Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5788–5791, December 1978
Biochemistry

Epidermal growth factor: Biological activity requires persistent occupation of high-affinity cell surface receptors

(epidermal growth factor antibodies/down regulation/hormone receptors/DNA synthesis)

YORAM SHECHTER, LYDIA HERNAEZ, AND PEDRO CUATRECASAS

Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

Communicated by George H. Hitchings, August 21, 1978

ABSTRACT **The enhancement of DNA synthesis by epidermal growth factor (EGF) in cultured human fibroblasts is demonstrable 24 hr after incubation of the cells at 37°C with very low concentrations (0.83 nM) of the hormone for very short periods (30 min) followed by thorough washing of the cells to remove the free hormone in the medium. This effect must result from persistent, extraordinarily tight binding of the hormone to surface receptors, because the addition of specific, purified anti-EGF IgG as late as 8 hr after initial hormone exposure can completely reverse the biological effects of the hormone. This causes only a slight (but significant) increase in the rate of dissociation at 37°C of the cell-bound ^{125}I-labeled EGF at low occupancy. Together with the fact that in the presence or absence of antibody virtually all of the demonstrable cell-bound ^{125}I-labeled EGF can be shown to dissociate from the cell during a period as short as 2–3 hr, the data suggest the possibility that the biological effects of this hormone may be mediated by occupation of only a negligible fraction of very high affinity binding sites. Thus, the processes of hormone internalization, degradation, and "down regulation" may be irrelevant to the effects of the hormone on DNA synthesis. For this effect the crucial and limiting processes appear to be strictly related to the continuous and persistent occupation of cell surface receptors.**

Recent evidence suggests that several peptide hormones may become internalized after complexing with cell surface receptors. For example, such a process has been described for β-melanotropin in mouse melanoma cells (1), for human chorionic gonadotropin in granulosa cells,* and for insulin in hepatocytes† and lymphocytes (2).

Carpenter and Cohen (3) have presented data on the binding of human epidermal growth factor (EGF) to fibroblasts which appear to demonstrate rapid internalization and degradation of the hormone after specific receptor binding. Upon incubation of cells with ^{125}I-labeled EGF (^{125}I-EGF) at 37°C, there is a rapid (i.e., by 2 hr) and profound (i.e., 80–90%) loss of cell-associated radioactivity and a concomitant, apparent loss of receptor sites ("down regulation") (4). In addition, when prelabeled cells are washed before incubation at 37°C, virtually all the cell-bound radioactivity dissociates rapidly (within 2 hr) and is recovered in the medium as a low molecular weight component as a result of an energy-dependent process suggestive of internalization and degradation by lysosomal enzymes. Recently, fluorescent analogs of EGF have been shown by a photo-bleaching recovery method to move laterally (at 23°C) on the surface of fibroblasts with a diffusion coefficient of $3–5 \times 10^{-10}$ cm^2/sec (5). By fluorescence microscopy, the initially diffuse staining undergoes rapid (within minutes) aggregation (patching) and internalization at 37°C (6). Although there is no direct evidence to link these phenomena to the biological activity of the hormone, the data appear to suggest such a possible relationship. Notably, the hormone concentration for inducing down regulation (3) is nearly identical to that for DNA stimulation (7, 8). Das and Fox (9) have suggested that the effects of EGF on DNA synthesis may be mediated by a peptide fragment originating from proteolytic cleavage of the internalized hormone-receptor complexes.

The present studies indicate that in order to elicit a biological response, cell-bound EGF must remain attached to the cell surface for protracted time periods (at least 8 hr). Furthermore, selective intervention of the receptor-bound EGF by specific antisera after the major phases of down regulation and internalization have occurred (i.e., at 8 hr) can still totally reverse the biological response.

EXPERIMENTAL PROCEDURE

Human fibroblasts were explanted and routinely grown at 37°C in Eagle's minimal essential medium with Earles' balanced salts, containing 2 mM glutamine, 100 units of penicillin per ml, and 100 units of streptomycin per ml, supplemented with 10% fetal calf serum, and equilibrated with 5% CO_2 in air. Cells were subcultured with 0.25% trypsin/0.02% EDTA. After trypsinization the parent confluent monolayer cells were plated in 1.5-cm diameter disposable multidish trays (Linbro Chemical Co., New Haven, CT) in 1 ml of medium containing 10% fetal calf serum. Trypsin was eliminated by two subsequent changes of medium. After 24–48 hr, the medium was changed to medium containing 5% serum and a further change was made 48–72 hr after the initial seeding. The cells were then grown to confluency (4–7 days). Passages 6 to 10 were used in this study.

Thymidine incorporation into confluent monolayers was measured as described (10) by using a 1-hr pulse with 1 μCi (1 Ci = 3.7×10^{10} Bq) of [*methyl*-^{3}H]thymidine per ml, started 24 hr after the addition of the mitogen. Under the conditions used in these studies, maximal thymidine incorporation was obtained at 1 ng of EGF per ml. Thymidine incorporation was 400–500% of control values in the absence of added mitogen.

The IgG fraction from rabbit antisera to EGF was purified as described by Fahey and Terry (11). The dialyzed antiserum (3 ml) was first purified by chromatography on a DEAE-cellulose column (DE-52, 30 × 0.8 cm) which was equilibrated and eluted with 0.02 M phosphate buffer (pH 7.8). The protein fraction was precipitated with an equal volume of cold, saturated $(NH_4)_2SO_4$. The precipitate was redissolved and reprecipitated twice more and finally dialyzed. The purified fraction (A_{280} = 3.8) precipitated 50% of ^{125}I-EGF (200 Ci/g; 10,000

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: EGF, epidermal growth factor.

* Amsterdam, A., Hollander, Z., Mimrod, A., Reisel, R. & Kohen, F. (1977) *The Seventeenth Annual Meeting American Society for Cell Biology*, San Diego, CA, p. 222a (abstr.).

† Levine, G., Sikstrom, R., Nadler, N. J., Hopriwa, B. & Posner, B. I. (1977) *The Seventeenth Annual Meeting American Society for Cell Biology*, San Diego, CA, p. 182a (abstr.).

cpm/tube) at a final dilution of 1:5000 as determined by radioimmunoassay.

Mouse submaxillary EGF was purified to homogeneity as described by Savage and Cohen (12). ^{125}I-EGF (200 Ci/g) was prepared as described (13) by using the method of Hunter and Greenwood (14). Binding to intact fibroblast monolayers (1.5-cm diameter, approximately 100 μg of protein or 3×10^5 cells per monolayer) was performed as described (7).

RESULTS

Incubation of cells with low concentrations (0.83 nM) of EGF for 30 min at 37°C followed by extensive washing of the cells and replacement with a medium free of EGF results in the retention of a significant biological effect upon subsequent incubation for an additional 24 hr (Table 1). This strikingly persistent effect after only a short exposure to the hormone could not have resulted from binding to and slow desorption from the culture dish. Incubation of the cells with 5 ng of ^{125}I-EGF per ml (0.83 nM, 5×10^5 cpm/ng) for 30 min at 37°C followed by extensive washing revealed that a total of only 8000 cpm (<0.02 ng) was bound to cells and dish together. If this amount of EGF had been totally adsorbed to the dish and were to have been released entirely into the medium, the maximal concentration that could have been achieved would have been 3.3 pM, which is two orders of magnitude lower than that required for half-maximal effects (7).

The use of specific antiserum against EGF was examined as a possible means of distinguishing whether these persistent effects of EGF were the result of tightly bound, externally localized hormone, or whether these biological effects were the result of some irreversible process (e.g., internalization) which was subsequently independent of the continued presence of the native hormone. As expected, rabbit anti-EGF serum had intrinsic DNA synthesis-stimulating activity and thus was unsuitable for use without further purification. Hence, the antiserum was subjected to fractionation to obtain a purified IgG fraction that was completely devoid of DNA synthesis-stimulating activity. This specific anti-EGF immunoglobulin could inhibit completely the DNA synthesis stimulated by EGF but not that stimulated by insulin (Table 2).

The effect of EGF on DNA synthesis can be completely reversed by anti-EGF IgG even when this is added to the cells as long as 8 hr after the addition of and incubation with EGF (at 37°C) (Fig. 1). Significant reversal by the antibody is seen even after incubating the cells with EGF for 12–16 hr. A similar reversal of the EGF effect can be achieved when the cells are incubated with EGF for 30 min at 37°C and washed (as described in Table 1) and the IgG is added after an additional 8-hr period of incubation at 37°C (not shown).

Table 1. Stimulation of thymidine incorporation by EGF with and without a washing step

Addition	Washing step included*	[^{3}H]Thymidine incorporation,† cpm
None	−	5,000
None	+	5,500
EGF (0.83 nM)	−	27,000
EGF (0.83 nM)	+	13,000

* The cells were washed after a 30-min period of incubation with the hormone three times with medium/0.1% albumin and then again incubated for 24 hr at 37°C in the same medium.

† Incorporation of thymidine (1 μCi/ml) during a 1-hr pulse, 24 hr after the addition of EGF, is expressed as counts per min per monolayer (average of three replicate determinations).

Table 2. Stimulation of thymidine incorporation by EGF and insulin in the presence and absence of a purified IgG fraction from rabbit anti-EGF serum

Addition	[^{3}H]Thymidine incorporation,* cpm/monolayer
None	5,300
Antibody (40 μl)	5,200
EGF (0.166 nM)	22,500
EGF (0.83 nM)	24,700
EGF (1.66 nM)	26,800
EGF (0.166 nM) + antibody (5 μl)	5,300
EGF (0.83 nM) + antibody (10 μl)	5,100
EGF (1.66 nM) + antibody (40 μl)	5,200
Insulin (2 μM)	23,000
Insulin (2 μM) + antibody (40 μl)	24,000
Insulin (2 μM) + EGF (0.83 nM) + antibody (40 μl)	25,000

* [^{3}H]Thymidine incorporation was carried out as described in Table 1.

It can be readily demonstrated that the antibody preparation can inhibit the binding of ^{125}I-EGF to cells at the concentrations of IgG used (Table 3). Acceleration by the antibody of the rate of dissociation of the cell-bound hormone was examined at two temperatures, either at low or high occupancy of receptor sites (Fig. 2). At 37°C and at high occupancy (Fig. 2*A*), no effect of anti-EGF IgG on the rate or extent of dissociation of cell-bound hormone was detectable. However, at low occupancy a small but significant increase in the rate of dissociation was demonstrable (Fig. 2*B*). At 24°C the enhancement in the rate of dissociation was more pronounced at either high or low receptor occupancy (Fig. 2 *C* and *D*, respectively).

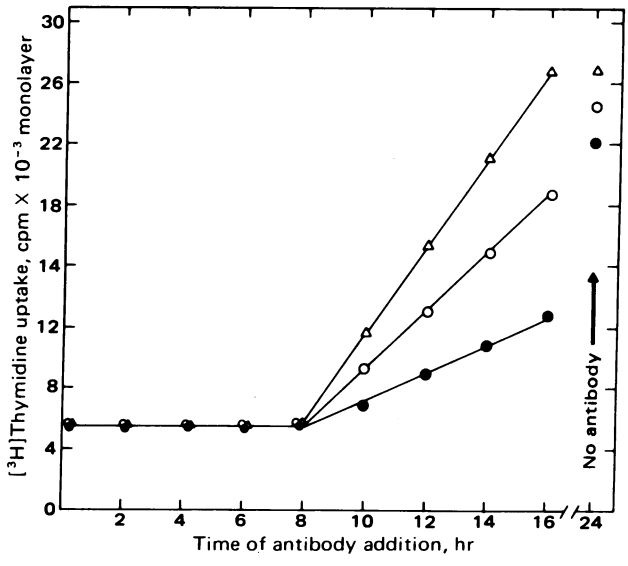

FIG. 1. Effect of addition of anti-EGF antibody at increasing time intervals on the DNA synthesis stimulated by mouse EGF in human fibroblasts. The cells were incubated with three different concentrations of EGF for 24 hr. The incorporation of [^{3}H]thymidine (1 μCi/ml) was then measured during a 1-hr pulse. The values are averages of three replicate determinations. The antibody was added at increasing time intervals during the incubation period. EGF concentrations were 0.166 nM (●), 0.833 nM (○), and 1.66 nM (△). The amount of antibody added was 5, 10, and 40 μl, respectively. The extent of stimulation at the various EGF concentrations in the absence of antibody is given in Table 3 and on the right margin of the figure.

Table 3. Binding of ^{125}I-EGF to fibroblasts in the presence and the absence of anti-EGF IgG

Additions		Cell-bound radioactivity, cpm/dish
^{125}I-EGF, nM	Antibody, μl	
0.83	—	41,000
0.83	10	2,050
1.66	—	50,300
1.66	40	3,600

Monolayers were incubated with ^{125}I-EGF (418,000 cpm/ng) in the presence or the absence of antibody for 1 hr at 37°C. The cells were then washed free of unbound radioactivity, dissolved in alkali, and measured for their radioactive content. Results are expressed as radioactivity bound per monolayer (average of two replicate determinations).

DISCUSSION

The fact that a short period (30 min) of exposure of cells to EGF is sufficient to elicit a partial biological response 24 hr later, in the absence of further addition of free hormone to the medium, indicates that persistent effect can be established early through fixation of the hormone (or a part thereof) or through some irreversible process triggered by the initial receptor interaction. This effect must be mediated by the presence of EGF at the cell surface because the effect can be reversed by specific anti-EGF immunoglobulin added many hours later. The data suggest extremely avid and persistent binding of EGF to some biologically relevant surface receptors. Furthermore, these receptors must remain continually occupied for many hours to generate the DNA synthetic response because potentially dissociated hormone would result in concentrations too low to allow appreciable rebinding.

It is clear that generation of a biological response requires that the receptor-bound EGF exist intact at least for some hours after the process of down regulation is complete, because this process is maximal after 3–4 hr at 37°C (5). Although it is theoretically possible that these hormone-receptor complexes may be slowly and continually internalized in a manner that would be obligatory for the action on DNA synthesis, no evidence or compelling reason for such a proposition exists. In addition to the total lack of a kinetic correlation with down regulation, the data indicate that the limiting and crucial step is one of occupation of the plasma membrane receptor. Clearly, the data are not consistent with the possible generation of an active fragment of EGF that is internalized and subsequently acts in a persistent manner to mediate the long term effects (i.e., DNA synthesis or mitogenesis) of the hormone in the absence of continued occupation of the receptor by the native hormone.

The persistent activity of cell-bound EGF appears to conflict with the abundant evidence (ref. 5; unpublished data) that nearly all (i.e., 90% or greater) of the ^{125}I-EGF bound to cells under similar conditions can be seen to dissociate into the medium as proteolytic fragments of the hormone. At present this discrepancy can perhaps be best rationalized by postulating that only a very small (perhaps negligible) proportion of the sites occupied by ^{125}I-EGF are relevant to the biological effects of the hormone. These occupied sites, however, must be accessible and sensitive to antibodies. It is also inherently obvious from these arguments that the apparent, marked degradation of the hormone observed during dissociation experiments must be irrelevant to hormonal action.

The fact that only a small enhancement in the rate of dissociation of the cell-bound hormone by antibodies is demonstrable (and only at low occupancy) under conditions (37°C) that totally reverse the biological effects could be consistent with the possibility that a negligible fraction of high-affinity binding sites are relevant. In addition, the possibility exists that the antibodies bind to receptor-occupied EGF and antagonize its action without causing dissociation.

The possibility that a very small subfraction of the total binding sites are relevant for the action of EGF is consistent with the persistence of the biological effects discussed earlier, which suggests a population of receptors with a high affinity for the hormone. The affinities estimated from ^{125}I-EGF binding studies (7, 13), which are supported by the marked rate and extent of dissociation of cell-bound hormone (e.g., Fig. 2), are certainly not sufficiently great to account for this apparent protracted binding. However, it is difficult to reconcile such a high affinity system with the affinities estimated from biological dose-response curves, which appear to be similarly low (7).

An intriguing alternative hypothesis is that, subsequent to binding of ^{125}I-EGF to intact cells, a specific proteolytic cleavage of the hormone occurs (at 37°C) which releases into the medium a small peptide that contains the ^{125}I label but leaves at the receptor a nonlabeled, biologically competent fragment. The latter would remain tightly bound to the re-

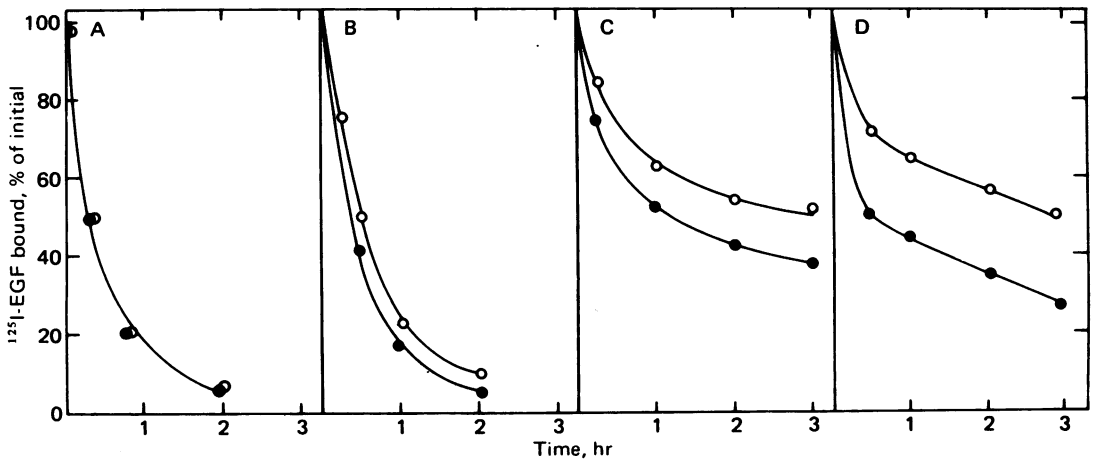

FIG. 2. Dissociation of cell-bound ^{125}I-EGF in the presence and the absence of anti-EGF IgG. Fibroblasts in monolayers were incubated with ^{125}I-EGF (400,000 cpm/ng) at 0.5 ng/ml (low occupancy, *B* and *D*) or 5 ng/ml (high occupancy, *A* and *C*) for 1 hr at 37°C. The cells were then washed free of unbound radioactivity and 1.0 ml of medium, pH 7.4/0.1% bovine albumin was added alone (○) or with antibody (50 μl) (●). The cells were incubated at either 37°C (*A* and *B*) or 24°C (*C* and *D*) and aliquots of the medium were withdrawn for determination of the radioactivity dissociated.

ceptor, although it would not be detectable by the present tracer labeling techniques, and it would be capable of interacting with antibody in the medium. At this point, there is no compelling reason for speculating that if such a process were to occur it would be obligatory for the biological function of the hormone. This possibility, although highly speculative, could explain a number of the unusual properties of EGF binding to intact cells described here and elsewhere.

Other recently described experimental data are in agreement with the major conclusions drawn in this work with respect to the importance of externally present EGF for the biological activity of the hormone (8, 15–17). However, the recent accumulation of substantial data concerning down regulation, internalization, and degradation of various polypeptide hormones at their target tissues (1–3, 6, 9, 18,*†) has shifted the focus of interest toward phenomena that may be irrelevant mechanistically to hormonal stimulation, as suggested in the present work.

The helpful discussions and suggestions provided by Drs. Kwen-Jen Chang, Steven Jacobs, Joseph Schlessinger, and James Niedel and the technical assistance provided by Joanna T. Rogers are gratefully acknowledged. Y.S. is a recipient of the Chaim Weizmann Research Training Fellowship.

1. Varga, J. M., Mollemann, G., Fritsch, P., Godawska, E. & Lerner A. B. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 559–562.
2. Carpenter, J. L., Gorden, P., Omherdt, M., van Obberghen, E., Kahn, C. R. & Orci, L. (1978) *J. Clin. Invest.* **61,** 1057–1070.
3. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71,** 159–171.
4. Raff, M. (1976) *Nature (London)* **259,** 265–266.
5. Shechter, Y., Schlessinger, J., Jacobs, S., Chang, K. J. & Cuatrecasas, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2135–2139.
6. Schlessinger, J., Shechter, Y., Willingham, M. C. & Pastan, I. (1978) *Proc. Natl. Acad. Sci. USA.* **75,** 2659–2663.
7. Hollenberg, M. D. & Cuatrecasas, P. (1975) *J. Biol. Chem.* **250** 3845–3853.
8. Carpenter, G. & Cohen, S. (1976) *J. Cell. Physiol.* **88,** 227–238.
9. Das, M. & Fox, C. F. (1978) *Proc. Natl. Acad. Sci. USA.* **75,** 2644–2648.
10. Hollenberg, M. D. & Cuatrecasas, P. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2964–2968.
11. Fahey, J. L. & Terry, E. W. (1973) in *Immunochemistry,* ed. Weir, D.M. (Blackwell Scientific Publications, Oxford), Vol. 1, pp. 71–74.
12. Savage, C.R., Jr. & Cohen, S. (1972) *J. Biol. Chem.* **247,** 7609–7611.
13. O'Keefe, E., Hollenberg, M. D. & Cuatrecasas, P. (1974) *Arch. Biochem. Biophys.* **164,** 518–526.
14. Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194,** 495–496.
15. Aharonov, A., Pruss, R. M. & Herschman, H. R. (1978) *J. Biol. Chem.* **253,** 3970–3977.
16. Vlodavsky, T., Brown, K. D. & Gospodarowicz, D. (1978) *J. Biol. Chem.* **253,** 3744–3750.
17. Lindgren, A. & Westermark, B. (1976) *Esp. Cell. Res.* **99,** 357–362.
18. Anderson, R. G. W., Goldstein, J. L. & Brown, U. S. (1977) *Nature (London)* **270,** 695–699.