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Immunocytochemical localization of pheromone-binding protein in moth antennae

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Summary. Odorant-binding proteins are supposed to play an important role in stimulus transport and/or inactivation in olfactory sense organs. In an attempt to precisely localize pheromone-binding protein in the antenna of moths, post-embedding immunocytochemistry was performed using an antiserum against purified pheromone-binding protein of *Antheraea polyphemus*. In immunoblots of antennal homogenates, the antiserum reacted exclusively with pheromone-binding protein of *A. polyphemus*, and cross-reacted with homologous proteins of *Bombyx mori* and *Autographa gamma*. On sections of antennae of male *A. polyphemus* and *B. mori*, exclusively the pheromone-sensitive sensilla trichodea are labelled; in *A. gamma*, label is restricted to a subpopulation of morphologically similar sensilla trichodea, which indicates that not all pheromone-sensitive sensilla contain the same type of pheromone-binding protein and accounts for a higher specificity of pheromone-binding protein than hitherto assumed. Within the sensilla trichodea, the extracellular sensillum lymph of the hair lumen and of the sensillum-lymph cavities is heavily labelled. Intracellular label is mainly found in the trichogen and tormogen cells: in endoplasmic reticulum, Golgi apparatus, and a variety of dense granules. Endocytotic pits and vesicles, multivesicular bodies and lysosome-like structures are also labelled and can be observed not only in these cells, but also in the thecogen cell and in the receptor cells. Cell membranes are not labelled except the border between thecogen cell and receptor cell and the autojunction of the thecogen cell. The intracellular distribution of label indicates that pheromone-binding protein is synthesized in the tormogen and trichogen cell along typical pathways of protein secretion, whereas its turnover and decomposition does not appear to be restricted to these cells but may also occur in the thecogen and receptor cells. The immunocytochemical findings are discussed with respect to current concepts of the function of pheromone-binding protein.

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Key words: Olfaction – Sensillum trichodeum – Odorant-binding protein – Cryofixation – Freeze-substitution – Immunogold labelling – *Antheraea polyphemus*, *Bombyx mori*, *Autographa gamma* (Insecta)

The antennae of moths are among the most intensively studied olfactory sense organs, because of their accessibility for electrophysiological techniques and because these moths communicate via pheromones, identified chemical stimuli produced by conspecifics eliciting characteristic behaviour reactions (reviewed by Schneider 1984; Kaissling 1986, 1987). Male silk moths perceive the sex-attractant pheromone of conspecific females at threshold concentrations of about 1000 molecules per cm^3 air, and a single pheromone molecule is sufficient to elicit an action potential (Kaissling and Priesner 1970). The receptors for the pheromone are located in the sensilla trichodea (Schneider et al. 1964; Boeckh et al. 1965), which by far outnumber all other sensillum types on the antenna (Schneider and Kaissling 1957; Boeckh et al. 1960; Steinbrecht 1970). The complex fine structure of the sensilla trichodea (s. trichodea) is now well known (Steinbrecht 1973; Steinbrecht and Gnatzy 1984; Gnatzy et al. 1984; Keil 1984a), but the functional interpretation of the sensillar *Bauplan* is still speculative in many respects (reviewed by Keil and Steinbrecht 1984; Steinbrecht 1984, 1987).

In order to understand fully the receptor events during stimulus transduction, it is necessary to understand in addition the 'peri-receptor events' (Getchell et al. 1984) of stimulus transport and stimulus inactivation. The interest in these peri-receptor events has substantially increased since odorant-binding proteins were discovered in both the sensillum lymph of insects (Vogt and Riddiford 1981) and the mucus of vertebrates (Pelosi et al. 1982; reviewed by Pevsner and Snyder 1990).

The first odorant-binding protein found in an insect was the pheromone-binding protein (PBP) of *A. polyphemus* (Vogt and Riddiford 1981). It was isolated from

antennal homogenates and later also directly from the sensillum lymph of male moths (Klein 1987). Pheromone-binding proteins were also discovered in the moths *Antheraea pernyi, Hyalophora cecropia, Bombyx mori, Manduca sexta, Lymantria dispar* and *Orgyia pseudotsugata* (Vogt and Riddiford 1981; Vogt et al. 1989, 1991). In addition to these PBPs, other antenna-specific proteins were discovered in both sexes, and were 'called general odorant-binding proteins' (GOBPs; Vogt et al. 1991; see also Breer et al. 1990). Direct evidence as to their binding of odorants or their association with olfactory receptor neurons, however, is still lacking. The amino-acid sequence of PBPs and GOBPs shows striking across-species homology for the PBPs and even more for the GOBPs; yet between PBPs and GOBPs there are larger differences (Györgyi et al. 1988; Raming et al. 1990; Vogt et al. 1991). The odorant-binding proteins of vertebrates, on the other hand, belong to a group of proteins fundamentally different from all odorant-binding proteins so far observed in insect antennae (Pevsner et al. 1988), and might, therefore, represent an example of convergent evolution.

The PBP of *A. polyphemus* is a soluble, non-glycosylated protein of about 15 kD with a low isoelectric point of 4.7; its extremely high concentration in the sensillum lymph has been estimated to be ~10 mM (Vogt and Riddiford 1981; Klein 1987). In polyacrylamide gels under non-reducing conditions, PBP is found in two bands indicative of two subpopulations (Klein 1987). Raming et al. (1989) deciphered the full amino acid sequence: there is a high proportion of sulfur-containing amino acids (8 methionine and 6 cysteine residues); they also calculated the local hydrophobicity along the polypeptide chain and predicted six strands of β-sheets and seven α-helical segments (see also Raming et al. 1990).

Concerning the function of PBP, several hypotheses are presently discussed: (1) PBP binds pheromone to inactivate the stimulus rapidly (Vogt and Riddiford 1981; Kaissling 1986, 1987); (2) PBP acts as a carrier or solubilizer in stimulus transport (Vogt and Riddiford 1986; Vogt 1987); (3) PBP is effective in both of these functions (Van den Berg and Ziegelberger 1991).

Immunocytochemical localization of PBP has been started in an attempt to find out whether PBP is exclusively located in the pheromone-sensitive sensilla trichodea or is present in other sensillum types as well. Moreover, localization of PBP in antennal compartments other than the sensillum lymph should give information about production and turnover of PBP. Ultimately, this might help to answer questions concerning the function of PBP. In this paper, we report on the immunocytochemical localization of PBP in three species of moths, the saturniid *Antheraea polyphemus*, the bombycid *Bombyx mori*, and the noctuid *Autographa gamma*, using an antiserum against purified PBP of *A. polyphemus*. A preliminary report of these results has been published elsewhere (Steinbrecht et al. 1991).

Materials and methods

1. Animals

Moths of *Antheraea polyphemus* Cramer and *Bombyx mori* L. were obtained from pupae raised by various breeders. *Autographa (Plusia) gamma* L. were caught wild with a light trap at Seewiesen. *A. polyphemus* was kept at 10° C after emergence and used 3–8 days old; *B. mori* was kept at 10° C or 20° C and sacrificed on the first to fourth day after emergence. The age of *A. gamma* was unknown.

2. Purification of PBP and preparation of antisera

For PBP purification, antennal side branches of male *A. polyphemus* were homogenized in 50 mM TRIS (pH 7.4), and subjected to preparative polyacrylamide gel electrophoresis (PAGE) in 15% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970) but without SDS. Part of the gel was stained with Coomassie brilliant blue R250. PBP was – according to the stain – cut out from the unstained, native gel. After electroelution (Bio-Trap, Schleicher & Schüll, Dassel, FRG), the protein concentration was determined by the method of Bradford (1976) using BSA as standard and the purity checked by analytical SDS-PAGE (see below).

Antisera were prepared by injecting 2 adult rabbits s.c. and i.m. with 120 µg PBP per animal on days 1, 12, and 47. The purified PBP was emulsified with an equal volume of Freund's complete adjuvant for the first injection or of Freund's incomplete adjuvant for further injections. Animals were bled 13 and 17 days after the last injection, the sera pooled and stored at −25° C without further purification of the antibodies (henceforth referred to as anti-PBP (Apo)).

3. PAGE and blotting

SDS-PAGE was carried out under reducing conditions in 15% polyacrylamide slab gels (Laemmli 1970). Proteins were stained with Coomassie brillant blue R250 or alternatively transferred to nitrocellulose membranes for 2 h by the semi-dry blotting procedure of Kyhse-Andersen (1984). After blocking with 0.05% Tween 20 in phosphate-buffered saline (PBS) for 1 h at room temperature (Batteiger et al. 1982), the nitrocellulose membranes were incubated with anti-PBP(Apo) at a dilution of 1:3000 for 2–3 h. Specific antigens were detected by goat anti-rabbit IgG-horseradish peroxidase conjugate using n-chloronaphthol as substrate. All washing procedures and dilutions were performed in PBS-Tween. All electrophoresis reagents were purchased from BioRad Laboratories, Richmond, Calif., USA.

For dot blotting, purified PBP was dissolved in PBS and aliquots applied to a nitrocellulose membrane using a suction device (BioDot, BioRad). Some of the dots were fixed in raising concentrations of formaldehyde and glutaraldehyde before performing the immunoreactions as described above.

4. Immunocytochemistry

Antennae were either chemically fixed or cryofixed and freeze-substituted. Chemical fixation was done by immersion of antennae into a mixture of paraformaldehyde (2.8%) and glutaraldehyde (3%) in PBS (pH 7.2) and followed by dehydration in an ethanol series. For cryofixation, specimens were plunged into super-cooled propane (−180° C) (Steinbrecht 1980; for details, see Steinbrecht 1992c), and transferred into pure acetone at −79° C for freeze-substitution. After ≥2 days, the temperature was slowly raised overnight to −30° C and the medium replaced by 3% glutaraldehyde in acetone (GA-acetone) for another 24 h. This medium was prepared by adding 6 ml of 50% aqueous glutaraldehyde (# 23116,

Serva, Heidelberg, FRG) to 100 ml pure analytical grade acetone (# 7328, Roth, Karlsruhe, FRG) and storing this mixture for at least 2 days over molecular sieve (# 8471, Roth, Karlsruhe, FRG) before using. Alternatively, freeze-substitution in acetone containing 2% OsO_4 (Os-acetone) was carried out as described by Steinbrecht (1980).

After warming to room temperature, the substitution medium was replaced by two changes of pure acetone and further by three changes of 100% ethanol. Embedding in LR White (Taab, Aldermaston, Berks, UK) was done for both chemically fixed and cryofixed specimens in the same way through mixtures of 2:1, 1:1, 1:2 of 100% ethanol and LR White. Specimens remained in pure LR White overnight at 4° C. Polymerization was accomplished at 60° C in tightly closed gelatine capsules, filled completely with the resin monomere. To avoid deleterious fluctuations of the polymerization temperature, the gelatine capsules were fitted into an aluminium block.

Ultrathin sections were cut with a diamond knife on a Reichert OMU2 ultramicrotome and mounted on Formvar-coated single hole grids. For immunocytochemistry, the sections were floated on 30 µl droplets of the following solutions: 2 × 2 min on PBS containing 50 mM glycine for masking free aldehyde groups, 2 × 10 min on PBG (PBS containing 0.05% gelatine and 0.5% bovine serum albumine), overnight on the primary antiserum diluted with PBG, 6 × 2 min on PBG, 1 h on the secondary antibody diluted 1:20 with PBG, 2 × 2 min PBS-glycine, 2 × 2 min H_2O, 10 min on 2% uranyl acetate, 6 × 2 min H_2O. Sections were blotted dry and investigated in a Zeiss EM 10A electron microscope at 80 kV.

The primary antiserum was used in dilutions of 1:300–1:1000000, standard dilution was 1:3000. The secondary antibody was anti-rabbit IgG, coupled to 10-nm colloidal gold (Amersham Buchler, Braunschweig, FRG). As a control, the primary antiserum was replaced either by pure PBG or by pre-immune serum at the same dilution.

For better visibility of the label at low magnification, silver intensification according to Danscher (1981) was performed on some grids. 15-min intensification was usually sufficient for increasing the particle size to some 40 nm. These grids were stained afterwards with uranyl acetate and lead citrate.

The number of animals studied by immunolabelling was 18 males and 2 females in *Bombyx mori*, 5 males in *Antheraea polyphemus*, and 4 males in *Autographa gamma*.

Results

1. Blotting experiments

Antennal proteins from male and female *Antheraea polyphemus, Bombyx mori*, and *Autographa gamma* were separated in SDS-gels and visualized by Coomassie staining (Fig. 1a) or by immunostaining with anti-PBP serum. At a dilution of 1:3000, the antiserum excluvisely labels the PBP (Fig. 1b). The antiserum is not selective for PBP of *A. polyphemus* but cross-reacts with the PBP of *B. mori*, and *A. gamma*. In *A. polyphemus*, the PBP is restricted to male antennae; however, in *B. mori*, also female antennae contain PBP, although in smaller amounts. In *A. gamma*, both sexes show equal immunostaining.

Dot blots showed that the specific staining is not significantly reduced if the antigen is fixed with formaldehyde and/or glutaraldehyde, even at a concentration of 3%. This feature of PBP is highly convenient for immunocytochemistry, because cross-linking not only improves general tissue preservation, but also reduces the danger of extraction or translocation of PBP as a soluble and relatively small protein.

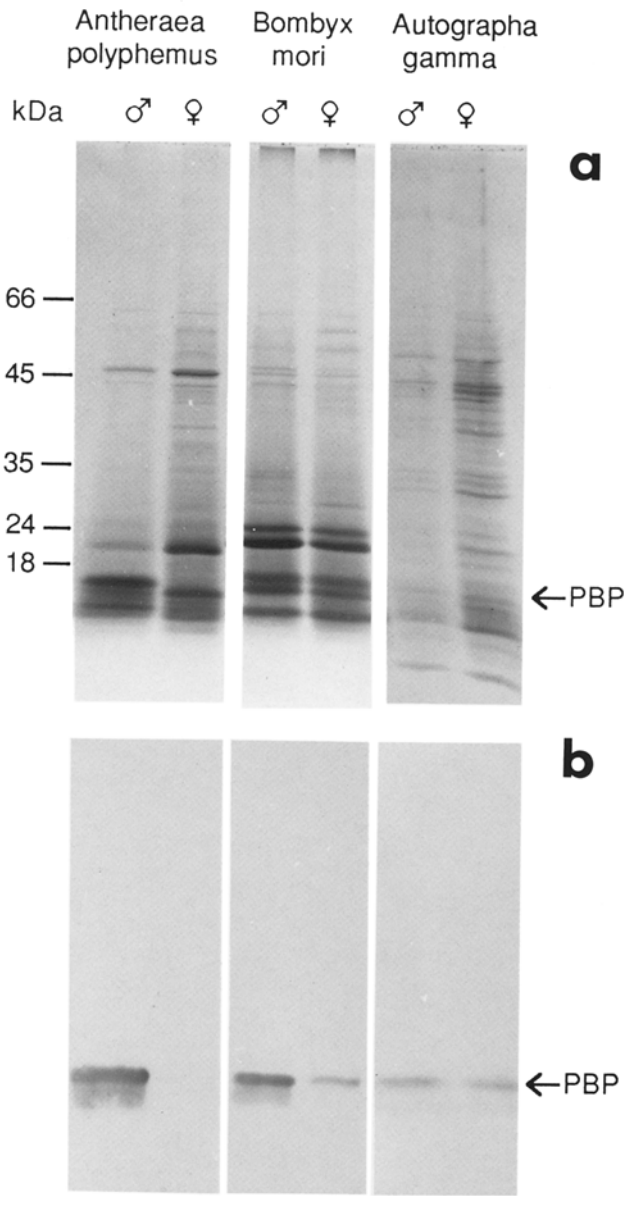
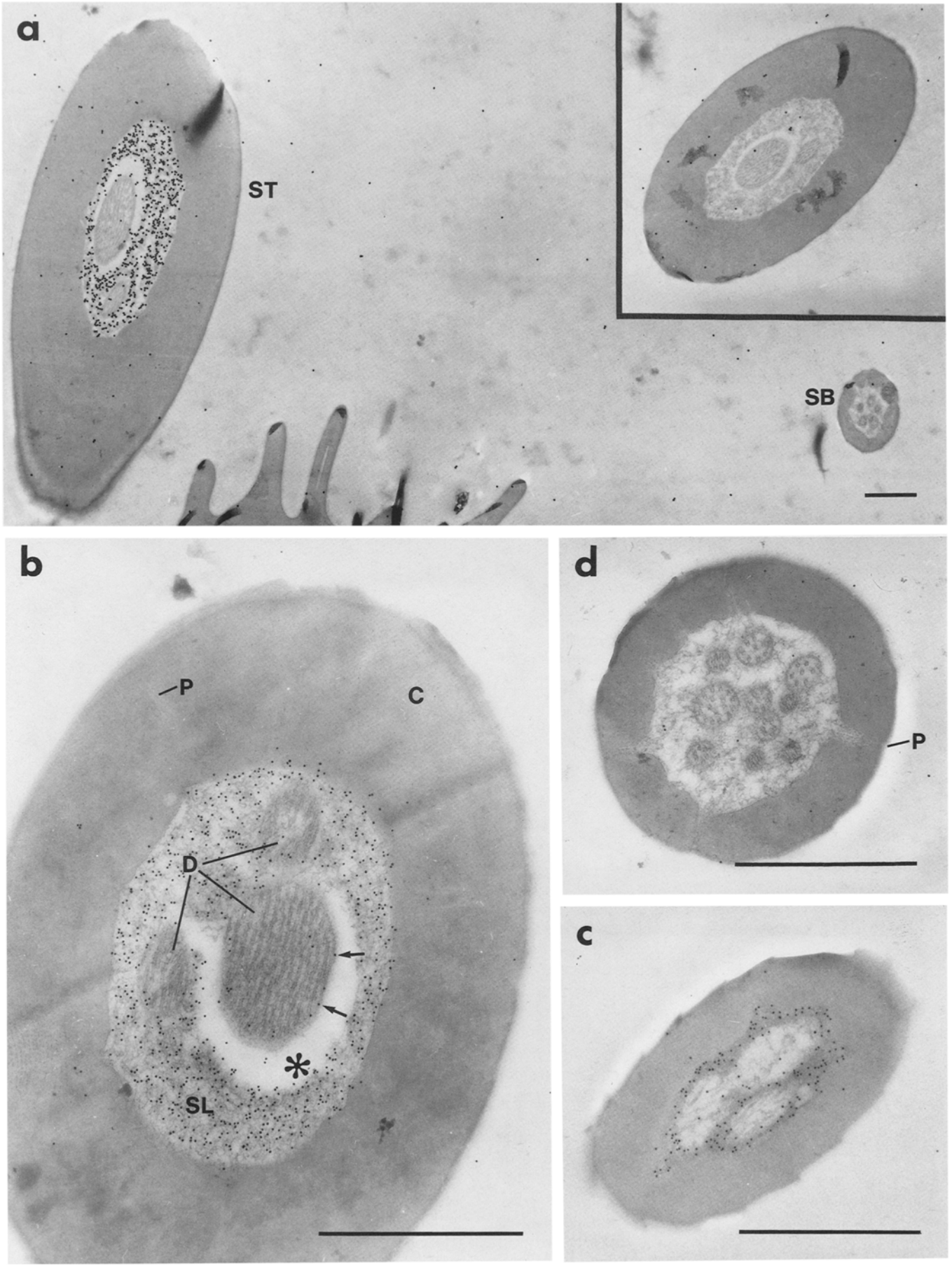


Fig. 1a, b. Analysis of male and female antennal proteins from three moth species. **a** SDS-PAGE stained with Coomassie Blue. **b** Immunoblot stained with anti-PBP (Apo), dilution 1:3000

2. Comparative immunocytochemistry of sensillum lymph

a) Controls. If the first antibody is omitted, there is practically no gold label on the sections except for unspecific adsorption at wrinkles or dirt particles. If pre-immune serum is used instead of the antiserum, some unspecific labelling is observed, in particular on the cuticle and the surrounding resin (Fig. 2a). Interference with non-specific background could be kept at a minimum by using higher dilutions of the antiserum (1:3000–1:30000).

The labelling of the sensillum lymph of the long s. trichodea of *A. polyphemus* can be regarded as a positive control. It is extremely strong except at sites where no


a
ST
SB
b
P
C
D
SL
*
d
P
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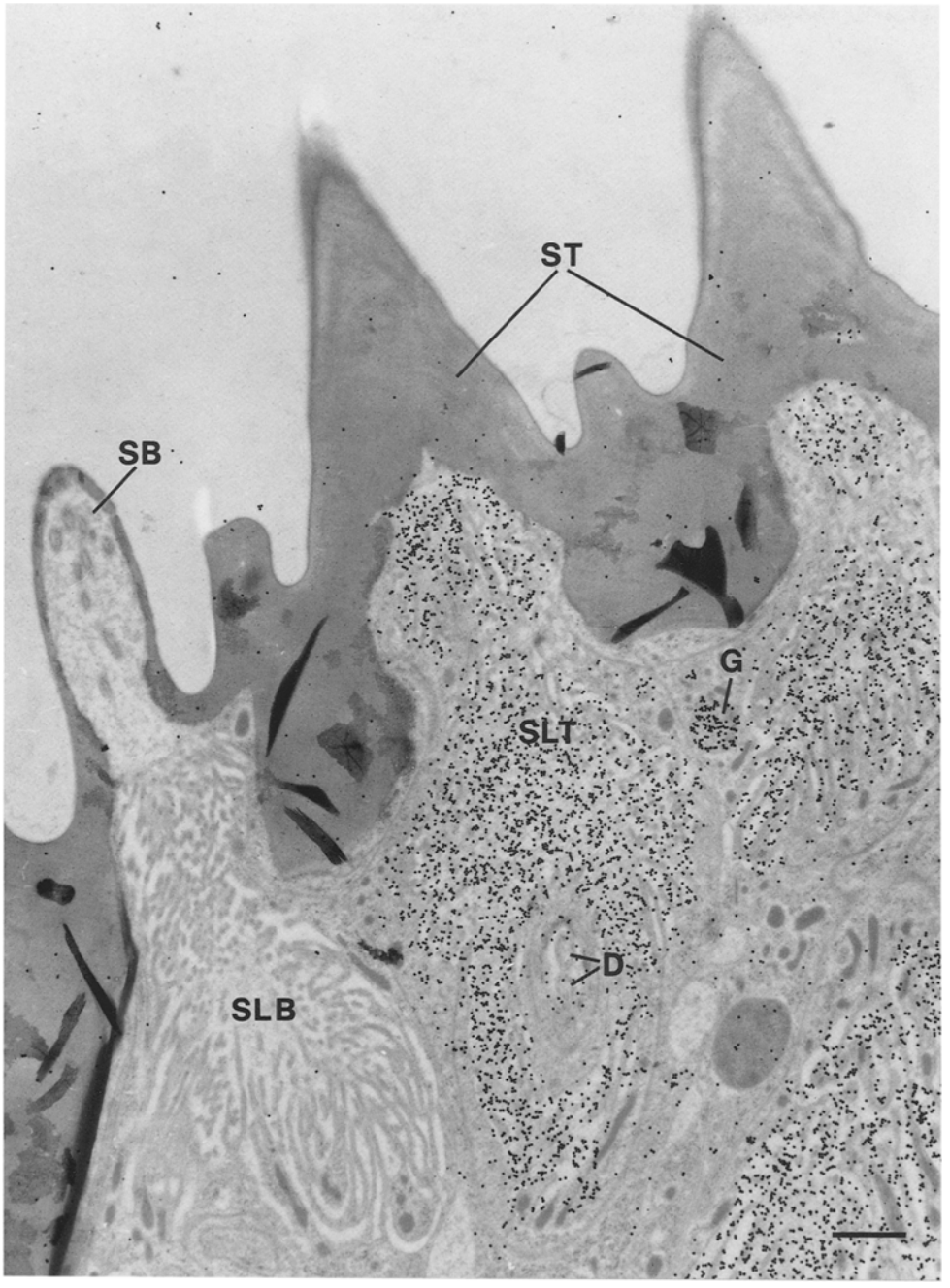

Fig. 3. *Bombyx mori*; oblique section through base of two s. trichodea (*ST*) and one s. basiconicum (*SB*). The sensillum lymph of the pheromone-sensitive sensilla trichodea (*SLT*) is labelled by anti-PBP(Apo), that of the sensillum basiconicum (*SLB*) is not. *D* Dendritic outer segments surrounded by labelled inner sensillum-lymph cavity; *G* heavily labelled granule in auxiliary cell. Specimen freeze-substituted in GA-acetone; anti-PBP(Apo) 1:30000; silver intensified. *Bar*: 1 μm; ×9800

Fig. 2a–d. *Antheraea polyphemus*, antennal sensilla after labelling with anti-PBP (Apo). **a** Cross sections of long s. trichodeum (*ST*) and s. basiconicum (*SB*) on the same micrograph; only the pheromone-sensitive sensillum trichodeum is labelled. *Inset*: When preimmune serum is used instead of primary antibody, no specific labelling of s. trichodea is observed. **b, c** Oblique sections of s. trichodea through proximal and distal parts of sensory hair, respectively. The sensillum lymph (*SL*) is heavily labelled; the dendrites (*D*) and the cuticle (*C*) show only a few unspecific gold particles. In **b** the sensillum lymph has been artificially separated from the large dendrite by shrinkage (*asterisk*), but still some gold particles are found on the dendritic membrane (*arrows*). **d** Only very few gold particles are found on sensillum lymph of sensillum basiconicum. *P* Stimulus-conducting pore-tubule systems. All specimens freeze-substituted in GA-acetone except **c** (chemically fixed). Dilution of primary antibody (or pre-immune serum) was 1:30000 in **a** and 1:3000 in **b–d**. Secondary antibody was goat anti-rabbit IgG coupled with 10-nm colloidal gold; silver intensification of gold particles was done in **a** (and *inset*). *Bar* in **a–d**: 1 μm; **a** ×9200; **b** ×38500; **c, d** ×34500

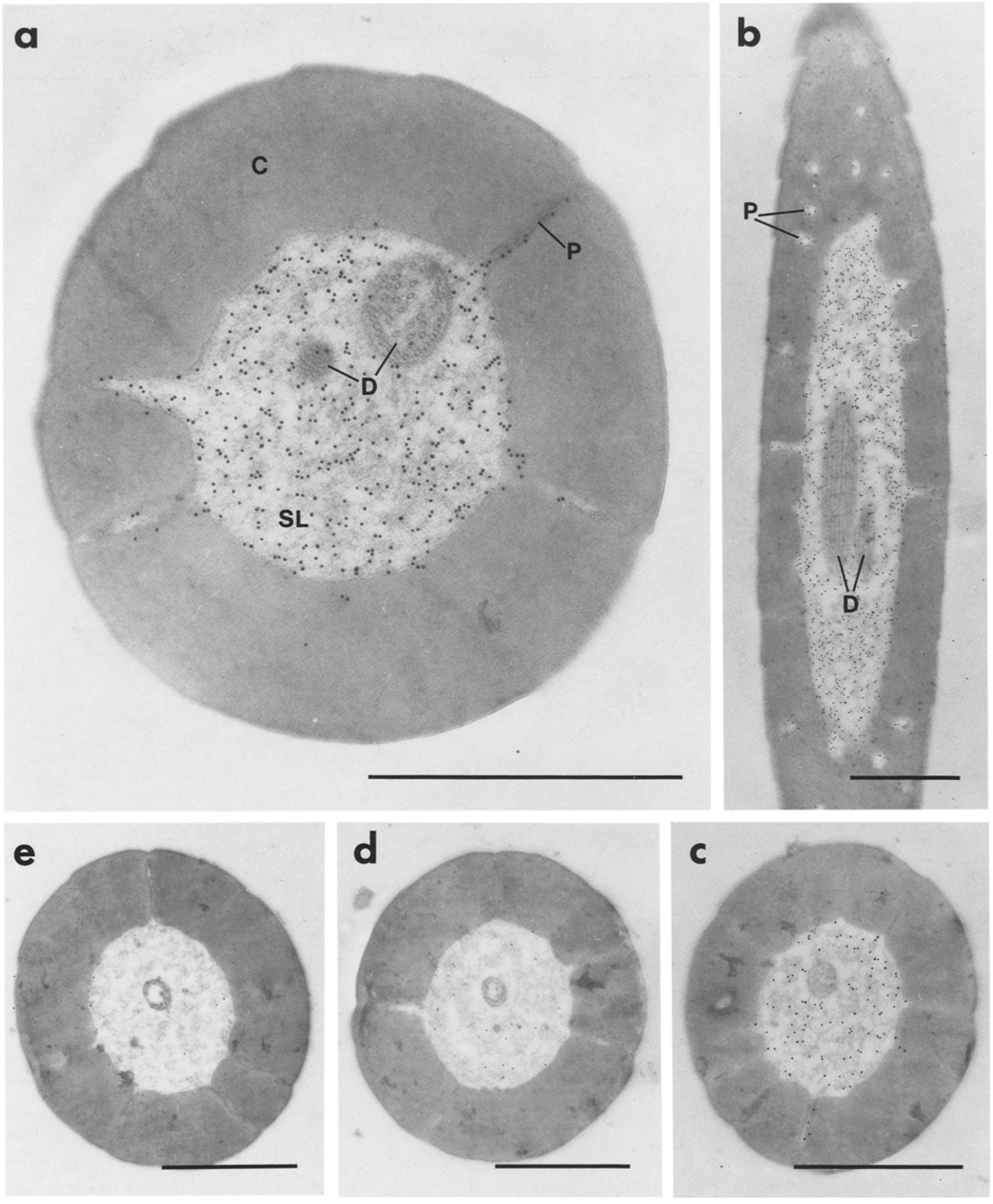

Fig. 4a–e. *Bombyx mori*, long s. trichodea after labelling with anti-PBP (Apo). **a** Cross section, **b** oblique section of sensory hairs freeze-substituted in GA-acetone, primary antibody diluted 1:3000. The sensillum lymph (*SL*) is heavily labelled, the dendrites (*D*) and the cuticle (*C*) are not. Label is also found in the lymph of the wall channels containing the stimulus-conducting pore-tubule systems (*P*). **c–e** Specimen freeze-substituted in Os-acetone, primary antibody diluted 1:3000 in **c**, 1:100000 in **d**; in **e** pre-immune serum was used instead of anti-PBP (Apo) at a dilution of 1:3000. Although by the osmium-treatment the antigenicity of the sensillum lymph is slightly reduced (compare **c** with **a, b**), even at a dilution of 1:100000 the reaction is still significantly above background. *Bar* in **a–e**: 1 μm; **a** ×56000; **b** 19500; **c** ×30000; **d, e** ×24000

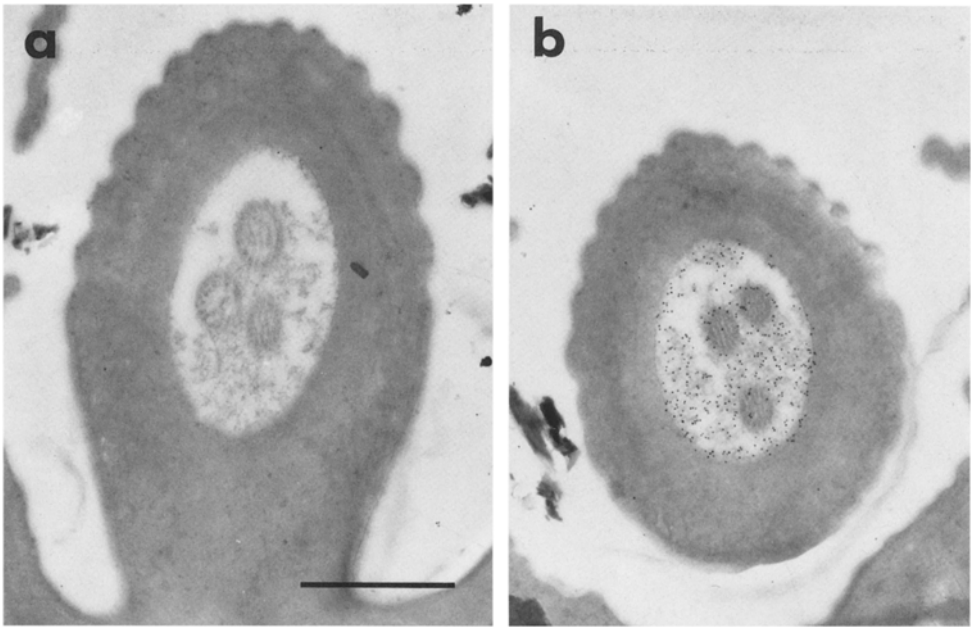

Fig. 5a, b. *Autographa gamma*, sensilla trichodea after labelling with anti-PBP(Apo). The cross sections shown in **a** and **b** are from the same section and both have been incubated with the primary antibody diluted 1:3000, but the characteristic labelling of the sensillum lymph is observed only in the sensillum shown in **b**. *Bar*: 1 μm; ×20000

sensillum lymph is left because of excessive shrinkage. Significant positive labelling is observed even with a dilution of the primary antiserum of 1:1000000. There is no noteworthy difference in labelling intensity between specimens freeze-substituted in GA-acetone and those conventionally fixed in glutaraldehyde (Figs. 2b, c), whereas freeze-substitution in osmium-acetone reduces the extent of labelling (Figs. 4b, c). In general, cryofixed material was preferred because of its superior tissue preservation as long as freezing damage was negligible.

b) Antheraea polyphemus. A strong positive immunoreaction is observed with the pheromone-sensitive s. trichodea of male moths which are absent in females (Figs. 2a–c). Both subtypes, the long and small s. trichodea, are labelled. There is no reaction with the sensilla basiconica which perceive general odours (Figs. 2a, d). S. basiconica are easily distinguished from s. trichodea by the evenly thin cuticular hair wall, the high number and different structure of their pore-tubule systems, and the large number of dendritic processes.

c) Bombyx mori. Strong labelling is observed with the sensillum lymph of the long s. trichodea (Figs. 3, 4). The label density is about the same as in *A. polyphemus*. No labelling was observed with the s. basiconica (Fig. 3) and the s. coeloconica. The antennae of two female *B. mori* were also studied. Although s. trichodea are also present on female antennae in this species, so far no labelled sensilla were detected.

d) Autographa gamma. Only male antennae were studied. No detailed morphology of the sensilla of this species being available, we only roughly distinguish between thick-walled trichoid sensilla and thin-walled basiconic sensilla (see above). Most of the sensilla are not labelled, even among the long s. trichodea. However, a small subpopulation of the trichoid sensilla shows intensive labelling similar to the long s. trichodea of male *Bombyx mori* (Fig. 5). The percentage of labelled s. trichodea varies considerably on different sections (0–30%). Their distribution and morphology has not yet been studied in detail, but from inspection of several antennal regions of the antennae of 4 animals, we could not yet find any particular morphological feature allowing a distinction of the labelled sensilla from the unlabelled ones (Fig. 5). Thus, at present, the labelled sub-populations can be characterized by immunocytochemistry only.

3. Localization of PBP in the labelled s. trichodea

Because *Bombyx mori* is most suitable for cryofixation, this species has been studied most extensively but, so far, we have not observed a differing distribution of label in the other species.

a) The sensory hairs. While the sensillum lymph is heavily labelled, neither the dendritic cytoplasm nor the cuticle of the hair wall shows more than a few unspecific gold grains (Figs. 2b, c, 4a, b).

b) The sensillum-lymph cavities. The sensillum lymph within the sensillum-lymph cavities below the hair base is as heavily labelled as in the hair lumen (Figs. 3, 6a). There is no difference in density of label between the outer and the inner sensillum-lymph cavity or between different regions of the outer sensillum-lymph cavity,

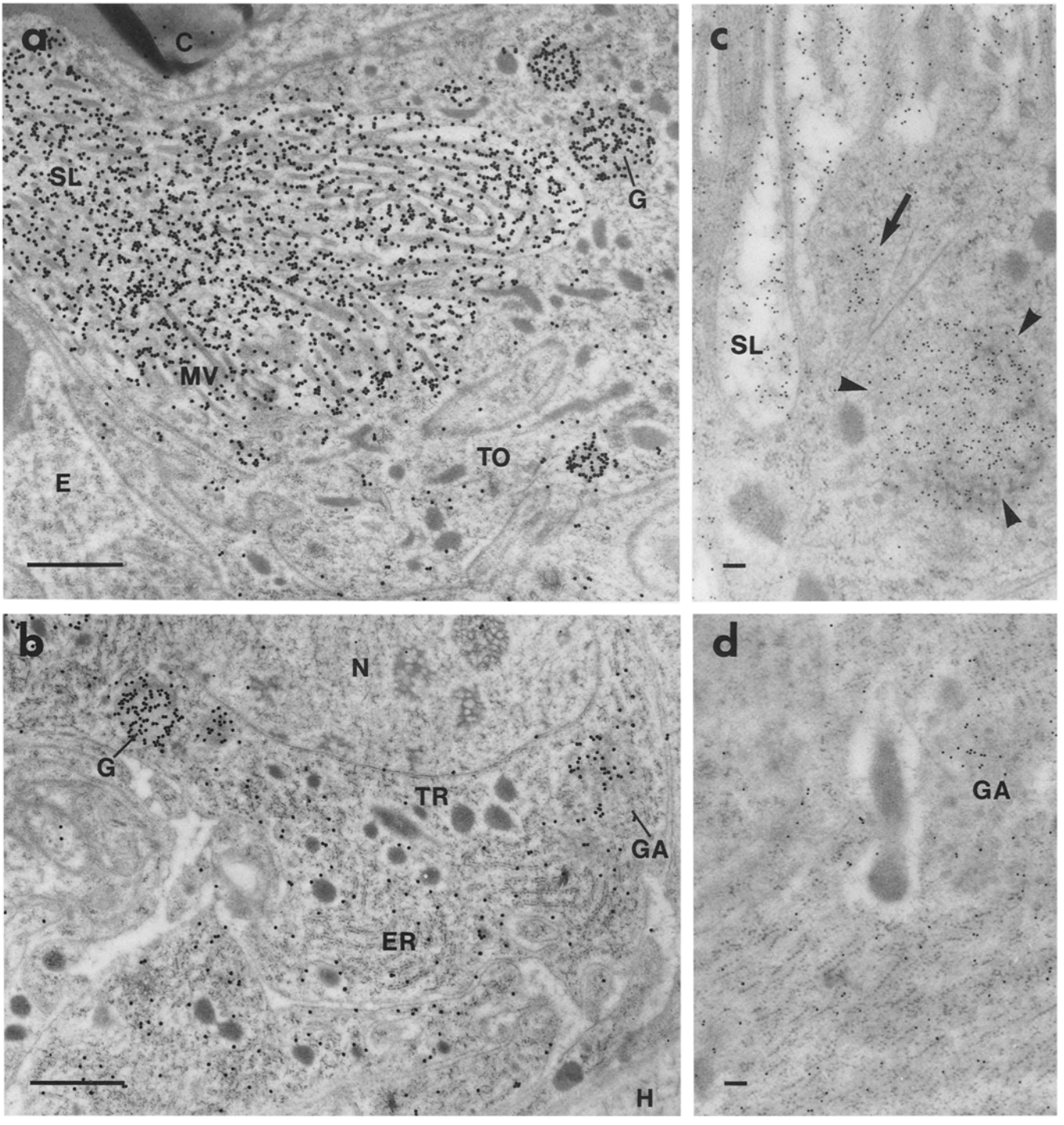

Fig. 6a–d. *Bombyx mori*, auxiliary cells of pheromone-sensitive sensilla trichodea labelled with anti-PBP(Apo). **a** Below the base of the sensory hair, the sensillum-lymph cavity (*SL*) is bordered by the tormogen cell (*TO*), the apical plasma membrane of which is enlarged by numerous microlamellae and microvilli (*MV*). Surrounding epidermal cells (*E*) are not labelled. **b** Trichogen cell (*TR*) showing typical features of protein synthesis. Rough endoplasmic reticulum (*ER*) and Golgi apparatus (*GA*) are labelled; dense granules (*G*) in the cytoplasm show the highest density of gold particles. **c** Detail of apical border region of tormogen cell. The sensillum lymph (*SL*) in between the microlamellae and microvilli is heavily labelled. In the apical cytoplasm we often observe smaller accumulations of gold label (*arrow*) which are not always associated with a distinct organelle; some of the dense granules have an expanded aspect, they are larger and less dense than usual (*between arrowheads*). **d** Detail of Golgi apparatus (*GA*) and endoplasmic reticulum of auxiliary cell; usually the former is more heavily labelled than the latter. *C* Cuticle; *H* haemolymph; *N* nucleus. Dilution of primary antibody was 1:30000 in **a, b** and 1:3000 in **c, d**. Silver intensification was carried out in **a** and **b**; all specimens freeze-substituted in GA-acetone. *Bar* in **a** and **b**: 1 μm; ×15800; in **c** and **d**: 100 nm; ×38500

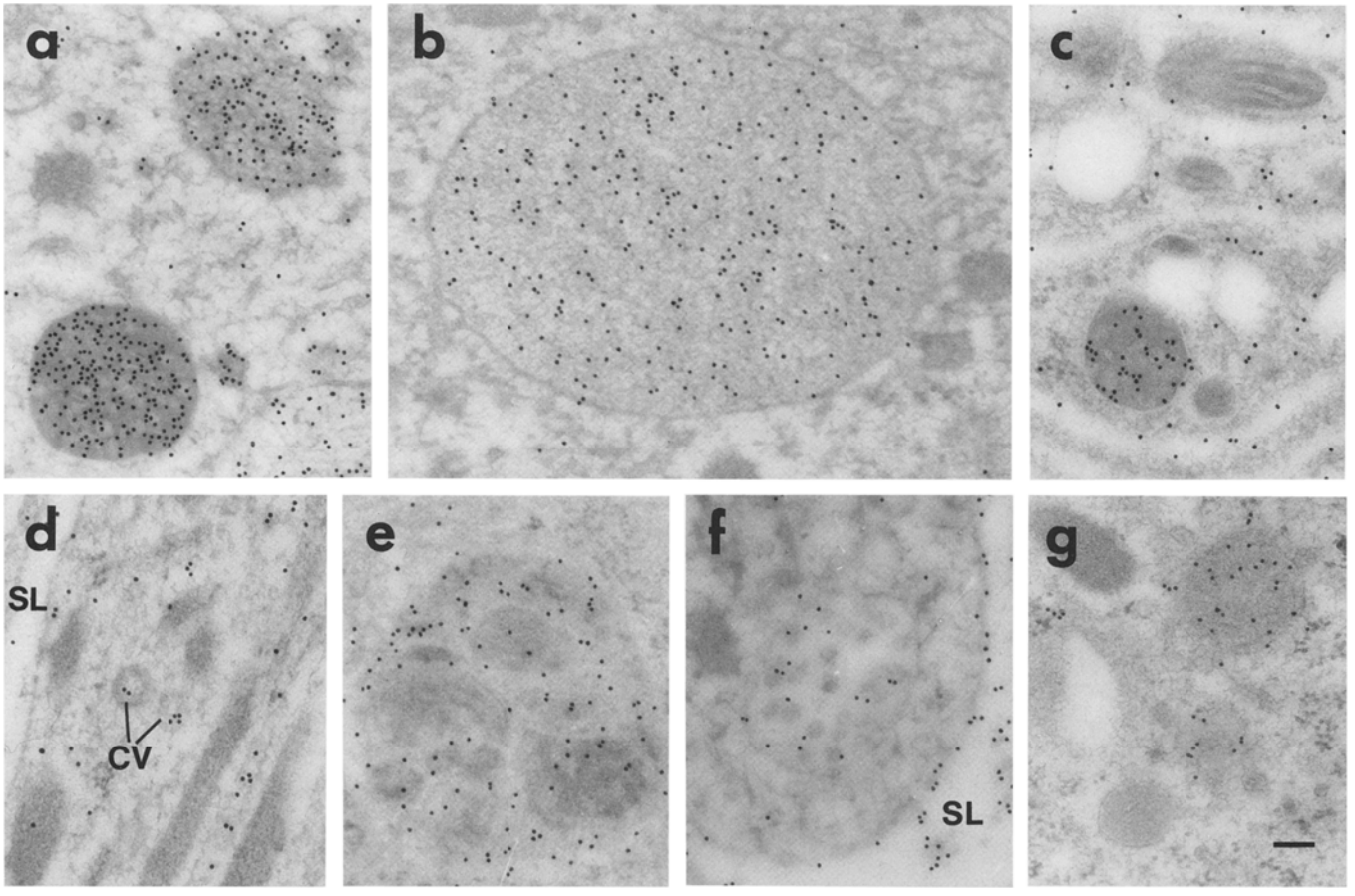

Fig. 7a–g. Various labelled organelles in auxiliary (**a–e**) and receptor (**f, g**) cells of s. trichodea. All specimens freeze-substituted in GA-acetone, except **b** and **c**. **a–c** Labelled dense granules of auxiliary cells vary considerably in size, density, and label density. After freeze-substitution in GA-acetone (**a**), a limiting membrane is not discernible; however, after freeze-substitution in Os-acetone (**b**) and after chemical fixation omitting OsO_4 (**c**), a limiting membrane is sometimes visible in positive and in negative contrast, respectively. **d** Labelled vesicles (*CV*) in trichogen cell with faint bristle-coating; **e** lysosome-like body in tormogen cell; **f** multivesicular body in inner dendritic segment; **g** dense granules in receptor cell soma. *SL* sensillum lymph. *Bar*: 100 nm; × 50000

e.g., the trichogen pouch (Steinbrecht and Gnatzy 1984) or the parts bordered mainly by the tormogen cell.

c) The tormogen and trichogen cells. Among the three sensillar auxiliary cells, i.e., the tormogen, trichogen and thecogen cell, the former two display a similar fine structure (Steinbrecht and Gnatzy 1984) and also show a similar intracellular labelling pattern. The nuclei and some cytoplasmatic areas are practically free of gold particles. Some regions of the granular endoplasmic reticulum (ER) show slight or medium labelling, in particular within the ER cisternae (Figs. 6b, d); the Golgi apparatus, which in these cells consists of an accumulation of vesicles without extensive cisternae, may show a somewhat higher labelling density (Figs. 6b, d).

Particular electron-dense granules are the most intensively labelled components in the cytoplasm. This is the only compartment where the density of labelling inside the cell is as high or even higher than in the extracellular sensillum lymph (Figs. 6a, b). The size and structure of the labelled granules varies considerably. Many granules are spherical with a diameter of ~0.5 μm. The substructure of the granules and also the distribution of the gold grains may be homogeneous (Figs. 7a–c) or inhomogeneous (Fig. 7e). Occasionally, the electron density of the granules is very much reduced and distinction from the surrounding ground cytoplasm is possible only by virtue of the label (Fig. 6c). After freeze-substitution in GA-acetone the granules appear not to be membrane-bounded (Fig. 7a), but after chemical fixation or after freeze-substitution with Os-acetone, an outer limiting membrane can be observed (Figs. 7b, c).

A particularly high frequency and variety of granules is found in the vicinity of the apical cell membrane of the auxiliary cells, which borders the sensillum-lymph cavity (Figs. 6a, c). In this region, there is also some diffuse labelling of the cytoplasm (Fig. 6c). Structures resembling coated pits and coated vesicles are observed; some of them are labelled (Fig. 7d).

d) The thecogen cell. This auxiliary cell differs from the other two auxiliary cells by forming a complete and tight envelope around the receptor cell somata and inner dendritic segments. It borders the inner sensillum-lymph cavity; in olfactory sensilla of moths its volume is comparatively small; endoplasmic reticulum or Golgi areas

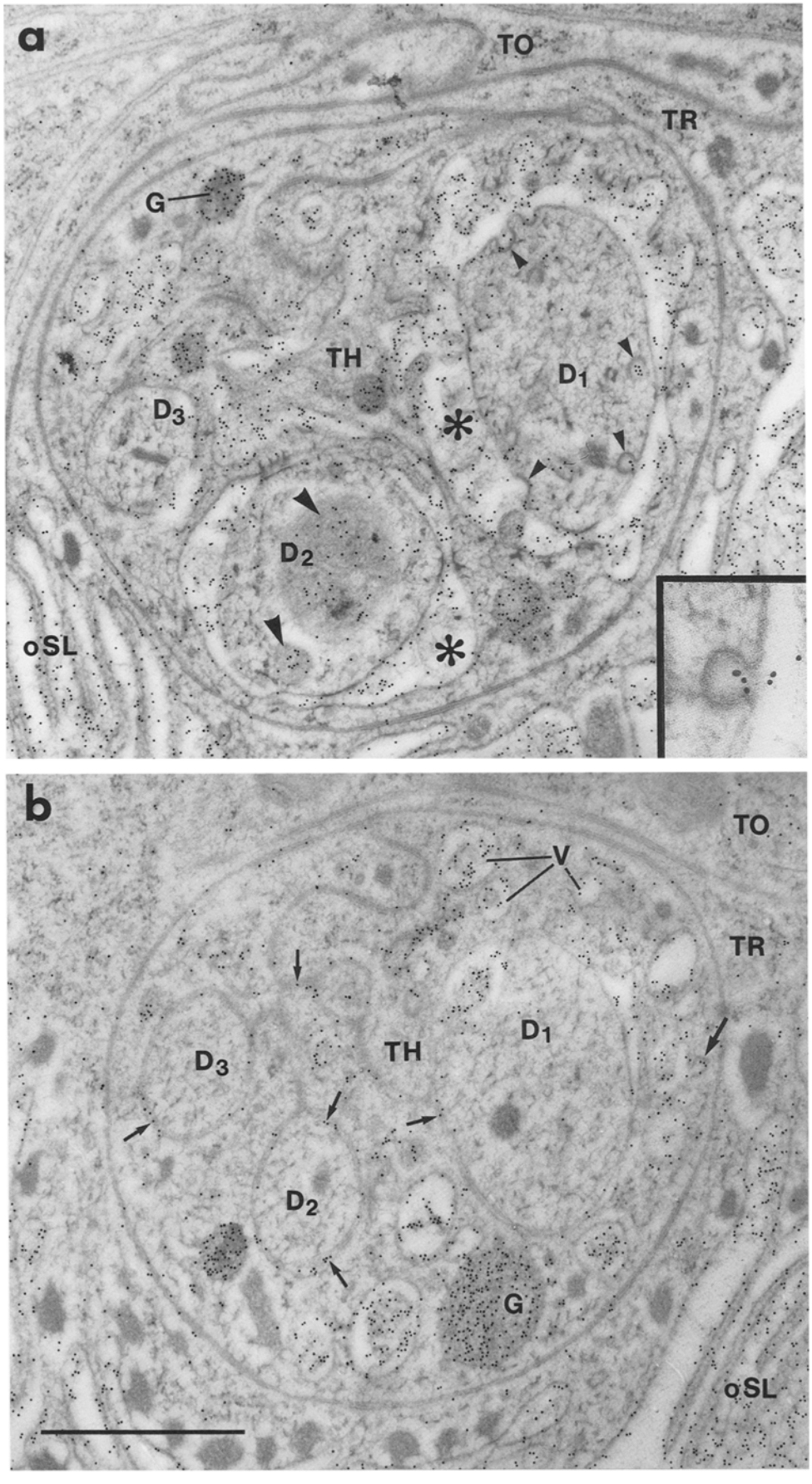

Fig. 8a, b. *Antheraea polyphemus*, cross sections through inner dendritic segments of a sensillum trichodeum close to ciliary segment; section shown in **b** is about 1 μm more proximal than that in **a**. The three sensory dendrites (D_1, D_2, D_3) are concentrically ensheated by the thecogen (*TH*), trichogen (*TR*), and tormogen (*TO*) cell. In **a**, the inner dendritic segments of D_1 and D_2 are not yet tightly wrapped by the thecogen cell but are surrounded by sensillum lymph of the inner sensillum-lymph cavity (*asterisks*) which is heavily labelled; in D_1 endocytotic pits and vesicles (*small arrowheads*), in D_2 a larger and a smaller dense body (*large arrowheads*) are labelled. *Inset*: Endocytotic pit at higher magnification showing a faint bristle-coating. In **b**, the inner sensillum-lymph space around the dendrites has almost vanished. The cytoplasm of the thecogen cell contains numerous small dense granules (*G*) and vesicles (*V*) of varying size, the contents of the latter have the same density as the sensillum lymph; both types of organelles are densely labelled. *Large arrow* points to a labelled endocytotic pit in the apical membrane of the thecogen cell. Note that the circumference of the inner dendritic segments as well as the autojunction ("mesaxon") of the thecogen cell membrane is labelled (*small arrows*). *oSL* Part of the large outer sensillum-lymph cavity. Specimen freeze-substituted in GA-acetone (some freezing damage is unavoidable at this level (~15 nm below surface)). Anti-PBP (Apo) 1:3000. *Bar*: 1 μm; ×32000; *inset* ×85000

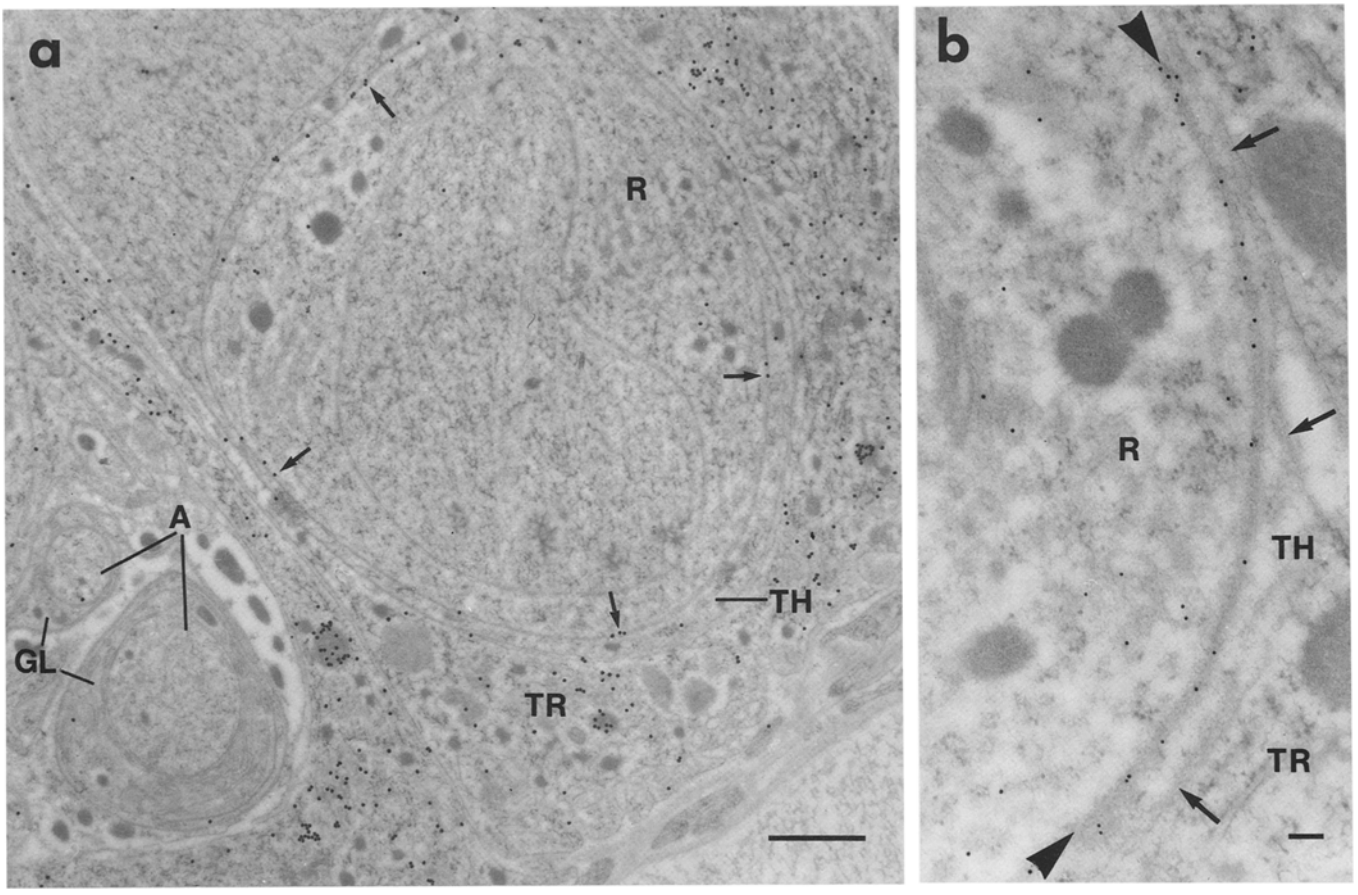

Fig. 9a, b. The receptor cell soma (*R*) of s. trichodea is labelled by anti-PBP(Apo) along the cell membrane. **a** Only the receptor cell membrane is labelled (*small arrows*), other cell membranes, e.g., those of the axons (*A*), glia cells (*GL*), or trichogen cells (*TR*), are not. Note the thin envelope formed by the thecogen cell (*TH*) completely surrounding the receptor cell. **b** 10-nm gold particles are found on both membranes of the receptor-thecogen cell junction (*arrowheads*), but not on the outer membrane of the thecogen cell envelope (*arrows*). **a** *Antheraea polyphemus*, anti-PBP(Apo) 1:30000, silver-intensified; **b** *Bombyx mori*, anti-PBP(Apo) 1:3000, both specimens freeze-substituted in GA-acetone. *Bar* in **a**: 1 μm; ×12600; in **b**: 100 nm; ×46300

are not conspicuous (Steinbrecht and Gnatzy 1984; Gnatzy et al. 1984).

In particular, the apical parts of the thecogen cell bordering the inner dendritic segments and the inner sensillum-lymph cavity contain numerous labelled dense granules and vesicles (Fig. 8). The dense granules have a diameter of ~0.2 μm and, therefore, are smaller than those in the other two auxiliary cells. The contents of the labelled vesicles have the same density as the sensillum lymph. Sometimes, label was observed on structures resembling coated pits and vesicles. The cell membrane adjacent to the receptor cell membrane, and also the autojunction ("mesaxon") formed by wrapping of the inner dendritic segments, exhibit a distinct but not very extensive labelling (Figs. 8b, 9).

e) The receptor cells. The cytoplasm of the outer dendritic segments and of the ciliary segments of the sensory cells is not labelled. In the inner dendritic segments and in the somata, however, occasionally labelled granules are found, which differ in size and appearance from those in the tormogen and trichogen cells. The diameter usually is ~0.2 μm, and a membrane is more easily discerned (Figs. 7g, 8a). Sometimes labelled multivesicular bodies are found (Fig. 7f). The endoplasmic reticulum and the Golgi apparatus, although prominent in the somata, are never labelled.

In the subciliary region, where the inner dendritic segments are still surrounded by the inner sensillum-lymph cavity, labelled pits and vesicles are observed (Fig. 8a). Sometimes, the outer surface of these pits bears an indistinct bristle-coating (Fig. 8a, inset).

The cell membrane of the perikarya and of the inner dendritic segments is labelled where it is bordered by the thecogen cell (see above, Fig. 9). The membrane of the axons, however, is not labelled (Fig. 9a).

f) Other antennal tissue. Except for a few unspecific gold particles, no label is observed in the haemolymph, the antennal nerve, the epidermis or in cells belonging to sensilla other than the s. trichodea as described above. Also the intercellular clefts between these cells are free of label.

Discussion

1. Biosynthesis and degradation of PBP

The trichogen and tormogen cells of insect sensilla – as their name implies – have important functions during sensillar development. Nevertheless, they do not degenerate after completion of ontogeny but display typical features of protein synthesis. Therefore, speculation about the secretory activity of these cells arose in analogy to the function of the sustentacular cells of the vertebrate olfactory mucosa (Ernst 1969; Steinbrecht 1969). The intracellular localization of PBP in secretory organelles of these auxiliary cells now gives direct support to this notion and for the first time enables us to discuss more precisely the possible biosynthetical pathway of this protein. Quite obviously, the tormogen and trichogen cells, and not the receptor cells, are the main site of its production (Fig. 10).

Precursor amino acids are taken up from the haemolymph, which is not labelled by anti-PBP(Apo). As could be shown by autoradiography (Steinbrecht 1992a and unpublished), the auxiliary cells avidly take up ^{3}H-leucin from the haemolymph and are heavily labelled as soon as 5 min after application. The observation that density of label increases from granular endoplasmic reticulum via Golgi apparatus to dense granules fits well to the classical pathway of protein synthesis. As in other examples of protein secretion (for review, see Palade 1975; Kelly 1985), there may be a final condensation of the protein in secretory granules, thus accounting for the extremely high labelling density in many granules. Nevertheless, the population of labelled granules is highly inhomogeneous, and at present it is difficult to distinguish between secretory granules and organelles involved in the degradation of PBP (see below).

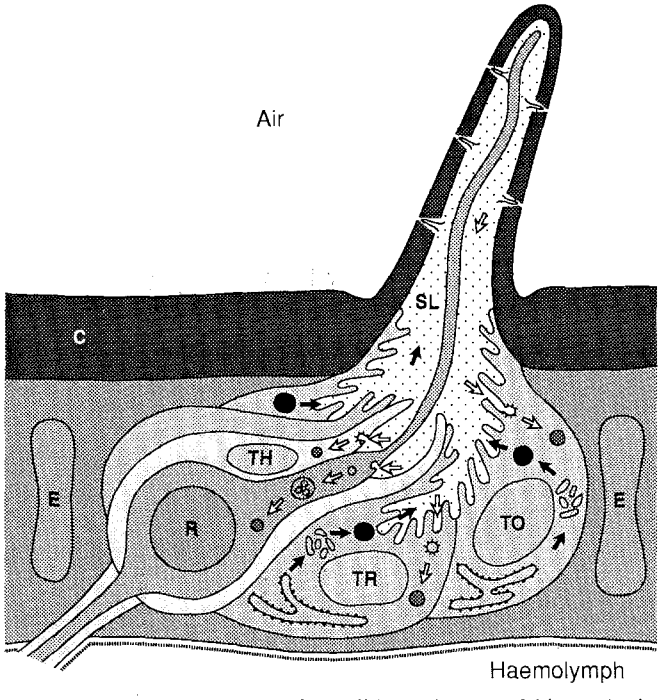

Fig. 10. Schematic drawing of possible pathways of biosynthesis and degradation of PBP in pheromone-sensitive sensilla trichodea. Biosynthesis and exocytosis (*filled arrows*) occurs in the trichogen (*TR*) and tormogen (*TO*) cell following the conventional pathway of protein secretion via granular endoplasmic reticulum, Golgi apparatus and secretory granules (*black*). Endocytosis and turnover (*open arrows*) probably occur in all sensillar cells via coated or non-coated pits and vesicles, multivesicular bodies and lysosomes (*stippled*). *C* cuticle and sensory hair with pores; *E* epidermal cell; *R* receptor cell with axon and sensory process, the outer segment of which extends into the hair and is surrounded by sensillum lymph (*SL*); *TH* thecogen cell, completely ensheathing receptor cell soma and inner dendritic segment

Unfortunately, the actual secretion process is still obscure. Probably the contents of the dense granules are exported into the sensillum lymph via exocytosis. The fact that we have not yet observed such cases of exocytosis is not contrary to this idea. Firstly, the apical membrane is heavily folded so that the verification of an exocytotic structure on morphological grounds is very difficult. Secondly, these events are very rapid and possibly quite rare, so that they may elude the observer, unless massive simultaneous exocytosis can be artificially triggered (for review, see Plattner 1989).

Preliminary immunocytochemical observations in pupae (Steinbrecht et al. 1991, and unpublished) show that biosynthesis of PBP starts only during the last days before emergence of the adult, at the same time when the sensillum-lymph cavities develop by invagination of the apical membranes of the auxiliary cells (Keil and Steiner 1991). Obviously, the production of PBP does not cease when development is completed but continues during the life of the imago. This is in accordance with Vogt et al. (1989), who found by in-vivo labelling methods that PBP synthesis in *Lymantria dispar* starts 4 days before emergence, increases drastically during the following days, and remains at a high level in the imago. These authors further concluded that there must be extensive breakdown of PBP as well, because the total amount of PBP stays constant; the steady-state turnover was estimated at 8×10^7 molecules/h/sensillum.

There are several indications of PBP-disintegrating pathways in our specimens (Fig. 10). In the tormogen and trichogen cells we observe coated pits and coated vesicles which sometimes – although not always – are labelled; in addition, labelled lysosome-like structures are found in these cells. The thecogen cell is very small and does not exhibit well-developed organelles of protein synthesis. There is, however, extensive endocytosis in the apical part of the thecogen cell which borders the inner sensillum-lymph cavity. Thus, the labelled organelles in this cell appear to belong to the decomposing rather than the biosynthetic pathway. The same must be assumed for the receptor cells which display an endoplasmic reticulum as well as a Golgi apparatus, but these structures are never labelled. Again, numerous labelled endocytotic pits and vesicles are found in the inner dendritic segments just below the ciliary constriction. Multivesicular bodies – known as organelles of the endocytotic pathway – are labelled as well; they are abundant in the inner dendritic segments and somata of olfactory receptor cells and are much less common in mechanoreceptive sensilla (Keil and Steinbrecht 1984). Endocytosis

has been observed previously in tracer studies applying lanthanum ions or cationized ferritin to the hair lumen (Keil and Steinbrecht 1987; T.A. Keil unpublished). Whether this endocytosis is clathrin-dependent or not, deserves further clarification. Keil (1989) reported particularly extensive vesicle budding in the subciliary segments of s. trichodea of *Manduca sexta* and speculated that this might be a site of membrane turnover of the receptor membrane. The significance of the internalization of PBP for the sensillar function is still unknown, as well as the specific role of the auxiliary and the receptor cells in this process. Of particular interest would be an answer to the question whether it depends on ligand binding by the PBP.

Even less clear is the reason for the labelling of the cell border around the inner dendritic segments and somata of the receptor cells. Whether the membrane of the outer dendritic segments is also labelled, cannot be decided, because this is masked by the high labelling density of the sensillum lymph (however, see Fig. 2b). Transport of bound PBP from the outer segment to the inner segment and perikaryon by membrane flow is unlikely, as this should be impeded by the belt of septate junctions proximal to the ciliary segment. Because single gold particles on sections may be found at least 20 nm apart from the antigenic site (Kemler and Schwarz 1989), we cannot decide whether the antigen resides with both or only one membrane, or with the cleft between the two which is only 6 nm wide (Fig. 9b; thecogen-receptor cell junction; Steinbrecht 1980). PBP on the thecogen cell membrane alone would be sufficent to explain the data; this would conform with the labelling of the autojunction of the thecogen cell (Fig. 8b) and with the lack of label at the axon hillock, where the thecogen sheath is replaced by a glia sheath (Fig. 9a).

In conclusion, we can now ascribe one more function to the auxiliary sensillar cells in these sensilla. In addition to their morphogenetic role established already by Henke (1953), and to their activity in regulating ion transport and electrochemical events (Thurm and Küppers 1980), these cells build up and break down specific odorant-binding proteins.

2. Distribution of PBP among sensillum types and species

Immunocytochemistry for the first time has proven that PBP is a specific component of the pheromone-sensitive sensilla trichodea; it is not present in other olfactory sensilla (Steinbrecht et al. 1991, and this paper). In male *Antheraea polyphemus*, the long and the small sensilla trichodea contain receptors for the female pheromone (Meng et al. 1989), and both types are labelled with the antiserum. The s. basiconica are never labelled; they perceive other, more general odours (Schneider et al. 1964). It was speculated that the 'general odorant-binding proteins' (GOBPs), which have been isolated from the antennae of male and female moths, would be localized in these sensilla (Vogt et al. 1991).

In vertebrates, localization of odorant-binding proteins (OBP) is not so specific. Pevsner et al. (1986) produced polyclonal antisera against bovine OBP and performed immunohistochemistry at the light-microscopical level. They observed specific immunoreactivity not only in Bowman's glands of the olfactory mucosa but also in mucus-secreting glands underlying the respiratory mucosa. Moreover, odorant binding was demonstrated by these authors in nasal mucus and lacrymal secretions but not in saliva. In any case, a more restricted and specialized localization of OBP would not make sense, because in vertebrates the olfactory receptor cells extend their sensory processes into a common mucus layer which is constantly flowing across the olfactory mucosa. In insects, however, every sensillum has its own separate sensillum lymph surrounding the dendrites of only a few receptor cells.

Taking into account the high degree of homology between PBPs of different moth species (Vogt et al. 1991; Raming et al. 1990), the labelling of s. trichodea of male *B. mori* with anti-PBP(Apo) is not surprising. On the other hand, sections of female s. trichodea of this species did not show labelling, although the immunoblots with antennal homogenates were positive (Fig. 1b, see also Maida et al., in preparation). Possibly, the immunoreactivity resides with antennal regions which have not yet been examined in the two animals studied. In this context it is important to know that the female sensilla trichodea do not respond to the pheromone components bombykol and bombykal but to the totally different compounds linalool and benzoic acid (Priesner 1979; Heinbockel and Kaissling 1990), so that PBP has not been expected in female *Bombyx mori*.

Our observations in male *Autographa gamma* show that only a small fraction of the s. trichodea is labelled with the antibody against PBP of *A. polyphemus*. This result is particularly important, because it shows that the population of morphologically identical s. trichodea can be inhomogeneous as to the kind of PBP they contain. *A. gamma* is known to produce and perceive several pheromone components (Dunkelblum and Gothilf 1983). In fact, most moth species use a mixture of compounds as sex attractant pheromone (Arn et al. 1986), and the receptor cells perceiving the different components of this bouquet often are located in physiologically different but morphologically similar s. trichodea (Priesner 1980). Thus, the perspective arises for a much higher specificity of PBP than hitherto assumed. Two different PBPs were also observed in *Lymantria dispar* (Vogt et al. 1989) and *Antheraea pernyi* (Krieger et al. 1991), but whether these belong to different sub-populations of s. trichodea or co-occur in the same sensillum is not yet known. Immunocytochemistry with monoclonal antibodies against different PBPs in electrophysiologically characterized sensilla would permit a direct check of any correlation between the selectivity of the receptor cells and the particular PBP in a given sensillum.

3. Function of PBP

As mentioned in the "Introduction", the function of PBP remains controversial. Originally, Vogt and Riddi-

ford (1981) supposed a role in the rapid inactivation of the stimulus, an idea that was shared by Kaissling (1986, 1987), because enzymatic degradation of pheromone on the sensory hairs and the antennae as studied by Kasang and Kaissling (1972; see also Kasang et al. 1988, 1989) is too slow to account for the almost immediate cessation of the receptor potential when an odour stimulus ends. When Vogt et al. (1985) examined the sensillar esterase which occurs in the sensillum lymph of *A. polyphemus* together with PBP, they observed an extremely rapid turnover rate of the purified esterase in vitro and attributed the inactivator function to this enzyme (but see below).

PBP is now postulated to act as a carrier or solubilizer helping the lipophilic pheromone molecules to cross the barrier of the aqueous sensillum lymph after they have penetrated the cuticular wall via the pores and pore tubules in this wall. In fact, the high concentration of PBP in the sensillum lymph may drastically change the solubility of lipophilic substances. Lipophilic tracer molecules have been observed to "dissolve" in the sensillum lymph when applied in bulk to the hair surface (Steinbrecht 1992a). Moreover, Van den Berg and Ziegelberger (1991) performed electrophysiological experiments on sensilla in which the lumen was perfused by Ringer solution. In this preparation it was possible to stimulate the receptor from the sensillum-lymph side when the Ringer contained pheromone. The stimulating effect was increased about hundredfold when the electrolyte contained pheromone plus PBP as compared to pheromone alone. This solubilizing effect, however, was rather unspecific and could be mimicked by bovine serum albumine.

Before the discovery of PBP, another mode of stimulus transport has been proposed: Stimulus molecules reach the dendritic membrane by direct contacts of the pore tubules with the receptor membrane (Steinbrecht and Müller 1971; see also Steinbrecht 1973, 1987; Keil 1982, 1984b). This hypothesis can still not be ruled out, since even the low number of contacts actually observed would be sufficient for excitation within the measured latency of the receptor cells (Kaissling and Priesner 1970; Kaissling 1986, 1987). Movements of the dendrites might further increase the contact probability in a given time (Williams 1988; T.A. Keil, unpublished). In this model, stimulus transport and inactivation would occur in two different 'perireceptor compartments' (Steinbrecht 1992b), the pore tubules and the sensillum lymph, respectively. In the model of Vogt (1987), however, both perireceptor events would occur in the same compartment.

At the moment, the major problem resides with explaining stimulus inactivation, which according to Kasang and Kaissling (1972; see also Kanaujia and Kaissling 1985; Kasang et al. 1988, 1989) comprises a rapid, non-enzymatic first step. The rapid turnover of the sensillar esterase (Vogt et al. 1985) is substantially slowed down in the presence of PBP (Vogt and Riddiford 1986), a fact that might account for the in-vitro and in-vivo data. However, can PBP work as a rapid inactivator, if pheromone bound to PBP is not prevented from stimulating the receptor, as shown by Van den Berg and Ziegelberger (1991)? Taking into account the two bands of PBP as regularly found in non-denaturating polyacrylamide gels, Van den Berg and Ziegelberger (1991) proposed a new hypothesis assuming two configurations of PBP, one serving in stimulus transport, the other in stimulus inactivation. This idea obtains further support from data of G. Ziegelberger and R. Maida (in preparation) who observed an affinity of PBP, not only to the pheromone but also to a membrane protein obtained from isolated sensory hairs. Since this protein also binds pheromone, it could be a candidate for the receptor protein in the dendritic membrane. It is conceivable that not only the receptor protein, but also the pheromone-binding protein undergoes a conformational change upon stimulus transduction. Possibly, these changes require a constant renewal of PBP in the sensillum lymph as supported by our immunolabelling data.

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