure at −70 °C was 30 h for testis and 3 h for retina. With probe C, no signal was seen in testis even after an exposure for 5 days. C, PCR amplification of CNG3- and CNG4-specific sequences. For each tissue two separate PCR reactions were performed using either CNG3- or CNG4-specific primer pairs as described under “Experimental Procedures.” One-tenth of a CNG3- and CNG4-specific amplification reaction was combined and separated on a 5% polyacrylamide gel. The identity of the amplified sequences was ascertained by sequencing of the respective bands.

Effect of Ca2+-calmodulin on Homo- and Heterooligomeric Channels—Previous work with olfactory (27) and rod photoreceptor channels (32) showed that these channels are modulated by Ca2+-calmodulin. The homooligomeric CNG3 channel and the heterooligomeric CNG3/CNG4d channel are regulated in a similar way by Ca2+-calmodulin (Fig. 4). Twenty µM cGMP induced normal current in the presence of 50 µM Ca2+ in the bath solution (Fig. 4, upper panels). The addition of 230 nM calmodulin and 50 µM Ca2+ to the intracellular side of the patch reduced the current amplitude of the heterooligomeric CNG3/CNG4d channel and the homooligomeric CNG3 channel by 80% at 20 µM cGMP (Fig. 4, lower panels) but not at a saturating cGMP concentration of 300 µM (data not shown). The decrease in current amplitude was caused by a Ca2+-calmodulin-dependent increase of the apparent Kd values for cGMP from 12.4 to 25.8 µM and from 18.1 to 60.3 µM for the CNG3/CNG4d- and CNG3 channel, respectively (Table I). The Hill coefficients for activation of the channels by cGMP were not changed by Ca2+-calmodulin (Table I), suggesting that Ca2+-calmodulin did not affect the cooperativity of cGMP-dependent activation of the channels.

Potentiation of the Channel Block by Ca2+-calmodulin—Previous work showed that several drugs, including l-cis-diltiazem, pimozone, and calmidazolium, block the native photoreceptor and olfactory channels in the micromolar range (33–35). These compounds have multiple effects and inhibit activation of several calmodulin-regulated enzymes. It was possible that calmodulin affected also the channel block by these substances. Therefore, we tested the effect of these substances on CNG3/CNG4d and CNG3 channel-mediated current in the absence and presence of calmodulin (Fig. 5). These experiments were carried out in the presence of 300 µM cGMP, which activates maximally the channels. Calmodulin itself had no effect on the current at this cGMP concentration, l-cis-Diltiazem (10 µM), calmidazolium (3 µM), and pimozone (10 µM) blocked the current through the CNG3/CNG4d channel by 72, 42, and 12% at +60 mV in the absence of calmodulin (Fig. 5A). Application of the drugs together with 230 nM Ca2+-calmodulin increased significantly the block to 92, 90, and 62%. The block induced by l-cis-diltiazem either alone or in combination with Ca2+-calmodulin was more pronounced at positive membrane potentials whereas the block produced by calmidazolium and pimozone revealed no voltage dependence. Since Ca2+-calmodulin did not affect the current at a saturating cGMP concentration, the enhanced block cannot be explained by the sum of the block induced by the drug plus that induced by Ca2+-calmodulin. Therefore, the results suggest that calmodulin potentiated the channel block.

The same experiments were repeated with the homooligomeric CNG3 channel. Pimozone blocked the CNG3-mediated current to the same extent as that through the heterooligomeric CNG3/CNG4d channel (Fig. 5B). l-cis-Diltiazem reduced
Modulatory Subunit of Cyclic Nucleotide-gated Channel

TABLE I

Apparent $K_a$ values and Hill coefficients ($n$) of CNG3/CNG4d and CNG3 currents activated by cGMP and cAMP in the absence and presence of 50 $\mu$M Ca$^{2+}$ and 230 nM calmodulin.

| MP       | CNG3/CNG4d | CNG3 |
|----------|------------|------|
| mV       | $K_a$ ($\mu$M) | $n$ | $K_a$ ($\mu$M) | $n$ |
| cAMP -Ca$^{2+}$/CaM | 432 ± 20 | 1.6 ± 0.1 | 10 | 1860 ± 28 | 4.3 ± 0.3* |
| +cAMP | 314 ± 40 | 1.3 ± 0.1 | 10 | 1920 ± 25 | 4.2 ± 0.2* |
| cGMP -Ca$^{2+}$/CaM | 12.6 ± 0.9 | 2.0 ± 0.2 | 10 | 225 ± 2.5 | 1.9 ± 0.4 |
| +cGMP | 12.4 ± 1.5 | 1.5 ± 0.1 | 10 | 181 ± 1.7 | 1.8 ± 0.3 |
| cGMP +Ca$^{2+}$/CaM | 32.1 ± 1.5 | 2.0 ± 0.1 | 9 | 59.3 ± 6.0 | 1.5 ± 0.2 |
| +cGMP | 25.8 ± 1.8 | 1.8 ± 0.1 | 9 | 60.3 ± 5.6 | 1.5 ± 0.2 |

*Values are from Biel et al. (21).

![Fig. 4. Inhibitory effect of Ca$^{2+}$-calmodulin on the heterooligomeric CNG3/CNG4d channel and the homooligomeric CNG3 channel.](http://www.jbc.org/)

The current through the CNG3 channel only by 5% at +60 mV in the absence of calmodulin. However, 10 $\mu$M L-cis-diltiazem blocked the current through the CNG3 channel by 83% in the presence of Ca$^{2+}$-calmodulin (Fig. 5B). This block was quantitatively similar to the block of the CNG3/CNG4d channel by L-cis-diltiazem. In contrast to the heterooligomeric channel, the block of CNG3 by L-cis-diltiazem was not voltage-dependent, indicating that CNG4d was responsible for the voltage dependence of the block of the heterooligomeric channel.

DISCUSSION

Three splice variants of a modulatory $\beta$ subunit of the CNG3 channel, CNG4c, CNG4d, and CNG4e, have been cloned from bovine testis. The COOH-terminal part of the CNG4 sequence (residues 116–939), which contains the transmembrane segments, the pore region, and the cGMP binding domain, is homologous to the second subunit of the human rod photoreceptor channel hRNCN2b (22). By contrast, the NH$_2$-terminal part (residues 1–115) reveals only weak homology to the sequence of hRNCN2b but contains a sequence (residues 10–115) that is essentially identical with a COOH-terminal portion of the retinal 65-kDa glutamic acid-rich protein (GARP, Ref. 25). The first nine residues of CNG4 are unique to this protein and show no homology to either GARP or hRNCN2b. The partial identity between GARP and CNG4 suggests that both proteins are derived from the same gene either by alternative splicing or by transcription from different promoters.

Northern analysis detected CNG4-specific transcripts of different size in tests (3.3 and 3.5 kb) and retina (7.5 kb). These transcripts might represent alternatively spliced variants of CNG4 expressed in these tissues. The full-length GARP is expressed in significant amounts only in retina. At present, the function of GARP is not known, but it is tempting to speculate that GARP and CNG4 modulate the same function in retina. CNG4-specific mRNA was detected in small amounts in several other tissues. Interestingly, bovine heart and kidney did not contain significant amounts of CNG4 transcripts, suggesting that the CNG3 channels present in these tissues are either homooligomeric channels or contain another modulatory subunit which has not been identified so far.

Similar to $\beta$ subunits of other CNG channels (22–24), each of the three CNG4 variants modulated the properties of the CNG3 channel, but failed to induce CAMP-dependent currents when expressed alone in HEK 293 cells. The modulated properties included a shift in the apparent $K_a$ values for cGMP and cAMP, single-channel flickering, and a sensitive block by L-cis-diltiazem in the absence of calmodulin. The $IC_{50}$ value of 10 $\mu$M and the time and the voltage dependence of the L-cis-diltiazem block were indistinguishable from that reported for the native cone channel (30). CNG4 did not change substantially the current-voltage relation in the absence of divalent cations. By contrast, it decreased the outward rectification of the current observed in the presence of extracellular Ca$^{2+}$, indicating that CNG4 influenced the voltage dependence of the block. These results strongly support the notion that CNG4 and CNG3 form heterooligomeric channels in HEK cells and in intact tissues.

The activation of both homooligomeric and heterooligomeric channels is modulated by Ca$^{2+}$-calmodulin. At physiological concentrations, Ca$^{2+}$-calmodulin increased about 2–3-fold the $K_a$ value for cGMP in both channels. The increase is qualitatively similar to the Ca$^{2+}$-calmodulin-dependent increase observed in native rod photoreceptor channels (32). However, in rod photoreceptor channels the effects of calmodulin required the presence of the long version of the second subunit hRNCN2b (29). Calmodulin had no effect on the homooligomeric CNG1 channel. The direct inhibition of CNG3 activation resembles the direct effect of Ca$^{2+}$-calmodulin on the olfactory ion conducting CNG2 subunit (27, 36), although calmodulin shifted the apparent $K_a$ values only by 2–3-fold compared with a 10–20-fold shift in the olfactory channel. In support of these electrophysiological results, CNG3, but not CNG1, contains a sequence in the amino terminus that is similar to the Ca$^{2+}$-calmodulin binding region identified in CNG2 (36).

The major effect of calmodulin observed was the potentiation of channel block at saturating cGMP concentrations, L-cis-Diltiazem, pimozide, and calmidazolium, which are also known to block native olfactory and photoreceptor channels (33–35), blocked the homo- and heterooligomeric channels. Most strikingly, the current induced by CNG3 alone became sensitive to
which together form a functional channel, strongly supports the notion that heterooligomeric CNG channels are not restricted to sensory cells. CNG4 transcripts were not identified in all tissues containing CNG3 mRNA. It is therefore likely that CNG3 forms homooligomeric channels in some cells as has been proposed for the olfactory (23) and the rod photoreceptor channel (37). Future investigations will be necessary to study the functional implications of these differences in subunit composition.

After submission of this manuscript the dosing and functional expression of the full-length β subunit (βRCNGCβ) from bovine rod outer segment has been reported (38). βRCNGCβ is identical with the calmodulin binding 240-kDa protein that copurifies with the rod photoreceptor CNG1 channel protein (39–41). Both CNG4 and βRCNGCβ have a bipartite structure consisting of a COOH-terminal β′-part, which is homologous to hRCNG2b, and a NH2-terminus, which is homologous to GARP. Whereas CNG4 contains only a small portion of GARP (residues 466–571), almost the complete GARP sequence (residue 1–571) is present in βRCNGCβ. In addition, βRCNGCβ lacks the NH2-terminal nine amino acids of CNG4. Within the common region CNG4 and βRCNGCβ differ at five positions: residues 26 (Gln), 827 (Ala), 833 (Ala), and 880 (Glu) are replaced in βRCNGCβ by Arg, Ser, Arg, and Asp, respectively, and residue 881 (Ala) is missing in βRCNGCβ. It is likely, that CNG4 and βRCNGCβ are splice variants of the same primary transcript. CNG4c and βRCNGCβ have been expressed together with the CNG3 and CNG1 channels, respectively. These β subunits modulate several properties of the cGMP-induced current (i.e. single channel flickering, sensitivity to l-cis-diltiazem) in a comparable manner, being consistent with the finding (38) that the GARP part of βRCNGCβ has no major influence on the electrophysiological properties of the rod CNG channel. However, the effect of the β subunit might depend on the type of α subunit which is coexpressed. Unlike the CNG1 channel, the homooligomeric CNG3 channel is inhibited by calmodulin in the absence of a β subunit. Coexpression of CNG4 does not induce an additional effect. For this reason it cannot be deduced from our experiments whether CNG4 is like βRCNGCβ a target for calmodulin. Further experiments will be necessary to identify the location of the calmodulin binding site.

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Fig. 5. Potentiation of the inhibitory effect of channel blockers by Ca2+-calmodulin. A, CNG3/CNG4d; B, CNG3. The left panels represent the current-voltage relation of individual patches perfused with 50 μM free Ca2+-solution in the absence of cGMP (●), followed by 0.3 mM cGMP (■), followed by 0.3 mM cGMP plus the indicated channel blocker (▲) and followed by cGMP plus channel blocker plus 230 nM calmodulin (▼). Dilt, 10 μM l-cis-diltiazem; Calm, 3 mM calmidazolium; Pimo, 10 μM pimozide. The steady-state effects of blockers and calmodulin were taken 30 s after application of blockers or 60 s after coapplication of blocker + calmodulin. The right panels show the extent of block in the absence (□) and presence of 230 nM calmodulin (■) at ±60 mV. ▲, currents in the absence of calmodulin and blocker. The values for current block (number of experiments/percent of normalized current at ±60 mV/percent of normalized current at +60 mV) were as follows. CNG3/CNG4d: l-cis-diltiazem (8/46.6 ± 4.3/28.4 ± 2.9), l-cis-diltiazem + Ca2+-calmodulin (8/27.4 ± 5.6/7.9 ± 1.1), calmidazolium (10/57.7 ± 7.7/58.2 ± 7.6), calmidazolium + Ca2+-calmodulin (10/12.3 ± 4.6/10.5 ± 3.6), pimozide (8/9.1 ± 7.5/88.2 ± 7.2), pimozide + Ca2+-calmodulin (8/43.2 ± 8.9/38.2 ± 8.6). CNG3: l-cis-diltiazem (7/87.7 ± 3.2/97.3 ± 4.4), l-cis-diltiazem + Ca2+-calmodulin (7/13.9 ± 5.4/17.6 ± 4.7), pimozide (6/83.1 ± 4.3/61.4 ± 5.1), pimozide + Ca2+-calmodulin (6/93.0 ± 9.6/30.3 ± 9.7). L-cis-diltiazem in the presence of Ca2+-calmodulin. In contrast to the heterooligomeric channel, the extent of the block of CNG3 by l-cis-diltiazem was almost identical at negative and positive membrane potentials, indicating that CNG4 was responsible for the voltage-dependent l-cis-diltiazem block. The molecular basis of the observed calmodulin-dependent potentiation is unclear, but probably involves a conformational change of the channel protein induced by Ca2+-calmodulin that increases the affinity of the channel for the used compounds.

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