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Attenuation of the Production of Inositol 1,4,5-Trisphosphate in the Mouse Vomeronasal Organ by Antibodies Against the $\alpha_{q/11}$ Subfamily of G-Proteins

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Abstract

The social and reproductive behaviors of most mammals are modulated by pheromones, which are perceived by the vomeronasal organ (VNO). Vomeronasal transduction in vertebrates is activated through G-protein–coupled receptors, which in turn leads to the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by the activity of phospholipase C. DAG has been shown to gate the transient receptor potential channel 2, whereas IP_3 may play a role in stimulating the release of calcium from the endoplasmic reticulum store. To investigate the role of the alpha subunits of $G_{q/11}$ in the transduction process, microvillar membranes from female mice VNO were preincubated with a selective C-terminal peptide antibody against $G\alpha_{q/11}$ and then stimulated with adult male urine. Incubation of VNO membranes with antibodies against $G\alpha_{q/11}$ blocked the production of IP_3 in a dose-dependent manner. We were also able to impair the production of IP_3 when we stimulated with 2-heptanone or 2,5-dimethylpyrazine in the presence of antibodies against the alpha subunit of $G_{q/11}$. 2-Heptanone is a known pheromone that has been linked to VIR receptors. Thus, our observations indicate that the alpha subunits of $G_{q/11}$ play a role in pheromonal signaling in the VNO.

Key words: 2,5-dimethylpyrazine, G-proteins, 2-heptanone, IP_3, pheromones, vomeronasal

Introduction

The social and reproductive behaviors of most mammals are modulated by pheromones, which are chemical signals among conspecifics. The perception of pheromones is mainly mediated by the vomeronasal organ (VNO) which is located at the base of the nasal septum. The VNO contains a lumen that communicates via a duct with the oral or nasal cavity. Therefore, chemical stimuli in urine and glandular secretions when inhaled can act upon the dendritic microvilli of bipolar chemosensory neurons in the VNO. These chemosensory neurons express two classes of putative pheromone receptor gene families, V1R and V2R, segregated in zones (Halpern *et al.*, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996; Wekesa and Anholt, 1999; Dulac, 2000; Pantages and Dulac, 2000). The V1R family of putative pheromone receptors is coexpressed with the G-protein $G\alpha_{i2}$ on the apical zone of the vomeronasal neuroepithelium that projects to the anterior aspect of the accessory olfactory bulb. The V2R family of receptors are coexpressed with the G-protein $G\alpha_o$ in the basal zone that projects to the posterior aspect of the accessory olfactory bulb (Halpern *et al.*, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996; Wekesa and Anholt, 1999; Dulac, 2000; Pantages and Dulac, 2000). V1R receptors are structurally similar to olfactory receptors although evolutionarily unrelated, and there are estimated to be about 150 different types (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Rodriguez *et al.*, 2002). V2Rs on the other hand have a long extracellular N-terminal region believed to be involved in ligand binding and are similar to metabotropic glutamate receptors. There are estimated to be 100 V2Rs in rodents, arrayed into several subfamilies (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997).

Electrophysiological and calcium-imaging studies have shown that the V1R class of receptors respond to volatile chemicals including 2-heptanone and 2,5-dimethylpyrazine (Leinders-Zufall *et al.*, 2000; Boschat *et al.*, 2002), whereas the V2R receptors respond to larger nonvolatile compounds such as MHC class I peptides (Krieger *et al.*, 1999; Leinders-Zufall *et al.*, 2004). 2-Heptanone found in both male and female urine extends the estrous cycle in female mice, whereas 2,5-dimethylpyrazine found only in female urine acts to delay puberty in females (Novotny *et al.*, 1985; Jemiolo

et al., 1989; Novotny, 2003). MHC class I peptides are involved in social recognition signals that convey information about genetic individuality (Leinders-Zufall *et al.*, 2004).

The transduction cascade in the VNO of vertebrates is distinct and is driven by phospholipase-C (PLC)–induced production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), and the subsequent increase in intracellular calcium concentration (Luo *et al.*, 1994; Inamura *et al.*, 1997a,b; Wekesa and Anholt, 1997; Holy *et al.*, 2000; Inamura and Kashiwayanagi, 2000; Leinders-Zufall *et al.*, 2000; Cinelli *et al.*, 2002; Wekesa *et al.*, 2003). DAG gates the transient receptor potential channel 2 (TRPC2), whereas IP_3 may a play a role in stimulating the release of calcium from the endoplasmic reticulum store (Lucas *et al.*, 2003).

Several G-protein subunits have been identified in the vomeronasal neurons, including $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_{q/11}$, $\beta\gamma_2$, $G\gamma_2$, and $G\gamma_8$ (Berghard *et al.*, 1996; Jia and Halpern, 1996; Tirindelli and Ryba, 1996; Runnenburger *et al.*, 2002; Wekesa *et al.*, 2003), but the G-protein alpha subunits that may be involved in pheromonal signaling have yet to be clearly shown. In order to determine which of the G-protein alpha subunits plays a role in the activation of PLC, we used antibodies prepared against the C-terminus. This approach is based on the evidence that the C-terminal region of the G-protein alpha subunits is involved in receptor coupling (Birnbaumer *et al.*, 1990), and therefore, antibodies that bind selectively to this region can disrupt alpha subunit activity. These antibodies uncouple G-proteins from their receptors *in situ* and have previously been used to selectively block activation of transducin, G_s, G_{i2}, G_o, and G_q (Cerione *et al.*, 1988; Mackenzie *et al.*, 1988; McFadzean *et al.*, 1989; Simonds *et al.*, 1989; McClue and Milligan, 1990).

Here we show that antibodies against $G\alpha_{q/11}$ can block the production of IP_3 in both VIR and V2R receptors in the female VNO when stimulated by whole adult male urine or isolated urinary compounds such as 2-heptanone or 2,5-dimethylpyrazine.

Materials and methods

Animals

CD-1 mice were originally obtained from Charles River Laboratories (Kingston, NY) 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 National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. Mice were kept in Nalgene cages 26 × 21 × 14 cm, at 25°C room temperature, and a 12:12 h light:dark cycle. Food and water were provided *ad libitum*.

Membrane preparations

VNOs from female mice, up to 4 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 40,000 rpm 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 as standard. The procedure used for the preparation of microvillar membranes is modeled after well-established methods (Anholt, 1995; Wekesa and Anholt, 1999; Wekesa *et al.*, 2003). These preparations have been previously characterized (Anholt *et al.*, 1986; Anholt 1995; Wekesa and Anholt, 1997) and are sufficiently enriched in chemosensory membranes. Membranes from the liver were prepared by homogenizing the tissue in PBS with a Teflon homogenizer. Membranes were collected by centrifugation, washed once, and suspended in PBS.

Second messenger assays

The chemicals 2-heptanone, 2,5-dimethylpyrazine, and angiotensin II (AngII) were purchased from Aldrich Chemical Co. (Milwaukee, WI). For IP_3 assays, reactions were incubated for 1 min at 37°C in 25 mM Tris-acetate buffer pH 7.2, 5 mM Mg-acetate, 1 mM dithiothreitol, 0.5 mM adenosine triphosphate, 0.1 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 10 µM guanosine triphosphate, and 20 µg VNO membrane proteins. Reactions were terminated by the addition of 1 M trichloroacetic acid. IP_3 was measured with a kit from Perkin Elmer, Inc. (Boston, MA) according to the manufacturer's instructions and is based on displacement of [^{3}H] IP_3 from a specific IP_3 binding protein. Differences between experimental and control animals were analyzed by analysis of variance.

Antibodies

The antibodies against alpha subunits of G-proteins were purchased from Calbiochem (La Jolla, CA). The C-terminus of the G alpha subunits is a defined receptor contact site, and the overexpression of peptides corresponding to this region competitively blocks interaction between G-protein–coupled receptors (GPCRs) and the targeted G-proteins (Gilchrist *et al.*, 2001). Consistent with this, the last five C-terminal amino acids of $G\alpha_{q/11}$ are important for maintaining receptor selectivity and signaling capacity (Gilchrist *et al.*, 2001). The antibody against the alpha subunit of $G\alpha_{q/11}$ was raised against the last 12 amino acids on the carboxy-tail peptide (QLNLKEYNLV). The antibody against the alpha subunit of G_{i2} was raised against the C-terminal peptide KNNLDCGLF.

Results

Inhibition of AngII-mediated stimulation of IP_3 in membranes from liver

The peptide hormone AngII regulates vasoconstriction, water and salt balance, and neuromodulation and cellular growth through actions on two types of AngII receptors type 1, AT_1, and type 2, AT_2 (de Gasparo *et al.*, 1995). The AT_1 receptor is the principal mediator of biological actions of AngII. AT_1 receptors are GPCRs and activate G-proteins through the membrane-proximal regions of the third cytoplasmic loop and C-terminus in the receptor (Sano *et al.*, 1997; Kai *et al.*, 1998). AT_1 activation by AngII results in the generation of IP_3 and DAG, via a PLC–coupled G-protein, $G\alpha_{q/11}$, to cause release of calcium from intracellular stores and activation of protein kinase C (Griendling *et al.*, 1997). Previous experiments using antibodies against $G\alpha_{q/11}$ impaired the production of IP_3 in this well-studied system (Gutowski *et al.*, 1991). In order to demonstrate that our antibodies can indeed block $G\alpha_{q/11}$, we used the AngII system in the liver. Incubation of liver membranes with AngII resulted in a robust increase of IP_3 ($P < 0.05$; Figure 1), whereas liver membranes that had been preincubated with 100-fold dilution of antibodies against $G\alpha_{q/11}$ did not show an increase in IP_3. This experiment confirmed that our antibodies were able to disrupt the activity of the alpha subunit of $G_{q/11}$.

Dose-dependent inhibition of the production of IP_3 in female VNO membranes by antibodies against $G\alpha_{q/11}$

To study transduction pathways activated by pheromonal stimuli, we developed preparations enriched in microvillar membranes from VNOs of females. Incubation of microvillar VNO membranes from females with adult male urine results in a significant increase in the production of IP_3 ($P < 0.05$; Figure 2). In order to determine if $G\alpha_{q/11}$ mediated the activation of PLC, we preincubated our VNO membranes in varying concentrations of antibodies against $G\alpha_{q/11}$. We observed that stimulation of VNO membranes with male urine in the presence of antibodies against $G\alpha_{q/11}$ impaired the production of IP_3 in a dose-dependent manner.

Female VNO response to male urine is not impaired by antibodies against $G\alpha_{i2}$

In order to determine if the alpha subunits of G_{i2} have the same effects as $G\alpha_{q/11}$, we preincubated our VNO membranes with antibodies against $G\alpha_{i2}$. Incubation of $G\alpha_{i2}$ antibodies in the presence of male urine did not impair the production of IP_3 (Figure 3).

Female VNO response to urinary chemicals is impaired by antibodies against $G\alpha_{q/11}$

The stimulation of microvillar membranes from VNOs of female mice with 2-heptanone and 2,5-dimethylpyrazine resulted in an increase in the production of IP_3 as compared to the control ($P < 0.05$; Figure 4). This effect was blocked by preincubation of the VNO membranes with antibodies against $G\alpha_{q/11}$.

Discussion

Understanding the signal transduction cascade in the VNO is an important step in understanding how this system encodes

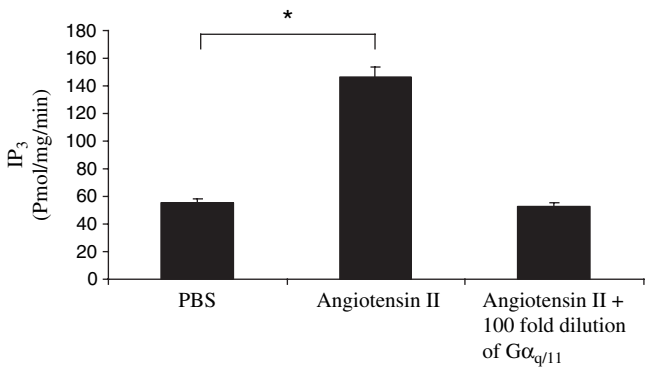

Figure 1 Inhibition of AngII-mediated stimulation of IP_3 in membranes from liver. Reactions were performed without stimulus, in the presence of 1 μM AngII, and with 1 μM AngII with 100-fold dilution of antibodies against $G\alpha_{q/11}$. Significant stimulation compared to basal activity (PBS) is observed in the presence of 1 μM AngII (*$P < 0.05$) but not in the presence of AngII with antibodies against $G\alpha_{q/11}$. The data points and error bars represent the averages and standard errors of at least five independent experiments, each consisting of duplicate measurements.

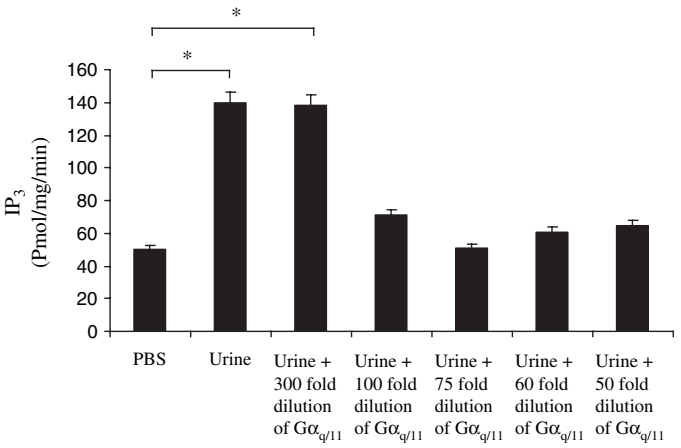

Figure 2 Dose-dependent inhibition of the production of IP_3 in female VNO membranes by antibodies against $G\alpha_{q/11}$. Reactions were performed without urine, in the presence of 10% male urine with varying concentrations of antibodies against $G\alpha_{q/11}$. Significant stimulation compared to basal activity (PBS) is observed when stimulated with urine alone and with low concentrations of the antibody (*$P < 0.05$). There was no significant difference detected between 100-fold dilution and 50-fold dilution of antibodies against $G\alpha_{q/11}$. The data points and error bars represent the averages and standard errors of at least five independent experiments, each consisting of duplicate measurements.

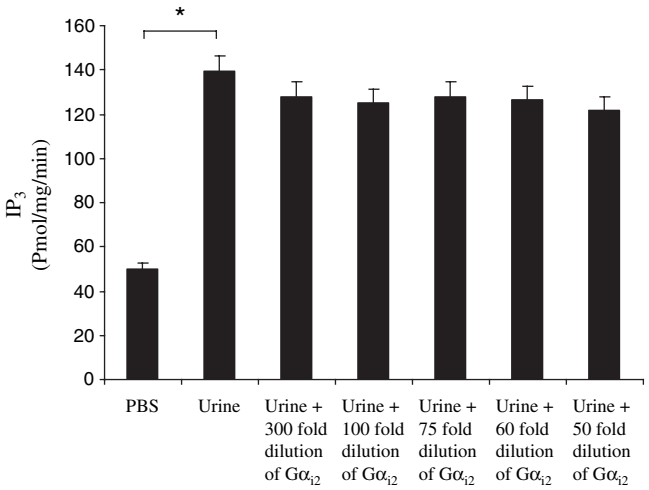

Figure 3 Female VNO response to male urine is not impaired by antibodies against $G\alpha_{i2}$. Reactions were performed without urine, in the presence of 10% male urine, and in the presence of 10% male urine with varying concentrations of antibodies against $G\alpha_{i2}$. Incubation of VNO membranes with varying concentration of $G\alpha_{i2}$ did not significantly impair the production of IP_3. The data points and error bars represent the averages and standard errors of at least five independent experiments, each consisting of duplicate measurements.

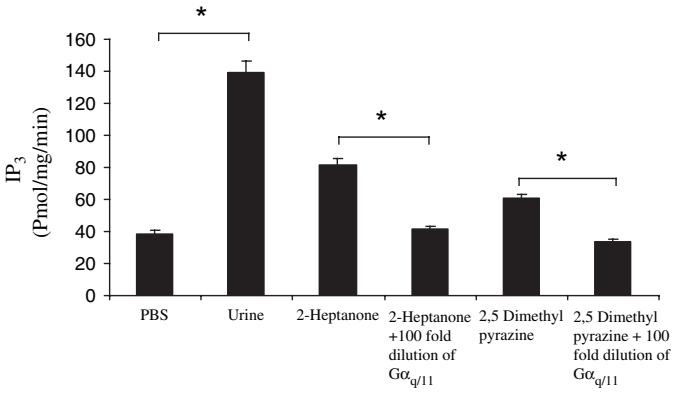

Figure 4 Female VNO response to urinary chemicals are impaired by antibodies against $G\alpha_{q/11}$. Reactions were performed without stimulus, in the presence of 10 μM 2-heptanone, 10 μM 2,5-dimethylpyrazine, 10 μM 2-heptanone together with 100-fold dilution of antibody against $G\alpha_{q/11}$, and 10 μM 2,5-dimethylpyrazine together with 100-fold dilution of antibody against $G\alpha_{q/11}$. Significant stimulation compared to basal activity is observed in the presence of 2-heptanone and 2,5-dimethylpyrazine ($*P < 0.05$). This stimulation is impaired when the VNO membranes are incubated with antibodies against $G\alpha_{q/11}$. The data points and error bars represent the averages and standard errors of at least five independent experiments, each consisting of duplicate measurements.

pheromonal information which is important for regulating reproductive behaviors and physiology. There is a lot of information on the molecular entities (receptors and G-proteins) that may be involved in pheromonal signaling but how these different molecules actually interact is not clear. Several studies using immunohistochemical techniques or *in situ* hybridization have shown that there are three G-proteins 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 $G\alpha_{i2}$ (Ohta *et al.*, 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 (Watkins *et al.*, 1992; Wong *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). In contrast, $G\alpha_q$ and $G\alpha_{11}$ are specialized for only one signal transduction function, activation of PLC (Taylor *et al.*, 1991; Lee *et al.*, 1992).

In our studies, we have been able to show that incubation of VNO membranes with antibodies against $G\alpha_{q/11}$ was able to impair the production of IP_3 when stimulated with 2-heptanone and 2,5-dimethylpyrazine (Figure 4). Although the response to these two compounds was higher than basal levels, it was significantly lower than stimulation with whole urine (Figure 4). This would suggest that these two compounds stimulate a subset of the VNO neurons, whereas urine which contains a mixture of different pheromones would stimulate either all or a higher subset of pheromone receptors. Previous experiments using electrophysiological and biochemical techniques have shown that 2-heptanone and 2,5-dimethylpyrazine stimulates V1R receptors (Boschat *et al.*, 2002; Thompson *et al.*, 2004). 2-Heptanone induces aggression in males and puberty in females (Novotny *et al.*, 1985, 1999), and the receptors that are responsible for this response are the VIRb2 receptors (Boschat *et al.*, 2002). These data would therefore suggest that even though V1R receptors are coupled to $G\alpha_{i2}$, the alpha subunit of $G\alpha_{q/11}$ plays a role in the transduction cascade.

Although there is a significant amount of data showing the receptors (V1Rs, V2Rs) and G-protein subunits ($G\alpha_o$, $G\alpha_{i2}$, $G\alpha_{q/11}$, $G\beta\gamma$) that may be involved in the pheromonal signaling, there are almost no studies showing the interactions of these proteins in the VNO. Studies using membrane preparations and electrophysiology have clearly shown that PLC plays a major role in the transduction cascade (Krieger *et al.*, 1999; Holy *et al.*, 2000; Lucas *et al.*, 2003; Wekesa *et al.*, 2003). Furthermore, blocking the G-protein alpha subunits of G_o and G_{i2} by adenosine diphosphate ribosylation using pertussis toxins does not block PLC-mediated production of IP_3 (Wekesa *et al.*, 2003). Although the previous study showed that the alpha subunits of G_o and G_{i2} do not play a role in the transduction cascade, it did not conclusively show that blocking the alpha subunit of $G\alpha_{q/11}$ impairs the activation of PLC and therefore impair the production of IP_3. In this current study, we show that by using

antibodies against the C-terminus of $G\alpha_{q/11}$, we can block the production of IP_3 in female VNOs that are stimulated with adult male urine or specific pheromones such as 2-heptanone and 2,5-dimethylpyrazine (Figures 2 and 3).

Several questions regarding signal transduction in the VNO are still unanswered, such as the role of IP_3 and the role of calcium once it has entered the cell. Studies by Liman (2003) show that VNO microvilli have a calcium-activated cation channel which could be opened by calcium ions that either enter the cell through the TRPC2 channel or from the endoplasmic reticulum. Opening of this channel would enhance the receptor potential and therefore amplify the signal. Several studies show that IP_3 levels increase in the VNO membrane preparations during stimulation with urinary pheromones (Kroner *et al.*, 1996; Wekesa and Anholt, 1997; Sasaki *et al.*, 1999; Wekesa *et al.*, 2003). Furthermore, injection of IP_3 into rat VNO neurons induces inward current responses under whole-cell voltage clamp conditions (Inamura *et al.*, 1997b). Ruthenium red, an IP_3 receptor inhibitor, reduces action potential firing induced by urine in rat VNO (Inamura *et al.*, 1997a). Also immunohistochemical studies by Brann *et al.* (2002) show the presence of IP_3 receptors throughout the sensory neuroepithelium of the rat VNO. All these studies support the notion that IP_3 plays a role in pheromonal signaling.

In summary, we conclude that the production of IP_3 in the VNO by urine, 2 heptanone, and 2,5-dimethylpyrazine is mediated by the alpha subunit of $G_{q/11}$.

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