STUDIES ON FREE AMINO ACIDS IN THE PANCREATIC β-CELLS

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This thesis is based on the following publications, which will be referred to by their Roman numerals.

I *Gylfe, E:* Changes of free amino acids in pancreatic β-cells after starvation and substrate deprivation. *Acta endocr (Kbh)* 75, 105 (1974).

II *Gylfe, E and Hellman, B:* Role of glucose as a regulator and precursor of amino acids in the pancreatic β-cells. *Endocrinology* in press.

III *Gylfe, E:* Glucose oxidation and contents of free amino acids in pancreatic β-cells stimulated by a non-metabolizable leucine analogue. *Biochim Biophys Acta* in press.

IV *Gylfe, E, Hellman, B, Sehlin J and Täljedal, I-B:* Amino acid conversion into 5-hydroxytryptamine in pancreatic β-cells. *Endocrinology* 93, 932 (1973).

INTRODUCTION

Several amino acids are insulin secretagogues (FAJANS *et al* 1967; LAMBERT *et al* 1969; MALAISSE 1969; MILNER 1970), whereas others act as inhibitors of glucose-stimulated insulin release (LERNMARK 1971 *a;* ROSSINI & BUSE 1973). Amino acids are also important as precursors in insulin biosynthesis. Although amino acids are thus intimately involved in β-cell physiology, there have been few attempts to measure free amino acids in the β-cell. This is doubtlessly due to the methodological difficulties involved in experimentation with the small amounts of tissue represented by pure mammalian pancreatic islets. The aim of the studies forming the basis of this thesis was consequently:

1 To develop a technique sensitive enough to allow measurements of the contents and fluctuations of different free amino acids in isolated pancreatic islets.

2 To study the contents of free amino acids in β-cell-rich pancreatic islets representing various states of metabolic and secretory activity, with special attention to whether stimulation of insulin release is mediated by altered β-cell concentrations of certain naturally occurring amino acids.

3 To study glucose conversion into amino acids in connection with stimulation and inhibition of insulin release.

4 To study whether a non-metabolizable insulin-releasing amino acid might stimulate insulin release through enhanced glucose metabolism.

5 To study how the metabolic conversion of amino acids into 5-hydroxytryptamine might be related to inhibition of insulin release.

GENERAL DESIGN OF EXPERIMENTS

Source and isolation of tissue samples representative of β-cells.

Non-inbred obese-hyperglycemic mice (gene symbol *ob/ob*) were taken from a local colony (HELLMAN 1965). The pancreatic islets from these animals consist to more than 90 % of β-cells (GEPTS *et al* 1960; HELLMAN 1961) which are known to respond adequately to various secretagogues (LERNMARK 1971 *b*).

β-cells representative of the *in vivo* situation (I) were obtained by microdissection from freeze-dried pancreas sections (*cf* LOWRY 1953). When metabolically active samples of β-cells were required for *in vitro* experiments (I—IV), islets were microdissected freehand (HELLERSTRÖM 1964) from fresh pancreas submerged in Krebs-Ringer bicarbonate buffer (KRB).

Incubation procedures and weighing of islet samples.

All incubations were performed at 37° C with KRB as basal medium. In most experiments batches of islets were first preincubated for 30 min (II—IV). They were then either incubated in closed vials (I—IV) or were perifused according to IDAHL (1972) (I). Incubated islets were freeze-dried overnight (—40° C, 0.1 Pa) and weighed on an electrobalance or on a quartz-fibre balance (LOWRY 1953).

TECHNIQUES FOR MEASURING SMALL AMOUNTS OF AMINO ACIDS IN BIOLOGICAL MATERIALS

Several methods are available for qualitative and quantitative determinations of amino acids. Measurements of free amino acids in the β-cells are complicated by the small size and heterogeneity of pancreatic islets. The conversion of glucose and fructose into amino acids in the so-called principal islets of the bony fish *Cottus quadricornis L* has been studied with the aid of paper chromatography (HELLMAN & LARSSON 1961). However, that technique did not allow quantitation of the islet content of amino acids. The advantage of using

islets from *ob/ob*-mice for amino acid measurements is not only related to their mammalian origin but also to the fact that they essentially consist of insulin-producing β-cells. Since mammalian islets are small, a very sensitive amino acid assay is required. Enzymatic techniques are specific and sufficiently sensitive, especially if they are coupled to enzymatic cycling. At present, however, such methods are restricted to certain amino acids, only one of which can be determined in each analysis. DANIELSSON *et al* (1970) measured the concentrations of glutamic acid in β-cells by using glutamate dehydrogenase. With micro column chromatography in association with colorimetric determination with ninhydrin PANTEN *et al* (1972) were able to assay 6 different amino acids in *ob/ob*-mice. Although that technique made it possible to study several amino acids simultaneously, as many as 50 pancreatic islets had to be used for each analysis. The sensitivity of that method can be expected to be improved by fluorometry with fluorescamine as recently described (UDENFRIEND *et al* 1972). Gas chromatographic systems do not seem to have been applied for amino acid determinations in pancreatic islets, although the sensitivity appears to be adequate (ZUMWALT *et al* 1971). A technique based on the reaction of amino acids with radioactively labelled dansyl chloride and the subsequent separation of dansylated products by two-dimensional thin-layer microchromatography (NEUHOFF *et al* 1969) allows quantitation of amino acids in the range of 10^{-12} moles. Such sensitivity would make it possible to determine amino acids in as little as 1 μg of dry islet tissue. In contrast to most other methods, the microchromatographic approach with dansylation also allows simple tracer studies of amino acid formation. The dansylation method is technically rather difficult, but on the other hand is quite rapid and does not require expensive chromatographic apparatus.

Our first application of the dansylation method to pancreatic islets from *ob/ob*-mice resulted in preliminary estimates of the β-cell contents of 12 different free amino acids (BRIEL *et al* 1972). The dansylation technique has now been improved to yield more accurate estimates (I). The sensitivity was increased by using ^{3}H-dansyl chloride of higher specific activity than the previously used ^{14}C-labelled compound. Alanine and proline, which were incompletely separated, were excluded from the analyses. When measuring glutamic acid, particular attention was paid to a spot consisting of N-dansyl-5-oxo-2-pyrrolidine carboxylic acid, a cyclic reaction product formed from glutamic acid (SEILER *et al* 1971). It has recently been reported that N-terminal glutamic acid and glutamine in peptides could form identical cyclic dansyl derivatives (TAMURA *et al* 1973). Since this could introduce an overestima-

tion of the glutamic acid concentration, it was checked whether free glutamine, like glutamic acid, forms N-dansyl-5-oxo-2-pyrrolidine carboxylic acid in the dansylation system employed. Small amounts of uniformly ^{14}C-labelled glutamine were added to islet extracts which were dansylated with unlabelled dansyl chloride. After chromatographic separation of the dansylated extract, the spots of dansyl-glutamine, dansyl-glutamic acid, N-dansyl-5-oxo-2-pyrrolidine carboxylic acid and the remainder of the chromatogram were scraped off for scintillation counting *(cf* I, II). The percentage recoveries of radioactivity (mean values ± SEM) in 4 experiments were as follows: dansyl-glutamine 80.9 ± 1.5, dansyl-glutamic acid 0.4 ± 0.1 and N-dansyl-5-oxo-2-pyrrolidine carboxylic acid 0.1 ± 0.0. The minute amounts of radioactivity found in dansyl-glutamic acid and N-dansyl-5-oxo-2-pyrrolidine carboxylic acid are due to the contamination of the ^{14}C-glutamine preparation with glutamic acid and 5-oxo-2-pyrrolidine carboxylic acid as specified by the manufacturer. There was consequently no detectable formation of N-dansyl-5-oxo-2-pyrrolidine carboxylic acid from glutamine under the conditions of dansylation used.

THE CONTENTS OF FREE AMINO ACIDS

The islet contents of free amino acids were fairly stable under the conditions tested (I—III). In all cases the amino acid pattern was characterized by the presence of γ-aminobutyric acid (GABA) and large amounts of taurine. The GABA concentrations observed can hardly be explained by the nervous component of islet tissue since the islets in rodents are sparsely innervated (LACY 1960; FALCK & HELLMAN 1963). However, the GABA content may possibly be related to a neural crest origin of islet cells as proposed by PEARSE *et al* (1973). Intact islets isolated for *in vitro* studies contained more leucine and valine than specimens obtained from freeze-dried pancreatic sections representing the *in vivo* situation (I). Protein catabolism might explain the high concentrations of these essential amino acids, because no amino acids were added to the isolation medium. When microdissected islets were incubated in substrate-free medium the concentrations of leucine and valine remained high, whereas aspartic acid decreased (I). However, perifusion of the isolated islets in the substrate-free medium depressed the concentrations of glycine, leucine, lysine, phenylalanine and valine (I). Starvation increased the β-cell concentrations of GABA and leucine but reduced the contents of aspartic acid and glutamic acid (I).

The β-cell concentrations of aspartic acid and GABA decreased after glucose stimulation of insulin release (II). Both the insulin-releasing b(—) isomer and the secretorily inactive b(+) isomer of the non-metabolizable leucine analogue 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH) reduced the islet contents of aspartic acid, GABA and glutamic acid but increased that of phenylalanine (III). The effects of glucose and BCH on the contents of aspartic acid, GABA and glutamic acid may be due to increased metabolism of these amino acids. Glucose has been shown to increase glutamic acid oxidation (SEHLIN 1972) and BCH stimulates the activity of glutamate dehydrogenase (GYLFE 1974). Stimulation of insulin release by glibenclamide and inhibition of glucose-stimulated release by epinephrine or diazoxide were not associated with altered amino acid concentrations (II). The results lend no support to the idea that stimulation of insulin release by glucose, BCH or glibenclamide is mediated by altered β-cell concentrations of certain naturally occurring amino acids.

GLUCOSE CONVERSION TO AMINO ACIDS

Most amino acid radioactivity was recovered in aspartic acid, GABA and glutamic acid after incubating microdissected islets with uniformly ^{14}C-labelled D-glucose (II). The incorporation of glucose carbon into glutamic acid increased when the β-cell metabolism and insulin release were stimulated by raising the glucose concentration to 20 mM. This increase may be related to the enhanced glutamic acid oxidation previously observed after exposing β-cells to high glucose concentrations (SEHLIN 1972). The high glucose concentration tended to reduce the size of the amino acid pool and was consequently also associated with increased incorporation per mole of aspartic acid, GABA and glutamic acid. The amino acid formation from glucose was not affected by the addition of glibenclamide — a secretagogue — or by epinephrine or diazoxide — inhibitors of glucose-stimulated insulin release. The specific incorporation of glucose carbon into GABA was 2—4 times higher than into glutamic acid, suggesting compartmentation of glutamic acid, which is the precursor of GABA. The probable existence of amino acid compartmentation raised the question whether conversion of glucose into aspartic acid and glutamic acid is part of the mechanism by which this substance stimulates the biosynthesis of insulin.

EFFECTS OF A NON-METABOLIZABLE INSULIN-RELEASING AMINO ACID ON GLUCOSE OXIDATION

The formation of radioactive CO_2 from uniformly ^{14}C-labelled D-glucose was determined in isolated islets exposed to stereoisomers of the non-metabolizable leucine analogue BCH (HELLMAN *et al* 1971 *a*).The insulin-releasing isomer b(—) appeared to stimulate glucose oxidation, whereas the non-releasing isomer b(+) was without effect. Although the stimulatory action of b(—)-BCH on glucose oxidation was small, the enhanced glucose metabolism may be part of the mechanism by which this non-metabolizable amino acid stimulates insulin release.

CONVERSION OF EXOGENOUS AMINO ACIDS INTO 5-HYDROXYTRYPTAMINE

Experiments were designed to measure the uptake of potential amino acid precursors of 5-hydroxytryptamine (HELLMAN *et al* 1971 *b, c*) and their conversion into amines. Both tryptophan and 5-hydroxytryptophan were readily taken up, resulting in an accumulation of these amino acids in the microdissected islets. Tryptophan was found not to be utilized as a precursor for tryptamine or 5-hydroxytryptamine. However, 5-hydroxytryptophan was rapidly converted into 5-hydroxytryptamine, which appeared in the islets in much greater amounts than the precursor. Previous fractionation studies with differential centrifugation have indicated that islets incubated with ^{14}C-labelled 5-hydroxytryptamine incorporate a considerable portion of the radioactivity into a particle fraction containing the bulk of insulin (HELLMAN *et al* 1972). The present studies with ^{14}C-labelled 5-hydroxytryptophan revealed incorporation of substantial amounts of radioactivity into the same particle fraction. It is suggested that 5-hydroxytryptophan inhibits glucose-stimulated insulin release by increasing the amount of stored 5-hydroxytryptamine in the β-cell secretory granules. Such an inhibition might under normal conditions conceal a stimulatory effect of 5-hydroxytryptophan itself.

CONCLUSIONS

1 A thin-layer microchromatographic technique including dansylation was characterized and applied for measuring the contents and formation of amino acids in β-cell-rich pancreatic islets.

2 The islets were characterized by the presence of γ-aminobutyric acid (GABA) and large amounts of taurine. The contents of free amino acids were fairly stable in different states of β-cell function. The results lend no support to the idea that stimulation of insulin release by glucose, 2-aminobicyclo-(2, 2, 1)heptane-2-carboxylic acid (BCH) or glibenclamide is mediated by altered β-cell concentrations of certain naturally occurring amino acids.

3 After incubating the microdissected islets with uniformly ^{14}C-labelled D-glucose, most amino acid radioactivity was recovered in aspartic acid, GABA and glutamic acid. A higher specific incorporation into GABA than into glutamic acid suggested compartmentation of the latter amino acid. The probable existence of amino acid compartmentation raises the question whether conversion of glucose into aspartic acid and glutamic acid is part of the mechanism by which this substance stimulates the biosynthesis of insulin.

4 It is possible that insulin release induced by a non-metabolizable leucine analogue (BCH) is at least in part due to enhanced glucose metabolism.

5 5-Hydroxytryptophan may inhibit glucose-stimulated insulin release by increasing the amount of stored 5-hydroxytryptamine in the β-cell secretory granules.

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