Journal of Supramolecular Structure
and Cellular Biochemistry 17:377-387 (1981)

# Long-Term Epidermal Growth Factor-Receptor Internalization and Processing in Quiescent Human Fibroblasts

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Epidermal growth factor is internalized into cells and concomitantly induces a massive clearance of up to 90% of its total surface receptors. The hormone-receptor complex is delivered to lysosomes and degraded or inactivated. Lysosomotropic alkylamines block the degradation but not the binding or internalization of ligand-receptor complexes and thus their presence results in a marked potentiation of intracellular accumulation of epidermal growth factor. We have used these alkylamines as pharmacological tools to trap internalized $^{125}$I-labeled epidermal growth factor and now report that the residual population of epidermal growth factor receptors remaining on human fibroblasts after completion of the receptor clearance process is not only accessible for ligand binding but also directs the continued internalization and degradation of this growth factor over prolonged periods of time. We also show that down regulation of epidermal growth factor receptors does not result in desensitization of cells to the mitogenic response.

**Key words: down regulation, pinocytosis, lysosomotropic drugs, desensitization**

Epidermal growth factor (EGF) and its receptors are rapidly internalized into cells by adsorptive pinocytosis [1,2]. The pinosomes formed are translocated to lysosomes [2] after a short delay [3,4] where both EGF [1-5] and its receptors [5,6] are degraded or inactivated by proteolysis. EGF receptors are, furthermore, internalized and inactivated even in the absence of binding of the ligand [1,5,7] as a result of basal turnover processes ($t_{1/2} \sim 6$ hr). Although there is a rapid stimulation of the removal of surface receptors upon exposure of cells to EGF, this enhanced rate is only transient and persists for only about 90-120 min [4,5]. After this time, the remaining receptors have an apparent half-life equivalent to the basal turnover rate [4,5], even though medium EGF is largely undegraded and the residual receptor population is seemingly occupied by EGF [1-5].

Received June 24, 1981; accepted August 18, 1981.

0275-3723/81/1704-0377$03.50 

A great deal of controversy has surrounded the meaning of the process of hormone-induced surface receptor "down regulation." It has been suggested that this process is a means for achieving desensitization of cells to subsequent hormone exposure [8]. However, for EGF, the known mitogenic potency of this peptide [1–7,9] correlates best with the concentration dependence for receptor internalization and not with receptor occupation [6]. Furthermore, in order for EGF to elicit a mitogenic response in quiescent fibroblasts, it must be present continuously in the medium external to cells for at least 6 to 8 hr [1,9,10]. Removal of EGF by simple washing [1] or by immunoinactivation by specific antisera [9,10] prior to this time eliminates the subsequent stimulation in DNA synthesis. These results suggest that the critical feature of EGF action takes place at the plasma membrane, but that the underlying processes are more complex than simple binding, which is maximal for EGF after a 1-hr exposure [1–5], or the resultant rapid internalization and degradation of the hormone, which are seemingly complete after 2 to 3 hr [1–5]. We now report that when cells have prior exposure to EGF, their surface receptor capacity is reduced, but that no mitogenic desensitization to subsequent exposures occurs.

We [4,5,10,11] and others [6,7] have suggested that the mitogenic activity of EGF may depend on the translocation of the hormone or its receptor into cells, and that lysosomal processing of the ligand receptor complex may result in the production of a cytoplasmic intracellular second messenger capable of interacting directly with nuclear components. In support of this hypothesis, we have found that lipophilic alkylamines which block lysosomal function [2–5,11], without affecting the binding or internalization of EGF [3–5], are potent inhibitors of EGF-induced mitogenic activity in quiescent fibroblasts [11].

In this study, we have used alkylamine lysosomal inhibitors as pharmacological tools to trap internalized EGF-receptor complexes [3,5]. In doing so, we hoped to understand further the molecular processes required for mitogenic induction by EGF. The results of this study show that EGF continues to be translocated into cells even after its initial complement of surface receptors has been largely degraded (or inactivated). Thus, the EGF-receptor internalization and degradation processes persist for up to 24 hr and are not limited to the massive receptor clearance phenomenon associated with "down regulation" (complete within 2–3 hr). This is the first conclusive evidence that EGF is continuously endocytosed and degraded throughout the extended incubations required for the potentiation of DNA synthesis. Furthermore, we show that the "down regulation" process does not mediate cellular desensitization to the mitogenic response. These data, in conjunction with those showing that lysosomotropic agents inhibit the mitogenic activity of EGF, provide circumstantial evidence that the prolonged requirement for EGF in the cellular medium may be a manifestation of a requirement for prolonged and continuous internalization and processing of EGF or its receptors.

## MATERIALS AND METHODS

### Cell Culture

Human foreskin fibroblast explants (HF) were grown as described [12]. After 24 to 48 hr, cells were changed from 10% fetal bovine serum (FBS) to 0.5% FBS for 24 hr prior to determination of $^{125}$I-EGF binding or DNA synthesis.

### $^{125}$I-EGF Accumulation

Mouse EGF was prepared in our laboratory [13] and iodinated as described [12]. Time courses of $^{125}$I-EGF association were performed at 37°C in DMEM, 0.1% bovine serum albumin (BSA) [5] with 10 mM methylamine ($MeNH_2$). This concentration of $MeNH_2$ inhibits the degradation of $^{125}$I-EGF (5 ng/ml) by > 95% [2–5,11]. In some experiments, cultures were pretreated for 4–6 hr with 5 ng/ml unlabeled EGF to induce maximal down regulation of receptors before the incubation with $^{125}$I-EGF was begun. Details of the exact incubation conditions are given in the respective figure legends.

### DNA Synthesis

Confluent monolayers were pulsed for 1 hr with [methyl-$^3$H]thymidine (1.0 $\mu$Ci/ml) 20 hr after the addition of mitogen. Unless otherwise indicated, the cells were not washed nor was the medium changed before pulsing. The assays were ended by washing the cells twice with cold Eagle's medium/0.1% BSA. To determine the radioactivity remaining in the $Cl_3CCOOH$-insoluble faction, the cells were trypsinized (0.5 ml, 0.25%) for 10 min, and an equal volume of 10% $Cl_3CCOOH$ at 4°C was added. The precipitates were collected onto glass-fiber filters (Whatman, GF/B) and washed twice with 10 ml of 5% $Cl_3CCOOH$ and once with 5ml of MeOH.

## RESULTS

### Is Receptor "Down Regulation" a Means for Cellular Desensitization to EGF?

It has been suggested that hormone-induced internalization of surface receptors is a means for desensitization of cells to subsequent hormonal challenges [1,8,14]. While this may be true for some of the immediate metabolic effects produced by peptide hormones [14,15], it is paradoxical that the conditions leading to receptor "down regulation" are just those optimal for the delayed actions of peptide hormones (exposure to hormone for prolonged periods of time), especially enhanced macromolecular synthesis. To determine whether the receptor clearance phenomenon is an important element in mediation of cellular refractoriness to the EGF response, we pretreated quiescent fibroblasts with 5 ng/ml EGF for 2 hr to produce cultures with reduced receptor binding capacity. For these cells a half-maximal reduction in receptor binding occurs at 1.5 ng/ml EGF. Maximal loss occurs at 3 ng/ml EGF with about an 80% reduction (not shown). These cells were then washed extensively in order to remove unbound EGF. After this, either DMEM, 0.1% BSA, or DMEM, 0.1% BSA containing EGF (0.1 to 20 ng/ml) was added back to the cultures for 14 hr. Then, cells were incubated with 1 $\mu$Ci/ml [$^3$H]thymidine for 4 hr to determine the rate of DNA synthesis. When EGF was removed from the cell medium after 2 hr of exposure, no potentiation in DNA synthesis was observed subsequently. This is consistent with previously obtained evidence [1,9,10]. In contrast, if EGF was added back to the cells, then DNA synthetic activity was essentially identical to that obtained when cells were exposed continuously to EGF (Fig. 1). These results demonstrate that the EGF-induced clearance of surface receptors does not result in a significant diminution of the cellular response at the EGF concentrations tested.

## Effect of $MeNH_2$ on Time Course of Association of $^{125}$I-EGF With Cells

After EGF induces a massive surface receptor loss and internalized $^{125}$I-EGF is degraded, there remains a population of receptors that are seemingly and inexplicably resistant to degradation [1,2,10]. This low level of EGF association persists throughout the duration of its period of mitogenic action, and thus, has been proposed to be the receptor population that mediates this effect. We wanted to study the nature of this long-term, low level of association of $^{125}$I-EGF with cells and to determine whether $^{125}$I-EGF was internalized subsequent to the primary process leading to down regulation of surface receptors. To do so, cells were incubated with 2.5 ng/ml $^{125}$I-EGF and the amount of $^{125}$I-EGF associated with cells over time was monitored. At different times after the initiation of the incubation with $^{125}$I-EGF, 10 mM $MeNH_2$ was added to the remaining cultures in order to prevent lysosomal degradation and thereby trap any $^{125}$I-EGF which accumulates intracellularly. Thus, the further association of $^{125}$I-EGF was determined in the presence of $MeNH_2$.

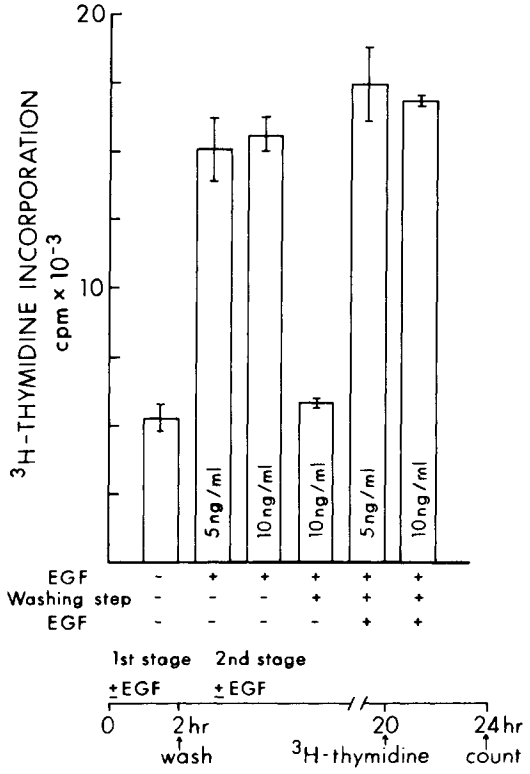


Fig. 1. Effect of prior exposure of cells to EGF on EGF-induced mitogenesis. At $t_0$, confluent monolayers of cells were exposed to 5 ng/ml unlabeled EGF at 37°C for 2 hr. This first stage of the incubation was used to induce surface receptor internalization [1-7]. The cells were then washed and in the second stage of the incubation, varying concentrations of EGF (5 and 10 ng/ml) were added. After 20 hr, the cultures were pulsed with a 1 $\mu$Ci/ml [$^3$H]thymidine for 4 hr. Some cultures only received the first incubation with EGF, whereas others received only the second stage of the incubation.

Whether $MeNH_2$ was added at 2 or 3 hr (Fig. 2A), or at 2, 8 or 18 hr (Fig. 2B), there was a time-dependent enhancement in cell-associated $^{125}$I-EGF when compared to their untreated counterparts. This $MeNH_2$-enhanced $^{125}$I-EGF associated with cells resists dissociation, is insensitive to trypsinization, and the potentiation does not occur at 4°C nor in the presence of EGF antisera (not shown). Thus, the $MeNH_2$-enhanced cell-associated $^{125}$I-EGF is most likely caused by an intracellular accumulation of undegraded radioligand. Under these conditions, $MeNH_2$ is added after the initial complement of receptors has been reduced and degradation of internalized EGF has partially proceeded (Fig. 2). The accumulation of $^{125}$I-EGF is stimulated approximately twofold over that of similar untreated cultures. These results demonstrate that cells may have the ability to internalize $^{125}$I-EGF even after their surface receptor capacity is severely reduced and suggest that EGF is continuously

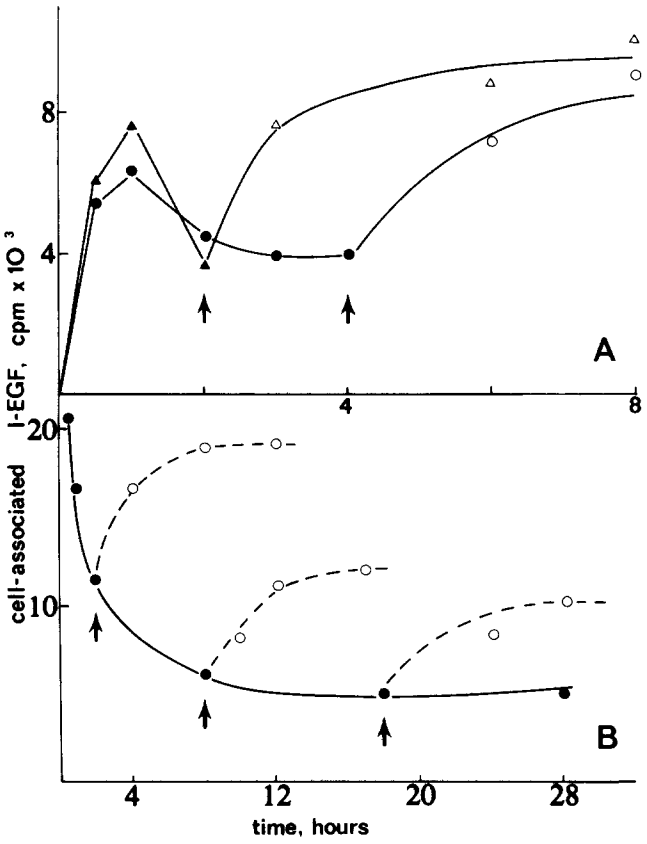


Fig. 2. Effect of $MeNH_2$ on association of $^{125}$I-EGF with cells. Time courses of the association of $^{125}$I-EGF were performed as described in Figure 1 with the addition of 10 ng/ml $^{125}$I-EGF initiating the experiments. The cultures were untreated in the beginning of the experiment except for the radiolabeled EGF, and the association of $^{125}$I-EGF was monitored in these untreated cultures (●,▲). At the times indicated by the arrows, 10 mM $MeNH_2$ was added to the remaining cultures and the association of $^{125}$I-EGF in the presence of $MeNH_2$ was now determined (△,○). (A) $MeNH_2$ added at 2 and 4 hr. (B) $MeNH_2$ added at 2, 8, and 18 hr. Specificity was determined with 5 μg/ml unlabeled EGF.

internalized for many hours or days. These results also clearly show that the capacity of cellular uptake of $^{125}$I-EGF measured at times later than 1 hr in the absence of lysosomotropic agents is a considerable underestimate and suggest that this is so because of an on-going process of degradation.

## Time Course of Association of $^{125}$I-EGF After Receptor Down Regulation

To be certain that the accumulation of $^{125}$I-EGF in cells treated with $MeNH_2$ after the apparent loss of surface receptors (Fig. 2) is not due to an artifact caused by the presence of the alkylamine itself, we pretreated cells for 3 hr with a concentration of unlabeled EGF (5 ng/ml) which maximally induces receptor loss in these cells and then determined their ability to accumulate $^3$H-thymidine now in the absence of $MeNH_2$ (Fig. 3A). These cells which have lost ~ 75% of their surface receptors are still capable of accumulating $^{125}$I-EGF in a time-dependent manner and do so to the same extent as observed when the incubation was initiated by the addition of $^{125}$I-EGF and down regulation of receptors and degradation of prebound ligand were allowed to proceed for 3 to 4 hr (Fig. 2). These results demonstrate that preexposure of cells to unlabeled EGF does not result in persistent occupation of a significantly large number of high affinity receptors [10]. Furthermore, the residual receptors remain accessible to the cell surface for prolonged periods of time and the accumulation of $^{125}$I-EGF in cells after prior exposure to unlabeled EGF is quantitatively identical to that observed when the radiolabel was present throughout the incubation (Fig. 2).

We have previously found that the presence of $MeNH_2$ in the cellular medium has no effect on the accumulation of $^{125}$I-EGF into cells during the first hour of incubation. We interpreted these results to suggest that $^{125}$I-EGF is not degraded during this period and that translocation of the hormone to lysosomes may exhibit a delay of about 1 hr [4]. Morphological evidence with ferritin-labeled EGF has confirmed this delay [3]. However, the results of Figure 2 suggest that the addition of $MeNH_2$ to the cellular medium at later times immediately potentiates the cellular accumulation of $^{125}$I-EGF. To study this further, $MeNH_2$ was added to cells which had been pretreated with unlabeled EGF to down regulate surface receptors. $MeNH_2$ stimulates both the apparent rate and extent of accumulation of $^{125}$I-EGF over those observed in the absence of the alkylamine (Fig. 3B). The extent of $^{125}$I-EGF accumulation reaches a plateau after about 30 min whether $MeNH_2$ is present or not. Interestingly, the steady-state levels of $^{125}$I-EGF associated with cells is identical to those observed when $^{125}$I-EGF was present continuously in the incubation (Fig. 2).

The $^{125}$I-EGF uptake capacity in cells whose receptors have been down regulated by unlabeled EGF reaches an initial plateau (Fig. 3), but after 2 to 3 hr of incubation, the cellular capacity to accumulate $^{125}$I-EGF drops to an even lower value in these experiments and reaches a new minimum after 6–8 hours of incubation (Fig. 4). This loss of $^{125}$I-EGF uptake capacity is not observed if $MeNH_2$ is present (Fig. 2). These results suggest that even after the initial surface receptor population has been drastically reduced by previous exposure to EGF, $^{125}$I-EGF is continuously being internalized and processed.

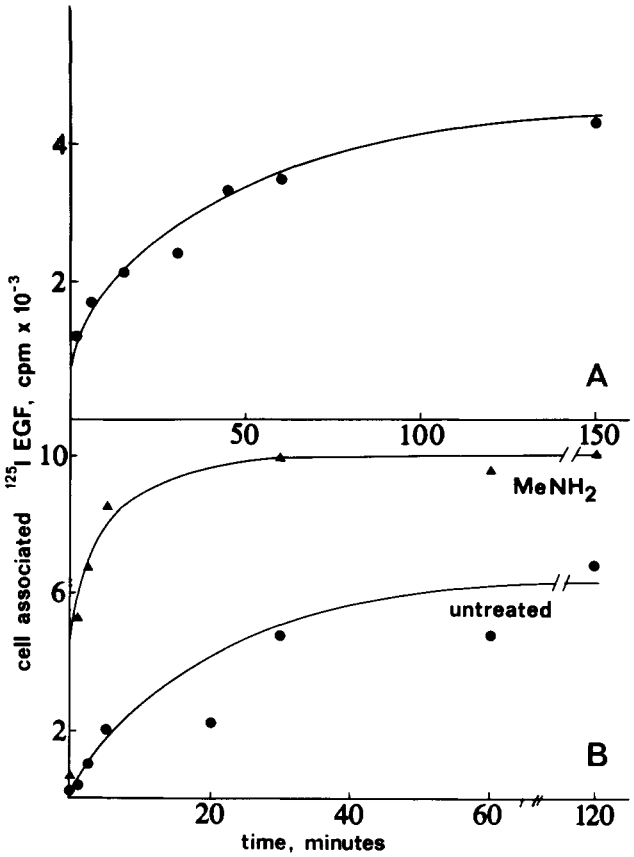


Fig. 3. Time course of association of $^{125}$I-EGF after receptor down regulation. Cells were pretreated with 5 ng/ml unlabeled EGF for 3 hr similarly to that described in Figure 2 in order to down regulate surface receptors maximally. After the cells were washed, they were incubated in DMEM, 1% FBS containing 10 ng/ml $^{125}$I-EGF, and the specific radioactivity associated with cells determined at the indicated times. (A) No additions to the incubation; (B) during the time course, cells were untreated (●), or 10 mM $MeNH_2$ was included in the incubation (▲).

## Comparison of Initial Cellular $^{125}$I-EGF Uptake With That Remaining After Receptor Down Regulation

The uptake capacity of cells before and after the initial receptor clearance by EGF was quantitatively compared by determining the extent of $^{125}$I-EGF taken into cells for a brief incubation period (15 min) at 37°C. The accumulation of $^{125}$I-EGF is linear during this period, and the short incubation ensures that the surface receptor concentration does not become limiting during the assay. All incubations contained 10 mM $MeNH_2$, so that artifacts due to ligand degradation would not interfere with the measurements. Figure 5 is a double reciprocal plot of the uptake of $^{125}$I-EGF into quiescent cells which either had never been previously exposed to EGF (A) or had been pretreated for 4 hr with 5 ng/ml unlabeled EGF (B). The maximal

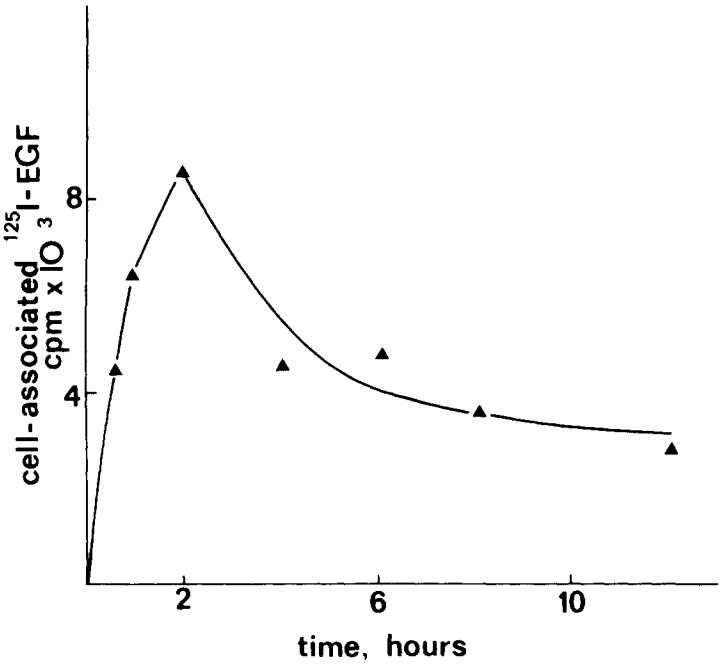


Fig. 4. Long-term association of $^{125}$I-EGF with cells previously down regulated. Surface receptor loss was induced by pretreating cells as described in Figure 3, and the time course of association of 10 ng/ml $^{125}$I-EGF at 37°C was determined from 1 to 12 hr.

uptake for untreated cells in the presence of $MeNH_2$ is 0.27 fmol/min/culture, whereas that for cells whose receptors had been cleared from the surface by previous exposure to EGF is 0.019 fmol/min/culture. The apparent Michaelis constant ($K_m$) for each condition was similar, ranging from 1.75 to 2.2 nM in four separate experiments.

## DISCUSSION

In the present report, we have used alkylamines to establish that EGF is continually taken up into cells by adsorptive pinocytosis even after down regulation of surface receptors is seemingly complete. Methylamine traps all internalized ligand-receptor complexes into dense intracellular vesicles [3,5] and prohibits the usual lysosomal degradation of radiolabeled EGF [3–5]. Thus, inclusion of $MeNH_2$ in cultures treated with $^{125}$I-EGF should result in a stimulation of $^{125}$I-EGF associated with cells as compared to untreated cells if the $^{125}$I-EGF is normally internalized and degraded. Figures 2 and 3A show that the association of $^{125}$I-EGF is further enhanced in the presence of $MeNH_2$, and we conclude that both the continuous internalization (Fig. 2 and 3) and degradation (Fig. 4) of $^{125}$I-EGF may be directed by a residual cell surface receptor population even after the initial process of receptor down regulation is complete.

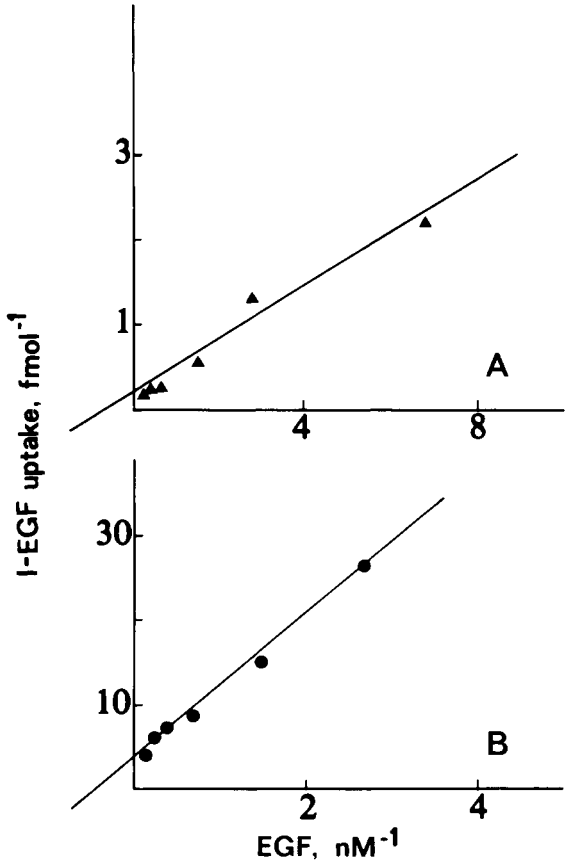


Fig. 5. Comparison of the concentration dependence of initial cellular uptake of $^{125}$I-EGF with that remaining after receptor down regulation. (A) Cells were incubated for 15 min at 37°C with varying concentrations of $^{125}$I-EGF (0.1–20 ng/ml) and the specific radioactivity associated with cells was determined. The association of $^{125}$I-EGF with cells at 15 min is within the linear range at all concentrations tested. The data are plotted in the double reciprocal form to determine the apparent association constant ($K_m$ app) and the maximal attainable velocity ($V_{max}$). (B) Same as in A, but the cells were pretreated with 5 ng/ml unlabeled EGF as described in Figure 1 in order to induce surface receptor loss.

The best characterized biological activity of EGF is its in vitro capacity to stimulate mitogenesis in cultured human and murine fibroblastic and epithelial cells [1,9–12,14]. In order for EGF to elicit a mitogenic response, it must be present in the culture medium during prolonged periods of time [1,9,10]. The results presented in this report suggest that EGF may be continually taken up by the cells, and that at least a portion of the EGF internalized at these late times is degraded. These results may thus indicate that the 6- to 8-hr requirement for extracellular EGF may be a manifestation for a need of the continual internalization of EGF or its receptor [5,11]. It is clear that brief exposure of cells to EGF, which is not sufficient to induce mitogenesis but which does induce surface receptor clearance, is not part of a

process which makes cells refractory to subsequent exposure to the hormone (Fig. 1). It was proposed by Gavin et al that the down regulation of surface receptors could be a possible mechanism for desensitization of cells to subsequent hormone challenges [8]. However, we find that prior exposure of cells to EGF is not a sufficient criterion for reduced responsiveness. In fact, it is known that the mitogenic response to EGF is highly dependent on a long-term exposure of cells to the peptide, and so the requirements for EGF activation of cells may be exactly those which have been shown to cause desensitization of cells to other hormone systems which mediate immediate alterations in cellular metabolism.

In the past, it has been assumed that EGF interacts with a single class of receptor macromolecules, because Scatchard plots of "binding" data were found to be linear in most cell types. Lately, however, it has become increasingly apparent that many cell lines may express heterogeneous EGF receptor types and that Scatchard plots may be concave. This is particularly true for rat fibroblast cell lines [16] and many epithelial cells [17–19]. It is clear that the association of $^{125}$I-EGF with cultured cells at 37°C does not meet the equilibrium conditions required for Scatchard analysis [20]. For this reason, we have analyzed the parameters of pinocytosis by double reciprocal plots. These data reveal that the concentration of apparent half-saturation ($K_m$) of the internalization process is the same before and after receptor down regulation (2 nM), but that there is a substantial (~ 90%) reduction in the maximal velocity ($V_{max}$), as expected (Fig. 5). It is noteworthy that the $K_m$ values for pinocytosis in cultured human fibroblasts are significantly higher than estimates of the receptor binding affinity (0.2 nM) obtained by Scatchard analysis at 37°C in these same cells [1,2]. Furthermore, there is an approximately 90% diminution in the maximal pinocytic capacity ($V_{max}$) of cells whose receptors have been preexposed to unlabeled EGF, as compared to only a 50% reduction observed with Scatchard plots [1]. Thus, double reciprocal plots provide better quantitative analysis of the parameters of this system, since an 80–90% reduction in binding capacity is usually observed in human fibroblast cell lines exposed to EGF. The discrepancy in apparent receptor affinity between these two types of plots may be explained by artifacts introduced in determination of these parameters for systems which characteristically do not reach equilibrium such as those exemplified by receptor-mediated internalization processes. This may be a particular problem for analysis of EGF-receptor dynamics where significant internalization occurs prior to delivery of the hormone to lysosomes and release of degradation products [2–4]. This results in a shift of bound/free ratios to higher values due to trapping of the radioligand in cells and gives apparent affinity constants which are far too high.

Recently, several reports have appeared suggesting that the internalization of EGF could be an important element in mediation of its mitogenic activity on cells [6,7,10,11,21,22]. We have lately found that primary and tertiary lysosomotropic alkylamines inhibit the mitogenic activity of EGF, insulin, and serum [11]. These amines do not inhibit the internalization of $^{125}$I-EGF or its receptor [3–5] but are potent inhibitors of lysosomal function [23] and EGF degradation [2,3,5]. These results suggest that lysosomal processing of EGF (and other growth promoting agents) may be important in production of in vitro biological activity. In contrast, agents which are not known to cause a shift in the intralysosomal pH but do inhibit intracellular protein degradation, like leupeptin and antipain, have only minimal

inhibitory effects (20–30%) on the EGF-induced mitogenic response of quiescent cells [24]. These results may indicate that exposure of EGF to an intracellular acid compartment like lysosomes is an important element for production of an activator of the mitogenic response. If the intracellular accumulation of EGF or its receptors are important for mediation of its mitogenic acitivity, then it is clear that a continued process of uptake over protracted periods of time is a critical feature. Such a long-term requirement could be explained if only a minor portion of the EGF-receptor complex escapes total degradation in lysosomes, and a threshold concentration of an activated component is required for activity.

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