

## Immunocytochemical Evidence for the Occurrence of Insulin in the Frontal Ganglion of a Lepidopteran Insect, the Tobacco Hornworm Moth, *Manduca sexta* L.

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The frontal ganglion of the adult forms of the tobacco hornworm, *Manduca sexta*, was investigated immunocytochemically for the occurrence of the gastro-entero-pancreatic (GEP) neurohormonal peptides, namely insulin, nerve growth factor, epidermal growth factor, insulin C-peptide, somatostatin, glucagon, glicentin, pancreatic polypeptide (PP), polypeptide YY (PYY), secretin, vasoactive intestinal peptide (VIP), gastric inhibitory peptide (GIP), gastrin, cholecystokinin (CCK), enkephalin,  $\alpha$ - and  $\beta$ -endorphins, substance P, neurotensin, bombesin, motilin, ACTH, serotonin, and calcitonin. Among all the antisera tested, positive immunostaining was obtained with anti-insulin B-chain serum only. The insulin B-chain immunoreactivity was localized in 4-6 large (30-40  $\mu$ m) neurons, in the neuropile, and in the recurrent nerve. It is speculated that the insulin-like immunoreactive material may be transported to the neurohaemal organ (corpora cardiaca) through the nervi cardiaco-somatogastrici.

The frontal ganglion is a component of the sympathetic (somatogastric) nervous system of insects (Lane, 1974). This ganglion has been found to contain neurosecretory cells in a number of insect species (cf. Lane, 1974). In a recent investigation of the tobacco hornworm, *Manduca sexta*, several gastro-entero-pancreatic (GEP) neurohormonal peptides have been localized in neurosecretory cells within the brain (El-Salhy *et al.* 1983). The present investigation was performed in order to see whether some of the GEP neurohormonal peptides occur also in the neurosecretory cells of the frontal ganglion of the tobacco hornworm.

### MATERIAL AND METHODS

About 350 adults of both sexes of the tobacco hornworm moth, *M. sexta* L., were used. The insects were

reared as described in detail elsewhere (Kramer *et al.*, 1977). The animals were anesthetized by cooling to 4°C and the frontal ganglion was dissected out under Bouin's fluid and then fixed in the same solution overnight. After paraffin embedding, the specimens were cut serially at 4  $\mu$ m.

The serially cut frontal ganglia were stained by haematoxylin-eosin to examine the histological structure, by acetaldehyde fuchsin to detect neurosecretory material (Buehner *et al.*, 1979), and by three silver stains (Sevier and Munger, 1965; Grimelius, 1968; Singh, 1964) which are known to selectively stain the GEP neuroendocrine cells (Grimelius and Wilander, 1980).

The peroxidase-antiperoxidase (PAP) method (Sternberger, 1979) was used to detect the GEP neurohormonal peptides. The antisera used were the same as those used previously (El-Salhy *et al.*, 1983). They included guinea-pig antiserum against porcine insulin No. 912 (a gift from L. Wide, Department of Clinical Chemistry, University Hospital, Uppsala, Sweden), guinea pig antisera against A-chain of bovine insulin No. Ma 47, and against B-chain of bovine insulin No. Ma 37 (both were gifts from P. Westermark, Department of Pathology, University of Uppsala, Sweden). These antisera were used at the dilution of 1:1000, 1:500, and 1:2000, respectively. The other antisera tested were anti-nerve growth factor, anti-epidermal

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growth factor, anti-somatostatin, anti-glucagon (eight antisera with different sequence specificities), anti-glicentin, anti-pancreatic polypeptide, anti-polypeptide YY, anti-secretin (three antisera), anti-VIP, anti-GIP (two antisera), anti-gastrin (two antisera; one specific for the C-terminus and the other for the sequence 6-13), anti-CCK (two antisera; one specific for the sequence 9-20 and the other for the sequence 19-25), anti-enkephalin, anti- $\alpha$ -endorphin, anti- $\beta$ -endorphin (two antisera), anti-substance P (two antisera), anti-neurotensin, anti-bombesin (three antisera), anti-motilin, anti-ACTH (three antisera), anti-serotonin (two antisera) and anti-calcitonin. Frontal ganglia from four to seven insects were utilized to test each antiserum. The controls used were essentially the same as those described previously (El-Salhy *et al.*, 1983). Briefly, alternate sections from the serially cut sections were treated with one of the negative controls (see below). Moreover, serially cut sections from one frontal ganglion were treated with one or the other of the negative controls described below and then immunostained by active antiserum (Duve and Thorpe, 1981). The negative controls included (a) replacing the first layer antiserum by normal rabbit or guinea pig serum, and (b) preincubating the antiserum with the corresponding peptide or related peptide(s) for 24 hr at 4°C, in case of obtaining positive immunostaining. In addition, the antisera were preincubated with 1:50 diluted rabbit anti-human C1q complement (Dako Copenhagen, Lot No. 03813) for 24 hr at 4°C when immunoreactive cells were seen (Buffa *et al.*, 1979). As positive controls, sections of mammalian tissues, known to contain the peptides under study, were included in each staining experiment.

## RESULTS

The histological structure of the frontal ganglion was the same as that described previously by Bell *et al.*, (1974). Briefly, the frontal ganglion is formed from a centrally located complicated network of nerves (neuropile) surrounded by large and small neurons. Despite the fact that no acetaldehyde fuchsin-positive cells were observed, acetaldehydefuchsinophil material was seen in the neuropile as well as in some nerve fibers in the recurrent nerve. All the silver stains tested were negative.

Insulin-immunoreactive cells were found to occur in the dorsal side of the frontal ganglion (Fig. 1). These cells amounted to 4-6 large, round cells with a diameter ranging between 30 and 40  $\mu\text{m}$ . Insulin-like

immunoreactive material was found in the neuropile and in some nerve fibers in the recurrent nerve. The insulin immunoreactivity was detected by anti-insulin B-chain serum (No. Ma 37), but not by anti-insulin No. 912 (specific for the insulin A-chain), or anti-insulin A-chain No. Ma 47. All the other antisera failed to reveal any immunoreactive cells or nerve fibers.

## Specificity Controls

No immunostaining was obtained when the anti-insulin B-chain was replaced by normal guinea pig serum. The anti-insulin B-chain was completely inactivated after the incubation with 100  $\mu\text{g}$  porcine insulin (Vitrum, Stockholm, Sweden, Lot No. 1800L) per milliliter diluted antiserum or of bovine insulin B-chain (Sigma Chem., St. Louis, Mo., U.S.A., Lot No. 39C-8005). Preincubation of the anti-insulin B-chain serum with 100  $\mu\text{g}$  bovine insulin A-chain (Boehringer, Mannheim, F.G.R., Lot No. 1040320) per milliliter diluted antiserum had no effect on the antiserum activity. The preincubation of the anti-insulin B-chain with anti-human C1q complement had no effect on the immunostaining obtained.

## DISCUSSION

Several GEP neurohormonal peptides have been reported to occur in the brain and other nervous tissues of insects (cf. El-Salhy, 1981; Hansen *et al.*, 1982; Falkmer and Van Noorden, 1983; Kramer, 1983). Among these GEP neurohormonal peptides insulin-like substance has been the most intensively studied. Insulin-like immunoreactive material has been observed in acid-ethanol extracts of the heads and whole bodies of several species of insects (Tager *et al.*, 1976; Duve *et al.*, 1976; Le Roith *et al.*, 1981). This substance has been reported to be similar to mammalian insulin in the molecular size, physicochemical properties, and biological activities (Tager *et al.*, 1976; Duve *et al.*, 1979; Kramer *et al.*, 1980, 1982; Le Roith *et al.*, 1981). The



FIG. 1. Insulin B-chain immunoreactive neurons in the frontal ganglion of the tobacco hornworm. Immunoreactive material can also be seen in the neuropile (arrow head) and in the recurrent nerve (arrow).  $\times 450$ .

cellular site of production of insulin-like substances has been localized in some of the median neurosecretory cells of the brain (Duve and Thorpe, 1979; El-Salhy *et al.*, 1980, 1983; Yui *et al.*, 1980) and in some of the lateral neurosecretory cells of *M. sexta* (El-Salhy *et al.*, 1983).

In the frontal ganglion of *M. sexta* two aldehyde fuchsinophil neurosecretory cells have been observed (Bell *et al.*, 1974) in fasted larvae and during pupal diapause, but not in fed larvae or adults. At the ultrastructural level, these neurosecretory cells have been found to contain electron-dense granules with a halo between the core and the limiting membrane (Borg *et al.*, 1973). The present observation that no aldehyde fuchsinophil cells could be detected in the frontal ganglion of adult forms of *M. sexta* is in line with the earlier findings of Bell *et al.* (1974). On the other hand, in the present study with the sensitive PAP procedure not only insulin-immunoreactive cells could be demonstrated in the adult *Manduca* frontal ganglion, but also the number of these cells

exceeded that observed previously by the aldehyde fuchsin method in fasted larvae and diapausing pupae (Bell *et al.*, 1974).

Similarly to the findings in the *Manduca* brain (El-Salhy *et al.*, 1983), the insulin-immunoreactivity in the frontal ganglion was detected by an antiserum specific to the B-chain. The sequence 22–26 of the insulin B-chain is said to represent the active (receptor-binding site) needed for the biological activities (Sabeson, 1980). It seems reasonable, therefore, to assume that the insulin B-chain is the most conserved part of the insulin molecule during the course of evolution.

The present observation of insulin-immunoreactive material in the neuropile and in the recurrent nerve suggests that this material may be transported to the corpora cardiaca (CC) via nervi cardiaco-somato-gastrici (a nerve emerges from the recurrent nerve to CC). In favour of this assumption is the finding that numerous insulin B-chain immunoreactive nerve fibres are located in the *Manduca* CC (El-Salhy *et al.*, 1983).

In conclusion, the present study showed another cellular site for the production of the insulin-like material detected by radioimmunoassay in the heads of *M. sexta* (Tager *et al.*, 1976). Moreover, it suggests a possible pathway of this material to CC.

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