

A Large Contribution of a Cyclic AMP-independent Pathway to Turtle Olfactory Transduction

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ABSTRACT Although multiple pathways are involved in the olfactory transduction mechanism, cAMP-dependent pathway has been considered to contribute mainly to the transduction. We examined the degree of contribution of cAMP-independent pathway to the turtle olfactory response by recording inward currents from isolated cells, nerve impulses from cilia and olfactory bulbar responses. The results obtained by the three recordings were essentially consistent with each other, but detail studies were carried out by recording the bulbar response to obtain quantitative data. Application of an odorant cocktail to the isolated olfactory neuron after injection of 1 mM cAMP from the patch pipette elicited a large inward current. Mean amplitude of inward currents evoked by the cocktail with 1 mM cAMP in the patch pipette was similar to that without cAMP in the pipette. Application of the cocktail after the response to 50 μ M forskolin was adapted also induced a large inward current. Application of the odorant cocktail to the olfactory epithelium, after the response to 50 μ M forskolin was adapted, brought about an appreciable increase in the impulse frequency. The bulbar response to forskolin alone reached a saturation level around 10 μ M. After the response to 50 μ M forskolin was adapted, 11 species of odorants were applied to the olfactory epithelium. The magnitudes of responses to the odorants after forskolin were 45–80% of those of the control responses. There was no essential difference in the degree of the suppression by forskolin between cAMP- and IP₃-producing odorants classified in the rat, suggesting that certain part of the forskolin-suppressive component was brought about by nonspecific action of forskolin. Application of a membrane permeant cAMP analogue, cpt-cAMP elicited a large response, and 0.1 mM citralva after 3 mM cpt-cAMP elicited 51% of the control response which was close to the response to citralva after 50 μ M forskolin. A membrane permeant cGMP analogue, db-cGMP elicited a small response and the response to 0.1 mM citralva was unaffected by db-cGMP. It was concluded that cAMP-independent (probably IP₃-independent) pathway greatly contributes to the turtle olfactory transduction.

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INTRODUCTION

The interaction of an odorant with the receptive membrane of olfactory neurons initiates a sequence of molecular and membrane events leading to sensory transduction, action potential initiation and the transmission of odor information to the olfactory bulb (Getchell, 1986). It is now generally believed that binding of chemical stimuli to specific olfactory receptors located on the plasma membranes of the cilia leads to stimulation of the G-protein-linked cAMP pathway (Pace, Hasnski, Salomon, and Lancet, 1985; Sklar, Anholt, and Snyder, 1986; Boekhoff, Tarelius, Strotman, and Breer, 1990). An increase of intraciliary cAMP level activates cAMP-gated cation channels (Nakamura and Gold, 1987), causing cell depolarization and triggering the discharge of action potentials (Suzuki, 1989; Kurahashi, 1990; Frings and Lindemann, 1990*b*; Firestein, Darrow, and Shepherd, 1991*a*).

In isolated cilia preparations from the bullfrog and the rat, many, but not all, olfactory stimuli enhanced GTP-dependent cAMP accumulation (Sklar et al., 1986; Boekhoff et al., 1990). Breer and Boekhoff (1991) demonstrated that, in the rat, odorants which did not increase cAMP concentration in the cilia preparation increased IP₃ concentration. Recent studies suggested that there are IP₃ receptors and IP₃ gated ion channels in cilia and soma of the catfish, the rat, the cattle and the bullfrog olfactory cells (Kalinowski, Aldinger, Boyle, Huque, Marecek, Prestwich, and Restrepo, 1992; Restrepo, Miyamoto, Bryant, and Teeter, 1990; Restrepo, Teeter, Honda, Boyle, Maracek, Prestwich, and Kalinoski, 1992; Kahn, Steiner, and Snyder, 1992; Suzuki, 1992). It was also pointed out that other transduction pathways such as direct activation of K-channels (Vodyanoy and Murphy, 1983), Ca-activated Cl channels (Kleene and Gesteland, 1991; Kurahashi and Yau, 1993; Lowe and Gold, 1993) and NO-cGMP cascade (Breer and Shepherd, 1993) may contribute to the olfactory transduction. Thus, there are multiple transduction pathways.

The present study aims to examine to what extent cAMP-independent pathway contributes to *in vivo* olfactory transduction in the turtle. cAMP pathway seems to be fully activated or desensitized when sufficient amounts of cAMP is injected from the patch pipette to the olfactory cell or forskolin, an activator of adenylate cyclase, 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of phosphodiesterase, or 8-(4-chlorophenylthio)-adenosine 3',5'-cyclic monophosphate (cpt-cAMP), a membrane permeant nucleotide, are applied to the olfactory cells from external solutions. We have found that large olfactory responses to all the odorants examined appear even after the responses to cAMP, forskolin, IBMX or cpt-cAMP itself are saturated, suggesting that cAMP-independent pathway greatly contributes to the *in vivo* olfactory transduction in the turtle.

METHODS

Animals

Turtles, *Geoclemys reevesii*, weighing 150–300 g, were obtained from commercial suppliers and maintained at 22°C. Animals were fed porcine and bovine liver *ad libitum*. For isolation of olfactory neurons or ciliary recording, animals were cooled to 0°C and decapitated. The nasal cavities were opened, and the olfactory epithelia were quickly removed.

Isolation of Olfactory Neurons

The epithelia were cut into slices of ~ 300 μm thickness in normal Ringer solution at 4°C and stored at 4°C . Slices were incubated for 10–30 min at 37°C in Ca-free Ringer solution for the isolation of olfactory neurons. Immediately before recording, one slice of the epithelium was placed in 500 μl normal Ringer solution in the recording chamber and shaken. No enzymes or proteases were added. Once cells had settled on the bottom of the chamber and a tight seal had been established on a cell, the chamber was continuously perfused with normal Ringer solution. Turtle olfactory neurons were readily distinguished from other types of cells such as respiratory and basal cells based on their characteristic morphology (Fig. 1A).

Whole-Cell Recordings

Recordings were made with an Axopatch 1C amplifier (Axon Instruments, Inc., Burlingame, CA) using patch electrodes of borosilicate glass. Electrodes with resistance of 5–10 $\text{M}\Omega$ were manufactured on a Narishige PP83 puller (Narishige Co., Tokyo, Japan) using a double stage pull. Giga ohm seals were obtained by applying negative pressure (-30 to -100 $\text{cm H}_2\text{O}$). The whole-cell configuration was attained by application of additional negative pressure. The current signal was digitized and stored on video tape.

Ciliary Recording

Ciliary recordings were taken according to the method described by Frings and Lindemann (1990a). Briefly, the recording chamber (500 μl) was a glass microscope slide to which a flat silicon ring was affixed. Two needles (200 μm in diameter) held the tissue, which was folded around one of them such that the mucosal surface was accessible. Patch pipettes fire-polished with resistance of 10–20 $\text{M}\Omega$ were filled with normal Ringer solution. For recording of electrical signals, a patch clamp amplifier (List EPC-7, Darmstadt, Germany) was set to voltage clamp mode, a pipette potential of 0 mV, and a gain of 100–200 mV/A. The current signal recorded from the cilia was stored on tape and digitized by the P-CLAMP (Axon Instruments, Inc.). The digitized data were analyzed by a discriminator/rate meter which was kindly supplied by Prof. Lindemann.

Recording of Olfactory Bulbar Response

Olfactory bulbar responses were recorded essentially as described previously (Taniguchi, Kashiwayanagi, and Kurihara, 1992). In brief, turtles were weakly anesthetized with the necessary and minimum amount of urethane to lessen pain in the operation of the animal, immobilized by an injection of *d*-tubocurarine chloride (450 $\mu\text{g}/100$ g body wt), and locally anesthetized with lidocaine at the wounded and head-fixation points. The olfactory bulb was exposed using a dental drill, and the dura mater on the olfactory bulb was removed carefully. The stimulant-induced brain waves (bulbar responses) were recorded by attaching a pair of silver electrodes to the medial part of the anterior bulb. The responses were amplified by DC-amplifier, filtrated into 3–300 Hz and integrated by electric integrator (time constant 0.3 s). All experiments were carried out at $20 \pm 3^\circ\text{C}$.

Stimulation

To stimulate cAMP-gated channels by cAMP directly, cAMP was injected from the patch pipette into olfactory neurons. Isolated olfactory neurons and olfactory cilia were stimulated by extracellular solutions according to the method described by Frings and Lindemann (1990a). Gravity was used to deliver a constant stream of Ringer solution from the stimulating tube. A Ringer solution was switched to 50 μM forskolin solution or odorant cocktail solution by three

electrically actuated valves. The stimulating tube with a lumen 160–200 μm in diameter was placed under visual control within ~ 500 μm of the cell. The delay due to dead space was 1–40 s, depending on the flow rate, volume of dead space and distance between neurons and the tip of the tube. Figs. 1, 2, and 3 represent the recordings taken at the beginning of stimulation immediately after the valve was switched.

To record the olfactory bulbar responses, the stimulating solutions were applied to the olfactory epithelium through a stainless steel tube at a flow rate of 27 ml/min. After application of 30 ml Ringer solution, the stimulating solution was applied. The time of the beginning of summated olfactory bulbar response is illustrated in Figs. 4 and 8. After the end of the experiments, 0.1 M ZnSO_4 solution was applied to the epithelium for 5–10 min to confirm that all olfactory neurons were stimulated. Application of odorant after the ZnSO_4 treatment did not induce the olfactory response in any of the cases examined (data not shown). This indicated that the stimulating method employed in the present study was suitable for stimulation of the turtle olfactory epithelium.

Preparation of Solutions

Normal Ringer solution contained (mM) 116 NaCl, 4 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES-NaOH (pH 7.4). Ca-free solution contained (mM) 116 NaCl, 4 KCl, 2 MgCl_2 , 10 HEPES-NaOH (pH 7.4). For the whole-cell and the ciliary recordings, 15 mM glucose and 5 mM sodium pyruvate were added to the Ringer solution. Patch pipettes for the whole-cell recording were filled with an internal solution (mM): 115 KCl, 5 NaCl, 2 MgCl_2 , 5 ATP, 10 HEPES-KOH (pH 7.4). For the stimulation with cAMP from the patch pipette, 1 mM cAMP was dissolved in the internal solution.

IBMX and membrane permeant cyclic nucleotide analogues were dissolved in normal Ringer solution. Forskolin was dissolved in ethanol to give a stock concentration of 10 mM. All odorants were dissolved in ethanol to prepare stock solutions at a concentration of 0.1 M (except triethylamine). Concentration of triethylamine stock solution was 1 M. Concentration of stock solution of each odorant for the mixture was 1 M. These stocks were added to normal Ringer solution to give indicated concentrations of odorants. The odorant solution used for the whole-cell recording and the ciliary recording consisted of a mixture of 0.2 mM each of citralva, hedione, eugenol, *l*-carvone and cineole in normal Ringer solution. Ethanol alone, at a concentration of 0.5%, had no effect on the spike rate of olfactory neuron or summated olfactory bulbar response.

Chemicals

All odorants were kindly supplied from Takasago International (Tokyo, Japan). Forskolin and IBMX were obtained from Wako Pure Chemical Industries (Osaka, Japan). cAMP and cpt-cAMP were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). N_6 , 2'-O-Dibutyryl guanosine 3',5'-cyclic monophosphate (db-cGMP) was obtained from Yamasa Shoyu Co., Ltd. (Choshi, Japan).

RESULTS

Responses of Isolated Olfactory Neurons to Odorants after Injection of cAMP

Olfactory neurons with motile cilia (Fig. 1A) were used for the whole-cell recording experiments. The probability of gigaohm formation was nearly 60%. More than hundred cells were successfully voltage-clamped by the whole-cell patch method. Voltage-clamped olfactory neurons, which displayed time- and voltage-dependent

inward and outward membrane currents in response to depolarizing voltage steps, were stimulated by odorants and forskolin.

Application of the odorant cocktail composed of 0.2 mM each of citralva, hedione, eugenol, *l*-carvone, and cineole induced inward currents in neurons when the holding potential was -50 mV (Fig. 1 *B*). The peak amplitudes of currents ranged from -421 to 29 pA. Fig. 1 *C* shows an inward current induced by 1 mM cAMP injected from the patch pipette. Inward currents were induced after the rupture of patch membrane

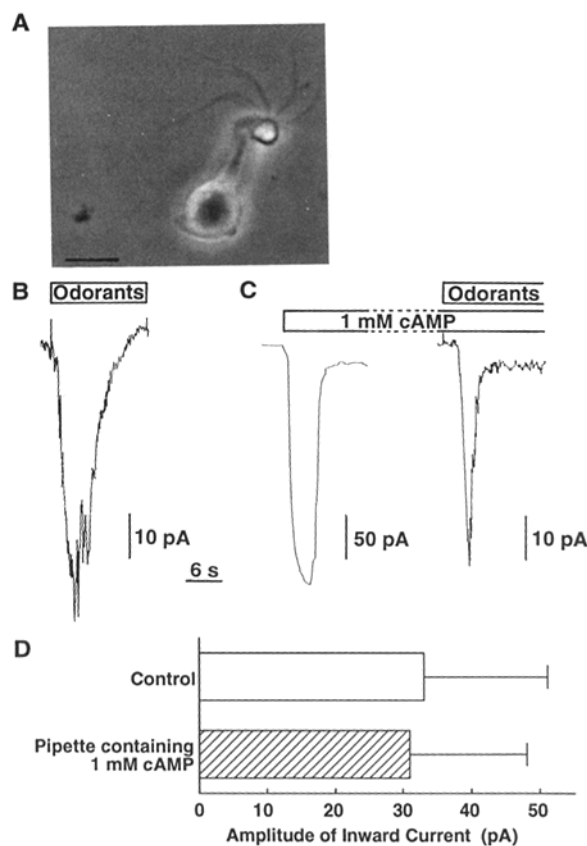


FIGURE 1. (A) Photomicrograph of a receptor neuron isolated from the turtle olfactory mucosa. The cells had characteristic morphology of the receptor neuron, such as cilia, dendrite and soma. Bar, 10 μ m. (B) Inward current induced by a mixture of the odorants when the patch pipette was filled with the normal inner solution. (C) Inward currents induced by 1 mM cAMP and the mixture of odorants when the pipette contained 1 mM cAMP. Rupture of the patch membrane induced an inward current. After the response to cAMP was adapted, odorants was applied. Columns on the top of the records represent duration of application of the odorant cocktail or 1 mM cAMP. (D) Mean amplitude of inward currents when the patch pipettes contained no cAMP (open column) and 1 mM cAMP (shaded column). Numbers of preparations were 25 and 26 for without and with cAMP, respectively. Error bars show SE. Holding potential was -50 mV.

when the pipette solution contained 1 mM cAMP. The peak amplitudes and durations of currents ranged from $-1,000$ to -40 pA and 6 to 66 s, respectively. Mean amplitude and duration of cAMP-induced current were -252 ± 30 pA (SE, $n = 31$) and 26 ± 4 s (SE, $n = 22$), respectively. When the normal pipette solution was used, current was unchanged or outward current (~ 22 pA) was induced by the rupture (data not shown). After the cAMP-induced inward current was adapted, the odorant cocktail was applied. The odorant cocktail induced an inward current even

when high concentration of cAMP was continuously supplied from the patch pipette as shown in Fig. 1 C. The peak amplitude of currents ranged from -404 to 60 pA. Fig. 1 D shows mean amplitudes of inward currents in response to the odorant cocktail when the patch pipette contained no cAMP and 1 mM cAMP. Mean amplitudes of inward currents induced by the odorant cocktail recorded by the pipette without and with 1 mM cAMP were -33 ± 18 pA (SE, $n = 25$) and -31 ± 17 pA (SE, $n = 26$), respectively.

Odor Responses of Isolated Olfactory Neurons after Adaptation to Forskolin

To explore effects of an increase in cAMP concentration on in vivo odor response, forskolin is an useful chemical because intracellular cAMP concentration is increased when forskolin is applied extracellularly. In the experiment for Fig. 2 A, $50 \mu\text{M}$

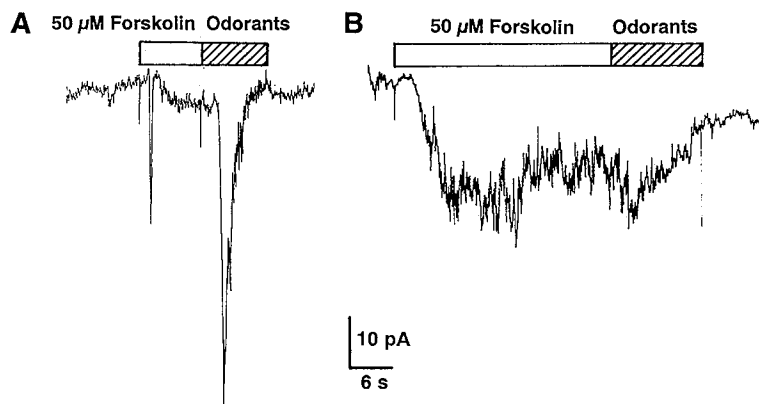


FIGURE 2. Inward currents in response to a mixture of the odorants after an inward current induced by $50 \mu\text{M}$ forskolin was adapted. An isolated olfactory cell was first stimulated by application of $50 \mu\text{M}$ forskolin. After the response to forskolin was adapted, a solution was changed to a solution containing the odorant mixture. Columns on the top of the records represent duration of application of $50 \mu\text{M}$ forskolin or odorants. A and B were recorded from different neurons. Holding potential was -60 mV.

forskolin applied first induced a transient inward current. After its response was adapted, the odorant cocktail was applied. As seen from the figure, an inward current in response to the odorant cocktail was induced even after forskolin. In the recording of Fig. 2 A, the response to forskolin was easily adapted, but the time course of adaptation varied from one cell to another. In Fig. 2 B, the inward current induced by forskolin was not easily adapted. In this case, the odorant mixture was applied when adaptation to forskolin had reached a steady level. As seen from the figure, an inward current in response to the odorant cocktail appeared after forskolin. Forskolin (2 – $50 \mu\text{M}$) induced inward currents at -60 mV (holding potential) in 16 receptor neurons. The forskolin-induced currents were heterogeneous in terms of amplitude and time course. The peak amplitude of the response to $50 \mu\text{M}$ forskolin ranged from -55 to -6 pA and the mean peak amplitude was -23 ± 6 pA (mean \pm SE; $n = 7$). The peak amplitude of response to the odorant cocktail after the response to

50 μM forskolin was adapted ranged from -55 to -4 pA. The mean amplitude was -18 ± 4 pA (mean \pm SE; $n = 7$).

Increase in Impulse Frequency in Response to Odorants after Adaptation to Forskolin

To investigate the effect of forskolin on in situ odor response, impulse frequency changes in response to the odorant mixture after forskolin were measured by recording action potentials from olfactory cilia. Fig. 3 shows a spike rate against time and a spike recording. Forskolin applied first increased impulse frequency after delay in a similar manner to that reported by Frings and Lindemann (1990b) in the frog olfactory epithelium. It is noted that the delay between switching time and the beginning of response in the frog was ~ 20 s. The delay in the ciliary recordings was longer than that in the whole-cell clamp experiments (Fig. 2). The difference in the delay seems to come from the difference in the mode of contact between forskolin and the cell. That is, in the ciliary recording, forskolin is incorporated only from the

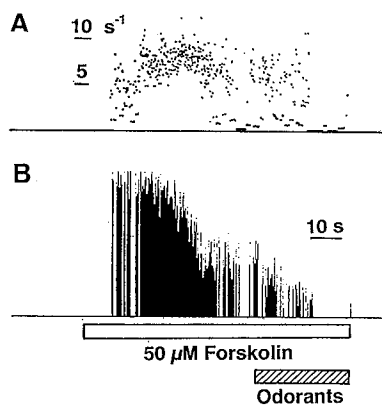


FIGURE 3. (A) Plot of spike rate against time. Each point represents the inverse of the time interval between two spikes. (B) Increase in impulse frequency in response to the odorant mixture containing 50 μM forskolin after an increase in firing induced by 50 μM forskolin was adapted. Impulses were recorded from olfactory cilia by using the ciliary recording technique. These impulses were elicited from one olfactory neuron, which was confirmed by high speed scanning of the trace (not shown).

cilia and the knob, while forskolin is incorporated from the entire cell surface in the isolated neuron.

After the response to forskolin was adapted to a spontaneous level, the odorant mixture containing 50 μM forskolin was applied. The duration of the response to forskolin recorded from the cilia was longer than those to forskolin and cAMP recorded from the isolated neurons. These results are consistent with those reported by Kurahashi (1990) and Frings and Lindemann (1990b); duration of an inward current induced by application of cAMP from the patch pipette was ~ 3 s in the newt olfactory cell, while an increase in impulse frequency recorded from the frog olfactory cilia was prolonged over 150 s.

Application of the odorant mixture increased impulse frequency after forskolin. Spike amplitude in the latter part of the forskolin response and the odor response were much smaller than that at an earlier part of the forskolin response or that of spontaneous discharges. Frings and Lindemann (1990b) explained that the decrease in spike amplitude is caused by strong and sustained membrane depolarization. Of

ten cells which reversibly responded to forskolin, six cells responded to the odorant mixture after 50 μ M forskolin.

Forskolin-independent Olfactory Bulbar Response

The whole-cell and ciliary recordings offer direct information on olfactory transduction in a single cell, but the properties of the cells and epithelium isolated may not be identical to in vivo systems. To obtain in vivo information, we examined the effects of forskolin on odor responses by recording olfactory bulbar responses. Although the olfactory bulbar responses give less direct information on receptor events, there are a number of advantages. The information which is transmitted to the olfactory bulb is significant for the olfactory system because the olfactory bulb is the site of the first synaptic interaction in the olfactory pathway. To examine effects of drugs systematically on odor response in isolated cells, we have to measure the effects of various species and varying concentrations of drugs using a single cell. Function of isolated cells to respond to odorants is decline by repeated application of drugs such as forskolin. Hence it is almost impossible to obtain quantitative data by a long series of experiment from a single cell. On the other hand, the preparation for the measurement of bulbar response is in vivo system and is very stable for 4–7 d. We can obtain reproducible and quantitative data on the effects of various drugs on odor response. In addition, it has been demonstrated that there is a good correlation between EOG, the olfactory neural response and the bulbar response (Caprio, 1977; Byrd and Caprio, 1982; Hara, 1982).

Fig. 4 shows typical olfactory bulbar responses to 0.1 mM citralva alone (*A*) and 0.1 mM citralva solution containing 50 μ M forskolin after adaptation to 50 μ M forskolin to a spontaneous level (*B*). It is noted that citralva is a typical odorant which increases cAMP level in the rat and the bullfrog olfactory cilia preparations (Sklar et al., 1986; Breer and Boekhoff, 1991). Fig. 4 *C* shows that the peak heights of the responses to forskolin alone and 0.1 mM citralva + forskolin after adaptation to forskolin are a function of the forskolin concentrations. The response to forskolin was increased with an increase in forskolin concentration and plateaued at 10–20 μ M. The response to 0.1 mM citralva plus forskolin after forskolin adaptation decreased with an increase in forskolin concentration up to 5 μ M and reached a plateau level. The magnitude of response to 0.1 mM citralva after 50 μ M forskolin, where the response to forskolin itself was sufficiently saturated, was $56 \pm 4\%$ (mean \pm SE; $n = 27$) of the response to citralva alone.

It was reported that lilial did not increase cAMP level in the rat and the bullfrog olfactory cilia preparations but increased IP₃ level in the rat preparation (Sklar et al., 1986; Breer and Boekhoff, 1991). Fig. 3 *D* shows that the magnitudes of the responses to 0.1 mM lilial + forskolin after adaptation to forskolin are a function of forskolin concentrations. As with the response to citralva, the response to lilial decreased as the forskolin concentration increased.

Fig. 5 summarizes the effects of 50 μ M forskolin on the responses to various species of odorants. The value in the figure represents relative magnitude of the response to an odorant after 50 μ M forskolin when the magnitude of the response to the odorant alone is taken as unity. The reduction of the odor response ranged from 45 to 80% of the control response. The odorants listed in the columns in the upper

group such as eugenol, citronellal, hedione, geraniol, citralva, cineole, and *l*-carvone are known to increase the cAMP level in rat and bullfrog olfactory cilia preparations. The odorants listed in the middle group such as lylal, liliat, triethylamine, and ethyl vanillin did not increase cAMP in the rat and the bullfrog preparations, but increased the IP₃ level in the rat preparation (Sklar et al., 1986; Breer and Boekhoff, 1991). There was no essential difference in the degree of the reduction of the response by

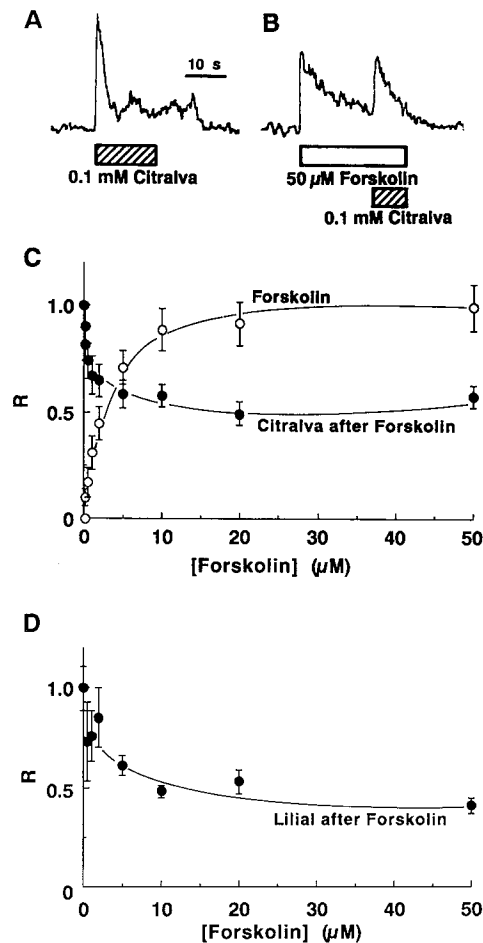


FIGURE 4. Effects of varying concentrations of forskolin on the responses to citralva and liliat. (A) Summated olfactory bulbar response to 0.1 mM citralva. (B) 50 μM forskolin was first applied and 0.1 mM citralva solution containing 50 μM forskolin was applied after adaptation to forskolin. (C) Relative magnitude of the olfactory bulbar responses to forskolin alone (○) and 0.1 mM citralva solutions containing forskolin of varying concentrations (●) as a function of forskolin concentration. Magnitudes of the responses to forskolin and citralva were measured from a basal level and a level immediately before the rise of summated value to the peak, respectively. Magnitudes of the responses to 50 μM forskolin (○) and 0.1 mM citralva alone (●) are taken as unity, respectively. Mean magnitude of the response to 50 μM forskolin was $73 \pm 5\%$ ($n = 25$) of that to 0.1 mM citralva alone. Each point is mean \pm SE of data obtained from at least four preparations. (D) Relative magnitude of the olfactory bulbar response to 0.1 mM liliat solutions containing forskolin of varying concentrations as a function of forskolin concentrations. Magnitude of the response to 0.1 mM liliat alone is taken as unity. Each point is mean \pm SE of data obtained from four preparations.

forskolin between two groups. The degree of the reduction was neither correlated with ability of cAMP-formation in the rat ($r = -0.39$), the bullfrog ($r = 0.39$) olfactory cilia nor IP₃-formation in the rat olfactory cilia ($r = -0.21$). The lowest column indicates an effect of 50 μM forskolin on a response to 1 mM cpt-cAMP, a membrane permeant analogue of cAMP. Application of cpt-cAMP induced large olfactory responses in the turtle olfactory system as described latter; magnitude of a

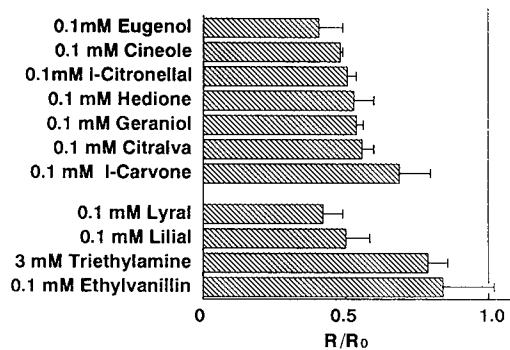


FIGURE 5. Responses to various odorants and cpt-cAMP after adaptation to 50 μ M forskolin. Odorants of upper and lower groups represent cAMP- and IP₃-dependent odorants, respectively (Sklar et al., 1986; Breer and Boekhoff, 1991). Each column represents relative magnitude of the response (R/R_0) when magnitude of the response to each odorant alone (R_0) is taken as unity. Column at the bottom represent relative magnitude of the response when magnitude of

the response to 1 mM cpt-cAMP alone is taken as unity. The data are mean \pm SE of those obtained from at least three preparations. Mean relative magnitude of the response to each odorant, where magnitude of the response to 0.1 mM citralva is taken as unity, is as follows: 0.1 mM Eugenol, 0.64 ± 0.07 (3); 0.1 mM cineole, 1.25 ± 0.04 (4); 0.1 mM citronellal, 1.04 ± 0.03 (3); 0.1 mM hedione, 0.99 ± 0.11 (4); 0.1 mM geraniol, 0.86 ± 0.07 (3); 0.1 mM carvone, 0.96 ± 0.10 (7); 0.1 mM lyral, 0.77 ± 0.07 (4); 0.1 mM lilial, 0.80 ± 0.06 (7); 3 mM triethylamine, 0.68 ± 0.10 (7); 0.1 mM ethyl vanillin, 0.33 ± 0.07 (7); 1 mM cpt-cAMP, 1.40 ± 0.32 (4); mean \pm SE (n).

response to 1 mM cpt-cAMP was 140 ± 32 (mean \pm SE, $n = 4$) of that to 0.1 mM citralva. As shown in the Fig. 5, 1 mM cpt-cAMP + 50 μ M forskolin induced only a small response after 50 μ M forskolin.

In the above experiments, relatively high concentrations of odorants were used. Hence, there is a possibility that the forskolin-independent responses are induced only by high concentrations of odorants. To test this possibility, the effects of forskolin on the responses to citralva of varying concentrations were examined. As shown in Fig. 6, low concentrations of citralva (1–10 μ M) elicited appreciable responses after 50 μ M forskolin, indicating that the cAMP-independent pathway contributes to the response to the odorant of low concentrations as well as high concentrations.

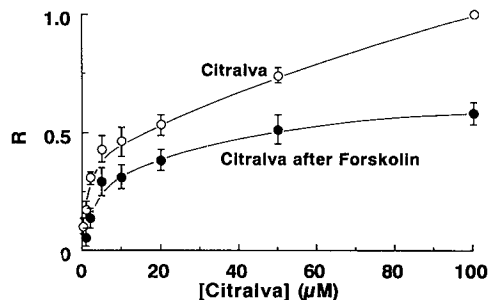


FIGURE 6. Effects of 50 μ M forskolin on dose-response relationships of citralva. Relative magnitudes of responses to citralva solutions alone (\circ) and those containing 50 μ M forskolin after adaptation to 50 μ M forskolin (\bullet) are plotted as a function of citralva concentrations. Magnitude of the response to 0.1 mM citralva alone is taken as unity. Each point is mean \pm SE of data obtained from four preparations.

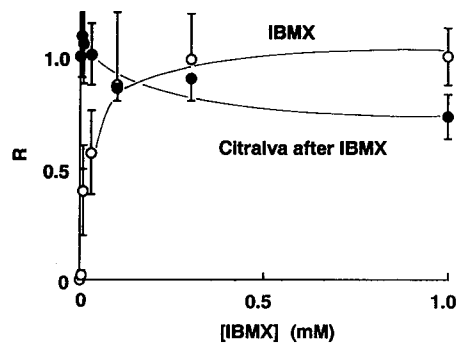


FIGURE 7. Effect of IBMX on the response to citralva. Relative magnitudes of responses to IBMX solution (○) and 0.1 mM citralva solutions containing IBMX of varying concentrations after the response to IBMX was adapted (●) are plotted as a function of IBMX concentrations. Magnitude of the response to 1 mM IBMX (○) and 0.1 mM citralva (●) are taken as unity, respectively. Each point is the mean \pm SE of data obtained from four preparations. Mean magnitude of response to 1 mM IBMX was $142 \pm 5\%$ (SE; $n = 4$) of that to 0.1 mM citralva.

Effects of IBMX and Membrane Permeant Cyclic Nucleotide Analogues on Odor Responses

It is expected that application of IBMX to the olfactory epithelium leads to an increase of cAMP level in the olfactory cells by inhibition of phosphodiesterase. Fig. 7 shows that the magnitudes of responses to IBMX itself, and 0.1 mM citralva solution containing IBMX after adaptation to IBMX, are a function of its concentration. The response to IBMX increased with an increase in IBMX concentration and plateaued at 0.3–1 mM. The response to 0.1 mM citralva after IBMX decreased with an increase in IBMX concentration and plateaued. The magnitude of the response to 0.1 mM citralva after 1 mM IBMX, where response to IBMX itself was saturated, was $73 \pm 10\%$ (mean \pm SE; $n = 4$) of 0.1 mM citralva alone. These results are consistent with

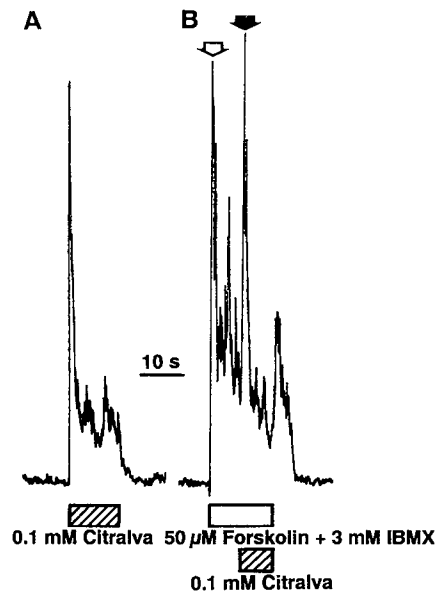


FIGURE 8. Typical bulbar responses to 0.1 mM citralva applied alone (A), and 0.1 mM citralva solution containing 50 μ M forskolin and 3 mM IBMX (black arrow) after the response to forskolin and IBMX (white arrow) was adapted (B).

those reported by Firestein et al. (1991a); IBMX reduced the sensory current induced by the odorant mixture (1 mM cineole, amyl acetate, acetophenone) by 35–50% in the salamander olfactory neurons.

To increase cAMP in the olfactory cells to a maximal level, a mixture of IBMX and forskolin was applied followed by application of an odorant. In Fig. 8, a mixture of 3 mM IBMX and 50 μ M forskolin was first applied followed by application of a 0.1 mM citralva solution containing the mixture. As seen from the figure, a large response to the odorant appeared after application of the mixture of IBMX and forskolin. The average magnitude of the response to 0.1 mM citralva after the mixture was $58 \pm 3\%$ (mean \pm SE, $n = 4$) of the odorant applied alone, which was similar to the value

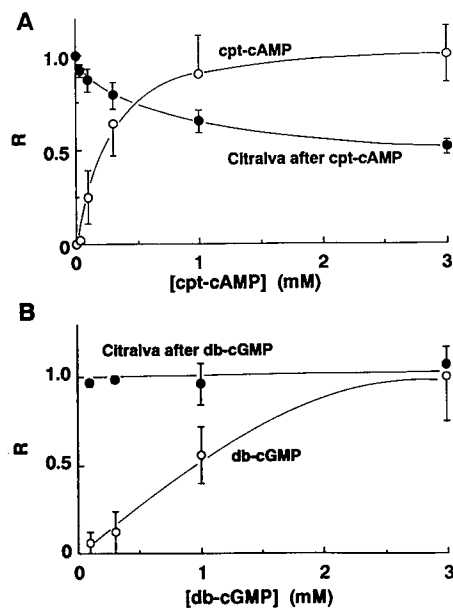


FIGURE 9. Effects of membrane permeant cyclic nucleotides on bulbar responses. (A) Relative magnitudes of responses to cpt-cAMP solution (○) and 0.1 mM citralva solutions containing cpt-cAMP of varying concentrations after the response to cpt-cAMP was adapted (●) are plotted as a function of cpt-cAMP concentrations. Magnitudes of the responses to 3 mM cpt-cAMP (○) and 0.1 mM citralva (●) are taken as unity, respectively. Each point is mean \pm SE of data obtained from four preparations. Mean magnitude of response to 3 mM cpt-cAMP was $155 \pm 24\%$ (SE; $n = 4$) of that to 0.1 mM citralva. (B) Relative magnitudes of responses to db-cGMP (○) and 0.1 mM citralva solutions containing db-cGMP of varying concentrations after the response to db-cGMP was adapted (●) are plotted as a function of db-cGMP concentrations. Magnitudes of responses to 3 mM db-cGMP (○) and 0.1 mM citralva (●) are taken as unity. Each point is the mean \pm SE of data obtained from eight preparations. Mean magnitude of response to 3 mM db-cGMP was $25 \pm 6\%$ (SE; $n = 8$) of 0.1 mM citralva.

obtained after 50 μ M forskolin (Fig. 4). That is, 50 μ M forskolin brought about a maximal effect on the odor response and the addition of 3 mM IBMX did not enhance the effect.

Fig. 9A shows the effects of a membrane permeant nucleotide, cpt-cAMP on the response to 0.1 mM citralva. The response to cpt-cAMP itself plateaus at ~ 1 mM. The response to 0.1 mM citralva after cpt-cAMP was decreased as the concentration of cpt-cAMP increased. The magnitude of response to citralva after 3 mM cpt-cAMP was $51 \pm 4\%$ (mean \pm SE; $n = 4$) of citralva applied alone.

To test the possibility that cGMP is a second messenger in the turtle olfactory system, the effects of a membrane permeant nucleotide, db-cGMP on the response to

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To test the possibility that cGMP is a second messenger in the turtle olfactory system, the effects of a membrane permeant nucleotide, db-cGMP on the response to

citralva were examined. Fig. 9 B shows that the magnitudes of the responses to db-cGMP alone and 0.1 mM citralva + db-cGMP after adaptation to db-cGMP are a function of db-cGMP concentration. The response to db-cGMP alone increased with concentration, but the magnitude of the response to 3 mM db-cGMP was only $25 \pm 6\%$ (mean \pm SE, $n = 8$) of 0.1 mM citralva, whereas the response to 3 mM cpt-cAMP was $155 \pm 24\%$ (mean \pm SE, $n = 7$) of 0.1 mM citralva. The response to 0.1 mM citralva was unaffected by prior application of db-cGMP (Fig. 9 B).

DISCUSSION

Responses to cAMP and Forskolin in the Turtle Olfactory Neuron

Injection of 1 mM cAMP induced inward currents in the turtle olfactory neuron (-252 pA, holding potential -50 mV). The amplitude of inward currents was smaller than the currents in the frog (-426 pA, holding potential -50 mV), the bullfrog (-500 pA, holding potential -75 mV) and the newt (319 pA, holding potential -50 mV) olfactory neurons, but similar to that in the salamander olfactory neuron (-226 pA, holding potential -50 mV) in response to 0.5 mM cAMP (Trotier, Rosen, and MacLeod, 1989; Suzuki, 1989; Kurahashi, 1990). These results indicate that activity of cAMP-gated channels in the turtle is similar to that in the salamander.

Application of forskolin also induced inward currents and increased frequency of impulses of the olfactory neurons, indicating that there exists adenylate cyclase in the turtle olfactory neuron. The olfactory bulbar responses to forskolin were saturated at 10 μ M and the concentration necessary to induce a half maximum response was 2.5 μ M. The half maximum concentration of forskolin for adenylate cyclase type III, which was reported to be a specific adenylate cyclase in the rat olfactory cell (Bakalyar and Reed, 1990) was 20 μ M (Choi, Xia, and Storm, 1992). The discrepancy may be due to differences in the animals used, and/or the measurements; we measured the turtle olfactory response and Choi et al. (1992) measured cAMP formation by the rat adenylate cyclase type III. In any case, the present results that stimulation with 1 mM cpt-cAMP after 50 μ M forskolin elicited only a small response suggest that 50 μ M forskolin, which was used throughout in the present study, is sufficient to bring about maximum cAMP level in the olfactory cells. cpt-cAMP is a hydrophobic chemical as is similar to odorants. Hence there is a possibility that cpt-cAMP stimulates receptors for odorants and produces a response via cAMP-independent pathway as is similar to odorants. The small response induced by cpt-cAMP after 50 μ M forskolin seems to be produced by the above mechanism.

Odor Responses Recorded with Pipette Containing cAMP

Kurahashi reported that inward current induced by injection of cAMP from the pipette plateaued at 0.5 mM in the newt olfactory neuron (1990). The response of cAMP-gated channels in inside-out patches to cAMP in the frog, rat and catfish plateaued at 3, 50, and 2 μ M, respectively (Nakamura and Gold, 1987; Frings, Lynch, and Lindemann, 1992; Goulding, Ngai, Kramer, Colicos, Axel, Siegelbaum, and Chess, 1992). These results suggest that 1 mM cAMP is sufficient to induce a maximum response via cAMP-gated channels. In the present study, we recorded inward currents induced by cAMP-dependent odorant cocktail even during continuous

application of 1 mM cAMP. Mean amplitude of response to the odorant cocktail when the pipette contained 1 mM cAMP was similar to that recorded without cAMP, indicating that the odorant cocktail induced an odor response not only via cAMP-dependent pathway but also via cAMP-independent pathway.

Suppressive Effects of Forskolin

Although stimulation of turtle olfactory neurons with 1 mM cAMP elicited an inward current in 100% of the cells tested, stimulation with 50 μ M forskolin elicited only 30% of the cells tested. Amplitude of inward currents induced by 50 μ M forskolin (-23 pA) was smaller than that induced by 1 mM cAMP (-252 pA). These results are consistent with those of the salamander olfactory neurons reported by Trotier et al. (1989); amplitude of inward current in response to 50 μ M forskolin, which was applied extracellularly, and 0.5 mM cAMP, which was injected from the patch pipette, were -37 and -226 pA, respectively. Amplitude of odor responses of the turtle olfactory neurons was also partially suppressed by 50 μ M forskolin as shown in the present study. In some catfish olfactory neurons, adaptation of the isolated neuron to 10 μ M forskolin for 5 min abolished the response to amino acids (Miyamoto, Restrepo, and Teeter, 1992). Similar results were observed with responses recorded from olfactory cilia; 13 of 23 cells did not respond to odorants or had no spontaneous discharges when forskolin was applied for 5 to 10 min (data not shown).

It is known that forskolin inhibits a number of membrane transport proteins and channel proteins through a mechanism that does not involve the production of cAMP (Laurenza, Sutkowski, and Seamon, 1989). When isolated cells are treated with forskolin by the bath application, forskolin acts not only on the olfactory cilia and knob but also on the dendrite, the cell soma and the axon. This might bring out the irreversible effect on the neurons. On the other hand, in the recording of the olfactory bulbar responses, repeated application of forskolin to the olfactory epithelium under in vivo conditions did not bring about any irreversible effect. For example, a large response to citralva (50% of the original response) appeared even after repeated application of 50 μ M forskolin during a whole day experiment. Olfactory epithelium is held in normal in vivo condition when the bulbar responses are recorded because sufficient oxygen and nutrition may be supplied from the blood vessel and chemical agents including odorants incorporated in the olfactory neurons may be removed by blood flow. Thus, the recording of the bulbar response has the advantage of being able to examine the forskolin effect on the olfactory responses.

Effects of Forskolin on Responses to cAMP- and IP₃-dependent Odorants

It was reported that many species of odorants induced cAMP accumulation in frog (Pace et al., 1985), bullfrog (Sklar et al., 1986), and rat (Boekhoff et al., 1990) olfactory cilia preparations, but some odorants did not accumulate cAMP. Breer and Boekhoff (1991) demonstrated that odorants which did not activate adenylate cyclase induced IP₃ accumulation in the rat olfactory cilia preparations. However, for all the odorants tested by them, there was no case where activation of both the cAMP and IP₃ pathways was observed. Odorants which activated adenylate cyclase in the rat olfactory cilia preparations also stimulated adenylate cyclase in the frog and the

bullfrog. Hence, it is highly probable that similar species of odorants activate adenylate cyclase in the turtle olfactory system.

The present results showed that forskolin suppressed the responses to the odorants by 45–80%. There was no essential difference in the degree of the suppression between cAMP- and IP₃-producing odorants reported in the rat. This was consistent with the results observed with the bullfrog; forskolin suppressed the electroolfactograms to all odorants examined including both cAMP-dependent and -independent odorants to a similar extent (Lowe, Nakamura, and Gold, 1989). These results suggest that the suppression by forskolin may be brought about partly by nonspecific effects of forskolin discussed above. Changes in ion concentrations in olfactory cells induced by influx and efflux of cations through cAMP-gated channels during sustained depolarization by forskolin may also contribute to the suppression. If so, then it cannot be concluded that all of the forskolin-suppressive components in the responses to cAMP-producing odorants are cAMP-dependent components.

Origin of cAMP-insensitive Response

The most important result in the present study is that the cAMP-insensitive pathway greatly contributes to the turtle olfactory transduction. Mean amplitude of inward currents in response to odorant cocktail when 1 mM cAMP was added to the internal solution was similar to that recorded by the pipette containing no cAMP. Citralva (0.1 mM), after 50 μ M forskolin, induced responses as high as 56% of the control response. The magnitude of the forskolin-insensitive response was unchanged when IBMX was used together with forskolin, suggesting that forskolin (50 μ M) was sufficient to bring about a maximum cAMP level. A membrane permeant cAMP analogue, cpt-cAMP, induced a large response, and after the response to a sufficiently high concentration of cpt-cAMP (3 mM) was adapted, 0.1 mM citralva induced responses as high as 51% of the control response. All these data indicate that at least 50% of the response to the odorant is a cAMP-independent component.

Recently, relative levels of expression during rat development were determined for olfactory-specific genes by quantitative polymerase chain reaction (PCR), starting at embryonic day 15 (E15) and ending at postnatal day 35 (P35) (Margalit and Lancet, 1993). Although odor responses were recorded from rat olfactory neurons starting at embryonic days E14–E16 (Gesteland, Yancey, and Farbman, 1982), the onset of expression for olfactory receptors and the olfactory cyclic nucleotide-gated channel were at embryonic day 19 (E19). These results suggest that the odor responses appear neither via the olfactory receptors nor the cAMP-gated channel in the early stage of development in the rat. The cAMP-independent pathway observed in the present study may be related to the pathway observed with rat fetal olfactory cells.

There are number of possibilities that may explain out results. One possibility is that the cAMP-insensitive component of the response to the odorant, reported as a cAMP-dependent component, is induced by activation of the IP₃ pathway. If this is true, it is also conceivable that all the odorants examined activate IP₃ pathway. This is not consistent with the results observed with the rat as described above (Breer and Boekhoff, 1991).

One may consider that the forskolin-independent component appears only at high concentrations (0.1 mM). The present study, however, showed that the forskolin-

insensitive component appeared in response to low concentrations of the odorant. For example, the magnitude of response to 5 μM citralva after 50 μM forskolin was 67% of that to citralva applied alone, suggesting that the cAMP-independent odor responses are also induced by low concentrations of odorant. In addition, it is noted that olfactory neurons function physiologically when odorants of high concentration such as 0.1 mM are applied to the neurons; the turtle olfactory system discriminates the difference in odor quality of 0.1 mM optical isomers and in odor strength at concentrations around 0.1 mM (Taniguchi et al., 1992). It is more probable that the cAMP-insensitive component of responses to cAMP-dependent and IP_3 -independent odorants in the rat is induced by activation of a pathway other than the cAMP- and IP_3 -dependent pathways.

db-cGMP induced only a small bulbar response and did not affect the response to citralva. Hence, it is unlikely that cGMP is second messenger in the turtle olfactory transduction. The pathway of Ca-activated chloride channels (Kleene and Gesteland, 1991; Kleene, 1993) is one of the candidates for the cAMP-insensitive pathway. Kurahashi and Yau (1993) reported that the odorant-induced current significantly contained Ca-activated Cl-current in newt and salamander olfactory neurons. There is also a possibility that changes in the phase boundary potential are the origin of the cAMP-insensitive component (Kashiwayanagi and Kurihara, 1984; Nomura and Kurihara, 1987).

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