Protein Targeting via the "Constitutive-like" Secretory Pathway in Isolated Pancreatic Islets: Passive Sorting in the Immature Granule Compartment

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Abstract. We have suggested the existence of a novel "constitutive-like" secretory pathway in pancreatic islets, which preferentially conveys a fraction of newly synthesized C-peptide, insulin, and proinsulin, and is related to the presence of immature secretory granules (IGs). Regulated exocytosis of IGs results in an equimolar secretion of C-peptide and insulin; however an assay of the constitutive-like secretory pathway recently demonstrated that this route conveys newly synthesized C-peptide in molar excess of insulin (Arvan, P., R. Kuliawat, D. Prabakaran, A.-M. Zavacki, D. Elahi, S. Wang, and D. Pilkey. *J. Biol. Chem.* 266:14171–14174). We now use this assay to examine the kinetics of constitutive-like secretion. Though its duration is much shorter than the life of mature granules under physiologic conditions, constitutive-like secretion appears comparatively slow ($t_{1/2} \cong 1.5$ h) compared with the rate of proinsulin traffic through the ER and Golgi stacks. We have examined whether this slow rate is coupled to the rate of IG exit from the *trans*-Golgi network (TGN). Escape from the 20°C temperature block reveals a $t_{1/2} \leqslant 12$ min from TGN exit to stimulated release of IGs; the time required for IG formation is too rapid to be rate limiting for constitutive-like secretion. Further, conditions are described in which constitutive-like secretion is blocked yet regulated discharge of IGs remains completely intact. Thus, constitutive-like secretion appears to represent an independent secretory pathway that is kinetically restricted to a specific granule maturation period. The data support a model in which passive sorting due to insulin crystallization results in enrichment of C-peptide in membrane vesicles that bud from IGs to initiate the constitutive-like secretory pathway.

We have been studying the existence of a unique pathway of protein secretion that exists in cells that also exhibit exocytotic discharge of secretory granules. This route is clearly distinct from true constitutive secretion in that it conveys content proteins that have been sequestered within the lumen of immature granules, and exhibits the same polarized targeting as the regulated secretory pathway (Arvan and Castle, 1987; Arvan and Lee, 1991). Nevertheless, this route is detected most readily under unstimulated conditions. As demonstrated by electron microscope autoradiography, this pathway seems to be related to the presence of early granule forms (immature secretory granules [IGs][1]; Salpeter and Farquhar, 1981) rather than mature granules (Arvan and Chang, 1987). We have termed this novel route the constitutive-like pathway, in an attempt to distinguish it both from regulated exocytosis as well as true constitutive secretion (Arvan et al., 1991). The constitutive-like pathway appears to account for observations of regulated protein secretion from two parallel intracellular pools (Rothman and Issenman, 1974; Roberge and Beaudoin, 1982), as well as preferential unstimulated release of newly synthesized peptide hormones over those stored in mature granules (Dudek, 1984). Nevertheless, a complete understanding of the constitutive-like pathway is still lacking.

According to one model, constitutive-like secretion could simply represent IGs released by the same regulated pathway as mature granules, but exhibiting preferential cytoskeletal delivery to exocytotic sites (Rhodes and Halban, 1987). A shortcoming of this model is its difficulty in explaining findings in exocrine systems where constitutive-like secretion is perfectly evident (Arvan and Castle, 1987), the intermixing of older and younger granules is limited (Neutra and Leblond, 1966; Castle et al., 1972), yet older granules actually are released in preference to younger granules (Sharoni et al., 1976; Singh, 1982). Further, the constitutive-like pathway would appear to continue under specialized conditions in which regulated exocytosis is virtually undetectable (Arvan et al., 1991; Arvan and Chang, 1987). Alternatively, we have proposed that constitutive-like secretion might orig-

1. *Abbreviations used in this paper*: IG, immature secretory granules; TGN, *trans*-Golgi network.

inate from the budding of membrane vesicles from IGs. This hypothesis makes three testable predictions: (*a*) peptides will be sampled by these membrane vesicles to the extent that they can enter the fluid phase of the bud, rather than in proportion to their overall concentration within the IG; (*b*) vesicular budding is a function restricted to IGs (i.e., this ability is lost as granules mature); and (*c*) despite the common origin of constitutive-like and regulated secretory pathways, the pathways are independent of one another (i.e., constitutive-like secretion is not obligatory in preparing granules for regulated discharge).

To test these predictions, we have examined the cellular handling of insulin in pulse-labeled pancreatic islets, which has served as an extremely favorable paradigm for studying secretory protein targeting (Halban, 1990). In pancreatic *B* cells as in other regulated peptide-secreting endocrine cells, sorting of the precursor polypeptide, proinsulin, occurs in the *trans*-Golgi network (TGN) (Orci et al., 1987*a*). After rapid transport through the ER and Golgi stacks, newly synthesized proinsulin molecules are then sequestered in condensing vacuoles/IGs with pale contents, as observed in the electron microscope (Orci, 1982). Given the possible activity of furin/PACE or related proteases in the TGN (Barr, 1991), initiation of prohormone cleavage theoretically might occur before sorting into IGs (Schnabel et al., 1989). However, both in vivo and in isolated islets, completion of the two distinct endoproteolytic and four carboxypeptidase cleavages (to produce equimolar amounts of C-peptide and insulin) are thought to require the low pH and high [Ca^{2+}] environment found in IGs (Orci et al., 1985, 1987*b*), Davidson et al., 1988; for review see Hutton, 1989). In this compartment, the ratio of proinsulin to (C-peptide + insulin) changes steadily as the IGs mature (Orci et al., 1986; Davidson et al., 1988). The IGs are also responsible for generating inhomogeneity of the peptide products within the granule lumen, with C-peptide dissolving at the perimeter while insulin crystallizes into a core (Orci, 1982; Michael et al., 1987). When expressed at high levels in transgenic mice, a mutation encoding a noncrystallizing proinsulin results in a reduced ability to form granule cores as well as increased "unregulated" protein secretion (Carroll et al., 1988) consistent with discharge via the constitutive-like pathway.

We have reported recently that at chase times after true constitutive secretion is thought to be completed (a route taken by only ~0.5% of proinsulin) (Rhodes and Halban, 1987), newly synthesized insulin-containing peptides in islet IGs exhibit hybrid secretory features: they may be conveyed either by direct IG exocytosis upon secretory stimulation, or to a lesser extent, by constitutive-like secretion (Arvan et al., 1991). The chase period corresponding to constitutive-like secretion appears to encompass the time when condensing vacuoles detach from the TGN and insulin compacts into electron-dense cores. Further, we have shown at glucose concentrations within the physiological (less stimulated) range, that the *B* cell secretes newly synthesized C-peptide and insulin with a stoichiometry different from that existing in the granule compartment, supporting the first prediction listed above. In this report, we have utilized the latter assay to examine precisely the kinetics of constitutive-like secretion, and to test the other predictions of our model.

Materials and Methods

Materials

Collagenase was from Worthington Biochemical Corp., Freehold, NJ. Hypaque, human serum albumin, TPCK-treated trypsin, soybean trypsin inhibitor, protein A–agarose, cycloheximide, and canavanine were from Sigma Chemical Co. (St. Louis, MO). Calf serum and antibiotic–antimycotic solution were from Gibco Laboratories (Grand Island, NY), [^{3}H]-leucine and [^{35}S]methionine and cysteine were from New England Nuclear (New Bedford, MA), antisera to porcine insulin (cross reacting with rat insulins I and II) and rat C-peptide were from LINCO Research Inc. (St. Louis, MO).

Isolation and Pulse–Chase Studies of Rat Pancreatic Islets

Islets from 225 g male Sprague-Dawley rats (fasted overnight) were isolated essentially as described previously (Arvan et al., 1991) with pancreatic digestion by perfusion with collagenase, flotation on a hypaque gradient, picking of individual islets, and recovery overnight in DME containing 10% calf serum plus 1% penicillin-streptomycin-amphotericin. Islets were washed twice with leu-free (or met-free, cys-free) DME before pulse-labeling for up to 15 min at 37°C in the same medium containing up to 300 μCi of [^{3}H]leucine (or [^{35}S]methionine and cysteine). In some experiments, preincubation of up to 75 min with 2 mM canavanine in arg-free DME was conducted before labeling with [^{3}H]leucine or ^{35}S-amino acids and chasing with canavanine in arg-free DME. All labeling and chase media also contained 0.5 mg/ml human serum albumin and 0.005% soybean trypsin inhibitor.

Chase incubations for batches of islets were performed using porous bottom incubation chambers. As described previously (Arvan et al., 1991), these incubation chambers allowed for precise collection of sequential, noncumulative hour-long chase periods while requiring no physical manipulations of the islets that might potentially induce mechanical injury. In the final chase period, islets were stimulated (22 mM glucose, 1 mM tolbutamide, and 1 mM isobutylmethylxanthine). An antiprotease cocktail containing aprotinin (1 mU/ml), leupeptin (0.1 mM), pepstatin (10 μM), EDTA (5 mM), and diisopropylfluorophosphate (1 mM) was added to the collected chase media. Islets were then lysed by sonication as described (Rhodes and Halban, 1987) in the presence of the same antiprotease cocktail listed above. All samples were spun in a microfuge for 5 min at 4°C to remove debris before electrophoresis or immunoprecipitation.

20°C TGN-Exit Block

To ensure that all samples had an identical efficiency of labeling, islets prepared and recovered overnight were pulse labeled as a batch with ^{35}S-amino acids for 10 min and then divided into individual 100-islet aliquots. The islets were cooled to 19.5°C immediately after pulse for 2 h, to block secretory protein exit from the TGN (Griffiths et al., 1985; Chanat and Huttner, 1991), so that labeled proinsulin accumulated therein. At this time individual aliquots of islets were warmed to 37°C for various time periods up to 30 min. To permit synchronous stimulation of all samples, after timed escape from the TGN-exit block, islets were again chilled to 19.5°C until 3 h of chase. At this time, all aliquots of islets were exposed to near-maximal stimulation for 5 min (see Fig. 3) or 10 min (see Fig. 4) at 37°C, in parallel with labeled control islets that had been incubated for 160 min at 37°C (plus 20 min at 19.5°C) to permit maximal transfer of newly synthesized proinsulin into IGs. (Preliminary experiments [not shown] indicated that 160 min was ample time for all pulse-labeled proinsulin to arrive in IGs.) At 180 min of chase, all islets were exposed to the combination secretagogue listed above plus 1 μM Phorbol-12-myristate, 13-acetate. The secretion of insulin-containing peptides was measured by quantitative immunoprecipitation (Halban, 1982). For the calculation of half-time of proinsulin transfer from the TGN to the stimulated IG compartment, stimulated secretion from control islets was defined as 100%; for all experimental samples, secretion was compared with this reference value.

Trypsin Digestion of Proinsulins

Samples of cell lysates incubated either with arginine or canavanine were pulse labeled with ^{35}S-amino acids for 15 min and then lysed immediately

by sonication in 0.1 M HCl. Debris was removed by centrifugation and the lysates were adjusted to pH 7.6. Lysates were then digested for 15 min at 37°C with TPCK-treated trypsin at a final concentration of 5 μg/ml. At the end of the digestion, gel sample buffer was added and the samples boiled for 60 s, before analysis by SDS-PAGE.

SDS-PAGE, Fluorography, and Quantitation

To examine proinsulin and insulin independently, samples of media and cell lysates were analyzed by 15% acrylamide SDS-PAGE plus urea using a Tricine buffer system (Schagger and von Jagow, 1987). In this system, proinsulins I and II were not resolved from one another; nor were insulins I and II. Gels were fixed in 20% TCA without alcohol, rinsed briefly with water to remove the TCA, and then rinsed briefly with 1 M sodium salicylate before a 20-min incubation with more of the same solution. Gels were dried under vacuum and exposed to preflashed XAR film (Eastman Kodak Co., Rochester, NY) at −70°C. To quantitate the extent of proinsulin processing, fluorographs were read by flatbed scanning and this information was analyzed by the ImageQuant software package (Molecular Dynamics, Benton, NJ). Pixel number and intensities from digitized images (see Fig. 5 *A*) representing proinsulin, conversion intermediates, and insulin, were directly integrated to generate the quantitative analysis. (see Fig. 5 *B*).

Labeled C-peptide: Insulin Ratios

Each [^{3}H]leucine-labeled sample was first precleared by exhaustive immunoprecipitation in the presence of protein A–agarose with an excess of a polyclonal antiserum to either insulin or C-peptide, similar to a previously published method (Ward et al., 1986). As described (Arvan et al., 1991), both antibodies also preclear proinsulin and conversion intermediates. Thus, at the conclusion of the preclearing with anti-insulin, radiolabeled C-peptide remained in the supernatant; after preclearing with anti–C-peptide, radiolabeled insulin remained in the supernatant. As tested by the manufacturer, there was no cross-reactivity of these sera with the opposite peptide. Finally, labeled insulin and C-peptide were directly precipitated from the precleared supernatants with the appropriate antisera. Several controls were used to insure reliable results. First, trial precipitations were performed with each sample to insure that approximately equal insulin counts were used in quantitative measurements of all samples. Second, the final direct precipitation for each sample was repeated with fresh antiserum and immunoabsorbent to insure that the immunoprecipitations were indeed quantitative. Third, total counts recovered in all peptides (precleared plus direct precipitations) differed by only 10% when comparing sequential precipitations in reverse order. Fourth, nonimmune precipitations recovered negligible counts when used in this assay. Finally, fractional recovery of insulin was confirmed by independent measurements of selected ^{35}S-labeled samples using scanning densitometry of fluorographs after SDS-PAGE, and by HPLC of insulin (not shown). To obtain molar ratios of C-peptide:insulin (C/I ratio), C-peptide counts were multiplied by 6/5, a factor which corrects for the relative leucine content of these peptides.

Results

Kinetics of Constitutive-like Secretion

We have previously described constitutive-like secretion of immunoprecipitable insulin-containing peptides from pulse-labeled pancreatic islets as a phase of release whose peak conveys a complex profile of proinsulin, insulin, and conversion intermediates (Arvan et al., 1991). Using SDS-PAGE and fluorography, Fig. 1 *A* shows the secretion of ^{35}S–pulse-labeled insulin-containing peptides as a function of time (containing secretion from the constitutive-like pathway under "euglycemic" [5.5 mM glucose] conditions), as well as regulated secretion during a subsequent stimulatory period. As shown, proinsulin (and one or more conversion intermediates, *open arrowhead*) represented a significant fraction of the phasic discharge during the initial chase hours. It must be noted that the first chase hour is the sole collection period which potentially may be contaminated with very small amounts proinsulin released by the true constitutive pathway (Rhodes and Halban, 1987). The data in Fig. 1 *A* indicate that after the first chase hour, insulin-containing peptides in the constitutive-like phase were derived from a compartment in which processing progressed steadily. This shifting, mixed-peptide profile generally matches the description of the IG, in which the degree of proinsulin conversion is constantly changing (Orci et al., 1986).

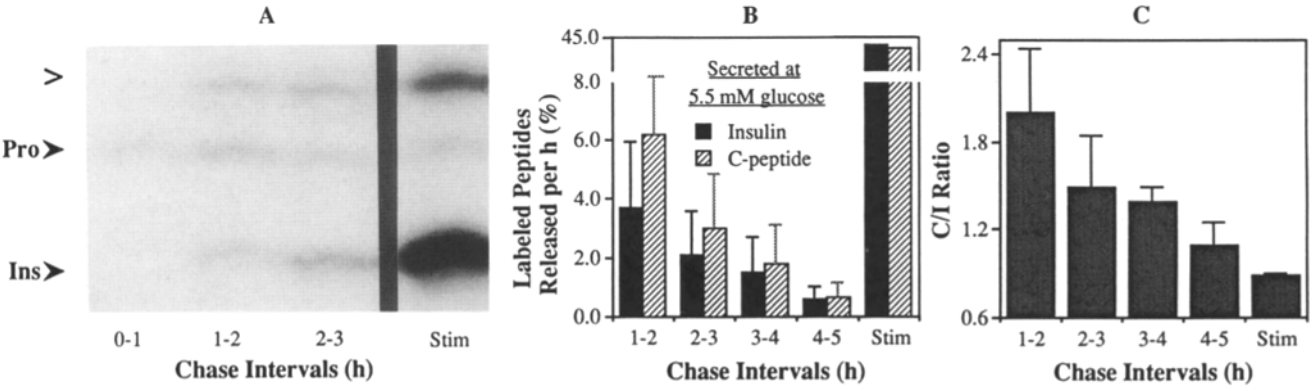

Figure 1. The constitutive-like pathway at 5.5 mM glucose results in phasic secretion of insulin-containing peptides and the nonstoichiometric release of newly synthesized C-peptide. (*A*) SDS-PAGE/fluorograph of immunoprecipitated insulin-containing peptides secreted during each of the first three chase hours and during a subsequent glucose stimulation period from rat pancreatic islets. The isolated islets were pulse-labeled for 7 min with [^{35}S]methionine and cysteine. Labeled protein secretion is evident, with a progression from uncleaved to fully processed insulin. The position of proinsulin and insulin standards is shown (*filled arrowheads*); a band migrating slower than proinsulin in this gel system appears to correspond to a conversion intermediate (*open arrowhead*, and data not shown). Insulin is the predominant form secreted in the stimulation period (*Stim*). (*B*) The recovery of [^{3}H]leucine-labeled insulin and C-peptide in the medium during each hour was expressed as a fraction of the total peptide recovered by immunoprecipitation from the combination of media plus cell lysates. The stimulation period was 5–6 h of chase, and the stimulated secretion (off scale) was 38 ± 10% for C-peptide; 44 ± 14% for insulin. Each bar represents the mean of three experiments. (*C*) Recovered counts in C-peptide and insulin were compared and corrected for leucine content to obtain molar (C/I) ratios at each time point shown in *B*. The error bars represent standard deviations.

Fortunately, the 1:1 stoichiometry of production of C-peptide and insulin is independent of the degree of prohormone conversion (Hutton, 1989; Steiner et al., 1989). Direct exocytosis of granules is accompanied by release of equimolar amounts of C-peptide and insulin (Rubenstein et al., 1969; Polonsky et al., 1984). By contrast vesicle budding from the IG perimeter is expected to sample soluble C-peptide disproportionately over less soluble, crystallizing insulin, creating an elevated "C/I ratio" in the constitutive-like secretion (Arvan et al., 1991). Under euglycemic chase conditions, [^{3}H]leucine-labeled C-peptide and insulin (measured by specific immunoprecipitation) (see Materials and Methods) were each released in a secretory phase (Fig. 1 *B*), and the C/I ratio significantly exceeded unity (Fig. 1 *C*). Importantly, this elevated ratio was not maintained as the chase progressed. In these experiments, the phase of labeled protein secretion and the C/I ratio both achieved peak values by 2 h of chase (although in some islet preparations these peaks occurred in the third chase hour)[2]. By 4–5 h of chase when the phase was nearly complete (Fig. 1 *B*), the C/I ratio approached 1.0 (Fig. 1 *C*). From these kinetic data, we have estimated that insulin secretion via the constitutive-like pathway exhibits a $t_{1/2} \cong$ 1.5 h.

Effect of Glucose Concentration on the Duration of Constitutive-like Secretion

In these experiments, islets pulse labeled as a batch at 5.5 mM glucose were divided and chased at various glucose concentrations to achieve different levels of secretory stimulation. The collected chase media were compared to examine labeled C/I ratios at selected intervals during the secretory phase. Since IGs are bona fide members of the regulated secretory pathway, we reasoned that greater stimulation of direct IG exocytosis should drive the C/I ratio towards unity. Indeed, at the peak of the secretory phase (1–2 h of chase), the secreted C/I ratio decreased progressively from 4.6 at 2.0 mM glucose to ~1.4 at 6.5 mM glucose (Fig. 2)[2]. When a near-maximal secretory stimulus (see Materials and Methods) was applied at this early chase time, a ratio of unity was obtained (not shown). The data indicate that during the 5-h secretory phase, the C/I ratio obtained was largely dependent upon the prevailing level of *B* cell stimulation by glucose, consistent with previous observations (Arvan et al., 1991).

More importantly, as the chase progressed, a decline of the C/I ratio towards unity was observed in a manner that appeared independent of glucose concentration (Fig. 2). A ratio approximating unity also was obtained for all samples (Fig. 2) upon a period of secretagogue stimulation at the end of the experiment (discussed further, below). In fact, at chase times later than 5 h, the C/I ratio never rose regardless of the degree of stimulation. Thus, despite the extended period encompassing the constitutive-like secretory pathway, these findings suggest strongly that this form of secretion is limited to a specific stage of granule maturation.

2. Some of the measurements obtained in the present study differ slightly from those described in our preliminary report (Arvan et al., 1991). We attribute these differences to (*a*) variations between different preparations of islets, and (*b*) improvements in the quantitative recovery of C-peptide by immunoprecipitation, including reprecipitation of supernates from the first immunoprecipitation (see Materials and Methods).

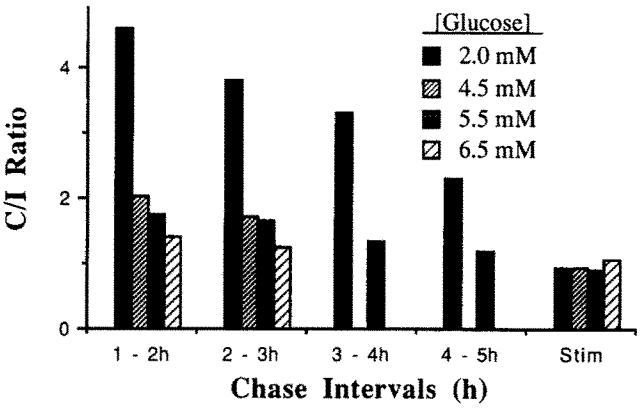

Figure 2. Molar ratios of newly synthesized C-peptide:insulin during the secretory phase at different glucose concentrations and during subsequent stimulation. Rat islets were isolated, pulse-labeled with [^{3}H]leucine, chased, and media immunoprecipitated as in Fig. 1. The data represent the mean of two experiments, each using ~1,200 islets.

Rate of Transfer of Proinsulin from the TGN to IG Compartments

In intracellular traffic through the constitutive secretory pathway, protein export from the ER is the rate-limiting step (Lodish et al., 1983), whereas for the regulated secretory pathway under unstimulated conditions, granule exocytosis is rate limiting (Mains et al., 1987). The rate of constitutive-like secretion in several tissues appears relatively slow (exocrine pancreatic zymogens: $t_{1/2} \cong$ 2.4 h [Arvan and Castle, 1987]; endocrine pancreatic insulin: $t_{1/2} \cong$ 1.5 h [this report]), yet the rate-limiting step in this route is unknown. Autoradiographic studies of pulse-labeled proinsulin molecules suggest a transport rate ($t_{1/2}$) through the ER and Golgi stacks of ~7 and 13.5 min, respectively (based on Orci, 1982), leaving a large amount of time in the constitutive-like pathway ($\geq$1 h) unaccounted for.

Many condensing vacuoles/IGs that in single electron microscope sections appear as "free" in the cytoplasm, can in serial sections be shown to be tethered to the TGN; accordingly, they are not available for regulated exocytosis (Tooze and Tooze, 1986). Thus, the formation of free IGs is difficult to measure by conventional electron microscope autoradiography. Although the rate of IG detachment from the TGN in vitro has been reported comparable to the rapid formation of constitutive secretory vesicles (Tooze and Huttner, 1990), it is not clear if this step might account for the slow overall constitutive-like secretory kinetics observed in vivo. Therefore, we examined detachment of IGs from the TGN and their acquisition of regulated secretory character, by exploiting the reversible 20°C blockade of TGN exit that is shared by both constitutive (Griffiths et al., 1985) and regulated (Chanat and Huttner, 1991) secretory cells.

Pulse-labeled islets were chased for 2 h at 19.5°C to allow proinsulin to accumulate at the site of the 20°C block (presumably the TGN), and then individual islet aliquots were warmed for varying short periods at 37°C to permit escape from the block. In parallel, labeled control islets were incubated at 37°C to permit maximal transfer of newly synthesized proinsulin into IGs (160 min, see Materials and

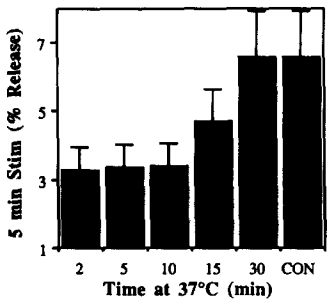

Figure 3. Escape of labeled proinsulin from the 20°C block leads to recovery of 5-min stimulated secretion. Islets were pulse-labeled, chased at 19.5°C, and then warmed to 37°C and stimulated for 5 min as described in Materials and Methods. The secretion of labeled insulin-containing peptides was quantitated by immunoprecipitation (Halban, 1982). The last bar shown (*CON*) represents control islets that were chased at 37°C continuously for 160 min. Note that there was very little recovery of stimulated release during the first 10 min of warming (see text). The data represent the mean of four experiments. The bars represent standard deviations.

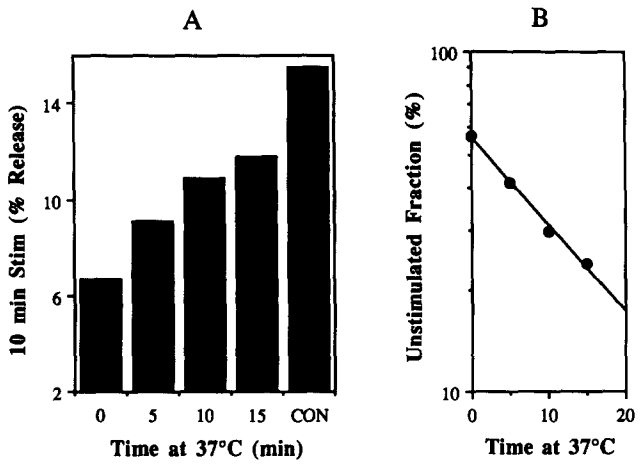

Figure 4. Time course of escape from the 20°C block as measured by stimulated release during a 10-min period. (*A*) Islets were pulse labeled, chased at 19.5°C, and warmed to 37°C for various times as in Fig. 3. The islets were then stimulated for 10 min as described in Materials and Methods. Unstimulated release at each of these times was ~3%. The final bar (*CON*) represents control islets that were chased at 37°C continuously for 160 min before stimulation. Note the apparent lack of a lag time in the recovery of stimulated release (see text). (*B*) Semi-log plot used to calculate the half-time of transfer from the 20°C block to the stimulation compartment (see Materials and Methods). The data represent the mean of two experiments, in which values differed by ~10%. $t_{1/2}$ = 11.9 min.

Methods). Subsequently, all aliquots of islets were exposed to near-maximal stimulation for 5 min at 37°C; this extremely brief period of stimulation was chosen to minimize the effect of the secretion period itself (at 37°C) on the intracellular transfer of labeled proinsulin. Fig. 3 shows that secretion of labeled insulin-containing peptides from samples escaping the 20°C block for only 2, 5, or 10 min demonstrated minimal ability to be stimulated by secretagogue. By contrast, at 15 min, partial recovery was evident, and by 30 min, stimulated release was equivalent to that seen in controls. Although complicated by a lag time seen during the first 10 min of warming, we could roughly estimate a $t_{1/2}$ of ~15 min for transfer from the temperature block to the stimulation compartment. However in this protocol, the fractional secretion of insulin-containing peptides, even in control islets, appeared quite modest. Further, since islets are clusters of ~1,000 cells, it seemed possible that insufficient warming of the islets during the stimulation period (see Materials and Methods) might contribute to the lag time observed.

For these reasons, we re-examined our protocol and doubled the secretagogue exposure period from 5 to 10 min. In this case, the level of secretory stimulation was significantly higher (Fig. 4 *A*). Further, the lag time seen in Fig. 3 was essentially eliminated. These data were conductive to a more precise calculation of $t_{1/2}$; a value of ≤12 min was obtained (Fig. 4 *B*) which was fairly similar to the rough estimate obtained from the 5-min stimulation assay (Fig. 3). However, given the possible time required for thermal equilibration of islet tissue, the $t_{1/2}$ value of 12 min (Fig. 4 *B*) is likely to be overestimated. In fact, it seemed possible that IG arrival might even occur at 19.5°C and that half-times of recovery might reflect only temperature sensitivity of the exocytotic machinery, since stimulation of control islets at 20°C results in very little regulated exocytosis of secretory granules (Provoda, C., and P. Arvan, unpublished data). Since completion of proteolytic conversion to insulin is thought to occur nearly quantitatively after arrival in IGs (Orci et al., 1985, 1987*b*; Davidson et al., 1987; for review see Hutton, 1989), we wished to confirm that recovery from the 20°C block characterized proinsulin transport from TGN to IG by examining the degree of labeled proinsulin processing in islets escaping the temperature block.

Fig. 5, *A* and *B* indicates that in control islets (170 min ≅ 14 half-times, Fig. 5 *A*, lane *4*), the extent of proinsulin processing was nearly complete. By contrast, islets permitted to escape the 20°C block for 10 and 25 min (~1 and ~2 half-times, respectively) had markedly reduced proinsulin processing (Fig. 5 *A*, lanes *1* and *2*, and Fig. 5 *B*), despite the fact that they had been at 19.5°C for >2 h. These data suggest that insulin is not formed in the TGN; thus constitutive-like secretion measured by C/I ratio probably originates in IGs. Even after four half-times, proinsulin conversion was <50% complete (Fig. 5) although by two half-times, islets exhibit stimulation approaching control levels. Therefore, the half-time of acquisition of regulated secretory behavior (Fig. 4 *B*) reflects a step that precedes insulin production from proinsulin; this most likely represents transfer from TGN to IG. Although it is possible that cooling could slow the rate of proinsulin processing in vivo, if this were the case, it would imply that arrival in IGs might be even faster than that which we have estimated. Thus, it is unlikely that detachment of IGs from the TGN is rate-limiting in constitutive-like secretion.

Constitutive-like Secretion of Labeled Proteins Is Independent of Protein Synthesis

Ongoing protein synthesis is not required to complete transport of nascent secretory proteins through the regulated secretory pathway during a 2-h chase under stimulated conditions (Jamieson and Palade, 1971). We wished to see if detection of phasic, constitutive-like secretion (under less stimulated conditions) of pulse-labeled insulin-containing peptides requires normal levels of synthesis of nascent chains, either during pulse labeling or during the first 2-h

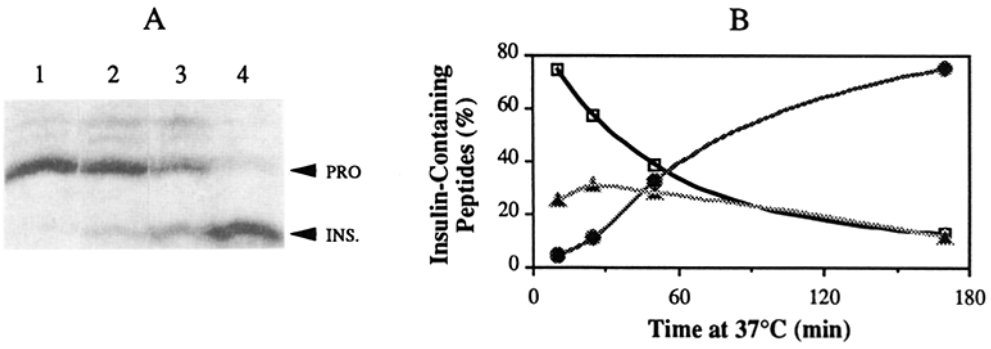

Figure 5. Labeled proinsulin blocked at 20°C has not arrived at the IG, based on its lack of conversion to insulin. Islets were pulse labeled, chased at 19.5°C, and warmed to 37°C for 10 min (~1 half-time of transfer from TGN to IG based on Fig. *4* b, lane *1*), 25 min (~2 half-times, lane *2*), or 50 min (~4 half-times, lane *3*). Control islets were chased at 37°C for 170 min (lane *4*). At each chase time the islets were lysed and mixed with their bathing media to account for any peptides that might have been secreted. The samples were then assayed by SDS-PAGE and fluorography to measure cleavage to mature insulin as an assay for time spent in the IG compartment. (*A*) The fluorographs were read by a flatbed scanner and the images digitized as described in Materials and Methods. The positions of proinsulin (*PRO*) and insulin (*INS.*) are indicated; the upper bands represent presumptive conversion intermediates. (*B*) The bands encoded in the digital images were integrated directly by computer (see Materials and Methods); radiolabel contained in conversion intermediates were summed and treated as one entity (*triangles*) as was material in the proinsulin (*squares*) and insulin (*circles*) bands.

postpulse. Islets were pretreated (45 min) and pulse labeled in the presence of 1 μg/ml cycloheximide and then chased in cycloheximide at 0.1 μg/ml; these conditions sustain a constant 80% inhibition of islet protein synthesis (not shown). During cycloheximide treatment, constitutive-like secretion proceeded in the usual phasic manner (Fig. 6). In other experiments we observed that extended inhibition of protein synthesis throughout five chase hours was similarly without effect on the phasic secretory pattern (not shown).

Canavanine Treatment Selectively Inhibits Constitutive-like Proinsulin Secretion

Since recent evidence has suggested the possibility that insulin crystallization may play a role in its retention within the IG compartment (Carroll et al., 1988), and proinsulin conversion is a prelude to insulin crystallization, we wished to test the effect of inhibition of proinsulin cleavage in situ on the magnitude of constitutive-like secretion. For this purpose we used canavanine, a fungal amino acid which remarkably resembles arginine, can be coupled to arginyl-tRNA, and is incorporated into protein. Rat proinsulin contains arginine within both critical dibasic cleavage sites that potentially can be replaced with canavanine. Previous studies using canavanine in pancreatic *B* cells have suggested that it results in the synthesis of a structurally normal proinsulin which is not cleaved by prohormone processing enzymes (Rosenzweig and Yip, 1979; Noe, 1981). Although canavanine replacement for arginine in the culture medium results in a reproducible 60–80% reduction in overall protein synthesis (not shown), this level of protein synthesis inhibition per se does not interfere with islet secretory protein targeting (Fig. 6). Fig. 7 *A* confirms that we were able to introduce canavanine into these cells, since canavanine replacement for arginine in the medium bathing isolated islets (see Materials and Methods) markedly decreased the conversion of newly synthesized proinsulin to insulin found in islets (compare Fig. 7 *A*, lanes *1* and *2*) and secretion (Fig. 7 *A*, lanes *3* and *4*) during the course of a 5-h chase period. On the other hand,

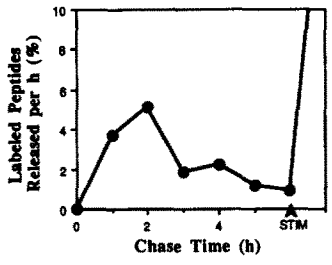

Figure 6. Phasic release of newly synthesized insulin from the constitutive-like pathway is not inhibited by cycloheximide. Islets were preincubated for 45 min, pulse labeled, and washed in the presence of 1 μg/ml cycloheximide. The labeled islets were then chased for 2 h in the presence of cycloheximide at 0.1 μg/ml (to inhibit protein synthesis 80% relative to control values), at which time cycloheximide was removed. Media and cell lysates were analyzed by quantitative immunoprecipitation with antibody to insulin. Stimulated secretion (off scale) was ~18%. The data represent the mean of two experiments.

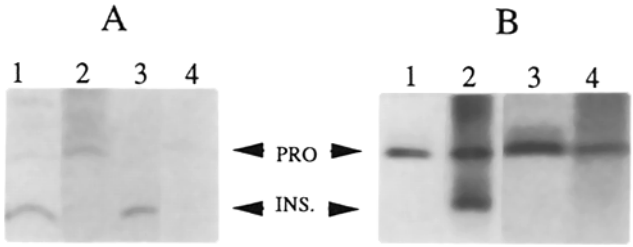

Figure 7. Arginine replacement with canavanine results in the synthesis of a proinsulin which is largely uncleavable. (*A*) Aliquots of pancreatic islets pulse labeled for 15 min with ^{35}S-amino acids and chased for 5 h (lanes *1* and *2*), or media secreted during the interval 4–5 h (lanes *3* and *4*) from cells treated either with arginine (lanes *1* and *3*) or canavanine (lanes *2* and *4*) were analyzed by SDS-PAGE and fluorography (see Materials and Methods). The position of proinsulin and insulin standards are shown. Note that endogenous conversion to mature insulin was substantially inhibited in the canavanine-treated islets. (*B*) Lysates from islets pulse labeled as in *A* either were not digested (lanes *1* and *3*) or were briefly incubated with low-dose trypsin (lanes *2* and *4*). Lanes *1* and *2*, arginine-treated controls; lanes *3* and *4*, canavanine-treated. Although carboxypeptidase activities have not been added, the tryptic cleavage product from control cells migrates close to the insulin position. The proinsulin synthesized in canavanine-treated islets is substantially resistant to tryptic cleavage.

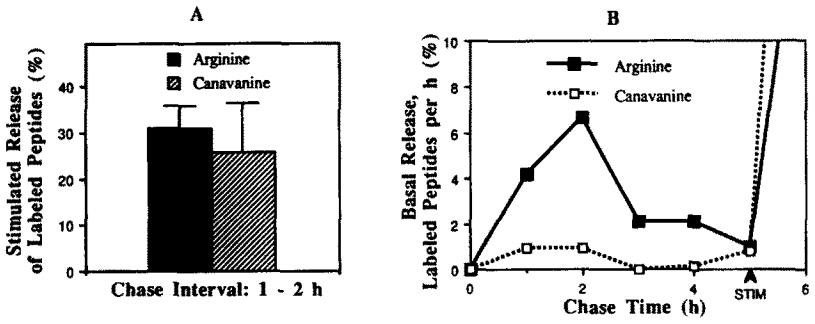

Figure 8. Canavanine treatment blocks phasic release of newly synthesized insulin from the constitutive-like pathway, yet permits normal secretion from the regulated pathway. (*A*) Arginine or canavanine-treated islets were pulse labeled as in Fig. 5, chased for 1 h, and then stimulated for 60 min with secretagogues. As shown, secretion of immunoprecipitable proinsulin synthesized in canavanine-treated islets was stimulated to the same extent as that in control islets, indicating that proinsulin arrival in the IG compartment is unaffected by canavanine treatment. The data represents the mean of three experiments. (*B*) Isolated islets were divided and treated with arginine or canavanine as in Fig. 5. Pulse labeling, noncumulative chase collections at 5.5 mM glucose, and insulin immunoprecipitations were performed as in Materials and Methods. Constitutive-like phasic secretion, evident from control islets, was blocked in the canavanine-treated islets. By contrast, stimulated release in the last chase hour (off scale) from canavanine treated islets was ~35%; from arginine-treated controls, ~25%. The data represent the mean of two experiments.

arginine–proinsulin synthesized for 7 min in islets maintained (before and after) in canavanine, was processed to insulin to a similar extent as that observed in control islets (not shown). Further, in vitro incubation of canavanine–proinsulin with low-dose trypsin (Fig. 7 *B*, lanes *3* and *4*) showed significant protease resistance relative to arginine-proinsulin (Fig. 7 *B*, lanes *1* and *2*).

Since the sorting of newly synthesized prohormones into the regulated secretory pathway does not depend upon their proteolytic cleavage (Halban, 1982; Tooze et al., 1987; Powell et al., 1988), it was not unexpected that when canavanine-treated islets were pulse labeled with [^{3}H]leucine, chased for 60 min and then stimulated with secretagogues, canavanine–proinsulin was secreted equally well to insulin-containing peptides from arginine-treated control islets (Fig. 8 *A*). Surprisingly however, under conditions of canavanine treatment, the constitutive-like pathway as monitored by phasic secretion (Fig. 8 *B*) was largely blocked. Further, even after protracted blockade, the subsequent ability to stimulate release of canavanine–proinsulin from the regulated secretory pathway still was preserved (Fig. 8 *B*). Thus, the use of canavanine demonstrates selective inhibition of the constitutive-like secretory pathway, a process which does not seem to be obligatory in preparation of granules for regulated discharge.

Discussion

Constitutive-like Secretion Is a Novel Pathway of Membrane Traffic Originating in IGs

Two complementary approaches used for a molecular dissection of secretory protein sorting and targeting are the use of mutational analysis of secretory proteins to define domains that confer specific targeting, and the development of new assays that permit sophisticated analysis of the routing of wild-type and mutant proteins. We recently have focussed attention on assays to delineate the constitutive-like pathway, demonstrating that this route begins after TGN sequestration into condensing vacuoles/IGs (Arvan and Chang, 1987), since it conveys granule proteins that in many instances have undergone IG-specific processing (Arvan et al., 1991; this report, Figs. 1, 2, and 5). A serious problem in analyzing constitutive-like secretion is that it exhibits significant kinetic overlap with both true constitutive secretion (at early chase times) and direct granule exocytosis (at later chase times). In fact, the IG itself is a member of the regulated secretory pathway (Arvan and Castle, 1987; Arvan et al., 1991; Tooze et al., 1991), leading to the proposal that constitutive-like secretion is a specialized portion of the regulated pathway involved in direct IG exocytosis (Rhodes and Halban, 1987). Despite this, we previously demonstrated the presence of constitutive-like secretion of granule proteins in the fetal exocrine pancreas at a time in development when stimulus-dependent secretion had not yet appeared (Arvan and Chang, 1987), and in adult pancreatic islets under unstimulated conditions in which regulated exocytosis was virtually eliminated (Arvan et al., 1991). Further, in the present report we have shown reciprocal inhibition with canavanine-treatment such that phasic, constitutive-like secretion can be largely blocked without inhibiting regulated IG exocytosis (discussed further, below).

Our data in pancreatic islets (Fig. 2) suggest that as the level of secretory stimulation is raised, IGs are secreted to a progressively greater extent by direct exocytosis, driving the C/I ratio down towards unity. Thus, the high glucose concentrations used in many earlier pulse–chase studies of pancreatic islets may have obscured detection of the constitutive-like pathway. Such an outcome is likely since only a relatively small fraction (~10%) of all peptides immunoprecipitable with anti-insulin serum is secreted during the 5-h phasic process under euglycemic conditions (Arvan et al., 1991). However, since these immunoprecipitates include proinsulin (and conversion intermediates), and since the major cellular pool of proinsulin (largely restricted to IGs) is considerably smaller than the pool of cellular insulin (mature granules plus IGs), it is evident that the constitutive-like pathway is likely to be a quantitatively significant route of cellular proinsulin secretion. A direct measurement of constitutive-like proinsulin secretion in physiological and pathological states therefore will be of considerable value.

Constitutive-like Secretion Involves Passive Sorting in the IG Compartment

We propose that the constitutive-like pathway is initiated by the budding of membrane vesicles from IGs at glucose con-

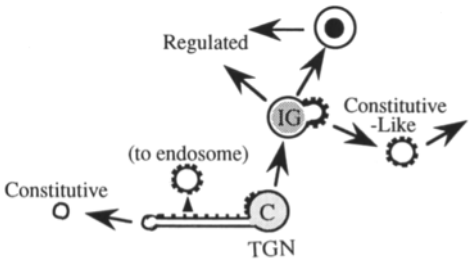

Figure 9. Schematic model of the origin of the constitutive-like pathway. As vesicles bud from the TGN for lysosomal enzyme targeting and true constitutive secretion, condensing vacuoles (*C*) bud to form IGs. IGs undergo direct exocytosis in response to secretagogue stimulation as do mature granules (shown containing dense core). Under less stimulated conditions, IGs mature over time, condensing their core. Constitutive-like secretion is envisioned to originate by vesicle budding during IG maturation.

centrations within the physiologic range (Fig. 9). Secretion of C:I with a stoichiometry in excess of that existing in the granule compartment (Figs. 1 and 2), supports this hypothesis. Our data imply that protein crystallization is important for intracellular insulin retention, whereas solubility of C-peptide seems to be a major factor in its exit via the constitutive-like pathway. Thus, a passive form of molecular sorting would appear to be occurring within IGs. Constitutive-like C-peptide secretion is consonant with the reported nonstoichiometric loss of intracellular C-peptide from islet *B* cells (Rhodes and Halban, 1988). If, as we estimate, ~6% of the labeled C-peptide and ~3% insulin (C/I ~2) is normally released in the constitutive-like secretory phase under euglycemic conditions (plus secretion of proinsulin and conversion intermediates adding up to ~10% [proinsulin + insulin] released), the remaining granules should then contain ~87% labeled C-peptide and ~90% insulin. This latter C/I ratio (0.967) should then be released upon secretory stimulation from mature granules, a value difficult to distinguish from 1.0. Averaging data from islets chased initially at 2.0–5.5 mM glucose, a C/I ratio was obtained in the final stimulation period of 0.94 ± 0.09 ($n = 9$), whereas for islets chased initially at >6.5 mM glucose, the final stimulated C/I ratio was 1.13 ± 0.11 ($n = 5$). (The average of all samples combined was 1.01 ± 0.13 [$n = 14$].) From these data, a trend can be seen that the stimulated C/I ratio is slightly lower in samples which exhibited a higher ratio during the first 5 h.

Our results must be distinguished in two ways from those recently reported by Quinn et al. (1991), who described regulated secretion of both crystallizing and noncrystallizing (pro)insulin variants transfected into AtT-20 cells. (*a*) From their studies of long-term (16 h) labeling of transfected cells, the fraction of (pro)insulin which entered IGs but was not retained therein (i.e., released into the long-term labeling medium) cannot be estimated because it was not analyzed. Therefore, it may be that the ability of proinsulin to oligomerize does not affect its entrance into IGs, but if granule content sorting is mediated in part by intragranular protein retention after its entrance, then the conclusions of Quinn et al. (1991) must be considered tentative. (*b*) While insulin expressed in islets is known to crystallize (Michael et al., 1987), such homo-crystallization is uncertain after transfection in heterologous cell lines, even when normal insulin is expressed. In this case, it is just as likely that heterophilic associations of both normal and mutant insulins with endogenous AtT-20 granule proteins might account for the observations of Quinn et al. (1991).

Constitutive-like Secretion Is Self-limited

Our model suggests that vesicular budding is a function of IGs as well as the TGN. Indeed, the data following the secreted C/I ratio kinetically (Figs. 1 and 2), suggest that the vesicular budding which initiates constitutive-like secretion is restricted to the period of granule maturation. Given the presence of this third secretory route, it is quite likely that results of earlier studies interpreted as demonstrating constitutive secretion of granule proteins (i.e., from the TGN) may be explained at least in part by the constitutive-like pathway from IGs. Further, the hypothesis that agents which raise intraorganellar pH cause rerouting of granule proteins to the constitutive pathway (Moore et al., 1983) may need to be reconsidered. An alternate hypothesis is that some or all of the observed rerouting may represent a consequence of abnormal peptide condensation in the IG compartment (von-Zastrow et al., 1989), with polypeptide loss from IGs via constitutive-like secretion.

The Constitutive-like Secretory Pathway May Use Clathrin-coated Vesicles

Use of canavanine was selected because it represents the only means thus far shown to block proinsulin cleavage in wild-type pancreatic islets in situ. Our initial thought was that inhibition of proinsulin cleavage might lead to reduced insulin crystallization, which potentially could augment constitutive-like proinsulin secretion. Surprisingly, despite the inhibition of conversion to mature insulin, constitutive-like secretion appeared blocked. This effect was not due to a generalized reduction in protein synthesis, since cycloheximide inhibition of protein synthesis to comparable levels had no inhibitory effect on the constitutive-like secretory phase. Further, regulated secretion of the same granules was entirely unaffected, indicating that constitutive-like secretion is not an obligatory step in preparation of granules for stimulus-dependent, regulated secretion.

Since canavanine replaces arginine in every protein whose turnover is sufficiently high, great caution must be used in drawing conclusions about its particular site of action with respect to constitutive-like secretion. With this caveat firmly in mind, one extremely interesting study involving electron microscope autoradiography examined pulse-labeled control pancreatic islets and those treated with canavanine (and lysine analogs, [Orci et al., 1984]). Unlike the control islets, whose IGs detached from the TGN with a clathrin coat which then was shed, the canavanine-treated islets showed a block in IG maturation such that IGs detached from the TGN, but subsequently were unable to shed their clathrin coats. Since these images would appear to represent the morphological correlate of our current biochemical findings, such observations raise the possibility that the constitutive-like vesicles which bud from IGs may be clathrin coated.

Previous workers have suggested that clathrin-coated vesicles may be used in direct transport from the TGN to the cell surface (Fishman and Fine, 1987); however, no definitive evidence yet exists to show that clathrin-coated vesicles can

serve as direct exocytotic carriers from the IG compartment. The kinetic data shown herein suggest a $t_{1/2}$ of ~1.5 h for the overall constitutive-like secretion of insulin. However, the steps including ER exit, transport across the Golgi stacks, condensing vacuole formation, and IG detachment from the TGN (Figs. 3 and 4), together account for less than half of this time. Thus it would appear that another step after vesicle budding from IGs is likely to be rate limiting in the constitutive-like secretory pathway.

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