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The Orientation of Insulin Receptors in the Red Cell Membrane

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High-affinity binding of insulin to receptors in human erythrocyte membranes occurred at the external surface, but not at the cytoplasmic surface of the plasma membrane, as assessed by insulin binding to right-side-out and inside-out membrane vesicles. Even after prolonged (3 h) incubation at 22°C, binding at the cytoplasmic membrane aspect remained negligible. The data indicate that the insulin receptor displays its hormone-binding site exclusively toward the extracellular space and that transmembrane mobility ("flip-flop") of the receptor from one to the other membrane leaflet is severely restricted.

Key words: **insulin receptors, receptor asymmetry, inverted membrane vesicles, red cell membrane, cytoplasmic membrane surface**

The first step in peptide hormone action is believed to involve binding to high-affinity receptors in the plasma membrane of target cells. Present concepts view the hormone-binding site as necessarily oriented toward the extracellular space. However, little information exists about other potential hormone-binding sites on the receptor molecule. The extent to which receptors penetrate the membrane core is not known. Although lateral movement of receptor molecules within the membrane is well documented [1–3], the possibility of transmembrane movement ("flip-flop") of receptors from one leaflet of the lipid bilayer to the other has not been examined. It is known that some lipid constituents of the plasma membrane exchange very rapidly between the two membrane leaflets [4]. A similar transmembrane movement of peptide hormone receptor could be related to internalization of hormone-receptor complexes [5, 6] or to the generation of intracellular mediators, as in the case of insulin [7–9].

The mature erythrocyte provides an excellent model system to probe some of these questions since 1) it contains insulin receptors; 2) its plasma membrane can be prepared in very pure form, free of contaminating intracellular membranes; 3) vesicles can be prepared with normally oriented (right-side-out) or inverted (inside-out) membrane; and 4) proteolytic activity is minimal. We have used vesicles derived from human red cells to compare insulin binding directly to the exterior and cytoplasmic surfaces of the plasma membrane.

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MATERIALS AND METHODS

Materials

Porcine insulin was a gift of Dr M. Root, Eli Lilly Co, Indianapolis. $Na^{125}I$ was purchased from Amersham, Arlington Heights, Illinois. Insulin was monoiodinated with ^{125}I according to the method of Freychet et al [10]. Dibutylphthalate was purchased from Aldrich, Milwaukee.

Preparation of Right-side-out and Inside-out Erythrocyte Vesicles

Erythrocytes were prepared from 50 ml heparinized blood drawn from human subjects fasted overnight. Cells were centrifuged at 4°C and washed free of plasma and leukocytes by repeated (3×) washing with phosphate-buffered saline, pH 8.0, and aspiration of the buffy coat. Alternatively, mononuclear leukocytes were separated from erythrocytes by Ficoll-Hypaque gradient centrifugation [11]. The resulting erythrocyte preparation was >99.99% pure. The cells were lysed in 5 mM Na-phosphate, pH 8.0, and right-side-out and inverted (inside-out) membrane vesicles were prepared according to the technique of Steck and Kant [12]. Aliquots of intact red cells and of both types of vesicles were prepared from each subject in order to minimize individual differences. Membrane sidedness of vesicles was verified in all preparations by measuring acetylcholinesterase activity in the presence and absence of Triton X-100 [12,13] (Table I). The resulting cells or vesicles were either used immediately or stored at 4°C for periods not exceeding 24 h.

Insulin Binding to Membrane Vesicles and Whole Erythrocytes

Right-side-out vesicles, inside-out vesicles, and intact erythrocytes were suspended in "buffer G" (50 mM Tris, 50 mM Hepes, 50 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM glucose, 2 mM EDTA, 1 mg/ml bovine serum albumin, pH 8.0) at appropriate concentrations, and insulin binding was determined at 22°C for 3 h according to Gambhir et al [14]. Porcine insulin was used as both the unlabeled and labeled species. Separation of free from bound radioactivity was effected by centrifugation at ~4°C at 9,000g through a layer of dibutylphthalate in the case of whole red cells [14] and through a layer of cold buffer G in the case of the vesicles, as the vesicles had a density lower than that of dibutylphthalate. Since the three preparations (intact cells, and right-side-out and inside-out vesicles) contain vastly different amounts of hemoglobin, phospholipid rather than protein was used as an estimate of membrane surface. Membrane phospholipid phosphorus was determined by Folch extraction [15] followed by digestion in perchloric acid and measure-

TABLE I. Acetylcholinesterase Activity (nMoles of Product/min/μg Lipid Phosphorus) (Mean ± SE)

	Without Triton X-100	With 0.2% (v/v) Triton X-100
Right-side-out vesicles (n = 7)	55.7 ± 8.6	43.9 ± 8.9
Inside-out vesicles (n = 7)	7.4 ± 1.4	33.4 ± 4.0

ment of inorganic phosphate [16]. In order to exclude the influence on insulin binding of the different procedural complexities needed to produce the three preparations, we equalized the number of diluting and washing cycles in all three preparations. We found that this did not affect our results.

RESULTS AND DISCUSSION

Figure 1 depicts the insulin-binding data obtained on three preparations. Insulin binding to inside-out vesicles was negligible and was not detectable in several preparations. Residual binding in some preparations may be due to minimal contamination of the vesicles with unsealed ghosts or right-side-out vesicles. It should be noted that cholinesterase activity was also approximately 13% accessible in inside-out vesicles (Table I). Insulin binding to whole erythrocytes and to right-side-out vesicles was similar. The somewhat lower binding to right-side-out vesicles is of borderline statistical significance by analysis of variance ($0.1 > P > 0.05$). Scatchard analysis indicates a slightly lower binding capacity in right-side-out vesicles than in whole erythrocytes, with similar binding affinity. It is possible that some receptors were either lost or masked during the preparation of the vesicles.

Maximum binding of ^{125}I-insulin and theoretical maximal insulin-binding capacities for the three preparations are listed in Table II. For intact erythrocytes, this corresponds to approximately 50 insulin-binding sites per cell, in agreement with other published data [17–19].

If the insulin-binding site in inside-out vesicles is inaccessible simply because it is enclosed on the inside of the vesicle, it should be possible to recover binding activity by disruption of the vesicles. Indeed, sonication of inside-out vesicles (5 sec) resulted in recovery of insulin binding to 78% (42.2 + 6.8 fmoles/mg phospholipid

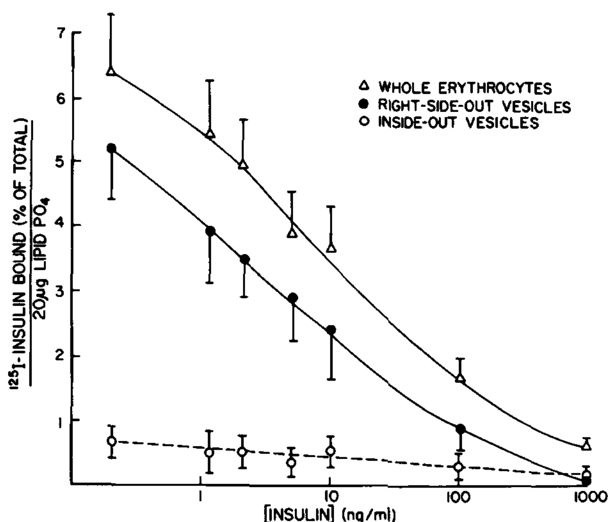

Fig. 1. Specific binding of ^{125}I-insulin to human erythrocytes or erythrocyte membrane vesicles as a function of unlabeled insulin concentration. "Nonspecific binding," defined as radioactivity bound in the presence of 50 μg/ml unlabeled insulin, is subtracted from all data points. The data shown are derived from seven inside-out, seven right-side-out, and six whole preparations. Bars denote SEM.

TABLE II. Insulin Binding per Unit Membrane Surface (femtomoles/mg Lipid Phosphorus)

	Maximum ^{125}I-insulin bound[a] (mean ± SE)	Maximum binding capacity[b]
Erythrocytes	64.4 ± 13.4	9,000
Right-side out vesicles	54.1 ± 7.7	5,350
Inside-out vesicles	5.0 ± 2.2	500

[a]At an ^{125}I-insulin concentration of 0.2 ng/ml.
[b]Derived from Scatchard plots of groups means.

phosphorus) of that observed in right-side-out vesicles. This indicates that binding sites were present but not accessible in inside-out vesicles.

Our data provide direct evidence for the widely held view that the plasma membrane is asymmetrical with respect to peptide hormone binding. In the erythrocyte, the binding site for insulin is exhibited on the outer surface of the membrane, whereas the cytoplasmic surface is virtually devoid of binding sites. We have chosen the erythrocyte as a model since techniques to examine the cytoplasmic surface of the plasma membrane are available for that simplest of cells. It is not entirely clear whether our data are representative for other, more critical cells for insulin action. However, the properties of the red cell insulin receptor are comparable to that found on other cells [14]. Moreover, Bennet and Cuatrecasas [20] have reached similar conclusions with adipocyte membrane vesicles, although the vesicle preparations they obtained from fat cells were less pure and less well defined. Our inability to demonstrate insulin binding to inside-out vesicles argues against significant transmembrane movement ("flip-flop") of the insulin receptor, at least at 22° C. This contrasts with the apparently very rapid exchange of membrane lipid constituents between the two leaflets of the bilayer at either 15°C or 37° C [4, 21]. Our findings provide further support for the concept that the insulin receptor is an integral membrane protein which displays its binding region exclusively toward the extracellular space.

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