Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4947–4951, July 1986
Neurobiology

Olfactory GTP-binding protein: Signal-transducing polypeptide of vertebrate chemosensory neurons

(sensory cilia/adenylate cyclase/odorant receptors/hormone receptors/photoreceptors)

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Communicated by Carl Pfaffmann, February 4, 1986

ABSTRACT **The sense of smell involves the stimulation of sensory neurons by odorants to produce depolarization and action potentials. We show that olfactory responses may be mediated by a GTP-binding protein (G protein), a homolog of the visual, hormonal, and brain signal transducing polypeptides. The olfactory G protein is identified in isolated dendritic membranes (olfactory cilia preparations) of chemosensory neurons from three vertebrate species and is shown to mediate the stimulation by odorants of the highly active adenylate cyclase in these membranes. The G protein of olfactory neurons is most similar to G_s, the hormonal stimulatory GTP-binding protein. Its α subunit has a molecular weight of about 42,000, and it undergoes ADP-ribosylation catalyzed by cholera toxin that leads to adenylate cyclase activation. The slight difference in molecular weights of the frog olfactory and the liver G_s α subunits and the higher sensitivity of olfactory adenylate cyclase to nonhydrolyzable GTP analogs are consistent with the possible existence of different G_s variants. Signal amplification due to the olfactory G protein may be responsible for the unusual acuity of the sense of smell.**

The molecular mechanisms of olfactory reception and transduction, while raising much interest, have been elusive (1–5). It has been suggested that receptor proteins, possibly constituting a family of gene products that specifically recognize various odorants (5–7), trigger a common transductory chain of events resulting in membrane depolarization and firing of action potentials (8–10). Whereas the receptor molecules have not yet been conclusively identified, a transduction membrane component with which they may interact has been described (11). This is an odorant-sensitive adenylate cyclase, which we have shown to be present in dendritic extensions (olfactory cilia) of frog chemosensory neurons. Odorant stimulation of the enzyme was GTP dependent, suggesting the involvement of a guanine nucleotide binding protein, G protein. We report here the identification of the olfactory G protein in three vertebrate species and describe in more detail the properties of olfactory G protein from frogs. It is suggested that the olfactory G protein is a member of the family of signal-transducing polypeptides, most similar to G_s, the hormonal stimulatory GTP binding protein.

MATERIALS AND METHODS

Animals. Live frogs (*Rana ridibunda*) and toads (*Bufo viridis*) were supplied by S. Hayt (Maale Efraim, Israel). Rats of the strain BN/Mai were obtained from the Weizmann Institute animal breeding center.

Reagents. Guanosine 5′-[γ-thio]-triphosphate ([S]pppG) was purchased from Boehringer Mannheim and forskolin was from Calbiochem. *n*-amyl acetate, 1,8-cineole, *l*-carvone, and citral (mixture of *cis* and *trans* isomers) were obtained from Aldrich. Cholera toxin and islet activating protein (pertussis toxin) were purchased from List Biological Laboratories (Campbell, CA). [^{32}P]NAD was bought from New England Nuclear and [^{32}P]ATP from Amersham. All the other reagents were from Sigma.

Membrane Preparations. Nasal olfactory epithelia and respiratory epithelia from palate (frog and toad) or from the nasal cavity (rat) were freshly dissected, and cilia preparations, obtained by the calcium-shock procedure as described (7, 12, 13), were kept at −70°C. Frog liver membranes were prepared according to Northup *et al.* (14). Frog brain membranes were prepared as described (11) and kept at −180°C.

ADP-Ribosylation. ADP-ribosylation for radiolabeling of G proteins was carried out according to the procedure of Schleifer *et al.* (15). Membranes or cilia (200 μg/ml) were incubated at 37°C in 20 mM Tris·HCl (pH 7.6) in the presence of 30 mM thymidine, 1 mM ATP, 0.1 mM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5 μM [^{32}P]NAD (10–20 Ci/mmol; 1 Ci = 37 GBq), 3 mM phospho*enol*pyruvate, pyruvate kinase at 5 units/ml, 0.05% Triton X-100, and cholera or pertussis toxin at 10 μg/ml. The reaction was stopped after 1 hr by adding electrophoresis sample buffer and boiling. The toxins were activated by preincubation with 20 mM dithiothreitol at 37°C for 15 min. ADP-ribosylation for G-protein activation was similarly conducted at 30°C with 1 mM NAD and no detergent for 30 min. The reaction was stopped by cooling to 0°C and centrifuging the cilia at 27,000 × *g* for 20 min. The pellet was resuspended and assayed for adenylate cyclase activity as described below.

Gel Electrophoresis and Autoradiography. Electrophoresis in $NaDodSO_4$/10% polyacrylamide slab gels was performed by the method of Laemmli (16). Silver staining of the gels was carried out according to Merril *et al.* (17), and polypeptides were quantitated by densitometry on a Beckman DU-8 spectrophotometer. For autoradiographic identification of ^{32}P-labeled polypeptides, gels were dried and exposed to Agfa Curix or Kodak X-AR x-ray film.

Gel Exclusion Chromatography. Frog olfactory cilia were ADP-ribosylated with [^{32}P]NAD and cholera toxin, and the reaction was stopped by cooling at 0°C. After incubation in 1% Triton X-100 for 15 min at 0°C the material was centrifuged at 10,000 × *g*. The supernatant was then loaded on a 150 × 9 mm Sephacryl S-300 (Pharmacia) column equilibrated with Tris·acetate (25 mM, pH 7.6), 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 100 mM NaCl, and 0.1% Triton X-100, and fractions of 350 μl were collected. The protein content of each fraction was precipitated by adding ice cold acetone,

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Abbreviations: G_s, stimulatory GTP-binding protein; G_i, inhibitory GTP-binding protein; [S]pppG, guanosine 5′-[γ-thio]-triphosphate; p[NH]ppG, guanosine 5′-[β,γ-imido]-triphosphate; G protein, guanine nucleotide binding protein.

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collected by centrifuging at 10,000 × g, and electrophoresed in NaDodSO$_4$/10% polyacrylamide gel.

Adenylate Cyclase Assay. Adenylate cyclase was assayed as described (11) by the method of Salomon *et al.* (18), in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Odorant activation was achieved by adding to the reaction mixture an equimolar mixture of the following odorants: *N*-amyl acetate, *l*-carvone, 1,8 cineol, and citral. The final total concentration of odorants was 1 mM (0.25 mM for each odorant).

RESULTS

Olfactory cilia preparations from three species (frog, toad, and rat) have been examined for adenylate cyclase activity. As reported (11), the frog preparation has a very high basal activity of the enzyme (around 20 nmol of cAMP per mg of protein per min, Fig. 1). This is at least 10 times higher than levels measured in other adenylate cyclase-rich membranes—e.g., from brain (19) or whole olfactory epithelium (11, 20). The toad and rat olfactory cilia preparations have activities similar to that of the frog (Fig. 1). In all three species, odorants alone have no significant effect on adenylate cyclase; GTP alone stimulates about 2-fold; but odorants plus GTP are relatively potent, giving rise to activities four to six times higher than basal levels (Fig. 1). It is evident that, in all vertebrate species tested, adenylate cyclase of olfactory cilia is sensitive to odorants and that stimulation by the physiological ligands involves the GTP-binding protein G protein, since GTP is essential for activation.

The GTP-binding protein of olfactory neurons was identified by subjecting the isolated dendritic membranes (olfactory cilia preparations) to [^{32}P]ADP-ribosylation catalyzed by cholera toxin. The cilia have a prominent 42-kDa polypeptide substrate for this reaction (Fig. 2). This polypeptide is of similar molecular weight to that of the α subunit of the stimulatory GTP-binding protein (G_s) in liver and other membranes that are also ADP-ribosylated by cholera toxin (refs. 21–23 and Fig. 2). Quantitative densitometry of the silver stained gels and of the autoradiograms of the ADP-ribosylated species show that olfactory cilia have 5–10 times higher amounts of [^{32}P]ADP incorporation per total protein compared to liver membranes. The molecular size of the polypeptide related to G_s in olfactory cilia is about 0.5 kDa higher than its liver counterpart (Fig. 2*A*). Toad and rat also have a polypeptide species specifically ADP-ribosylated by cholera toxin in the same molecular weight range, although the relative amounts vary from one species to another (Fig. 2*B*). The small amount of labeled G_s in rat olfactory cilia may have to do with the observation that the mammalian preparation is not as enriched in sensory membranes as is the amphibian preparation (unpublished observation). Control nonsensory cilia from respiratory epithelium have practically no G_s in all three species.

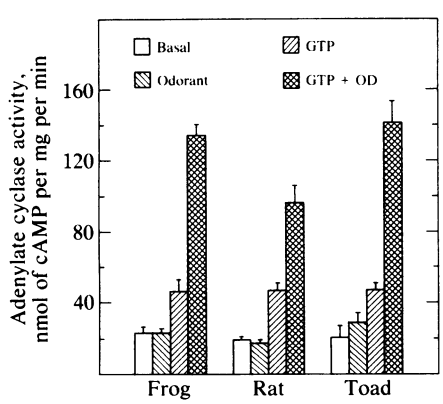

FIG. 1. Effect of odorant mixture (OD) in the presence or absence of 10 μM GTP on adenylate cyclase activity of olfactory cilia preparations from frog, rat, and toad. Assays were conducted with ciliary protein at 3–7 μg/ml.

Olfactory cilia also contain a doublet of polypeptides (at about 39 and 40 kDa) that undergoes ADP-ribosylation catalyzed by pertussis toxin (Fig. 2*B*). These are putatively identified as α subunit variants of the inhibitory GTP-binding protein (G_i), and possibly the brain GTP-binding protein, G_o (22–26). The pertussis toxin substrates are not unique to olfactory cilia and appear also in the respiratory organelles.

The olfactory G_s is more definitely identified by the NaDodSO$_4$/polyacrylamide gel electrophoresis of fractions from a Sephacryl S-300 column of the [^{32}P]ADP-ribosylated olfactory cilia proteins shown in Fig. 3. The G_s-related protein is identified by comparing the stained gel (*Top*) with its autoradiographic representation (*Bottom*). It is seen to correspond to a polypeptide band in Fig. 2 that constitutes about 1% of the silver stained proteins of the olfactory cilia preparation, as determined by quantitative densitometry. Olfactory G_s elutes at fractions 32–37, yielding a Stokes radius of 4.4 ± 0.4 nm, calculated from a calibration curve as described (27), using serum albumin (3.5 nm), catalase (5.2 nm), and ferritin (7.8 nm) as standards. This radius corresponds to an apparent molecular weight (for a globular protein) of nearly 100,000, close to the expected molecular weight (80,000) for the heterotrimer of G proteins (23, 28).

The slight difference in electrophoretic mobility between classically defined G_s α subunit in liver membranes and the olfactory counterpart prompted us to examine possible functional differences between the olfactory GTP-binding protein and its homologs. As shown in Fig. 4*A*, adenylate cyclase activity of olfactory cilia is enhanced 8–10 times by the nonhydrolyzable GTP analogs [S]pppG and guanosine 5'-[β,γ-imido]-triphosphate (p[NH]ppG), with effective K_d values of about 1 nM and 0.1 μM, respectively. These apparent affinity values are 30- to 100-fold higher than typical ones found for guanine nucleotide activation of adenylate cyclase in other systems (24, 29). The guanine nucleotide dependence of adenylate cyclase in one such control membrane preparation from frog brain is shown in Fig. 4*B*.

The olfactory G protein may be functionally defined as belonging to the stimulatory type, since odorants increase rather than decrease adenylate cyclase activity in the sensory membranes and because cholera toxin catalyzes the specific labeling of a G_s-related α subunit in the olfactory cilia preparation. To substantiate the relation between these observations, we studied the effect of the cholera toxin-catalyzed ADP-ribosylation of olfactory cilia on their adenylate cyclase activity (Fig. 5*A*). A dose-dependent activation of such reaction was observed both in the absence and the presence of odorants. Cholera-toxin treatment was found to decrease the percent activation of the adenylate cyclase by odorants as described for other agonists (30) but did not affect the maximally activated enzyme in the presence of forskolin or [S]pppG.

The pertussis toxin labeling data show that all olfactory cilia preparations also contain a G_i-related protein. To examine the possible interaction of G_i with adenylate cyclase of olfactory cilia, the enzyme was fully activated with forskolin, a ligand that interacts directly with the cyclase catalytic unit, and various concentrations of [S]pppG were added (Fig. 5*B*). Inhibition of the activated enzyme by [S]pppG would indicate G_i function. This was not observed, suggesting that the G_s-related protein rather than the G_i-related protein is dominant in the modulation of adenylate cyclase in the olfactory cilia preparation under the conditions tested.

DISCUSSION

The finding that isolated olfactory cilia preparations from three vertebrate species contain large amounts of odorant-

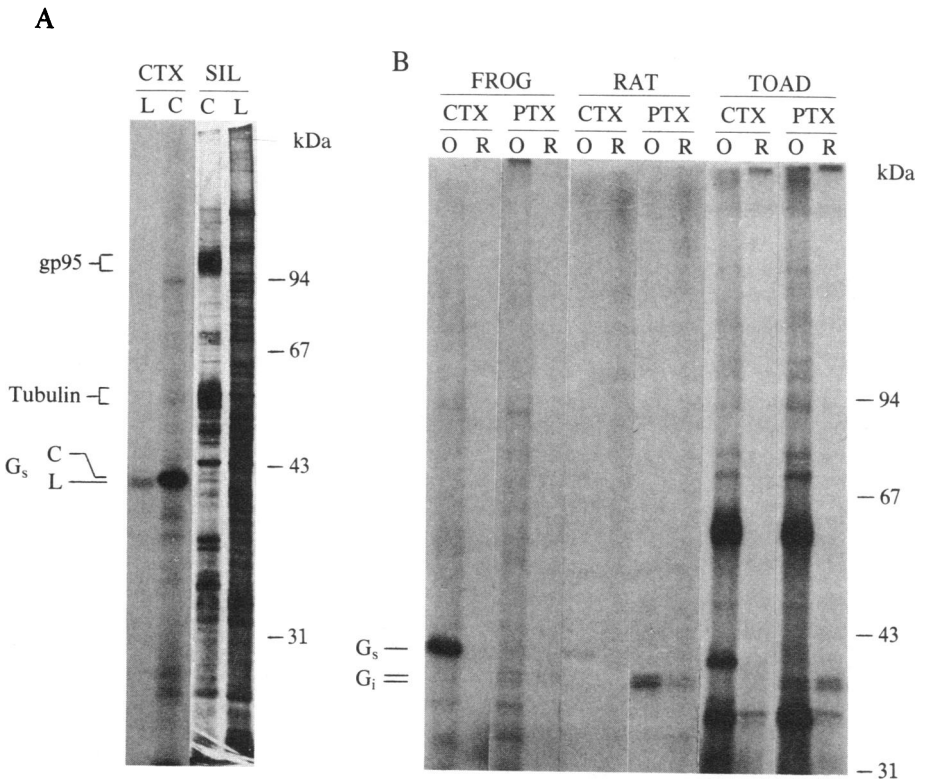

FIG. 2. (*A*) Autoradiograph of ADP-ribosylation of olfactory cilia (C) and liver (L) membranes catalyzed by cholera toxin (CTX). The silver staining (SIL) of the same gel lanes is also shown. (*B*) ADP-ribosylation patterns in olfactory (O) and respiratory (R) cilia preparations from frog, rat, and toad catalyzed by cholera toxin (CTX) or pertussis toxin (PTX). Polypeptides labeled in both the cholera and pertussis toxin lanes undergo toxin-independent nonspecific ADP-ribosylation. G-proteins, molecular size standards, tubulin and a specific protein of the olfactory cilia (gp95, see refs. 7 and 13) are marked.

sensitive, GTP-dependent adenylate cyclase complements our results (11) that a GTP-dependent odorant-sensitive adenylate cyclase may play a central role in frog olfactory reception. The following observations are consistent with the

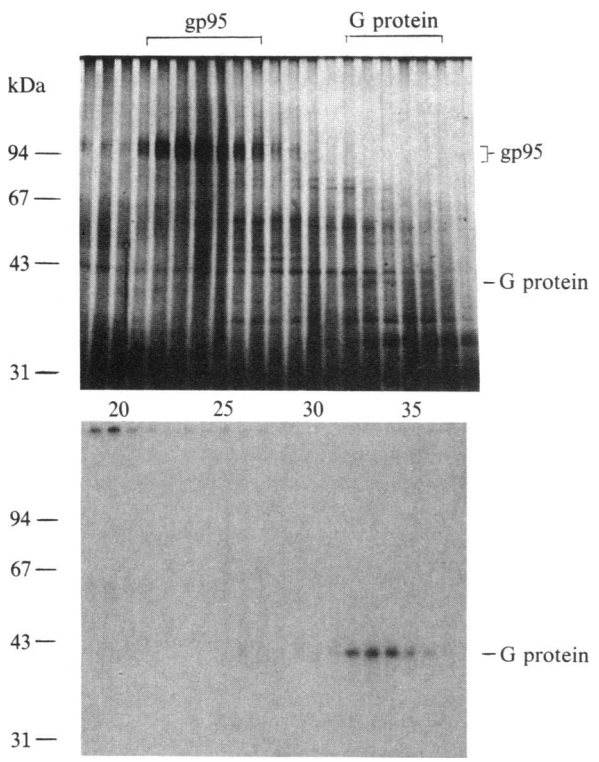

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of Triton X-100 soluble proteins of cholera toxin [^{32}P]ADP-ribosylated olfactory cilia, separated by gel exclusion chromatography on Sephacryl S-300. Column fractions are designated by number in the center of the figure, fractions 18 and 55, respectively, being the excluded and included volumes. (*Top*) Silver staining of the ciliary proteins. (*Bottom*) Autoradiographic visualization of [^{32}P]ADP-ribosylated polypeptides on the same gel. Polypeptide identification is as in Fig. 2.

claim that the G_s-related protein of olfactory cilia preparations is a functional chemosensory component: (*i*) odorants activate rather than inhibit adenylate cyclase in the sensory neuronal membranes; (*ii*) GTP is required for the *in vitro* odorant response; (*iii*) the cholera toxin-labeled G_s-related protein is enriched in the sensory membrane organelles relative to nonsensory counterparts or to membranes of olfactory epithelium after cilia removal (11); (*iv*) ADP-ribosylation with cholera toxin activates adenylate cyclase in olfactory cilia while decreasing the relative activation by odorants.

An interesting corroboration of the possible role of G_s in olfaction comes from two independent studies carried out in other laboratories. Patients with pseudohypoparathyroidism, a genetic disease that involves resistance to parathyroid and other hormones, have been reported to have impaired olfactory sensitivity (31, 45). It has been independently demonstrated that pseudohypoparathyroidism, at least in some patients, is accompanied by a G_s deficiency (32, 33). Together, these results are consistent with the involvement of G_s in olfactory transduction.

The G_s protein in olfactory cilia is slightly different in apparent molecular weight from the liver polypeptide. Such a difference is quite small compared to the range of molecular sizes reported for various subspecies of G_s α subunit (between 42 and 52 kDa, refs. 21 and 22). Another notable difference is the higher apparent affinity towards GTP analogs displayed by olfactory cilia relative to other membrane preparations. These results are consistent with olfactory G_s being a unique polypeptide variant. However, since the human genetic results point to a common genetic origin, it is possible that such G_s subspecies result from different posttranslational modifications or from alternative mRNA splicing.

GTP-binding proteins play a central role in a diverse ensemble of transmembrane signal transfer events, including hormone and neurotransmitter reception (23, 34), cell division, and tumorigenesis (23, 35). Olfaction appears to join this growing family of membrane receptor mechanisms. Of particular interest is the molecular analogy between olfaction and photoreception (28, 36). As for transducin in the visual

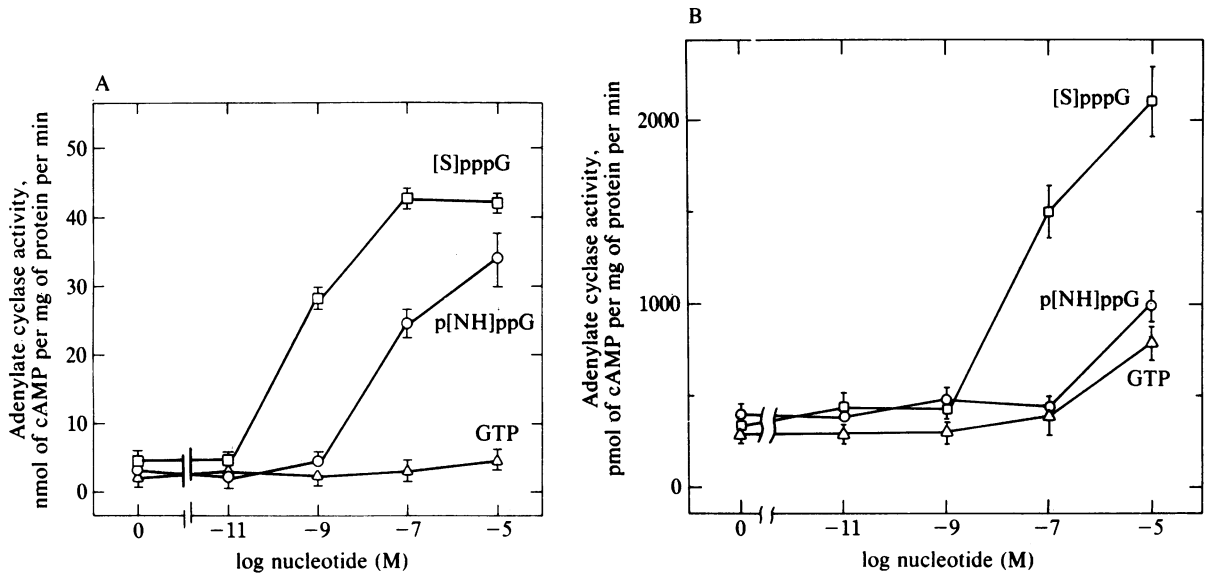

FIG. 4. Activation of frog olfactory ciliary (*A*) and frog brain (*B*) membrane adenylate cyclase by guanine nucleotides at various concentrations. Note that ordinate scale is 25 times larger in *A*.

system (cf. refs. 28 and 37), the G protein of olfactory neurons may play a role in signal amplification that could underlie the extreme sensitivity of olfactory detection (cf. refs. 5 and 38). An enzymatic cyclic nucleotide cascade is consistent with the relatively slow time constants (0.1–1.0 sec) reported for odorant activation (4, 5).

Olfactory cilia contain (but are not specifically enriched in) G_i-related pertussis toxin substrates. While no guanine nucleotide inhibition of the fully stimulated olfactory adenylate cyclase can be seen, it is possible that the G_i-related proteins of the olfactory cilia preparation help modulate the chemosensory response through the action of yet unidentified inhibitory ligand(s). It is worth noting that olfactory neurons are unique in having G_s as their dominant GTP-binding protein, other neurons being much richer in G_i-related proteins (25, 26, 34). Thus, olfactory cells could constitute a valuable system for studies of the role of stimulatory G protein(s) in dendritic signal transduction.

There is ample evidence for the existence of odorant-detecting receptor proteins in the sensory neurons (1, 2, 4, 5), and candidate polypeptides have been identified (1, 7, 13, 39, 40, 43). The discovery of a G protein, that presumably interacts directly with the putative receptor molecule, can help in future receptor identification and isolation (cf. refs. 5, 11, 13, 41, and 43). It would be interesting to find out whether olfactory receptor proteins with different odorant specificities (cf. refs. 3, 5–7) all interact with G_s and whether one or several G_s types are involved in transducing the diverse odor signals. The molecular cloning of G_s in brain (42) and in olfactory epithelium (44) could help address such questions.

We thank Dr. Zvi Farfel for his most helpful advice on the relationship between pseudohypoparathyroidism, G_s, and smell. We are grateful to Dr. Yoram Salomon, Dr. Emanuel Hanski, and Ms. Zehava Chen for much advice and help, and to Mr. Michael Greenberg for expert technical assistance. This work was supported by grants from the National Institutes of Health (NS22063), from the United

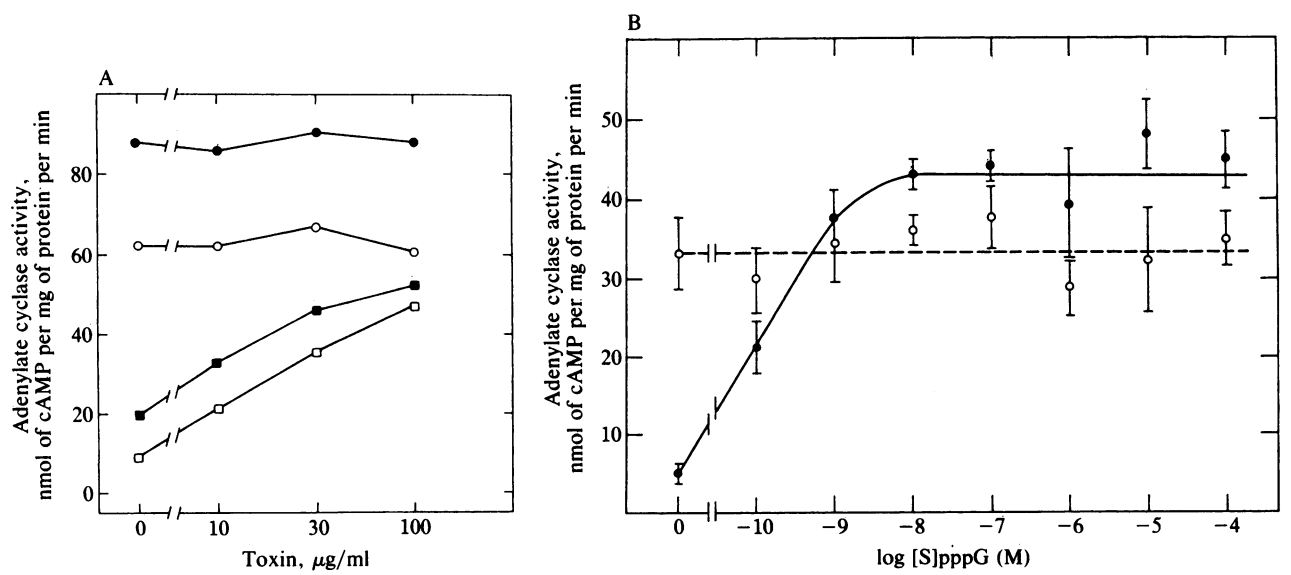

FIG. 5. (*A*) Effect of preincubation with various concentrations of cholera toxin on adenylate cyclase activity measured in frog olfactory cilia preparation in the presence of 10 μM GTP. □, No effector (basal); ■, 1 mM odorants; ○, 10 μM forskolin; ●, 10 μM [S]pppG. Relative activation by odorants (vs. basal level) at various cholera toxin concentrations: 112%, 50%, 31%, and 11%. (*B*) Effect of various concentrations of [S]pppG on basal (●) and 10 μM forskolin-stimulated (○) adenylate cyclase activity of frog olfactory cilia.

States–Israel Binational Science Foundation (Jerusalem), from the Gutwirth Foundation and from the Minerva Foundation (Munich). D.L. is incumbent of the Recanati Career Development Chair.

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