Insulin-degrading Activity of Plasma Membranes from Rat Skeletal Muscle
Its Isolation, Characterization, and Biologic Significance

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SUMMARY

We isolated the plasma membrane from rat skeletal muscle without using drastic procedures such as extraction by salts or other agents. As a result of the purity of our preparations, evaluated by enzymatic markers, lipid composition, ^{125}I-insulin specific binding, and morphologic examinations, we were able to use the plasma membrane to study insulin degradation.

Isolated plasma membranes were capable of degrading insulin, but 95% of total degrading activity was found in the cytosol fraction. The membranes proteolytically degraded the hormone with a high degree of specificity and a pH optimum of 7.0. The extent of degradation depended on time, temperature, and protein concentration. The apparent K_m for insulin was 1.7 $\times 10^{-7}$ M. N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB) markedly inhibited insulin degradation by membranes, whereas glutathione (GSH) and dithiothreitol (DTT) were stimulatory. These characteristics of insulin-degrading activity in the plasma membrane (membrane-IDE) were similar to those of the partially purified insulin-degrading enzyme from the cytosol fraction (cytosol-IDE).

To clarify the biologic significance of IDE, we examined the cytosol- and membrane-IDE activities under various conditions in rats grouped as follows: fed, fasted for 1 day, fasted for 3 days, refed, refed + actinomycin D, diabetic, and hyperinsulinemic (insulinoma). A positive correlation with a high coefficient ($r = 0.674$, $P < 0.001$) was demonstrated between cytosol-IDE and the plasma insulin concentration but not between membrane-IDE and insulin levels. While actinomycin D had no effect on membrane-IDE levels, it abolished the insulin-mediated rise in cytosol-IDE activity, suggesting that the latter was dependent on RNA synthesis.

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Extensive studies on insulin metabolism have shown that the hormone is rapidly cleared from the circulation, probably through the action of enzymes with a high degree of specificity. In most tissues of mammals, the existence of insulin-degrading enzymes has been demonstrated, and many reports concerning the purification and characterization of these enzymes have appeared.[1–4]

Previously, we reported preliminary results of the partial purification and characterization of an insulin-degrading enzyme (cytosol-IDE) extracted from the cytosol fraction of rat skeletal muscle by ammonium sulfate precipitation and by Sephadex G-200 column chromatography. These studies showed that cytosol-IDE proteolytically degraded insulin with a high degree of specificity. The optimum pH was 7.0 and the K_m for insulin in the kinetic studies was 1.2×10^{-7} M, as estimated from Lineweaver-Burk plots. The enzyme activity was activated by Ca^{++}, Mg^{++}, GSH, or DTT and was inhibited by Cu^{++}, Zn^{++}, NEM, or PCMB.[5–7]

On the other hand, with the advance of studies on insulin receptors, it has been recognized that plasma membranes are also capable of degrading insulin. Many investigations concern the specificity of insulin-degrading systems associated with cell membranes[8,9] and the interaction of insulin binding and degradation.[10–12]

Skeletal muscle is clearly a major target organ of insulin, but previous studies of insulin binding to this tissue were not successful.[13,14] This was a result of the unavailability of an adequate plasma membrane preparation. Recently, methods were described for obtaining plasma membranes from skeletal muscle[15] without subjecting membrane proteins to the denaturing conditions of high salt concentrations or extraction at 37 °C in the presence of alkali. By introducing minor modifications of these subcellular separation techniques, we were able to isolate the plasma membrane from rat skeletal muscle. Furthermore, by evaluating the purity of plasma membranes by enzymatic markers, lipid composition, ^{125}I-insulin specific binding, and morphologic examinations, we obtained respectable results.

In the present investigation, we isolated the plasma membrane from rat skeletal muscle and compared the insulin-degrading system in this fraction (membrane-IDE)

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with that in the cytosol (cytosol-IDE). Furthermore, to clarify the biologic significance of the enzyme(s) localized in the intracellular site and on the cell's surface, we measured cytosol-IDE and membrane-IDE activities in muscle tissue from rats of different nutritional and hormonal status.

MATERIALS AND METHODS

Materials. ^{125}I-labeled porcine insulin (specific activity, 150 to 200 μCi/μg) was purchased from Dainabott RI Laboratories, Tokyo. Pork monocomponent insulin, pork proinsulin, pork A-chain, pork B-chain, and highly purified pork glucagon were obtained from Novo Research Institute, Copenhagen. Pork C-peptide was the product of Daiichi Radioisotope Laboratories, Tokyo. Human growth hormone and rat TSH were kindly supplied by the National Institutes of Health, Bethesda, Md. Human gamma globulin, bacitracin, cytochrome C (horse heart), Na-ATP, and 5'-AMP were products of Schwarz/Mann, Orangeburg, N.Y. Bovine serum albumin was obtained from Sigma Chemical, St. Louis, Mo. Fluorescamine was purchased from Hoffman-LaRoche Diagnostics, Nutley, N.J. Sephadex G-50 and G-200 were manufactured by Pharmacia Fine Chemicals, Piscataway, N.J. Streptozotocin was purchased from Upjohn Overseas, Kalamazoo, Mich. Actinomycin D was purchased from Japan Merk-Banyu, Tokyo. Other chemicals were of reagent grade.

Animals. Male Wistar rats were categorized as fed, fasted for 1 day, fasted for 3 days, refed, refed + actinomycin D, diabetic, and hyperinsulinemic groups. Each rat (except fasted rats) was fed standard rat chow ad libitum until 1 h before sacrifice. Animals were killed by decapitation, and blood was collected for the determination of blood glucose and plasma insulin concentrations. Refed rats were fed for 72 h after fasting for 72 h. In refed + actinomycin D rats, the agent, diluted with sodium chloride, was injected once a day intraperitoneally for 3 days in a dose of 4 μg/100 g body wt. The first injection was done 30 min before refeeding. For the production of streptozotocin (STZ) diabetes, STZ (65 mg/kg body wt in 0.02 M sodium citrate buffer, pH 3.8) was administered intravenously to rats fasted for about 24 h. Two weeks later, diabetic rats were selected by testing for glycosuria with Tes-Tape (Lilly-Shionogi Pharmaceutical). Hyperinsulinemic animals received STZ injection about one year previously and were diagnosed by blood glucose, plasma insulin, and histologic examination.

Preparation of plasma membranes and cytosol-IDE from skeletal muscle. Preparation of the membrane and the cytosol fraction is schematically depicted in Figure 1. Animals were killed by decapitation, hind-leg muscles were removed, trimmed of fat and connective tissue, minced, and then placed in 8% (wt/vol) sucrose (5 ml/g wet tissue) and homogenized with a Potter-Elvehjem homogenizer (five passes of the pestle). The homogenate was filtered through Kidwai's assembly.[16] The filtration device, which consists of five different mesh sizes of wire cloths (16–80 mesh), was placed on a Büchner funnel with an acrylic cylinder for a support and the Büchner flask was connected to a vacuum line. The filtrate was centrifuged at 100,000 *g* for 60 min, and the supernatant was used as the cytosol fraction. The cytosol-IDE was partially purified from this cytosol fraction by 40 to 70% ammonium sulfate precipitation and by Sephadex G-200 column chromatography, as previously

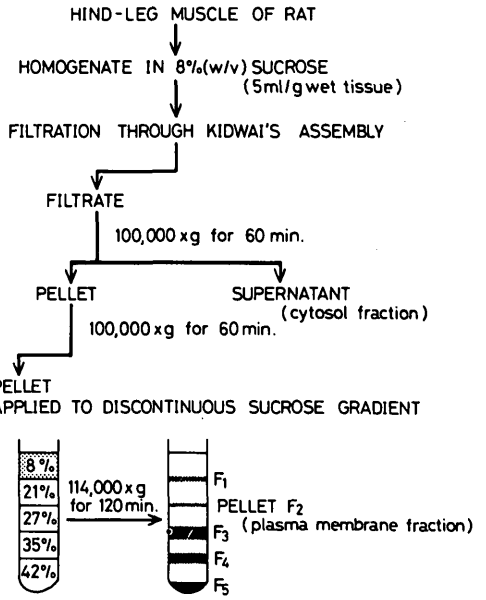


FIGURE 1. Procedure for isolation of plasma membrane and cytosol fractions from rat skeletal muscle.

reported.[7] On Sephadex G-200 column chromatography, peak insulin-degrading activity was eluted at the front shoulder of the protein peak and then cytosol-IDE activity was determined in five pooled fractions of peak enzymatic activity. The resulting pellet was washed by homogenizing it in 8% sucrose with a microhomogenizer and was then repelleted at 100,000 *g* for 60 min. The washed pellet was resuspended in 1 ml of 8% sucrose and was then applied to a discontinuous sucrose gradient (1 ml each of 42, 35, 27, and 21% sucrose) by use of a Beckman SW 25-5 type rotor at 114,000 *g* avg for 120 min. Five fractions, collected individually, were diluted to 8% sucrose with cold, distilled water and recentrifuged at 100,000 *g* for 60 min; the pellets (F1–F5) were stored at −20 °C until studied. All procedures were done at 4 °C. After the initial membrane characterization studies were completed (see below), only the F2 fraction was collected and used as membrane-IDE.

Insulin-degrading studies. The insulin-degrading activity was estimated by determining the quantity of trichloroacetic acid (TCA)–soluble radioactivity produced in incubation with ^{125}I-insulin. The incubation mixture contained each subcellular fraction, membrane-IDE or cytosol-IDE, 1×10^{-10} M ^{125}I-insulin, 0.1 M phosphate buffer (pH 7.0), and 0.15% bovine serum albumin (BSA). The total volume was 1.0 ml. After completion of incubation at 37 °C, 1 ml of 15% TCA was added. The percentage of insulin degraded was determined as the net amount of supernatant radioactivity. Experimental results were expressed as specific activity (mol insulin degraded/h/mg protein).

Insulin-binding studies. Binding studies were carried out by the method of Cuatrecasas[17] with minor modification. Briefly, plasma membranes (150 to 200 μg protein/ml) were incubated with 0.1 nM ^{125}I-insulin in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.1% bovine serum albumin and 1 mM N-ethylmaleimide. Incubations were performed for 48 h at 4 °C, then stopped by adding 0.1% (vol/vol) human gamma globulin in 0.1 M sodium phosphate buffer (pH 7.4) and 25% (wt/vol) polyethylene glycol (final concentration, 10%).

The microtubes were vibrated, centrifuged at 3000 rpm for 45 min, and the radioactivity of the total and the precipitate was counted. Nonspecific binding was defined as radioactivity associated with the pellet in the presence of 50 μg/ml native insulin. Specific binding was obtained by subtracting the nonspecific from the total radioactivity bound.

Other analytic procedures. Protein was determined by the Lowry method,[18] with bovine serum albumin used as standard. Na^+-K^+-ATPase and 5′-nucleotidase were assayed by the method described by Wallach[19] and Heppel and Hilmoe,[20] respectively, and liberated Pi was measured by the method of Fiske and Subbarrow.[21] Units of activity were defined as micromoles of liberated Pi/mg protein/60 min. Lactic dehydrogenase (LDH) was measured by the method of Babson-Phillips using nitro-tetrazolium blue.[22] Cytochrome-C oxidase was measured by the method of Cooperstein and Lazarow.[23] Cholesterol was measured according to Zlatkis-Zak[24] and phospholipid by the method of Chen et al.[25] Blood glucose was determined by the method of Hoffman using an AutoAnalyzer. Plasma insulin concentrations, measured by the modified two-antibody immunoassay of Morgan and Lazarow,[26] were expressed as immunoreactive insulin (IRI).

RESULTS

CHARACTERIZATION OF PLASMA MEMBRANE PREPARATION

Na^+-K^+-ATPase and 5′-nucleotidase activities were measured at each step of the purification as markers of the plasma membrane. Both enzymes were found to be concentrated in the F2 fraction (Na^+-K^+-ATPase, 10.92 ± 1.62; 5′-nucleotidase, 2.77 ± 0.51 μmol Pi/mg protein/h). The F2 fraction was enriched 15 times with Na^+-K^+-ATPase and 33.7 times with 5′-nucleotidase when compared with the filtrate. LDH activity, representing cytosol distribution, was located almost entirely in the cytosol fraction (22,013 ± 1932 U/mg protein), while its activity in the F2 fraction was 1422 ± 267 U/mg protein. Only 0.02% of the total LDH activity was contained in the plasma membrane. Cytochrome-C oxidase, as a mitochondrial marker, was present almost exclusively in the F4 fraction (26.85 ± 1.45 at 550 nm/mg protein/h), with slight activity demonstrable in the F2 fraction (1.84 ± 0.41 at 550 nm/mg protein/h). The yield of protein in this fraction was 0.8 ± 0.1 mg/10 g wet muscle, a recovery of 0.3% (Table 1).

It is well known that there is a difference in the lipid composition of various membranes.[27,28] Therefore, we determined total cholesterol and total phospholipid in each fraction to differentiate between plasma membrane and sarcoplasmic reticulum. In Table 2, the F2 fraction had a higher cholesterol content as compared with the F3 and F4 fractions. Phospholipid content was not much different. Thus, the cholesterol-phospholipid ratio was highest in the plasma membrane. This observation is consistent with others.[15,28]

Electron microscopic findings in the F2 fraction revealed

TABLE 1
Distribution of protein, marker enzymes, and insulin-degrading activity in filtrate, 100,000*g* supernatant (cytosol fraction), and F2 fraction (plasma membrane fraction)

	Filtrate	100,000*g* supernatant (cytosol fraction)	F_2 Fraction (plasma membrane)
Protein			
yield	330 ± 7 (6)	261 ± 10 (6)	0.8 ± 0.1 (8)
% recovery	100	79	0.3
Na^+-K^+-ATPase			
specific activity	0.73 ± 0.28 (5)	0.08 ± 0.02 (5)	10.92 ± 1.62 (5)
purity	1.0	0.1	15.0
% recovery	100	9	3.8
5′-Nucleotidase			
specific activity	0.08 ± 0.01 (4)	0.05 ± 0.03 (4)	2.77 ± 0.51 (4)
purity	1.0	0.6	33.7
% recovery	100	49	8.2
Lactic dehydrogenase			
specific activity	17,873 ± 978 (5)	22,013 ± 1932 (5)	1422 ± 267 (5)
purity	1.0	1.2	0.08
% recovery	100	97	0.02
Cytochrome-c oxidase			
specific activity	4.49 ± 0.56 (5)	0.12 ± 0.04 (5)	1.84 ± 0.41 (5)
purity	1.0	0.03	0.4
% recovery	100	2	0.1
Insulin-degrading activity			
specific activity	64 ± 5 (6)	78 ± 4 (6)	132 ± 9 (6)
purity	1.0	1.2	2.1
% recovery	100	95	0.5

Protein contents are expressed as milligrams per 10 g wet wt of the tissue. Specific activities of Na^+-K^+-ATPase and 5′-nucleotidase are expressed as μmoles released Pi/mg protein/h. LDH are expressed as a specific activity (Wróblewski U/mg protein) and cytochrome-c oxidase activity as a reduction in absorbance at 550 nm/mg protein/h. Insulin-degrading activities are expressed as 10^{-15} mol of insulin degraded per milligram protein per hour. Numbers in parentheses are the number of experiments performed on separate preparations, and values are expressed as mean ± SEM.

TABLE 2
Lipid composition of skeletal muscle subcellular fractions

Fractions	Total cholesterol (μmol/mg protein)	Total phospholipid (μmol/mg protein)	Cholesterol / Phospholipid (molar ratio)
F_2	0.34 ± 0.06 (4)	0.47 ± 0.01 (4)	0.73
F_3	0.13 ± 0.04 (4)	0.44 ± 0.01 (4)	0.31
F_4	0.11 ± 0.03 (4)	0.37 ± 0.04 (4)	0.29

Numbers in parentheses are the number of experiments performed on separate preparations. Values are expressed as mean ± SEM.

TABLE 3
Effect of muscle plasma membrane purity on the amount of ^{125}I-insulin specific binding

	^{125}I-Insulin specific binding mol × 10^{-16} / 200 μg protein	Specific act. of 5′-nucleotidase Pi μmol / mg protein/h	Relative ratios binding / 5′-nucleotidase
100,000 *g* PPT	1.2 ± 0.4 (6)	0.25 ± 0.04 (4)	1.00
F_2	19.1 ± 2.9 (6)	2.77 ± 0.51 (4)	1.52
F_3	5.7 ± 1.4 (5)	1.20 ± 0.19 (4)	1.05
F_4	1.7 ± 0.4 (5)	0.28 ± 0.06 (4)	1.31

Specific binding and enzyme activity were measured as described in MATERIALS AND METHODS. Results are expressed as mean ± SEM. Numbers in parentheses are the number of experiments.

vesicles of various shapes and sizes. It is likely that these vesicles are plasma membrane fragments, which were demonstrated in the literature cited by Kidwai et al.[15]

At 4 °C and with ^{125}I-insulin at 1.0×10^{-10} M, 19.1 ± 2.9 × 10^{-16} mol insulin was bound per 200 μg protein in the F2 fraction (Table 3). As compared with the 100,000 *g* pellet, this represented a 16-fold increase in binding activity. Nonspecific binding represented 25 to 35% of the total amount bound. Though insulin-binding activity was also seen in the F3 and F4 fractions, the ratio of specific insulin binding to the activity of 5′-nucleotidase, a marker of plasma membranes, was less than that observed in the F2 fraction.

ENZYMATIC CHARACTERIZATION OF MEMBRANE-IDE

Subcellular distribution of insulin-degrading activity. At 37 °C, with ^{125}I-insulin at 1×10^{-10} M, 132 ± 9 × 10^{-15} mol (mean ± SEM) insulin was degraded per hour per milligram protein in the plasma membrane (membrane-IDE) and 78 ± 4 × 10^{-15} mol insulin was degraded per milligram protein in the cytosol fraction. However, total insulin-degrading activity was 2112 × 10^{-15} mol/1 g muscle in the filtrate, 2035 × 10^{-15} mol/1 g muscle in the cytosol fraction, and 10.6 × 10^{-15} mol/1 g muscle in the plasma membrane. In other words, 95% of total insulin-degrading activity was located in the cytosol fraction and only about 0.5% in the plasma membrane (Table 1). These results are similar to those reported for insulin degradation in isolated fat cells by Hammond and Jarett[29] and in kidney by Duckworth.[30]

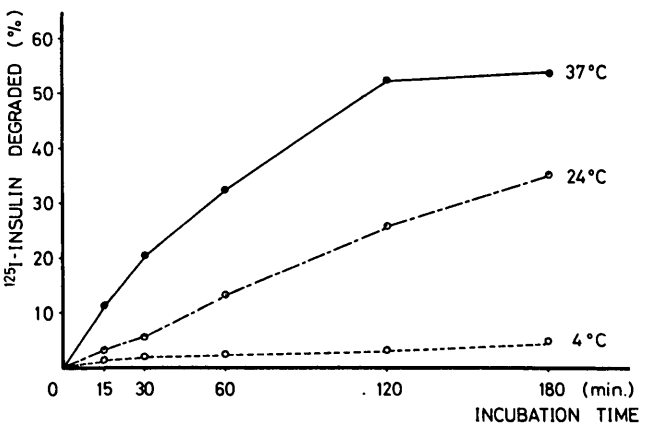
Dependence on membrane concentration, time, and temperature. When insulin was incubated with various concentrations of plasma membranes, a linear relationship was observed between hormone degradation and the membrane protein concentration within the range of 12 to 180 μg. As shown in Figure 2, when the incubation mixture contained 1×10^{-10} M ^{125}I-insulin and 120 μg protein plasma membrane, the degradation reached a plateau after 120 min at 37 °C. Degradation was reduced at 24 °C, and insulin was not degraded at 4 °C even after incubation for 180 min. Similar results were observed in liver plasma membranes[10] and isolated hepatocytes.[9] To determine the optimum pH of the system, membrane-IDE activity was studied over a pH range of 5.8 to 9.4. A relatively narrow pH-activity curve was obtained, with optimal activity occurring at pH 7.0 (data not shown).

Degradation as a function of substrate concentration. A kinetic analysis of insulin degradation by rat muscle plasma membrane is presented in Figure 3. The K_m for insulin was 1.7×10^{-7} M; this value is of the same order of magnitude as those reported by Baba et al. in the skeletal muscle[7] and by Freychet et al. in liver plasma membranes.[10]

Studies of various effectors on insulin degradation. To determine the specificity of membrane-IDE, inhibitory effects of native insulin, proinsulin, or various other peptides on degradation of ^{125}I-insulin were tested (Table 4A). In the presence of 100 nM native insulin, the degradation of ^{125}I-insulin was inhibited to 46% of the control, whereas only slight inhibition was noted with the other peptides tested. This suggests that membrane-IDE has marked specificity for the degradation of insulin. Table 4B shows the influence of various agents on the enzyme activity. Cu^{++}, Zn^{++}, EDTA, and Trasylol were relatively weak inhibitors of membrane-IDE while NEM and PCMB were strong inhibitors. GSH and DTT increased the enzyme activity. These observations suggest that a sulfhydryl group in the enzyme is necessary for enzymatic activity. Cytosol-IDE behaved in the same manner as membrane-IDE in these studies, except that proinsulin and B-chain were more inhibitory and Ca^{++} and Mg^{++} slightly activated the enzyme.

Mode of insulin degradation by membrane-IDE. The nature of the degradation products was studied by gel filtration on a Sephadex G-50 column that was calibrated with monocomponent insulin, A-chain, bacitracin (mol wt

FIGURE 2. Effect of time and temperature on insulin degradation by muscle plasma membranes. Muscle membranes (0.12 mg/ml) were incubated with ^{125}I-insulin (final concentration, 1×10^{-10} M) at 4 °C, 24 °C, and 37 °C, respectively. Insulin degradation was determined as described in MATERIALS AND METHODS.



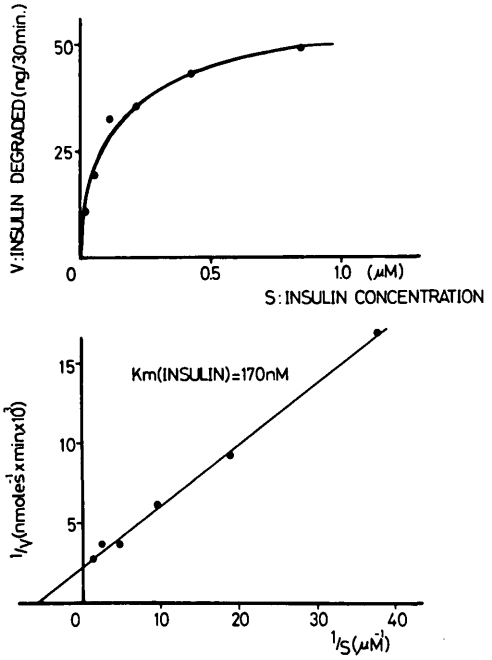


FIGURE 3. Degradation of insulin as a function of substrate concentration. Membranes (100 μg) were incubated for 30 min at 37 °C in phosphate buffer (pH 7.0) containing 0.15% BSA, 0.1 nM of ^{125}I-insulin, and various concentrations of unlabeled insulin (0.025–0.85 μM). (top) The velocity of the reaction, expressed as nanograms of insulin degraded during 30 min, was measured by the TCA method. (bottom) Lineweaver-Burk plot of the same data.

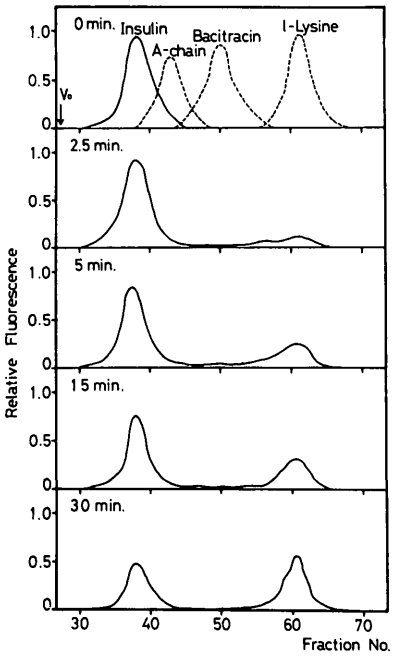


FIGURE 4. Elution patterns of insulin-degrading products. The upper panel (0 min) shows the calibration of the Sephadex G-50 column. In the other panels, elution profiles of relative fluorescence after 2.5, 5, 15, and 30 min of incubation are indicated; 0.65 ml of eluate was mixed with 1 ml of 0.1 M borate buffer (pH 8.6); 50 μl of fluorescamine in acetone (0.5 mg/ml) was added and immediately mixed. The fluorescence was measured on a Farrand spectrofluorometer with excitation wavelength at 390 nm and emission at 475 nm. Vo, void volume.

TABLE 4
Effect of various agents on insulin degradation by membrane-IDE or cytosol-IDE. (A) Substrate specificity of membrane-IDE or cytosol-IDE. (B) Influence of various agents on the membrane-IDE or cytosol-IDE activity.

	Substance	Concentration	Relative activity (%) Membrane-IDE	Relative activity (%) Cytosol-IDE
	None	—	100	100
(A)	MC-Insulin	100 nM	46	42
	Proinsulin	100 nM	79	68
	A-chain	100 nM	88	83
	B-chain	100 nM	82	71
	C-peptide	100 nM	90	98
	Glucagon	100 nM	94	95
	TSH	100 nM	92	86
	GH	100 nM	95	94
	Human γ-globulin	100 nM	98	97
(B)	Ca^{++}	1 mM	99	110
	Mg^{++}	1 mM	100	114
	Cu^{++}	1 mM	42	37
	Zn^{++}	1 mM	65	73
	NEM	1 mM	4	0
	PCMB	1 mM	0	0
	GSH	1 mM	134	128
	DTT	1 mM	165	151
	EDTA	1 mM	64	64
	Trasylol	10,000 KIU	74	67

Membrane-IDE (85 μg) or cytosol-IDE (60 μg) and 0.1 nM ^{125}I-insulin were incubated in the absence and in the presence of various agents at the concentrations indicated in the table. Relative activity is expressed as the percentage of activity of IDE incubated without additions.

1450), and L-lysine (Figure 4). Monocomponent insulin (75 μg/ml) was incubated with membrane-IDE (0.4 mg/ml protein) at 37 °C for 2.5, 5, 15, and 30 min, respectively. Incubations were terminated by centrifugation, and the supernatant was applied to a Sephadex G-50 column. The degradation products eluted from the column were measured by fluorescamine, which is known to be a highly specific and sensitive reagent for the detection of amino acids, peptides, and proteins.[31] The fluorescence peak first appeared at the insulin position. This had decreased after 2.5 min of incubation, at which time another peak began to appear at the position of L-lysine. With increasing time of incubation the insulin peak further decreased and the formation of small molecular weight materials increased. Though small amounts of intermediates appeared, an obvious peak was not detectable. At no time did products eluting at the A-chain position appear. This shows that membrane-IDE, like the cytosol enzyme, degrades insulin in a proteolytic manner. This mode of degradation of insulin is similar to that reported by Terris and Steiner, utilizing isolated hepatocytes[12] and perfused livers.[32]

CORRELATION BETWEEN CYTOSOL- OR MEMBRANE-IDE ACTIVITIES AND PLASMA INSULIN CONCENTRATIONS

In order to clarify the biologic significance of IDE, the correlation between the enzyme activities in the cytosol or the plasma membrane and plasma insulin concentrations was examined. The data are summarized in Table 5 and are shown graphically in Figure 5. In Figure 5(A) is shown the effect of fasting and subsequent refeeding on the levels of cytosol and membrane-IDE activity in rat skeletal muscle.

TABLE 5

Summary of the experimental data concerning the correlation between the IDE activity and plasma insulin levels. (A) Effect of fasting, refeeding, and actinomycin D. (B) Effect of diabetes and hyperinsulinemia.

Conditions	N	Body weight (g)	Blood glucose (mg/dl)	IRI (μU/ml)	IDE Activity (10^{-15} mol/mg protein)	
					Cytosol	Membrane
(A) Fed	16	277 ± 12	135 ± 5	31 ± 2	760 ± 21	132 ± 9
Fasted for 1 day	9	220 ± 17	88 ± 4	15 ± 2	713 ± 15	133 ± 3
Fasted for 3 days	9	194 ± 4	36 ± 6	10 ± 2	519 ± 42	130 ± 6
Refed	5	223 ± 21	158 ± 6	25 ± 2	651 ± 15	132 ± 18
Refed + Actinomycin D	8	206 ± 11	163 ± 4	23 ± 1	564 ± 25	135 ± 10
(B) Diabetic	14	173 ± 4	278 ± 37	11 ± 1	509 ± 19	
Hyperinsulinemic	4	530 ± 46	51 ± 8	134 ± 28	1067 ± 26	

Experimental conditions were as described in MATERIALS AND METHODS. Plasma insulin levels are expressed as IRI (μU/ml), cytosol- and membrane-IDE activity as specific activity (10^{-15} mol insulin degraded/h/mg protein). Each value is the mean ± SEM.

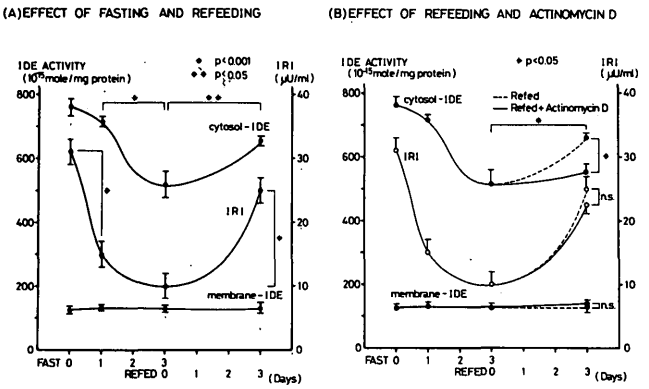
Cytosol-IDE activity and insulin levels decreased with increasing time of fasting. Both parameters rose again with refeeding. In contrast, no changes were seen in membrane-IDE levels under these conditions.

Figure 5(B) shows the effect of actinomycin D on the IDE activities. Plasma insulin concentrations were almost normal in actinomycin D–treated, refed rats, but the levels of cytosol-IDE activity were significantly lower compared with refed control animals. Membrane-IDE levels were unaffected by this agent.

In diabetic rats, cytosol-IDE activity and plasma insulin levels were significantly lower than in normal, fed rats ($P < 0.001$). On the other hand, the enzyme activity was significantly higher than normal in hyperinsulinemic rats ($P < 0.001$) (Table 5B).

Figure 6 shows the correlation between enzyme activity and plasma insulin levels. A positive correlation with high coefficient ($r = 0.674$, $P < 0.001$) was demonstrated between cytosol-IDE activity and plasma insulin levels. However, between membrane-IDE activity and insulin levels, no correlation was seen ($r = 0.112$).

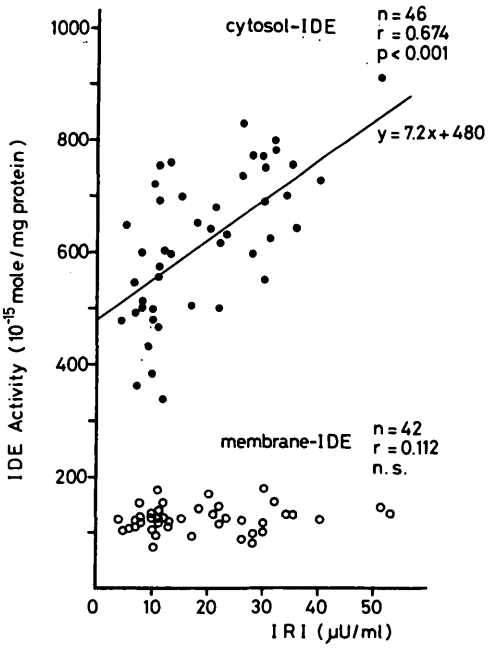
FIGURE 5. Effect of fasting, refeeding, and actinomycin D on the levels of cytosol and membrane-IDE activity in the rat skeletal muscle. Each value was as described in Table 5(A). The horizontal axis shows the time course (day), vertical axis shows the specific activity (10^{-15} mol/h/mg protein) of IDE and IRI (μU/ml). Cytosol- and membrane-IDE activities are indicated by closed circles on upper and lower curves, respectively. IRI is indicated by open circles. n.s.: not significant.



DISCUSSION

Despite many studies concerning insulin degradation in plasma membrane isolated from various tissues, skeletal muscle, which is clearly a major target organ of insulin, has not been examined because an adequate plasma membrane preparation is unavailable. The present method had the advantage of being mild and fast, and it yields a fairly pure plasma membrane preparation. The purity and percentage recovery of plasma membranes were similar to those reported by Kidwai[15] and Olefsky.[33] Furthermore, the extent of specific binding for insulin became higher in proportion to the specific activity of 5′-nucleotidase, a marker of the plasma membrane. Therefore, we have been able to use the plasma membrane isolated by this method to study insulin degradation and binding.

FIGURE 6. Correlation between IDE activity and plasma insulin level. Each value is obtained from the experimental rats (except actinomycin D group).



The insulin-degrading system associated with skeletal muscle plasma membranes (membrane-IDE) proteolytically degraded insulin with a high degree of specificity and a pH optimum of 7.0. The K_m for insulin was 1.7×10^{-7} M. As a result of the effects of various agents on insulin degradation, the active site of membrane-IDE seemed to contain a thiol residue. These enzymatic characteristics are similar to those of the soluble cellular enzyme (cytosol-IDE) partially purified from rat skeletal muscle.[6,7] Recently, cytosol-IDE was further purified from pig skeletal muscle by ultracentrifugation, ammonium sulfate precipitation, Bio-Gel P-200 filtration, DEAE-cellulose chromatography, and finally, ampholine electrofocusing. With pig skeletal muscle homogenate used as the starting material, a 411-fold purification was obtained. The enzyme protein was almost purified to a single component on a polyacrylamide gel electrophoresis.[34]

Many reports are currently available concerning the similarity between intracellular enzymes and cell-surface enzymes for insulin degradation. Hammond and Jarett[29] have demonstrated that insulin degradation in isolated fat cells occurs both in the cytosol and in the plasma membrane. Varandani[35] has also reported that glutathione-insulin transhydrogenase (GIT) exists in plasma membranes isolated from rat liver as well as in microsomes. However, they have not sufficiently evaluated the purity of plasma membranes isolated by their method. Therefore, the findings of markedly lower, degrading activity in the plasma membrane than in the intracellular elements have raised the question as to whether these activities are an intrinsic component of plasma membranes or whether they represent contamination with intracellular elements. We are unable to answer this question with certainty, but using a number of purification criteria, we have tried to avoid contamination with intracellular components, especially the cytosol fraction. As shown in Table 1, though membrane-IDE was enriched 2.1 times as compared with the filtrate, LDH as a cytosolic marker in the F2 fraction was 8% of the filtrate. Percent recovery of insulin-degrading activity was 25 times higher than that of LDH activity in the F2 fraction. Furthermore, when we measured LDH activity in the 100,000 *g* pellet and in the F1 fraction, it was 5876 ± 1900 and 10,362 ± 1802 U/mg protein, respectively. The F1 fraction was enriched in LDH activity 7.3 times that in the F2 fraction (10,362 ± 1802 vs 1422 ± 267 U/mg protein). Thus, cytosolic contamination was concentrated in the F1 fraction during a sucrose gradient. Accordingly, we believe that the membrane-bound enzyme is not a reflection of cytosolic contamination of the plasma membrane.

There have been several reports concerning the correlation of insulin-degrading activities and plasma insulin concentrations. Morgan and his co-workers[36] reported that the insulinase activity in rat liver homogenates is regulated by available insulin and that actinomycin D inhibits the renewal of insulinase activity. Consequently, they concluded that insulin is an inducer of insulinase biosynthesis. Varandani[37] has shown that the changes in the hepatic levels of GIT in the rat respond to changes in plasma insulin concentrations. However, there is, as yet, no report concerning the correlation between insulin-degrading activity in the plasma membrane and plasma insulin concentrations. The present experiments may help to clarify the question as to whether insulin is degraded in the intracellular site or on the plasma membrane. We have demonstrated that cytosol-IDE activity varies with changes in plasma insulin levels, while membrane-IDE is not influenced by insulin. If the cytosol-IDE is one of the factors that regulates the blood level of insulin, hormone degradation would have to occur in the cytoplasm. This process would require the entrance of insulin into cells. Recently, several groups have suggested that insulin enters the cytosol and then binds to the nucleus,[38] mitochondria,[39] and other structures.

Actinomycin D abolished the insulin-mediated increment of cytosol-IDE, while it had no effect on membrane-IDE levels. However, the mechanism whereby the latter enzyme becomes associated with the cell membrane is not known.

In conclusion, IDE plays an important role in insulin metabolism in skeletal muscle, and it is likely that both cytosol and membrane-IDE, acting either individually or in concert, constitute a physiologic mechanism by which the cellular response to insulin is terminated.

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