Volume 211, number 2, 169–174 FEB 04356 January 1987

Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut

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Received 23 October 1986; revised version received 20 November 1986

By hydrophobic gel permeation and high pressure liquid chromatography we isolated from pig intestinal mucosa a peptide which corresponds to proglucagon 78–107 as suggested by chromatography and determination of its N-terminal sequence. Natural and synthetic proglucagon 78–107 dose dependently and potently increased insulin secretion from the isolated perfused pig pancreas. Proglucagon 78–107 also secreted by the small intestine may participate in the hormonal control of insulin secretion.

Glucagon-like peptide; Incretin; Monobasic cleavage

1. INTRODUCTION

The mammalian glucagon precursor (proglucagon) is a 180 amino acid peptide. Besides glucagon itself it contains two glucagon-like sequences, originally designated 'glucagon-like peptides 1 and 2' (GLP-1 and GLP-2), which are separated by a 13 amino acid spacer sequence [1,2]. The glucagon-like sequences (GLP-1 shown in fig.1), which are about 50% homologous with glucagon, are flanked by pairs of basic amino acids, putative processing sites. Proglucagon appears to be processed differently in the mammalian pancreas and small intestine [3–5]. In the pig and rat pancreas the following peptides are produced and secreted upon appropriate stimulation: (i) glucagon; (ii) glicentin-related pancreatic peptide (GRPP) corresponding to proglucagon 1–30; (iii) a large peptide that contains both the GLP-1 and the GLP-2 sequences [4–6]. In the pig small intestine the major secreted products are the 69 amino acid glucagon-containing peptide, glicentin, and the two glucagon-like sequences GLP-1 and GLP-2 as separate peptides, not as parts of one large peptide [4]. Gel filtration studies have shown that the glucagon-like peptides secreted from the pig small intestine have approximately the same size as synthetic replicas of the two glucagon-like peptides synthesized according to their structure as predicted from the proglucagon sequence [4]. The exact structure of the ileal glucagon-like peptides is not known, however. We therefore isolated the naturally occurring glucagon-like peptide 1 from acid-ethanol extracts of pig small intestinal mucosa and determined part of its sequence. In addition, we studied biological effects of the peptide as well as the effects of a peptide synthesized according to the structure of the natural peptide.

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2. MATERIALS AND METHODS

2.1. *Isolation of proglucagon 78–107*

Ileal mucosa was excised from anaesthesized pigs and immediately frozen. Acid ethanol-extracts were prepared according to method II in [7]. In short, frozen tissue was homogenized in 4 vols acid ethanol and centrifuged. 5 vols cold diethyl ether were added to the supernatant and the aqueous

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00145793/87/$3.50

phase was isolated at −50°C. The resulting precipitate was then dissolved in distilled water containing 8 mol/l urea. The GLP-1 immunoreactive peptide was isolated in 4 consecutive steps. The procedure was monitored with a radioimmunoassay developed for synthetic GLP-1 (proglucagon 71–107) [4], using antiserum 1953 raised against synthetic GLP-1, ^{125}I-labeled synthetic GLP-1, and synthetic GLP-1 (1–37 amide, code no.7166, Peninsula, Belmont, CA, USA) for standards. Determined with this radioimmunoassay extracts of the pig ileum mucosa contain 88 ± 4 pmol/g of immunoreactive GLP-1. Extract of 1 kg mucosa corresponding to ileal mucosa from 3 pigs of 40–45 kg was applied to a 3 × 15 cm glass column packed with Techoprep C18, 40–63 μm (HPLC Technology, England), and eluted with a gradient of 20–80% ethanol in water containing in addition 0.01% trifluoroacetic acid (TFA, Pierce, Rockford, IL). From the GLP-1 immunoreactive fractions the ethanol was removed by evaporation and the pool was applied to a 50 × 1000 mm (K 50/100) column packed with Sephadex^{R} G-50, fine grade (Pharmacia, Uppsala, Sweden) and eluted with 0.5 M acetic acid at a flow rate of 1 ml/min at 4°C. GLP-1 immunoreactive fractions were then subjected to reverse-phase high-pressure liquid chromatography on an 8 × 250 mm Nucleosil^{R} C18 column employing LKB pumps and detectors (LKB, Bromma, Sweden). The column was eluted with water containing 0.1% TFA and a gradient of acetonitrile (grade S, Rathburn Chemicals, Ltd, Walkerburn, Scotland) from 0 to 80%. Finally, GLP-1 immunoreactive fractions were subjected to isocratic HPLC with 43% ethanol in water containing in addition 0.01% TFA as the mobile phase.

2.2. *Sequence determination*

Solvent was removed under vacuum from the HPLC purified peptide. It was reconstituted in 0.06 ml of 10% TFA in water and subjected to automated sequence analysis by sequential Edman degradation on an Applied Biosystems 470A gas-phase sequenator with the 02 NVAC program (batch 1) or the 02RPTH program (batch 2), programmes modified from Hunkapillar et al. [8], available at Applied Biosystems, Foster City, CA, USA. All chemicals were purchased from Applied Biosystems. The phenylthiohydantoin derivatives of amino acids were either (batch 1) characterized by HPLC on a Hewlett-Packard 1084 liquid chromatograph with a 0.45 × 25 cm column of CN (5 μm particles, IBM Instruments) and a sodium acetate/acetonitrile gradient elution system as described [9], or, in the case of batch 2, the samples from the sequenator were methylated before HPLC by treating the dried derivatives with acidified methanol (1 M HCl in methanol; Applied Biosystems) for 10 min at 50°C. The amino acid derivatives were then characterized on an Applied Biosystems PTH column, 2.1 × 22 cm, using the elution system recommended by the manufacturer. Aminobutyric acid was used as internal standard during the HPLC for correction of elution time and for quantifying the amino acid derivatives.

2.3. *Effect of natural and synthetic GLP-1 on insulin secretion*

Synthetic proglucagon 78–108 amide was obtained from Peninsula Laboratories (San Carlos, CA) by custom synthesis (lot no.008802; peptide purity by amino acid analysis 72%). Before the peptide was used in physiological studies, it was purified to homogeneity by isocratic HPLC as described above. Sequence determination of synthetic peptide confirmed the structure as being proglucagon 78–107 amide. The biological effect of both the synthetic proglucagon 78–107 fragment and the isolated natural peptide was studied using perfused porcine pancreas, prepared and perfused as described in [10]. The pancreas was isolated together with the supplying arteries and veins and perfused with an artificial medium consisting of Krebs-Ringer bicarbonate buffer, containing 15% washed bovine erythrocytes, 0.1% human serum albumin (reinst, trocken, Behringwerke Marburg, FRG), 5 or 7 mmol/l glucose, 5% dextran T-70, aprotinin 100000 KIU/l (Trasylol^{R}, Bayer, Leverkusen, FRG), and 5 mmol/l of a mixture of amino acids (Amodex Asa, Pharmacia, Uppsala, Sweden). The medium was gassed with 95% oxygen and 5% CO_2. Synthetic proglucagon 78–107 was infused intra-arterially for 5 or 10 min periods in doses corresponding to final perfusate concentrations of 10^{-10}–10^{-8} mol/l. Effluent fractions were collected every minute and kept on ice until centrifugation. The supernatants were stored at −20°C until assay. Insulin was measured in all fractions as described earlier [10].

3. RESULTS AND DISCUSSION

The results of the isolation procedure are shown in fig.2. A homogeneous peak of immunoreactive GLP-1 was eluted from the C18 Techoprep^{R} column, and was further purified by gel filtration and repeated HPLC. The sequence of the first 17 amino acids is shown in table 1. This sequence corresponds to proglucagon 78–94; in other words, a truncated form of GLP-1. By gel filtration on Sephadex G 50 columns the natural peptide eluted exactly at the position of synthetic proglucagon 78–107 amide. In addition, on analytical reverse-phase HPLC using a 24–48% acetonitrile gradient over 45 min the natural and the synthetic peptide had the same retention time (26 min). This strongly suggests that the natural peptide corresponds to proglucagon 78–107. In the present study we could not determine whether the natural peptide has a free α-carboxyl group or is amidated. An amidated form was chosen for the synthetic peptide because of the presence in proglucagon of a glycine residue after the C-terminal dibasic flanking sequences of GLP-1; in many propeptides this sequence gives rise to carboxyamidation during the posttranslational processing [11].

According to our results, the cleavage of proglucagon to release GLP-1 does not occur at the expected site of the pair of basic amino acids (69 and 70) but after the single basic amino acid at position 77. Similar monobasic proteolytic processing is found for many precursors as recently reviewed [12]. The natural porcine GLP-1, which according to the present study corresponds to proglucagon 78–107 amide, is thus very similar to anglerfish, catfish and salmon GLP-1 ([13–15]; see fig.1). In fact, the six amino acids between the classical dibasic cleavage and the monobasic cleavage site are not encoded for by the anglerfish gene [13]. After removal of the 6 N-terminal amino acids of GLP-1 the sequence homology of the remaining peptide with glucagon is even more pro-

Species	Residue no. in human proglucagon																		
	72						78												90
Human	His	Asp	Glu	Phe	Glu	Arg	His	Ala	Glu	Gly	Thr	Phe	Thr	Ser	Asp	Val	Ser	Ser	Tyr
Ox	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Hamster	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Rat	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Guinea pig	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Anglerfish	-	-	-	-	-	-	"	"	Asp	"	"	"	"	"	"	"	"	"	"
Salmon	+	+	+	+	+	+	"	"	Asp	"	"	Tyr	"	"	Asn	"	"	Thr	"
Catfish	+	+	+	+	+	+	"	"	Asp	"	"	Tyr	"	"	"	"	"	"	"

Species	Residue no. in human proglucagon																		
	91									100							107		110
Human	Leu	Glu	Gly	Gln	Ala	Ala	Lys	Glu	Phe	Ile	Ala	Trp	Leu	Val	Lys	Gly	Arg	Gly	Arg
Ox	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Hamster	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Rat	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Guinea pig	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"	"
Anglerfish	"	Lys	Asp	"	"	Ile	"	Asp	"	Val	Asp	Arg	"	Lys	Ala	"	Gln	Val	"
Salmon	"	Gln	"	"	"	"	"	Asp	"	Val	Ser	"	"	Lys	Ser	"	"	Ala	+
Catfish	"	Gln	"	"	"	"	"	Asp	"	"	Thr	"	"	Lys	Ser	"	Gln	Pro	Lys

Fig.1. Sequences from 8 species of proglucagon regions containing glucagon-like peptide 1. Glucagon-like peptide-1 containing sequences of proglucagon from all species whose proglucagon structure has been determined [1,2,13–15,23,24]. The peptide which was originally designated GLP-1 (of hamster proglucagon) corresponds to proglucagon 72–108. Amino acids which occupy the same position in the glucagon molecule are encircled. Amino acids 72–77 are not coded for in the anglerfish gene. (" ") Same amino acid as in human proglucagon; (+ +) gene structure not known [14,15]; (– –) amino acids not encoded for in anglerfish gene [13]. Circle around amino acids indicates amino acids in same positions as human glucagon. Vertical line between amino acids 77 and 78 indicates position of N-terminal amino acid in glucagon-like peptide 1 from pig, anglerfish, salmon and catfish.

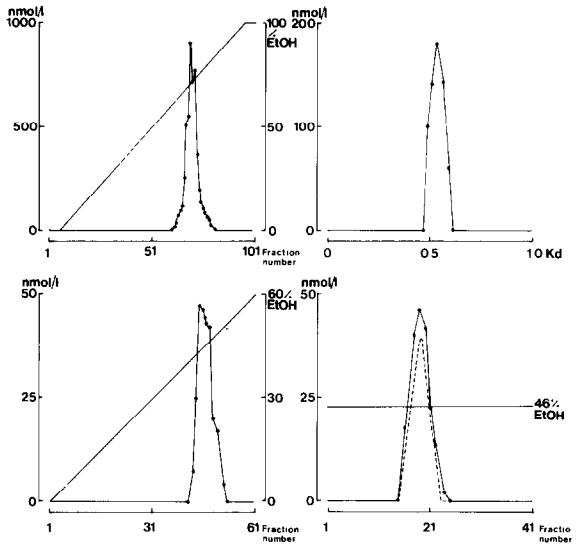

Fig.2. Isolation of porcine natural GLP-1. The concentration of natural GLP-1 was monitored by radioimmunoassay. Upper left part shows the results of hydrophobic chromatography. Extract of 1 kg mucosa was applied to the column and eluted with a gradient of ethanol in water (right ordinate scale). Upper right part shows gel filtration of peak fractions from the first step. Lower left part shows reverse-phase HPLC of peak fractions from the gel filtration. The column was eluted with a gradient of acetonitrile in water (right ordinate scale). The lower right part shows the results of isocratic HPLC (43% ethanol in water). The dotted line shows UV-absorption at 280 nm.

nounced (see fig.1). Since the N-terminal part of the glucagon molecule is essential for its biological activity [16], proglucagon 78–107 might be expected to show glucagon-like effects. So far, only few studies on the biological effects of the glucagon-like peptides GLP-1 and GLP-2 have been reported. Ghiglione et al. [17] found no effect of synthetic GLP-1 (1–36 amide, Peninsula) on blood glucose or insulin levels in fasting rabbits in what was considered as pharmacological doses. Schmidt et al. [18] found a weak insulinotropic effect of synthetic GLP-1 (1–36 amide, code no.7166, Peninsula), but not of synthetic GLP-2 (1–34, code no.7156) on isolated rat islet at rather high concentrations of GLP-1 and GLP-2 and in the presence of 10 mmol/l glucose. We [19] recently demonstrated inhibition of gastric acid secretion in healthy volunteers during submaximal pentagastrin stimulation when GLP-1 (1–36 amide) was infused at a rate of 400 ng/kg × h. This infusion rate increased the plasma levels of immunoreactive GLP-1 from 90 to about 600 pmol/l. We found no effect of synthetic GLP-1 (code 7166) or GLP-2 (code 7156; both from Peninsula) on the isolated perfused pancreas in concentrations up to 10^{-8} M either on the exocrine or the endocrine secretion [4]. By contrast, natural GLP-1, isolated from pig intestinal mucosa strongly increased insulin secretion from the same preparations. At a concentration of 10^{-10} mol/l of natural GLP-1 (determined by radioimmunoassay)

Table 1

Amino acid in mammalian proglucagon 78–96	Sequence cycle	Amino acid	Yield of amino acid phenylthiohydantoin derivative (pmol)	
			Batch 1	Batch 2
His	1	His	37	–
Ala	2	Ala	108	18
Glu	3	Glu	76	105
Gly	4	Gly	81	54
Thr	5	Thr	43	–
Phe	6	Phe	80	64
Ser	7	X	–	–
Ser	8	Ser	22	–
Asp	9	Asp	–[a]	32
Val	10	Val	64	47
Ser	11	X	–	–
Ser	12	X	–	–
Tyr	13	Tyr	46	24
Leu	14	Leu	–	14
Glu	15	Glu		16
Gly	16	X		–
Gln	17	Gln		16
Ala	18	X		–

[a] The unmethylated PTA-Asp could be identified close to the injection artifact but was not quantified

Sequence determination of the GLP-1 immunoreactive peptide isolated from porcine intestinal mucosa. The results of Edman degradation of two independent batches of peptide are shown. Identification and quantitation of the phenylthiohydantoin derivatives were performed on a Hewlett-Packard 1084 (1) or 1090A (2) chromatograph. The amino acid derivatives were methylated before identification in batch 2. (–) Uncertain identification

and 7 mmol/l glucose in the perfusate insulin secretion increased from 21.9 ± 2.6 (average ± SE of 5 min basal secretion in 2 preparations) to 31.6 ± 1.1 pmol/min (average of 5 min stimulated secretion); and at 10^{-9} mol/l insulin secretion increased from 32.0 ± 1.1 to 73.6 ± 2.9 pmol/min. A similar increase in insulin secretion was observed after administration of synthetic proglucagon 78–107 amide (figs 3,4). After arterial infusion of synthetic proglucagon 78–107 at 10^{-10} mol/l insulin secretion approximately doubled and increased more than 4-fold after 10^{-9} mol/l. Thus this peptide is at least as potent and effective as gastric inhibitory polypeptide (GIP), hitherto the most potent intestinal insulinotropic hormone [20]. At 5.0 mmol/l glucose in the perfusate the insulinotropic effect was less conspicuous; but the relative increase in insulin secretion was approximately the same (not shown).

On this background it might be suggested that the reported effects of GLP-1 in high doses could be due to enzymatic conversion of GLP-1 to proglucagon 78–107 in the medium. Trypsin-like enzymatic activity, which may be difficult to avoid in pancreatic tissue incubation studies, might be responsible for such conversion, due to the presence of a basic amino acid residue at position 76.

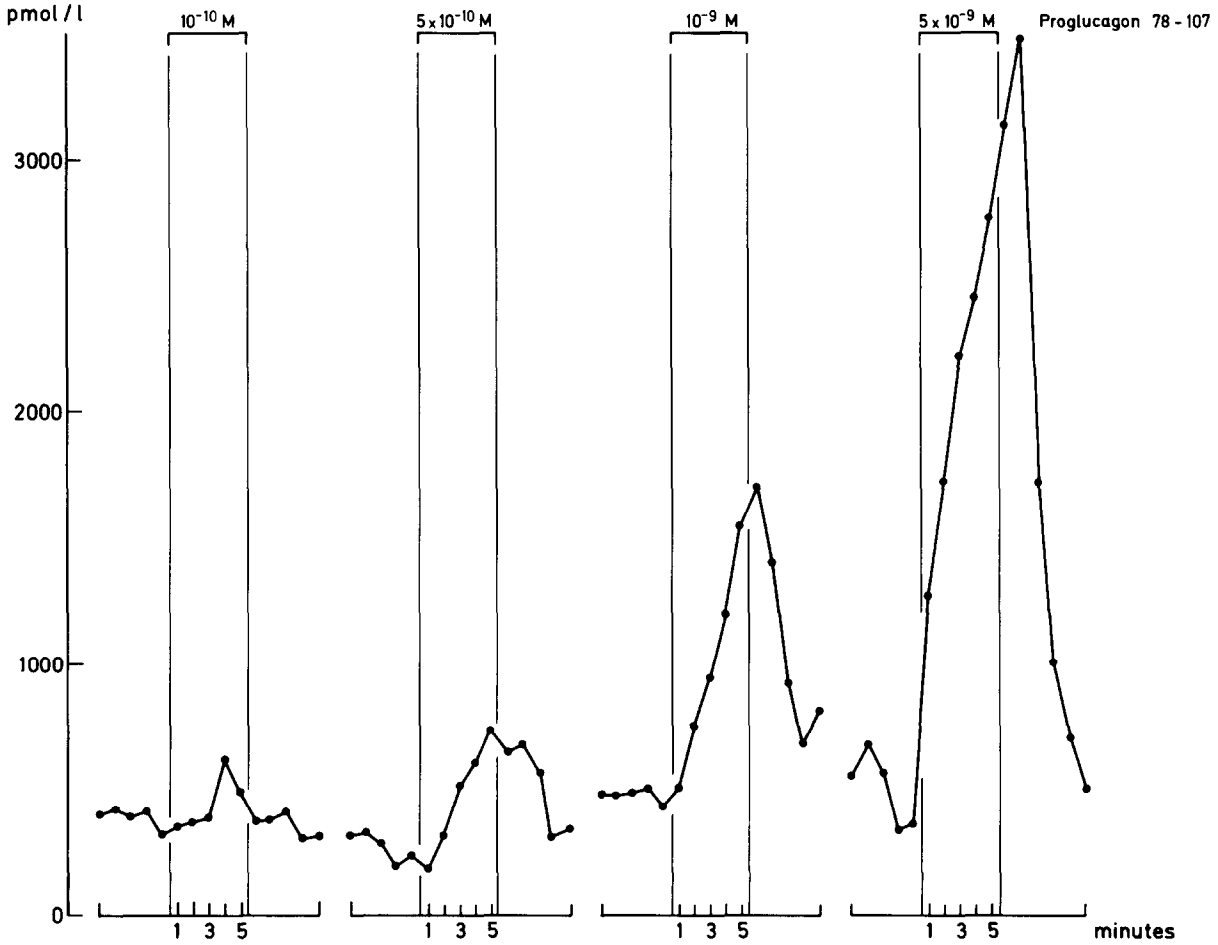

Fig.3. Insulin secretion of the isolated perfused pig pancreas in response to infusion of synthetic proglucagon 78–107 amide. The ordinate shows the concentration of insulin in the effluent. The final perfusate concentrations of proglucagon 78–107 amide are indicated in boxes. Results of a single representative experiment. Perfusate glucose concentration was 7.0 mmol/l.

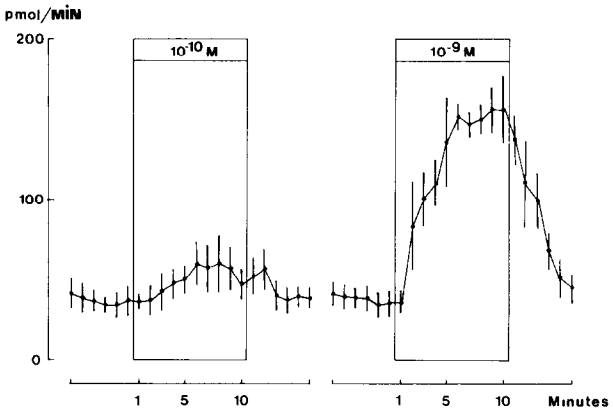

Fig.4. Insulin secretion (pmol/min) of the isolated perfused pig pancreas in response to infusion of synthetic proglucagon 78–107 amide at 10^{-10} and 10^{-9} mol/l. Glucose concentration in perfusate was 7 mmol/l. Mean ± SE, $n = 4$.

Previously, we found that the concentration of immunoreactive GLP-1 in plasma rose in response to a mixed meal in human volunteers [21]. Lauritsen et al. [22] have shown that the lower intestine contributes significantly to the insulinotropic effect of luminal versus intravenous glucose administration (the incretin effect). Our results suggest that proglucagon 78–107 may be the incretin of the lower gut.

REFERENCES

[1] Bell, G.I., Santerre, R.F. and Mullenbach, G.T. (1983) Nature 302, 716–718.
[2] Heinrich, G., Gros, P., Lund, P.K. and Habener, J.F. (1984) J. Biol. Chem. 259, 14082–14087.
[3] Holst, J.J. (1983) Gastroenterology 84, 1602–1613.
[4] Ørskov, A.C., Holst, J.J., Knuhtsen, S., Baldissera, F.G.A., Poulsen, S.S. and Nielsen, O.V. (1986) Endocrinology 119, 1467–1474.
[5] Patzelt, C. and Schiltz, E. (1984) Proc. Natl. Acad. Sci. USA 81, 5007–5011.
[6] Moody, A.J., Holst, J.J., Thim, L. and Jensen, S.L. (1981) Nature 289, 514–516.
[7] Newgard, C. and Holst, J.J. (1981) Acta Endocrinol. (Kbh.) 98, 564–572.
[8] Hunkapillar, M.W., Hewick, R.M., Dreyer, W.J. and Hood, L.M. (1983) Methods Enzymol. 91, 399–413.
[9] Hunkapillar, M.W. and Hood, L.E. (1983) Methods Enzymol. 91, 493–496.
[10] Jensen, S.L., Fahrenkrug, J., Holst, J.J., Kuhl, C., Nielsen, O.V. and Schaffalitzky de Muckadell, O.B. (1978) Am. J. Physiol. 235, E381–E386.
[11] Bradbury, A.F., Finnie, M.D.A. and Smyth, D.G. (1982) Nature 298, 686–688.
[12] Schwartz, T.W. (1986) FEBS Lett. 200, 1–10.
[13] Lund, P.K., Goodman, R.H., Montminy, M.R., Dee, P.C. and Habener, J.F. (1983) J. Biol. Chem. 258, 3280–3284.
[14] Plisetskaya, E.M., Pollock, H.G. and Kimmel, J.R. (1986) Can. J. Physiol. Pharmacol., July 1986, 29.
[15] Andrews, P.C. and Ronner, P. (1985) J. Biol. Chem. 260, 3910–3914.
[16] Holst, J.J. (1983) Handb. Exp. Pharmacol. 66, 245–261.
[17] Ghiglione, M., Uttenthal, L.O., George, S.K. and Bloom, S.R. (1984) Diabetologia 27, 599–603.
[18] Schmidt, W.E., Siegel, E.G. and Creutzfeldt, W. (1984) Diabetologia 28, 704–711.
[19] Scholdager, B., Ørskov, C., Holst, J.J. and Christiansen, J. (1986) Abstract presented at the World Congress of Gastroenterology, at Sao Paulo, 1986, Dig. Dis. Sci., issue of September.
[20] Lindkær Jensen, S., Holst, J.J., Vagn Nielsen, O. and Lauritsen, K.B. (1981) Acta Physiol. Scand. 111, 223–238.
[21] Ørskov, C., Holst, J.J., Baldissera, F.G.A. and Kirkegaard, P. (1986) Can. J. Physiol. Pharmacol., July 1986, 151–152.
[22] Lauritsen, K.B., Moody, A.J., Christensen, K.C. and Jensen, S.L. (1980) Scand. J. Gastroenterol. 15, 833–840.
[23] Lopez, L.C., Frazier, M.L., Su, C.-J., Kumar, A. and Saunders, G.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5485–5489.
[24] Seino, S., Welsh, M., Bell, G.I., Chan, S.J. and Steiner, D.F. (1986) FEBS Lett. 203, 25–30.