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The Dissociation and Degradation of Internalized Insulin Occur in the Endosomes of Rat Hepatoma Cells*

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We have studied the intracellular processing of insulin in the rat hepatoma cell line Fao. Fao cells internalized cohorts of surface-bound ^{125}I-insulin or ^{125}I-insulin-like growth factor II within 3–5 min. Degraded ^{125}I-insulin-like growth factor II did not appear in the medium until 20–30 min after uptake, consistent with a time course of lysosomal delivery. In contrast, internalized insulin was completely degraded within 7–10 min. The half-times for dissociation and degradation of internalized insulin were identical at 37 °C (3 min), suggesting that the two processes occurred in the same compartment. Subcellular fractionation of Fao cells showed that a pulse of internalized insulin was largely intact after 3 min and associated with a light membrane fraction devoid of lysosomal markers. After an additional 4 min, the amount of insulin in this compartment decreased by 40%, with an increase in degraded insulin in the cytosol; no transfer of intact insulin to lysosomes or the cytosol was detected. The relationship between insulin-receptor dissociation and insulin degradation was further studied with inhibitors of insulin processing. Monensin blocked both dissociation and degradation of internalized insulin, as did incubation of the cells at 20 °C, suggesting that both endosomal acidification and endosomal fusion were required for insulin processing. At 25 °C, dissociation ($+t_{1/2}$ = 12.9 min) preceded degradation ($+t_{1/2}$ = 15.8 min). Inhibitors of lysosomal proteases were without effect on the half-time for either process. In contrast, bacitracin, an inhibitor of insulin degradation, caused a 2-fold increase in the half-times for both dissociation and degradation. Thus, intracellular insulin dissociation and degradation are tightly coupled endosomal processes in Fao cells, and insulin degradation facilitates the dissociation of insulin from its receptor inside the cell.

Insulin and the related insulin-like growth factors (IGF)[1] I and II stimulate cellular growth and metabolism through cell-surface receptors. Insulin and IGF-I bind preferentially to homologous receptors which contain a tyrosine-specific protein kinase (reviewed in Ref. 1). Activation of the tyrosyl kinase is thought to play a central role in transmission of the regulatory signals. In contrast, the mechanism by which IGF-II stimulates cellular growth is less clear. IGF-II binds with high affinity (K_D = 0.2 nM) to a receptor which is identical to the cation-independent mannose 6-phosphate receptor (2–4). The IGF-II/Man-6-P receptor is unrelated to the receptors for insulin and IGF-I and consists of a large extracellular domain with a 164-amino acid cytoplasmic tail. It is unclear whether this receptor mediates any of the physiological responses of IGF-II; IGF-II may produce some of its biological effects through low affinity binding to the IGF-I receptor.

The IGF-II/Man-6-P receptor internalizes constitutively in the absence or presence of ligand (5). The disposition of internalized IGF-II has been thought to involve lysosomal delivery, based on the sensitivity of its degradation to inhibitors of lysosomal proteases (6). Furthermore, the role of the mannose 6-phosphate receptor, which is identical to the IGF-II/Man-6-P receptor, in the delivery of ligands to the lysosome is well-established (3, 4, 7). Insulin degradation, on the other hand, has remained a complex issue (8). The binding of insulin to its receptor stimulates internalization of the receptor-ligand complex, with subsequent degradation of insulin and recycling of the receptor (9). A number of studies (reviewed in Ref. 8) have shown the presence of insulin-degrading activity in the cytosol of various cells. Alternatively, several studies (10–13) have demonstrated insulin degradation products and insulin-degrading activity in endosomal preparations from rat liver. Hepatic insulin processing is complicated by the existence of multiple pathways of both insulin internalization and processing, and the quantitative roles of cytosolic and endosomal degradation in hepatic insulin processing have not been clearly established (14, 15).

In this study, we have examined the kinetics of processing of insulin and IGF-II in insulin-sensitive Fao hepatoma cells. Fao cells contain insulin and IGF-II/Man-6-P receptors, but no IGF-I receptors. Although insulin and IGF-II are internalized at a similar rate by Fao cells, the degradation of IGF-II proceeds significantly more slowly than that of insulin. The similar kinetics of intracellular dissociation and degradation of insulin as well as the inhibition by bacitracin of both processes suggest that these processes occur in the same endosome and are tightly coupled. Our data suggest that in Fao cells the degradation of insulin (but not of IGF-II) occurs in the endosome and that endosomal degradation accelerates the intracellular dissociation of insulin from its receptor.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Steady-state Internalization of Insulin and IGF-II—When Fao cells were incubated with ^{125}I-insulin or ^{125}I-IGF-II, total

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[1] The abbreviations used are: IGF, insulin-like growth factor; CHO, Chinese hamster ovary.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 7–10) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

cell-associated radioactivity had reached steady state by 1 h (Fig. 1, *A* and *B*). Based on the acid-wash procedure, most of the cell-associated ^{125}I-IGF-II was in an intracellular compartment, whereas only 35% of the total cell-associated ^{125}I-insulin was intracellular. Internalized ^{125}I-insulin was degraded and excreted into the medium more rapidly than ^{125}I-IGF-II, which explains the relatively low intracellular accumulation of insulin by the Fao cells (Fig. 1, *A* and *B*). Thus, Fao cells process insulin about 10-fold faster than IGF-II.

Single Cohort Internalization and Degradation of Insulin and IGF-II—The influx of insulin and IGF-II was determined by measuring the internalization and degradation of a single cohort of ligand. ^{125}I-Insulin or ^{125}I-IGF-II was bound to the surface of Fao cells during incubation at 4 °C and then was allowed to internalize during incubation at 37 °C. Fao cells rapidly internalized the single cohort of ^{125}I-insulin or ^{125}I-IGF-II, reaching a maximum after 3–5 min (Fig. 2, *A* and *B*). The amount of internalized insulin declined rapidly between 3 and 10 min, whereas the maximum amount of intracellular IGF-II was ~50% greater than that of insulin and remained constant for nearly 20 min. This difference is due to the release of degraded insulin into the medium (Fig. 2*A*). Degradation of the insulin cohort showed an initial lag of 3 min, but then rapidly increased over the next 7 min. In contrast, the degradation of IGF-II was nearly undetectable before 10 min and then increased gradually. Since the internalization rates of insulin and IGF-II were the same, the differences in the disposition of these ligands were dependent on post-internalization processing.

The 20-min time lag between the internalization of IGF-II and the appearance of degradation products in the medium is consistent with the time required for lysosomal delivery and was similar to the kinetics of low density lipoprotein degradation by Fao cells (data not shown). We chose to focus the remainder of this study on insulin because its rapid degradation suggested the presence of alternative pathways of ligand processing.

Intracellular Dissociation and Degradation of Insulin—To evaluate the processing of insulin in Fao cells, the dissociation of internalized insulin from its receptor inside the cell was directly studied by measuring the polyethylene glycol precipitability of intracellular ^{125}I-insulin. Intracellular insulin was initially >80% polyethylene glycol-precipitable, suggesting that most of the insulin was receptor-bound immediately after internalization. After 1 min, the polyethylene glycol precipitability of the internalized insulin decreased rapidly, with <20% of the internalized insulin bound to receptor after 7.5 min (Fig. 3). The intracellular dissociation of insulin appeared first-order on a semilogarithmic plot, with $t_{1/2}$ = 3 min (data not shown).

To compare the kinetics of the intracellular dissociation and degradation, the trichloroacetic acid precipitability of the internalized insulin was measured (Fig. 3). Intracellular insulin was initially 90% trichloroacetic acid-precipitable, suggesting that little if any degradation occurred prior to internalization. However, after 1 min, the trichloroacetic acid precipitability of the internalized insulin fell rapidly, with $T_{1/2}$ = 3 min (data not shown). Thus, the half-times for intracellular insulin dissociation and degradation at 37 °C were the same.

Analysis of Insulin Uptake and Degradation by Subcellular Fractionation—The close coupling between insulin dissocia-

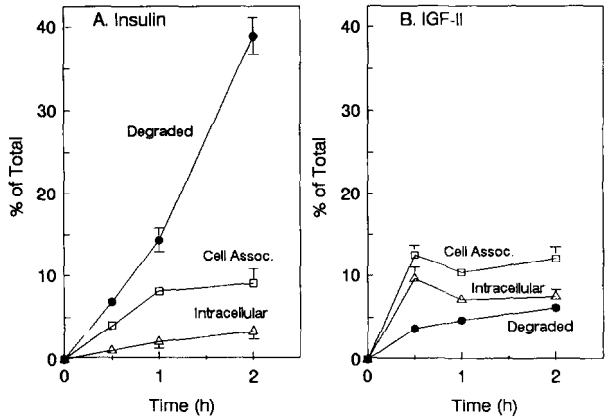

FIG. 1. **Uptake and degradation of ^{125}I-insulin and ^{125}I-IGF-II by Fao cells.** Fao cells were incubated at 37 °C in medium containing ^{125}I-insulin (*A*) or ^{125}I-IGF-II (*B*) (5 × 10^6 cpm/ml). At various times, the medium was removed, and the amount of trichloroacetic acid-soluble radioactivity was determined. The cells were washed at neutral or acidic pH and solubilized to determine the amount of total cell-associated and intracellular radioactivity, respectively.

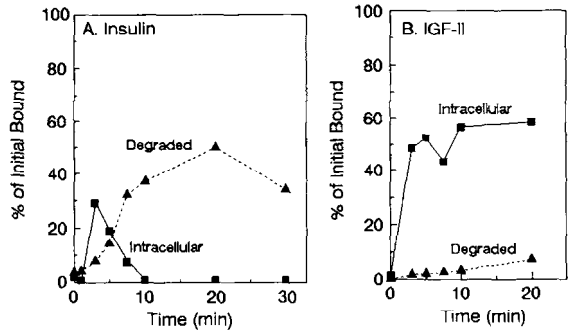

FIG. 2. **Single cohort uptake and degradation of ^{125}I-insulin and ^{125}I-IGF-II by Fao cells.** Fao cells were allowed to bind ^{125}I-insulin (*A*) or ^{125}I-IGF-II (*B*) for 2 h at 4 °C. After neutral washes of 4 °C to remove unbound ligand, the cells were rapidly warmed to 37 °C. At various times, the medium was removed to determine release of trichloroacetic acid-soluble radioactivity, and the cells were rapidly chilled. After acidic washes to remove residual surface-bound ligand, the cells were solubilized and counted to determine internalized radioactivity.

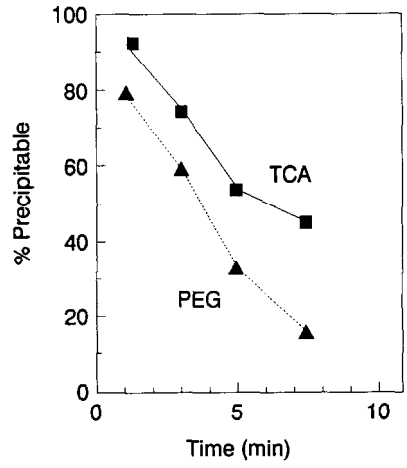

FIG. 3. **Rates of intracellular dissociation and degradation of internalized insulin.** Fao cells were allowed to internalize a cohort of surface-bound ^{125}I-insulin as described for Fig. 2. At various times, the cells were rapidly chilled and subjected to an acid wash to remove residual surface-bound insulin. The cells were solubilized in buffer containing 0.1% Triton X-100. After centrifugation to remove unsolubilized material, the amounts of polyethylene glycol (*PEG*)-precipitable radioactivity, representing receptor-bound insulin, and trichloroacetic acid (*TCA*)-precipitable material, representing intact insulin, were determined as described under "Experimental Procedures."

tion and degradation suggested that insulin degradation occurred early in the endosomal pathway. To define the subcellular compartment in which this degradation took place, Fao cells were allowed to internalize a pulse of ^{125}I-insulin. After an acid wash to remove surface-bound insulin, the cells were homogenized and separated into cytosolic and particulate fractions (Fig. 4*A*). Internalized insulin was located primarily in the particulate fraction, with maximal accumulations at 3 min. Trichloroacetic acid precipitation suggested that the bulk of this insulin was intact. Intracellular insulin in this fraction subsequently declined, and this was accompanied by a gradual increase in trichloroacetic acid-soluble material in the cytoplasm. No transfer of intact insulin between the particulate and cytoplasmic pools was observed.

To further characterize the compartment in which the internalized insulin resided, Fao cells were allowed to internalize surface-bound ^{125}I-insulin at 37 °C, acid-washed to remove residual surface-bound insulin, homogenized, and fractionated on Percoll gradients. In control cells which were subjected to this procedure without warming, only a trace amount of ^{125}I-insulin migrated with endosomal markers (Fig. 4*B*, *solid line*). In contrast, when the cells were warmed to 37 °C for 3 min prior to acid washing, a marked increase in internalized ^{125}I-insulin was detected in this fraction (Fig. 4*B*, *dashed line*). A small amount of internalized insulin (<15%) co-migrated with the lysosomal markers. The insulin associated with the low density membranes was largely intact as judged by trichloroacetic acid precipitability (data not shown). After 7 min at 37 °C (Fig. 4*B*, *dotted line*), the amount of ^{125}I-insulin in light membrane fractions decreased by ~40%. No increase in the amount of insulin associated with lysosomes was observed, suggesting that internalized insulin was degraded without any observable association with lysosomal fractions. We were unable to detect any increase in trichloroacetic acid-soluble degradation products in either low or high density fractions, suggesting that this material moved readily out of the membrane compartments.

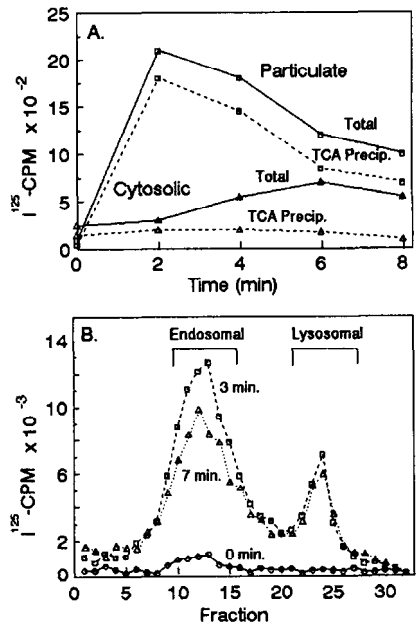

FIG. 4. **Subcellular fractionation of Fao cells after internalization of ^{125}I-insulin.** Fao cells were allowed to internalize a cohort of surface-bound ^{125}I-insulin as described for Fig. 2. At various times, the cells were rapidly chilled and subjected to an acid wash to remove residual surface-bound insulin. The cells were homogenized as described under "Experimental Procedures." *A*, post-nuclear supernatants were sedimented at 100,000 × *g* for 60 min to prepare particulate and cytosolic fractions. Total and trichloroacetic acid (*TCA*)-precipitable radioactivity in each fraction were determined. *B*, post-nuclear supernatants were layered over 28 ml of 10% Percoll in buffer A on a cushion of 4 ml of 2.5 M sucrose and centrifuged in a Sorvall SV-288 vertical rotor at 28,000 × *g* for 50 min. Fractions (1 ml) were collected and counted. The positions of an endosomal marker (dialyzed fluorescein isothiocyanate-dextran) and lysosomal markers (β-hexosaminidase and acid phosphatase) are indicated. Percoll fractionation of cells incubated at 37 °C for 0, 3, and 7 min is shown.

Perturbations of Insulin Processing Have Similar Effects on Dissociation and Degradation—The relation between insulin dissociation and degradation, studied under a number of conditions designed to affect different aspects of the endocytic machinery, is summarized in Table I. Treatment of Fao cells with monensin strongly inhibited both dissociation and degradation, as would be expected for processes dependent on endosomal acidification (Fig. 7 and Table I). The use of (B26)-^{125}I-insulin, which binds to the insulin receptor with a 2-fold higher affinity than does (A14)-^{125}I-insulin in a number of cell types and a 1.6-fold higher affinity in Fao cells (16), had no effect on the half-time for either process (Table I). In contrast, reductions in temperature slowed both processes. At 20 °C, both dissociation and degradation of internalized insulin were undetectable (Table I). At 25 °C, the rate of dissociation (12.9 min) was slightly faster than that of degradation (15.8 min), suggesting that dissociation preceded degradation by a small increment which was not detectable at 37 °C (Fig. 8 and Table I).

The effect of several protease inhibitors on insulin processing was examined. Leupeptin and pepstatin, inhibitors of lysosomal proteases, had no effect on either the dissociation or degradation of internalized insulin (Table I). In contrast, bacitracin, a potent inhibitor of insulin degradation in Fao and other cells (17), caused a 2-fold increase in the half-time for intracellular insulin degradation (Fig. 5 (*solid lines*) and Table I) and had similar effects on the release of degraded insulin into the medium (data not shown). Surprisingly, bacitracin also inhibited the intracellular dissociation of insulin from its receptor, with a 2-fold increase in the half-time for this process (Fig. 5 (*dashed lines*) and Table I). The inhibition of intracellular insulin dissociation by bacitracin was not due to changes in insulin binding affinity or the pH sensitivity of insulin binding (Fig. 9) and did not reflect a general disruption of endocytic processes (Fig. 10). The similar inhibition of both degradation and intracellular dissociation of internalized insulin by bacitracin suggests that the two processes are tightly coupled and that insulin degradation increases the rate of

TABLE I

Perturbations of intracellular dissociation and degradation of insulin

The kinetics of intracellular insulin degradation and dissociation were analyzed as described for Fig. 5, with modifications as indicated. Half-times are the means ± S.E. of three to seven separate experiments except for the leupeptin/pepstatin experiment, which was only done once and showed no effect. Where indicated by figure numbers, the data are presented in full in the main text or Miniprint. Half-times were not determined (ND) in cases where dissociation and degradation were undetectable.

Ligand	*T*	Agent	Half-time		Fig.
			Dissociation	Degradation	
	°C		*min*		
A14	37	None	3.3 ± 0.7	4.1 ± 0.6	3
A14	37	Monensin	ND	ND	7
A14	20	None	ND	ND	
A14	25	None	12.9 ± 3.6	15.8 ± 4.8	8
B26	37	None	3.2 ± 0.2	3.7 ± 0.1	
A14	37	Leupeptin, pepstatin	2.4	2.7	
A14	37	Bacitracin	7.5 ± 1.4	10.5 ± 3.7	5

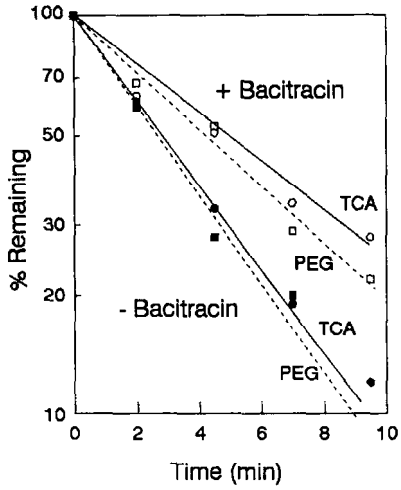

FIG. 5. **Bacitracin inhibits intracellular dissociation and degradation of insulin.** Fao cells were allowed to take up a cohort of surface-bound ^{125}I-insulin as described for Fig. 2 in the absence or presence of 100 units/ml bacitracin. Intracellular dissociation and degradation of internalized insulin in the absence or presence of bacitracin were determined by measuring the decline in polyethylene glycol (*PEG*)- and trichloroacetic acid (*TCA*)-precipitable radioactivity, respectively, as described for Fig. 2. Semilogarithmic plots are shown of the decline in polyethylene glycol- and trichloroacetic acid-precipitable intracellular radioactivity from cells treated in the absence or presence of bacitracin.

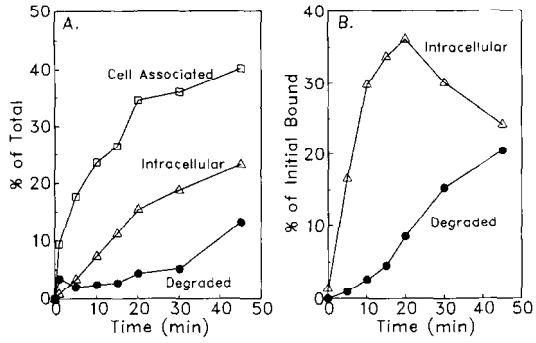

FIG. 6. **Insulin processing by CHO cells expressing human insulin receptors.** *A*, CHO/IR cells were incubated at 37 °C in medium containing ^{125}I-insulin. At various times, the medium was removed, and the amount of trichloroacetic acid-soluble radioactivity was determined. The cells were washed at neutral or acidic pH and solubilized to determine the amount of total cell-associated and intracellular radioactivity, respectively. *B*, CHO/IR cells were allowed to bind ^{125}I-insulin for 2 h at 4 °C. After neutral washes at 4 °C to remove unbound ligand, the cells were rapidly warmed to 37 °C. At various times, the medium was removed to determine release of trichloroacetic acid-soluble radioactivity, and the cells were rapidly chilled. After acidic washes to remove residual surface-bound ligand, the cells were solubilized and counted to determine internalized radioactivity.

ligand dissociation by reducing the endosomal concentration of insulin.

Insulin Processing by Chinese Hamster Ovary (CHO) Cells Expressing Human Insulin Receptor—The rapid degradation of insulin in hepatoma cells could reflect the physiological role of the liver as one of the major organs of insulin catabolism. To investigate the possibility that the endosomal degradation of insulin is cell type-specific, we examined the processing of insulin in CHO cells transfected with the human insulin receptor (Fig. 6). CHO cells express ~3,000 endogenous receptors; the selection of a clonal cell line expressing 80,000 human receptors (CHO/IR) has been previously described (18). When CHO/IR cells were incubated with ^{125}I-insulin at 37 °C, the cells accumulated ligand over a 45-min time course; intracellular radioactivity also increased and represented ~50% of total cell-associated radioactivity (Fig. 6*A*). However, the CHO/IR cells did not begin to degrade appreciable amounts of ^{125}I-insulin until 20–30 min. The time lag for insulin degradation was more clearly seen when CHO/IR cells were allowed to internalize a single cohort of surface-bound ^{125}I-insulin (Fig. 6*B*). Like Fao cells, CHO/IR cells rapidly internalized surface-bound insulin. However, unlike Fao cells, the levels of intracellular insulin in the CHO/IR cells remained elevated after 45 min. Moreover, the degradation of internalized insulin was initially slow, but increased markedly after 15–20 min. Thus, CHO cells transfected with the human insulin receptor were unable to degrade insulin with the rapid kinetics characteristic of Fao cells. The time lag for the initiation of insulin degradation by the CHO/IR cells is consistent with the time required for lysosomal delivery (19), and the rate of degradation was similar to that observed for IGF-II degradation in Fao cells. These data suggest that the rapid endosomal degradation of insulin may be a cell type-specific phenomenon.

DISCUSSION

We have examined the internalization and intracellular dissociation and degradation of insulin in Fao rat hepatoma cells. The overall rate of insulin processing was compared to that of IGF-II, whose receptor is known to deliver ligands to the lysosome (7). Fao cells rapidly internalize prebound ^{125}I-insulin and ^{125}I-IGF-II, with maximal intracellular levels reached at 3–5 min for both ligands. The time course of post-internalization processing, however, differs for the two ligands. Insulin is degraded within 4–5 min after internalization, whereas a lag of 20–30 min occurs between the internalization of IGF-II and the appearance of degradation products in the medium. The kinetics of IGF-II degradation are compatible with the delivery of IGF-II to the lysosomes for degradation since previous electron microscopy and cell fractionation studies have suggested that the delivery of internalized EGF to the lysosome requires 20–30 min (19, 20). In contrast, the rapid intracellular degradation of insulin by Fao cells is incompatible with the time required for the delivery of various ligands and fluid-phase markers to the lysosome and suggests the existence of an alternative degradative pathway.

The striking similarity in the kinetics of dissociation and degradation of internalized insulin suggests that the two processes are tightly coupled and occur in an early endosomal compartment. Immediately after internalization, insulin is intact and bound to its receptor. However, within 3 min, the polyethylene glycol and trichloroacetic acid precipitability of the internalized insulin begins to fall. At this point, internalized insulin is primarily associated with a light membrane fraction which cosediments with markers for fluid-phase endocytosis and presumably represents endosomal vesicles. Over the next 5 min, the dissociation and degradation of the internalized insulin proceed with identical half-times of ~3 min at 37 °C, and the amount of insulin associated with the light membrane fraction decreases. No increase in the amount of ligand which cosediments with lysosomal markers is observed. Notably, intact insulin is never observed in the cytosol. Instead, a decline in membrane-associated trichloroacetic acid-precipitable insulin is followed by the appearance of trichloroacetic acid-soluble material in the cytosol and medium. Although these data and those of others (11–13) suggest that insulin processing occurs in the endosomal fraction, the possibility of transport out of the endosome followed by extremely

rapid cytosolic degradation cannot be ruled out by kinetic experiments alone.

Bacitracin slows the rates of both intracellular dissociation and degradation of internalized insulin. This inhibition of both processes provides a second line of evidence consistent with the endosomal degradation of internalized insulin. The most direct explanation for this effect is that bacitracin blocks insulin degradation in the endosome, which raises the concentration of insulin in this compartment. This shifts the insulin binding equilibrium in the endosome, thereby increasing the amount of receptor-bound insulin. Thus, a 2-fold increase in the amount of endosomal insulin results in a 2-fold increase in bound insulin and a concomitant lengthening of the half-time for dissociation. This argument is consistent with electron microscopy studies (18) which show that the fusion of endocytosed material with large vesicles does not occur until 10–25 min after internalization; a similar argument has been proposed by Doherty *et al.* (11) based on the extremely small estimated endosomal volume (10^{-17} liter). Bacitracin has complex effects on insulin metabolism (21). However, in this context the effect of bacitracin on insulin dissociation is almost certainly mediated by its inhibition of insulin degradation. It is not due to a slight augmentation of insulin binding affinity caused by bacitracin since the slightly larger increase in affinity introduced by the use of (B26)-^{125}I-insulin does not affect endosomal dissociation or degradation. It is also not due to nonspecific effects on the endocytic machinery since the processing of β-galactosidase, low density lipoprotein, and IGF-II by Fao cells is not affected.

Our data indicate that insulin is degraded within the same compartment in which receptor-ligand dissociation occurs. If insulin degradation occurred after the transit of insulin into a receptor-free compartment, an inhibitor of insulin degradation would not affect insulin dissociation. Similarly, if insulin degradation occurred after transport of insulin from the endosome to the cytosol, then bacitracin should cause an accumulation of intact cytosolic insulin. In fact, an increase in membrane-associated insulin, but no increase in intact cytosolic insulin, is observed after bacitracin treatment (data not shown). The possibility that bacitracin is an inhibitor of insulin transport across the endosomal membrane cannot be formally excluded by these studies. In this case, its effect on insulin dissociation would not be directly related to its inhibition of insulin degradation, which could still be a cytoplasmic event. However, no such endosomal transport system for internalized insulin has yet been demonstrated. Thus, the inhibition of insulin dissociation by bacitracin suggests that dissociation and degradation occur in the endosome.

Endosomal degradation of insulin requires the presence of a protease inside the endosomal lumen. It is not clear how the protease gains access to this compartment. Insulin degradative activities associated with liver plasma membranes have been reported, and such an activity could be internalized with the insulin receptor (14, 22). Alternatively, early endosomes containing internalized insulin receptor-insulin complexes could fuse with a proteolytically active compartment. The fact that degradation of internalized insulin is blocked at 20 °C is consistent with this hypothesis since temperatures of 16–20 °C inhibit the fusion of early and late endosomes (23). The inhibition of insulin dissociation by bacitracin further suggests that receptor-bound insulin may be the substrate for an endosomal insulin protease. In this regard, the purified insulin protease can degrade insulin cross-linked to the insulin receptor, and *in vitro* insulin degradation in rat liver endosomal fractions is not inhibited by disruption of the endosomal membrane (12, 24). However, the kinetics of insulin endocytosis at 25 °C, at which temperature both internalization and processing are markedly slowed, suggest that dissociation may precede degradation. The ability of monensin to block both processes is most easily explained by this ordering of the two events, although both dissociation and proteolysis could be acidification-dependent. Thus, whereas the ordering of endosomal dissociation and degradation cannot be determined by this study, the data suggest that they are tightly coordinated.

Evidence for the nonlysosomal degradation of internalized insulin has accumulated for a number of years (25–28), and rapid degradation of internalized insulin has been reported in other systems (29). Recent work, however, has been divided on the exact site of insulin degradation. Microinjection studies using antibodies to the major insulin protease have suggested a cytosolic localization, as have attempts to locate the protease using immunocytochemical techniques (30, 31). In contrast, several groups (10–12, 26) have demonstrated the presence of degraded insulin in endosomes from rat liver as well as the ability of isolated endosomes to degrade insulin *in vitro*. Furthermore, Pease *et al.* (12) have calculated that the rate of insulin degradation in hepatic endosomes is consistent with the rate of insulin clearance *in vivo*. The insulin degradation products produced by hepatocytes *in vivo* and by isolated endosomes *in vitro* are identical to those produced by incubation of insulin with purified insulin protease (10, 32). However, *in vitro* degradation of insulin by an endosomal fraction from rat liver was insensitive to inhibitors of the insulin protease, suggesting that other proteases may be involved (12). The requirement for endosomal acidification in insulin degradation has also been controversial (10, 11). This study suggests that endosomal degradation quantitatively accounts for the degradation of internalized insulin in Fao cells and that endosomal acidification is required for this process.

The bulk of well-studied endocytosed ligands, such as low density lipoproteins and asialoglycoproteins, depend on lysosomal delivery for their degradation. In this study, the processing of insulin is markedly different from that of IGF-II, β-galactosidase, and low density lipoprotein. Differences between the processing of insulin and other ligands have also been reported in other systems (6, 33, 34). Whereas the observed differences in the processing rates of insulin and other ligands in Fao cells raise the possibility of sorting to different endosomal compartments, the presence of an insulin-specific protease within the endosome could adequately explain their differential rates of degradation. The phenomenon of endosomal catabolism of endocytosed ligands may in fact be general. Stahl and co-workers (35, 36) have demonstrated the presence of cathepsin D in macrophage endosomes, suggesting that molecules other than insulin may be degraded in this compartment, and have reported the endosomal degradation of parathyroid hormone. This study, which shows endosomal degradation of insulin in hepatoma cells, could reflect the physiological role of the liver as one of the major organs of insulin catabolism. In this regard, the processing of insulin by CHO cells transfected with the human insulin receptor cDNA is much slower than that observed in Fao cells. CHO cells have few endogenous receptors and may not be expected to express proteases required for rapid insulin degradation. Similarly, the half-time for intracellular insulin dissociation in rat fibroblasts, determined using methods similar to those used here, was 3 times as long (37). The presence of endosomal proteases capable of degrading insulin may therefore be cell type-specific.

The inhibition of endosomal insulin dissociation by bacitracin suggests that insulin degradation increases the rate of

insulin dissociation inside the cell; a similar suggestion has been made in a recent study (11) on insulin degradation in isolated rat liver endosomes. During insulin stimulation of Fao cells, 70–80% of internalized insulin receptors are tyrosyl-phosphorylated (38). It is not clear whether the rapid dissociation of insulin affects the tyrosyl phosphorylation of the intracellular receptor pool. However, the rapid dissociation of insulin from internalized receptors would render these receptors particularly susceptible to inactivation by cellular phosphotyrosine-protein phosphatases. In Fao cells, insulin does in fact stimulate phosphotyrosine-protein phosphatases which are active toward the insulin receptor.[3] Thus, the degradation-driven dissociation of insulin in the endosome may facilitate the regulation of intracellular insulin receptor activity by dephosphorylation.

In summary, insulin and IGF-II are both rapidly internalized by Fao cells, but are degraded in different compartments. Kinetic and pharmacological data suggest that insulin dissociation and degradation both occur in the endosome, where insulin degradation facilitates insulin dissociation. This rapid processing of internalized insulin may play a role in modulating the activity of internalized insulin receptors.

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Supplementary Material to

THE DISSOCIATION AND DEGRADATION OF INTERNALIZED INSULIN OCCURS IN THE ENDOSOMES OF RAT HEPATOMA CELLS

by

Jonathan M. Backer, C. Ronald Kahn and Morris F. White

EXPERIMENTAL PROCEDURES

Materials--[^{125}I](B-26)insulin and [^{125}I](A-14)insulin and [^{125}I]iodine (350-600 Ci/ml) were purchased from Amersham. [^{125}I]-labeled β-galactosidase was a gift from Dr. G.G. Sahagian, Tufts School of Medicine, Boston, MA. [^{125}I]-labeled low-density lipoprotein (LDL) was a gift from Dr. M. Krieger, M.I.T., Cambridge, MA. Recombinant human IGF-II was a gift from Eli Lilly (Indianapolis, IN).

Cell Culture-- Fao cells are derived from the Reuber H35 rat hepatoma, and have a high concentration of insulin receptors and many insulin-stimulated responses (38). The cells were maintained as previously described (39). Chinese hamster ovary (CHO) cells expressing the human insulin receptor (CHO/IR) were transfected, selected and maintained as previously described (18). Cells were used on the first day of confluence after an overnight incubation in serum-free RPMI-1640 medium (Fao) or F-12 medium (CHO).

Radio-labeling of ligands-- Recombinant human IGF-II was labeled with ^{125}I using a modified chloramine-T method (6). Briefly, 1 μg IGF-II in 20μl of 186mM glycine/290mM NaCl pH 7.4 was incubated at 22°C with 1mCi carrier-free [^{125}I]iodine and 5μl chloramine-T (2 mg/ml in glycine buffer) for 5 min. An additional 5μl chloramine-T was added, followed immediately by 5μl sodium metabisulfite (0.2 mg/ml in glycine buffer) and 100μl 1M acetic acid containing 0.1% bovine serum albumin (BSA). The mixture was subjected to gel exclusion chromatography on Sephadex G25 (Pharmacia) in 1M acetic acid/0.1% BSA. The specific activity of [^{125}I]IGF-II was 820μCi/nmole.

Binding and uptake of [^{125}I]insulin, [^{125}I]IGF-II or [^{125}I]β-galactosidase-- Confluent monolayers of Fao cells were grown in 35 mm 6-well dishes (Nunc). The cells were washed twice in phosphate-buffered saline pH 7.4 (PBS) and incubated at 37°C in "binding buffer" (RPMI-1640 containing 50 mM HEPES pH 7.4, 0.1% bovine serum albumin (BSA), and 5 x 10^6 CPM/ml [^{125}I]insulin, [^{125}I]IGF-II or [^{125}I]β-galactosidase. At various times, the medium was removed and intact ligand was precipitated by the addition of 10% trichloroacetic acid (TCA). The cells were rapidly chilled by immersion in ice-cold PBS, and washed twice with either PBS to determine total cell associated radioactivity or acidic PBS (pH 3.5) to determine internalized radioactivity. The cells were then solubilized in 0.1% SDS/0.1 N NaOH and counted in a GammaTrac 1290 γ-counter (TM Analytic). Protein content was determined by the method of Bradford (40).

Measurement of Dissociation and Degradation during a Single Cohort Internalization of [^{125}I]insulin or [^{125}I]IGF-II.--Fao cells were washed twice in PBS containing 0.1% BSA, then incubated at 4°C in "binding buffer" containing 10^5 cpm [^{125}I]insulin (A-14 or, when indicated, B-26) for 2 hours. The cells were then washed in cold PBS to remove unbound ligand and rapidly warmed by the addition of "binding buffer" at 37°C or, where stated, at other temperatures. In some experiments, binding buffer also contained 100 units/ml bacitracin, 1 μg/ml leupeptin and 2 μg/ml pepstatin, or 25μM monensin. At the indicated time intervals, the media was removed and saved and the cells were washed two times in acidic PBS, pH 3.0, containing 0.1% BSA and once in neutral PBS, pH 7.4. The cells were then solubilized in 2 ml. of 0.1% Triton X-100, 150 mM NaCl, 25mM HEPES pH 7.4, and 0.1 mg/ml aprotinin. Triton X-100 at a concentration of 0.1% was shown to solubilize >90% of internalized receptors, and has minimal effects on insulin binding affinity (41). Insoluble material was removed by centrifugation at 4°C for 30 minutes in an Beckman Microfuge B. The supernatant from each sample was removed and divided into two equivalent portions for determination of insulin dissociation and degradation.

The amount of receptor bound, PEG-precipitable radioactivity was determined by a modification of the method of Cuatrecasas (42); the use of PEG-solubility to measure insulin degradation products and intracellular insulin-receptor complexes has been previously reported (36,43). An aliquot (400μl) of the 0.1% Triton X-100 extract was incubated with 1 ml of 25% PEG plus 100μl of 0.4% γ-globulins for 30 minutes at 4°C. The mixture was centrifuged for 5 minutes at 13,000 x g in a Beckman Microfuge B and the supernatant was removed. 0.5 ml 12.5% PEG was added to each tube and the centrifugation was repeated. The radioactivity in the combined supernatants was counted as described above to determine PEG soluble radioactivity which represents free insulin. The pellet was drained and counted to determine PEG precipitable radioactivity which represents receptor bound insulin. The degradation of internalized insulin was measured by assessing the solubility in 10% TCA/1% BSA of a second aliquot of the Triton X-100 extract. Finally, degradation of insulin released into the medium was measured by assessing solubility in 10% TCA/1% BSA.

Subcellular fractionation of Fao cells. Confluent monolayers of Fao cells in 15 cm. dishes (4 dishes per time point) were allowed to take up a single cohort of [^{125}I]insulin at 37°C for various times as described above. The medium was then removed and the cells rapidly chilled by immersion in ice-cold PBS. The cells were washed twice in acidic PBS as described above, once in neutral PBS and once in buffer A (10 mM HEPES pH 7.4, 250 mM sucrose, 1 mM EDTA). The cells were scraped into buffer A, centrifuged at 600 x g for 10 min. at 4°C, and resuspended in a total of 1 ml. buffer A containing 0.35 mg/ml phenylmethylsulfonyl fluoride, 0.1 mg./ml aprotinin, and 1 μg/ml leupeptin. The resuspended cells were homogenized on ice with 15 stokes of a glass-teflon homogenizer, and centrifuged at 600 x g for 10 min. The supernatant was reserved and the pellet resuspended in 1 ml. buffer A containing protease inhibitors, homogenized for 10 strokes, and centrifuged again for 10 min. at 600 x G. The post-nuclear supernatants were pooled; homogenization was 85% complete as judged by recovery of β-galactosidase activity. In some experiments, the supernatant was centrifuged at 100,000 x G at 4°C for 60 min. to yield cytosol (supernatant) and total particulate (pellet) fractions. Aliquots were precipitated with 10 % TCA for determination of intact and degraded [^{125}I]insulin. In other experiments, 1.5 ml. of post-nuclear supernatant was layered over 28 ml. of 10 % Percol in Buffer A on a cushion of 4 ml. 2.5 M. sucrose, and centrifuged at in a SV-288 vertical rotor (Sorval) at 28,000 x G for 50 min. The gradients were fractionated using an Isco fractionator; aliquots were precipitated with 10 % TCA for determination of intact and degraded [^{125}I]insulin.

Subcellular marker enzymes. Aliquots from the Percol gradients were assayed for total protein by the method of Bradford (40). The lysosomal markers β-hexosaminidase and acid phosphatase, the plasma-membrane marker 5'-nuclotidase and the endoplasmic reticulum marker glucose-6-phosphatase were assayed as described (44). The position of the endosomal fraction was determined by incubating CHO cells for 30 min at 37°C with 5 mg/ml dialyzed 70 kDa FITC-dextran; subcellular fractionation on 10% Percol gradients generated a light, β-hexosaminidase-free fraction (endosomes) as well as a dense, β-hexosaminidase-containing fraction (lysosomes).

Analysis of insulin binding affinity. Analysis of insulin binding was performed on confluent monolayers in 24-well dishes. The cells were washed twice with PBS and incubated at 4°C overnight in binding buffer containing 20,000 CPM/ml A-14 [^{125}I]insulin, B-26 [^{125}I]insulin, or A-14 [^{125}I]insulin plus 100 u/ml bacitracin. The cells were then washed twice in PBS containing 0.1% BSA and once in PBS, solubilized in 0.1% SDS/0.1 N NaOH, and counted as described above. The relative binding affinity of [^{125}I]labeled insulin in each case was compared to that of A-14 [^{125}I]insulin as described by Linde *et al* (16).

Measurement of insulin dissociation from surface receptors. Confluent monolayers of Fao cells were grown in 6-well dishes. The cells were incubated with A-14 [^{125}I]insulin or B-26 [^{125}I]insulin for 16 h. at 4°C and then were washed twice in PBS at 4°C to remove unbound ligand. Dissociation was initiated by immersing the cells in neutral (pH 7.4) or acidic (pH 6.0) binding buffer at 37°C in the absence or presence of 100 u/ml bacitracin. At various times the cells were rapidly chilled by immersion in ice-cold PBS, washed twice in PBS at 4°C, solubilized and counted as described above.

pH dependence of insulin binding. Confluent monolayers of Fao cells were grown in 6-well dishes. After two washes in PBS, the cells were incubated at 4°C for 16 h. in binding buffer, containing [^{125}I]insulin without or with 100 u/ml bacitracin, which had been adjusted to varying pH. The cells remained stably attached throughout the incubations. The cells were then washed, solubilized and counted as described above.

Uptake and degradation of [^{125}I]LDL. Cellular processing of LDL was determined as described by Krieger (45). Cells were grown in RPMI-1640 containing 10 % human lipoprotein-deficient serum for 48 h., and changed to serum-free RPMI 1640 16 h. before use. The cells were washed twice in PBS at 4°C, and incubated for 2 h. at 4°C in binding buffer containing 10 μg/ml [^{125}I]LDL; nonspecific binding was determined in the presence of 400 g/ml LDL. The cells were then washed twice with PBS at 4°C, and internalization was initiated by the addition of binding buffer at 37°C. At various times, the medium removed and proteins precipitated with 20% TCA at 4°C. After centrifugation, 700μl of supernatant was mixed with added to 7 μl 40% potassium iodide, and 28 μl H^3O^2 was added while vortexing. After 5 min. at 22 °C, 1 ml. chloroform was added, and the samples vortexed and then centrifuged for 5 min. at 2000 RPM. 0.5 ml of the upper phase was removed and counted for determination of degraded [^{125}I]LDL.

RESULTS

Effect of acidotropic agents--Monensin, a carboxylic ionophore which disrupts intracellular ion gradients, was a potent inhibitor of both insulin dissociation and degradation inside the cell. The internalization of surface bound insulin was slowed slightly by monensin, although significant levels of internalized ligand accumulated within 3-5 min (Fig. 7a). In contrast to control cells, however, the level of internalized insulin remained elevated and degraded insulin appeared in the medium at low levels only after 20-30 minutes. Furthermore, the intracellular dissociation and degradation of insulin was barely detectable in the presence of monensin (Fig. 7b), presumably due to the disruption of endosomal acidification.

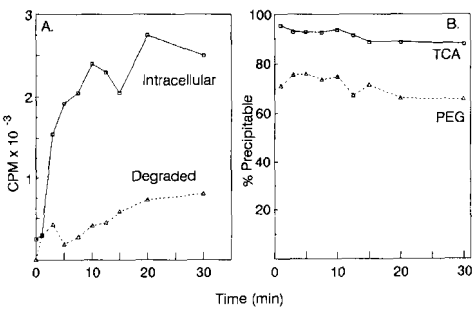

Fig. 7. Inhibition of intracellular dissociation and degradation of internalized insulin by monensin. Fao cells were allowed to bind [^{125}I]insulin for 2 h 4°C. After washes at 4°C to remove unbound insulin, the cells were rapidly warmed in the presence of 25μM monensin. At various times, the medium was removed and the cells rapidly chilled. (A) Total intracellular radioactivity and TCA-soluble radioactivity in the medium were determined as described in Fig. 2. (B.) Intracellular degradation and dissociation were determined as described in Fig. 3.

Temperature Dependence of Insulin Processing--To examine the relationship between insulin dissociation and degradation inside the cell, the effect of low temperature of both processes was examined. At 20°C, the internalization of surface bound insulin was slow, with maximal levels obtained by 7-8 min (data not shown). Both dissociation and degradation of internalized ligand were undetectable up to 15 min at this temperature. At 25°C, insulin internalization was maximal by 7.5 min, with a subsequent decrease in internalized ligand and an appearance of degraded insulin in the medium during continued incubation at 25°C (Fig. 8A.). Intracellular insulin dissociation and degradation at 25°C were slow relative to 37°C., with half-times of 12.9 and 15.8 min. respectively (Fig. 8B,8C).

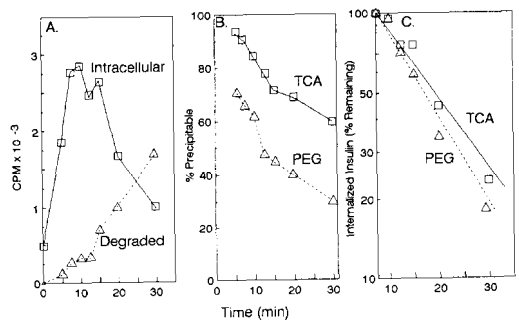

Fig. 8. Insulin processing by Fao cells at 25°C. Fao cells were allowed to bind [^{125}I]insulin for 2 h 4°C. After washes at 4°C to remove unbound insulin, the cells were rapidly warmed to 25°C. At various times, the medium was removed and the cells rapidly chilled. (A) Total intracellular radioactivity and TCA-soluble radioactivity in the medium were determined as described in Fig. 2. (B.) Intracellular degradation and dissociation were determined as described in Fig. 3. (C). Semilogarithmic plot of the data in (B).

Effects of bacitracin on intracellular insulin processing are not due to changes in insulin binding affinity or inherent dissociation rate--Bacitracin has been reported to increase the affinity of insulin for its receptor in a variety of cells (17). To examine the possibility that the inhibition of intracellular ligand dissociation by bacitracin was due to altered binding affinity, insulin binding was measured in the absence or presence of bacitracin and relative binding affinities were determined as described by Linde *et al.* (16). Bacitracin caused a 1.5-fold increase in the binding affinity of A-14 [^{125}I]insulin (data not shown). To determine whether a change in binding affinity of this magnitude would affect intracellular insulin processing, we examined the uptake and processing of B-26 [^{125}I]insulin, whose binding affinity to the insulin receptor in Fao cells is 1.6-fold greater than that of A-14 [^{125}I]insulin (data not shown). The rates of intracellular dissociation and degradation of B-26 insulin were not significantly different than that of A-14 insulin (Table I). Thus the inhibition of intracellular insulin dissociation by bacitracin was not due to altered binding affinity.

The dissociation of internalized ligands from their receptors is thought to be driven primarily by the acidification of the endosomal lumen (46). To rule out he possibility that bacitracin was altering the pH-dependent dissociation of insulin from its receptor, the dissociation of bound insulin from its receptor was evaluated at both pH 7.4 and ph 6.5, the pH of early endosomes (47). At pH 7.4 (Fig. 9a.), bacitracin caused a slight increase in the rate of A-14 insulin dissociation. At pH 6.5, however, the dissociation of insulin from its receptor in the absence

or presence of bacitracin was identical (Fig. 9b). These data are confirmed by the fact that pH dependence of equilibrium insulin binding was also unaffected by bacitracin (Fig. 9c). Thus the inhibition of intracellular insulin dissociation by bacitracin was not due to a change in the pH dependence of insulin binding or dissociation.

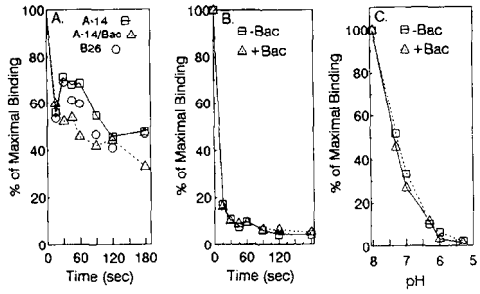

Fig. 9. **Effect of bacitracin on insulin binding to intact Fao cells.** (A). Fao cells were allowed to bind [^{125}I]insulin for 2 h 4°C. After washes at 4°C to remove unbound insulin, the cells were rapidly warmed to 37°C in the presence or absence of 100 u/ml bacitracin in medium at pH 7.4. At various times, the cells were chilled by immersion in ice-cold PBS, solubilized and counted. Squares, A-14 [^{125}I]insulin. Circles, B-26 [^{125}I]insulin. Triangles, A-14 [^{125}I]insulin in 100 u/ml bacitracin. (B.) Dissociation of bound A-14 [^{125}I]insulin in the presence or absence of 100 u/ml bacitracin was measured as above at pH 6.5. (C.) Fao cells were allowed to bind A-14 [^{125}I]insulin in the absence or presence of 100 u/ml bacitracin for 16 h at 4°C at varying pH. The cells were then washed with PBS, solubilized and counted.

Bacitracin does not inhibit the internalization or processing of other ligands--Although bacitracin had no effect on the pH dependent dissociation of insulin from its receptor *in vitro*, it is possible that bacitracin inhibited endosomal acidification or some other aspect of receptor mediated endocytosis *in vivo*. If this were the case, however, bacitracin would be expected to affect the processing of other ligands such as IGF-II, β-galactosidase (a lysosomal enzyme) and low density lipoprotein (LDL) by their respective receptors. Bacitracin had no effect on the internalization of surface-bound IGF-II by Fao cells; receptor-ligand complexes were efficiently transported into the cell within 3-5 minutes in the absence or presence of bacitracin. (Fig 10a). Degradation of IGF-II was slow, with less than 3% of internalized IGF-II released as TCA-soluble material after 10 min, and was also unaffected by bacitracin (inset). The uptake and degradation of β-galactosidase, an alternative ligand of the IGF-II/M6P receptor, was similarly unaffected during a 3 h incubation with or without bacitracin (Fig. 10b). Finally, the processing of internalized LDL, a receptor mediated process with an established lysosomal site of degradation, was unchanged in the absence or presence of bacitracin (Fig. 10c). Thus the inhibition of insulin dissociation from its receptor inside the cell by bacitracin does not reflect a general disruption of endocytic processes.

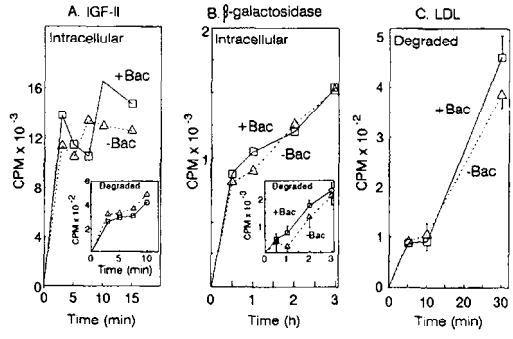

Fig. 10. **Bacitracin does not alter endocytic uptake and processing of other ligands.** (A). Fao cells were allowed to bind [^{125}I]IGF-II for 2 h 4°C. After washes at 4°C to remove unbound insulin, the cells were rapidly warmed to 37°C in the presence or absence of 100 u/ml bacitracin. Total intracellular radioactivity and TCA-soluble radioactivity in the medium (inset) were determined as described in Fig. 2. (B.) Fao cells were incubated at 37°C with [^{125}I]β-galactosidase for varying times. The cells were rapidly chilled and total intracellular radioactivity and TCA-soluble radioactivity in the medium (inset) were determined as described in Fig. 1. (C.) Fao cells were incubated at 37°C with [^{125}I]LDL for varying times. The cells were rapidly chilled and TCA-soluble radioactivity in the medium were determined as described in *Experimental Procedures*.