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Adenylyl Cyclase Activity in Turtle Vomeronasal and Olfactory Epithelium

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Many vertebrates have two olfactory systems such as the main olfactory organ and the vomeronasal organ. To compare the transduction mechanism in both systems, we measured adenylyl cyclase activity in turtle vomeronasal and olfactory epithelium preparations. Whereas forskolin and GTP induced cAMP accumulation in vomeronasal preparations, common odorants, which induced cAMP accumulation in olfactory preparations and electrophysiological responses in vomeronasal organs, did not induce cAMP accumulation in vomeronasal preparations. The present results suggest that the cAMP-mediated transduction pathway in the vomeronasal organ is not involved in transduction for common odorants and probably plays a role in perception of specific chemosignals.

Many vertebrates including humans have two olfactory systems such as the main olfactory organ and the accessory olfactory organ called as the vomeronasal organ (1). The vomeronasal organ is a chemosensory system having different roles from the main olfactory system in relating social and sexual behavior and is preferentially sensitive to pheromones and attractants from the prey (2–4). In addition, vomeronasal systems of the turtle and garter snake respond to common odorants as similar to main olfactory systems (5, 6).

In main olfactory systems, common odorants stimulate second messenger pathways involving adenylyl cyclase and phospholipase C via GTP-binding proteins in generation of cAMP and IP_3 (7). The second messengers activated cAMP-gated and IP_3-gated cation channels, respectively (8, 9). Odorants were classified into two types (10, 11); one type of odorants induced cAMP accumulation in rat and bullfrog olfactory cilia preparations and other type of ones did not induce cAMP, but induced IP_3 accumulation. The turtle is a good experimental animal for studies on olfactory mechanisms and many electrophysiological data using the turtle have been accumulated (5, 12–14), but no biochemical data on the second messengers have been available. In the present study, we examined whether classification of odorants on cAMP dependence determined in the rat and bullfrog holds in the turtle. We also examined whether common odorants induce cAMP accumulation in the turtle vomeronasal epithelia preparations. The results showed that any odorant did not induce cAMP accumulation, suggesting that the responses of the vomeronasal receptor cells to common odorants are mediated by cAMP-independent pathways.

METHODS

Preparation of vomeronasal and olfactory membranes. Vomeronasal and olfactory sensory epithelia of turtles (*Geoclemys reevesii*) were isolated immediately after animals anesthetized by cooling to 0°C were killed and soaked in Ringer's solution to remove superficial blood and debris as described by Shirley *et al.* (15). The epithelia in HEPES buffer solution were sonicated at 0°C for 25 min with a sonicator. The suspension was centrifuged at 15000g for 20 min at 0°C. The supernatant was withdrawn. The pellet was used as membrane preparations.

Measurement of cAMP. Membrane preparations (100–200 μg proteins/ml) were incubated at 25°C for 10 min with various agents in the assay solution. cAMP in the supernatant was measured by the sensitive radioimmunoassay procedure as described by Honma *et al.* (16) using the kits for cAMP radioimmunoassay.

Solutions. Ringer's solution contained 116 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES-NaOH (pH 7.4). The HEPES buffer solution contained 250 mM sucrose, 0.1 mM EDTA and 20 mM HEPES-NaOH (pH 7.5). The

Abbreviations: GTPγS, Guanosine 5′-3-*O*-(thio)triphosphate; IBMX, 3-isobutyl-l-methylxanthine.

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assay solution contained 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.3 mM IBMX, 0.5 mM ATP, 5 mM creatine phosphate, 30 unit/ml creatinephosphokinase, 1.5 mg/ml bovine serum albumin and 18.8 mM HEPES-NaOH (pH 7.5). The final concentration of each odorant in cAMP-dependent odorant cocktails I (citralva, hedione, eugenol, *l*-carvone and cineole) and II (*l*-citronellal, geraniol and menthone), which were reported to increase cAMP concentration in rat and bullfrog cilia preparations but unchanged IP_3 concentration in rat ones (10, 11), was 200 μM. The final concentrations of odorants in the IP_3-dependent odorant cocktail, which were reported to increase IP_3 concentration in rat cilia preparations but unchange cAMP concentration in rat and bullfrog ones (10, 11), were 20 μM lilial, 20 μM lyral and 10 μM ethyl vanillin.

RESULTS AND DISCUSSION

First, we measured cAMP accumulation in turtle vomeronasal epithelium preparations in response to forskolin. As shown in Fig. 1a, cAMP accumulation is proportional to forskolin concentration and was not saturated in whole forskolin concentration used. Application of forskolin to turtle olfactory epithelium preparations also induced cAMP accumulation (Fig. 1b). In this case, cAMP accumulation reached a saturation level around 3 to 10 μM forskolin. Similar dose-response relation was electrophysiologically observed in the turtle olfactory bulbar responses to varying concentrations of forskolin (13); the response to forskolin was increased with an increase in forskolin concentration and plateaued at 10–20 μM.

Next, we examined effects of GTP and GTPγS on basal activity of adenylyl cyclase. As shown in Fig. 2a, GTP activated cAMP accumulation in vomeronasal epithelium preparations in a dose-dependent manner. Concentration of GTP to give half-maximal cAMP accumulation was 0.2 μM. The dose-dependence of olfactory epithelium preparations was different from that in vomeronasal ones (Fig. 2b); an increase in GTP concentration rather slowly increased cAMP accumulation to about 0.5 μM, and a second increase appeared from 10 μM to 20 μM. A further increase in GTP concentration suppressed the cAMP accumulation. As shown in Fig. 3, 10 and 100 μM GTPγS increased the activity approximately 12 and 11-fold the basal level in vomeronasal epithelium preparations, respectively, suggesting adenylyl cyclase was activated through the binding of GTPγS to Gs. Increases of cAMP accumulation by GTPγS in olfactory epithelium preparations were smaller than those in vomeronasal ones; 10 and 100 μM GTPγS enhanced 6.5- and 6-fold the basal activity, respectively.

Figure 4 shows cAMP-accumulation induced by odorants in turtle vomeronasal and olfactory epithelium preparations. Application of odorant cocktails without GTP slightly induced cAMP accumulations in olfactory epithelium preparations but these effects were not significant. In the presence of 2 μM GTP, cAMP-dependent odorant cocktails I and II greatly induced cAMP accumulation in olfactory epithelium preparations, while IP_3-dependent odorant cocktail did not practically induce cAMP accumulation. Nevertheless, the magnitudes of current responses to cAMP-dependent odorant cocktail I and IP_3-dependent odorant cocktail were similar in isolated turtle olfactory cells under the voltage-clamp condition (Kashiwayanagi and Kurihara, unpublished

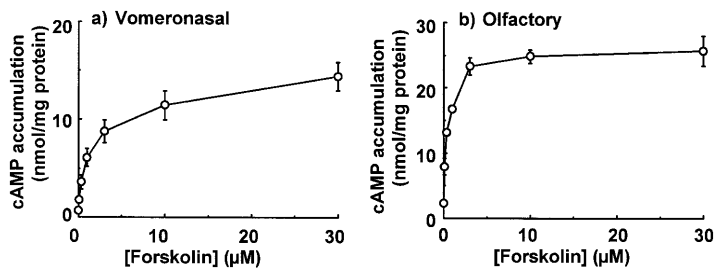

FIG. 1. Effect of forskolin on adenylyl cyclase activities in vomeronasal (a) and olfactory (b) epithelium preparations. Cell membranes were assayed for adenylyl cyclase activity in the presence of varying concentrations of forskolin. Data points and error bars represent the averages and standard errors of the mean of three independent experiments, each consisting of duplicate measurements.

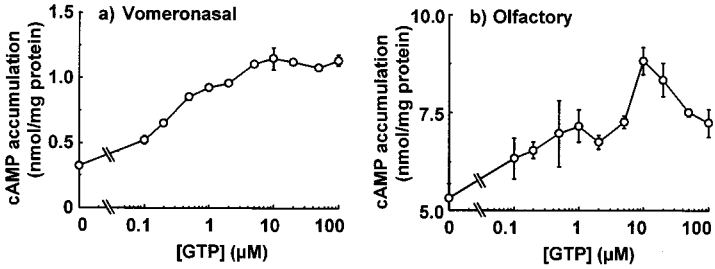

FIG. 2. Effect of GTP on adenylyl cyclase activity in vomeronasal (a) and olfactory (b) epithelium preparations. Data points and error bars represent the averages and standard errors of the mean of triplicate measurements. Similar results were obtained from three independent experiments.

data). This indicated that the IP_3-dependent odorants induce the responses via cAMP-independent pathways in turtle olfactory cells. The present results showed that the classification of the cAMP-dependent and independent odorants, which were determined in the rat and bullfrog, also holds in the turtle.

In vomeronasal epithelium preparations, any odorant cocktails did not practically induce cAMP accumulation even in the presence of GTP (Fig. 4b), while these odorants induced accessory bulbar responses (17). The odorant induced accessory bulbar responses even after complete desensitization of the cAMP-dependent pathway archived by pre-application of high concentrations of forskolin, indicating that common odorants induced responses not via the cAMP-dependent pathway but via cAMP-independent pathways (17).

In previous studies, we showed that the component via cAMP-independent pathways is also contained in the responses of the olfactory cells to cAMP-dependent odorants (13, 14). This was proposed by the fact that the cAMP-dependent odorants induced inward currents in isolated turtle olfactory cells after complete desensitization of the cAMP-dependent pathway by dialysis of high concentration of cAMP to olfactory cells from the patch pipette (13, 14). Thus the component via cAMP-independent pathways exists both in the vomeronasal and olfactory responses and, in the vomeronasal cells the responses induced by common odorants are fully composed of the component via cAMP-independent pathways.

In a separate study, we measured the responses of the turtle olfactory bulb and accessory olfactory bulb to 50 μM forskolin, 0.1 and 0.2 mM citralva (Taniguchi, Kanaki, Kashiwayanagi and Kurihara, unpublished data). The magnitude of the response to 0.1 mM citralva is much larger than that to forskolin in the olfactory bulb, but that to forskolin is much larger than that to 0.2 mM

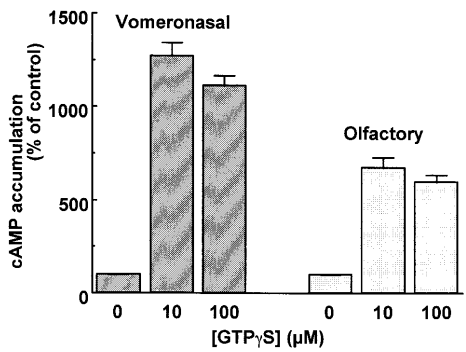

FIG. 3. Effect of GTPγS on adenylyl cycles activity in vomeronasal and olfactory epithelium preparations. Basal activities were taken as 100. Data points and error bars represent the averages and standard errors of the mean of three independent experiments, each consisting of duplicate measurements.

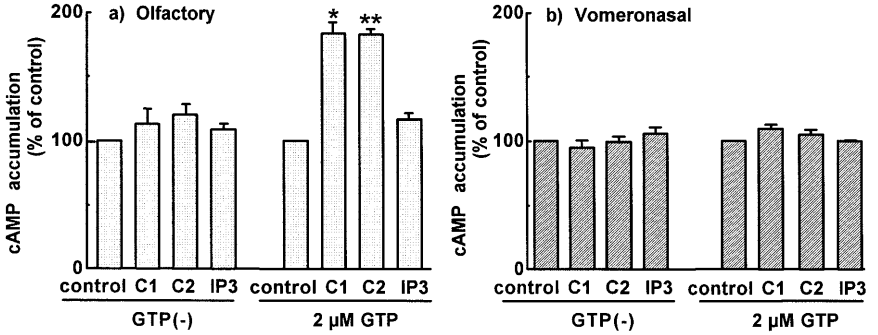

FIG. 4. Effect of odorants on adenylyl cyclase activity in olfactory (a) and vomeronasal (b) epithelium preparations with and without GTP. Basal activities in the absence or in the presence of 2 μM GTP were taken as 100. Data points and error bars represent the averages and standard errors of the mean of three independent experiments, each consisting of duplicate measurements. Cl, cAMP-dependent odorant cocktail I; C2, cAMP-dependent odorant cocktail II; IP3, IP_3-dependent odorant cocktail. *, $P < 0.01$; **, $P < 0.001$.

citralva in the accessory olfactory bulb. In addition, dialysis of the vomeronasal cell with cAMP and IP_3 induced the inward current, suggesting the existence of cAMP- and IP_3-gated cation channels in the cell (18, 19). These results suggest that there are functional cAMP- and IP_3-mediated pathways in the vomeronasal cells as well as the olfactory cells. It is generally considered that the vomeronasal organ is a system to perceive chemosignals emitted by conspecifics or prey (2–4). In garter snake vomeronasal epithelium, a glycoprotein (ES20) extracted from earthworm, which is a chemoattractant for the garter snake, decreased the basal level of cAMP (20). Hence it is possible that the cAMP-mediated pathway in the turtle vomeronasal cell is involved in the transduction of these chemosignals, although such chemicals have not been identified yet.

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