BBA 76033

INSULIN-STIMULATED PLASMA MEMBRANES FROM RAT ADIPOCYTES: THEIR PHYSIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES

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(Received February 21st, 1972)
(Revised manuscript received June 9th, 1972)

SUMMARY

Using methods previously described, we have isolated plasma membranes from insulin-treated adipocytes of the rat epididymal fat pad. Such membranes showed an accelerated uptake and release of D-glucose when compared with similar preparations from cells not exposed to the hormone. The only change observed was in the rate of D-glucose uptake; at equilibrium, D-glucose space was identical in both preparations. Vigorous alkaline hydrolysis of the insulin resulted in loss of its typical effect on both intact cells and derived plasma membranes. Anti-insulin antiserum, potent enough to inhibit the effect of the hormone when both were added to intact cells, did not prevent the insulin effect when added to plasma membranes prepared from insulin-treated cells. Addition of insulin directly to plasma membranes was without effect; exposure of cells prior to rupture was required.

Studies of infrared spectra, native membrane protein fluorescence, and fluorescence of 8-anilino-1-naphthalene sulfonate added to the membranes, showed no differences between control plasma membranes and those prepared from insulin-treated cells.

We conclude that: (1) plasma membranes can be prepared from insulin-treated fat cells which retain an enhanced glucose transport; (2) the effect of insulin on glucose transport does not involve large scale changes in the structure of the plasma membrane; (3) the insulin unresponsiveness of isolated plasma membranes, as well as the resistance to anti-insulin serum of membranes prepared from insulin-treated cells, appears to result from an uncoupling of insulin binding from glucose transport, the basis of which requires further exploration.

INTRODUCTION

Recent work has indicated that many of the effects of insulin derive from the interaction of the hormone with the surface membrane of the target cell. This con-

Abbreviation: ANS, 8-anilino-1-naphthalene sulfonate.

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clusion is strongly supported by the observation that insulin covalently bound to large agarose polymers is capable of stimulating glucose uptake and inhibiting free fatty acid release by isolated adipose cells to an extent similar to native insulin, despite the inaccessibility of the insulin–sepharose complex to the cell interior[1].

The insulin activation of transmembrane glucose transport in adipocytes appears to proceed in a number of distinguishable steps. First, insulin is non-covalently but specifically bound to a surface receptor site[2]. This saturable, high affinity binding parallels closely the activation of glucose transport. The binding of insulin can, however, be dissociated from the subsequent metabolic effects[3]. Digestion of adipocytes with a variety of neuraminidase preparations, under certain restricted conditions, results in abolition of the insulin stimulated increment in glucose uptake without alteration in insulin binding, basal glucose uptake or cellular leakiness. Thus, the sialic acids residues cleaved under these circumstances are not critical to the insulin recognition function, but are intimately involved in the "transduction" of insulin binding into accelerated glucose transport. The nature of the molecular species involved in this "transduction" and the manner in which they function are unknown. On the basis of earlier, indirect data[4] it had been suggested that insulin may lead to a rearrangement in the structure of regions of the plasma membrane, with consequent alterations in glucose transport and other membrane functions.

We have previously reported that microsomes, prepared from disrupted isolated fat cells, contain an intact glucose transport system that can be measured by a simple assay[5]. Furthermore, microsomes prepared from cells exposed to insulin prior to rupture showed an enhanced rate of glucose uptake as compared to control microsomal preparations[6]. Recently this glucose transport system has been demonstrated to reside in the plasma membrane fraction, as expected[7]. In this report, we describe the properties of insulin stimulated glucose transport in purified adipocyte plasma membrane vesicles. Furthermore, using a number of spectroscopic techniques, we have attempted to compare the structural characteristics of plasma membrane particles which have been functionally activated by insulin with those not exposed to the hormone.

MATERIALS AND METHODS

Isolated rat epididymal fat cells were prepared by the method of Rodbell[8]. Fat cell plasma membrane was isolated as described by McKeel and Jarett[9] with minor modifications[10]. The preparation and properties of sonicated fat cell plasma membranes are detailed elsewhere[7]. The measurement of glucose uptake and release by fat cell plasma membrane particles was accomplished by a millipore filtration technique previously described (Fig. 1 or ref. 5). In experiments comparing the glucose transport and/or spectroscopic characteristics of plasma membranes from insulin-treated cells with similar preparations from fat cells not exposed to insulin, the following procedure was employed: a single pool of isolated adipocytes was divided into two portions, one portion was exposed to insulin (1 munit/ml) at 37 °C for 15 min, the other portion was incubated in parallel in the absence of insulin. Both cell suspensions were then washed twice in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), resuspended and homogenized. Plasma membranes were isolated and subjected to brief sonication[7] except where indicated. A portion of the plasma membrane fraction from

both control and insulin treated cells ("insulin membranes") was taken for assay of glucose uptake. The remainder was utilized for spectroscopic experiments. In all preparations used for spectroscopic comparisons, an enhanced glucose uptake in the insulin exposed membranes, relative to the parallel control membranes, was demonstrated.

Infrared spectra were obtained with a Perkin Elmer Model 221 spectrophotometer, from plasma membrane in the freeze-dried state. Unsonicated plasma membrane preparations were washed free of sucrose by suspension in 5 mM sodium phosphate (pH 8.0) and centrifuged at 100000 × *g* for 30 min. This procedure was repeated twice. The pellet from the last wash was resuspended in the same buffer to a concentration of 1–2 mg protein per ml. 25 μl was placed on a AgCl disc (Harshaw Chemical Co.) in a rectangular film, and the disc was immediately immersed in liquid nitrogen. The frozen films were then lyophilized. The freeze-dried films were stored overnight *in vacuo* over anhydrous $CaSO_4$.

Fluorescence measurements were performed with a Hitachi Perkin Elmer spectrofluorometer, MPF 2K, at 23 °C. Excitation and emission spectra were obtained of plasma membrane vesicles suspended in 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) or membranes in this solution added to Krebs–Ringers phosphate buffer. Excitation spectra were recorded with emission and excitation slits of 2 and 6 nm, respectively. Emission spectra were obtained with excitation and emission slits of

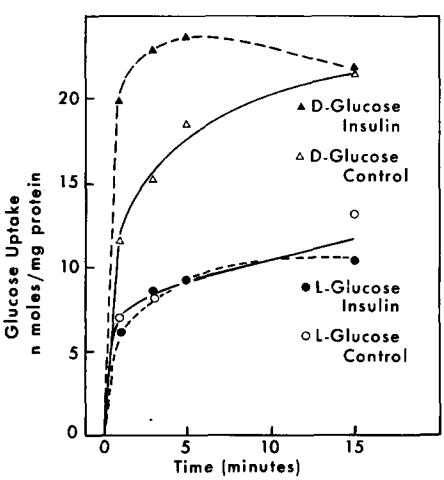

Fig. 1. Retention of insulin effect in plasma membranes. Isolated membranes from insulin-treated and control cells were assayed for uptake of D-[^{3}H]glucose and L-[^{14}C]glucose. All incubations were carried out at 20 °C except where indicated. To 0.225 ml of membrane suspension (approximately 0.3 mg/ml, in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4), 0.025 ml of an equimolar solution of radioactive D- and L-glucose was added: (final concentration of 5 mM each). At the times indicated aliquots (0.05 ml) of the suspension were filtered rapidly over a pre-chilled millipore filter (pore size 0.45 μm). The pellet was then washed rapidly on the filter with 10 drops of ice cold Krebs–Ringer phosphate buffer with one-half the usual concentration of calcium[12]. The filters were then transferred directly to counting vials. The membranes were dispersed in 0.2 ml distilled water, and the radioactivity determined in 10 ml of Buhler's scintillation solution[13]. Specific activities were determined by counting appropriate dilutions of the labelled sugars. In the experiment shown, D-glucose uptake had reached equilibrium at 15 min, whereas L-glucose uptake continued for several hours.

4 and 4 nm, respectively, unless otherwise specified. Spectra presented are uncorrected for variations in excitation energy or phototube sensitivity.

Estimation of the binding and fluorescence enhancement of 8-anilino-1-naphthalene sulfonate (ANS) by adipocyte plasma membranes were made as follows. Single 70–300 μl aliquots of sonicated or unsonicated plasma membranes (as noted in each experiment) suspended in 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) were added to fluorescence cuvettes containing 1.7 ml of Krebs–Ringers phosphate buffer (pH 7.4) and the final volume was brought to 2.0 ml by the addition of sucrose Tris buffer. Stock solutions of ANS in water were added in 10-μl aliquots and the cuvettes were mixed by inversion. Excitation wavelength was 360 nm (slit width, 6 nm) and fluorescence was recorded at 470 nm (slit width, 10 nm) using a 43 filter. Appropriate corrections were made for light scattering and dilution. To determine the maximal enhancement of ANS fluorescence by adipocyte plasma membrane, double reciprocal plots of membrane protein concentration *versus* fluorescence were constructed at a variety of ANS concentrations between 3–29 μM. Extrapolation to infinite protein concentration yielded the fluorescence of totally bound ANS. Using these values in terms of fluorescence per μmole bound fluor, for each protein concentration, the amounts of free and bound ANS, at a number of different total ANS concentrations, were calculated from the observed fluorescence. To obtain the association constant and number of ANS binding sites, this data was analyzed by the method of Scatchard[11]. All data was analyzed by least squares analysis.

ANS was obtained from Eastman Chemical Co. and recrystallized 4 times from water. Porcine insulin (lot No. PJ 5589) was kindly provided by Dr Mary Root of Eli Lilly Company. Anti-insulin antiserum was obtained from Arnel Products.

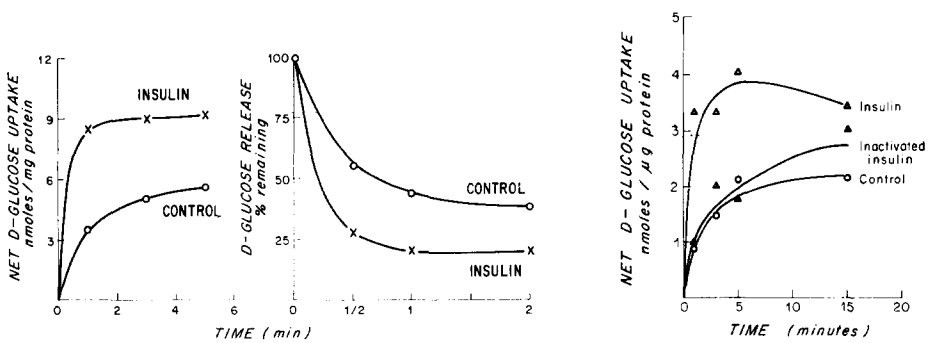

Fig. 2. The effect of insulin on net D-glucose uptake and release from plasma membranes. Left: isolated plasma membranes from insulin-treated and control cells were assayed for uptake of D-[^{3}H]glucose and L-[^{14}C]glucose[7] as in Fig. 1. The excess of D- over L-glucose or "net D-glucose uptake", for each time point is shown. Right: aliquots of the same membrane preparations were preloaded for 1 h with D-[^{3}H]glucose and the rate of glucose release measured following dilution in buffer with no glucose[7]. Figures are given as the percentage remaining in each preparation at the indicated time following dilution.

Fig. 3. Loss of insulin effect following alkaline hydrolysis. Insulin (10 units/ml) was made 1 M in NaOH and heated to 80 °C for 2 h, then cooled and neutralized with 1 M HCl. Intact adipocytes were exposed to this preparation (1 munit/ml), untreated insulin (1 munit/ml) or no insulin for 15 min at 37 °C, disrupted, and the plasma membranes isolated and assayed for net D-glucose uptake.

RESULTS

Effects of insulin on plasma membrane preparation

Although the yield of plasma membrane protein varied from day to day, within a single experiment the yield of plasma membrane protein from control and insulin treated adipocytes did not differ significantly. In the seven experiments in which total recovery of plasma membrane protein was measured, this was 0.371 mg for control cells, 0.313 mg for insulin-treated cells (evaluated as paired samples, $P > 0.1$). Furthermore, polyacrylamide-gel electrophoresis in 1% sodium dodecylsulfate of plasma membrane preparations from control and insulin treated adipocytes yielded identical peptide staining patterns[12].

Effects on glucose transport

Pre-incubation of isolated intact adipose cells with insulin led to an accelerated rate of both D-glucose uptake (Fig. 1, Fig. 2, left) and release (Fig. 2, right) by the isolated plasma membrane vesicles. The effect was specific for D-glucose since neither the uptake (Fig. 1) nor release of L-glucose was significantly changed in the insulinized membranes as compared to the control membranes.

This effect of insulin appeared to be entirely on the rate at which D-glucose was taken up and not on its final volume of distribution, since with incubations longer than 10 min (Fig. 1), the final or equilibrium level of D-glucose taken up per mg of protein was the same for plasma membrane vesicles from insulin-treated and control adipocytes. Similarly, in incubations carried out to 5 h (because several hours were required for L-glucose uptake to reach equilibrium), we observed that D- and L-glucose eventually reached equal concentrations in control and insulin-treated membranes.

To demonstrate the requirement for "native" hormone, we carried out prolonged alkaline hydrolysis of insulin. Whole cells exposed to this preparation showed essen-

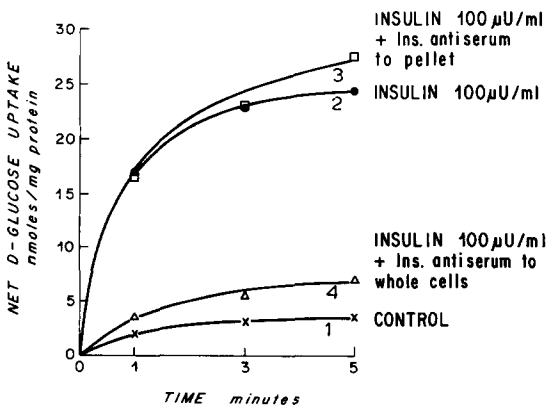

Fig. 4. Effect of anti-insulin antiserum on insulin-stimulated glucose uptake by adipocyte plasma membranes. Intact adipocytes were divided into four equal aliquots and treated as follows: (1) no insulin; (2 and 3) insulin (100 μnits/ml) for 15 min at 37 °C; (4) insulin (100 μnits/ml) for 5 min, then excess antiserum added and incubation continued for 10 min. All cells were disrupted and plasma membranes isolated. To No. 3, excess antiserum was added to the plasma membranes 5 min before the uptake assay (incubation at 20 °C). All membranes were then assayed for uptake of D-[^{3}H]glucose and L-[^{14}C]glucose. Excess or net D-glucose uptake is shown.

tially no stimulation of glucose uptake as measured by $^{14}CO_2$ production from D-[U-^{14}C]glucose (data not shown). Plasma membranes prepared from cells exposed to degraded hormone likewise showed no acceleration of the rate of entry of D-glucose (Fig. 3), whereas native insulin resulted in the usual stimulatory effect.

To evaluate the reversibility of the change(s) induced by insulin, membranes prepared from cells exposed to insulin were incubated with anti-insulin antiserum prior to measurement of glucose uptake. There was no inhibition of insulin-stimulated glucose uptake when compared with insulin membranes not exposed to antiserum (Fig. 4). The potency of the antibody was proved by exposing insulin-treated whole cells to the antiserum prior to disruption; under these conditions, anti-insulin serum abolished the insulin-induced increment in glucose transport both in the intact fat cells as well as in plasma membranes prepared from these cells.

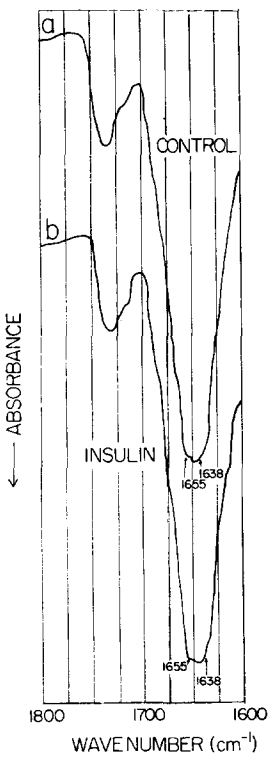

Fig. 5. Infrared spectra of the Amide I band of adipocyte plasma membrane. (a) Membrane preparations from cells not exposed to insulin. (b) Membrane preparation from insulin-exposed cells.

We have made many attempts to observe an effect of insulin on glucose transport when added directly to plasma membrane preparations; these have been uniformly unsuccessful. Membranes suspended in isotonic NaCl, isotonic KCl, Krebs–Ringer bicarbonate, or the usual sucrose–phosphate medium fail to respond to high levels of the hormone (1–100 munits/ml). Addition of a lyophilized, redissolved high speed supernate (100000 × *g*, 30 min) prepared from fat cell homogenates did not cause

the membranes to be responsive to the hormone. Only when insulin was added to whole cells prior to disruption was there a stimulation of glucose transport in plasma membrane vesicles.

Spectroscopic studies

(*1*) *Infrared spectroscopy*. An infrared spectrum in the region of the Amide I band of fat cell plasma membrane is shown in Fig. 5a. The salient features are the peak near 1650 cm^{-1} with shoulders at 1655 cm^{-1} and 1638 cm^{-1}. In Fig. 5b is shown the infrared spectrum of fat cell plasma membrane prepared from a part of the same pool of adipose cells as in Fig. 5a but exposed to insulin prior to disruption. While the membrane fraction from the insulin-treated cell showed a significantly greater glucose uptake, there were no alterations in the appearance of the Amide I band compared to control. This finding was confirmed in three additional experiments.

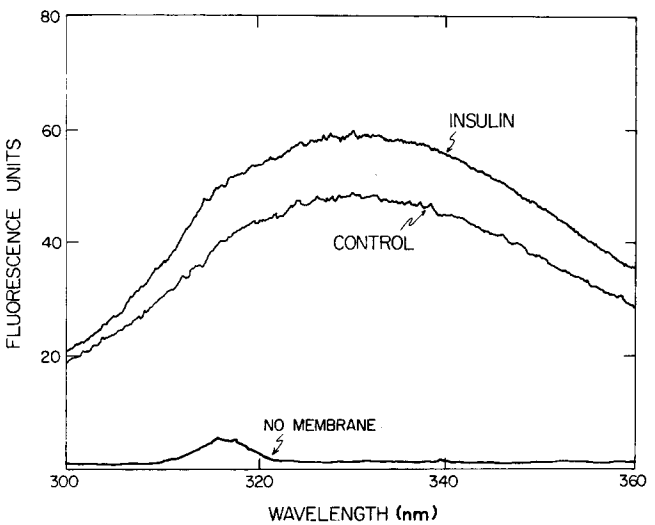

Fig. 6. Comparison of the emission spectra of control *vs* insulin-exposed plasma membrane preparations. Excitation is at 287 nm (spectra uncorrected).

(*2*) *Fluorescence spectroscopy*. (a) Native membrane protein fluorescence. Emission spectra of adipocyte plasma membrane (unsonicated) were obtained with excitation at 275, 287 and 292 nm. These spectra all showed a single broad peak at approximately 332 nm, without evidence of a shoulder in the region of 300–315 nm (Fig. 6). In addition, with excitation at these wavelengths, the fluorescence emission intensity measured at 308 nm changed in a parallel fashion to that measured at 332 nm (and 350 nm). At the same protein concentrations, emission spectra obtained in 0.02 M Tris–HCl (pH 7.4) and Krebs–Ringer phosphate buffer (pH 7.4) were essentially identical (Fig. 7a,b). Emission spectra of adipocyte plasma membrane suspended in Krebs–Ringer phosphate buffer, obtained from insulin-treated and control fat cells, showed identical characteristics in three experiments (Fig. 6). When normalized for protein concentration or arbitrary light scattering units, the fluorescence emission intensities at 332 nm were the same. The insulinized membrane vesicles did, however, show increased glucose uptake with respect to control preparations.

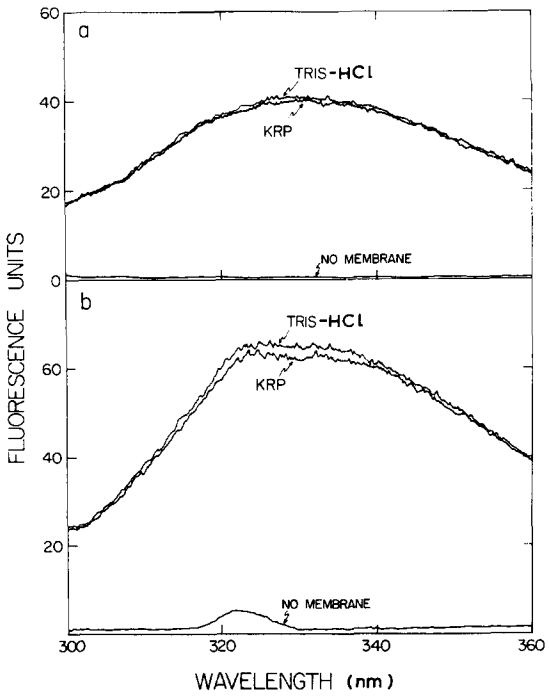

Fig. 7. Fluorescence emission spectra of adipocyte plasma membrane suspended in 0.02 M Tris–HCl (pH 7.4) and Krebs–Ringer phosphate buffer (pH 7.4). (a) Excitation at 275 nm. (b) Excitation at 292 nm. The shoulder near 323 nm is seen in (b) is due to Raman scattering of the buffer and particulate suspension (spectra uncorrected).

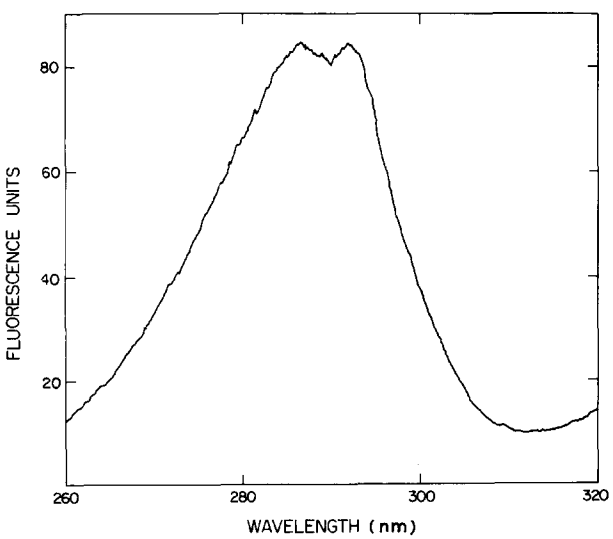

Fig. 8. Excitation spectrum of adipocyte plasma membrane suspended in 0.02 M Tris–HCl (pH 7.4). Emission recorded at 332 nm (spectrum uncorrected).

Insulin (final concentration 100 μunits/ml), when added to adipocyte plasma membranes previously unexposed to the hormone, did not alter the membrane emission spectrum.

Excitation spectra of adipocyte plasma membrane vesicles showed 2 peaks, at 287 nm (corrected: 278 nm) and 292 nm (corrected: 284 nm) (Fig. 8), whether the emission was recorded at 332 nm or 308 nm.

(b) Fluorescence probe studies. The addition of adipocyte plasma membrane vesicles to an aqueous solution of ANS resulted in a marked enhancement of ANS fluorescence and a shift of the emission maximum from 515 to 470 nm. At a given ANS and protein concentration, the fluorescence was further increased by increasing ionic strength (up to 0.2 M NaCl), increasing divalent cation concentration (up to 0.005 M $CaCl_2$) and decreasing pH. The effect of these parameters on ANS binding was not systematically investigated. Limiting enhancement of ANS fluorescence was estimated by extrapolation of a series of double reciprocal plots of fluorescence *versus* membrane protein concentration to infinite protein concentration, where all the fluor would be bound (Fig. 9). These results indicated that the fluorescence of membrane bound ANS was increased approximately 155-fold over that in aqueous solution.

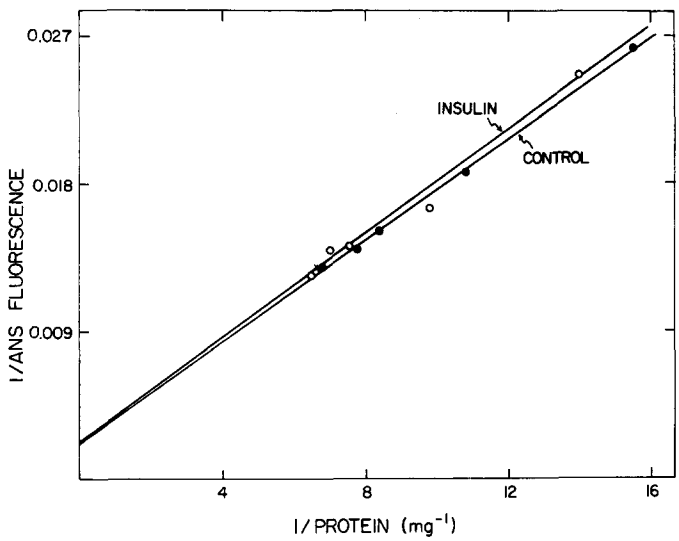

Fig. 9. Double reciprocal plot of ANS fluorescence *vs* membrane protein concentration for insulin-exposed (○) and control (●) plasma membrane preparations. Data shown was obtained at 9.3 μM ANS. Identical experiments were also performed at 3.1, 6.2, 12.3, 15.3 and 28.7 μM ANS.

This value was the same for sonicated and unsonicated plasma membrane preparations. Scatchard plots were used to compute the binding parameters for ANS and adipocyte plasma membrane (Fig. 10). Values obtained at a variety of protein concentrations are summarized in Table I. Comparison of plasma membrane preparations from control and insulin-treated fat cells with respect to degree of fluorescent enhancement of ANS, the number of ANS binding sites and the apparent association constant did not reveal any differences.

TABLE I

BINDING PARAMETERS FOR ANS AND ADIPOCYTE PLASMA MEMBRANES

For each experiment, values for n (number of ANS binding sites/g of protein) and K (association constant) were calculated for control and insulin-exposed membranes from Scatchard plots performed at six protein concentrations. The mean and S.D. was calculated for each set of six values; results from control and insulin exposed membranes were compared by Student's t test. None of the paired values were significantly different at the 0.05 level. Limiting enhancement of fluorescence was obtained from plots such as in Fig. 9. Each value is the mean of the extrapolated fluorescence of five such plots at different ANS concentrations. In Exp. 1, the plasma membrane was not sonicated; in Expt 2, both insulin and control fractions were sonicated.

	n (*mean* $\pm$ *S.D.*)	K (μM) (*mean* $\pm$ *S.D.*)	*Limiting fluorescence enhancement (-fold)*
Expt 1			
Control	70.5 $\pm$ 6.7	35.5 $\pm$ 4.4	152
Insulin	74.8 $\pm$ 6.6	33.0 $\pm$ 3.0	177
Expt 2			
Control	52.7 $\pm$ 6.6	51.2 $\pm$ 7.1	155
Insulin	49.0 $\pm$ 3.1	47.2 $\pm$ 2.6	140

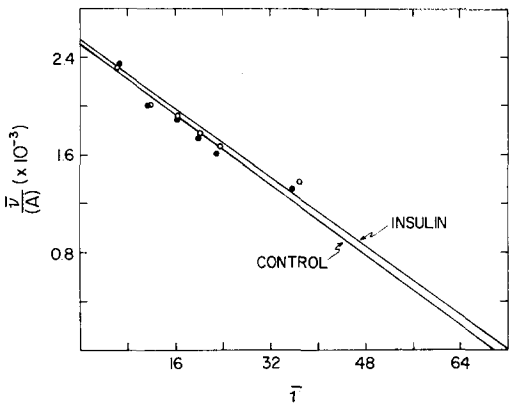

Fig. 10. Scatchard plot of ANS binding to control (0.092 mg/ml) (●) and insulin-exposed (0.102 mg/ml) (○) plasma membrane preparations.

DISCUSSION

We have previously shown[5] that isolated adipocyte membranes retain their ability to transport glucose without further metabolising the sugar. The plasma membrane preparation used in the present studies, which is in the form of small closed vesicles, is able to transport glucose in a manner similar to that observed in intact cells[7]. Insulin pretreatment of the intact cells resulted in an enhanced rate of entry of D-glucose into the intravesicular space of the subsequently isolated plasma membrane particles (Fig. 1) without affecting the final concentration achieved at equilibrium (Fig. 1). This was anticipated, since D-glucose transport in intact adipose cell appears to involve simple facilitated diffusion without the capacity to concentrate against a gradient[13].

Several observations strongly indicate that this effect reflects changes in D-glucose transport *per se*, rather than alterations in the composition, gross morphology or non-specific permeability of the "insulinized" membranes:

(1) The essentially identical recovery of plasma membrane protein and electrophoretic pattern of membrane peptides demonstrate that insulin treatment of adipocytes does not detectably alter the fractionation of the plasma membrane or the protein complement of the isolated membrane vesicles.

(2) The following considerations indicate that the average vesicle volume is similar for control and "insulinized" plasma membrane preparation: At equilibrium, the intravesicular glucose concentration equals that in the bathing medium, and the total intravesicular glucose content is a direct function of the total intravesicular volume. Under these conditions, the amount of glucose retained on the millipore filter reflects the total intravesicular volume retained. Furthermore, in the absence of variable contamination by cytoplasmic proteins (a contingency excluded by the electrophoretic analyses) membrane protein content is a reasonable approximation of membrane surface area. Thus at equilibrium, the ratio of intravesicular glucose content per mg membrane protein is a direct function of average vesicle volume. As can be seen in Fig. 1 the D-glucose uptake per mg protein at equilibrium is the same for control and "insulinized" plasma membranes.

(3) L-Glucose enters the fat cell by passive diffusion. Insulin treatment of adipocyte does not selectively alter the non-specific permeability of the fat cell plasma membrane to sugars, since the rate of entry of L-glucose into control and "insulinized" plasma membrane vesicles is identical (Fig. 1). The stimulation of glucose uptake we observe in "insulinized membranes", thus appears to be the same phenomenon as the effect of the hormone on intact adipose cells[14].

The data presented in Fig. 2 indicate that insulin stimulated both the uptake and exit of sugar from plasma membranes vesicles. This is consistent with the enhancement of bidirectional glucose flux seen in the intact cell[15]. However, our efflux experiments are open to another possible interpretation. In the case of red blood cell ghosts[16], it has been shown that plasma membrane vesicles may form with a "right side out" or "inside out" orientation by exocytosis or endocytosis, respectively, according to the conditions under which the ghosts are incubated. In the absence of specific markers for the external surface of the adipose cell, we have no way of determining the orientation of vesicles formed from adipocyte plasma membrane; if there is a significant fraction of these vesicles in the "inside out" orientation, the enhanced exit rate observed with the "insulinized membranes" might still reflect transport from the outer to the inner surface. The insulin binding site may provide a marker suitable for the resolution of this question[2].

When plasma membrane glucose transport is enhanced by insulin treatment of intact fat cells, the new functional state exhibits considerable stability; insulin augmented glucose transport survives cell disruption and, when examined in subsequently isolated plasma membrane vesicles, can no longer be reversed by anti-insulin serum. While the basis for this stability is unknown (see below), the ability to prepare purified membrane fractions which retain the insulin effect has permitted us to compare insulin-activated membranes with those in the basal state for structural differences.

A number of laboratories[17–19] have reported that rather non-specific perturba-

tions of the fat cell plasma membrane including partial digestion by lipolytic and proteolytic enzymes, can mimic insulin action in terms of stimulating glucose metabolism and inhibiting fatty acid release. This, *plus* the multiple "primary" effects of the hormone, has led Rodbell *et al.*[20] to postulate that insulin may act by causing a transformation of membrane phospholipids from a laminated to a micellar form, "possibly because of alterations in the conformation of the protein layer of the membrane". We have examined the composite protein secondary structure in adipocyte plasma membrane vesicles by infrared spectroscopy, and have attempted to monitor changes in the hydrophobic regions of the membrane by the use of the fluorescent probe ANS. In addition, we have looked for changes in the micro-environment of the membrane protein aromatic residues as reflected in their fluorescence properties. No differences have been detected by these means between plasma membrane fractions which have been prepared from fat cells treated with insulin and which continue to show its functional effect, and membranes prepared from untreated cells. To assess the significance of these findings, the limitations and assumptions in these methods must be mentioned.

The infrared absorption referred to as the Amide I band arises primarily from the C=O stretch vibration of the amide group of the peptide bond[21]. Its position is known to be dependent on the protein secondary structure, with α-helical and unordered conformations absorbing at 1650–1660 cm^{-1}, while β conformations have this band near 1630–1635 cm^{-1} (refs 22 and 23). Adipocyte plasma membrane appear to be composed of all three conformations; detailed evidence for this has been presented elsewhere[24]. While infrared spectra yield specific molecular information on protein structure, three factors should be considered in their evaluation. First, the requirements for sample preparations are rather vigorous. This is necessitated by the strong absorption of H_2O in the region of interest[22]. Also, the plasma membrane contains a mixture of proteins, and the spectra give only the average conformation. Finally, this technique is insensitive to helix-coil transitions[21]. However, infrared spectroscopy has been used to monitor conformational changes in mitochondrial membrane proteins in association with transitions in their respiratory state[25]. Also changes in the conformation of proteins in the red blood cell membrane have been described in association with ATPase activity[26]. The infrared changes in both these instances have been primarily the transition to β structure, especially anti-parallel β (1690 cm^{-1}). With the above reservations, the action of insulin does not appear to involve such a change in the average secondary structure of the membrane protein.

The fluorescence emission spectra of adipocyte plasma membranes are typical of tryptophan in proteins[27]. The presence of the peak at 332 nm (uncorrected, compare with 348 nm for free tryptophan at the same instrumental settings) indicates that a large number of these residues are in an apolar environment. The spectra provide no evidence of tyrosine fluorescence, since the form of the emission spectrum is unchanged by excitation at 275, 287 or 292 nm[27]. The excitation spectra, however, indicate the presence of two aromatic species contributing to the fluorescence. Several explanations are possible. The two species may be tyrosine and tryptophan; the absence of tyrosine fluorescence may be due to transfer of excitation energy from tyrosine to tryptophan. Alternatively, since no data is currently available on the amino acid composition of adipocyte plasma membrane proteins, the possibility must be considered that tyrosine is not present or if present, its fluorescence is quenched. In

either case, the two absorbing species may be different populations of trytophan residues. These possibilities cannot be further distinguished at present.

The fluorescence properties of aromatic residues in proteins are highly sensitive to their environment. Thus, the monitoring of native protein fluorescence has been extensively used to follow conformational perturbations induced in a variety of proteins (for review, see ref. 27). The emission spectra of insulin-treated adipocyte plasma membrane and control preparations are identical in form. Because of the difficulty in comparing the fluorescence intensity of two different membrane preparations, small ($< 10\%$) differences in emission intensity cannot be ruled out. Despite this reservation, the results indicate that no major change occurs in the environment of membrane peptide aromatic residues in association with the action of insulin.

Certain fluorescent probes such as ANS fluorescence relatively weakly in aqueous solution, but show a marked increase in quantum yield of fluorescence and a shift to the blue of their emission spectra upon solution in media of low dielectric constant[28]. When proteins, aqueous dispersions of phospholipids or biological membranes are added to solutions of ANS, a large increase in fluorescence occurs[29,30]. This presumably corresponds to the binding of a portion of the ANS to low dielectric (hydrophobic) regions of these structures, otherwise unspecified. While the concept of a "low dielectric" region on the molecular level is open to debate[31], it is likely that membrane-bound ANS is highly restricted in its interaction with the aqueous environment. In general, changes in membrane structure may result in changes in the quantum yield of bound ANS and/or changes in ANS binding parameters[32]. The measurement of the change in fluorescence enhancement, while very non-specific in terms of information concerning molecular structure, is very sensitive to changes in the environment of the probe. It is possible, however, that bound ANS may itself disturb membrane organization. We have no evidence on this point with respect to adipocyte plasma membrane. Fluorescent probes have been used to follow changes in axonal membranes associated with the passage of action potentials[33] as well as respiration-related membrane changes in mitochondria[34]. Adipocyte plasma membrane interacts with ANS in a manner analogous to that described for other membrane preparations. The maximal fluorescence enhancement and calculated number of binding sites is similar to that of erythrocyte membranes[35]. Membranes from insulin-treated fat cells show identical fluorescence enhancement and ANS binding when compared with control preparations. This does not support the suggestion that insulin action is associated with widespread alterations in the structure of the hydrophobic regions of the membrane.

The ability to demonstrate the presence of a physiological effect of insulin in a relatively purified plasma membrane preparation has allowed examination of this membrane for hormone-related, large scale changes in structure using spectroscopic techniques. No evidence for such changes has been found. The possibility that insulin's action results in structural changes in relatively circumscribed areas of the plasma membrane, such as near its binding site, has not been ruled out. The structural basis of the enhanced glucose transport in plasma membranes derived from insulin treated cells is under continued investigation.

We have been unable to demonstrate any effect on glucose uptake by the addition of high concentrations of insulin directly to isolated plasma membrane preparations. Only when intact cells are exposed to the hormone prior to disruption is the

effect observed. The ineffectiveness of insulin added after cellular disruption is consistent with earlier studies on a variety of insulin-sensitive cellular functions[36-38]. As noted above, the ability to reverse the insulin stimulation of glucose transport by the addition of anti-insulin serum is lost in going from intact cell to isolated membrane. These observations require further analysis. The purified plasma membrane fraction used in the present studies retains the glucose transport function *per se*. Thus, the inability of this preparation to respond to insulin is due either to a compromise in the binding of insulin, or in the processes which couple this binding to glucose transport. Cautrecasas[2] has reported that the insulin binding function of intact fat cells is quantitatively recovered in a particulate, non-nuclear fraction after cellular homogenization. Furthermore, Crofford[39] has demonstrated that fat cell plasma membranes, prepared by a technique[9] essentially identical to that used in the present studies, were able to remove native insulin from the medium, presumably through binding. While we have not yet determined the insulin binding activity or orientation ("sidedness") of our membrane preparation, we consider it unlikely that insulin binding has been lost.

The intactness of the insulin binding function does not in itself ensure that a hormonal response will be elicited. Sialidase treatment of fat cells can abolish the insulin stimulated component of glucose transport without affecting insulin binding[3]. This indicates that some step involved in the transmission of insulin binding to glucose transport has been perturbed. The failure of anti-insulin serum to reverse insulin stimulated glucose transport, when added to the isolated membranes, also suggests some dissociation of insulin binding from glucose transport in these particles. In what way might cellular disruption and membrane isolation accomplish such a dissociation? Pertinent to this question is an examination of the properties of fat cell ghosts[40]. This preparation can bind insulin[2] and respond to the hormone with enhanced glucose transport[41]. The hypotonic lysis employed in the preparation of fat cell ghosts, while adequate to release the neutral lipid particles, results in a relatively mild disruption of the fat cell[40]. The resealed vesicles are large, and highly contaminated with enclosed residual intracellular material, including mitochondria, vesicles of endoplasmic reticulum and small amounts of cytoplasmic constituents*. Thus, several explanations are consistent with the above data, and are currently under investigation:

(1) Membrane isolation may lead to changes in instrinsic membrane structure, involving a site critical to insulin responsiveness, but independent of glucose transport (and perhaps insulin binding) *per se*.

(2) The isolated membranes may lack some intracellular constituent critical to the response to insulin. Our inability to restore insulin responsiveness by a variety of additions, including reconstituted cytosol, does not support this hypothesis. However, transmembrane potential, intracellular membranous components, *etc.* are less easily replaced and may be of importance.

(3) The membrane vesicles bearing the insulin receptor may be isolated largely in the inside-out orientation. This would prevent access of the insulin receptor to medium insulin as well as to anti-insulin antibody.

* Even with this relatively limited fractionation, insulin responsiveness is not uniformly retained by the ghosts. Approximately 1/3 of ghost preparations fail to show enhanced glucose transport on addition of insulin[41].

(4) The insulin binding and glucose transport functions may actually be spatially dissociated, *i.e.* simply fractionated into separate populations of vesicles. For example, if the $1.1 \cdot 10^4$ insulin receptor sites per fat cell[2] are evenly distributed on the surface of a 100-μm diameter cell, less than 5 % of plasma membrane vesicles of the size obtained in the present studies (approximately 2000 Å diameter[7]) will bear an insulin receptor. The number and relative distribution of insulin sensitive glucose transport sites is unknown.

We anticipate that the study of insulin activated adipocyte plasma membrane particles will yield considerable insight into the nature of the membrane species which bind insulin and couple it to glucose transport.

ACKNOWLEDGMENTS

We acknowledge with gratitude the excellent technical assistance of Miss Marianne Milligan and Mrs Judy Croll.

Supported in part by Grants N.I.A.M.D. No. AM 13774-02 and AM05205-12, N.S.F. No. BO19636, and a Research Grant from the American Diabetes Association.

Joseph Avruch is a Special Fellow (5FO3AM 43519) of the U.S. Public Health Service.

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