

A Second Subunit of the Olfactory Cyclic Nucleotide-Gated Channel Confers High Sensitivity to cAMP

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Summary

Sensory transduction in olfactory neurons is mediated by intracellular cAMP, which directly gates a nonselective cation channel. A cDNA encoding a cyclic nucleotide-gated (CNG) ion channel subunit (rOCNC1) has been cloned previously from rat olfactory epithelium. However, differences between the functional properties of rOCNC1 and the native olfactory CNG channel suggest that the native channel could be composed of several distinct subunit types. Here, we report the cloning and characterization of a cDNA encoding a second olfactory CNG channel subunit (rOCNC2) that is 52% identical to rOCNC1 and that is expressed specifically in olfactory sensory neurons. Expression of rOCNC2 alone in *Xenopus* oocytes does not lead to detectable CNG currents. However, coexpression of rOCNC2 with rOCNC1 results in a CNG conductance that differs from that detected upon expression of rOCNC1 alone and more closely resembles the native conductance in several respects, including its sensitivity to cAMP. This suggests that the native olfactory CNG channel is a hetero-oligomer composed of rOCNC1 and rOCNC2 subunits.

Introduction

Sensory transduction in olfactory neurons appears to involve the generation of intracellular cyclic AMP (cAMP) and the opening of a cyclic nucleotide-gated (CNG) channel (Firestein, 1992; Reed, 1992; Ronnett and Snyder, 1992). Transduction is initiated by the binding of odorants to specific odorant receptors on the cilia of the olfactory neurons (Rhein and Cagan, 1980, 1983). These receptors couple to a GTP-binding protein that stimulates adenylate cyclase and the synthesis of cAMP (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Breer et al., 1990). cAMP acts directly on a CNG channel, the opening of which allows an influx of Na^+ and Ca^{2+} ions (Nakamura and Gold, 1987; Kurahashi and Shibuya, 1990; Firestein et al., 1991; Lowe and Gold, 1993). Biochemical studies (Pace et al., 1985; Sklar et al., 1986; Boekhoff et al., 1990; Breer et al., 1990), patch clamp recordings (Nakamura and Gold, 1987; Firestein et al., 1991; Lowe and Gold, 1993), and, more recently, molecular cloning have lent support to this mechanism. Many of the components of this transduction pathway have been cloned, including a family of odorant receptors (Buck and Axel, 1991), a G protein (Jones and Reed, 1989), an adenylate cyclase (Bakalyar and Reed, 1990), and a CNG channel (Dhallan et al., 1990; Ludwig et al., 1990; Goulding et

al., 1992). Each is homologous to related proteins in other signaling pathways; for instance, the CNG channel is much like that mediating phototransduction in the retina (Kaupp et al., 1989). An alternative pathway has been proposed that involves the generation of 1,4,5-trisphosphate inositol (IP_3), but the details of this mechanism are as yet poorly understood (Boekhoff et al., 1990).

Olfactory CNG channels were first detected in patch-clamp recordings from the cilia of freshly dissociated toad olfactory sensory neurons (Nakamura and Gold, 1987) and have since been studied in a number of species, including frog (Frings et al., 1992; Kolesnikov et al., 1990), salamander (Zufall et al., 1991), catfish (Bruch and Teeter, 1990; Goulding et al., 1992), and rat (Frings et al., 1992). The CNG channels in olfactory sensory neurons of all species share a common feature: a high sensitivity to cAMP, with concentrations for half-maximal activation ($K_{1/2}$) on the order of 2–20 μM . The $K_{1/2}$ for cGMP activation of these channels is only slightly lower (1–4 μM). In the retina, in contrast, cGMP mediates sensory transduction (Yau and Baylor, 1989), and retinal CNG channels have a $K_{1/2}$ for cGMP that is 30-fold lower than their $K_{1/2}$ for cAMP (Tanaka et al., 1989; Altenhofen et al., 1991). Other properties of the olfactory CNG channels are not as well conserved. Single-channel conductance varies widely across species, from 12–19 pS for rat and frog (Kolesnikov et al., 1990; Frings et al., 1992) to 45 pS for salamander and catfish (Bruch and Teeter, 1990; Zufall et al., 1991).

Based on homology with the sequence of a retinal CNG channel from cow (Kaupp et al., 1989), cDNAs encoding CNG channels have been cloned from olfactory epithelium of rat (Dhallan et al., 1990), cow (Ludwig et al., 1990), and catfish (Goulding et al., 1992). The olfactory CNG channels are highly homologous to one another and to the retinal CNG channels. In addition, a surprising homology has been found between these ligand-gated ion channels and voltage-gated ion channels (Jan and Jan, 1990). The homology, which is most notable in the proposed pore-lining and voltage-sensing regions of the channel sequences (Heginbotham et al., 1992; Goulding et al., 1992), suggests that the two classes of proteins share a common transmembrane topology. A cyclic nucleotide-binding domain, comprising a stretch of 80–100 amino acids near the carboxyl terminus of the channel, has been identified by homology with the cGMP-binding domain of cGMP-dependent kinases (Kaupp et al., 1989). Recently, the assignments of the pore region and the cyclic nucleotide-binding domains have gained support through examination of the effects of specific mutations on channel function (Altenhofen et al., 1991; Heginbotham et al., 1992; Goulding et al., 1993; Root and MacKinnon, 1993; Eismann et al., 1994; Kramer et al., 1994). The stoichiometry of the CNG

channels is at present unknown. The homology with K⁺ channels, which are tetramers (MacKinnon, 1991; Liman et al., 1992), suggests that the CNG channel is also composed of four subunits. The activation of the channels by cyclic nucleotide is fit with a Hill equation with a coefficient of 1–3. This finding, along with the observation that each subunit contains a single cyclic nucleotide-binding domain (Kaupp et al., 1989), suggests that at least three, and probably four or more subunits contribute to the channel.

Cloned olfactory CNG channels have been functionally expressed in *Xenopus laevis* oocytes and mammalian cell lines (Dhallan et al., 1990; Ludwig et al., 1990; Goulding et al., 1992). In these systems, the channels are presumably formed as homo-oligomers of the subunit encoded by the cDNA. Although the functional properties of the cloned channels resemble in many ways those of the native olfactory CNG channels (Nakamura and Gold, 1987; Frings et al., 1992; Goulding et al., 1992), some significant differences are apparent. The most striking of these is a difference in sensitivity to cAMP: the cloned channels are ~30-fold less sensitive to cAMP than are the native channels (Nakamura and Gold, 1987; Dhallan et al., 1990; Frings et al., 1992; Goulding et al., 1992). This discrepancy could be due to differences between the olfactory neurons and the heterologous expression systems in posttranslational processing or in the phosphorylation state of the channels. Alternatively, it could be that additional subunits of the CNG channel are missing from the expression system.

Recently, it has been found that retinal rods express two distinct genes for CNG channel subunits and that the native rod channel is likely to be a hetero-oligomer of the two subunits (Chen et al., 1993). The second subunit cloned (hRCNC2) has the interesting property of not forming functional channels when expressed alone in a heterologous system. However, when coexpressed with the previously identified rod subunit (hRCNC1), channel activity is detected that differs from that seen with hRCNC1 alone and more closely resembles that of the native channel (Chen et al., 1993).

We have used the polymerase chain reaction (PCR) to look for the existence of additional subunits of the olfactory CNG channel. Here, we report the cloning of a cDNA from rat olfactory epithelium that encodes a new member of the family of CNG channels (rOCNC2). Using the *Xenopus* oocyte expression system, we find that, like the second retinal rod subunit, this subunit is unable to form a functional homo-oligomer. However, hetero-oligomeric channels are formed between rOCNC2 and rOCNC1 that display many of the properties of the native olfactory CNG channels.

Results

Cloning of a Second Rat Olfactory CNG Channel Subunit

We designed degenerate PCR primers that were ex-

pected to recognize all previously identified members of the CNG channel family (Kaupp et al., 1989; Dhallan et al., 1990, 1992; Ludwig et al., 1990; Biel et al., 1993; Bönigk et al., 1993; Chen et al., 1993). The primers corresponded to highly conserved sequences of amino acids in the proposed cyclic nucleotide-binding domain of CNG channels. These primers were used in PCR reactions with template cDNA prepared from mouse olfactory epithelium RNA (Buck and Axel, 1991). A restriction digest of the PCR products revealed the existence of at least two distinct species. Subcloning of the PCR products and sequence analysis of the recombinants demonstrated the presence of a product encoding a protein that has a high degree of homology with members of the CNG channel family. Screening of a rat olfactory epithelium cDNA library with this PCR product yielded a cDNA clone, λrOCNC2.

λrOCNC2 encodes a protein of 575 amino acids, whose sequence is aligned in Figure 1 with the amino acid sequence of the previously identified rat olfactory CNG channel, rOCNC1. The rOCNC2 sequence shares 52% amino acid identity with the rat and bovine olfactory CNG channels (Dhallan et al., 1990; Ludwig et al., 1990), 49% identity with the catfish olfactory channel (Goulding et al., 1992), 51% identity with the bovine rod channel (Kaupp et al., 1989), and 30% identity with a second subunit of the rod channel (Chen et al., 1993). The putative transmembrane domains of rOCNC1 and rOCNC2 were assigned on the basis of sequence homology with voltage-activated K⁺ channels, as in Goulding et al., 1992. In Figure 1, these are labeled S1–S6, and the putative pore-forming region is denoted by "p." The most striking feature of the rOCNC2 sequence is its very short amino terminus, which extends only 33 amino acids beyond the beginning of the first proposed transmembrane domain. By comparison, the amino-terminal sequences of other members of the CNG channel family vary between 115 and >300 amino acids in length, with the sole exception of a splice variant of the second rod channel (Chen et al., 1993). A portion of this sequence may be subject to proteolytic cleavage (Molday et al., 1991). The amino terminus has been shown to play a role in subunit assembly of voltage-activated K⁺ channels (Li et al., 1992; Shen et al., 1993) and in coupling of ligand binding to channel opening in CNG channels (Goulding et al., 1994, *Biophys. J.*, abstract). Thus, a lack of important functional domains in the amino terminus of the rOCNC2 sequence may explain a failure of the rOCNC2 subunits to form detectable homo-oligomeric channels, as described below.

Based on homology with other proteins and on mutagenesis studies, functional roles have been assigned to many regions of CNG channels. An acidic residue in the pore region of the channel has been shown to contribute to a divalent cation-binding site (Root and MacKinnon, 1993; Eismann et al., 1994). The conservative substitution in the sequence of rOCNC2 (marked with an asterisk) at this position may thus have implications for the permeation and blocking affinity of

rOCNC2									
rOCNC1 MMTEKSNGVKSSPANNHHPPPSIKANGKDDHRRGSRPQSVAADDTSPELQRLAEMDTPRRGFFQRIVRLGVIRDWANKNFRREEEPRPDSFLERF	100								
rOCNC2MSQDGKVKTTESTPPAPTKARKWLPVLDPSGDYYYWWLNTMVFIMYNLIIIVCRACFPDQLQHSYLVWFVLDYTS DLLYLLDICVRFH G	92								
rOCNC1 RGPELQTVTTHQGDDKGKDGEKGKTFKLFLVLDPAGDWYYRWLFVIAMPVLYNWCLLVARACFSDLQRNYFVWLVDYFSDTVYIADLIRLRTGF	200								
	S3	S4	S5						
rOCNC2 LEQGILVVVKGMIA SR YR TWSFLLDLASLVP TDAAYVQLGPHIP[TLRLRNRFLRVPRLFEAFDRTE]TRTAYPNAFR[AKLMLYI FVVIHWN SCLYFAL S R	192								
rOCNC1 LEQGLLVVKDPKKLRDNY[TLQFKLDVASIPTDLYFAVG IHSPEVRFNRLLHARMFFFDRTE]TRTSYPNIF[SNLVLVYI LVI I HWNACI YYVISK	300								
	p	*	S6						
rOCNC2 YLGFG RDAWVYPPA QPGF ERLR RQYLYSFYFSTL LTTVGDTPLPDREEEYL FMVGDFELLAVMGFATIMGSMSSVIYNMNTADA AFY PDHALVKKY M K L	292								
rOCNC1 SIGFGVDTWVYPNITDPEGYLA REYI CYLWSTLT LTTIGETPPV[KDEEYLFVIFDFLIGV LIFATIVGNVGS M IS MNATRAEFQAKIDAVKH YM QF	400								
rOCNC2 QHV NKR LERR VIDI WYQHLQINKKMTNE VAI LQHL PERL RAEV AVA SVHL STLSRVQIFQNCEASLLEELV LK LQ P QTYSPGEYVCRKG D I G R E M Y I I R E Q Q	392								
rOCNC1 RKVS KDM EAKV KWF DYLW TNK KVTD EREV LK NL PA KLR A EIA IN VH LST LKK V RIF QDCEA GLL V EL VL KLR PQV FSPG DY I CRKG D I G K E M Y I I K EG K	500								
	**								
rOCNC2 LAVV ADDG VTO A VLGAG LYFGE I S I I N I K G N M S G N R R T A N I K S L G Y S D L F C L S K E D L R E V L S E Y P Q A Q A V M E E K G R E I I L K M N K L D V N A E A E I A L Q E A	492								
rOCNC1 LAVV ADDG VTO A VLGAG LYFGE I S I I N I K G N M S G N R R T A N I R S L G Y S D L F C L S K D D L M E A V T E Y P D A K K V L E E R G R E I L M K E G L D E N E V A A S M E V D .	600								
rOCNC2 TESRLKG LDQQ LDD LQTKFARLLA ELESSALKI AYRIERLEWQ TREW PMP ED MG EAD DEAE PGE GTSKD GEKG KAG QAG P SGIE	575								
rOCNC1 VQE KLE QLET NM D TLYTRF ARLLA EYTGA QQKL KQ RITV L ETKM KQ NHEDD Y L S D G I N T P E P T A E E	664								

Figure 1. The Dededuced Amino Acid Sequence of rOCNC2 Aligned with That of rOCNC1

The sequence for rOCNC1 was deduced by Dhallan et al. (1990). Vertical bars and colons between the two sequences indicate identical residues and conservative substitutions, respectively. By homology with the voltage-gated K^+ channels, the proposed transmembrane domains (enclosed in boxes) are labeled S1-S6, and the proposed pore region is indicated by a "p" (Jan and Jan, 1990; Goulding et al., 1992). The horizontal bar over the sequence indicates the cyclic nucleotide-binding domain (Kaupp et al., 1989). Residues of proposed functional significance that are conserved among other members of the CNG channel family but are not found in rOCNC2 are marked with asterisks (Kaupp et al., 1989; Dhallan et al., 1990; Ludwig et al., 1990; Goulding et al., 1992; Bönigk et al., 1993). The percentage of sequence identity reported in the text is for the region of overlap. For a comparison between rOCNC1 and rOCNC2, this is amino acids 1-557 of the rOCNC2 sequence.

divalent cations (Root and MacKinnon, 1993; Eismann et al., 1994). In the fourth transmembrane domain, the proposed voltage sensor of voltage-dependent channels, rOCNC2 has a net charge of +2, which is more net positive charge than is found in the S4 region of rOCNC1 and may affect the voltage dependence of gating of the channel. Surprisingly, amino acid residues in the cyclic nucleotide-binding domain of the channel are highly conserved. The threonine residue in the sequence GNRRTAN is thought to stabilize the binding of cGMP but not cAMP (Weber et al., 1989), and mutant channels in which this residue has been replaced by an alanine show a reduced affinity for cGMP relative to cAMP (Altenhofen et al., 1991). Thus, substitution of this residue might have been expected in the sequence of an olfactory channel. Although this residue is conserved in rOCNC2, sequence divergence at nearby residues might alter the conformation of the cyclic nucleotide-binding site to reduce the selectivity for binding of cGMP.

Analysis of the Pattern of Expression of rOCNC2

The tissue specificity of rOCNC2 expression was examined by Northern blot analysis with a ^{32}P -labeled probe prepared from the coding and noncoding regions of the rOCNC2 cDNA. Figure 2 shows that hybridization of this probe was evident only to mRNA

from olfactory epithelium and not to mRNA derived from a number of other tissues. Two hybridizing species were present in olfactory epithelium RNA, a major species of ~2.7 kb and a minor species of ~3.3 kb.

In situ hybridization experiments further demonstrated the expression of the rOCNC2 transcript in the olfactory epithelium. Figure 3 shows coronal sections through the nasal cavity of a 7-day-old rat that were hybridized to ^{35}S -labeled antisense RNA probes prepared from cDNAs encoding rOCNC2, rOCNC1, or olfactory marker protein (OMP). OMP is expressed by all mature olfactory neurons (Danciger et al., 1989), and thus, the full extent of the olfactory epithelium was delineated by the hybridization signal obtained with the OMP probe. The rOCNC2 probe clearly hybridized throughout the entire extent of the olfactory epithelium. Moreover, the pattern of expression of rOCNC2 was indistinguishable from that of rOCNC1. No hybridization was seen with control sense strand probes. Higher magnification of a section hybridized with rOCNC2 and counterstained with an Hoechst nuclear dye is also shown in Figure 3 (lower right). The hybridization to rOCNC2 is seen only in the layer of the epithelium that contained the cell bodies of mature olfactory neurons. Note that neither the apical cytoplasm of supporting cells nor stem cells at the base of the epithelium are labeled. These data indicate

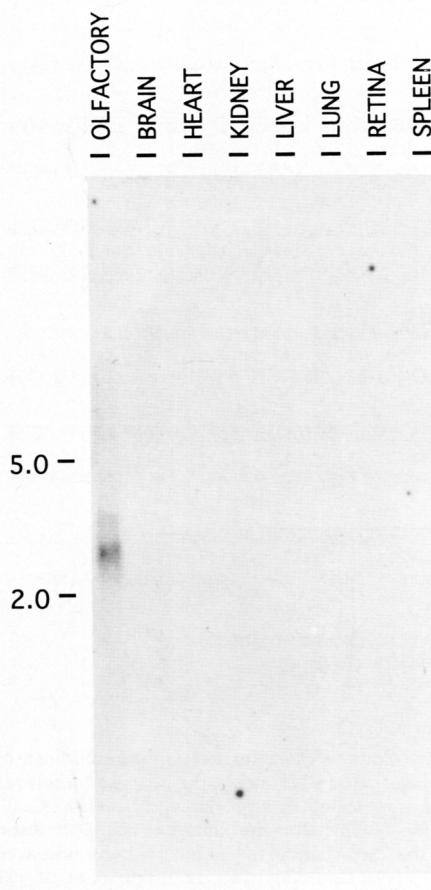


Figure 2. Tissue-Specific Expression of *rOCNC2*

A Northern blot probed with 32 P-labeled *rOCNC2* cDNA shows hybridization only to olfactory epithelium RNA, with a major hybridizing species of ~ 2.7 kb and a minor species of ~ 3.3 kb. Each lane contains 1 μ g of poly(A)⁺ RNA.

that *rOCNC2* is expressed specifically in olfactory sensory neurons and that single neurons are likely to express both *rOCNC1* and *rOCNC2*.

Formation of Functional Channels as Hetero-Oligomers of *rOCNC2* and *rOCNC1*

To study the functional properties of *rOCNC2*, *Xenopus* oocytes were injected with *in vitro* transcribed *rOCNC2* RNA. Oocytes were assayed for the expression of CNG channels by patch-clamp recording. Excised inside-out patches from these oocytes failed to respond to bath application of 1 mM cGMP. This result was observed for 2 distinct clones of *rOCNC2* and in many experiments. *In vitro* translation indicated that the *rOCNC2* RNA was competent to direct the synthesis of a protein of the expected molecular weight (data not shown). Thus, failure to observe expression of CNG channels was likely due either to failure of the *rOCNC2* protein to incorporate into the oocyte membrane or to form a functional homo-oligomeric channel.

The structural homology between CNG channel subunits and voltage-activated K⁺ channel subunits

suggests that the functional CNG channel is an oligomer, composed of identical or nonidentical subunits. Thus, to test whether *rOCNC2* could function as a subunit of a hetero-oligomeric channel, we coexpressed it with *rOCNC1*. Oocytes were injected with approximately equal amounts of *rOCNC2* and *rOCNC1* RNA (*rOCNC1* + *rOCNC2*; Figure 4), and the resulting currents were compared to those recorded from oocytes injected with *rOCNC1* RNA alone (*rOCNC1*). Excised patches from oocytes injected with either *rOCNC1* or *rOCNC1* + *rOCNC2* RNAs responded with large macroscopic currents to bath application of cAMP or cGMP. Figure 4 shows currents evoked from a *rOCNC1* and a *rOCNC1* + *rOCNC2* patch in response to a 1–2 s application of 10 or 500 μ M cAMP to the intracellular surface of the patch. Two differences between the currents are immediately apparent from this figure: *rOCNC1* + *rOCNC2* currents are more sensitive to cAMP, and they show desensitization at high cAMP concentrations. This suggests that hetero-oligomeric channels are formed upon cojunction of the two RNAs.

Cyclic Nucleotide Sensitivity of *rOCNC1* + *rOCNC2* Hetero-oligomers

Native rat olfactory CNG channels and the expressed *rOCNC1* channels differed in sensitivity to cyclic nucleotide. To examine whether this discrepancy might be due to the expression of the *rOCNC2* subunit by olfactory neurons, we measured the sensitivity to cyclic nucleotide of the *rOCNC1* + *rOCNC2* currents. Ramp depolarizations from -80 to $+80$ mV were applied in the presence of varied concentrations of cAMP or cGMP in the solution bathing the intracellular surface of the patch. For currents that decayed with time (see Figure 4), ramp depolarizations were recorded only during the maintained phase of the current. A family of ramp depolarizations at different concentrations of cAMP is shown in Figure 5B for a *rOCNC1* + *rOCNC2* patch and is compared with currents recorded from a *rOCNC1* patch (Figure 5A). Large currents were apparent in the *rOCNC1* + *rOCNC2* patch upon exposure to concentrations of cAMP as low as 3.3 μ M, whereas the *rOCNC1* currents were detected only at a much higher concentration of cAMP (33 μ M). Also apparent from this figure is a difference in outward rectification of the currents, which is discussed below.

Dose-response curves for cAMP were obtained by measuring the evoked current at a $V_m = -80$ or $+80$ mV and normalizing this value to the amplitude of the current obtained at saturating concentrations of cAMP. The mean data for $V_m = -80$ mV are plotted in Figure 6A for a set of *rOCNC1* + *rOCNC2* patches and are compared with the mean data for a set of *rOCNC1* patches. Least squares fitting of the data from individual experiments to the Hill equation gave the parameter values listed in Table 1. Also listed in Table 1 are values for the $K_{1/2}$ obtained at $+80$ mV. At -80 mV, the $K_{1/2}$ for the *rOCNC1* + *rOCNC2* currents

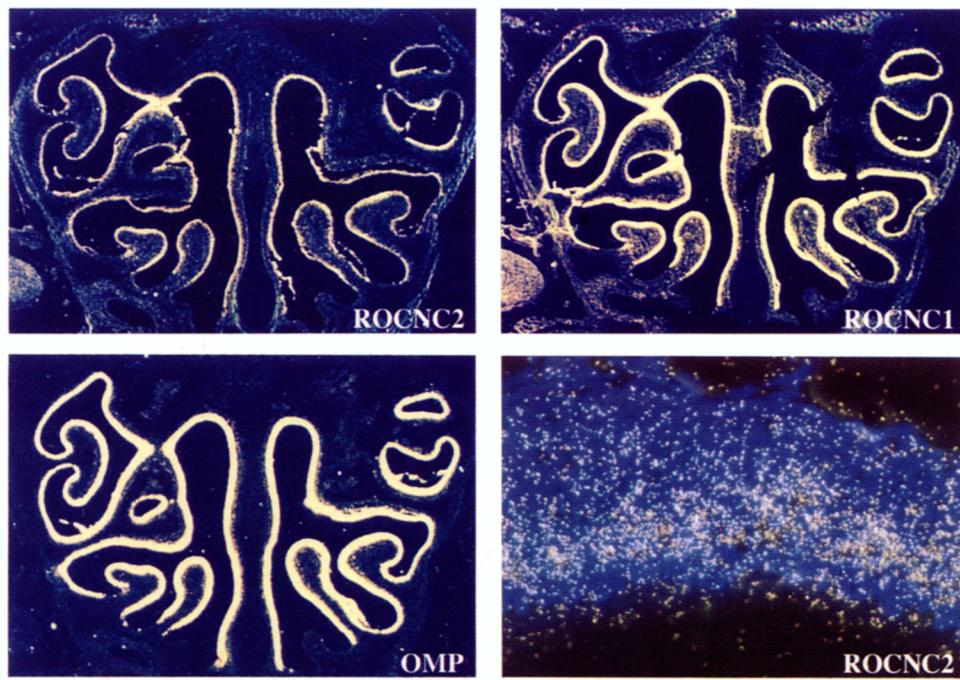


Figure 3. Distribution of rOCNC2 Transcript within the Rat Olfactory Epithelium

Coronal sections ($8 \mu\text{m}$) through the rat nasal cavity were hybridized with ^{35}S -labeled antisense RNA probes prepared from DNAs encoding rOCNC2, rOCNC1, or OMP. The nasal septum is in the center with the two nasal cavities on either side. The scale bar at bottom right equals $1 \mu\text{m}$. A higher magnification view of a section hybridized with the rOCNC2 probe and then stained with the nuclear dye (Hoechst No. 33258) is shown in the lower right panel. Grains indicative of hybridization are concentrated in the layer of the epithelium that contains mature olfactory neurons in the 7-day-old rat. The nasal lumen is at the bottom of the photograph. For this panel the scale bar equals $31 \mu\text{m}$.

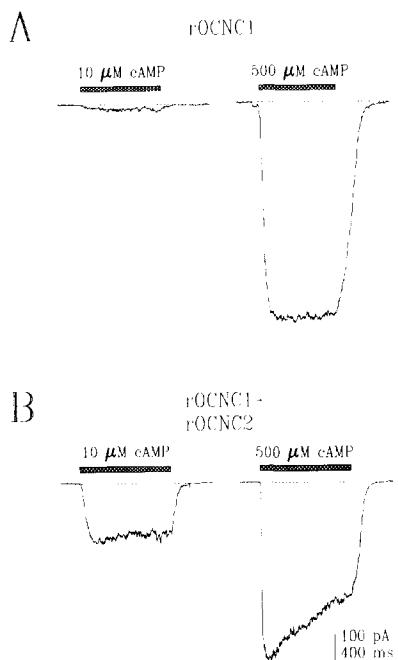


Figure 4. Macroscopic rOCNC1 and rOCNC1 + rOCNC2 Currents

(A) Inward currents were recorded from an inside-out patch excised from an oocyte injected with rOCNC1 RNA in response to bath application of $10 \mu\text{M}$ or $500 \mu\text{M}$ cAMP. The striped bar

is $16 \mu\text{M}$. This is four times lower than the value of $62 \mu\text{M}$ for the rOCNC1 currents and is not far from the $K_{1/2}$ of the native olfactory CNG channel ($3\text{--}4 \mu\text{M}$; Nakamura and Gold, 1987; Frings et al., 1992).

The sensitivity to cGMP was also measured. For both rOCNC1 and rOCNC1 + rOCNC2 patches, the maximal current evoked with saturating concentrations of cGMP was not significantly different from the maximal current evoked with saturating concentrations of cAMP. Dose-response curves obtained at $V_m = -80 \text{ mV}$ are shown in Figure 6B, and the means of the fits to the Hill equation for $V_m = -80 \text{ mV}$ and $+80 \text{ mV}$ are given in Table 1. The rOCNC1 + rOCNC2 channels were slightly less sensitive to cGMP ($K_{1/2} = 5.2 \mu\text{M}$) than were the rOCNC1 currents ($K_{1/2} = 2.3 \mu\text{M}$).

A distinctive feature of the native olfactory CNG channels is that they have a similar affinity for cAMP as for cGMP, with a $K_{1/2}$ ratio of 2:1, respectively (Frings et al., 1992; Nakamura and Gold, 1987). The rOCNC1 + rOCNC2 channels resembled the native olfactory channels in this respect, with a ratio of 3:1. The rOCNC1 currents, on the other hand, had a ratio of

indicates the approximate time of application of the cyclic nucleotide. The holding potential was -80 mV .

(B) Same as in (A) for patch excised from an oocyte injected with rOCNC1 + rOCNC2 RNA.

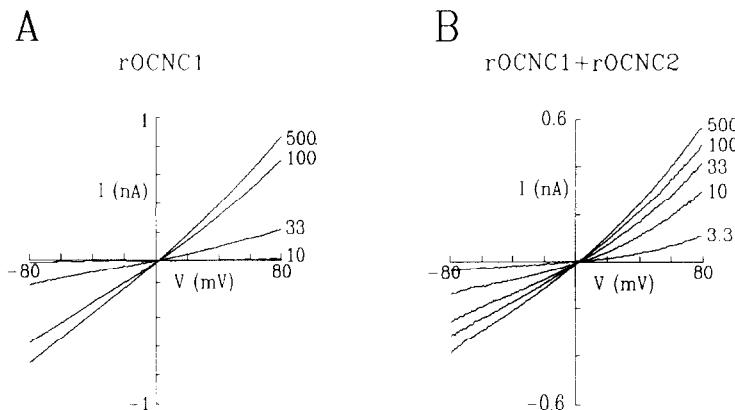


Figure 5. Current-Voltage Relations for rOCNC1 and rOCNC1 + rOCNC2 Currents at Different Concentrations of cAMP

(A and B) The traces show the response to a ramp depolarization from -80 to 80 mV (time for ramp was 500 ms) in the presence of varying micromolar concentrations of cAMP (indicated beside each trace). Each trace is the average of 5 – 10 ramps and has been corrected for leakage current. For concentrations of cAMP at which the rOCNC1 + rOCNC2 currents displayed desensitization, the ramps were obtained during the maintained phase of the current.

30:1. Thus, the coexpression of the two subunits can fully account for the cyclic nucleotide specificity of the native olfactory channel.

As seen in Figure 4, rOCNC1 + rOCNC2 currents desensitized, and therefore cAMP and cGMP dose-response curves were also determined by measuring the peak current at each concentration of cyclic nucleotide, after a rapid solution exchange (10–50 ms). Mean data from these experiments are plotted in Figure 6 (p). A small increase in $K_{1/2}$ for both nucleotides was observed under these conditions (for cAMP, $K_{1/2} = 25 \pm 4 \mu\text{M}$; $n = 4$; for cGMP, $K_{1/2} = 12 \pm 1 \mu\text{M}$; $n = 3$), but the ratio between them was still 2:1.

Interestingly, the rOCNC1 + rOCNC2 channels displayed a more shallow dependence on cyclic nucleotide concentration than did the rOCNC1 channels. The fits to the Hill equation give coefficients of 0.9–1.3 for rOCNC1 + rOCNC2, whereas the coefficient for rOCNC1 is between 2.3 and 2.8. The value for the native channel is 1.3–1.8 (Frings et al., 1992; Nakamura and Gold, 1987). A decrease in the slope of the dose-response curve for the rOCNC1 + rOCNC2 channels is not surprising. If the CNG channel is a tetramer, then these patches may contain channels with up to four distinct subunit compositions (4rOCNC1, 3rOCNC1 + 1rOCNC2, 2rOCNC1 + 2rOCNC2, and 1rOCNC1 + 3rOCNC2). Each of these channels is likely to have a different sensitivity to cyclic nucleotide. Thus, the measured dose-response curve would reflect the sum of the individual dose-response curves, which is expected to be less steep. Alternatively, it is possible that a particular stoichiometry of the two subunits is favored. In this case, a difference in affinities of the individual subunits to cyclic nucleotide might also produce a more shallow dose-response curve.

Macroscopic Properties of rOCNC1 + rOCNC2 Currents

The macroscopic properties of the rOCNC1 + rOCNC2 currents differed in two further ways from those of the rOCNC1 currents. The rOCNC1 + rOCNC2 currents displayed more outward rectification, and they desensitized.

A close inspection of Figure 5B reveals that the rOCNC1 + rOCNC2 currents were outwardly rectifying in Ca^{2+} -free solution, whereas the rOCNC1 currents were almost linear (Figure 5A). The rectification

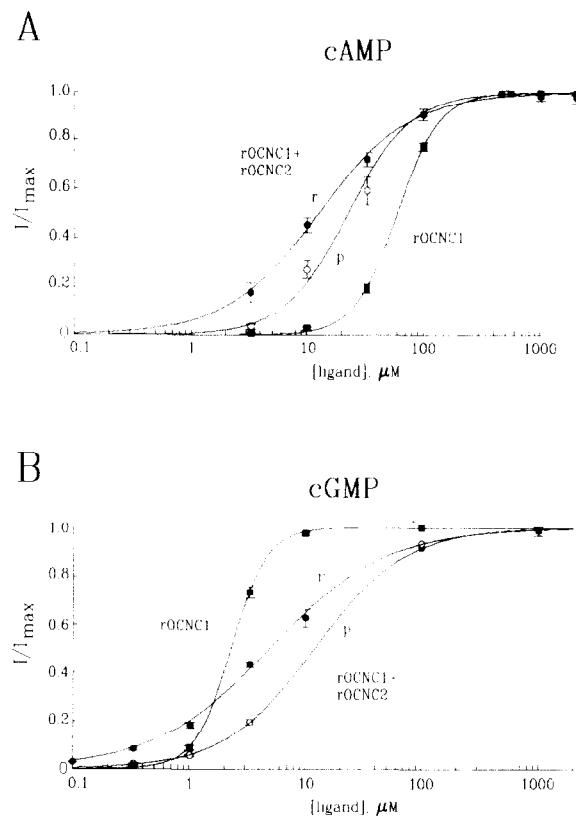


Figure 6. cAMP and cGMP Dose-Response Curves for rOCNC1 and rOCNC1 + rOCNC2 Currents

(A and B) Data for rOCNC1 channels (squares) were obtained from voltage ramps at the points where $V_m = -80$ mV. Data for rOCNC1 + rOCNC2 channels (circles) were either from ramps (r) or from the peak current after a rapid application of cyclic nucleotide (p). Each point is the mean \pm SEM from 4–7 patches. The data were normalized to the response at a saturating concentration of cAMP ($500 \mu\text{M}$) or cGMP ($100 \mu\text{M}$ for rOCNC1; 1 mM for rOCNC1 + rOCNC2). Curves are the fits of the plotted points to the Hill equation $I/I_{\max} = 1/[1 + (K_{1/2}/[\text{cyclic nucleotide}])^n]$.

Table 1. Comparison of Cyclic Nucleotide Sensitivity for rOCNC1, rOCNC1 + rOCNC2, and Native Rat CMG Channels

Channel	V_m	cAMP		cGMP	
		$K_{1/2}$	n	$K_{1/2}$	n
rOCNC1	+80	59 ± 2	2.3 ± 0.1	2.2 ± 0.1	2.8 ± 0.1
	-80	62 ± 3	2.4 ± 0.1	2.3 ± 0.1	2.9 ± 0.1
rOCNC1 + rOCNC2	+80	11 ± 2	1.1 ± 0.2	3.8 ± 0.3	1.3 ± 0.1
	-80	16 ± 3	1.1 ± 0.2	5.3 ± 0.7	0.9 ± 0.1
Native rat ^a	+50	2.5	1.8	1.0	1.3
	-50	4.0	1.8	1.8	1.3

Values of $K_{1/2}$ and n were determined from least squares fitting of the data to the Hill equation: $I/I_{max} = 1/[1 + (K_{1/2}/[\text{cyclic nucleotide}])^n]$. Data from rOCNC1 and rOCNC1 + rOCNC2 represent the mean values ± SEM from 4–7 patches.

^a Frings et al., 1992.

was observed at all concentrations of cAMP but was most prominent at low concentrations (see Figure 5B). In the presence of 100 μM cAMP, the ratio of current at +80 and -80 mV for rOCNC1 + rOCNC2 was 1.6 ± 0.1 (n = 7), which is significantly greater than the value for rOCNC1 of 1.2 ± 0.1 (n = 6; p < .05; Student's t test). Similar results were found in the presence of 100 μM cGMP (for rOCNC1 + rOCNC2, 1.8 ± 0.1; n = 4; for rOCNC1, 1.1 ± 0.1; n = 6). The degree of rectification seen for the rOCNC1 + rOCNC2 currents is similar to that reported for CNG currents recorded from olfactory sensory neurons (Nakamura and Gold, 1987; Frings et al., 1992). The rectification could be due to an increase in open probability (P_o), or in single-channel conductance at positive potentials. Voltage jumps applied in the presence of cyclic nucleotide showed a relaxation consistent with an increase in P_o at positive potentials (data not shown). However, our inability to measure the single-channel conductance, detailed below, prevented further study of the rectification.

rOCNC1 + rOCNC2 currents in excised patches desensitize, a feature that is not shared by the native conductance (Nakamura and Gold, 1987; Frings et al., 1992). As shown in Figure 4B, the rOCNC1 + rOCNC2 current decayed to approximately 60% of its peak value in response to a 1.6 s application of 500 μM cAMP, whereas the rOCNC1 current remained constant (Figure 4A). In this figure, the desensitization was close to completion, as longer applications of cAMP did not elicit further desensitization. Desensitization was observed in all patches expressing rOCNC1 + rOCNC2 channels. For saturating concentrations of cAMP (250 μM to 1 mM), the currents relaxed to 52% ± 3% of their peak amplitude (n = 10; peak amplitude = 297 ± 68 pA; range = 22–680 pA). In contrast, rOCNC1 currents showed no evidence of desensitization in the presence of 500 μM cAMP (n = 4; peak amplitude = 428 ± 101 pA; range = 254–720 pA; Dhallan et al., 1990). The relaxation of the currents was not due to ion accumulation, as voltage steps to 0 mV during the maintained phase of the rOCNC1 + rOCNC2 currents showed no evidence of a shift in the reversal potential (data not shown), and

the degree of desensitization was not correlated with the magnitude of the currents. A similar degree of desensitization was seen in the presence of 100 μM cGMP and at positive potentials. These data suggest that the desensitization is an intrinsic property of the rOCNC1 + rOCNC2 channels rather than the result of cAMP- or Ca²⁺-induced modulation. Similar desensitization is not seen for native olfactory CNG channels (Nakamura and Gold, 1987; Frings et al., 1992). This discrepancy could be due to differences between olfactory neurons and Xenopus oocytes in phosphorylation state or in posttranslational processing of the

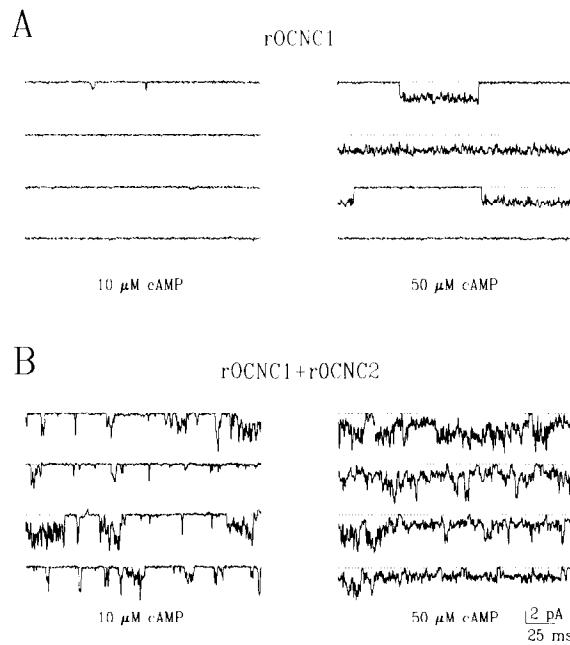


Figure 7. Single-Channel Recordings from Oocytes Injected with rOCNC1 RNA or rOCNC1 + rOCNC2 RNAs. Inside-out patches were excised and superfused with 10 μM or 50 μM cAMP. Consecutive sweeps are shown. (A) Openings of a single rOCNC1 channel were seen in the presence of 50 μM cAMP but not 10 μM cAMP. (B) Flickery openings of hetero-oligomeric rOCNC1 + rOCNC2 channels were seen at both 10 μM and 50 μM cAMP.

channels, in the ratio of the two subunits expressed or in the expression of additional channel subunits. Alternatively, the discrepancy may be the result of differences in experimental conditions. Interestingly, desensitization is not observed when rOCNC1 and rOCNC2 are coexpressed in a mammalian cell line (Kai Zinn, personal communication). This does not, however, allow us to discriminate among the possible explanations for the observed desensitization of the rOCNC1 + rOCNC2 channels in *Xenopus* oocytes.

Single-Channel Properties

To examine the unitary properties of rOCNC1 + rOCNC2 channels, patch-clamp recordings were obtained from oocytes expressing low densities of channels. Figure 7A shows consecutive traces for a patch expressing an rOCNC1 channel and a patch expressing several rOCNC1 + rOCNC2 channels at -80 mV. Openings of a single rOCNC1 channel were seen in the presence of 50 μ M cAMP but not 10 μ M cAMP, consistent with the macroscopic dose-response curve. In the presence of 50 μ M cAMP, the rOCNC1 channel exhibited stable open events that were tens to hundreds of milliseconds in duration. At higher concentrations of cyclic nucleotide (500 μ M cAMP), the channel rarely closed and had a P_o near 1 (data not shown). Noisy open channels showed some evidence of subconductance states, but these could not be resolved in amplitude histograms (data not shown). The amplitude of single channels was ~2.8 pA, as determined by measuring the peak corresponding to the fully open state in an amplitude histogram. The single channel current-voltage curve (-100 to +60 mV) was linear with a slope conductance of 35 pS (data not shown), which is substantially larger than the value of 12–15 pS for the conductance of the native rat olfactory CNG channel (Frings et al., 1992).

In contrast, rOCNC1+rOCNC2 unitary events were brief and flickery at concentrations of 10 or 50 μ M cAMP, with the latter evoking more frequent openings (Figure 7B). Amplitude histograms (data not shown) did not reveal the existence of discrete open states, indicating that the lifetime of the open state of these channels is too brief to be resolved. Given the flickery nature of the opening events, a determination of the number of channels in this patch was not possible. The amplitude of the flickery events could not be measured; however, a long-lived channel opening was observed occasionally (Figure 7B, bottom trace), and the amplitude of these events (~1.5 pA at -80 mV) was generally smaller than that of the rOCNC1 channel openings (~2.8 pA) and more similar to that of the native channel events (Frings et al., 1992).

Discussion

The data presented here indicate that rat olfactory sensory neurons express two distinct CNG channel subunits (rOCNC1 and rOCNC2) and strongly suggest

that the native olfactory CNG channel is a hetero-oligomer of these subunits. These findings can explain some of the discrepancies between the properties of the native channel (Frings et al., 1992) and those of the previously cloned rat CNG channel protein (rOCNC1; Dhallan et al., 1990), including differences in sensitivity to cyclic nucleotide, degree of outward rectification, and single-channel conductance.

The observation that both *rOCNC1* and *rOCNC2* subunits are expressed throughout the olfactory epithelium is interesting in light of recent work mapping the distribution of odorant receptors (Ressler et al., 1993; Vassar et al., 1993). These studies show that the olfactory epithelium is divided into several distinct spatial zones in which different odorant receptors are expressed. Furthermore, it has been suggested that odorant receptors may use one of two second messenger pathways, one mediated by cAMP and the other mediated by IP₃ (Boekhoff et al., 1990). Thus, the expression of both CNG channel subunits in all zones indicates that the zonal organization of the epithelium is unlikely to be related to a second messenger pathway.

Channels formed from rOCNC1 and rOCNC2 subunits have a selectivity for cyclic nucleotide very similar to that of the native olfactory channels (Frings et al., 1992; Nakamura and Gold, 1987). Interestingly, the absolute values of K_{on} for cAMP and cGMP for the rOCNC1+rOCNC2 channels are ~4-fold higher than those for the native channel. A similar observation has been made for the cloned catfish CNG channel, which has a 15-fold higher value of K_{on} for cAMP and for cGMP than does the native catfish olfactory channel but has similar selectivity between the two cyclic nucleotides (Goulding et al., 1992). One possible explanation for these differences is that P_o at saturating cyclic nucleotide concentrations is higher for the native channels than for the cloned channels. Cloned catfish olfactory channels show abrupt shifts in gating, from a high P_o mode to a low P_o mode, and thus it is possible that the high cyclic nucleotide sensitivity of the native channel comes about because it spends more time in the high P_o mode (Goulding et al., 1992). For the rOCNC1+rOCNC2 channels, a measurement of P_o was not feasible under the conditions of these experiments, and no measurement of P_o for the native rat olfactory channel is yet available.

A comparison of the properties of rOCNC2 and a second subunit of the retinal rod CNG channel reveals some striking similarities. Neither subunit forms a functional channel as a homo-oligomer, but both can contribute to hetero-oligomeric channels in concert with the previously cloned subunits. The hetero-oligomeric channels are similar, in that the open state of the single channels is flickery (Chen et al., 1993). The flickery channel openings in photoreceptors and olfactory neurons may enhance the signal to noise ratio, as the current carried by any one channel is reduced and the rapid current fluctuations are smoothed by the membrane capacitance (Mathews

and Watanabe, 1988). This is similar to the proposed role of Ca^{2+} block of these channels, in which signal to noise is increased by reducing the current carried by a single-channel opening (Yau and Baylor, 1989).

Expression of two functionally distinct ion channel subunits that can form a hetero-oligomer provides a mechanism for fine tuning the response characteristics of a cell. Thus, the sensitivity to cAMP of the native olfactory CNG channel could be adjusted by regulation of the relative levels of expression of rOCNC1 and rOCNC2 subunits. This predicts that differences in subunit composition might be found in the olfactory neuron population. Indeed, patch-clamp recordings of CNG currents from olfactory sensory neurons reveal a heterogeneity among cells in sensitivity to cyclic nucleotide (Nakamura and Gold, 1987) and to block by amiloride (Frings et al., 1992). In addition, two subunits may confer properties that cannot be achieved with a single subunit, such as a broader dose-response curve. It will be interesting to see whether additional CNG subunits are expressed in other cell types, such as retinal cones (Bönigk et al., 1993) and aorta (Biel et al., 1993), or in other species, such as catfish (Goulding et al., 1992), where only a single CNG subunit type has been identified, and, if so, to examine the consequences of hetero-oligomer formation in these systems.

Experimental Procedures

Reverse Transcription PCR

RNA was extracted from olfactory epithelia of C57BL/6J mice using RNAzol B (TM Cinna Scientific). Following treatment of the RNA with DNase I (Pharmacia), random-primed cDNA was generated (Buck and Axel, 1991). The cDNA was used as template in PCR (Buck and Axel, 1991) with the degenerate primers AAA/GGGIGAIATIGGIC/AG/AIGAG/AATGTA and AC/TG/ATTIGCIG-TICG/TICG/TG/ATTICC, corresponding to amino acid sequences that are common to the cyclic nucleotide-binding domains of a number of proteins. The amplified DNA was cloned into pBluescript SK (Stratagene), and individual clones were sequenced. A clone that encoded a protein similar but not identical to other CNG channel proteins was used as a probe to screen a rat olfactory epithelium cDNA library (Buck and Axel, 1991). Of 37 positive clones isolated, 2 with inserts of ~2.8 kb were purified and subjected to nucleotide sequence analysis. The sequences of the 2 were identical over the region sequenced (5' untranslated and coding regions), except that 1 had an additional 18 nucleotides at its 5' end.

Northern Blot Analysis

Poly(A)⁺ RNA (1 μg) isolated from each of the rat tissues indicated was size fractionated in formaldehyde-agarose and blotted onto Hybond N (Amersham). Hybridization was at high stringency with a ³²P-labeled probe corresponding to the entire coding and noncoding region of the rOCNC2 cDNA (Buck and Axel, 1991). Examination of 28S and 18S ribosomal RNAs confirmed the integrity of the RNAs used.

In Situ Hybridization

Tissue preparation, probe synthesis, hybridization, and autoradiography were performed as previously described (Ressler et al., 1993), except that following fixation, the tissue was decalcified for 1 week in 250 mM EDTA, 4% paraformaldehyde at 4°C (Ryan et al., 1991). The DNA encoding rOCNC1 was generously provided by R. Reed (Dhallan et al., 1990), and that encoding OMP was the kind gift of F. Margolis (Danciger et al., 1989). Images

obtained from photographs of the hybridized sections viewed under dark-field illumination alone or in combination with fluorescence illumination were processed with Adobe Photoshop software; adjustments in color balance, intensity, or contrast were performed simultaneously on images of rOCNC1 and rOCNC2 hybridized sections.

Oocyte Expression

DNA containing the coding and noncoding regions of rOCNC1 and rOCNC2 were subcloned into the EcoRI site of the high expression vector, pGEMHE (Liman et al., 1992), which contains untranslated regions of the Xenopus β -globin gene. Transcription of RNA and injection of oocytes was as previously described (Koren et al., 1990). Approximately equal concentrations of rOCNC1 and rOCNC2 RNAs, as estimated by agarose gel electrophoresis, were mixed for coinjection experiments.

Electrophysiology

Patch electrodes were pulled from borosilicate glass, firepolished (1–2 $M\Omega$ resistance), and coated with beeswax (for single-channel recordings). Currents were recorded with an Axopatch-1c patch-clamp amplifier (Axon Instruments) and low pass filtered with a 4 pole Bessel filter. Patch and bath solutions contained 130 mM NaCl, 3 mM HEPES, and 0.5 mM EDTA (pH 7.6). Stock solutions (10 mM cAMP and 10 mM cGMP; Sigma) were stored frozen and freshly diluted to the appropriate concentrations on the day of use. Application of different concentrations of cyclic nucleotide was accomplished by positioning the excised patch in front of a linear array of microcapillary tubes. For single-channel recording, records were sampled at 1 kHz and filtered at 2 kHz.

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Note Added in Proof

Results similar to those reported here have been obtained by Bradley et al. (*Proc. Natl. Acad. Sci. USA*, in press).