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The Location of VIP in the Pancreas of Man and Rat

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Summary. VIP has powerful stimulatory effects on both endocrine and exocrine pancreas but its localisation within the gland has not been established. In this study, human pancreas was obtained fresh at surgery (eleven) or within four hours of death (seven). The pancreas was also removed from rats (twenty-two). Immunocytochemical staining showed VIP to be present in fine nerve fibres in all areas of the pancreas. Many fibres were seen in the exocrine pancreas, running between the acini, and around ducts and blood vessels. In addition, dense networks of fibres were observed forming meshes around islets and occasional ganglia were found containing immunoreactive cell bodies. In general, there were fewer VIP fibres in the rat pancreas than in the human, but overall distribution was identical. The mean VIP content of whole human pancreatic tissue was 42 ± 10 pmol/g wet weight (38 ± 9 pmol/g in head, 49 ± 6 pmol/g in body and 42 ± 11 pmol/g in tail). Whole rat pancreatic tissue contained 28 ± 7 pmol/g wet weight while preparations of isolated islets were found to contain 374 ± 30 pmol/g. It is possible that the heavy VIP innervation of the islets described here indicates a role in the regulation of islet hormone release.

Key words: Immunocytochemistry, radioimmunoassay, pancreas, gut hormones, vasoactive intestinal polypeptide.

Vasoactive intestinal polypeptide (VIP) is a basic, twenty-eight amino acid peptide, first isolated and purified by Said and Mutt in 1972 [1]. It has a widespread distribution throughout the body where it appears to be predominantly localised in nervous tissue [2, 3, 4]. In peripheral tissue VIP is found mostly in fine varicose nerve fibres, for example in the gastrointestinal tract [5], salivary glands [6] and genital tract [7, 8, 9, 10].

VIP has a range of biological activities including systemic vasodilation and the stimulation of gut motility and secretion [11]. It also influences both the endocrine and exocrine pancreas stimulating the release of somatostatin [12], glucagon [13, 14, 15] insulin [13, 14, 15] and pancreatic polypeptide [14] and the secretion of alkaline bicarbonate juice [14, 16].

In view of these potent effects, and the recent expansion [17, 18] of the earlier brief reports of the presence of VIP nerves in the human pancreas [2, 3] further, more detailed investigation seemed warranted.

A combined immunocytochemical and radioimmunoassay study was used to establish both the exact quantities of VIP present and the precise localisation of the peptide within the various pancreatic structures in man. In addition, the rat pancreas was examined because of the possibility of obtaining isolated islets for extraction.

Materials and Methods

Source of Tissue

Human. Eighteen fresh surgical specimens of pancreas were obtained during the removal of carcinomas of the exocrine pancreas (two) and APUDomas [19] (six insulinomas and one as yet unidentified). One normal pancreas was obtained during a therapeutic splenectomy and one "normal" specimen was taken from a patient suspected of having an endocrine tumour but in whom, on conventional histological examination, no tumour was found.

In addition, five whole non-diseased specimens were taken within four hours of death at post-mortem examination.

Rat. A total of twenty-two male and female Sprague-Dawley rats, weighing between 200–300 g was used. Whole pancreas was removed from twelve rats. In addition, islets were obtained from a further ten Sprague-Dawley rats (weight 200–250 g) [20]. The

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pancreas was taken and distended with bicarbonate buffered medium [21], chopped and treated with collagenase for 10–15 min to free the islets. The islets were then removed, using a fine pipette, and frozen in a small amount of buffered medium in batches of 200 for extraction.

Immunocytochemistry

The tissues were all fixed in p-benzoquinone, either in solution or in vapour form [22]. Care was taken to ensure that the pancreata studied were completely normal, on conventional histological examination.

Samples of pancreas, measuring no more than 0.5 × 0.5 × 0.5 cm, were fixed by immersion in 0.4% benzoquinone in phosphate buffered saline (PBS) 10 nmol/l, pH 7.1–7.4) for 2h at 4° C [23]. Fixation was followed by thorough rinsing in sucrose in PBS (0.2 mol/l). The fixed tissue was then mounted on cork mats and snap frozen in Arcton (Freon) pre-cooled (−145° C) in liquid nitrogen. Sections of 15 μ were cut in a cryostat at −20° C, mounted on slides previously coated with a solution of gelatin and formalin (1:1 v/v, 1% gelatin:2% formalin) and air-dried for at least 30 min. For fixation in benzoquinone vapour, samples of pancreas, no larger than 1 × 1 × 0.3 cm. were rapidly frozen in melting Arcton and freeze-dried overnight at −40° C. The tissue was exposed to benzoquinone vapour for three hours at 60° C and infiltrated with paraffin wax for 90 min [22]. Sections of 10 μ were cut and mounted on uncoated slides.

Immunocytochemistry was carried out by the use of indirect immunofluorescence and by the unlabelled antibody enzyme (peroxidase-anti-peroxidase or PAP) method [24]. For the indirect immunofluorescence technique, sections were incubated with VIP antiserum (dilution 1:300) for 16–20 h in a moist atmosphere at 4° C. A second layer of fluorescein conjugated goat anti-rabbit globulin was applied at a dilution of 1:150 for one hour at room temperature. Sections were mounted in PBS-glycerine (1:9 v/v PBS:glycerine) and examined under a Leitz ultra-violet microscope.

The PAP technique involved the pre-treatment of sections by immersion in 0.03% (v/v) hydrogen peroxide in PBS for 30 min to remove endogenous peroxidase activity. Possible background staining was also removed by the application of normal goat serum, dilution 1:30, for 30 min at room temperature. The VIP antiserum was used at a dilution of 1:1200 and incubation was for 16–20 h at 4° C. The second layer of unconjugated goat anti-rabbit globulin (Miles Laboratories) was used at a dilution of 1:50 for 30 min. The final layer consisted of PAP complex (UCB Laboratories), dilution 1:300, and incubation was again for 30 min at room temperature. Visualisation of the PAP complex was achieved by the diaminobenzidine method of Graham and Karnovsky [25]. When developed the sections were dehydrated, mounted in DPX and examined under a Leitz transmitted light microscope.

Controls were those proposed by Sternberger [24] including the use of VIP antiserum which had previously been incubated with excess VIP (0.1 nmol/ml of diluted antiserum). Non-immune rabbit serum was used as first layer and either the fluorescein conjugated globulin or the PAP complex was used alone.

A semi-quantitative assessment of VIP fibre populations in human and rat pancreas was carried out using an arbitrary counting method. This involved the counting of the points of intersection of VIP fibres and the lines on an eye-piece graticule which consisted of a grid of 100 equal sized squares. Five different fields were examined on two sections from four randomly chosen human and rat specimens. The sections were immunostained using the peroxidase-anti-peroxidase technique and examined under the × 25 objective of a Leitz transmitted light microscope. The results were analysed by the application of an unpaired Student's t-test.

Radioimmunoassay

For the whole pancreas material the tissues were kept deep frozen until the time of extraction. They were then weighed while still deep frozen and plunged into vigourously boiling water for 5 min, cooled, homogenised in 5 ml of water/g of tissue and then centrifuged at 2500 g for 10 min. The tissue pellet was then re-extracted in a similar manner using 4 ml of water/g wet weight and the two supernatants were pooled. The gross tissue pellets were then re-extracted a further two times with 5 ml and 4 ml/g of 0.1 mol/l formic acid. After final centrifugation, acid extracts were pooled and water and acid extracts were assayed separately [26].

Each batch of isolated islets was weighed whilst still deep frozen and then boiled for 2 min in 0.5 ml of water. After cooling the islets were homogenised in 0.5 ml of water and centrifuged for 10 min at 2500 g. The resulting supernatant was removed and saved and the tissue debris was re-extracted with a further 0.5 ml of water. Both water extracts were pooled for assay. The tissue debris was then re-extracted a further two times in 0.1 mmol/l0.1 mol/l formic acid under identical conditions as for the water extraction, and the extracts were pooled and assayed. The VIP antiserum was used at a final dilution of 1:320,000 to give 50% binding of 1 fmol ^{125}I-VIP in 800 ml after 3 days incubation at 4° C. Under these conditions the assay was sensitive to changes of 3 fmol/assay tube (95% confidence limit) using pure porcine VIP as standard.

Radioactive VIP label was prepared by a modification of the lacto-peroxidase method. A trace iodination technique was employed followed by high resolution ion exchange chromatography for purification of the monoiodinated VIP. The resulting ^{125}I-VIP label had a specific activity of between 1.6 and 1.0 mCi/mol and remained stable for up to three months at −20° C [26].

Statistical examination of the results was carried out by analysis of variance and by the application of the unpaired Student's t-test.

VIP Antisera

The VIP antisera for immuncytochemistry and radioimmunoassay were raised in New Zealand white rabbits against natural porcine VIP coupled to bovine serum albumin by the carbodiimide method [26].

The antisera reacted with C-terminal synthetic VIP fragments (but not N-terminal fragments) and showed no cross-reactivity with secretin, glucagon or gastric inhibitory peptide, which are structurally related to VIP, or with any of the known neuropeptides: bombesin [27], gastrin [28], cholecystokinin [29], neurotensin [30], somatostatin [31], substance P [32] and enkephalin [33].

Chromatography

For gel chromatography 2 ml of human pancreas extract was loaded on to a 1.5 × 90 cm Sephadex G50 superfine column precalibrated with dextran blue (Vo), cytochrome C, natural porcine VIP and Na^{125}I(Vt) as molecular size markers. The column was eluted with 0.1 mmol/l formic acid and, with the exception of porcine VIP, the above markers were included in each column load sample of pancreas extracts.

Results

Immunocytochemistry

In the human pancreas, immunocytochemistry, by both indirect immunofluorescence and the unlabelled antibody enzyme method, localised VIP to fine, vari-

cose nerve fibres which showed a widespread distribution throughout the organ. Fibres were seen in the exocrine pancreas, between acini (Fig. 1) and around ducts and blood vessels. The endocrine cells were found to have a particularly rich supply of VIP fibres which enmeshed the islets forming peri-insular networks (Fig. 2). This neuro-insular complex was a consistent finding in all the specimens. VIP fibres could also be seen to be in contact with, and sometimes appeared to terminate on, the pancreatic endocrine cells.

Occasional large nerves, containing parallel aligned VIP fibres, were observed and these were often in proximity to ganglia containing immunoreactive and non-immunoreactive cell bodies (Fig. 3). No appreciable differences were noted in the number and distribution of VIP fibres when comparative studies were carried out between (a) the three anatomical areas (head, body and tail) and (b) a normal non-tumourous pancreas and pancreas surrounding either carcinomas or any of the types of endocrine tumour. These subjective immunocytochemical findings were fully corroborated by the radioimmunoassay determination of the VIP content of the various specimens (Table 1).

A similar overall distribution of VIP fibres was seen in the rat pancreas although there appeared to be fewer fibres than in the human tissue. A semi-quantitative assessment of the immunocytochemical findings showed that the number of VIP fibres in the human pancreas was almost twice that in the rat pancreas. This difference was statistically significant ($p < 0.001$).

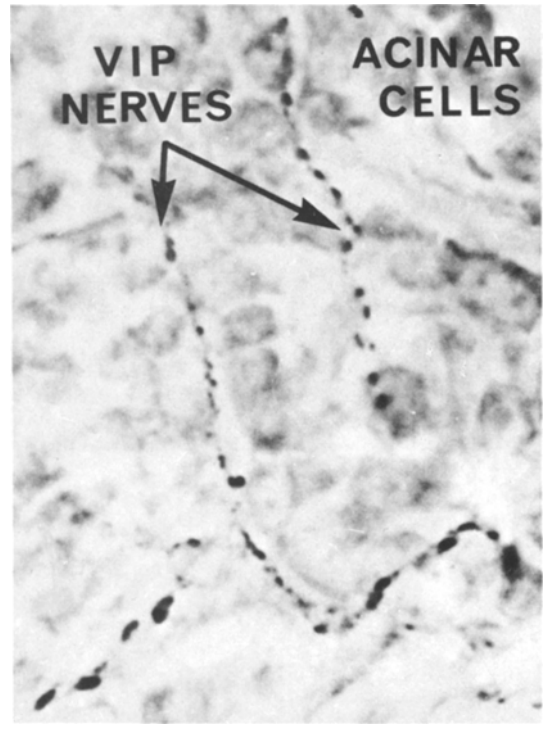

Fig. 1. VIP containing fibres, immunostained by the unlabelled antibody enzyme method (PAP), running through acinar tissue of the human pancreas. Tissue was fixed in p-benzoquinone and the 15 μ sections were not counterstained (× 650)

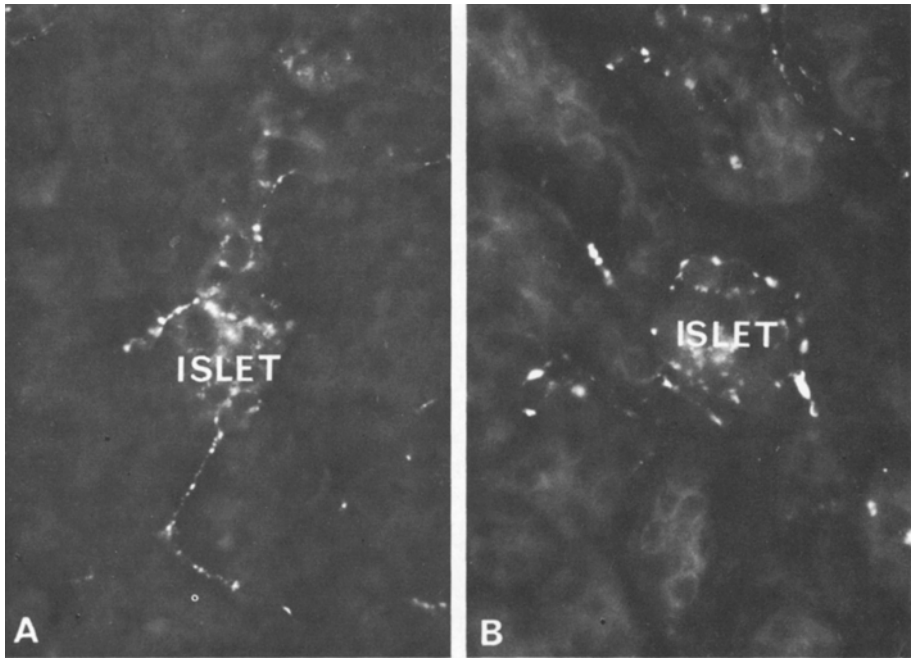

Fig. 2 A and B. VIP fibres, immunostained by the indirect immunofluorescence technique, in 15 μ sections of tissue fixed as for Figure 1. **A** Penetrating into an islet (× 300), **B** Surrounding and enmeshing an islet (× 420)

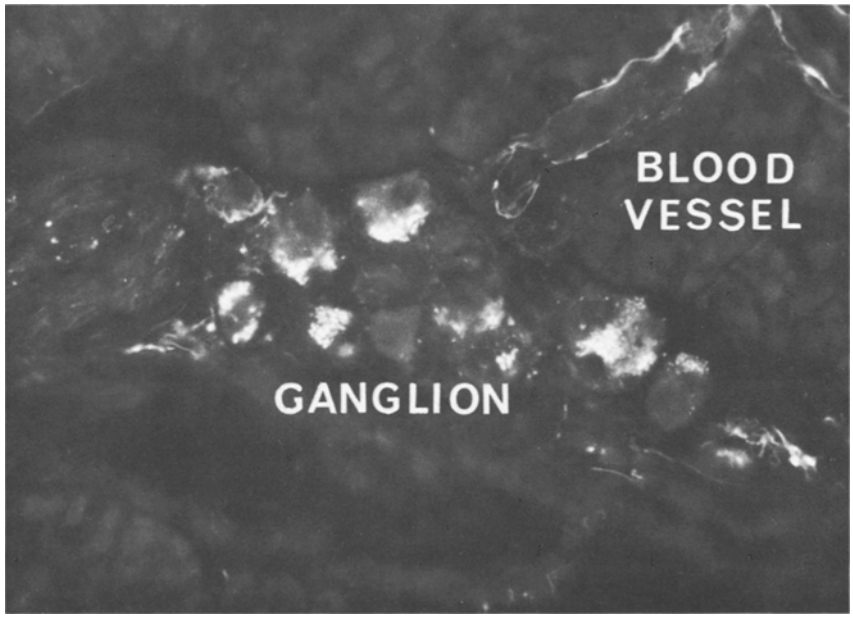

Fig. 3. Human pancreatic ganglion in 10 μ section of tissue fixed and immunostained as for Fig 2. VIP immunoreactive and non-immunoreactive ganglion cells and few VIP fibres can be seen within the ganglion. A blood vessel lies adjacent to the ganglion (× 430)

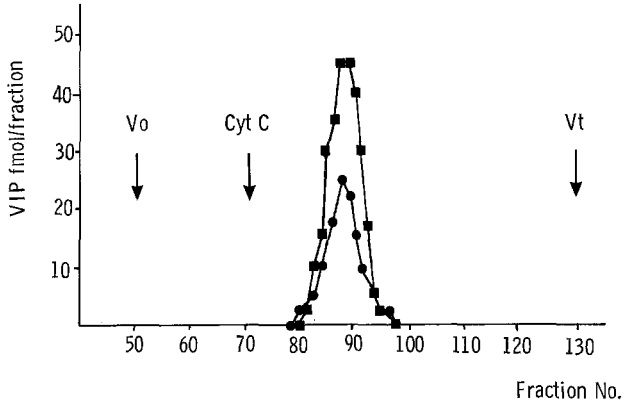

Fig. 4. Chromatography of VIP extracted from human pancreas (■—■) and porcine VIP standard (●—●)

Table 1. VIP content of human and rat pancreata. Numbers of samples shown in parentheses

Tissue	VIP content (pmol/g) wet weight ± SEM	
Human (18):		
Pancreas (exocrine and endocrine) – comprised of groups shown below)	41.6 ± 10	(18)
"Normal" pancreas surrounding APUDoma	56 ± 11	(7)
"Normal" pancreas surrounding carcinoma	38, 34	(2)
Normal surgical pancreas	29, 37	(2)
Post mortem: Head	38 ± 9	(7)
Body	49 ± 6	(7)
Tail	42 ± 11	(7)
Rat (22):		
Whole pancreas	28 ± 7	(12)
Isolated islets	374 ± 30	(10)

Radioimmunoassay (Table 1)

Determination of VIP content by radioimmunoassay of tissue extracts revealed that (a) similar values were obtained for each specimen of human pancreas and for each region (head, body and tail) examined. Analysis of variance showed there was no significant difference between the values. (b) Human pancreas contained approximately 50% more VIP than rat pancreas. This difference approached significance ($p < 0.1$).

Chromatography

In all cases (Fig. 4) a single major molecular form of VIP immunoreactivity was demonstrated in the chromatographs of human pancreatic extracts. This peak of VIP immunoreactivity eluted in an identical position to the standard natural porcine VIP used to calibrate the column, indicating considerable physico-chemical similarity between porcine intestinal and the human pancreatic VIP-like immunoreactivity.

Discussion

There appears to be a considerable quantity of VIP in the human and rat pancreas and this has been localised to fine, varicose nerve fibres. The VIP fibres were found to be in close contact with cells in both the exocrine and endocrine pancreas. As in other mammals, such as cat and pig [18] and dog [17], VIP immunoreactive ganglion cells were observed in the human pancreas. Thus, there may be an intrinsic sys-

tem of peptidergic nerves in the pancreas, analogous to that known to exist in the gut [34].

The existence of VIP containing cells has been reported in dog, guinea-pig and human islets [35]. However, the immunostaining was further found to be abolished by the prior addition of glucagon to the VIP antiserum [36].

Under the conditions of the present study, using a C-terminally directed antiserum which did not cross react with glucagon, no VIP containing cells were detected.

Hickson [38] first showed that stimulation of the vagus causes bicarbonate secretion from the pancreas and that this effect is unaltered by atropine, suggesting the presence of a non-cholinergic neural mechanism. VIP is known to cause pancreatic alkaline juice flow [14, 16] and can also be released by stimulation of the vagus [39, 40]. If VIP acts as a neurotransmitter, as has been widely suggested [41], then it is possible that the VIP fibres present in the acinar tissue are involved in the local control of exocrine pancreatic function. This postulate has been supported by the reported presence of specific receptors for VIP on the membranes of acinar cells [42].

Recently, evidence has accumulated for a role for VIP as an agent in the control of the endocrine, as well as exocrine, pancreas. Infusion of VIP into isolated dog pancreas releases somatostatin [12], insulin, glucagon and pancreatic polypeptide [15]. Similarly, perfusion of the isolated cat and pig pancreas with VIP has been shown to release both insulin and glucagon [13, 14]. It is of considerable interest that the amounts of VIP required to release these two hormones, between 1 and 50 nmol/l, are never attained in the circulation under normal physiological conditions. However it is possible that such concentrations could be reached locally by release of VIP directly on to the islets from peri-insular VIP fibres.

The unity between the structure and function of nerves and islets was first inferred by Van Campenhout when he referred to islets and adjacent autonomic nerve cell bodies as a "complex neuro-insulinaire" [43]. The original division of the autonomic nervous system into two components, cholinergic and adrenergic [44], has been challenged by the discovery of a considerable number and variety of peptidergic autonomic nerves. Recent immunocytochemical studies have localised VIP in some of the non-cholinergic, non-adrenergic autonomic fibres previously described as containing neurosecretory granules [45]. These nerves correspond to those in p(peptidergic)-type nerves, first described by Baumgarten et al. in 1970 [46]. Thus VIP nerves appear to be part of the autonomic nervous system and the finding of a high degree of VIP innervation of the islets further emphasises the close relationship between the autonomic nervous system and the endocrine pancreas.

The presence of VIP, a potent insulinotropic agent, in nerve fibres associated with the islets of Langerhans suggests, but provides no direct evidence for, a functional role. The investigation of the possible importance of the VIPergic innervation of the islet requires the development of new techniques to measure the local release of peptides from nerves or of specific VIPergic blocking agents to establish its significance particularly in the development of diabetes mellitus.

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