RAPID STIMULATION OF PINOCYTOSIS IN HUMAN CARCINOMA CELLS A-431 BY EPIDERMAL GROWTH FACTOR

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ABSTRACT

Horseradish peroxidase (HRP) uptake was used to measure fluid-phase pinocytosis in monolayers of human epithelioid carcinoma cells (A-431). Histochemistry confirmed that cell-associated HRP was restricted to intracellular vesicles. Biochemical methods showed that HRP uptake in control cultures was directly proportional to the duration of exposure. The addition of low concentrations of epidermal growth factor (EGF) to the incubation media produced a 10-fold increase in the initial rate of pinocytosis. The EGF effect was rapid (within 30 s) but transient; the rate of pinocytosis returned to control levels within 15 min. Metabolic inhibitors reduced the EGF-stimulated rate of pinocytosis by >90%.

A conjugate of EGF and ferritin (F:EGF) was used to simultaneously compare the intracellular locations of EGF and HRP. Much of F:EGF was internalized in ~100-nm vesicles, while most of the HRP was located in much larger vesicles (range 0.1–1.2 μm) which also contained F:EGF.

The tumor-promoter 12-0-tetradecanoyl-phorbol-13-acetate, which shares several biological activities with EGF, was also effective in stimulating an increase in the rate of pinocytosis.

KEY WORDS hormone receptors · growth factor · endocytosis · phorbol esters

Most eukaryotic cells can internalize soluble material by both fluid-phase (bulk) and adsorptive pinocytosis (see reference 26 for a review). During fluid-phase pinocytosis, cells engulf droplets of solution and the uptake of a solute is directly related to its concentration in the extracellular fluid. Adsorptive pinocytosis is a selective, saturable process that involves the binding of a ligand to a cell-surface receptor before internalization. Recently, it has become clear that a number of polypeptide hormones are internalized by their target cells by the process of adsorptive pinocytosis (1, 3, 15, 22, 25). As a first step toward determining the molecular mechanism of action of the mitogenic polypeptide epidermal growth factor (EGF),[1] we have studied its internalization in detail at both the biochemical and morphological level (3, 13, 14). These studies indicate that cell-bound EGF, along with its receptor (7, 19), is internalized into endocytic vesicles and eventually degraded in lysosomes. Detailed morphological studies of the interaction of a biologically active,

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; F:EGF one-to-one conjugate of ferritin and EGF; HBSS, Hank's balanced salts solution; HBSS-glu, glucose-free HBSS; HRP, horseradish peroxidase; ^{125}I-EGF, ^{125}I-labeled EGF; TPA, 12-0-tetradecanyl-phorbol-13-acetate.

one-to-one conjugate of EGF and ferritin (F:EGF) show that, after binding to human epithelioid carcinoma cells A-431, F:EGF is rapidly redistributed in the plane of the plasma membrane to form small groups (14). These groups are internalized by pinocytic vesicles which eventually fuse with multivesicular bodies or lysosomes. By 15 min, 83% of cell-bound F:EGF is internalized. Although these studies on the binding and internalization of EGF describe the hormone's metabolic fate in detail, they do not establish the role that internalization plays in the biological activity of EGF.

Steinman and Cohn (27) and Steinman and coworkers (28) have developed methods that use horseradish peroxidase (HRP) as a biochemical and morphological marker of fluid-phase pinocytosis. In this communication, we have used these methods to study the effect of EGF on the rate of fluid-phase pinocytosis in monolayers of A-431 cells in an attempt to more clearly define the events that occur while cells are internalizing the hormone.

Because the phorbol esters share several biological properties with EGF (including promotion of chemically induced carcinogenesis [16], stimulation of the proliferation of cultured cells [9, 29], and induction of plasminogen activator [17], the effects of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) on pinocytosis were also tested.

MATERIALS AND METHODS

Mouse EGF was isolated from the submaxillary gland (24). ^{125}I-labeled EGF (^{125}I-EGF) and F:EGF were prepared by published methods (3, 14). HRP (Type II) and poly-L-lysine (Type V-A, M_r ~ 30,000) were purchased from Sigma Chemical Co. (St. Louis, Mo). Phorbol-13-diacetate and TPA were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Cationized ferritin was obtained from Polysciences, Inc. (Warrington, Pa.) and α_2-macroglobulin was a gift from Ira Pastan.

Human epithelioid carcinoma A-431 cells (10) were propagated in 10% fetal calf serum (13). In all experiments, ~1.8–3.0 × 10^5 cells were plated into each 35-mm Petri dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum. Cultures (6–9 × 10^5 cells/dish; ~85% confluent) were used 3–4 d after plating. The rates of pinocytosis were determined by measuring the uptake of HRP by the method of Steinman and Cohn (27) and Steinman et al. (28), using the following general experimental format: Cultures were washed twice with Hanks' balanced salts solution (HBSS) and then incubated for 15 min at 37° in DMEM/0.1% bovine serum albumin (BSA). This solution was replaced with 1.5 ml of DMEM/0.1% BSA containing HRP (1 mg/ml) and, when indicated, EGF. The cultures were incubated at 37°C for the indicated time and rapidly washed twice at 37°C with HBSS/0.1% BSA followed by six washes at 4°C with HBSS/0.1% BSA over a 30-min period. The cultures were lysed in 0.7 ml of 0.1% Triton X-100, and the amount of HRP in the lysate was measured by the enzymatic assay of Steinman and Cohn (27) and Steinman et al. (28). A 0.1-ml aliquot of the lysate was pipetted into 1 ml of an assay buffer containing 0-dianisidine and H_2O_2. The rate of the reaction was measured by following the change in absorbancy at 460 nm in a Gilford Recording Spectrophotometer (Gilford Instruments Laboratories Inc., Oberlin, Ohio), and it was linear over the 2-min assay period. This rate was converted to nanograms of HRP by comparison to a standard curve. The standard curve was linear from 1 to 10 ng/ml of HRP and had a slope of ~0.028 Δ OD_{460}/min/ng HRP. Lysates of these cells contained low levels (1–2 ng HRP/10^6 cells) of endogenous peroxidase like activity. Incubation with EGF did not alter the amount of endogenous activity. The amount of HRP taken up during a 30-min test period was linear with respect to HRP concentration from 0.5 to 2.0 mg/ml. Cells incubated with HRP at 4°C took up negligible amounts of enzyme.

To determine the cellular location of internalized HRP, monolayers were washed rapidly twice with HBSS/BSA (4°C), once with cacodylate buffer (4°C), and then fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate (pH 7.4) at room temperature for 5 min. After washing (twice with cacodylate buffer and three times with phosphate buffer), HRP was histochemically localized by a modification of the method of Graham and Karnovsky (12). The cells were incubated for 10 min at room temperature with 50 mg/100 ml diaminobenzedine in 0.1 M phosphate buffer (pH 7.3) containing 0.01% hydrogen peroxide. Cells were postfixed in 1% osmium tetroxide and processed for transmission electron microscopy as described (14).

RESULTS

Effect of EGF on Pinocytosis Rate

The rate of pinocytosis in monolayers of A-431 cells as measured by HRP uptake is constant over a 1-h test period (Fig. 1). One million cells internalized 37.5 ng of HRP/h. This corresponds to a pinocytosis rate of 37.5 nl/10^6 cells/h. This value is in good agreement with the basal pinocytosis rates reported in mouse L cells (28) and Swiss 3T3 cells (8).

However, when EGF (100 ng/ml) was added with the HRP solution, the initial pinocytosis rate was increased to 378.1 nl/10^6 cells/h. (Fig. 1). After the initial burst of pinocytic activity the rate of pinocytosis decreased; after 15 min it had re-

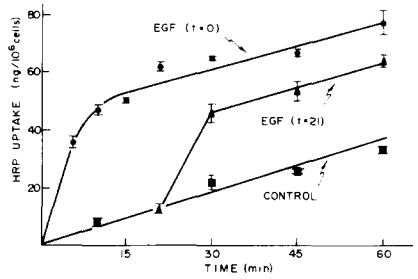

FIGURE 1 Kinetics of HRP uptake. A solution (37°C) of HRP (1 mg/ml in DMEM/0.1% BSA), with (●——●) and without (■——■) EGF (100 ng/ml), was added to monolayers of A-431 cells at t = 0. In one set of replicate cultures (▲——▲) EGF was added 21 min after the HRP. Each point represents the average of duplicate incubations. The bars extend to the individual value of each incubation.

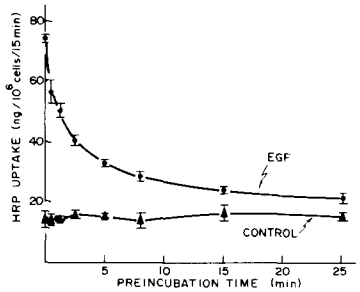

FIGURE 2 Effect of preincubation with EGF on the EGF stimulation of pinocytosis. EGF (100 ng/ml) was added to monolayers of A-431 cells and the cultures were preincubated at 37°C for the indicated times. After the preincubation, HRP (final concentration = 1 mg/ml) was added and cultures were incubated with HRP and EGF for 15 min at 37°C. EGF preincubation (●——●); no EGF preincubation (▲——▲). The points are the average of duplicate incubations. The bars extend to the individual value of each incubation.

turned to the basal rate. A similar response was observed when EGF was added to control cells 21 min after the addition of HRP (Fig. 1). The presence of serum in the medium was not required to produce the EGF response.

The time course of HRP uptake under EGF stimulation (Fig. 1) suggests that the effect of EGF on pinocytosis is short lived. To more clearly define the temporal relationship between EGF binding and stimulation of pinocytosis, the following experiment was performed: HRP uptake was measured over a constant interval (15 min) preceded by various periods of preincubation with EGF. As described above, stimultaneous addition of EGF and HRP (no preincubation) resulted in a large stimulation of HRP uptake. Brief incubation with EGF before the addition of HRP, however, caused rapid and dramatic declines in the rate of pinocytosis (Fig. 2). Preincubation with EGF for 1.8 min was sufficient to cause a 50% reduction in the EGF-stimulated HRP uptake. A 25-min preincubation resulted in a pinocytosis rate very near control levels. These results confirm that the effect of EGF on pinocytosis is rapid but transient.

The influence of EGF concentration on the stimulation of pinocytosis was determined. EGF and HRP were added simultaneously to A-431 cells at 37°C and the amount of HRP internalized was measured over a 15-min incubation period. As shown in Fig. 3 top, maximal stimulation (Sevenfold) was produced by 50 ng/ml of EGF; higher concentrations produced a submaximal stimulation.

A similar dose response curve was obtained by preincubating cells with varying concentrations of EGF at 4°C for 45 min. The cultures were washed

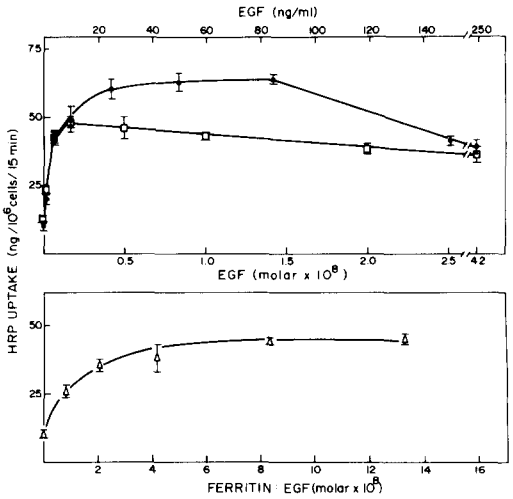

FIGURE 3 Effect of EGF concentration on HRP uptake. A solution (37°C) of HRP (1 mg/ml) and increasing concentrations of EGF were added to monolayers of A-431 cells, and the amount of HRP internalized was measured after a 15-min incubation at 37°C (●——●). Replicate cultures were incubated at 4°C with increasing concentrations of EGF (□——□) or ferritin-conjugated EGF (△——△) for 45 min, washed to remove unbound hormone, then incubated at 37°C for 15 min in a solution containing HRP (1 mg/ml). The points are the average of duplicate incubations. The bars extend to the individual value of each incubation.

at 4°C to remove unbound EGF, and the cells were then incubated for 15 min with HRP at 37°C. Although there was no EGF in the medium during the incubation with HRP, the cell-bound EGF was sufficient to stimulate pinocytosis (Fig. 3). Maximal stimulation (4.5-fold) was produced when cells were preincubated with 10 ng/ml EGF. Preincubation with F:EGF at 4°C also produced a 4.5-fold maximal stimulation of HRP uptake although higher molar concentrations were required (Fig. 3). The requirement for higher concentrations is consistent with earlier studies that showed F:EGF to be ~8% as efficient in binding to cellular receptors as native EGF (14).

Effect of EGF Concentration on Binding

To compare the binding of EGF with the pinocytosis dose-response curve, monolayers were incubated with increasing concentrations of ^{125}I-EGF under conditions identical to those used in Fig. 3, and the amount of cell-bound ^{125}I-EGF was measured. Comparison of Figs. 3 and 4 shows that maximal pinocytosis stimulation occurred when only a small fraction of the EGF receptor sites were occupied. At 37°C, one-half-maximal binding occurred at an EGF concentration of 50 ng/ml while only 3 ng/ml gave a half-maximal pinocytosis stimulation. Occupancy of only ~4% of the receptors produced a half-maximal stimulation of pinocytosis. At 4°C, 65 ng/ml of EGF gave half-maximal binding while preincubation at 4°C with 2 ng/ml EGF gave a half-maximal stimulation of pinocytosis. It should be noted that in these experiments the incubation times were not long enough to allow the attainment of binding equilibrium at low concentrations of EGF.

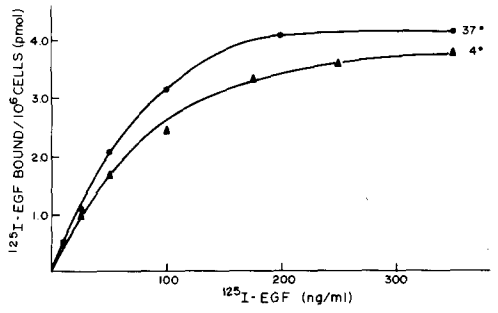

FIGURE 4 Effects of ^{125}I-EGF concentration on binding at 37° and 4°C. The indicated concentrations of ^{125}I-EGF (1,420 cpm/ng) were added to duplicate cultures of A-431 cells, and the specific binding was determined after a 15-min incubation at 37°C (●——●) or a 40-min incubation at 4°C (▲——▲) by methods previously described (3).

Metabolic Inhibitors Decrease Pinocytosis

The effect of metabolic inhibitors on the basal and EGF-stimulated rates of pinocytosis was examined. These experiments were performed in glucose-free HBSS (HBSS-glu). Metabolic inhibitors do not decrease the binding of EGF to its cellular receptor (3). Table I shows that low concentrations of drugs (sodium azide or dinitrophenol) that inhibit ATP production by the respiratory pathway strongly inhibited HRP uptake in both control and EGF-stimulated cultures. The inhibition of pinocytosis by these drugs was reversed by washing with drug-free medium. This suggests that the inhibition was due to a decrease in the availability of ATP rather than a general toxic effect.

Effect of Plasma Membrane Ligands on Pinocytosis Rates

It has been clearly demonstrated that EGF is internalized by adsorptive pinocytosis (14). Because polylysine (23), cationized ferritin (11), and α_2-macroglobulin (21) are internalized by adsorptive endocytosis in certain cell cultures, these polypeptides were tested for their ability to stimulate HRP uptake in monolayers of A-431 cells. Polylysine produced a slight increase (1.3-fold) during the 20-min test period (Table II). The other polypeptides tested and 10% calf serum were without effect (Table II).

A variety of polypeptide hormones and growth factors, including insulin (100 ng/ml), growth hormone (100 ng/ml), prolactin (100 ng/ml), and multiplication stimulating activity (500 ng/ml), were tested for their ability to stimulate HRP uptake. None of these polypeptides produced any significant effect on the pinocytosis rate.

Effects of Pharmacological Agents on Pinocytosis Rates

Table III summarizes the results of experiments in which pharmacological inhibitors and analogues were used to determine whether cyclic nucleotides, microtubules, microfilaments, or the lysosomal degradation of EGF were involved in the pinocytosis response. Cyclic nucleotides (8-bromo cyclic AMP and 8-bromo cyclic GMP) and the phosphodiesterase inhibitor 1-methyl-3-isobu-

TABLE I

Effects of Metabolic Inhibitors on Pinocytosis Rate

Addition	Pinocytic rate		Inhibition of		Ratio of rate
	Basal	Plus EGF	Basal rate	EGF-stimulated rate	EGF:basal
	ng HRP internalized/10^6 cells/15 min		%	%	
None	8.6	43.4	—	—	5.05
Na azide (0.4 mM)	7.8	22.9	9	47	2.94
Na azide (2.0 mM)	3.0	4.0	65	91	1.33
Dinitrophenol (0.2 mM)	3.4	4.0	60	91	1.17
Dinitrophenol (1.0 mM)	2.6	2.5	70	94	0.96

Monolayers of A-431 cells were washed twice with glucose-free Hanks (HBSS-glu); then the cells were preincubated in HBSS-glu/BSA (37°C) containing the indicated inhibitor for 15 min. EGF (75 ng/ml) and HRP (1 mg/ml) were added and the cultures were incubated for an additional 15 min at 37°C. The cells were then washed with HBSS-glu/BSA and assayed for HRP uptake as described in Materials and Methods. The uptake values are the averages of duplicate incubations.

TABLE II

Effects of Plasma Membrane Ligands on Pinocytosis Rate

Treatment	Concentration	HRP internalized	
		20 min	45 min
		ng/10^6 cells	
None	—	9.5 ± 0.3	21.2 ± 2.8
EGF	75 ng/ml	40.4 ± 2.6	46.2 ± 3.3
Poly-L-lysine	50 μg/ml	12.0 ± 0.5	20.1 ± 1.1
α_2-macroglobulin	60 μg/ml	9.6 ± 1.1	18.1 ± 1.5
Cationized ferritin	200 μg/ml	7.9 ± 0.4	18.7 ± 0.2
Ferritin	200 μg/ml	9.0 ± 1.2	20.6 ± 0.8
Calf serum	10% (vol/vol)	9.2 ± 0.7	22.0 ± 1.3

Monolayers of A-431 cells were washed and preincubated as described in Materials and Methods. HRP (1 mg/ml) and the indicated polypeptide were then incubated with the cells in DMEM/BSA for either 20 or 45 min. Cells were washed and assayed for HRP uptake. The uptake values are the averages of triplicate incubations ± SD.

tylxanthine did not alter the basal or EGF-stimulated rate of pinocytosis. These results suggest that the cyclic nucleotides do not mediate the EGF effect. Microtubules do not appear to be involved because nocadazol was without effect. Cytochalasin B stimulated the basal rate of pinocytosis without significantly altering the EGF-stimulated rate. Compounds that inhibit the degradation of cell-bound EGF (chloroquine and procaine) did not change the amount of HRP internalized.

Maxfield et al. (20) have reported that primary amines such as *n*-butylamine block the patching of EGF in 3T3 cells. Although *n*-butylamine stimulated the basal rate of pinocytosis in A-431 cells (1.8-fold), it did not alter the EGF-stimulated rate (Table III).

Phorbol Esters

It has been reported that the tumor promoter TPA and EGF induce certain similar biological responses (9, 16, 17, 29) and that TPA can inhibit the binding of EGF to its cellular receptor in HeLa cells (18). Because of these apparent similarities between EGF and phorbol esters, TPA was tested for its ability to stimulate pinocytosis in A-431 cells. Increasing concentrations of TPA were added to cultures with HRP and incubated at 37°C for 25 min. Fig. 5 shows that there was a dose-dependent stimulation of HRP uptake with a maximal stimulation of 2.7-fold above the basal rate. Under these conditions, EGF (75 ng/ml) produced a maximal stimulation of 3.9-fold (81.1

TABLE III

Effects of Pharmacological Agents on Pinocytosis Rate

Treatment	Concentration	HRP internalized	
		Basal	EGF
		ng/10⁶ cells/15 min	
None	—	11.0	55.2
n-butylamine	30 mM	19.6	50.3
Nocadazol	20 μm	11.3	56.3
Cytochalasin B	5.0 μg/ml	31.2	49.4
Chloroquine	0.2 mM	12.7	56.4
Procaine	2.0 mM	11.3	48.9
8-bromo cyclic AMP	1.0 mM	9.2	53.6
8-bromo cyclic GMP	1.0 mM	9.8	56.7
1-methyl-3-isobutylxanthine	1.0 mM	11.1	52.2

Monolayers of A-431 cells were washed and preincubated for 15 min at 37°C with the indicated pharmacological agent as described in Materials and Methods. HRP (1 mg/ml) and the indicated pharmacological agent were then incubated with the cells in DMEM/BSA for 15 min both in the presence and in the absence of EGF (75 ng/ml). Cells were washed and assayed for HRP uptake. The uptake values are the averages of duplicate incubations.

± 2.1 ng HRP internalized/10^6 cells/25 min). When cultures were incubated with optimal concentrations of EGF (75 ng/ml) and TPA (60 ng/ml), the resulting rate of pinocytosis was the same as with EGF alone. Suboptimal amounts of EGF (1.5 ng/ml) and TPA (5 ng/ml) produced additive increases in HRP uptake.

Phorbol-12, 13-diacetate, which is not an efficient tumor promoter, was without effect in stimulating pinocytosis even when used at high concentrations (250 ng/ml).

Visualization of Internalized HRP

Cytochemical experiments were performed to determine the location of cell-associated HRP. Endocytic vesicles containing HRP were clearly visualized in monolayers of A-431 cells that were incubated with EGF and HRP (1.5 mg/ml) for as short a period as 30 s (Fig. 6). The vesicles were of varying sizes and shapes ranging from 0.1 to 1.2 μm in diameter. When the incubation time was <10 min, most vesicles had electron-transparent centers and a peripheral rim of electron-opaque reaction product. There was no striking difference in the size and shape of HRP-containing vesicles in unstimulated cultures, although, as expected from the biochemical studies (Fig. 1), the number of vesicles containing reaction product was greatly reduced. Reaction product was not seen on the cell surface, indicating that HRP was not adsorbed to the plasma membrane.

The following experiments were performed to simultaneously compare intracellular locations of F:EGF and HRP. Cultures were incubated at 4°C with F:EGF, washed to remove unbound hormone, and then incubated with HRP (1.5 mg/ml) at 37°C for various time periods between 1 and 20 min. Much of the internalized F:EGF was located in 100-nm diameter vesicles. Although some of the 100-nm vesicles contained both F:EGF and HRP, no HRP could be detected in many of the 100-nm vesicles which contained F:EGF. Most of the HRP localized to much larger vesicles of the same size and shape as were observed in cells treated with native EGF. Some of the F:EGF also was detected in these large HRP-containing vesicles. Fig. 7 shows F:EGF in small vesicles that do not contain HRP reaction product (small arrows) and in larger vesicles that contain HRP (large arrows). Control experiments showed that HRP did not affect the binding or internalization of F:EGF.

DISCUSSION

The uptake of HRP has been used both as a biochemical and as a morphological marker of fluid-phase pinocytosis in monolayers of human epithelioid carcinoma cells A-431. Several lines of evidence establish that these methods do measure fluid-phase pinocytosis in A-431 monolayers

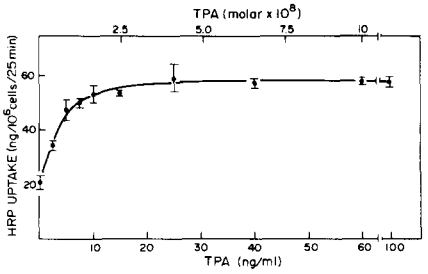

FIGURE 5 Effect of TPA concentration on HRP uptake. A solution (37°C) of HRP (1 mg/ml) and increasing concentrations of TPA was added to monolayers of A-431 cells, and the amount of HRP internalized was measured after a 25-min incubation. Each point represents the average of triplicate incubations ± SD. TPA (1 mg/ml in DMSO) was diluted in DMEM before addition. The DMSO had no effect on the pinocytosis rate.

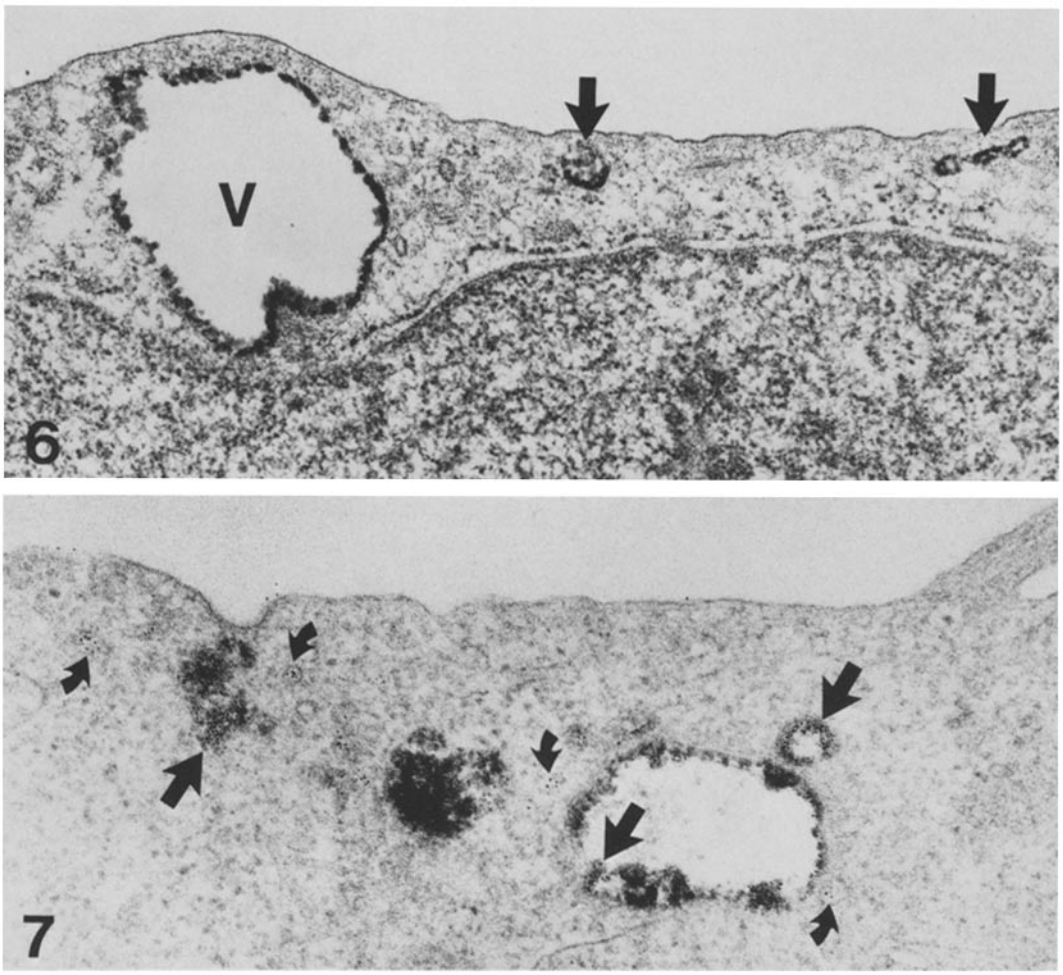

FIGURE 6 Location of HRP in EGF-treated cell. EGF (100 ng/ml) and HRP (1.5 mg/ml) were added to monolayers of A-431 cells, and the cultures were incubated at 37°C for 30 s. HRP reaction product is located in a vacuole (*V*, 1 μm Diam) as well as in vesicles (arrows). Section was stained with uranyl acetate and lead citrate. × 40,000.

FIGURE 7 Simultaneous localization of HRP and F:EGF. Monolayers of A-431 cells were incubated at 4°C with F:EGF (100 nM) for 45 min, washed to remove unbound hormone, then incubated at 37°C for 20 min with HRP (1.5 mg/ml). Both labels are present in pinocytic vacuoles and large vesicles (large arrows); however, small vesicles (<100 nm Diam) contain only F:EGF (small arrows). Unstained section. × 66,000.

rather than adsorption of the HRP: (*a*) The uptake of HRP was linear with respect to time and extrapolated to zero uptake at zero time, (*b*) the uptake of HRP was linear with respect to the concentration of HRP in the solution, (*c*) HRP uptake was greatly reduced or absent in the presence of metabolic inhibitors or at 4°C, and (*d*) morphological studies did not visualize any HRP in intercellular spaces or on the cell surface. F:EGF bound to cellular receptors and was internalized normally in the presence of HRP (1.5 mg/ml), which suggests that HRP did not interfere with the interaction between EGF and its plasma membrane receptor.

We have previously reported on the interaction of F:EGF with monolayers of A-431 cells (14). The initially dispersed cell-bound F:EGF redistributed in the plane of the plasma membrane to

form small groups which were subsequently internalized into small pinocytic vesicles. The internalization of F:EGF into densely packed vesicles and the rapidity of the process suggests that EGF is selectively pinocytosed rather than being internalized during a process of general membrane turnover. The biochemical data presented in this report suggest, at first glance, that EGF is stimulating its own internalization because there is a 10-fold increase in the initial rate of pinocytic activity in cells incubated with EGF (Fig. 1). Much cell-bound F:EGF is internalized via 100-nm diameter vesicles which frequently do not contain HRP reaction product. Considering the thickness of the membrane ~10 nm) and glycocalyx (~20 nm), a 100-nm (outer diameter) vesicle has an inner diameter of ~40 nm. A sphere of 40-nm diameter contains 3.4×10^{-17} ml of fluid. A 1.5 mg/ml solution of Sigma Type II HRP contains $\sim 1.2 \times 10^{16}$ molecules of HRP/ml. Therefore the average 100-nm vesicle contains only ~0.4 molecules of HRP. These calculations clearly illustrate how cells can internalize a ligand such as EGF by adsorptive endocytosis while excluding all but insignificant quantities of extracellular fluid.

Previous studies with F:EGF have shown that the predominant pathway of uptake of cell-bound F:EGF involves the formation of small clusters in the plane of the membrane followed by selective internalization of the clusters into ~100-nm Diam vesicles (14). Data presented in this report show that during the same time interval EGF stimulates fluid-phase pinocytosis as measured by HRP uptake and that the HRP was predominantly located in larger vesicles. Some F:EGF was observed bound to the membrane of the large vesicles, and it was at approximately the same density as on the cell surface in general. Thus, it appears that two pathways exist by which EGF can be internalized: (*a*) EGF is clustered and selectively internalized into small vesicles, and (*b*) EGF that is randomly bound to areas of the plasma membrane that are destined to form large vesicles also is internalized.

The primary physiological purpose of the EGF-stimulated uptake of large vesicles may be (*a*) to take in droplets of extracellular fluid, (*b*) to internalize cell-bound hormone, or (*c*) to possibly serve some as yet undetermined function.

Previous studies (4) have shown that the initial interaction (binding) of EGF with the cell surface is not sufficient to stimulate DNA synthesis. In contrast, the rapidity of the EGF effect on the pinocytosis rate suggests that EGF exerts its effect while on the cell surface. It seems possible that the binding of EGF to its cell surface receptor may be sufficient to induce some early effects (such as membrane phosphorylation [6], increased transport [see reference 5 for a review], and pinocytosis) whereas the mitogenic response requires the internalization of the hormone:receptor complex.

It is of interest that TPA, like EGF, stimulated pinocytosis in A-431 cells. However, under a variety of experimental conditions TPA did not compete with ^{125}I-EGF for cellular binding sites in A-431 monolayers (unpublished results). Therefore, it seems unlikely that TPA produces its effect by binding to the EGF receptor.

Previous morphological studies on monolayers of glial cells have shown that 5 h after the addition of EGF there was an increase in ruffling activity (2). The uptake of large pinocytic vacuoles frequently occurred at areas of ruffling activity. In A-431 cells, we have observed a rapid but transient increase in ruffling at the light and scanning electron microscope levels in response to EGF. The ruffling occurs during the same time period (1–15 min) that the increased pinocytosis occurs (M. Chinkers, J. A. McKanna, and S. Cohen. *J. Cell. Biol.* In press).

Davies and Ross (8) recently reported an increased pinocytosis rate in cultures of smooth muscle cells and 3T3 cells 6 h after the addition of platelet-derived growth factor. It seems likely that in these instances, the induction of increased pinocytosis by growth factors several hours after addition (2, 8) is one of many late events occurring in response to amplified and propagated signals generated by the binding and/or internalization of the mitogens. In contrast, the rapidity of the response of A-431 cells to EGF suggests that the increased pinocytosis rate reported herein is more closely associated with the cells' initial response to the hormone. The rapid pinocytosis response of A-431 cells to EGF may be a reflection of the relatively large number of EGF receptors present in these cells ($2–3 \times 10^6$ receptors/cell) and the corresponding generation of a massive signal for cellular pinocytosis. These same early events may occur in other mitogen-stimulated cells but go unnoticed because the response is greatly reduced in magnitude.

Recent studies have shown that the addition of EGF to a cell-free membrane preparation from A-431 cells produces a marked stimulation of the phosphorylation of endogenous membrane-associated proteins (6). Because phosphorylation re-

actions participate in the regulation of many metabolic pathways, it is tempting to speculate that the EGF-dependent phosphorylation of endogenous membrane proteins may be one of the molecular signals that results in an increased pinocytosis rate.

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