Blocking Adenylyl Cyclase Inhibits Olfactory Generator Currents Induced by “IP$_3$-Odors”

SHAN CHEN, ANDREW P. LANE, ROLAND BOCK, TRESE LEINDERS-ZUFALL, AND FRANK ZUFALL

Department of Anatomy and Neurobiology and Program in Neuroscience, University of Maryland, Baltimore, Maryland 21201

Received 7 March 2000; accepted in final form 7 April 2000

Chen, Shan, Andrew P. Lane, Roland Bock, Trese Leinders-Zufall, and Frank Zufall. Blocking adenylyl cyclase inhibits olfactory generator currents induced by “IP$_3$-odors.” J Neurophysiol 84: 575–580, 2000. Vertebrate olfactory receptor neurons (ORNs) transduce odor stimuli into electrical signals by means of an adenylyl cyclase/cAMP second messenger cascade, but it remains widely debated whether this cAMP cascade mediates transduction for all odors or only certain odor classes. To address this problem, we have analyzed the generator currents induced by odors that failed to produce cAMP in previous biochemical assays but instead produced IP$_3$ (“IP$_3$-odors”). We show that in single salamander ORNs, sensory responses to “cAMP-odors” and IP$_3$-odors are not mutually exclusive but coexist in the same cells. The currents induced by IP$_3$-odors exhibit identical biophysical properties as those induced by cAMP odors or direct activation of the cAMP cascade. By disrupting adenylyl cyclase to block cAMP formation using two potent antagonists of adenylyl cyclase, SQ22536 and MDL12330A, we show that this molecular step is necessary for the transduction of both odor classes. To assess whether these results are also applicable to mammals, we examine the electrophysiological responses to IP$_3$-odors in intact mouse main olfactory epithelium (MOE) by recording field potentials. The results show that inhibition of adenylyl cyclase prevents EOG responses to both odor classes in mouse MOE, even when “hot spots” with heightened sensitivity to IP$_3$-odors are examined.

INTRODUCTION

In ciliated olfactory receptor neurons (ORNs) of the vertebrate main olfactory epithelium (MOE), transduction of odors into excitatory electrical signals involves the activation of a G-protein-coupled adenylyl cyclase/cAMP second messenger cascade leading to the sequential opening of Ca$^{2+}$-permeable cAMP-gated channels and Ca$^{2+}$-activated chloride channels (for reviews see Firestein 1996; Frings et al. 2000; Reed 1992; Ronnett and Snyder 1992; Schild and Restrepo 1998; Zufall et al. 1994). Several reports have shown that odors sometimes also cause formation of inositol-1,4,5-trisphosphate (IP$_3$) (Boekhoff et al. 1990; Breer and Boekhoff 1991; Huque and Bruch 1986; Ronnett et al. 1993), and it has been suggested that IP$_3$, via the subsequent gating of plasma membrane channels in the olfactory cilia, mediates an alternative transduction pathway causing ORN depolarization (Okada et al. 1994; Restrepo et al. 1990). Other studies, however, were unable to confirm a significant contribution of IP$_3$ to the excitatory odor responses of ciliated ORNs (Belluscio et al. 1998; Brunet et al. 1996; Firestein et al. 1991a; Kleene et al. 1994; Lowe and Gold 1993; Lowe et al. 1989).

Very few data are available for the electrical responses to IP$_3$-producing odors (IP$_3$-odors), especially at the level of single ORNs, making an assessment of these conflicting reports difficult (Schild and Restrepo 1998). Therefore a primary goal of this study was to provide a biophysical and pharmacological analysis of olfactory generator currents induced by IP$_3$-odors. Our results show that there are no detectable differences between the electrical responses to IP$_3$-odors and those induced by direct activation of the cAMP cascade. By disrupting adenylyl cyclase to block cAMP formation using two potent antagonists of adenylyl cyclase, SQ22536 (Harris et al. 1979) and MDL12330A (Guei, et al. 1977), we show that this molecular step is necessary for the transduction of a wide variety of odors including those that produced IP$_3$ in biochemical assays.

METHODS

ORNs were acutely dissociated from the nasal epithelium of adult tiger salamanders (Ambystoma tigrinum) as described previously (Leinders-Zufall et al. 1996). Odor responses were recorded under voltage clamp applying the perforated patch technique with amphoterin B. If not otherwise stated, the holding potential was $-60$ mV. Current recordings, data acquisition, and online analyses were controlled by an EPC-9 patch-clamp amplifier controlled by Pulse software (HEKA Electronic, Lambrecht/Pfalz, Germany). Focal stimulation of olfactory cilia was obtained by pressure ejecting the odor solutions from multibarrel glass pipettes (Leinders-Zufall et al. 1996). ORNs were continuously superfused with Ringer solution containing (in mM) 115 NaCl, 2.5 KCl, 1.0 CaCl$_2$, 1.5 MgCl$_2$, 4.5 HEPES, and 4.5 Na-HEPES (pH 7.6, adjusted to 240 mOsm). The pipettes contained (in mM) 17.7 KCl, 92.3 KOH, 82.3 methanesulfonic acid, 5.0 EGTA, and 10 HEPES [pH 7.5 (KOH), adjusted to 220 mOsm]. Data analyses and calculations were performed as described previously (Leinders-Zufall et al. 1999).

For the electro-olfactogram (EOG) recordings from MOE, CD-1 mice (2-mo-old, either sex) (Charles River, Wilmington, MA) were killed using CO$_2$. Following decapitation, the nasal septum was removed to expose the endoturbinate system. The apical surface of the epithelium was superfused continuously with oxygenated saline (95% O$_2$,5% CO$_2$) containing (in mM) 120 NaCl, 25 NaHCO$_3$, 5 KCl, 5...
BES, 1 MgSO₄, 1 CaCl₂, and 10 mM glucose. Odor stimulation was performed using the same methods as described above. Field potentials were recorded using glass pipettes (resistance, 1 MΩ; tips filled with extracellular solution in 1% agar) that were connected via an Ag/AgCl wire to a differential amplifier (DP-301, Warner Instruments, Hamden, CT). A second Ag/AgCl wire connected to an agar bridge served as indifferent electrode. The output signal was digitized, low-pass-filtered (8-pole Bessel; corner frequency, 60 Hz), and analyzed using Pulse software.

The following odorants were used in this study: acetophenone (1-phenylethanone, Sigma); n-amylacetate (Sigma); isoamylacetate (Sigma); cineole (eucalyptol), 1,3,3-trimethyl-2-oxabicyclo(2,2,2)-octane (Sigma); citral (3,7-dimethyl-2,6-octadienenitrile, Aldrich); ethylvanillin, 3-ethoxy-4-hydroxybenzaldehyde (Aldrich); 2-heptanol (Aldrich); isovaleric acid (Aldrich); lilial, 4-(1,1-dimethylethyl)-a-methylbenzenepropanol (Givaudan Roure); (-)-limonene, (R)-4-isopropenyl-1-methyl-1-cyclohexene (Sigma); lyral (cyclohexanal), 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxyaldehyde (Givaudan Roure); menthone, 5-methyl-2-(1-methylethyl)cyclohexanone (Sigma); octanal (Sigma); pyrazine (Aldrich); and 3-ethyl 2-methylpyrazine (Aldrich). Odorants were of the highest purity grade available. Odorant stock solutions were prepared in dimethylsulfoxide (DMSO) and diluted to the final concentration with extracellular solution. The final concentration of DMSO was 0.1% (vol/vol).

The adenylyl cyclase inhibitors 9-(tetrahydro-2-furyl)-adenine (SQ22536) and cis-4-N-(2-phenylcyclopentyl)-azacyclotridecan-2-imine-hydrochloride (MDL12330A) were obtained from Research Biochemicals International (Natick, MA). Both compounds were water soluble but stock solutions were sonicated for several minutes. 3-isobutyl-1-methyl-xanthine (IBMX, Sigma) was prepared as described previously (Leinders-Zufall et al. 1999).

RESULTS

Lyral and lilial-induced generator currents in salamander ORNs

The first series of experiments was designed to analyze generator currents induced by IP₃-odors at the level of single ORNs. The results are based on a total of 242 ORNs of which 97 were tested with the aldehydes lyral and lilial, two odorants...
that have failed to produce cAMP in biochemical assays (Breer and Boekhoff 1991; Sklar et al. 1986). Of these 97 cells, 25 responded to lyral, 9 to lilial, and 5 to both odorants. In the example of Fig. 1A, lyral (300 μM) induced the activation of a large transient inward current with an overall amplitude, shape, and time course typical of excitatory odor currents in amphibian ORNs (Firestein and Werblin 1989). Lilial was ineffective in evoking a detectable current in this cell. The same neuron responded also to cineole (Fig. 1A), which is known to stimulate CNG channel activation via cAMP (Firestein et al. 1991a) and to limonene, another odorant that has failed to affect adenylyl cyclase activity in biochemical assays (Sklar et al. 1986). Application of the phosphodiesterase inhibitor IBMX, which bypasses odor receptor activation and leads to CNG channel activation because of the high basal activity of olfactory adenylyl cyclase (Firestein et al. 1991b), produced a similar inward current as lyral, cineole, and limonene (Fig. 1A).

Figure 1B shows response profiles from 21 ORNs that were stimulated sequentially with six different odorants (lyral, lilial, cineole, n-amylacetate, acetophenone, and limonene) and with IBMX. These profiles make two important points with respect to the mechanisms underlying the lyral/lilial-induced currents. The first is that all ORNs that reacted to lyral or lilial were also responsive to IBMX. This indicates that lyral or lilial responses occurred in ORNs that contained an intact cyclic nucleotide second messenger cascade. A second point made by the response profiles is that lyral or lilial can be recognized by the same neurons that also respond to general cAMP-producing odors such as cineole, n-amylacetate, or acetophenone. Thus molecular recognition and subsequent signal transduction of cAMP-odors and IP₃-odors is not mutually exclusive in salamander ORNs.

These results point to the possibility that lyral and lilial are transduced by these ORNs via the same molecular mechanism as cineole and n-amylacetate. To investigate this further, we examined the biophysical properties of the sensory currents in more detail. First, we analyzed the response kinetics by superimposing the onset phase of the lyral and cineole-induced current traces (Fig. 1C). Both responses exhibited identical activation time courses \( (n = 11) \). The onset time course of the limonene-induced current also matched the responses induced by lyral and cineole (Fig. 1C). Second, we elicited voltage ramps at the peak of the odor-induced currents to examine the corresponding conductance change (Fig. 1D). Figure 1E illustrates that the current-voltage \( (I-V) \) curves induced by lyral or lilial were almost indistinguishable from the curves obtained with cineole or IBMX, yielding nearly identical reversal potential values [lyral, \(-19.8 \pm 7.0 \text{ (SD) mV, n = 6;} \) lilial, \(-18.9 \pm 2.9 \text{ mV, n = 4;} \) cineole, \(-19.2 \pm 3.9 \text{ mV, n = 6;} \) IBMX, \(-17.5 \pm 5.3 \text{ mV, n = 4;} \) LSD: \( P = 0.49-0.93 \) ]. This suggests that all four responses were mediated by the same or very similar ionic mechanisms. Third, we bath-applied 6-anilino-5,8-quinolinedione (LY83583, 20 μM), a potent inhibitor of ion fluxes through CNG channels (Leinders-Zufall and Zulfah 1995). LY83583 completely but reversibly inhibited the lyral and lilial-induced current \( (n = 3, \text{ data not shown).} \) All of these results are consistent with the notion that lyral and lilial caused the generation of electrical currents via the same second messenger system as cineole and IBMX.

Reversible disruption of odor transduction by inhibition of adenylyl cyclase

To test directly whether lyral and lilial affected the activity of adenylyl cyclase, we attempted to disrupt cAMP formation by applying two widely used adenylyl cyclase inhibitors, SQ22536 (Harris et al. 1979) and MDL12330A (Guellaen et al. 1977). Because the actions of the two antagonists have not been investigated systematically in ORNs, we first tested their...
usefulness and specificity (see also Sato et al. 1996). Figure 2A shows that in the presence of SQ22536 (300 μM, added to the bath solution) the cineole-induced response was completely abolished, an effect that could be reversed after washing the tissue for 10 min with drug-free extracellular solution. A pulse of odor-free extracellular solution containing 0.1% DMSO was unable to produce a detectable response ruling out that the potentials were caused by movement artifacts or other nonspecific effects (data not shown). The curve is constructed by pooling several individual measurements (n = 13). The data are well-fitted with a Langmuir function

\[
\frac{I}{I_{\text{max}}} = \left(1 + \frac{[\text{SQ22536}]}{K_d}\right)^{-1}
\]

indicating that the drug binds to a specific molecular site in a one-to-one manner, with an apparent equilibrium dissociation constant \(K_d\) of 2.5 μM. If this site represents the olfactory adenylyl cyclase, then SQ22536 should also inhibit the response induced by IBMX. Moreover, one would expect SQ22536 to block the IBMX current with the same potency as the cineole response. The curve of Fig. 2B (closed symbols) shows that this was the case. We also examined the effect of MDL12330A and found that it, too, inhibited the cineole and IBMX-induced responses with almost identical \(K_d\) values (Fig. 2C). Compared with SQ22536, MDL12330A was nearly twofold more potent (\(K_d = 1.3\) μM; Fig. 2C). Enzyme inhibitors sometimes can directly affect the olfactory CNG channels. For example, it has been shown that neomycin can block CNG channel activity (Ma and Michel 1998). To rule out that SQ22536 and MDL12330A influenced the CNG channels, we analyzed cGMP-activated currents in the absence or presence of both drugs. There was no significant difference (Fig. 2, D and E). Taken together, these data rule out that SQ22536 and MDL12330A acted upstream or downstream from adenylyl cyclase under the experimental conditions used here.

We next examined whether the two inhibitors would also block the responses to lyral and lilial. The inhibition curves of Fig. 2, F and G show that this was the case. The curves could be fitted according to Eq. 1 using almost identical \(K_d\) values as in Fig. 2, B and C. Thus lyral and lilial-induced currents were inhibited by SQ22536 and MDL12330A with the same potency as the cineole and IBMX-induced responses. We also tested the effects of both inhibitors at much lower concentrations of lyral or lilial (10 μM each, n = 4) but there was no difference in their blocking potency (data not shown).
EOG-responses to IP$_3$-odors in mouse main olfactory epithelium depend on adenyl cyclase

To assess whether these results are also applicable to mammalian ORNs, we examined the responses to IP$_3$-odors in mouse MOE by recording extracellular field potentials from the epithelial surface. This measurement, the EOG (Ottosson 1956), registers summed local activities of ORNs in intact epithelium. Lyral- and lilial-induced EOG responses were observed at several locations on the endoturbinates IIa, IIb, and III. (1–11, 14, 16; Fig. 3A), although with varying relative sensitivities. Initially, we focused on hot spots exhibiting heightened sensitivity to lyral or lilial. At these locations, we recorded large negative field potentials at relatively low stimulus concentrations, ranging from 100 nM to 1 µM (Fig. 3B). When we added SQ22536 to the bath solution (100 µM for 10 min), activation of both lyral and lilial-induced responses was completely prevented, an effect that could be reversed after washing the tissue for 10 min with drug-free extracellular solution (Fig. 3B). As in the salamander, this inhibition depended on the concentration of SQ22536, with an apparent $K_d$ of 1 µM but was independent of the odor concentration (data not shown). Almost identical results were obtained using a panel of 14 structurally diverse odorants (Fig. 3C). SQ22536 disrupted transduction of odorants which have been reported to elicit increases in cAMP (amylacetate, cineole, citralva, and menthone) as well as those which have been reported to elicit IP$_3$ (ethylvanillin, isovaleric acid, lilial, lyral, and pyrazine). There was no significant difference in the effect of SQ22536 at the various recording sites. As a control, we tested the effect of SQ22536 on field potentials caused by IBMX application (100 µM, n = 5; Fig. 3C). Application of MDL12330A (30 µM for 10 min) also eliminated the responses to both lyral/lilial and n-amylacetate/cineole (Fig. 3C). Finally, to rule out species differences, we analyzed EOG responses in rat MOE using various concentrations of the odors listed in Fig. 3C and found that these responses, too, were abolished by SQ22536 (data not shown).

**DISCUSSION**

Our data show that generator currents induced by IP$_3$-odors exhibit identical biophysical and pharmacological properties as those activated by cAMP-odors or direct activation of the cAMP cascade. Pharmacological blockade of cAMP formation prevented the activation of the responses to both odor classes in isolated salamander ORNs and intact mouse MOE. This blockade was also seen when epithelial hot spots with heightened sensitivity to IP$_3$-odors were examined. Several tests were conducted to show that the effects of the two adenyl cyclase inhibitors used here, SQ22536 and MDL12330A, are specific and selective. Our results, using effective pharmacological blockade, and those of previous gene knock out studies (OCNC-1: Brunet et al. 1996; $G_{a}$: Belluscio et al. 1998) are conclusive evidence that in a majority of ciliated ORNs of the MOE, $G_{a}$-mediated adenyl cyclase activation and CNG channel opening are essential for the transduction of a wide variety of odors including those that produced IP$_3$ in biochemical assays.

We must emphasize, however, that we cannot rule out the possibility that there are small subsets of ORNs in the MOE that use second messengers other than cAMP for odor transduction. Two emerging possibilities should be noted here. First, there is mounting evidence that microvillous ORNs are present in the MOE of diverse vertebrates, including most teleost fishes, salamanders, and some mammals including man (see Morita and Finger 1998 and references therein). Microvillous ORNs seem to express odor receptors that share similarity with the vomeronasal receptors (Cao et al. 1998; Speca et al. 1999). Although the precise transduction pathways in these cells remain to be determined, it seems likely that they respond to chemostimulation with phosphatidyl inositol turnover, resulting in the production of IP$_3$ (see discussion in Speca et al. 1999). Isolated “cilia preparations” used for biochemical measurements of odor-induced second messenger formation may have contained both cilia and microvilli. Second, given the identification of a subset of ORNs lacking type III adenyl cyclase but instead expressing a receptor guanylyl cyclase (GC-D) in their cilia (Julifs et al. 1997), it seems highly likely that these cells, too, respond to odors in a cAMP-independent manner. This notion is further supported by the result that, in OCNC1-null mice, tyrosine hydroxylase expression in the olfactory bulb, which reflectsafferent activity, is reduced in the majority of periglomerular neurons but retained in the “necklace” glomeruli which receive afferent input from the GC-D expressing receptor neurons (Baker et al. 1999). The pharmacological approach developed here should add an important tool for the identification of ORNs exhibiting odor responses that are independent of cAMP-formation. It should also be useful for examining the role of activity-dependent processes in the development of the olfactory system.

We thank Givaunin Roire for the generous gift of lyral and lilial and M. Shipley and F. Margolis for comments on earlier versions of this manuscript.

This work was supported in part by National Institutes of Health Grants NS-37748 to F. Zufall and DC-03773 to T. Leinders-Zufall. A. P. Lane was the recipient of NIH Training Grant DC-00054.

Present address of S. Chen: Synaptic Physiology Unit, National Institute of Neurological Diseases and Stroke, Bldg. 36, Rm. 2C-09, National Institutes of Health, Bethesda, MD 20892.

**REFERENCES**


