The Odorant-sensitive Adenylate Cyclase of Olfactory Receptor Cells

Differential Stimulation by Distinct Classes of Odorants*

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We have characterized odorant-stimulated adenylate cyclase activity in isolated chemosensory cilia prepared from frog and rat olfactory epithelium. Cilia from both species exhibit high levels of adenylate cyclase activity. Basal activity is stimulated approximately 2-fold by GTP and approximately 5-fold by guanosine 5'-[3-O-thio]triphosphate and forskolin. Odorants augment enzyme activity 30–65% above the basal level in a tissue-specific and GTP-dependent manner. Calcium reduces GTP-stimulated activity with a 50% effective concentration at 10 μM. Odorants vary in their influence upon olfactory adenylate cyclase activity. Most fruity, floral, minty, and herbal odorants stimulate the enzyme. 3,7-Dimethyl-2,6-octadienonitrile (citralva), menthone, d-carvone, l-carvone, and 2-isobutyl-3-methoxypyrazine display similar potencies in activating the adenylate cyclase up to concentrations of 100 μM. Putrid odorants, such as isovaleric acid, triethylamine, pyridine, thiazole, and methoxy pyrazine, and odorous chemical solvents, do not stimulate enzyme activity. In homologous series of pyrazines, thiazole, and pyridine odorants, compounds with the longest hydrocarbon side chains are best able to enhance enzyme activity. The failure of certain odorants to affect adenylate cyclase activity suggests that additional transduction mechanisms besides the formation of cAMP are involved in olfaction.

Olfactory reception in vertebrates is mediated via chemosensory receptor cells located in the olfactory epithelium. These cells encode the molecular structures of odorants into patterns of afferent neuronal activity (1–6). These patterns are then decoded and processed in the central nervous system where different chemical structures are perceived as distinct odor qualities (for review, see Refs. 7–9). In contrast to the visual and auditory systems, little is known about the primary events that underlie recognition and transduction in the olfactory system. Olfactory receptor cells are bipolar neurons which extend an axon into the olfactory bulb of the brain and a dendrite toward the nasal lumen. This dendrite carries at its apex a group of chemosensory cilia which project into the mucus lining the neuroepithelium. Several lines of evidence suggest that the cilia represent the sites where the initial chemosensory recognition and transduction events take place (10–16). Cilia can be detached and isolated from the olfactory epithelium providing an in vitro system amenable to biochemical studies of olfactory reception (16, 17).

Some evidence suggests that olfactory transduction may involve cAMP as a second messenger. High levels of adenylate cyclase activity occur in rabbit olfactory epithelium (18) and cAMP and phosphodiesterase inhibitors modulate the summed receptor potentials elicited by odorants in the olfactory epithelium (19, 20). Odorant-stimulated adenylate cyclase activity was measured directly by Pace et al. (16) and found to be tissue-specific and GTP-dependent.

In the present study we provide a detailed characterization of odorant interactions with the olfactory adenylate cyclase. We report that adenylate cyclase stimulation occurs primarily with fruity, floral, minty, and herbaceous odorants, while putrid odorants fail to influence the olfactory adenylate cyclase.

**Experimental Procedures**

Materials—[α-32P]ATP (800 Ci/mmol) and [3H]cAMP (31.1 Ci/mmol) were obtained from New England Nuclear-DuPont (Boston, MA). ATP, cAMP, GTP, creatine phosphate, and creatine phosphokinase were from Sigma. GTP•PS was obtained from Boehringer-Mannheim and forskolin from Behring Diagnostics. Odorants used in the adenylate cyclase assay were kindly provided and monitored for chemical purity by glass capillary gas chromatography by International Flavors and Fragrances (Union Beach, NJ) and are >98% pure. Odorants were stored under nitrogen at 4 °C. Odorant pyrazines and thiazoles were purchased from Pyrazine Specialties (Atlanta, GA).

Isolation of Cilia—Rana catesbeiana were supplied by Amphibians of North America (Nashville, TN) or Acadian Biological (Rayne, LA). Frogs were killed by decapitation and the ventral and dorsal sheets of pigmented olfactory epithelia were dissected. Cilia were detached from the epithelium as described by Anholts et al. (17). Briefly, cilia were detached by a calcium shock (10 mM CaCl₂) after preincubation in 2 mM EDTA supplemented Ringers (2 mM HEPES, 112 mM NaCl, 3.4 mM KCl, 2.4 mM NaHCO₃, pH 7.4) to dissolve the mucus. The detached epithelia were removed by centrifugation in a serological centrifuge and the supernatant containing the detached cilia was layered on a 45% (w/w) sucrose cushion and centrifuged for 30 min at 350,000 × g in a Beckmann SW 55-Ti rotor. The white band of partially purified cilia was collected from the interface on top of the sucrose cushion and diluted in an equal volume of Ringers solution. The cilia were pelleted by centrifugation at 4 °C for 15 min at 350,000 × g in the SW 55-Ti rotor and resuspended in a small volume of Ringers solution containing 2 mM EGTA unless otherwise indicated.

1 The abbreviations used are: GTPyS, guanosine 5’-[(3-O-thio)triphosphate; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; EGTA, N-ethylenbis-(oxyethyl)eneminitrile]lutarate acid; see Table I, Footnote b, for odorant nomenclature.

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In experiments where the calcium concentration (CaCl2) was varied, cilia were resuspended in Ringers buffer without EGTA. Ringers buffer was prepared in water chromatographed over a Chelex 100 (Bio-Rad) column to remove divalent cations.

Rat olfactory cilia were prepared by a similar method. Male Sprague-Dawley rats were killed by decapitation. Nasal turbinates were dissected, cilia were resuspended in Ringers buffer without EGTA. Ringers (Bio-Rad) column to remove divalent cations.

Shaker for 20 min at 4 °C. The deciliated epithelia were removed by centrifugation for 5 min at 6,000 × g. The supernatant containing the detached cilia was centrifuged for 10 min at 12,000 × g and the resulting pellet containing the isolated cilia was washed twice in 10 mM Tris-HCl, 3 mM MgCl2, 1 mM EDTA, pH 8.0. The final cilia pellet was resuspended in 100 mM Tris-HCl, pH 8.0. The bathing medium was supplemented with CaCl2 to a final concentration of 10 mM and agitated gently on an end over end shaker for 20 min at 4 °C. The deciliated epithelia were removed by centrifugation for 5 min at 6,000 × g. The supernatant containing the detached cilia was centrifuged for 10 min at 12,000 × g and the resulting pellet containing the isolated cilia was washed twice in 10 mM Tris-HCl, 3 mM MgCl2, 1 mM EDTA, pH 8.0. The final cilia pellet was resuspended in a small volume of 10 mM Tris.HCl, 3 mM MgCl2, 1 mM EDTA, pH 8.0.

The concentrated frog and rat cilia were aliquoted and stored at -70 °C. No loss of activity was detected for up to 6 months. Protein concentrations of the cilia preparations were measured according to the method of Lowry et al. (21) using bovine serum albumin as standard.

Adenylate Cyclase Assay—Adenylate cyclase activity was assayed according to the method of Salomon (22). All solutions were prepared in water purified by filtration through a Milli-Q Water System (Millipore, Milford, MA) to remove divalent cations. Assays were carried out in triplicate in a final volume of 50 μl. The incubation mixture contained 25 mM Tris, pH 8.0, with 1 mM [32P]ATP (1.5-2.5 × 105 cpm/assay), 5 mM Mg acetate, 50 μM cAMP, 1 mM diethylthioketone, 0.5 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, and 85 units/ml creatine phosphokinase supplemented with 10 μM GTP as indicated in the figures. The reaction was initiated by the addition of cilia to a final protein concentration of 50-100 μg/ml. The tubes were incubated for 25 min at 30 °C. Reactions were terminated by the addition of 100 μl of 2% sodium dodecyl sulfate, 10 mM Tris, 50 mM EDTA, and 10 μM GTPyS or forskolin in isolated olfactory cilia.

The adenylate cyclase in frog olfactory cilia is sensitive to calcium ions (Fig. 2). One millimolar calcium suppresses cyclase activity by an order of magnitude. Reduction of the GTP-stimulated activity becomes evident at ~3 μM calcium. This activity is suppressed to the level found in the absence of GTP by 100 μM calcium with a 50% effective concentration.
adenylate cyclase in olfactory cilia (Table I). Many floral, fruity, herbaceous, and minty odors are potent stimulators of the enzyme. Citralva, a substituted terpenoid odorant having a fruity odor quality, is one of the most potent cyclase stimulators. We have, therefore, arbitrarily assigned a standard value of 100% stimulation to the cyclase activity elicited by 100 μM citralva. Maximal stimulation by citralva corresponds to a 55% increase in activity over the GTP-stimulated basal level (Fig. 3A). Odors structurally related to citralva, such as citral dimethyl acetal and citronellol, stimulate the enzyme to ~69% and ~56% of the citralva-stimulated level, respectively (Table I). Many odorants which are structurally related to citralva, such as menthone, d-carvone, l-carvone, isomenthone, l-cinnamic aldehyde, and coniferan are also potent stimulators of the olfactory adenylate cyclase. We designate odors that stimulate adenylate cyclase activity to greater than 50% of the citralva-stimulated level as potent cyclase stimulators. Those odorants that increase activity to less than 20% of the citralva-stimulated level are considered poor stimulators. Several fruity, floral, minty, and herbaceous odorants stimulate activity to an intermediate level. Examples include amylsalicylate (40%), dimethylloctanol (33%), eucalyptol (45%), eugenol (47%), and cinnamic aldehyde (34%). Interestingly, the classes of fruity, floral, minty, and herbaceous odorants also contain some nonstimulating odorants such as limonene, lyral, phenylethyl alcohol, lilial, and ethyl vanillin (Table I).

We compared the concentration-response behavior of several odorants. Citralva, menthone, the stereoisomers d-carvone and l-carvone, and the potent bell-pepper odorant, 2-isobutyl-3-methoxyisopropyl, stimulate the enzyme in a concentration-dependent manner between 1 μM and 1 mM with similar potencies (Fig. 4A). The concentration-response curves for many odorants do not reach saturation as limited solubility precludes testing concentrations above 1 mM.

In contrast to the fruity, floral, minty, and herbaceous odorants a number of putrid odorants and odorous chemical solvents do not stimulate adenylate cyclase activity. Putrid odorants such as isovaleric acid and triethylamine, and odorous chemical solvents such as pyridine do not enhance enzyme activity at concentrations up to 1 mM (Fig. 4B). The apparent lack of adenylate cyclase stimulation by this group of odorants does not represent adverse effects on the enzyme, since the basal activity is unaffected. Moreover, in the presence of 1 mM triethylamine, 100 μM citralva elicits the same stimulation as when tested alone. Reduction of the basal activity is apparent only at concentrations of triethylamine above 1 mM.

**Activation of Adenylate Cyclase by Homologous Series of Odorants**—To gain insight into the molecular parameters that determine the potency of an odorant as a cyclase stimulator, we investigated homologous series of structurally related odorants including the pyrazines, thiazoles, and pyridines. Stimulation of adenylate cyclase activity can be detected only when the parent compound, methoxyphenylpyrazine, thiazole, or pyridine has a hydrocarbon chain attached (Table II). Thus,
Odorant-sensitive Adenylyl Cyclase

Stimulation by odorant pyrazines, thiazoles, and pyridines of the GTP-dependent adenylyl cyclase in frog olfactory cilia

Pyrazine, thiazole, and pyridine odorants were tested at 100 µM in the presence of 10 µM GTP. The data are expressed as a percentage of the activity observed in the presence of 100 µM citralva. Citralva stimulation was 17 and 22% of the stimulation by 2 µM forskolin and 10 µM GTP·γ·S, respectively. Values are expressed as the mean ± S.E. of (n) experiments.

<table>
<thead>
<tr>
<th>Odorant Stimulation Log p*</th>
<th>100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxypyrazines</td>
<td></td>
</tr>
<tr>
<td>Methoxypyrazine</td>
<td>–5 ± 3 (5)</td>
</tr>
<tr>
<td>2-Methyl-3-methoxypyrazine</td>
<td>9 ± 3 (3)</td>
</tr>
<tr>
<td>2-Ethyl-3-methoxypyrazine</td>
<td>20 ± 8 (5)</td>
</tr>
<tr>
<td>2-Isopropyl-3-methoxypyrazine</td>
<td>36 ± 8 (5)</td>
</tr>
<tr>
<td>2-Isobutyl-3-methoxypyrazine</td>
<td>53 ± 4 (10)</td>
</tr>
<tr>
<td>Alkylpyrazines</td>
<td></td>
</tr>
<tr>
<td>2-Ethylpyrazine</td>
<td>–7 ± 8 (2)</td>
</tr>
<tr>
<td>2,3-Dimethylpyrazine</td>
<td>–6 ± 8 (3)</td>
</tr>
<tr>
<td>2,3,5-Trimethylpyrazine</td>
<td>–5 ± 1 (3)</td>
</tr>
<tr>
<td>2-Ethyl-3-methylpyrazine</td>
<td>–2 ± 7 (3)</td>
</tr>
<tr>
<td>Pyrazine</td>
<td>0</td>
</tr>
<tr>
<td>2,3,5,6-Tetramethylpyrazine</td>
<td>0 ± 11 (3)</td>
</tr>
<tr>
<td>2-Methylpyrazine</td>
<td>7 ± 6 (3)</td>
</tr>
<tr>
<td>2,3-Diethyl-5-methylpyrazine</td>
<td>16 ± 9 (3)</td>
</tr>
<tr>
<td>Pyridines</td>
<td></td>
</tr>
<tr>
<td>Pyridine</td>
<td>4 ± 10 (4)</td>
</tr>
<tr>
<td>2-Hexylpyridine</td>
<td>107 ± 8 (3)</td>
</tr>
<tr>
<td>3-Hexylpyridine</td>
<td>118 ± 10 (3)</td>
</tr>
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<td>Thiazoles</td>
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</tr>
<tr>
<td>Thiazole</td>
<td>–18</td>
</tr>
<tr>
<td>2-Acethlthiazole</td>
<td>3 ± 7 (6)</td>
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<tr>
<td>2,4-Dimethyl-5-acetyltiazole</td>
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</tr>
<tr>
<td>2,4,5-Trimehytiazole</td>
<td>25 ± 2 (2)</td>
</tr>
<tr>
<td>2-Isobutythiazole</td>
<td>69 ± 5 (4)</td>
</tr>
</tbody>
</table>

TABLE II

*a NC, not calculated.

FIG. 3. GTP dependence of the odorant-stimulated adenylyl cyclase. Adenylyl cyclase activity in: A, frog olfactory cilia; B, rat olfactory cilia. Citralva, 2-isobutyl-3-methoxypyrazine, menthone, n-carvone, and L-carvone were tested at 100 µM in the absence (open bars) or presence (hatched bars) of 10 µM GTP. Data are expressed as the percentage stimulation over the basal level found in the absence or presence of 10 µM GTP. Basal enzyme activity was 1 nmol/mg/min in the absence of GTP and to 2 nmol/mg/min in the presence of 10 µM GTP. Values are the mean ± S.E. of 2-24 experiments.

2-isobutyl-3-methoxypyrazine and 2-isobutylthiazole stimulate activity to 53% and 59% of the citralva-stimulated level, respectively, while methoxypyrazine and thiazole are inactive. The most striking enhancement of stimulation occurs when aliphatic chains are attached to pyridine. Pyridine itself does not augment adenylyl cyclase activity. Derivatives of pyri-
Bradford (24) which detects about 3–5-fold lower values than measured by the method of Lowry et al. (21) using bovine serum albumin as standard. We have found that olfactory cilia from Rana ridibunda, kindly donated to us by Drs. Pace and Lancet (Department of Membrane Research, Weizmann Institute of Science, Rehovot), display the same adenylate cyclase activity as our cilia preparations from R. catesbeiana when assayed for protein and adenylate cyclase activity under the same experimental conditions. It is of note, however, that there is no disagreement between laboratories that cyclase levels measured in olfactory cilia are at least an order of magnitude greater than those detected in other tissues. We find a smaller increase in activity in the presence of odorants than others (16). This slight discrepancy most likely results from differences in assay conditions.

Micromolar concentrations of calcium ions inhibit GTP-stimulated adenylate cyclase activity. The potency of calcium suggests that it may be a physiologic regulator of the odorant-sensitive adenylate cyclase as appears to be the case for other hormone-sensitive adenylate cyclases (25).

Our data indicate that odorants may be categorized into two groups, those that stimulate the olfactory adenylate cyclase and those that have no effect on the enzyme. In general, stimulation of adenylate cyclase is most apparent with odorants which are fruity, floral, minty, or herbaceous. Although many odorants from these groups stimulate adenylate cyclase activity, some exceptions are evident, including odorants like limonene, lyral, and lilial which do not stimulate cyclase activity.

The inability of putrid odorants such as isovaleric acid and triethylamine to stimulate adenylate cyclase activity cannot be explained by adverse effects of these odorants on the enzyme, since these odorants do not significantly decrease the basal GTP-stimulated adenylate cyclase activity up to concentrations of 1 mM. Although we cannot fully exclude the possibility that stimulation by these odorants is below the level of detection in our assay, extracellular recordings from individual receptive units indicate that putrid odorants stimulate similarly sized populations of olfactory neurons as fruity, floral, minty, and herbaceous odorants (3).

Putrid odorants are frequently polar, charged molecules, whereas hedonic odorants are mostly nonpolar. We tested odorants from three homologous series and found that compounds with the longest hydrocarbon side chains were best able to stimulate adenylate cyclase activity. These results suggest that one factor which determines the potency of an odorant to activate the enzyme may be its hydrophobicity. Odorant hydrophobicity, as measured by a calculated octanol/water partition coefficient (23), correlates with adenylate cyclase activity within the series of methoxypropyrazine odorants ($r = 0.59, n = 5$). However, for odorants from varying odor classes, hydrophobicity is not closely correlated with cyclase activation ($r = 0.54, n = 56$). Several hydrophobic odorants, for example, limonene, lilial, and α-pinene do not stimulate enzyme activity. Thus, hydrophobicity alone is not the only determinant of adenylate cyclase activation.

Among the pyrazines evaluated, there is some parallel between human odorant detection thresholds and adenylate cyclase stimulation. Thus 2-isobutyl-3-methoxypropyrazine and 2-isopropyl-3-methoxypropyrazine, which have the lowest detection thresholds (~2 parts/trillion; $1.2 \times 10^{-11}$ M and $1.3 \times 10^{-11}$ M, respectively), stimulate adenylate cyclase activity while methoxypropyrazine, 2-ethyl-3-methoxypropyrazine, 2-ethylpyrazine, 2,3,5-trimethylpyrazine, and 2,3,5,6-tetramethylpyrazine, with detection thresholds 100,000 times higher, do not stimulate the enzyme. However, micromolar concentra-

Odorant-sensitive Adenylate Cyclase