RELEASE OF IMMUNOREACTIVE AND RADIOACTIVELY PRELABELLED ENDOGENOUS (PRO-)INSULIN FROM ISOLATED ISLETS OF RAT PANCREAS IN THE PRESENCE OF EXOGENOUS INSULIN

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SUMMARY

To study the influence of insulin on its own secretion, collagenase-isolated islets of rat pancreas were prelabelled with [3H]leucine for 2 h. After washing the islets, (pro-)insulin release was stimulated by glucose in the presence or absence of exogenous insulin (up to 2·5 mu./ml).

Hormone release was unchanged by the presence of exogenous insulin as judged by determination of both immunoreactive insulin and radioactivity incorporated into the proinsulin and insulin fractions of the medium. No direct feedback mechanism for insulin secretion was apparent from this study.

INTRODUCTION

Insulin secretion appears to be regulated mainly by the glucose level in the circulation. Therefore, much attention has been paid to a possible direct feedback of insulin on its own secretion (see Frerichs, 1975). The literature in this field is controversial, apparently due to methodological difficulties. When insulin is administered in vivo, there is usually the disadvantage of interference from a lowered blood glucose level (Turner & Johnson, 1973; Horwitz, Rubenstein, Reynolds, Molnar & Yanaihara, 1975) which, itself, changes the release of insulin (but cf. Sando, Kanazawa & Kuzuya, 1970; Liljenquist, Chiasson, Jennings, Horwitz & Rubenstein, 1975). On the other hand, inaccuracies may arise in vitro when insulin secretion is calculated as the difference between levels of total immunologically measurable insulin and the amount of exogenous insulin. Radioactive prelabelling of isolated pancreatic islets, followed by stimulation of insulin release in the presence or absence of added, non-radioactive insulin should allow exact differentiation between endogenous and exogenous hormone.

MATERIAL AND METHODS

Islets of Langerhans were isolated from the pancreas of male rats (strain Lemgo-Kirchberg-Biberach) by digestion of the exocrine tissue with collagenase (Serva, Heidelberg). Batches of 25 islets were incubated for 2 h with [4,5-3H]leucine (50 μCi, 59 Ci/mmol; Radiochemical Centre, Amersham) in 1 ml Krebs–Ringer bicarbonate buffer, pH 7·4, supplemented with 17 naturally occurring amino acids (20 μg of each amino acid/ml, leucine excluded), bovine
serum albumin (2 mg/ml, Behringwerke, Marburg) and glucose (3 mg/ml). The samples were kept under a constant gas phase of O2:CO2 (95:5 %, v/v); a metabolic shaker was used at a temperature of 37 °C. After 2 h, the islets were separated from the incubation medium and washed three times in 3 ml of fresh buffer medium containing 0.5 mg glucose/ml. Afterwards, the islets were again incubated for 2 h in 1 ml fresh medium devoid of [³H]leucine at a glucose concentration of 2 mg/ml; insulin was added to the incubation medium of half of these batches during the second incubation period. Control islets were incubated with 1 mg glucose/ml only. Porcine insulin (10x crystallized) was tested at concentrations of 2.5 µu./ml and 200 µu./ml; rat insulin, available in only limited amounts, was tested at a concentration of 200 µu./ml. Both insulin preparations were donated by Novo Industri, Copenhagen. Immunologically measurable insulin was estimated in aliquots (20 µl) of the incubation media before and after labelling as well as before, during and after the second incubation period (0, 1 and 2 h). For the immunoassay, an antibody against porcine insulin and porcine or rat insulin standards were used. The avidity of this antibody for rat insulin amounted to about 85 % compared with porcine insulin.

The control measurements of insulin release during the labelling period at 3 mg glucose/ml showed no difference between the batches of islets. Total radioactivity was determined before, during and after the second incubation period in 20 µl samples of the incubation media using a liquid scintillation counter.

At the end of the second incubation period, the islets were separated from the incubation medium and trichloroacetic acid was added to the islets as well as to the medium, yielding a final concentration of 10 % (w/v). The samples were then sonicated for 15 s, the precipitates dissolved in 0.5 ml 1 m-acetic acid and fractionated on a calibrated Sephadex G-50 fine column, 52 x 1.2 cm, in 1 m-acetic acid. Fractions of 1 ml were collected for measurement of radioactivity. Total radioactivity was determined for the proinsulin and insulin peaks. Estimations carried out with labelled hormone for recovery after precipitation and gel filtration revealed no significant intra-assay variations and no major variations from day to day. Mean recovery (± s.e.m.) amounted to 54.2 ± 3.7 %. Since comparison was made only between paired experiments performed on the same day no corrections for loss were necessary. Elution profiles from the Sephadex column showing good separation of the three protein peaks (high molecular weight proteins, proinsulin and insulin) as well as a more detailed description of all procedures used here, including the identification procedures performed for the proinsulin and insulin peaks, have been presented elsewhere (Schatz, Maier, Hinz, Nierle & Pfeiffer, 1973; Schatz, Nierle & Pfeiffer, 1975; Schatz, 1976). Mean values ± s.e.m. were calculated from seven and eight experiments for rat and porcine insulin respectively. Paired Student's t-test was used for statistical evaluation.

RESULTS

Immunologically measurable insulin released during the second incubation period in response to glucose stimulation is shown in Figs 1 and 2. Insulin secretion stimulated by 2 mg glucose/ml was not significantly altered by the presence in the incubation medium of high or low concentrations of porcine insulin or 200 µu. rat insulin/ml.

The total radioactivity released into the incubation medium was not significantly influenced by addition of insulin (Figs 1 and 2). Extraction and fractionation of proinsulin from the incubation media revealed that there were also no significant differences between the amount of total hormone (proinsulin + insulin)-bound radioactivity released in the absence and presence of exogenous insulin (Table 1). After addition of 2.5 µu. porcine insulin/ml, a significant 15 % reduction of radioactivity was found in the insulin fraction of the incubation medium. However, the corresponding islets also contained less radioactivity
Fig. 1. Immunologically measurable insulin (a) and total radioactivity (b) in the incubation media during 2 h of incubation of 25 prelabelled isolated pancreatic rat islets with 2 mg glucose/ml only (●) or also with porcine insulin (200 μu./ml (△) or 2.5 μu./ml (○)) or with 1 mg glucose/ml only (▲). The islets were prelabelled with [3H]leucine for 2 h in 3 mg glucose/ml. Samples of the incubation media for both 0 time values were taken 5 min after contact of the islets with the medium. Thus the early phase of insulin release is responsible for the immunoreactivity as well as the radioactivity found in these samples. Statistical evaluation revealed no significant influence of exogenous porcine insulin on release of both endogenous insulin and total radioactivity. Values are means ± S.E.M. of eight determinations.

incorporated into hormone, although not to a significant extent (Table 1). When the radioactivity of the released hormonal peptides was expressed as a percentage of the radioactivity incorporated into proinsulin and insulin, no difference was found.

**DISCUSSION**

No influence of insulin on its own secretion can be deduced from this study. In early experiments using pieces of rat pancreas (Frerichs, Reich & Creutzfeldt, 1965), inhibition of insulin output was achieved by exogenous insulin only at the very high concentration of 10 μu./ml whereas at 2.5 μu. insulin/ml release appeared not to be hindered (cf. also Hahn & Michael, 1970). On the other hand, it was also reported that much lower insulin concentrations, ranging from 180 to 1500 μu./ml, reduced hormone release in vitro (Sodoyez, Sodoyez-Goffaux & Foà, 1969; Iversen & Miles, 1971; Loreti, Dunbar, Chen & Foà, 1974; Dunbar, McLaughlin, Walsh & Foà, 1976).

In the experiments presented here endogenous hormone release was not significantly reduced by exogenous insulin concentrations of 200 μu./ml and 2.5 μu./ml as judged by two parameters. No major variations occur in the amount of newly synthesized (pro-)insulin released when prelabelled islets are stimulated with glucose (Sando, Borg & Steiner, 1972) or other secretagogues (Schatz et al. 1975). Therefore, as taken in this study, the secretion of radioactive (pro-)insulin can be regarded as a true index of total endogenous hormone release, besides that of immunologically measurable insulin. Measurement of
Fig. 2. Immunologically measurable insulin (a) and total radioactivity (b) in the incubation media during 2 h of incubation of 25 prelabelled isolated pancreatic rat islets with 2 mg glucose/ml only (●) or also with 200 μl rat insulin/ml (△) or with 1 mg glucose/ml only (▲). The islets were prelabelled with [3H]leucine for 2 h with 3 mg glucose/ml. No influence of exogenous rat insulin on release of both endogenous insulin and total radioactivity was found. Values are means ± S.E.M. of seven determinations (see also legend to Fig. 1).

Table 1. Radioactivity (c.p.m.) incorporated into the proinsulin and insulin fractions (for details see text) of both incubation media and islets after 2 h of incubation with 2 mg glucose/ml in the absence or presence of exogenous insulin

<table>
<thead>
<tr>
<th>Incubation media</th>
<th>Glucose (2 mg/ml)</th>
<th>(2-5 mu./ml)</th>
<th>(200 μl./ml)</th>
<th>Glucose (2 mg/ml)</th>
<th>(200 μl./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinsulin</td>
<td>4320 ± 800</td>
<td>4210 ± 990</td>
<td>3680 ± 710</td>
<td>4440 ± 680</td>
<td>4260 ± 280</td>
</tr>
<tr>
<td>Insulin</td>
<td>14310 ± 420</td>
<td>12080 ± 600**</td>
<td>13470 ± 1680</td>
<td>17440 ± 3270</td>
<td>13920 ± 2760</td>
</tr>
<tr>
<td>Islets</td>
<td>17460 ± 2120</td>
<td>12860 ± 1380*</td>
<td>14600 ± 1640</td>
<td>20820 ± 4820</td>
<td>20480 ± 3320</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>117300 ± 8860</td>
<td>97380 ± 16340</td>
<td>12640 ± 5380</td>
<td>154580 ± 15520</td>
<td>132020 ± 12500</td>
</tr>
<tr>
<td>Insulin</td>
<td>153390 ± 8100</td>
<td>126530 ± 17640</td>
<td>158210 ± 7120</td>
<td>197280 ± 18780</td>
<td>170680 ± 15500</td>
</tr>
<tr>
<td>Total c.p.m.</td>
<td>18630 ± 890</td>
<td>16290 ± 1250</td>
<td>17150 ± 2050</td>
<td>21880 ± 3840</td>
<td>18180 ± 2820</td>
</tr>
<tr>
<td>Incubation media</td>
<td>Total hormone-bound c.p.m. released</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation media</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Islets</td>
<td>12.1 ± 1.5</td>
<td>12.8 ± 3.3</td>
<td>10.8 ± 1.8</td>
<td>11.0 ± 2.3</td>
<td>10.6 ± 1.9</td>
</tr>
</tbody>
</table>

Each incubation comprised 25 isolated rat islets which had been prelabelled for 2 h with [3H]leucine in 3 mg glucose/ml. The experiments with porcine and rat insulin were not paired. Values are means ± S.E.M. of seven and eight experiments with rat and porcine insulin respectively.

* P = 0.05;  ** P < 0.005: compared with controls incubated with 2 mg glucose/ml only.
connecting (C)-peptide release should also offer a suitable approach for in-vitro studies of insulin secretion in the presence of exogenous insulin. However, species specificity of C-peptide would require experiments with either human islets or the use of an homologous animal C-peptide assay. Our results, i.e. the lack of influence of insulin on its own release, agree with those of several other authors (Sando et al. 1970; Malaisse, Malaisse-Lagae, Lacy & Wright, 1967; Grodsky, Curry, Bennett & Rodrigo, 1968). The differences from the authors cited above who described a direct feedback inhibition of insulin release might be explained by variations in both methodology and species, e.g. Dunbar et al. (1976) used islets from Syrian hamsters.

In vivo, studies in man with human C-peptide assay systems at constant blood glucose levels have yielded conflicting results concerning the possible existence of a direct feedback inhibition of insulin secretion (Liljenquist et al. 1975; Bottermann, Zilker & Ermler, 1976; Morishita, Shima, Tanaka & Tarui, 1976). It should be remembered that in experiments in vitro and in vivo addition or administration of exogenous insulin may not result in the same localized insulin concentrations at the membrane of the $\beta$ cells within the islets as exist under physiological conditions during insulin release in vivo (cf. Schatz, Otto, Hinz, Maier, Nierle & Pfeiffer, 1974). A decrease of insulin receptor sites on cultured human lymphocytes has been found after 5–16 h at an insulin concentration of $10^{-4}$ mol/l (Gavin, Roth, Neville, de Meyts & Buell, 1974). Since insulin receptors have also been demonstrated in isolated cells of pancreatic islets (Fussgaenger, Suessmann, Hager, Heinze, Schleyer & Pfeiffer, 1976) it would be of special interest to determine whether increased levels of insulin, for prolonged periods, result in a reduced number of insulin-binding sites at the membrane of the $\beta$ cells. It has also been reported that exogenous proinsulin inhibits insulin release from isolated islets of hamster pancreas. This effect of proinsulin is even greater than that of insulin itself whereas exogenous C-peptide does not influence insulin secretion according to Dunbar et al. (1976). On the other hand, glucose-stimulated insulin output from the isolated perfused rat pancreas was reduced by homologous synthetic rat C-peptide in the experiments of Toyota, Abe, Kudo, Kimura & Goto (1975). Thus the definite role of the peptides secreted from the $\beta$ cells in the regulation of insulin release still remains to be clarified.

Some amounts of intrainsular $[3^H]$leucine might still become incorporated into pro-insulin after the labelling period as discussed elsewhere (Schatz et al. 1975). On the other hand, it has been shown that insulin (Hinz, Schatz, Maier, Nierle & Pfeiffer, 1971; Malaisse, Pipeleers & Levy, 1974), as well as proinsulin (Dunbar et al. 1976), is without significant effect on (pro-)insulin biosynthesis in vitro. Therefore, the slight reduction of $[3^H]$-labelled (pro-)insulin within the islets at the end of the experiments (Table 1) cannot be explained by an inhibitory action of 2-5 mu. exogenous insulin/ml on (pro-)insulin biosynthesis. Total radioactivity within the incubation medium during the second incubation period was not greater at insulin concentrations of 2-5 mu. and 200 $\mu$u./ml than in the absence of exogenous hormone (Figs 1 and 2). Thus an augmented release of non-hormone-bound $[3^H]$leucine also seems to be unlikely. An increased secretion of (pro-)insulin during the incubation of the islets with 2-5 mu. insulin/ml can be ruled out by the results obtained. One explanation would be that degradation of $[3^H]$-labelled endogenous (pro-)insulin already within the islet cell itself was enhanced to a small extent by the high concentration of exogenous insulin. The radioactivity of these degradation products might not have been precipitated with trichloroacetic acid, resulting in the somewhat lower radioactivity of hormonal islet proteins found at the end of incubations.

The unchanged percentage of hormone-bound radioactivity released in the presence and absence of exogenous insulin (Table 1) further illustrates the lack of influence of exogenous insulin on hormone release in our experiments.
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Insulin release in the presence of insulin

