The Journal of Experimental Biology 206, 827-832

doi:10.1242/jeb.00174

Involvement of $G_{q/11}$ in signal transduction in the mammalian vomeronasal organ

Kennedy S. Wekesa*, Stephanie Miller and Audrey Napier

Alabama State University, Biomedical Research and Training Programs, Montgomery, AL 36104-0271, USA

*Author for correspondence (e-mail: kwekesa@asunet.alasu.edu)

Accepted 26 November 2002

Summary

Social behaviors of most mammals are profoundly affected by pheromones. Pheromones are detected by G-protein coupled receptors in the vomeronasal organ (VNO). To investigate the role of $G\alpha_{q/11}$ in vomeronasal signal transduction pathways, microvillar membranes from murine VNO were prepared. Incubation of such membranes from prepubertal females with adult male urine results in an increase in production of inositol-(1,4,5)-trisphosphate (IP_3). This stimulation is mimicked by GTPγS, blocked by GDPβS and is tissue specific. Furthermore, use of bacterial toxins such as pertussis that lead to ADP-ribosylation of the G-protein alpha subunits of G_o and G_{i2} do not block the increase in IP_3 levels but U-73122, a PLC inhibitor, blocks the production of IP_3. Studies with monospecific antibodies revealed the presence of three G-proteins, $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$-related protein, in vomeronasal neurons, concentrated on their microvilli. Our observations indicate that pheromones in male urine act on vomeronasal neurons in the female VNO *via* a receptor-mediated, $G\alpha_{q/11}$-protein-dependent increase in IP_3 levels.

Key words: pheromone, $G_{q/11}$, IP_3, signal transduction, mammal, vomeronasal organ.

Introduction

Responding to chemical signals is essential for the survival and reproduction of most organisms. The nasal cavity of mammals contains two independent sets of olfactory systems, the main olfactory system and the vomeronasal system. Olfactory neurons of the main olfactory system are located in the posterior recess of the nasal cavity and detect a large variety of small volatile chemicals that are carried by the air during breathing. This information is then processed in the main olfactory bulb (MOB) and in multiple cortical centers of the brain, leading to the cognitive and emotional responses to smell. Pheromones are mainly detected in the vomeronasal organ (VNO, a bilateral sensory structure found at the base of the nasal septum in mammals). VNO neurons send axonal projections to the accessory olfactory bulb (AOB) located dorsal to the most posterior portion of the MOB. Axons of AOB neurons indirectly project to specific nuclei of the amygdala and of the ventromedial hypothalamus that are involved in reproductive and aggressive behaviors (Winans and Scalia, 1970; Wysocki and Meredith, 1991; Kaneko et al., 1980). Information coded from chemical communication cues detected by the VNO can thus readily influence the central control of reproductive physiology and behavior (Meredith, 1983; Winans et al., 1982).

The initial events of pheromonal detection require the activation of specific receptors by pheromones and the transduction of the stimulus. Molecular evidence has led to the isolation of three independent families of vomeronasal receptor genes (VR), known as V1Rs (Dulac and Axel, 1995), V2Rs (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) and V3Rs (Pantages and Dulac, 2000) that encode putative pheromone receptors. A new classification of vomeronasal receptor genes has been proposed whereby the V1Rs and the V3Rs are consolidated into one family (Rodriguez et al., 2002). These vomeronasal receptors are unrelated to olfactory receptors. It has also been shown that V1Rs and V3Rs are expressed in the same population of VNO neurons, whereas V2Rs are expressed in a distinct subpopulation. These populations are non-overlapping and there is evidence to suggest that individual VNO neurons are likely to express only one receptor gene. Neurons lining the apical half of the VNO neuroepithelium express V1Rs (Dulac and Axel, 1995), whereas neurons in the basal half express V2Rs (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). In mice and rats these neurons also express G-proteins in the axonal projections. V1Rs and V3Rs express the alpha subunit of $G\alpha_{i2}$ and project to the anterior region of the AOB, whereas V2Rs express the alpha subunit of $G\alpha_o$ and project to the posterior regions of the AOB (Halpern et al., 1995; Wekesa and Anholt, 1999; Rodriguez et al., 1999). The expression of three types of pheromone receptors supports the idea that they might be involved in detection of different types of chemosensory information.

Vomeronasal chemoreception is mediated by narrowly tuned high-affinity receptors (Leinders-Zufall et al., 2000), and transduction involves release of inositol-1,4,5-trisphosphate (IP_3) (Taniguchi et al., 2000; Wekesa and Anholt, 1997; Inamura et al., 1997; Luo et al., 1994). In the garter snake *Thamnophis sirtalis*, it has been shown that a chemoattractant isolated from its prey induced the generation of inositol-1,4,5-trisphosphate in the VNO (Jiang et al., 1990; Luo et al., 1994). It has been shown that dialysis of IP_3 into the turtle *Geoclemys reevesii* and rat *Rattus norvegicus* VNO induces inward currents (Inamura et al., 1997; Taniguchi et al., 1995). We have also previously shown that female porcine VNO can be stimulated by male urine and seminal fluid to cause an increase of IP_3 (Wekesa and Anholt, 1997). These results suggest that pheromonal information is mediated *via* the IP_3-dependent pathway in the vomeronasal receptor neurons.

Although there is some consensus that IP_3 is the second messenger in the vomeronasal system, there is no consensus as to which G-protein(s) links receptor activation to the hydrolysis of phosphatidyl inositol bisphosphate (PIP_2). In the present study, we looked at the effects of G-protein bacterial toxins and phospholipase C inhibitors on responses to urine in the mouse vomeronasal organ in order to obtain information about the signal transduction pathways. We now provide evidence in support of signal transduction in the mammalian VNO being mediated by a member of the $G\alpha_{q/11}$ family of G-proteins *via* the IP_3-dependent transduction pathway.

Materials and Methods

Animals

CD-1 mice *Mus musculus* L. were originally obtained from Charles River Laboratories (Kingston, NY, USA) and maintained in a breeding colony in the Department of Biological Sciences at Alabama State University. Animals were housed in Institutional Animal Care and Use Committee-inspected and approved facilities and cared for according to the NIH Guide for Care and Use of Laboratory Animals (1997).

Membrane preparations

Adult male urine was freshly collected on a daily basis and stored as samples under argon at –80°C until used. VNOs from female mice, up to 2 weeks old, were dissected from their crevices in the nasal cavity, removed from the cartilaginous capsule, and frozen on dry ice. The tissues were then minced with a razor blade and subjected to sonication for 2–5 min in ice-cold phosphate-buffered saline (PBS). The resulting suspension was layered on a 45% (w/w) sucrose cushion and centrifuged at 4°C for 30 min at 3000 ***g*** in a Beckman SW55Ti rotor. The membrane fraction on top of the sucrose was collected and centrifuged as before for 15 min to pellet the membranes. The membranes were resuspended in 100 µl of ice-cold PBS. Protein was then determined according to the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard.

Second messenger assays

Non-hydrolysable forms of G-protein guanosine 5′-O-(3-thiotriphosphate) (GTPγS), inhibitor guanosine 5′-O-(2-thiodiphosphate) (GDPβS) and U-73122 were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Bordella Pertussis Toxin was purchased from Calbiochem (La Jolla, CA, USA). For IP_3 assays, reactions were incubated for 1 min at 37°C in 25 mmol l^{-1} Tris-acetate buffer pH 7.2, 5 mmol l^{-1} magnesium acetate, 1 mmol l^{-1} dithiothreitol (DTT), 0.5 mmol l^{-1} ATP, 0.1 mmol l^{-1} $CaCl_2$, 0.1 mg ml^{-1} BSA, 10 µmol l^{-1} GTP and 20 µg VNO membrane protein. Reactions were terminated by the addition of 1 mol l^{-1} trichloroacetic acid (TCA). IP_3 concentration was measured with a kit from Perkin Elmer, Inc. (Boston, MA, USA) according to the manufacturer's instructions, based on displacement of $[^3H]IP_3$ from a specific IP_3 binding protein. In experiments using bacterial toxins, 1 µg ml^{-1} pertussis (PTX) was incubated with membranes for 1 h. To assess the effect of ADP-ribosylation by pertussis toxin on phosphoinositide hydolysis, experiments were performed by a procedure similar to that described by Schleifer et al. (1980). The toxin was activated by preincubation with 20 mmol l^{-1} DTT at 37°C for 15 min. The VNO membranes from prepubertal female mice at a concentration of 0.2 mg ml^{-1} were exposed to 10 µg ml^{-1} of the activated toxin for 1 h at 37°C in 20 mmol l^{-1} Tris-HCl, pH 7.6, in the presence of 30 mmol l^{-1} thymidine, 1 mmol l^{-1} ATP, 0.1 mmol l^{-1} GTP, 5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 3 mmol l^{-1} phosphoenolpyruvate, 5 units ml^{-1} pyruvate kinase, 15 µg ml^{-1} saponin and 0.2 mmol l^{-1} NAD. Control membranes were incubated in the same reaction mixture but in the absence of the toxin. Differences between experimental and control animals were analyzed by analysis of variance (ANOVA).

Antibodies

Antibodies against α subunits of G-proteins were obtained from Calbiochem (La Jolla, CA, USA). The antibody against the α subunit of G_{i2} was raised against the C-terminal decapeptide KNNLDCGLF of G_{i2}. The antibody against the α subunit of G_o was raised against the C-terminal peptide KNNLKECGLY of $G\alpha_o$.. The antibody against $G\alpha_{q/11}$ was raised against the C-terminal peptide. Antibodies reactive with $G\alpha_{i2}$ and $G\alpha_o$ do not crossreact.

Western blotting

VNO membrane samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, followed by electrophoretic transfer onto a nitrocellulose membrane. Strips of the membrane, containing approximately 20 µg protein, were probed with a 1000-fold dilution of normal rabbit serum or 1000-fold dilutions of rabbit antisera against specific G protein subunits (Calbiochem, La Jolla, CA, USA). Bound antibody was visualized *via* a biotinylated goat-anti-rabbit secondary antibody complexed with avidin and biotinylated horseradish peroxidase (HRP), using Amersham's chemiluminescent

ECL detection system (Amersham, Arlington Heights, IL, USA). Migration distances were calibrated against Kaleidoscope prestained molecular mass markers (BioRad, Richmond, CA, USA).

Immunohistochemistry

Mice were given a lethal injection of sodium pentobarbital (50 mg kg^{-1}, i.p.) and perfused intracardially with PBS, followed by extensive perfusion with 10% paraformaldehyde. The nasal cavities were dissected and fixed overnight in 10% paraformaldehyde. Decalcification of nasal tissue was performed for 3 days at ambient temperature using the formic acid–sodium citrate method (Luna, 1968). 5 μm thick sections through nasal tissue, formalin-fixed and paraffin-embedded, were deparaffinized in xylene and rehydrated through graded alcohols. The sections were pretreated with 0.1% pepsin (Sigma, St Louis, MO, USA) in 0.01 mol l^{-1} HCl, pH 2.3, for 20 min to facilitate epitope access. Following pepsin treatment, the sections were blocked for 30 min with BEAT blocking solutions A and B (Zymed Laboratories, San Francisco, CA, USA). They were then incubated with a 250-fold dilution of normal rabbit serum or antiserum against $G\alpha_{i2}$, $G\alpha_o$ or $G\alpha_{q/11}$ in PBS, 0.05% Triton X-100 (Boehringer Mannheim, Indianapolis, IN, USA) overnight at 4°C. Following incubation with the primary antibody, sections were washed extensively in PBS, 0.05% Triton X-100, and incubated for 10 min at room temperature with affinity-purified biotinylated goat anti-rabbit antibody. Following a 10 min incubation with HRP-conjugated streptavidin, antibody complexes were visualized using 3′-amino-9′-ethylcarbazole as chromogenic substrate. This generates a red deposit at the site of antibody binding. Sections were counterstained with Hematoxylin, and viewed and photographed under a Zeiss Axiophot microscope. They were then processed using Adobe Photoshop 6.0.

Results

Identification of G-proteins on the microvillar surface of the VNO

Using subunit-specific rabbit antisera against unique peptide sequences of $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$, we confirmed in our microvillar membrane preparation the presence of $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$, as previously reported (Fig. 1). We further investigated whether the $G\alpha_{q/11}$ protein is localized to the microvillar surface of the VNO. The apical region of the murine vomeronasal neuron contains a dense group of microvilli, in contrast to supporting cells that carry smaller groups of microvilli (Adams, 1992). Immunohistochemical staining of coronal sections through the VNO with $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$ antiserum revealed staining of microvillar tufts at the surface of the vomeronasal lumen (Fig. 2). To verify the specificity of this staining, adjacent sections were incubated either without the primary antibody or with normal rabbit serum at the same concentration. Under these conditions, no staining was detected.

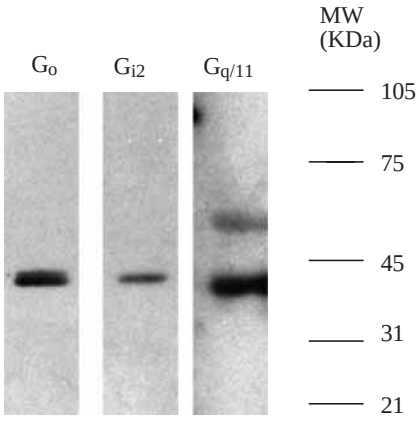

Fig. 1. Identification of G-protein subunits in the vomeronasal organ (VNO) membranes. Each strip contained 20 μg of VNO membrane protein and was probed with a 1000-fold dilution of monospecific rabbit antisera against subunits of G-proteins, as indicated. The positions of molecular mass markers (MW) are indicated.

Increase in IP_3 levels induced by male urine in VNO membranes from prepubertal female mice

To study transduction pathways activated by pheromonal stimuli from the male, we developed a preparation enriched in microvillar membranes from VNOs of prepubertal female mice. Incubation of microvillar membranes with male urine results in a robust increase in IP_3 as compared to the control ($P<0.05$). This response is mimicked by GTPγS and blocked by GDPβS (Fig. 3). We therefore concluded that female VNO membranes respond to stimuli in male urine with an increase in IP_3 *via* a G-protein-coupled pathway. In order to determine whether the production of IP_3 was mediated by phospholipase C, we incubated our membranes with a highly selective, cell-permeable inhibitor of PLC (U-73122, 10 μmol l^{-1}). We observed that incubation of VNO membranes with a PLC inhibitor blocked the production of IP_3 ($P<0.05$). In order to determine which G-protein mediated the activation of PLC, we incubated our VNO membranes in pertussis toxin before stimulation. We observed that incubation of VNO membranes with PTX in the presence of male urine still resulted in an increase of IP_3 levels. This response was not different from that of the control membranes, which were only stimulated with male urine in the absence of PTX. We concluded that pheromonal stimulation of VNO membranes results in an increase of IP_3 levels *via* activation of PLC by the alpha subunit of the $G_{q/11}$ class of G-proteins.

Discussion

Several studies using immunohistochemical techniques or *in situ* hybridization have shown that there are three G-proteins concentrated on the microvillar surface of the VNO, $G\alpha_{i2}$, $G\alpha_o$ and $G\alpha_{q/11}$ (Wekesa and Anholt, 1997; Berghard and Buck, 1996). Roles for activation of phosphoinositide hydrolysis have been reported for $G\alpha_{i2}$ (Brandt et al., 1985; Ohta et al.,

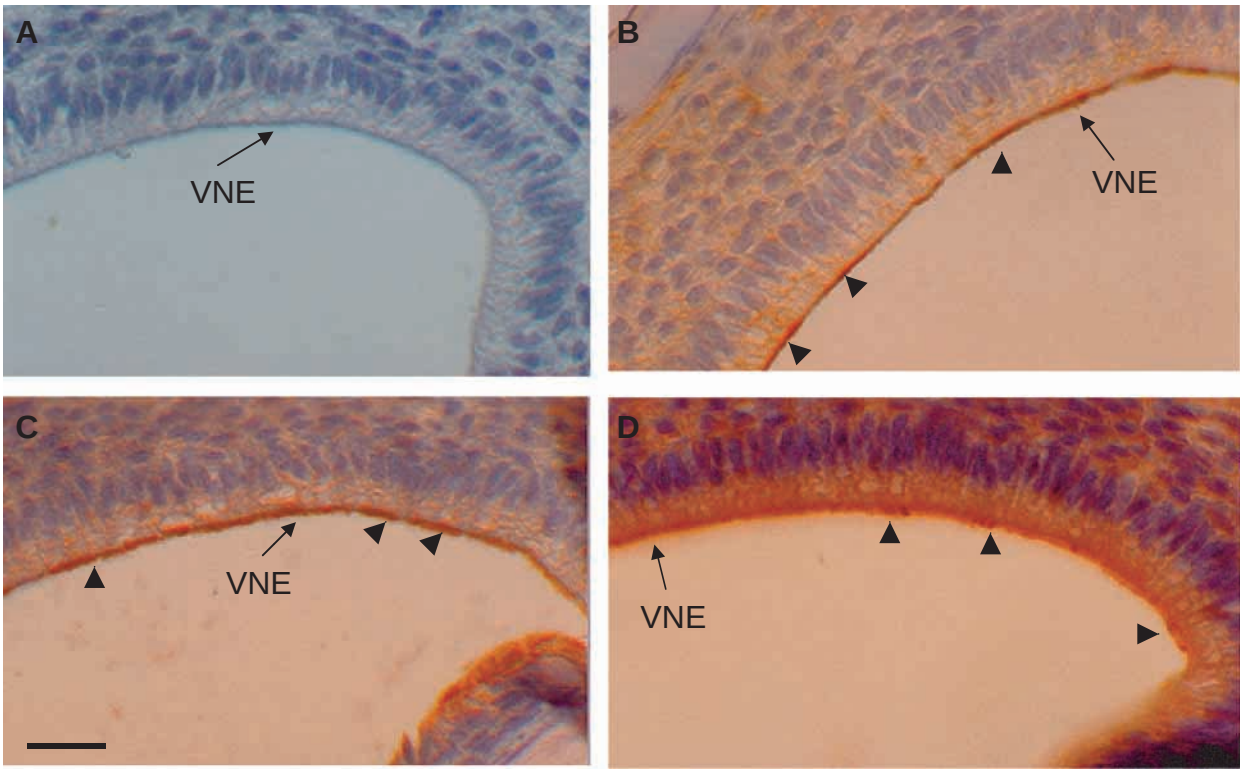

Fig. 2. Immunohistochemical localization of $G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$ to the microvillar surface of the vomeronasal organ. (A) A section stained with normal rabbit serum. (B) Section stained with a 250-fold dilution of antiserum against $G\alpha_o$. (C) Section stained with a 250-fold dilution of antiserum against $G\alpha_{i2}$. (D) Section stained with a 250-fold dilution of antiserum against $G\alpha_{q/11}$. Note the presence of staining within the neuroepithelium (arrowheads) for all G-protein antibodies ($G\alpha_o$, $G\alpha_{i2}$ and $G\alpha_{q/11}$). Scale bar, 100 μm. VNE, vomeronasal neuroepithelium.

1985) and for $G\alpha_o$ (Moriarty et al., 1990; Blitzer et al., 1993). $G\alpha_{i2}$ has been implicated in inhibition of adenylyl cyclase (Wong et al., 1992; Watkins et al., 1992; Taussig et al., 1993), potassium channel activation (Yatani et al., 1988; Kobayashi et al., 1990) and modulation of calcium channels (Linder et al., 1990). The principal functions of $G\alpha_o$ appear to be inhibition of neuronal calcium channels (Linder et al., 1990; Kleuss et al., 1991) and activation of potassium channels (Van Dongen et al., 1988; Kobayashi et al., 1990). By contrast, $G\alpha_q$ and $G\alpha_{11}$ are specialized for only one signal transduction function, namely activation of phospholipase C-β (Taylor et al., 1991; Lee et al., 1992).

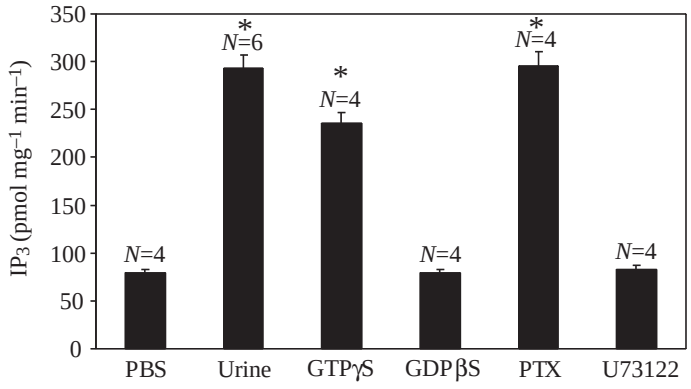

Fig. 3. The production of IP_3 in female mouse vomeronasal organ (VNO) membranes by male mouse urine. Reactions were performed without stimuli (PBS), in the presence of 10% male urine, 100 μm GTPγS, 10% urine with GDPβs, 10% male urine with pertussis toxin (PTX) and 10% male urine with U73122. *Significantly different from control (PBS) value (P<0.05).

In the present study we looked at the effects of phospholipase C inhibitors and G protein toxins in the signal transduction pathways. By use of bacterial toxins, which are commonly used to classify G proteins, we were able to show that VNO microvilli membranes incubated with pertussis toxin (PTX) are capable of being stimulated by urine to produce IP_3. Members of the $G\alpha_q$ family, e.g. $G\alpha_{11}$ and $G\alpha_q$, lack a recognition site for ribosylation by PTX (Pang and Sternweis, 1990; Strathmann and Simon, 1990; Blank et al., 1991; Sternweis et al., 1992) whereas $G\alpha_i$ and $G\alpha_o$ have a recognition site and undergo ADP ribosylation in the presence of PTX. Since we do not see any changes in production of IP_3 in the presence of PTX, we conclude that the main G-protein involved in the production of IP_3 is PTX-insensitive, such as the $G\alpha_{q/11}$ class of G-proteins.

The presence of G-protein, recognized by anti-$G\alpha_q$ on western blots of VNO microvillar membrane preparations, indicates that a substrate is present to explain the physiological effect of this antibody and is consistent with

the possibility suggested by the ribosylation experiments that the G-proteins are members of the $G\alpha_q$ family. These results are consistent with the observation that $G\alpha_q$ activates PLC and causes an increase in IP_3 in our membrane preparations. The ability to block this response with a PLC inhibitor such as U-73122 (Bleasdale et al., 1990; Inamura et al., 1997) further confirms the $G\alpha_q$ mediated pathway. It has also been shown that when U-73122 is applied to the neuroepithelium it blocks spiking in response to urine (Holy et al., 2000). $G\alpha_q$ and its close relatives activate the β1, β2 and β3-isoforms of PLC and, in contrast to $G\alpha_{i2}$ and $G\alpha_o$, are not inhibited by pertussis toxin (Taylor et al., 1991; Lee et al., 1992). In addition to the α subunits of G proteins, βγ subunits can in some instances mediate inhibition or activation of adenylate cyclase (Tang and Gilman, 1991; Clapham and Neer, 1993) or stimulation of phospholipase C, especially the β2-isoform (Camps et al., 1992; Boyer et al., 1992; Katz et al., 1992; Clapham and Neer, 1993) and we cannot exclude a role for the βγ subunits in mediating vomeronasal signal transduction. However, our experiments are designed primarily to determine the relative contributions of the distinct α subunits of G_{i2}, G_o and $G_{q/11}$ to activation of phospholipase C in the VNO.

Pheromonal-induced increases in IP_3 levels imply a role for calcium in vomeronasal signal transduction. The phosphoinositide cycle responds to the actions of an agonist at the receptor level by hydrolysis of PIP_2, resulting in the generation of IP_3 and diacylglycerol (DAG) (Neer and Clapham, 1988). DAG enhances the activity of protein kinase C (PKC) by rendering it more sensitive to stimulation by calcium (Nishizuka, 1988), while IP_3 stimulates the release of calcium from the endoplasmic reticulum stores (Berridge, 1987; Berridge and Irvine, 1989). Previous experiments by Leinders-Zufall et al., 2000) using slice preparations showed that pheromone application to VN results in a robust and reproducible increase in calcium levels. These results are consistent with other studies showing that urine stimulation activates a calcium-permeable cation-selective inward current in rat vomeronasal neurons (Inamura and Kashiwayanagi, 2000). A role for IP_3 signaling pathways is also suggested by the presence of the transient receptor potential (TRP) channel family, TRP2 in the VNO (Liman et al., 1999). This supports the idea that the increases in IP_3 levels are $G\alpha_q$ related. In *Drosophila* it has been shown that during the photoisomerization process, rhodopsin, a G_q class molecule, is activated, which triggers an IP_3 signaling cascade and leads to the opening of cation-sensitive channels dTRP and dTRPL. It is possible that pheromonal transduction might act in a similar manner. Although it is unclear how TRP2 is activated, members of the TRP channel family are often coupled to PLC activation and can be gated by products of the PIP_2 second messenger cascade. Consistent with these findings, VNO responses to components of urine have been blocked by pharmacological inhibitors of PLC (Holy et al., 2000). Therefore it possible that activation of pheromone receptors by $G\alpha_{q/11}$ leads to the stimulation of PLC. Elevation of IP_3 or DAG would then lead to the activation of TRP2 channels and membrane depolarization.

The authors would like to thank Demetria Rivers for the animal care. We thank Kroshona Tabb, Michal Stallworth and Besstina Lyles for helping with the embedding and sectioning of paraffin tissues. This work was supported by a grant from the National Institutes of Health (NIGMS-MBRS SCORE GM08219) to K.S.W.

References

Adams, D. R. (1992). Fine structure of the vomeronasal and septal olfactory epithelia and of glandular structures. *Microbiol. Res. Tech.* **23**, 86-97.

Berghard, A. and Buck, L. B. (1996). Sensory transduction in vomeronasal neurons: evidence for G_o, G_{i2}, and adenylyl cyclase II as major components of a pheromone signaling cascade. *J. Neurosci.* **16**, 909-918.

Berridge, M. J. (1987). Inositol triphosphate and diacylglycerol: two interacting second messengers. *Annu. Rev. Biochem.* **56**, 159-193.

Berridge, M. J. and Irvine, R. F. (1989). Inositol phosphates and cell signaling. *Nature* **341**, 197-204.

Blank, J. L., Ross, A. H. and Exton, J. H. (1991). Purification and characterization of two G-proteins that activate the β1 isozyme of phosphoinositide-specific phospholipase C. *J. Biol. Chem.* **266**, 18206-18216.

Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J. and Bunting, S. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils *J. Pharmacol. Exp. Ther.* **255**, 756-768.

Blitzer, R. D., Omri, G., De Vivo, M., Carty, D. J., Premont, R. T., Codina, J., Birnbaumer, L., Cotecchia, S., Caron, M. G., Lefkowitz, R. J., Landau, E. M. and Iyenger, R. (1993). Coupling of the expressed α_{1B}-adrenergic receptor to the phospholipase C pathway in *Xenopus* oocytes: The role of Go. *J. Biol. Chem.* **268**, 7532-7537.

Boyer, J. L., Waldo, G. L. and Harden, T. K. (1992). βγ-subunit activation of G-protein regulated phospholipase C. *J. Biol. Chem.* **267**, 25451-25456.

Brandt, S. J., Douhgherty, R. W., Lapetina, E. G. and Niedel, J. E. (1985). Pertussis toxin inhibits chemotactic peptide-stimulated generation of inositol phosphates and lysosomal enzyme secretion in human leukemic (HL-60) cells. *Proc. Natl. Acad. Sci. USA* **82**, 3277-3280.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J. and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipaseC-β_2 by G protein βγ-subunits. *Nature* **360**, 684-686.

Clapham, D. E. and Neer, E. J. (1993). New roles for G-protein βγ-dimers in transmembrane signaling. *Nature* **365**, 403-406.

Dulac, C. and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83**, 195-206.

Halpern, M., Shapiro, L. S. and Jia, C. (1995). Differential localization of G proteins in the opossum vomeronasal system. *Brain Res.* **677**, 157-161.

Herrada, G. and Dulac, C. (1997). A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* **90**, 763-773.

Holy, T. E., Dulac, C. and Meister, M. (2000). Responses of vomeronasal neurons to natural stimuli. *Science* **289**, 1569-1572.

Inamura, K. and Kashiwayanagi, M. (2000). Inward current responses to urinary substances in rat vomeronasal sensory neurons. *Eur. J. Neurosci.* **12**, 3529-3536.

Inamura, K., Kashiwayanagi, M. and Kurihara, K. (1997). Inositol-1,4,5-trisphosphate induces responses in receptor neurons in rat vomeronasal sensory slices. *Chem. Senses* **22**, 93-103

Jiang, X. C., Inouchi, J., Wang, D. and Halpern, M. (1990). Purification and characterization of a chemo-attractant from electric shock-induced earthworm secretion, its receptor binding, and signal transduction through the vomeronasal system of garter snakes. *J. Biol. Chem.* **265**, 8736-8744.

Kaneko, N., Debski, E. A., Wilson, M. C. and Whitten, W. K. (1980). Puberty acceleration in mice. II. Evidence that the vomeronasal organ is a receptor for the primer pheromone in male mouse urine. *Biol. Reprod.* **22**, 873-878.

Katz, A., Wu, D. and Simon, M. I. (1992). Subunits βγ of heterotrimeric G protein activate β_2 isoform of phospholipase C. *Nature* **360**, 686-689.

Kleuss, C., Hescheler, J., Ewel, C., Rosentahl, W., Schultz, G. and Wittig,

B. (1991). Assignments of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature* **353**, 43-48.

Kobayashi, I., Shibasaki, H., Takahashi, K., Tohyama, K., Kurachi, Y. and Ito, H. (1990). Purification and characterization of five different α subunits of guanine-nucleotide-binding proteins in bovine brain membranes: Their physiological properties concerning the activities of adenylate cyclase and atrial muscarinic potassium ion channels. *Eur. J. Biochem.* **191**, 499-506.

Lee, C. H., Park, D., Wu, D., Rhee, S. G. and Simon, M. I. (1992). Members of the G_q α subunit gene family activate phospholipase C β isozymes. *J. Biol. Chem.* **267**, 16044-16047.

Leinders-Zufall, T., Lane, A. P., Puche, A., Ma, W., Novotny, M. V., Shipley, M. T. and Zufall, F. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* **405**, 792-796.

Linder, M. E., Ewald, D. A., Miller, R. J. and Gilman, A. G. (1990). Purification and characterization of $G_{o\alpha}$ and three types of $G_{i\alpha}$ after expression in *Escherichia coli*. *J. Biol. Chem.* **265**, 8243-8251.

Liman, E. R., Corey, D. P. and Dulac, C. (1999). TRP2: A candidate transduction channel for mammalian pheromone sensory signaling. *Proc. Natl. Acad. Sci. USA* **96**, 5791-5796.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Luna, L. G. (ed.) (1968). Preparation of Tissues. *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, pp.1-12. New York: McGraw-Hill.

Luo, Y., Lu, S., Chen, P., Wang, D. and Halpern, M. (1994). Identification of chemoattractant receptors and G proteins in the vomeronasal system of garter snakes. *J. Biol. Chem.* **269**, 16867-16877.

Meredith, M. (1983). Sensory physiology of pheromone communication. In *Pheromones and Reproduction in Mammals* (ed. J. G. Vandenbergh), pp. 95-112. New York, NY: Academic Press.

Matsunami, H. and Buck, L. (1997). A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* **90**, 775-784.

Moriarty, T. M., Padrell, E., Carty, D. J., Omri, G., Landau, E. M. and Iyengar, R. (1990). G_o protein as signal transducer in the pertussis toxin-sensitive phosphatidylinositol pathway. *Nature* **343**, 79-82.

Neer, E. J. and Clapham, D. E. (1988). Roles of G protein subunits in transmembrane signaling. *Nature* **333**, 129-134.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

Ohta, H., Okajima, F. and Ui, M. (1985). Inhibition by islet-activating protein of a chemotactic peptide-induced early breakdown of inositol phospholips and calcium mobilization in guinea pig neutrophils. *J. Biol. Chem.* **260**, 15771-15780.

Pang, I. H. and Sternweis, P. C. (1990). Purification of unique alpha subunits of GTP-binding regulatory proteins (G proteins) by affinity chromatography with immobilized beta-gamma subunits. *Proc. Natl. Acad. Sci. USA* **86**, 7814-7818.

Pantages, E. and Dulac, C. (2000). A novel family of candidate pheromone receptors in mammals. *Neuron* **28**, 835-845.

Rodriguez, I., Feinstein, P. and Mombaerts, P. (1999). Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell* **97**, 199-208.

Rodriguez, I., Del Punta, K., Rothman, A., Ishii, T. and Mombaerts, P. (2002). Multiple new and isolated families within the mouse superfamily of V1R vomeronasal receptors. *Nature Neurosci.* **5**, 134-140.

Ryba, N. J. P. and Tirindelli, R. (1997). A new multigene family of putative pheromone receptors. *Neuron* **19**, 371-379.

Schleifer, L. S., Garrison, J. C., Sternweis, P. C., Northup, J. K. and Gilman, A. G. (1980). The regulatory component of adenylate cyclase from uncoupled S49 lymphoma cells differ in charge from the wild type protein. *J. Biol. Chem.* **255**, 2641-2644.

Sternweis, P. C. and Smrcka, A. V. (1992). Regulation of phospholipase C by G proteins. *Trends Neurosci.* **17**, 502-506.

Strathmann, M. and Simon, M. I. (1990). G protein diversity: a distinct class of α subunits is present in vertebrates and invertebrates. *Proc. Natl. Acad. Sci. USA* **87**, 9113-9117.

Tang, W. J. and Gilman, A. G. (1991). Type-specific regulation of adenylyl cyclase by βγ subunits. *Science* **254**, 1500-1503.

Taniguchi, M., Wang, D. and Halpern, M. (2000). Chemosensitive conductance and inositol 1,4,5 trisphosphate-induced conductance in snake vomeronasal receptor neurons. *Chem. Senses* **25**, 67-76.

Taniguchi, M., Kashiwayanagi, M. and Kurihara, K. (1995). Intracellular injection of inositol 1,4,5-trisphosphate increases a conductance in membranes of turtle vomeronasal receptor neurons in the slice preparation. *Neurosci. Lett.* **188**, 5-8.

Taussig, R., Iniguez-Lluhi, J. A. and Gilman, A. G. (1993). Inhibition of adenylyl cyclase by $G_{i\alpha}$. *Science* **261**, 218-221.

Taylor, S. J., Chae, H. Z., Rhee, S. G. and Exton, J. H. (1991). Activation of the β1 isozyme of phospholipase C by the α subunits of the G_q class of G proteins. *Nature* **350**, 516-518.

Van Dongen, A. M. J., Codina, J., Olate. J., Mattera, R., Joho, R., Birnbaumer, L. and Brown, A. M. (1988). Newly identified brain potassium channels gated by the guanine nucleotide binding proteion G_o. *Science* **242**, 1433-1437.

Watkins, D. C., Johnson, G. L. and Malbon, C. C. (1992). Regulation of the differentiation of teratocarcinoma cells into primitive ectoderm by $G\alpha_{i2}$. *Science* **258**, 1373-1375.

Wekesa, K. S. and Anholt, R. R. H. (1997). Pheromone-regulated production of inositol-(1,4,5)-trisphosphate in the mammalian vomeronasal organ. *Endocrinol.* **138**, 3497-3504.

Wekesa, K. S. and Anholt, R. R. H. (1999). Differential expression of G proteins in the mouse olfactory system. *Brain Res.* **837**, 117-126.

Winans, S. S. and Scalia, F. (1970). Amygdaloid nucleus: new afferent input from the vomeronasal organ. *Science* **170**, 330-332.

Winans, S. S., Lehman, M. N. and Powers, J. B. (1982). Vomeronasal and olfactory CNS pathways which control male hamster mating behavior. In *Olfaction and Endocrine Regulation* (ed. W. Breipohl), pp. 23-34. London: IRL Press Limited.

Wong, Y. H., Conklin, B. R. and Bourne, H. R. (1992). G_{i2}-mediated hormonal inhibition of cyclic AMP accumulation. *Science* **255**, 339-342.

Wysocki, C. J. and Meredith, M. (1991). The vomeronasal system. In *Neurobiology of Taste and Smell* (ed. T. E. Finger and W. L. Silver), pp. 125-150. New York, NY: John Wiley & Sons.

Yatani, A., Hamm, H., Codina, J., Mazzoni, M. R. Birnbaumer, L. and Brown, A. M. (1988). A monoclonal antibody to the alpha subunit of Gk blocks muscarinic activation of atrial K^+ channels. *Science* **241**, 828-831.