Distinct Endocytotic Pathways in Epidermal Growth Factor–stimulated Human Carcinoma A431 Cells

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Abstract. Addition of EGF to human epidermoid carcinoma A431 cells increases the rate of fluid-phase pinocytosis 6–10-fold as measured by horseradish peroxidase uptake (Haigler, H. T., J. A. McKanna, and S. Cohen. 1979. *J. Cell Biol.* 83:82–90). We show here that in the absence of extracellular Na^+ or in the presence of amiloride the stimulation of pinocytosis by EGF is substantially reduced. Amiloride had no effect on the endocytosis of EGF itself or of transferrin, demonstrating that the receptor-mediated endocytotic pathway operated normally under conditions that blocked stimulated pinocytosis. Amiloride blocked EGF-stimulated pinocytosis in both HCO_3^--containing and HCO_3^--free media. The EGF-stimulated pinocytotic activity can frequently be localized to areas of the cell where membrane spreading and ruffling are taking place.

These results demonstrate that (*a*) EGF induces a distinct amiloride-sensitive endocytotic pathway on A431 cells; (*b*) occupied EGF receptors do not utilize this pathway for their own entry; (*c*) endocytosis of occupied EGF receptors is not in itself sufficient to stimulate pinocytosis.

A variety of cellular activities are stimulated after the addition of growth factors or growth-promoting agents to a wide variety of cell types. Receptor tyrosine kinase activity, protein kinase C activity, and Na^+/H^+ exchange are rapidly stimulated (for reviews see Rozengurt, 1986; Schlessinger, 1986), as are several membrane-related phenomena such as pinocytosis (Haigler et al., 1979*b*) and membrane ruffling (Chinkers et al., 1979). These changes are usually observed within seconds or minutes although in some cell type/stimulus combinations several hours of exposure are required. For example, membrane ruffling and pinocytosis in human glial cells was stimulated only after 4 h of exposure to EGF (Brunk et al., 1976) and when H-ras proteins were microinjected into quiescent rat embryo fibroblasts pinocytosis was stimulated ~30 min later (Bar-Sagi and Feramisco, 1986).

Human epidermoid carcinoma A431 cells provide a particularly striking example of the changes in cellular response which can follow occupation of EGF receptors. Within a minute there is a dramatic stimulation of fluid-phase pinocytosis (Haigler et al., 1979*b*) and membrane ruffling activity (Chinkers et al., 1979) and on approximately the same time scale there is an amiloride sensitive stimulation of Na^+ influx and H^+ efflux, leading, in HCO_3-free buffers, to an elevation in cytoplasmic pH of ~0.1–0.2 U (Rothenberg et al., 1983*a*,*b*).

The best-characterized endocytotic pathway is mediated by cell surface coated pits and is responsible for the receptor-mediated uptake of a wide variety of nutrients, growth factors, and viruses (reviewed by Helenius et al., 1983; Goldstein et al., 1985; Wileman et al., 1985). The question often arises as to whether all of the observed uptake of fluid-phase markers can be accounted for by the activity of coated pits and, if not, what alternative routes for fluid-phase pinocytosis there might be. This has been difficult to resolve because (*a*) the precise volume available to the fluid phase in an endocytotic coated vesicle is uncertain and (*b*) it is difficult to be certain that the markers used are truly confined to the fluid phase. Some studies strongly suggest that the activity of coated pits can account for the observed pinocytotic uptake (e.g., Marsh and Helenius, 1980), while others indicate that there may be additional pinocytotic routes. For example, cell surface pits apparently devoid of a clathrin coat have been observed on a number of cell types (Palade, 1953; Fawcett, 1965; Hopkins et al., 1985) and have been suggested as the sites of internalization of certain toxins (Montesano et al., 1982) as well as cross-linked class I MHC antigens (Huet et al., 1980). A recent report demonstrated that, under certain conditions, acidification of the cytosol can block receptor-mediated endocytotic activity without affecting uptake of ricin toxin or Lucifer yellow, again indicating the existence of a second pathway (Sandvig et al., 1987). However, other recent data indicate that both receptor-mediated and fluid-phase endocytosis are blocked to a similar extent in cells acidified to different pH values (Cosson et al., 1989).

Since pinocytosis, as assayed by fluid-phase uptake, is greatly stimulated in A431 cells treated with EGF we have investigated (*a*) which endocytotic pathways were being

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stimulated under these conditions and (*b*) whether there might be distinct requirements for the operation of different endocytotic pathways. The results demonstrate that a distinct pinocytotic pathway operates in EGF-stimulated A431 cells which can be selectively blocked by inhibitors of Na^+/H^+ exchange.

Materials and Methods

Reagents

EGF was a generous gift from Dr. K. D. Brown, AFRC Babraham, Cambridge, U.K., or was purchased from Sigma Chemical Co. (Poole, U.K.). Cell culture media was from Northumbria Biologicals, Northumberland, U.K., and ^{125}I was from Amersham International, Amersham, U.K. All other reagents were from Sigma Chemical Co.

Cells

A431 cells were obtained at passage 35 from Professor Colin Hopkins, Imperial College, London, and were maintained in DME supplemented with 10% FCS and antibiotics. Cells were not generally used beyond passage 45. All experiments were performed on cells which had almost reached confluency in 30-mm diam dishes (Sterilin Ltd., Feltham, England) following subculture 2 d previously. Before an experiment the growth medium was removed and the cells were incubated for at least 2 h at 37°C in serum-free DME buffered with Hepes, pH 7.4, containing 2 mg/ml BSA.

Measurement of EGF-stimulated Pinocytosis

After serum-free preincubation each dish was incubated at 37°C in 0.75 ml of medium A (137 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mg/ml BSA, and 20 mM Hepes, pH 7.4) containing 2 mg/ml horseradish peroxidase (HRP)[1] (type II; Sigma Chemical Co.) and 50 ng/ml EGF where indicated. In some experiments NaCl in medium A was replaced with KCl. After various times at 37°C the medium was removed, the cells were quickly rinsed in 2 ml medium A, then immersed quickly in two 300-ml volumes of PBS containing 2 mg/ml BSA held at 37°C, and finally transferred to a third large beaker containing ice cold PBS/BSA. At the end of an experiment each dish was washed six times over a 30-min period at 4°C with PBS/BSA. The cells were lysed at room temperature in 0.5 ml of 0.2% Triton X-100 with gentle rocking. The lysate was removed and centrifuged for 5 min at 13,000 *g*. At 4°C HRP activity was stable in cell lysates for at least 24 h.

HRP was assayed essentially as described by Steinman and Cohn (1972). 0.01 ml 0.3% H_2O_2 and 0.1 ml 1 mg/ml o-dianisidine, both freshly prepared, were added to 0.79 ml of 0.1 M Na phosphate buffer, pH 5.0. The reaction was initiated by the addition of 0.1 ml of cell lysate prepared as described above and the rate of change in absorbance at 460 nm followed using a spectrophotometer (model DU-40; Beckman Instruments, Inc., Palo Alto, CA). A standard curve was constructed by preparing lysates containing known amounts of HRP. This was linear in the range 0.25–5.0 ng HRP/ml (final concentration) and gave a slope = 0.0443 A_{460} U/min per ng HRP. To assess the background of HRP bound to the cell surface, medium A containing HRP was added to control dishes at 37°C, then immediately removed, and these dishes were washed as described above. Alternatively cells were held in medium A containing HRP at 0°C and then washed exhaustively. Both protocols gave a similar low background level of HRP, which could not be removed from the surface of the cells, equivalent to ~1 ng/10^6 cells. This background was determined in each experiment and subtracted from all other values.

Amiloride

Amiloride was dissolved in DMSO to a concentration of 0.6 M, diluted 20-fold in the appropriate incubation buffer, and then added to the cells to a final concentration of 3 mM or as indicated. Cells were preincubated for 5 min in the presence of amiloride or in medium containing an equivalent amount of DMSO (0.5%) before the addition of EGF.

1. *Abbreviation used in this paper*: HRP, horseradish peroxidase.

Acidification after NH_3 Loading

A431 cells were acidified essentially as described by Sandvig et al. (1987). Briefly, the last 30 min of the incubation in serum-free medium (see above) was performed in DME/BSA/Hepes which also contained 25 mM NH_4Cl. This medium was then replaced with either Na^+-containing or Na^+-free medium A for 5 min before the addition of EGF and HRP.

Endocytosis of ^{125}I-Transferrin and ^{125}I-EGF

Ferritransferrin and EGF were labeled with ^{125}I by the Iodogen method (Fraker and Speck, 1978) to a specific activity of ~7×10^3 cpm/ng and 1.2×10^5 cpm/ng, respectively. ^{125}I-Transferrin was added simultaneous to the addition of EGF and HRP. Where endocytosis of EGF was to be measured, ^{125}I-EGF was mixed with unlabeled EGF to a final specific activity of ~4.8×10^4 cpm/ng and this mixture was used to stimulate cells.

Each monolayer was processed as described above to measure cell-associated HRP but, before solubilization in Triton X-100, the cells were washed in 0.5 M NaCl, 0.2 M acetic acid, pH 2.5 (Hopkins and Trowbridge, 1983), to remove surface-bound ^{125}I-EGF or ^{125}I-transferrin. Internalized ^{125}I-labeled ligand was taken to be the sum of that present in the Triton lysate and in a subsequent wash in 0.5 ml 10% SDS which removed any remaining cellular material from the dish.

Microscopy

For light microscopy, cells were grown on glass coverslips, exposed to 50 ng/ml EGF in the presence of 10 mg/ml HRP for the times indicated, and then washed extensively in PBS/BSA as described above. The cells were briefly rinsed in PBS and fixed for 20 min in PBS containing 0.5% glutaraldehyde. HRP activity was revealed by incubation in PBS containing 0.5 mg/ml diaminobenzidine and 0.01% H_2O_2. Coverslips were mounted on glass slides and viewed in a photomicroscope (model III; Carl Zeiss, Inc., Thornwood, NY) under both phase contrast and bright field illumination. Photographs were taken on HP5 (Ilford, Ltd., Basildon, Essex, England) and Ektachrome ASA 160 film (Eastman Kodak Co., Rochester, NY).

For electron microscopy, cells were grown on gelatin-coated blocks of araldite and stimulated with 300 ng/ml EGF in growth medium. The cells were washed in Dulbecco's PBS and labeled at 0°C with a rabbit antitransferrin receptor antiserum and subsequently with 10 nm colloidal gold conjugated to affinity-purified goat anti-rabbit Ig (Sigma Chemical Co.). The cells were prepared for electron microscopy as described by Bretscher and Thomson (1983), and the number of gold particles found in coated pits was scored.

Results

Stimulation of Pinocytosis by EGF

As originally reported by Haigler et al. (1979*b*), when monolayers of A431 cells are incubated with 50 ng/ml EGF in serum-free medium there is a dramatic stimulation of pinocytotic activity as measured by HRP uptake. The basal rate of pinocytosis was 7.8 nl/h per 10^6 cells, while in the presence of EGF the rate of pinocytosis measured over the first 3 min increased to 75 nl/h per 10^6 cells (Fig. 1). As reported by Haigler et al. (1979*b*), this increased rate was transient and 6–10 min after the addition of EGF pinocytotic activity had returned to the basal rate. The extra HRP taken in during the pinocytotic burst was, however, retained within the cells (Fig. 1).

EGF-stimulated Pinocytosis Is Blocked under Conditions That Acidify the Cytosol

To explore the possible involvement of coated pits in the increased uptake, we tested a recently described method of blocking this pathway by acidifying the cytosol (Sandvig et al., 1987), as a result of imposing an outward gradient of NH_3/NH_4^+ across the plasma membrane (Boron and de

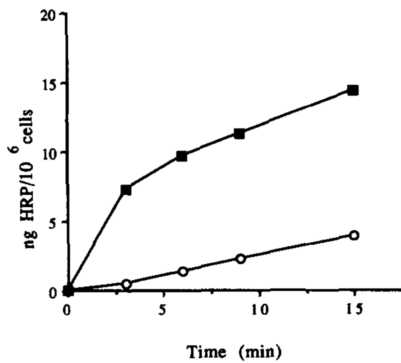

Figure 1. Stimulation of pinocytosis by EGF. A431 cells were incubated with 2 mg/ml HRP at 37°C in the presence (■) or absence (○) of 50 ng/ml EGF for various times and were then washed and assayed for HRP activity as described in Materials and Methods.

Weer, 1976). We confirmed the observations of Sandvig et al. (1987) that under these conditions the uptake of ^{125}I-transferrin is effectively blocked (87% inhibition after preloading with 25 mM NH_4Cl; data not shown). To test whether EGF could still induce a burst of pinocytosis in cells whose cytosol had been acidified, cells were loaded with 25 mM NH_4Cl for 30 min, washed free of external NH_4Cl, and incubated in either Na^+-containing or Na^+-free medium for 5 min before the addition of HRP and EGF. After an additional 6 min the cells were processed as usual to assess the uptake of HRP.

The results in Fig. 2 show that conditions that acidify the cytosol dramatically inhibited the ability of EGF to stimulate pinocytosis in these cells. It appeared that it was acidification of the cytosol rather than a direct effect of NH_3/NH_4^+ that was responsible for the block in stimulated pinocytosis because cells loaded with NH_3 but then returned to Na^+-containing medium during the EGF stimulation showed substantial EGF-stimulated pinocytosis (Fig. 2). In Na^+-containing buffers acidification of the cytosol stimulates exchange of intracellular H^+ for extracellular Na^+, thus normalizing cytosolic pH (reviewed in Pouysségur, 1985; Moolenaar, 1986).

On the basis of sensitivity to conditions that induce acute cytosolic acidification, stimulated pinocytosis could not be distinguished from endocytosis through coated pits. In addition, we noted that stimulated pinocytosis and endocytosis through coated pits, as indicated by ^{125}I-transferrin uptake, had a similar temperature dependency (data not shown).

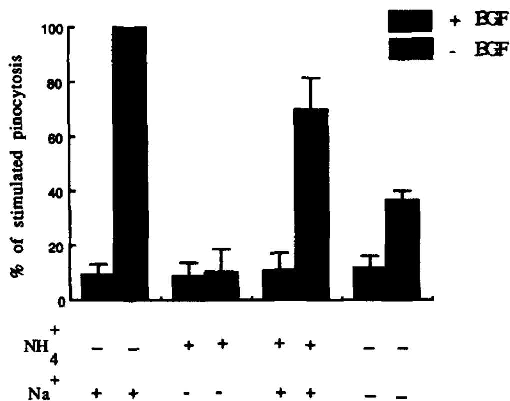

Figure 2. Intracellular acidification blocks EGF-stimulated pinocytosis. Cells were acidified after an NH_4^+/NH_3 prepulse as described by Sandvig et al. (1987) and in Materials and Methods. After loading with NH_3/NH_4^+, cells were transferred to either Na^+-containing or Na^+-free medium as indicated and incubated with EGF for 6 min in the presence of HRP which was assayed as described in Materials and Methods. Results from four different experiments are expressed as a percentage (means ± SEM) of the EGF-stimulated pinocytosis.

Stimulation of Pinocytosis Requires Active Na^+/H^+ Exchange

An additional control included in the above acidification experiments gave an unexpected result. Cells which had not been loaded with NH_4Cl but which, like the loaded cells, were then transferred to Na^+-free medium before HRP and EGF addition showed a considerably diminished level of EGF-stimulated HRP uptake (Fig. 2). In Na^+-free medium the additional pinocytotic activity induced by EGF was only 26% of that in Na^+-containing medium. We were particularly interested in this unexpected cation requirement for EGF-stimulated pinocytosis because the basal rate of pinocytosis appeared to be unaffected by the presence or absence of Na^+ (Fig. 2).

The presence of extracellular Na^+ is also required for the operation of the Na^+/H^+ exchange system, an important regulator of cytosolic pH whose activity is stimulated in a variety of cells by growth factors and phorbol esters (Smith and Rozengurt, 1978; Moolenaar et al., 1982; Rothenberg et al., 1983*a*). The requirement for extracellular Na^+ raised the possibility that the operation and perhaps stimulation of this Na^+/H^+ exchange system was required for EGF-stimulated pinocytosis and might therefore be inhibitable with amiloride which blocks Na^+/H^+ exchange (L'Allemain et al., 1984; Zhuang et al., 1984 and references therein).

Cells were incubated in a medium containing HRP plus amiloride with or without EGF. Uptake of HRP was measured as before, after a 6 min incubation period. The data in Fig. 3 show that 3 mM amiloride caused a marked decrease in the stimulation of HRP uptake even in medium con-

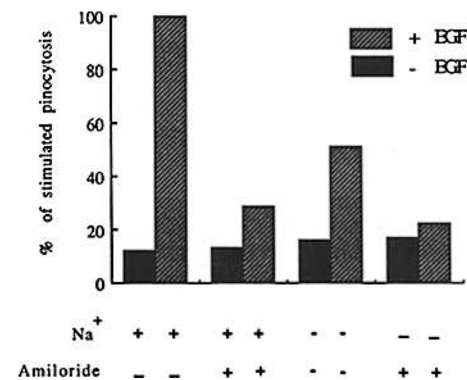

Figure 3. Amiloride blocks EGF-stimulated pinocytosis. A431 cells were incubated for 5 min in 3 mM amiloride in Na^+ or Na^+-free buffer before stimulation in the same buffer with 50 ng/ml EGF for 6 min in the presence of 2 mg/ml HRP. The data are the means of two experiments and are expressed as a percentage of the EGF-stimulated pinocytosis.

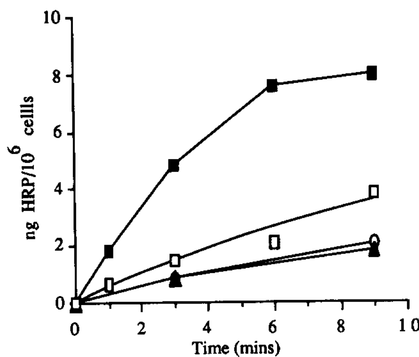

Figure 4. Time course of pinocytosis in the presence of amiloride. Cells were incubated with HRP for the times indicated in the presence (■, □) or absence (▲, ○) of EGF with (□, ○) or without (■, ▲) amiloride. Endocytosis of HRP was determined as described in Materials and Methods.

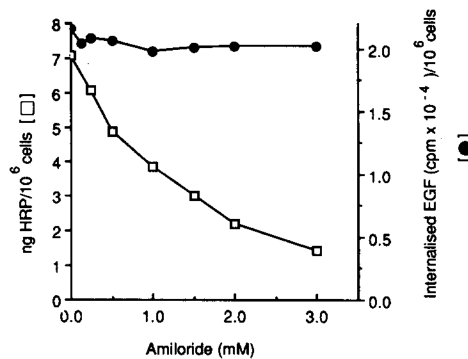

Figure 5. Amiloride blocks EGF-stimulated pinocytosis but not receptor-mediated endocytosis of EGF. Cells were stimulated with ^{125}I-labeled EGF for 9 min in the presence of HRP and various concentrations of amiloride as shown. Intracellular (acid-resistant) ^{125}I-EGF (●) and pinocytosed HRP (□) were determined for each dish of cells as described in Materials and Methods. The presence of a 100-fold excess of unlabeled EGF reduced binding and internalization of ^{125}I EGF by 97%. The acid wash in this experiment removed 93% of ^{125}I-EGF bound to the cell surface at 4°C.

taining Na$^+$. Under these conditions the additional pinocytotic activity induced by EGF was only 17% of that observed in the absence of amiloride. In Na$^+$-free medium the effect of amiloride was even more striking, virtually abolishing the stimulation of pinocytosis (Fig. 3). Taken together, the requirement for extracellular Na$^+$ and the inhibition by amiloride strongly suggest that ongoing Na$^+$/H$^+$ exchange is required for the rapid stimulation of pinocytosis by EGF.

When the kinetics of pinocytosis were examined in the presence of amiloride a clear inhibition of stimulated pinocytosis was observed at all time points (Fig. 4). The basal rate of pinocytosis (i.e., in the absence of EGF) was essentially unaffected by antagonists of Na$^+$/H$^+$ exchange (Figs. 3 and 4), which suggested that at least two distinct endocytotic pathways might be operating.

Receptor-mediated Endocytosis Is Unaffected by Amiloride

We were concerned that the block in EGF-stimulated pinocytosis observed in the presence of amiloride might be due to a direct effect of amiloride on EGF binding and/or endocytosis. To test this possibility directly and, at the same time, assess whether the receptor-mediated endocytotic pathway was operating normally we measured the endocytosis of EGF itself in the presence of amiloride. Monolayers of A431 cells were incubated in the presence of various concentrations of amiloride and were then stimulated with 50 ng/ml of ^{125}I-EGF for 9 min in the presence of HRP. For each monolayer of cells we measured the endocytosis of both EGF and HRP. The results showed that while there was a concentration-dependent inhibition of EGF-stimulated pinocytosis by amiloride there was no effect on the binding and endocytosis of EGF itself (Fig. 5). Over the range of amiloride concentrations tested, the amount of EGF becoming acid-resistant remained the same and corresponded to 64 ± 0.67% (SEM, $n = 9$) of the total cell-associated EGF. This result demonstrates that (*a*) the pinocytotic pathway stimulated by EGF must be distinct from the endocytotic pathway taken by the EGF receptor, and (*b*) endocytosis of occupied EGF receptors per se is not sufficient to stimulate pinocytosis.

Since occupied EGF receptors become endocytosed predominantly through coated pits (Haigler et al., 1979*a*; Hopkins et al., 1985), the above result strongly suggested that in cells treated with amiloride this endocytotic pathway was operating normally over the time course of the experiment and that the extra pinocytosis induced by EGF must enter the cells by some other route. Further evidence for the normal operation of the coated pit route in cells stimulated with EGF in the presence of amiloride was obtained by measuring the endocytosis of the transferrin receptor. Cells were incubated with or without EGF in the presence of HRP and ^{125}I-transferrin in amiloride-containing or control buffers as appropriate. For each cell monolayer the amount of ^{125}I-transferrin endocytosed as well as bound to the surface was

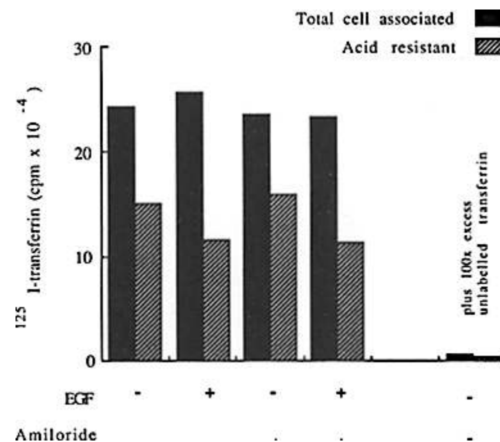

Figure 6. Endocytosis of transferrin in the presence of EGF and/or amiloride. Cells were incubated at 37°C in the presence of HRP and 0.2 μg/ml ^{125}I-ferritransferrin for 5 min in the presence and absence of EGF and amiloride as indicated. For each condition the total cell-associated and intracellular (acid-resistant) transferrin was determined for duplicate dishes of cells at the same time as pinocytosed HRP (data not shown). The acid wash in this experiment removed 98% of ^{125}I-transferrin bound to the cell surface at 0°C. ^{125}I-Transferrin binding and uptake in the presence of a 500-fold excess of unlabeled transferrin is indicated in the rightmost columns.

Table I. Amiloride Blocks EGF-stimulated Pinocytosis in HCO_3^--containing Media

	− EGF	+ EGF
− Amiloride	0.95	13.34
+ Amiloride	0.99	2.44

A431 cells were incubated in bicarbonate-buffered DME containing 2 mg/ml HRP and 2 mg/ml BSA under a 5% CO_2/95% air atmosphere in the presence and absence of 50 ng/ml EGF and 3 mM amiloride as indicated. Pinocytosed HRP was assayed after 6 min as described in Materials and Methods. Results from duplicate dishes are expressed in nanograms HRP pinocytosed per 10^6 cells.

measured alongside the amount of HRP pinocytosed. The results of a representative experiment are shown in Fig. 6. It can be seen that the presence of amiloride made little difference to the amount of transferrin endocytosed in the 5-min period after EGF stimulation. As judged by this assay the operation of coated pits was normal under these various conditions whereas the endocytosis of HRP into the same cells was substantially inhibited as expected (data not shown). There did appear to be a small but reproducible fall in the amount of endocytosed (acid-resistant) transferrin in EGF-stimulated versus control cells (Fig. 6). A similar fall in specific internalization of transferrin on A431 cells stimulated with EGF was recently observed by Wiley (1988).

EGF-stimulated Pinocytosis Is Amiloride Sensitive in HCO_3^--containing Media

Recent reports have demonstrated that growth factor stimulation of Na^+/H^+ exchange which in HCO_3^--free medium raises cytosolic pH may not do so in HCO_3^--containing buffers (Cassel et al., 1985; Ganz et al., 1989). Experiments to test if amiloride was still an effective inhibitor of EGF-stimulated pinocytosis in HCO_3^--containing media showed that stimulated pinocytosis was reduced by the presence of amiloride to 12% of the control level when the experiment was conducted in medium buffered solely with HCO_3^- (Table I) demonstrating that amiloride is at least as effective in HCO_3^--containing as in HCO_3^--free media.

Localization of EGF-induced Pinocytotic Activity

The results presented so far demonstrate that a distinct pinocytotic process is induced by EGF in A431 cells. Pinocytosis through coated pits is thought to occur uniformly over the cell surface (e.g., Bretscher and Thomson, 1983). To determine which parts of the cell surface were involved in taking

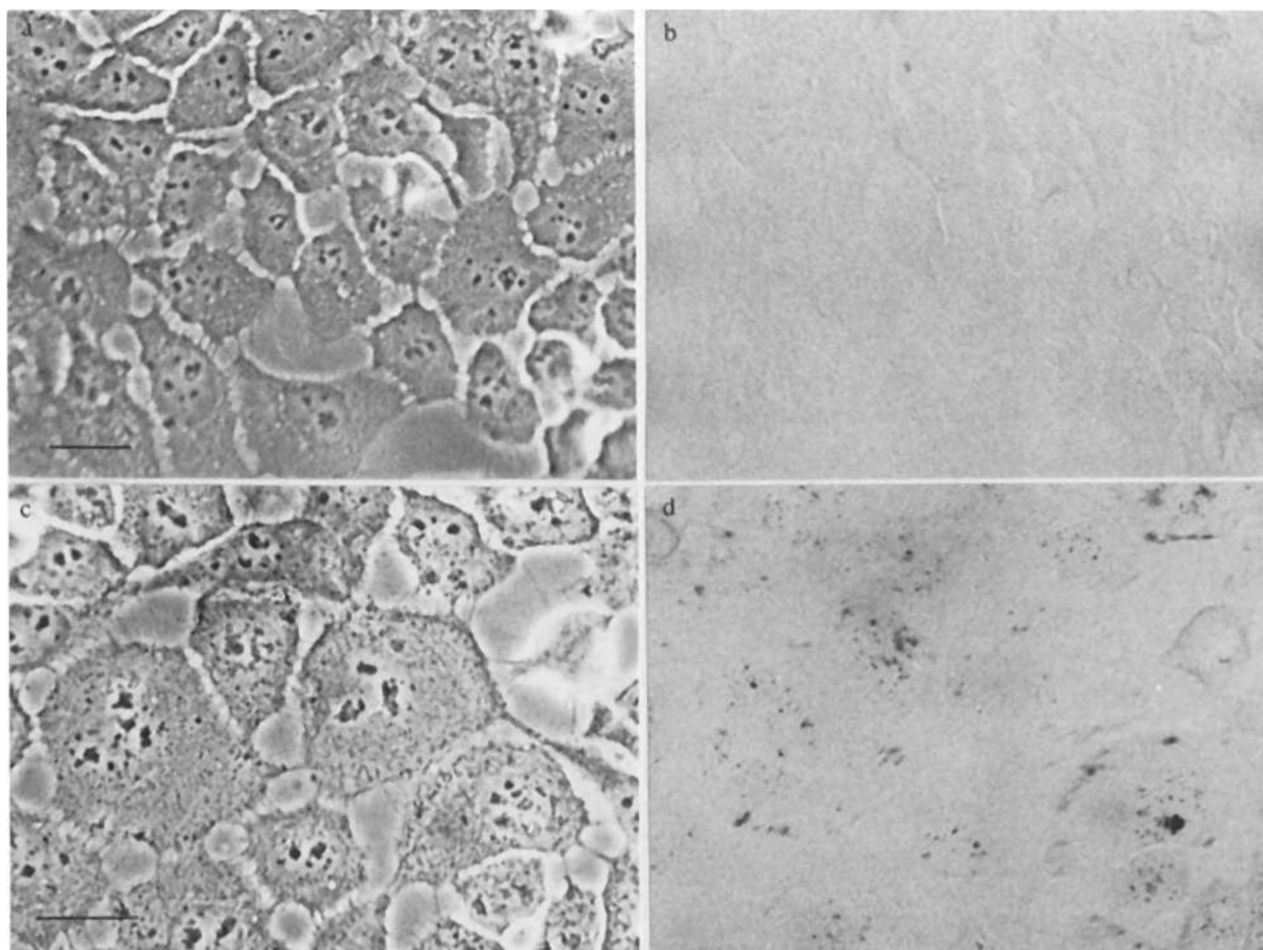

Figure 7. Light microscopy of A431 cells stimulated with EGF for 5 min. Cells were incubated with 10 mg/ml HRP in the absence (*a* and *b*) or presence (*c* and *d*) of 50 ng/ml EGF and processed as described in Materials and Methods. The same field of cells is shown under phase contrast (*a* and *c*) and bright field (*b* and *d*) illumination. HRP uptake can only be detected in stimulated cells. Bar, 30 μm.

up HRP in response to EGF stimulation, we grew A431 cells on glass coverslips and stimulated them with EGF for up to 6 min in the presence of HRP and then processed them for light microscopy. The cells were fixed and HRP activity was revealed by standard cytochemical procedures. Intracellular vesicles filled with reaction product were clearly visible after as little as 2 min when EGF was present (Fig. 8) and by 6 min most cells were positively stained. In contrast, in the absence of EGF, very few cells showed any visible HRP reaction product after a 6-min incubation (Fig. 7). There was considerable heterogeneity in both the size and number of HRP-filled structures in the stimulated cells. While some cells displayed a few large and irregularly shaped bodies others had a larger number of smaller and more regularly shaped vesicles (Figs. 7 and 8, and data not shown). Although EGF-stimulated pinocytosis was not confined to cells with free edges we noticed that cells with well-isolated spreading margins frequently had the bulk of their reaction product confined to vesicles that were close to these "leading edges" indicative of a relationship between the pinocytotic activity and ruffling activity occurring at these margins (Fig. 8).

Discussion

Our first experiments were designed to see if there was any relationship between the EGF-stimulated pinocytotic activity and endocytosis mediated by the coated vesicle pathway. Since the EGF receptor is thought to become clustered in coated pits only upon binding of EGF (Haigler et al., 1979*a*) it seemed possible that the large number of EGF receptors (2–3 × 10^6/cell) might, once occupied, induce the formation of additional endocytotic pits which might then mediate at least some of the additional fluid uptake. Consistent with such a model are earlier studies on PC12 cells which revealed a two- to threefold increase in the number of coated

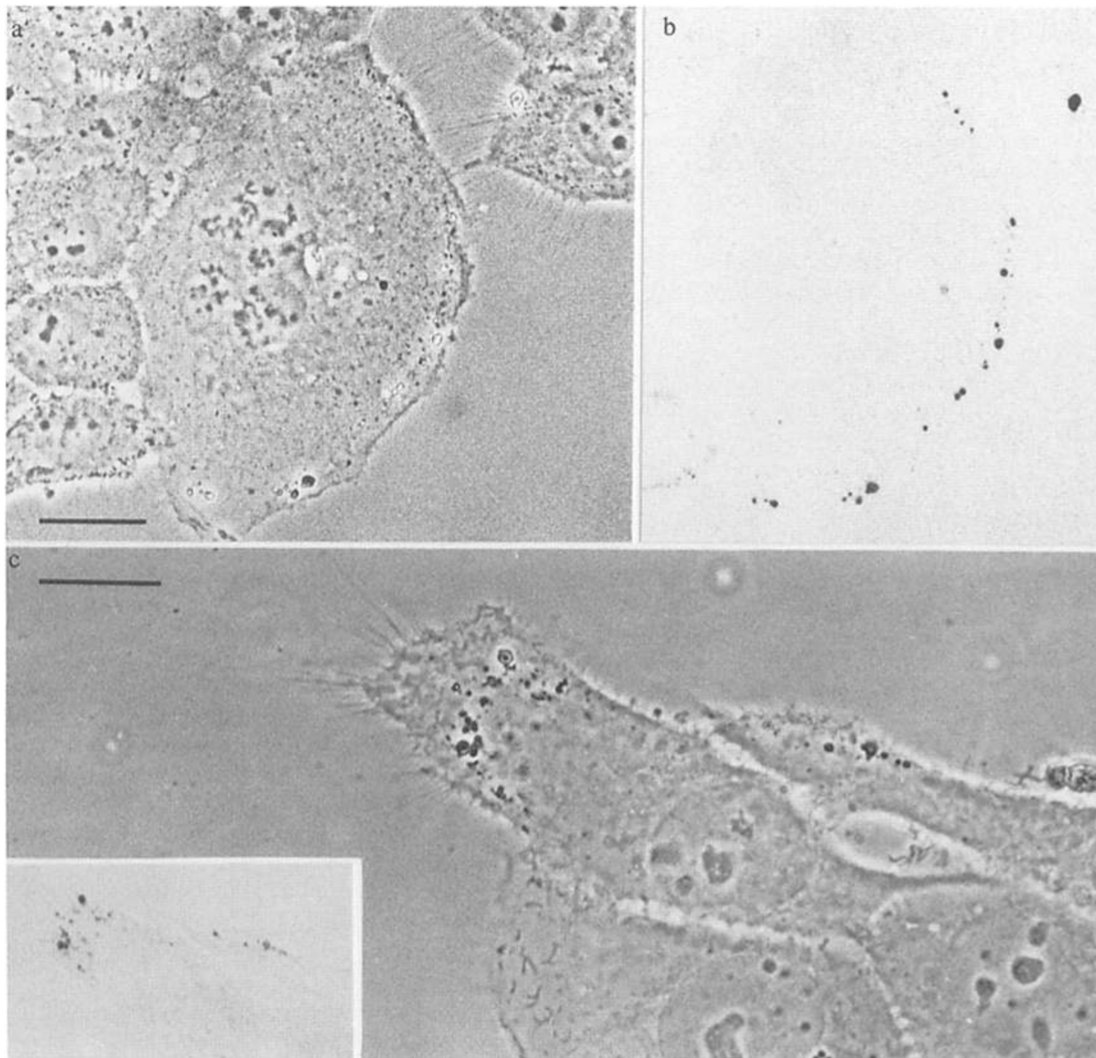

Figure 8. EGF-stimulated pinocytosis seen at spreading/ruffling edges of some cells. Cells were stimulated with EGF for 6 min (*a* and *b*) or 2 min (*c*) and processed as described in Materials and Methods. *a* and *b* show phase contrast and bright field images of the same large cell. (*Inset*) Bright field image at lower magnification for field shown in *c*. HRP-filled vacuoles can be seen in close association with the cell margin (*a* and *b*) and with a ruffling edge (*c*). Bars (*a* and *b*) 30 μm; (*c*) 15 μm.

pits upon EGF or NGF addition (Connolly et al., 1984), and recent studies on A431 cells which showed that the rate of fluid-phase uptake is proportional, although not linearly, to the number of occupied EGF receptors (Wiley, 1988, and our unpublished results). However, several factors rule out the possibility that the EGF-induced pinocytosis can, to any significant extent, be accounted for by increase in the rate of formation and budding of coated pits on A431 cells.

First and foremost, the data presented here demonstrate that EGF-stimulated pinocytosis can be selectively blocked when Na^+/H^+ exchange activity is inhibited, while under the same conditions endocytosis via coated pits, as measured by the uptake of both transferrin as well as EGF itself, proceeded normally.

Secondly, the magnitude of the pinocytotic burst (6-10-fold above basal) would require a very large increase, either in the number of coated pits, or in their rate of formation and invagination which might be expected to affect the density of receptors clustered in coated pits. In fact the rate of transferrin uptake was only slightly altered during EGF stimulation (Wiley, 1988, and Fig. 6). Such a result could still be obtained if the same number of transferrin receptors were distributed among more coated pits. For an increase in coated pit activity to account for the extra pinocytosis the density of transferrin receptors per pit would be expected to fall ~6-10-fold. We estimated the density of transferrin receptors in coated pits on cells which had or had not been stimulated with EGF. The results shown in Fig. 9 demonstrate that there was no measurable difference in transferrin receptor density in coated pits on the two cell types. Combined with the ^{125}I-transferrin endocytosis data (Fig. 6) this argues against coated pits being the vehicles for the extra fluid uptake.

Thirdly, on well-spread cells the localization of the pinocytotic vacuoles to the margins of the cell suggests an uptake route other than coated pits which are more or less uniformly distributed on these as on other cells (Bretscher and Thomson, 1983).

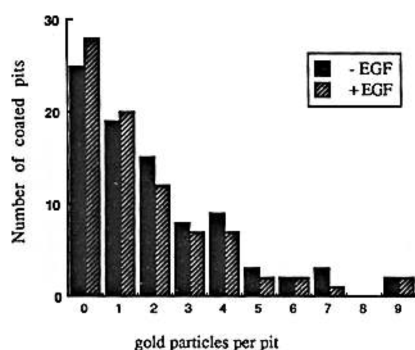

Figure 9. Transferrin receptor distribution in coated pits on EGF-stimulated A431 cells. Control cells and cells stimulated with EGF for 1 min were labeled at 0°C with an antitransferrin receptor antibody and subsequently with goat anti-rabbit Ig conjugated to 10-nm colloidal gold. The cells were prepared for electron microscopy and the frequency of gold particles in coated pits was analyzed. On a sample where the antireceptor antibody was omitted there were no gold particles in 23 coated pits observed, demonstrating the specificity of labeling.

Smooth flask-shaped invaginations about the same size as coated pits have been observed on A431 cells (Hopkins et al., 1985), Vero cells (Sandvig et al., 1987), fibroblasts (Huet et al., 1980), hepatocytes (Montesano et al., 1982), and endothelial cells (Palade, 1953). Smooth pits of this kind have been implicated in the uptake of cholera, tetanus, and ricin toxins (Montesano et al., 1982; Sandvig et al., 1987) and in the uptake of EGF itself (Hopkins et al., 1985). Since uptake of ricin toxin was only slightly reduced by acidification of the cytosol in several cell types including A431 (Sandvig et al., 1987), whereas the stimulated pinocytosis was completely abolished (Fig. 2) under the same conditions, these smooth pits are not likely to be responsible for the extra fluid-phase pinocytosis. However, it appeared that acidification of the cytosol did not abolish the basal level of HRP uptake measured over a 6-min period (Fig. 2) consistent with the data of Sandvig et al. (1987) who reported that fluid-phase pinocytosis on several cell types was resistant to acidification. Comparing basal uptake under acidified and nonacidified conditions we found there was considerable variation from experiment to experiment because the amounts of HRP taken up over a short time period are small. The quantitation of HRP uptake under acidified conditions would need to be more carefully investigated before one could conclude that most fluid-phase pinocytosis under basal conditions is mediated by a pathway other than coated pits. Nonetheless it is possible that up to three different endocytotic pathways may exist in EGF-stimulated A431 cells.

Since the conditions we used to acidify the cytosol required omitting Na^+ from the medium can we be certain that the EGF-stimulated pinocytosis is really sensitive to acidification? Perhaps the best evidence that it is sensitive derives from the observation that inhibition of EGF-stimulated pinocytosis was more effective when cells were first loaded with NH_3 and then transferred to Na^+-free medium (Fig. 2).

It appears most likely that the stimulated pinocytotic activity is related in some way to the vigorous membrane ruffling activity also induced by EGF and which is most obvious at the spreading margins (Brunk et al., 1976; Chinkers et al., 1979). Consistent with this in EGF-stimulated cells, we frequently observed HRP-filled pinocytotic vacuoles close to cell margins although cells in the middle of a group also show both EGF-induced ruffling activity (Chinkers et al., 1979) and pinocytotic vacuoles (Fig. 7). A relationship between membrane ruffling and "macropinocytosis" was suggested in earlier studies where droplets of fluid sometimes appeared to be taken up at sites where membrane ruffling activity was most evident (e.g., Lewis, 1931; Abercrombie and Ambrose, 1958; Fawcett, 1965; Brunk et al., 1976). Stimulated pinocytotic activity is likely to be a consequence of ruffling because if ruffling developed as a consequence of stimulated pinocytosis – e.g., as pinocytosed membrane was returned locally to the cell surface – then one might expect the pinocytosed fluid to be rapidly regurgitated instead of being retained within the cell (Haigler et al., 1979*b*, and Figs. 1, 7, and 8). However, exocytosis of intracellular membrane pools can be triggered by growth factors (Davis and Czech, 1986; Davis et al., 1987) and might be involved in the ruffling/pinocytosis response.

What is the basis of the differential sensitivity of endocytotic pathways on A431 cells to amiloride? There are many studies on a wide variety of cell types which demon-

strate that stimulation of Na^+/H^+ exchange activity by growth factors and tumor promotors can lead to cytosolic alkalinization. This has been proposed to be a key element in the transduction of external stimuli into metabolic responses ultimately leading to cell proliferation (e.g., Moolenaar et al., 1983, 1984). One possibility, considering its sensitivity to amiloride, is that the EGF-stimulated pinocytotic burst requires the permissive conditions created by cytosolic alkalinization. However, some recent reports indicate that in the presence of HCO_3^-, growth factors fail to raise cytoplasmic pH although Na^+/H^+ exchange activity is stimulated to the same extent as HCO_3^--free buffers (Cassel et al., 1985; Ganz et al., 1989). Since we have shown that EGF-stimulated pinocytosis is sensitive to amiloride in the presence or absence of HCO_3^- (Table I) alkalinization of the cytosol may not be a crucial element in the induction of the pinocytotic response.

As well as blocking growth factor-induced alkalinization amiloride can also induce a progressive acidification of the cytosol (Moolenaar et al., 1983; Rothenberg et al., 1983*b*; Zhuang et al., 1984). A possible explanation for our results is that mild acidification, induced by amiloride, blocks the stimulated pinocytotic pathway but not the receptor-mediated pathway which is not arrested until the cytosolic pH falls below ~6.5 (Sandvig et al., 1987; Davoust et al., 1987). Davoust et al. (1987) have reported that acidification of the cytosol to pH 6.8 was compatible with endocytosis via coated pits but interfered with net accumulation of fluid-phase markers indicating that steps along the endocytotic pathway can be differentially sensitive to lowering the cytosolic pH. The existence of a pinocytotic pathway linked to membrane ruffling and sensitive to intracellular pH changes is consistent with a recent report by Heuser (1989). He noted that membrane ruffling was arrested on acidified cells but recovered vigorously during subsequent alkalinization after washout of the acidifying agent. One hypothesis is that permissive conditions for EGF-stimulated pinocytosis are found only over a narrow range of intracellular pH and are consequently sensitive to perturbation of Na^+/H^+ exchange system by growth factors and/or amiloride. A possible difficulty with such a scheme is that amiloride was still an effective inhibitor of stimulated pinocytosis when the cells were maintained in HCO_3^--containing buffers (Table I) which might be expected to supress fluctuations in intracellular pH.

Our current data strongly suggest that amiloride acts by blocking Na^+/H^+ exchange and not by some nonspecific means. The concentrations of amiloride required to block pinocytosis on the one hand and Na^+/H^+ exchange on the other are similar (Fig. 5, and Rothenberg et al., 1983*a*). Moreover, the analogue dimethylamiloride which inhibits the exchange system at much lower concentrations (Zhuang et al., 1984; L'Allemain et al., 1984) also blocked EGF-stimulated pinocytosis at similar low concentrations (data not shown). We do not yet know if stimulation of the exchange system is actually required or whether it is sufficient simply for it to be operational. Nor can we exclude the possibility that it is Na^+ influx rather than H^+ efflux which is the important element. Miyata et al. (1989) recently reported that ruffling and pinocytosis stimulated by growth factors on KB cells were inhibited under conditions which raised cytosolic Ca^{2+} or cAMP levels. How these observations relate to the effects of amiloride is not yet clear.

In summary, we draw three conclusions from the work presented here. (*a*) Although further experiments are necessary to establish exactly how amiloride is able to selectively block the stimulated pinocytotic pathway, this drug clearly allows us to differentiate two distinct endocytotic pathways in EGS-stimulated A431 cells, the coated pit pathway and an amiloride-sensitive pathway which appears to involve regions of the cells engaged in membrane ruffling activity. (*b*) The EGF-induced pathway is not, to any significant extent, used by the EGF receptor for its own uptake. (*c*) Endocytosis of occupied EGF receptors is not, in itself, sufficient to trigger the events leading to stimulated pinocytosis.

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