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 [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 [Ca^{2+}] in HEK293 cells transiently transfected with plasmids containing cDNA for single fish odorant receptors. Diacetyl evoked a transient increase in intracellular [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 (Brunet *et al.*, 1996).

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_{2a}) 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 $5HT_3R$ 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 *Hin*dIII–*Xba*I and the resulting 123 bp fragment was subcloned in pRc/CMV (Invitrogen) previously digested with *Hin*dIII–*Xba*I.

pI6/6, pI2/13, pI4/3 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 *Xba*I and subcloned in pSMyc previously digested with *Xba*I. The plasmids pI6/6 (ZOR8C), pI2/13 (ZOR3C), pI4/3 (ZOR2A) and pODR10 were identified by restriction enzyme analysis and completely sequenced using the Abi system.

P1: GCTCTAGATTCAGGTCCTCCTCACTGATCAGC-TTCTGCTCCATGTTAACTTCTCCTTGTGCCAGGGA
P2: CCCAAGCTTGCCACCATGGTGGTGTGGCTCC-AGCTG
P3: GCTCTAGAGATGGAAAATAATACCAATTTTAA-CTTCATG
P4: CGTCTAGAGATGGTGATTGTCAGAGCTGTTA-ATCTGTTG
P5: GCTCTAGAGATGCTGTATTTTATACACACAGA-AAGAGGA
P6: ACCACAGAAGTAAGGTTCCTTCACAAAGATCC
P7: CGTCTAGAGTCGGGAGAATTGTGGATT
P8: GCTCTAGACATTCTCATGACAAGTCCAATG

*Xba*I 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% CO_2 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 Lämmli (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 (Lämmli, 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 P1, 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 P1. Blots were than 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 P1.

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.

Ca^{2+}-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 $CaCl_2$, 1.0 mM $MgCl_2$, 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 Ca^{2+}-free standard solution was used: 140 mM NaCl, 5.4 mM KCl, 2 mM EGTA, 1.0 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose,

pH 7.4. The dishes were placed in the incubator for 35 min. Thereafter the cultures were washed with an excess of fura-2/AM-free solution and placed in the incubator for another 60–100 min in order to allow for cleavage of the ester (Oles *et al.*, 1997). The culture dish was placed on the stage of the microscope and the cells were superfused at 250 µl/min with standard solution at 35°C. Dual wavelength measurements of fura-2 fluorescence were performed using a set-up based on a Zeiss Axiovert microscope to which a custom-made dual-wavelength excitation device was adapted (Bals *et al.*, 1990). The microscope was equipped with a long working distance objective (Achroplan 40, 0.6 corr.; Zeiss, Köln, Germany). Fluorescence was recorded from a single cell by means of a photomultiplier tube (Hamamatsu R4632, Munich, Germany). The wavelength for excitation of the dye was alternated between 340 and 380 nm usually at 5–10 s^{-1}. Photomultiplier signals were background corrected and the ratio (f_{340}/f_{380}) was calculated. Fluorescence signals and the ratio were stored on the hard disc of an AT-computer. Online analysis of the signals was performed by means of custom-made software. Spatio-temporal Ca^{2+}-distributions were investigated using a highly sensitive CCD camera (Photometrics, PXL37). Acquisition and calculation of the fluorescence images were done by a Macintosh-based program, IPlab 3.0. The calculated fluorescence values were displayed in pseudo colors.

Agonists were applied by means of a solenoid-switch-operated superfusion device. The tip diameter of the common outlet tube was 0.5 mm. The half-time of exchange between two solutions achieved by the local perfusion device was ~500 ms.

Odors

Five grams of TetraMin fish food was extracted in 100 ml standard solution for 1 h at 37°C. The solution was centrifuged for 20 min at 20 000 *g*. The supernatant was filtered through a 0.45 µm filter. The pH was adjusted to 7.4 and the osmolarity to 310 mOsm. For experiments in which components with different molecular masses were to be compared, the TetraMin fish food solution was fractionated with Nap10 columns (Pharmacia) according to the manufacturer's instructions.

Diacetyl (Sigma) was made up in stock solutions of 10 mM and stored frozen. Dilutions of that solution were made freshly every day.

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

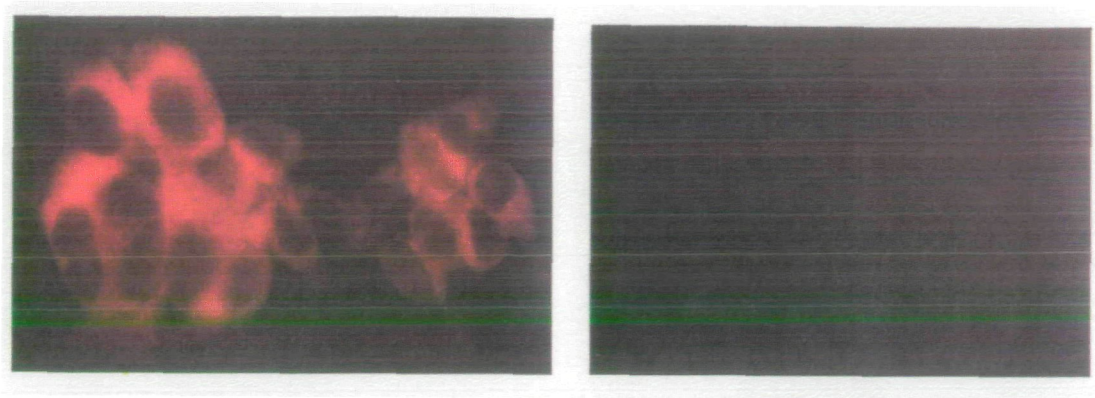

Figure 1 Immunolocalization of the *myc*-epitope fused to the olfactory receptors with the Myc1-9E10 antibody Left HEK-OR, right: HEK-M.

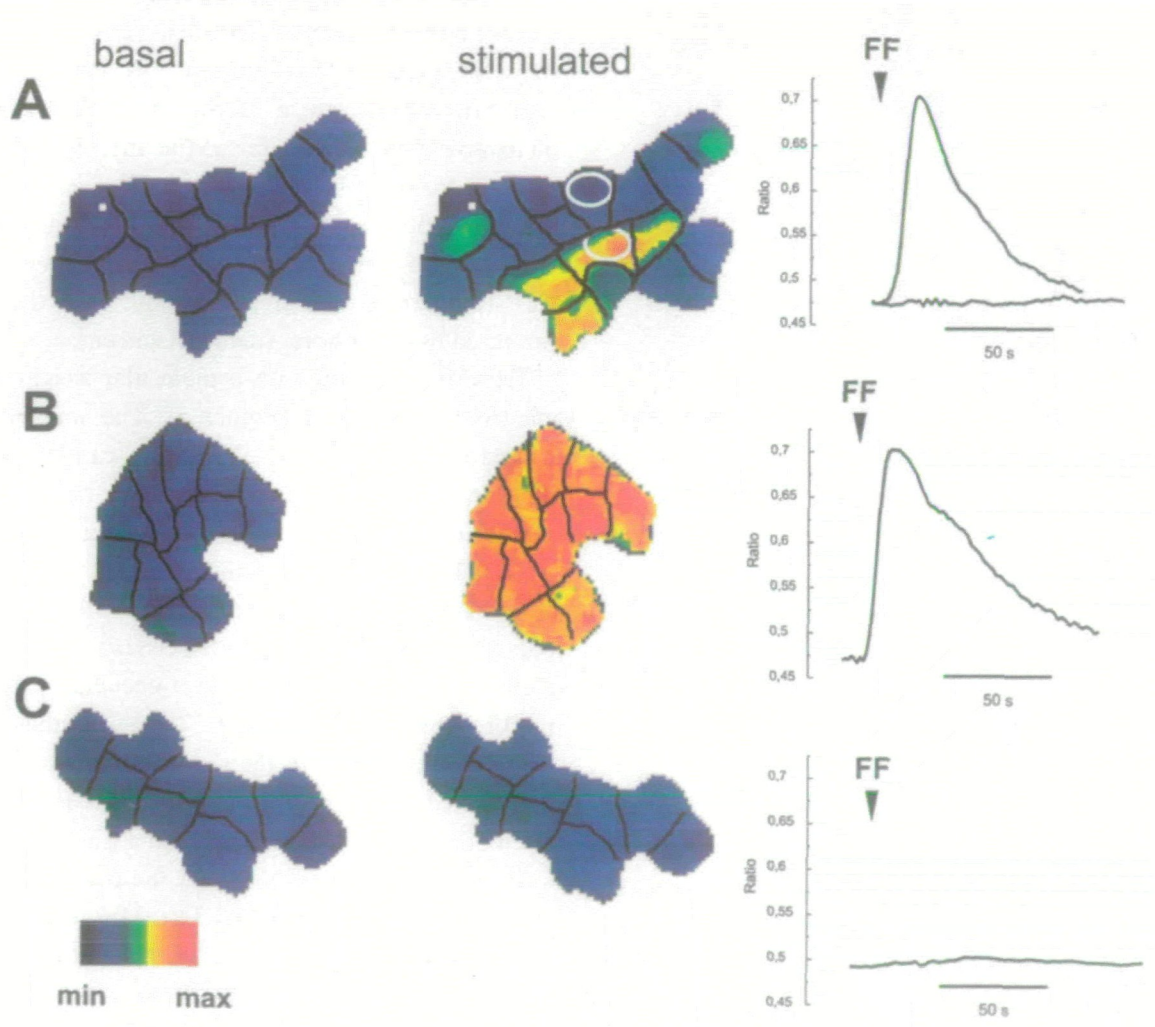

Figure 3 Fish food solution-induced Ca^{2+}-changes in various transfected cells. The different shapes of the signals reflect the arrangement of HEK cell clusters in a dish Individual cells are marked. Ca^{2+}-changes are shown in pseudo colors. Basal Ca^{2+}-distribution (left) is compared with a TetraMin stimulated state at maximum Ca^{2+} response (middle). The integrated fluorescence ratio (f_{340}/f_{380}) 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)** Ca^{2+}-transients in transiently transfected HEK293 (pI6/6); **(B)** Ca^{2+}-measurement in stably transfected HEK-OR, **(C)** in stably transfected HEK-M cells fish food extract failed to activate a Ca^{2+}-signal

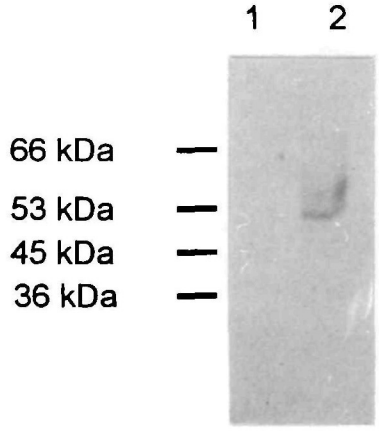

Figure 2 Western blot analysis of the immunoprecipitated olfactory receptors Lane 1 HEK-M, lane 2: HEK-OR.

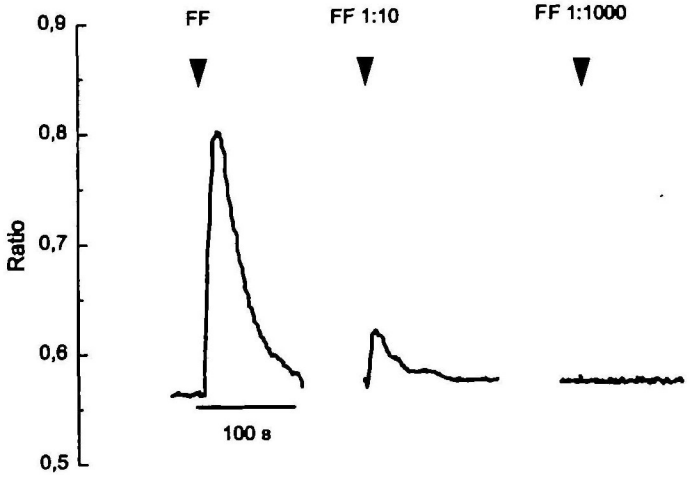

Figure 4 Effect of fish food dilution on the Ca^{2+}-signal The amplitude of the Ca^{2+}-signal decreases with lower fish food extract concentrations. The Ca^{2+}-signals are shown as a function of time for three different dilutions of TetraMin (1:1, 1 10, 1:1000). Only the very high dilution (1·1000) failed to elicit a response The arrows indicate the beginning of the fish food extract (FF) application (2 s).

be seen leading to an increase in cAMP and Ca^{2+} (for reviews see Ache, 1994; Hatt, 1996). Because both second messenger pathways can mediate an increase in the cytosolic $[Ca^{2+}]$, measuring the intracellular $[Ca^{2+}]$ changes allowed us to detect odor responses irrespective of the particular signal transduction pathway.

In a first set of experiments we applied TertraMin 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 Ca^{2+}-signal: a more rapid intracellular Ca^{2+}-increase, followed by a slower decay phase to the basal level (see Figure 3B). After 3–4 min washout the

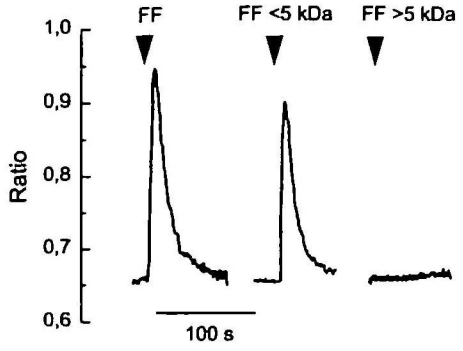

Figure 5 Test of fractionated fish food extract. Only the <5 kDa fraction induced a measurable Ca^{2+}-signal, whereas the >5 kDa fraction showed no Ca^{2+}-transient The arrows indicate the beginning of the fish food extract (FF) application (2 s)

Ca^{2+}-signals were fully reproducible (data not shown). With a lower concentration of TetraMin (1:10) the amplitude of the Ca^{2+}-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 Ca^{2+}-signal induced with fish food solution was similar in shape to those elicited by Ca^{2+}-ionophores and reached ~80% of the amplitude produced by ionophores (data not shown).

TetraMin solution with a molecular weight of <5 kDa elicited a Ca^{2+}-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 Ca^{2+}-signal, each containing a single receptor-encoding plasmid (pl6/6, pl2/13 and pl4/3) (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 Ca^{2+}-signal.

To exclude the possibility that the plasmid itself induces the Ca^{2+}-increase, we applied TetraMin to cells transfected with pSMyc without receptor cDNA. In none of 92 cells measured could we detect any increase of $[Ca^{2+}]$ in response to TetraMin application (see Figure 3C).

As a control for the ability of such cells to increase $[Ca^{2+}]$ in general, we applied ATP (100 μM), activating presumably the P_{2y} type of ATP receptor, which is coupled to the IP_3 pathway (Hansen *et al.*, 1993). A strong Ca^{2+}-signal was regularly seen (n = 4) (data not shown).

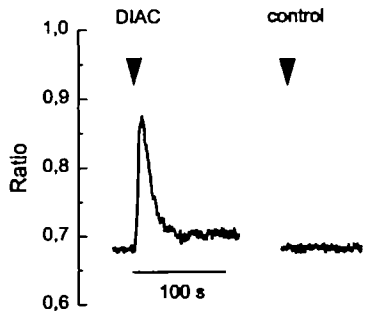

Figure 6 In HEK293 cells, transiently transfected with pODR10 (*C elegans*), superfusion with diacetyl (100 μM) causes a significant increase in $[Ca^{2+}]$. Control cells (HEK-M) showed no Ca^{2+}-signal in response to application of diacetyl The arrows indicate the beginning of the diacetyl (DIAC) application (2 s)

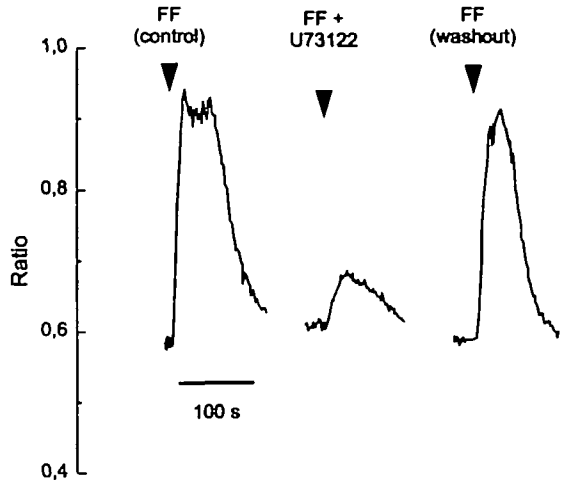

Figure 7 U73122, a specific inhibitor of PLC, reduced the Ca^{2+}-signals induced by fish food extract After a control measurement with fish food extract the cultures were incubated for 10 min with U73122 (10 μM), the resulting significant reduction of the Ca^{2+}-response was reversible after 10 min washout. The arrows indicate the beginning of the fish food extract (FF) application (2 s)

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 μM) and in mixture: the L-amino acids alanine, proline, leucine, phenylalanine, serine, lysine and threonine, the bile acids litocholine and taurocholine, 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 μM) induced a strong increase in the $[Ca^{2+}]$ in about 10% of the cells (Figure 6).The number of responding cells correlates with the transfection rate. This is a clear indication that the ORD10 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 μM) 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 [Ca^{2+}] in cells carrying the receptor protein in the membrane. A transient increase of [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 Myc1-9E10 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 [Ca^{2+}] are thought to control several elements of the transduction cascade (Tareilus *et al.*, 1995). Therefore we monitored increased 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 [Ca^{2+}]. This would, however, be expected to decrease the response amplitudes of [Ca^{2+}] and should not affect the general validity of the [Ca^{2+}] increase, we observed. The [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 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 (Tarelius *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 Ca^{2+}-concentration. In culture dishes with the stable cell line HEK-OR all cells showed a 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 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 [Ca^{2+}] of transfected cells, even in millimolar concentrations. 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 phospodiesterase 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, $G_{olf\alpha}$ 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 (unpublished 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 opportunity to obtain detailed insights into second messenger pathways and the specificity of ORPs.

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REFERENCES

Ache, B.W. (1994) Towards a common strategy for transducing olfactory information. *Cell Biol.*, **5**, 55–63.

Bals, S., Bechem, M., Paffhausen, W. and Pott, L. (1990) Spontaneous and experimentally evoked $[Ca^{2+}]_i$-transients in cardiac myocytes measured by means of a fast Fura-2 technique. *Cell Calcium*, **11**, 385–396.

Brunet, L.J., Gold, G.H. and Ngai, J. (1996) General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. *Neuron*, **17**, 681–693.

Buck, L. and Axel, R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell*, **65**, 175–187.

Evan, G I., Lewis, G.K., Ramsay, G. and Bishop, M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610–3616.

Fukuyama, P.M., Costas, K., Meurer, E., Xu, X., Muallem, S., Behar, V., Chorev, M., Rosenblatt, M., Tashjian, A.H. and Suva, L.J. (1996) Inositol 1-,4-,5-trisphosphate-dependent Ca^{2+} signaling by the recombinant human PTH/PTHrP receptor stably expressed in a human kidney cell line. *Bone*, **18**, 381–389.

Gierasch, L.M (1989) Signal sequences *Biochemistry*, **28**, 923–930.

Gudermann, T., Kalkbrenner, F. and Schultz, G. (1996) Diversity and selectivity of receptor-G protein interaction. *Annu Rev. Pharmacol. Toxicol*, **36**, 429–459.

Hansen, M, Boitano, S., Dirksen, E R and Sanderson, M J. (1993) Intercellular calcium signaling induced by extracellular adenosine 5'-triphosphate and mechanical stimulation in airway epithelial cells. *J. Cell Sci.*, **106**, 995–1004.

Hatt, H (1996) Molecular mechanisms of olfactory processing in the mammalian olfactory epithelium. *ORL J. Otorhinolaryngol. Relat. Spec.*, **58**, 183–194.

Kang, J and Caprio, J. (1995) *In vivo* responses of single olfactory receptor neurons in the channel catfish, *Ictalurus punctatus J Neurophysiol*, **73**, 172–177

Krieger, J, Schleicher, S, Strotmann, J, Wanner, I., Boekhoff, I, Raming, K., de Geus, P. and Breer, H. (1994) Probing olfactory receptors with sequence-specific antibodies *Eur J. Biochem*, **219**, 829–835 .

Lämmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophge T4. *Nature*, **227**, 680–685.

Michel, W C and Lubomudrov, L.M. (1995) Specificity and sensitivity of the olfactory organ of the zebrafish, *Danio rerio J Comp Physiol.*, **177**, 191–199.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M, Edmondson, J and Axel, R. (1996) Visualizing an olfactory sensory map *Cell*, **87**, 675–686

Mukerji, J., Haghighi, A. and Séguéla, P (1996) Immunological characterization and transmembrane topology of 5-hydroxy-tryptamine3 receptors by functional epitope tagging. *J Neurochem.*, **66**, 1027–1032.

Ngai, J., Dowling, M.M., Buck, L., Axel, R. and Chess, A. (1993) The family of genes encoding odorant receptors in the channel catfish. *Cell*, **72**, 657–666

Oles, M., Ewert, M, Meyer, T. and Pott, L. (1997) Rapid down regulation of Ca^{2+} signals by endotheline-1 in a human bronchial cell line. *Cell Calcium*, **21**, 221–231.

Pace, U., Hanski, E., Saloman, Y and Lancet, D (1985) Odorant-sensitive adenylate cyclase may mediate olfactory reception *Nature*, **316**, 255–258.

Raming, K., Krieger, J, Strotmann, J, Boekhoff, I, Kubick, S, Baumstark, C. and Breer, H. (1993) Cloning and expression of odorant receptors. *Nature*, **361**, 353–356.

Restrepo, D., Miyamoto, T., Bryant, B.P. and Teeter, J.H. (1990) Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science*, **249**, 1166–1168

Restrepo, D, Boekhoff, I and Breer, H. (1993) Rapid kinetic measurements of second messenger formation in olfactory cilia from channel catfish *Am. J Physiol.*, **264**, C906-C911.

Restrepo, D., Teeter, J.H and Schild, D (1996) Second messenger signaling in olfactory transduction. *J. Neurobiol*, **30**, 37–48

Sengupta, P., Chou, J.H. and Bargmann, C I. (1996) *odr-10* encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl *Cell*, **84**, 899–909

Tareilus, E, Noé, J and Breer, H (1995) Calcium signals in olfactory neurons *Biochim Biophys. Acta Mol. Cell Res*, **1269**, 129–138

Towbin, H., Stachlin, T. and Gordon, J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc Natl Acad. Sci USA*, **76**, 4350–4364.

Watts, VJ and Neve, K A (1996) Sensitization of endogenous and recombinant adenylate cyclase by activation of D2 dopamine receptors. *Mol Pharmacol*, **40**, 966–976

Weth, F., Nadler, W. and Korsching, S (1996) Nested expression domains for odorant receptors in zebrafish olfactory epithelium *Proc Natl Acad. Sci USA*, **93**, 13321–13326.

Wu, H, James-Kracke, M.R and Halenda, S.P. (1992) Direct relationship between intracellular calcium mobilization and phospholipase D activation in prostaglandin E-stimulated human erythroleukemia cells *Biochemistry*, **31**, 3370–3377.

Zufall, F., Hatt, H. and Firestein, S. (1993) Rapid application and removal of second messengers to cyclic nucleotide-gated channels from olfactory epithelium. *Proc Natl Acad Sci. USA*, **90**, 9335–9339.

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