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The effects of structural modifications on the insulin-releasing activity of β-cell-tropin

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The minimal effective concentration of the pituitary insulin secretagogue β-cell-tropin (β-CT) on the *in vitro* perfused pancreas was established and the effects of various modifications of the peptide on its potency were tested: iodination with ^{127}I and acetylation reduced the insulin-releasing activity of β-cell-tropin, and the *C*-terminal fragments β-CT-(2–18), β-CT-(3–18) and β-CT-(6–18) were all less potent than the intact molecule; β-CT-(1–6) was not active and did not inhibit β-CT-induced insulin release.

INTRODUCTION

β-Cell-tropin (β-CT) is a peptide which was originally isolated from perifusates (superfusates) of the isolated pituitary neurointermediate lobe of the genetically obese (*ob/ob*) mouse (Beloff-Chain *et al.*, 1980). β-CT has a range of biological activities. (1) It stimulates the release of insulin, at substimulatory concentrations of glucose, from the isolated perfused rat pancreas (Dunmore & Beloff-Chain, 1982) and from perifused islets of Langerhans (Billingham *et al.*, 1982) eliciting a monophasic response. (2) It potentiates the biphasic release of insulin induced by high concentrations of glucose from the perfused pancreas (Beloff-Chain *et al.*, 1981). (3) It has an insulin-like action in promoting lipogenesis in adipose tissue (Watkinson & Beloff-Chain, 1984). (4) Preliminary studies have shown that glucagon release is also stimulated by β-CT (S. J. Dunmore, unpublished work).

β-CT has been characterized as ACTH-(22–39) (Fig. 1) (Beloff-Chain *et al.*, 1983) and evidence of its hormonal nature is provided by its presence in plasma of the obese mouse (Billingham *et al.*, 1982) and human plasma (Salvatoni *et al.*, 1986).

In the present study the relationship of the structure of β-CT to its stimulation of the monophasic insulin release from the perfused pancreas was studied, testing various modifications of the molecule. As the closely related peptide corticotropin-like intermediate-lobe peptide (CLIP) [ACTH-(18–39)] is inactive (Beloff-Chain *et al.*, 1983), it seems likely that a free *N*-terminus is necessary for activity, and in preliminary reports this concept was supported by the finding that acetylation of intact β-CT reduced its activity (Dunmore *et al.*, 1983, 1984). It was also shown in preliminary studies that β-CT-(6–18) is active at relatively high concentrations, and that iodination of the tyrosine residue [the penultimate residue at the *N*-terminal end of the molecule (Fig. 1)] reduced its biological activity (Dunmore *et al.*, 1984).

These preliminary observations have been extended to establish the minimal effective concentration of β-CT required to produce biological activity compared with β-CT-(2–18), β-CT-(3–18) and β-CT-(6–18). The *N*-terminal fragment β-CT-(1–6) was also tested for insulin-releasing activity and as a possible inhibitor of intact β-CT.

MATERIALS AND METHODS

Materials

Natural mouse β-CT was obtained from incubated *ob/ob* mouse neurointermediate lobes, purified by

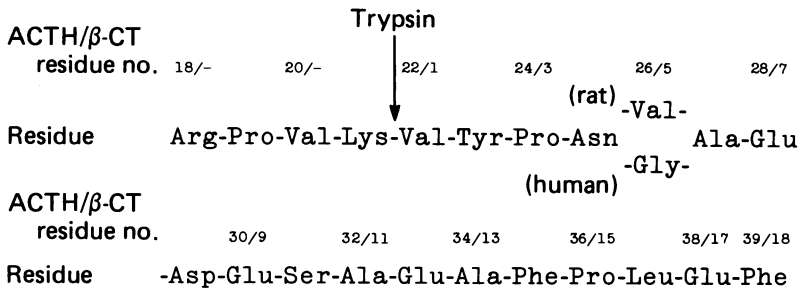

Fig. 1. Sequence of CLIP and β-CT

The arrow indicates the point of cleavage of CLIP by mild treatment with trypsin to produce β-CT (see the Materials and methods section). Numbering of ACTH is indicated by the figure preceding the solidus and that of β-CT by the figure following the solidus.

Abbreviations used: β-CT, β-cell-tropin; ACTH, adrenocorticotropin; CLIP, corticotropin-like intermediate-lobe peptide; α-MSH, α-melanotropin; CCK, cholecystokinin.

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Bio-Gel P-2/P-6 gel filtration and reverse-phase h.p.l.c. as has been previously described (Beloff-Chain *et al.*, 1981).

Synthetic β-CT was prepared from commercially produced synthetic 'human'-sequence CLIP (UCB-Bioproducts, Brussels, Belgium) by mild digestion with trypsin followed by reverse-phase h.p.l.c., as detailed in Beloff-Chain *et al.* (1983).

β-CT-(6–18) was a gift from Dr. B. Riniker (Ciba-Geigy Pharmaceuticals).

β-CT-(1–6) was synthesized by Dr. J. Humphries (Beecham Research Division) using the techniques described for whole β-CT synthesis in Humphries *et al.* (1983).

β-CT-(2–18) and β-CT-(3–18) were produced from β-CT (synthetic) by manual Edman degradation using 5% phenylisothiocyanate in pyridine. The coupling reaction was performed at 45 °C for 1 h under an N_2 atmosphere. Cleavage was carried out with trifluoroacetic acid at 45 °C for 10 min. The products were separated by reverse-phase h.p.l.c. using acetic acid/propan-1-ol gradient elution as described in Morris *et al.* (1980), and their structures confirmed by fast atom bombardment m.s. on a VG ZAB high field mass spectrometer, equipped with an H-SCAN fast atom bombardment gun (Morris *et al.*, 1981).

Acetylated β-CT was prepared from natural β-CT by reaction with acetic anhydride in methanol (1:4, v/v), followed by purification on reverse-phase h.p.l.c. (Beloff-Chain *et al.*, 1981).

^{127}I-β-CT was prepared from tryptic β-CT using the chloramine T method of Hunter & Greenwood (1962), followed by reverse-phase h.p.l.c. purification.

Successful acetylation or iodination were confirmed by M_r determination on fast atom bombardment m.s. (Beloff-Chain *et al.*, 1983). The modified β-CT samples were quantified by u.v. absorption and CLIP radioimmunoassay (Salvatoni *et al.*, 1986).

All the peptides described above appeared to be homogeneous by reverse-phase h.p.l.c.

Pancreas perfusion

Pancreata from rats weighing approx. 250 g were perfused as previously described (Dunmore & Beloff-Chain, 1982). Peptide samples for testing were dissolved to the required concentration in perfusion buffer containing 5.6 mM-glucose. After a 15–20 min stabilization period, 5 min of 1 min fractions were collected to establish the basal secretion rate at 5.6 mM-glucose, followed by 5 min of the buffer containing the peptide sample (sufficient time to allow for the characteristic 2–3 min monophasic stimulation of insulin secretion evoked by β-CT). After this, buffer containing 16.7 mM-glucose was perfused for 10 min; a normal biphasic response of insulin release confirmed the viability of the preparation. Insulin concentration was measured by a standard double-antibody radioimmunoassay.

RESULTS

Potency of β-CT

Intact ('synthetic') β-CT was tested (in the perfused pancreas, for effects on insulin release) at concentrations of 2 ng/ml (1 nmol/l); 0.4 ng/ml (200 pmol/l); 0.04 ng/ml (20 pmol/l) and 0.01 ng/ml (5 pmol/l) (Fig. 2). β-CT was equally potent at the two highest concentrations. It was less active at 0.04 ng/ml (although not significantly so). At 0.01 ng/ml β-CT caused only a small stimulation of insulin secretion.

Acetylated and iodinated β-CT

Both *N*-acetylated and iodinated β-CT, tested at concentrations of 1.3 ng/ml (0.64 nmol/l) and 1.0 ng/ml (0.47 nmol/l) respectively, gave stimulations of insulin release that were significantly smaller than those caused by β-CT at a similar concentration of 2 ng/ml (1 nmol/l) or at a lower level of 0.4 ng/ml (0.2 nmol/l) (Fig. 2).

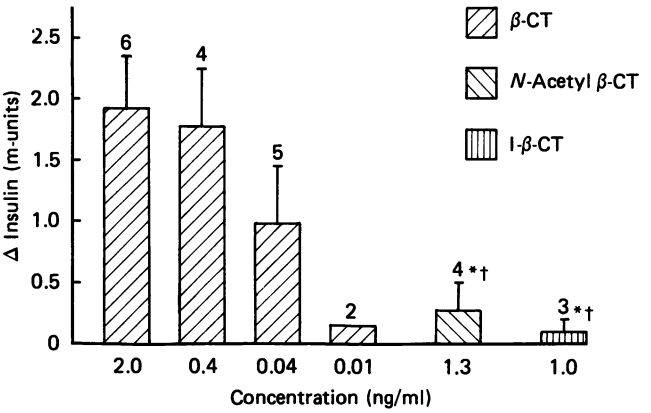

Fig. 2. Effect of various concentrations of intact β-CT, and of *N*-acetyl β-CT and ^{127}I-iodinated β-CT, on insulin release from perfused pancreas

Δ Insulin is the total insulin secreted in excess of basal in the monophasic peak. Columns and bars show means and S.E.M. values, respectively; the number of experiments is indicated above the bar. Mean basal secretion rate was 128.0±11.3 μunits/min ($n = 24$) *Significant difference ($P < 0.05$) between peptide and intact β-CT at 2.0 ng/ml; †significant differences between peptide and intact β-CT at 0.4 ng/ml.

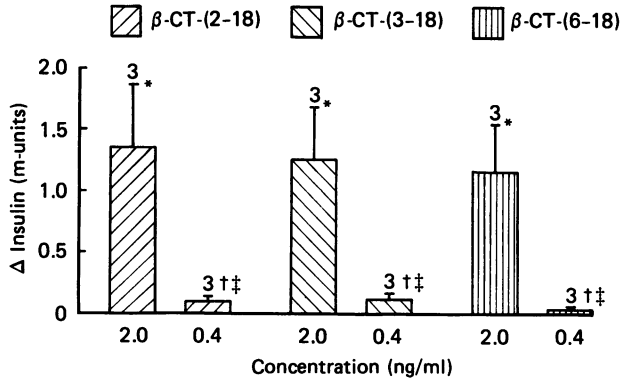

Fig. 3. Effect of β-CT-(2–18), β-CT-(3–18) and β-CT-(6–18) (each at 2.0 and 0.4 ng/ml) on insulin release from perfused pancreas

Δ Insulin is the total insulin secreted in excess of basal in the monophasic peak. Columns and bars show means and S.E.M. values, respectively; the number of experiments is indicated above the bar. Mean basal secretion rate was 118.8±11.2 μunits/min ($n = 18$). *Significant difference between peptide at 2.0 and 0.4 ng/ml; †significant difference between peptide at 0.4 ng/ml and intact β-CT at 2.0 ng/ml (see Fig. 2); ‡significant difference between peptide at 0.4 ng/ml and intact β-CT at 0.4 ng/ml (see Fig. 2).

β-CT-(2–18), β-CT-(3–18) and β-CT-(6–18)

Stimulations of insulin secretion by all three of these *C*-terminal peptides were not significantly different from those caused by β-CT at 2 ng/ml (Fig. 3), but all evoked much less insulin secretion at 0.4 ng/ml than did the whole peptide.

β-CT-(1–6)

Synthetic β-CT-(1–6) was tested at 2 ng/ml (2.8 nmol/l) but did not cause any increase in insulin secretion.

The peptide, used at the same concentration, did not appear to inhibit natural mouse β-CT at a concentration of 0.4 ng/ml (0.2 nmol/l). In two experiments the mean total insulin release (Δ insulin) following introduction of the natural β-CT was 1.36 m-unit, which is in the range obtained using intact β-CT alone at 0.4 ng/ml (see Fig. 1).

DISCUSSION

It is apparent from the results above that β-CT is a very potent insulin secretagogue, able to increase insulin secretion substantially at concentrations similar to those found recently in human plasma (Salvatoni *et al.*, 1986) and certainly at the levels measured previously in *ob/ob* mouse plasma (Billingham *et al.*, 1982).

Acetylation of a number of peptide hormones is known to occur physiologically and has been shown to be of importance in modulating the activity of these substances. For example, various biological activities of α-MSH alter according to its acetylation state. The principal form of α-MSH is acetylated on the free amino group of the *N*-terminal serine, but it is recognized that the precursor, desacetyl-α-MSH (not acetylated), is the main type of α-MSH in the brain, and is generally less active than monoacetyl α-MSH or another form, 'diacetyl α-MSH' (Rudman *et al.*, 1983). Another peptide of the pituitary pars intermedia, β-endorphin, has been shown to be inactivated by acetylation (Smyth *et al.*, 1979). Acetylated forms of CLIP or β-CT are not known to occur naturally (although phosphorylated and glycosylated CLIPs have been isolated; Bennett *et al.*, 1982). Since CLIP is inactive it was considered possible that artificial acetylation of the *N*-terminal of β-CT could affect its activity, and the results detailed above show that this is indeed the case, *N*-acetyl β-CT being much less potent than β-CT.

Introduction of a large atom, iodine-127, onto the tyrosine at residue 2 of β-CT (see Fig. 1) is shown by the data in Fig. 2 to inactivate the peptide. This is similar to the effect of β-CT iodination on its stimulation of lipogenesis in isolated adipocytes, which has been described by Watkinson & Beloff-Chain (1984). It seems, therefore, that the *N*-terminal of β-CT has an important role in its biological activity, any obstruction of this end of the molecule reducing its insulin-releasing activity. The *N*-terminal fragment β-CT-(1–6) is not an active secretagogue and furthermore appears to have no capacity to inhibit the activity of β-CT, which suggests that this part of the molecule does not bind to the receptor. Moreover, *C*-terminal fragments β-CT-(2–18), -(3–18) and -(6–18) are all active although much less potent than the entire molecule. It may be surmised, then, that receptor binding and activation sequences occur towards the *C*-terminal portion of the peptide, but that the *N*-terminal is important in determining the configuration of the whole molecule which allows full activation. It is also possible that any increase in the size of the β-CT molecule may affect its 'fit' to receptor.

Other examples of insulin secretagogues which are fragments of a larger peptide hormone are CCK-8 and CCK-4 (Frame *et al.*, 1975; Rehfeld *et al.*, 1980), which consist of the *C*-terminal portions of the parent peptides CCK-33 and CCK-39, which also have insulin-releasing activity, whereas ACTH, the 39-residue peptide of which β-CT is a *C*-terminal fragment, has been shown only to have insulin-releasing activity at very high and unphysiological concentrations, as previously discussed in Beloff-Chain *et al.* (1975).

Future studies will be directed at the elucidation of the precise sites of receptor binding and activation on the β-CT molecule. The perfused pancreas has been found to be the most sensitive method for structure–function studies on β-CT; however, it is envisaged that isolated perifused islets and isolated adipocytes may prove to be more suitable systems for such receptor studies.

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