

Transactivation of the Epidermal Growth Factor Receptor Mediates Cholinergic Agonist-Induced Proliferation of H508 Human Colon Cancer Cells¹

Kunrong Cheng, Piotr Zimniak, and Jean-Pierre Raufman²

Division of Gastroenterology, VA Maryland Health Care System and Program in Oncology, University of Maryland Greenebaum Cancer Center, Baltimore, Maryland 21201 [K. C., J.-P. R.], and Central Arkansas Veterans Healthcare System and Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205 [P. Z.]

ABSTRACT

Some human colon cancer cell lines (e.g., H508 cells) express M3 subtype muscarinic receptors that are activated by cholinergic agonists. The objective of the present study was to determine the cellular mechanisms underlying M3 muscarinic receptor-mediated proliferation of H508 human colon cancer cells. In H508 cells, but not in SNU-C4 cells that do not express muscarinic receptors, acetylcholine stimulated calcium-dependent phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) and p90 ribosomal S6 kinase and consequent cell proliferation. Atropine or inhibitors of MAPK phosphorylation blocked these effects. Conversely, the actions of epidermal growth factor (EGF) on H508 cells were neither calcium dependent nor mediated by cholinergic mechanisms. Both acetylcholine- and EGF-induced phosphorylation of p44/42 MAPK was abolished in the presence of EGF receptor (EGFR) inhibitors (AG1478 and PD168393). In Chinese hamster ovary cells transfected with the rat M3 muscarinic receptor, which lack EGFR, acetylcholine-induced MAPK phosphorylation was not altered in the presence of EGFR inhibitors. In H508 cells, protein kinase C inhibitors did not alter acetylcholine- or EGF-induced MAPK phosphorylation. Finally, inhibition of EGFR activation abolished acetylcholine-induced H508 cell proliferation. These data indicate that, in H508 human colon cancer cells, cholinergic ligand interaction with M3 muscarinic receptors results in transactivation of EGFR, thereby stimulating cellular proliferation.

INTRODUCTION

Post-receptor signaling cascades are crucial for ligand-receptor interaction to result in changes in cell function, including modulation of gene expression, stimulation of the movement of zymogen granules to the cell surface, and a host of others. The complexity of these cascades permits interaction between signaling pathways (so-called “cross-talk”), thereby allowing “fine-tuning” of the response to different ligands alone or in combination. This also allows for amplification of the signal, qualitatively and quantitatively. One form of “cross-talk” between signaling pathways, designated transactivation, occurs when a neighboring receptor is activated by components of the signaling cascade stimulated by ligand binding to a different class of receptor. The elements of these signaling cascades and their interactions vary from cell to cell, even for the same class of receptor. In fact, depending on the cell type, a particular interaction between receptor signaling cascades can result in stimulatory or inhibitory effects. This is of particular interest in unraveling the sequence of events that leads to normal and abnormal cellular proliferation.

The muscarinic cholinergic family of GPCRs³ includes five mus-

carinic receptor subtypes designated M1–M5 (1, 2). GPCRs share several characteristics including seven membrane-spanning domains with intracellular domains that are coupled to specific G proteins. Peripheral M3 receptors, which are very common in the gastrointestinal tract, are coupled to a G protein designated G_{q11} that activates phospholipase C, resulting in inositol phosphate formation and a rise in cellular calcium (3). Activation of M3 receptors in the gastrointestinal tract results in enzyme secretion, fluid and electrolyte transport, smooth muscle contraction, and other actions. It is apparent that some cancer cells express muscarinic receptors. For example, H508 colon cancer cells express M3 muscarinic receptors (4, 5). Activation of these receptors results in stimulation of H508 cell proliferation (5). The cellular pathways that transmit to the nucleus the signal caused by interaction of acetylcholine with muscarinic receptors, thereby resulting in cell proliferation, have not been elucidated.

In contrast to GPCRs, EGFR, a member of the growth factor receptor family, has a single membrane-spanning domain and intrinsic tyrosine kinase activity. Activation of receptors in this family results in protein tyrosine phosphorylation and the formation of phosphotyrosine-dependent intracellular protein complexes. These events are connected to cellular proliferation in a number of cell types, including cancer cells (6). Transactivation of EGFRs by other plasma membrane receptors has been linked to mitogenic signaling by multiple GPCR agonists including endothelin-1, lysophosphatidic acid, thrombin, and others (7, 8). Activation of muscarinic receptors has been reported to transactivate the EGFR, thereby resulting in phosphorylation of components of the EGFR signaling cascade (3, 9–11). That is, interaction of muscarinic agonists with their receptors may cause activation of EGFR in the absence of EGF. Hence, we thought it possible that muscarinic stimulation of H508 human colon cancer cell proliferation might be mediated by transactivation of EGFR.

The objective of the present study was to determine the cellular mechanisms underlying cholinergic agonist-induced H508 cell proliferation. In particular, we focused on a possible interaction between H508 and EGFRs that would explain the proliferative effects of muscarinic stimulation of these cells. Our findings support the novel observation that acetylcholine-induced proliferation of H508 human colon cancer cells is mediated by transactivation of EGFR.

MATERIALS AND METHODS

Chemicals and Reagents. DMEM, MEM nonessential amino acids, penicillin, streptomycin, and G418 were from Life Technologies, Inc.; BAPTA-AM, H7, GF109203X, PD98059, and AG1478 were from Alexis; EGTA-AM, pp2, PD168393, wortmannin, LY294002, and carbamylcholine (carbachol) were from Calbiochem. All other chemicals were obtained from Sigma or Fisher.

Antibodies and Immunoblotting. Rabbit polyclonal anti-MAPK, mouse monoclonal anti-phospho-MAPK, rabbit polyclonal anti-phospho-EGFR, and rabbit polyclonal anti-phospho-90RSK were from Cell Signaling (Beverly, MA). Phosphorylation of p44/42 MAPK, EGFR, and p90RSK was determined

protein kinase C; CHO, Chinese hamster ovary; FBS, fetal bovine serum; rM3, rat M3; SRB, sulforhodamine B; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3'-kinase.

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² To whom requests for reprints should be addressed, at Division of Gastroenterology and Hepatology, University of Maryland School of Medicine, Division of Gastroenterology, 22 South Greene Street, N3W62, Baltimore, MD 21201. Phone: (410) 328-8728; Fax: (410) 328-8315; E-mail: jraufman@medicine.umaryland.edu.

³ The abbreviations used are: GPCR, G protein-coupled receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RSK, ribosomal S6 kinase; PKC,

by methods described previously (12). Briefly, cells were subcultured in 6-well plates (10^6 cells/well). After a 24-h incubation at 37°C, the cells were serum-starved for an additional 24 h, washed with PBS, and allowed to recover in PBS for 1 h at 37°C before adding test agents. After a 10-min incubation with test agents, the reaction was terminated by adding lysis buffer [150 mM NaCl, 10 mM Tris-HCl, 1% (w/v) deoxycholic acid, 1% (v) NP40, 0.1% (w/v) SDS, 4 mM EDTA, 1 mM Na_3VO_4 , 20 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, 250 $\mu\text{g}/\text{ml}$ nitrophenyl phosphate, and 1 mM phenylmethylsulfonyl fluoride (pH 8)]. When inhibitors (H7, GF109203X, PD98059, AG1478, pp2, PD168393, wortmannin, LY294002, and atropine) were used, they were added 30 min before test agents. Cell lysates were subjected to SDS-PAGE (10% gel; Invitrogen). Resolved proteins were transferred electrophoretically to nitrocellulose membranes (Micron Separations) and probed with anti-phospho-p44/42 (ERK 1/2) MAPK, anti-phospho-EGFR, or anti-phospho-90RSK. Bound antibody was detected by chemiluminescence (Supersignal kit; Pierce). To verify that equal amounts of protein were added to each lane, the blots used with anti-phospho-p44/42 MAPK were stripped and reprobed with anti-p42.

Cell Culture. Colon cancer cell lines (H508 and SNU-C4) were grown in RPMI 1640 (American Type Culture Collection) supplemented with 10% FBS (Biowhittaker). Adherent cultures were passaged weekly at subconfluence after trypsinization. Cultures were maintained in incubators at 37°C in an atmosphere of 5% CO_2 and 95% air. CHO cells expressing rM3 subtype muscarinic receptors were obtained from American Type Culture Collection. These cells do not express EGFR (13). Cells were grown in DMEM supplemented with 10% FBS, 1 \times MEM nonessential amino acids, penicillin (50 units/ml), streptomycin (50 mg/ml), and G418 (0.1 mg/ml).

Cytotoxicity Assays. Before proceeding with these studies, we evaluated potential cytotoxic actions of test agents on H508, SNU-C4, and CHO-M3 cells by trypan blue exclusion (Sigma assay kits). At the concentrations used in the following experiments, none of these agents altered trypan blue exclusion.

Cell Proliferation Assay. Cell proliferation was determined using the SRB colorimetric assay (14). Cells were seeded in 96-well plates (Corning Glass Works, Corning, NY) at approximately 10% confluence and allowed to attach for 24 h. The growth medium was removed, and fresh medium without FBS and containing the indicated concentration of test agent was added. Cells were incubated for the described period of time at 37°C in an atmosphere of 5% CO_2 and 95% air. After incubation, cells were treated for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Protein-bound dye was extracted with unbuffered 10 mM Tris base. Absorbance was measured at 560 nm using a computer-interfaced, 96-well microtiter plate reader.

Caspase-3 Assay. H508 and SNU-C4 cellular caspase-3 activity was measured using a kit from Sigma. Cells ($2\text{--}4 \times 10^5$ cells/ml) were seeded in T75 flasks. Growth medium was removed after 24 h, and cells were incubated with the indicated agent, containing no added FBS, for 3 or 6 days at 37°C in an atmosphere of 5% CO_2 and 95% air. After incubation, cells were washed with PBS and lysed. Caspase-3 activity was examined with 96-well plates in duplicate for 90 min, and absorbance was measured at 405 nm using a computer-interfaced 96-well microtiter plate reader. As a positive control, H508 and SNU-C4 cells were incubated with deoxycholic acid (250 μM) for 1.5 h and 30 min, respectively.

RESULTS

Effects of Acetylcholine on Phosphorylation of p44/42 MAPK and p90RSK in H508 Human Colon Cancer Cells. Important steps in the muscarinic receptor signaling cascade include p44/42 MAPK, also referred to ERK 1/2 (3, 15), and a 90-kDa transcription factor designated p90RSK (16). p90RSK regulates gene expression and cell cycle regulation (16). We considered it likely that, in H508 cells, p90RSK would play a role in mediating acetylcholine-induced cell proliferation. To determine the elements of the signaling cascade downstream of the M3 receptor in H508 cells, we examined the effects of muscarinic stimulation on the phosphorylation of p44/42 MAPK and p90RSK. As shown in Fig. 1A, acetylcholine (300 μM) stimulated phosphorylation of p44/42 MAPK and p90RSK. These results support the hypothesis that to promote cell proliferation, mus-

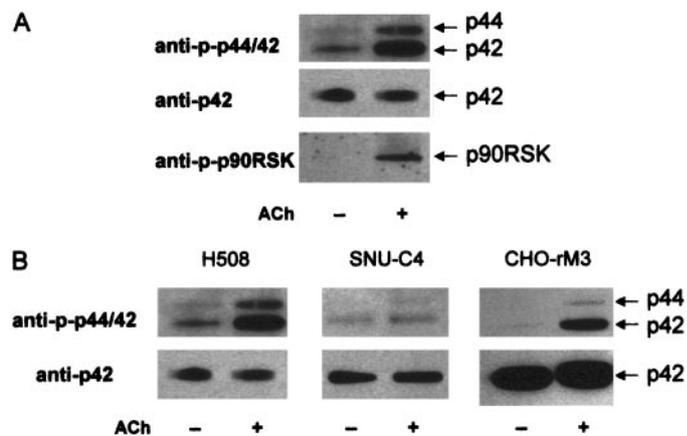


Fig. 1. A, acetylcholine-induced phosphorylation of MAPK and p90RSK in H508 human colon cancer cells. B, comparison of acetylcholine-induced MAPK phosphorylation in H508 and CHO cells that express M3 receptors and SNU-C4 cells that do not express muscarinic receptors. H508, SNU-C4, and CHO-rM3 cells were treated with or without acetylcholine (300 μM) for 10 min at 37°C. p44/42 MAPK and p90RSK activities were determined by immunoblotting with antibodies specific for phosphorylated MAPK and p90RSK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK (p42). Results shown are representative of three experiments.

carinic receptor activation stimulates the p44/42 MAPK cascade including activation of p90RSK.

Comparison of the Actions of Acetylcholine on p44/42 MAPK Phosphorylation in H508, SNU-C4, and CHO-rM3 Cells. To confirm that the phosphorylation events shown in Fig. 1A were a specific consequence of activation of M3 receptors, we examined acetylcholine-induced p44/42 MAPK phosphorylation in a human colon cancer cell line, SNU-C4, that does not express muscarinic receptors (4, 5). We also examined the actions of acetylcholine on CHO cells transfected with rat M3 receptors (CHO-rM3 cells).

As shown in Fig. 1B, acetylcholine induced p44/42 MAPK phosphorylation in H508 and CHO-rM3 cells, which express M3 muscarinic receptors, but not in SNU-C4 cells, which do not express M3 receptors (4). Specifically, upon exposure to acetylcholine, the phosphorylation of p44/42 increased 14-fold in H508 and 27-fold in CHO-rM3 cells but only 1.6-fold in SNU-C4 cells (Fig. 1B). These findings indicate that the effects of acetylcholine on phosphorylation of p44/42 MAPK are dependent on the expression of muscarinic receptors.

Actions in H508 Cells of Atropine, BAPTA, EGTA, and PD98059 on Acetylcholine-Induced Phosphorylation of p44/42 MAPK and p90RSK. To further explore the links between muscarinic receptor activation and phosphorylation of p44/42 MAPK and p90RSK, we examined the actions of atropine, a muscarinic receptor antagonist; BAPTA and EGTA, chelators of intracellular calcium (17–19); and PD98059, an inhibitor of MEK. MEK is a term used to describe MAPK kinase the regulatory protein just upstream of p44/42 MAPK.

Acetylcholine-induced phosphorylation of p44/42 MAPK and p90RSK in H508 cells was reduced in the presence of atropine, BAPTA, EGTA, and PD98059 (Fig. 2). These findings support the hypothesis that activation of the muscarinic receptor by acetylcholine (antagonized by atropine) causes calcium-dependent phosphorylation of p44/42 MAPK and p90RSK.

Actions of Atropine, BAPTA, and PD98059 on Acetylcholine-Induced Proliferation of H508 Cells. To strengthen the evidence for a link between muscarinic receptor activation, MAPK phosphorylation, and H508 cell proliferation, we examined the actions of atropine, BAPTA, and PD98059 on proliferation. Cell proliferation was deter-

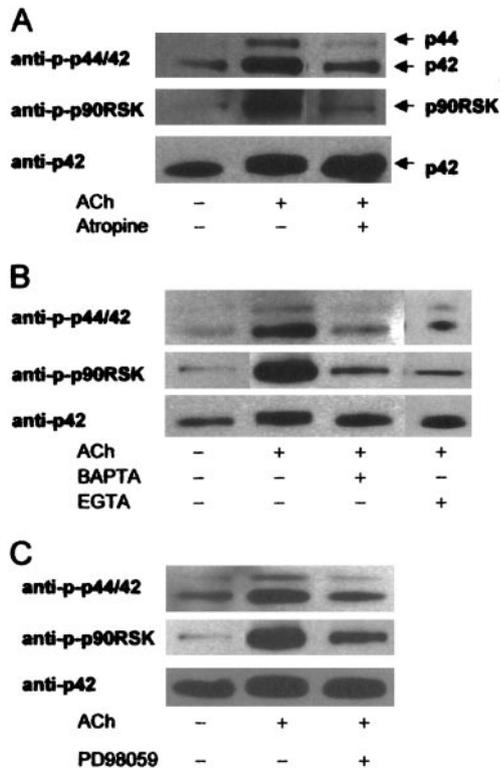


Fig. 2. Actions of atropine, BAPTA, EGTA, and PD98059 on acetylcholine-induced phosphorylation of MAPK and p90RSK in H508 cells. H508 cells were treated with or without acetylcholine (300 μ M) for 10 min at 37°C in the absence or presence of (A) atropine (5 μ M), (B) BAPTA (25 μ M) and EGTA (100 μ M), and (C) PD98059 (25 μ M). MAPK and p90RSK activities were determined by immunoblotting with antibodies specific for phosphorylated MAPK and p90RSK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK. Results shown are representative of three experiments.

mined using a validated colorimetric assay (14). As shown in Fig. 3, acetylcholine-induced proliferation of H508 cells was reduced by the muscarinic antagonist atropine and, to a lesser degree, by the calcium chelator BAPTA and the MEK inhibitor PD98059. Acetylcholine did not stimulate proliferation of SNU-C4 cells, which do not express muscarinic receptors (data not shown). Moreover, to exclude acetylcholine-induced apoptosis as a possible contributing factor to differences in proliferation, we examined caspase-3 activation in H508 and SNU-C4 cells. Caspase-3 activation is a key step in the regulation of apoptosis (20, 21). Caspase-3 activation was not altered by muscarinic activation under any of the conditions tested in the present study (data not shown).

These findings indicate that the same agents that inhibit acetylcholine-induced p44/42 MAPK and p90RSK phosphorylation inhibit H508 colon cancer cell proliferation. This provides additional evidence supporting the link between activation of the muscarinic receptor and cellular proliferation.

Actions of EGFR Inhibitors, PD168393 and AG1478, on Acetylcholine- and EGF-Induced p44/42 MAPK Phosphorylation in H508 and CHO-rM3 Cells. To determine whether transactivation of the EGFR plays a role in mediating the actions of acetylcholine on H508 cell proliferation, we used two inhibitors of the EGFR, PD168393 and AG1478 (22–25). As a control, in these experiments we also examined the actions of acetylcholine, EGF, and the EGFR inhibitors on CHO-rM3 cells. CHO-rM3 cells do not express EGFR (13, 26).

As shown in Fig. 4A, in H508 cells, EGFR inhibitors caused a decrease in acetylcholine- and EGF-induced p44/42 MAPK phosphorylation. In contrast, in CHO-rM3 cells, acetylcholine-induced p44/42

MAPK phosphorylation was not altered in the presence of PD168393 or AG1478 (Fig. 4B). Moreover, in CHO-rM3 cells, EGF did not alter p44/42 MAPK phosphorylation (Fig. 4B). These results indicate that, in H508 human colon cancer cells that express M3 muscarinic and

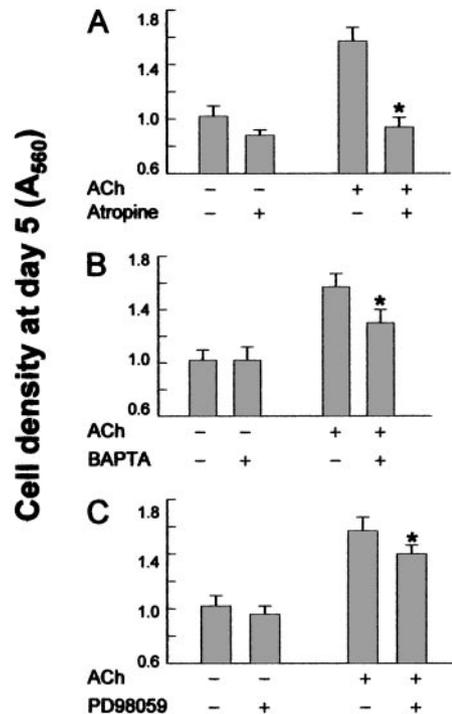
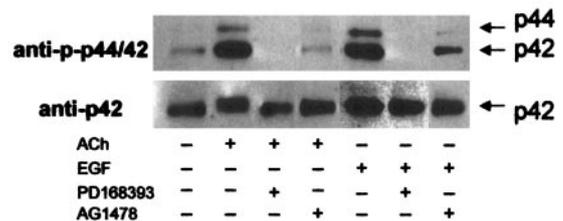


Fig. 3. Actions of atropine, BAPTA, and PD98059 on acetylcholine-induced proliferation of H508 cells. H508 cells were treated with or without acetylcholine (300 μ M) for 5 days at 37°C in the absence or presence of (A) atropine (5 μ M), (B) BAPTA (1 μ M), and (C) PD98059 (25 μ M). Cellular proliferation was determined by the SRB colorimetric assay (14) as described in "Materials and Methods." Results shown are mean \pm SE of three experiments. *, value significantly different than that observed with no inhibitor ($P < 0.05$).

A. H508



B. CHO-rM3

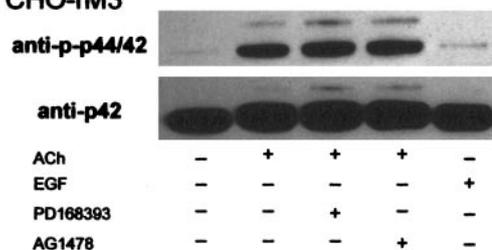


Fig. 4. Actions of EGFR inhibitors, PD168393 and AG1478, on acetylcholine- and EGF-induced phosphorylation of MAPK in H508 and CHO cells expressing M3 receptors (CHO-rM3). H508 (A) or CHO-rM3 (B) cells were treated with 300 μ M acetylcholine and 10 ng/ml EGF for 10 min at 37°C in the absence or presence of PD168393 (1 μ M) or AG1478 (0.1 μ M). MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK (p42). Results shown are representative of three experiments.

EGFRs, inhibitors of EGFR block acetylcholine-induced p44/42 MAPK phosphorylation. This finding is consistent with transactivation of EGFR by interaction of acetylcholine with the M3 receptor.

Effect of BAPTA, Atropine, and PD98059 on EGF-Induced p44/42 MAPK Phosphorylation in H508 Cells. To determine the conditions under which direct stimulation of EGFR by EGF would activate the MAPK cascade, we examined the actions of a calcium chelator, a muscarinic antagonist, and a MAPK kinase (MEK) inhibitor. As shown in Fig. 5, BAPTA and atropine did not alter EGF-induced MAPK phosphorylation. In contrast, EGF-induced p44/42 MAPK phosphorylation was decreased in the presence of the MEK inhibitor PD98059 (Fig. 5). These results indicate that unlike the actions of acetylcholine, the direct actions of EGF on EGFR are not mediated by a calcium-dependent or cholinergic mechanism.

Role of PKC in Acetylcholine- and EGF-Induced MAPK Phosphorylation in H508 Cells. In several cell types, PKC isozymes play a role in mediating the actions of GPCRs (3). PKC inhibitors and down-regulation of PKC activity reduce the actions of muscarinic activation on some muscarinic receptor subtypes, including the M3 subtype (3, 10). In contrast, signaling downstream of EGFR appears to be PKC independent (7, 15). In H508 cells, acetylcholine- and EGF-induced p44/42 MAPK phosphorylation (Fig. 6, A and B, respectively) was not altered by addition of the PKC inhibitors H7 or GF109203X.

Addition of AG17 (5 μ M), an inhibitor of PYK2, a protein that is part of the PKC signaling cascade, did not alter acetylcholine- and EGF-induced p44/42 MAPK phosphorylation (data not shown). Lack of PYK2 involvement in acetylcholine-induced phosphorylation of p44/42 MAPK in H508 cells is, in part, confirmatory of the absence of a role for PKC. Moreover, in other cells, PYK2 activation has been linked to M1 but not M3 muscarinic receptor subtype signaling (27, 28).

In contrast to these negative findings, the EGFR inhibitor AG1478 reduced acetylcholine- and EGF-induced p44/42 MAPK phosphorylation. These observations support the hypothesis that the actions of acetylcholine are mediated by transactivation of EGFR.

Role of Src in Acetylcholine- and EGF-Induced p44/42 MAPK and EGFR Phosphorylation in H508 Cells. Activation of EGFR results in phosphorylation of specific tyrosine residues in EGFR (Tyr⁹⁹², Tyr⁸⁴⁵, Tyr¹⁰⁴⁵, and Tyr¹⁰⁶⁸). Of these, we were only able to detect phosphorylation of Tyr⁹⁹² in response to addition of acetylcholine or EGF (data not shown).

In some cells, Src family kinases are reported to play a central role in GPCR-mediated assembly of membrane-associated mitogenic signaling complexes (29). We used an inhibitor of Src, pp2, to examine the actions of acetylcholine and EGF on phosphorylation of p44/42 MAPK and Tyr⁹⁹² in EGFR. As shown in Fig. 7, pp2 reduced

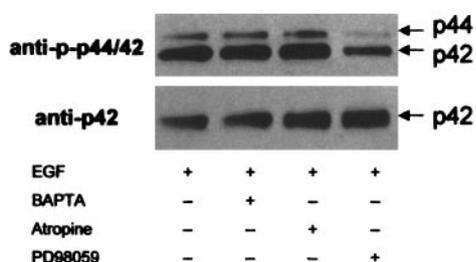


Fig. 5. Actions of atropine, BAPTA, and PD98059 on EGF-induced phosphorylation of MAPK in H508 cells. H508 cells were treated with EGF (10 ng/ml) for 10 min at 37°C in the absence or presence of BAPTA (20 μ M), atropine (100 μ M), or PD98059 (25 μ M). MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK. Results shown are representative of three experiments.

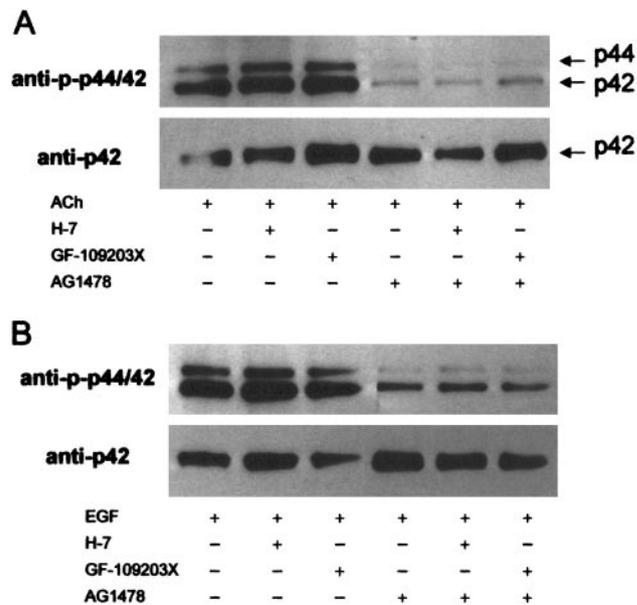


Fig. 6. Actions of PKC inhibitors, H7 and GF109203X, and the EGFR inhibitor, AG1478, on (A) acetylcholine- and (B) EGF-induced MAPK phosphorylation in H508 cells. H508 cells were treated with 300 μ M acetylcholine or 10 ng/ml EGF for 10 min at 37°C in the absence or presence of H7 (15 μ M), GF109203X (0.5 μ M), or AG1478 (0.1 μ M). MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK. Results shown are representative of three experiments.

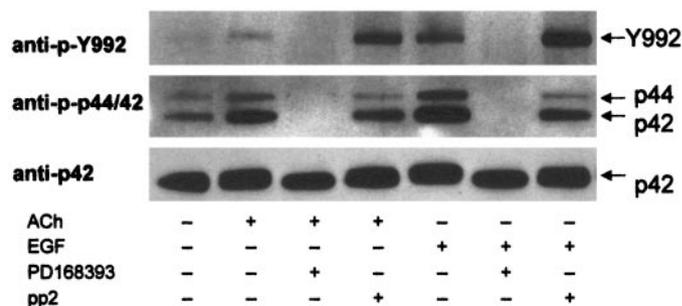


Fig. 7. Actions of the Src inhibitor, pp2, and the EGFR inhibitor, PD168393, on acetylcholine- and EGF-induced phosphorylation of MAPK and EGFR in H508 cells. H508 cells were treated with 300 μ M acetylcholine or 10 ng/ml EGF for 10 min at 37°C in the absence or presence of PD168393 (1 μ M) or pp2 (10 μ M). Transactivation of the EGFR was determined by immunoblotting with antibodies specific for phosphorylated Tyr⁹⁹² in the EGFR and phosphorylated MAPK as described in "Materials and Methods." Amounts of protein added were verified by immunoblotting with antibodies specific for nonphosphorylated MAPK. Results shown are representative of three experiments.

acetylcholine- and EGF-induced p44/42 MAPK phosphorylation (Fig. 7). In contrast, in the presence of pp2, acetylcholine- and EGF-induced phosphorylation of Tyr⁹⁹² in EGFR was increased (Fig. 7). These findings indicate that, in H508 cells, acetylcholine, like EGF, causes phosphorylation of a specific tyrosine residue in EGFR. Moreover, blockade of signaling downstream of the muscarinic receptor appeared to augment acetylcholine-induced signaling that is mediated by transactivation of EGFR.

Role of PI3K in Acetylcholine- and EGF-Induced p44/42 MAPK Phosphorylation in H508 Cells. PI3Ks are implicated in mediation of EGF-stimulated cell proliferation (30). To determine whether PI3Ks play a role in acetylcholine- or EGF-stimulated H508 cell proliferation, we examined the actions of two inhibitors, wortmannin and LY294002. As shown in Fig. 8, both PI3K inhibitors reduced acetylcholine-induced phosphorylation of p44/42 MAPK. EGF-induced phosphorylation of p44/42 MAPK was also reduced by

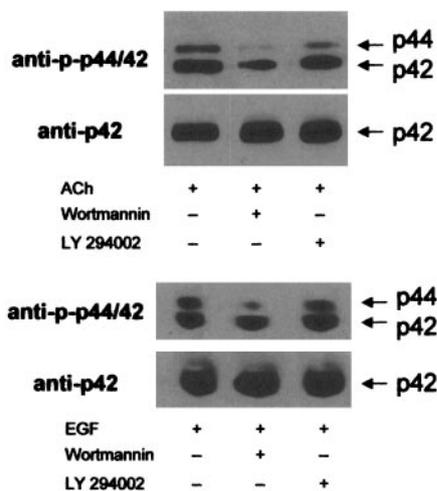


Fig. 8. Actions of PI3K inhibitors, wortmannin and LY294002, on acetylcholine- and EGF-induced phosphorylation of p44/42 MAPK in H508 cells. H508 cells were treated with 300 μ M acetylcholine or 10 ng/ml EGF for 10 min at 37°C in the absence or presence of wortmannin (50 nM) or LY294002 (50 μ M). MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK as described in "Materials and Methods." Results shown are representative of three experiments.

wortmannin, but LY294002 had only a minor effect (Fig. 8). The weaker inhibition seen with LY294002 may be a consequence of the 1000-fold greater potency of wortmannin (31–35). These findings indicate that transactivation of EGFR, by means of activating PI3K signaling, can bypass p44/42 MAPK signaling (30). This may explain the observation that inhibition of MEK, which resulted in reduction of p44/42 MAPK phosphorylation, provided only partial inhibition of cell proliferation (Fig. 3C).

Actions of AG1478 on Acetylcholine-Induced Proliferation of H508 Cells. Finally, to complete the link between acetylcholine-induced transactivation of EGFR and H508 cell proliferation, we examined the actions of the EGFR inhibitor AG1478 on proliferation. As shown in Fig. 9, acetylcholine-induced proliferation of H508 cells was reduced to basal levels by the EGFR inhibitor. These findings confirm that transactivation of EGFR is necessary for acetylcholine-induced proliferation of H508 human colon cells.

DISCUSSION

In the United States, in men and women, colorectal cancer is the second most common cause of cancer death (36). The majority of these cancers arise by a progression from normal colonic mucosa to adenomatous polyps to cancer that is associated with the accumulation of somatic gene modifications. These modifications include oncogene and suppressor gene mutations that alter function (37). Despite these advances in our understanding of the genetic causes of colon cancer, it is evident that additional factors play a role in oncogenesis and tumor progression (growth and dissemination). Possible exogenous factors include a multitude of dietary components (38–40). Endogenous factors that have been proposed to promote colorectal cancer formation and/or progression include fecal bile acids and tissue growth factors (41). It is likely that the interplay of these factors determines whether cancer will occur and how rapidly it will progress.

Cancers of various types in many organs express receptors for potential growth factors, including hormones, neurotransmitters, and others. Although the bulk of investigation of growth factors has been with *in vitro* systems, there is much interest in applying this information to the clinical treatment of cancer. In the last decade, Frucht *et al.* (4, 5) observed that some human colon cancer cell lines, particularly H508 cells, express cholinergic muscarinic receptors. Specifically,

these were identified as M3 subtype muscarinic receptors (5). Moreover, these receptors are functional in the sense that addition of a cholinergic agonist results in activation of the appropriate post-muscarinic receptor signaling cascade (increased inositol phosphate formation and cellular calcium concentration) and stimulation of cell proliferation (4, 5). Hence, although there is no evidence that muscarinic activation plays a role in neoplastic transformation, these data support the notion that such stimulation favors the growth and possible dissemination of a pre-existent cancer. However, the mechanism whereby M3 muscarinic receptor activation in H508 human colon cancer cells leads to cellular proliferation was unknown.

The link between activation of GPCRs in general, and muscarinic receptors in particular, and cellular proliferation varies from cell to cell. For example, some cancer cells (*e.g.*, 1311N1 astrocytoma cells) express muscarinic receptors that activate phospholipid turnover but not gene expression (42, 43). In other cells (*e.g.*, ventricular myocytes) muscarinic stimulation results in cell growth, but the intermediate steps linking receptor activation to gene expression are unclear (44). Over the past few years, investigators have found that activation of GPCRs may result in transactivation of receptor tyrosine kinases, like EGFR (25, 45). In Rat-1 cells transfected with the bombesin/gastrin-releasing peptide receptor, bombesin-induced transactivation of EGFR results in DNA synthesis (25). With regard to the effects of muscarinic receptor activation, results have been conflicting regarding whether transactivation of other receptors has a stimulatory or inhibitory effect on cell growth (3, 45, 46). For example, in neuroblastoma cells, activation of muscarinic receptors inhibits EGF-induced signaling cascades (45). In contrast, in HEK cells transfected with M3 receptors, muscarinic activation results in PKC-independent transactivation of EGFR and activation of the MAPK signaling cascade (3). From these observations, it is evident that the response of a particular cell to muscarinic activation depends on a number of factors, including muscarinic receptor expression (and the particular muscarinic receptor subtype expressed) and the expression of other plasma membrane receptors (*e.g.*, EGFR). Based on these observations, we considered the possibility that the link between activation of muscarinic receptors on H508 human colon cancer cells and cellular proliferation was transactivation of EGFR.

In the present study, we provide evidence that in H508 human colon cancer cells, activation of M3 muscarinic receptors results in transactivation of EGFR, thereby stimulating cellular proliferation. In H508 cells, but not in SNU-C4 cells, which do not express muscarinic

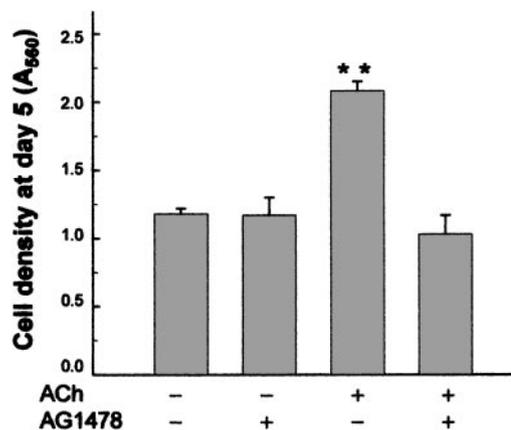


Fig. 9. Actions of AG1478 on acetylcholine-induced proliferation of H508 cells. H508 cells were treated with or without acetylcholine (300 μ M) for 5 days at 37°C in the absence or presence of AG1478 (0.15 μ M). Cellular proliferation was determined by the SRB colorimetric assay (14) as described in "Materials and Methods." Results shown are mean \pm SE of three experiments. **, value significantly different than that observed in the presence of EGFR inhibitor ($P < 0.005$).

receptors, acetylcholine stimulates calcium-dependent phosphorylation of MAPK and p90RSK and consequent cell proliferation. These effects are attenuated in the presence of atropine or a MEK inhibitor. Conversely, the actions of EGF are not calcium dependent and are not mediated by cholinergic mechanisms. In H508 cells, acetylcholine- and EGF-induced phosphorylation of MAPK is abolished in the presence of EGFR inhibitors (AG1478 and PD168393). In CHO cells transfected with the rat M3 muscarinic receptor, which lack EGFR, acetylcholine-induced MAPK phosphorylation is not altered in the presence of EGFR inhibitors. In H508 cells, addition of PKC inhibitors does not alter either acetylcholine- or EGF-induced MAPK phosphorylation. In contrast, inhibition of Src using pp2 appeared to enhance signaling through the EGFR pathway (increased phosphorylation of Tyr⁹⁹² in EGFR in response to acetylcholine or EGF). This suggests that, by an unknown mechanism, blocking the distal aspects of the M3 receptor signaling cascade increases signaling (transactivation and direct activation) via EGFR.

In the following discussion, methodological considerations require that we be somewhat circumspect regarding the conclusions that can be drawn from the above findings. Issues regarding the specificity of the inhibitors used in our studies must be considered. The specificity of inhibitors of MEK, EGFR, and PKC that we used has been validated in previous studies (7, 10, 22–25, 44, 47). Nevertheless, to increase the level of confidence in our observations, wherever possible we used two different inhibitors of the same function. In addition, verification of the actions of inhibitors was obtained by comparing these findings to measurement of the post-intervention phosphorylation of relevant substrates (*e.g.*, MAPK, p90RSK, and EGFR). We also examined the effect of interventions on H508 cell proliferation. The results of these three approaches were concordant.

Based on our observations, we hypothesize that acetylcholine or other cholinergic ligands interact with M3 muscarinic receptors on the plasma membrane, thereby initiating a post-receptor signaling cascade that results in activation of phospholipase C, a G protein-dependent step, with a resultant increase in cellular calcium. Moreover, a series of phosphorylations ensues from Src to MEK to MAPK and eventually to p90RSK that can act as a transcription factor (16).

Likewise, EGFR activation also initiates a series of phosphorylations. Based on the work of others, it is likely that convergence of the M3 receptor and EGFR cascades occurs at the Ras protein, with apparently the same steps for both cascades distal to that mediator (8, 16, 29). Our findings indicate that an intact EGFR signaling cascade is necessary for acetylcholine-induced signaling to result in H508 cell proliferation. Inhibition of EGFR (PD168393 and AG1478) or MEK reduces acetylcholine-induced MAPK phosphorylation and cellular proliferation.

These results raise intriguing questions that must be addressed in future studies. Although the calcium-dependent link between the M3 receptor and EGFR is proximal in the M3 signaling cascade, the actual point of interaction is unknown. Likewise, the kinase responsible for acetylcholine-induced Tyr⁹⁹² phosphorylation in EGFR is unknown. It is possible that an increase in calcium stimulates an undefined kinase or stimulates autophosphorylation of EGFR (8). Moreover, the implications of PI3K activation must be explored further.

Our findings have potentially important implications regarding mitogenic signaling and proliferation of colon and other cancers. Tumor biology will differ depending on exposure to mitogenic factors and on the expression of relevant receptors. SNU-C4 cells that do not express muscarinic receptors have no proliferative response to cholinergic agonists (5). Likewise, CHO-M3 and astrocytoma cells (13, 26, 43) that lack EGFR do not proliferate in the presence of cholinergic agonists. Full expression of cholinergic agonist-induced proliferation appears to require coexpression of the appropriate muscarinic

receptor and a transmembrane protein kinase receptor. For cancer management, these findings expand potential targets for antiproliferative therapy. That is, either M3 muscarinic or EGFRs and components of their post-receptor signaling cascades can be targeted.

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Kunrong Cheng, Piotr Zimniak and Jean-Pierre Raufman

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