Chymotrypsin Substrate Analogues Inhibit Endocytosis of Insulin and Insulin Receptors in Adipocytes

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Abstract. To explore the possible role of proteolytic step(s) in receptor-mediated endocytosis of insulin, the effects of inhibitors of various classes of proteases on the internalization process were studied in isolated rat adipocytes. Intracellular accumulation of receptor-bound ^{125}I-insulin at 37°C was quantitated after rapidly dissociating surface-bound insulin with an acidic buffer (pH 3.0). Of the 23 protease inhibitors tested, only chymotrypsin substrate analogues inhibited insulin internalization. Internalization was decreased 62–90% by five different chymotrypsin substrate analogues: *N*-acetyl-Tyr ethyl ester, *N*-acetyl-Phe ethyl ester, *N*-acetyl-Trp ethyl ester, benzoyl-Tyr ethyl ester, and benzoyl-Tyr amide. The effect of the substrate analogues in inhibiting insulin internalization was dose-dependent, reversible, and required the full structural complement of a chymotrypsin substrate analogue. Cell surface receptor number was unaltered at 12°C. However, concomitant with their inhibition of insulin internalization at 37°C, the chymotrypsin substrate analogues caused a marked increase (160–380%) in surface-bound insulin, indicating trapping of insulin–receptor complexes on the cell surface. Additionally, 1 mM *N*-acetyl-Tyr ethyl ester decreased overall insulin degradation by 15–20% and also prevented the chloroquine-mediated increase in intracellular insulin, further indicating that surface-bound insulin was prevented from reaching intracellular chloroquine-sensitive degradation sites. The internalization of insulin receptors that were photoaffinity labeled on the cell surface with B2(2-nitro-4-azidophenylacetyl)-des-PheB^1-insulin was also inhibited 70–90% by the five chymotrypsin substrate analogues, as determined by the effects of the analogues on the accumulation of trypsin-insensitive (intracellular) 440-kD intact labeled receptors. In summary, these results show that chymotrypsin substrate analogues efficiently inhibit the internalization of insulin and insulin receptors in adipocytes and implicate a possible role for endogenous chymotrypsin-like enzyme(s) or related substances in receptor-mediated endocytosis of insulin.

INTERNALIZATION of ligands selectively bound to cell surface receptors occurs through the process of receptor-mediated endocytosis. Ligands for which this process has been widely studied are polypeptide growth factors and hormones, including insulin, nutrient transport proteins such as low density lipoprotein and transferrin, asialoglycoproteins and immunoglobulins (reviewed in references 5, 17, 36, 43, 44). For ligands such as low density lipoprotein, transferrin, and asialoglycoproteins, endocytosis serves the function of delivering extracellular molecules to the cell interior for further metabolic usage or degradation. However, the physiologic role of internalization of insulin and other polypeptide hormones remains incompletely understood. One important function of the internalization of insulin–receptor complexes is believed to be mediation of the insulin-induced receptor loss (down-regulation) that has been extensively demonstrated in various cell types, including adipocytes (15, 18, 24, 29). Other potential functions of the internalization process may include the termination of insulin action by removing the hormone from the cell surface and transporting it to intracellular degradation sites and/or mediating the intracellular effects of the hormone (5, 34, 40). Thus, characterization of the internalization of insulin–receptor complexes is of importance in further understanding its role in the physiologic regulation of insulin receptors and insulin action.

In various studies of receptor-mediated endocytosis, several factors that inhibit the endocytotic process have been described. These include inhibitors of metabolic energy (27, 33, 36), depletion of intracellular potassium (26, 27), sulfhydryl reagents (22, 42), hypertonic media (11), and transglutaminase inhibitors (12, 19). Although energy depletion, potassium depletion, sulfhydryl reagents, and hypertonic media effectively inhibit endocytosis, they pose the possible problem of nonselectively affecting multiple cellular processes. Although potentially more selective, transglutaminase inhibitors, which have been reported to block internalization of several other ligands (12, 19), do not inhibit insulin internalization in isolated rat adipocytes (14). Additionally, many of the effects of these agents have been attributed to inhibition of receptor recycling, rather than to inhibition of endocytosis (41).

To further explore the mechanism(s) of receptor-mediated insulin internalization and to identify agents that block the process in the adipocyte, a major insulin-sensitive cell, we have examined the effects of protease inhibitors. Protease inhibitors were considered possible blockers of endocytosis since they inhibit other cellular processes dependent upon vesicular movement and membrane fusion. For example, inhibitors of metalloendoproteases prevent the fusion of myoblasts into myotubules (10) as well as the exocytotic release of transmitters from presynaptic nerve terminals (3), histamine from mast cells, and catecholamines from adrenal cells (31). Synthetic tripeptide aldehyde protease inhibitors have also been shown to block the exocytotic release of prolactin from cultured pituitary cells (32). Since endocytosis also involves vesicular movements and requires a membrane fusion event, the joining of segments of the plasma membrane to form endocytotic vesicles, we hypothesized that a proteolytic step may be required during the endocytosis of insulin–receptor complexes. To test this hypothesis, we studied the effects of inhibitors of various classes of proteases on the internalization of insulin and insulin receptors in isolated rat adipocytes. Our results show that compounds that are competitive inhibitors of chymotrypsin-like enzymes prevent the internalization of insulin–receptor complexes in adipocytes.

Materials and Methods

Materials

$Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, IL); collagenase from Worthington Biochemical Corp. (Freehold, NJ); Eagle's minimum essential medium (MEM) from Gibco (Grand Island, NY); bovine serum albumin (BSA, Fraction V) from Armour Pharmaceutical Co. (Tarrytown, NY); silicone oil from Arthur H. Thomas Co. (Philadelphia, PA); IODO-GEN from Pierce Chemical Co. (Rockford, IL); phosphoramidon from Enzyme Systems Products (Livermore, CA); and dimethyl sulfoxide, monodansylcadaverine, and methylamine from Sigma Chemical Co. (St. Louis, MO). Porcine monocomponent insulin was supplied by Dr. Ronald Chance of Eli Lilly & Co. (Indianapolis, IN). All other protease inhibitors and protease substrate analogues were obtained from Vega Biotechnologies, Inc. (Tucson, AZ) or Sigma Chemical Co. The photoreactive insulin derivative, B2 (2-nitro-4-azidophenylacetyl)-des-$PheB^1$–insulin (NAPA-DP-insulin),[1] which has been well characterized and is fully biologically active (8, 13), was kindly provided by Dr. Dietrich Brandenburg of Deutsches Wollforschungsinstitut, Aachen, Federal Republic of Germany.

Iodination of Insulin and Photoreactive Insulin Derivative

Insulin and NAPA-DP-insulin were each iodinated to a specific activity of ~200 μCi/ug using the water-insoluble oxidizing agent, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN), essentially as we have described previously (6, 7). ^{125}I-insulin was chromatographed on a Sephadex G-50 column to remove free ^{125}I. NAPA-DP-^{125}I-insulin was dialyzed overnight at 4°C in a dark room to remove free ^{125}I and the iodinated product was stored protected from light at 4°C and used within 3 wk.

Preparation of Isolated Adipocytes

Isolated adipocytes were prepared by collagenase digestion (35) of epididymal fat pads obtained from male Sprague-Dawley rats weighing 200–240 g. The cells were filtered through a 250-μm nylon mesh, washed four times, and suspended in a binding buffer (pH 7.6) consisting of MEM, 10 mM Hepes, and 1% BSA. Adipocyte counts were performed after fixing the cells in 2% osmium tetroxide/0.05 M collidine/0.9% NaCl buffer using a model ZB Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) as previously described (7, 21).

1. *Abbreviation used in this paper*: NAPA-DP-insulin, B2(2-nitro-4-azidophenylacetyl)-des-$PheB^1$–insulin.

Measurement of Total, Intracellular, and Surface-Bound ^{125}I-Insulin

Isolated rat adipocytes (~400,000 cells/ml) were incubated with 0.3 ng/ml ^{125}I-insulin in MEM, 10 mM Hepes, 1% BSA, pH 7.6, at 37°C in the presence or absence of protease inhibitors and substrate analogues. Most of the protease substrate analogues were first dissolved in dimethyl sulfoxide, producing 0.1–0.5% dimethyl sulfoxide in the final incubation. Dimethyl sulfoxide at this concentration had no effect on insulin binding, internalization, or degradation. Nonspecific binding was determined by performing parallel incubations in the presence of 50 μg/ml unlabeled porcine insulin. At the indicated times, three 200-μl aliquots were withdrawn for determination of total cell-associated radioactivity. Each 200-μl aliquot of cell suspension was added to a plastic microfuge tube containing 150 μl of silicone oil and the tubes were centrifuged for 30 s in a microfuge (model 11; Beckman Instruments, Inc., Fullerton, CA). This results in the separation (by the silicone oil) of the cell layer from the aqueous layer containing free unbound ^{125}I-insulin. The cell plug (top layer) was then removed and the radioactivity measured. Since total cell-associated ^{125}I-insulin at 37°C represents both surface (receptor) bound and internalized insulin, the amount of ^{125}I-insulin in each of these cellular compartments was determined using a modification (21, 33) of an acid extraction procedure (19) that rapidly and quantitatively removes surface-bound insulin, leaving behind intracellular insulin. 1 ml of cell suspension was centrifuged in a 17 × 100-mm polystyrene tube for 30 s at 200 rpm, after which the infranatant was removed and replaced with an equal volume (850 μl) of ice-cold barbital sodium acetate buffer, pH 3.0, containing 28 mM Na acetate, 20 mM Na barbital, and 117 mM NaCl. After further incubation for 6 min at 4°C, three 200-μl aliquots were removed and the remaining cell-associated (nonextractable) ^{125}I-insulin was measured after centrifugation of the cells through silicone oil. Extractable ^{125}I-insulin was then calculated by subtracting nonextractable binding from total binding. By this method, the acid-extractable radioactivity represents ^{125}I-insulin bound to cell surface receptors while the nonextractable radioactivity represents insulin that has been internalized.

Measurement of ^{125}I-Insulin Degradation

The degradation of ^{125}I-insulin was determined by assessing the precipitability of ^{125}I-material from the incubation medium with 10% trichloroacetic acid. With this method, trichloroacetic acid–precipitable material is considered to be predominantly intact insulin and trichloroacetic acid–soluble material is considered to be degraded insulin (16, 28).

Photoaffinity Labeling of Cell Surface Insulin Receptors and Internalization of the Labeled Insulin–Receptor Complexes

Suspensions of isolated adipocytes (1×10^6 cells/ml) were incubated in the dark at 14°C with 60 ng/ml of NAPA-DP-^{125}I-insulin. After 30 min of incubation, the cells were transferred to 60 × 15-mm plastic petri dishes and photolysis was carried out for 3 min by exposure to a long wave (366-nm) ultraviolet lamp (Blak-Ray; Ultra-Violet Products, Inc., San Gabriel, CA) placed 10 cm from the cell suspension. These procedures covalently cross-link 25% of the specifically bound NAPA-DP-^{125}I-insulin to the cell surface insulin receptors without altering cell viability (6, 7). To initiate internalization of the labeled insulin–receptor complexes, adipocytes photolabeled at 14°C were washed and incubated in fresh buffer at 37°C. Aliquots of the cells were taken at various times and exposed to 200 μg/ml of trypsin for 2 min at 37°C. This trypsinization procedure affords an effective means of distinguishing between labeled receptors remaining on the cell surface that are sensitive to tryptic proteolysis and those that have been internalized and, therefore, become insensitive to the extracellular trypsin (6, 7). After the 2-min exposure to trypsin, soybean trypsin inhibitor (final concentration, 400 μg/ml) was added and the cells were then washed three times and solubilized with a solution consisting of 3% SDS, 2 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride, and 5 mM ethylenediaminetetraacetic acid.

Identification of Solubilized Photoaffinity-labeled Insulin Receptors

The solubilized proteins from the photoaffinity-labeled cells were analyzed under nonreducing conditions by SDS PAGE according to the method of Laemmli (25), using porous acrylamide gels as described previously (6, 7). The gels were stained with Coomassie Brilliant Blue, destained, dried, and

autoradiographed at −70°C using X-Omat AR film (Eastman Kodak Co., Rochester, NY) and Cronex Lightning Plus (Dupont Co., Diagnostic & Bio-Research Systems, Wilmington, DE) intensifying screen. The relative molecular mass (M_r) markers used were fibronectin (440 kD), myosin (200 kD), β-galactosidase (116.5 kD), phosphorylase B (92.5 kD), bovine serum albumin (66 kD), and ovalbumin (45 kD).

Results

Effects of Protease and Transglutaminase Inhibitors on Insulin Internalization

Using the acid–barbital extraction technique for quantitation of intracellular insulin, a variety of inhibitors of different classes of proteases (2) and transglutaminase inhibitors (12, 19) were studied for their effects on the internalization of ^{125}I-insulin in adipocytes incubated at 37°C (Table I). The concentrations of the compounds used in these initial studies were determined from the known inhibitory concentrations of these agents as used in various other studies. The first group of compounds tested were competitive inhibitors of metalloendoproteases (Table I, group 1 compounds), agents that have previously been shown to inhibit exocytosis (3, 31). The substrate analogues (Cbz-Gly-Phe amide, Cbz-Leu amide) and the nonsubstrate competitive inhibitor (phosphoramidon) of metalloendoproteases did not inhibit insulin internalization in adipocytes but instead caused increased intracellular accumulation of ^{125}I-insulin (Table I). Similarly, a series of competitive inhibitors of trypsin-like serine proteases and thiol cathepsins (Table I, group 2 compounds) also did not inhibit insulin internalization but caused variable degrees of increase in the intracellular accumulation of ^{125}I-insulin. Among this group of inhibitors, benzoyl-Arg ethyl ester and tosyl-Arg methyl ester have previously been shown to inhibit various insulin effects in adipocytes, including insulin stimulation of receptor phosphorylation, glucose transport, glucose oxidation, and lipogenesis (4, 9, 30, 39) at concentrations (5 mM) identical to those used in the present studies. Leupeptin and pepstatin are microbial-derived nonsubstrate competitive inhibitors of serine/thiol and carboxyl proteases, respectively, and neither of these compounds influenced the amounts of intracellular ^{125}I-insulin in adipocytes (Table I, group 3).

Of the various competitive inhibitors of endoproteases tested, only *N*-acetyl-Tyr ethyl ester, a substrate analogue of chymotrypsin-like serine proteases (20), markedly decreased the intracellular accumulation of ^{125}I-insulin in adipocytes (Table I, group 4). In the presence of 1 mM *N*-acetyl-Tyr ethyl ester, intracellular insulin accumulated to only 15% of values reached in parallel control incubations.

A series of inhibitors of exoproteases (2) were also studied for their effects on insulin internalization. As shown in Table I, competitive inhibitors (substrate analogues) of aminopeptidases (group 5), carboxypeptidases (group 6), and di- and tripeptidases (group 7) did not inhibit insulin internalization but caused variable increases in the amount of intracellular ^{125}I-insulin. Similarly, the transglutaminase inhibitors dansylcadaverine and methylamine did not inhibit the internalization of insulin into adipocytes.

The results summarized in Table I show that of the various inhibitors tested, only *N*-acetyl-Tyr ethyl ester, a competitive inhibitor (substrate analogue) of chymotrypsin-like enzymes inhibited the receptor-mediated endocytosis of insulin in adipocytes. Therefore, the effects of this agent on the internalization process were further characterized.

Table I. Effects of Protease and Transglutaminase Inhibitors on Insulin Internalization in Rat Adipocytes

Inhibitors	Concentration	Internalized insulin (percent of control)
	mM	
Competitive inhibitors of endoproteases		
None (control)		100
1. Cbz-Gly-Phe amide	5.0	354 ± 40
Cbz-Gly-Leu amide	5.0	227 ± 8
Phosphoramidon	0.1	301 ± 21
2. Tosyl-Arg methyl ester	5.0	156 ± 5
Benzoyl-Arg ethyl ester	5.0	136 ± 3
Benzoyl-Arg amide	5.0	130 ± 17
Benzoyl-Arg-nitroanilide	2.0	338 ± 46
Benzoyl-DL-Arg-β-napthylamide	0.3	122 ± 6
3. Leupeptin	0.1	99 ± 4
Pepstatin	0.003	99 ± 1
4. *N*-acetyl-Tyr ethyl ester	1.0	15 ± 1
Competitive inhibitors of exoproteases		
5. Leu-NH_2	5.0	156 ± 15
Cbz-Gly-Gly amide	5.0	124 ± 3
6. Cbz-Glu-Tyr	5.0	181 ± 3
Cbz-Pro-Phe	5.0	158 ± 8
7. Gly-Leu	5.0	105 ± 14
Ala-Gly	5.0	115 ± 8
Ser-Met	5.0	112 ± 5
Gly-Gly-Gly	5.0	97 ± 6
Transglutaminase inhibitors		
8. Methylamine	15.0	174 ± 24
Dansylcadaverine	0.2	115 ± 4

Adipoctyes were incubated for 60 min at 37°C with 0.3 ng/ml of ^{125}I-insulin either in the absence or presence of the various inhibitor compounds shown. The amount of internalized insulin was then quantitated after dissociation of the surface-bound insulin using acid–barbital buffer. The results, expressed as percent of the amount of insulin internalized in control cells, represent the mean ± SEM of three to four separate experiments.

Effect of N-acetyl-Tyr ethyl ester Concentration on Insulin Binding and Internalization

Fig. 1 shows the effects of increasing concentrations of *N*-acetyl-Tyr ethyl ester on total, surface-bound, and intracellular cell-associated insulin. At concentrations of *N*-acetyl-Tyr ethyl ester greater than 0.25 mM, large dose-dependent decreases in the intracellular accumulation of ^{125}I-insulin were observed. During the 60-min incubation, the amount of internalized insulin decreased by 50 and 87% in the presence of 0.55 mM and 1.0 mM *N*-acetyl-Tyr ethyl ester, respectively. Nonspecific binding was not changed in the presence of 1 mM *N*-acetyl-Tyr ethyl ester during the incubation period. Additionally, cell viability as assessed by stability of adipocyte counts and the ability of the cells to exclude trypan blue remained unaltered during the same incubation period. Internalization remained suppressed at concentrations of

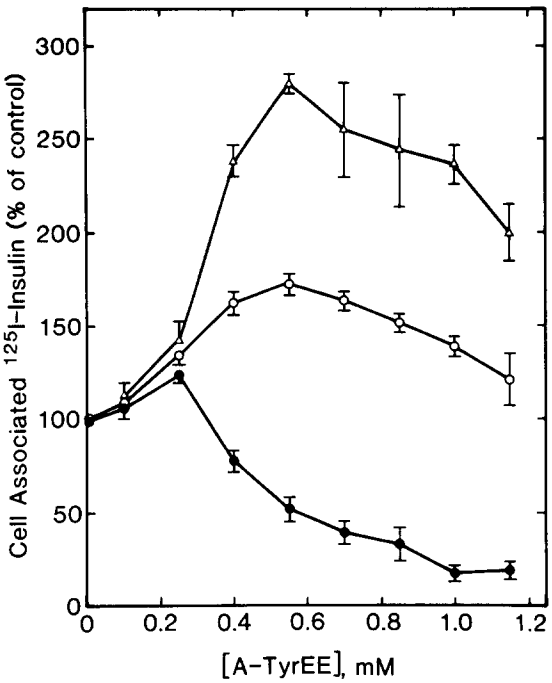

Figure 1. Effect of *N*-acetyl-Tyr ethyl ester concentration on total, intracellular, and surface-bound insulin. Adipocytes were incubated for 60 min at 37°C with 0.3 ng/ml ^{125}I-insulin in the absence or presence of the indicated concentrations of *N*-acetyl-Tyr ethyl ester. Total (○), intracellular (●), and surface-bound (△) cell-associated insulin were then determined in triplicate and corrected for nonspecific binding. The mean ± SEM of three separate experiments are shown.

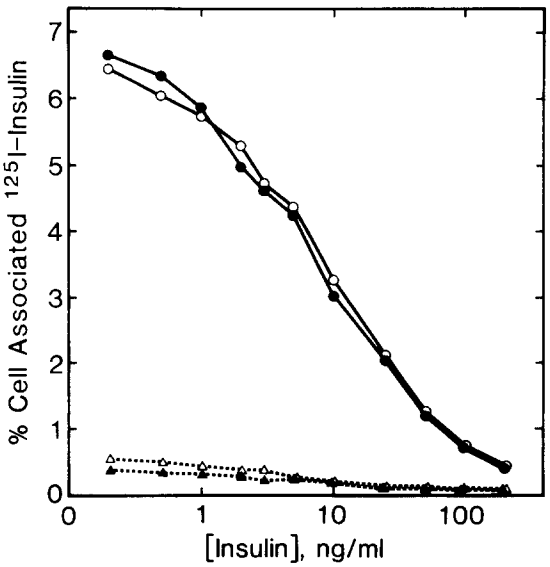

Figure 2. Lack of effect of *N*-acetyl-Tyr ethyl ester on binding of ^{125}I-insulin to adipocytes at 12°C. Adipocytes were incubated for 30 min at 37°C in the absence (○, △) or presence (●, ▲) of 1 mM *N*-acetyl Tyr ethyl ester. The cells were then cooled to 12°C and 0.2 ng/ml ^{125}I-insulin plus sufficient unlabeled insulin were added, giving the indicated final concentrations of the hormone. After further incubation at 12°C for 150 min, total, intracellular and surface-bound ^{125}I-insulin were determined in triplicate. The data shown represent the mean of cell surface bound (○, ●) and intracellular (△, ▲) ^{125}I-insulin from three separate experiments.

N-acetyl-Tyr ethyl ester higher than 1 mM; however, prolonged incubation with these higher concentrations caused increased nonspecific binding. Total cell-associated ^{125}I-insulin was consistently increased in the presence of the various concentrations of *N*-acetyl-Tyr ethyl ester, including those that resulted in submaximal inhibition of insulin internalization. Surface-bound insulin was also markedly increased with the maximal increase (280%) occurring at 0.55 mM *N*-acetyl-Tyr ethyl ester, the concentration at which intracellular insulin accumulation was decreased by 50%.

To determine if *N*-acetyl-Tyr ethyl ester has any effect on cell surface insulin-binding independent of its effect on internalization, adipocytes were first incubated at 37°C for 30 min in the absence or presence of 1 mM *N*-acetyl-Tyr ethyl ester. After cooling the cells to 12°C, insulin binding (total, intracellular, and cell surface) was determined at this temperature in the presence of increasing hormone concentrations. As shown in Fig. 2, intracellular insulin accumulation was small at 12°C, reflecting the slow internalization of insulin at this temperature (33). Surface-bound ^{125}I-insulin was the same in treated and control cells. Scatchard analysis of surface binding (data not shown) indicated that cell surface receptor number and affinity were identical in the presence or absence of *N*-acetyl-Tyr ethyl ester.

Time Course of the Effect of N-acetyl-Tyr ethyl ester on Insulin Binding and Internalization

Fig. 3 shows the time course of the effects of 1 mM *N*-acetyl-Tyr ethyl ester on total, intracellular, and surface-bound insulin in adipocytes incubated at 37°C. Total binding (Fig. 3 *A*) became maximal after 15–30 min of incubation in both control and treated cells. Levels of total binding were similar in the two groups between 5 and 30 min of incubation, after which total binding was greater in cells treated with *N*-acetyl-Tyr ethyl ester. Intracellular ^{125}I-insulin accumulation (Fig. 3 *B*) was rapidly and markedly reduced in *N*-acetyl-Tyr ethyl ester–treated cells such that during the first 30 min of incubation it was only 5% of that attained in control cells. Subsequently, a gradual small rise occurred in intracellular insulin in the treated cells, possibly as a consequence of hydrolysis of a fraction of *N*-acetyl-Tyr ethyl ester in the incubations such that its effective concentration is lowered to a level that would be submaximal in inhibiting internalization (See Fig. 1). Surface insulin binding was maximal between 15 and 30 min in both *N*-acetyl-Tyr ethyl ester–treated and untreated cells, but the amount of surface-bound insulin was approximately doubled in the *N*-acetyl-Tyr ethyl ester–treated cells (Fig. 3 *C*). Thus, with the inhibition of insulin internalization by *N*-acetyl-Tyr ethyl ester (*B*), a large increase in the amount of surface-bound insulin (*C*) was observed.

Effect of N-acetyl-Tyr ethyl ester on Insulin Degradation

Fig. 4 shows the time course of insulin degradation in the incubation medium in the presence and absence of *N*-acetyl-Tyr ethyl ester, as determined by trichloroacetic acid precipitability of ^{125}I-insulin. During the 90 min of incubation at 37°C, insulin degradation was consistently lower in the *N*-acetyl-Tyr ethyl ester–treated cells as compared with the control cells. The percent insulin degradation in the control incubations represented 16.9 ± 2.7% at 60 min and 23.2 ± 1.7% at 90 min, while in the presence of *N*-acetyl-Tyr

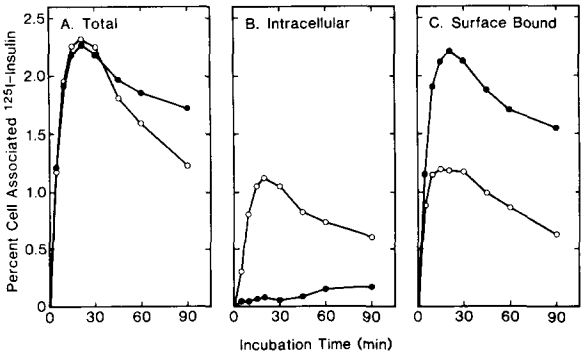

Figure 3. Effect of *N*-acetyl-Tyr ethyl ester on the time course of insulin binding and internalization. Adipocytes were incubated at 37°C with 0.3 ng/ml of ^{125}I-insulin in the absence (○) or presence (●) of 1 mM *N*-acetyl-Tyr ethyl ester. Aliquots of the cells were removed at the indicated times for measurement of total (*A*), intracellular (*B*), and surface-bound (*C*) specific cell-associated radioactivity. Each data point represents the mean ± SEM of the percent ^{125}I-insulin specifically associated with 4×10^5 adipocytes in three separate experiments.

ethyl ester, the corresponding amounts were 14.1 ± 2.2 and 19.5 ± 1.9% at 60 and 90 min, respectively. The differences in insulin degradation between the control and treated cells at each of these two incubation periods were statistically significant at $P < 0.01$ level.

Effects of N-acetyl-Tyr ethyl ester in Inhibiting the Chloroquine-mediated Increase in Intracellular Insulin

To further determine the effect of *N*-acetyl-Tyr ethyl ester on the internalization and intracellular processing of insulin, experiments were performed in the absence or presence of chloroquine, a lysosomotropic agent that blocks the processing of internalized insulin and causes a marked increase in intracellular insulin accumulation (21, 28, 33). Thus, under conditions in which insulin internalization is inhibited, a chloroquine-mediated increase in intracellular insulin should not occur. This is shown in Fig. 5. During a 60-min incubation of adipocytes at 37°C in the absence of *N*-acetyl-Tyr ethyl ester, chloroquine (0.1 mM) caused a 250% increase in intracellular ^{125}I-insulin. By contrast, in the presence of *N*-acetyl-Tyr ethyl ester, insulin internalization was inhibited and chloroquine failed to cause an increase in intracellular insulin (Fig. 5).

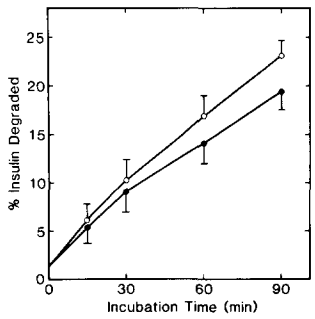

Figure 4. Effect of *N*-acetyl-Tyr ethyl ester on insulin degradation by rat adipocytes at 37°C. Adipocytes were incubated at 37°C with 0.3 ng/ml ^{125}I-insulin in the absence (○) or presence (●) of 1 mM *N*-acetyl-Tyr ethyl ester. At the times shown, the percent of ^{125}I-insulin degraded (trichloroacetic acid-soluble) was determined. The data shown represent the mean ± SEM of five separate experiments.

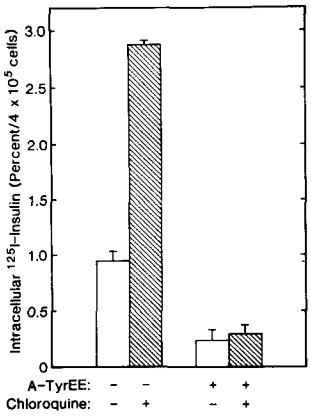

Figure 5. Effect of *N*-acetyl-Tyr ethyl ester on the chloroquine-mediated increase of intracellular ^{125}I-insulin. Adipocytes were incubated with 0.3 ng/ml of ^{125}I-insulin at 37°C with: no further additions, chloroquine (100 μM), *N*-acetyl-Tyr-ethyl ester (1 mM), or chloroquine (100 μM) plus *N*-acetyl-Tyr-ethyl ester (1 mM). After 1 h of incubation, nonextractable (intracellular) cell-associated radioactivity was determined in triplicate. Data shown represent mean ± SEM of three separate experiments.

Reversibility of the N-acetyl-Tyr ethyl ester–induced Inhibition of Insulin Endocytosis

To determine the reversibility of the inhibition of insulin internalization by *N*-acetyl-Tyr ethyl ester, adipocytes were first incubated for 30 min at 37°C with 1 mM *N*-acetyl-Tyr ethyl ester. Under these conditions insulin internalization was inhibited to 10% of that in control cells (Fig. 6). After removal of *N*-acetyl-Tyr ethyl ester by washing and resuspension of the cells in fresh buffer, the adipocytes gradually regained most of their ability to internalize insulin, such that 4 h after washing the treated adipocytes internalized insulin with ~70% of the efficiency of control cells (Fig. 6).

Structural Requirements for Inhibition of Insulin Endocytosis by N-acetyl-Tyr ethyl ester

It was of importance to determine whether the full structural complement of the chymotrypsin substrate analogue, *N*-acetyl-Tyr ethyl ester, is required for its inhibition of insulin internalization or whether fragments of the molecule have any effect. Therefore, the effects on insulin binding and internalization of fragments of *N*-acetyl-Tyr ethyl ester (*N*-acetyl-Tyr, Tyr, and Tyr ethyl ester) were measured. *N*-acetyl-Tyr and Tyr are not substrates since they lack a hydrolyzable bond on the carboxyl group and since Tyr ethyl ester has a free unblocked amino group and is a weak substrate for chymotrypsin, an enzyme with predominantly endoprotease ac-

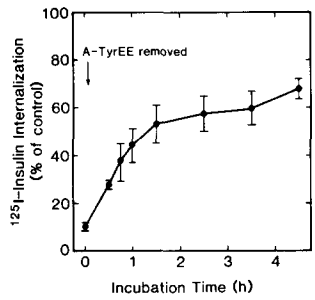

Figure 6. Reversibility of the effect of *N*-acetyl-Tyr ethyl ester. Adipocytes were incubated for 30 min at 37°C in the absence (control) or presence of 1 mM *N*-acetyl-Tyr-ethyl ester. The untreated and treated cells were then washed three times and resuspended in fresh buffer at 37°C. At the indicated times after washing, aliquots of cells were taken and incubated for 30 min at 37°C with 0.3 ng/ml ^{125}I-insulin, after which intracellular insulin was determined. At each time point intracellular insulin accumulation in cells previously exposed to *N*-acetyl-Tyr ethyl ester was compared with that found in the parallel control incubation.

Table II. Structural Requirements for Inhibition of Insulin Internalization by N-acetyl-Tyr ethyl ester

Addition	Cell-associated insulin (percent of control)		
	Total	Intracellular	Surface
N-Acetyl-Tyr ethyl Ester (1 mM)	96 ± 12	4 ± 2	145 ± 5
N-Acetyl-Tyr (5 mM)	103 ± 21	105 ± 18	105 ± 23
Tyrosine (5 mM)	101 ± 20	113 ± 17	93 ± 17
Tyr ethyl ester (2 mM)	89 ± 4	89 ± 8	90 ± 2

Adipocytes were incubated for 30 min at 37°C with 0.3 ng/ml of ^{125}I-insulin in the presence or absence of the various modified tyrosine compounds. Total, surface-bound, and intracellular insulin were then quantitated. Results shown represent the mean ± SEM of three separate experiments.

tivity (2, 20). Table II shows that while the intact *N*-acetyl-Tyr ethyl ester molecule inhibited insulin internalization to 4% of that in control cells, its fragment compounds did not significantly decrease internalization even when these compounds were used at concentrations higher than that of *N*-acetyl-Tyr ethyl ester.

Effects of Different Chymotrypsin Substrate Analogues on Insulin Internalization

To determine if other chymotrypsin substrate analogues besides *N*-acetyl-Tyr ethyl ester inhibit insulin internalization, the effects of four additional substrate compounds were studied. Table III shows that *N*-acetyl-Phe ethyl ester, *N*-acetyl-Trp ethyl ester, and benzoyl-Tyr ethyl ester, compounds which are all chymotrypsin substrate analogues (20), inhibited insulin internalization by 83–92%. This magnitude of inhibition was similar to that which occurred with *N*-acetyl-Tyr ethyl ester, although the concentrations required for maximal inhibition differed among the various agents. Benzoyl-Tyr amide, another chymotrypsin substrate analogue (20), was tested at 10 mM, the highest concentration at which it is soluble, and resulted in a 62% decrease in insulin internalization. Thus, chymotrypsin substrate analogues with Tyr, Phe, or Trp as the amino acid, *N*-acetyl or benzoyl groups as the amino-terminal blocking group, and ethyl ester or amide as the carboxyl-terminal blocking group were all effective inhibitors of internalization (Table III).

Effects of Chymotrypsin Substrate Analogues on the Internalization of Photoaffinity-labeled Insulin Receptors

The experiments described thus far examined the effects of the chymotrypsin substrate analogues on the internalization of surface-bound ^{125}I-insulin. If these effects are due to inhibition of receptor-mediated endocytosis, then internalization of the cell surface insulin receptors should also be inhibited. To verify this, the effects of the various substrate analogues on the internalization of photoaffinity-labeled cell surface insulin receptors were studied. After photoaffinity-labeling of the surface insulin receptors with ^{125}I-NAPA-DP-insulin at 14°C, endocytosis was initiated by warming the cells to 37°C. The subsequently internalized insulin–receptor complexes were distinguished from the complexes remaining on the cell surface by the sensitivity of the latter to tryptic digestion (6, 7). Fig. 7 shows the time course of internalization of the specifically photoaffinity-labeled nonreduced cell surface in-

Table III. Effects of Chymotrypsin Substrate Analogues on Insulin Binding and Internalization in Rat Adipocytes

Addition	Cell-associated insulin (percent of control)		
	Total	Intracellular	Surface
N-acetyl-Tyr ethyl ester (1 mM)	98 ± 12	4 ± 2	179 ± 14
N-acetyl-Phe ethyl ester (0.75 mM)	95 ± 10	8 ± 4	160 ± 2
N-acetyl-Trp ethyl ester (0.5 mM)	132 ± 14	9 ± 6	285 ± 71
Benzoyl-Tyr ethyl ester (0.2 mM)	96 ± 17	17 ± 6	166 ± 23
Benzoyl-Tyr amide (10 mM)	163 ± 23	38 ± 11	308 ± 53

Adipocytes were incubated for 30 min at 37°C with 0.3 ng/ml of ^{125}I-insulin in the presence or absence of the chymotrypsin substrate analogues. Total, surface-bound, and intracellular insulin were then quantitated. Results shown represent the mean ± SEM of three or four separate experiments.

sulin receptors (M_r 440,000). In the absence of *N*-acetyl-Tyr ethyl ester, progressive receptor internalization occurred as demonstrated by the time-dependent increase in the intensities of the trypsin-insensitive (intracellular) M_r 440,000 receptor bands (Fig. 7 *A*, lanes *1–5*). In contrast, in the presence of 1 mM *N*-acetyl-Tyr ethyl ester, the intensities of the trypsin-insensitive M_r 440,000 bands were markedly decreased (Fig. 7 *A*, lanes *6–10*), indicating inhibition of receptor internalization. Although not directly quantitated, it is also evident from the autoradiogram in Fig. 7 *A* that the M_r 140,000 receptor proteolytic fragments generated by trypsin from the labeled receptors remaining on the cell surface are more intense in the presence of *N*-acetyl-Tyr ethyl ester (lanes *6–10*) than in its absence (lanes *1–5*). This indicates that more of the labeled receptors were trapped on the cell surface by *N*-acetyl-Tyr ethyl ester. Direct quantitation of the internalized M_r 440,000 receptors (Fig. 7 *B*) demonstrates that *N*-acetyl-Tyr ethyl ester decreased the internalization of photoaffinity-labeled cell surface insulin receptors by ~90%.

Fig. 8 shows the effects of the various chymotrypsin substrate analogues on the endocytosis of photoaffinity-labeled insulin–receptor complexes. *N*-acetyl-Tyr ethyl ester, *N*-acetyl-Phe ethyl ester, *N*-acetyl-Trp ethyl ester, benzoyl-Tyr ethyl ester, and benzoyl-Tyr amide all inhibited the intracellular translocation of insulin–receptor complexes by ~70–90% in direct proportion to the ability of these agents to inhibit ^{125}I-insulin internalization as determined by the acid–barbital extraction technique (Table III).

Discussion

From the data presented in this study, several lines of evidence indicate that synthetic peptides that are specific substrate analogues of chymotrypsin-like enzymes inhibit the endocytotic uptake of insulin–receptor complexes into isolated rat adipocytes. First, of the various protease and transglutaminase inhibitors tested, only the chymotrypsin substrate analogues inhibited insulin internalization (Tables I and III). Secondly, as determined for the analogue that we studied in detail, *N*-acetyl-Tyr ethyl ester, inhibition of internalization required the full structural complement of a chymotrypsin substrate analogue (20), since fragments of the

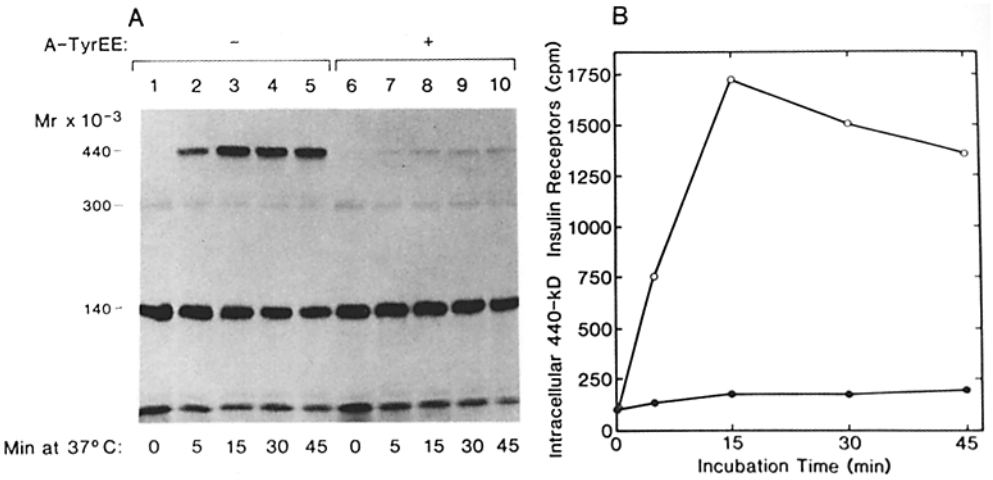

Figure 7. Effect of *N*-acetyl-Tyr ethyl ester on the time course of internalization of photoaffinity-labeled insulin receptors. (*A*) Insulin receptors on the surface of isolated rat adipocytes were photoaffinity-labeled with ^{125}I-NAPA-DP insulin at 14°C. The labeled cells were washed and then incubated at 37°C to initiate internalization. At the indicated times, the cells were trypsinized to degrade surface receptors, washed, and solubilized. The samples were then analyzed by electrophoresis and autoradiography under disulfide nonreducing conditions. These procedures were performed in the absence (lanes *1–5*) or presence (lanes *6–10*) of 1 mM *N*-acetyl-Tyr ethyl ester. (*B*) The areas of the dried gel containing the trypsin-insensitive (intracellular) M_r 440,000 receptor bands were cut out from the dried gel and the radioactivity measured in a gamma counter for the control (○) and 1 mM *N*-acetyl-Tyr ethyl ester–treated cells (●).

N-acetyl-Tyr ethyl ester molecule were all without effect on internalization (Table II). Thirdly, while decreasing intracellular insulin accumulation, the substrate analogues caused large increases in surface-bound insulin (Figs. 1 and 3; Table III), indicating sequestration of receptor-bound insulin on the cell surface. Additionally, *N*-acetyl-Tyr ethyl ester inhibited the chloroquine-mediated increase in intracellular insulin (Fig. 5), thus indicating that the substrate analogues prevented the delivery of surface-bound insulin to intracellular chloroquine-sensitive sites. Fourth, the studies with receptor photoaffinity labeling (Figs. 7 and 8) clearly demonstrate that the chymotrypsin substrate analogues inhibit internalization of covalent insulin–receptor complexes. Furthermore, the degree of this inhibition by the different substrate analogues (Fig. 8) closely parallels the degree to which they inhibited ^{125}I-insulin internalization as determined by the independent technique of acid–barbital extraction of surface-bound insulin (Table III). This direct correlation between inhibition of ligand and receptor internalization strongly indicates that the chymotrypsin substrate analogues inhibit receptor-mediated endocytosis.

The inhibition of internalization by the chymotrypsin substrate analogues is unlikely to be due to nonspecific cell toxicity, since cell viability, as ascertained by adipocyte counts, trypan blue exclusion, and nonspecific insulin binding, was unaltered in the presence of the agents. Additionally, the analogue-induced inhibition of internalization was largely reversible (Fig. 6). While the reason for the incomplete recovery of internalization is not clear, one possibility may be the persistent interaction of a residual amount of the inhibitor with adipocyte target proteins. Alternatively, after maximal inhibition, the complete recovery of internalization may depend on slow cellular processes, such as new synthesis of protein and/or lipid components of the internalization apparatus. The fact that several of the compounds that are not substrates for chymotrypsin (Table I), but are esters or amides like the chymotrypsin substrate analogues, did not inhibit insulin internalization is further evidence against nonspecific cell toxicity as the cause of the observed inhibition of internalization. Thus, of the ester compounds tested, the chymotrypsin substrate analogues, *N*-acetyl-Tyr ethyl ester, *N*-acetyl-Phe ethyl ester, and benzoyl-Tyr ethyl ester, all inhibited insulin internalization (Table III) while the nonsubstrate esters, tosyl-Arg methyl ester and benzoyl-Arg ethyl ester, failed to inhibit (Table I, group 2). Similarly, of the amide compounds tested, benzoyl-Tyr amide, a chymotrypsin substrate analogue (20), inhibited insulin internalization

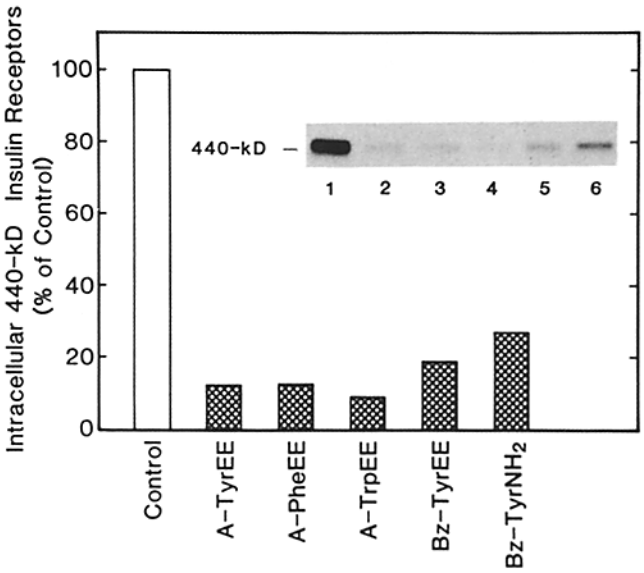

Figure 8. Effect of chymotrypsin substrate analogues on the intracellular accumulation of photolabeled insulin receptors. Isolated rat adipocytes were photolabeled at 14°C, washed, and further incubated in fresh buffer at 37°C with: no additions (control, lane *1*), 1 mM *N*-acetyl-Tyr ethyl ester (A-TyrEE, lane *2*); 0.75 mM *N*-acetyl-Phe ethyl ester (A-PheEE, lane *3*); 0.5 mM *N*-acetyl Trp ethyl ester (A-TrpEE, lane *4*); 0.2 mM benzoyl-Tyr ethyl ester (B_Z-TyrEE, lane *5*); and 10 mM benzoyl-Tyr amide (B_Z-Tyr NH_2, lane *6*). After 30 min the cells were trypsinized, solubilized, and the solubilized samples were then analyzed by SDS PAGE and autoradiography. The inset shows the autoradiogram of the trypsin-insensitive (intracellular) 440-kD insulin receptors and the bar graph shows quantitative profile of the radioactivity in the receptor bands as determined by gamma counting of areas of the gel containing the 440-kD bands.

(Table III) while several of the other amides that are not substrate analogues for chymotrypsin-like enzymes failed to inhibit insulin internalization (Table I, amides in group 1, 2, and 5). While these series of data argue against nonspecific cell toxicity being the reason for inhibition of internalization by the chymotrypsin substrate analogues, they do not rule out the possibility that these agents may also be influencing multiple cellular processes in addition to their inhibition of endocytosis.

The effect of *N*-acetyl-Tyr ethyl ester on surface-bound insulin is of interest in that surface binding was approximately doubled under conditions where insulin internalization was inhibited (Figs. 1 and 3). Inhibition of endocytosis in alveolar macrophages by phenylarsine oxide similarly causes large increases in the number of surface receptors for several ligands and this increase was attributed to intracellular receptor pools being recruited to the cell surface (22). The increase in surface insulin binding that we observed is unlikely to be the result of a similar fusion of intracellular insulin receptor pools with the cell surface for two reasons. Insulin binding was not increased when cells were first incubated with *N*-acetyl-Tyr ethyl ester at 37°C and then cooled to 12°C to determine cell surface receptor number and affinity (Fig. 2). In addition, the size of intracellular insulin receptor pool, ~10–15% of total cellular receptors in adipocytes (18, 29, 38), is not large enough to account for the twofold increase in surface binding observed in the presence of chymotrypsin substrate analogues. The increase in surface binding is also not likely to be the result of a direct effect of chymotrypsin substrate analogues on cell surface receptors, since binding was not increased at 12°C in the presence of *N*-acetyl-Tyr ethyl ester (Fig. 2). A more likely explanation is that most of the increase in surface binding is the result of inhibiting the insulin-induced redistribution of surface receptors to intracellular sites, a process that normally occurs in adipocytes (18, 29, 38). The additional possibility also exists that inhibition of endocytosis may be causing an indirect increase in apparent receptor affinity as predicted from mathematical modeling of receptor-mediated endocytosis (23).

The effects of *N*-acetyl-Tyr ethyl ester on insulin processing and degradation (Figs. 4 and 5) lend further support to the view that this compound inhibits receptor-mediated insulin internalization. Chloroquine, a lysosomotropic agent (28, 33), was without effect on intracellular insulin accumulation in *N*-acetyl-Tyr ethyl ester–treated cells, while it tripled intracellular insulin in nontreated cells (Fig. 5). This finding indicates that by inhibiting endocytosis, *N*-acetyl-Tyr ethyl ester prevented the delivery of extracellular insulin to intracellular chloroquine-sensitive sites and also demonstrates that *N*-acetyl-Tyr ethyl ester acts by a mechanism different from that of chloroquine, a drug that inhibits insulin processing intracellularly (28, 33). The 15–20% decrease in insulin degradation caused by *N*-acetyl-Tyr ethyl ester (Fig. 4) reflects that portion of overall degradation that is mediated by endocytosis and intracellular processing. In this regard, our finding is consistent with previous reports showing that overall insulin degradation in rat adipocyte suspensions occurs predominantly by nonreceptor-mediated processes (16).

The fact that several of the compounds that did not inhibit endocytosis caused significant increases in intracellular insulin (Table I) is of interest. As a group, this effect was most marked for the metalloendoprotease inhibitors, Cbz-Gly-Phe amide, Cbz-Gly-Leu amide, and phosphoramidon (Table I, group 1) agents that have previously been shown to inhibit exocytotic processes (3, 31). Since a portion of internalized insulin in adipocytes is processed by retroendocytosis (28), which involves an exocytotic step, it is possible that the effects of the metalloendoprotease substrates in causing an increase in the internalized pool of insulin (Table I) may be a consequence of their inhibiting the retroendocytosis of internalized insulin.

It is also of interest to note that the substrate analogues of trypsin-like proteases, tosyl-Arg methyl ester and benzoyl-Arg ethyl ester, which did not inhibit insulin internalization (Table I, group 2), have previously been shown to inhibit phosphorylation of the insulin receptor (39). In contrast, the chymotrypsin substrate analogue, *N*-acetyl-Phe ethyl ester, which is shown in the present study as inhibiting internalization of insulin (Table III) and insulin receptors (Fig. 8), had previously been found to have little effect on insulin receptor phosphorylation (39). This apparent divergence between effects on internalization of insulin–receptor complexes and phosphorylation of the receptors suggests that insulin receptor phosphorylation is not a necessary step for its internalization. This view is further supported by the recent demonstration that phosphorylation is not required for receptor-mediated internalization of growth hormone in IM-9 lymphocytes (1).

The transglutaminase inhibitors methylamine and dansylcadaverine did not block receptor-mediated uptake of insulin into adipocytes (Table I), confirming recent similar observations (14). Since transglutaminase inhibitors may prevent the endocytosis of other ligands into different cell types (12, 19), their lack of effect in adipocytes indicates that there are differences in the mechanisms of internalization of different ligands by different cell types. In this regard, the low density lipoprotein receptor is initially clustered in clathrin-coated pits on the cell surface of fibroblasts and is internalized in a constitutive manner, that is, the receptor is internalized in the presence or absence of ligand (17). In contrast, the insulin receptor on adipocytes is distributed more diffusely on the cell surface and a role for clathrin-coated pits remains to be demonstrated (37). Furthermore, the internalization of the insulin receptor in adipocytes is a partly inductive process, since the number of intracellular insulin receptors is increased in the presence of insulin (18, 29, 38). Whether the chymotrypsin substrate analogues exert their effects only on the endocytosis of insulin and insulin receptors in adipocytes because of such differences or whether they prove to be inhibitors of the endocytosis of other ligands in different cell types remains to be determined.

Although the basic mechanism by which the chymotrypsin substrate analogues inhibit internalization of insulin–receptor complexes is still unknown, certain general conclusions can be made based on the available data. Receptor-mediated endocytosis requires multiple steps (17, 43, 44), consisting of binding of the ligand to its receptor on the cell surface, translocation of the ligand–receptor complexes to areas of the plasma membrane that form endocytotic vesicles, pinching off of the endocytotic vesicle from the plasma membrane, and finally, movement of the vesicle to its intracellular destination(s). Clearly, the inhibition of internalization by the chymotrypsin substrate analogues was not a consequence of decreased binding since surface binding was markedly in-

creased in the presence of these agents (Figs. 1 and 3; Tables II and III). It is also not likely that the agents acted at a step distal to the pinching and closing off of the endocytotic vesicle from the cell surface, since insulin and its receptor would not be sensitive to the acid–barbital extraction procedure or to tryptic digestion after this step. Thus, the chymotrypsin substrate analogues most likely inhibit the step(s) leading to the formation of endocytotic vesicles from the plasma membrane, or any step proximal to complete vesicle formation but distal to the initial binding of insulin. The fact that the five compounds that inhibited the internalization of insulin (Table III) and insulin receptors (Fig. 8) are all chymotrypsin substrate analogues (20) suggests that their effect may be mediated by inhibition of an endogenous chymotrypsin-like protease whose activity is required in the endocytotic process, possibly in the initial formation or pinching off of endocytotic vesicles from the plasma membrane. However, until the presence of a chymotrypsin-like protease can be demonstrated in the plasma membrane of adipocytes, the possibility remains that the substrate analogues could be inhibiting internalization by other mechanism(s). Regardless of the molecular mechanism involved, chymotrypsin substrate analogues efficiently block internalization of insulin–receptor complexes and should prove useful in studying the functional consequences of insulin internalization and in further investigations into the possible role of endogenous chymotrypsin-like enzymes or related molecules in receptor-mediated endocytosis of insulin.

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