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Autonomic innervation of the pancreas in diabetic and non-diabetic rats. A new view on intramural sympathetic structural organization

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Abstract

Using histochemical and immunocytochemical methods the intramural neural tissue of the pancreas was investigated in non-diabetic and in alloxan-diabetic rats. It was demonstrated that the non-diabetic pancreas contains an average of 2.71 cells/mm³ tissue that react positive for activity of acetylcholinesterase and 2.38 cells/mm³ tissue that show monoamine oxidase activity. Both cholinergic and monoaminergic cells are found as solitary cells and in clusters of various sizes. All these cells are embedded in the exocrine tissue. Both histochemical methods revealed the presence of intra-insular fiber plexuses. Treatment with alloxan resulted in disappearance of intra-insular cholinergic and monoaminergic activity and also in a 68% reduction of the cholinergic cells and 54% of the monoaminergic cells in the diabetic pancreas. Application of immunocytochemical methods employing antibodies against norepinephrine and dopamine demonstrated the noradrenergic character of at least some of the monoaminergic cell groups. It is discussed how the present data and data from previous innervation studies provide evidence for an intramural ganglionic organization of the sympathetic innervation of the rat pancreas.

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Introduction

In the past decade a vast body of evidence has accumulated that the central nervous system exerts a powerful influence on glucose levels in the general circulation as a result of the nervous control of insulin and glucagon release. By a large number of investigations it was demonstrated that the release of pancreatic hormones can be manipulated by stimulation or lesioning of structures in the hypothalamus, medulla oblongata, and the sympathetic and parasympathetic branches of the autonomic nervous system [1,2,18,20]. As part of the analysis of the substrate that underlies the above mentioned physiological processes, we have recently investigated the preganglionic innervation of the endocrine pancreas by means of retrograde axonal transport of horseradish peroxidase (HRP) [22]. In these experiments we studied the labeling of cell bodies in the central nervous system after injection of HRP in the pancreas of non-diabetic and alloxan-induced diabetic rats. Retrogradely labeled preganglionic parasympathetic somata were demonstrated in the dorsal motor vagus and ambiguus nuclei of the lower medulla and in the ventral horns of segments 3 and 4 of the cervical spinal cord. As may be expected from the destructive effect of alloxan on the islet B-cells and their parasympathetic innervation [22,27], HRP applied to the alloxan-diabetic pancreas resulted in a considerably reduced number of labeled parasympathetic neurons.

In contrast to what might be expected in view of the classical sympathetic innervation of the pancreas, in the same HRP experiments a substantial labeling of preganglionic neurons was observed in the sympathetic areas of the intermediolateral and ventral horns of the thoracolumbar cord [22,24]. This finding implies, at least for a part, intramural contacts between the preganglionic sympathetic terminals and postganglionic somata and, consequently, the presence of intramural sympathetic, noradrenergic cell bodies within the pancreas. Transfer of HRP from cell bodies, over the synaptic cleft to presynaptic terminal boutons has as yet never been observed nor described. The presence of intramural postganglionic sympathetic cells is considered normal for the innervation of the adrenal medulla [14] and has also been demonstrated for the sympathetic innervation of the urinary bladder and urethra [15,24], but has not been reported before for pancreatic innervation.

Assuming the noradrenergic character of sympathetic postganglionic neurons and the cholinergic nature of parasympathetic postganglionic neurons, the pancreas tissue of the rat was investigated with histochemical methods. The occurrence of monoaminergic sympathetic somata was tested with monoamine oxidase (MAO) histochemistry, whereas cholinergic cell groups were determined by the presence of acetylcholinesterase (AChE) activity. With respect to the latter method it should be born in mind that also non-cholinergic cells may display AChE activity [5,11]. Since the monoamine degrading enzyme MAO is likely to be present in relatively high concentrations both in noradrenergic and dopaminergic cells, pancreatic tissue was treated in several cases with specific antibodies against norepinephrine or dopamine [12]. Application of histofluorescent procedures, as were performed in the initial stages of this investigation, provided extensive information on the distribution and morphology of noradrenergic nerve fibers [7,8], but not on the occurrence of cell bodies.

Furthermore, to determine the effects of alloxan treatment on intramural autonomic innervation of the pancreas, all procedures were applied to both normal and alloxan-induced diabetic pancreas tissue.

Materials and Methods

In the initial phase of this study various histochemical procedures were performed on the pancreatic tissue of 15 male albino Wistar rats weighing approx. 300 g. The aims of these experiments were (1) to develop a highly reproducible histochemical procedure for optimal visualization of monoaminergic and cholinergic somata and (2) to select an area of pancreatic tissue that can be considered as representative for the entire organ. The procedure for acetylcholinesterase activity as described by Coupland [4] was found to result in a precipitate in peripheral visceral tissues which was best suited for analysis. Cholinergic somata appeared as dark brown-black dots, whereas fibers could be observed in their gross morphological appearance against a faint brownish background. Monoaminergic innervation was studied with the MAO procedure of Glenner et al. [13,26] and by the histofluorescence technique of Falck-Hillarp [8]. The MAO procedure proved to be the most appropriate for demonstration of monoaminergic somata. In this material positively reacting somata stand out as clearly blue cells against minor background precipitate, but, however, with only faint staining of monoaminergic neurites. The Falck-Hillarp procedures on the other hand appeared to be powerful techniques to show fiber morphology, but failed to reveal a reliable image of somata. Furthermore, the Falck-Hillarp procedures demand a tissue fixation regime, which does not permit optimal cholinergic staining in the same material.

With respect to the selection of a representative part of pancreatic tissue we have compared the results in various parts of the dorsal and ventral lobes of the pancreas. The impression was gained that there is a rather even distribution of nerve cells over the entire organ. Based on that observation 3 parts were selected in the ventral lobe embedded in the first duodenal loop, which are indicated by a-c in Fig. 1. Quantitative analysis of AChE-positive cells was carried out on part 'a', whereas the MAO procedures were performed on part 'b'. In the initial stage of this study histofluorescence procedures were applied to area 'c'. These latter data, however, were not considered in the quantitative analysis.

In the final stages of this investigation an attempt was made to define the transmitter nature of the MAO-positive cells. For that purpose the pancreas of several animals were studied immunocytochemically (ICC) with antibodies against norepinephrine (NE) or dopamine (DA).

Induction of diabetes. To induce diabetes by destruction of the endocrine pancreatic B cells, 10 animals were starved for 24 h prior to an i.p. injection of alloxan in distilled water (15 mg alloxan per 100 g b.wt.), followed by another 24 h of food deprivation. Urine production and urine glucose concentrations were measured daily. Animals were considered diabetic at a urine production of 100 ml/24 h with a glucose concentration of at least 0.5%. To obtain complete destruction of B-cells and

Fig. 1. Drawing indicating the stomach and first duodenal loop and the position of the pancreas in a ventral view. The ventral lobe of the pancreas that follows the duodenal curve is divided in 3 parts (a-c) referring to the 3 pancreatic sections treated according to the various procedures described in the text.

their innervation, the animals received a subcutaneous injection of 6 IU of protamin zinc insulin (Organon) every other day during a survival period of 6 weeks.

Histochemical procedures. Both diabetic and non-diabetic animals were transcardially perfused with warm (37°C) saline containing 10 IU heparin/ml. In those cases in which part of the pancreas was treated for histofluorescence the perfusate was cooled to 0°C. Tissue treated for AChE activity was cut in 40 μ m sections on a cryostat microtome. Sections were thaw-mounted on gelatin-coated slides and incubated for 16 h at 37°C in a buffered copper-glycine solution (pH = 5.0) containing 1.53 mg/ml acetylcholineiodide (Sigma) and 0.07 mg/ml ethopropazine. After incubation sections were treated successively for one minute each with sodium sulphide and silver nitrate.

For the MAO procedure 40 μ m sections were thaw-mounted and pre-incubated for 1 h at 37°C in buffered (pH = 8.0) sodium sulphate solution (0.3% w/v), followed by an incubation for 45–60 min at 37°C in a buffered (pH = 7.6) solution containing 5 mg/ml 5-hydroxytryptamine (Sigma) and 0.5 mg/ml nitrobluetetrazolium. Both MAO and AChE sections were then fixed in formaldehyde, dehydrated and coverslipped.

Tissue treated for histofluorescence was frozen immediately after perfusion and also sectioned by the cryostat microtome. Sections were mounted on slides, held at -20°C, then vacuum-dried with phosphopentoxide, treated with paraformaldehyde at 60% relative humidity for 1 h at 80°C and coverslipped. The sections were studied with a Zeiss fluorescence microscope with excitation between 390–410 nm. Since the histofluorescent material only provides data on the morphology of fibers as has been described by others [7] these data will not be further discussed.

TABLE I

Numbers of cholinergic and noradrenergic somata per mm³ of pancreatic tissue in non-diabetic and in alloxan-induced diabetic animals

—, determination unsuccessful for technical reasons.

Non-diabetic			Diabetic		
Case	AChE - positive cells/mm ³	MAO - positive cells/mm ³	Case	AChE - positive cells/mm ³	MAO - positive cells/mm ³
15	—	2.17	42	—	0.88
19	2.06	2.55	43	1.62	1.15
23	2.52	2.55	45	0.76	1.04
24	2.90	2.48	46	0.78	0.77
25	—	1.83	49	0.37	0.62
29	1.49	2.74	52	0.72	0.70
34	3.67	2.46	53	0.99	2.19
35	3.62	2.28	54	—	1.39
Mean	2.71	2.38	Mean	0.87	1.09
S.E.M.	0.35	0.10	S.E.M.	0.17	0.18

Immunocytochemistry. Immunocytochemical demonstration of norepinephrine and dopamine was carried out on two animals. They were transcardially perfused with a buffered 5% glutaraldehyde solution under high pressure. Both brain and pancreas were dissected, immersed in 20% sucrose and cut at 15 μ m sections on a cryostat microtome. The sections were thaw-mounted on gelatin-coated slides and incubated overnight at 4°C with 1:3000 rabbit-anti-NE or rabbit-anti-DA, kindly donated by Dr. M. Greffard from Lab. Biochem. Cell., Bordeaux, France. The sections were then incubated for 2 h in goat-anti-rabbit IgG, followed by 1 h in rabbit peroxidase-anti-peroxidase. In all incubations and rinses the solvent was made up of 0.05 M Tris buffer (pH = 7.6), 0.9% sodium chloride and 0.5% triton X-100. The peroxidase complex was visualized in a diaminobenzidine (DAB) solu-

Fig. 2. Histograms with numbers of cells/mm³ reacting positively for acetylcholinesterase (ACh) and monoamine oxidase (NE) in normal and diabetic pancreas tissue.

tion (40 mg/100 ml Tris buffer, pH = 7.4) containing 0.9 ml H₂O₂ 1.5% for 10–30 min. After staining the sections were dehydrated and coverslipped.

Quantitative analysis In the tissue parts studied, somata that reacted positively in the AChE or MAO procedures were counted in subsequent sections and compared for diabetic and non-diabetic cases. The numbers obtained were corrected for double-counting by application of the formula of Konigsmark [21]. The surface area and volume of all sections were measured and determined with the aid of an IBM morphometric program. For comparison all cell numbers were expressed as the number of cells per mm³ of pancreatic tissue (Table I) (Fig. 2).

Results

Cholinergic pancreas innervation in non-diabetic and diabetic animals. In the non-diabetic pancreas positively reacting somata and fibers of cholinergic nerve cells can be discerned. AChE-positive somata with a measured average diameter of 27 μ m either appear as single solitary cells or in clusters of up to 12 cells. As has been depicted in the cluster frequency graph (Fig. 3), solitary cells and small clusters of 2–3 cells were by far the most common. The frequency of larger cholinergic neuron clusters becomes smaller. Very large cell clusters are rare and never contain more than 12 cells. All cholinergic somata are embedded in the connective tissue of the exocrine pancreas or in the connective tissue that accompanies the vasculature or the ductal system. Fragments of networks can be observed all over the exocrine pancreas, which is quite different from the conspicuous morphology of the islet

Fig. 3. Occurrence of cholinergic and noradrenergic neurons in normal (open) and diabetic (stippled) columns expressed in frequency of various cluster sizes.

innervating terminal fibers (see ref. 4 for details). The staining does not permit the visualization of fine terminal structures, but shows a rather diffuse precipitate around the presumed B-cells that occupy the islet core. In several cases connections were observed between the islet innervating networks and nearby situated perikarya.

The mean number of AChE-positive somata in the pancreas of non-diabetic animals was determined as 2.71 cells/mm³ tissue (S.E.M. = 0.35; n = 6). In diabetic cases there was a dramatic change in the cholinergic innervation of the pancreas, as can be concluded from both the reduction of cholinergic somata and the fiber pattern. In contrast to normal animals there was a complete lack of staining product in the islets of Langerhans. On the other hand, the fiber innervation of the exocrine tissue did not appear to be seriously affected. The effect of alloxan on cholinergic innervation is most clearly demonstrated by the impressive decrease of AChE-positive somata, which resulted in a mean number of 0.87 cells/mm³ (S.E.M. = 0.17; n = 6) in the diabetic pancreas. This implicates a reduction of cholinergic cells to 32% as compared to the non-diabetic animals (Fig. 4).

Fig. 4. Series of photomicrographs illustrating acetylcholinesterase precipitates in pancreatic tissue. a: solitary AChE-positive neuron in diabetic pancreas. Scale bar = 50 μ m. b: survey picture of cholinergic innervation of several islets of Langerhans and in the exocrine tissue of a normal pancreas. Scale bar = 450 μ m. c: detail of soma adjacent to the islet. Bar = 50 μ m. d: slightly larger magnification of AChE precipitate in an islet of Langerhans. Normal pancreas. Scale bar = 250 μ m.

Monoaminergic pancreas innervation in non-diabetic and diabetic animals. The occurrence of MAO-positive somata in the pancreatic tissue may be regarded as the most striking finding in the present investigation. In non-diabetic animals we counted an average of 2.38 monoaminergic cell bodies/mm³ tissue with a relatively low standard deviation (S.E.M. of 0.10) (Table I). The MAO-positive cells appeared as clear blue, oval-shaped somata with an average diameter of 23.4 μ m. Comparing the numbers of MAO-positive cells with cholinergic cells it is obvious that both types of cells occur in the same quantitative range. The number of MAO cells occurring solitary or in small clusters, however, is much smaller. The frequency of larger clusters is higher than is the case with the cholinergic somata (Fig. 3).

The visualization of fiber morphology in the MAO-stained material is far less outstanding than in the AChE-stained sections. Larger fiber bundles can be observed as faintly blue granular precipitates. A comparable staining occurs in the islets of Langerhans, which is slightly stronger than the more evenly blue staining precipitate in the exocrine tissue (Fig. 5).

Fig. 5. a, b and d are photomicrographs of monoamine oxidase-positive cells in normal (a, d) and alloxan-diabetic (b) pancreas tissue. The MAO-positive cell may appear as solitary (d), or in small-clustered (a) or large-clustered ganglion like configurations. c: photograph of faintly staining MAO-positive islets of Langerhans in the non-diabetic pancreas. Scale bar in a and d = 50 μ m; bar in b = 100 μ m, in c = 300 μ m.

In the diabetic cases the following changes occur. Most striking is the disappearance of the MAO-positive intra-insular fiber networks as a result of the alloxan treatment. The induction of diabetes by alloxan also has a very strong effect on the number of MAO-positive somata and the cell cluster size. In the group of alloxan-diabetic animals the mean number of monoaminergic cells was decreased to 1.09 cells/mm³ (S.E.M. = 0.18) which is a reduction to 54% of the number of monoaminergic cells. It is obvious that alloxan treatment affected both smaller and larger clusters to approximately the same extent.

Immunocytochemistry (ICC). An important question that remains to be answered is the nature of positively reacting somata in the MAO-stained material. In the MAO procedure a chemical reaction indicated the presence of the enzyme monoamineoxidase, which is present in relatively high concentrations in both dopaminergic and noradrenergic neurons. In order to discriminate between DA and NE somata, pancreatic tissue was treated with specific antibodies against dopamine or norepinephrine that have recently become available [12]. The major result of the ICC procedures with anti-DA and anti-NE was the demonstration of labeled somata in the sections treated with anti-NE. The anti-DA-treated material did not show stained perykarya but presented evidence of positively reacting fibers apparently of extramural origin.

Discussion

By application of histochemical procedures it was demonstrated that the rat pancreas contains considerable amounts of cells that react either MAO- or AChE-positive.

Especially the occurrence of MAO-positive somata, which by application of immunocytochemical methods were shown to be noradrenergic in nature, is a striking finding that is in contrast to the classical concept of sympathetic autonomic organization (Fig. 6). In this view, the preganglionic cholinergic cells [23] synapse in the extramural, celiac ganglion. From there the noradrenergic postganglionic fibers penetrate the organs to reach their cellular targets. The present data suggest that, apart from the above described sympathetic circuit, another organization of sympathetic innervation, that at least may exist parallelly, bears a strong resemblance to the parasympathetic innervation. These findings are consistent with our previously described data on labeling of sympathetic preganglionics in the intermediolateral column of the thoracic spinal cord after HRP application to the pancreas [22].

There was a dramatic decrease in numbers of AChE-positive somata in the diabetic pancreas, as might have been expected from the decrease of labeled, parasympathetic neurons after HRP injections in alloxan-induced diabetic animals. This decrease of postganglionic cells, however, was much more than proportional as compared to the decrease of parasympathetic preganglionic cells. Previously we calculated that alloxan treatment leads to an overall loss in labeling of 27% of the parasympathetic preganglionic neurons. The same alloxan treatment, however, results in a decrease of 68% of AChE-positive cells. Alloxan treatment also had a

Fig. 6. a and b: two photomicrographs of groups of cells that react positively with antibodies against norepinephrine. PAP procedure. Scale bar = 50 μ m.

profound effect on the occurrence of MAO-positive cells, namely a reduction of 54%. Although this reduction is not as large as the decrease of cholinergic somata, it does not match the data obtained with HRP injection in the normal and alloxan-diabetic pancreas. In these tracing experiments we were unable to establish any significant negative effect of alloxan treatment on the amount of preganglionic, sympathetic neurons as determined by retrogradely transported HRP. This discrepancy between reductions of preganglionic and postganglionic cells after treatment with the B-cell toxin alloxan may be explained in various ways. One explanation is that only part of the intramural AChE- and MAO-positive cell groups function as postganglionic autonomic neurons being connected with CNS preganglionic cell populations. Speculating on the character of such cells not directly related to the CNS, one might think of nerve cells analogous and homologous to the enteric nervous system which is considered as the third division of the autonomic nervous system [10,11]. In this respect we might also point out the possibility that part of the MAO-positive cells may be serotonergic in nature, although we were able to demonstrate a substantial quantity of noradrenergic cells by means of ICC methods. Evidence exists, however, that serotonin-containing cells and fibers are involved in the innervation of pancreatic tissue [19,20]. Adrenergic cell bodies in the enteric plexus of the proximal colon were previously demonstrated by Furness and Costa [9] who also partly favour a sympathetic origin of those neurons.

A major consequence of intramural sympathetic postganglionic organization, as suggested by the present data, refers to the interpretation of *in vitro* studies of pancreatic function. A frequently used model in such studies is the *in vitro* perfusion of the isolated pancreas [2,17,28]. In case of a sympathetic intramural ganglionic organization one should take into account the possibility that cholinergic stimulation in such a pancreas preparation not only leads to parasympathetic insulin release, but may induce a purely sympathetic glucagon secretion as well. The latter phenomenon, commonly observed in experimental set ups, has thus far been attributed to parasympathetic rather than to orthosympathetic mechanisms [17,28].

An unequivocal conclusion that may be drawn concerning the effect of alloxan on the pancreas is that this toxin not only results in a total destruction of the insulin

producing B-cell [3], but also has a powerful effect on the innervation of the islets of Langerhans. It was a striking feature of our material that in the alloxan-diabetic pancreas the islet tissue was completely devoid of AChE-positive and MAO-positive fiber precipitates, whereas the fiber morphology in the exocrine tissue appeared to be largely unaffected. Consequently, although treatment with alloxan — and reportedly also with streptozotocin — is an effective method to destroy the B-cells of the endocrine pancreas [3,16,27], one should take into account a larger damage than is usually found in spontaneously or genetically diabetic animals [7].

In summary, it may be concluded that the rat pancreas contains noradrenergic ganglionic cell groups as demonstrated by (immuno-) histochemical methods. This occurrence of NE-containing cells points out an intramural sympathetic organization of the pancreas, which was already suggested by labeling of sympathetic areas in the spinal cord after intrapancreatic injections of HRP. Currently, experiments employing anterograde, intra-axonal tracer techniques [29] are being carried out to reveal direct nervous connections from sympathetic preganglionic cell groups to the pancreas.

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