Vol. 117, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 28, 1983 Pages 823-834

CHARACTERIZATION OF INSULIN BINDING TO THE ERYTHROLEUKEMIA CELL LINE K 562

Tang Xin-Zhi, Michael Tally†, Mikael Jondal* and Kerstin Hall

Department of Endocrinology, Karolinska Hospital and
*Department of Immunology, Karolinska Institute,
S-104 01 Stockholm, Sweden

Received September 26, 1983

SUMMARY. The K 562 is a transformed human erythroid stemcell and is used as a target cell for NK-T-cells. In this study the presence of insulin receptors in K 562 is established.

The best binding and negative cooperativity was found in the two Hepes containing buffers whereas no cooperativity was obtained in the Krebs-Ringer buffer. The calculated affinity constants and receptor number per cell varied according to the buffer. Preincubation with insulin caused a down-regulation of the insulin binding capacity. 10 ng/ml caused a lowering of the affinity, with an unchanged number of receptors. 100 ng/ml caused a decrease in receptor number with unchanged affinity. These results were found in both Hepes and Krebs-Ringer phosphate buffer. IGF-I shows cross-reactivity with the insulin receptor, with a potency of 12 and 100 times less than insulin in Krebs-Ringer phosphate buffer and G-buffer respectively. However, no specific IGF-I receptors were found.

The presence of receptors on K 562 cells suggests a biological role for insulin. The different results in the different buffers, indicate that a buffer containing Hepes and/or Tris, is required to expose negative cooperativity and make the receptors more accessible to insulin.

INTRODUCTION. Peripheral blood leucocytes have been used in studies of insulin-binding for several years (1). The advantage of such cells is that they are easily accessible and therefore can be used as markers for changes in receptor characteristics. Monocytes are considered to reflect acute changes in cells in other tissues (2). Erythrocytes, although their receptor concentrations are low, have been studied in order to observe long-term changes in receptor affinity and concentration (3,4). Human derived cellines have also been used as models for cells of human origin (5-8).

In this study we establish the presence of insulin receptors in a transformed celline derived from erythroid stemcells. This celline, K 562, isolated from a patient (9), contains no hemoglobin, grows in suspension and is quite stable. It is used as a standard target cell for human natural killer

†To whom correspondence should be addressed.

0006-291X/83 $1.50

cells (10). Monoclonal antibodies have been produced directed against a number of different antigens on the cell surface of K 562 cells (11). The K 562 celline show binding of insulin comparable to other insulin-binding systems, and provide a tool for studying insulin receptors and their modulation by extracellular factors.

MATERIAL AND METHODS. RPMI 1640 culture medium, and fetal calf serum were purchased from Flow Laboratories Ltd, Great Britain. Hepes (N-2-Hydroethyl-piperazine-N-2-ethanesulfonic acid), Tris (tris (hydroxymethyl) amino methane) and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, Missouri, USA). $Na^{125}I$ (carrier free) was obtained from Amersham, Great Britain. Crystalline porcine insulin was from Nordic Insulin Laboratories, Denmark, and human synthetic proinsulin was a generous gift from the Eli Lilly Co. Insulin-like growth factor I (IGF-I) was kindly supplied as a gift from Prof Rene Humbel, Switzerland. All chemicals were of reagent grade.

Buffers used. Four buffers were used in the binding studies. 1) Krebs-Ringer Phosphate buffer (KRP) pH 7.4. 2) Krebs-Ringer Bicarbonate buffer (KRB) pH 7.4 (12). 3) Hepes Buffer; NaCl 135 mM, KCl 4.8 mM, $MgSO_4$ 1.7 mM, $CaCl_2$ 2.5 mM, NaH_2PO_4 1.0 mM, Hepes 10 mM, pH 7.4 (13). 4) G-buffer; NaCl 50 mM, Kcl 5 mM, $MgCl_2$ 10 mM, $CaCl_2$ 10 mM, Hepes 50 mM, TRIS 50 mM, EDTA 2 mM, Dextros 10 mM, pH 8.0 (3). All buffers were used with or without 1% BSA.

Iodination of insulin. Porcine insulin was iodinated according to the lactoperoxidase method of Thorell and Johansson (14). The iodinated hormone was separated from ^{125}I on a carboxymethyl cellulose column. This iodination method has been proved to give a label with high specific binding to receptors (15).

Growth of cultured human K 562 cells. Human K 562 cells were maintained at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Binding studies were always performed 3 days after change of medium. Cell viability, monitored by trypan blue exclusion (16), exceeded 90% in all experiments. K 562 cells were washed twice in binding buffer and centrifuged at 500 x g for 1 min in between washings before immediate use.

Preincubation, washing and insulin binding procedure. Pretreated cells were preincubated in RPMI 1640 culture media, with or without 10 or 100 ng/ml insulin for 3 h at 37°C. The preincubation was terminated by centrifugation at 500 x g for 1 min. Cells incubated without insulin were washed twice with 10 ml of buffer for 30 min.

Cells pretreated with 10 ng/ml insulin were washed three times, 1 x 5 ml x 20 min and 2 x 10 ml x 20 min. Cells pretreated with 100 ng/ml insulin were washed four times, 2 x 10 ml x 10 min and 2 x 25 ml x 20 min. Cells were separated by centrifugation for 1 min at 500 x g after each washing. All washing and final resuspensions were done in the buffer used in each of the binding experiments. Viability was determined before and after each experiment.

The binding studies were performed in polysterene tubes coated with 1% BSA. Each tube contained 1.8×10^6 cells, ^{125}I-insulin (172 pM, appr. 1×10^{-7} Ci/ml), increasing amounts of unlabelled porcine insulin, proinsulin or IGF-I, in a total volume of 300 µl buffer with 1% BSA. Duplicates were done for each concentration. Incubations were carried out at 37°C for 15 min whereafter 2 x 100 µl were aspirated off from each tube and the 100 µl remaining in the incubation tube was used as reference. The aspirated 100 µl was immediately overlayered and centrifuged through 250 µl of a mixture of

phtalic acid dibutyl ester and phtalic acid dinonyl ester (3:2 δ = 1.022) at 12 000 x g from 1 min. Both hydrophilic and hydrophobic supernatant were aspirated off carefully and the tip of the centrifugation tube containing the cells was excised and its radioactivity determined.

Nonspecific binding and degradation. Specific binding, expressed per 6 x 10^6 cells per ml/6 x 10^6 was obtained by subtracting unspecific binding, determined in the presence of 3 µg/ml unlabelled insulin, from total bound radioactivity. The nonspecific binding was 5-20% of the total binding. Insulin degradation was determined by measuring precipitation of radioactivity in the medium with 12% (w/v) trichloracetic acid (17). The amount of insulin degraded at 37°C was about 5 and 10% after 15 and 30 min respectively.

Calculations. Scatchard analyses were performed according to Scatchard (18), and affinity profile according to de Meyts (19). Dissociation rates were determined according to de Meyts (20).

RESULTS

Binding kinetic studies. Binding of insulin to K 562 cells was performed at 37°C in four different buffers. The increase in insulin-binding with increasing incubation time is shown in fig. 1. Maximal binding in Hepes buffer and

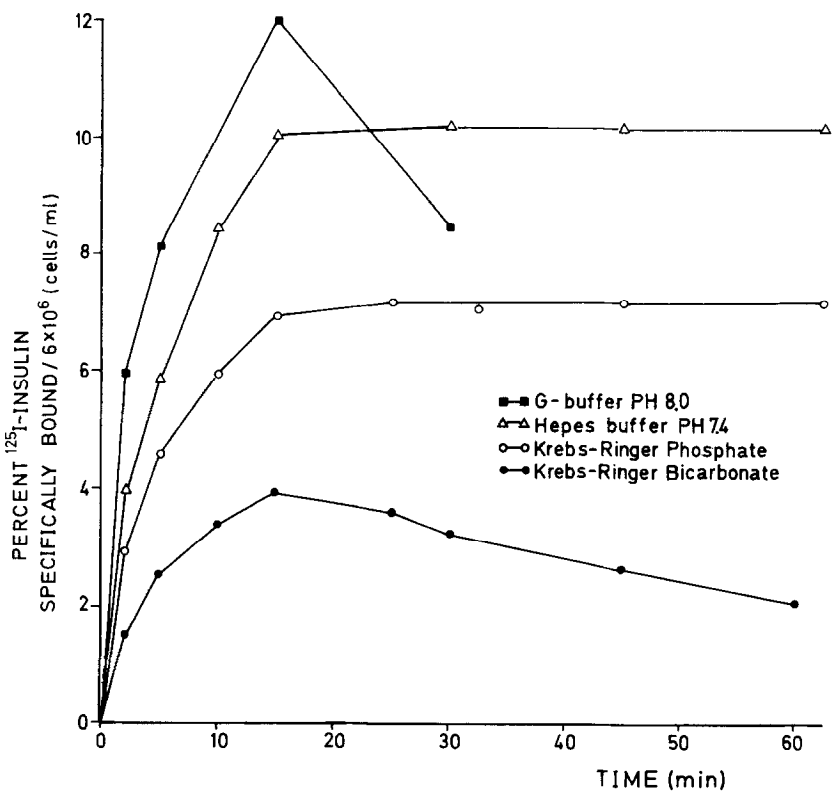

Fig. 1 Time-dependency of ^{125}I-insulin binding to K562 cells in four different buffers at 37°C. Specific binding is expressed per 6x10^6 cells/ml.

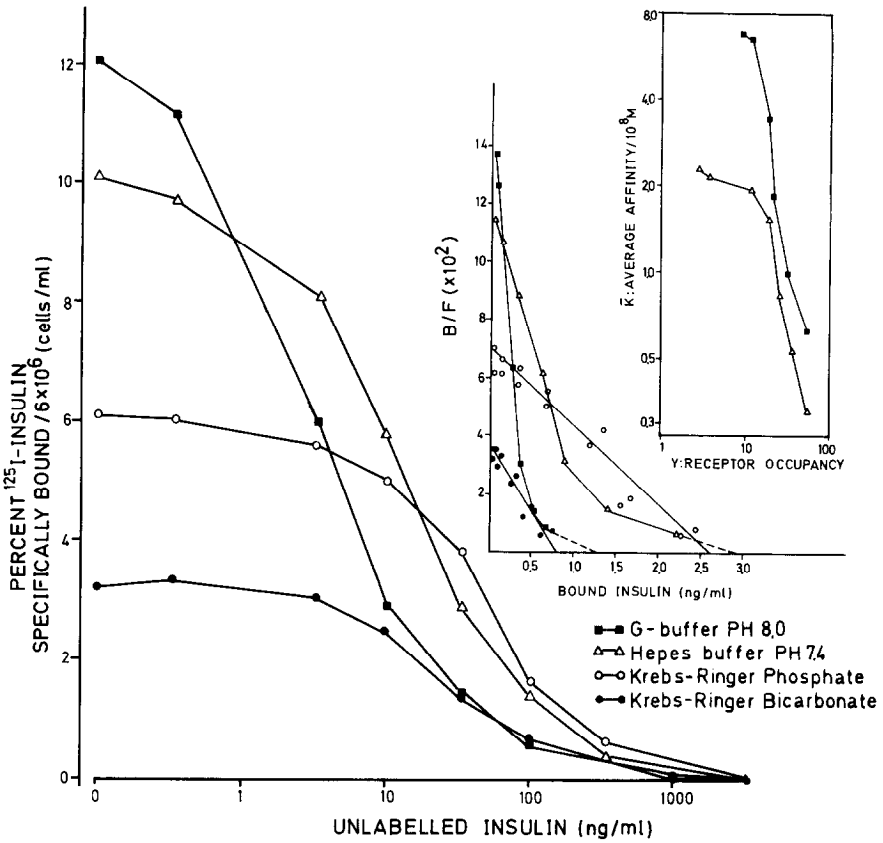

Fig. 2 Displacement of ^{125}I-insulin binding to K562 cells by insulin in four different buffers. Cells were incubated with labelled insulin (172 pM) with increasing amounts of unlabelled insulin. Corrections have been made for unspecific binding. Inset panels show Scatchard plot and affinity profile derived from these data.

KRP-buffer was reached after approximately 15 min and remained unchanged for 60 min. In KRB- and G-buffer, however, a steady state was not obtained but maximal binding was also here reached at the same time as in the two other buffers. An incubation time of 15 min was therefore used in all following experiments.

The specific binding varied between 3% and 12% with the buffer used. The highest specific binding of labelled insulin (12%) was reached in G-buffer. The competition curves with porcine insulin in the four different buffers are shown in fig. 2. The insulin concentration required for 50% displacement varied between 5.3 and 41.7 ng/ml with the buffer used. Scatchard analysis

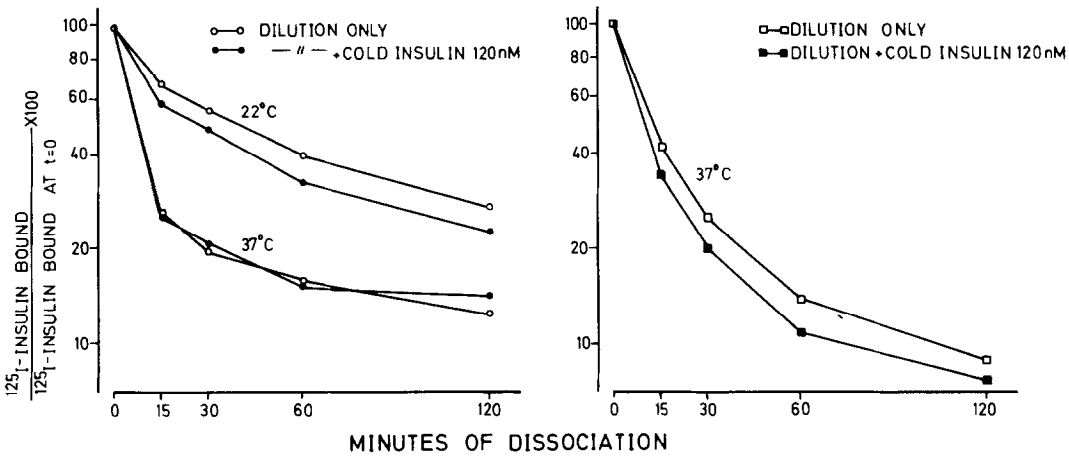

Fig. 3 Dissociation rate of ^{125}I-insulin prebound to K562 cells. 1.4×10^7 cells/ml were preincubated with 172 pM labelled insulin. Cells were centrifuged gently and resuspended with a 100-fold dilution with or without 1.2×10^{-7} M unlabelled insulin present. Preincubation and dissociation in Krebs Ringer phosphate buffer is shown in left and in G-buffer the right panel.

of these results disclosed curvelinear plots in G-buffer and Hepes buffer whereas linearity was obtained in the KR-buffers.

Dissociation rate of bound labelled insulin was determined in G-buffer and KRP-buffer as is shown in fig. 3. Addition of insulin in the concentration of approximately 600 ng/ml at 37°C enhanced the dissociation rate in G-buffer but not in KRP-buffer. At 22°C a slight enhancement was seen also in KRP-buffer. This result supports the interpretation that the curvelinear Scatchard plot is due to negative cooperativity.

The former result can either be interpreted as negative cooperativity or the presence of more than one type of receptor affinities. The KRB- and KRP-buffer curves, however, indicate the non-cooperative binding site. In G-buffer the average affinity (K) is higher than in Hepes-buffer. In the average affinity profile, cells incubated in G-buffer have an overall higher affinity level than cells incubated in Hepes-buffer. The calculated affinity constants as well as receptor concentrations, for insulin on K 562 cells in different buffers are given in Table 1.

Table I. Specific binding, affinity constants and binding capacity of insulin to K562 cells(6 x 10^6 cells/ml) in four different buffers.

Buffer	% maximal ^{125}I-insulin specific binding	50% of displacement Mx10^9	Ka $M^{-1}x10^{-8}$	$\bar{K}e$ $M^{-1}x10^{-8}$	$\bar{K}f$ $M^{-1}x10^{-8}$	α $\frac{\bar{K}f}{\bar{K}e}$	Binding sites/cell x 10^{-4}
G *	12 ± 1.5	0.93 ± 0.5		6.5± 0.5	0.63± 0.3	0.1 ± 0.03	2.2 ± 0.4
Hepes	10.5	1.8		2.3	0.34	0.148	5.3
KRP	6.2	7.5	1.59				4.4
KRB	3.2	4.2	2.56				1.4

* The results are expressed as mean ± SEM in 12 separate experiments.

Specificity of insulin receptors. The ability of proinsulin and IGF-I to compete with labelled insulin on its receptor on K 562 cells in KRP- and G-buffer is shown in fig. 4. IGF-I was 12 times and 100 times less potent than

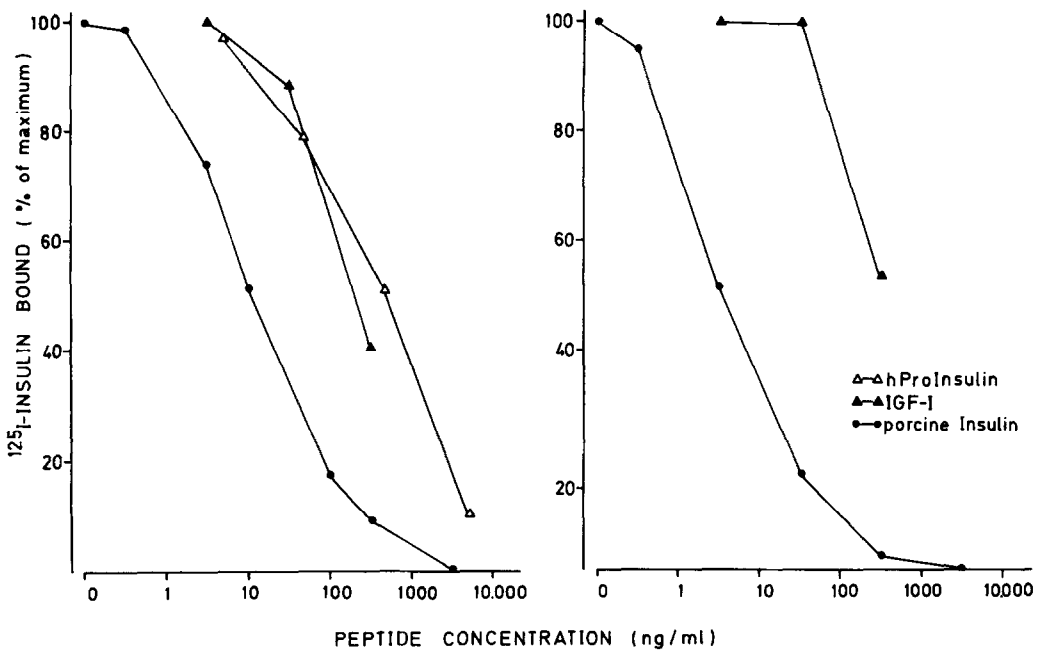

Fig. 4 Competitive inhibition of ^{125}I-insulin binding by human proinsulin, insulin-like growth factor I (IGF-I) and porcine insulin. Cells were incubated with labelled insulin (172 pM), with increasing amounts of indicated peptide. Incubations performed in Krebs Ringer phosphate buffer are shown in left panel and in G-buffer in right panel.

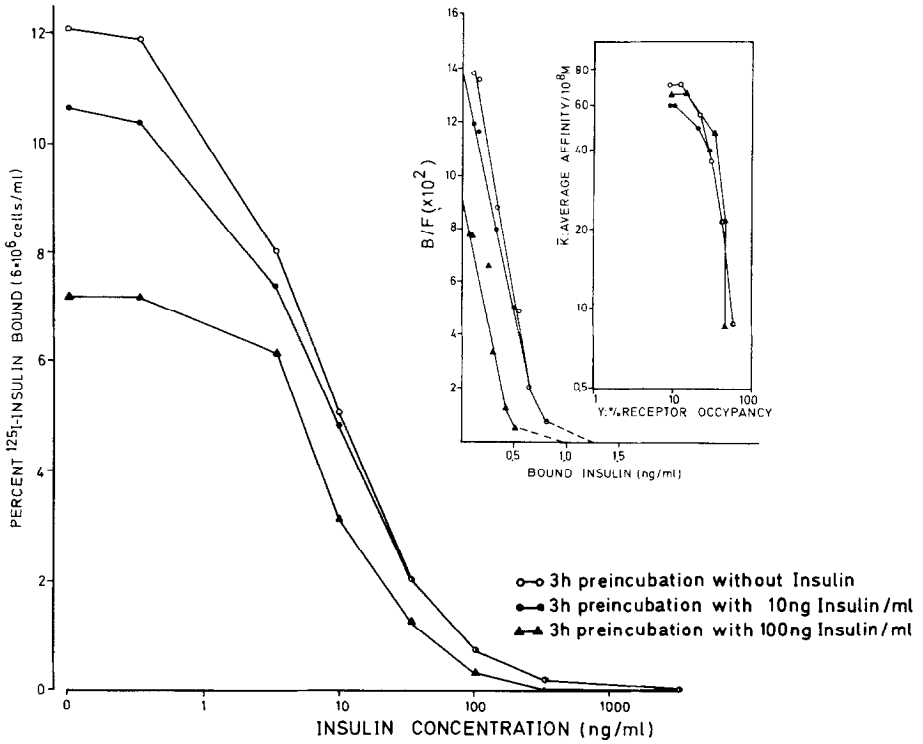

Fig. 5 Effect of preincubation with insulin on insulin binding to K562 cells. Cells were preincubated in culture medium (RPMI 1640) with or without 10 or 100 ng/ml insulin for 3 hours, and washed before binding assay in G-buffer. Inset panels show Scatchard plot and affinity profile derived from these data.

insulin in KRP-buffer and G-buffer respectively. This discrepancy in potency ratio was mainly due to a higher sensitivity to insulin in G-buffer. No specific binding sites for IGF-I or somatomedin A were found on K 562 cells.

Insulin-induced loss of insulin receptors. K 562 cells were preincubated with 2 different concentrations of insulin for 3 hours. In G-buffer (fig. 5) the exposure of K 562 cells to 10 ng/ml of insulin caused a 12% decrease in the specifically bound labelled insulin. Scatchard analysis of the binding data derived from the competetive-inhibition curves were curvelinear. A decrease in high affinity binding with no alteration in lower affinity was found and Ke decreased from 7.0×10^8 M^{-1} to 5.9×10^8 M^{-1} with no change in receptor concentration. Preincubation with insulin in a concentration of

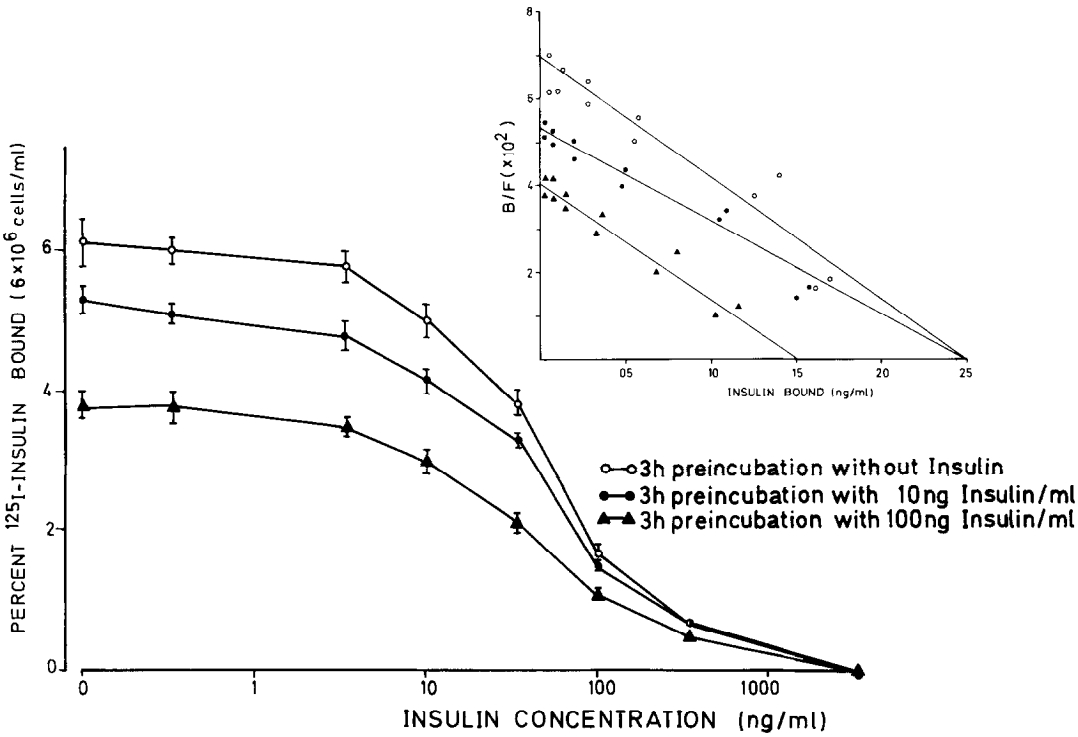

Fig. 6 Effect of preincubation with insulin on insulin binding to K562 cells. Cells were preincubated in culture medium (RPMI 1640) with or without 10 or 100 ng/ml insulin for 3 hours and washed before binding assay in Krebs Ringer phosphate buffer. Inset panel shows Scatchard plot derived from these data.

100 ng/ml, caused a 40% decrease of specific binding and the total receptor concentration (Ro) decreased 35%. Cell-surface receptor-number was reduced from 2.2×10^4 to 1.3×10^4, with no significant change in receptor affinity. The ratios of $\frac{Kf}{Ke}$ are nearly the (Table 2).

The results when KRP-buffer was used as assay buffer are shown in fig. 6. Pretreatment of K 562 cells with 10 ng/ml or 100 ng/ml of insulin resulted in a 14% and 38% decrease in specific binding respectively. The Scatchard analysis confirmed the straight line pattern. The change in binding found after 3 hours preincubation with 10 ng/ml of insulin is primarily due to altered receptor affinity. After preincubation with 100 ng/ml insulin, the receptor concentrations showed a 40% decrease in cell-surface receptor number, but with no significant change in receptor affinity. This fact is re-

Table II. Effects of preincubation with unlabelled insulin for 3 h at 37°C on the binding characteristics (see Methods).

Buffer (washing & assay)	Insulin conc. during pre-incubation (ng/ml)	% specific ^{125}I-insulin binding	Insulin binding capacity, Ro (nm/6x10^6 cells)	Ka $M^{-1}x10^{-8}$	$\bar{K}e$ $M^{-1}x10^{-8}$	$\bar{K}f$ $M^{-1}x10^{-8}$	$\alpha = \frac{\bar{K}f}{\bar{K}e}$	Binding sites per cell x 10^{-4}
G	0	12.1	0.219		7.0	0.88	0.125	2.2
	10	10.6	0.219		5.9	0.88	0.150	2.2
	100	7.2	0.132		6.7	0.87	0.129	1.3
KRP	0	6.2	0.439	1.59				4.4
	10	5.3	0.439	1.25				4.4
	100	3.8	0.263	1.52				2.6

The results are derived from experimental data shown in Figure 5 and 6.

flected in no significant change in the affinity constant (Ka) between the control, and the 100 ng/ml insulin treated cells.

DISCUSSION. Human erythrocytes have been used extensively to determine insulin-binding in different patient material (4, 21-26) and the red cells have been thought to act as a mirror of long-term physiological situations. The concentration of receptors are, however, extremely low compared to other tissues, and biological action has only been seen on the plasma membrane level (Löw, Crane, Grebing, Hall in preparation) and not on the whole cell. The presence of receptors in these cells raised the question whether insulin had any biological significance in the earlier stages of erythrocyte development but the inconvenience of using human material has made such studies impossible. The K 562 celline, however, provides a tool for these studies as they are erythroblastoid transformed cells of human origin (9). They grow in suspension and do not form any cell clusters. They contain normally no hemoglobin but can, after stimulation with different agents, start production of different forms of hemoglobin (27), and as we have shown here they have insulin receptors. In preliminary experiments, insulin also slightly enhances the uptake of 3-0-Methyl-D (1-^{3}H) glucose (not publ.). These indications of

a true biological effect of insulin on these cells are supported by the down-regulating action of insulin presented in this study. The fact that the K 562 cells grow in suspension of a great advantage since it is possible to avoid the use of enzymatic or mechanical methods to obtain an even distribution, and the viability is therefore high.

The binding kinetics reveals differences between the buffers. Curvelinear Scatchard plots were only found in buffers containing Hepes. In the Hepes-buffer used in this study the concentration of Hepes is 10 mM which is enough to give curvelinearity and good binding in the lower concentrations of insulin. The effect of G-buffer is larger than the effect of Hepes-buffer both on sensitivity and curvelinearity, but in this buffer the concentration of Hepes is 5-fold higher and furthermore, Tris is present in a 50 mM concentration. The effect of Tris on the binding kinetics and exposing or causing down-regulating effects of insulin on adipocytes have previously been discussed by others (28,29). These results might indicate that the presence of other zwitterions, not only Tris, make the receptors more accessible to insulin. In the Krebs-Ringer buffers the insulin binding in the lower concentrations is lower and the Scatchard plots are linear, indicating that some kind of structural difference is at hand acting on the receptor or the hormone in these buffers. The dissociation results support the finding that the negative cooperativity only is seen in the zwitterion-containing buffers at 37^{o}C.

In our down-regulation experiments all the preincubations were done in cell culture media (RPMI 1640) containing 5 mM Hepes. The results, when assaying in G-buffer or Krebs-Ringer phosphate buffer, are approximately the same considering their specific binding kinetics; the Scatchard analysis is curvelinear in G-buffer and linear in Krebs-Ringer phosphate buffer. Preincubation for 3 hours in 10 ng/ml insulin causes a slight decrease in binding but this seems to be due primarily to a lowering of receptor affinity, more than a decrease in the number of receptors. Preincubation with 100 ng/ml insulin under the same conditions does not seem to change the affinity but rather lower the number of receptors markedly. The insulin induced down-

regulation of insulin receptors suggests a turnover rate on these cells supporting the assumption that insulin has a biological activity. The physiological role of insulin on K 562 is unclear.

The presence of Hepes during the preincubation is not a prerequisite for the down-regulation of receptors. In later experiments preincubation in both Krebs-Ringer phosphate- and G-buffer results in a good down-regulation which is dependent of the insulin concentration. The only difference is that the effect is much more expressed in the lower (10 mg/ml) concentration of insulin in G-buffer than in Krebs-Ringer phosphate (KRP-buffer). Insulin-like growth factor I and human proinsulin cross-react with the insulin receptor but IGF-I has no detectable receptors of its own (not shown). The fact that the IGF-I concentration for 50% displacement is almost the same in both G-buffer and Krebs-Ringer phosphate buffer, implies that the zwitterionic effect works over a site not accessible to IGF-I.

ACKNOWLEDGEMENTS. This study was supported by grants from the Swedish Medical Research Council no 4224 and the Nordic Insulin Foundation, Denmark.

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