We have examined the role of tyrosine phosphorylation in regulation of calcium-dependent chloride secretion across T84 colonic epithelial cells. The calcium-mediated agonist carbachol (CCh, 100 μM) stimulated a time-dependent increase in tyrosine phosphorylation of a range of proteins (with molecular masses ranging up to 180 kDa) in T84 cells. The tyrosine kinase inhibitor, genistein (5 μM), significantly potentiated chloride secretory responses to CCh, indicating a role for CCh-stimulated tyrosine phosphorylation in negative regulation of CCh-stimulated secretory responses. Further studies revealed that CCh stimulated an increase in both phosphorylation and activity of the extracellular signal-regulated kinase (ERK) isoforms of mitogen-activated protein kinase. Chloride secretory responses to CCh were also potentiated by the mitogen-activated protein kinase inhibitor, PD98059 (20 μM). Phosphorylation of ERK in response to CCh was mimicked by the protein kinase C (PKC) activator, phorbol myristate acetate (100 nM), but was not altered by the PKC inhibitor GF 109203X (1 μM). ERK phosphorylation was also induced by epidermal growth factor (EGF) (100 ng/ml). Immunoprecipitation/Western blot studies revealed that CCh stimulated tyrosine phosphorylation of the EGF receptor (EGFr) and increased co-immunoprecipitation of the adapter proteins, Shc and Grb2, with the EGFr. An inhibitor of EGFr phosphorylation, tyrphostin AG1478 (1 μM), reversed CCh-stimulated phosphorylation of both EGFr and ERK. Tyrphostin AG1478 also potentiated chloride secretory responses to CCh. We conclude that CCh activates ERK in T84 cells via a mechanism involving transactivation of the EGFr, and that this pathway contributes an inhibitory signaling pathway by which chloride secretory responses to CCh may be negatively regulated.

Chloride secretion across intestinal epithelial cells is under close regulation by hormonal, neural, paracrine, and endocrine factors. Such factors stimulate chloride secretion by binding to specific receptors on the surface of epithelial cells and consequently stimulating elevations in the levels of intracellular second messengers, such as cyclic nucleotides and calcium. In turn, increases in the levels of intracellular messengers activate the transport proteins that comprise the chloride secretory mechanism (1). However, even though both calcium and cyclic nucleotides are both capable of stimulating epithelial chloride secretion, it has been known for many years that these two classes of intracellular messengers stimulate temporally distinctive chloride secretory responses. While responses to cyclic nucleotide-mediated agonists, such as vasoactive intestinal peptide, are sustained, secretory responses to calcium-mediated agonists, such as the muscarinic agonist, carbachol (CCh), are transient, even though levels of intracellular calcium remain elevated after the secretory response has resolved (2–4). This implies that negative signals exist within intestinal epithelial cells that limit, or “switch off” epithelial secretory responses to calcium-mediated agonists. Since epithelial cells are continuously exposed, in vivo, to endogenously generated hormones and neurotransmitters that stimulate epithelial secretion via calcium-dependent mechanisms (5), such inhibitory signals may represent an intrinsic epithelial “braking” mechanism by which excessive chloride, and consequently water, secretion may be prevented.

Previous work from our laboratory has identified several intracellular messengers which appear to negatively influence calcium-mediated chloride secretion. Influx of extracellular calcium, generation of inositol (3,4,5,6)-tetrakisphosphate, and activation of protein kinase C (PKC), have all been shown to inhibit secretory responses to calcium-dependent agonists (1). More recently we have shown that receptor tyrosine kinase-dependent signaling pathways, such as those activated by epidermal growth factor (EGF) also activate intracellular pathways that are inhibitory to calcium-dependent secretion (6, 7). Furthermore, since influx of extracellular calcium (8) and stimulation of inositol (3,4,5,6)-tetrakisphosphate production (9) in response to calcium-dependent secretagogues have both been demonstrated to be dependent upon stimulation of tyrosine kinase activity, tyrosine kinases appear to play important role(s) in negative regulation of epithelial secretory processes. Indeed, recent studies demonstrate that tyrosine kinases may function in the regulation of apical cystic fibrosis transmembrane conductance regulator CI− channels (10–13) and basolateral K+ channels (13), both of which are transport proteins essential for epithelial chloride secretion.

* This work was supported in part by National Institutes of Health Grant DK29835 (to K. E. B.). These studies were presented in part at the 1997 annual meeting of the American Gastroenterological Association, and have been published in abstract form (Keely, S. J., Uribe, J. M., and Barrett, K. E. Gastroenterology (1997) 112, A375). 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.

† Recipient of a Predoctoral Fellowship DK07202 from an Institutional Training Grant in Digestive Diseases.

‡ Faculty member, Biomedical Science Ph.D. Program, University of California, San Diego, School of Medicine. To whom correspondence and reprint requests should be addressed: UCSD Medical Center, 8414, 200 West Arbor Dr., San Diego, CA 92103. Tel.: 619-543-3726; Fax: 619-543-6969; E-mail: kbarrett@ucsd.edu.

1 The abbreviations used are: CCh, carbachol; GPCR, G-protein-coupled receptor; EGF, epidermal growth factor; EGFr, EGF receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; Isc, short circuit current; PMA, phorbol myristate acetate; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; 4-DAMP, 4-diphenylacetoxyl-N-methylpipеридин.
Carbachol Activates ERK in Colonic Epithelial Cells

Although it has been demonstrated previously that calcium-dependent secretagogues stimulate intestinal epithelial tyrosine kinase activity (8), there is little known of the signaling pathways involved in such processes. However, some insight into how G-protein-coupled receptors (GPCRs), such as the muscarinic receptor for CCh on colonic epithelia, might be linked to alterations in intracellular tyrosine kinase activity comes from studies in other models. In recent years, there has been a growing body of evidence to suggest that many GPCR agonists can activate the MAPK signaling cascade in a variety of cell types including epithelial cells (14–19). The MAPK signaling cascade is classically involved in mediating the mitogenic actions of growth factors, such as EGF, and the mechanisms by which the EGF receptor (EGFR) is coupled to MAPK activation have been well elucidated (20). Upon ligand binding the EGFr becomes autophosphorylated and recruits the adapter molecules, Shc and Grb2, which bind to tyrosine-phosphorylated residues on the receptor by virtue of their SH2 domains. Formation of the EGFr-Shc-Grb2 complex leads to activation of a guanine nucleotide exchange factor, mSOS, which in turn activates the low molecular weight G-protein, p21ras. This, in turn brings about activation of the upstream components of the MAPK cascade, Raf and MEK, ultimately leading to the stimulation of the ERK isoforms of MAPK. The mechanisms by which GPCRs stimulate ERK activation are less well understood but current evidence suggests the involvement of two predominant pathways which may be differentially activated depending on the nature of receptor coupling to heterotrimeric G-proteins (21–23). G1 protein-coupled receptors (G1,PCR) appear to stimulate ERK activation predominantly by a mechanism involving increases in intracellular tyrosine kinase activity, and which may be mediated by transactivation of the EGFr receptor. In contrast, Gq protein-coupled receptors (Gq,PCR) are believed to stimulate ERK predominantly via a tyrosine kinase-independent pathway mediated by PKC. However, recent studies indicate that these two pathways for ERK activation may not be mutually exclusive and that in some systems they may converge into a common p21ras-mediated pathway (24).

In the present study we sought to elucidate further the involvement of tyrosine kinase-dependent signaling pathways in negative regulation of calcium-dependent chloride secretion across T84 colonic epithelial cells. We employed CCh as a prototypic calcium-mediated secretagogue, which acts via Gq, protein-coupled M3 muscarinic receptors on colonic epithelial cells to stimulate chloride secretion (25, 26). In particular, we set out to investigate a possible role for the ERK isoforms of MAPK in regulation of CCh-stimulated secretion, and to determine possible mechanisms underlying CCh stimulation of ERK activity in T84 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbachol, vasoactive intestinal polypeptide, and genistein were obtained from Sigma. Tyrothasin AG1478, GF 902103X, and PD 98059 were obtained from Calbiochem, San Diego, CA. Epidermal growth factor was obtained from Genzyme, Cambridge, MA. Mouse anti-human EGF receptor and mouse anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology Inc., Lake Placid, NY. Rabbit anti-phospho-ERK antibodies were obtained from New England Biolabs. Proteins were then detected using an enhanced chemiluminescence detection kit (Boehringer Mannheim, Indianapolis, IN). Densitometric analysis of Western blots was carried out using NIH Image software.

**MAP Kinase Assays**—Cells were stimulated, lysed, and immunoprecipitated with anti-phospho-ERK as described above in the immunoprecipitation and Western blotting protocol. ERK activity was determined using a commercially available assay kit (New England Biolabs). Brieﬂy, immunoprecipitates were washed twice in lysis buffer and twice in MAPK assay buffer. Washed immunoprecipitates were then incubated for 30 min in 100 µM ATP and 1 µg of Elk1 substrate in 50 µl of assay buffer. Reactions were stopped by the addition of 50 µl of 2 X gel loading buffer and samples were boiled for 3 min prior to SDS-PAGE. Phosphorylation of Elk1 by MAPK was determined by Western blotting (as described above) with antibodies specific for the phosphorylated form of Elk1.

**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-test were used to compare mean values as appropriate. p values < 0.05 were considered to represent significant differences.

**RESULTS**

**CCh Stimulated Tyrosine-phosphorylation Negatively Regulates CCh-stimulated Chloride Secretion in T84 Cells**—We first set out to examine the effects of CCh on protein tyrosine phosphorylation in T84 cells. Western blot analysis of T84 cell lysates revealed that, when stimulated with CCh (100 µM) on the basolateral domain, a rapid-onset tyrosine phosphorylation of several T84 cell proteins, with molecular masses ranging up to approximately 180 kDa, occurred (Fig. 1).
To determine if the observed increases in protein tyrosine phosphorylation stimulated by CCh are involved in regulation of CCh-stimulated chloride secretion, we next examined the effects of the general tyrosine kinase inhibitor, genistein, on $I_{sc}$ responses to CCh across voltage-clamped monolayers of T84 cells. Pretreatment of T84 cells with genistein (5 μM), which partially reversed CCh-stimulated protein tyrosine phosphorylation (Fig. 2, inset), significantly potentiated subsequent chloride secretory responses to basolateral CCh (100 μM). Maximal responses to CCh were 30.8 ± 7.7 μA/cm² in control monolayers compared with 67.8 ± 7.7 μA/cm² in monolayers pretreated with genistein (n = 10; p < 0.01). Data are expressed as mean ± S.E. increases in $I_{sc}$ ($\Delta I_{sc}$) induced by CCh addition at time 0.

**CCh Stimulates ERK Activity in T84 Cells**—Two of the proteins showing increased tyrosine phosphorylation in response to CCh are involved in regulation of CCh-stimulated chloride secretion, we next examined the effects of the general tyrosine kinase inhibitor, genistein, on $I_{sc}$ responses to CCh across voltage-clamped monolayers of T84 cells. Pretreatment of T84 cells with genistein (5 μM), which partially reversed CCh-stimulated protein tyrosine phosphorylation (Fig. 2, inset), significantly potentiated subsequent $I_{sc}$ responses to CCh (100 μM; Fig. 2), suggesting a possible role for CCh-stimulated tyrosine kinase activity in limiting the extent of CCh-stimulated secretory responses.

**CCh Stimulates ERK Activity in T84 Cells**—Two of the proteins showing increased tyrosine phosphorylation in response to CCh had calculated molecular masses of 44 and 42 kDa, corresponding to the molecular masses of the ERK1 and ERK2 isoforms of MAPK, respectively. We therefore decided to investigate a possible role for ERK in negative regulation of CCh-stimulated secretory responses. Western blot experiments were first conducted which verified that ERK is phosphorylated in T84 epithelial cells in response to CCh stimulation (Fig. 3A). Furthermore, kinase assays, in which we measured the ability of anti-phospho-ERK immunoprecipitates to phosphorylate the ERK substrate, Elk, demonstrated that CCh-stimulated increases in ERK phosphorylation were closely accompanied by increases in ERK activity (Fig. 3B).

**CCh-stimulated ERK Activation Is Mediated via Muscarinic $M_3$ Receptors**—In order to determine if CCh stimulation of ERK phosphorylation is a specific effect mediated by activation of cholinergic receptors, we examined the effect of the muscarinic $M_3$ receptor antagonist, 4-DAMP, on CCh-stimulated responses. 4-DAMP (100 nM) abolished $I_{sc}$ responses to CCh across voltage-clamped T84 cells. Control responses to CCh were 21.5 ± 7.5 μA/cm² compared with 0.4 ± 0.2 μA/cm² in 4-DAMP-pretreated cells (n = 4). 4-DAMP (100 nM) also abolished ERK activation in response to CCh. ERK phosphorylation in CCh-stimulated cells was 27.5 ± 8.2-fold that in unstimulated cells compared with a 1.4 ± 1.0-fold stimulation in 4-DAMP-pretreated cells (n = 3).

**CCh-stimulated ERK Activity Negatively Regulates CCh-stimulated Chloride Secretion in T84 Cells**—In order to test the hypothesis that CCh-stimulated ERK activation may be involved in regulation of CCh-stimulated chloride secretion, we examined the effects of a specific inhibitor of ERK activation, PD 98059, on both ERK phosphorylation and $I_{sc}$ responses to CCh. Pretreatment of T84 cell monolayers with PD 98059 caused a concentration-dependent reduction in phosphorylation of ERK in response to CCh (Fig. 4A). Furthermore, PD 98059 (20 μM), which reduced CCh-stimulated ERK phosphorylation by 53.7 ± 19.9% (n = 3; p < 0.05), potentiated subsequent $I_{sc}$ responses to CCh across voltage-clamped monolayers of T84 cells (Fig. 4B). Maximal responses to CCh were 31.2 ±
Transactivation of the EGFr Mediates CCh-stimulated ERK Activation in T84 Cells—To date two mechanisms have been proposed by which GPCRs may bring about ERK activation. One is mediated by a tyrosine kinase-dependent pathway, which may involve transactivation of the EGFr, while the other is a PKC-mediated tyrosine kinase-independent pathway (21–23). Both pathways for ERK activation appear to be present and functional in T84 cells since both EGF (100 ng/ml) and the phorbol ester, PMA (100 nM), stimulate T84 cell ERK phosphorylation, albeit with different kinetics and to different extents than does CCh (Fig. 5, compare with Fig. 3). In the next series of experiments we set out to investigate the extent to which either, if any, of these pathways is involved in mediating the effects of CCh on ERK activity. Since one of the proteins displaying increased phosphorylation in response to CCh was a 180-kDa protein corresponding to the molecular mass of the EGFr (Fig. 1), we first examined a possible role for the EGFr in mediating the effects of CCh on ERK activation. Immunoprecipitation studies were carried out which verified that CCh (100 μM) stimulated a time-dependent increase in tyrosine phosphorylation of EGFr (Fig. 6A). The time course of the response was similar to that observed for CCh stimulation of ERK phosphorylation (cf. Fig. 3). CCh also stimulated an increase in co-immunoprecipitation of the adapter proteins, Shc and Grb2 with EGFr (Fig. 6, B and C). As expected, EGF itself (100 ng/ml) also stimulated phosphorylation of the EGFr and increased co-immunoprecipitation of both Shc and Grb2 with the EGFr (Fig. 6, A-C). Taken together, these data imply that CCh stimulates transactivation of EGFr in T84 cells, and, in a fashion similar to that of EGF, stimulates the formation of Shc-Grb2-EGFr complexes. Formation of such complexes is normally a prerequisite for ERK activation in many cell types.

We next examined the effects of a specific inhibitor of EGFr activation, tyrphostin AG1478, on CCh-stimulated ERK phosphorylation. Pretreatment of T84 cells with tyrphostin AG1478 (1 μM) abolished the effects of CCh on EGFr phosphorylation and significantly inhibited CCh-stimulated ERK activation (Fig. 7A). In the presence of tyrphostin AG1478 CCh-stimulated ERK phosphorylation was reduced by 51.8 ± 4.0% (n = 7; p < 0.01). Thus, transactivation of the EGFr appears to mediate a significant proportion of the effects of CCh on ERK activation in T84 cells. Furthermore, we found that, similar to findings with the ERK inhibitor PD 98059, tyrphostin AG1478 also potentiated Isc responses to CCh across voltage-clamped monolayers of T84 cells (Fig. 7B), supporting a role for transactivation of the EGFr, with subsequent activation of ERK, in negative regulation of CCh-stimulated epithelial secretion.

PKC Is Not Involved in CCh-stimulated ERK Activation in T84 Cells—In order to determine a possible role for PKC in the effects of CCh on ERK activation, we examined the effects of a cell-permeable inhibitor of PKC activity, GF109203X, on CCh-stimulated ERK phosphorylation. Pretreatment of T84 cells with GF109203X (1 μM) significantly inhibited ERK phosphorylation responses to PMA. Responses in cells treated with PMA in the presence of GF109203X were 58.8 ± 5.0% of those in cells stimulated with PMA alone (n = 3; p < 0.05). In contrast, GF109203X did not alter CCh stimulation of ERK phosphorylation when used alone, nor did it have any additional effect above that induced by the EGFr inhibitor, tyrphos-
tin AG1478 when both inhibitors were used in combination (Fig. 8). These data imply that neither GF109203X-sensitive isoforms of PKC nor other potential nonspecific targets of GF109203X, are involved in mediating the effects of CCh on ERK activity in T84 epithelial cells.

DISCUSSION

In the present study we have investigated a possible role for tyrosine kinase-dependent signaling pathways in negative regulation of calcium-mediated epithelial chloride secretion. In agreement with previous studies in another colonic epithelial cell line, HT-29 (8), we found that the prototypic calcium-mediated secretagogue, CCh, stimulated tyrosine phosphorylation of several proteins in T84 cells. Increased tyrosine phosphorylation was rapid in onset, occurring within 1 min, with maximal tyrosine phosphorylation occurring after approximately 5 min, a time corresponding to that at which CCh-stimulated secretory responses return to basal levels. This increase in tyrosine kinase activity appears to be involved in regulation of calcium-mediated epithelial chloride secretion. In agreement with previous studies in another colonic epithelial cell line, HT-29 (8), we found that the prototypic calcium-mediated secretagogue, CCh, stimulated tyrosine phosphorylation of several proteins in T84 cells. Increased tyrosine phosphorylation was rapid in onset, occurring within 1 min, with maximal tyrosine phosphorylation occurring after approximately 5 min, a time corresponding to that at which CCh-stimulated secretory responses return to basal levels. This increase in tyrosine kinase activity appears to be involved in regulation of calcium-mediated epithelial chloride secretion. In agreement with previous studies in another colonic epithelial cell line, HT-29 (8), we found that the prototypic calcium-mediated secretagogue, CCh, stimulated tyrosine phosphorylation of several proteins in T84 cells. Increased tyrosine phosphorylation was rapid in onset, occurring within 1 min, with maximal tyrosine phosphorylation occurring after approximately 5 min, a time corresponding to that at which CCh-stimulated secretory responses return to basal levels. This increase in tyrosine kinase activity appears to be involved in regulation of calcium-mediated epithelial chloride secretion.

Since two of the proteins displaying increased tyrosine phosphorylation in response to CCh had molecular weights corresponding to those of the ERK 1 and 2 isoforms of MAPK, we extended our studies to investigate a possible role for these enzymes in regulation of epithelial secretion. Although MAPK activation is classically associated with regulation of cell growth and differentiation, our studies also reveal an apparent acute role for ERK in negative regulation of calcium-mediated chloride secretion across intestinal epithelial cells. This conclusion stems from the observations that not only does CCh rapidly stimulate increases in ERK phosphorylation and activity in T84 cells, but pretreatment of T84 cells with PD 98059 leads to increases in both the magnitude and duration of CCh-stimulated secretory responses. PD 98059 is reported to be a highly specific inhibitor of the upstream component of the MAPK cascade, MEK, and has been shown reliably to inhibit ERK activation in a variety of systems (15, 29, 30). The idea that CCh simultaneously activates both prosecretory and antisecretory signaling pathways is supported by the finding that the muscarinic M3 receptor antagonist, 4-DAMP, not only inhibited CCh-stimulated I\textsubscript{sc} responses, but also abolished the effects of CCh on ERK phosphorylation. Thus, it appears that net CCh-stimulated secretory responses are determined not only by the extent of stimulation of prosecretory pathways, but also by the extent to which negative signaling intermediates, such as ERK, are activated.

The mechanisms by which GPCRs stimulate ERK activation are complex and appear to be dependent not only on the nature of receptor coupling to heterotrimeric G-proteins, but also upon
pathway normally favored by Gi-PCR (21–23), in mediating the effects of CCh on ERK activation. Several lines of evidence support this hypothesis. First, CCh stimulates tyrosine phosphorylation of the EGFr with a time course similar to that for ERK activation. Second, CCh stimulates recruitment of the adapter proteins, Shc and Grb2, to the EGFr. Association of these proteins with receptor tyrosine kinases is well documented as being an early step in growth factor-mediated activation of the MAPK cascade (20). Finally, at concentrations which have been demonstrated to block autophosphorylation of the EGFr, but not that of other EGFr family members (34, 35), we found that tyrphostin AG1478 abolished CCh-stimulated EGFr phosphorylation in T84 cells, but also significantly reduced CCh-stimulated ERK phosphorylation. Furthermore, similar to the ERK inhibitor, PD 98059, tyrphostin AG1478 potentiates ERK phosphorylation in T84 cells, but also significantly reduced CCh-stimulated ERK phosphorylation. Fig. 8. PKC does not mediate CCh stimulation of ERK activity. A, T84 cells were stimulated with basolateral CCh (100 μM, 2 min) in the absence or presence of bilateral tyrphostin AG1478 (1 μM, 20 min), bilateral GF 902103X (1 μM, 20 min), or a combination of both inhibitors. Cell lysates were analyzed for ERK phosphorylation by Western blotting with anti-phospho-ERK antibodies. Panel A shows densitometric analysis of the data represented in panel B combined with three similar studies, expressed in arbitrary units (a.u.). As described in the legend to Fig. 7, tyrphostin AG1478 significantly reduced ERK phosphorylation in response to CCh. However, GF 902103X was without effect on CCh-stimulated ERK phosphorylation either when used alone or in combination with tyrphostin AG1478 (n = 4 throughout). Statistical analysis was carried out by ANOVA followed by Student-Newman-Keuls post-test. Asterisks represent significant differences from control, unstimulated cells (*, p < 0.05; ***, p < 0.001). # represents significant differences from CCh-stimulated cells.

Fig. 7. Tyrphostin AG1478 inhibits CCh-stimulated phosphorylation of the EGFr and ERK and potentiates CCh-stimulated secretory responses. A, cells were stimulated with basolateral CCh (100 μM, 2 min) in the presence or absence of tyrphostin AG1478 (1 μM, bilateral, 20 min). For determination of ERK phosphorylation, whole cell lysates were analyzed by Western blotting with anti-phospho-ERK antibodies. For determination of EGFr phosphorylation, cell lysates were immunoprecipitated with anti-EGFr and subsequent Western blots were probed with anti-phosphotyrosine. The extent of EGFr and ERK phosphorylation was determined by densitometric analysis and expressed in arbitrary units (a.u.). Tyrphostin AG1478 abolished CCh stimulation of EGFr phosphorylation and significantly reduced CCh-stimulated ERK phosphorylation (n = 7). Statistical analysis was carried out by ANOVA followed by Student-Newman-Keuls post-test. Asterisks denote significant differences from control, unstimulated cells (*, p < 0.05; ***, p < 0.001). # and ## denote significant differences from CCh-stimulated cells (p < 0.05 and p < 0.01, respectively). B, bilateral pretreatment of voltage-clamped monolayers of T84 cells for 20 min with tyrphostin AG1478 (1 μM), significantly potentiated subsequent chloride secretory responses to basolateral CCh (100 μM). Maximal responses to CCh in control monolayers were 29.2 ± 2.7 μA/cm² compared with 57.9 ± 10.5 μA/cm² in tyrphostin AG1478-treated cells (n = 5; p < 0.05). Data are expressed as mean ± S.E. increases in Isc (ΔIsc) induced by carbachol addition at time 0.

In summary, these results indicate that stimulation of PKC can be linked to ERK activation in T84 cells, this pathway does not appear to represent a mechanism by which CCh stimulates ERK activity in these cells. Thus, GF 902103X, which inhibited PMA-stimulated T84 cell ERK activation, and which has been shown to inhibit GPCR-stimulated ERK activation in other cells (14), had no effect on ERK phosphorylation in response to CCh. Rather, our data strongly support a role for transactivation of the EGFr, a
ruration remained in the presence of the antagonist, implying more than one pathway may exist by which CCh stimulates ERK activation. This residual, EGFr-independent ERK phosphorylation does not appear to be mediated by PKC since GF 902103X was not only ineffective in reducing CCh-stimulated ERK phosphorylation when used alone, but was also without effect when used in combination with tyrophostin AG1478. Further studies are required in order to identify this apparent PKC- and EGFr-independent mode of T84 cell ERK activation in response to CCh.

It is not yet known how CCh-stimulated transactivation of the EGFr and subsequent ERK activation might interact with epithelial transport pathways to bring about inhibition of chloride secretion. Some studies implicate cystic fibrosis transmembrane conductance regulator chloride channels, which are believed to be an important exit pathway for chloride in response to calcium-dependent secretagogues, as a possible target for inhibitory tyrosine kinase-dependent signals (36, 37). Moreover, recent studies demonstrate roles for M1 muscarinic receptor-mediated transactivation of the EGFr in regulation of K+ channels in kidney cells (38), and for ERK in regulation of K+ channel function in neuronal cells (39). Further studies are required to determine if similar processes are involved in ERK-mediated inhibition of CCh-stimulated secretion in T84 cells.

Likewise, additional studies are required to define possible interactions between CCh-stimulated ERK activity and other signals believed to be involved in negative regulation of calcium-dependent chloride secretion, such as agonist-stimulated influx of extracellular calcium (4). Of particular note in this regard are the recent findings of Rosen and Greenberg (40) who have demonstrated that influx of calcium through voltage-sensitive calcium channels stimulates EGFr phosphorylation, association of Grb2 and Shc with the EGFr, and ERK activation in PC12 neuronal cells. Similarly, it has been demonstrated that calcium influx and a novel calcium-dependent tyrosine kinase, PYK-2, are important in mediating the effects of GPCR receptor activation on ERK activity and subsequent K+ channel inhibition in HEK-293 kidney cells (24, 39, 41). Although a possible role for CCh-stimulated calcium influx in mediating the effects of the agonist on ERK activation in T84 cells has yet to be investigated, preliminary studies from our laboratory indicate that T84 cells not only express PYK-2, but that the enzyme becomes associated with the EGFr upon stimulation with CCh.2 Thus, CCh-stimulated influx of extracellular calcium may represent an early step in a signaling cascade which ultimately results in stimulation of ERK activity via transactivation of the EGFr.

In summary, we have shown that CCh stimulates activation of the ERK isoforms of MAPK in colonic epithelial cells, and that this appears to be involved in limiting the extent of CCh-stimulated chloride secretory responses. Stimulation of ERK activity by CCh occurs via a pathway independent of PKC, but which involves transactivation of the EGFr. We speculate that since chloride is the predominant ion driving fluid secretion in the intestine (5), agonist-stimulated transactivation of the EGFr and subsequent activation of ERK may represent a physiological braking mechanism to prevent excessive electrolyte and fluid loss when the levels of calcium-dependent secretagogues are elevated within the intestinal mucosa.

Acknowledgment—We thank Glenda Wheeler for assistance with manuscript submission.

REFERENCES

1. Barrett, K. E. (1997) Am. J. Physiol. 273, C1069–C1076
2. Dharbowski, A., Grohlewski, G. E., Schafer, C., Guan, K.-U., and Williams, J. A. (1997) Am. J. Physiol. 274, C1472–C1479
3. Wilkie, N., Morton, C. M., Ng, L. L., and Boarder, M. R. (1996) J. Biol. Chem. 271, 32447–32453
4. Corbett, K. E. (1998) Cell Signal. 9, 353–351
5. Luttrell, L. M., van Biesen, T., Hawes, B. E., Della-Rocca, G. J., Luttrell, D. K., and Leffkowitz, R. J. (1998) J. Biol. Chem. 273, 12444–12452
6. Heasley, L. E., Senkfo, S. I., Winitz, S., Strasheim, A., Teitelbaum, I., and Beri, T. (1994) Am. J. Physiol. 267, F366–F373
7. Nakamura, K., Zhou, C.-J., Parente, J., and Chew, C. S. (1996) Am. J. Physiol. 271, 6040–6049
8. Zou, Y., Komuro, I., Yamazaki, T., Aikawa, R., Sumiyoshi, K., Hiroi, Y., Mizuno, Y., and Yazaki, Y. (1996) J. Biol. Chem. 271, 33592–33597
9. Kii, Y., and Krebs, E. G. (1995) FASEB J. 9, 276–285
10. van Biesen, T., Luttrell, L. M., Hawes, B. E., and Leffkowitz, R. J. (1996) Endocr. Rev. 17, 698–714
11. Sugden, P. H., and Clerk, A. (1997) Cell Signal. 9, 353–351
12. Luttrell, L. M., van Biesen, T., Hawes, B. E., Della-Rocca, G. J., Luttrell, D. K., and Leffkowitz, R. J. (1998) Adv. Pharmacol. 42, 466–470
13. Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Leffkowitz, R. J. (1997) J. Biol. Chem. 272, 19125–19132
14. Dickinson, K. E., Frizzell, R. A., and Chandra Sekar, M. (1992) Am. J. Physiol. 263, F366–F373
15. Liao, D.-F., Monia, B., Dean, N., and Berk, B. C. (1997) J. Biol. Chem. 272, 737–745
16. Reenstra, W. W. (1995) Adv. Pharmacol. 269, C914–C922
17. Aikawa, R., Sumiyoshi, K., Shimjo, I., Hiroi, Y., Mizuno, Y., and Yazaki, Y. (1996) J. Biol. Chem. 271, 33592–33597
18. Dikic, I., Tokiwa, G., Lev, S., Countneidge, S. A., and Schlessinger, J. (1996) Nature 382, 547–550

2. J. Keely, L. S. Bertelsen, and K. E. Barrett, unpublished observations.