NEURO PHARMACOLOGY

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NANC NEUROTRANSMISSION

NO AND SYNAPTIC PLASTICITY

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Expression of Cyclic Nucleotide-gated Cation Channels in Non-sensory Tissues and Cells

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Summary—Using a combination of PCR based cloning and Northern blot analysis we have investigated the tissue expression of cyclic nucleotide-gated (CNG) cation channels in a variety of non-excitable tissues and cells. Partial sequences of all three known CNG channels and of an auxiliary subunit of the rod photoreceptor channel were determined. The expression of CNG channel genes is both tissue and species specific. Southern blot analysis with human genomic DNA revealed specific patterns of hybridization with probes derived from the different CNG channel types indicating that they are encoded by distinct genes. Analysis of human genomic sequences showed that all three genes are derived from a common ancestral gene and might have a similar gene structure. We were not able to identify additional genes encoding CNG channels. The CNG3 channel, which was originally cloned from bovine kidney may be expressed also in bovine cone photoreceptor cells. These data suggest that some of the effects of cGMP in peripheral tissues and cells might involve the activation of CNG channels.

Keywords—Cyclic nucleotide-gated channel, cation channel, tissue expression, genomic, northern blot.

Nitric oxide is a widely used hormonal signal which stimulates soluble guanylyl cyclase in a variety of tissues. Cyclic GMP produced by this enzyme modulates the activity of cGMP-dependent protein kinases, phosphodiesterases and cyclic nucleotide-gated (CNG) cation channels. CNG channels represent a novel class of ion channels which play an important role in sensory signal transduction. Functionally they belong to the class of ligand gated channels because they are activated by the binding of a ligand (cGMP or cAMP) to the cyclic nucleotide binding site. Structurally however they show almost no similarity with ligand-gated channels, but share some important features with voltage-gated channels. Up to now three types of CNG channels have been identified in sensory cells: the cone photoreceptor channel (chcone), the rod photoreceptor channel (CNG1) and the olfactory channel (CNG2). Rod photoreceptor and olfactory channel cDNAs have been cloned from a variety of species [CNG1: bovine (Kaupp *et al.*, 1989), human (Dhallan *et al.*, 1992; Pittler *et al.*, 1992), mouse (Pittler *et al.*, 1992) and chicken (Bönigk, *et al.*, 1993); CNG2: bovine (Ludwig, *et al.*, 1990), rat (Dhallan *et al.*, 1990) and catfish (Goulding *et al.*, 1992)], whereas only the chicken nucleotide sequence (Bönigk *et al.*, 1993) is known for the cone photoreceptor channel.

CNG channels consist of about 640–740 amino acids and possess 6 transmembrane segments. The regions best conserved among channels and species are the pore, a loop between the S5 and the S6 segment, and the cyclic nucleotide binding site in the carboxyterminal tail. The most variable part of the nucleotide sequence of CNG channels is the aminoterminus.

The olfactory channel is about 25-fold more sensitive to both cAMP and cGMP than the rod photoreceptor channel. Both channels are 30–40-fold more sensitive to cGMP than to cAMP (Altenhofen *et al.*, 1991).

An auxiliary subunit to the rod photoreceptor channel, hRCNC2, termed here CNG4, has been cloned from human retina (Cheng *et al.*, 1993). It shows only 30% overall sequence identity with CNG1. The two splice variants of this subunit differ only in the length of the aminoterminus. CNG4 shows no channel activity when expressed alone. However, coexpression with CNG1 gives rise to functional channels with properties more similar to the native rod photoreceptor channel than expression of CNG1 alone.

We have cloned a new member of the CNG channel family, CNG3, from bovine kidney (Biel *et al.*, 1994). Amino acid sequence comparison revealed an overall sequence identity of 60% to CNG1 and of 62% to

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CNG2. Activation constants for both cGMP and cAMP were in the range of those reported for CNG1 (Altenhofen *et al.*, 1991). In addition, we have identified a CNG channel from rabbit aorta, rACNG (Biel *et al.*, 1993). rACNG showed 93.7% amino acid identity with the bovine olfactory channel, its main difference being an elongated aminoterminus of 68 amino acids. This strong homology indicated that rACNG and CNG2 may be derived from the same gene.

In the present study we have used a combination of PCR techniques and Northern analysis to demonstrate that CNG channels are expressed in a variety of non-sensory tissues and cells from different species. Additionally, we show that the expression of the auxiliary subunit, CNG4, is not limited to sensory cells either. We also present evidence that CNG1–3 and the auxiliary subunit of the rod photoreceptor channel are the products from four different genes. The genes encoding CNG1, CNG2 and CNG3 may be derived from a common ancestral gene.

METHODS

Preparation of RNA, genomic DNA and cDNA

Poly $(A)^+$ RNA was isolated from various bovine rabbit and rat tissues and human, hamster and monkey cell lines either by the guanidinium thiocyanate method on a CsCl gradient followed by oligo dT cellulose chromatography or by using commercial mRNA isolation kits from Dynal or Invitrogen. Genomic DNA was isolated from human peripheral leukocytes (Quiagen, genomic DNA extraction kit). mRNA was transcribed into first strand DNA using oligo dT primers and SuperScript^{tm} RNase$^-$ reverse transcriptase (BRL).

PCR amplification and analysis of PCR products

Two pairs of degenerate oligonucleotide primers flanking highly conserved regions of CNG channels were synthesized for amplification of CNG channel specific sequences [Fig. 1(a) and (c)].

(1) P1 (5′CGGGAATTCTGGTT(C/T)GA(C/T)TA (C/T)(C/T)TGTGGAC(A/C/G/T)AA(C/T)AA3′)

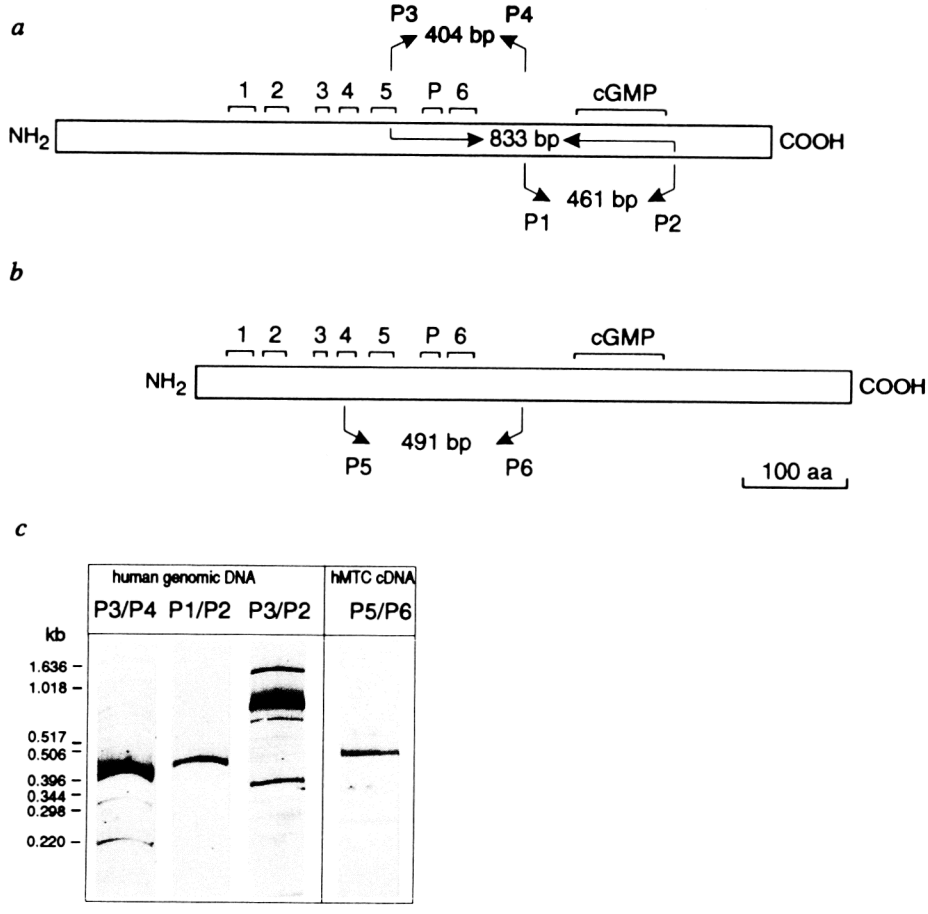

Fig. 1. PCR amplification of CNG channel and subunit specific sequences. (a) Scheme of the bovine rod photoreceptor channel. For amplification of CNG channel specific sequences three combinations of degenerate oligonucleotide primers (P1/P2, P3/P4, P3/P2) were used. P: Pore region, cGMP: cGMP binding site (b) Scheme of the auxiliary subunit of the human rod photoreceptor channel (hRCNC2 (Cheng *et al.*,1993), termed here CNG4). For amplification of CNG4 specific sequences degenerate oligonucleotide primers P5/P6 were used. (c) Polyacrylamid electrophoresis of amplified PCR fragments from human genomic DNA (primer pairs P3/P4, P1/P2, P3/P2) and from cDNA of hMTC cells (primer pair P5/P6). Size of marker DNA (lkb ladder, BRL) is given in kb. Fragments of the predicted size were excised, subcloned and sequenced.

corresponding to the peptide W_{435} FDYLWTNK and P2 (5′CGGGAATTCGC(C/T)TCCAT (A/G)AG(A/G)TC(A/G)TC(C/T)TT3′) corresponding to the peptide K_{576} DDLMEA of the bovine rod photoreceptor channel flanking the putative cGMP binding region (Kaupp *et al.*, 1989).

(2) P3 (5′AT(T/C/A)AT(T/C/A)CA(T/C)TGGAA (T/C)GC(T/C/A/G)TG3′) corresponding to the peptide I_{308} IHWNAC and P4 (5′TT(A/G/T/C) G(T/C)CCACA(G/A)(A/G)TA(G/A)TC(A/G)A-ACC3′) corresponding to the peptide W_{435}FDYLWTN of the bovine rod photoreceptor channel flanking the pore region.

Three combinations of primers (P3/P4,P1/P2,P3/P2) were used to generate a 404, 461 or 833 bp fragment, respectively.

Two degenerate oligonucleotide primers (P5, P6) yielding a 491 bp fragment were used for amplification of CNG4 specific sequences [Fig. 1(b) and (c)]. Sequences of these primers were as follows:

P5 (5′CGGGAATTCTA(C/T)ATGGC(A/G/C/T)TT (C/T)TT(C/T)GA(G/A)TT3′) corresponding to the peptide Y_{141}MAFFEF and P6 (5′CGGGAATTC-TGCCA(T/G/A/C)GT(G/A)TA(C/T)TC(G/A)TAC-CA3′) corresponding to the peptide W_{292}YEYTWH of hRCNC2a (termed here CNG4a) cloned from human retina (Cheng *et al.*, 1993).

With bovine retina CNG3 specific PCR products of 428 bp were amplified using CNG3 specific primers P7 (5′ACGGCCAACATCAGGAG3′) corresponding to the peptide T_{584}ANIRS of CNG3, P8 (5′GGCGC-AGCGTCGCACTG3′) comprising the nucleotides 2161–2177 of pCGK26 (Biel *et al.*, 1994). PCR reactions of 50 μl contained 10 ng first strand cDNA or 100 ng genomic DNA, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, dGTP, dATP, dTTP, dCTP 0.2 mM each, 25 pmol of forward and reverse primer 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. Control reactions without first strand DNA or genomic DNA, respectively, were included in each PCR amplification experiment. For each mRNA controls were run without reverse transcriptase to ensure that results were not due to amplification of any DNA contaminants. When genomic DNA was used as template, the first cycle was preceeded by an additional denaturing step (10 min, 95°C). Blunt end PCR products were subcloned into pUC 18. From each PCR a number of clones was analyzed by restriction analysis and sequenced on both strands.

Northern blot analysis

Poly $(A)^+$ RNA from various tissues was separated on a 1.5% agarose gel, transferred to a nylon membrane (Biodyne, Pall) and hybridized as previously described (Hullin *et al.*, 1992). Three different α^{32}P-labelled cDNA probes derived from CNG1, CNG2 and CNG3, respectively were used for hybridization: the *Hinc*II (267)-*Nsi*I (1259) fragment of pRCG1 (Kaupp *et al.*, 1989), the 2.2 kb *Hind*III (vector)-*Hind*III (2162) fragment of pCG 357 (Biel *et al.*, 1993), the *EcoR*I (913)–*EcoR*I (2199) fragment of pCGK26 (Biel *et al.*, 1994).

Southern blot analysis

*EcoR*I and *Hind*III digested genomic DNA from human blood lymphocytes was resolved on a 0.75% agarose gel, denatured, neutralized and transferred to a Nylon membrane (Biodyne, Pall). The following cDNA probes were α^{32}P-labelled by random priming and used for hybridization: the *Hinc*II (267)–*Asp*718 (2128) fragment of pRCGl (Kaupp *et al.*, 1989), the 2.4 kb *Hind*III (vector)–*Hind*III (2366) fragment of pCG153 (Biel *et al.*, 1993), the 2.3 kb fragment *Hind*III (vector)–*Xba*I (2264) fragment of pCGK26 (Biel *et al.*, 1994) and a CNG4 specific 491 bp fragment amplified from first strand cDNA of hMTC (human Medullary Thyroid Carcinoma) cells using primers P5/P6. Hybridization was done in 5 × SSC, 10 × Denhardt's solution, 1% SDS, 150 μg/ml denatured salmon sperm DNA at 50°C for 16 hr. Filters were washed with 3 × SSC, 0.1% SDS followed by 0.3 × SSC, 0.1% SDS at 60°C and autoradiographed at −80°C.

RESULTS

PCR analysis

We used a PCR based approach to identify CNG channel derived sequences from a variety of excitable and non-excitable tissues and cell lines. Using three combinations of degenerate oligonucleotide primers P1–P4 [Fig. 1(a) and (c)] PCR products of 404 bp (primers P3 and P4), 461 bp (primers P1 and P2) and 833 bp (primers P3 and P2), respectively, could be amplified. Restriction enzyme analysis and sequencing of the different fragments allowed in each case the classification to either CNG1, CNG2 or CNG3 (Table 1). The expression of CNG1-3 is tissue and species specific. For example rabbit heart expresses only CNG2, whereas bovine heart expresses CNG3. Surprisingly and in contrast to recent reports (Ahmad *et al.*, 1990; Ahmad *et al.*, 1992), none of the known CNG channels could be amplified from rat heart and kidney. However, a CNG channel specific sequence could be detected only in one of all tested rat tissues (testis) suggesting that the lack of PCR amplification might be due to primer incompatibilities or other intrinsic reasons. The same argument might explain the lack of CNG2 specific sequences in bovine tissues. However it should be noted that the absence of specific PCR products in rat and bovine tissues demonstrates that the used approach was specific and not caused by contamination of the probes with cloned CNG sequences or genomic DNA. Interestingly expression of all three CNG channels could be detected in COS-, and human Medullary Thyroid Carcinoma

Table 1. Tissue distribution of CNG channels. (+) indicates that identity of PCR products was confirmed by subcloning and sequencing. (−) indicates that a specific transcript could not be identified

		PCR		
Species	Tissue	CNG1	CNG2	CNG3
Rabbit	Heart	−	+	−
	Aorta	−	+	−
	Brain	−	+	−
	Cerebellum	−	+	−
	Pancreas	+	−	−
	Colon	−	−	+
Bovine	Heart	−	−	+
	Cerebellum	−	−	−
	Pineal Gland	+	−	+
	Kidney	+	−	+
	Testis	−	−	+
	Adrenal Gland	+	−	+
	Retina	+	−	+
Rat	Heart	−	−	−
	Brain	−	−	−
	Kidney	−	−	−
	Testis	+	−	−
	RBL cells	−	−	−
	Endothelium	−	−	−
Hamster	Ovary cells	+	−	−
Monkey	COS cells	+	+	+
Human	hMTC cells	+	+	+
	HEK 293 cells	−	−	−
	genomic	+	+	+

(hMTC) cells. Since both cell lines are tumor cells this finding might be related to a relaxation of gene expression in carcinoma cells.

Using degenerate primers only CNG1 specific sequences could be detected in bovine retina, whereas CNG3 specific sequences could not be identified. This finding is probably caused by an overrepresentation of CNG1 specific transcripts in bovine retina mRNA, since the use of CNG3 specific primers P7/P8 resulted in the detection of CNG3 specific PCR products. Despite the use of degenerate oligonucleotide primers we were not able to identify additional members of the CNG channel family. However, we have amplified many nucleotide sequences yet unknown representing CNG1, CNG2 or CNG3 in different species. As can be seen from the alignment (Fig. 2), nucleotide sequence identity among PCR fragments coding for one type of channel amplified from different mammal species is about 90%. In contrast, comparison of the PCR fragments coding for different types of channel within the same species show only 70–77% nucleotide sequence identity. By the use of degenerate oligonucleotide primers P5/P6 a CNG4 specific fragment of 491 bp [Fig. 1(b) and (c)] could be amplified from hMTC cells. Human genomic DNA or cDNA from any bovine (testis, right atrium, kidney), rat (cerebellum, endothelium) or rabbit (brain) tissue, failed as template for specific amplification of a CNG4 related fragment.

Northern blot analysis

Northern blot analysis was performed to identify tissues containing larger amounts of messenger RNA encoding CNG1, CNG2 or CNG3. Previously we have shown that CNG3 specific mRNA (a major species of 4.2 kb and a minor species of 3.0 kb) is present predominantly in bovine testis, but also in bovine kidney cortex and medulla and bovine heart (right atrium, left ventricle) (Biel *et al.*, 1994). Figure 3 shows that hybridization of rabbit colon mRNA with a probe derived from bovine CNG3 resulted in a message slightly larger than in bovine tissues (4.5 kb) and much less abundant than the message in bovine testis. Hybridization of rat heart mRNA with both a CNG3 and a CNG1 specific probe yielded faint signals at 6.4 and 8.4 kb. No signal was observed with the following tissues using probes for CNG1-3: rabbit liver, bovine adrenal gland, bovine diencephalon, rat brain, rat kidney, rat pancreas.

Southern blot analysis

Southern blot analysis (Fig. 4) revealed four different patterns of hybridization, when human genomic DNA digested either with *EcoR*I or *Xba*I was hybridized with probes encoding CNG1, CNG2, CNG3 or CNG4, respectively.

DISCUSSION

We have determined partial sequences of three different CNG channel genes from a variety of excitable and non-excitable tissues and cells (Table 1 and Fig. 2) indicating that CNG channels apart from their role in sensory cells may also play an important role in tissues and cells not involved in sensory signal transduction.

Sequence alignment of PCR fragments coding for one type of channel showed that the nucleotide sequence from different species is about 90% identical (Fig. 2). In contrast, nucleotide sequences of PCR fragments coding for different channels within the same species are only 70–77% identical. This finding supports the hypothesis that despite considerable sequence homology CNG1, CNG2 and CNG3 are encoded by three different genes. Southern analysis of human genomic DNA (Fig. 4) further confirms this view. Hybridization with a CNG1 and a CNG3 specific probe yielded signal patterns completely different from each other. Hybridization with a probe encoding the olfactory channel yielded a combination of signals obtained by hybridization with CNG1 and CNG3 specific probes, but in this case signals were much fainter. The hybridization pattern obtained with the olfactory channel probe is most likely caused by cross hybridization with CNG1 and CNG3 specific DNA fragments. Apart from the signals caused by cross hybridization no further bands were detectable. The finding of one large band by hybridization of *EcoR*I digested human genomic DNA with a rod photoreceptor probe and the lack of a signal when using an olfactory channel probe are in agreement with the results of Dhallan *et al.* (1990).

PCR amplification is a very sensitive method allowing the detection of only a few transcripts per cell. This

CNG1

```
HumGen: TGTGTTCTACTCTATTTCTAAAGCTATTGGATTTGGAAATGATACATGGGTCTACCCTGATATTAATGATCCTGAATTTGGCCGTTTGGCTAGAAAATACGTATACAGCCTTTACTGGTC
Rab   : -----A----------G----------------------G-------A--------CG-----------------T-----------------T--C-----T--G--T-----
Rat   : -----A------C--C--A------C----------G-----C-----------------G-----------------------------------C----------T-----

HumGen: TACACTGACTTTGACTACCATTGGTGAAACACCCCCTCCCGTGAGGGATTCTGAGTATGTCTTTGTGGTGGTTGATTTCCTAATTGGAGTGTTAATTTTTGCTACCATCGTTGGTAACAT
Rab   : ----------C--------------------A-----T---CT-----------------------------C---T-----------------------------T--------T--
Rat   : ---CT---------G--------A-----C--A--C------CT------C--------------------A--C---T----------T--------C--C-----T--C--------

HumGen: AGGTTCTATGATTTCCAACATGAATGCAGCCAGAGCAGAATTTCAAGCAAGAATTGATGCTATCAAGCAATATATGCATTTTCGAAATGTAAGCAAAGATATGGAAAAGAGGGTTATTAA
Rab   : ------------------------C-----T--G----------G------G-C--C--------------C---A----C--C--C--------G--------------A--------
Rat   : ---C--C----------T------------C-G-----------T-----G------------A--G--C---A------------G--------C----------A--------

HumGen: ATGGTTTGACTACCTGTGGACCAACAAAAAAACAGTTGATGAGAAAGAAGTCTTAAAGTATCTACCTGATAAACTAAGAGCAGAAATTGCCATCAACGTTCACTTAGACACATTAAAAAA
Rab   : G----------T----------------G----------A----------G--------------------G----------C--------T----------------------
Rat   : --------------------T--------G-----C-------G------TC-G-GA--C--C-----C-----C--G-----G----------T------C-------G--------
COS   : ————————————————————----------------------------------------------------------------------------T----------------------
CHO   : ————————————————————---------------------G-------GA--C--G-----C--------G----------T-----T-----T----------G-----

HumGen: GGTACGCATTTTTGCTGATTGTGAAGCTGGTCTGTTGGTGGAGTTGGTCTTGAAATTGCAACCCCAAGTCTACAGTCCTGGAGATTATATTTGCAAGAAAGGGGATATCGGACGAGAGAT
Rab   : ---G-------------C--------A-------------A----------G--A--G----------T-----------------------------------T----------
Rat   : ---T--T--C--------C-----G----------------------G--------A--------G--G----------C--A-------------C--T--G--G-----
COS   : ----------------------------------------------------------------------------------------------------------T----------
CHO   : ------A--C--------C-----------------------------G------C-A--------G--G----------C--A-------------C--T--G--------

HumGen: GTACATTATCAAGGAAGGCAAACTCGCTGTGGTGGCAGATGATGGAGTCACTCAGTTTGTGGTATTGAGCGATGGCAGCTACTTCGGTGAGATCAGCATTCTTAACATTAAAGGGAGCAA
Rab   : ------C----------------------------------------A----------GC----T-------------T-----------------------------C-----
Rat   : ------C----------------T-------------C--C---A----A----------G-----T--C----------T--C-------------------C-----C-----
COS   : ----------------------------------------------------------A----------C----------T--------------------T-------------
CHO   : ------C--A--A----------T--------A--C---------A----A----------G-----T-----------------------------C--T--C-----C-----

HumGen: AGCTGGCAATCGAAGAACGGCCAATATTAAAAGTATTGGCTACTCAGACCTGTTCTGTCTCTCA
Rab   : ------------------A----------------------T----------------------
Rat   : G--------C--------A----------G--C----------G----------C------
COS   : ---C-------------C-----------------------------------------------
CHO   : G--------C--C--G--A-------------C----------------------C------
```

CNG2

```
HumGen: CATCTATTATGCCATCTCCAAATCCATAGGCTTTGGGGTCGACACCTGGGTTTACCCAAACATCACTGACCCTGAGTATGGCTACCTGGCTAGGGAATACATCTATTGCCTTTACTGGTC

HumGen: CACACTGACTCTCACTACCATTGGGGAGACACCACCCCCTGTAAAGGATGAGGAGTACCTATTTGTCATCTTTGACTTCCTGATTGGCGTCCTCATCTTTGCCACCATCGTGGGAAATGT

HumGen: GGGCTCCATGATCTCCAACATGAATGCCACCCGGGCAGAGTTCCAGGCTAAGATCGATGCCGTGAAACACTACATGCAGTTCCGAAAGGTCAGCAAGGGGATGGAAGCCAAGGTCATTAG

HumGen: GTGGTTTGACTACTTGTGGACCAACAAGAAGACAGTGGATGAGCGAGAAATTCTCAAGAATCTGCCAGCCAAGCTCAGGGCTGAGATAGCCATCAATGTCCACTTGTCCACACTCAAGAA
COS   : ————————————————————----------------------G----------------------------------------------------------------------T-----

HumGen: AGTGCGCATCTTCCATGATTGTGAGGCTGGCCTGCTGGTAGAGCTGGTACTGAAACTCCGTCCTCAGGTCTTCAGTCCTGGGGATTACATTTGCCGCAAAGGGGACATCGGCAAGGAGAT
COS   : ----------------------------------------------------T----------------A--------------------------T--------------------------

HumGen: GTACATCATTAAGGAGGGCAAACTGGCAGTGGTGGCTGATGATGGTGTGACTCAGTATGCTCTGCTGTCGGCTGGAAGCTGCTTTGGCGAGATCAGTATCCTTAACATTAAGGGCAGTAA
COS   : ----------------------G----------------------G--------------C-----C-T------------------T--------------------------A-----

HumGen: AATGGGCAATCGACGCACAGCTAATATCCGCAGCCTGGGCTACTCAGATCTCTTCTGCTTGTCC
COS   : ------------------G-----------------------------C---------------
```

CNG3

```
HumGen: CATCTACTTTGCCATTTCCAAGTTCATTGGTTTTGGGACAGACTCCTGGGTCTACCCAAACATCTCAATCCCAGAGCATGGGCGCCTCTCCAGGAAGTACATTTACAGTCTCTACTGGTC
Rab   : ---------------C-----------A-----C---------------------------------C-AG------T-C-----T----------------------C----------

HumGen: CACCTTGACCCTTACCACCATTGGTGAGACCCCACCCCCCGTGAAAGATGAGGAGTATCTCTTTGTGGTCGTAGACTTCTTGGTGGGTGTTCTGATTTTTGCCACCATTGTGGGCAATGT
Rab   : ----C-------G--------C--G--------------T-----G--C--------------C--------C------C-------A--G--------C--------C--------C--

HumGen: GGGCTCCATGATCTCGAATATGAATGCCTCACGGGCAGAGTTCCAGGCCAAGATTGATTCCATCAAGCAGTACATGCAGTTCCGCAAGGTCACCAAGGACTTGGAGACGCGGGTTATCCG
Rab   : ---------------C--C-----C-----G----------------------C--C--------A----------------------G--------------------C--G-----

HumGen: GTGGTTTGACTACCTGTGGGCCAACAAGAAGACGGTGGATGAGAAGGAGGTGCTCAAGAGCCTCCCAGACAAGCTGAAGGCTGAGATCGCCATCAACGTGCACCTGGACACGCTGAAGAA
COS   : ————————————————————----------------------------------------------------------C----------------------------------------
Rab   : --————————————————————————————————————————————————————————————————————————————————————————————————————————————————

HumGen: GGTTCGCATCTTCCAGGACTGTGAGGCAGGGCTGCTGGTGGAGCTGGTGCTGAAGCTGCGACCCACTGTGTTCAGCCCTGGGGATTATATCTGCAAGAAGGGAGATATTGGGAAGGAGAT
COS   : ------------------------A--G----------------------A-----A--G-----------------------------------------------C----------

HumGen: GTACATCATCAACGAGGGCAAGCTGGCCGTGGTGGCTGATGATGGGGTCACCCAGTTCGTGGTCCTCAGCGATGGCAGCTACTTCGGGGAGATCAGCATTCTGAACATCAAGGGGAGCAA
COS   : ------------T--------------------A--C-----------------------------------------------------------------C----------------

HumGen: GTCGGGGAACCGCAGGACGGCCAACATCCGCAGCATTGGCTACTCAGACCTGTTCTGCCTCTCA
COS   : A-----------------------------------------------------T--------C
```

Fig. 2. Aligned nucleotide sequences of PCR products coding for the same type of channel from different species. CNG1 specific fragments were amplified from human genomic DNA, rabbit pancreas and rat testis first strand cDNA using primers P3/P2 and from COS and CHO cell first strand cDNA using primers P1/P2. CNG2 specific fragments were amplified from human genomic DNA using primers P3/P2 and from COS cell first strand DNA using primers P1/P2. CNG3 specific primers were amplified from human genomic DNA using primers P3/P2, from rabbit colon first strand DNA using primers P3/P4 and from COS cell first strand DNA using primers P1/P2. The sequences of oligonucleotide primers used in PCR reactions have not been included in this figure. HumGen: human genomic DNA; rab: rabbit; COS: COS cells (monkey cell line); CHO: Chinese hamster ovary cells; ---: Identity with the respective sequence from human genomic DNA; ——: Sequence not amplified.

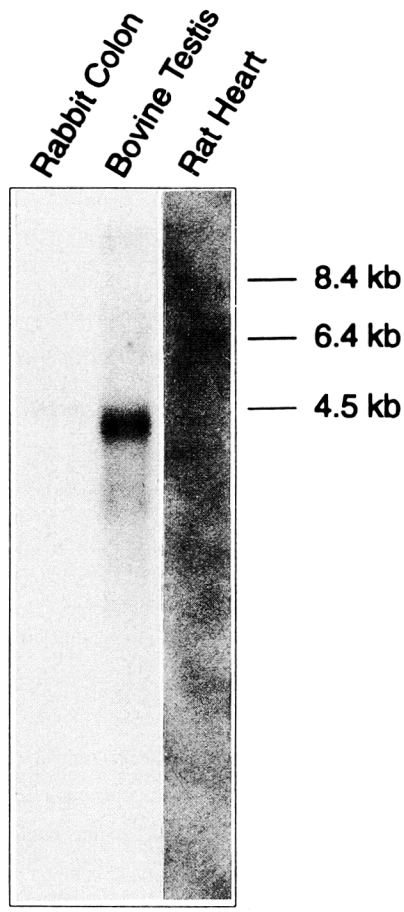

Fig. 3. Northern blot analysis of CNG channel expression. Ten μg rabbit colon mRNA and 4 μg bovine testis mRNA were hybridized with α^{32}P-labelled probe derived from bovine CNG3. Ten μg rat heart mRNA was hybridized with a mixture of bovine CNG1 and CNG3 specific probes. Exposure time was 6 days with rat heart and rabbit colon mRNA and overnight with bovine testis mRNA.

implies that the identification of a sequence by PCR does not necessarily prove the functional importance of the respective gene product in the cell. To identify tissues in which CNG channel specific transcripts are frequent we therefore performed Northern blot analysis (Fig. 3) which is a much less sensitive detection system. Recently we have shown that CNG3 specific transcripts are expressed mainly in bovine testis, kidney and heart (Biel *et al.*, 1994). This study shows that a probe derived from bovine CNG3 also detects a mRNA species of 4.5 kb in rabbit colon. Sequencing of PCR products derived from rabbit colon cDNA confirmed that the transcript is derived from rabbit CNG3 gene (Fig. 2). Biochemical assays and immuno-histochemical studies will be necessary to confirm the expression of functional CNG3 channels at the protein level. Hybridization of rat heart mRNA with both a CNG1 and a CNG3 specific probe yielded signals at 6.4 and 8.4 kb, which is much larger than the transcripts expected for CNG1 (3.2 kb, Pittler *et al.*, 1992) and CNG3 (4.2 kb, Biel *et al.*, 1994). Since PCR analysis of rat heart mRNA did not yield CNG1 or CNG3 specific fragments the signals obtained with rat heart mRNA might not be related to the genes encoding CNG1 or CNG3.

Despite the use of degenerate oligonucleotide primers which fit to all sequences of CNG channels yet known in mammals, all PCR products amplified encoded CNG1, CNG2 or CNG3. From this it is likely that apart from the three genes coding for CNG1, CNG2 and CNG3 there are no further genes belonging to the same gene family. However, it should be noted that there may still be genes differing significantly in terms of gene structure and nucleotide sequence. We cannot exclude that additional members of the CNG channel family could not be detected by PCR due to primer mismatch or other intrinsic reasons. The rod and the cone photoreceptor channel has been cloned from chicken retina (Bönigk *et al.*, 1993). Both channels are only 66.8% homologous to each other. This is in the range of amino acid identity among CNG1, CNG2 and CNG3 and suggests that like CNG1, CNG2 and CNG3 chicken rod and cone photoreceptor channels are the products of two different genes. Possibly CNG3 is also expressed in bovine cone photoreceptor cells which is supported by the following findings: (1) we were only able to amplify PCR fragments encoding CNG1, CNG2 and CNG3 and did not succeed in finding a novel sequence that might correspond to the sequence of the cone photoreceptor channel in mammals. (2) Bovine CNG3 and the chicken cone photoreceptor channel differ in about 22% of their amino acids. As chicken and bovine rod photoreceptor channels also differ in 24.3% of their amino acid sequence, the sequence differences between chicken cone photoreceptor channel and bovine CNG3 can be explained by the distant relationship between birds and mammals. (3) By the use of degenerate primers all PCR fragments amplified from bovine retina encoded CNG1. However, using specific primers P7/P8 we were able to amplify also PCR fragments coding for CNG3 proving that CNG3 specific transcripts are present in bovine retina. The fact that specific primers are necessary to amplify PCR products encoding CNG3 reflects the anatomy of mammal retina, where rods are much more frequent than cones.

By the use of degenerate primers we were able to amplify homologous sequences encoding all three channels also from human genomic DNA (Fig. 2). This shows that within the sequences amplified none of the three genes possesses an intron. The human rod photoreceptor channel contains at least 10 exons (Dhallan *et al.*, 1992). One large exon encodes the carboxyterminal two thirds of the protein comprising also the region amplified by primers P1–P4, whereas seven small exons encode the aminoterminal one third of the protein. Our results suggest that the gene structure of CNG2 and CNG3 may be similar. Using degenerate primers P5/P6 we could amplify a PCR fragment encoding the auxiliary subunit CNG4 from hMTC cells. This indicates that also in peripheral tissues CNG channels may be associated with an auxiliary subunit.

PCR amplification of CNG4 was not possible from

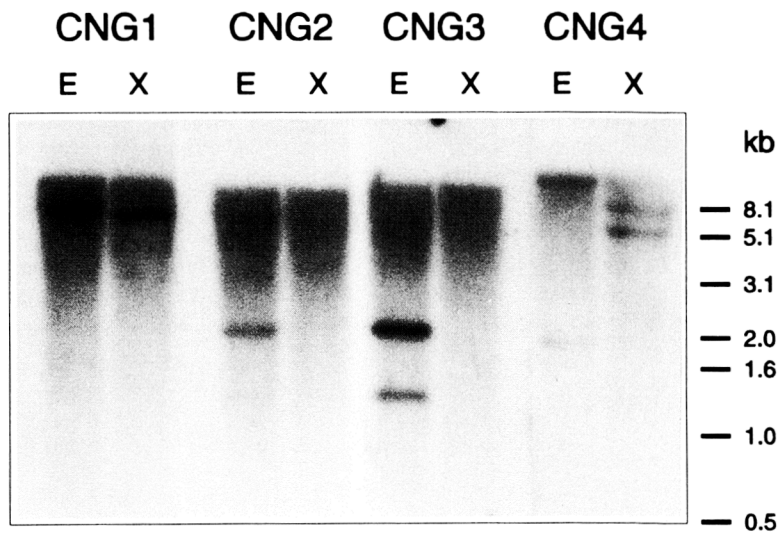

Fig. 4. Southern blot analysis: Ten μg of human genomic DNA digested with either *EcoR*I(E) or *Xba*I (X) were hybridized with α^{32}P-labelled CNG1, CNG2, CNG3 or CNG4 specific probe,respectively. Exposure time was 6 days.

the tested bovine, rabbit or rat tissues. This may be due to primer incompatibility since only the human nucleotide sequence of CNG4 is known. From human genomic DNA CNG4 could not be amplified either suggesting the presence of an intron within the sequence.

Hybridization of human genomic DNA digested either with *EcoR*I or *Xba*I with the PCR product amplified from hMTC cells using primers P5/P6 generates a hybridization pattern completely different from the patterns obtained by hybridization with probes coding for CNG1, CNG2 and CNG3 (Fig. 4). The presence of a distinct gene encoding CNG4 is in accordance with the big difference in amino acid sequence of CNG4 compared to CNG1, CNG2 and CNG3.

The finding that CNG channels are expressed in such a variety of tissues not involved in sensory signal transduction suggests that they might play a functional role in these tissues as well. Such role would be expected especially in tissues expressing CNG channel specific transcripts in abundance, which for CNG3 is the case with testis, kidney, heart and colon. In all these tissues cGMP is established as an important second messenger. In addition there is evidence for the presence of nitric oxide in these tissues which activates soluble guanylyl cyclase. Thus it is tempting to speculate that a NO-mediated increase in cGMP concentration besides stimulating cGMP dependent kinases and phosphodiesterases exerts some of its effects by activating a CNG cation channel.

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