In Vitro Characterization of Biosynthetic Human Proinsulin

D. A. PODLECKI, B. H. FRANK, AND J. M. OLEFSKY

SUMMARY

The binding and biologic properties of human proinsulin produced by recombinant DNA technology have been determined. The biosynthetic human proinsulin was iodinated using lactoperoxidase and subsequently purified by HPLC to yield the [(^{125}I)Tyr^{A14}]-proinsulin isomer.

Using isolated rat adipocytes, biosynthetic human proinsulin was shown to have approximately 11% of the binding potency of native insulin. At 16°C and 37°C, the ED_{50} values of biosynthetic human proinsulin were 3.7 nM and 15 nM, respectively, which was significantly different from the insulin values of 0.4 nM and 1.7 nM, respectively. Kinetic analysis suggested that the decreased affinity of biosynthetic human proinsulin was due primarily to a decreased association rate rather than an increased dissociation rate. Similar to insulin, biosynthetic human proinsulin exhibited a decreased half-time of dissociation in the presence of insulin (16.7 nM) or proinsulin (111 nM); however, this negative cooperative effect was lost in the presence of high concentrations of proinsulin (11 μM).

Biologic potency, assessed by measuring glucose transport in rat adipocytes, showed that biosynthetic human proinsulin had 10% of the biologic activity of insulin, suggesting close coupling between binding to receptors and membrane generated cellular response.

By extracting cell surface bound proinsulin with acidic buffer, the amount of ^{125}I-proinsulin that internalized following binding to surface receptors was measured. At equilibrium, 55% of the cell-associated radioactivity was internalized at 37°C. When chloroquine-treated (200 μM) cells were incubated with ^{125}I-proinsulin at 37°C, a 1.5-fold increase in the amount of intracellular proinsulin was observed at 1 h. This suggests that like insulin, chloroquine treatment augments the intracellular accumulation of proinsulin by inhibiting intracellular processing of internalized proinsulin.

In conclusion, (1) biosynthetic human proinsulin exhibits ~ 10% of the binding and biologic potency of native insulin in adipocytes, (2) despite its decreased affinity for surface receptors, proinsulin is readily internalized by adipocytes, where it is degraded by a chloroquine-sensitive pathway. DIABETES 33:111–118, February 1984.

From the Department of Medicine, Division of Endocrinology, University of Colorado Health Sciences Center, Denver, Colorado (D.A.P. and J.M.O.) and Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana (B.H.F.).
Address reprint requests to Jerrold M. Olefsky, M.D., University of California, San Diego, Department of Medicine, M-023E, La Jolla, California 92093.
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Porcine and bovine proinsulin have been shown to have qualitatively similar but reduced binding and biologic potency when compared with native insulin. As reviewed by Chance[1] and Kitabchi,[2,3] these proinsulins have been demonstrated to exhibit activity in the range of 1–15% of native insulin. Since the advent of recombinant DNA technology, large quantities of human proinsulin have recently become available and have allowed further characterization of this hormone. Unlike human insulin, which varies only by one and three amino acids from porcine and bovine insulins, respectively, human proinsulin varies by 11 and 17 amino acids from porcine and bovine proinsulins, respectively.[4–6] Although structural homology exists, these amino acid differences may have accounted for some of the decreased activity reported and may not reflect the true activity of human proinsulin. Preliminary reports suggest, however, that like porcine and bovine proinsulins, biosynthetic human proinsulin has a marked reduction in binding potency.[7]

Human proinsulin accounts for approximately 10% of the total immunoreactive insulin secretion of B-cells in normal individuals,[8] but may account for a larger percentage in certain disease states such as insulinomas, diabetes with pregnancy, diabetes with obesity, and insulin resistance due to anti-insulin receptor antibodies.[8–11] Theoretically, even with its reduced activity, proinsulin may have significant biologic effects when present in large amounts. With these clinical

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observations in mind, this study sought to characterize the binding and biologic properties of biosynthetic human proinsulin.

MATERIALS AND METHODS

Materials. Porcine insulin was a gift from Dr. R. E. Chance of the Eli Lilly Laboratories. Eagle's minimal essential medium with Hanks' salts was purchased from GIBCO Laboratories (Santa Clara, California). Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Company (Phoenix, Arizona). Collagenase was purchased from Worthington Biochemical Corporation (Freehold, New Jersey), and chloroquine was purchased from Sigma Chemical Co. (St. Louis, Missouri).

Preparation of biosynthetic human proinsulin. The preparation and characterization of biosynthetic human proinsulin has been described.[12]

Preparation and purification of [(^{125}I)Tyr^{A14}]-proinsulin and [(^{125}I)Tyr^{A14}]-insulin.* The preparation and isolation of [(^{125}I)Tyr^{A14}]-biosynthetic human proinsulin has been described by Frank.[13] The preparation of [(^{125}I)Tyr^{A14}]-insulin has been published elsewhere.[14] Verification of the location of the labeled tyrosine in human proinsulin was accomplished by transformation of the tracer to insulin[15] and then demonstrating coelution with [(^{125}I)Tyr^{A14}]-insulin of HPLC. In addition, the transformed tracer was subjected to amino acid sequencing and the release of the ^{125}I was found to coincide with the A-14 position.[16]

Preparation of isolated adipocytes. Isolated adipocytes were prepared from the epididymal fat pads of Sprague-Dawley rats (180–220 g) by the method of Rodbell.[17] Once isolated, these adipocytes were washed three times with buffer consisting of Eagle's minimal essential medium with Hanks' salts, 4% bovine serum albumin, and 25 mM Hepes (N-2-Hydroxyethyl-piperazine-N-2-ethanesulfonic acid) adjusted to pH 7.8. This buffer was used in all subsequent studies unless otherwise stated.

Binding, degradation, and biologic activity. Measurements of insulin binding to isolated adipocytes were performed as previously described in detail.[18] Measurements of proinsulin binding were done similarly but, in general, larger numbers of adipocytes were required.

Degradation was determined by measuring the ability of ^{125}I-insulin or ^{125}I-proinsulin to precipitate in 10% trichloroacetic acid. Both were greater than 98% precipitable before the start of each experiment and more than 85% precipitable at the conclusion of each experiment, unless otherwise stated.

Glucose transport studies were performed in isolated adipocytes as previously described in detail.[19]

Kinetic analysis. Association rates were determined as previously described.[20] Dissociation rates were determined by first preincubating at 16°C, either adipocytes (4×10^5 cells/ml) and ^{125}I-insulin (67 pM) for 3 h or adipocytes (1×10^6 cells/ml) and ^{125}I-proinsulin (44 pM) for 5 h in the absence and presence of insulin (17 μM). Each incubation mixture was then centrifuged rapidly for 30 s and the infranatant removed. The adipocytes were rapidly washed, twice, by diluting to the original volume with buffer at 4°C and repeating the centrifugations and aspirations. After the third aspiration, the cells were diluted to the original volume with buffer alone or buffer to which either insulin or proinsulin was added. The specific cell-associated radioactivity was then determined at the indicated times.

Acid extraction procedure. Acid extraction was performed according to a modification of the method of Haigler[21] as previously described.[22] Isolated adipocytes (1×10^6 cells/ml) were incubated with ^{125}I-proinsulin (44 pM) at 37°C in the absence and presence of porcine insulin (17 μM). At each time point, specific binding was determined. For each time point, parallel total and nonspecific binding tubes were rapidly centrifuged and the infranatant removed. The buffer was replaced by an equal volume of barbital-sodium acetate buffer adjusted to pH 3.0 at 4°C. After 6 min, the amount of cell-associated radioactivity was measured in the extracted cell suspension. The percent of specific binding remaining after extraction represents the nonextractable fraction of cell-associated radioactivity. Previous studies[22] have shown that this procedure removes essentially all cell-surface-bound insulin and that nonextractable radioactivity represents intracellular material.

RESULTS

Steady-state binding. To determine when steady-state binding conditions were achieved, the time course of binding for ^{125}I-proinsulin (22 pM) was studied at 16°C (Figure 1A). At this temperature, internalization and degradation are minimal and therefore binding primarily reflects association of proinsulin to cell surface receptors. ^{125}I-proinsulin reached approximately 52% of its maximal binding at 60 min and maximal binding at 240 min. This contrasts with the more rapid attainment of steady state by ^{125}I-insulin (Figure 1B). Under similar conditions, ^{125}I-insulin (33 pM) reached 93% of its maximal binding at 60 min and maximal binding at 120 min. At equilibrium 0.92% of ^{125}I-proinsulin was bound compared with approximately 9.5% of ^{125}I-insulin per 5×10^5 adipocytes.

Kinetic analysis. To assess whether the decreased affinity of ^{125}I-proinsulin for insulin receptors was due to a change in association or dissociation rates, kinetic studies were performed. Again, these studies used adipocytes incubated at 16°C to avoid the inaccurate results that are obtained from kinetic studies performed at higher temperatures where internalization and degradation are significant factors. The inset of Figure 1A shows the second-order rate plot of the data between 4 and 20 min shown in Figure 1A. The calculated association rate constant was $4.7 \times 10^5\ M^{-1}\ min^{-1}$. Under similar conditions the association rate constant of insulin was calculated to be $3.9 \times 10^7\ M^{-1}\ min^{-1}$ as shown in the inset of Figure 1B. Parallel dissociation experiments (Figure 2A and 2B) showed that ^{125}I-proinsulin and ^{125}I-insulin dissociated with half-time of 28 and 32 min, respectively. This suggests that the decrease in association rate is the major factor that reduces the affinity of biosynthetic human ^{125}I-proinsulin for the insulin receptor.

*The numerical sequence of the carboxy terminal amino acids of proinsulin are larger than insulin because of the connecting peptide. Our iodinated proinsulin product is [(^{125}I)Tyr^{79}]-proinsulin but if the connecting peptide is removed, this amino acid becomes the A-14 tyrosine residue of insulin. To reinforce the concept that our monoiodination preparations of proinsulin and insulin contain the ^{125}I-atom attached to the same tyrosine residue, we prefer to use the term, [(^{125}I)Tyr^{A14}]-proinsulin, rather than the chemically precise term, [(^{125}I)Tyr^{79}]-proinsulin.

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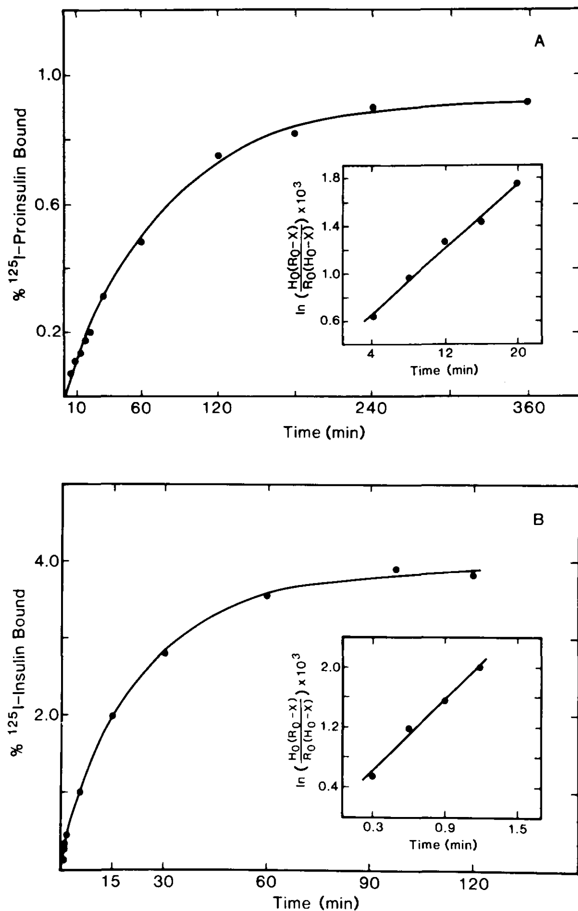

FIGURE 1. Time course of biosynthetic human ¹²⁵I-proinsulin and ¹²⁵I-insulin binding. (A) Rat adipocytes (5 × 10⁵/ml) and ¹²⁵I-proinsulin (22 pM) were incubated at 16°C with and without insulin (17 μM). At the indicated time points, triplicate 300-μl aliquots were removed and the binding determined. All points have been corrected for nonspecific binding. The inset represents the second order rate plot of the data shown between 4 and 20 min in Figure 1A. (B) Rat adipocytes (2 × 10⁵/ml) and ¹²⁵I-insulin (33 pM) were incubated and processed as above. The inset represents the second order rate plot of the data shown between 0.3 and 1.2 min in Figure 1B.

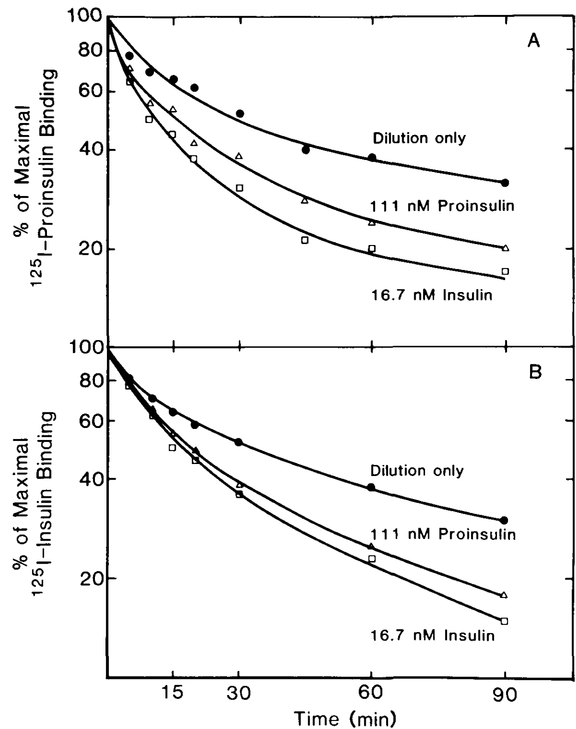

FIGURE 2. Time course of biosynthetic human ¹²⁵I-proinsulin and ¹²⁵I-insulin dissociation. (A) Rat adipocytes (1 × 10⁶/ml) and ¹²⁵I-proinsulin (44 pM) were incubated at 16°C with and without insulin (17 μM). After 5 h, the cells were rapidly washed twice at 4°C and resuspended to the original volume with buffer alone (●), buffer and 111 nM of proinsulin (△), or buffer and 16.7 nM insulin (□) at 16°C. Specific binding was then determined at the indicated time points and expressed as a percent of the maximal amount of ¹²⁵I-proinsulin bound. (B) Rat adipocytes (4 × 10⁵/ml) and ¹²⁵I-insulin (67 pM) were incubated at 16°C with and without insulin (17 μM). After 3 h, the cells were washed and diluted as above; in buffer only (●), buffer and 111 nM of proinsulin (△), or buffer and 16.7 nM of insulin (□). Specific binding was determined at the given time points and expressed as a percent of the maximal amount of ¹²⁵I-insulin bound.

Interestingly, both unlabeled insulin (16.7 nM) and biosynthetic human proinsulin (111 nM), accelerated the half-time of dissociation of ^{125}I-proinsulin from 28 min, due to dilution only, to 15 min and 10 min, respectively. The half-time of dissociation of ^{125}I-insulin was similarly accelerated from 32 min to 19 min and 16 min, by unlabeled insulin (16.7 nM) or proinsulin (111 nM), respectively. Thus, both proinsulin and insulin are able to accelerate the dissociation of previously bound ^{125}I-proinsulin or ^{125}I-insulin, indicative of negative cooperative effects.

Figure 3 shows the dose-response curves for proinsulin's ability to accelerate ^{125}I-proinsulin dissociation and insulin's effect to accelerate ^{125}I-insulin dissociation. The half-maximal effect occurred with 13 nM proinsulin and 2.3 nM insulin, respectively, whereas the maximal effects occurred with 900 nM proinsulin and 100 nM insulin, respectively. These dose-response curves also demonstrate that proinsulin at 11.1 μM or insulin at 1.7 μM are less effective at accelerating the dissociation of labeled hormone than are lower concentrations of these hormones. This has been reported for insulin previously,[23] and this observation may be due to the formation of insulin dimers at these higher concentrations, which do not exert negative cooperative effects.[24] Proinsulin also dimerizes in solution[25] and a similar mechanism could be responsible for its decreased ability to accelerate the dissociation of bound ^{125}I-proinsulin at high concentrations.

Competition studies. Competition studies were performed by incubating adipocytes with either ^{125}I-proinsulin or ^{125}I-insulin and various concentrations of biosynthetic human proinsulin or porcine insulin, for 5 h at 16°C or 1 h at 37°C. Figure 4A and 4C shows that at 16°C unlabeled biosynthetic human proinsulin competes poorly for either labeled ligand. This resulted in a rightward shift of the proinsulin competition curve when compared with the insulin competition curves. Proinsulin exhibited an ED_{50} value of 3.6 nM when ^{125}I-proinsulin was the trace and an ED_{50} value of 3.8 nM when ^{125}I-insulin was used. Insulin exhibited ED_{50} values of 0.4 nM and 0.3 nM, respectively. This suggests that biosynthetic human proinsulin has 10% of the binding potency of insulin in adipocytes incubated at 16°C.

At 37° (see Figure 4B and 4D), maximal binding of both ligands was reduced when compared with the binding seen at 16°C. Thus, maximal binding of biosynthetic human ^{125}I-proinsulin was only 0.3% per 1 × 10^6 cells and ^{125}I-insulin

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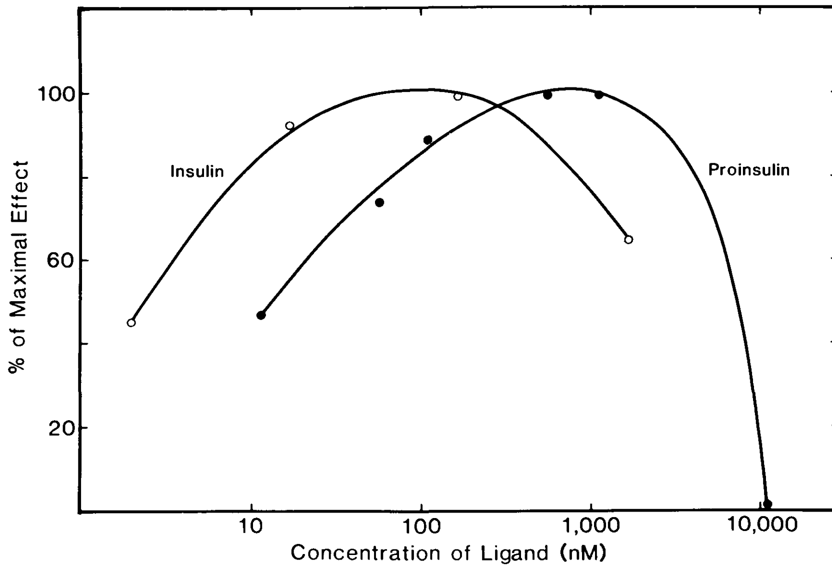

FIGURE 3. Effects of biosynthetic human proinsulin and insulin to accelerate their own dissociation. Biosynthetic human ^{125}I-proinsulin or ^{125}I-insulin were preincubated with adipocytes and processed as described in the legend of Figure 2A and B, except various concentrations of proinsulin (●) or insulin (○) were used in the final step. The data are expressed as a percent of the maximal increase in dissociation seen in the presence of ligand compared with dilution alone.

was 1.7% per 2 × 10^5 cells. This temperature-dependent decrease in average affinity was reflected by larger ED$_{50}$ values as well. Thus, competition curves using unlabeled proinsulin and ^{125}I-proinsulin or ^{125}I-insulin yielded ED$_{50}$ values of 16 nM and 14 nM, respectively. The corresponding ED$_{50}$ values for insulin were 1.8 nM and 1.6 nM, respectively. These data suggest that although the average affinity of both ligands is decreased at 37°C, biosynthetic human proinsulin still exhibits 12% of the binding potency of native insulin.

Scatchard analysis (see Figure 5A) of the proinsulin com-

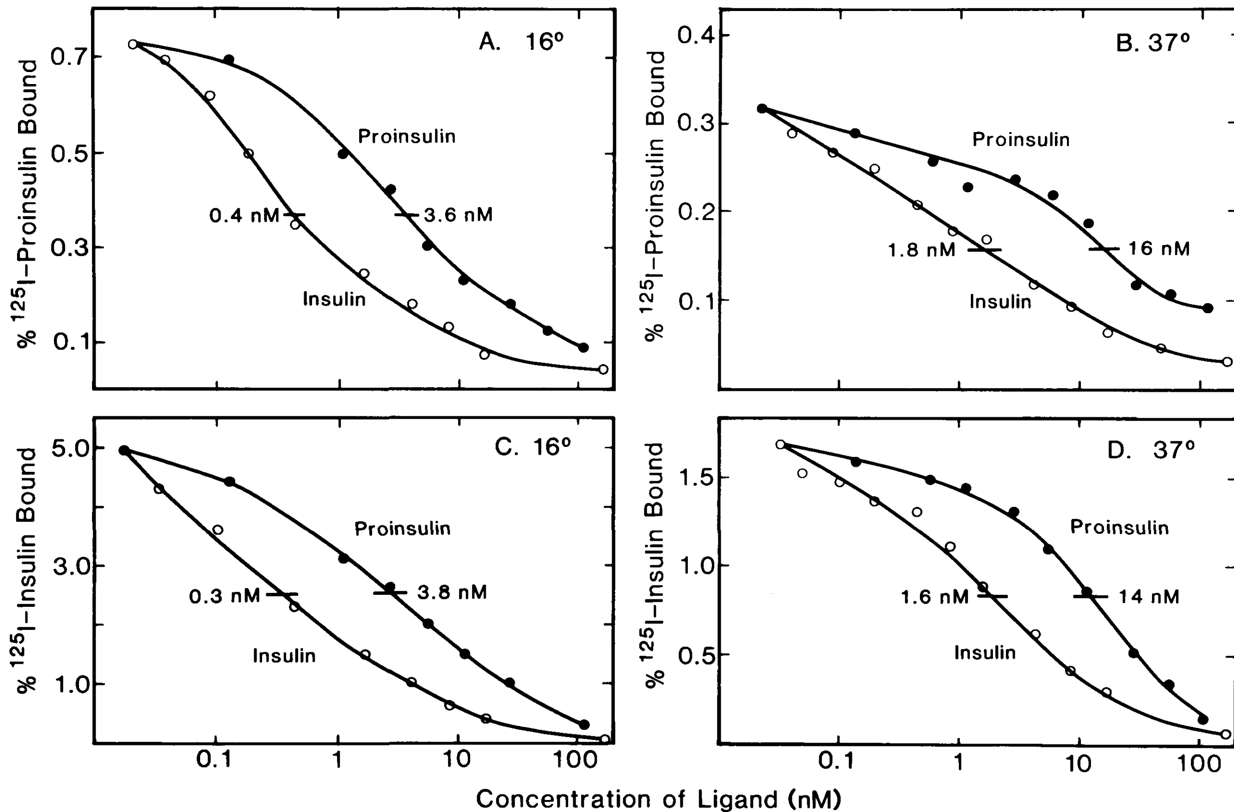

FIGURE 4. Ability of biosynthetic human proinsulin and insulin to inhibit ^{125}I-proinsulin or ^{125}I-insulin binding in adipocytes. (A) Adipocytes (5 × 10^5/ml) were incubated with ^{125}I-proinsulin (22 pM) and various amounts of either biosynthetic human proinsulin (●) or porcine insulin (○). Specific binding was determined at 5 h. Degradation did not exceed 8.0%. (B) Similar to above but cells (1 × 10^6 cells/ml) were incubated for 1 h at 37°C. Degradation did not exceed 12%. (C) Adipocytes (2 × 10^5/ml) were incubated at 16°C with ^{125}I-insulin (33 pM) and various amounts of either biosynthetic human proinsulin (●) or insulin (○). Specific binding was determined after 3 h. Degradation did not exceed 2%. (D) Similar to above but incubation was for 1 h at 37°C. Degradation did not exceed 6%. ED$_{50}$ values for each curve are shown.

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petition curve in Figure 4A and the insulin competition curve in Figure 4C reveals both to be curvilinear. Although the cause of curvilinear Scatchard plots is complex, it is interesting that the abscissal intercepts yield a similar number of receptor sites per cell when the data are corrected for adipocyte number. For proinsulin this was 72 fmol and for insulin it was 86 fmol, indicating that both ligands bind to a similar number of receptors.

This is more clearly seen in Figure 5B. With this analysis, the B/F values are expressed as a percent of the maximum B/F value for each Scatchard curve. This method of analysis has been used for comparing ligands with differing affinities[26] and avoids the obvious graphic disparity between the Scatchard plots of high- and low-affinity ligands as seen in Figure 5A.

Biologic activity. The biologic potency of biosynthetic human proinsulin was assessed by measuring glucose uptake in isolated adipocytes. This technique utilizes 2-[1,2-^{3}H] deoxyglucose which is transported, phosphorylated, and then trapped within cells because it is not further metabolized.

As shown in Figure 6, proinsulin reaches maximal activity at 11 nM with half-maximal stimulation achieved at 0.5 nM. Insulin had higher potency, requiring only 0.8 nM for maximal stimulation and 0.05 nM for half-maximal stimulation. Human

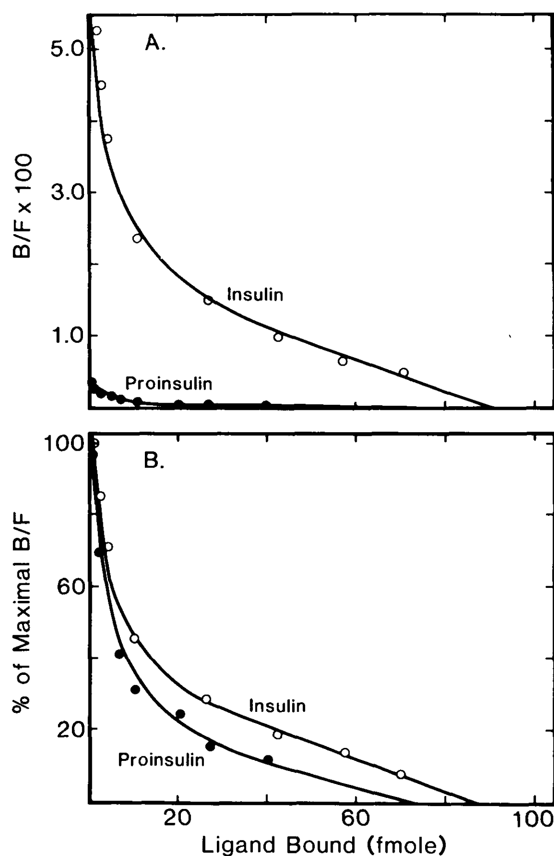

FIGURE 5. (A) Scatchard analyses of the biosynthetic human proinsulin (●) binding data from Figure 4A and the insulin (○) binding data from Figure 4C corrected to 2 × 10^5 cells. (B) Scatchard analysis of the data presented in Figure 5A with B/F values expressed as a percent of the (B/F)$_0$ values for biosynthetic human proinsulin (●) and insulin (○).

synthetic proinsulin thus has approximately 10% of the biologic potency of native insulin. Furthermore, close coupling between the binding of biosynthetic human proinsulin and its membrane generated cellular response is suggested by the similarity between the measured binding potency (10% at 16°C and 12% at 37°C) and the measured biologic potency (10% at 37°C).

An analytical approach to the coupling between binding and biologic activity is seen in Figure 7. In this graph the amount of 2-deoxyglucose uptake is plotted as a function of the amount of ligand bound to the cell-surface receptors. Cell-surface-bound hormone was determined by first incubating adipocytes with various concentrations of ^{125}I-proinsulin or ^{125}I-insulin and then exposing the cells to barbital sodium acetate buffer (pH 3.0) for 6 min at 4°C. Exposure of the cells to this acidic environment causes rapid dissociation of surface-bound radioactivity (extractable), while internalized radioactivity is not affected (nonextractable).[22] By determining the amount of specific cell-associated radioactivity before (total) and after acid extraction, one can quantitate the proportion of cell-associated radioactivity that is on the surface (extractable) or intracellular (nonextractable).

The proinsulin curve (Figure 7) is slightly shifted to the left but closely parallels the insulin curve, suggesting that proinsulin and insulin have similar coupling efficiencies. In the case of proinsulin, however, a very large amount of ligand must be present to achieve an equivalent receptor occupancy compared with insulin.

Internalization of biosynthetic human proinsulin. The time course of ^{125}I-proinsulin and ^{125}I-insulin internalization was investigated at 37°C in isolated adipocytes using the acid extraction technique described above. Biosynthetic human ^{125}I-proinsulin reached maximum binding at 30 min and this was constant for an additional 60 min (Figure 8B). Insulin also reached maximum total binding at 30 min but then exhibited a gradual decline in cell-associated radioactivity at all subsequent time points (Figure 8A). The amount of total cell-associated radioactivity was markedly different for the two ligands. At 60 min, the binding of ^{125}I-proinsulin was only 0.21% (0.019 fmol/2 × 10^5 cells) compared with 2.76% for insulin (1.4 fmol/2 × 10^5 cells). The amount of cell-associated radioactivity that was nonextractable (intracellular) was also different for the two ligands. At 60 min, 55% of the cell-associated radioactivity was nonextractable for proinsulin and 44% for insulin. This suggests that proinsulin-occupied receptors appear to be internalized to a greater extent than insulin-occupied receptors; however, the actual amount of proinsulin internalized was only 0.010 fmol compared with 0.62 fmol for insulin.

Internalization was also demonstrated by incubating adipocytes in the presence or absence of chloroquine. This agent has been shown to increase cell-associated ^{125}I-insulin by inhibiting the intracellular processing of the hormone.[27] In the presence of chloroquine, cell-associated ^{125}I-proinsulin radioactivity was markedly increased at all time points without reaching steady state (Figure 8C). This increase in cell-associated ^{125}I-proinsulin was due exclusively to the accumulation of intracellular ligand since acid-extractable radioactivity (surface bound) was the same in the presence or absence of chloroquine. At 60 min, total binding was 0.29%, an increase of 140% compared with untreated cells. Non-

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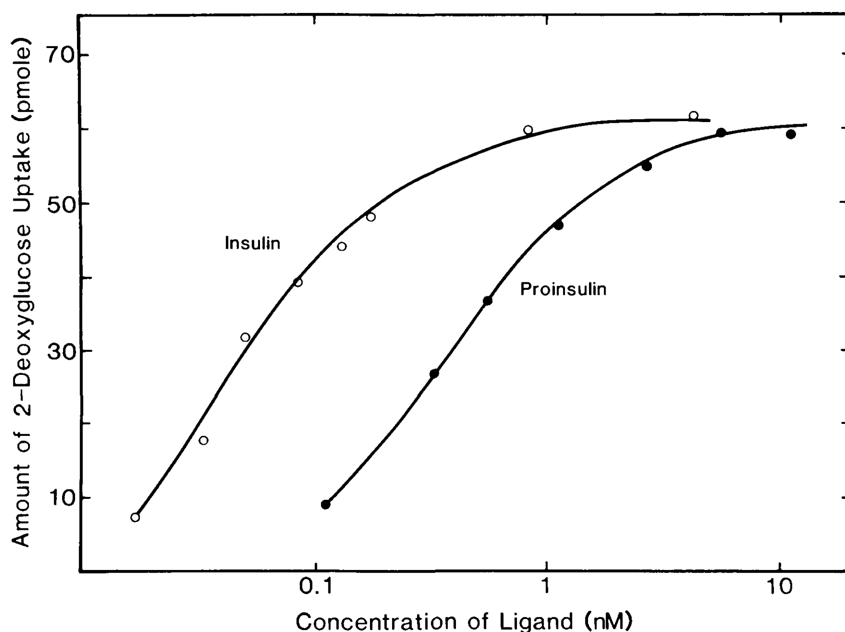

FIGURE 6. Stimulation of 2-deoxyglucose uptake by biosynthetic human proinsulin and insulin. Adipocytes (2×10^5 cells/ml) were preincubated at 37°C for 1 h with various concentrations of biosynthetic human proinsulin (●) or insulin (○). Then 0.1 mM 2-deoxyglucose and 0.2 μCi of 2-[1,2-^{3}H]deoxyglucose were added and the amount of uptake determined after a 3-min incubation.

extractable (intracellular) radioactivity accounted for 66% of this total binding which represented internalization of 0.017 fmol of proinsulin. This was an increase of 0.007 fmol above control, suggesting that chloroquine inhibits the intracellular processing of proinsulin.

Degradation of biosynthetic human proinsulin. Degradation of ^{125}I-proinsulin was not a limiting factor despite the larger number of adipocytes needed for the binding studies. Thus, after 180 min at 16°C, 3.8% of the extracellular proinsulin was degraded (as measured by solubility in 10% TCA) per 5×10^5 cells/ml while degradation of ^{125}I-insulin was 4.8% per 5×10^5 cells/ml. Degradation at 37°C was accelerated for both ligands. After 60 min only 12% of the ^{125}I-proinsulin was degraded per 1×10^6 cells/ml but ^{125}I-insulin degradation was 32% per 1×10^6 cells/ml. Thus, both ligands are degraded by temperature-dependent mechanisms and at 37°C, proinsulin does not become degraded as rapidly as insulin.

DISCUSSION

In the present paper, we have characterized and compared the binding and biologic properties of biosynthetic human proinsulin with insulin. Although an initial study has suggested that biosynthetic human proinsulin is similar to porcine and bovine proinsulins in that it has markedly reduced binding activity to peripheral lymphocytes,[7] enough structural differences exist to question the validity of extending

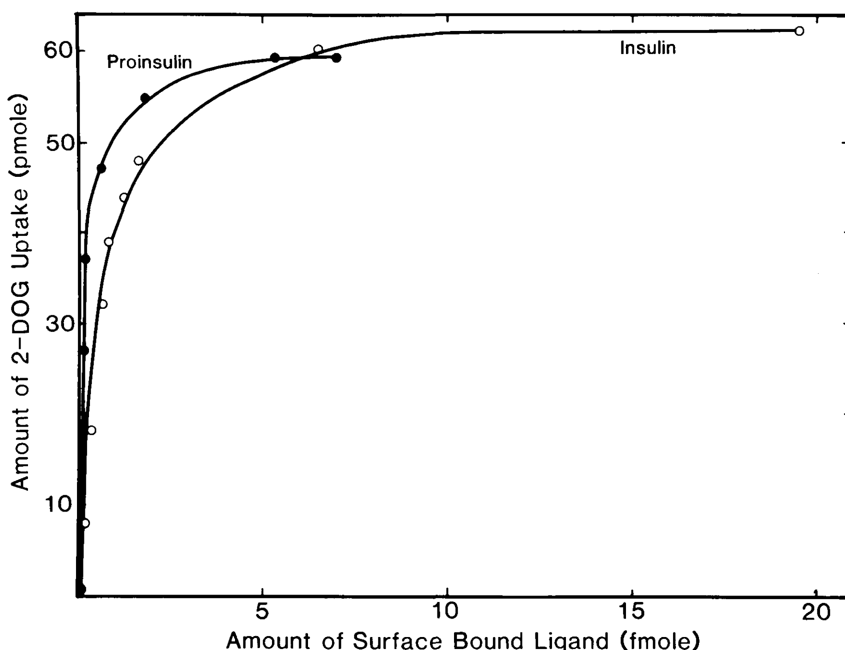

FIGURE 7. The amount of 2-deoxyglucose uptake as a function of surface-bound ligand. Adipocytes (2×10^5/ml) were incubated for 1 h at 37°C with ^{125}I-proinsulin (0.11–11.1 nM) or ^{125}I-insulin (0.02–4.2 nM) in the absence or presence of unlabeled insulin (17 μM). Specific total binding was determined. Additional aliquots of cells were incubated in parallel and extracted with barbital-sodium acetate buffer at each concentration to release all cell surface bound radioactivity as described in MATERIALS AND METHODS. Specific nonextractable (intracellular) binding was determined and subtracted from the specific total binding to give surface binding. The amount of 2-deoxyglucose uptake for each concentration from Figure 6 was then plotted as a function of the corresponding amount of surface-bound proinsulin (●) or insulin (○).

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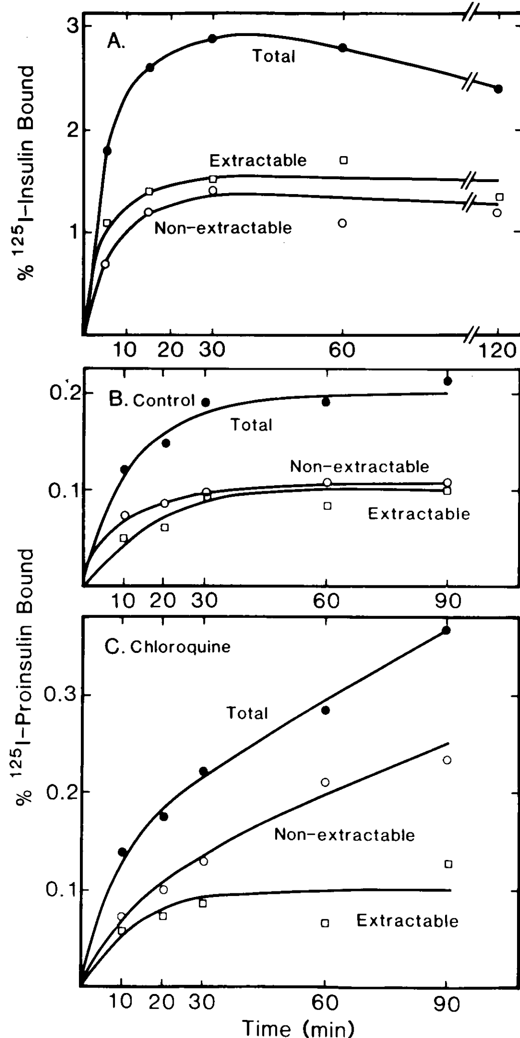

FIGURE 8. Time course of biosynthetic human ^{125}I-proinsulin and ^{125}I-insulin binding in adipocytes showing total (●), extractable (□), and nonextractable (○) cell-associated radioactivity. (A) Adipocytes (2×10^5/ml) and ^{125}I-insulin (50 pM) were incubated at 37°C and specific binding was determined at the given time points. Additional aliquots of cells were incubated in parallel and extracted with barbital-sodium acetate buffer at each point to release all cell-surface-bound radioactivity as described in MATERIALS AND METHODS. All points have been corrected for nonspecific binding. (B) Adipocytes (1×10^6/ml) were incubated at 37°C with ^{125}I-proinsulin (44 pM) and treated as in A. (C) Adipocytes (1×10^6/ml) were incubated at 37°C in the presence of 200 μM chloroquine and treated as in A.

other observations made with porcine and bovine proinsulins to biosynthetic human proinsulin.

Binding and biologic studies were performed in isolated rat adipocytes. The binding to these cells of biosynthetic human ^{125}I-proinsulin reached equilibrium after 4 h at 16°C, but the maximum amount of proinsulin bound was greatly reduced when compared with the amount of insulin bound at equilibrium. Kinetic studies performed at 16°C showed that the decreased affinity of biosynthetic human proinsulin was due primarily to a decrease in its association rate constant with little change in the rate of dissociation of ^{125}I-proinsulin compared with ^{125}I-insulin. Our data concerning the decreased affinity of biosynthetic human proinsulin are qualitatively similar to the data of Freychet, who reported that porcine proinsulin has a 20-fold lower association rate constant that accounted for its decreased affinity to liver membrane receptors.[28]

At 16°C, both native ligands were capable of accelerating the dissociation of either ^{125}I-proinsulin or ^{125}I-insulin in a dose-dependent manner. The maximum effective concentration of biosynthetic human proinsulin was 10-fold greater than the maximum effective insulin concentration. These data suggest that proinsulin is able to exhibit negative cooperative effects in a similar but weaker manner than insulin. At very high hormone concentrations, the negative cooperative effect is diminished, and for insulin this has been attributed to dimerization of insulin molecules.[24] It is interesting to speculate that the same reasoning will hold for proinsulin since it too is capable of forming dimers.[25]

Competition studies verified the decreased affinity of proinsulin. At 16°C, biosynthetic human proinsulin had 10%, and at 37°C, had 12% of the binding potency of insulin. Similar ED_{50} values were found for both proinsulin and insulin using either biosynthetic human ^{125}I-proinsulin or ^{125}I-insulin at either temperature. This supports the idea that both ligands compete for the same population of receptors and is further supported by Scatchard analyses which suggest that the hormones bind to a similar number of receptors.

Using the uptake of 2-deoxyglucose as a measure of biologic activity in adipocytes, biosynthetic human proinsulin was approximately 10% as potent as insulin. This value correlates closely with the value of 12% observed for the binding potency of proinsulin observed at 37°C. Although the observed binding and biologic potencies suggest close coupling between these events, a direct comparison between the uptake of 2-deoxyglucose and the amount of surface bound ligand showed that proinsulin and insulin had similar coupling efficiencies. Thus, if equimolar amounts of either ligand were bound, a similar amount of 2-deoxyglucose would be transported. Because of its decreased affinity, however, biosynthetic human proinsulin must be present in much larger amounts to achieve an equivalent receptor occupancy compared with insulin.

Despite its decreased affinity for the insulin receptor, biosynthetic human proinsulin is readily internalized by adipocytes. Similar to insulin, proinsulin is internalized by a time- and chloroquine-sensitive mechanism, but at 37°C, the percent of bound biosynthetic human proinsulin that is internalized is somewhat greater than insulin.

Our studies suggest that the relative potency for biosynthetic human proinsulin is similar to the relative potencies reported for porcine and bovine proinsulins, despite the relatively large differences that exist in the amino acid sequences of their connecting peptide (see refs. 1–3). When compared with insulin, biosynthetic human proinsulin can be demonstrated to possess many of the characteristics of insulin but because of its reduced affinity, larger concentrations are necessary for these proinsulin effects to be observed.

Finally, internalization of bound biosynthetic human proinsulin may have physiologic significance. We have reported that internalization of proinsulin appears to proceed to a greater degree than does insulin. Furthermore, we have observed that the degradation of proinsulin is markedly reduced compared with insulin. Once insulin is internalized, it

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is sequentially degraded. The first step of its degradation may be mediated by "insulin specific protease," which has a negligible ability to degrade proinsulin.[3] This suggests that the biochemical pathway for the degradation of proinsulin may be different from that of insulin.

These observations lead one to speculate that if the process of internalization or the presence of intracellular insulin, receptor, or insulin-receptor complex triggers any biologic effect, then the internalization of proinsulin may also be an effective stimulus because once bound it appears to be internalized to a greater degree and once internalized it may be less susceptible to degradation than insulin. Furthermore, proinsulin represents a significant proportion of the normal B-cell secretion, and in certain clinical conditions this portion is markedly increased.[8-11] If its internalization rate is increased and its intracellular degradation rate is decreased, then physiologic concentrations of circulating proinsulin may have biologic importance.

In conclusion, biosynthetic human proinsulin has approximately 10% of the binding and biologic potency of insulin as determined in isolated adipocytes. Despite its reduced binding affinity, biosynthetic human proinsulin is internalized and the significance and fate of the internalized proinsulin-insulin receptor remains unknown.

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