A Novel Calcium-Regulated Membrane Guanylate Cyclase Transduction System in the Olfactory Neuroepithelium†

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ABSTRACT: This report defines the identity of a calcium-regulated membrane guanylate cyclase transduction system in the cilia of olfactory sensory neurons, which is the site of odorant transduction. The membrane fraction of the neuroepithelial layer of the rat exhibited Ca^{2+}-dependent guanylate cyclase activity, which was eliminated by the addition of EGTA. This indicated that the cyclase did not represent a rod outer segment guanylate cyclase (ROS-GC), which is inhibited by free Ca^{2+}. This interpretation was supported by studies with the Ca^{2+} binding proteins, GCAPs (guanylate cyclase activating proteins), which stimulate photoreceptor ROS-GC in the absence of Ca^{2+}. They did not stimulate the olfactory neuroepithelial membrane guanylate cyclase. The olfactory neuroepithelium contained a Ca^{2+} binding protein, neurocalcin, which stimulated the cyclase in a Ca^{2+}-dependent fashion. The cyclase was cloned from the neuroepithelium and was found to be identical in structure to that of the previously cloned cyclase termed GC-D. The cyclase was expressed in a heterologous cell system, and was reconstituted with its Ca^{2+}-dependent activity in the presence of recombinant neurocalcin. The reconstituted cyclase mimicked the native enzyme. Immunocytochemical studies showed that the guanylate cyclase coexists with neurocalcin in the apical region of the cilia. Deletion analysis showed that the neurocalcin-regulated domain resides at the C-terminal region of the cyclase. The findings establish the biochemical, molecular, and functional identity of a novel Ca^{2+}-dependent membrane guanylate cyclase transduction system in the cilia of the olfactory epithelium, suggesting a mechanism of the olfactory neuroepithelial guanylate cyclase regulation fundamentally distinct from the phototransduction-linked ROS-GC.

Odorant transduction is a biochemical process by which the olfactory neuroepithelial layer generates electric signals in response to odorant molecules (reviewed in refs *1*−*4*). This process occurs in the ciliated apical border of sensory neurons located in the epithelial layer. Many molecular details of this process are lacking. There is, however, a general consensus that the odorant induces an elevation in the level of cyclic AMP and enhances the opening of a cyclic AMP/cyclic GMP-gated cation channel (*5*−*7*). This results in a spike in the level of intracellular Ca^{2+} and depolarization of the ciliary plasma membrane (*8*). Odorant signal recognition occurs by an odorant-specific receptor, which resides in the cilia. A very large family of odorant receptors ensures odor discrimination. These receptors are members of the group of seven transmembrane-spanning proteins that couple with G_{olf} proteins and activate type III adenylate cyclase. On the basis of the finding that a membrane guanylate cyclase (mGC)[1] also exists in the ciliary membrane, a separate mechanism for depolarization of the cilia has also been proposed (*9*). In this mechanism, the olfactory neuroepithelial guanylate cyclase, termed GC-D (*9*), is a direct receptor of the odorant. The odorant, upon binding to its receptor, generates cyclic GMP and depolarizes the cilia (*9*). It has been postulated that the receptor region of the cyclase resides in its extracellular domain and the direct interaction of the odorant and the receptor activates GC-D (*10*). The mechanism has been supported by the findings which show that the GC-D-containing neurons also contain two other cyclic GMP-specific signaling components, cyclic GMP-stimulated phosphodiesterase (PDE2) and the α2 subunit of a cyclic GMP-selective cyclic nucleotide-gated channel (CNG) (*11*).

In the study present here, through biochemical, functional, and immunological approaches, the identity of the native Ca^{2+}-dependent mGC transduction system in the sensory neurons of olfactory epithelium at a molecular level has been disclosed. With the recombinant components, the reconsti-

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; GCAP, guanylate cyclase activating protein; mGC, membrane guanylate cyclase; PBS, phosphate-buffered saline; ROS-GC, rod outer segment membrane guanylate cyclase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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tuted system mimics the native Ca^{2+}-sensitive mGC system. The Ca^{2+}-signaling domain involved in cyclase regulation has been defined. The findings provide a novel molecular model, which does not involve direct regulation of the cyclase by the odorant ligand. Instead, the cyclase is regulated by the intracellular spikes of Ca^{2+} generated by the odorant in the cilia. These spikes are sensed by neurocalcin, which, in turn, acts at an intracellular domain and activates the cyclase. The model explains how the neuroepithelial mGC acts as a common processor for all incoming odorant molecules and depolarizes the cilia, an effect that is the opposite of that for hyperpolarization produced by the phototransduction-linked rod outer segment guanylate cyclase (ROS-GC).

EXPERIMENTAL PROCEDURES

Reagents. GCAP1, GCAP2, and neurocalcin (the neurocalcin δ form) were cloned, expressed, and purified as described previously (*12*, *13*). S100β was obtained commercially (Sigma Chemical Co.).

Antibodies. Characterization of highly specific antibodies raised against GCAP1, GCAP2, ROS-GC1, and ROS-GC2 has been described previously (*12*, *14*). Monospecific antibody against the olfactory neuroepithelial mGC was raised in rabbits against a specific 12-amino acid region in the C-terminus of the protein. A polyclonal antibody against neurocalcin was raised with the purified recombinant protein as an antigen. All antisera were tested for their specificity through the enzyme-linked immunosorbent assay (ELISA) and by Western blotting according to standard protocols. The neurocalcin antibody was specifically tested for cross-reactivity with GCAP1 and GCAP2. No cross-reactivity was observed with either of the GCAPs. After the specificity of the reaction had been established, antibodies were enriched by precipitating the immunoglobulin fraction using ammonium sulfate. ELISA and Western blots were used to determine the titer of the enriched antibodies. A 1:10000 dilution was generally used for colorimetric detection of the antigen on Western blots. A monoclonal antibody against S100β (clone SH-B1) was purchased from Sigma Chemical Co. (St. Louis, MO).

Molecular Cloning of the Neuroepithelial mGC. The rat olfactory neuroepithelial cDNA library was the source of the cloned neuroepithelial mGC. Poly(A)$^+$ RNA was isolated from the rat olfactory epithelium following the "Dynal-kit" protocol (Dyna Labs), and the cDNA library was constructed using a Superscript cDNA synthesis kit (Gibco-BRL). The cDNA was isolated in two fragments: fragment 1 and fragment 2. Fragment 1 (1937 bp) represented the 5′ part (nucleotides 46−1982), and fragment 2 (2773 bp) represented the 3′ part (nucleotides 569−3431) (*9*). These fragments were individually ligated into the pBluescript vector. To assemble the full-length neuroepithelial mGC cDNA, an internal *Bam*HI restriction site (at position 1683) was used. The assembled construct was sequenced to verify proper ligation. For expression studies, the cDNA was subcloned into the pcDNA3 vector.

Construction of the Neuroepithelial mGC Deletion Mutants. Three deletion mutants were constructed: Δ75−466, Δ521−823, and the double deletion mutant, Δ75−466/Δ521−823. For generation of the Δ75−466 mutant, two *Hpa*I restriction sites were introduced into the guanylate cyclase cDNA, between codons corresponding to amino acids 74 and 75, and 466 and 467, using the "Quick Change" mutagenesis kit (Stratagene). The *Hpa*I fragment was excised, and the remaining part was religated, resulting in deletion of the extracellular domain (amino acids residues 75−466). Two *Sma*I restriction sites were created between codons corresponding to amino acid residues 520 and 521, and 823 and 824, followed by *Sma*I digestion and religation to obtain deletion mutant Δ521−823. To generate the double deletion mutant, the *Bam*HI−*Not*I fragment of the Δ75−466 mutant was substituted with the *Bam*HI−*Not*I fragment of the Δ521−823 mutant. All constructs were sequenced to confirm their identities.

Expression Studies. COS-7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the wild-type recombinant mGC expression constructs by the calcium phosphate coprecipitation technique (*15*). Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5) and 10 mM buffer, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed several times with the same buffer. The resulting pellet represented crude membranes.

Fractionation of the Olfactory Tissues. Rat olfactory epithelium and bulb were purchased from Zivic-Miller. Membrane and soluble fractions from the olfactory epithelium and bulb were isolated according to the protocol described previously (*12*, *14*). Briefly, the tissue was homogenized in buffer containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), and protease inhibitors (Genotech). The postmitochondrial supernatant, obtained after centrifugation at 400*g* followed by centrifugation at 10000*g*, was centrifuged at 40000*g*. The pellet was designated as the membrane fraction and the supernatant as the soluble fraction. A similar procedure was adapted to isolate membrane and soluble fractions from the bovine retina, which served as positive controls for Western blot analyses.

Guanylate Cyclase Assay. Membrane fractions were assayed for guanylate cyclase activity as described previously (*16*). Briefly, membranes were preincubated on an ice bath with or without GCAP1, GCAP2, and neurocalcin in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5) adjusted to the appropriate free Ca^{2+} concentrations with precalibrated Ca^{2+}/EGTA solutions (Molecular Probes). The total assay volume was 25 μL. The reaction was initiated by addition of the substrate solution containing 4 mM $MgCl_2$ and 1 mM GTP and continued by incubation at 37 °C for 10 min. The reaction was terminated by addition of 225 μL of 50 mM sodium acetate buffer (pH 6.2) followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP that formed was determined by a radioimmunoassay using 100 μL aliquots of the diluted reaction mixtures (*17*).

Western Blot. Western blotting and analyses were carried out exactly according to the protocol described previously (*12*, *14*, *18*). Briefly, protein samples were transferred onto nitrocellulose membranes after electrophoresis. The membranes were incubated in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% bovine serum albumin

(BSA) for 1 h at room temperature followed by incubation for 1 h in the same solution containing the primary antibody. After washing with TBS-T, incubation was continued for the same period of time in TBS-T containing 3% BSA and the secondary antibody. After washing in TBS-T, visualization of the immunoreactive protein bands was carried out according to the manufacturer's (Vector Labs) protocol.

Immunohistochemical Analyses. Rat olfactory epithelial samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. Dehydration and embedding were carried out according to standard procedures (*14*, *19*). Sections (4 μm) were used for immunohistochemical analyses according to the procedure described previously (*19*). Briefly, paraffin was removed with xylene, and the sections were rehydrated through a graded series of washes in decreasing concentrations of ethanol. Antigens were then retrieved by microwave activation in Target Citrate Buffer (Dako). Endogenous peroxidase activity was quenched by treating sections for 30 min with 3% hydrogen peroxide in water. After nonspecific protein binding had been blocked in PBS containing preimmune serum, 2% BSA, and 0.1% Tween 20, sections were incubated with primary antibodies in the same solution for 60 min at 37 °C in a humid chamber and then washed for 60 min at room temperature with PBS containing 0.1% Tween 20. Detection of immunolabeled proteins was carried out using the Vectastain Elite system (Vector Labs) and washing conditions were as recommended by the manufacturer. The chromogenic reaction employed was 3,3′-diaminobenzidine-4 HCl and hydrogen peroxide (Biomeda). Specimens were then dehydrated and mounted in Permount (Vector Labs). Some specimens were first counterstained to reveal nuclei using hematoxylin. Immunostaining was visualized with a Nikon FXA microscope. Images were recorded on Fuji color film (ASA100) or acquired using a Princeton Instruments cooled CCD camera. Digital images were processed using commercially available software (ImagePro Plus; Phase3 Imaging Systems). Controls included detection reactions carried out under identical conditions except that either ROS-GC1 antibody or preimmune serum was added. Staining was insignificant in either case. Alternatively, frozen sections were obtained as follows. After being fixed overnight in formaldehyde at 4 °C, the tissue was cryoprotected in 25% sucrose overnight at 4 °C and 4 μm thin sections were prepared (Leica cryostat). After endogenous peroxidase had been quenched, these sections were probed with the antibodies as described above. Fluorescent secondary antibodies were not used since autofluorescence of the tissue was observed.

RESULTS

The Olfactory Neuroepithelium Contains a Ca^{2+}-Dependent mGC. The particulate fraction of the rat olfactory epithelium was assayed for guanylate cyclase activity. The specific activity was 1.5 ± 0.2 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}. To determine whether the activity of the epithelial mGC is regulated in a Ca^{2+}-sensitive manner, the particulate fraction was isolated and assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. The mGC activity exhibited a Ca^{2+} dose-dependent stimulation. The maximal stimulation was ~5-fold over the basal value and was achieved at ~5 μM Ca^{2+}. The EC_{50} value for Ca^{2+} was ~0.8 μM (Figure 1). Therefore, the olfactory neuroepithelium contains a Ca^{2+}-dependent mGC, and its behavior is the opposite of that of the phototransduction-linked ROS-GC, which is inhibited by Ca^{2+} spikes.

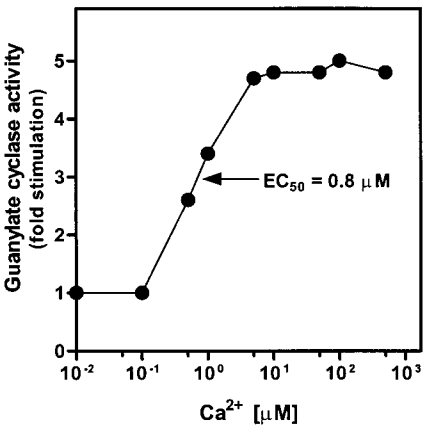

FIGURE 1: Ca^{2+}-dependent guanylate cyclase activity in membranes of rat olfactory epithelium. Membranes of rat olfactory epithelium were isolated as described in Experimental Procedures and assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. The basal (unstimulated) cyclase activity was 1.5 ± 0.2 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}; the stimulated cyclase activity was 7.2 ± 0.6 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}. The experiment was carried out in triplicate and repeated three times. The results that are presented are from one representative experiment. Error bars are within the size of the symbols. The EC_{50} value was determined graphically.

Olfactory Neuroepithelial mGC Is Not a Known Member of the ROS-GC Subfamily. There are two known Ca^{2+}-sensitive mGCs; both are members of the photoreceptor ROS-GC subfamily: ROS-GC1 and ROS-GC2 (reviewed in ref *20*). Both ROS-GCs are stimulated at 10 nM Ca^{2+} via GCAPs and are inhibited at high Ca^{2+} concentrations (*21*−*27*). To directly assess whether the native olfactory neuroepithelial mGC was one of these ROS-GCs, the particulate fraction of the neuroepithelium was incubated with recombinant GCAP1 or GCAP2 in the presence of 10 nM free Ca^{2+}. There was no stimulation of the olfactory neuroepithelial mGC. However, the recombinant GCAP1 stimulated the activity of ROS-GC1, and the recombinant GCAP2 stimulated the activity of ROS-GC1 and ROS-GC2 (Figure 2). Thus, it is concluded that the olfactory neuroepithelial mGC is neither ROS-GC1 nor ROS-GC2.

In Addition to the Neuroepithelial mGC, the Olfactory Neuroepithelium Contains Neurocalcin. Recent evidence indicates that one of the ROS-GCs, ROS-GC1, also exists outside photoreceptor cells in the retinal bipolar neurons (*28*, *29*), in pinealocytes (*12*), and in the mitral cells of the olfactory bulb (*14*). In some of these cells, for instance, in a subset of pinealocytes (*12*), it is stimulated in a Ca^{2+}-dependent fashion via Ca^{2+}-binding proteins. To distinguish these proteins from GCAPs, they have been named Ca^{2+}-dependent guanylate cyclase activating proteins (CD-GCAPs). There are two known ROS-GC1 stimulating CD-GCAPs: S100β and neurocalcin (*13*, *30*−*32*). To determine if the Ca^{2+}-dependent activation of the neuroepithelial mGC (Figure 1) is mediated by any of these CD-GCAPs in the neuroepithelial layer, their direct presence in this tissue was scrutinized by Western blotting. Analysis with the neuro-

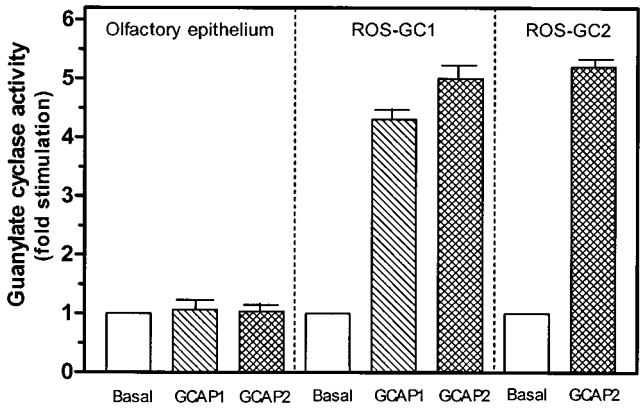

FIGURE 2: Effect of GCAP1 and GCAP2 on rat olfactory neuroepithelial mGC. Membranes of rat olfactory neuroepithelium were isolated as described in Experimental Procedures. These were assayed for the guanylate cyclase activity in the absence (Basal) or presence of 4 μM GCAP1 (GCAP1) or 20 μM GCAP2 (GCAP2) and 10 nM Ca^{2+}. The experiment was carried out in triplicate and repeated three times for reproducibility. The results that are depicted (means ± standard deviation) are from one representative experiment. Membranes of COS cells expressing ROS-GC1 or ROS-GC2 were assayed in parallel and served as positive controls.

calcin-specific antibody showed the presence of neurocalcin in both the membrane and soluble fractions of the olfactory neuroepithelium (Figure 3A, EPITHELIUM, SOLUBLE and MEMBRANE). The mobility of the immunoreactive band in each lane was the same as that observed in the lane containing purified, recombinant neurocalcin (Figure 3A, rNEUROCALCIN). These results are in agreement with the earlier reports describing the presence of neurocalcin in rat olfactory neuroepithelium (*33*, *34*). The results clearly show that neurocalcin is present in the membrane fraction, where the neuroepithelial mGC resides, and, therefore, can function as its Ca^{2+}-dependent activator. Detection of neurocalcin also in the soluble fraction may be either due to a loss of the membrane-bound neurocalcin during isolation of the soluble and membrane fractions or due to membrane association of neurocalcin synthesized as a soluble protein in vivo.

No band was observed with the S100β antibody probe in either the membrane or soluble fraction (Figure 3B, EPITHELIUM); the antibody readily reacted with purified S100β (Figure 3B, S100β). Thus, neurocalcin, and not S100β, was the potential regulator of the neuroepithelial mGC, an interpretation consistent with the biochemical and reconstitution data presented below.

Neurocalcin Stimulates Native Neuroepithelial mGC in a Ca^{2+}-Dependent Fashion. To determine if neurocalcin is a natural Ca^{2+} regulator of the neuroepithelial mGC, the membrane fraction of the neuroepithelium was incubated with incremental concentrations of bovine recombinant neurocalcin (expressed in bacterial cells and purified) at the saturating Ca^{2+} concentration. Neurocalcin stimulated the mGC activity 4-fold above the basal value. The stimulation was in a dose-dependent fashion (Figure 3C). The half-maximal stimulation was achieved at ~1 μM and saturation at ~4 μM. Thus, submicromolar to micromolar levels of neurocalcin mediate Ca^{2+} signaling of the mGC. The ability of neurocalcin to stimulate the mGC in the olfactory epithelial membranes beyond the point achieved through the addition of Ca^{2+} alone to these membranes indicates that

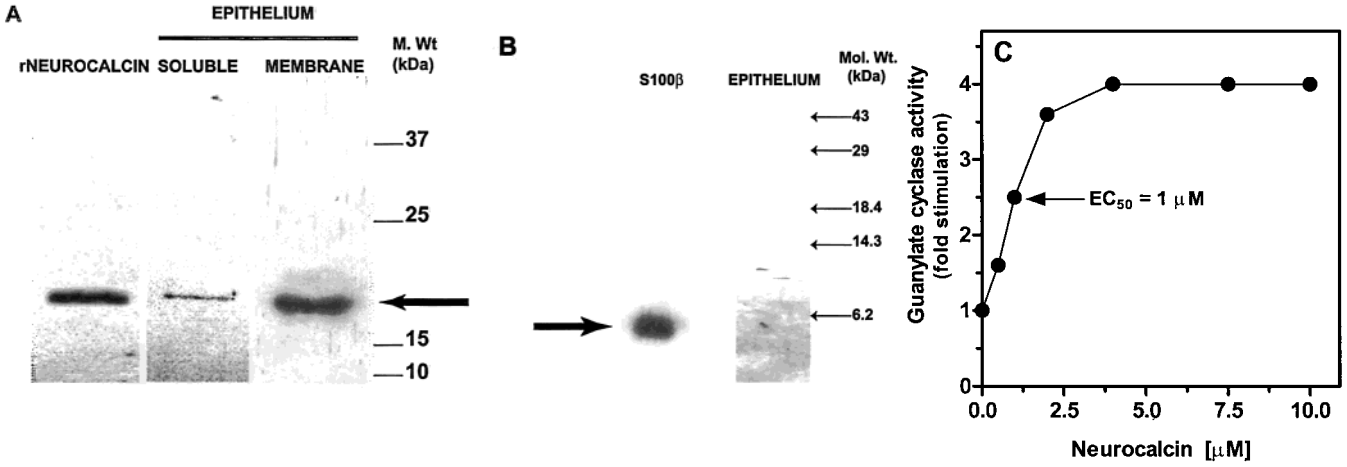

FIGURE 3: Rat olfactory neuroepithelial mGC is regulated by neurocalcin. (A) Presence of neurocalcin in the olfactory neuroepithelium. Membrane and soluble fractions were isolated from the olfactory ciliary epithelium as described in Experimental Procedures. The membrane fraction was solubilized and used to purify neurocalcin. Western analysis was performed with 4 μg of this protein (EPITHELIUM, MEMBRANE) along with 100 μg of protein from the soluble fraction of the olfactory epithelium (EPITHELIUM, SOLUBLE) and 1 μg of bacterially expressed, purified recombinant neurocalcin (rNEUROCALCIN). Proteins were loaded on a SDS−polyacrylamide (10%) gel, electrophoresed, transferred onto Nytran membranes, and probed with antibodies against neurocalcin. The immunoreactive band is indicated by a solid arrow. The positions of the molecular size markers are given alongside. (B) Absence of S100β in the olfactory neuroepithelium. One hundred micrograms of protein from the soluble fraction of the olfactory epithelium (EPITHELIUM) and 1 μg of commercial S100β (S100β) were independently electrophoresed on a SDS−polyacrylamide gel (15%), transferred, and subjected to Western analyses with antibodies against S100β according to previously described protocols (*18*). The positions of the molecular size markers are given alongside. The immunoreactive band, present in the S100β lane, is indicated by an arrow. No reaction was observed in the epithelial soluble fraction. A similar result was obtained when epithelial membrane fractions were probed. (C) Effect of neurocalcin on rat olfactory neuroepithelial mGC. Membranes of rat olfactory neuroepithelium were isolated as described in Experimental Procedures and were assayed for guanylate cyclase activity in the presence of incremental concentrations of neurocalcin and 100 μM Ca^{2+}. The basal (no neurocalcin added) cyclase activity was 7.1 ± 0.6 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}; the maximally stimulated cyclase activity was 28 ± 2.3 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}. Each experiment was carried out in triplicate and repeated three times for reproducibility. The results that are depicted are from one representative experiment. The error bars are within the size of the symbols. The EC_{50} value was determined graphically.

the levels of neurocalcin present in these membrane preparations are substantially lower than that needed for maximal activation of the cyclase.

Cloning of the Olfactory Neuroepithelial mGC and the Specificity of Its Antibody. To characterize and determine the Ca^{2+}-regulated features of the neuroepithelial mGC at the molecular level, the cyclase was cloned from the rat olfactory epithelium. Its structure was found to be identical to that of a previously reported cyclase, named GC-D, which was cloned from the total rat olfactory cDNA library (*9*). Thus, GC-D and the neuroepithelial mGC are identical mGCs, and because the cyclase in this report has been directly cloned from the olfactory neuroepithelial layer, GC-D is functionally Ca^{2+}-modulated olfactory neuroepithelial mGC.

To test the degree of immunological identity between the recombinant and the native neuroepithelial mGC, an antibody probe was generated using a unique peptide sequence from the C-terminus of the cloned, recombinant neuroepithelial mGC as the antigen. The specificity of this antibody was tested against both native and denatured neuroepithelial mGC, ROS-GC1 and ROS-GC2. The reactivities of the antibody against native neuroepithelial mGC, ROS-GC1, and ROS-GC2 were assessed through an ELISA. Equimolar amounts of the peptide antigen derived from the neuroepithelial mGC, a corresponding peptide fragment from ROS-GC2, and the entire catalytic domain of ROS-GC1, which was bacterially expressed and purified, were used (*35*). The results presented in Figure 4A clearly demonstrate that the antibody specifically recognizes the neuroepithelial mGC, but not either of the ROS-GCs. Western analyses were carried out to affirm the specificity of this antibody toward the recombinant neuroepithelial mGC under denaturing conditions. ROS-GC1 and ROS-GC2 were used as negative controls. The results presented in Figure 4B shows that the antibody specifically recognizes recombinant neuroepithelial mGC (indicated by an arrow), but not rROS-GC1 or rROS-GC2 (compare lanes rROS-GC1, rROS-GC2, and rNEmGC). When the membrane fraction from the olfactory epithelium was subsequently probed with this antibody by Western blotting, a single immunoreactive band was observed (Figure 4B, EPITHELIUM). A single band of the same mobility was observed with COS cell membranes expressing the recombinant neuroepithelial mGC (Figure 4B, rNEmGC). The molecular size of the immunoreactive band (~120 kDa) matches the expected size based on the primary amino acid sequence of the neuroepithelial mGC. No band was observed in the membrane fraction isolated from the olfactory bulb (Figure 4B, BULB) or retina (Figure 4B, RETINA). This leads to three important conclusions: (1) The neuroepithelial mGC antibody is highly specific and does not cross-react with the bulb or retinal mGCs: atrial natriuretic factor-receptor guanylate cyclase (ANF-RGC), C-type natriuretic peptide-receptor guanylate cyclase (CNP-RGC), ROS-GC1, or ROS-GC2; (2) The recombinant neuroepithelial mGC is immunologically identical to the native olfactory neuroepithelial mGC; (3) Expression of the neuroepithelial mGC is unique to the olfactory neuroepithelium and is not found in the olfactory bulb or the retina.

The Recombinant Neuroepithelial mGC, upon Reconstitution, Mimics the Native Olfactory Neuroepithelial mGC. To determine whether the recombinant neuroepithelial mGC mimics the biochemical features of the native form, it was expressed in COS cells. Membranes isolated from the transfected cells exhibited guanylate cyclase activity. The specific activity of the recombinant neuroepithelial mGC was ~100 pmol of cyclic GMP min^{-1} (mg of protein)$^{-1}$. Kinetic analysis of the dependence of the enzyme on GTP yielded an apparent K_m of 71 μM for GTP (data not shown). The native neuroepithelial mGC under saturating Ca^{2+} conditions is stimulated by submicromolar to micromolar concentrations of neurocalcin (Figure 3C). To test if the recombinant form also exhibits this characteristic, COS cells expressing the mGC were recombined with a series of concentrations of purified recombinant neurocalcin at a constant, saturating Ca^{2+} concentration. Under these conditions, neurocalcin stimulated the neuroepithelial mGC in a dose-dependent fashion, with half-maximal activation occurring at 1 μM, and saturation at ~4 μM (Figure 4C). Previously, neurocalcin has been demonstrated to activate ROS-GC1 in a Ca^{2+}-dependent fashion (*13*). However, the maximal activation of ROS-GC1 by neurocalcin is only ~2-fold (*13*). These results show that neurocalcin is far more effective in enhancing the saturating activity of the neuroepithelial mGC. The maximum achievable level of stimulation of the neuroepithelial mGC by neurocalcin is 6-fold (Figure 4C).

The stimulation of the neuroepithelial mGC by neurocalcin was Ca^{2+}-dependent. It progressively increased as the concentration of free Ca^{2+} increased with a half-maximal activation at 1 μM (Figure 4D). No stimulation of cyclase activity was observed in the presence of Ca^{2+} alone (Figure 4D). The maximal activation in the presence of neurocalcin was reached at 5 μM and maintained at higher concentrations of free Ca^{2+} (Figure 4D). These results show that the recombinant neuroepithelial mGC is a Ca^{2+}-regulated cyclase, that it mimics the native cyclase in its Ca^{2+} regulation, that the regulation by Ca^{2+} is not direct and is mediated by the Ca^{2+} -binding protein, neurocalcin, and that no other components are needed to reconstitute the Ca^{2+} regulation of the enzyme. It is, thus, concluded that the native form of the olfactory neuroepithelial mGC has been cloned and has been reconstituted to reproduce its native Ca^{2+}-dependent features.

The Olfactory Neuroepithelial mGC and Neurocalcin Are Expressed Together in the Cilia of the Olfactory Neuroepithelium. The olfactory neuroepithelial layer is composed of the ciliated sensory neurons, the site of odor transduction, and the supporting and basal cells. For neurocalcin to be a physiological modulator of the neuroepithelial mGC in the epithelium, these two molecules must be colocalized within the same cell. To address this issue, immunohistochemical analyses were carried out with highly specific antibodies against the neuroepithelial mGC and neurocalcin. Antibodies against ROS-GC1 were used as a control. Consecutive paraffin sections of olfactory neuroepithelium (4 μm thick) were probed with these antibodies as described in Experimental Procedures. Olfactory bulb, which expresses ROS-GC1 (*14*) but not the neuroepithelial mGC (Figure 4B), was used as a control. The results are presented in Figure 5. The results obtained with the neuroepithelial mGC, ROS-GC1, and neurocalcin antibodies are presented in the NEmGC, ROS-GC1, and NEUROCALCIN panels, respectively. The tissue (BULB or EPITHELIUM) is also indicated in each panel. Identical regions in the NEmGC-EPITHELIUM and

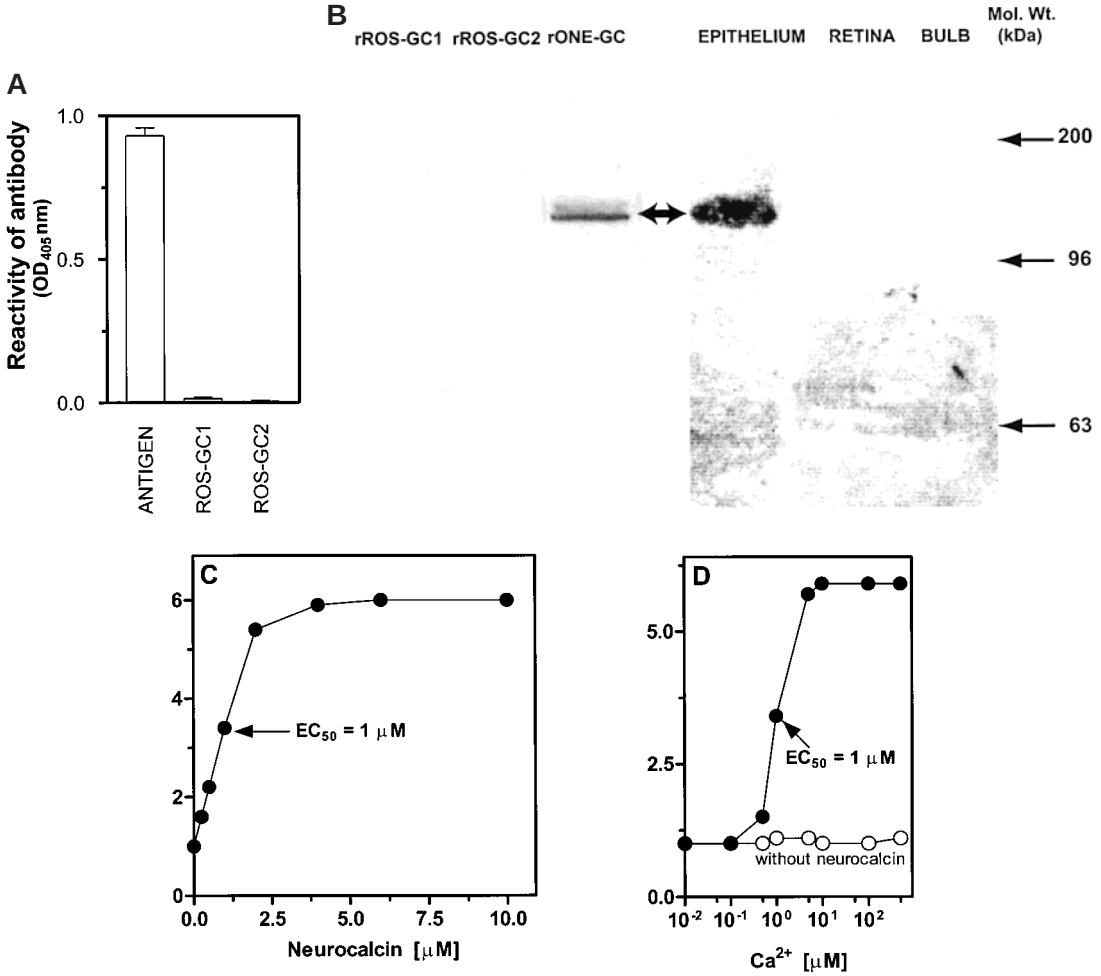

FIGURE 4: Recombinant neuroepithelial mGC mimics native neuroepithelial mGC. (A and B) Immunological identity of recombinant and native olfactory neuroepithelial mGC. Monospecific antibodies were raised against a peptide sequence unique to neuroepithelial mGC. The specificity of the antibody was tested against native cyclases through an ELISA (A). The reactivity of the antibody was assessed against the peptide antigen (ANTIGEN), a peptide corresponding to the region from ROS-GC2 (ROS-GC2), and the entire catalytic region of ROS-GC1 expressed in bacteria (ROS-GC1). Equal amounts of protein were coated. Standard protocols were used, and the results obtained with the antibody at a 1:5000 dilution are presented as OD at 405 nm. Experiments were carried out in duplicate and repeated for reproducibility. The specificity of the antibody was also tested by Western blotting (B). Membrane fractions were isolated as described in Experimental Procedures. Equal amounts of protein from the membrane fractions of COS cells expressing recombinant neuroepithelial mGC (rNEmGC), ROS-GC1 (rROS-GC1), and ROS-GC2 (rROS-GC2) were loaded on a SDS−polyacrylamide gel (8%), electrophoresed, transferred onto Nytran membranes, and probed with the neuroepithelial mGC antibody. Reaction was observed only with recombinant neuroepithelial mGC (indicated by arrow). Similar analyses with membrane fractions from olfactory epithelium (EPITHELIUM), bulb (BULB), and the retina (RETINA) were carried out. A solid arrow indicates the immunoreactive band. The positions of the molecular size markers are given alongside. (C and D) Biochemical identity of recombinant neuroepithelial mGC with the native cyclase—the response to neurocalcin. COS cells were transfected with the neuroepithelial mGC cDNA, and their membranes were prepared as described in Experimental Procedures. These were assayed for guanylate cyclase activity in the presence of incremental concentrations of neurocalcin and 100 μM Ca^{2+} (C) or in the presence of increasing concentrations of free Ca^{2+} and a constant concentration (4 μM) of neurocalcin (D). Each experiment was carried out in triplicate and repeated three times for reproducibility. The results that are depicted are from one representative experiment. The error bars are within the size of the symbols.

NEUROCALCIN-EPITHELIUM panels have been boxed. The boxed regions have been enlarged, presented side by side individually (NEmGC, NEUROCALCIN) as well as merged (MERGE) in the COMPOSITE-EPITHELIUM panel. The ROS-GC1-EPITHELIUM panel provides the control. All EPITHELIUM panels are oriented so that the ciliary layer is toward the top. The cilia, nuclei, and the basal region of the neuroepithelial layer have been indicated in the ROS-GC1-EPITHELIUM panel. The pseudostratified appearance of the olfactory epithelium is evident from the columnar appearance of the nuclei, which are stained blue in all EPITHELIUM panels. Positive staining for the protein appears brown in all panels.

In the BULB panels, specific staining of mitral cells (indicated by black arrows) and a population of smaller neurons is obtained with the ROS-GC1 antibody (ROS-GC1-BULB), as reported previously (*14*). No specific staining is observed with the neuroepithelial mGC antibody (NEmGC-BULB). Thus, the results obtained with the olfactory bulb are in agreement with those obtained through Western blotting (Figure 4B); neuroepithelial mGC is not expressed in the olfactory bulb.

In olfactory epithelium, results with the neuroepithelial mGC antibodies show that the cyclase is expressed uniformly throughout the ciliary region (Figure 5, NEmGC). The staining is more intense in the apical regions, a distinct dark

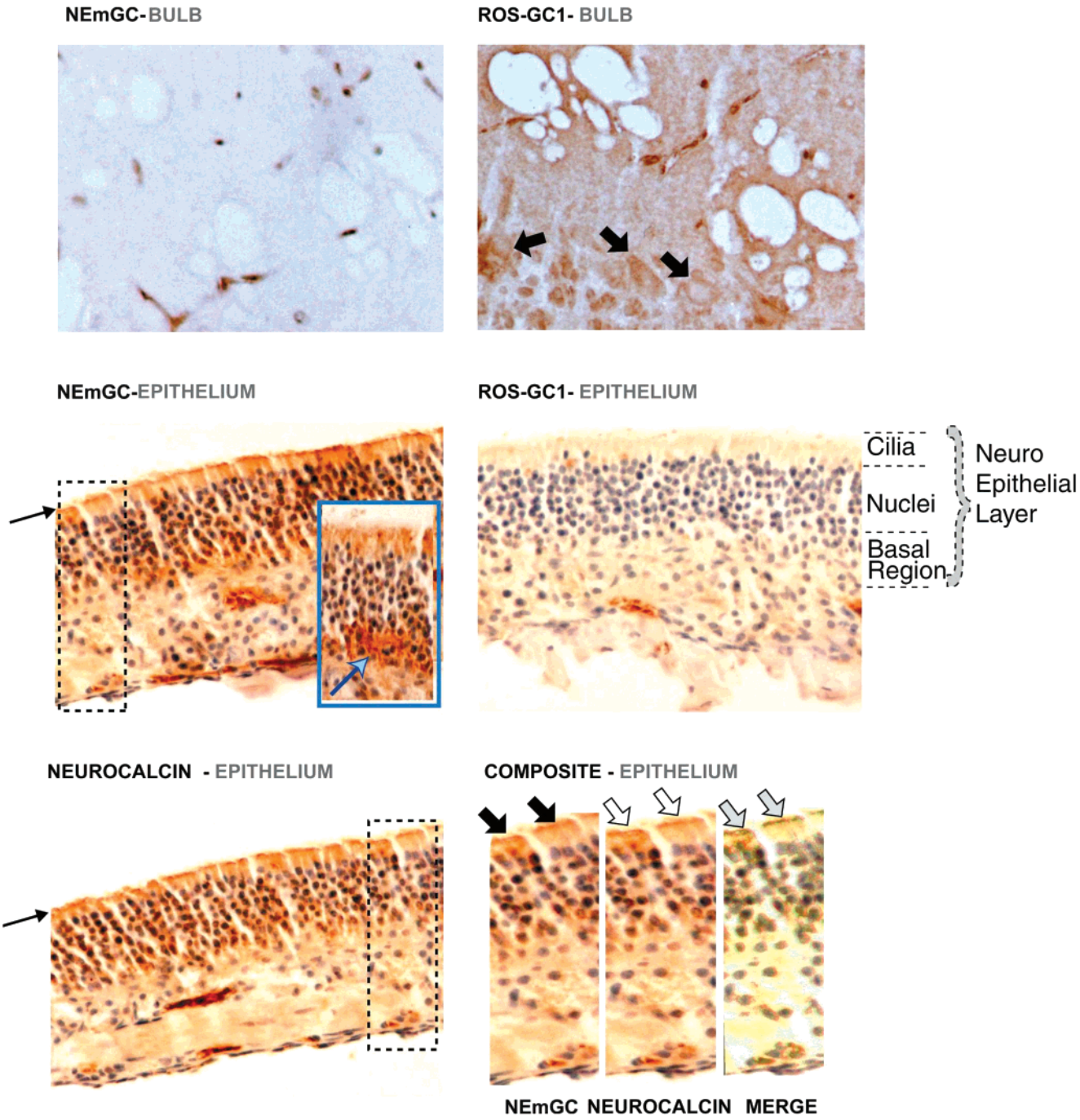

FIGURE 5: Immunolocalization of neuroepithelial mGC and neurocalcin in the olfactory neuroepithelium. Consecutive paraffin sections of the olfactory bulb (BULB) or neuroepithelium (EPITHELIUM) were subjected to immunohistochemical analyses to localize the neuroepithelial mGC and neurocalcin as described in Experimental Procedures. The antibody against ROS-GC1 was used as a control. Results with the antibodies are provided in their respective panels, along with the tissue presented in that panel. In the NEmGC-BULB panel, no specific staining is observed, as opposed to the ROS-GC1-BULB panel, where mitral cells (indicated by black arrows) and smaller neurons are stained positive for ROS-GC1. In the NEmGC-EPITHELIUM panel, the distinct apical staining, observed uniformly throughout the ciliary region, is indicated by an arrow. Basal staining for the neuroepithelial mGC, observed in restricted locations, is depicted in the inset, and the staining is indicated by a blue arrow. In the NEUROCALCIN-EPITHELIUM panel, uniform apical staining of the cilia is indicated by an arrow. Overlapping regions between the consecutive sections in NEmGC-EPITHELIUM and NEUROCALCIN-EPITHELIUM were identified (boxed regions in the respective panels), cropped, and used to generate a composite. The COMPOSITE-EPITHELIUM panel contains three segments: NEmGC, NEUROCALCIN, and MERGE. Block arrows indicate identical locations of staining and are colored black (NEmGC), white (NEUROCALCIN), or gray (MERGE). Antibodies against ROS-GC1 were used as a control, and the result is presented (ROS-GC1-EPITHELIUM). The cilia, nuclei, and the basal region of the neuroepithelial layer have been denoted. No staining was observed with the preimmune serum or when the primary antibody was omitted.

brown color at the top of the ciliary layer (indicated by an arrow). Very little staining is visible in the perinuclear region. The specific staining of the cilia, together with the lack of staining in the perinuclear region, leads to the conclusion that the neuroepithelial mGC is specifically expressed in the sensory neurons and not in the supporting cells. Some reaction with the neuroepithelial mGC antibody is also visible in the basal region; it is, however, localized to isolated areas

in the neuroepithelium. One such region is presented as an inset (Figure 5, NEmGC-EPITHELIUM; inset boxed in blue; staining is indicated with a blue arrow). Thus, specific and distinct staining is observed with the neuroepithelial mGC antibody (Figure 5, NEmGC-EPITHELIUM). No such staining is obtained with the control antibody (Figure 5, ROS-GC1-EPITHELIUM).

Immunolocalization with the neurocalcin antibody shows that this protein is also expressed uniformly throughout the cilia, especially in the apical region (Figure 5, NEUROCALCIN-EPITHELIUM; indicated by an arrow). Like with the neuroepithelial mGC, very little staining is observed around the nuclei. In contrast to that with the neuroepithelial mGC, there is no staining observed in the basal region. Thus, the expression of the neuroepithelial mGC and neurocalcin is colocalized in the apical region of the ciliary layer (compare staining indicated by arrows in NEmGC-EPITHELIUM vs NEUROCALCIN-EPITHELIUM). This is highlighted in the COMPOSITE-EPITHELIUM panel. Identical regions from the NEmGC and NEUROCALCIN panels (boxed) have been cropped, enlarged, labeled, and presented side by side. After color conversion, the image registration procedure (ImagePro software) was used to register the images to achieve an exact overlap. The merged image is presented (Figure 5, COMPOSITE-EPITHELIUM; MERGE) alongside. The regions where the expression of the neuroepithelial mGC and neurocalcin is colocalized within the same cell are indicated by solid arrows: black arrows in NEmGC, white arrows in NEUROCALCIN, and gray in MERGE. The results show that the apical region of the cilia exhibits precise colocalization of these two molecules within the same cell. This demonstrates that the neuroepithelial mGC and neurocalcin coexist in the apical region of the ciliary epithelium, which is the site of odorant transduction.

To confirm these conclusions, an alternate technique, cryosectioning, which better preserves antigenicity, was also used. The results obtained were identical to those obtained with paraffin sections (data not shown).

The Neurocalcin-Regulated Domain Resides at the C-Terminus of the Neuroepithelial mGC. The sequence of the cDNA clone indicates that the unprocessed neuroepithelial mGC consists of 1110 amino acids (Figure 6A and ref *9*). Hydropathy analysis suggests that the mGC is synthesized as a prepeptide with a leader sequence of 66 residues. The 408 N-terminal amino acids of the mature guanylate cyclase represent the presumed extracellular domain. In the receptor guanylate cyclase subfamily, this domain harbors the hormone receptor. However, in the ROS-GC subfamily, no function has been assigned to this domain, which is relatively divergent in all mGCs. The extracellular domain in the neuroepithelial mGC is ~40% identical with the corresponding ROS-GC1 and ROS-GC2 domains but only ~20% identical with those of ANF-RGC and CNP-RGC. A transmembrane (TM) domain of 26 amino acids separates the extracellular domain from the intracellular region, which contains at its C-terminus a 213-residue catalytic domain (amino acids 877−1089) and 20 residues of a C-terminal extension (CTE). A 46-amino acid domain (DD) presumed to be necessary for the cyclase dimerization separates the catalytic domain from the rest of the N-terminal 330-amino acid intracellular region. In ROS-GC1, this region is modular, containing the Ca^{2+}-regulated module (*36*, *37*). The topographical representation of the neuroepithelial mGC is depicted in Figure 6A.

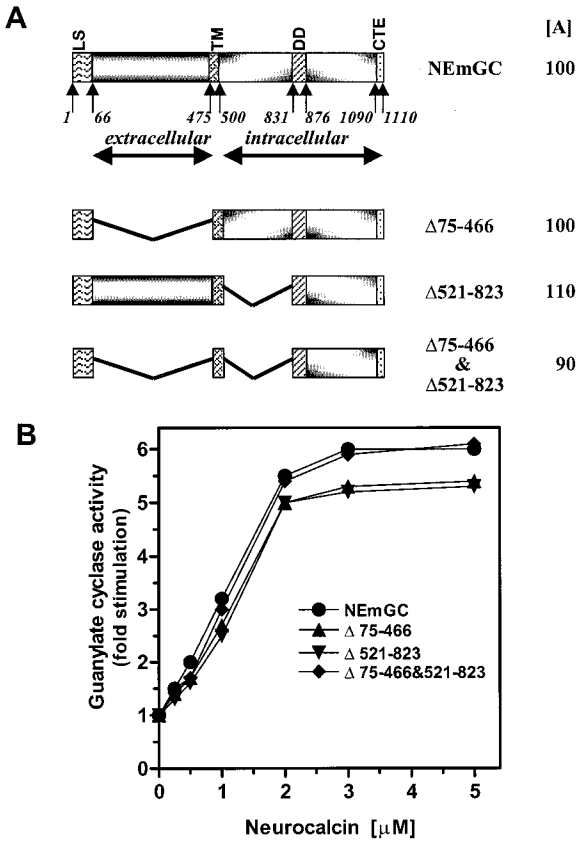

FIGURE 6: (A) Schematic representation of the neuroepithelial mGC, its deletion mutants, and their specific activities in COS cell particulate fractions. The topography of the neuroepithelial mGC is illustrated within the gray box. The amino acid regions encompassing the leader sequence (LS), the transmembrane domain (TM), the putative dimerization domain (DD), and the C-terminal extension (CTE) are indicated. The mutants are represented below the gray box. The deleted regions are indicated, and the positions of the deleted amino acid are given alongside for each of the mutants. The right-hand column [A] depicts the specific cyclase activity (picomoles of cGMP per milligram of protein per minute) of these expressed proteins as measured in crude membranes of COS cells. (B) Effect of neurocalcin on the cyclase activity of the neuroepithelial mGC mutants. Three deletion mutants were constructed. These and the wild-type neuroepithelial mGC were individually expressed in COS cells as described in Experimental Procedures. Particulate fractions of COS cells expressing wild-type recombinant neuroepithelial mGC or its mutants were assayed for guanylate cyclase activity in the presence of increasing concentrations of neurocalcin and 100 μM Ca^{2+}. The results that are depicted are from one representative experiment. The error bars are within the size of the symbols.

To locate the neurocalcin-regulated domain, three deletion mutants of the neuroepithelial mGC were constructed: Δ75−466 (deleted extracellular domain), Δ521−823 (deleted N-terminal region of the intracellular domain), and Δ75−466/Δ521−823 (double deletion, deleted extracellular domain and N-terminal region of the intracellular domain), which are schematically represented in Figure 6A. In all these mutants, the leader sequence and the transmembrane domain were retained to ensure proper translocation of the expressed protein to the cell membrane. The mutants were transiently expressed in COS cells. The membrane fractions were isolated, appropriately treated, and analyzed for their cyclase activities. At a fixed Ca^{2+} concentration of 100 μM,

neurocalcin stimulated the wild type and all the mGC mutants in a dose-dependent fashion with comparable EC_{50} values of ~1 μM (Figure 6B). The enzyme saturation of the wild-type and mutant cyclases also occurred at comparable neurocalcin concentrations; the saturation values of the Δ75−466 and Δ521−823 mutants were ~10% lower than that of the wild type. These results show that the neurocalcin-regulated domain does not reside in the segment of amino acids 76−823. The segment of amino acids 1−66 represents the leader sequence. Hence, the regulated domain must reside at the C-terminus, between amino acids 824 and 1110.

DISCUSSION

In the study presented here, analysis of the olfactory neuroepithelial region reveals that the plasma membrane fraction of its cilia contains a guanylate cyclase, neuroepithelial mGC, which is regulated by Ca^{2+}. This cyclase has been cloned and reconstituted to establish its identity with the native cyclase. Like the native form, the recombinant form does not respond to the GCAPs at nanomolar free Ca^{2+} concentrations; it, however, specifically responds to neurocalcin-regulated Ca^{2+} signaling. That neurocalcin is a natural regulator of neuroepithelial mGC is supported by the following facts: (1) it stimulates the cyclase within the physiological, submicromolar to micromolar, concentrations of free Ca^{2+}; (2) the EC_{50} values for Ca^{2+} in the native and reconstituted system with the added recombinant neurocalcin are almost identical (compare Figures 1 and 4D); and (3) the kinetic profiles of neurocalcin activation of the cyclase in the native membranes are identical with that of the reconstituted enzyme in the heterologous system where only the recombinant forms of the neuroepithelial mGC and neurocalcin are used. In both cases, the EC_{50} value is 1 μM and enzyme saturation is achieved at ~2.5 μM neurocalcin.

The immunocytochemical studies show that the neuroepithelial mGC and neurocalcin coexist in the ciliated apical border of sensory neurons located in the epithelial layer, which is the site of odorant transduction. This finding suggests a molecular model of odorant transduction. In this model, Ca^{2+} increments in the cilia are the result of an interaction between the seven-transmembrane-spanning protein receptor and the odorant ligand. These increments are sensed by neurocalcin, which binds Ca^{2+} and activates the cyclase. As a consequence, the production of cyclic GMP is accelerated, which then directly modulates the activity of its purported channel (*11*). The model predicts that a rise in the cyclic GMP will keep the channel open, and its decline will result in its closure. The "open state" of the channel will cause the entry of Ca^{2+} through it and result in depolarization of the ciliary plasma membrane; the "closed state" will cause hyperpolarization.

Several key elements of the model are supported by the previously established facts. (1) The odorant receptor is a seven-transmembrane-spanning protein receptor (*38*−*43*). To date, no member of the Ca^{2+}-modulated membrane guanylate cyclase family has been found to be a receptor for an extracellular ligand. In contrast, all, including neuroepithelial mGC, are regulated by the intracellular increases in Ca^{2+} concentration. (2) The activation of odorant receptor results in increments of free Ca^{2+} concentration (reviewed in refs *1*−*4*). (3) A channel specifically gated by cyclic GMP exists in the cilia of the neuroepithelium (*11*).

There are at least two conceptual gaps in the model: (1) why the Ca^{2+} concentration spikes induced by the opening of cyclic AMP-gated channels would promote opening of the cyclic GMP-gated channels and (2) why the cilia are not depolarized by the Ca^{2+} entering via the cyclic AMP-gated channels. It is anticipated that future experimentation on the model will aid in closing these gaps.

A striking feature of the odorant transduction model is that its operational principle is the opposite of that of the phototransduction model. In the latter model, ROS-GC1 and GCAP1 function as key components in the recovery phase of phototransduction. Upon illumination, the Ca^{2+} concentration rapidly drops to ~50 nM due to closure of cyclic GMP-gated channels resulting from the hydrolysis of cyclic GMP. This causes hyperpolarization of the photoreceptor membranes and stimulation of the cyclase (reviewed in ref *20*). As a result, the cyclic GMP concentration is elevated which, in turn, opens the cyclic GMP-gated channels and leads to the recovery of the dark state, where photoreceptor membranes are restored to their resting potential from a hyperpolarized state (reviewed in ref *20*). Hence, the ROS-GC transduction system is Ca^{2+}-inhibited, and its driving components are GCAPs. In contrast, the neuroepithelial mGC transduction system is Ca^{2+}-stimulated and its driving component is neurocalcin.

The subcellular localization of the neuroepithelial mGC in the neuroepithelium has been reported previously (*10*). Consistent with that study (*10*), this study shows the presence of the cyclase in the cilia of the sensory neurons. The earlier study shows that the guanylate cyclase-containing neurons do not overlap with the cAMP-signaling neurons and they also contain the cyclic GMP-specific gated channel (*11*). Taken together with the findings presented here, these neurons also contain neurocalcin. Thus, the complete membrane guanylate cyclase transduction machinery comprising of the Ca^{2+} sensor component (neurocalcin), neuroepithelial mGC, and cGMP-gated channel is all housed in the ciliary region of the sensory neurons.

There is one significant difference between the findings of this study and the earlier study (*10*). The earlier study found that the presence of the neuroepithelial mGC is restricted to a subpopulation of the sensory neurons where it is uniformly distributed within the cilia (*10*). Similar results were reported by another group (*11*). Both groups, however, used the same antibody to localize the cyclase (*10*, *11*). Our study shows that the neuroepithelial mGC is expressed in all sensory neurons and is specifically enriched in the apical region of the cilia. The difference in the findings may be a consequence of the antibody probes used in the two studies. In the study presented here, the specificity of the antibody probe has been demonstrated against native as well as denatured neuroepithelial mGC through ELISA and Western analysis. The antibody does not cross-react with two other Ca^{2+}-sensitive cyclases: ROS-GC1 and ROS-GC2 (Figure 4A,B). In Western analyses, the neuroepithelial mGC antibody detects a single protein in the native epithelium, which comigrates with the recombinant form, but not in the olfactory bulb or retina (Figure 4B). The results with the olfactory bulb are also confirmed through immunohistochemical studies; no staining is observed in this tissue with the neuroepithelial mGC antibody. Thus, the localization of the neuroepithelial mGC has been carried out with its highly

specific antibody probe in the present study. No such data for the antibody specificity have been provided in the other studies (*10*, *11*). Given the absence of these details, a precise comparison of that study with the present one is not possible. It is conceivable that the other antibody recognizes only a very restricted epitope.

In one study, an attempt has been made to localize a Ca^{2+}-dependent membrane guanylate cyclase in the neuroepithelium layer of the rat (*44*). Through kinetic analysis, the authors suggested the presence of two membrane guanylate cyclases in the cilia (*44*). These hypothetical cyclases had respective K_m values of 4.4 and 1700 μM for GTP. The cyclase with the K_m of 4.4 μM was suggested to be Ca^{2+}-sensitive (*44*). In the same study, the authors showed that the mGC is not restricted to a subpopulation of the sensory neurons by using an antibody of broad specificity against a general epitope of membrane guanylate cyclases (*44*). The immunocytochemical conclusion of the study agrees with the present study, that the membrane guanylate cyclase is localized in the general population of cilia and is not restricted to a specific subpopulation. However, the kinetic analysis of neuroepithelial mGC (K_m of 71 μM for GTP) indicates that it is not identical with any of the two hypothetical guanylate cyclases of the previous report (*44*).

In conclusion, this study describes the discovery of a new Ca^{2+}-regulated neuroepithelial mGC transduction system in the cilia of the olfactory epithelium. This discovery has enabled the investigators to construct a molecular model of odorant transduction. The model operates in a fashion that is the opposite of that of the known ROS-GC-linked phototransduction model. In the phototransduction model, Ca^{2+} spikes inhibit ROS-GC; in the odorant transduction model, they stimulate neuroepithelial mGC. Furthermore, the present model, contrary to the direct-ligand (odorant) model (*9*−*11*), envisions regulation of the neuroepithelial mGC through the intracellular spikes of Ca^{2+} in the cilia. An attractive possibility is that the generation of the spikes is through the seven-transmembrane-spanning odorant receptors, which are linked with the adenylate cyclase system. Thus, Ca^{2+} couples that adenylate and membrane guanylate cyclase transduction systems.

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