**Gₙ Protein-coupled Receptor Agonists Induce Transactivation of the Epidermal Growth Factor Receptor in T₈₄ Cells**

**IMPLICATIONS FOR EPITHELIAL SECRETORY RESPONSES**

Received for publication, October 22, 2003, and in revised form, November 17, 2003

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We have previously shown that Gₙ protein-coupled receptor (GₙPCR) agonists stimulate epithelial growth factor receptor (EGFr) transactivation and activation of mitogen-activated protein kinases (MAPK) in colonic epithelial cells. This constitutes a mechanism by which Cl⁻ secretory responses to GₙPCR agonists are limited.

In the present study we examined a possible role for the EGFr in regulating Cl⁻ secretion stimulated by agonists that act through GₙPCRs. All experiments were performed using monolayers of T₈₄ colonic epithelial cells grown on permeable supports. Protein phosphorylation and protein-protein interactions were analyzed by immunoprecipitation and Western blotting. Cl⁻ secretion was measured as changes in short-circuit current (ΔIsc) across voltage-clamped T₈₄ cells. The GₙPCR agonist, vasoactive intestinal polypeptide (VIP; 100 nM), rapidly stimulated EGFr phosphorylation in T₈₄ cells. This effect was mimicked by a cell-permeant analog of cAMP, Bt₆cAMP/AM (3 µM), and was attenuated by the protein kinase A (PKA) inhibitor, H-89 (20 µM). The EGFr inhibitor, tyrphostin AG1478 (1 µM), inhibited both Bt₆cAMP/AM-stimulated EGFr phosphorylation and lᵢᵢᵢᵢ, responses. VIP and Bt₆cAMP/AM both stimulated ERK MAPK phosphorylation and recruitment of the p85 subunit of phosphatidylinositol 3-kinase (PI3K) to the EGFr in a tyrphostin AG1478-sensitive manner. The PI3K inhibitor, wortmannin (50 nM), but not the ERK inhibitor, PD 98059 (20 µM), attenuated Bt₆cAMP/AM-stimulated secretory responses. We conclude that GₙPCR agonists rapidly transactivate the EGFr in T₈₄ cells by a signaling pathway involving cAMP and PKA. Through a mechanism that likely involves PI3K, transactivation of the EGFr is required for the full expression of cAMP-dependent Cl⁻ secretory responses.

The transport of fluid across the intestinal epithelium is a passive process that is driven by the active transport of ions. Under normal circumstances, net absorption of ions and fluid predominates, which prevents excessive loss of water from the body along with the feces. However, some conditions, for example, the presence of food or bacterial-derived antigens in the lumen, can trigger rapid conversion of the intestine from its normally absorptive to a secretory phenotype. Under normal circumstances it is thought that this process is protective in that it constitutes an innate defense mechanism by which toxic substances are flushed from the intestine before they gain access to the systemic circulation. However, in addition to inhibiting absorptive processes, certain pathological conditions can induce overexpression of this defense mechanism resulting in excessive and prolonged secretion of Cl⁻ by the epithelium. Since Cl⁻ secretion is the predominant driving force for fluid secretion in the intestine, this can lead to the clinical manifestation of diarrhea, a prominent and disabling symptom of several intestinal disorders, including inflammatory bowel diseases and enteric bacterial infections (1).

The molecular mechanisms by which epithelial Cl⁻ secretion is regulated are only partly understood. Typically, Cl⁻ secretion is promoted by neuroimmune mediators that bind to specific G protein-coupled receptors (GPCRs) on the cell surface to elevate intracellular levels of second messengers. GₙPCR agonists elevate intracellular levels of Ca²⁺, whereas GₙPCR agonists activate adenylate cyclase and stimulate accumulation of cAMP. In turn, elevations in the levels of intracellular messengers bring about alterations in the activity of the ion transport proteins that constitute the epithelial secretory machinery. Transepithelial Cl⁻ secretion is followed by passive, paracellular movement of cations, particularly Na⁺, and the net accumulation of salt in the intestinal lumen creates an osmotic driving force for fluid secretion to occur (2).

Although neuroimmune agonists that activate either G₉ or GₙPCRs both promote intestinal Cl⁻ secretion, they do so with distinctive kinetics. Although GₙPCR-induced secretory responses are typically slow in onset and sustained, those induced by activation of G₉PCRs and elevations in intracellular Ca²⁺ are rapid in onset and transient, even in the continued presence of agonist. These observations previously led us to speculate that inhibitory signaling mechanisms may exist within epithelial cells that serve to limit the extent and duration of Ca²⁺-dependent secretory responses. In this regard, our previous studies have shown that, at the same time they stim-

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*This work was supported by a First Award from the Crohn’s and Colitis Foundation of America (to S. J. K.) and by National Institutes of Health Grant DK28305 (to K. E. B.). This work was presented, in part, at the 1998 meeting of the American Gastroenterological Association and has been published in abstract form (Gastroenterology (1998) 114, A515). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GPCR, G protein-coupled receptor; Bt₆cAMP/AM, dibutyryl cyclic AMP/acetoxymethyl ester; CFTR, cystic fibrosis transmembrane conductance regulator; EGF, epidermal growth factor; EGFr, EGFr receptor; ERK, extracellular signal-regulated protein kinase; GₙPCR, Gₙ protein-coupled receptor; lᵢᵢᵢᵢ, short circuit current; ΔIsc, change in short circuit current; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; VIP, vasoactive intestinal polypeptide; PBS, phosphate-buffered saline; FSK, forskolin.

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ulate Cl− secretion, Ca2+-dependent GPCR agonists, typified by the muscarinic agonist carbachol, stimulate transactivation of the epidermal growth factor receptor (EGFr) with subsequent activation of the ERK and p38 isoforms of mitogen-activated protein kinase (MAPK) (3–5). This signaling pathway appears to limit the extent of Ca2+-dependent secretory responses, and we proposed that it may act as a physiological braking mechanism to prevent excessive Cl− and, consequently, fluid loss from the body in conditions where neuroimmune mediators that act through GPCRs are increased within the intestinal mucosa.

Indeed, the role that the EGFr plays in modulating epithelial responses to various physiological and pathophysiological stimuli has been the subject of much research interest in recent years (6, 7). In addition to GPCR agonists (3, 8–10), a wide array of substances, including bile acids (11), bacteria (12–14), steroids (15), and growth factors (16, 17), have all been shown to have the capability to transactivate the EGFr and thereby recruit associated effector signaling pathways. However, as yet, there is little information regarding the role of EGFr-dependent signaling mechanisms in regulating cellular responses to agonists that bind to GPCRs thereby eliciting elevations in intracellular cAMP. Data that do exist in this regard are conflicting. Although studies in COS-7 and neuronal PC12 cells indicate that cAMP-dependent agonists have the capability to induce EGFr transactivation (18–20), agents that elevate cAMP in fibroblasts and corneal epithelium appear to inhibit signaling via the EGFr (21, 22). Thus, it seems likely that heterogeneity exists in the role that EGFr-dependent signaling mechanisms play in modulating GPCR-induced responses in different systems. In the present study we have investigated a potential role for transactivation of the EGFr in regulating Cl secretary responses to neuroimmune agonists that activate GPCRs in intestinal epithelial cells. The data we present indicate a novel role for the EGFr in mediating the full expression of cAMP-dependent epithelial secretory responses.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dibutyryl cyclic AMP, acetoxyethyl ester (Bt2cAMP/VAM) was obtained from Molecular Probes Inc. (Eugene, OR). Vasoactive intestinal polypeptide and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO). Tyrophostin AI4178, PP2, PD 98059, wortmannin, LY 294002, and PD 153035 were from Calbiochem (San Diego, CA). Anti-phosphotyrosine (PY20; monoclonal IgG2bk) was obtained from BD Transduction Laboratories (Beverly, MA). Enhanced chemiluminescence detection kits were from Amersham Biosciences (Piscataway, NJ). In T84 cells it has been shown that increases in I_{sc} are wholly reflective of increases in electrogenic Cl− secretion (24). I_{sc} measurements were carried out in Ringer’s solution containing (in mM): 154 NaCl, 4.7 KCl, 1.2 Ca2+, 0.8 Mg2+, 119.8 Cl−, 25 HCO3−, 2.4 H2PO4−, 5 glucose. Results were normalized and expressed as ΔI_{sc} (μA/cm2).

**Statistical Analysis**—All data are expressed as mean ± S.E. for a series of n experiments. Student’s t tests were used to compare paired data. One-way analysis of variance with the Student Neuman-Keuls post-test was used when three or more groups of data were compared. p values < 0.05 were considered to be statistically significant.

**RESULTS**

**VIP Stimulates Tyrosine Phosphorylation of the EGFr in T84 Cells**—Using vasoactive intestinal polypeptide (VIP) as a prototypical agonist, we first set out to determine if GPCR agonists induce transactivation of the EGFr in T84 cells. Monolayers of T84 cells were stimulated with VIP (100 nM) for various times (0.5–30 min) before lysis. At this concentration VIP is known to maximally induce epithelial secretory responses (25). Cell lysates were then immunoprecipitated with anti-EGFr antibodies, and immunoprecipitates were Western-blotted with antiphosphotyrosine. As shown in Fig. 1, basolateral addition of VIP stimulated tyrosine phosphorylation of the EGFr in a time-dependent fashion. The effects of VIP were rapid in onset with maximal EGFr phosphorylation occurring within 0.5 min after addition of the peptide. A less prominent effect of VIP on EGFr phosphorylation was observed after ~15 min, but this did not achieve statistical significance. Fig. 1B, which depicts densitometric analysis of the time course over which VIP stimulates EGFr phosphorylation in comparison to that of its effects on Cl− secretion, shows that peak EGFr phosphorylation occurs prior to the onset of VIP-stimulated secretory responses.

**VIP Stimulates EGFr Phosphorylation through Elevations in Intracellular cAMP and Activation of Protein Kinase A**—In intestinal epithelial cells VIP is known to exert its prosecretory effects by elevating intracellular levels of cAMP and activation of protein kinase A (PKA). We were therefore interested in
In the time course of VIP-stimulated chloride secretion, we found that, similar to VIP, a cell-permeant analog of cAMP, Bt2cAMP/AM (3 μM), also stimulated tyrosine phosphorylation of the EGFr (Fig. 2A). At this concentration Bt2cAMP/AM is maximally effective in stimulating Cl− secretory responses across voltage-clamped monolayers of T84 cells mounted in Ussing chambers (open circles; n = 5). Data are expressed as mean ± S.E. increases in EGFr phosphorylation or ΔIsc (ΔIsc) induced by VIP added at time 0.

Inhibition of the EGFr Attenuates cAMP-dependent Chloride Secretion in Colonic Epithelial Cells—Next, to determine if transactivation of the EGFr plays a role in regulation of cAMP-dependent Cl− secretion, we examined the effects of an EGFr inhibitor, tyrphostin AG1478, on ΔIsc responses to Bt2cAMP/AM across voltage-clamped monolayers of T84 cells. Pretreatment of T84 cells with tyrphostin AG1478 (1 μM), which reduced Bt2cAMP/AM-stimulated EGFr phosphorylation by 78.5 ± 8.7% (n = 3; p < 0.01) (Fig. 3A), significantly attenuated subsequent ΔIsc responses to Bt2cAMP/AM (3 μM) (Fig. 3B). Control responses to Bt2cAMP/AM were 40.8 ± 5.5 μA/cm² compared with 21.3 ± 4.5 μA/cm² in tyrphostin-pretreated cells (n = 6; p < 0.01). In similar experiments secretory responses to VIP in T84 cells were also attenuated, albeit to a lesser extent, by tyrphostin AG1478 (1 μM). Control responses to VIP were 51.3 ± 7.3 μA/cm² compared with 43.2 ± 6.5 μA/cm² in tyrphostin AG1478-pretreated cells (n = 6; p < 0.01). To confirm these findings we examined the effects of another inhibitor of the EGFr, PD 153035, on ΔIsc responses to VIP. In this case, responses to VIP in PD 153035 (1 μM)-pretreated cells were 34.3 ± 4.3 μA/cm² compared with 46.3 ± 5.1 μA/cm² in control cells (n = 10; p < 0.01).

cAMP-dependent Agonists Transactivate the EGFr in Epithelial Cells—Because we have previously shown that transactivation of the EGFr in response to GsPCR agonists leads to subsequent activation of the ERK isoforms of MAPK, we next set out to determine if ERK may also be activated as a consequence of GsPCR-induced EGFr transactivation. We found that stimulation of cells with basolateral VIP (100 nM) resulted in phosphorylation of both the 42- and 44-kDa isoforms of ERK (Fig. 4, A and C). The kinetics of VIP-stimulated ERK phosphorylation lagged somewhat behind those of VIP-stimulated EGFr phosphorylation with significant phosphorylation of ERK not being observed until 5–15 min after addition of the peptide (compare with Fig. 2). Similarly, Bt2cAMP/AM (3 μM) also stimulated phosphorylation of ERK MAPK. In this case, ERK phosphorylation was maximal 5 min after the addition of agonist (Fig. 4, B and C). Furthermore, we found that when cells were pretreated with
the EGFr inhibitor, tyrphostin AG1478 (1 μM), Bt2cAMP/AM-stimulated ERK phosphorylation was reduced by 73.7 ± 10.5%, (n = 5, p < 0.001) indicating this response is likely due to activation of the EGFr (Fig. 4D).

cAMP-dependent Agonists Stimulate Recruitment of the p85 Subunit of PI3K to the EGFr—Because, similar to ERK, PI3K has also been implicated in mediating the downstream effects of EGFr activation in a variety of cell types, we also examined the possibility that activation of the EGFr in response to cAMP-dependent agonists in T84 cells may also lead to PI3K activation. Treatment of T84 cells with basolateral VIP (100 nM) resulted in recruitment of the p85 subunit of PI3K to the EGFr (Fig. 5, A and C). The kinetics with which VIP stimulated p85 recruitment to the EGFr closely followed those for VIP-stimulated EGFr phosphorylation (compare with Fig. 2) with a peak response occurring 1 min after addition of the agonist. VIP-stimulated recruitment of the p85 subunit of PI3K was accompanied by phosphorylation of a downstream substrate of PI3K, AKT. After 1 min stimulation with VIP, AKT phosphorylation was increased 3.6 ± 0.3-fold over control, unstimulated cells (n = 5; p < 0.01). This effect was blocked by the PI3K inhibitor, wortmannin (50 nM), significantly reduced subsequent I_{sc} responses to Bt2cAMP/AM (3 μM) by 63.7 ± 9.2% (n = 5, p < 0.01). However, pretreatment of voltage-clamped T84 cells with PD 98059 (20 μM) did not alter subsequent I_{sc} responses to Bt2cAMP/AM (Fig. 6A) or to VIP (data not shown). In contrast, pretreatment of voltage-clamped T84 cells with the PI3K inhibitor, wortmannin (50 nM), significantly reduced subsequent I_{sc} responses to Bt2cAMP/AM (3 μM) (Fig. 6B). Maximal responses to Bt2cAMP/AM were 41.7 ± 6.7 μA/cm² in controls and 15.7 ± 2.6 μA/cm² in cells pretreated with wortmannin (n = 5, p < 0.01). Wortmannin (50 nM) also reduced I_{sc} responses to VIP. Responses to VIP in control cells were 51.0 ± 3.6 μA/cm² compared with 41.1 ± 1.3 μA/cm² in wortmannin-pretreated cells (n = 5; p < 0.01). In addition, secretory responses to VIP, Bt2cAMP/AM, and the adenylyl cyclase activator, forskolin (FSK) were also attenuated by another inhibitor of PI3K, LY 290042. Responses to VIP (100 nM), Bt2cAMP/AM (3 μM), and FSK (10 μM), in the presence of LY 290042 (20 μM), were 75.2 ± 5.6% (n = 5; p < 0.05), 65.1 ± 5.1% (n = 11; p < 0.001), and 79.6 ± 5.3% (n = 12; p < 0.01) of those in control cells, respectively. Finally, to confirm the role of PI3K in regulating cAMP-dependent intestinal Cl⁻ secretion, we also analyzed the effects of LY 290042 on I_{sc} responses to FSK in another colonic epithelial cell line, HT-29 (clone 19A) cells. Similar to its effects in T84 cells, LY 290042 reduced responses to FSK in HT-29 cells. Responses to FSK in control cells were 74.5 ± 5.8 μA/cm² compared with 55.8 ± 12.1 μA/cm² in LY 290042-pretreated cells (n = 4; p < 0.05). Together, these data imply that PI3K, but not ERK, is involved in mediating the full expression of cAMP-dependent Cl⁻ secretory responses in T84 cells.

Inhibition of PI3K, but Not ERK, Attenuates cAMP-dependent Cl⁻ Secretion in T84 Cells—Finally, we examined a possible role for ERK and PI3K in regulation of cAMP-dependent epithelial Cl⁻ secretion. First we examined the effects of the ERK inhibitor, PD 98059, on I_{sc} responses to Bt2cAMP/AM across voltage-clamped monolayers of T84 cells. As shown in the inset to Fig. 6A, PD 98059 (20 μM) inhibited ERK phosphorylation in response to Bt2cAMP/AM (3 μM) by 63.7 ± 9.2% (n = 5, p < 0.01). However, pretreatment of voltage-clamped T84 cells with PD 98059 did not alter subsequent I_{sc} responses to Bt2cAMP/AM across voltage-clamped monolayers of T84 cells.

**Fig. 2.** Bt2cAMP/AM stimulates PKA-dependent EGFr phosphorylation in T84 cells. A, cells were stimulated with bilateral Bt2cAMP/AM (3 μM) for the times indicated, and cell lysates were immunoprecipitated with antibodies to the EGFr. Immunoprecipitated proteins were analyzed by Western blotting with antiphosphotyrosine. B, densitometric analysis of several similar experiments. Data are expressed as mean ± S.E. increases in EGFr phosphorylation in response to Bt2cAMP/AM (n = 6), measured in arbitrary units (a.u.). Asterisks denote significant differences from control, unstimulated cells (**, p < 0.01; *** p < 0.001). C, cells were treated with H-89 (bilateral; 10 μM) for 30 min prior to stimulation with Bt2cAMP/AM (3 μM; 0.5 min). Cell lysates were then immunoprecipitated with anti-EGFr antibodies, and immunoprecipitated proteins were analyzed by Western blotting with antiphosphotyrosine. D, densitometric analysis of several such experiments in comparison to similar experiments in which the effects of H-89 on EGFr-induced (100 nM; 2 min) EGFr phosphorylation was examined. Data are expressed as mean ± S.E. increases in EGFr phosphorylation in response to Bt2cAMP/AM (n = 9) or EGFr (n = 7) in the absence or presence of H-89, measured in arbitrary units (a.u.). Asterisks denote significant differences from control, unstimulated cells (**, p < 0.001). # denotes significant differences from Bt2cAMP/AM-stimulated cells (###, p < 0.01).
GsPCR Agonists Transactivate the EGFr in Epithelial Cells

It is becoming increasingly apparent that, in addition to its classic role as a growth factor receptor, the EGFr also serves as an important signaling intermediate in regulation of cellular responses induced by GPCR agonists in epithelial cells. Although our previous studies have shown that recruitment of the EGFr by GsPCR agonists serves as an inhibitory mechanism to limit the extent and duration of secretory responses to such agonists (3), in the present study we present data that are consistent with a contrasting role for the EGFr in mediating the full expression of GsPCR-stimulated Cl− secretion. First, we found that treatment of intestinal epithelial cells with VIP rapidly stimulates tyrosine phosphorylation of the EGFr. Because it has been shown that both epithelial receptors for VIP are coupled to Gs proteins, stimulation of adenylate cyclase, and production of cAMP (27), we examined if elevations in intracellular cAMP alone were sufficient to stimulate phosphorylation of the EGFr. We found that a cell-permeant analog of cAMP, Bt2cAMP/AM, which stimulates qualitatively similar Cl− secretory responses to VIP, also mimicked the effects of the peptide on EGFr phosphorylation, implying that these effects are mediated by elevations in intracellular cAMP. Furthermore, the observation that cAMP-induced phosphorylation of the EGFr is attenuated by the PKA inhibitor, H-89, suggests that PKA is also likely to be a component of the signaling pathway linking GsPCRs to the EGFr. In other systems where EGFr transactivation has been demonstrated to occur in response to cAMP-dependent agonists, PKA has also been implicated in this process (18, 19). Indeed, in COS-7 cells, it has been proposed that β2-adrenergic agonists stimulate phosphorylation of the EGFr by an indirect mechanism involving a PKA-induced switch of coupling by the normally Gs protein-coupled β2-adrenoceptor to a Gi protein. This brings about activation of Src family kinases, which, in turn, activate the EGFr (18). Whether such a mechanism underlies the effects of GsPCR-induced transactivation of the EGFr in intestinal epithelial cells is currently under investigation. Similarly, whether matrix metalloproteinases and extracellular shedding of EGFr ligands are involved in mediating GsPCR-induced EGFr transactivation, as they in mediating transactivation of the EGFr in response to GsPCR agonists (7, 28–31), is a subject for further investigation.

In the present study, cAMP-induced phosphorylation of the EGFr was practically abolished by pretreatment of T84 cells with the EGFr inhibitor, tyrphostin AG1478, which at the concentrations employed, did not block agonist-induced responses but selectively inhibited those responses (30, 31). Together, these data imply that in T84 epithelial cells, transactivation of EGFr is required for the full expression of GsPCR-stimulated Cl− secretory responses. Because it is in direct contrast to its antisecretory influence on GsPCR-induced secretory responses, this role for the EGFr in mediating responses to GsPCR agonists is intriguing, because it is not clear how the precise physiological significance of these opposing actions on epithelial secretion remains unclear, it is evident that the EGFr plays a central role in modulating the extent and duration of epithelial secretory responses to neuroimmune secretagogues that act at GPCRs.

Having demonstrated a role for the EGFr in regulation of GsPCR-stimulated Cl− secretion, we were next interested in identifying potential signaling mechanisms that might lie downstream of EGFr. We first focused our attention on PI3K and ERK MAPK, both of which commonly act downstream of the EGFr in other systems. We found that cAMP-induced transactivation of the EGFr led to the activation of both ERK and PI3K. However, the kinetics for activation of these two pathways were found to be markedly different. PI3K activation occurred rapidly in response to both VIP and Bt2cAMP/AM and temporally correlated with the kinetics of EGFr phosphorylation, whereas ERK activation occurred at later time points with responses to VIP and Bt2cAMP/AM being observed only after 15 and 5 min incubation, respectively. Why the time course of VIP-stimulated ERK activation is delayed in comparison to that of Bt2cAMP, even though they activate the EGFr with similar kinetics is not yet known but may reflect the influence of additional, as yet unidentified, signaling mechanisms that regulate ERK phosphorylation in response to receptor occupancy by VIP. Nevertheless, our data indicate that, even though cAMP-dependent agonists have the capability to activate ERK MAPKs, ERK does not appear to be involved in regulation of cAMP-induced secretory responses. This is based on the observation that the ERK inhibitor, PD 98059, which effectively blocked cAMP-stimulated ERK phosphorylation, did not alter Ise responses to Bt2cAMP/AM across voltage-clamped T84 cells.

**Fig. 3.** Tyrphostin AG1478 inhibits cAMP-induced EGFr phosphorylation and chloride secretion in T84 cells. A, cells were stimulated with Bt2cAMP/AM (3 μM; bilateral; 0.5 min) either in the absence or presence of pretreatment with tyrphostin AG1478 (1 μM; bilateral; 20 min), and cell lysates were immunoprecipitated with antibodies to the EGFr. Immunoprecipitated proteins were analyzed by Western blotting with anti-phosphotyrosine. Asterisks denote significant differences from control, unstimulated cells (**, p < 0.01). # denotes significant differences from cells stimulated with Bt2cAMP/AM in the absence of tyrphostin AG1478 (#, p < 0.01). B, bilateral pretreatment of voltage-clamped monolayers of T84 cells with tyrphostin AG1478 (1 μM; 20 min) significantly attenuated subsequent Cl− secretory responses to Bt2cAMP/AM (3 μM). Data are expressed as mean ± S.E. increases in Ise (ΔIse) induced by Bt2cAMP/AM addition at time 0 (n = 6).

**DISCUSSION**

It is becoming increasingly apparent that in addition to its classic role as a growth factor receptor, the EGFr also serves as an important signaling intermediate in regulation of cellular responses induced by GPCR agonists in epithelial cells. Although our previous studies have shown that recruitment of the EGFr by GsPCR agonists serves as an inhibitory mechanism to limit the extent and duration of secretory responses to such agonists (3), in the present study we present data that are consistent with a contrasting role for the EGFr in mediating the full expression of GsPCR-stimulated Cl− secretion. First, we found that treatment of intestinal epithelial cells with VIP rapidly stimulates tyrosine phosphorylation of the EGFr. Because it has been shown that both epithelial receptors for VIP are coupled to Gs proteins, stimulation of adenylate cyclase, and production of cAMP (27), we examined if elevations in intracellular cAMP alone were sufficient to stimulate phosphorylation of the EGFr. We found that a cell-permeant analog of cAMP, Bt2cAMP/AM, which stimulates qualitatively similar Cl− secretory responses to VIP, also mimicked the effects of the peptide on EGFr phosphorylation, implying that these effects are mediated by elevations in intracellular cAMP. Furthermore, the observation that cAMP-induced phosphorylation of the EGFr is attenuated by the PKA inhibitor, H-89, suggests that PKA is also likely to be a component of the signaling pathway linking GsPCRs to the EGFr. In other systems where EGFr transactivation has been demonstrated to occur in response to cAMP-dependent agonists, PKA has also been implicated in this process (18, 19). Indeed, in COS-7 cells, it has been proposed that β2-adrenergic agonists stimulate phosphorylation of the EGFr by an indirect mechanism involving a PKA-induced switch of coupling by the normally Gs protein-coupled β2-adrenoceptor to a Gi protein. This brings about activation of Src family kinases, which, in turn, activate the EGFr (18). Whether such a mechanism underlies the effects of GsPCR-induced transactivation of the EGFr in intestinal epithelial cells is currently under investigation. Similarly, whether matrix metalloproteinases and extracellular shedding of EGFr ligands are involved in mediating GsPCR-induced EGFr transactivation, as they in mediating transactivation of the EGFr in response to GsPCR agonists (7, 28–31), is a subject for further investigation.

In the present study, cAMP-induced phosphorylation of the EGFr was practically abolished by pretreatment of T84 cells with the EGFr inhibitor, tyrphostin AG1478, which at the concentrations employed, is well documented to be specific for the EGFr over other members of the ErbB family of receptor tyrosine kinases (32, 33). Furthermore, when voltage-clamped T84 cells were treated with tyrphostin AG1478, subsequent Cl− secretory responses to Bt2cAMP/AM and VIP were significantly attenuated. Secretory responses to VIP were also inhibited by pretreatment of the cells with another selective EGFr inhibitor, PD 153035 (34, 35). Together, these data imply that in T84 epithelial cells, transactivation of EGFr is required for the full expression of GsPCR-stimulated Cl− secretory responses. This role for the EGFr in mediating responses to GsPCR agonists is intriguing, because it is in direct contrast to its antisecretory influence on GsPCR-induced secretory responses. Thus, although the precise physiological significance of these opposing actions on epithelial secretion remains unclear, it is evident that the EGFr plays a central role in modulating the extent and duration of epithelial secretory responses to neuroimmune secretagogues that act at GPCRs.

Having demonstrated a role for the EGFr in regulation of GsPCR-stimulated Cl− secretion, we were next interested in identifying potential signaling mechanisms that might lie downstream of EGFr. We first focused our attention on PI3K and ERK MAPK, both of which commonly act downstream of the EGFr in other systems. We found that cAMP-induced transactivation of the EGFr led to the activation of both ERK and PI3K. However, the kinetics for activation of these two pathways were found to be markedly different. PI3K activation occurred rapidly in response to both VIP and Bt2cAMP/AM and temporally correlated with the kinetics of EGFr phosphorylation, whereas ERK activation occurred at later time points with responses to VIP and Bt2cAMP/AM being observed only after 15 and 5 min incubation, respectively. Why the time course of VIP-stimulated ERK activation is delayed in comparison to that of Bt2cAMP, even though they activate the EGFr with similar kinetics is not yet known but may reflect the influence of additional, as yet unidentified, signaling mechanisms that regulate ERK phosphorylation in response to receptor occupancy by VIP. Nevertheless, our data indicate that, even though cAMP-dependent agonists have the capability to activate ERK MAPKs, ERK does not appear to be involved in regulation of cAMP-induced secretory responses. This is based on the observation that the ERK inhibitor, PD 98059, which effectively blocked cAMP-stimulated ERK phosphorylation, did not alter Ise responses to Bt2cAMP/AM across voltage-clamped T84 cells.
This finding is intriguing in light of our previous studies, which demonstrate that ERK activation by GPCR agonists limits the extent and duration of secretory responses elicited through this receptor subtype (2) and raises the question as to how ERK differentially modulates epithelial secretory processes when activated in response to different classes of GPCR agonist. One potential explanation lies in the possibility that downstream effectors of ERK activation might have the capability to differentially interact with the various transport proteins that comprise cAMP and Ca\(^{2+}\)-dependent secretory pathways. For example, arachidonic acid, which is produced in response to ERK-induced phospholipase A\(_2\) activity in a variety of cell types (36–38), has the capability to differentially modulate different classes of intestinal epithelial K\(^{+}\) channels (39, 40). Because separate K\(^{+}\) channel populations are known to regulate Ca\(^{2+}\)- and cAMP-dependent Cl\(^{-}\) secretion (2), these channels could present a target for differential regulation of G\(_s\)- and G\(_q\)PCR-induced secretory responses by ERK MAPK. Another possibility is that additional signaling intermediates might modify the ability of ERK to interact with the Cl\(^{-}\) secretory mechanism. Further studies investigating mechanisms by which EGF\(r\)-dependent signaling pathways interact with the Cl\(^{-}\) secretory mechanism will likely shed more light on this intriguing subject. Likewise, even though it does not appear to be involved in regulating Cl\(^{-}\) secretion, the role that ERK does play in regulating physiological responses to cAMP-dependent hormones remains to be determined. However, previous studies in HT-29 colonic epithelial cells indicate that it may be involved in regulation of mitogenesis (42).

Although our current data do not support a role for ERK in regulation of G\(_s\)PCR-induced secretory responses, they do support a role for PI3K. This conclusion is based on multiple observations. First, both VIP and Bt\(_{2}\)cAMP/AM rapidly stimulated recruitment of the regulatory p85 subunit of PI3K to the EGFr, an effect we have previously shown to be a reliable index of PI3K activation (43). Second, VIP stimulated phosphorylation of the PI3K substrate, AKT. Third, inhibition of PI3K with either wortmannin or LY 290042 attenuated Bt\(_{2}\)cAMP/AM-dependent secretory responses across voltage-clamped monolayers of T\(_{84}\) cells. At the concentrations employed in this study, both wortmannin and LY 290042 are believed to be specific for PI3K (44, 45). We also found that LY 290042 significantly inhibited secretory responses to VIP and to the adenylate cyclase activator, FSK. Thus, although our studies imply a role
for PI3K in regulation of cAMP-dependent secretory responses, they are in direct contrast to those from another laboratory in which the effects of PI3K inhibitors on cAMP-stimulated Cl− secretion were examined (46, 47). In these previous studies it was found that, at concentrations similar to those employed in our present study, wortmannin (100 nM) and LY 290042 (15 μM) were without effect on Cl− secretion in response to FSK across voltage-clamped T84 cells. Only when higher concentrations of wortmannin (200–500 nM) were used was an inhibitory effect on FSK-induced responses observed. The authors postulated that, at such high concentrations, inhibition of FSK-induced secretion was due to a nonspecific effect of wortmannin in blocking ERK MAPK activation, rather than its effects on PI3K. At present we cannot offer a satisfactory explanation for the discrepancies between our current findings and those of Ecay and co-workers (46, 47) and can only speculate that they may be related to differences in cell passage, serum components, or other culture conditions. However, because we found that low concentrations of PI3K inhibitors were effective in reducing VIP-, forskolin-, and Bt2cAMP/AM-dependent secretory responses, not only in T84 cells, but also in HT-29 cells, and because inhibition of ERK MAPK was without effect on secretory responses to cAMP-dependent agonists, we believe that, under the standard culture conditions employed in our studies, our data strongly implicate PI3K as an regulator of cAMP-dependent Cl− secretion in intestinal epithelial cells.

The mechanisms by which PI3K regulates cAMP-dependent Cl− secretion are currently not yet known. One possibility is that PI3K may alter the activity of one or more of the transport proteins that comprise the Cl− secretory machinery, and in this regard PI3K has been shown to alter ion channel activity in some cell types (48, 49). However, previous studies from our laboratory suggest an inhibitory effect of PI3K on intestinal epithelial K+ channels (50), an effect that does not explain our current data because inhibition of K+ channel function would likely lead to attenuation of Cl− secretory responses (2). It is also important to note, however, that these previous studies related to secretory responses elicited by Ca2+/CaM-dependent agonists and, perhaps, to a separate population of K+ channels to those involved in cAMP-mediated secretory responses. Another possibility is that PI3K may regulate trafficking of transport proteins to and from the plasma membrane. In support of this hypothesis it has been previously shown that PI3K is intimately involved in cytoskeletal reorganization and membrane trafficking in a number of systems, including intestinal epithelial cells (51–55). Furthermore, a growing body of evidence suggests that epithelial secretory responses to cAMP-dependent agonists are not only dependent on phosphorylation and activation of CFTR channels resident in the apical membrane but are also dependent on rapid trafficking of the channel between endosomes and the plasma membrane (56–59). That cAMP-dependent secretagogues induce recruitment of PI3K to the EGFr with kinetics that precede Cl− secretory responses supports this hypothesis. Thus, studies are currently underway in this laboratory to determine if altered trafficking of CFTR by PI3K regulates cAMP-dependent secretion in intestinal epithelial cells.

Together with our previous findings (3), the data presented...
transactivation of the receptor in response to GsPCR agonists appears to result in formation of EGFr homodimers (61). Our current data with cAMP-dependent agonists are somewhat reminiscent of the effects of EGF itself on PI3K and ERK activation, where EGF rapidly (<1 min) stimulates activation of PI3K with activation of ERK occurring only after several minutes incubation with the growth factor (3, 43). These observations may provide clues as to the mechanisms by which signals are propagated through the EGFr in response to neuroimmune mediators that activate GPCRs, and experiments are currently underway to more fully elucidate the nature of ErbB receptor signaling complexes formed in response to such agonists.

In summary, our current studies underline the central role that the EGFr plays in regulating intestinal epithelial transport. In addition to limiting the extent of GqPCR-induced Cl– secretion, the EGFr appears also to be rapidly activated in response to GsPCR agonists and, through a mechanism which likely involves PI3K, is required to mediate the full expression of secretory responses to such agonists. Agonists that act through GqPCRs are diverse and plentiful within the intestinal mucosa and include bioactive peptides, eicosanoids, neurotransmitters, and immune cell mediators that are undoubtedly important in regulating intestinal epithelial secretion both in health and disease (2). It is therefore our hope that a greater understanding of how EGFr-dependent signaling pathways are involved in regulation of epithelial secretory processes will ultimately lead to the development of new strategies for the treatment of fluid and electrolyte transport abnormalities associated with intestinal disease.

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