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Identification of vertebrate volatiles stimulating olfactory receptors on tarsus I of the tick *Amblyomma variegatum* Fabricius (Ixodidae)

I. Receptors within the Haller's organ capsule

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Abstract. Gas chromatography-coupled electrophysiological recordings (GC-EL) from olfactory sensilla within the capsule of Haller's organ of the tick *Amblyomma variegatum* indicate the presence of a number of stimulants in rabbit and bovine odours, and in steer skin wash. Some of these stimulants were fully identified by gas chromatography-mass spectrometry analysis and by matching electrophysiological activity of synthetic analogues as: 1) hexanal, 2-heptenal, nonanal, furfural, benzaldehyde, and 2-hydroxybenzaldehyde (in all extracts); 2) heptanal, 2-, 3-, and 4-methylbenzaldehyde, and γ-valerolactone (only in bovine and rabbit odour). Careful examination of the electrophysiological responses permit characterization of 6 receptor types: 1) a benzaldehyde receptor, 2) a 2-hydroxybenzaldehyde receptor, 3) three types of receptors responding differently to aliphatic aldehydes, and 4) a lactone receptor.

Key words: Tick – Haller's organ – Olfactory receptors – Benzaldehyde – 2-Hydroxybenzaldehyde – Aliphatic aldehydes – γ-Valerolactone

Introduction

Adults of the tropical bont tick, *Amblyomma variegatum* (Acari, Ixodidae), lie in wait in the litter zone for hosts such as domestic and wild bovidae. The presence of a vertebrate in the vicinity arouses adults of this tick species to initiate active search on the ground in order to locate the host. At the end of the dry season, males of *A. variegatum* are first to find a suitable host, and feed for few days before emitting an aggregation-attachment pheromone (Schoeni et al. 1984), which in turn enhances attractivity of the host for conspecifics (Norval et al. 1989; Barré et al. 1991). This favours meeting of the sexes on the host. While the aggregation-attachment pheromone together with host odour seems crucial for host-seeking and attachment by females (Barré 1989; Barré et al. 1991), host odour alone is important for infestation of the host by pioneer males.

This paper deals with olfactory receptors housed in wall-pore single-walled sensilla within the capsule of Haller's organ on the tarsus of the leg pair I, considered to contain some of the main host-odour receptors in ticks. This supposition was confirmed by the behavioural bioassay of Lees (1948) on *Ixodes ricinus*, and in electrophysiology experiments in which mouse odour was used to stimulate capsule receptors in *Hyalomma asiaticum* (Sinitsina 1974). In addition, breath components CO_2 and H_2S have been clearly identified as olfactory stimulants for receptors in the capsule of Haller's organ of *A. variegatum* (Steullet and Guerin 1992a, b). The present study aims to extend our knowledge on other olfactory receptors (specificity spectrum) responding to host odour within wall-pore single-walled sensilla of the Haller's organ in this tick species. Gas chromatography-coupled electrophysiology recordings of host-odour receptors are then employed to isolate active constituents in vertebrate odour concentrates.

Materials and methods

Tick rearing. *A. variegatum*, originating from the Ivory Coast (Adiopodoumé), have been reared since 1981 at the Agricultural Research Centre of Ciba-Geigy Ltd. (St. Aubin, Switzerland). All stages (immature and adult) are fed on Simmental calves at 22 to 24°C and then kept under constant darkness during moult at 28°C/80–90% RH. Unfed males foreseen for these experiments were maintained in an environmental cabinet: 10 h light at 25°C/85% RH, 10 h darkness at 18°C/95% RH separated by 2 h dusk and dawn periods.

Electrophysiology. Unfed male *A. variegatum* (under 7 months old) were immobilized on a perspex holder with double-sided sticky tape. Pedal nerves of the anterior leg pair were destroyed by pinching the coxa with fine forceps to prevent muscle activity during

Abbreviations: GC-EL, gas chromatography-coupled electrophysiological recording; GC-MS, gas chromatography-coupled mass spectrometry

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electrophysiological recordings. The narrow opening of the capsule (a slit across dorsal side of the tarsus 50 µm long and 5 µm wide) was enlarged to provide better access to the 7 wall-pore sensilla within by using a piece of razor blade mounted on a Leitz micromanipulator.

Recordings from olfactory receptors were accomplished with glass electrodes connected to a high-input impedance preamplifier and an AC/DC amplifier (UN-03, Syntech, The Netherlands). The reference electrode, filled with 0.2 *M* NaCl, was inserted into the coxa of one of the anterior legs, whereas the recording electrode (tip diameter <5 µm), filled with 0.2 *M* KCl and 1% polyvinylpyrrolidon 90 K (Fluka, Switzerland), was mounted on a Leitz micromanipulator and gently introduced into the dissected capsule until cell activity was captured. Contact between the electrode tip and the pore-wall of a sensillum was sufficient to capture cell activity. Recordings from different sensilla within the capsule were made by varying the orientation of the recording electrode in the capsule. Cell activity could thus be consistently recorded at 6 distinct locations (Fig. 1). AC and DC signals were stored on video tapes as in Steullet and Guerin (1992a). AC signals were also fed into a IBM-compatible computer and visually analysed using the view option of the spike analysis programme SAPID (Smith et al. 1990), and displayed on paper with a laser printer.

Stimulus delivery. Air scrubbed through charcoal and silicagel, and humidified to 80% RH at 22°C ± 1°C passed continuously at 40 cm/s over the preparation from a 8 mm diameter glass tube, the outlet of which was about 10 mm from the tarsus. Stimulation was achieved by applying a charcoal-filtered air stream to a 5-ml polypropylene syringe containing the stimulus. A solenoid valve permitted displacement of 2 ml of the syringe content in 1 s into the humidified air stream through a septum-covered hole in the glass tube at 3 cm from its outlet. To prevent changes in air flow during stimulation, a solenoid-controlled charcoal-filtered air flow (2 ml/s) was delivered continuously through a blank syringe into the humidified air stream during stimulus off. Stimulations followed at 3 min intervals.

Different concentrates of host odours and the following synthetic chemicals were at first used to study the specificity of receptors located in the different parts of the capsule: ammonia (3.5% and 0.35% NH_4OH in distilled H_2O), acetone (10^{-3} *M* and 10^{-2} *M* in distilled H_2O), 3-pentanone, 4-heptanone, γ-butyrolactone, γ-valerolactone, 6-caprolactone, pentanol, 1-octen-3-ol, propanoic acid, 2-methylpropanoic acid, butanoic acid, 3-methylbutanoic acid, pentanoic acid, heptanoic acid, L-lactic acid, and 4-methylphenol (all vertebrate-associated volatiles); nonanoic acid, 2-nitrophenol, 2,6-dichlorophenol and methylsalicylate (tick pheromone components); and 1-octene, octylamine, hexyl acetate (others); dichloromethane and distilled H_2O (solvent blanks). Except for ammonia and acetone, all these chemicals (> 98% pure as indicated by GC) were dissolved in dichloromethane (Merck, analytical grade) and tested at 10^{-3} and 10^{-2} *M* dilutions (levels normally evoking clear responses in most responsive receptors). When a receptor responded to a tested chemical, graded dilutions from 10^{-5} to 10^{-2} *M* were delivered to the preparation to determine a dose-response curve. A 10 µl aliquot of the stimulus solution was deposited on a piece of filter paper and placed in the stimulus cartridge after evaporation of the organic solvent. Separate cartridges were employed for each stimulus and each concentration. Each cartridge was only used once. Three min were arbitrarily allowed for stimulus evaporation inside the syringes prior to delivering the volatile to the preparation. CH_4 (neat from the mains) and CO_2 (from a gas cylinder of 5% CO_2/95% O_2) were also tested; stimulus syringes were then directly filled with these gases.

Host-odour stimuli. Human breath, human axillary secretion, and extracts of bovine and rabbit odours were employed as host-odour stimuli. Human breath was blown into a 5-ml syringe used as stimulus cartridge (for further details, see Steullet and Guerin 1992b). Human axillary secretion was collected with a dry-acetone-washed cotton pad (7 × 7 cm) rubbed on the axillary area of a 28 year-old male and then enclosed in a stimulus cartridge. The axillary region was not treated with deodorants or perfumes, and was not washed for 24 h prior to secretion collection. The stimulus blank consisted of a dry-acetone-washed cotton pad.

Air from a metallic cage containing a single tick-naive rabbit (New Zealand), a white strain sometimes used in this laboratory to feed *A. variegatum*, was pumped for 24 h at 500 ml/min through ca. 600 mg of conditioned Porapak Q (60–80 mesh) packed in a glass tube 7 cm long × 4 mm diameter (Steullet and Guerin 1992b). The cage was located in an animal room with 20 other rabbits of the same strain. Volatiles were desorbed with 3 ml dichloromethane (Merck, analytical grade) and the extract was then slowly concentrated under N_2 to ca. 50 µl. One or 10 µl of the concentrated extract was enclosed on filter paper in the stimulus cartridge. Air from adjacent rooms without animals (blank control) was also collected as described above on Porapak and analysed by GC-MS.

Air from a 30 m^3 stall occupied by 2 tick-naive Simmental steers (about 200 kg each), a race frequently used to rear *A. variegatum*, was pumped for 24 h at 500 ml/min through 600 mg of conditioned Porapak Q. Solvent desorbtion and concentration were achieved as for rabbit odour and 1 or 10 µl of the concentrated extract was used as stimulus. Extract of air from a washed stall unoccupied for a month was used as a blank control and analysed by GC-MS. Collection of rabbit and bovine odour was undertaken on several occasions with different rabbits and steer. The concentrated extracts smelled very similar to the natural odours.

Different body parts (head, shoulder, side, dewlap, chest, belly, legs, armpit, and perianal area) of two tick-naive Simmental steers were rubbed with acetone-washed cotton pads (7 × 7 cm) soaked with dichloromethane (analytical grade). Gloves were used for this operation. The cotton pads were placed in a 500-ml gas-wash bottle, held at 70°C, through which N_2 passed for 1 h at 100 ml/min. Released volatiles were held up in a cold trap (4 mm diameter, 20 cm long glass U-tube steeped in a dry ice/acetone mixture) in a Dewar flask. Dichloromethane (2 ml, analytical grade) was used to extract trapped volatiles, water was removed by lowering the extract to −10°C and removing the solvent from the ice. The extract was subsequently concentrated under N_2 to ca. 50 µl. One or 10 µl of the concentrate was then used as stimulus.

Gas chromatography-coupled electrophysiological recordings (GC-EL). Olfactory receptors, characterized as responding to vertebrate odours, were subsequently employed to locate active product(s) among the many constituents of odour extracts by GC-EL. Components of an active extract (bovine or rabbit odours collected on Porapak, skin wash of steer) were separated on a high-resolution capillary gas chromatography column (chromatograph: Carlo Erba Instruments HRGC 5160 with an on-column injector; fused-silica column: 30 m DBWAX, internal diameter 0.32 mm or 0.25 mm, 0.25 µm film thickness, G&W Scientific, USA; carrier gas: H_2 at 0.5 m/s at 40°C; temperature programmed: 60°C for 5 min, 8°C/min to 230°C, and held for 10 min). The column effluent was split (glass Y-splitter), 2/3 being sent to the flame ionisation detector (FID) and 1/3 (longer arm) to an electrophysiological preparation with receptor(s) sensitive to host odour (biological detector). An air stream (1 l/min), maintained at ca. 80% RH and 22 ± 1°C in a 7 mm diameter glass water-jacketed tube, swept one third of the column effluent to the tick preparation 30 cm away from a heated transfer line (250°C) in the wall of the chromatograph. The outlet of the glass tube (reduced outlet of 3 mm diameter) was 5 mm from the tick tarsus where the air speed was 1.5 m/s. Column effluent was thus simultaneously monitored by the FID and the activity of the receptors recorded to locate possible active component(s) of the extracts being analysed (Wadhams 1982).

All spikes from what usually amounted to multicellular recordings (AC signal) were sorted from background noise with a level discriminator incorporated in the UN-03 amplifier, and the sum of the frequencies of all firing cells was continuously converted to a voltage (time constant of the frequency to voltage converter: 1 s). This signal was printed on a multichannel chart-recorder simultaneous with the FID response. An electrophysiological response was

indicated by a sudden change in the overall activity of the olfactory cells recorded. Time delay (about 3 s) between the FID response and the biological response, due to the added travel time of substances in the longer arm of the splitter and delivery tube to the electrophysiological preparation, was estimated by recording activity of an easily accessible 2-nitrophenol-sensitive receptor located in a sensillum on the anterior pit of Haller's organ during elution of 10 ng 2-nitrophenol (Diehl et al. 1991). A difference in the delay between the FID response and the receptor response was never observed between the synthetic compounds tested. Variation in the response latency of the different receptors studied was negligible compared to the passage time of the substances to the preparation. The Kovat's index for each active component detected was calculated with reference to alkanes (C_{10} to C_{20}) injected under the same GC conditions.

Gas chromatography-coupled mass spectrometry (GC-MS). Extracts analysed by GC-EL to locate active constituents were subsequently concentrated about 5 times and analysed on the same GC phase by GC-MS (Hewlett Packard 5890 series I I chromatograph – mass selective detector 5971A) to identify the active products. One µl of extract was injected on-column to the DBWAX capillary column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, G&W Scientific, USA) connected via 1 m of deactivated fused-silica capillary to the MS (ionisation chamber temperature 180°C; ionisation energy 70 eV). Helium was used as carrier gas under constant pressure (velocity 0.3 m/s at 40°C) and separation was achieved with the same temperature programme as in GC-EL. Active components of the extracts located by GC-EL were relocated in GC-MS from the calculated Kovat's index, and by comparison of the chromatogram profiles obtained in GC-EL and GC-MS.

Identification of the active components. Identification of an electrophysiologically active peak in an extract was first based on the match of its mass spectrum with that of a known product stored in a computer-based library of the GC-MS. The mass spectrum and the calculated Kovat's index of the extract-unknown were then compared with those of the library-proposed synthetic analogue injected under the same conditions. Biological activity of the identified product was subsequently tested with the synthetic analogue by electrophysiology experiments on the olfactory receptor concerned. Full identification based on the mass spectrum was not always feasible because of the small amount of compound present and/or because of coeluting products which obscured the spectrum. In some extracts, compounds either suspected or clearly identified in other extracts, such as methylbenzaldehyde isomers and γ-valerolactone, were nevertheless detected by searching at the retention times of the volatiles in question for some of their characteristic fragment ions whose mass to charge ratios (M/Z) were 65, 91, 119, and 120 for methylbenzaldehyde isomers and 56, 85, and 100 for γ-valerolactone. The following synthetic chemicals were employed as standards in GC-MS and electrophysiology: hexanal, heptanal, nonanal, (E)-2-heptenal, furfural, benzaldehyde, 2-methylbenzaldehyde, 3-methylbenzaldehyde, 4-methylbenzaldehyde, 2-hydroxybenzaldehyde, and γ -valerolactone. In addition, (E)-2-hexenal, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 1-phenylethanone and cyclohexanone were used because of their relatedness to suspected active products. Dilutions of these chemicals (≥ 98% purity) from 10^{-5} to 10^{-2} *M* in dichloromethane were tested on the receptors concerned as described above.

Blank extracts (air from rooms without rabbits or steer) were also analysed by GC-MS to check for the possible presence of stimuli previously identified in bovine and/or rabbit odour extracts. For this purpose, 2-bromophenol (1.64 µg) was added as internal standard to 1.5 ml rabbit, bovine, and blank extracts before concentration. Using the single ion monitoring facility (SIM) of the mass selective detector, the presence of a stimulus was searched for, at the retention time of the synthetic analogue, by one of its characteristic fragment ions with a mass to charge ratio (M/Z) of 72 for hexanal, 70 for heptanal, 83 for (E)-2-heptenal, 98 for nonanal, 96 for furfural, 106 for benzaldehyde, 119 for methylbenzaldehyde isomers, and 122 for 2-hydroxybenzaldehyde. Quantification was achieved by peak integration for the characteristic fragment ion chosen. Abundance of each stimulus was then normalized with reference to the amount of 2-bromophenol calculated from the peak area of one of its characteristic fragment ions (M/Z = 172 corresponding to M^+–1).

Olfactory receptor characterization. After identification of the host-odour stimulants, attention was focused on the olfactory receptors concerned. Dose-response curves based on the first 200 ms of the response to synthetic stimulants and thus the specificity spectrum of the responsive receptors were studied. To discriminate between different receptor cells of similar spike shape and amplitude in multicellular recordings, double successive stimulation was sometimes necessary. This consisted of delivering an active compound A for a few s to the preparation interrupted for 1 s by stimulation with active compound B, and vice versa. No change in response, decrease or increase in firing of cell(s), cessation of firing in cell(s) or excitation of new cell(s) during the transition from compound A to B indicates if the same cell is responsive to the two substances or if different cells are excited. Possible cross-adaptation between receptor cells was not examined.

Results

Activity of olfactory receptors was captured at 6 distinct locations in the capsule of Haller's organ as revealed by hundreds of recordings. Results presented here deal with receptors recorded from within regions I and VI of the capsule (Fig. 1). Whereas one receptor in region I was

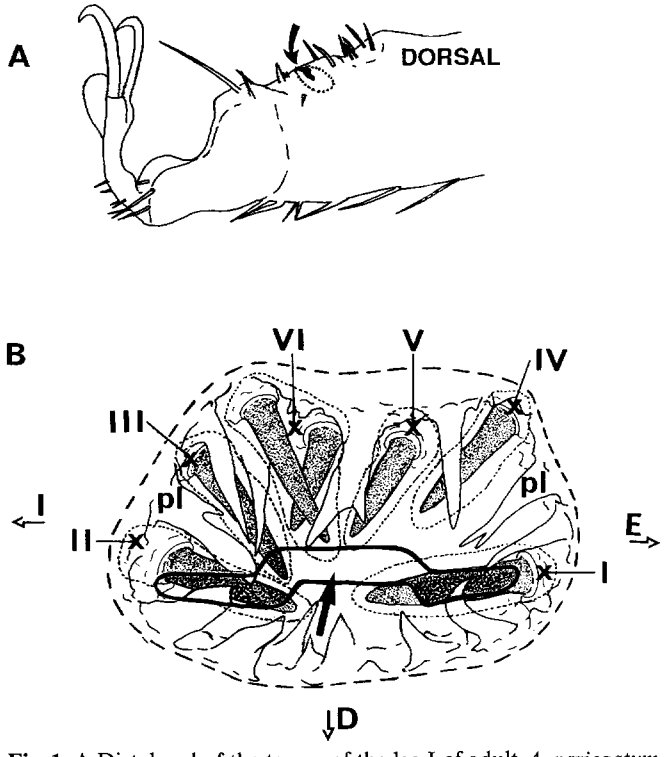

Fig. 1. **A** Distal end of the tarsus of the leg I of adult *A. variegatum* with the slit opening (*arrow*) to olfactory sensilla within the capsule of Haller's organ. **B** Diagrammatic view of the lay-out of olfactory sensilla in the capsule as shown by microscopic studies. The capsule is divided here into 6 regions (I-VI) from which olfactory responses were obtained. An outline of the slit opening is surimposed (see *arrow*). *D* distal; *E* external; *I* internal; *pl* pleomorph (for more details, see also Steullet and Guerin 1992a)

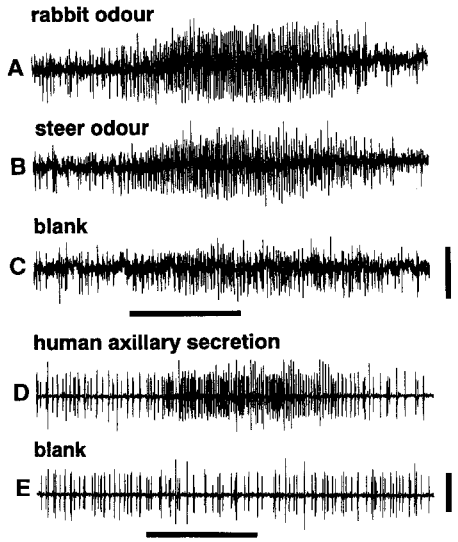

Fig. 2A–E. Responses recorded from olfactory sensilla in region VI (Fig. 1) of the capsule of Haller's organ in male *A. variegatum* where several receptors are excited by vertebrate odours. In preparation one: responses to rabbit odour (**A**), steer odour (**B**) both collected on Porapak Q, and to a wash of conditioned but unused Porapak Q (blank odour) (**C**). The response to stimulation with the blank odour was much lower than that due to either bovine or rabbit odour. In preparation two: responses to human armpit secretion collected on cotton (**D**), and to cotton alone (blank) (**E**). A slight inhibition occurs during stimulation with the blank. *Horizontal bars* 1 s stimulation; *vertical bars* 1 mV

sensitive to γ-valerolactone, a compound detected in traces in bovine and rabbit odours, the other receptors stimulated by bovine and rabbit odours collected on Porapak (Fig. 2A–C) were all found in the proximal region of the capsule (region VI in Fig. 1). Receptors within the latter region were also regularly excited by human axillary secretion (Fig. 2D, and E), but did not respond clearly to either human breath or to physiological relevant levels of the synthetic chemicals cited in Materials and methods under stimulus delivery.

Identification of some vertebrate volatiles stimulating olfactory receptors in region VI of the capsule

Gas chromatography-coupled electrophysiological recordings (GC-EL) revealed that several components of bovine and/or rabbit odours stimulated olfactory receptors in region VI of the capsule (Table 1 and Figs. 3, 4, and 5). Gas chromatography-coupled mass spectrometry (GC-MS) subsequently permitted identification of some of these active volatiles, often in minor amounts in both bovine and rabbit odours collected on Porapak. These volatiles were hexanal, heptanal, 2-heptenal, nonanal, furfural, benzaldehyde, 2-, 3-, and 4-methylbenzaldehyde, and 2-hydroxybenzaldehyde (Table 1). Hexanal, 2-heptenal, nonanal, furfural, benzaldehyde, and 2-hydroxybenz-

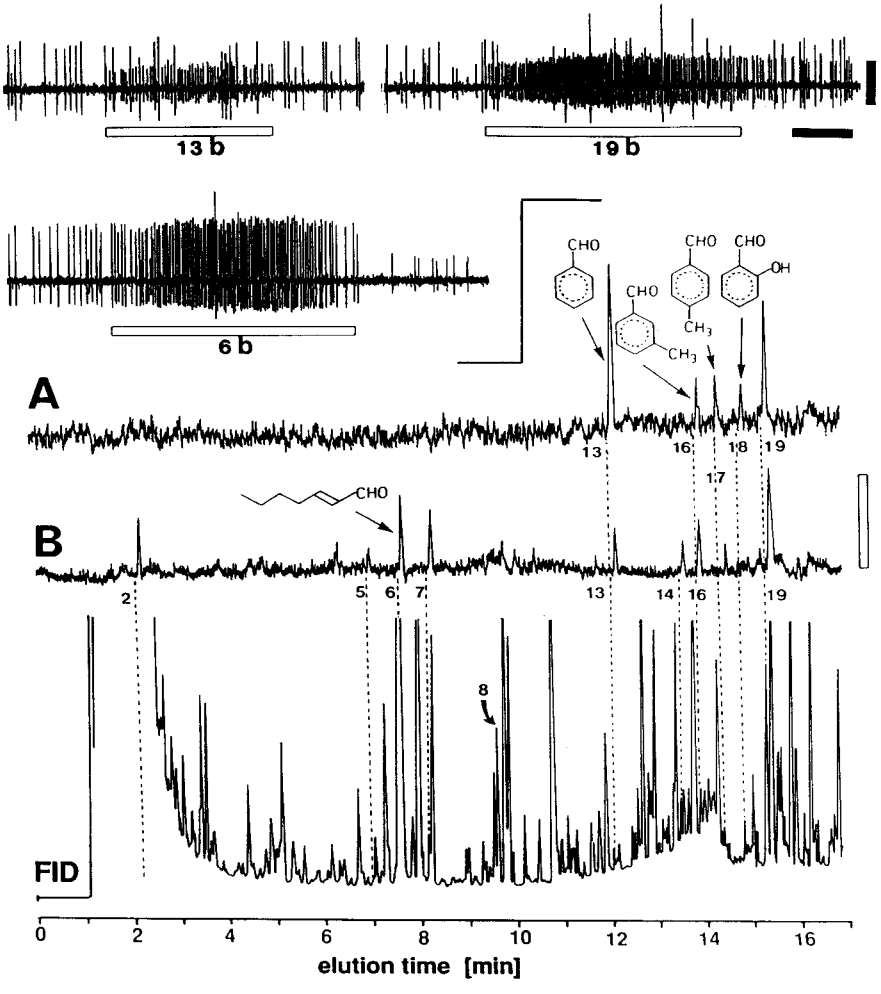

Fig. 3A, B. Analysis of a bovine odour, collected on Porapak, by gas chromatography-coupled electrophysiology of olfactory receptors within region VI of the capsule of Haller's organ in male *A. variegatum*. The *bottom trace* is the flame ionisation detector response (FID). The *upper traces* **A** and **B** represent the summed frequency of all firing cells (frequency to voltage converted signal) in two different recordings; hollow vertical bar is 50 impulses/s. Difference in response to the odour constituents between **A** and **B** was due to the different population of olfactory receptors captured in each case, although the recording electrode was placed in the same region of the capsule for both analyses. Two olfactory sensilla occur in this region of the capsule (Fig. 1). GC-MS analyses of the active peaks indicated that responses were recorded in both **A** and **B** for benzaldehyde (13), 3-methylbenzaldehyde (16), and the unknown peak 19, but only in **A** for 4-methylbenzaldehyde (17) and 2-hydroxybenzaldehyde (18). Receptors responding to (E)-2-heptenal (6) and the unknown peaks 2, 5, 7, and 14 were present in **B** but not in **A**. Other minor responses recorded once or only very occasionally were not accounted for. Active constituents are numbered as in Table 1. The FID trace indicated 0.5 to 1 ng for benzaldehyde (13) in the odour extract injected. Spike trains generated in **B** during elution of (E)-2-heptenal (6b), benzaldehyde (13b), and the unknown peak 19b are given at the top of the figure. *Hollow horizontal bars* depict the approximate time taken by the products to elute from the GC column; *solid horizontal bar* 1s; *solid vertical bar* 1 mV

Table 1. Identified constituents of bovine and rabbit odour which stimulated olfactory receptors located within the proximal-internal region VI of the capsule of Haller's organ in male *A. variegatum*

Peak number	Olfactory stimulant	a) Identification criteria	b) Odour source	c) Kovat's index in GC-EL	d) Kovat's index in GC-MS	e) Kovat's index of standards in GC-MS	f) Response occurrence
3	hexanal	MKE	steer	1085±0	1077	1079	2/9
			rabbit	1084±8	1081	1079	6/10
4	heptanal	MKE	steer	–	1188	1186	0/9
			rabbit	1184±5	1184	1184	3/10
6	2-heptenal	MKE #	steer[a]	1336±0	1337	1336	4/9
			rabbit	1338±4	1338	1336	5/10
8	nonanal	MKE	steer	1400±0	1398	1394	3/9
			rabbit	1396	1396	1396	1/10
11	furfural	MKE	steer[a]	1475±7	1469	1469	2/9
			rabbit	1469±7	1470	1469	3/10
13	benzaldehyde	MKE	steer[a]	1535±5	1531	1532	5/9
			rabbit	1528±5	1532	1532	6/10
15	2-methylbenzaldehyde	MKE	steer	–	1622	1621	0/10
			rabbit	1600	1622	1621	1/10
16	3-methylbenzaldehyde	MKE	steer	1623±3	1627	1626	2/9
			rabbit	1611±5	1628	1626	3/10
17	4-methylbenzaldehyde	MKE	steer	1657±3	1656	1653	2/9
			rabbit	1643±3	1655	1653	3/10
18	2-hydroxybenzaldehyde	MKE	steer[a]	1677±3	1686	1682	5/9
			rabbit	1675±5	1689	1682	10/10

This table is based on gas chromatography-coupled electrophysiology (GC-EL) and gas chromatography-coupled mass spectrometry (GC-MS) analyses of bovine and rabbit odour collected on Porapak Q, and the skin wash of steer (both types of analyses were made on the same GC phase DBWAX). **a)** Different criteria on which identification of a particular vertebrate volatile was based: M – matching mass spectra, K – matching Kovat's index, and E – matching electrophysiological activity with that of the synthetic analogue (# the trans isomer of 2-heptenal was employed). **b)** Analyses were made of bovine or rabbit odour as collected on Porapak, and [a] indicates that the active compound was also detected by GC-MS in a bovine skin wash. **c)** Mean Kovat's index (±standard deviation) of active peaks in GC-EL analyses. **d)** Kovat's index of the active peak located in GC-MS. **e)** Kovat's index of the synthetic product corresponding to that of the biologically active peak in GC-MS. **f)** Number of GC-EL analyses in which a response was observed/out of the total number of analyses with receptors from within region VI of the capsule. Other components of bovine and/or rabbit odours (GC peaks 1, 2, 5, 7, 9, 10, 12, 14, and 19 listed in the text and figures) occasionally activated receptors in GC-EL analyses, but could not be identified by GC-MS

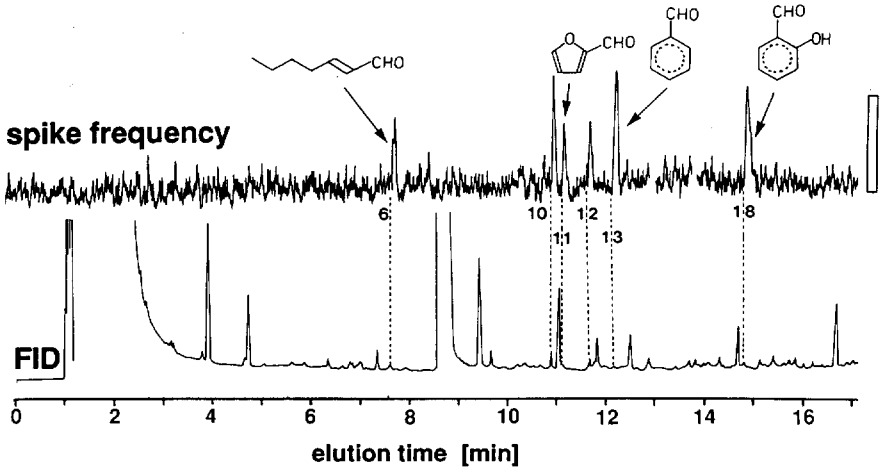

Fig. 4. Analysis of a bovine skin wash by gas chromatography-coupled electrophysiology of olfactory receptors within the proximal region VI of the capsule of Haller's organ in a male *A. variegatum*. The *lower trace* is the flame ionisation detector response (FID) and the *upper trace* is the summed frequency of all firing cells (frequency to voltage converted signal). *Hollow vertical bar* is 50 impulses/s. Active components are numbered as in Table 1. GC-MS analysis of the active peaks indicated that responses were obtained for (E)-2-heptenal (6), furfural (11), benzaldehyde (13), and 2-hydroxybenzaldehyde (18). Active constituents 10 and 12 were not characterized. Although the mass spectrum and retention time of peak 10 suggests 1-octen-3-ol, response to the synthetic analogue was never recorded from this region of the capsule. Furthermore, the response to component 12 always accompanied that to furfural (11) in GC-EL experiments. The FID trace indicated (E)-2-heptenal (6) at 0.1–0.5 ng in the extract injected

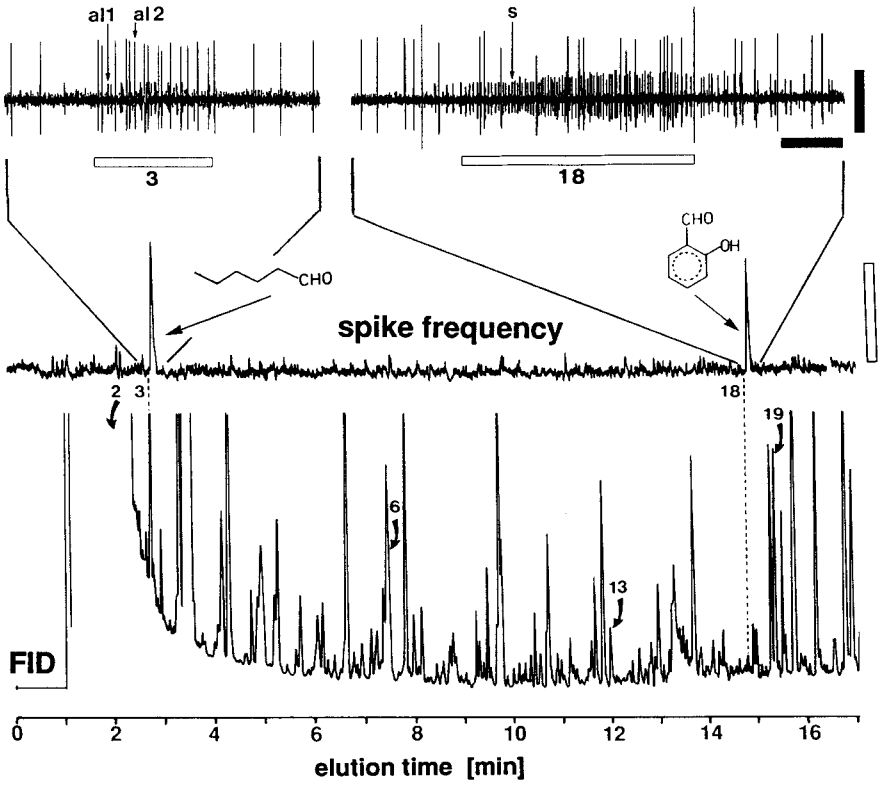

Fig. 5. Analysis of a rabbit odour, collected on Porapak, by gas chromatography-coupled electrophysiology of olfactory receptors within the proximal region VI of the capsule of Haller's organ in a male *A. variegatum*. The *lower trace* is the flame ionisation detector response (FID); the *middle trace* is the summed frequency of all firing cells (frequency to voltage converted signal), *hollow vertical bar* is 25 impulses/s; the *upper traces* are the actual spike trains generated during elution of hexanal (3) and 2-hydroxybenzaldehyde (18). The aliphatic aldehyde receptors type 1 (al1) and type 2 (al2) were both stimulated by hexanal (3), but only the 2-hydroxybenzaldehyde receptor (s) responded to 2-hydroxybenzaldehyde (18). *Hollow horizontal bars* indicate the elution time of the active peaks. *Solid horizontal bar* 1 s; *solid vertical bar* 1 mV. Active components are numbered as in Table 1. Receptors sensitive to 2-heptenal (6), benzaldehyde (13), and the unidentified peak 19, known to occur in this region of the capsule, were not captured in this recording

Table 2. Olfactory receptor types in the capsule of Haller's organ of *A. variegatum* which responded to specific classes of vertebrate volatiles

Receptor type	Location in the capsule	Best stimulant	Number of observations[a]
Benzaldehyde receptor	region VI	benzaldehyde	25
2-hydroxybenzaldehyde receptor	region VI	2-hydroxybenzaldehyde	23
Aliphatic aldehyde receptor 1	region VI	hexanal	12
Aliphatic aldehyde receptor 2	region VI	heptanal	11
Aliphatic aldehyde receptor 3	region VI	(E)-2-heptenal	13
Lactone receptor	region I	γ-valerolactone	15

[a] Indicates the number of times the receptor was recorded from in either gas chromatography-coupled electrophysiology analyses of vertebrate volatiles or in classic single-unit recordings

aldehyde were also discovered in steer skin wash. Although hexanal, nonanal, furfural, and benzaldehyde were also detected in blanks, they were more abundant in areas permeated with vertebrate odours, i.e. 2 to 4 times more benzaldehyde and furfural, >4 times more nonanal, and >20 times more hexanal than in blanks.

Characterization of receptors responding to the identified vertebrate volatiles

The responses recorded to the host-odour components by GC-EL recordings from region VI of the capsule were sometimes very variable. Olfactory receptor(s) responding to 2-heptenal (6) and the unidentified components 2, 5, 7, 14 of bovine odour in recording B of Fig. 3 were absent in recording A, whereas activity of the receptor sensitive to 2-hydroxybenzaldehyde (18) was only captured in recording A. In another GC-EL experiment (Fig. 5), no responses were recorded from receptors for 2-heptenal (6), benzaldehyde (13), and the unidentified components 2 and 19, although these constituents of this rabbit extract proved active on olfactory receptors from approximately the same location within the capsule in other GC-EL recordings. This was due to the fact that the different types of olfactory receptors from within region VI of the capsule from which recordings were obtained were probably distributed in two adjacent sensilla (Fig. 1), and the chance of picking up activity of any given receptor was dependent on the exact location of the electrode. In this study, we were unable to determine to which sensilla the different receptors captured within region VI of the capsule belong. Careful examination of spike shape and amplitude of receptors responding to

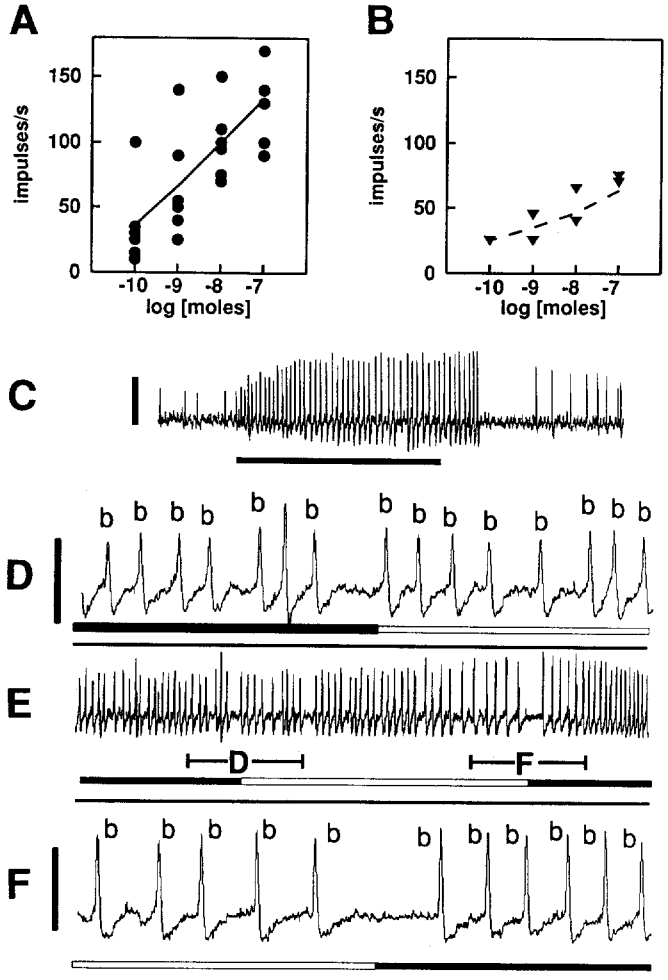

Fig. 6. **A–C** Response of a benzaldehyde-sensitive receptor in the proximal region VI of the capsule of Haller's organ in male *A. variegatum* to benzaldehyde and furfural. **A** Dose (moles in stimulus cartridge) of benzaldehyde (n = 6 different ticks) and **B** dose of furfural (n = 2 different ticks) plotted against spike frequency of the responding receptor calculated from the first 200 ms of the response. *Trend lines* connect mean values. In **A** and **B** the response of each receptor increased with increasing doses throughout the range tested. **C** Representative response of the receptor to benzaldehyde at 10^{-9} moles in the stimulus cartridge. **D–F** Double successive stimulation of the benzaldehyde-sensitive receptor (*b*) with 10^{-8} moles benzaldehyde in the stimulus cartridge (*solid horizontal bar*) interrupted with 10^{-7} moles furfural in a second stimulus cartridge (*hollow horizontal bar*). Absence of a second excited cell at onset of stimulation with furfural suggests that furfural activated the same receptor as benzaldehyde. **D** Spike train illustrating response to last 200 ms of stimulation with benzaldehyde and corresponding period from onset of stimulation with furfural. **F** Spike train resulting from reverse of situation in **D**, i.e. last 200 ms of stimulation with furfural and restart of stimulation with benzaldehyde. *Solid horizontal bar* in **C** and *hollow bar* in **E** represent 1 s; *vertical bars* 1 mV

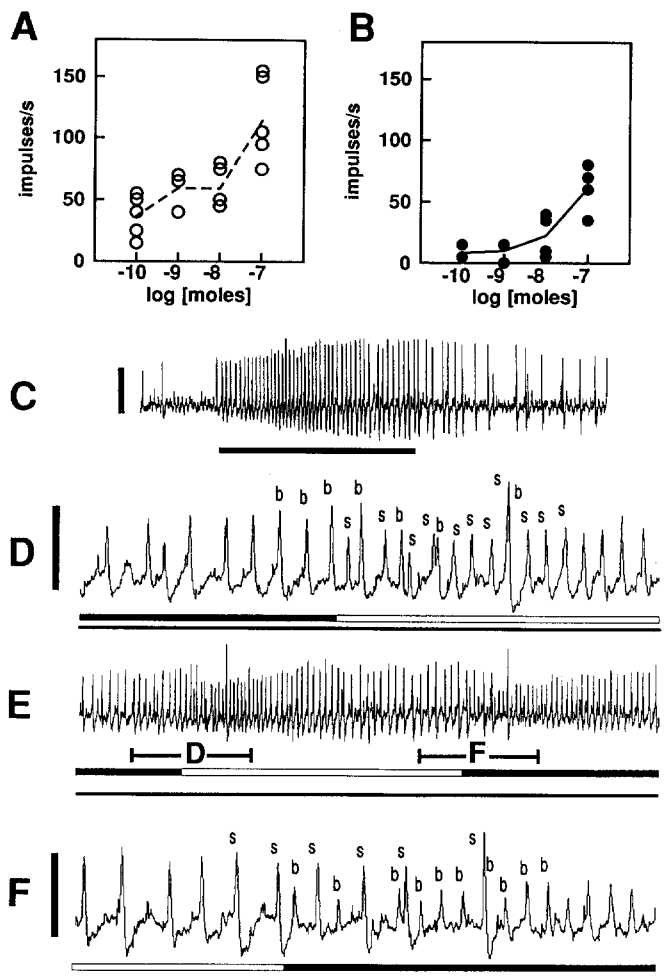

Fig. 7. **A–C** Response of receptors sensitive to 2-hydroxybenzaldehyde and benzaldehyde in the proximal region VI of the capsule of Haller's organ in *A. variegatum*. **A** Dose (moles in stimulus cartridge) of 2-hydroxybenzaldehyde (n = 5 different ticks) and **B** dose of benzaldehyde (n = 4 different ticks) plotted against spike frequency of the responding receptor calculated from the first 200 ms of the response. *Trend lines* connect mean values. In **A** and **B** the response of each receptor increased with increasing doses throughout the range tested. **C** Representative response of the receptor to 2-hydroxybenzaldehyde at 10^{-9} moles in the stimulus cartridge. **D–F** Double successive stimulation while recording from a receptor responding to benzaldehyde (*solid horizontal bar*) interrupted for 1 s (*hollow horizontal bar* in **E**) by stimulation with 2-hydroxybenzaldehyde (both products at 10^{-8} moles in separate stimulus cartridges). A phasic burst in spike frequency of a second excited cell at onset of stimulation with 2-hydroxybenzaldehyde and at restart with benzaldehyde indicated presence of separate receptors (*b* and *s* respectively, in **D** and **F**) for these two products in region VI of the capsule of Haller's organ. **D** Spike train illustrating response to last 200 ms of stimulation with benzaldehyde and corresponding onset period with 2-hydroxybenzaldehyde. **F** Spike train resulting from reverse of situation in **D**, i.e. last 200 ms of stimulation with 2-hydroxybenzaldehyde and restart of stimulation with benzaldehyde. *Solid horizontal bar* in **C** and *hollow bar* in **E** represent 1 s; *vertical bars* 1 mV

single and/or double stimulations with synthetic analogues of the identified stimulants (benzaldehyde, 2-hydroxybenzaldehyde, furfural, and aliphatic aldehydes) permitted us to clearly discriminate at least five types of receptors in the region VI of the capsule (Table 2). Proper characterization of receptors responding to methylbenzaldehyde isomers was not undertaken because of the great difficulty in obtaining reproducible recordings. GC-EL analyses and double successive stimulations permitted characterization of separate receptors for the aromat-

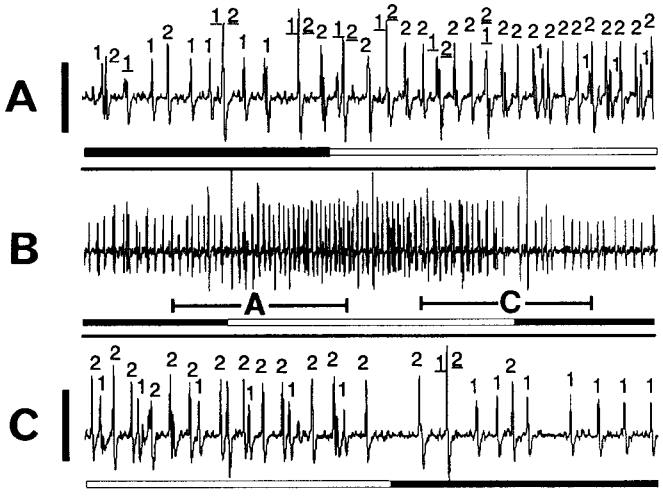

Fig. 8A–C. Response of olfactory receptors located within the proximal region VI of the capsule of Haller's organ in a male *A. variegatum* to the short-chain aliphatic aldehydes (E)-2-hexenal and hexanal (each at 10^{-8} moles in the stimulus cartridge). **B** Double successive stimulation with (E)-2-hexenal (*solid bar*), which stimulated both receptor 1 and receptor 2 weakly, was interrupted for 1 s by stimulation with hexanal (*hollow bar*) which activated receptor 2 more than (E)-2-hexenal did. Sensitivity of receptor 1 to both aldehydes was approximately the same. **A** Spike train illustrating response to last 300 ms of stimulation with (E)-2-hexenal and corresponding period from onset of stimulation with hexanal. **C** Spike train resulting from reverse of the situation in **A**, i.e. last 300 ms of stimulation with hexanal and restart of stimulation with (E)-2-hexenal. Spikes with underlined numbering are overlapping events; *vertical bars* 1 mV

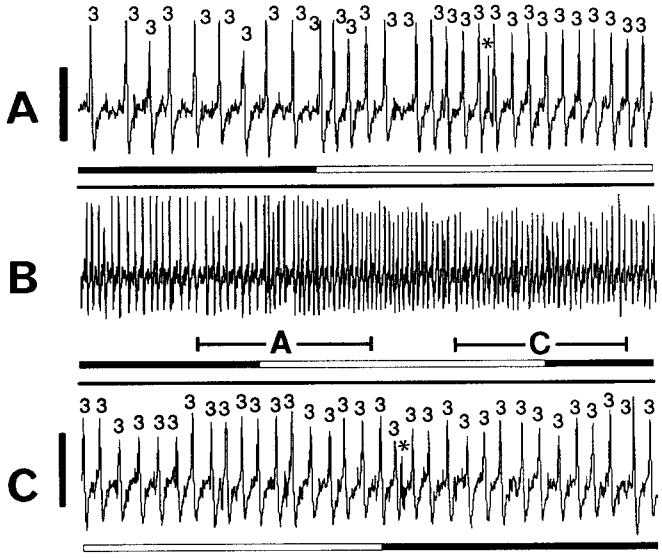

Fig. 9A–C. Response of an olfactory receptor (aldehyde receptor type 3) located within the proximal region VI of the capsule of Haller's organ in a male *A. variegatum* to the unsaturated aldehydes (E)-2-hexenal and (E)-2-heptenal (each at 10^{-8} moles in the stimulus cartridge). **B** Double successive stimulation with (E)-2-heptenal (*solid bar*) interrupted for 1 s by stimulation with (E)-2-hexenal (*hollow bar*). Absence of a second responding unit at onset of stimulation with (E)-2-hexenal or at restart of stimulation with (E)-2-heptenal indicated that both products activated the same receptor. This receptor type did not respond to short-chain saturated aliphatic aldehydes. **A** Spike train illustrating response to the last 300 ms of stimulation with (E)-2-heptenal and corresponding period from onset of stimulation with (E)-2-hexenal. **C** Spike train resulting from reverse of the stimulation in **A**, i.e. last 300 ms of stimulation with (E)-2-hexenal and restart of stimulation with (E)-2-heptenal. At least one other receptor with a spike of smaller amplitude (*star*) occurs in these traces. *Vertical bars* 1 mV

ic aldehydes (benzaldehyde and 2-hydroxybenzaldehyde) and for different aliphatic aldehydes.

Benzaldehyde receptor. One type of receptor in region VI of the capsule was sensitive to benzaldehyde (Fig. 6A and C) and to furfural as suggested by double successive stimulations (Fig. 6D–F). The dose-response curve for furfural was however much flatter than that for benzaldehyde (Fig. 6A and B). Since in GC-EL an estimated concentration of ca. 6×10^9 molecules of benzaldehyde/cm^3 air arriving at the tick preparation increased the base firing of this receptor from 5 to ca. 50 impulses/s (calculated on a spike train of 1 s during the peak of the response), the threshold of this receptor for benzaldehyde is still much lower than this level. Concentration of benzaldehyde at the preparation was estimated on the amount injected onto the GC column, the width of the benzaldehyde eluting peak, the fraction of the column effluent directed to the preparation, and the dilution factor in the air stream carrying the product to the preparation. Furthermore, responses to furfural were always accompanied by responses to a later eluting product in GC-EL experiments (peak 12 in Fig. 4) which excited spikes of the same shape and amplitude as furfural and benzaldehyde. This suggested that the unidentified component 12 may stimulate the same receptor as benzaldehyde and furfural, and thus share common chemical properties with these two volatiles.

2-Hydroxybenzaldehyde receptor. Another receptor in region VI of the capsule was strongly stimulated by 2-hydroxybenzaldehyde (Fig. 7A and C). Double successive stimulation with benzaldehyde and 2-hydroxybenzaldehyde permitted a clear distinction between the 2-hydroxybenzaldehyde receptor and the benzaldehyde receptor which were sometimes both captured in the same recordings (Fig. 7D–F). However, the 2-hydroxybenzaldehyde receptor also had the capacity to respond to very high doses of benzaldehyde (at least 10^{-8} moles in the stimulus cartridge) (Fig. 7B).

Three types of aliphatic aldehyde receptors. Three types of aliphatic aldehyde receptors with different specificity spectra were distinguished in region VI of the capsule (Figs. 8 and 9). One receptor (type 1) was particularly sensitive to saturated and unsaturated C_6 aliphatic aldehydes, but was only slightly sensitive to heptanal and hardly to nonanal (Fig. 10A and B). Another receptor (type 2) responded mainly to saturated aliphatic aldehydes such as hexanal and heptanal (Fig. 10C and D), whereas a third receptor (type 3) was excited by unsaturated aliphatic aldehydes such as (E)-2-hexenal and (E)-2-heptenal (Fig. 10E and F). These aliphatic aldehyde re-

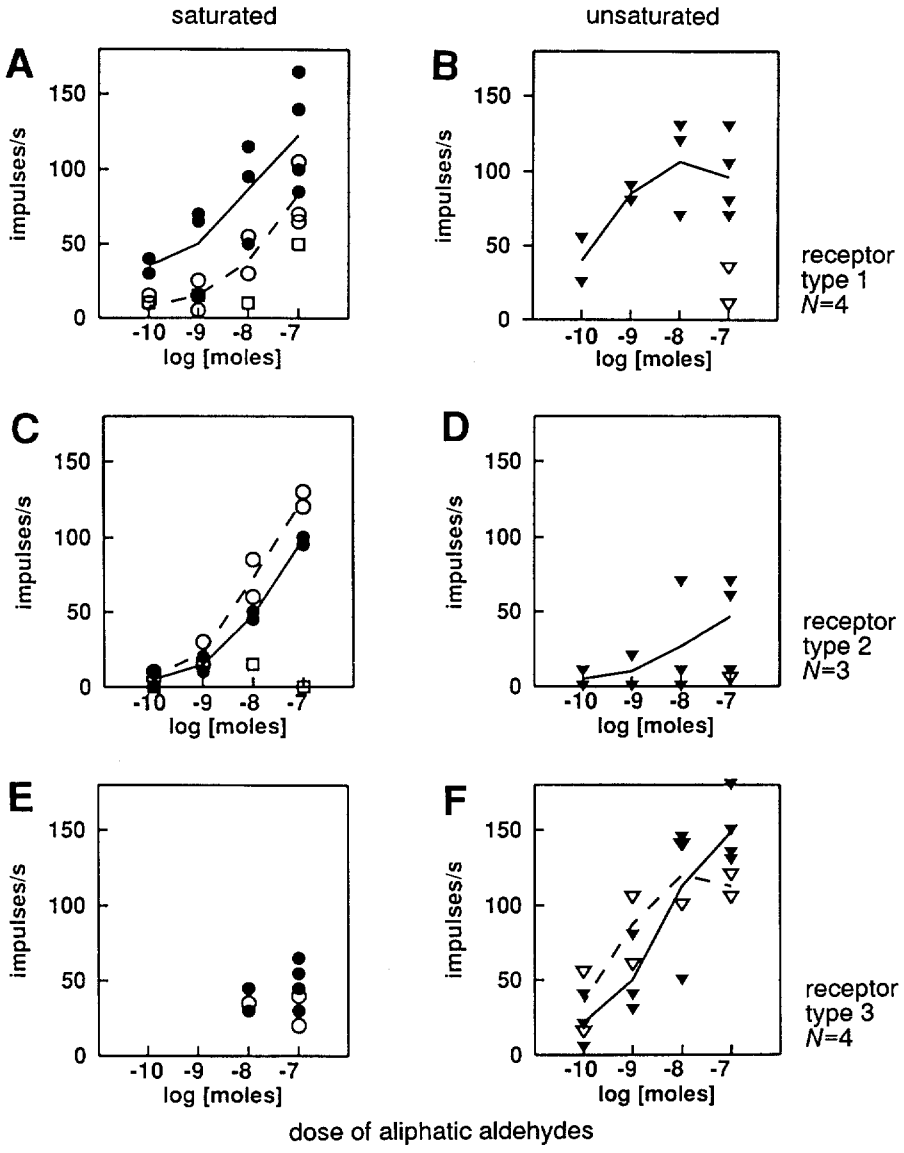

Fig. 10A–F. Representative dose response-curves of olfactory receptors located within the proximal region VI of the capsule of Haller's organ in male *A. variegatum* to saturated and unsaturated aliphatic aldehydes. *Solid circle* hexanal; *hollow circle* heptanal; *square* nonanal; *solid triangle* (E)-2-hexenal; *hollow triangle* (E)-2-heptenal. *Ordinates* in **A–F** are spike frequencies of the responding receptors calculated from the first 200 ms of the response, and *abscissas* dose of products tested (moles in stimulus cartridge). *Trend lines* connect mean values. In each case, the response of the receptors increased with increasing doses throughout the range tested. **A** and **B** Aldehyde receptor type 1 responded equally well to saturated and unsaturated C_6 aldehydes (**A** and **B** were established with the same receptors, $n = 4$); **C** and **D** aldehyde receptor type 2 was more sensitive to the saturated C_6 and C_7 aldehydes than to the corresponding unsaturated products (**C** and **D** were established with the same receptors, $n = 3$); **E** and **F** aldehyde receptor type 3 which was selectively stimulated by unsaturated aldehydes (**E** and **F** were established with the same receptors, $n = 4$) (see also Figs. 9 and 10)

ceptors seemed less sensitive than the benzaldehyde receptor since in GC-EL analyses an estimated concentration of ca. 3×10^{11} molecules of hexanal/cm^3 air arriving at the preparation was required to evoke responses of ca. 25 impulses/s from a base frequency of ca. 5 impulses/s in receptor type 1, and ca. 15 impulses/s from a base frequency of ca. 5 impulses/s in receptor type 2. In GC-EL analyses, responses to hexanal were due to either receptor type 1 or 2, or occasionally both (Fig. 5), heptanal and nonanal mostly excited receptor type 2, and (E)-2-heptenal stimulated receptor type 3 (Fig. 3). (E)-2-hexenal was not detected in any of the host-odour extracts analysed in this study. Responses to two unidentified components of bovine odour collected on Porapak (peaks 5 and 7 in Fig. 3B) always accompanied responses to (E)-2-heptenal, and spike shapes and amplitudes of the excited receptors were very similar. This suggests that components 5 and 7 may share common chemical properties with 2-heptenal and may likewise excite the aliphatic aldehyde receptor type 3. Nevertheless, we were unable to identify both peaks 5 and 7 by mass spectrometry.

Lactone receptor. Finally, when the glass electrode was introduced within the exterio-anterior part of the capsule (region I in Fig. 1), a receptor responsive to γ-valerolactone was systematically found among other sensory cells (Fig. 11). This receptor also responded weakly to γ-butyrolactone and 6-caprolactone, hardly at all to bovine odour extracts, and not at all to human breath and human axillary secretion. Nevertheless in GC-MS, a compound with three fragment ions (with mass to charge ratios of 56, 85, and 100) characteristic for γ-valerolactone and eluting at the same retention time as the synthetic analogue was detected in bovine and rabbit odour collected on Porapak. This suggested that these extracts may contain traces of γ-valerolactone but in insufficient quantity to clearly stimulate the lactone receptor in GC-EL.

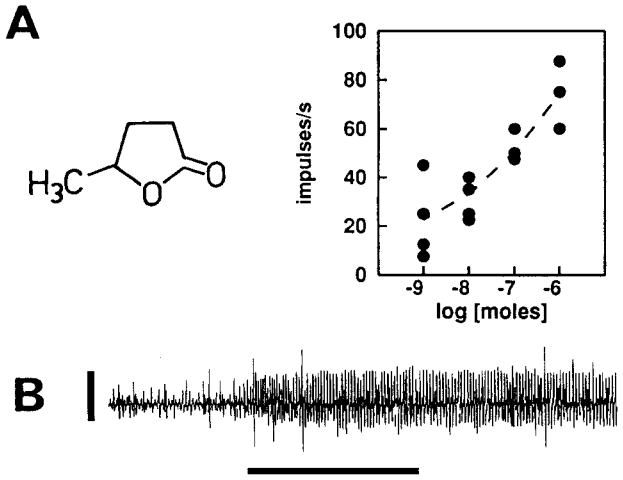

Fig. 11A, B. Response of an olfactory receptor within the exterio-anterior region I (Fig. 1) of the capsule of Haller's organ in male *A. variegatum* to γ-valerolactone. **A** Dose (moles in stimulus cartridge) of γ-valerolactone plotted against spike frequency of the responding receptor calculated from the first 200 ms of the response ($n = 5$ different ticks). *Trend line* connects mean values. **B** Representative response of the receptor to γ-valerolactone at 10^{-7} moles in the stimulus cartridge. *Horizontal bar* 1 s stimulation; *vertical bar* 1 mV

Discussion

The following components of rabbit and/or bovine odour stimulate olfactory receptors within chemosensilla in regions I and VI of the capsule of Haller's organ in *A. variegatum*: short-chain saturated and unsaturated aliphatic aldehydes, furfural, benzaldehyde, methylbenzaldehyde isomers, 2-hydroxybenzaldehyde, and γ-valerolactone. Except for saturated aliphatic aldehydes, these volatiles were remarkably minor components of the collected vertebrate odours.

Aliphatic and aromatic aldehydes as well as lactones are not known as kairomones for other haematophagous arthropods. Only propanal-sensitive neurons have been described in tsetse flies (Bogner 1992). These volatiles are furthermore not specific to vertebrate odours. Many phytophagous but also some scavenger arthropods are known to bear olfactory receptors sensitive to aliphatic aldehydes (i.e. constituents of the green leaf odour) or to benzaldehyde (Sass 1976; Seelinger 1977; Visser 1986). Benzaldehyde and 2-hydroxybenzaldehyde produced by different plants can act as repellents for several insects (Wallace and Mansell 1976). Finally, aliphatic aldehydes, benzaldehyde, 2-hydroxybenzaldehyde, and lactones also occur in the defensive secretions of various arthropods (Tschinkel 1975; Blum 1981).

In *A. variegatum*, benzaldehyde stimulates a receptor within region VI of the capsule. High amounts of this compound also excite another receptor most sensitive to 2-hydroxybenzaldehyde. Benzaldehyde has already been identified as a component of the aggregation-attachment pheromone of the related species *A. hebraeum* (Apps et al. 1988), but not in *A. variegatum* (Diehl et al. 1991; Diehl, unpublished). This aromatic aldehyde has been identified from a number of vertebrate sources: preorbital glands of the muskox (Flood et al. 1989), chin glands of the rabbit (Goodrich 1983), human vaginal secretion (Preti et al. 1977), and mouse urine (Andreolini et al. 1987). Benzaldehyde is also reported as a common volatile in air (Welsch and Watts 1990) and in this study was detected in the air of empty stalls (blanks), but at lower amounts than in rooms with steer or rabbits. The benzaldehyde receptor is also stimulated by furfural, a component of both bovine and rabbit odours collected on Porapak, bovine skin wash, and rabbit excrement, but also in human effluvia (Ellin et al. 1974), in human vaginal secretion (Preti et al. 1977) and in human urine (Zlatkis and Liebich 1971).

2-Hydroxybenzaldehyde strongly stimulates another receptor in the region VI of the capsule of Haller's organ. This product, present as a minor constituent of bovine and rabbit odours collected on Porapak and of the bovine skin wash, but absent in our blank odour, was once identified in extracts of replete female *A. variegatum* (Wood et al. 1975), but a physiological role was never proposed. Presence of 2-hydroxybenzaldehyde in vertebrate odours is nevertheless not well-documented, although it has been reported in the anal glands of the beaver (Lederer 1946).

Aliphatic aldehydes, found in our concentrates of vertebrate odour, are detected by three different types of receptors in region VI of the capsule of Haller's organ of *A. variegatum*. The type 1 responds best to both saturated and unsaturated C_6 aliphatic aldehydes, the type 2 to saturated C_6 and C_7 aliphatic aldehydes, and the type 3 to unsaturated C_6 and C_7 aliphatic aldehydes. The aliphatic aldehydes were generally present in our odour extracts at levels well above those of the aromatics. Saturated and unsaturated aliphatic aldehydes, as well as the corresponding ketones, alcohols, and fatty acids are well-documented commonly occurring volatiles in vertebrate odours. Some are also common air-pollutants (Welsh and Watts 1990), so it was not surprising to find traces of hexanal and nonanal in our blanks. Unsaturated aliphatic aldehydes are reported from goats (Smith et al. 1984), human breath (Krotoszynski et al. 1977), and the rabbit (Goodrich 1983), and saturated aliphatic aldehydes occur in the dog anal sac (Natynczuk et al. 1989), coyote urine (Schultz et al. 1988), skin glands of various bovidae (Burger et al. 1981), anal and chin glands of the rabbit (Goodrich 1983), human effluvia (Ellin et al. 1974; Goetz et al. 1988; Krotoszynski et al. 1977), and human axillary secretion (Labows et al. 1979b). The electrophysiological responses reported here for the human axillary secretion may be due to its aliphatic aldehyde content. Equipped with three aliphatic aldehyde receptor types, *A. variegatum* would be able to discriminate between volatile mixtures characteristic of different vertebrates. The ratio between responses of the different aldehyde receptors could thus serve as an odour-specific coding parameter as proposed by Tichy and Loftus (1983) for perception of alcohols by the stick insect, *Carausius morosus*.

γ-Valerolactone, present here in traces in bovine and rabbit odour, excites a receptor located in region I of the capsule of *A. variegatum*. Lactones have been reported from a variety of species within the Bovidae, Camelidae,

and Primates. Yeasts of the genus *Pityrosporum* are responsible for the production of different γ-lactones in areas rich in sebaceous glands of humans (Labows et al. 1979a). Several lactones are also reported in human scalp and urine (Goetz et al. 1988; Zlatkis and Liebich 1971), in occipital glands of *Camelus bactrianus* (Ayorinde et al. 1982), in preorbital glands of the muskox (Flood et al. 1989), blue and grey duikers (Burger and Pretorius 1987; Burger et al. 1990), and in pedal glands of bontebock (Burger et al. 1977). As adult *A. variegatum* mostly feed on Bovidae, lactones present in many of these vertebrates could possibly function as cues for host-finding and/or feeding-site selection.

In this study, receptor activity was recorded from six distinct regions within the capsule of Haller's organ of *A. variegatum*, corresponding to sensillum locations observed in microscopy. Recordings from five of these locations were highly reproducible in terms of the number of cells captured, spike shapes and amplitudes, spontaneous activity, and stimulus response, i.e. a situation typical for single sensillum recordings (Steullet and Guerin 1992a). By contrast, the recordings were not very reproducible in the posterio-interior region VI of the capsule. Here, the number of cells captured, and spike shapes and amplitudes varied from one preparation to another. We imagine that depending on the exact location of the tip of the glass recording electrode within region VI, different populations of receptors from one or both of the two closely associated sensilla in this region of the capsule were being sampled.

The seven wall-pore single-walled sensilla within the capsule carry between 21 and 35 receptor cells according to Hess and Vlimant (1982). About half of these cells have now been properly characterized: a CO_2-excited and a CO_2-inhibited receptor (Steullet and Guerin 1992a), two sulfide receptors (Steullet and Guerin 1992b), a methylsalicylate receptor (component of the aggregation-attachment pheromone, Hess and Vlimant 1986), and as described here benzaldehyde and 2-hydroxybenzaldehyde receptors, three aliphatic aldehyde receptors, and a lactone receptor. The capsule of the Haller's organ of *A. variegatum* is thus a very elaborate sense organ containing many olfactory receptors, each with a distinct sensitivity spectrum and capable of responding to various vertebrate odours such as breath, human axillary secretion, skin wash of steer, and to bovine and rabbit odour extracts.

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