Role of protein phosphatase 2A in calcium-dependent chloride secretion by human colonic epithelial cells

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Chow JYC, Barrett KE. Role of protein phosphatase 2A in calcium-dependent chloride secretion by human colonic epithelial cells. Am J Physiol Cell Physiol 292: C452–C459, 2007. First published August 9, 2006; doi:10.1152/ajpcell.00034.2006.—EGF inhibits carbachol-induced chloride secretion by regulating a basolateral potassium channel via phosphatidylinositol 3-kinase (PI 3-kinase) and PKCe activation. Although both EGF and carbachol cause tyrosine phosphorylation of p85 of PI 3-kinase, only EGF activates the enzyme. Serine phosphorylation of p85 is thought to suppress the lipid kinase of PI 3-kinase. Our present study examined whether the differential effects of carbachol and EGF on PI 3-kinase activity correspond to varying phosphorylation of p85, and the mechanisms and consequences. T84 colonic epithelial cells were treated with either EGF or carbachol. Cell lysates were immunoprecipitated with p85 antibody and blotted with either phosphotyrosine or phosphoserine antibodies. Protein phosphatase (PP) 1 and 2A activities were also measured. Both tyrosine and serine residues of p85 were phosphorylated by carbachol, whereas EGF induced only tyrosine phosphorylation. Moreover, EGF abolished carbachol-induced serine phosphorylation of p85 and activated PP2A without affecting PP1. Carbachol did not affect either phosphatase. Calyculin A or okadaic acid pretreatment reversed the inhibitory action of EGF on carbachol-induced chloride secretion and restored serine phosphorylation of p85. Although carbachol recruits p85, it phosphatases both serine and tyrosine residues so that the lipid kinase of PI 3-kinase is inhibited. EGF results in p85 tyrosine phosphorylation as well as dephosphorylation of serine residues via the activation of PP2A. This explains the differential induction of PI 3-kinase enzyme activity in response to EGF and/or carbachol and has functional implications. Our data provide further insights into negative signals that regulate chloride secretion and into the molecular basis of signaling diversification in the intestinal epithelium.

epithelial secretion; PI 3-kinase; EGF

FLUID TRANSPORT ACROSS intestinal epithelial cells is a complex and tightly regulated process with net absorption prevailing under normal physiological conditions. Chloride secretion plays an important role in mucosal hydration in health throughout the gastrointestinal tract. It also contributes to body fluid homeostasis by driving fluid secretion. Epithelial fluid secretion may also be upregulated in response to a wide range of physiological stimuli. Fluid secretion can be advantageous because it aids the smooth passage of digested materials throughout the intestine and the removal of waste products from the intestinal tract before they can gain access to the systemic circulation. Nevertheless, under pathological conditions such as during bacterial infections, an uncontrolled upregulation of epithelial fluid secretion can occur, leading to secretory diarrhea.

The ion transport pathways comprising the chloride secretory mechanism of T84 cells, a line of human colonic epithelial cells with a crypt cell phenotype, have been well defined (12, 13). Chloride is taken up across the basolateral membrane of the cells via a Na+/K+-2Cl⁻ cotransporter and exits the cell across the apical membrane via chloride channels. An important member of such chloride channels is the cystic fibrosis transmembrane conductance regulator. Basolateral potassium channels support chloride secretion by allowing for potassium recycling, whereas energy for the process is supplied by the activity of a basolateral Na⁺,K⁺-ATPase. Primary control for the overall transport process occurs at the level of apical chloride channels and basolateral potassium channels, in response to agonists that elevate positive second messengers for chloride secretion, namely, cyclic nucleotides and cystosolic calcium. Signaling mechanisms intrinsic to the epithelium can also inhibit secretion. Thus the muscarinic agonist carbachol initially activates secretion in a calcium-dependent fashion and then renders cells refractory to additional stimulation. Furthermore, growth factors such as epidermal growth factor (EGF) inhibit secretion induced by calcium-dependent agonists, including carbachol, without themselves serving as positive effectors of secretion.

Previously, work in our laboratory (35) has shown that the inhibitory effect of EGF on chloride secretion in T84 cells is due to its ability to stimulate phosphatidylinositol (PI) 3-kinase and the production of 3-phosphorylated lipids. However, whereas both carbachol and EGF recruit PI 3-kinase, only EGF increases the activity of this enzyme. Nuclide efflux studies showed that EGF reduced calcium-stimulated basolateral ⁸⁶Rb⁺ efflux, but not apical ¹²⁵I⁻ efflux, suggesting that a basolateral potassium channel constitutes the target of the PI 3-kinase-dependent negative signaling pathway. PI 3-kinase is a ubiquitous lipid kinase that phosphorylates the 3-position of the inositol ring of inositol phospholipids to generate such lipid messengers as phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. The exact role and molecular targets of these lipid products have not been fully elucidated, although increasing evidence suggests that they may serve as intracellular second messengers (14, 29). PI 3-kinase is a heterodimer consisting of a p85 regulatory subunit with SH2 domains and a p110 catalytic subunit (34). Regulation of the p85/p110 PI 3-kinase is complex. Membrane recruitment of p85 can be stimulated by growth factors or other stimuli that activate...
tyrosine kinase activity, presumably via SH2 binding. p85 likewise has been shown to bind p110 via the inter-SH2 region (10) once p85 is activated. Activation of the p85 subunit requires tyrosine phosphorylation to permit binding and activation of p110. However, Dhand et al. (11) additionally identified serine 608 in the inter-SH2 domain of p85 as playing a critical role as an inhibitory regulatory site. Phosphorylation of this site apparently converts p110 into a protein kinase.

In this study, we hypothesized that carbachol and EGF exhibit different effects on PI 3-kinase activity because they differentially alter the balance of serine and tyrosine phosphorylation of the p85 subunit. Furthermore, we predicted that this would alter chloride secretion. We have determined that EGF reduces serine phosphorylation of p85, likely by activating a protein phosphatase that in turn regulates PI 3-kinase activity and its functional consequences.

MATERIALS AND METHODS

Materials. The following materials were purchased from the sources indicated: okadaic acid and calycin A from Alexis (San Diego, CA); [γ-32P]ATP from NEN New England Biolabs (Beverly, MA); EGF from Genzyme (Cambridge, MA); Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F12) from JRH (Lex- nexa, KS); monoclonal antibodies directed against the p85 subunit of PI 3-kinase and positive controls (Jurkat, human fibroblast, and macrophage cell extracts) and goat anti-mouse horseradish peroxidase secondary antibody from Transduction Laboratories (Lexington, KY); Hybond ECL polyvinylidene difluoride (PVDF) membrane, Kodak X-ray films, and ECL Plus detection kit from Amersham Pharmacia Biotech (Piscataway, NJ); cell culture membrane inserts (Millipelle, 0.45-μm-pore size mixed cellulose ester) from Millipore (Bedford, MA); Me2SO, Tween-20 (EIA grade), dithiothreitol, glycine, Trizma, sodium fluoride, and 1 nM EDTA in PBS) for 45 min. Cells were then scraped into microcentrifuge tubes and spun at 12,000 rpm for 10 min, and the pellet was discarded. Samples were analyzed for protein content (Bio-Rad protein assay kit) and adjusted so that each sample contained an equal amount of protein. For immunoprecipitation studies, lysates were incubated with immunoprecipitating antibody, per the manufacturer’s instructions, 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 washed in PBS (3 ×) and resuspended in 2× gel loading buffer (50 mM Tris, pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, and 20% glycerol), boiled for 5 min, and then loaded onto a polyacrylamide gel for resolution. Separated proteins were transferred overnight at 4°C onto a PVDF membrane. After transfer, the membrane was preblocked with a 1% solution of blocking buffer for 30 min followed by a 1-h incubation with the appropriate concentration of primary antibody in 1% blocking buffer. After washing (4 × 10 min) in Tris-buffered saline with 1% Tween (TBST) membranes were incubated for 30 min in horseradish peroxidase-conjugated secondary antibody in 1% blocking buffer. After washing in TBST (4 × 10 min), immunoreactive proteins were detected using an enhanced chemiluminescent detection kit. In all studies, blots were stripped and reprobed with the immunoprecipitated antibody to ensure adequate loading of the protein of interest in each lane. Densitometric analysis of the Western blots was performed using NIH Image digital imaging software.

Protein phosphatase activity assay. The procedures were described in the manufacturer’s instruction manual. Briefly, a kinase reaction was initiated by adding 0.5 mM of [γ-32P]ATP to the phosphorylation reaction buffer that contained Fospholipase A (a substrate of protein phosphatases). The reaction was incubated at 30°C for 1 h and was terminated by addition of diluted ammonium sulfate solution. The precipitated protein was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was discarded, and the protein pellet was washed five times with ice-cold diluted ammonium sulfate solution. The protein pellet was then dissolved in solubilization buffer (50 mM Tris·HCl, 0.1 mM EDTA, 15 mM caffeine, and 1% β-mercaptoethanol). The solution was then applied to a concentrator and purified by centrifugation at 5,000 × g for 30 min at 20°C. The retentate containing radiolabeled phosphorylase A was then stored at 4°C until use in the protein phosphatase assay. Twenty microliters of protein extract were mixed with 20 μl of protein phosphatase assay buffer (2 mM EDTA, 20 mg/ml BSA, 400 mM imidazole·HCl, and 2% β-mercaptoethanol), and the reaction was initiated by addition of 20 μl of radioactive phosphorylase A. The reaction was allowed to proceed at 30°C for 10 min and was terminated by addition of TCA solution. The mixture was centrifuged at 12,000 × g for 3 min at 4°C, and the supernatant was used to determined the amount of radioactivity released as free 32P. Protein phosphatases 1 and 2A are the two most prominent types of protein phosphatases found in mammalian cells and can be distinguished from type 2B and 2C phosphatases by their preference for protein phosphatases 1 and 2A are the two most prominent types of protein phosphatases found in mammalian cells and can be distinguished from type 2B and 2C phosphatases by their preference for protein phosphatases 1 and 2A are the two most prominent types of protein phosphatases found in mammalian cells and can be distinguished from type 2B and 2C phosphatases by their preference for.
To confirm this finding, we first pretreated T84 cells with either EGF and/or carbachol, followed by immunoprecipitation of the cell lysates with an anti-p85 antibody and Western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 1, EGF stimulated tyrosine phosphorylation of p85 as early as 1 min after addition, with the level of phosphorylation sustained at least for 5 min. Similarly, carbachol also possessed the ability to stimulate tyrosine phosphorylation of p85 at 1 min after its addition. However, unlike EGF, the effect of carbachol on tyrosine phosphorylation of p85 appeared somewhat more transient (Fig. 2).

Effects of EGF and/or carbachol on serine phosphorylation of p85. Regulation of PI 3-kinase activity depends on the association of p85 and p110. If they do not interact with each other, then the enzyme will not function as a lipid kinase. In addition to tyrosine phosphorylation, regulation of the association of these two subunits also has been shown to be regulated by the balance of phosphorylated serine and tyrosine residues on p85. We therefore examined the ability of EGF and carbachol to induce serine phosphorylation of p85. Again, cells were pretreated with either EGF and/or carbachol, and the lysates were immunoprecipitated with an antibody against the p85 protein followed by Western blotting with an anti-phosphoserine antibody. EGF treatment alone did not induce serine phosphorylation of p85 (data not shown). Carbachol, however, caused serine phosphorylation of p85 as early as 1 min after addition, an effect that was sustained for at least 5 min (Fig. 3). Interestingly, in cells pretreated with EGF for 15 min, followed by acute treatment with carbachol, there was no longer any evidence of serine phosphorylation of p85 (Fig. 4). This finding suggests that EGF may block the ability of carbachol to induce serine phosphorylation of p85 and/or may induce dephosphorylation of serine residues of p85 via the activation of phosphatase(s). We therefore first examined possible candidates that mediate p85 serine dephosphorylation in response to EGF.

Effects of EGF and/or carbachol on activities of protein phosphatases. Protein phosphatase 2A is a serine/threonine phosphatase. Regulation of this enzyme is not fully understood and controversial. It has been shown to be induced by growth factors, including EGF, in some studies, although it also has been shown to be inactivated by EGF via tyrosine phosphor-
ylation (6). In our cell system, immunoprecipitates of the protein phosphatase 2A catalytic subunit did not appear to undergo tyrosine phosphorylation in response to EGF (data not shown). If tyrosine phosphorylation is a key step to inactivate the phosphatase, then the activity of this enzyme in our system is apparently not negatively affected by EGF. We then studied the effect of either EGF or carbachol on the activities of protein phosphatases 1 and 2A. Lysates from T84 cells treated with EGF or carbachol were incubated with radiolabeled phosphorylase A, a substrate of these protein phosphatases. Protein phosphatases 1 and 2A are the two most prominent types of protein phosphatases found in mammalian cells and can be distinguished from type 2B and 2C phosphatases by their preference for phosphorylase A as a substrate (8, 17). Carbachol did not affect protein phosphatase 2A activity at any time point examined (Fig. 5). On the other hand, EGF caused a significant increase in the activity of protein phosphatase 2A as early as 1 min after addition of the growth factor (Fig. 6). To assess the specificity of this finding, we also assessed the ability of EGF to activate protein phosphatase 1. However, the activity of this enzyme was unchanged following EGF treatment (Fig. 7). We concluded that protein phosphatase 2A is an EGF target that may sustain PI 3-kinase signaling in growth-factor treated cells secondary to serine dephosphorylation of p85, and thus would be predicted to play a role in regulating chloride secretion.

Effects of okadaic acid or calyculin A on the inhibitory action of EGF on carbachol-induced chloride secretion. To assess whether protein phosphatase 2A plays a role in the inhibitory effect of EGF on chloride secretion, we examined the effects of two protein phosphatase inhibitors on carbachol-induced secretory responses. Calyculin A is a general protein serine/threonine phosphatase inhibitor that acts on both protein phosphatases 1 and 2A. At concentrations of 0.5 and 5 nM, calyculin A did not significantly affect chloride secretion by itself and also did not alter carbachol-induced chloride secretion (Fig. 8). A higher concentration of calyculin A, 50 nM, significantly potentiated carbachol-induced chloride secretion. This may reflect sustained phosphorylation of components of the chloride secretory machinery. We therefore used 0.5 and 5 nM calyculin A to test whether these concentrations, which did not potentiate the response to carbachol by themselves, could nevertheless counteract the inhibitory effect of EGF on carbachol-induced chloride secretion. In fact, whereas 0.5 nM calyculin A did not alter the inhibitory effect of EGF on carbachol-induced chloride secretion, the higher concentration (5 nM) completely reversed this inhibitory effect. We concluded that the inhibitory effect of EGF on chloride secretion rests, at least in part, on the ability of the growth factor to activate a phosphatase.

Fig. 6. Effect of EGF on PP2A activity in T84 cells. Monolayers were treated with EGF (100 ng/ml) basolaterally for the times indicated. Monolayers were then lysed as described in MATERIALS AND METHODS, and cell lysates were then used in the protein phosphatase activity assay. Data are means ± SE for 9 experiments. *P < 0.05, significant increase in phosphatase activity. Statistical effects were determined by 1-way ANOVA with Student-Newman-Keuls post hoc test.
to alter the activity of different targets (25). Although both carbachol and EGF cause inhibition of subsequent calcium-dependent chloride secretion, they do so by inhibiting an apical chloride channel vs. a basolateral potassium channel, respectively (20). Likewise, EGF, but not carbachol, appears to mediate inhibitory effects via PI 3-kinase and PKCe (7). The molecular basis for this differential signaling from the EGF receptor was, however, largely unknown. In the present study, we focused on the basis for discordant activation of PI 3-kinase between EGF and carbachol.

Significant information is available regarding the regulation of PI 3-kinase activity in cells stimulated with a variety of agonists. The enzyme is a heterodimer consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. p85 is capable of interacting with a variety of proteins by virtue of the fact that it contains SH2, SH3, and proline-rich domains. It has been proposed to act as an adaptor protein, in a manner analogous to Shc, and also presumably serves to recruit the p110 subunit to its substrates in the plasma membrane following activation (15). The regulatory subunit p85 is activated by tyrosine phosphorylation (19).

The PI 3-kinase holoenzyme is a lipid kinase responsible for the production of 3-phosphorylated lipids and has been impli-

**DISCUSSION**

Both carbachol and EGF activate the EGF receptor in T84 cells, presumably at a relatively proximal point in their respective signaling cascades (21). However, we have shown that thereafter, the signaling events induced by each ligand diverge and utilize different intracellular mechanisms and messengers...
regulated by opposing kinases and phosphatases (16). Although both EGF and carbachol induced tyrosine phosphorylation of p85, carbachol also induced serine phosphorylation of this protein. EGF failed to induce an increase in the level of serine phosphorylation, and interestingly, EGF pretreatment markedly reduced carbachol-induced serine phosphorylation of p85. These data strongly suggest that the differential effect of phosphorylation plays a key role in many cellular processes. The phosphorylation state of a target protein is regulated by opposing kinases and phosphatases (16). It is hypothesized that p85, when not combined with the p110 subunit, displays serine kinase activity. Therefore, serine residues on p85 will be constitutively phosphorylated. On the other hand, EGF treatment does not cause serine phosphorylation, secondary to its ability to activate PP2A, which can also reverse p85 serine phosphorylation induced by carbachol.
carbachol or EGF on PI 3-kinase activation, as well as on chloride secretion, may stem from their differential effect on p85 serine phosphorylation. Although it has been postulated that p110 could serve as a protein serine kinase that acts on p85, it was not clear how serine on p85 becomes dephosphorylated after cells are treated with EGF.

Protein phosphatase 2A is a major cytoplasmic serine/threonine phosphatase that plays an important role in the regulation of cell growth and a diverse set of cellular proteins, including metabolic enzymes, ion channels, hormone receptors, and kinase cascades (9, 27). There is presently little information on the regulation of protein phosphatase 2A. It has been postulated that phosphorylation of protein phosphatase 2A by EGFR kinase activity of the holoenzyme is activated. Our data like- on p85, and as a result, p110 associates with p85 and the lipid kinase activity of PI 3-kinase is not activated. EGF treatment, on the other hand, causes protein tyrosine phosphorylation, and therefore the lipid kinase activity of PI 3-kinase is not activated. This likely inhibits association of p110 and p85, as reported by an intrinsic protein-serine kinase activity by phosphoinositides, and of lipid kinase activity by Mn2+ (27). Biochim Biophys Acta 1267: 139–144, 1995.

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**GRANTS**

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