Association of Newly Synthesized Islet Prohormones with Intracellular Membranes

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ABSTRACT Results from recent studies have indicated that pancreatic islet prohormone converting enzymes are membrane-associated in islet microsomes and secretory granules. This observation, along with the demonstration that proglucagon is topologically segregated to the periphery within alpha cell secretory granules in several species, led us to investigate the possibility that newly synthesized islet prohormones might be associated with intracellular membranes. Anglerfish islets were incubated with [^{3}H]tryptophan and [^{14}C]isoleucine for 3 h, then fractionated by differential and density gradient centrifugation. Microsome (M) and secretory granule (SG) fractions were halved, sedimented, and resuspended in the presence or absence of dissociative reagents. After membrane lysis by repeated freezing and thawing, the membranous and soluble components were separated by centrifugation. Extracts of supernatants and pellets were chromatographed by gel filtration; fractions were collected and counted. A high proportion (77–79%) of the newly synthesized proinsulin and insulin was associated with both M and SG membranes. Most of the newly synthesized proglucagons and prosomatostatins (12,000-mol-wt precursors) were also membrane-associated (86–88%) in M and SG. In contrast, glucagon- and somatostatin-related peptides exhibited much less membrane-association in SG (24–31%). Bacitracin, bovine serum albumin EDTA, RNAse, α-methylmannoside, *N*-acetylglucosamine, and dithiodipyridine had no effect on prohormone association with membranes. However, high salt (1 M KCl) significantly reduced membrane-association of prohormones. Binding of labeled prohormones to SG membranes from unlabeled tissue increased with incubation time and was inhibited by unlabeled prohormones. The pH optimum for prohormone binding to both M and SG membranes was 5.2. It is suggested that association of newly synthesized prohormones with intracellular membranes could be related to the facilitation of proteolytic processing of prohormones and/or transport from their site of synthesis to the secretory granules.

Two lines of suggestive evidence led us to investigate the possibility that islet prohormones might be associated with intracellular membranes. The first was the demonstration that islet prohormone converting enzymes may be associated with secretory granule membranes (1, 2). More recently, membrane-association of converting enzymes has been demonstrated in secretory granules from pituitary neurointermediate lobe (3), anterior lobe (4), and in hypothalamic synaptosomes (5). The second line of suggestive evidence was derived from the results of Ravazzola and Orci (6) and Garaud et al. (7). These investigators employed Protein A–gold immunohistochemistry to demonstrate the apparent segregation of glucagon precursors at the periphery of alpha cell secretory granules and relatively higher concentrations of glucagon at the granule core. If there is a non-uniform distribution of prohormones and products within secretory granules, then there must be a mechanism for maintaining differential distribution. It is thus possible that newly synthesized precursors maintain an association with secretory granule membranes. Moreover, it is possible that this association might be initiated more proximally, in the membranes of the rough endoplasmic reticulum (ER)[1] and/or the Golgi complex. We have therefore examined the possibility that newly synthesized islet prohormones may

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; ER, endoplasmic reticulum; S-14, somatostatin-14; S-28 (anglerfish) somatostatin-28. NaAc-HAc, 40 mM sodium acetate–acetic acid (pH 5.2).

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become associated with the inner aspect of microsome and secretory granule membranes.

MATERIALS AND METHODS

Materials: L-^{3}H-amino acid mixture (185 mCi/mg) and L-[^{14}C]isoleucine (300 mCi/mmol) were obtained from ICN (Irvine, CA). L-[^{3}H]tryptophan (8.0 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Bio-Gel P-2 and P-30 (100–200 mesh) and Protein Assay Reagent were obtained from Bio-Rad Laboratories (Rockville Center, NY). Bovine serum albumin (BSA),[1] bacitracin, RNAse, α-methylmannoside, *N*-acetylglucosamine, and 2,2′-dithiodipyridine were purchased from Sigma Chemical Co. (St. Louis, MO). National Diagnostics (Somerville, NJ) was the source of Monofluor scintillation cocktail.

Incubation and Fractionation of Islet Tissue: Much of the detailed methodology employed to incubate (1, 2, 8) and fractionate (2, 9, 10) anglerfish islet tissue has been published previously. Fractions prepared by the procedure employed have been characterized morphologically (9), monitored for prohormone and hormone content (1, 10) and examined for plasma membrane and lysosomal enzyme markers (2). In the present study, 2-mm^3 pieces of decapsulated islet tissue were incubated 3 h in the presence of 100 μCi [^{3}H] tryptophan and 20 μCi [^{14}C]isoleucine in 280 mosm Kreb's Ringer bicarbonate. All procedures subsequent to islet incubation were performed at 4° C. After washing three times with Kreb's Ringer bicarbonate, the tissue was suspended in 1.0 ml 0.25 M sucrose, 1 mM $MgCl_2$, homogenized, and fractionated as described (2, 9). The two secretory granule fractions from each preparation were combined and concentrated by centrifugation (150,000 *g*, 0.5 h). Microsome fractions were concentrated in separate tubes during the same centrifugation. The secretory granule and microsomal precipitates were both suspended in 5.0 ml of 40 mM sodium acetate-acetic acid (NaAc-HAc), pH 5.2, and transferred to 5-ml centrifuge tubes. After refluxing thoroughly, each suspension was divided equally into two 5 ml centrifuge tubes and re-precipitated (150,000 *g*, 0.5 h).

To prepare ^{3}H-labeled prohormones for membrane-association studies, 110–150 mg of islet tissue was incubated 5 h in Kreb's Ringer bicarbonate with 500 μCi of a mixture of 15 ^{3}H-amino acids. After the incubation, the tissue was washed three times with Kreb's Ringer bicarbonate, then homogenized and extracted in 2 M acetic acid. Extracts were desalted on Bio-Gel P-2 and chromatographed on columns of Bio-Gel P-30. Details of these procedures are given in references 8–10. The portion of the eluates containing the M_r 8,500–15,000 peptides were recovered and lyophilized.

Analysis of Membrane—Association of Newly Synthesized Prohormones and Hormones: One half of each secretory granule and microsome preparation was suspended in 0.8 ml of pH 5.2 NaAc-HAc (controls) and the other half was suspended in 0.8 ml of the same buffer containing the appropriate concentration of the potential dissociative reagent to be tested (experimentals). The particulate fractions were dispersed in a hand-held siliconized homogenizer, transferred to siliconized glass tubes and the membranes were disrupted by repeated freezing and thawing (6 ×) using a dry ice-acetone bath. In experiments in which the effects of detergents on membrane-association of prohormones were tested, the freeze-thaw lysis was omitted.

After membrane lysis, each preparation was transferred to a 2-ml centrifuge tube and membranous components were separated from soluble components (150,000 *g*, 0.5 h). Supernatants were aspirated, placed in siliconized glass tubes and extracted by adding 0.8 ml 4 M acetic acid. Precipitates were extracted two times with 0.8 ml 2 M acetic acid with the insoluble material being removed by centrifugation. Before gel filtration, 10 mg bacitracin was added to each extract to reduce nonspecific adsorption of prohormones and hormones to the gel matrix. Extracts were subjected to gel filtration on matched columns of Bio-Gel P-30 (1.6 × 96 cm) in 2 M acetic acid. Alternate 1.5-ml fractions were assayed for radioactivity by liquid scintillation spectroscopy.

Computation of Membrane—Association of Newly Synthesized Polypeptides: It has been demonstrated that glucagon-related peptides (2, 8, 10), somatostatin-14 (S-14) (1, 11, 12), somatostatin-28 (S-28) (13, 14), and their respective precursors become selectively labeled with [^{3}H]-tryptophan in anglerfish islets. Proinsulin and insulin can be selectively labeled with [^{14}C]isoleucine in this system (2, 8, 11, 12). It is thus possible to monitor the relative amounts of prohormones and hormones in tissue extracts by assessing the distribution of [^{3}H]tryptophan- and [^{14}C]isoleucine-labeled peptides in gel filtration eluates. The prohormones for the glucagons and somatostatins elute collectively and form a distinct peak of ^{3}H-radioactivity at M_r 12,000. These have been designated as "12,000-mol-wt precursors." Glucagon-related peptides and S-28[2] form a broad peak of ^{3}H-label between M_r 2,500 and M_r 4,500. S-14 produces a ^{3}H peak at M_r 1,600. Proinsulin and insulin produce ^{14}C-labeled peaks at M_r 10,000 and M_r 6,000, respectively (1, 2, 8, 10–14).

To assess membrane-association of the various polypeptides in microsomes and secretory granules, we added the total ^{3}H- or ^{14}C-radioactivity that was attributable to proinsulin, 12,000-mol-wt precursors, or products found in extracts of soluble and membranous components. Percent membrane-association was then calculated by dividing the radioactivity in the membranous component by this sum:

% membrane-association = DPM ^{3}H- or ^{14}C-labeled peptide in precipitates/ DPM ^{3}H- or ^{14}C-labeled peptide in precipitates plus supernatants,

where DPM refers to disintegrations per minute.

Association of Labeled Prohormones with Secretory Granule Membranes from Unlabeled Tissue: Secretory granules were prepared from unlabeled islet tissue, lysed by repeated freezing and thawing in NaAc-HAc, 1 M KCl (to dissociate membrane-associated proteins), concentrated (150,000 *g*, 0.5 h), and the resulting granule membranes were washed three times in NaAc-HAc without KCl. The washed membranes were suspended in 1.0 ml NaAc-HAc and dispersed in a hand-held homogenizer. Binding studies were performed in 1.5-ml conical polypropylene centrifuge tubes using a total volume of 100 or 125 μl. The buffer was 40 mM NaAc-HAc, pH 5.2, containing 0.1% BSA. To assess membrane-association of prohormones, we combined 8.0 μl of the dispersed membranes ($\approx$64 μg protein) and prohormones labeled with a ^{3}H-amino acid mixture (426 mCi/mmol; final concentration = 2.6×10^{-6} M) for incubation at 4°C. Nonspecific binding to the polypropylene tubes was assessed by omitting granule membranes. Incubations containing membranes were performed in triplicate; those lacking membranes were performed in duplicate. After incubation, samples were centrifuged 5 min in a microcentrifuge at 4°C. Supernatants were removed and precipitates were suspended in 1.5 ml of 2.0 M acetic acid. Radioactivity in precipitates was assayed using 3.6 ml of Monofluor. Prohormone association with membranes was expressed as counts per minute or CPM (or picomoles) bound. CPM bound was derived by subtracting the nonspecific binding CPM from the CPM found in membrane precipitates.

Determination of pH Optimum for Prohormone Association with Membranes: Membranes from both secretory granules and microsomes were prepared and washed with 1 M KCl as described above. Aliquots of dispersed secretory granule (80 μg protein) and microsomal (12 μg protein) membranes were incubated 30 min with prohormones labeled with a ^{3}H-amino acid mixture (426 mCi/mmol; final concentration, 1.3×10^{-6} M) at 4°C in buffers of varying pH. The buffers were NaAc-HAc at pH 3.7 or pH 5.2; sodium phosphate at pH 6.2 or 7.2; and sodium carbonate at pH 9.2. The concentration of all buffers was 40 mM. Incubations were terminated by centrifugation and prohormone association with membranes was assessed as described above.

RESULTS

Newly synthesized prohormones were found to be associated with the membranous components of both microsomes and secretory granules. The two panels on the left side of both Figs. 1 and 2 are representative gel filtration chromatograms that show the relative distribution of prohormones and hormones between membranous and soluble components immediately after membrane lysis in the absence of dissociative agents. The ^{3}H-labeled 12,000-mol-wt precursors and ^{14}C-labeled proinsulin were found predominantly in the membrane precipitates of both microsomes and secretory granules. Most of the insulin recovered was also found in the membranous fractions. The two panels on the right side of Figs. 1 and 2 show the change in the pattern of membrane-association that was observed when membrane lysis was performed in the presence of 1 M KCl, a treatment known to reduce noncovalent interactions between proteins. Incubation with high salt concentrations caused a dissociation of the prohormones and insulin from the membranes.

The data in Figs. 3 and 4 summarize the results from a number of experiments. In the absence of dissociative agents, 78% of the proinsulin recovered in both secretory granule and microsome fractions was associated with the membranous components. Proglucagon(s) and prosomatostatin(s) were also associated predominantly with secretory granule (86%) and

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[2] Noe, B., and Spiess, J., 1983, Abstracts 65th Annual Meeting of the Endocrine Society, p. 85; also, manuscript in preparation.

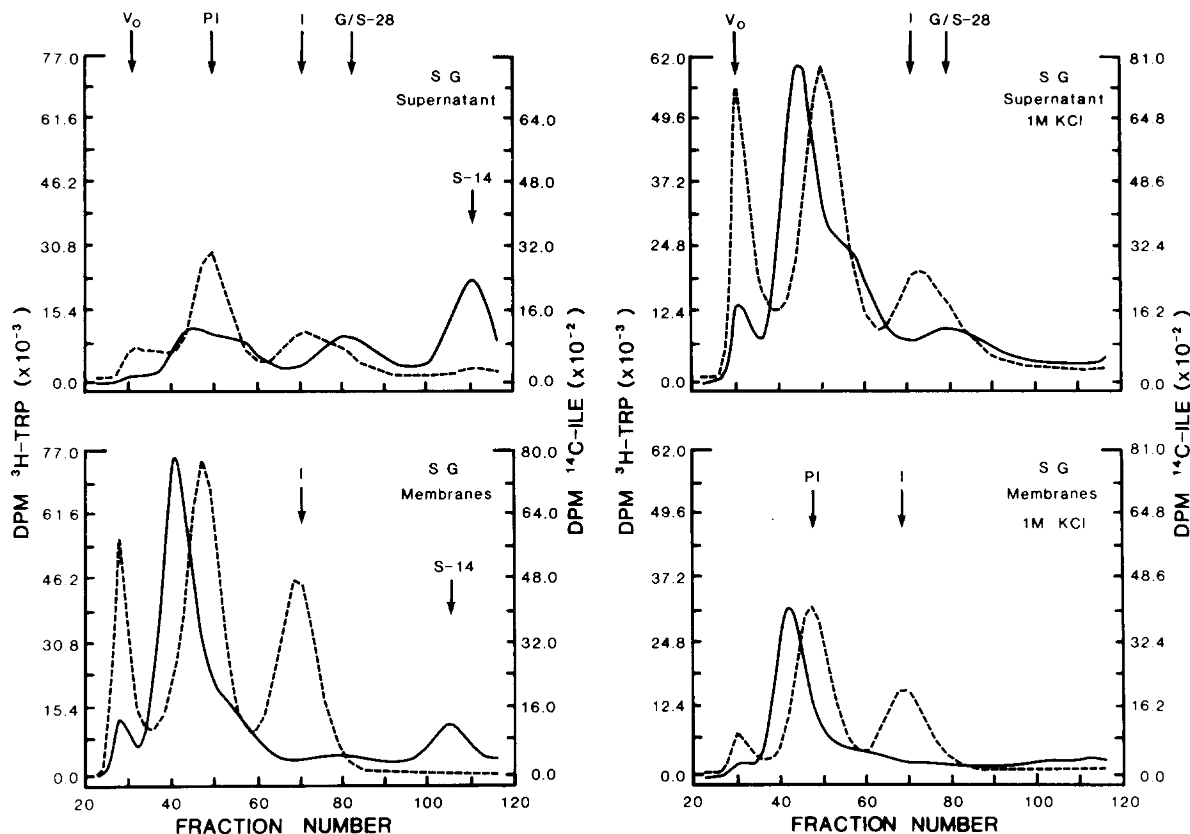

FIGURE 1 Association of islet prohormones and hormones with islet secretory granule membranes and dissociation by 1 M KCl. The procedures for fractionation of labeled islet tissue, division and treatment of the granule fractions, and gel filtration of subfraction extracts are described in Materials and Methods. These gel filtration chromatograms show the distribution of ^{3}H-labeled 12,000-mol-wt precursor and products (——) and ^{14}C-labeled proinsulin and insulin (- - -) between islet secretory granule membranes (bottom) and supernatants (top) disrupted in the absence (left) and presence (right) of 1 M KCl. Elution markers: V_o, void volume; *PI*, proinsulin; *I*, insulin; *G/S-28*, glucagon(s) and somatostatin-28; *S-14*, somatostatin-14.

microsome (88%) membranes. The association of insulin with membranes was also high in the absence of dissociative agents; 79% in secretory granules and 77% in microsomes. In contrast, the association of glucagon-related peptides, S-28 and S-14 with intracellular membranes was significantly lower. Glucagon and S-28 association with granule membranes was 31% and with microsomal membranes was 46%. Similarly, only 24% of the S-14 recovered was associated with secretory granule membranes. No data for S-14 association with microsomal membranes was generated since no S-14 was recovered from microsome fractions (Fig. 2). Partial solubilization of membranes with lysolecithin resulted in a significant reduction of the amount of islet polypeptides which were found in the precipitates (Figs. 3 and 4). This suggests that the presence of granule or microsomal membranes was specifically required to achieve precipitation of the hormone-related molecules and excludes the possibility that some soluble factor from microsomes or granules caused the polypeptides to precipitate. If granule or microsome lysis was performed in the presence of KCl, membrane-association of islet polypeptides was reduced. A significant reduction in the membrane-association of the 12,000-mol-wt precursors, but not glucagon- and somatostatin-related peptides, was induced by 100 mM KCl (Fig. 4). At higher salt concentrations (1 M KCl), membrane-association of all islet polypeptides was reduced significantly (Figs. 3 and 4).

Since KCl was effective in promoting dissociation of the prohormones and insulin from intracellular membranes, it was assumed that the associative mechanism was noncovalent in nature. In an attempt to further define the nature of the interaction between the islet polypeptides and membranes, several potential dissociative agents other than KCl were tested. Because it is known that some of the anglerfish islet prohormone converting enzymes are membrane-associated cysteine proteinases (1, 2), we wanted to determine whether prohormone association with membranes might be mediated via an enzyme-substrate interaction. Therefore, freshly prepared granules and microsomes were lysed in the presence of dithiodipyridine, a reagent known to be a potent competitive inhibitor of islet prohormone conversion (2). At a concentration that was very effective in inhibiting prohormone conversion (300 μM), dithiodipyridine had no effect on the association of islet polypeptides with intracellular membranes (Figs. 3 and 4). Similarly, dithiodipyridine at 1.0 mM had no effect. It was also found that EDTA (10 mM), α-methylmannoside (500 mM) and *N*-acetyl glucosamine (1.0 M) caused no reduction in prohormone association with either microsomal or secretory granule membranes (data not shown). These results indicate that neither divalent cations nor branched chain oligosaccharides are involved in mediating prohormone association with intracellular membranes.

Of the potential dissociative reagents tested, only KCl proved to be effective in promoting dissociation of islet prohormones from secretory granule and microsomal mem-

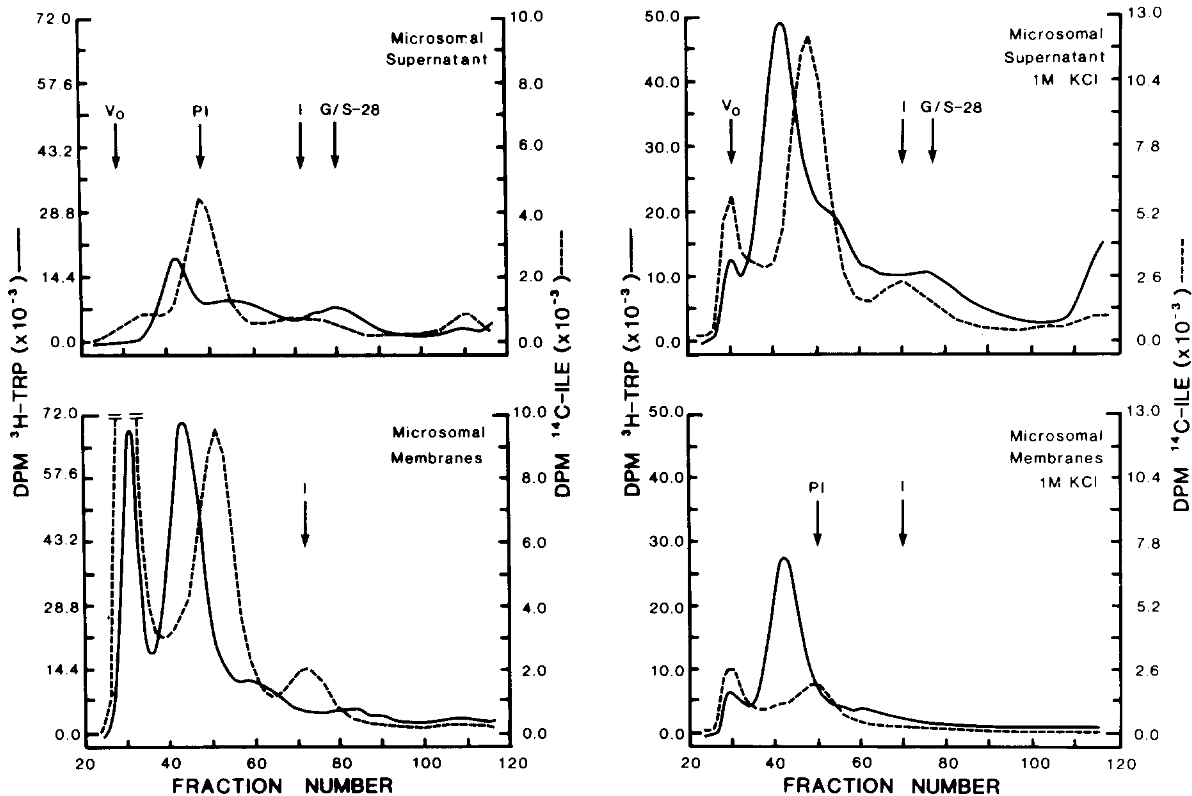

FIGURE 2 Association of islet prohormones and hormones with islet microsomal membranes and dissociation by KCl. Same experimental protocol and presentation of data as in Fig. 1, except data are from microsomal membranes and supernatants. Elution markers: V_o, void volume; *PI*, proinsulin; *I*, Insulin; *G/S-28*, glucagon(s) and somatostatin-28; *S-14*, somatostatin-14.

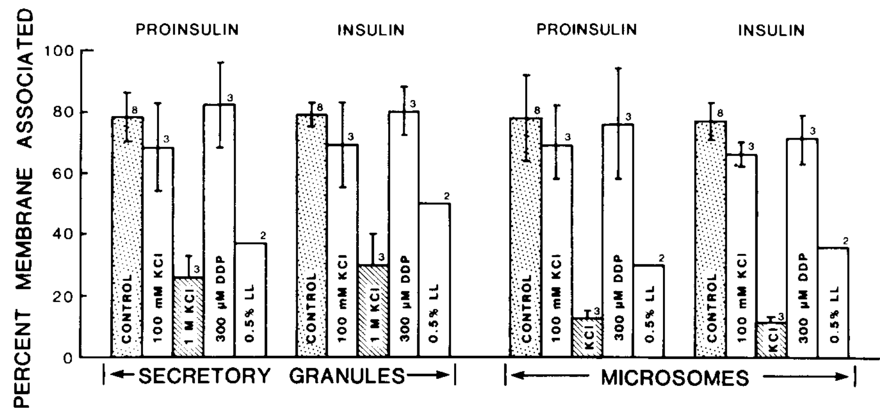

FIGURE 3 Membrane-association of proinsulin and insulin. Experiments were performed as in Figs. 1 and 2. Percent ^{14}C-labeled proinsulin and ^{14}C-labeled insulin that remained associated with membranes in controls (untreated) and reagent treated fractions were calculated as described in Materials and Methods. Data are mean ± S.D. of the number of determinations indicated. *DDP*, dithiodipyridine; *LL*, lysolecithin.

branes. Membrane-association of hormone products was also reduced by KCl (Figs. 1–4). There was no significant difference in the relative reduction of membrane-association of proinsulin and insulin caused by KCl. However, the relative reduction in membrane association of the 12,000-mol-wt precursors was significantly greater than that of both glucagon- and somatostatin-related peptides ($P \leq 0.05$). This was verified using arc sin transformation in conjunction with a two-tailed Student's t test to compare the relative percent reduction in membrane-association of precursors vs. products.

The results from these initial experiments (Figs. 1–4), were consistent with the possibility that the association observed may be mediated via membrane binding sites analogous to hormone receptors on the cell surface. To test this hypothesis, we monitored the time course of association of ^3H-labeled prohormones (2.6×10^{-6} M) with KCl washed secretory granule membranes from unlabeled islets. The data in Fig. 5 present the results from a typical experiment. Association of the prohormones with secretory granule membranes increased rapidly with increasing duration of incubation and came to a plateau after ~30 min. Prolongation of incubation up to 12 h did not result in enhanced prohormone association with the membranes. Addition of unlabeled prohormones (10^{-4} M) inhibited the association of labeled prohormones in a time-dependent manner. Addition of BSA, bacitracin, RNAse, or bovine insulin at 10^{-4} M had no effect on the association of labeled prohormones. Addition of anglerfish insulin (10^{-4} M) caused a reduction in prohormone association. Association was reduced to 44.9% of the control levels by 30 min after addition of anglerfish insulin. This was a significant reduction but not as great as that observed when

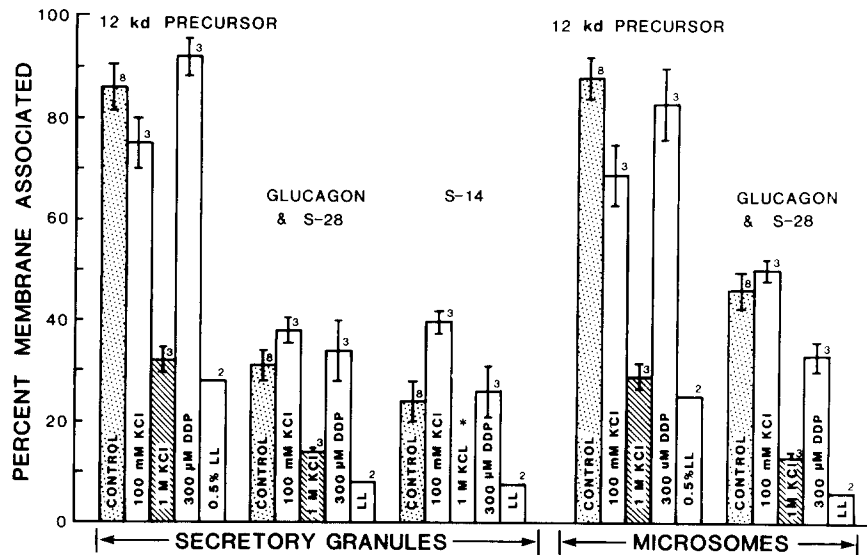

FIGURE 4 Membrane association of proglucagon(s), prosomatostatin(s), and their products. Data are from the same experiments as in Fig. 3 with the percent membrane association of the ^{3}H-labeled precursors and products presented (mean ±S.D. (*) of the number of determinations indicated). No SS-14 was recovered from secretory granule lysates treated with 1 M KCl (Fig. 1).

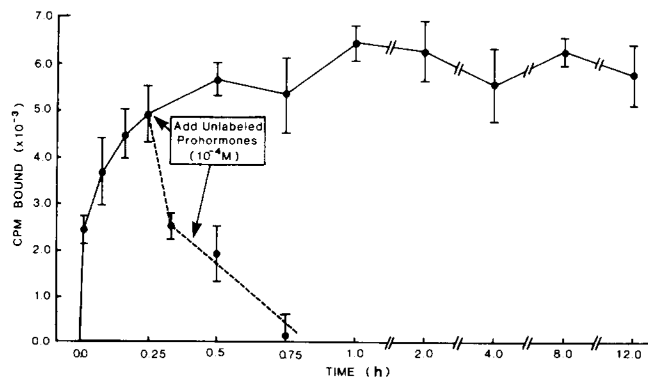

FIGURE 5 Association of labeled prohormones with secretory granule membranes. Prohormones (10^{-6} M) labeled with a mixture of ^{3}H-amino acids were incubated with secretory granule membranes prepared from unlabeled tissue. At the times indicated, triplicate samples were removed for precipitation and determination of prohormone association (——). At 15 min, unlabeled prohormones (final concentration 10^{-4} M) were added to three sets of duplicate incubations. These were removed for assessment of binding at 20, 30, and 45 min of incubation (----). Data are mean ±S.D. of three determinations.

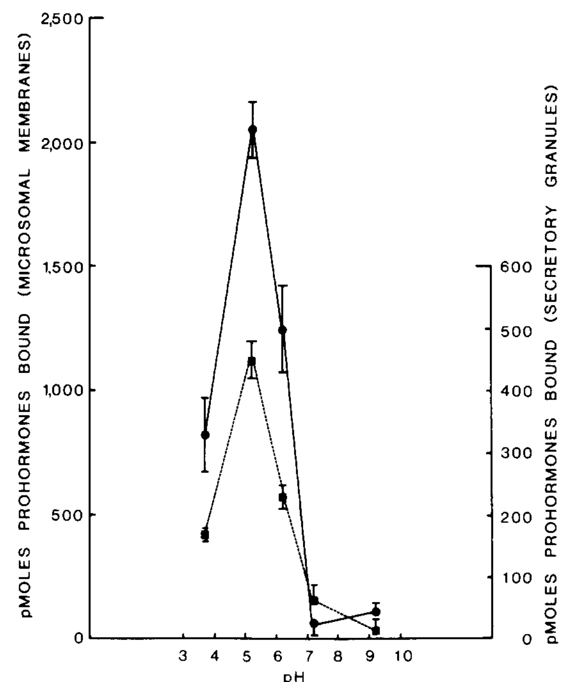

FIGURE 6 Determination of pH optimum for prohormone binding to intracellular membranes. ^{3}H-labeled prohormones were incubated 30 min with microsome or secretory granule membranes in buffers of varying pH as indicated. The amount of labeled prohormones which became associated with membranes was calculated. The data are expressed as picomoles prohormones bound/mg membrane protein; each point is the mean ±S.D. of three or more determinations. (■) Secretory granules. (●) Microsomes.

unlabeled prohormones were added (Fig. 5). These results indicate that the secretory granule membranes have a selective affinity for the prohormones.

The effect of pH on membrane-association of prohormones was also tested. Optimal binding of prohormones to both secretory granule and microsomal membranes was at pH 5.2 (Fig. 6). The apparent capacity of microsomal membranes for associated prohormones was higher than that of secretory granule membranes.

DISCUSSION

The results indicate that newly synthesized islet prohormones and insulin, may be membrane-associated in ER, Golgi complex, and secretory granules of islet cells (Figs. 1-4). When one considers the possibility that membrane-associated polypeptides may be dislodged during membrane disruption by repeated freezing and thawing, it seems reasonable to propose that the proportion of the prohormones (and possibly insulin) which is membrane-associated in vivo may be even higher than was monitored in the present study. The implication is that, since the amounts of prohormones bound after membrane lysis were quite high (78% for proinsulin and 86–88% for the 12,000-mol-wt precursors), nearly all of the newly synthesized prohormones could be membrane-associated in the intact cells. It should be emphasized that association of prohormones to secretory granule membranes was maintained despite very high concentrations of endogenous islet hormones. From radioimmunoassay data (1), insulin concentrations were estimated at 3.5×10^{-4} M in intact granules

and 1.1 × 10^{-5} M in suspensions subjected to lysis. The concentrations of somatostatin and glucagon were estimated to be two and ten times lower, respectively, than insulin. This suggests that insulin, glucagon-related, and somatostatin-related product peptides do not effectively inhibit membrane-association of newly synthesized islet prohormones.

Of all the islet hormone products, only insulin was membrane-associated to the extent of being equivalent with the level of association of the prohormones. This could be due to mediation of the membrane-association of proinsulin via the recognition of the insulin-containing portion of the proinsulin molecule. This suggestion is supported by the observation that addition of 10^{-4} M purified anglerfish insulin caused a 45% reduction in prohormone association with granule membranes. Since the labeled prohormones employed consisted of a mixture of these peptides and addition of unlabeled prohormones at 10^{-4} M resulted in complete inhibition of association (see below), the reduction in association induced by anglerfish insulin may reflect competition with only the labeled proinsulin in the mixture.

The membrane-association of the glucagon- and somatostatin-related peptides was much lower than that of insulin (Figs. 1–4). It was assumed that the level of "association" of the glucagon and somatostatin-related peptides represented nonspecific adsorption to membranes. Thus, the association of these peptides provided an internal control with which to compare levels of prohormone association. It is noteworthy that high salt treatment reduced proinsulin and 12,000-mol-wt precursor association with membranes to a significantly greater extent than glucagon-related peptides and S-28 (Fig. 4). These observations imply that microsomal and secretory granule membranes have a selective affinity for the prohormones and insulin.

The results in Figs. 5 and 6 indicate that the component(s) of the intracellular membranes that are responsible for mediating the membrane-association of prohormones have receptor-like characteristics. Prohormone association with membranes increased rapidly, appeared to be saturable and was competitively inhibited by addition of excess unlabeled prohormones (Fig. 5). Addition of nonhormonal proteins at the same concentration had no inhibitory effect on association of prohormones with membranes. Bovine insulin, which is significantly different in primary structure from anglerfish insulin, also had no inhibitory effect. This is in marked contrast to the effect of anglerfish insulin that promoted a significant reduction in prohormone association. Moreover, the association between prohormones and membranes was pH-dependent, with optimum association being observed at pH 5.2 (Fig. 6). All of these results suggest strongly that secretory granule membranes have specific binding sites for islet prohormones. It is recognized, however, that these experiments were performed using less than ideal conditions. The labeled ligand employed to assess membrane-association in vitro consisted of a mixture of prohormones. This mixture includes anglerfish proinsulin (8, 10, 15), at least two forms of proglucagon (2, 8, 10, 16, 17), and at least two forms of prosomatostatin (1, 11, 14, 18, 19). It is therefore not known whether all of the labeled polypeptides in the prohormone pool became membrane-associated or whether only several were bound. The data in Figs. 3 and 4 suggest that all of the prohormones and insulin are membrane-associated in vivo. However, to further characterize any potential membrane receptors will require the use of purified islet prohormones as ligands. At the present time, none of these prohormones has been purified in sufficient quantities to use for this purpose.

It is unlikely that the association of prohormones with secretory granule and microsomal membranes is mediated by cell surface hormone receptors that are normally present in microsomes and granules or that contaminate microsome and secretory granule fractions. Results from previous studies have demonstrated that 25% of the total plasma membrane marker (5′-nucleotidase) recovered was found in the microsome fraction but only 7.7% was in the secretory granule fractions (2). As seen in Fig. 4, the level of membrane-association of glucagon-related peptides and S-28 to microsomal membranes is only 1.5 times that found in secretory granule membranes. This difference is not sufficient to be caused by differential contamination by plasma membrane receptors. In contrast, the level of prohormone association with microsomal and secretory granule membranes was not only much greater than that of glucagon-related peptides and S-28, but was nearly identical in both membrane fractions (Figs. 3 and 4). Moreover, it has long been recognized that the affinity of cell surface hormone receptors for hormone precursors is poor (20, 21).

It is of interest that the pH optimum for prohormone association with intracellular membranes was 5.2 (Fig. 6). This pH is the optimum for islet prohormone conversion (1, 2). In addition, the intragranular pH of secretory granules from a number of tissues (22–24), including islet B cells (25), has been found to be in the range of 5.0 to 6.2. The pH within secretory granules would therefore be appropriate to facilitate association of prohormones with secretory granule membranes. Whether prohormones would also be membrane-associated with ER and Golgi membranes under physiologic conditions in vivo remains to be determined. If the intracisternal pH of ER and Golgi complex is at 7 or above, membrane association of prohormones may be minimal (Fig. 6).

How would maintenance of a mechanism that promotes polypeptide association with intracellular membranes be functionally advantageous to the cell? Three hypotheses are proposed:

(*a*) If conditions in the intact cell were appropriate to allow association of prohormones with ER and Golgi membranes, this association may in some way facilitate transport of the prohormones to the Golgi complex and secretory granules. On the basis of results that demonstrated differential rates of movement of separate secretory proteins from ER to Golgi complex, the existence of ER membrane receptors for secretory proteins has been postulated previously for several different cell types (26–28). Results from several studies have indicated that microtubules may be involved in transport of secretory proteins from ER to Golgi complex. Use of inhibitors of microtubule function has been shown to slow or stop transport of hormone or enzyme precursors from the rough ER in parathyroid (29, 30), exocrine pancreas (31) and pancreatic islets (32). It is possible that association of newly synthesized prohormones (or proenzymes) with some membrane components, such as transmembrane intramembranous proteins, provides a mechanism whereby microtubules on the cytoplasmic side of the rough ER might mediate transport of these precursors.

(*b*) The association of prohormones with intracellular membranes may serve to route peptides destined for secretion to secretory granules rather than lysosomes. It is now fairly well established that lysosomal enzymes are directed to pri-

mary lysosomes by phosphomannosyl receptors (33–35). No parallel mechanism for directing polypeptides that are destined for export from their cells of origin to secretory granules has been established. Sequence data for the anglerfish islet (pre)prohormones (15–19) indicate the lack of acceptor sites (asparagine-x-threonine) for the addition of branched chain oligosaccharides. This observation, along with the inability of α-methymannoside and *N*-acetylglucosamine to promote prohormone dissociation from intracellular membranes, indicates that the membrane-association of these precursors is mediated by a mechanism that does not require carbohydrate for recognition. It is possible that the membrane acceptors involved are instrumental in routing prohormones to secretory granules.

(*c*) Finally, the newly synthesized prohormones may become membrane-associated to keep them in juxtaposition with converting enzymes(s). The membrane-association of prohormone converting enzymes has been demonstrated in secretory granules from several different tissues and species (1–5). Maintenance of prohormones near the granule periphery, as has been demonstrated for proglucagon (6, 7), may facilitate prohormone conversion. Conditions favoring prohormone association with secretory granule membranes should be particularly appropriate in view of the optimum pH of 5.2 for association (Fig. 6) and the fact that intragranular pH in many tissues has been shown to be in the pH 5.0–6.0 range (22–25). That membrane-association would be favored in granules is consistent with the observation that the secretory granule is the predominant site of prohormone conversion in most tissues (36, 37).

Confirmation or refutation of each of these hypotheses will require extensive additional investigation.

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Requests for reprints should be addressed to Dr. Noe.

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Note Added in Proof: Orci et al. (1983, *C. R. Acad. Sci. Paris*, 297: 467–470) recently reported results from an immunocytochemical study that demonstrate the association of insulin immunoreactive determinants with the cisternal membranes of the Golgi complex in rat pancreatic beta cells. This observation suggests that (pro)hormones may be associated with the membranes of the endoplasmic reticulum and/or Golgi complex in mammalian islets as well.

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