RESULTS

1 Targets of odor inhibitory action

1.1 Odor inhibition of IBMX-induced depolarization

Anisole has a floral flavor and has been used as an odorant in many psychological, behavioral, and physiological studies (Laing et al., 1984). The anisole was able to elicit excitation in also newt olfactory receptor neurons. For example, potential recordings from 38 cells show that application of 1 mM anisole to 38 cells caused membrane depolarization in 12 cells; the mean amplitude of depolarization was 21.6 ± 5.46 mV (mean ± SE). The reason why excitatory responses to an odorants are not equivalent among cells is thought to that olfactory receptor proteins are not equivalent among cells (see Introduction). In order to avoid the difficulties of analysis and examine specifically the inhibitory effect by odorants, I chose cells in which odorants did not elicit depolarization or depolarizing currents in following experiments.

In eight cells that responded to IBMX, a membrane-permeable inhibitor of phosphodiesterase (PDE), and did not respond to 1 mM anisole (Figure 11), application of 0.25 mM IBMX caused a depolarization of 16.4 ± 3.36 mV. During the depolarization, addition of 1 mM anisole produced membrane hyperpolarization in all the tested cells (n=8) (Figure 11). The mean amplitude of membrane hyperpolarization was 10.4 ± 3.29 mV. This odor-induced hyperpolarization occurred with very short latencies (64.0 ±3.30 ms; n=8). IBMX inhibits the basal activity of PDE, and thus elevates intracellular cAMP (Firestein et al., 1991). Because the odorant did not cause hyperpolarization in the absence of IBMX-induced depolarization (Figure 11), the hyperpolarization is likely due to suppression of the cAMP-induced conductance, which depolarizes the cell.

Figure 11B shows potential recordings, from the same cell as in Figure

11A, during repetitive application of a hyperpolarizing current. Odor stimulation caused hyperpolarization and decreased the amplitude of the injected hyperpolarizing voltage, indicating that the hyperpolarization was due to a decrease in conductance. In order to determine whether odor stimulation can suppress the IBMX-induced current, I examined the effect of odorants on the current and measured reversal potentials of the current in voltage-clamp mode, shown in Figure 12. For focusing on suppression by odorant, I also used only cells that did not respond to the odorant.

1.2 Odorants inhibit IBMX-induced conductance, but not cell's basal K^+ conductance

In a subset of six cells that responded to IBMX but not to 1 mM anisole, the mean peak amplitude of current induced by 0.25 mM IBMX was 156 ± 23.2 pA at the holding potential +25 mV. The application of 1 mM anisole for 2 s suppressed the current in all the cells at +25 mV (Figure 12). The mean peak amplitude of the current suppression is 78.3 ± 14.7 pA at +25 mV in the six cells. The ratio of the suppressed current to the induced current was 0.53 ± 0.08 (n=6) (Figure 15). In the six cells, the mean reversal potential of the conductance suppressed by the odorant was 6.64 ± 1.84 mV, which was remarkably similar to that of the conductance induced by IBMX (6.04 ± 2.23 mV; $p>0.68$, paired t-test).

Olfactory receptor neurons have basal potassium conductance, which keeps the cell hyperpolarized (Trotier, 1986; Lowe and Gold, 1991; Kleene, 1992). While odor stimulation suppressed the conductance induced by IBMX, the basal potassium conductance, which was inhibited by 10 mM TEA, was not suppressed by the odorant (Figure 12A). The fact that the odor stimulation did not suppress the TEA-suppressive current but did suppress

IBMX-induced current is indicating that the odor suppression of the transduction conductance could cause the hyperpolarization (Figure 11). Furthermore, when using 10 μM forskolin instead of IBMX to activate the transduction conductance, I was able to observe the suppression of the current and hyperpolarization by odorants (n=4; data not shown).

1.3 Odorants inhibit cAMP-induced current

Receptor proteins are not involved in the IBMX-induced increase in intracellular cAMP in olfactory receptor neurons. Thus, the fact that odor stimulation suppressed IBMX-induced current suggested that a target of the odor inhibitory effect on cAMP-system is downstream to receptors. To investigate the target further, I used intracellular perfusion to introduce cAMP into the cell directly and examined the inhibitory effect on cAMP-induced current.

Introduction of 0.5 mM cAMP into six cells caused an outward current of 544 ± 161 pA at +25 mV. An application of the odorant suppressed currents (145 ± 18.3 pA at +25 mV) in all six cells after the introduction of 0.5 mM cAMP (Figure 13B and D), while it did not cause suppression before the introduction of 0.5 mM cAMP (Figure 13A and C). The mean ratio of the suppressed current to the induced current was 0.39 ± 0.09 (n=6)(Figure 15). The reversal potential of the suppressed conductance was almost identical to that of conductance induced by introducing cAMP (Figure 13E). Odor stimulation was even able to suppress the current induced by intracellular perfusion of the cAMP, suggesting that the odorant inhibited events after increase in intracellular cAMP.

1.4 Odorants inhibit 8-Br-cGMP induced current

Next, to examine the possibility that the inhibition of cAMP-induced conductance involved the degeneration of cAMP, I tested the inhibitory effect of an odorant on current induced by 8-Bromo-cyclic GMP (8-Br-cGMP), a membrane-permeable and unhydrolysable (Zimmerman et al., 1985; Nawy, 1999) analogue of cyclic GMP that activates the CNG-channel in olfactory receptor neurons by bath application (Firestein, 1991).

Of the three cells that responded to 1 mM 8-Br-cGMP but not to 1 mM anisole, the mean peak amplitude of current induced by 8-Br-cGMP was 543 ± 31.5 pA at +25 mV. While the odorant did not cause suppression before application of 8-Br-cGMP, it did suppress the current (263 ± 157 pA at +25 mV) after application (Figure 14). The mean ratio of the suppressed current to the induced current was 0.47 ± 0.26 (n=3)(Figure 15). Together with the fact that odor stimulation suppressed the transduction current during application of IBMX (Figure 12), the result that the odorants suppressed the 8-Br-cGMP induced current (Figure 14) would seem to contradict the possibility that inhibition of the cAMP-induced conductance involved breakdown of cAMP. A more likely possibility can be the direct inhibitory action of the odorant on the CNG-channel directly.

2 Property of odor inhibitory effect

2.1 Dose-dependence of odor inhibition

Odor suppression of the transduction current was dose dependent (Figure 16). Application of 0.25 mM IBMX to five cells elicited a 98.2 ± 55.2 pA inward current at -45 mV, which was suppressed by anisole in a dose dependent manner. In two of five cells, 123 μM anisole suppressed the IBMX-induced current by about 10 % and 10 mM anisole suppressed nearly

all the current. The mean IC_{50} value was 1.37 ± 0.21 mM (n=5).

2.2 Interaction between odor excitatory and inhibitory effects

Next, I examined interplay between the excitatory and the inhibitory effects. Isoamyl acetate suppressed a forskolin-induced current in a dose dependent manner, shown in Figure 17. On the other hand, an increase in forskolin concentration made the suppression less (Figure 17). Such interplay between the excitatory and the inhibitory effects on transduction current was observed in two other cells. To check whether an increase in intracellular cAMP causes the antagonistic suppression, I examined the suppression of a current caused by introduction of cAMP. I observed that odorants suppressed the cAMP-induced current (Figure 18A), and then compared the peak amplitude of current suppression by an odorant mixture (1.1 mM cineole, isoamyl acetate, anisole, and limonen) on cells intracellularly dialyzed with 0.1 mM cAMP and those with 10 mM cAMP (Figure 18). The amplitude of current suppression by the odorant mixture on the cells dialyzed with 10 mM cAMP was significantly smaller than in the cells dialyzed with 0.1 mM cAMP ($p<0.01$)(Figure 18).

2.3 Non-cell-specific suppression by odorants

An inhibitory effect on the transduction current was not found only with anisole. Of the cells that responded with depolarization to 0.25 mM IBMX at -45 mV but did not show an excitatory response to each test chemical, I examined the suppression of IBMX-induced current by four odorants (1 mM isoamyl acetate, cineole, limonene, and isovaleric acid) and an amino acid (1mM glutamate). The suppression was observed in all the four odorants as shown in Figure 19.

Studies on profiles of excitatory responses in olfactory receptor neurons (ORNs) have shown that chemical properties of odorants are reflected in activated neural pattern of ORNs: a set of odorants whose chemical properties are dissimilar exhibits dissimilar activated neural patterns each other (Mori and Yoshihara, 1995). For example, a set of odorants (cineole, isoamyl acetate, anisole, and limonene), whose chemical properties are known to be quite different, were found to be clearly distinguished by ORNs (Figure 20B)(Duchamp-Viret et al., 1990; Sicard and Holley 1984; Duchamp et al., 1974). It is showed that values of Pearson's 'r' correlation coefficient for responses to each pair of odorants are low, indicating that these odorants cause excitation in less overlapping population of ORNs (Duchamp-Viret et al., 1990).

Nevertheless, general profiles of odor suppression by this set of odorants in ORNs were similar, compared to those of odor excitation. Figure 20 shows profile difference between suppression and excitation. To evaluate the similarities between responses, I calculated the Pearson's 'r' correlation coefficient for current responses to each pair of odorants. The 'r' values for all the 6 pairs were over 0.85 (Figure 21B), which is well above the highly significant level of $p<0.001$ ('r'= 0.91 ± 0.02). Shown in Figure 21A, all the 'r' values for the 66 pairs of receptor cells were distributed above 0.55 ('r'= 0.89 ± 0.01). The high 'r' values for the pairs of the odorants and of receptor cells demonstrate that the odorants were hardly distinguished in the suppression, and the profiles of ORNs in the suppression were almost uniform.

3 Mutual suppression caused by odor inhibitory effect

3.1 Odorants inhibit odor-induced depolarization

The above results demonstrate that odorants were able to have an

inhibitory effect on cells in which cAMP-system was activated by application of IBMX, 8-Br-cGMP, or injection of cAMP. This suggests that the application of an odorant to a cell could inhibit the depolarization caused by another odorant. In the next experiment, I examined this possibility.

Of 5 cells that responded to 1 mM isoamyl acetate but not to 1 mM anisole, mean amplitude of depolarization caused by isoamyl acetate was 26.6 ± 10.9 mV. Addition of anisole caused membrane hyperpolarization when the cells were depolarized by background application of isoamyl acetate in all the cells (Figure 22). The mean amplitude of hyperpolarization was 18.8 ± 10.6 mV.

The results in Figure 22 corresponded with that in Figure 11 and demonstrate that odor stimulation is able to inhibit depolarization even when the depolarization was caused by another odorant.

3.2 Mixture suppression is dependent on individual odor excitation

As shown in Figure 17 and 18, there was interplay between inhibitory and excitatory effects on transduction current. According to the result in Fig 18, odor inhibitory effect was antagonized more strongly as intracellular cAMP increased. Thus it is suggested that degree of inhibition by odorants would be dependent on amplitudes of excitatory responses, which is assumed to reflect the total production of cAMP.

In Figures 23 and 24, I compared a response to a mixture odorant with responses to individual odorants, and examined relation between amplitude of responses to mixture odorant and that to individual odorants. Figure 23 shows typical relation between responses to mixture odorants and individual component odorants; and, Figure 24 exhibits collected results. All cells shown in Figures 23 and 24 (n=14) responded to 0.5 mM isoamyl acetate excitatory (282 ± 37.3 pA; mean ± SE), while their responses to 0.5 mM

anisole were varied (vertical values of plots in Figure 24 show relative amplitude of responses to anisole. The values are distributed from 1 to 0), as shown in Figure 24.

According to the previous expectations, addition of anisole would more greatly suppress excitatory responses to isoamyl acetate as anisole elicits the less excitatory responses. A result shown in Figure 24 illustrates this expectation. When a cell did not respond to anisole, addition of 0.5 mM anisole suppressed the responses to 0.5 mM isoamyl acetate by about 75 % in the cell. On the other hand, when anisole elicited an excitatory current in another cell, the suppression caused by addition of anisole was less (Figure 24). In order to check the relation between the extent of suppression and amplitude of anisole responses, I examined the correlation between relative amplitudes of responses to anisole and the extent of suppression by the addition of anisole in 14 cells that responded to 0.5 mM isoamyl acetate with excitation. The addition of anisole suppressed the current much more as anisole caused a smaller excitatory response than isoamyl acetate did (Fig. 24). There was a significant negative correlation between the relative amplitudes of responses to anisole and the extent of suppression by adding anisole in 14 cells that responded to 0.5 mM isoamyl acetate excitatory ($r = -0.89$, $p<0.00002$)(Fig. 24). This result corresponds to result shown in Figure 17 and 18, and means that excitation that caused by odorants as well as forskolin or cAMP could antagonize odor inhibitory effect.

3.3 Mutual suppression observed in EOG

As shown in the previous section, mixture application of odorants leads to mutual suppression. To ensure the mutual suppression of ORNs' responses in olfactory epithelium, I recorded odorant-evoked changes in the voltage

across the olfactory epithelium (electro-olfactorgrams, EOG) of the newt; the EOG is the extracellular field potential that locally sums cells' responses to odorants. The amplitude of EOG is thought to reflect the summation of responses of ORNs. Furthermore, I also aimed to examine odorant-pair specificity in mixture suppression in the next experiment. As described in the previous section, addition of an odorant suppressed responses to another odorant more greatly when the added odorant alone did not caused excitatory response. Thus an odorant-pair that caused excitation in the less overlapping population should lead to the greater mixture suppression. In order to investigate this hypothesis, I compared extent of mixture suppression between dissimilar odorant pair and similar odorant pair.

I measured EOG responses to odorants (5 mM isoamyl acetate, anisole, and benzene; see also the legend of Figure 25 legend) and to the binary mixtures of the above three odorants (Figure 25). A combination of benzene and anisole is reported to excite similar populations of ORNs, while the other combination of isoamyl acetate and anisole excites dissimilar, with less overlapping populations (Duchamp et al., 1974; Sicard and Holley, 1984). By comparison of the magnitudes of responses to odorants, I found that an addition of anisole to isoamyl acetate elicited a response with about 75% smaller than isoamyl acetate did alone, while an addition of anisole to benzene elicited a response with 75% larger than benzene did alone. Because no interplay between ORNs in the olfactory epithelium was found, the reduction in response appears to indicate the mutual suppression between different components of odorants in individual ORNs. An addition of anisole to isoamyl acetate reduced the magnitude of the response to a statistically significant extent ($p<0.002$), while the effect of an addition of anisole to benzene was not significant ($p>0.6$) (Fig. 25B). The difference of

the effect of added anisole to the two different odorants was significant ($p<0.03$). This odor pair-specific suppression was also observed with a mixture of cineole and isoamyl acetate, a dissimilar pair, and a mixture of isoamyl acetate and amyl acetate, a similar pair (Fig. 25C).