Inhibition of Proinsulin to Insulin Conversion in Rat Islets Using Arginine and Lysine Analogs

LACK OF EFFECT ON RATE OF RELEASE OF MODIFIED PRODUCTS*

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Rat pancreatic islets were exposed to a combination of analogs of arginine (3 mM canavanine) and lysine (3 mM thialysine) for 2 h and then labeled with [3H]leucine in the continued presence of the analogs. Control islets were incubated in parallel without analogs. Prelabelled islets were then incubated for a 3-h chase period without analogs. Incorporation of the analogs blocked conversion of newly synthesized, radioactive, proinsulin to insulin. No untoward toxic effects of the analogs were found on nonradioactive insulin as measured by radioimmunoassay. The rate of release of the modified proinsulin was no different from that found for proinsulin newly synthesized in the absence of analogs and, as such, susceptible to the normal action of the enzymes responsible for proinsulin to insulin conversion. These results confirm the previously untested hypothesis that the β-granule is the minimal functional unit of release; granules are thus handled by the B-cell and released at a rate which is independent of the physicochemical nature of their contents.

Release of secretory products from the pancreatic B-cell is thought to occur uniquely by fusion of the limiting membrane of β-granules with the plasma membrane leading to exocytosis of granule contents (1). To date, there is no convincing evidence for an alternative, nongranule-mediated mode of release.

It is believed that during passage through the Golgi apparatus, proinsulin molecules are selectively packaged into β-granules, and that the enzymatic conversion of proinsulin to insulin occurs within the granules themselves (2), the halftime for conversion being ~30 min (2) and independent of the rate of release from the B-cell (3). The proportion of proinsulin released from the pancreas relative to insulin is, thus, presumably a reflection of the time of residence of granules within the B-cell.

Implicit in this working model of the mechanism of insulin (and proinsulin) release is that the β-granule is, in essence, the minimal functional unit. Handling of granules should be quite independent (within certain limits) of the physicochemical nature of granule contents. A granule containing proinsulin, thus, should be released at the same rate and be subject to the same regulatory forces as one containing only native insulin. The present study was designed to probe this concept by arresting the conversion of proinsulin to insulin in β-granules of rat islets. This was achieved by the incorporation of analogs of arginine and lysine (the two residues recognized by the enzyme(s) responsible for proinsulin to insulin conversion (2)) into nascent proinsulin molecules, a method used previously with success in angelfish islets (4).

MATERIALS AND METHODS

Isolation of Islets and Incubations—Rat pancreatic islets were isolated by the collagenase method (5) and subsequently maintained in tissue culture for 3 days (6) in Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, NY), 10% newborn calf serum, 8.3 mM glucose. At the end of the maintenance period, the islets were washed 3 times in a modified Krebs-Ringer bicarbonate buffer (6), containing 10 mM Hepes, 8.3 mM glucose, and 2.5 mg/ml of bovine serum albumin (KRB-Hepes buffer).

In order to prequilibrate islet cells with the lysine and arginine analogs, the islets (200 islets/2 ml) were then incubated for 2 h at 37°C in KRB-Hepes buffer containing 3 mM S-2-aminoethyl cysteine (4-thialysine) and 3 mM canavanine (both obtained from Sigma). Control islets were incubated in parallel in the absence of the amino acid analogs. At the end of the preincubation period, the islets were labeled (30 min, 37°C) in KRB-Hepes buffer (200 islets/1 ml) in the continued presence or absence of 3 mM thialysine and canavanine, and supplemented with 100 µCi/ml of [4,5-3H]leucine (Radiochemical Centre, Amersham, Bucks, United Kingdom, specific radioactivity 85 Ci/mmol). The labeled islets were then washed 3 times in KRB-Hepes buffer in order to remove unincorporated [3H]leucine.

Three batches of 20 islets each were suspended in 1 ml of 0.2 M glycine, 2.5 mg/ml of BSA (glycine/BSA buffer) for subsequent extraction. The remaining islets were distributed in batches of 20 in 1 ml of Dulbecco’s modified Eagle’s medium, 10% newborn calf serum, 8.3 mM glucose, for a 3-h postlabeling (chase) incubation at 37°C in a humidified, gassed atmosphere (95% air, 5% CO₂). At the end of the chase period, the islets were washed 3 times in KRB-Hepes buffer and transferred to 1 ml of glycine/BSA buffer, and the culture media collected. Following sonication of the islets (7), both the islet sonicates and the culture media samples were ultracentrifuged (7) to remove cell debris. The supernatants were kept frozen prior to analysis.

Analytical Methods—In order to determine the radioactivity incorporated into proinsulin and insulin, aliquots of the processed islets and culture media were subjected to quantitative immunoprecipitation, using guinea pig anti-insulin serum (Miles, Rehovot, Israel) to bind the antigens and protein A-Sepharose (Pharmacia, Zurich, Switzerland) to precipitate the immune complexes. This method has been described in detail elsewhere (7, 8). The excess of antibody used in this method (8) ensures quantitative binding (and subsequent precipitation) of both proinsulin and of insulin. In order to determine the relative percentage of immunoprecipitable radioactivity in the form of proinsulin or insulin, aliquots of islets and media were subjected to immunoprecipitation by the above method, and bound material then displaced and subjected to column chromatography on Sephadex G-50 (Pharmacia, Zurich, Switzerland) as described previously (3).

Radioactivity in all samples was determined in a liquid scintillation counter following the addition of Biofluor (New England Nuclear, Dreieich, Federal Republic of Germany) as the scintillant.

For polyacrylamide gel electrophoresis, 20% slab SDS-gels were prepared and run, and radioactive bands subsequently visualized by fluorography, according to the methods adopted by Tartakoff and Vassali (9). Prior to gel electrophoresis, islets were extracted in acid ethanol (absolute ethanol/H₂O/concentrated HCl (v/v/v) (150/47/3)) and aliquots of such extracts were electrophoresed without prior immunoprecipitation.

* The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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The total (labeled and unlabeled) insulin in islets and that released into the culture medium was determined by a standard radioimmunoassay technique as described previously (7, 10).

Calculations, Presentation of Data, and Statistical Analyses—The conversion of proinsulin to insulin involves the removal of 5 leucine residues in the C-peptide (11). Native rat 1 and 2 insulin contain 6 leucine residues (11), and the intact proinsulin molecule contains 11 leucine residues. All data for immunoprecipitable radioactivity are expressed in order to take this change into account. Thus, for any given sample, the percentage of radioactive insulin and proinsulin was estimated by column chromatography, and the absolute immunoprecipitable radioactivity in the form of proinsulin then corrected by a factor of 6/11 in order to enable all samples to be compared on a quantitative basis regardless of the extent of proinsulin to insulin conversion.

All data are expressed as the mean ± S.E., and the level of significance for differences between groups was assessed by Student's t-test for unpaired groups.

RESULTS

Effects of Analogs on Total, Immunoreactive Insulin—The total (unlabeled and labeled) insulin content of islets and that released into the medium during the chase period was determined by radioimmunoassay. At the end of the 2-h preincubation and the 30-min labeling periods, it was found that there was no significant difference in the immunoreactive insulin content of islets treated with 3 mM canavanine and thialysine compared with controls (Table I, left-hand column). Similarly, no difference was observed at the end of the 3-h chase incubation (Table I, center column). By contrast, it was found that there was a small, but significant, increase in immunoreactive insulin release during the chase period from islets which had been pretreated with analogs (Table I, right-hand column).

Effects of Analogs on Proinsulin Biosynthesis and on Conversion of Proinsulin to Insulin—After the 2-h preincubation and the 30 min labeling periods, batches of islets were set aside and processed to determine the radioactivity incorporated into immunoprecipitable (proinsulin) and trichloroacetic acid precipitable (total protein) products. It was found that the presence of 3 mM canavanine and thialysine resulted in a reduced incorporation of radioactivity (Table II). Both the incorporation of radioactivity into proinsulin and trichloroacetic acid precipitable products were reduced by 77-78% in the presence of analogs. Thus, there was no apparent specific reduction of biosynthesis of proinsulin relative to other islet proteins.

At the end of the labeling period, immunoprecipitable radioactive products were, as expected, predominantly in the form of proinsulin (not shown) since the labeling time does not permit significant processing of the newly synthesized proinsulin to insulin (2). During the chase period, however, such conversion would indeed be anticipated. Samples of sonicated islets and of culture medium at the end of the 3-h chase period were subjected to immunoprecipitation. The precipitated material was then dissociated from the antibody-protein A-Sepharose complex with acetic acid, and applied to a G-50 Sephadex column. Representative elution profiles of such columns for culture media samples are shown in Fig. 1. For control islets, it was found that 30-40% of the immunoprecipitable radioactivity eluted in the position of proinsulin, whereas the remaining 60-70% eluted with native insulin (Fig. 1A). For islets which had been preincubated and labeled in the presence of analogs, the immunoprecipitable radioactive material released into the medium during the chase period was found to elute from the column almost exclusively in the volume characteristic of proinsulin (Fig. 1B). The relative percentages of labeled proinsulin and insulin for islets and culture media are given in Table III. Exposure to the analogs resulted in an almost complete inhibition of conversion of proinsulin to insulin. The small percentage of radioactivity, eluting in the void volume of the columns, consists, most probably, of nonspecifically immunoprecipitated radioactivity (3) unrelated to proinsulin or insulin, and has not been considered in the data shown in Table III.

Since incorporation of analogs into peptides may alter their properties upon gel chromatography (thereby potentially rendering modified proinsulin and any putative conversion products unseparable by Sephadex G-50), radioactive products were characterized further by SDS-polyacrylamide gel electrophoresis. When islets which had been preincubated and labeled in the presence of analogs were electrophorized under nonreducing conditions, a single major radioactive band migrating with a mobility of 0.88 relative to pork insulin and of 1.04 relative to beef proinsulin was found both in material extracted from 30 min labeled islets and from islets after a 3-h chase. By contrast, material extracted from control islets following a 3-h chase showed, in addition to a residual band of proinsulin, the generation during the chase period of a new major radioactive band with a mobility of 1.03 relative to pork insulin. When these samples were run in parallel under reducing conditions (β-mercaptoethanol), again for islets prelabeled in the presence of analogs only one major radioactive band was observed (mobility 1.05 relative to beef proinsulin standard also run under reducing conditions) even after the 3-h chase. For control islets, a minor band corresponding to proinsulin was still visible following a 3-h chase, but the radioactive insulin band was, as expected, altered by reduction, now appearing as two bands, presumably reflecting the generation of the A and B chains. It is concluded from the above that the radioactive material under consideration following chromatography by Sephadex G-50 is indeed modified proinsulin with no evidence for the generation of modified insulin molecules by either gel chromatography or by gel electrophoresis.

In order to confirm that these effects of analogs on conversion of proinsulin to insulin were related to their incorporation into nascent labeled proinsulin molecules, islets were prein-

### Table I

| Immunoreactive (total) insulin content of islets and that released into medium during 3-h chase incubation | After 3-h chase |
|---------------------------------------------------------------|-----------------|
| Immunoactive insulin (ng/islet)                              |                 |
| **Prelabeled islets**                                        | **Culture medium** |
| Controls                                                     | 21.9 ± 1.4 (10) | 20.2 ± 1.7 (10) | 3.7 ± 0.3 (11) |
| Prelabeled in presence of analogs                           | 23.2 ± 2 (11)   | 20.3 ± 0.8 (11) | 4.5 ± 0.3 (11)* |

* p < 0.1 versus controls.
cubated and labeled in the combined presence of 3 mM analogs and of 10 mM arginine and 10 mM lysine. Under such conditions, incorporation of \( ^{3}H \)leucine into immunoprecipitable products was only 80 ± 10 (4) dpm/islet (compared with 390 dpm/islet when analogs were present alone (see Table II), in keeping with the known inhibitory effects of these amino acids at high concentrations on proinsulin biosynthesis (12). However, the presence of native arginine and lysine during the prelabeling and labeling periods resulted in a percentage conversion of proinsulin to insulin during the subsequent 3-h chase period which was not significantly different from controls (Table III).

**Effects of Analogs on Release of Prelabeled Proinsulin and Insulin from Islets during the Chase Period**—The fate of the labeled immunoprecipitable material was followed during the 3-h chase period (Fig. 2). Since in the presence of analogs the rate of incorporation of \( ^{3}H \)leucine into islet proteins (including proinsulin) was reduced (Table II), the data have been expressed as a percentage of the immunoprecipitable radioactivity found in the corresponding prelabeled islets. In addition, all data have been corrected, where appropriate, to take into account the loss of leucine residues due to conversion of proinsulin to insulin (see "Materials and Methods").

![Column chromatography (G-50 Sephadex) of immunoprecipitated radioactive products from 3-h chase culture media samples. Following immunoprecipitation (anti-insulin serum) immunoprecipitable products were dissociated from the immune complex and then subjected to column chromatography. The radioactivity eluting in each fraction was then determined. The elution volume of markers are as shown. Vo, void volume (Dextran blue); PI = proinsulin; I = insulin. Islets had been preincubated and labeled prior to the chase period either in the absence (A) or in the presence (B) of 3 mM canavanine and thialysine.](image)

**Table III**

| Percentage of radioactivity | Proinsulin | Insulin |
|-----------------------------|------------|---------|
| Islets after 3-h chase       |            |         |
| Controls                     | 19 ± 2 (4) | 81 ± 2 (4) |
| Prelabeled with analogs      | 52 ± 3 (4) | 8 ± 3 (4) |
| Prelabeled with analogs + 10 mM arginine + 10 mM lysine | 24 ± 3 (4) | 76 ± 3 (4) |
| Culture medium after 3-h chase |            |         |
| Controls                     | 32 ± 3 (4) | 68 ± 3 (4) |
| Prelabeled with analogs      | 96 ± 1 (4) | 4 ± 1 (4) |
| Prelabeled with analogs + 10 mM arginine + 10 mM lysine | 37 ± 2 (4) | 63 ± 2 (4) |

**DISCUSSION**

It has recently been shown that incorporation of amino acid analogs into nascent hormone precursors can inhibit conversion of the prohormone to the prohormone (13) and of the prohormone to the hormone (4, 14). In confirmation of a previous study on the inhibition of proinsulin to insulin conversion by incorporation of arginine and lysine analogs in anglerfish islets (4), an effective inhibition of such conversion was achieved in rat islets in the present study following...
preincubation and labeling in the presence of 3 mM canavanine and thialysine. That this inhibition arose as a consequence of the incorporation of the analogs into newly synthesized (and, as such, labeled) peptides was confirmed by the observation that when islets were labeled in the combined presence of the analogs and of native arginine and lysine, the labeled proinsulin was then converted to insulin during the subsequent chase period, suggesting that the native amino acids successfully competed with the analogs for incorporation into nascent peptides (including proinsulin).

A critical observation is that exposure of islets to the analogs did not result in any pronounced defects in islet secretory mechanism, despite the fact that total protein biosynthesis was severely inhibited during the time of exposure to the analogs, thereby possibly limiting availability of potentially critical, rapidly turning over, proteins. This was shown by considering handling of immunoreactive insulin, which will consist, in major part, of unlabeled, unmodified molecules. There was no change in islet immunoreactive insulin content and the small change in the amount of immunoreactive insulin released during the chase period (after removal of analogs) was not a consistent finding in all experiments, albeit statistically significant when all experiments performed are considered together. The reason for the increase when it is observed remains to be explained.

The direct consequences of the incorporation of analogs into proinsulin can, however, only be evaluated when the fate of labeled material is considered. In confirmation of previous studies, nearly 50% of newly synthesized insulin (and proinsulin) was released from the islets, as compared with less than 20% of stored, unlabeled, insulin (as measured by radioimmunoassay), during the chase period (15). It was found that the inhibition of proinsulin to insulin conversion by preexposure of islets to analogs did not have any significant effect on the rate of release of immunoprecipitable products, despite the fact that, whereas up to 80% of proinsulin labeled in the absence of analogs had been converted to insulin during the chase period, less than 10% of the proinsulin labeled in the presence of analogs had been converted to insulin during the same period. Incorporation of amino acid analogs could change the behavior of proteins on separating systems. In the absence of complete peptide mapping of a modified protein, attribution of identity, therefore, must remain tentative when based uniquely upon elution from a column. This is not the case in the present study, however, for the following reasons. Firstly, samples were subjected to immunoprecipitation prior to gel chromatography. Any modification to the tertiary structure of the proinsulin molecule does not, therefore, appear to affect recognition by anti-insulin antibodies. Secondly, in a previous study on modification of anglerfish hormones by canavanine and thialysine, no apparent changes in chromatographic properties were noted for proinsulin, proglucagon, or prosomatostatin (4) (although these products were not characterized by immunoprecipitation). Finally, when analyzed by SDS-polyacrylamide gel electrophoresis, only one major radioactive band was observed in islets prelabeled in the presence of analogs even after a 3-h chase period, and the mobility of this band relative to a beef proinsulin marker (rat proinsulin not being available) remained unchanged following reduction, in keeping with a proinsulin-like molecule (as opposed to insulin, where such reduction generates the insulin A and B chains).

The results of this study confirm directly for the first time an essential theory in the current working model of the mechanism of insulin release. This is that the rate of proinsulin and of insulin release are identical under the conditions studied. The secretory mechanism of the B-cell thus acts uniquely upon β-granules regardless of their contents. In the present study, the granules containing labeled proinsulin following exposure to the arginine and lysine analogs, in fact, contain modified proinsulin rather than the native product. Indeed, rat proinsulin (1 and 2) contain 5 arginine residues (11), of which only 3 are involved in the conversion to insulin (2). There are 3 lysine residues in rat 1 proinsulin and 2 in the rat 2 proinsulin (11). Only 1 lysine residue is involved in the processing of proinsulin to insulin (2). Since neither arginine nor lysine are involved in the peptide bond(s) cleaved during conversion of proproinsulin to insulin (11), exposure to the analogs used in this study should not affect the normal rapid conversion to the prohormone to proinsulin (16). The fact that even modified proinsulin is released at the same rate as native proinsulin and insulin further confirms the hypothesis that granule contents remain essentially “incognito” within the protected environment of the β-granule until released from the B-cell. It will be of interest to measure the rate of intracellular degradation (8) of modified proinsulin to see whether the enzymes involved are in any way specific for proinsulin or insulin. In other cell types, modified proteins existing in the cytoplasm (and not within granules) are degraded more rapidly than the native proteins (17).

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