Studies on Amino Acid Responses in Olfactory Receptor Neurons of the Newt, *Cynops pyrrhogaster*

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Table of Contents

ABSTRACT

Amphibians are capable of smelling both volatile and water-soluble (e.g., amino acids) odorants. Volatile substances are well-known odorants. The olfactory signal transduction in response to volatile odorants through a G-protein-coupled cascade uses cAMP as second messenger. However, amino acid responses in olfactory receptor neurons (ORNs) including their signal transduction remain to be completely explained. First of all, I observed intracellular Ca^{2+} changes induced by four different amino acid solutions (arginine, glutamic acid, alanine, and proline; all at 250 μM) in the ORNs of Japanese newt (*Cynops pyrrhogaster*) using Fura-2 calcium imaging method. To examine responsiveness of individual ORNs, I tested fifteen ORNs by the amino acids. The individual ORNs differed in responsiveness to these amino acids. They clearly showed their own response pattern. On the other hand, the results indicate that the amino acids tested in the experiments did not show significant specificities on the responses (by GLMM: generalized mixed linear model). I also tested 2 μM forskolin on five ORNs which responded to some/all amino acids tested. The ORN that responded to amino acid also responded to forskolin, an adenylate cyclase activator, which implies that the signal transduction of the amino acid response is mediated by the cAMP pathway. Second, I examined unique biological characteristics of amphibians in their

olfactory responses. Adult Japanese newts mostly live in water except during hibernation. To examine olfactory responses of the newts to adaptation to short-term stay on land (land adaptation), I measured the magnitude of the olfactory response at five different time points (land adaptation time: 0, 30, 54, 90, and 114 h) after transfer from aquatic to terrestrial habitat using electro-olfactogram (EOG) recording. Statistical analysis indicated that the time to land adaptation had a significant effect on the magnitude of EOG induced by 1 μM and 10 μM amino acid mixtures (by weighted linear model: $P < 0.05$). Further, the slope estimates of the weighted linear model were significantly positive ($P < 0.05$). These results indicate that the magnitude of EOG response to amino acid mixtures (arginine, alanine, proline, and glutamic acid) is significantly increased with land adaptation time. On the other hand, I observed no significant relationship between the magnitude of EOG response induced by 0.05% volatile odorant mixture (isoamyl acetate, n-amyl acetate, cineole, and limonene) and land adaptation time. My results indicate that olfactory sensitivity to amino acids is significantly increased with land adaptation time in adult Japanese newts.

GENERAL INTRODUCTION

Odorants include volatile and water-soluble odorous substances. Volatile substances are well-known odorants. Odor detection by animals is dependent on the habitat of the animals. Terrestrial vertebrates including humans use their olfactory system to detect only volatile odorants, while aquatic vertebrates such as fish use their olfactory system to detect only water-soluble odorants (Hara, 1994). Amphibians have adapted to both terrestrial and aquatic life, and they are capable of olfaction of both volatile odorants and amino acids. In the study of vertebrate olfactory sense, amphibians have been used as model animals (e.g., Nakamura and Gold, 1987; Kurahashi, 1990). Vertebrate olfactory receptor neurons (ORNs) acquire olfactory signal transduction in response to volatile odorants through a G-protein-coupled cascade that uses cAMP as a second messenger (Pace et.al., 1985; Nakamura and Gold, 1987; Firestein and Werblin, 1989; Kurahashi, 1990). However, the properties of amino acid response in ORNs and the signal transduction mechanism are still unclear.

In this study, I have investigated amino acid responses in ORNs of amphibians, using Japanese newt (*Cynops pyrrhogaster*). In Part I, I examine the properties of amino acid responses using calcium imaging method. In Part II, I focus attention on short-term habitat change of the newts and their unique olfactory responses. I employed

electro-olfactogram (EOG) recording method to measure magnitudes of olfactory responses, and examined the effects of “land adaptation” (short-term stay on land) on the olfactory responses.

Part I

1 INTRODUCTION

1.1 Olfactory signal transduction

The process of olfaction in many species of amphibians and terrestrial vertebrates begins in the cilia of ORNs (reviewed in Kleene, 2008). Volatile odorants (OD) that dissolved in the mucus bind to specific olfactory receptors (ORs) which locate at the plasma membrane of olfactory cilia (Buck and Axel, 1991). Subsequently, the activation of OR leads to an activation of adenylate cyclase; the subsequent accumulation of cAMP opens cyclic nucleotide-gated ion channels (CNG channels). As a result, Na^+ and Ca^{2+} enter the cell through the CNG channels, producing a membrane depolarization. In addition, Ca^{2+} activates chloride channels, leading to an influx of Cl^- that further depolarizes the cell (Kurahashi and Yau, 1993; Lowe and Gold, 1993, Fig. 1, 2). On sufficient depolarization, the ORN can generate one or more action potentials in the sensory axon (Gold, 1999). The opening of CNG channels causes Ca^{2+} influx localized in the cilia and hence the Ca^{2+} concentration is supposed to rise in the cilia and the dendrite.

1.2 Olfactory receptor neurons (ORNs)

There are three types of the cells in the olfactory epithelium (Fig. 3). Supporting cells and glandular cells secrete mucus that coats the luminal surface of the epithelium. ORN is bipolar nerve cell. One process of each neuron is a single, unbranched axon that synapses in the olfactory bulb of the brain. The second process of each neuron is a dendrite that projects into the mucus and terminates in an olfactory knob. From each knob, olfactory cilia emanate into the mucus.

In fact, there are two classes of ORNs in vertebrate olfactory organ (Ichikawa and Ueda, 1977), ciliary ORN (Fig. 4A) and microvillar ORN (Fig. 4B). In general, the length of microvilli is shorter than that of cilia. Aquatic vertebrates like fish (Muller and Marc, 1984) and several species of amphibians like *Xenopus* (Hansen et al., 1998) have both ciliary and microvillar ORNs in their olfactory epithelium. However, in many species of amphibians and terrestrial vertebrates, ciliary ORNs populate the olfactory epithelium whereas microvillar ORNs lie in the vomeronasal organ (VNO) or the region equivalent for VNO (e.g., the olfactory epithelium of the lateral nasal sinus region in the newt: Toyoda and Kikuyama, 2000). The study of olfactory response of fish showed that both ciliary ORNs and microvillar ORNs respond to amino acids (Lipschitz and Michel, 2002; Hansen et al., 2003). Yamada and Nakatani (2001) suggested that ciliary ORNs of

the newt could respond both volatile odorants and amino acids using patch-clamp techniques.

In this section, I examine the properties of amino acid responses using calcium imaging method.

2 MATERIALS AND METHODS

2.1 Animals

Adult Japanese newts, *Cynops pyrrhogaster*, were purchased from a local animal supplier. The newts were kept in a plastic container that had both terrestrial and aquatic condition, at room temperature (23-26°C). In the container, the animals were able to move freely. They were fed pellet food once a week, except for the day before experiments.

2.2 Calcium imaging

2.2.1 Cell preparation and solutions

The newts were anesthetized by cooling on ice, decapitated and pithed. Olfactory epithelia were excised in normal saline (110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 5 mM Na^+-HEPES, 5 mM Glucose; pH = 7.6) under a dissection microscope. To obtain isolated olfactory receptor neurons (ORNs), cells were treated with enzymes. The olfactory epithelia were incubated in a Ca^{2+} and Mg^{2+}-free solution (110 mM $NaCl_2$, 2.5 mM KCl, 5 mM Na^+-HEPES; pH = 7.6) containing 0.7-0.8% collagenase (Wako Pure chemical Industries, Osaka, Japan) for 10 min at 35°C. The tissue was rinsed with normal saline and cut carefully into small pieces (< 1 mm^3) with

forceps. Those pieces were incubated in the Ca^{2+} and Mg^{2+}-free solution containing 0.5 μg/ml DNAse (Sigma) for 10 min at room temperature, and then collected with a pipette. The pieces in the saline were gently triturated by passing them several times through fire-polished tip of a Pasteur pipette. The procedure of cell isolation is illustrated in Fig. 5. Cell suspension was stored at 4°C until use (up to 10 h). Isolated cells were placed in a recording chamber coated with 20% polyornithine (Sigma) to immobilize cells. ORNs can be clearly identified by its characteristic morphology, having a single dendrite with an olfactory knob from which olfactory cilia emanate. I chose the cells that retained olfactory cilia and responded to high K^+-solution (112.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 5 mM Na^+-HEPES, 5 mM Glucose; pH = 7.6) for the experiment.

2.2.2 Dye loading and calcium imaging techniques

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured by dual wavelength ratiometric method using Fura-2 AM (DOJINDO, Japan), a membrane-permeate fluorescent calcium indicator.

Cells were incubated in the dark and at room temperature (~23ºC) for 30 min with 5 μM of the acetoxymethyl ester forms of the Ca^{2+}-sensitive probe Fura-2. Non-internalized dye was then washed away with normal saline, and the cells were

ready for imaging.

Fluorescence imaging experiments were carried out on the stage of an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan), using a xenon continuous arc light source (Polychrome II; Hamamatsu Photonics, Hamamatsu, Japan), a × 20 Plan objective lens (NA 0.75, WD 0.55 mm; UApo20×/340, Olympus), and a cooled CCD camera (Hamamatsu Photonics). The ratio F_{340} /F_{380} (ΔF) of Fura-2 fluorescence was obtained by exciting sequentially at 340 and 380 nm and measuring emission at 500 nm. A computer-controlled filter changer (Hamamatsu Photonics) was used to switch excitation wavelength. The system was controlled by ARGUS software (Hamamatsu Photonics).

Fluorescence images were sampled at a resolution of 0.5-10 Hz depending on exposure time. The images were analyzed with ARGUS/HISCA calcium imaging processing system (Hamamatsu Photonics). The calcium imaging setup is illustrated in Fig. 6. All recordings were performed at room temperature.

2.2.3 Perfusion system and test solutions

Bath solution (normal saline) was applied by gravity and flowed through an application tube into the recording chamber. The bath solution was used for flushing the test solutions after each application. The test solutions were flowed through a different application tube placed near the cell. Each test solution (3 ml) was applied manually at a rate of 150 μl/sec (Fig. 7). I used arginine (Sigma), glutamic acid (Sigma), alanine (Wako, Osaka, Japan) and proline (Peptide Institute, Osaka, Japan) as test odorants. These amino acids are L-form and the concentration of each amino acid was 250 μM. I have also used 2 μM forskolin to activate adenylate cyclase. All test solutions were dissolved in the saline. The pH of the solutions was adjusted to 7.6.

2.2.4 Statistical analysis

I used generalized linear mixed model (GLMM) to identify whether tested amino acids showed the specificities on the responses of individual ORNs or not. The binary response variable was the response, or no response, of individual ORN to the test solution. The “response” means that the peak fluorescence ratio was larger than the fluorescence rate at rest, and “no response” means that no detectable signal was present in response to the test solution. The species of test solutions were as fix effects, and

individual cells were as random effects. I followed backward stepwise procedures to fit a minimal adequate model containing explanatory variables. Significance was determined at $P < 0.05$. All statistical analyses were conducted using the statistical package R (version 2.7.2, http://www.r-project.org/).

3 RESULTS

In order to analyze cell functions in response to amino acid stimulus, I have measured intracellular calcium concentration using calcium imaging technique and Fura-2 calcium-specific fluorescent dye. In these experiments, I only used intact ORNs that had typical ORN morphology and retained olfactory cilia. Calcium fluorescence in the cilia and the dendrite were below the resolution limit of the imaging setup. Therefore, I measured average fluorescence intensity of whole ORN (indicated by green frame in Fig. 8A) except for Fig. 12. In the end of each experiment, the cell was tested by applying high K^+ -solution to prove that it was alive. Unresponsive cells were discarded from the data.

Fig. 8 shows intracellular Ca^{2+} changes in an ORN (Cell 12 in Fig. 10) induced by applying 250 μM arginine solution. The fluorescence image of the ORN at resting condition is shown in Fig. 8A, showing that the fluorescence ratio (ΔF) of the whole ORN was low level. After stimulus triggering, intracellular Ca^{2+} concentration of the ORN increased (Fig. 8B). The Ca^{2+} level recovered after wash out (Fig. 8C). The fluorescence ratio was plotted against time in Fig. 8D. The fluorescence ratio rose quickly to a transient peak after stimulation and then reduced to a low level. After wash out the test solution, the fluorescence ratio recovered to near resting level. Fig. 9 shows

the fluorescence ratio changes in response to three different amino acid solutions (glutamic acid: Fig. 9A, alanine: Fig. 9B, proline: Fig. 9C). It is indicated that the ORN responded to all amino acids tested in the experiments.

Similarly, fifteen ORNs were tested with four different amino acids (Fig. 10). The peak fluorescence ratio was larger than the fluorescence rate at rest that means the cell responded to the test solution. Seven ORNs showed intracellular Ca^{2+} increase in response to all the test solutions. One cell showed no responses. The rest of the cells responded to one to four amino acids. In other words, each cell had its own response pattern for the amino acids. However, the results indicate that the amino acids tested in the experiments did not show significant specificities on the responses (by GLMM).

I also tested 2 μM forskolin on five ORNs which responded to some/all amino acids tested. All of them showed increases in intracellular Ca^{2+} after application of forskolin. An example is shown in Fig. 11.

Fig. 12 showed the fluorescence images successively recorded every 1.174 sec. These images were taken from the response to the glutamic acid solution as shown in Fig. 9A. After stimulus triggering (the fifth image in Fig. 12), intracellular Ca^{2+} concentration transiently increased at the dendritic knob region. In the Fig. 13, I compared a change in Ca^{2+} concentration of two areas of the ORN, one is dendritic knob

(Fig. 13A) and another is soma (Fig. 13B). The amplitude of Ca^{2+} transient at the knob was 1.25, while 0.35 at the soma. The intracellular Ca^{2+} elevation induced by glutamic acid in the knob was higher than that in the soma. Similar results were obtained from the cells that responded to all other amino acids tested. The ORNs whose fluorescence images of the knob were clearly identified also showed similar results (n = 2).

4 DISCUSSION

4.1 Responsiveness of isolated ORN to amino acids

In the present study, I have tested arginine, glutamic acid, alanine and proline that belong in different categories. Arginine is basic, glutamic acid is acidic, alanine is short chain neutral, and proline is long chain neutral. It is noted that the individual ORNs differed in responsiveness to these amino acids (Fig 10). Some cells responded to all the tested amino acids, while the other cells responded to some of the four species of amino acid. Although most of the ORNs responded to all or multiple kinds of amino acid, they clearly showed their own response pattern. Therefore, an ORN may be able to distinguish amino acids. More experiments using all other amino acids are necessary to elucidate response pattern of ORNs to amino acids.

4.2 Ca^{2+} transients induced by amino acids

The intracellular Ca^{2+} elevation induced by amino acid was in a transient manner (Fig. 8 and Fig.9). It is known that volatile odorant response in the newt adapt quickly and the mechanism of this adaptation has been reported (Kurahashi and Menini, 1997). It is implied that the Ca^{2+} transient may be due to an adaptation.

The signal transduction mechanism of volatile odorant response is well studied,

while that of amino acid response is not known at all. In the present study, I found clues of signal transduction mechanism of amino acid response. The ORN that responded to amino acid also responded to forskolin, an adenylate cyclase activator (Fig. 11), which implies that the signal transduction of the amino acid response is mediated by the cAMP pathway.

In this study, I have measured intracellular Ca^{2+} concentration as an indication of the cell activity. In order to find out where the Ca^{2+} come from, I have compared the Ca^{2+} elevation at two different part of the cell. After stimulation with amino acid (glutamic acid), the Ca^{2+} elevation in the knob was about 3.6 times higher than that in the soma (Fig. 12, Fig. 13), implying that the Ca^{2+} concentration increases in the knob first and then the Ca^{2+} diffused into the soma. I deduced from the results that initial event of amino acid odor detection begins in the cilia as volatile odor detection does. The Ca^{2+} transients are dependent primarily on Ca^{2+} entry through activated CNG channels (Leinders-Zufall et al., 1998).

Part II

1 INTRODUCTION

Terrestrial vertebrates including humans use their olfactory system to detect only volatile odorants, while aquatic vertebrates such as fish use their olfactory system to detect only water-soluble odorants (Hara, 1994). Amino acids are water-soluble odorants. Amino acids are used as feeding stimuli by several species of aquatic predators, such as giant tiger prawn (Coman et al., 1996) and some teleosts (Hara, 2006).

In the study of vertebrate olfactory sense, amphibians have been used as model animals. The unique olfactory response of amphibians has been studied extensively. Amphibians have adapted to both terrestrial and aquatic life. Early behavioral studies suggest that amphibians are capable of olfaction of both volatile and water-soluble odorants (Reese, 1912; Copeland, 1913). Recent molecular studies on ORs reveal that amphibians have two classes of ORs, class I receptors for water-soluble odorants and class II receptors for volatile odorants (Freitag et al., 1998; Mezler et al., 2001). Yamada and Nakatani (2001) used patch-clamp recordings to demonstrate that ORNs in Japanese newt, *Cynops pyrrhogaster*, respond to both volatile odorants and amino acids.

The unique biological characteristics of amphibians correlate to changes in their

olfactory responses. Olfactory response characteristics change during metamorphosis in tiger salamander (Arzt et al., 1986). It has been reported that oscillatory potential changes in the olfactory epithelium of Japanese toad during breeding period (Nakazawa et al., 2000). There is species-specific difference in the lifestyle of adult amphibians. Adult Japanese newts mostly live in water except during hibernation. When newts are transferred from water to land, they begin to grow olfactory cilia (Shibuya and Takagi, 1963). After a short-term stay on land, the olfactory system of the newts may adapt to terrestrial lifestyle. It is not completely understood, however, how adaptation to short-term stay on land (land adaptation) influences olfactory response of the newts.

I employed electro-olfactogram (EOG) recording method to measure the magnitude of olfactory response, and examined the effects of land adaptation on the olfactory response by fitting to a weighted linear model. My results indicate that olfactory sensitivity to amino acids is significantly increased with land adaptation time.

2 MATERIALS AND METHODS

2.1 Animals and preparation

Adult male Japanese newts, *Cynops pyrrhogaster*, were purchased from a local animal supplier. The animals were kept in a tank filled with water about 10 cm deep at a temperature of about 20°C. They were fed pellet food twice a week, except for the day before the experiments. In order for the animals to adapt to a terrestrial habitat, I transferred the animals from the water tank to a plastic container with no water (land adaptation). They were kept in the plastic container, in which the humidity is maintained in order to prevent them from being dried up, for 30–114 h depending on the experiment.

2.2 EOG recording

The newts were pithed and decapitated. Olfactory epithelium was exposed by cutting the roof of the nasal cavity. Procedure for recording EOG responses to test solutions (Fig. 14) was similar to that described in Shibuya and Takagi (1963). A glass pipette electrode with a tip diameter of 0.1 mm was used to record EOG. The pipette was filled with normal saline (110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 5 mM Na-HEPES; pH = 7.6) and connected through an Ag/AgCl wire to a

conventional amplifier. The electrode was placed in the center of the olfactory epithelium. The head of the animal was mounted on a recording chamber, and solutions flowed continuously through an application tube into the chamber. Solutions were applied by gravity into a four-way valve that was operated pneumatically under remote control by computer. Normal saline was applied continuously during the experiments except when test solutions were applied. All recordings were performed at room temperature (23-26°C).

2.3 Test solutions

Two kinds of odorous fluids (water-soluble and volatile) were used as test solutions. One was an amino acid mixture containing four amino acids (arginine, glutamic acid, alanine and proline) at each concentration of 1 μM or 10 μM. The other was a volatile odorant mixture containing four arbitrary volatile odorants (isoamyl acetate, n-amyl acetate, cineole, and limonene). Each of the volatile odorants was dissolved in DMSO and used at a final concentration of 0.05%. All test solutions were dissolved in normal saline. The pH of all solutions was adjusted to 7.6.

2.4 Statistical analysis

To examine the effects of land adaptation, weighted linear modeling was performed using weighted least squares (Quinn and Keough, 2002). The variance of the magnitude of EOG varied for different time points (0, 30, 54, 90, and 114 h). Therefore, I weighted each observation by the reciprocal of an estimate of its variance. I compared the two slope estimates (Table 1) by Student's *t*-test to determine differences between the magnitude of EOG response to the amino acid mixture at concentrations of 1 μM and 10 μM. I compared the intercept estimates to test solutions (Table 1) also by *t*-test, and determined whether the magnitude of EOG response at 0 h was different for the odorants. Significance was determined at $P < 0.05$. All statistical analyses were conducted using the statistical package R (version 2.7.0, http://www.r-project.org/).

3 RESULTS

To examine the effects of terrestrial adaptation, I recorded EOG response, an extracellular field potential where amplitude reflects the summed response of individual ORNs (Ottoson, 1956). Maximal response of EOG was referred to as the magnitude of EOG (indicated by arrows in Fig. 15). This method allowed me to measure activities of many cells in olfactory epithelium simultaneously.

In preliminary studies, I measured the amplitude of EOG responses to test solutions among different animals kept on terrestrial habitat in increments of six hours for 6 to 126 h, to obtain a rough time course of amplitude change (data not shown). On the basis of this result, I determined five different time points (land adaptation time: 0, 30, 54, 90, and 114 h after transfer from aquatic to terrestrial habitat) at which to record EOG responses.

Fig. 15 shows the EOG response to odorants of the newts that were kept on terrestrial habitat for 0, 30, 54 90, and 114 h. For each recording, an electrode was placed on one olfactory epithelia and test solutions were applied to the same side. Slow potentials were evoked by application of test solutions to olfactory epithelium (amino acid mixture: Fig. 15A; volatile odorant mixture: Fig. 15B). The magnitudes of EOG induced by amino acid mixtures were greater at later time points.

In order to perform statistical analyses, I recorded the amplitude of EOG response to odorants from at least four samples at each time point. Fig. 16 shows the mean magnitude of EOG to odorants plotted against land adaptation time. The magnitude of EOG (mean ± SE) induced by 1 μM amino acid mixture at each time point was 423.62 ± 40.61, 952.61 ± 300.06, 577.30 ± 103.12, 800.13 ± 156.97, and 608.01 ± 157.34 μV, respectively (Fig. 16A). Statistical analysis indicated that the time to land adaptation had a significant effect on the magnitude of EOG (weighted linear model; $F_{1, 37}$ = 6.5002, P = 0.01506). Further, the slope estimate of weighted linear model was significantly positive (slope estimate = 2.703, t = 2.55, P = 0.0151; Table 1). These results indicate that the magnitude of EOG response to 1 μM amino acid mixture is significantly increased with land adaptation time. Similarly, the magnitude of EOG induced by 10 μM amino acid mixture is significantly influenced by the time course of land adaptation (weighted linear model: $F_{1, 37}$ = 17.737, $P < 0.001$) and significantly increased with land adaptation time (slope estimate = 7.573, t = 4.212, $P < 0.001$; Table 1). The magnitude of EOG (mean ± SE) induced by 10 μM amino acid mixture at each time point was 397.91 ± 94.54, 899.30 ± 242.71, 897.56 ± 147.09, 1007.60 ± 149.83, and 1780.95 ± 625.22 μV, respectively (Fig. 16B). Comparison of two slope estimates of the weighted linear model showed a significant effect (t-test: $P < 0.05$), indicating

that increase in the magnitude of EOG with land adaptation time was significantly greater for exposure to 10 μM amino acid mixture compared with exposure to 1 μM amino acid mixture.

Fig. 16C shows the mean magnitude of EOG induced by the volatile odorant mixture. The magnitude of EOG (mean ± SE) at each time point was 570.24 ± 89.44, 778.70 ± 154.40, 695.84 ± 90.18, 480.29 ± 217.57, and 1014.98 ± 236.31 μV, respectively. The results indicate that there is no significant relationship between the magnitude of EOG response to the volatile odorants and land adaptation time (weighted linear model: $F_{1, 36} = 1.4726$, $P = 0.2328$), which are distinct from the results for the magnitudes of EOG response to the amino acid mixture.

All intercept estimates of weighted linear model (Table 1) showed no significant differences (*t*-test). In other words, at 0 h for the newts that were kept in a water tank until just before the dissection, there was no significant difference between the magnitudes of EOG induced by the test solutions in the present study.

4 DISCUSSION

4.1 Effects of land adaptation on EOG responses

Statistical analysis indicates that the magnitude of EOG induced by amino acid mixture increased significantly with time to land adaptation (Fig. 16A, B). During the experiments, the olfactory epithelium was constantly perfused with test solutions, a condition similar to that experienced by animal olfaction in aquatic conditions, just after returning from terrestrial conditions. Amino acids are effective feeding stimuli. Several aquatic and semi-aquatic vertebrates including newts search for food in water using their olfactory sense. The basic amino acid, arginine, was the most stimulatory compound in the California newts (*Taricha torosa*) foraging activity and the response threshold for the activity was estimated as 8.3×10^{-1} μM in the behavioral study (Ferrer and Zimmer, 2007). The magnitude of EOG induced by the amino acid mixture increased in a dose-dependent manner after land adaptation (as determined by comparison of the slope estimates at 1 μM and 10 μM using *t*-test), while there was no significant difference between the magnitude of EOG response to 1 μM and 10 μM amino acid mixtures at 0 h as determined by comparison of the intercept estimates using *t*-test. The difference between the slopes estimated at 1 μM and 10 μM indicate that the effects of land adaptation are increased in a dose-dependent manner. The reason is

unknown, however, it may be attributed to condition change of epithelial mucosa and/or fasting. In any case, land adaptation may have beneficial effects in immediate foraging activity for the newts returning to water.

It has been reported that the olfactory epithelium of the newt does not respond to odorous vapors at all in water (Shibuya and Takagi, 1963). In the present study, we used volatile odorants as odorous fluids, so we could record EOG responses to volatile odorants under aquatic conditions (Fig. 15B, 16C; 0 h). Recent electrophysiological studies on olfactory responses used volatile odorants as test solutions (e.g., Kurahashi and Yau, 1993). Each molecule of a volatile or water-soluble odorant permeates the olfactory epithelial mucosa. Therefore, there is no difference between the EOG responses to volatile vapors under terrestrial conditions and those to volatile fluids under laboratory conditions. Unlike the response to mixture of amino acids, land adaptation had no significant effects on the magnitude of EOG induced by volatile odorants (Fig. 16C), indicating that expected EOG responses to odorous vapors are not influenced by land adaptation.

4.2 Land adaptation and modulation effects on olfactory responses

It is possible that EOG responses to odorants are modulated by various factors. As mentioned above, the difference between the slopes estimated at 1 μM and 10 μM that may be attributed to condition change of epithelial mucosa and/or fasting. Some researchers advocate that olfactory responses are modulated by the condition of epithelial mucosa (Oka et. al, 2006; Iwasa et. al, 2008). Olfactory binding proteins expresses in the mucosa, which bind odor molecules and carry the molecules to the olfactory receptors. The condition change of the mucosa may effect on the binding proteins. It has also been reported that endocrine substances (e.g., adrenaline: Kawai et. al, 1999) modulate olfactory responses to odorants. However, there have been no reports about amphibian's endocrine changes after adaptation to land.

Mousley and her co-workers (2006) found that axolotl neuropeptide Y (NPY) modulates EOG responses evoked by glutamic acid, a food-related odorant, only in hungry animals. They tested axolotls that had been fed either 1 or 10 d before testing ("well fed" and "hungry", respectively). Bath application of synthetic axolotl NPY enhanced EOG responses elicited by glutamic acid in hungry animals but not in well fed animals. It raises a possibility that endocrine substances induced by fasting may affect my results in the newt. In my experiments, the newts in water (land adaptation time: 0h)

were not fed for 48-96 h (2-4 d), and the newts after the transfer were not fed for 30-114 h before recordings. In preliminary studies, I measured the amplitude of EOG responses to test solutions among different animals, which were not fed and kept in aquatic habitat at five different time points (0, 30, 54, 90, 114 h) to obtain a rough time course of amplitude change (data not shown). There seemed to be no difference between the EOG responses. Furthermore, possible effects by endocrine substances can be excluded because the newt was decapitated and the head was perfused with normal saline during the recordings.

4.3 Olfactory cilia and land adaptation

It has been reported that aquatic and semi-aquatic vertebrates like fish (Muller and Marc, 1984) and *Xenopus* (Hansen et al., 1998) have ciliary and microvillar ORNs, but many species of amphibians including newts have only ciliary ORNs as mentioned above (Part I). Using whole cell patch-clamp technique, Yamada and Nakatani (2001) showed that ciliary ORNs respond to both volatile odorants and amino acids in isolated ORNs of the newt.

Olfactory signal transduction takes place in the olfactory cilia. ORs are localized on the plasma membrane of olfactory cilia and olfactory transduction channels (CNG

channels and Ca^{2+}-activated Cl^- channels) are present on the cilia (Takeuchi and Kurahashi, 2008). When newts are transferred from water to land, their olfactory cilia begin to grow. At 108 h after transfer, the length of the cilia reached 22 μm (more than five times as long as those of newts in water), while the ciliary length of newts in water did not change (Shibuya and Takagi, 1963). The mechanism and function of the growth of olfactory cilia upon transfer from aquatic to terrestrial habitat is still unclear. My present results suggest a correlation between the magnitude of EOG induced by amino acids and growth of cilia caused by land adaptation. One possible explanation underlying the increase in sensitivity as measured by EOG by land adaptation is that growth of cilia is accompanied by an increase in surface area resulting in an increase in the number of ORs, and consequently resulting in an increase in the magnitude of EOG. However, in a recent report by Takeuchi and Kurahashi (2008), the total ciliary currents do not differ much between 15 and 30 μm long cilia. Moreover, my results indicate that no significant effects of land adaptation on the magnitude of EOG were induced by volatile odorants (Table 1). Therefore, the length of the cilia may not play an important role in the magnitude of EOG response.

My results suggest that expression of amino acid receptors but not volatile odorant receptors increase with land adaptation time. The reason for selective expression of

amino acid receptors by land adaptation is unknown. As I mentioned previously, amino acids are effective stimuli for feeding behavior. Terrestrial habitats may be unusual for adult newts and the increase in olfactory sensitivity to amino acids may be important for their survival on land.

GENERAL DISCUSSION

In this study, I showed the properties of amino acid response of ORN in two different aspects.

In Part I, I focused on the general properties of the amino acid response. The ORN that responded to amino acid also responded to forskolin, which implies that the signal transduction of the amino acid response is mediated by the cAMP pathway. The difference between the newt and other aquatic vertebrates is that the ciliary ORN of the newt responds to both volatile odorants and amino acids. Furthermore, it is implies that the signal transduction mechanisms of amino acid response and volatile odorant response are mediated by the cAMP pathway. In other words, the newt uses one type of ORN (ciliary ORN) and common signal transduction pathway for both volatile odorant and amino acid responses.

In part II, I focused on the changes of the response property depending on the habitat. Shibuya and Takagi (1963) reported that the ciliary length of the newt changed when they were transferred from water to land. They hypothesized that growth of the cilia correlated with the changes in EOG responses to odorous fluid (extracts of dried worm) and some odorous vapors (e.g., amyl acetate). However, the recent study by Takeuchi and Kurahashi (2008) revealed that the total ciliary currents do not differ

much between 15 and 30 μm long cilia. This discrepancy raises a question about the correlation between response magnitude and ciliary length caused by land adaptation. In my results, only amino acid response increased after land adaptation while volatile odorant response did not. Therefore, I deduce that the magnitude of EOG response does not depend on the length of the cilia, but on expression rates of olfactory receptor proteins.

In this study, I concluded that the signal transduction mechanism of amino acid response is same as volatile odorant response. On the other hand, the expression of olfactory receptor protein induced by habitat change is different between amino acid receptor and volatile odorant receptor. For amphibians like newts, the ability to detect amino acid by olfactory receptor is crucial to search foods, and it may be beneficial for them to change expression rate of amino acid receptors depending on their habitat.

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TABLE

(1 table)

Table 1. Estimated parameters of weighted linear model.

* $P < 0.05$.

Test odorant	Estimate		SE	*t*	*P*
1 μM Amino Acid Mix	Intercept	431.326	40.176	10.74	< 0.001*
	Slope	2.703	1.06	2.55	0.0151*
10 μM Amino Acid Mix	Intercept	429.294	88.99	4.824	< 0.001*
	Slope	7.573	1.798	4.212	< 0.001*
Volatile Odorant Mix	Intercept	590.56	80.89	7.3	< 0.001*
	Slope	1.99	1.64	1.213	0.233

FIGURES AND LEGENDS

(16 figures)

Fig. 1. The model of olfactory signal transduction in vertebrate. On olfactory cilia membrane, odorants bind to olfactory receptors (OR), which interact with G-proteins (G). G-proteins stimulate adenylate cyclase (AC), causing cAMP increases in the cilia. The cAMP opens cyclic nucleotide-gated channels (CNG channels), leading to a change in membrane potential. Ca^{2+} influx through the open CNG channels activate chloride channels that lead to an increase in the inward Cl^- currents that further depolarize the cell. On sufficient depolarization, the ORN can generate one or more action potentials in the axon.

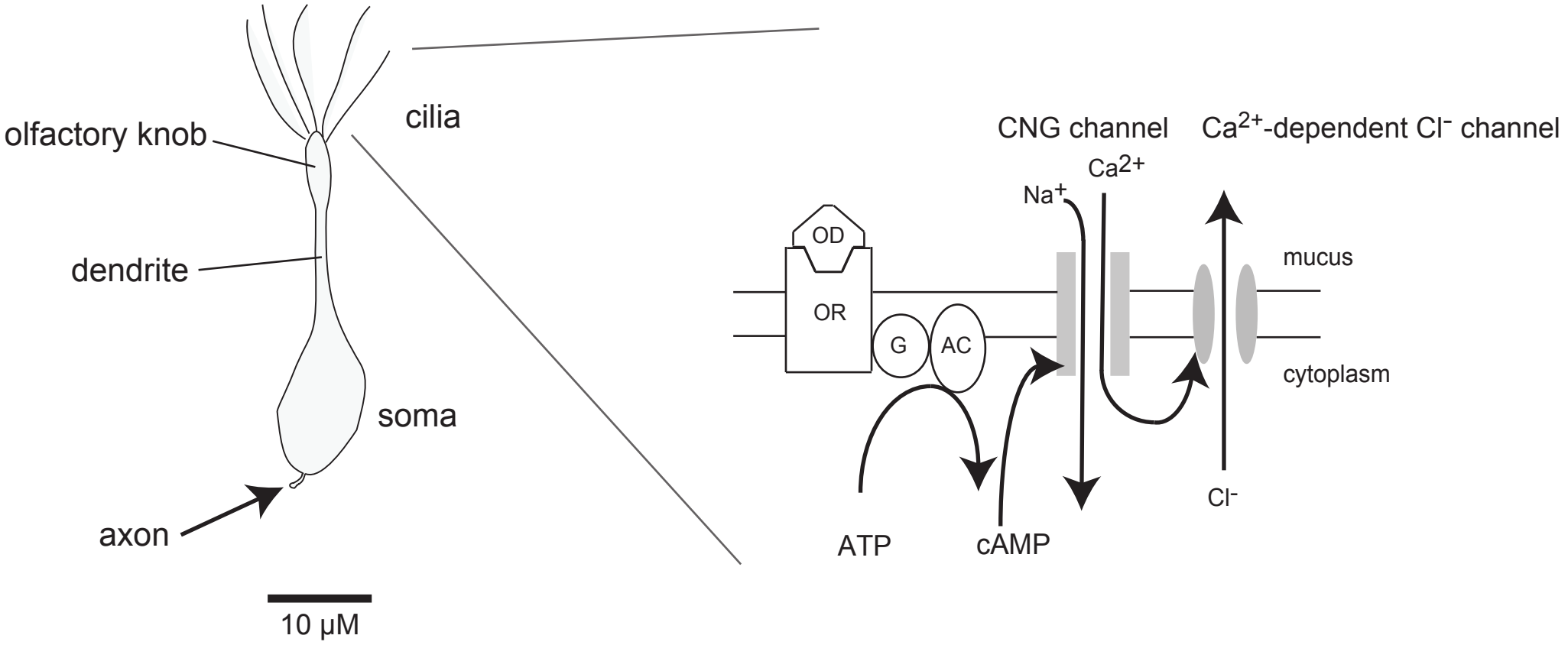

olfactory knob
cilia
dendrite
soma
axon
10 µM
CNG channel
Ca2+-dependent Cl- channel
Ca2+
Na+
OD
OR
G
AC
mucus
cytoplasm
Cl-
ATP
cAMP

Fig. 2. An example of current responses induced by volatile odorant mixture. Recording was performed under whole cell voltage clamp at – 80 mV from an isolated olfactory receptor neuron of the newt. Solid bar indicates timing of application of the volatile odorant mixture that was the same solution used in the experiments of Part II.

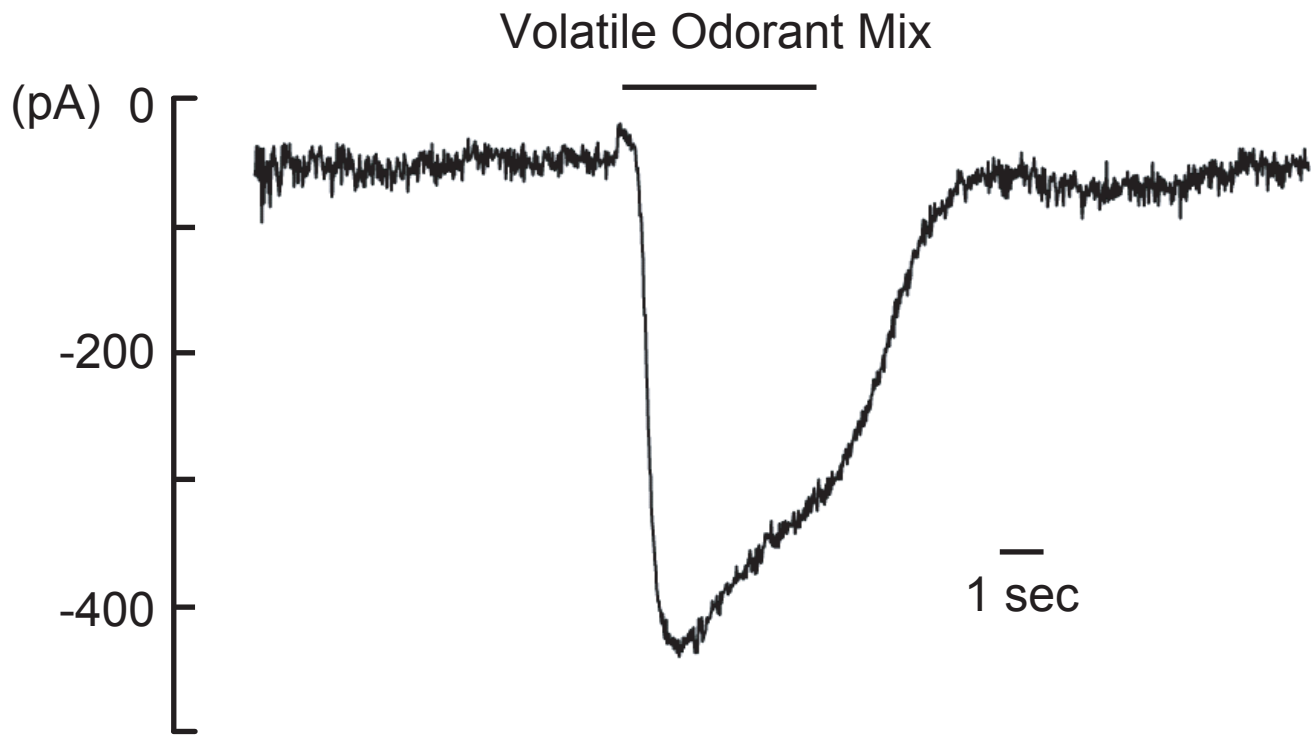

Volatile Odorant Mix
(pA) 0
-200
-400
1 sec

Fig. 3. A schematic drawing of vertebrate olfactory epithelium. Olfactory receptor neurons (ORNs) lie in the olfactory epithelium. ORNs are shown in green**.** Each ORN projects a dendrite to the apical (luminal) surface of the epithelium (top of the figure). The surface is covered with mucus that surrounds the olfactory knob and olfactory cilia. The ORN that has not reached the apical surface is immature. The mucus is formed by secretions of the supporting (sustentacular) cells (shown in yellow) and the Bowman's glands (shown projecting a duct through the epithelium). Basal progenitor cells are shown in orange at the base of the epithelium.

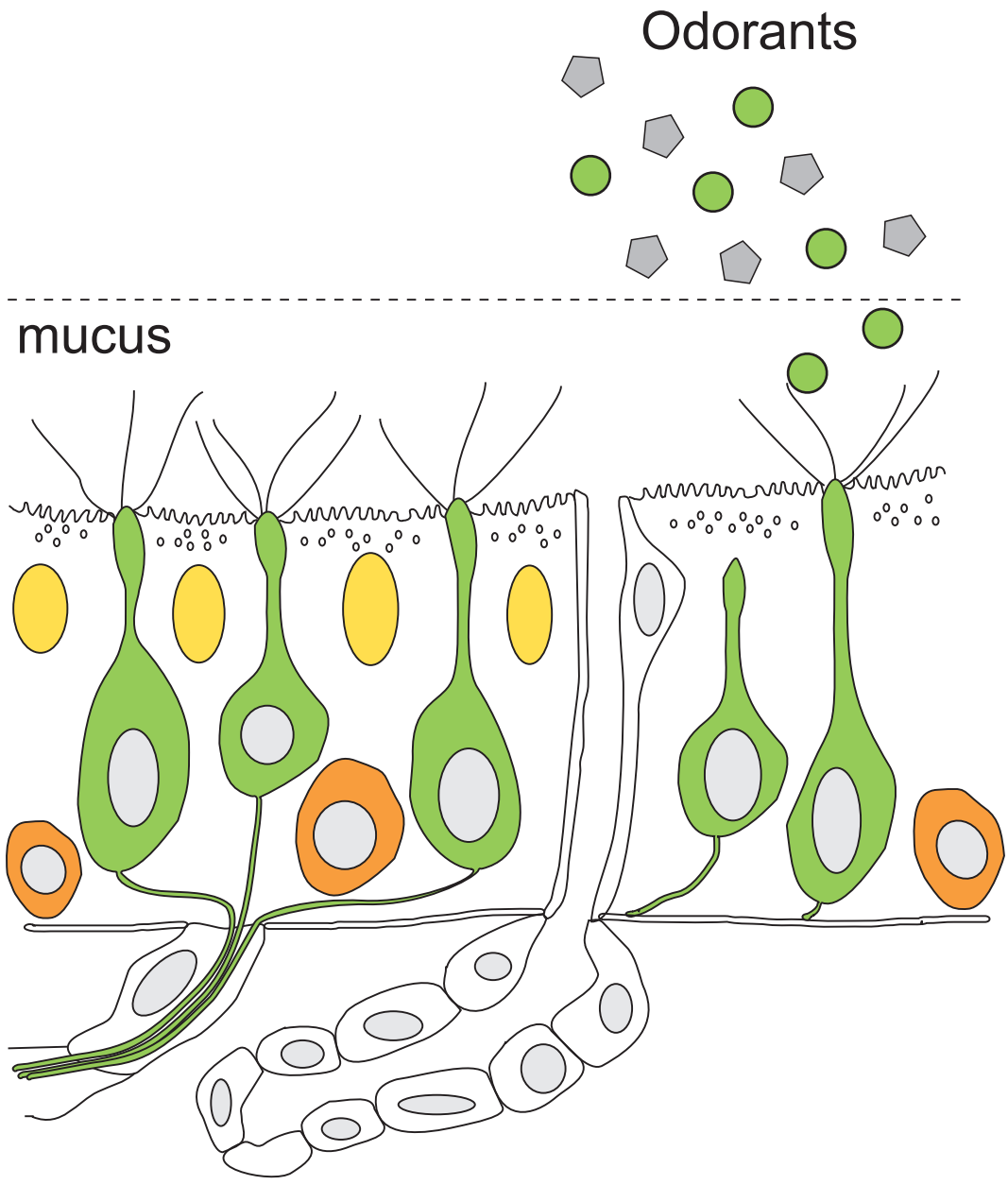
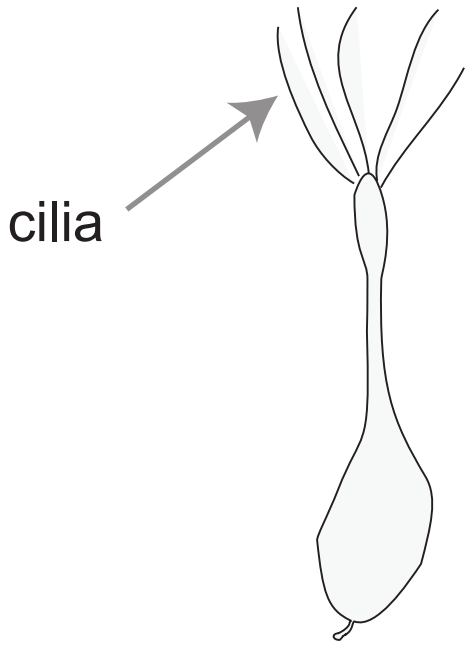

Odorants
mucus

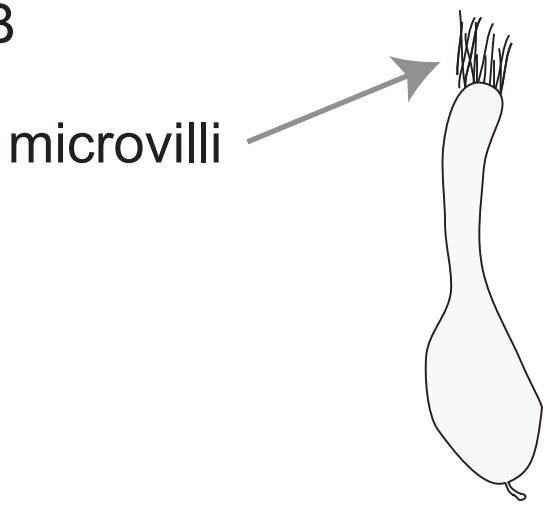
Fig. 4. Schematic drawings of two classes of olfactory receptor neurons (ORNs). (A) Ciliary ORN (indicated as green cell in Fig. 3). (B) Microvillar ORN, which mainly lies in olfactory organs of aquatic vertebrates like fish and in the vomeronasal organ (VNO) of amphibians and terrestrial vertebrates.

A



ciliary olfactory receptor neuron

B

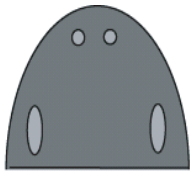


microvillar olfactory receptor neuron

Fig. 5. Outline of the isolation procedures of olfactory receptor neurons (ORNs). After decapitation, the nasal cavity was opened, and then the olfactory epithelium was treated with enzymes to obtain isolated ORNs.

Cell isolation procedure

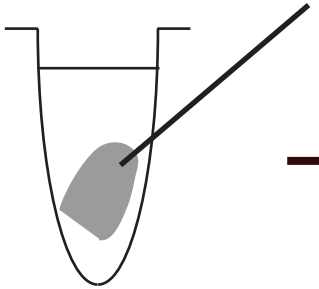
1.



newt head

2.

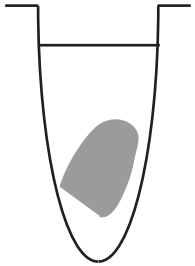
olfactory epithelium



enzyme treatment with collagenase

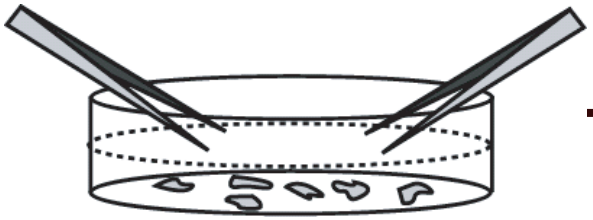
35℃・10 min

3.



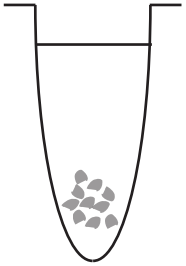
rinse with the normal saline

4.



cut into small pieces (< 1mm^3)

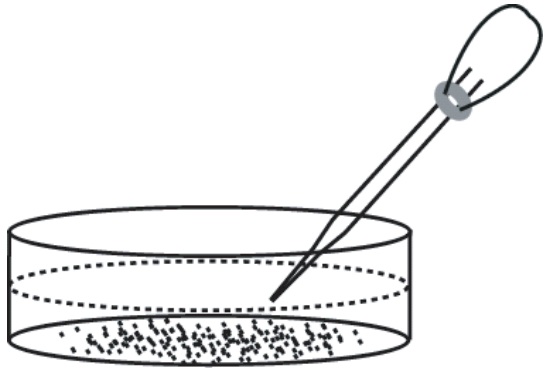
5.



enzyme treatment with DNAse

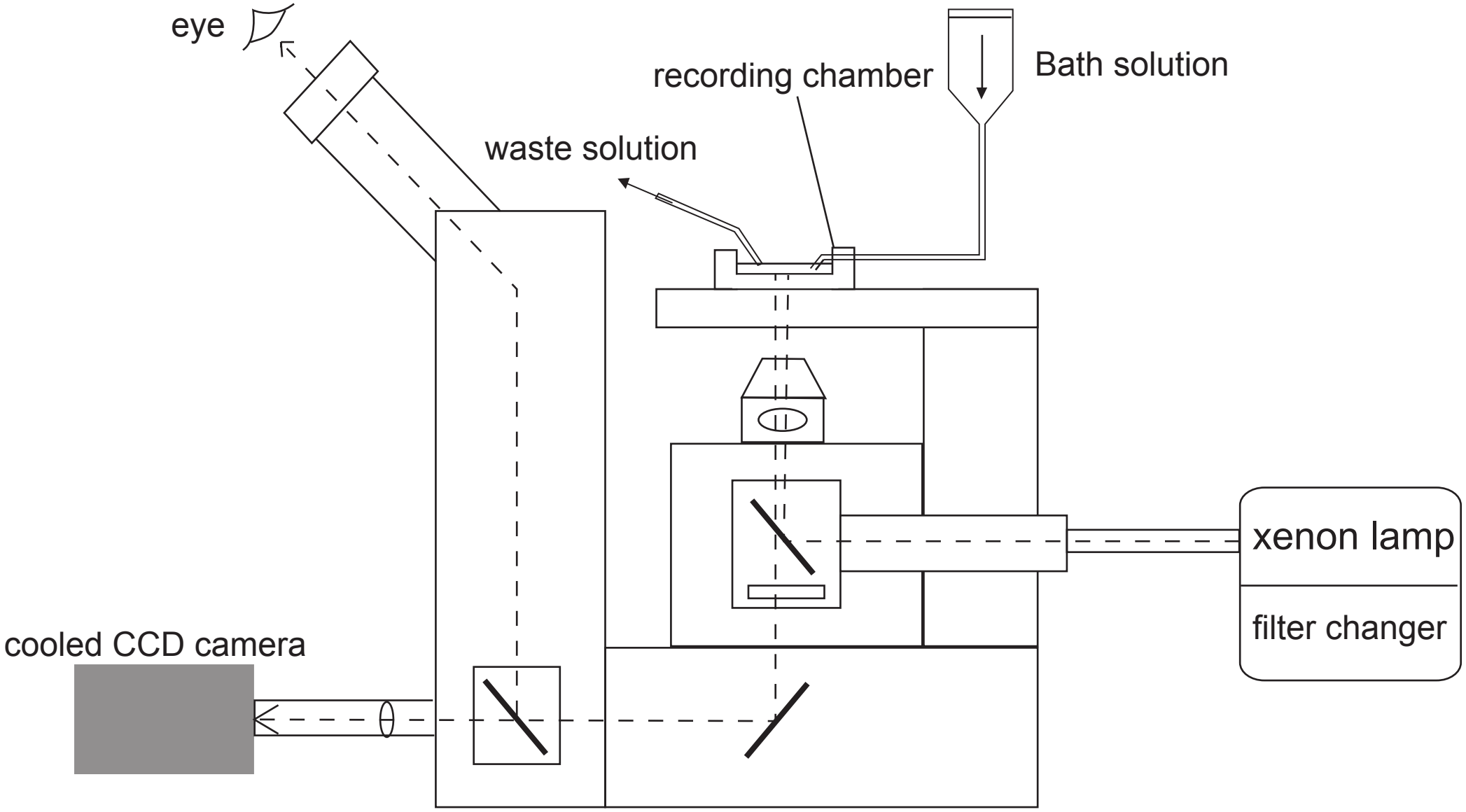
room temperature・10 min

6.



trituration

Fig. 6. A diagram of calcium imaging setup.



inverted fluorescence microscope

Fig. 7. Outline of application procedures of test solutions in the calcium imaging.

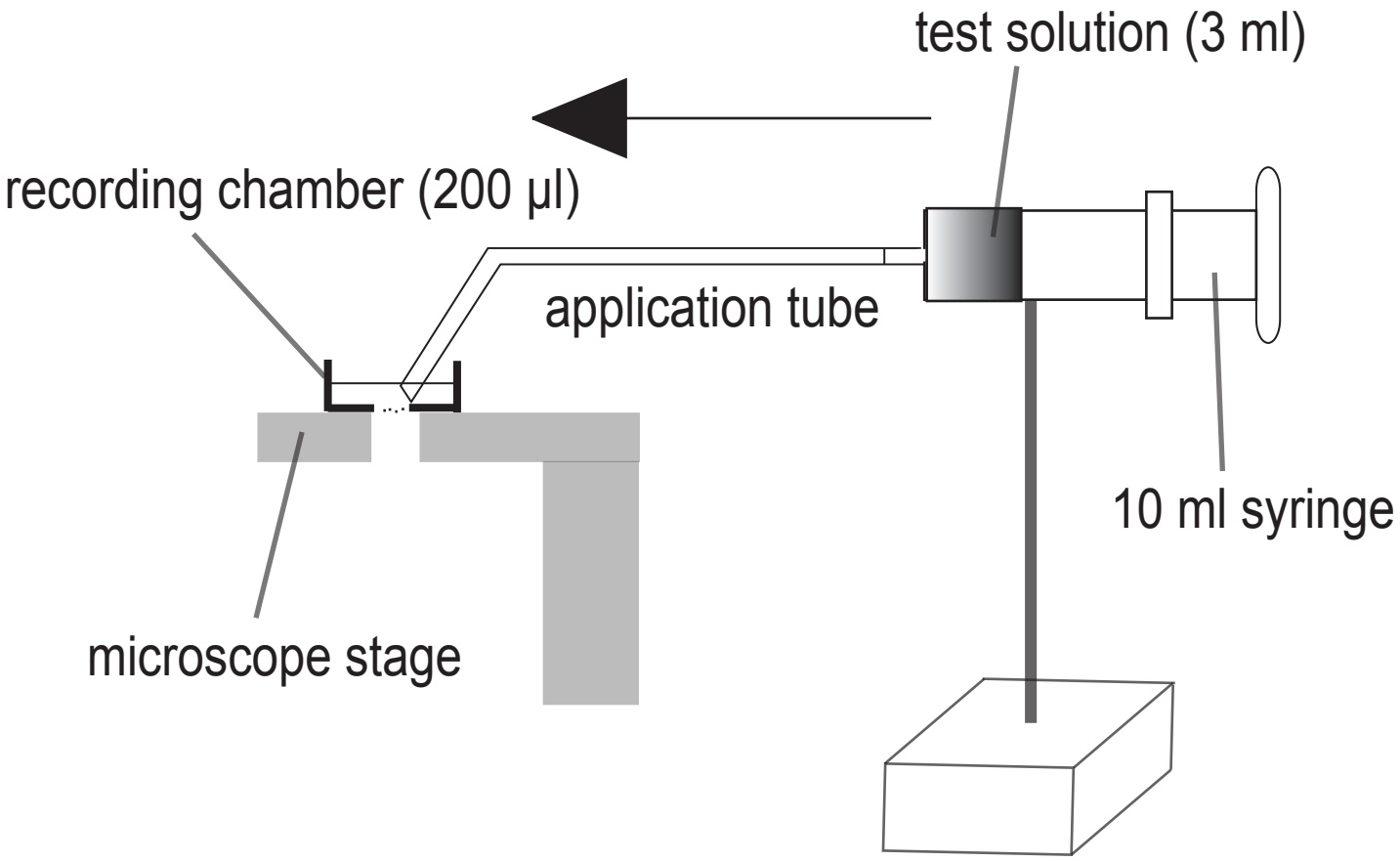

test solution (3 ml)
recording chamber (200 µl)
application tube
10 ml syringe
microscope stage

Fig. 8. Optical imaging reveals intracellular Ca^{2+} changes in ORN after the application of amino acid solution (250 μM arginine). Fluorescence images (pseudocolor scale) at 340 nm were taken at rest in the absence of the test solution (A), peak fluorescence after triggering the test solution (B), after washing (C). The color in pseudocolor scale represents relative calcium concentration, and top of the color scale (red) shows the highest concentration. (D) Time course of a Ca^{2+} transient induced by the test solution, which is taken from the whole ORN labeled by an analysis frame (indicated by green frame). The first arrow represents the timing of the stimulus, and the second arrow represents the beginning of wash out.

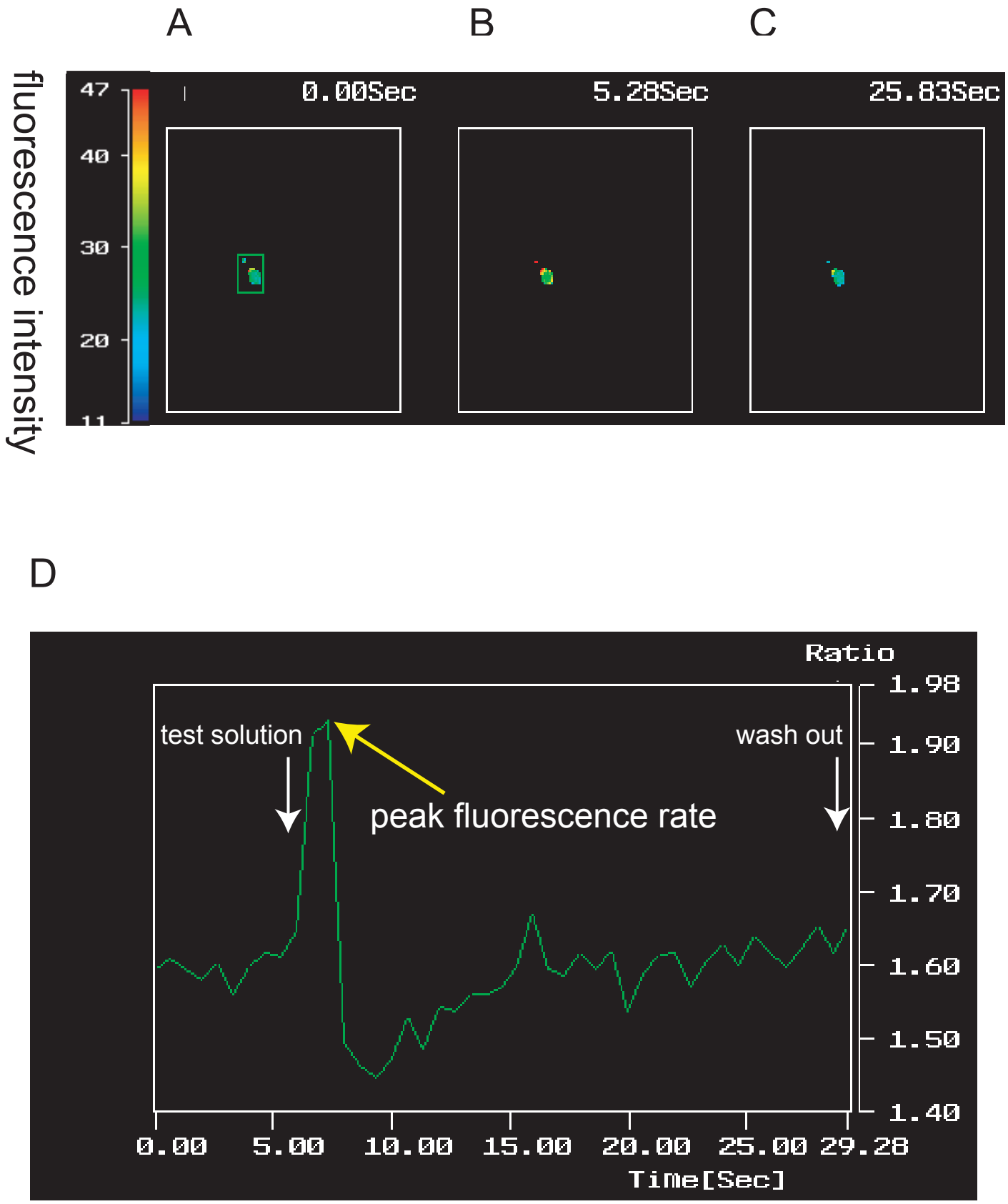

A
B
C
fluorescence intensity
47
40
30
20
11
0.00Sec
5.28Sec
25.83Sec
D
Ratio
1.98
1.90
1.80
1.70
1.60
1.50
1.40
test solution
wash out
peak fluorescence rate
0.00
5.00
10.00
15.00
20.00
25.00
29.28
Time[Sec]

Fig. 9. Time course of a Ca^{2+} transients induced by each amino acid solution (A: 250 μM glutamic acid, B: 250 μM alanine, C: 250 μM proline), which is taken from the whole ORN labeled by the analysis frame in Fig. 8. The first arrow represents the timing of the stimulus, and the second arrow represents the beginning of wash out.

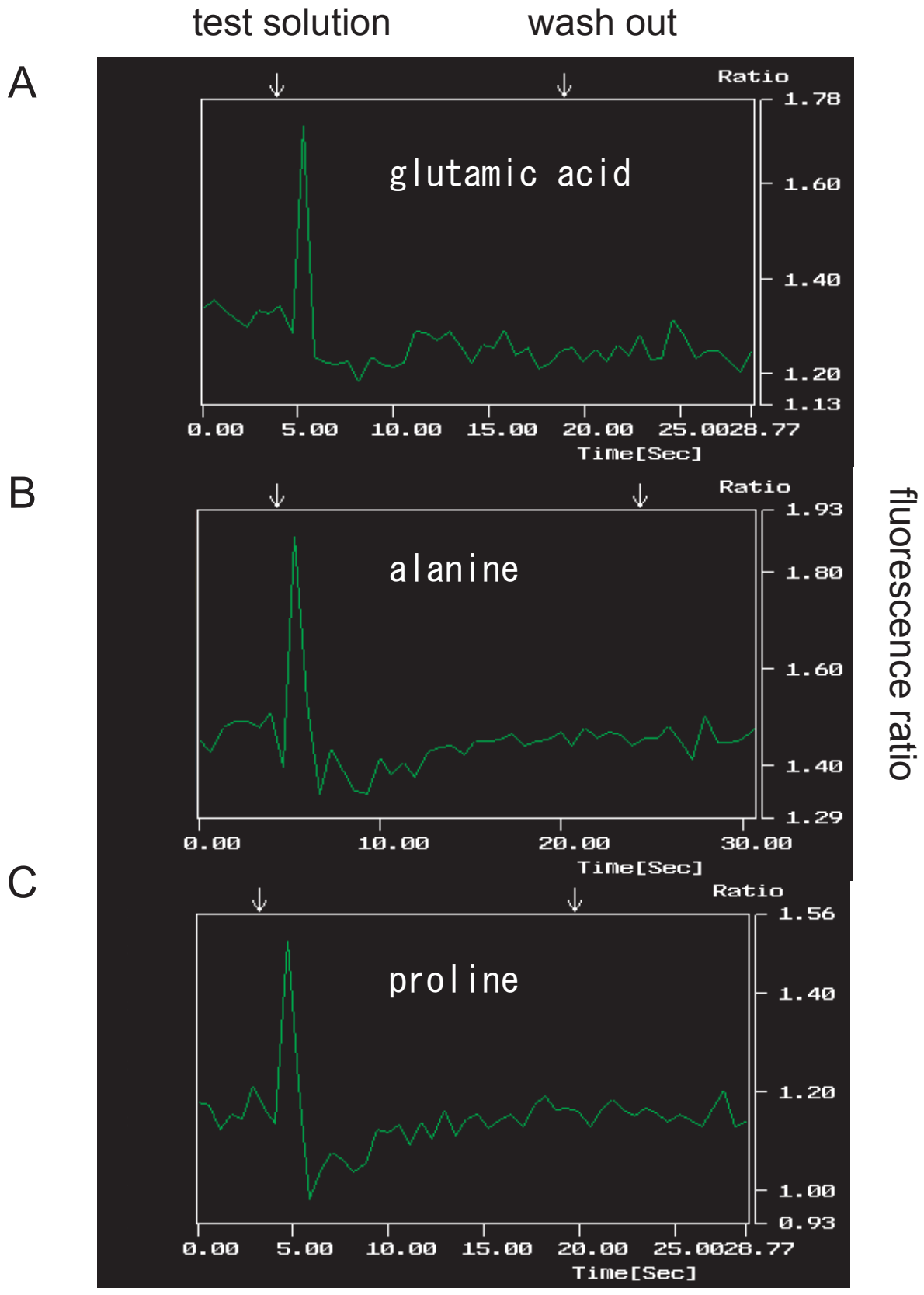

test solution
wash out
A
Ratio
1.78
1.60
1.40
1.20
1.13
glutamic acid
0.00
5.00
10.00
15.00
20.00
25.00
28.77
Time[Sec]
B
Ratio
1.93
1.80
1.60
1.40
1.29
alanine
0.00
10.00
20.00
30.00
Time[Sec]
C
Ratio
1.56
1.40
1.20
1.00
0.93
proline
0.00
5.00
10.00
15.00
20.00
25.00
28.77
Time[Sec]
fluorescence ratio
Time (sec)

Fig. 10. Response profiles to a series of amino acids in different cells. Four different amino acids (arginine, glutamic acid, alanine, and proline; all at 250 μM) were tested in 15 individual ORNs. Each cell was also stimulated with high K^+-solution to prove that it was alive. Circle represents intracellular calcium elevation induced by the test solution in the ORN. The minus sign indicate no response.

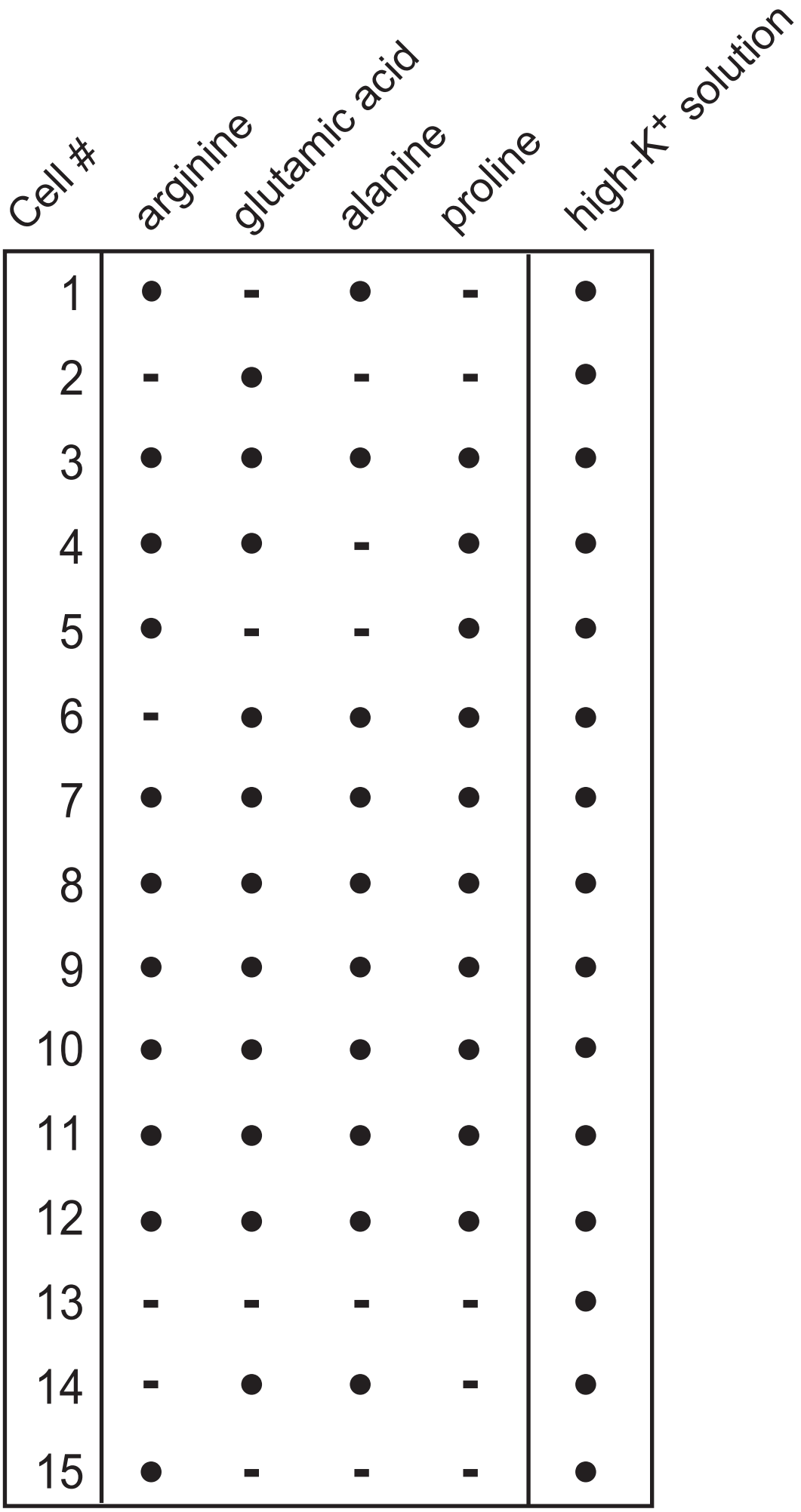

Cell #
arginine
glutamic acid
alanine
proline
high-K+ solution
1 ● - ● - ●
2 - ● - - ●
3 ● ● ● ● ●
4 ● ● - ● ●
5 ● - - ● ●
6 - ● ● ● ●
7 ● ● ● ● ●
8 ● ● ● ● ●
9 ● ● ● ● ●
10 ● ● ● ● ●
11 ● ● ● ● ●
12 ● ● ● ● ●
13 - - - - ●
14 - ● ● - ●
15 ● - - - ●

Fig. 11. Time course of a Ca^{2+} transient induced by 2 μM forskolin, which can directly activate cAMP pathway. The tested ORN responded to all tested amino acid solution (Cell 10 in Fig. 10). The fluorescence is taken from the whole ORN. The first arrow represents the timing of the stimulus, and the second arrow represents the beginning of wash out.

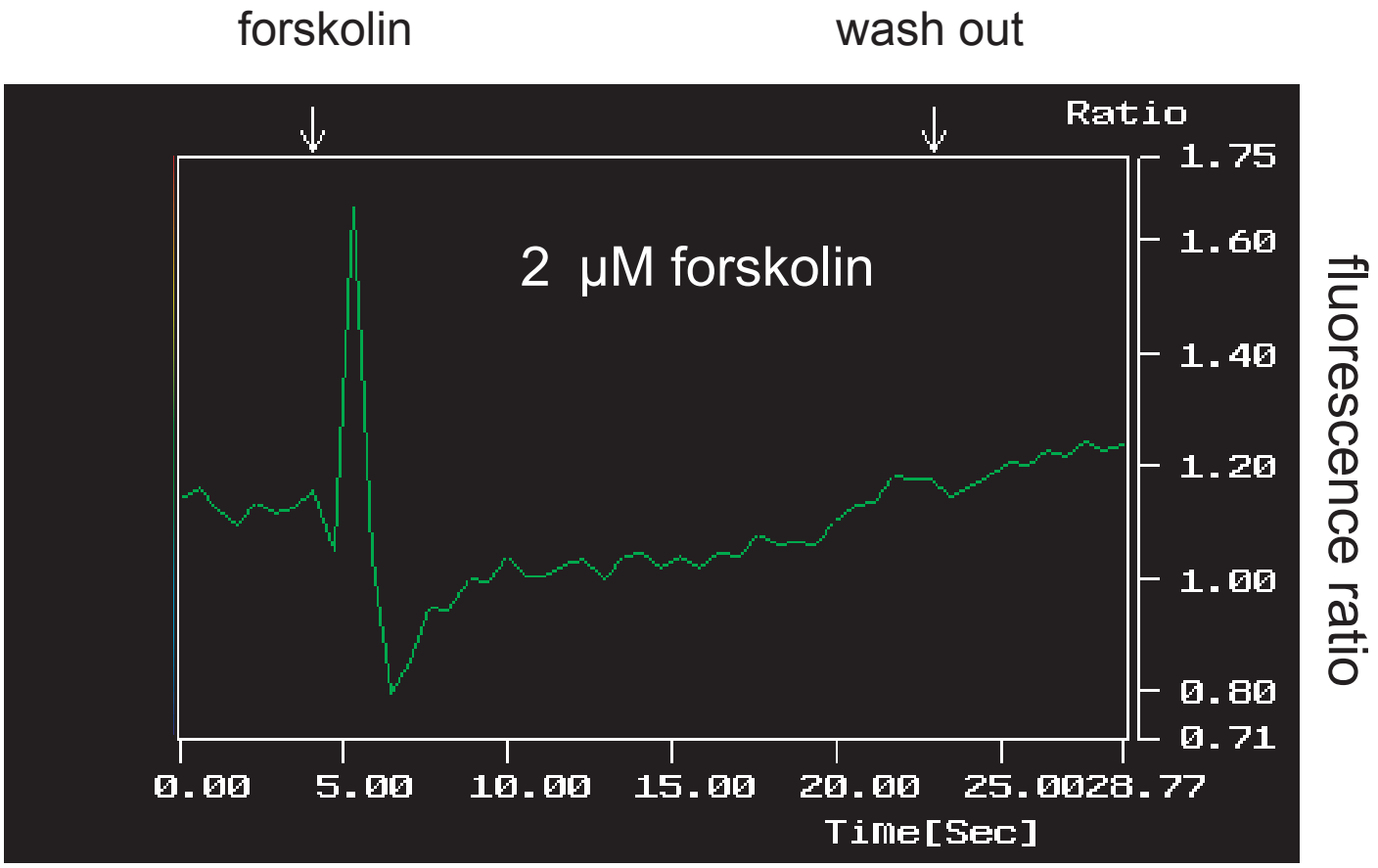

forskolin
wash out
Ratio
1.75
1.60
1.40
1.20
1.00
0.80
0.71
2 μM forskolin
0.00
5.00
10.00
15.00
20.00
25.00
28.77
Time[Sec]
fluorescence ratio
Time (sec)

Fig. 12. Optical imaging, which was recorded every 1.174 sec, revealed Ca^{2+} elevations in ORN after the application of 250 μM glutamic acid. These images were taken from the response to the glutamic acid solution as shown in Fig. 9A. After stimulus triggering (the fifth image), intracellular Ca^{2+} concentrations showed localized increase in the knob.

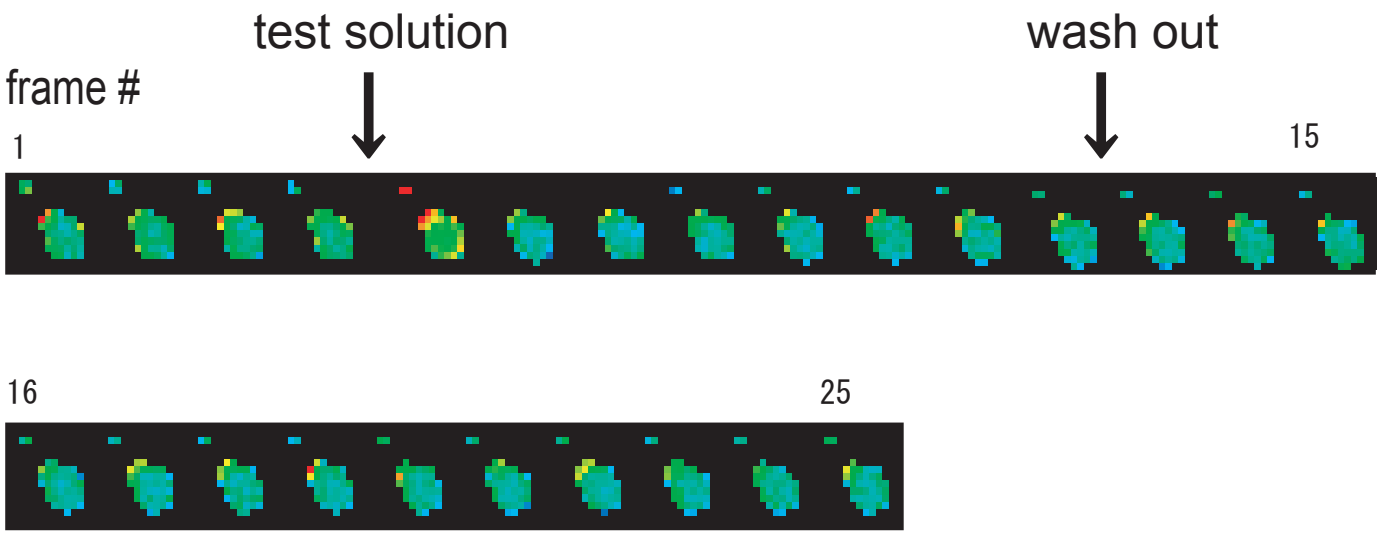
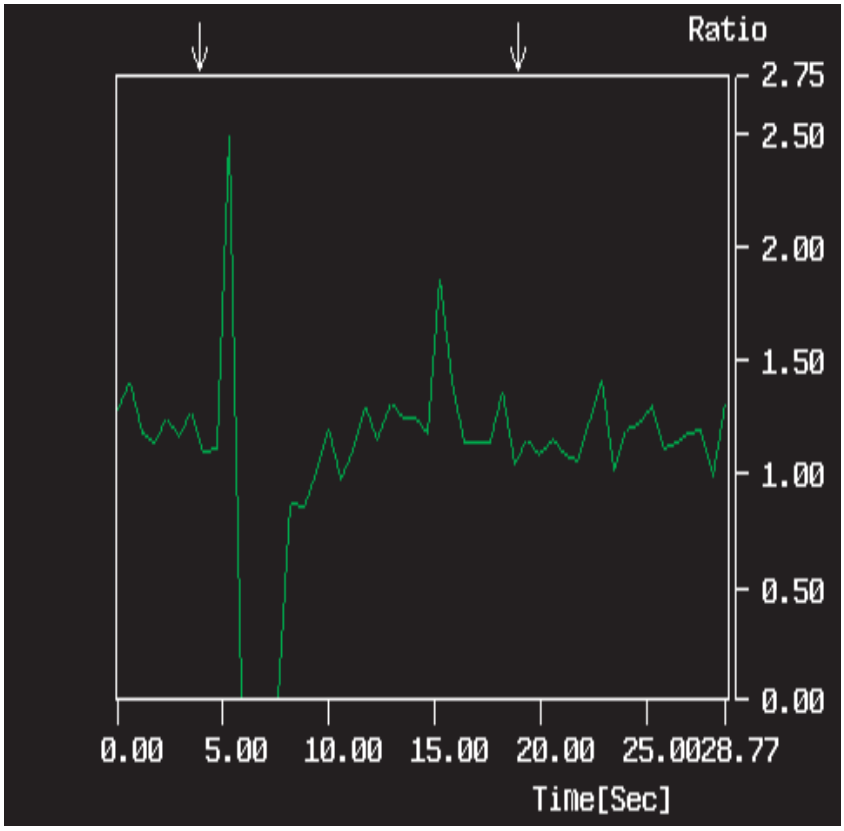
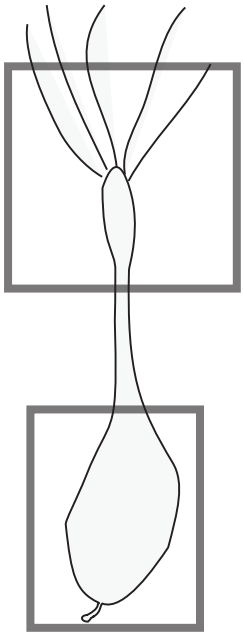

test solution
wash out
frame #
1
15
16
25

Fig. 13. Comparison of Ca^{2+} transients between the knob and the soma. The images were taken from the response to the glutamic acid solution as shown in Fig. 9A.

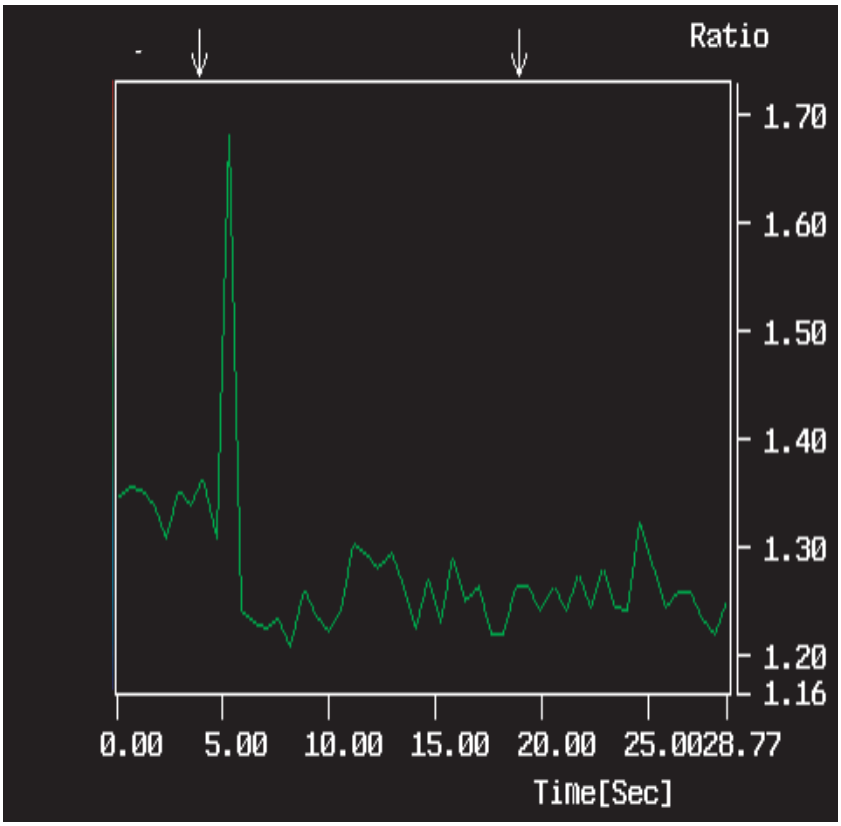
A



analysis frame: knob



B



analysis frame: soma

Fig. 14. A diagram of EOG recoding setup. An electrode was placed in the center of the olfactory epithelium. The head of the newt was mounted on a recording chamber, and solutions flowed continuously through an application tube into the chamber. Solutions were applied by gravity into a four-way valve that was operated pneumatically under remote control by computer. The abbreviation, Amp., represents a conventional amplifier.

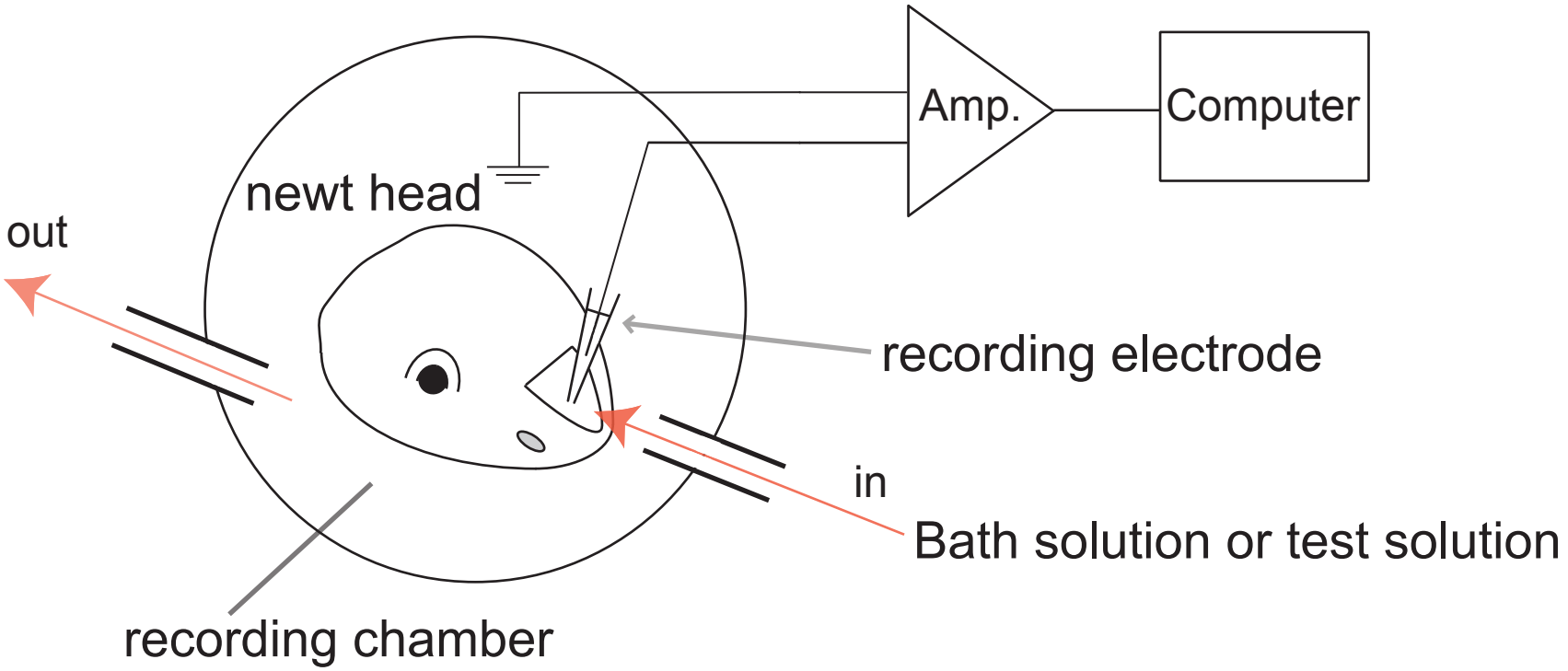

Amp.
Computer
newt head
out
recording electrode
in
Bath solution or test solution
recording chamber

Fig. 15. Olfactory response of Japanese newts after land adaptation. (A) EOG response to amino acid mixture (arginine, glutamic acid, alanine, and proline). In these figures, the amino acid mixtures (1 μM, 10 μM) were applied to the animal at each time point (land adaptation time: 0, 30, 54, 90, and 114 h). The increased response in one recording was the EOG response to 10 μM amino acid mixture. (B) EOG response to volatile odorant mixture (isoamyl acetate, n-amyl acetate, cineole, and limonene). Numbers on the left indicate land adaptation time. Solid bars represent the time of application of the test solutions.

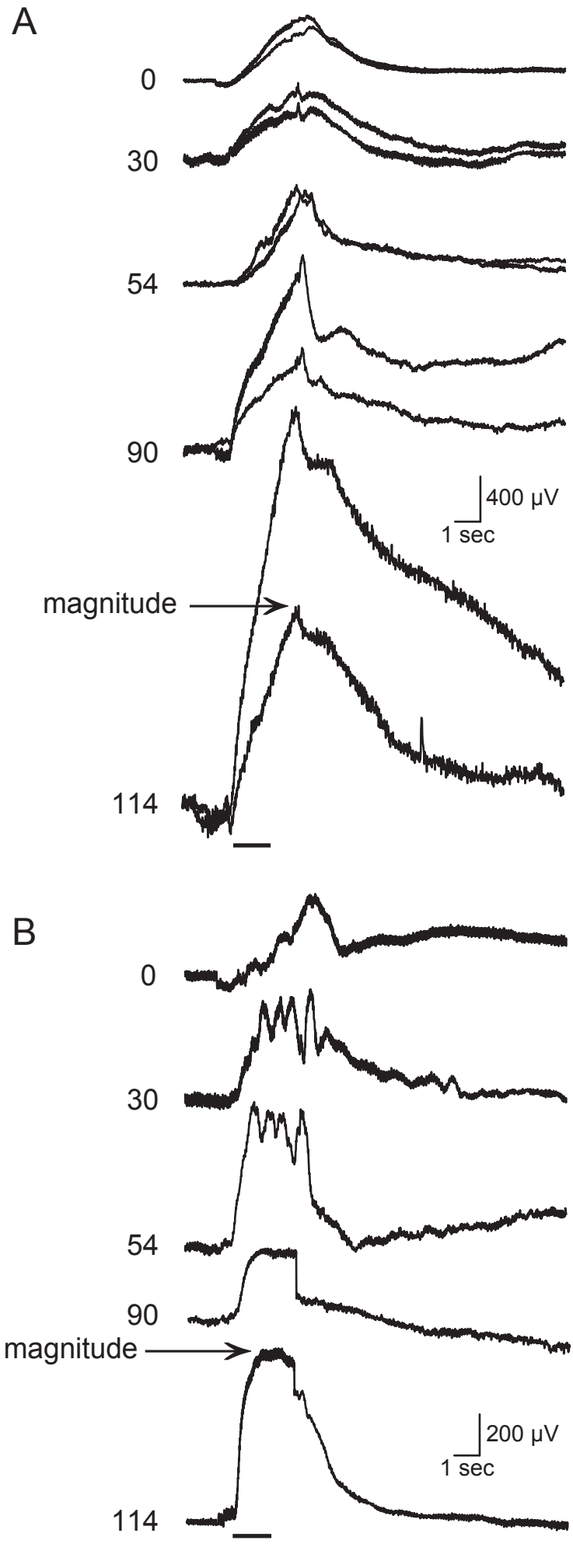
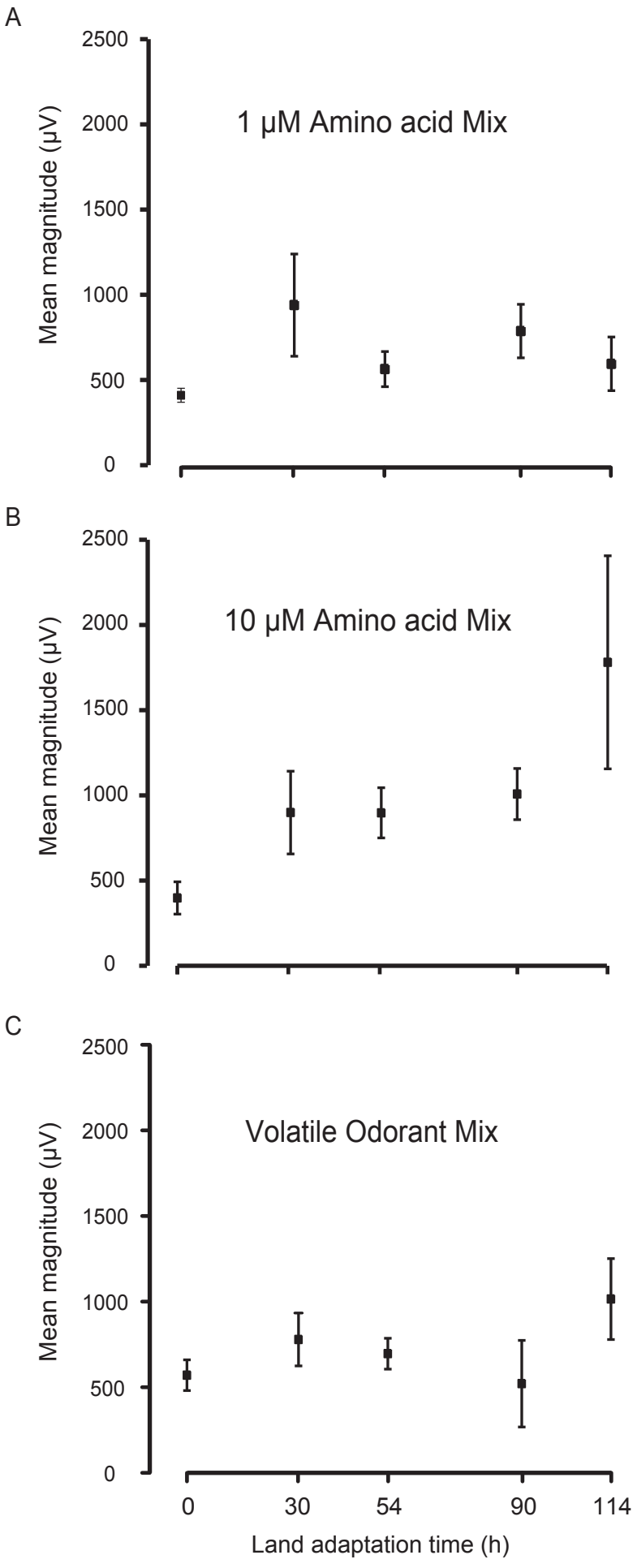

A
0
30
54
90
400 µV
1 sec
magnitude
114
B
0
30
54
90
magnitude
200 µV
1 sec
114

Fig. 16. Mean magnitude of EOG induced by test odorants at land adaptation time of 0, 30, 54, 90, and 114 h. The mean magnitude of EOG induced by (A) 1 μM amino acid mixture (the number of animals tested at each time point were 4, 11, 7, 8, and 9, respectively); (B) 10 μM amino acid mixture (the number of animals tested at each time point were 5, 11, 8, 8, and 7, respectively). The magnitude of EOG induced by amino acid mixtures was significantly affected by the time to land adaptation (by weighted linear model: $P < 0.05$). The slope estimates of weighted linear model (Table 1) indicate that the magnitude of EOG to 1 μM and 10 μM amino acid mixtures was significantly increased with land adaptation time ($P < 0.05$). (C) The mean magnitude of EOG to volatile odorant mixture (the number of animals tested at each time point were 8, 7, 5, 7, and 11, respectively). There was no significant relationship between the magnitude of EOG response to the volatile odorants and land adaptation time (by weighted linear model). The plot at each time point represents mean ± SE.


A
2500
2000
1500
1000
500
0
Mean magnitude (μV)
1 μM Amino acid Mix
B
2500
2000
1500
1000
500
0
Mean magnitude (μV)
10 μM Amino acid Mix
C
2500
2000
1500
1000
500
0
Mean magnitude (μV)
Volatile Odorant Mix
0
30
54
90
114
Land adaptation time (h)