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Abstract

Insulin and human erythrocyte cell membrane interactions were studied with respect to binding and dissociation. The per cent of specific binding of 125I-labeled insulin to erythrocytes was directly proportional to the cell concentration. The optimum pH for binding was 8.1. The initial binding rate was directly proportional to, and the steady state insulin binding was reversely proportional to, the incubation temperature. The per cent of specific binding of 125I-labeled insulin was 12.10 +/- 1.13 per cent (mean +/- SD)/4 X 10(9) cells (n = 10) at 0.8 ng/ml insulin. Native insulin competed with 125I-labeled insulin for binding and showed almost complete inhibition at 10(4) ng/ml. The Scatchard plots were upward concave. Maximum binding capacity was 230 binding sites per cell. The average affinity constant decreased as the per cent of fractional occupancy increased. Affinity constants for the empty and filled sites were 1.49 and 0.16 X 10(8) M-1 respectively. Bound insulin was displaced by native insulin. The dissociation rate by "dilution + native insulin" was higher than that by "dilution only". The dissociation rate was accelerated even by the physiological concentration of insulin and maximum at 100 ng/ml. It is concluded that human erythrocytes have insulin binding sites which are indistinguishable from insulin receptors on the target tissues for insulin.

KEYWORDS: insulin binding, human erythrocyte.

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CHARACTERISTICS OF HUMAN ERYTHROCYTE INSULIN BINDING SITES

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Abstract. Insulin and human erythrocyte cell membrane interactions were studied with respect to binding and dissociation. The per cent of specific binding of ^{125}I-labeled insulin to erythrocytes was directly proportional to the cell concentration. The optimum pH for binding was 8.1. The initial binding rate was directly proportional to, and the steady state insulin binding was reversely proportional to, the incubation temperature. The per cent of specific binding of ^{125}I-labeled insulin was 12.10±1.13 per cent (mean ±SD)/4 × 10^9 cells (n = 10) at 0.8 ng/ml insulin. Native insulin competed with ^{125}I-labeled insulin for binding and showed almost complete inhibition at 10^4 ng/ml. The Scatchard plots were upward concave. Maximum binding capacity was 230 binding sites per cell. The average affinity constant decreased as the per cent of fractional occupancy increased. Affinity constants for the empty and filled sites were 1.49 and 0.16 × 10^8 M^{-1} respectively. Bound insulin was displaced by native insulin. The dissociation rate by "dilution + native insulin" was higher than that by "dilution only". The dissociation rate was accelerated even by the physiological concentration of insulin and maximum at 100 ng/ml. It is concluded that human erythrocytes have insulin binding sites which are indistinguishable from insulin receptors on the target tissues for insulin.

Key words : insulin binding, human erythrocyte.

The first step of insulin action is binding of insulin to specific receptors on the cell surface (1). In 1971, a procedure for the direct study of specific insulin-receptor interactions using radioactive insulin and rat adipose tissues was reported (2). Since then, insulin-receptor interactions in several tissues such as liver (3), adipose tissue (4), placenta (5) and brain (6) have been studied.

The demonstration and characterization of specific insulin binding sites in circulating human lymphocytes (7, 8), later proved to be monocytes rather than lymphocytes (9), have made it practical to study the change of insulin receptors in pathological states in man. Insulin binding sites in human erythrocytes have also been demonstrated by Cuatrecasas (2) and Gavin *et al.* (10). The relatively small amount of specific binding in their system made the use of erythrocytes to study of the insulin receptors questionable. Nevertheless Gambhir *et al.* (11, 12) recently described an improved assay procedure for study of insulin-erythrocyte interactions.

In the present study, the physical characteristics of insulin binding sites in

human erythrocytes are described.

MATERIALS AND METHODS

Radiolabeling of Insulin. Crystalline porcine insulin was purchased from Sigma Chemical Co. and radiolabeled with ^{125}I (Amersham, Bucks, U.K.) in the presence of chloramine-T by a method of Hunter and Greenwood (12) modified as follows. To 0.5 nmol of porcine insulin in 18 μl of 0.5 M sodium phosphate buffer (pH 7.4), 5 μl of carrier free $Na^{125}I$ (0.5 mCi) and 5 μl of chloramine-T (40 μg/ml) were added in a plastic tube and mixed at room temperature for 3 min. Then 15 μl of sodium metabisulfite (40 μg/ml) were added, followed by the addition of 0.2 ml of 2.5 % bovine serum albumin. Radiolabeled insulin was purified through a Sephadex G-50 column (0.8 × 50 cm) at 4 °C. Appropriate factions were kept frozen in a stock solution (20 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 145 mM NaCl, 2 % bovine serum albumin, adjusted to pH 7.4 with NaOH). ^{125}I-labeled insulin thus obtained had a specific activity of 100 to 150 mCi/mg and could be used for at least 4 weeks without loss of binding activity.

Preparation of the erythrocyte suspension. Venous blood was drawn into a tube containing heparin (250 units/ml blood). After centrifugation at 400 × g for 5 min at 20 °C, the plasma, the buffy coat and the upper portion of erythrocyte layer were aspirated. The erythrocyte pellet was washed twice more in the same ways as above after being suspended in 4 vol of the ice cold incubation buffer at 4 °C. The washed erythrocytes were adjusted with buffer to a concentration of 4×10^9 cells/ml. This suspension had a leukocyte contamination of less than 10^5 cells/ml.

Binding of ^{125}I-labeled insulin. Erythrocytes (about 1.6×10^9 cells) were incubated with ^{125}I-labeled insulin (0.4 ng) in the presence of 0 to 10^5 ng/ml native insulin in 0.5 ml of the incubation buffer (50 mM Hepes, 50 mM Tris, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 2 mM EDTA, 5 mM KCl, 10 mM Glucose, 50 mM NaCl and 0.5 % bovine serum albumin and adjusted to pH 8.0) in a water bath at 15 °C for 150 min (11). After the incubation, duplicate 200 μl aliquots of the reaction mixture were layered onto 0.3 ml of dibutyl phthalate and 0.2 ml of the buffer in the prechilled plastic microcentrifuge tubes (Fisher Scientific Co.). After centrifugation at 4,000 × g for 5 min at 4 °C in a Fisher Microfuge and counting total radioactivities, the buffer and the dibutyl phthalate layers were aspirated. The cell pellets were excised and bound radioactivities were counted.

Bound ^{125}I-labeled insulin in the presence of 10^5 ng/ml native insulin was considered nonspecific binding, which was about 3 per cent of the initial radioactivities. Specific insulin binding was calculated by subtracting nonspecific binding from total binding. The results were normalized to per cent specific ^{125}I-labeled insulin binding per 4×10^9 erythrocytes. Degradation of ^{125}I-labeled insulin during incubation, determined by 5 % trichlor acetic acid precipitability, was negligible.

Dissociation of ^{125}I-labeled insulin bound to erythrocytes. Dissociation studies were conducted as designed by De Meyts *et al.* (14, 15) with some modifications. Erythrocytes were associated with radiolabeled insulin so that a small portion of insulin binding sites in erythrocytes were occupied. At the end of the binding raction, the aliquots were transferred to a 30-fold vol of fresh buffer with (dilution + native insulin) or without (dilution only) 100 ng/ml of native insulin. At various time intervals, aliquots were removed to monitor the dissociation of bound insulin from erythrocytes. Nonspecific binding after the dissociation phase was determined using erythrocytes associated with ^{125}I-labeled in-

sulin in the presence of 10^5 ng/ml native insulin.

Effects of dilution and insulin concentration on dissociation. The experiments were performed in the same way as described in *"Dissociation of ^{125}I-labeled insulin bound to erythrocytes"* except that (a) dilutions was varied from 10 to 100-fold to see the effect of dilution and (b) the native insulin concentration in the fresh medium was varied from 1 to 10^4 ng/ml to see the effect of insulin concentration on dissociation.

RESULTS

Binding of ^{125}I-labeled insulin as a function of erythrocyte concentration. Erythrocytes in a final concentration of 0.35 to 5.6 × 10^9 cells/ml were incubated with ^{125}I-labeled insulin (1 ng/ml) in the absence or presence of 10^5 ng/ml native insulin at 15 °C for 150 min. The per cent of ^{125}I-labeled insulin bound specifically to hman erythrocytes was directly proportional to the cell numbers over the entire range of erythrocyte concentrations employed (Fig. 1).

The pH dependence of insulin binding. Buffers with pH ranging from 6 to 9 were prepared by varying the molar ratio of Tris to Hepes but keeping the total molarity of both compounds at 100 mM. Erythrocytes were washed and suspended in appropriate buffers, then assayed for insulin binding. Specific binding of ^{125}I-labeled insulin showed a sharp pH optimum at 8.1 (Fig. 2), which is similar to those reported for other tissues (4, 7, 8).

Effects of incubation time and temperature on insulin binding. The binding of

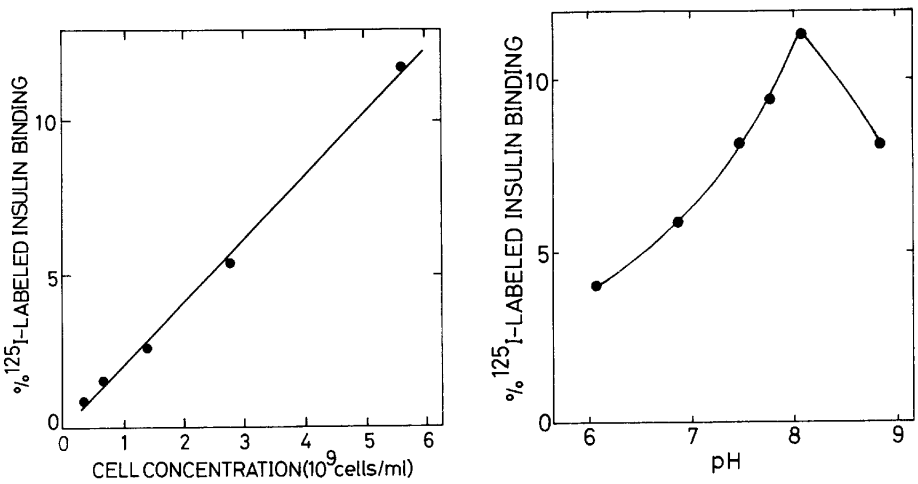
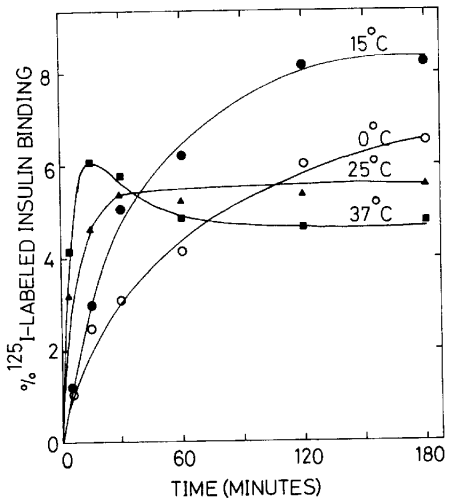


Fig. 1.*(left)* Specific binding of ^{125}I-labeled insulin as a function of the concentration of human erythrocytes. Erythrocytes in 0.5 ml of the buffer were incubated with 1 ng/ml ^{125}I-labeled insulin with or without 10^5 ng/ml native insulin at 15 °C for 150 min. Specific binding of ^{125}I-labeled insulin was determined as described in the text.

Fig. 2. *(right)* pH dependency of the specific binding of ^{125}I-labeled insulin. Preparation of the erythrocyte suspension and binding assay were performed in buffer adjusted to the appropriate pH.

radiolabeled insulin to human erythrocytes was dependent on incubation time and temperature (Fig. 3). The initial binding rate was directly proportional to, while the binding at steady state was reversely proportional to, the incubation temperature. The maximum steady state binding was found at 15 °C after 120 min and was maintained for at least 60 min. At 37 °C, the peak was obtained at 15 min, then the per cent of binding lowered and reached a steady state by 60 min. At steady state, ^{125}I-labeled insulin binding at 15 °C was about two times more than that at 37 °C. A steady state was not attained within three hours at 0 °C.

Binding of ^{125}I-labeled insulin as a function of insulin concentration. Fig. 4 shows that the specific binding of insulin to human erythrocytes seems to be saturated



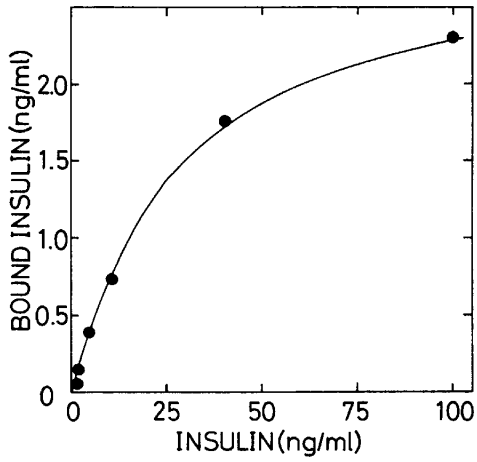


Fig. 3. *(left)* Effects of incubation time and temperature on the specific binding of ^{125}I-labeled insulin. Erythrocytes (2.5×10^9 cells/ml) were incubated with 0.8 ng/ml ^{125}I-labeled insulin for 150 min at various temperatures as indicated in the figure. The specific binding of ^{125}I-labeled insulin was determined at the time indicated.

Fig. 4. *(right)* Amount of insulin bound to human erythrocytes as a function of the concentration of insulin. Erythrocytes were incubated with increasing amounts of insulin at 15 °C for 150 min. Specifically bound insulin was determined and expressed in units per 4×10^9 erythrocytes.

with respect to insulin concentration. Though the amount of boud insulin did not reach a plateau under the experimental conditions, the slope of the binding curve became more and more gentle as the insulin concentration increased.

^{125}I-labeled insulin binding to erythrocytes. At the insulin concentration of 0.8 ng/ml, 12.10±1.13 per cent (mean±SD, n = 10) of ^{125}I-labeled insulin bound specifically to 4×10^9 erythrocytes (Fig. 5a). Unlabeled insulin at physiological concentration already inhibited ^{125}I-labeled insulin binding. More than 50 per

cent inhibition was observed at 10 ng/ml native insulin. Glucagon had no inhibitory effect. Displacement of ^{125}I-labeled insulin was almost complete at 10^4 ng/ml native insulin. Nonspecific binding was about 3 per cent of the initial radioactivities.

Scatchard analysis (16) of the binding data shows a curvelinear plot with an upward concave configuration that is consistent with site-site binding sites interactions of the negative cooperative type (Fig. 5b). The maximum binding

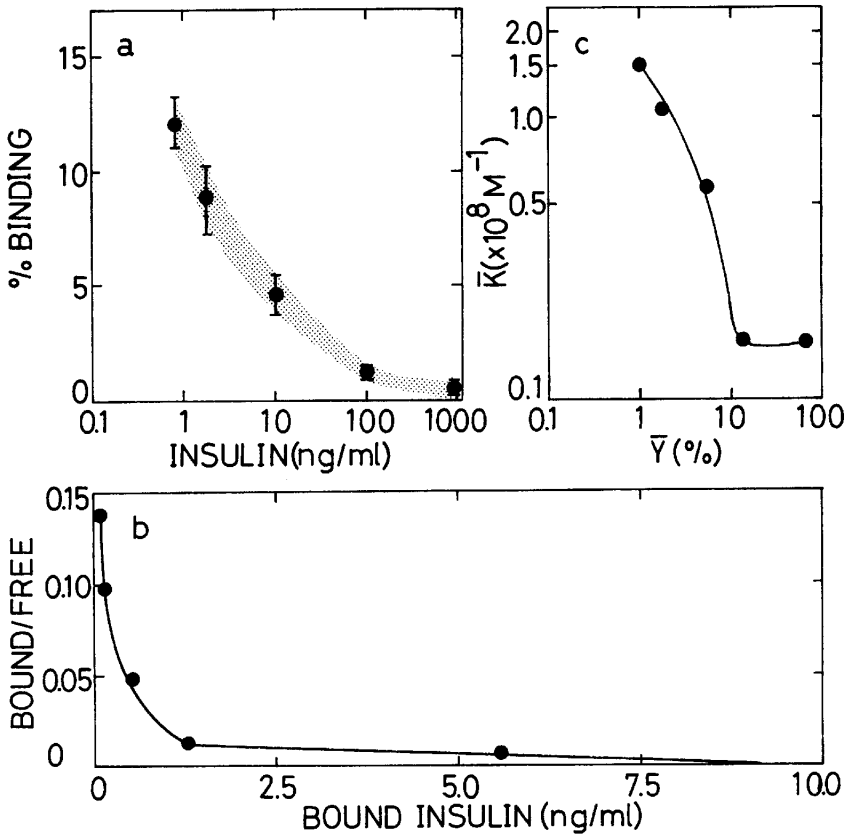


Fig. 5. ^{125}I-labeled insulin binding to human erythrocytes. (a) Competition of native insulin with the binding of ^{125}I-labeled insulin to human erythrocytes. Erythrocytes were incubated with 0.8 ng/ml ^{125}I-labeled insulin in the presence of native insulin ranging from 0 to 1000 ng/ml at 15 °C for 150 min. ^{125}I-labeled insulin binding was determined as described in "Materials and Methods". Results are expressed as specific binding ^{125}I-labeled insulin/4 $\times 10^9$ erythrocytes (mean ± SD for 10 separate experiments). (b) Scatchard plots of insulin binding to human erythrocytes. Data from the experiments in (a) were replotted according to the method of Scatchard (16). (c) Average affinity as a function of fractional binding site occupancy. Data from the experiments in (a) were replotted according to the method of De Meyts *et al.* (17). The average affinity ($\bar{K}$) is (B/F)/(Ro - B) and the fractional occupancy ($\bar{Y}$) is (B/Ro) × 100, where B, F and Ro are the bound insulin, the free insulin and the total binding site concentration respectively.

capacity (Ro) calculated from data at insulin concentrations of 10^2 and 10^3 ng/ml was 9.32 ng/4 × 10^9 cells or 230 binding sites/erythrocyte (Table 1).

Average affinity analysis of De Meyts *et al.* (17) showed that the affinity of the binding site decreased as the binding site occupancy increased up to 10 per cent (Fig. 5c). The affinity was fixed at higher binding site occupancy. The affinities of the empty site ($\bar{K}e$) and the filled site ($\bar{K}f$) were calculated as 1.49 and 0.16 × 10^8 M^{-1} respectively.

Dissociation of bound insulin. After a steady state of ^{125}I-labeled insulin binding was attained, excess cold insulin (10^5 ng/ml) was added to the reaction mixture, which was then incubated further at 15 °C. At various time intervals, aliquots were removed to determine the dissociated fraction of bound ^{125}I-labeled insulin from erythrocytes. The initial velocity of dissociation rate was higher, and slowed down gradually (Fig. 6). Thirty per cent of bound insulin were dissociated in first 15 min and 5 per cent in next 15 min. In 150 min, about 60 per cent of bound insulin were dissociated.

TABLE 1. *IN VITRO* ^{125}I-LABELED INSULIN BINDING TO HUMAN ERYTHROCYTES

Specific ^{125}I-labeled insulin binding	Receptor concentration		Average affinity constant	
			$\bar{K}e$	$\bar{K}f$
%/4 × 10^9 cells	ng/4 × 10^9 cells	Binding sites/cell	10^8 M^{-1}	
12.10±1.13	9.3	230	1.49	0.16

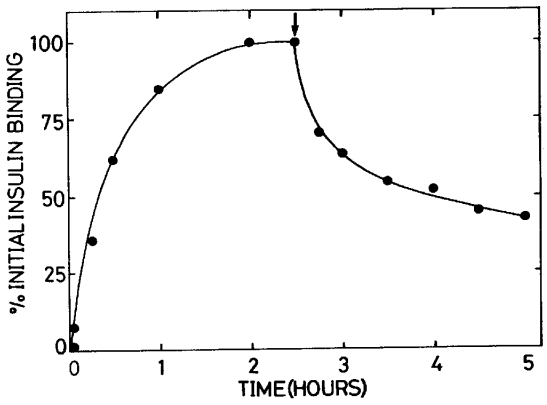


Fig. 6. Time course of dissociation of the ^{125}I-labeled insulin from human erythrocytes by an excess amount of native insulin. After erythrocytes were associated with ^{125}I-labeled insulin, 10^4 ng/ml native insulin were added. Bound ^{125}I-labeled insulin was determined at the indicated times. The results are expressed as a percentage of the bound radioactivities at the end of the association phase.

Effects of dilution and native insulin on dissociation. Experimental procedures designed by De Meyts *et al.* (14, 15) to demonstrate site-site receptor interactions of hormone receptors were used with some modification. Radiolabeled insulin was associated with erythrocytes so that a minority of erythrocyte binding sites

for insulin was occupied. After steady state was obtained, one part of the reaction mixture was diluted with fresh buffer (dilution only), while another was diluted with fresh buffer containing native insulin (dilution + native insulin). At various time intervals, dissociation of bound ^{125}I-labeled insulin was monitored in both sets as decribed in "Materials and Methods". The difference between "dilution only" and "dilution + native insulin" was constant at dilution higher than 20-fold dilution (Fig. 7).

The dissociation rate in "dilution only" was dependent on time and temperature (Fig. 8). There was no dissociation at 0 °C for 150 min. At higher temperatures, dissociation curves are concave with the initial higher dissociation rate. At 15 °C, 60 per cent of bound insulin dissociated in 150 min. At 37 °C, dissociation was almost complete in 60 min.

Dissociation of bound ^{125}I-labeled insulin from erythrocytes by "dilution + native insulin" was also dependent on time and temperature (Fig. 8). Dissociation with a near first order dissociation rate was observed even at 0 °C. At higher temperatures, the dissociation curve became concave with a higher dissociation rate.

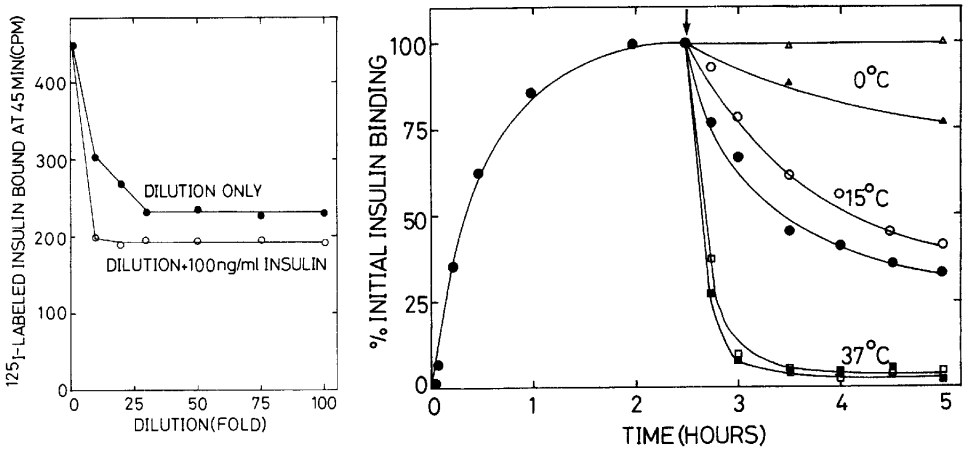


Fig. 7. *(left)* Effect of dilution on dissociation of ^{125}I-labeled insulin from human erythrocytes. After erythrocytes were associated with ^{125}I-labeled insulin, aliquots were diluted as indicated in the figure with the fresh buffer containing or not containing 100 ng/ml native insulin. After 45 min at 15 °C, bound and free insulin were separated and bound radioactivities was plotted as a function of the dilution factor.

Fig. 8. *(right)* Dissociation of bound ^{125}I-labeled insulin from human erythrocytes. Erythrocytes (3×10^9 cells/ml) were associated with 0.8 ng/ml ^{125}I-labeled insulin, after which aliquots were diluted 30 times with fresh medium with (solid symbols) or without (open symbols) 100 ng/ml native insulin. Incubation was conducted at 0 °C (triangles), 15 °C (circles) or 37 °C (squares). Aliquots were withdrawn and radioactivities determined at various time intervals. The results are expressed as a percentage of the radioactivities bound at the end of the association phase.

The dissociation rate in "dilution + native insulin" was higher than that in "dilution only" (Fig. 8). The difference was more marked at lower temperatures.

Fig. 9 shows the effects of varying the concentration of native insulin on dissociation of bound insulin from erythrocytes. The magnitude of the effect was dependent on the concentration of native insulin. At physiological concentrations of native insulin, a cooperative effect was apparent. The maximum cooperative effect was observed at 100 ng/ml native insulin. At much higher concentrations of native insulin, the cooperative effect was less marked.

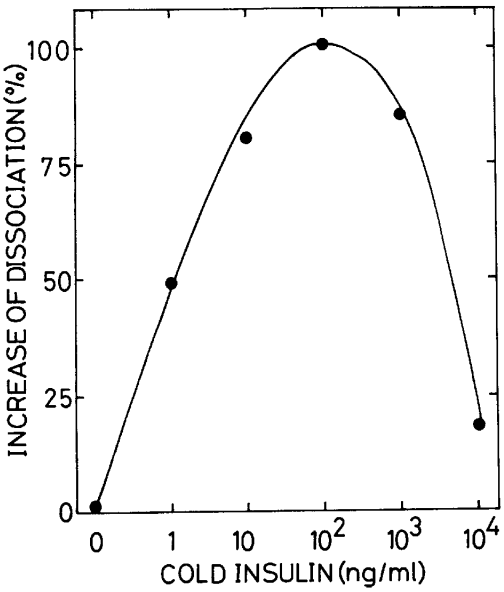


Fig. 9. Effect of native insulin concentration on dissociation of ^{125}I-labeled insulin from human erythrocytes. Dissociation studies were performed as described in the legend to Fig. 8 in the presence of increasing amounts of native insulin (from 0 to 10^4 ng/ml). The difference in the bound radioactivities between "dilution only" and "dilution + native insulin" at the indicated insulin concentration is expressed as a percentage of the difference observed at 100 ng/ml native insulin.

DISCUSSION

The results here show that human erythrocytes have specific insulin binding sites whose physical characteristics are similar to those of insulin receptors in target tissues. Since the physiological action of insulin on matured human erythrocytes is unknown and questionable, the term "insulin binding site" rather than "insulin receptor" is preferred in this report.

The binding of ^{125}I-labeled insulin to human erythrocytes dependents on parameters such as cell concentration, pH, temperature and time (Figs. 1-3). There is a linear increase in the specific binding of ^{125}I-labeled insulin over 10-fold increase in cell concentration. To obtain appreciable binding of insulin, more than 10^9 erythrocytes/ml were needed as reported (10-12). Cell concentrations binding a comparable amounts of insulin were 10^7 cells/ml for monocytes (9, 18) and 10^5 to 10^6 cells/ml for adipocytes (19). These differences correspond well to the differences in concentration of insulin binding sites in these cell types.

The calculated insulin binding sites in the present study were 230 binding sites/cell (Table 1). Monocytes were around 20,000/cell (18) and adipocytes were around 250,000/cell (19) respectively.

Binding of insulin to human erythrocytes shows a sharp pH dependency as described for other insulin receptors (4, 7, 8). The optimum pH is 8.1 and seems somewhat higher than those for other insulin receptors. Differences in the physicochemical properties of matured erythrocyte membrane or structural alterations of insulin binding protein on erythrocytes may be the cause of this change in pH optimum.

Insulin binding to human erythrocytes was directly proportional to the incubation temperature. Steady state binding was reversely proportional to the incubation temperature and attained a maximum value at 15 °C in accordance with previous reports for several tissues (4, 7, 10, 12). In human mononuclear cells, initial binding is not always proportional to the temperature (8, 20). The maximum binding at steady state occurred at higher tempratures in several membrane preparations (4, 21). The reason for these discrepancies is unclear. At higher temperatures, ^{125}I-labeled insulin binding reaches a maximum in 15 min, then declines and reaches a steady state in human erythrocytes as with isolated tissue cells and mononuclear blood cells (7, 22-25). This time dependency of insulin binding at higher temperatures is not observed in fat or liver cell membrane preparations (4, 21), so it is not explained by increased degradation or dissociation of insulin at higher temperatures (25). Changes in cellular integrity and membrane fluidity may be factors. Binding of insulin may cause redistribution or conformational changes in insulin receptors on the surface of cells (26), leading to decrease in the amount of bound insulin with longer incubation.

Scatchard analysis (16) of insulin binding to human erythrocytes shows an upward concave curve (Fig. 5b), just the same as reported for other insulin receptors (7, 12, 15, 19). In the present study, it was calculated that one mature human erythrocyte has an average of 230 binding sites, which is in good agreement with the reported number (27). However this figure must be accepted with reservation as determination of the number of insulin binding sites depends on the highest insulin concentrations used for extrapolation in the Scatchard plots. Curvelinear plots in Scatchard analysis of insulin-receptor interactions can be explained by two or more types of binding sites (7, 8) or by uniform binding sites exerting negative cooperative type site-site interactions (14, 15). In the present study, the procedure proposed by De Meyts *et al.* (14, 15) to prove negative cooperativity was used for human erythrocytes (Figs. 7-9). The effects of native insulin, temperature and insulin concentration on the dissociation of bound ^{125}I-labeled insulin from human erythrocytes were similar to those reported for insulin receptors in target tissues (14, 15, 20). Evidence for two kinds of insulin binding sites on human erythrocytes reported recently (28) raises doubts about the explanation of enhanced dissociation by native insulin proposed by De Meyts

et al. (29), so no conclusions can be made about the number and site-site interactions of insulin binding sites from those dissociation studies only.

Physiological actions of insulin on mature human erythrocytes have not been proved. The following evidence suggests alterations in insulin binding sites during cell differentiation: Insulin receptors decrease in number during Friend erythroleukemia cell differentiation (24), increase during 3T3-L1 preadipocyte differentiation to adipocyte (30), and loss of insulin binding sites occurs on reticulocyte maturatuion (31, 32). These observations indicate that insulin has some effect on erythrocyte precursors during erythropoiesis. The number of insulin receptors on erythrocyte precursors may be regulated genetically, decreasing gradually with cell differentiation. In peripheral blood, erythrocyte insulin binding sites are probably lost mechanically or enzymatically as are other cellular components (33).

In conclusion, peripheral mature human erythrocytes have insulin binding sites which may be remnants of insulin receptors and which may have some physiological role during erythropoiesis. Their physical characteristics are indistinguishable from those of insulin receptors in target tissues.

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