

Insect olfactory receptors are heteromeric ligand-gated ion channels

Koji Sato¹, Maurizio Pellegrino², Takao Nakagawa¹†, Tatsuro Nakagawa¹, Leslie B. Vosshall² & Kazushige Touhara¹

In insects, each olfactory sensory neuron expresses between one and three ligand-binding members of the olfactory receptor (OR) gene family, along with the highly conserved and broadly expressed Or83b co-receptor^{1–9}. The functional insect OR consists of a heteromeric complex of unknown stoichiometry but comprising at least one variable odorant-binding subunit and one constant Or83b family subunit^{10–16}. Insect ORs lack homology to G-protein-coupled chemosensory receptors in vertebrates¹⁷ and possess a distinct seven-transmembrane topology with the amino terminus located intracellularly^{10,18}. Here we provide evidence that heteromeric insect ORs comprise a new class of ligand-activated non-selective cation channels. Heterologous cells expressing silkworm, fruitfly or mosquito heteromeric OR complexes showed extracellular Ca²⁺ influx and cation-non-selective ion conductance on stimulation with odorant. Odour-evoked OR currents are independent of known G-protein-coupled second messenger pathways. The fast response kinetics and OR-subunit-dependent K⁺ ion selectivity of the insect OR complex support the hypothesis that the complex between OR and Or83b itself confers channel activity. Direct evidence for odorant-gated channels was obtained by outside-out patch-clamp recording of *Xenopus* oocyte and HEK293T cell membranes expressing insect OR complexes. The ligand-gated ion channel formed by an insect OR complex seems to be the basis for a unique strategy that insects have acquired to respond to the olfactory environment.

To examine the mechanism of signal transduction of multimeric OR + Or83b complexes, we expressed ORs with known ligand specificities from silkworm (*Bombyx mori*), fruitfly (*Drosophila melanogaster*) and the malaria vector mosquito (*Anopheles gambiae*), together with members of the Or83b co-receptor family from each species (BmOr-2, Or83b and GPROR7, respectively) in heterologous cells and performed Ca²⁺ imaging and electrophysiology experiments. HeLa cells expressing *Drosophila* Or47a + Or83b or *Anopheles* GPROR2 + GPROR7 showed ligand-dependent and dose-dependent^{7,12,19} increases in intracellular Ca²⁺ that required the Or83b-class co-receptor^{10–13} (Fig. 1a, b, and Supplementary Fig. 1). Inward current responses to pentyl acetate (PA) or 2-methyl phenol (2-MP) were observed by whole-cell patch-clamp recordings of HeLa cells expressing Or47a + Or83b or GPROR2 + GPROR7, respectively, at a holding potential of –60 mV (Fig. 1c, d). The current showed a slight outward rectification similar to that previously observed for *Bombyx* BmOr-1 + BmOr-2 expressed in *Xenopus* oocytes¹². Reversal potentials of -1.9 ± 0.8 and $+0.2 \pm 1.1$ mV (means \pm s.e.m.) for Or47a + Or83b and GPROR2 + GPROR7, respectively, were measured (Fig. 1e–h). Waveforms of outward and inward currents were completely symmetrical (Fig. 1e, f), suggesting that odorants activate a non-selective cation conductance through the insect OR complex.

We next characterized the source of increase in intracellular Ca²⁺ when insect ORs are stimulated by odour ligands (Fig. 2). Chelating

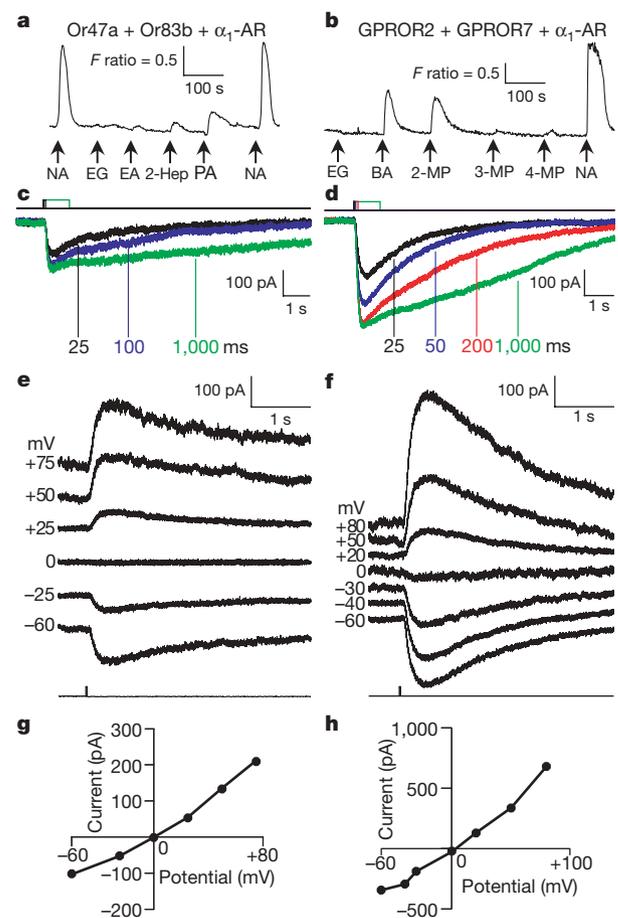


Figure 1 | Odorants activate a non-selective cation conductance in HeLa cells expressing multimeric insect ORs. **a, c, e, g,** Recording from a HeLa cell expressing Or47a, Or83b and α_1 -adrenergic receptor (α_1 -AR) on stimulation with 100 μ M pentyl acetate (PA), ethyl acetate (EA), 2-heptanone (2-Hep), eugenol (EG) and 100 nM noradrenaline (NA). **b, d, f, h,** Recording from a HeLa cell expressing GPROR2, GPROR7 and α_1 -AR on stimulation with 100 μ M 2-methyl phenol (2-MP), 3-methyl phenol (3-MP), 4-methyl phenol (4-MP), EG, benzaldehyde (BA) and 100 nM NA. **a, b,** Fura-2 acetoxyethyl ester (Fura-2/AM) Ca²⁺ imaging in response to ligand stimulation for 10 s (arrows indicate onset). **c, d,** Inward current responses with various stimulus durations of PA (**c**) and 2-MP (**d**). **e, f,** Current responses to stimulus (bottom traces indicate onset) at various holding potentials ($n = 14$ and 4, respectively). **g, h,** Current–voltage (I – V) relationship; the respective peak current of each response in **e** and **f** is plotted.

¹Department of Integrated Biosciences, The University of Tokyo, Chiba 277-8562, Japan. ²Laboratory of Neurogenetics and Behavior, The Rockefeller University, New York, New York 10065, USA. †Present address: Laboratory of Neurogenetics and Behavior, The Rockefeller University, New York, New York 10065, USA.

extracellular Ca^{2+} with EGTA or bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) strongly diminished the increase in Ca^{2+} mediated by Or47a + Or83b odour activation but had no effect on the increase in Ca^{2+} stimulated by activation of the $\text{G}\alpha_q$ -coupled α_1 -adrenergic receptor (α_1 -AR) by noradrenaline (Fig. 2a, b). This suggests that most of the increase in intracellular Ca^{2+} observed when insect ORs are activated derives from the influx of extracellular Ca^{2+} . *Drosophila* olfactory sensory neurons show receptor-dependent spontaneous activity in the absence of odour ligands^{4,7,8,20,21}, and we examined whether such spontaneous activity can be detected in heterologous cells expressing insect ORs. In Or47a + Or83b-expressing cells, the resting intracellular Ca^{2+} level was higher than that in mock-transfected cells (Supplementary Fig. 2a) and this was strongly dependent on the extracellular Ca^{2+} concentration (Supplementary Fig. 2b, c). Basal intracellular Ca^{2+} levels were decreased by extracellular application of EGTA in Or47a + Or83b-expressing cells (Fig. 2a and Supplementary Fig. 2b), suggesting that insect ORs mediate spontaneous Ca^{2+} influx in the absence of odour ligand.

To examine whether this odour-evoked activation requires known intracellular signalling cascades, we performed pharmacological experiments. No current response was observed in whole-cell patch-clamp experiments using a recording pipette filled with intracellular solution containing cyclic AMP, cyclic GMP or inositol-1,4,5-trisphosphate (IP_3) (Supplementary Fig. 3a). U73122, an inhibitor of phospholipase C, abolished α_1 -AR-mediated Ca^{2+} responses but did not affect increases in Ca^{2+} mediated by Or47a + Or83b (Fig. 2b, d). OR complex activation is therefore largely independent of $\text{G}\alpha_q$ /PLC signalling (Fig. 2b, d). Moreover, insect OR activation fails to stimulate the $\text{G}\alpha_s$ /cAMP pathway, because we did not detect increases in intracellular cAMP in HEK293T cells expressing Or47a + Or83b, BmOr-1 + BmOr-2 or GPROR2 + GPROR7 after stimulation with odour (Fig. 2c and Supplementary Fig. 4a). Control cells expressing the mouse OR, mOR-EG, showed robust increases. cAMP produced by stimulation of mOR-EG activated cyclic nucleotide-gated channel A2 (CNCA2), but activation of Or47a + Or83b with pentyl acetate had no effect on this channel (Supplementary Fig. 4b, c). Application of 8-bromo-cGMP or 8-bromo-cAMP failed to produce Ca^{2+} responses in HEK293T cells or oocytes expressing Or47a + Or83b or GPROR2 + GPROR7

(Supplementary Fig. 5). Although we failed to detect activation of a $\text{G}\alpha_s$ /cAMP pathway by ligand stimulation of Or47a + Or83b, BmOr-1 + BmOr-2 or GPROR2 + GPROR7, we did observe that some OR complexes showed a small, ligand-independent sensitivity to cyclic nucleotides (Supplementary Fig. 5b). The basis of this sensitivity was not investigated further in this study. To examine whether G-protein signalling was necessary for insect OR activation, we performed experiments with GDP- β S, a non-hydrolysable form of GDP that inhibits G-protein-coupled signalling. GDP- β S had no effect on the current response of BmOr-1 + BmOr-2 to bombykol (Fig. 2e) or the response of Or47a + Or83b to pentyl acetate (Supplementary Fig. 3d) when these receptor complexes were expressed in oocytes, but GDP- β S significantly inhibited Ca^{2+} -activated Cl^- current responses induced by the activation by noradrenaline of α_1 -AR/ $\text{G}\alpha_q$ /PLC- and cystic fibrosis transmembrane conductance regulator (CFTR) currents elicited by stimulation of β_2 -adrenergic receptor (β_2 -AR)/ $\text{G}\alpha_s$ /adenylyl cyclase pathways by isoprenaline (isoproterenol) (Fig. 2e). These results make it unlikely that G-protein-signalling cascades underlie responses of the insect OR complex.

To examine the response kinetics of an OR heteromultimer, we recorded increases in intracellular Ca^{2+} and electrical responses in the same cell. A HeLa cell expressing Or47a + Or83b and α_1 -AR showed a Ca^{2+} response to both pentyl acetate and noradrenaline, but showed an electrical response to pentyl acetate only, not to noradrenaline (Fig. 3a). The latency of the Or47a + Or83b-mediated Ca^{2+} response was less than one-tenth that of the α_1 -AR/ $\text{G}\alpha_q$ /PLC-mediated Ca^{2+} response (240 ± 46 ms versus $3,300 \pm 400$ ms). The latencies of current responses of Or47a + Or83b (Fig. 3b) and GPROR2 + GPROR7 (Fig. 3c) were 17.9 ± 3.1 and 28.5 ± 1.9 ms, respectively. This is considerably shorter than the about 50–200-ms latency of the G-protein-coupled olfactory response in vertebrate olfactory neurons^{22,23}, in which the loading of G proteins is the rate-limiting step²³. The time course of Ca^{2+} responses of Or47a + Or83b and GPROR2 + GPROR7 was also distinct from those of α_1 -AR (Fig. 3f) and mOR-EG (Fig. 3g). The slope of the odorant-induced insect OR response was not modulated by the duration of stimulation (Fig. 3d, e), whereas the slope of noradrenaline-induced α_1 -AR or eugenol-induced mOR-EG responses was sharper when the stimulation period was prolonged (Fig. 3f, g).

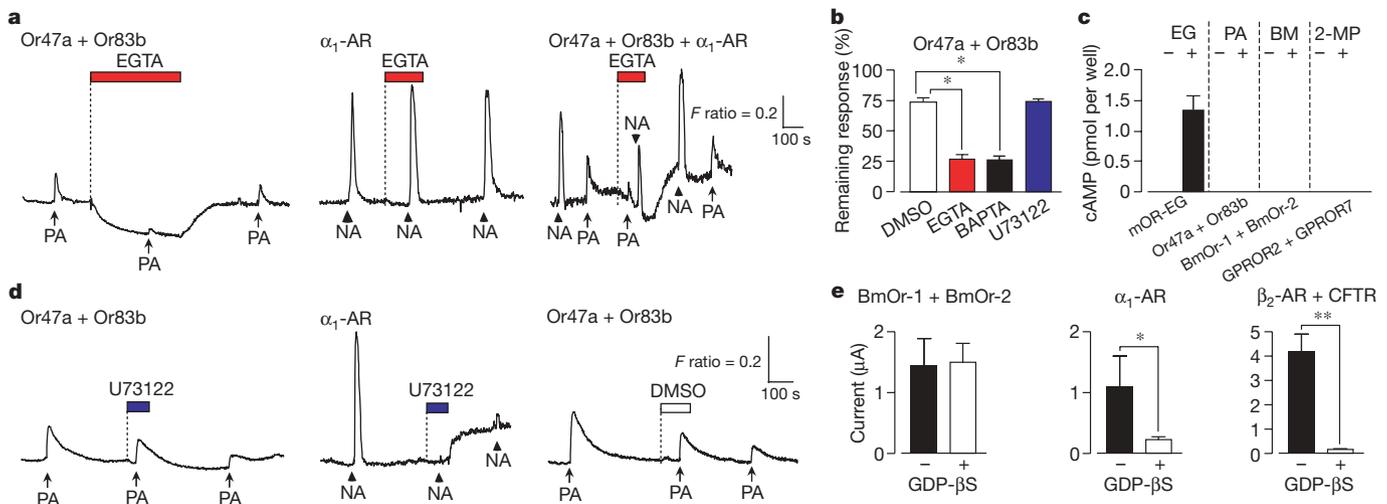


Figure 2 | Insect OR activity is independent of G protein signalling. **a**, Ca^{2+} responses of HeLa cells expressing Or47a + Or83b, α_1 -AR or Or47a + Or83b + α_1 -AR to a 10-s stimulation with 100 μM pentyl acetate (PA) or 100 nM noradrenaline (NA) with application of 10 mM EGTA (red bar) or 5 mM BAPTA (black bar). **b**, Effects of Ca^{2+} chelators (10 mM EGTA and 5 mM BAPTA) and U73122 (PLC inhibitor; containing 0.1% DMSO) on Or47a + Or83b Ca^{2+} response (DMSO control, $n = 28$; U73122, $n = 18$; EGTA, $n = 10$; BAPTA, $n = 20$). Significance assessed by *t*-test: asterisk, $P < 0.001$. **c**, cAMP production in HEK293T cells expressing mOR-EG, Or47a + Or83b,

BmOr-1 + BmOr-2 or GPROR2 + GPROR7 stimulated with eugenol (EG; 1 mM), PA (100 μM), bombykol (BM; 10 μM) or 2-methyl phenol (2-MP; 100 μM), respectively. **d**, As in **a**, but with application of 10 μM U73122 (filled bar) or 0.1% DMSO (open bar). **e**, Effect of GDP- β S on ligand-induced inward currents in *Xenopus* oocytes expressing BmOr-1 + BmOr-2 (30 μM BM), α_1 -AR (1 μM NA) or β_2 -AR + CFTR (10 μM isoprenaline). Significance assessed by *t*-test: asterisk, $P < 0.05$; two asterisks, $P < 0.01$; $n = 5$ each. Data are shown as means \pm s.e.m.

If the heteromultimer combination of the insect OR complex determines ion selectivity, we might expect that the ion permeability of odour-gated currents would differ with different subunit compositions. Indeed, both the reversal potential and outward rectification of Or47a + Or83b and Or47a + BmOr-2 differed slightly (Supplementary Fig. 6a, b). Ruthenium red, a cation-channel blocker, which completely inhibits odour-evoked inward current responses of BmOr-1 + BmOr-2 in *Xenopus* oocytes¹², inhibited both bombkol-evoked inward current and basal Ca^{2+} levels (Supplementary Fig. 6c, d) of BmOr-1 + BmOr-2-expressing cells but had no effect on HEK293T cells expressing GPROR2 + GPROR7 (data not shown). This suggests that insect OR subunit composition affects the degree of inhibition by ruthenium red.

To obtain evidence that currents are produced directly by the insect ORs, outside-out patch-clamp recordings were performed on oocyte membranes containing Or47a + Or83b or GPROR2 + GPROR7. First we established that oocytes expressing GPROR2 + GPROR7 showed high-affinity ligand-specific macroscopic currents that depended on the expression of both subunits (Supplementary Fig. 7a) and required extracellular Na^+ (Supplementary Fig. 7b, d). Patches from uninjected control oocytes did not show current responses to two different odour stimuli or oocyte Ringer's solution (Supplementary Fig. 7e), indicating that odorants do not induce non-specific currents by interacting with oocyte membrane or

membrane proteins. Outside-out patch preparations of oocytes injected with Or47a + Or83b or GPROR2 + GPROR7 showed transient unitary currents whose frequency increased with the application of the cognate ligands pentyl acetate or 2-methyl phenol (Fig. 4 and Supplementary Fig. 7f–h). The channel conductance of the Or47a + Or83b complex was 1.2 ± 0.03 pA ($n = 4$) at a holding potential of -80 mV (Fig. 4d). The magnitude of odour-evoked currents was voltage-dependent and increased when the holding potential was progressively changed from $+40$ mV to -120 mV (Supplementary Fig. 7h). The frequency of these odour-evoked events was concentration-dependent (Fig. 4e). There was no difference in pentyl-acetate-evoked conductance from membranes expressing Or47a + Or83b in the presence or absence of ATP (1 mM)/GTP (100 μM) in the recording pipette, suggesting that ATP/GTP-mediated signal transduction components are not involved in the primary conductance of this channel (without ATP/GTP shown in Fig. 4a–c; with ATP/GTP shown in Supplementary Fig. 3b, c).

Similar results were obtained in outside-out patch-clamp recording of HEK293T membranes expressing Or47a + Or83b or GPROR2 + GPROR7 (Fig. 5). In contrast with the bath application

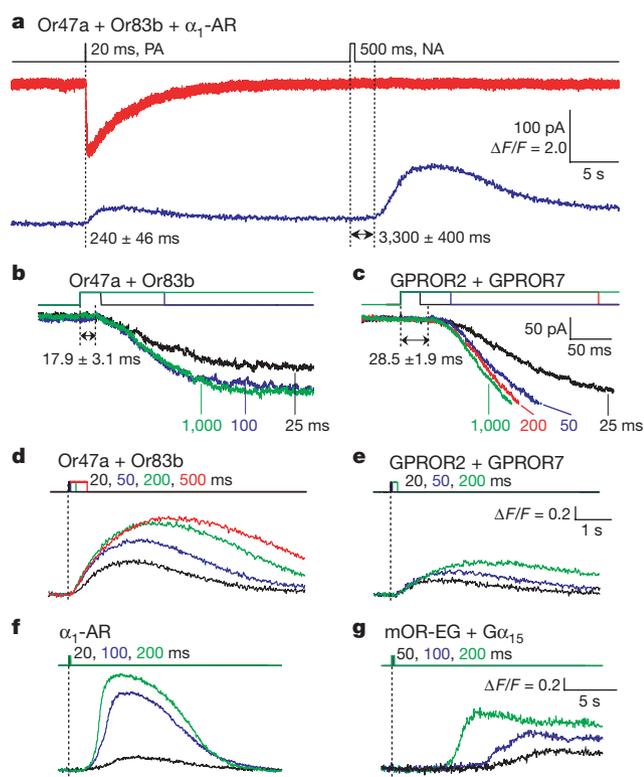


Figure 3 | Kinetic analysis of Ca^{2+} and current responses of insect ORs in HeLa cells. **a**, Simultaneous recording of whole-cell current (red trace) and intracellular Ca^{2+} (Fluo-4-4K; blue trace) from a HeLa cell expressing Or47a + Or83b + α_1 -AR. Stimulus durations of pentyl acetate (PA; 20 ms) and noradrenaline (NA; 500 ms) are indicated at the top. Ca^{2+} response latency is shown as mean \pm s.e.m. ($n = 8$). **b**, **c**, Onset of inward current responses of Or47a + Or83b to PA (**b**) and GPROR2 + GPROR7 to 2-methyl phenol (2-MP) (**c**) applied with various stimulus durations as indicated by the different coloured curves. The latency of current response is shown as mean \pm s.e.m.; scales for **b** and **c** are shown at the right in **c** (**b**, $n = 14$; **c**, $n = 4$). **d**–**g**, Onset of Fluo-4-acetoxymethyl ester (Fluo-4/AM)-based Ca^{2+} responses of Or47a + Or83b to PA (**d**), GPROR2 + GPROR7 to 2-MP (**e**), α_1 -AR to NA (**f**), and mOR-EG along with $\text{G}\alpha_{15}$ to EG (**g**), with stimuli applied for various durations as indicated. Scales for **d** and **e** are shown at the right in **e**, and for **f** and **g** at the right in **g**.

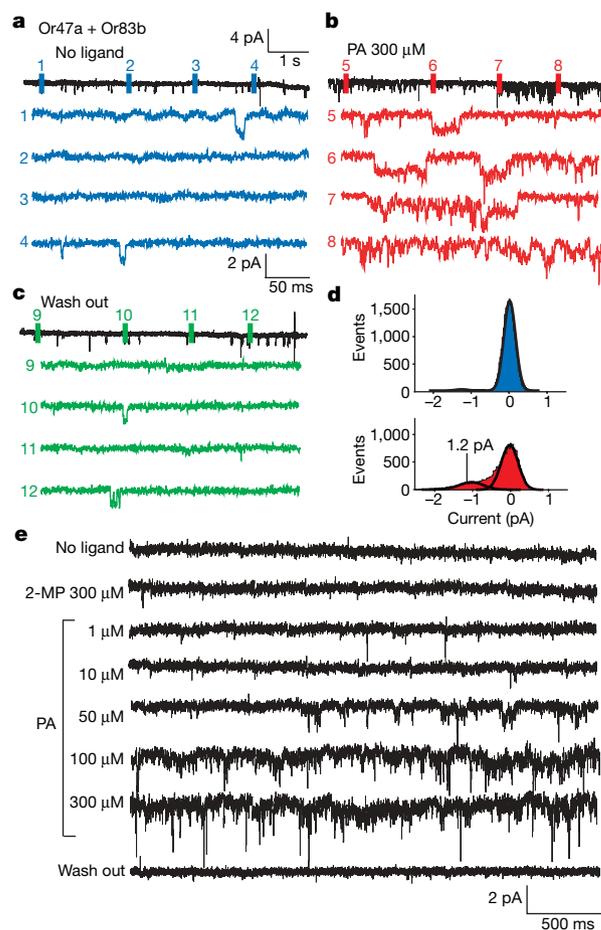


Figure 4 | Excised outside-out patch-clamp recording of Or47a + Or83b currents measured in oocyte membranes. **a**–**c**, Outside-out patch-clamp recording of a *Xenopus* oocyte membrane expressing Or47a + Or83b before stimulation (**a**), during stimulation with 300 μM pentyl acetate (PA) (**b**), and after wash out (**c**). The bottom traces of each panel indicate expansions of 300-ms current traces of single-channel recording at the positions indicated by the numbers. The data for **a**–**c** were obtained from the same cell with voltage clamped at -80 mV. Scales for **a**–**c** are indicated at the top in **a** and scales for the expansions are at the bottom in **a**, **d**. All-point current histograms of unitary events before (blue) and during (red) application of the ligand PA in **b** and **c**. Amplitude distributions were fitted with two gaussian components (black lines). **e**, Dose–response and ligand selectivity of Or47a + Or83b currents with voltage clamped at -90 mV.

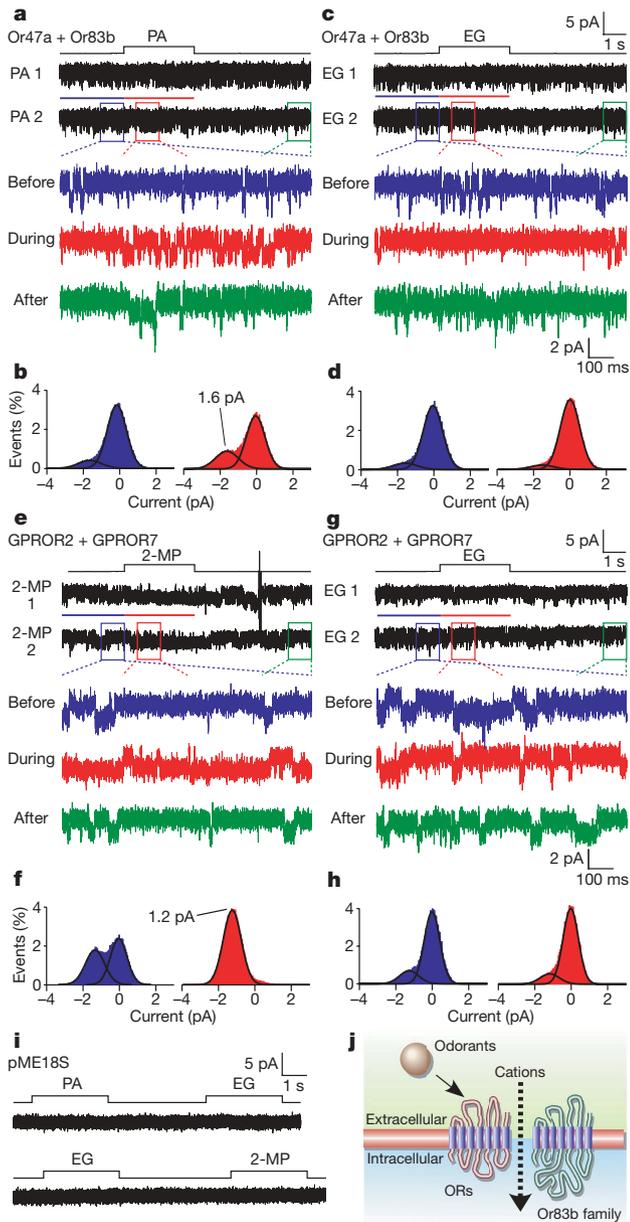


Figure 5 | Excised outside-out patch-clamp recording of insect OR currents measured in HEK293T membranes. **a–d**, Or47a + Or83b-expressing HEK293T membranes stimulated for 3 s with pentyl acetate (PA; 300 μ M) (**a**, **b**) or the non-agonist eugenol (EG; 300 μ M) (**c**, **d**). The bottom traces show expansions of the current trace of the single-channel recording at the positions indicated by coloured boxes on trace 2 before (blue), during (red) and after (green) stimulation with PA (**a**) or EG (**c**). All-point current histograms were obtained from the region indicated on trace 1 by the blue bar without stimulation (blue histogram, left) and by the red bar for stimulation with PA (**b**) or EG (**d**) (red histogram, right). Mean peak current level (1.6 pA) was obtained from the fitted gaussians. Data in **a–d** were obtained from the same cell. Scales for **a** and **c** are indicated at the top in **c** and scales for the expansions are at the bottom in **c**. The scale on the y axis indicates the relative number of current events with a bin width of 0.05 pA. **e–h**, As in **a–d**, but using GPROR2 + GPROR7-expressing HEK293T membranes stimulated for 3 s with 2-methyl phenol (2-MP; 300 μ M) (**e**, **f**) or the non-agonist EG (300 μ M) (**g**, **h**). Scales for **e** and **g** are indicated at the top in **g** and scales for the expansions are at the bottom in **g**. Data in **e–h** were obtained from the same cell. **i**, Outside-out patch-clamp recording of vector-transfected HEK293T membranes stimulated for 3 s with 300 μ M PA, EG and 2-MP. Membranes in **a–i** were at a holding potential of -60 mV. **j**, Schematic model for ligand-gated channel properties of the OR + Or83b complex.

used for oocyte experiments in Fig. 4, we used a puff application of odour ligands. Pentyl acetate evoked unitary currents of 1.5 ± 0.06 pA ($n=4$) in outside-out patch membranes expressing Or47a + Or83b and held at -60 mV (Fig. 5a, b, and Supplementary Fig. 8a–c). We calculated a slope conductance of 27 pS for Or47a + Or83b from a current–voltage (I – V) curve (Supplementary Fig. 8b). Stimulation of Or47a + Or83b-expressing cell membrane with the non-ligand eugenol did not increase currents (Fig. 5c, d). The same experiments performed in membranes expressing GPROR2 + GPROR7 produced unitary currents of 1.2 ± 0.07 pA ($n=3$) with a conductance of 20 pS at -60 mV in response to the cognate ligand 2-methyl phenol (Fig. 5e, f, and Supplementary Fig. 8d, e). As seen for Or47a + Or83b, the non-ligand eugenol did not stimulate GPROR2 + GPROR7 currents (Fig. 5g, h). The presence of currents in the absence of cognate ligand suggests that both Or47a + Or83b and GPROR2 + GPROR7 are spontaneously active, which is consistent with our other results in Figs 2a, 4a and Supplementary Figs 3 and 6d. Untransfected HEK293T membranes did not show currents on stimulation with pentyl acetate, eugenol or 2-methyl phenol (Fig. 5i). In both oocytes (Fig. 4) and mammalian cells (Fig. 5), single-channel events predominated. We attribute this either to low expression levels of these insect ORs in heterologous cell membrane patches or to unstable characteristics of the multimeric receptor in these cells. Nevertheless, these outside-out patch-clamp experiments using two different membrane preparations provide initial evidence that the insect OR complex possesses ligand-gated channel properties.

Taken together, these data provide compelling evidence that a heteromeric complex of a conventional insect OR and the highly conserved Or83b family co-receptor has the characteristics of a cation non-selective ion channel directly gated by odour or pheromone ligands (Fig. 5j). We conclude that G-protein-mediated signalling is negligible in producing the current elicited by odour activation of insect OR heteromultimers. Our findings provide insight into long-argued insect olfactory transduction mechanisms and may explain the lack of clear consensus on the role of second messengers in this process²⁴. The insect ORs share no homology with any previously described ion channel and do not contain any known ion selectivity filter motifs²⁵. We observed that insect OR activity was not inhibited by Gd^{3+} (data not shown), a lanthanide that is a broad-spectrum ion channel inhibitor^{26–28}. Therefore, although the ionic permeability reported here for Na^+ , K^+ and Ca^{2+} would be consistent with the properties of non-selective cation channels, a molecular basis for this novel ionotropic activity remains to be elucidated. The spontaneous activity of the OR complex found here seems to account for previous observations that olfactory sensory neurons exhibit bipolar electrical activity and become electrically negative on the deletion of Or83b *in vivo*^{4,7,8}. Given that there are 62 and 79 potential ligand-binding OR subunits in *Drosophila* and *Anopheles*, respectively, the insect ORs may represent the largest single family of ion-channel-like proteins in any organism. Our work also raises the intriguing possibility that the insect gustatory system, which senses bitter and sweet tastants as well as carbon dioxide, shares this ionotropic coupling mechanism with the insect ORs. In fact, an ionotropic sugar-gated channel in fleshfly taste cells has previously been reported²⁹. Our finding offers the caveat that other orphan receptors classified as G-protein-coupled receptors merely because of their putative seven-transmembrane topology may instead possess ligand-gated channel activities, as has been shown previously for light-activated channelrhodopsin³⁰. This work has important implications for worldwide efforts to identify specific inhibitors for the insect ORs, which may prove useful in controlling host-seeking behaviours of disease-vector insects such as mosquitoes.

Note added in proof: The authors of an accompanying paper³¹ also conclude that insect ORs possess ion channel-like properties, but they report G_s -dependent cyclic-nucleotide activation of Or83b alone, which we fail to observe.

METHODS SUMMARY

Odorant stocks and stimulation. Stock solutions of bombykol, isoprenaline, forskolin, 3-isobutyl-1-methylxanthine, ruthenium red, and U73122 were prepared in dimethylsulphoxide (DMSO). Other stimuli in Figs 1–3 and 5 were added directly to Ringer's solution. Stimuli in Fig. 4 were prepared in DMSO as 1 M stocks, which were then diluted into the extracellular solution. For patch-clamp recording and kinetic analysis, odorants were applied focally to the recording cell with the use of a handmade pressure-ejection system and a TTL (transistor–transistor logic) pulse generator. For other experiments, odorants and inhibitors were applied by perfusing the bath solution.

Electrophysiology and Ca²⁺ imaging. Two-electrode voltage-clamp recording from *Xenopus laevis* oocytes was performed as described previously¹². Ca²⁺ imaging from mammalian cell lines was performed as described previously³².

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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- Clyne, P. J. *et al.* A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* **22**, 327–338 (1999).
- Vosshall, L. B., Amrein, H., Morozov, P. S., Rzhetsky, A. & Axel, R. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* **96**, 725–736 (1999).
- Couto, A., Alenius, M. & Dickson, B. J. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* **15**, 1535–1547 (2005).
- Dobritsa, A. A. *et al.* Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* **37**, 827–841 (2003).
- Elmore, T., Ignell, R., Carlson, J. R. & Smith, D. P. Targeted mutation of a *Drosophila* odor receptor defines receptor requirement in a novel class of sensillum. *J. Neurosci.* **23**, 9906–9912 (2003).
- Fishilevich, E. & Vosshall, L. B. Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr. Biol.* **15**, 1548–1553 (2005).
- Hallem, E. A. & Carlson, J. R. Coding of odors by a receptor repertoire. *Cell* **125**, 143–160 (2006).
- Hallem, E. A., Ho, M. G. & Carlson, J. R. The molecular basis of odor coding in the *Drosophila* antenna. *Cell* **117**, 965–979 (2004).
- Goldman, A. L., Van der Goes van Naters, W., Lessing, D., Warr, C. G. & Carlson, J. R. Coexpression of two functional odor receptors in one neuron. *Neuron* **45**, 661–666 (2005).
- Benton, R., Sachse, S., Michnick, S. W. & Vosshall, L. B. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors *in vivo*. *PLoS Biol.* **4**, e20 (2006).
- Larsson, M. C. *et al.* Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43**, 703–714 (2004).
- Nakagawa, T., Sakurai, T., Nishioka, T. & Touhara, K. Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* **307**, 1638–1642 (2005).
- Neuhaus, E. M. *et al.* Odorant receptor heterodimerization in the olfactory system of *Drosophila melanogaster*. *Nature Neurosci.* **8**, 15–17 (2005).
- Jones, W. D., Nguyen, T. A., Kloss, B., Lee, K. J. & Vosshall, L. B. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr. Biol.* **15**, R119–R121 (2005).
- Krieger, J., Klink, O., Mohl, C., Raming, K. & Breer, H. A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. [A]* **189**, 519–526 (2003).
- Pitts, R. J., Fox, A. N. & Zwiebel, L. J. A highly conserved candidate chemoreceptor expressed in both olfactory and gustatory tissues in the malaria vector *Anopheles gambiae*. *Proc. Natl Acad. Sci. USA* **101**, 5058–5063 (2004).
- Wistrand, M., Kall, L. & Sonnhhammer, E. L. A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Protein Sci.* **15**, 509–521 (2006).
- Lundin, C. *et al.* Membrane topology of the *Drosophila* OR83b odorant receptor. *FEBS Lett.* **581**, 5601–5604 (2007).
- Hallem, E. A., Nicole Fox, A., Zwiebel, L. J. & Carlson, J. R. Olfaction: mosquito receptor for human-sweat odorant. *Nature* **427**, 212–213 (2004).
- de Bruyne, M., Clyne, P. J. & Carlson, J. R. Odor coding in a model olfactory organ: the *Drosophila* maxillary palp. *J. Neurosci.* **19**, 4520–4532 (1999).
- de Bruyne, M., Foster, K. & Carlson, J. R. Odor coding in the *Drosophila* antenna. *Neuron* **30**, 537–552 (2001).
- Sato, K. & Suzuki, N. The contribution of Ca²⁺-activated Cl⁻ conductance to amino-acid-induced inward current responses of ciliated olfactory neurons of the rainbow trout. *J. Exp. Biol.* **203**, 253–262 (2000).
- Firestein, S., Shepherd, G. M. & Werblin, F. S. Time course of the membrane current underlying sensory transduction in salamander olfactory receptor neurons. *J. Physiol. (Lond.)* **430**, 135–158 (1990).
- Kaissling, K. E. Peripheral mechanisms of pheromone reception in moths. *Chem. Senses* **21**, 257–268 (1996).
- Zagotta, W. N. Membrane biology: permutations of permeability. *Nature* **440**, 427–429 (2006).
- Lacampagne, A., Gannier, F., Argibay, J., Garnier, D. & Le Guennec, J. Y. The stretch-activated ion channel blocker gadolinium also blocks L-type calcium channels in isolated ventricular myocytes of the guinea-pig. *Biochim. Biophys. Acta* **1191**, 205–208 (1994).
- Kanzaki, M. *et al.* Molecular identification of a eukaryotic, stretch-activated nonselective cation channel. *Science* **285**, 882–886 (1999).
- Halaszovich, C. R., Zitt, C., Jungling, E. & Luckhoff, A. Inhibition of TRP3 channels by lanthanides. Block from the cytosolic side of the plasma membrane. *J. Biol. Chem.* **275**, 37423–37428 (2000).
- Murakami, M. & Kijima, H. Transduction ion channels directly gated by sugars on the insect taste cell. *J. Gen. Physiol.* **115**, 455–466 (2000).
- Nagel, G. *et al.* Channelrhodopsin-1: a light-gated proton channel in green algae. *Science* **296**, 2395–2398 (2002).
- Wicher, D. *et al.* *Drosophila* odorant receptors are both ligand-gated and cyclic nucleotide-activated cation channels. *Nature* doi:10.1038/nature06861 (this issue).
- Katada, S., Nakagawa, T., Kataoka, H. & Touhara, K. Odorant response assays for a heterologously expressed olfactory receptor. *Biochem. Biophys. Res. Commun.* **305**, 964–969 (2003).

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METHODS

Patch-clamp experiments in mammalian cell lines. Full-length complementary DNAs for silkworm ORs (BmOr-1 and BmOr-2), mosquito ORs (GPROR1, GPROR2 and GPROR7) and fruitfly ORs (Or47a and Or83b) were cloned into the pME18S vector. ORs were transiently transfected into HeLa or HEK293T cells with Lipofectamine 2000 reagent (Invitrogen). α_1 -AR and GFP or mRFP were co-transfected as a control. Whole-cell currents were amplified with a patch-clamp amplifier (CEZ-2400; Nihon-koden), and were digitized with PowerLab (AD Instruments). The extracellular solutions (normal Ringer's solution) contained (in mM): 140 NaCl, 5.6 KCl, 5 HEPES, 2.0 pyruvic acid sodium salt, 1.25 KH_2PO_4 , 2.0 CaCl_2 , 2.0 MgCl_2 , 9.4 D-glucose (pH 7.4). The electrode solution contained (in mM): 140 KCl, 10 HEPES, 5 EGTA-2K, 10 D-glucose (pH 7.4). To record the shift in reversal potential and in equilibrium potential for a specific ion, the following external (bath) and internal (electrode) solution was used: low- Na^+ (N-methyl-D-glutamine; NMDG) external solution (in mM: 190 NMDG, 40 HEPES, 5.6 KCl, 2.0 pyruvic acid sodium salt, 1.25 KH_2PO_4 , 2.0 CaCl_2 , 2.0 MgCl_2 , 9.4 D-glucose; pH 7.4); low- K^+ (NMDG) internal solution (in mM: 202 NMDG, 40 HEPES, 5 EGTA-2K, 10 D-glucose; pH 7.4). For the outside-out recording, normal Ringer's solution was used for the electrode solution. The data were sampled at 20 kHz and filtered at 2 kHz.

Xenopus oocyte electrophysiology. Oocytes were microinjected with 25 ng of complementary RNA (cRNA) for a conventional OR and 25 ng of cRNA for the Or83b family. Whole-cell currents were recorded with a two-electrode voltage-clamp filled with 3 M KCl, and were amplified with an OC-725C amplifier (Warner Instruments), low-pass filtered at 50 Hz and digitized at 1 kHz. Outside-out patch-clamp recordings were performed 18–26 h after injection. After removal of the vitelline layer, oocytes were transferred to a Petri dish with a bath solution of oocytes Ringer's solution containing (in mM): 82.5 NaCl, 2 KCl, 1 MgCl_2 , 5 HEPES, 1.8 CaCl_2 (pH 7.5). Pipettes (4–7 M Ω) were covered with Sylgard (Dow Chemical Company) and filled with intracellular solution containing (in mM): 100 KOH, 10 HEPES, 1 EGTA, 100 sulphamic acid (pH 7.6). After seal formation (more than 5 G Ω), patches were excised and transferred to the recording chamber, where the extracellular side was continuously superfused with extracellular solution containing (in mM): 100 NaOH, 10 HEPES, 1 MgCl_2 , 100 sulphamic acid (pH 7.5); this was supplemented, where indicated, by the odorants. Solutions were switched by computer-driven electric valves (General Valve Corp.). Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Inc.), low-passed at 1 kHz (eight-pole Bessel; Frequency Devices), digitized at 10 kHz by means of an ITC-16 interface (Instrutech Corporation) and saved to a PC hard disk with PULSE v8.11 acquisition software (HEKA Elektronik). Data were analysed with Clampfit 9.0 (Axon Instruments, Inc.) and Origin PRO 7 (Origin Lab). The *I*-*V* curves showing ion permeability were produced with low- Na^+ solution (Na^+ in oocyte Ringer's solution replaced by the impermeable cation NMDG $^+$) and Cl^- -free solution (Cl^- in oocyte Ringer's solution replaced by sulphamic acid). These experiments used bath application of ligands, precluding any measurement of the response latency of these currents.

Ca^{2+} imaging. Insect ORs were transfected into HeLa or HEK293T cells, which were loaded with 2.5 μM Fura-2/AM or Fluo-4/AM for 30 min. Fluorescence was measured with an Aquacosmos Ca^{2+} imaging system (Hamamatsu Photonics). For the simultaneous recording of whole-cell currents and intracellular Ca^{2+} levels, the electrode solution (in mM: 140 KCl, 10 HEPES, 5 EGTA-2K, 10 D-glucose, 0.05 Fluo-4-pentapotassium salt (Fluo-4-4K); pH 7.4) was used. To synchronize the patch-clamp amplifier, odorant stimulator and charge-coupled device camera (ORCA-ER; Hamamatsu), all devices were under the control of a peripheral interface controller (PIC16F877; Microchip Technology, Inc.).

cAMP assay. HEK293T cells transfected with mOR-EG, Or47a + Or83b, BmOr-1 + BmOr-2 or GPROR2 + GPROR7 were incubated with 1 mM 3-isobutyl-1-methylxanthine for 30 min. The cells were then exposed to the indicated concentration of odorant solution for 15 min. cAMP levels were determined with an enzyme-linked immunosorbent assay kit (Applied Biosystems) in accordance with the manufacturer's directions.