Another member of the cyclic nucleotide-gated channel family, expressed in testis, kidney, and heart

cGMP/ion channel/cloning/calciu

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ABSTRACT Cyclic nucleotide-gated cation channels are essential in visual and olfactory signal transduction. An additional member of the cGMP-gated channel family, termed CNG-3, has been cloned from bovine kidney. Its deduced amino acid sequence is 60% and 62% identical with the CNG-channel proteins from bovine rod outer segment and bovine olfactory epithelium, respectively. Northern analysis and sequences amplified by the PCR showed that the CNG-3 mRNA is present in testis, kidney, and heart. Calcium permeated the expressed channel in the presence of extracellular Mg²⁺ and Na⁺ at membrane potentials from −100 to +45 mV. It is likely that CNG-3 protein is responsible for cGMP-induced Ca²⁺ entry in cells other than sensory cells.

Cyclic GMP is a key regulatory molecule of specific cellular functions such as the visual transduction process (1, 2), relaxation of smooth muscle (3), intestinal secretion of water and salt (4), and reabsorption of Na⁺ and water in the distal tubule of the nephron (5). Overproduction of cGMP has been associated with neuronal degeneration, endothox shock, diarrhea, and loss of Na⁺ and water. These diverse cellular effects of cGMP are mediated by several distinct receptors: cGMP-stimulated and -inhibited phosphodiesterases (6), cGMP-dependent protein kinases (3), and cGMP-gated cation channels (1, 2). Cyclic nucleotide-gated (CNG) channels are essential in visual and olfactory signal transduction. These proteins [CNG-1 (7) and CNG-2 (8–10)] are encoded by two different genes. The CNG-1 channel is activated at 40-fold higher cGMP concentrations than the CNG-2 channel. An additional subunit confers high-affinity inhibition by L-cis-diltiazem to the rod photoreceptor channel (11). The native and expressed channels are nonspecific cation channels that are highly permeable to monovalent cations and are blocked by the divalent cations Mg²⁺ and Ca²⁺. In the presence of extracellular Mg²⁺ and Na⁺, Ca²⁺ permeates these channels at hyperpolarizing membrane potentials (see refs. 1, 2, and 12 for further discussion).

So far, these channels seemed not to be expressed to a significant level in other tissues, although partial sequences of homologous channels have been amplified by PCR from kidney (13) and heart (14). A functional olfactory CNG-2-like channel has been cloned from a rabbit aorta library (10), but its tissue and cellular localization remained unsolved. However, a few reports suggested that cGMP-gated Ca²⁺ entry was mediated by CNG channels in nonsensory cells at depolarized membrane potentials (15–17). Apparently, a CNG-gated channel mediated Ca²⁺ entry in calcium-depleted pancreatic acinar cells (15). Cultured renal epithelial A6 cells have a cGMP-stimulated cation-channel activity (16). Atrio-natriuretic factor, which stimulates cGMP production in the kidney, increased glomerular filtration by relaxation of preglomerular arterioles and constriction of postglomerular arterioles (17, 18). This communication supports these reports and shows that bovine kidney expresses another member of the CNG-channel family, one which is permeable to Ca²⁺ at a wide range of membrane potentials.

METHODS

Cloning Strategy. Two degenerate oligonucleotide primers (primer 1: 5’-CGGGAATTCTGGTTYGAYTAYYTGGACNAAYA-3’; primer 2: 5’-CGGGAATTCCYTCCCATRAGRTRCTCYTT-3’) were synthesized on the basis of the amino acid sequence of two conserved regions in CNG-1 and CNG-2 [W435FDYLWTKN and K373DDLMEA, numbering amino acid residues corresponding to CNG-1 (7)]. These primers were used to amplify cDNA from bovine kidney by the PCR. Reaction conditions were as described (10). Amplified DNA was digested with EcoRI, purified by gel electrophoresis, and subcloned into pBluescript (Stratagene). Seventeen clones were analyzed by restriction analysis and sequencing. One clone (pCGK7) was obtained with a 443-bp insert that is identical to the sequence of CNG-1. Sixteen clones contained a 443-bp insert that had 91% amino acid sequence identity and 73% nucleotide sequence identity to CNG-1. Screening of a directional, oligo(dT)-primed bovine kidney cDNA library constructed in the pSPORTI vector (GIBCO/BRL) with the 3'P-labeled insert of one of these clones (pCGK5) yielded two clones, pCGK25 and pCGK26. The sequence derives from clone pCGK26 (3954 bp, excluding the poly(A) tail), which was fully sequenced on both strands using either 35S-labeled dATP or a laser fluorescence DNA sequencer.

Transfection of Human Embryonic Kidney 293 Cells. Recombinant plasmids pCGK26/CVM and pCG357/CVM were used for transient expression (19) of CNG-3 and of the rabbit aorta CNG (rACNG) channel (10), in human embryonic kidney 293 cells. These cells do not express endogenous CNG channels (8). pCGK26/CVM was constructed as follows: PCR was done with pCGK26 as template and the following primers: a 5' adapter primer containing a HindIII restriction site followed by a consensus sequence for initiation of translation in vertebrates and the first 20 nt from the coding region of pCGK26 and a gene-specific 3' primer. The PCR product was digested with HindIII and Bgl II to yield an 83-bp fragment. The 83-bp fragment and the 2.2- kb Bgl II-Xba I fragment from pCG26 were ligated with the HindIII-Xba I fragment from the pRC/CVM vector (Invitrogen), yielding pCGK26/CVM. The recombinant plasmid

Abbreviations: CNG, cyclic nucleotide-gated; rACNG, rabbit aorta CNG.

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The sequence reported in this paper has been deposited in the GenBank data base (accession no. X76485).
pCG357/CMV was constructed by subcloning the cDNA insert of pCG357 (10) into the HindIII site of pRC/CMV.

Northern Analysis. Poly(A)^+ RNA was prepared from rabbit ileum, aorta, and brain and bovine tongue, cardiac atrium, cardiac ventricle, renal cortex, renal medulla, and testis. Ten micrograms each was separated on a 1.2% agarose gel, transferred to Biodyne nylon membranes (Pall), and hybridized as described (20). The probe used was the 32P-labeled EcoRI (915)–EcoRI (2199) fragment derived from pCG26.

Electrophysiology. Patch-clamp recordings were made 2–4 days after transfection by using inside-out patches and the whole-cell recording mode (21). The following solutions were used: solution A, 10 mM glucose/140 mM NaCl/5 mM KCl/10 mM NaHepes/0.5 mM NaEGTA/0.5 mM NaEDTA (pH 7.2); solution B, 10 mM glucose/140 mM NaCl/5 mM KCl/10 mM NaHepes (pH 7.2); solution C, 140 mM potassium aspartate, 8 mM NaCl/10 mM EGTA/10 mM NaHepes (pH 7.2); solution D, 140 mM NaCl/2.8 mM KCl/10 mM CaCl₂/2 mM MgCl₂/10 mM NaHepes (pH 7.2).

RESULTS AND DISCUSSION

Two degenerate oligodeoxynucleotide primers designed after highly conserved peptide sequences in bovine CNG cation channels, CNG-1 (7) and CNG-2 (9), were used in a PCR to amplify homologous sequences from bovine kidney cDNA. PCR clones obtained could be assigned to two classes. One of the clones (pCGK7) was identical to the sequence of CNG-1. Clones of the other class showed 91% amino acid identity and 73% nucleotide sequence identity to CNG-1, indicating that these sequences potentially represented an additional CNG channel, referred to as CNG-3. To obtain a full-length clone of CNG-3 one of these clones (pCGK5) was used to screen a cDNA library from bovine kidney. Two clones were identified, the largest of which (pCGK26) was fully sequenced. The longest open reading frame encodes a 706-aa protein of calculated Mr 81,132 with a putative transmembrane topology similar to that of related CNG channels (Fig. 1). Amino acid-sequence comparison reveals an overall sequence identity of 60% to CNG-1 and 62% to CNG-2. The largest divergence occurs at the amino-terminal region preceding the first putative transmembrane domain.

The electrophysiological properties of CNG-3 were investigated by using inside-out patches excised from human embryonic kidney 293 cells transfected with a eukaryotic expression vector containing CNG-3-specific cDNA (Fig. 2). The CNG-3 channel was compared with rACNG channel (10), which has been cloned from rabbit aorta and is an olfactory-like channel. cGMP and cAMP activated both channels at positive and negative membrane potentials. cAMP induced 80% and 100% of the maximal current observed in the presence of cGMP in cells expressing CNG-3 and rACNG channels, respectively. In control experiments a total of 25 patches were excised from nontransfected cells; none of these responded to cyclic nucleotides. The apparent $K_a$ values of CNG-3 for cGMP (18 µM ± 40 mV) and cAMP (1.9 mM ± 40 mV) (Fig. 2b and Table 1) are similar to those reported for CNG-1 (23). The difference in affinity between cGMP and cAMP is slightly larger for CNG-3 (100-fold) than for CNG-1 (40-fold). CAMP activated both channels with an apparent Hill coefficient of $\approx$2 (Table 1). In contrast, cAMP activated the CNG-3 channel with an apparent Hill coefficient of 4 or even higher, suggesting that the channel is composed of five identical subunits (2). The apparent $K_a$ values and Hill coefficients of rACNG channel are similar to those determined in Xenopus oocytes injected with rACNG channel-specific cRNA (10), indicating that the different affinities of CNG-3 and rACNG channel for cGMP and cAMP are not determined by the expression system but are intrinsic properties of each channel.

The ion selectivity of CNG-3 was determined by measuring the reversal potential under symmetrical bionic conditions (Fig. 3a). On the basis of the Goldman–Hodgkin–Katz equation, permeability ratios $P_i/P_{Na}$ yielded the following series of ion selectivity: $N\text{a}^+ > H\text{a}^+ > K^+ > Li^+ > Rb^+ > Cs^+$ with $P_i/P_{Na} = 2.32:1.0:0.93:0.91:0.62:0.34$. These values are similar to those found with other CNG channels (1, 8, 11, 24). CNG-3 showed outward rectification when the divalent cations $Mg^{2+}$ and $Ca^{2+}$ were present in the pipette solution (Fig. 3b and c), reflecting the known (see refs. 1, 2, 12) voltage-dependent block of $Na^+$ conductance. The blocking effect was more pronounced in the presence of 1 mM Mg$^{2+}$ than in the presence of 2 mM Ca$^{2+}$ (Fig. 3b). The reversal potential in the presence of 1 mM Mg$^{2+}$ and 2 mM Ca$^{2+}$ ranged between +6 mV and +15 mV. In five experiments, 100 µM l-cis-diltiazem blocked the current only by 16.8 ± 2.0%.
As stated above CNG channels have been implicated in the mediation of Ca^{2+} entry in Ca^{2+}-depleted cells at high membrane potentials (15). Furthermore it has been shown that the photoreceptor (1) and the olfactory channel (25) are permeable to Ca^{2+}. To test this hypothesis, CNG- and cAMP-activated currents were measured under the ionic conditions used to measure the Ca^{2+} current in Ca^{2+}-depleted cells (26)—i.e., in the presence of 2 and 10 mM extracellular Mg^{2+} and Ca^{2+}, respectively. Under these conditions, cGMP induced an inward current at 0 mV in an excised inside-out patch (Fig. 3c), which was not observed in the absence of divalent cations. The current at negative and positive potentials had a significantly decreased amplitude compared with the current in the absence of divalent ions and showed outward rectification. The reversal potential of the current was +45 mV (range: +37 to +58 mV, four experiments), being consistent with the theoretical reversal potential of +47 mV for these ionic conditions (27). This finding suggests that the current was carried, at least in part, by Ca^{2+}. The calculated permeability ratio $P_{Ca^{2+}}/P_{Na^{+}}$, at 0 mV according to Lewis (27) was 23.6, being in line with Ca^{2+} permeabilities determined for photoreceptor channels (1, 28).

To verify the significance of this inward current, similar experiments were done in the whole-cell configuration. CNG-3-expressing cells were dialyzed with 1 mM cGMP in the absence and presence of Ca^{2+} and Mg^{2+} (Fig. 4a). The cells were initially superfused for 1 min with a Ca^{2+}-free solution. Under these conditions, no inward current could be detected while the membrane potential was clamped to 0 mV. Exchange of the Ca^{2+}-free solution by a solution containing 2 and 10 mM extracellular Mg^{2+} and Ca^{2+}, respectively, resulted in a sustained inward current at 0 mV that was reversibly blocked by extracellular Mn^{2+} (10 mM). A symmetrical, linear current-voltage relation was obtained in the absence of divalent cations (data not shown). The divalent cations Mg^{2+} and Ca^{2+} induced an outward rectifying current (Fig. 4b and d). Replacement of Ca^{2+} by Mn^{2+} completely blocked the inward current as inferred from the absence of an inward current at 0 mV (Fig. 4b and d). The inward current observed at negative membrane potentials was small in comparison with the current in the absence of Mn^{2+}. Nontransfected cells dialyzed with 1 mM cGMP had a small linear current that showed no rectification (Fig. 4c and d). A similar current was observed in nontransfected cells in the absence

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**Table 1.** Apparent $K_\alpha$ values and Hill coefficients ($\nu$) of currents activated by cGMP and cAMP at membrane potentials ($V_m$) of +40 mV and -40 mV

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<thead>
<tr>
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<th>cGMP</th>
<th>cAMP</th>
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<tr>
<td></td>
<td>$K_\alpha$, $\mu$M</td>
<td>$\nu$</td>
</tr>
<tr>
<td>CNG-3</td>
<td>10</td>
<td>18.00 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>-40</td>
<td>21.70 ± 2.33</td>
</tr>
<tr>
<td>rACNG</td>
<td>9</td>
<td>0.45 ± 0.04</td>
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<tr>
<td></td>
<td>-40</td>
<td>0.46 ± 0.02</td>
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Entries are means ± SEMs; $n$, number of experiments.
of divalent cations of CaC12, 1 mM of Mg2+ (solution A), and both 1 mM Mg2+ and 2 mM Ca2+ (solution B) at the extracellular side. The bath solution contained solution A; the pipette contained solution B supplemented with divalent cations as indicated. (c) Relation in the presence of 2 mM Mg2+ and 10 mM Ca2+ at the extracellular side. The bath and the pipette contained solution C and D, respectively.

of CNG-3 for Ca2+ determined in whole-cell configuration. Before cells were used for whole-cell measurement inside-out patches were tested for expression of CNG-3. Serial resistance was compensated as much as possible (>60%). Leak current has not been subtracted. (a) Whole-cell current recorded at a holding potential of 0 mV. After an initial superfusion for 1 min with Ca2+-free solution (solution A) the cell was superfused with solution D containing 10 mM CaCl2 and 2 mM MgCl2. The current trace in the absence of Ca2+ is only partially shown. Beginning of superfusion with solution D is indicated by an arrow. The pipette contained solution C supplemented with 1 mM cGMP. Establishment of the whole-cell mode is indicated by an asterisk. Solution D with 10 mM MnCl2 instead of CaCl2 was superfused for the time indicated by the horizontal line. Cell capacitance was 35 pF. (b) Currents obtained with the same cell as in a using voltage steps at increments of ±15 mV from a holding potential of 0 mV in the presence of divalent cations (solution D or solution D with 10 mM MnCl2 instead of CaCl2, as indicated). The pipette contained solution C supplemented with 1 mM cGMP. Only current traces obtained at −90 mV, −60 mV, −30 mV, 0 mV, +30 mV, +60 mV, and +90 mV are shown. (c) Currents of a nontransfected cell (cell capacitance = 42 pF) in the presence of divalent cations on the extracellular side as indicated. The pipette contained solution C supplemented with 1 mM cGMP, and the bath contained solution D. Voltage pulses were as described in b. (d) Current–voltage relation of the currents shown in b (a, 10 mM Ca2+ and 2 mM Mg2+; β, 10 mM Mn2+ and 2 mM Mg2+) and c (γ).
determined in whole-cell configuration is slightly less positive than that of the cGMP-stimulated current of acinar cells (+45 mV), reflecting the property of CNG-3 to be permeable for monovalent and divalent cations.

The tissue distribution of the mRNA encoding CNG-3 was determined by Northern analysis (Fig. 5). A major mRNA species of 4.2 kb is predominantly expressed in testis and kidney cortex and medulla. A weak signal was also found in cardiac atrium and ventricle. The mRNA species found in testis and heart are derived from the same gene as that encoding CNG-3 because partial sequences identical with CNG-3 could be amplified by PCR from the mRNA of these tissues (data not shown). No positive signals were seen with mRNA from ileum, tongue, aorta, cerebellum, and total brain. A smaller transcript of 3.0 kb was detected in kidney and testis and may represent a polyadenylation variant of the CNG-3 transcript.

In addition to CNG-3, a sequence has been amplified by PCR from kidney that is identical with CNG-1. However, the predominant species of bovine kidney appears to be CNG-3 because CNG-3 and CNG-1 were amplified in a 16:1 ratio. Previously, sequences of the rod photoreceptor channel have been identified in mouse cortical collecting-duct cells and rat cardiac atrium and ventricle. The mRNA species found in these tissues (data not shown). No positive signals were seen with mRNA from ileum, tongue, aorta, cerebellum, and total brain. A smaller transcript of 3.0 kb was detected in kidney and testis and may represent a polyadenylation variant of the CNG-3 transcript.

Under physiological conditions, CNG-3 should be activated by cGMP. Its electrophysiological properties are similar to those reported for the retinal CNG-1 channel (7, 23), although CNG-3 shows a similar overall homology to both the photoreceptor and olfactory channels. CNG-3 may allow Ca2+ permeation not only at negative but also at positive membrane potentials. However, CNG-3 is not responsible for ICRAC [calcium release-activated calcium current (26, 29)] or similar currents (30, 31) because these currents are not activated by cGMP.

The potential physiological role of CNG-3 in testis, kidney, and heart is probably related to the regulation of cellular calcium at a wide range of membrane potentials. CNG-3 could represent the equivalent of a receptor-operated Ca2+ channel, which allows neurotransmitter-controlled Ca2+ entry. Chemotaxis of sperm cells may be controlled during fertilization by a mechanism similar to olfactory signal transduction because testis expresses members of the olfactory-receptor gene family (32). Sea urchin sperm has a membrane-bound guanylate cyclase that is activated by resact and speract and mediates chemotaxis of the sperm (33). The strong expression of CNG-3 in testis favors the idea that the chemotactic movement of the sperm may be mediated by the cGMP-activated channel. Regardless of the mechanism by which CNG-3 protein increases cytosolic Ca2+—i.e., by allowing Ca2+ permeation at hyperpolarizing or depolarizing voltages or by Na+-induced depolarization and opening of voltage-dependent Ca2+ channels, its identification as a component of nonsensory cells is important because it indicates that some of the effects of NO and atrio-natriuretic factor may be mediated by an increase and not a decrease (for further review, see refs. 34–36) in cellular Ca2+.

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![Fig. 5. Northern analysis of CNG-3 expression. Ten micrograms of poly(A)+ RNA of each tissue was used. Autoradiographic exposure was 1 day for testis, aorta, cerebellum, and brain and 3 days for ileum, tongue, atrium, ventricle, renal cortex, and renal medulla. No signal was observed in aorta, cerebellum, brain, ileum, and tongue even after an exposure for 7 days.](image-url)