Lack of Correlation between Changes in Polyphosphoinositide Levels and Actin/Gelsolin Complexes in A431 Cells Treated with Epidermal Growth Factor

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Abstract. The polyphosphoinositides, PIP and PIP₂, have been proposed to regulate actin polymerization in vivo because they dissociate actin/gelsolin complexes in vitro. We tested this hypothesis by comparing the ability of EGF and bradykinin to affect PI metabolism and the actin cytoskeleton in A431 cells. EGF, but not bradykinin, was found to induce ruffling and dissociation of actin/gelsolin complexes in these cells. However, both EGF and bradykinin stimulated the accumulation of inositol phosphates in [³H]inositol-labeled cells indicating that stimulation of PI turnover is not sufficient for the induction of changes in actin/gelsolin complex levels. EGF stimulated a twofold increase in the level of PIP in A431 cells. Other phosphoinositide levels were not markedly altered. Treatment of the cells with cholera toxin abrogated the EGF-induced rise in PIP levels without altering the dissociation of actin from gelsolin. These data indicate that increases in PIP and/or PIP₂ are not necessary for dissociation of actin/gelsolin complexes. Overall, these experiments suggest that in A431 cells, the effects of EGF on the actin cytoskeleton are unlikely to be mediated through changes in PIP or PIP₂ levels.

The stimulation of phosphoinositide turnover has been shown to be a central event in the transduction of signals via receptors for a variety of agents. Hormone-stimulated cleavage of the polyphosphoinositide, PIP₂, yields two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (29). Diacylglycerol activates protein kinase C (21) while inositol 1,4,5-triphosphate induces the release of calcium from intracellular stores (3). For many hormones, the production of these two second messengers is thought to be the principal mechanism used by the hormone to bring about changes in the functions of intracellular proteins and enzymes.

Recently, several reports have appeared that have contradicted the current dogma that the only metabolic effects of phosphoinositides are due to their role as substrates for breakdown by phospholipase C. For example, the activity of the sarcoplasmic reticulum Ca²⁺-ATPase appears to be stimulated directly by binding of PIP or PIP₂ (15). The function of two cytoskeletal proteins; profilin and gelsolin, also appears to be regulated by polyphosphoinositides. Profilin forms a 1:1 complex with actin monomers inhibiting polymerization (32). Polyphosphoinositides dissociate the profilin/actin complexes, promoting actin polymerization (25, 27). Gelsolin is an 87-kD protein that severs, caps, and nucleates actin filaments in vitro in a calcium-dependent fashion. Each of these processes results in gelsolin bound to the barbed end of an actin filament (32). This actin/gelsolin complex is stable to EGTA but is dissociated by polyphosphoinositides (6, 7, 20, 23).

The effects of polyphosphoinositides on profilin and gelsolin in vitro may serve to regulate actin polymerization in vivo. Treatment of platelets with thrombin results in the stimulation of PI turnover (33, 44) and is associated with changes in actin filament concentration and distribution (16, 27). Lind et al. (27) demonstrated that in thrombin-stimulated platelets gelsolin and profilin are rapidly converted from a free form to one in which they are bound to actin in high affinity 1:1 complexes. After several minutes, these complexes dissociate and the gelsolin and profilin are once again free in the cytosol. More recent work by Hartwig et al. (17) suggests that thrombin may induce the association of gelsolin with the cell membrane, positioning it in proximity to the regulatory polyphosphoinositides.

Based on these findings, a model has been proposed to explain the thrombin-induced changes in the actin cytoskeleton of platelets (27, 28, 38). In this model, gelsolin is first activated via the rapid increase in intracellular calcium resulting from the breakdown of PIP₂ and the generation of inositol 1,4,5-trisphosphate. Gelsolin then caps, nucleates, or severs actin filaments resulting in the formation of actin/gelsolin complexes in which gelsolin is bound to the barbed end of actin filaments. A subsequent rise in polyphosphoinositide levels due to resynthesis of metabolized lipids releases the...
gelsolin from the actin filaments, permitting actin polymerization at the newly created free barbed ends. Consistent with this model is the observation that the membranes of neutrophils exhibit an increased ability to nucleate actin polymerization after treatment with a chemotactic peptide (8).

EGF induces membrane ruffling (12) and a change in the distribution of the actin-based cytoskeleton in A431 cells (35), a human epidermoid carcinoma cell line. EGF also stimulates turnover of phosphoinositides, activates a PI kinase, and induces a rise in the levels of PIP (31, 43). In light of these observations, we tested the hypothesis that EGF-induced changes in polyphosphoinositide levels affect the actin cytoskeleton and cell motility by altering actin/gelsolin and actin/profilin interactions. We report here that although EGF induces an increase in PIP levels and a concomitant decrease in actin/gelsolin complexes, the two effects of the growth factor can be completely dissociated in cholera toxin–treated cells. In addition, bradykinin stimulates more extensive PI turnover than EGF but does not cause dissociation of actin/gelsolin complexes indicating that breakdown of polyphosphoinositides is not always associated with changes in the actin cytoskeleton.

Materials and Methods

Materials

EGF was purified according to the method of Savage and Cohen (34). Myo-[3H]inositol was from American Radiolabeled Chemicals, Inc., St. Louis, MO. Rhodamine-phalloidin was from Molecular Probes Inc., Eugene, OR. Anti-gelsolin monoclonal antibody 2C4 (9) (Sigma Chemical Co., St. Louis, MO) was purified and coupled to CNBr-Sepharose (5). Rabbit plasma gelsolin was a generous gift of Dr. Carl Frieden (Washington University, St. Louis, MO).

Cell Culture

A431 cells were maintained in Dulbecco’s modified Eagle’s medium containing 3% fetal calf serum and 7% newborn calf serum. For all experiments, cells were cultured so as to be 60–90% confluent at the time of use.

Fluorescence Microscopy of A431 Cells Stained with Rhodamine-Phalloidin

A431 cells grown on glass coverslips were incubated with EGF or bradykinin at 37°C for various times. Cells were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 5 min, and blocked by a 30-min incubation in PBS containing 1% bovine serum albumin and 10 mM Tris, pH 7.2. The cells were incubated with 0.165 μM rhodamine-phalloidin in PBS for 10 min, washed three times with PBS, and mounted in PBS. A Zeiss IM-35 microscope with a 40x Planapo phase-contrast objective, NA 0.65, was used for both phase and fluorescence microscopy.

Assay for Cell Content of Filamentous Actin

This assay is modified from that of Howard and Oresajo (19). A431 cells in 96-well micro-titer plates were incubated with EGF or bradykinin at 37°C. The cells were fixed with 3.7% formaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. The fixed, permeabilized cells were incubated with 100 μl of 1.65 μM rhodamine-phalloidin in PBS for 15 min which was sufficient to saturate the filamentous actin in these samples. The wells were washed three times with PBS and dissolved in 0.5 ml 0.1 N NaOH and the fluorescence of each sample was determined using an SLM8000 fluorimeter (SLM Instruments, Inc., Urbana, IL).

Immunoprecipitation of EGTA-stable Actin/Gelsolin Complexes

Actin/gelsolin complexes were measured as described (9). A431 cells grown on 150-mm culture dishes were incubated with 30 nM EGF or 10 μM bradykinin at 37°C and then extracted with Triton extraction buffer (9). Immunoblots showed that all the gelsolin from both control and hormone-treated cells was in the soluble fraction. This supernatant was mixed with 20 μl of anti-gelsolin antibody-Sepharose beads and incubated for 1–2 h at 4°C. The beads were washed once with Triton extraction buffer containing 5 mM ATP, and then washed three times with 0.5 ml of 50 mM Tris, pH 7.4, 0.5 M NaCl, 1 mM EGTA, and 0.3 M MgCl2. Proteins associated with the beads were analyzed by SDS-PAGE (24) except that piperazone diacyrlamide cross-linker was substituted for the N,N-dimethyl-bis-acrylamide cross-linking agent to reduce background upon silver staining. The positions of the actin and gelsolin bands were determined by comigration with authentic samples of these proteins and by Western blotting with anti-actin and anti-gelsolin antibodies. Actin and gelsolin protein bands were quantitated by scanning laser densitometry. The molar ratio of actin to gelsolin was estimated by comparing the ratio of the absorbance of the actin band to that of the gelsolin band with the same ratio obtained under conditions which promoted the formation of a 1:1 complex of actin/gelsolin. 1:1 complexes of actin/gelsolin were prepared by lysing cells and incubating the Triton-soluble fraction with anti-gelsolin antibody-Sepharose in the presence of 5 mM Ca2+ followed by washing the beads with buffer containing EGTA.

Isolation and Quantitation of Profilin/Actin Complexes

Profilin/actin complexes were isolated and analyzed using polyproline Sepharose as described (27). The ratio of profilin to actin was calculated assuming that on a per microgram basis profilin Coomassie blue stains with 90% the intensity of actin (37).

Measurement of Inositol Phosphates and Inositol Phospholipids

A431 cells were grown in 35-mm plates and labeled overnight in Dulbecco’s modified Eagle’s medium containing 2 μCi/ml myo-[3H]inositol, 25 mM Hepes, pH 7.4, 7% dialyzed newborn calf serum, and 3% dialyzed fetal calf serum. 30 min before treatment with hormone, 10 mM LiCl was added to the cultures. EGF (30 nM) or bradykinin (10 μM) were added to the cultures for the times indicated and the cells incubated at 37°C. For inositol phosphate analysis, the incubations were terminated by aspirating the medium from the cultures and precipitating the monomers with cold 5% trichloroacetic acid. Samples were then analyzed by chromatography on Dowex columns as described previously (13). For analysis of inositol phospholipids, incubations were terminated by aspirating the medium and adding 2.25 ml methanol/conc. HCl (1/10). Lipids were extracted with chloroform and analyzed by thin layer chromatography as described (31).

Results

Morphological Changes in A431 Cells

As shown in Fig. 1, unstimulated A431 cells exhibited a spread morphology with a thin peripheral region of cytoplasm that lacks vesicles. Occasional ruffles were observed at the periphery of cells. Within 30 s after the addition of EGF, the size and number of ruffles increased. The response peaked between 2 and 5 min. By 10 min the ruffling had subsided and the cell margins had begun to retract. Fixation of the cells followed by staining with rhodamine-phalloidin confirmed the presence of filamentous actin in these ruffles (data not shown). By contrast, no change in the extent of cell ruffling was observed for up to 20 min after the addition of bradykinin.

Changes in the Actin Cytoskeleton

To determine whether EGF or bradykinin stimulated changes
in the association of actin with gelsolin, EGTA-stable actin/ gelsolin complexes from hormone-treated A431 cells were isolated as described in Materials and Methods. Fig. 2 shows a silver-stained SDS–polyacrylamide gel of the complexes present in A431 cells treated with EGF. While the level of gelsolin was relatively constant throughout the time course, a decrease in the level of actin, which is proportional to the level of actin/gelsolin complexes, was apparent as early as 15 s after the addition of EGF. The levels of actin remained low for the remainder of the time course.

EGF, but not bradykinin, decreased the level of actin/gelsolin complexes (Fig. 3). In untreated control cells, the average molar ratio of actin/gelsolin was 0.5 but ranged from 0.3 to 0.9 in different experiments. EGF induced a rapid and sustained 40–50% decrease in the level of EGTA stable actin/gelsolin complexes. Paired t-test analysis of the five experiments with EGF indicated that the observed changes in actin were significant at the $P < 0.05$ level. Bradykinin stimulated no consistent change in the level of the actin/gelsolin complexes in A431 cells. Additional experiments demonstrated that treatment of A431 cells with bradykinin for 10, 20, or 30 s did not result in a decrease in actin/gelsolin complexes (data not shown). Thus, bradykinin induced neither short-term nor long-term changes in actin/gelsolin complex levels.

Using polyproline agarose to isolate profilin and as-

Figure 2. Effect of EGF on EGTA stable actin/gelsolin complexes in A431 cells. A431 cells were treated with 30 nM EGF for the times indicated and Triton X-100-soluble extracts prepared as described in Materials and Methods. The free gelsolin and actin/gelsolin complexes were isolated by adsorption to anti-gelsolin antibody-coupled Sepharose. The immunoprecipitates were analyzed by SDS-PAGE and the protein bands visualized by silver staining. The antibody only lane is a control in which the anti-gelsolin antibody-coupled Sepharose beads have not been exposed to cell extract before electrophoresis. The positions of the actin and gelsolin bands are indicated by $A$ and $G$. Numbers on the right refer to the positions of molecular mass markers in kilodaltons.

Figure 1. The effect of EGF (left) and bradykinin (right) on A431 cell morphology. A431 cells were grown on coverslips and maintained at 37°C in Dulbecco’s modified Eagle’s medium during microscopy. Numbers in the upper left corner indicate the time in minutes after the addition of 30 nM EGF or 10 μM bradykinin. Bar, 10 μm.
EGF and bradykinin elicited markedly different effects on the levels of phosphoinositides in A431 cells. As shown in Fig. 6, left EGF stimulated a significant increase in the level of PIP (31) that peaked at 2 min, well after the changes in actin/gelsolin complexes or F-actin content. The levels of PI increased slightly over the time course but the levels of PIP2 remained essentially constant. Bradykinin failed to significantly alter the levels of either PI or PIP, although there was a small, transient decrease in the level of PIP2 (Fig. 6, right).

**Effect of Cholera Toxin on PIP and Actin/Gelsolin Complex Levels**

We have reported previously that cholera toxin ablates the ability of EGF to stimulate a rise in PIP levels in A431 cells but does not alter the ability of the hormone to stimulate PI turnover (31). Treatment of cells with cholera toxin blocked

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**Figure 3.** Time course of the effect of EGF and bradykinin on EGTA-stable actin/gelsolin complexes. Triton-soluble extracts were prepared from A431 cells incubated with 30 nM EGF (A) or 10 μM bradykinin (B) for the times indicated. EGTA-stable actin/gelsolin complexes were immunoprecipitated from the extracts and analyzed by SDS polyacrylamide gel electrophoresis. The data are presented as the percent change in the actin/gelsolin ratio and represent the mean ± standard error of five separate experiments for EGF and four separate experiments for bradykinin.

**Figure 5.** Time course of the effect of EGF and bradykinin on inositol phosphate accumulation in A431 cells. A431 cells were labeled for 16-20 h with myo-[3H]inositol. Cells were then incubated with 30 nM EGF (left) or 10 μM bradykinin (right) for the times indicated. The soluble inositol phosphates were then isolated and analyzed as described in Materials and Methods. Values represent the mean ± standard deviation of quintuplicate determinations from a representative experiment.

**Figure 4.** Time course of the effect of EGF and bradykinin on F-actin content of A431 cells. A431 cells grown in 24-well dishes were incubated with 30 nM EGF or 10 μM bradykinin for the times indicated. The cells were then fixed and permeabilized and the binding of rhodamine-phalloidin assessed as described in Materials and Methods. Values represent the mean ± standard deviation of data from two separate experiments performed in quadruplicate.

**Figure 6.** Time course of the effect of EGF and bradykinin on phosphoinositide levels in A431 cells. A431 cells were labeled for 16-20 h with myo-[3H]inositol. Cells were then incubated with 30 nM EGF (left) or 10 μM bradykinin (right) for the times indicated. The inositol phospholipids were then isolated and analyzed as described in Materials and Methods. Values represent the mean ± standard deviation of quintuplicate determinations from a representative experiment.
the ability of EGF to stimulate a rise in PIP levels (Fig. 7A) but did not affect the ability of EGF to induce dissociation of actin/gelsolin complexes (Fig. 7B). Microscopic observation of control and cholera toxin-treated cells demonstrated that both cultures ruffled in response to EGF (data not shown).

**Discussion**

Changes in polyphosphoinositide levels have been proposed to mediate hormone-induced alterations in the cytoskeleton of platelets (27), neutrophils (18), and macrophages (10, 17) through alterations in actin/gelsolin interactions. In vitro, gelsolin can cap, nucleate, or sever actin and each process results in gelsolin bound to the barbed end of an actin filament. Actin/gelsolin complexes, as detected in our assay, are most likely derived from gelsolin bound to the barbed end of actin filaments in cells. Release of gelsolin from the barbed ends is detected in our assay as a decrease in levels of actin/gelsolin complexes. This creates a free barbed end that can grow or shrink and a free gelsolin that may be reactivated by loss of bound phosphoinositide.

We investigated the effects of EGF and bradykinin on both phosphoinositide metabolism and changes in the actin cytoskeleton in A431 cells, a cultured cell system. Our data on the actin cytoskeleton in A431 cells are in general agreement with the findings in other systems (10, 17, 18, 27). EGF altered the motility and shape of A431 cells, increased the F-actin content, and decreased the actin/gelsolin complex level. Our findings are consistent with the hypothesis that the dissociation of gelsolin from the barbed ends of actin fila-

**Figure 7.** Effect of cholera toxin treatment on the ability of EGF to stimulate changes in the levels of PIP (A) and actin/gelsolin complexes (B). For determination of PIP levels, A431 cells were labeled with [³H]inositol for 16-20 h. Half the cultures were treated with 5 µg/ml cholera toxin for 3 h at 37°C. Half of the control and cholera toxin–treated cultures were then incubated for 4 min with 30 nM EGF. The inositol phospholipids were isolated and analyzed as described in Materials and Methods. Data shown represent the mean ± standard deviation of sextuplicate determinations from a representative experiment. For determination of actin/gelsolin complex levels, cultures of A431 cells were treated in the absence or presence of 5 µg/ml cholera toxin for 3 h at 37°C and then half of the cultures were treated with 30 nM EGF for 4 min. Triton-soluble extracts were prepared from the cultures and actin/gelsolin complexes isolated and analyzed as described in Materials and Methods. Data shown represent mean ± standard deviations from triplicate determinations from a representative experiment.

Despite the fact that only EGF stimulated a change in the level of actin/gelsolin complexes, both EGF and bradykinin stimulated PI turnover in A431 cells. Thus, there appears to be no direct correlation between the ability of a hormone to stimulate the production of diacylglycerol and inositol trisphosphate and its ability to induce dissociation of gelsolin from actin. The fact that EGF, but not bradykinin, induced changes in actin/gelsolin complexes argues against the hypothesis that flux through the PI pathway is sufficient to induce changes in these complexes. If this were the case, bradykinin would be expected to be more effective than EGF in inducing complex dissociation since the former stimulates a more rapid and robust turnover of PI.

Examination of the inositol phospholipid levels in cells stimulated with EGF demonstrated that the levels of PIP increased during the time that actin-gelsolin complexes were becoming dissociated. However, the effect of EGF on polyphosphoinositide levels was somewhat slow compared to its effect on actin/gelsolin complexes. In addition, the effects of EGF on PIP levels could be dissociated from the effects of this hormone on actin/gelsolin complexes by treatment of cells with cholera toxin. This toxin blocked the ability of EGF to induce the rise in PIP levels without altering the effects of the growth factor on actin/gelsolin complexes. Although effects mediated through changes in lipid aggregation and/or localization cannot be ruled out, the most straightforward interpretation of these data is that the ability of EGF to induce dissociation of actin/gelsolin complexes is not related to the ability of this growth factor to stimulate changes in PIP or PIP₂ levels.

Several alternative hypotheses can be considered. It is possible that the effect of EGF may be mediated by another form of polyphosphoinositide such as the recently discovered phosphatidylinositol 3,4,5-trisphosphate. This compound has been identified in activated neutrophils (39), thrombin-stimulated platelets (22), CSF-1–stimulated macrophage cell lines (42), and PDGF-stimulated smooth muscle cells (2). Consistent with this possibility is the recent report of a correlation between increases in phosphatidylinositol trisphosphate levels and actin polymerization in chemoattractant-stimulated neutrophils (14). Alternatively, the EGF receptor is known to possess intrinsic tyrosine protein kinase activity (40, 41) that is required for signal transduction (11, 28). Bradykinin is not known to stimulate the phosphorylation of proteins on tyrosine residues. Given the differences between the response of A431 cells to EGF and bradykinin, it is possible that the EGF-induced changes in the actin cytoskeleton involve the phosphorylation on a tyrosine of a cytoskeletal protein. Several cytoskeletal proteins including vinculin (36), spectrin (1, 4), talin (30), and ezrin (4) are known to be substrates for tyrosine kinases. Further experiments will be required to distinguish between these two hypotheses.
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