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Odorant-induced Hyperpolarization and Suppression of cAMP-activated Current in Newt Olfactory Receptor Neurons

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Abstract

Although many studies have reported that odorants can elicit inhibitory responses as well as excitatory responses in vertebrate olfactory receptor neurons, the cellular mechanisms that underlie this inhibition are unclear. Here we examine the inhibitory effect of odorants on newt olfactory receptor neurons using whole cell patch clamp recording. At high concentrations, odorant stimulation decreased the membrane conductance and inhibited depolarization. Various odorants (anisole, isoamyl acetate, cineole, limonene and isovaleric acid) suppressed the depolarizing current in a dose-dependent manner. Furthermore, one odorant could suppress the depolarization caused by another odorant. The depolarization caused by isoamyl acetate was inhibited by anisole in cells that were excited by isoamyl acetate but not by anisole. Odorants were able to hyperpolarize cells that were depolarized by cAMP-induced conductance. Given that this inhibitory effect of odorants can affect excitation caused by other odorants, we suggest that it might play a role in coding odorants in olfactory receptor neurons.

Introduction

The mechanism of excitatory olfactory receptor transduction has been intensely studied. It is now well established that odorants bind to receptor proteins and cause the activation of adenylate cyclase via a receptor-coupled G protein (Sklar *et al.*, 1986; Lowe *et al.*, 1989; Bakalyar and Reed, 1990; Breer *et al.*, 1990). As a result, intracellular cAMP levels increase, causing the opening of a cyclic nucleotide-gated, non-selective cation channel (CNG channel) on the plasma membrane of the cilia (Nakamura and Gold, 1987; Kurahashi, 1990; Firestein *et al.*, 1991; Frings and Lindemann, 1991; Kleene and Gesteland, 1991a; Lowe and Gold, 1991; Kramer and Siegelbaum, 1992). Cation influx through the open CNG channels produces a membrane depolarization, giving rise to action potentials (Firestein and Werblin, 1989; Kurahashi, 1989). In addition, calcium influx through the CNG channels activates a chloride channel that leads to an inward Cl^- current that further depolarizes the cell (Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993; Reuter *et al.*, 1998).

On the other hand, studies on olfactory receptor neurons from a wide variety of vertebrate species [frog (Duchamp *et al.*, 1974); salamander (Getchell and Shepherd, 1978a,b); catfish (Kang and Caprio, 1995); rat (Duchamp-Viret *et al.*, 1999)] have reported that odorants can elicit inhibitory responses as well as excitatory responses. These odorant-evoked inhibitory and excitatory responses can be observed in a single cell (Duchamp *et al.*, 1974; Morales *et al.*, 1994; Kang and Caprio, 1995), suggesting that olfactory receptor neurons possess both excitatory and inhibitory mechanisms. While the mechanism of the excitatory response is well established, little is known about the inhibitory mechanism [reviewed in (Getchell, 1986; Ache and Zhainazarov, 1995)].

Furthermore, in behavioral [monkey (Laska and Hudson, 1993); rat (Laing *et al.*, 1989b); lobster (Daniel and Derby, 1991)] and psychological (Cain, 1975; Laing *et al.*, 1984, 1989a; Berglund and Olsson, 1993a,b) studies it has been shown that responses to odorants were smaller when the odorants were applied simultaneously than when the odorants were applied individually. Mutual suppression by odorants was shown to take place in individual olfactory receptor neurons (Bell *et al.*, 1987). Thus, one of the factors proposed to account for suppression at the behavioral and psychological levels is mutual suppression by odorants in olfactory receptor neurons (Jinks and Laing, 1999a,b). A series of olfactory studies on lobster seems to demonstrate convincingly the idea that lobster olfactory neurons have dual transduction systems, inhibitory and excitatory. In addition, it was shown that mutual suppression is due to the co-existence of inhibitory and excitatory pathways that have different odorant specificities (Michel *et al.*, 1991; Fadool and Ache, 1992; Michel and Ache, 1992) [reviewed in (Ache and Zhainazarov, 1995)]. Knowledge of mutual suppression in vertebrate olfactory receptor neurons, however, is limited. Because the transduction system of vertebrate olfactory receptor neurons is different from that of invertebrate cells,

the lobster olfactory system cannot necessarily be used as a model for vertebrates (Ache and Zhainazarov, 1995).

In order to explore other mechanisms of mutual suppression, we examined the possibility that odorant suppression of the depolarizing conductance causes hyperpolarization in newt olfactory receptor neurons. Odorant suppression of the current was first reported by Kurahashi *et al.* (Kurahashi *et al.*, 1994). They showed that responses to a pulse of odorant were suppressed by the second pulse of odorant in a double pulse stimulation protocol, indicating that odorants have inhibitory as well as excitatory effects in newt olfactory receptor neurons. In this study we also observed that simultaneous application of odorants suppressed depolarizing responses to odorants and examined the underlying mechanism of inhibition of depolarization caused by other odorants. Our results suggest that the response pattern of olfactory receptor neurons in the olfactory epithelium is formed by inhibitory as well as excitatory effects of odorants, which may play a role in mutual odorant suppression in vertebrates.

Materials and methods

Animals and cell preparation

Receptor neurons were dissociated enzymatically from the olfactory epithelium of the newt *Cynops pyrrhogaster*. The animals were anesthetized by cooling on ice, decapitated and pithed. Nasal cavities were opened and the olfactory mucosae were excised under a dissection microscope. The mucosae were incubated in a Ca^{2+} and Mg^{2+}-free solution (110 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 5 mM glucose, pH 7.6) containing 0.2% collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 10 min at 35°C. The tissue was then rinsed with Ringer's solution (110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, 5 mM HEPES, 5 mM glucose, pH 7.6) and kept at 4°C for 1 h. This step appeared to facilitate obtaining dissociated cells with intact cilia. The tissue was then teased with forceps and gently triturated in a fire-polished Pasteur pipette. The cell suspension was stored at 4°C until use (up to 10 h later). This procedure was modified from Kurahashi (Kurahashi, 1989).

Electrophysiology

Recording pipettes with a highly tapered shank were pulled from borosilicate glass capillaries of 1.2 mm outer diameter and 0.6 mm inner diameter (A-M Systems, Carlsborg, WA) using a pipette puller (PP-83; Narishige, Tokyo, Japan). The pipette tip was trimmed with a microforge. Only pipettes that had smooth tips with an inner diameter of 5–7 m were used in experiments. The pipette resistance was 2–5 MΩ when filled with Ringer's solution. Before use, the pipette tip was dipped into a mixture of molten Parafilm (American National Can, Greenwich, CT), light mineral oil and heavy mineral oil so that a high seal resistance (1–10 GΩ) could be obtained. This step was modified from that used by Collins and co-workers, who reported that high resistance seal formation with large diameter pipettes is facilitated by hydrocarbon coating (Collins *et al.*, 1992). Pipettes with a large tip diameter (5–6 μm) have decreased access resistance and enhanced mechanical stability during recording.

Approximately 500 μl of suspension containing dissociated cells were transferred to a recording chamber. Experiments were performed under a phase contrast inverted microscope (IMT-2; Olympus, Tokyo, Japan). Solitary olfactory receptor neurons could be easily identified by their unique morphology (see Kurahashi, 1989). Receptor neurons with intact cilia were chosen for the experiments. Electrical recordings from olfactory receptor neurons were made with a patch clamp amplifier (EPC7; List, Darmstadt, Germany) in the whole cell recording configuration (Hamill *et al.*, 1981). The data were sampled at 24 kHz by a 12 bit analog-to-digital converter (Digidata 1200; Axon Instruments, Foster City, CA) linked to a PC and stored in a DAT data recorder (RD-120TE; TEAC, Tokyo, Japan). Data analysis was performed on a PC using pCLAMP 6.0 (Axon Instruments) and Origin 4.0 software (Microcal Software, Northampton, MA). Voltage ramps and voltage steps in the voltage clamp mode were controlled with pCLAMP. Membrane potentials were corrected for the ~–5 mV junction potential between the internal and external solutions. All recordings were performed at room temperature (20–25°C). Data are expressed as means ± SE.

Perfusion system

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 from an electronic pulse generator or PC. The solutions then flowed through an application pipette into the recording chamber. After a patch pipette was sealed to a cell, the cell was moved near the outlet of the application pipette. In this way, complete solution change around the recorded cell could be achieved in 178 ± 6.5 ms (n = 10), as judged from junction currents (Hodgkin *et al.*, 1984). The timings of stimulation were estimated from the junction currents and are indicated symbolically by a horizontal bar in the figures.

Intracellular perfusion was carried out with a commercial pipette perfusion system (2PK+; Adams & List Associates, Westbury, NY) with modifications. In order to stabilize the cell, slight positive and negative pressures were applied until zero net pressure across the pipette tip was achieved by successive approximation.

Solutions

Standard Ringer's solution contained 110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$ and 5 mM HEPES, pH 7.6. The standard pseudo-intracellular solution contained 100 mM KCl, 2 mM $MgCl_2$, 1.263 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES and 0.05 mM Na_2-GTP, pH

7.6. The free Ca^{2+} concentration in this solution was 10 nM, calculated with BAD4 software (Brooks and Storey, 1992). In all experiments except for that in Figure 3, we added 1 mM ATP to the standard pseudo-intracellular solution. All odorants [cineole, anisole, isoamyl acetate, (+)-limonene and isovaleric acid] were diluted in standard Ringer's solution. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless noted otherwise.

Results

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) (data not shown). In order to specifically examine the inhibitory effect of odorants, we chose cells that did not show an excitatory response to the odorant.

For the eight cells that responded to IBMX, a membrane-permeable inhibitor of phosphodiesterase (PDE) (Firestein *et al.*, 1991), and did not respond to 1 mM anisole (see Figure 1), application of 0.25 mM IBMX caused a depolarization of 16.4 ± 3.36 mV (mean ± SE). During depolarization, addition of 1 mM anisole produced membrane hyperpolarization in all the tested cells ($n = 8$) (see Figure 1). The mean amplitude of membrane hyperpolarization was 10.4 ± 3.29 mV. This odor-induced hyperpolarization occurred with very short latencies (64 ± 3.3 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 1), hyperpolarization is likely due to suppression of cAMP-induced conductance, which depolarizes the cell.

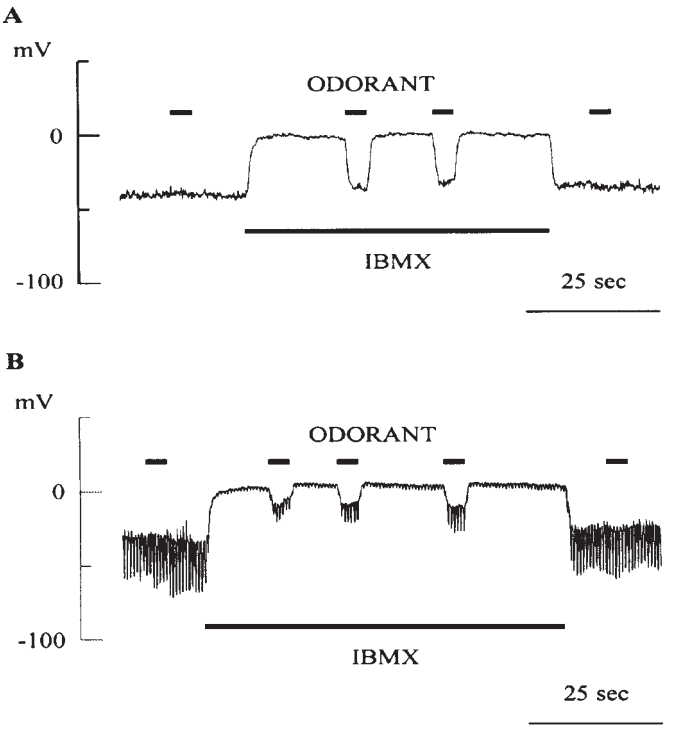

Figure 1 Odorant-inhibited membrane depolarization caused by application of IBMX. **(A)** Responses to 1 mM anisole before, during and after application of 0.25 mM IBMX under whole cell current clamp mode. The application of chemicals (odorant, 1 mM anisole; IBMX, 0.25 mM) are indicated by solid bars (see Materials and methods). **(B)** Responses to 1 mM anisole with superimposed hyperpolarizing voltage pulses. The recording was obtained after the experiment in (A) from the same cell. The hyperpolarizing voltage pulses were caused by repetitive injection of a 20 pA current pulse.

Figure 1B shows potential recordings, from the same cell as in Figure 1A, during repetitive application of a hyperpolarizing current. Odor stimulation caused hyperpolarization and decreased the amplitude of the applied hyperpolarizing voltage (Figure 1B), indicating that hyperpolarization was due to a decrease in conductance. In order to determine whether odor stimulation can suppress the IBMX-induced current, we examined the effect of odorants on the current and measured reversal potentials of the current, shown in Figure 2. In order to focus on suppression by odorants, we used only cells that did not respond to the odorant.

In another subset of six cells that responded to IBMX and not to 1 mM anisole the mean peak amplitude of current induced by 0.25 mM IBMX was 155.8 ± 23.19 pA at +25 mV. The application of 1 mM anisole for 2 s suppressed the current in all six cells at +25 mV (Figure 2). The mean peak amplitude of current suppression was 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 ($n = 6$). Odor stimulation suppressed the depolarizing conductance (Figure 2D). 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 the conductance induced by IBMX (6.04 ± 2.23 mV, $P > 0.68$, paired t-test).

Olfactory receptor neurons have a 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 2A). The fact that odor stimulation did not suppress the hyperpolarizing conductance but did suppress the transduction conductance illustrates that odor suppression of the transduction conductance caused the hyperpolarization (see Figure 1). Furthermore, when using 10 μM forskolin instead of IBMX to activate the transduction conductance we were able to observe suppression of the current and hyperpolarization by odorants (data not shown).

Receptors are not involved in the IBMX-induced increase in intracellular cAMP in olfactory receptor neurons. Thus, the fact that odor stimulation suppressed the IBMX-induced current suggests that a target of the odor inhibitory effect on the cAMP system is downstream of the receptors.

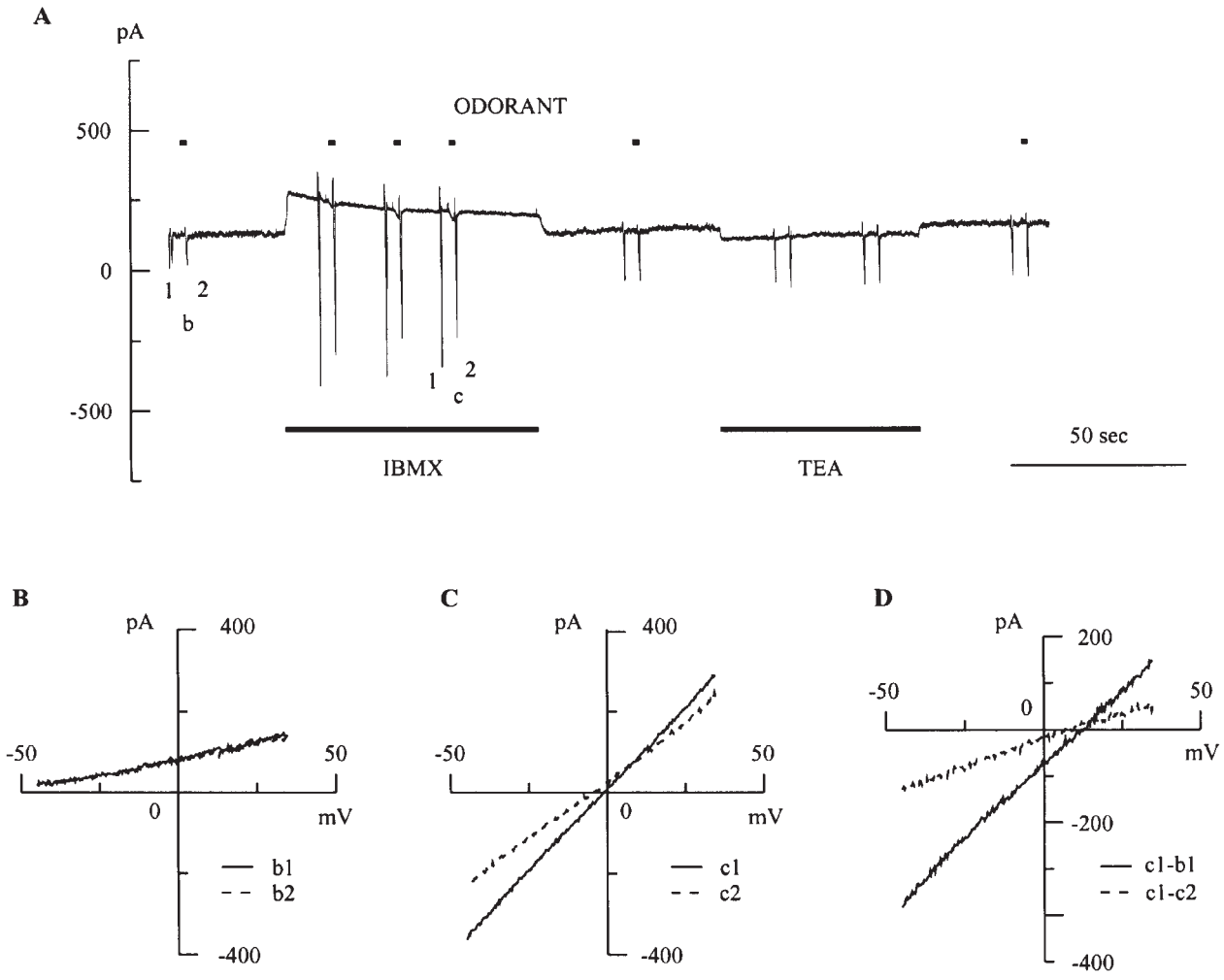

Figure 2 Suppression of the IBMX-induced current by odor stimulation. **(A)** Current recordings under whole cell voltage clamp conditions at +25 mV. Solid bars indicate the application of chemicals (IBMX, 0.25 mM; odorant, 1 mM anisole; TEA, 10 mM). Spikes in the trace represent the application of voltage ramps (35 to –45 mV). Outward membrane current is plotted on the vertical axis. **(B)** Current–voltage (*I–V*) relationship obtained from the experiment in (A) in the absence (b1, solid line) or presence (b2, dotted line) of 1 mM anisole. **(C)** *I–V* relationship obtained from the experiment in (A) in the absence (c1, solid line) or presence (c2, dotted line) of 1 mM anisole during application of 0.25 mM IBMX. **(D)** *I–V* relationship of the IBMX-induced current (c1 – b1, solid line), which is the difference between c1 in (C) and b1 in (B), and *I–V* relationship of the odor-suppressed current (c1 – c2, dotted line), which is the difference between c1 and c2 in (C).

To investigate the target further, we used intracellular perfusion to introduce cAMP into the cell directly and examined the inhibitory effect on the cAMP-induced current.

Introduction of 2.5 mM cAMP into six cells caused a 543.5 ± 160.5 pA outward current at +25 mV. While application of an odorant did not cause suppression before the introduction of 2.5 mM cAMP (Figure 3A and C), the odorant suppressed currents (144.8 ± 18.27 pA at +25 mV) in all six cells after the introduction of cAMP (Figure 3B and D). The mean ratio of the suppressed current to the induced current was 0.39 ± 0.09 (n = 6). The conductance suppressed by odor stimulation was a depolarizing conductance and the reversal potential of the suppressed conductance was almost identical to that of the conductance induced by introducing cAMP (Figure 3E). Odor stimulation was even able to suppress the current induced by intracellular perfusion of cAMP, suggesting that the odorant inhibited events after the increase in intracellular cAMP.

Next, to examine the possibility that inhibition of the cAMP-induced conductance involved breakdown of cAMP, we tested the inhibitory effect of the odorants on currents induced by 8-bromo-cGMP (8-Br-cGMP), a membrane-permeable and unhydrolyzable (Zimmerman *et al.*, 1985; Nawy, 1999) analog of cGMP that activates the CNG channels in olfactory receptor neurons, by bath application (Firestein, 1991).

Of the three cells that responded to 1 mM 8-Br-cGMP and not to 1 mM anisole, the mean peak amplitude of the current induced by 8-Br-cGMP was 543 ± 31.5 pA at

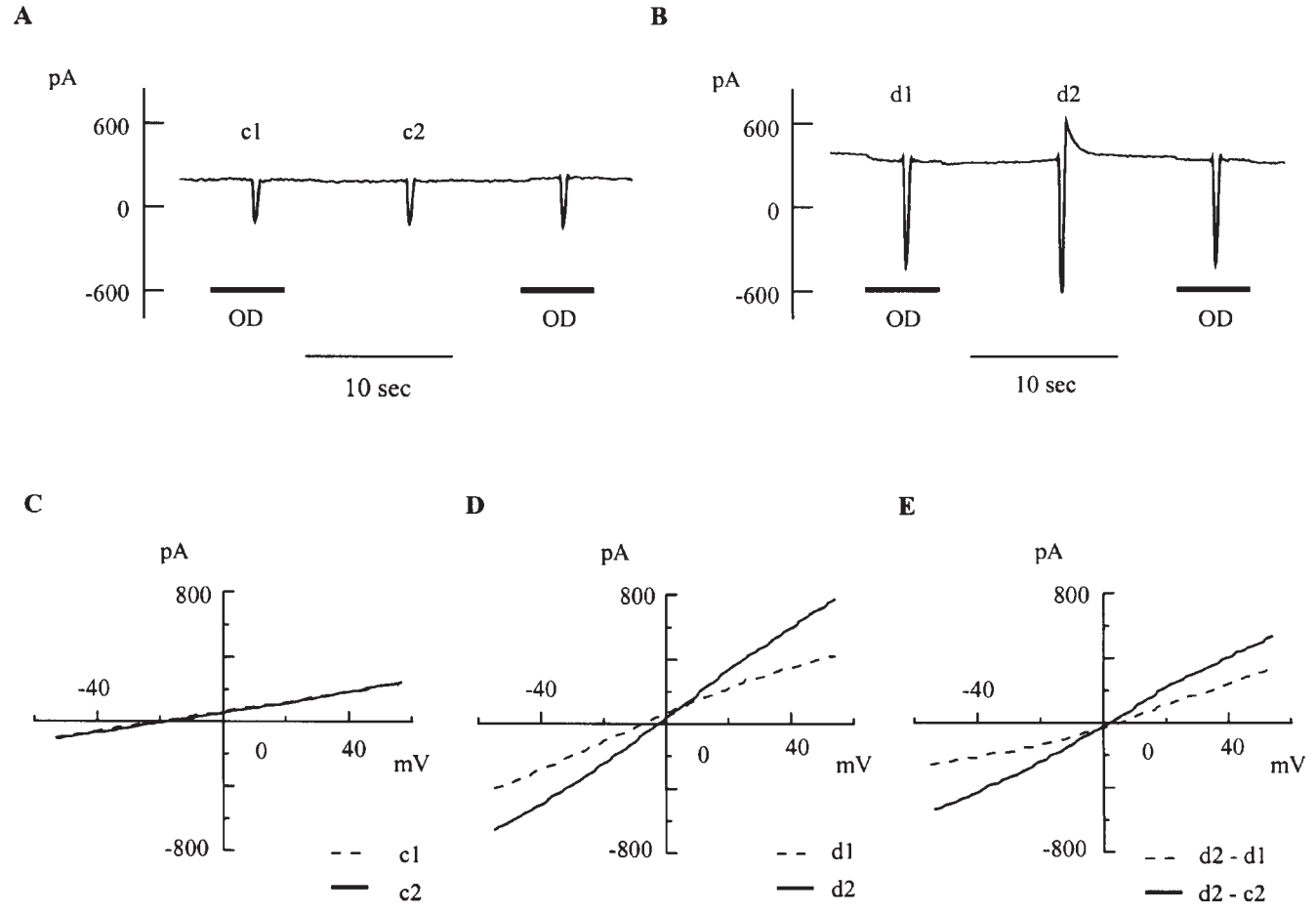

Figure 3 Suppression of the cAMP-induced current by odorant. Current recordings at +25 mV were performed before **(A)** and during **(B)** intracellular perfusion with 0.5 mM cAMP. Spikes in the trace represent the application of voltage ramps (–55 to +55 mV). Bars below the trace indicate odorant stimulation (a mixture of 1.3 mM *n*-amylacetate, 1.3 mM isoamylacetate and 0.25 mM limonene). The recording pipette was filled with standard pseudo-intracellular solution before perfusion (A). **(C)** *I–V* relationship obtained from the results of the experiment in (A) in the presence (c1) and absence (c2) of odorant mixture. **(D)** *I–V* relationship obtained from the experiment in (B) in the presence (d1) and absence (d2) of odorant mixture during intracellular cAMP perfusion. **(E)** *I–V* relation of the current suppressed by odorants (dotted line), which is the difference between d2 and d1 in (D) and *I–V* relation of the cAMP-induced current (solid line), which is the difference between the *I–V* relations d2 in (D) and c2 in (C).

+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 4). The mean ratio of the suppressed current to the induced current was 0.47 ± 0.26 (n = 3). Together with the fact that odor stimulation suppressed the transduction current during application of IBMX (Figure 2), this result that odorants suppressed the current induced by 8-Br-cGMP (Figure 4) would seem to contradict the possibility that inhibition of the cAMP-induced conductance involved breakdown of cAMP. A more likely possibility may be that odorants inhibit the CNG channel directly (Kurahashi *et al.*, 1994).

This suppression of the transduction current by an odorant was dose-dependent (Figure 5). 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 the five cells 123 μM

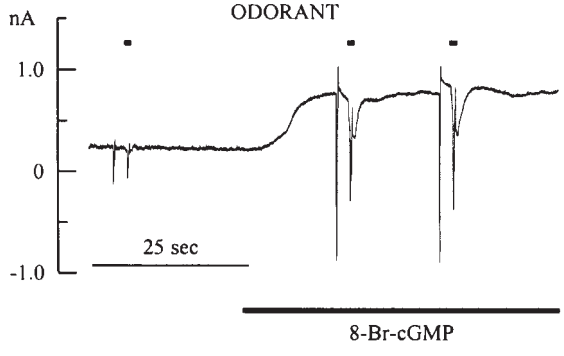

Figure 4 Suppression of the 8-Br-cGMP-induced current by odorant. Current recording was performed under whole cell voltage clamp conditions at +25 mV. Solid bars indicate the application of chemicals (8-Br-cGMP, 1 mM; odorant, 1 mM anisole). Spikes in the trace represent application of voltage ramps (45 to –55 mV).

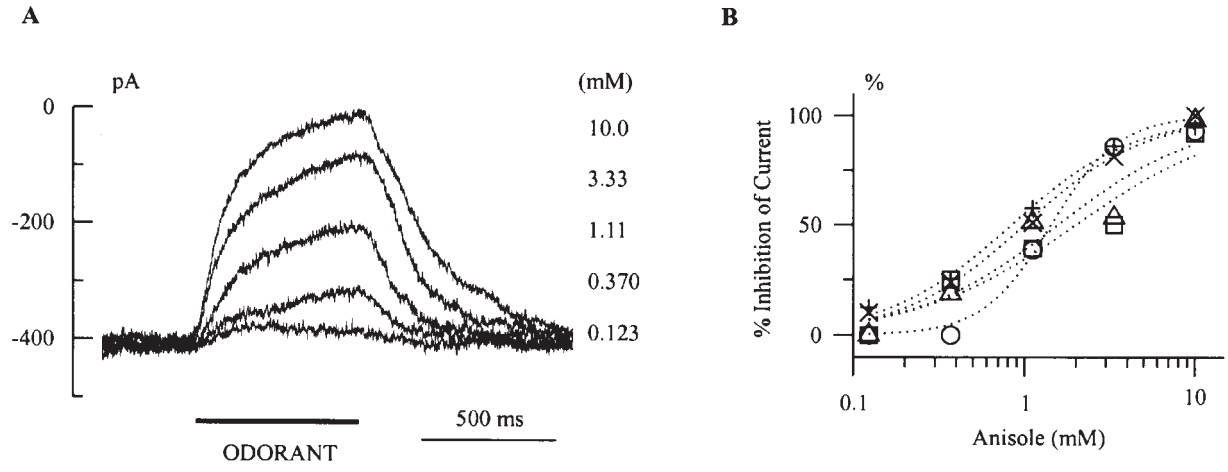

Figure 5 Dose-dependent suppression of the IBMX-induced current by odorant. **(A)** Suppression of the 0.25 mM IBMX-induced current by 10, 3.33, 1.11, 0.370 and 0.123 mM anisole. Recording was performed under whole cell voltage clamp conditions at –50 mV in cells that did not respond to the tested odorants alone. After eliciting an inward current by application of 0.25 mM IBMX, the cell was placed sequentially in one of a series of solutions with decreasing concentration of odorant for 600 ms. **(B)** Collected dose–response curves from five cells. The vertical axis shows the percentage current suppression during odor stimulation relative to the IBMX-induced current in each cell; the horizontal axis shows the odorant concentration. Dotted curves are the best fit of the Hill equation: $y = E_{max}[x^n/(x^n + IC_{50}{}^n)]$, where E_{max} is the maximal percentage inhibition, which was normalized to 100%. Values of n and IC_{50} were as follows: (+) n = 1.2, IC_{50} = 0.84 mM; (○) n = 2.2, IC_{50} = 1.4 mM; (△) n = 1.0, IC_{50} = 1.6 mM; (□) n = 0.93, IC_{50} = 2.0 mM; (×) n = 1.2, IC_{50} = 1.0 mM.

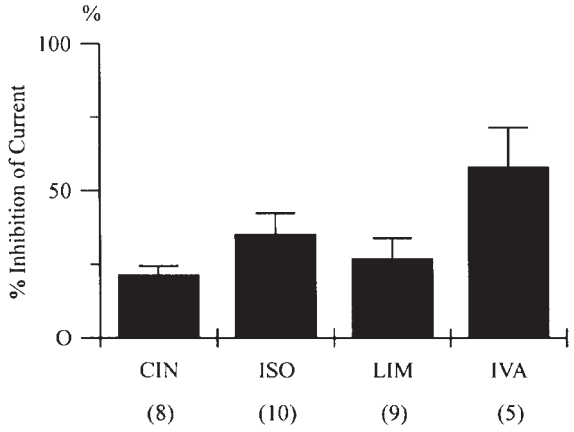

Figure 6 Inhibitory effect of various odorants on the IBMX-induced current. Recordings were performed under the same conditions as for Figure 5. Bar graphs show the average percentage suppression of the IBMX-induced current during odor stimulation (CIN, 1 mM cineole; ISO, 1 mM isoamyl acetate; LIM, 1 mM (+)-limonene; IVA, 1 mM isovaleric acid). Short vertical bars represent the SE; the number of cells is shown under the graph. While we also examined the inhibitory effect of 1 mM sodium glutamate, it did not affect the IBMX-induced current in 11 tested cells (data not shown).

anisole suppressed the IBMX-induced current by ~10% and 10 mM anisole suppressed nearly all the current. The mean IC_{50} value was 1.37 ± 0.21 mM (n = 5). Furthermore, an inhibitory effect on the transduction current was not only found with anisole. Of the cells that responded to 0.25 mM IBMX at –45 mV and did not show an excitatory response to each test chemical, we examined suppression of the IBMX-induced current by four odorants (1 mM isoamyl acetate, cineole, limonene and isovaleric acid). Figure 6 shows the collected results. As shown, suppression was observed with all four odorants.

The above results demonstrate that odorants have an inhibitory effect on cells in which the cAMP system has been activated by application of IBMX or 8-Br-cGMP or injection of cAMP. This suggests that application of an odorant to a cell could inhibit the depolarization caused by another odorant. In the next experiment we examined this possibility.

Of the five cells that responded to 1 mM isoamyl acetate and not to 1 mM anisole, the mean amplitude of depolarization caused by isoamyl acetate was 26.6 ± 10.9 mV. In all these cells addition of anisole caused membrane hyperpolarization when the cells were depolarized by a background application of isoamyl acetate (Figure 7). The mean amplitude of hyperpolarization was 18.8 ± 10.6 mV. The results in Figure 7 correspond with that in Figure 1 and demonstrate that odor stimulation is able to inhibit depolarization even when the depolarization was caused by another odorant.

Discussion

Mechanism of odor suppression of the cAMP-induced current

Kurahashi and co-workers showed that suppression of the transduction current by odorants has a short latency and suggested that odorants suppress CNG channels directly (Kurahashi *et al.*, 1994). A short latency of suppression was also observed in our study (Figure 5). Some additional results provide evidence that odorants inhibit CNG channels directly. First, odor stimulation suppressed the

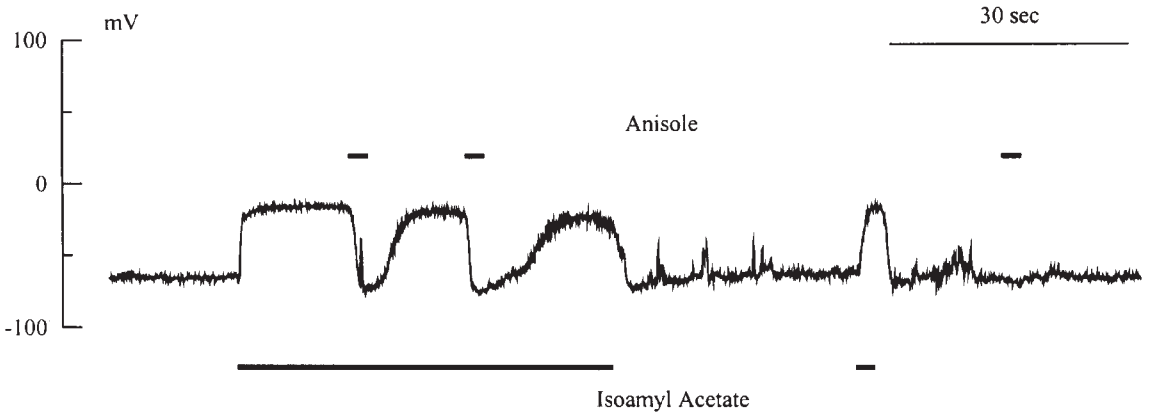

Figure 7 Odor inhibition of odor excitation under whole cell current clamp conditions. Solid bars indicate the application of odorants (isoamyl acetate, 1 mM; anisole, 1 mM).

IBMX-induced current (Figure 2). Competitive effects of stimulus–receptor binding have been proposed to be a mechanism of suppression of odor-induced currents by odorants (Derby *et al.*, 1991). Odor suppression of the IBMX-induced current, however, suggests that suppression cannot only be due to competitive effects and that the target of suppression is downstream of receptor binding in the cAMP pathway. Second, the conductance induced by intracellular injection of cAMP was also suppressed by odorants (Figure 3). While application of IBMX raises the intracellular concentration of cAMP by inhibiting breakdown of cAMP in the cell, injection of cAMP raises the concentration directly. Thus, odor suppression of the current induced by injection of cAMP demonstrates that the suppression cannot only be caused by inhibition of adenylate cyclase and shows that the target of suppression is after accumulation of intracellular cAMP. Finally, odor stimulation suppressed the current induced by application of 8-Br-cGMP (Figure 4). 8-Br-cGMP is a membrane-permeable and unhydrolyzable analog of cGMP (Zimmerman *et al.*, 1985; Nawy, 1999) and has been reported to activate CNG channels in olfactory receptor neurons (Firestein *et al.*, 1991). Therefore, suppression of the current induced by application of 8-Br-cGMP rules out the possibility that this suppression was caused only by breakdown of intracellular cAMP and means that suppression of the transduction current was due to suppression of activation of the CNG channel itself. Furthermore, the ratio of suppressed current when the current was activated by application of 8-Br-cGMP (0.47 ± 0.26, $n = 3$) was very similar to that when the current was activated by application of IBMX (0.53 ± 0.08, $n = 6$, $P > 0.75$, one-way ANOVA). Thus inhibition of the CNG channel seems to be a major cause of suppression of the cAMP-induced current.

While a Ca^{2+}-activated chloride current contributes to the transduction current, the result that 10 mM anisole suppressed almost all the IBMX-induced current (Figure 5) means that anisole also decreased the chloride current as well. However, we cannot conclude that odorants suppress chloride conductance directly, because inhibition of the CNG channel alone would cause a decrease in intracellular calcium and inactivate chloride conductance. Results in this study cannot determine whether or not the decrease in the chloride current was due to suppression of the chloride channel.

While odor stimulation suppresses the transduction current, it does not suppress the basal conductance that maintains the cell in a hyperpolarized state. As shown in Figure 2, odor stimulation did not suppress the TEA-inhibited basal potassium conductance. If odorants did suppress the basal potassium conductance, application of odorants to resting cells would cause depolarization. Such depolarization, however, has not been observed. As shown in Figure 1, odor stimulation did not cause such a depolarization even when it was able to suppress the current induced by IBMX.

Odor representation

It has been generally accepted that the representation of an odor is the pattern of activated neurons in the olfactory epithelium and that the activation of an olfactory receptor neuron is determined by the excitatory specificity of a particular odor [reviewed in (Mori and Yoshihara, 1995; Hildebrand and Shephered, 1997)]. On the other hand, although some vertebrate studies have described an odorant inhibitory effect and suggested that the inhibitory effect plays a part in forming the odor representation (Duchamp *et al.*, 1974; Dionne, 1992), there has been no evidence that the inhibitory effect of an odorant modulates the response of the cell to another odorant.

Our results might provide support for the hypothesis that odor representation is not formed solely by odorant-induced excitation. For example, 1 mM anisole caused excitation in only 12 of 38 cells, but it was able to inhibit excitatory

responses induced by isoamyl acetate (Figure 7). This suggests that the inhibitory effect of an odorant is involved in forming odor representation by modulating the excitation caused by other types of odorants.

It has been reported that olfactory neurons are sensitive to various odorants in concentrations that range from millimolar to picomolar (Getchell and Shepherd, 1978a,b; Getchell, 1986; Firestein *et al.*, 1993). However, we observed an inhibitory effect of anisole on IBMX-induced currents in the high dose range (Figure 5). There are several possible explanations for the difference between the dose ranges of inhibition and excitation. One possibility is that the inhibitory effect of an odorant might work functionally at a higher dose than the excitatory response. In the case of the lobster (Ache and Zhainazarov, 1995) the dose ranges of the excitatory and inhibitory systems are nearly identical (Michel and Ache, 1994) and both systems involve dual second messenger pathways and work in parallel to form the cell's response potential. On the other hand, the inhibitory effect in newt olfactory neurons appears to work only by inhibiting the excitatory transduction pathway, as shown in this study. Thus the inhibitory effect seems simply to play a supporting role in the formation of odor representation. The inhibitory effect may be restricted to high dose ranges in newt olfactory receptor neurons.

Another possible cause of a higher dose range may be the nature of our experiments. We have attempted to explore the dose-dependency of odor suppression of a large steady current induced by continuous application of 0.25 mM IBMX (Figure 5) in order to observe the suppression clearly. In this case the dose range of suppressive odorant may be inappropriate for estimating the physiological dose range, because excess cAMP was continuously produced by IBMX application. In order to determine the physiological dose range of the inhibitory effect of odorants, further study will be needed under more physiological conditions. In particular, we will need to compare responses to mixtures of odorants and to individual odorant components of this mixture (Figure 7) and estimate the inhibitory effect of an odorant on responses to the other odorants, as studied in the lobster (Derby *et al.*, 1991; Michel and Ache, 1994).

Inhibitory responses have been identified as an inhibition of excitation in the cell [reviewed in (Getchell, 1986; Ache and Zhainazarov, 1995)]. Some recent studies in amphibians have described an odor-induced hyperpolarizing current, which is triggered by an increase in intracellular calcium, as the cellular mechanism that causes inhibition (Morales *et al.*, 1994, 1995, 1997). Studies on excitatory transduction system in vertebrates, however, have shown that increases in intracellular calcium exclusively elicit chloride conductance (Kleene and Gesteland, 1991b) and cause depolarization of the cilia (Kurahashi and Yau, 1993). The cause of the discrepancy between these studies remains unclear, but one hypothesis is that the hyperpolarizing current might not co-exist in the same cell with a standard cAMP cascade (Vogler and Schild, 1999). On the other hand, the results of this study suggest the possibility that odorant-induced hyperpolarization might be due to odor suppression of the cAMP-induced current. It is difficult to determine whether the entire hyperpolarization is due to this mechanism. Nevertheless, when the cells have a high level of intracellular cAMP, which could be caused by either background stimulation by the odorant (Figure 7) or by a high basal activity of adenylate cyclase (Kleene, 1994), odor suppression would elicit hyperpolarization of the olfactory receptor neuron.

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