PREPARATION OF SOLUBILIZED INSULIN RECEPTORS FROM HUMAN LYMPHOCYTES

by

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SUMMARY. Specific insulin receptors from human lymphocytes in culture have been prepared using the nonionic detergent NP-40. Receptors were solubilized from intact cells and from crude plasma membrane fractions. Insulin receptors prepared in this manner retain many of the characteristics of the receptors studied in intact cells.

We have recently demonstrated the presence of specific insulin receptors in human circulating cells (1). The insulin receptors in isolated peripheral lymphocytes displayed characteristics very similar to those studied in fat cells and liver membranes of rats (2,3,4). Several reports have appeared on the isolation and characterization of solubilized insulin receptors from fat cells and liver from rats, providing evidence that these receptors are localized in the plasma membrane fraction of these tissues (5,6,7). The presence of receptors on cultured human lymphocytes and the availability of large numbers of these cells make it possible to study insulin receptors in tissue of human origin. This report describes the isolation of a solubilized insulin receptor from cultured human lymphocytes using the nonionic detergent NP-40.

MATERIALS AND METHODS

Cultured lymphocytes (RPMI 4265) were grown under conditions previously

described (8) and were separated from growth media by centrifugation at 600 xg for 10 minutes at 4°. The supernatant was decanted; the cell pellet was washed twice with cold 0.01M phosphate-buffered saline (PBS), pH 7.6. The cells (approx. 10^9 total) were then suspended in 10ml of PBS containing 0.5% Shell Nonidet P-40(NP-40) for 10 minutes at 4°C; under these conditions, the cell membranes can be dissolved without breakage of the nucleus (9). The suspension was centrifuged at 600xg to remove unbroken cells and nuclei. The supernatant was then centrifuged at 20,000xg for 60 minutes at 4°C and afterwards was dialyzed for 36-48 hours against several changes of PBS, pH 7.6, at 4°C. The dialysate was then centrifuged at 105,000xg for 2 hours before use in an insulin-binding assay. Alternatively, solubilized receptor was prepared in the following manner: lymphocytes (10^9 total) were suspended in PBS and lysed by rapid freezing in an ethanol-dry ice bath followed by thawing at 37°C three times. The broken cell suspension was then centrifuged at 800xg for 10 minutes at 4°C (10). The resultant supernatant was centrifuged at 20,000xg for 30 minutes in order to obtain a crude plasma membrane pellet as described by Allan and Crumpton (11). Under these conditions, 90% of the insulin binding activity in the "freeze-thaw" supernatant was recovered in the 20,000xg pellet. This pellet was dissolved in PBS containing 0.5% NP-40 at 4°C, exhaustively dialyzed against PBS, pH 7.6, and then centrifuged at 105,000xg for 90 minutes before assay for specific insulin-binding activity. Protein concentrations were determined by the method of Lowry, et al. (12); appropriate controls indicated that NP-40 in concentrations up to 0.5% did not interfere with color development in the Lowry procedure in these studies.

Biologically-active monoidoinsulin was prepared as previously described (1). To determine specific insulin binding, the receptor preparations were incubated with 10^{-11}-10^{-10}M ^{125}I-insulin for 90 minutes at 23°C. "Bound ^{125}I-insulin was separated from "free" by filtration on G-50 Sephadex columns or by adsorption to 50mg tablets of talc (13).

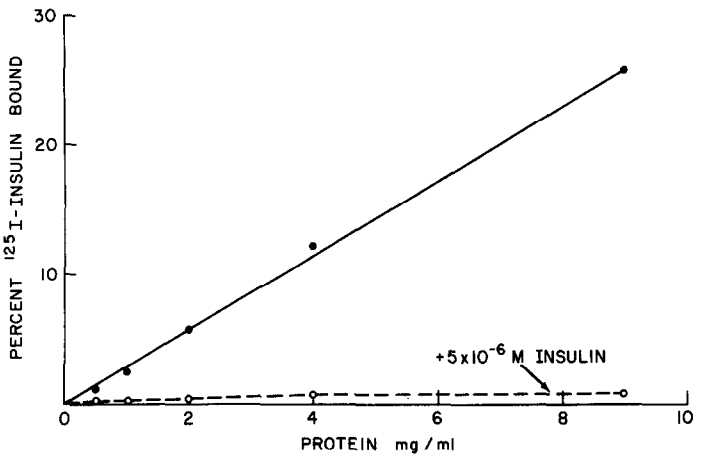

Figure 1. Effect of increasing NP-40 solubilized insulin receptor on specific binding of ^{125}I-insulin. Protein was incubated with labeled insulin for 90 minutes at 23°C in a total volume of 0.3ml. Samples were then diluted to 2ml by the addition of cold PBS, pH 7.6. A 50mg tablet of talc was added to each tube and the mixture was thoroughly vortexed. The tubes were centrifuged for 10 minutes at 2500 rpm at 4°. Supernatants were carefully removed from the packed talc pellets and both were counted in an automatic well-type gamma counter to determine distribution of radioactivity. Under these conditions, the "free" hormone adsorbs to the talc (13). In parallel experiments, PBS alone or PBS containing solubilized receptor protein was thoroughly mixed with 50mg talc before addition of labeled hormone at 4°. Results from these controls indicated that none of the protein concentrations used in these experiments interferred with the adsorption of "free ^{125}I-insulin to talc under the conditions described.

RESULTS AND DISCUSSION

Insulin receptors prepared by both methods outlined above displayed identical characteristics in their interactions with insulin. Specific binding of ^{125}I-insulin was proportional to protein concentration over a twenty-fold range; when native insulin ($5x10^{-6}$M) was included in the incubations, complete inhibition of ^{125}I-insulin binding was observed (Fig. 1). When the receptor preparation was incubated with 10^{-11}M ^{125}I-insulin and the mixture then filtered on a column of G-50 Sephadex (Fig. 2), a significant fraction of the radioactivity appeared in the void volume of the column, suggesting formation of a complex similar to that described by Cuatrecasas (5). Formation of this complex was completely inhibited by the presence of 10^{-5}M unlabeled insulin in an identical experiment (Fig. 2). Identical incubations were performed except that talc tablets were used to separate the insulin-receptor complex from unreacted ^{125}I-

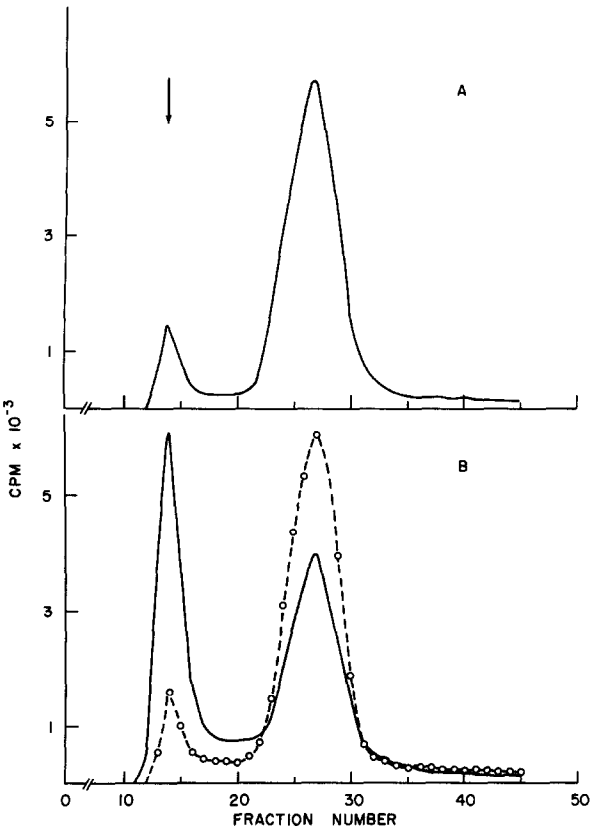

Figure 2. Sephadex G-50 chromatography of labeled insulin bound to NP-40 solubilized receptors from human lymphocytes. Approx. 3mg of protein were incubated with 75,000 cpm ($5x10^{-11}M$) ^{125}I-insulin for 90 minutes at 23°C in a total volume of 0.3ml. The sample was made 3% in sucrose and applied to a column (1x30cm) of Sephadex G-50 previously equilibrated at 4°C with Krebs-Ringer-phosphate buffer containing 0.1% serum albumin. The flow rate was 5ml/hr. ^{125}I-insulin was filtered alone (A), and after incubation with receptor protein (B, solid line). Similarly, protein and ^{125}I-insulin were incubated in the presence of $10^{-5}M$ native insulin and applied to the column (B, broken line). When the unretarded peak (B, solid line, Fractions 13-15) was refiltered on this column, 80% of the radioactivity applied emerged in the void peak, and the remainder appeared in the peak corresponding to ^{125}I-insulin (data not shown). The arrow marks position of void volume determined with Blue Dextran 2000 (Pharmacia). The small amount of radioactivity appearing in the void upon filtration of labeled insulin alone is most likely damaged hormone and did not fluctuate significantly.

insulin (Table 1). Good agreement was found between the two methods.

The biologic specificity of the insulin binding interaction was examined by incubating receptor with insulin, insulin derivatives, and other peptides. The data indicated that the ability of a preparation to inhibit the binding of

Table 1. Gel filtration vs. talc adsorption in the separation of "bound" and "free" ^{125}I-insulin after incubation with solubilized receptor.

Expt.	Method	^{125}I-insulin(cpm)	Receptor(mg)*	Bound(cpm)
1	G-50	2×10^4	0.35	1.45×10^3
	Talc	2×10^4	0.35	1.32×10^3
2	G-50	7.2×10^4	3.75	1.73×10^4
	Talc	7.2×10^4	3.75	1.88×10^4

*Represents crude solubilized receptor preparation from whole cells or from crude plasma membrane as described in Methods.
Receptor was incubated with labeled insulin for 90 min at 23°. The mixtures were then applied to a column of Sephadex G-50 as described in figure 2 or diluted with cold PBS and treated with 50 mg talc as described in figure 1.

^{125}I-insulin to solubilized receptor varied in a manner proportional to its biologic activity. These results were indistinguishable from those obtained in studies with intact cells (1), suggesting that the solubilized receptors retain many, if not all, of the characteristics of the receptors found on the unbroken cell.

The receptor described in this report was not sedimented upon prolonged sedimentation at 200,000xg nor was it retained by repeated passage through Millipore filters with 0.2μm pores (5). The data suggest that the insulin binding interactions described here closely resemble those observed in whole cells and in similar detergent-solubilized extracts from fat and liver cell membranes (5,6,7). The cultured human lymphocytes provide a readily-available source of tissue for the isolation of the insulin receptor and perhaps for the specific receptors of other peptide hormones as well (8). The simplicity of the solubilization process using NP-40 and intact cells makes this a very convenient

Table 2. Effect of insulin, insulin derivatives, and other peptides on binding of ^{125}I-insulin to NP-40 solubilized receptor from RPMI 4265 human lymphoid cells.

Peptide	Conc. (ng/ml)	^{125}I-insulin Bound (cpm)
None	-	6304
Insulin	3	4520
	10,000	310
Desoctapeptide	50	6250
	500	4300
Desala. Desasp.	50	6430
	500	4150
Pork Proinsulin	100	3185
Pentagastrin	10,000	6510
ACTH	10,000	6350
A-Chain	10,000	6210
B-Chain	10,000	6120

3mg of protein were incubated with 10^{-11}M ^{125}I-insulin in the presence of peptides listed for 90 min at 23° in a total volume of 0.3 ml. Samples were diluted to 2ml with cold PBS, pH 7.6, and treated with talc as described in the legend of figure 1. Results of two experiments were combined.

procedure for preparation of solubilized receptors, although the effect of residual detergent bound to the soluble material is not known at this time. It is of interest that the detergent solubilized insulin receptor from lymphocytes did not precipitate upon extensive dialysis in PBS. This is in contrast to the findings reported for liver and fat cell receptors (5,6,7). The reason for this difference is not known.

While these studies on detergent solubilized insulin receptors from lympho-

cytes were in progress, we obtained evidence in other studies suggesting that these receptors could be prepared in soluble form without the use of detergents or related compounds (14). To date, we have prepared small quantities of receptor in the latter fashion and it may soon be possible to compare directly receptors from the same tissue isolated with and without the use of detergents. It is hoped that these studies will further contribute to our understanding of the nature of the interaction of insulin with its native specific receptor as the first step in its mechanism of action.

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