Chemosensory neurons in the vomeronasal organ (VNO) detect pheromones related to social and reproductive behavior in most terrestrial vertebrates. Current evidence indicates that the chemo-electrical transduction process is mediated by G protein-coupled second messenger cascades. In the present study, attempts were made to identify the G protein subtypes which are activated upon stimulation with urinary pheromonal components. G protein-specific antibodies were employed to interfere specifically with inositol 1,3,4-trisphosphate formation induced by urinary stimuli and to immunoprecipitate Gα-subunits, activation dependent labeled with [α-32P]GTP azidoanilide. The results of both experimental approaches indicate that stimulation of female VNO membrane preparations with male urine samples induces activation of Gα as well as Gβ subtypes. Experiments using different fractions of urine revealed that upon stimulation with lipophilic volatile odorants, only Gα proteins were activated, whereas Gα activation was elicited by α2u-globulin, a major urinary protein, which is a member of the lipocalin superfamily. Since each G protein subtype is stereotypically coexpressed with one of the two structurally different candidate pheromone receptors (V1R and V2R), the results provide the first experimental evidence that V1Rs coexpressed with Gα may be activated by lipophilic probably volatile odorants, whereas V2Rs coexpressed with Gβ seem to be specialized to interact with pheromonal components of proteinaceous nature.

Terrestrial vertebrates detect chemical signals via sensory neurons located in two anatomically distinct systems: the nasal olfactory epithelium (OE) and the vomeronasal organ (VNO). While the main olfactory system is responsible for the “conventional” sense of smell, the VNO appears to specifically detect pheromones, thereby inducing a distinct social or sexual behavior or endocrine response (1–3). In rodents, the major source of pheromones seems to be the urine, however, only few volatile (4) and non-volatile (5) urinary substances producing a definite endocrine or behavioral response have been identified. The best characterized non-volatile urinary components in male mouse urines are the “major urinary proteins” (6). Major urinary proteins have been suggested to be involved in puberty acceleration (5, 7, 8), an endocrinological effect prevented by lesions of the VNO or the accessory olfactory bulb (1). Major urinary proteins of mice and the rat equivalent α2u-globulin (9) belong to the superfamily of lipocalins, a structurally homologous but diverse family of extracellular proteins, characterized by their ability to bind small, principally hydrophobic molecules (10). Although recent structural studies have characterized the naturally bound volatile substances (11), major urinary proteins lacking the natural volatiles remain active (8) suggesting that proteins themselves act as pheromones.

The molecular mechanisms responsible for the detection of pheromones and the signal transduction processes in the VNO are not well understood. Initially, it has been suggested that the transduction mechanisms in the VNO would resemble those in the olfactory epithelium, but some of the specific molecular components involved in the transduction of odorants in receptor neurons of the OE, like Gαolf adenyllyl cyclase (AC) subtype III, and the α-subunit of the cyclic nucleotide-gated cation channel could not be detected in the neuroepithelium of the VNO (12, 13). Moreover, recent molecular biology advances implicate that neurons in the VNO utilize another set of receptor genes than the olfactory epithelium. Two novel and structurally unrelated multigene families of putative pheromone receptors have been identified (V1R and V2R) which are exclusively expressed in neurons of the VNO (14–17). Although receptors of both families represent members of G protein-coupled receptors, the structural differences especially in the N-terminal region led to the concept that each receptor family may be tuned to recognize a distinct class of ligands.

Receptors of each family show a distinct nonoverlapping expression pattern within two major laminar zones. Whereas V1Rs are expressed in the apical half of the neuroepithelium, V2Rs are restricted to the basal region of the epithelium. An identical spatial expression pattern has also been observed for two types of G protein α-subunits. Whereas Gα is expressed only in apical neurons, Gβ expression is restricted to neurons in the basal half of the VNO neuroepithelium (13, 14, 18). The stereotypical distribution of both receptor families with distinct G protein subtypes implies a linkage between both components.

The present study aimed to characterize the G protein subtypes in the rat vomeronasal organ activated upon stimulation with male urine by performing photoaffinity labeling experiments. Furthermore, antibodies for different G protein subtypes were employed to interfere with the urine-induced IP3 signaling. To evaluate whether there is any correlation between the biochemical properties of applied urinary stimuli and the activation of distinct G protein subtypes, stimulation ex-
periments were performed with different fractions of urine as well as with purified α2u-globulin.

**EXPERIMENTAL PROCEDURES**

**Materials**

Male and female adult Sprague-Dawley rats were purchased from Charles River, Sulzfeld, Germany. The odorant methone was provided by DROM (Baierbrunn, Germany), amyl acetate was purchased from Sigma (Deisenhofen, Germany). Hydroxyapatite Type I was obtained from Bio-Rad (München, Germany), the Centricron concentrators were purchased from Millipore (Eschborn, Germany), enterokinase was from Boehringer (Mannheim, Germany). The radioligand assay kits for cAMP (adenosine 3',5'-cyclic phosphate) and myo-[3H]inositol 1,4,5-trisphosphate determination were provided by Amersham; [α-32P]GTP was purchased from NEN Life Science Products Inc. Sources of other materials have been described (19).

**Methods**

**Antiseria**—Antisera against G protein subunits were obtained after injection of synthetic peptides representing subtype-specific regions of different subunits into rabbits as described previously (20) or provided by Santa Cruz Biotechnology, Santa Cruz, CA. The peptide sequences used to raise the antisera are shown in Table I. Urine Collection and Purification—Urine from male fertile rats (12–14 weeks old) was collected, pooled, centrifuged to remove cells (5 min, 5, 500 × g), and stored as aliquots at −70 °C until use. To extract hydrophobic volatile odorants, a 2-ml volume of pooled male urine was treated with 2 ml of dichloromethane; following separation of the organic and water phase by centrifugation (10 min, 6,000 × g), both phases were collected and stored at −70 °C.

**Purification of α2u-Globulin**—Urine was collected from fertile male rats, pooled, and stored immediately at −70 °C. After thawing to 4 °C, a 50-ml pool was centrifuged at 5,000 × g for 30 min at 4 °C, and the clear urine was dialyzed against 4 mM sodium phosphate buffer, pH 6.8. The purification of α2u-globulin was performed according to Holmqist et al. (21). Briefly, after addition of phenylmethylsulfonyl fluoride to a final concentration of 0.1 mM, the samples were applied to a 1.5-ml column of hydroxyapatite with a flow rate of 0.5 ml/min. The column was washed with 10 times column volumes of 4 mM sodium phosphate buffer, pH 6.8. Elution was performed using a linear 4–400 mM sodium phosphate buffer gradient, pH 8.0. Total protein was evaluated by using the A280, corresponding fractions were pooled and analyzed by SDS-PAGE. Fractions containing the 18-kDa proteins were collected and analyzed by gelatin zymography with 5 μl of supernatant in a 15 × 10-cm gel containing 1% gelatin. GTP gamma S incorporation by α2-globulin (diluted in reaction buffer) was measured using 10 μl of VNO membrane preparations in hypotonic buffer (10 mM Tris/HCl, 3 mM MgCl2, 2 mM EGTA, pH 7.4) and stored at −70 °C. Olfactory cilia of the OE were obtained using the calcium-shock method as described previously (24).

**Stimulation Experiments and Second Messenger Determination**—Stimulation experiments, performed with male rat urine, separated fractions of urine, isolated α2u-globulin of male urine, and recombinant α2u-globulin were performed as described previously (20).

Briefly, 300 μl of reaction buffer (200 mM NaCl, 10 mM EGTA, 50 mM Mops, 2.5 mM MgCl2, 1 mM dithiorethiol, 0.05% sodium cholate, 1 mM ATP, and 4 mM GTP, pH 7.4) including 12 nm free calcium calculated and adjusted as described (25), was prewarmed at 37 °C with or without odorants. The reaction was started by the addition of 60 μl of VNO membrane preparations (equivalent to 2 μg of membrane protein) for 2 min at 37 °C and stopped by the addition of 7% ice-cold perchloric acid (200 μl) before the concentration of IP3 was determined according to Palmer et al. (26). To prevent degradation of IP3, stimulation experiments were performed in the presence of 10 mM LiCl (final concentration during incubation). To determine the influence of the subtype-specific G protein α-subunit antisera on the efficiency of urine-induced second messenger responses, membrane preparations of α2u-globulin were preincubated with the indicated dilutions of the specific antisera and subsequently stimulated with urine or purified fractions of urine.

**Photo labeling of Activated G Proteins**—[α-32P]GTP azidoanilide was synthesized and purified as described (27); receptor-dependent G protein labeling was performed as described previously (20). Briefly, frozen VNO membrane preparations in hypotonic buffer were centrifuged (10 min, 12,000 × g, 4 °C) and resuspended in double concentrated labeling buffer (60 mM HEPES, 5 mM MgCl2, 200 mM NaCl, 200 μM EDTA, 500 μM GDP, pH 7.4). 10 μl of urine, general odorants, separated urine fractions (diluted in water), or α2u-globulin (diluted in reaction buffer) were adapted to 37 °C with 20 μl of [α-32P]GTP azidoanilide (4 × 105 cpm/tube). The reaction was started by adding 30 μl of VNO membrane preparations (1–2 μg/ml) and continued for 2 min at 37 °C the reaction was terminated by cooling the samples to 4 °C. Excess [α-32P]GTP azidoanilide was removed by centrifugation (5 min, 12,000 × g, 4 °C). The pellet was resuspended in labeling buffer containing 2 mM dithiothreitol, placed on a Parafilm-coated metal plate (4 °C), and irradiated for 30 s with a 254-nm UV lamp (150 W, VI-100, Gwel-Herbol, Rottendorf, Germany). 5 μl of the indicated, undiluted subtype-specific G protein α-subunit antisera was added.
After 1 h at 4 °C under constant rotation, 60 µl of Protein A-Sepharose (3 mg) was added and the samples were incubated overnight at 4 °C. Thereafter the Sepharose beads were pelleted (1 min, 12,000 × g, 4 °C) and washed twice with 1 ml of washing buffer A (50 mM Tris/HCl, 600 mM NaCl, 0.5% SDS, 1% Tergitol Nonidet P-40, pH 7.4) and twice with washing buffer B (100 mM Tris/HCl, 300 mM NaCl, 10 mM EDTA, pH 7.4). Preparation of the samples for SDS-PAGE was performed as described Laugwitz et al. (20). Incorporated [α-32P]GTP azidoanilide was determined densitometrically after gel-exposure to a PhosphorImager (Fuji).

RESULTS

Based on a recent study indicating that treatment of vomeronasal organ slice preparations of rats with urine dilutions elicit an increased impulse frequency of sensory neurons which can be blocked by phospholipase C inhibitors (28), we set out to explore whether urine-induced IP3 responses in membrane preparations of VNOs from female rat are mediated by a G protein-controlled reaction cascade. IP3 responses induced upon stimulation with different concentrations of male urine (0.1 to 4%, v/v) were determined in the presence of GTP or GDP. The results in Fig. 1 demonstrate that male urine elicited a dose-dependent increase in the formation of IP3; this response was only observed in the presence of GTP indicating that urine-induced phospholipase C activation in the VNO is mediated via G proteins.

Immunohistochemical studies (18, 19, 20) and in situ hybridization experiments (13) have demonstrated that different G proteins α-subunits (Gαs, Gαo, and Gα12/13) are expressed in neurons of the VNO. Toward an identification of distinct G protein subtypes which are activated upon stimulation with urine, a photoaffinity labeling approach was employed using the photoreactive, hydrolysis resistant [α-32P]GTP azidoanilide (27, 20). Female VNO membrane preparations were stimulated with two different concentrations of male urine in the presence of [α-32P]GTP azidoanilide, and subsequently distinct G protein α-subunits were immunoprecipitated (24) using subtype-specific antibodies for different G protein α-subtypes (Table I).

Fig. 2 shows that urine addition significantly enhanced incorporation of [α-32P]GTP azidoanilide into Gαs as well as Gαo subtypes. Quantification of the labeling intensity indicates that stimulation with male urine (0.2%, v/v) induced a 57% increase in Gαs labeling and a nearly 100% increase in the [α-32P]GTP azidoanilide incorporation into Gαo compared with control samples. In contrast, proteins precipitated with an antisera specific for Gα2 and Gαq (AS 348) as well as Gα12 subtypes (AS 368) show only very weak incorporation of [α-32P]GTP azidoanilide under control conditions, and no increase upon urine stimulation was registered (data not shown).

Antibodies for distinct G protein α-subtypes have been successfully used to selectively inhibit ligand-induced phosphatidylinositol bisphosphate hydrolysis (24, 31). Accordingly, membrane preparations of female VNOs from rat were pretreated with different concentrations of subtype-specific antibodies and subsequently stimulated with a 2% (v/v) dilution of male urine. As demonstrated in Fig. 3A, preincubation with the Gαo common antibody C-10 or with the Gαq-specific antibody K-20 attenuated the urine-induced IP3 response in a concentration-dependent manner; 50% inhibition was obtained at a 1:500 dilution of either antibody. In contrast, antibody AS 348 specific for Gαq subtypes as well as AS 368 selective for the Gαq subfamily did not affect the responsiveness significantly (Fig. 3B). These results indicate that the IP3 response in female VNO preparations induced upon stimulation with a complex pheromonal mixture (male urine) is mediated via Gαo as well as Gαq proteins.

Functional and chemical analysis of urine from rodents have shown that pheromonal components are found in both the volatile as well as non-volatile fraction (5, 32). The volatile components can be extracted from whole male urine using dichloromethane (δ). Using this procedure, the dichloromethane-extractable urinary components as well as the remaining aqueous fraction were subsequently analyzed for their ability to induce IP3 responses in VNO membrane preparations. As shown in Fig. 4, compounds extracted with dichloromethane induced a concentration-dependent increase in the level of IP3, indicating that this fraction contained active ligands; in contrast, the aqueous fraction did not induce any changes in the concentration of IP3 compared with control samples (Fig. 4). Although it is unclear why the aqueous fraction is inactive it is conceivable that the proteins may be denatured by dichloromethane.

In order to address the issue of which G protein subtypes are labeled upon stimulation with the volatile urinary components, additional photoaffinity labeling experiments were performed employing different concentrations of the dichloromethane extract (0.02–0.4%, v/v). As demonstrated in Fig. 5, the organic extract induced a dose-dependent incorporation of [α-32P]GTP azidoanilide into Gαq, whereas a stimulus-induced labeling of Gα2 was not detectable (Fig. 5). These results indicate that Gq proteins but not G2 proteins are activated upon stimulation with volatile urinary components.

To confirm this observation, the effects of Gαq, as well as Gαo antibodies on dichloromethane extract-induced IP3 signaling

| TABLE I |
| --- |
| **Peptide sequences used for generation of antibodies** |
| **Antiserum** | **Peptide sequence** | **Specificity** | **Amino acids** | **Ref. or source** |
| Gαs (AS 348) | (C)RMHLRQYELL | Gα35–39Gα60 | 385–394 | 45 |
| Gαs (AS 266) | (C)LNREDGEKAAEVR | Gα11–22, Gα23 | 22–34 | 45 |
| Gα1 (C-10) | KNNLKEQGLY | Gα23–35Gα35 | 345–354 | —e |
| Gαq (K-20) | KVCNDVSRMDTEPSAE | Gα11–35Gα13 | 105–124 | —e |
| Gαq (AS 368) | (C)LQLNKLKEYNVL | Gα11–35Gα13 | 349–359 | 46 |

*C Santa Cruz Biotechnology.*
were analyzed. VNO preparations were pretreated with different concentrations of each of the two subtype-specific antibodies and subsequently stimulated with a 2% (v/v) dilution of the organic urinary extract. Whereas the dichloromethane extract-induced IP$_3$ formation was only slightly affected upon pretreatment with high concentrations of the Ga$_o$ antibody (1:250 dilution), Ga$_i$ antibodies caused already a strong inhibition at very low antibody concentrations: at a 1:2000 dilution of the Ga$_i$ antibody, the dichloromethane extract-induced IP$_3$ response was blocked by 70% (Fig. 6).

Previous electrophysiological experiments have demonstrated that the vertebrate VNO system is not only selective to a narrow range of pheromonal components, but in addition is sensitive to various general odorants normally known to be reorganized by the olfactory epithelium (33). In order to address the question which G protein subtypes are activated upon stimulation with volatile odorants, amyl acetate and menthol, which elicit electrical responses in turtle VNO neurons (33), were applied in photolabeling experiments. Membrane preparations of rat VNOs were stimulated with different concentrations of organic urinary substances of male rat urine extracted with dichloromethane or the remaining aqueous urinary components were applied to VNO membrane preparations. Basal levels of IP$_3$ obtained upon incubating of VNO preparations only with reaction buffer were 24.03 ± 7.3 pmol/mg of protein; treatment of samples with a 5% (v/v) dilution of pure dichloromethane did not affect the concentration of IP$_3$ (26.0 ± 4.8 pmol/mg of protein). Data are presented as ligand-induced IP$_3$ concentration in picomole/mg of protein and are the mean values of three experiments ± S.D.
concentrations of the two general odorants and subsequently G\textsubscript{i}, as well as G\textsubscript{o}, subtypes were precipitated by means of subtype-specific antibodies. Application of menthone (Fig. 7) as well as amyl acetate (not shown) induced a concentration-dependent labeling of G\textsubscript{i}, however, neither menthone (Fig. 7) nor amyl acetate (not shown) induced a significant incorporation of the GTP analog into G\textsubscript{o}.

The observation that neither organic components from male urine nor general volatile odorants induced G\textsubscript{o} activation led to the idea that urinary proteins may be ligand candidates for G\textsubscript{o} stimulation. In urine of fertile male rats, androgen-dependent α\textsubscript{2u}-globulin, a member of the lipocalin superfamily (10), is the quantitatively major protein (9). Therefore, α\textsubscript{2u}-globulin was purified from male urine as described by Holmquist et al. (21) and assessed for its capability to induce IP\textsubscript{3} formation. The results of these experiments (Fig. 8) indicate that purified α\textsubscript{2u}-globulin induced a very strong concentration-dependent increase in the concentration of IP\textsubscript{3}.

Since the procedure for isolating native α\textsubscript{2u}-globulin, such as gel permeation and dialysis purification steps, are not sufficient to separate noncovalently bound volatile urinary compounds (34), recombinant α\textsubscript{2u}-globulin, heterologously expressed in E. coli, was employed. As shown in Fig. 8A, application of recombinant α\textsubscript{2u}-globulin also induced an elevated IP\textsubscript{3} response; however, the efficacy was considerably smaller compared with native purified urinary protein. Nevertheless, the α\textsubscript{2u}-globulin-induced second messenger response is specific since stimulation of olfactory cilia from the OE did not

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**Fig. 5.** Effect of increasing concentrations of an organic extract of male rat urine on photolabeling of G\textsubscript{i} and G\textsubscript{o}. Membrane preparations of VNOs of female rats were stimulated with different concentrations of a dichloromethane extract of male rat urine ranging from 0.0 to 0.4% (v/v) (indicated as numbers) in the presence of \[^{32}\text{P}\]GTP azidoanilide. G\textsubscript{i} was precipitated with the antibody AS 266 (top panel), whereas G\textsubscript{o} was precipitated with the antibody R-20 (bottom panel). The autoradiograms showing the 40-kDa region of a SDS-PAGE are representative of three independent experiments with similar results. Quantification of incorporated \[^{32}\text{P}\]GTP azidoanilide was determined by densitometric analysis of autoradiograms presented as photosimulated luminescence (PSL) and calculated as percentage of the basal photolabeling obtained in the presence of 0.4% (v/v) dichloromethane.
show any changes in IP$_3$ concentration (Fig. 8B). To characterize the G protein subtype involved in recombinant $\alpha_{2u}$-globulin-induced IP$_3$ signaling, the effect of subtype-specific antibodies on IP$_3$ formation was analyzed. As documented in Fig. 9, Go$_i$ antibodies did not alter the responsiveness to $\alpha_{2u}$-globulin, even at rather high concentrations. In contrast, antibodies against Go$_o$ significantly attenuate IP$_3$ formation in a concentration-dependent manner. This observation was confirmed in photoaffinity labeling experiments; stimulating VNO membrane preparations with 1 $\mu$M recombinant $\alpha_{2u}$-globulin led to a significant incorporation of [$\alpha$-$^{32}$P]GTP azidoanilide into Go$_o$, whereas labeling of Go$_i$ subtypes was not changed (Fig. 10).

**DISCUSSION**

In the present study, G protein subtypes involved in urine-induced IP$_3$ formation in VNO preparations were characterized by two different experimental approaches. Subtype-specific G protein antibodies have been used as tools to selectively inhibit urine-induced IP$_3$ responses and to immunoprecipitate G protein subtypes photoaffinity labeled with the hydrolysis resistant GTP analogue [$\alpha$-$^{32}$P]GTP azidoanilide. The results of both experimental approaches indicate that whole male urine induces activation of Gi, as well as Go subtypes, suggesting a role for both G protein subtypes in transducing pheromonal responses mediated by urinary components. Activation of Gi and Go subtypes in the VNO appears to be realized by two structurally different classes of odorants. Whereas Gi activation was only observed upon stimulation with lipophilic, possibly volatile components, Go activation was elicited by $\alpha_{2u}$-globulin, a major urinary protein, which is a member of the lipocalin superfamily. Previous studies have shown that lipocalin proteins, like $\alpha_{2u}$-globulin and mouse urinary proteins, share the ability to bind volatile pheromones (34), suggesting that both proteins act as carrier proteins for hydrophobic ligands; however, the results of the present study suggest that $\alpha_{2u}$-globulin is not merely a carrier of volatile pheromones but is able to induce receptor-mediated Go activation. This observation is in line with previous behavioral studies indicating that members of the lipocalin superfamily may by themselves be active in VNO-mediated pheromonal responses. It has been demonstrated that mouse urinary proteins separated from volatile ligands, either by extraction or competitive displacement, were still active in bioassays (8). Furthermore, recombinant aphrodisin, a lipocalin-like protein from the female golden hamster vaginal discharge, expressed in E. coli induced a specific copulatory response in male golden hamster (35). This observation indicates that lipocalins can act as pheromones by themselves. However, a functional role for small ligands cannot be excluded, since recombinant aphrodisin only gained its full activity when it was supplemented with organic extracts of the vaginal discharge (35), suggesting that ligands may modulate the activity of lipocalins by altering its conformation.

A main result of the present study is the observation that both Gi and Go subtypes, activated by different classes of urinary stimuli, led to the activation of phospholipase C and the generation of IP$_3$. Thus, it is the question whether or not there is a physiological significance of having two populations of VNO neurons with distinct activated G protein subtypes that activate the same downstream effector. Activation of phospholipase C by pertussis toxin-sensitive G protein subtypes, like Gi and Go, appears to be mediated by $\beta\gamma$-subunits of the trimeric G protein (36). Therefore one might speculate whether the simultaneously released $\alpha$-subunits of Gi and Go may affect pathways not monitored in this study, suggesting a bifunctional action of these G proteins. Go$_o$-subunits are well known to modulate the activity of Ca$^{2+}$ channels (37); thus a Go$_o$-
regulated channel may contribute to govern the membrane potential in this subset of neurons. In the context of a bifunctional role for \( \alpha_i \)-subunits, it is interesting to note that vomeronasal neurons express adenylyl cyclase subtype II (13). \( \gamma_i \)-subunits inhibit AC II (38), thus, it is conceivable that activation of \( G_i \) by volatile pheromonal components may not only lead to phospholipase C activation mediated by \( \beta\gamma \)-subunits, but in addition to \( G_i \) controlled inhibition of AC II. Interestingly, an attenuation of cAMP levels in VNO preparations from female rats were stimulated with 1 \( \mu \)M recombinant protein pheromone \( \alpha_{2\mu}-\text{globulin} \); and subsequently the concentration of IP$_3$ was determined.

![Image](image-url)

**Fig. 8.** Tissue specificity of the production of IP$_3$ by \( \alpha_{2\mu}-\text{globulin} \) in female VNO preparations. A, concentration-response curves of \( \alpha_{2\mu}-\text{globulin} \)-induced IP$_3$ formation. Membrane preparations of female rat VNOs were stimulated with different concentrations of either \( \alpha_{2\mu}-\text{globulin} \) purified from male rat urine or recombinant protein expressed in *E. coli*. Data are calculated as ligand-induced IP$_3$ formation; the basal level in the absence of the lipocalin was 23.6 ± 8.8 pmol/mg of protein. Data are the means of three independent experiments ± S.D. B, effect of recombinant \( \alpha_{2\mu}-\text{globulin} \) on IP$_3$ formation in olfactory cilia of the OE. Isolated olfactory cilia were incubated either with reaction buffer (control) or stimulated with recombinant \( \alpha_{2\mu}-\text{globulin} \) (50 \( \mu \)M), respectively, the nonhydrolyzable GTP analogue GTP-$\gamma$S (1.6 \( \mu \)M), and subsequently the concentration of IP$_3$ was determined.

![Image](image-url)

**Fig. 9.** Selective blockade of \( \alpha_{2\mu}-\text{globulin} \) induced IP$_3$ formation by \( G_o \)-specific antibodies. Membrane preparations of female rat VNOs were pretreated for 10 min on ice with different concentrations of \( G_o \) (C-10) or \( G_o \) (K-20)-specific antibodies and subsequently stimulated with 50 \( \mu \)M recombinant \( \alpha_{2\mu}-\text{globulin} \). Data are calculated as % of ligand-induced IP$_3$ formation (51.2 ± 5.0 pmol/mg of protein); the basal level of IP$_3$ in the absence of the lipocalin was 31.7 ± 4.7 pmol/mg of protein. Data are the means of three independent experiments ± S.D.

![Image](image-url)

**Fig. 10.** Photoaffinity labeling of \( G_o \) and \( G_i \) subtypes upon stimulation with recombinant \( \alpha_{2\mu}-\text{globulin} \). VNO membrane preparations from female rats were stimulated with 1 \( \mu \)M recombinant \( \alpha_{2\mu}-\text{globulin} \); and subsequently \( G_o \) (top panel) as well as \( G_i \) (bottom panel) were precipitated with subtype-specific antibodies (\( G_o \), C-10; \( G_i \), K-20). The autoradiograms, showing the 40-kDa region of a SDS-PAGE, are representative of three independent experiments with similar results.

![Image](image-url)

The extremely large highly variable N-terminal domain, may recognize high molecular weight nonvolatile pheromones. Alternatively, as V2Rs-related \( G \) protein-coupled receptors, like metabotropic glutamate receptors (41) and the extracellular calcium sensing receptors (42) recognize very small ligands by means of the large N-terminal domain (43), it appears conceivable that V2Rs may bind small pheromonal components via a "venus flytrap" mechanism as suggested for the metabotropic glutamate receptors, whereas V1Rs, resembling ORs in the olfactory epithelium, may recognize volatile odorants presented by members of the lipocalin family, e.g. odorant-binding proteins (44). The result of the present study demonstrating that \( G_i \) coexpressed with V1Rs is activated by volatile ligands, whereas \( G_o \) coexpressed with V2Rs is activated upon stimulation with the protein pheromone \( \alpha_{2\mu}-\text{globulin} \), provide the first experimental evidence that each of the two VNO-receptor families is indeed activated by a distinct class of ligands. The emerging concept that V1Rs are activated by small volatile compounds, whereas V2Rs are specialized for interacting with protein pheromones can now be tested in experimental studies analyzing heterologously expressed receptors.
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