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Olfactory Receptor Neuron Profiling using Sandalwood Odorants

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Abstract

The mammalian olfactory system can discriminate between volatile molecules with subtle differences in their molecular structures. Efforts in synthetic chemistry have delivered a myriad of smelling compounds of different qualities as well as many molecules with very similar olfactive properties. One important class of molecules in the fragrance industry are sandalwood odorants. Sandalwood oil and four synthetic sandalwood molecules were selected to study the activation profile of endogenous olfactory receptors when exposed to compounds from the same odorant family. Dissociated rat olfactory receptor neurons were exposed to the sandalwood molecules and the receptor activation studied by monitoring fluxes in the internal calcium concentration. Olfactory receptor neurons were identified that were specifically stimulated by sandalwood compounds. These neurons expressed olfactory receptors that can discriminate between sandalwood odorants with slight differences in their molecular structures. This is the first study in which an important class of perfume compounds was analyzed for its ability to activate endogenous olfactory receptors in olfactory receptor neurons.

Key words: olfactory receptor neuron; olfactory receptor; sandalwood; calcium imaging

Introduction

Smelling is a very complex event in which molecules from the environment are inhaled through the nose and dissolved in the mucus of the olfactory neuroepithelium where they subsequently bind to olfactory receptors (ORs). Once a receptor has been activated, a cascade of events is initiated that transforms the chemical-structural information contained in the odorous stimulus into a neural signal, i.e. a membrane potential. This signal is projected to the first relay place in the brain, the olfactory bulb, from where it is transported to higher regions of the brain.

In mammals there are ~1000 different ORs, which belong to the large gene family of G protein-coupled receptors (Buck and Axel, 1991). Covering ~1% of the genome, OR genes form the largest gene family, which is organized in clusters and present on most chromosomes (reviewed by Mombaerts, 1999).

Recent studies from a number of laboratories have provided evidence for the code of smell, but all these findings only scratch at the surface of the paradigm to understand the sense of smell (Krautwurst *et al.*, 1998; Zhao *et al.*, 1998; Malnic *et al.*, 1999; Wetzel *et al.*, 1999; Araneda *et al.*, 2000, 2004; Kajiya *et al.*, 2001; Bozza *et al.*, 2002; Gaillard *et al.*, 2002; Spehr *et al.*, 2003; Oka *et al.*, 2004). Nevertheless, these findings have started the transition of biological approaches in the fragrance industry from a distant dream to a feasible high number screening for new structural leads.

The fruitful phase of chemical synthesis, which started almost 100 years ago, has allowed fragrance research groups to accumulate thousands of odor compounds. Only a limited number of these compounds have been introduced into the perfumer's palette. The availability of this vast number of molecules is an invaluable resource of substances for characterization of the receptive range of olfactory receptors.

The lack of a robust *in vitro* expression system for ORs makes it difficult to identify ligands for a large number of ORs. An alternative approach to identify receptor-ligand pairs is the analysis of primary olfactory receptor neurons (ORNs) with a specific family of odorants to identify the appropriate ORs. This approach is possible because each ORN expresses only one or a small number of ORs. In the past, aldehydes have often been used to study their interaction pattern with ORs in ORNs (Zhao *et al.*, 1998; Araneda *et al.*, 2000, 2004; Kaluza and Breer, 2000; Bozza *et al.*, 2002). We have chosen a different group of odorants,

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belonging to the sandalwood family, to study the activation of ORs in dissociated primary rat ORNs.

Sandalwood oil from the East Indian sandalwood tree (*Santalum album* L.) has been used as a precious ingredient since the beginning of perfumery, and woody compounds reminiscent of sandalwood oil are heavily used in modern perfumery. In the 1970s sandalwood oil became scarce and expensive. Chemists in fragrance companies therefore put considerable efforts into synthesizing cheaper substitutes that have similar odor qualities. Sandalwood oil consists mainly of α- and β-santalol (~70%), which also give the oil its woody scent. Over the years, development of synthetic molecules as substitutes for sandalwood oil has led to a series of successful compounds, including the ones that have been used in this study (Sandalore®, Ebanol®, Radjanol® and Javanol®, all produced by Givaudan), all derived from α-campholenic aldehyde. Extensive structure–odor relationship (SOR) studies helped in the design of sandalwood oil substitutes with improved properties (low threshold, high substantivity). The available SOR data from a series of active and inactive stereoisomers were used to generate sandalwood olfactophore hypotheses. The quality of the sandalwood scent depends on the length and substitution pattern of the aliphatic chain between a hydroxyl group and a bulky lipophilic moiety. In general, the introduction of a double bond in this aliphatic chain enhances the odor intensity (Fráter *et al.*, 1998; Bajgrowicz and Fráter, 2000; Gautschi *et al.*, 2001).

Different sandalwood odorants were used in the present study, to learn about the OR activation patterns elicited by these compounds. Odorant stimuli were applied to dissociated primary rat ORNs and the activation profile of responding neurons analyzed.

Materials and methods

Cell preparation and culture

Dissociated primary cultures of rat ORNs were prepared using a previously described procedure (Vargas and Lucero, 1999) with minor modifications. Briefly, adult Wistar rats (150–200 g) were sacrificed by decapitation (conducted by veterinary assistants at the Institute of Veterinary Physiology, University of Zurich, Switzerland). The olfactory neuroepithelium was dissected and placed in 5 ml of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Switzerland) containing 9.6 mg/ml HEPES, 4 mg/ml dispase II (Roche Molecular Biochemicals, Switzerland), 1 mg/ml collagenase (Roche Molecular Biochemicals), 1 mg/ml hyaluronidase and 60 μg/ml dioxyribonuclease I. The enzymatic digestion was carried out with gentle shaking at 37°C for 60 min. The tissue was transferred to 2 ml DMEM containing 10% fetal bovine serum (Invitrogen) and triturated using a fire-polished Pasteur pipette. The resulting cell suspension was filtered using a 70 μm cell strainer (Falcon) and plated onto laminin-coated (10 μg/ml; BD Biosciences, Bedford, MA) glass coverslips.

Intracellular calcium measurements

For calcium imaging recordings, the cultured cells were loaded with 4 μM fura-2/AM (Molecular Probes Europe BV, Leiden, The Netherlands) for 30 min at room temperature. Dual-wavelength measurements at 510 nm (340 and 380 nm excitations) were performed on an inverted fluorescent microscope (Axiovert S100, Zeiss, Germany) and a digital CCD camera (Hamamatsu, Japan). Images were recorded and analyzed using the software Open*lab* (Improvision, Coventry, UK). A ligand gradient of 0–25 μM final concentration over 8 s was applied to the cells using a gradient pump (BioRad, Switzerland) with a washout time of 10 min between ligand applications.

Chemicals

Odorant molecules were from Givaudan (Schweiz AG). All other chemicals were obtained from Sigma Chemical Company (St Louis, MO) unless stated otherwise.

Results and discussion

ORN profiling by measuring internal calcium fluxes

Dissociated cultures of mature rat ORNs were analyzed by immunocytochemistry using an antibody to neuron-specific β-tubulin isotype III (Pixley, 1992). This showed that 24 h post-plating ~80% of the attached cells were neurons. Many neurons on the coverslips were bipolar ORNs, which still contained intact axons and dendrites (data not shown). The cultures were analyzed by calcium imaging within 48 h post-culturing. To test the quality and viability of the cultures, the calcium flux following KCl application was measured. This revealed that over 90% of the attached ORNs were viable. In ORN profiling studies, the activation of endogenous ORs was analyzed by recording the internal calcium fluxes following the application of odor molecules. The actual concentration of the ligand molecules, to which the cells were exposed during calcium imaging measurements, was 25 μM as determined by gas chromatography (Fisons Instruments GC 8000 with a Restek DB-5 column). The quality of the ligand molecules was analyzed periodically by gas chromatography mass spectrometry to ensure that highly pure molecules were used throughout the entire study. ORN profiling was carried out using the molecules shown in Figure 1. The main molecules of sandalwood oil are α- and β-santalol, which also give the oil its woody character. The synthetic sandalwood oil substitutes used in this study were Javanol®, Radjanol®, Sandalore® and Ebanol®. The molecule of 5α-androst-16-en-3α-ol was chosen because it has been reported to possess a characteristic creamy, sandalwood scent, besides having a strong urinous, animalic odor (Fráter *et al.*, 1998; Bajgrowicz and Fráter, 2000). Octanal was used as negative control with a

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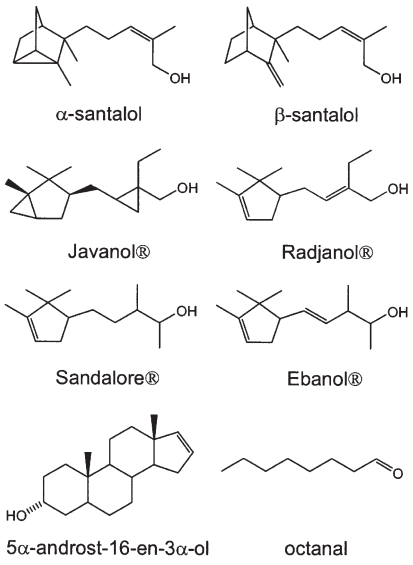

Figure 1 Molecular structures of odorants used in this study. The two major compounds of sandalwood oil are α- and β-santalol which also give the oil its woody character. Javanol®, Radjanol®, Sandalore® and Ebanol® are four synthetic molecules with a sandalwood note. 5α-androst-16-en-3α-ol was applied because it has occasionally been described in the literature to have a sandalwood character whereas octanal was used as negative control.

chemical structure and an odor quality that are quite different from the sandalwood molecules.

An average ORN preparation was plated onto eight glass coverslips. Under the microscope the area that could be recorded by calcium imaging during each ligand application was equivalent to 0.1% of the coverslip area. Each coverslip was only exposed to ligands for a maximum of 10 times (different areas) unless cells were identified that responded to sandalwood molecules. Thus, only ~1% of the cells from each preparation were actually analyzed, corresponding to ~8000 viable neurons. The number of dissociated ORNs, which were responding to sandalwood molecules, was very low. It was not unusual to screen a whole ORN preparation without finding a responding cell. On average, 1–2 ORNs per preparation were identified that responded to at least two sandalwood stimuli. Since several areas were analyzed on the same coverslip, sandalwood-responding neurons which were identified towards the end of the analysis of a coverslip must have responded to the previous stimuli which could not be recorded. Sandalwood-responding ORNs could be stimulated up to eleven times (Figure 2). During the recording, the signal intensities resulting from a given odorant remained constant over several applications until the sudden total disappearance of the cell response. ORNs that were activated by members of the sandalwood family did not respond to the application of 5α-androst-16-en-3α-ol or the control molecule octanal. ORNs, which could be activated at least twice by the sandalwood family, were always responding to applications of sandalwood oil,

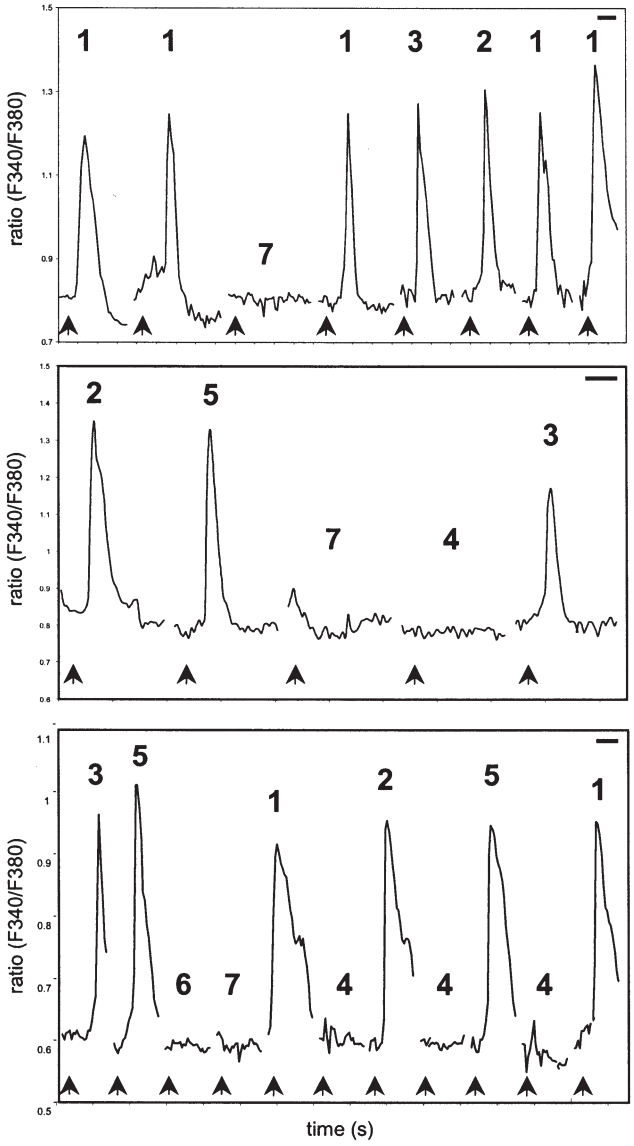

Figure 2 ORN profiling using sandalwood compounds. Dissociated primary ORN cultures were studied by calcium imaging following application of sandalwood molecules. The odorants used were **1** sandalwood oil; **2** Javanol®; **3** Radjanol®; **4** Sandalore®; **5** Ebanol®; **6** 5α-androst-16-en-3α-ol; **7** octanal. The odors were added at the time points indicated by an arrow. Between the applications there was a washout period of 10 min. The data shown in each graph was collected from a single ORN. The panels show the recorded profiles for ORNs 1, 4 and 7 from Figure 3. The cells were specifically responding to sandalwood odorants but not to 5α-androst-16-en-3α-ol or octanal. Bar = 60 s.

Javanol®, Radjanol® or Ebanol® but never to Sandalore®, 5α-androst-16-en-3α-ol or octanal (Figure 3). To exclude the possibility that Sandalore® was less soluble in the aqueous buffer, and that therefore the final concentration in the calcium imaging experiment was lower than for other sandalwood molecules, the actual ligand concentrations were analyzed by gas chromatography, showing that all molecules had equal final concentrations of 25 μM during

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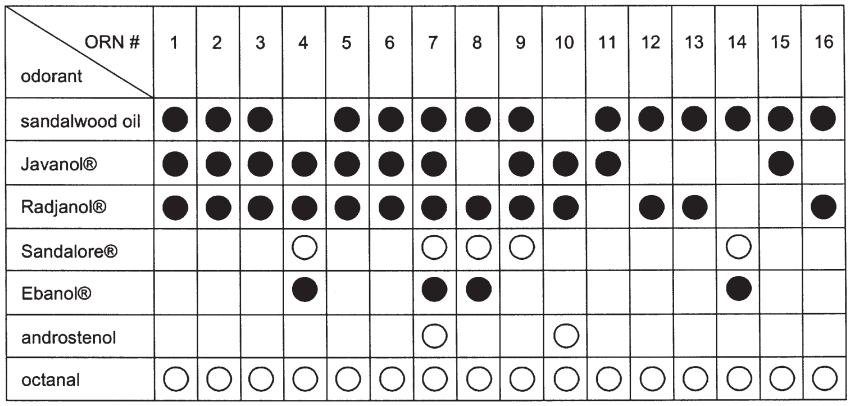

Figure 3 Summary of ORNs responding to two or more sandalwood stimuli. The identified ORNs did respond to applications of sandalwood oil, Javanol®, Radjanol® or Ebanol® but never to Sandalore®, 5α-androst-16-en-3α-ol or octanal. OR activation is indicated by a filled circle and non-activation by an open circle.

the experiments (data not shown). Even at higher concentrations (up to 250 μM) did Sandalore® not activate the sandalwood-responding ORNs. Still higher Sandalore® concentrations could not be tested because of its low solubility in aqueous buffers. Approximately 6% of the neurons did respond to applications of octanal, which is similar to previously reported data (Araneda *et al.*, 2004).

Structure–odor relationship

Why do not all members of the tested sandalwood odorants activate the same OR? First, it needs to be mentioned that sensory analyses have been done with human panellists only (Bajgrowicz and Fráter, 2000), and that no sensory psychophysical studies were conducted with rats to determine whether rats are likely to perceive the tested sandalwood compounds as being olfactively similar. However, taking into account that the rat OR repertoire is 2–3 times larger than the human one, there is a high chance that rats can smell the compounds and that at least some ORs respond to previously identified sandalwood olfactophores (Godfrey *et al.*, 2004).

β-Santalol is the main odor vector of natural sandalwood oil, and its building blocks are a bulky rigid moiety, a flexible spacer and an OH group at the other end of the spacer. It is known that the size, substitution and possibly the electronic density within the spacer have an impact on both the intensity as well as the quality of sandalwood-like compounds derived from campholenic aldehyde (Fráter *et al.*, 1998; Bajgrowicz and Fráter, 2000). In the search for more potent synthetic substitutes for sandalwood oil as perfume ingredient, the double bond in the spacer region was replaced with a cyclopropane ring (as in Javanol®). Introduction of this ring allowed the rigidification of the structure, while keeping an electron-rich structural feature, isoelectronic to a double bond present in other commercially available sandalwood substitutes (Ebanol®, Radjanol®). In this study, we have identified endogenous ORs present in rat ORNs, for which receptor activation was dependent on the presence of a double bond or its substitution with a cyclopropane ring within the spacer region. Sandalore®, despite having a sandalwood character, was the sole member of the sandalwood family that did not activate these particular ORs. So far, Sandalore® is the only tested molecule without that electron-rich structural feature in the spacer and more compounds need to be tested to draw final conclusions. However, the difference in chemical structure and electron density puts Sandalore® in a different subgroup of sandalwood odorants and this may well correlate with targeting a different subset of ORs. It is possible that there are rat ORs, which respond only to Sandalore® or to all of the above sandalwood compounds. Additional studies will provide that information. On the other hand, it is also possible that Sandalore® is a weak activator of the identified ORNs and the agonistic nature of the odorant would become apparent once it is possible to conduct dose-response curves. In fact, human panellists rate Sandalore® as much weaker than any other synthetic sandalwood compounds (threshold 14-, 23- and 171-times higher than Ebanol®, Radjanol® and Javanol®, respectively; Givaudan internal database). Based on the combinatorial receptor coding system (Malnic *et al.*, 1999), each sandalwood compound activates a set of ORs representing a specific 'fingerprint' of the scent. As the tested sandalwood odorants can be olfactively discriminated, this indicates that the receptor activation patterns are unique, although it is likely that some or most of the sandalwood molecules activate a similar set of ORs.

This is the first study using a family of perfume compounds with a relatively rigid chemical backbone as agonists of olfactory receptors. While heterologous approaches do not allow yet to routinely deorphanize this special group of G protein-coupled receptors, the use of ORNs has the advantage to be closer to physiology and there is no need to worry about the presence of essential components of the signal transduction cascade. The identification of ORNs expressing receptors that respond to odorants sharing a common olfactophore provides an attractive

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starting material to be used for cloning of rat sandalwood receptors for heterologous expression and additional analyses. In order to make further conclusions and compare receptor data with modelling hypotheses in a more sophisticated way, more odorants need to be tested with single, identified receptors.

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