Corrections and Retraction

CORRECTIONS

IMMUNOLOGY. For the article “Gene therapy with a single chain interleukin 12 fusion protein induces T cell-dependent protective immunity in a syngeneic model of murine neuroblastoma” by Holger N. Lode, Torsten Dreier, Rong Xiang, Nissi M. Varki, Angray S. Kang, and Ralph A. Reisfeld, which appeared in number 5, March 3, 1998, of Proc. Natl. Acad. Sci. USA (95, 2475–2480), the authors note the following correction. Figs. 1A and B, the legend to Fig. 1B, and lines 5–9 on page 2476 erroneously indicate that an OKT3 leader sequence was introduced upstream from the p35 gene to assure secretion in eukaryotic cells. The κ chain leader sequence of the anti-HIV-1 p24 monoclonal antibody was used instead. The leader sequence is as follows.

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10 20  30
ATGAGTGTGC CCACTCAGGT CCTGGGGTTG
40 50  60
CTGCTGCTGT GGCTTACAGA TGCCAGATGT
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NEUROBIOLOGY. For the article “Functional identification and reconstitution of an odorant receptor in single olfactory neurons” by Kazushige Touhara, Shintaro Sengoku, Koichiro Inaki, Akio Tsuboi, Junzo Hirono, Takaaki Sato, Hitoshi Sakano, and Tatsuya Haga, which appeared in number 7, March 30, 1999, of Proc. Natl. Acad. Sci. USA (96, 4040–4045), the authors note the following correction. In Fig. 4, the methyl group at the 3’ position is missing in the chemical structures of hydroxycitronellol (HC) and hydroxycitronellal dimethyl acetal (HCA). The International Union of Pure and Applied Chemistry (IUPAC) nomenclature for HC and HCA is 3,7-dimethyloctan-1,7-diol and 3,7-dimethyl-7-hydroxyoctan-1-al dimethyl acetal, respectively.

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Correction published online before print: Proc. Natl. Acad. Sci. USA, 10.1073/pnas.070046597. Text and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.070046597
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PHYSIOLOGY. For the article “Corticotropin-releasing hormone deficiency unmasks the proinflammatory effect of epinephrine” by Katia P. Karalis, Effie Kontopoulou, Louis J. Muglia, and Joseph A. Majzoub, which appeared in number 12, June 8, 1999, of Proc. Natl. Acad. Sci. USA (96, 7093–7097), the authors note that in addition to those mentioned in the Acknowledgments, Dr. K. Rice is thanked for his synthesis of the corticotropin-releasing hormone antagonist antalarmin.

RETRACTION

ECOLOGY. For the article “Monarch butterflies (Danaus plexippus L.) use a magnetic compass for navigation” by Jason A. Etheredge, Sandra M. Perez, Orley R. Taylor, and Rudolf Jander, which appeared in number 24, November 23, 1999, of Proc. Natl. Acad. Sci. USA (96, 13845–13846), the authors note the following. “The positive response to magnetic fields in two experiments cannot be repeated. Further experiments show the false positives in these tests result from a positive taxis by the butterflies to the light reflected off the clothing of the observers. We therefore retract our report. We regret the inconvenience that publication of this study may have caused.”
Functional identification and reconstitution of an odorant receptor in single olfactory neurons

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Edited by Richard Axel, Columbia University, New York, NY, and approved February 2, 1999 (received for review June 1, 1998)

ABSTRACT The olfactory system is remarkable in its capacity to discriminate a wide range of odors through a series of transduction events initiated in olfactory receptor neurons. Each olfactory neuron is expected to express only a single odorant receptor gene that belongs to the G protein coupled receptor family. The ligand–receptor interaction, however, has not been clearly characterized. This study demonstrates the functional identification of olfactory receptor(s) for specific odorant(s) from single olfactory neurons by a combination of Ca²⁺-imaging and reverse transcription–coupled PCR analysis. First, a candidate odorant receptor was cloned from a single tissue-printed olfactory neuron that displayed odorant-induced Ca²⁺ increase. Next, recombinant adenovirus-mediated expression of the isolated receptor gene was established in the olfactory epithelium by using green fluorescent protein as a marker. The infected neurons elicited external Ca²⁺ entry when exposed to the odorant that originally was used to identify the receptor gene. Experiments performed to determine ligand specificity revealed that the odorant receptor recognized specific structural motifs within odorant molecules. The odorant receptor-mediated signal transduction appears to be reconstituted by this two-step approach: the receptor screening for given odorant(s) from single neurons and the functional expression of the receptor via recombinant adenovirus. The present approach should enable us to examine not only ligand specificity of an odorant receptor but also receptor specificity and diversity for a particular odorant of interest.

The olfactory systems of vertebrates have a remarkable capacity to recognize and discriminate thousands of different odorant molecules (1–5). This process of discrimination, which results in sensory perception, begins with a series of signal transduction steps that occur within the olfactory neurons. The initial step in the process of odorant perception is the recognition of volatile odorant molecules by odorant receptors, members of the G protein coupled receptor superfamily, that are expressed on the surface of olfactory neuronal cilia (6, 7). The ligand(s)–receptor interactions then cause an increase in concentration of the intracellular messenger, cAMP, or inositol trisphosphate (1–5). Odorant-evoked elevations in cAMP are thought to directly activate a cation-selective cyclic nucleotide-gated channel, which causes external Ca²⁺ influx, leading to membrane depolarization and the generation of action potentials.

It is widely accepted that each olfactory neuron selectively expresses only one of hundreds of odorant receptor genes. Olfactory neuron cells expressing a given receptor are randomly dispersed within one of four distinct zones found in the olfactory epithelium (8, 9). The receptive range of each olfactory neuron is thought to be determined by the specific odorant receptor expressed by the neuron (10). Convergence of olfactory neurons expressing the same receptor onto two specific glomeruli (11, 12) suggests that the function of the glomeruli, which is characterized by the type of olfactory neurons connected to it, is to achieve finer tuning for given odors at the level of the olfactory bulb (4). This notion is consistent with physiological studies of recordings taken from mitral cells responding to stimulation by various odorants (13). The link between the unique expression patterns of odorant receptors and the precise topographic map of olfactory neurons likely allows the olfactory system to establish the complex tuning mechanisms necessary for distinguishing a wide variety of structurally diverse odors.

How is the enormous diversity of aromas recognized by only hundreds of odorant receptors? To understand the nature of odorant–odorant receptor interactions, several attempts have been made to determine the ligand specificity of individual receptor types. The odorant sensitivity was determined for a rat odorant receptor, OR5, which was expressed in Sf9 cells (7) or Escherichia coli (14), and a nonmammalian odorant receptor, the nematode ODR10, which was expressed in mammalian tissue cultures (15). Most recently, a rat odorant receptor I7 was functionally expressed by using an adenovirus-mediated expression system (16). Although these functional expressions of cloned odorant receptors allowed the determination of the chemical receptive ranges of receptor molecules in olfactory neurons, the conventional ligand screening steps have been a laborious task because of the enormous range of odors. To overcome this problem, we herein demonstrate the functional cloning of an odorant receptor gene in an agonist-directed manner from single olfactory neurons by combining Ca²⁺ recording and single cell reverse transcription–coupled PCR techniques. Next, we adopt an adenovirus-mediated expression system to reconstitute the cloned receptor to ascertain the reliability of this approach and to enable further biochemical analyses. The present studies have tremendous potential to examine specificity and diversity of odorant receptors that recognize a particular odorant of interest.

MATERIALS AND METHODS

Isolation of Olfactory Receptor Neurons. Olfactory epithelium was dissected out into Ringer’s solutions (138 mM NaCl/5.6 mM KCl/2 mM CaCl₂/2 mM MgCl₂/2 mM sodium pyruvate/9.4 mM glucose/5 mM Hepes, pH 7.4) from 3- to 11-day-old rats (9). Briefly, the olfactory epithelium was isolated using the procedure of Reiner et al. (10) and placed in a beaker with a solution of collagenase type II (12 mg/mL; Sigma), and protease type X (0.01 mg/mL; Sigma) to dissociate the epithelium into single cells. The mixture of epithelium was incubated for 15 min at 37 °C and neutralized with tissue culture medium (200 mL). The epithelium was then washed 3 times with tissue culture medium.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: RT-PCR, reverse transcription–coupled PCR; GFP, green fluorescent protein; MOR, mouse olfactory receptor; MA, myrc aldehyde; HC, hydroxycitronellol; IRES, internal ribosome entry site.

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4-week-old BALB/c CrSLc mice (Japan SLC, Hamamatsu, Japan) that were anesthetized by mioblock injection containing 20 µg/ml of bromopanocodein (Sankyo). The epithelial pieces were treated with Ca^{2+}-free Ringer’s for 10 min, 0.025% trypsin (Sigma) for 9–11 min at 37°C, 0.025% trypsin inhibitor (Sigma) for 10 min, and 0.1 mg/ml DNase (Sigma) for 2.5 min. The trypsinized olfactory epithelium cells then were tissue-printed on Cell-TAK (Collaborative Biomedical Products, Bedford, MA)-coated cover glass as described (10). In brief, superficial quasidissociated cells of the treated pieces were printed continuously on the coated glass floor by rolling the piece so that the lateral side maintained gentle contact with the floor by the use of an elastic glass pipette.

**Fura-2 Ca^{2+}-Imaging of Odorant Responses.** The cover glass was mounted on a teflon recording chamber, and 5 µM fura-2/AM (Molecular Probes) was loaded in tissue printed cells for 30 min. The fura-2 fluorescence at 510 nm by excitation at 340 or 380 nm was measured by use of inverted fluorescent microscope [Nikon TMD300 or Olympus (New Hyde Park, NY) IMT-2] and ICCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Samples were illuminated by a xenon lamp, and a computer-controlled filter changer was used to switch the excitation wavelength. Images were recorded at 1.3-sec intervals and were analyzed with argus50 software.

**Generation and Application of the Adenovirus Vectors.** Recombinant adenovirus vectors were generated by cosmid-terminal protein complex method as described (21). Recombinant adenovirus vectors then were amplified further to larger scale in HEK293. For the bicistronic adenovirus vector, the expression of the bicistronic unit was turned on by a C-tag, directing recombination at the final step of amplification, which was confirmed by Southern blot analysis. A total of 2.5–5.0 µl of adenovirus vector solution at a titer of 10^{9} pfu/ml was injected by pipetting at 0.25–0.5 µl/min into a nostril of 3–4-week-old C57BL/6Ncrj mice (Charles River Breeding Laboratories).

**RESULTS AND DISCUSSION**

Disruption of molecular signals in the olfactory system consists of the initial detection of odors by olfactory neurons followed by the subsequent processing in the olfactory bulb and olfactory cortex. Molecular receptive range in olfactory neurons and bulbs are based on the information coded within odorant molecules such as differences in chain length, terminal groups, and positions of functional groups (4, 10). Direct insight into odorant–olfactory receptor interactions, however, has not yet been obtained because of the difficulty in expressing functional receptors necessary for the screening of thousands of odorant molecules. To approach a detailed understanding of the dynamic tuning mechanisms involved in discriminating a variety of odors at receptor level, we reasoned that the combination of odorant response assay with single cell RT-PCR analysis (23) would lead to the determination of the types of receptors expressed by single olfactory neurons and a correlation with the physiological responses to those receptors.

**Molecular Cloning of the Mouse Olfactory Receptor (MOR) 23 Coding Sequence.** The entire coding region of MOR23 was amplified by PCR from the mouse MOR23 genomic clone with the upstream primer 5’-CAGACCGTGACATGTCGACAGAATAACTTC-3’ and the downstream primer 5’-GATATCAGAAAGTGTTCCTGCCA-3’. The amplified fragment was then cloned into pGEM-T vector (Promega), and the MOR23 sequence was verified by sequencing.
Olfactory receptor neurons suitable for simultaneous recording of odorant responses in several cells were isolated by use of tissue printing method (10) from mouse olfactory epithelium located on the upper septum and roof designated as zone 1 among four distinct spatial receptor zones (9). Odorant receptor-mediated elevations in cAMP or inositol-1,4,5-trisphosphate trigger Ca^{2+} influx through a cation-selective cyclic nucleotide-gated channel or an as-yet uncharacterized inositol trisphosphate-gated ion channel, respectively (1–5). Tissue-printed olfactory neurons were thus subjected to fura-2 based Ca^{2+} imaging to measure increases in intracellular Ca^{2+} levels in response to odorant stimulations. Sequential applications of various odorants by use of the recording chamber shown in Fig. 1a allowed identification of cells responding to certain odorant(s).

For example, shown in Fig. 1b is a Ca^{2+}-level recording of a single cell that responds only to the odorous aldehyde, lyral, at 100 μM concentrations among 11 sequentially applied odorants (the structure of lyral is shown in Fig. 4). Viability of the cell was confirmed by high potassium chloride buffer-induced membrane depolarization. Of ~3,500 tissue-printed viable olfactory neurons, we could identify a total of 226 positive cells that responded to some of the 11 applied odorants. For example, 29 cells responded to eugenol, 44 responded to ethylvanillin, 2 responded to geraniol, 16 responded to lilial, 41 responded to cresol, and 6 responded to lyral. Some cells showed responses to more than one odorant (data not shown). Each responsive cell was subsequently picked in a microcapillary tube and was subjected to single cell RT-PCR using degenerate oligonucleotides designed on the basis of the known conserved sequences among the olfactory receptor superfamily (6, 7). The DNase treatment of column-purified RNA samples and the first PCR by using the oligo(dT)-adapter sequence eliminated contaminating PCR products derived from genomic DNA (Fig. 1c). Thus, no PCR product was observed in the sample that had not undergone the reverse transcriptase reaction (Fig. 1c). Sequence analysis of the two-round PCR-amplified fragment obtained from one lyral responsive cell (Fig. 1b) revealed it to be identical to the mouse olfactory receptor MOR23 (24) (Fig. 1d). Consistent with previous implication (8, 9), it was found that only one type of odorant receptor was isolated from a single cell. We have also isolated 15 additional odorant receptor genes from cells that responded to other odorants used in this study such as eugenol and cresol, although the characterization of these single cell RT-PCR products is in progress.

Among them, the MOR23 is of particular interest because it has been relatively well characterized and has distinct regulatory expression patterns in olfactory neurons and testis tissues (24, 25). It was confirmed by an in situ hybridization experiment that the expression of the endogenous MOR23 is restricted to epithelium of the upper septum and roof defined as expression zone 1 (9), the same region used for the tissue printing (Fig. 2a). At this stage, although lyral was a candidate ligand for MOR23, a functional reconstitution study was required to verify lyral-MOR23 interaction.

We then targeted the olfactory neuron itself as an expression system for the reconstitution of the odorant-receptor interaction. Thus, recombinant adenovirus vectors containing a bicistronic expression unit for MOR23 and GFP, and a monocistronic one for GFP alone as a negative control vector, were constructed (Fig. 2b). When these vectors were applied to nasal tissues, the presence of the GFP protein was detectable in both cases by fluorescent microscopy (Fig. 2c and d). In each infection—i.e., the bicistronic MOR23 vector and the monocistronic GFP vector—coronal sections in the middle part of the epithelium exhibited robust fluorescence within the dorso-lateral recessed area at the confronted surfaces between the turbinates and lower septum, none of which is a part of zone 1, where the endogenous MOR23 is expressed. The same
observations were obtained throughout the anterior–posterior axis (data not shown), thereby ruling out the possibility for a meeting of the endogenous MOR23 and the transgene in the same cell. There was no significant difference in expressed zones between the MOR23 and GFP constructs (Fig. 2c), suggesting that the extent of the viral infection seems to depend on how much longer the peripheral space can retain the viral solution within its cavity.

To test odorant responses at single cell level, the MOR23 adenovirus-infected fluorescent cells were individually isolated by the tissue printing method from the GFP-fluorescent olfactory epithelium. The antisense RNA for the MOR23 coding region hybridization of the MOR23 transcript in coronal sections of the olfactory epithelium. The antisense RNA for the MOR23 coding region was used as a probe. The presence of the transcripts was indicated by dispersed focal staining shown by arrows. The expression of MOR23 is totally restricted to the expression zone 1 at every section. (Bars = 0.5 mm (left), 250 μm (middle), and 100 μm (right)) (b) Schematic drawings of the expression units of the recombinant adenovirus vectors used in this study. Regions corresponding to the mature transcripts are indicated by boxes. The bicistronic adenovirus expression unit (Upper) contains MOR23 gene, IRES sequence, and GFP gene followed by the rabbit β-globin polyadenylation signal (GpA). The monocistronic expression unit (Lower) contains the GFP gene followed by GpA. The IRES allows internal translation initiation, resulting in expression of both MOR23 and GFP. These individual expression units are located at the E1A-deleted region and the bicistronic (Right) recombinant adenovirus vectors. (Left) Under light microscopy. (Center and Right) Under fluorescent illumination. There are no significant differences in the infected regions throughout the olfactory epithelium between two constructs. (Bars = 0.5 mm.) (d) Higher magnification of MOR23 virus-infected cells. The fluorescent cells are predicted to be olfactory neurons. (Bar = 100 μm.) (e) Tissue-printing of the GFP fluorescent olfactory epithelium result in identification of MOR23-infected individual single olfactory neurons under fluorescence illumination. (Bar = 100 μm.)

myrcenol share a common tertiary alcohol unit in lyral molecules. None of these compounds was recognized by MOR23 in Ca\textsuperscript{2+}-responsive assays at either 100 μM (Fig. 4) or 1 mM concentrations (data not shown) except lyral whereas HC and HC dimethyl acetal evoked Ca\textsuperscript{2+} increase at 10 mM concentrations (Fig. 4). These results implicate that ligand specificity of MOR23 is highly defined to the presence of both functional units in lyral, although the main functional group recognized by the MOR23 binding pocket is the tertiary alcohol moiety. It should be noted, however, that quantitative comparison of the affinities of these ligands is difficult because of the differences in solubilities of these compounds.

To determine the origin of Ca\textsuperscript{2+} increase triggered by lyral-MOR23 interaction, Ca\textsuperscript{2+} recordings in infected neurons were performed in the absence of external Ca\textsuperscript{2+}. Forskolin-mediated Ca\textsuperscript{2+} increase was significantly impaired without external Ca\textsuperscript{2+} (data not shown). Similarly, the Ca\textsuperscript{2+}-free condition completely abolished the lyral-MOR23-mediated elevation of intracellular Ca\textsuperscript{2+} whereas the same cell responded to lyral after the exchange of buffer to Ca\textsuperscript{2+} containing Ringer’s solution (Fig. 5). These results indicate that lyral binding to the MOR23 receptor leads to external Ca\textsuperscript{2+} influx through cell surface channels. The treatment of the cells with thapsigargin, a Ca\textsuperscript{2+}-ATPase inhibitor that depletes intracellular Ca\textsuperscript{2+} stores, did not affect the Ca\textsuperscript{2+} increase without external Ca\textsuperscript{2+} (data not shown). The Ca\textsuperscript{2+}-free condition completely abolished the lyral-MOR23-mediated elevation of intracellular Ca\textsuperscript{2+} whereas the same cell responded to lyral after the exchange of buffer to Ca\textsuperscript{2+} containing Ringer’s solution (Fig. 5). These results indicate that lyral binding to the MOR23 receptor leads to external Ca\textsuperscript{2+} influx through cell surface channels. The treatment of the cells with thapsigargin, a Ca\textsuperscript{2+}-ATPase inhibitor that depletes intracellular Ca\textsuperscript{2+} stores, did not affect the Ca\textsuperscript{2+} increase without external Ca\textsuperscript{2+} (data not shown).

Conventional expression studies to screen ligands recognized by a receptor of interest have been a laborious task in the case of olfactory receptors because of the vast range of odorant molecules. In this study, functional expression and ligand screening of odorant receptor was greatly facilitated by the fact that the candidate ligand was already specified during the case of olfactory receptors because of the vast range of odorant molecules. In this study, functional expression and ligand screening of odorant receptor was greatly facilitated by the fact that the candidate ligand was already specified during the functional identification of the receptor from single olfactory neurons. In addition, our approach enables the screening of receptors for an odorant of interest at single cell level. Therefore, isolation of several receptors, which respond to a certain odorant, and their homology search will provide information on not only ligand-specificity of the receptors but also aspects

Fig. 2. Localization of the endogenous MOR23 gene and expression of the recombinant adenovirus-mediated transgene in olfactory epithelium. (a) In situ hybridization of the MOR23 transcript in coronal sections of the olfactory epithelium. The antisense RNA for the MOR23 coding region was used as a probe. The presence of the transcripts was indicated by dispersed focal staining shown by arrows. The expression of MOR23 is totally restricted to the expression zone 1 at every section. (Bars = 0.5 mm (left), 250 μm (middle), and 100 μm (right)) (b) Schematic drawings of the expression units of the recombinant adenovirus vectors used in this study. Regions corresponding to the mature transcripts are indicated by boxes. The bicistronic adenovirus expression unit (Upper) contains MOR23 gene, IRES sequence, and GFP gene followed by the rabbit β-globin polyadenylation signal (GpA). The monocistronic expression unit (Lower) contains the GFP gene followed by GpA. The IRES allows internal translation initiation, resulting in expression of both MOR23 and GFP. These individual expression units are located at the E1A-deleted region and the bicistronic (Right) recombinant adenovirus vectors. (Left) Under light microscopy. (Center and Right) Under fluorescent illumination. There are no significant differences in the infected regions throughout the olfactory epithelium between two constructs. (Bars = 0.5 mm.) (d) Higher magnification of MOR23 virus-infected cells. The fluorescent cells are predicted to be olfactory neurons. (Bar = 100 μm.) (e) Tissue-printing of the GFP fluorescent olfactory epithelium result in identification of MOR23-infected individual single olfactory neurons under fluorescence illumination. (Bar = 100 μm.)
of evolution of thousands of olfactory receptor proteins. We have not been able to identify additional odorant receptor genes from cells responding to lyral. But it is reasonable to think that there exist more receptors that recognize lyral with different ligand specificities. Further analysis of lyral-responsive cells is necessary to address structural aspects of the ligand-receptor interaction and tuning mechanisms that allow determination of the specific aroma.

In addition to the MOR23 gene, we have so far cloned 15 other odorant receptor genes from the 220 cells that responded to some of the 11 tested odorants such as eugenol, cresol, and ethyl vanillin. Reconstitutions of these receptors are currently in progress. There are a couple of reasons for being unable to isolate odorant receptors from all responsive cells. First, we had to eliminate 25% of samples because of contamination by neighboring cells during picking up of the responsive cell. Second, according to the PCR analyses using glucose-3-phosphate dehydrogenase, RNA isolation and subsequent reverse transcription were not successfully performed in 40% of samples. Third, even though we used degenerate

![Figure 3](image1.png)

**FIG. 3.** Characterization of odorant responses in MOR23-adenovirus infected cells. (a) Lyral-mediated Ca\(^{2+}\) increase in MOR23 adenovirus-infected cells shown by changes in fura-2 fluorescence intensity ratios (340/380 nm) (1 mM lyral application for 10 sec at the indicated bars). (Upper) Bicistronic MOR23-IRES-GFP. (Lower) Monocistronic GFP. High KCl buffer (HK) and forskolin (FK) (10\(^{-5}\) M) were applied at indicated bars (5 sec) to assess cell viability and olfactory neuronal properties. (b) Ca\(^{2+}\) increase in MOR23-adenovirus infected cells was induced by lyral-containing odorant mixture (mix + LY) but not by the mixture without the lyral (mix – LY). The mixture contains the same odorants used in Fig. 1b (10 or 100 \(\mu\)M). (c) Dose-dependent Ca\(^{2+}\) increase as a percentage of the response at 10 mM lyral. Data ±SE (\(n = 16\) for 1 mM, \(n = 5\) for others).

![Figure 4](image2.png)

**FIG. 4.** Ligand specificity of MOR23. Ca\(^{2+}\) increase in MOR23-expressing cells was induced by some odorants structurally similar to lyral at higher concentrations. MA, myraec aldehyde; DM, dihydro-myricerol; HC, hydroxycitronellol; HCA, hydroxycitronellol dimethyl acetal; TM, tetrahydromyrcenol. MOR23 responds to HC and HC dimethyl acetal (HCA) at 10 mM but not at 100 \(\mu\)M. Common structural motifs are shown by boxes. LY is a mixture of two isomers: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene carbaldehyde and 3-(4-hydroxy-4-methylpentyl)-3-cyclohexene carbaldehyde. MA is also a mixture of two isomers, which are the dehydrated forms of LY (hydroxy MA). Only 4-substituted isomers of LY and MA are depicted in the figure.

<table>
<thead>
<tr>
<th>high KCl*</th>
<th>Forskolin†</th>
<th>Lyral response</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>+</td>
<td>0 ((n = 17))</td>
</tr>
<tr>
<td>MOR23</td>
<td>+</td>
<td>0 ((n = 14))</td>
</tr>
<tr>
<td>+Thapsigargin</td>
<td>+</td>
<td>16 ((n = 22))</td>
</tr>
<tr>
<td>+Thapsigargin</td>
<td>+</td>
<td>0 ((n = 19))</td>
</tr>
<tr>
<td>+Thapsigargin</td>
<td>+</td>
<td>7 ((n = 11))</td>
</tr>
</tbody>
</table>

GFP, monocistronic GFP adenovirus vector; MOR23, bicistronic MOR23-IRES-GFP vector.

*High KCl-responsive viable cells are 30–60% of total tissue-printed fluorescent cells, depending on preparation.

†The 50–60% of high KCl-responsive cells elicit Ca\(^{2+}\) increase on forskolin stimulation (10\(^{-5}\) M).
of odorant receptor from single olfactory neurons, avoided the presence of the endogenous receptor for the odorant in the olfactory neurons. Caution has to be taken herein to avoid the presence of the endogenous receptor for the odorant response observed in the original olfactory neuron and to confirm the correlation of the cloned receptor with the odorant response. Both their study and ours clearly support the notion that an adenovirus vector is a useful tool to functionally express odorant receptors in the olfactory neurons. Caution has to be taken herein to avoid the presence of the endogenous receptor for the odorant under study. In our study, the number of the neurons expressing the transgene were clearly distinct from those of the endogenous receptor. Thus, the possibility of mistaking endogenous signaling in infected cells was minimized.

In summary, this study demonstrates the functional identification of odorant receptor from single olfactory neurons, using Ca\(^{2+}\) imaging and single cell RT-PCR followed by functional expression of the receptor using biestronic recombinant adenoviruses. The reconstitution of MOR23 in olfactory neuron receptors proved that the lyral response, which was originally observed in the tissue-printed single olfactory neuron, was indeed derived from endogenous MOR23-lyral interaction and that the type of an odorant receptor expressed by a single olfactory neuron correlates with the physiological response of the cell to the specific odorants. MOR23 also recognized compounds structurally related to lyral, suggesting that odorant receptors possess a receptive range based on structural determinants in odorant molecules. This strategy for identifying receptors for a particular odorant of interest should prove to be a powerful method for studying complex odorant(s)-receptor interactions. The present approach also could apply to other receptor systems in which potential subtypes of the receptor are differentially expressed in tissues containing heterogenous cell types. Further, agonist-directed screening of the receptor could lead to identification of new receptor subtypes with different pharmacological properties. Currently, we are screening more receptors from single neurons that respond to specific odorant(s) to define tuning mechanisms that allow various odorants to be discriminated by the odorant receptors in the olfactory neurons.

**Note Added in Proof.** After this paper was submitted, Krautwurst *et al.* (26) reported functional expression of a chimeric–receptor library in HEK293. Both their approach and ours enable us to study receptor–odorant interactions by providing a system for isolating receptors that are specific to a certain odorant molecule.

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