Insulin Receptors in Rabbit Erythrocyte

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Synopsis

The existence of insulin receptors in rabbit erythrocytes was studied by evaluating the specific binding of ^{125}I -insulin to erythrocyte membranes. The binding of ^{125}I -insulin was pH, time and temperature dependent. Maximal binding was achieved by incubation for 20 hr at 0°C . The optimum pH was 7.4. Treatment with cations and enzymes enhanced the specific binding except for with trypsin, the treatment which greatly reduced the binding. Unlabeled insulin over a wide range of concentrations competitively inhibited the binding of ^{125}I -insulin, while the binding was little affected by structurally unrelated hormones. Scatchard plot was represented as a concave curve. Binding sites of relatively high affinity ($K_1=0.9\times 10^9\text{ M}^{-1}$) and low capacity ($8.0\times 10^{13}/\text{g}$ protein) could be distinguished from those of lower affinity ($K_2=0.8\times 10^7\text{ M}^{-1}$) and higher capacity ($1.8\times 10^{15}/\text{g}$ protein). Hill's analysis and dissociation of ^{125}I -insulin from membranes demonstrated the characteristics of negative cooperation between receptor sites.

Both incorporation of $\text{H}_3^{32}\text{PO}_4$ to erythrocyte membranes and uptake of ^{45}Ca were significantly reduced by the addition of unlabeled insulin. Unlabeled insulin produced no effect on uptake of ^{45}Ca into trypsin-treated erythrocytes.

On the basis of these results, it was suggested that rabbit erythrocytes might possess biologically significant insulin receptors located on the cell membranes.

It has been suggested that receptor sites for insulin might exist on membranes of isolated fat and liver cells of mammals (Cuatrecasas *et al.*, 1971; Gammeltoft and Glieman, 1973) and that receptors might be initial sites of interaction of insulin molecules with cells. Gavin *et al.* (1972) demonstrated the specific binding of insulin to human circulating cells such as granulocytes and lymphocytes, and suggested that the binding assay of insulin might be applicable to the clinical diagnosis of diabetes mellitus. The binding experiment with erythrocyte membranes should be more important to clinical application than that

with other circulating cells or isolated cells, since erythrocytes are circulating cells easily available in large quantities and contain no demonstrable subcellular organelles, thus providing a supply of pure plasma membranes. At the present time, however, the binding of insulin to erythrocytes is less studied and it still remains unclear whether or not erythrocytes of mammals without nucleus may be insulin-sensitive cells.

The present work describes the characteristics and the biological significance of insulin binding to erythrocyte membranes, partly on the basis of the similarity of insulin receptor in the erythrocyte to that in the fat cell of rats.

Materials and Methods

Reagents: Crystalline pork insulin was a gift from Shimizu Pharmaceutical Co., Japan. ^{125}I -insulin (199–234 mCi/mg) was purchased from Dainabot Radioisotope Laboratory, Japan. γ - ^{32}P ATP (2.1 Ci/mmol), ^{32}P H_3PO_4 (carrier-free) (80 mCi/ml) and $^{45}\text{CaCl}_2$ (80 mCi/ml) were from the Radiochemical Center Amersham, England. PCS and NCS solubilizers were from Amersham Co., USA. Dibutylryl cyclic adenosine 3'; 5'-monophosphate (DiBucAMP), glucagon, thyrotropic hormone (TSH), adrenocorticotrophic hormone (ACTH) and neuraminidase (0.31 U/mg protein) were from Sigma Chemical Co., USA. Trypsin (Trypsin 1:250) was from Difco Laboratories, USA. Phospholipase C (Lot No. 610-8) was a product of P-L Biochemicals Inc., USA. Bovine serum albumin (BSA, fraction V) was obtained from the Armour Pharmaceutical Co., USA. Adenosine triphosphate (ATP) was from Yamasa Shoyu Co., Japan. Dinonylphthalate, dibutylphthalate, ethylene glycol bis(β -aminoethylether)-N, N, N', N'-teraacetic acid (EGTA), trichloroacetic acid (TCA) and other reagents were products of Wako Pure Chemical Co., Japan. All reagents used were of analytical grade, and the purity of ^{125}I -insulin used was 85–95% on Sephadex G-50 chromatography.

Preparation of rabbit erythrocyte membrane

The preparation of rabbit erythrocyte membranes was carried out according to a modification of the method described by Ginsberg *et al.* (1976).

The stock buffer used for hemolysis of cells consisted of 85 mM Tris, 30 mM NaCl, 10 mM glucose and 1 mg/ml BSA (buffer A) and was adjusted at pH 7.4 by the addition of HCl. The stock solution was diluted with 10 volumes of distilled water just before use (buffer A/10). The freshly heparinized blood was collected from three to four of male rabbits weighing 3 to 5 Kg to minimize the experimental source of error. Erythrocyte cells were prepared by centrifuging the blood at $400\times g$ for 10 min and washing cells three times with equal volumes of saline. Removal of buffy coat was repeated at every end of centrifugations. Hemolysis was initiated by rapidly mixing washed cells with three volumes of cold buffer A/10 supplemented with 2 mM MgCl_2 . Membranes were centrifuged at $1500\times g$ for 10 min under cooling and washed six to seven times with buffer A/10. The washed membranes were rinsed with buffer A/10 containing 0.2 mM MgCl_2 three times and collected by centrifugation at $3000\times g$ for 10 min. The membranous ghosts were frozen at -60°C until use for the binding assay.

Procedures for the binding of insulin to erythrocyte membranes

In the present experiments, we used the same buffer as Ginsberg *et al.* (1976) employed for the binding of insulin to turkey erythrocyte membranes. The buffer consisted of 150 mM Tris, 125 mM NaCl, 100 mM glucose and 2.5 mg/ml BSA (buffer B) and was adjusted at pH 7.4 by the addition of HCl. Membranes were first washed in buffer A prior to the binding assay. In a typical binding assay, 200 μl of the membrane suspension was incubated at 0°C in 100 μl of ^{125}I -insulin (0.011 $\mu\text{Ci}/100\text{ }\mu\text{l}$) and 50 μl of buffer B and parallel incubation was performed using 50 μl of buffer B containing 36 $\mu\text{g}/\text{ml}$ of unlabeled insulin. After a 20-hr incubation, 3 ml of cold buffer A was added to the incubation mixture and each tube was centrifuged at $50000\times g$ for 2 min at 4°C . The radioactivity in the pellet was counted in a γ scintillation counter (Shimazu Seisakusho Co., Japan). The total binding of ^{125}I -insulin referred to the counts in the absence of unlabeled insulin and the nonspecific binding in its presence. The specific binding was obtained by subtracting the nonspecific from the total binding. The displacement curve was obtained by varying the concentration of unlabeled insulin from 0 to 36 $\mu\text{g}/\text{ml}$. The concentration of membrane protein was determined by the method of Lowry *et al.* (1951). The concentration of membrane protein used in the present experiments ranged from 0.5 to 1.0 mg/ml.

Incorporation of ^{32}P H_3PO_4 into erythrocytes and fat cells

Fat cells employed were prepared from epididymal tissues of Sprague-Dawley rats, weighing 200 to 300 g, made to fast overnight by the collagenase digestion technique of Rodbell (1966). The incubation medium used was PO_4^{3-} -free Krebs-Henseleit bicarbonate buffer. The medium was prepared by substituting equivalent amounts of KCl for KH_2PO_4 and equilibrated with a mixed gas of O_2 and CO_2 (95:5) just before use (pH 7.4).

Five hundred μl of packed fat cells or erythrocytes were incubated in 500 μl of media containing ^{32}P - H_3PO_4 (10^5 cpm/10 μl) and appropriate additives for 10 min at 37°C under the condition of shaking with frequency of 60 strokes and amplitude of 5 cm. Using fat cells, a portion (200 μl) of the reaction mixture was first transferred to 2 ml of cold 5% TCA and centrifuged at $50000\times g$ for 5 min. One hundred μl of 1 N NaOH and subsequently 3 ml of cold 5% TCA were added to the pellet and each tube was centrifuged at $50000\times g$ for 5 min. The pellets were dissolved in 10 ml of PCS containing 10% NCS and their radioactivities were counted in a liquid scintillation counter (Packard 3385, Packard Instrument Co., USA). With erythrocytes, a portion (200 μl) of the reaction mixture was first hemolyzed

in 2 ml of cold buffer A/10 and membranes were collected by centrifugation at $50000\times g$ for 5 min. Labeled membranes were washed twice with buffer A/10 and subsequently with 1 N NaOH and cold 5% TCA as described in the case of fat cells and dissolved in 10 ml of PCS.

Determination of Na, K-ATPase and Mg-ATPase activities

Na, K-ATPase and Mg-ATPase activities were assayed according to a modification of the method of Lernmark *et al.* (1976). The reaction mixture contained 300 μ l of 30 mM histidine buffer (pH 7.4) containing 1.0 mM MgCl_2 , 0.1 mM EGTA, 130 mM NaCl, 20 mM KCl and 0.13 mM γ -[^{32}P]ATP (4000 cpm/nmol) and 100 μ l of the membrane suspension (approximately 300 μ g of protein). Incubation was carried out with or without ouabain at 1 mM of a final concentration at 37°C for 60 min. Ca-ATPase activity was assayed in 300 μ l of a final volume of incubates, which contained 200 μ l of 60 mM Tris buffer (pH 7.4) containing 2.5 mM MgCl_2 , 50 mM KCl, 0.5 mM CaCl_2 and 0.15 mM γ -[^{32}P]ATP (4000 cpm/nmol) and 100 μ l of the membrane suspension (approximately 300 μ g protein). Incubations were carried out with or without EGTA at 0.05 mM of a final concentration at 37°C for 60 min. In both of experiments examined, parallel incubations were performed in the presence of 100 $\mu\text{U/ml}$ of unlabeled insulin in order to assess the effect of insulin on the activity of those enzymes. Each reaction was terminated by adding 1 ml of 0.1 N HCl containing 4% (W/V) activated charcoal, 0.2 mg/ml BSA, 1 mM Na phosphate and 1 mM Na pyrophosphate. All samples were mixed and allowed to stand in ice for 30 min to adsorb unreacted ATP before being centrifuged at 4°C for 5 min by an Eppendorf Centrifuge (Eppendorf Centrifuge 5412, Eppendorf Co., W-Germany). A portion (500 μ l) of the supernatant fluid was pipetted into 10 ml of H_2O and the radioactivity was counted in a liquid scintillation counter. Mg-ATPase activity was estimated from the activity obtained in the presence of ouabain or EGTA in a series of two experiments. Na, K-ATPase and Ca-ATPase activities were estimated from the differences between activities obtained with and without ouabain, and between those obtained with and without EGTA, respectively.

Uptake of ^{45}Ca into erythrocytes and fat cells

The medium used was Krebs-Henseleit bicarbonate buffer and equilibrated against a mixed gas of O_2 and CO_2 (95:5) (pH 7.4) just before use.

Five hundred μ l of packed fat cells and erythrocytes were incubated in 500 μ l of media containing $^{45}\text{CaCl}_2$ (4×10^4 cpm/10 μ l) and appropriate additives at 37°C under the same condition of shaking as noted above. After a 10-min incubation, a portion

(200 μ l) of the reaction mixture was transferred to plastic microtubes (Beckman Instrument Inc., USA) containing 200 μ l of dinonylphthalate (fat cells) or dibutylphthalate (erythrocytes) and centrifuged at $400\times g$ for 2 min under cooling. A part of the microtube containing erythrocytes or fat cells was cut off and the radioactivity was counted in a liquid scintillation counter after those cells were dissolved with 10 ml of PCS containing 10% NCS and 1% of hydrogen peroxide solution.

Kinetic analyses for insulin binding

The number of insulin binding sites and affinity constants were estimated by Scatchard plot (Scatchard, 1949). Hill's constant was calculated according to Hill's equation (Hill, 1910). Calculation of the average affinity was performed according to the method of De Meyts and Roth (1975).

Calculation

The statistical comparison was performed by Student's *t* test.

Results

Effect of pH on insulin binding

Fig. 1 demonstrates that the optimum pH for specific insulin binding is 7.4. The binding slightly decreased at a range of pH lower or higher than 7.4.

Effect of temperature on insulin binding

Fig. 2 show changes in specific binding of ^{125}I -insulin to erythrocyte membranes (1.09 mg/ml protein) under four different temperatures of 0, 15, 26 and 37 °C with time. At 0 °C, specific binding increased progressively with time of incubation. By the end of 20 min the binding was 2.9 % of the total ^{125}I -insulin concentration and the first equilibrium state was achieved for 60 to 180 min. At 8 hr, the binding reached the second equilibrium state continuing up to 20 hr. After a 20-hr incubation, specific binding was 15.7 % of the total ^{125}I -insulin concentration. At 15 °C, association of ^{125}I -insulin to erythrocyte membranes proceeded in the initial stage rapidly and reached a plateau by the end of 60 min. After a 8-hr incubation, the

Fig. 1. Effect of pH on the specific binding of ¹²⁵I-insulin to rabbit erythrocyte membranes. The incubation was carried out according to the standard binding assay described in the text. Each point shows the mean value of triplicates.

binding decreased to 2.5 % of the total ¹²⁵I-insulin concentration. When the incubation temperature was raised to 26 or 37°C, the binding reached a transient plateau followed by rapid decline within 30 min after commencing the incubation. Nonspecific binding was unaffected by any condition of incubation temperatures tested, being 5 to 6 % of the total ¹²⁵I-insulin.

Effects of cations and enzymes on insulin binding

The effect of various cations on insulin binding was studied in buffer B supplemented with Ca²⁺, Mg²⁺, K⁺ and Na⁺ of 10 mM or higher concentrations. As shown in Table 1, the addition of Ca²⁺ and Mg²⁺ caused a significant enhancement of specific binding. On the other hand, K⁺ and Na⁺ of 10 mM concentration produced no effect on the binding, but at higher concentrations both cations significantly enhanced the binding.

Effects of enzyme treatments on insulin binding are also summarized in Table 1. The specific binding was significantly diminished to 13 % of the control by pretreatment of membranes with trypsin. Contrariwise, phospholipase C, neuraminidase

Fig. 2. Effect of temperature on the specific binding of ¹²⁵I-insulin to rabbit erythrocyte membranes. The incubation was carried out under several different temperatures according to the standard binding assay. Each point shows the mean value of triplicates.

and the combination of them produced only a slightly increased effect in either case.

Displacement curve and Scatchard analysis

As demonstrated in Fig. 3A, even low concentrations of unlabeled insulin displaced the binding of ^{125}I -insulin to erythrocyte membranes (0.82 mg/ml protein). At a concentration of 0.25 ng/ml, insulin displaced 5.7 % of the initial binding. The competitive reaction proceeded until the concentration of unlabeled insulin attained 36 $\mu\text{g/ml}$.

Scatchard analysis demonstrated the existence of two types of binding sites, one relatively high affinity ($0.9 \times 10^9 \text{ M}^{-1}$) low capacity ($80 \times 10^{13}/\text{g}$ protein) receptors and the other low affinity ($0.8 \times 10^7 \text{ M}^{-1}$) high capacity ($1.8 \times 10^{15}/\text{g}$ protein) receptors. The number of receptors per one erythrocyte cell was estimated to be 35 and 780 respectively, since 1 g of membrane protein was prepared from 2.3×10^{12} cells (Fig. 3B)

Dissociation of ^{125}I -insulin binding

As shown in Fig. 4, the rates of dissociation of ^{125}I -insulin in the absence of un-

Table 1. Effect of ions and enzyme treatments on specific insulin binding to rabbit erythrocyte membranes

Ion and enzyme	Conc.	Specific binding (per cent control) (n)
None		100.00 $\pm$ 10.20 (4)
Ca^{2+}	10 mM	164.59 $\pm$ 4.48a) (4)
Mg^{2+}	10 mM	141.83 $\pm$ 15.71b) (4)
Na^+	10 mM	99.71 $\pm$ 1.66 (4)
	2 M	246.05 $\pm$ 4.18a) (4)
K^+	10 mM	88.87 $\pm$ 4.27 (4)
	1 M	208.48 $\pm$ 3.83a) (4)
None		100.00 $\pm$ 3.54 (8)
Phospholipase C	100 $\mu\text{g/ml}$	116.38 $\pm$ 0.83 (3)
	200 $\mu\text{g/ml}$	87.44 $\pm$ 4.30 (3)
Neuraminidase	50 $\mu\text{g/ml}$	120.10 $\pm$ 8.17 (3)
Neuraminidase	50 $\mu\text{g/ml}$	120.85 $\pm$ 0.26 (3)
+Phospholipase C	100 $\mu\text{g/ml}$	
Neuraminidase	50 $\mu\text{g/ml}$	112.24 $\pm$ 3.90 (3)
+Phospholipase C	200 $\mu\text{g/ml}$	
None		100.00 $\pm$ 4.83 (4)
Trypsin	60 $\mu\text{g/ml}$	13.34 $\pm$ 6.01a) (4)

Procedures for treatment of membranes with enzymes are as follows; erythrocyte membranes (1.4 mg protein) were incubated in 1.5 ml of buffer B containing indicated concentrations of trypsin, neuraminidase and phospholipase C at 37°C. After 15 min (trypsin and neuraminidase), 45 min (phospholipase C) and 60 min (neuraminidase+phospholipase C) of incubation, each reaction was stopped by adding 3 ml of cold buffer B and centrifuging at $50000 \times g$ for 5 min. Each pellet was resuspended in 1.4 ml of buffer B. The binding of ^{125}I -insulin was performed according to the standard binding assay described in the text. Each value represents the mean ($\pm$ SEM) of n experiments noted in parentheses in the table. Statistical comparison was against control incubations. a) $p < 0.001$, b) $p < 0.05$.

Fig. 3A. Displacement of the binding of ^{125}I -insulin to rabbit erythrocyte membranes by unlabeled insulin and other hormones. The incubation was carried out according to the method described in the text. Small inset in this figure represents the saturation curve calculated from the displacement curve. Each point shows the mean value of triplicates. This experiment was repeated three times with results similar to those shown here.

Fig. 3B. Scatchard analysis for the binding of insulin to rabbit erythrocyte membranes. Scatchard plot was obtained from one of the experiments noted in Fig. 3A. Small inset in this figure represents Hill's plot, which was calculated using the mean values ($[B]$) and the maximum value ($[B_{\max}]$) of insulin binding obtained from Scatchard plot. Each point shows the mean value of triplicates.

labeled insulin were dependent on temperature and remarkably fast dissociation was observed at 37 °C. The addition of 68 μ g of unlabeled insulin accelerated dissociation of 125 I-insulin from membranes and 22 % of the initial binding was liberated after 10 min.

Negative cooperation

Fig. 5A demonstrates the presence of receptor-site interaction of negative cooperation type. Aliquots of 125 I-insulin-membrane complex were diluted with 100 volumes of cold buffer B or of cold buffer B containing 63 μ g of unlabeled insulin and dissociation of 125 I-insulin was measured at 37 °C. The addition of excess insulin to a 1:100 dilution significantly enhanced dissociation of 125 I-insulin from membranes by 22, 24 and 8 % of the control at 10, 40 and 90 min.

Fig. 5B shows the average affinity profile. The empty sites affinity, $\bar{K}_e$, was below $0.9 \times 10^8 \text{ M}^{-1}$. The average affinity, $\bar{K}$, began to decrease when 0.6 % of the total sites were occupied and afterwards continued to decrease until $\bar{K}$ reached $0.2 \times 10^8 \text{ M}^{-1}$. The average affinity reached a minimum ($\bar{K}_f$)

when only 15 to 20 % of receptors were occupied.

Incorporation of $[^{32}\text{P}]\text{H}_3\text{PO}_4$ and uptake of ^{45}Ca into erythrocytes and fat cells

Incorporation of ^{32}P into intact erythrocyte membranes was significantly reduced to 49 and 58 % of the control by the addition of insulin and DiBucAMP respectively. Both of these substances significantly inhibited uptake of ^{45}Ca into erythrocytes. Using fat cells, ^{32}P incorporation and ^{45}Ca uptake were diminished by insulin, while DiBucAMP reduced ^{32}P incorporation and contrariwise enhanced ^{45}Ca uptake remarkably. The inhibitory effect of insulin was more remarkable for fat cells than for erythrocytes (Table 2).

Treatment with trypsin significantly reduced both uptake of ^{45}Ca and specific binding of ^{125}I -insulin to erythrocytes and insulin produced no effect on ^{45}Ca uptake into trypsin-treated erythrocytes (Figs. 6A and 6C). Moreover, trypsin-treatment enhanced ^{45}Ca binding to erythrocyte membranes and insulin produced no significant effect on ^{45}Ca binding to either intact or trypsin-treated membranes (Fig. 6B).

Fig. 4. Effect of temperature on the dissociation of ^{125}I -insulin from rabbit erythrocyte membranes. After incubation for 20 hr at 0°C , the incubation was continued under temperatures of 0, 15 and 37°C . Triplicate aliquots were removed for investigating the dissociation of ^{125}I -insulin from membranes. Each point is represented as the percentage of the initial binding obtained at the end of 20-hr incubation. Small inset in this figure shows the dissociation of ^{125}I -insulin from membranes after the addition of $68\text{ }\mu\text{g}$ of native insulin to the incubation mixture of ^{125}I -insulin and membranes. Triplicate aliquots were removed at various intervals.

Fig. 5A. Dissociation of ^{125}I -insulin from rabbit erythrocyte membranes in a 100-fold dilution in the absence and in the presence of $63\text{ }\mu\text{g/ml}$ of unlabeled insulin. Each point shows the mean value ($\pm\text{SEM}$) of triplicates. Statistical comparison was against control incubations. a) $p < 0.01$, b) $p < 0.05$.

Fig. 5B. Average affinity profile of ^{125}I -insulin binding data shown in Fig. 3A. The average affinity, $\bar{K}$, calculated as $([\text{B}]/[\text{F}])/([\text{R}_0] - [\text{B}])$ was plotted as a function of $\log ([\text{B}]/[\text{R}_0])$; $\log ([\text{B}]/[\text{R}_0])$ is represented as $\log \bar{Y}$. $\bar{Y}$: fractional occupancy. $[\text{R}_0]$: total receptor sites calculated from Scatchard analysis. Each point was calculated using the mean value shown in Fig. 3A.

Table 2. Incorporation of [32 P]H $_3$ PO $_4$ and uptake of 45 Ca

	Erythrocyte		Fat cell	
	32 P incorporation (cpm/ml RBC)	45 Ca uptake (nmol/ml RBC)	32 P incorporation (cpm/100 mg TG)	45 Ca uptake (nmol/100 mg TG)
None	326227 $\pm$ 27324 (4)	134.93 $\pm$ 7.19 (4)	151751 $\pm$ 7348 (4)	5.81 $\pm$ 0.61 (4)
Insulin (100 μ U/ml)	159078 $\pm$ 16592 ^b (3)	107.41 $\pm$ 5.24 ^c (3)	20029 $\pm$ 832 ^a (4)	4.19 $\pm$ 0.22 ^d (4)
DiBucAMP (1 mM)	189395 $\pm$ 22855 ^b (4)	112.98 $\pm$ 10.22 (4)	19958 $\pm$ 262 ^a (4)	28.02 $\pm$ 0.68 ^a (4)

Each value of 32 P incorporation and 45 Ca uptake was obtained by incubating erythrocytes and fat cells with [32 P]H $_3$ PO $_4$ and 45 CaCl $_2$ for 10 min at 37°C. Each value is represented as the mean ($\pm$ SEM) of the number of experiments noted in parentheses in the table. RBC represents red blood cell and TG tri-glyceride, which was measured by the glycerol kit (Glycerol and Neutral Fat Test, Boehringer-Mannheim-Yamanouchi Co. Ltd., Japan). Statistical comparison was against control incubations.

a) $p < 0.001$, b) $p < 0.01$, c) $p < 0.02$, d) $p < 0.05$.

Fig. 6A. Uptake of 45 Ca in intact and trypsin-pretreated erythrocytes of rabbit. Treatment with trypsin at 120 μ g/ml of concentration was performed on erythrocytes in KHB buffer (pH 7.4) for 15 min at 37°C. The cells were washed with KHB buffer prior to experiments of 45 Ca uptake and the binding of 125 I-insulin. Effect of insulin on 45 Ca uptake was determined in the presence of 500 μ U/ml insulin and in its absence.

Fig. 6B. Binding of 45 Ca to intact and trypsin-pretreated membranes of rabbit erythrocytes. Treatment with trypsin at 120 μ g/ml of concentration was performed on erythrocyte membranes in KHB buffer for 15 min at 37°C. The binding of 45 Ca to membranes was carried out in KHB buffer containing 45 Ca (40000 cpm/10 μ l) for 10 min at 37°C. Effect of insulin on 45 Ca binding was determined in the presence of 1.2 mU/ml insulin and in its absence.

Fig. 6C. Binding of 125 I-insulin to intact and trypsin-pretreated erythrocytes. Incubations for the binding of 125 I-insulin (0.011 μ Ci/100 μ l) to intact and trypsin-pretreated erythrocytes were carried out at 0°C for 20 hr in the presence of 36 μ g/ml of native insulin and in its absence. Separation of erythrocytes from free 125 I-insulin was performed using dibutylphthalate. In Fig. 6, open columns represent intact cells or membranes and columns with shadow trypsin-pretreated cells or membranes. Each column represents the mean ($\pm$ SEM) of 6 to 7 experiments.

Na, K-ATPase, Ca-ATPase and Mg-ATPase activities

Na, K-ATPase and Ca-ATPase activities in erythrocyte membranes were 5.50 ± 0.39 nmol/mg protein/hr and 2.36 ± 0.09 nmol/mg protein/hr, respectively. The addition of $100 \mu\text{U/ml}$ insulin reduced Na, K-ATPase activity to 79 % of the control and Ca-ATPase activity to 56 %. The activity of Mg-ATPase was different between both of assay methods by which Na, K-ATPase and Ca-ATPase activities were determined. In the former, the activity of Mg-ATPase was estimated to be 53.71 ± 0.27 nmol/mg protein/hr and in the latter 17.66 ± 0.37 nmol/mg protein/hr. The addition of $100 \mu\text{U/ml}$ insulin reduced Mg-ATPase activity to 98 % of the control in the former and contrariwise enhanced it to 106 % of the control in the latter.

Discussion

Insulin binding was pH, time and temperature dependent. The pH range over which the binding occurred was rather broad for erythrocyte membranes. This is in contrast to fat cell membranes where the binding is known to occur sharply at pH 7.5 (Cuatrecasas, 1971 b). The specific binding of ^{125}I -insulin to erythrocyte membranes occurred rapidly even at a concentration of insulin as low as 40 pM , a concentration within the physiologic range for insulin in the peripheral blood. The binding curve showed a profile progressively rising by two steps at 0°C and at higher temperatures a transient plateau followed by rapid or gradual decline in the binding. Temperature dependency of the binding has been observed in other types of cells such as human monocytes (Bar *et al.*, 1976) and rat hepatocytes, in which the number of receptor sites decreased at 30°C (Kahn *et al.*, 1974). In view of the observations that the radioactivity in TCA-precipitates of supernatant when rabbit erythrocyte mem-

Fig. 7. Effect of insulin on ATPase activities in erythrocyte membranes Na, K-ATPase (A), Ca-ATPase (B) and Mg-ATPase activities in rabbit erythrocyte membranes were determined with the use of γ - ^{32}P ATP in the presence of $100 \mu\text{U/ml}$ insulin and in its absence according to the methods described in the text respectively. Each column shows the mean value ($\pm$ SEM) of 5 experiments.

branes were incubated in the medium containing ^{125}I -insulin at 37°C decreased with time but was not diminished by the presence of 1 mM N-ethylmaleimide (unpublished data), it seems possible that erythrocyte membranes may contain an insulin-degrading enzyme. A rapid decline in the binding at higher temperatures may be due to the degradation of either insulin or receptors, or both. In our previous study, we demonstrated that the binding of insulin to intact rabbit erythrocytes declined rapidly at 37°C but at 0 and 25°C and that the binding curve rose by two steps (Shinomiya *et al.*, 1975). These results were quite different from those of human erythrocytes (Gambhir *et al.*, 1978) and therefore more detailed, comparative studies will be required for the binding of insulin to both human and rabbit erythrocytes in order to clarify this conflict.

Divalent cations such as Ca^{2+} and Mg^{2+} showed a tendency to increase in the binding of insulin to membranes and high ionic media containing Na^+ or K^+ doubled the binding of insulin to rabbit erythrocyte membranes, as observed in fat and liver cell membranes (Cuatrecasas *et al.*, 1971; Cuatrecasas, 1971 b). However, Gavin *et al.* (1973) demonstrated no effects of divalent cations on insulin binding to cultured lymphocytes. The binding of insulin to rabbit erythrocyte membranes is quite similar to that to turkey erythrocyte membranes, human erythrocytes and human placental membranes, in view of the effects of cations (Ginsberg *et al.*, 1976; Gambhir *et al.*, 1978; Ponser, 1974).

A decrease in insulin binding by trypsin treatment coincides well with the result from fat cells (Kono, 1969), suggesting that insulin receptor may be a protein. Phospholipase C and neuraminidase produced a small effect on the binding of insulin to erythrocyte membranes of rabbits. This has been observed in human placenta membranes and was in contrast to fat cell

membranes where phospholipase C remarkably enhanced the binding (Cuatrecasas, 1971 a).

Both high and low affinity binding sites could be distinguished by Scatchard analysis. The number of receptor sites on erythrocyte membranes varies from species to species. The binding sites of insulin on rabbit erythrocyte membranes were estimated to be approximately 800 per one cell. Those of human and turkey erythrocytes per one cell were reported to be 2000 and 3000, respectively (Gambhir *et al.*, 1978; Ginsberg *et al.*, 1977). It is therefore obvious that the binding sites of insulin on erythrocytes are remarkably less than those on liver and fat cells of rats (Kahn *et al.*, 1974; Hammond *et al.*, 1972). Moreover, Scatchard analysis demonstrated two types of binding sites with low and high affinities, while the study of dissociation of ^{125}I -insulin suggested that insulin receptors on rabbit erythrocytes might be of negative cooperation type. This property of receptors was supported from the observations that Hill's constant was lower than one unit, 0.7477 and the average affinity fell rapidly when 0.6% of the total binding sites were occupied. This has been observed in human and turkey erythrocytes.

Erythrocytes, like many other types of cells, contain a very active Ca^{2+} pump which keeps intracellular free Ca^{2+} concentrations at the micromolar range. It is interesting to note that in rabbit erythrocytes insulin diminished both the activity of Ca-ATPase and ^{45}Ca uptake. These findings suggest that insulin may cause a redistribution of Ca^{2+} between the cell membrane and cytoplasmic fluid so as to reduce the activity of Ca-ATPase. The activation of Ca-ATPase has been known to be associated with the increase in the binding of Ca^{2+} to membranes (Ca^{2+} -binding protein) and in the concentration of Ca^{2+} in the extracellular fluid. Even if it is not yet defined whether this effect of insulin occurred directly

through insulin receptors, disappearance of insulin effect on ^{45}Ca uptake in trypsin-treated erythrocytes seems to support the involvement of insulin receptors in the mechanism for both the activation of Ca-ATPase and ^{45}Ca uptake.

Forn and Greengard (1976) suggested that the action of insulin, an antilipolytic agent, might occur through the alteration of the level of phosphorylation of proteins in fat cell membranes. On the analogy of this biological role of receptors in fat cells, insulin receptors on rabbit erythrocytes may be associated with metabolism of phosphorus in cells, including protein phosphorylation and incorporation of phosphorus into lipids in membranes.

On the other hand, ^{45}Ca uptake into erythrocytes was reduced by insulin and DiBucAMP, while in fat cells DiBucAMP, which can induce lipolysis, remarkably enhanced ^{45}Ca uptake in contrast to insulin. This conflict may reflect the difference in an important biological role of Ca^{2+} between fat and erythrocyte cells.

In conclusion, the present results demonstrate the existence of insulin receptors on rabbit erythrocyte membrane, and suggest that insulin receptor may function in the regulation of metabolism of calcium and phosphorus in rabbit erythrocytes.

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