

UC Irvine

UC Irvine Previously Published Works

Title

Electric field-induced redistribution and postfield relaxation of epidermal growth factor receptors on A431 cells.

Permalink

<https://escholarship.org/uc/item/47k7d21j>

Journal

Journal of Cell Biology, 104(5)

ISSN

0021-9525

Authors

Giugni, TD
Braslau, DL
Haigler, HT

Publication Date

1987-05-01

DOI

10.1083/jcb.104.5.1291

Peer reviewed

Electric Field–induced Redistribution and Postfield Relaxation of Epidermal Growth Factor Receptors on A431 Cells

Terrence D. Giugni, Daniel L. Braslau, and Harry T. Haigler

Department of Physiology and Biophysics, University of California, Irvine, California 92717

Abstract. The lateral mobility of the epidermal growth factor (EGF) receptor in the plane of the plasma membrane of cultured A431 cells was investigated using direct and indirect fluorescent probes to measure the generation and relaxation of electric field–induced receptor asymmetry. A steady electric field of 15 V/cm for 30 min at 23°C induced a redistribution of the unoccupied EGF receptor such that there was approximately a three-fold higher concentration of receptors at the cathode-facing pole. After termination of the field, the unoccupied receptors back diffused at 37°C with a rate corresponding to a diffusion coefficient of $2.6\text{--}3.5 \times 10^{-10}$ cm²/s. No diffusion was detected at 4°C. Formation of the hormone–recep-

tor complex is known to induce receptor clustering and internalization. By inhibiting internalization with metabolic poisons, we were able to study the cell surface mobility of clusters of the hormone–receptor complex. The same degree of asymmetry was induced when the occupied receptor was exposed to an electric field and the rate of back diffusion of clusters of the hormone–receptor complex corresponded to a diffusion coefficient of $0.68\text{--}0.95 \times 10^{-10}$ cm²/s. Although the unoccupied receptor is somewhat more mobile than the hormone–receptor complex, it was still far less mobile than one would predict for an unconstrained protein imbedded in a phospholipid bilayer.

To perform their physiological tasks, many integral membrane proteins must diffuse within the plane of the membrane to interact with other macromolecules. An accurate determination of the lateral diffusion rate of these proteins is important to understand these interactions. Most information gathered to date has used the technique of fluorescence photobleaching recovery (FPR)¹ to study cell surface receptors occupied with fluorescently labeled ligands. A small spot is bleached by an intense beam from a laser and the diffusion coefficient (*D*) is calculated from the rate of recovery of fluorescence as the unbleached receptor–ligand complex from adjacent areas diffuses into the bleached spot. *D* values determined by FPR for a number of receptors were in the order of $10^{-11}\text{--}10^{-10}$ cm²/s. These diffusion rates are ~100-fold slower than one might expect from theoretical calculations (Saffman and Delbruck, 1975; Saffman, 1976) and measured values for integral membrane proteins reconstituted into phospholipid bilayers (Peters, 1981; Tank et al., 1982). Artifacts of the FPR method have been considered as a possible explanation of this discrepancy, but, at least in certain model systems, the results of FPR experiments have been verified by independent methods (Koppel and Sheetz, 1981).

Address reprint requests to Harry T. Haigler.

1. *Abbreviations used in this paper:* B-EGF, biotin-conjugated epidermal growth factor; *D*, lateral diffusion coefficient; EGF, epidermal growth factor; FI-EGF; fluorescein-conjugated EGF; FPR, fluorescence photobleaching recovery; TXR-avidin, Texas red-conjugated streptavidin.

One possible explanation for the unexpectedly slow *D* values determined in cells is that, by binding, the ligand induces interactions between its receptor and other cellular macromolecules that impede movement of the ligand–receptor complex. Unfortunately, the FPR method does not permit measurement of the *D* of unoccupied receptors. One method to study the rate of diffusion of unoccupied receptors uses in situ electrophoresis to induce an asymmetric distribution of the membrane components in intact cells followed by measuring the postfield relaxation of asymmetry of the receptor in question (Poo, 1981). Using this method, the unoccupied low density lipoprotein receptor was determined to have a *D* of 1.1×10^{-9} cm²/s at 22°C (Tank et al., 1985), while *D* for the occupied receptor as determined by FPR was 1.4×10^{-11} cm²/s (Barak and Webb, 1982). It is not known if the occupied receptor diffused slower because of direct effects of the large low density lipoprotein particle or because of ligand-induced interactions of the receptor with cellular components. In studies of other receptors, the *D* values measured by postfield relaxation were both larger (Poo et al., 1979) and approximately the same (McCloskey et al., 1984) as the *D* values for the occupied receptors determined by FPR (Schlessinger et al., 1976; Axelrod et al., 1978; Wolf et al., 1980).

In this study, we have measured the rate of back diffusion of the occupied and unoccupied epidermal growth factor (EGF) receptor after inducing asymmetry by application of a steady electric field. Several considerations make this a good model system for studying the effects of receptor oc-

cupancy on diffusion in the plane of the plasma membrane. EGF is a small univalent polypeptide that binds with high specificity and affinity to a receptor of known structure. The mature receptor contains a 621-amino acid extracellular domain that is separated by a single 26-amino acid hydrophobic transmembrane domain from a 542-amino acid cytosolic domain (reviewed by Hunter, 1984). The cytosolic domain contains several phosphorylation sites and a tyrosine protein kinase activity that is activated when EGF binds to the extracellular domain. The unoccupied receptor is randomly distributed on the cell surface, but upon binding EGF the hormone-receptor complex rapidly forms clusters that are internalized via coated pits (reviewed by Haigler, 1983). The internalized hormone-receptor complex is degraded in lysosomes. Although the molecular signals that trigger clustering and internalization are not yet known, experiments with metabolic inhibitors have established that internalization, but not clustering, requires cellular energy input (Haigler et al., 1979; McKanna et al., 1979). The cytosolic region of the receptor containing the tyrosine kinase activity appears to be required for receptor internalization (Prywes et al., 1986).

Lateral diffusion of the EGF receptor is required for clustering and internalization of the hormone. It is also possible that the hormone-receptor complex must diffuse within the plane of the membrane to interact with other occupied receptors in order to activate the kinase activity associated with the receptor (Zidovetzki et al., 1981). The rapid stimulation of ion flux that is stimulated by EGF could also require diffusion-dependent interaction of the occupied receptor with ion channels. *D* values of $2-8 \times 10^{-10}$ cm²/s have been determined for the occupied EGF receptor by FPR (Shechter et al., 1978; Hillman and Schlessinger, 1982; Rees et al., 1984). These values are considerably lower than one would expect from viscous drag of the membrane lipid on the transmembrane segment of the receptor. The low value does not seem to be the result of the interaction of the cytosolic domain of the receptor with cellular structures such as the cytoskeleton because a mutant form of the receptor lacking all but nine of the amino acids in the cytoplasmic domain had approximately the same *D* value as the intact receptor (Livneh et al., 1986). To determine if the constrained lateral mobility is a ligand-induced property of the receptor, we have estimated the *D* value for the occupied and unoccupied EGF receptor after redistribution of the receptors by an electric field. The diffusion rate of the EGF receptor decreased when occupied by EGF but only between three- and fourfold.

Materials and Methods

Synthesis and Characterization of EGF Conjugates

EGF was isolated (Savage and Cohen, 1972) from mouse submaxillary glands and iodinated ($1.8-2.1 \times 10^5$ cpm/ng) by published procedures (Wiley and Cunningham, 1982). Fluorescein-conjugated EGF (Fl-EGF) was prepared as previously described (Haigler et al., 1978).

Biotinylated-EGF (B-EGF) was prepared by reacting EGF (1.0 mg in 0.5 ml sodium bicarbonate, 0.2 M, pH 8.75) with a fivefold molar excess of *N*-hydroxysuccinimidobiotin (Pierce Chemical Co., Rockford, IL; 11.3 μ l of a 25-mg/ml solution in dimethylformamide) with gentle stirring for 1 h at 20°C. The conjugate was separated from unreacted *N*-hydroxysuccinimidobiotin by gel filtration on a Sephadex G-25 column (0.9 \times 25 cm) equilibrated with water. The protein in the excluded peak gave a single band that migrated toward the anode more rapidly than unlabeled EGF on native gel electrophoresis (7% polyacrylamide, pH 9.5) as described (Carpenter et al., 1975) and indicated that the reaction with the single free amino group

of EGF went to completion. B-EGF adsorbed to a previously described DEAE ion exchange column (Carpenter et al., 1975) more tightly than native EGF and eluted as a single symmetrical peak. However, this purification step was not routinely used since the reaction appeared to go to completion. A fluorescamine assay indicated that B-EGF had <0.05 mol free amine per mol protein.

The following experiment was performed to determine if B-EGF would react with avidin. B-EGF was prepared using a tracer amount of ¹²⁵I-EGF and the radioactive conjugate was mixed with a 10-fold molar excess of avidin. The resulting solution was chromatographed on a Sephadex G-100 column and all detectable radioactivity co-migrated with avidin. Aliquots of the high molecular weight radioactive peak were incubated in the presence or absence of a 500-fold molar excess of biotin and chromatographed again on the Sephadex G-100 column. A biotin-dependent shift in the elution position of the radioactivity from one corresponding to that of avidin to one corresponding to that of native EGF was observed.

B-EGF was indistinguishable from native EGF in competitive binding assays using human fibroblasts and ¹²⁵I-EGF by previously described methods (Haigler et al., 1979). Half-maximal stimulation of [³H]thymidine incorporation by human fibroblasts (Haigler et al., 1979) was elicited by ~ 0.2 nM B-EGF compared to ~ 0.1 mM native EGF.

In Situ Electrophoresis

A431 cells were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum, HEPES buffer (20 mM), penicillin, and streptomycin. Experimental cultures were plated on glass coverslips at a density of 7,000 cells/cm². 1 d after plating, the coverslips were rinsed and incubated in modified Steinberg's solution (145 mM NaCl, 7 mM KCl, 0.42 mM Ca(NO₃)₂, 1.3 mM MgSO₄, 4.6 mM Tris-HCl, pH 7.8) in a 0.5-mm deep electrophoresis chamber formed on a microscope slide as described (Poo et al., 1978). A current (~ 2.6 mA) was applied through a pair of agar bridges to produce a steady 15-V/cm field that was maintained for 30 min at $\sim 23^\circ\text{C}$.

Fluorescent Labeling of Cells

Unless otherwise indicated, cultures were labeled at 4°C with either Fl-EGF or B-EGF by removing the coverslips from the electrophoresis chamber and incubating in 1.0 ml of DME containing HEPES buffer (20 mM, pH 7.4), BSA (1 mg), and the indicated derivative of EGF (200 ng). At this concentration of EGF, the occupancy of the EGF receptor was essentially 100% (Haigler et al., 1978). After a 30-min incubation, the cultures were rinsed five times at 4°C with DME/HEPES/BSA. Cultures labeled with Fl-EGF were fixed by adding cold formaldehyde (3%) in PBS and incubating 10 min at room temperature. The cultures were rinsed three times with PBS and mounted in 90% glycerol buffered with Tris-HCl (4 mM, pH 7.2). Before fixation, cultures labeled with B-EGF were incubated in 1 ml of DME/HEPES/BSA containing Texas red-conjugated streptavidin (TXR-avidin) (Bethesda Research Laboratories, Bethesda, MD) for 60 min at 4°C followed by six rinses with DME/HEPES/BSA. Micrographs of the fluorescent stain were taken with Ilford XP-1 film with exposures of 1 min.

The following experiments establish the specificity of labeling by these methods. The amount of staining was reduced by greater than 90% as measured by microfluorimetry (see below) when cultures were labeled as above except a 200-fold excess of native EGF was added to the incubation with Fl-EGF or B-EGF. A similar reduction in staining of B-EGF-labeled cells was observed if cells were incubated with TXR-avidin in the presence of biotin (1.0 mM) or if cells were not exposed to B-EGF before incubation with TXR-avidin.

If A431 cells were labeled with B-EGF and TXR-avidin at 4°C and then warmed to 37°C for 20 min before fixation, the majority of the staining was in bright dots that we interpreted to be endocytic vesicles because their plane of focus was lower than that of the cell surface. This interpretation was supported by the fact that they could not be extracted by an acetic acid wash that has been shown to remove cell-surface ¹²⁵I-EGF but not internalized hormone (Haigler et al., 1980). However, if B-EGF-labeled cells were warmed to 37°C for 20 min before staining at 4°C with TXR-avidin, very little staining was observed. These results establish that TXR-avidin will not bind to B-EGF after it is internalized.

Back Diffusion of EGF Receptors

To measure the rate of back diffusion of the unoccupied receptor after electrophoresis, cultures were incubated for the indicated time at 37°C in DME containing HEPES buffer after terminating the electric field. The cultures

then were labeled at 4°C with either FI-EGF or B-EGF as described above. To measure the rate of back diffusion of occupied EGF receptors, cultures were labeled immediately after electrophoresis with either FI-EGF or B-EGF at 4°C as described above except the incubation medium lacked glucose and contained 2-deoxyglucose (30 mM) and sodium azide (2 mM). After washing to remove unbound hormone conjugates, the cultures were incubated at 37°C for the indicated time in DME/Hepes/BSA/2-deoxyglucose/sodium azide, and then FI-EGF-stained cultures were fixed and B-EGF-labeled cultures were labeled at 4°C with TXR-avidin followed by fixation and mounting in buffered glycerol as described above.

Measurement of Asymmetry Index

The cellular distribution of fluorescent stain was measured by a microfluorimetric method using a universal Zeiss microscope fitted with epiillumination (III RS) optics and a PMI photomultiplier (Carl Zeiss, Inc., Thornwood, NY). Single cells that were not in direct contact with other cells were located and the intensity of the circumferential ring staining at the pole of the cell facing the cathode (180°) and the anode (0°) of the applied field was measured through an aperture that subtended an 8- μ m diameter spot when using a 40 \times objective. Background fluorescence intensity, measured by shifting the measuring aperture to an adjacent cell-free region, was subtracted from all ring stain intensity measurements. Photometer readings of formaldehyde-fixed cells that had not been exposed to the fluorescent probes were <10% higher than background values. This insignificant level of autofluorescence was neglected in asymmetry index calculations. The asymmetry of fluorescence stain on each cell was normalized by determining the asymmetry index (Poo et al., 1979) described by the following formula: $A = (I_{180} - I_0)/(I_{180} + I_0)$. Each cover slip containing labeled cells was scanned linearly and every isolated cell that was encountered was measured. We alternated between sampling the cathodal or anodal pole first.

Within experimental error, the logarithm of the asymmetry index was a linear function of the relaxation time and the slope of this line was determined by least squares analysis with the line constrained to pass through the experimentally determined asymmetry index value at zero time. The 1/e decay time (t_d) was determined from this curve and used to calculate the diffusion coefficient (D) according to the relationship (Poo, 1981) $D = r^2/2t_d$. The average cell radius (r) was 12.5 μ m (SD = 3.9) as determined by measuring the diameter of unfixed cells with a reticle calibrated slide micrometer. The radius of unfixed cells was used in the calculation of the diffusion coefficient rather than the radius of formaldehyde-fixed cells because cells were fixed after back diffusion.

¹²⁵I-EGF Binding and Internalization

Binding was measured by placing coverslips containing cells in 1 ml of DME containing Hepes buffer (20 mM, pH 7.4), BSA (1 mg), and ¹²⁵I-labeled EGF (100 ng). After incubating for 1 h at 4°C, the coverslips were washed five times with the same buffer and bound radioactivity was determined with a gamma counter. Nonspecific binding was determined in paral-

lel incubations containing a 500-fold excess of unlabeled hormone. Internalization of ¹²⁵I-labeled EGF was determined as previously described (Haigler et al., 1980).

Results

As expected from previous studies (Haigler et al., 1978), A431 cells labeled at 4°C with either FI-EGF (not shown) or B-EGF followed by TXR-avidin (Fig. 1 A) showed uniform cell surface fluorescent staining that gave the appearance of ring staining because the membrane was viewed end-on at the circumference of the cell. However, if the cells were exposed to a 15-V/cm electric field for 30 min at 23°C before staining, the label was asymmetrically distributed. In >80% of the cells, the pole of the cell facing the cathode was strikingly brighter (Fig. 1 B). The distribution of the stain was measured by microfluorimetry using an 8- μ m-diameter aperture to collect photons from the anode- and cathode-facing poles of the cells. The asymmetry index of cells labeled with FI-EGF and B-EGF/TXR-avidin was 0.44 and 0.51, respectively, (Figs. 2 and 3) indicating that approximately threefold more stain was at the pole of the cell facing the cathode. The induction of asymmetry by the electric field did not require cellular metabolic activity because essentially the same asymmetry was obtained (FI-EGF = 0.42; B-EGF/TXR-avidin = 0.47) when cells were preincubated and exposed to the electric field in medium containing the energy inhibitors sodium azide (2 mM) and 2-deoxyglucose (30 mM).

The asymmetric distribution decayed if the electric field was terminated and the cells were incubated at 37°C before labeling. A typical cell showing no asymmetric staining after incubation for 9 h postfield before labeling is shown in Fig. 1 C.

Only isolated cells that were growing such that they were not in contact with an adjacent cell were investigated. Cells growing in clumps also showed accumulation of stain at the cathode-facing pole, but it was more difficult to quantify the asymmetry in these cells. No gross morphological changes due to the electric field were detected by phase-contrast microscopy.

The effects of an electric field on the distribution of receptors that were occupied with hormone also was investigated.

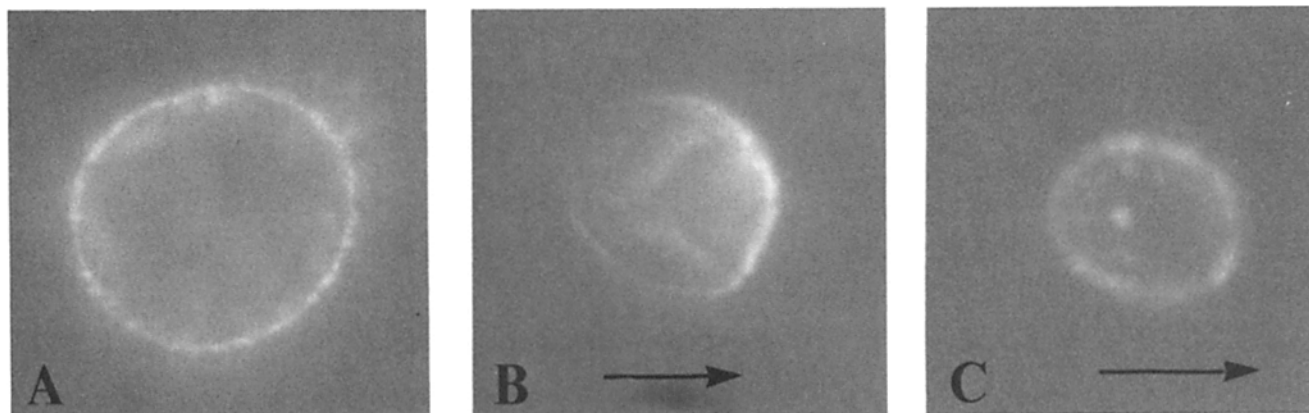


Figure 1. Electric field induction of asymmetric distribution of EGF receptors followed by postfield relaxation. A431 cells were labeled with B-EGF and TXR-avidin as described in Materials and Methods. The control cell was not exposed to an electric field (A) while experimental cells (B and C) were exposed to a 15-V/cm field for 30 min at 23°C. Experimental cells were labeled immediately after terminating the field (B) or after a 9-h incubation at 37°C (C). The arrows indicate the direction of the electric field. The ring staining is at the edge of the cell. The average cell was 25 μ m diam.

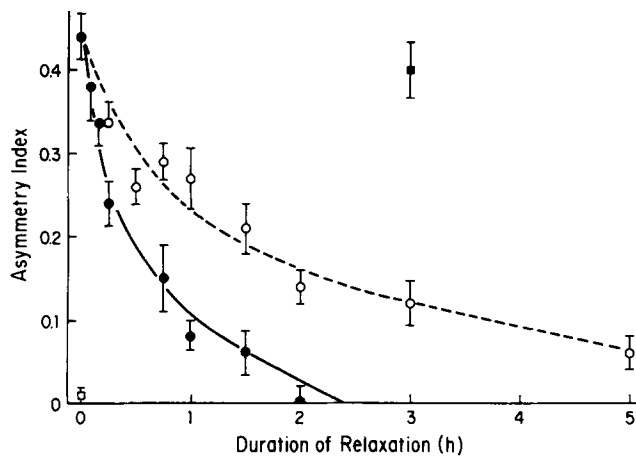


Figure 2. Time course of postfield relaxation of asymmetry in occupied and unoccupied EGF receptors measured with FI-EGF. Cultures were exposed to a 15-V/cm field for 30 min at 23°C and the asymmetry index was measured using FI-EGF as described in Materials and Methods. To measure the back diffusion of the unoccupied receptor, the field was terminated, the cultures were incubated for the indicated time at 37°C (closed circles) or 4°C (closed square), and then labeled with FI-EGF at 4°C as described in Materials and Methods. To measure the back diffusion of the occupied receptor, the field was terminated, the cultures were labeled with FI-EGF at 4°C, washed to remove unbound hormone, incubated at 37°C for the indicated time, and then fixed with formaldehyde (open circles). Control cultures (open square) were labeled without exposure to an electric field. The asymmetry index was measured for greater than 40 cells in several separate experiments as described in Materials and Methods for each experimental point and the error bars represent 95% confidence limits. The lines are single exponential fits of the data that were constrained to the asymmetry index determined at $t = 0$.

In these experiments glucose-free solutions containing sodium azide (2 mM) and 2-deoxyglucose (30 mM) were used to minimize endocytosis of the occupied hormone-receptor complex during the procedures (Haigler et al., 1979). Cells were labeled with either FI-EGF or B-EGF at 4°C, washed to remove unbound hormone, then exposed to a 15-V/cm electric field for 30 min at 23°C. The cells labeled with B-EGF were incubated at 4°C with TXR-avidin after the electrophoresis so that any internalized B-EGF would not be labeled by the probe. The asymmetry index for cells labeled with FI-EGF and B-EGF was 0.42 and 0.46, respectively, and was within experimental error of the values obtained when cells with unoccupied receptors were exposed to an electric field.

The effects of an electric field on internalized EGF also was investigated. Cultures were labeled with FI-EGF or B-EGF/TXR-avidin at 4°C, washed to remove unbound hormone, then incubated at 37°C for 20 min. Previous ultrastructural studies showed that the hormone was located in endocytic vesicles under these conditions (Haigler et al., 1979) and the appearance of the fluorescent labels was consistent with this location (data not shown). Analysis of the internalization of ^{125}I -EGF in parallel cultures under these conditions indicated that >90% of the hormone was internalized. Cultures containing internalized F-EGF or B-EGF/TXR-avidin were exposed to a 15-V/cm electric field for 30 min

at 23°C then fixed and observed. No asymmetry was detected by either gross observation or by microfluorimetry.

The possibility that exposure to the electric field induced selective removal and/or insertion of new receptors was investigated. Binding of ^{125}I -EGF was measured as described in Materials and Methods in control cells, in cells immediately after exposure to an electric field, and in cells exposed to an electric field followed by a 3-h postfield relaxation period at 37°C. Less than 10% difference in ^{125}I -EGF binding was detected in these cultures indicating that no gross change occurred in receptor number.

Since the above experiments indicate that the electric field-induced changes are due to redistribution of EGF receptors, the rate of back diffusion was measured in order to estimate the diffusion coefficient of the occupied and unoccupied receptor. To measure the back diffusion of the unoccupied receptor, cells were incubated for increasing times at 37°C after termination of the electric field. The cells were then labeled with FI-EGF or B-EGF/TXR-avidin, fixed, and the asymmetry index was measured by microfluorimetry. The rate of back diffusion as measured by both methods was similar and, within experimental error, appeared to be a single exponential decay with complete relaxation of asymmetry (Figs. 2 and 3). The $1/e$ relaxation times were 36 and 49 min and corresponded to lateral diffusion coefficients of 3.5 and 2.6×10^{-10} cm²/s, respectively (Table I). No detectable change in the electric field-induced asymmetry was detected at 4°C (Fig. 2).

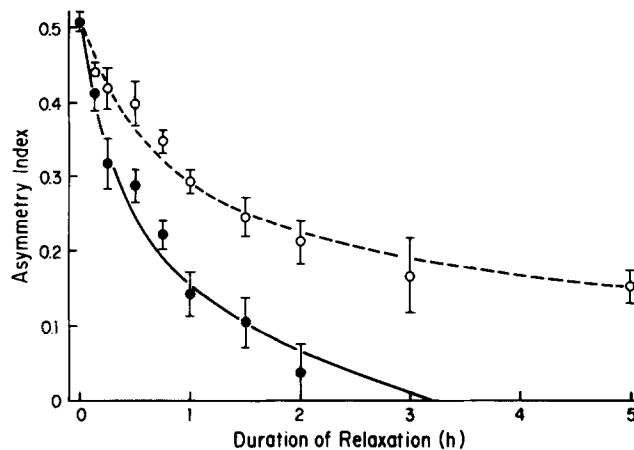


Figure 3. Time course of postfield relaxation of asymmetry in occupied and unoccupied EGF receptors measured with B-EGF and TXR-avidin. Cultures were exposed to a 15-V/cm field for 30 min at 23°C and the asymmetry index was measured using B-EGF and TXR-avidin as described in Materials and Methods. To measure the back diffusion of the unoccupied receptor, the field was terminated, the cultures were incubated for the indicated time at 37°C, and then labeled with B-EGF and TXR-avidin at 4°C as described in Materials and Methods (closed circles). To measure the back diffusion of the occupied receptor, the field was terminated, the cultures were labeled with B-EGF at 4°C, washed to remove unbound hormone, incubated at 37°C for the indicated time, and then incubated at 4°C with TXR-avidin (open circles). The cells were fixed and the asymmetry index was measured for greater than 40 cells in several separate experiments as described in Materials and Methods for each experimental point. Error bars represent 95% confidence limits. The lines are single exponential fits of the data that were constrained to the asymmetry index determined at $t = 0$.

Table I. Back Diffusion of the EGF Receptor

Ligand	Receptor occupancy	t_d	D
		min	$\text{cm}^2/\text{s} \times 10^{-10}$
Fl-EGF	Unoccupied	36	3.5
B-EGF	Unoccupied	49	2.6
Fl-EGF	Occupied	137	0.95
B-EGF	Occupied	192	0.68

The $1/e$ relaxation time (t_d) for back diffusion of the occupied and unoccupied EGF receptor was determined from the exponential decay curves as measured using Fl-EGF or B-EGF/TXR-avidin (Figs. 2 and 3). The lateral diffusion coefficient (D) was calculated from the equation (Poo, 1981) $D = r^2/2t_d$, where r , the radius of a cell, was 12.5 μm .

The following experiment showed that cellular metabolism did not significantly influence the postfield back diffusion of the unoccupied receptors. Cells were exposed to an electric field, then incubated at 37°C for either 30, 60, or 120 min in either DME or in glucose-free DME containing 2-deoxyglucose (30 mM) and sodium azide (2 mM). The cells were labeled with B-EGF/TXR-avidin and the asymmetry indices were determined. At each time point, the asymmetry indices differed by <15% in the presence or absence of energy inhibitors.

To measure the back diffusion of the occupied receptor, cells were exposed to an electric field, labeled with either Fl-EGF or B-EGF at 4°C, and then incubated for increasing times at 37°C. After back diffusion, the Fl-EGF-labeled cells were fixed. B-EGF-labeled cells were incubated at 4°C with TXR-avidin before fixation. The asymmetry index, as measured by microfluorimetry, decreased in a time-dependent manner that approximated a single exponential with complete relaxation of asymmetry (Figs. 2 and 3). The $1/e$ relaxation times were 137 and 192 min and corresponded to lateral diffusion coefficients of 0.95 and $0.68 \times 10^{-10} \text{ cm}^2/\text{s}$, respectively (Table I).

One concern with the measurement of back diffusion of the occupied receptor was that endocytosis of the hormone-receptor complex might contribute to the asymmetry relaxation curve even though the experiment was performed in glucose-free medium containing energy inhibitors. No bright dots of fluorescent stain were detected at any point in the back diffusion experiment using Fl-EGF. However, parallel cultures were subjected to a more sensitive assay using ^{125}I -EGF and an acid wash to selectively remove cell surface hormone. The results of these experiments indicated that at the 3-h back diffusion point, ~30% of the cell-bound hormone had been internalized. Thus the slightly different values for the diffusion coefficient obtained with Fl-EGF and B-EGF/TXR-avidin may reflect a small error introduced by internalization. Since internalized B-EGF would not be detected and Fl-EGF would be detected, the errors introduced by the two methods should be of a different nature.

Discussion

Exposure of viable A431 cells to an electric field induced an asymmetric distribution of the EGF receptor as determined by postfield labeling with direct and indirect fluorescent probes (Fig. 1). The highest concentration of receptor was at the pole of the cells facing the cathode thus indicating that the receptor was less negatively charged than other mem-

brane components (McLaughlin and Poo, 1981). In cells that had been treated with metabolic poisons to inhibit internalization of the hormone-receptor complexes, we demonstrated that the occupied receptor also was redistributed by an electric field. Previous studies have shown that, under the conditions of this experiment, the occupied receptor formed tightly packed clusters containing 10–15 hormone-receptor complexes that tended to accumulate at coated pits (Haigler et al., 1979; McKanna et al., 1979). Since the occupied receptors were mobile in the electric field, it is clear that they are not irreversibly tethered to the coated pit. As previously reported by others (Tank et al., 1985), we did not note any electric field-induced redistribution of coated pits as measured by fluorescent microscopy using antiserum against clathrin (Braslau, D. L., and H. T. Haigler, unpublished results). It also was found that internalized EGF was immobile in the electric field.

The rate of lateral diffusion of the occupied EGF receptor ($D = 0.68\text{--}0.95 \times 10^{-10} \text{ cm}^2/\text{s}$) was estimated by measuring the rate of relaxation of electric field-induced asymmetry in cells that were labeled with EGF before back diffusion at 37°C (Figs. 2 and 3). The D value for the occupied EGF receptor as previously determined by FPR ($2.3\text{--}8.5 \times 10^{-10} \text{ cm}^2/\text{s}$) (Schechter et al., 1978; Hillman and Schlessinger, 1982; Livneh et al., 1986) was significantly higher than this value. The discrepancy between these two methods is even more pronounced at low temperature. At 4°C the receptor had no detectable mobility as measured by the postfield recovery method (Fig. 2) while the FPR method determined that D was $\sim 3 \times 10^{-10} \text{ cm}^2/\text{s}$ (Zidovetzki et al., 1981; Hillman and Schlessinger, 1982). A number of factors could contribute to this discrepancy. One concern is that the electric field will cause a lateral heterogeneity in the distribution of membrane proteins and lipids that could alter the rate of back diffusion particularly immediately after relaxing the field. However, the rate of back diffusion could be approximated by a single exponential indicating that this factor did not grossly affect the determination of the value of D . Nonetheless, this method is not sensitive enough to rule out the possibility that the relaxation curve is the sum of subpopulations of receptors with somewhat different mobilities.

Alternatively, the discrepancy between D as determined either by FPR or by in situ electrophoresis and back diffusion may be a reflection of physiological parameters that are involved in one method and not the other. The FPR method involves the measurement of the diffusion of receptors into a small ($\sim 1 \mu\text{m}$ diam) spot while the postfield recovery method involves the measurement of the diffusion of receptors over the entire diameter of the cell. Since the apparent rate of diffusion measured by the latter method is slower, additional cellular mechanisms may impede the diffusion of the occupied EGF receptor as it leaves its local environment.

Rees et al. (1984) found a subpopulation of EGF receptors that were immobile at 37°C. These immobile receptors corresponded to high affinity binding sites that comprised ~5% of the total number of receptors. Since the experiments described herein were done at saturating concentrations of EGF, the small contribution of this subpopulation of immobile receptors would not be detected.

Although significantly different D values for the occupied EGF receptors are obtained by postfield relaxation and FPR methods, the D values determined by both methods are

smaller than the D value of $\sim 0.5\text{--}1.0 \times 10^{-8}$ cm²/s expected from theoretical calculations (Saffman and Delbruck, 1975; Saffman, 1976) and experiments on purified membrane proteins in lipid bilayers (Peters, 1981; Smith et al., 1979, 1980; Criado et al., 1982; Tank et al., 1982). One of the few cell surface receptors in which the D values of both the occupied and unoccupied states are known is the low density lipoprotein receptor. The D value (2×10^{-9} cm²/s) of the unoccupied receptor, as determined by back diffusion following in situ electrophoresis (Tank et al., 1985), approached the value expected for unhindered lateral diffusion while the D value of the occupied receptor, as measured by FPR (Barak and Webb, 1982), was ~ 100 -fold lower. The constrained diffusion rate was proposed to be due to ligand-induced interactions between the occupied receptor and an unidentified cellular component. The cellular component may be the cytoskeleton because membrane blebs that were separated from the cytoskeleton contained low density lipoprotein receptors with a D value of $\sim 10^{-9}$ cm²/s (Barak and Webb, 1982). However, the difference in D values for occupied and unoccupied low density lipoproteins receptors does not appear to be a general rule; the occupied and unoccupied IgE-Fc receptors diffused laterally at nearly the same rate (McCloskey et al., 1984).

The low density lipoprotein studies raised the possibility that the constrained lateral diffusion rate of the occupied EGF receptor was induced by the ligand. Although we found that the D value for the unoccupied EGF receptor ($2.6\text{--}3.5 \times 10^{-10}$ cm²/s) was slightly higher than the D value of the occupied receptor, it still was an order of magnitude removed from the value expected for an unhindered protein. The cellular or molecular basis of the nearly fourfold difference in the D values between the occupied and unoccupied EGF receptor is not clear. The occupied receptor would have been in the form of tightly packed clusters during back diffusion (Haigler et al., 1979). It is not known if the clusters diffused as a stable group or if they dissolved to allow diffusion of monomeric hormone-receptor complexes. Since lateral diffusion theory (Saffman and Delbruck, 1975; Saffman, 1976) predicts, and experiments show (Vaz et al., 1982), that D depends only logarithmically on the molecular weight of the diffusing particle, D should decrease less than two-fold upon formation of a diffusing cluster containing 10–15 hormone-receptor complexes.

Since the calculated D value is proportional to the square of the cellular radius, a small error in choosing the correct value for the radius may have a large effect on D . Since A431 cells are normally flat polygonal cells that exhibit only small membrane folds (Chinkers et al., 1979) we calculated D assuming the cell surface is planar. Previous experiments have shown that D as determined by the FPR method is effected to a significant extent by nonplanar forms of membranes (Wolf et al., 1982; Aizenbud and Gershon, 1982). However, cell geometry may have a greater effect when back diffusion over the entire cell surface is considered. It has also been shown that treatment of A431 cells with EGF induces extensive ruffling (Chinkers et al., 1979). This ruffling could contribute to the increased asymmetry index relaxation time observed in EGF-treated cultures (Figs. 2 and 3).

It is unlikely that the single 26-amino acid transmembrane region of the EGF receptor experiences sufficient drag from the lipid of the plasma membrane to hinder the rate of lateral

diffusion to this extent unless it is via interaction with other membrane macromolecules. This seems unlikely since the rotational diffusion of the EGF receptor before clustering is approximately what one might expect for an unhindered membrane protein (Zidovetzki et al., 1981). The hindered lateral diffusion rate does not appear to be due to interactions of the cytosolic domain of the receptor with the cytoskeleton because the D value is approximately the same when all but nine of the amino acids of the cytosolic domain are deleted (Livneh et al., 1986). Thus interactions between cellular components and the extracellular domain of the EGF receptor appear to be the most likely source of the hindered lateral diffusion. The extracellular matrix has previously been implicated in hindering the lateral diffusion of major histocompatibility antigens in cultured fibroblasts (Wier and Edidin, 1986).

We thank Drs. Michael McCloskey and Mu-Ming Poo for advice.

This work was supported by grant CD-240 from the American Cancer Society and grant CA 31279 from the United States Public Health Service.

Received for publication 10 November 1986, and in revised form 12 January 1987.

References

- Axelrod, D., A. Wight, W. Webb, and A. Horwitz. 1978. Influence of membrane lipids on acetylcholine receptor and lipid probe diffusion in cultured myotube membrane. *Biochemistry*. 17:3604–3609.
- Aizenbud, B. M., and N. D. Gershon. 1982. Diffusion of molecules on biological membranes of nonpolar form. A theoretical study. *Biophys. J.* 38: 287–293.
- Barak, L. S., and W. W. Webb. 1982. Diffusion of low density lipoprotein-receptor complex on human fibroblasts. *J. Cell Biol.* 95:846–852.
- Carpenter, G., K. J. Lembach, M. M. Morrison, and S. Cohen. 1975. Characterization of the binding of ¹²⁵I-labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* 250:4297–4304.
- Chinkers, M., J. A. McKanna, and S. Cohen. 1979. Rapid induction of morphological changes in human carcinoma cells A431 by epidermal growth factor. *J. Cell Biol.* 83:260–265.
- Criado, M., W. L. C. Vaz, F. J. Barrantes, and T. M. Jovin. 1982. Translational diffusion of acetylcholine receptor (monomeric and dimeric forms) of *Torpedo marmorata* reconstituted into phospholipid bilayers studied by fluorescence recovery after photobleaching. *Biochemistry*. 21:5750–5755.
- Haigler, H. T. 1983. Epidermal growth factor: cellular binding and consequences. In *Growth and Maturation Factors*, Vol. I. G. Guroff, editor. John Wiley & Sons, Inc., New York. 117–154.
- Haigler, H. T., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. *Proc. Natl. Acad. Sci. USA.* 75: 3317–3321.
- Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. 1980. Dantylcadaverine inhibits internalization of ¹²⁵I-epidermal factor in BALB 3T3 cells. *J. Biol. Chem.* 255:1239–1241.
- Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. *J. Cell Biol.* 81:382–395.
- Hillman, G. M., and J. Schlessinger. 1982. Lateral diffusion of epidermal growth factor complexed to its surface receptors does not account for the thermal sensitivity of patch formation and endocytosis. *Biochemistry*. 21:1667–1672.
- Hunter, T. 1984. The epidermal growth factor receptor gene and its product. *Nature (Lond.)*. 311:414–416.
- Koppel, D. E., and M. P. Sheetz. 1981. Fluorescence photobleaching does not alter the lateral mobility of erythrocyte membrane glycoproteins. *Nature (Lond.)*. 293:159–161.
- Livneh, E., M. Benveniste, R. Prywes, S. Felder, Z. Kam, and J. Schlessinger. 1986. Large deletions in the cytoplasmic kinase domain of the epidermal growth factor receptor do not affect its lateral mobility. *J. Cell Biol.* 103:327–331.
- McCloskey, M. A., Z.-Y. Liu, and M.-M. Poo. 1984. Lateral electromigration and diffusion of FcE Receptors on rat basophilic leukemia cells: effects of IgE binding. *J. Cell Biol.* 99:778–787.
- McKanna, J. A., H. T. Haigler, and S. Cohen. 1979. Hormone receptor topology and dynamics: morphological analysis using ferritin-labeled epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 76:5689–5693.
- McLaughlin, S., and M.-M. Poo. 1981. The role of electro-osmosis in the elec-

- tric field-induced movement of charged macromolecules on the surfaces of cells. *Biophys. J.* 34:85-93.
- Peters, R. 1981. Translational diffusion in the plasma membrane of single cells as studied by fluorescence microphotolysis. *Cell Biol. Int. Rep.* 5:737-760.
- Poo, M.-M. 1981. *In situ* electrophoresis of membrane components. *Annu. Rev. Biophys. Bioeng.* 10:245-276.
- Poo, M.-M., J. W. Lam, N. Orida, and A. W. Chao. 1979. Electrophoresis and diffusion in the plane of the cell membrane. *Biophys. J.* 26:1-22.
- Poo, M.-M., W.-J. H. Poo, and J. W. Lam. 1978. Lateral electrophoresis and diffusion of concanavalin A receptors in the membrane of embryonic muscle cell. *J. Cell Biol.* 76:483-501.
- Prywes, R., E. Livneh, A. Ullrich, and J. Schlessinger. 1986. Mutations in the cytoplasmic domain of EGF receptor affect EGF binding and receptor internalization. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2179-2190.
- Rees, A. R., M. Gregoriou, P. Johnson, and P. B. Garland. 1984. High affinity epidermal growth factor receptors on the surface of A431 cells have restricted lateral diffusion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1843-1847.
- Saffman, P. G. 1976. Brownian motion in thin sheets of viscous fluid. *J. Fluid Mech.* 73:593-602.
- Saffman, P. G., and M. Delbruck. 1975. Brownian motion in biological membranes. *Proc. Natl. Acad. Sci. USA.* 72:3111-3113.
- Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative. *J. Biol. Chem.* 247:7609-7611.
- Schlessinger, J., W. Webb, E. L. Elson, and H. Metzger. 1976. Lateral motion and valence of Fc receptors on rat peritoneal mast cells. *Nature (Lond.)* 264:550-552.
- Shechter, Y., J. Schlessinger, S. Jacobs, K.-J. Chang, and P. Cuatrecasas. 1978. Fluorescent labeling of hormone receptors in viable cells: preparation and properties of highly fluorescent derivatives of epidermal growth factor and insulin. *Proc. Natl. Acad. Sci. USA.* 75:2135-2139.
- Smith, L. M., J. W. Parce, B. A. Smith, and H. M. McConnell. 1979. Antibodies bound to lipid haptens in model membranes diffuse as rapidly as the lipids themselves. *Proc. Natl. Acad. Sci. USA.* 76:4177-4179.
- Smith, L. M., J. L. R. Rubenstein, J. W. Parce, and H. M. McConnell. 1980. Lateral diffusion of M-13 coat protein in mixtures of phosphatidylcholine and cholesterol. *Biochemistry.* 19:5907-5911.
- Tank, D. W., W. J. Fredericks, L. S. Barak, and W. Webb. 1985. Electric field-induced redistribution and postfield relaxation of low density lipoprotein receptors on cultured human fibroblasts. *J. Cell Biol.* 101:148-157.
- Tank, D. W., E.-S. Uw, P. R. Meers, and W. Webb. 1982. Lateral diffusion of gramicidin C in phospholipid multilayers: effects of cholesterol and high gramicidin concentration. *Biophys. J.* 40:129-135.
- Vaz, W. L. C., M. Criado, V. M. C. Madeira, G. Schoellmann, and T. M. Jovin. 1982. Size dependence of the translational diffusion of large integral membrane proteins in liquid-crystalline phase lipid bilayers. A study using fluorescence recovery after photobleaching. *Biochemistry.* 21:5608-5612.
- Wier, M. L., and M. Edidin. 1986. Effects of cell density and extracellular matrix on the lateral diffusion of major histocompatibility antigens in cultured fibroblasts. *J. Cell Biol.* 103:215-222.
- Wiley, H. S., and D. D. Cunningham. 1982. The endocytic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. *J. Biol. Chem.* 257:4222-4229.
- Wolf, D. E., M. Edidin, and P. R. Dragsten. 1980. Effect of bleaching light on measurement of lateral diffusion in cell membranes by the fluorescence photobleaching recovery method. *Proc. Natl. Acad. Sci. USA.* 77:2043-2045.
- Wolf, D. E., A. H. Handyside, and M. Edidin. 1982. Effect of microvilli on lateral diffusion measurements made by the fluorescence photobleaching recovery technique. *Biophys. J.* 38:295-297.
- Zidovetzki, R., Y. Yarden, J. Schlessinger, and T. M. Jovin. 1981. Rotational diffusion of the epidermal growth factor complexed to cell surface receptors reflects rapid microaggregation and endocytosis of occupied receptors. *Proc. Natl. Acad. Sci. USA.* 78:6981-6985.