

Identification of Second Messenger Mediating Signal Transduction in the Olfactory Receptor Cell

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ABSTRACT One of the biggest controversial issues in the research of olfaction has been the mechanism underlying response generation to odorants that have been shown to fail to produce cAMP when tested by biochemical assays with olfactory ciliary preparations. Such observations are actually the original source proposing a possibility for the presence of multiple and parallel transduction pathways. In this study the activity of transduction channels in the olfactory cilia was recorded in cells that retained their abilities of responding to odorants that have been reported to produce InsP_3 (instead of producing cAMP, and therefore tentatively termed “ InsP_3 odorants”). At the same time, the cytoplasmic cNMP concentration ($[\text{cNMP}]_i$) was manipulated through the photolysis of caged compounds to examine their real-time interactions with odorant responses. Properties of responses induced by both InsP_3 odorants and cytoplasmic cNMP resembled each other in their unique characteristics. Reversal potentials of currents were 2 mV for InsP_3 odorant responses and 3 mV for responses induced by cNMP. Current and voltage (I-V) relations showed slight outward rectification. Both responses showed voltage-dependent adaptation when examined with double pulse protocols. When brief pulses of the InsP_3 odorant and cytoplasmic cNMP were applied alternatively, responses expressed cross-adaptation with each other. Furthermore, both responses were additive in a manner as predicted quantitatively by the theory that signal transduction is mediated by the increase in cytoplasmic cAMP. With InsP_3 odorants, actually, remarkable responses could be detected in a small fraction of cells ($\sim 2\%$), explaining the observation for a small production of cAMP in ciliary preparations obtained from the entire epithelium. The data will provide evidence showing that olfactory response generation and adaptation are regulated by a uniform mechanism for a wide variety of odorants.

KEY WORDS: olfactory receptor cell • signal transduction • second messenger • cAMP • caged compound

INTRODUCTION

Olfactory sensation starts at the ciliary membrane of the olfactory receptor cell. It has long been believed that this signal transduction is mediated by two parallel pathways (Ache and Zhainazarov, 1995; Schild and Restrepo, 1998). The generally accepted view is that the olfactory receptor cell selects either cAMP or InsP_3 as a second messenger, depending on the species of odorant molecules. In the cAMP pathway, odorant binding to the receptor protein triggers an activation of G-protein (G_{olf}), and in turn activates adenylyl cyclase (AC), which produces cAMP intracellularly. Cytoplasmic cAMP activates cyclic nucleotide-gated (CNG) cation channels directly. In addition, the resultant Ca^{2+} influx triggers the opening of Ca^{2+} -activated Cl^- channels (for review see Gold et al., 1990; Kurahashi and Yau, 1993; Firestein and Shepherd, 1995; Schild and Restrepo, 1998; Gold, 1999; Frings et al., 2000; Firestein, 2001). In contrast, the InsP_3 pathway uses completely independent molecules. The signal of receptor activation is transmitted to PLC via a G protein (presumably

G_q). As a result, the cytoplasmic InsP_3 concentration increases, which finally leads to the opening of plasma membrane cationic channels. Plasma membrane ion channels that are activated by cytoplasmic InsP_3 is unusual, but its presence has been reported for olfactory receptor cells in a wide variety of animals spanning from the invertebrate (lobster, Fadool and Ache, 1992) to the vertebrate (catfish, Restrepo et al., 1990; *Xenopus*, Schild et al., 1995; bullfrog, Suzuki, 1994; Kashiwayanagi et al., 1996; rat, Kaur et al., 2001).

The signaling pathway involving cAMP is now well established (Kurahashi and Yau, 1993; Firestein and Shepherd, 1995; Schild and Restrepo, 1998; Gold, 1999; Frings et al., 2000), but the involvement of InsP_3 is still controversial (Gold, 1999; see also Vogl et al., 2000; Kaur et al., 2001). This is because, especially at the single-cell level, there is very limited information regarding responses to odorants that have been shown to produce InsP_3 exclusively (tentatively termed “ InsP_3 odorants” throughout the text). The technical limitation has been mainly the low probability of single cell sensitivities to InsP_3 odorants (Lowe and Gold,

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Abbreviations used in this paper: AC, adenylyl cyclase; CNG, cyclic nucleotide-gated.

1993; Chen et al., 2000). Moreover, the fine structure of sensory cilia (0.2 μm diameter) has made experimental manipulations extremely difficult. In the present study, we show that sensory responses induced by InsP_3 odorants are actually generated by an increase in cytoplasmic cAMP. The activity of transduction channels in olfactory cilia was recorded while the cytoplasmic cNMP concentration ($[\text{cNMP}]_i$) was freely manipulated through the photolysis of caged compounds. This allowed us to examine cross-interactions between InsP_3 odorants and cytoplasmic cNMPs directly in real time. Responses induced by both stimulants were homologous in their characteristic properties and showed cross-adaptation with each other. Furthermore, both responses were additive in a manner as predicted precisely by the theory that signal transduction is mediated by cAMP. The present results provide strong evidence at the single-cell level that olfactory signal transduction is mediated by cAMP for a wide variety of odorants.

MATERIALS AND METHODS

Preparation

Olfactory receptor cells were dissociated enzymatically from the olfactory epithelium of the newt, *Cynops pyrrhogaster*. The experiments were performed under the latest ethical guidelines for animal experimentation at Osaka University, based on international experimental regulations. Dissociation protocols have been described elsewhere (Kurahashi, 1989). In short, the animal was cooled on ice and double pithed. The mucosae excised from the olfactory cavity were incubated for 5 min at 37°C in a solution which contained 0.1% collagenase (Sigma-Aldrich) with no added Ca^{2+} and Mg^{2+} (in mM): 110 NaCl, 3.7 KCl, 10 HEPES, 15 glucose, 1 pyruvate. All solutions were adjusted to pH 7.4 with NaOH. The tissue was rinsed three times with a normal Ringer's solution (in mM): 110 NaCl, 3.7 KCl, 3 CaCl_2 , 1 MgCl_2 , 10 HEPES, 15 glucose, 1 pyruvate, and triturated. Isolated cells were plated on the concanavalin A-coated glass coverslip. Cells were maintained at 4°C until use. In the present study, we selected olfactory receptor cells having more than five cilia.

Recording Procedures

Membrane currents were recorded with the whole-cell recording configuration (Hamill et al., 1981). Patch pipettes were made of borosilicate tubing with filament (outer diameter, 1.5 mm; World Precision Instruments) by using a two-stage vertical patch electrode puller (PP-830, Narishige Scientific Instruments). The recording pipette was filled with a solution containing (in mM): 119 CsCl, 1 CaCl_2 , 5 EGTA, 10 HEPES (pH was adjusted for 7.4 by CsOH) to suppress K channels that cause large current fluctuations. Normal Ringer's solution (for composition, see above) was used as the external solution for all recordings. The pipette resistance was 10–15 M Ω . The recording pipette was connected to a patch clamp amplifier (Axopatch 1D; Axon Instruments, Inc.). The signal was low-pass filtered at 0.5 kHz, digitized by an A/D converter (sampling frequency, 1 kHz) connected to a computer (PC9821; NEC). Simultaneously, signals were monitored on an oscilloscope and recorded on a chart recorder. Light and odor stimuli and the data acquisition were regulated by the same

computer using an original program. The results were analyzed by an offline computer and plotted by using Microcal Origin 6.0 software (OriginLab Corporation). For curve drawings, data sampled at 1/16 kHz were used. Experiments were performed at room temperature (23–25°C).

Odorant Stimulation

Lilial and lylal (donated by Takasago Co., Japan) were dissolved in DMSO and then diluted in the bath solution at 1 mM concentration with final DMSO concentration 0.2%. A possibility that DMSO (and/or its breakdown product) causes nonselective effects on the cell membrane has been excluded from two powerful bases; the presence of higher concentration of DMSO in the cytoplasm (see below) and heterogeneous responsiveness between lilial and lylal stimuli (see RESULTS). The stimulus solution was pressure applied to the cilia from puffer pipettes having a tip diameter of 1 μm . The tip of the puffer pipette was situated 20 μm from the tip of the dendrite. The pressure (maximum 150 kPa) was controlled by a computer-regulated pressure ejection system developed in our laboratory (Ito et al., 1995). There was a time lag between the actual chemical stimulation of the cell and the application of the TTL pulse to the magnetic valve. This time lag, being 60 ms on average, was estimated from the liquid junction current caused by the same application system.

Photolysis of Caged Compounds

Caged cAMP and caged cGMP (Dojin; Calbiochem) were used in the present study. As described before (Kurahashi and Menini, 1997; Takeuchi and Kurahashi, 2002), we saw no remarkable differences between cAMP and cGMP responses. The only difference was that a lower intensity of light was sufficient to evoke responses by caged cGMP than to evoke responses of similar amplitude by caged cAMP, presumably due to a higher sensitivity of CNG channels to cGMP than to cAMP. Caged cNMP was dissolved in DMSO and stored at -20°C in complete darkness. Under this condition caged cNMP can be stored up to 180 d without degradation. These stocks were diluted by Cs-containing pipette solution before each experiment, and the solution was put into the recording pipette. Final DMSO concentration was 1% in the pipette. After the establishment of whole-cell recording configuration, the cell interior was loaded with caged compounds by free diffusion. The presence of cytoplasmic DMSO (and its diffusion through the membrane) provided an experimental confidence excluding a possibility that external application of DMSO-containing stimuli (see above) did not cause nonselective effects on the cell membrane. The UV component from a Xenon lamp (100W, XPS-100; Nikon) was used for photolysis. The timing and duration of light illumination were controlled by a computer-regulated magnetic shutter (Copal). The intensity of the light was controlled by a neutral-density wedge filter having approximately a 2-log unit range. The wedge filter was connected to a pulse motor (UPK569H-NAC, 5 phases; Oriental Motor). To avoid vibration from movement of the shutter or the wedge filter, the light source, shutter and wedge filter were mechanically isolated from the microscope, and the light stimulus was guided by a quartz fiber connected to the entrance of the epi-fluorescence system, focused on the cell under recording (objective, 60 \times). By adjusting the diaphragm, the illuminated area was set at 40 μm in diameter, covering only the ciliary region. Light and odor stimuli were, unless otherwise indicated, applied with >20-s intervals in order to avoid adaptation of the system and depletion of the caged compound. The light intensities indicated in the text are relative values in reference to the maximum intensity being 1.0.

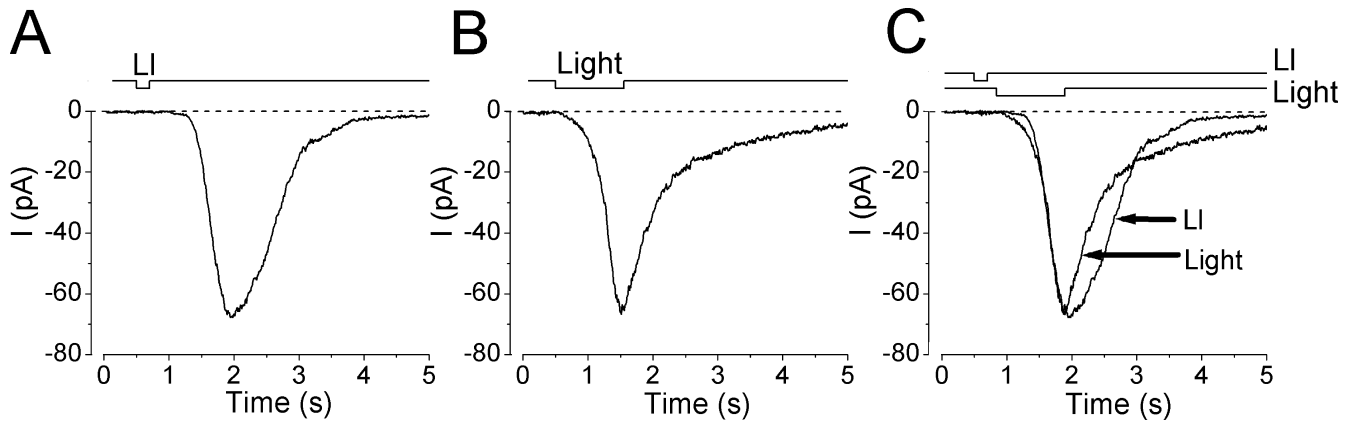


FIGURE 1. Membrane responses induced by InsP_3 odorant (lilial; abbreviated as LI) and light stimulation. (A) Whole-cell current induced by lilial. Holding potential (V_h), -54 mV. The cell was loaded with 1 mM caged cAMP. Downward deflection of the upper trace indicates the timing and duration of the odor stimulation (pressure 10 kPa, duration 200 ms). (B) Response induced by light (intensity 0.42, duration 1050 ms). Same cell as in A. (C) Superposition of data from A and B.

RESULTS

Responses Induced by InsP_3 Odorants and Cytoplasmic cNMPs

Whole-cell voltage-clamp recordings (Hamill et al., 1981) were made on isolated, single olfactory receptor cells that were intracellularly dialyzed by caged cyclic nucleotides (caged cNMPs). Sensory cilia of the cell

were stimulated by both UV light for photolysis and puff-applied odorants (lilial and/or lyral; both are known as InsP_3 odorants). All cells expressed a large inward current when $[\text{cNMP}]_i$ was elevated by UV illumination (Fig. 1; see also, Kurahashi and Menini, 1997; Takeuchi and Kurahashi, 2002). As has been reported previously (Lowe and Gold, 1993), however, a very small fraction of cells showed significant responses to

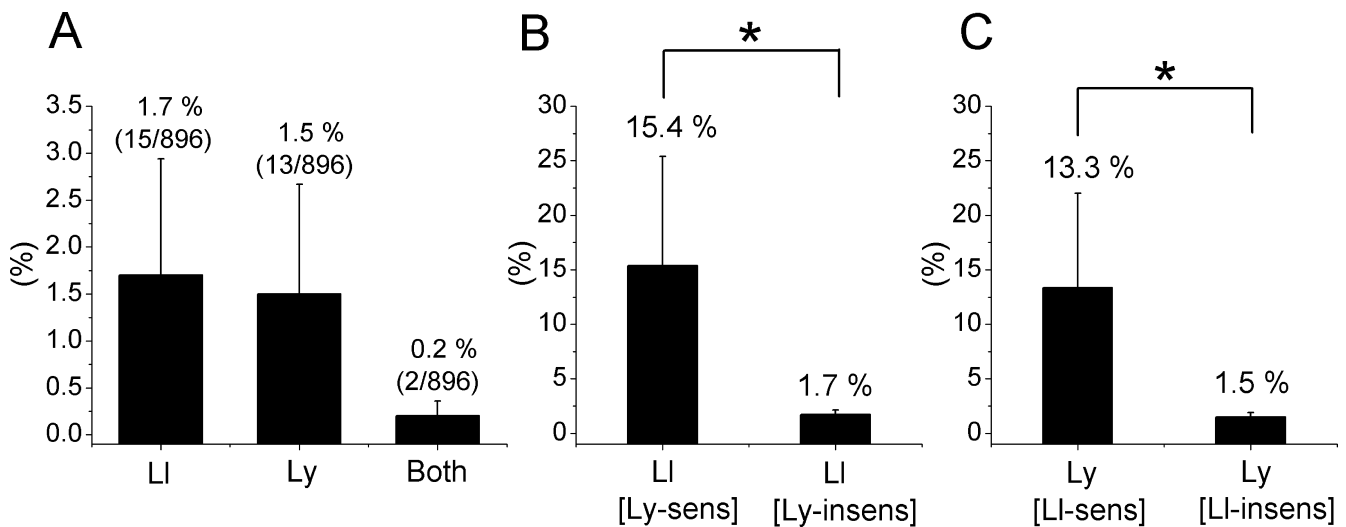
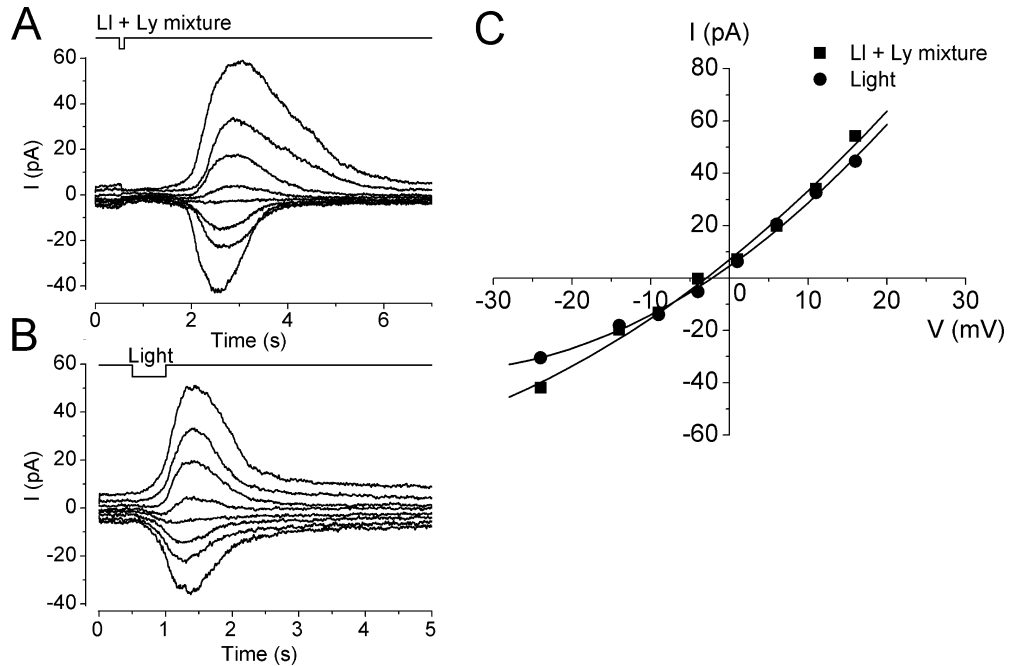


FIGURE 2. Response generative probability for two odorant stimuli including InsP_3 odorants (1 mM lilial and 1 mM lyral). Numbers in parentheses plated on the bars indicate the number of sensitive cells and total number of cells examined. Response generation was defined as an induction of inward current under whole-cell voltage-clamp condition (V_h , -54 mV), regardless of the absolute amplitude of the inward current (nor cells' sensitivity). Error bars indicate SD (σ) that was obtained by $\sigma = (pq/n)^{1/2}$, where p is the probability, $q = 1 - p$ and n is the number of cells tested. (A) Heterogeneous responsiveness of cells when stimulated by two different InsP_3 odorants (LI, cells responded to lilial alone; Ly, cells responding to lyral alone). (B) Probability of lilial-response occurrence in lyral-sensitive (Ly-sens) and lyral-insensitive cells (Ly-insens). Note that population of cells responding to lilial is larger in lyral-sensitive group than in lyral-insensitive group. In this kind of plot, it is assumed that there is a positive correlation when the left bar is bigger than the right one. In an opposite case (the left was smaller than the right), there is a negative correlation. The asterisk indicates the presence of statistical significances between these conditional probabilities with $p.c. < 0.05$. (C) Response probability to lyral in lilial-sensitive (LI-sens) and lilial-insensitive (LI-insens) cells. Data in B and C were obtained from A.

FIGURE 3. Voltage dependences of currents induced by InsP_3 odorants and photolysis of cytoplasmic caged cAMP. Mixture of 1 mM lilyal and 1 mM lylal was put into the puffer pipette. (A) Odorant-induced current recorded at various membrane potentials. V_h was changed from -24 to 16 mV. Downward deflections of the top trace indicate the timing and duration of the stimuli (pressure, 60 kPa, duration 100 ms). (B) Light-induced currents at various membrane potentials (intensity 0.27, duration 500 ms). The light condition was adjusted to induce a similar size of response as the odorant-induced response at -24 mV. (C) Current-voltage (I - V) relation of conductances activated by odor and light. Data from A and B.



lilyal (1.6%, examined in 1,569 cells, 1 mM concentration in the puffer pipette, with the real concentration stimulating the cells being much lower than this value), or to lylal (1.7%, in 896 cells, 1 mM in the pipette). When the same cell was stimulated by both lilyal and lylal, cell sensitivities were very heterogeneous (Fig. 2 A). Such heterogeneous responsiveness is obviously related to olfactory discrimination, and, therefore, provides convincing evidence showing that the inward current responses observed here are physiologically relevant. It is also interesting to note that lilyal responses were observed more frequently in lylal-sensitive cells than in lylal-insensitive cells (Fig. 2 B), and, vice versa, lylal responses were recognized more frequently in lilyal-sensitive cells than in lilyal-insensitive cells (Fig. 2 C). These results may suggest that there is a positive cross-correlation between lilyal and lylal sensitivities among cells.

Temporal Kinetics of Responses

By adjusting timing, intensity, and duration of the light pulse, we could obtain a light-induced current with a very similar rising phase as the odorant-induced response (Fig. 1 C). To do so, we had to set the onset of the light stimulation 302 ± 142 ms ($n = 7$, 60-ms delay has been subtracted) later than the onset of the odorant pulse. This is actually a unique property found in the cAMP system in which the time difference is responsible for the activation kinetics of receptor-cyclase enzymatic reactions. Besides, it has been known in the cAMP system that the waveform of current responses is

determined by the properties of the underlying enzymes. The rising phase is determined by the rate of cAMP formation, and by the very high cooperativity of the cAMP-triggered machinery (Takeuchi and Kurahashi, 2002). A slight delay in the odorant-induced current may be responsible for the suppressive effect of odorant molecules on the odorant-induced current (Kurahashi et al., 1994; see also Kawai et al., 1997).

The odorant-induced current lasted longer than the response induced by light (Fig. 1 C). A similar result was obtained in a similar kind of experiment with a cAMP odorant (Takeuchi and Kurahashi, 2002). For the cAMP enzyme system, it is assumed that the falling phase of the current induced by the cAMP odorant is specified by (a) the termination of AC activity, (b) the rate of cAMP hydrolysis by phosphodiesterase (PDE), and (c) by the Ca^{2+} -feedback system to the CNG channel (Chen and Yau, 1994; Kurahashi and Menini, 1997; Takeuchi and Kurahashi, 2002). Among these possibilities, (b) and (c) are common for both odorant and cAMP responses. Since the action of light stimulation is equivalent to the activity of AC, the difference in the falling phases has been explained in terms of the odorant-activated AC activity returning to its basal level gradually rather than by a sudden shutdown.

Current and Voltage Relationships

Fig. 3 shows the voltage dependence of odorant-induced and cAMP-induced currents obtained from the same cell. For both currents, the polarity was inward at negative potentials and reversed beyond ~ 0 mV. In

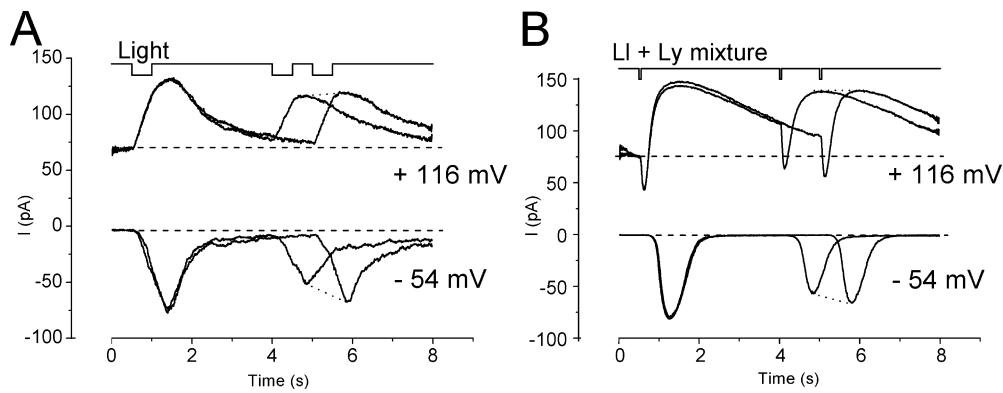


FIGURE 4. Adaptation of responses induced by light and InsP_3 odorants. (A) Two successive light pulses were applied with different intervals at -54 and $+116$ mV. Caged cGMP. Light intensity, 0.27. Duration, 200 ms. Note that the reduction in the response amplitude is not remarkable at $+116$ mV where no Ca^{2+} influx is expected. (B) Same kind of experiments as in A except that odorant (mixture of 1 mM

lilyl and 1 mM lyral) was used as stimuli. Different cell from A. At $+116$ mV, an inward current was induced at the beginning of the stimulation. This is due to the suppressive effect of odorants on basal current (Kawai et al., 1997), and on odorant-induced current (Kurahashi et al., 1994). Pressure 30 kPa. Duration, 50 ms.

general, the reversal potential is specified by the ion selectivity of ion channels underlying the system, and was actually very similar to each other (InsP_3 odorants, 1.5 ± 8.3 mV, $n = 8$; cNMPs, 2.8 ± 3.5 mV, $n = 11$). This suggests that both currents are carried by the same species of ions. Furthermore, the shape of the current-voltage (I-V) relation was very similar, showing a slight outward rectification. It seems likely that the electrical properties of ion channels underlying InsP_3 odorant responses and cNMP responses are the same.

Voltage-dependent Adaptation

It has been known that odorant responses mediated by the cAMP pathway show adaptation caused by Ca^{2+} feedback to CNG channels (Kurahashi and Menini, 1997) (see also Fig. 4 A). With InsP_3 odorants, response adaptation was observed as well (Fig. 4 B). Double

odorant pulses reduced the amplitude of secondary response at the negative potential. At $+116$ mV where very little Ca^{2+} influx is expected, however, the duration of current became prolonged and double odorant pulses caused similar amplitudes of the responses that were independent from the time intervals of double pulses. These observations suggest that InsP_3 odorant responses express adaptation with very similar properties as the cAMP response.

Cross-adaptation between Responses Induced by Odorant and cNMP

So far, we compared properties of responses induced by InsP_3 odorants and cytoplasmic cNMPs from several viewpoints. Very high similarities found in characteristic properties strongly suggest that both responses are mediated by the same enzymatic pathway. Next, two

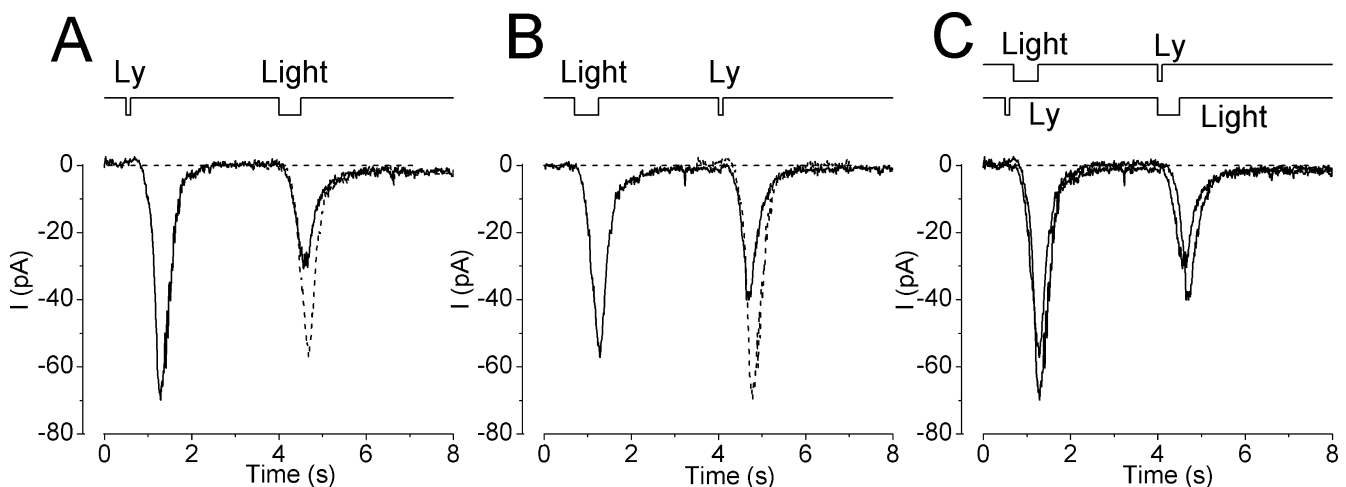


FIGURE 5. Cross-adaptation between InsP_3 odorant and light. With 1 mM caged cGMP. $V_h = -54$ mV. (A) Light stimulation was applied after lyral stimulation. A wave drawn by dashed line shows a light-induced current observed in the absence of conditioning pulse. (B) Lyral stimulation was applied after light stimulation. (C) Superimposition of data from A and B.

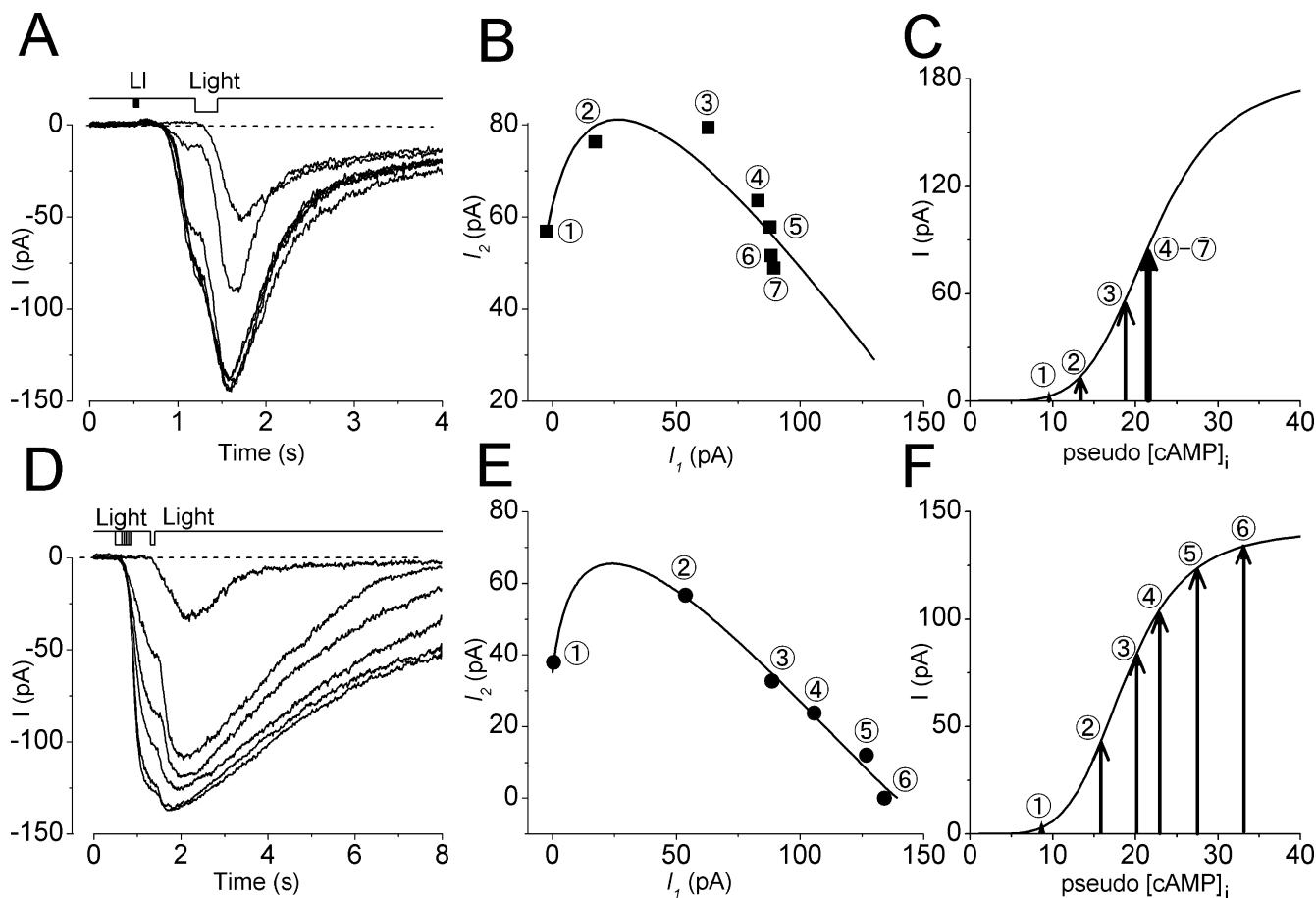


FIGURE 6. Summation of responses induced by InsP_3 odorant and light. The cell was loaded with 1mM caged cAMP. $V_h = -54$ mV. (A) Light stimulation was applied at the peak of the odorant-induced response. The light intensity (0.78) was kept constant, while the dose of the odorant stimuli was varied. Odorant used was liliac. (B) Amplitudes of light-induced responses (I_2) were plotted against the amplitude of the odorant-induced current (I_1). The solid curve was drawn by a formula (Eq. 2) in the text. (C) Dose-response relation of cAMP-triggered current. The curve was drawn by the Hill equation with $n_H = 5$ (obtained from Takeuchi and Kurahashi, 2002). In native cells $K_{1/2}$ is unknown, but we tentatively adopted 22 μM that has been obtained from excised-patch experiments (Kurahashi and Kaneko, 1991). Best data fitting was obtained when C_2 was 8 μM . Arrows indicate the estimated $[\text{cAMP}]_i$ that odorant stimulation produced. (D) Light stimulation was applied at the peak of the light-induced response. The intensity and duration of secondary light stimuli were kept constant (intensity 0.78, duration 100 ms), while the duration of the first stimuli were varied. Different cell from A. (E) Amplitudes of secondary light-induced responses (I_2) were plotted against the amplitude of the first one. The solid curve was drawn by the formula (Eq. 2) in the text. (F) The dose-response curve was drawn by the same protocol as in C. Arrows indicate the estimated $[\text{cAMP}]_i$ that the first light stimuli elevated.

kinds of experiments were performed in order to specify the actual second messenger mediating the response induced by InsP_3 odorants.

In one series of experiments, we observed cross-adaptation by applying both odorant and light pulses to the same cell alternatively (Fig. 5, A and B). First, an odorant pulse was applied to induced inward current response. Immediately after the termination of the odorant-induced current, a light pulse was applied to increase $[\text{cNMP}]_i$. The light-induced current was reduced to $\sim 55\%$ of its original amplitude. Similarly, the conditioning pulse using the light step reduced the odorant-induced response. If the odorant-induced response was mediated by the InsP_3 system that utilizes completely different molecules from the cAMP sys-

tem, such cross-reaction would not be observed. The straightforward interpretation is that the current induced by the InsP_3 odorant is actually evoked by cytoplasmic cAMP.

Nonlinear Summation

A direct relation between odorant- and cNMP-induced currents was further confirmed by another set of experiments in which $[\text{cAMP}]_i$ was elevated at the peak of the odorant-evoked response (Fig. 6 A). If both responses were generated independently, it was expected that light-induced currents would be unaffected by the odorant stimulation. First, we applied light stimulation causing a small amplitude of response at the basal level. Next, a small amount of odorant was applied to induce

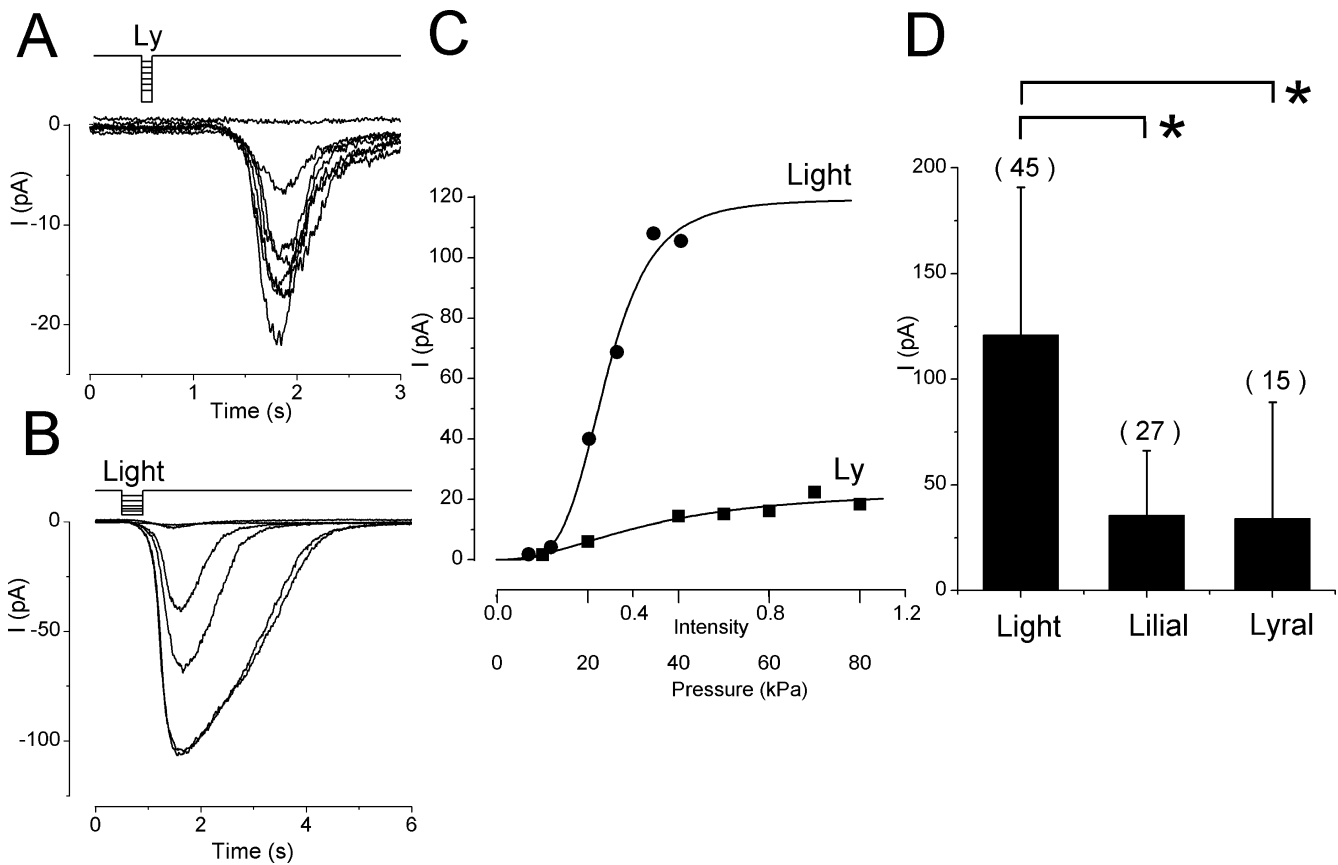


FIGURE 7. Dose dependence of the InsP_3 odorant- and light-induced responses in the same cell. The cell was loaded with 1 mM caged cGMP. V_h -54 mV. (A) Dependence of the odorant response on the pressure applied to the puffer pipette that contained 1 mM lyral. Downward deflections of the top trace indicate the amount of pressure, timing, and duration applied to the puffer pipette. (B) Intensity dependence of the light-induced current. (C) Pressure-response relation and intensity-response relation. Peak amplitudes of responses obtained in A and B were plotted against the pressure and the light intensity. The smooth lines were drawn by least square fittings of the data points by the Hill equation, $I = I_{max} \times P^{n_H} / (P^{n_H} + K_{1/2}^{n_H})$, where I is the current, P is the pressure of odorants or intensity of the light, $K_{1/2}$ is the half maximum intensity, and n_H is Hill coefficient. $I_{max} = 23$ pA, $K_{1/2} = 33.8$ kPa, and $n_H = 2.0$ for odorant-response and $I_{max} = 119$ pA, $K_{1/2} = 0.22$, and $n_H = 4.6$ for light-induced response. (D) Averaged amplitudes of maximum responses induced by light, lillal, and lyrall. Numbers appeared in parentheses are numbers of cells examined. Asterisks indicate the presence of statistical significance when examined with t test (p.c. < 0.05).

small amplitude of response, and, at the peak of the odorant-induced current, the same intensity of light was applied. Interestingly, the light-induced current was boosted in comparison with that induced at the basal level. However, further increase of odorant dose in turn caused a reduction in the light-induced current component. It thus seems likely that there are direct and nonlinear interactions between odorant-induced current and increases in $[\text{cAMP}]_i$. Actually, this nonlinear summation can be quantitatively accounted for by the fact that membrane current increases nonlinearly as a function of $[\text{cAMP}]_i$ (Lowe and Gold, 1993; Takeuchi and Kurahashi, 2002). The relation between current amplitude, I , and $[\text{cAMP}]_i$, C , is fitted by the Hill equation

$$I = I_{max} \times \frac{C^{n_H}}{C^{n_H} + K_{1/2}^{n_H}}, \quad (1)$$

where I_{max} is the maximum amplitude, n_H is Hill coefficient of the cAMP triggered system, and $K_{1/2}$ is the concentration causing half activation of the system. The parameter n_H has been found by our previous experiments to be five (Takeuchi and Kurahashi, 2002). Based on this, the relation between odorant-induced current (I_1) and light-activated current (I_2) is fitted by the formula

$$I_2 = -I_0 - I_1 + \frac{I_{max}}{1 + \left\{ C_2 + \frac{K_{1/2}^{n_H}}{\left(\frac{I_{max}}{I_0 + I_1} - 1 \right)^{1/n_H}} \right\}^{n_H}}, \quad (2)$$

where I_0 is the basal current and C_2 is the change in $[cAMP]_i$ by photolysis (Lowe and Gold, 1993). The relation between I_1 and I_2 showed an inverse U-shape and was fitted by this relation (Fig. 6 B). To confirm if such nonlinear summation actually occurs in the cAMP-mediated system in the olfactory cilia, double light stimuli were applied (Fig. 6 D). The relation again showed an inverse-U-shape and the data points could be actually fitted by the relation used for the previous analysis (Fig. 6 E). Nonlinear summation was observed when the same kind of experiment was made at +116 mV, where very little Ca^{2+} influx is expected (e.g., Takeuchi and Kurahashi, 2002). This observation excludes a possibility that nonlinear summation is caused by an additional effect of cytoplasmic Ca^{2+} (for detail, see DISCUSSION).

One may find it puzzling that the $InsP_3$ odorant-induced current did not reach the maximum amplitude of the cAMP-activated current even with a very strong stimulus. When we examined dose (for odor) and intensity (for light) dependences in the same cells, the maximum amplitude was actually smaller for odorant-induced current (Fig. 7). This can be simply explained, however, by the fact that $InsP_3$ odorants may activate AC only partially, presumably because of the low affinity of $InsP_3$ odorants for the receptor protein. To support this idea, we never saw that the odorant response exceeds the maximum amplitude of the light-activated current in the same cell. Even with cAMP odorants, we frequently saw that the maximum amplitude of the odorant-induced response did not reach the maximum amplitude of light-induced currents. Actually, the result obtained in the experiment of Fig. 6 A could be very well explained by the fact that the dose range for $[cAMP]_i$ elevated by the $InsP_3$ odorant was using only a partial region within the full dynamic range (Fig. 6 C compare with Fig. 6 F).

DISCUSSION

The present study shows that olfactory responses that have been thought to be mediated by PLC- $InsP_3$ pathway are actually mediated by the increase in $[cAMP]_i$. In this work, we employed a combination of patch clamp recordings and photolysis of cytoplasmic-caged compounds. The biggest advantage of using this combined technique is that contribution of cytoplasmic cAMP is evaluated in single, living cells that have retained their intrinsic functions. Fundamental properties (response kinetics, reversal potential, I-V relation, and Ca^{2+} -dependent adaptation) of responses induced by both cytoplasmic cAMP and $InsP_3$ odorants resembled each other in their unique characteristics. Furthermore, both responses expressed direct interactions; namely, symmetrical cross-adaptation and nonlinear summation.

The idea that the olfactory transduction system uses multiple pathways was originally derived from biochemical observations showing that certain types of odorant molecules do not produce cAMP when tested with a ciliary preparation fractionated from the whole olfactory sensory epithelia of the frog (Sklar et al., 1986). Later, those odorants that did not produce cAMP were found to produce $InsP_3$ instead (Boekhoff et al., 1990). In the present study, however, it was shown that the number of cells responding to $InsP_3$ odorants was extremely minor. Furthermore, the response amplitude was relatively small in comparison with that induced by cytoplasmic cAMP. Therefore, it is reasonable to think that the small increase in cAMP formation within entire epithelium when measured by a biochemical assay can be attributed to the small number (2%) of cells responding to $InsP_3$ odorants and to the partial activation of AC. With cAMP-producing odorants (e.g., cineole), in fact, the probability becomes as high as 25% (Takeuchi et al., 2003). Thus, the initial biochemical data for little cAMP production have now been very well explained with the cAMP theory at the single cell level. Actually, this idea has been proposed in a previous work done by Lowe et al. (1989). They have measured electro-olfactogram responses (total electrical activities of the olfactory mucosa, including the receptor potential of the olfactory receptor cell) to a wide variety of odorants, and have found that there is a positive correlation between the cAMP production rate and the amplitude of the electro-olfactogram.

Of course, however, the present data or results by Lowe et al. (1989) do not exclude the possibility that $InsP_3$ odorants do produce $InsP_3$ in the olfactory cilia. Those $InsP_3$ may contribute to some other functions of the olfactory receptor cell. It is also possible that $InsP_3$ is used in other chemosensory cells including vomeronasal organs or olfaction to amino acids in certain types of animals (e.g., lobster, Fadool and Ache, 1992; *Xenopus*, Iida and Kashiwayanagi, 1999; Manzini and Schild, 2003; fish, Restrepo et al., 1990; Ivanova and Caprio, 1993). Actually, the present experimental logic would provide powerful tools to evaluate the involvement of possible second messenger substances in those chemosensory cells.

The Total Numbers of Functional Receptor Proteins

We observed that cells responding to both lily and lilyal were only 0.2%. This statistical value may provide the absolute number of receptor proteins functionally expressed in the newt olfactory sensory cilia, based on the assumption that individual receptor cells express only one type of gene as the odorant receptor (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994). The simplest estimation is established when one assumes that the receptor protein that can

recognize both lily and lilyl is only one out of entire receptor proteins. In this case, the total number of receptor proteins is estimated to be 500.

Critically, however, this number may still be overestimation, because some fraction of dissociated cells may have lost their abilities of responding to any odorants; the total number of receptor protein becomes smaller, as the real response probability becomes higher. In the present experiments, the criteria we set for confirming cells' survivals were that (a) the standard morphology (especially, the presence of cilia), (b) responsiveness to cytoplasmic cNMPs, and (c) physiological expression of voltage-activated membrane currents. In fact, we still have to pay particular attention to the possibility that some steps of the transduction machineries have been destroyed in some fraction of cells examined. In addition, of course, the estimation for the total number is also dependent on the variation of receptor proteins that can recognize both lily and lilyl.

Although there are, thus, a number of assumptions in such an estimation, the value (e.g., ~ 500) seems to be quite consistent with those obtained from molecular biological experiments, especially when the variation among animal species are taken into account (several hundreds to 1,000 in rodents, Buck and Axel, 1991; 100 in fish, Freitag et al., 1998; 350 in human, Glusman et al., 2001; Zozulya et al., 2001)

Cross-correlation for Odorant Responses in Single Olfactory Receptor Cells

In the present study we observed that there was a positive cross-correlation between lily and lilyl sensitivities among cells; lily responses were observed much more frequently in lily-sensitive cells than in lily-insensitive cells, and vice versa lilyl responses were more frequently observed in lily-sensitive cells than in lily-insensitive cells. Interestingly, a similar cross-correlation in sensitivity has been found between the InsP_3 odorant (lily) and the cAMP odorant (lilyl, Takeuchi et al., 2003). The correlation within InsP_3 odorants is, however, much higher than that observed between lily and lilyl. Therefore, odorant molecules that have been subgrouped into InsP_3 odorants may have a strong positive cross-correlation in terms of the binding affinity to receptor proteins. Again, however, this kind of analysis is also dependent on how many cells sampled are physiologically functional in their transduction activities. In addition, unfortunately, the analyses were made only on a very limited number of odorant species. It would be worthwhile to further examine cross-correlations for responsiveness of cells to a wide variety of odorants, especially in relation to molecular structures and to qualities of smells.

Cross-adaptation between InsP_3 Odorant and Cytoplasmic cNMP

In previous experiments done by Kashiwayanagi et al. (1996), InsP_3 odorants and cAMP odorants did not show any cross-adaptation in the bullfrog olfactory receptor cell. Based on this observation, they concluded that responses induced by odorants like lily or lilyl were independent from the cAMP pathway in amphibia. In our present experiments, in contrast, responses induced by InsP_3 odorants and cytoplasmic cAMP expressed cross-adaptation with each other. Furthermore, our previous experiments have shown that InsP_3 and cAMP odorants do cause symmetrical cross-adaptations (Takeuchi et al., 2003). One remarkable difference in the experimental protocols is that our experiments were all done after we confirmed their dynamic ranges to avoid saturation, whereas their work did not pay attention to this point. If one uses an odorant dose at over-saturation, adaptational reduction in amplitude cannot be observed (see DISCUSSION of Takeuchi and Kurahashi, 2002). It would be important to reexamine bullfrog olfactory receptor cells after the odorant dose was set to a subsaturating level.

Involvement of Ca^{2+} -activated Cl^- Channels

One may think that Ca^{2+} -activated Cl^- channels are used as a common element between the cAMP and InsP_3 pathways. Because both CNG channels and InsP_3 -gated channels are thought to be Ca^{2+} permeable, it is possible that Cl^- channels located in the ciliary membrane can be driven by Ca^{2+} influx regardless of the species of underlying ionic channels. If there are common (or overlapping) elements between two systems, it is qualitatively possible that nonlinear summation is observed as has been observed in the present study. However, such a possibility could be excluded by the fact that nonlinear summation was observed when the same kind of experiment was made at +116 mV, where very little Ca^{2+} influx was expected (e.g., Takeuchi and Kurahashi, 2002). Actually, it is very unlikely that the kinetics of the total current become identical between the cAMP-mediated and the InsP_3 -mediated systems, even when they use common Cl^- channels as final outputs. Again, we emphasize that both signal transduction systems have been thought to be mediated by totally different enzymes.

Temporal Kinetics of Uncaging Responses

By looking at current waves evoked by light stimuli, one may realize that the current rising phase sometimes becomes steeper even after the light stimulus is shutdown (exampled in Fig. 7 B). It is therefore highly likely that cNMP production is still kept in the darkness. This may simply be due to (a) a dark reaction for uncaging. An-

other possibility is that (b) caged cNMP is present in the cell membrane with higher concentration than in cytoplasm, which causes delayed emission of free cNMP into the cytoplasm (Lowe and Gold, 1993). Ca^{2+} may also be responsible for this phenomenon, because the delayed influx of Ca^{2+} through CNG channels may cause a delayed activation of Ca^{2+} -activated Cl^- channels. However, Ca^{2+} dynamics alone cannot explain the phenomenon, because current rising after the cessation of light was observed even at +116 mV (Fig. 4 A). More systematic experiments would be necessary to understand the relation between uncaging and the current development.

cAMP as a Universal Second Messenger Mediating Olfactory Transduction

Since individual olfactory receptor cells express one type of receptor protein on their ciliary membranes and since two pathways have been thought to be activated by different odorous molecules, it has been generally believed that the AC-cAMP system and the PLC- InsP_3 system are segregated to different cells. Recently, Touhara et al. (1999) have shown that a certain type of olfactory receptor protein that recognizes lylal (named MOR23) can become functional by an adenovirus expression in olfactory receptor cells that are sensitive to forskolin. Forskolin is known as a specific activator for AC, and therefore it is interpreted that those cells that expressed MOR23 have intrinsic AC-cAMP systems. If the AC-cAMP system and the PLC- InsP_3 system were segregated to different cells, it is reasonable to think that MOR23 becomes functional having interaction with the AC-cAMP system.

There have been several approaches that have tried to identify the second messenger mediating olfactory transduction. In a series of studies using gene-targeting methods, electro-olfactogram responses to InsP_3 odorants were abolished or reduced in animals that lacked individual elemental proteins constituting the cAMP pathway (CNG channel, Brunet et al., 1996; G_{olf} ; Belluscio et al., 1998; type III AC; Wong et al., 2000). It has been always concerned, however, that protein disruption may have side effects to the function of receptor cells or animal bodies. In addition, it has been shown that the inhibitor of AC reduces the response induced by InsP_3 odorants (Chen et al., 2000). But the specificity of the inhibitor needs to be clarified. In contrast, in the present study the effects of cytoplasmic cNMPs in sensory cilia were directly examined in cells that retained their response abilities to odorants (including InsP_3 odorants). Furthermore, we have shown previously that responses induced by both cAMP and InsP_3 odorants resemble each other and show almost perfect symmetrical cross-adaptation (Takeuchi et al., 2003, see also commentary by Barry, 2003). The degree of cross-

adaptation between the cAMP odorant and the InsP_3 odorant matched exactly the same, which cannot be explained simply by coincidences in parallel systems. All together, it is highly likely that the main stream of olfactory signal transduction is uniformly mediated by cAMP for a wide variety of odorants through a universal second messenger.

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