Gated Conductances in Native and Reconstituted Membranes from Frog Olfactory Cilia

Tadashi Nakamura,*‡ Hyung-Hwan Lee,* Hirokatsu Kobayashi,* and Tomo-Oki Satoh*
*Department of Applied Physics and Chemistry, Faculty of Electro-Communications, and ‡Department of Information Network Science, Graduate School of Information Systems, University of Electro-Communications, Chofu, Tokyo 182, Japan

ABSTRACT Although cAMP is well established as a second messenger for olfactory transduction in vertebrates, the role of inositol 1,4,5-trisphosphate (IP_3) in this process remains controversial. We addressed this issue by comparing currents evoked by cAMP and IP_3 in native and reconstituted membranes from olfactory cilia. We detected only a cyclic nucleotide-gated conductance in the native membrane but both cyclic nucleotide-gated and IP_3-gated conductances in the reconstituted membrane. The magnitudes of the cyclic nucleotide- and IP_3-gated conductances were not correlated with each other in reconstituted membranes, suggesting that cyclic nucleotide- and IP_3-gated channels originate in different cellular compartments.

INTRODUCTION

cAMP is well established as an excitatory second messenger in vertebrate olfactory transduction. Odorants activate a G-protein-coupled adenylyl cyclase (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986; Breer et al., 1990), which causes the activation of depolarizing cyclic nucleotide-gated channels in the ciliary plasma membrane (see, e.g., Nakamura and Gold, 1987, 1988; Kolesnikov et al., 1990; Kurahashi, 1990; Bruch and Teeter, 1990; Firestein et al., 1991a, b; Kurahashi and Kaneko, 1991; Frings and Lindemann, 1991; Kleene and Gesteland, 1991a; Miyamoto et al., 1992; Kleene et al., 1994; Lowe and Gold, 1993a, b; Nakamura et al., 1994, 1995).

Ca^{2+} influx via the cyclic nucleotide-gated channels activates an additional inward current via Ca^{2+}-dependent Cl^- channels (Kleene and Gesteland, 1991b; Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993b; Kleene et al., 1994; Nakamura et al., 1995).

IP_3 has also been proposed to contribute to olfactory transduction based on the existence of G-protein-coupled, odorant-activated phospholipase C (Breer et al., 1990; Huque and Bruch, 1986; Boekhoff et al., 1990). Thus far, however, observations of the effects of IP_3 on membrane conductance have been variable, with some investigators reporting no significant IP_3-gated conductance in excised cilia (Kleene et al., 1994; Nakamura et al., 1995) or during whole-cell recordings (Firestein et al., 1991a; Lowe and Gold, 1993a; Nakamura et al., 1994) and others reporting a low density of IP_3-gated channels in excised patches of somatic membrane (Suzuki, 1994), small IP_3-evoked currents during whole-cell recording (Miyamoto et al., 1992), or IP_3-evoked currents in reconstituted ciliary membranes (Restrepo et al., 1990, 1992). Thus far, there is no satisfactory explanation for these disparate findings. Many of the data on IP_3-gated conductances were obtained with reconstituted membranes from the olfactory cilia of catfish (Restrepo et al., 1990) and rats (Restrepo et al., 1992). We have now compared the cyclic nucleotide-gated and IP_3-gated conductances detected in native and reconstituted frog ciliary membranes to gain insight into the sources of variability in the effects of IP_3.

MATERIALS AND METHODS

Bullfrogs anesthetized by cooling on ice were quickly decapitated and pithed, and their olfactory epithelia were dissected out. The conductance of the native ciliary membrane or the reconstituted membrane was measured in excised cilia (Kleene and Gesteland, 1991a) or by the tip-dip technique (Suarez-Isla et al., 1983; Coronado and Latorre, 1983) (electrode tip diameter ~0.5 μm). Membrane currents were measured with a patch clamp amplifier (CEZ-2300, Nihonkohden, Tokyo) during voltage ramps from −50 to +50 mV. The command voltage and the current sampling were controlled by a personal computer (PC-9801, NEC, Tokyo) equipped with analog-to-digital converters.

Native membrane

The olfactory epithelium was incubated with 0.07% papain (Calbiochem, San Diego, CA) for 10 min at 37°C under low divalent cation conditions (Nakamura and Gold, 1987). The tissue was then triturated to release solitary cells. The cells were plated onto a glass cover slip on the bottom of the recording chamber. The simplified saline (in mM: NaCl 120, HEPES 5, pH 7.4) was used as a control solution for the bath and the patch pipettes, unless stated otherwise. An entire cilium was sucked into the electrode as described by Kleene and Gesteland (1991a). Then we excised the cilium from the cell by briefly raising the electrode tip out of the solution.

Reconstituted membrane

A ciliary suspension was obtained from olfactory epithelia of a frog by the Ca^{2+} shock method (Chen et al., 1986). Briefly, the olfactory epithelia from one frog were stirred slowly in 5 ml of low Ca^{2+} solution (in mM:

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Address reprint requests to Dr. Tadashi Nakamura, Department of Applied Physics and Chemistry, University of Electro-Communications, Chofu, Tokyo 182, Japan. Tel.: 81-424-83-2161; Fax: 81-424-89-4666; E-mail: tad@is.uec.ac.jp.

Mr. Satoh's current address: Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Company Ltd., Tsukuba, Ibaraki 305, Japan.

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NaCl 100, EDTA 2, HEPES 30, pH 7.4) containing leupeptin (10 μg/ml) for ~2 min. Then the Ca^{2+} concentration was raised to 10 mM, and the stirring was continued for another 18 min. The detached cilia were collected by centrifugation (17,500 × g, 20 min) and resuspended in 0.5 ml of the bath solution (in mM: NaCl 110, KCl 3.5, $MgCl_2$ 1.6, $CaCl_2$ 1, MOPS 5.0, pH 7.4). Light microscopic observation revealed that more than half of the membrane fragments in the suspension kept the shape of the cilia. The suspension was sonicated (50 W, 40 s), 300 μl was transferred to a small Teflon chamber (6-mm diameter, 8-mm depth), and 30–40 μl of phospholipid (azolectin, Sigma Chemical Co., St. Louis, MO) solution (40 mg/ml in hexane) was then added to form a lipid monolayer at the air–water interface. Reconstituted bilayers were then formed from this monolayer by the tip-dip method (Suarez-Isla et al., 1983; Coronado and Latorre, 1983), i.e., by moving the patch electrode up and down across the monolayer. The electrode contained the bath solution.

RESULTS

Native membrane

Excised cilia responded to cAMP applied to the cytoplasmic surface by means of the bath solution. Figure 1 *a* shows representative records of conductance changes. We obtained each I-V curve by averaging 4–10 traces recorded consecutively. The control trace (Fig. 1 *a*, trace *1*) was recorded in the low Ca^{2+}, Mg^{2+} solutions (in mM: NaCl 118, EGTA 0.5, EDTA 0.5, HEPES 5, pH 7.4). Next, application of 200-μM cAMP caused a reversible increase in the conductance (Fig. 1 *a*, trace *2*). A cyclic nucleotide-gated conductance was observed in all stable excised cilia ($n > 400$). 50-μM IP_3 was then applied by means of the bath. The olfactory IP_3-gated channels are reported to be permeable to monovalent cations (Restrepo et al., 1992). Therefore, we tested 50-μM IP_3 in the absence of divalent cations (Fig. 1 *a*, trace *3*). However, IP_3 did not cause a significant increase in conductance. We tested a total of 15 cilia, none of which responded to IP_3 under these conditions. IP_3-gated channels are often tested with Ba^{2+} to increase the current (Kuno and Gardner, 1987; Restrepo et al., 1992; Suzuki, 1994). Therefore, we also tested IP_3 in the presence of 8.6-mM Ba^{2+} (cytoplasmic side). Ba^{2+} alone increased the conductance (Fig. 1 *b*, trace *2*), apparently by activating the Ca^{2+}-dependent Cl^- conductance in olfactory cilia (Kleene and Gesteland, 1991b). However, addition of IP_3 did not cause a further conductance increase (Fig. 1 *b*, trace *3*). In addition, we tested IP_3 in the presence of 10–50-μM Ca^{2+} because Ca^{2+} may be required for binding of IP_3 to the IP_3-gated channels (Kalinoski et al., 1992). Again we obtained I-V curves similar to those in Fig. 1 *b*, showing a Ca^{2+}-activated Cl^- conductance but no IP_3-gated conductance.

The lack of an observable effect of IP_3 under these conditions could conceivably be explained if the large Cl^- conductance prevented the IP_3 conductance from being detected. To test this possibility we added niflumic acid, a Cl^- channel blocker (Kleene, 1993), to the test solutions. In Fig. 1 *c* the niflumic acid blocked the Ba^{2+}-activated conductance (trace *3*), confirming that the Ba^{2+}-activated conductance was the Cl^- conductance. In the presence of both Ba^{2+} and niflumic acid, IP_3 again had no effect (trace *4*, and five other cilia).

Thus, we detected two conductances in the native ciliary membrane, the cyclic nucleotide-gated conductance and the Ca^{2+}- or Ba^{2+}-gated conductance, but failed to detect an IP_3-gated conductance in either the presence or the absence of Ca^{2+} or Ba^{2+}.

Reconstituted membrane

In 128 of 495 trials, we obtained stable lipid bilayers by the tip-dip method. The seal resistance of bilayers was 1.1–6.6 GΩ in the control solution (Fig. 2 *a*, trace *1*). The conductance of some of these membranes increased reversibly when 200-μM cAMP was applied through the perfusing solution (Fig. 2 *a*, trace *2*). This response to cAMP was

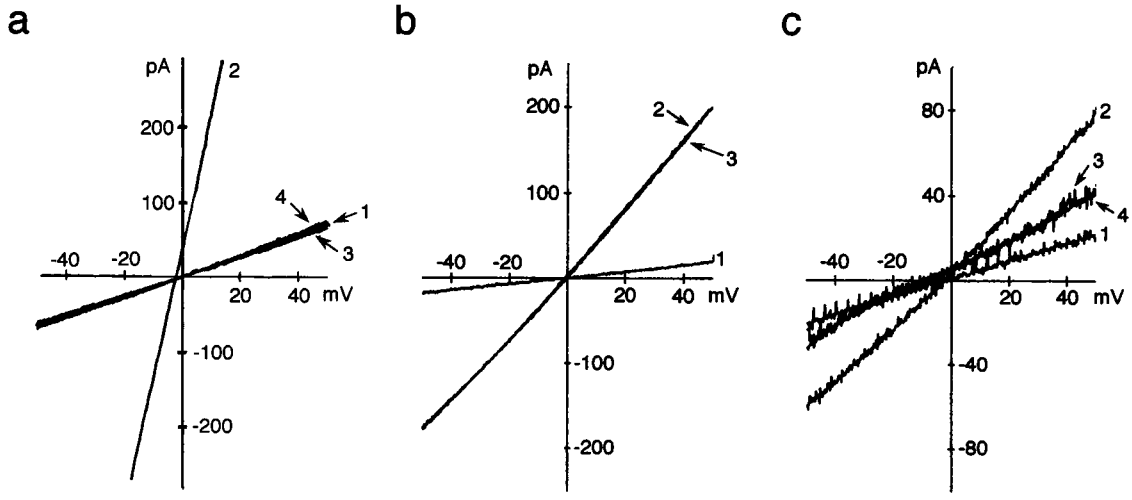

FIGURE 1 IV curves showing the gated conductances in a native ciliary membrane: (*a*) measured in the presence of 200-μM cAMP (*2*), 50-μM IP_3 (*3*), and control (*1* and *4*); (*b*) measured in the presence of 8.6-mM Ba^{2+} (*2*), 8.6-mM Ba^{2+} plus 50-μM IP_3 (*3*), and control (*1*); (*c*) measured in the presence of 8.6-mM Ba^{2+} (*2*), 8.6-mM Ba^{2+} plus 500-μM niflumic acid (*3*), 8.6-mM Ba^{2+}, 500-μM niflumic acid plus 50-μM IP_3 (*4*), and control (*1*).

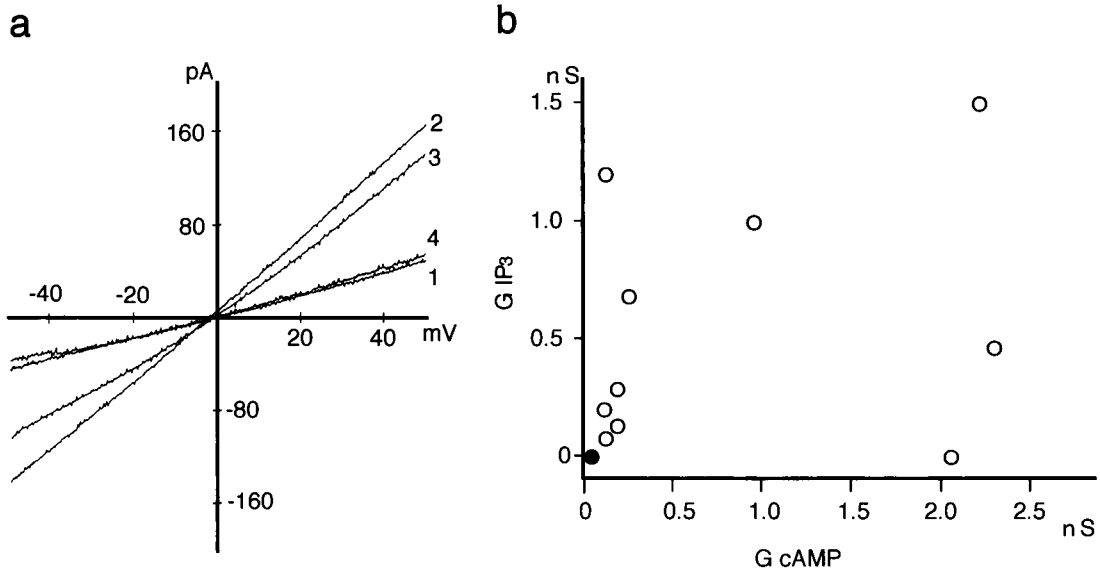

FIGURE 2 Gated conductances in reconstituted membranes from cilia-rich membrane fraction. (*a*) IV curves showing the conductances in a membrane. Measured in the presence of 1-mM cAMP (*2*), 50-μM IP_3 (*3*), and control (*1* and *4*). (*b*) Relationship between conductances gated by 200-μM cAMP (GcAMP) and 50-μM IP_3 (GIP_3). Data (12 points) are from Table 1. The filled circle represents two points within a very short distance.

observed in 16 of 128 bilayers (success rate 13%). Twelve of the cAMP-sensitive membranes were stable long enough to permit us to test IP_3 as well, and IP_3-activated currents were observed in eleven of them. IP_3 activated a conductance reversibly (Fig. 2 *a*, trace *3*), and the IP_3 dose-response curve showed saturation at more than 50 μM (data not shown), which is consistent with the previous study of reconstituted membranes (Restrepo et al., 1992).

In Fig. 2 *a*, the conductance increase by 50-μM IP_3 was similar to that caused by 200-μM cAMP. Table 1 lists the conductances observed in the 12 patches that were tested with both 200-μM cAMP (G-cAMP) and 50-μM IP_3 (G-IP_3). The magnitudes of the conductances activated by 50-μM IP_3 and by 200-μM cAMP were not correlated (r = 0.34; see also Fig. 2 *b*, which plots G-IP_3 versus G-cAMP) for each membrane. Because 1-mM Ca^{2+} was necessary to maintain the bilayer, we could not determine whether the reconstituted membranes also contained a Ca^{2+}-activated conductance.

TABLE 1 Conductance changes of the reconstituted bilayer membranes from the cilia-rich membrane fraction induced by 200-μM cAMP (GcAMP) or by 50-μM IP_3 (GIP_3)

Membrane No.	G cAMP* (pS)	G IP_3* (pS)	GIP_3/GcAMP‡
1	240	680	(2.84)
2	170	290	(1.70)
3	940	1000	(1.08)
4	97	197	(2.03)
5	170	128	(0.75)
6	18	5	(0.28)
7	2200	1500	(0.69)
8	110	1200	(11.5)
9	2280	460	(0.20)
10	20	10	(0.50)
11	106	77	(0.72)
12	2040	0	(0)

*Calculated from slopes of the IV curves.
‡The ratios indicate relative quantities of the two types of conductance incorporated into the bilayer.

DISCUSSION

In confirmation of earlier work (Kleene et al., 1994; Nakamura et al., 1995), we report that frog olfactory cilia contain a cyclic nucleotide-gated and a Ca^{2+}-dependent Cl^- conductance but not an IP_3-gated conductance. This argues against IP_3 playing a direct role in olfactory transduction in the frog, such as that shown for cyclic AMP in amphibians (Lowe et al., 1989; Kurahashi, 1990; Frings and Lindemann, 1991; Firestein et al. 1991a, b; Lowe and Gold, 1993a; Nakamura et al., 1994) and mammals (Lowe and Gold 1993b).

In contrast, we observed both cyclic nucleotide-gated and IP_3-gated conductances in reconstituted membranes (the presence of Ca^{2+}-dependent Cl^- channels was not tested for in the reconstituted membranes). Cyclic nucleotide-gated channels are known to be localized to the ciliary membrane (Kurahashi, 1990; Lowe and Gold, 1993a) and are present at high density in this part of the cell (Nakamura and Gold, 1988; Kurahashi and Kaneko, 1991). Therefore, the presence of cyclic nucleotide-gated channels in the reconstituted membranes may serve as a marker for the incorporation of ciliary membrane proteins into the lipid bilayer. If this is so, the lack of a correlation between the magnitudes of cyclic nucleotide- and IP_3-gated conductances in individual patches suggests that the IP_3-gated channels are not of ciliary origin. This interpretation pro-

vides a potential explanation for the failure of several groups to observe IP_3-gated conductance in the plasma membrane of olfactory receptor cells (Firestein et al., 1991a; Nakamura et al., 1994; Kleene et al., 1994; Lowe and Gold, 1993a; Nakamura et al., 1995). This interpretation is, in addition, consistent with previous evidence of nonciliary membranes in membranes isolated by the Ca^{2+} shock technique (Boyle et al., 1987; Anholt et al., 1986). Bruch (1989) has reported that a microsomal fraction contained IP_3-gated channels, and IP_3 receptors have also been demonstrated in supporting cells and within the olfactory epithelium (Cunningham et al., 1993). There is also evidence of IP_3-evoked Ca^{2+} release from intracellular stores (Sato et al., 1991; Nakamura et al., 1994). Thus, there are several potential nonciliary sources of IP_3-gated channels. Our procedure for cell isolation was essentially the same as that used for recording the IP_3-induced currents in whole-cell (Miyamoto et al., 1992) and in inside-out (Suzuki, 1994) configurations. Therefore, it is unlikely that our failure to observe an IP_3-gated conductance is due to our isolation technique.

Our results are consistent with reports of IP_3-gated channels in excised patches from somata of frog (Suzuki, 1994) and rat (Lischka et al., 1995) olfactory receptor cells. However, the odorant-evoked transduction current has been localized to the cilia in tiger salamander (Lowe and Gold, 1991), so the presence of IP_3-gated channels in the soma does not necessarily address their involvement in transduction. Cyclic nucleotide- and IP_3-gated channels have been shown to coexist in the plasma membrane of cultured lobster olfactory receptor cells, where the IP_3-gated channels mediate an inward (excitatory) current and the cyclic nucleotide-gated channels mediate an outward (inhibitory) current (Fadool and Ache, 1992). Although it was previously proposed that IP_3 mediates excitatory odorant responses (Restrepo et al., 1990), recent evidence suggests that IP_3 may mediate inhibitory odorant responses instead (Morales et al., 1995; Lischka et al., 1994). If this is so, then an analogous situation may exist in vertebrate and invertebrate olfactory receptors, with cAMP and IP_3 mediating responses of opposite polarity in each phylum but with cAMP mediating excitation and IP_3 mediating inhibition in vertebrates. It is not certain why we observed only an IP_3-evoked inward current, but the ionic channels that we observed in reconstituted membranes may not be the same ones that were observed by Lischka et al. In any case, if IP_3 is shown to mediate inhibitory odorant responses, this would support our hypothesis that the adenylnyl cyclase pathway mediates excitatory transduction for a wide variety of odorants (Lowe et al., 1989).

An IP_3-binding protein (IP_3 receptor) has been localized to the cilia both by photoaffinity labeling (Restrepo et al., 1992) and by immunohistochemistry (Cunningham et al., 1993). It remains possible that this protein is an IP_3-gated channel that is inactive under the experimental conditions that we and several others have used to test for the existence of IP_3-gated conductances. Future studies of the structure and function of this protein are needed to establish the function of IP_3 in olfactory transduction.

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