Difference in Behavior Between Responses to Forskolin and General Odorants in Turtle Vomeronasal Organ

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Abstract

To elucidate the signal transduction mechanisms in the turtle vomeronasal receptor neurons, the effects of forskolin, changes in mucosal Ca^{2+} concentrations and ruthenium red on the responses of the accessory olfactory bulb to general odorants were examined. Forskolin elicited a large response, suggesting that there are cAMP-gated channels in the vomeronasal neurons. On the other hand, the dependence of the responses to general odorants on Ca^{2+} concentrations was different from that of the response to forskolin. A large response to an odorant (n-amyl acetate) appeared after the cAMP-mediated pathway was fully desensitized by application of 50 μM forskolin. These results suggest that the cAMP-mediated pathway does not contribute significantly to generation of the response to general odorants. A concentration of 50 μM ruthenium red significantly reduced the responses to n-amyl acetate alone and after 50 μM forskolin desensitization, suggesting that the inositol triphosphate-mediated pathway contributes partly to generation of the responses to general odorants in the vomeronasal neurons. **Chem Senses 21: 763–771, 1996.**

Introduction

Most terrestrial vertebrates detect chemosignals by two types of olfactory systems: the main olfactory organ and an accessory olfactory organ called the vomeronasal organ. A number of behavioral, biochemical and anatomical studies have demonstrated that the vomeronasal system plays important roles in the perception of chemical stimuli related to animal behavior (Halpern, 1987; Wysocki and Meredith, 1987). Despite the increasing interest in the structure and biological functions of the vomeronasal system, the molecular basis of recognition of chemical stimuli remains little understood.

The turtle vomeronasal organ responds sensitively to many kinds of general odorants (Tucker, 1963, 1965; Hatanaka *et al.*, 1988; Shoji and Kurihara, 1991). Recently, Inouchi *et al.* (1993) reported that the vomeronasal organ of garter snakes also responded to general odorants. In the vertebrate olfactory system, an olfactory response is considered to be induced by binding of an odorant to receptor proteins, followed by G-protein-mediated increases in the internal cAMP and inositol-1,4,5-triphosphate (IP_3) concentrations to open cyclic nucleotide- and IP_3-activated cation channels on the plasma membrane, respectively (Restrepo *et al.*, 1990; Breer *et al.*, 1994). Hence, it is possible that the second messenger-dependent pathway may be

involved in the vomeronasal transduction mechanism for general odorants. In a previous paper, we examined properties of adenylyl cyclase activity in the membrane preparations from turtle vomeronasal epithelia (Okamoto *et al.*, 1996). The activity was appreciably activated by GTPγS or forskolin, but was not activated by the odorant cocktails, while the adenylyl activity in the membrane preparations from the olfactory epithelia was appreciably activated by odorant cocktails.

In the present study, we examined whether a cAMP-dependent pathway contributes to generation of the vomeronasal responses to general odorants *in vivo* by measuring the turtle accessory olfactory bulbar responses. It was shown that calcium dependence of the responses to some general odorants was evidently different from that to the response to forskolin which is induced via a cAMP-dependent pathway. The response to a general odorant, n-amyl acetate, was not practically suppressed by previous application of a high concentration of forskolin. The results obtained suggest that the responses to general odorants are generated via a cAMP-independent pathway.

Materials and methods

Recording of accessory olfactory bulbar response

Turtles (*Geoclemys reevesii*) weighing 140–240 g were used in the present study. Accessory olfactory bulbar responses were recorded by following a modification of the procedure described previously (Taniguchi *et al.*, 1992). Briefly, turtles were weakly anesthetized with the necessary and minimum amount of urethane to lessen pain during the operation, immobilized by i.m. injection of *d*-tubocurarine chloride (450 mg/100 g body wt), and administered lidocaine as a local anesthetic at the wound and head-fixation points. The accessory olfactory bulb was exposed using a dental drill, and the dura mater on the accessory olfactory bulb was removed carefully. To eliminate the possible effects of the main olfactory bulb activities, the olfactory nerve was cut off before entry to the main olfactory bulb. The stimulant-induced brain waves (bulbar responses) were recorded by attaching a pair of silver bipolar electrodes to the medial part of the anterior bulb. The responses were amplified with a DC amplifier, filtered into 3–300 Hz and then integrated using an electric integrator (time constant 0.3 s).

Chemical stimulation

The adapting and stimulating solutions were applied to the vomeronasal epithelium through a stainless steel tube. Before application of the stimulating solution, the vomeronasal epithelium was irrigated with corresponding adapting solution for ~10 min. Stimulating solution, which was prepared by dissolving odorants in the corresponding adapting solution, was applied to the epithelium at a flow rate of 31 ± 7 ml/min. After each application of the stimulating solution on the epithelium, the epithelium was rinsed with the adapting solution. About 10 min were interposed between successive stimulations. All experiments were carried out at 20 ± 3°C.

Preparation of solutions

Ringer's solution without Ca^{2+} consisted of (mM), 116 NaCl, 4 KCl, 2 $MgCl_2$, 10 HEPES–NaOH (pH 7.4). For Ca^{2+}-free Ringer's solution, 2 mM EGTA was added to Ringer's solution without Ca^{2+}. Ringer's solutions containing 0.1, 2 (i. e. normal Ringer's solution) and 20 mM Ca^{2+} were prepared by adding 0.1, 2 and 20 mM $CaCl_2$ respectively to Ringer's solution without Ca^{2+}. Eighty millimolar Ca^{2+} Ringer's solution consisted of (mM) 80 $CaCl_2$ and 10 HEPES–NaOH (pH 7.4). Forskolin stock solution was prepared by dissolution in ethanol at 10 mM, and appropriate volumes were added to Ringer's solution to give the desired concentrations. Ruthenium red was dissolved in normal Ringer's solution. These forskolin and ruthenium red solutions were prepared daily. Stimulating solutions of n-amyl acetate were prepared by direct dissolution in Ringer's solution containing Ca^{2+} at the desired concentrations. The other odorants were dissolved in ethanol to give a stock concentration of 0.1 M. These stock solutions were added to Ringer's solution to give the indicated concentrations of odorants. The final concentration of ethanol never exceeded 0.5%—a level that had no measurable effect on summated accessory olfactory bulbar response (data not shown).

Chemicals

Lilial, geraniol, lyral, citralva and hedione were kindly supplied by Takasago International (Tokyo, Japan). n-Amyl acetate, forskolin and ruthenium red were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). All chemicals used were of the highest grade available.

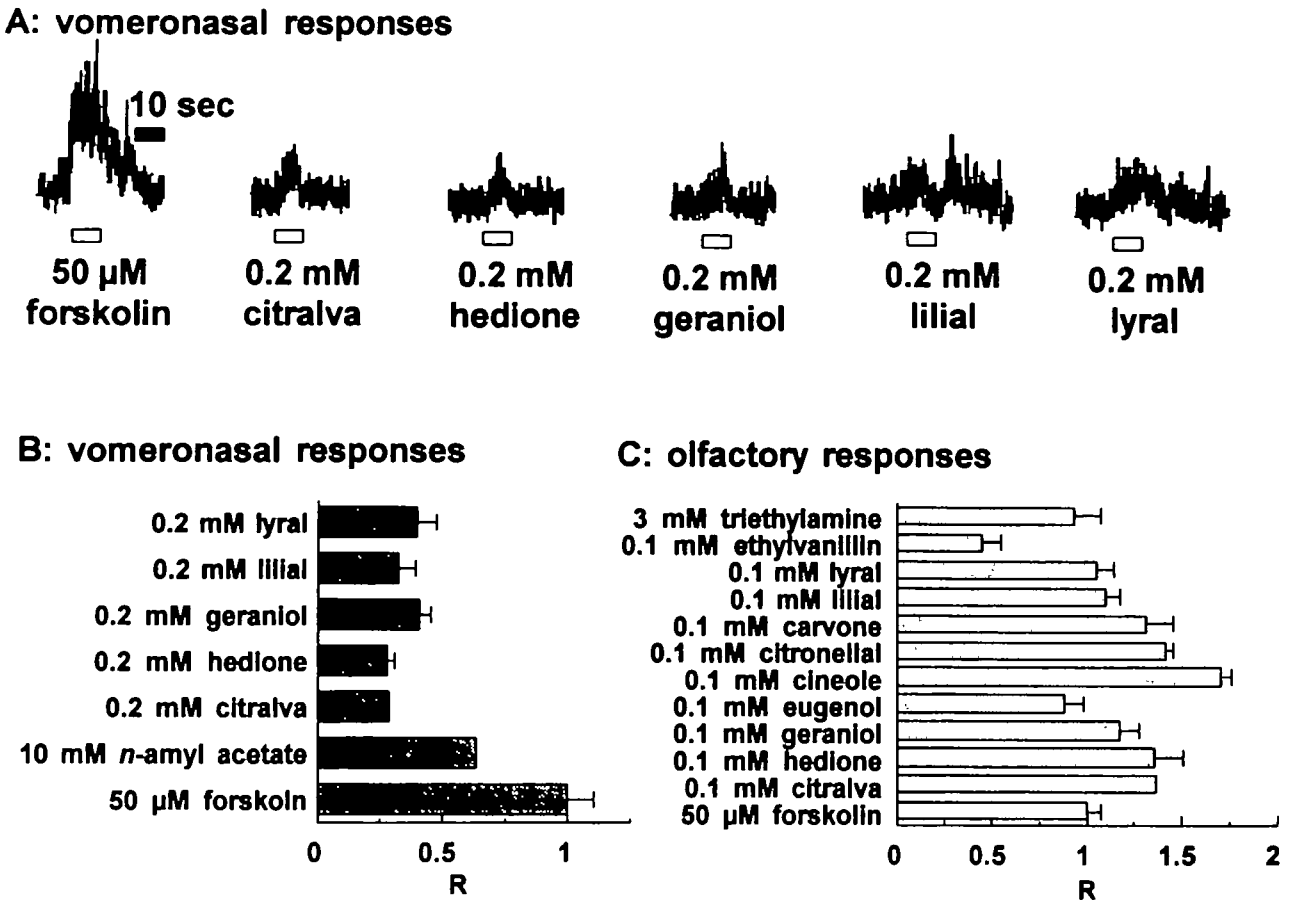

Figure 1 The concentration of n-amyl acetate used here was different from that to other odorants because of the differences in their solubilities in Ringer's solution. (**A**) Typical records of the summated accessory olfactory bulbar responses to various stimuli. The ordinate represents the relative magnitudes of the responses. Bars under the records represent duration of exposure to the various chemicals. (**B**) Relative magnitudes of the accessory olfactory bulbar responses to various stimuli (**C**) Relative magnitude of the turtle main olfactory bulbar responses to various stimuli. The magnitudes of the accessory and main olfactory responses were calculated relative to those to 50 μM forskolin. Points represent the means ± SEM of data obtained from at least three preparations. The data shown in (C) are taken from Kashiwayanagi *et al.* (1994).

Results

Figure 1(A) shows the summed accessory olfactory bulbar responses to various stimuli dissolved in normal Ringer's solution. In this study, the peak height of the summed bulbar response was taken as the magnitude of the response. Figure 1(B) shows relative magnitudes of the vomeronasal responses to various stimuli where the magnitude of the response to 50 μM forskolin was taken as unity. As shown in the figure, the relative magnitudes of the vomeronasal responses to 0.2 mM odorants were much less than 1.0. This differs markedly from the case of the turtle main olfactory bulbar responses where the relative magnitude of the response to 50 μM forskolin was smaller than or comparable to those to 0.1 mM odorants such as lyral, lilial, geraniol, hedione and citralva (Figure 1C).

Figure 2 shows relative magnitude of the accessory

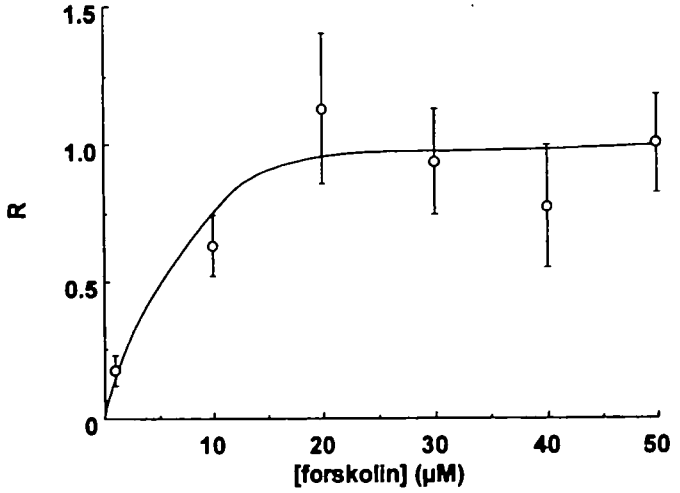

Figure 2 Relative magnitudes of the accessory olfactory bulbar responses to forskolin of varying concentrations as a function of its concentration. The magnitude of the response to 50 μM forskolin was taken as unity.

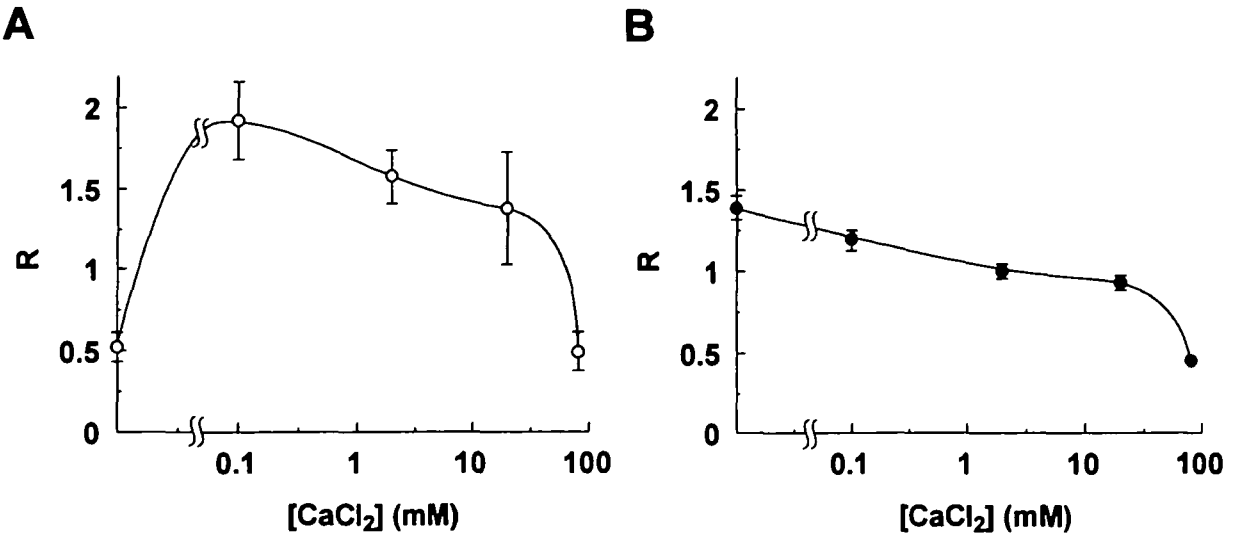

Figure 3 The relative magnitudes of the accessory olfactory bulbar responses to 50 μM forskolin (**A**) and 10 mM n-amyl acetate (**B**) as a function of $CaCl_2$ concentration. The magnitude of the response to 10 mM n-amyl acetate dissolved in normal Ringer's solution was taken as unity for each dose–response curve. Points represent the means ± SEM of data obtained from at least three preparations.

olfactory response to forskolin as a function of concentration. The response to forskolin increased with increases in concentration and reached a plateau at 10~20 μM similarly to the turtle main olfactory response to forskolin (Kashiwayanagi *et al.*, 1994).

It has been reported that divalent cations directly block the cAMP-activated cation channels in vertebrate olfactory neurons (Nakamura and Gold, 1987; Suzuki, 1989; Kurahashi, 1990; Frings *et al.*, 1991; Zufall *et al.*, 1991). To examine the effects of Ca^{2+} on the accessory olfactory responses, the epithelium was perfused with an adapting solution containing a given concentration of Ca^{2+} and then forskolin and an odorant dissolved in the adapting solution were applied to the epithelium. Figure 3 shows the magnitudes of the responses to forskolin (A) and n-amyl acetate (B) as a function of Ca^{2+} concentrations in perfusing solution. The magnitude of the response to forskolin increased with increasing $CaCl_2$ concentration from 0 to 0.1 mM and was greatly decreased at 80 mM $CaCl_2$. Figure 3(B) shows that the magnitude of the response to 10 mM n-amyl acetate decreased gradually with increase in $CaCl_2$ concentration from 0 to 20 mM. The response to 10 mM n-amyl acetate was greatly decreased at 80 mM $CaCl_2$, similarly to the response to 50 μM forskolin. Figure 3 shows that dependence of the response to n-amyl acetate on Ca^{2+} concentration differs from that to forskolin at the low Ca^{2+} concentration.

We examined the effects of increases in Ca^{2+} concentration on the responses to other odorants. Figure 4 shows

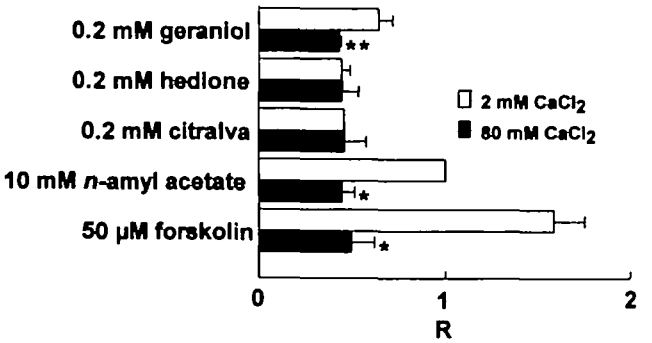

Figure 4 Relative magnitudes of the responses to various chemical stimuli in 80 mM Ca^{2+} solution. The magnitude of the response to 10 mM n-amyl acetate in normal Ringer's solution (i.e. $CaCl_2$ concentration 2 mM) was taken as unity. Data for 10 mM n-amyl acetate and 50 μM forskolin were taken from Figure 3. The responses to 10 mM n-amyl acetate and 50 μM forskolin were reduced by increasing Ca^{2+} concentration (*$P < 0.001$). The response to 0.2 mM geraniol was slightly reduced by increasing Ca^{2+} concentration (**$P < 0.05$). Points represent the means ± SEM of data obtained from at least three preparations.

the relative magnitudes of the responses to various odorants dissolved in normal Ringer's solution (2 mM $CaCl_2$) and an adapting solution containing 80 mM $CaCl_2$. The responses to n-amyl acetate and geraniol were significantly suppressed by increasing $CaCl_2$ concentration to 80 mM, while those to hedione and citralva were not affected.

To examine to what extent cAMP-mediated pathway contributes to *in vivo* vomeronasal transduction for general odorants, we recorded the vomeronasal responses to n-amyl acetate after the cAMP-mediated pathway had been desensitized by forskolin. Figure 5 illustrates typical records

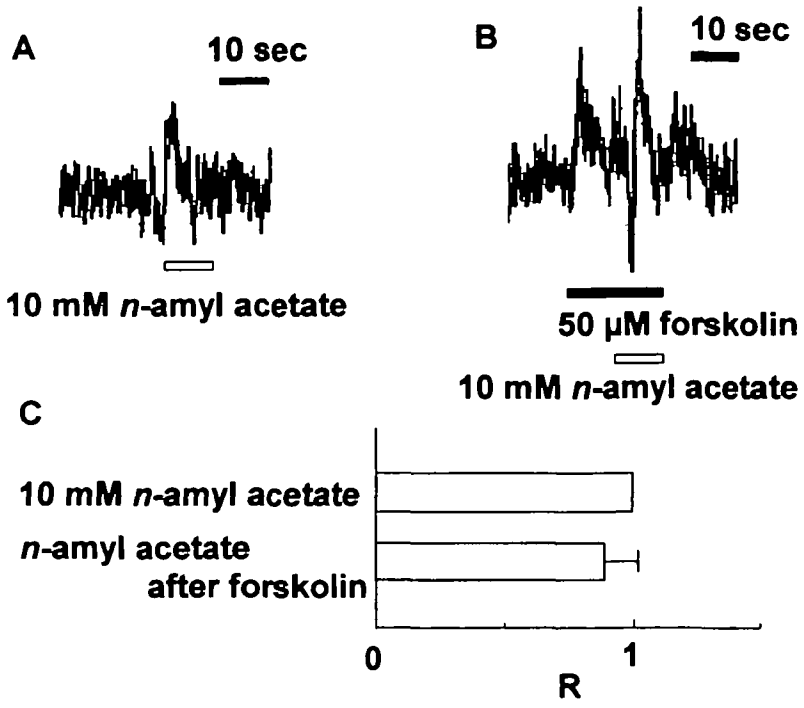

Figure 5 Effects of 50 μM forskolin on the response to 10 mM n-amyl acetate. (**A**) Summed accessory olfactory bulbar response to 10 mM n-amyl acetate applied alone. (**B**) 50 μM forskolin was applied first followed after adaptation by 10 mM n-amyl acetate solution containing 50 μM forskolin. (**C**) Relative magnitudes of the accessory olfactory bulbar responses to 10 mM n-amyl acetate applied alone and 10 mM n-amyl acetate solution containing 50 μM forskolin after adaptation to 50 μM forskolin. Magnitudes of the responses to 10 mM n-amyl acetate alone and after adaptation to forskolin were measured from the basal level to the peak and from a level immediately before the rise of the summed value to the peak respectively. The magnitude of the response to 10 mM n-amyl acetate applied alone was taken as unity. Points represent the means ± SEM of data obtained from at least six preparations.

of the accessory olfactory responses to 10 mM n-amyl acetate applied alone (A) and 10 mM n-amyl acetate solution containing 50 μM forskolin after the response to 50 μM forskolin had adapted to a spontaneous level (B). As shown in the figure, the response to n-amyl acetate was distinct even after adaptation to 50 μM forskolin. Figure 5(C) shows the relative magnitudes of the response to 10 mM n-amyl acetate before and after application of 50 μM forskolin. The response to 10 mM n-amyl acetate was only partially but not significantly suppressed by forskolin. The magnitude of the response to 10 mM n-amyl acetate after 50 μM forskolin was 89 ± 13% (mean ± SEM; n = 8) of the response to n-amyl acetate applied alone. This suggests that the cAMP-mediated pathway does not contribute significantly to generation of the vomeronasal response to a general odorant such as n-amyl acetate.

The effects of the IP_3 channel blocker ruthenium red on the magnitude of the vomeronasal responses were examined (Figure 6). The responses to 10 mM n-amyl acetate alone and 10 mM n-amyl acetate plus 50 μM forskolin after adaptation to 50 μM forskolin were significantly reduced by 50 μM ruthenium red, suggesting that the IP_3-mediated pathway contributes somewhat to the vomeronasal response

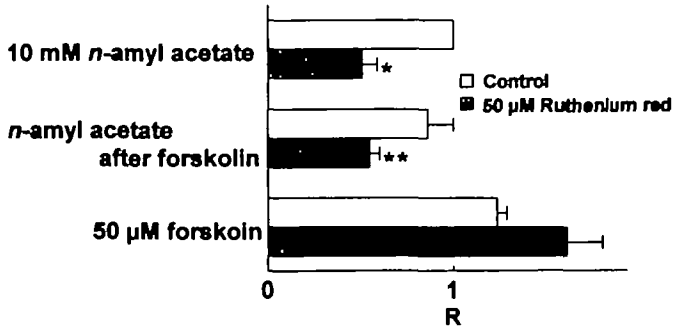

Figure 6 Effects of 50 μM ruthenium red on accessory olfactory bulbar responses to 10 mM n-amyl acetate alone and after exposure to forskolin, and 50 mM forskolin alone. The magnitude of the response to 10 mM n-amyl acetate applied alone in normal Ringer's solution was taken as unity. Note that the magnitude of the response to 50 μM forskolin was slightly enhanced by ruthenium red while the magnitudes of the responses to 10 mM n-amyl acetate alone and after adaptation to 50 μM forskolin were reduced by ruthenium red (*P < 0.001, **P 0.05 respectively). Points represent the means ± SEM of data obtained from at least five preparations.

to n-amyl acetate. In contrast to the responses to n-amyl acetate alone and after forskolin, slight enhancement of the magnitude of the response to 50 μM forskolin applied alone was observed in the presence of 50 μM ruthenium red.

Discussion

Accessory olfactory bulbar response

In the present study, we measured the accessory olfactory bulbar responses to examine the sensitivity of the vomeronasal system to forskolin and the effects of changes in environmental Ca^{2+} concentration on the accessory olfactory bulbar responses to forskolin and general odorants. Previously, using the whole-cell configuration of the patch-clamp technique, we successfully recorded the responses induced by intracellular dialysis of cAMP (Taniguchi *et al.*, 1996) or IP_3 (Taniguchi *et al.*, 1995) into turtle vomeronasal receptor neurons in slice preparations. We also observed with cell-attached recordings that extracellular application of 10 μM forskolin caused a single turtle vomeronasal neuron to show a transient increase in spike rate (Taniguchi *et al.*, 1996). However, no response to any general odorant was recorded using the whole-cell configuration of the patch-clamp technique. On the other hand, the accessory olfactory bulbar responses to odorants can be measured stably and reproducibly and offer quantitative and reproducible data over a long period (e.g. 3 days).

Vomeronasal response to forskolin and cAMP-dependent pathway in the receptor neurons

The present results indicated that forskolin elicited conspicuously large responses in the turtle vomeronasal system, suggesting the existence of adenylyl cyclase in turtle vomeronasal receptor neurons (Figure 1). This is consistent with previous observations that forskolin increased cAMP level in the vomeronasal tissue membrane preparations of garter snakes and turtles (Luo *et al.*, 1994; Okamoto *et al.*, 1996). Our previous study demonstrated that intracellular application of cAMP into turtle vomeronasal receptor neurons evoked an inward current, which is evidence for the existence of cAMP-activated conductance in the receptor membranes of these neurons (Taniguchi *et al.*, 1996). Therefore, forskolin should accumulate cAMP in the turtle vomeronasal receptor neurons and elicit responses via cAMP-gated channels. Thus, the present results together with above observations suggest that the membranes of turtle vomeronasal receptor neurons possess a sufficient number of cAMP-gated channels to elicit a large response to forskolin.

In the turtle main olfactory system, the response to 50 μM forskolin was increased with an increase in the Ca^{2+} concentration from 0 to 0.1 mM and decreased monotonically with a further increase in Ca^{2+} concentrations up to 80 mM (Kashiwayanagi *et al.*, 1996). The Ca^{2+} dependence of the turtle vomeronasal response to 50 μM forskolin shown in Figure 3(A) was similar to that of the turtle main olfactory response to 50 μM forskolin. Our previous study using whole-cell recording demonstrated that the currents evoked by intracellular dialysis of cAMP into turtle vomeronasal receptor neurons started to appear between 0 and 0.1 mM, increased with increasing cAMP concentration, and reached a plateau at 1 mM. This dose dependence was similar to that in isolated olfactory neurons of the newt (Kurahashi, 1990) and turtle (Kashiwayanagi and Kurihara, 1995). In addition, our previous study revealed that intracellular dialysis of cAMP and cGMP evoked inward currents, the reversal potentials of which were estimated to be 10 mV for cAMP and –4 mV for cGMP, respectively (Taniguchi *et al.*, 1996), suggesting that the two nucleotides act on the same channels as seen in cyclic nucleotide-gated channels in olfactory neurons (Trotier and MacLeod, 1986; Nakamura and Gold, 1987; Suzuki, 1989; Bruch and Teeter, 1990; Firestein *et al.*, 1992). Thus, the present results, together with our previous findings, support the idea that the properties of cyclic nucleotide-gated channels in turtle vomeronasal receptor neurons are similar to those in some of the vertebrate olfactory neurons.

Ca^{2+} dependence of *in vivo* vomeronasal responses to odorants

The responses to hedione and citralva were unchanged by increases in mucosal Ca^{2+} concentration, suggesting that the responses to these odorants are not generated through a cAMP-dependent pathway. On the other hand, the vomeronasal response to geraniol as well as n-amyl acetate was inhibited by high concentrations of Ca^{2+} (Figure 4). In olfactory neurons, the cyclic nucleotide-gated channels were blocked by extracellular Ca^{2+} (Nakamura and Gold, 1987; Kurahashi, 1990; Frings *et al.*, 1991; Zufall *et al.*, 1991). However, the present results do not support the idea that the vomeronasal responses to odorants such as n-amyl acetate and geraniol are generated partly via a cAMP-dependent pathway as follows.

Since the vomeronasal response to forskolin was saturated at concentrations >10 μM (see Figure 2) and the response to the high concentration of forskolin was adapted, the cAMP-mediated pathway is likely to be desensitized

completely. On the other hand, the response to n-amyl acetate remained at >89% of that applied alone even after application of high concentrations of forskolin (Figure 5C), suggesting that the cAMP-dependent pathway does not contribute significantly to the generation of the vomeronasal response to general odorants such as n-amyl acetate. This is consistent with previous results; odorant cocktails did not practically induce cAMP accumulation even in the presence of GTP in turtle vomeronasal epithelium preparations (Okamoto *et al.*, 1996), while these odorants induced accessory bulbar responses.

In previous studies, we showed that the cAMP-dependent odorants induced inward currents in isolated turtle olfactory cells after complete desensitization of the cAMP-dependent pathway by dialysis of a high concentration of cAMP to olfactory cells from the patch pipette (Kashiwayanagi *et al.*, 1994; Kashiwayanagi and Kurihara, 1995). Thus the component via the cAMP-independent pathway exists both in the vomeronasal and olfactory responses and, in the vomeronasal cells, the responses induced by general odorants are fully composed of the component via the cAMP-independent pathway.

Effects of ruthenium red on odor responses

In the garter snake, binding of the chemoattractant ES20 to its receptors was suggested to be coupled with the G-proteins (G_s, G_i and G_o) and increased IP_3 levels (Luo *et al.*, 1994) suggesting the existence of IP_3-mediated transduction pathways in the vomeronasal receptor neurons. We found previously that intracellular application of 0.1 mM IP_3 to turtle vomeronasal receptor neurons elicited an inward current with an increase in membrane conductance with a peak amplitude of 90 pA, which was greatly reduced to 18 pA by bathing the neurons in 10 μM ruthenium red solution (Taniguchi *et al.*, 1995). This demonstrated that the membranes of the turtle vomeronasal receptor neurons possess IP_3-activated conductance which can be blocked by external application of ruthenium red (Taniguchi *et al.*, 1995), as reported elsewhere in olfactory neurons of catfish (Restrepo *et al.*, 1990) and lobster (Fadool and Ache, 1992). As shown in Figure 6, the responses to 10 mM n-amyl acetate alone and after the application of forskolin were significantly reduced by ruthenium red. Hence, it is possible that the IP_3-mediated pathway contributes to the *in vivo* response to odorants such as n-amyl acetate. It is, however, as yet unknown whether general odorants induce elevation of IP_3 level in the vertebrate vomeronasal receptor neurons. The decrease in the magnitude of the response to n-amyl acetate could have been due to non-specific inhibition by ruthenium red. However, this possibility can be excluded because the presence of ruthenium red did not reduce the magnitude of the response to 50 μM forskolin applied alone.

Recently, Halpern *et al* showed that $G_{i\alpha}$ and $G_{o\alpha}$ proteins were localized in vomeronasal neurons of the opossum (1995). An olfactory GCR was shown to exist in the mouse vomeronasal epithelium (Kishimoto *et al.*, 1994), but the main GCRs in the vomeronasal cells were found to be a new family of GCRs that differed from the olfactory GCRs (Dulac and Axel, 1995). mRNA encoding adenylyl cyclase type II and cyclic nucleotide gated channel (II subunit) were also found in the rat vomeronasal epithelium (Berghard and Buck, 1996; Berghard *et al*, 1996). As mentioned above, the vomeronasal response to forskolin was much larger than that to a general odorant. For instance, the magnitude of the vomeronasal response to 50 μM forskolin was five times that to 100 μM citralva (data not shown). In the main olfactory system of the turtle, the magnitude of the response to 50 μM forskolin was much smaller than that to 100 μM citralva (Kashiwayanagi *et al.*, 1994). Behavioral studies have demonstrated that the vomeronasal system plays important roles in the perception of chemical stimuli related to feeding, social and reproductive behavior. These observations support the idea that the cAMP-mediated pathway in the turtle vomeronasal receptor neurons may be involved in signal transduction for special chemical stimuli such as chemoattractants or pheromones.

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