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Quantitative determination of the lateral diffusion coefficients of the hormone–receptor complexes of insulin and epidermal growth factor on the plasma membrane of cultured fibroblasts

(hormone receptors/fluorescent hormones/receptor mobility/fluorescence photobleaching recovery)

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ABSTRACT **Fluorescent derivatives of insulin and epidermal growth factor bound to 3T3 mouse fibroblasts are *mobile* on the cell surface, with similar diffusion coefficients, $D \approx (3\text{–}5) \times 10^{-10}$ cm²/sec at 23°C. Increasing the temperature to 37°C results in rapid receptor immobilization. The immobilization is attributed to aggregation of hormone–receptor complexes, their internalization, or a combination of both processes.**

Insulin is a small polypeptide hormone that plays a major role in the regulation of the metabolism of sugars, fats, and amino acids, and also stimulates the growth of many cultured cells (1, 2). The rapid actions of insulin such as stimulation of glucose and amino acid transport originate shortly after its interaction with its plasma membrane receptor. Its delayed effect on the synthesis of DNA and RNA may require either the production of an intracellular second messenger or the entry of insulin or its fragments into the interior of the cell. In fact, several recent reports propose that insulin can be internalized (3) or bind to the nucleus of human lymphocytes (4).

Epidermal growth factor (EGF) is another small polypeptide hormone. It is isolated from the submaxillary glands of mice (5). It is a potent mitogen for both epidermal and epithelial tissues and also many tissue culture cells lines (6, 7). EGF initially binds tightly to a specific receptor; binding is followed by internalization and degradation (8). It is not clear how EGF produces the mitogenic responses.

Any model proposed for insulin action must take into consideration the diversity of its cellular effects and the progressive timing of the action of the hormone. Similarly, any proposed model for the action of EGF must explain its rapid internalization and degradation and identify the step in which the mitogenic response is triggered.

Several authors have already proposed that the fluidity of the plasma membrane plays a role in the mechanism of hormone action (9–12). Lateral motion of the hormone receptor in the plasma membrane would enable it to interact with various effector molecules, some of which might be mobile, and others located at fixed sites. The existence of a mobile receptor is consistent with the fact that hormones that bind to different receptors can apparently activate the same adenylate cyclase molecule. Furthermore β-adrenergic receptors from turkey erythrocytes can activate the adenylate cyclase of other cell types after the two cell types are fused by Sendai virus (13).

In this paper we report on direct measurements of the mobility of biologically active fluorescently labeled derivatives of EGF and insulin attached to their specific receptors on the cell surface. Insulin was coupled at lysine B-29 with a single lactalbumin molecule previously labeled with 7–8 rhodamine molecules (R-lact-insulin). Another insulin derivative was prepared that contained a single rhodamine molecule directly coupled to lysine B-29 (R-insulin). In a similar manner, the α-amino group of EGF was coupled to rhodamine lactalbumin (giving R-lact-EGF) or directly to rhodamine (giving R-EGF). Both types of analogues retain considerable binding affinity as determined by radioreceptor assays (14) and also biological activity (14). Full details about the synthesis, purification, and biological activity of these derivatives are reported elsewhere (14).

Using these fluorescent hormones, Schlessinger *et al.* (15) have recently shown that the hormone receptor complexes are initially homogeneously distributed on the cell surface, but that at 23°C or 37°C the occupied receptors aggregate into patches on the cell membrane. The fluorescent patches soon internalize, and the labeled hormones appear in endocytic vesicles within the cell. The internalization step is sensitive to temperature and requires metabolic energy (15).

Receptor mobility was measured by the fluorescence photobleaching recovery (FPR) method (16–20). In FPR a brief, intense, focused laser light pulse irreversibly bleaches the fluorescence in a small region (≈3 μm²) of the cell membrane. The time course for recovery of fluorescence in the bleached region due to replenishment by fresh fluorophores from adjacent regions of the cell membrane was recorded as previously described (18). The mobilities of the receptor-hormone complexes are expressed as diffusion coefficients D (cm²/sec) calculated from recovery data. As previously described, we interpret incomplete recovery as an indication that a fraction of the fluorophores is immobile on the time scale of the experiments (16–18, 21). The fractional recovery therefore represents the fraction of mobile fluorophores.

MATERIALS AND METHODS

Reagents. Pork insulin was a product of Eli Lilly. EGF was purified from male mouse submaxillary glands according to the procedure of Savage and Cohen (22). Full details about the synthesis, purification, and activity of all the hormone derivatives are reported elsewhere (14).

Cells. The cells studied were mouse BALB 3T3 clone c/3. All measurements were performed on stationary confluent cells, which have more receptor sites for insulin and EGF than

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Abbreviations: EGF, epidermal growth factor; R-insulin, insulin labeled with rhodamine at lysine B-29; R-lact-insulin, insulin labeled at lysine B-29 with α-lactalbumin that is labeled with 7–8 rhodamine molecules; R-EGF, EGF labeled with rhodamine at the α-amino group; R-lact-EGF, EGF labeled with α-lactalbumin that is labeled with 7–8 rhodamine molecules; FPR, fluorescence photobleaching recovery.

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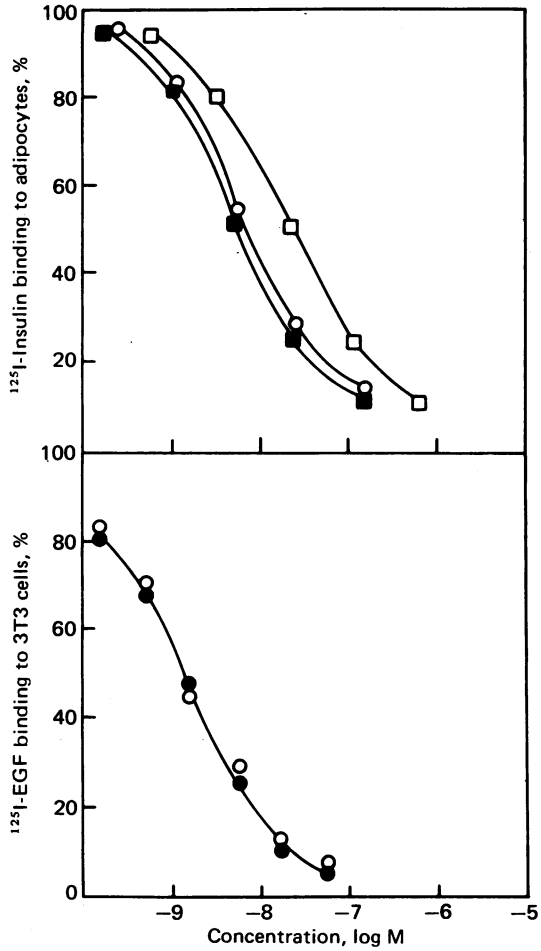

FIG. 1. Inhibition by insulin or EGF and their analogues of ^{125}I-insulin or ^{125}I-EGF binding to cell membrane. Binding is given as percent of maximum. (*Upper*) Isolated rat adipocytes (3.6×10^5 cells) were incubated with ^{125}I-insulin at 1 ng/ml and various concentrations of native insulin (■), R-insulin (○), or R-lact-insulin (□) in 0.25 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, with 1% bovine serum albumin for 45 min at 32°C. Insulin binding was determined by floating the adipocytes over silicone oil in a Beckman Microfuge. (*Lower*) 3T3 fibroblasts were grown to confluency. To remove the cells from the monolayer, the medium was replaced with phosphate-buffered saline and after incubation at 37°C for 15 min the cells were gently scraped with a rubber policeman. The cells were pelleted by centrifugation for 5 min at $300 \times g$, washed with Krebs–Ringer bicarbonate buffer, pH 7.4, 1% albumin, and resuspended in the same buffer to give 2.2×10^6 cells per ml of fibroblast suspension. The suspension was then incubated at 15°C with 27 pg of ^{125}I-EGF and with various concentrations of native EGF (●) or R-lact-EGF (○). After 2 hr the amount of ^{125}I-EGF bound was determined by using EAWP Millipore filters (26). Similar results were obtained when the radioreceptor assay was performed on confluent 3T3 cells attached to plastic tissue culture dishes (27).

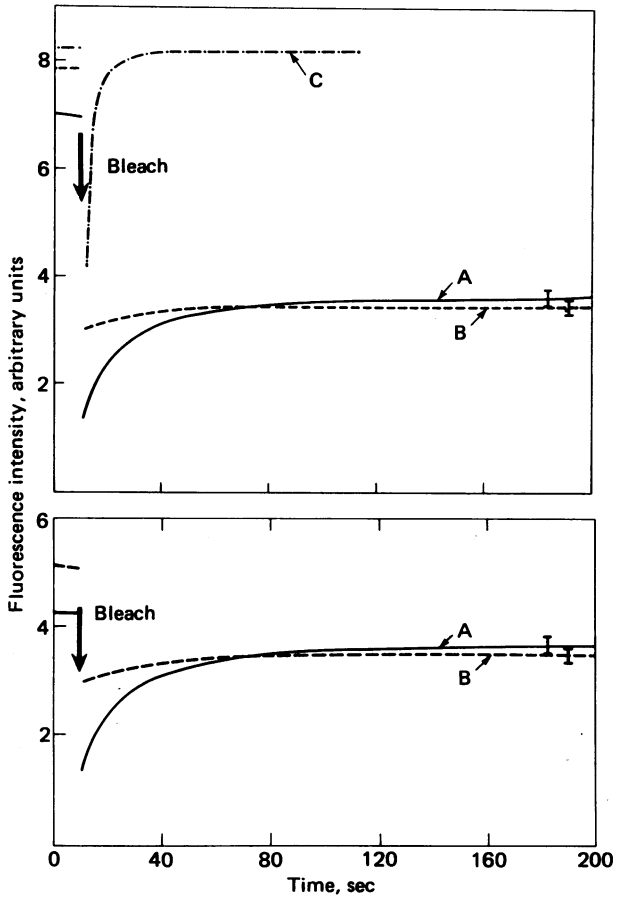

FIG. 2. (*Upper*) Photobleaching recovery curves of 3T3 cells labeled with R-lact-insulin at 23°C in the presence of 10 mM sodium azide (curve A). Other cells were prelabeled at 23°C with R-lact-insulin and then incubated for 20 min at 37°C in the presence of medium containing serum (curve B). For comparison some cells were labeled with the lipid probe dil (3,3-dioctadecylindocarbocyanine iodide) as previously described (21). The diffusion coefficient of dil equals 10^{-8} cm^2/sec (curve C), as previously reported (21). (*Lower*) The recovery curves A and B present the same data as curves A and B *Upper* after the component of the nonspecific fluorescence has been subtracted. This nonspecific fluorescence was determined under the same experimental conditions as the recovery curves. The error bars indicate the degree of noise in the FPR experiments.

growing cells (12, 23). The cells were plated at 10^5 cells per dish in 2 ml of Dulbecco–Vogt modified Eagle's medium with 10% fetal calf serum in 35-mm plastic tissue culture dishes under 95% air/5% CO_2 humidified atmosphere at 37°C. The cells were fed after 24 hr and used after 96 hr. Under this condition 3T3 cells possess approximately 20,000 receptor sites for insulin (24) and 40,000–100,000 receptor sites for EGF (23, 25). To obtain maximal fluorescence from specific labeling over background fluorescence, a high enough concentration of fluorescent hormone (50 ng/ml for 15–30 min at 23°C) was employed to occupy most of the receptor sites on the cells. All FPR experiments were performed at 23°C.

Procedures. The following procedures were used with no modifications: binding of ^{125}I-labeled insulin (^{125}I-insulin) to fat cells (26) and binding of ^{125}I-EGF to fibroblasts (23). The binding activity of the various analogues was tested in a radioreceptor assay. That all derivatives are active is demonstrated in Fig. 1, which shows competition curves of the different fluorescent analogues with ^{125}I-insulin (*upper*) or ^{125}I-EGF (*lower*).

RESULTS

The goal of this study is to determine the lateral diffusion coefficient of the occupied receptors for insulin and EGF on viable cells.

Insulin receptor mobility

Typical measurements of the mobility of R-lact-insulin bound to its specific membrane receptor are illustrated by curve A in Fig. 2, which shows recovery curves of BALB/c 3T3 fibroblasts incubated with insulin at 50 ng/ml in Dulbecco's phosphate-buffered saline with bovine serum albumin at 1 mg/ml for 20 min at 23°C. From this and similar curves a diffusion coefficient of $(4.8 \pm 1.6) \times 10^{-10}$ cm^2/sec was calculated for R-

Table 1. Lateral mobility of R-lact-insulin and R-lact-EGF on 3T3 cells

Hormone derivative and binding conditions	D, $cm^2/sec \times 10^{10}$	Mobile fraction, % recovery
R-lact-insulin, 20 min, 23°C	4.0 ± 0.8	40–80
R-lact-insulin, 20 min, 23°C, 10 mM sodium azide	4.8 ± 1.6	50–80
R-lact-insulin, 20 min, 37°C	0.1–1	<10
R-lact-insulin, 20 min, 23°C; incubated 30 min more, 37°C	0.1–1	<10
R-lact-EGF, 20 min, 23°C, 10 mM sodium azide	3.4 ± 0.5	50–85
R-lact-EGF, 20 min, 23°C; incubated 30 min more, 37°C	0.1–1	<10

Values of lateral diffusion coefficients measured by FPR were obtained from the average $\tau_{1/2}$ of FPR curves taken from 10 different measurements; generally two measurements were obtained per each cell. The errors in the table and text are derived by propagating the standard deviation of the measured $\tau_{1/2}$ values and reflect this relative accuracy. The mobile fraction is expressed as the range of measured values.

lact-insulin. The mobile fraction was 40–80%. A similar diffusion coefficient was obtained when the cells were first incubated for 30 min (23°C) with 10 mM sodium azide and then washed twice and incubated with R-lact-insulin at 50 μg/ml for 20 min at 23°C. Sodium azide was added to prevent internalization of hormone–receptor complex (15).

However, when the cells were incubated with R-lact-insulin at 37°C instead of 23°C for 20 min a different result was obtained. The mobile fraction fell to 10% or less, and the diffusion coefficient of the remaining mobile receptors also was reduced to 10^{-11}–10^{-10} cm^2/sec (Table 1). A similar result was obtained when the cells were exposed to R-lact-insulin for 20 min at 23°C, washed, and then incubated at 37°C for 20 min in complete medium with 10% serum before measurements were made (Table 1).

EGF receptor mobility

3T3 cells were incubated for 30 min with 10 mM sodium azide and then washed twice and incubated with R-lact-EGF at 30 ng/ml in Dulbecco's phosphate-buffered saline with bovine serum albumin at 1 mg/ml for 20 min at 23°C. Typical FPR measurements of the mobility of R-lact-EGF bound to its receptor are illustrated by curves A in Fig. 3 at 23°C. From these and similar curves, a diffusion coefficient of $(3.4 \pm 05) \times 10^{-10}$ cm^2/sec was calculated for R-lact-EGF. The mobile fraction was 50–85%. Results were different, however, when cells were first labeled with R-lact-EGF for 20 min at 23°C and then washed and incubated for 20 min at 37°C in the presence of medium containing serum. As with insulin, the mobile fraction fell to 10% or less and the diffusion coefficient of the remaining mobile receptors also was reduced to 10^{-11}–10^{-10} cm^2/sec (Table 1).

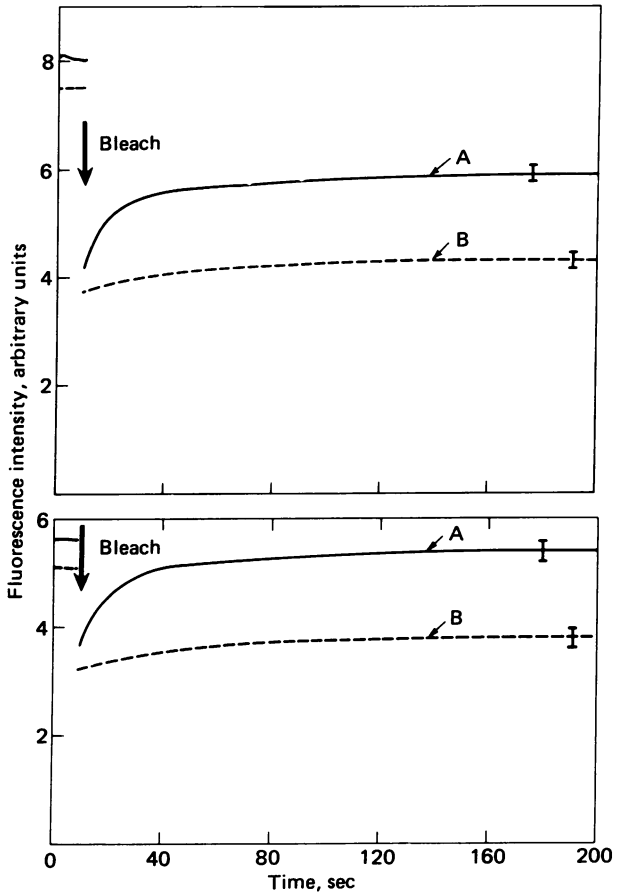

FIG. 3. (*Upper*) The cells were either labeled with R-lact-EGF at 23°C in the presence of 10 mM sodium azide (curve A) or labeled with R-lact-EGF at 23°C without azide and then washed and incubated for 20 min at 37°C in the presence of medium containing serum (curve B). All FPR experiments were performed at 23°C approximately 10 min after the end of incubation. (*Lower*) The recovery curves A and B present the same data as curves A and B *Upper* after the component of the nonspecific fluorescence has been subtracted. The nonspecific fluorescence was determined under the same experimental conditions as the recovery curves. The error bars indicate the degree of noise in the FPR experiments.

Mobility of a fluorescent lipid probe

The cells were incubated with 1 ml of dil (3,3-dioctadecylindocarbocyanine iodide) in Hanks' balanced salt solution containing 1% ethanol for 10 min at 37°C and then washed twice. The diffusion coefficient of dil was measured to be 10^{-8} cm^2/sec and the mobile fraction was close to 100% (21).

Analysis of mobility data

The nonspecific fluorescence emitted from the labeled cells can affect the measurement of the diffusion coefficient (D) and the fractional recovery. In order to solve this problem, we have developed a simple procedure for subtracting the fluorescence intensity that is due to the nonspecific binding of the fluorescent markers. Usually we use this procedure when the nonspecific fluorescence intensity exceeds 10% of the total fluorescence intensity. The level of nonspecific fluorescence of the fluorescent hormone analogues is defined as the fluorescence intensity measured from the cells after pretreating them with insulin or EGF at 250 ng/ml (20 min at 23°C) and then incubating the cells with the corresponding fluorescent derivatives at 50 ng/ml for 20 min in the presence of the native hormone. The background fluorescence intensity is measured from

nonlabeled cells under the same experimental conditions used for measurement of the total or nonspecific fluorescent intensities. Note the nonspecific fluorescence includes the background fluorescence. The fluorescence intensities in all of these experiments were measured from an area of 3 μm^2 of the cell membrane. The fluorescence intensity values are recorded by the photomultiplier of the photobleaching apparatus (18) under completely identical conditions. This way we can quantitatively evaluate the ratio of the specific to nonspecific fluorescence. We find that the nonspecific fluorescence of cells labeled with either R-lact-EGF or R-lact-insulin is approximately 30–40% of the total fluorescence intensity (ten measurements on five cells). Approximately a third of the nonspecific intensity is due to the background fluorescence emitted from nonlabeled cells under the same experimental conditions. Cells labeled with R-insulin or R-EGF were not adequate for FPR experiments because of low ratio of specific to nonspecific fluorescence. Cells treated with R-lactalbumin alone (10^{-9}–10^{-6} M) showed slightly higher fluorescence values than the background fluorescence but less than the nonspecific fluorescence. We find that the nonspecific fluorescence intensity (including background fluorescence) of *all* markers used so far recovers at a very slow rate after photobleaching (28). The apparent diffusion coefficient for the recovery of the nonspecific fluorescence corresponds to $D < 10^{-12}$ cm^2/sec, which is in the time domain of "immobile" molecules in the FPR experiments (16–18, 21, 28). Therefore, the nonspecific fluorescence does not have any significant effect on the measurement of the diffusion coefficients. The nonspecific fluorescence does have an effect on the measurement of the amount of mobile molecules.

The mobile fraction was calculated after subtracting the nonspecific fluorescence from the total fluorescence intensity. The diffusion coefficient $D = (w^2/4\tau_{1/2})\gamma$, in which w is the e^{-2} radius of a Gaussian laser beam (≈ 1 μm), $\tau_{1/2}$ is the half-time for recovery, and γ is an experimental parameter that accounts for the amount of bleaching and beam profile (γ = 1.2–1.4) (17). Diffusion coefficients that are smaller than 3×10^{-12} cm^2/sec are considered to indicate immobility on the time scale of the experiments. Recovery curves of cells labeled with either R-lact-insulin or R-lact-EGF are presented in Fig. 2 *upper* and Fig. 3 *upper*. Fig. 2 *lower* and Fig. 3 *lower* depict the same data after the nonspecific components have been subtracted. The diffusion coefficients describe an average of ten measurements of five different cells.

DISCUSSION

The interpretation of the difference in mobility data at 23°C and 37°C was clarified by studying the distribution of the fluorescent hormone derivatives on the cell membrane with a sensitive image-intensified video camera (15). When the 3T3 cells were labelled at 37°C with either the insulin derivatives (R-lact-insulin or R-insulin) or EGF derivatives (R-lact-EGF or R-EGF), the receptor–hormone complexes rapidly aggregated into patches on the cell surface and soon thereafter appeared in endocytic vesicles within the cell. The aggregation process did not require metabolic energy but was sensitive to the temperature. At 4°C the hormone receptor complexes appeared diffusely distributed at the resolution level of light microscopy. As the incubation temperature was raised to 23°C, aggreates (patches) of fluorescent hormone began to form, and they formed even more rapidly at 37°C (15). The FPR experiments were performed at 23°C, conditions under which some of the hormone–receptor complexes were already aggregated. We do not know the fraction of receptors aggregated or the effect of aggregate size on the diffusion coefficient in membranes. According to the theory of Saffman and Delbrück (29), the dependence on size should be very weak. Nevertheless, the real diffusion coefficient of the unperturbed free receptor could be higher than the values obtained at 23°C. With the equipment now available we are unable to measure diffusion coefficients at low temperature, at which gross patching is less evident. The diffusion coefficients of both hormone–receptor complexes are somewhat higher than those previously measured for nonselectively labeled proteins and surface antigens (19–21). However, the diffusion coefficients of both hormone receptor complexes are substantially lower than the diffusion coefficient of a fluorescent lipid incorporated in the plasma membrane (21). The large variability in the fractional recovery of insulin and EGF does not allow us to draw major conclusions about the amount of mobile receptors. This is mainly due to low ratio of specific to nonspecific fluorescence (see data analysis). Still, the mobile fraction seems to be relatively high. The immobility of EGF or insulin receptor complexes after labeling or incubation at 37°C is consistent with previous observations which suggest that EGF can be internalized under these conditions (8). Similarly, it was suggested that insulin can also internalize or compartmentalize under similar conditions (3, 4, 30). Our results show that the aggregated or internalized hormone–receptor complexes appear to be immobile on the experimental time scale. We do not know yet if the aggregation process alone can immobilize the receptor complexes, because at 37°C we could have a mixed population of aggregated and internalized complexes (15). It is also unclear what fraction of the total hormone receptor complexes can aggregate and internalize.

Employing biologically active fluorescent derivatives of insulin and EGF, we draw the following conclusions: (*i*) The fluorescence intensity of the labeled hormone derivatives on cells is significantly above the nonspecific background fluorescence. (*ii*) Insulin and EGF receptor complexes *are mobile on the cell surface* with similar diffusion coefficients [$(3–5) \times 10^{-10}$ cm^2/sec]. Their diffusion coefficients are much lower than the diffusion coefficient of a lipid probe. (*iii*) A large fraction of the hormone–receptor complexes remain mobile under conditions at which a significant fraction is aggregated. (*iv*) Increasing the cell temperature to 37°C yields pronounced immobilization. The immobilization is presumably due to hormone–receptor complex aggregation, internalization, or a combination of both (15).

This work presents a number of factors that are important in considering models for the action of insulin, EGF, or perhaps other polypeptide hormones. Hormone receptors can *move laterally* in the plasma membrane of the cell at a high rate. The unoccupied hormone receptors appear to be mobile and homogeneously distributed (15) over the cell surface [we cannot exclude the existence of clusters composed of two to six receptor molecules such as have recently been reported (31)]. At 37°C the occupied receptors became *immobilized* within a few minutes. Apparently binding of hormones to their receptors leads to receptor aggregation. The aggregation does not require metabolic energy, and it seems to depend on the rate of diffusion of the occupied receptors. We do not know how our observations are related to the mechanism of hormone action. Most of the biological responses to insulin are very rapid (e.g., glucose uptake, glucose oxidation, etc.), but the mitogenic effects of insulin and EGF are substantially slower. Although the observed immobilization and aggregation appear to be too slow to account for the rapid responses to insulin, the aggregation of a very few receptors could be the first step in the events leading to the rapid insulin responses. If we assume that receptors are initially homogeneously distributed and they move in a random walk with $D = 4 \times 10^{-10}$ cm^2/sec, then two receptors would collide in $\approx$50 msec. Therefore limited aggregation is fast enough to account for even the rapid insulin responses (32).

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