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# Adenylate Cyclase Mediates Olfactory Transduction of Amino Acid Responses in the Newt

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**ABSTRACT**—It has been reported that amphibians can smell not only airborne odorants but also amino acids. It is not clear, however, whether the signal transduction pathway of the amino acid responses is same as that of volatile odorant responses. In this study, we use patch-clamp recordings of newt olfactory receptor neurons to show that amino acid (200  $\mu$ M glutamic acid, acidic; 200  $\mu$ M arginine, basic; 200  $\mu$ M alanine or cysteine, neutral) responses are accompanied by inducing depolarizing currents. Moreover, responses to both amino acids and forskolin, a stimulator of adenylate cyclase, were observed in the same cells, which indicates that the cells responding to amino acids possess the cAMP-system. In addition, our EOG (electro-olfactogram) studies show that forskolin attenuates not only responses to volatile odorants, but also those to amino acids. These data provide evidence that the cyclic AMP system might underlie the signal transduction pathway of amino acid responses in addition to volatile odorant responses.

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## INTRODUCTION

While land animals use their olfactory system to perceive various airborne odorants, fishes sense water-soluble odorants, e.g. amino acids (Hara, 1994). On the other hand, it is known from early behavioral studies that amphibians, which are adapted to both aquatic and terrestrial life, are capable of smelling both volatile and water-soluble substances (Resses 1912; Copland, 1913). Recent studies on olfactory receptor proteins (ORP) using the techniques of molecular biology have provided evidence that amphibians have two distinct classes of gene-encoded ORPs; one group of ORPs is closely related to those of mammals and seem to be for the specific detection of volatile odorants, whereas another group is similar to those of fish and seem to be for the specific detection of water-soluble odorants (Freitag *et al.*, 1995; Freitag *et al.*, 1998).

It is unclear, however, whether the signal transduction cascade of the amino acid response is different from that of the response to volatile odorants. The mechanism of the electrical response to volatile odorants in olfactory receptor neurons (ORNs) has been intensely studied in wide variety of vertebrate species, including amphibians. It is known that odorants bind to ORPs and cause the activation of adenylate cyclase; the subsequent accumulation of cAMP opens cyclic nucleotide-gated ion channels (CNG channels). As a result,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  enter the cell through the CNG channels, producing a membrane depolarization. In addition,  $\text{Ca}^{2+}$  activates chloride channels, leading to an influx of  $\text{Cl}^-$  that further depo-

larizes the cell (Kurahashi and Yau, 1993; Lowe and Gold, 1993). It is now well established that cyclic AMP (cAMP) system underlies the response to volatile odorants (reviewed in Gold, 1999).

In contrast, how amino acids evoke responses in vertebrate ORNs is controversial. According to some studies in fish, odorants stimulate phospholipase C (PLC) activity and inositol trisphosphate ( $\text{IP}_3$ ) production (catfish: Restrepo *et al.*, 1990; Restrepo *et al.*, 1993; salmon: Lo *et al.*, 1994), and odorants do not stimulate rapid cyclic nucleotide synthesis at least in catfish (Restrepo *et al.*, 1993). These reports suggest that the  $\text{IP}_3$  system might underlie the amino acid response exclusively. Nevertheless, in zebrafish, a component of the cAMP system is reported to be present, and ORNs have a cAMP-conductance (Barth *et al.*, 1996; Ma and Michel, 1998). In the water nose of *Xenopus laevis*, it was reported that amino acid responses are not mediated by the cAMP transduction pathway (Iida and Kashiwayanagi, 2000). Iida and Kashiwayanagi (2000) show that ORNs in water nose have an  $\text{IP}_3$ -induced conductance, but amino acid responses are not accompanied by a conductance.

To study amino acid responses in amphibians, we have used newt, which is reported to respond to water-soluble odorants as well as to volatile odorants in an electro-olfactogram (EOG) study (Shibuya and Takagi, 1963). We have employed the EOG-recording method to show that forskolin attenuates the EOG response to amino acids. Forskolin directly stimulates adenylate cyclase (reviewed in Seamon and Daly, 1986), and hence the amplitude of odor responses should decrease based on the cAMP model of olfactory transduction (Gold, 1999; Nakamura and Gold, 1987; Lowe *et al.*, 1989). In the experiment using the whole-cell patch-clamp method, our

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results indicate that amino acids induce membrane depolarization and increase the depolarizing conductance of the cell.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Data is expressed as mean  $\pm$  standard error.

### Whole-cell patch-clamp recordings

The experimental procedure was similar to that described in Kurahashi (Kurahashi, 1989). Newts, *Cynops pyrrhogaster*, were obtained from commercial suppliers and kept at room temperature. After decapitation under anesthesia with ice water, whole olfactory epithelia were excised. The epithelia were treated with 0.2% collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 10 min at 35°C in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free saline of composition as described below except for Ca<sup>2+</sup> and Mg<sup>2+</sup>. After rinsing with normal saline (in mM: 110 NaCl, 2.5 KCl, 1.0 CaCl<sub>2</sub>, 1.6 MgCl<sub>2</sub>, 5 Na-HEPES; pH 7.6), the tissue was teased with forceps and gently triturated in a fire-polished Pasteur pipette. Cells were scanned on a microscope with phase-contrast optics and recordings were made with the whole-cell patch-clamp method. The patch pipette had a tip inner diameter of ca. 3  $\mu$ m and a resistance of 2 to 5 M $\Omega$ . Seal resistance upon establishment of a membrane patch was  $\sim$  10 G $\Omega$ . The standard pseudo-intracellular solution contained (in mM) 100 KCl, 2 MgCl<sub>2</sub>, 1.263 CaCl<sub>2</sub>, 5 EGTA, 0.05 Na<sub>2</sub>-GTP, 1 ATP and 10 HEPES (pH 7.6). Membrane potentials were corrected for the ca. -5 mV junction potential between internal and external solutions. After rupture of the patch membrane, the pseudo-intracellular solution diffuses into the cell, which causes the resting potential of the cell to drift (Tachibana and Kaneko, 1986). Thus, we measured the resting potential immediately after the rupture of the patch membrane. Recordings of odorant responses were made after the cell's membrane potential stabilized. The perfusion system was modified from the design of Hodgkin *et al.* (Hodgkin *et al.*, 1984). Solutions were fed by gravity into a four-way valve that was operated pneumatically under remote control by computer. The solution flowed continuously through an application pipette into an experimental chamber. After a patch pipette sealed onto a cell, the cell was moved into the outlet of the application pipette. The cells were continuously perfused with normal saline during the experiments except when the test solutions were applied. The test odorants were dissolved in normal saline. In this system, a complete solution change around the recorded cell could be achieved in 178  $\pm$  6.5 ms (n=10) as estimated from junction currents.

### EOG recordings

Procedure of recording EOG responses to water-soluble odorants was similar to that described in Shibuya and Takagi (Shibuya and Takagi, 1963). The newts were pithed and decapitated. The roof of a nasal cavity was removed, exposing olfactory epithelium. The EOG (Ottoson, 1956) was recorded with a glass pipette electrode that contacted on the center of olfactory epithelium. One EOG record was obtained from each animal. An electrode with tip diameter of about 100  $\mu$ m was filled with normal saline, and connected through an Ag/AgCl wire to a conventional amplifier. The head of the animal was mounted on a recording chamber, and solutions were applied via the four-way valve (described above) onto olfactory epithelium. The normal saline was applied during the experiments except when the test solutions were applied. All the odorants were dissolved in the normal saline.

## RESULTS

We obtained whole-cell patch-clamp recordings from 157 cells; the resting potential observed was -54.1  $\pm$  1.53 mV

(n=157). As reported in previous studies (e.g. Kurahashi, 1989), volatile odorants elicited membrane depolarization, and in addition, we observed amino acid induced excitation in a fraction of newt olfactory receptor neurons (Fig. 1). All the amino acids that we tested (200  $\mu$ M glutamic acid, acidic; 200  $\mu$ M arginine, basic; 200  $\mu$ M alanine or cysteine, neutral) elicited excitation, and patterns of the responses appeared to be cell- and odorant- specific (Fig. 1a and b). No morphological difference was observed between the cells that responded to amino acids and the cells that responded to volatile odorants.

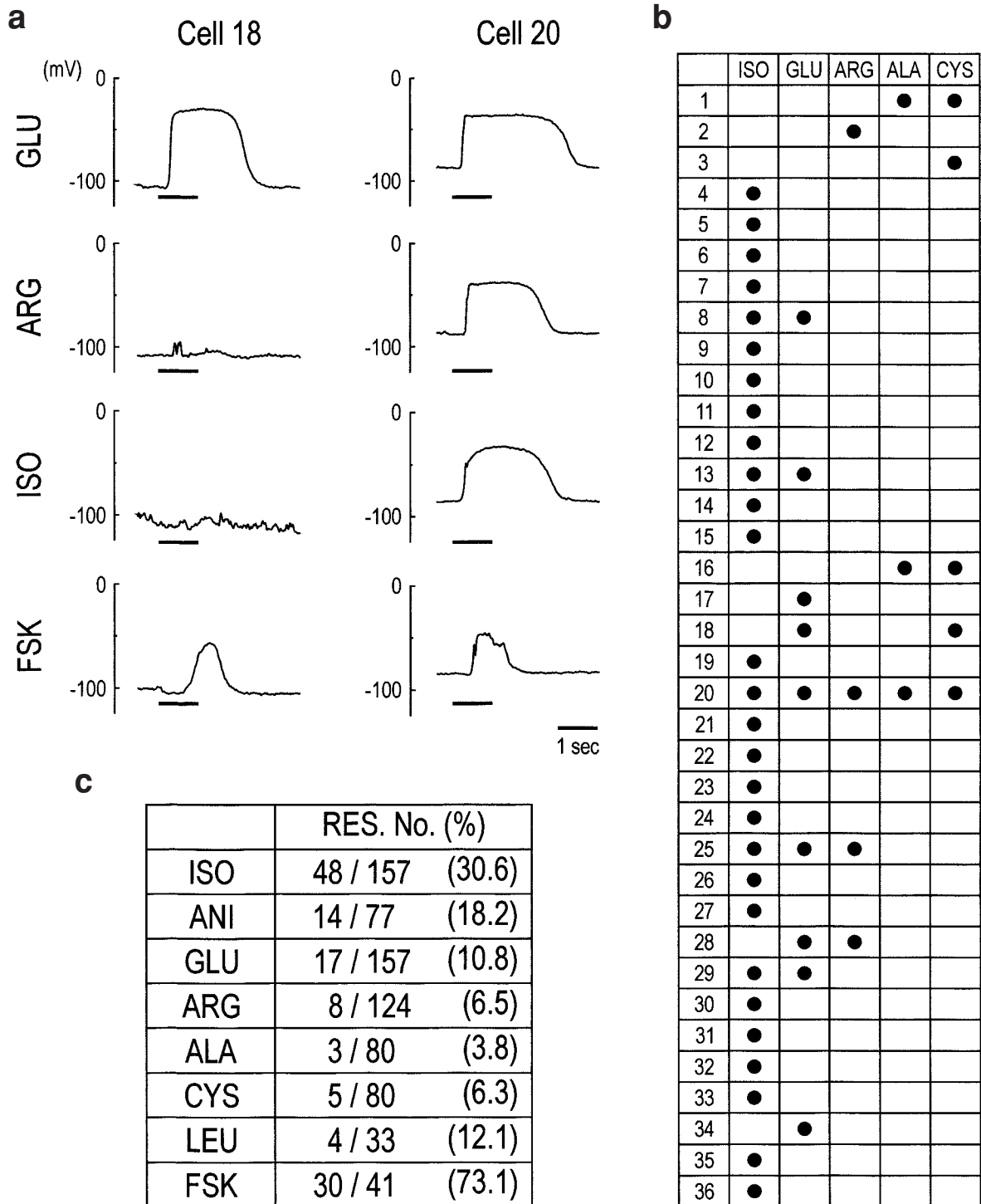
As shown in Fig. 1a and b, 5 cells out of 36 cells that responded to odorants responded to both amino acids and volatile odorants, while the other cells responded only to a volatile odorant (isoamyl acetate) or amino acid(s). Solid bars under the traces in Fig. 1a indicate the timings of odorant application, which was estimated from junction currents (see Materials and Methods). For example, Cell #18 in Fig. 1a responded only to glutamic acid while Cell #20 responded to glutamic acid, arginine, and isoamyl acetate. The mean latency of responses to glutamic acid was 133.6  $\pm$  81.03 ms (n=17).

Intracellular perfusion of cAMP elicited depolarizing current in ORNs (data not shown; study in newt previously reported by Kurahashi, 1990). In addition to odorant responses, application of 2  $\mu$ M forskolin also elicited membrane depolarization (Fig. 1a and c). The application of the adenylate cyclase activator forskolin elicited depolarization in all the cells that responded to amino acids (5 cells tested; e.g. "Cell 20" in Fig. 1a) or volatile odorants (12 cells tested). The responses to extracellularly applied forskolin showed a longer delay, because of the time needed for forskolin to permeate the cell membrane. The results suggest that the cells responding to odorants possess adenylate cyclase and a cAMP-induced conductance.

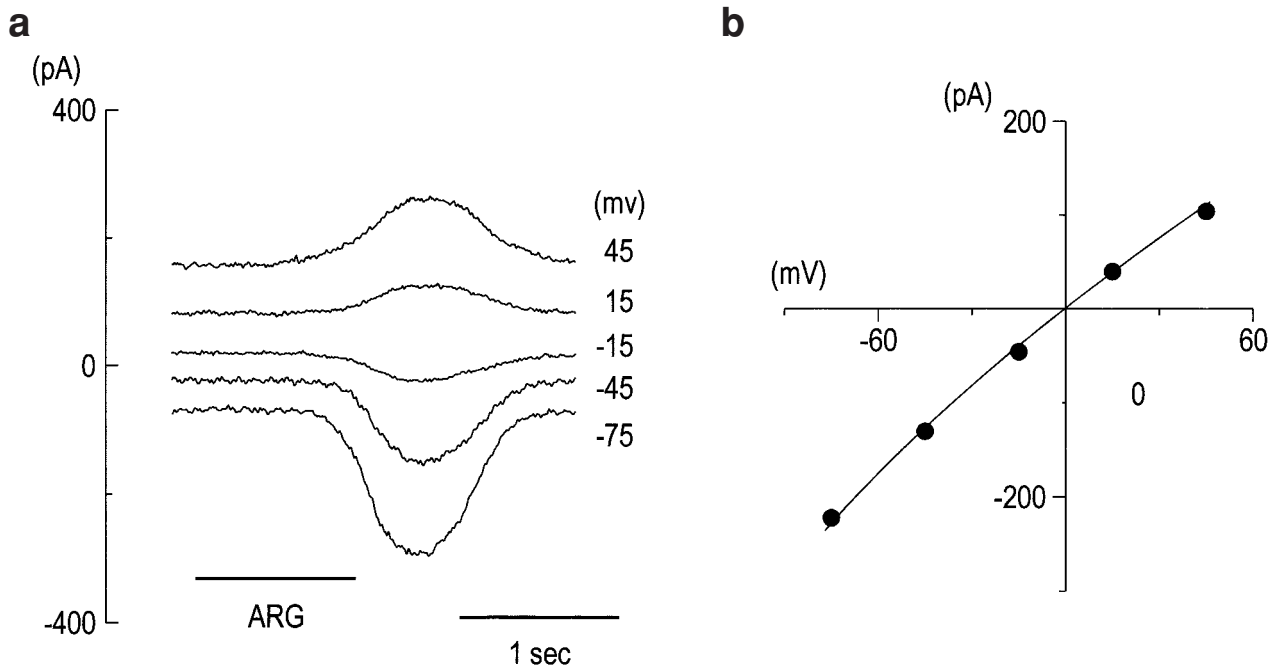
Furthermore, the amino acid responses were accompanied by a depolarizing conductance. In the experiment shown in Fig. 2, we employed the voltage-clamp method, and observed currents evoked by amino acids. Amino acids elicited depolarizing currents with a reversal potential of 2.8  $\pm$  3.9 mV (n=6) (The amino acids used were 200  $\mu$ M glutamic acid or arginine).

In Fig. 3, we recorded electro-olfactogram (EOG) (Ottoson, 1956), an extracellular field potential where amplitude reflects the summed responses of individual ORNs. In order to examine the effects of forskolin on responses to amino acids, we employed the EOG recording method, which allowed us to measure simultaneously activities of many cells in olfactory epithelium.

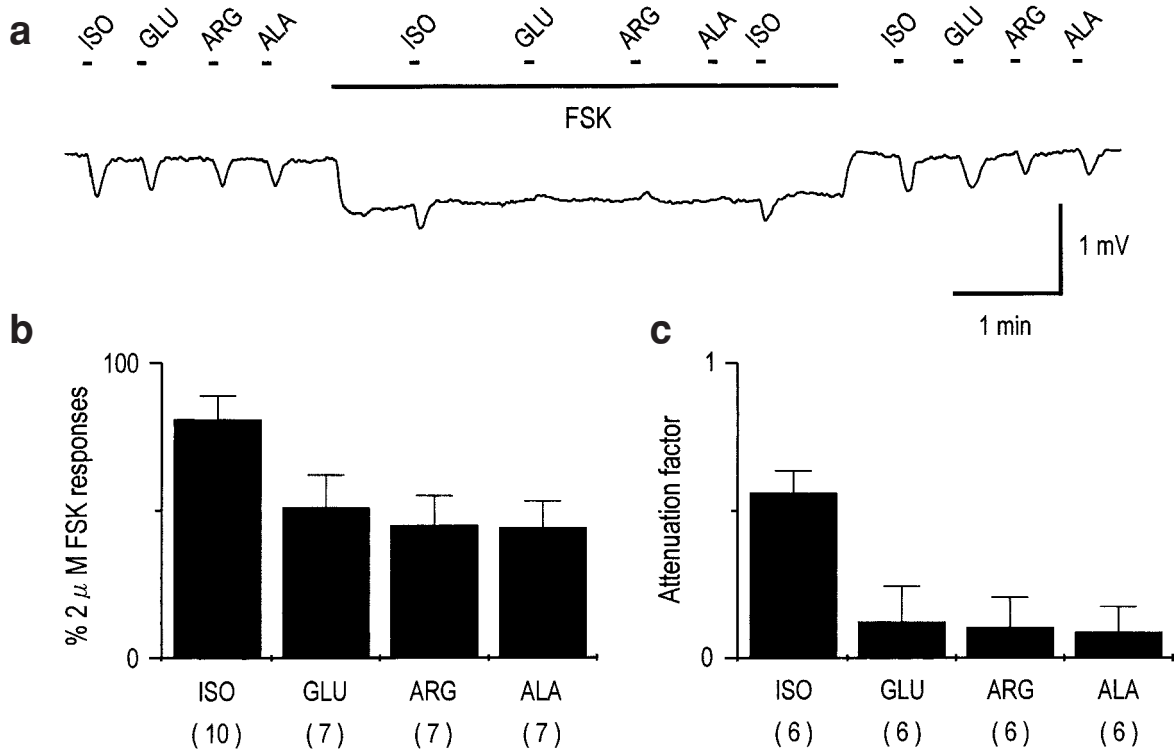
Consistent with the results of patch-clamp recordings in Fig. 1, responses to amino acids or volatile odorants were accompanied by responses to forskolin in all the records. The amplitude of the responses to 2  $\mu$ M forskolin were larger than those to amino acids or isoamyl acetate in all the experiments. This is thought to be reasonable because forskolin activates ORNs non-specifically whereas isoamyl acetate and amino acids activate only a small subset of the cells.



**Fig. 1.** Responses to amino acids, volatile odorants, and forskolin recorded from dissociated olfactory receptor neurons using the patch-clamp method. (a) Membrane potential recordings from two cells. Solid bars indicate timings of application of the chemicals, which was estimated from the junction current (see Method). The cell numbers correspond to the numbers in (b). (b) Response profiles to a series of odorants in 36 cells. Voltage responses for a series of odorants were obtained by the same procedure as in (a). Closed circles indicate that the response over 10 mV depolarization was observed. Blank spaces indicate no response (or less than 10 mV). We set this criterion because the fluctuation of membrane potential reached several millivolts. (c) Collected results of percentages of cells responding to amino acids, volatile odorants, and forskolin. "Res. No."-column shows number of tested cells (denominators), number of cells responding to individual chemicals (numerators), and percentages of responsive cells. Abbreviations for applied chemicals and the concentrations (in  $\mu\text{M}$ ): [volatile odorants] 500 isoamyl acetate (ISO), 500 anisole (ANI); [amino acids] 200 glutamic acid (GLU), 200 arginine (ARG), 200 alanine (ALA), 200 cysteine (CYS), 200 leucine (LEU); 2 forskolin.



**Fig. 2.** Whole-cell currents induced by 200  $\mu$ M arginine (ARG) at different holding potentials. (a) Current traces at different holding potentials. The holding potentials are indicated beside the individual traces. (b) Current-voltage relationship. The peak current of each response shown in (a) was plotted against the holding potential. The curve was drawn by eye. The reversal potential determined from this curve was 0 mV.



**Fig. 3.** The effects of forskolin on odorant responses by means of electro-olfactogram (EOG). (a) Typical electrical responses to odorants before, during, and after application of 2  $\mu$ M forskolin. The trace shows successive negative voltage responses elicited by 5 sec application of individual odorants. Application of forskolin caused a steady shift in the baseline and attenuated magnitude of the odorant responses. (b) Average ( $\pm$ SE) relative stimulatory effectiveness of individual odorants. (c) Average ratio ( $\pm$ SE) of the EOG responses to odorants before and during forskolin application. In the all recordings, forskolin attenuated the response to individual odorants. Numbers under the panels indicate the number of recordings.

Fig. 3 shows the effects of forskolin on odorant responses in EOG recording. Whereas volatile odorant responses were reported to be decreased by forskolin (Lowe *et al.*, 1989), application of forskolin also attenuated the magnitude of responses to amino acids (Fig. 3a and 3c). The trace in Fig. 3a is a typical recording showing the attenuation by forskolin application. In this experiment, amino acid responses were observed in normal saline, and then the bath solution was switched to normal saline containing 2  $\mu$ M forskolin. As a result, forskolin induced a stable increase in membrane potential, which was thought to reflect a sustained increase in cAMP. During the steady forskolin effect was observed, the amplitude of amino acid responses was reduced. Such attenuation was observed in all the odorants tested including the amino acids (Fig. 3c).

## DISCUSSION

### Amino acid responses in ORNs

Early behavioral studies on newt have shown that newts can sense water-soluble odorants as well as airborne odorants; in addition, Shibuya and Takagi (1963) reported that the olfactory epithelium of newts responds to both airborne odorants and water-soluble odor stimuli (extracts of dried silkworm pupae and meats). Furthermore, recent molecular biological studies indicate that amphibians in general might have two classes of odorant receptor proteins (ORPs); one group of ORPs is thought to be specialized for detecting volatile odorants, whereas the other group may be for recognizing water-soluble odorants (Freitag *et al.*, 1995, 1998). In this study, we showed for the first time that ORNs in newt, the species that has been widely used for investigating olfactory transduction (e.g. Kurahashi 1989, 1990), can respond to both amino acids and volatile odorants.

In studies of *Xenopus levis*, putative ORPs for amino acids are exclusively expressed in the ORNs of the lateral diverticulum (Freitag, 1995), which are reported to respond to amino acids (Iida and Kashiwayanagi, 2000). Iida and Kashiwayanagi (2000) suggested the possibility that both IP<sub>3</sub>-dependent and IP<sub>3</sub>-independent pathways contribute to the generation of responses to amino acids in the water nose. This system seems to be different from the signal transduction pathway of responses to amino acids in newt ORNs. It has been no evidence, however, that newt has a specialized region in the olfactory epithelium like *Xenopus*. While our study clearly showed the existence of ORNs that respond to amino acids, morphological studies will be needed to localize the ORNs that possess ORPs for amino acids.

It is known that odorants can elicit inhibitory responses as well as excitatory responses. Recently, it has been suggested that the inhibitory effects of odorant on CNG-channels might underlie the inhibitory responses, and that these inhibitory effects play a part in forming odor representation in ORNs (Jinks and Laing, 1999a; Yamada and Nakatani, 2001). Thus, the question of whether amino acids can elicit inhibitory responses seems important. In this study, however, we did

not determine whether amino acids have an inhibitory effect on cell's transduction cascade. Although we observed small inhibitory responses in several cells (c.a. amino acids responses during forskolin stimulation in Fig 3a), further experiments are needed to answer this question.

### Transduction cascade of amino acid responses

In this study, we attempted the two types of experiment to investigate the signal transduction cascade of the amino acid responses. In the first experiment, we employed the patch-clamp recording method. Responses to both amino acids and forskolin were observed in the same cell (e.g. Fig. 1a), which supports the hypothesis that the cAMP cascade is involved in the transduction of amino acid responses as well. Because forskolin is membrane-permeable stimulator of adenylate cyclase (Seamon and Daly, 1986), the coexistence of amino acid responses and forskolin responses in a given cell indicates that cells that responded to amino acids possessed the cAMP system. The patch-clamp experiments also showed that the application of amino acids elicited depolarizing currents with a reversal potential of near 0 mV (Fig. 2), which is consistent with the reversal potential of currents induced by volatile odorants- and cAMP reported by Kurahashi (1989, 1990).

In the second experiment, we employed the EOG recording method. Because it is a summed response, the EOG provides information about all classes of ORNs. Consequently, the EOG provides suitable electrophysiological measurement for investigating the generality of the olfactory transduction system. The study on the signal transduction cascade of volatile odorant responses in ORNs by means of EOG recording showed that forskolin attenuated the EOG responses for volatile odorants and indicated that adenylate cyclase mediates signal transduction for volatile odorant responses (Lowe *et al.*, 1989). This is because forskolin, according to the cAMP model (Nakamura and Gold, 1987; Gold, 1999), should attenuate odor responses through its effect both on adenylate cyclase and basal cAMP concentration (Lowe *et al.*, 1989). The effects of forskolin on adenylate cyclase would cause a decrease in the number of enzyme molecules to be activated by odorants. On the other hand, the effects of forskolin on cAMP levels would cause an increase in the basal cAMP concentration and hence partially saturate the transduction conductance, so that there would be little conductance available for an additional odorant response.

As shown in Fig. 3, forskolin attenuated all the responses to amino acids and to isoamyl acetate, a volatile odorant, in animals that we tested. As previously discussed, this result suggests that adenylate cyclase mediates both isoamyl acetate responses and amino acid responses. In Fig 3c, the difference in the amplitudes of the attenuation between isoamyl acetate responses and amino acids responses seems to be inconsistent with the idea that forskolin equally attenuates the isoamyl acetate responses and the amino acid responses. However, unlike the experiments in single cells, attenuation factor in EOG experiments cannot be compared directly, because the amplitude of EOG reflects numbers of activated

cells as well as amplitude of activation of the individual cells, and the magnitude of adenylate cyclase activation in individual cells is thought to be vary widely.

In this study, we observed that forskolin attenuated all responses to amino acids and isoamyl acetate (Fig. 3). Along with our patch-clamp results described above, this result also suggests that the cAMP pathway underlies the amino acid responses, as does the cAMP pathway of volatile odorant responses.

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