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N-terminal glycation of cholecystokinin-8 abolishes its insulinotropic action on clonal pancreatic B-cells

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Abstract

Monoglycated cholecystokinin octapeptide (Asp^1-glucitol CCK-8) was prepared under hyperglycaemic reducing conditions and purified by reverse phase-high performance liquid chromatography. Electrospray ionisation mass spectrometry and automated Edman degradation demonstrated that CCK-8 was glycated specifically at the amino-terminal Asp^1 residue. Effects of Asp^1-glucitol CCK-8 and CCK-8 on insulin secretion were examined using glucose-responsive clonal BRIN-BD11 cells. In acute (20 min) incubations, 10^{-10} mol/l CCK-8 enhanced insulin release by 1.2–1.5-fold at 5.6–11.1 mmol/l glucose. The stimulatory effect induced by 10^{-10} mol/l CCK-8 was abolished following glycation. At 5.6 mmol/l glucose, CCK-8 at concentrations ranging from 10^{-11} to 10^{-7} mol/l induced a significant 1.6–1.9-fold increase in insulin secretion. Insulin output in the presence of Asp^1-glucitol CCK-8 over the concentration range 10^{-11}–10^{-7} mol/l was decreased by 21–35% compared with CCK-8, and its insulinotropic action was effectively abolished. Asp^1-glucitol CCK-8 at 10^{-8} mol/l also completely blocked the stimulatory effects of 10^{-11}–10^{-8} mol/l CCK-8. These data indicate that structural modification by glycation at the amino-terminal Asp^1 residue effectively abolishes and/or antagonises the insulinotropic activity of CCK-8.

Keywords: Cholecystokinin-8; Glycation; Insulin secretion; BRIN-BD11 cell

1. Introduction

Cholecystokinins (CCKs) constitute a family of hormonal and neuronal peptides that exhibit pleiotropic effects in the brain, gut and pancreas [1,2]. Molecular forms of CCK ranging from 4 to 58 amino acids have been identified and the dominant forms found in the circulation after feeding are CCK-8, CCK-22, CCK-33, CCK-39 and CCK-58 [3]. The C-terminal sulphated octapeptide CCK-8 is well conserved across species and is the smallest form to retain a full range of biological activities [2,4].

CCK is secreted by endocrine cells in the duode-

Abbreviations: CCK-8, sulphated cholecystokinin-8; ESI-MS, electrospray ionisation mass spectrometry; GIP, gastric inhibitory polypeptide; *m/z*, mass-to-charge ratio; NIDDM, non-insulin-dependent diabetes mellitus; TFA, trifluoroacetic acid; tGLP-1, glucagon-like peptide 1(7–36)amide

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num and proximal jejunum of the small intestine in response to absorption of digested food components such as amino acids [5]. The feeding-induced release of CCK is indicative of an important role as a physiological regulator of digestion and post-prandial nutrient homeostasis. Indeed, many studies have demonstrated that CCK is an important satiety factor, regulator of intestinal motility and key stimulus for gall bladder contraction [1,2]. It also stimulates the secretion of pancreatic enzymes and insulin from the B-cells of the islets of Langerhans [1,2]. The action on the pancreatic B-cells [6,7] is notable as CCK is one of the strongest stimulators of islet phospholipase C, leading to activation of signal transduction pathways involving inositol trisphosphate, diacylglycerol, protein kinase C and phospholipase A_2 [8,9]. Thus, CCK together with conjoint parasympathetic nerve activation and release of enteroinsular hormones, such as gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (tGLP-1), are considered key determinants of the early 'cephalic' insulin response to meal ingestion [10,11].

Recent studies have indicated that structural modifications of peptide hormones by glycation can alter their biological potencies [12–16]. Whereas N-terminal glycation of insulin and tGLP-1 compromises their actions on target cells [12–14], glycation of GIP is associated with a considerable augmentation of insulin releasing activity both in vitro and in vivo [16,17]. We have recently demonstrated that glycation of CCK-8 markedly enhances its satiating activity when administered to mice prior to refeeding [15]. This action appears to be due in part to the fact that N-terminal glycation affords significant protection of the peptide against proteolytic degradation and inactivation by serum peptidases [15].

Since CCK-8 is an effective inhibitor of feeding in humans [18,19], these observations raise the possibility that glycated CCK-8 administration may have a potential therapeutic use in the management of obesity [15]. Furthermore, there is evidence that CCK-8 administration may be of benefit in patients with NIDDM [9,20]. The purpose of the present study was to examine the effects of long-acting Asp^1-glucitol CCK-8 on insulin secretion from clonal pancreatic B-cells to assess whether glycation modifies the insulinotropic activity of the peptide.

2. Materials and methods

2.1. Materials

Cholecystokinin sulphated octapeptide (CCK-8; Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-amide) was purchased from the American Peptide Company (Sunnyvale, CA, USA). High performance liquid chromatography (HPLC) grade acetonitrile was obtained from Rathburn (Walkersburn, UK). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, UK). All water was purified using a Milli-Q Water Purification System (Millipore Corporation, Millford, MA, USA). RPMI 1640 tissue culture medium, foetal calf serum, penicillin and streptomycin were purchased from Gibco Life Technologies, Paisley, Strathclyde, UK. $Na^{125}I$ (100 mCi/ml) for iodination of insulin was from Amersham International plc, Aylesbury, UK. Rat insulin standard was obtained from Novo Industria, Copenhagen, Denmark. All other chemicals were purchased from Sigma, Poole, UK.

2.2. Preparation of glycated CCK-8

Glycated CCK-8 was prepared and purified by HPLC as described previously [15]. In brief, CCK-8 was incubated with glucose under reducing conditions in 10 mmol/l sodium phosphate buffer pH 7.4 for 24 h. Control CCK-8 (non-glycated) was prepared in an identical manner except for the exclusion of glucose from the incubation mixture. The reaction was stopped by addition of 0.5 mol/l acetic acid and the sample mixture applied to a 250×4.6 mm Vydac C-18 analytical column (The Separations Group, Hesperia, USA) and gradient elution conditions were established using aqueous/TFA and acetonitrile/TFA solvents, as described previously [15]. Pooled glycated or control CCK-8 fractions were concentrated under vacuum (AES 1000 Savant, Life Sciences International, Runcorn, UK). They were then purified to homogeneity on a 150×4.6 mm Supelcosil C-8 column (Supelco, Poole, UK). Control and glycated CCK-8 samples were quantified using the Supelco C-8 column by comparing the peak areas with a standard curve constructed from known concentrations of CCK-8.

2.3. Structural characterisation

Glycated CCK-8 and control CCK-8 were dissolved (approximately 400 pM) in 100 μl of water and analysed by electrospray ionisation mass spectrometry (ESI-MS). Samples were applied to the LC/MS equipped with a microbore C-18 HPLC column (150×2.0 mm, Phenomenex UK, Macclesfield, UK). Samples (30 μl direct loop injection) were applied at a flow rate of 0.2 ml/min, under isocratic conditions 35% acetonitrile/water. Mass spectra were recorded on a Finnigan LCQ benchtop mass spectrometer (Finnigan MAT, Hemel Hempstead, UK) and spectra were obtained from the quadrupole ion trap mass analyser. Spectra were collected using full ion scan mode over the mass-to-charge (*m*/*z*) range 150–2000. The primary structure of glycated CCK-8 was determined by automated Edman degradation, using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH) amino acids under gradient elution conditions [15].

2.4. Culture of insulin-secreting cells

BRIN-BD11 cells were cultured in RPMI 1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mmol/l glucose. The production and characterisation of BRIN-BD11 cells are described elsewhere [21]. Cells were maintained in sterile tissue culture flasks (Corning, Glass Works, UK) at 37°C in an atmosphere of 5% CO_2 and 95% air using a LEEC incubator (Laboratory Technical Engineering, Nottingham, UK).

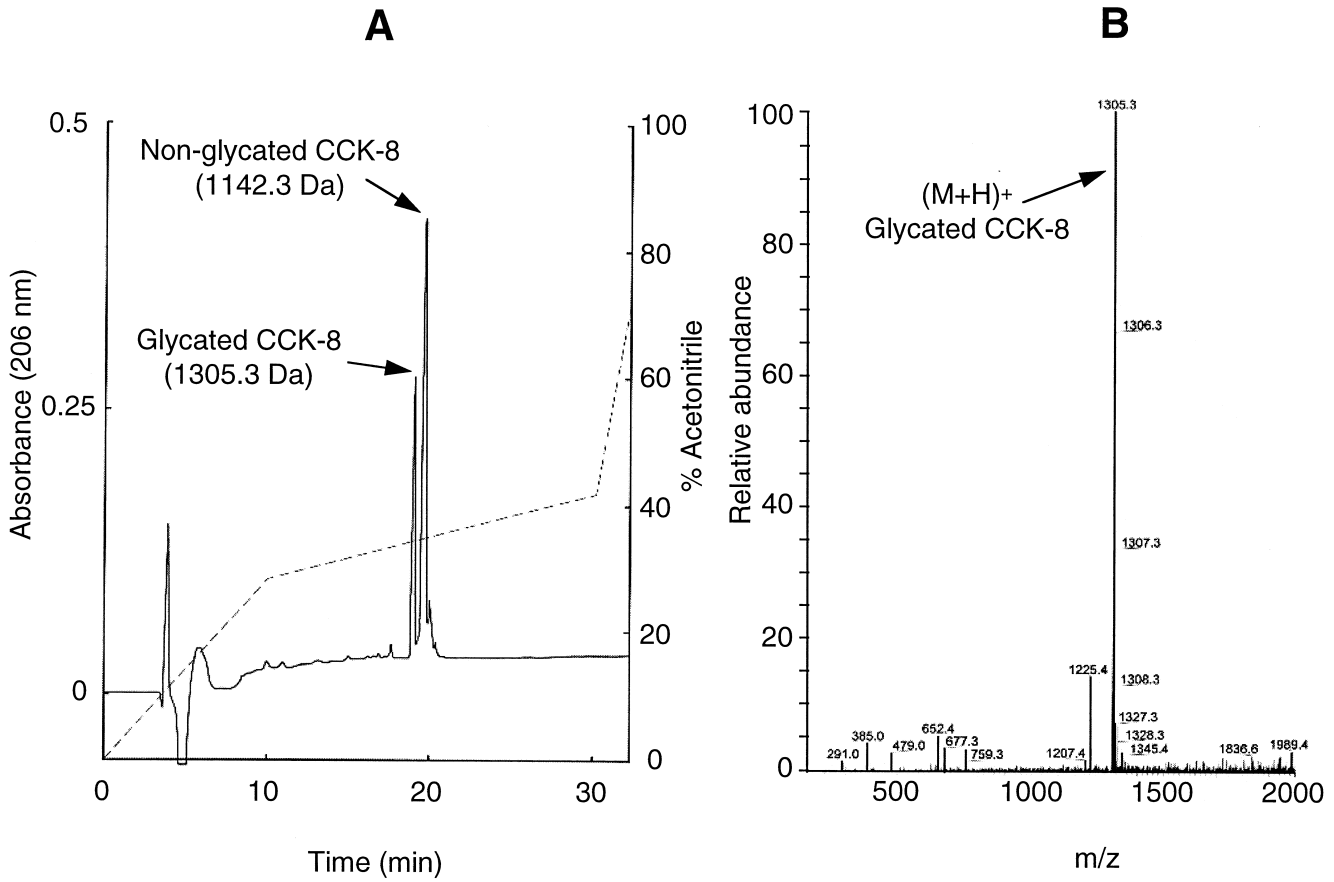

Fig. 1. (A) Reverse phase (RP)-HPLC profile for the separation of glycated and non-glycated CCK-8 on a Vydac C-18 column. (B) Molecular mass determination of glycated CCK-8. Glycated CCK-8 was seen to elute at 18.84 min and to be followed at 19.51 min by CCK-8. After completion of the HPLC purification, the molecular masses of CCK-8 (M_r 1142.3) and glycated CCK-8 (M_r 1305.3) were determined by ESI-MS.

2.5. Acute tests for insulin secretion

Insulin release was determined using monolayers of BRIN-BD11 cells. The cells were harvested with the aid of trypsin/EDTA (Gibco Life Technologies, Paisley, Strathclyde, UK), seeded into 24-well plates (Nunc, Roskilde, Denmark) at a density of 2.5×10^5 cells per well and allowed to attach during overnight culture. Acute studies of insulin release were preceded by 40 min pre-incubation at 37°C in 1.0 ml Krebs Ringer bicarbonate buffer (115 mmol/l NaCl, 4.7 mmol/l KCl, 1.28 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 10 mmol/l $NaHCO_3$ and 0.1% (w/v) bovine serum albumin, pH 7.4) supplemented with 1.1 mmol/l glucose. Test incubations were performed at 37°C using the same buffer supplemented with varying glucose concentrations, structurally modified CCK-8 or control CCK-8 as indicated in the figures. After 20 min incubation, the buffer was removed from each well and aliquots were stored at −20°C for subsequent measurement of insulin by radioimmunoassay [22].

2.6. Statistical analysis

Groups of data are presented as means ± S.E.M. Statistical evaluation was performed using Student's unpaired *t*-test. Differences were considered to be significant if $P<0.05$.

3. Results

3.1. HPLC separation and structural characterisation of glycated and non-glycated CCK-8

Glycated CCK-8 and non-glycated CCK-8 were clearly resolved by separation on a Vydac C-18 HPLC column with retention times of 18.84 min and 19.51 min, respectively (Fig. 1A). The molecular masses of glycated CCK-8 and control CCK-8 determined by ESI-MS were M_r 1305.3 and M_r 1142.3, respectively. The difference between the observed molecular masses (163.0 Da) indicated that the glycated peptide contained a single glucitol adduct (theoretical mass 164 Da) corresponding to monoglycated CCK-8 (Fig. 1B). Automated Edman degradation of monoglycated CCK-8 gave a yield of PTH-amino acids (in pmol) in consecutive cycles of: Asp (6), Tyr (283), Met (348), Gly (205), Tyr (155), Met (286), Asp (189) and Phe (65). The yield in the first cycle shows that Asp[1] was almost completely absent, indicating that the amino terminus was blocked. This evidence combined with the data from mass spectrometry clearly indicates that CCK-8 is glycated at the amino terminal Asp[1] residue.

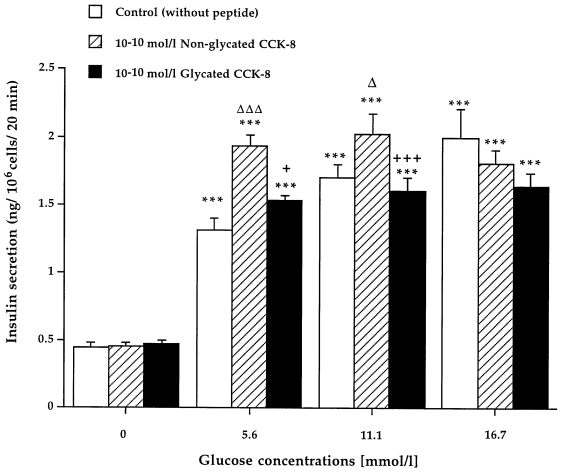

Fig. 2. The effects of glucose and 10^{-10} mol/l Asp[1]-glucitol CCK-8 or CCK-8 on insulin secretion. After 40 min of preincubation, the effects of glucose in the absence and presence of 10^{-10} mol/l peptide were tested during 20 min incubation. Values are means ± S.E.M. for eight separate observations. $^{***}P<0.001$ compared with 0 mmol/l glucose alone (control). $^{\Delta}P<0.05$, $^{\Delta\Delta\Delta}P<0.001$ compared to the same glucose concentration without peptide, $^{+}P<0.05$, $^{+++}P<0.001$ compared with CCK-8 at the same glucose concentration.

3.2. Effects of Asp[1]-glucitol CCK-8 and CCK-8 on insulin secretion

As indicated in Fig. 2, 5.6–16.7 mmol/l glucose induced a significant 3.0–4.5-fold stepwise stimulation of insulin secretion. Addition of 10^{-10} mol/l CCK-8 significantly enhanced insulin release by 1.2–1.5-fold at 5.6–11.1 mmol/l glucose. In contrast, 10^{-10} mol/l Asp[1]-glucitol CCK-8 failed to stimulate insulin release at all glucose concentrations tested. Compared with CCK-8, insulin secretion at 5.6–11.1 mmol/l glucose was 21% less in the presence of the structurally modified peptide.

Concentration-dependent studies of CCK-8 (10^{-14}–10^{-7} mol/l) conducted at 5.6 mmol/l glucose revealed a 1.6–1.9-fold stimulation of insulin release

at concentrations of 10^{-11} mol/l and above (Fig. 3). Glycation of CCK-8 abolished the stimulatory effects of the peptide when tested over the range 10^{-11}–10^{-7} mol/l compared with 5.6 mmol/l glucose alone.

To evaluate possible interactions between Asp^1-glucitol CCK-8 and CCK-8, peptide combinations were tested. As shown in Fig. 4, 10^{-8} mol/l Asp^1-glucitol CCK-8 decreased by 7–24% insulin release induced by 10^{-11}–10^{-7} mol/l CCK-8. The stimulatory effects of the peptide at all but the highest concentration (10^{-7} mol/l) were abolished by Asp^1-glucitol CCK-8.

4. Discussion

CCK-8 was glycated in vitro under hyperglycaemic reducing conditions which permitted purification of the stable Asp^1-glucitol adduct of CCK-8. This novel peptide has recently been shown to be a potent inhibitor of food intake [15], raising interesting questions concerning its possible therapeutic, physiological and pathophysiological roles. Further studies are required to clarify these issues and to determine whether Asp^1-glucitol CCK-8 occurs in vivo. However, it is notable that insulin and tGLP-1 which can be glycated in vitro also exist naturally in pancreas or intestine [14,23–25].

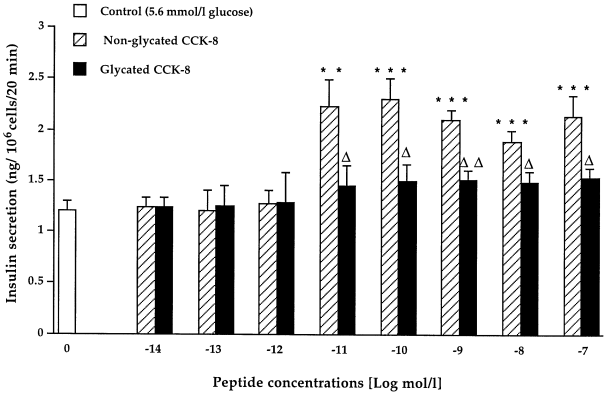

Fig. 3. The effects of various concentrations of Asp^1-glucitol CCK-8 or CCK-8 on insulin release at 5.6 mmol/l glucose. After 40 min preincubation, the effects of various concentrations of peptide (10^{-14}–10^{-7} mol/l) were tested during a 20 min incubation period. Values are means ± S.E.M. for eight separate observations. $^{**}P<0.01$, $^{***}P<0.001$ compared with 5.6 mmol/l glucose alone (control). $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ compared with CCK-8 at the same peptide concentration.

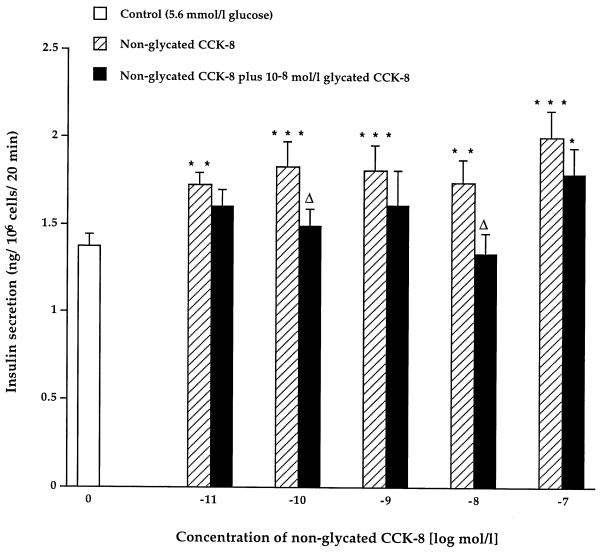

Fig. 4. The effects of 10^{-8} mol/l Asp^1-glucitol CCK-8 on insulin secretion induced by 10^{-11}–10^{-7} mol/l CCK-8 at 5.6 mmol/l glucose. After 40 min pre-incubation, the effects of 10^{-11}–10^{-7} mol/l CCK-8 were tested in the presence and absence of 10^{-8} mol/l Asp^1-glucitol CCK-8 during a 20 min incubation period. Values are means ± S.E.M. for eight separate observations. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with 5.6 mmol/l glucose alone (control). $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ compared with the same CCK-8 concentration without added Asp^1-glucitol CCK-8.

The current study examined the effects of CCK-8 and Asp^1-glucitol CCK-8 on insulin secretion using the clonal pancreatic BRIN-BD11 cell line. This relatively new cell line, derived from electrofusion of rat pancreatic B-cells with immortal RINm5F cells, is glucose sensitive and exhibits good responsiveness to a wide range of peptides and neurotransmitter substances [14,16,21,26,27]. Consistent with this view, CCK-8 exhibited insulinotropic effects on BRIN-BD11 cells at peptide concentrations from 10^{-11} to 10^{-7} mol/l. As noted elsewhere, this action was glucose-dependent [6,7,28], being demonstrable with the clonal B-cells only at moderate glucose concentrations of 5.6 and 11.1 mmol/l.

The mechanism of CCK-8 stimulated insulin release is complementary to the main pathways induced by glucose and it appears to involve binding to a specific subtype of CCK_A receptors on the B-cell [6,28,29]. This triggers a complex series of events involving stimulation of phospholipase C and the production of inositol trisphosphate and diacylglycerol [8,30–32]. The former augments intracellular Ca^{2+} by

affecting endoplasmic reticulum calcium stores whereas diacylglycerol activation of protein kinase C induces protein phosphorylation to enhance the Ca^{2+} sensitivity of the insulin exocytotic machinery [33]. Recent studies also indicate that CCK-8 promotes the generation of arachidonic acid through activation of both Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A_2 [9]. The glucose sensitivity of the CCK-8 effect may reflect dependence on glucose metabolism to elevate intracellular Ca^{2+} or influence other key elements of the phospholipase C pathway [30]. Maximal effects of CCK-8 on insulin release are known to occur at concentrations around 10 mmol/l glucose [6,7,28]. However, the mechanism underlying the relative ineffectiveness of CCK-8 at 16.7 mmol/l glucose is unclear. Interestingly, such an effect has been noted recently for tGLP-1 [14,34,35], indicating that both peptides are particularly well equipped to influence insulin secretion over a restricted physiological glucose concentration range.

A key finding of the present paper is that glycation of CCK-8 at the α-amino Asp^1 site severely compromised the insulinotropic action of the peptide. Activity was decreased by up to 35% compared with CCK-8 and the Asp^1-glucitol CCK-8 failed to stimulate insulin release over the entire concentration range of 10^{-11}–10^{-7} mol/l. This decrease of biological activity extends previous observations that glycation of peptides can significantly alter their biological potencies [12–16]. However, the decreased insulinotropic activity of Asp^1-glucitol CCK-8 contrasts sharply with our recent observation that glycation of CCK-8 considerably augments its potency as a peripherally acting satiety agent [15]. Thus, although part of the in vivo effect may be due to increased resistance of Asp^1-glucitol CCK-8 to serum aminopeptidase degradation [15], receptor-mediated effects on the central nervous system are clearly intact. It appears therefore that glycation of CCK-8 specifically impairs the function of the CCK_A receptor subtype involved in insulin secretion [28] without greatly affecting receptor binding and signal transduction pathways involved in feeding at target cells endowed with other CCK_A receptor subtypes or CCK_B receptors [28,36,37].

Further studies including observations using human islets, are clearly warranted to elucidate the mechanism underlying the glycation-induced decrease of insulinotropic potency. CCK-7 is considerably less potent than CCK-8 in stimulating insulin release [38], suggesting that N-terminal modification of the parent peptide may interfere with receptor binding. In fact, the present demonstration that Asp^1-glucitol CCK-8 was able to effectively abolish the insulin stimulatory action of 10^{-11}–10^{-7} mol/l CCK-8 suggests that the modified peptide may act as an antagonist at the CCK_A receptor subtype on pancreatic B-cells. This possibility contrasts with the enhanced insulinotropic activity of Tyr^1-glucitol GIP [16], but it strongly parallels the antagonistic properties of some N-terminally modified analogues of GLP-1(7–36)amide at the islet tGLP-1 receptor [14,39–43].

In summary, this paper demonstrates that glycation of CCK-8 at the α-amino group of Asp^1 abolishes/antagonises the insulinotropic action of the peptide. Thus unlike long-acting analogues of tGLP-1 and GIP which are under consideration for NIDDM therapy [17,42], the aminopeptidase resistant Asp^1-glucitol adduct of CCK-8 offers no promise as a novel long-acting insulin-releasing drug. However, the observation that glycation of CCK-8 markedly enhances its peripheral satiety effect without influencing insulin secretion, and thereby avoiding any threat of mounting insulin resistance or hypoglycaemia, favours a therapeutic potential of this structurally modified form of CCK-8 as a novel anti-obesity agent [15].

Acknowledgements

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