cGMP-dependent ADP depolymerization of actin mediates estrogen increase in cervical epithelial permeability

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Gorodeski, George I. cGMP-dependent ADP depolymerization of actin mediates estrogen increase in cervical epithelial permeability. Am J Physiol Cell Physiol 279: C2028–C2036, 2000.—Estrogen increases secretion of cervical mucus in women, and the effect depends on fragmentation of the cytoskeleton. The objective of the present study was to understand the molecular mechanism of estrogen action. Treatment of human cervical epithelial cells with 17β-estradiol, sodium nitroprusside (SNP), or 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) increased cellular monomeric G-actin and decreased polymerized F-actin. The effects of estradiol were blocked by tamoxifen, by the guanylate cyclase inhibitor LY-83583, and by the cGMP-dependent protein kinase inhibitor KT-5823. The effects of SNP were blocked by LY-83583 and KT-5823, while the effects of 8-Br-cGMP were blocked only by KT-5823. Treatment with phalloidin decreased paracellular permeability and G-actin. Treatment with 17β-estradiol, SNP, or 8-Br-cGMP attenuated SNP-induced phosphorylation of [32P]adenylate NAD in vitro: tamoxifen blocked the effect of estrogen; LY-83583 blocked the effect of SNP but not that of 8-Br-cGMP, while KT-5823 blocked effects of both SNP and 8-Br-cGMP. These results indicate that estrogen, nitric oxide (NO), and cGMP stimulate actin depolymerization. A possible mechanism is NO-induced, cGMP-dependent protein kinase augmentation of ADP-ribosylation of monomeric actin.

paracellular permeability; transepithelial transport; cervical mucus; nitric oxide; guanosine 3′,5′-cyclic monophosphate; monomeric G-actin

CERVICAL EPITHELIAL CELLS regulate secretion of the cervical mucus. The cervical mucus is important for reproduction and for a woman’s health (16). The major component of the cervical mucus, the water-soluble cervical plasma, originates by transudation of fluid and solutes from the blood into the cervical canal via the paracellular pathway (16). Estrogens increase secretion of cervical plasma in women (16). Studies using cultures of cervical epithelial cells have shown that estrogen increases epithelial paracellular permeability by decreasing the resistance of the lateral intercellular space (R_{LIS}) (17, 18). The R_{LIS} is determined by the proximity of the plasma membranes of neighboring cells and by the length of the intercellular space from the tight junctions to the basal lamina (28, 48). In an intact epithelium, these functions depend on the ability of epithelial cells to change their size in response to stimuli (17, 27).

The cytoskeleton determines the ability of cells to change their shape in response to environmental and intrinsic stimuli. Cells with a flexible cytoskeleton can conform more readily to decreases in size compared with cells with a rigid cytoskeleton (19, 45). Actin filaments, called F-actin, are the major component of the cytoskeleton in eukaryotic cells, and they determine cell structure (19). Actin filaments are made of oriented globular monomeric G-actin molecules that are maintained in a dynamic state of remodeling (9, 33, 41, 43). The density of actin filaments depends on equilibrium between polymerization of monomeric G-actin and depolymerization of filamentous F-actin (4, 10, 11, 13, 37, 42, 49). Actin filaments are polar molecules with two different ends: a slow-growing “pointed end” and a faster growing “barbed end”. During actin polymerization, the growth of the barbed end is balanced at steady state by loss of actin molecules at the pointed end. Thermodynamically, actin polymerization is preferred to actin depolymerization, but most cells possess at least two mechanisms to inhibit filament formation. Filament nucleation sites might be blocked so that the monomers have nothing to grow off, or actin monomers might be sequestered in a nonpolymerizable form so that filaments have nothing to grow with (4, 10, 11, 13, 37, 42, 49). Enhanced polymerization of G-actin to form F-actin is usually associated with a more dense and rigid cytoskeleton, whereas depolymerization of F-actin is associated with a more dynamic cytoskeleton. In human cervical epithelial cells, estrogen increases G-actin, suggesting that the estrogen increase in permeability involves fragmentation of the cytoskeleton (18).

The effect of estrogen on permeability is mediated by nitric oxide (NO) and cGMP (19). At the present time, relatively little is known about the molecular mechanism of estrogen action on G-actin. Previous studies showed that NO can increase G-actin by stimulating ADP-ribosylation of monomeric actin (3, 5, 6, 29, 38,
ADP-ribosylated actin is no longer capable of polymerization, but it can act as a capping protein that binds to the barbed ends of actin filaments to inhibit polymerization (50). This sequesters G-actin and reduces its intracellular pool for polymerization. The net effect is a decrease in F-actin content (12) and fragmentation of the cytoskeleton.

Since estrogen increases NO activity (19), it is possible that NO directly stimulates sequestration of G-actin. However, the effect of NO on permeability in human cervical epithelial cultures depends on cGMP and on cGMP activation of cGMP-dependent protein kinase (19). Those findings do not support a direct role for NO in the modulation of actin; instead, they suggest that the ADP-ribosylation of monomeric actin is triggered by cGMP-dependent protein kinase. The objective of this study was to determine the mechanism by which NO and/or cGMP mediate the estrogen-induced increase in G-actin.

**METHODS**

**Cell cultures.** Two types of cell cultures were used. 1) Human ectocervical epithelial cells (hECE), a model of the stratified ectocervical epithelium, were used. Cells were obtained from minces of ectocervix and used in third passage (26). 2) CaSki cells, a stable line of transformed cervical epithelial cells that express phenotypic markers of the endocervix (26), were also used. Cells were grown and maintained in a culture dish at 37°C in a 91% O2-9% CO2 humidified incubator and plated on filters for experiments (26). Cells were routinely tested for mycoplasma. For experiments with estrogen, cells on filters were shifted to steroid-free medium for 3 to 5 days (18). Before experiments, filters that contained cells were washed three times and preincubated for 15 min at 37°C in a modified Ringer buffer (26).

Changes in paracellular permeability were determined in terms of changes in transepithelial electrical conductance. Changes in transepithelial electrical conductance (GTE) were determined continuously across filters mounted vertically in a modified Ussing chamber, from successive measurements of transepithelial electrical current (ΔI), and of the transepithelial potential difference (ΔPD, lumen negative): GTE = ΔI/ΔPD. Thorough descriptions of the method, including conditions for optimal determinations of GTE across low-resistance epithelia, calibrations and controls, potential pitfalls, and appropriate measures to prevent artifacts were published (25). All reagents used for the Ussing chamber experiments were added from concentrated stocks (×300–1,000) of either 1% ethanol, dimethyl sulfoxide, or saline to both the luminal and subluminal solutions.

**DNase I inhibition assay.** Cells on filters were lysed in situ, and deoxyribonuclease (DNase) I activity in the lysate was assayed by measuring DNase I-dependent degradation of DNA as described (18). Total actin was measured by the guanidine-HCl method after depolymerization of F-actin to monomeric G-actin (18).

**Rhodamine-phalloidin assay for F-actin.** F-actin was quantified in cells attached on filters by binding of rhodamine-phalloidin to actin filaments in permeabilized and formaldehyde-fixed cells (46). Cells grown on filters were washed three times with stabilization buffer [75 mM KCl, 3 mM MgSO4, 1 mM EGTA, 0.2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM imidazole, and 10 mg Aprotinin/mL, pH 7.2] and permeabilized with 0.03% saponin in stabilization buffer for 10 min at room temperature. Cultures were fixed in freshly prepared 3% formaldehyde in stabilization buffer for 20 min at room temperature, washed twice, and stained in the dark with 0.175 μg/ml rhodamine-phalloidin in stabilization buffer for 30 min. After washing three times with stabilization buffer, extraction of rhodamine-phalloidin was initiated by adding ice-cold HPLC-grade methanol for 30 min at −20°C. Thereafter, the cells were scraped off with a rubber policeman and extraction was continued overnight at −20°C. Aliquots were obtained for measurements of DNA, and the suspension was centrifuged for 10 min at 10,000 g. Rhodamine in the supernatant was determined by means of a rhodamine-phalloidin standard curve (30, 46), using an Aminco-Bowman spectrophotofluorometer (542 nm/563 nm excitation-emission ratio), and expressed per milligram of cellular DNA. Total cellular actin content was determined in lysates of cells in parallel filters by measuring the optical density at 290 nm. Actin content was calculated by using the extinction coefficient ε290 = 26,460 mol−1·cm−1 (47). Positive fluorescence stain of cells was visually verified using Zeiss epifluorescence microscopy.

**Actin ADP-ribosylation assay.** Cells on filters were harvested, resuspended in iced phosphate buffer, and homogenized in a Dounce homogenizer (B-pestle; Kontes, Vineland, NJ). Lysates were spun for 10 min at 4°C at 10,000 g, and 100 μl of the supernatant was incubated with 200 μl buffer (10 mM triethanolamine-HCl, pH 7.5, 10 mM thymidine, 2 mM EDTA, 10 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml pepstatin) that contained 3 μM [32P]adenylate NAD plus 1 mM sodium nitroprusside (SNP) for 30 min at 37°C. [32P]Adenylate ADP-ribosylated cytoplasmic actin was extracted by using DNase I beads. DNase I beads (10 μl) were added to the reaction mixture and rotated end-over-end for 60 min at 4°C. Beads were washed with PBS, and actin was released with 0.1% SDS. The released actin was boiled in sample buffer and subjected to SDS-PAGE in a 12% gel with subsequent autoradiography, as we have described (22). The amount of actin per lane was normalized to milligrams of total protein. Preparation of DNase I beads (36): DNase I beads [5-ml latex beads (polystyrene beads, 2 μm, 25% wt/vol); Polysciences, Warrington, PA] were washed three times in 100% ethanol and three times in PBS and mixed with DNase I (10 mg dissolved in 0.2 M sodium phosphate, pH 7.5) and tresyl chloride-activated agarose (1 g, washed with 0.2 M sodium phosphate). The mixture was incubated at 4°C overnight with gentle mixing. Cellular DNA and total protein were measured as described (20, 22).

**Densitometry.** X-Ray films were analyzed with a laser densitometer Sciscan 5000 (United States Biochemical, Cleveland, OH).

**Statistical analysis of the data.** Data are presented as means ± SD, and significance of differences among means was estimated by ANOVA. Trends were calculated using GB-STAT version 5.3 (Dynamic Microsystems, Silver Spring, MD) and analyzed with ANOVA. Best fit of regression equations (least-squares criterion) was achieved with SlideWrite Plus (Advanced Graphics Software, Carlsbad, CA), which uses the Levenberg-Marquardt algorithm, and was analyzed using ANOVA.

**Chemicals and supplies.** Anocell (Anocell-10) filters were obtained from Anotec (Oxon, UK). Fluorescent microspheres (FluoresBrite beads, calibration grade) were obtained from Polysciences. [32P]Adenylate NAD was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).
**RESULTS**

*Estrogen increases the permeability of cultured cervical epithelia.* Baseline $G_{TR}$ levels across cultures of CaSki and hECE grown in steroid-free medium ranged from 30 to 48 mS·cm$^{-2}$ (Fig. 1, ~35 Ω·cm$^2$). These levels confirm our previous results (17, 18, 23, 26) and indicate that human cervical epithelial cells form a relatively permeable epithelium on filters (39). Treatment with 10 nM 17β-estradiol increased $G_{TR}$ across cultures of both cell types, indicating that treatment with physiological concentrations of estradiol increases the permeability of cultured human cervical epithelia.

$cGMP$ mediates the estrogen-induced increase in G-actin and permeability. In human cervical epithelial cells, the effect of estrogen on permeability involves the estrogen receptor α, NO, and cGMP (19, 24). Both NO and cGMP can upregulate G-actin and increase permeability (19), but relatively little is known about the mechanism of action of these mediators on actin steady state. The objectives of the first experiment were to test the degree to which NO and cGMP mediate the estrogen increase in G-actin and the degree to which the effect of NO depends on cGMP.

The strategy was to determine the degree to which increases in cellular cGMP are sufficient to increase G-actin and necessary for NO to increase G-actin and the permeability. Two groups of drugs were used in these experiments with known mechanisms of action in human cervical epithelial cells. The first group included agents that block the estrogen increase in permeability. They included tamoxifen (which blocks estrogen-dependent transcription of estrogen receptor α (24)), LY-83583 (which blocks guanylate cyclase), and KT-5823 (which blocks cGMP-dependent protein kinase (15, 19, 31, 35)). The second group included agents that bypass the estrogen receptor α and directly increase the permeability. They included SNP (a NO donor) and 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP; cell-permeable analog of cGMP). Changes in G-actin were expressed as the ratio of G-actin/total actin (18).

In cultures of CaSki and hECE grown in steroid-free medium, neither tamoxifen, LY-83583, nor KT-5823 had significantly modulated G-actin (Fig. 1). Treatment with 17β-estradiol, SNP, or 8-Br-cGMP increased G-actin (Fig. 1). Tamoxifen blocked the increase in G-actin induced by 17β-estradiol, but not by SNP or 8-Br-cGMP; LY-83583 blocked the increases in G-actin induced by 17β-estradiol and by SNP, but not by 8-Br-cGMP; KT-5823 blocked the increase in G-actin induced by 17β-estradiol, SNP, and 8-Br-cGMP (Fig. 1). 17β-estradiol, SNP, 8-Br-cGMP, tamoxifen, LY-83583, and KT-5823 had no significant effect on total cellular actin, which ranged 125 ± 12 pg/mg cellular DNA.

$cGMP$ mediates decreases in F-actin induced by estrogen and NO. In most cells, the density of actin filaments depends on equilibrium between polymerization of monomeric G-actin and depolymerization of filamentous F-actin (19, 45). Enhanced polymerization of G-actin to form F-actin is associated with a more dense and rigid cytoskeleton, whereas depolymerization of F-actin is associated with a more dynamic (fragmented) cytoskeleton (19, 45). The experiment shown in Fig. 1 utilized the DNase I inhibition test to determine changes in G-actin in response to treatments with 17β-estradiol, SNP, and 8-Br-cGMP. To confirm that 17β-estradiol, SNP, and 8-Br-cGMP modulate actin steady-state equilibrium, experiments were also done using the rhodamine-phalloidin binding assay to determine changes in F-actin. Phalloidin, a heptapeptide toxin from the poisonous mushroom *Amanita phalloides*, binds tightly and specifically to polymerized actin; it decreases the critical concentration for actin polymerization by reducing the dissociation rate constant of monomers from filament ends (10). The rhodamine-phalloidin binding assay is based on the high binding affinity of phalloidin to F-actin. Cells are permeabilized and exposed to a buffer with phalloidin-bound rhodamine. After lysis of cells, the amount of bound rhodamine is assayed fluorometrically, and it reflects cellular F-actin (10). Changes in F-actin were expressed as the ratio of F-actin/total actin.

In cultures of CaSki and hECE grown in steroid-free medium, neither tamoxifen, LY-83583, nor KT-5823 had a significant effect on F-actin (Fig. 2). Treatment with 17β-estradiol, SNP, or 8-Br-cGMP decreased...
Tamoxifen blocked the decrease in F-actin induced by 17β-estradiol, but not by SNP or 8-Br-cGMP; LY-83583 blocked the decrease in F-actin induced by 17β-estradiol and by SNP, but not by 8-Br-cGMP; KT-5823 blocked the decrease in F-actin induced by 17β-estradiol, SNP, and 8-Br-cGMP (Fig. 2). These results are similar, although in the opposite direction, to the effects of 17β-estradiol, SNP, 8-Br-cGMP, tamoxifen, LY-83583, and KT-5823 on G-actin (Fig. 1).

17β-estradiol, SNP, 8-Br-cGMP, tamoxifen, LY-83583, and KT-5823 had no significant effect on total cellular actin, as determined fluorimetrically by measuring the optical density at 290 nm. Levels of total cellular actin ranged 172 ± 12 pg/mg cellular DNA among the different experiments and were in the range observed using the DNase I inhibition test.

Collectively, the results shown in Figs. 1 and 2 confirm that 17β-estradiol, SNP, and 8-Br-cGMP fragment the cytoskeleton and support the hypothesis that NO-induced increase in cGMP and cGMP activation of cGMP-dependent protein kinase are critical steps in estrogen increase in G-actin.

Phalloidin blocks increases in G-actin and permeability. It was previously shown that increases in G-actin lead to increases in permeability (24). To clarify this effect, cells were treated with the cytoskeleton-modifying agent phalloidin (10). In cells treated with phalloidin, total cellular actin was not significantly different from control cells not treated with phalloidin (not shown), indicating that incubations with phalloidin up to 60 min do not significantly affect total actin (47). In contrast to a lack of an effect on total cellular actin, phalloidin decreased G-actin in a time- and concentration-related manner (Fig. 3). Addition of 10 ng/ml phalloidin to estrogen-treated cells decreased the ratio of G-actin/total cellular actin from 70 to 10% within 60 min (Fig. 3A, closed circles, P < 0.01). In cells grown in steroid-free medium, 10 ng/ml phalloidin decreased G-actin/total cellular actin from 35 to 10% (Fig. 3A, open circles, P < 0.01). The effect of phalloidin was concentration dependent: in estrogen-treated cells, decreases in the ratio of G-actin/total cellular actin began at 0.1 ng/ml phalloidin and reached saturation at ~10 ng/ml phalloidin (Fig. 3B). In estrogen-treated cells not treated with phalloidin, levels of the ratio of G-actin/total cellular actin remained stable at ~70% for the duration of the 60-min experiments (Fig. 3A, inverted triangles).

The experimental design shown in Fig. 3 was also used to determine the effects of phalloidin on G-actin in cells treated with SNP or 8-Br-cGMP and to correlate...
changes in G-actin with changes in permeability. As shown in Fig. 4, phalloidin lowered $G_{TE}$ and G-actin both in CaSki cells and in hECE, regardless of whether or not cells were treated with 17β-estradiol, SNP, or 8-Br-cGMP. Furthermore, phalloidin lowered $G_{TE}$ and G-actin to levels that were observed in cells grown in steroid-free medium for 5 days. After mounting the filters in an Ussing chamber, 1 mM SNP or 50 μM 8-Br-cGMP were added for 30 min before experiments. *Experiments done in the absence of phalloidin; △, cells treated with 10 nM phalloidin for 60 min before experiments. Following determinations of $G_{TE}$, filters were used for G-actin determinations as in Fig. 2. Total cellular actin was determined in parallel filters, and it measured 131 ± 11 pg/mg cellular DNA. Levels are means ± SD of 3–5 filters at each point.

ADP-ribosylation of actin in vitro. The objective of the next set of experiments was to test the hypothesis that estrogen increases G-actin by a mechanism that involves NO/cGMP-dependent ADP-ribosylation of monomeric actin. The background for the hypothesis was that ADP-ribosylation of monomeric actin could sequester G-actin and decrease its availability for polymerization (5, 6, 38, 44). The assay used in these experiments was NO-induced ADP-ribosylation in vitro, in broken cell preparations (5, 6, 38, 44). The reaction was initiated by adding the NO donor SNP and monitored by preincubation of cell lysates with $[^{32}P]$adenylate NAD, which is a substrate for ADP-ribosylation (8, 34).

To test the efficacy of this assay in human cervical epithelial cells, CaSki cells were lysed, and lysates were incubated with $[^{32}P]$adenylate NAD in the presence of 0.5 and 1 mM of the NO donor SNP. As shown in Fig. 5, SNP augmented in vitro phosphorylation of $[^{32}P]$adenylate NAD in a concentration-related manner. Densitometry analysis of four experiments revealed that $[^{32}P]$adenylate NAD phosphorylation in vitro was 3.1 ± 0.3- and 8.5 ± 0.5-fold greater in lysates of cells treated with 0.5 and 1 mM SNP, respectively, than in lysates not treated with SNP ($P < 0.01$). The results in CaSki cells are similar to studies in other cell types (5, 6, 8, 34, 38, 44) and indicate that the assay can yield semiquantitative data.

To confirm that the effect is the result of NO generated by the SNP, the experiment was repeated in the presence of the NO scavenger hemoglobin (7). Addition of 50 μM hemoglobin blocked the effect of SNP, and the degree of phosphorylation of $[^{32}P]$adenylate NAD in vitro was similar to that observed in lysates without the added SNP (Fig. 5). This result indicates that the

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**Fig. 4.** Effects of phalloidin on G-actin and on transepithelial electrical conductance ($G_{TE}$) in CaSki cells (A) and in hECE (B). Cells were plated on filters for 8 h and then shifted to and maintained in steroid-free medium for 5 days. There were a number of different treatment schedules. Some filters that contained cells were treated with 10 nM 17β-estradiol (Estradiol) or the vehicle (C, control) for 2 days before experiments. Other treatments were done on cells grown in filters in steroid-free medium for 5 days. After mounting the filters in an Ussing chamber, 1 mM SNP or 50 μM 8-Br-cGMP were added for 30 min before experiments. •, experiments done in the absence of phalloidin; △, cells treated with 10 nM phalloidin for 60 min before experiments. Following determinations of $G_{TE}$, filters were used for G-actin determinations as in Fig. 2. Total cellular actin was determined in parallel filters, and it measured 131 ± 11 pg/mg cellular DNA. Levels are means ± SD of 3–5 filters at each point.

**Fig. 5.** Effects of SNP and hemoglobin (Hb) on phosphorylation of $[^{32}P]$adenylate NAD in vitro in lysates of CaSki cells. Cells were plated on filters for 8 h and then shifted to steroid-free medium. After 5 days, cells were lysed in situ on the filters, and lysates were incubated with $[^{32}P]$adenylate NAD in the presence of 0.5 or 1 mM of SNP and in the absence or presence of 50 μM hemoglobin. Following the incubations, identical amounts of whole cell extract (10^4–10^5 cells/20 μl) were separated by gel electrophoresis. The experiment was repeated 4 times.
SNP-augmented phosphorylation of \[^{32}\text{P}]\text{adenylate NAD}

SNP, and 8-Br-cGMP modulate ADP-ribosylation of actin. The rationale was that the degree of SNP-induced in vitro phosphorylation of \[^{32}\text{P}]\text{adenylate NAD}

Subsequently, the degree of SNP-induced in vitro phosphorylation of \[^{32}\text{P}]\text{adenylate NAD}

In lysates obtained from CaSki and hECE treated with 17\(\beta\)-estradiol, SNP produced significantly less phosphorylation of \[^{32}\text{P}]\text{adenylate NAD}

Treatment of intact cells with SNP or 8-Br-cGMP also produced less SNP-induced phosphorylation of \[^{32}\text{P}]\text{adenylate NAD}

**Table 1. Modulation of in vitro SNP-induced \[^{32}\text{P}]\text{adenylate NAD phosphorylation**}

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment Before Lysis</th>
<th>Est</th>
<th>TMX</th>
<th>Est + TMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaSki (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.2 ± 0.1*</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>hECE (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.1 ± 0.1*</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CaSki (n = 4)</td>
<td>Control</td>
<td>1</td>
<td>0.1 ± 0.1*</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>hECE (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.3 ± 0.4*</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>CaSki (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.1 ± 0.2*</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>hECE (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.3 ± 0.4*</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>hECE (n = 3)</td>
<td>Control</td>
<td>1</td>
<td>0.2 ± 0.2*</td>
<td>0.3 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SD of densitometry of n filters in each category normalized to Control category in each group. The experiments are described in Figs. 7 and 8. *P < 0.05. Est, 17\(\beta\)-estradiol; TMX, tamoxifen; B/cGMP, 8-Br-cGMP; SNP, sodium nitroprusside; hECE, human ectocervical epithelial cells.
Fig. 7. Modulation of in vitro SNP-induced $[^{32}P]\text{adenylate NAD}$ phosphorylation in hECE. Cells were plated on filters for 8 h and then shifted to steroid-free medium for 5 days. Thirty minutes before experiments, cells were treated with 1 mM SNP, 50 μM 8-Br-cGMP, 25 μM LY-83583, or 25 μM KT-5823, alone or in combination. Following treatments, cells were lysed in situ on the filters and lysates were incubated with $[^{32}P]\text{adenylate NAD}$ in the presence of 1 μM SNP, and identical amounts of whole cell extract (10$^4$–10$^5$ cells/20 μl) were separated by gel electrophoresis. The experiments were repeated 3 times. Data are summarized in Table 1.

The degree of SNP-induced $[^{32}P]\text{adenylate NAD}$ phosphorylation in vitro was similar to that in cells treated with only 8-Br-cGMP, and, in both cases, it was smaller than in control cells not treated with these agents (Fig. 7, Table 1). This result indicates that blocking guanylate cyclase with LY-83583 does not modulate the effect of 8-Br-cGMP on SNP-induced $[^{32}P]\text{adenylate NAD}$ phosphorylation in vitro.

A different result was obtained in cells treated with SNP plus KT-5823 (Fig. 7). In lysates of hECE that were treated with SNP before lysis, the SNP-induced in vitro phosphorylation of $[^{32}P]\text{adenylate NAD}$ was significantly smaller than in lysates of cells not treated with SNP and was similar to the result shown in Fig. 6A. In contrast, in lysates of hECE that were treated before lysis with SNP plus KT-5823, the SNP-induced in vitro phosphorylation of $[^{32}P]\text{adenylate NAD}$ was similar to that observed in control cells not treated with these agents (Fig. 7, Table 1). This result indicates that blocking cGMP-dependent protein kinase with KT-5823 abrogates the effect of SNP on the in vitro SNP-induced $[^{32}P]\text{adenylate NAD}$ phosphorylation.

DISCUSSION

Estrogen increases paracellular permeability across human cervical epithelial cells. The effect is mediated by the estrogen receptor α (24), NO, and cGMP (19) and involves an increase in G-actin (18, 19, and present results). The present experiments suggest that one of the mechanisms of action of cGMP involves cGMP-dependent, protein kinase-induced ADP-ribosylation of monomeric actin; it sequesters G-actin and decreases its availability for polymerization. This shifts actin steady-state equilibrium from F-actin to G-actin and leads to depolymerization of actin filaments and fragmentation of the cytoskeleton.

The present results provide two groups of novel data. First, experiments that used F-actin assays confirmed that estrogen upregulates G-actin. Treatments with 17β-estradiol, SNP, or 8-Br-cGMP did not significantly affect total cellular actin, but all three agents decreased F-actin. The effect of estrogen, SNP, and 8-Br-cGMP on G-actin and the permeability could be blocked by treatment with the cytoskeleton-modifying agent phalloidin. Furthermore, in estrogen-treated cells, phalloidin decreased G-actin and $G_{\text{TR}}$ to levels that prevailed in cells grown in steroid-free medium. Phalloidin binds with high affinity to polymerized actin, and the high binding affinity is greater than the dissociation rate constant of monomeric G-actin from filament ends (10). As a result, phalloidin sequesters G-actin within actin filaments and reduces cellular G-actin. These results support the hypothesis that the estrogen increase in permeability depends on a shift in actin steady-state equilibrium from F-actin to G-actin and on fragmentation of the cytoskeleton.

It was previously reported that treatment with cytochalasin D, an F-actin modulator, also regulates paracellular permeability across human cervical epithelial cells, but, in contrast to phalloidin, cytochalasin D increases paracellular permeability (21). A possible explanation for these disparate effects is that phalloidin abrogates F-actin depolymerization, and, therefore, shifts actin steady-state equilibrium toward G-actin (12). In contrast, cytochalasin D possesses F-actin-disrupting properties (12, 42), and, like estrogen, would tend to shift actin steady-state equilibrium toward G-actin. Subsequently, phalloidin would stimulate formation of a rigid cytoskeleton that would decrease the $R_{\text{LIS}}$ and increase the permeability (17). Unlike estrogen, cytochalasin D also abrogates the tight junctional resistance, probably by disruption of F-actin at sites that interact with tight junctional elements (21).

The second novel finding was that estrogen increases G-actin by augmenting depolymerization of actin, possibly by ADP-ribosylation of monomeric actin. The experiments utilized assays of NO-induced ADP-ribosylation of actin in broken cell preparations, with $[^{32}P]\text{adenylate NAD}$ as the substrate for phosphorylation (6, 8, 34, 38, 44). The results show that the assay can yield semiquantitative data using SNP as the NO donor. In some experiments, cells were also treated with SNP before lysis. A concern was that NO generated from SNP before lysis of the cells could add to that which is generated by the SNP used in the reaction mixture in vitro and interfere with data interpretation. This concern is rather theoretical, because before lysis, cells were washed to remove excess SNP from the extracellular medium. In addition, intracellular NO would have a negligible effect on the in vitro reaction since NO is a short-lived volatile gas, and any NO that may have accumulated inside the cell would have been eliminated following the lysis of cells.
The present results show that 17β-estradiol, SNP, and 8-Br-cGMP augment ADP-ribosylation of monomeric G-actin. Tamoxifen blocked the effect of 17β-estradiol, similar to blocking the estrogen increase in G-actin (present results) and permeability (24), probably by inhibiting the estrogen receptor α-induced increase in NO and cGMP (24). LY-83583, an inhibitor of guanylate cyclase (15, 19, 31, 35), blocked the effect of SNP but not of 8-Br-cGMP. These responses resemble the effect of LY-83583 on the SNP increase in G-actin (present results) and permeability (24) and the lack of inhibition of 8-Br-cGMP increase in G-actin and permeability. A possible explanation is that both NO and cGMP can augment ADP-ribosylation of monomeric G-actin, but upregulation of cGMP is necessary for NO to exert its full effect. KT-5823, an inhibitor of cGMP-dependent protein kinase (15, 19, 31, 35), blocked the SNP- and 8-Br-cGMP-augmented increase in ADP-ribosylation of monomeric G-actin. These responses resemble the inhibitory effects of KT-5823 on the SNP- and 8-Br-cGMP-induced increase in G-actin (present results) and in permeability (24).

In the past, conversion of actin into a capping protein by ADP-ribosylation was considered a pathophysiolog-ical reaction. For example, ADP-ribosylation of actin catalyzed by bacterial toxins can lead to inhibition of actin assembly and to cytoskeletal rearrangements (32, 40). More recent studies suggest a physiological role for ADP-ribosylation of G-actin (1, 2). The present results suggest that ADP-ribosylation of monomeric G-actin may occur physiologically in human cervical epithelial cells in response to estrogen.

On the basis of the results of the present study and our previous studies (18, 19, 24), we propose a novel model of estrogen action in the cervix that could explain how estrogen increases secretion of cervical mucus. Estrogen increases NO activity by activation of estrogen receptor α and upregulation of endothelial cell NO synthase. NO acts on guanylate cyclase and stimulates an increase in cGMP; cGMP, acting via cGMP-dependent protein kinase, stimulates depolymerization of actin, possibly by ADP-ribosylation of monomeric G-actin, and shifts actin steady state toward G-actin. This fragments the cytoskeleton and renders cells more sensitive to decreases in cell size in response to stimuli that occur in vivo, such as the prevailing blood pressure (17). A decrease in cell size leads to an increase in the volume of the intercellular space and a decrease in the R1,IS, increase the permeability (17). It should be emphasized that ADP-ribosylation of monomeric actin is one (and perhaps not the sole) explanation for the effect of cGMP and that ADP-ribosylation of actin also probably reflects the available pool of G-actin.

This model may have clinical and pharmacological significance. Until recently, relatively little was known about regulation of cervical permeability, and most cases of abnormal cervical mucus were attributed to defective estrogen production (16). This explanation was met with difficulties, because treatment with estrogen may not improve mucus production in all women. The present study revealed a complex machin-ery of estrogen-dependent modulation of cervical permeability. The results suggest that regulatory sites distal to the estrogen receptor may be involved with defective mucus production. Pharmacological agents can modulate these sites, and it may be possible to target steps downstream to the estrogen receptor to modulate cervical permeability and cervical mucus production in women.

The discovery that estrogen can regulate actin polymerization in cervical epithelial cells may also improve our understanding of how other tissues adapt to hor-mononal changes during different phases of a woman’s life. For instance, during labor, the uterine cervix softens, dilates, and becomes effaced. Until recently, these changes were attributed to effects of estrogen on extra-cellular matrix (16). The present results suggest that the effect of estrogen may involve structural changes also in cervical stromal and smooth muscle cells. However, more studies are required to understand the implications of estrogen regulation of the cytoskeleton in uterine tissues of the woman.

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