Negatively Calcium-Modulated Membrane Guanylate Cyclase Signaling System in the Rat Olfactory Bulb†

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ABSTRACT: The mechanism by which the individual odor signals are translated into the perception of smell in the brain is unknown. The signal processing occurs in the olfactory system which has three major components: olfactory neuroepithelium, olfactory bulb, and olfactory cortex. The neuroepithelial layer is composed of ciliated sensory neurons interspersed among supportive cells. The sensory neurons are the sites of odor transduction, a process that converts the odor signal into an electrical signal. The electrical signal is subsequently received by the neurons of the olfactory bulb, which process the signal and then relay it to the olfactory cortex in the brain. Apart from information about certain biochemical steps of odor transduction, there is almost no knowledge about the means by which the olfactory bulb and cortical neurons process this information. Through biochemical, functional, and immunohistochemical approaches, this study shows the presence of a Ca^{2+}-modulated membrane guanylate cyclase (mGC) transduction system in the bulb portion of the olfactory system. The mGC is ROS-GC1. This is coexpressed with its specific modulator, guanylate cyclase activating protein type 1 (GCAP1), in the mitral cells. Thus, a new facet of the Ca^{2+}-modulated GCAP1−ROS-GC1 signaling system, which, until now, was believed to be unique to phototransduction, has been revealed. The findings suggest a novel role for this system in the polarization and depolarization phenomena of mitral cells and also contradict the existing belief that no mGC besides GC-D exists in the olfactory neurons.

Three neural compartments are involved in the perception of smell in mammals. The first is the olfactory epithelium, where the sensory cells for olfaction reside; the second is the olfactory bulb where the axons emanating from sensory cells project, and the third is the olfactory cortex, which is linked with the olfactory bulb via the olfactory tract (Figure 1). The odor signal is sensed within the olfactory epithelium, and smell perception occurs within the olfactory cortex. Thus, the sensory neurons of the olfactory epithelium are internetted with central cortical neurons via the intermediary neurons in the olfactory bulb.

Olfactory signaling is initiated at the ciliated apical border of sensory cells of the epithelial layer. The odorant binds to its surface membrane receptor, and this binding ultimately generates an electrical signal. The biochemical process involved in the conversion of odorant receptor binding into generation of electrical signal is called odorant transduction. The molecular details of this process are not known. Available evidence indicates that odorant receptors belong to the family of G-protein-coupled receptors (*1*−*4*). Two different mechanisms have been proposed for odorant transduction: one involving cyclic AMP and the other inositol triphosphate (IP_3). In the cyclic AMP pathway, the

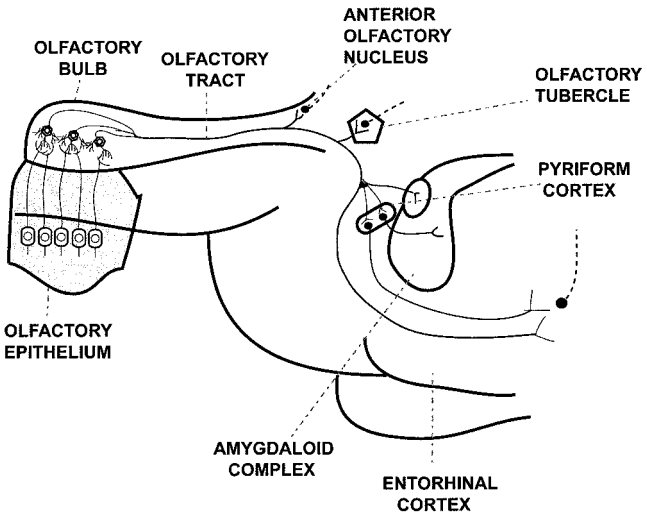

FIGURE 1: Schematic diagram of the olfactory system. The major areas involved in the perception of smell are illustrated. The odor molecules are initially sensed by the ciliated sensory neurons of the olfactory epithelium. This sensation is transmitted to the olfactory bulb, where the signal is processed. The olfactory tract carries the processed information from the olfactory bulb to the five regions: anterior olfactory nucleus, olfactory tubercle, pyriform cortex, amygdaloid complex, and entorhinal cortex. From these areas, the signal is further sent out (indicated by dashed lines) to the limbic system.

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odorant−receptor interaction generates cyclic AMP, opens the cyclic AMP-gated channel, and generates an electrical signal by causing an influx of Ca^{2+} and Na^{+}, which is reinforced by an additional Ca^{2+}-activated Cl^{-} influx (*5*−*8*). The net result is a depolarization of the ciliary plasma membrane (*6*−*8*). The details of the IP_3 pathway have not been delineated so well (*9*−*11*; reviewed in ref *12*). On the basis of the finding that a membrane guanylate cyclase (mGC),[1] GC-D, also exists in the ciliary membrane, the site of odorant transduction, an alternative, additional mechanism for depolarization of cilia has also been proposed (*13*, *14*). In this hypothesis, cyclic GMP, generated by GC-D, regulates the cyclic AMP-gated channel via cyclic GMP-stimulated phosphodiesterase 2 (*14*). Because to date no ligand has been identified for GC-D, it is considered as an orphan receptor (*13*). It has been, however, suggested that activation of GC-D occurs by the direct interaction of an as yet unidentified odorant molecule with its receptor, which resides in the extracellular domain of the cyclase (*15*).

Studies from another laboratory, however, propose that the neuroepithelial mGC is involved in secondary events rather than direct odorant binding (*16*). In this scenario, the mGC serves to amplify an odorant-induced cAMP response, rather than odorant binding. A role for mGC in events downstream of odorant binding has also been proposed in other systems. In *Caenorhabditis elegans*, ODR1 (mGC) has been demonstrated to mediate odor discrimination and adaptation (*17*). In salmon, a role for mGC has been proposed in odor imprinting (*18*). Thus, while the involvement of mGC in odorant transduction in the neurosensory epithelium has been amply demonstrated, its precise role remains to be elucidated.

Even less is known about the neural pathways beginning with the "depolarization of the cilia" and ending in the olfactory cortex. The odorant−receptor interaction results in the transfer of this information from olfactory receptor cells to the olfactory bulb via a series of action potentials. The incoming axons from the olfactory receptor cells terminate in synaptic areas termed glomeruli (*19*). Therein, the information is modulated and relayed via the intermediary neurons of the olfactory bulb which include mitral, tufted, granule, and periglomerular cells (*20*, *21*). The information is received by the dendrites of these neurons, processed in the soma, and transmitted, eventually, via the olfactory tract to the five specified areas of the olfactory cortex which include the anterior olfactory nucleus, olfactory tubercle, pyriform cortex, amygdaloid complex, and entorhinal complex. These areas decode the sensory input and translate the original odorant signal into the perception of the smell at the olfactory cortical centers. There is almost no information about the identity of the biochemical principles of this post-odorant transduction−signal processing machinery.

In the study presented here, biochemical and immunological tools have been used to establish the presence of a Ca^{2+}-modulated mGC (ROS-GC1) and its modulator, guanylate cyclase activating protein type 1 (GCAP1), in the olfactory bulb membranes. In situ hybridization and immunohistochemistry show that both the cyclase and its modulator reside within mitral cells. These findings provide a novel molecular explanation for the Ca^{2+}-dependent depolarization and polarization of mitral cells, which act as "microprocessors".

EXPERIMENTAL PROCEDURES

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 (wt-r) ROS-GC1 or ROS-GC2 expression constructs by the calcium phosphate coprecipitation technique (*22*). Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$ 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 tissues were purchased from Zivic-Miller. Membrane and soluble fractions from the olfactory epithelium and bulb were isolated (*23*). 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 (*24*). Briefly, membranes were preincubated on an ice bath with or without GCAP1, GCAP2, or S100β 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 maintained by incubation at 37 °C for 10 min. Termination was effected 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 formed was determined by radioimmunoassay (*25*).

Expression and Purification of Recombinant GCAP1 and GCAP2. GCAP1 and GCAP2 were cloned, expressed, and purified as described previously (*23*).

Western Blot. After being boiled in gel-loading buffer [62.5 mM Tris-HCl (pH 7.5), 2% SDS, 5% glycerol, 1 mM β-mercaptoethanol, and 0.005% bromophenol blue], samples were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in a buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. The proteins were transferred to Immobilon membranes (Millipore) in the same buffer but containing the appropriate concentration of methanol. The blot was incubated in Tris-buffered saline (TBS, pH 7.5) containing 100 mM Tris-HCl, 0.9% NaCl, and 0.05% Tween-20 (TBS-T) with 5% powdered, nonfat Carnation milk (or 3% bovine serum albumin) (blocking buffer) overnight at 4 °C and rinsed with TBS-T.

[1] Abbreviations: ANF, atrial natriuretic factor; CNP, C-type natriuretic peptide; ROS, rod outer segment; GCAP, ROS guanylate cyclase activating protein; mGC, membrane guanylate cyclase; NeuN, neuronal nuclear antigen; PBS, phosphate-buffered saline; ROS-GC, ROS membrane guanylate cyclase; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; STa, heat-stable enterotoxin; STa-RGC, heat-stable enterotoxin receptor guanylate cyclase; TBS, Tris-buffered saline; wt-r, wild-type recombinant.

Primary antibodies were added at appropriate dilutions in the blocking buffer, and incubation was continued for 1 h. After being rinsed with TBS-T, blots were treated with the secondary antibody conjugated to horseradish peroxidase (or alkaline phosphatase) in blocking buffer (1:10000) for 1 h. Finally, visualization of the immunoreactive protein bands was carried out according to the manufacturer's (Vector labs) protocol.

Antibodies. GCAP1 expressed in bacterial cells and subsequently purified was used to raise antibodies against this protein in rabbits. The ROS-GC1 antibody was raised against a 44-amino acid C-terminal domain expressed in *Escherichia coli* as a fusion protein. Monospecific antibodies against ROS-GC2 and GCAP2 were raised in rabbits using synthesized peptides from the C-terminal region of the respective proteins. All antisera were tested for their specificity through the enzyme-linked immunosorbent assay (ELISA) and by Western blotting according to standard protocols. 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 dilution of 1:10000 was generally used for colorimetric detection of the antigen on Western blots. ROS-GC1 antisera kindly provided by A. Yamazaki (Wayne State University, Detroit, MI; for immunohistochemical studies) and K.-W. Koch (Institut für Biologische Informationverarbeitung, Jülich, Germany; for Western blots) were used to verify the results obtained with the ROS-GC1 antisera raised by this laboratory. A monoclonal antibody against S100β (clone SH-B1) was purchased from Sigma Chemical Co. (St. Louis, MO). The NeuN antibody was purchased from Chemicon.

Immunohistochemical Analyses. Rat olfactory bulbs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight. The bulbs were dehydrated and embedded in paraffin according to standard procedures. Four micrometer sections were used for immunohistochemical analyses according to the procedure described previously (*26*). 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 in PBS containing preimmune serum, 2% BSA, and 0.1% Tween-20 had been blocked, 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 those recommended by the manufacturer. The chromogenic reaction included 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 the primary antibody either was not added or was replaced by preimmune serum. Staining was insignificant in either case.

In Situ Hybridization. Biotinylated probes for ROS-GC1 and GCAP1 were obtained by PCR amplification as described previously (*23*). A probe for ROS-GC2 was used as a control. Sections were obtained and prepared as described above and washed in 2× SSC with 0.1% SDS. Hybridization was performed in Hybrisol VI overnight at 37 °C in a humid atmosphere, and transcripts were detected with the Vectastain Elite kit (Vector Labs) as described above. Imaging was carried out using a Nikon phase-contrast microscope and a Princeton Instruments cooled CCD camera. Images were processed as described above. No significant signal was detected when the probe was omitted.

RESULTS

Until recently, it was believed that retinal neurons are the sole domain of a signaling mechanism in which mGC is modulated by calcium ions. The calcium-sensitive ROS-GC transduction system has been assumed to be exclusively linked to phototransduction and, possibly, to retinal synaptic activity (reviewed in ref *27*). This belief, however, was invalidated by the recent discovery of the ROS-GC1 signal transduction system in pineal neurons where, as in retina, both positive and negative Ca^{2+} signals are sensed by ROS-GC1 (*23*). This finding opened up the possibility that a calcium-modulated ROS-GC signal transduction system may be operating in other neurons as well.

Olfactory Bulb but Not Olfactory Epithelial Cell Membranes Expresses a Guanylate Cyclase with the Biochemical Attributes of ROS-GC1. Particulate fractions of the rat olfactory epithelium and olfactory bulb were isolated and tested for guanylate cyclase activity. Both fractions exhibited cyclase activity, although there were quantitative differences. The specific cyclase activity exhibited by the epithelial layer was ~1 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}, whereas that of the olfactory bulb was ~10 pmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1} (Figure 2A).

A signature feature of ROS-GC subfamily members (ROS-GC1 and ROS-GC2) is their Ca^{2+}-sensitive modulation by GCAPs (reviewed in refs *27* and *28*). To determine whether ROS-GC(s) contributes to the guanylate cyclase activity in the olfactory epithelium and bulb, the effect of GCAPs on the olfactory mGC was tested. When GCAP1, at a saturating concentration (5 μM), was recombined with the particulate fractions of olfactory bulb in the presence of 10 nM free Ca^{2+}, the mGC activity was stimulated 3-fold over the basal value (Figure 2B). Bulb mGC was also stimulated by GCAP2. Saturating concentrations of GCAP2 (20 μM) caused a 4-fold increase in bulb mGC activity at 10 nM Ca^{2+} (Figure 2B). No stimulation of the epithelial cyclase activity was observed with either GCAP under identical conditions (Figure 2B). These results suggest that a ROS-GC is expressed in membranes of the olfactory bulb but not in the epithelial layer. Therefore, further analyses were focused on the olfactory bulb.

To determine if one or both ROS-GCs are expressed in the olfactory bulb, advantage was taken of the selectivity of

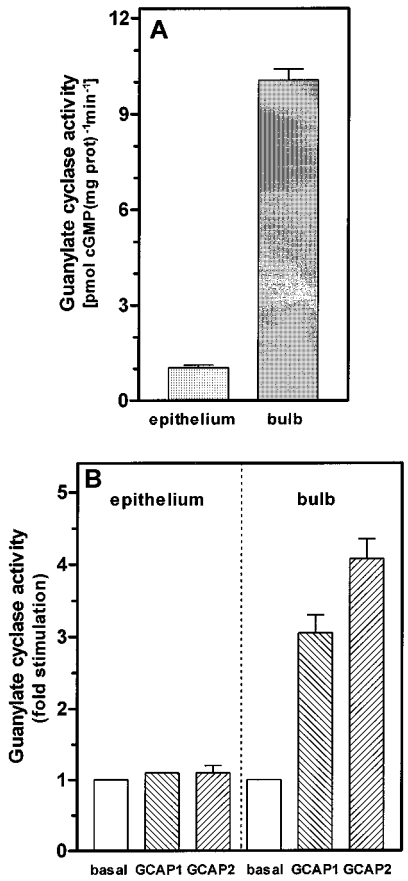

FIGURE 2: Guanylate cyclase activity in membranes of rat olfactory epithelium and bulb. Membranes of rat olfactory epithelium and bulb were isolated as described in Experimental Procedures and assayed for guanylate cyclase activity in the absence (A) or presence (B) of 10 nM Ca^{2+} and 5 μM GCAP1 or 20 μM GCAP2. The experiment was carried out in triplicate and repeated three times. The results (means + SD) are from one representative experiment.

ROS-GC modulators: GCAP1, GCAP2, and S100β. In the presence of Ca^{2+} concentrations of <50 nM, GCAP1 stimulates ROS-GC1, but not ROS-GC2 (*29*, *30*). The half-maximal stimulation of wt-rROS-GC1 occurs at ~1 μM GCAP1 (*29*). GCAP2, like GCAP1, exhibits its stimulatory effect in the presence of low Ca^{2+} concentrations. It appears to be the primary modulator of ROS-GC2 activity with an EC_{50} value of ~1 μM (*30*). GCAP2 can also stimulate wt-rROS-GC1, but the half-maximal stimulation occurs at a higher concentration, ~8 μM, of GCAP2 (*31*, *32*). In contrast to the GCAPs, S100β elicits its effect at micromolar ranges of free Ca^{2+} and it stimulates wt-rROS-GC1 with an EC_{50} value of ~1 μM, whereas wt-rROS-GC2 with EC_{50} of ~8 μM (*33*−*35*). Thus, the precise functional identification of the ROS-GC type, based on the distinct stimulatory profiles of GCAP1, GCAP2, and S100β, is possible. Therefore, dose−response curves for the bulb mGC with GCAP1, GCAP2, and S100β were obtained. The membrane fraction of COS cells expressing wt-rROS-GC1 or ROS-GC2 served as the control.

In 10 nM Ca^{2+}, GCAP1 stimulated the bulb mGC activity in a dose-dependent manner (Figure 3A). The maximal achieved stimulation was about 3-fold (Figure 3A). The EC_{50} value was ~1 μM, identical to that for wt-rROS-GC1 (Figure 3A). The GCAP2 stimulatory effect was also dose-dependent, and again the EC_{50} value of ~8 μM was the same as that for wt-rROS-GC1, but not for wt-rROS-GC2 (Figure 3B). In the presence of 500 μM free Ca^{2+}, the bulb mGC responded to increasing concentrations of S100β (Figure 3C). The half-maximal stimulation was achieved at 0.8 μM S100β, and the maximal stimulation was approximately 2.5-fold. This pattern of stimulation with S100β is consistent with the bulb mGC being ROS-GC1. Thus, through all functional criteria, it is established that the membranes of the olfactory bulb express ROS-GC1 and that this cyclase form is not present in the olfactory epithelium.

Relevant to olfactory epithelium, in one report, the presence of two kinetically distinct forms of mGC in the olfactory cilia of rat has been shown (*16*). The molecular identities of these forms have not been determined, but their biochemical features do not match those well-established for ROS-GC1 (*16*, *29*, *31*, *32*, *36*, *37*; reviewed in ref *27*). Neither ciliary mGC responds to GCAP1 or GCAP2 at ≤50 nM Ca^{2+} (*16*), a characteristic feature of ROS-GC1 (*29*, *31*, *32*, *36*, *37*; reviewed in ref *27*). In contrast, one of the ciliary mGCs is modestly stimulated (~45% above the basal value) by GCAP1 at 10 μM Ca^{2+} (*16*). This feature of the ciliary mGC is also the opposite of that of ROS-GC1, which is inhibited by GCAP1 at micromolar Ca^{2+} concentrations (*36*). Thus, unlike the olfactory bulb mGC, the ciliary mGC is not ROS-GC1.

Olfactory Bulb Membranes Express Peptide Receptor Guanylate Cyclases Also. There are two subfamilies of mGCs: peptide receptors and ROS-GCs. Members of the peptide receptor subfamily are regulated by the binding of peptides to the extracellular domain of the cyclase (reviewed in ref *28*). The ROS-GCs are regulated by Ca^{2+}. This is mediated through binding of EF-hand regulatory proteins to the intracellular domain of the cyclase (*29*, *37*−*39*; reviewed in ref *27*). Having established the existence of a ROS-GC in bulb membranes, we carried out experiments to investigate if any peptide receptor cyclase was also expressed. To determine whether ANF-RGC (atrial natriuretic factor receptor guanylate cyclase) and/or CNP-RGC (C-type natriuretic factor receptor guanylate cyclase) is expressed, bulb membranes were exposed to 10^{-8} and 10^{-6} M ANF or CNP in the presence of 0.4 mM ATP. The cyclase activity was stimulated by both ANF and CNP. The stimulation was dose-dependent with a maximum of about 2-fold (Figure 3D). It is also noted that the enterotoxin (STa) receptor membrane guanylate cyclase (STa-RGC) has been cloned from the bovine olfactory bulb, and the sequence has been deposited (*40*). Thus, in bulb membranes, the basal cyclase activity observed is the sum of the activities of several mGCs: ROS-GC1, ANF-RGC, CNP-RGC, and STa-RGC. Among these, evidence for the existence of ANF-RGC alone has been provided by previous studies (*41*, *42*). Since our study was oriented toward Ca^{2+}-modulated mGC, further analyses were focused on ROS-GC.

The Ca^{2+}-Modulated GCAP1−ROS-GC1 and Not the GCAP2−ROS-GC2 Signal Transduction System Is Expressed in Olfactory Bulb. The results presented above demonstrate the functional presence of a Ca^{2+}-modulated mGC in the olfactory bulb and further suggest that the mGC is ROS-GC1. To determine the direct biochemical presence of ROS-GC1 in the olfactory bulb membranes, Western analyses were carried out. Membranes from the rat olfactory bulb were isolated, electrophoresed, transferred onto nitrocellulose

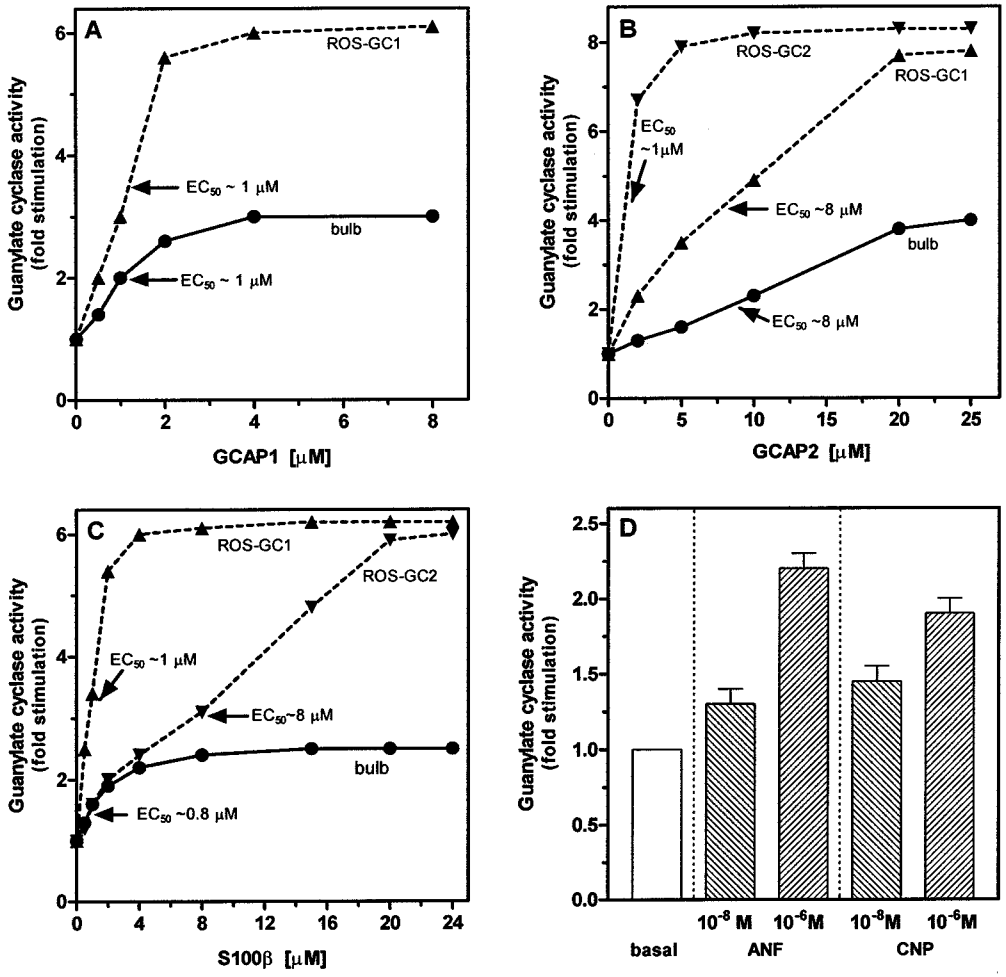

FIGURE 3: Biochemical characterization of rat olfactory bulb mGC. Membranes of rat olfactory bulb were isolated as described in Experimental Procedures. These were assayed for the guanylate cyclase activity in the presence of incremental concentrations of (A) GCAP1 and 10 nM Ca^{2+}, (B) GCAP2 and 10 nM Ca^{2+}, (C) S100β and 500 μM Ca^{2+}, or (D) ANF or CNP and 0.4 mM ATP. Each experiment was carried out in triplicate and repeated three times for reproducibility. The results (means + SD) are from one representative experiment. In panels A−C, error bars are within the size of the symbols.

membrane, and probed with specific antibodies against ROS-GC1 or ROS-GC2. A single band was detected with the ROS-GC1 antibody (ROS-GC1 in Figure 4A). The band exhibited mobility (118 kDa) identical to that obtained with the retinal membranes (ROS-GC1 in Figure 4A). No band was detected in the olfactory neuroepithelial membranes (ROS-GC1 in Figure 4A). Thus, ROS-GC1 is present in olfactory bulb membranes, but not in the sensory epithelium. Identical reactions carried out with the ROS-GC2 antibody, however, failed to detect any protein in the olfactory bulb membranes (ROS-GC2 in Figure 4A). The reactions readily detected ROS-GC2 in retinal membranes used as a positive control. Thus, ROS-GC1 is the sole ROS-GC expressed in the olfactory bulb, and it is not expressed in the neuroepithelial compartment of the olfactory system.

Since ROS-GC1 is modulated by GCAP1 and GCAP2 in a Ca^{2+}-sensitive manner and olfactory bulb membranes contain ROS-GC1, it was important to examine if GCAP1 and/or GCAP2 was also expressed. Western analyses showed that the GCAP1 antibody detected a single band in the membrane fraction isolated from the olfactory bulb (GCAP1 in Figure 4A), but not in the soluble fraction (data not shown). No reaction was observed when the GCAP2 antibody was used (data not shown). Thus, the olfactory bulb houses GCAP1, the specific modulator of ROS-GC1, but not GCAP2.

To investigate if ROS-GC1 and GCAP1 are functionally linked in a Ca^{2+}-sensitive fashion, olfactory bulb membranes were assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. At nanomolar Ca^{2+} levels, GCAP1 stimulates ROS-GC1 activity and progressively higher Ca^{2+} concentrations cause a decrease in this activity (*29*). The results depicted in Figure 4B show that increasing amounts of Ca^{2+} do indeed cause a dose-dependent decrease in mGC activity, with an IC_{50} value of ~90 nM. This is in agreement with the previously determined value of 100 nM Ca^{2+} for half-maximal inhibition of GCAP1-stimulated wt-r ROS-GC1 activity (*29*). These results demonstrate that a Ca^{2+}-sensitive GCAP1−ROS-GC1 signaling system operates in the olfactory bulb membranes. In addition, they also indicate that ROS-GC1 and GCAP1 are in proximity on bulb membranes.

Expression of ROS-GC1 and GCAP1 Is Colocalized in Mitral Cells of the Olfactory Bulb. The mitral cells are an important component in the transduction of odorant signals from the sensory epithelium to the olfactory cortex. These cells are also readily identified, based on their lack of staining for the neuronal nuclear antigen (NeuN) (*43*). Hence, mitral

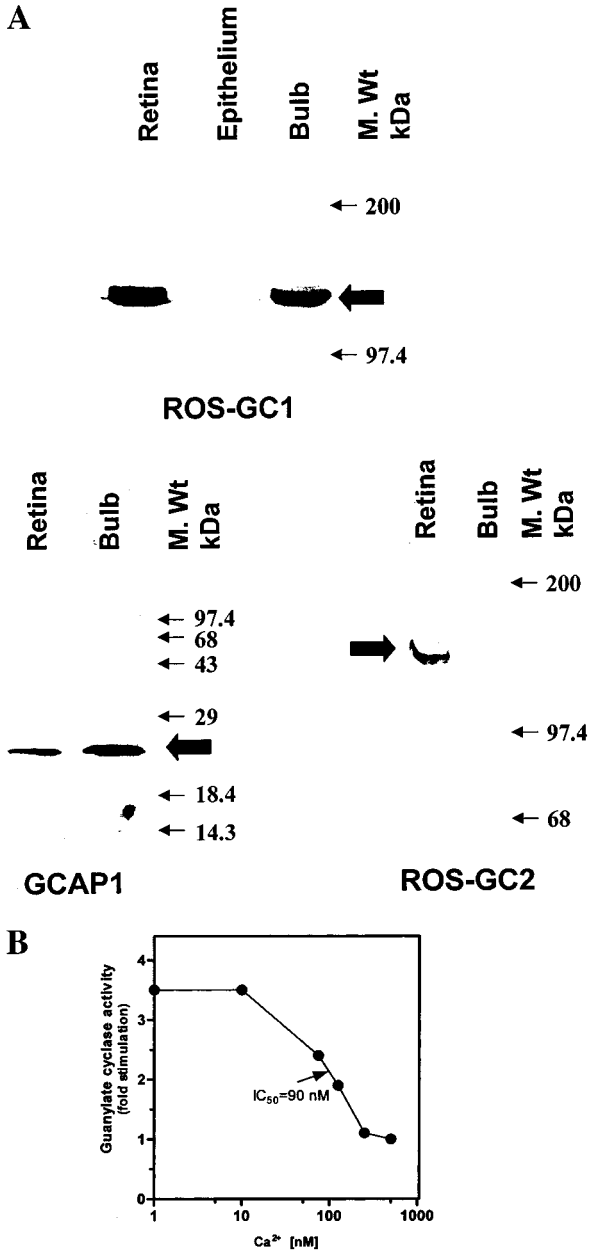

FIGURE 4: Characterization of the rat olfactory bulb ROS-GC system. (A) Western blot. Membrane fractions from retina, olfactory neuroepithelium, and olfactory bulb were isolated and subjected to Western analyses as described in Experimental Procedures. Equal amounts of proteins were compared. The result obtained with each of the specific antibodies against ROS-GC1, GCAP1, and ROS-GC2 is provided in the respective panel. The panel for ROS-GC1 has membrane fraction from the olfactory neuroepithelium (Epithelium) in addition to those from retina (Retina) and olfactory bulb (Bulb). Molecular size markers are provided alongside, and the position of the immunoreactive band is indicated by a solid arrow in each panel. (B) Ca^{2+} effect on olfactory bulb mGC activity. Membranes of rat olfactory bulb were isolated as described in Experimental Procedures. They were assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. The experiment was carried out in triplicate and repeated twice. The results are from one experiment.

cells became the focus of analysis for the presence of ROS-GC1 and GCAP1. Serial sections of olfactory bulb were stained with antibodies against NeuN, ROS-GC1, and GCAP1. Results obtained with each antibody are presented in the respective panels (NeuN, ROS-GC1, and GCAP1) in Figure 5. The rows of large neurons (indicated by arrows) that correspond to the mitral cells have been "boxed" in each panel. Comparable regions have been provided for NeuN and ROS-GC1. It is evident that the large row of mitral cells located below the external plexiform layer stains positive for ROS-GC1 as does a row of smaller neurons lying adjacent to mitral cells. However, the NeuN antibody, while heavily staining the underlying row of smaller neurons, does not stain mitral cell nuclei. The boxed region in each panel has been magnified and provided as an inset on the top of each panel to highlight this fact. Results with the GCAP1 antibody demonstrate the expression of GCAP1 in mitral cells (indicated by arrows; panel GCAP1 of Figure 5). Thus, ROS-GC1 and GCAP1 are colocalized in mitral cells. Neither protein can be detected within the nucleus, but they are colocalized in the cell body.

To determine if these proteins were synthesized endogenously in mitral cells, serial sections were used for in situ hybridization analyses. Both ROS-GC1 and GCAP1 transcripts are detected in the mitral cells (indicated by arrows), which are identified on the basis of their size and location (panels ROS-GC1 and GCAP1 of Figure 6). These regions have been magnified to illustrate the presence of transcripts in the larger neurons. Thus, both the transcripts for ROS-GC1 and GCAP1 and their encoded proteins are colocalized in the mitral cells (Figure 6).

It is also noted that the expression of ROS-GC1 is widespread in the olfactory bulb neurons. From the periphery of the tissue (corresponding to the external plexiform layer) to the core of the olfactory bulb (comprising the granular cells, small interneurons, and white matter), ROS-GC1 expression was detected (panel ROS-GC1 of Figure 5). Whether it is colocalized with GCAP1 in each of these neurons remains to be investigated.

DISCUSSION

The objective of this study was to investigate the presence of a Ca^{2+}-modulated ROS-GC transduction system in the olfactory epithelium and bulb, and to determine if the system mimics the ones present in rod outer segments of the retinal neurons (*29*, *32*; reviewed in ref *27*) and a subpopulation of pinealocytes (*23*). The rationale for this objective was that a role for Ca^{2+} signaling has been demonstrated in odor perception (reviewed in refs *44* and *45*). Our study shows that a functional, negatively Ca^{2+}-modulated ROS-GC transduction system exists in the olfactory bulb, but not in the epithelium. This signaling system mimics the systems established earlier in the rod outer segment (ROS) of the retinal neurons where phototransduction occurs (*29*, *31*, *32*, *36*, *37*; reviewed in ref *27*) and in the pineal gland (*23*). In ROS, ROS-GC exists in two isoforms: ROS-GC1 and ROS-GC2; therein, both forms are Ca^{2+}-modulated via GCAPs, GCAP1 and GCAP2 (reviewed in ref *27*). In contrast to the phototransduction system, in olfactory bulb neurons ROS-GC exists in only one form, that of ROS-GC1, and its modulator also exists in one form, that of GCAP1. In situ and immunohistochemical studies show that these two molecules colocalize in the mitral cell neurons of the bulb. The results demonstrate that ROS-GC1 and its Ca^{2+}-dependent modulator, GCAP1, are synthesized and expressed in the mitral neurons; therein, they are the sole components of the Ca^{2+}-dependent mGC system. Thus, the mitral cell

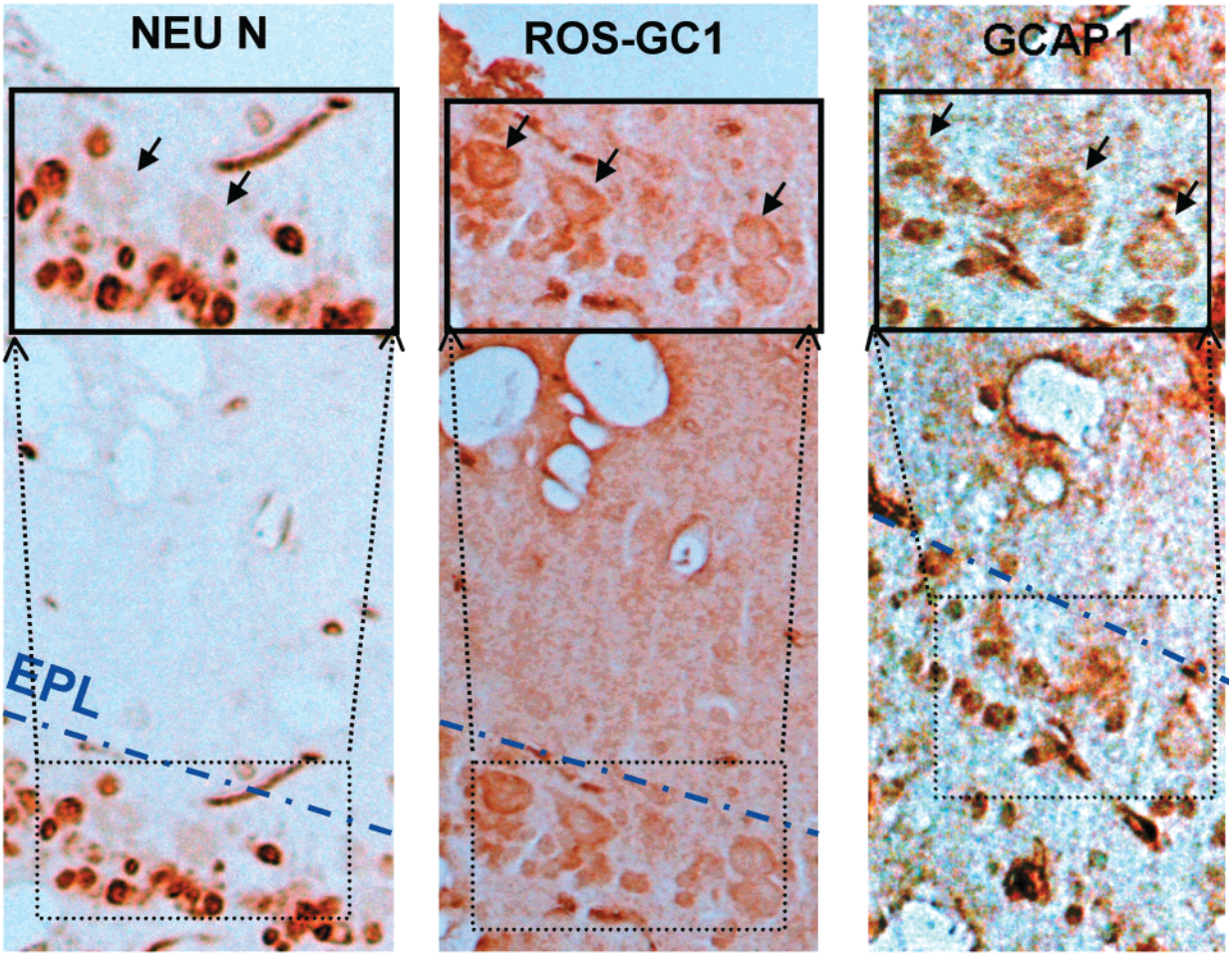

FIGURE 5: Immunohistochemical analysis of colocalization of ROS-GC1 and GCAP1 in mitral cells. Paraffin sections of rat olfactory bulb were probed for the localization of neuronal nuclear antigen, ROS-GC1, and GCAP1 as described in Experimental Procedures. The results are presented in panels NeuN, ROS-GC1, and GCAP1, respectively. Consecutive sections showing the same field have been presented in NeuN and ROS-GC1. Fields containing the external plexiform layer (EPL) and the mitral cell layer are presented in all panels, and the interphase between the two layers is marked by a dashed line. In each panel, the region containing the mitral cells has been boxed and the boxed region is magnified and presented as an inset. Arrows in the inset denote mitral cells.

ROS-GC1 transduction system is solely modulated by Ca^{2+} via GCAP1.

Until now, the GCAP1−ROS-GC1 signaling system has been regarded as unique to phototransduction (reviewed in ref *27*). In the current phototransduction model, ROS-GC1 and GCAP1 function as key elements in the recovery phase of phototransduction. In the dark state, when Ca^{2+} levels are around 500 nM, ROS-GC1-bound GCAP1 keeps the cyclase activity at a basal level. After illumination, the Ca^{2+} concentration rapidly decreases 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 (reviewed in ref *27*). Driven by the decreased Ca^{2+} concentration, ROS-GC1-bound GCAP1 stimulates cyclase activity. 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 *27*). Hence, a Ca^{2+}-dependent oscillation of ROS-GC1 activity, which in turn modulates polarization in the presence of cyclic nucleotide-gated channels, is achieved in the presence of GCAP1. With this perspective, what may be the physiological relevance of the ROS-GC1−GCAP1 transduction system in the mitral cells?

To construct a physiological model, the following established features of mitral cells have been considered. (1) The cells receive excitatory signals from the olfactory sensory cells (*19*−*21*). (2) They receive inhibitory signals from the neighboring cells (*46*, *47*). (3) In addition, they undergo autoexcitation (*48*). (4) The integration of these signals occurs in the soma. Mitral cells express both voltage-dependent and cyclic nucleotide-gated Ca^{2+} channels (*49*−*53*). With these considerations and the placement of the GCAP1−ROS-GC1 signal transduction system in the highly fluctuating Ca^{2+} environment, the following principles of the model are envisioned. After odorant stimulation, the neurosensory cells send excitatory signals to the mitral cells. These signals are integrated with the dendrodendritic inhibitory signals received from neighboring cells (*46*, *47*). As a result, the excitatory input is either stopped or modulated (*48*, *54*). The site of integration of these signals is the mitral cell soma, where the process of self-excitation also occurs. The latter happening causes a release of glutamate. Glutamate binds to NMDA autoreceptors located on the dendrites and cell body (*48*). As a consequence, the soma environment is constantly in a state of Ca^{2+} flux. The GCAP1−ROS-GC1 signal

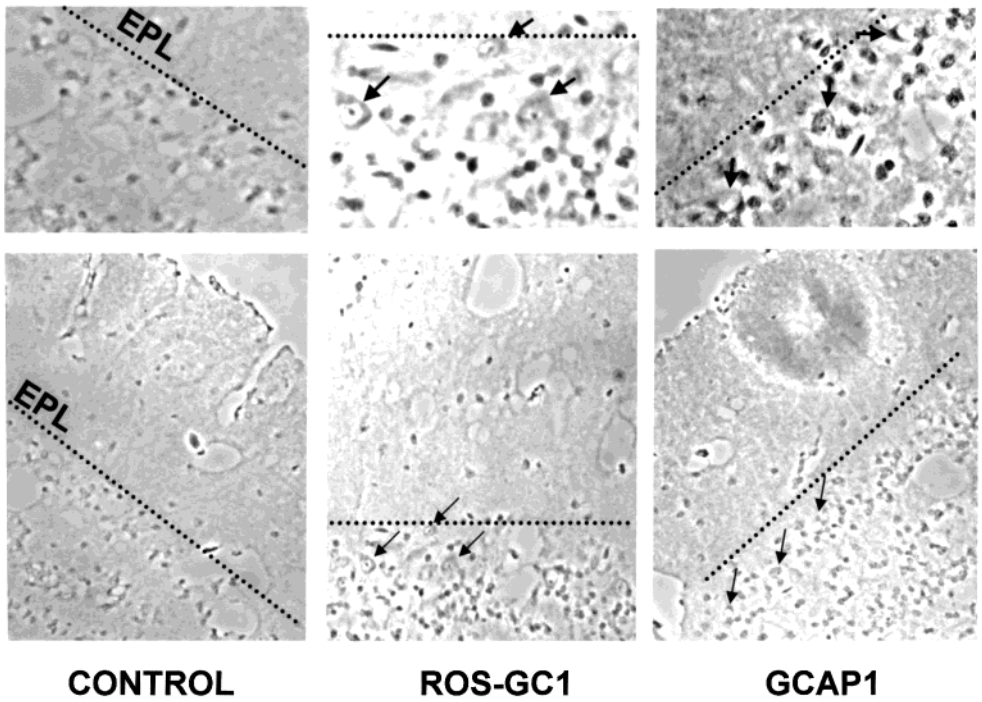

FIGURE 6: Mitral cells express transcripts for ROS-GC1 and GCAP1. Paraffin sections of rat olfactory bulb were probed for ROS-GC1 or GCAP1 mRNA as described in Experimental Procedures. The probe for ROS-GC2 served as the control. The results are presented in panels Control, ROS-GC1, and GCAP1. Fields containing the external plexiform layer (EPL) and the mitral cell layer are presented in all panels, and the interphase between the two layers is marked by a dashed line. For all panels, a low-magnification picture is provided in the bottom row and a higher-magnification picture in the top row. Arrows in both rows indicate identical, positively labeled mitral cells.

transduction system localized in the mitral cell body senses the Ca^{2+} fluxes and dampens or activates the cyclase. Thus, proportionate levels of cyclic GMP regulate the channel activity.

In the specific case of autoexcitation, glutamate release from the cell bodies is triggered by Ca^{2+} influx. On the basis of the envisioned model, a low-Ca^{2+} environment ($<$50 nM) within mitral cells will signal activation of ROS-GC1 through GCAP1. Cyclic GMP formed in response to activation of ROS-GC1 by GCAP1 will facilitate the regenerative calcium spikes by regulating the activity of calcium channels. In addition to directly regulating cyclic nucleotide-gated channels through binding, cyclic GMP might also play a role in modulating the activity of voltage-activated calcium channels, which are regulated by phosphorylation (*55*) via cyclic GMP-dependent protein kinase. Although this model is highly speculative, it is quite amenable to experimental verification, since the features of the GCAP1−ROS-GC1 system are well-defined. The enticing aspect of this model is that it provides a simple molecular explanation for the phenomena of polarization and depolarization of mitral cells.

In summary, this study has established the presence of the GCAP1−ROS-GC1 signal transduction system in mitral cells of the olfactory bulb. The system mimics the Ca^{2+} signaling in photoreceptors and in a subpopulation of pinealocytes. Thus, the system is not restricted to photic modulation, and has the makings of a unified theme by which Ca^{2+} signaling controls the sensory and processing activities of neurons. It is noteworthy that the GCAP2−ROS-GC2 system, which is also involved in phototransduction, has no role in the neuronal processes occurring in pinealocytes and mitral cells.

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