Effects of insulin, glucagon, and epinephrine on the plasma membrane of the white adipose cell: a freeze-fracture study

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Abstract A quantitative study of the adipocyte plasma membrane under the influence of lipolytic or lipogenic hormones has been carried out by freeze-fracture. The number of plasma membrane invaginations (so-called pinocytosis) and of intramembranous particles was evaluated. Under the influence of either insulin, glucagon, or epinephrine, the membrane invaginations were significantly increased with respect to control adipocytes. Insulin was shown to increase the number of intramembranous particles of the plasma membrane whereas glucagon or epinephrine had an opposite effect. These data point to a hormonal modulation of the fine-structural organization of the adipocyte plasma membrane.

Supplementary key words adipocyte · membrane · ultrastructure

Present knowledge of the relationship between the function and the fine structure of adipocytes is based mostly upon nonquantitative electron microscopic examination of conventional thin section preparations (1–11). The introduction of the technique of freeze-fracturing has made it possible to study large areas of the cell membrane, thereby revealing its general organization and allowing quantitative studies of its components (12, 13). Indeed, freeze-fracture exposes the interior of the membrane (14) and reveals a number of structural elements, the intramembranous particles, which are believed to represent, at least in part, membrane proteins (15).

In the present study, this technique has been applied to the adipocyte in order to reassess the number of membrane invaginations (so-called pinocytosis) with respect to various hormonal stimulations (insulin, glucagon, epinephrine) and to quantify the intramembranous particle content of the membrane under these experimental conditions.

MATERIALS AND METHODS

Animals and preparation of tissue

Epididymal fat pads of six young (200–250 g) male Wistar albino rats were used for each experiment. The rats, fed ad libitum on Purina chow were killed by decapitation and the fat pads of the tail of the epididymis were quickly removed with a minimum of handling. Pieces weighing about 100 mg each were used for incubation. In each animal, fat pads from either side (right or left) were used indifferently; one was incubated in the presence of one hormone while the other was incubated in a control medium.

Solutions and incubations

Fat pads were incubated in a freshly prepared Krebs-Ringer bicarbonate (KRB) buffer containing 3.0 mg/ml of glucose and 4% bovine serum albumin fraction V (General Biochemicals, Chagrin Falls, Ohio). After the addition of a given hormone (see below) the medium was equilibrated for a period of 5 min with 95% O_2 and 5% CO_2. The pH of the medium was then adjusted to 7.4 with dilute HCl or NaOH.

A stock solution of insulin was prepared by dissolving 20 mg of insulin (monocomponent crystalline pork insulin, Novo, Copenhagen, Denmark) in 5 ml of acetic acid (0.3%). The stock solution was diluted in KRB at the time of the experiment and the diluted solution was added to the incubation vials to yield 1000 μU/ml.

Glucagon solution (100 μg/ml) was freshly prepared by dissolving crystalline porcine glucagon (Eli

Abbreviations: KRB, Krebs-Ringer bicarbonate buffer

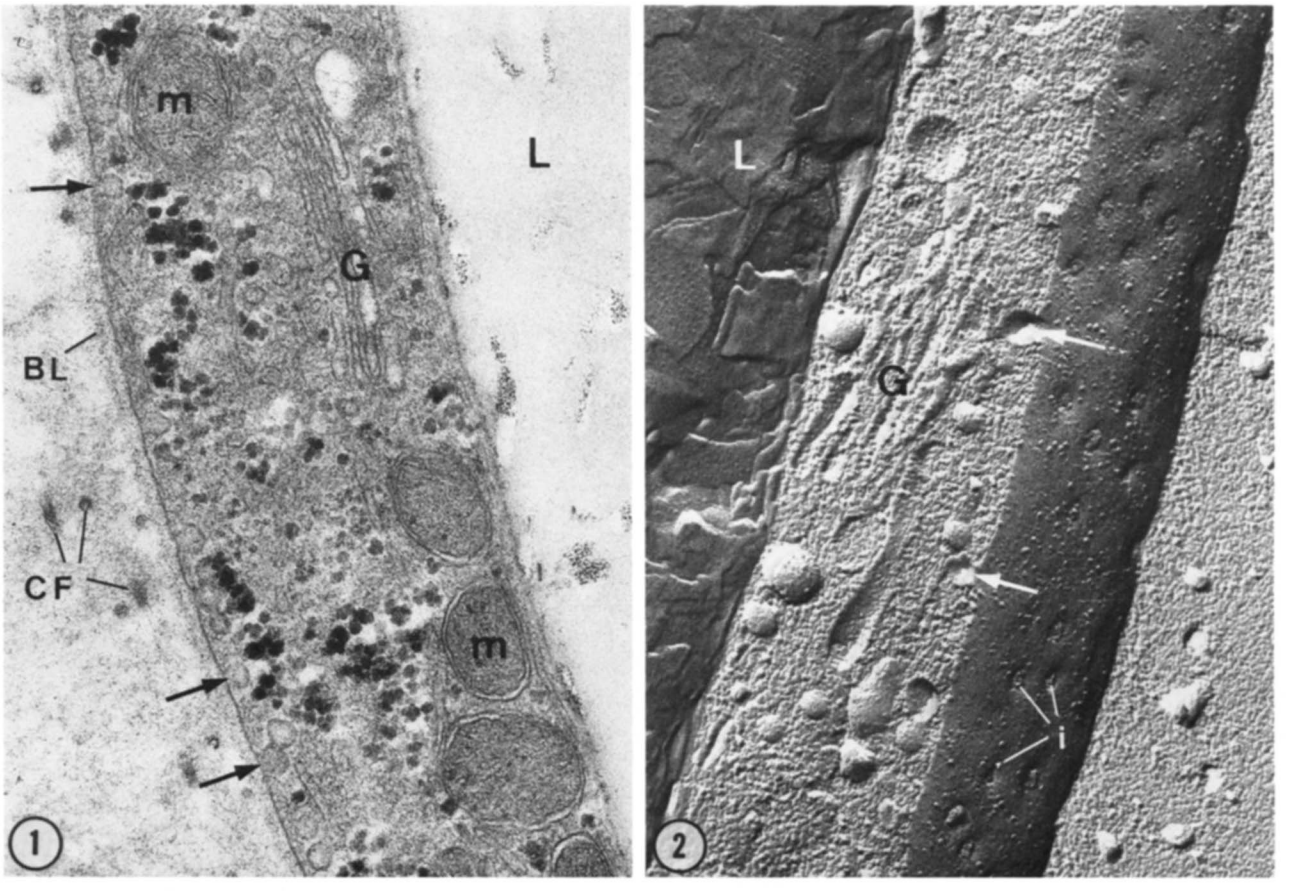


Fig. 1. Adipose cell cytoplasm and plasma membrane as viewed on thin section. The cytoplasm surrounding the central lipid droplet (L) contains a Golgi apparatus (G), mitochondria (m). The arrows point to several microvesicular invaginations. A well-defined basal lamina (BL) and collagen fibers (CF) underlie the cell membrane. ×42,000.

Fig. 2. Freeze-fracture replica showing an area comparable to that in Fig. 1. The lipid droplet (L), intracellular organelles and the plasma membrane appear as distinct fracture faces. The fracture face of the plasma membrane (A-face) shows the intramembranous particles and circular depressions corresponding to the opening of the surface invaginations (i). At the arrows, depressions are half-fractured and show the wall of the corresponding microvesicular invaginations. ×66,000.

Lilly, Indianapolis, Ind.) in 0.02 M glycine buffer (pH 8.8). This stock solution of glucagon was diluted in KRB and the diluted solution was added to the incubation vials to give a final concentration of 5 μg/ml.

A commercial solution of epinephrine (1 mg/ml) (Vifor, Geneva, Switzerland) was diluted in KRB and the diluted solution was added to the incubation vials to a final concentration of 1.0 μg/ml. In order to prevent degradation of epinephrine, ascorbic acid (0.5 mg/ml) was added to the test and control media. The relatively high doses of hormone used were chosen to produce a maximal effect on the adipose cell (3, 16).

After an incubation of 45 min at 37°C, fat pads were removed quickly and minced in a 4% glutaraldehyde solution in phosphate buffer, pH 7.4, at room temperature. At the end of a 2 hr fixation, small pieces of tissue were immersed in 30% glycerol in phosphate buffer for at least 30 min, rapidly frozen in Freon 22, cooled in liquid nitrogen, fractured, and shadowed in a Balzers BAF 301 apparatus according to the technique of Moor and Mühlethaler (12).

The freeze-fracture replicas were cleaned in a solution of sodium hypochlorite (Chlorox) for 2 hr; some replicas were soaked overnight in dimethylformamide (Merck, Darmstadt, West Germany); others were immersed for a few minutes in dimethylformamide, followed by two immersions of 20 min each in a freshly prepared mixture of 20 ml of chloroform (Merck, Darmstadt, West Germany) and 10 ml of methanol (Merck, Darmstadt, West Germany) and finally three immersions of 30 min each in dimethylformamide. Dimethylformamide treatment, with or without the chloroform–methanol step, is of utmost importance for obtaining clean, fat-free replicas. The replicas were finally rinsed in distilled water, mounted

on copper grids and examined in a Philips EM 300 electron microscope (Philips Industries, Einolhoven, The Netherlands). Magnifications were calibrated with a reference grid (Fullam, Inc., Schenectady, N.Y. 2160 lines/mm). For thin section electron microscopy, the glutaraldehyde-fixed fragments were postfixed after brief washing in phosphate buffer for a minimum of 2 hr in 2% phosphate-buffered OsO_4, dehydrated in graded ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Quantitative evaluation of freeze-fracture replicas

For each animal, twelve membrane faces present in a minimum of three different replicas were used. They were carefully selected for flatness. In each membrane face, four pictures were taken at a fixed magnification of 35,000 ×. Measurements of membrane invaginations and membrane particles were carried out on 3-times enlarged positive prints by the same person, who did not know of the experimental conditions. All measurements were performed with the aid of an 8× magnifier containing a reticle calibrated in tenths of a millimeter. The number of membrane invaginations and of intramembranous particles was expressed per 1 micron square of membrane. For intramembranous particles, the numbers obtained were corrected to subtract the surface occupied by membrane invaginations[1].

RESULTS

Organization of adipose cell plasma membrane

Thin section. As amply documented in earlier studies by several authors (1–3, 5, 6, 8–11) adipocyte plasma membrane shows numerous invaginations about 35 nm in diameter and is surrounded by a continuous layer of granular and filamentous material (so-called basal lamina) (**Fig. 1**). Collagen fibers are scattered outside the basal lamina.

Freeze-fracture replicas. Upon freeze-fracture, the plasma membrane is split along the inner phospholipid matrix and yields two complementary halves or fracture faces (14). One of these corresponds to the membrane's inner leaflet and is called by convention the A-face (or the P-face as recently suggested by Branton et al. [17]), the other corresponds to the membrane's outer leaflet and is called the B-face (or E-face in the newly proposed terminology [17]). On the A-faces, invaginations of the adipocyte plasma membrane appear as circular depressions of the surface (**Figs. 2** and **3**) while on B-faces they look like elevated craters (**Fig. 4**). The distribution, size, and frequency of the invaginations are similar on both faces. In addition to these differentiations, both fracture faces of the adipocyte plasma membrane contain globular subunits, the so-called intramembranous particles, randomly distributed (**Figs. 5** and **6**). Their size is similar on both faces (see below), but they are more numerous on A- than on B-faces.

Influence of hormones on membrane invaginations

Table 1 shows the modifications induced by each of the three hormones tested on the number of membrane invaginations. Insulin increased the number of membrane invaginations as did glucagon and epinephrine. As shown in **Fig. 7**, no change in the mean size or in the size distribution of membrane invaginations occurred as the result of incubation with any of the hormones tested.

Influence of hormones on intramembranous particles

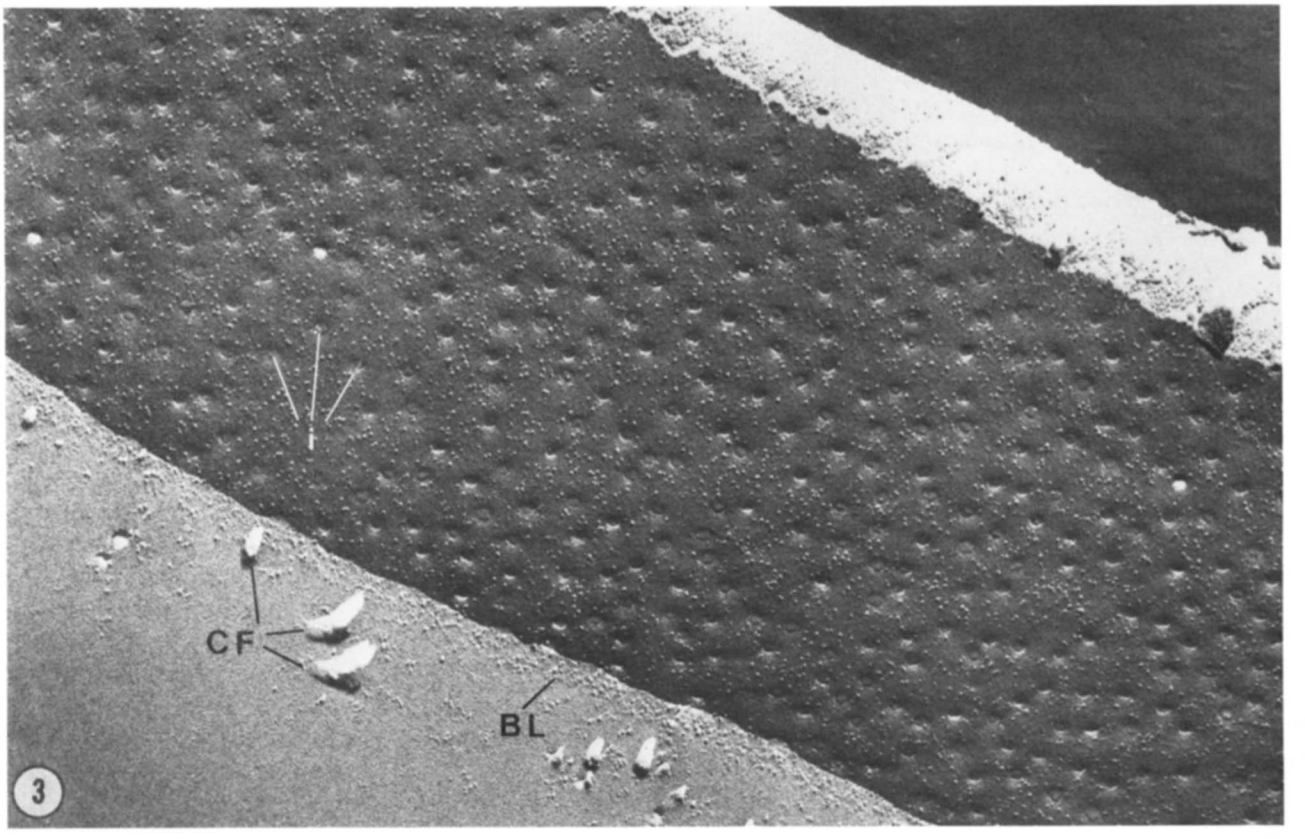
The changes produced by insulin, glucagon, and epinephrine on the number of intramembranous particles are summarized in **Table 2**. Insulin increased the number of intramembranous particles on the A-faces as well as on the B-faces, while glucagon reduced their number on both A- and B-faces. Epinephrine also decreased the frequency of intramembranous particles, but on A-faces only; on B-faces the decrease was not significant. The mean size and size distribution of intramembranous particles did not seem to be affected by any of the hormones studied (**Fig. 8**).

DISCUSSION

This study has shown the feasibility of freeze-fracturing of adipose tissue and the possibilities of a quantitative approach to the structure–function relationship of the adipose cell membrane.

First, our data confirm previous suggestions (1, 3) that the surface invaginations of the plasma mem-

[1] In order to exclude a change in the adipocyte surface due to shrinking or swelling of the cells during the experimental procedure, we tried to estimate the surface by measuring the diameter (D) of the cells in thick (1 μm) sections of embedded material (the surface of a sphere is equal to πD^2). The mean diameter (μm ± SEM) of adipocytes is 71.3 ± 2.4 (control insulin); 70.7 ± 3.0 (control glucagon); 71.4 ± 2.3 (control epinephrine) versus 70.7 ± 1.5 (insulin); 69.9 ± 1.7 (glucagon); 70.2 ± 1.6 (epinephrine). Since none of the experimental values was found significantly different from its respective control, it is concluded that the diameter (and hence, the surface) is not modified by the experimental conditions.



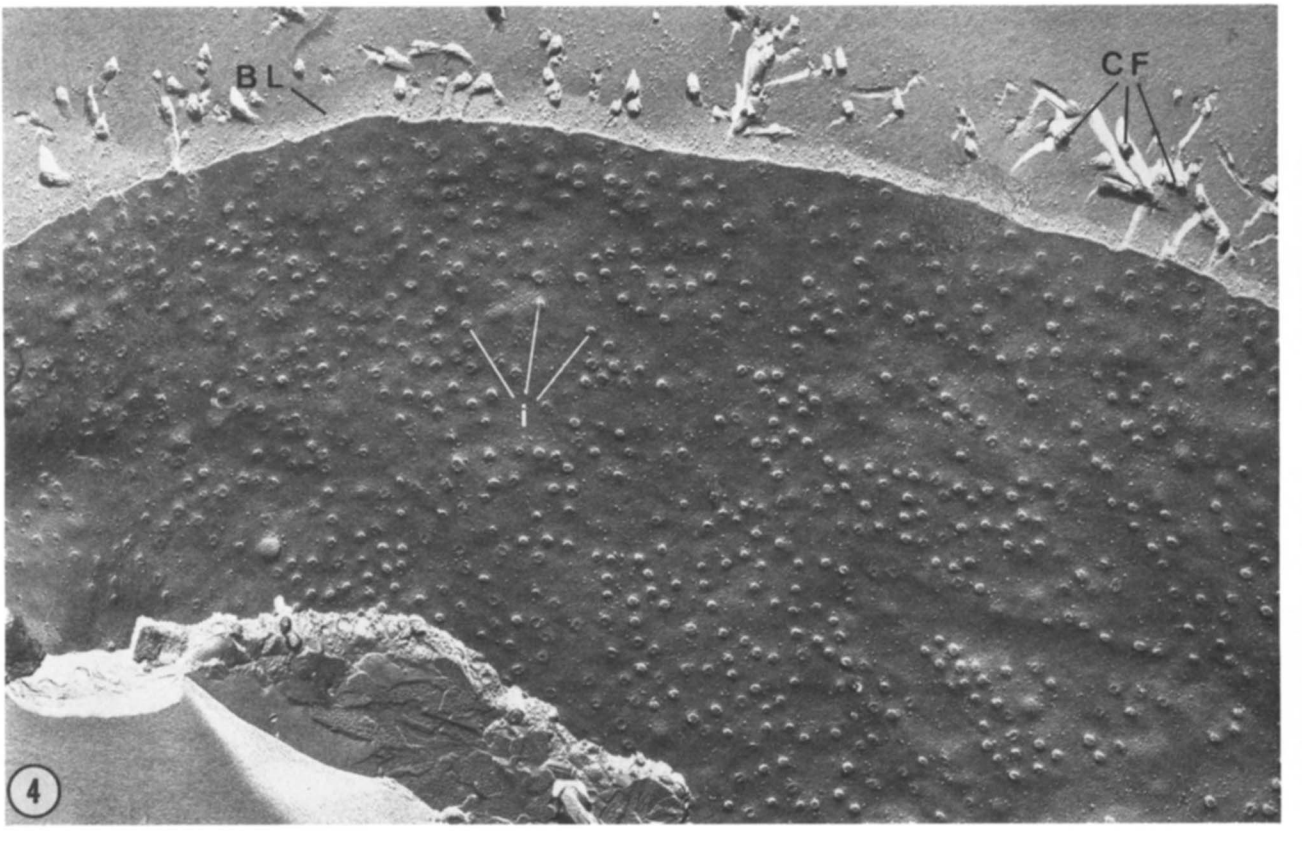
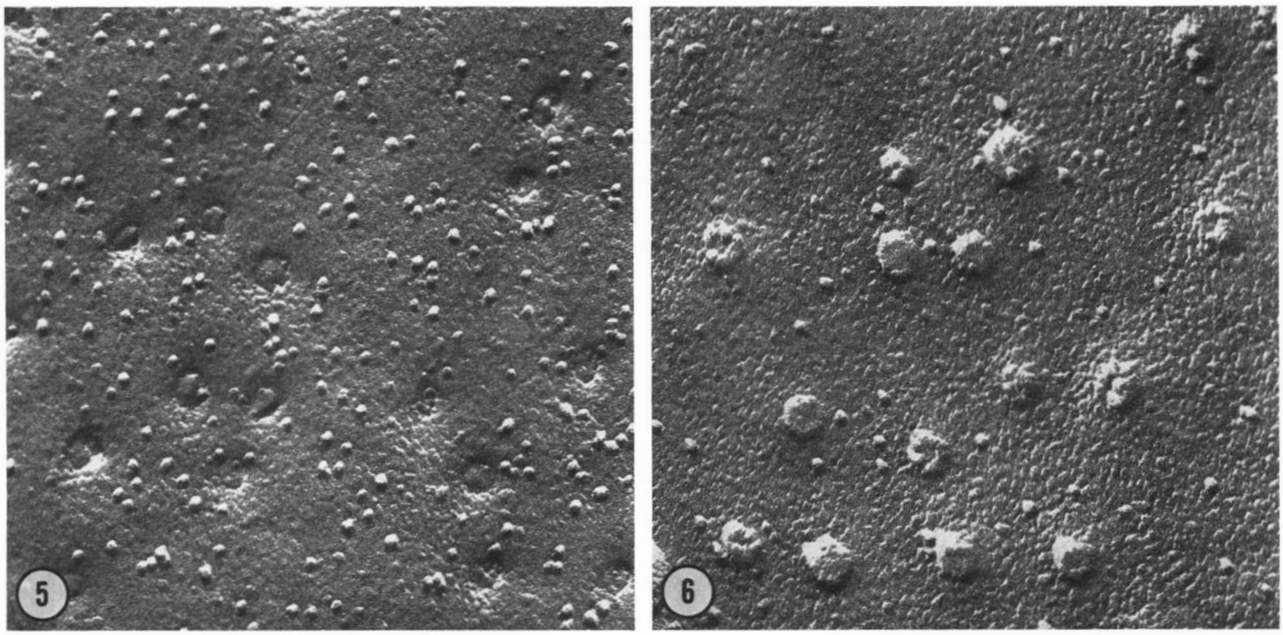


Fig. 3. General view of the A-face (inner leaflet) of the adipocyte plasma membrane. Note the numerous circular depressions (invaginations) (i) and the intramembranous particles. BL, basal lamina; CF, collagen fibers. ×43,000.

Fig. 4. General view of the B-face (outer leaflet) of the adipocyte plasma membrane. In this face, membrane invaginations (i) appear as elevated craters and the intramembranous particles are less numerous than in the A-face. BL, basal lamina; CF, collagen fibers. ×41,000.



Figs. 5 and 6. Details of both A- and B-faces seen at high magnification. The reverse appearance of the membrane invaginations as well as the unequal number of intramembranous particles are clearly evidenced. ×126,000.

brane can be modulated by lipolytic or lipogenic hormones; secondly, they reveal for the first time changes induced by such hormones in the fine organization of the membrane.

As far as membrane invaginations are concerned, we have shown that all three hormones tested increase the number of invaginations. In view of the different technical approaches used in earlier studies, it is difficult to find a valid basis of comparison for our results. This is evident, for example, in the data of Barnett and Ball (1) that indicates an increase in surface invaginations induced by insulin (as judged in thin sectioned material) and in those of Cushman (3) suggesting no effect of insulin but a positive effect of epinephrine on the number of membrane invaginations (as estimated by the uptake of radioactive colloidal gold). A further uncertainty lies in the fact that the changes measured by quantitative morphology are within 10–15% of the control values, a variation which could have easily been overlooked or falsely identified in thin sections. If we take for granted, as suggested by our results, that an increase of membrane invaginations does occur following insulin, glucagon or epinephrine treatment, its functional implication is nevertheless entirely open to discussion.

Indeed, whereas the surface invaginations were formerly considered as endocytotic and/or exocytotic (1, 3, 6, 11, 18), such a role has recently been questioned by Jarett and Smith (19) who found functional evidence for them being permanently-open, finger-like recesses of the surface with probably no function in the internalization of extracellular material or externalization of intracellular substances. The answer to the question of why the lipolytic and lipogenic hormones tested increase these differentiations is linked therefore to the elucidation of their definitive role.

The demonstration of an increase of the intramembranous particles under the influence of a lipogenic hormone, insulin, and a reduction by two lipolytic hormones, glucagon and epinephrine, de-

TABLE 1. Membrane invaginations

	Frequency/μm^2	A & B Faces[a]
Control	50.0 ± 1.2[b]	n = 72[c]
Insulin (1000 μU/ml)	56.8 ± 1.3	n = 72
		$P < 0.001$[d]
Control	48.7 ± 1.0	n = 72
Glucagon (5 μg/ml)	52.2 ± 1.0	n = 72
		$P < 0.02$
Control	47.3 ± 0.9	n = 72
Epinephrine (1 μg/ml)	52.8 ± 1.0	n = 72
		$P < 0.001$

[a] The number of membrane invaginations/μm^2 was identical on A- and B-faces.
[b] Results are expressed as means ± SEM.
[c] n = number of membrane faces studied.
[d] Control values were not found to be statistically different when compared to each other. Statistical significance was determined with the Student's t test.

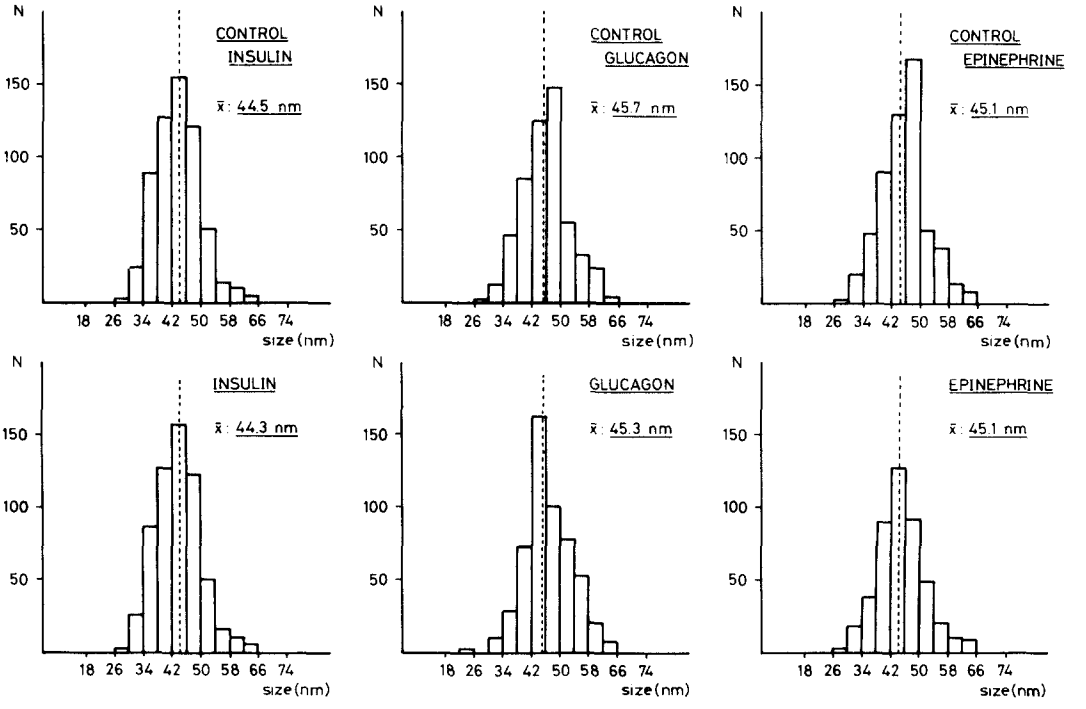


Fig. 7. Size distribution of membrane invaginations on A- and B-faces of the adipocyte plasma membrane under the different experimental conditions. The mean size ($\bar{x}$) is represented by the vertical dotted line. N = number of invaginations in each class.

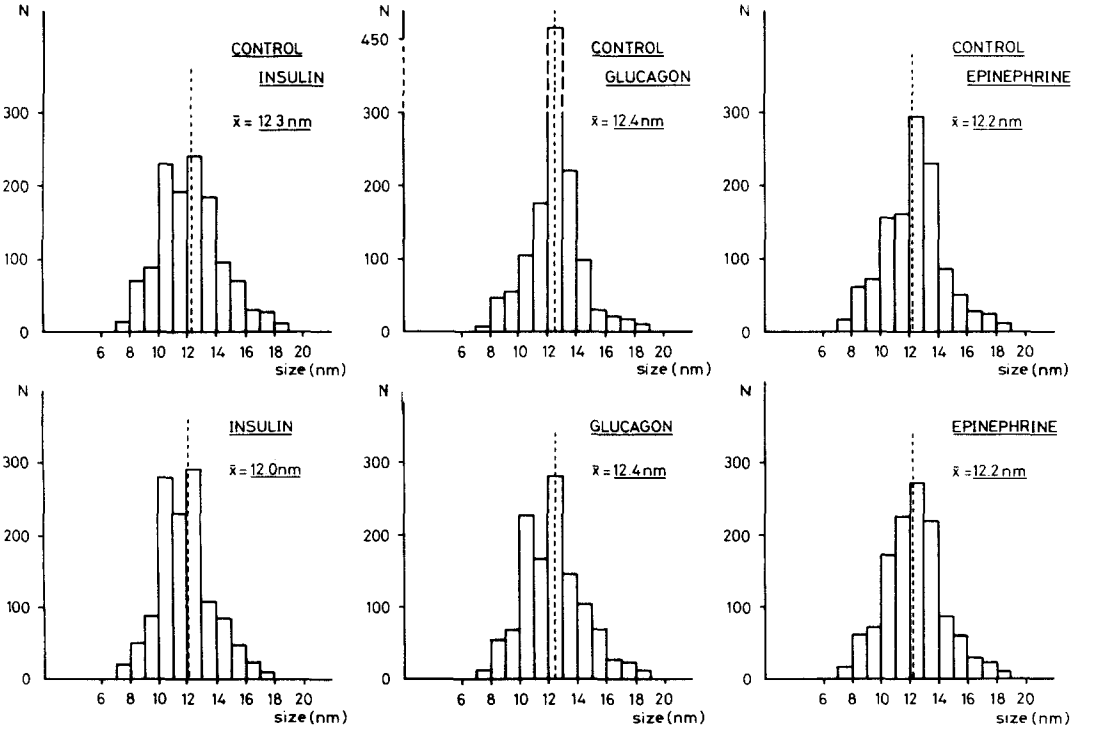


Fig. 8. Size distribution of intramembranous particles on A- and B-faces of the adipocyte plasma membrane under the different experimental conditions. The mean size ($\bar{x}$) is represented by the vertical dotted line. N = number of particles in each class.

TABLE 2. Intramembranous particles

	Frequency/μm^2			
	A-Face		B-Face	
Control	507.9 ± 19.7[a]	n = 30[b]	150.2 ± 8.2	n = 30
Insulin (1000 μU/ml)	567.9 ± 17.7	n = 30	201.0 ± 9.3	n = 24
		$P < 0.05$[c]		$P < 0.001$
Control	550.1 ± 15.0	n = 30	191.9 ± 7.5	n = 30
Glucagon (5 μg/ml)	444.0 ± 16.2	n = 30	164.6 ± 7.6	n = 30
		$P < 0.001$		$P < 0.02$
Control	549.0 ± 17.6	n = 30	170.0 ± 4.9	n = 30
Adrenaline (1 μg/ml)	479.9 ± 20.8	n = 30	154.5 ± 7.5	n = 30
		$P < 0.02$		

[a] Results are expressed as means ± SEM.
[b] n = number of membrane faces studied.
[c] Although no significant differences were noted between control values in A-faces, all three values were significantly different in B-faces. Statistical significance was determined with the Student's *t* test.

serves some comments. Since intramembranous particles are believed to represent, at least in part, the proteins of the membrane and among them probably receptors (20, 21) and membrane-bound enzymes (15, 22), a change in the protein content of the adipocyte membrane could reflect hormonal modulation of the membrane organization with possible functional implications.

In conclusion, this study reveals quantitative changes in the adipose cell membrane when adipose cells are exposed to pharmacologic concentrations of hormones that influence their function. How these changes are determined and what their functional significance is remain to be established.

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