Isolation and partial characterization of pancreatic polypeptide-like material in the brain of the blowfly *Calliphora vomitoria*

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Using 10^6 flies (5 kg of heads) a pancreatic polypeptide-like material has been partially purified from the blowfly *Calliphora vomitoria*. The isolated material was eluted on Sephadex G-50 similarly to bovine pancreatic polypeptide and had an R_F on polyacrylamide-gel electrophoresis that was identical with that of the bovine hormone. The material diluted linearly and showed parallelism with bovine standards in a bovine pancreatic polypeptide immunoassay. In specificity controls the immunoreactivity was not abolished by trasylol and no cross-reactivity was discerned in assays for glucagon, proangiotensin and cyclic AMP. These data suggest that the pancreatic polypeptide material in the brain of the blowfly has close structural similarity to the mammalian hormone.

Avian pancreatic polypeptide (chicken) was isolated and sequenced by Kimmel *et al.* (1975). Since then human, porcine, ovine and bovine pancreatic polypeptides have been purified and their primary structures determined (Floyd *et al.*, 1977). Avian pancreatic polypeptide and beef pancreatic polypeptide have homology at 16 out of the 36 amino acid residues, whereas the mammalian polypeptides differ by not more than 2–3 amino acids. Pancreatic polypeptide immunoreactive material has been tentatively shown by immunocytochemistry to occur in the brain of mammals (Loren *et al.*, 1979), which may imply that pancreatic polypeptide is one of the series of gastroenteropancreatic/brain peptides. Recent immunocytochemical studies have shown that pancreatic polypeptide-like immunoreactive material is present in the brain of several insect species: *Calliphora erythrocephala* (Duve & Thorpe, 1980); *Bombyx mori* (Yui *et al.*, 1980); *Eristalis aeneus* (El-Salhy *et al.*, 1980); and in the annelid *Lumbricus terrestris* (Sundler *et al.*, 1977).

The physiological role of pancreatic polypeptide in vertebrates (either pancreatic or neurally located) is equivocal, and in insects its function is unknown. The purification of pancreatic polypeptide-like material from *Calliphora* brain was initiated to obtain sufficient pure material for chemical characterization and physiological studies.

Experimental and results

Insects

Pupae (10^6) of *Calliphora vomitoria* were obtained in batches of 2×10^5 from the Blackwater Bait Co., Stone, Essex, U.K. After eclosion, flies were maintained on sucrose and water for 5–6 days. Heads were separated from bodies as previously described (Duve *et al.*, 1979) and were kept at -20°C before extraction. A total of 5 kg of fly heads were processed.

Tissue extraction

The extraction procedure conserved both insulin- and pancreatic polypeptide-like peptides. Work was carried out in an insulin- and pancreatic polypeptide-free laboratory and stringent precautions were taken to eliminate contamination by both insulin and mammalian pancreatic polypeptide. All glassware, columns and chemicals were new.

Heads were placed in a freezing mixture of ethanol and solid CO_2 and minced in a commercial meat-mincing machine (Bauknecht, Stuttgart, Germany). The minced heads (2.5 kg batches) were extracted overnight at 4°C in 16 litres of 65% ethanol acidified to pH 2.8 with conc. phosphoric acid. After centrifugation, the residue was extracted with a further 11 litres of acidified alcohol for 2 h.

The supernatants were combined and filtered through a Hyflo bed. The brown filtrate was evaporated at 26–30°C under vacuum to 2.2 litres by means of a large-scale cyclic still. The resulting solution was clarified by passage through another Hyflo bed.

Column chromatography

Gel filtration of the extract was performed on a Sephadex G-15 column (14 cm × 100 cm) eluted with

1M-acetic acid (15 ml/min) at 22°C. Fractions (495 ml) were collected and a conductivity and absorbance profile at 280 nm were performed. Duplicate samples (50 µl of each fraction were freeze-dried and tested for both insulin and pancreatic polypeptide immunoreactivity (Fig. 1a). Fractions 2–8 were pooled and freeze-dried, reconstituted in 1M-acetic acid and filtered on Sephadex G-50 superfine. Fractions containing the pancreatic polypeptide and insulin immunoreactivities (116–

Fig. 1. Filtration on Sephadex G-15 (F) (a) and Sephadex G-50 (SF) (b)

(a) shows filtration on a Sephadex G-15 (F) column (100 cm × 14 cm) of heads (10^6) of *Calliphora vomitoria*, extracted in acid alcohol (65% ethanol/ H_3PO_4) (flow rate, 15 ml/min; 495 ml fractions). Samples (50 µl) of each fraction were immunoassayed for pancreatic polypeptide and insulin. Protein absorbance at 280 nm (●) and conductivity (□) were measured. The histogram indicates insulin immunoreactivity. The pancreatic polypeptide immunoreactive profile (result not shown) was similar. (b) shows filtration on Sephadex G-50 (SF) column PA (100 cm × 14 cm) of reconstituted freeze-dried immunoreactive fractions (2–8) from Sephadex G-15 in (a) (flow rate, 15 ml/min; 75 ml fractions). Samples (50 µl) of each fraction were immunoassayed for pancreatic polypeptide and insulin. Protein absorbance at 280 nm (●) was measured. The histogram indicates pancreatic polypeptide (PP) immunoreactive fractions. Insulin immunoreactivity extends from fractions 116 to 142. The insets show linear dilution of both immunoreactive materials, in their respective immunoassays. The parallelism with their respective standards (■) shows identity of antigenic determinants. Abbreviation used: B/F, bound/free concentration.

143) were pooled and freeze-dried. Both the pancreatic polypeptide- and insulin-immunoreactive materials produced linear dilution curves that showed parallelism with the bovine standards (Fig. 1b). Total yield of protein containing immunoreactivity from 5 kg of fly heads was 960 mg of an off-white powder. The immunoreactive content of both pancreatic polypeptide- and insulin-like materials was approx. 60 ng/mg.

Immunoassays

Throughout the purification, a homologous bovine pancreatic polypeptide immunoassay employing charcoal separation was used to measure the fly pancreatic polypeptide-like material. Two different bovine pancreatic polypeptide antisera were used (Duve & Thorpe, 1980) and both gave similar cross-reactivity. Insulin immunoreactivity was assayed by using a double-antibody radioimmunoassay method with bovine insulin standards as described previously (Duve *et al.*, 1979).

To test for specificity of cross-reactivity with bovine insulin and pancreatic polypeptide antibodies immunoassays for cyclic AMP (New England Nuclear), proangiotensin (Travenol Labs. Inc., Cambridge, MA, U.S.A.) and glucagon (O'Connor & Lazarus, 1976) were carried out on the isolated material.

Separation of pancreatic polypeptide and insulin immunoreactivities

Efforts were not directed to separating the two immunoreactive species. In a pilot experiment freeze-dried material (20 mg) was chromatographed on a QAE-A25 Sephadex column (Fig. 2a). Pancreatic polypeptide immunoreactivity was eluted with 0.05 M-Tris, pH 9.0, and the insulin immunoreactivity was displaced with 60% ethanol in 0.1 M-HCl. Complete separation of the immunoreactive materials was also obtained on polyacrylamide-gel electrophoresis. Gels were prepared in glass columns (0.5 cm × 22 cm). The running gel contained 10% polyacrylamide and the spacer gel 4% acrylamide. The material (3 mg) was reconstituted in 400 μ l of 0.1 M-NH₄HCO₃ buffer, pH 8.0, and diluted 1:1 with 40% (w/v) sucrose before application to the gel. Gels were sliced in 2 mm sections and extracted overnight in 0.5 ml of 0.1 M-NH₄HCO₃ buffer, pH 8.0. Samples (25 μ l) of the eluate were immunoassayed for pancreatic polypeptide and insulin immunoreactivity.

The fly pancreatic polypeptide-like material and the fly insulin-like material electrophoresed similarly to bovine pancreatic polypeptide and bovine insulin standards (Fig. 2b). The pancreatic polypeptide-like material was well separated from insulin immunoreactivity (R_F 0.22 compared with R_F 0.74).

Fig. 2. QAE-Sephadex A25 chromatography (a) and polyacrylamide-gel electrophoresis (b)

(a) shows QAE-Sephadex A25 chromatography of 20 mg of freeze-dried immunoreactive material from Fig. 1(b) after desalting on Sephadex G-15 (K 50/100). Column dimensions 0.5 cm × 2 cm; 1 ml fractions were collected. Fractions 1–15 eluted with 0.05 M-Tris, pH 9.0; fractions 16–25 eluted with 60% ethanol/0.1 M-HCl (arrow). All fractions were immunoassayed for pancreatic polypeptide (PP) and insulin. Protein absorbance at 280 nm (●) was measured. Pancreatic polypeptide immunoreactive fractions are shown in the open histogram, and insulin immunoreactive fractions in the stippled histogram. (b) shows polyacrylamide-gel electrophoresis of 4 mg of freeze-dried immunoreactive material from Fig. 1(b) after desalting on Sephadex G-15 (K 50/100), reconstituted in 400 μ l of NH₄HCO₃ buffer and immunoassayed for insulin and pancreatic polypeptide (PP). The positions of standard bovine peptides are indicated by the arrows.

Discussion

Immunocytochemical studies have demonstrated the presence of various mammalian-like hormones in invertebrate tissues. These include gastrin (cf. Duve & Thorpe, 1981a), pancreatic polypeptide and insulin. However, identification of peptides by immunocytochemical techniques is of limited value in identifying the molecular species that is cross-reacting with the antibody. Identification by immunoassay is also not straightforward, on the basis of two recent reports. Thus Maruo *et al.* (1979) found that the apparent immunoreactivity of a human choriogonadotropin-like factor in the crab *Ovalipes ocellatus* was due to an interfering proteinase and Julliard *et al.* (1980) showed that an immunoglobulin G molecule contained some of the same antigenic determinants as β -endorphin. In both these studies the material under examination differed substantially from the substance that was ostensibly being studied. In the present experiments we have used several criteria to show the similarity of the hormone under study with its mammalian counterpart. Since we have characterized the insulin-like immunoreactive material in a previous publication (Duve *et al.*, 1979) we concentrate here on the pancreatic polypeptide-like material. The peptide is isolated from fly brain, where it was demonstrated by immunocytochemistry (Duve & Thorpe, 1980). It can be separated from the insulin-like material by QAE-Sephadex chromatography and by polyacrylamide-gel electrophoresis. The migration properties of the pancreatic polypeptide-like material are equivalent to those of the bovine species and they elute on Sephadex G-50 in the same position as a bovine marker. Trasylol did not inhibit the immunoreactivity, assays for cyclic AMP, proangiotensin and glucagon were all negative and insulin-like immunoreactivity was not shown by the separated pancreatic polypeptide-like material, all these observations being indicative that the immunoreactivity was specific. Furthermore, the material diluted

linearly and showed parallelism with bovine standards in the immunoassay.

It is of interest that both the mammalian-like hormones characterized exist in the brain of the blowfly. It is possible that the function of the pancreatic polypeptide-like material was originally linked to central-nervous-system regulation and only recently has the metabolic role of the hormone been developed. Some evidence for this is shown by the fact that we were unable to detect pancreatic polypeptide immunoreactivity in the haemolymph despite being able to measure the insulin-like material (Duve & Thorpe, 1981b). Further experiments are necessary to clarify the position.

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