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PROTEOLYTIC ACTIVITY IN THE INSULIN RECEPTOR

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SUMMARY. When a highly purified preparation of rat liver insulin receptor is incubated with trypsin, the receptor develops hydrolytic activity towards Nα-benzoyl-L-arginine ethyl ester, Nα-p-tosyl-L-arginine methyl ester, and Nα-benzoyl-DL-arginine-p-nitroanilide, (compounds which are synthetic substrates of trypsin). The activity toward Nα-benzoyl-DL-arginine-p-nitroanilide is inhibited by soybean trypsin inhibitor but not by Nα-p-tosyl-L-lysil chloromethyl ketone. These data are consistent with the presence of proteolytic activity in the insulin receptor specific for the bonds whose carbonyl functions are provided by arginine but not by lysine. Furthermore we found that the esterase activity toward Nα-benzoyl-L-arginine ethyl ester in the presence of trypsin is enhanced by insulin, and that the concentration of insulin that produced the half maximum stimulation is of the same magnitude as the dissociation constant for the soluble receptor. These data suggest that the insulin receptor is a zymogen, activated by trypsin, and based on its specific activity, may be the protease which releases a peptide chemical mediator of intracellular action of insulin.

The mechanism of action of insulin remains unknown although insulin is one of the best studied proteins. There are many hypotheses to explain its multiple effects on the cell.

It has been reported that trypsin at low concentrations, mimics the actions of insulin (1-3), and that trypsin added to adipocyte plasma membranes causes the generation of a chemical mediator for insulin action (2-4).

It is apparent that the binding of insulin to its receptor enhances the release of a low molecular weight glycopeptide that mediates some of the

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The abbreviations used are: BAEE, Nα-benzoyl-L-arginine ethyl ester; TAME, Nα-p-tosyl-L-arginine methyl ester; BAPNA, Nα-benzyol-DL-arginine-p-nitroanilide; TLCK, Nα-p-tosyl-L-lysylchloromethyl ketone.

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intracellular actions of insulin (2-8). An intrinsic plasma membrane protease is involved in the release of this glycopeptide. This protease is inhibited by synthetic substrates with arginine esters but not by lysine or other amino acid esters (2). Therefore, it has been suggested (2) that the insulin-receptor interaction activates an endogenous arginine-specific protease that acts on an unspecified substrate to release the chemical mediator. The nature of this protease, the source of the chemical mediator, as well as the mechanism by which the binding of the hormone to its receptor leads to the release of this putative mediator is not yet known.

The insulin receptor is a tyrosine-specific protein kinase, which is stimulated by insulin (3,9), and it has been suggested that the release of the insulin mediator is stimulated by a phosphorylation reaction, similar to insulin-stimulated receptor tyrosine kinase phosphorylation (10), this phosphorylation is specifically inhibited by the arginine esters BAEE and TAME (11).

Clusters of insulin receptors are required for the hormone response (12-14), therefore, it is conceivable that the clusters are involved in the generation and transport of the chemical mediator, and that the protease responsible for the mediator release may be an activated receptor acting on other receptors in the cluster.

The present study was performed to investigate whether the insulin receptor contains any proteolytic activity, and we report here that the purified insulin receptor incubated with trypsin, hydrolyzes the arginine esters BAEE and TAME and the arginine amido compound BAPNA. These hydrolytic activities are inhibited by soybean trypsin inhibitor but not by TLCK (a specific inhibitor of proteases on the peptide bond whose carbonyl is provided by lysine). We discuss the possibility that the insulin receptor is the protease that releases the glycopeptide mediator, and a possible mechanism involving the tyrosine kinase activity, for triggering this proteolytic effect.

MATERIAL AND METHODS

Materials. Wistar rats (200-250g) were used. ^{125}I was obtained from Amersham. 1,3-diaminodipropylamine and 2,4,6 trinitrobenzensulfonic acid were purchased from Kodak; ethanolamine (scintillation grade) and succinic anhydride were from Fisher Scientific Company; dioxan from Merck; crystalline porcine insulin was a gift from Eli Lilly, Sephadex G10 and Sepharose 4B-CL were from Pharmacia Fine Chemicals. Diethylaminoethyl cellulose microgranular DE52 was from Whatman. All reagents for sodium dodecyl sulphate/polyacrylamide gel electrophoresis and comassie blue G-250 were from Bio-Rad. BAEE, BAPNA, TAME, TLCK, porcine trypsin type IX (15,700 and 16,500 BAEE units[1]) and all the remaining reagents were from Sigma Chemical Corp. The spectrophotometer was a Beckman model 35.

[1] One BAEE unit is defined as an increase of 0.001 O.D. per min. per mg of protein, at 253 nm, at pH 7.7 and 35° C, with BAEE as substrate in a reaction volume of 3.2 ml (1 cm light path).

Purification of insulin receptor from rat liver. Liver membranes were prepared by differential centrifugation (15) and solubilized with 2% Triton X-100; insulin receptor was purified by sequential chromatogragy on DEAE cellulose, insulin-agarose and concanavalin A-agarose (16). The [^{125}I]Insulin was prepared according to Posner et al.(17). Protein was measured by the procedure of Bradford (18) using bovine serum albumin as the standard.

Assay of esterase activity. The BAEE assay was performed as described by Schwert and Takenaka (19) (in a quartz microcell) with minor modifications: the mixture to be assayed described in table 1 and fig 1, was added to 500 ul of 1mM BAEE in 50 mM Tris-HCl pH 7.7; the activity was followed spectrophotometrically by recording the changes in absorbance at 253 nm of BAEE over 7.5 minutes and rates were estimated from the recorded slopes. Walsh (20) described the procedure for TAME, that we followed with modifications, and essentially is the BAEE assay described above but with TAME substituting for BAEE and a wave length of 247 nm to follow the activity.

Amidase assay. The rate of reaction was estimated from the slope of electronic recordings. The method of Erlanger et al. (21) was followed using BAPNA as substrate, in the volume and conditions of the precedent assays at a wave length of 410 nm.

RESULTS

Esterase activity could not be detected when mixtures of freshly prepared purified insulin receptor or the receptor plus insulin were assayed with BAEE as the substrate (Table 1). However, if the receptor mixture was first incubated with trypsin, a 2.5 fold increase above the initial activity of trypsin alone was obtained. When insulin was added to the above mixture, an additional

TABLE 1

ESTERASE ACTIVITY OF PURIFIED INSULIN RECEPTOR ON BAEE

Additions	Relative esterase activity			
Trypsin	100	±	12	(3)
Trypsin + Insulin	106	±	3	(3)
Insulin receptor	0			(3)
Insulin receptor + Insulin	0			(3)
Trypsin + Insulin receptor	252	±	52	(5)*
Trypsin + Insulin receptor + Insulin	388	±	60	(5)**

The proteins indicated were added successively to 500 μl of BAEE (1 mM in 50 mM Tris-HCl pH 7.7). After each addition, the spectrophotometric activity was followed for 7.5 min at 253 nm. The additions were: 33 ng of trypsin in 3 μl; 2 μg of insulin receptor in 100 μl; 3 μl of insulin to reach 1 μM final concentration. The results are the mean of the number of experiments (in parenthesis) ± S.D. The insulin receptor was obtained from three independent purifications. The actual values for insulin receptor + trypsin with the values corrected by volume and the basal trypsin activity subtracted were 287 ± 60 BAEE units, which increased to 542 ± 112 BAEE units in the presence of insulin.

* $p < 0.002$ with respect to trypsin.

** $p < 0.001$ with respect to trypsin + insulin receptor.

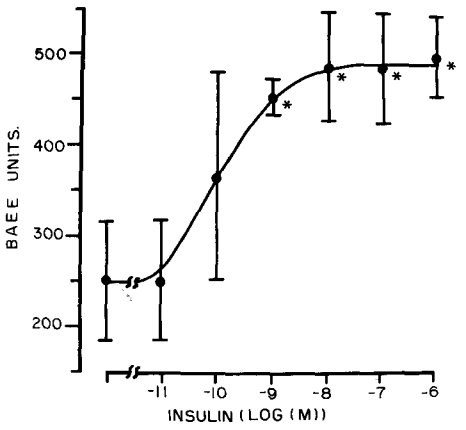

Fig. 1. Dose response of the effect of insulin on esterase activity of the mixture of trypsin + Insulin receptor. Trypsin (33 ng in 3 µl) were added to 500 µl of BAEE (1 mM in Tris-HCl pH 7.7). The trypsin activity was monitored spectrophotometrically by recording the changes in absorbance of BAEE at 253 nm for 7.5 minutes. Then 2 µg of insulin receptor in 100 µl were added and the recording continued for another 7.5 minutes. To the above mixture successive increasing doses of insulin were added, and the activity was recorded at the same time interval after each addition. Each point represents the mean ± S.D. of 5 experiments. The insulin receptor for the experiments shown was from 3 independent purifications. From all points the value of initial trypsin activity has been subtracted.

* $p < 0.001$ with respect to the mixture trypsin + insulin receptor, with no insulin.

increment to 3.9 fold greater than the initial activity with trypsin was achieved. If three week old preparations of the receptor were used, these increases in activity were not observed (data not shown). Insulin by itself (in the absence of the receptor) does not increase the activity of trypsin.

The BAEE esterase activity of trypsinized insulin receptor was determined as a function of insulin concentration (Fig.1). Esterase activity increased with increasing insulin concentrations up to 10^{-8}M; a half maximal response was at 1.2×10^{-10}M.

Purified insulin receptor also increases the esterase activity of trypsin with TAME as a substrate; the increase was rather modest in comparison to the increment described with BAEE. Trypsin (33 ng/500 µl of 1 mM TAME) provided 0.0325 ± 0.001 (S.D.) O.D. after ten minutes; in the presence of 2 µg of insulin receptor this value increased to 0.0485 ± 0.001 ($n=3$, $p < 0.001$).

TLCK completely abolished the trypsin response in the BAEE esterase assay, but only in the absence of insulin receptor. On the other hand, in the presence of the insulin receptor, 284 units of BAEE esterase activity was observed. This value is very close to that obtained by subtracting the trypsin activity in the absence of receptor in the experiment shown in table 1 (see legend in table). We also used BAPNA as a substrate which overcomes amplitude noise encountered in BAEE esterase assay in the presence of TLCK. With BAPNA

TABLE 2

EFFECT OF TLCK AND SOYBEAN TRYPSIN INHIBITOR ON AMIDASE ACTIVITY OF PURIFIED INSULIN RECEPTOR

ADDITIONS	O.D. (10 min)
Trypsin	0.0076 ± 0.0001
Trypsin + TLCK	0.0018 ± 0.0001
Trypsin + Insulin receptor + TLCK	0.0065 ± 0.0007
Trypsin + Insulin receptor + TLCK + Soybean trypsin inhibitor	0

Either 33 ng of trypsin in 103 μl (Trypsin) or the same amount of trypsin treated for 1 minute with 10 μl of TLCK, final concentration 1 mM (Trypsin + TLCK) or 2 μg of insulin receptor in 100 μl incubated with the above quantity of trypsin for one minute and then treated with TLCK (Trypsin + Insulin receptor + TLCK) were added to 500 μl of BAPNA (1 mM in 50 mM Tris-HCl pH 7.7) The activity was monitored by recording spectrophotometrically the changes in absorbance at 410 nm for 7.5 minutes. To the last group, after the recording, 10 μg of soybean trypsin inhibitor were added. The results are the mean ± S.D. from 3 experiments.

as substrate, TLCK inhibits 78% of trypsin activity (Table 2), however, activity of trypsin plus insulin receptor was not affected. This amidase activity was completely inhibited by soybean trypsin inhibitor. When the mixture of trypsin plus insulin receptor was treated with TLCK, further addition of insulin did not increase the remaining activity of the mixture, either with BAPNA or with BAEE, as substrates (data not shown).

DISCUSSION

The increase in BAEE esterase activity when freshly prepared insulin receptor is added to trypsin, and the lack of this response with an old preparation of insulin receptor, strongly suggests that trypsin is acting on the receptor, and supports the idea that the insulin receptor may be a protease. Furthermore, the coincidence of the esterase activity in the experiments shown in Table 1 and the activity obtained when TLCK inhibits trypsin in the BAEE assay, seems to suggest that the increase in the esterase activity is due to an activation of protease activity in the insulin receptor.

A direct effect of insulin on the esterase activity of the insulin receptor is suggested by the failure of insulin to modify the esterase activity of trypsin unless insulin receptor was present. Also noteworthy is the coincidence between the concentration of insulin that produces a half maximal stimulatory activity in the BAEE assay and the dissociation constant value reported for the soluble insulin receptor (1.3×10^{-10}M, 22).

According to Seals and Czech (2), we may expect that if the insulin receptor is the protease which releases the chemical mediator, the protease

should have hydrolytic activity towards arginine substrates and this activity should not be inhibited by TLCK, but should be inhibited by the soybean trypsin inhibitor. Our results completely support this idea, since we found activity of the insulin receptor towards BAEE, BAPNA and TAME. The insulin receptor activity with BAEE or BAPNA as substrates was not inhibited by TLCK, and disappeared when soybean trypsin inhibitor was added.

That a protective effect of the receptor on trypsin autolysis might have significantly influenced our results seems to us unlikely. In the present study, the activity produced by trypsin was linear with time up to the point when the receptor was added and, in other instances, it also retained its linearity well beyond that point in time. These observations suggest that, under the conditions of our experiments, significant trypsin autolysis did not occur. Moreover, protection against even major trypsin autolysis would at best be expected to restore, not increase the slope produced by trypsin alone. Therefore, modulated trypsin autolysis could not account for the sharply increased curves generated by the addition of receptor and insulin.

BAEE and TAME are inhibitors of the insulin receptor autophosphorylation, and the esters of lysine, alanine or phenylalanine are much less efficient in inhibiting that process (11). The coincident esterase activity of insulin receptor towards BAEE and TAME, with the same abilities of the latter to inhibit the autophosphorylation process, suggests that a relationship may exist between these effects. If the esterase activity of the receptor versus trypsin activity is expressed on a molar basis, we find that the insulin receptor is 4.3 times less active with BAEE and 8.4 times less active with TAME. This represents 1.95 times less active with TAME than with BAEE, a value very close to the ratio of 2.5 found for the inhibition of the insulin receptor autophosphorylation by both substrates at 1.0 mM (11).

Since we did not detect any esterase activity in the receptor, unless preincubated with trypsin, and since the phosphorylation of the receptor is less sensitive to inhibition by TAME, we propose that the autophosphorylation of the receptor occurs first; this in turn activates the proteolytic activity in the receptor which then releases the peptide mediator, either from the receptor itself or from another protein. The mediator may in turn increase the kinase activity of the receptor. Such a mechanism may explain the ability of trypsin to increase the phosphorylation of the insulin receptor (23), the release of the peptide mediator (2, 24, 25), and the stimulated release of the mediator by a tyrosine kinase phosphorylation (10). Also the increased tyrosine kinase activity of the receptor may phosphorylate other plasma membrane proteins, like G protein(s) (26, 27), which in turn may activate other protein(s) like the proposed phospholipase C (28, 29) which releases from membrane several other proteins, linked to phosphatidylinositol by a glycan anchor (30), and all leading to pleiotropic effects.

The insulin receptor is reported to be more sensitive to degradation by trypsin in the presence of insulin (31). We found that once tryspin is inhibited by TLCK no increment of esterase activity of the insulin receptor could be observed. This result suggests that the interaction of insulin with its receptor induces a conformational change that makes a fragment accessible. When the fragment is removed the esterase activity increases. Another possibility is that the interaction of the hormone with inactive receptors exposes particular segments, the scission of these segments induces the proteolytic activity of the cluster.

In conclusion, our results provide the first direct evidence that the insulin receptor is a zymogen, and therefore may be the protease which releases a mediator for insulin action.

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