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Report

Characterization of Epidermal Growth Factor Receptors on Plasma Membranes Isolated from Rat Gastric Mucosa[1]

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The binding of human epidermal growth factor (hEGF), β-urogastrone, to plasma membranes isolated from rat gastric mucosa was studied to characterize gastric EGF receptors. The binding of [^{125}I]hEGF was temperature dependent, reversible, and saturable. A single class of binding sites for EGF with a dissociation constant of 0.42 n*M* and maximal binding capacity of 42 fmol/mg protein was suggested. There was little change in the binding of [^{125}I]hEGF upon addition of peptide hormones (secretin, insulin), antiulcer drugs (cimetidine), or an ulcer-inducing reagent (aspirin). Cross-linking of [^{125}I]hEGF to gastric plasma membranes with the use of disuccinimidyl suberate resulted in the labeling of a protein of 150 kDa. These results indicate the presence of EGF receptors on plasma membranes of rat gastric mucosa.

KEY WORDS: epidermal growth factor (EGF); urogastrone; receptor; rat gastric mucosa; plasma membranes; cross-linking; antiulcer agent.

INTRODUCTION

Epidermal growth factor (EGF), a 53-amino acid polypeptide, was found to be identical to β-urogastrone isolated from human urine (1,2). This peptide has several effects on gastrointestinal function: (a) inhibition of gastric secretion in several species including men and rats (3–6), (b) stimulation of the proliferative process in digestive tract tissues (7,8) and (c) promotion of the healing of experimental gastric or duodenal ulcers (9–12).

These effects on EGF on cellular functions are thought to be mediated via specific plasma membrane receptors. The existence of specific receptors for EGF has been demonstrated in various tissues and cell lines (13–18). The EGF receptors in gastric and intestinal glands have been isolated from guinea pigs (19) and rat intestinal epithelial cells (20), respectively. Recently, specific receptors for EGF in rat intestinal microvillus membranes were also reported (21). However, details of EGF binding to its receptors in gastric subcellular fractions have not been clarified.

In our previous study (22,23) the interaction of secretin, an enterogastrone in humans and dogs, with the receptors for secretin in the stomach and pancreas was investigated. In this paper we have studied the specific receptors for EGF on the plasma membranes isolated from rat gastric mucosa to further development of EGF as an antiulcer drug.

MATERIALS AND METHODS

Materials

Highly purified human EGF (more than 99% of purity) prepared from a genetically engineered *E. coli* host was kindly provided by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Highly purified mouse EGF (mEGF) and bovine fibroblast growth factor (bFGF) were obtained from Toyobo Co. (Osaka, Japan). Human EGF was radioiodinated by the chloramine-T method and purified by gel filtration using Sephadex G-50. The specific activity of [^{125}I]hEGF was about 160–260 μCi/μg. Secretin was provided by Eisai Co. (Tokyo), insulin by Novo Industri A/S (Copenhagen, Denmark), cimetidine by Fujisawa Pharmaceutical Co. (Osaka, Japan), cetraxate by Daiichi Pharmaceutical Co. (Tokyo), and proglumide sodium salt by Kaken Pharmaceutical Co. (Tokyo). Carrier-free Na^{125}I was purchased from Green Cross Co. (Tokyo); somatostatin, cholecystokinin octapeptide (CCK8), and pepstatin A from Peptide Institute (Osaka, Japan); prostaglandin E_2 and indomethacin from Sigma Chemical (St. Louis, MO); theophylline from Nakarai Chemicals (Kyoto, Japan); and Sephadex G-50 and Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden). Thin-layer electrophoresis plates were purchased from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals were obtained from commercial sources and were reagent grade.

Preparation of Gastric Plasma Membranes

Plasma membranes were isolated from the gastric mu-

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cosa of male Wistar albino rats (180–260 g) by density-gradient centrifugation as previously described (22). Briefly, the stomachs were removed and rinsed with ice-cold buffer containing 0.25 *M* sucrose, 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 0.1 m*M* phenymethylsulfonyl fluoride (PMSF), 100 U/ml Trasylol, and 0.1 μ*M* pepstatin (buffer A). The gastric mucosa was scraped and homogenized in ice-cold buffer A (1:7, w/v) with a Dounce homogenizer. Then the homogenate was centrifuged and a crude plasma membrane (CPM) fraction was obtained. The CPM fraction was mixed with Percoll (10%, v/v) in buffer A. This mixture (total volume 30 ml) was centrifuged at 48,000*g* for 30 min. On the basis of the distribution of (Na^+-K^+)-ATPase activity, an 8–13 ml aliquot from the top was used as the plasma membrane fraction. After removing the Percoll particles by centrifugation, the plasma membranes were resuspended in a buffer containing 100 m*M* Tris–HCl (pH 7.5), 0.1 m*M* PMSF, 100 U/ml Trasylol, and 0.1 μ*M* pepstatin (buffer B). All these procedures were performed at 0–4°C. Protein was assayed according to the method of Lowry *et al.* (24) using bovine serum albumin (BSA) as a standard.

Binding of [^{125}I]hEGF to Plasma Membranes

Binding studies were performed at 0, 25, and 37°C in buffer B containing 2% BSA (incubation medium). In the regular assay, the binding reaction was initiated by adding 50 μl of membrane suspension (30–50 μg protein) in buffer B to 150 μl of incubation medium, which contained [^{125}I]hEGF (ca. 30,000–50,000 cpm), with or without the competing ligand preincubated for 5 min. The incubation was stopped at the indicated times by diluting the reaction mixture with 1 ml of ice-cold stop solution (buffer B containing 2% BSA) and the tube contents were immediately poured onto Millipore filters (type EHWP, 0.5 μm, 25-mm diameter) and washed once with 7 ml ice-cold buffer B. The filter radioactivity was determined by an automatic gamma counter.

Nonspecific adsorption to the filters was determined by adding 1 ml of ice-cold stop solution to labeled substrate mixture (200 μl). This value was less than 0.05% of the total radioactive hEGF added. To determine the nonspecific binding of hEGF, 100 n*M* of unlabeled hEGF was added to the parallel incubations. Nonspecific binding was 0.1–0.2% of the total radioactive hEGF added.

The linearity of the relationship between [^{125}I]hEGF binding and the amount of membrane protein was tested in the range of 12.5–100 μg protein/tube (total volume, 0.2 ml) as in the binding study described above.

Dissociation of Bound [^{125}I]hEGF from Gastric Plasma Membranes

Plasma membranes (about 30 μg protein) were first incubated with [^{125}I]hEGF (about 45,000 cpm) for 60 min at 25°C. At the end of the first incubation, the plasma membranes were added with 100 n*M* unlabeled hEGF and incubated for an additional 30 min at 25°C.

Cross-Linking of EGF Receptor

Cross-linking of [^{125}I]hEGF to gastric plasma membranes was performed by a modification of the method of Mukku (25).

Membranes (190 μg protein) were incubated at 25°C for 60 min in the presence of [^{125}I]hEGF (0.5 μCi) with and without 4 μ*M* unlabeled hEGF in a total volume of 0.1 ml of binding buffer containing 50 m*M* HEPES (pH 7.5), 1 m*M* PMSF, 100 U/ml Trasylol, 0.1 μ*M* pepstatin, and 2% BSA, followed by centrifugation at 10,000*g* for 5 min at 4°C. The pellets were then resuspended in 23 μl of binding buffer lacking BSA and 2 μl of 6.25 m*M* disuccinimidyl suberate dissolved in dimethyl sulfoxide. After 10 min at 25°C, 5 μl of 0.5 *M* glycine was added to terminate the cross-linking reaction. The mixture was boiled for 3 min with sample buffer containing 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, and 62.5 m*M* Tris–HCl (pH 6.8). The samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% gradient acrylamide gel). The radiolabeled bands were detected by autoradiography of the dried gel.

RESULTS

The binding of [^{125}I]hEGF to rat gastric plasma membranes was proportional to the amount of membrane protein up to 100 μg/tube (total volume, 0.2 ml) (Fig. 1). Figure 2 shows the effect of temperature on the binding of [^{125}I]hEGF to gastric plasma membranes. At 37°C the binding of [^{125}I]hEGF to gastric plasma membranes was half-maximal by 10 min and maximal by 60 min. Reducing the temperature to 25°C decreased the binding of [^{125}I]hEGF. Both the rate and the amount of [^{125}I]hEGF bound was diminished at 4°C. In this series of experiments, nonspecific binding was approximately 10% of total binding throughout the incubation period. To ascertain whether [^{125}I]hEGF was stable under the incubation conditions, the sample incubated for 60 min at 25°C was analyzed by gel filtration (Sepadex G-50). More than 90% of the radioactivity in the incubation mixture appeared at the position of intact hEGF.

To examine the dissociation of the bound hEGF, plasma membranes were first incubated with [^{125}I]hEGF at 25°C for 60 min, then 100 n*M* unlabeled hEGF was added and incubated for an additional 30 min (Fig. 3). The radioactivity bound to the membranes was displaced by unlabeled EGF;

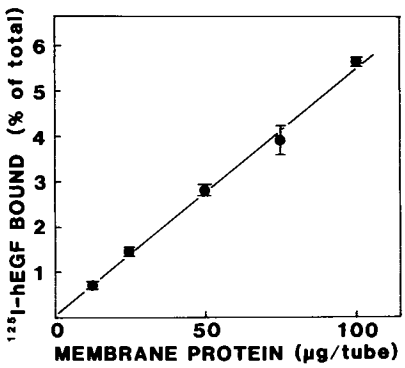

Fig. 1. Binding of [^{125}I]hEGF as a function of membrane protein. Plasma membranes (12.5–100 μg/tube) were incubated at the indicated concentrations with [^{125}I]hEGF for 60 min at 25°C. Each point is the mean ± SE of three determinations.

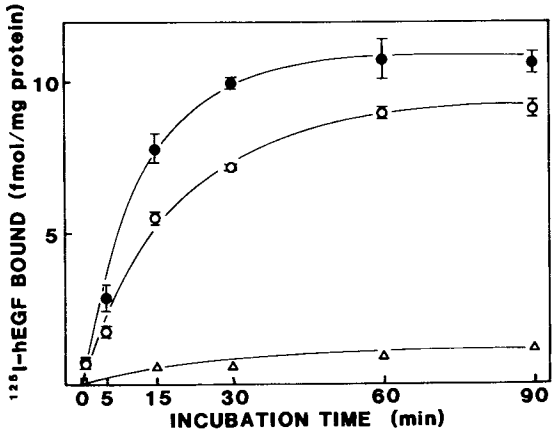

Fig. 2. Time course of [^{125}I]hEGF binding to plasma membranes. Plasma membranes (31 μg protein) were incubated with [^{125}I]hEGF (160 p*M*) at 0 (△), 25 (○), and 37°C (●). Each point is the mean ± SE of three determinations.

the dissociation half-life of bound [^{125}I]hEGF was 38 min (k_{off} = 0.019 min^{-1}). The association rate constant (k_{on}) was 0.092 nM^{-1} · min^{-1}. The dissociation constant, K_d, obtained from the ratio (k_{off}/k_{on}) was 0.20 n*M*.

Competitive inhibition of the binding of [^{125}I]hEGF to gastric plasma membranes was studied at equilibrium by adding increasing amounts of unlabeled human and mouse EGFs to a fixed concentration of [^{125}I]hEGF. Figure 4 shows both unlabeled EGFs inhibit the binding of [^{125}I]hEGF, whereas bFGF had no effect. Unlabeled hEGF caused detectable inhibition of the binding of [^{125}I]hEGF at 0.03 n*M* and half-maximal inhibition at 0.5 n*M*. The inhibition curve for mEGF showed half-maximal inhibition at 1 n*M*, slightly higher than hEGF. When results from the experiment in which the ability of unlabeled hEGF to inhibit the binding of [^{125}I]hEGF were examined by Scatchard analysis, a single class of binding sites was suggested with a K_d of 0.42 n*M* and a maximum binding capacity of 42 fmol/mg protein (Fig. 5).

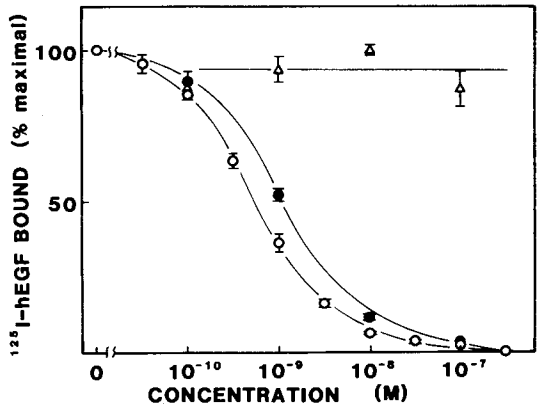

Fig. 4. Effect of unlabeled EGFs and bFGF on [^{125}I]hEGF binding. Plasma membranes (42–49 μg protein) were incubated for 60 min at 25°C with [^{125}I]hEGF (110 p*M*) and indicated concentrations of hEGF (○), mEGF (●), and bFGF (△). Saturable binding of [^{125}I]hEGF is expressed as the percentage of radioactivity bound in the absence of added peptides. Each point is the mean ± SE of three determinations.

Furthermore, various agents were tested for their abilities to change the binding of [^{125}I]hEGF (Table I). Peptide hormones (secretin, insulin, somatostatin, CCK8, or tetragastrin) did not inhibit the binding of [^{125}I]hEGF. Antiulcer agents (cimetidine, cetraxate, proglumide, or prostaglandin E_2) or ulcer inducers such as aspirin, indomethacin, or theophylline also did not alter the binding of [^{125}I]hEGF.

[^{125}I]hEGF was covalently linked to the membranes using disuccinimidyl suberate, a cross-linking agent, and the iodinated proteins were then separated by SDS-PAGE. Figure 6 shows an autoradiogram of the results. A labeled band of 150 kDa was observed and this band was abolished when the membranes were incubated in the presence of 4 μ*M* unlabeled hEGF. These results suggest that the observed band represents specific receptors for EGF on gastric plasma membranes.

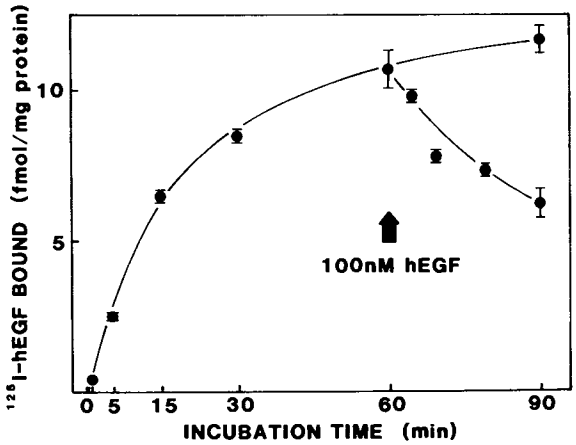

Fig. 3. Dissociation of bound [^{125}I]hEGF from plasma membranes. Plasma membranes (48 μg protein) were first incubated with 144 p*M* [^{125}I]hEGF for 60 min at 25°C. At the end of the first incubation, 100 n*M* unlabeled hEGF was added, and incubation continued for an additional 30 min at 25°C. Each point is the mean ± SE of three determinations.

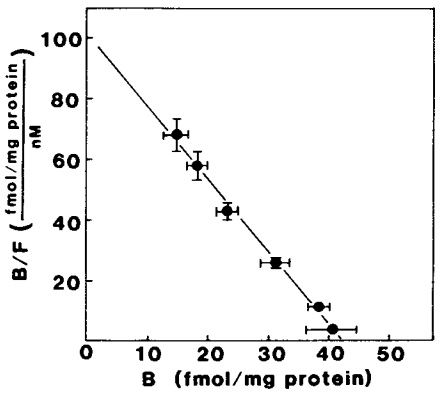

Fig. 5. Scatchard plot of [^{125}I]hEGF binding to plasma membranes. Plasma membranes were incubated for 60 min at 25°C with [^{125}I]hEGF plus various concentrations of unlabeled hEGF. The concentration range of EGF was 0.03–30 n*M*, and the protein concentration of plasma membranes was 0.16–0.25 mg/ml. The value of the ordinate was normalized according to protein concentration. Each point is the mean ± SE of six separate experiments.

Table I. The Effect of Various Agents on the Binding of [^{125}I]hEGF to Gastric Plasma Membranes[a]

Agent added	[^{125}I]hEGF bound (% of control)
Secretin (1 μM)	98.2 ± 2.7
Insulin (1 μM)	91.1 ± 3.6
Somatostatin (1 μM)	92.0 ± 1.8
CCK-8 (1 μM)	99.1 ± 2.7
Tetragastrin (0.1 μg/ml)	100.0 ± 5.4
Cimetidine (0.1 mM)	92.7 ± 2.5
Cetraxate (0.1 mM)	92.7 ± 4.0
Proglumide (0.1 mM)	97.7 ± 1.1
Prostaglandin E_2 (10 μM)	93.8 ± 3.5
Aspirin (0.1 mM)	94.7 ± 3.5
Indomethacin (0.1 mM)	91.2 ± 2.7
Theophylline (0.1 mM)	93.8 ± 5.3

[a] Plasma membranes were incubated with [^{125}I]hEGF and the indicated agents for 60 min at 25°C. Results are expressed as the percentage of saturable binding of [^{125}I]hEGF without addition of agents. Each value is the mean ± SE of three determinations.

DISCUSSION

This study is the first report concerning specific receptors for EGF on plasma membranes from the stomach. In the present study we have demonstrated the characteristics of binding sites for EGF on rat gastric plasma membranes. It was reported that EGFs from mouse, rat, and human are recognized by the same receptors on human placenta, but the number of EGF binding sites for homologous human EGF is slightly higher than that for mice or rats (26). Since the complex of EGF and its receptor is rapidly internalized by cells (15), we performed the experiments under conditions that allowed detection of EGF binding to cell surface receptors in the absence of internalization of the bound EGF by the use of isolated plasma membrane fraction.

The present study demonstrates that the binding of [^{125}I]hEGF to gastric plasma membranes is saturable, reversible, specific, and temperature dependent. Scatchard analysis of the data suggested the presence of a single class of binding sites. However, the presence of two classes of EGF binding sites has been reported in gastric glands isolated from guinea pigs (19). The binding affinity (K_d = 0.42 nM) and the maximum binding capacity in rat gastric plasma membranes (42 fmol/mg protein) are fairly close to those of the high-affinity binding sites observed in the isolated gastric glands from guinea pigs. The dissociation constant obtained by the ratio (k_{off}/k_{on}) from Fig. 3 was 0.20 nM, which is comparable to the value (0.42 nM) obtained with saturation isotherms and Scatchard analysis. The isolated guinea pig gastric gland binding data of the suggesting two binding sites (19) may represent not only cell surface binding sites, which can interact easily with EGF in the medium, but also intracellular EGF binding sites, which are less available to interact with EGF in the medium. A recent study (21) indicated that the binding of EGF to intestinal microvillus membranes of adult rat shows a single class of EGF receptors on the membranes with a dissociation constant of 0.43 nM and a binding capacity of 98 fmol/mg protein. The dissociation constant of gastric membranes is almost the same as that of intestinal membranes (21).

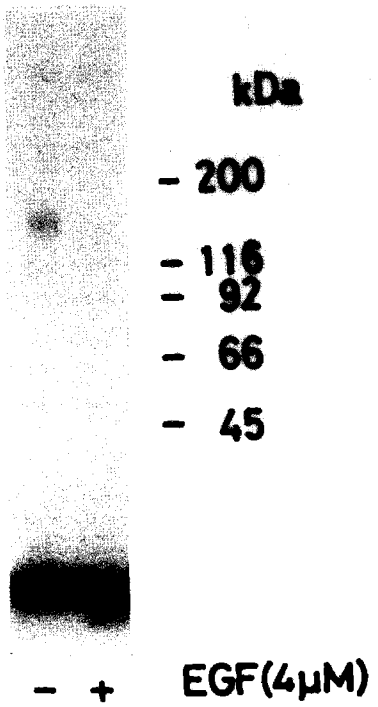

Fig. 6. Cross-linking of [^{125}I]hEGF to gastric mucosal plasma membranes. Plasma membranes were incubated with [^{125}I]hEGF (0.5 μCi) in the absence (−) or presence (+) of 4 μM unlabeled EGF. Cross-linking of bound [^{125}I]hEGF in the pellet was carried out with disuccinimidyl suberate. The reaction mixture was analyzed by SDS-PAGE and autoradiography.

In a previous study (22), the association of radioiodinated secretin to gastric plasma membranes was rapid, and a quick dissociation of the iodinated secretin from the membranes was observed. This study showed the binding of EGF to gastric plasma membranes reached a plateau at 60 min. These findings suggest that the binding properties of EGF to gastric plasma membranes is different from that of secretin.

Mouse EGF, which shows a high degree of homology to hEGF, with 37 of the 53 residues of mEGF corresponding to those of hEGF (2), interacts with the gastric plasma membrane binding sites. However, the receptors did not interact with bFGF or various other peptide hormones such as secretin, insulin, CCK8, somatostatin, or tetragastrin. These results indicate that the [^{125}I]hEGF binding receptors are highly selective for EGF. The antiulcer drugs and the inducers of ulcer did not alter the binding of [^{125}I]hEGF directly, which suggests that their biological activity, healing or inducing ulcers, is not directly mediated via EGF receptors.

The EGF receptor contains tyrosine-specific kinase activity, which has been postulated to be a part of mechanism of EGF action (25). We observed that the 150-kDa protein may be covalently linked to [^{125}I]hEGF in gastric plasma membranes by the use of disuccinimidyl suberate. The mo-

lecular weight of EGF receptors on rat intestinal microvillus (luminal) membranes was indicated to be 170 kDa using a similar method (21). It may not be appropriate to compare directly these differences in receptors because we used the basolateral membrane enriched fraction of gastric mucosa as plasma membranes (22). In a preliminary study, comparable binding of [^{125}I]hEGF to an apical membrane fraction from rat gastric mucosa was observed. The receptors may be present on membranes of the apical (luminal) and basolateral (blood vascular) sides. Freidenberg *et al.* (27) found specifically labeled bands at 170 and 150 kDa in affinity-labeling studies on rat liver membranes, indicating that these bands represented the EGF receptors. Furthermore, they noted that over 90% of the autophosphorylation occurred in the 170-kDa protein, with less than 10% in the 150-kDa protein. However, the significance of this phosphorylation in EGF modulation of various cellular functions is not clear. In our preliminary study we did not observe a significant autophosphorylation of the 150-kDa band (unpublished data). These findings may indicate that there are differences in the EGF receptors of gastric mucosa and liver.

These results are important for the development of EGF and related peptides as antiulcer drugs and for the investigation of gastric ulcer formation. Studies of the modulation of EGF receptors during fasting or submandibular dissection will also provide useful information about the physiological role of EGF in gastric function and about a diversity of EGF receptors in various tissues.

In conclusion, the present study demonstrates the presence of specific receptors for EGF on plasma membranes isolated from rat gastric mucosa and that the receptor's molecular weight is approximately 150 kDa.

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