Newly Synthesized Proinsulin/Insulin and Stored Insulin Are Released from Pancreatic B Cells Predominantly via a Regulated, Rather Than a Constitutive, Pathway

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Abstract. The pancreatic B cell has been used as a model to compare the release of newly synthesized prohormone/hormone with that of stored hormone. Secretion of newly synthesized proinsulin/insulin (labeled with [³H]leucine during a 5-min pulse) and stored total immunoreactive insulin was monitored from isolated rat pancreatic islets at basal and stimulatory glucose concentrations over 180 min. By 180 min, 15% of the islet content of stored insulin was released at 16.7 mM glucose compared with 2% at 2.8 mM glucose. After a 30-min lag period, release of newly synthesized (labeled) proinsulin and insulin was detected; from 60 min onwards this release was stimulated up to 11-fold by 16.7 mM glucose. At 180 min, 60% of the initial islet content of labeled proinsulin was released at 16.7 mM glucose and 6% at 2.8 mM glucose. Specific radioactivity of the released newly synthesized hormone relative to that of material in is-

lets indicated its preferential release. A similar degree of isotopic enrichment of released, labeled products was observed at both glucose concentrations. Quantitative HPLC analysis of labeled products indicated that (*a*) glucose had no effect on intracellular proinsulin to insulin conversion; (*b*) release of both newly synthesized proinsulin and insulin was sensitive to glucose stimulation; (*c*) 90% of the newly synthesized hormone was released as insulin; and (*d*) only 0.5% of proinsulin was rapidly released (between 30 and 60 min) in a glucose-independent fashion. It is thus concluded that the major portion of released hormone, whether old or new, processed or unprocessed, is directed through the regulated pathway, and therefore the small (<1%) amount released via a constitutive pathway cannot explain the preferential release of newly formed products from the B cell.

T HE general outline for the sequence of steps involved in the biosynthesis, processing, packaging, storage, and release of secretory products (including polypeptide hormones) has been known for many years (31, 33). The final step in this sequence is the extrusion of secretory granule contents by exocytosis. It has often been reported that there is preferential release of newly synthesized product over that which is stored intracellularly. This observation has been made for a variety of secretory products including insulin (6, 11, 22, 38), placental lactogen (43, 47), parathyroid hormone (26), alkaline phosphatase (4), pancreatic amylase (39), prolactin (35, 44), vasopressin (36), gonadotrophin (20), and also for some neurotransmitters, including acetylcholine (46), brain catecholamines (34), and serotonin (3). Within a given secretory cell type the total population of secretory granules will consist of a mixture of newly formed and older stored granules. Preferential release of a newly

synthesized product indicates that instead of random secretion of these two granule pools there is a defined subpopulation of recently formed granules destined for preferential release instead of storage.

It has recently been suggested that there are two pathways for the release of a secretory product (24), the so-called regulated and constitutive pathways. For the regulated pathway, a product is concentrated into secretory granules which can accumulate in the cytoplasm and in which processing of any precursor can occur. The granules have a half-life of hours or days and the release of their products arises in response to a stimulus. For the constitutive pathway, newly synthesized products are packaged into membrane-limited vesicles which, unlike secretory granules, are destined for rapid release in a nonregulated fashion. There is only a short transit time between the Golgi complex and the plasma membrane, no cytoplasmic storage pool, and no processing of precursors. Since some cells display both pathways for the same product (27, 41, 44) it is tempting to speculate that preferential release of a newly synthesized hormone involves commitment of some of this material to the constitutive pathway.

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In this study we have used B cells in isolated rat pancreatic islets as a model system for studying the regulation of release of newly synthesized prohormone/hormone compared with that of a stored, processed hormone. There have been several previous studies showing the phenomenon of preferential release of newly synthesized over stored insulin (6, 22, 38). However, these studies did not address the central issue of whether a portion of newly synthesized material was being directed to the putative constitutive pathway thereby explaining its rapid, preferential release.

Materials and Methods

Materials

Male Sprague-Dawley rats (180–250 g) were purchased from Charles River Laboratories Inc., Wilmington, MA. L-[4,5-^{3}H]leucine (141–153 Ci/mmol) was obtained from Amersham International, Amersham, Buckinghamshire, United Kingdom, and Biocount scintillation cocktail from Research Products International Corp., Mount Prospect, IL. Tissue culture medium TC 199, RPMI 1640, and newborn calf serum were all from Irvine Scientific, Santa Ana, CA. Ficoll-400 was obtained from Pharmacia P. L. Biochemicals, Uppsala, Sweden, and collagenase (type IV, 165 U/mg) was from Cooper Biomedical, Malvern, PA. All other chemicals were purchased from Fisher Scientific, Lexington, MA or Sigma Chemical Co., St. Louis, MO.

Pancreatic Islet Isolation

A modified procedure of Gotoh et al. (8) and Noel et al. (30) was followed for isolation of rat pancreatic islets. The pancreas of an anesthetized rat was distended via the pancreatic duct with 15 ml of TC 199 medium containing 10% vol/vol newborn calf serum and 1.5 mg/ml collagenase. The distended pancreas was then excised and digested at 37°C with shaking (100 strokes per min) for 20–30 min, until an even, homogenous suspension of particulate material was observed. The digest was washed three times with TC 199/10% newborn calf serum and then filtered through a cellector (85 ml capacity, 40 mesh; Thomas Scientific, Swedesboro, NJ). The filtered tissue was then centrifuged (600 g for 5 min), and the pellet resuspended in 23% wt/vol Ficoll in TC 199 (no serum) in a 50-ml conical tube (0.5 ml tissue per 10 ml Ficoll solution). This was then overlayered sequentially with 10 ml of 20.5% and 5 ml each of 17 and 11% Ficoll in TC 199 (no serum) to form a discontinuous Ficoll gradient which was centrifuged for 20 min at 800 g at 4°C (RT-6000 centrifuge, swinging head rotor; DuPont Co., Sorvall Instrument Div., Newtown, CT). Isolated islets appear at the 20.5/17% interface of the gradient and exocrine tissue pellets at the bottom of the tube. The isolated islets were removed and washed three to four times in TC 199/10% newborn calf serum. They were then maintained in RPMI 1640 containing 8.3 mM glucose, 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) for 48 h before use in subsequent experiments. Between 400 and 600 rat islets were routinely isolated per rat pancreas with this procedure.

Pulse/Chase Radiolabeling of Isolated Islets

Approximately 2,000 isolated rat islets were taken after the 48-h tissue culture period and washed in Krebs-Ringer bicarbonate buffer, 0.1% wt/vol BSA, and 10 mM Hepes, pH 7.4 (KRB-BSA) with 2.8 mM glucose. At time zero, the islets were pulse-labeled at 37°C for 5 min in a 15-ml plastic conical tube in 100 μl KRB-BSA containing 16.7 mM glucose and 1.0 mCi L-[4,5-^{3}H]leucine. This concentration of glucose was used to stimulate proinsulin biosynthesis (42) and thus to get maximum [^{3}H]leucine incorporation into newly synthesized endogenous proinsulin during the 5-min radiolabeling period. Pulse-labeling of the islets was stopped by washing in ice-cold KRB-BSA, 2.8 mM glucose. The islets were split into two batches of 1,000 islets each in 3 ml KRB-BSA in 35 × 10-mm petri dishes (Falcon Labware, Oxnard, CA) for a 175-min postlabel (chase) incubation at 37°C. The only difference between these two groups of islets was that one was at a glucose concentration for basal insulin release (2.8 mM) and the other at a stimulatory glucose concentration (16.7 mM) (see reference 18 for review). The period of pulse labeling was considered as time zero to 5 min, and the chase period a further 175 min, for a total of 180 min. At the 15-, 30-, 45-, 60-, 90-, 120-, and 180-min time points, a 200-μl aliquot of the chase

medium and 50 islets (in a 20-μl aliquot) were removed from each of the two islet groups. After removal of the aliquot of medium it was centrifuged in a 1.5-ml microfuge tube (Bio-Rad Laboratories, Richmond, CA) at 800 g for 10 min (to pellet any cell debris). The pellet was discarded and the supernatant stored at −20°C pending analysis. The islets were washed once with 500 μl ice-cold PBS containing 0.1% wt/vol BSA, pH 7.4 (PBS-BSA) by centrifugation (1500 g, 1 min, 1.5-ml plastic microfuge tube). The supernatant was removed and discarded, and the pelleted islets resuspended in 100 μl PBS-BSA. The islet aliquots were then sonicated (25 W, 10 s, sonifier model B15-P; Branson Sonic Power Co., Danbury, CT), centrifuged (10,000 g, 2 min), and stored at −20°C pending analysis.

Analysis of Islet Sonicates and Chase Medium

The total immunoreactive insulin in the aliquots of chase medium and in the islet sonicate samples was assayed by radioimmunoassay (19).

The amount of [^{3}H]proinsulin and [^{3}H]insulin in the chase medium and islet sonicate samples was analyzed by HPLC (15). To compensate for the five leucine residues that are lost in rat C-peptides when rat proinsulins are converted to rat insulins, a correction factor of 11/6 was applied (13). The use of this HPLC technique was found to be quantitative in that in a given sample aliquot, the total radioactive counts recovered in the [^{3}H]proinsulin and [^{3}H]insulin peaks of an HPLC profile were equal (±5%) to the radioactivity recovered by previously described quantitative anti-insulin immunoprecipitation techniques (16, 17). Aliquots of an islet sonicate or islet incubation medium were added directly to the HPLC system (model 332; Beckman Instruments, Inc., San Ramon, CA) without prior purification. An Altex Ultrasphere ODS 5 μm column (length, 25 cm; internal diameter, 4.6 mm; Beckman Instruments, Inc., Altex Scientific Operations, Berkeley, CA) was used with the following buffers: (a) buffer A (TEAP): phosphoric acid (50 mM), triethylamine (20 mM), sodium perchlorate (50 mM) adjusted to pH 3.0 with sodium hydroxide; (b) buffer B: 90% vol/vol acetonitrile, 10% vol/vol water. Both of the rat insulins were eluted together isocratically at 36% buffer B at a flow rate of 1 ml/min (12–13 min after the sample was injected into the system). At 20 min the percentage of buffer B was increased linearly from 36 to 39% over 15 min and maintained at 39% for an additional 25 min, allowing for isocratic elution of both the rat proinsulins at 40 min after sample injection. The peaks were collected in 1-min fractions (1 ml vol) in tubes containing 100 μl of 0.5 mM sodium borate, 1% wt/vol BSA, pH 9.3, in order to have carrier protein present and to neutralize the acidity of the elution buffer. Radioactivity in the samples was detected by adding 10 ml of Biocount scintillation cocktail to the fractions in scintillation vials and by counting in a liquid scintillation counter (1215 Rackbeta II; LKB Instruments, Gaithersburg, MD) with 30–40% counting efficiency.

The [^{3}H]leucine incorporation into total protein in aliquots of islet sonicates or chase medium was determined by precipitation with 10% wt/vol TCA as previously described (2). The TCA precipitates were resuspended in 500 μl of 1 M acetic acid/1% BSA, and transferred to scintillation vials. Then 10 ml of Biocount scintillation cocktail was added and the samples counted as previously described for HPLC fractions.

Presentation of the Results

All data are expressed as the mean ± SEM, with the number of independent observations indicated in parentheses or in the figure legends. The data are also corrected for the media removed for sampling during the course of the experiments. The level of significance for differences between groups was assessed by Student's t test for unpaired groups.

Results

The Release of Total Immunoreactive Insulin

The total immunoreactive insulin released from islets with time at 2.8 mM or 16.7 mM glucose is shown in Fig. 1. At 15 min, 16.7 mM glucose stimulated release of insulin fourfold ($P < 0.001$) above the basal release rate at 2.8 mM glucose. This stimulation increased to eightfold ($P < 0.001$) at 180 min, at which time 15.7 ± 1.3% of the average islet insulin content (22.4 ± 2.0 ng insulin/islet, $n = 48$) had been released at 16.7 mM glucose compared with 1.8 ± 0.3% at 2.8 mM glucose. The average islet insulin content did not
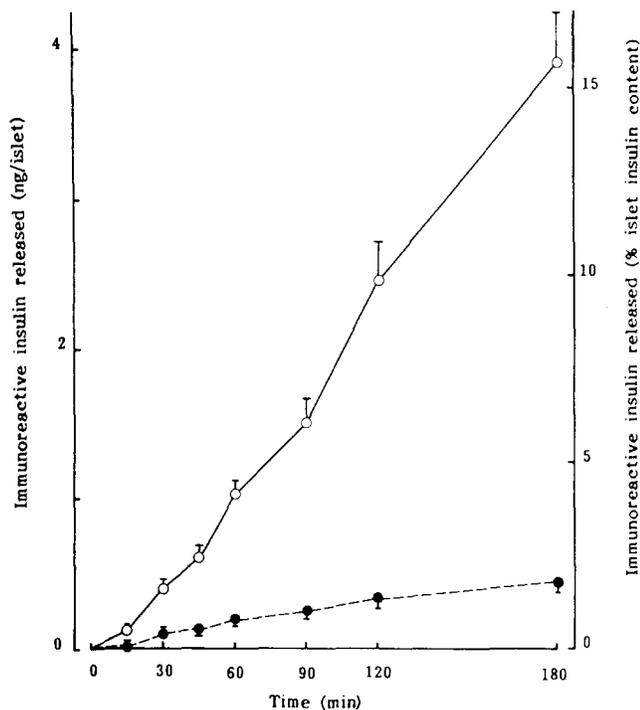
Figure 1. The release of total stored immunoreactive insulin from isolated rat pancreatic islets. Islets were chased for 175 min at 2.8 mM glucose (*solid circles*) or 16.7 mM glucose (*open circles*) after a 5-min pulse labeling period. The results are shown as a mean ± SEM of at least three individual observations.

significantly alter at 2.8 or 16.7 mM glucose throughout the experiment.

The Fate of Newly Synthesized Proinsulin and Insulin during the Chase Period

The fate of [^{3}H]proinsulin during the chase period is shown in Fig. 2. There was no detectable newly synthesized [^{3}H]proinsulin released into the medium until the 45-min

time point (Fig. 2, left). By 60 min, 0.47 ± 0.04(3)% of the newly formed proinsulin had been released at basal, 2.8 mM, glucose and 0.46 ± 0.05(3)% at 16.7 mM glucose. There was a significant stimulation of [^{3}H]proinsulin release by 16.7 mM glucose from 90 min onwards (fourfold [$P < 0.005$] at 90 min, rising to sixfold [$P < 0.001$] by 180 min). By 180 min, 5.8 ± 0.8(3)% of the initial islet pool of [^{3}H]proinsulin had been released as [^{3}H]proinsulin when chased at 16.7 mM glucose, compared with 0.92 ± 0.14(3)% at 2.8 mM glucose. The [^{3}H]proinsulin that was retained in the islets (Fig. 2, right) decreased with time, reflecting both its release and the intracellular conversion of proinsulin to insulin and C-peptide (31, 42; also see Fig. 3 and 6) so that by 180 min only 5.9 ± 0.8(3)% (2.8 mM glucose) and 3.5 ± 0.8(3)% (16.7 mM glucose) of the initial islet content of [^{3}H]proinsulin remained as the nonconverted prohormone.

The fate of [^{3}H]insulin resulting from the conversion of [^{3}H]proinsulin during the chase period is shown in Fig. 3. The data for [^{3}H]insulin have been corrected for the loss of [^{3}H]leucine associated with the conversion of proinsulin (11 leucines) to insulin (6 leucines) and C-peptide (5 leucines) as described in Materials and Methods. There was no detectable [^{3}H]insulin release into the medium until 60 min. At 90 min there was a ninefold stimulation ($P < 0.005$) of release of newly synthesized [^{3}H]insulin by 16.7 mM glucose compared with 2.8 mM glucose. This effect increased to an 11-fold stimulation ($P < 0.001$) by 180 min, resulting in 53.3 ± 3.5(3)% of the initial islet content of [^{3}H]proinsulin being released as [^{3}H]insulin at 16.7 mM glucose and 4.8 ± 0.6(3)% at 2.8 mM glucose (Fig. 3; left).

The [^{3}H]insulin retained in the islets (Fig. 3, right) increased during the early time points of the chase, reflecting its generation caused by the intracellular conversion of proinsulin to insulin and C-peptide (31, 42), which was mirrored by the intracellular decrease in [^{3}H]proinsulin previously discussed (Figs. 2 and 5). The intracellular [^{3}H]insulin in islets was first detectable at the 30-min time point, and between 30 and 90 min there was no effect of glucose on its rate of appearance. At the later times, at 2.8 mM glucose, [^{3}H]in-
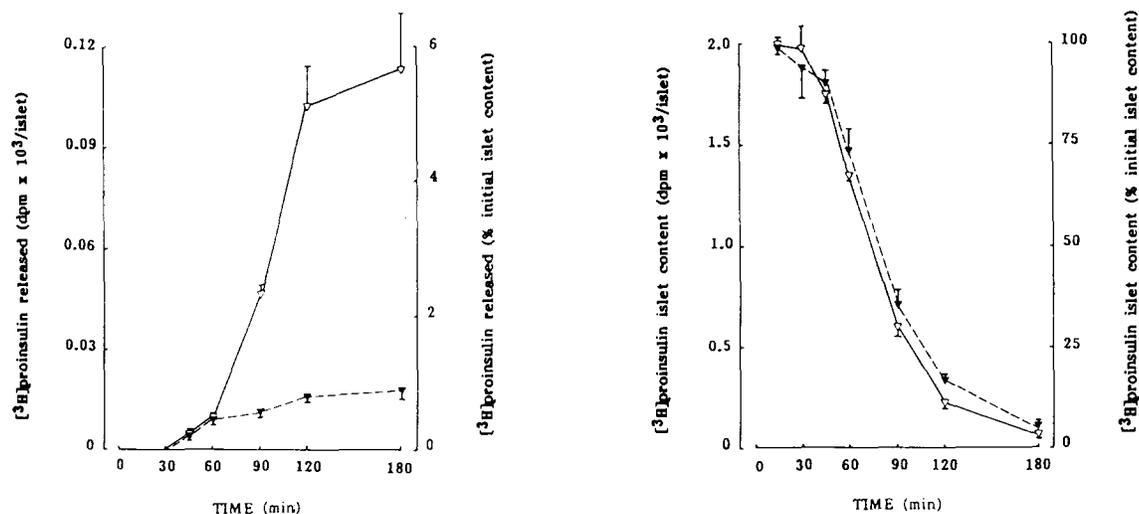

Figure 2. The fate of newly synthesized [^{3}H]proinsulin. Islets were chased for 175 min at 2.8 mM glucose (*solid triangles*) or 16.7 mM glucose (*open triangles*) after a 5-min pulse labeling period. (*Left*) [^{3}H]Proinsulin released into the medium; (*right*) [^{3}H]proinsulin retained in the islets. The results are shown as a mean ± SEM of at least three individual observations.
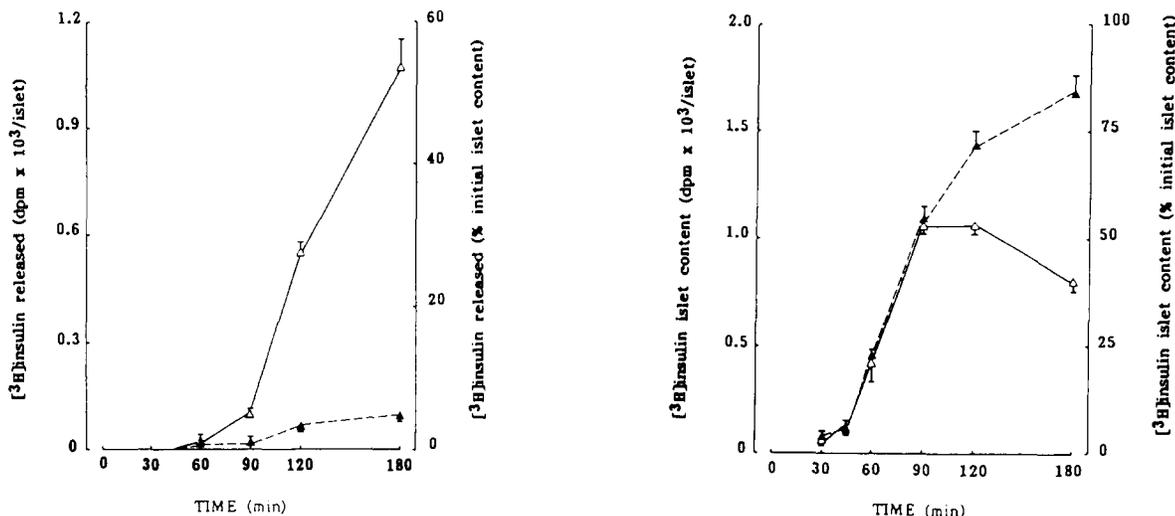
Figure 3. The fate of newly synthesized [³H]insulin. Islets were chased for 175 min at 2.8 mM glucose (*solid triangles*) or 16.7 mM glucose (*open triangles*) after a 5-min pulse labeling period. (*Left*) [³H]Insulin released into the medium; (*right*) [³H]insulin retained in the islets. The results are shown as a mean ± SEM of at least three individual observations. The data have been corrected for the loss of [³H]leucine residues associated with the conversion of proinsulin to insulin and C-peptide (see Materials and Methods).

sulin in islets continued to increase, resulting in 83.5 ± 3.3(3)% of the original [³H]proinsulin synthesized being retained in the islets in the form of [³H]insulin at 180 min. At 16.7 mM glucose, there was a significant decrease in [³H]insulin retained in the islets compared with the 2.8 mM glucose chase. Of the original [³H]proinsulin synthesized only 53.4 ± (3)% at 120 min (*P* < 0.005) and 40.8 ± 2.6(3)% at 180 min (*P* < 0.001) was retained in islets as [³H]insulin (Fig. 3, right). The different patterns of islet [³H]insulin contents are an immediate result of glucose stimulation of [³H]insulin release during the chase.

The Preferential Release of Newly Synthesized Insulin during the Chase Period

The highest specific radioactivity (expressed as disintegrations per minute [³H]proinsulin or [³H]insulin per nanogram immunoreactive insulin) of the newly synthesized [³H]proinsulin in the islets at the 15-min time point was 90.8 ± 3.6(3) and 90.9 ± 5.8(3) dpm/ng for islets being chased at 2.8 or 16.7 mM glucose. This is an expected finding, since, at this time, no newly synthesized proinsulin has been either released or converted to insulin. It should also be recalled that the islets used for the two chase condi-
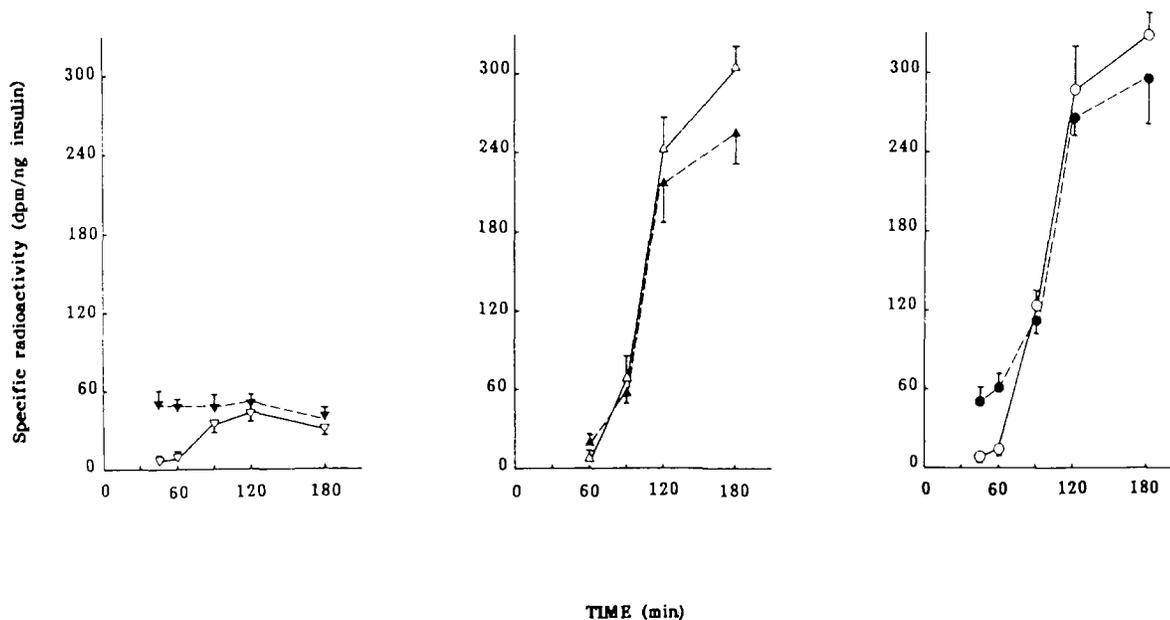

TIME (min)

Figure 4. The specific radioactivities of newly synthesized [³H]proinsulin and [³H]insulin released from isolated rat pancreatic islets. Islets were chased for 175 min at 2.8 mM glucose (*solid triangles*) or 16.7 mM glucose (*open triangles*) after a 5-min pulse labeling period. The specific radioactivities are shown for: [³H]proinsulin, *left*; [³H]insulin, *center*; and [³H]proinsulin + [³H]insulin, *right*. Results are presented as in Fig. 3.
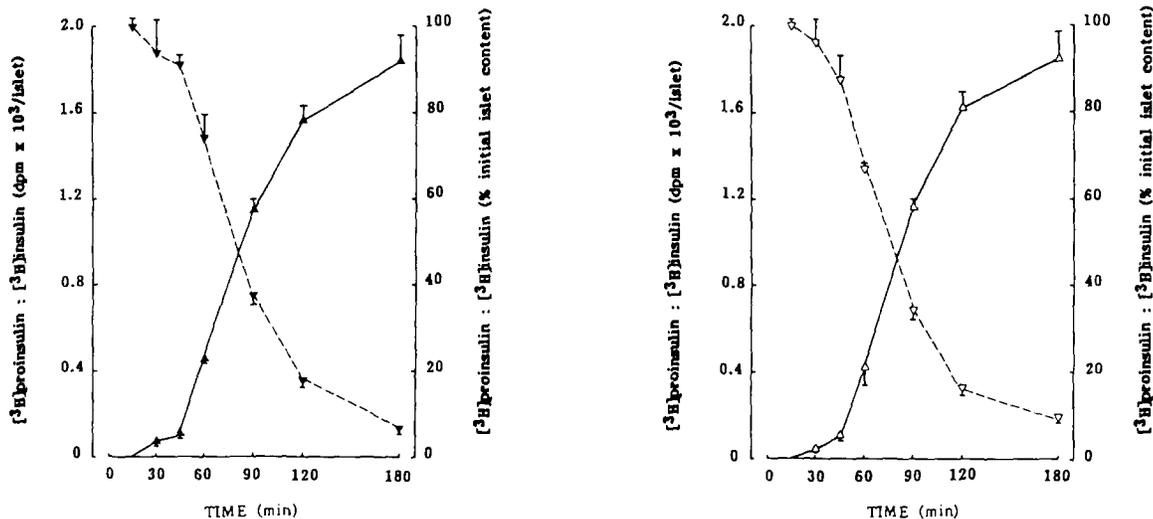
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Figure 5. The conversion of [³H]proinsulin (*broken line*) to [³H]insulin (*solid line*) in isolated rat pancreatic islets. Isolated islets were chased at either 2.8 mM (*left*) or 16.7 mM (*right*) glucose after a 5-min pulse labeling period. The results are presented as in Fig. 3.

tions were obtained from the same common pool of 5-min pulse-labeled islets. Any increase in specific radioactivity for material released during the chase compared with that of the islets at 15 min indicates an isotopic enrichment and, thus, preferential release of newly synthesized hormone over that of the unlabeled stored hormone.

The results for [³H]proinsulin (Fig. 4, left) must be considered with caution. The radioimmunoassay system used does not specifically discriminate between proinsulin and insulin, but the affinity of the antibody towards proinsulin is only ∼50% of that for insulin. The majority of the immunoreactive material in islets and medium will, in addition, be insulin rather than proinsulin. The specific radioactivity for [³H]proinsulin is thus, in reality, radioactivity measured specifically in proinsulin relative to immunoreactivity predominantly arising from insulin. During the first 60 min of

the chase period, there was only a small amount of [³H]proinsulin released (see Fig. 2), with no stimulation by glucose. From 60 min onward there was pronounced glucose stimulation. In contrast, immunoreactive insulin release was stimulated by glucose throughout the chase period (Fig. 1). The combined result of these observations is that there is no apparent isotopic enrichment of [³H]proinsulin in the chase medium (Fig. 4, left) and, indeed, the specific radioactivity at high glucose is lower than that at low glucose at the early time points. Without a specific radioimmunoassay for proinsulin, however, it is not possible to determine whether newly synthesized proinsulin is released in preference to the very small amount of stored proinsulin.

The specific radioactivity of [³H]insulin that had been released into the medium (Fig. 4, center) represents a significant isotopic enrichment at the 120- ($P < 0.01$) and 180-
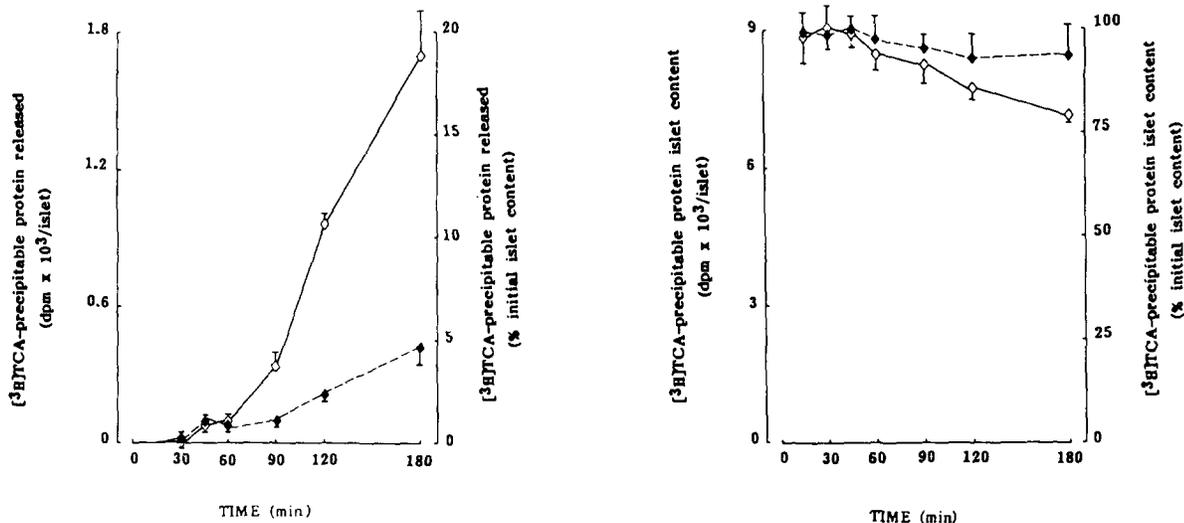

Figure 6. The fate of newly synthesized [³H] total protein (TCA precipitable) from isolated rat pancreatic islets. Islets were chased for 175 min at 2.8 mM glucose (*solid triangles*) or 16.7 mM glucose (*open triangles*), after a 5-min pulse labeling period. (*Left*) ³H total protein released into the medium; (*right*) ³H total protein retained in the islets. The results are shown as a mean ± SEM of at least three individual observations.

($P < 0.001$) min time points two- to threefold above that observed in islets at 15 min at both basal (2.8 mM) and stimulatory (16.7 mM) glucose chase concentrations. This suggests that during the later times of the chase period there was preferential release of newly synthesized [^{3}H]insulin relative to that of stored (total immunoreactive) insulin irrespective of the chase glucose concentration. When the specific radioactivities of released [^{3}H]proinsulin and [^{3}H]insulin are considered together (Fig. 4, right), the isotopic enrichment is also observed from 90 min onwards (1.5-fold increase). However, here again consideration should be given to the limited cross-reactivity of the immunoassay system for proinsulin and insulin.

The Conversion of Proinsulin to Insulin during the Chase Periods at Different Glucose Concentrations

When the amount of either [^{3}H]proinsulin or [^{3}H]insulin in the islets and medium (Figs. 2 and 3) were combined at each time point during the chase period, an indication of intracellular [^{3}H]proinsulin conversion was obtained (Fig. 5). After an initial lag phase of ~30 min, [^{3}H]proinsulin was converted with a half-time of the order of 45–60 min irrespective of the ambient glucose concentration (compare both panels of Fig. 5).

The Fate of Total Newly Synthesized Protein during the Chase Period

The fate of newly synthesized ^{3}H total protein (radioactivity precipitable with TCA) during the chase is shown in Fig. 6. Small quantities of ^{3}H total protein could be found in the medium at 30 min (Fig. 6, left). There was no significant effect of 16.7 mM glucose on ^{3}H total protein released between 30 and 60 min compared with a 2.8-mM glucose chase. However, the release of ^{3}H total protein from 90 min onwards was stimulated by 3.5-fold at 16.7 mM glucose ($P < 0.01$), so that by 180 min 19.1 ± 2.5(3)% of the initial islet content of ^{3}H total protein had been released at 16.7 mM glucose chase, and only 4.8 ± 0.7(3)% at 2.8 mM glucose. This was reflected in the ^{3}H total protein retained in the islets by 180 min (79.9 ± 1.0[3]% at 16.7 mM glucose and 93.3 ± 7.2[3]% at 2.8 mM glucose; Fig. 6, right).

^{3}H Total protein comprises both insulin-related and non-related material. When the contribution of [^{3}H]proinsulin and [^{3}H]insulin is discounted from the values for ^{3}H total protein released during the chase period (Fig. 7) it is apparent that there was no significant effect of glucose on the release of such non–insulin-related proteins. Furthermore, the quantity of such material released by 180 min amounted to <4% of the initial islet content of [^{3}H]TCA-precipitable products.

Discussion

The Pathway of Hormone Release

The results described show that glucose-stimulated release of unlabeled insulin stores (immunoreactive insulin) was similar to that previously observed (17, 21). This secretion pattern is, by definition, typical for a regulated pathway (24) in that the product is processed from a precursor (before its release) and is concentrated into secretory granules that ac-
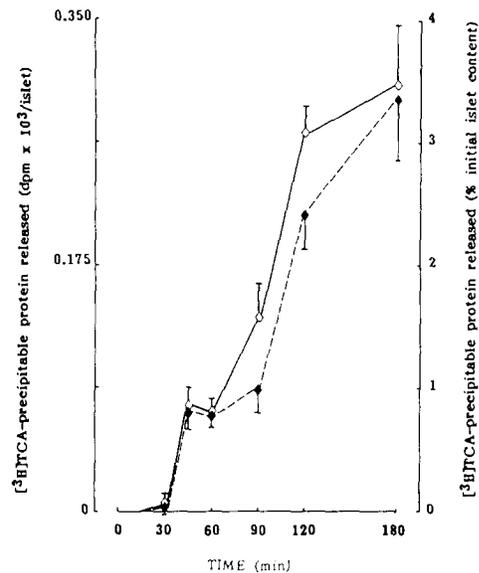

Figure 7. The release of newly synthesized ^{3}H total protein, other than [^{3}H]proinsulin and/or [^{3}H]insulin, from isolated rat pancreatic islets. Islets were chased for 175 min at 2.8 mM glucose (*solid triangles*) or 16.7 mM glucose (*open triangles*), after a 5-min pulse labeling period. The results are shown as a mean ± SEM of at least three individual observations.

cumulate in the cytosol until their release is triggered by a stimulus (e.g., glucose in this particular instance). The pancreatic B cell, therefore, represents a good model for regulated release of a stored hormone.

Newly synthesized hormone or prohormone were only released from the B cells after an initial 45-min lag period. This lag period before release is due to the time required from synthesis on the rough endoplasmic reticulum to passage through the Golgi complex, packaging into secretory granules, and transit to the plasma membrane for exocytosis (23, 31, 33). The major component (90%) of newly synthesized products released to the medium was the processed hormone, insulin. The release of [^{3}H]insulin was glucose stimulated, suggesting its release via the regulated pathway. Even though the release of newly synthesized proinsulin was only a minor component of released newly synthesized products (10%), the majority of this released [^{3}H]proinsulin (from 60 min onwards) was also subject to glucose regulation, and was therefore also presumed to be released via the regulated pathway. This confirms a previous observation that even when proinsulin conversion is inhibited its mode of release appears unchanged (12). The small amount of newly synthesized proinsulin that was rapidly released between 30 and 60 min, however, was not subject to glucose regulation and might therefore represent hormone that was released via the constitutive pathway (24). Our data are also consistent with the presence of a constitutive release pathway active for non–insulin-related proteins synthesized in islet cells.

Our results show that 99% of newly synthesized proinsulin/insulin from pancreatic B cells is released via the regulated pathway and 1% is potentially released via the constitutive pathway. A single secretory cell product is usually only released via a constitutive or regulated pathway (9, 10, 24). However, it has been suggested (from observations made

mostly in tumor-derived cell lines) that under some circumstances both constitutive and regulated pathways of release for one particular prohormone/hormone product may exist in the same cell (24, 27). Indeed, this has been suggested for insulin in virally transformed pancreatic B cells (HIT cell line [28]), or B cell tumor-derived cells (5), where a much greater proportion of newly synthesized proinsulin was released via a constitutive pathway than observed in this study. Similar observations have been made for ACTH release from pituitary-derived AtT20 cells (27). When the human insulin gene was transfected into AtT20 cells the subsequently synthesized proinsulin/insulin was released in a fashion similar to that in HIT cells as, indeed, was the native hormone of AtT20 cells (ACTH) (28). It is possible that such observations of constitutive and regulated release for one product reflect the partial dedifferentiation of transformed cells since the highly differentiated pancreatic endocrine cells used in this study show prohormone/hormone release that appears to be mediated predominantly via the regulated pathway.

The Preferential Release of Newly Synthesized Hormone

The results show that from 85 min after the 5-min pulse-label period there was preferential release of newly synthesized proinsulin/insulin over that of stored insulin. Similar observations have been made previously for insulin (6, 11, 22, 38) and also other hormones (3, 4, 20, 26, 35, 36, 40, 41, 43, 44, 46, 47). The present study does, however, include two important and novel observations. First, 99% of the released newly synthesized proinsulin/insulin was subjected to glucose regulation, and second, the major proportion (at least 90%) of the released newly synthesized hormone was in the form of processed [^{3}H]insulin. It can therefore be concluded that preferential release of newly synthesized hormone is mediated via the regulated pathway only and is not a reflection of constitutive release. Also, the specific radioactivities of released newly synthesized insulin (and insulin and proinsulin together) indicate that preferential release of newly synthesized hormone occurs to the same extent whether islets are incubated at basal or stimulatory glucose concentrations. Thus, even though there was elevated insulin release in glucose-stimulated islets, the proportion of newly synthesized hormone released relative to that of stored hormone was similar under basal or stimulatory conditions.

Cellular heterogeneity has been proposed as a possible explanation for the preferential release of newly synthesized hormone (45). Even though there is some evidence for islet B cell heterogeneity (37), it will be necessary to isolate and characterize the two putative B cell populations in order to establish whether such heterogeneity underlies preferential release of newly synthesized insulin. Our data would suggest that even a subpopulation of B cells displaying rapid insulin synthesis and release would still be operating almost entirely via the regulated release pathway.

Two pools of secretory granules rather than two separate secretory pathways have also been suggested as a possible mechanism for the preferential release of newly synthesized hormone (11, 14, 26, 44). According to these theories, the newly formed secretory granules compose a granule pool that is more readily available for release than the pool of older stored granules. Grodsky and co-workers have sug-

gested that in the B cell glucose "marks" granules destined for preferential release during their production in the Golgi complex (5, 6). Such granule heterogeneity generated by a stimulus signal has not been proposed for other secretory cell types and our present and previous (11) data do not support this concept. In particular, we observe preferential release of newly synthesized proinsulin/insulin even during a chase at basal 2.8-mM glucose. We therefore propose a new hypothesis to explain both our findings and those of others. This hypothesis is based on the present data as well as two other observations concerning the function of the B cell. (a) Exposure of B cells to glucose results in activation of the cell cytoskeleton by microfilament contraction and tubulin polymerization leading to increased granule movement towards the plasma membrane and a consequent increase in exocytosis (21). (b) The Golgi complex of pancreatic B cells appears to be restricted to a specific intracellular location (25), which may suggest an association with the B cell cytoskeleton. Indeed, an association between microtubules and Golgi complex membrane has been shown in rat liver cells (1).

We now propose that newly formed β-granules, immediately after budding off from the trans elements of the Golgi complex, become rapidly associated with the cytoskeleton or, alternatively, are already associated with it as they form. Older, stored β-granules are less available for cytoskeleton attachment due to their more remote location in the cytosol. This more intimate association between new granules and the cytoskeleton would result from apparent anchorage of the Golgi complex to the cytoskeleton, and would result in rapid exocytosis of such granules, thereby accounting for preferential release of newly formed proinsulin/insulin. Glucose activation of the cytoskeleton, leading to stimulated release, would, in our model, be as effective for any granule already associated with the cytoskeleton (i.e., new or old). Newly formed granules not released from the B cell would detach from the cytoskeleton to become part of the stored pool. Glucose may play an additional role in stabilizing anchorage of granules to the cytoskeleton. It has been clearly shown that newly formed granules are characterized by a clathrin coat (so-called immature or coated granules; reference 31). It has furthermore been shown that proinsulin appears to be restricted to immature rather than mature granules (31, 32). The immature and mature granules, with their different properties and contents, could have represented the two pools of new and old granules. The immature coated granule may thus have been the substrate for rapid, possibly constitutive, release of newly formed proinsulin. Our data do not support this concept since glucose was found to regulate the release of newly synthesized proinsulin. We therefore suggest that, in terms of their secretory fate, new and old granules are not handled differently. Preferential release simply reflects rapid association of new granules with the cytoskeleton. Granule maturation (uncoating) and proinsulin conversion would arise in granules despite their association with the cytoskeleton. Thus, newly formed proinsulin and insulin would both be rapidly released and would both be sensitive to glucose stimulation. According to our model, the data of Gold et al. (5, 6) could be accounted for by a greater percentage of newly formed granules being retained on the activated and more available cytoskeleton at high glucose (21), rather than by marking of the granules themselves.

The Conversion of Prohormone

The kinetics of proinsulin conversion shown in this study are in agreement with previous studies (31, 42). The rate and extent of proinsulin conversion to insulin (and C-peptide) were similar at basal or stimulatory concentrations of glucose. Since proinsulin cannot be converted to any significant extent once released from the B cell, the total extent of conversion reflects not only intrinsic B cell conversion activity per se but also the rate and extent of release of proinsulin from B cells (equivalent to its rate of escape from the conversion site), which was minimal even at 16.7 mM glucose. The apparent discrepancy between the results of those suggesting secretagogue regulation of proinsulin conversion (7, 29) and our own data lies in a fundamental difference in experimental design. Thus, Nagamatsu et al (29) found that after prolonged exposure of islets to glucose there was an acceleration in the subsequent conversion of proinsulin to insulin. Their data support our conclusion that glucose has no effect on conversion of preformed proinsulin after an acute exposure of islets to the sugar.

Conclusion

Taken together, our data show that in a highly specialized secretory cell, such as the pancreatic B cell, the release of the major secretory products occurs via a regulated pathway. This applies to both proinsulin and insulin. The preferential release of newly synthesized proinsulin/insulin occurs to the same extent at both stimulatory and nonstimulatory glucose concentrations. Although the precise mechanism responsible for preferential release of newly synthesized proinsulin/insulin remains to be determined, we suggest that the preferential association of newly formed granules with the cytoskeleton may explain this phenomenon. Our data do not exclude the existence of a constitutive release pathway in islet cells. Indeed, non-insulin-related proteins would appear to be released via such a pathway, albeit to a limited extent. For proinsulin and insulin, however, the contribution of a constitutive routing is indeed small, accounting for <1% of newly synthesized proinsulin or insulin released from the B cell. It is, finally, possible that in transformed cells (i.e., secretory cell lines) there is a shift from the regulated to the constitutive pathway resulting in a more pronounced nonregulated secretion of prohormone.

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