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Odorant-sensitive adenylate cyclase: Rapid, potent activation and desensitization in primary olfactory neuronal cultures

(olfaction/cell culture)

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ABSTRACT **Using primary olfactory neuronal cultures, we have demonstrated rapid, potent increases in cAMP levels and adenylate cyclase [AC; ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in response to odorants. Isobutylmethoxypyrazine is active at 1 nM. Odorant enhancement is dependent on Ca^{2+} concentration with maximal effects at 10–100 μM. Biphasic temporal and concentration-related effects occur with all odorants. All odorants examined elicit desensitization with AC responses abolished when odorants are reapplied immediately after removal. When reapplied 1 min after removal, odorants elicit an AC response greater than on first exposure, implying a cellular "memory" for odorants.**

Olfactory signal transduction in mammals appears to involve an odorant-sensitive adenylate cyclase [AC; ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] (1–3). Recently, Breer and associates (4) have used rapid mixing techniques to demonstrate potent enhancement of cAMP levels in isolated preparations of rat olfactory cilia. Cellular disruption may interfere with receptor–effector coupling, especially in the AC system (5). To evaluate olfactory signal transduction in intact cells, we have developed a primary culture of highly enriched neonatal rat olfactory neurons (6). Using this system, we now demonstrate potent and rapid increases in cAMP levels and AC activity by odorants. We also demonstrate desensitization of the AC response by odorants.

METHODS

Primary cultures of rat olfactory neurons were prepared as described (6). RIA of cAMP levels was performed with an RIA kit (NEN/Dupont), with modifications as described (6). To assay cells for AC activity *in situ*, staphylococcal α-toxin (7, 8) was used to permeabilize cells. Monolayers were rinsed with phosphate-buffered saline and the normal culture medium was then replaced with an intracellular buffer (ICB) containing 150 mM KCl, 20 mM Tris·HCl, 1 mM $MgCl_2$, a variable concentration of free calcium, and 100 units of α-toxin per ml at a final pH of 7.4. Cells were treated with ICB containing α-toxin for 30 min prior to experimentation. This concentration was found to permeabilize >90% of cells by trypan blue. The concentration of free calcium in each experiment was measured by a calcium electrode (Biomedical Instrumentation Group, Philadelphia), using solutions containing known amounts of free calcium as standards (W-P Instruments, New Haven, CT). All odorants were prepared freshly as a 50 mM stock in absolute ethanol each day of experimentation. Odorants were obtained from International Flavors and Fragrances (Union City, NJ).

AC was assayed by a modification of the method of Salomon (9). Briefly, cell monolayers that had previously received 0.25 ml of ICB containing α-toxin were exposed to 0.25 ml of ICB with or without various concentrations of odorants. At intervals of 0–60 sec thereafter, or as dictated by individual protocol, the medium was aspirated and replaced with ICB containing 5 mM magnesium acetate, 100 μM ATP, 100 μM GTP, 500 μM cAMP, 1 mM dithiothreitol, phosphocreatine at 2 mg/ml, creatine phosphokinase and [α-^{32}P]ATP at 1 mg/ml (NEN/DuPont). After 15 sec, the reaction was stopped with solution containing ATP (20 mg/ml), 2% (wt/vol) sodium dodecyl sulfate, and 1.3 mM cAMP. [^{3}H]cAMP was used as a tracer in the stop solution to calculate recovery. Cell monolayers were then scraped and the material was processed as described (9).

RESULTS

Odorant Effects on cAMP Levels. Odorants potently and rapidly increase cAMP levels in olfactory neuronal cultures (Fig. 1). Olfactory neuronal cells exposed to isobutylmethoxypyrazine (IBMP) demonstrate a slight increase at 1 nM odorant (Fig. 1*A*). At 10 nM, cAMP levels double with a further increase at 100 nM. Odorant responses progressively decline at 1 and 10 μM but increase somewhat at 100 μM. The response of cAMP levels to odorants is rapid, with increases evident at 20 sec and maximal enhancement at 1 min, while levels at 2 min are substantially lower. At most concentrations of odorants, cAMP levels return to baseline levels by 2–5 min.

Similar patterns of response are apparent for citralva (Fig. 1*B*) and isovaleric acid (Fig. 1*C*). For isovaleric acid, a small increase in cAMP is detected at 1 nM with a doubling at 10 nM and more than a tripling at 100 nM. cAMP responses then decline at 1 μM, but, at 10 μM, cAMP levels are as high as those observed at 100 nM. At 100 μM isovaleric acid, cAMP responses again decline. With citralva, a 50% increase in cAMP levels occurs at 1 nM with a greater response at 10 nM and a maximal 2.5-fold increase at 100 nM. As with IBMP and isovaleric acid, citralva responses decline at 1 μM. A further decline occurs for citralva at 10 μM, but cAMP levels are again increased at 100 μM.

Thus, with IBMP, isovaleric acid and citralva biphasic dose–response curves are apparent with one peak of cAMP augmentation at 10–100 nM and another peak at 10–100 μM. All three odorants display a similar time course.

D-Carvone and coniferan are less potent stimulants of cAMP levels. With both of these odorants, cAMP increases are first apparent at ≈1 μM and maximal increases are only 50–100%. However, the time course of responses for D-carvone and coniferan is similar to the other three odorants.

Odorant Effects on AC. The rapid increases and decreases in cAMP levels in olfactory neuronal cultures and in isolated cilia (4) are consistent with an activation of AC and possibly

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Abbreviations: AC, adenylate cyclase; IBMP, isobutylmethoxypyrazine.

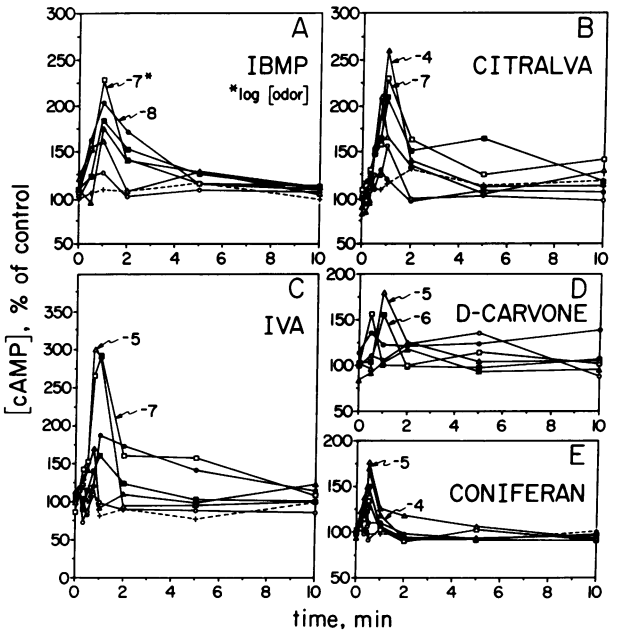

FIG. 1. Effect of odorants on intracellular cAMP levels in cultures of rat olfactory neurons. Cell monolayers were exposed to medium containing odorants at concentrations ranging from 10^{-10} to 10^{-4} M. At various times thereafter, the incubation mixture was quenched with trichloroacetic acid and cells were processed. The amount of cAMP present is shown as percentage of control. Odorants used were IBMP (*A*), citralva (*B*), isovaleric acid (IVA) (*C*), D-carvone (*D*), and coniferan (*E*). The level of cAMP at each time point is shown at odor concentrations of 10^{-10} M (+), 10^{-9} M (○), 10^{-8} M (●), 10^{-7} M (□), 10^{-6} M (■), 10^{-5} M (△), and 10^{-4} M (▲).

a subsequent deactivation of the enzyme. Since cellular disruption might interfere with such a complex system, we conducted our AC assays in the intact cells. The cells were treated for 30 min with α-toxin to facilitate entry of radiolabeled ATP and other reaction components. In preliminary experiments, odorants elicited rapid effects on AC no longer evident after a few minutes. To detect such transient alterations of AC, enzyme activity was assayed with incubation times of 15 and 30 sec. With this brief incubation time, enzyme activity is linear between 15 and 30 sec. Amounts of [^{32}P]cAMP formed in typical experiments increase from 100 cpm at time 0 to ≈900 cpm at 15 sec and 1800 cpm at 30 sec. Enzyme activity is stimulated by both GTP[γS] and forskolin.

In our earlier studies of AC in olfactory cilia, free calcium levels were submicromolar, while Breer and associates (4) maintained a free calcium concentration in cilia at 0.02 μM. With calcium levels of 0.1 μM or less, we detect very low AC activity in intact cells and no evidence of stimulation by the potent odorant IBMP (Fig. 2). With 10 and 100 μM free calcium, we observe a striking stimulation of AC by IBMP. The stimulation is biphasic with a peak response at 10–100 nM IBMP, a lesser increase with 1 μM, and a subsequent further increase at 10 and 100 μM. All subsequent experiments have used 100 μM free calcium.

We evaluated the response of AC to several odorants at seven concentrations of odorants and at six time intervals (Fig. 3). At 0.1 nM, IBMP does not affect AC, while at 1 nM a 4-fold stimulation is evident at 20 sec. Maximal 6- to 7-fold stimulation of AC activity occurs with 10–100 nM IBMP at 10 sec. AC activity declines at 1 μM IBMP and increases again at 10 and 100 μM. A suggestion of two temporal phases for IBMP responses is evident with effects of lower concentrations of IBMP occurring earlier than effects of higher concentrations.

The biphasic time course in odorant effect also takes place with isovaleric acid and citralva. Citralva (Fig. 3*B*) displays

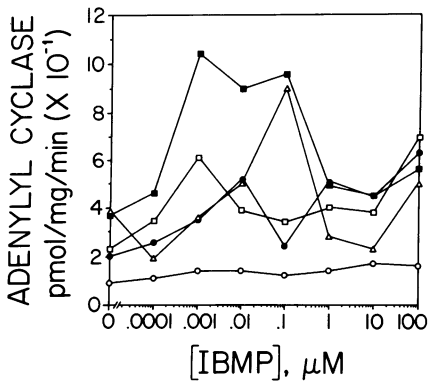

FIG. 2. Effect of calcium concentration on basal and odorant-stimulated AC activity. Cell monolayers were pretreated with ICB containing 100 units of α-toxin per ml for 30 min. At the end of that preincubation, cells were exposed to ICB containing various calcium concentrations at a variety of concentrations of IBMP. At various times thereafter, the ICB was aspirated and replaced with a reaction mixture containing [^{32}P]ATP. After incubation for 30 sec, the reaction mixture was quenched with stop buffer. AC activity is represented as pmol of cAMP formed per mg of protein in the reaction mixture per min. Free calcium concentrations used were 0.1 μM (○), 1 μM (●), 10 μM (□), 100 μM (■), and 1 mM (△).

an even more complex, multiphasic concentration–response pattern. Stimulation of enzyme activity is detected at 1 nM, with a lesser response at 10 nM, a large increase of enzyme activity at 0.1 and 1.0 μM, followed by another decline at 10 μM, but an increase at 100 μM. The time course of citralva responses is also complex, with clearly discernable and

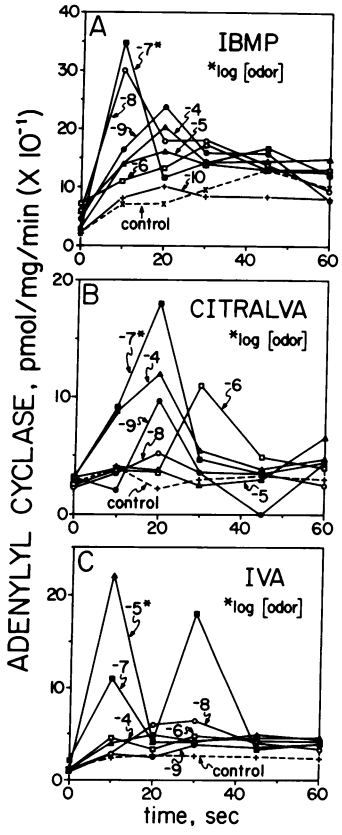

FIG. 3. Effect of odorants on AC activity. Cell monolayers were treated with α-toxin and exposed to odorants, and AC activity was monitored. Odorants used were IBMP (*A*), citralva (*B*), and isovaleric acid (IVA) (*C*). Odorant concentrations are none added (×), 10^{-10} M (+), 10^{-9} M (●), 10^{-8} M (○), 10^{-7} M (■), 10^{-6} M (□), 10^{-5} M (▲), and 10^{-4} M (△).

differentiable peaks at 10 and 30 sec. Isovaleric acid (Fig. 3*C*) is less potent that IBMP with no AC activation at 1 nM and only a modest increase at 10 nM. At 100 nM, a biphasic increase in enzyme activity is observed with peaks at 10 and 30 sec. As with IBMP, 1 μM isovaleric acid produces a lesser response, while a further augmentation of enzyme activity is evident at 10 μM with another decline at 100 μM.

For D-carvone and coniferan, both potency and magnitude of response of AC are less than with the other odorants (Fig. 4). Some activation of the enzyme occurs with 10 nM D-carvone, while no increase of enzyme activity is detected with <100 μM coniferan.

Desensitization of Odorant Responses. One of the most striking aspects of olfaction is the rapid desensitization to odorants in both humans and animals (10, 11). To evaluate possible desensitization at the receptor level, we exposed neuronal cultures to odorants for 60 sec, removed the odorants, and reapplied them either immediately, after 1 min (Fig. 5), or after 5 min (data not shown). With IBMP, immediate reexposure to odorants produces a pronounced reduction in AC response at all odorant concentrations examined. When the cells are reexposed to IBMP 1 min after removal of the odorant, the resultant increase in AC activity is substantially greater than with the first application of odorant. By contrast, 5 min after the initial exposure the enzyme response to odorants is the same as with the initial exposure.

We have also evaluated desensitization to isovaleric acid, D-carvone, coniferan, and citralva (data not shown). With these odorants, enzyme response was only evaluated after immediate reexposure to odorant. With all four of these odorants, a marked decline in response is evident upon reexposure.

DISCUSSION

In the present study, we detect rapid and potent stimulation of AC in olfactory neuronal cultures with several odorants. Odorant response cannot be detected at calcium concentrations <1 μM, $\approx$10 times higher than physiologic intracellular calcium and similar to levels of calcium that occur after activation of the inositol phospholipid second messenger

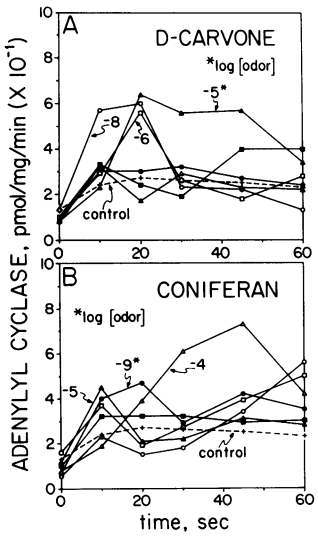

FIG. 4. Effect of less potent odorants on AC activity. Cell monolayers were treated with α-toxin and exposed to odorants, and AC activity was monitored. Both D-carvone (*A*) and coniferan (*B*) were found to stimulate AC to a lesser degree in generally higher concentrations than other odorants tested. Odorant concentrations shown include no added odor (control) (+), 10^{-9} M (●), 10^{-8} M (○), 10^{-7} M (■), 10^{-6} M (□), 10^{-5} M (▲), and 10^{-4} M (△).

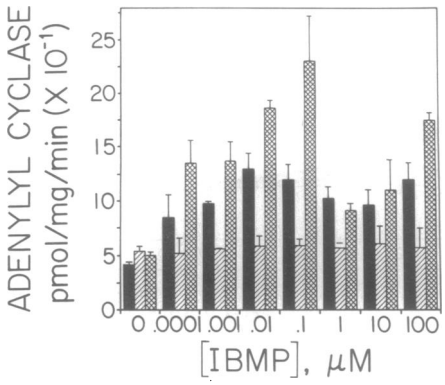

FIG. 5. Desensitization of the AC response upon reexposure of cells to IBMP. Cell cultures were pretreated with α-toxin. At time 0, monolayers were exposed to various concentrations of IBMP, ranging from 0 to 10^{-4} M. At the end of 1 min, monolayers were rinsed and received either fresh ICB containing the same concentration of odorant (acute reexposure) or ICB without odorant (recovery). Those monolayers receiving acute reexposure to odorant were allowed to incubate for various times ranging from 0 to 60 sec, at which time AC activity was monitored. Those cells allowed to recover for 1 min were then rinsed and reexposed to the same concentration of odorant again for intervals ranging from 0 to 60 sec, after which AC activity was monitored. AC activity was calculated as average stimulated peak height for basal odorant stimulation (■), acute reexposure to odorant (▨), and cells reexposed to odorant after 1 min of recovery (▣).

system. These observations suggest that elevation of $[Ca^{2+}]$ to micromolar levels by the inositol phospholipid system may initiate AC activation in olfactory transduction. In the same neuronal cultures, we have observed stimulation of inositol phospholipid turnover by odorants with the same absolute and relative potencies that occur with AC activation (12). Interestingly, the inositol phospholipid response is more rapid, with increased turnover maximally evident by 1 sec and responses declining at 5–10 sec, which might provide the endogenous calcium levels required for an AC response to odorants. Other investigators (4, 13, 14) have also demonstrated increased inositol phospholipid turnover upon odorant exposure.

We have monitored AC in intact cells with incubation times of 10–30 sec, as odorant stimulation of enzyme activity declines at later time points. This suggests that the rapidly activated enzyme is also rapidly inactivated. This time course is much too rapid to involve protein synthesis. Because of the importance of calcium for AC activity, signal transduction in our cells may involve odorant stimulation of inositol phospholipid turnover, increasing intracellular calcium and thereby activating AC. Enzyme activity would decline as free calcium levels fall. Alternatively, calcium might activate the enzyme by stimulating a calcium calmodulin-dependent protein kinase activity. It is also conceivable that the inositol phospholipid and AC responses are independent.

The AC responses to odorants are often biphasic with respect to both time and odorant concentration. Mechanisms responsible for the biphasic patterns are not readily apparent. Interestingly, intracellular calcium oscillations in response to inositol phospholipid cycle stimulation display a time course similar to the biphasic AC responses. The biphasic concentration–response patterns for AC might related to psychophysical evidence that some odorants change the quality of their sensory properties at different concentrations.

The rapid and potent effects of odorants upon AC in neuronal cells and in cilia (4) differ from earlier results with odorant-sensitive AC measured in olfactory cilia in which odorant effects required 1000 times higher concentrations and were detected at 15 min (2). Our present findings suggest

that the lesser potencies observed in earlier studies reflect desensitization. The time course and potencies of odorants in intact cells resemble the results of Breer and co-workers (4) in cilia, but effects of specific odorants vary. In cilia (4), individual odorants stimulate accumulation of cAMP or inositol trisphosphate but do not mediate both responses, whereas in our cell cultures odorants display similar effects on both inositol phospholipid turnover and AC. Reasons for such discrepancies are not apparent but may related to the different experimental systems used. Our cultures derive from neonatal tissue, whereas Breer and associates use adult, isolated cilia preparations maintained at extremely low calcium levels. In addition, we utilize intact cells; cellular disruption may interfere with coupling of elements of the transduction pathway.

A striking finding of our study is the desensitization of AC responses to odorants. The pronounced, immediate desensitization fits with psychophysical evidence of immediate desensitization to odorants (10, 11). Of particular interest is the augmented response of AC to odorants applied 1 min after first exposure. This suggests that an alteration within the cells initiated by the odorants is sustained for at least 1 min and vanishes by 5 min, when AC response to odorant returns to baseline levels. This "memory" of the cells at 1 min argues against enzyme activation being solely dependent on elevated free calcium.Calcium-dependent phosphorylation of AC fits better with this response; as with many proteins, phosphorylation and dephosphorylation occur over a period of a few minutes. Whether AC or some other protein is phosphorylated to produce AC activation is unclear.

Odorant desensitization may provide a means to identify classes of odorants that act upon the same or closely similar receptors. Odorants that act upon the same receptors should manifest cross-desensitization. By evaluating the presence or absence of cross-desensitization among a large number of odorants, we hope to obtain insight into numbers of discrete classes of odorant receptors.

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