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Primary structure and functional expression of a cyclic nucleotide-gated channel from rabbit aorta

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Sequences specific for cyclic nucleotide-gated channels (CNG channels) have been amplified by PCR from cDNA of heart, aorta, sino-atrial node, cerebellum, C-cells and kidney. The complete amino acid sequence of a CNG channel from rabbit aorta has been deduced by cloning and sequence analysis of the cDNA. Synthetic RNA derived from this cDNA induces the formation of a functional CNG channel in *Xenopus* oocytes.

Cyclic nucleotide-gated channel; Aorta; Primary structure; Expression

1. INTRODUCTION

Cyclic nucleotide-gated cation channels (CNG channels) play a central role in the signal transduction of vertebrate photoreceptor cells and olfactory neurons (see [1] for review). The primary structure of these channels has been determined by molecular cloning of the corresponding cDNAs [2–8]. Recently, a novel subunit of the rod photoreceptor channel has been identified [9]. In addition, partial sequences being highly homologous to photoreceptor and olfactory channels have been amplified by PCR from kidney [10] and heart [11], respectively.

Using PCR techniques we have now amplified CNG channel-specific sequences from cDNA of heart, aorta, sino-atrial node, cerebellum, C-cells and kidney. We have used the amplified DNA from aorta to isolate a full length cDNA from a rabbit aorta cDNA library. The cDNA encodes a polypeptide which is highly homologous to cloned olfactory channels, indicating that the channels expressed in olfactory epithelium and aorta are derived from the same primary transcript.

2. MATERIALS AND METHODS

2.1. *Polymerase chain reaction*

10 ng of oligo(dT) primed cDNA (rabbit heart, aorta, sino-atrial node and cerebellum, bovine kidney and human C-cells) and 50 pmol of the degenerate oligonucleotide primers P1 and P2 were incubated in 100 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, dATP, dCTP, dGTP, dTTP 0.2 mM each, and 2.5 U *Taq* DNA polymerase. Forty cycles (94°C, 1.0 min; 45°C, 1.0 min; 72°C, 1.5 min) were performed with a programmable thermocycler. As a control, a reaction without cDNA was also performed. One tenth of the reaction products were analysed by gel electrophoresis. The amplified fragment obtained from aorta was digested with *Eco*RI, subcloned into pBluescript (Stratagene) and sequenced [12]. Sequences of primers were as follows: P1, 5′- CGGGAATTCTGGTT(C/T)GA(C/T)TA(C/T)(C/T)TGTGGAC-(ACGT)AA(CT)AA-3′ corresponding to the peptide W_{435}FD-YLWTNK and P2, 5′-CGGGAATTCGC(C/T)TCCAT(A/G)AG-(A/G)TC(A/G)TC(C/T)TT-3′ corresponding to the peptide K_{576}DD-LMEA of the bovine rod outer CNG channel (see [2]).

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2.2. *Generation and screening of a rabbit aorta cDNA library*

A directional, oligo(dT) primed cDNA library was constructed in the pSPORT1 vector (GIBCO BRL life Technologies). After electroporation in *E. coli* XL1-blue (STRATAGENE) ~3×10^5 clones were obtained. Screening ($3 \times$ SSC, $10 \times$ Denhardt's solution, 150 μg/ml sheared denatured salmon sperm DNA at 50°C; washing in $0.3 \times$ SSC, 0.1% SDS at 50°C) with the radiolabelled PCR fragment amplified from aorta yielded one positive clone, pCGA1 (~ 5.5 kb). The coding region, the complete 5′ untranslated and parts of the 3′ untranslated region of pcGA1 were sequenced on both strands.

2.3. *Construction of recombinant plasmids pCG153 and pCG357*

The recombinant plasmid pCG153 carrying the entire protein-coding sequence of pCGA1 was constructed as follows. PCR was done with pCGA1 as template and the following primers: a 5′ adapter primer (containing *Xho*I and *Hin*dIII restriction sites followed by a consensus sequence for initiation of translation in vertebrates [13], and the first twenty nucleotides from the coding region of pCGA1) and a gene-specific 3′ primer. The PCR product was digested with *Xho*I and *Asp*718 to yield a 144-bp fragment. The 144-bp PCR fragment and the 2.2-kb *Asp*718–*Hin*dIII fragment from pCGA1 were ligated with the *Xho*I–*Hin*dIII fragment from pSP72 (containing the 0.26-kb *Bgl*II–*Bam*HI fragment carrying a poly(dA) tract from pSPCA1 [14] at the *Bam*HI site) yielding pCG153.

The recombinant plasmid pCG357 was constructed in the same way as pCG153 with the only difference that the open reading frame starts

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with the third in-frame ATG triplet encoding Met^{69}, which is used as initiating methionine of bovine olfactory channel.

2.4. *Functional expression in* Xenopus *oocytes*

Capped cRNA specific for the cyclic nucleotide-gated channel from aorta was synthesized in vitro using *Bam*HI-cleaved pCG153 or pCG357, respectively. Injection of cRNA into *Xenopus* oocytes and current measurements were as described [15].

3. RESULTS AND DISCUSSION

We used PCR to identify novel members of the cyclic nucleotide-gated cation channel family. cDNA derived from a variety of tissues was amplified using a pair of degenerate oligodeoxynucleotid primers. These primers have been designed according to highly conserved sequences flanking the putative cyclic nucleotide-binding region of known CNG channels (Fig. 1a). Specific amplification products of the predicted size (461 bp) were obtained from rabbit heart, aorta, sino-atrial node, cerebellum, bovine kidney and to a smaller amount from a human thyroid C cell line [16] (Fig. 1b). The DNA amplified from aorta was analysed by restriction analysis and found to consist of a single DNA species. Sequencing of the DNA revealed high sequence similarity (75–90%) to sequences of known CNG channels.

The full length 5.5 kb clone pCGA1 was identified by screening of a rabbit aorta cDNA library with the radiolabelled PCR fragment. Fig. 2 shows the first 2,669 bp of the pCGA1 containing the 5′ untranslated region, the complete coding region and portions of the 3′ untranslated sequence.

The translation initiation site was assigned to the first ATG which appears downstream of a stop codon found in-frame. Since this ATG is not surrounded by a perfect ribosome-binding consensus sequence [13] we cannot exclude the possibility that another ATG is actually used as starting point for protein translation. The third in-frame ATG which is located 204 bp downstream of the first ATG fulfils the requirements for initiation of protein translation. This ATG is used as start codon of rat and bovine olfactory channels and could therefore also serve as start codon of the CNG channel encoded by pCGA1. In contrast to the sequences of rat and bovine olfactory channels, the third in-frame ATG of pCGA1 is not preceded by a stop codon indicating that the first in-frame ATG will be used as start codon.

The open reading frame of pCGA1 encodes for a protein of 732 amino acids with a calculated M_r of 84,043. Amino acid sequence comparison reveals that the CNG channel from aorta (rACNG) is highly homologous to the bovine olfactory channel (93.7% homology), whereas the homology to the CNG channel cloned from bovine rod outer segment (56.7%) is much smaller. rACNG mainly differs from olfactory channels in hav-

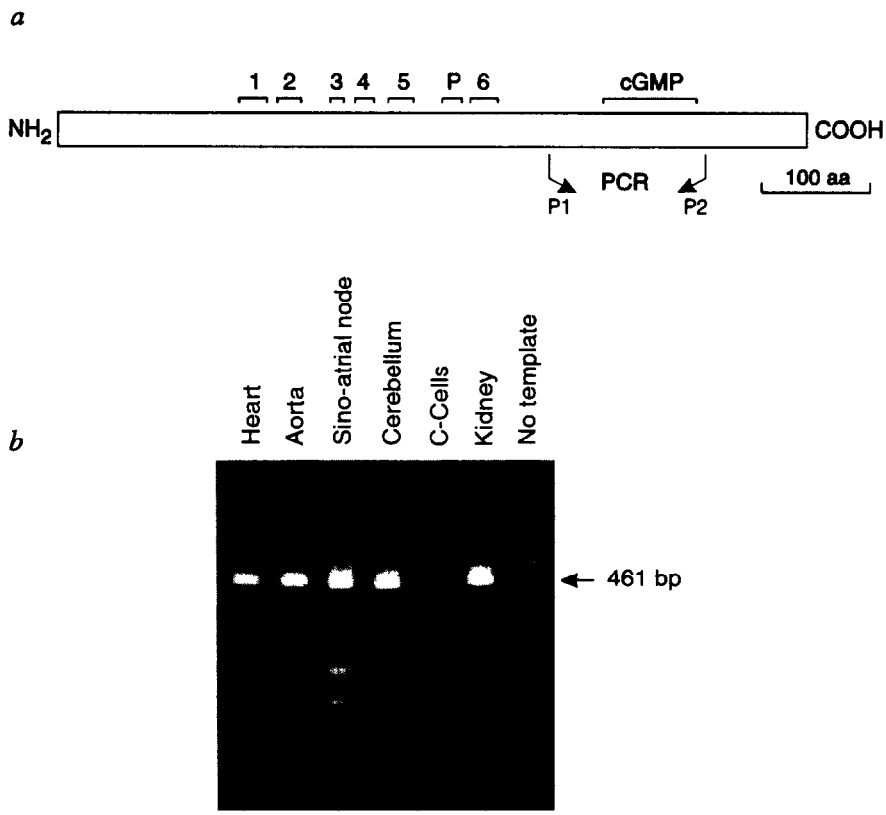

Fig. 1. (a) Scheme of bovine rod photoreceptor channel. The putative transmembrane domains (1–6), the putative ion-conducting pore (P) and the cGMP-binding region are indicated. The positions of the degenerate primers P1 and P2 used for PCR amplification are marked by arrows. (b) Analysis of amplified PCR fragments by polyacrylamide electrophoresis. Specific bands of 461 bp are indicated by a an arrow.

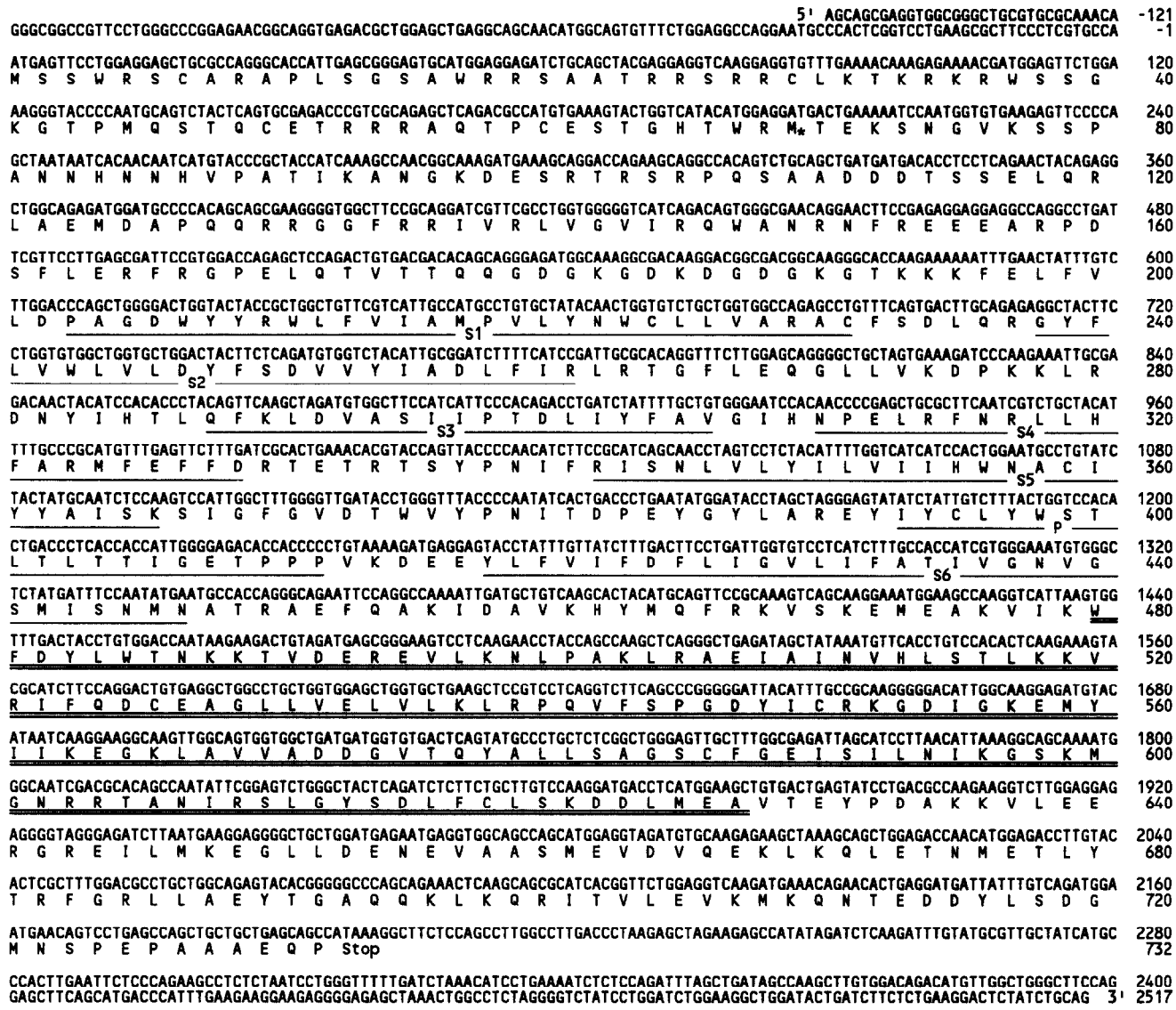

Fig. 2. Nucleotide and deduced amino acid sequence of rACNG. The putative transmembrane segments S1 to S6 and the putative ion-conducting pore (P) are shown. The sequence of the PCR fragment amplified from aorta is double-underlined. The third methionine, which corresponds to the initiating methionine of the bovine olfactory channel, is marked by an asterisk.

ing an elongated N-terminal region of 68 amino acids resulting from the assignment of the initiating methionine to the first ATG of the open reading frame. The strong homology to the olfactory channel suggests that CNG channels from aorta and olfactory epithelium are derived from the same gene. This is supported by the finding that partial sequences identical with rACNG could be amplified by PCR from mRNA of rabbit olfactory epithelium (data not shown). Thus, the amino acid exchanges as well as the elongated N-terminal region might reflect the evolutionary distance between rabbit and cow.

To examine the electrophysiological properties of rACNG, specific cRNA derived from clone pCG153 which contains the complete open reading frame of pCGA1 and from clone pCG357 which starts at the third in-frame ATG (Met^{69}) corresponding to the initiating methionine of bovine olfactory channel were injected into *Xenopus* oocytes. Inside-out patches of plasma membrane were excised from injected oocytes and tested for sensitivity to bath-applied cAMP and cGMP. Typical current–voltage relations obtained with cRNA specific for pCG153 at increasing cGMP and cAMP concentrations in the presence of symmetrical solution containing 100 mM KCl, 10 mM EGTA-KOH and 10 mM HEPES-KOH (pH 7.2) are shown in Fig. 3a,b. Maximum currents obtained in the same patch at saturating cGMP and cAMP concentrations were similar (Fig. 3b). The channel induced by pCG153 is ~40-fold less sensitive to cAMP than to cGMP. Apparent K_a values for activation by cGMP (1.7 μM at +80 mV) and cAMP (59.5 μM at +80 mV) and the Hill coefficients

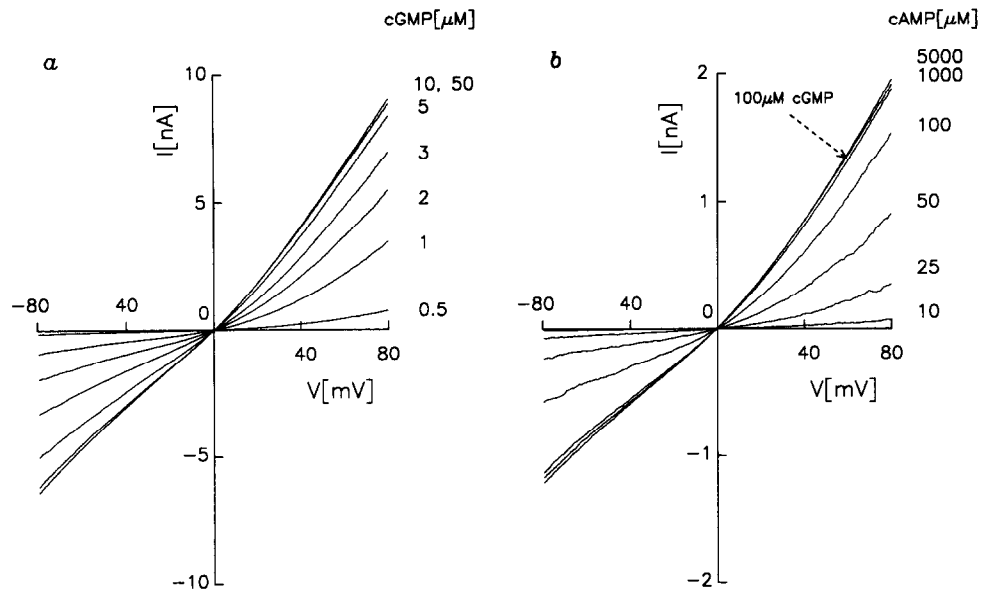

Fig. 3. Current responses in inside-out patches from *Xenopus* oocytes injected with cRNA specific for pCG153. (a) Series of current–voltage (*I–V*) recordings in the presence of increasing cGMP concentrations. (b) Series of *I–V* recordings in the presence of increasing cAMP concentrations. For comparison the *I–V* curve of the same patch for 100 μM cGMP (saturating concentration) is shown.

(2.2 for cGMP and 1.8 for cAMP) (Table I) are similar to values reported for cloned olfactory channels [5,15]. Currents induced by cRNA specific for pCG357 were not significantly different from those obtained with pCG153 (Table I).

The ion selectivity of rACNG was examined by measurement of the reversal potential under symmetrical biionic conditions (Fig. 4 and Table II). On the basis of the Goldman–Hodgkin–Katz equation permeability ratios P_i/P_K yielded the following series of ion selectivity:

$$NH_4^+:K^+:Na^+:Li^+:Rb^+:Cs^+ = 3.04:1:0.99:0.97:0.79:0.48$$

These relative ion permeabilities agree quite well with those of expressed olfactory and photoreceptor channels [2,5].

In summary we have cloned a cyclic nucleotide-gated channel which is highly homologous to the olfactory channel suggesting that both channels are derived from the same gene. When expressed in *Xenopus* oocytes the channel reveals the basic properties found in other cyclic nucleotide-gated cation channels. The sensitivity to cAMP and cGMP, the cooperativity and the ion selectivity of the channel is similar to those of expressed olfactory channels being consistent with the close structural relationship of both channels. The photoreceptor channel and the channels cloned from olfactory epithelium and aorta differ in their affinity to cAMP and cGMP. In all three channels the nucleotide-binding region is very similar (see [1] for further discussion) indicating that other parts of the channel molecules may determine the differences observed. By analogy to the N-terminal 'ball' which mediates inactivation of voltage-dependent potassium channels by interacting with a region on the cytoplasmic face of the channel [17] it seems attractive to speculate that the N-terminus of CNG channels might interact with the region involved

Table I

Apparent K_a and Hill coefficients (υ) of currents activated by cGMP and cAMP at membrane potentials (V_m) of +80 mV and −80 mV

	n	V_m (mV)	K_a (μM)	υ
cGMP				
pCG153	7	+80	1.7 ± 0.4	2.2 ± 0.3
		−80	2.9 ± 0.6	2.3 ± 0.2
pCG357	15	+80	1.9 ± 0.5	2.7 ± 0.6
		−80	3.3 ± 1.0	2.5 ± 0.5
cAMP				
pCG153	7	+80	59.5 ± 10.6	1.8 ± 0.2
		−80	112.8 ± 29.6	1.8 ± 0.4
pCG357	10	+80	45.2 ± 12.8	1.6 ± 0.3
		−80	79.3 ± 21.4	1.6 ± 0.2

The entries are mean ± S.D. *n*, number of experiments.

Table II

Reversal potentials of expressed pCG153 in the presence of different ion species measured under biionic conditions

	V_{rev} (mV)	*n*
NH_4^+	−28.06 ± 6.6	13
K^+	0	15
NA^+	0.13 ± 2.1	13
Li^+	0.73 ± 1.31	15
Rb^+	5.89 ± 1.27	13
Cs^+	18.74 ± 1.5	10

The entries are mean ± S.D.; *n*, number of experiments.

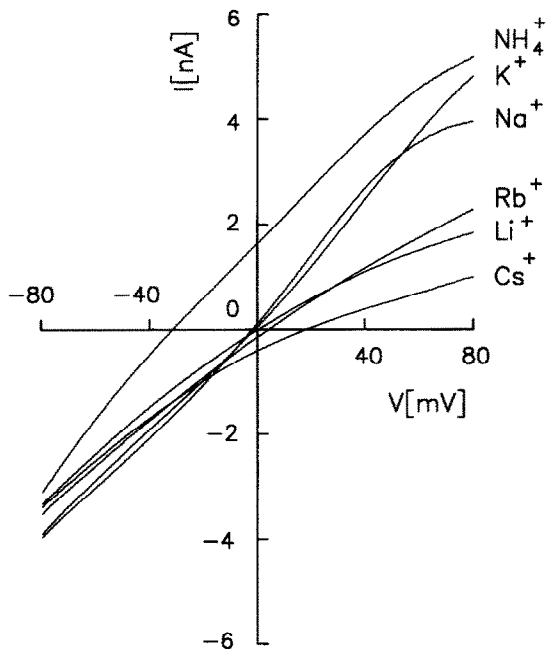

Fig. 4. Determination of relative ion permeabilities of pCG153. *I–V* recordings under symmetrical biionic conditions at 50 μM cGMP. The pipette solution contained 100 mM KCl, and the perfusion solution contained the respective cation at 100 mM.

in binding of cyclic nucleotides. In this context it is noteworthy that the longer amino-terminal region of rACNG does not influence the ligand sensitivity because no significant difference in apparent K_a for cGMP or cAMP was observed between the 'short' and the 'long' form of the channel. In rabbit tissues the ligand affinity may be modified by the presence of additional channel subunits. Recently, a new subunit of the photoreceptor channel has been identified [9] which affected the sensitivity of the photoreceptor channel for L-*cis*-diltiazem. Possibly other CNG channels also have a hetero-oligomeric structure. However, it remains to be established that the differences in cyclic nucleotide sensitivity are not caused by the small differences of the amino acid composition of the putative cyclic nucleotide binding region observed between different CNG channels.

Transcripts for rACNG could not be detected in Northern blot experiments with mRNA of rabbit aorta. In contrast to Barnstable et al. [18] we were also unable to obtain a positive signal in Northern blots with mRNA from rabbit total heart and bovine cardiac sinus node using either probes derived from pCGA1 or bovine photoreceptor channel. Taken together these findings suggest that rACNG may be present only in specific cells of the aorta. A possible location could be the endothelium. Recently it was shown that cGMP depolarized cultured rat coronary endothelial cell layers [17]. The low abundant expression of the cGMP-gated channel in endothelial cells would be in line with the fact that endothelial cells couple excellently and may need therefore very few channels to regulate the membrane potential.

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