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The Natural Occurrence of Insulin Receptors in Groups on Adipocyte Plasma Membranes As Demonstrated With Monomeric Ferritin-Insulin

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This study was designed to document whether the reported distribution of insulin receptors in small groups of receptor sites randomly distributed in the glycocalyx of adipocytes and isolated adipocyte plasma membranes was a naturally occurring phenomena or due to artifacts. Possible artifacts include: 1) oligomeric forms of ferritin in the ferritin-insulin preparation, 2) an uneven distribution of the glycocalyx on the plasma membrane, or 3) ligand-induced aggregation of occupied receptor complexes. Biogel A 1.5m chromatography of the ferritin-insulin conjugate revealed the ferritin in the ferritin-insulin complex to consist of 55% monomers, 15% dimers, and 30% oligomers. The monomer peak was purified (> 95%) for use in these studies. Cationic ferritin, a glycocalyx marker, when incubated with paraformaldehyde-fixed plasma membranes, was found to be uniformly distributed on the surface of the plasma membrane indicative of uniformly distributed glycocalyx. The ability to demonstrate and inhibit ligand-induced aggregation on the isolated plasma membrane was established with a multivalent ligand, ferritin-concanavalin A. More than 66% of the ferritin-concanavalin A receptors were found in large clusters of 5 or more and 34% as singletons or clusters of up to 4 when incubated at 24°C with fresh membranes. Only 38% of the ferritin-concanavalin A receptors were in large clusters; 62% were singletons or clusters up to 4 on membranes prefixed with paraformaldehyde before incubation. The distribution of the monomeric ferritin-insulin was similar on both adipocytes and purified adipocyte plasma membranes and was consistent with earlier reports with ferritin-insulin. The quantitative distribution of the monomeric ferritin-insulin as singletons or in groups of 2–6 was comparable between the intact cells and isolated membranes incubated at 24°C. The binding of 500 μUnits monomeric ferritin-insulin per ml to the isolated plasma membranes was studied under incubation conditions similar to those used with ferritin-concanavalin A. Under all three conditions, fresh membranes at 24°C and 0–4°C and prefixed membranes at 24°C, the pattern of distribution of the monomeric ferritin-insulin as singletons or groups of 2–6 was identical, indicating that the ligand was not causing aggregation into clusters as did the concanavalin A. Thus, the occurrence of insulin receptors in small groups appears to be a natural phenomenon in the plasma membrane structure of adipocytes.

Key words: adipocyte, insulin receptor, ferritin-insulin, ferritin-con A, plasma membrane

Abbreviations used are: PBS, phosphate buffered normal saline, pH 7.4; Fm-I, monomeric ferritin-insulin; F-I, ferritin-insulin; F-Con A, ferritin concanavalin A; NaCac, sodium cacodylate-HCl buffer, pH 7.4; MSH, melanocyte-stimulating hormone.

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The insulin receptor was shown to be located in the glycocalyx coating of the plasma membrane of adipocytes by electron microscopic techniques using a covalently linked, biologically and immunologically active ferritin-insulin complex (1–3). These findings confirmed biochemical data suggesting that the receptor was associated with a glycoprotein (4, 5). The ferritin-insulin occupied receptors were observed to be randomly and irregularly dispersed as single complexes or in small groups of 2–6 molecules. Similar findings have been reported by Orci et al. (6) and Siess et al. (7) using two different techniques for labeling the bound insulin molecule.

The apparent arrangement of insulin receptors in groups is important in providing a structural model for the biochemical data concerning hormone-receptor interactions and, in particular, possible site-to-site interactions involved in negative cooperativity as proposed by DeMeyts and colleagues (8–11). However, none of the ultrastructural studies to date have established that the apparent groups of insulin receptors and their distribution on the plasma membrane are not the result of one of several artifacts. These include the possibilities that: 1) the groups are due to oligomeric forms of ferritin conjugated to only one insulin molecule which is bound to a receptor, 2) the glycocalyx coating is not evenly distributed but patchy, or 3) the groups are due to ligand-induced aggregation of the occupied receptors.

In none of the studies to date has the presence of oligomeric forms of the ligand been excluded. Although Jarett and Smith (2) purified the ferritin-insulin complex to a 1:1 molar ratio, they did not rule out the presence of oligomeric forms of the ligand. The ferritin-insulin complexes of Orci et al. (6) could have contained oligomeric ferritin or insulin or both, since each molecule was independently linked to a common dextran molecule. Siess et al. (7) used a ferritin-labeled anti-insulin antibody which had not been purified to yield monomeric forms of ferritin or monovalent antibody. In addition, these insulin-receptor ultrastructural studies were not performed under conditions which would inhibit ligand-induced aggregation of the receptor sites in the plane of the membrane.

The present study was designed to document the natural distribution of the insulin receptor on the adipocyte plasma membrane using a monomeric ferritin-insulin complex as well as other ultrastructural membrane markers (cationic ferritin and ferritin concanavalin A and biochemical or physical alterations of the membrane. It was found that the insulin receptors are in naturally occurring groups of up to 6 receptor sites randomly distributed on the plasma membrane in association with an evenly distributed glycocalyx. These groups of insulin receptors were shown not to be the result of ligand-induced aggregation or clustering as is the case for concanavalin A.

MATERIALS

The materials used in these studies and their respective sources were: horse spleen ferritin (6 × recrystallized), cationic ferritin and ferritin concanavalin A, Miles Laboratories, Elkhardt, IN; Biogel A 1.5m agarose, Bio Rad Laboratories, Richmond, CA; porcine insulin was a gift from Dr. R. Chance, Eli Lilly and Co., Indianapolis, IN; collagenase and bovine serum albumin, Sigma Chemical Co., St. Louis, MO; glutaraldehyde, Fisher

Chemical Co., St. Louis, MO; and methyl α-D-mannopyranoside, Calbiochem, San Diego, CA. All other reagents were of the highest purity available from commercial sources.

METHODS

Preparation of Monomeric Ferritin-Insulin

The conjugation procedure has been extensively modified from the previously published report (2). The reaction was carried out in a polypropylene beaker by dissolving 5 mg porcine insulin in 1 ml of 0.05 M KCl-HCl, pH 2.0, followed by an additional 4.5 ml of the KCl-HCl buffer and 75 mg ferritin. This was stirred for 3–5 min at 24°C and 4 × 125 μl aliquots of a 1:75 dilution of 50% glutaraldehyde was added at 10 min intervals with constant stirring. The mixing was continued for 60 min at 24°C and an additional 90 min at 4°C. The reaction was stopped by adding 150 μl of 1 M lysine-HCl and stirring for 30 min at 24°C. Any aggregates (always less than 1%) were removed by a 10,000g centrifugation for 10 min. The supernatant was applied to a Sephadex G-200 column (2.5 × 30 cm) and eluted with KCl-HCl buffer. The ferritin peak, containing both ferritin and ferritin-insulin, and the free insulin peaks were collected separately. The ferritin peak was neutralized by slow addition of 1 N NaOH to pH 6.0 and 0.1 N NaOH to pH 7.4. Infrequently the solution turned muddy brown between pH 5.5 and 6.5. This was reversed by raising the pH to 8.0 and back titrating to pH 7.4 with 0.1 M $NaPO_4$ buffer, which provided a clear ruby colored solution. This solution (about 10 ml) was dialyzed to equilibration with 50 mM $NaPO_4$, pH 7.4, applied to a Biogel A 1.5m column (5 × 50 cm) and eluted with the $NaPO_4$. The monomeric ferritin-insulin peak was collected and concentrated by centrifugation at 150,000 g for 2 hr (see Results). The pellet was resuspended in $NaPO_4$ and stored at 4°C but never frozen.

Determination of the Biological and Immunological Insulin Activity of Fm-I*

The biological activity of the conjugate was assessed by comparing its effectiveness to that of unreacted porcine insulin in stimulating glucose oxidation by intact fat cells as described previously (2). The immunological activity was determined by radioimmunoassay as previously reported (1).

Preparation and Processing of Intact Adipocytes and Adipocyte Plasma Membranes for Conventional Thin-Section Electron Microscopy

Isolated adipocytes were prepared by collagenase digestion of epididymal fat pads (12) and purified plasma membranes were prepared from isolated cells as previously described (13). In some experiments an aliquot of the membrane suspension was prefixed for 30 min in PBS buffered 2% paraformaldehyde at 4°C. The membrane suspension was then diluted 20-fold with PBS, centrifuged at 12,000 g for 10 min at the pellet resuspended to the 20-fold dilution volume. The suspension was allowed to stand 30 min at 4°C and was recentrifuged and the membranes resuspended in PBS. Specific details of the incubation conditions are given in the legends to the tables and figures; the following is the general protocol used. Fresh or prefixed plasma membranes resuspended to about 1.5 mg protein per ml PBS were incubated with Fm-I, cationic ferritin, or F-Con A at

0–4°C or 24°C. Control conditions in the Fm-I studies included incubation of membranes with either an equal concentration of unconjugated ferritin or Fm-I plus 1,000-fold excess unlabelled insulin; nonspecific binding of F-Con A was determined in incubation mixtures containing 50 mM methyl-α-D-mannopyranoside.

After incubation the various membrane mixtures were diluted fivefold with PBS at 0–4°C, washed twice by centrifugation at 12,000 g for 15 min, and resuspended. The final pellets were fixed with 2% glutaraldehyde in 0.1 M NaCac buffer, pH 7.4, for 60 min at 4°C. Post fixation in 2% OsO_4-0.1 M NaCac for 60 min was followed by en bloc staining with 1% uranyl acetate. After dehydration through ethanol the pellets were embedded in Spurr resin (14).

Intact adipocytes were incubated at 24° in Krebs-Ringer phosphate buffer, pH 7.4, with 3% bovine serum albumin and 100 mg% dextrose with the desired concentration of ligand. The cells were washed, fixed in 2% glutaraldehyde in 0.1 M NaCac, embedded in 2% agar, and prepared for thin sectioning as previously described (2).

Thin sections were examined in a Philips 200 electron microscope without on-grid staining.

RESULTS

Purification of Monomeric Ferritin-Insulin

The ferritin-insulin conjugate used in the previous report (2) was chromatographed on a Biogel A 1.5m column and revealed a heterogeneous ferritin population (Fig. 1), composed of approximately 55% monomers (fraction III), 15% dimers (fraction II), and 30% oligomers (fraction I). Although there was an average molar ratio of ferritin to insulin

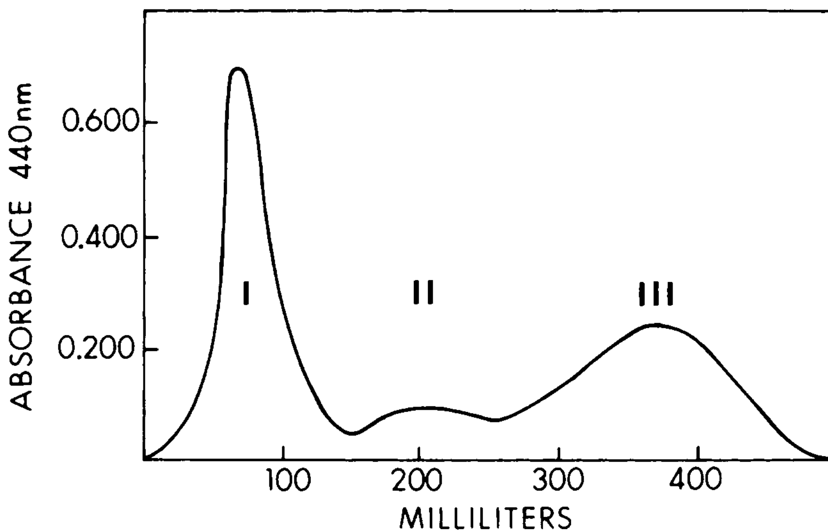

Fig. 1. Biogel A 1.5m chromatography of F-I. Ferritin-insulin utilized in previous reports (1–3) was applied to a 5 × 50 cm Biogel A 1.5m column (exclusion limit of 1,500,000) and eluted with phosphate buffered saline, pH 7.4. Fractions of 5 ml each were collected after the void volume and the absorbance at 440 nm were determined and plotted. The oligomeric ferritin (peak I) consisting of trimers or greater of ferritin constituted 30% of the total ferritin-insulin, while 15% of the ferritin-insulin was dimeric ferritin (peak II). Peak III corresponds to the region of the monomeric ferritin and accounted for 55% of the total ferritin-insulin.

of 1, with no detectable free insulin (1, 2), the possibility that multiple ferritins could be observed as the result of a single occupied receptor necessitated purifying the ferritin-insulin to eliminate the oligomeric forms of ferritin in the conjugate.

Ferritin-insulin was prepared as described in Methods and chromatographed on Biogel A 1.5m, yielding a pattern qualitatively similar to Fig. 1. Fractions from peak III were collected as the monomeric ferritin-insulin peak. This monomeric ferritin-insulin peak was rechromatographed after storage at 4°C for 2 weeks, yielding a Gaussian distribution corresponding to the original peak III. There was no detectable formation of oligomers caused by the concentrating or storage procedures. The conjugate appeared to be greater than 95% ferritin monomer and could contain at most a few percent as dimers but certainly no higher oligomers. There was no free insulin and the monomeric ferritin-insulin had equal insulin potencies in both radioimmunoassay and bioassay, producing concentration curves superimposable to unreacted porcine insulin in both types of assays (data not shown), similar to the ferritin-insulin previously characterized (1–3).

Additional studies revealed that ferritin alone could be chromatographed on the Biogel A 1.5m in large quantities and the monomeric peak collected and stored at 4°C for at least 6 months. Aliquots of this monomeric ferritin could be used in the conjugation procedure without modification of the monomeric state of the ferritin since chromatography of the ferritin-insulin conjugate revealed a single peak identical to that in Fig. 2.

Binding of Cationic Ferritin

The uniformity of the glycocalyx coating of the plasma membrane was investigated with the use of cationic ferritin, a known glycocalyx marker (15). Incubation of paraformaldehyde prefixed adipocyte plasma membranes with cationic ferritin at 24°C resulted in a continuous and evenly spaced layer of ligand of the entire membrane surface (Fig. 3) indicating a continuous glycocalyx coating.

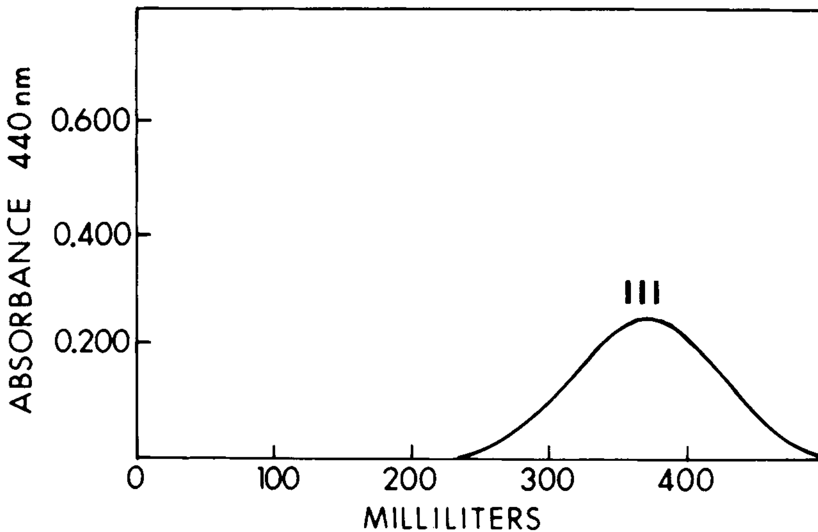

Fig. 2. Biogel A 1.5m chromatography of F-I. Monomeric ferritin-insulin was prepared as described in Methods. After 2 weeks of storage at 4°C the material was rechromatographed on a Biogel A 1.5m column, and the absorption at 440 nm was determined and plotted as described in Fig. 1. The single eluted peak corresponded to monomeric ferritin with less than 5% dimeric ferritin contamination.

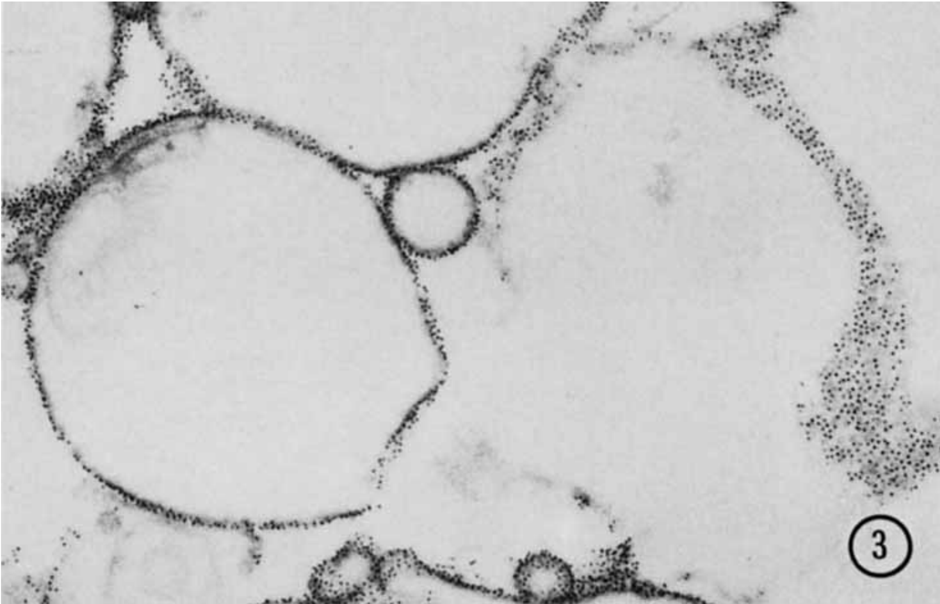

Fig. 3. Cationic ferritin binding to prefixed adipocyte plasma membranes. Isolated adipocyte plasma membranes were prepared and prefixed with 2% buffered paraformaldehyde as described in Methods. The membranes were incubated with 1 mg cationic ferritin per ml at 24°C for 30 min, washed and prepared for thin-section analysis as described in Methods. The cationic ferritin was thinly and evenly distributed along the outside edge of cross-sectioned membrane vesicles and randomly distributed over the surface of tangentially cut membrane fragments. × 63,500.

Binding of Ferritin Concanavalin A

When F-Con A (50 μg/ml) was incubated with fresh plasma membranes at 24°C, there were many large clusters of F-Con A complexes observed (Fig. 4). Incubation of paraformaldehyde prefixed membranes with the same concentration of ligand prevented the formation of these large clusters (Fig. 5). The quantitative differences between the distribution of the F-Con A in various size clusters of the two conditions are graphically demonstrated in Fig. 6. It can be seen that on the prefixed membranes approximately 25% of the total F-Con A exist as single entities with 62% of the F-Con A in clusters of up to 4. Only 38% of the F-Con A was found in larger clusters of up to 15 or more on prefixed membranes. The distribution pattern was quite different when F-Con A was incubated with fresh membranes at 24°C. Only 5% was found as single entities, 34% in clusters of up to 4, and more than 66% in larger clusters of up to 15 or more. Results obtained by incubating F-Con A with membranes at 0–4°C were similar to those found with prefixed membranes except for one difference. The 0–4°C incubations allowed an increase in small clusters of 2–3 F-Con A and a diminution of singletons compared to the prefixed membranes. However, the total F-Con A observed in clusters of up to 4 was 62% in both conditions and 38% in larger clusters of up to 15 or more. Thus, both prefixation and 0–4°C incubation prevented large-scale aggregation of the multivalent ligand. Binding of F-Con A was totally inhibited under all conditions with 50 mM methyl-α-D-mannopyranoside (data not shown).

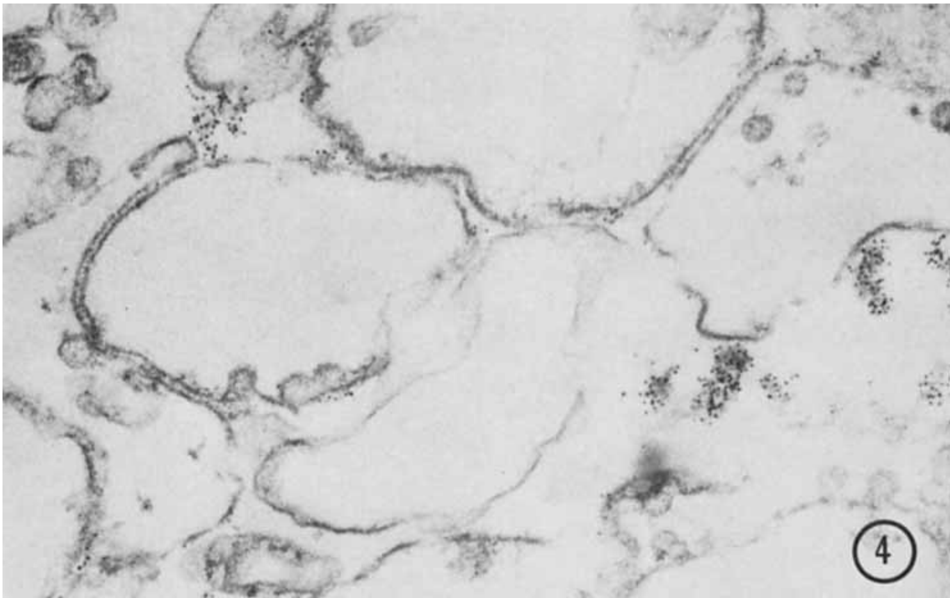

Fig. 4. F-Con A binding to adipocyte plasma membranes. Fresh isolated adipocyte plasma membranes were incubated with 50 μg F-Con A per ml at 24°C for 30 min, washed and prepared for thin-section analysis as described in Methods. F-Con A was found predominantly in large clusters, many having 15 or more visible ferritin molecules, with few molecules found as single receptor sites. A large proportion of the membrane surface revealed no F-Con A. × 71,000.

Binding of Monomeric Ferritin-Insulin

Incubation of isolated adipocytes with Fm-I as described in a previous publication (2) revealed a similar pattern of distribution of the Fm-I as was seen when the adipocytes were incubated with F-I which contained various oligomeric forms. Figure 7 is a composite of different regions of the adipocyte selected to show the various size groups of Fm-I particles and their distribution both on the cell surface and on the extracellular side of the surface-connected vesicles, all in association with glycocalyx material. The density of distribution of insulin receptors suggested by these side-by-side micrographs of the Fm-I dose not represent the actual random distribution that occurs, since there are numerous areas of the cell surface devoid of any Fm-I. Figure 8 is a schematic representation of the Fm-I distribution on an adipocyte sectioned through the plane of the nucleus. The spatial distribution along the cell surface and in the surface-connected vesicles is typical of that found in the 100–150 micrographs needed to complete the entire cell surface.

The large size of the adipocyte, the small amount of plasma membrane available for examination on any given grid, and the small number of insulin receptors per square micron of plasma membrane as found both biochemically (3, 16, 17) and morphologically (3, 7), makes quantitative electron microscopic studies on intact cells difficult. It was decided to test the isolated plasma membrane as a valid representataive of the intact cell for the Fm-I studies, since this would markedly increase the concentration of plasma membrane per grid and the visible Fm-I. The feasibility of this approach was supported by biochemical evidence that the binding characteristics of ^{125}I-insulin to the plasma membrane were similar to those of whole cells (13, 16), and that the insulin receptors for either

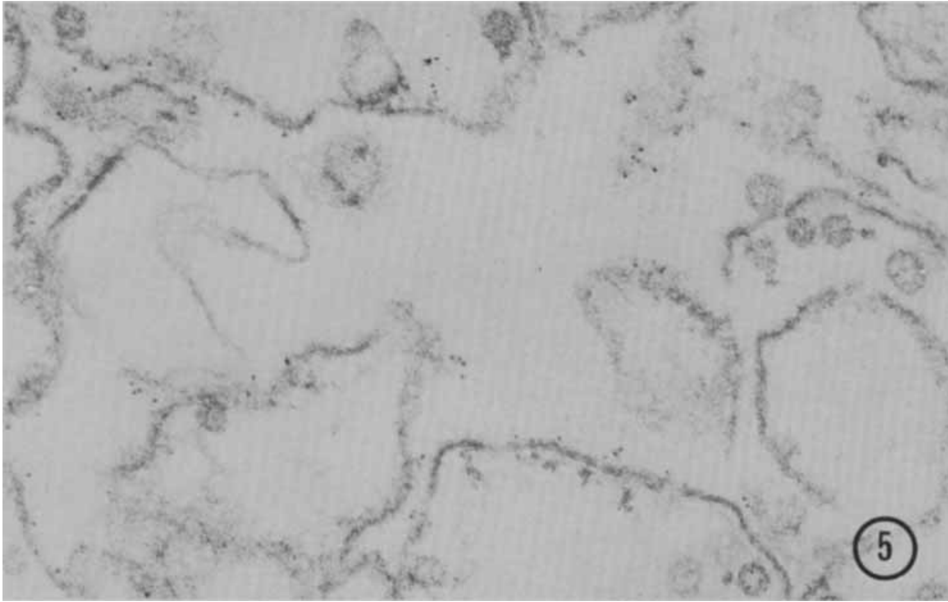

Fig. 5. F-Con A binding to prefixed adipocyte plasma membranes. Isolated adipocyte plasma membranes were prefixed with 2% buffered paraformaldehyde as described in Methods. The membranes were incubated with 50 μg F-Con A per ml at 24°C for 30 min, washed, and prepared for thin-section analysis as described in Methods. F-Con A was found randomly distributed over most membrane surfaces in single molecules and in clusters of from 2 to more than 15 receptor sites. There was no indication of F-Con A clusters of the size shown in Fig. 4. X 93,000.

^{125}I-insulin or F-I were specifically associated with the plasma membranes during subcellular fractionation (1–3, 13, 16, 18). In addition the responsiveness of biological systems in the intact cell and of the ATPase activity of the isolated plasma membranes was indistinguishable whether insulin or F-I or Fm-I was used (1, 2). It was still necessary to show that the distribution of the insulin receptors as determined by the Fm-I was not different between the intact adipocyte and the isolated plasma membranes.

Plasma membranes were incubated at 24°C with 500 μUnits Fm-I per ml for 60 min, the same as were adipocytes. Figure 9 is a representative section showing the random distribution of the Fm-I in association with the glycocalyx coating. The Fm-I was found on cross-sectional areas of the membranes to consist of singletons or in groups of 2–6 ferritin molecules similar to the findings on the adipocyte. Tangential sections of the membranes better illustrated the groupings. The quantitative distribution of the Fm-I into groups of various sizes was determined on both the adipocyte and plasma membrane preparations. The data in Table I illustrates that the distribution was similar in both preparations, indicating that no alterations in the distribution of insulin receptors occurred during the isolation of the plasma membranes. Incubation of either cells or plasma membranes with Fm-I plus a 1,000-fold excess of insulin prevented binding to the membranes or cells (not shown) as previously shown using F-I (1–3). Unconjugated ferritin did not bind to either preparation as previously reported (1–3). These biochemical and quantitative morphological studies justified the use of plasma membranes in subsequent studies.

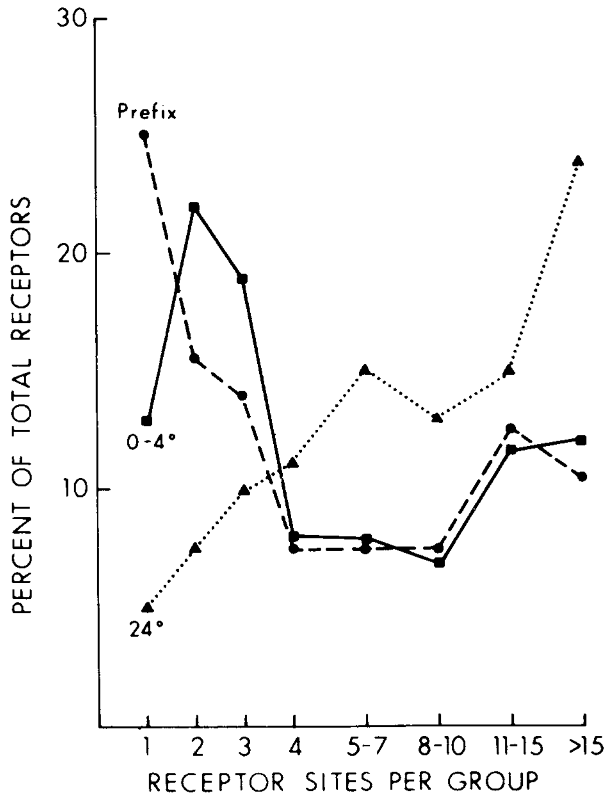

Fig. 6. Quantitative analysis of F-Con A distribution on adipocyte plasma membranes. Isolated adipocyte plasma membranes were prepared and an aliquot prefixed with 2% buffered paraformaldehyde as described in Methods. Fresh membranes were incubated at 0–4° and 24°C, and the prefixed membranes were incubated at 24°C, each with 50 μg F-Con A per ml for 30 min. The samples were prepared for electron microscopic analysis as described, and at least 24 micrographs from randomly selected areas were taken of each incubation condition. The sizes of the groups were classified according to the number of F-Con A molecules which appeared to be closely associated. The number of groups were tabulated and the total number of receptors mathematically determined. Groups larger than 15 were arbitrarily assigned 16 receptors for calculation purposes. The data is presented showing the percentage of the total receptors found in each size group. The differences in the distributions of the receptors clearly demonstrates that prefixation as well as 0–4°C incubation prevented the formation of the larger F-Con A clusters.

The distribution of the Fm-I on the isolated plasma membranes was studied under conditions siimilar to those used to determine the ability of F-Con A to cause aggregation of its receptors, namely at 24°, 0–4°, and 24°C on prefixed membranes. Figure 10 is a representative electron micrograph of the Fm-I distribution on plasma membranes incubated at 0–4°C. The various sized groups and their random distribution are qualitatively indistinguishable from the results found with 24°C incubated membranes. The same is true for sections of prefixed membranes incubated at 24°C (not shown). The results of quantitative measurements of the distribution of the Fm-I in various sized groups are shown in Fig. 11. It can be seen that no differences were found between the distributions in the 3 conditions studied in contrast to the results found for F-Con A (Fig. 6).

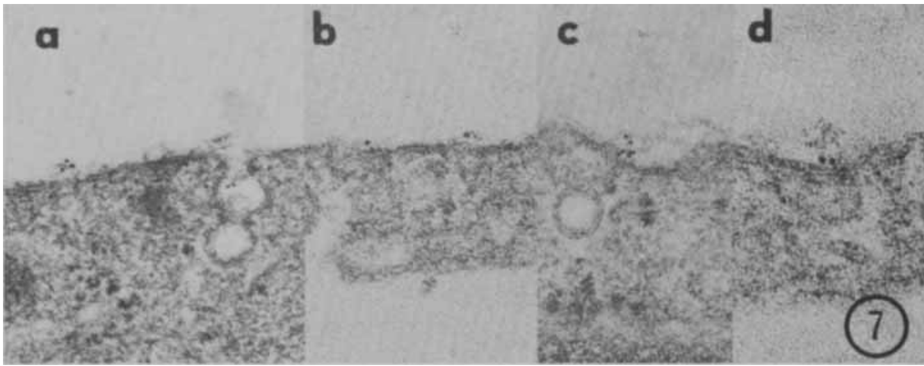

Fig. 7. Fm-I binding to adipocytes. This composite was prepared to show the various groupings of Fm-I found. The isolated adipocytes were incubated with 500 μUnits Fm-I per ml for 60 min at 24°C and prepared for thin-section analysis as described in Methods. Fm-I was found in the glycocalyx on the membrane surfaces as well as in surface-connected vesicles (a) in groups of 2 (d) to 6 (c) as well as single (b) molecules. × 95,000.

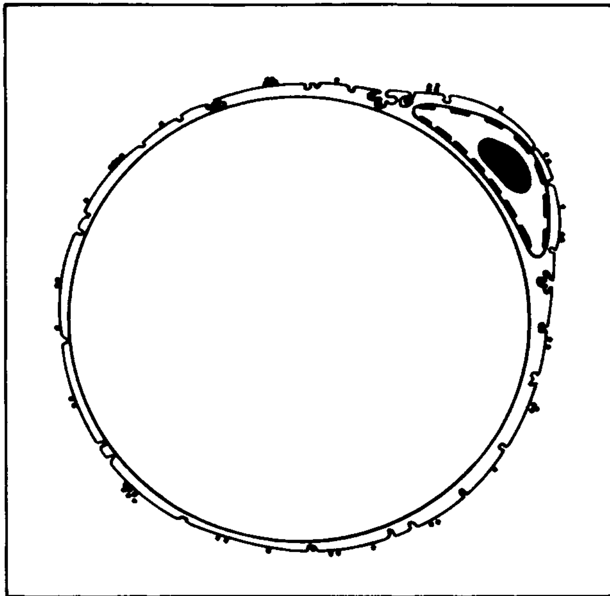

Fig. 8. Schematic representation of Fm-I binding to adipocytes. This schematic drawing of a typical adipocyte, with all cytoplasmic structures except the nucleus omitted, is presented to give a representative picture of the Fm-I distribution on the cell surface and in the surface-connected vesicles. The dark spots representing ferritin are not drawn to scale, but the distribution of the various size groups and spacing of the ferritin molecules around the cell surface were reconstructed from micrographs such as those in Fig. 7. The magnification of this diagram is approximately × 1,000.

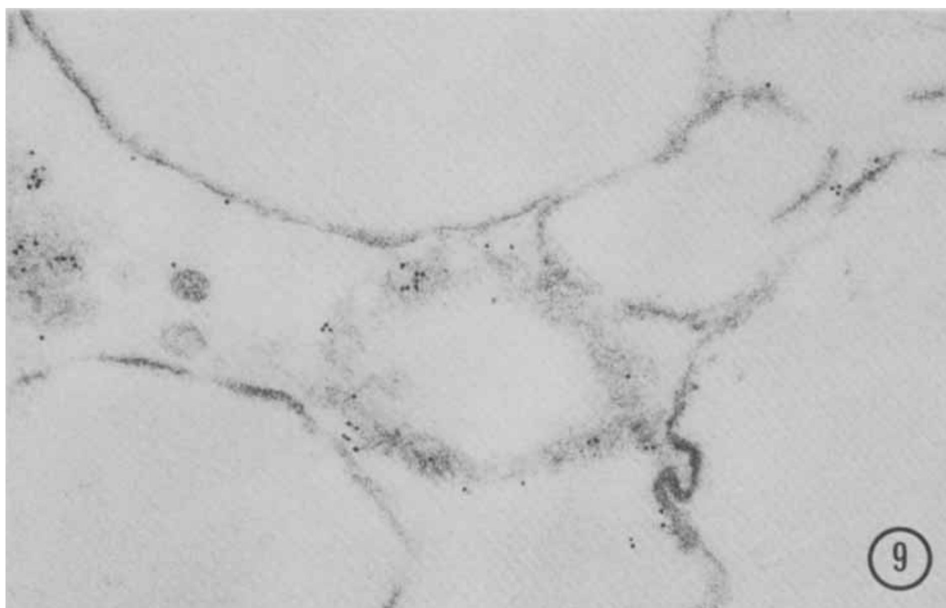

Fig. 9. Fm-I binding to adipocyte plasma membranes. Isolated plasma membranes were incubated with 500 μUnits Fm-I per ml for 60 min at 24°C, washed, and prepared for thin-section analysis as described in Methods. Fm-I was found on the outside of cross-sectioned plasma membrane vesicles in the glycocalyx as single molecules. Tangential portions of the membrane showed the grouped and individual molecules. × 83,000.

TABLE I. Distribution of Fm-I in Various Sized Groups on Intact Adipocytes and Isolated Plasma Membranes

Group size (number of receptors/group)	% of Total Receptors Adipocytes	Plasma membranes
1	24.8	28.7
2	24.4	24.9
3	18.6	20.4
4	13.4	12.8
5	9.0	9.4
6	9.8	3.8

Adipocytes and isolated plasma membranes were prepared as described in Methods and incubated with 500 μUnits Fm-I per ml at 24° for 60 min. The samples were prepared for thin-section analysis, and quantitative analysis was performed on 100 micrographs of adipocytes and 60 micrographs of plasma membranes as described in the legend to figure 6. There was no apparent difference between the adipocyte and plasma membrane distribution patterns for Fm-I receptors.

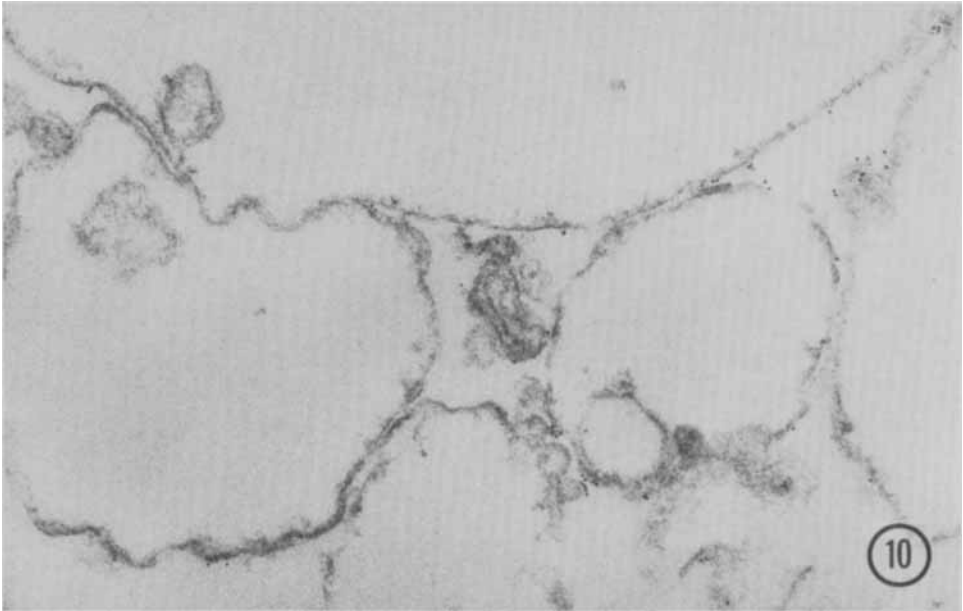

Fig. 10. Fm-I binding to adipocyte plasma membranes at 4°C. Isolated plasma membranes were incubated with 500 μUnits Fm-I per ml for 60 min at 4°C, washed, and prepared for thin-section analysis as described in Methods. The distribution of Fm-I molecules was similar in all respects to that illustrated in Fig. 9. × 80,000.

DISCUSSION

The present study has documented that the ferritin-insulin used in previous studies from this laboratory (1–3) contained oligomeric forms of ferritin which could have given an artifactual representation of the insulin receptors occurring in groups. The purification of the ferritin-insulin to a monomeric form of ferritin allowed reinvestigation of the topography of the insulin-receptor on adipocytes and plasma membranes. These studies have confirmed the earlier observations. It was found that Fm-I, and, therefore, the insulin receptors represented by the Fm-I, were distributed randomly in association with the glycocalyx coating of the adipocyte. Most significantly, groups of up to 6 ferritin particles, as well as singletons, were observed.

The earlier studies have been extended by showing that the groups of insulin receptors occurred naturally on the membrane and were not artifacts due to an uneven distribution of the glycocalyx or to ligand-induced aggregation of the receptors. The continuous and even distribution of the glycocalyx as demonstrated by cationic ferritin, a known glycocalyx marker (15), strongly suggests that the observed insulin receptor distribution is not due to any alterations of the glycocalyx of the cell membrane by any of the preparative procedures. Previous biochemical studies on insulin binding and biological responsiveness by adipocytes and isolated plasma membranes (1–3, 13, 16, 19) coupled with the present quantitative morphological comparison of the topographical distribution of the Fm-I on the adipocytes and isolated plasma membranes demonstrates that the insulin receptors have survived the isolation procedures in their native topographical distribution.

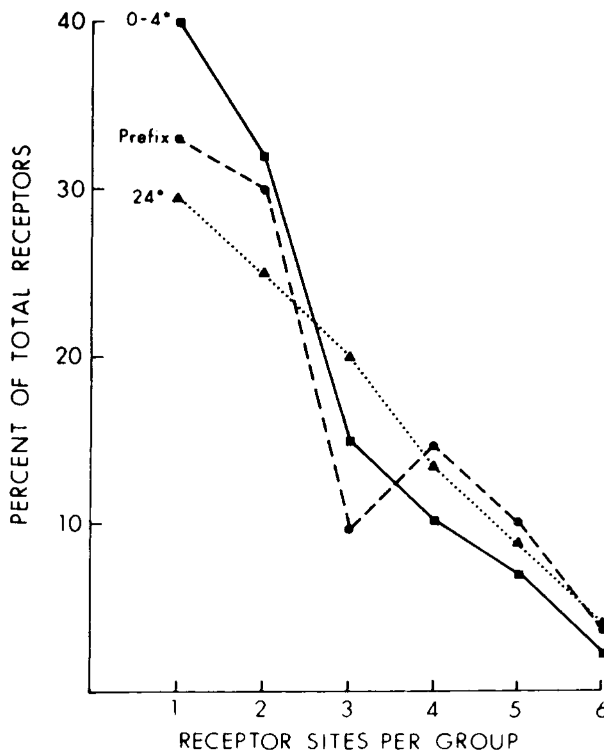

Fig. 11. Quantitative analysis of Fm-I distribution on adipocyte plasma membranes. Isolated adipocyte plasma membranes were prepared and an aliquot prefixed with 2% buffered paraformaldehyde as described in Methods. Fresh membranes were incubated at 0–4° and 24°C, each with 500 μUnits Fm-I per ml for 60 min. The samples were prepared for electron microscopic analysis as described, and at least 60 micrographs from randomly selected areas were taken of each incubation condition. Quantitative analysis of receptor distribution was performed as described in the legend to Fig. 6. There were no appreciable differences in receptor distributions between any of the incubation conditions.

The experiments performed with ferritin Con A provide several important pieces of data. The finding that the tetravalent ligand formed clusters and aggregates of receptor sites at 24°C on the isolated membranes demonstrates that the lectin receptors have retained their mobility. The clustering, although greatly inhibited, was not totally prevented by 0–4°C incubation, suggesting that receptors are still mobile, at least within confined spatial limits, at this temperature. This is similar to findings of Bornens et al. (20) on Con-A binding to thymocytes. The paraformaldehyde prefixation of the membrane used in these studies was sufficient to prevent virtually all ligand-induced aggregation and therefore presumably represents the native distribution of the lectin receptor on the adipocyte plasma membrane.

By analogy to the F-Con A studies, the grouped ferritin-insulin receptors seen on prefixed membranes represent the native distribution of the insulin receptor. The inability to detect any qualitative or quantitative changes in the distribution of the insulin receptors into larger groups during an hour-long incubation at 24° or 0–4°C compared to the prefixed membranes is not surprising since Fm-I is a monovalent and monomeric ligand and

would not be expected to cause ligand-induced aggregation or clustering of receptors. Thus, the natural occurrence of groups of insulin receptors on the adipocyte is distinctly different from the formation of patches or clusters described for other membrane receptors and antigens (21–23) related to multivalent ligands such as Con A. These findings for the insulin receptor are not unique in that Varga et al. (24) have reported that the receptors for MSH are displayed in groups prior to and independent of their exposure to the hormone.

The present study has not been able to answer the question as to whether the insulin receptors, as singletons or groups, are mobile. Certainly mobility was not necessary for the groups to occur, since they are naturally occurring. In order to answer this important question, a second multivalent ligand will have to be used, such as an antibody to ferritin or insulin. Preliminary attempts at such an approach using antiferritin antibody have been inconclusive due to the antibody removing the Fm-I from the membrane (unpublished observations). This may be related to the rapidity of dissociation of the insulin from its receptor compared to the slow dissociation of Con A (25) and the density of insulin receptors and their occupancy compared to Con A.

The morphological data reported in the present study has as its major physiological importance the providing of a possible structural model for the biochemical data concerning the interaction of insulin with its receptor and for understanding subsequent responses. responses. DeMeyts et al. (8–11) have presented mathematical models as well as biochemical data which suggest that insulin's interactions with its receptor are of the negative cooperative type. Levitzki has presented two possible theoretical models for negative cooperativity (26). One model required the ligand-occupied receptor to aggregate, allowing for interactions, while the other pictured the receptors as naturally grouped so that occupancy of one site would allow interaction with and alterations of adjacent sites. The present data is consistent with this last model. Further support for this, if negative cooperativity does explain the behavior of insulin-receptor interactions, is the finding that the temperatures which DeMeyts et al. (8–11) have commonly used to demonstrate most easily negative cooperativity are low enough that mobility would be greatly restricted. Thus, having the receptors already in naturally occurring groups would circumvent the need for mobility.

Jacobs and Cuatrecases have offered an alternative mathematical interpretation to negative cooperativity for the insulin-binding data (27), which they call the mobile receptor model. This intriguing hypothesis has been based on the behavior of receptors that cluster and subsequently cap (28). However, there is no data available, including that from the present study, which demonstrates that insulin receptors are mobile, and the mobile receptor model has not taken into consideration that the receptors are in naturally occurring groups.

The present studies have demonstrated the necessity for morphological studies characterizing the properties of the insulin receptor if an accurate structural model is to be developed to explain biochemical data. It is clear that analogies to other receptors such as immunoglobulins and lectins cannot always be made. Not only is it critical to determine whether the insulin receptors, either singly or in groups, are mobile, but the relationship to an effector must be established. Other areas of insulin action pertinent to morphological investigation are the phenomena of "down regulation" (29, 30), the relationship of receptors from thin and obese, normal and diabetic, etc. As more differences are documented

between the characteristics and behavior of insulin and lectin receptors, the more intriguing it becomes to determine the commonality which produces many of the same physiological responses (19, 31, 32). The Fm-I complex should prove useful in such studies.

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