Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants

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The molecular mechanisms mediating the chemo-electrical signal transduction in olfactory receptor cells are still elusive. In this study odor induced formation of second messengers in rat olfactory cilia was monitored in a subsecond time range using a rapid kinetic device. Application of micromolar concentration of citralva induced a rapid, transient elevation of the cyclic adenosine monophosphate level, whereas the concentration of inositol trisphosphate was not affected. In contrast, pyrazine caused a rise in the concentration of inositol trisphosphate, not affecting the level of cyclic adenosine monophosphate. Analysis of the kinetic parameter for the odorant induced reaction indicated that apparently two systems are operating simultaneously. The activating effects of odorants appear to be mediated via different G-proteins. Thus, at least two different second messenger pathways appear to be involved in olfactory signal transduction.
Key words: G-protein/kinetic/olfaction/second messengers/transduction

Introduction

Chemosensory receptor cells located in the olfactory epithelium encode the molecular structure of odorant molecules into afferent neuronal activity. Olfactory receptor cells are bipolar neurons extending an axon into the olfactory bulb and a dendrite toward the nasal lumen. The dendrite tip carries a group of cilia which are supposed to be the sites of initial recognition and transduction events in olfactory reception (Getchell, 1986; Lancet, 1986). These organelles can be detached and isolated from the olfactory epithelium providing an *in vitro* system amenable to biochemical studies of olfactory reception (Anholt *et al.*, 1986; Chen *et al.*, 1986).

In contrast to the well understood reaction cascade underlying the visual signal transduction, little is known about the molecular basis of recognizing specific molecules and the primary events mediating the transduction of the molecular signal into an electrical response. Several lines of evidence suggest that olfactory transduction may involve cAMP as second messenger. Phosphodiesterase inhibitors and cAMP modulate the summated receptor potential elicited by odorants in the olfactory epithelium (Minor and Sakina, 1973) and cAMP-gated ion channels were observed in excised patches of ciliary plasma membranes (Nakamura and Gold, 1987); furthermore, high levels of adenylate cyclase

activity were detected in the olfactory epithelium and this enzyme was found to be stimulated by odorants (Pace *et al.*, 1985; Sklar *et al.*, 1986). However, in these studies it was never demonstrated that the level of cAMP actually changed due to odorant application and it is in particular unclear if cAMP concentrations are changed in the short term range relevant for the transduction process. Furthermore, a number of odorants obviously did not affect adenylate cyclase activity suggesting that besides the formation of cAMP, additional transduction mechanisms may be involved in olfaction (Sklar *et al.*, 1986). In the present study we provide evidence that different odorants induce the formation of different second messengers in rat chemosensory cilia and that changes in second messenger levels occur on a millisecond time scale.

Results

Using a fast kinetic methodology two representatives of different odorant classes were assayed for their potential to induce a rapid (ms) formation of second messengers, putatively involved in the chemo-electrical signal transduction cascade: firstly, citralva (3,7-dimethyl-2-,6-octadiene-nitrile), a substituted terpenoid having a fruity (citrus) odor quality which was found to be one of the most potent stimulants of the odor-sensitive adenylate cyclase in frog olfactory ciliary membranes (Sklar *et al.*, 1986) and secondly, pyrazine, the parent compound of many highly potent odorants, including the bell pepper odorant (2-isobutyl-3-methoxypyrazine). As can be seen in Figure 1A the application of 1 μM citralva induced a rapid formation of cAMP. After 50 ms the level of cAMP was elevated from 50 pmol/mg protein to 460 pmol/mg. Thereafter, the cAMP concentration rapidly returned to the basal level within 100 ms. The concentration of inositol trisphosphate (IP$_3$) was not affected by this odorant. Applying 1 μM pyrazine to a ciliary preparation caused the basal level of IP$_3$ (70 pmol/mg protein) to rise: the concentration of IP$_3$ rapidly increased reaching a peak of 770 pmol/mg protein within 25 ms. The elevated IP$_3$ concentration declined to the basal level within the subsequent 100 ms (Figure 1B). Thus, both odorants induced a fast, transient molecular signal as expected for olfactory signal transduction. However, different second messenger pathways were selectively activated by the fruity odorant citralva and the putrid odorant pyrazine.

In Figure 2 the rapid kinetics of second messenger formation induced by different concentrations of the odorants is demonstrated. It is interesting to note that the onset-kinetic for accumulation of both second messengers is very similar for all concentrations of odorants applied. However, the offset-kinetic is quite different, depending on the concentration of stimulants: at low odorant concentrations (1 μM) the level of the respective second messenger returned to the basal concentration very rapidly; the time course for decaying almost matched that of activation. In contrast, at higher con-
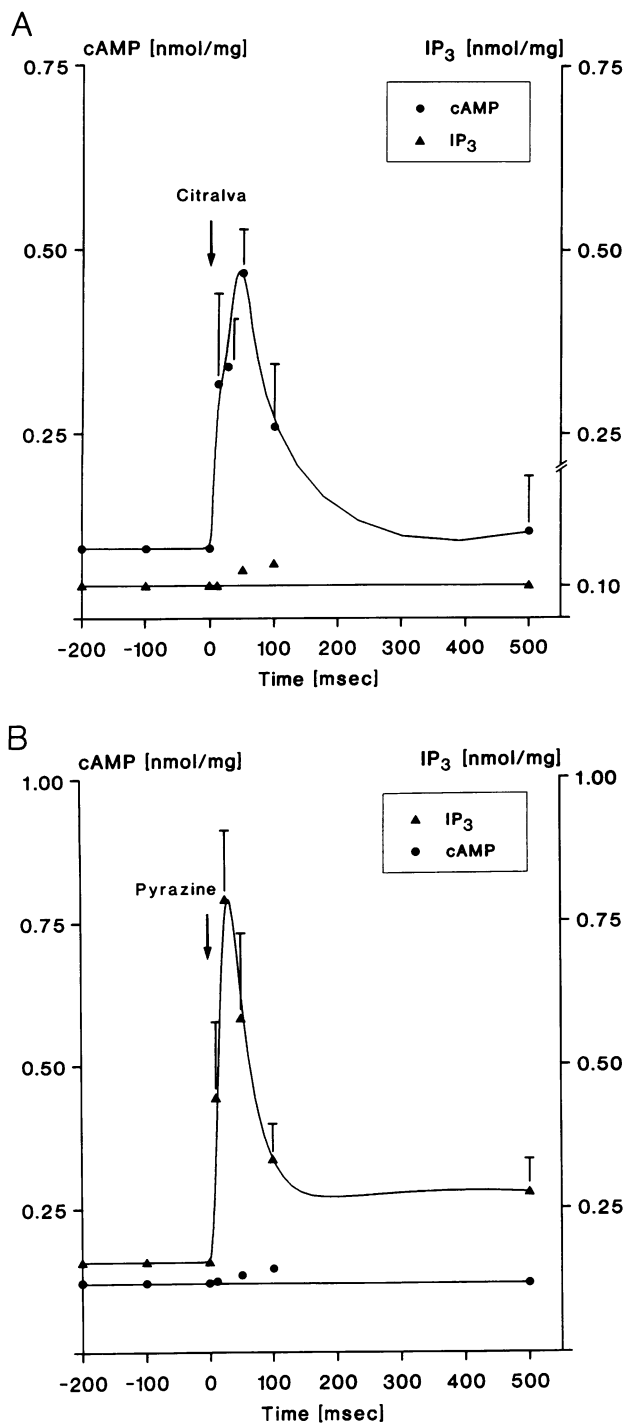
Fig. 1. The odorant dependent increase of second messenger (cAMP, IP₃) concentrations in rat olfactory cilia measured in rapid time course experiments. Samples of olfactory cilia preparations from rat were incubated with micromolar concentrations of odorants and the reactions were quenched after appropriate incubation intervals by addition of perchloric acid. Data are the mean of 3−5 experiments ±SD. (A) Application of 1 μM citralva (arrow) to olfactory cilia induced an immediate acculumation of cAMP reaching a maximum level after 50 msec; thereafter the elevated cAMP concentration rapidly decayed, returning to the basal level within a few hundred ms. The concentration of IP₃ in the cilia was not affected by citralva. (B) Stimulation of olfactory cilia with 1 μM pyrazine caused a rise in IP₃ concentration; a peak level was detected already after 25 ms. Subsequently the level rapidly decayed. The concentration of cAMP was not changed by pyrazine.
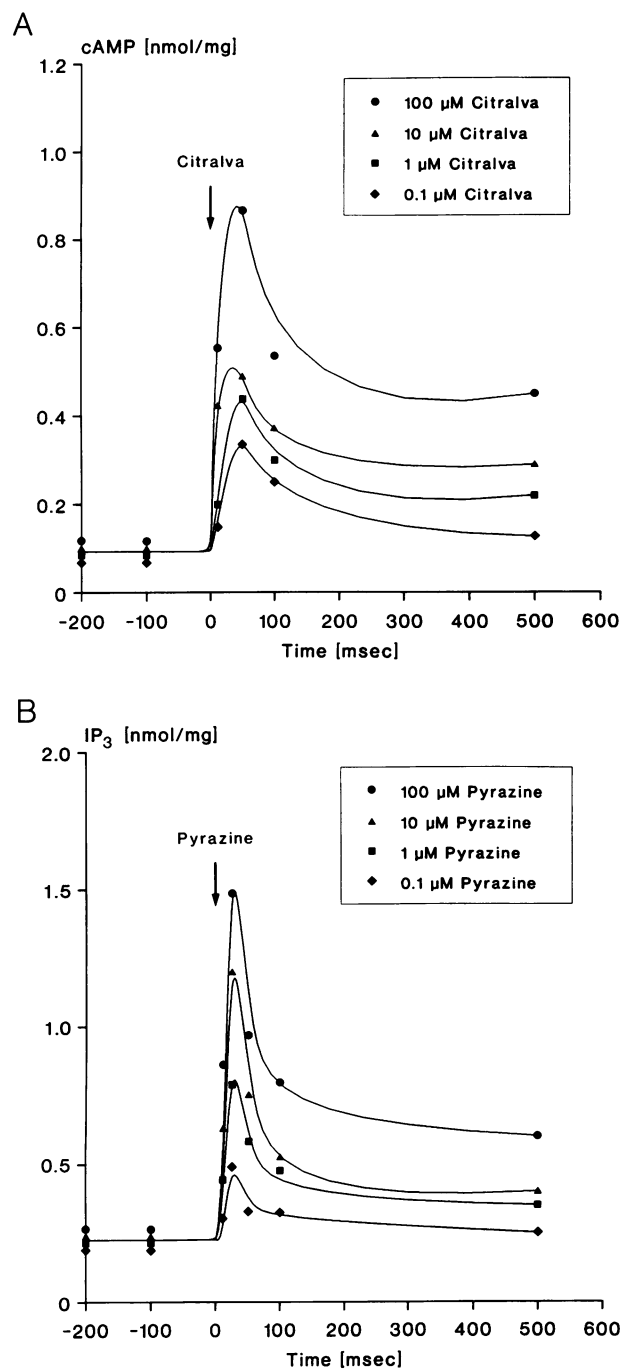
Fig. 2. Time course of second messenger formation and decaying after application of four different concentrations of odorants. Note: although the peak level of cAMP is clearly concentration dependent, the time intervals to reach the maximal concentration is the same at all concentrations (cAMP: 50 ms; IP₃: 25 ms). However, the decay time was considerably prolonged at higher concentrations of stimulants. (A) Formation of cAMP induced by different concentrations of citralva. (B) Changes of the IP₃ level induced by different concentrations of pyrazine.

centrations the decay time of the elevated level of cAMP and IP₃ was considerably prolonged.

The maximal level of cAMP induced by citralva after 50 ms or the maximal level of IP₃ induced by pyrazine (after 25 ms), was in each case proportional to the odorant concentration actually applied. However, even at the highest
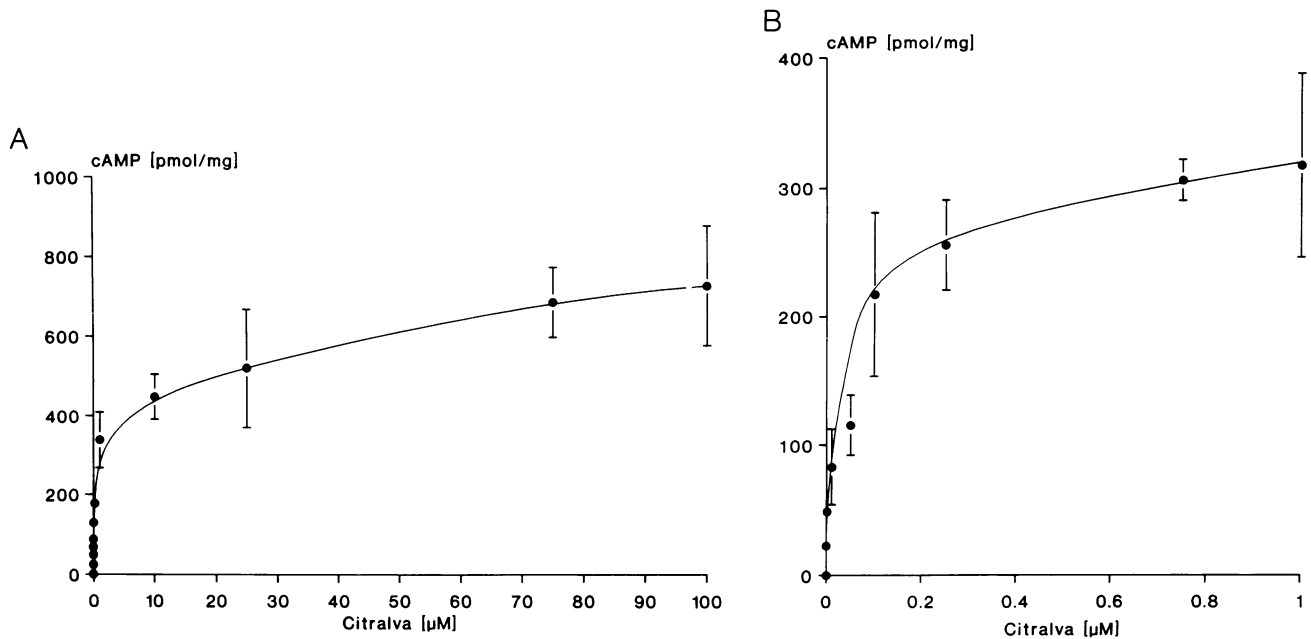
Fig. 3. Dose—response curve of cAMP formation in rat olfactory cilia induced by citralva. The concentration of cAMP was determined 50 ms after stimulus application. Data are the mean of 3—5 determinations ±SD. (**A**) Formation of cAMP induced by citralva monitored over a large concentration range. A concentration range of 0.01—100 μM is displayed. (**B**) Elevation of cAMP levels induced by low concentrations of citralva. A concentration range of 100 pM—1 μM is displayed.

odorant concentration, the second messenger level never reached the maximal value that could be induced by the stable GTP analog, GTPγS (cAMP: 1503 ± 510 pmol/mg protein; IP$_3$: 1529 ± 326 pmol/mg protein).

Monitoring the stimulant-induced formation of second messengers over a large concentration range allowed the construction of dose-response curves (Figure 3). The lowest concentration at which a significant response could be detected was 1 nM. The amount of cAMP determined 50 ms after application of citralva increased with increasing stimulant concentration. The reaction showed an apparent saturation at ~1 μM citralva (Figure 3B). However, at much higher odorant concentrations (1—100 μM) there was a further increase in activity, but with a quite different rate (Figure 3A) suggesting the existence of two systems which may have different affinities for the stimulant. Kinetic analysis of these data using the double reciprocal plot procedure (Lineweaver and Burke, 1954) gave points which were curvilinear in distribution, which suggests that two systems of different affinities were operating simultaneously. This situation is not uncommon in biological systems and a good example is the phosphorylation of glucose by two hexokinases of different affinities in rat liver homogenates (Walker, 1963).

Graphical estimates of the kinetic constants for cAMP formation induced by citralva have accordingly been made on the assumption that two systems are operating and using Walker's (1963) mathematical treatment. At low odorant concentration the low affinity system can be assumed to make a negligible contribution and the kinetic constants of the high affinity system, K_{m1}, V_{max1}, may be determined directly from the experimental results (Figure 3B). At higher odorant concentrations both systems contribute to the formation of cAMP and the graphically determined values for K_m and V_{max} represent combined values designated K_{mc} and V_{maxc}. Values for K_{m2} and V_{max2}, the constants for the low affinity

system, can be calculated from the knowledge of K_{m1}, V_{max1}, K_{mc} and V_{maxc} and the following equations: $V_{max2} = V_{maxc} - V_{max1}$ and $K_{m2} = V_{maxc} \times K_{mc}/V_{max2}$. This treatment of the kinetic analysis gave the following kinetic constants: $K_{m1} = 50$ nM, $K_{m2} = 6.2$ μM, $V_{max1} = 280$ pmol/mg/50 ms, $V_{max2} = 440$ pmol/mg/50 ms. After replotting the values of the dose—response curve in a Hofstee diagram (Hofstee, 1959) the data points could be fitted by two straight lines, confirming the existence of two systems with different affinities (Figure 4). The affinity constants obtained by this analysis are consistent with the results obtained by the procedure of Walker (1963).

The shape of the dose—response curve for the pyrazine induced formation of IP$_3$ was reminiscent of the previous one. Analysis of the data by Walker's procedure (1963) supported the existence of two activating systems having widely different K_m values: $K_{m1} = 18$ nM, $K_{m2} = 2.2$ μM, $V_{max1} = 360$ pmol/mg/25 ms, $V_{max2} = 1040$ pmol/mg/25 ms. Thus, an odorant may activate its second messenger pathway via high and low affinity routes.

In order to approach the question of whether the rapid accumulation of second messengers induced by odorants was receptor mediated via specific GTP binding proteins the effects of bacterial toxins which are known to modify specific G-proteins (Gilman, 1987) were analyzed. As can be seen from Figure 5, cholera toxin, which persistently activates the stimulating G-protein of the adenylate cyclase, the G$_s$-protein, induced an accumulation of cAMP in olfactory cilia preparation but had no effect on the IP$_3$ level. On the other hand, pertussis toxin which modifies and inactivates the G$_i$- and G$_o$-proteins affected neither the levels of cAMP nor the basal level of IP$_3$. However, the accumulation of IP$_3$ induced by pyrazine was completely prevented (Figure 5B). These observations suggest the possibility that the formation of cAMP induced by citralva may be mediated by G$_s$-
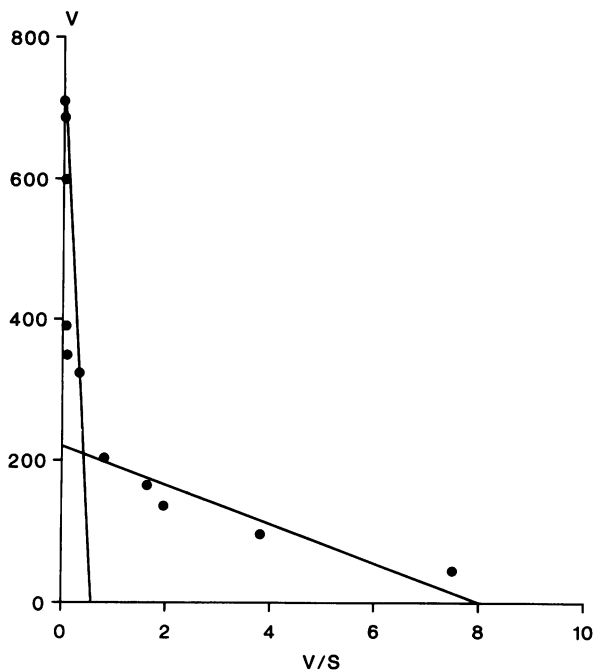
Fig. 4. Hofstee plot of the odorant induced cAMP formation in rat olfactory cilia. The Y axis (V) represents the rate of cAMP formation (pmol/mg protein/50 ms); the X axis represents the quotient of the enzyme rate and the odorant concentration (S). Y intercepts represent the V_{max} and the slopes of the lines the K_D values. Two profoundly different systems can be distinguished by virtue of an apparently different affinity for citralva.

proteins and indicate that a pertussis toxin sensitive G-protein is involved in the reaction cascade activated by pyrazine.

Discussion

There is some circumstantial evidence that the chemo-electrical signal transduction in olfactory receptor cells is mediated via membrane receptors and brought about by second messenger cascades (Minor and Sakina, 1973; Pace *et al.*, 1985; Lancet *et al.*, 1986a,b; Sklar *et al.*, 1986; Nakamura and Gold, 1987). It has been demonstrated that the adenylate cyclase of olfactory cilia is activated by odorants (Pace *et al.*, 1985; Sklar *et al.*, 1986) with a potency that correlates with their effectiveness in electro-olfactogram recordings (Lowe *et al.*, 1989). Furthermore, ion channels gated by cyclic nucleotides have been detected in olfactory neurons (Nakamura and Gold, 1987). However, it was always a controversy whether the odorant induced formation of cyclic nucleotides is fast enough to cause the permeability changes responsible for the electrical responses recorded by microelectrodes. However, the response latency of several hundred milliseconds lend support to the concept that second messenger systems are involved in the transduction process (Firestein and Werblin, 1989).

The application of a rapid kinetic methodology in this study has now allowed the demonstration that the odorant induced formation of second messengers in olfactory cilia occurs in the millisecond time range and thus clearly precedes the generator current of the receptor cells which can be recorded only after a latency of 150−500 ms (Firestein and Werblin, 1989). Furthermore, the data presented in this paper indicate that the odorant induced accumulation of second messengers exhibit a short-term transient nature. At low stimulus con-
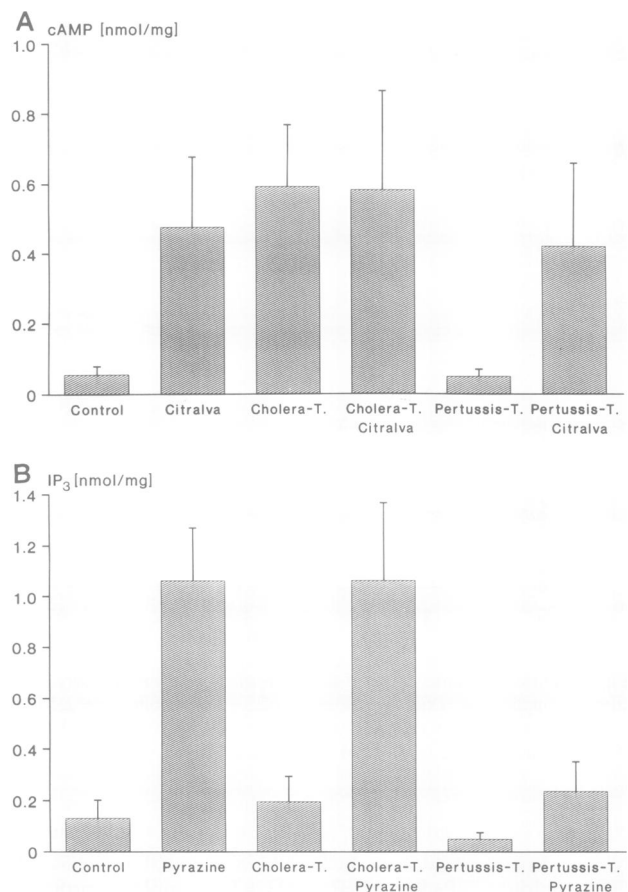

Fig. 5. Effects of bacterial toxins on the endogenous and odorant induced levels of cAMP and IP$_3$ in cilia preparations from rat olfactory epithelia. Olfactory cilia were incubated with pre-activated cholera toxin or pertussis toxin for 15 min at 37°C followed by 15 min at 4°C. Untreated and pretreated cilia were assayed for cAMP (**A**) and IP$_3$ (**B**) with and without stimulation with odorants. The concentration of IP$_3$ was determined after 25 ms; cAMP was estimated 50 ms after stimulus application. Data are the mean of 3−5 determinations ± SD.

centrations the increased second messenger level decayed within 100 ms. Such a transient nature of the molecular signals involved in the transduction process is essential for receptor cells, like olfactory neurons, which can repeatedly be stimulated.

Compared with the adenylate cyclase measurements (Pace *et al.*, 1985; Sklar *et al.*, 1986) it is interesting to note that at the short-time range measured in this study the molecular response of the olfactory cilia can be detected at a much lower, more physiological concentration of odorants (Dionne, 1988).

Besides the rapid kinetic of the odorant induced accumulation of second messengers it is interesting to note that different odorants obviously activate different second messenger pathways in rat olfactory cilia. Based on the results of adenylate cyclase measurements it has been suggested that odorants may be categorized into two groups, those that stimulate the olfactory adenylate cyclase and those that have no effect on the enzyme. From these observations it has been concluded that weak cyclase activators may act via a different transduction pathway (Sklar *et al.*, 1986), however, it has also been argued that non-detectable adenylate cyclase activation in the test tube may reflect poor physiological odorant potency (Lancet *et al.*, 1989). The data

presented in this paper clearly favor the first alternative; pyrazine was found to be unable to activate adenylate cyclase (Sklar *et al.*, 1986) and did not change the concentration of cAMP (Figure 1), however, this compound was very potent in inducing the formation of IP_3 (Figure 1). Thus, in olfactory receptor cells of rats at least two alternative second messenger pathways can be activated by different odorants. This conclusion is supported by the results of ongoing studies, analyzing a large number of different odorants. Each odorant tested activated only either cAMP of IP_3. The selective stimulation of one second messenger pathway or the other by odorous molecules, as demonstrated for citralva and pyrazine in this study, thus, appears to be a more general phenomenon.

This observation may be taken as a first indication that the transduction machinery in the olfactory system of vertebrates may be more complex than for example in insects. The activity of adenylate cyclase in antennal preparation from various insect species was found to be very low and furthermore all pheromones and odorants analyzed so far were found to stimulate the phospholipase C and induce the accumulation of IP_3 (Boekhoff *et al.*, 1990; Breer *et al.*, 1990). At present it is unclear if even more transduction mechanisms are involved in vertebrate olfactory transduction, like the cGMP pathway or direct ion channel gating. Studies in progress exploring the effect of various odorants from different classes may contribute to the elucidation of the physical or chemical cues of odorant molecules responsible for activating alternative second messenger pathways.

Evaluation of the kinetic parameters for the odorant induced formation of second messengers revealed that two systems of widely different affinities are operating simultaneously. A high affinity system with a K_m values in the nanomolar range and a low affinity system having affinities in the micromolar range. Although the physiological implications of these observations are presently unclear, the data support the view that olfactory receptor cells may be equipped with specific receptor molecules which recognize odorous compounds with particular chemical structure or physicochemical properties. In addition, at higher concentrations odorants may be able to activate receptor molecules which are tailored for compounds that are structurally different but related.

The kinetic data for the odorant induced formation of second messengers, cAMP as well as IP_3, indicate that the reaction cascades involved in these processes do not show any kind of cooperativity. This observation is of particular interest in view of the recent findings that dose—response curves of odorant induced electrophysiological phenomena, like the generator current and the receptor potential, gave a Hill coefficient of ~ 2.7 (Firestein and Werblin, 1989); these data clearly indicate a positive cooperativity of the whole transduction process. The non-cooperativity of the second messenger formations suggests that a reaction further down the cascade provides the cooperativity of the whole reaction. In this context it is interesting to note that for the gating of cation channels in the plasma membranes of olfactory cilia a positive cooperativity has been observed (Nakamura and Gold, 1987) which is reminiscent of the cooperativity of cGMP gated channels in the membranes of rod outer segments (Cook *et al.*, 1987).

Bacterial toxins, such as cholera toxin and pertussis toxin, are known to modify specific G-proteins via ADP ribosylation and are proven to be valuable tools in evaluating the type of G-protein involved in particular signal transduction cascades (Gilman, 1987). The results of experiments determining the formation of cAMP and IP_3 induced by citralva or pyrazine in the presence of these two toxins indicate that the activation of different second messenger pathways by odorants is mediated by specific G-proteins (Figure 5): G_s-proteins appear to be involved in the formation of cAMP (Pace *et al.*, 1985) whereas pertusssis toxin sensitive G-proteins (G_o or G_i) mediate the odorant induced accumulation of IP_3. As all bacterial toxin sensitive G-proteins appear to be stimulated via ligand activation of specific receptor proteins, the toxin sensitivity of second messenger pathways in the olfactory ciliary membranes supports the concept that odorants are recognized and perceived by specific membrane receptor proteins which upon activation stimulate intracellular effector systems via specific G-proteins (Dodd and Persaud, 1981).

Materials and methods

Materials

Sprague−Dawley rats were supplied by the 'Zentralinstitut für Versuchstierzucht−Hannover'. cAMP and IP_3 radioligand assay kits were purchased from Amersham. Cholera toxin was received from Sigma; pertussis toxin from List Biological Laboratories. Citralva was generously provided by International Flavors and Fragrances (Union Beach, NJ); pyrazine was obtained from Sigma. Odorants were dissolved in DMSO giving a stock solution of 100 mM. Different volumes were added to the stimulant buffer to obtain the approximate final odor concentrations. Both solutions were thoroughly mixed in an ultrasonic water bath at 37°C and used immediately. Controls were performed using the highest DMSO concentration (never exceeding 0.05%); at this concentration DMSO did not affect the reactions.

Isolation of olfactory cilia

Cilia preparation from rat olfactory epithelia were detached and isolated as described by Anholt *et al.* (1986) and Chen *et al.* (1986). All operations were performed at 0−4°C. Rat olfactory epithelia were dissected and washed in Ringer solution (120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 7.5 mM glucose, pH 8.0). The olfactory cilia were detached by a calcium shock procedure raising the Ca^{2+} concentration in the Ringer solution to 10 mM. After agitating in an end-over-end shaker (20 min, 4°C) the deciliated epithelia were removed by centrifugation for 15 min at 6000 *g*. The supernatant was collected and the pellet was incubated again with a solution containing 10 mM $CaCl_2$ for 20 min. The deciliated epithelia was precipitated by centrifugation and the supernatants containing the detached cilia were combined. Cilia were isolated by centrifugation of the supernatant for 15 min at 12 000 *g*. The resulting pellet containing the olfactory cilia was washed twice in 10 mM Tris−HCl, 3 mM $MgCl_2$, 2 mM EDTA, pH 8.0. Samples of concentrated cilia were suspended in a small volume and stored at −70°C.

Measurement of short term changes in cAMP and IP_3 concentrations using a rapid quench device

A rapid quench device equipped with three syringes was utilized to measure subsecond changes in cAMP and IP_3 concentration in olfactory cilia preparations. Syringe I contained the olfactory cilia (50−100 μg protein/ml) in 70 μl buffer solution (10 mM Tris−HCl, pH 8.0, 3 mM $MgCl_2$, 2 mM EDTA). Syringe II was filled with 360 μl buffer composed of 200 mM NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM $MgCl_2$, 1 mM DTT, 0.05% Na-cholate, 1 mM ATP, 1 μM GTP and $CaCl_2$ to give a free Ca^{2+} concentration of 0.02 μM containing odorants at appropriate concentrations. All solutions were adapted to 37°C. Syringe III contained perchloric acid (10%) cooled to 0°C. The reaction was started by mixing the contents of syringes I and II. After an appropriate incubation time the reaction was stopped by quenching the sample by ejecting 350 μl perchloric acid from syringe III. The quenched samples were collected and cooled on ice before analysis for second messenger concentrations. The equipment used was capable of quenching samples at time intervals ranging from 8 to 500 ms. The activation of the three different syringes was controlled by an IBM AT computer.

Determination of cAMP and IP₃ concentration

The quenched and cooled samples were vortexed and centrifuged for 5 min at 2500 g at 4°C. 400 μl of the supernatants were transferred to a separate tube containing 100 μl of 10 mM EDTA (pH 7.0). The samples were neutralized by adding 500 μl of a 1:1 (v/v) mixture of 1,1,2 trichlorotrifluoroethane and tri-n-octylamine, followed by thorough mixing. After centrifugation for 2 min at 500 g three phases were obtained; the upper phase, which contained all water soluble components, was used for estimating the concentrations of cAMP and IP₃. The concentration of cAMP was determined following the procedure of Steiner et al. (1972). IP₃ was estimated according to the procedure of Palmer et al. (1989).

Before determining the effect of bacterial toxins, pre-activated promoters of cholera toxin and pertussis toxin were prepared by incubating cholera toxin (300 μg/ml) for 30 min at 37°C in 15 mM Tris—HCl (pH 7.5) containing 25 mM dithiothreitol and pertussis toxin (100 μg/ml) with 1 mM ATP and 20 mM dithiothreitol for 30 min at 37°C. Cilia preparations (100 μl) were pre-incubated with either 20 μl pre-activated cholera toxin (30 μg/ml) or pre-activated pertussis toxin (1 μg/ml) in the presence of 1 mM NAD for 15 min at 37°C followed by 15 min at 4°C.

Protein concentration of the cilia preparation was measured according to the method of Bradford (1986) using bovine serum albumin as standard.

Data are expressed as nmol (pmol) of cAMP or IP₃ per mg protein.

Acknowledgements

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