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Responses to Putative Second Messengers and Odorants in Water Nose Olfactory Neurons of *Xenopus laevis*

Akio Iida and Makoto Kashiwayanagi

Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

Correspondence to be sent to: Makoto Kashiwayanagi, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan. e-mail: yanagi@hucc.hokudai.ac.jp

Abstract

Using the whole-cell mode of the patch-clamp technique, we attempted to record inward currents in response to cAMP, inositol 1,4,5-trisphosphate (IP_3) and odorants from sensory neurons in the olfactory epithelium of the *Xenopus laevis* lateral diverticulum (water nose). Dialysis of 100 μM of IP_3 induced inward currents, while dialysis of 1 mM of cAMP into olfactory neurons did not induce any response under the voltage-clamp conditions. Changes in membrane conductance were examined by applying ramp pulses. The slope of the current–voltage (*I–V*) curve during the IP_3-induced response was steeper than that after the response, indicating that IP_3 increased the membrane conductance. The water nose olfactory neurons have been shown to respond to both amino acids and volatile odorants. The slopes of *I–V* curves during responses to amino acids and a volatile odorant, lilial, were similar to those before the responses, suggesting that the total membrane conductance was not changed during responses to amino acids and the volatile odorant.

Introduction

The olfactory organs of aquatic animals respond to water-soluble odorants such as amino acids. Biochemical experiments indicated that amino acids induced inositol-1,4,5-trisphosphate (IP_3) accumulation in the catfish olfactory cilia (Restrepo *et al.*, 1993). In contrast, amino acids did not change cAMP levels in the catfish olfactory cilia even at high concentration such as 1 mM (Restrepo *et al.*, 1993). On the other hand, a gene-encoding cAMP-activated channel was cloned from the carp olfactory epithelium (Goulding *et al.*, 1992) and activities of the channel were observed in the plasma membrane of the carp olfactory neuron (Kolesnikov and Losolapov, 1993). Thus the transduction mechanism of amino acids has remained unclear. To explore the transduction mechanism of amino acids, it is important to measure the odor responses to amino acids directly from olfactory neurons of aquatic animals. To date, however, only a few such direct recordings have been obtained (Suzuki, 1977; Ivanova and Caprio, 1993).

In *Xenopus laevis*, the olfactory organ is compartmentalized into two independent subregions, the medial diverticulum and the lateral diverticulum. Anatomical observation has indicated that the olfactory sensory epithelium in the medial diverticulum comes into contact with air only, while the epithelium in the lateral diverticulum is in contact with water only (Altner, 1962). The latter, therefore, is known as the water nose. Olfactory sensory neurons in the water nose possess a gene family encoding olfactory G-protein-coupled receptors related to the receptors of fish (Freitag *et al.*, 1995). Previously, we recorded responses to amino acids from the water nose olfactory neurons of *X. laevis* under whole-cell voltage-clamp conditions (Iida and Kashiwayanagi, 1999). In addition, we found that the water nose responded to volatile odorants. In the present study, we explore the transduction mechanism of the responses to amino acids and a volatile odorant in olfactory neurons of the *Xenopus* water nose.

Materials and methods

Slice preparation of Xenopus laevis *olfactory sensory epithelium*

Slices of olfactory sensory epithelium were prepared as described in a previous paper (Taniguchi *et al.*, 1996). *Xenopus laevis* frogs were obtained from commercial suppliers and maintained at 15°C. The animals were fed porcine liver *ad libitum*. For the preparation of olfactory epithelial slices, animals were cooled to 0°C to anesthetize them completely and then decapitated. The olfactory epithelia were quickly removed from the decapitated frogs and cut into slices ~120 μm thick with a vibrating slicer (DTK-1000, D.T.K., Kyoto, Japan) in normal Ringer solution at 0°C and stored at 4°C. Epithelial slices were fixed on the glass at the bottom of a recording chamber, permitting access by patch pipette to neurons on the surface of the slice. The preparations were viewed under an upright

microscope (model Optiphot, Nikon, Tokyo, Japan) using a ×40 water immersion lens.

Data recording and analysis

The conventional whole-cell patch-clamp method was used to measure transmembrane currents (Hamill *et al.*, 1981). Patch pipettes with resistances of 5–10 MΩ were made from borosilicate glass capillaries with an inner filament (GD-1.5, Narishige Co., Tokyo, Japan) using a two-stage electrode puller (PP853, Narishige Co.); the pipettes were heat-polished. Gigaohm seals were obtained by applying negative pressure (–30 to –100 cm H_2O). The whole-cell configuration was attained through the application of additional negative pressure. Membrane currents (holding potential –70 mV) and voltages were recorded in the whole-cell configuration. Data were recorded continuously using an EPC-7 patch-clamp amplifier (List, Darmstadt, Germany) and stored on videocassette via a digital audio processor (PCM-501, Sony, Tokyo, Japan). All recordings were carried out at room temperature. Membrane currents were low-pass filtered with a two-pole, Bessel filter of EPC-7. The filter frequency was 10 kHz. Analysis was carried out on a personal computer using pCLAMP software (Axon Instruments, Foster City, CA). All values are given as mean ± SEM. A liquid junction potential was measured as described by Neher (Neher, 1992). The liquid junction potential never varied more than several mV in the altered solutions. All data were corrected for the liquid junction potential.

Stimulation

Olfactory neurons were stimulated with extracellular solutions by bath application from the outlet of the stimulating tube. Ringer solution, delivered by gravity, was alternated with odorant solutions by means of eight electrically actuated valves.

Solutions

The Ringer solution consisted of the following (in mM): 116 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES–NaOH (pH 7.4). Patch pipettes were usually filled with an internal solution (in mM): 115 KCl, 2 $MgCl_2$, 2 EGTA, 10 HEPES–KOH (pH 7.4). Stock solutions of lilial and L-alanine were prepared at 1 mM and 0.1 M, respectively, and stored at 4°C. These solutions were added to Ringer solution to give the concentrations of odorants indicated. The final concentration of each amino acid in the amino acid cocktail (L-alanine, L-arginine, L-glutamic acid and L-methionine) was 1 mM. The deviations in pH in the stimulating solutions were within 0.2. Lilial was vigorously stirred with a magnetic stirrer for >30 min at room temperature.

Chemicals

Amino acids (L-alanine, L-arginine, L-glutamic acid and L-methionine) were obtained from Wako (Tokyo, Japan). Lilial was kindly supplied by Takasago International (Tokyo, Japan). All chemicals used were of the highest grade available.

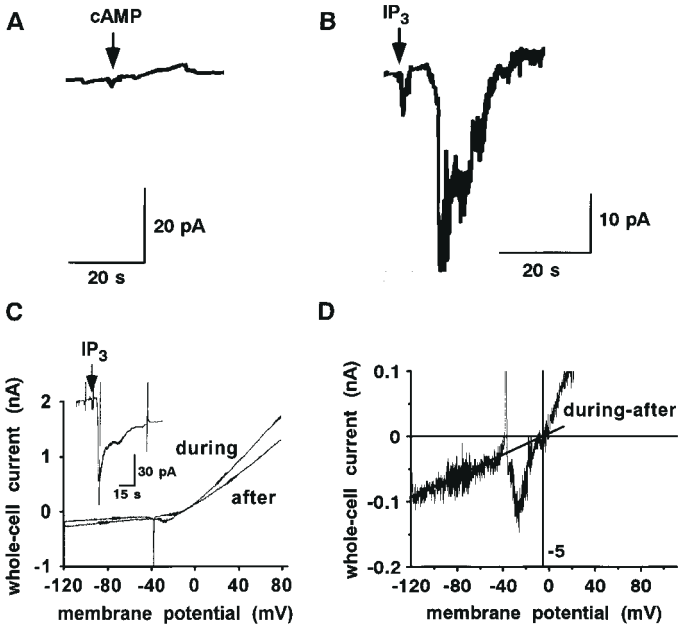

Figure 1 Responses to putative second messengers of the water nose olfactory neuron. Membrane currents at the breakthrough measured with patch pipettes filled with inner solutions containing 1 mM cAMP **(A)** and 100 μM IP_3 **(B)**. Whole-cell current–voltage relationships for the current induced by 100 μM IP_3 **(C)**. The current was measured by applying a voltage ramp (500 mV/s) from –120 to 80 mV before and during the response. The subtraction of the *I–V* curve during the response from that before the response **(D)**. The holding potential was –70 mV.

Results

Dialysis of cAMP into olfactory neurons

To examine the electrophysiological effects of cAMP on olfactory sensory neurons of the *Xenopus* water nose, a high concentration of cAMP (1 mM), which induced maximum responses in olfactory sensory neurons of the newt and turtle (Kurahashi, 1990; Kashiwayanagi and Kurihara, 1995), was dialyzed from the patch pipette into the neuron. Upon the breaking of the patch, cAMP did not practically induce current changes in 94 cells examined under the whole-cell patch-clamp conditions (Figure 1A). These results suggest that cAMP is not a primary second messenger in the odor reception of the *Xenopus* water nose.

Current responses to IP_3

One hundred micromolar IP_3 was dialyzed from the patch pipette into the olfactory neurons. Upon the breaking of the patch, IP_3 induced inward currents in 49 out of 87 cells (Figure 1B). The peak amplitude of inward currents in response to 100 μM IP_3 ranged, typically, from 0 to 200 pA.

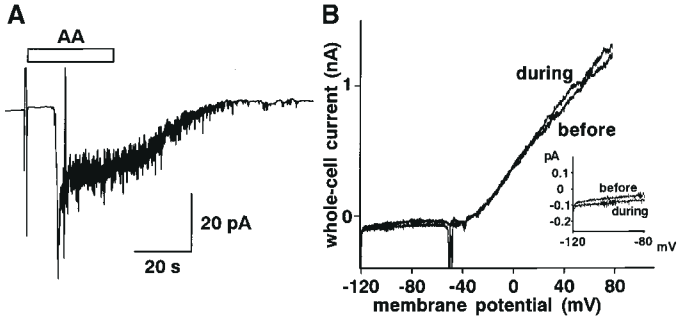

Figure 2 Current–voltage relationship during responses to amino acid cocktail. Inward current in response to the amino acid cocktail **(A)**. Whole-cell current–voltage relationships for the current induced by the amino acid cocktail **(B)**. The current was measured by applying a voltage ramp (500 mV/s) from –120 to 80 mV before and during the response. The holding potential was –70 mV.

The mean amplitude of inward currents was 27.6 ± 4.6 pA (n = 88).

The voltage dependence of membrane currents induced by 100 μM IP_3 was examined by applying a voltage ramp from –120 to 80 mV (500 mV/s) to voltage-clamped neurons near the peak of the response and after the desensitization of responses (Figure 1C). Figure 1D shows subtracted current after the response from that during the response. The mean reversal potential was estimated to be –0.3 ± 1.7 mV (n = 7).

Voltage dependence of responses to amino acids and a volatile odorant

The voltage dependence of currents in response to an amino acid cocktail was examined by applying the voltage ramp before odor stimulation and near the peak of the response (Figure 2). The slope of the I–V curve below –60 mV during inward current responses to the cocktail was not essentially changed from that before the response.

Similarly, I–V curves before stimulation with 1 mM L-alanine and during the response to L-alanine were parallel below –60 mV (Figure 3A). The voltage dependence of currents in response to a volatile odorant, lilial, was also examined (Figure 3B). The slope of the I–V curve during the response to 100 μM lilial below –60 mV was similar to that before the stimulation. I–V curves during odor responses were measured from 14 cells. Changes in the slope of the I–V curve during the response to amino acids and lilial were observed in only two cells, and no changes in the slope were observed from 12 cells.

Effects of ruthenium red on odor responses

Ruthenium red has been shown to inhibit responses induced by IP_3 in olfactory neurons and vomeronasal neurons (Miyamoto *et al.*, 1992; Honda *et al.*, 1995; Taniguchi *et al.*, 1995; Inamura *et al.*, 1997). Figure 4 shows the response of a neuron to an amino acid cocktail. After application of

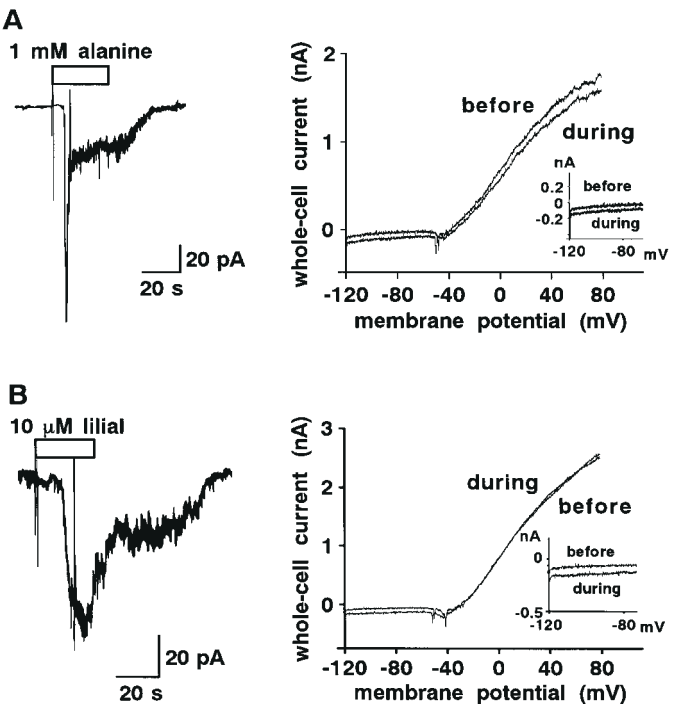

Figure 3 Current–voltage relationships during responses to alanine and lilial. The current was measured by applying a voltage ramp (500 mV/s) from –120 to 80 mV before and during the response to 1 mM alanine **(A)** and to 10 μM lilial **(B)**. The holding potential was –70 mV.

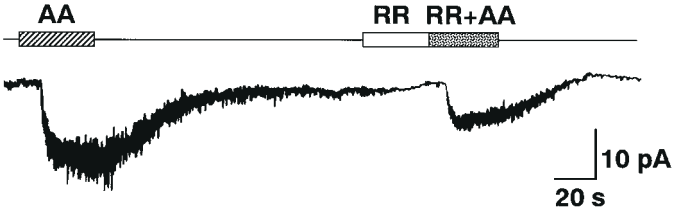

Figure 4 Effect of ruthenium red on the odor response to the amino acid cocktail. After application of 10 μM ruthenium red, the amino acid cocktail containing 10 μM ruthenium red was applied. The holding potential was –70 mV.

10 μM ruthenium red, the amino acid cocktail containing ruthenium red induced an inward current, although the magnitude of the response with ruthenium red was smaller than that without ruthenium red. Similar results were obtained from three cells.

Discussion

In the present study, we examined responses to putative second messengers in sensory neurons of the water nose of *Xenopus laevis* using the whole-cell voltage-clamp technique and showed that the water nose olfactory neurons responded to IP_3 but not to cAMP.

Odorants, which induce cAMP accumulation in the olfactory epithelium of the bullfrog, induce cAMP accumu-

lation in the olfactory epithelium of the rat, sheep and turtle (Sklar *et al.*, 1986; Breer and Boekhoff, 1991; Fabbri *et al.*, 1995; Okamoto *et al.*, 1996). These volatile odorants induce responses in the sensory neurons of the *Xenopus* water nose (Iida and Kashiwayanagi, 1999). Hence, it is possible that responses to volatile odorants are mediated via the cAMP-dependent pathway in the water nose olfactory neurons. However, dialysis of 1 mM of cAMP did not induce inward currents in the neurons, which suggests that odor responses of the water nose olfactory neurons to volatile odorants are generated via a cAMP-independent transduction pathway. In addition, the water nose olfactory neurons also responded to amino acids, indicating that responses to amino acids are not mediated via cAMP. This is consistent with biochemical measurements showing that amino acids, even in high concentrations, do not induce cAMP accumulation in catfish olfactory cilia (Restrepo *et al.*, 1993).

As shown in the present study, dialysis of IP_3 into the water nose olfactory neurons induces inward currents. Hence, it is possible that IP_3 mediates responses to amino acids. Inward current responses to amino acids are, however, not completely inhibited by ruthenium red, an inhibitor of IP_3-activated channels, suggesting that the IP_3-independent transduction pathway, in addition to the IP_3-dependent pathway, contributes to the generation of odor responses to amino acids in the water nose.

The slopes of *I–V* curves during odor response to amino acids and a volatile odorant were similar to those before odor stimulation. It is possible that an increase in membrane conductance by activation of IP_3-activated channels is accompanied by a decrease in membrane conductance due to closing of other ion channels. There is another possible mechanism for odor reception in the water nose. Olfactory responses of the chum salmon to amino acids in fresh water are similar to those to amino acids in sea water (Shoji *et al.*, 1994), suggesting that changes in membrane conductance at the receptive membrane of the olfactory neuron do not contribute to the generation of responses to amino acid in the chum salmon. In the *Xenopus* water nose, the mechanism accompanying without changes in membrane conductance, such as changes in the phase boundary potential (Kashiwayanagi and Kurihara, 1993), may also contribute to the generation of odor responses to amino acids and a volatile odorant.

As shown in previously (Iida and Kashiwayanagi, 1999) and in the present paper, the water nose sensory neurons of *Xenopus* responded sensitively to various volatile odorants, although the neurons have no mammalian-type, G-protein-coupled receptors (Freitag *et al.*, 1995). In addition, the sensory neurons did not respond to cAMP dialyzed to the neurons. These results suggest that volatile odorants induce responses in the sensory neurons via neither known mammalian-type receptors, nor the cAMP-dependent pathway. Such a pathway may contribute to the generation of odor responses in other animals. For example, the responses of turtle olfactory neurons to volatile odorants were induced via both the cAMP-dependent and cAMP-independent pathways (Kashiwayanagi *et al.*, 1994; Kashiwayanagi and Kurihara, 1995). The cAMP-independent pathway observed in the turtle may be a similar pathway to that observed in the sensory neurons of the *Xenopus* water nose.

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