STUDIES ON INSULIN RELEASE FROM THE ISOLATED MOUSE ISLET

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This thesis is a survey based on the following papers:

I.   The β-cell capacity for insulin secretion in microdissected pancreatic islets from obese-hyperglycemic mice (in collaboration with B. Hellman). Life Sciences 8: 53, 1969.

II. Effect of epinephrine and mannoheptulose on early and late phases of glucose-stimulated insulin release (in collaboration with B. Hellman). Metabolism 19: 614, 1970.

III. Isolated mouse islets as a model for studying insulin release. Acta Diabet. Lat. 8: 649, 1971.

IV. The significance of 5-hydroxytryptamine for insulin secretion in the mouse. Horm. Metab. Res. 3: 305, 1971.

V.   Specificty of leucine-stimulation of insulin release. Hormones 3: 1972.

VI. Effects of neutral and dibasic amino acids on the *in vitro* release of insulin. Hormones 3: 1972.

These papers will be referred to in the text by their Roman numerals.

INTRODUCTION

Evidence has accumulated indicating that deficient β-cell function with respect to the synthesis, storage and, especially, the release, of insulin may be a primary factor in the pathogenesis of diabetes mellitus. When exploring the complicated mechanisms which govern insulin release from the pancreatic β-cells, *in vitro* techniques offer obvious advantage over *in vivo* studies. Insulin release has been studied with pieces of pancreas incubated in a physiological buffer (Coore and Randle, 1964, Malaisse et al., 1967). Procedures have also been described for isolating intact mammalian pancreatic islets by microdissection (Hellerström, 1964, Keen et al., 1965) or by collagenase digestion of the exocrine parenchyma (Moskalewski, 1965, Lacy and Kostianovsky, 1967). These techniques make it possible to avoid degradation of released insulin by lytic factors originating from the exocrine pancreas.

The available procedures for studying insulin release from isolated islets are subject to criticism. This is especially true of the commonly used collagenase-isolation procedure in which the exposure to collagenase may affect the function of the islet cells (Atkins and Matty, 1969). Expressing the rate of insulin release in relation to the number of islets (Howell and Taylor, 1968, Lacy et al., 1968, Coll-Garcia and Gill, 1969) may not always give comparable results, owing to the variations in islet size (Hellman, 1959). On the other hand, the alternative of using the islet protein content as a reference (Martin and Gagliardino, 1967, Hahn et al., 1970) limits further ultramicrochemical analyses of the incubated islets.

In the present investigation an *in vitro* system has been developed employing free-hand microdissected mouse islets and permitting a direct correlation between insulin from a single islet and the islet metabolism. The rate of insulin release can be expressed per unit islet dry weight after freeze-drying and weighing each incubated islet. The aim of the studies on which this thesis is based were:

1. to develop the *in vitro* system to a routine procedure for studying insulin release from the isolated pancreatic islets.

2. to study regulation of insulin release in response to naturally occurring substances considered to be of physiological importance for the β-cell function. Glucose, amino acids and biogenic amines were selected for these investigations.

DESIGN OF THE *IN VITRO* TECHNIQUE

Analytical procedures.

Insulin was measured by radioimmunoassay, free and antibody bound insulin being separated by filtration (Hales and Randle, 1963) or ethanol precipitation (Heding, 1966). Mouse insulin was found to differ from human insulin in its ability to displace ^{125}I-insulin from guinea-pig anti-insulin serum (III). Crystalline mouse insulin was therefore used as reference. A routine check of whether the various test substances interferred with the insulin assay became necessary after the observation that the shape of the standard curve differed depending on whether Krebs-Ringer bicarbonate buffer fortified with glucose or a phosphate buffer was used (III). Moreover, batches of the commercially available insulin binding reagent (The Radiochemical Centre, Amersham, England) had to be carefully selected, since they differed in their capacity to bind mouse insulin (II).

The amounts of insulin released were expressed per unit islet dry weight. The freeze-dried islets were weighed on a quartz fibre balance (Lowry, 1953). The level of the glycolytic intermediate fructose 1,6-diphosphate was assayed in oil wells (Idahl, 1971) using an enzymatic procedure in combination with enzymatic cycling (Matschinsky et al., 1968). The presence of 5-hydroxytryptamine in pancreatic sections or in free islet cell preparations was demonstrated histochemically with the formaldehyde condensation technique of Falck et al. (1962).

Choice of animals.

Islets from obese-hyperglycemic mice were preferred for the present studies since they consist to more than 90 % of β-cells. These cells respond adequately to a glucose stimulus when compared with islets of lean mice (I). The use of islets from obese-hyperglycemic mice for studies of insulin release is also attractive because their metabolic characteristics are fairly well known. This type of islet has been used in extensive studies on the enzymes involved in glucose metabolism (Matschinsky and Ellerman, 1968, Täljedal, 1970), the levels of various glycolytic intermediates (Hellman and Idahl, 1969, Hellman, 1970, Idahl 1971),

oxygen consumption (Hellerström, 1967) and the membrane transport and oxidation of glucose (Hellman et al., 1969, Hellman et al., 1971a, 1972b) and amino acids (Hellman et al., 1971b, 1972a, Christensen et al., 1971).

Very large islets from obese-hyperglycemic mice were found to release less insulin than smaller islets (I). Technical difficulties connected with the isolation of the very small islets may have some relevance for this observation. If the dissection procedure damages the cells in the islet periphery there may be leakage of insulin. To reduce the influence of islet size, most analyses were restricted to islets within a certain weight range. The fact that the proteinase inhibitor Trasylol® did not influence the amounts of insulin released (I) is consistent with the absence of contaminating exocrine pancreas.

Effects of various physico-chemical parameters on insulin release.

Optimum conditions for insulin release from the microdissected islets were explored (III). Several types of incubation vessels containing volumes of media varying from 15 µl to 1 ml were used. Estimation of the diffusion rate indicated that even samples drawn from the larger volumes were representative of the total amount of insulin released. Shaking the incubation vessel enhanced the rate of insulin release at both low and high glucose concentrations. These changes are consistent with the sensitivity of the insulin releasing mechanisms to mechanical influences as amply demonstrated when including the islets in a microperifusion system (Idahl, personal communication).

When defining conditions necessary for glucose stimulation of insulin release, the following main observations were made. The optimum temperature for the glucose-stimulated insulin release was 37° C. When the islets were transferred from media of this temperature to media of lower temperatures there was considerable leakage of insulin, which stresses the importance of working at physiological temperatures throughout the experiments. Glucose did not stimulate insulin release when the incubations were performed at pH 5 or 6. A 50 % reduction of the osmolality also abolished glucose-stimulated insulin release, whereas a 30 % increase had no effect. The secretory response to 17 mM glucose was the same irrespective of whether iso-osmolality was maintained by altering the sodium chloride concentration. Reduction of oxygen tension from 95 % to 70 % did not affect glucose stimulation of insulin release. Complete substitution of nitrogen for oxygen abolished the glucose effect and induced a considerable leakage of insulin from the β-cells.

Effects of collagenase on insulin release.

Isolation of islets is facilitated by previous exposure of the pancreas to collagenase (Moskalewski, 1965, Lacy and Kostianovsky, 1967). Whether the exposure to collagenase affects the β-cell function is a matter of discussion (Atkins and Matty, 1969, Hahn and Michael, 1970).

Microdissected mouse islets were exposed to collagenase and, for comparison, to lecithinase C or trypsin (III). In the light microscope the collagenase-treated islets appeared well preserved except for the breakdown of their delicate surrounding capsule. Pretreatment with collagenase did not alter the response to glucose, although the basal insulin release became significantly higher when the islets were pretreated with any of the above-mentioned enzymes. Irrespective of whether the islets were pretreated with collagenase, their content of fructose 1,6-diphosphate increased with increasing glucose concentrations. Some collagenase interference with glucose metabolism was, however, apparent from the increased level of fructose 1,6-diphosphate in islets incubated at a low glucose concentration. The present data suggest that, when possible, exposure of the islets to collagenase should be avoided.

STUDIES ON THE REGULATION OF INSULIN RELEASE

Effects of glucose.

Glucose is the major natural stimulant of insulin release from the β-cells. A sigmoidal dose-response curve was obtained, both during the short initial (early phase) and the second prolonged (late phase) period of glucose stimulation (III). In the early phase maximum stimulation was achieved with 10 mM glucose, while the corresponding value for the late phase was 20 mM. In the latter case the rate of insulin release was no less than 13—14 times greater than the release at 1 mM glucose. The sigmoidal curve obtained for the isolated mouse islets agrees well with that shown for the isolated rat pancreas (Malaisse et al., 1967). A similar relationship has also been demonstrated with mouse islets when studying the respiratory rate (Hellerström and Gunnarsson, 1970), glucose oxidation (Ashcroft et al., 1970) and the induction of "action" potentials (Dean and Matthews, 1970).

In islets from fasted lean mice the insulin release in response to glucose was

significantly increased if the islets were previously exposed to a high glucose concentration during 60 minutes of incubation (III). In rats fasted for 48 hours it has been reported that the ability of glucose to stimulate insulin release is markedly decreased (Grey et al., 1970). This impaired β-cell responsiveness to glucose could, however, be restored by refeeding or by injections of small amounts of this sugar. The authors suggested that their findings could be a result of changes in a glucose-inducible enzyme system in the β-cells. The present data would seem to support this hypothesis if the enzymes involved are rapidly inducible.

There are two hypothetical models for the mechanism of glucose-stimulated insulin release. The glucose molecule itself might serve as the signal, possibly by binding to a receptor in the β-cell membrane. Alternatively, glucose may give rise to a metabolite triggering the release of insulin. The existence of a mediated glucose transport into the β-cells has been demonstrated (Hellman et al., 1971a).Recent observations after including phlorizin, an inhibitor of glucose transport, in the present *in vitro* system (Hellman et al., 1972b) indicate that insulin release is not governed by the total uptake of glucose. Stimulation of insulin release might rather be elicited by the binding of glucose to a membrane receptor which accounts for a minor fraction of the total glucose uptake.

Mannoheptulose is a seven carbon-sugar which inhibits glucose-induced insulin release (Coore et al., 1963). In the present system mannoheptulose inhibited not only the late phase but also the initial early phase of glucose stimulation (II). It has been suggested that the mannoheptulose inhibition of insulin release is due to interference with membrane transport (Matschinsky et al., 1970) or with phosphorylation (Ashcroft and Randle, 1968, Malaisse et al., 1968) of glucose. That such mechanisms might have significance, at least for the late phase, is evident from the observation that mannoheptulose prevented the increase of islet fructose 1,6-diphosphate normally seen with increasing glucose levels (II). Recent studies on the rate of mannoheptulose uptake by the β-cells is compatible with the idea that this compound interferes with the intracellular metabolism of glucose (Hellman et al., 1972c).

Effects of amino acids.

It is well documented that certain amino acids stimulate insulin release. In the present *in vitro*-system L-leucine had a stimulatory action irrespective of whether glucose was present (V). The maximum stimulation of insulin release

was reached with a concentration of 20 mM L-leucine. The L-configuration was essential for leucine to be recognized by the β-cells as an insulin secretagogue. The mode of action by which amino acids elicit insulin release does not seem to be related to their metabolism in the β-cells (Fajans et al., 1964, Hellman et al., 1971b). It has been suggested that insulin release is rather triggered by the binding of the amino acid to a specific transport molecule in the β-cell plasma membrane (Christensen and Cullen, 1969, Christensen et al., 1971). However, L-isoleucine alone or in combination with L-leucine did not affect insulin release (V). It therefore seems unlikely that all sites for L-leucine transport serve as receptors for stimulation of insulin release.

The amino acids L-alanine and a-aminoisobutyric acid are believed to be transported into the β-cell by the alanine-preferring uptake route (Hellman et al., 1972a). These amino acids were found not to stimulate insulin release (VI). Although 5—20 mM L-arginine had no effect on β-cell function in the presence of 3 mM glucose, there was a gradual increase in the amount of insulin released when the experiments were performed at 10 mM glucose. This suggests that a critical glucose concentration must be reached before L-arginine can acts as an insulin secretagogue. A similar glucose dependence has been observed with a non-metabolizable arginine analogue used for studying the transport of dibasic amino acids (Christensen et al., 1971). N-methyl-L-arginine, which is inert with respect to the transport system for dibasic amino acids (Christensen, personal communication), did not stimulate insulin release in the present *in vitro* system (VI). The available data are thus compatible with the idea that the site triggering insulin release in response to arginine may be a transport receptor site for this amino acid.

Effects of biogenic amines.

Epinephrine is a potent inhibitor of insulin release *in vivo* (Porte et al., 1966) an *in vitro* (Coore and Randle, 1964). In the present system this catecholamine significantly reduced not only the sustained late phase but also the initial early phase of glucose stimulation (II).

Histochemical fluorescent technique (Cegrell et al., 1964, Cegrell, 1968) and autoradiography (Ritzén et al., 1965, Ericson and Ekholm, 1970) revealed that tryptaminergic mechanisms operate in the β-cells of the mouse. Depletion of the monoamines by reserpinization of lean mice led to elevated serum insulin levels (IV). However, islets microdissected from these animals released less insulin in response to glucose than did the controls. Marked inhibition of glucose-stimul-

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ated insulin release was noted with islets isolated from mice injected with 5-hydroxytryptophan in combination with a monoamine oxidase inhibitor. The islets from these animals displayed an intense histochemical reaction for 5-hydroxytryptamine. Inhibition of glucose-stimulated insulin release was also noted after the β-cell rich islets of obese-hyperglycemic mice had accumulated and decarboxylated 5-hydroxytryptophan *in vitro*. The latter observation is consistent with recent findings in the isolated rabbit pancreas (Tjälve, 1970).

Extended studies in our laboratory (Hellman et al., 1972d) on the subcellular distribution of 5-hydroxytryptamine are in accordance with previous reports (Falck and Hellman, 1964, Jaim-Etcheverry and Zieher, 1968, Ericson and Ekholm, 1970) that this amine may co-exist with insulin in the β-cell secretory granules. However, neither glucose nor the sulfonylurea derivative glibenclamide could be shown to mobilize granule-bound 5-hydroxytryptamine from the intact β-cells. It therefore seems possible that co-storage of insulin and 5-hydroxytryptamine in the β-granules reduces the secretory capacity of the β-cells.

GENERAL CONCLUSIONS

1. An *in vitro* system using microdissected mouse islets has been developed for studying insulin release. After freeze-drying and weighing each of the incubated islets, the rate of insulin release could be expressed per unit islet dry weight. Optimum conditions for insulin release with respect to islet size and various physico-chemical parameters were explored. The *in vitro* system is useful for rapid screening and characterization of various substances with respect to their effects on the release of insulin, and permits a direct correlation between insulin release from a single islet and the islet metabolism. Islets from obese-hyperglycemic mice were considered particularly useful in view of their high content of adequately functioning β-cells. Reproducible values were obtained for both the early and the late phases of glucose-stimulated insulin release, provided that the basal insulin release was kept low. Introduction of collagenase to the *in vitro* system tended to increase the basal rate of insulin release.

2. When using this *in vitro* system for studies of the regulation of insulin release by naturally occurring substances, the following main observations were made:

a) *Effects of glucose.* Insulin release induced by glucose displayed a sigmoidal

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dose-response curve. In the prolonged late phase of incubation maximum stimulation was achieved with 20 mM glucose. Glucose-stimulated insulin release was significantly increased in islets previously adapted to this sugar. Mannoheptulose inhibited not only the late phase but also the initial early phase of glucose-stimulated insulin release and prevented the increase in islet fructose 1,6-diphosphate normally seen with increasing glucose levels.

b) *Effects of amino acids.* L-leucine stimulated insulin release independent of glucose with maximum effect at a concentration of 20 mM. D-leucine and L-isoleucine had no effect, indicating that not all sites for L-leucine transport into the β-cells are identical with receptor sites for stimulation of insulin release. L-alanine and *a*-aminoisobutyric acid were found not to stimulate insulin release. A critical glucose concentration must be reached before the dibasic amino acid L-arginine can act as an insulin secretagogue. N-methyl-L-arginine lacked stimulatory capacity, indicating that there may be a close relationship between the transport site for L-arginine and a receptor triggering insulin release.

c) *Effects of biogenic amines.* Glucose-stimulated insulin release was strongly inhibited after exogenous exposure of the β-cells to epinephrine or after endogenous formation of 5-hydroxytryptamine.

ACKNOWLEDGEMENTS

REFERENCES

Ashcroft, S. J. H., C. J. Hedeskov and P. J. Randle, Biochem. J. 118: 143, 1970.

Ashcroft, S. J. H. and P. J. Randle, Biochem. J. 107: 599, 1968.

Atkins, T. and A. J. Matty, J. endocr. 46: XVII, 1969.

Cegrell, L., Acta Physiol.scand., suppl. 314, 1968.

Cegrell, L., B. Falck and B. Hellman, In: The Structure and Metabolism of the Pancreatic Islets, Eds. S. E. Brolin, B. Hellman and H. Knutsson, Pergamon Press, Oxford, p. 429, 1964.

Christensen, H. N. and A. M. Cullen, J. Biol. Chem. 244: 1521, 1969.

Christensen, H. N., B. Hellman, Å. Lernmark, J. Sehlin, H. S. Tager and I.-B. Täljedal, Biochim. Biophys. Acta 241: 341, 1971.

Coll-Garcia, E. and J. R. Gill, Diabetologia 5: 61, 1969.

Coore, H. G. and P. J. Randle, Biochem. J. 93: 66, 1964.

Coore, H. G., P. J. Randle, E. Simon, P. F. Kraicer and M. C. Shelesnyak, Nature (Lond.) 197: 1264, 1963.

Dean, P. M. and E. K. Matthews, J. Physiol. 210: 255, 1970.

Ericson, L. E. and R. Ekholm, J. Ultrastruct. Res., in press, 1970.

Fajans, S. S., J. C. Floyd Jr., R. F. Knopf and J. W. Conn, J. Clin. Invest. 43: 2003, 1964.

Falck, B. and B. Hellman, Acta endocr. (Kbh.) 45: 133, 1964.

Falck, B., N.-Å. Hillarp, G. Thieme and A. Torp, J. Histochem. Cytochem. 10: 348, 1962.

Grey, N. J., S. Goldring and D. M. Kipnis, J. Clin. Invest. 49: 881, 1970.

Hahn, H. J., H. G. Lippman and D. Schultz, Acta biol. med. germ. 25: 421, 1970.

Hahn, H. J. and R. Michael, Endokrinologie 56: 69, 1970.

Hales, C. N. and P. J. Randle, Biochem. J. 88: 137, 1963.

Heding, L. G., In: Labelled Proteins in Tracer Studies, Eds. L. Donato et al., Euratom, Brussels, p. 345, 1966.

Hellerström, C., Acta endocr. (Kbh.) 45: 122, 1964.

Hellerström, C., Endocrinology 81: 105, 1967.

Hellerström, C. and R. Gunnarsson, Acta diabet. Lat. 7 (suppl. 1): 127, 1970.

Hellman, B., Acta endocr. (Kbh.) 31: 91, 1959.

Hellman, B., Diabetologia 6: 110, 1970.

Hellman, B. and L.-Å. Idahl, Endocrinology 84: 1, 1969.

Hellman, B., L-Å. Idahl, Å. Lernmark, J. Sehlin, E. Simon and I.-B. Täljedal, Mol. Pharmacol. in press, 1972 c.

Hellman, B., Å. Lernmark, J. Sehlin and I.-B. Täljedal, Metabolism, im press, 1972b.

Hellman, B., Å. Lernmark, J. Sehlin and I.-B. Täljedal, Biochem. Pharmacol, in press, 1972d.

Hellman, B., J. Sehlin and I.-B. Täljedal, Med. exp. 19: 351, 1969.

Hellman, B., J. Sehlin and I.-B. Täljedal, Biochim.Biophys. Acta 241: 147, 1971a.

Hellman, B., J. Sehlin and I.-B. Täljedal, Biochem. J. 123: 513, 1971b.

Hellman, B., J. Sehlin and I.-B. Täljedal, Endocrinology, in press, 1972a.

Howell, S. L. and K. W. Taylor, Biochem. J. 108: 17, 1968.

Idahl, L.-Ã., Hormones, in press, 1971.

Jaim-Etcheverry, G. and L. M. Zieher, Endocrinology, 83: 917, 1968.

Keen, H., R. Sells, and R. J. Jarrett, Diabetologia 1: 28, 1965.

Lacy, P. E. and M. Kostianovsky, Diabetes, 16: 35, 1967.

Lacy, P. E., D. A. Young and C. J. Fink, Endocrinology, 83: 1155, 1968.

Lowry, O. H., J. Histochem. Cytochem. 1: 420, 1953.

Martin, J. M. and J. J. Gagliardino, Nature (Lond.) 213: 630, 1967.

Matschinsky, F. M. and J. E. Ellerman, J. Biol.Chem. 243: 2730, 1968.

Matschinsky, F. M., J. E. Ellerman, R. Landgraf, J. Krzanowski, J. Kotler-Brajtburg and R. Fertel, In: Recent Advances in Quantitative Histo- and Cytochemistry, Eds. U. C. Dubach and U. Schmidt, Hans Huber Publishers, Bern, p. 180, 1971.

Matschinsky, F. M., J. V. Passonneau and O. H. Lowry, J. Histochem. Cytochem. 16: 29, 1968.

Malaisse, W. J., M. A. Lea and F. Malaisse-Lagae, Metabolism 17: 126, 1968.

Malaisse, W. J., F. Malaisse-Lagae and P. H. Wright, Endocrinology 80: 99, 1967.

Moskalewski, S., Gen.comp. Endocr. 5: 342, 1965.

Porte, Jr., D., A. Graber, T. Kuzuya and R. H. Williams, J. Clin. Invest. 45: 228, 1966.

Ritzén, M., L. Hammarström and S. Ullberg, Biochem. Pharmacol. 14: 313, 1965.

Tjälve, H., Acta Physiol, Scand., suppl. 360, 1971.

Täljedal, I.-B., In: The Structure and Metabolism of the Pancreatic Islets, Eds. S. Falkmer, B. Hellman and I.-B. Täljedal, Pergamon Press, Oxford, p. 233, 1970.