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ODORANT- AND GUANINE NUCLEOTIDE-STIMULATED PHOSPHOINOSITIDE TURNOVER IN OLFACTORY CILIA

Taufiqul Huque and Richard C. Bruch*

Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104

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SUMMARY: Isolated olfactory cilia from the channel catfish (Ictalurus punctatus) exhibited phosphatidylinositol-4,5-bisphosphate phosphodiesterase (E.C.3.1.4.11) activity. The phosphodiesterase activity was stimulated in the presence of an odorant for the catfish, namely the amino acid L-alanine. The enzyme activity was also stimulated in the presence of GTP and its nonhydrolyzable analogues. The activation of the phosphodiesterase by guanine nucleotides, in combination with the identification of guanine nucleotide-binding protein(s) in the isolated cilia, indicate the probable participation of a guanine nucleotide-binding protein in stimulation of phosphoinositide turnover in the olfactory receptor neuron.

The biochemical mechanisms underlying olfaction are poorly understood (1,2). Recent biochemical evidence indicates that the, as yet unidentified olfactory receptors and associated transduction elements are localized in the cilia on the dendritic termini of the sensory cells (3-6). However, the sequence of molecular events between the initial interaction of odorants with olfactory receptors and generation of an action potential remains speculative. The possible participation of adenylate cyclase in olfactory transduction has been suggested previously, since olfactory tissue contains high basal levels of adenylate cyclase activity (7,8). Pace et al. (5) have shown that the enzyme is stimulated in the presence of odorants. Furthermore, the regulatory components of adenylate cyclase, the stimulatory and inhibitory guanine nucleotide-binding proteins, G_s and G_i, have also been identified in olfactory cilia (5).

Since many cells respond to external stimuli by undergoing increased phosphoinositide turnover (9), we have investigated the possibility that olfactory neurons may also respond by this pathway following odorant-receptor interaction. Isolated

* To whom correspondence should be addressed.

Abbreviation used: PIP_2-PDE, phosphatidylinositol-4,5-bisphosphate phosphodiesterase (E.C.3.1.4.11).

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olfactory cilia preparations from the catfish exhibit PIP_2-PDE activity. This enzyme catalyzes the rapid hydrolysis of phosphatidylinositol-4,5-bisphosphate to form the potential second messengers diacylglycerol and inositol-1,4,5-trisphosphate in response to agonist occupation of appropriate receptors (9). We report here that phosphoinositide turnover in the isolated cilia is stimulated in the presence of an odorant and guanine nucleotides. These results indicate that olfactory receptor occupancy stimulates phosphoinositide turnover by a mechanism that probably depends on the participation of a guanine nucleotide-binding protein.

MATERIALS AND METHODS

Isolation of Olfactory Cilia - Cilia were detached from the olfactory epithelium by a modified method using calcium shock treatment in the absence of ethanol (4,10,11). The isolation and biochemical characterization of the isolated cilia preparations will be presented in detail elsewhere (12). The average yield of cilia was 155 ug protein/g tissue (standard deviation = 9.3 ug/g for 5 preparations). Protein was determined by the Bradford method (13).

PIP_2-PDE Activity Measurements - Following incubation of olfactory tissue fractions with 0.25 mM phosphatidylinositol-4,5-bisphosphate (Sigma) in the presence of 1 mM $CaCl_2$, the formation of inositol-1,4,5-trisphosphate (as organic phosphorus) was used as the criterion of enzyme activity as described by Tou et al. (14) and Low and Weglicki (15). Phosphate was determined by the method of Chen et al. (16). Duplicate assays routinely varied by less than 5%.

Odorant Binding Measurements - Olfactory cilia were assayed for [2,3-^{3}H]-L-alanine (ICN Radiochemicals) binding activity as previously described (6,10). Total binding was determined in duplicate samples with 1 uM radiolabel. Nonspecific binding was determined in parallel samples containing 20 mM unlabeled alanine. Specific binding, calculated by subtraction of nonspecific from total binding, was linearly dependent on the amount of ciliary protein up to at least 50 ug.

Polyacrylamide Gel Electrophoresis and Western Blotting - Cilia were solubilized in sample buffer containing 125 mM Tris·HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.01% bromphenol blue and were boiled 5 min. The samples were applied on 10% separation gels with 4% stacking gels (17). Electrophoresis was performed at 30 ma in a water-cooled apparatus. The resolved proteins were transferred to nitrocellulose (18) at 1 amp for 45 min. The nitrocellulose membrane was incubated at room temperature for 1 hr with 10 mM Tris·HCl, pH 7.4 containing 0.1 M NaCl, 1% bovine serum albumin, and 0.1% Tween 20, followed by overnight incubation in the same buffer containing rabbit antiserum (1:100 dilution) to the common β-subunit of the guanine nucleotide-binding proteins (19). Antiserum to the guanine nucleotide-binding protein β-subunit and partially purified β-subunit (purity about 60%) were generous gifts from D. Manning (Univ. of PA). Following washing of the membrane with 10 mM Tris·HCl, pH 7.4 containing 0.1 M NaCl, the membrane was incubated for 2 hrs with the same buffer containing 1% bovine serum albumin, 0.1% Tween 20, and peroxidase-conjugated goat anti-rabbit IgG (Sigma, 1:500 dilution). Labeled protein components were visualized using peroxidase substrate solution (Kirkegaard and Perry Laboratories, Cat. No. 50-62-00). Polyacrylamide gels were stained with silver (20) to assess the completeness of protein transfer to nitrocellulose.

RESULTS AND DISCUSSION

PIP_2-PDE Activity in Olfactory Cilia - The results of PIP_2-PDE activity measurements in isolated olfactory preparations from the catfish are shown in Table I.

Table I. PIP_2-PDE Activity of Catfish Olfactory Tissue[a]

Fraction	Specific Activity[b]	% of Total Activity
Whole epithelium	29.8	100
Deciliated epithelium	6.9	75
Cilia		
Freshly isolated	21.3	5.5
-15°C, 2 weeks	18.5	

[a] Values are averages of duplicate determinations from a representative experiment using olfactory tissue from 6 fish.

[b] nmoles organic P/min/mg protein. Protein was determined by the Lowry method (21).

The phosphodiesterase activity of the catfish olfactory epithelium before deciliation was similar to that of rat (15 nmoles organic P/min/mg/protein). In the rat, about 92% of the total enzyme activity was recovered in a 100,000 x g supernatant fraction, in agreement with the observation that the phosphodiesterase is a cytosolic enzyme (22). As indicated in Table I, the catfish olfactory cilia retained comparable activity to that of the whole epithelium, whereas the activity decreased significantly in the deciliated tissue. Furthermore, the enzyme appears to be stable, since about 90% of the initial activity was retained after storage of the cilia at -15° C for 2 wks. The activity measurements in Table I were obtained with cilia prior to sucrose gradient centrifugation (10,12). In these preparations, the phosphodiesterase specific activity was increased 3-fold over that of the deciliated epithelium. Following sucrose gradient centrifugation (12), the resulting cilia preparation contained virtually all of the original activity placed on the gradient. Thus, the phosphodiesterase specific activity of the purified cilia was further increased to an average of 4.4-fold (± 0.4 for 3 preparations) over that of the deciliated epithelium. In combination, these observations indicate that olfactory tissue contains the key enzyme, PIP_2-PDE, required to initiate phosphoinositide turnover.

Odorant-Stimulated Activation of Olfactory PIP_2-PDE - The demonstration of PIP_2-PDE activity in the isolated cilia preparations suggested the working hypothesis that olfactory transduction is mediated by odorant-receptor interaction which stimulates phosphoinositide lipid metabolism. Evidence supporting this hypothesis was obtained by PIP_2-PDE activity measurements in isolated cilia in the presence of the odorant L-alanine (23,24). Aliquots of the cilia preparations were preincubated on ice for 1 hr in

the absence and presence of various concentrations of the odorant. These conditions are identical to those used for measurement of equilibrium binding of odorants to olfactory membranes (6,10,25). The odorant binding activity of the isolated cilia using 1 uM radiolabeled L-alanine was about 50 pmoles [^{3}H]-L-alanine bound/mg protein. Odorant binding studies in both membrane fractions derived from olfactory homogenates (25,26) and in isolated cilia (6,10) have shown that the interaction of odorant amino acids with the binding sites is rapid, reversible and saturable. These characteristics of odorant interaction with the binding sites are consistent with generally accepted criteria for the interaction of a ligand with a physiologically relevant membrane receptor protein (27).

Representative results of olfactory PIP_2-PDE activity measurements following preincubation with L-alanine are shown in Table II. These results indicate that the enzyme was stimulated in the presence of the odorant. However, enzyme stimulation was maximal at 5 uM L-alanine and decreased at higher odorant concentrations. The decreased response at high odorant concentration may have resulted from receptor desensitization or activation of an inhibitory receptor site. Alternatively, since the rate of PIP_2-PDE catalyzed hydrolysis is rapid (9,22), the decreased activity may result from substrate depletion. Although these results are not sufficient to support any one of these interpretations, the concentration-dependent effect of the odorant on olfactory PIP_2-PDE activity in the isolated cilia preparations was reproducible.

Guanine Nucleotide-Binding Protein Participation in Activation of Olfactory PIP_2-PDE - Recent evidence indicates that a guanine nucleotide-binding protein is involved in receptor-mediated catabolism of phosphoinositide lipids (28-30). Two fundamental criteria should be satisfied if phosphoinositide turnover is dependent on a guanine nucleotide-binding protein. These criteria are: 1) a guanine nucleotide-binding

Table II. Odorant-Stimulated Activation of Olfactory PIP_2-PDE[a]

L-Alanine Added (μM)	% Stimulation
0	0
5	57
10	25
20	0
100	2

[a] Values are averages of duplicate determinations from a representative experiment using olfactory cilia from 6 fish.

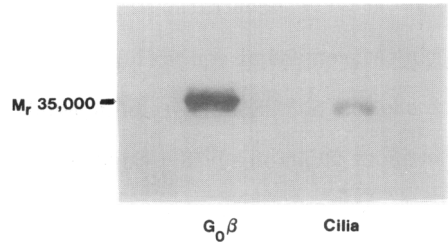

Figure 1. Identification of Guanine Nucleotide-Binding Protein β-Subunit in Olfactory Cilia. About 0.3 μg of purified $G_o\beta$-subunit ($G_o\beta$) and 10 μg of ciliary protein were applied on a 10% polyacrylamide gel. After electrophoresis and protein transfer, the nitrocellulose membrane shown was processed as described in Materials and Methods. Only the M_r 35,000 region of the blot is shown since no additional components were observed.

protein must be located in the receptor membrane, and 2) the turnover of phosphoinositide lipids should be stimulated by guanine nucleotides. Both criteria were satisfied in the isolated cilia preparations. The presence of guanine nucleotide-binding protein(s) in these preparations was demonstrated by Western blotting using polyclonal antiserum to the common β-subunit (19). As shown in Figure 1, a polypeptide of M_r 35,000 which comigrates with the purified β-subunit of G_o (the guanine nucleotide-binding protein isolated from the brain (31)) was specifically labeled by the antibody in the cilia. Although these results demonstrate the presence of a guanine nucleotide-binding protein(s) in the cilia (5), additional data are required to identify the individual guanine nucleotide-binding proteins in the ciliary membranes. Activation of PIP_2-PDE by guanine nucleotides (criterion 2) was demonstrated by enzyme activity measurements in the absence and presence of GTP. The results (Table III) indicate the enzyme was stimulated at low concentrations of GTP (<1 uM). As observed in the presence of the odorant (Table II), the activity decreased at higher nucleotide concentrations. In

Table III. Nucleotide-Stimulated Activation of Olfactory PIP_2-PDE[a]

GTP Added (μM)	% Stimulation
0	0
0.1	39
1	28
10	5
100	0

[a] Olfactory cilia were incubated in the absence and presence of the indicated amounts of GTP at 37°C for 5 min prior to assay of the enzyme activity.

addition, the maximal stimulation elicited by the odorant (57% increase) and the nucleotide (39% increase) was similar. Activation of olfactory PIP_2-PDE was also observed in the presence of the nonhydrolyzable GTP analogues guanosine 5'-(ß, γ-imino) triphosphate and guanosine 5'-(3-0-thio) triphosphate. At 0.1 uM, these analogues were equally effective in their ability to activate PIP_2-PDE, but were 78% as effective as GTP.

In combination, the results reported here indicate that olfactory cilia contain PIP_2-PDE activity which is sensitive to odorants and guanine nucleotides. The molecular mechanism by which phosphoinositide turnover is activated is not known (9), although guanine nucleotide-binding protein involvement has been indicated (28-30). The results of this study, previously unreported in the olfactory system, indicate that olfactory receptor occupancy also stimulates phosphoinositide turnover by a mechanism that depends on a guanine nucleotide-binding protein.

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