NEURONAL INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR LOCALIZED TO THE PLASMA MEMBRANE OF OLFACTORY CILIA

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Abstract—Both the cyclic adenosine 3',5'-monophosphate and the phosphoinositide second messenger systems are involved in olfactory signal transduction. The inositol 1,4,5-trisphosphate receptor is one of the principal intracellular calcium channels responsible for mobilizing stored calcium. The precise location of the 1,4,5-trisphosphate receptor (endoplasmic reticulum vs surface) and its role in the events of olfactory signal transduction need to be defined. By light microscopical and confocal immunohistochecmy we show expression of the olfactory-enriched G-protein, Gαo, associated with cyclic AMP responses, and of the inositol 1,4,5-trisphosphate receptor in the dendritic projections and cilia of essentially all olfactory receptor neurons, indicating that individual neurons express components of both second messenger systems. By immunoelectron microscopy, we demonstrate that the inositol 1,4,5-trisphosphate receptor is localized to the surface membrane of rat olfactory cilia.

This is the first morphological demonstration of the surface membrane localization of inositol 1,4,5-trisphosphate receptor in olfactory cilia. Our findings, taken in conjunction with electrophysiological data from other workers, are supportive of the inositol 1,4,5-trisphosphate receptor playing a novel role in regulating calcium flux at the ciliary surface membrane.

Olfactory signal transduction, which occurs in the cilia of olfactory receptor neurons, appears to proceed through the generation of the intracellular second messengers, cyclic adenosine 3',5'-monophosphate (cAMP) and inositol 1,4,5-trisphosphate (InsP3).7,8,9,40,45,50,52,60 The membrane receptors, which are presumed to transduce the odorant stimulus, are members of a large gene family.10 The disposition of the two second messenger pathways in individual sensory neurons is unknown, but the potential for interactions between cascades within a cell is intriguing. Intracellular cAMP initiates the electrophysiological response by directly activating a non-specific cation channel located in the plasma membrane of the sensory cilia.13,16,32,43 The mechanism by which InsP3 elicits an electrophysiological response in this system may be through analogous channels in the plasma membrane gated by InsP3.50,51

In the brain and peripheral tissues, receptor-mediated stimulation of phospholipase C generates InsP3, which releases intracellular calcium (Ca2+). Abundant evidence indicates that InsP3 receptors are associated with non-mitochondrial Ca2+ stores localized to compartments of the endoplasmic reticulum (ER). Immunohistochemical analysis at the electron microscopic level has revealed that the InsP3 receptor is confined to the endoplasmic reticular membranes in Purkinje cells of the cerebellum.18,41,57,63 Heterogeneity in InsP3 receptor proteins has been described and arises from multiple genes.39,55,61 as well as alternatively spliced forms.12,42 In addition to an ER localization, studies from several laboratories have supported the existence of plasma membrane forms of InsP3 receptor in lymphocytes36,27 hepatic cells8 and most recently in lobster olfactory cilia.14

In the present study, we employ immunohistochemistry at the light, confocal and electron microscopic levels to determine the localization of InsP3 receptor in rat olfactory neuroepithelium and its potential association with the structures known to contain other components of the odorant transduction pathway, the olfactory cilia. Olfactory cilia consist of microtubules with a surrounding cytoplasmic matrix enclosed by a surface membrane.34,36 They lack ER or other internal membranous structures. We found that InsP3 receptor is enriched in the ciliary layer of the neuroepithelium and by immunoelectron microscopy confirmed that it was expressed by the ciliary structures rather than the microvillar processes of the non-neuronal cells. Our localization of InsP3 receptor, an integral membrane

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Abbreviations: cAMP, cyclic adenosine 3',5'-monophosphate; DAB, 3,3-diaminobenzidine tetrahydrochloride; EDTA, ethylenediaminetetra-acetate; ER, endoplasmic reticulum; Gαo, olfactory-enriched G protein; InsP3, inositol 1,4,5-trisphosphate; ORN, olfactory receptor neuron; P95, phosphate-buffered saline; PFA, paraformaldehye; SDS, sodium dodecyl sulfate.
protein, to olfactory cilia implies that this is a surface membrane form of the molecule. Our findings are consistent with the work of Fadool and Ache, who have recently reported functional studies of InsP-gated ion channels in the plasma membrane of cultured tobrus olfactory neurons. Taken together, the evidence suggests a novel mechanism of InsP action in signal transduction in the olfactory system.

**EXPERIMENTAL PROCEDURES**

**Immunohistochemistry**

Adult male Sprague-Dawley rats, 150–200 g (Charles River Laboratories, Wilmington, MA), were anesthetized and perfused transcardially with ice-cold phosphate-buffered saline (PBS) and then 4% paraformaldehyde (PFA) in PBS. Olfactory turbinates were dissected, postfixed for 1 h in 4% PFA and then immersed in 15% (w/v) sucrose overnight. Blocks were imbedded in Tissue-tek (Miles Laboratories) and stored at −70°C until sectioned at 8 μm on a cryostat.

For light microscopy, sections were rehydrated and blocked for 20 min in 10% normal goat serum then incubated with the primary antibody, affinity-purified rabbit polyclonal antibody raised to the cerebellar InsP receptor (Jackson Immuno Research Laboratories) and stored at −70°C until sectioned at 8 μm on a cryostat.

For double-labeling immunofluorescence, frozen sections were rehydrated, blocked in normal donkey serum and incubated simultaneously with the two primary antibodies: anti-goat immunoglobulin (H + L) and anti-rabbit immunoglobulin (H + L) both at 1:50 dilution (Jackson Immuno Research Laboratories). Control sections were incubated with preimmune sera in place of the primary antibodies. After washing, sections were mounted in 10% glycerol in PBS with 1 mg/ml p-phenylenediamine dihydrochloride. The confocal information was analysed using a Nikon Optiphot microscope module connected to a laser scanning confocal imaging system (Biorad MRC600). Computer-assisted analysis was taken simultaneously for two channels with a 514 nm argon ion laser excitation path between the two filters.

**Immunoelectron microscopy**

Animals were perfused as described above and dissection was performed to expose the nasal septum in the midline adjacent to the cribiform plate and olfactory bulb. A sheet of olfactory membrane measuring 2 mm by 2 mm was gently teased away from the underlying cartilage and placed in 4% PFA for 1 h postfixing. For DAB detection, tissue rinsed in PBS was immunostained intact in enzyme-linked immunosassay tray wells using the InsP receptor and Gαi rabbit polyclonal antibodies described above and the Vectastain Elite ABC Kit (Vector Laboratories). As controls, tissues were incubated with non-immune rabbit serum at 1:500 dilution instead of the primary antibody. After washing, the tissue was osmicated in 1% osmium tetroxide in maleate buffer, pH 5.2, for 1 h and washed in buffer for 30 min and subsequently treated with 0.2% polyethylene glycol for 1 h prior to dehydration in a graded series of methanol. The tissue was placed in 1:1 Epon in propylene oxide for 1 h and then in 100% Epon for 12 h. The tissue was flat embedded, hardened and the sections reoriented at 180° in a BEEM capsule to enable sectioning through the epithelium. Semithin plastic sections were taken until the region of interest was reached and then ultrathin sections were cut out with a diamond knife. Sections were collected on Formvar-coated slotted grids and examined using a Jelel 100 CX electron microscope.

For immunogold detection, a sheet of olfactory epithelium fixed in 4% PFA was dissected as described above and put through the osmication, dehydration and embedding process. Ultrathin sections were collected on nickel grids and etched in 10% H2O2 for 15 min. After washing, non-specific binding was blocked by incubation in 10% normal goat serum in PBS for 1 h. Primary antibodies were the InsP3 receptor and Gαi rabbit polyclonal antibodies described above used at concentrations of 30 μg/ml. As controls, sections were incubated with non-immune rabbit serum used at 1:500 dilution. As an additional control, immunoadsorption of each antibody against the insP receptor (0.2 ng) were solubilized in 1% SDS and 1% β-mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis on a 17% gel. The separated proteins were transferred to Immobilon (Millipore) according to the method of Towbin et al. and the membrane was probed with affinity-purified rabbit anti-InsP receptor antibody used at a concentration of 0.3 μg/ml. The secondary antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin, labeled with either 15 nm or 20 nm gold particles. AuProbe EM GAR (Amersham) used at 1:15 dilution. The sections were postfixed in 2% glutaraldehyde—hydrogen peroxide for 2 min, stained with 7% uranyl acetate and examined using a Jelel 100 CX electron microscope.

**Immunoblotting**

Total homogenates of adult rat olfactory tissue (225 μg), cerebellum (4 μg), a suspension of primary cultures of neonatal olfactory cells (30 μg) and purified cerebellar InsP receptor (0.2 μg) were solubilized in 1% SDS and 1% β-mercaptoethanol and subjected to SDS polyacrylamide gel electrophoresis on a 17% gel. The separated proteins were transferred to Immobilon (Millipore) according to the method of Towbin et al. and the membrane was probed with affinity-purified rabbit anti-InsP receptor antibody used at a concentration of 0.3 μg/ml. The secondary antibody was peroxidase-conjugated goat anti-rabbit immunoglobulin labeled with either 15 nm or 20 nm gold particles. AuProbe EM GAR (Amersham) used at 1:15 dilution. The sections were postfixed in 2% glutaraldehyde—hydrogen peroxide for 2 min, stained with 7% uranyl acetate and examined using a Jelel 100 CX electron microscope.

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[Jackson Immuno Research Laboratories]
Northern analysis

Poly(A)+ selected RNA (1 µg) was electrophoresed on a 1% agarose gel, blotted to Nytran (Schleicher and Schuell) and hybridized at 42°C for 20 h with a random primed 32P-labeled 1.8 kb polymerase chain reaction fragment containing 3'-carboxy terminal coding region of rat cerebellar InsP3 receptor (5908-7737 bp in the cDNA sequence) or a tubulin cDNA probe. The membrane was rinsed once at room temperature and then twice in 0.5 x standard saline citrate and 0.1% SDS at 65°C for 20 min. The blot was exposed at -70°C to X-ray film with an intensifying screen.

Inositol 1,4,5-trisphosphate binding assays

Binding assays were performed as described previously but with several modifications. Cerebellar tissue was placed in 50 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.4, and the following protease inhibitors: 0.2% Trasylol, 2 µg/ml leupeptin, 4 µg/ml antipain, 2 µg/ml chymostatin, 1 µg/ml pepstatin and 0.06% (w/v) phenylmethylsulfonyl acid. Tissue was homogenized in a polytron (Brinkman) and spun for 15 min at 17,000 r.p.m. in an SS-34 rotor. The membrane fraction was resuspended in Tris buffer, respun and resuspended to a final concentration of 0.3 mg/ml. Rat olfactory cilia were prepared as described previously but in the presence of protease inhibitors. The final cilia pellet was resuspended in Tris buffer containing protease inhibitors to a concentration of 0.3 mg/ml. Binding assays were performed in a 500 µl volume containing 50 µl [3H]InsP3 (NEN-DuPont) at the designated concentration, 50 µl unlabeled inositol phosphate as indicated, 250 µl of cerebellar membranes or cilia and 150 µl of Tris buffer. Incubation was for 10 min on ice in a Biovial (Beckman), after which the samples were spun at 12,000 r.p.m. for 15 min in an SS-24 rotor. The supernatant was aspirated and the pellets were solubilized in 0.2 ml of 1% SDS and counted in Formula 963 scintillation fluid.

RESULTS

Localization of inositol 1,4,5-trisphosphate receptor and Gαi in olfactory neuroepithelium

Gαi and the InsP3 receptor were localized in olfactory epithelium to assess the possible co-expression of components of both the cAMP and pertussis toxin-sensitive phosphoinositide pathways within the neuroepithelium. The antibody against Gαi was an antipeptide antibody described previously. Two polyclonal antibodies produced against purified cerebellar InsP3 receptor, one made in rabbit and the other in goat, were employed for InsP3 receptor immunohistochemistry. 54,55 For light microscopic immunohistochemistry, Gαi and InsP3 receptor immunoreactivity was examined in consecutive serial sections collected from representative regions of the olfactory neuroepithelium.

The olfactory neuroepithelium consists of three major cell types: olfactory receptor neurons (ORNs), the basal cells, which are a form of stem cell capable of replacing ORNs as they senesce, and the supporting or sustentacular cells. The ORN has a bipolar morphology, extending to the receptive surface a single dendritic projection terminating in a dendritic knob from which emanate non-motile cilia.

Within the olfactory epithelium, InsP3 receptor was found to be most highly enriched in the ciliary layer, which is visible bordered by the layer of dendritic knobs (Fig. 1A). The dendritic knobs were also immunoreactive. Immunoreactivity was also evident, but at lower levels, in the outer third of the epithelium, a region containing sustentacular cells as well as the dendritic projections of ORNs. We also observed very light levels of immunoreactivity in association with the cell bodies of some ORNs in the neuroepithelium and immunoreactivity of axon bundles just beneath the basal lamina.

Overall the distribution of Gαi was quite similar, being highly enriched in the ciliary layer (Fig. 1B). The most notable difference was that significant Gαi immunoreactivity did not extend beneath the ciliary layer into the underlying region of the neuroepithelium. Some cell bodies in the ORN layer were lightly immunoreactive for Gαi and axon bundles were faintly immunoreactive (Fig. 1B).

The localization of InsP3 receptor to the ciliary layer indicated that it must be present in the neuronal cilia or the microvilli of the sustentacular cells, or both, as these are the only structures present in this layer. To further address this question, we examined InsP3 receptor expression in neuron-depleted epithelium subsequent to surgical bullectomy. InsP3 receptor immunoreactivity was absent from the ciliary layer under these conditions, supportive evidence for a ciliary rather than microvillar localization (data not shown). The fainter immunoreactivity present beneath the ciliary layer was preserved in neuron-depleted epithelium, suggesting that this is contributed to by the apical regions of the sustentacular cells. In contrast, Gαi immunoreactivity is abolished by bullectomy.

In an extensive examination of the olfactory neuroepithelium, we found InsP3 receptor and Gαi to be distributed uniformly over the ciliary surface without evidence for regionalization of expression of either component. Additionally, there were no regions of the neuroepithelium found to be non-immunoreactive for either Gαi or InsP3 receptor. These results are consistent with findings for other second messenger components of the olfactory signal transduction cascade: the olfactory-specific, type III adenyl cyclase is also found to be uniformly distributed across the luminal surface of the neuroepithelium (Cunningham A. M. and Reed R. R., unpublished observations).

Co-expression of inositol 1,4,5-trisphosphate receptor and Gαi in olfactory neuroepithelium

To perform high resolution co-localization studies of Gαi and InsP3 receptor, we used a goat antibody against InsP3 receptor and a rabbit antibody against Gαi for double-labeling immunofluorescence. In phase confocal photomicrographs, the ciliary layer was found to be particularly clear and distinctive (Fig. 1C). InsP3 receptor was expressed in the ciliary layer across the neuroepithelium, but the immuno-
reactivity in the region of the epithelium just beneath the ciliary layer was comparatively more prominent using this goat antibody and immunofluorescent method of detection (Fig. 1D) than with DAB detection (Fig. 1A). In contrast, G_gap was found to be predominantly localized to the ciliary layer, with some light immunoreactivity also found in ORN cell bodies (Fig. 1E).

The co-expression of these components, as determined by confocal and light microscopy, in a pattern which was regular and continuous across the ciliary surface layer, strongly suggests that olfactory receptor cells in the epithelium express both InsP3 receptor and G_gap.

Localization of inositol 1,4,5-trisphosphate receptor to cilia by immunoelectron microscopy

Localization at the level of light and confocal microscopy demonstrated the presence of InsP3 receptor in the ciliary layer of the neuroepithelium, a region comprised of cilia emanating from the dendritic knobs of the sensory neurons and the microvilli of the sustentacular cells. To directly distinguish between a ciliary or microvillar immunolocalization, we utilized immunoelectron microscopy with both DAB and gold detection techniques.

Olfactory cilia are not homogeneous along their length, but consist of distinct proximal portions which are 2–3 μm long and ~0.3 μm in diameter, and much longer, 50–60 μm, thinner distal portions which are ~0.1 μm in diameter. Functional differences between these ciliary regions have been suggested on the basis of enzymatic cytochemical and immunoelectron microscopic localization studies of components of the transduction apparatus.

Immunoelectron microscopy using pre-embedding staining and DAB detection demonstrated InsP3 receptor immunoreactivity prominently in the dendritic knobs and their projecting thicker proximal ciliary segments as well as the thinner distal portions of cilia (Fig. 2B). Immunoreactivity was also found in the apical ER of the sustentacular cells. Similarly, G_gap immunoreactivity was found evenly distributed between the proximal thick and distal thin ciliary structures and the dendritic knobs, but was, in contrast, not present in sustentacular cells (Fig. 2C).

Neither G_gap or InsP3 receptor immunoreactivity was found in the microvilli. A control section which was incubated with non-immune rabbit serum at 1:500 dilution instead of the primary antibody is shown (Fig. 2A).

In our extensive study of this tissue, which was derived exclusively from the neuroepithelium lining the midline septum, we did not identify any dendritic knobs which were non-immunoreactive for the antigen being studied, either InsP3 receptor or G_gap. This finding is supportive of the fact that all dendritic knobs, and hence all ORN cells, in the regions examined express both InsP3 receptor and G_gap.

Although useful in determining patterns of expression at comparatively low magnifications, and in particular for confirming that all dendritic knobs in the section under study were immunoreactive, the relatively diffuse pattern of reaction product obtained with DAB detection did not enable us to determine the subcellular localization of immunoreactivity, in particular any association with the surface membranes. Accordingly, immunogold labeling was combined with a postembedding staining method. This procedure resulted in a significant loss of antigenicity for both InsP3 receptor and G_gap, as labeling with gold particles was light in comparison with the intensity of staining obtained with DAB, even at significantly higher antibody concentrations. This circumstance was most problematic for the InsP3 receptor antibody, where preservation of antigenicity was difficult, even for light microscopic immunohistochemistry, unless conditions were optimal. Nevertheless, levels

Fig. 1 Comparative localization of InsP3 receptor and G_gap in the olfactory neuroepithelium by DAB immunohistochemical light microscopy and double-labeling immunofluorescent confocal microscopy. (A) InsP3 receptor immunoreactivity visualized as gold/brown reaction product is most prominent in the superficial, wavy ciliary layer. The layer of dendritic knobs (indicated by three small arrows) is immunoreactive and the region of the epithelium just inward to this layer, containing the dendritic processes and the sustentacular cells, is also lightly immunoreactive. Deeper in the neuroepithelium just below the basal lamina, immunoreactivity is present in association with axon bundles. Light immunoreactivity is also present in some ORN cell bodies. (B) Ciliary layer; dks, dendritic knobs; ORN, olfactory receptor neuron cell bodies; bar, basal lamina. (B) G_gap immunoreactivity is predominantly confined to the ciliary layer with very light immunoreactivity of some cell bodies deep in the epithelium. A lightly immunoreactive axon bundle is present in the lower right region of the photomicrograph. A and B are not consecutive serial sections. (C) Phase-contrast photomicrograph corresponding to double-labeling immunofluorescence shown in D and E, demonstrating clearly the distinctive ciliary surface layer. (D) InsP3 receptor immunoreactivity (green) is present in the ciliary region, but there is also significant immunoreactivity in the epithelial inward to the ciliary layer, an area containing the bodies of sustentacular cells and the dendrites of ORNs. Faint immunoreactivity is also detectable in the ORN bodies deeper in the epithelium. It should be noted that in double-labeling experiments a different polyclonal antibody to the InsP3 receptor, produced in goat, was used compared to that shown in A. This goat antibody also showed significant immunoreactivity in the region of the sustentacular cells when used with light microscopy and DAB detection. (E) G_gap immunoreactivity (red) is highly localized to the surface ciliary layer with some faint immunoreactivity in the region of the ORN cell bodies. Scale bar in B = 10 μm (A, B); bar in E = 10 μm (C–E).
of labeling were sufficient to support a ciliary rather than microvillar localization which, together with the localization already provided by the light and confocal studies, supplies definitive evidence for a ciliary localization.

Immunogold visualization confirmed that InsP$_3$ receptor was localized to the dendritic knobs and cilia and was not present in the microvilli of sustentacular cells. All dendritic knobs identified in the sections were labeled for InsP$_3$ receptor. When cross-sections of thick proximal regions of cilia were identified and counted, 181 unlabeled cilia cross-sections and 42 labeled cilia cross-sections were identified in 38 fields (each with dimensions of 6.3 x 4.5 µm) selected at random. This analysis revealed that ~ 19% of cross-sections of cilia were labeled, which is consistent with our observation that generally every cluster of cross-sections identified (usually comprised of five to six cilia) showed one labeled cross-section. With labeled cilia, it was a consistent finding that gold particles were usually found in association with the surface membrane. Thinner distal regions of cilia were also able to be identified, particularly in longitudinal section, and were also labeled. The microvilli of the surrounding sustentacular cells were totally devoid of label for InsP$_3$ receptor, even when the concentration of the primary antibody was increased four-fold.

Some representative examples of our findings are presented in Fig. 3. A control section which was incubated with non-immune rabbit serum was devoid of labeling. This result is shown (Fig. 3A) with a dendritic knob situated between two sustentacular cells and both cilia and microvilli being evident. Immunoadsorption controls were devoid of labeling (data not shown). Gold particle labeling for InsP$_3$ receptor, although at low levels, was present throughout the dendritic knob and is seen on proximal cilia emanating from the knob (Fig. 3D). The thinner continuation of a ciliary process is present in the top part of the photomicrograph and is clearly labeled. In this figure, most of the remaining gold labeling is associated with fragments of cilia. Microvilli are seen

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Fig. 2. Localization of InsP$_3$ receptor and G$_o$ in olfactory cilia by immunoelectron microscopy using pre-embedding immunoperoxidase staining and DAB detection. (A) Electron micrograph of a control section incubated with non-immune rabbit serum instead of the primary antibody. Two dendritic knobs are present (one indicated by a large arrow) characterized by their electron-dense basal bodies and projecting thick proximal cilia. The somata of sustentacular cells and ORNs are present inferiorally. The network of fine structures around the dendritic knobs is composed of thick proximal cilia (small arrow), identifiable in cross-section by the characteristic microtubules arrayed inside, and the numerous smaller structures comprised of the thin parts of distal cilia and the microvilli of the sustentacular cells. Two distal cilia cut in cross-section with identifiable inner microtubules are indicated (arrowheads). (B) InsP$_3$ receptor immunoreactivity, indicated by the diffuse dark reaction product, is present in the dendritic knob (large arrow) and is prominent in the thick proximal cilia seen in cross-section (small arrows), as well as thinner distal segments of cilia seen in cross-section (arrowheads). Microvilli, which can be identified in the lower left of the figure projecting from the cell surface between cross-sections of thick proximal cilia, are non-immunoreactive. Additionally, significant amounts of immunoreactivity are present in the dendritic projections of ORNs and the apical cytoplasm of sustentacular cells. (C) G$_o$ immunoreactivity is present in the dendritic knobs (large arrow) and in both the large proximal (small arrow) and smaller distal (arrowheads) cross-sections of cilia. Scale bar = 1 µm.
Inositol 1,4,5-trisphosphate receptor in olfactory cilia

Expression of inositol 1,4,5-trisphosphate receptor in olfactory tissue by immunoblotting and northern analysis

Immunoblot analysis of olfactory epithelium with the rabbit polyclonal antibody revealed a single 260,000 mol. wt band which is similar in size to the cerebellar protein, suggestive evidence that this antibody interacts selectively with InsP, receptor in olfactory tissue (Fig. 5a). InsP, receptor antigen was far more abundant in cerebellum than olfactory tissue, cerebellum being the richest source of the protein identified. Included in the analysis is purified InsP, receptor protein, against which the polyclonal antibody was produced, and primary cultures of neonatal olfactory neurons which have been shown to express InsP, receptor.11 Northern analysis detected a single 10 kb InsP, receptor mRNA in olfactory tissue (Fig. 5b), which co-migrates with the message in cerebellum. Consistent with the immunoblot analysis, we found InsP, receptor mRNA to be far less abundant in olfactory tissue than in cerebellum.

Characterization of tritiated inositol 1,4,5-trisphosphate binding in olfactory cilia

Preparations of olfactory cilia have been shown by biochemical analysis to be essentially devoid of plasma membrane contamination.1 In addition, enzymatic assays were performed to compare ciliary preparations and various subcellular fraction components. These assays demonstrated that ciliary preparations are essentially free of plasma membrane or ER contamination (Ronnett G. V., unpublished observations).

The inositol phosphate binding specificity of InsP, receptor was examined in olfactory cilia and cerebellar membranes (Fig. 6). The data are derived from averages of duplicates from a single assay which was representative of experiments performed three times. [3H]InsP, binding was assayed at pH 7.4, where InsP, has a lower affinity for cerebellar InsP, receptors than at pH 8.3.12 [3H]InsP, was found to bind saturably to olfactory cilia. Scatchard analysis indicated a single binding site with a Kd of 160 nM, whereas in parallel experiments the Kd for cerebellar membranes was 60 nM. The inositol phosphate specificity of the binding in cilia was the same as in cerebellum, with Ins(1,3,4)P, Ins(2,4,5)P, and InsP, failing to significantly inhibit binding at 2 μM concentration. The relative Bmax for cilia is ~ 1/30 that of cerebellar tissue.

DISCUSSION

The most important finding of this study is the localization of InsP, receptor to olfactory cilia and its association with the ciliary surface membrane. This
Fig. 3. Immunogold electron microscopy showing the association of InsP₃ receptor with the surface membrane of olfactory cilia. (A) A control section which was incubated with non-immune serum shows no labeling with gold particles. A single dendritic projection and dendritic knob are illustrated centrally with sustentacular cells on either side. Thick proximal cilia project from the dendritic knob and a proximal portion of a cilium cut in cross-section is indicated with an arrow. Microvilli are seen projecting from the surface of the sustentacular cells. dp, dendritic projection; sc, sustentacular cell; c, proximal cilium; m, microvilli. As an additional control, incubation was performed with antibody preadsorbed with the InsP₃ receptor protein, and this resulted in no labeling with gold particles (data not shown). (B) InsP₃ receptor labeling with gold was sparse. Gold particles are seen scattered over the dendritic knob and on proximal cilia projecting from it. The thinner continuation of a ciliary process is present in the top part of the photomicrograph and is clearly labeled with a cluster of gold particles. Microvilli, which are present on both sides of the micrograph projecting from the surface of the sustentacular cells, were always unlabeled. There was some labeling of the apical regions of sustentacular cells present deeper in the cell bodies, not included in this electron micrograph. (C) Cilia cut in longitudinal or oblique section often showed gold particle labeling in association with the surface membrane (arrowheads). In addition, four gold particles are seen in association with a grazing cut through a ciliary membrane. (D–F) Similarly, thick proximal cilia cut in cross-section showed distinctive labeling in association with the surface membrane of the cilium (arrowheads). Microvilli, which are visible coursing between the cilia in D, and in a dense cluster (G), were always unlabeled. Scale bar = 0.4 μm (A, B); 0.25 μm (C–G).
localization suggests a novel mechanism for InsP₃-mediated signalling in the olfactory system. Our results provide the first demonstration of a neuronal form of InsP₃ receptor definitively immunolocalized to a site outside the ER or associated internal organelles.

In the CNS, InsP₃ receptors have been immunolocalized to the ER and associated structures. An initial report showed P₄₀₀, which is analogous to InsP₃ receptor, in the ER, postsynaptic densities and plasma membrane in mouse cerebellum, however, subsequent studies were unable to confirm a plasma membrane localization. Nonetheless, there is a growing body of evidence for a plasma membrane localization of InsP₃ receptors in non-neuronal cells. Whole cell patch recordings of lymphocytes demonstrated an InsP₃-gated calcium conductance in the plasma membrane. In addition, subcellular fractionation studies of liver revealed an enrichment of InsP₃ receptor in plasma membrane fractions. In agreement with these electrophysiological and biochemical findings, InsP₃ receptor has recently been localized to the plasma membrane in Jurkat cell T lymphocytes at the light microscopic level and is capped following concanavalin A stimulation. Our immunolocalization of InsP₃ receptor to the plasma membrane of cilia in conjunction with the recent report of functional InsP₃-activated channels in lobster ORNs convincingly demonstrates a unique role for InsP₃ receptor in this system in mobilizing extracellular Ca²⁺. These findings illustrate the necessity for us to revise our notion as to how this second messenger pathway may function in olfactory signal transduction, and provides a new level of complexity in the potential for interaction between the cAMP and phosphoinositide cascades within a given cell.

How might a plasma membrane InsP₃ receptor in olfactory cilia specifically participate in olfactory signal transduction? In signal transduction involving the phosphoinositide system, an initial release of intracellular Ca²⁺ is usually followed by a phase of Ca²⁺ entry from extracellular sources (for review see Ref. 5). Irvine and colleagues have suggested that InsP₃ is responsible for the movement of Ca²⁺ across the plasma membrane, while in some cells, it has been proposed previously that InsP₃ may act directly at the plasma membrane. Olfactory cilia are bathed in mucus containing millimolar Ca²⁺, thereby providing a large ionic gradient for Ca²⁺ flux. The
absence of ER or other intracellular membranes in cilia, coupled with the presence of InsP₃ receptor in association with the ciliary membrane, suggests that InsP₃ activates receptor-associated channels in the ciliary membrane and permits Ca²⁺ to enter from the external environment. The cell would then be depolarized in a concentration-dependent manner. This proposal is in keeping with the recent electrophysiological findings in lobster cilia. In addition, Ca²⁺ may play a vital role in either integrative or negative feedback controls of signal generation, as has been outlined previously. For instance, stimulation of the InsP₃ pathway and the subsequent increase in intracellular Ca²⁺ could block cAMP-induced cell depolarization via a Ca²⁺-activated phosphodiesterase, providing a negative feedback loop.

InsP₃ may feasibly play an analogous role in mobilizing external Ca²⁺ at other sites in the nervous system besides the olfactory neuron. In the retina and in various brain regions, InsP₃ receptor is known to be concentrated in nerve terminals, but a plasma membrane localization has not been established. In these areas, a surface InsP₃ receptor might potentially be found to play a role in Ca²⁺ fluxes associated with neurotransmitter release.

We also found InsP₃ receptor in the apical ER of the sustentacular cells. These cells are enigmatic and might function as glial-like cells or help in supportive functions such as maintaining the balance of electrolytes in the mucous layer. An olfactory-specific cytochrome P₄₅₀ and a UDP glucuronosyl transferase, thought to be involved in odorant clearance, have also been localized to the apical portion of the sustentacular cells. Our findings illustrate that there are at least two major separate pools of InsP₃ receptor proteins in the outer region of the olfactory epithelium: one associated with the cilia of the ORNs and the other in the sustentacular cell ER.

It is of interest to compare our pattern of localization of Gₛₐ with that reported by Menco et al. in a study that used the same Gₛₐ antibody, which was produced in our laboratory. Using a freeze-
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Fig. 6. Binding isotherms for the interaction of \([{}^{3}H]\text{InsP}_3\) with cerebellar membranes and isolated olfactory cilia. Binding analysis demonstrates that unlabeled \(\text{InsP}_3\) can displace \([{}^{3}H]\text{InsP}_3\) from cerebellar membranes with a \(K_D\) of 60 nM (○), while in cilia the \(K_D\) is 160 nM (●). Other inositol phosphates, \(\text{InsP}_4\) (□), \(\text{Ins}(1,3,4,5)\text{P}_4\) (■) and \(\text{Ins}(2,4,5)\text{P}_3\) (△) as indicated, cannot effectively displace \([{}^{3}H]\text{InsP}_3\) binding from ciliary or cerebellar membranes at 2 μM inositol phosphate (InsPO,). CRBLM, cerebellum.

Data are derived from a single assay performed in duplicate.

substituted, postembedding technique designed to preserve the delicate, distal parts of cilia, these authors compared the immunoreactivity of the distal vs the proximal ciliary segments for \(G_{\alpha}\). They concluded that there was significantly more antigenicity distally, hypothesizing that this may represent the primary site of signal transduction. Somewhat surprisingly, they found the dendritic knobs to label very lightly, at 20–40% the level of distal cilia, even though the dendritic knobs are intensely immunoreactive at the light microscopic level. For the purpose of our study, we were most interested in preserving the morphology and antigenicity of the dendritic knobs and their immediate surrounding structures, rather than contrasting regional differences in the cilium, and for this our technique proved very valuable. It is apparent that the technique used by Menco et al. may well be preferable for the selective preservation of regions of distal cilia. As we did not exhaustively compare the degree of labeling distally to proximally and distal ciliary structures were infrequent compared to proximal, we cannot exclude the possibility that \(G_{\alpha}\) may be expressed at a higher level distally. Certainly, the pattern of \(G_{\alpha}\) labeling we found by immunogold detection was consistent with the results we obtained with the DAB immunoelectron microscopic technique.

Binding studies using \([{}^{3}H]\text{InsP}_3\) binding to rat cerebellar membranes and isolated olfactory cilia were conducted to further characterize the receptor species localized in olfactory neuroepithelium. The rat cerebellar \(\text{InsP}_3\) receptor demonstrated a \(K_D\) of 60 nM in intact membranes at physiological pH, similar to that reported previously. The somewhat higher \(K_D\) of 160 nM for \(\text{InsP}_3\) binding in isolated cilia preparations is more in keeping with that seen in the plasma membrane of Jurkat lymphocytes. Should the \(\text{InsP}_3\) binding activity represent contamination from the ER fraction, one might expect the \(K_D\) to be similar to that seen in cerebellar membranes, or to indicate the presence of multiple forms. The lack of these findings suggests that the binding demonstrated in the ciliary preparations predominantly represents the binding activity in cilia. As found with the cerebellar form, \(\text{InsP}_3\) and other \(\text{InsP}_3\) analogs do not effectively compete for binding. Recently, an inositol phosphate binding activity has been reported in isolated catfish olfactory cilia. In this case, the relative affinities for \(\text{InsP}_3\) and \(\text{InsP}_4\) were similar and the apparent molecular weight of the species identified by photoaffinity labeling was 107,000. Similarly, in isolated rat olfactory cilia an inositol phosphate binding activity has been found and a species of mol wt 120,000 identified by photoaffinity labeling. The significance of the discrepancy between the catfish results and our data remains uncertain, but could at least in part be due to interspecies differences. The apparent lower molecular weight and higher \(K_D\) corresponding to the binding activity found by others in rat might reflect proteolytic degradation, as \(\text{InsP}_3\) receptors in different tissues of multiple species all have a mol. wt. above 200,000. Our finding that the olfactory form of the \(\text{InsP}_3\) receptor is represented on immunoblot by a band of similar size to the cerebellar form is in keeping with the recent work of Fadool and Ache. In cultured lobster ORNs, they identified a band migrating with an apparent mol. wt above 200,000 using an antibody produced to a mammalian \(\text{InsP}_3\) receptor, and additionally provide evidence that this antibody can selectively increase odor-evoked inward currents in their system. Multiple variants of \(\text{InsP}_3\) receptor have been reported and in olfactory neuroepithelial RNA, a splice variant, the short form, predominates (Cunningham A. M., unpublished observations). There is no evidence, however, based on our immunoblotting and northern data, for a unique species being present in olfactory tissue. These experiments included both the ciliary and the ER-associated pools of \(\text{InsP}_3\) receptor present in the neuroepithelium. Further studies are required, therefore, to determine if the surface membrane forms of the receptor might represent a distinct class of \(\text{InsP}_3\) receptor proteins.

The description by Buck and Axel of a large family of putative olfactory odorant receptor molecules has initiated intense interest in the expression of these receptors by olfactory sensory neurons. Previous evidence had suggested that both the cAMP and phosphoinositide systems were involved in signal transduction initiated by some odorants, although other workers have reported that a particular odorant stimulates one, but not both, pathways. A recent report has provided the first direct evidence that a member of this family of receptors can respond to...
odrant stimulation with generation of InSP$_3$.

Studies of 2-deoxyglucose uptake in olfactory bulb after stimulation with specific odorants, summarized by Shepherd and Firestein, have shown regionalization of odorant responsivity in the bulb. If a parallel regionalization of receptor expression exists in the neuroepithelium, it is feasible that components of second messenger pathways might also show some compartmentalization of expression. For this reason, we were interested in establishing the patterns of expression of InSP$_3$, receptor and G$_{olf}$ in the neuroepithelium. Double-labeling experiments established that both InSP$_3$, receptor and G$_{olf}$ were evenly distributed and co-expressed throughout the neuroepithelium, without evidence for regionalization of either component. We did not, however, specifically address the question whether an individual olfactory receptor neuron co-expresses both InSP$_3$, receptor and G$_{olf}$. It is feasible that individual neurons expressing either InSP$_3$, receptor or G$_{olf}$, but not both, could be randomly mingles in the neuroepithelium, and present the immunohistochemical appearance we found by light and confocal microscopy. We consider this circumstance highly unlikely since at the electron microscopic level we did not identify any dendritic knobs which were InSP$_3$, receptor- or G$_{olf}$-negative. Hence we can conclude that, in the areas of the neuroepithelium examined by electron microscopy, there are individual neurons which do co-express InSP$_3$, receptor and G$_{olf}$.

**CONCLUSIONS**

Our study provides the first direct evidence for a ciliary surface form of the InSP$_3$, receptor in the olfactory sensory neuron. These findings are consistent with the idea that this receptor plays a direct role in the entry of extracellular Ca$^{2+}$ during the initiation of the olfactory signal. Apart from the novel surface immunolocalization of InSP$_3$, receptor, our observation that InSP$_3$, receptor and G$_{olf}$ appear to occur in the same cells has important implications for olfactory signal transduction. It supports the notion that the large multigene family of G protein-coupled odorant receptors reported by Buck and Axel may utilize both second messenger pathways. The existence of multiple second messenger pathways in an individual neuron might reflect the expression of more than one odorant receptor gene in a single cell, or the fact that a single odorant binding to its receptor may potentially activate dual second messenger systems. Specificity in olfactory signal transduction may be enhanced by the interaction between second messenger pathways within a given cell.

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