Rat pancreas contains the proglucagon(64–69) fragment and arginine stimulates its release

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Rat proglucagon(64–69) corresponding to the C-terminal hexapeptide of putative rat glicentin sequence in the precursor was synthesized. A glicentin C-terminal hexapeptide specific radioimmunoassay, using the synthetic hexapeptide as standard, demonstrated the presence in rat pancreas of a peptide identified with the synthetic rat proglucagon(64–69): H-Asn-Arg-Asn-Asn-Ile-Ala-OH. The hexapeptide was released concomitantly with glucagon by arginine stimulation from the isolated perfused rat pancreas. The results indicate that the pancreas co-stores and possibly co-releases the hexapeptide with glucagon as one of the processing products of proglucagon.

Rat proglucagon *Glicentin(64–69)* *Isolated rat pancreas perfusion* *Radioimmunoassay* *Arginine-stimulated glucagon release* *Peptide synthesis*

1. INTRODUCTION

A recent study of the rat glucagon gene deduced an amino acid sequence for rat preproglucagon [1]. This information has made it possible to examine molecular processing of the rat glucagon precursor in pancreatic tissue employing synthetic peptides. This study demonstrates that rat pancreas contains a peptide identified with synthetic H-Asn-Arg-Asn-Asn-Ile-Ala-OH corresponding to putative rat glicentin(64–69); arginine stimulation enhanced the release of this hexapeptide and glucagon from isolated perfused rat pancreas.

2. MATERIALS AND METHODS

2.1. *Peptides*

Rat proglucagon(64–69) corresponding to putative rat glicentin(64–69) was newly synthesized by a solid-phase technique [2] employing a Beckman 990B peptide synthesizer. Purity of the hexapeptide was assessed by routine analytical criteria. Amino acid ratios in an acid hydrolysate (6 N HCl, 110°C, 24 h) of the peptide were: Asp, 3.02; Ala, 1.00; Ile, 1.04; Arg, 0.93; and in aminopeptidase M digest Asn, 2.83; Ala, 1.08; Ile, 1.05; Arg, 1.03. Other synthetic peptides used were those described previously [3].

2.2. *Tissue extraction*

Male Wistar rats (200–250 g) were decapitated and the pancreas was removed. Pancreatic tissue was homogenized with a 5-fold excess of ice-cold 0.1 M acetic acid. The homogenate was heated in a boiling water bath for 5 min and cooled after which the final acetic acid concentration was adjusted to 1 M. The homogenate was centrifuged at 1400 × *g* for 20 min at 4°C. The precipitate was washed with 1 M acetic acid and the combined supernatant and washing were lyophilized.

2.3. *Gel filtration*

The crude extract of rat pancreas was chromatographed upon Sephadex-G50F (column

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1.5 × 91 cm) eluted with 3 M acetic acid. Column fractions (2.5 ml) were collected, lyophilized and reconstituted with assay buffer.

2.4. *High performance liquid chromatography (HPLC)*

Reverse-phase HPLC was performed on a Toyo-Soda SP8700 high speed liquid chromatograph. Rat pancreas crude extract and synthetic peptides were chromatographed on a Nucleocil $5C_{18}$ column (M. Nagel, Düren, FRG) (4.6 × 250 mm) in a gradient solvent system of CH_3OH/0.01 N HCl (0:100–10:90, v/v, for 30 min and 10:90–50:50 for 40 min) at a flow rate of 1 ml/min. The eluate was collected every min, lyophilized and reconstituted with assay buffer.

2.5. *Isolated perfused rat pancreas*

Overnight-fasted male Wistar rats (200–250 g) were anesthetized with sodium pentobarbital. The pancreas was removed and perfused according to Grodsky and Fanska [4]. The pancreas was perfused with Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4), supplemented with 4% Dextran T70 (Pharmacia, Uppsala, Sweden) and 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, MO). The perfusion medium was equilibrated with 95% O_2:5% CO_2 and the flow rate was maintained at 1.9 ml/min. During the first 25 min, glucose (final concentration 5.5 mM) was introduced via a side-arm syringe at a flow rate of 0.1 ml/min. Glucose was replaced with arginine (final concentration 15 mM) for a 20 min period. The pancreas was perfused with glucose again for the last 10 min. After the first 15 min equilibration period with glucose, the perfusate was collected every min in tubes containing 1000 KIU Trasylol (Bayer, Leverkusen, FRG). Hexapeptide and pancreatic glucagon contents of each perfusate sample were determined by specific radioimmunoassays. In the other experiment, isolated rat pancreas was perfused with arginine (final concentration 10 mM) in the presence of glucose (final concentration 5.5 mM) for 20 min after 25-min preperfusion with glucose (final concentration 5.5 mM).

2.6. *Radioimmunoassay*

Radioimmunoassay for rat glicentin C-terminal hexapeptide was performed according to [3] using antiserum R4804 [5], ^{125}I-3-(4-hydroxyphenyl)propionyl-porcine glicentin(62–69) as tracer and rat glicentin(64–69) as standard.

Pancreatic glucagon specific radioimmunoassay was carried out as described using antisera OAL-123 [6].

3. RESULTS

In the radioimmunoassay specific for glicentin(64–69), the newly synthesized putative rat glicentin(64–69) displaced the tracer in a manner parallel to that of porcine glicentin(64–69) (fig.1). The relative cross-reactivity of the rat peptide was 37.8% of the porcine hexapeptide in this system. Crude extracts of 3 rat pancreases all possessed dilution curves parallel to the standard curve of synthetic rat glicentin(64–69) (fig.1). The gel filtration profile of the immunoreactivity in crude extract of rat pancreas is shown in fig.2a. The integrated immunoreactivity in the main peak

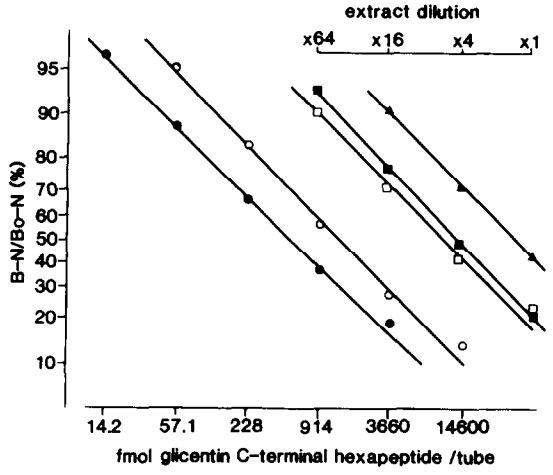

Fig.1. Dose-response curves of synthetic rat and porcine glicentin(64–69) fragments and dilution curves of crude extracts of 3 different rat pancreas specimens in radioimmunoassay system with antiserum R4804 and ^{125}I-3-(4-hydroxyphenyl)propionyl-porcine glicentin-(62–69) as tracer. (○—○) Synthetic rat glicentin(64–69), (●—●) synthetic porcine glicentin(64–69) and (□—□, ■—■, ▲—▲) rat pancreatic crude extracts.

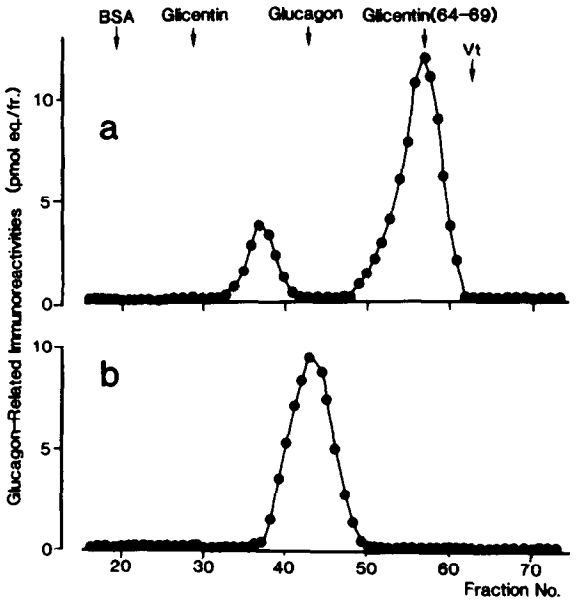

Fig.2. Gel filtration profiles of (a) immunoreactive hexapeptide and (b) immunoreactive pancreatic glucagon in crude rat pancreatic extracts.

fractions corresponding to the hexapeptide accounts for 81.5% of the total immunoreactivity detected in the crude extract and it corresponded to 132%, on a molar basis, of pancreatic glucagon immunoreactivity in the same extract which was calculated from the elution profile shown in fig.2b. The hexapeptide peak fractions were collected and successively analyzed by HPLC. The immunoreactivity eluted as a single peak (fig.3a). The retention time was exactly the same as that of synthetic rat glicentin(64–69) (fig.3c) and distinctly different from that of synthetic porcine glicentin(64–69) (fig.3b).

Fig.4 shows arginine-stimulated release of glicentin C-terminal hexapeptide immunoreactivity from the isolated perfused rat pancreas. Release of the hexapeptide immunoreactivity in the presence of 15 mM arginine was biphasic and parallel to the release of glucagon. Glucose suppressed the release of both hexapeptide and glucagon. Total response of the hexapeptide was 105%, on a molar basis, of the total pancreatic glucagon released. No appreciable release of the hexapeptide immunoreactivity was observed when 10 mM arginine was infused in the presence of 5.5 mM glucose.

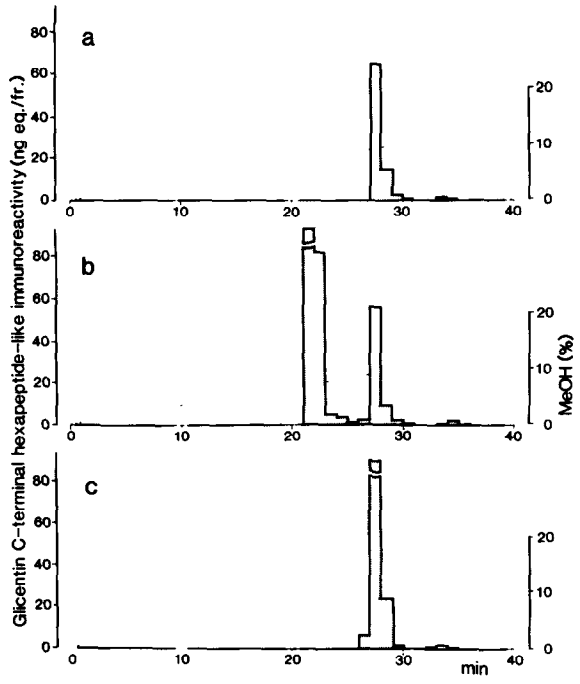

Fig.3. Identification in HPLC of the hexapeptide in rat pancreas extract with synthetic peptides. HPLC profiles of (a) the hexapeptide-like immunoreactive component in rat pancreas (80 ng equivalent), (b) a mixture of the hexapeptide-like immunoreactive component in rat pancreas (80 ng equivalent) and synthetic porcine glicentin(64–69) (200 ng) and (c) a mixture of the hexapeptide-like immunoreactive component in rat pancreas (80 ng equivalent) and synthetic rat glicentin(64–69) (200 ng).

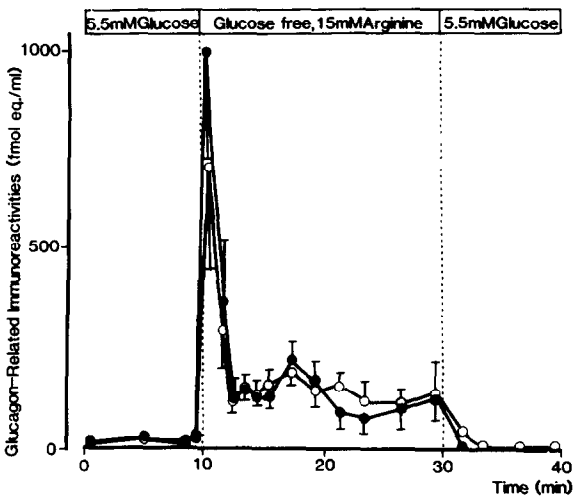

Fig.4. Arginine-stimulated release of (●—●) glicentin C-terminal hexapeptide and (○—○) pancreatic glucagon from isolated perfused rat pancreas. $n = 5$.

4. DISCUSSION

In a previous study [7], we isolated a glicentin(64–69) fragment from porcine pancreas using a radioimmunoassay specific for the hexapeptide [3] and presented evidence for the existence of the hexapeptide fragment in porcine pancreas with glucagon on approximately an equimolar basis. Recent presentation of a putative amino acid sequence for rat preproglucagon, deduced from the gene sequence [1], promoted the rat as an experimental model for studying molecular processing of proglucagon. Based on the proposed structure of the precursor, we synthesized a putative rat glicentin(64–69) and developed a radioimmunoassay for the rat hexapeptide. The assay system measured rat hexapeptide-related materials.

The hexapeptide in rat pancreas was identified with synthetic H-Asn-Arg-Asn-Asn-Ile-Ala-OH by HPLC and differed from the porcine peptide H-Asn-Lys-Asn-Asn-Ile-Ala-OH. The hexapeptide was the major component in a crude rat pancreatic extract measurable with the current hexapeptide assay and only a small amount of glicentin- and oxyntomodulin-like components was detected. The molar ratio of the hexapeptide and pancreatic glucagon in the same extract which was calculated from the gel filtration profile was approx. 1:0.75. The extraction efficiency of glucagon, but not the hexapeptide, in rat pancreas with hot dilute acetic acid was found to be lower, approx. 64%, when compared with acid-alcohol extraction as has been observed with porcine pancreas [5,7]. On the basis of this figure, the hexapeptide in rat pancreatic extract was assumed to correspond to 85% pancreatic glucagon on a molar basis. This was compatible with our previous result with porcine pancreas [7] and suggests approximately equimolar production of the hexapeptide and glucagon in rat pancreas.

From the isolated perfused rat pancreas, the hexapeptide was released concomitantly with pancreatic glucagon by 15 mM arginine stimulation. The total response of the hexapeptide and glucagon indicated concomitant release of approximately equimolar amounts of both peptides under the condition used. Based on the specificity of the hexapeptide radioimmunoassay employed and the values measured, the immunoreactivity recognized in the perfusates by the hexapeptide-specific assay system was reasonably attributable mainly to the hexapeptide itself.

We have demonstrated [5] immunohistochemically the existence, in the α granules of human pancreatic A cells, of glicentin C-terminal immunoreactivity stained with antiserum R4804. Together with the present results, it seems reasonable to conclude that in addition to the N-terminal fragment of glicentin, GRPP [8], the pancreas co-stores and possibly co-releases the hexapeptide with glucagon as one of the processing products of proglucagon. Whether the hexapeptide and glucagon are released under physiological conditions and a potential physiological role for the hexapeptide itself remain to be investigated.

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