

Rapid Rounding of Human Epidermoid Carcinoma Cells A-431 Induced by Epidermal Growth Factor

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ABSTRACT Epidermal growth factor (EGF) induces rapid rounding of A-431 human epidermoid carcinoma cells in Ca^{++} -free medium. Cell rounding is not induced by a variety of other polypeptide hormones, antiserum to cell membranes, local anesthetics, colchicine, cytochalasin B, or cyclic nucleotides. However, trypsin, like EGF, induces rounding of A-431 cells in the absence of Ca^{++} . Both trypsin- and EGF-induced rounding are temperature dependent, appear to be energy dependent, and are inhibited by cytochalasins, suggesting the active participation of microfilaments in cell rounding. However, a medium transfer experiment suggests that EGF-induced rounding is not attributable to secretion of a protease, and a number of serine protease inhibitors have no effect on the EGF-induced rounding process. Cell rounding is not attributable to the slight stimulation by EGF of the release of Ca^{++} that is observed in Ca^{++} -free medium, as stimulation of such release by the ionophore A23187 neither induces cell rounding nor blocks EGF-induced rounding.

Cells that have rounded up after treatment with EGF or trypsin spread out upon addition of Ca^{++} to the medium, even in the continuing presence of EGF or trypsin. Like the cell-rounding process, the cell-spreading process is temperature dependent, appears to be energy dependent, and is inhibited by cytochalasin B. Thus, EGF does not destroy the ability of the cell to spread; rather, in the presence of EGF (or trypsin), cell spreading and the maintenance of the flattened state become dependent on external Ca^{++} . Because untreated cells remain flattened in the absence of Ca^{++} , the data suggest that EGF may disrupt Ca^{++} -independent mechanisms of adhesion normally present in A-431 cells.

Cells grown in monolayer culture may become rounded in response to a wide variety of substances. These include hormones, cyclic nucleotides (23, 28, 29, 31, 43), proteases (12, 32, 36), and antisera raised against membrane proteins (13, 41, 42). In addition, cells normally round up during mitosis. Cell rounding presumably occurs because of reorganization of the cytoskeleton, but the biochemical mechanisms leading to such reorganization are not yet known.

Several recent studies concerned with the mechanism of action of epidermal growth factor (EGF) have employed the human epidermoid carcinoma cell line A-431. The unusually high number of receptors for EGF present on these cells has facilitated visualization of the binding of EGF and clustering and endocytosis of the EGF/receptor complex (16, 18, 25), as well as demonstration of the rapid stimulation of the rates of fluid pinocytosis in whole cells (19) and protein phosphorylation in isolated membranes (6, 7) by EGF. In addition, we have recently reported that A-431 cells undergo a sequence of shape

changes in response to EGF (8). Within 1 min of exposure to EGF, numerous ruffles and filopodia are formed. This effect is transient, peaking at 2 min of treatment, and is followed by a gradual retraction from the substrate at the edges of colonies. After several hours, the monolayer culture has reorganized into multilayered colonies.

This sequence of morphological changes, originally described for cells growing in Dulbecco's Modified Eagle's Medium (DMEM) containing serum, may also be observed in simple buffered saline solutions containing millimolar levels of Ca^{++} (our unpublished observations). However, as reported in this paper, the omission of Ca^{++} from the medium drastically alters the morphological response of A-431 cells to EGF. In Ca^{++} -free medium, EGF induces rapid rounding of A-431 cells. Because this response is easily monitored by phase-contrast microscopy, we have been able to investigate the mechanism of EGF-induced cell rounding by observing the effects of a variety of experimental manipulations on this process.

MATERIALS AND METHODS

Cell Culture

A-431 human epithelioid carcinoma cells were grown in DMEM containing 10% fetal calf serum (FCS). Cultures were routinely prepared in 35-mm Falcon culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). For SEM, cells were grown on 22-mm square cover glasses in 35-mm dishes.

Assay for Cell Rounding

Cells were washed once with 1 ml Dulbecco's phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.9 mM CaCl₂, 0.5 mM MgCl₂), once with 1 ml Ca⁺⁺-/Mg⁺⁺-free Dulbecco's phosphate-buffered saline (CMF), and incubated at 25°C in 1.0 ml CMF in the presence or absence of 100 ng EGF and/or various substances being tested for their effects on cell rounding. Except where indicated in the text, such substances and EGF were added to the cultures simultaneously. After 20 min, the living cells were examined by phase-contrast microscopy. Appropriate controls were run to test the effects of the solvents in which stock drug solutions were prepared.

Light Microscopy

Routine observations of living cells were made at × 100 on a Wild M40 inverted phase-contrast microscope. Phase-contrast photomicrography of living cells was performed on a Leitz microscope using a Leitz Orthomat camera and Kodak Plus X film.

SEM

Cells were fixed for 1 h with 4% glutaraldehyde in CMF, washed three times for 10 min each with 0.1 M sodium phosphate, pH 7.1, and postfixed for 2 h with 1% OsO₄ in 0.1 M sodium phosphate, pH 7.1. The cultures on cover slips were then rinsed several times with distilled water, dehydrated through a graded ethanol series, and critical point dried, using the phase-separation method of alcohol/CO₂ substitution to minimize the effects of convection currents on surface ultrastructure (26). Cover slips were cut into quarters, which were mounted on aluminum stubs with graphite paste and sputter coated with Au/Pd. Fixation, washing, and dehydration were performed at room temperature.

Samples were viewed on a Hitachi S-500 microscope operated at 20 kV. Kodak Min-R X-ray film, developed with a Kodak RP X-Omat processor, was used for micrography.

Measurement of Cellular Ca⁺⁺ Release

Cells were preincubated for 20 h at 37°C in 2.0 ml DMEM containing 10% FCS and 2.8 μCi [⁴⁵Ca]CaCl₂ (New England Nuclear, Boston, Mass.). Cultures were then washed as described for the standard rounding assay (once with 2 ml PBS and once with 1 ml CMF) and incubated at 25°C in 1.0 ml CMF in the presence or absence of EGF (100 ng/ml) or A23187 (10 μM). After various times, duplicate cultures were washed rapidly three times with 1.5 ml of ice cold CMF, bringing the total number of washes after loading with ⁴⁵Ca⁺⁺ to six. Remaining cell-associated radioactivity was determined by solubilizing the cells in 1.0 ml 0.5 N NaOH, mixing with 10 ml of Aquasol (New England Nuclear), and counting in a liquid scintillation counter (LS-3133P; Beckman Instruments, Inc., Irvine, Calif.). To obtain a value for cell-associated radioactivity at zero time (no incubation in CMF), cells were treated exactly as described above except that the "incubation" in CMF at 25°C consisted of only a rapid wash with CMF.

Measurement of Cellular ATP

ATP in perchloric acid extracts of A-431 cells was determined by use of a firefly luciferase assay (22).

Materials

Mouse EGF (33) and DEAE-purified antisera to mouse EGF (9, 14) and to A-431 cell membranes (17) were prepared as previously described. The A-431 cell line was obtained from Dr. J. DeLarco, National Institutes of Health.

RESULTS

EGF-induced Rounding

Rapid cell rounding occurs when monolayer cultures of A-431 cells are treated with EGF in Ca⁺⁺-free media. This phenomenon is illustrated in Fig. 1, using phase-contrast microscopy of living cells. A-431 cells incubated for 20 min at

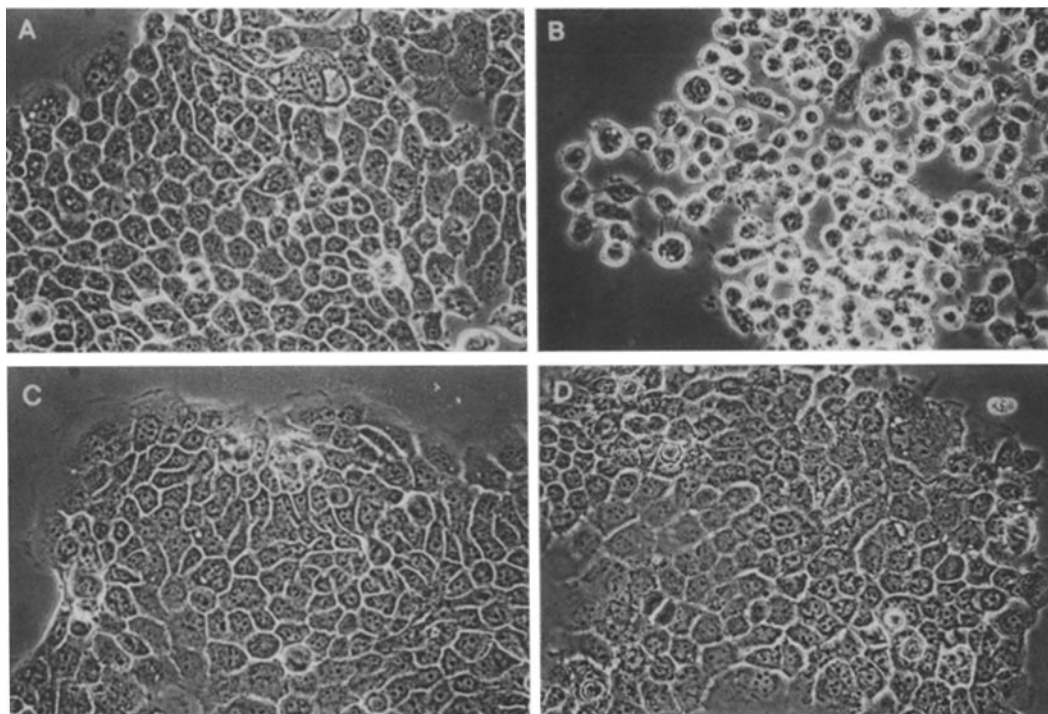


FIGURE 1 Effect of EGF on A-431 cell shape in the presence or absence of Ca⁺⁺. Phase-contrast micrographs of living cells at × 160. All incubations were for 20 min at 25°C. (A) Incubation in CMF. Cells remain flattened. (B) Incubation in CMF containing 100 ng/ml EGF. Cells are rounded. (C) Incubation in CMF supplemented with 1 mM CaCl₂. (D) Incubation in CMF supplemented with 1 mM CaCl₂ and 100 ng/ml EGF. No cell rounding has occurred in the presence of Ca⁺⁺.

25°C in Ca^{++} -/ Mg^{++} -free CMF remain flattened (Fig. 1A). However, when EGF (100 ng/ml) is present during such an incubation, most cells become highly rounded (Fig. 1B). This phenomenon occurs only in the absence of added Ca^{++} ; in the presence of 1 mM Ca^{++} , A-431 cells remain flattened in either the absence (Fig. 1C) or the presence (Fig. 1D) of EGF. Thus, it appears that either Ca^{++} inhibits EGF-induced rounding, or the absence of Ca^{++} permits EGF to have this effect. In

contrast, EGF-induced rounding may occur in the presence of 1 mM Mg^{++} .

The observations have been confirmed and extended by use of scanning electron microscopy (Fig. 2). Untreated A-431 cells (Fig. 2A) are flattened and covered with small plicae, as previously described (8). The cells have a similar, though somewhat less flattened, appearance after a 20-min incubation in CMF (Fig. 2B). After 20 min in CMF containing EGF, the

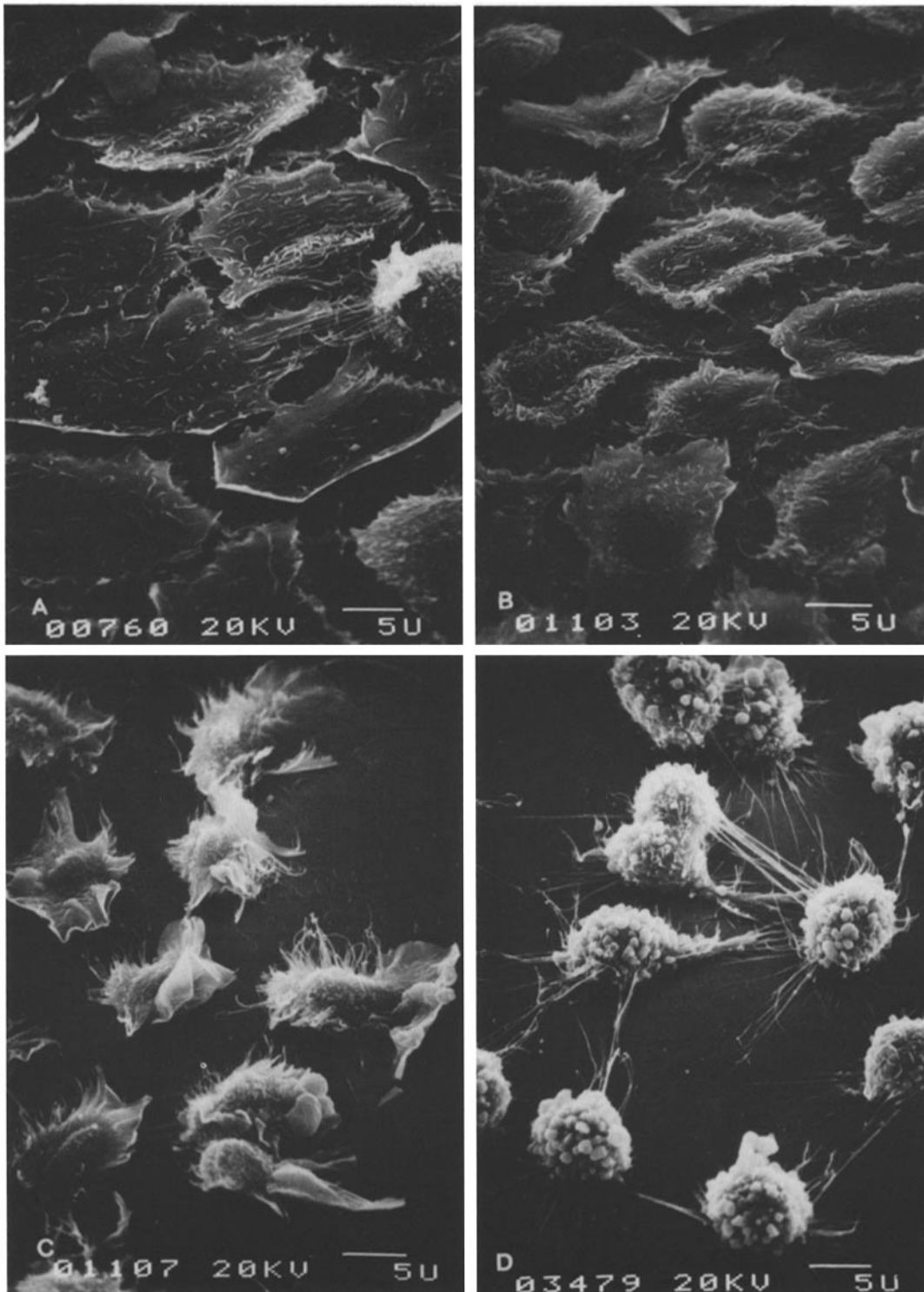


FIGURE 2 EGF-induced rounding of A-431 cells. (A) Untreated culture. Cells are flattened and display many plicae. (B) Culture incubated in CMF for 20 min at 20°C. Cells have contracted slightly. (C) Culture incubated for 10 min at 25°C in CMF containing 100 ng/ml EGF. Large ruffles and filopodia may be observed. (D) Incubation for 20 min at 25°C in CMF containing 100 ng/ml EGF. Cells are rounded and display many blebs and retraction fibers. Scanning electron micrographs at $\times 1,400$.

rounded cells display many blebs and are connected to the substrate and to each other by retraction fibers (Fig. 2D). Partly rounded cells (Fig. 2C, 10-min treatment with EGF in CMF) may display large ruffles and filopodia. Stimulation of ruffle and filopodium formation by brief treatment with EGF in the presence of Ca^{++} has previously been reported (8).

Whether A-431 cells respond to EGF by rapidly rounding or by rapidly ruffling and gradually retracting and piling up on one another as described previously (8) depends only on the absence or presence of Ca^{++} . The other differences in incubation conditions (37°C in DMEM + 10% serum in the previous report vs. 25°C in PBS in the experiments described herein) have little effect on the morphological response. That is, EGF induces A-431 cell rounding either in CMF at 25°C or in DMEM containing 10% serum and 2 mM EDTA at 37°C , whereas the previously reported ruffling/retraction/piling up sequence occurs either in Ca^{++} -containing PBS at 25°C or in DMEM containing 10% serum at 37°C .

Experiments were performed to determine the concentration of EGF required to produce optimal cell rounding. Concentrations of EGF as low as 5 ng/ml caused complete rounding of many cells, but at least 50 ng/ml was required to produce rounding of nearly all cells in a culture. This suggests that some variability exists among A-431 cells with regard to responsiveness to EGF. For our standard rounding assay (see Materials and Methods) we selected an EGF concentration (100 ng/ml) that reproducibly yielded rounding of virtually all of the cells in a culture during a 20-min incubation in CMF at 25°C .

The responses illustrated in Figs. 1 and 2 were typical; however, in several experiments unknown factors resulted in significant cell rounding in CMF in the absence of EGF. Even in these experiments, however, the relative effect of EGF was apparent because the CMF + EGF-treated cells were much more rounded than those treated with CMF only.

Specificity of the EGF Effect

To test the specificity of the rounding effect of EGF on A-431 cells, we substituted the following polypeptide hormones for EGF in the cell-rounding assay: insulin, glucagon, follicle-stimulating hormone (FSH), thyrotropin, growth hormone, prolactin (all at 67 $\mu\text{g}/\text{ml}$), or fibroblast growth factor (133 ng/ml). Neither a 20-min incubation with these hormones in CMF at 25°C nor a 1-h incubation at 37°C had any effect on the shape of the A-431 cells as visualized by phase-contrast microscopy.

Although this experiment established that the mere presence of a variety of peptide hormones would not cause cell rounding, we do not know whether A-431 cells bind any of the above hormones. Therefore, we tested the ability of concanavalin A (Con A) and of antiserum to A-431 membranes to produce cell rounding. Because both of these substances block the binding of EGF to its receptor (5, 17), they presumably bind at or near the EGF binding site. Incubation with Con A (100 $\mu\text{g}/\text{ml}$) or DEAE-purified antiserum to A-431 membranes (160 $\mu\text{g}/\text{ml}$) for 20 min at 25°C or 1 h at 37°C did not produce any cell rounding. Thus, the effect of EGF on A-431 cell shape appears to be highly specific.

Role of Ca^{++}

Because EGF induces cell rounding only in the absence of added Ca^{++} , it seemed possible that the rounding might be caused by leakage of Ca^{++} from the cells, and that this leakage might be accelerated by treatment with EGF. This hypothesis

was tested by preloading cells with $^{45}\text{Ca}^{++}$ and measuring the loss of cell-associated radioactivity under standard rounding conditions (Fig. 3). Although EGF may cause a slight stimulation of Ca^{++} efflux, it is unlikely that this is the cause of cell rounding, because stimulation of Ca^{++} efflux by the ionophore A23187 (Fig. 3) did not produce cell rounding during a 20-min incubation at 25°C . Similarly, treatment with EGTA (1 mM), which would be expected to accelerate Ca^{++} efflux, did not mimic treatment with EGF in the rounding assay. Thus, the mere loss of cellular Ca^{++} appears to be insufficient to cause rounding. Although it could be hypothesized that A23187 causes a flux of Ca^{++} similar to that caused by EGF, plus other changes that block rounding, this seems unlikely, as A23187 does not block the rounding induced by EGF. The ionophore neither caused nor blocked cell rounding at doses ranging from 10 nM to 10 μM .

Displacement of Ca^{++} from the plasma membrane has been suggested as a mechanism whereby local anesthetics induce the rounding of some cell types (30). Therefore, we tested lidocaine (1 mM, 5 mM), procaine (1 mM, 10 mM), cocaine (1 mM, 10 mM), and tetracaine (0.1 mM, 1 mM) in the standard rounding assay. None were able to produce appreciable rounding of A-431 cells, again attesting to the specificity of the EGF-induced morphological alterations.

Metabolic Activity and Cell Rounding

To investigate the possible involvement of cellular metabolic activity in the EGF-induced rounding process, we examined the effects of metabolic inhibitors on cell rounding.

Uncouplers of oxidative phosphorylation were used to investigate the ATP requirements of the rounding process. Cellular ATP levels were reduced by preincubating cultures for 15 min at 37°C in PBS containing Ca^{++} and Mg^{++} and 5×10^{-4} M azide or 5×10^{-5} M 2,4-dinitrophenol (Table I). The treated cells were then assayed for rounding as described in Materials

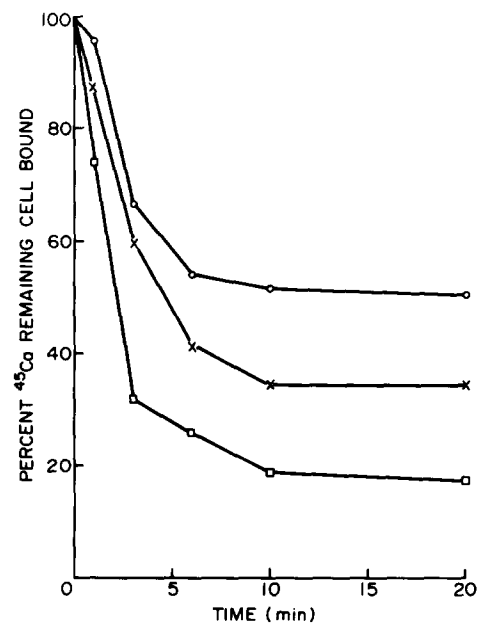


FIGURE 3 Effect of EGF on the release of Ca^{++} from A-431 cells during incubation in CMF. Cells were preloaded with ^{45}Ca and assayed for the release of Ca^{++} as described in Materials and Methods. Counts are expressed as a percentage of cell-associated counts at $t = 0$ (6,300 cpm). \circ , control; \times , EGF (100 ng/ml); \square , A23187 (10 μM).

and Methods, adding fresh azide or dinitrophenol simultaneously with the EGF. In contrast to the results obtained under our standard conditions, no cell rounding was observed during a 20-min incubation at 25°C (Fig. 4A). However, the addition

TABLE I
Effects of Energy Inhibitors on ATP Levels in A-431 Cells

Treatment	ATP levels nmol per dish ± SE
A. None	58.5 ± 2.2
B. 15-min preincubation at 37°C in PBS	62.1 ± 2.9
C. 15-min preincubation at 37°C in PBS containing dinitrophenol	35.1 ± 2.2
D. 15-min preincubation at 37°C in PBS containing azide	43.8 ± 1.4
E. As C but further incubated at 25°C for 20 min in CMF containing dinitrophenol and EGF (rounding assay) followed by addition of glucose for 20 min at 25°C.	61.2 ± 2.8
F. As E except NaF added simultaneously with glucose	1.5 ± 0.1
G. As D but further incubated at 25°C for 20 min in CMF containing azide and EGF (rounding assay) followed by addition of glucose for 20 min at 25°C.	63.0 ± 1.6
H. As G except NaF added simultaneously with glucose	7.8 ± 0.1

A-431 cells, at $\sim 7.5 \times 10^6$ cells per 100-mm dish, were treated as indicated in duplicate and the cell monolayers were extracted on ice with 3 ml of cold 0.5 N perchloric acid. Concentrations of reagents were: 2,4-dinitrophenol, 5×10^{-5} M; sodium azide, 5×10^{-4} M; EGF, 100 ng/ml; glucose, 1 mg/ml; NaF, 10 mM. Rounding assays and determination of ATP in perchloric acid extracts were performed as described in Materials and Methods.

of glucose (1 mg/ml) to the medium at the end of this 20-min incubation resulted, after a further 20 min, in cell rounding in the EGF-treated cultures (Fig. 4B); upon the addition of glucose, cellular ATP concentrations returned to control levels (Table I). Rounding was prevented if the medium was adjusted to contain 10 mM NaF at the time of addition of glucose (not shown); the presence of NaF further lowered the cellular ATP level and prevented the glucose-induced increase (Table I).

These data suggest that uncouplers of oxidative phosphorylation may block EGF-induced cell rounding in the glucose-free CMF medium by partially depleting cellular ATP. It is puzzling that the uncouplers blocked cell rounding under conditions in which ATP levels were reduced by only 30–40%; this raises the possibility that dinitrophenol and azide block rounding through side effects unrelated to inhibition of ATP production. However, this seems unlikely for two reasons: (a) lowering cellular ATP levels with either of two structurally unrelated inhibitors blocks cell rounding, and (b) raising and lowering ATP levels by several means correlates with permission or prohibition, respectively, of EGF-induced cell rounding. Lowering ATP with dinitrophenol or azide blocks rounding. Adding glucose to the medium permits cell rounding and concurrent regeneration, presumably through glycolysis, of ATP, even in the continued presence of the uncouplers. Finally, the glycolytic inhibitor NaF prevents the glucose effects on both cell rounding and ATP regeneration.

The effect of temperature on the EGF-induced rounding process was examined. A-431 cells treated with EGF (100 ng/ml) in CMF rounded up within 20 min of addition of the hormone to the medium at 25°C and within 10 min at 37°C. At 0°C, however, the same treatment produced no cell rounding during a 2-h incubation, as observed by phase-contrast

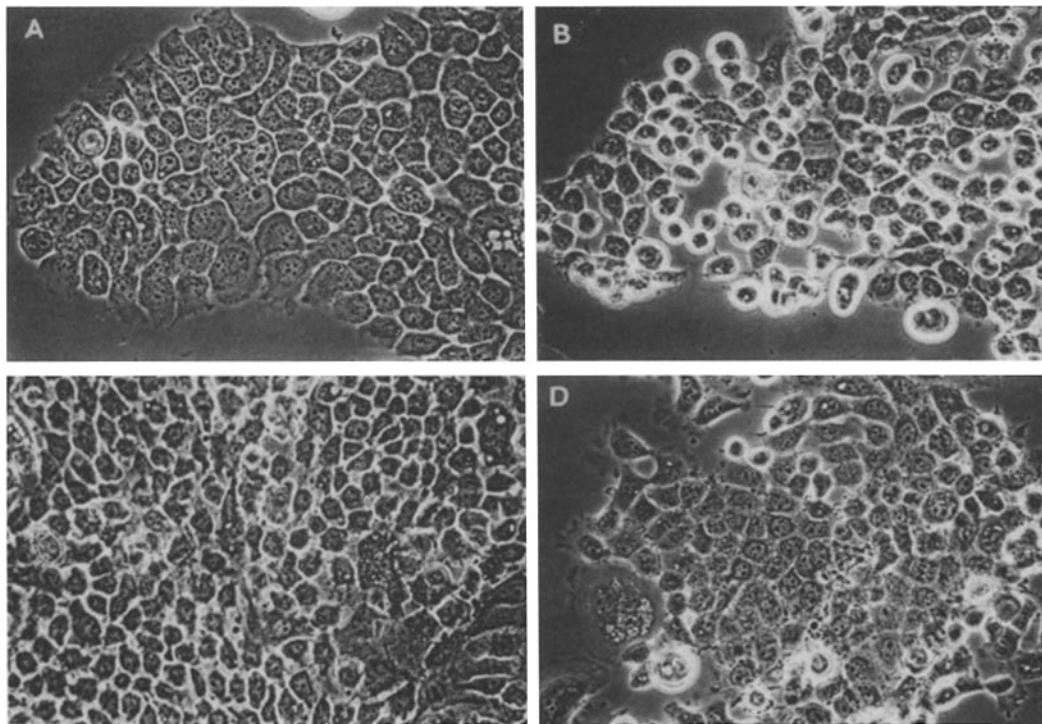


FIGURE 4 Inhibition and reversal of EGF-induced rounding. (A) Cells incubated for 20 min in CMF containing 100 ng/ml EGF and $50 \mu\text{M}$ 2,4-dinitrophenol. Cells remain flattened. (B) After treatment as in A, glucose (1 mg/ml) was added to the medium and the cells were incubated for 20 min. Cells are rounded. (C) Incubation in CMF containing 100 ng/ml EGF and $10 \mu\text{g/ml}$ cytochalasin B. Cell rounding is partially inhibited. (D) After a 20-min incubation in CMF containing 100 ng/ml EGF, 1 mM CaCl_2 was added to the rounded cells. After a further 30-min incubation, most of the rounded cells have spread. All incubations at 25°C. Phase-contrast micrographs of living cells at $\times 160$.

microscopy, even though A-431 cells are known to bind EGF at 0°C (16, 18, 19). The temperature dependence of the rounding process provides additional evidence that metabolic activity, or perhaps membrane fluidity, is required for this process.

The possible role of protein synthesis in the rounding process was then examined. Cycloheximide (20 µg/ml) does not block EGF-induced cell rounding in the standard rounding assay, although the incorporation of amino acids into TCA-precipitable materials is inhibited >95% under these conditions (data not shown). Thus, although certain metabolic activities seem necessary for cell rounding (above), protein synthesis does not appear to be required.

Role of Microtubules and Microfilaments in the Cell-rounding Process

Because microtubules and microfilaments are known to be involved in the control of cell shape, the role of these structures in the EGF-induced rounding process was studied, using colchicine and cytochalasin B.

Because rounded cells may contain fewer or less well organized microfilaments and microtubules (15, 21, 24, 40), we first tested the hypothesis that A-431 cells would round up after the disruption of these structures. However, colchicine (10⁻⁵ M), cytochalasin B (10 µg/ml), and the combination of the two drugs were all unable to induce A-431 cell rounding in the standard rounding assay. This result suggests that disruption of microtubules and/or microfilaments is insufficient to cause rounding of A-431 cells, and, therefore, that EGF does not act by such a disruptive mechanism.

We next tested the ability of these drugs to inhibit EGF-induced cell rounding. Cytochalasin B inhibits EGF-induced rounding (Fig. 4C), suggesting that intact actin-containing microfilaments play an important role in the EGF-induced rounding process. Identical results were obtained using cytochalasin A, D, or E. Colchicine had no effect on the rounding process under standard assay conditions, even if the cultures were preincubated with the drug for 2 h and then assayed for rounding in the presence of EGF and colchicine.

Reversibility of EGF-induced Rounding

The following experiments were performed to examine the conditions under which cells, rounded in the presence of EGF, could regain a flattened morphology.

Because rounding occurs only in the absence of Ca⁺⁺, we tested the ability of Ca⁺⁺ to reverse the rounding process. Cells were rounded by treatment with EGF (100 ng/ml) in CMF for 20 min at 25°C. Rounding was monitored by phase-contrast microscopy. After this 20-min incubation, CaCl₂ was added to the medium to a final concentration of 1 mM. Within 40 min of addition of Ca⁺⁺ at 25°C, most of the EGF-rounded cells were once again well-flattened (Fig. 4D). Thus, the EGF-induced rounding process is reversible simply upon addition of Ca⁺⁺ to the medium, even in the continuous presence of EGF.

This cell-spreading process, like the EGF-induced rounding process, appears to be energy dependent. If cells were treated as described in the preceding paragraph except that the medium was adjusted to contain 5 × 10⁻⁴ M azide or 5 × 10⁻⁵ M 2,4-dinitrophenol immediately before the addition of Ca⁺⁺, no cell spreading was observed after a 40-min incubation at 25°C in the presence of Ca⁺⁺. However, if 1 mg of glucose was added to each culture after this 40-min incubation, the cells spread out within another 40 min at 25°C. Adjusting the medium to

contain 10 mM NaF immediately before the addition of glucose prevented cell spreading. NaF did not block cell spreading in the absence of the uncouplers. For reasons similar to those discussed above with regard to the effects of azide and dinitrophenol on EGF-induced rounding, it appears that ATP production is required for the spreading process.

Like EGF-induced rounding, cell spreading does not appear to require protein synthesis. If cells are rounded by treatment with EGF (100 ng/ml) in CMF at 25°C, subsequent addition of both cycloheximide (20 µg/ml) and 1 mM CaCl₂ to the medium permits cell spreading to occur at the same rate as when CaCl₂ alone is added.

Cell spreading is largely blocked by adding cytochalasin B (10 µg/ml) to the medium at the time of Ca⁺⁺ addition, but not by adding colchicine (10⁻⁵ M). Spreading does not occur at 0°C.

Thus, the pharmacological sensitivities of the cell-spreading process are similar if not identical to those of the EGF-induced rounding process. Both processes are temperature dependent, appear to be energy dependent, and are cytochalasin sensitive; neither are affected by cycloheximide or colchicine. Both cell spreading and cell rounding appear to be active processes that involve the system of actin containing microfilaments.

Protease-induced Rounding of A-431 Cells

Because proteases such as trypsin are known to induce cell rounding (12, 32, 36), we investigated the possible role of proteases in the EGF-induced rounding process.

Like EGF, trypsin (5 µg/ml) causes A-431 cells in CMF to round up within 20 min at 25°C. In experiments identical to those described above for EGF-induced rounding, it was determined that trypsin-induced rounding may be blocked or reversed by 1 mM Ca⁺⁺. In addition, the trypsin-induced rounding process is reversibly inhibited by azide or dinitrophenol, and is partially blocked by cytochalasin B, as determined in experiments identical to those with EGF. Thus, a number of similarities exist between the rounding processes induced by EGF and trypsin.

The possibility that EGF causes cell rounding by stimulating secretion of a protease was examined by a medium-transfer experiment. Cells were rounded up by treatment with 100 ng EGF in 1 ml CMF for 20 min at 25°C. EGF in the medium was then inactivated by addition of an excess (1.4 mg) of DEAE-purified antiserum to EGF. This medium was then transferred to a dish of untreated cells. However, this medium from EGF-treated cells did not cause cell rounding, as monitored by phase-contrast microscopy. In a control experiment, DEAE-purified serum (1.8 mg) was used in place of anti-EGF; this control medium was able to stimulate rounding in a fresh dish of cells, presumably by virtue of the EGF present. This experiment suggests that EGF-induced rounding is not mediated by a secreted protease, but it does not rule out the involvement of a cell-associated protease in the rounding process.

However, experiments using inhibitors of serine proteases suggest that EGF-induced rounding is not mediated by such an enzyme. Soybean trypsin inhibitor (1 mg/ml), bovine pancreatic trypsin inhibitor (1 mg/ml), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (10 µg/ml), L-1-tosylamide-α-phenylethyl chloromethyl ketone (10 µg/ml), phenylmethylsulfonyl fluoride (1 mM), and *p*-nitrophenyl-guanidobenzoate (0.5 mM) all failed to inhibit the EGF-induced rounding process in the cell rounding assay.

In summary, although fundamental similarities may exist between EGF-induced and protease-induced cell rounding, no evidence has been obtained indicating that EGF-induced rounding involves the action of a protease.

Lack of Effect of Cyclic Nucleotides on Cell Rounding

Because cyclic nucleotides have been reported to induce flattening (21, 39) or rounding (23, 28, 29, 31, 43) of cells growing in tissue culture, we investigated the possibility that EGF caused cell rounding by altering cyclic nucleotide levels. We tested the effects of the 8-bromo derivatives of cyclic AMP and cyclic GMP at concentrations of 0.1 mM and 1 mM, using our standard rounding assay. These substances neither caused cell rounding nor blocked EGF-induced cell rounding, as observed by phase-contrast microscopy after a 20-min incubation at 25°C. Identical results were obtained when the cyclic nucleotide analogues were tested in the presence of the cyclic nucleotide phosphodiesterase inhibitor methyl isobutylxanthine (1 mM). Thus, it appears unlikely that cyclic nucleotides are involved in the rounding response to EGF.

DISCUSSION

EGF induces rapid morphological changes in A-431 human epidermoid carcinoma cells in monolayer culture. Although the final cell shape obtained after treatment with EGF depends on whether or not Ca^{++} is present in the medium, ruffling and then retraction from the substrate are observed within minutes of addition of EGF to the medium in either the presence (8) or the absence of Ca^{++} . In the absence of Ca^{++} , rapid cell rounding is then observed, whereas in the presence of Ca^{++} , a more complex series of morphological changes ensues (8).

Because EGF induces rapid cell rounding only in the absence of Ca^{++} , the role of Ca^{++} in this process is of interest. Rounding does not appear to be the result of a Ca^{++} flux, as the Ca^{++} ionophore A23187 can neither cause cell rounding nor block it. Treatment of A-431 cells with EGF may result in decreased cellular adhesiveness to the substrate such that maintenance of a flattened state becomes increasingly dependent on extracellular Ca^{++} . This loss of adhesiveness might account for the gradual retraction from the substrate observed even in the presence of Ca^{++} , and for the rapid rounding observed in Ca^{++} -free medium. This possibility is further supported by the observation that A-431 cells treated with EGF in the presence of Ca^{++} are more prone to artifactual detachment during extraction with detergent or processing for electron microscopy than are untreated cells.

It is of interest to compare our results with those of Lawrence et al. (23), who reported that treatment of cultured ovarian granulosa cells with FSH led to cell rounding after 1 h at 37°C. Although this cell rounding process resembles the EGF-induced rounding of A-431 cells in its apparent requirement for ATP and its independence of protease action, protein synthesis, Ca^{++} fluxes, and microtubule function, the responses differ in several important respects: (a) Unlike EGF-induced rounding, FSH-induced rounding may occur in the presence of Ca^{++} . (b) FSH-induced rounding may be mimicked by treatment with cytochalasin B. Cytochalasin B inhibits EGF-induced rounding. (c) Cyclic AMP, which appears to mediate FSH-induced rounding of granulosa cells, appears to be uninvolved in the EGF-induced rounding of A-431 cells. Thus, although these hormones may produce grossly similar morphological changes

in their target cells, their mechanisms of action seem quite different.

Carpenter et al. (6, 7) have reported that EGF rapidly stimulates phosphorylation of isolated A-431 membranes by an endogenous cAMP-independent protein kinase. We speculate that a phosphorylation event may be responsible for the morphological alterations induced by EGF. It may also be speculated that in systems in which cell rounding is mediated by cAMP, cAMP-dependent protein kinases may be involved. The hypothesis that morphological changes may be induced by changes in the state of phosphorylation of cytoskeletal proteins is consistent with the energy dependence of the shape changes discussed above, as well as with observations that different states of phosphorylation of spectrin relate to changes in erythrocyte shape (3), that full activity of nonmuscle myosin requires phosphorylation of the myosin light chain (1), and that several other cytoskeletal proteins may be phosphorylated (4, 27, 34, 37). The observation that the product of the *src* gene of avian sarcoma virus, which induces ruffling and cell rounding in chick fibroblasts (2, 38), is a protein kinase (10) is further evidence that cell shape may be regulated by protein phosphorylation/dephosphorylation reactions.

Because both the *src*-coded kinase (20, 11) and the EGF-stimulated kinase from A-431 cells (35) specifically phosphorylate tyrosine, it is possible that phosphorylation of tyrosine in certain cytoskeletal proteins may be responsible for ruffling and rounding induced either by viral transformation or by EGF.

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