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NADPH DIAPHORASE ACTIVITY IN THE ANTENNAE OF THE HAWKMOTH *MANDUCA SEXTA*

MONIKA STENGL AND RITA ZINTL
Institut für Zoologie, Universität Regensburg, Universitätsstraße 31, D-93053 Regensburg, Germany

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Summary

Biochemical and physiological studies suggested that increases in the levels of cyclic GMP in insect antennal receptor cells play a role in olfactory adaptation. As inositol-trisphosphate-dependent Ca^{2+} influx appears to precede the increase in intracellular cyclic GMP levels, it was hypothesized that a Ca^{2+}-dependent mechanism might stimulate the guanylyl cyclase. The present study used histochemical staining for NADPH diaphorase to examine whether antennal receptor neurones of male *Manduca sexta* could contain nitric oxide synthase. This Ca^{2+}/calmodulin-dependent enzyme is a prerequisite for nitric-oxide-dependent stimulation of guanylyl cyclase and possesses NADPH diaphorase activity. It was found that a subpopulation of olfactory receptor neurones as well as mechano-, thermo- and hygroreceptors on the moth antenna are NADPH-diaphorase-positive. Staining was also seen in non-neuronal cells. In the developing antenna, the NADPH-diaphorase-dependent staining was first observed at pupal stage 13–14, at approximately the same time as the antennal receptor neurones became physiologically active. The number and location of stained receptor cells was highly variable, and significantly more pheromone-sensitive sensilla were NADPH-diaphorase-positive in pheromone-stimulated antennae. This suggests that the enzyme is transiently activated by pheromone rather than being continuously active.

Key words: NADPH diaphorase, nitric oxide synthase, antennal receptor neurones, olfactory transduction, insects, antennae, *Manduca sexta*.

Introduction

Females of *Manduca sexta* and other species of moth attract conspecific males *via* the release of a species-specific blend of sex pheromones (Starratt *et al.* 1979; Tumlinson *et al.* 1989). The males detect these pheromones with specialized olfactory receptor neurones (ORNs) innervating long trichoid sensilla (type I) present on the flagellum of the antennae (Sanes and Hildebrand, 1976; Schweitzer *et al.* 1976; Kaissling and Thorson, 1980; Kaissling, 1987; Keil, 1989; Christensen *et al.* 1989; Kaissling *et al.* 1989; Lee and Strausfeld, 1990).

Biochemical and electrophysiological techniques have been used to examine how pheromones elicit depolarizing receptor potentials in the ORNs. It has been shown that after pheromone stimulation in insect antennae there is a transient rise in inositol trisphosphate ($InsP_3$) levels (Breer *et al.* 1990), which opens first $InsP_3$-dependent Ca^{2+} channels (Stengl, 1994*a*) and then Ca^{2+}- and protein-kinase-C-dependent cation channels in the ORNs (Zufall and Hatt, 1991; Zufall *et al.* 1991; Stengl *et al.* 1992*a*,*b*; Stengl, 1993, 1994*a*,*b*). After strong pheromone stimulation, cyclic GMP levels rise slowly, modulating cation channels in the ORNs (Ziegelberger *et al.* 1990; Boekhoff *et al.* 1993; Zufall and Hatt, 1991; Zufall *et al.* 1991; Stengl, 1994*b*). While the mechanisms leading to an $InsP_3$-dependent rise in intracellular $[Ca^{2+}]$ are largely understood, the events that lead to a rise in cyclic GMP levels are still obscure. Since the guanylyl cyclase in insect ORNs is not directly Ca^{2+}-dependent (Ziegelberger *et al.* 1990), it is conceivable that cyclic GMP levels increase *via* a Ca^{2+}-dependent rise in nitric oxide (NO) levels, as is assumed in some vertebrate ORNs (Breer *et al.* 1992; Lischka and Schild, 1993). The activation of NO synthase (NOS) is Ca^{2+}/calmodulin-dependent, producing NO and citrulline from L-arginine and oxygen, and requires NADPH (Moncada *et al.* 1991; Vincent and Hope, 1992; Bredt and Snyder, 1992; Bredt *et al.* 1991).

Evidence for a possible involvement of NO in olfactory transduction in vertebrates has been provided by Breer *et al.* (1992) and Lischka and Schild (1993). It has also been shown that mammalian NOS contains nicotinamide adenine dinucleotide phosphate diaphorase (NADPH diaphorase) activity which can be visualized using a histochemical staining technique (Dawson *et al.* 1991; Hope *et al.* 1991).

Therefore, in the present study, we have looked for NADPH-diaphorase-dependent staining in sensilla trichoidea type I of *M. sexta* antennae to test whether NOS or a related enzyme might be present in pheromone-sensitive olfactory receptor neurones.

Materials and methods

Animals

The moths *Manduca sexta* (Johannson) (Lepidoptera: Sphingidae), reared from eggs on an artificial diet (modified from that of Bell and Joachim, 1976), were kept on a long-day photoperiod regimen (17 h:7 h light:dark, with lights on at 06:00 h) at 25–28 °C and 50–60 % relative humidity. The experiments were started in the morning and were performed after anaesthetizing the moths using CO_2 or by cooling. For some experiments, only parts of the flagellum were removed and the animal was returned to the mating cage after the operation.

All chemicals used were from Sigma Chemical Co. (Deisenhofen, Germany) unless stated otherwise.

NADPH diaphorase staining procedure

A total of 295 antennal preparations (mostly 8 μm longitudinal sections through five flagellar annuli per preparation) from adult *Manduca sexta* males and from two females were used for the NADPH diaphorase staining procedure on cryostat sections. The scapus and pedicellus of the antenna, which house mechanoreceptors, were not inspected. The histochemical staining procedure resulted in a dark-blue reaction product where the NADPH diaphorase was located. The identity of stained cells was determined at the light-microscopic level in longitudinal and transverse sections through the median portion of the flagellum on the basis of their specific location on the annulus (Lee and Strausfeld, 1990) and the overlying cuticular specializations (e.g. long trichoid sensilla). The nomenclature of the sensilla used here follows the terminology of Altner and Prillinger (1980), Steinbrecht (1984), Zacharuk (1985) and Lee and Strausfeld (1990).

The antennal flagella were fixed in 4 % paraformaldehyde or Zamboni's fixative, usually for 2–4 h. Tissue was cryoprotected in 30 % sucrose for 4 h. Thirty-one of the fixed antennae were embedded in cubes of boiled liver (Zimmermann, 1992) and sliced with a cryostat. The liver-embedding allowed fixation and staining of the preparations on the same day and thus decreased the fixation times. All other preparations were embedded in gelatin–albumin, fixed in 10 % formalin overnight, mounted and frozen in melting isopentane at −160 °C. Longitudinal and transverse sections (8–30 μm thick) were cut in a cryostat (Frigocut 2800, Reichert-Jung) at −28 °C. The liver-embedded sections were collected on coverslips coated with 0.05 % aqueous poly-L-lysine; the gelatin–albumin-embedded sections were collected in multi-well plates containing 0.05 mol l^{-1} Tris–HCl buffer. The sections were incubated for at least 30 min at room temperature in 1 mmol l^{-1} NADPH (BIOMOL Feinchemikalien GmbH, Hamburg), 0.5 mmol l^{-1} Nitroblue Tetrazolium and 0.2 % Triton X-100 in 0.05 mol l^{-1} Tris–HCl buffer. The formation of blue formazan deposits indicated the presence of NOS or a related enzyme in the flagella of the antennae.

In 56 antennal preparations, the staining was performed at pH 8.0, 8.4 or 8.6 to examine the pH-dependence of the formazan production. All other preparations were stained at pH 7.4.

Pheromone-stimulated (N=56) and unstimulated moths were compared using identical experimental conditions (pH 7.4, 2–4 h fixation) to investigate stimulation-dependent changes in enzyme activity. Male moths were reared together with females until pupal stage 17–18 (about 1 day before eclosion) and were then kept in isolation from females until they eclosed. The antennae were used for histochemistry within 1–7 days of eclosion.

Three groups of animals were distinguished according to the duration (seconds, minutes or days) and method of pheromone stimulation. The stimulation conditions were chosen to match conditions used for physiological experiments. The first group (N=13) was stimulated with 10 μl of female pheromone gland extract stock solutions (three female glands extracted for 2 min in 100 μl of hexane) on filter paper. The animals were allowed to sit on the pheromone-impregnated filter paper for 5–10 min before the antennae were cut off and immediately dropped into fixative. The second group (N=22) was stimulated with 3 μl of synthetic bombykal (0.1 μg ml^{-1}). The pheromone was applied to filter paper, which was stuck into a 10 ml syringe. For 5–15 s the pheromone-containing air was slowly blown over the antennae of the moths before they were cut off and fixed. The third group (N=21) of male moths was taken directly from the mating cage where they had been exposed for periods ranging from hours to days to the continuous presence of pheromone released by the female moths. For the first two groups, one antenna of the isolated animals was used as a non-stimulated control, while the other antenna was stimulated with high pheromone concentrations. Stimulated antennae of the third group were compared with non-stimulated antennae of isolated males. All non-stimulated antennal preparations were pooled (N=30). The comparison between stimulated and non-stimulated antennal preparations was carried out on matching flagellar segments. One antennal preparation usually comprises five annuli (annuli 9–13). The number of stained pheromone-sensitive type I trichoid sensilla from all antennal preparations of one group was counted and divided by the number of preparations examined, thus giving the arithmetic mean of stained sensilla per five annuli. Population standard deviations were calculated using standard statistical software. Because large standard deviations were obtained, indicating that the groups do not express a normal standard distribution, the medians were calculated. The data were displayed in histograms to illustrate the significant differences in the numbers of stained sensilla trichoidea in unstimulated antennae *versus* antennae stimulated for 5–10 min with pheromone (see Fig. 4).

Development of NADPH-diaphorase-dependent staining

To determine at what stage NADPH diaphorase activity is first present in the developing antenna, antennae from pupal stage 2 to early stage 9 (25 antennal preparations from 11 different animals), late stage 9 to 13 (30 antennal preparations

from 9 different animals) and stage 14 to 17 (9 antennal preparations from 6 different animals) were stained at pH 7.4 using the procedure described above.

Controls

Various controls were performed to determine whether formazan production depended on the presence of NOS or a related enzyme. Sections were incubated in a staining solution containing either no NADPH (N=3, N always indicates the number of antennal preparations) or no tetrazolium salt (N=2). Since NOS is directly inhibited by the arginine analogues L-N^G-nitroarginine and L-N^G-monomethylarginine, 0.1–50 mmol l^{-1} L-N^G-nitroarginine (N=12) or 0.1–10 mmol l^{-1} D-N^G-nitroarginine (N=6) was added to the staining solution. Because NO is known to down-regulate NOS (Rengasamy and Johns, 1993), sections from two antennal preparations were preincubated with 5–10 mmol l^{-1} nitroprusside (which produces NO) during the staining procedure.

Haemoglobin assay

To test whether NADPH diaphorase activity in moth antennae might be due to NOS-dependent release of NO, or to haem-oxygenase-dependent release of CO, the haemoglobin oxygenase assay (Feelisch and Noak, 1987) was employed by Dr Uli Müller (Berlin) as described previously (Müller 1994; Müller and Hildebrandt, 1995). The method is based on the quantitative oxidation of oxyhaemoglobin to methaemoglobin by NO (or related substances) formed. The oxidation is linked to a change in the absorbance characteristics of haemoglobin, which is assessed by continuous recording of the extinction difference (Δe) between 401 and 411 nm. For a detailed definition of Δe, please see Feelisch and Noak (1987). An NADPH-independent redox activity was separated using ion-exchange columns (Müller and Hildebrandt, 1995). The animals were stimulated with 10 μl of pheromone gland extracts (three female glands extracted for 2 min in 100 μl of hexane). The pheromone was applied to filter paper, which was stuck into a 10 ml syringe. For 10–20 min, the pheromone-containing air was slowly blown over the antennae of the moths before the antennae were cut off and processed.

Results

NADPH-diaphorase-positive sensilla

There was considerable variability in the number of neuronal and non-neuronal cells stained in the antennae under all conditions tested. Staining was concentrated in certain parts of the sensilla.

On the sensillar side and on the scale side of the flagellum, staining was frequently associated with the cavity at the base of hair shafts of different types of sensilla, apparently due to staining of non-neuronal supporting cells (Figs 1A, 2B, 3A,C). Staining was less often observed in somata and was possibly seen in the inner dendrites of the receptor neurones (Figs 1B,C, 2A). It was never seen in the outer dendrites, which are the only parts of the receptor neurones to extend uncovered into

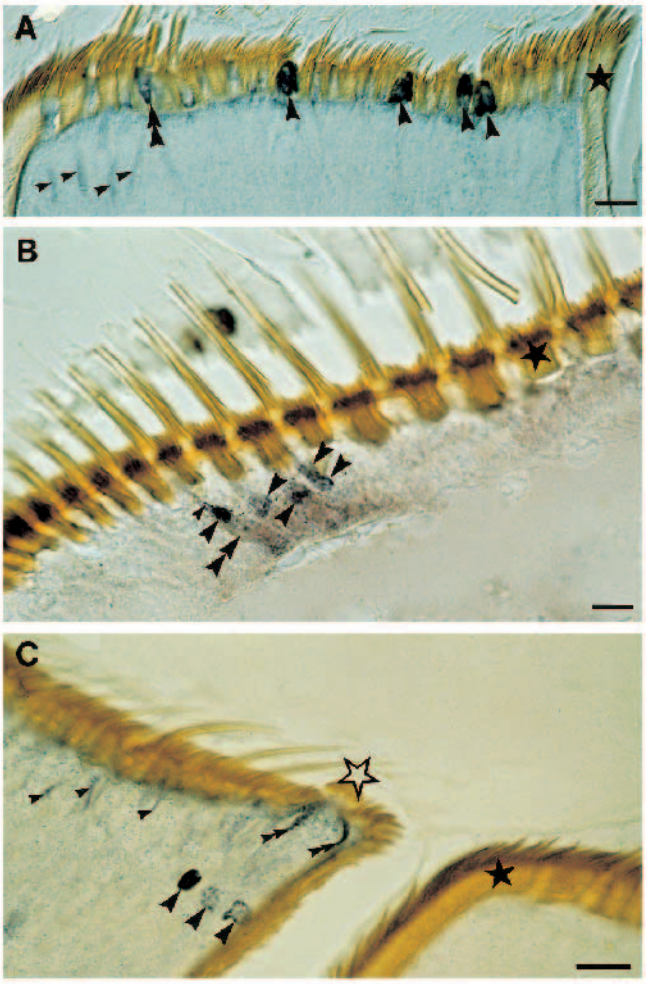

Fig. 1. (A–C) Longitudinal sections (A,C) and a cross section (B) of the dorsal sensillar side of antennal flagella of pheromone-exposed *Manduca sexta*. For a diagram of the antenna with the distribution of different types of sensilla, please see Lee and Strausfeld (1990). A is from a sixteenth-stage male pupa (2 days before eclosion); B and C are from adult males. (A) Four sensilla coeloconica (large arrowheads) are NADPH-diaphorase-positive among non-stained sensilla trichoidea type II and sensilla basiconica. The staining is strongest in the cavity at the base of their hair shafts. Fainter staining is seen in the base of a sensillum chaeticum (double arrowhead). Some axon bundles (small arrowheads) are weakly stained. (B) A cluster of four of the 13 type I sensilla trichoidea shown are NADPH-diaphorase-positive. Staining is present in the somata (arrowheads) and the inner dendrites (small arrowhead), but not in the outer dendrites of the pheromone-sensitive receptor neurones. In addition, the non-neuronal supporting cells of the sensillum (double arrowhead) express weak staining. (C) Three NADPH-diaphorase-positive somata (large arrowheads) innervate the cuticular dome (only partly cut in this section) of the styliform complex. Several stained processes are seen in the same annulus (small arrowheads), two of which (double arrowheads) originate from the styliform complex (open star). Note the entirely unstained annulus to the right. The histochemistry was performed at pH 8.0 in B, and at pH 7.4 in A and C, with gelatin–albumin embedding. The dark-brown cuticle (black stars) with the cuticularized hairs is unstained in all preparations. Scale bars, 20 μm.

the sensillar lymph of the hair shaft (Keil, 1989). In addition, non-neuronal somata (Figs 1A,B, 3A) and parts of receptor axon bundles (Fig. 1A,C) appeared to be faintly stained.

Some of the stained sensilla could be clearly identified as pheromone-sensitive type I trichoid sensilla (Fig. 1B), which have characteristically long hairshafts and a specific location on the annulus. In some cases it was possible to distinguish clearly the stained receptor neurone (with a 10 μm diameter soma and processes) from the stronger non-neuronal staining in the cavity of the hair shaft, which appeared to result from stained non-neuronal supporting cells, because it was traced to a faintly staining larger soma (more than 20 μm in diameter, Fig. 1B). Other olfactory sensilla, such as sensilla coeloconica (Figs 1A, 3A, which were observed most frequently), type II sensilla trichoidea (Fig. 1C) and sensilla basiconica (Figs 1C, 3A), also showed NADPH-diaphorase-positive hairshafts. These sensilla could not always be identified unequivocally because they occupied overlapping positions on the annulus and sometimes only somata or processes with no visible direct contact with cuticular hairshafts were stained (Figs 1A,C, 3A). Thus, except for type I trichoid sensilla, quantification of the various stained sensilla was not possible.

Among non-olfactory sensilla, some sensilla chaetica with the typically ring-necked shafts expressed NADPH diaphorase activity in the hairshafts (Fig. 1A). Furthermore, at least three stained somata per annulus extended processes into the cuticular dome of the styliform complex (Fig. 1C).

On the scale side of the flagellum, staining was observed in at least 21 somata per annulus. These cells lacked recognizable processes and lay close to unstained cuticular canals (Fig. 2A). In addition, staining was seen in cuticular canals and shafts of sensilla chaetica and scales (Figs 2B, 3C). Staining was also detected in campaniform sensilla extending along the intersegmental membranes between annuli (Fig. 2C).

Variability of the NADPH-diaphorase-dependent staining

The NADPH-diaphorase-dependent staining, especially of the base of the hair shafts, was only seen in subpopulations of different types of sensilla and was highly variable in terms of location within different annuli (Figs 1–3). Often, but not always, staining of sensilla was observed at apparently similar locations in neighbouring annuli (Fig. 3A). Furthermore, the stained sensilla often appeared to cluster (Figs 1A–C, 3A). Some annuli were completely devoid of staining (Fig. 1C). Others showed an increased background level with staining of neighbouring sensilla and this often included staining of sensilla of various modalities within the same annulus (Figs 1A,C, 3A).

To estimate how the duration of fixation influenced the staining pattern, the histochemical reaction was performed after fixation for 10 min, 2–4 h and overnight. With the liver-embedding procedure (N=31), staining took place on the same day as fixation (in contrast to the gelatin–albumin-embedding procedure with overnight fixation, N=264). This procedure produced a stronger contrast between the background staining in annuli or intersegmental regions containing stained somata and regions containing no stained somata (Fig. 2B). While the intensity of staining and the total number of cells stained varied considerably between the different procedures, both resulted in the same variable pattern of staining of the various sensilla

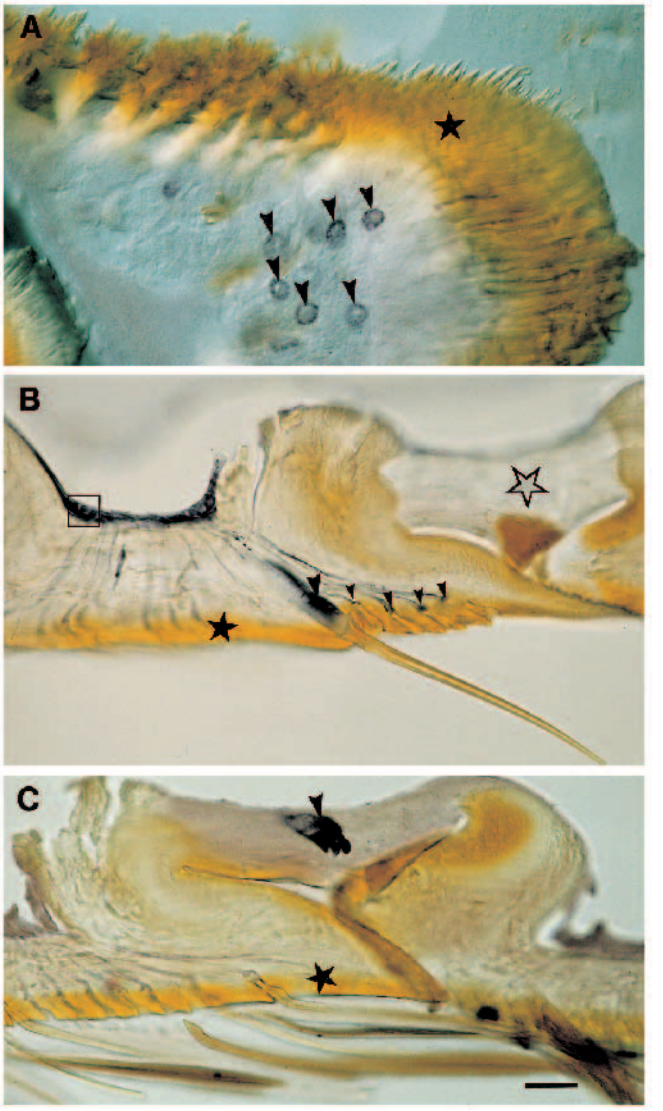

Fig. 2. (A–C) Longitudinal sections of the scale side of the antennal flagellum. (A) Six somata without processes in the cell body layer lining the cuticle of the scale side exhibit NADPH-diaphorase-positive staining (arrowheads). (B) The cuticular canal and hair shaft of a sensillum chaeticum (large arrowhead) as well as cuticular canals and shafts of the scraped-off scales (small arrowheads) show NADPH-diaphorase-positive staining. Liver-embedded tissue showed enhanced staining (possibly due to the shorter fixation times compared with those for the gelatin–albumin-embedded preparations) and increased background staining in the cell body layer (open square) lining the cuticle, which appeared to contain many small stained somata. No stained cells and no background stain were found in the intersegmental region (open star). (C) A campaniform sensillum in the intersegmental region between two annuli (arrowhead) is NADPH-diaphorase-positive. The background staining of the tissue is increased at pH 8.6 (A,C with gelatin–albumin embedding) and pH 8.4 (B with embedding in liver). The brown cuticle is always unstained (black stars). Scale bar, 20 μm.

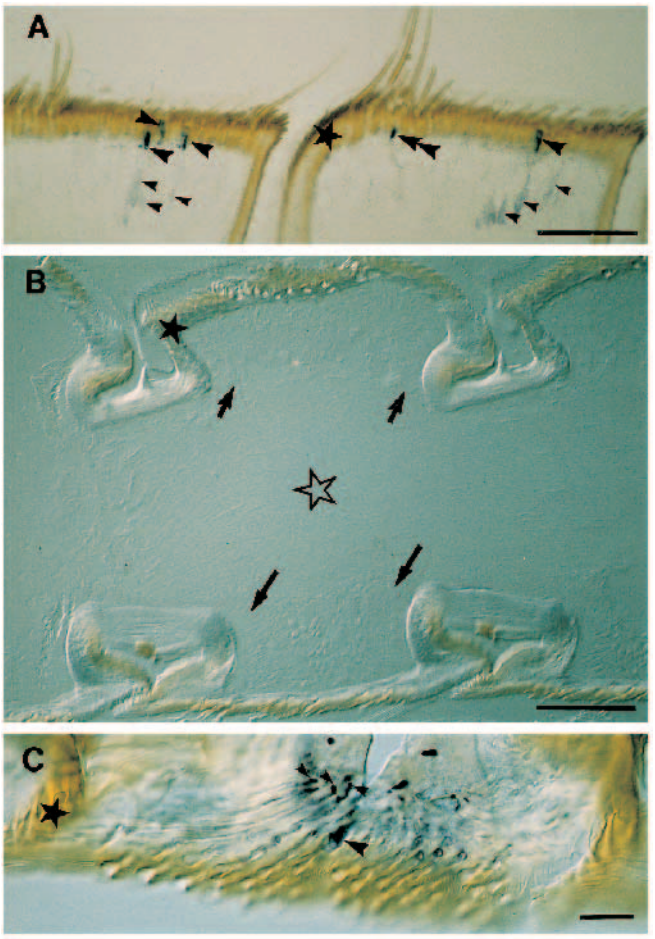

Fig. 3. (A–C) Longitudinal flagellar sections stained at pH 7.4 with gelatin–albumin embedding. (A) The NADPH-diaphorase-specific staining is still apparent after addition of 1 mmol l^{-1} L-N^G-nitroarginine to a pheromone-exposed adult male flagellum. The staining is clustered in two consecutive annuli at about the same location within each annulus. The arrowheads point to staining in the shafts of sensilla coeloconica (large arrowheads), and possibly also sensilla basiconica, to stained supporting cells (small arrowheads) and to staining of the shaft of a type I trichoid sensillum (double arrowhead). (B) This complete longitudinal section shows no staining at pupal stage 12 in the soma layer underneath the cuticle of the sensillar side (between the short arrows), of the scale side (between the long arrows) or of the receptor axon bundles (open star) that project to the antennal lobe. (C) At pupal stage 15, the NADPH-diaphorase-specific staining of the shafts (large arrowhead) and cuticular canals (small arrowheads) of the scales is as strong as in the adult moth. The brown cuticle is always unstained (black stars). Scale bars, 100 μm (A,B) and 20 μm (C).

shafts. However, on the scale side, the somata without processes invariably stained with all procedures.

To determine whether changes in pH play a role in the non-uniform staining pattern, staining was performed at pH 7.4 (N=41) and pH 8.0, 8.4 or 8.6 (N=56). The enzyme reaction was strongly pH-dependent: both staining intensity and the background level of staining increased with increasing pH (compare Figs 1–3). The background staining was lowest at pH 7.4 and, therefore, this was selected as the usual staining procedure. In some animals, the somata of apparently all sensory neurones appeared to stain at pH 8.6 after fixation for 15 min (not shown). The strongest staining was always seen in some sensilla shafts and showed the same pattern of variability irrespective of the pH tested.

NADPH-diaphorase-dependent staining in developing pupal antennae

No NADPH-diaphorase-dependent staining (Fig. 3B) was seen in antennal preparations of pupal stage 12 or younger (N=52). At pupal stage 13–14, faint staining was observed on the scale side in the shafts of scales and campaniform sensilla (N=4). This staining became much stronger at stage 15–16 (Fig. 3C), when staining of sensilla chaetica on the sensilla side (Fig. 1A) and sensilla campaniformia in the intersegmental region was first observed (N=5). In one preparation of a stage 14 pupa, sensilla coeloconica were stained, and this pattern of staining was observed more regularly at stage 15–16 (Fig. 1A, N=5). Staining of pheromone-sensitive trichoid sensilla was first observed in adults shortly after eclosure.

Comparison between pheromone-stimulated and unstimulated antennae

The numbers of stained shafts or somata of type I trichoid sensilla were compared in pheromone-stimulated (N=56) *versus* non-stimulated moths (N=30) to determine whether the variability in the number of stained sensilla resulted from a specific pheromone-dependent activation of the NADPH diaphorase activity (Table 1; Fig. 4). In animals which were stimulated with the complete pheromone blend for 5–10 min (N=13), 1–73 (mean 17.9±19.1, S.D.; median 13) stained type I trichoid sensilla per antennal preparation (five annuli) were counted, compared with 0–7 (mean 1.1±2.2, S.D.; median 0) stained sensilla per five annuli of the pooled non-stimulated antennae (N=30). Thus, a maximum of 8 % (33) of all type I trichoid sensilla per annulus were stained and there was a 16-fold increase in the number of stained sensilla after exposure to the pheromone blend for several minutes. After stimulation with the synthetic pheromone component bombykal for 5–15 s (N=22), 0–23 (mean 2.6±5.6, S.D.; median 0) stained type I trichoid sensilla per five annuli were counted. Directly after

Table 1. *Number of stained type I trichoid sensilla per five annuli before and after pheromone stimulation for different durations*

Duration of stimulation with pheromone	Number of type I trichoid sensilla (mean±S.D.)	Median	N
No stimulation	1.1±2.2	0	30
5–15 s	2.6±5.6	0	22
5–10 min	17.9±19.1	13	13
Hours to days	5.7±8.1	1	21

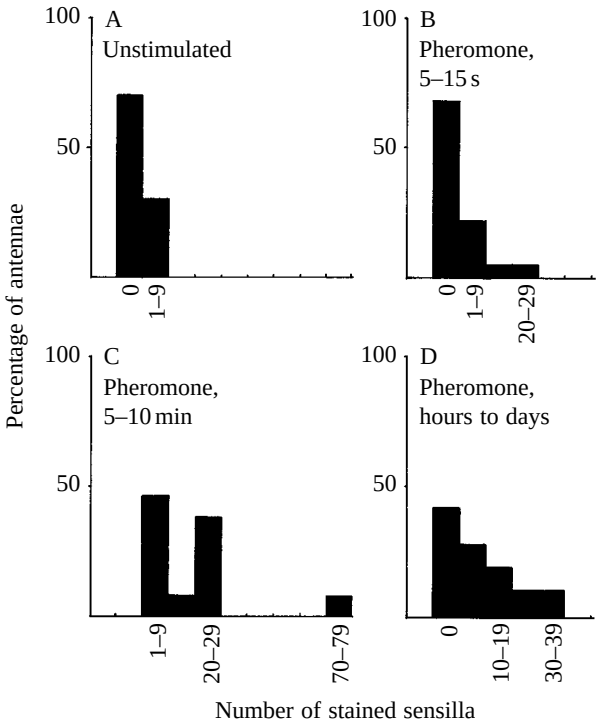

Fig. 4. (A–D) Diagrams showing the percentage of antennae, without pheromone (A) or with pheromone present for different durations (B–D), containing different numbers of NADPH-diaphorase-positive trichoid sensilla. (A) In unstimulated antennae (*N*=30), 70 % of all antennae tested showed no stained trichoid sensilla, while 30 % of all antennae showed 1–9 stained sensilla. (B) After application of pheromone for 5–15 s, 68 % of all antennae tested showed no stained trichoid sensilla, 22 % of all antennae showed 1–9, 5 % showed 10–19 and 5 % showed 20–29 stained trichoid sensilla (*N*=22). (C) After application of pheromone for 5–10 min, 46 % of all antennae tested showed 1–9, 8 % showed 10–19, 38 % showed 20–29 and 8 % showed 70–79 stained trichoid sensilla (*N*=13). (D) After application of pheromone for periods ranging from several hours to days, 43 % of the antennae showed no stained trichoid sensilla, while 28 % showed 1–9, 19 % showed 10–19, 5 % showed 20–29 and 5 % showed 30–39 stained trichoid sensilla (*N*=22).

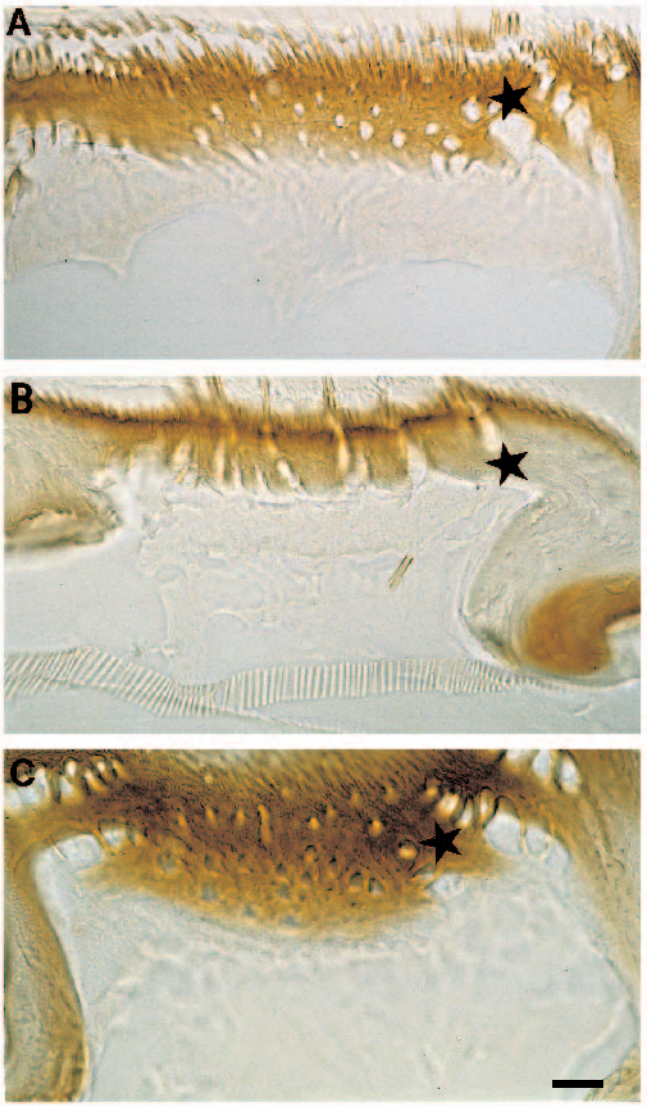

Fig. 5. (A–C) 10 μm longitudinal sections of the sensillar side of the antennal flagellum. No NADPH-diaphorase-specific staining was seen under all control conditions. In A, no NADPH, and in B no Nitroblue Tetrazolium, was added to the staining solution. In C, preincubation with 50 mmol l^{-1} L-N^G-nitroarginine, a specific inhibitor of NOS, suppressed all staining. All sections were stained at pH 7.4 with gelatin–albumin embedding. Black stars, cuticle. Scale bar, 20 μm.

removal from the mating cage (where they had contact with the female sex pheromones for periods ranging from several hours to days), male moths showed 0–30 (mean 5.7±8.1, S.D.; median 1) stained type I trichoid sensilla per five annuli (*N*=21).

Despite the fact that this procedure is only semi-quantitative (the 8 μm sections tear or fold easily), and despite the large standard deviations, the medians indicate that significantly more pheromone-sensitive sensilla appeared to be stained in flagella stimulated for several minutes with pheromone than in flagella of non-stimulated moths (Table 1; Fig. 4).

Controls

All dark-blue staining disappeared in the soma layer underneath the cuticle and the base of the cuticular shafts if NADPH (*N*=3) or tetrazolium salt (*N*=2) was omitted in the staining procedure (Fig. 5A,B). The NADPH-diaphorase-dependent stain became fainter but was still visible with increasing concentrations of L-N^G-nitroarginine (from 0.1 to 10 mmol l^{-1}) in the staining solutions (*N*=6) (Fig. 3A). After preincubation with 50 mmol l^{-1} L-N^G-nitroarginine and addition of 10 mmol l^{-1} L-N^G-nitroarginine to the staining solution (*N*=2), NADPH-diaphorase-dependent staining disappeared completely (Fig. 5C). No staining was detected after preincubation with 10 mmol l^{-1} nitroprusside (*N*=2) (not shown). The staining was not affected by addition of 0.1–10 mmol l^{-1} D-N^G-nitroarginine, a physiologically non-functional analogue (not shown, *N*=6).

Pheromone-dependent activity detected in the haemoglobin assay is not inhibited by NOS inhibitors

To determine whether pheromone-dependent NADPH diaphorase activity in moth antennae might be due to NOS-dependent release of NO, or to release of CO, the haemoglobin assay (Feelisch and Noak, 1987) was employed (Müller, 1994; Müller and Hildebrandt, 1995). Antennae stimulated for 10–20 min with pheromone gland extracts (see Materials and methods) prior to measurement revealed a Δe of 0.033±0.01 (N=12), which significantly differed from the value of Δe for unstimulated antennae (0.010±0.008; N=8; P<0.01, t-test). These activities in pheromone-stimulated antennae were not significantly inhibited by 200 μmol l^{-1} each of the NOS inhibitors L-N^G-monomethylarginine and L-N^G-nitroarginine (Δe=0.029±0.01; N=12; P=0.35, t-test). Thus, the signal detected in the pheromone-stimulated moth antennae as revealed by the haemoglobin assay is probably due to an enzyme activity other than NOS.

Discussion

In the antennal flagellum of the moth *Manduca sexta*, sensilla of different modalities including pheromone-sensitive type I trichoid sensilla were NADPH-diaphorase-positive. In antennae stimulated for several minutes with pheromone, the number of stained type I trichoid sensilla was significantly higher than that in non-stimulated animals, as judged by calculation of the arithmetic means and the medians.

The identification of the NADPH-diaphorase-positive cells within the antennal flagellum of *M. sexta* has been greatly helped by the thorough study of Lee and Strausfeld (1990), who mapped the different sensilla types at specific locations within an annulus. Wherever the NADPH-diaphorase-positive stain extended into the cavity of cuticular hairs, it was possible to distinguish between NADPH-diaphorase-positive type I and II trichoid sensilla, sensilla basiconica, sensilla coeloconica and sensilla chaetica. Both type I and II trichoid sensilla are olfactory sensilla. Both types can be unequivocally distinguished according to hair length and their specific location on the annulus (Lee and Strausfeld, 1990). Type I trichoid sensilla are the only male-specific pheromone-dependent sensilla, with about 443 sensilla per annulus (Kaissling *et al.* 1989; Lee and Strausfeld, 1990). Since only light microscopy was used, it was not possible to make further distinctions between sensilla basiconica types I and II (which are both thought to be olfactory sensilla, responding to general odours), between sensilla coeloconica type I (possibly chemosensitive) and type II (possibly thermosensitive) or between sensilla chaetica type I (possibly chemo- and mechanosensitive) and type II (mechanosensitive).

Neuronal as well as non-neuronal elements appeared to stain in a subpopulation of all these sensilla. Since we only used light microscopy, we could not determine whether the staining of hairshafts was also extracellular. It is likely that the strong staining in the base of hairshafts is in the trichogen or tormogen cells, which together form the sensillum lymph space, since in some preparations the stain could be traced to a faintly staining larger soma. After excessive odour stimulation, it is possible that non-neuronal cells show up-regulation of enzymes with NADPH diaphorase activity, and these might play a role in preventing stimulus-dependent damage of the respective sensillum.

The faint staining of axon bundles could not always be unequivocally distinguished from faint staining of non-neuronal somata such as the trichogen cells, because they occurred at similar positions in the annulus. However, such faint staining appeared to be specific, since it occurred rather rapidly during the staining process, was never seen in the controls and expressed the same pattern of staining as the strong staining of hairshafts.

The stained somata without processes on the scale side might belong to sensilla scolopophora, because they appeared to lie close to cuticular canals. Since cuticular canals and shafts of scales were also stained, *M. sexta* appears to have innervated scales. These sensilla squamiformia may be mechanosensitive or chemosensitive (Zacharuk, 1985).

The finding that NADPH diaphorase in *M. sexta* is not confined to chemoreceptors differs from observations in *Drosophila melanogaster* where NADPH-diaphorase-dependent staining was found only in the olfactory portion of the antenna and the antennal nerve, whereas the mechanosensory parts appeared to be devoid of staining (Müller and Buchner, 1993). Müller and Buchner (1993) have reported that only a proportion of olfactory receptor neurones were stained in *D. melanogaster*. This was also observed in *M. sexta*, where it was found that, regardless of large variations of pH, fixation time and other parameters of the histochemical procedures, the same sensilla types, but not all of the cells of one type, were stained. It is possible that only a subpopulation of the receptor cells contains NADPH diaphorase activity. Since neighbouring annuli sometimes showed staining of sensilla in the same location, and because in some preparations with shorter fixation times and higher pH all sensory somata appeared to stain, it seems unlikely that the observed variability is entirely due to the fact that only a proportion of the sensilla contained NADPH diaphorase activity.

Since the recent discovery of NO as a second messenger (Bult *et al.* 1990; Moncada *et al.* 1991; Vincent and Hope, 1992; Bredt and Snyder, 1992; Garthwaite, 1991; Kishimoto *et al.* 1993) and the finding that NADPH diaphorase is identical to NOS in most vertebrate preparations (Dawson *et al.* 1991; Hope *et al.* 1991), considerable effort has been put into understanding the functions of this signalling system. The specific features of NO, which is a short-lived, highly diffusible molecule that can easily cross cell membranes, have stimulated speculations about its possible functions as an intra- and intercellular messenger activating soluble guanyly cyclase (Bredt and Snyder, 1992; Breer and Shepherd, 1993). NOS has recently been identified in insect tissues, such as the olfactory system (Elphick *et al.* 1993, 1994, 1995; Elofsson *et al.* 1993; Müller, 1994; Müller and Buchner, 1993; Müller and Bicker, 1994; Müller and Hildebrandt, 1995; Ribeiro *et al.*

1993), but the function of NO in invertebrates is still unknown. Thus, we attempted to test, using NADPH diaphorase histochemistry, whether NOS might be present in antennal receptor neurones in order to examine the function of NO in pheromone-dependent receptor neurones.

Chemosensitive and other sensilla types in the antennal flagellum of *M. sexta* were found to be NADPH-diaphorase-positive, but application of L-N^G-nitroarginine, which specifically inhibits NOS in locusts (Elphick *et al.* 1993), completely inhibited staining only at very high concentrations of 10–50 mmol l^{-1}. However, since the NADPH diaphorase activity of NOS is not inhibited by application of L-N^G-nitroarginine in bees (U. Müller, personal communication), it remains unclear whether the specific staining pattern is caused by NOS or by a related enzyme. Nitroprusside, which frees NO, completely inhibited NADPH-diaphorase-dependent staining, which would be consistent with a possible down-regulation of NOS by NO (Rengasamy and Johns, 1993). However, since no specific antibody is available against insect NOS, no specific anatomical test is possible to determine whether the observed NADPH-diaphorase-dependent staining is indicative of NOS. The preliminary biochemical studies shown here (performed by Dr Uli Müller, Berlin) indicated that NOS-like activity was elicited in moth antennae after several minutes of pheromone stimulation; however, this activity was not blocked by NOS-specific inhibitors. Therefore, it is probable that NADPH diaphorase activity in *M. sexta* antennae is not due to NOS but rather to an enzyme such as haem oxygenase (Verma *et al.* 1993) or NADPH–P450 oxidoreductase (Kishimoto *et al.* 1993). Further biochemical analysis (which is beyond the scope of this paper) is necessary to test this hypothesis.

The observed increase in the number of stained pheromone-dependent trichoid sensilla in stimulated *versus* non-stimulated antennae indicates that the non-homogeneous staining depends at least partly on specific activation of the NADPH diaphorase. The enzyme activity might be up-regulated *via* a pheromone-dependent increase of intracellular [Ca^{2+}] (Stengl, 1993, 1994*a*,*b*) in those receptor neurones receiving stimulation for several minutes, but not in those that are stimulated for seconds or for days. This hypothesis is further supported by the preliminary biochemical studies reported here and by preliminary Fura studies, which indicated that after pheromone stimulation cultured olfactory receptor neurones show increases in intracellular [Ca^{2+}] which appear to spread, possibly *via* an unknown diffusible messenger, after several minutes of stimulation to a subpopulation of other olfactory neurones (M. Stengl and B. Lindemann, unpublished observations). A possible role for such a diffusible messenger in insect antennae is a synchronization of the adaptational state of all sensilla of one type on the antenna. A diffusible messenger could prevent changes in stimulus concentration in some parts of the antenna (e.g. caused by changes in the wind velocity) from confusing the qualitative and quantitative coding of odours in olfactory sensilla. Furthermore, the clustered staining even in sensilla of different modalities might indicate that neighbouring mechanosensory and chemosensory sensilla might influence each other. Future studies will test whether this is accomplished *via* a diffusible messenger such as NO or CO (Verma *et al.* 1993).

Down-regulation of the NADPH diaphorase activity, e.g. *via* end-product inhibition (as is known for NOS, Rengasamy and John, 1993), might occur after several hours of stimulation, because under these conditions there was no apparent significant difference in the number of stained trichoid sensilla compared with the number of non-stimulated sensilla. This observation correlates with results from patch-clamp studies, which have shown that physiological responses cease after very long and strong pheromone stimulation (Stengl *et al.* 1992*b*).

An activity-dependent function of NADPH diaphorase in antennal receptor neurones is further suggested by the correlation between the appearance of the NADPH-diaphorase-dependent staining in developing antenna and the physiological maturation of antennal receptor neurones. It has been shown previously by Schweitzer *et al.* (1976) in electroantennogram recordings of *M. sexta* that mechanical responses start at stage 14, before the responses to general odorants at stage 15, and that responses to pheromone occur at stage 17, just before eclosion. These findings correlate with the observation that NADPH-diaphorase-dependent staining is first seen in mechanosensory receptors at pupal stage 13–14, before it is observed in general chemosensory receptors (at stage 14–16) and pheromone-sensitive trichoid sensilla (in pharate adults). No correlation was observed between NADPH-diaphorase-dependent staining in antennal receptor neurones and the processes of programmed cell death in the antenna at stage 9–11, pathway finding (at stage 3–9, where receptor axons enter the antennal lobe) or synapse maturation of receptor axon terminals in the antennal lobe (stage 8–12; Hildebrand, 1985).

In conclusion, our study of the NADPH-diaphorase-dependent staining in antennal receptors of *M. sexta* shows that there is a pheromone-dependent enzyme present in neuronal and non-neuronal cells that could possibly be used for activity-dependent staining procedures.

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