Functional Reconstitution of the Olfactory Membrane: Incorporation of the Olfactory Adenylate Cyclase in Liposomes†

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ABSTRACT: Chemosensory cilia isolated from the olfactory epithelium of *Rana catesbeiana* were solubilized with Lubrol PX in the presence of supplementary lipid, forskolin, and sodium fluoride. Subsequent removal of the detergent by adsorption onto Biobeads SM2 results in the formation of proteoliposomes that display forskolin- and GTPγS-sensitive adenylate cyclase activity. Sucrose gradient centrifugation of liposomes formed in the presence of fluorescently labeled phosphatidylcholine demonstrates association between the olfactory adenylate cyclase and the exogenously added lipid. Forskolin stimulates the enzyme in reconstituted membranes with the same potency as in native membranes (EC_{50} = 1–2 μM). However, GTPγS is 350-fold more potent in native membranes (EC_{50} = 4.0 ± 0.5 nM) than in reconstituted membranes (EC_{50} = 1.4 ± 0.3 μM). These studies represent a first step toward the functional reconstitution and molecular dissection of the olfactory membrane.

Chemosensory cilia on the dendritic tips of olfactory receptor cells mediate odorant recognition and subsequent signal transduction events that lead to excitation of the olfactory receptor cell (Getchell et al., 1984; Getchell, 1986; Lancet, 1986; Anholt, 1987). These organelles can be detached from the olfactory epithelium and isolated as a partially purified preparation of chemosensory membranes (Chen & Lancet, 1984; Anholt et al., 1986a; Chen et al., 1986). Biochemical characterization of such preparations has shown that they are enriched in adenylate cyclase activity and in addition contain a stimulatory GTP binding protein, Gs[1] (Pace et al., 1985; Sklar et al., 1986; Anholt et al., 1987; Jones & Reed, 1987). This adenylate cyclase is activated by forskolin, nonhydrolyzable GTP analogues, such as GTPγS, and micromolar concentrations of some, but not all, odorants (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986). Presently, the role of this enzyme in mediating and regulating the response to odorants is not clear. Although a role for cAMP as a second messenger in olfactory transduction has been proposed (Lancet, 1986; Gold & Nakamura, 1987), its precise function in the olfactory process has not yet been defined. Furthermore, it remains to be determined whether stimulation of the olfactory adenylate cyclase by odorants occurs via as yet unidentified odorant receptor proteins or as a result of the interaction between odorants and the lipid membrane (Nomura & Kurihara, 1987a,b).

Patch-clamp studies using excised patches of the ciliary membrane have demonstrated direct activation of ion channels by cyclic nucleotides (Nakamura & Gold, 1987; Gold & Nakamura, 1987). However, activation of these channels by odorants was not reported in these studies. Olfactory cilia also contain channels that are activated directly by nanomolar concentrations of odorants (Labarca et al., 1987). In addition, they contain a Na/K-dependent ATPase (Anholt et al., 1986a) and a Ca-dependent ATPase (Farmer, Karavanich, and Anholt, unpublished observations).

It is not clear how these different transduction systems mediate and control the response of the chemosensory membrane to odorants. Functional reconstitution studies may elucidate the link between odorant binding and subsequent enzyme and/or channel activation. This study describes the functional reconstitution of the olfactory adenylate cyclase and its associated GTP binding protein as a first step toward determining whether odorants activate this enzyme through a specific odorant receptor protein or by interacting with the lipid membrane.

Materials and Methods

Materials. Bullfrogs (*Rana catesbeiana*) were obtained from Acadian Biological (Rayne, LA). Lubrol PX, cholic acid, and saponin were from Sigma Chemical Co. (St. Louis, MO). Cholic acid was recrystallized as the sodium salt according to the method of Kagawa and Racker (1971). Crude soybean lipid (L-α-phosphatidylcholine type IIS; Sigma) was used without further purification and prepared daily as a 100 mg/mL stock suspension sonicated to optical clarity in a Bransonic bath sonicator under nitrogen. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Triton X-100 (membrane grade), ATP, cAMP, creatine phosphate, creatine kinase, GTP, and GTPγS were purchased from Boehringer Mannheim. Octyl β-D-glucopyranoside and forskolin were obtained from Calbiochem (La Jolla, CA). Fluorescent 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine was purchased from Avanti Polar Lipids (Birmingham, AL). Odorants were generously donated by International Flavors

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gs, guanine nucleotide binding protein that mediates stimulation of adenylate cyclase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; NBD-PC, 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine; Ringer's solution, 2 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, 112 mM NaCl, 3.4 mM KCl, and 2.4 mM $NaHCO_3$, pH 7.4; EGTA–Ringer's solution, Ringer's solution supplemented with 2 mM EGTA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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and Fragrances, Inc. (Union Beach, NJ). All other chemicals were reagent grade. All reagents were prepared in double glass distilled water.

Preparation of Olfactory Cilia. Frogs were killed by decapitation, and olfactory cilia were detached and isolated from the olfactory epithelium as described previously (Anholt et al., 1986a). Protein was assayed according to the method of Lowry et al. (1951). Adenylate cyclase activity was measured according to the method of Salomon (1979) as described previously (Sklar et al., 1986), except that dithiothreitol was deleted from the assay mixture. Olfactory cilia displayed a (155 ± 36)-fold (n = 3) increase in forskolin-stimulated adenylate cyclase activity as compared to membranes prepared from the rest of the olfactory epithelium. Cilia were resuspended in frog Ringer's solution and stored at −80 °C.

Solubilization and Reassembly of the Olfactory Adenylate Cyclase. Olfactory cilia were solubilized on ice at a protein concentration of 0.75–1.2 mg/mL in 1% Lubrol PX, supplemented with 2 mg/mL soybean lipid, 10 mM NaF, and 10 μM forskolin in Ringer's solution, unless indicated otherwise. After incubation for 15 min on ice, the solubilization mixture was centrifuged for 30 min in a microcentrifuge at 16600g at 4 °C. The supernatant was collected, and the lipid concentration in the supernatant was adjusted to 25 mg/mL, after which the detergent was removed by adsorption onto Biobeads SM2. The mixture was added to Biobeads SM2 (Bio-Rad) at a volume:weight ratio of 2:1 and shaken end-over-end at 4 °C for 6–8 h. An equal volume of Ringer's solution was then added to the preparation. The beads were allowed to sediment, and the turbid supernatant containing reconstituted membranes was collected. The proteoliposomes were dialyzed for 16–18 h at 4 °C against 500 volumes of Ringer's solution with one buffer change to remove the added NaF and forskolin. The reconstituted membranes could be stored at −80 °C without loss of activity.

Sucrose Gradient Centrifugation. Sucrose gradients consisted of 4.4 mL of a linear 5–20% (w/w) gradient on top of 0.5 mL of a 45% (w/w) sucrose cushion in EGTA–Ringer's solution. Reconstituted membranes were prepared from soybean lipid containing 1% (w/w) fluorescent NBD-PC, and 100 μL of sample was applied to each gradient. The gradients were centrifuged for 6 h at 4 °C in a Beckman SW50.1 rotor at 40000 rpm. Fractions of 200 μL were collected after the tube was punctured. The fractions were assayed for adenylate cyclase activity, and fluorescence was measured as luminescence in arbitrary units using a Farrand Optical A-4 System fluorometer (primary filter, 7-54; secondary filter, 3-70). Sucrose concentrations in the fractions were measured with a refractometer.

Results

Initial reconstitution studies investigated the effect of several detergents on the recovery of adenylate cyclase activity. These included cholate, CHAPS, octyl β-D-glucopyranoside, Triton X-100, and Lubrol PX. Treatment of olfactory cilia with 0.6% cholate or CHAPS followed by removal of the detergent via dialysis in the presence of supplementary soybean lipid allowed subsequent recovery of 35 ± 12% (n = 3) and 50 ± 15% (n = 3) of the initial adenylate cyclase activity, respectively. Sucrose gradient centrifugation revealed, however, that the enzyme failed to associate with exogenous lipid although it was present in membranes banding at 20.5 ± 0.5% (n = 5) sucrose, significantly lighter than native membranes that band at 37 ± 3% (n = 6) sucrose (data not shown). These observations suggested that at these low concentrations of detergent peripheral membrane protein is stripped off and that membrane fragments may be formed that reseal without detectable incorporation of exogenous lipid. Higher concentrations of cholate or CHAPS, even in the presence of supplementary lipid, result in loss of enzyme activity although protein incorporates into liposomes as evident from the appearance of a fluorescent shoulder on the leading edge of the liposome peak of the gradient.

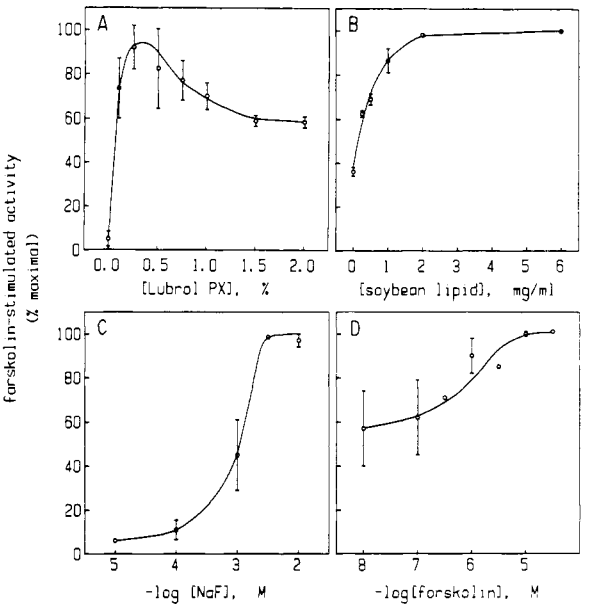

FIGURE 1: Solubilization and reconstitution of the olfactory adenylate cyclase. (A) Solubilization of the olfactory adenylate cyclase by Lubrol PX. Olfactory cilia were treated with the indicated concentrations of Lubrol PX at a constant detergent:soybean lipid ratio of 5:1 (w/w) in the presence of 10 mM NaF and 10 μM forskolin. Forskolin-stimulated adenylate cyclase activity was measured after reassembly as described under Materials and Methods. The data were compiled from three separate experiments, each consisting of triplicate measurements. In each experiment, data were standardized as a percentage of the highest activity (picomoles per minute) recovered, usually at 0.25% or 0.5% Lubrol PX. At these concentrations, adenylate cyclase activity recovered was 30 ± 19% higher in the detergent-treated samples than in the original native membranes. This enhancement of enzyme activity at low concentrations of Lubrol PX may be similar to the effects observed after treatment of native ciliary membranes with saponin, described in Table I and discussed in the text. (B) Protection of the olfactory adenylate cyclase by supplementary soybean lipids during solubilization with Lubrol PX. Olfactory cilia were solubilized with 1% Lubrol PX at the indicated concentrations of supplementary soybean lipid in the presence of 10 mM NaF and 10 μM forskolin and reconstituted as described under Materials and Methods. Data were compiled from two experiments each consisting of triplicate measurements. (C) Protection of the olfactory adenylate cyclase by NaF during solubilization with Lubrol PX. Olfactory cilia were solubilized with 1% Lubrol PX in the presence of 2 mg/mL supplementary soybean lipid, 10 μM forskolin, and the indicated concentrations of NaF followed by reassembly as described under Materials and Methods. Data were compiled from two experiments each consisting of triplicate measurements. (D) Protection of the olfactory adenylate cyclase by forskolin during solubilization with Lubrol PX. Olfactory cilia were solubilized with 1% Lubrol PX in the presence of 2 mg/mL supplementary soybean lipid, 10 mM NaF, and the indicated concentrations of forskolin followed by reassembly as described under Materials and Methods. Data were compiled from three experiments each consisting of triplicate measurements. The maximum level of forskolin-stimulated adenylate cyclase activity in these experiments was 7.2 ± 2.2 pmol/min (n = 7). Error bars are deleted where they are smaller than the symbol.

Subsequent pilot studies that evaluated the effect of octyl β-D-glucopyranoside, Triton X-100, and Lubrol PX established Lubrol PX as the best detergent for the solubilization and functional reassembly of the olfactory adenylate cyclase. A concentration of 1% Lubrol PX was chosen for further experiments (Figure 1A). Lower concentrations of Lubrol PX

are similar to cholate and CHAPS in that they do not enable the incorporation of adenylate cyclase activity in liposomes but instead give rise to the formation of lighter resealed ciliary membranes. Higher concentrations of Lubrol PX (up to 2%) result in a ~10% lower recovery of enzyme activity.

To stabilize the activity of the adenylate cyclase during solubilization of the membranes with Lubrol PX, it is necessary to provide supplementary soybean lipid, sodium fluoride, and forskolin (Figure 1B–D). In the absence of supplementary lipid, approximately 35% of the maximally recoverable adenylate cyclase activity is retained, most likely due to protection by endogenous membrane components (Figure 1B). Maximal protection by supplementary lipid is obtained at a detergent:lipid ratio of 5 (2 mg/mL lipid in 1% Lubrol PX). In addition to supplementary lipid, inclusion of sodium fluoride in the solubilization mixture is essential for recovery of enzyme activity. Only 6% of the maximally extractable adenylate cyclase activity is retained when solubilization is performed in the absence of NaF, and maximal protection occurs at 10 mM NaF (Figure 1C). The additional inclusion of forskolin in the solubilization mixture doubles the subsequent recovery of adenylate cyclase activity (Figure 1D).

Association of adenylate cyclase activity with exogenously added soybean lipid was analyzed by performing the reconstitution procedure in the presence of fluorescent NBD-PC followed by sucrose density gradient centrifugation. Native ciliary membranes band at 37 ± 3% sucrose (n = 6), whereas liposomes formed in the absence of protein band at 11.7 ± 0.4% sucrose (n = 6; Figure 2A). Treatment of the ciliary membranes with 0.25% Lubrol PX results, after removal of the detergent, in the formation of several populations of lighter membranes that band at ~23% and ~15% sucrose (Figure 2B). Solubilization of the membranes with 1% Lubrol PX reveals, after removal of the detergent, a peak of adenylate cyclase activity at ~14.5% sucrose comigrant with a distinct shoulder on the leading edge of the fluorescent lipid peak. Integration of the area under this adenylate cyclase associated lipid peak, assuming symmetry of the fluorescent shoulder, indicates that ~22% of the total lipid comigrates with ~91% of the adenylate cyclase activity (Figure 2C). The lipid:protein ratio (w/w) in this population of membranes is approximately 8-fold higher than in native ciliary membranes, where it is estimated to be near unity. Reconstitution of the enzyme after solubilization of the chemosensory membranes in 2% Lubrol PX reveals a shoulder on the leading edge of the fluorescent peak, that now represents ~45% of the total lipid and coincides with a symmetrical peak that contains ~97% of the adenylate cyclase activity at a density of ~13.6% (Figure 2D). The lipid:protein (w/w) ratio in this population of membranes is about 12-fold higher than in the native membrane.

The functional properties of the adenylate cyclase per se remain intact during the solubilization/reassembly process as evident from the dose–response behavior of forskolin-stimulated enzyme activity. Forskolin stimulates the enzyme in reconstituted membranes with the same potency as in native membranes with an EC_{50} of 1–2 μM (Figure 3A). In contrast, stimulation of adenylate cyclase activity via Gs appears altered in the reconstituted system. GTPγS activates the reconstituted enzyme with an EC_{50} of 1.4 ± 0.3 μM, ~350-fold higher than the concentration required for half-maximal stimulation in native membranes (4.0 ± 0.5 nM; Figure 3B).

The reconstituted adenylate cyclase is not stimulated by odorants such as citralva (3,7-dimethyl-2,6-octadienenitrile), menthone, and 3-isobutyl-2-methoxypyrazine up to concentrations of 0.1 mM even in the presence of 100 μM GTP. This

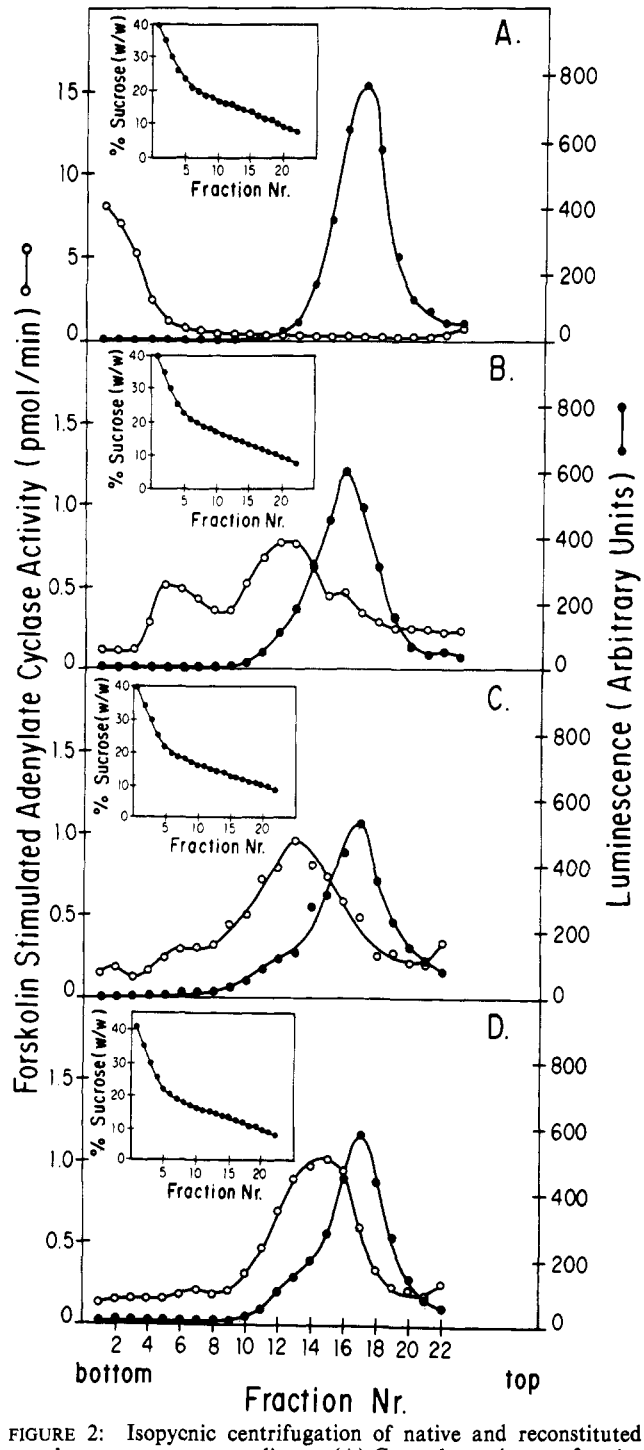

FIGURE 2: Isopycnic centrifugation of native and reconstituted membranes on sucrose gradients. (A) Control: a mixture of native membranes (adenylate cyclase trace) and liposomes made in the absence of ciliary protein (fluorescent NBD-PC trace). (B) Reconstituted membranes formed after solubilization of olfactory cilia with 0.25% Lubrol PX. (C) Reconstituted membranes formed after solubilization of olfactory cilia with 1% Lubrol PX. (D) Reconstituted membranes formed after solubilization of olfactory cilia with 2% Lubrol PX. In each case, solubilization was performed at a detergent:soybean lipid ratio of 5:1 (w/w) in the presence of 10 mM NaF and 10 μM forskolin at a protein concentration of 1.2 mg/mL. The insets to each panel show the sucrose concentrations in each fraction.

may be due to inactivation of putative odorant receptor proteins or to altered interactions between odorants and the lipid bilayer. Some support for the latter possibility comes from the observation that treatment of native membranes with saponin at concentrations at which the detergent inserts itself into the membrane without solubilizing it (Froehner et al., 1983)

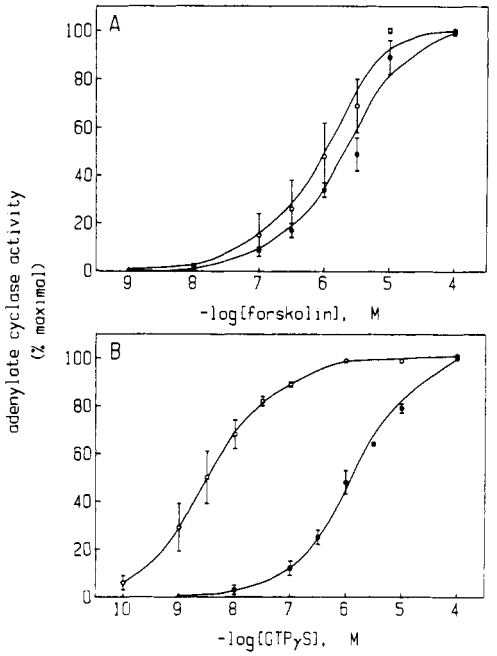

FIGURE 3: Stimulation of the olfactory adenylate cyclase by forskolin and GTPγS in native and reconstituted membranes. (A) Stimulation by forskolin in native (open symbols) and reconstituted (closed symbols) membranes. (B) Stimulation by GTPγS in native (open symbols) and reconstituted (closed symbols) membranes. The curves were obtained by subtracting the basal rate in the absence of forskolin or GTPγS from each data point and standardizing each point with respect to the plateau level at which stimulation was maximal. Data were compiled from three experiments each consisting of triplicate measurements. Error bars are deleted where they are smaller than the symbol. The EC_{50} values for stimulation by forskolin are 1.0 ± 0.4 μM and 2.1 ± 0.4 μM for native and reconstituted membranes, respectively. The EC_{50} values for stimulation by GTPγS are 4.0 ± 0.5 nM and 1.4 ± 0.3 μM for native and reconstituted membranes, respectively. Maximal stimulated enzyme activities by forskolin and GTPγS were similar in native membranes and are 9 ± 2 times higher than the basal rate. Forskolin-stimulated activity at plateau levels was (7 ± 1)-fold higher than the basal activity in reconstituted membranes. In reconstituted membranes, the maximal activity obtained by stimulation with 0.1 mM GTPγS was 44 ± 5% (n = 3) of the maximal activity obtained by stimulation with 0.1 mM forskolin in the same membranes. Dose–response curves for forskolin and GTPγS obtained after solubilization of ciliary membranes in 2% Lubrol PX were identical with those obtained after solubilization of the membranes in 1% Lubrol PX.

doubles forskolin- and GTPγS-stimulated enzyme activity, while eliminating sensitivity of the enzyme to odorants (Table I). Enhancement of forskolin- or GTPγS-stimulated adenylate cyclase activity is also observed after treatment of reconstituted membranes with saponin albeit to a slightly lesser extent (~1.7-fold, data not shown). Stimulation by odorants of the olfactory adenylate cyclase is still evident in native membranes when assayed in the presence of sonicated soybean lipid in amounts equivalent to those employed during reconstitution.

Discussion

This paper describes the incorporation of the olfactory adenylate cyclase in liposomes after solubilization of the ciliary membrane with Lubrol PX in the presence of supplementary lipid, NaF, and forskolin. Soybean lipid provides effective protection for the enzyme at a concentration of 2 mg/mL in 1% Lubrol PX. These conditions quantitatively resemble the conditions required to stabilize other transmembrane proteins during solubilization, such as the nicotinic acetylcholine receptor channel (Anholt et al., 1981) and the mitochondrial benzodiazepine receptor (Anholt et al., 1986b). In the case

Table I: Effect of Saponin Treatment on Adenylate Cyclase Activity in Olfactory Cilia

stimulant	act. [nmol min^{-1} (mg of protein)$^{-1}$] ± SEM: control membranes (n = 6)	saponin-treated membranes[a] (n = 4)
basal rate[b]	1.42 ± 0.24	0.81 ± 0.14
GTPγS (10 μM)	7.02 ± 1.66	17.78 ± 2.63[c]
forskolin (10 μM)	9.93 ± 1.28	17.13 ± 2.25[d]
odorants (0.1 mM)[b]		
citralva	2.63 ± 0.42[e]	1.15 ± 0.20
3-isobutyl-2-methoxypyrazine	2.20 ± 0.31[f]	1.00 ± 0.17
menthone	2.20 ± 0.32[f]	1.03 ± 0.18

[a] Membranes were preincubated for 10 min with 0.2% saponin. [b] The assays were performed in the presence of 10 μM GTP. [c] $p < 0.005$ versus control membranes. [d] $p < 0.025$ versus control membranes. [e] $p < 0.05$ versus basal rate. [f] $p < 0.10$ versus basal rate.

of the nicotinic acetylcholine receptor, it has been proposed that the protecting lipid preserves an annulus of immobilized lipid around the transmembrane region of the protein, allowing it to be carried as a lipoprotein complex in the detergent solution (Anholt et al., 1981).

The dependence on NaF and forskolin for preservation of enzyme activity during solubilization indicates that the active conformation of the adenylate cyclase is more stable during solubilization and reassembly than its resting conformation. It is likely that Gs and the catalytic moiety of the adenylate cyclase remain associated as a complex during solubilization under these conditions. Previous studies have shown that Gs may remain associated with the adenylate cyclase from rabbit myocardial membranes during solubilization of the guanosine 5′-(β,γ-imidotriphosphate) activated enzyme in Lubrol PX and its subsequent purification by affinity chromatography on forskolin–Sepharose (Pfeuffer et al., 1985).

Analysis of the reconstituted membranes by isopycnic sucrose gradient centrifugation indicates that treatment with increasing concentrations of Lubrol PX results in the formation of progressively lighter membranes. Native ciliary membranes band at ~37% sucrose, slightly higher than the isopycnic density of acetylcholine receptor rich membranes from torpedo electric organ (Elliott et al., 1980). This high isopycnic density reflects a high protein:lipid ratio (near unity) in line with the high density of intramembranous particles observed in freeze–fracture electron photomicrographs (Menco, 1984). The behavior of reconstituted membranes on sucrose density gradients suggests that low concentrations of Lubrol PX (0.25%) lead to the removal of peripheral membrane protein and/or the formation of membrane fragments that reseal. Higher concentrations of the detergent lead to solubilization of the olfactory membrane and allow reassembly of the solubilized material with exogenously added soybean lipid.

Adenylate cyclase activity per se in the reconstituted membranes remains intact as judged from the dose dependence of stimulation by forskolin. The lowered potency with which GTPγS activates the reconstituted enzyme reflects a lowered binding affinity of Gs for GTPγS and/or a less effective coupling between this regulatory G-protein and the adenylate cyclase due to the reduced protein:lipid ratio in the reconstituted membranes which could lower the probability of interaction between the G-protein and the adenylate cyclase by lateral diffusion.

The β-adrenergic receptor appears to associate transiently with its Gs–adenylate cyclase complex and to dissociate in the presence of GTPγS (Stadel et al., 1981). By analogy, putative odorant receptor proteins may also associate only transiently

with the olfactory Gs–adenylate cyclase system, and this interaction may be disrupted in the reconstituted system. Thus, it is perhaps not surprising that odorant-mediated stimulation of the enzyme via Gs, which is only 30–65% above the basal level in native membranes (Sklar et al., 1986), is not at all detectable in the reconstituted system. The lack of odorant sensitivity of the reconstituted enzyme may result from denaturation of a putative odorant receptor protein and/or its uncoupling from the Gs–adenylate cyclase system perhaps as the result of residual Lubrol PX in the reconstituted membranes. Alternatively, it may result from altered interactions between odorants and the lipid membrane (Nomura & Kurihara, 1987a,b). It is here of interest to note that frog melanophores which, like olfactory cilia, contain a high concentration of Gs exhibit a rise in intracellular cAMP levels concomitant with dispersion of their pigment in response to the same types and concentrations of odorants that stimulate the adenylate cyclase in olfactory cilia (Lerner et al., 1988). The observation that insertion of saponin into the native membrane can enhance stimulation by GTPγS and forskolin of the enzyme while at the same time abolishing sensitivity to odorants indicates that perturbations of the bilayer can modulate adenylate cyclase activity. Enhancement of adenylate cyclase activation by saponin could be explained by facilitated access of substrates to the enzyme. This is, however, unlikely since the membranes are in the assay already exposed to hypoosmotic conditions and since stimulation by GTPγS, a polar compound, is not significantly different from that by forskolin, an apolar readily permeant agent (Table I).

The studies described here represent a first step toward the molecular dissection of the olfactory membrane. An adenylate cyclase linked system reconstituted from purified components, as has been described for the β-adrenergic receptor (May et al., 1985; Lefkowitz et al., 1985), will eventually help clarify whether odorants modulate the olfactory adenylate cyclase via defined odorant receptor proteins or via membrane-mediated effects on Gs.

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Registry No. GTPγS, 37589-80-3; Lubrol PX, 9002-92-0; NaF, 7681-49-4; adenylate cyclase, 9012-42-4; forskolin, 66575-29-9.

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