In many systems, the integration of converging regulatory signals that relay on G protein-coupled receptor (GPCR) activation into functional cellular pathways requires the involvement of receptor tyrosine kinase. In this report, we provide evidence that activation of GPCR by β-adrenergic agonist leading to stimulation in gastric mucin secretion requires epidermal growth factor receptor (EGFR) participation. Using [3H]glucosamine-labeled gastric mucosal cells, we show that stimulatory effect of β-adrenergic agonist, isoproterenol, on mucin secretion was inhibited by EGFR kinase inhibitor, PD153035, as well as wortmannin, a specific inhibitor of PI3K. Both inhibitors, moreover, blunted the mucin secretory responses to β-adrenergic agonist-generated second messenger, cAMP as well as adenylate cyclase activator, forskolin. The gastric mucin secretory responses to isoproterenol, furthermore, were inhibited by PP2, a selective inhibitor of tyrosine kinase Src responsible for ligand-independent EGFR autophosphorylation, but not by ERK inhibitor, PD98059. The inhibition of ERK, moreover, did not cause attenuation in mucin secretion in response to cAMP and forskolin. The findings underline the role of EGFR as a convergence point in gastric mucin secretion triggered by β-adrenergic GPCR activation, and demonstrate the requirement for Src kinase in EGFR transactivation.

Key words: gastric mucin; secretion; β-adrenergic GPCR activation; Src kinase; EGFR transactivation.
INTRODUCTION

A unique property of gastric epithelium is its ability to withstand a wide variety of insults by exogenous agents and noxious luminal contents. Among the mucosal components to which this protective function is most often ascribed are the viscous layer of mucus that tenaciously adheres to the epithelial surfaces, the cell membranes of gastric epithelium, and the mucosal blood flow (1, 2). Although it is accepted that the maintenance of mucosal homeostasis relays on the multitude of interactions occurring within the three defense elements, the initial brunt of luminal insult falls on the protective layer of mucus that constitutes the pre-epithelial element of gastric mucosal defense (2-4). The gastric mucus layer is not a single entity, but a heterogeneous mixture of proteins, glycoproteins, and lipids in the form of a gel imbibed with water and electrolytes. The integrity and strength of the mucus coat as well as its performance relays mainly on its mucin component. This large, highly glycosylated glycoprotein plays a major role in the maintenance of viscoelastic, permselective and hydrophobic properties of the layer, and the disturbances in mucin synthesis are associated with the loss of mucus coast continuity, mucosal injury, and the onset of gastric disease (3, 5). Moreover, as a constituent of the mucus coat, the glycoprotein remains in intimate contact with the underlying epithelial surfaces, and exerts important modulatory influence on the processes occurring within the epithelial perimeter of gastric mucosal defense (6, 7).

The functional efficiency of gastric mucin depends on a delicate balance of factors that control the processes of the glycoprotein assembly and secretion. Hence, an orderly and synchronized processing of signaling clues affecting mucin synthesis and secretion plays a major role in the preservation of gastric mucosal integrity as well as inherent resistance of the mucosa to injury. Among the factors of paramount importance to the control of gastric mucin secretion are those derived through sympathetic and parasympathetic innervation, and gastric mucosa possesses receptors capable of responding to neurotransmitter signals from either system (8, 9). Indeed, an increase in gastric mucus gel hydration was reported to occur in response to stimulation by cholinergic agonists, while β-adrenergic agonists have been shown to affect the mucus glycoprotein production (8, 10, 11). Moreover, previous study from our laboratory revealed that gastric mucosal cells in culture respond to cAMP β-adrenergic agonist simulation by the increase in phospholipid secretion (12). Of the three known subtypes of β-adrenergic receptors (β1, β2, and β3), the β3 receptor, although expressed in stomach and intestine (13, 14), has the most significant effect on lipid metabolism in adipose tissue (15), while the activation of β1,2-adrenergic receptors is associated with stimulation of macromolecular constituents of gastric mucus, including its principal glycoprotein, mucin (8).

Interestingly, more recent evidence indicates that the cellular effects of G protein-coupled receptor (GPCR) agonists such as isoproterenol and vasoactive
intestinal peptide (VIP) occur in association with epidermal growth factor receptor (EGFR) transactivation (16, 17). This ligand-independent EGFR activation involves selective phosphorylation of the tyrosine residues, which are not autophosphorylation sites associated with EGF stimulation, by GPCR activated Src kinase (16, 18). In this study, using gastric mucosal cells, we have investigated the role of EGFR transactivation in regulation of the signaling pathways leading to gastric mucin secretion in response to stimulation by β-adrenergic agonist, isoproterenol.

MATERIALS AND METHODS

Gastric mucosal cell isolation and mucin labeling

The study was conducted with Sprague-Dawley rats in compliance with the institutional Animal Care and Use Committee. The gastric mucosal cells collected by scraping the mucosa with a blunt spatula on ice-cold glass plate. The scrapings were minced and suspended in 5 volumes of Dulbecco's modified (Gibco) Eagle's minimum essential medium (DMEM), supplemented with fungizone (50 µg/ml), penicillin (50 U/ml), streptomycin (50 g/ml) and 2% albumin. The cells were gently dispersed by trituration with a syringe, settled by centrifugation at 300 x g for 5 min, and, following three consecutive rinsing with DMEM, resuspended in the medium to a concentration of 3 x 10^6 cell/ml (12). Aliquots of the cell suspension (1 ml) were transferred to DMEM in culture dishes containing [3H]glucosamine (110 µCi), and incubated for 4 h under 95% O_2/5% CO_2 atmosphere at 37°C (16). The cells were the centrifuged at 300 x g, washed three times with DMEM containing 5% albumin to remove free radiolabel, and resuspended in a fresh DMEM free of albumin.

Mucosal cell incubation

To assess the effect of β-adrenergic agonist on the gastric mucosal cell mucin secretory responses, the cells were preincubated for 30 min either with saline diluent or indicated concentrations of β-adrenergic antagonist, alprenolol (Sigma) or cholinergic antagonist, atropine (Sigma), followed by 30 min incubation with 0 - 15 µM isoproterenol (Sigma). In the experiments on the effect of EGFR inhibitor, PD153035 (Calbiochem), ERK1/2 inhibitor, PD98059 (Calbiochem),PI3K inhibitor, wortmannin (Sigma), Src kinase inhibitor, PP2 (Calbiochem), protein kinase A (PKA) inhibitor, H89 (Sigma), and adenylate cyclase inhibitor, SQ22536 (Sigma), the cells prior to addition of the isoproterenol were first incubated for 30 min with the indicated concentration of the agent. The effects of dibutyryl-cAMP (Sigma) and forskolin (Sigma) were assessed following 30 min preincubation with PD153035, wortmannin, H89 or saline diluent. At the end of 30 min incubation period, the cells were centrifuged at 300 x g for 5 min and washed three times with fresh DMEM. The medium and washes were combined, and used for [3H]glucosamine labeled mucin assay (19). Cell preparations before and during the experimentation were assessed for viability and cellular integrity using Trypan blue dye exclusion assay and the determination of lactate dehydrogenase released into the medium (12).

Mucin analysis

The combined cell wash and incubation medium containing 3H-labeled mucin were treated at 4°C with 10 volumes of 2% phosphotungstic acid in 20% trichloroacetic acid for 4 h and the formed precipitates were collected by centrifugation (20). The crude glycoprotein precipitates were dissolved
in 6 M urea and chromatographed on Bio-gel A-1.5 column (0.9 x 110 cm). The eluted fractions were monitored spectrophotometrically for protein and for radioactivity by liquid scintillation spectrometry. The mucus glycoprotein fractions eluted in the excluded volume were pooled, dialyzed, lyophilized, and subjected to analysis for total incorporation of radiolabel and protein content (19).

Data analysis

All experiments were carried out using duplicate sampling and the results are expressed as mean ± SD. Analysis of variance (ANOVA) was used to determine significance, and the significance level was set at P > 0.05.

RESULTS

The role of EGFR transactivation in mediation of the signaling pathways involved in gastric mucin secretion in response to β-adrenergic agonist stimulation was investigated using [3H]glucosamine-labeled rat gastric mucosal cells exposed to isoproterenol. Under the employed incubation conditions, the cell viability remained over 98% as judged by Trypan blue uptake, and the lactate dehydrogenase assays indicated only negligible if any (0.7%) cellular damage. The 3H-labeled mucin secretion by the gastric mucosal cells showed a dose-

![Fig. 1. Effect of β-adrenergic agonist, isoproterenol, on mucin secretion by rat gastric mucosal cells in culture. The [3H]glucosamine-labeled cells, pretreated with saline diluent (A) or indicated concentrations of β-adrenergic antagonist, alprenolol, or cholinergic antagonist, atropine (At), (B), were stimulated for 30 min with the indicated concentrations of isoproterenol and the medium was analyzed for radiolabel mucin. Values represent the means ± SD of five experiments. *P < 0.05 compared with that of control.](image)
dependent increase with the isoproterenol concentration up to 10µM, at which point a 3.2-fold increase in mucin release over that of controls was attained (Fig. 1A). The prosecretory effect of isoproterenol on mucin secretion was countered in a dose-dependent manner by a specific β-adrenergic antagonist, alprenolol, which at the optimal concentration of 15 µM produced a 94.8% impedance of the isoproterenol stimulatory effect (Fig. 1B). However, the stimulatory effect of isoproterenol on mucin secretion was not affected by cholinergic antagonist, atropine (Fig. 1B). This indicated that the observed increase in mucin secretion by gastric mucosal cells occurred in response to β-adrenergic agonist stimulation.

We next examined the gastric mucosal cell mucin secretory responses to isoproterenol in the presence of a specific EGFR kinase inhibitor, PD153035. The results revealed that pretreatment with PD153035 led to a dose-dependent suppression in the isoproterenol effect, which at PD153035 concentration of 100nM produced an 86.4% decrease in the isoproterenol-stimulated mucin secretion. (Fig. 2A). Moreover, the stimulatory effect of isoproterenol on gastric mucin secretion was also inhibited by PP2, a selective inhibitor of tyrosine kinase Src responsible for ligand-independent EGFR transactivation. The effect of PP2 was concentration-dependent and at 15µM produced an 83.1% reduction of the isoproterenol-stimulated mucin secretion (Fig. 2B).

![Fig. 2. Effect of EGFR inhibitor, PD153035, and Src kinase inhibitor, PP2, on gastric mucosal cells mucin secretion in response to stimulation by isoproterenol. The [3H]glucosamine-labeled cells, preincubated with the indicated concentrations of PD153035 (A) or PP2 (B), were stimulated for 30 min with 10µM isoproterenol (Iso) and the medium was analyzed for radiolabel mucin. Values represent the means ± SD of five experiments. *P < 0.05 compared with that of Iso.](image-url)
As the prosecretory effects of isoproterenol are mediated through elevation of intracellular levels of cAMP, we further investigated the effect of EGFR inhibitor PD153035 on the gastric mucosal cell mucin secretory responses to a cell permeable analog of cAMP, dibutyryl-cAMP (DBcAMP), and adenylate cyclase activator, forskolin. The data revealed that in the absence of PD153035, both agents evoked a dose-dependent stimulation in mucin secretion, with dibutyryl-cAMP at 100 µM increased by a 2.9-fold (Fig. 3A) and with forskolin at 20 µM by a 2.8-fold (Fig. 3B). Pretreatment with EGFR kinase inhibitor, PD153035, reduced the stimulatory effect of dibutyryl-cAMP on the gastric mucosal cell mucin secretion by a 75.2% (Fig. 3A) and that of forskolin by a 69.3% (Fig. 3B). Furthermore, the mucin stimulatory effects of dibutyryl-cAMP and forskolin were countered by an 82.4% and 75.6%, respectively, by a specific inhibitor of PKA, H89, which also caused a 73.1% reduction in the isoproterenol-induced mucin secretion (Fig. 4). The prosecretory effect of isoproterenol on mucin secretion, moreover, was subject to inhibition (64%) by SQ22536, an inhibitor of adenylate cyclase activity (Fig. 4).

As the downstream effects of EGFR transactivation are mediated by PI3K, we further examined the isoproterenol-induced gastric mucosal cell mucin secretory responses in the presence of PI3K inhibitor, wortmannin. The results revealed that

![Graph](image-url)
pretreatment with wortmannin at 100 nM evoked an 85.2% reduction in mucin secretion in response to isoproterenol (Fig. 5). Furthermore, wortmannin caused the inhibition in gastric mucosal cell mucin secretory responses to a cell permeable analog of cAMP, dibutyryl-cAMP and adenylate cyclase activator, forskolin, which decreased by 78.1% and 74.5%, respectively (Fig. 5). No significant alterations in the mucosal cell mucin secretory responses to isoproterenol, dibutyryl-cAMP or forskolin, however, were observed with the inhibitor of ERK pathway, PD98059 (Fig. 5), thus indicating that ERK is not involved in mediation of isoproterenol-induced mucin secretory responses in gastric mucosa.

**DISCUSSION**

The integrity and strength of the protective layer of mucus, constituting pre-epithelial element of gastric mucosal defense, relays mainly on its mucin component and hence the factors that control this glycoprotein elaboration are of direct relevance to the preservation of gastric mucosal homeostasis. Generally, the
control of gastric mucus secretion and its makeup occurs through neurotransmitters, released by sympathetic and parasympathetic innervation, that bind specific GPCRs on the gastric mucosal cells and trigger the generation of soluble second messengers, Ca\(^{2+}\), PKC, and cAMP (8, 9). Although the secretory responses to Ca\(^{2+}\)- and PKC-mediated cholinergic agonists are rapid in onset, they are transient in duration, while those induced by cAMP-dependent β-adrenergic agonists are considerably more sustained and affect the secretion of macromolecular constituents of gastric mucus, including its principal functional component, mucus glycoprotein (8, 10, 11). Moreover, the cellular responses mediated by GPCR signaling systems that relay on β-adrenergic agonist-generated soluble cAMP messenger occur in association with EGFR transactivation (16, 17).

Indeed, activation of GPCR by β-adrenergic agonist, isoproterenol, leading to EGFR transactivation with the involvement of PKA-induced stimulation of ERK and Src kinases has been reported in transformed kidney cells, (16), and colonic epithelial cells respond to VIP stimulation with GPCR-mediated signaling pathway through cAMP- and PKA-dependent EGFR transactivation that involves PI3K (17). This ligand-independent EGFR activation, apparently, involves selective phosphorylation of the tyrosine residues that are distinctly

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**Fig. 5.** Effect of PI3K inhibitor, wortmannin (Wt), and ERK inhibitor, PD98059 (PD) on gastric mucosal cells mucin secretion in response to isoproterenol (Iso), dibutyryl-cAMP (DB-cAMP), and forskolin (Fsk). The [\(^3\)H]glucosamine-labeled cells, preincubated with 100 nM Wt or 40 µM PD, were stimulated for 30 min with 10 µM Iso or 100 µM DB-cAMP, or 20 µM Fsk and the medium was analyzed for radiolabel mucin. Values represent the means ± SD of five experiments. *P < 0.05 compared with that of agonist alone.
different from those associated with EGF stimulation (16, 18). Hence, in our study, we examined the role of EGFR transactivation in regulation of gastric mucin secretion in response to stimulation by isoproterenol.

The results demonstrated that exposure of gastric mucosal cells to isoproterenol led to stimulation in mucin secretion, and that the effect was sensitive to inhibition by β-adrenergic antagonist, alprenolol, that is known to block β1/2-receptors, but not the β3 receptor (15, 21). Hence, the gastric mucosal cells responded with the increase in mucin secretion specifically to β1/2-adrenergic effector. Indeed, our further data revealed that the mucosal cell mucin secretory responses were also stimulated by cAMP cell permeable analog, dibutyryl-cAMP, as well as adenylate cyclase activator, forskolin, and that prosecretory effect of isoproterenol on mucin secretion was subject to suppression by a specific inhibitor of adenylate cyclase activity, SQ22536 and the PKA inhibitor, H89. Furthermore, the stimulatory effect of isoproterenol on mucin secretion was inhibited by a specific inhibitor of EGFR kinase activity, PD153035, as well as wortmannin, a specific inhibitor of PI3K. Both inhibitors also caused the reduction in the gastric mucosal cell mucin secretory responses to cAMP analog, dibutyryl-cAMP, and forskolin. These findings thus provide the evidence for the role of EGFR in mediation of β-adrenergic agonist prosecretory action on gastric mucin secretion, and point to PI3K as a critical regulator of cAMP-dependent secretory responses.

Based on the recent literature data with VIP and cell permeable cAMP analog, dibutryrl-cAMP, it appears that the secretory responses to cAMP-dependent secretagogues require the recruitment of a p85 regulatory subunit of PI3K to the EGFR (17). This process apparently is associated with autophosphorylation of tyrosine residue within the regulatory subunit and leads to an increase in catalytic activity of p110 subunit of PI3K towards membrane inositol lipids that trigger protein kinase B/Akt activation (22, 23). Indeed, in the intestinal epithelial cells, the inhibition of PI3K with wortmannin attenuated secretory responses not only to VIP and dibutyryl-cAMP, but also to the adenyl cyclase activator, forskolin (17). Moreover, Akt activation and PI3K-dependent generation of phosphatidylinositol phosphates have been shown to be sensitive to an inhibitor of Src kinase, PP2 (22).

Indeed, studies into the mechanism of GPCR-mediated transactivation of the EGFR assign the central role in the activation of the receptor tyrosine kinase process to the Src kinase (16, 18, 24). While EGFR is known to undergo autophosphorylation at five tyrosine residues upon EGF ligand stimulation, the phosphorylation sites that are dependent on Src kinase and triggered by β-adrenergic agonist engagement of GPCR involve Tyr-845 and Tyr-1101 (18, 25). Apparently, the phosphorylation of these two tyrosine residues is associated with EGFR activation through dimerization and triggers the formation of a heterocomplex between Src and activated EGFR that results in an enhanced phosphorylation of receptor substrates (16, 18, 25, 26). Interestingly in many systems, GPCR-mediated EGFR transactivation is also associated with the activation of ERK MAPK, and the Src kinase inhibitors impair EGFR phosphorylation as well as ERK activation (27,
28). Hence, we focused further on the role of ERK and Src kinases in mediation of gastric mucin secretion in response to stimulation by isoproterenol.

The results revealed that while the inhibition of Src kinase with a selective pharmacological inhibitor, PP2, evoked a significant reduction in mucin secretion in response to isoproterenol, the ERK inhibitor, PD98059, caused only marginal alterations in subsequent mucin secretory responses to isoproterenol. The inhibition of ERK, furthermore, did not attenuate mucin secretion in response to dibutyryl-cAMP and forskolin. These findings are thus consistent with the recent data obtained with T_{44} colonic epithelial cells and demonstrating that, although cAMP-dependent agonist-induced EGFR transactivation is accompanied by ERK phosphorylation, the kinase is not involved in mediation of cAMP-dependent secretory responses (17). However, while our results herein and the data obtained by others (17, 27, 29), implicate PKA as an integral part of the signaling pathway linking cAMP-dependent β-adrenergic agonist-activated GPCR to the EGFR transactivation, there are also indications that cAMP is capable of ERK1/2 activation in a PKA independent fashion (30).

Indeed, activation of ERK1 and ERK2 members of MAPK family plays important role in the regulation of a wide variety of cellular responses, including gene expression, protein synthesis, and cell growth and differentiation (24, 28, 31). ERK phosphorylation has been observed after ligand activation of GPCRs associated with malignant cell proliferation as well as protein kinase C mediated EGFR transactivation (31, 32). Moreover and perhaps most fittingly, it has been pointed out recently that ERK activation is dependent on Src kinase, as well as EGFR activation, depending on cell type, and the type and duration of the stimulus, and that the characteristics of cells containing overexpressed ectopic receptors and signaling proteins may differ from those of cells expressing endogenous GPCRs (27). It is also conceivable that EGFR has the capability to differentially modulate and propagate intracellular signals of the cAMP-dependent responses, or may reflect the differences in the signaling events at the level of receptor activation.

Taken together, the results of our study underline the central role of EGFR in mediation mucosal secretory processes, and demonstrate the requirement for Src kinase-dependent EGFR transactivation in the regulation of gastric mucin secretion in response to β-adrenergic GPCR activation.

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