Odorants Selectively Activate Distinct G Protein Subtypes in Olfactory Cilia*

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Chemoelectrical signal transduction in olfactory neurons appears to involve intracellular reaction cascades mediated by heterotrimeric GTP-binding proteins. In this study attempts were made to identify the G protein subtype(s) in olfactory cilia that are activated by the primary (odorant) signal. Antibodies directed against the α subunits of distinct G protein subtypes interfered specifically with second messenger responses elicited by defined subsets of odorants; odor-induced cAMP-formation was attenuated by Goα antibodies, whereas Goα antibodies blocked odor-induced inositol 1,4,5-trisphosphate (IP3) formation. Activation-dependent photolabeling of Go subunits with [α-32P]GTP azidoanilide followed by immunoprecipitation using subtype-specific antibodies enabled identification of particular individual G protein subtypes that were activated upon stimulation of isolated olfactory cilia by chemically distinct odorants. For example odorants that elicited a cAMP response resulted in labeling of a Goα-like protein, whereas odorants that elicited an IP3 response led to the labeling of a Goq-like protein. Since odorant-induced IP3 formation was also blocked by Goα antibodies, activation of olfactory phospholipase C might be mediated by βγ subunits of a Gq-like G protein. These results indicate that different subsets of odorants selectively trigger distinct reaction cascades and provide evidence for dual transduction pathways in olfactory signaling.

Chemoelectrical signal transduction is considered to be mediated via intracellular reaction cascades triggered by G protein-coupled receptors (1). Biochemical studies over the last decade have revealed that odorants elicit the formation of either cAMP or IP3 in olfactory preparations (2–6). Whereas the functional implications of the dual transduction pathways in the crustacean olfactory system are well established (7, 8), the relative importance of the two pathways in olfactory signaling remains controversial (9, 10). Heterotrimeric GTP-binding proteins play a key role in signal transduction processes, coupling activated receptors to the appropriate effector system. A variety of different Go subtypes have been identified in vertebrate olfactory epithelium including Goa short.

Gq11, Gq12, Gq13, Goα, and Gq9 (11–17). Even an olfactory-specific isoform of Goa (Goq11) has been discovered (18). However, it is currently unclear how many and which type of G proteins are involved in olfactory signal transduction. To approach the question of which G protein subtype(s) mediate the transduction processes in olfactory sensory cells, it is necessary to identify the G protein that is activated upon stimulation with distinct odor ligands. This can be accomplished by an activation-dependent labeling procedure (19), in which receptor-activated G protein α subunits are photolabeled using the hydrolysist-resistant GTP-analogue [α-32P]GTP azidoanilide. Subsequent immunoprecipitation of Go subunits with subtype-specific antibodies permits identification of G protein subtypes that are labeled upon stimulation of olfactory cilia preparations with distinct odorants. The data indicate that cAMP- and IP3-inducing odorants result in labeling of different G protein subtypes.

EXPERIMENTAL PROCEDURES

Materials

Sprague-Dawley rats were purchased from Charles River, Sulzdorf. The odorants citralva (3,7-dimethyl-2,6-octadiennitrile), hedione (3-oxo-2-pentyl cyclopentanoneacetic acid methyl ester), eugenol (2-methoxy-4-(2-propenyl)phenol), lilial (4-(4-hydroxy-4-methyl pentyl)-3-cyclohexene-10-carboxylic acid), and ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde) were provided by DROM, Baierbrunn. Isosalicic acid (3-methylbutyric acid) and pyrrolidine (tetrahydropyrrole) were purchased from Sigma. The radioligand assay kits for cAMP and myo-[3H]inositol 1,4,5-trisphosphate determination as well as the enhanced chemoluminescence system (ECL) were provided by Amersham Corp. [α-32P]GTP was purchased from NEN Life Science Products. All other chemicals were obtained from Sigma.

Methods

Antisera—Antisera against G protein subunits were obtained either after injection into rabbits of synthetic peptides representing subtype-specific regions of different subunits using procedures described previously (19, 20), or from Santa Cruz Biotechnology (Santa Cruz, CA). In both cases, the peptide sequences used to raise the antisera are shown in Table I.

Isolation of Olfactory Cilia—Olfactory cilia preparations were obtained using the calcium-shock method (21, 22). Briefly, after a short wash of the olfactory epithelium in ice-cold saline solution (120 mM NaCl, 5 mM KCl, 1.6 mM KH2PO4, 25 mM NaHCO3, 7.5 mM glucose, pH 7.4), the tissue was subjected to Ringer’s solution containing 10 mM calcium and gently stirred for 5 min at 4 °C. Detached cilia were isolated by three sequential centrifugation steps for 5 min at 7,700 × g. The supernatants were collected, and the resulting pellets were resuspended in Ringer’s solution containing 10 mM calcium and gently stirred for 5 min at 4 °C. Detached cilia were isolated by three sequential centrifugation steps for 5 min at 7,700 × g. The supernatants were collected, and the resulting pellets were resuspended in Ringer’s solution containing 10 mM CaCl2 as described above. The cilia preparation was obtained after a final centrifugation step of all the pooled supernatants for 15 min at 27,000 × g. The resulting pellet containing the cilia was resuspended in hypotonic buffer (10 mM Tris, 3 mM MgCl2, 2 mM EGTA, pH 7.4) and stored at −70 °C. The yield of cilia was around 0.5 mg per rat.

Stimulation Experiments and Second Messenger Determination—To determine the influence of the subtype-specific G protein α subunit antisera on the efficiency of odorant-induced second messenger re-
sponges, isolated cilia were preincubated with the indicated dilutions of specific antisera and subsequently stimulated with an odorant mixture.

Stimulation experiments were performed at 37 °C for 2 min in the presence of 1 mM isobutylmethylxanthine when cAMP formation was determined, or 1 mM LiCl when the IP3 response was measured. Specifically, 205 l were adapted to 37 °C for 2 min with 20 mM EDTA, pH 7.4, and resuspended in labeling buffer containing 2 mM dithiothreitol, placed on a Parafilm-coated metal plate (4 °C), and irradiated for 1 min, 4 °C. The reaction was started by the addition of 30 l of labeling buffer containing 0.4–1 l/μl of olfactory cilia and stopped by addition of 7% ice-cold perchloric acid (100 mM). The samples were centrifuged (4 min, 12,000 g) and washed twice with 1 ml of washing buffer A (50 mM Tris/HCl, 600 mM NaCl, 0.5% SDS, 1% Tergitol NP-40, pH 7.4) and twice with washing buffer B (10 mM Tris/HCl, 300 mM NaCl, 10 mM EDTA, pH 7.4). Samples were then prepared for SDS-PAGE (19). Incorporation of [32P]GTP-azidoanilide was determined densitometrically after gel exposure to a phosphoimager (Fuji).

**SDS-PAGE and Western Blot Analysis**—Membrane preparations were prepared for SDS-PAGE as described previously (27), subjected to 12.5% acrylamide gel electrophoresis, and analyzed using the Laemmli buffer system (28). For Western blot analysis, the separated proteins were transferred onto nitrocellulose using a semidry blotting system (Pharmacia Biotech Inc.). The blot was stained with Ponceau S and stored at 4 °C until use. For Western blot analysis, nonspecific binding sites were blocked with 5% nonfat milk powder (Naturafflor) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.05% Tween 20). The blots were incubated overnight with specific antibodies against the different G protein subunits diluted in TBST, containing 3% nonfat milk powder. After three washes with TBST, a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution in TBST with 3% milk powder) was applied, and the ECL system was used to monitor immunoreactivity.

**RESULTS**

The antisera used in this study were generated against synthetic peptides derived from sequence domains that are unique for a particular class of G protein α subunits (see Table I). Western blot analyses using specific antisera were performed to determine the relative distribution of distinct Ga subunits within (a) a preparation of olfactory sensory cilia, (b) total olfactory epithelium, and (c) a cerebral cortex preparation (Fig. 1). The antisera AS 348, which recognizes a decapetidic corresponding to the C terminus of Ga as well as Ga (19), detected a single 44–45-kDa band. The immunoreactive polypeptide was found to be highly enriched in olfactory cilia compared with the whole olfactory epithelium. The antibody C10, which recognized a C-terminal decapetidic of Ga, was highly enriched in olfactory cilia compared with the whole olfactory epithelium.
and Gαi-3, stained a single 40/41-kDa protein band in all three preparations with similar intensity. Thus Gαi subtypes are apparently not enriched in the cilia. Similarly, labeled bands observed in both olfactory fractions were also detected upon application of Gαq-specific antiserum. Western blot analysis with a Gαo-specific antibody directed against an epitope corresponding to the sequence common to both Gαo-1 and Gαo-2 revealed an immunoreactive band at 40 kDa that was enriched in the cilia (see Fig. 1).

Antibodies specific for distinct G proteins have been used successfully in functional studies. For example specific inhibi-

FIG. 2. Concentration-response curves for the selective blockade of odorant-induced second messenger signaling by Gαi antibodies. Isolated olfactory cilia were incubated for 10 min on ice with different concentrations of a Gαi-specific antiserum (AS 348) raised against a peptide common to both Gαi and Gαall. Subsequently the samples were stimulated with odorants, and the second messenger concentrations were determined. Only the odor-induced cAMP formation was blocked in a concentration-dependent manner by Gαi antibodies. The odor-induced IP3 signal was not affected by this subtype-specific antiserum. Values are the means of triplicate determinations ± S.D. Panel A shows stimulation with a mixture of odorants inducing cAMP formation (citralva, hedione, and eugenol, each 1 μM). The basal cAMP level was 1733 ± 163 pmol/mg of protein; in the presence of the highest concentration of the Gαi antibodies (1:100) the level of cAMP under control conditions was 1615 ± 118 pmol/mg of protein. Panel B shows stimulation with odorants eliciting an IP3 response (lilial, lyral, and ethylvanillin, each 1 μM). The basal level of IP3 was 227 ± 67 pmol/mg of protein; pretreating cilia with a 1:100 dilution of the antibody did not affect the concentration of IP3 (216 ± 47 pmol/mg protein).

FIG. 3. Effects of different Gα antibodies on odor-induced second messenger formation. Aliquots of isolated olfactory cilia were preincubated with different concentrations of affinity-purified subtype-specific Gα antibodies (C-10 for Gαi, K-20 for Gαo, and AS 368 for Gαq); subsequently samples were stimulated with an odorant mixture. A, effects of stimulation with a mixture of citralva, hedione, and eugenol (each 1 μM) inducing cAMP formation. The concentration of cAMP under control conditions (1723 ± 299 pmol/mg) was not affected upon pretreating cilia with the different G protein antibodies. At the highest antiserum concentration (1:100), the level of cAMP was 1663 ± 189 for the Gαi antibody, 1685 ± 218 pmol/mg for the Gαo antibody, and 1710 ± 295 pmol/mg for the antibody recognizing Gαq. Data represent the mean values of cAMP formation (pmol/mg of protein) of triplicate determinations; the S.D. was ± 10% or less. B, effects of stimulation by an odorant mixture eliciting an IP3 response (lilial, lyral, and ethylvanillin; each 1 μM). The basal IP3 level of 268 ± 25 pmol/mg of protein was unaffected, even by the highest concentration of the G protein antibodies (Gαi, 268 ± 14 pmol/mg; Gαq, 275 ± 445 pmol/mg; and Gαo, 242 ± 87 pmol/mg). Values are the means of triplicate determinations ± S.D.
tion of (a) ligand-induced, α subunit GTPase activity (29, 30) and (b) α-phosphatidylinositol 4,5-diphosphate hydrolysis (31) have been described. Evidence for two second messenger pathways in olfactory signaling suggests that more than one G protein subtype may be involved in mediating olfactory transduction (8, 5, 32). Therefore attempts were made to determine if the utility of subtype-specific antibodies could be used as tools to identify G protein subtypes that are active in olfactory signaling cascades. Isolated olfactory cilia were pretreated with different concentrations of subtype-specific antibodies and subsequently stimulated with odorant mixtures, which elicit either

**FIG. 4.** Influence of GDP on photoaffinity labeling of Gαs. Membranes of isolated olfactory cilia were photolabeled with [α-32P]GTP azidoanilide at various GDP concentrations in the absence (control) and presence (odor) of a mixture of citralva, hedione, and eugenol (each 1 μM). Labeled membranes were solubilized, and proteins were immunoprecipitated with the Gαs common antiserum AS 348 and subsequently subjected to SDS-PAGE. Note that, in the presence of low GDP concentrations, photolabeling of Gαs is similar under control conditions and in the presence of odorants. Only upon application of high GDP concentrations (1 mM), was a significant odor-induced enhancement of photolabeling of Gαs observed.

**FIG. 5.** Time course of ligand-induced Gαs photoaffinity labeling in isolated olfactory cilia and human platelet membranes. Membranes were incubated with [α-32P]GTP azidoanilide in the presence and absence of either an odorant mixture containing 1 μM of citralva, hedione, and eugenol or the hormone cicaprost (1 μM) for the times indicated in the abscissa. The basal level of Gαs constantly increased during the whole time course, reflecting the accumulation of G protein α subunits liganded with [α-32P]GTP azidoanilide which is poorly hydrolyzed by α subunits (data not shown). After solubilization, membrane proteins were immunoprecipitated with an Gαs common antiserum (AS 348). The data represented as ligand-induced stimulation of photolabeling of Gαs calculated as percent of maximal incorporation are a representative of three independent experiments with identical results. Data for Gαs labeling of human platelet membranes under cicaprost stimulation were taken from Laugwitz et al. (19).

**FIG. 6.** Concentration dependence of the citralva effect on photolabeling of Gαs. A, isolated cilia were stimulated in the presence of 1 mM GDP with increasing concentrations of the fruity odorant citralva (1 pM to 10 μM); subsequently Gαs was immunoprecipitated with the Gαs common antiserum AS 348. The autoradiogram showing the 45-kDa region of a SDS-PAGE is a representative of three independent experiments each giving very similar results. B, concentration-response curve of citralva-induced incorporation of [α-32P]GTP azidoanilide. The autoradiogram of photolabeled Gαs in A was densitometrically evaluated. The inset C shows the logarithm scale of odorant concentration of the dose-response curve in B. Note that a half-maximal labeling was accomplished at about 50 nM of citralva. Data indicate the photostimulated luminescence of Gαs by citralva as a percentage of maximal incorporation.
cAMP (citralva, hedione, and eugenol) or IP₃ formation (lilial, lyral, and ethylvanillin).

The effect of increasing concentrations of the anti-Gαs serum on odor-induced cAMP or IP₃ formation is shown in Fig. 2. Whereas odor-induced cAMP formation was blocked in a concentration-dependent manner reaching about 45% inhibition at a 1:100 dilution (Fig. 2A), IP₃ formation elicited by appropriate odorants was not significantly affected (Fig. 2B).

Activation of phospholipase Cβ subtypes is mediated by members of either the pertussis toxin-insensitive Gq family or by pertussis toxin-sensitive Gi and Go proteins (33). Fig. 3 shows the effect of increasing concentrations of Goα, Goβ, and Gαs antibodies on odor-induced second messenger responses. Pretreatment of cilia preparations with any of the three subtype-specific antibodies did not alter the responsiveness to the odorants citralva, hedione, and eugenol, known to induce a cAMP signal (Fig. 3A). The IP₃ response elicited by appropriate odorants was not affected by anti Gαs serum (Fig. 3B). In contrast, antibodies against Gαs significantly attenuated odor-induced IP₃ formation in a concentration-dependent manner; inhibition was 65% at a 1:250 dilution and more than 75% at a 1:100 dilution. Gαs antibodies gave a significant inhibition only at the highest concentration (1:100).

With the aim of identifying directly G protein subtypes that are activated upon odor stimulation, a photoaffinity labeling approach was employed using the photoactive GTP analogue [α-32P]GTP azidoanilide (26, 34). Previous studies have shown that monitoring receptor-stimulated binding of GTP analogues require addition of exogenous GDP (19, 35, 36). Since G protein subtypes display different basal nucleotide analogues require addition of exogenous GDP (19, 35, 36). Since G protein subtypes display different basal nucleotide exchange rates (37–39), it was necessary to determine the appropriate GDP concentration that allows visualization of odor-induced G protein labeling. In the first set of experiments, conditions were optimized toward an odorant-induced photolabeling of Goα proteins. Isolated olfactory cilia were incubated with [α-32P]GTP azidoanilide in the presence of different concentrations (0–1 mM) of exogenous GDP, and incubation was continued for 20 s at 37 °C upon application of a mixture of three odorant compounds (citralva, hedione, and eugenol, each 1 μM). Gαs subunits were immunoprecipitated using an antiserum directed against Goα subtypes, separated on SDS-PAGE, and the incorporated [α-32P]GTP azidoanilide label was determined by autoradiography. The results of a representative experiment (n = 3) are shown in Fig. 4. The immunoprecipitate gave a single photolabeled band with an apparent molecular mass of 44–45 kDa, a size identical to the molecular mass of the protein visualized in immunoblot experiments (see Fig. 1). However, the Goα common antiserum AS 348 used to immuno-

![Fig. 7. Photolabeling of Gαs upon stimulation with distinct odorants.](image)

![Fig. 8. Photoaffinity labeling of individual G protein α subunits upon stimulation with an odorant mixture inducing IP₃ formation.](image)
precipitate G subunits does not allow us to distinguish whether the Golf (44.7 kDa) or the Gs short isoform (44.2 kDa), both of which are expressed in the olfactory system, is labeled upon odorant stimulation. Comparing the intensity of \[^{32P}\text{-GTP}\] labeling, it was clear that, at low GDP concentrations, photolabeling is similar under control conditions and in the presence of odorants. However, upon application of rather high GDP concentrations (1 mM), significantly enhanced labeling was detected in stimulated samples. This observation contrasts with studies on photolabeling of G\textsubscript{a} in membrane preparations of human platelets, where agonist-induced labeling was detectable in the presence of 1 mM GDP (19).

In view of the rapid kinetics of olfactory reaction cascades, time course experiments on agonist-induced labeling of G\textsubscript{a} were performed, in which cilia preparations were photolabeled upon incubation with an odorant mixture (citralva, hedione, and eugenol) for different time intervals. Application of odorants elicited a rapid incorporation of the labeled GTP analogue (Fig. 5). The ratio of agonist-stimulated to basal photolabeling of the G\textsubscript{a}-like protein was highest at short incubation times; the relative labeling was fully saturated after 10 s. In contrast, hormone-induced incorporation of \[^{32P}\text{-GTP}\] into G\textsubscript{a} of human platelets has been shown to follow a very different time course (see Fig. 5). Maximal labeling is reached after about 10 min.

To determine the potency of individual odorants, photolabeling experiments were performed using different concentrations of citralva. As demonstrated by the autoradiogram in Fig. 6A, incorporation of \[^{32P}\text{-GTP}\] into G\textsubscript{a}-like protein increased in a concentration-dependent manner. In addition, it is clear that even very low doses (picomolar) of the odorant are sufficient to induce a significant labeling of G\textsubscript{a}. The intensity of the labeling, evaluated densitometrically, was used to construct a concentration-response curve (Fig. 6B). In conformity with the results of many similar olfactory stimulation experiments, we did not detect saturation; an approximately half-maximal activation at about 50 nM was estimated. These results are in line with previous experiments monitoring odor-induced second messenger responses (5).

Odorants showed different potencies when stimulation of adenylyl cyclase was examined (2). To explore whether less potent adenylyl cyclase activators also induce labeling of the G\textsubscript{a}-like protein, cilia preparation were stimulated with the odorant eugenol, which shows only 47% of adenylyl cyclase activation compared with citralva. Stimulation of cilia preparations with 1.6 mM eugenol caused a significant incorporation of \[^{32P}\text{-GTP}\] into G\textsubscript{a} (Fig. 7A). Thus this procedure also allows for
the determination of odorant-dependent Goα labeling by less potent adenylyl cyclase activators.

Several odorants have been shown not to induce a detectable cAMP signal but rather the formation of IP3 (32). However, it might be possible that adenylyl cyclase activation was not detected due to the insufficient sensitivity of the method used. By photolabeling Go subunits, we were able to detect odor-induced activation of Goα at very low odorant concentrations (see Fig. 6). Therefore, we examined the labeling of Goα by individual phospholipase C-stimulating odorants, i.e. lyral, isovaleric acid, and pyrrolidine. The results depicted in Fig. 7, B and C, show that application of citralva at different concentrations induced a concentration-dependent increase in Goα labeling, whereas neither low (16 nM) nor high (1.6 μM) concentrations of isovaleric acid and pyrrolidine (Fig. 7C) or lyral (Fig. 7A) affected labeling of Goα.

Different G protein types are known to link receptors to phospholipase C (33). To evaluate which G protein subtype might be involved in odor-induced IP3 formation, photolabeling studies were performed with a stimulating odorant mixture (lilial, lyral, and ethylvanillin, each 1 μM) followed by immunoprecipitation with subtype-specific antibodies for Goα/Goβ1, Goαi, and Goβ isoforms (AS 6) (34). In all cases the different antibodies precipitated photolabeled proteins with molecular masses identical to those found in the immunoblotting experiment (see Fig. 1). However, proteins precipitated with Goα or with Goαi antibodies did not show any increase in [α-32P]GTP azidoanilide incorporation upon odorant stimulation, neither in the presence of a low GDP concentration (not shown) nor in the presence of high GDP levels (Fig. 8, 500 μM GDP). Nevertheless, for Goβ, the results were different. Whereas at low concentrations of GDP (0–100 μM) no differences were detected in photolabeling of Goβ when compared with control samples (data not shown), at high exogenous GDP concentrations (500 μM; see Fig. 8), an odorant-induced increase in [α-32P]GTP azidoanilide incorporation was detected in proteins precipitated with Goαi antibodies. This indicates that the “IP3 odors” activating Goβ-like protein may have a similarly high nucleotide exchange rate as the Goαi-like G protein, which is labeled upon application of “CAMP-odors” (see Fig. 4).

The potency of odorants in activating the Goα-like protein is demonstrated in Fig. 9A; as shown for Goβ, activation (see Fig. 6). Very low odor concentrations were sufficient to induce significant labeling of Goα. A densitometric evaluation of the
photoaffinity labeled Goα-like protein is presented in Fig. 9B; the concentration-response curve revealed an apparent half-maximal labeling of Go at odorant concentrations of about 200 nM.

To evaluate the specificity of Goα labeling, we analyzed the effects of individual odorants that have been shown to activate phospholipase C. Stimulation with the “IP3-odorants” lyral (Fig. 10A; 1.6 μM) or isovaleric acid (Fig. 10, B, 16 nM, and C, 1.6 μM) induced an enhanced photolabeling of Goα. Analyzing the intensity of the labeling densitometrically, it was clear that isovaleric acid induced a concentration-dependent increase in [α-32P]GTP azidoanilide incorporation. In contrast, the application of high odor concentrations of the cAMP compound eugenol did affect Goα labeling (see Fig. 10A; 1.6 μM); even stimulation with high concentrations of the very potent adenyl cyclase activators citralva or hedione failed to induce a significant incorporation of the GTP analogue (see Fig. 10C, 1.6 μM).

As Go and Gs subtypes usually activate phospholipase C through their βγ subunits (33), experiments were performed to explore whether the βγ subunit of the identified Goα-like G protein is mediating phospholipase C activation. Isolated olfactory cilia were pretreated with an antiserum selective for the protein is mediating phospholipase C activation. Isolated olfactory-induced formation of IP3. Thus, we have demonstrated that odor stimulation remains to be determined. Phospholipases of the pertussis toxin-sensitive Gi/Go family (33). The involvement of the G proteins that release the activating intracellular receptors via IP3 and Ca2+

DISCUSSION

The present study shows that subtype-specific antibodies attenuate odor-induced second messenger responses and immunoprecipitate activation-dependent photolabeled Goα subunits. Both approaches indicate that a Goα-like protein mediates odor-induced cAMP, whereas a Gβγ-like protein controls odor-induced formation of IP3. Thus, we have demonstrated that different subsets of odorants selectively activate one of the two G proteins. This finding is consistent with previous biochemical studies indicating that odorants elicit either a CAMP or an IP3 response in olfactory cilia preparations. Therefore, these results provide further evidence that the phenomenon of dual transduction pathways in olfactory signaling, which is well established for the lobster (8), is also found in vertebrates. However, it is presently unclear how these biochemical results can be reconciled with the observation that transgenic mice displaying general anosmia (10). The Gs common antiserum AS2 I. Boekhoff, unpublished results.

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