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Epidermal Growth Factor Facilitates Epinephrine Inhibition of $P2X_7$-Receptor-Mediated Pore Formation and Apoptosis: A Novel Signaling Network

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Abstract

Epidermal growth factor (EGF), epinephrine, and the $P2X_7$ receptor system regulate growth of human uterine cervical epithelial cells, but little is known about how these systems intercommunicate in exerting their actions. The objective of this study was to understand the mechanisms of EGF and epinephrine regulation of growth of cervical cells. Treatment of cultured CaSki cells with 0.2 nM EGF increased cell number via a PD98059-sensitive pathway. Treatment with 2 nM epinephrine increased cell number, and the effect was facilitated by cotreatment with EGF. Whereas the effect of EGF alone involved up-regulation of [^{3}H]-thymidine incorporation and an increase in cell proliferation, the effect of epinephrine was mediated by inhibition of apoptosis. Epinephrine inhibited apoptosis induced by the $P2X_7$ receptor ligand 2′,3′-0-(4-benzoylbenzoyl)-ATP, by attenuation of $P2X_7$ receptor plasma membrane pore formation. Cotreatment with EGF facilitated epinephrine effect via a phosphoinositide 3-kinase-dependent mechanism. CaSki cells express the β2-adrenoceptor, and the epinephrine antiapoptotic effect could be mimicked by β2-adrenoceptor agonists and by activators of adenylyl cyclase. Likewise, the effect could be blocked by β2-adrenoceptor blockers and by the inhibitor of protein kinase-A H-89. Western immunoblot analysis revealed that epinephrine decreased the levels of the glycosylated 85-kDa form of the $P2X_7$ receptor and increased receptor degradation, and that EGF potentiated these effects of epinephrine. EGF did not affect cellular levels of the β2-adrenoceptor. In contrast, EGF, acting via the EGF receptor, augmented β2-adrenoceptor recycling, and it inhibited β2-adrenoceptor internalization via a phosphoinositide 3-kinase-dependent mechanism. We conclude that, in cervical epithelial cells, EGF has a dual role: as mitogen, acting via the MAPK/MAPK kinase pathway, and as an antiapoptotic factor by facilitating epinephrine effect and resulting in greater expression of β2-adrenoceptors in the plasma membrane. These findings underscore a novel signaling network of communication between the receptor tyrosine kinases, the G protein-coupled receptors, and the purinergic $P2X_7$ receptor.

Proper function of the epithelia of the female genital tract depends on coordinated growth and differentiation of the epithelial cells, and deviations from these well-controlled functions can cause infertility and lead to dysplasia and cancer. The uterine cervical epithelium is maintained by a balance between proliferation of the basal layer of cells, and death of cells in upper layers. Our current state of knowledge suggests three levels of growth-control of cervical epithelial cells: proliferation, controlled by mitogenic signals [*e.g.* estrogen and epidermal growth factor (EGF)] *vs.* growth inhibitory factors (*e.g.* TGFβ) (1); terminal differentiation, controlled mainly

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by estrogen (2); and senescence of cells evading growth control and terminal differentiation due to the erosion of telomeres (3). Recently it was reported that growth of human cervical epithelial cells is also regulated by apoptosis; the effect involves predominantly the $P2X_7$ receptor as its proximal signaling arm, and it uses the mitochondrial apoptotic pathway (4). However, relatively little is known about regulation of these effects and the mechanisms involved. Because apoptosis functions to eliminate abnormal cells (5) and dysregulation of apoptotic cell-death has been implicated in the premature death of cells, loss of tissue, aging, states of disease, and neoplastic transformation (6), elucidation of these data could be important for our understanding of cervical cell biology and tumorigenesis.

Both EGF (7) and epinephrine (8) are added routinely to cultures of squamous epithelial cells to enhance cell-growth. EGF and members of the EGF receptor family influence cell survival predominantly by stimulating proliferation (9), including of human cervical epithelial cells (1). Until recently, no mechanistic explanation was given for the growth-promoting effect of epinephrine. Recently we found that epinephrine increases the number of human cervical epithelial cells in culture, and that EGF potentiates the epinephrine effect. Because epinephrine, in contrast to EGF alone, inhibited apoptosis of cervical cells, we hypothesized that EGF, in addition to its mitogenic role, also potentiates epinephrine antiapoptotic effect.

The specific objective of the present study was to understand the mechanisms of EGF and epinephrine antiapoptotic effects. The results suggest that the epinephrine effect is mediated by β2-adrenoceptor-dependent inhibition of $P2X_7$ receptor pore formation. The results also suggest a dual role for EGF: as mitogen, using the MAPK/MAPK kinase (MAPK/MEK) signaling pathway, and as facilitator of epinephrine effect. We also found that the effect of EGF involves facilitated, phosphoinositide 3-kinase (PI3K)-dependent inhibition of β2-adrenoceptor internalization, and facilitated β2-adrenoceptor recycling, thereby increasing the level of β2-adrenoceptors in the plasma membrane. These findings underscore a novel signaling network that could be a general paradigm for functional communication between the receptor tyrosine kinases (RTKs), the G protein-coupled receptors (GPCRs), and the purinergic $P2X_7$ receptor.

Materials and Methods

Cell cultures

The experiments used CaSki cells, a line of transformed cervical epithelial cells that were obtained from the ATCC (Manassas, VA) and were previously characterized as stably expressing phenotypic characteristics of human cervical epithelial cells (7). Cells were grown and subcultured in RPMI-1640 supplemented with 8% fetal calf serum, 0.2% $NaHCO_3$, nonessential amino acids (0.1 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml) at 37 C in a 91%O_2-9%CO_2 humidified incubator. Cultures were routinely tested for mycoplasma. Preliminary experiments were conducted on cells plated on culture plates, and definitive experiments were repeated on cells plated on filters. The latter method improves culturing conditions and promotes epithelial cell polarization and differentiation (7). Filters were either Anocell (Anocell-10, Oxon, UK, obtained through Sigma Chemicals, St. Louis, MO), Transwell (Costar Corporation, Cam-bridge, MA), or Millicell-CM (Millipore, Bedford, MA). Filters were coated on their upper (luminal) surface with 3–5 $\mu g/cm^2$ collagen type IV and incubated at 37 C overnight. The remaining collagen solution was aspirated, and the filter was dried at 37 C. Before plating, both sides of the filters were rinsed three times with Hanks' balanced salt solution (HBSS). Cells were plated on the upper surface of the filter at 3×10^5 cells/cm^2. By plating at this high density, the cultures become confluent and polarized within 12 h after plating (7). All solutions and agents were added to both the luminal and subluminal bathing solutions.

DNA synthesis

DNA synthesis was measured by [^{3}H]thymidine incorporation. Six hours before assays, cells attached on filters were labeled for 4 h with 1 μM methyl-[^{3}H]-thymidine (1 μCi/ml; specific activity, 87 Ci/mmol; Amersham, Piscataway, NJ) and maintained for 2 additional hours in medium devoid of radioactive thymidine. After labeling, the cells were washed several times in ice-cold PBS, lysed in 200 μl 0.1 N NaOH, and precipitated in 2 ml ice-cold 10% trichloroacetic acid for 2 d at 4 C. The radioactivity [dpm/mg protein, determined by Bio-Rad (Hercules, CA) Protein Assay solution] of triplicated samples was determined by β-scintillation counting (LS1801 scintillation counter; Beckman, Fullerton, CA).

DNA solubilization assay

Twenty-four hours before the end of the experiment, cells were labeled with [^{3}H]thymidine (specific activity, 93 Ci/mmol; 5 μCi/1 × 10^6 cells) for 16 h. The medium was removed, and cells were washed three times with fresh medium lacking [^{3}H]thymidine and were incubated in the same medium for an additional 6 h. At the end of incubation, the supernatant was stored, and cells were lysed in 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.5; plus 1 mM EDTA–0.2% Triton X-100) for 1 h at 4 C. The intact chromatin was separated from the fragmented DNA by 5 min centrifugation at 4 C in Eppendorf microcentrifuge at 12,000 × g. The supernatant (lysate) was stored, while the pellet was resuspended in 0.5 ml of 1% sodium dodecyl sulfate (SDS). The radioactivity contained in the supernatants, lysates, and pellets was counted in a scintillation counter, and DNA fragmentation was calculated as: [% solubilized DNA] = ([cpm supernatant + cpm lysates]/[cpm supernatant + cpm lysate + cpm pellet]) × 100.

Binding studies

Binding studies of the adrenoceptors and the EGF receptor used a similar method. Two hundred fifty microliters of binding medium (serum-free DMEM, 0.1% BSA, 10 mM HEPES) containing 10–1000 pM of the radioligand were added to the apical container of the filter, and 500 μl of the same solution to the basolateral container; parallel tubes ontained an additional 200-fold excess radioinert ligand. The cells were then incubated at 4 C for 3 h; at the completion of incubations cells were washed three times with ice-cold HBSS containing 0.1% BSA. Cells were solubilized in 0.2 N NaOH, and the bound ligand was separated on glass fiber filters (Whatman, GF/C, Tonawanda, NY) by vacuum filtration. The associated radioligand was counted either in a β-scintillation counter (for [^{3}H] associated ligands) or in a γ-counter (for [^{125}I] associated ligands). The following radioligands were used: [^{3}H]prazosin and [^{3}H] tamsulosin (α-adrenoceptor radioligand) and [^{125}I]cyanopin-dolol [β-adrenoceptor radioligand (10)] (all at 1 μCi/ml; specific activity, about 90 Ci/mmol; Amersham), and [^{125}I]EGF (5 μCi/ml; specific activity, about 100 Ci/mmol; Biomedical Technology, Stoughton, MA). The α- and β2-adrenoceptor radioinert ligands were phenylephrine and zinterol (Sigma Chemicals). Human recombinant EGF was from R&D Systems (Minneapolis, MN). Specific receptor binding was expressed per milligram DNA, and data were analyzed by Scatchard plot as described (11).

Fluorescence changes

Determinations of fluorescence changes in cells attached on filters were published (12). For measurements of cytosolic calcium, cells on filters were incubated with 7 μM fura-2/acetoxymethyl ester plus 0.25% pluronic F12 (12). Measurements of fluorescence were conducted in a custom-designed fluorescence chamber (12). In this apparatus, a filter with cells is placed horizontally in an enclosed dark chamber maintained at a fixed temperature, under conditions that permit selective perfusion of the luminal (0.2 ml) and subluminal compartments (0.5 ml) at rates of 1–1.5 ml/min. Cells were illuminated over the apical surface, and the intensity of the emitted light from the apical surface was measured. Agents were added to the

luminal and subluminal solutions, and changes in cytosolic calcium were determined by switching the excitation filters to record the maximal (340/510 nm excitation/emission) and minimal (380/510 nm) fluorescence. Levels of cytosolic calcium were determined by the formula $[Ca^{2+}]_i$ (nM) = $[(R - R_{min})/(R_{max} - R)]\cdot K_d\cdot(S_{f2}/S_{b2})$, where $[Ca^{2+}]_i$ is the level of cytosolic calcium, R is the ratio of fluorescence excitation measurements at 340 to 380 nm, R_{min} and R_{max} are the experimentally determined minimum and maximum calcium measurement ratios, 340 nm to 380 nm respectively, K_d is the dissociation constant for fura-2 (224 nM), and S_{f2}/S_{b2} is the ratio of fluorescence value at 380 nm excitation determined at R_{min} (0 calcium) and R_{max} (maximal calcium). Maximal calcium fluorescence was obtained by adding 10 μM ionomycin in the presence of 10 mM $CaCl_2$, and minimal calcium fluorescence was obtained by competing calcium from fura-2 with 2.5 mM $MnCl_2$. This method was also used for experiments with the nuclear stain ethidium bromide (molecular mass, 394 Da). Ethidium bromide was added to the perfusing solutions from a concentrated stock (×100) at a final concentration of 5 μM. Upon influx into cells, the dye binds to nuclear chromatin and elicits specific fluorescence. Changes in fluorescence were measured on-line at wavelengths 518/605 nm (excitation/emission).

Western blot analysis

The postnuclear supernatant of cells was solubilized in lysis buffer (50 mM Tris-HCl, pH 6.8; 1% 3-[(3-cholamidopropyl)dimethyl-ammo-nio]-1-propanesulfonate; 5 mM EDTA, pH 8.0) containing 50 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml benzamidine, 10 μg/ml bacitracine, 10 μg/ml leupeptin, and 2 μg/ml aprotinine. Aliquots (about 45 μl), normalized to 15 μg protein, were loaded on 10% polyacrylamide SDS gel, and vertical electrophoresis was conducted at 50 mA for 1.5 h. Gels were transferred onto Immobilon membrane (Millipore) at 200 V for 1.5 h, membranes were blocked in 5% milk, and receptor polypeptides were visualized using 1.5 μg/ml rabbit anti-$P2X_7$ antibody at 4 C overnight. Membranes were washed three times in PBS and fluorescent stained for 1 min using an enhanced chemiluminescence kit of antirabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For protein deglycosylation, 20 μg total lysate proteins containing 1% SDS was denatured by heating for 5 min at 100 C. The mixture was incubated overnight at room temperature after addition of 1 U fresh *N*-glycosidase F (GlycoTech, Rockville, MD) in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 50 mM NaCl. Samples were analyzed by Western blot as described above.

Cellular cAMP

Cellular cAMP was assayed in total homogenates of cells attached on filters. For assays, cells were incubated in HEPES-buffered Krebs-Ringer's bicarbonate buffer (pH 7.4) containing 1 mg/ml BSA and 2.5 mM glucose (Krebs bicarbonate Ringer) at 37 C for 30 min. The reactions were terminated by adding 12% trichloroacetic acid (1:1 vol/vol). Cells were scraped off the filters and centrifuged at 3,000 × *g* for 15 min. Supernatants were extracted with 2 ml water-saturated diethylether three times. The concentration of cAMP in the aqueous phase was determined in duplicate by RIA using a commercially available kit (Amersham).

*β*2-Adrenoceptor internalization (endocytosis) assays

Fifteen minutes before assays, cells were shifted to ice-cold medium and labeled with 1 μCi/ml [^{125}I]cyanopindolol for 30 min at 4 C. After three washes with ice-cold medium lacking the radioligand, cells were shifted to fresh, warm (37 C) medium and treated with 1 μM zinterol. At time intervals of 0, 15, 30, and 60 min, cells were washed with ice-cold medium. Surface-bound and internalized *β*2-adrenoceptors were distinguished according to Haigler *et al.* (13), with some modifications, as we have described (1). Briefly, cells were washed three times with ice-cold HBSS to remove unbound radioactivity. Cultures were then incubated for 10 min at

4 C in 0.2 M acetic acid plus 0.5 M NaCl (pH 2.5) (250 and 500 μl, respectively, in the apical and basolateral containers of the filter). The filters were then rinsed with 250 and 500 μl, respectively, of the same solution, and the washes (defined as the surface bound fraction) were collected. The remaining cell-associated radioactivity was removed by incubating the cells for 60 min at 37 C with 1 N NaOH. The percent membrane bound [^{125}I]cyanopindolol was determined relative to membrane bound [^{125}I]cyanopindolol in cells not exposed to zinterol.

*β*2-Adrenoceptor recycling assays

Fifteen min before assays, cells were shifted to ice-cold medium and labeled with 1 μCi/ml [^{125}I]cyanopindolol for 30 min at 4 C. After three washes with ice-cold medium lacking the radioligand, cells were incubated at 4 C for 15 min with 1 μM zinterol, and then shifted to 37 C and incubated for an additional 60 min in the same medium. At the completion of incubation, cells were washed three times at 37 C with the same medium, lacking zinterol, to remove unbound zinterol, and internalized processed zinterol was released into the medium. After the wash, cells were incubated further at 37 C for 0, 0.5, 3, 4, 8, 16, and 24 h, and the amount of membrane bound [^{125}I]cyanopindolol was determined as above. Data about recycled receptor are presented in terms of percent control bound [^{125}I]cyanopindolol (compared with cells not exposed to zinterol).

Densitometry

Densitometry was done using AGFA Arcus II scanner (AGFA, New York, NY) and Un-Scan-It gel automated digital software (Silk Scientific, Orem, OR).

Statistical analysis of the data

Data are presented as means (± SD), and significance of differences among means was estimated by Student's *t* test. Trends were calculated using GB-STAT V5.3, 1995 (Dynamic Microsystems Inc., Silver Spring, MD) and analyzed with ANOVA.

Chemicals and supplies

Ethidium bromide and fura-2/AM were obtained from Molecular Probes (Eugene, OR). Benzyloxy-valine-alanine-aspartate-O-methyl-fluoromethylketone (zVAD-FMK) was from Calbiochem (La Jolla, CA). All other chemicals, unless specified otherwise, were obtained from Sigma Chemicals. The anti-$P2X_7$ receptor pAb was from Alomone Laboratories (Jerusalem, Israel).

Results

Effects of epinephrine and EGF on proliferation and apoptosis of cervical cells

Near-confluent cultures of CaSki cells on filters were shifted for 14 h to serum-free medium supplemented with 1% BSA and were treated in the same medium with 0.2 nM EGF, 2 nM epinephrine, or both for an additional 24 h. These concentrations were chosen because they are within the physiological range *in vivo* (14–16). At the completion of treatments, cells were harvested off the filters and counted. Treatments with EGF or with epinephrine alone increased the number of cells in culture (Fig. 1A). However, treatment with EGF plus epinephrine increased cell number more than did each drug alone (Fig. 1A), suggesting that EGF facilitates the effect of epinephrine.

To better understand the effects of EGF and epinephrine on the growth of cervical cells, two additional experiments were done. First, the effects of EGF and epinephrine on proliferation were determined in terms of DNA synthesis. EGF, but not epinephrine, increased [^{3}H] thymidine incorporation (Fig. 1B), confirming the mitogenic properties of EGF in human

cervical epithelial cells (1). In the second experiment, effects of EGF and epinephrine on apoptosis were determined in terms of modulation of changes in DNA solubilization induced by the $P2X_7$ receptor-specific agonist 2′,3′-0-(4-benzoylbenzoyl)-ATP (BzATP). It was recently shown that human cervical epithelial cells undergo apoptosis constitutively and that the effect involves the purinergic $P2X_7$ receptor mechanism (4). Because the degree of constitutive apoptosis is low and does not allow for experimental manipulations, BzATP was added to increase the apoptosis. As is shown in Fig. 1C, treatment with 100 μM BzATP for 9 h increased DNA solubilization significantly from the baseline level of 1.9% to 7.8%. Pretreatment for 24 h with 0.2 nM EGF alone had little effect on the BzATP-induced apoptosis (Fig. 1C). In contrast, pretreatment for 24 h with 2 nM epinephrine decreased DNA solubilization to 4.6%, and cotreatment with EGF facilitated the epinephrine effect (2.9%). The control for this experiment was zVAD-FMK, a pan-caspase inhibitor that was added 30 min before assays at a concentration of 50 μM. Treatment with zVAD-FMK decreased the BzATP-induced DNA solubilization to levels that were lower than baseline apoptosis (about 1.4%, Fig. 1C). Collectively, the data in Fig. 1 indicate that epinephrine increases cell number by attenuation of apoptosis, whereas EGF has a dual role: as mitogen, and as facilitator of epinephrine antiapoptotic effect.

Mechanism of epinephrine effect

Involvement of the β2-adrenoceptor mechanism—The low concentration of epinephrine that inhibited apoptosis (2 nM) suggested an effect mediated by adrenergic receptor(s). Epinephrine is a relatively nonspecific β-adrenergic agonist with some α-adrenergic potential. To understand the mechanism of epinephrine effect, three experiments were done. First, cells were tested for the expression of α- and β-adrenoceptors. Binding assays revealed no specific binding of the β-adrenoceptor radioligands [^{3}H]prazosin or [^{3}H] tamsulosin (not shown), ruling out expression of functional β-adrenoceptors. In contrast, CaSki cells bound, with high affinity, the β-adreno-ceptor radioligand [^{125}I]cyanopindolol (Fig. 2A, K_d ~ 0.3 nM), and the binding activity was about 15 fmol/mg DNA (~10,000 receptors/cell). The radiolabel could be displaced by coincubation with the β2-adrenoceptor ligands zinterol and terbutaline (Fig. 2A) but not with the β1-adrenoceptor ligand isoproterenol (not shown). These data indicate that CaSki cells express the β2-adrenoceptor.

The second and third experiments tested the degree of which activation and inhibition, respectively, of β2-adrenoceptor modulate BzATP-induced apoptosis. Pretreatment for 24 h with either zinterol or terbutaline (1 μM) inhibited BzATP-induced apoptosis (Fig. 2B). Similarly, pretreatment for 14 h with 10 μM forskolin [in the presence of 0.5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxan-thine, to activate directly adenylyl cyclase] also inhibited BzATP-induced apoptosis (Fig. 2B). In contrast, pretreatments with 10 μM of the α1-adrenoceptor ligand isoproterenol or with 100 μM of the α1-adrenoceptor ligand phenyl-ephrine had no effect on BzATP-induced apoptosis (Fig. 2B).

Cotreatment for 24 h with either 10 μM propranolol (β-adrenoceptor antagonist), 100 nM ICI 118,551 (β2-adreno-ceptor antagonist), or 30 μM of H-89 dihydrochloride (protein kinase A inhibitor) blocked the zinterol (1 μM)-induced inhibition of BzATP-induced apoptosis (Fig. 2C). Collectively, the data in Fig. 2 indicate that epinephrine-dependent attenuation of BzATP-induced apoptosis is mediated by the β2-adrenoceptor. The data also suggest that the effect involves cAMP and protein kinase A as downstream mediators.

Modulation of the mitochondrial pathway—Apoptosis can be induced by the cell-surface receptor and/or mitochondrial pathways. The former usually involves the Fas and TNF mechanisms followed by activation of caspase-8 (6,17,18). The mitochondrial pathway involves the release of cytochrome-c (19) and activation of caspase-9 (20). The cell-surface

receptor and mitochondrial-dependent pathways integrate at the level of the effector caspases, so that activation of caspases 9 and 8 triggers the nonreversible execution of apoptosis by caspases 6, 7, and 3 (5,6). In CaSki cells, apoptosis induced by ligation of the $P2X_7$ receptor mechanism involves predominantly the mitochondrial pathway (4). This knowledge was used to dissect the effects of epinephrine on BzATP-induced apoptosis.

CaSki cells were treated with either 100 μM BzATP for 9 h or with 10 μM TNFα for 14 h, and effects on DNA solubilization were determined. These concentrations produce a submaximal degree of apoptosis in human cervical epithelial cells, and their effects involve, respectively, the cell-surface receptor and mitochondrial pathways (4). Either of these two agents significantly increased DNA solubilization (Fig. 3), confirming our previous results (4) and the results in Figs. 1 and 2 regarding BzATP effects. Pretreatment for 24 h with 1 μM zinterol inhibited BzATP-induced increase in DNA solubilization, but it had no effect on the increase in DNA solubilization induced by TNFα (Fig. 3A). Pretreatment for 24 h with 0.2 nM EGF alone had no significant effect on BzATP- or TNFα-induced increase in DNA solubilization (Fig. 3B).

Inhibition of $P2X_7$ receptor pore formation—One of the explanations for the results in Fig. 3 is that the β2-adrenoceptor-dependent antiapoptotic signaling pathway interacts with proximal steps of the $P2X_7$ receptor signaling cascade, possibly at the level of the $P2X_7$ receptor. Activation of the $P2X_7$ receptor can induce apoptosis by a number of known mechanisms, including IL-1β (21), TNFα-TNF-related apoptosis-inducing ligand (22), and the p38, c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (23) and NF-κB pathways (24). However, the $P2X_7$ receptor is unique in its ability to form pores in the plasma membrane in the continued presence of the ligand (25). Plasma membrane pore formation depends on the long C terminus of the receptor (26), and oligomerization of neighboring molecules is believed to cause progressive dilatation of the pore to a diameter of approximately 4 nm and an increase in the permeation path to molecules of molecular mass of 400–900 Da (27). In its final size, the pore is relatively permeable to Ca^{2+}, but it remains selective to other cations and is impermeable to anions (28). Uncontrolled influx of Ca^{2+} and the sustained increases in cytosolic calcium are believed to trigger the apoptotic mitochondrial pathway (28). The objective of the following experiments was to test the hypothesis that epinephrine inhibits apoptosis by blocking $P2X_7$ receptor pore formation.

The first experiment tested the degree of which pretreat-ment with epinephrine blocks BzATP-induced Ca^{2+} influx. We used fura-2-loaded CaSki cells attached on filters housed in a fluorescence chamber as described in *Materials and Methods* and in our previous publication (12). Under these conditions, treatment with BzATP induced 1-[N,O-bis(5-iso-quinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62)-sensitive, time-dependent sustained Ca^{2+} influx that began about 10 min after adding the ligand (Fig. 4A). EGF alone had no significant effect, and pretreatment with epinephrine attenuated the BzATP-induced increase in cy-tosolic calcium (*inset*, Fig. 4A). Pretreatment with epinephrine plus EGF attenuated the BzATP-induced increase in cytosolic calcium to a degree that was observed in cells treated with the $P2X_7$ receptor modifier KN-62 (*inset*, Fig. 4A).

The second experiment used the same design, but it tested more directly $P2X_7$ receptor pore formation. Cells attached on filters were treated with BzATP in the presence of 5 μM ethidium bromide (molecular mass, 394 Da). The rationale was that, in cells with intact plasma membrane, influx of the dye is minimal. As is shown in Fig. 4B, under baseline conditions, there were no changes in fluorescence at 518/605 nm (excitation/emission), indicating no influx of ethidium bromide. Treatment with BzATP resulted in time-related increase in the fluorescence (Fig. 4B), indicating nuclear binding of ethidium bromide and suggesting that the nucleotide induced influx of ethidium bromide. The increase in fluorescence began about 10

min after adding the agonist, and it correlated with the sustained increase in cytosolic calcium (Fig. 4, A and B). Similar to the effect on cytosolic calcium, the BzATP-induced influx of ethidium bromide could be blocked by pretreatment with KN-62 (*inset*, Fig. 4B). Pre-treatment with EGF alone had no significant effect; pretreatment with epinephrine attenuated the BzATP-induced influx of ethidium bromide, and pretreatment with epinephrine plus EGF inhibited the BzATP-induced influx of ethidium bromide, similar to the effect of KN-62 (Fig. 4B and *inset*). Collectively, the results in Fig. 4 suggest that epinephrine inhibited ligation-induced $P2X_7$ receptor pore formation; EGF alone had no significant effect, but it facilitated the effect of epinephrine.

Modulation of $P2X_7$ receptor expression—The objective of the experiments in this section was to determine epinephrine effect on the expression of the $P2X_7$ receptor. Expression of the $P2X_7$ receptor protein was analyzed using the Alomone polyclonal rabbit anti-$P2X_7$ receptor antibody as the primary antibody. This antibody was raised against the purified peptide (C)KIRK EFPKT QGQYS GFKYP Y (corresponding to residues 576–595 of rat $P2X_7$ with an additional N-terminal cysteine (29), and our data (see below) and data by others (30) support its usefulness for the objectives of the present study.

Western immunoblot analysis of total homogenates from CaSki cells revealed specific reactivities to 85-kDa, 65-kDa, and 18-kDa forms that were abolished in the presence of the antigen used to raise the antibody (Fig. 5A). The 65-kDa is most likely the native form of the $P2X_7$ receptor corresponding to the reported $P2X_7$ receptor 70-kDa isoform, whereas the 18-kDa is a degradation product (28,31–33). To better understand the role of the 85-kDa form, two additional experiments were done. First, the effects of length in culture on the responses to BzATP were compared with the cellular expression of the receptor. The levels of BzATP-induced Ca^{2+} influx and of BzATP-induced influx of ethidium bromide were greater in cells cultured for 6 d than in cells cultured for 2 d (Fig. 5B). Length in culture also affected the levels of the $P2X_7$ receptor forms: in d-2 cells, the predominant form was the 65-kDa; in contrast, in d-6 cells, the levels of the 85-kDa and 18-kDa forms were greater than in d-2 cells (Fig. 5D, left lanes). These results suggest that the mature and functional form of the $P2X_7$ receptor protein is the 85-kDa form. This hypothesis was further supported by the results shown in Fig. 5C. Incubation *in vitro* of cell lysates with *N*-glycosidase F showed a decrease in the density of the 85-kDa form along with an increase in the density of the 65-kDa form (Fig. 5C). Densitometry analysis of two experiments indicates that the ratio of 65-kDa/85-kDa increased more than 3-fold after treatment *in vitro* with *N*-glycosidase F. These data are in general agreement with previous studies in the field (29,30,32) and indicate that the 85-kDa form is the glycosylated form of the $P2X_7$ receptor.

Pretreatment of CaSki cells with EGF alone had only mild effect on the levels of the $P2X_7$ receptor forms, increasing mainly the 18-kDa form in d-6 cells (Fig. 5 and Table 1). Pretreatment of d-2 cells with epinephrine decreased the 65-kDa form; whereas in d-6 cells, it decreased the 85-kDa and 65-kDa forms and increased the 18-kDa form (Fig. 5 and Table 1). Cotreatment of d-2 cells with EGF plus epinephrine increased the 18-kDa form 50-fold; cotreatment of d-6 cells decreased the 85-kDa form and increased the 18-kDa isoform 35-fold. In addition, in d-6 cells, treatment with epinephrine plus EGF resulted in the appearance of an intermediate form of 75-kDa (Fig. 5, Table 1). Collectively, the data in Fig. 5 and Table 1 indicate that EGF facilitates epinephrine-induced decrease of the glycosylated 85-kDa form of the $P2X_7$ receptor, and of $P2X_7$ receptor degradation. The data also suggest that EGF, acting in concert with epinephrine, induces deglycosylation of the $P2X_7$ receptor.

Mechanism of EGF-dependent facilitation of epinephrine effect

EGF facilitated epinephrine antiapoptotic effect (Figs. 1, 4, and 5), but treatment with EGF alone had little effect on apoptosis or on the density level of the β2-adrenoceptor (Fig. 2A). These data suggested that the EGF effect involves modulation of β2-adrenoceptor function. To better understand the effect of EGF, we assayed the functionality of the EGF receptor in CaSki cells. CaSki cells bound EGF specifically and with high affinity (Fig. 6A). Scatchard analysis of the specific [^{125}I]EGF binding suggested two populations of EGF receptors: high-affinity sites (K_d ~ 0.5 nM, binding activity of about 50 fmol/mg DNA, ~50,000 receptors/cell); and low-affinity sites (K_d ~ 2.1 nM). These data confirm previous results in normal human cervical epithelial cells (1) and in human papillomavirus 16 (immortalization human ectocervical epithelial cells) (34,35). Treatment with epinephrine did not affect EGF receptor binding capacity or binding affinity (Fig. 6A). The positive control for the experiment was estrogen, whereby treatment of cells with 100 nM 17β-estradiol for 24 h increased binding capacity of the high-affinity EGF receptor sites to about 85 fmol/mg DNA (Fig. 6A) without affecting binding affinity.

Functionality of the EGF receptor was determined by incubating CaSki cells with the EGF receptor inhibitor AG1478. In cells treated for 24 h with 0.2 nM EGF, cotreatment with 5 μM AG1478 blocked EGF-increase in [^{3}H]thymidine incorporation (Fig. 6B). Similarly, cotreatment with 10 μM of the MAPK/MEK inhibitor PD98059 blocked EGF-induced increase in [^{3}H]thymidine incorporation (Fig. 6B). In contrast, cotreatment with 10 μM of the PI3K inhibitor wortmannin had no significant effect on [^{3}H]thymidine incorporation compared with that observed in cells treated only with EGF (Fig. 6B). These data indicate that the EGF-induced increase in [^{3}H]thymidine incorporation (and probably in cell number) is mediated by the EGF receptor and the MAPK/MEK pathway.

The results in Fig. 2, B and C, suggested to us that epinephrine inhibition of BzATP-induced apoptosis involves activation of adenylyl cyclase and protein kinase A, and therefore that epinephrine uses cAMP as the downstream messenger of the β2-adrenoceptor. These data were used to dissect the mechanism by which EGF facilitates epinephrine action. Treatments with 2 nM epinephrine or with 1 μM zinterol increased cellular cAMP by about 2-fold, and co-treatment with 0.2 nM EGF augmented the responses to both epinephrine and zinterol (Fig. 7A). These data suggest that EGF exerts its effect at a level proximal to the adenylyl cyclase.

The next set of experiments tested the hypothesis that EGF modulates plasma membrane levels of β2-adrenoceptor. The experiments looked into effects of EGF on plasma membrane β2-adrenoceptor steady-state. To test the degree of which EGF modulates β2-adrenoceptor internalization, CaSki cells were incubated at 4 C with [^{125}I]cyanopindolol, shifted to 37 C, and treated with zinterol. As shown in Fig. 7B and summarized in Table 2, pretreatment for 24 h with 0.2 nM EGF attenuated the time-related zinterol-induced decrease in the membrane-bound [^{125}I]cyanopindolol. One explanation for this result is that EGF inhibits internalization of the β2-adrenoceptor. An additional explanation is that EGF accelerates recycling of the ligated β2-adrenoceptor receptor and stimulates its restoration into the plasma membrane. To study this question more directly, CaSki cells were labeled with [^{125}I]cyanopindolol and treated with zinterol at 4 C, and then shifted to 37 C. As shown in Fig. 7C and summarized in Table 2, the increase in the membrane-bound [^{125}I]cyanopindolol was faster in cells pretreated with EGF than in control cells. Collectively, the data in Fig. 7, B and C, and in Table 2 suggest that treatment with EGF increases plasma membrane β2-adrenoceptor levels, possibly by attenuating receptor internalization and facilitating recycling of the receptor.

To better understand the mechanism of EGF modulation of plasma membrane β2-adrenoceptor levels, the above experiments were repeated in the presence of PD98059 or wort-mannin. Cotreatment with PD98059 had no significant effect on EGF modulation of plasma membrane

β2-adrenoceptor steady-state (Table 2). Treatment with wortmannin alone had no significant effect on the internalization and recycling of the membrane-bound [^{125}I]cyanopindolol or on DNA solubilization (not shown). Cotreatment with EGF plus wort-mannin also had no significant effect on the EGF-induced facilitated β2-adrenoceptor recycling (Table 2). In contrast, coincubation with wortmannin inhibited the EGF-induced attenuation of β2-adrenoceptor internalization and the EGF-induced decrease in DNA solubilization (Table 2).

Discussion

The present study reports novel data about epinephrine- and EGF-regulation of apoptosis in human cervical epithelial cells. We discovered that EGF facilitates β2-adrenoceptor-dependent inhibition of $P2X_7$ receptor-mediated apoptosis. EGF mechanism of action involves up-regulation of plasma membrane β2-adrenoceptor levels by acceleration of β2-adrenoceptor recycling, and by PI3K-depedent inhibition of β2-adrenoceptor internalization. β2-Adrenoceptor-dependent modulation of apoptosis has been previously described, but effects vary and depend on cell type and on the experimental conditions. Previous studies in cardiac myocytes reported that β2-adrenocep-tors exert antiapoptotic effects via an effect mediated by the caspase 9 mitochondrial pathway (36–38). These data could be relevant to the present study because, in human cervical epithelial cells, activation of β2-adrenoceptors blocks $P2X_7$ receptor-dependent apoptosis (present results), which also uses a caspase 9 mitochondrial pathway (4).

In human cervical epithelial cells, activation of the $P2X_7$ receptor depended on membrane expression of the glyco-sylated 85-kDa form (Fig. 5), which is the mature and functional form of the $P2X_7$ receptor protein. Previous groups described the expression and function of the glycosylated $P2X_1$ and $P2X_2$ receptors (39–41), as well as the $P2X_4$ (42) and $P2X_6$ receptors (43). Our novel data suggest that membrane expression and functionality of the $P2X_7$ receptor also depend on glycosylation of the receptor, because receptor de-glycosylation was detrimental for receptor function and pore formation. The $P2X_7$ receptor possesses five putative *N*-glycosylation sites: Asn^{187}, Asn^{202}, Asn^{213}, Asn^{241}, and Asn^{284} (29); but at present, little is known about the role of the attached *N*-glycans for the expression and function of the $P2X_7$ receptor. The present data suggest that epinephrine, in an action mediated by β2-adrenoceptor activation of protein kinase A, stimulates deglycosylation of the $P2X_7$ receptor and receptor degradation. The molecular mechanism of this novel protein kinase A effect is unknown. One possibility is an effect at the level of the endoplasmic reticulum/Golgi, namely inhibition of receptor processing trafficking and membrane expression. Another explanation is an effect at the level of the plasma membrane, *i.e.* enhanced degradation of previously expressed receptor.

The effect of epinephrine was mediated by the GPCR β2-adrenoceptor. The expression of β2-adrenoceptors in uterine cervical and vaginal cells was previously reported, both in the myometrial and uterine smooth muscle cells (44,45), as well as in uterine epithelial cells. Tolszczuk and Pelletier (46) described the expression of β2-adrenoceptors in rat endo-metrial cells. Whitaker *et al.* (47) showed intense labeling of [^{125}I]cyanopindolol over glands and surface columnar epithelium of the human cervix, and moderate labeling over the squamous cervical/vaginal epithelium, and surrounding the basal layer. In females, plasma levels of epinephrine range from 0.1–3 nM and change relatively little throughout life (14). In addition, plasma levels of epinephrine are within the range of the K_d of the cervical β2-adrenoceptor (Fig. 2A). Collectively, these data suggest that epinephrine tonically exerts antiapoptotic effect in the cervix *in vivo*.

We also found that EGF facilitated epinephrine antiapoptotic effect. EGF is a multifunctional factor whose actions are mediated by a RTK linked to multiple downstream cascades such as

protein kinase C, PI3K, JNK, and the MAPK signaling pathways (recently reviewed in Ref. 48). All cascades have been described in human cervical cells, and the MAPK signaling pathway is usually associated with mitogenic properties of EGF (Ref. 1 and Fig. 1B). Interactions between EGF-induced signals and GPCR signaling pathways have been reported (49–54). In some systems, the early convergence of signals originating from GPCRs and RTKs reflects GPCR-mediated transactivation of an RTK (55). However, only limited observations have been related to effects of EGF on adenylyl cyclase activation via β-adrenergic or other stimulatory GPCRs (56). EGF can increase transcription of β2-adrenoceptor (57) and cause rapid changes of adenylyl cyclase activities in a variety of *in vitro* systems (50,58). The present findings are novel, in that EGF blocked β2-adrenoceptor internalization via a PI3K-dependent mechanism and facilitated receptor recycling via a PI3K-independent mechanism. The molecular mechanisms of EGF action are, at present, unknown; but based on studies in other systems, they could involve modulation of signaling along the classical GPCR cascade of endocytosis, resensitization, recycling, and degradation (59).

Based on these findings, our hypothesis is summarized in Fig. 8, and it depicts that, in the cervix, EGF has a dual role regarding the regulation of growth of cervical epithelial cells. Acting via the MAPK cascade pathway, EGF stimulates mitosis and proliferation, and it is probably the mediator of estrogen effect (11). EGF also facilitates β2-adrenoceptor-dependent stimulation of deglycosylation and degradation of the $P2X_7$ receptor. EGF up-regulates plasma membrane β2-adrenoceptor levels via two possible mechanisms: facilitated recycling of the β2-adrenoceptor, and attenuation of β2-adrenoceptor internalization by a PI3K-dependent mechanism. These effects of EGF would tend to increase the pool of the receptor that is available for activation upon ligand binding. Our novel findings underscore a new signaling network that could be a general paradigm for functional communication among RTKs, GPCRs, and the purinergic $P2X_7$ receptor.

The present results are physiologically relevant because human cervical epithelial cells express the EGF receptor (Ref. 1 and Fig. 6A) and the β2-adrenoceptor (Ref. 47 and Fig. 2A). Moreover, in women, plasma EGF concentrations range from 0.1–5 nM (15,16) and are within the range of EGF binding by the EGF receptor (6A). Furthermore, in the cervix, EGF is released from stromal cells on which cells of the basal layer of the cervical epithelium rest (60). We conclude that, in the cervix, the observed EGF effects of stimulated epithelial growth are the combination of EGF mitogenic and EGF antiapoptotic effects, and the latter are the result of EGF-dependent facilitation of β2-adrenoceptor inhibition of $P2X_7$ receptor-mediated apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BzATP
: 2′,3′-0-(4-Benzoylbenzoyl)-ATP

EGF
: epidermal growth factor

GPCR
G protein-coupled receptor

HBSS
Hanks' balanced salt solution

JNK
c-Jun N-terminal kinase

KN-62
1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine

MAPK/MEK
MAPK/MAPK kinase

PI3K
phosphoinositide 3-kinase

RTK
receptor tyrosine kinase

SDS
sodium dodecyl sulfate

zVAD-FMK
benzyloxy-valine-alanine-aspartate-O-methyl-fluoromethylketone

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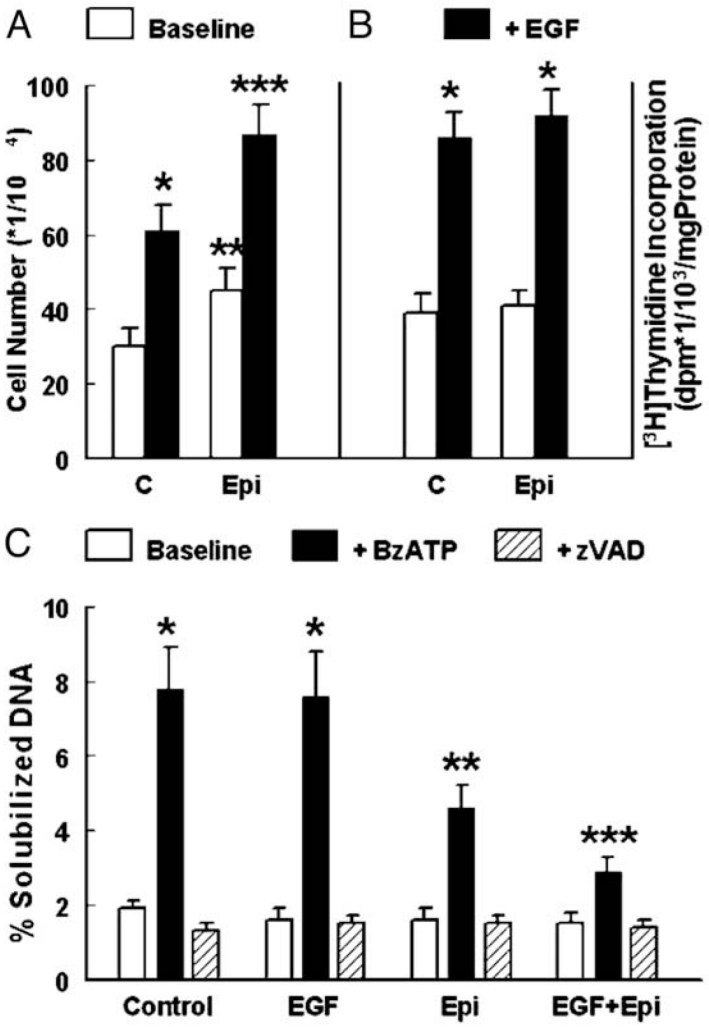

Fig. 1.

Means (± SD, three to five independent experiments) of the effects of EGF and epinephrine (Epi) on number of CaSki cells in culture (panel A), DNA synthesis (panel B), and BzATP-induced DNA solubilization (panel C). In A: *, $P < 0.01$ (EGF *vs.* Baseline); **, $P < 0.03$ [Baseline, Epi *vs.* C (control)]; ***, $P < 0.03$ (EGF, Epi *vs.* C). In B: *, $P < 0.01$ (EGF *vs.* Baseline, in both C and Epi groups). In panel C: *, $P < 0.01$ (BzATP *vs.* Baseline, Control and EGF groups); **, $P < 0.01$ (BzATP, Epi *vs.* Control and EGF groups); ***, $P < 0.03$ (BzATP, EGF+EPI *vs.* Epi groups).

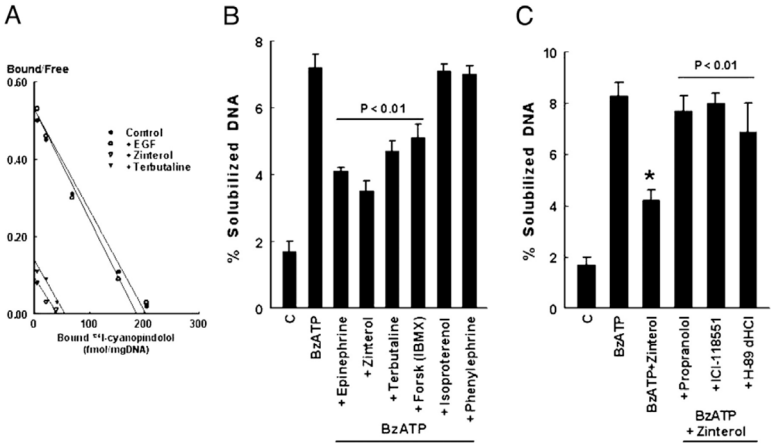

Fig. 2.

A, Scatchard analysis of [^{125}I]cyanopindolol binding to CaSki cells, and the effects of EGF, zinterol, and terbutaline. B, Modulation of BzATP-induced DNA solubilization by adrenoceptor agonists and by activators of adenylyl cyclase forskolin (Forsk., in the presence of 3-isobutyl-1-methylxanthine). C, Modulation of zinterol-dependent inhibition of BzATP-induced DNA solubilization by β2-adrenoceptor antagonists and by the protein kinase A inhibitor H-89 dihydrochloride (H-89-dHCl). Data in B and C are means (± SD) of three to four independent experiments. *, $P < 0.01$ (BzATP+Zinterol *vs.* BzATP).

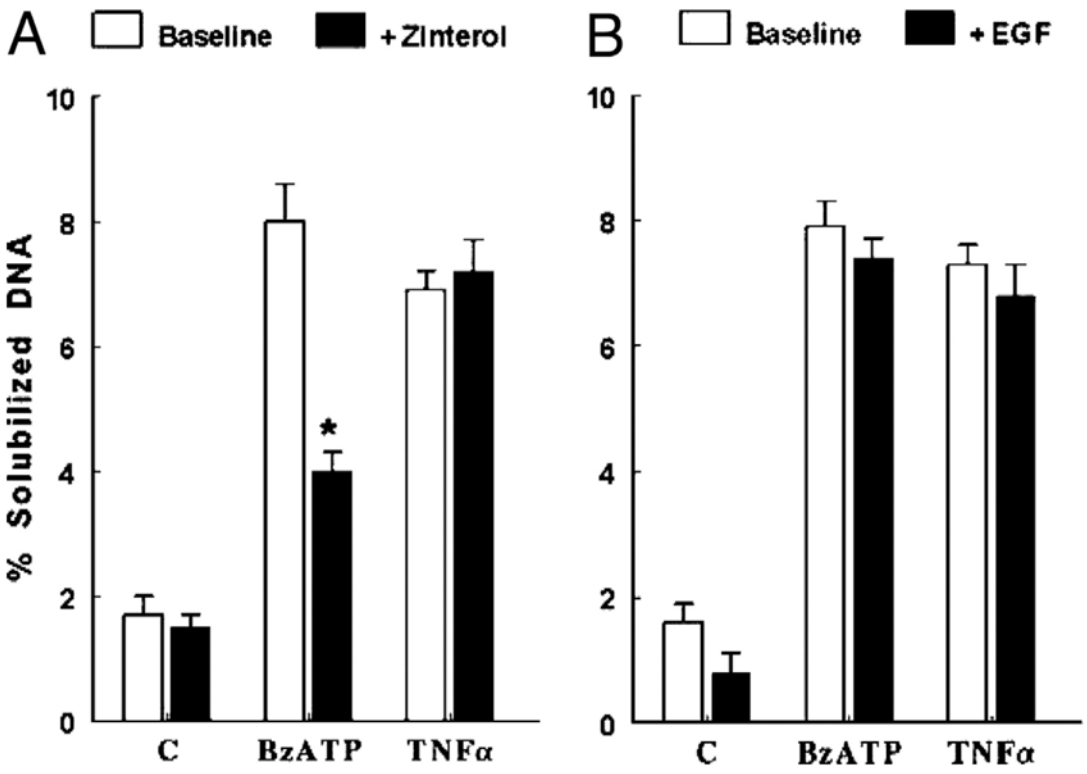

Fig. 3.

Effects of zinterol (A) and EGF (B) on DNA solubilization induced by BzATP and by TNF*α*. Data are means (± SD) of three independent experiments. *, $P < 0.01$ (BzATP group, Zinterol *vs.* Baseline).

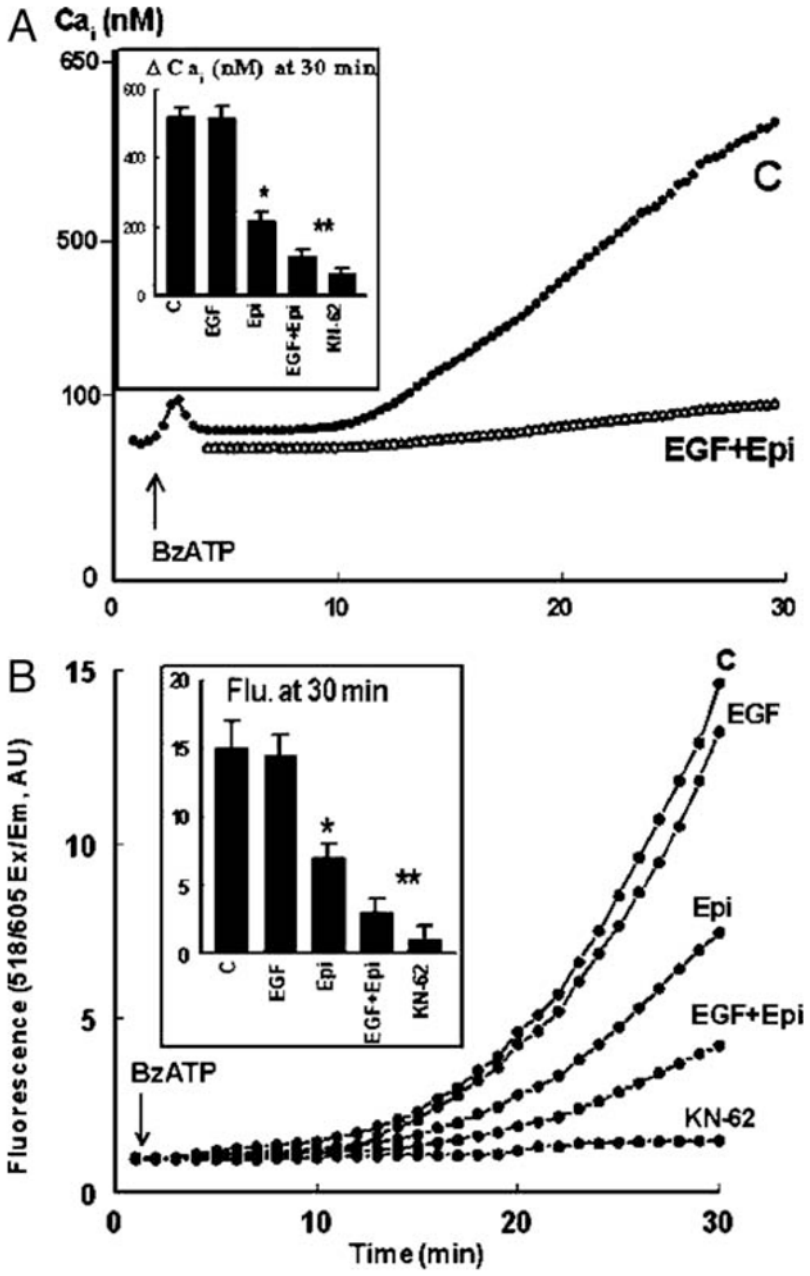

Fig. 4.

EGF and Epi modulation of BzATP-induced changes in cytosolic calcium (Ca_i) (A) and of BzATP-induced influx of ethidium bromide (B) in CaSki cells attached on filters. AU, Arbitrary units of fluorescence (Flu.) at 518/605 nm (excitation/emission). *Insets*, Means (± SD, three to five independent experiments) of the respective increases in Ca_i and in intracellular ethidium bromide Flu., 30 min after adding the BzATP. KN-62 was added at 100 nM, 15 min before adding BzATP. In *both insets*: *, $P < 0.01$ [Epi *vs.* C (control) and EGF groups]; **, $P < 0.03$ (EGF+Epi and KN-62 *vs.* Epi).

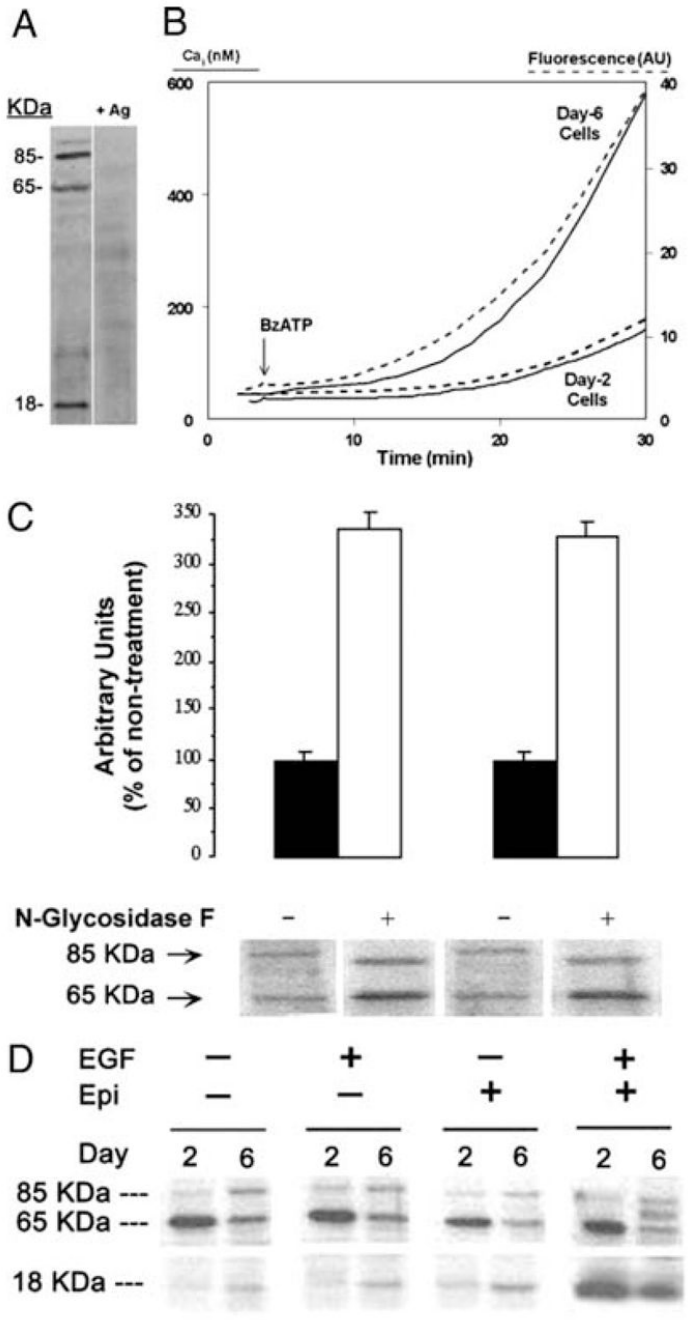

Fig. 5.

A, Western immunoblot analysis of $P2X_7$ receptor protein using total homogenates from d-6 cultured CaSki cells. + Ag denotes coincubation with the $P2X_7$ antigen. B, Effects of length in culture on BzATP (100 μM)-induced influx of Ca^{2+} (*solid lines*, determined in terms of the increase in Ca_i, and on BzATP-induced influx of ethidium bromide (*broken lines*). C, Effects of incubation of CaSki-cell lysates *in vitro* with *N*-glycosidase F. The *filled* and *empty bars* represent the densitometric ratio of the 65-kDa/85-kDa forms before and after treatment with *N*-glycosidase F, respectively. D, Effects of day in culture, and of treatments with EGF and Epi on the cellular expression of the $P2X_7$ receptor. Western immunoblot analysis was done on total homogenates of CaSki cells grown on filters for 2 or 6 d. Where indicated, cells were

treated with EGF, epinephrine, or both as described in the text. Results of three experiments are summarized in Table 1.

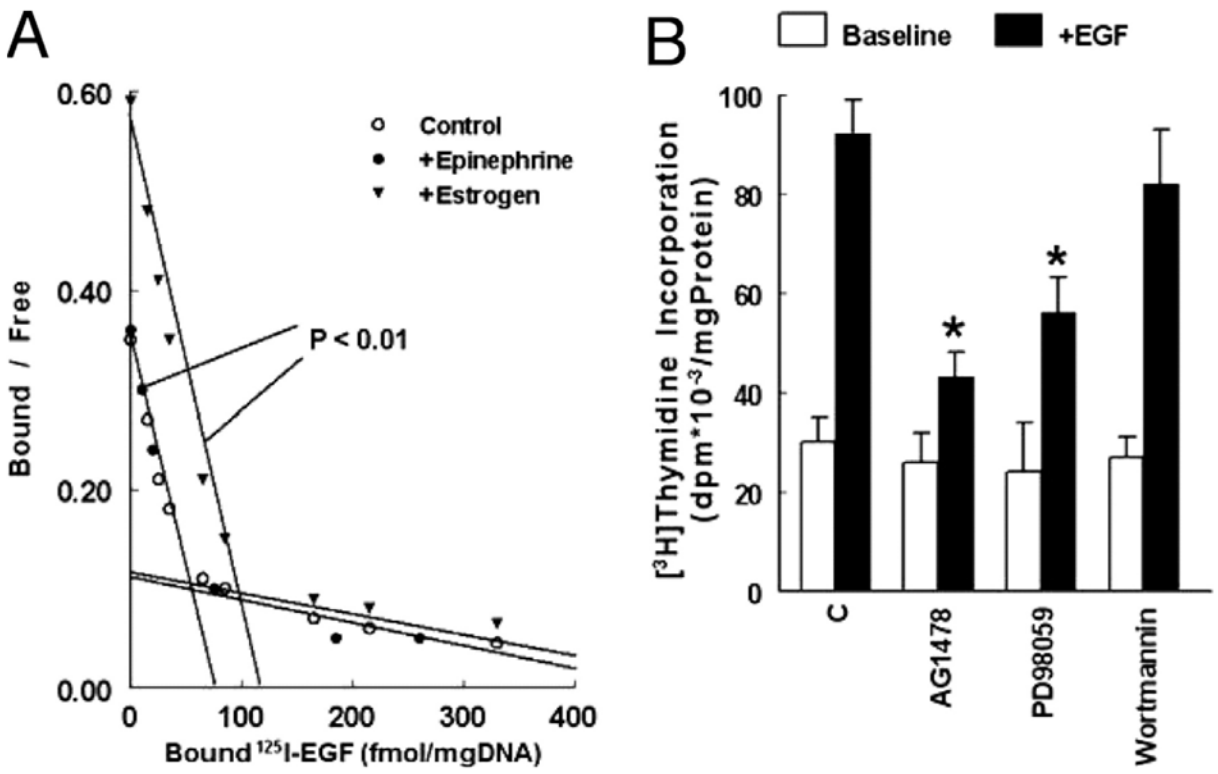

Fig. 6.

A, Scatchard analysis of [^{125}I]EGF binding to CaSki cells and the effects of epinephrine and 17β-estradiol. B, Modulation of EGF-induced DNA synthesis by AG1478, PD98059, and wortmannin. Data are means ± SD of three independent experiments. *, $P < 0.01$ [EGF category, for both AG1478 *vs.* C (control) and PD98059 *vs.* C].

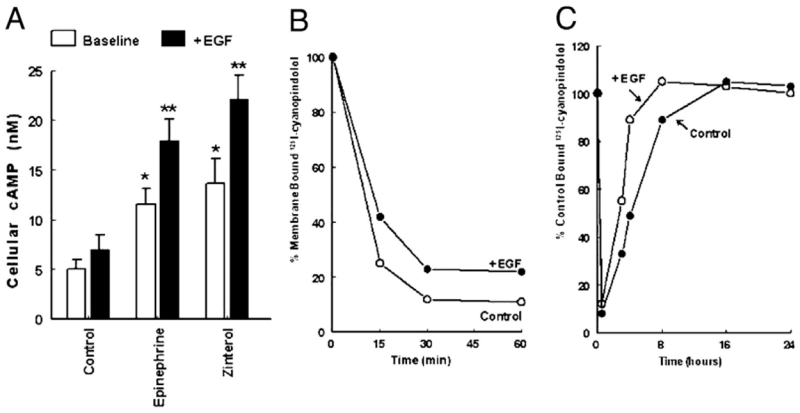

Fig. 7.

A, EGF-dependent modulation of epinephrine- and zinterol-induced increase in cellular cAMP. *, $P < 0.01$ (Baseline category, for both epinephrine *vs.* control, and zinterol *vs.* control); **, $P < 0.01$ (EGF category, for both epinephrine *vs.* control, and zinterol *vs.* control). B and C, EGF and wortmannin modulation of zinterol-induced changes in membrane-bound [^{125}I] cyanopindo-lol. B, Effects on [^{125}I]cyanopindolol internalization. C, Effects on [^{125}I] cyanopindolol recycling. The experiments are described in the text.

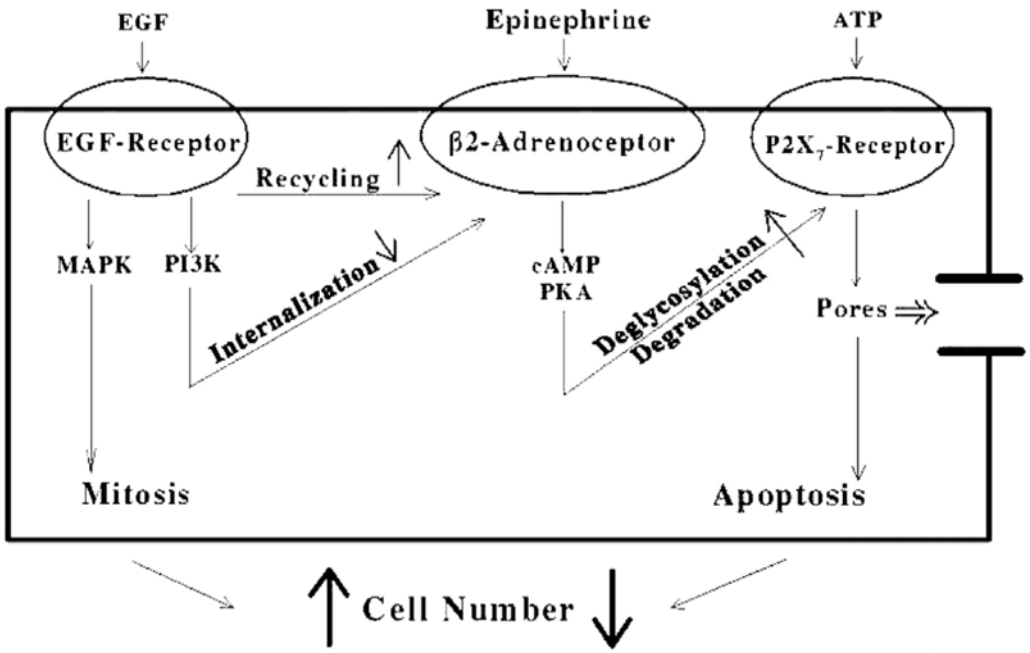

Fig. 8.

A novel signaling network communication among RTKs, GPCRs, and the purinergic $P2X_7$ receptor. In human cervical epithelial cells, EGF, acting via the MAPK cascade pathway, stimulates mitosis and proliferation. EGF also facilitates β2-adrenoceptor-dependent stimulation of deglycosylation and degradation of the $P2X_7$ receptor. The effect of EGF involves attenuation of β2-adrenoceptor internalization by a PI3K-dependent mechanism and facilitated recycling of the β2-adrenoceptor by a PI3K-independent mechanism. The EGF effects on β2-adrenoceptor endocytosis and recycling would tend to up-regulate β2-adrenoceptor responsiveness by increasing the pool of the receptor that is available for activation upon ligand binding. The composite effects of EGF and epinephrine would tend to facilitate inhibition of $P2X_7$ receptor-dependent pore formation and apoptosis. The combined EGF mitogenic and antiapoptotic effects would lead to an increase in cell number and to epithelial growth. PKA, Protein kinase A.

TABLE 1

Effects of day in culture and of treatments with EGF and epinephrine on the expression of the $P2X_7$ receptor

EGF	−		+		−		+	
Epinephrine	−		−		+		+	
Day in culture	2	6	2	6	2	6	2	6
$P2X_7$ receptor forms (kDa)								
85	10	80 ± 15[a]	10 ± 5	80 ± 5	10 ± 5	20 ± 5[b]	10 ± 5	5 ± 2[b]
75								10 ± 3
65	10	3 ± 2	10 ± 2	3 ± 2	5 ± 2[b]	1 ± 1[b]	20 ± 5	1 ± 2[b]
18	10	70 ± 10	10 ± 5	110 ± 10[b]	10 ± 5	150 ± 25[b]	500 ± 15[b]	350 ± 30[b]

Data are means (± SD) of densitometry analyses of three experiments (see Fig. 5).

[a] Arbitrary densitometric values normalized to the expression of the various P2X7 receptor forms in d-2 cells not treated with EGF or epinephrine.

[b] $P < 0.05$–0.01 compared with bands' densities in d-2 cells not treated with EGF or epinephrine.

TABLE 2

Modulation of EGF effects by wortmannin a and PD98059

	Control	EGF	EGF +PD98059	EGF +Wortmannin
Membrane bound [^{125}I] cyanopindolol (% at 60 min)	15.0 ± 1.2	22.5 ± 2.1[a]	23.2 ± 2.4	18.0 ± 1.3[b]
$t_{1/2}$ of increase in [^{125}I] cyanopindolol (min)	7.5 ± 0.6	4.1 ± 0.4[a]	4.2 ± 0.3[a]	4.5 ± 0.5[a]
% Solubilized DNA (in the presence of BzATP, epinephrine)	4.9 ± 0.3	3.6 ± 0.3[a]	3.6 ± 0.3[a]	4.3 ± 0.3[b]

The experiments are described in Figs. 7, B and C, and 1C, respectively. Data are means (± SD) of three to five independent experiments in each panel. The $t_{1/2}$ for increase in [^{125}I]cyanopindolol was determined from the curves (Fig. 7C).

[a] $P < 0.05$–0.01 compared with control.

[b] $P < 0.05$–0.01 compared with control, EGF, or EGF + PD98059 categories.