

Immunohistochemistry of β -neoendorphin and dynorphin in the endocrine pancreas of rat and man*

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Summary. Serial sections from araldite-embedded rat and man pancreata were investigated immunohistochemically for the presence of prodynorphin-related peptides and α -endorphin. Immunoreactivities were visualized by the avidin/biotin-peroxidase complex (ABC) technique.

In the human pancreas, none of the endocrine cells could be immunostained for prodynorphin-, proopiomelanocortin-related peptides and enkephalins. In the rat pancreas, however, all glucagon cells exhibited immunoreactivities for both β -neoendorphin and dynorphin A. In addition, these cells contain α -endorphin-like immunoreactivity but no immunoreactivities for corticotropin, melanotropin, 16 K-fragment, α -N-acetyl- α -endorphin and enkephalins.

All specificity controls confirmed that the rat endocrine pancreas might be an other source of dynorphin and endorphin with a biosynthetic pathway different from that in the pituitary or in other locations. However, concerning synthesis or degradation of peptide precursor substances interspecies differences may exist.

Introduction

Since the discovery of Met- and Leu-enkephalin (Hughes et al. 1975), many other opioid peptides have been isolated from various organs. These are derived from three different prohormones: 1) proopiomelanocortin, containing the sequences for ACTH, MSH, 16 K-fragment and endorphin (Nakanishi et al. 1979); 2) proenkephalin, the common precursor of both Met- and Leu-enkephalin (Gubler et al. 1982; Noda et al. 1982); 3) prodynorphin, which contains three main fragments, i.e. α -neoendorphin, dynorphin A and dynorphin B which all have a Leu-enkephalin sequence at their amino-terminal extension (Kakidani et al. 1982).

The identified fragments of these prohormones are widely distributed in the brain and in a variety of peripheral organs. Within the endocrine pancreas immunoreactivities for endorphin, ACTH, 16 K-fragment, enkephalin and the molluscan cardioexcitatory peptide FMRF-NH₂ have been demonstrated by immunohistochemical methods (Forssmann et al. 1977; Larsson et al. 1977; Grube et al. 1978; Grube and Weber 1979; Sorenson et al. 1984). Moreover, biochemical studies localized endorphin, endorphin-like po-

lypeptides and high-molecular enkephalin containing polypeptides in the pancreas (Bruni et al. 1979; Houck et al. 1981; Smyth and Zakarian 1982; Stern et al. 1982). Experimental investigations have shown that opioid peptides may be involved in the function of the endocrine pancreas (Ipp et al. 1978; Green et al. 1983; Schusdziarra et al. 1984).

We now report on immunohistochemical findings suggesting that in the pancreas of rat α -endorphin and prodynorphin-derived peptides coexist in the glucagon immunoreactive cells.

Materials and methods

Peptides. Synthetic β -neoendorphin (code 8727), synthetic porcine dynorphin A (code 8730), synthetic human β -endorphin (code 8616), synthetic human α -N-acetyl- β -endorphin (code 8640) and synthetic β -LPH 61–69 (code 8632) were obtained commercially from Peninsula Lab., Inc. (San Carlos, USA). Synthetic glucagon was purchased from Serva (Heidelberg, FRG).

Antisera. Antisera against pancreatic hormones: anti-pork insulin guinea-pig serum LAA and anti-pork glucagon rabbit serum K 4023 were commercially obtained from Novo (Denmark). An antiserum against somatostatin was kindly supplied by Dr. Etzrodt (Ulm, FRG).

The antisera against various fragments of opioid peptide prohormones and their specificities are listed up in Table 1. The avidin/biotin-peroxidase complex (ABC-) kit for immunostaining was commercially obtained from Vector Lab. (Burlingame, USA).

Tissue preparation. Small specimen from surgically removed human pancreata and from pancreata of normal Wistar rats were snap-frozen in liquid Freon 22, freeze-dried and fixed by vapor-phase paraformaldehyde and embedded in araldite. Serial semithin sections were cut at 0.5 μ m and mounted on microscope slides by heat (90 °C for 30 min); subsequently the resin was removed with sodium methoxide (Mayor et al. 1961). Rat pituitary and bovine adrenal medulla which had been prepared in the same way served as reference organs for the antisera used during the immunohistochemical procedure.

Immunohistochemical staining. After rehydration, the sections were incubated in 2% non-immune goat serum for 30 min and rinsed in 0.01 M phosphate-buffered 0.15 M saline (PBS) pH 7.3 (the same buffer was used as diluent for the antisera and as rinsing solution during the immunohistochemical procedure). Subsequently adjacent sections were immunostained for various opioid peptides and for the "established" pancreatic hormones by the ABC-technique (Hsu et al. 1981).

Incubation steps were as follows:

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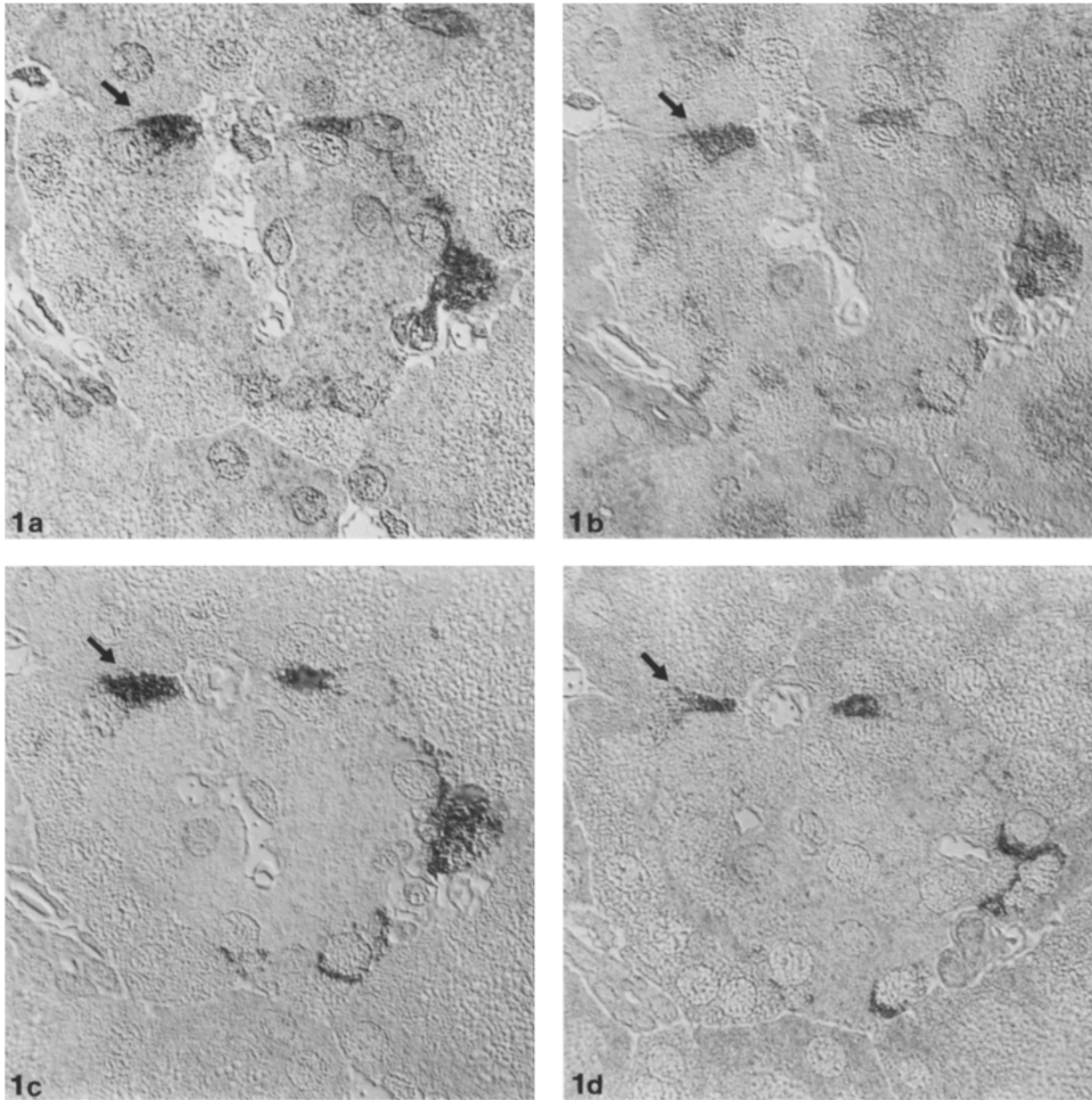


Fig. 1a–d. Four serial semithin sections (0.5 µm) from the rat pancreas, immunostained (ABC method) for dynorphin A (**1a**), β-neoendorphin (**1b**), glucagon (**1c**), and for endorphin (**1d**). An immunoreactive cell, present in all four sections, is indicated by an arrow. Interference contrast (according to Nomarski). × 500

1) hormone antisera, diluted 1:1000–1:6000 with PBS, for 24 h at 4 °C; 2) biotin-labelled anti-rabbit IgG, diluted 1:100 with PBS, for 30 min at room temperature; 3) avidin/biotin-peroxidase complex, diluted 1:50 with PBS, for 30 min at room temperature. This complex was prepared 30 min before incubation by mixing avidin and biotin-labelled peroxidase (1:1/vol) and diluted 1:50 for incubation. After each step the sections were washed with PBS for 10 min. Staining for peroxidase was performed using 0.0125% diaminobenzidine-HCl and 0.002% H₂O₂ in 0.05 M Tris-HCl buffer pH 7.6.

The sections were examined in a Reichert-Jung Polyvar Microscope equipped with phase-contrast and interference-contrast optics.

Specificity controls. The method-specificity of the immunostaining was tested as recommended in the literature (Sternberger 1979; Grube 1980; Grube and Weber 1980):

- omission of single steps in the immunohistochemical protocol;
- use of ascending dilutions of the peptide antisera;
- use of PBS containing 0.5 M NaCl as rinsing solution to exclude binding of antibodies by non-immunologic mechanisms.

The results of these controls showed that immunostaining by the ABC-technique as performed in the present study did not interfere with method non-specificities.

The antibody-specificities were tested by preadsorption of the antisera with homologous and heterologous peptides (10–100 µg/ml) for 24 h at 4 °C. The adsorption tests were performed with the working dilution of the antisera (see Table 1). Examinations of reference organs in these tests served as supplementary control.

Results

All antisera against the main pancreatic hormones, including insulin, glucagon and somatostatin, immunostained the

Table 1. List of antibodies and their specificities

Antibodies against	Origin	Antigens recognized	Antigens not recognized	Working dilution
DYN 1-17 (DYN A)	Dr. Weber, Stanford	DYN 1-13, DYN 1-17	DYN 6-13, α -neoEND, ENDS, ENKs	1:1000
DYN 1-13	Dr. Seizinger, Munich	DYN 1-8, DYN 1-13	DYN 1-6, ENKs, β -END	1:1000
DYN 1-8	Dr. Weber, Stanford	DYN 1-8	DYN 1-17, neoENDs, Leu-ENK	1:2000
β -neoEND	Dr. Seizinger, Munich	α -neoEND, β -neoEND	DYNs, ENKs, β -END	1:1000
Leu-ENK	Dr. Weber, Stanford	Leu-ENK	Met-ENK, DYNs, ENDS	1:1000
Met-ENK	Dr. Weber, Stanford	Met-ENK, Leu-ENK	DYNs, ENDS	1:1000
α -END	Drs. Eipper & Mains, Denver	β -LPH, β -LPH 61-69, POMC, α -, β -, γ -END, α -N-acetyl- β -END	ENKs, DYNs, neoENDs, α -MSH, ACTH 1-39	1:2000
ACTH	Dr. Voigt, Berlin	ACTH 1-24, ACTH 1-39, POMC	α -MSH, ACTH 19-39, ACTH 25-39, β -END	1:2000
α -MSH	Dr. Voigt, Berlin	α -MSH, ACTH 1-13	ACTH 1-10, ACTH 1-39, ACTH 18-39, POMC, β -END	1:6000
16 K-fragm.	Drs. Eipper & Mains, Denver	16 K-fragment, POMC	other POMC-fragments, ENKs, DYNs, ENDS	1:2000
α -N-ac- α -END	Dr. Weber, Stanford	α -N-acetyl-ENDs	desacetyl-ENDs	1:1000

Abbreviations: POMC = proopiomelanocortin; ACTH = corticotropin; MSH = melanotropin; LPH = lipotropin; END = endorphin; DYN = dynorphin; ENK = enkephalin; GLU = glucagon

corresponding endocrine cell populations in human and rat pancreata.

In the human pancreas none of the endocrine cells exhibited immunoreactivities for proopiomelanocortin-, pro-enkephalin- or prodynorphin-derived peptides.

However, in the rat pancreas a subpopulation of endocrine cells showed immunoreactivities for dynorphin A and β -neoendorphin. These cells proved to be glucagon (A-) cells as revealed by sequential staining of consecutive sections by glucagon antiserum (Fig. 1a, b, c; Fig. 2a, b). A-cells lacked immunoreactivities towards shorter dynorphin-related peptides like dynorphin 1-8 or dynorphin 1-13. Likewise, neither A-cells nor other endocrine cells showed immunoreactivities towards Met- and Leu-enkephalin. – Out of the antisera against various fragments of proopiomelanocortin (cf. Table 1), only the antiserum against endorphin revealed immunostaining of A-cells (Fig. 1c, d; Fig. 3a, b), as already stated previously (Grube et al. 1978).

Thus, all glucagon cells in the rat pancreas contain immunoreactivities for the three different opioid peptides. Two of these peptides belong to the prodynorphin family (dynorphin A and β -neoendorphin) and the remaining one belongs to the proopiomelanocortin family (endorphin). – But none of the corresponding antisera or of all other antisera against opioid peptides immunostained insulin or somatostatin cells.

Specificity controls

All antisera that revealed immunoreactivities in rat pancreatic glucagon cells were preadsorbed by their corresponding peptides and by heterologous peptides. A “blocking” of the immunoreactivities was achieved when at least 10–20 μ g/ml diluted antiserum of the corresponding peptide was added to the antisera. The results of the preadsorption tests are listed up in Table 2.

Discussion

Our immunohistochemical findings show that all A-cells of the rat endocrine pancreas contain dynorphin A and

β -neoendorphin-like immunoreactivities. In addition, the same islet cells exhibit endorphin-like immunoreactivities. The coexistence of endorphin and dynorphin/neoendorphin-like immunoreactivities within the same endocrine cell is very striking and hitherto has not been reported.

Because A-cells are known to bind immunoglobulins unspecifically (Grube et al. 1978; Grube and Weber 1979), we performed extensive specificity controls. During these controls a non-specific binding of peptide antibodies, of the “bridge-antibody”, of avidin or of biotin-labelled peroxidase to the tissue could be excluded. Furthermore, the dilution range of the antisera used (1:1000 and higher) minimizes a possible non-specific staining of A-cells by non-immunologic binding mechanisms. Moreover, in preadsorption tests none of the various peptide immunoreactivities could be blocked by heterologous peptides. Only in the case of endorphin-like immunoreactivity the immunostaining could be blocked by addition of β -LPH 61–69 and α -N-acetyl- β -endorphin. And finally, the negative staining of human pancreatic endocrine cells by these antisera might be taken as a supplementary control.

Thus, from all specificity tests performed it may be concluded that immunostaining of rat pancreatic glucagon cells by the antisera against dynorphin A, β -neoendorphin and endorphin is caused by specific (immunologic) mechanisms. Hence, peptides of two different opioid peptide families seem to coexist within the same endocrine cell.

Concerning endorphin-like immunoreactivities in the mammalian endocrine pancreas, various authors presented either immunochemical (Houck et al. 1981; Smyth and Zakarian 1982; Stern et al. 1982; Tung and Cockburn 1984) or immunohistochemical findings (Grube et al. 1978; Watkins et al. 1980) which suggest the presence of endorphin in this organ.

However, discrepancies exist with respect to the endocrine cell type where endorphin is localized: in contrast to our findings Watkins et al. (1980) found endorphin-like immunoreactivity in the somatostatin (D-) cells of the rat, guinea-pig and man. This may be due to different specificities of the antibodies.

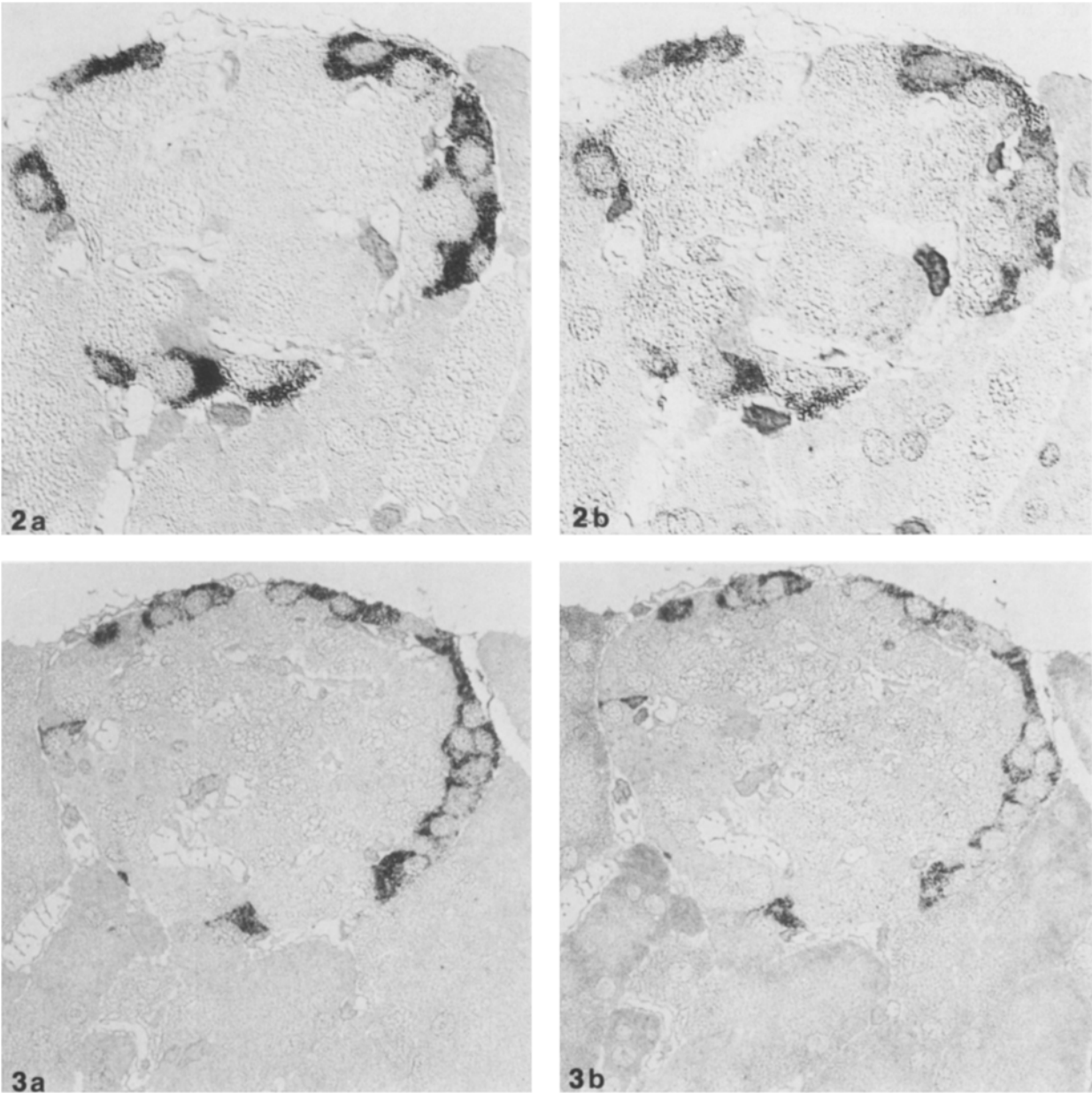


Fig. 2a, b. Two adjacent semithin sections (0.5 μ m) from the rat pancreas, immunostained (ABC method) for glucagon (2a) and dynorphin A (2b). All glucagon immunoreactive cells show also dynorphin immunoreactivities. Interference contrast. $\times 400$

Fig. 3a, b. Two adjacent semithin sections (0.5 μ m) from the rat pancreas, immunostained (ABC method) for glucagon (3a) and endorphin (3b). All glucagon immunoreactive cells show also endorphin immunoreactivities. Phase contrast. $\times 280$

Table 2. Immunohistochemical preadsorption tests

Peptides	Antisera			
	DYN A	β -neo-END	END	GLU
DYN 1-17	0	+	+	+
β -neo-END	+	0	+	+
β -END	+	+	0	+
β -LPH 61-69	+	+	0	+
α -N-acet- β -END	n.t.	n.t.	0	+
GLU	+	+	+	0

Symbols: + = immunostaining unaffected; 0 = complete blocking of the immunoreaction; n.t. = not tested

On the other hand, species-specific differences in the distribution of the opioid peptides should be mentioned. 16 K-fragment, as a part of proopiomelanocortin, has been localized mainly in the PP-cells and sporadically in glucagon cells of rat and dog pancreas (Grube and Weber 1979), and Met-enkephalin-like material in D-cells of dog and monkey pancreas (Forssmann et al. 1977); whereas in man neither ACTH nor endorphin-like immunoreactivities could be detected (Grube and Weber 1979).

An other question in this context is related to the molecular form of endorphin in the pancreas. Smyth and Zakarian (1982) found acetylated endorphin in addition to its desacetylated form in the pancreas of the rat, whereas in

our study the antiserum against α -N-acetyl- α -endorphin showed no immunoreaction in the endocrine pancreas.

Because rat pancreatic glucagon cells did not show any other immunoreactivities towards proopiomelanocortin-related peptides it may be concluded that pancreatic endorphin is synthesized by an other biochemical pathway than in the pituitary.

Concerning our findings of dynorphin and neoendorphin-like immunoreactivities in the rat pancreatic glucagon cells, we failed to find similar results in the literature. In immunochemical investigations of the guinea-pig pancreas a Leu-enkephalin-Arg peptide fragment has been described (Stern et al. 1982). Since this peptide sequence obviously is contained only in prodynorphin it might be speculated whether prodynorphin is present in pancreatic endocrine cells. However, during our immunohistochemical investigations we found no immunoreactivities for Leu-enkephalin, dynorphin 1–8 or dynorphin 1–13.

Summing up, our immunohistochemical findings give rise to the conclusion that rat pancreatic glucagon cells are an other source of opioid peptides. In this location, however, opioid peptides of different hormonal families were found in the same endocrine cell and other fragments of these peptide families could not be detected in glucagon cells. Therefore, we assume that the biosynthetic pathway of these opioid peptides is different from that in other locations.

Possibly, rat glucagon precursor substances in contrast to hamster and bovine preproglucagon (Bell et al. 1983; Lopez et al. 1983) may contain sequence homologies with these opioid peptides. – From the lack of any opioid peptide immunoreactivity in the human endocrine pancreas during our immunohistochemical investigations, it may be concluded that interspecies differences exist with regard to synthesis or degradation of these hormone precursor substances.

Apart from the outlined open questions related to the cellular distribution of pancreatic opioid peptides or their mode of synthesis, it should be emphasized that experimental investigations have shown that endorphin and dynorphin are involved in the regulation of “established” pancreatic hormones (Ipp et al. 1978; Green et al. 1983; Schusdziarra et al. 1984).

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