Rapid Constitutive Internalization and Externalization of Epidermal Growth Factor Receptors in Isolated Rat Hepatocytes

MONENSIN INHIBITS RECEPTOR EXTERNALIZATION AND REDUCES THE CAPACITY FOR CONTINUED ENDOCYTOSIS OF EPIDERMAL GROWTH FACTOR

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It was previously demonstrated that freshly isolated rat hepatocytes can internalize severalfold more epidermal growth factor (EGF) molecules than the number of surface EGF receptors, suggesting extensive reutilization of receptors during endocytosis (Gladhaug, I. P. & Christoffersen, T. (1987) Eur. J. Biochem. 164, 267–275). The present report attempts to explore the pathways involved in the internalization of EGF receptors. Incubation of hepatocytes at 37 °C in the absence of ligand increased the surface receptor pool by 50–100% within 45 min. Pretreatment with monensin inhibited the turnover of the surface EGF receptor pool by 50–60% within 10 min and blocked the temperature-dependent externalization of receptors. Cycloheximide caused a slower attenuation of the surface receptor pool, whereas tunicamycin and chloroquine did not significantly affect the exchange of receptor pools. Monensin reduced the surface receptor pool and the endocytic uptake in corresponding proportions, without affecting the internalization of prebound EGF. Endocytic uptake was unaffected by chloroquine and slightly reduced by cycloheximide. The internalization of unoccupied receptors and the endocytosis of prebound EGF followed similar kinetics (Rho 5–5 min), suggesting that unoccupied receptors are internalized at a rate comparable to that of occupied receptors. The results suggest that there is a rapid turnover of the surface pool of EGF receptors with constitutive internalization of unoccupied surface receptors and externalization of internal receptors. This is consistent with, but does not prove, a true recycling of the EGF receptors in the hepatocytes. The monensin-sensitive externalization pathway determines the capacity for continued endocytosis of EGF.

The receptor for epidermal growth factor (EGF) is a 170-kDa transmembrane glycoprotein (1–4) involved in growth control (5) and apparently in oncogenesis (6). The receptor shows homology with the erbB-1 oncogene product (7) and has a tyrosine-protein kinase activity in the internal domain which is activated by binding of EGF to the external domain (4, 8). The ligand-receptor complexes cluster in coated pits and are rapidly internalized by receptor-mediated endocytosis (9–11). Despite extensive studies the biochemical mechanisms and physiological significance of growth factor receptor translocations following ligand binding is still unclear. In other ligand-receptor systems, such as the receptors for nutrients and metabolic products including low density lipoprotein, transferrin, and asialoglycoproteins, there is evidence that receptors are recycled back to the plasma membrane after the intracellular dissociation of the ligand (for review, see Refs. 12–15). For the EGF receptor, the intracellular pathway during endocytosis is less clear. Biochemical studies in cultured fibroblasts and transformed human epithelial cell lines have indicated degradation of the receptor during endocytosis with no recycling of the receptor (16–19). However, data obtained in rat liver (20) and isolated rat hepatocytes (21) have suggested the presence of a pool of internal EGF receptors that functions during endocytosis. Recently, several immunocytochemical studies have shown the presence of intracellular EGF receptors in several types of cells (22–26). In rat liver an internal pool of approximately the same magnitude as the surface receptor number has been identified on sucrose gradients (24). In human liver the majority of the EGF receptors appear to be localized within the cytoplasm (26). We previously showed that the freshly isolated rat hepatocyte endocytoses EGF in severalfold excess of the available surface receptor number (21), suggesting a considerable recruitment of receptors to the plasma membrane during endocytosis. In the present work we have attempted to gain more information about the pathways involved in the externalization of EGF receptors. We have examined how agents known to interfere with intracellular routing and synthesis of receptors influence the surface expression of EGF receptors and the capacity for endocytosis of EGF. In particular we have investigated the effect of monensin, a carboxylic ionophore which disrupts intracellular traffic and recycling of many receptors (27–30). Monensin intercalates into membranes and abolishes transmembrane ion gradients (31–33). It disturbs the functions of several intracellular vesicles, particularly Golgi functions, probably by disrupting pH and calcium gradients across intracellular membranes (34–36).

Here we report that monensin rapidly depletes hepatocyte surface receptor activity for EGF and inhibits continuous endocytosis of EGF, suggesting that there is a constitutive process involving both internalization and externalization of receptors. This process determines the capacity for endocytosis of EGF.

EXPERIMENTAL PROCEDURES

Materials—125I-EGF (specific activity 119–140 Ci/g) was from Amersham International (Buckinghamshire. United Kingdom). Un-
labeled EGF (receptor grade) was from Collaborative Research (Lexington, MA). Collagenase (type 1), bovine albumin (fraction V), chloroquine, tunicamycin, and cycloheximide were from Sigma. Monensin was from Calbiochem. Dibutyl phthalate was from Fluka AG (Buch, Switzerland).

**Preparation of Hepatocytes**—Male Wistar rats (200–240 g) were fed ad libitum. Hepatocytes were isolated by in vitro collagenase perfusion and low speed centrifugations (37°C with modifications (21, 38)). Viability, measured by the ability to exclude trypan blue, was 93–97%. Cell counts were done in a Burker hemocytometer.

**Buffers and Incubations**—For all incubations hepatocytes (10⁶ cells/ml) were suspended in Krebs-Hepes-Ringer buffer (118.4 mM NaCl, 2.9 mM KCl, 2.5 mM KH₂PO₄, 1.2 mM MgSO₄, 2.7 mM CaCl₂, 24.6 mM Hepes) supplemented with 1% bovine albumin and 10 mM glucose, pH 7.4. The incubations were carried out during continuous gyratory shaking (125 rev/min) in sealed conical flasks (10–25 ml) at 37 or 0°C. Stock solutions of 25 mM monensin in 96% ethanol were kept at −20°C. Monensin was diluted with incubation medium immediately before use. The total amount of ethanol never exceeded 0.1% (v/v) of the incubation medium, and equivalent amounts of ethanol were added to the control suspensions in each experiment. At this concentration ethanol did not affect the binding or uptake of labeled EGF (data not shown). Incubation with monensin (1–100 μM) at the conditions indicated did not affect hepatocyte viability (data not shown).

**Binding of ¹²⁵I-EGF**—Binding to the hepatocyte surface EGF receptors was determined at 0°C (i.e. at conditions where internalization is negligible) as described previously (21). For saturation studies, labeled EGF was diluted with unlabeled EGF in a fixed ratio of 1:9, final concentration range 0.01–10 nM. Equilibrium binding data were analyzed according to Scatchard (39). Nonspecific binding (routinely 3–6%) was determined in the presence of 1000 excess of unlabeled EGF. The binding reaction was terminated by transferring aliquots of incubation mixture into polyethylene microtubes and centrifugation of the hepatocytes through a dibutyl phthalate oil layer at 9500 × g for 4 min (Beckman Microfuge 11) (40). The cell pellet and the medium (representing bound and free ligand, respectively) were counted in a γ-counter (Packard).

**Internalization of ¹²⁵I-EGF**—Uptake studies were performed at 37°C as described previously (21). At this temperature, the cell-associated radioactivity equals the sum of surface-bound and intracellular ligand. The acid/salt elution method of Haigler et al. (41) for dissociating surface-bound ligand was used to distinguish between surface-bound and intracellularly trapped ligand.

**RESULTS**

Constitutive Recruitment of EGF Receptors to the Cell Surface by Mechanisms Sensitive to Monensin and Cycloheximide—Saturation binding demonstrated that the freshly isolated rat hepatocyte expresses 100,000–150,000 EGF receptors/cell surface, and that the surface receptor number increases to a maximum (200,000–250,000) when the cells are incubated for 45–60 min at 37°C, indicating that the surface EGF receptor pool is in a state of dynamic turnover (Fig. 1 inset), indicating that its effect was not due to direct interference with the ligand-receptor interaction. Cycloheximide induced a slower attenuation of the surface binding (Fig. 2A), and apparently preserved the steady state binding characteristics of the surface receptors (Fig. 3A). Thus, whereas incubation with monensin at 37°C in the absence of ligand induced an acute reduction in the number of cell surface EGF receptors down to 50% below steady state level, incubation with cycloheximide under identical conditions slowly reduced the receptor number toward a steady state level.

To assess the role of the acidic compartments of the endocytic pathway in the maintenance of the surface EGF receptor number, hepatocytes were also incubated with chloroquine.

![Fig. 1. Effects of monensin, cycloheximide, and tunicamycin on the time-dependent acquisition of surface receptors.](image-url)
cycloheximide, or chloroquine. The cells were then chilled at was repeated once with an essentially similar result. The mean of duplicate measurements. Each experiment represents the mean of duplicate measurements. Each experiment was repeated three times for the last 15 min. The cells were then chilled at 0 °C for 15 min, and the surface receptors were assayed as described in Fig. 2A. Each point represents the mean of duplicate measurements. The experiment was repeated three times with essentially similar results. B, Scatchard analysis of the effect of monensin. Hepatocytes (10⁶ cells/ml) were preincubated at 0 °C (○) or 37 °C (▲) for 60 min, or at 37 °C for 60 min in the absence (●) or presence (▲) of 25 μM monensin for the last 15 min. The cells were then chilled (0 °C, 15 min) and incubated with labeled EGF (0 °C, 8 h) as described under "Materials and Methods." The results represent the mean with S.E. of values from three identical experiments each assayed in duplicate. Inset, lack of effect of monensin on the ligand-receptor binding reaction. 25 μM monensin (△, ▲) was added in the binding assay at 0 °C to control suspensions (●, ○) that had been preincubated for 60 min at 0 °C (△, ▲) or for 45 min at 37 °C and then chilled for 15 min at 0 °C (●, ▲).

As shown in Fig. 3B, incubation with chloroquine (100 μM) for 45 min did not significantly inhibit the increase in cell surface receptors. Thus, the surface receptor level was probably independent of maintenance of intravesicular low pH.

Monensin Reduces Endocytosis of EGF through Depletion of the Pool of Available Surface Receptors—Fig. 4 shows the effect of monensin and cycloheximide on internalization of EGF. The cells were incubated with a saturating concentration of EGF (5 nM), surface-bound EGF was removed by the acid/salt elution method, and the acid-resistant radioactivity representing intracellularly trapped internalized ligand was measured. The maximal surface binding capacity prior to endocytosis was also measured by saturation binding at 0 °C. During the initial 30 min of endocytosis, about three times as many EGF molecules were internalized as the number of available surface receptors. Pretreatment with monensin (25 μM) led to a reduction (61%) in the number of internalized EGF molecules/cell, closely corresponding to the monensin-induced decrease (67%) in surface receptors. The effect of monensin was half-maximal at 2 μM and maximal at 10 μM (data not shown). In view of the inhibitory effect of cycloheximide on receptor recruitment to the cell surface (Fig. 2A), we also examined the effect of pretreatment with cycloheximide on the internalization of EGF. In contrast to monensin, cycloheximide caused only a slight reduction (16%) in the number of internalized EGF molecules/cell.

Monensin Inhibits Endocytosis without Affecting Internalization of Prebound EGF—The data in Fig. 4 suggest that monensin reduces endocytosis through inhibition of a process that is required for membrane insertion of receptors, rather than affecting the internalization process per se. To test this hypothesis more directly, we monitored the effect of monensin on the endocytosis both during continuous internalization of a saturating concentration of labeled EGF and during internalization of a single wave of prebound labeled EGF. The cells were prebound with labeled EGF (5 nM) at 0 °C for 3 h, in the presence and absence of monensin for the last 45 min. The cells were warmed to 37 °C and the acid-resistant and acid-sensitive ligand was followed for 30 min. Fig. 5A shows that continuous uptake of EGF in excess of the initially surface-bound ligand (i.e. endocytosis which depends on a

FIG. 2. Effects of monensin and cycloheximide on the surface EGF receptor pool. A, time course. Hepatocytes (10⁶ cells/ml) were preincubated at 0 °C (○) or 37 °C (▲) for 45 min. At time 0 (arrow) 25 μM monensin (Mon), 5 μg/ml cycloheximide (Chx), or the combination of 25 μM monensin and 5 μg/ml cycloheximide were added to the suspensions kept at 37 °C. At the times indicated, cell batches were chilled at 0 °C for 15 min and the surface EGF binding was assayed as described in Fig. 1. Each point represents the mean of duplicate measurements. The experiment was repeated three times with essentially similar results. B, Scatchard analysis of the effect of monensin. Hepatocytes (10⁶ cells/ml) were preincubated at 0 °C (○) for 60 min, or at 37 °C for 60 min in the absence (●) or presence (▲) of 25 μM monensin for the last 15 min. The cells were then chilled (0 °C, 15 min) and incubated with labeled EGF (0 °C, 8 h) as described under "Materials and Methods." The results represent the mean with S.E. of values from three identical experiments each assayed in duplicate. Inset, lack of effect of monensin on the ligand-receptor binding reaction. 25 μM monensin (△, ▲) was added in the binding assay at 0 °C to control suspensions (●, ○) that had been preincubated for 60 min at 0 °C (△, ▲) or for 45 min at 37 °C and then chilled for 15 min at 0 °C (●, ▲).

FIG. 3. Scatchard analysis of the effect of cycloheximide and chloroquine on the surface EGF receptor pool. Hepatocytes (10⁶ cells/ml) were preincubated at 0 °C for 45 min (○), or (▲) at 37 °C for 45 min in the absence (●) or presence (▲) of 5 μg/ml cycloheximide, or (□) in the absence (●) or presence (▲) of 100 μM chloroquine. The cells were then chilled at 0 °C for 15 min, and the surface receptors were assayed as described in Fig. 2B. Each point represents the mean of duplicate measurements. Each experiment was repeated once with an essentially similar result.

FIG. 4. EGF surface binding and internalization: effects of monensin and cycloheximide. Hepatocytes (10⁶ cells/ml) were preincubated for 45 min at 37 °C in the absence (control) and presence of 25 μM monensin (Mon) or 5 μg/ml cycloheximide (Chx). Cell batches for measurement of surface binding were then chilled (0 °C, 15 min) and then incubated with labeled EGF (5 nM, 0 °C, 2 h) to assess maximal surface binding in control cells (open bar), cycloheximide-treated cells (hatched bar) and monensin-treated cells (filled bar). Internalization of labeled EGF (5 nM) at 37 °C was determined in parallel cell batches using the acid/salt elution method (41) to assay intracellularly trapped ligand, as described under "Materials and Methods." Each point represents the mean of duplicate measurements. One of two identical experiments.
EGF Receptor Recycling in Hepatocytes

**DISCUSSION**

The receptors for EGF have generally been considered to be a relatively stable plasma membrane population when not exposed to the ligand. Recently, it has been shown that receptors for transferrin (48) and insulin-like growth factor II (49) can recycle between the plasma membrane and the endosome compartment even when unoccupied by ligand. In this study we provide results which suggest that the surface pool of unoccupied EGF receptors in isolated hepatocytes is subject to rapid, constitutive turnover. This conclusion is based primarily on the ability of monensin to block the temperature-dependent acquisition of surface EGF receptors, (b) rapidly deplete the steady state receptor number, and (c) reduce the rate of EGF endocytosis. Although, theoretically, the possibility exists that the changes in surface receptor expression may be related to intramembrane alterations, as has been claimed for the asialoglycoprotein receptor (50), the most probable explanation for the present data is that a rapid, constitutive recruitment of preformed, unoccupied EGF receptors to the cell surface occurs concomitantly with a constitutive internalization.

Constitutive externalization of receptors was supported by two different lines of evidence. First, the increase in surface receptor expression that occurs on raising the temperature suggests recruitment of preformed intracellular receptors to the plasma membrane. Although the present data do not exclude the possibility that de novo receptor synthesis may contribute to this process, the lack of detectable effect of tunicamycin during the same time range as the rapid inhibitory effect of cycloheximide suggests that the increase in surface receptors was due to externalization of fully glycosylated, preformed receptors. Recent studies in our laboratory to further investigate the effect of cycloheximide have also shown that the inhibitory effect of cycloheximide on receptor recruitment can be abolished by amiloride. Second, monensin reduced the surface receptor number and endocytic capacity in corresponding proportions (Fig. 4), without affecting the internalization of prebound EGF as measured by the acid/salt elution method (Fig. 5, A and B). This suggests that monensin affects a rate-limiting process in the maintenance of the surface receptor pool, possibly by inhibiting surface membrane insertion of internal receptors involved in continued endocytosis.

The conclusion that a constitutive internalization of unoccupied EGF receptors takes place in the hepatocytes was based on the demonstration that the surface receptor number declined markedly (from 190,000 to 60,000 receptors/cell surface) upon treatment with monensin in the absence of ligand. A likely explanation for this phenomenon is that monensin, by blocking recruitment of receptors to the surface, demasks a constitutive internalization. The half-time for the loss of surface receptors (Fig. 2A) was roughly similar to the half-time of the internalization of labeled EGF prebound to the surface receptors at 0°C (Fig. 5A). Although the evidence is only indirect, the data indicate that at steady state conditions

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*1. L. P. Gladhaug and T. Christoffersen, unpublished data.*
in the absence of ligand about 50% of the total number of unoccupied EGF receptors leave the plasma membrane at a rate approximately equal to that of ligand-induced receptor internalization. Taken together the data suggest that in freshly isolated hepatocytes, at least a portion of the unoccupied EGF receptors are subject to constitutive internalization.

Previous biochemical evidence from cultured fibroblasts have shown that monensin causes intracellular accumulation of EGF-receptor complexes by preventing lysosomal proteolysis (51), with no effect on the long term rate of internalization of EGF (52), indicating that monensin inhibits EGF degradation without affecting endocytic capacity. In contrast, the present data from hepatocytes show that monensin lowered the level of surface receptors in parallel with a decreased capacity for endocytosis. This is also in accordance with the failure of all surface receptors to reach the cell surface in cultured fibroblasts.

The effect of monensin was only partial. In all experiments at least 30% of all surface receptors were unaffected by monensin even at concentrations up to 100 μM (data not shown). Similarly, monensin inhibited endocytosis only about 60%. Although we do not know the explanation for this, an obvious possibility is that there are two populations of EGF receptors traversing different intracellular pathways, only one of which is sensitive to monensin. It is interesting to note that similar results have been reported for the hepatic asialoglycoprotein receptor (28).

In conclusion the present results indicate a constitutive, concomitant externalization and internalization of EGF receptors in hepatocytes. The precise intracellular routes traversed by the receptors are not known. A true recycling of the internalized receptors is an attractive hypothesis that might explain the data reported here. However, the relative contribution of internalized as opposed to newly synthesized receptors to the internal pool from which externalization occurs cannot yet be decided.

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