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Ca^{2+} Sensor GCAP1: A Constitutive element of the ONE-GC modulated odorant signal transduction pathway

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Abstract

In a small subset of the olfactory sensory neurons the odorant receptor ONE-GC guanylate cyclase is a central transduction component of the cyclic GMP signaling pathway. In a two-step transduction model, the odorant, uroguanylin, binds to the extracellular domain and activates its intracellular domain to generate the odorant second messenger, cyclic GMP. The present study through comprehensive technology, including gene-deletion, live cell Förster Resonance Energy Transfer (FRET) and Surface Plasmon Resonance (SPR) spectroscopy documents the identity of a remarkably intriguing operation of a Ca^{2+} sensor component of the ONE-GC transduction machinery, GCAP1. In the ciliary membranes, the sites of odorant-transduction, GCAP1 is biochemically and physiologically coupled to ONE-GC. Strikingly, this coupling reverses its well-established function in ROS-GC1 signaling, linked with phototransduction. In response to the free Ca^{2+} range from nanomolar to semimicromolar it inhibits ROS-GC1; yet in this range it incrementally stimulates ONE-GC. These two opposite modes of signaling two SENSORY processes by a single Ca^{2+} sensor define a new transduction paradigm of membrane guanylate cyclases. This paradigm is pictorially presented.

Keywords

Odorant transduction; Calcium; ONE-GC; GCAP1; Membrane guanylate cyclase

The odorant signal is initiated at the ciliated apical border of the olfactory sensory neurons located in the main olfactory epithelium (MOE). Binding of an odorant to its receptor generates an electrical signal. The biochemical term for this process is odorant-transduction (reviewed in 1⁻4). It is a two-step process; in the first step, the signal generates its second messenger; in the second step, the second messenger transforms the signal into an electrical signal, which then becomes a means of signal transmission and the final perception of SMELL in the cortical layers of the brain.

Till recently, the only second messenger of the odorant signal was considered to be cyclic AMP (5⁻9). Although, it still remains to be the major messenger, it is not the sole second messenger.

In the incremental development of the field, it has now been established that cyclic GMP is also the second messenger of the odorant signal (10⁻14; reviewed in: 1, 15⁻17; also 18 for recent discussion). This signaling pathway resides in a small population of the olfactory

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receptor neurons (ORN) and is independent of the cyclic AMP signaling pathway (11, 12). The pathway begins with the ONE-GC membrane guanylate cyclase [also named GC-D (10)], which is co-present with the specific cyclic GMP-dependent components, cyclic GMP-specific cyclic nucleotide-gated channel subunit, CNGA3, and a cyclic GMP-dependent phosphodiesterase, PDE2 (11, 12).

Importantly, the *modi operandi* of these two odorant pathways are radically different. In contrast to the cyclic AMP, cyclic GMP pathway does not function through the GTP-binding protein, G_{olf}. It originates from ONE-GC, which is both the receptor for the odorants uroguanylin (19, 20) and green pepper (14, 21), and also the transducer through its guanylate cyclase activity. Thus, in line with the prototype ANF-RGC membrane guanylate cyclase signal transduction model (22), coexistence of the uroguanylin receptor and guanylate cyclase activities on a single transmembrane spanning polypeptide chain makes the cyclic GMP signal transduction pathway more direct and, theoretically faster.

Among the multiple membrane guanylate cyclase signal transduction mechanisms, the odorant-linked ONE-GC mechanism is unique in several aspects (reviewed in: 17). It does not fit into the two traditional transduction models represented by the two membrane guanylate cyclase subfamilies. Unlike the Ca^{2+}-modulated ROS-GC subfamily, it recognizes the signal through its extracellular domain. And, unlike the hormone receptor subfamily but like the ROS-GC subfamily, the odorant signal after its transmission to the intracellular domain undergoes multiple Ca^{2+}-modulated steps. These steps amplify the signal prior to its final translation at the catalytic site into the production of cyclic GMP, the odorant's second messenger.

ONE-GC in addition to being an odorant receptor and transducer possesses an additional intriguing feature. Indirectly, through carbonic anhydrase enzyme, its catalytic site senses atmospheric CO_2 and accelerates the production of cyclic GMP (23, 24). For these reasons, ONE-GC represents the third subfamily of membrane guanylate cyclases, which accounts for its hybrid features of the other two subfamilies: peptide hormone receptor and Ca^{2+}-modulated ROS-GC (18, 20).

In the current odorant, uroguanylin, two-step model, in step one, the odorant binds ONE-GC and primes it for stimulation, causing its partial activation. This step is Ca^{2+}-independent (25). In step two, Ca^{2+}-bound neurocalcin δ through a defined intracellular domain, saturates ONE-GC activity, and depolarizes the ciliary membranes (25).

Besides neurocalcin δ, two other Ca^{2+}-sensors co-exist with ONE-GC. They are hippocalcin (26) and GCAP1 (27). However, the applicability of the two-step model for these signal transducers has not been studied. This study investigates the role of GCAP1, at the biochemical and physiological level, in the odorant ONE-GC signal transduction. The findings demonstrate that it represents a new paradigm of signal transduction. This model is pictorially presented and it may become a prototype for certain other neurosensory processes.

EXPERIMENTAL PROCEDURES

Antibodies

The specificities of antibodies against ONE-GC and GCAP1 have been described previously (27, 28). The antibodies were affinity purified. PDE2 antibody was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Secondary antibodies conjugated to a fluorescent dye (DyLight 488 and DyLight 549) were purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA.

GCAPs knockout mice

GCAPs knockout ($GCAP1^{-/-}GCAP2^{-/-}$) mice used as the source of the retina and olfactory epithelium were kindly provided by Dr. Alexander Dizhoor (Salus University) with the permission of Dr. Jeannie Chen (University of Southern California).

Immunohistochemistry

Mice were sacrificed by lethal injection of ketamine/xylazine (the protocol approved by the Salus University IUCAC) and perfused through the heart, first with a standard Tris-buffered saline (TBS) and then with freshly prepared 4% paraformaldehyde in TBS. Tissues (MOE and retina) were fixed for 1–4 hours in 4% paraformaldehyde with TBS at 4°C, cryoprotected in 30% sucrose overnight at 4°C and cut into 20 μm sections using Hacker-Bright OTF5000 microtome cryostat (HACKER Instruments and Industries Inc., Winnsboro, SC). *ONE-GC or GCAP1 immunostaining*: The sections were washed with TBS, blocked in 10% preimmune donkey serum in TBS/0.5% Triton X-100 (TTBS) for 1hr at room temperature, washed with TTBS, incubated with ONE-GC or GCAP1 antibody (diluted 50:1) in blocking solution overnight at 4°C, washed with TTBS for and then incubated with DyLight 488-conjugated donkey anti-rabbit antibody (200:1) for 1 hr, washed with TTBS. *PDE2A immunostaining*: The sections were incubated with 10% goat serum in TTBS, washed with TTBS and incubated with PDE2A antibody (50:1) in blocking solution for 1 hr, washed with TTBS, and incubated with DyLight 549 conjugated goat anti-rabbit antibody (200:1) for 30 min, washed TTBS and covered with UltraCruz™ mounting medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Images were acquired using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system, and analyzed using Olympus FluoView FV10-ASW software. Digital images were processed using Adobe Photoshop software.

Recombinant YFP-tagged GCAP1 and CFP-tagged ONE-GC

The bovine GCAP1 sequence was amplified by PCR from a cDNA clone using a forward primer 5′-TCAGATCTCGAGGCAGCCATGGGGAACATTATGAGCG-3′ and reverse primer 5′-CCCAACAGAATTCGAGAGCCGTCGGCCTCC-3′ thus adding the Kozak's motif in front of the ATG translation start codon, mutating the STOP codon and adding Xho1 restriction sites at both ends. The amplified sequence was inserted into Xho1 digested pZsYellow1-N1 vector (Clontech-TaKaRa Bio Co, USA) using "Infusion" kit (Invitrogen, USA).

CFP labeled ONE-GC was obtained identically as YFP labeled GCAP1 except that the PCR amplified ONE-GC sequence (forward primer 5′-CCGGACTCAAGATCTCGAGGCCACCATGGCAGGTCTGCA-3′ and reverse primer 5′-TCGAAGCTTGAGCTCGAGTGAGCAGACTCTGGCGAGCTTTG -3′) was inserted into pAmCyan1-N1 vector (Clontech-TaKaRa Bio Co, USA). For both YFP-GCAP1 and CFP-ONE-GC proper ligation was verified by sequencing. The resulting constructs were used for transfection of COS cells.

Expression in COS cells

COS cells were grown in coverslip chambers (two 4-cm^2 chambers per slide) in DMEM medium supplemented with 10% fetal bovine serum and transfected with the GCAP1-YFP and/or ONE-GC-CFP expression constructs using the Ca^{2+}-phosphate co-precipitation technique (29). 72 hr after transfection the cells were viewed directly or fixed in 4% paraformaldehyde in Tris-buffered saline (TBS) for 15 min at room temperature.

Preparation of olfactory neuroepithelial membranes preincubated with uroguanylin

Mouse MOE was homogenized in 250 mM sucrose/10 mM Tris-HCl pH 7.4/1 mM $CaCl_2$ buffer containing protease inhibitors (Sigma-Aldrich, USA). The homogenate was centrifuged at 1,000*g* and the supernatant was incubated on ice for 10 min with 10^{-6} M uroguanylin. Following the incubation the homogenate was centrifuged at 100,000*g* and the pellet (membrane fraction) was washed three times with 50 mM Tris-HCl pH 7.4/10 mM $MgCl_2$ buffer; the pellet represented membranes fraction. Washed membranes were suspended in the same buffer. Control membranes were prepared identically except that the homogenate was incubated without the addition of uroguanylin.

Guanylate cyclase activity assay

Control, or preincubated with uroguanylin, olfactory neuroepithelial membrane fractions were assayed for guanylate cyclase activity as described previously (25). Briefly, membranes were incubated on ice-bath with or without GCAP1 in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg creatine kinase and 50 mM Tris-HCl, pH 7.5, adjusted to 10 μM free Ca^{2+} concentrations with pre-calibrated Ca^{2+}/EGTA solutions (Molecular Probes). The total assay volume was 25 μl. The reaction was initiated by addition of the substrate solution (4 mM $MgCl_2$ and 1mM GTP, final concentration) and maintained by incubation at 37 °C for 10 min. The reaction was terminated by the addition of 225 μl of 50 mM sodium acetate buffer, pH 6.2 followed by heating on a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by radioimmunoassay (30).

Expression of ONE-GC fragment aa 836-1110 and GCAP1

Expression and purification of ONE-GC fragment aa 836-1110 was as described previously (27) and of GCAP1 as described in (31).

SPR spectroscopy

Sensorgrams were recorded using BIAcoreX-100 system. For immobilization, the ONE-GC fragment aa 836-1110 was dissolved in 0.05 M sodium acetate buffer pH 4 and coupled to the CM5 sensor chip. Immobilization level was ~4ng/mm^2. An independent flow cell on the chip was subjected to a “blank immobilization” (no ONE-GC fragment immobilized) and used as a control flow cell. The running buffer contained 10 mM HEPES pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.005% surfactant P-20. GCAP1 was dissolved in the running buffer at varying concentrations (0.063 – 8 μM) and flushed over both cells. The sensor surface was regenerated after each cycle with 0.05 M glycine pH 2. Binding was observed as an increase in resonance units (RU) and analyzed by the BIAcoreX100 software.

RESULTS

A scattered population of olfactory neurons expresses GCAP1

Rat MOE expresses GCAP1 at the mRNA and protein level (27). To determine in what regions of the MOE GCAP1 is expressed, its localization pattern was analyzed by immunostaining. To critically validate the antibody specificity for this approach, GCAP1 immunostaining of the wild-type and the GCAP1/GCAP2 double-knockout (GCAPs knockout) mouse retinas was tested (Fig. 1A). Intense GCAP1 immunoreactivity was observed in the wild-type photoreceptor outer segments (Fig. 1A: “wild type”), easily recognized by their typical morphology and location and, as observed earlier (28), in the OPL regions. In contrast, the corresponding layers in the GCAPs knockout mice retina show no staining (Fig. 1A: panel “GCAPs knockout”). Thus, the antibody is highly specific for

GCAP1 detection and is ideal for screening the presence of GCAP1 in the mouse olfactory epithelium.

The GCAP1 immunolabeling was present in the thinly distributed population of the olfactory sensory neurons (Fig. 1B: right panel). The cilia (a few indicated with white arrows) of the neurons were intensely labeled; lower intensity labeling penetrated into the dendrites and soma (a few indicated with yellow arrows). The GCAPs knockout mice showed complete absence of staining (Fig. 1C: right panel).

These results establish the presence of GCAP1 in the scattered population of the olfactory sensory neurons. It is predominantly present in the cilia, the site of odorant-transduction, yet, in lesser quantity is present also in other regions of the olfactory neuron.

GCAP1 and ONE-GC are co-expressed with the PDE2

The pattern of GCAP1 labeling as shown in figure 1B is very much like the one established earlier for ONE-GC and cyclic GMP phosphodiesterase PDE2A in mouse and rat olfactory epithelia (11, 12). To assess if GCAP1, ONE-GC and PDE2A co-reside in the ONE-GC neurons of the mouse MOE the technique of double immunostaining was used.

Because antibodies against ONE-GC and GCAP1 used in this study were raised in rabbits the direct double ONE-GC/GCAP1 immunostaining was not feasible. Therefore, this goal was achieved indirectly. Although PDE2A has not been functionally linked with ONE-GC, it always coexists with ONE-GC (11). Therefore, the co-presence of GCAP1 and PDE2A was determined first, then, of ONE-GC and PDE2A, and from them, the co-presence of GCAP1 and ONE-GC was assessed.

Figure 2A “GCAP1” shows that the cilia of 7 neurons (a few indicated by arrows) in this section exhibit an intense signal (red) generated with the GCAP1 antibody. In most cases this signal with a lower intensity also continues in the dendrites and somas. Figure 2A “PDE2A” shows the same section with the green signal generated with the PDE2A antibody. The “merged” image of the two signals shows that the same neurons express GCAP1 and PDE2A. When the GCAPs knockout mouse MOE was analyzed (Fig. 2B), the GCAP1 signal disappeared (Fig. 2B “GCAP1”) but the PDE2A signal persisted (Fig. 2B, “PDE2A”). These results demonstrate that GCAP1 and PDE2A are co-expressed in the same population of the olfactory sensory neurons and that absence of GCAP1 expression does not affect the expression of PDE2A.

Consistent with the earlier mouse (11) and rat (12) MOE studies, the present study shows the co-expression of ONE-GC and PDE2A in the cilia of selected mouse olfactory neurons (Fig. 2C: arrows point out to 3, out of 15, immunostained in this section); the “ONE-GC” panel shows the red signal generated by the ONE-GC antibody and the “PDE2A” panel, green signal generated by the PDE2A antibody. Upon their mergence (panel “merged”) the red and green signals overlap completely. Thus, ONE-GC and PDE2A are co-present in selected olfactory neurons.

Because both GCAP1 and ONE-GC are co-expressed with PDE2A it is concluded that GCAP1 is present in the ONE-GC neurons. The presence of ONE-GC, GCAP1 and PDE2A is predominant in the cilia, yet, to a leaser degree it is also present in other regions of the olfactory neurons.

ONE-GC and GCAP1 physically interact in the reconstituted cell system

Based on the above results that GCAP1 and ONE-GC are physically coupled in the mouse ONE-GC olfactory neurons, it was important to know if this was the intrinsic property of

these two molecules or, it was bestowed upon them through the accessory protein/s of the olfactory sensory system. In the first situation, wherever these two molecules are co-present in a living cell they should be bound, in the second, they would be bound exclusively in the olfactory sensory neurons.

This issue was addressed by expressing GCAP1 and ONE-GC individually or together in the heterologous cell system of COS cells and analyzing their cellular localization using the same antibodies as for immunolocalization of GCAP1 and ONE-GC in the olfactory epithelium

Expressed alone GCAP1 was evenly scattered in all cellular compartments including nucleus (Fig. 3A). Identical pattern of GCAP1 distribution, when expressed alone in HEK cells, has been observed earlier (32). It is consistent with previous conclusion that although GCAP1 has some ability to bind to the membranes, when expressed alone it behaves as a soluble protein (32–36). As anticipated, ONE-GC immunoreactivity was localized exclusively to the cellular membranes, the plasma membrane and the endoplasmic reticulum (ER) (Fig. 3B). There was no signal in the nuclear region of the cell.

In the ONE-GC and GCAP1 co-expressing cells, the pattern of GCAP1 immunoreactivity was drastically changed (compare figure 3A with figure 3C). The GCAP1 antibody generated signal was no longer evenly scattered in all cellular compartments. It was now localized solely to the plasma and ER membranes, the pattern of expression identical to that of ONE-GC. As indicated earlier, co-localization experiment of GCAP1 and ONE-GC was not possible due to the same source of both antibodies (rabbit). Because GCAP1 co-expressed with ONE-GC follows the pattern of ONE-GC localization it is, thereby, concluded that the membrane bound ONE-GC anchors GCAP1 to itself. Thus, it is the intrinsic property of the ONE-GC and GCAP1 to be bound to each other and no accessory proteins are required for their interaction.

GCAP1 and ONE-GC are functionally interlocked

To assess if the physical co-localization of GCAP1 and ONE-GC advances to their functional interaction, the reconstituted COS cell system was analyzed through FRET. The principle of this technique is that if the distance between two proteins is less than 10Å (functional interaction distance) an excited donor will transfer its energy to the acceptor, causing it to fluoresce on the expense of the donor's fluorescence. Thus, the acceptor quenches the fluorescence of the donor. One of the most common FRET pairs is cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The CFP emission spectrum (λ_{max} 475 nm) overlaps with YFP absorption spectrum (λ_{max} 512 nm). Therefore, these two fluorescent proteins were used to tag GCAP1 and ONE-GC. GCAP1 was tagged with YFP and ONE-GC with CFP and the fusion proteins were expressed in COS cells

The initial experiments were designed to verify that the fluorescent tag does not affect the expression of the fusion proteins. When GCAP1-YFP was expressed alone in COS cells the fluorescence was dispersed evenly in the cell (Fig. 4A). This pattern of fluorescence distribution remained unchanged in repeated transfections despite varying amounts of GCAP1-YFP cDNA used for transfection and was virtually identical to that obtained with immunocytochemical analysis.

When GCAP1-YFP was co-expressed with ONE-GC in COS cells the distribution of GCAP1-linked YFP fluorescence changed drastically (Fig. 4B). It was now limited to the plasma and ER membranes, following the pattern of ONE-FC expression. It is thus concluded that the localization results obtained with immunostaining and intrinsic fluorescence of the fusion proteins are in complete agreement.

Based on these conclusions the fluorescence quenching experiment was performed. The COS cells were co-transfected with GCAP1-YFP and ONE-GC-CFP. The CFP was the donor and YFP was the acceptor-quencher. The cells were excited at 458 nm (CFP excitation wavelength) and the emitted fluorescence was observed at 480 nm (emission of CFP) and at the 530 nm (emission of YFP). The results are presented in figure 5. The presence of GCAP1-YFP together with ONE-GC-CFP resulted in practically non-existing fluorescence of CFP at 480 nm (Fig. 5A) and in significant emission of yellow fluorescence by YFP at 530 nm (Fig. 5B). The ability of GCAP1-linked YFP to quench the ONE-GC-linked CFP fluorescence and the fact that the extent of quenching is reversely proportional to the distance between the fluorophore and the quencher show that in a living COS cell ONE-GC and GCAP1 are co-localized within a distance allowing their physical interaction. It is, therefore, concluded that GCAP1 and ONE-GC constitute a physically locked transduction system.

GCAP1 binds to the aa836-1110 segment and stimulates ONE-GC activity

To define the GCAP1-ONE-GC transduction system in biochemical terms, the site and the binding kinetics of GCAP1 with ONE-GC were assessed through SPR spectroscopy and the direct functional analyses.

Previous studies have shown that GCAP1 binds to the aa 836-1110 fragment of ONE-GC (27). Therefore, a soluble construct representing this fragment of ONE-GC was expressed in bacteria and purified to homogeneity. The fragment was functionally active, containing intrinsic guanylate cyclase activity of 3 pmol cyclic GMP/min/mg protein. It also possessed Ca^{2+}-modulated GCAP1-dependent activity, responding to GCAP1 in a dose-dependent fashion in presence of the saturating amount of Ca^{2+} (10 μM) (Fig. 6A). Thus, this fragment was suitable for the direct binding analyses.

The fragment was immobilized on a sensor chip and incremental concentrations of GCAP1 were supplied in the mobile phase containing 10 μM Ca^{2+}. A representative set of sensorgrams is presented in figure 6B. The respective fitting curves, derived after fitting to a 1:1 Langmuir binding model are also shown in this figure. It is evident from the figure that the fitting curves deviate from the experimental data especially in the dissociation phase. Therefore, the binding kinetics was determined graphically. To determine the half-maximal binding (EC_{50}), the experimental RU values were plotted as a function of GCAP1 concentration (Fig. 6C). EC_{50} of GCAP1 was 0.5 μM. Scatchard analysis of the binding data (Fig. 6D) resulted in the K_D value of 0.65 μM. This K_D value for GCAP1 binding to the ONE-GC fragment aa 836-1110 is in agreement with the 0.5 μM EC_{50} value of GCAP1 for the wild-type ONE-GC activation (27). The calculated equilibrium association constant, K_A, is $1.5 \times 106\ M^{-1}$. Thus, GCAP1 binds ONE-GC with moderate affinity.

It is of interest to note that these binding kinetics between GCAP1 and ONE-GC are very similar to that of the other Ca^{2+} sensor of ONE-GC, neurocalcin δ (14).

GCAP1 is a Ca^{2+} sensor of the odorant-linked ONE-GC transduction system in the olfactory sensory neurons

In line with the past (11, 12) and the present findings that ONE-GC mouse MOE contains the ONE-GC membrane guanylate cyclase transduction system, it was assessed whether under the native conditions GCAP1 functions as a Ca^{2+} sensor of ONE-GC. The membrane fraction of the mouse MOE was incubated with incremental concentrations of GCAP1 in the presence of saturating amount (10 μM) of Ca^{2+} or in its complete absence (1 mM EGTA added to the reaction mixture). In the presence of Ca^{2+} GCAP1 stimulated the membrane guanylate cyclase activity in a dose dependent fashion. The half-maximal stimulation was at

~0.5 μM GCAP1 and the maximal stimulation of ~3 fold above the basal value was observed at 2 μM GCAP1 (Fig. 7: solid circles). In the absence of Ca^{2+} GCAP1 was totally ineffective (Fig. 7: open circles). These results demonstrate that similar to the recombinant ONE-GC (Figure 1A in 27), GCAP1 is a natural Ca^{2+}-senosr component of ONE-GC.

This conclusion was brought to the physiological level with the identical study carried out in the GCAPs knockout mouse membranes, which lacked GCAP1 and GCAP2. The membrane fraction of the MOE isolated from the wild type (control) and the $GCAP1^{-/-}GCAP2^{-/-}$ mice were assayed for the guanylate cyclase activity in the presence of 1 mM EGTA (Ca^{2+} depleted conditions) and 10 μM Ca^{2+}. For the wt membranes the specific activity was ~11 pmol cyclic GMP/min/mg protein in the absence of Ca^{2+} and ~56 pmol cyclic GMP/min/mg protein in the presence of Ca^{2+} (Fig. 8A). The difference between these two activities reflects the combined contributions of the Ca^{2+}-dependent modulators of ONE-GC activity in the olfactory neuroepithelium, GCAP1, neurocalcin δ and hippocalcin (14, 26). To directly assess the contribution of GCAP1-modulated ONE-GC pathway the membranes of the $GCAP1^{-/-}GCAP2^{-/-}$ olfactory neuroepithelium were assayed under the same conditions. The specific activity in the absence of Ca^{2+} was identical to that of the wt membranes, ~11 pmol cyclic GMP/min/mg protein; in the presence of Ca^{2+} the activity was ~37 pmol cyclic GMP/min/mg protein (Fig. 8A). Thus, the input of GCAP1 modulated ONE-GC activity is reflected in the difference between activity of the wt and $GCAP1^{-/-}GCAP2^{-/-}$ neuroepithelial membranes and constitutes approximately 34% of total Ca^{2+}-dependent ONE-GC activity.

This conclusion was validated by the reconstitution experiment. Membranes of the wt and GCAPs knockout olfactory neuroepithelium were reconstituted with exogenous GCAP1. The rationale for the experiment was that exogenous GCAP1 should bring the cyclase activity to approximately the same maximal level in both types of membranes. And, indeed, GCAP1 stimulated guanylate cyclase activity in a dose dependent manner (Fig. 8B). The activity of the wt membranes was stimulated ~3-fold above the basal level and of the $GCAP1^{-/-}GCAP2^{-/-}$ membranes, 4-fold reaching the maximal activity of ~170 pmol cyclic GMP/min/mg protein.

It is thereby concluded that GCAP1 is the physiological Ca^{2+} sensor of the odorant linked ONE-GC transduction system.

GCAP1 is a Ca^{2+}-modulator of the odorant uroguanylin signaling of ONE-GC activity

The co-presence of ONE-GC and GCAP1 in the olfactory cilia strongly suggested that GCAP1 is involved in the odorant signal transduction. Because ONE-GC responds to the odorant, uroguanylin, stimulation by activating a two-step, Ca^{2+} independent and Ca^{2+}-dependent, signaling cascade it was warranted to analyze if Ca^{2+} sensor GCAP1 is involved in the processing of the Ca^{2+}-dependent step.

Freshly isolated mouse olfactory neuroepithelium was homogenized under the Ca^{2+}-depleted conditions (1 mM EGTA). The homogenate was first pre-incubated with 10^{-6} M uroguanylin and then the membranes were tested for the guanylate cyclase activity in presence of increasing concentrations of GCAP1 at 10 μM Ca^{2+}. Control experiment was performed identically, except the homogenate was pre-incubated, without uroguanylin.

The basal guanylate cyclase activity was 45 pmol cyclic GMP/min/mg protein (Fig. 9). In the presence of increasing concentrations of GCAP1 the ONE-GC activity of mock-preincubated membranes increased by about 3.5-fold (Fig. 9: open circles). The picture, however, was different for membranes pre-incubated with uroguanylin. Here the GCAP1 dose-dependent Ca^{2+} signaling of ONE-GC resulted in more than 12-fold stimulation of

ONE-GC activity, from 45 to 560 pmol cyclic GMP/min/mg protein (Fig. 9: closed circles). Thus, the combined effects of uroguanylin and GCAP1 far exceed the sum of their individual effects; they are synergetic.

DISCUSSION

In the developing field of the mammalian membrane guanylate cyclase this study is a continuation in exposing its new mechanistic means of signal transduction. The study documents that: 1- GCAP1 is a constitutive part of the odorant linked ONE-GC transduction machinery; 2- it is its Ca^{2+} sensor component; 3- it functions in the opposite fashion to that in the phototransduction; and 4- the odorant-ONE-GC-GCAP1 transduction system operates through two-step signaling mechanism.

GCAP1, a constitutive part of the odorant linked ONE-GC transduction machinery

To date GCAP1 is considered exclusively as the Ca^{2+}-sensor component of the phototransduction system. Its function is to capture the incremental (nanomolar to semimicromolar) Ca^{2+} signals, inhibit ROS-GC1 activity and the production of cyclic GMP, the second messenger of LIGHT signal (37–41). With this mode in the rod/cone outer segments it modulates the recovery and adaption processes of phototransduction. In the identical mode it functions in the cone pedicles (28), olfactory bulb neurons (42), pinealocytes (43) and in spermatogenic cells of the testes (44).

The presented findings demonstrate that in the mouse olfactory cilia: 1- GCAP1 is a physiological constituent of the ONE-GC signaling pathway; thus, it is not solely a constituent of the phototransduction system; 2- GCAP1/ONE-GC transduction pathway is neither connected nor overlaps with the major cyclic AMP signaling pathway; and 3- besides PDE2A (11) and CNGA3 (12), GCAP1 is also a marker protein of the ONE-GC neurons.

GCAP1 is the positive Ca^{2+} sensor modulator of the ONE-GC

Like in the *in vitro* reconstitution and the rat olfactory neuroepithelium systems (27), in the mouse MOE GCAP1 stimulates ONE-GC activity in the presence of Ca^{2+}. Thus, its *modi operandi* in the photo- and odorant-transduction processes are in opposite fashions, and so are the consequences. The first *modus operandus* causes a decline in the production of cyclic GMP. This, via closure of the cyclic GMP-gated channels and continuous extrusion of Ca^{2+} through its exchanger causes hyperpolarization in the outer segments. The second mode causes acceleration in the cyclic GMP production and depolarization of the olfactory ciliary membranes (45).

How does GCAP1 exhibit reversible operation of structural homologues ROS-GC1 and ONE-GC? This riddle, as yet, is not solved. The present study, however, discloses two clues: 1- The signal transduction sites of GCAP1 in these two cyclases are different. In ROS-GC1 it resides in two short regions of its juxtamembrane domain, M445-L456 and L503-I522 (46); in ONE-GC, it is at the C-terminal domain, 836-1110 (27); 2- In phototransduction-linked ROS-GC1 initiation of the GCAP mode is indirect, via the Light signal-induced cascade occurring through G-protein, transducin; in ONE-GC, it is direct, it originates and is transmitted through ONE-GC.

GCAP1 is bound to ONE-GC in the native olfactory sensory neurons

Previous immunoprecipitation results demonstrate an important olfactory-relevant feature of GCAP1. It is that GCAP1 is permanently bound to ONE-GC in the olfactory neurons (27). Thus, only changes in free $[Ca^{2+}]_i$ but not GCAP1-ONE-GC association/dissociation cycle

define the velocity of the odorant-linked ONE-GC transduction machinery. This is identical to phototransduction where GCAP1 is permanently bound to ROS-GC (reviewed in: 47).

Consistent with its role of a physiological partner to the ONE-GC odorant transduction system, SPR studies show that it binds ONE-GC with moderate affinity (K_D of 0.67 μM; K_A, 1.5 × 106 M^{-1}).

Gene deletion and rescue operations demonstrate that GCAP1 contributes ~34% to the ONE-GC-signal transduction system. It can now be concluded that three Ca^{2+} sensors define ONE-GC modulation; they are neurocalcin δ, Hpca, and GCAP1; and their respective contributions in ONE-GC modulation are 27% for neurocalcin δ (26), 30% for hippocalcin (26) and 34% for GCAP1.

GCAP1-modulated odorant signal transduction model

The presented evidence demonstrates that the GCAP1-modulated uroguanylin signaling of ONE-GC activity is a two-step process. As shown earlier (25) and also now, the first process occurs at the ONE-GC extracellular domain by its interaction with the odorant. This step is Ca^{2+}-independent. It *PRIMES* ONE-GC, partially (~3-fold) activates it, and prepares it for the second GCAP1/Ca^{2+}-dependent step, which, causes full activation of the guanylate cyclase, 4-fold over the primed step, overall ~12-fold. This signal transduction mechanism is pictorially presented in figure 10.

In the formulation of this model the following features of ONE-GC are considered general, derived from other members of the membrane guanylate cyclase family: 1- ONE-GC is homo-dimer (48); 2- Its contact points for homo-dimerization reside in its extracellular and catalytic domains (48–50); 3- The monomers of the catalytic domains are anti-parallel (48, 49).

The Model. ***Basal state:*** The concentration of free $[Ca^{2+}]_i$ in the resting cilia is 60–100 nM (1); ONE-GC dimer is bound to Ca^{2+}-free GCAP1 and its activity is in the basal state. ***Primed, partially active state***: The odorant, uroguanylin, signaling begins by its binding to the extracellular receptor domain of ONE-GC (20). In a Ca^{2+}-independent fashion, it causes structural changes, which are successively transduced to the catalytic module, causing its partial activation of ~3-fold. The small amount of cyclic GMP produced as a result of this activation opens a fraction of the CNG3 channels and some influx of Ca^{2+}, which partially depolarizes the ONE-GC neuron's membrane. ***Fully* a*ctive state:*** The Ca^{2+} ions which entered the ONE-GC neuron as a result of partial ONE-GC activation bind to GCAP1. Ca^{2+}-bound GCAP1 causes full activation of ONE-GC; cyclic GMP formed opens maximal number of CNGA3 channels causing maximal influx of Ca^{2+} and depolarization of the ONE-GC neuron's membrane.

CONCLUSION

Besides disclosing an intriguing GCAP1-modulated odorant transduction mechanism, this study has begun to answer a seminal question related to the general role of cyclic GMP signaling pathway in the odorant signaling.

How can a single membrane guanylate cyclase transduce multiple forms of the odorant?

The emerging answer is that it is defined by the architecture of the odorant receptor guanylate cyclase. *Consider ONE-GC*. It is a transducer of three odorants: green pepper (14, 21), uroguanylin (19, 20, 25) and atmospheric CO_2 (23, 24). Studies with the last two odorants reveal that these odorants achieve specificity of ONE-GC signaling through their

divergent mechanisms by which they modulate their specific domains in ONE-GC (25). The chemosensory odorant, uroguanylin, starting from the extracellular domain of ONE-GC requires multiple signal transduction events, which are Ca^{2+}-independent and Ca^{2+}-dependent (25). But bicarbonate, the second messenger of CO_2, directly, in Ca^{2+}-independent fashion binds to the catalytic module of ONE-GC and activates it (25). In keeping with the present and earlier findings, uroguanylin signaling at the extracellular receptor domain can switch-on at least three Ca^{2+}-dependent transduction pathways, carried through GCAP1, neurocalcin δ and hippocalcin. Although, as yet, we do not understand the meaning of these multiple signaling pathways, it is clear that, in contrast to the indirect canonical cyclic AMP-linked pathway, the ONE-GC transduction pathway is extremely flexible, and with its multiple control devices is capable of being the common transducer of the divergent odorant signals.

ABBREVIATIONS

ANF-RGC	atrial natriuretic factor receptor guanylate cyclase
FRET	Förster resonance energy transfer
GCAP	guanylate cyclase activating protein
ONE-GC	olfactory neuroepthelial guanylate cyclase
ROS-GC	rod outer segment guanylate cyclase

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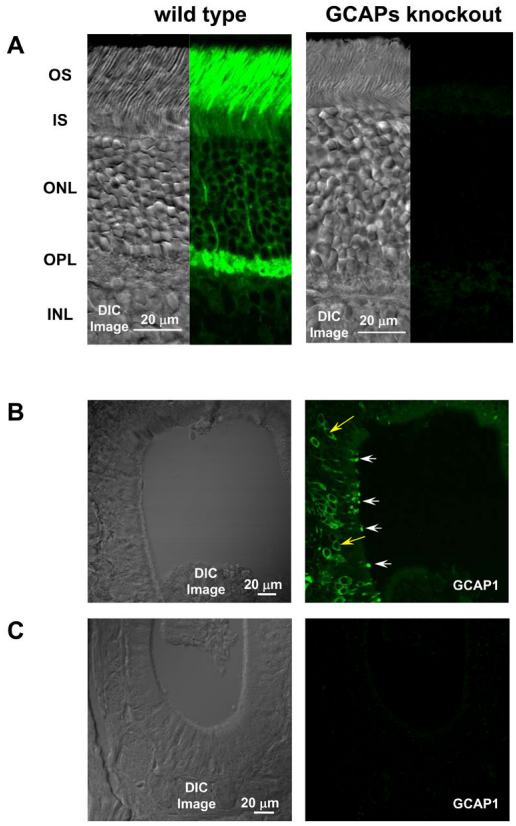

Figure 1. Immunoreactivity of GCAP1 in the mouse retina and olfactory neuroepithelium

(**A**) The affinity purified antibody was used to immunostain retinal cryosections from the wild type ("wild type") and GCAP1$^{-/-}$GCAP2$^{-/-}$ ("GCAPs knockout") mice. For each type of mice the left panel presents the differential interference contrast (DIC) image of the section showing the retinal layers (indicated to the left); the right panels show staining with GCAP1 antibody. In the retina from the wild type mouse the staining is strong in rod and cone outer segments and in the OPL. In the retina from the GCAPs knockout mouse there is no staining in either retinal layer. The same antibody was used to immunostain cryosections of the olfactory neuroepithelium from the wild type (**B**) and GCAPs knockout (**C**) mice. In both (B) and (C) the left panels show the DIC images and the right panels, immunostaining with GCAP1 antibody. In the olfactory neuroepithelial section from the wild type mouse (B) selected olfactory neurons show intense staining with GCAP1 antibody. The staining is intense in the cilia (four indicated by white arrows) and less intense in the dendrites and somas (two indicated by yellow arrows). The staining is absent in the GCAPs knockout mouse (C).

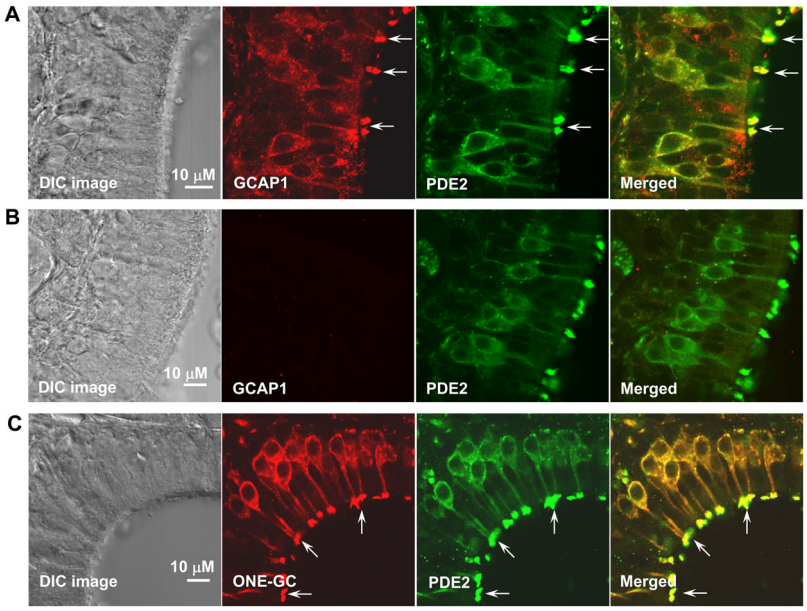

Figure 2. GCAP1 is expressed in the same as ONE-GC and PDE2A olfactory neurons

Cryosections of the olfactory neuroepithelium from the wild type and GCAPs knockout mice were immuno-stained with GCAP1 and PDE2A antibodies or ONE-GC and PDE2A antibodies as described in "Experimental Procedures". The DIC image showing the integrity of the olfactory neuroepithelium sections are presented at the left ("DIC image"). **(A)** Cryosection of the wild type mouse olfactory neuroepithelium was immunostained with GCAP1 and PDE2A antibodies. Intense staining with either antibody was observed in the cilia (3 intense signals are indicated with arrows) and of lower intensity in the dendrites and somas. **(B)** Cryosection of the GCAPs knockout mouse olfactory neuroepithelium was immunostained with GCAP1 and PDE2A antibodies. There was no immunoreactivity with the GCAP1 antibody whereas intense labeling of selected neurons with anti PDE2A antibody was observed. **(C)** Cryosection of the wild type mouse olfactory neuroepithelium was immunostained with ONE-GC and PDE2A antibodies. Strong signals with both antibodies were observed in the cilia (3 out of several signals are indicated with arrows). The dendrites and somas of the immunoreactive neurons were stained as well. The right-hand panels ("Merged") present the composite images of GCAP1 and PDE2 or ONE-GC and PDE2 staining and show that both GCAP1 and ONE-GC are co-expressed with PDE2A

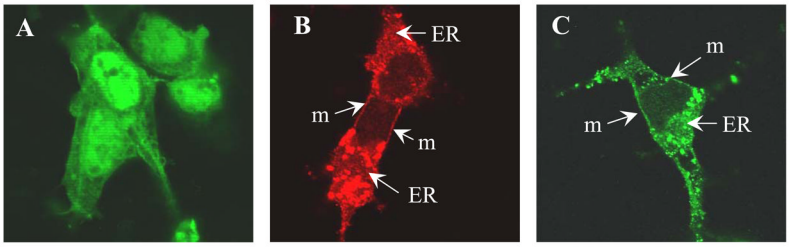

Figure 3. Cellular localization of GCAP1 and ONE-GC expressed in COS cells

(A) GCAP1 expressed in COS cells. (B) ONE-GC expressed in COS cells. (C) GCAP1 co-expressed with ONE-GC in COS cells. COS-cells were transfected with GCAP1 or/and ONE-GC cDNA. 72 hr after transfection the cells were fixed with 4% paraformaldehyde and incubated with GCAP1 or ONE-GC antibody followed by incubation with secondary antibodies conjugated with DyLight 488 (green) for GCAP1 immunolocalization and with DyLight 549 (red) for ONE-GC imunolocalization. The DyLight 488 was excited at 488 nm and DyLight 549, at 543 nm. The cells were viewed using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system. Indicated by arrows are: cell membrane (m) and membranes of endoplasmic reticulum (ER).

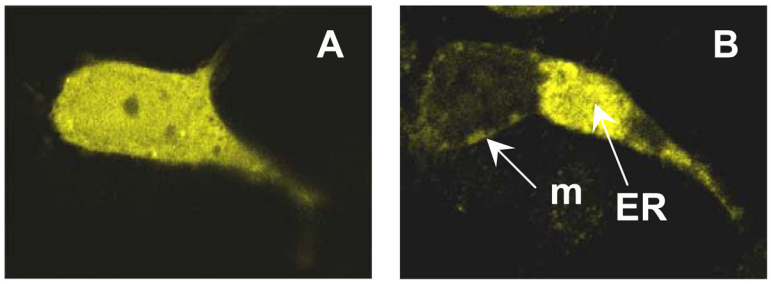

Figure 4. Co-localization of GCAP1-YFP with ONE-GC-CFP in live COS cells

(**A**) GCAP1-YFP was expressed in COS cells. Laser excitation was at 515 nm. GCAP1-YFP fusion protein is present in the entire cell. (**B**) GCAP1-YFP was co-expressed with ONE-GC in COS cells. Laser excitation was at 515 nm. In the presence of ONE-GC fusion protein GCAP1-YFP localizes to cellular membranes, plasma and ER membranes, the site of ONE-GC expression.

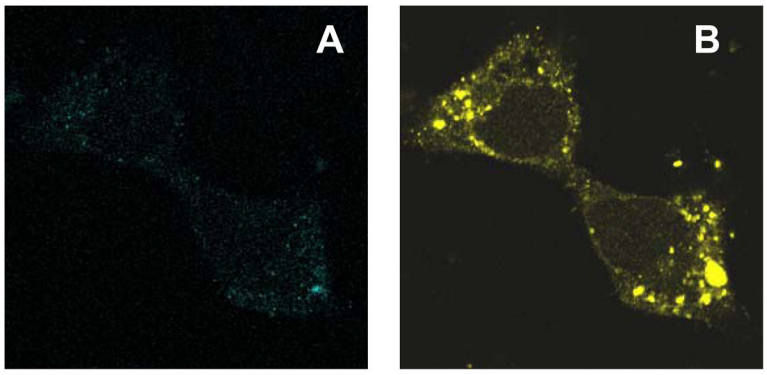

Figure 5. Co-expression of GCAP1-YFP with ONE-GC-CFP quenches CFP fluorescence

GCAP1-YFP and ONE-GC-CFP were co-expressed in COS cells. 72 hr after transfection the cells were observed under confocal microscope. Laser excitation was at 458 nm and the emission was observed at 480 nm (CFP) and at the 530 nm (CFP).

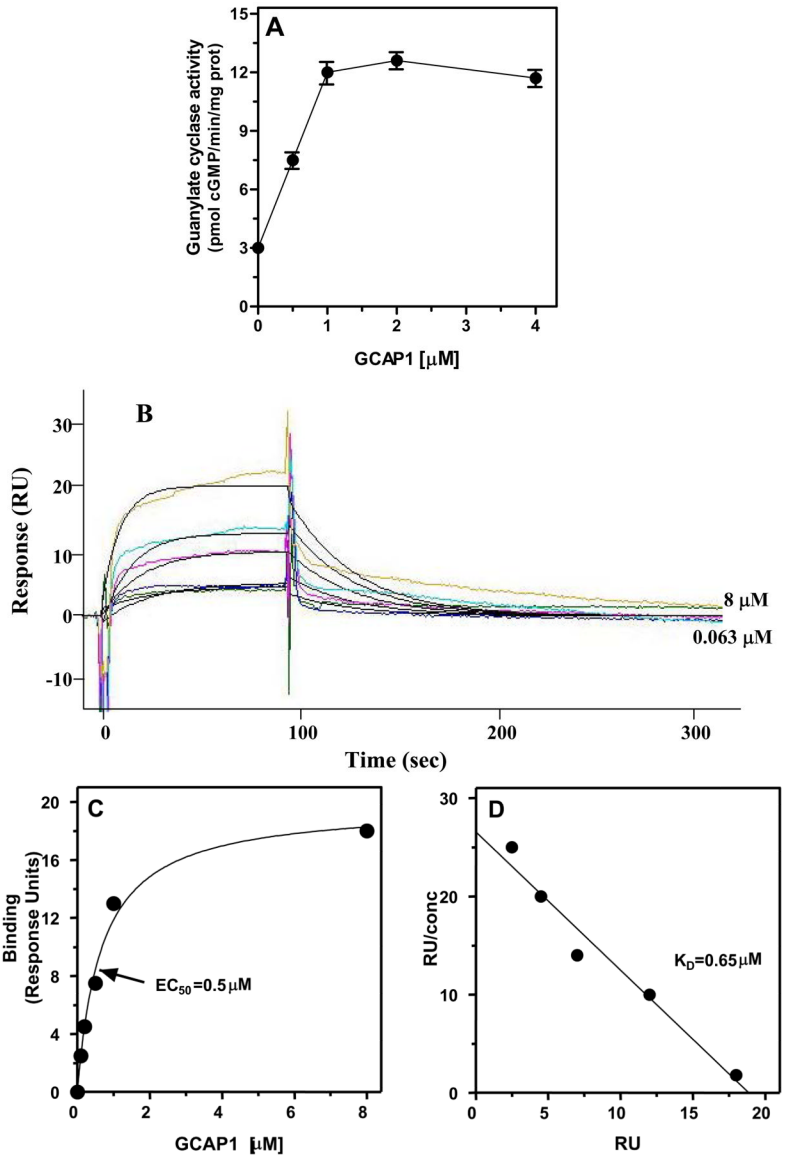

Figure 6. Binding of GCAP1 to the ONE-GC fragment aa 836-1110. SPR analysis

(**A**) ONE-GC fragment consisting aa residues 836-1110 was expressed in bacterial cells as a soluble protein as described in "Experimental Procedures" section and analyzed for GCAP1-dependent activity. Experiment was done in triplicate and repeated two times with separate preparations of the ONE-GC fragment. The results presented are mean ± SD from these experiments. (**B**) The ONE-GC fragment aa 836-1110 was immobilized on a CM5 sensor chip and GCAP1 was supplied in the mobile phase at concentrations between 0.063 and 8 μM in the running buffer. Typical set of overlaid sensorgrams together with fitting curves is shown. The curves presented were obtained after subtracting the effect of buffers and salts on resonance signals using the uncoated (blank) surface in flow cell 1 as the reference surface using BIAevaluation software. (**C**) Binding (RU) as a function of the concentration of GCAP1. (**D**) Scatchard transformation of the binding data. Experiment was repeated five times with different GCAP1 preparations. The results presented are from one typical experiment.

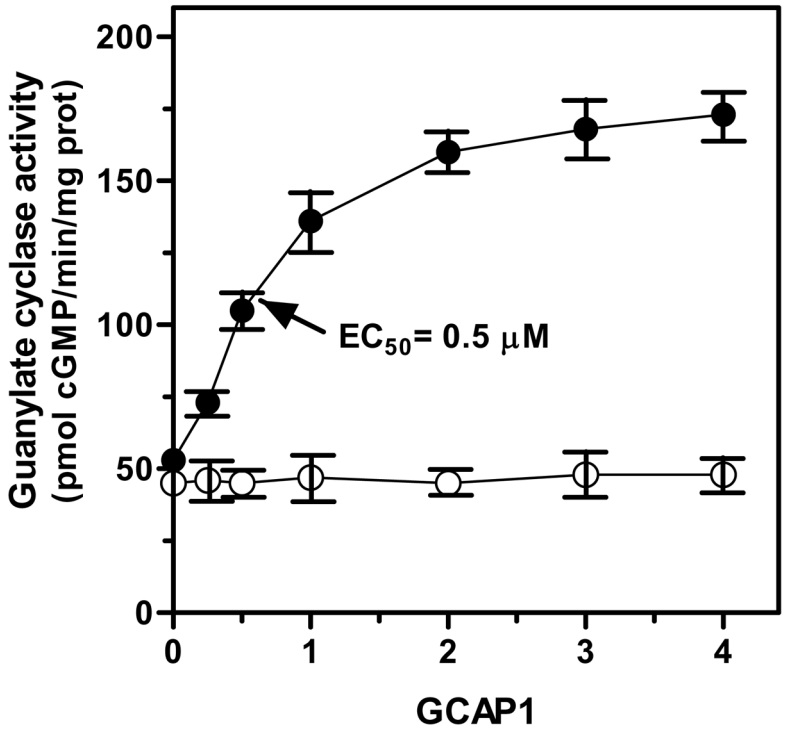

Figure 7. Ca^{2+}-dependent activation of ONE-GC by GCAP1

Olfactory neuroepithelium was isolated from wild type mice, homogenized in 250 mM sucrose/10 mMTris-HCl pH 7.5 buffer and the particulate fraction was isolated. This was analyzed for basal and GCAP1-dependent guanylate cyclase activity in the presence of 10 μM Ca^{2+} (closed circles) or 1 mM EGTA (open circles). The experiment was done in triplicate and repeated three times for the wild type mouse and two times for the knockout mouse. The results presented are the average ± SD from these experiments.

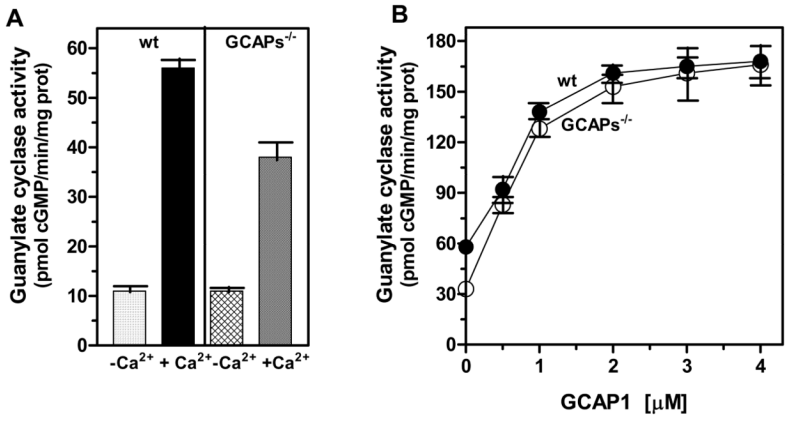

Figure 8. GCAP1-modulated ONE-GC system in the olfactory neuroepithelium

(**A**) Particulate fractions of the olfactory neuroepithelium were isolated from the wild type (wt) and GCAPs knockout (GCAPs$^{-/-}$) mice. Membranes were assayed for the guanylate cyclase activity in the presence of 1 mM EGTA ($-Ca^{2+}$) or 10 μM Ca^{2+} ($+Ca^{2+}$). (**B**) The membranes were assayed for guanylate cyclase activity in the presence of 10 μM Ca^{2+} and indicated concentrations of GCAP1. Cyclic GMP formed was measured by radioimmunoassay. The experiment was done in triplicate and repeated two times with separate membrane preparations. The results presented (mean ± SE) are from one experiment.

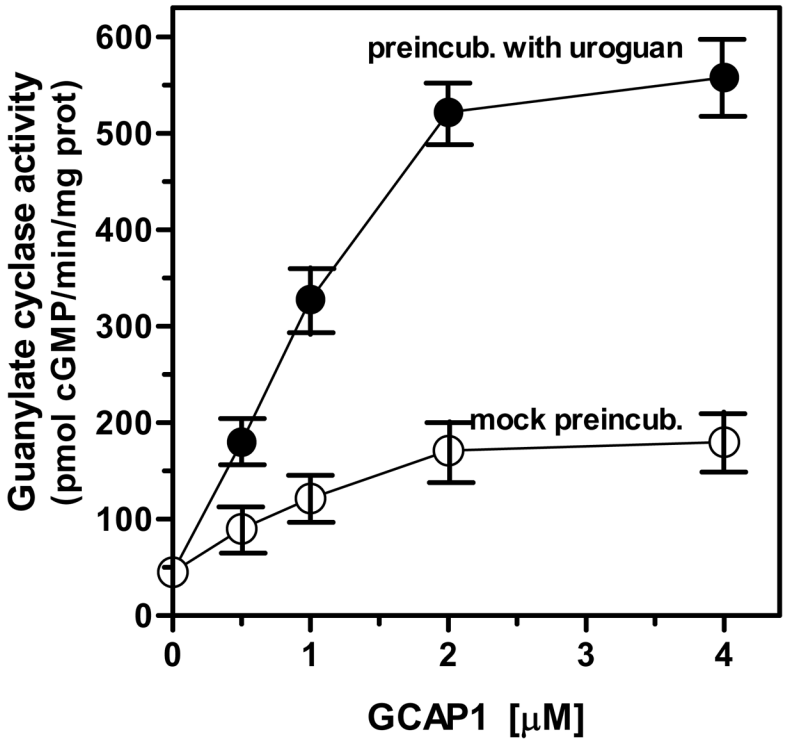

Figure 9. Synergetic effect of uroguanylin and GCAP1 on ONE-GC activity

Olfactory neuroepithelium from the wild type mouse was homogenized in the absence of Ca^{2+}, pre-incubated with 10^{-6} M uroguanylin, the membrane fraction was prepared, and assayed for guanylate cyclase activity in the presence of 10 μM Ca^{2+} and increasing concentrations of GCAP1. Membranes from mock-pre-incubated homogenates were treated as controls. The experiment was done in triplicate and repeated three times with separate homogenates. The results are mean ± SD from these experiments.

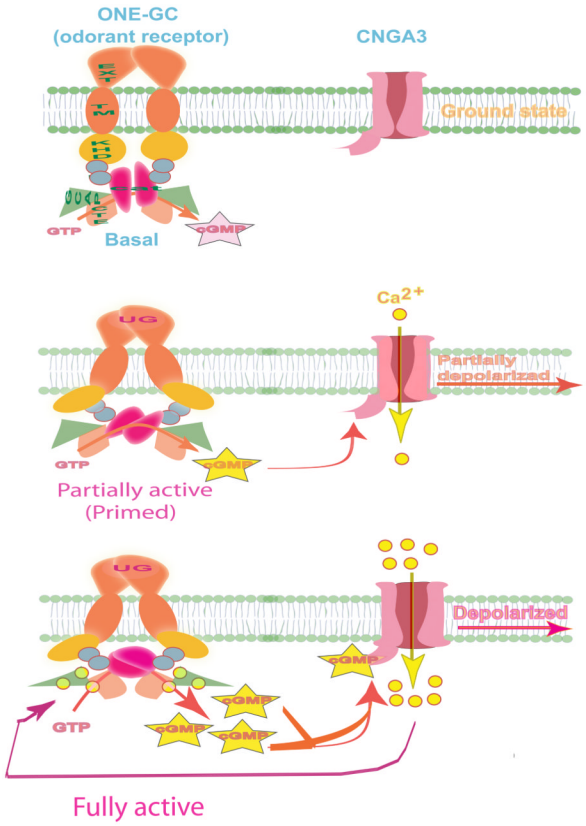

Figure 10. Two-step uroguanylin - GCAP1 - ONE-GC signal transduction model in the olfactory receptor neuron

The Model. *Basal*: Ground state. The concentration of free $[Ca^{2+}]_i$ in the resting cilia is 60–100 nM; ONE-GC dimer is bound to GCAP1 and its activity is in the Basal state. ***Primed, partially active state***: The odorant, uroguanylin, signaling begins by its binding to the extracellular receptor domain of ONE-GC. In a Ca^{2+}-independent fashion, it causes structural changes in the domain, which are transduced to the intracellular domain and finally to the catalytic module, causing its partial activation, of ~3-fold. The small amount of cyclic GMP produced as a result of this activation opens a fraction of the CNG3 channels and some influx of Ca^{2+}, which partially depolarizes the ONE-GC neuron's membrane. ***Fully active state:*** The Ca^{2+} ions which entered the ONE-GC neuron as a result of the partial ONE-GC activation bind to GCAP1 and create two physiological consequences: (1) cause full activation of ONE-GC; and (2) the resulting cyclic GMP opens maximal number of CNGA3 channels causing maximal influx of Ca^{2+} and depolarization of the ONE-GC neuron's membrane.