Evidence for the presence of
a neutral insulinotrophic peptide in the porcine duodenum

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Abstract. A crude mixture of thermostable peptides extracted from porcine duodenum was fractionated by
electrofocusing. A neutral fraction, different from the basic fractions of GIP, VIP, PHI and CCK was found to
promote insulin secretion when injected in vivo to normal rats. This neutral fraction, extracted from the crude
mixture by chromatography, stimulated insulin output from an isolated rat pancreas and enhanced glucose-
induced insulin release. The insulinotrophic effect of this partially purified duodeno-jejunal material disappeared
following digestion with trypsin. The insulin-releasing activity was found to correspond to a compound of
molecular weight higher than that of insulin (i.e. higher than 6000). No GIP-like immunoreactivity was found in
this neutral fraction indicating that the active peptide(s) are not GIP related compounds.

These observations suggest that porcine duodenum contains and incretin activity different from that of the
insulinotrophic factors already reported.

It has been known for many years that insulin secretion is modulated mainly by variations in
plasma glucose and by factors released from the gastrointestinal tract in response to carbohydrate
ingestion (McIntyre et al. 1965; Perley & Kipnis 1967; Mutt 1982). During the last decade, gastric
inhibitory polypeptide, now commonly referred to as glucose-dependent insulinotrophic polypeptide
(GIP), has been recognized as the main incretin-like peptide (Creutzfeldt 1979; Pederson & Brown
1976; Ebert et al. 1979). However, it has been claimed from experiments in animals and man that ‘incretins’ other than GIP do exist. Creutzfeldt (1979) and Ebert et al. (1979) have tested the role
of GIP as an incretin using an immunochemical approach and have shown that infusion with a
potent GIP antiserum in rats does not abolish the late incretin effect of GIP released by hydrochloric
acid. Studies by Levin et al. (1979, 1980) on insulin release following perfusion of an isolated rat pancreas
with the portal venous effluent of a perfused rat gastrointestinal tract, also suggested the existence of a distinct insulinotrophic substance secreted by the intestine.

It is apparent from the investigation in man by Sarson et al. (1982) that the insulin response pro-
vided by iv infusion of GIP and glucose is only about one third of that produced by the ingestion
of an oral glucose load, in spite of the similarity of the degree of hyperglycaemia observed. This

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study and that of Salera et al. (1982) also suggest that GIP is not the only factor involved in the insulin response to a glucose load in man.

Consequently, verification of the presence of insulinotropic peptides other than GIP in a crude extract of porcine duodenum was sought. We describe the first steps in purification of a fraction extracted from porcine upper small intestine, capable of stimulating insulin release from an in situ rat pancreas preparation as well as from a perfused rat pancreas.

Materials and Methods

**Biological assays for the measurement of insulin release**

The insulin releasing activity of the different fractions obtained from a concentrate of thermostable gut peptides was determined in two kinds of bioassays. The first was an in situ rat pancreas preparation which allowed the rapid screening of all the fractions obtained. The second was an isolated rat pancreas preparation used to evaluate the glucose-dependent activity of the more interesting fractions.

**In situ rat pancreas preparation**

The technique first described by Fasel et al. (1970) was adapted for our purpose. Male Wistar rats (Pathophysiologisches Institut, Bern, Switzerland), 250–300 g, were anaesthetized by ip injection of pentobarbital (50 mg/kg). After laparotomy, a cathether (0.86 mm diameter, 20 cm length) was inserted into the portal vein just above the junction with the pancreato-duodenal vein for blood removal and injection of the fraction to be tested. The peptides to be tested and the endogenous intestinal hormones thus followed the same physiological pathway. The catheter was then left in place for 15 min, before removing the first blood sample (0.6 ml) for the determination of basal insulin levels. The animals were then injected via the same route with a bolus (0.5 ml) containing the test fraction. Blood samples were drawn 1, 2, 5 and 10 min after injection for insulin determinations. 0.2 ml of physiological saline at body temperature was injected through the catheter after injection of the test fraction and 0.5 ml after each subsequent blood removal.

Immunoreactive insulin (IRI) was tested by radioimmunoassay (charcoal separation technique), using rat insulin as reference standard. For each hormone or peptide mixture tested, the maximum effect on insulin release was obtained 1 min after injection; a typical pattern is shown in Fig. 1. A dose response increase in portal insulinemia was obtained after bolus injections of increasing doses of GIP (Fig. 2).

**Isolated rat pancreas preparation**

Male Wistar rats (Laboratoire de Recherches Métaboliques, Genève, Switzerland), weighing 200–250 g, fed ad libitum, were anaesthetized by ip injection of pentobarbital (50 mg/kg), and the pancreas was isolated. The pancreas was perfused with a Krebs-Ringer bicarbonate solution containing 4 g/l human serum albumin (Swiss Red Cross, Bern, Switzerland), running by an open circuit - non-recycling perfusion system according to the method described by Grodsky et al. (1967) and modified by Assan et al. (1977). The pancreas was equilibrated by perfusion for 20 min before the beginning of the experiments. The perfusion rate was 3.0 ml/min.

Insulin levels are expressed as mean ± SEM. Non-paired and paired t-tests were used to evaluate the data statistically.

**Purification procedure**

Tissue extraction and preliminary fractionation were carried out according to the method described by Mutt (1978). The starting material was a methanol insoluble
fraction derived from a 66% ethanol soluble concentrate of thermostable intestinal peptides. This basal extract contained high amounts of GIP, cholecystokinin 33 (CCK 33) and cholecystokinin 39 (CCK 39). This material was further purified by ion-exchange chromatography and gel chromatography. The presence of active peptide(s) exhibiting a neutral isoelectric point was assessed by preparative isoelectric focusing of the starting material and of the fractions collected after each purification step.

a) Ion exchange chromatography. The starting material was eluted with phosphate buffer 0.05 M, pH 6.0 on an 1.6 x 30 cm CM-Sephadex column (Pharmacia). The fractions collected were lyophilized after desalting on a G-25 Sephadex column in 0.2 M acetic acid. The resolving power was controlled by preparative electrofocusing. GIP assay has been performed on the neutral active fraction obtained from this purification step (Sarson et al. 1980).

b) Gel chromatography. The neutral insulinotropic fractions collected from ion-exchange chromatography were dissolved in a 0.25 M phosphate buffer pH 8.0 containing 20% sucrose, and applied on an 0.9 x 60 cm Sephadex G-50 Superfine column (Pharmacia). The optical density of the eluted fractions was measured at 280 nm. Aliquots (equivalent to 10 µg proteins) were injected into the in vivo rat pancreas preparation described above.

c) Preparative electrofocusing. Qualitative analysis of the fractions obtained after each purification step was performed by flat-bed electrofocusing in a granulated gel using a LKB 2117 Multiphor apparatus (Bromma, Sweden). Sephadex IEF gel and Pharmalyte 3–10 amphotolines were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The separated zones were collected by sectioning the gel bed with a fractionation grid. Gel sections were re-suspended in NaCl 0.9% and the peptide content eluted. Aliquots (equivalent to 10 µg protein) were injected into the in situ rat pancreas preparation. All methods attempted to separate peptides from amphotolines on a preparative scale (ultrafiltration, dialysis, ion-exchange chromatography, TCA precipitation) were unsuccessful. It is generally admitted that amphotolines do not possess any biological activity of their own and that they do not affect the biological activity of peptides. This was confirmed on our in situ rat pancreas preparation: injection of amphotolines at all pH levels used did not stimulate insulin secretion or modify the effect of GIP on insulin output.

d) Reversed phase liquid chromatography. Analysis by high-performance liquid chromatography (HPLC) was performed on a Waters instrument (Waters Associates, Milford, MA) equiped with a µ-Bondapak C18 column, using 43% ethanol, 57% water, 5 mM ammonium acetate as eluant and determination at 215 nm.

e) Effect of trypsin. The effect of trypsin on the insulin-releasing activity of the active fractions eluted from the Sephadex G-50 column was tested by the following protocol: 500 µg of the active fraction was suspended in 12 ml phosphate buffer 0.067 M pH 7.6 and incubated for 1 h at 37°C with or without 1200 µg of bovine trypsin

Fig. 2.
In situ rat pancreas preparation: per cent change in insulin levels 1 min after injection with NaCl or various doses of GIP. Data are expressed as mean ± SEM of 7 animals per group.
(3.5 U/mg, Merck). Trypsin alone was also incubated in the same buffer. Aliquots (0.5 ml) of these incubation media were injected to the in situ rat pancreas preparation.

**Chemical and labelled hormones**

$^{125}$I insulin, $^{125}$I glucagon and $^{125}$I luteinizing hormone-releasing hormone (LRH) were obtained from Serono (Chavannes-de-Bogis, Switzerland). The other chemicals were purchased from Merck (Darmstadt, FRG).

**Results**

**Effect of the basal fraction**

The in situ rat pancreas preparation response to the injection of 10 µg of the starting material is shown in Fig. 1. Maximal response occurred 1 min after bolus injection, with 4-fold increments in portal insulinaemia (57.5 ± 10.0 to 280.0 ± 37.5 µU/ml, $P < 0.05$). Basal levels were restored 10 min after injection.

**Preparative electrofocusing of the starting material**

Fifty mg of the starting material were mixed with 3.33 ml ampholines in the IEF gel preparation (pH gradient 3.5—9.5). Running conditions were as follows: 1500 V, 25 W, 48 h. The gel was then divided into 30 fractions. Each fraction was eluted with 30 ml NaCl 0.9%. The response of the in situ rat pancreas preparation to the injection of 0.5 ml aliquots is shown in Fig. 3. Biological activity was revealed in three distinct pH areas. The more basic area around pH 9.4 can be attributed to vasoactive intestinal peptide (VIP), CCK 33 and CCK 39 and possibly other unknown peptides. The second one, between pH 8.3 and 8.0, to GIP and the porcine intestinal peptide (PHI), among others. The third one, constituted by neutral peptides, can be attributed to one or several original insulinotropic factors.

**Cation exchange chromatography of the starting material.**

In order to isolate the fraction constituted by neutral peptides, the starting material was submitted to partial purification using cation-exchange chromatography (Fig. 4). The eluted fractions were tested on the in situ rat pancreas preparation and
Cation exchange chromatography of the starting material: elution profile at optical density 277 nm. Qualitative analysis of the active fractions was performed by preparative electrofocusing. The results are summarized using the following symbols: ○: no activity; ● 0–200% stimulation; ●● 200–400%; ●●● 400–600%; ●●●● 600–800%; ●●●●● over 800%. The insulinotrophic activity of fractions 7 to 10 must be attributed to the neutral factor. After fraction 10, this factor coeluates with more basic active peptides (i.e. GIP).

Gel chromatography of the neutral insulinotrophic fraction. This fraction was recovered from the CM-Sephadex column and eluted on a 0.9 x 60 cm Sephadex G-50 superfine column in the presence of labelled insulin, glucagon and LHRH. The optical density of the eluted fractions was measured at 280 nm. The biological activity of groups of fractions was tested on the in situ rat pancreas preparation (symbols are as in Fig. 4). Elution of the neutral insulinotrophic factor prior to insulin indicates an apparent molecular weight for the active peptide higher than that of insulin.
the composition of the active fractions was evaluated by preparative electrofocusing. Although the yield of this separation step was small because of the difficulty in separating the neutral insulinotropic fraction from GIP, this method did allow the isolation of a group of active fractions containing only peptides with an isoelectric point close to pH 7.0. We shall refer to these peptides as the neutral insulinotropic fraction.

**Gel chromatography of the neutral insulinotropic fraction**

In order to evaluate the molecular weight (MW) of this (these) active neutral factor(s), the neutral fraction was eluted on a G-50 superfine gel in the presence of three labelled peptides: insulin (MW 5734), glucagon (MW 3483) and LRH (MW 1182). The active fraction eluted just before insulin (Fig. 5) suggesting that its apparent MW is slightly higher than that of insulin.

Incubation of the neutral fraction with bovine trypsin resulted in suppression of its biological activity.

The concentration of GIP-like immunoreactivity in the neutral insulinotropic fraction, was found to be below 20 fmol/μg. Analysis of the same fraction by reversed phase liquid chromatography did not reveal any GIP content (Fig. 6).

In addition to testing with the in situ rat pancreas preparation, the insulinotropic effect of the active fraction was also verified using an isolated rat pancreas preparation (Fig. 7a and b). The results show that the activity of the neutral insulinotropic factor is glucose-dependent. In the presence of 5.5 mM glucose, the active peptide doubled insulin output, which increased from approximately 3 ng/min to 8 ng/min. Insulin production in the presence of 10.0 mM glucose was approximately 20 ng/min and was also doubled by perfusion with the active peptide.
Isolated rat pancreas preparation: effect of the neutral insulinotrophic factor in the presence of basal glucose concentrations (5.5 mM · l⁻¹) in the perfusate. IRI output is significantly higher ($P < 0.05$) from 2 to 20 min of infusion with $1 \mu$g/min of the extract, compared to basal IRI output (mean of the four determinations at −3 to 0 min). $n = 5$.

Isolated rat pancreas preparation: effect of neutral insulinotrophic factor in the presence of elevated glucose concentrations (10.0 mM · l⁻¹) in the perfusate. The glucose-dependent activity of the extract is revealed by comparison with the results obtained in the presence of 5.5 mM glucose. $n = 6$. IRI output is significantly higher ($P < 0.05$) from 3 to 20 min of infusion with the extract, compared to controls (without duodenal extract).
Discussion

This study demonstrate through successive purification steps, the presence in the porcine duodenum of an insulinotrophic activity different from that of GIP and the other basic incretin candidates VIP, CCK and secretin. It supports the suggestion by several authors, based on experimental approaches, of the existence in the gastrointestinal tract of unidentified insulinotrophic factors (Creutzfeldt 1979; Ebert et al. 1979, 1983; Lauritsen et al. 1980; Sarson et al. 1982; Hedner, personal communication).

The biological activity of the partially purified neutral factor was evaluated using two different systems: the in situ rat pancreas and perfused isolated rat pancreas preparations. Results obtained with the latter showed the activity of this factor to be glucose dependent, as is the case with GIP (Pederson & Brown 1976). In our experimental conditions, insulin output was doubled by infusion with 1 μg/min of the extract. When insulin output was stimulated by elevated glucose concentrations in the perfusate, the active factor still doubled insulin production. Relatively high amounts of the extract were perfused in the preparation. However, it has to be considered that the degree of purity of the active factor cannot be evaluated and that its potential biological activity is not known. This could also result from differences in species specificity or in the possibility that the factor could be a precursor with restricted biological activity.

The difficulties encountered along the purification steps were caused by the presence of the known insulino trophic compounds in the starting material. The yield of some preparation steps like chromatography on CM-Sephadex was very low. However, the different observations reported suggest that the fractions obtained were free from the above mentioned insulino trophic factors. In contrast to other gastrointestinal peptides proposed as incretin candidates, the neutral factor was found to correspond to a compound of molecular weight higher than that of insulin, i.e. higher than 6000 daltons. It indicates that the original factor did not result from degradation or metabolism of other insulino trophic hormones. The peptic nature of the active factor was confirmed by the observation that tryptic digestion suppressed the activity of the preparation. This result could be expected since it is probable that most incretin-like factors are polypeptides, as has been suggested by Johnson (1981).

The low cross-reactivity in the GIP radioimmunoassay, which was shown to be highly specific (Sarson et al. 1980) suggests that this neutral peptide is not a GIP-structure related peptide, and is probably different from the high molecular form of GIP.

In conclusion, the present observation suggests the existence, in the porcine duodenum, of an incretin activity different from that of the insulino trophic factors already reported.

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