Characterization of a novel inositol 1,4,5-trisphosphate receptor in isolated olfactory cilia

D. Lynn KALINOSKI,*§ Scott B. ALDINGER,* Ardithanne G. BOYLE,† Taufiqul HUQUE,* James F. MARECEK,‡ Glenn D. PRESTWICH‡ and Diego RESTREPO*

*Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104, †Veterans Administration Medical Center, Philadelphia, PA 19104, and ‡State University of New York, Department of Chemistry, Stony Brook, NY 11794, U.S.A.

Inositol 1,4,5-trisphosphate (InsP_3), a product of G-protein-mediated receptor activation of phosphoinositide turnover, plays the role of a second messenger when olfactory neurons are stimulated with certain olfactory stimuli. In this paper we examine the specific binding of [^3H] InsP_3 to isolated olfactory cilia, microsomes and brain membranes from the channel catfish (*Ictalurus punctatus*) and, by photoaffinity labelling with an InsP_3 analogue (^{125}I -labelled 1-[3-(4-azidosalicyloxy)-aminopropyl]inositol 1,4,5-trisphosphate (^{125}I -ASA- InsP_3)), we tentatively identify the major InsP_3 -binding protein in catfish olfactory cilia. InsP_3 binding to ciliary membranes is specific and saturable, with a K_d of $1.10 \pm 0.31 \mu\text{M}$ and a maximum number of binding sites (B_{max}) of $17.6 \pm 5.8 \text{ pmol/mg}$. The rank order for potency of inhibition of [^3H] InsP_3 binding is $\text{Ins}(1,4)\text{P}_2 < \text{Ins}(1,3,4)\text{P}_3 < \text{Ins}(1,3,4,5)\text{P}_4 = \text{Ins}(1,4,5)\text{P}_3 < \text{Ins}(2,4,5)\text{P}_3$. Exposure of cilia membranes to u.v. light in the presence of ^{125}I -ASA- InsP_3 results in the labelling of a protein with apparent M_r 107000. Labelling is specifically prevented by $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, but not by $\text{Ins}(1,4)\text{P}_2$ or $\text{Ins}(1,3,4)\text{P}_3$. Both specific [^3H] InsP_3 binding and photoaffinity labelling of the M_r -107000 protein were displaced by heparin. The K_d and the inhibition of [^3H] InsP_3 binding and of photoaffinity labelling by inositol phosphates and heparin are consistent with the ability of micromolar concentrations of $\text{Ins}(1,4,5)\text{P}_3$ [but not $\text{Ins}(1,3,4)\text{P}_3$] to activate the InsP_3 -gated currents in patch-clamp experiments with olfactory neurons. These results suggest that InsP_3 binding to a M_r -107000 cilia membrane protein may represent binding to the olfactory InsP_3 -gated cation channel.

INTRODUCTION

A response to stimulation with certain olfactory stimuli in catfish and rat is an increase in intracellular $\text{Ins}(1,4,5)\text{P}_3$ (Huque & Bruch, 1986; Boekhoff *et al.*, 1990; Breer & Boekhoff, 1991), which presumably leads to an elevation of intracellular Ca^{2+} concentration through Ca^{2+} influx mediated by an InsP_3 -gated cation channel (Restrepo *et al.*, 1990). In other cell systems InsP_3 plays an important second-messenger function by coupling receptor activation in a GTP-dependent manner to an increase in intracellular Ca^{2+} caused by release of Ca^{2+} from internal stores mediated by opening of a microsomal InsP_3 -gated Ca^{2+} channel. Several laboratories have provided direct evidence for InsP_3 -receptor proteins by demonstrating saturable high-affinity binding sites for radiolabelled InsP_3 in permeabilized neutrophils and hepatocytes (Spat *et al.*, 1986) as well as in microsomal and/or plasma-membrane fractions from anterior pituitary (Guillemette *et al.*, 1987), liver (Guillemette *et al.*, 1988) and brain (Worley *et al.*, 1987b). An InsP_3 -binding protein has been purified from cerebellar membranes (Supattapone *et al.*, 1988a) and smooth muscle (Chadwick *et al.*, 1990), whose binding properties, assayed after reconstitution into lipid vesicles, are essentially the same as those of the membrane-bound receptor (Supattapone *et al.*, 1988a; Ferris *et al.*, 1989). A cDNA coding for a M_r -313000 InsP_3 -binding protein has been isolated from cerebellum (Furuichi *et al.*, 1989). Transfection of this cDNA clone into a fibroblast L-cell line increased the number of high-affinity InsP_3 -binding sites and induced Ca^{2+} -release activity (Miyawaki *et al.*, 1990).

Several studies suggest that an InsP_3 -receptor protein may be present in olfactory cilia of the channel catfish *Ictalurus punctatus*.

Some water-soluble L-amino acids, which are effective olfactory stimuli for the channel catfish (Caprio, 1978), elicit the rapid hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, resulting in transient accumulation of the second messenger InsP_3 (Huque & Bruch, 1986). The formation of InsP_3 is also activated by guanine nucleotides, suggesting G-protein mediation of the response, and time-course studies indicate that significant increases in InsP_3 are observed within less than 10 s of receptor activation. Studies with isolated olfactory cilia incorporated into artificial phospholipid bilayers and with whole-cell patch clamp of isolated olfactory neurons have identified a plasma-membrane conductance that is directly gated by InsP_3 (Restrepo *et al.*, 1990). Ca^{2+} and Na^+ influx carried by these channels could cause receptor-cell depolarization, suggesting that G-protein-linked phosphoinositide turnover may play a mediatory role in olfactory transduction through the opening of an InsP_3 -gated channel. In the present report we characterize the specific binding of radiolabelled InsP_3 to ciliary and microsomal membranes from the olfactory epithelium of the channel catfish and, by photoaffinity labelling with an InsP_3 analogue, we identify a novel M_r -107000 InsP_3 -binding protein in olfactory cilia. The properties of the ciliary binding site are different from those reported for binding of InsP_3 to microsomal fractions in other tissues. The specific binding of InsP_3 to the M_r -107000 protein in olfactory cilia may represent binding of InsP_3 to the ciliary InsP_3 -gated cation channel.

METHODS

Materials

D-[2- ^3H (n)]Inositol 1,4,5-trisphosphate (^3H] InsP_3) (10–30 Ci/mmol) was obtained from NEN Radiochemicals and Amersham

Abbreviations used: ASA- InsP_3 , 1-[3-(4-azidosalicyloxy)aminopropyl]inositol 1,4,5-trisphosphate; KHE medium, solution containing K^+ , Hepes and EGTA- Ca^{2+} (see the Methods section for composition); EC_{50} , concn. giving 50% inhibition of [^3H] InsP_3 binding.

§ To whom correspondence should be addressed.

Corp. L-[2,3-³H]Alanine (50 Ci/mmol) was obtained from ICN Radiochemicals. [U-¹⁴C]Sucrose (5–15 mCi/mmol) was procured from Amersham. Ins(1,3,4) P_3 , Ins(1,4,5) P_3 , Ins(1,3,4,5) P_4 , Ins(1,4) P_2 , low-molecular-mass heparin, pepstatin A, ionomycin and aprotinin were purchased from Calbiochem. Leupeptin and Ins(2,4,5) P_3 were purchased from Boehringer Mannheim Biochemicals. HAWP nitrocellulose filtration membranes were obtained from Millipore. All other reagents were purchased from standard suppliers.

Isolation of olfactory cilia

Cilia were detached from the olfactory epithelium of the channel catfish by the method of Boyle *et al.* (1987) by treatment with 10 mM-CaCl₂ in 20 mM-Na-Aces, pH 7.4, containing 0.3 M-sucrose, 10 μ g of leupeptin/ml, 76.8 nM-aprotinin, 0.7 μ M-pepstatin, 0.83 mM-benzamide, 0.23 mM-phenylmethanesulphonyl fluoride and 1 mM-iodoacetamide. Cilia were washed and resuspended in KHE medium, containing 110 mM-KCl, 1 mM-K₂EGTA, 0.2 mM-CaCl₂ and 10 mM-Hepes, pH 7.4. KHE contained 20 nM free Ca²⁺ at 4 °C, calculated by the procedure of Harrison & Bers (1989). For binding experiments this solution was supplemented with 50 μ g of digitonin/ml to permeabilize ciliary membranes.

Isolation of olfactory microsomes

The deciliated olfactory tissue contained in the 1000 g pellet (Boyle *et al.*, 1987) was homogenized in 20 mM-Aces buffer containing 0.3 M-sucrose, 5 mM-EDTA and 5 mM-EGTA, pH 7.4, by 10 strokes of a hand-driven glass/Teflon homogenizer and was centrifuged at 20000 g for 30 min. The supernatant was centrifuged at 100000 g for 1 h. The microsomal pellet was washed with KHE buffer, centrifuged a second time at 100000 g for 1 h, and was finally resuspended by homogenization in 25 ml of KHE for use in subsequent binding experiments.

Brain homogenate

Brains from three catfish were homogenized in 20 vol. of ice-cold KHE, with a glass/Teflon homogenizer. The homogenate was centrifuged for 10 min at 1000 g and the pellet was discarded. Brain membranes were collected by centrifugation at 30000 g for 20 min and were resuspended at a final concentration of 3–4 mg of protein/ml in KHE buffer.

Receptor-binding measurements by filtration

Assays for [³H]Ins P_3 binding to olfactory cilia or brain membranes were performed by the filtration assay of Worley *et al.* (1987a). Binding assays were performed in 3–10 replicate samples. Assays were in 0.5 ml final volume containing 5–15 nM-[³H]Ins P_3 in the absence and presence of various concentrations of unlabelled ligand or other additions. After the addition of olfactory cilia or microsomes (25–100 μ g of protein) or brain membranes (300 μ g), the samples were incubated on ice for 10 min. A temperature of 4 °C was used, to minimize degradation of the Ins P_3 . Samples were rapidly filtered under vacuum through nitrocellulose filters (0.45 μ m pore size; type HAWP, Millipore) and were washed quickly with 2 $\times$ 3 ml of ice-cold KHE.

Non-specific binding, which typically accounted for 50 % of the total binding in the experiments with olfactory cilia, was determined in parallel samples containing 50 μ M unlabelled ligand. As shown in Fig. 1, specific binding (total binding minus non-specific) was linearly dependent on the amount of protein in the sample, whereas non-specific binding was independent of the amount of protein. In addition, control experiments indicated that addition of larger amounts of unlabelled ligand (100–400 μ M) did not affect the non-specific binding and that non-specific binding was not affected by pH (in the range 5–9) or by addition

Fig. 1. Dependence of specific and non-specific binding on the amount of protein in the sample

Cilia were resuspended in 110 mM-KCl/1 mM-EGTA/0.2 mM-CaCl₂/10 mM-K-Hepes, pH 7.4, plus 7 nM-[³H]Ins P_3 and 50 μ g of digitonin/ml in the presence (non-specific binding) or the absence (total binding) of 50 μ M unlabelled Ins(1,4,5) P_3 . After a 10 min incubation at 4 °C, samples were filtered and washed as described in the Methods section. Results are from one experiment performed in quintuplicate samples. The lines shown are straight-line regressions. For the graph of specific binding versus protein, the slope is 0.141 ± 0.004 c.p.m./ μ g and the intercept 0.7 ± 0.3 c.p.m. (a two-tailed *t* test indicates that the slope is significantly different from zero at $P < 0.001$). For the graph of non-specific binding versus protein, the slope is 0.01 ± 0.03 c.p.m./ μ g and the intercept 8.9 ± 2.8 c.p.m. (the slope is not significantly different from zero, at $P < 0.8$).

of 100 μ g of heparin/ml or of 50 μ g of digitonin/ml. These facts suggest that non-specific binding represented mostly binding of [³H]Ins P_3 to the HAWP nitrocellulose filters. Non-specific binding was not decreased by further washing, by the use of glass-fibre filters or by presoaking of the filters with KHE solution containing 1 mg of BSA/ml.

Preliminary experiments were performed to examine the rates of association and dissociation for [³H]Ins P_3 binding to isolated olfactory cilia membranes, a rapid filtration method being used. The association of Ins P_3 is rapid. The number of [³H]Ins P_3 -binding sites occupied by 10 min is 100 % of the specific binding measured after 20 min. The rate of Ins P_3 dissociation was determined by displacement of [³H]Ins P_3 binding by addition of excess unlabelled Ins P_3 (50 μ M); 90 % of the specifically bound [³H]Ins P_3 dissociated by 2 min. Because of the extremely rapid dissociation, filters were washed rapidly with 2 $\times$ 3 ml of ice-cold KHE buffer (washing lasted 5–10 s overall). Using this wash procedure we could consistently measure Ins P_3 binding to cilia membranes; however, the rapid dissociation suggests that some bound Ins P_3 may dissociate during filtration and that our binding data probably underestimate the actual number of receptor sites in the ciliary membranes.

The filters were solubilized in 1 ml of 2-methoxyethanol (Fisher) and diluted with 10 ml of Universol before liquid-scintillation counting in a refrigerated Packard Tri-Carb 300C instrument. Specific binding (after subtraction of non-specific binding) typically amounted to 10–40 c.p.m. Radioactivity in each sample was counted twice for 30 min. Variation in quintuplicate samples was typically less than 15 %. Assays for radiolabelled L-alanine binding were performed as previously described (Kalinoski *et al.*, 1987; Rhein & Cagan, 1980). Assays were performed in triplicate in 0.5 ml final volume containing 1 μ M radioligand. Non-specific binding was determined in parallel

samples containing 20 mM unlabelled ligand. Assays were incubated on ice for 1 h, rapidly filtered, and washed as described above.

Receptor-binding measurements by centrifugation

To obtain independent measurements of InsP₃ binding that are not affected by the speed of dissociation, we performed centrifugation assays of [³H]InsP₃ binding to olfactory cilia. Cilia (0.15–0.2 mg) were incubated with 15 nM-[³H]InsP₃ in 0.5 ml of KHE at 4 °C for 10 min in the presence (non-specific binding) or absence (total binding) of 50 μM unlabelled InsP₃. Assays were terminated by centrifugation at 14000 *g* for 5 min (4 °C) in an Eppendorf Microfuge. Immediately after centrifugation the supernatant was discarded, and the pellets were dissolved by incubation for 30 min in 0.4 ml of 5% (w/v) trichloroacetic acid, followed by addition of 5 ml of Universol. [¹⁴C]Sucrose (0.6 μCi/ml) was added to the KHE in the assay to quantify carry-over of assay medium into the pellet. ³H and ¹⁴C radioactivity was determined after counting in a Packard Tri-Carb 300C instrument by using two energy windows of 0–25 and 25–156 keV. The amount of [³H]InsP₃ in the pellet was corrected by subtracting the amount of [³H]InsP₃ in the assay medium carried over with the pellet (5–8 μl, estimated from the amount of [¹⁴C]sucrose in the pellet). The amount of non-specific binding was not significantly different from zero, further suggesting that the non-specific binding measured in the filtration assay represents binding of [³H]InsP₃ to the filters.

⁴⁵Ca release from olfactory cilia and microsomes

Cilia and microsomes were resuspended at a protein concentration of 650 μg/ml in a solution containing 110 mM-KCl, 50 μM-EGTA and 10 mM-Hepes, titrated with KOH to pH 7.1 and supplemented with 3 μCi of ⁴⁵Ca (final Ca²⁺ concn. 50 μM). The free Ca²⁺ concentration, calculated in accordance with Harrison & Bers (1989), was approx. 3 μM. Cilia and microsomes were incubated in this solution for 1.5 h at room temperature. At 10 min before the ⁴⁵Ca-release assay, this solution was supplemented with 1 mM-ATP (sodium salt) and 2 mM-MgCl₂. For the ⁴⁵Ca release assay 0.6 ml of ⁴⁵Ca-loaded cilia or microsomes were added to 2.4 ml of KHE (pH 7.1 at room temperature, final free Ca²⁺ concn. 60 nM) with appropriate additions. Immediately after thorough mixing of these solutions, 0.4 ml samples were withdrawn every 1 min and were filtered through nitrocellulose filters (0.45 μm; type HAWP, Millipore) and washed with 10 ml of ice-cold KHE. Filters were solubilized and counted for radioactivity as described above under 'Receptor-binding measurements'.

Ins(1,4,5)P₃ phosphatase assay

Conversion of [³H]InsP₃ to [³H]InsP₂ was assayed in the absence of ATP by incubation of cilia at room temperature in media (0.2 ml) containing 25 mM-Mops, 3 mM-MgCl₂, 10 mM-NaCl, 80 mM-KCl, 0.6 mM-CaCl₂ and 2 mM-EGTA, pH 7.1, supplemented with [³H]InsP₃ (30000 c.p.m.; approx. 15 nM). Free Ca²⁺ concentration was 0.053 μM, as calculated by the method of Raaflaub (1956). Assays were started by addition of cilia (2–10 μg of protein) and incubated at room temperature for 10 min. Reactions were stopped by addition of 0.2 ml of 10% trichloroacetic acid, followed by 0.1 ml of 0.5% BSA. Tubes were left on ice for 10 min and then centrifuged at 1000 *g* for 10 min. The supernatant was removed, neutralized with 2.5 M-NaOH and loaded on to ion-exchange columns (Bio-Rad AG1X8). Inositol phosphates were eluted by stepwise gradient elution with increasing concentrations of ammonium formate/formic acid (Downes & Michell, 1982). Fractions (12 ml) were collected and samples counted for radioactivity. The amount of [³H]InsP₂

produced was calculated from the specific radioactivity of the [³H]InsP₃.

Labelling of ciliary proteins with ¹²⁵I-ASA-InsP₃

ASA-InsP₃ was synthesized as described by Prestwich *et al.* (1991). The radioiodinated probe, ¹²⁵I-ASA-InsP₃, was prepared by using Iodobeads (Pierce Chemical Co.) and Na¹²⁵I (Dupont NEN) as follows: two beads were added to 2 mCi of Na¹²⁵I (1.2 nmol) in 150 μl of 100 mM-Na₂HPO₄ (pH 7.5) and allowed to equilibrate at room temperature for 5 min. The remaining steps were performed under dim red light. ASA-InsP₃ [10 μl of a stock solution containing 2 μg (1.7 nmol)] was added and the reaction kept at room temperature for 20 min with occasional agitation. The reaction was terminated by removing the solution from the beads, which were then washed with 2 × 100 μl of water. The washes were added to the reaction solution. Analysis by h.p.l.c. showed that almost all of the radioactivity was in the ¹²⁵I-ASA-InsP₃ species. The molar ratio of mono- to di-iodinated product was 8:1. A sample evaluated by liquid-scintillation counting showed 1.32 mCi of recovered radioactivity in the sample. The specific radioactivity was about 800 Ci/mmol. The final volume of the solution was adjusted to give a radioactivity of 1 μCi/μl.

For photoaffinity labelling, cilia membranes were suspended at a final protein concentration of 0.5 mg/ml in TME medium (10 mM-Tris/HCl, 3 mM-MgCl₂, 2 mM-EGTA, pH 8.2) supplemented with 50 μg of digitonin/ml. Samples (100 μl) were divided into wells of a 96-well plate placed on ice. Inositol phosphates (50 μM) or heparin (100 μg/ml) were added from stock concentrated solutions. Then 4 μCi of ¹²⁵I-ASA-InsP₃ was added to each well under dim red light, and the cilia were allowed to equilibrate for 20 min in the dark. The membranes were exposed for 2 min to u.v. light (254 nm) provided by three RPR-1849 lamps (Southern New England Co., Hamden, CT, U.S.A.) placed 3 cm above the samples.

Labelled cilia were solubilized by dilution (1:1) in a sample buffer containing 125 mM-Tris/HCl, pH 6.8, 4% (w/v) SDS (Research Organics), 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.1% Bromophenol Blue, and were boiled for 5 min. The discontinuous system of Laemmli (1970) was used for electrophoresis, with 4%-acrylamide stacking gels at pH 6.8 and 7.5% separating gels at pH 8.8. To detect ¹²⁵I-labelled proteins, X-OMAT AR film (Kodak, Rochester, NY, U.S.A.) was exposed for 4–8 days at –70 °C by using Cronex intensifying screens (DuPont, Willmington, DE, U.S.A.).

Other methods

Glucose-6-phosphatase activity was measured by standard procedures (Morre, 1971). Phosphate was assayed by the method of Chen *et al.* (1956). Protein was determined as described by Bradford (1976). Non-linear regressions in Figs. 3 and 4 were performed with the program ENZFITTER from Elsevier-Biosoft (Amsterdam). This program provides best-fit estimates of the non-linear least-squares-fit parameters as well as estimates of their standard errors calculated by using the matrix-inversion method. Best-fit parameters are reported as (parameter ± s.e. of estimate).

RESULTS

pH- and Ca²⁺-dependencies for specific [³H]InsP₃ binding

InsP₃ binding to microsomal membranes in other tissues is highly dependent on pH and Ca²⁺ concentration. Specific [³H]InsP₃ binding in olfactory cilia was lowest at pH 6 and increased 6-fold as the pH was made more alkaline, reaching a

Fig. 2. pH-dependence of specific $[^3\text{H}]\text{InsP}_3$ binding to olfactory cilia at constant Ca^{2+} concentration ($< 1 \text{ nM}$)

Cilia, prepared as described in the Methods section, were resuspended in 110 mM-KCl/1 mM-EDTA/1 mM-Hepes, pH 7.4. Then 0.1 ml of cilia suspension (25–100 μg of protein) was added to 0.4 ml of a medium containing 8.5 nM- $[^3\text{H}]\text{InsP}_3$ in 110 mM-KCl buffered with 5 mM-potassium citrate, 5 mM-Mes, 5 mM-Hepes and 5 mM-Tris and supplemented with 5 mM-EDTA and 50 μg of digitonin/ml. pH was measured with a Ross pH electrode (Orion). Incubations were conducted at 4 °C for 10 min. Results are means $\pm$ S.E.M. of quadruplicate determinations from three experiments. In each experiment InsP_3 binding was normalized by dividing by the amount of specific InsP_3 binding at pH 7.

maximum at pH 9 (Fig. 2). In the physiological pH range (6.5–7.8), specific InsP_3 binding increased as a function of pH. On the other hand, specific binding of InsP_3 decreased by only 2-fold as $[\text{Ca}^{2+}]$ was decreased from 1 mM to $< 1 \text{ nM}$, with little change in the physiologically relevant range from 10 nM to 1 μM (results not shown). Subsequent studies of $[^3\text{H}]\text{InsP}_3$ binding were performed at pH 7.4 and a free Ca^{2+} concentration of 20 nM. These values for pH and $[\text{Ca}^{2+}]$ closely approximate to intracellular conditions (Restrepo & Teeter, 1990) and measurements of intracellular pH (D. Restrepo, unpublished work).

Inhibition of binding of $[^3\text{H}]\text{InsP}_3$ by unlabelled InsP_3

The affinity and number of binding sites for InsP_3 were evaluated by using inhibition-binding curves instead of the standard saturation-binding protocol, because of the cost of the $[^3\text{H}]\text{InsP}_3$. Specific $[^3\text{H}]\text{InsP}_3$ binding to olfactory cilia was measured in the presence of increasing concentrations of unlabelled InsP_3 (Fig. 3, $\blacktriangle$); 50% inhibition of $[^3\text{H}]\text{InsP}_3$ binding occurred at about 1 μM . The data were adequately fitted by an equation describing displacement of a trace amount of bound $[^3\text{H}]\text{InsP}_3$ by addition of unlabelled InsP_3 assuming a single binding site:

$$B^* = B^*_0 / (1 + ([\text{InsP}_3] / K_d)) \quad (1)$$

where B^* is the amount of labelled InsP_3 bound, B^*_0 is the amount of labelled InsP_3 bound in the absence of unlabelled InsP_3 , $[\text{InsP}_3]$ is the concentration of unlabelled InsP_3 and K_d is the dissociation constant for InsP_3 binding to the receptor. A Scatchard plot (inset, Fig. 3) was linear, indicating that olfactory cilia possess a single binding site for InsP_3 . The dissociation constant for InsP_3 , estimated from the fit of eqn. (1) to the data in Fig. 3, was $1.10 \pm 0.31 \mu\text{M}$, which is substantially higher than dissociation constants reported for microsomal InsP_3 receptors in other tissues (1–40 nM). As evidenced by the fact that 0.05 μM - InsP_3 did not inhibit binding of a significant amount of $[^3\text{H}]\text{InsP}_3$ (Fig. 3) and by the linearity of the Scatchard plot (Fig. 3, inset), olfactory cilia do not display a high-affinity binding site with an affinity at nanomolar concentrations. In contrast, InsP_3 binding to catfish brain membranes measured with the same filtration assay displayed high- and low-affinity sites with apparent K_d values of 9 nM and 0.8 μM respectively (Fig. 3, $\bullet$). The number of InsP_3 receptor sites was estimated by calculating the maximum

Fig. 3. Inhibition of $[^3\text{H}]\text{InsP}_3$ binding by unlabelled InsP_3 in olfactory cilia (Δ) and brain membranes ($\bullet$)

Binding assays included cilia (25–100 μg of protein) or brain membranes (300 μg) and 5–11 nM- $[^3\text{H}]\text{InsP}_3$ in 0.5 ml of 110 mM-KCl/1 mM- K_2EGTA /0.2 mM- CaCl_2 /10 mM-Hepes-K, pH 7.4, plus the indicated amounts of unlabelled InsP_3 and 50 μg of digitonin/ml. Incubations were for 10 min at 4 °C. Error bars shown are S.E.M. Olfactory cilia (Δ): data points were from multiple (3–10) determinations from ten separate experiments normalized within each experiment to the amount of $[^3\text{H}]\text{InsP}_3$ bound in the absence of unlabelled InsP_3 . Not all InsP_3 concentrations were assayed in each experiment. The number of experiments at each concentration are (n , InsP_3 concn. in μM): 4, 0.05; 3, 0.125; 3, 0.25; 4, 0.5; 6, 1.5; 4, 3.0; 3, 6; 3, 9; 3, 12. A fit of eqn. (1) to the data for olfactory cilia (continuous line) yields a K_d of $1.10 \pm 0.31 \mu\text{M}$. Brain membranes ($\bullet$): data points are from one experiment with triplicate samples per experimental condition. The data were fitted with an equation describing binding of InsP_3 to two binding sites with apparent K_d values of 9 nM and 0.8 μM (continuous line). Binding to the high-affinity site accounted for 83% of the total $[^3\text{H}]\text{InsP}_3$ binding in the absence of unlabelled InsP_3 . Inset: modified Scatchard plot. Eqn. (1) can be reorganized in the form

$$B^* = B^*_0 - [\text{InsP}_3] B^* / K_d$$

which is equivalent to a Scatchard equation. Because the data were normalized to the amount of binding in the absence of unlabelled InsP_3 , $B^*_0 = 1.0$. K_d can be estimated as minus the inverse of the slope or as the abscissa intercept. For the olfactory cilia (Δ), the data fell on a straight line, indicating the presence of one binding site for InsP_3 . The estimated K_d was 1.8 μM . The Scatchard data for the brain membranes ($\bullet$) were non-linear, indicating the presence of more than one binding site for InsP_3 . The continuous line shown is the best fit of a two-site model, with K_d values of 9 nM and 0.8 μM and in which the high-affinity binding site accounts for 83% of the total binding in the absence of unlabelled InsP_3 .

number of binding sites (B_{max} , in pmol/mg of protein) from the best-fit K_d (1.1 μM) and from the concentrations of $[^3\text{H}]\text{InsP}_3$ in the medium ($[\text{InsP}_3^*]$, in μM) and the amounts of $[^3\text{H}]\text{InsP}_3$ bound in the absence of unlabelled InsP_3 (B^*_0 , in pmol/mg of protein) determined in 17 independent experiments by using eqn. (2) (Spat *et al.*, 1986):

$$B_{\text{max}} = B^*_0 (K_d / [\text{InsP}_3^*]) \quad (2)$$

The receptor site density, calculated in this manner, was 17.6 ± 5.8 pmol/mg of protein (mean $\pm$ S.E.M., $n = 17$). In addition, specific binding measured in a centrifugation assay yielded a value of 169 $\pm$ 53 fmol/mg (mean $\pm$ S.E.M., from two experiments performed in quadruplicate) for $[^3\text{H}]\text{InsP}_3$ binding to cilia in the presence of 15 nM free $[^3\text{H}]\text{InsP}_3$. From eqn. (2), this corresponds to a receptor site density of 12.4 ± 4 pmol/mg, which is not significantly different from the estimate in the filtration assay (by a two tailed t -test, $P < 0.3$). The maximum

Table 1. Subcellular distribution of InsP₃ binding and markers

Ciliary and microsomal membranes were prepared as described in the Methods section. Assays (0.5 ml) contained either 7 nM-[³H]InsP₃ or 1 μM-L-[³H]alanine, and cilia or microsomes (25–100 μg of protein). Incubations were performed for 10 min at 4 °C. Data from one experiment are means ± S.E.M. for *n* determinations.

	Cilia	Microsomes	<i>n</i>
Glucose-6-phosphatase (pmol of P _i /min per mg)	−1.57 ± 1.44	5.70 ± 0.18	3
L-[³ H]Alanine (pmol/mg of protein)	13.2 ± 0.73	1.43 ± 0.15	5
[³ H]InsP ₃ (fmol/mg of protein)	52.7 ± 8.87	23.8 ± 2.96	5

Table 2. Displacement of specific [³H]InsP₃ binding by related compounds

Ciliary membranes were prepared as described in the Methods section. Assays (0.5 ml) included 7 nM-[³H]InsP₃, cilia (25–100 μg of protein) and various concentrations of competing substances. Incubations were performed at 4 °C for 10 min. Data are means ± S.E.M. from three experiments each performed in quintuplicate. EC₅₀ was estimated by fitting eqn. (1) to the data (with EC₅₀ replacing K_d). The standard errors of the estimate are given for EC₅₀ values estimated for Ins(1,4,5)P₃ and Ins(1,3,4)P₃, which were derived from the full inhibition curves in Figs. 4 and 5.

Compound	[³ H]InsP ₃ bound (%)		Estimated EC ₅₀ (μM)
	1.5 μM compound	15 μM compound	
Ins(2,4,5)P ₃	27 ± 5	18 ± 19	0.63
Ins(1,4,5)P ₃			1.10 ± 0.31
Ins(1,3,4,5)P ₄	42 ± 12	26 ± 7	1.3
Ins(1,3,4)P ₃	89 ± 9	66 ± 23	11 ± 2
Ryanodine		61 ± 16	23
Ins(1,4)P ₂	76 ± 1	69 ± 9	28

number of InsP₃-binding sites in brain membranes (calculated by using eqn. 2, from the data in Fig. 3) was 0.6 pmol/mg. It is important to stress that binding in cerebellum is expected to be several-fold higher than this whole-brain estimate because, except for the cerebellum and the hippocampus, other brain tissue possesses few InsP₃-binding sites (Worley *et al.*, 1987a).

Subcellular distribution of [³H]InsP₃ binding

Previous studies of InsP₃-receptor-binding sites have indicated that these receptors are located in membranes closely associated with, but distinct from, the endoplasmic reticulum (Rossier *et al.*, 1989). To determine the subcellular localization of the InsP₃ receptor(s) in olfactory tissue, we evaluated the relationship of specific binding of [³H]InsP₃ in isolated ciliary membranes and microsomes with specific binding of L-[³H]alanine, a potent olfactory stimulus for catfish, whose receptors are localized in ciliary membranes (Bruch & Rulli, 1988), and glucose-6-phosphatase, a standard marker enzyme for endoplasmic reticulum (Morre, 1971) (Table 1). Specific binding of [³H]InsP₃ was about 2-fold higher in ciliary membranes than in microsomes. It is unlikely that specific binding of InsP₃ to olfactory cilia was due to contamination with membranes from the endoplasmic reticulum, because there was virtually no glucose-6-phosphatase activity in the ciliary membranes. As expected, L-[³H]alanine

binding was about 10-fold higher in cilia than in microsomes. These data suggest the presence of an InsP₃ receptor located in the ciliary membranes of olfactory cells in addition to one localized to endoplasmic reticulum, and are in agreement with the presence of an InsP₃-gated Ca²⁺ current in reconstituted ciliary membrane vesicles (Restrepo *et al.*, 1990), as well as with InsP₃-stimulated release of Ca²⁺ from vesicles formed from the microsomal fraction derived from olfactory epithelium (Bruch, 1988).

Transmembrane orientation of the ciliary InsP₃-binding site

Electron-microscopic studies indicated that the isolated cilia preparation contains both broken cilia (with the intracellular side of the membrane exposed) and closed outside-out vesicles (Boyle *et al.*, 1987). Because the presumed location of the InsP₃-receptor-binding sites is an intracellular one, addition of the detergent digitonin should permit access of [³H]InsP₃ to the inside of the closed vesicles, thereby increasing the number of binding sites for InsP₃. We found that, in the presence of 50 μg of digitonin/ml, specific [³H]InsP₃ binding to isolated cilia was roughly 2-fold higher than binding in the absence of digitonin. As stated in the Methods section, assays were regularly conducted in the presence of digitonin.

Because the ciliary InsP₃-binding site is presumably facing inward in the outside-out cilia vesicles and because microsomal InsP₃-binding sites are expected to be facing outward in microsomal vesicles, we reasoned that InsP₃ should have different effects on ⁴⁵Ca release from cilia and microsomal vesicles. As previously demonstrated by Bruch (1988), addition of 15 μM-InsP₃ to olfactory microsomes that have been previously loaded with ⁴⁵Ca elicited a release of 25% of the ⁴⁵Ca sequestered. In contrast, and as expected if cilia vesicles are outside-out with the InsP₃-binding site facing inward, InsP₃ did not induce a release of ⁴⁵Ca from olfactory cilia under exactly the same conditions. As a control, the Ca²⁺ ionophore ionomycin (10 μM) released all ⁴⁵Ca in the cilia and microsome vesicles, indicating that these preparations contain vesicles capable of sequestering Ca²⁺. These observations are an indication that the InsP₃-binding sites in cilia and microsomal vesicles have different transmembrane orientations.

Specificity of [³H]InsP₃ binding

We found that the rank order for potency of inhibition of [³H]Ins(1,4,5)P₃ binding was Ins(1,4)P₂ < Ins(1,3,4)P₃ < Ins(1,3,4,5)P₄ = Ins(1,4,5)P₃ < the non-physiological analogue Ins(2,4,5)P₃ (Table 2 and Fig. 4). This selectivity for the InsP₃-binding site in olfactory cilia is different from that of InsP₃ receptors in either peripheral tissues or brain (Spat *et al.*, 1986; Worley *et al.*, 1987a; Guillemette *et al.*, 1987; Ferris *et al.*, 1989). Inositol triphosphates that differed only by the position of the phosphate group displayed large differences in EC₅₀ {the concentration of a given compound that caused 50% inhibition of [³H]Ins(1,4,5)P₃ binding}. The EC₅₀ for Ins(1,3,4)P₃ was 20-fold larger than the EC₅₀ for Ins(2,4,5)P₃. In contrast, there was little difference in EC₅₀ for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄. This suggests that the binding site for InsP₃ is not simply a non-specific site, because non-specific binding to membranes for a charged molecule such as Ins(1,4,5)P₃ would have been expected to be highly dependent on the number of charges on the molecule rather than on the orientation of the charged groups.

The binding site displays the stereospecificity required for a physiological InsP₃ receptor in olfactory cilia, because the relatively low affinity of Ins(1,3,4)P₃ for the Ins(1,4,5)P₃-binding site is consistent with the inability of intracellularly applied Ins(1,3,4)P₃ to cause membrane-potential depolarization in isolated olfactory neurons under whole-cell patch-clamp (Restrepo

Fig. 4. Inhibition of specific $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding by unlabelled $\text{Ins}(1,3,4)\text{P}_3$

Conditions of assay were as in Fig. 3. The EC_{50} for $\text{Ins}(1,3,4)\text{P}_3$, estimated from a fit of eqn. (1) to the data (with EC_{50} replacing K_d), was $11 \pm 2 \mu\text{M}$.

et al., 1991). In addition, $\text{Ins}(1,4)\text{P}_2$, the putative product of degradation of $\text{Ins}(1,4,5)\text{P}_3$, displayed the lowest affinity of all compounds tested. This property would be expected for a physiological receptor, because it would ensure that degradation of $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4)\text{P}_2$ would terminate the response.

Ryanodine, an antagonist of sarcoplasmic-reticulum Ca^{2+} channels, displayed the ability to inhibit partially InsP_3 binding to olfactory cilia at a concentration of $15 \mu\text{M}$.

Inhibition by heparin

Heparin has been shown to inhibit binding of $[^3\text{H}]\text{InsP}_3$ to microsomal membranes through a competitive-inhibition mechanism (Ghosh *et al.*, 1988) and has been utilized to purify the InsP_3 receptor from brain (Supattapone *et al.*, 1988a) and smooth muscle (Chadwick *et al.*, 1990) to homogeneity. We found that heparin inhibited the binding of InsP_3 in olfactory cilia in a dose-dependent manner, with an EC_{50} of $6.7 \pm 1.7 \mu\text{g/ml}$, or approx. $0.7 \mu\text{M}$ (result not shown). This value for the EC_{50} is the same as that for inhibition of InsP_3 binding to microsomal membranes in brain (Worley *et al.*, 1987b) and liver (Tones *et al.*, 1989). The fact that heparin competes for binding of InsP_3 to specific receptor sites, but does not inhibit inositol 5-phosphatase (Bootman *et al.*, 1990), supports the hypothesis that the $[^3\text{H}]\text{InsP}_3$ -binding site in olfactory cilia is a specific receptor protein and not a phosphatase enzyme.

Photoaffinity labelling with $^{125}\text{I}\text{-ASA-InsP}_3$

Although $^{125}\text{I}\text{-ASA-InsP}_3$ labelled several ciliary proteins, only one protein with apparent M_r 107 000 (band labelled 'a' in Fig. 5a) displayed labelling that was specifically prevented by co-incubation of $\text{Ins}(1,4,5)\text{P}_3$ ($50 \mu\text{M}$) with the $^{125}\text{I}\text{-ASA-InsP}_3$ during u.v. exposure. Using the same photoaffinity label, Mourey *et al.* (1991) have labelled the purified high- M_r $\text{Ins}(1,4,5)\text{P}_3$ receptor in purified and crude membrane fractions from rat cerebellum. In agreement with these observations, in control experiments we found that the $^{125}\text{I}\text{-ASA-InsP}_3$ used in the present study specifically labels the cerebellar InsP_3 receptor protein in rat, which displays an apparent M_r of 230 000 in our gel system (results not shown). Labelling by $^{125}\text{I}\text{-ASA-InsP}_3$ of the M_r -107 000 protein in olfactory cilia displayed the same specificity as the displacement of specific $[^3\text{H}]\text{InsP}_3$ binding. Thus, although $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$, which are relatively potent inhibi-

Fig. 5. Photoaffinity labelling of olfactory-cilia proteins with $^{125}\text{I}\text{-ASA-InsP}_3$

Olfactory-cilia membranes ($100 \mu\text{l}$; protein concn. 0.5 mg/ml) were incubated in the dark for 20 min in TME medium with $50 \mu\text{g}$ of digitonin/ml and $4 \mu\text{Ci}$ of $^{125}\text{I}\text{-ASA-InsP}_3$ in the presence or absence of inositol phosphates ($50 \mu\text{M}$). SDS/PAGE was performed after exposure of membranes to u.v. light (254 nm) for 2 min (see the Methods section). (a) Incubation in the presence of $\text{Ins}(1,4,5)\text{P}_3$ prevents labelling of the M_r -107 000 protein (a). Lanes: 1, control; 2, + $\text{Ins}(1,4,5)\text{P}_3$. (b) $^{125}\text{I}\text{-ASA-InsP}_3$ labelling of M_r -107 000 protein (lane 1, control) was prevented by addition of $\text{Ins}(2,4,5)\text{P}_3$ (lane 2) and $\text{Ins}(1,3,4,5)\text{P}_4$ (lane 5), but not by $\text{Ins}(1,4)\text{P}_2$ (lane 4) or $\text{Ins}(1,3,4)\text{P}_3$ (lane 3). Data are representative of the following numbers of experiments for each condition: $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ (4), $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ (3) and InsP_4 (1).

tors of specific $[^3\text{H}]\text{InsP}_3$ binding (Table 2), prevented $^{125}\text{I}\text{-ASA-InsP}_3$ labelling of the M_r -107 000 band (Fig. 5b), $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,3,4)\text{P}_3$, which are poor inhibitors of $[^3\text{H}]\text{InsP}_3$ binding (Table 2), did not prevent labelling of the M_r -107 000 (Fig. 5b). In addition, in three experiments labelling of the M_r -107 000 protein was prevented by incubation with $100 \mu\text{g}$ of heparin/ml (result not shown), which is consistent with the inhibition of specific $[^3\text{H}]\text{InsP}_3$ binding by heparin in olfactory cilia (see above).

Dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$

To explore whether the binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to ciliary membranes could represent binding to a degradative enzyme, we characterized the $\text{Ins}(1,4,5)\text{P}_3$ -concentration-dependence of the phosphatase enzyme responsible for conversion of InsP_3 to InsP_2 (presumably the 5-phosphatase). The rate of InsP_2 formation was related by a simple Michaelis-Menten curve to the concentration of InsP_3 in the assay. The Hill coefficient for this relationship was not significantly different from 1 (1.1 ± 0.1), indicating that a single process (and hence a single binding site for InsP_3) was involved in the dephosphorylation reaction. The K_m of $29 \pm 2.4 \mu\text{M-InsP}_3$ was over 20-fold larger than the K_d for InsP_3 binding to the ciliary receptor ($1.1 \mu\text{M}$), suggesting that the binding of $\text{Ins}(1,4,5)\text{P}_3$ to cilia does not represent binding of InsP_3 to the phosphatase enzyme. The maximum activity of the enzyme was substantial ($15.2 \pm 0.36 \text{ nmol/min per mg}$ of protein). The

kinetic parameters ($V_{\max}$ and K_m) are similar to those reported for the InsP₃ 5-phosphatase in other tissues (Connolly *et al.*, 1985; Downes *et al.*, 1982).

DISCUSSION

In the present study we have found that olfactory cilia possess a specific site for binding of [³H]InsP₃, and we have identified, by photoaffinity labelling with the InsP₃ analogue ¹²⁵I-ASA-InsP₃, a ciliary InsP₃-binding protein with an apparent M_r of 107 000. The K_d for the InsP₃-receptor site in olfactory cilia is of the same order of magnitude as the concentration of InsP₃ needed to open InsP₃-gated channels in reconstituted ciliary membranes (Restrepo *et al.*, 1991). In addition, Ins(1,3,4)P₃ fails to inhibit specific Ins(1,4,5)P₃ binding (Table 2) and to prevent photoaffinity labelling of the M_r -107 000 InsP₃-binding protein in cilia (Fig. 5b), in agreement with the ability of Ins(1,4,5)P₃, but not Ins(1,3,4)P₃, to trigger an inward current in olfactory neurons under whole-cell patch-clamp (Restrepo *et al.*, 1991). Furthermore, binding of [³H]InsP₃ to cilia membranes and photoaffinity labelling of the M_r -107 000 protein share the same specificity (Table 2 and Fig. 5), and, as expected for a physiological receptor, the putative product of degradation of Ins(1,4,5)P₃ [namely Ins(1,4)P₃] displays low affinity for the binding site (Table 2), ensuring termination of the response after degradation of the InsP₃. It is unlikely that the specific binding of InsP₃ measured by us represents binding to the InsP₃ phosphatase enzyme, because heparin, which has been demonstrated in other systems to inhibit [³H]InsP₃ binding to specific receptors, but not to the InsP₃ phosphatase (Bootman *et al.*, 1990; Worley *et al.*, 1987b), displaces [³H]InsP₃ binding to olfactory cilia and because, as shown in the Results section, the K_m for the dephosphorylation reaction is one order of magnitude higher than the K_d for InsP₃ binding to the ciliary site. For these reasons, we conclude tentatively that the specific binding of [³H]InsP₃ to olfactory cilia represents binding to a protein of apparent M_r 107 000, which is perhaps identical with the ciliary InsP₃-gated channel identified by us in olfactory cilia (Restrepo *et al.*, 1990).

Several facts suggest that the InsP₃-receptor protein (presumably the InsP₃-gated channel) is located in the ciliary plasma membrane. (1) Addition of 50 µg of digitonin/ml increased specific binding by 2-fold. This is expected if the binding site is located in the ciliary membrane with an intracellular orientation, because the cilia fraction includes many vesiculated cilia in the outside-out configuration (Boyle *et al.*, 1987). An increase in the number of binding sites upon addition of digitonin would not be expected if the binding sites were located on the cytoplasmic face of an intracellular organelle. In fact, in agreement with this premise, Supattapone *et al.* (1988b) have shown that addition of detergent to microsomes does not increase the number of InsP₃-binding sites. (2) If the ciliary binding site were located in intracellular membranes closely associated with the cilia, InsP₃ would have been expected to release ⁴⁵Ca from the ciliary vesicles, because microsomal InsP₃ receptors have the InsP₃-binding site facing the cytoplasm, contrary to the experimental results described above. (3) If the ciliary InsP₃-binding site were located in a contaminating membrane, this membrane structure would have to be highly enriched with this site, in order to be able to account for the results in Table 1. This is unlikely, because electron-microscopic studies of the isolated olfactory cilia have indicated that the ciliary fraction is highly enriched with cilia which do not appear to possess any intraciliary vesicular structures (Boyle *et al.*, 1987). A ciliary membrane location for the InsP₃ receptor would be consistent with the fact that intracellularly applied InsP₃ induces an inward transmembrane current in olfactory neurons under whole-cell patch-clamp (Rest-

repo *et al.*, 1990), and with the fact that odorants which induce InsP₃ formation in catfish (Huque & Bruch, 1986) trigger an increase in intracellular [Ca²⁺] in isolated olfactory neurons which is due to transmembrane entry of Ca²⁺ and not to release of Ca²⁺ from internal stores (Restrepo *et al.*, 1990).

The design of InsP₃-receptor photoaffinity labels has focused on modification of the 2-hydroxy (Hirata *et al.*, 1989) or the 1-phosphate (Schafer *et al.*, 1990; Prestwich *et al.*, 1991) group. Although steric modification of these positions lowers receptor binding affinity, specificity for the InsP₃-binding site is retained. In contrast with the low- M_r proteins labelled in pancreatic cells by ¹²⁵I-labelled aminoethyl-tethered ASA-InsP₃ by the Mayr group (Schafer *et al.*, 1990), the aminopropyl-tethered ¹²⁵I-ASA-InsP₃ labels the high- M_r (230 000) InsP₃ receptor in rat cerebellum (Mourey *et al.*, 1991; the present work). Interestingly, the iodinated ASA-InsP₃ (a mixture of 5-iodo- and 3,5-di-iodo-ASA-InsP₃) has higher affinity for the rat InsP₃ receptor than does uniodinated ASA-InsP₃ (Mourey *et al.*, 1991; Prestwich & Marecek, 1991).

There are striking differences between the olfactory InsP₃-binding site and that of the brain binding site in catfish or those reported for microsomal InsP₃-binding sites in other tissues. The affinity of InsP₃ for the olfactory site is 30–1000 times lower than those in other tissues, and the stereospecificity of the two sites is different [compare the data in Fig. 3 and Table 2 with those in Guillemette *et al.* (1987, 1988), Spat *et al.* (1986) and Worley *et al.* (1987b)]. In addition, the apparent M_r of the InsP₃-binding protein is 107 000 for the olfactory protein and 260 000 for the rat cerebellar receptor (Supattapone *et al.*, 1988a). These facts strongly suggest that the olfactory InsP₃-binding protein is different from the cerebellar microsomal protein. The olfactory protein may possess a lower affinity because, since cilia have a very small internal volume, it is quite possible that activation of PtdIns(4,5)P₂ phosphodiesterase by olfactory stimuli may lead to large localized increases in the concentration of InsP₃. The large concentrations of InsP₃ in the cilia would quickly be lowered both through diffusion into the cell body and through action of the InsP₃ phosphatase, which displays a robust activity in this tissue (see the Results section).

The number of InsP₃ receptor sites found in the isolated olfactory cilia (17 pmol/mg of protein) is among the highest reported in any tissue and parallels binding to cerebellar membranes, which are thought to be enriched with the microsomal InsP₃ receptor (23 pmol/mg of protein). These high levels indicate that the InsP₃ receptor is highly enriched in olfactory cilia. The presence in olfactory cilia of a large number of InsP₃-receptor sites and of an InsP₃-binding protein of apparent M_r 107 000 (the present study), the demonstration of an odour-stimulated PtdIns(4,5)P₂ phosphodiesterase (Huque & Bruch, 1986; Boekhoff *et al.*, 1990; Breer & Boekhoff, 1991), the immunolocalization of a putative InsP₃-receptor site in rat cilia (Wood *et al.*, 1990), the direct opening of a Ca²⁺ and Na⁺ channel by InsP₃ in isolated cilia from the catfish (Restrepo *et al.*, 1990) and in excised inside-out patches of frog olfactory neuron membrane (Suzuki, 1991), and the enrichment of olfactory cilia plasma membrane with Ca²⁺-ATPase (Lo *et al.*, 1990) support a functional role for InsP₃ and Ca²⁺ in olfactory transduction. In addition, our results suggest that the ciliary InsP₃ receptor characterized by us may be the InsP₃-gated cation channel responsible for mobilizing Ca²⁺ and Na⁺ across the plasma membrane of olfactory cilia, leading to depolarization of the olfactory neuron upon stimulation with certain odorants.

We thank Dr. Joseph G. Brand and Dr. John H. Teeter for their critical reading of the manuscript and for suggestions. G.D.P. and J.F.M. thank the Center for Biotechnology and the New York State

Center for Science and Technology for financial support. This work was supported by National Institutes of Health grants DC-00566, DC-00327, DC-01228, DC-00356 and BRSO S07RR05825 and by a grant from the Veterans Administration (to J. G. Brand).

REFERENCES

- Boekhoff, I., Tarelius, E., Strotmann, J. & Breer, H. (1990) *EMBO J.* **9**, 2453–2458
- Bootman, M. D., Pay, G. F., Rick, C. E. & Tones, M. A. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1334–1339
- Boyle, A. G., Park, Y. S., Huque, T. & Bruch, R. C. (1987) *Comp. Biochem. Physiol.* **88B**, 767–775
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Breer, H. & Boekhoff, I. (1991) *Chem. Senses* **16**, 19–29
- Bruch, R. C. (1988) in *G-Proteins and Calcium Mobilizing Hormones* (Nacchache, P. H., ed.), pp. 123–134, CRC Press, Boca Raton
- Bruch, R. C. & Rulli, R. D. (1988) *Comp. Biochem. Physiol.* **91B**, 535–540
- Caprio, J. (1978) *J. Comp. Physiol.* **123**, 357–371
- Chadwick, C. C., Saito, A. & Fleischer, S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2132–2136
- Chen, P. S., Jr., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
- Connolly, T. M., Bross, T. E. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 7868–7874
- Downes, C. P. & Michell, R. H. (1982) *Biochem. J.* **198**, 133–140
- Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
- Ferris, C. D., Haganir, R. L., Supattapone, S. & Snyder, S. (1989) *Nature (London)* **342**, 87–89
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) *Nature (London)* **342**, 32–38
- Ghosh, T. K., Eis, T. S., Mullaney, J. M., Ebert, C. L. & Gill, D. L. (1988) *J. Biol. Chem.* **263**, 11075–11079
- Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8195–8199
- Guillemette, G., Balla, T., Baukal, A. J. & Catt, K. J. (1988) *J. Biol. Chem.* **263**, 4541–4548
- Harrison, S. M. & Bers, D. M. (1989) *Am. J. Physiol.* **256**, C1250–C1256
- Hirata, M., Watanabe, Y., Ishimatsu, Y., Ikebe, T., Kimura, Y., Yamaguchi, K., Ozaki, S. & Koga, T. (1989) *J. Biol. Chem.* **264**, 20303–20308
- Huque, T. & Bruch, R. C. (1986) *Biochem. Biophys. Res. Commun.* **137**, 36–42
- Kalinoski, D. L., Bruch, R. C. & Brand, J. G. (1987) *Brain Res.* **418**, 34–40
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lo, Y. H., Bradley, T. M. & Rhoads, D. E. (1990) *Chem. Senses* **15**, 608 (abstr.)
- Miyawaki, A., Furuichi, T., Maeda, N. & Mikoshiba, K. (1990) *Neuron* **5**, 11–18
- Morre, D. J. (1971) *Methods Enzymol.* **22**, 130–140
- Mourey, R. J., Estevez, V. A., Marecek, J. F., Prestwich, G. D. & Snyder, S. H. (1991) *Abstr. Soc. Neurosci.* **17**, 423
- Prestwich, G. D. & Marecek, J. F. (1991) in *Inositol Phosphates and Related Compounds: Synthesis and Therapeutic Potential* (Reitz, A., ed.), pp. 122–131, American Chemical Society, Washington, DC
- Prestwich, G. D., Marecek, J. F., Mourey, R. J., Theibert, A. B., Ferris, C. D., Danoff, S. K. & Snyder, S. H. (1991) *J. Am. Chem. Soc.* **113**, 1822–1825
- Raaflaub, J. (1956) *Methods Biochem. Anal.* **3**, 301–325
- Restrepo, D. & Teeter, J. H. (1990) *Am. J. Physiol.* **258**, C1051–C1061
- Restrepo, D., Miyamoto, T., Bryant, B. P. & Teeter, J. H. (1990) *Science* **240**, 1166–1168
- Restrepo, D., Huque, T., Kalinoski, D. L., Kumazawa, T., Miyamoto, T. & Teeter, J. H. (1991) *Chem. Senses* **16**, 403 (abstr.)
- Rhein, L. D. & Cagan, R. H. (1980) in *Biochemistry of Taste and Olfaction* (Cagan, R. H. & Kare, M. R., eds.), pp. 47–68, Academic Press, New York
- Rossier, M. F., Capponi, A. M. & Vallotton, M. B. (1989) *J. Biol. Chem.* **264**, 14078–14084
- Schafer, R., Nehls-Shabandu, M., Grabowski, B., Dehlinger-Kremer, M., Schultz, I. & Mayr, G. (1990) *Biochem. J.* **272**, 817–825
- Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W. (1986) *Nature (London)* **319**, 514–516
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988a) *J. Biol. Chem.* **263**, 1530–1534
- Supattapone, S., Danoff, S. K., Thiebert, A., Joseph, S. K., Steiner, J. & Snyder, S. (1988b) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8747–8750
- Suzuki, N. (1991) *Chem. Senses*, in the press (abstr.)
- Tones, M. A., Bootman, M. D., Higgins, B. F., Lane, D. A., Pay, G. F. & Lindahl, U. (1989) *FEBS Lett.* **252**, 105–108
- Wood, S. F., Ronnett, G. V. & Snyder, S. H. (1990) *Chem. Senses* **15**, 655 (abstr.)
- Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987a) *Nature (London)* **325**, 159–161
- Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987b) *J. Biol. Chem.* **262**, 12132–12136

Received 22 April 1991/10 July 1991; accepted 19 August 1991