Functional Expression of Odorant Receptors of the Zebrafish *Danio rerio* and of the Nematode *C. elegans* in HEK293 Cells

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Abstract

Odorant receptors of zebrafish and *C. elegans* were functionally expressed in vertebrate kidney cells (HEK293) using the eucaryotic expression vector pSMyc. Receptor-encoding cDNA cloned into this vector was expressed as a fusion protein with the N-terminal membrane import sequence of the guinea-pig serotonin receptor followed by a myc tag. Immunocytochemical evidence indicates that this strategy directs a protein with the predicted immunoreactivity and approximate molecular weight to the plasma membrane. Fish food extract (TetraMin) evoked a transient increase in intracellular $[\text{Ca}^{2+}]$ in HEK293 cells transiently transfected with plasmids containing cDNA for three fish odorant receptors and converted to stable cell lines. The effect of the extract was concentration dependent and limited to the fraction of the extract <5 kDa. Pretreating the transfected cells with the PLC inhibitor U73122 reduced the odor-evoked signal. Fish food extract also evoked a transient increase in intracellular $[\text{Ca}^{2+}]$ in HEK293 cells transiently transfected with plasmids containing cDNA for single fish odorant receptors. Diacetyl evoked a transient increase in intracellular $[\text{Ca}^{2+}]$ in HEK293 cells transiently transfected with plasmids encoding the cDNA of ODR10, an odorant receptor of *C. elegans* suggested in other work to be specific for diacetyl. These results strongly imply that odorant receptors can be functionally expressed in HEK293 cells using this novel expression protocol. Chem. Senses 22: 467–476, 1997.

Introduction

Our knowledge about the mechanisms by which olfactory stimuli in vertebrates are detected and transduced into electrical cell signals has increased considerably in the last few years. The application of a variety of modern techniques has resulted in the identification and characterization of the molecular components of the signaling pathways. There is now overwhelming evidence that olfactory transduction for many odorants occurs via two different second messengers, the cAMP and the IP$_3$ systems (Pace *et al.*, 1985; Restrepo *et al.*, 1993), each of which includes a specific receptor protein, a G-protein, a specific enzyme, adenylate cyclase or phospholipase C (PLC), and a second messenger gated ion channel. Recent data from knockout experiments indicate that the cAMP...
pathway plays an essential role for the sense of smell in mice.

One of the most interesting components of the olfactory signal transduction cascade is the protein that interacts directly with odorant molecules. A multigene family that is likely to encode odorant receptor proteins (ORP) has been identified in the rat (Buck and Axel, 1991). Northern blot analysis showed that expression of this gene family is restricted to the olfactory epithelium. The putative ORPs share all characteristic features of the superfamily of G-protein-coupled receptors with seven transmembrane domains (Buck and Axel, 1991). The proof that these proteins are odorant receptors is still elusive. They have also been proposed to guide olfactory axons to their specific target glomeruli (Mombaerts et al., 1996).

Only limited attempts have been made to examine recombinant ORPs expressed in non-neuronal cells. ORPs of the rat cloned in the baculovirus system have been expressed in an SF9 cell line derived from the insect Spodoptera frugiperda. Membranes prepared from these cells showed an odor-induced increase in IP$_3$ in rapid quench experiments (Raming et al., 1993). So far, however, all attempts have failed to achieve a functional expression of members of the vertebrate odorant receptor gene family in intact vertebrate cells. In general, the functional expression of G-protein-coupled seven transmembrane receptors in HEK293 cells is possible and has been shown for dopaminergic (D2) (Watts and Neve, 1996), muscarinergic (m1 and m3) and adrenergic (α$_2$) receptors (for a review see Gudermann et al., 1996). The failure to detect physiological responses of recombinant ORPs might have several reasons: high specificity for ligands, the absence of odorant binding proteins, a lack of incorporation of the protein in the cell membrane or the absence of functionally essential compounds.

Therefore, we tried to establish a system in which most of these problems might be solved. We used an odorant receptor of C. elegans presumed from mutant studies to respond to the odorant diacetyl (Sengupta et al., 1996). We also investigated odorant receptors from fish since identification of the proper ligands ought to be easier due to the much smaller size of the odorant receptor gene family (Weth et al., 1996) and the availability of several classes of physiologically relevant odorants. The ORPs cloned so far do not possess an N-terminal membrane import sequence. Therefore the receptor-encoding cDNAs were connected to a membrane import sequence of the serotonin receptor to increase the probability of membrane incorporation. For expression a human embryonic kidney cell line (HEK293) was used. This cell line possesses the molecular components of both of the second messenger cascades, the cAMP and the IP$_3$ system (Fukuyama et al., 1996). In experiments with this expression system we were able to functionally express receptor proteins of both zebrafish and nematode, as evidenced by an increase in intracellular calcium.

**Materials and methods**

**Plasmid constructs**

$pSMyc$

A 130 bp PCR product encoding the membrane import sequence of the 5HT$_3$ receptor [23 amino acids (aa)] of the guinea-pig in frame with the 12 aa peptide MEQKLISEEDLN of the human c-myc gene (Evan et al., 1985) was obtained in a PCR containing Pfu-DNA polymerase chain reaction (PCR) buffer (Stratagene, Heidelberg, Germany), 1.5 mM MgCl$_2$, 0.2 mM of each dNTP, 1 ng 5HT$_3$ cDNA from guinea-pig cloned in pRc/CMV (Invitrogen, NV Leek, The Netherlands), 0.5 mM primer P1 and P2, and 2.5 U Pfu polymerase (Stratagene). PCR amplification was performed according to the following schedule: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, for 30 cycles.

The PCR product was digested with HindIII–XbaI and the resulting 123 bp fragment was subcloned in pRc/CMV (Invitrogen) previously digested with HindIII–XbaI.

pl6/6, pl2/13, pl4/3 and pODR10

PCR products of odorant receptors were obtained under the same PCR conditions as described above with plasmids ZOR3C, ZOR2A and ZOR8C coding for odorant receptors of the zebrafish as a template, gene specific primers 5′ (P3, P4, P5) and a plasmid specific primer 3′ (P6). The ODR10 encoding PCR product was obtained with cloned cDNA as a template and primer P7 5′ and primer P8 3′. The PCR was performed according to the following schedule: 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min for 30 cycles, with the final extension lengthened to 10 min. The PCR products were digested with XbaI and subcloned in pSMyc previously digested with XbaI. The plasmids pl6/6 (ZOR8C), pl2/13 (ZOR3C), pl4/3 (ZOR2A) and pODR10 were identified by restriction enzyme analysis and completely sequenced using the Abi system.
Expression of Zebrafish and Nematode Odorant Receptors

PI: GCTCTAGATTCAGGTCCTCCTCACTGATCAGC- 
TTCTGCTCCATGTTAACTTCTCCTTGTGCCAGGGA
P2: CCCAAGCTTGCCACCATGGTGGTGTGGCTCC-
AGCTG
P3: GCTCTAGAGATGGAAAATAATACCAATTTTAA-
CTTCATG
P4: CGTCTAGAGATGGTGATTGTCAGAGCTGTTA-
ATCTGTTG
P5: GCTCTAGAGATGCTGTATTTTATACACACAGA-
AAGAGGA
P6: ACCACAGAAGTAAGGTTCCTTCACAAAGATCC
P7: CGTCTAGAGTCGGGAGAATTGTGGATT
P8: GCTCTAGACATTCTCATGACAAGTCCAATG
Xbal sites are indicated by underlining.

Cell culture and transfection of HEK293 cells
HEK293 cells were grown at 37°C in MEM (Gibco) supplemented with 10% heat-inactivated fetal calf serum, in a humidified 95% air, 5% CO2 incubator. Semiconfluent cells were transfected in 35 mm dishes (Falcon) by using the CaP-precipitation technique as described previously (Zufall et al., 1993), using supercoiled plasmid coding for odorant receptors. Efficiency of transfection, typically <10%, was checked histochemically with reporter plasmid pCH 110 (Pharmacia, Freiburg, Germany) coding for β-galactosidase. Measurements were done 48–72 h post-transfection. The stable cell line HEK-OR was obtained by co-transfection of HEK293 cells with the plasmids pl6/6, pl2/13 and pl4/3 and selection by treatment with G418 (500 mg/l). The mock transfected stable cell line HEK-M was obtained under the same conditions as described above by transfection of HEK293 cells with the plasmid pSMyc (without the cDNA of ORPs).

Immunohistochemistry
Cells were fixed at 60°C for 20 min in 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde. Cells were incubated for 1 h with the Myc1-9E10 (Evan et al., 1985) antibody (2 µg/ml) and, after washing three times for 10 min with phosphate-buffered saline, for 1 h with the anti-mouse TRITC antibody (Sigma, Deisenhofen, Germany) (2 µg/ml).

To permeabilize the cells, they were incubated with Triton X-100 (0.05%) as described previously (Mukerji et al., 1996).

SDS–PAGE/Western blot
Protein samples in Lämml (62.5 mM Tris, pH 6.8, 10% glycerine, 2% SDS, 5% 2-mercaptoethanol, 0.1 g/l Pyronine Y) were incubated at 95°C for 3 min and centrifuged (13 000 g) for 5 min. The samples were subjected to a 10% SDS–PAGE (Lämml, 1970). Proteins were transferred to nitrocellulose as described previously (Towbin et al., 1979). Non-specific binding sites were blocked by incubation with blocking buffer (blocking reagent in PI, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5; Boehringer Mannheim). The incubation with the Myc1-9E10 antibody (20 µg/ml) was followed by two washes with buffer PI. Blots were then incubated with alkaline-phosphatase-conjugated goat anti-mouse antibody (1:30 000) and washed as described above. Bound antibodies were visualized by means of a substrate solution containing 0.015% nitro-blue tetrazolium and 0.007% 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris, 100 mM NaCl, pH 9.5. The reaction was stopped by incubation in PI.

Immunoprecipitation
Myc1-9E10 antibody was coupled to cyan bromide–Sepharose according to the manufacturer’s instructions (Pharmacia). Cells were broken by incubation in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 40 mM Na-fluoride, 5 mM EDTA, 5 mM EGTA, 1% P40, 0.1% Na-desoxycholate, 0.1% SDS) for 30 min on ice. Unbroken cells were removed by centrifugation for 5 min at 13 000 g. The supernatant was incubated with 10 µl of Sepharose-coupled antibody for 4 h at room temperature. After the pellet had been washed six times with lysis buffer it was treated with sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerine, 0.1 g/l Pyronine Y). The sample was analyzed by SDS–PAGE.

Ca2+-imaging: fura-2 measurements
Prior to an experiment the culture medium was removed and replaced by a standard experimental solution (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 10 mM HEPES, 5 mM glucose, pH 7.4) containing fura-2/AM (5–10 µM) (Molecular Probes Europe BV, Leiden, Netherlands). In some experiments a Ca2+-free standard solution was used: 140 mM NaCl, 5.4 mM KCl, 2 mM EGTA, 1.0 mM MgCl2, 10 mM HEPES, 5 mM glucose,
Results

Immunohistochemistry
To localize the receptor protein in the plasma membrane the cell line HEK-OR, stably co-transfected with the plasmids pl6/6, pl2/13 and pl4/3, each encoding for a different ORP, was used for immunohistochemical experiments. The cell line HEK-M, stably transfected with pSMyc, served as a control. Intact cells were treated with the Myc1-9E10 antibody raised against the N-terminal extracellular myc-epitope. The immunofluorescence signal was not visible throughout the cytosol, but rather formed a characteristic ring of labeling around the HEK-OR cell surface (n = 6) (see Figure 1, left). We detected no labeling signal in HEK-M cells (n = 5) (see Figure 1, right). Permeabilizing the HEK-OR cells by pretreatment with Triton X-100 caused the antibody to stain the cells more homogeneously (data not shown). These results also show that the receptor proteins are located in the plasma membrane with their N-terminus on the extracellular side.

Western blot experiments
To analyse the molecular mass of the expressed receptor proteins cells of HEK-OR were lysed and receptor proteins were enriched by immunoprecipitation. Samples were analyzed by SDS page and the proteins were transferred to nitrocellulose. Receptor proteins were visualized by incubation with the Myc1-9E10 antibody. In Western blot experiments of HEK-OR immunoprecipitations a band with an average molecular mass of 50–53 kDa (see Figure 2) was detected. Immunoprecipitation of the mock transfected cell line HEK-M showed no band.

Ca^{2+}-imaging

Functional expression
Using biochemical and immunohistochemical methods we were able to show that recombinant ORPs are expressed in the plasma membrane of HEK293 cells. It was therefore possible to analyze the function of these receptors by measuring the cell responses to chemical stimuli with the Ca^{2+}-imaging technique. Recent biochemical studies on isolated olfactory neurons of fish have shown that some odorants elicit a cAMP response whereas others induce IP_3 generation followed by a Ca^{2+}-increase (Restrepo et al., 1996). In addition, cross talk between the two systems could
Figure 1  Immunolocalization of the myc-epitope fused to the olfactory receptors with the Myc1-9E10 antibody. Left: HEK-OR, right: HEK-M.

Figure 3  Fish food solution-induced $\text{Ca}^{2+}$-changes in various transfected cells. The different shapes of the signals reflect the arrangement of HEK cell clusters in a dish. Individual cells are marked. $\text{Ca}^{2+}$-changes are shown in pseudo colors. Basal $\text{Ca}^{2+}$-distribution (left) is compared with a TetraMin stimulated state at maximum $\text{Ca}^{2+}$ response (middle). The integrated fluorescence ratio ($F_{540}/F_{580}$) for one cell (see white circle) measured over time is also shown (right). The arrows indicate the beginning of fish food application (2 s). (A) $\text{Ca}^{2+}$-transients in transiently transfected HEK293 (p6G6); (B) $\text{Ca}^{2+}$-measurement in stably transfected HEK-OR; (C) in stably transfected HEK-M cells fish food extract failed to activate a $\text{Ca}^{2+}$-signal.
be seen leading to an increase in cAMP and Ca²⁺ (for reviews see Ache, 1994; Hatt, 1996). Because both second messenger pathways can mediate an increase in the cytosolic [Ca²⁺], measuring the intracellular [Ca²⁺] changes allowed us to detect odor responses irrespective of the particular signal transduction pathway.

In a first set of experiments we applied TetraMin to HEK-OR, the stable cell line transfected with three odorant receptors. In all cells measured (n = 75) application (2 s) of TetraMin induced a transient Ca²⁺-signal: a more rapid intracellular Ca²⁺-increase, followed by a slower decay phase to the basal level (see Figure 3B). After 3–4 min washout the Ca²⁺-signals were fully reproducible (data not shown). With a lower concentration of TetraMin (1:10) the amplitude of the Ca²⁺-increase was reduced, as was the rise time. TetraMin diluted by a factor of 1000 was completely ineffective (Figure 4). TetraMin that had been heated (100°C) for 10 min was just as effective (n = 4) as the untreated solution. The Ca²⁺-signal induced with fish food solution was similar in shape to those elicited by Ca²⁺-ionophores and reached ~80% of the amplitude produced by ionophores (data not shown).

TetraMin solution with a molecular weight of <5 kDa elicited a Ca²⁺-signal (Figure 5). The smaller amplitude compared with the control solution can be explained by dilution of the stimulus concentration during the column separation. TetraMin solution with a molecular mass >5 kDa had no effect (Figure 5).

In contrast to the stably transfected cell line (HEK-OR), only a few transiently transfected cells showed a Ca²⁺-signal, each containing a single receptor-encoding plasmid (p16/6, p12/13 and p14/3) (Figure 3A). The low number of responding cells correlates with the weak transfection rate (<10%), determined by co-transfection of pCH110 (data not shown). Each of the three receptors induced a Ca²⁺-signal.

To exclude the possibility that the plasmid itself induces the Ca²⁺-increase, we applied TetraMin to cells transfected with pSMyc without receptor cDNA. In none of 92 cells measured could we detect any increase of [Ca²⁺] in response to TetraMin application (see Figure 3C).

As a control for the ability of such cells to increase [Ca²⁺] in general, we applied ATP (100 µM), activating presumably the P2Y type of ATP receptor, which is coupled to the IP₃ pathway (Hansen et al., 1993). A strong Ca²⁺-signal was regularly seen (n = 4) (data not shown).
Expression of Zebrafish and Nematode Odorant Receptors

Specific ligand
Next we tried to find out the specific ligand for the fish odorant receptors. Electrophysiological experiments on the olfactory organ of fish have shown that many amino acids, bile acids and some hormones are stimulatory (Kang and Caprio, 1995; Michel and Lubomudrov, 1995). Therefore we tested the following pure compounds, both individually (10 and 100 nM) and in mixture: the L-amino acids alanine, proline, leucine, phenylalanine, serine, lysine and threonine, the bile acids l-taurine and d-taurine, as well as progesterone. Mixtures of all of them were used as well as submixtures of 2–4 of those compounds. We did not elicit any Ca$^{2+}$-increase in HEK-OR or in transiently transfected cells with a single receptor expressed with any of these stimuli.

Because we were unable to detect a specific agonist for the fish receptors, we used the ODR10 receptor of C. elegans, for which diacetyl has been postulated to be agonistic (Sengupta et al., 1996). To generate pODR10 the cDNA of ODR10 was subcloned in pSMyc and transiently transfected into HEK293 cells and the response of the cells to diacetyl was tested. Application of diacetyl (100 nM) induced a strong increase in [Ca$^{2+}$] in about 10% of the cells (Figure 6). The number of responding cells correlated with the transfection rate. This is a clear indication that the ODR10 receptor is functionally expressed in HEK293 and the first demonstration that recombinant odorant receptors can be activated in a mammalian cell line by a specific ligand.

Transduction pathway
To evaluate the transduction pathway responsible for the Ca$^{2+}$-increase and to explore the source of the Ca$^{2+}$-ions that contribute to the odorant-induced elevation of intracellular Ca$^{2+}$-levels, various inhibitors were employed.

First we tested whether the Ca$^{2+}$-increase is based on release from intracellular stores or on an influx from the extracellular side via Ca$^{2+}$-permeable channels. When the standard extracellular solution was replaced by a Ca$^{2+}$-free solution, the HEK-OR cell response was not affected either in the amplitude of the TetraMin-induced Ca$^{2+}$-increase or in its time course (n = 2) (data not shown), suggesting that the [Ca$^{2+}$] is increased by release from intracellular stores.

One possible signal pathway for increasing [Ca$^{2+}$] involves the G-protein-induced activation of IP$_3$ via PLC. Incubation of HEK-OR with the specific PLC inhibitor U73122 (Wu et al., 1992) (10 nM) for 10 min led to a significant reduction of the Ca$^{2+}$-increase following TetraMin stimulation (Figure 7, n = 3). The effect was reversible within 10 min. Incubation of the HEK-OR cells with 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of the phosphodiesterase, which should produce a prolonged elevation of cAMP concentration, had no effect on the Ca$^{2+}$-signal (n = 4, data not shown).

Discussion
Our data provide the first documentation of the functional expression of ORPs in the plasma membrane of a vertebrate cell line. Using fish food (TetraMin) as the
stimulus, we activated odorant receptors of zebrafish expressed in human embryonic kidney cells (HEK293), as evidenced by a rapid, transient increase of the intracellular \( \text{Ca}^{2+} \) in cells carrying the receptor protein in the membrane. A transient increase of \( \text{[Ca}^{2+}] \) after odorant stimulation has also been seen in native chemosensory neurons isolated from the olfactory epithelium of fish (Restrepo et al., 1990), frog (Restrepo et al., 1996) and rat (Tareilus et al., 1995).

The first hurdle to be overcome for functional expression was to import the expressed ORPs into the plasma membrane in the proper orientation. It is known that an N-terminal signal sequence (Gierasch, 1989) is responsible for the import of directly gated ion channels into the plasma membrane. Having recently cloned the 5HT\(_3\) receptor from guinea-pig, we fused the import sequence from the 5HT\(_3\) receptor to the extracellular N-terminal of the odorant receptor in the hope that the serotonin receptor import sequence would function similarly in our expression system. In addition a myc tag sequence was fused, so that the location of the receptor could be determined by means of immunohistochemistry. It has been shown for the 5HT\(_3\) receptor that a myc tag fused to the N-terminal of the protein did not influence the functional properties (Mukerji et al., 1996). Labeling of intact HEK-OR cells using the Mycl-9E10 antibody showed staining restricted to the outer plasma membrane of all cells in a dish. To exclude the possibility that the antibody labels cytosolic receptor proteins because of cell damage caused by transfection or receptor expression, we compared intact and artificially damaged (permeabilized) HEK-OR cells. The labeling pattern of permeabilized cells was more homogeneous. The result demonstrates that unpermeabilized cells have an undamaged cell membrane and thus the staining of these cells is caused by labeling of an extracellular domain of the immunoreactive protein.

The Myc1-9E10 antibody has also been used to identify the heterologously expressed ORPs in Western blots of immunoprecipitated HEK-OR cells. The apparent molecular mass of the immunoreactive protein (50–53 kDa) was found to be significantly larger than the mass deduced from the amino acid sequence (34, 36 and 39 kDa for the three fish receptors cloned) predicted. This discrepancy might be due to a glycosylation. Interestingly, however, it is similar to that of ORPs in native cells of the rat olfactory epithelium (50 kDa) (Krieger et al., 1994). In our stable cell line HEK-OR, used in these experiments, three different odorant receptors were co-expressed to increase the probability of finding the specific ligand for activation. All three are immunoreactive to the same antibody. This may explain the indistinctness of the visible band in the Western blot.

Once it was established that the receptor protein was in the membrane, the next step was to show its functional activation by an odorant. Odor-induced increases in \( \text{[Ca}^{2+}] \) are thought to control several elements of the transduction cascade (Tareilus et al., 1995). Therefore we monitored increased \( \text{Ca}^{2+}\)-concentration as a measure of odorant receptor activation. Intracellular accumulation of fura-2/AM might lead to the AM-ester entering organelles, causing the ratios to reflect other than cytosolic \( \text{[Ca}^{2+}] \). This would, however, be expected to decrease the response amplitudes of \( \text{[Ca}^{2+}] \) and should not affect the general validity of the \( \text{[Ca}^{2+}] \) increase, we observed. The \( \text{[Ca}^{2+}] \) increases, as well as the corresponding decay time constants we measured (Figures 3–7) are slow. The reasons for this are unclear. Possibly \( \text{Ca}^{2+} \) is taken up by mitochondria during stimulation and slowly released afterwards. Odor-induced Ca-signals in native rat olfactory receptor neurons, however, are equally slow (Tareilus et al., 1995).

After application of fish food (TetraMin) cells transfected with an odorant receptor of zebrafish responded with a fast and transient increase of the intracellular \( \text{Ca}^{2+}\)-concentration. In culture dishes with the stable cell line HEK-OR all cells showed a \( \text{Ca}^{2+} \)-signal, whereas in dishes with cells transiently transfected with one of the three odorant receptors <10% did so, correlating with the low transfection rate.

Differences in the amplitude of the response of the three receptor types were never larger than those observed in the responses of any one type and fell within the range of variability assignable to differences in fura-2 loading. The three receptors would not necessarily be expected to differ in their sensitivity, however, given the complexity of the fish food extract. HEK293 cells without receptors or those transfected with the pSMyc (HEK-M) showed no \( \text{Ca}^{2+} \)-increase after TetraMin stimulation.

We used fish receptors for the expression studies because the number of agonists described on the basis of electrophysiology (Michel and Lubomudrov, 1995) (several amino acids, bile acids and some hormones) and the number of different receptors estimated by several techniques (Weth et al., 1996) seem to be an order of magnitude lower than in mammals. This was expected to increase the chance of finding a specific agonist. However, none of the amino acids...
tested in mixtures and as single components increased the 
\([\text{Ca}^{2+}]\) of transfected cells, even in millimolar concentra-
tions. The same is true for all the bile acids and the hormone
tested. The reason could be that fish respond to a large
number of unknown chemical stimuli and that only a few
(three out of ~100) receptors (Ngai et al., 1993; Weth et al.,
1996) were examined. We were unable to detect the chemical
components in the fish food that were responsible for the cell
response, other than to say that the stimulus substances
must have a molecular mass of <5 kDa and must be heat
resistant. Expression of additional receptor types and/or a
more detailed chemical analysis of the fish food may help to
identify a specific ligand for fish receptors in the future.

Our data on the activation of the receptor protein from
C. elegans using diacetyl showed clearly that our system is
able to express receptor proteins which can be activated by a
specific ligand, inducing a Ca\(^{2+}\)-signal. In the future the next
steps are to determine the sensitivity of the ODR10 receptor,
by constructing dose–response curves, and to establish its
specificity by testing various agonists structurally related to
diacetyl.

Besides its specificity and sensitivity, a functional receptor
protein is characterized by its ability to activate a second
messenger cascade. The chemo-electrical transduction in
olfactory cells typically proceeds through the generation of
two second messengers, notably cAMP and IP\(_3\) (or Ca\(^{2+}\)).
We have shown that the Ca\(^{2+}\)-increase is not due to an
extracellular influx of Ca\(^{2+}\) through Ca\(^{2+}\)-permeable ion
channels. The increase of [Ca\(^{2+}\)] is induced by the activation
of a second messenger cascade: in experiments using an
inhibitor for blockage of PLC, involved in the IP\(_3\) pathway,
the Ca\(^{2+}\)-signal was dramatically reduced, whereas IBMX,
an inhibitor of the phosphodiesterase involved in the cAMP
cascade, had no effect on the Ca\(^{2+}\)-response. These data
suggest that the cAMP pathway is not involved in the
generation of the Ca\(^{2+}\)-signal. In HEK293 cells the
cross-talk between the two second messenger pathways
seems not to be as pronounced as in native olfactory
neurons. In additional patch clamp experiments (whole cell
mode) on cells in which ORPs, Ga\(_{olf}\), and the CNG channel
were co-expressed, we did not elicit any activation of the
cAMP-gated channels by fish food. Forskolin or 8-bromo-
cAMP, however, elicited a strong inward current (un-
published data). These results cannot fully exclude the
activation of the cAMP pathway: cAMP might increase, but
not sufficiently to activate the CNG-channels.

Our data show the functional activation of ORPs of
vertebrates and invertebrates in human embryonic kidney
cell lines. It was demonstrated that odorants (fish food,
diacetyl) lead to an increase in the intracellular [Ca\(^{2+}\)] based
on the activation of specific receptors coupled to a second
messenger cascade. These results now provide an opportu-
nity to obtain detailed insights into second messenger
pathways and the specificity of ORPs.

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