ErbB2 and ErbB3 Receptors Mediate Inhibition of Calcium-dependent Chloride Secretion in Colonic Epithelial Cells*

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We have previously demonstrated that epidermal growth factor (EGF) inhibits calcium-dependent chloride secretion via a mechanism involving stimulation of phosphatidylinositol 3-kinase (PI3-K). The muscarinic agonist of chloride secretion, carbachol (CCh), also stimulates an antiserective pathway that involves transactivation of the EGF receptor (EGFR) but does not involve PI3-K. Here, we have examined if ErbB receptors, other than the EGFR, have a role in regulation of colonic secretion and if differential effects on ErbB receptor activation may explain the ability of the EGFR to propagate diverse signaling pathways in response to EGF versus CCh. Basolateral, but not apical, addition of the ErbB3/ErbB4 ligand α-hergulin (HRG; 1-100 ng/ml) inhibited secretory responses to CCh (100 μM) across voltage-clamped T₈₄ epithelial cells. Immunoprecipitation/Western blot studies revealed that HRG (100 ng/ml) stimulated tyrosine phosphorylation and dimerization of ErbB3 and ErbB2, but had no effect on phosphorylation of the EGFR. HRG also stimulated recruitment of the p85 subunit of PI3-K to ErbB3/ErbB2 receptor dimers, while the PI3-K inhibitor, wortmannin (50 nM), completely reversed the inhibitory effect of HRG on CCh-stimulated secretion. Further studies revealed that, while both EGF (100 ng/ml) and CCh (100 μM) stimulated phosphorylation and dimerization of ErbB3 and ErbB2, but had no effect on phosphorylation of the EGFR. HRG also stimulated recruitment of the p85 subunit of PI3-K to ErbB3/ErbB2 receptor dimers, while the PI3-K inhibitor, wortmannin (50 nM), completely reversed the inhibitory effect of HRG on CCh-stimulated secretion. Further studies revealed that, while both EGF (100 ng/ml) and CCh (100 μM) stimulated phosphorylation of the EGFR, only EGF stimulated phosphorylation of ErbB2, and neither stimulated ErbB3 phosphorylation. EGF, but not CCh, stimulated the formation of EGFR/ErbB2 receptor dimers and the recruitment of p85 to ErbB2. We conclude that ErbB2 and ErbB3 are expressed in T₈₄ cells and are functionally coupled to inhibition of calcium-dependent chloride secretion. Differential dimerization with other ErbB family members may underlie the ability of the EGFR to propagate diverse inhibitory signaling pathways in response to activation by EGF or transactivation by CCh.

The movement of water across intestinal epithelial cells is a passive process driven by the active transport of ions. Under normal circumstances sodium and chloride are actively absorbed from the intestine, creating an osmotic gradient favorable for fluid absorption. However, several pathological conditions are characterized by alterations in intestinal ion transport processes, consequently resulting in abnormalities of fluid transport (1). In such conditions, it is thought that the absorptive capacity of the intestine is overwhelmed by excessive fluid secretion, leading to the clinical manifestation of secretory diarrhea. Conversion of the epithelium from a net absorptive to a secretory state can occur through the actions of neuronal, hormonal, and immunologically derived substances that are released, for example, during conditions of bowel inflammation. Typically, such agents promote chloride secretion by binding to specific receptors on the surface of epithelial cells, thereby increasing levels of intracellular second messengers, such as cyclic nucleotides and calcium. This, in turn, activates prosecretory signaling pathways, which ultimately interact with, and activate, the transport proteins that comprise the chloride secretory mechanism (1, 2).

In addition to prosecretory signaling pathways, it is becoming evident that mechanisms also exist within epithelial cells that result in down-regulation of chloride secretion (3). For example, treatment of epithelial cells with epidermal growth factor (EGF) results in inhibition of subsequent chloride secretory responses to calcium-dependent agonists, such as carbachol (CCh) (4). This effect of EGF is mediated by activation of phosphatidylinositol 3-kinase (PI3-K) (5). Furthermore, we have recently demonstrated that, in addition to stimulating secretion, agonists such as CCh also stimulate tyrosine kinase-dependent signaling pathways that limit the extent of ongoing secretory responses (6). These pathways involve transactivation of the EGFR and subsequent activation of the extracellular signal-regulated kinase isoforms of mitogen-activated protein kinase. However, CCh-induced activation of the EGFR receptor (EGFR), unlike that induced by EGF itself, does not result in an increase in the lipid kinase activity of PI3-K (5). One possible explanation for this apparent differential propagation of signaling pathways by the EGFR in response to activation by EGF, or transactivation by CCh, respectively, may lie in the complex nature of molecular interactions between the EGFR and its related growth factor receptors, which together constitute the ErbB family of receptor tyrosine kinases.

The EGFR receptor (EGFR; ErbB1) is the prototypic member of the ErbB family. To date, three other members of this family have been identified: ErbB2 (p185<sup>erbB2</sup>), ErbB3 (p180<sup>erbB3</sup>), and ErbB4 (p180<sup>erbB4</sup>) (7–9). Despite the large degree of structural homology between them, ErbB family members differ from each other in their patterns of expression, ligand specificity, and intracellular substrates. Ligands that bind to and activate ErbB receptors can broadly be divided into two classes, 1

1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CCh, carbachol; HRG, hergulin; Iₛ, short circuit current; PI3-K, phosphatidylinositol 3-kinase; ANOVA, analysis of variance.
those that bind the EGFR, such as EGF and transforming growth factor-α, and those which bind to ErbB3 and ErbB4, the heresulins (HRG). ErbB2 has been classified as an orphan receptor due to the lack of a known ligand that binds this receptor. ErbB receptors also differ in their kinase activity; whereas ErbB3 lacks intrinsic kinase activity, ErbB2 is the most catalytically active member of the ErbB family. The transduction of extracellular signals via ErbB receptors is a complex process, involving what can be considered as both lateral and vertical signaling pathways. Generally, upon ligand binding, ErbB receptors undergo autophosphorylation and dimerization to form catalytically active homo- or heterodimers, with ErbB2 being the preferred partner for the other three activated receptors (10–12). Depending on the type of dimer formed, specific sets of SH2-containing proteins interact with the dimer complex, ultimately resulting in the activation of different intracellular effector proteins, such as mitogen-activated protein kinase, PI3-K, and phospholipase C-γ. The multiplicity of ErbB receptor ligands, possible combinations of ligand-stimulated receptor dimers, and the existence of multiple downstream effector proteins, confers this family of receptors with an enormous potential for regulation and diversification of intracellular signaling pathways (9, 11, 13–16).

Although overexpression of ErbB receptors, particularly ErbB2, has been correlated with the development of epithelial tumors (15, 17, 18), there is little information regarding potential roles for ErbB receptors in acute regulation of epithelial function. Therefore, in the present study, we have employed the T84 colonic epithelial cell line to examine a possible role for ErbB family members, other than the EGFR, in regulation of intestinal chloride secretion and to examine the possibility that differential formation of receptor dimer complexes may underlie the ability of the EGFR to propagate diverse inhibitory signals in response to activation of the receptor by EGF itself, or in response to transactivation by CCh.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human α-HRG (R&D Systems, Minneapolis, MN), epidermal growth factor (Genzyme, Cambridge, MA), mouse monoclonal anti-human EGFR mouse anti-recombinant rat ErbB3 rabbit polyclonal anti-human p85, and mouse monoclonal anti-phosphotyrosine antibodies (Upstate Biotechnology Inc., Lake Placid, NY), rabbit polyclonal antibodies against a peptide corresponding to the carboxyl terminus (1169–1186) of human ErbB2 (Santa Cruz Biotechnology, Santa Cruz, CA), and Tris-glycine electrophoresis gels (Bio-Rad) were obtained from the sources indicated. All other reagents were of analytical grade and were obtained commercially.

**Cell Culture**—Methods for maintenance of T84 cells in culture were as described previously (19). Briefly, T84 cells were grown in Dulbecco's modified Eagle's medium/F-12 medium (JRH, Lenexa, KS) supplemented with 5% newborn calf serum. Cells were passaged by trypsinization. For Ussing chamber/voltage clamp experiments, approximately 5 × 10⁵ cells were seeded onto 12-mm Millicell transwell polycarbonate filters. For Western blotting/immunoprecipitation experiments, approximately 10⁶ cells were seeded onto 30-mm Millicell transwell polycarbonate filters. Cells seeded onto filters were cultured for 10–15 days prior to use.

**Electrophysiological Studies**—Monolayers of T84 cells were cultured in Ussing chambers (window area = 0.6 cm²) and bathed in oxygenated (95% O₂, 5% CO₂) Ringer's solution at 37 °C. The composition of the Ringer's solution was (in mM): 140 Na⁺, 5.5 K⁺, 1.2 Ca²⁺, 0.8 Mg²⁺, 120 Cl⁻, 25 HCO₃⁻, 2.4 HPO₄²⁻, 0.4 HPO₄³⁻, and 10 glucose. Monolayers were voltage-clamped to zero potential difference by the application of short-circuit current (Iₛc). Under these conditions, changes in Iₛc in response to agonists are wholly reflective of electrogenic chloride secretion (20).

**Immunoprecipitations and Western Blotting**—T84 cell monolayers grown on filters were washed (twice) with Ringer's solution, allowed to equilibrate for 30 min at 37 °C, and then stimulated with agonists for the times indicated. The reaction was stopped by washing in ice-cold phosphate-buffered saline, and the cells were lysed in ice-cold lysis buffer (1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM Na₂-vanadate, 1 mM NaF, and 1 mM EDTA in phosphate-buffered saline) for 45 min. Cells were then scraped into microcentrifuge tubes and spun at 12,000 rpm for 10 min, and the pellets were discarded. Samples were adjusted for protein content (Bradford assay) and adjusted so that each sample contained an equal amount of protein. For immunoprecipitation studies, lysates were incubated with the appropriate diluted immunoprecipitating antibody for 1 h at 4 °C, followed by another 1-h incubation at 4 °C with protein A-Sepharose. Lysates were then centrifuged for 3 min at 15,000 rpm, and the supernatant was discarded. The pellets were then washed twice in lysis buffer and twice in phosphate-buffered saline and were then resuspended in 2× gel loading buffer (50 mM Tris, pH 6.8, 2% SDS, 200 mM dithiothreitol, 20% glycerol, 0.2% bromophenol blue). Samples were boiled for 3 min and then loaded onto a polyacrylamide gel, and proteins were separated by electrophoresis. Resolved proteins were transferred overnight at 4 °C onto a polyvinylidene membrane (NER Life Science Products). After transfer the membrane was preblocked with a 1% solution of blocking buffer (Upstate Biotechnology Inc.) for 30 min, followed by a 1-h incubation with the appropriate concentration of primary antibody in 1% blocking buffer. After washing (three times for 10 min each) in Tris-buffered saline with 1% Tween (TBST), membranes were then incubated for 30 min in horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG/ horseradish peroxidase; Transduction Laboratories, Lexington, KY) in 1% blocking buffer. This was followed by three 10-min washes in TBST. Immunoreactive proteins were detected using a chemiluminescence detection kit (Roche Molecular Biochemicals). Densitometric analysis was carried out using NIH Image software.

**Statistical Analyses**—All data are expressed as mean ± S.E. for a series of n experiments. Student's t tests or analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test were used to compare mean values as appropriate. p values < 0.05 were considered to represent significant differences.

**RESULTS**

**Heregulin Inhibits CCh-stimulated Chloride Secretion in T84 Cells**—First, we set out to determine if ErbB receptors, other than the EGFR, are functionally expressed in colonic epithelial cells. To do this, we examined the effects of the ErbB3/ErbB4 ligand, α-HRG, on Iₛc responses to CCh across voltage-clamped monolayers of T84 cells. Pretreatment of T84 cells with basolateral HRG (100 ng/ml; 20 min) resulted in significant attenuation of subsequent Iₛc responses to basolateral CCH (100 μM) (Fig. 1A). Maximal responses to CCh were 45.7 ± 4.7 μA/cm² and 24.8 ± 2.8 μA/cm² in the absence and presence of HRG, respectively (p < 0.001; n = 9). The effects of basolateral HRG were concentration-dependent, with a maximal effect occurring at approximately 100 ng/ml (Fig. 1B). Of note, a residual response to CCh persisted that was insensitive to even the highest concentration of HRG tested. These data are reminiscent of those previously reported for EGF (4). HRG was without effect on CCh-stimulated Iₛc when added to the apical side of the monolayer. Responses to CCh (100 μM) were 48.3 ± 4.5 μA/cm² and 44.7 ± 6.2 μA/cm² in the absence and presence of apical HRG (100 ng/ml), respectively (n = 6). HRG alone had no effect on basal Iₛc.

**Heregulin Stimulates Tyrosine Phosphorylation and Dimerization of ErbB2 and ErbB3 in T84 Cells**—We next examined the effects of the growth factor on protein tyrosine phosphorylation in T84 cells. Basolateral HRG (100 ng/ml) induced a time-dependent increase in the tyrosine phosphorylation of high molecular mass (~180 kDa) proteins (Fig. 2), with a maximal effect occurring at approximately 15 min. In order to determine if these protein bands corresponded to receptor dimerization in response to HRG, experiments were next carried out in which cells were stimulated with HRG (100 ng/ml); lysates were immunoprecipitated with antibodies to EGFR, ErbB2, or ErbB3; and Western blots were analyzed with anti-phosphotyrosine. HRG was found to stimulate tyrosine phosphorylation of both ErbB2 and ErbB3, but had no effect on tyrosine phos-
Phosphorylation of EGFR (Fig. 3). Expression of ErbB4 in T84 cells was not detected (data not shown). Further experiments were carried out in which cells were stimulated with HRG (100 ng/ml), lysates were immunoprecipitated with anti-ErbB3, and immunoprecipitated proteins were analyzed by Western blotting with anti-ErbB2. These experiments revealed that, in addition to stimulating tyrosine phosphorylation of ErbB3 and ErbB2, HRG also stimulates the formation of ErbB3/ErbB2 receptor dimers (Fig. 4).

**PI3-K Mediates the Inhibitory Effect of HRG on CCh-stimulated Isc**—We next went on to determine the signaling pathway mediating the effects of HRG on CCh-stimulated secretion. First, in voltage clamp experiments we found that the inhibitory effects of maximally effective concentrations of EGF (100 ng/ml) and HRG (100 ng/ml) on CCh-stimulated chloride secretion were not additive, implying EGF and HRG exert their antisecretory effects via a common signaling pathway (Fig. 5A). Since previous studies from our laboratory have demonstrated that PI3-K mediates the inhibitory effects of EGF on CCh-stimulated secretion, we therefore examined a possible role for PI3-K in also mediating the inhibitory effects of HRG. Cells were stimulated with HRG (100 ng/ml), cell lysates were immunoprecipitated with anti-ErbB3 or with anti-ErbB2, and immunoprecipitated proteins were analyzed by Western blotting with antibodies to the p85 subunit of PI3-K. The data demonstrate that HRG increased co-immunoprecipitation of p85 with both ErbB2 and ErbB3 (Fig. 5, C and D), indicating HRG likely stimulates activation of PI3-K. Thus, we examined the effects of the PI3-K inhibitor, wortmannin, on HRG-mediated inhibition of CCh-stimulated Isc (Fig. 5D). Of note, and as previously reported (5), wortmannin (50 nM) did not significantly alter responses to CCh alone.

**EGF and CCh Differentially Stimulate ErbB Receptor Tyrosine Phosphorylation, Dimerization, and Recruitment of p85 to ErbB Receptor Complexes in T84 Cells**—While both EGF and CCh stimulate activation of the EGFR in T84 cells, only EGF stimulates increases in PI3-K activity (5). We therefore set out to determine if differential stimulation of ErbB receptors might
underlie the ability of the EGFR to propagate diverse signals in response to stimulation by EGF versus CCh. Cells were stimulated with either CCh (100 μM) or EGF (100 ng/ml), and cell lysates were immunoprecipitated with antibodies to EGFR, ErbB2, or ErbB3. Immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine. As previously reported (6), both CCh and EGF stimulated EGFR phosphorylation (Fig. 6A). However, only EGF stimulated an increase in tyrosine phosphorylation of ErbB2 (Fig. 6B) and neither agonist appeared to have an effect on ErbB3 phosphorylation, although ErbB3 phosphorylation could readily be detected in HRG-stimulated cells (Fig. 6C).

In similar experiments, EGF- and CCh-stimulated T84 cell lysates were immunoprecipitated with anti-EGFR, followed by Western blotting with anti-ErbB2. Data from these studies revealed that only EGF, and not CCh, stimulated the formation of EGFR/ErbB2 receptor dimers (Fig. 7A). Finally, experiments were carried out to determine if the apparent difference in the ability of EGF and CCh to stimulate activation of ErbB2 might underlie the difference in their ability to stimulate PI3-K. T84 cell monolayers were stimulated with EGF (100 ng/ml) or CCh (100 μM), and lysates were immunoprecipitated with antibodies to EGFR or ErbB2. Western blots were then probed with antibodies to the p85 subunit of PI3-K. We found that, although both EGF and CCh stimulated recruitment of p85 to the EGFR (Fig. 7B), only EGF stimulated the recruitment of p85 to ErbB2 (Fig. 7C).

**DISCUSSION**

In the present study we provide further evidence that the ErbB family of growth factor receptors plays an important role in regulation of intestinal epithelial ion transport. In addition to the EGFR, we have shown that colonic epithelial cells also express ErbB3 and ErbB2 and that activation of these receptors with HRG, a growth factor that is expressed in the intestinal mucosa (21), results in inhibition of calcium-dependent chloride secretory responses. *In vivo*, such an effect on chloride secretion would be accompanied by a reduction in net fluid secretion into the intestinal lumen. The effects of HRG appear to be mediated by the formation of ErbB2/ErbB3 receptor dimers, since HRG increased tyrosine phosphorylation of both these receptor types and increased co-immunoprecipitation of ErbB2 with ErbB3. Since no known ligands bind directly to ErbB2, the formation of ErbB2/ErbB3 dimers is likely brought about by HRG first binding to ErbB3 followed by recruitment of catalytically active of ErbB dimer complexes (7, 18, 22–24). It is also noteworthy that, in T84 cells, HRG did not stimulate phosphorylation of the EGFR, indicating that there is a similar selectivity and specificity of growth factor/ErbB receptor interactions in the intestinal epithelium to that seen in other tissues (9, 14).

Similar to EGF (5), the effects of HRG in inhibiting CCh-stimulated chloride secretion appear to be mediated via stimulation of PI3-K activity, an enzyme that mediates the effects of HRG in several other cell types (18, 23–25). This conclusion is
FIG. 6. EGF and CCh differentially stimulate ErbB receptor phosphorylation in T84 cells. Cells were stimulated with CCh (100 μM) or EGF (100 ng/ml) for the times indicated and lysates were immunoprecipitated with antibodies to EGFR (A), ErbB2 (B), or ErbB3 (C). Immunoprecipitated proteins were then Western blotted with antiphosphotyrosine. Although both CCh and EGF stimulated phosphorylation of EGFR (p < 0.05 and p < 0.001, respectively, by ANOVA), only EGF was found to stimulate phosphorylation of ErbB2 (p < 0.005). Neither CCh nor EGF had any effect on ErbB3 phosphorylation, although, as can be seen in panel C, tyrosine phosphorylation of the receptor was readily detected in response to HRG (100 ng/ml; 15 min). Panels D and E show the densitometric analysis of these data (n = 3–6 for each experiment).

The present studies demonstrate that, depending on the activating agonist, stimulation of the EGFR results in the formation of different receptor dimer complexes. Activation of the EGFR by its cognate ligand, EGF, not only leads to increased tyrosine phosphorylation of the EGFR, but also results in increased phosphorylation of ErbB2, accompanied by the formation of EGFR/ErbB2 receptor dimer complexes. The formation of this complex is rapid, occurring within 1 min, is stable for prolonged periods of time, and thus mirrors the time course of the inhibitory effects of EGF on calcium-stimulated chloride secretion (4). In contrast, transactivation of the EGFR by CCh is not accompanied by appreciable stimulation of ErbB2 phosphorylation nor does it bring about the formation of EGFR/ErbB2 receptor dimer dimers. This is in contrast to previous studies in rat fibroblasts where G-protein-coupled receptor agonists, such as lysophosphatidic acid and thrombin, were found to stimulate both EGFR and ErbB2 phosphorylation (30). This apparent difference in the responsiveness of ErbB2 receptors to G-protein-coupled receptor-mediated tyrosine phosphorylation in fibroblasts and colonic epithelial cells underlines the inherently heterogeneous nature of signaling within the ErbB family of receptor tyrosine kinases.

It is not yet clear how activation of the EGFR by EGF itself, or transactivation by CCh, leads to differential phosphorylation of ErbB2 and recruitment to the EGFR. However, it is likely that this may be due to differential phosphorylation of tyrosine residues on the EGFR (13). Whereas the intrinsic tyrosine kinase activity of the EGFR mediates autophosphoryl-
in the intestinal epithelium may provide a means by which
As depicted in Fig. 8, we propose that ErbB receptor expression
by the recruitment of PI3-K to ErbB3/ErbB2 receptor dimers.
results in inhibition of subsequent calcium-dependent chloride
which showed that CCh increases the amount of both the p85
with the EGFR. This is in agreement with our previous data,
note that, even though it does not stimulate the lipid kinase
T84 colonic epithelial cells also express the ErbB2 and ErbB3
the physiological significance of this effect may be since, as
stimulates PI3-K recruitment to the EGFR, it is unclear what
inhibition by growth factors, such as EGF and
receptors and hormones. These studies, along with emerging
evidence to suggest a potential role for growth factors, such as
healing mucosal ulcers associated with intestinal
37–39), may provide the basis for novel approaches in the
treatment of intestinal inflammatory disorders.

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