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Interacting post-muscarinic receptor signaling pathways potentiate matrix metalloproteinase-1 expression and invasion of human colon cancer cells

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Abstract

M3 muscarinic receptor (M3R) expression is increased in colon cancer; M3R activation stimulates colon cancer cell invasion via cross-talk with epidermal growth factor receptors (EGFR), post-EGFR activation of mitogen-activated protein kinase (MAPK) ERK1/2, and induction of matrix metalloproteinase-1 (*MMP1*) expression. *MMP1* expression is strongly associated with tumor metastasis and adverse outcomes. Here, we asked whether other MAPKs regulate M3R agonist-induced *MMP1* expression. In addition to activating ERK1/2, we found that treating colon cancer cells with acetylcholine (ACh) stimulated robust time- and dose-dependent phosphorylation of p38 MAPK. Unlike ERK1/2 activation, ACh-induced p38 phosphorylation was EGFR-independent and blocked by inhibiting protein kinase C-α (PKC-α). Inhibiting activation of PKC-α, EGFR, ERK1/2, or p38-α/β alone attenuated but did not abolish ACh-induced *MMP1* expression, a finding that predicted potentiating interactions between these pathways. Indeed, ACh-induced *MMP1* expression was abolished by incubating cells with either an EGFR or MEK/ERK1/2 inhibitor combined with a p38-α/β inhibitor. Activating PKC-α and EGFR directly with the combination of phorbol 12-myristate 13-acetate (PMA) and EGF potentiated *MMP1* gene and protein expression, and cell invasion. PMA- and ACh-induced *MMP1* expression were strongly diminished by inhibiting Src and abolished by concurrently inhibiting both p38-α/β and Src, indicating that Src mediates the cross-talk between PKC-α and EGFR signaling. Using siRNA knockdown, we identified p38-α as the relevant p38 isoform. Collectively, these studies uncover novel functional interactions between post-muscarinic receptor signaling pathways that augment

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AUTHOR CONTRIBUTIONS

Anan H. Said, Shien Hu, Ameer Abutaleb, Tonya Watkins, Kunrong Cheng, and Panjamurthy Kuppusamy performed experiments and analyzed data. Ahmed Chahdi, Neeraj Saxena, Guofeng Xie, and Jean-Pierre Raufman developed the concepts. Anan Said, Shien Hu, and Jean-Pierre Raufman prepared and edited (pre-submission) the paper. All authors reviewed the results and approved the final version of the manuscript.

MMP1 expression and drive colon cancer cell invasion; targeting these potentiating interactions has therapeutic potential.

Keywords

Matrix metalloproteinases; colorectal cancer; p38 MAPK; cell invasion; cell signaling; muscarinic receptors

INTRODUCTION

In the United States, colon cancer is the third leading cause of cancer-related mortality and the most common cause of death from gastrointestinal disease [1]. Each year approximately 150,000 people are diagnosed with colon cancer and nearly 50,000 are anticipated to perish from the disease [2]. While early resection of primary cancers greatly improves prognosis, persons who succumb die primarily as a consequence of unresectable metastases [3]. Despite advances in elucidating the somatic gene mutations that cause progressive intestinal mucosal dysplasia and cancer, the mechanisms governing colon cancer cell invasion and dissemination are less certain [4, 5]. While novel mutations are not observed in metastases, growth factors promote colon cancer cell invasion into adjacent epithelium and blood vessels, and extravasation in other organs; these are key precursors to the establishment of metastatic foci [4, 6].

Whereas the molecular events driving colon cancer progression remain obscure, a growing body of evidence suggests that in addition to serving as growth factors, muscarinic receptor agonists modulate the expression and activation of key molecules that promote cancer cell dissemination [7]. Of the five muscarinic receptor subtypes, M1R, M3R, and M5R are conditional oncogenes when expressed in cells capable of proliferation [8]. M3R are expressed widely in the gut and both M3R and *CHRM3*, the gene encoding its expression, are over-expressed in colon cancer [7, 9–11]. We previously showed that M3R activation governs key hallmarks of colon cancer progression including cell proliferation, survival, migration, and invasion [12–17]. In mouse models, M3R activation augments, and M3R deficiency attenuates, intestinal neoplasia [18–20].

Many steps precede tissue invasion and metastasis in cancer, including loss of cell adhesion, extracellular matrix degradation, and induction of cell migration [21]. Members of the matrix metalloproteinase (MMP) family of enzymes play key roles in degrading the extracellular matrix and promoting invasion [22–24]. Within this family, expression of MMP1, a secreted enzyme that breaks down interstitial collagens, correlates strongly with advanced colon cancer, metastatic dissemination, and an adverse outcome [25, 26]. Thus our discovery that M3R activation selectively induces *MMP1* expression by approximately 50-fold identified a mechanism likely to be important for colon cancer progression [26]. Our finding that blocking MMP1 activity abolishes M3R agonist-induced colon cancer cell invasion lends further credibility to this hypothesis [27]. The focus of the current work was to understand more completely how M3R activation promotes MMP1 expression.

Our previous work suggested that transactivation of EGFR and downstream activation of MEK followed by extracellular signal-related kinase1/2 (ERK1/2) [also known as p44/42 mitogen activated kinase (MAPK)] represented a critical signal transduction pathway mediating post-M3R *MMP* gene induction [26]. However, whereas blocking this pathway suppressed M3R agonist-induced *MMP1* expression efficaciously, it did so incompletely – there remained residual acetylcholine-induced MMP1 expression. This led us to consider the possibility that other post-M3R signaling pathways play a role in regulating *MMP1* expression. Mammals possess two other MAPK's, c-Jun-N-terminal kinase (JNK) and p38 MAPK (p38), which are also focal points for phosphorylation cascades that transduce extracellular stimuli into cellular responses and play key roles in promoting cancer growth and dissemination [28]. We considered these MAPK's worthy of consideration, particularly p38 which mediates growth factor-induced cell migration and *MMP1* expression in other cancers [25, 29–31].

As reported here, this line of investigation led us to identify novel potentiating interactions between post-M3R signaling pathways, involving protein kinase C (PKC)-α and p38-α activation that are likely to provide malignant cells with a means of rescuing M3R agonist-induced *MMP1* gene induction when only EGFR/ERK signaling is blocked. We describe a gain of pro-invasive characteristics of human colon cancer cells following activation of this M3R/MMP1 axis and uncover intermediary factors and pathways that modulate these interactions between signaling pathways.

MATERIALS AND METHODS

Reagents and antibodies

The following antibodies were used: total and phospho-ERK1/2, total p38, phospho-p38, total JNK and phospho-JNK, and β-actin from Cell Signaling (Danvers, MA); p38-α and p38-β from Thermo Fisher (Waltham, MA). Chemicals were purchased as follows: ACh and phorbol 12-myristate 13-acetate (PMA) from Sigma-Aldrich; EGF from Cell Signaling. See Supplemental Table 1 for list and sources of the inhibitors used in this study.

Cell lines and cell culture

HT-29, H508, and Caco-2 human colon cancer cell lines were authenticated and purchased from American Type Culture Collection (ATCC). H508 cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) plus antibiotics. HT-29 cells were grown in McCoy's 5A medium (Life Technologies) supplemented with 10% FBS plus antibiotics. Caco-2 cells were grown in E-MEM (Quality Biological) supplemented with 10% FBS plus antibiotics. Cells were grown at 37°C, with 5% CO_2 in a humidified incubator and passaged weekly at subconfluence after trypsinization.

Immunoblot analysis

After the indicated treatments, cells were lysed in a solution containing 20 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM $NaVO_3$, 1 mM EDTA, 1% Triton X-100, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. Cell lysates were centrifuged at 15,000 × *g* at 4°C for 10 min. Supernatants were collected and

protein concentration was determined by the BCA method (Thermo Scientific). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes that were probed against total (Cell Signaling catalog #4695) and phospho-ERK1/2 for sites p-T202 and p-Y204 (Cell Signaling catalog #4376), total (Cell Signaling catalog #9212) and phospho-p38 for sites p-T180 and p-Y182 (Cell Signaling catalog #9211), total (Cell Signaling catalog #9252) and phospho-JNK for sites p-T183 and p-Y185 (Cell Signaling catalog #9251), p38-α (Thermo Fisher #PA1-41321) and p38-β (Thermo Fisher #PA5-14050). To confirm equal protein loading, blots were stripped and re-probed with antibody to β-actin. Image quantification was performed by scanning densitometry using NIH Image J 1.50i software. In most experiments, conditions were normalized and expressed relative to the positive control.

RNA extraction and real-time PCR

Total cellular RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. First strand cDNAs were synthesized using the Superscript III First Strand Synthesis System (Invitrogen). Real-time PCR was performed with ABI StepOnePlus real-time PCR System (Applied Biosystems) using Veriquest Sybr Green qPCR Master (Affymetrix) with specific primers for *MMP1* or *GAPDH*. PCR conditions were 2 min at 50°C, 10 min at 95°C, and then 40 cycles at 95°C for 15 s and 60°C for 60 s. PCR specificity was confirmed by melting-curve analysis. The standard cycle threshold ($2^{-\Delta\Delta Ct}$ CT) method was used to calculate relative levels of mRNA expression. Individual expression values were normalized by comparison to GAPDH. Sense and antisense PCR primers for RT-PCR were as follows: *MMP1*, 5′- AACTGCCAAATGGGCTTG -3′ and 5′- CCGTGTAGCACATTCTGTCC -3′. *GAPDH*, 5- CTCCTCACAGTTGCCATGTA -3′ and 5′- GTTGAGCACAGGGTACTTTATTG -3′.

MMP1 ELISA

After cells were treated with the indicated agents, aliquots of the supernatants were collected and assessed for MMP1 release using the Human MMP-1 ELISA Kit (Sigma-Aldrich) according to the manufacturer's instructions. Inhibitors were pre-incubated for 45 min at 37°C prior to 4-h stimulation with indicated agents. All results were normalized to the unstimulated control.

Cell transfection

Lipofectamine® RNAiMAX Reagent (Life Technologies) was used to transfect silencing RNA. Pre-designed MISSION siRNAs specific to human p38-α (esiRNA1, Sigma-Aldrich) were used to knock down p38-α expression. MISSION siRNAs consist of a heterogeneous mixture of siRNAs that are endoribonuclease-prepared siRNA pools targeting the same mRNA sequence. Cells were transfected with siRNAs for 72 h prior to treatment with designated agents.

Cell invasion assay

Invasion assays were performed using Biocoat Matrigel Invasion Chambers (Corning) following the manufacturer's instructions. Briefly, HT-29 and Caco-2 cells were trypsinized

and resuspended in serum-free medium and placed in the upper chamber of the Transwell inserts at a density of 5×10^4 cell/ml. Chemoattractant (fetal bovine serum) was placed in the lower chamber, and the cells were treated with the indicated agents. Cells were incubated for 48 h in a 37°C, 5% CO_2 humidified incubator. Non-invading cells were dislodged with a cotton-tipped swab. Invading cells were fixed and stained with 0.1% crystal violet (Sigma). Imaging of the invaded cells was acquired from an inverted light microscope, and analysis was performed with NIS-Elements imaging software (Nikon).

Statistical analysis

Data are presented as mean ± SE of at least three separate experiments, and analyzed by two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Treating human colon cancer cells with a M3R agonist stimulates p38 MAPK phosphorylation

First, we explored the possibility that in addition to ERK1/2 either JNK or p38 might play a role in M3R agonist-induced induction of MMP1 expression in colon cancer [28]. To do so, we employed two well-characterized human colon cancer cell lines which primarily and robustly express the M3R muscarinic receptor subtype; HT-29 and H508 cells, respectively, are derived from human male and female well-differentiated colon adenocarcinomas [9, 32]. Our published dose-response experiments revealed that changes in colon cancer cell signaling and function are first detected with 1 μM acetylcholine (ACh) and maximal with 100–300 μM ACh [26].

Treating HT-29 cells with maximal concentrations of ACh for 5 min stimulated 10- to 20-fold increases in p38 and ERK1/2 phosphorylation, but did not alter JNK phosphorylation (Figure 1A, B). No changes in total levels of p38, ERK1/2, or JNK were detected (Figure 1A). Pre-incubating cells with atropine (5 μM) consistently abolished ACh-induced p38 and ERK1/2 phosphorylation, confirming these actions were MR-dependent (Figure 1A, B). Similar results were observed with H508 cells; ACh stimulated 15-fold increased p38 phosphorylation with no change in total p38 (Figure 1C, D). p38 phosphorylation was rapid and reversible; detected within 5 min, maximal by 10 min, and nearly returned to basal levels within 1 h (Figure 1C, D). In accord with our previous ACh dose-response findings [26], p38 phosphorylation was detectable after 10-min incubation with 1 μM and maximal with 100–300 μM ACh (Figure 1E, F).

Regulation of M3R agonist-induced p38 activation

Next, to uncover the intermediary signaling molecules that regulate p38 activation in ACh-stimulated colon cancer cells we used a series of previously validated selective chemical inhibitors [12–14, 16–18]; we confirmed effects by using two separate inhibitors chosen by their selectivity for blocking the kinase of interest [33] (Supplementary Table 1). Control experiments with atropine and other selective inhibitors used in these and subsequent experiments did not alter basal p38 phosphorylation (Supplemental Figure 1A). As a positive control, pre-incubating HT-29 cells with 5 μM atropine before treatment with 100 μM ACh

for 5 min consistently inhibited both p38 and ERK1/2 activation (Figure 2A, B). Incubating cells with atropine alone did not alter levels of total or phospho-p38 or phospho-ERK1/2 (Figures 1, 2A, B). In contrast, neither MEK (10 μM PD98059, 10 μM U0126) nor EGFR (5 μM PD168393, 5 μM PD153035) inhibitors altered ACh-induced p38 phosphorylation (Figure 2A–D). Immunoblot controls verified MEK and EGFR inhibitors robustly blocked ACh-induced ERK1/2 phosphorylation (Figure 2A–D).

Previously, we showed that ACh-induced transactivation of EGFR activates downstream PI3K/AKT signaling [16, 18]. As anticipated by the failure of EGFR inhibitors to block ACh-induced p38 phosphorylation (Figure 2C, D), neither of two PI3K inhibitors tested (10 μM LY294002, 5 nM Wortmannin) altered p38 phosphorylation (Figure 2E, F). These results indicated unambiguously that ACh-induced p38 activation is not regulated by EGFR, ERK1/2, or PI3K/AKT activation.

Protein kinase C (PKC) is an important post-M3R signaling molecule [34, 35]. To determine whether PKC played a role in mediating post-M3R p38 activation in colon cancer cells we used two selective PKC-α/β1 inhibitors, Gö6976 and Gö6983. Notably, pre-incubating HT-29 cells with either 5 μM Gö6976 or 5 μM Gö6983 consistently attenuated ACh-induced p38 phosphorylation but did not alter levels of total p38 (Figure 3A, B). These findings were replicated in H508 colon cancer cells (Figure 3A, B). In these experiments, atropine and a MEK inhibitor (U0126) were used, respectively, as positive and negative controls. Others have reported that HT-29 colon cancer cells express little to no PKC-β [36–38]. Hence, our findings implicated PKC-α as the key intermediary between M3R and p38 activation in colon cancer.

To test the hypothesis that post-M3R PKC activation mediates downstream p38 phosphorylation, we examined the effects of activating PKC directly with a phorbol ester. We incubated HT-29 and H508 cells with phorbol 12-myristate 13-acetate (PMA) to activate PKC directly and employed selective inhibitors to show that these actions were neither MR- nor EGFR-dependent. As shown in Figure 3C and D, neither control (atropine or MEK inhibitor U0126) altered PMA-induced p38 phosphorylation. In contrast, in both cell lines, PKC-α/β1 inhibitors, Gö6976 and Gö6983, consistently inhibited PMA-induced p38 phosphorylation (Figure 3C, D). These findings confirmed that PKC-α regulates p38 activation in colon cancer cells and provided further evidence supporting the role of PKC-α as a key intermediary molecule between M3R and p38 activation.

Intrigued by the novel observation that M3R activation resulted in PKC-α-mediated activation of p38, we sought a functional role for this finding. We considered whether this signaling pathway could contribute to a more invasive phenotype. Previously, we reported that M3R signaling promoted colon cancer cell invasion by increasing expression and activation of MMP1 [26, 27]. Whereas stimulating colon cancer cells with ACh induced *MMP1* expression by activating EGFR/ERK1/2 signaling [26], it was conceivable that other post-M3R signaling pathways contributed to this action.

Interactions between post-M3R signaling pathways contribute to ACh-induced MMP1 expression

As shown in Figure 4, we found incubating HT-29 cells with 100 μM ACh stimulated a nearly 40-fold increase in *MMP1* mRNA expression, an action completely blocked by pre-incubating cells with atropine (Fig. 5A; 40.8 ± 0.7-fold increase in *MMP1* mRNA with ACh alone compared to 1.3 ± 0.2-fold increase with ACh plus atropine; $P < 0.05$). Pre-incubating HT-29 cells with inhibitors of PKC-α/β1, p38-α/β, EGFR, and MEK strongly attenuated but did not completely abolish ACh-induced *MMP1* expression (Figure 4). Notably, pre-incubating cells with the combination of a p38-α/β inhibitor plus either an EGFR or MEK inhibitor completely blocked ACh-induced *MMP1* expression; i.e., the levels of ACh-induced *MMP1* mRNA following pre-incubation with these combinations of inhibitors were significantly lower than those observed with these inhibitors acting alone and not different from control basal values (Figure 4). Pre-incubating cells with the triple combination of an EGFR, PKC-α/β1, and p38-α/β inhibitor caused no further reduction in *MMP1* mRNA levels (Figure 4). Similar findings were observed when we repeated this experiment using H508 instead of HT-29 cells (Supplemental Fig. 2). Control experiments showed atropine and other selective inhibitors used in these and subsequent experiments did not stimulate *MMP1* expression (Supplemental Figure 1B).

We hypothesized that the effects of inhibitor combinations on ACh-induced *MMP1* expression were most likely explained by potentiating interactions between post-M3R signaling pathways. In particular, a potentiating interaction between post-M3R PKC/p38 and EGFR/ERK signaling would explain the observations that blocking any component of these two signaling pathways attenuated ACh-induced *MMP1* expression and simultaneously blocking both pathways abolished it. To test this hypothesis we designed experiments to activate PKC/p38 and EGFR/ERK signaling directly and determined the impact on *MMP1* expression.

Simultaneous activation of PKC/p38 and EGFR/ERK signaling potentiates MMP1 gene and protein expression

Based on our findings that blocking activation of both PKC/p38 and EGFR/ERK signaling downstream of M3R modulated ACh-induced *MMP1* expression, we hypothesized that incubating HT-29 cells with a combination of PMA, a phorbol ester that directly activates PKC, and EGF, an EGFR agonist, would potentiate *MMP1* expression. First, we confirmed that the effects of PMA and EGF did not require activation of M3R; pre-incubating HT-29 cells with atropine alone did not alter *MMP1* expression whereas pre-treating cells with atropine blocked ACh-induced *MMP1* expression (Figure 5A). Treating HT-29 cells with PMA (50 nM) or EGF (10 ng/ml) increased levels of *MMP1* mRNA and, as predicted, these changes were not blocked by pre-incubating cells with atropine (Figure 5A).

To confirm that inducing *MMP1* mRNA expression resulted in similar effect on protein expression, we used ELISA to measure MMP1 protein levels in the culture media after stimulating HT-29 cells with test agents. As shown in Figure 5B, incubating cells with 100 μM ACh stimulated a greater than two-fold increase in the levels of secreted MMP1 ($P < 0.01$) that was completely blocked by pre-treating cells with 5 μM atropine. PMA and EGF

stimulated approximately four- and two-fold increases in MMP1 release, respectively, effects that were not altered by pre-incubating cells with atropine (Figure 5B). Collectively, these findings indicate that activating M3R, PKC, and EGFR directly induces MMP1 gene and protein expression.

We next examined the effects of simultaneous direct activation of PKC and EGFR signaling. Based on the robust increase in *MMP1* mRNA expression observed with independent activation of PKC and EGFR and the evidence for potentiating interactions suggested by the data in Figure 4, we hypothesized that simultaneously activating these two post-M3R signaling pathways would potentiate both *MMP1* gene and protein expression. Indeed, as expected, incubating HT-29 cells with 10 ng/ml EGF plus concentrations of PMA greater than 1 nM significantly augmented *MMP1* mRNA levels compared to the calculated additive value for those concentrations of PMA and EGF acting alone (Figure 5C). Moreover, the degree of potentiation appeared to increase in concert with increases in the concentration of PMA (Figure 5C). Similar findings were observed when we examined EGF- and PMA-induced changes in MMP1 protein release from HT-29 cells (Figure 5D). Augmented MMP1 release was first detected in cells incubated with 5 nM PMA plus 10 ng EGF/ml, and the degree of potentiation increased as the concentration of PMA was progressively increased (Figure 5D). These results indicate that direct co-stimulation of PKC and EGFR signaling potentiates increases in MMP1 mRNA and protein levels, a finding consistent with our hypothesis that potentiating interactions between these pathways mediate the effects of M3R activation and explain the findings shown in Figure 4.

PKC regulates p38 and EGR/ERK activation and potentiation of post-M3R *MMP1* expression

To investigate further the potentiating interactions that augment *MMP1* mRNA and protein expression we explored post-PKC signaling pathways. Prior studies reporting that PKC cross-talk with EGFR may be mediated by Src [39, 40] led us to consider the likelihood that Src plays an important intermediary role in post-M3R induction of *MMP1* expression in colon cancer. To address this we used chemical inhibitors to parse the relative contributions of EGFR, MEK/ERK1/2, p38, and Src signaling to *MMP1* mRNA expression in PMA-activated cells. As shown in Figure 6A, as we anticipated pre-incubating HT-29 cells with PKC-α/β1 inhibitors (5 μM Gö6976 and 5 μM Gö6983) abolished PMA-induced increases in *MMP1* gene expression. Pre-incubating cells with inhibitors of p38-α/β (10 μM SB202190, 10 μM SB203580), EGFR (5 μM PD168393, 5 μM PD153035), MEK (10 μM PD98059, 10 μM U0126), and Src (10 μM PP2) alone significantly attenuated PMA-induced increases in levels of *MMP1* mRNA (Figure 6A). These findings were consistent with two discrete actions of PKC, downstream activation of p38 and Src-mediated cross-talk with EGFR/MEK/ERK signaling. As predicted, PMA-induced *MMP1* gene expression was abolished when we tested this hypothesis by simultaneously blocking p38-α/β plus either EGFR or MEK/ERK1/2 signaling (Figure 6A).

To confirm the role of Src, we examined the actions of blocking this intermediary on ACh-induced *MMP1* gene expression. As shown in Figure 6B, pre-incubating cells with either a p38-α/β or Src inhibitor robustly attenuated ACh-induced *MMP1* gene expression. Notably,

pre-incubating cells with the combination of the p38-α/β and Src inhibitors abolished ACh-induced *MMP1* expression (Figure 6B), similar to the effects of combining the Src and p38-α/β inhibitors on PMA-induced *MMP1* expression (Figure 6A). Moreover, immunoblotting revealed that ACh treatment stimulated Src phosphorylation, an action blocked by pre-incubating cells with the Src inhibitor PP2 or the PKC-α/β1 inhibitor Gö6976; these interventions did not alter levels of total Src (Figure 6C, D). These findings strongly support the hypothesis that in human colon cancer cells Src serves as a key intermediary between PKC-α and EGFR/MEK/ERK signaling.

Collectively, these observations identify PKC-α as a central regulator of post-M3R signaling. Once activated, PKC-α stimulates Src-mediated activation of EGFR/MEK/ERK1/2 signaling that induces *MMP1* gene expression. By a separate, distinct mechanism PKC-α activates p38-α/β signaling that also induces *MMP1* gene expression. Simultaneous activation of EGFR/MEK/ERK1/2 and p38-α/β signaling downstream of PKC-α, as occurs following M3R activation, potentiates *MMP1* gene expression.

Identification of p38-α as the p38 isoform relevant to potentiating interactions in colon cancer cells

Since the inhibitors we used block both α and β isoforms of p38, we employed siRNA knockdown of p38-α to define more clearly the role of this isoform in mediating M3R agonist-induced increases in *MMP1* expression. As shown in Figure 7A, following HT-29 cell transfection with siRNA we were able to knock down p38-α expression by more than 90%; transfecting cells with non-targeting 'mock' siRNA did not substantially alter levels of total p38 or p38-α. Immunoblotting for p38-β resulted in a weak signal that was not substantially altered by siRNA knockdown of p38-α (Figure 7A). These findings identify p38-α as the predominant isoform in this cell line, a supposition confirmed by markedly reduced expression of total p38 following siRNA knockdown of p38-α (Figure 7A).

As shown in Figure 7B, transfecting HT-29 cells with mock or p38-α siRNA did not alter basal levels of *MMP1* mRNA. In contrast, transfecting cells with siRNA targeting p38-α significantly reduced both ACh- and PMA-induced expression of *MMP1* mRNA, an effect not observed following transfection with non-targeting mock siRNA (Figure 7B). These findings confirm the p38-α isoform mediates downstream actions of M3R and PKC activation on MMP1 expression.

Potentiating interactions between EGFR/ERK and PKC/p38 signaling augment colon cancer cell invasion

Lastly, we explored the functional consequences of the potentiating interactions we uncovered. Invasion is an important cellular attribute required for colon cancer cell dissemination to other organs such as the liver. Our previous work supported a role for M3R signaling in stimulating colon cancer cell invasion; blocking M3R activation with atropine or the actions of MMP1 with a neutralizing antibody abolished muscarinic agonist-induced colon cancer cell invasion [17, 27]. Here, we compared the impact of selectively blocking the post-M3R signal transduction pathways studied above on ACh-induced HT-29 cell

invasion. We measured cell invasion using a Boyden chamber/Matrigel assay and quantified changes with imaging software after 48-h incubation with test agents.

As shown in Figure 8A, we first confirmed that treatment with 100 μM ACh robustly stimulated HT-29 cell invasion and that this was completely blocked by pre-incubating cells with atropine. Then we confirmed that, as we observed for changes in *MMP1* mRNA (Figure 5C) and MMP1 protein (Figure 5D), the combination of PMA and EGF potentiated cell invasion (Figure 8B); that is, whereas PMA and EGF induced ~5- and 2-fold increases in cell invasion individually, when added together these PKC and EGFR agonists stimulated a 10-fold increase in cell invasion. The combination of PMA and EGF stimulated cell invasion to a greater degree than the additive value predicted from the results of these agents acting alone.

We sought to validate the results shown in Figure 8B with another colon cancer cell line but H508 cells are too large to pass through 8-micron pores in Boyden chambers. To address this technical limitation, we used smaller Caco-2 human colon cancer cells. Supplemental Figure 3 confirms that the combination of PMA plus EGF stimulates potentiation of *MMP1* expression in Caco-2 cells. As shown in Figure 8C, similar to our observations in HT-29 cells, both PMA and EGF stimulated significant increases in Caco-2 cell invasion that were augmented by treating cells with the combination of these agents. Altogether, these findings confirm that potentiating interactions between post-M3R signaling pathways augment colon cancer cell invasion.

Finally, we examined the actions of pre-incubating HT-29 cells with selective inhibitors of post-M3R signaling on ACh-induced cell invasion. None of the inhibitors used in the invasion assays altered basal cell invasion (Supplemental Figure 1C). As shown in Figure 8D, the results of this experiment were consistent with potentiating interactions between post-M3R signaling pathways. Moreover, the findings strongly suggested that these potentiating interactions are central to ACh-induced cell invasion. That is, whereas inhibiting only PKC-α, p38-α, EGFR, or MEK/ERK1/2 activation attenuated ACh-induced cell invasion, combining inhibitors to block EGFR plus p38-α, MEK plus p38-α, or Src plus p38-α nearly abolished cell invasion (Fig. 8D). Thus, achieving maximal ACh-induced colon cancer cell invasion appears to depend largely on potentiating interactions between post-M3R signaling pathways.

DISCUSSION

No specific genetic mutations convert a primary colon tumor into an invasive metastatic cancer [41]. Rather, it appears that cancer cells that already possess the ability to invade adjacent normal tissue have metastatic potential and the more invasive the phenotype the more likely tumor cells will metastasize to other organs. Matrix metalloproteinases, which encompass a family of zinc-dependent endopeptidases that play critical roles in a wide variety of physiological and pathological processes, are key drivers of cell invasion [42]. Whereas low levels of MMP1 are important for tissue remodeling, in cancer increased levels of MMP1 facilitate cell invasion and dissemination; hence, it is not surprising that the level

of *MMP1* expression correlates with advanced colon cancer stage, metastasis, and adverse outcome [43–45].

Our group showed that M3R agonists like acetylcholine that promote colon cancer cell proliferation, survival, migration, and invasion, are also highly efficacious regulators of *MMP1* expression [26]. In the colon, these actions may be mediated by neurocrine, paracrine, or autocrine release of acetylcholine [46, 47] or alternatively by the actions of luminal bile acids that may also interact functionally with muscarinic receptors on neoplastic epithelial cells and induce *MMP1* gene expression [27, 48]. In a mouse model, we found that bethanechol, another M3R agonist, robustly augmented colon neoplasia and expression of *Mmp13*, the murine homologue of *MMP1* [19]. Blocking either M3R or MMP1 activation in human colon cancer cells abolished M3R agonist-induced colon cancer cell invasion [27], confirming that MMP1 plays a critical role in mediating these effects of M3R activation. Overall, we acquired a strong body of *in vitro* and *in vivo* evidence supporting a functional *M3R/MMP1 axis* that promotes colon cancer cell invasion.

The present communication addresses the regulation of this *M3R/MMP1 axis* – we found that M3R agonist-induced transcriptional activation of *MMP1* is regulated by a complex set of potentiating interactions between post-M3R signaling pathways. To validate our findings, we used at least two selective inhibitors to block key molecules in at least two colon cancer cell lines, used selective activators of these signaling molecules to verify their roles, and confirmed a key finding using siRNA knockdown. In so doing we identified PKC-α as a focal point of the signaling cascade downstream of M3R activation. ACh-induced *MMP1* expression was abolished by blocking activation of PKC-α directly, or by blocking downstream activation of p38-α and either EGFR or ERK1/2.

As anticipated from these findings, direct co-activation of PKC and EGFR robustly potentiated both *MMP1* gene and protein expression, and augmented cell invasion. Consistent with reports that PKC cross-talk with EGFR may be mediated by Src, we found that ACh-induced *MMP1* expression and cell invasion were attenuated by inhibiting Src and totally blocked by concurrently inhibiting p38-α plus Src; similar findings were observed for ACh-induced *MMP1* expression. As expected from these findings, we also discovered that ACh-induced Src phosphorylation (activation) is blocked by inhibiting activation of PKC-α. Collectively, these studies uncover novel functional interactions between post-M3R signaling pathways involving PKC-α-mediated p38-α activation and Src-mediated cross-talk between PKC-α and EGFR/ERK1/2 signaling which augment expression of *MMP1*, and drive cell invasion (Figure 9).

Members of the PKC family regulate basic cellular processes including proliferation differentiation, survival, and migration [49]. In particular, activation of PKC-α has been reported to promote colon cancer progression [50–52] and resistance to chemotherapy [53]. Nonetheless, although PKC isozyme expression is altered in many types of cancer, the role of PKC in the initiation and progression of cancers remains uncertain [54]. Indeed, it appears that fundamental actions of PKC are isoenzyme- and context-dependent; in some cancer cells PKC isozymes act as tumor promoters whereas in others they are tumor suppressors [55]. In concert with our findings, in cell lines derived from cancers of the

breast, liver, brain, and skin, PKC-α stimulates cell invasion [55]. Our data show that indirect activation of PKC-α as part of a post-M3R signaling cascade or direct activation of PKC using the phorbol ester PMA stimulates colon cancer cell invasion. Moreover, we find that this effect is mediated by downstream activation of separate circuits involving both EGFR/ERK1/2 and p38-α signaling, the former mediated by Src activation (Figure 5). These findings highlight the key concept reviewed by Hannahan and Weinberg [5] – the mechanistic underpinnings of different stages in cancer progression (e.g. cell proliferation vs. cell invasion) may be very different and a particular molecule or pathway may be pro-neoplastic at one stage and anti-neoplastic at another. There are now several examples of the context-dependent, dual nature of key signaling molecules. A recent report suggests that reactive oxygen species (ROS) may promote cancer cell proliferation but inhibit cell invasion and dissemination [56]. TGF-β may be either pro-tumorigenic or tumor suppressive depending on Sox4 co-expression [57]. Likewise, PKC-α may play seemingly opposing roles in mediating cell proliferation and invasion.

Four p38 isozymes, designated α, β, γ, and δ, have been identified. The principal p38 inhibitor used in our studies is p38-α/β-selective and our siRNA knockdown data (Figure 7) provides strong evidence that the p38-α isoform is the downstream effector of PKC-induced *MMP1* gene expression in colon cancer cells. Although not previously linked to muscarinic receptor agonist-induced induction of matrix metalloproteinase genes in colon cancer, p38-α is reported to play a role in interleukin-1-induced *MMP1* gene transcription in fibroblasts and endothelial cells [58], plays a critical role in squamous epithelial cell invasion [59], and mediates an H-Ras-induced invasive phenotype in breast epithelial cells [60]. Moreover, PKC-α is reported to alter p38 activity in microglia [61], macrophages [62], vascular smooth muscle cells [63], and mast cells [64]. These findings in other cell types provide additional support for our findings that upstream activation of PKC-α regulates activation of p38-α in colon cancer cells.

Collectively, our results led us to propose the model shown in Figure 9 wherein signaling interactions downstream of M3R activation promote enhanced transcription of *MMP1*, increased release of MMP1 into the cell microenvironment, and augmented cell invasion. Activation of PKC-α provokes cooperative signaling that results from Src-dependent activation of the EGFR/ERK1/2 pathway and EGFR-independent activation of p38-α. The robust 30- to 50-fold increase in *MMP1* gene expression observed following ACh interaction with M3R can be mimicked by directly co-activating PKC-α and EGFR/ERK1/2. This amplified signaling network results in potentiated levels of *MMP1* mRNA and protein, and enhanced colon cancer cell invasion.

Cooperative interactions between transcription factors have long been implicated in gene regulation [65, 66]. Thus, we believe the most likely explanation for the findings described herein is that the coordinated recruitment of downstream transcription factors and cofactors to regulatory elements of the *MMP1* gene mediates the potentiating interaction of post-muscarinic receptor signaling pathways. Although beyond the scope of the present work, this speculation will be tested in future experiments.

Finally, although M1R and M3R may both be expressed by gastrointestinal epithelial cells, our work and the cartoon depicted in Figure 9 identifies M3R as the operative muscarinic receptor subtype that regulates the signaling cascades studied herein. This may be considered speculative as acetylcholine is a non-selective M3R agonist. Nonetheless, we believe this conclusion is justified based on the strong body of evidence that M3R regulates these signaling cascades in human colon cancer cells [13, 46, 48], the identification of M3R as the primary M3R subtype expressed in both HT-29 and H508 colon cancer cells [17], and the key finding in animal models that M3R deficiency robustly attenuates the development of colon neoplasia whereas M1R deficiency has no effect [11, 18, 20].

Medicinal approaches to treating cancer using broad-spectrum MMP inhibitors have met with failure and selective therapeutic targeting of MMP1 has not yet been achieved [42]. Whereas EGFR inhibitors have demonstrated efficacy for colon cancer, these effects are commonly transient and emerging drug resistance generally results in tumor resurgence, cancer dissemination, and death. Thus, understanding the mechanisms whereby colon cancer cells develop resistance to EGFR-based therapies is important. Several such mechanisms have been elucidated, including downstream activating mutations in RAS and BRAF, c-MET signaling, and the mutation, hyperactivation or over-expression of EGFR itself. Our findings suggest that convergence of signaling pathways with molecules downstream of EGFR may also play a role. In this instance, activation of PKC-α downstream of M3R stimulates *EGFR-independent* activation of p38-α and induction of *MMP1* gene expression, a series of events that favor cell invasion and dissemination. Our findings suggest that combining an agent that blocks EGFR activity with an agent that blocks either M3R, PKC-α, or p38-α activity may have therapeutic promise for preventing or retarding colon cancer cell metastasis or blocking cancer cell escape when resistance to one therapy (e.g. EGFR inhibitors) emerges. Our findings provide additional support for the notion that combination therapy simultaneously targeting several pathways is needed to incapacitate redundant post-receptor signal transduction mechanisms that regulate *MMP1* expression in colon cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACh	acetylcholine
EGFR	epidermal growth factor receptor
ERK	extracellular signal-related kinase

JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MMP1	matrix metalloproteinase-1
M3R	subtype 3 muscarinic receptors
p38	p38 MAPK
PI3K	phosphoinositol-3 kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
qPCR	quantitative real-time PCR

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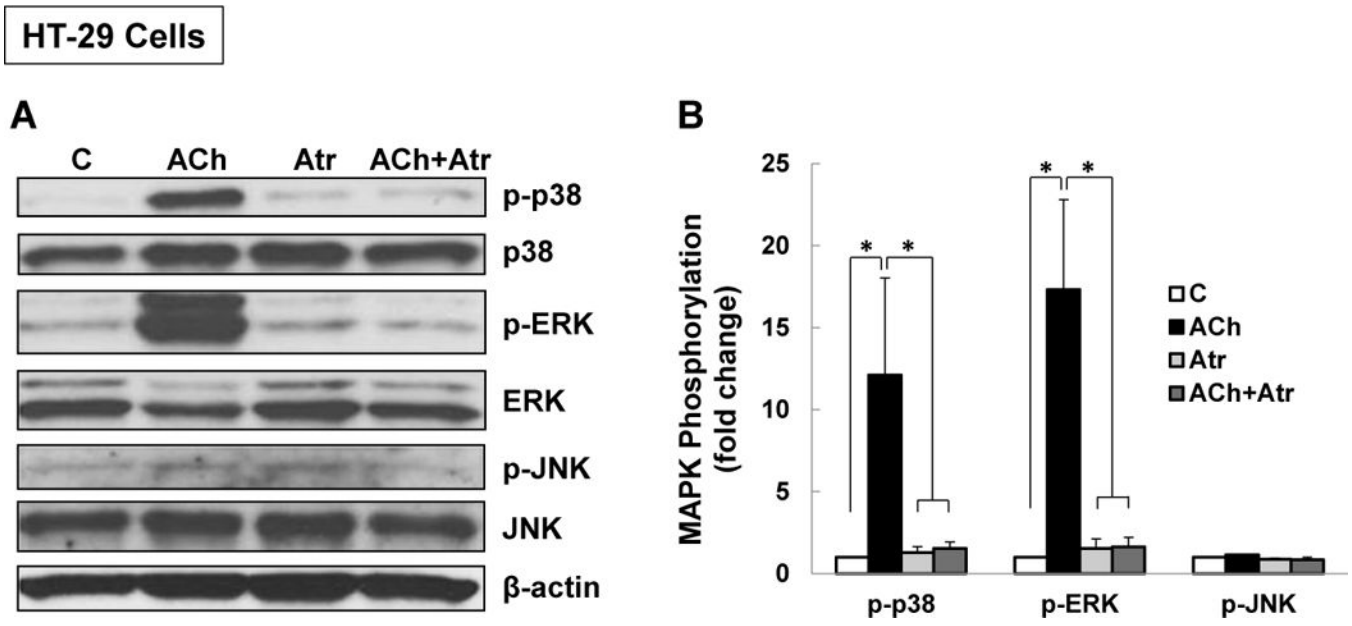


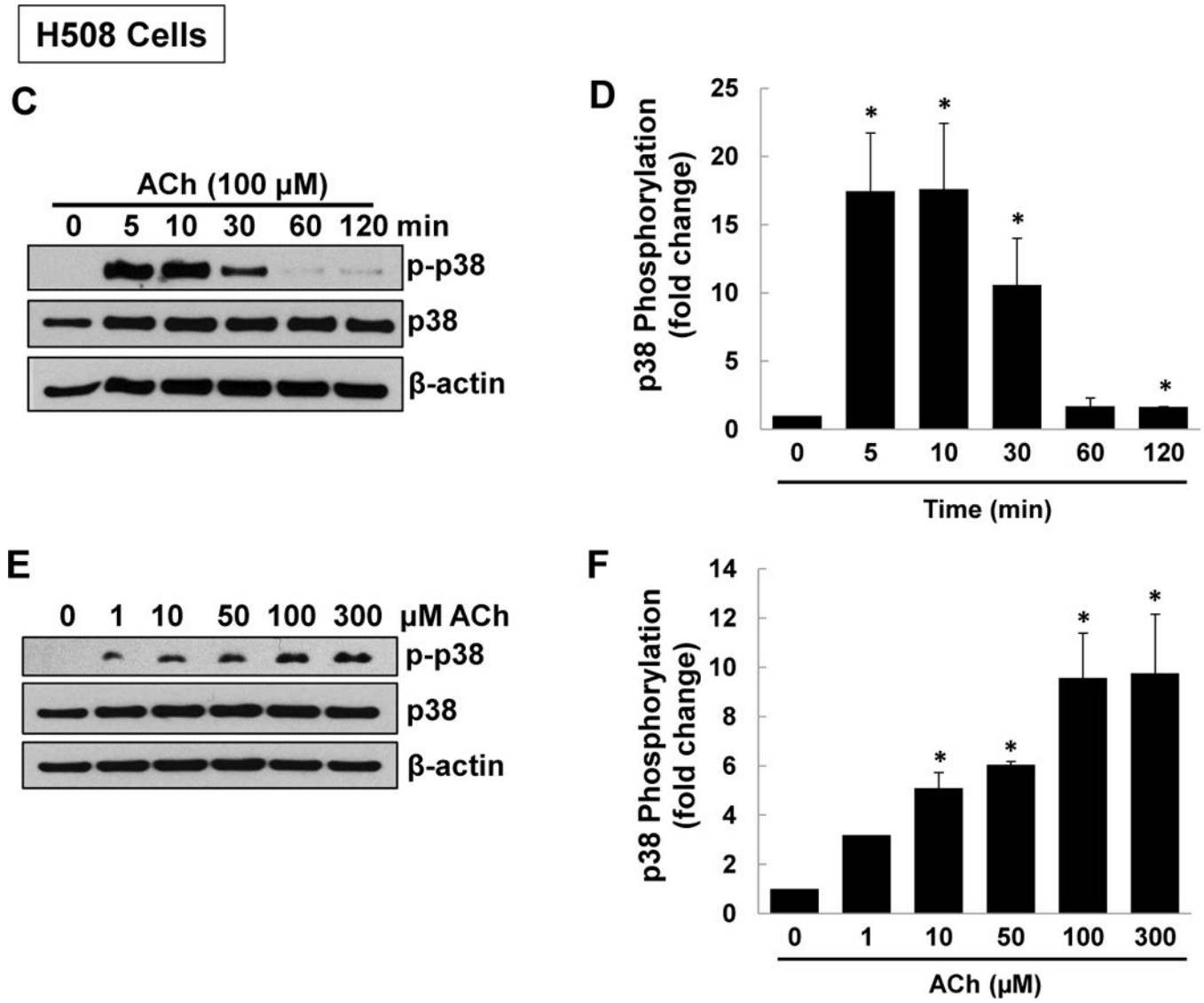
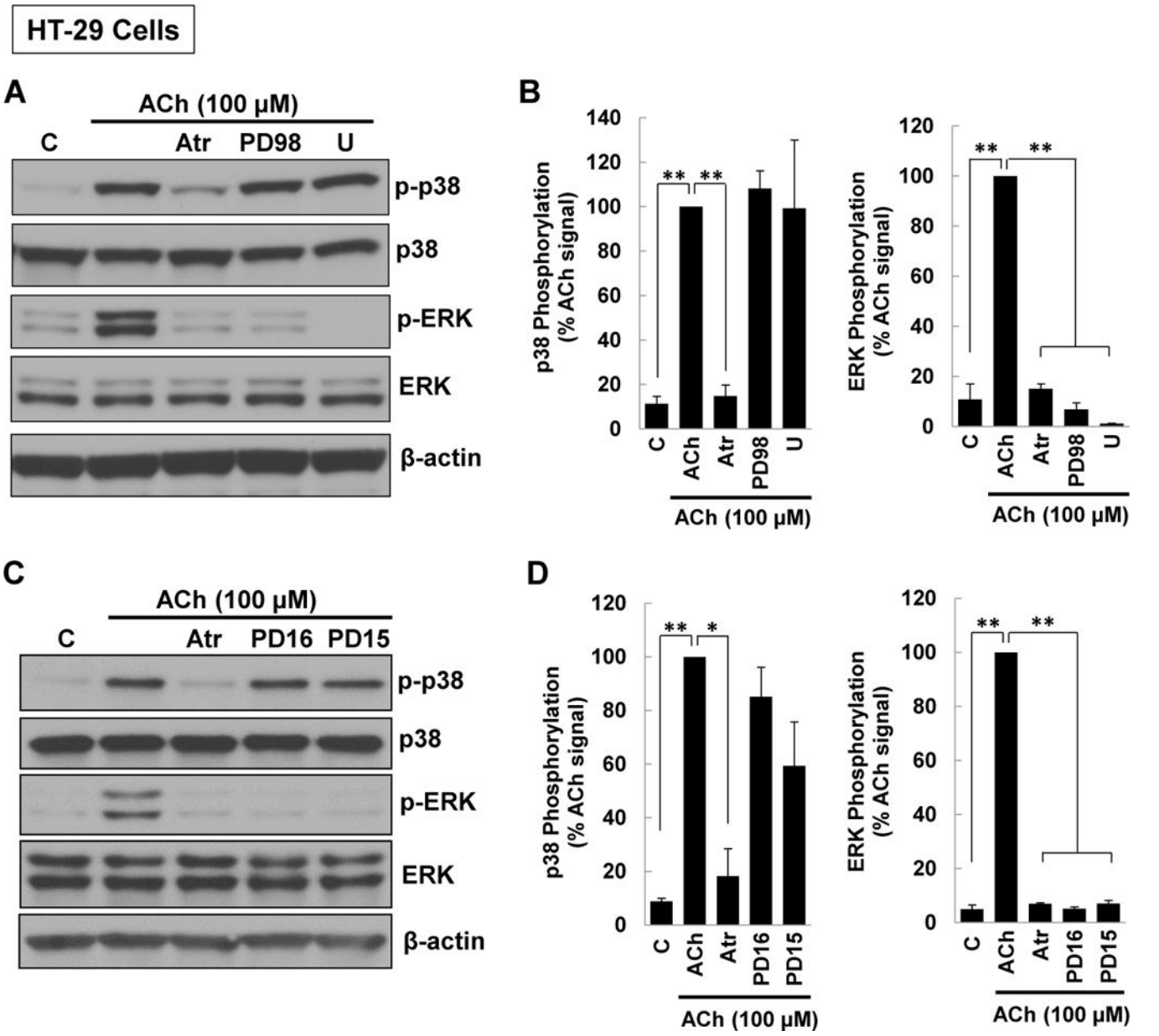


Figure 1. M3R activation stimulates p38 MAPK and ERK1/2 phosphorylation

(**A**) Acetylcholine (ACh) stimulates p38 and ERK1/2 phosphorylation. HT-29 colon cancer cells were incubated for 5 min with 200 μM ACh and cell extracts were immunoblotted with antibodies against phosphorylated and total p38, ERK1/2, and JNK. (**B)** Densitometry of immunoblots from four separate experiments show that ACh stimulated robust phosphorylation of p38 and ERK1/2, but did not alter levels of total or phosphorylated JNK, total ERK1/2 or total p38. The actions of ACh were blocked by pre-incubating cells with 5 μM atropine (Atr) for 45 min. C, untreated control; *$P < 0.05$. (**C**) ACh stimulates time-dependent p38 phosphorylation. H508 colon cancer cells were incubated with 100 μM ACh for the indicated times. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38. (**D**) Densitometry of immunoblots from three separate experiments show that ACh stimulated maximal p38 phosphorylation within 5 to 10 min which was nearly back to basal levels within 1 h. *$P < 0.05$ compared to Time 0. (**E**) ACh stimulates dose-dependent p38 phosphorylation. H508 colon cancer cells were incubated

with the indicated concentrations of ACh for 10 min. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38. (**F**) Densitometry of immunoblots from three separate experiments show ACh-induced p38 phosphorylation was detectable with 1 μM ACh and maximal with 100 to 300 μM ACh. *$P < 0.05$ compared to vehicle alone (no ACh). In all immunoblots, β-actin was used as a loading control.



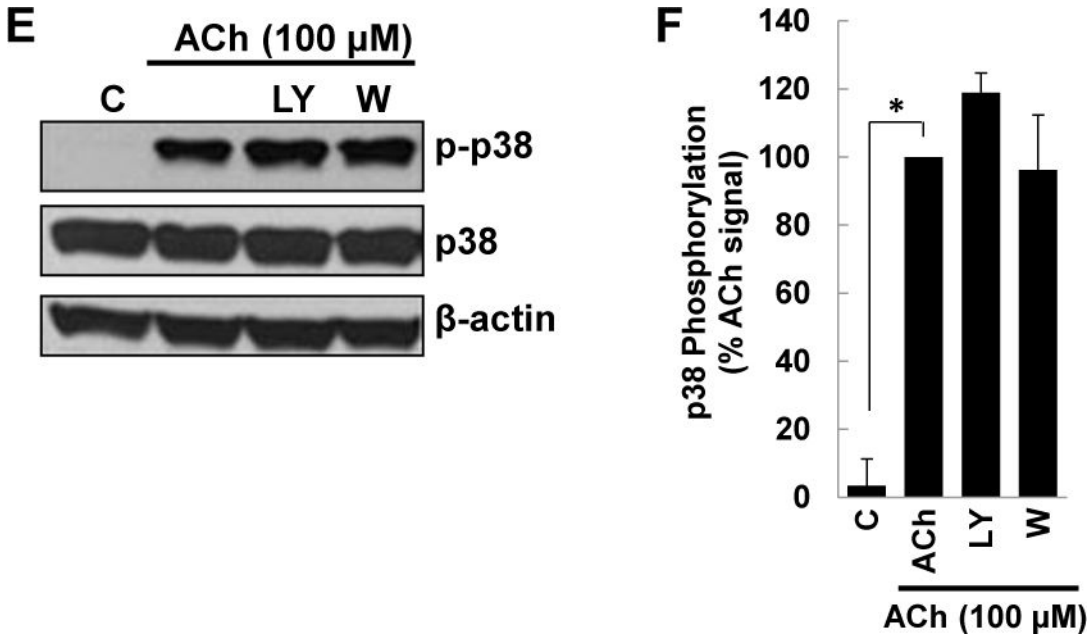
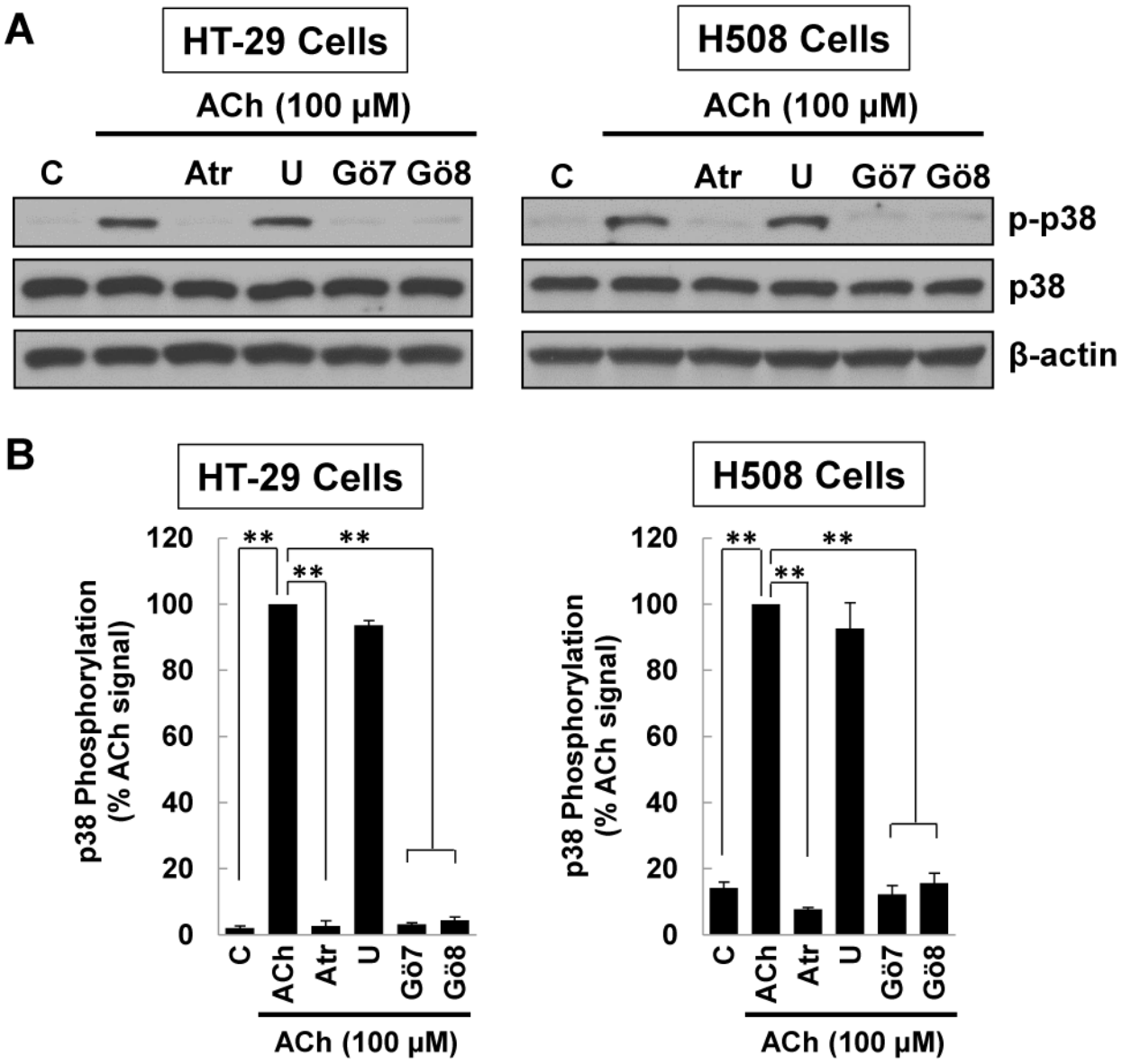


Figure 2. ACh-induced p38 phosphorylation is not altered by inhibiting MEK/ERK1/2, EGFR, or PI3K/AKT activation

(**A**) HT-29 cells were pre-incubated for 45 min with M3R or MEK/ERK1/2 inhibitors before adding ACh (100 μM) for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38 and ERK1/2. (**B**) Densitometry of immunoblots from three separate experiments shows ACh-induced p38 phosphorylation was blocked by pre-incubating cells with 5 μM atropine (Atr) but not by MEK (ERK1/2) inhibitors [10 μM PD98059 (PD98), 10 μM U0126 (U)]. As positive controls, atropine and MEK (ERK1/2) inhibitors consistently blocked ACh-induced ERK1/2 phosphorylation. $^{**}P < 0.01$ compared to 100 μM ACh alone. (**C**) HT-29 cells were pre-incubated for 45 min with inhibitors of M3R or EGFR inhibitors [5 μM PD168393 (PD16), 5 μM PD153035 (PD15)] before adding ACh (100 μM) for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38 and ERK1/2. (**D**) Densitometry of immunoblots from three separate experiments shows ACh-induced p38 phosphorylation was blocked by pre-incubating cells with atropine but not by EGFR inhibitors. As positive controls, atropine and EGFR inhibitors consistently blocked ACh-induced ERK1/2 phosphorylation. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 100 μM ACh alone. (**E**) HT-29 cells were pre-incubated for 45 min with PI3K/AKT signaling inhibitors [10 μM LY 294002 (LY), 5 nM Wortmannin (W)] before adding 100 μM ACh for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38. (**F**) Densitometry of immunoblots from three separate experiments shows ACh-induced p38 phosphorylation was not blocked by pre-incubating cells with PI3K/AKT inhibitors. $^{*}P < 0.05$ compared to 100 μM ACh alone.



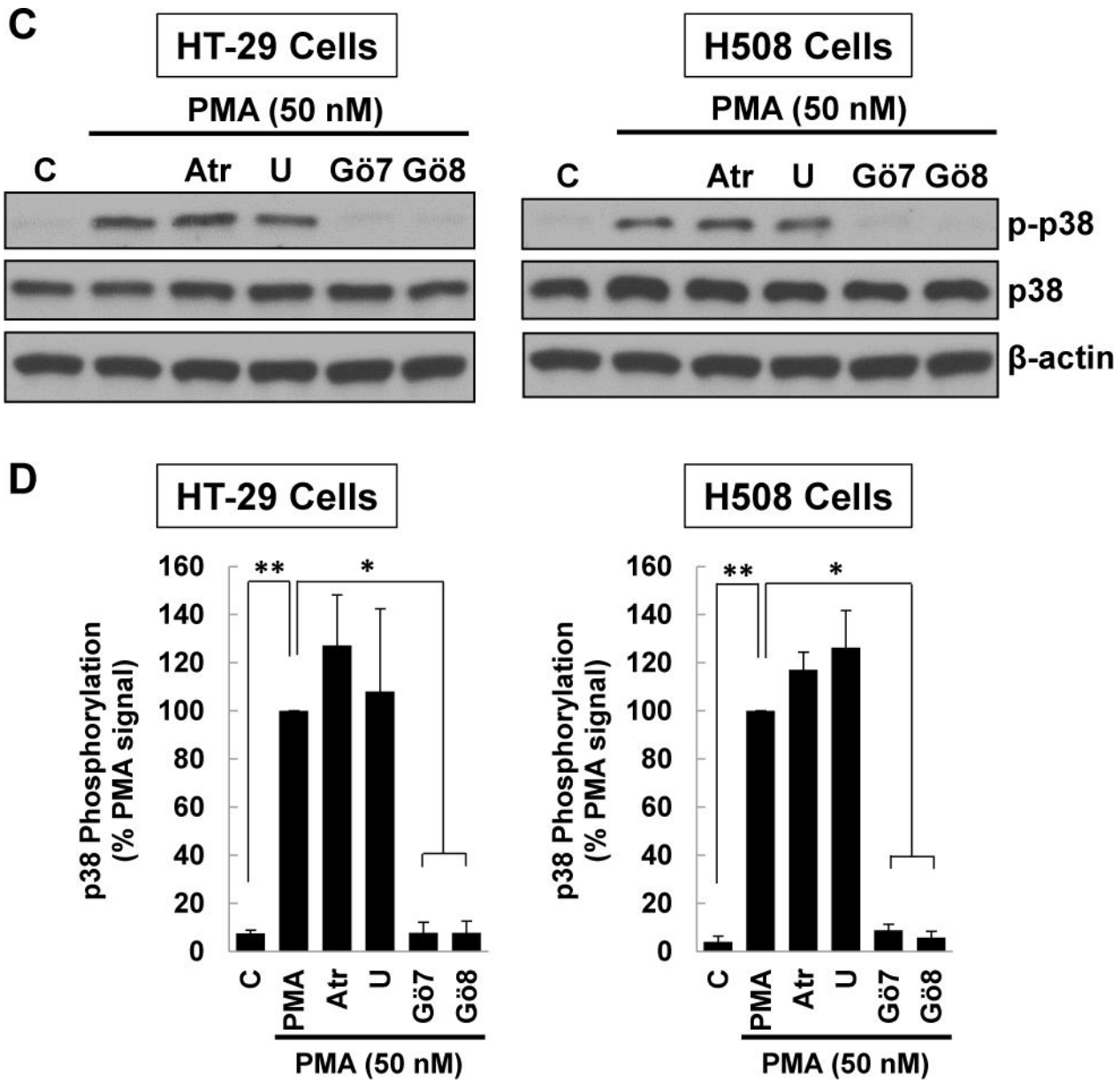


Figure 3. ACh-induced p38 phosphorylation is mediated by activation of PKC

(**A**) HT-29 and H508 cells were pre-incubated for 45 min with atropine, a MEK inhibitor [10 μM U0126 (U)] or PKC-α/β1 inhibitors [5 μM Gö6976 (Gö7), 5 μM Gö6983 (Gö8)] before adding 100 μM ACh for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38. (**B**) Densitometry of immunoblots from three separate experiments shows pre-incubating HT-29 and H508 cells with atropine or PKC-α/β1 inhibitors consistently attenuated ACh-induced p38 phosphorylation, but pre-incubation with atropine or the MEK inhibitor had no effect. $^{**}P < 0.01$ compared to 100 μM ACh alone. (**C**) HT-29 and H508 cells were pre-incubated for 45 min with atropine, a MEK inhibitor [10 μM U0126 (U)], or PKC-α/β1 inhibitors [5 μM Gö6976 (Gö7), 5 μM Gö6983 (Gö8)] before adding 50 mM PMA for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total p38. (**D**)

Densitometry of immunoblots from three separate experiments shows pre-incubating HT-29 and H508 cells with PKC-α/β1 inhibitors consistently attenuated PMA (50 nM)-induced p38 phosphorylation, but pre-incubation with atropine or the MEK inhibitor had no effect. *$P < 0.05$, **$P < 0.01$ compared to 50 nM PMA alone. β-actin was a loading control. C, untreated control.

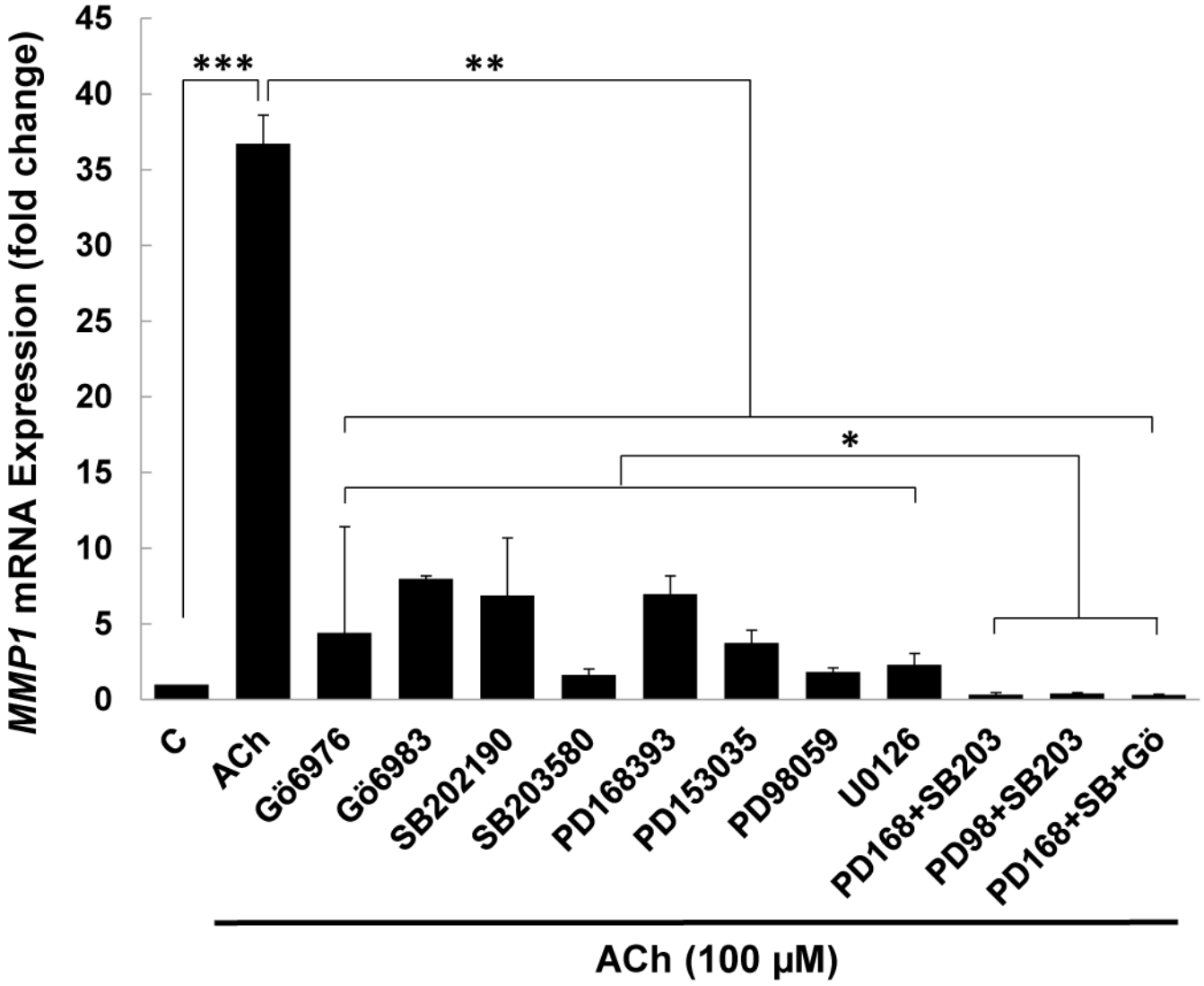
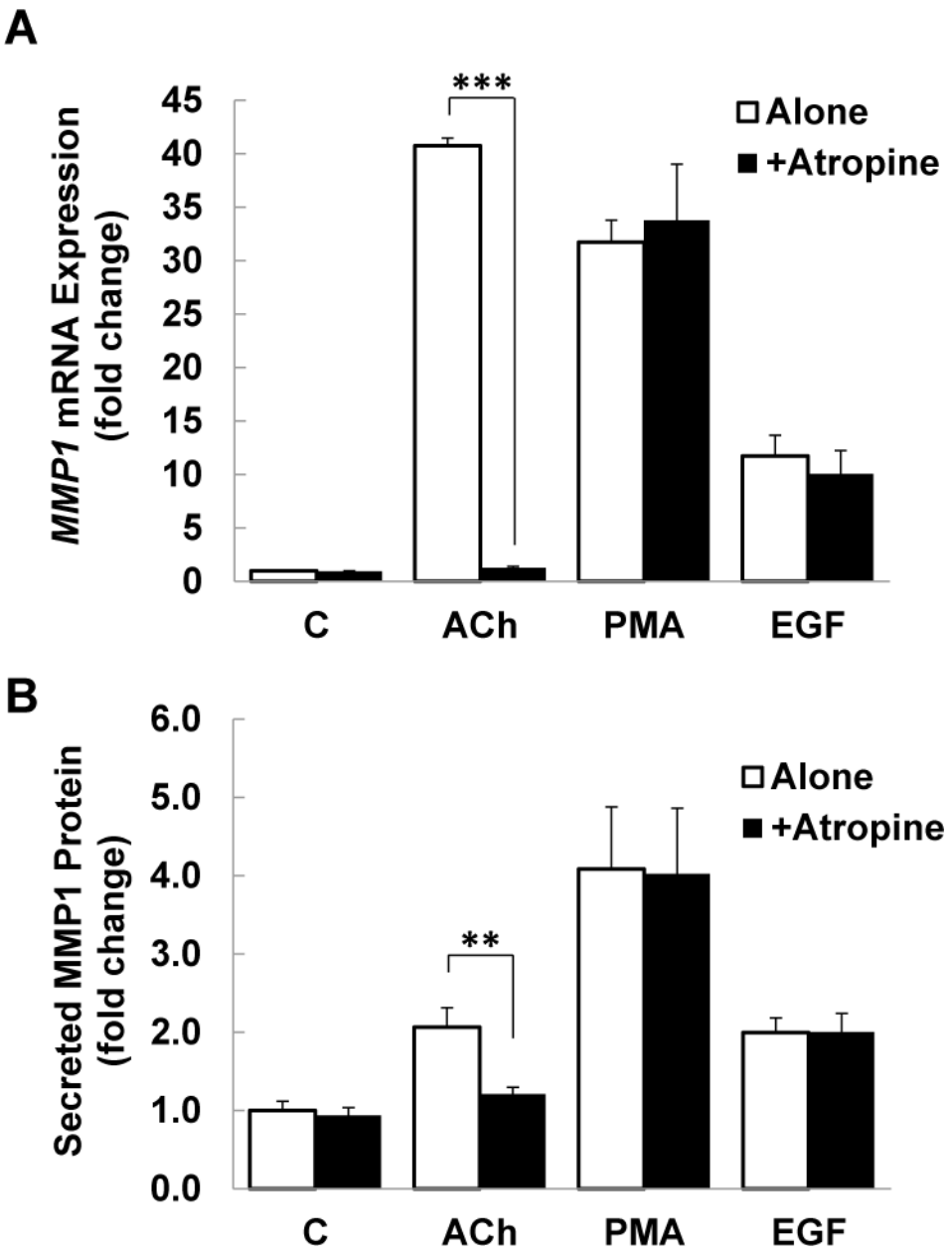


Figure 4. Potentiating interactions between post-M3R signaling pathways govern ACh-induced *MMP1* gene induction

HT-29 cells were pre-incubated for 45 min with PKC-α/β1, p38-α/β, EGFR, or MEK inhibitors, and then incubated for an additional 4 h with no additional test agents (C) or with ACh (100 μM). *MMP1* mRNA levels were measured by qPCR. ACh-induced *MMP1* expression was attenuated by inhibitors of PKC-α/β1 (5 μM Gö6976, 5 μM Gö6983), p38-α/β (10 μM SB202190, 10 μM SB203580), EGFR (5 μM PD168393, 5 μM PD153035), and MEK (10 μM PD98059, 10 μM U0126). Pre-incubating cells with a combination of p38-α/β plus EGFR inhibitors or p38-α/β plus MEK inhibitors abolished ACh-induced *MMP1* gene expression, as did pre-incubation with a combination of p38-α/β, EGFR and PKC-α/β1 inhibitors. qPCR data were normalized to GAPDH and are means of four separate experiments. C, untreated control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.



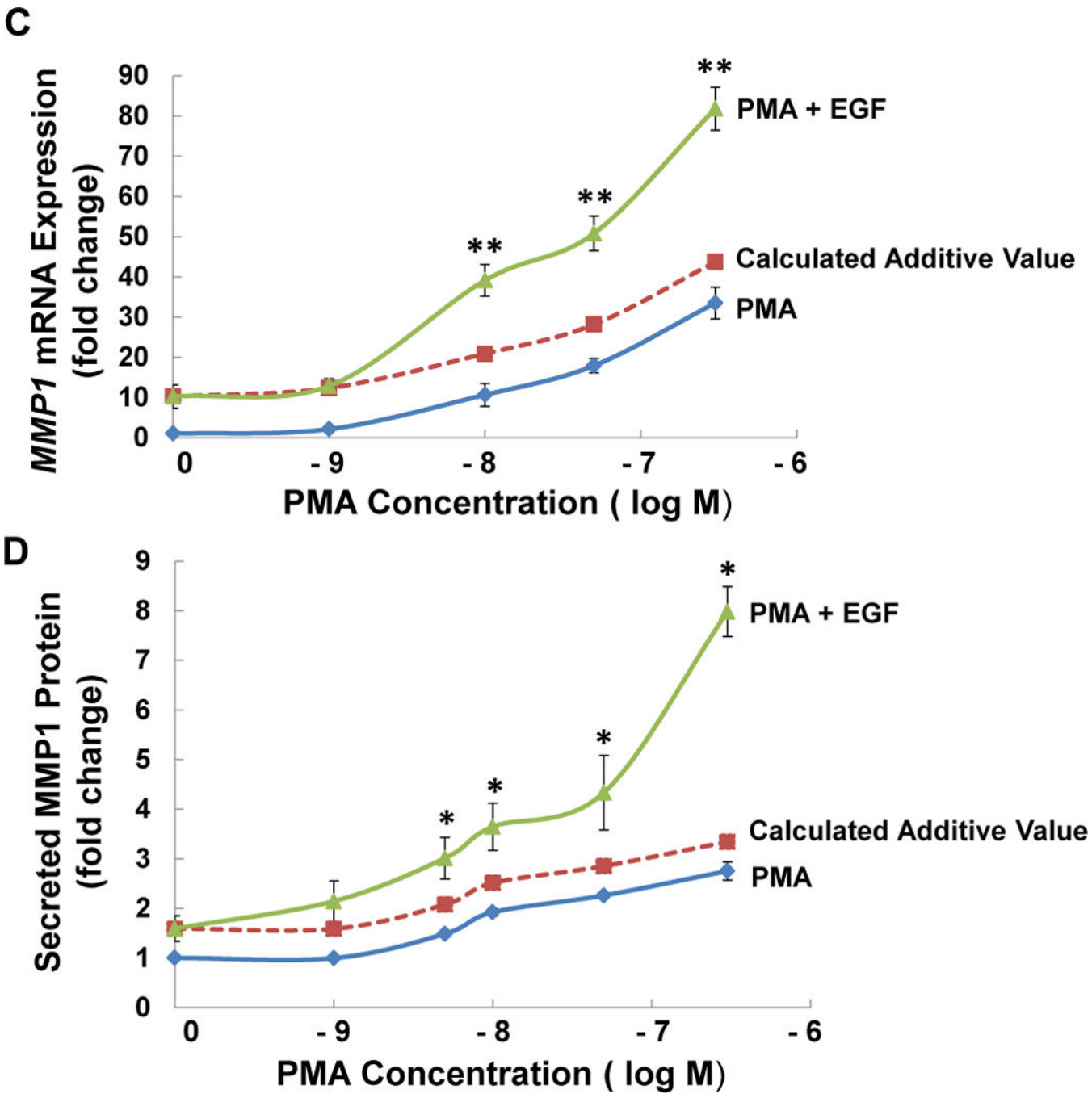
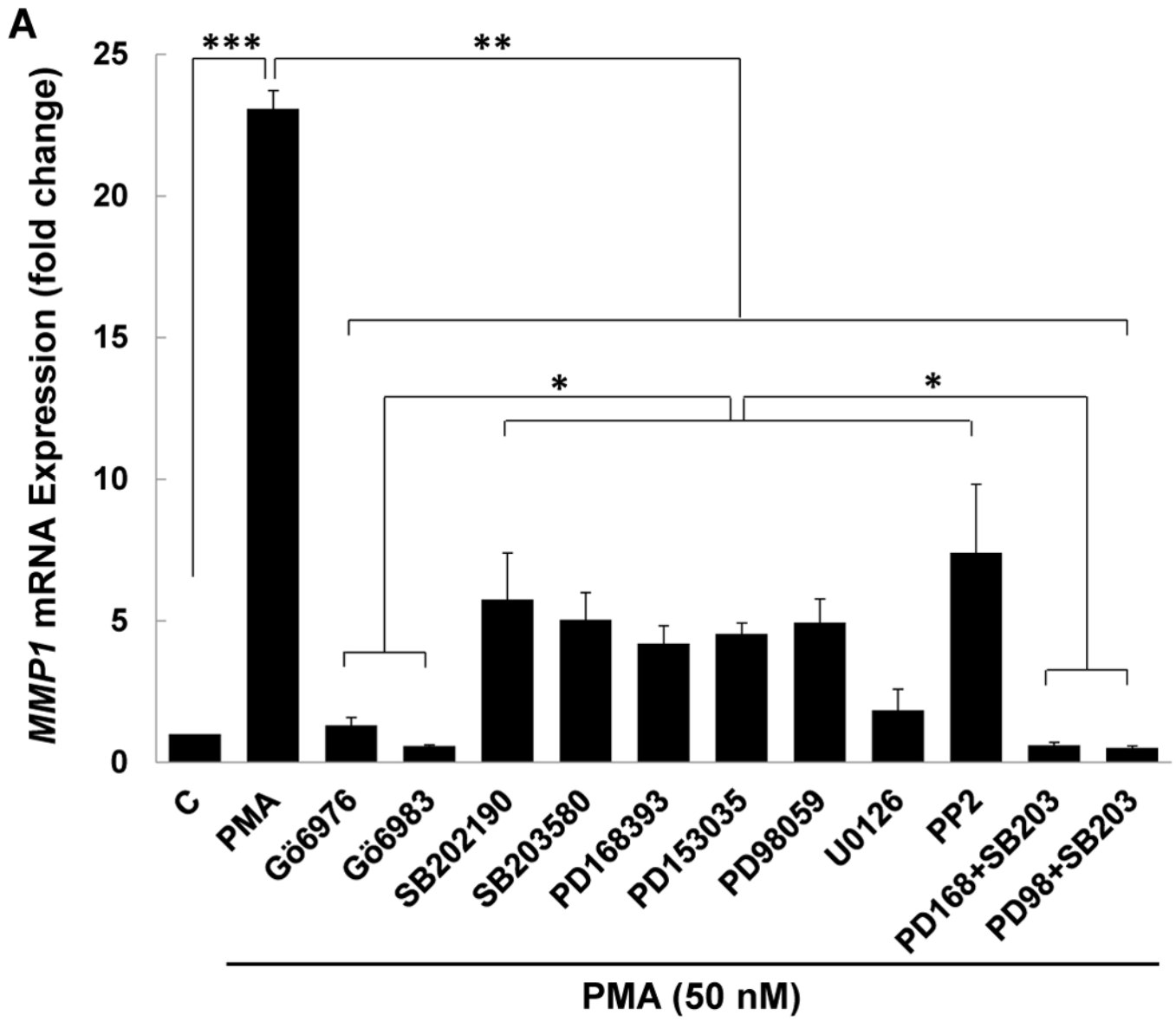


Figure 5. Simultaneous activation of PKC and EGFR signaling potentiates *MMP1* gene expression

(**A**) HT-29 cells were pre-incubated with 5 μM atropine (Atr) for 45 min and then incubated for an additional 4 h with no test agents (C), or with ACh (100 μM), a PKC activator (50 nM PMA), or an EGFR activator (10 ng/ml EGF). *MMP1* mRNA levels were measured by qPCR. Treating HT-29 cells with PMA or EGF increased levels of *MMP1* mRNA. Whereas the effects of ACh were blocked by pre-incubating cells with atropine, PMA- and EGF-induced changes in *MMP1* expression were unaffected. C, untreated control; ***$P < 0.001$.

(**B**) Similar findings were observed when HT-29 cells were treated under the same conditions and MMP1 protein expression was measured using ELISA. C, untreated control; $^{**}P < 0.01$. (**C**) HT-29 cells were treated with increasing concentrations of PMA, as indicated, for 4 h and *MMP1* gene expression was measured by qPCR. Increasing concentrations of PMA progressively increased *MMP1* gene expression. Adding 10 ng/ml of EGF to each concentration of PMA significantly potentiated *MMP1* gene expression. The dashed line represents the calculated additive values for 10 ng/ml of EGF plus the indicated concentration of PMA. (**D**) Similar findings were observed when HT-29 cells were treated under the same conditions and secreted MMP1 protein was measured using ELISA. qPCR data were normalized to GAPDH. Data represent mean ± SE of at least three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to calculated additive value.



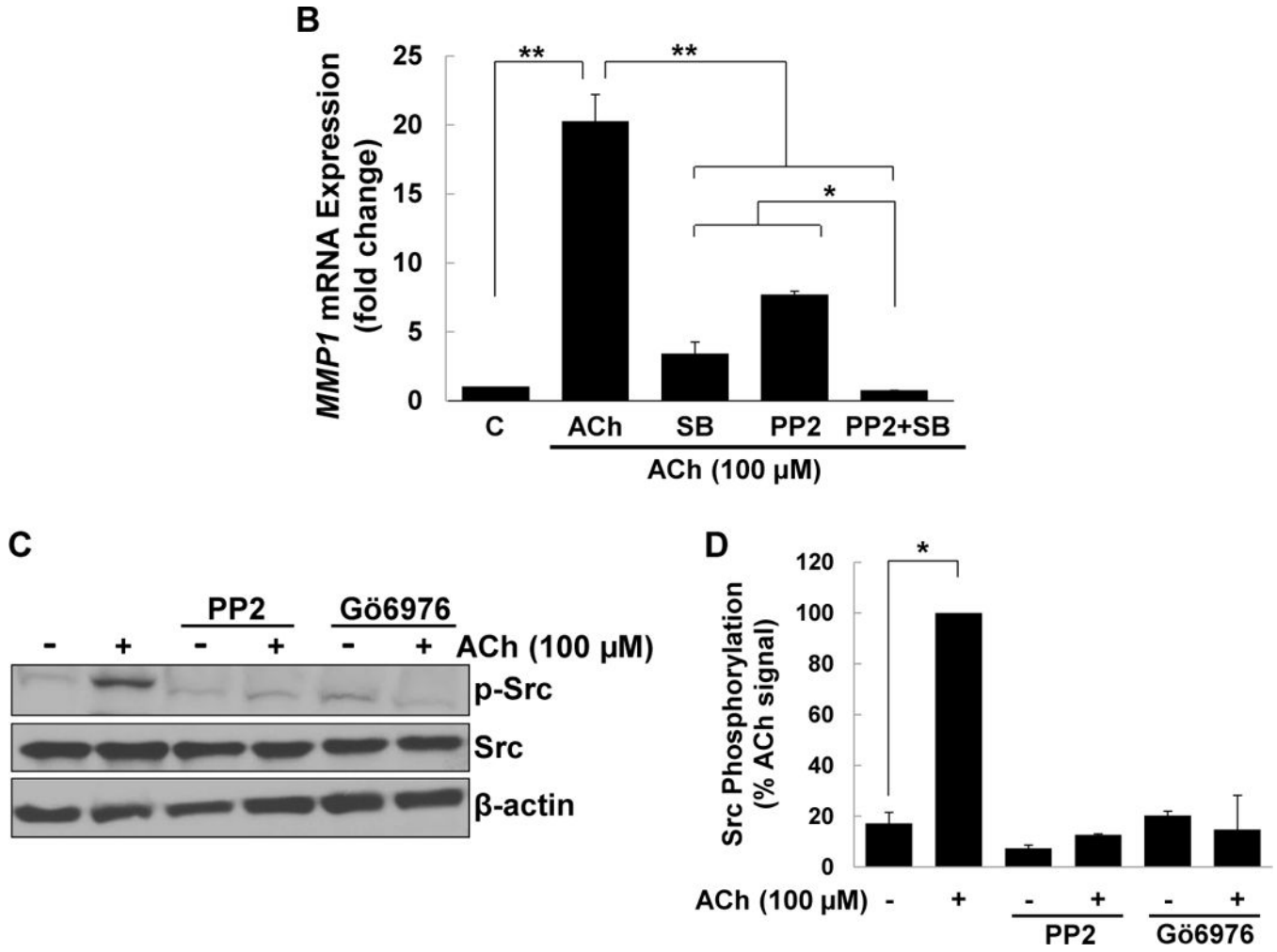


Figure 6. Potentiating interactions between post-PKC signaling pathways govern *MMP1* gene induction

(**A**) HT-29 cells were pre-incubated for 45 min alone or with PKC-α/β1 inhibitors (5 μM Gö6976, 5 μM Gö6983), p38-α/β inhibitors (10 μM SB202190, 10 μM SB203580), EGFR inhibitors (5 μM PD168393, and PD153035), MEK inhibitors (10 μM PD98059, 10 μM U0126), or a Src inhibitor (10 μM PP2), alone or in combination, before adding 50 nM PMA for an additional 4-h incubation. *MMP1* mRNA levels were measured by qPCR. PMA-induced *MMP1* gene expression was abolished by pre-incubating cells with PKC-α/β1 inhibitors and attenuated by pre-incubating cells with inhibitors of p38-α/β, EGFR, MEK, and Src. Simultaneously blocking p38-α/β and EGFR, p38-α/β and MEK, or p38-α/β and Src abolished PMA-induced *MMP1* gene expression. (**B**) HT-29 cells were pre-incubated for 45 min alone or with a p38-α/β (10 μM SB203580) or Src (10 μM PP2) inhibitor, alone or in combination, followed by an additional 4-h incubation with 100 μM ACh. *MMP1* mRNA levels were measured by qPCR. ACh-induced *MMP1* expression was attenuated by pre-incubation with p38-α/β and Src inhibitors and abolished by these inhibitors in combination. qPCR data were normalized to GAPDH and are the mean ± SE of three separate experiments. C, untreated control; $*P < 0.05$, $**P < 0.01$. (**C**) H508 cells were pre-incubated for 45 min with inhibitors of Src (10 μM PP2) and PKC-α/β1 (5 μM Gö6976) before adding

ACh (100 μM) for an additional 5-min incubation. Cell extracts were immunoblotted with antibodies against phosphorylated and total Src. (**D**) Densitometry of immunoblots from two separate experiments shows ACh-induced Src phosphorylation was blocked by pre-incubating cells with Src and PKC-α/β1 inhibitors. *$P < 0.05$ compared to 100 μM ACh alone.

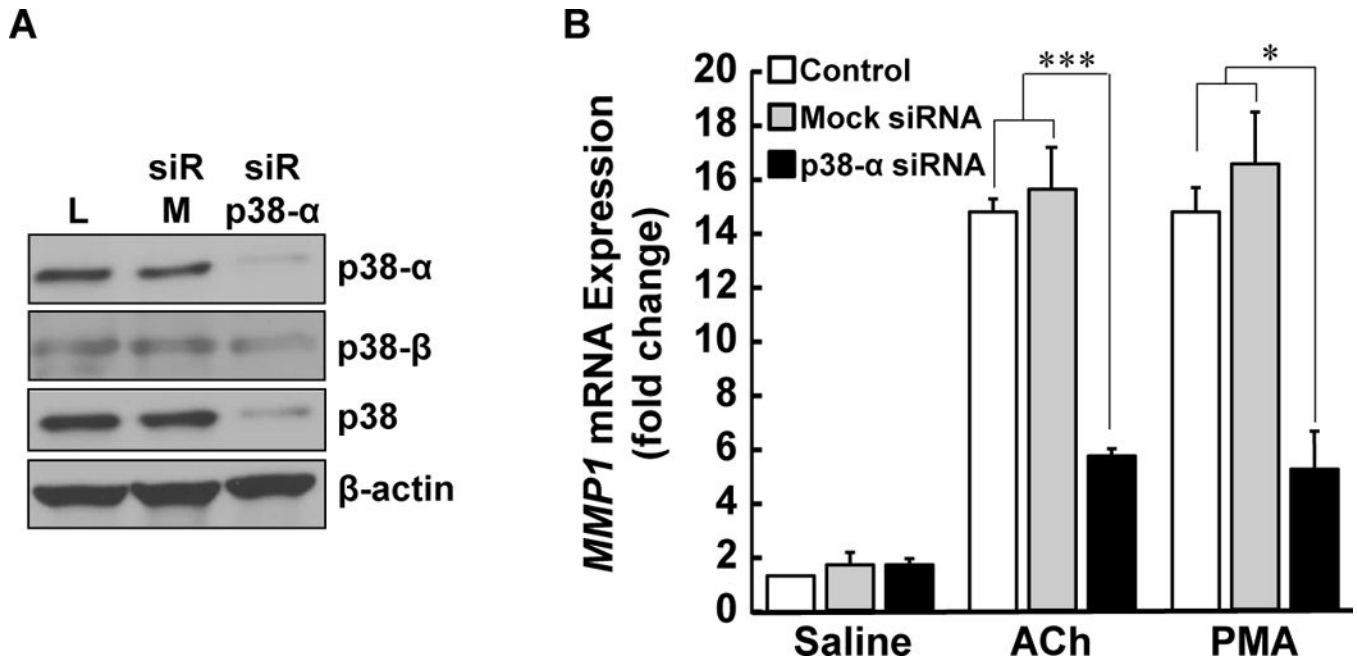
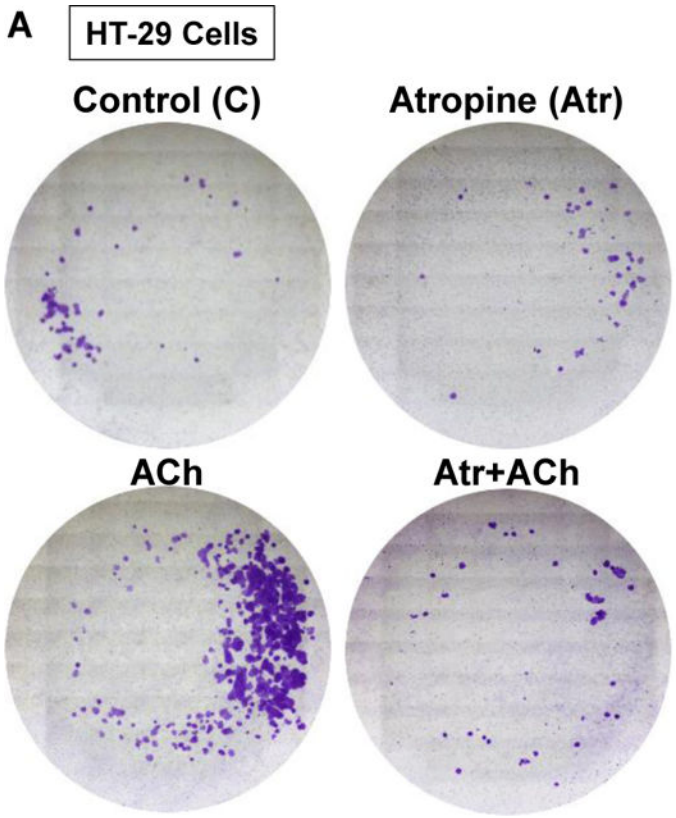
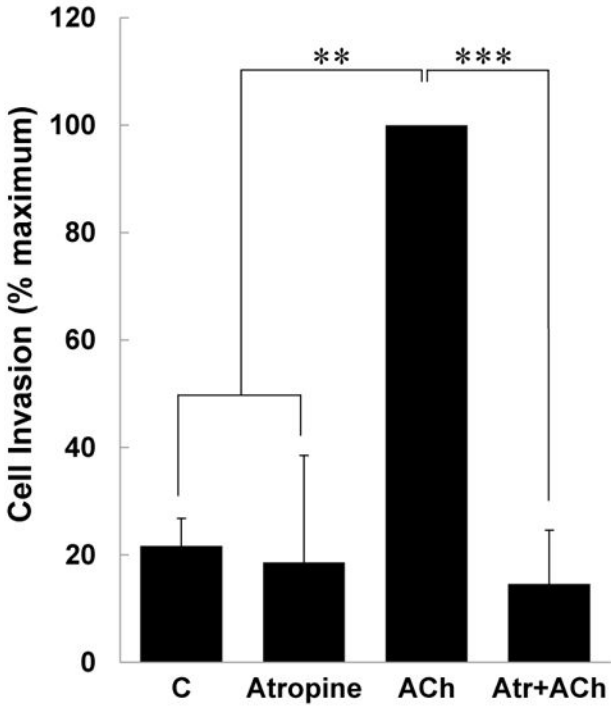
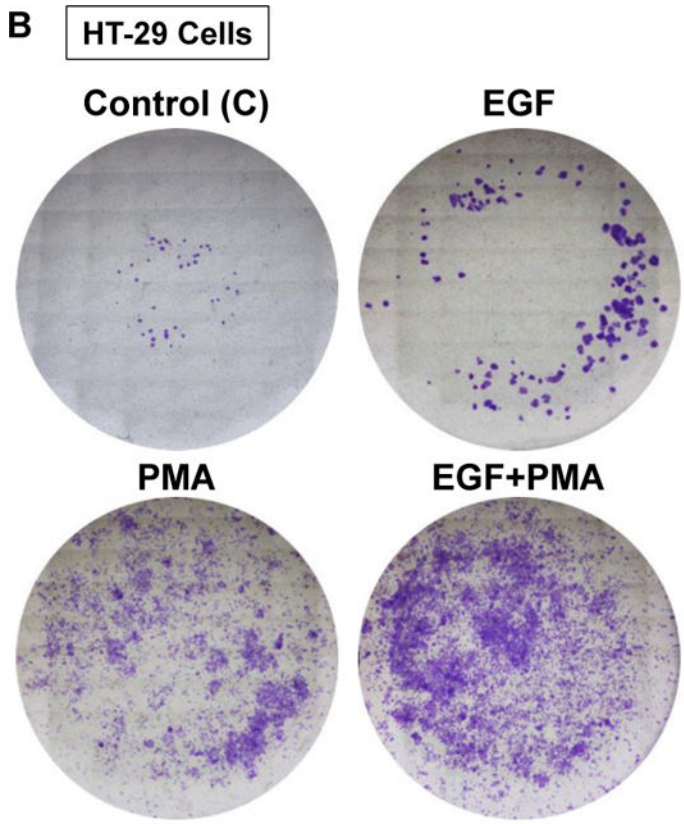


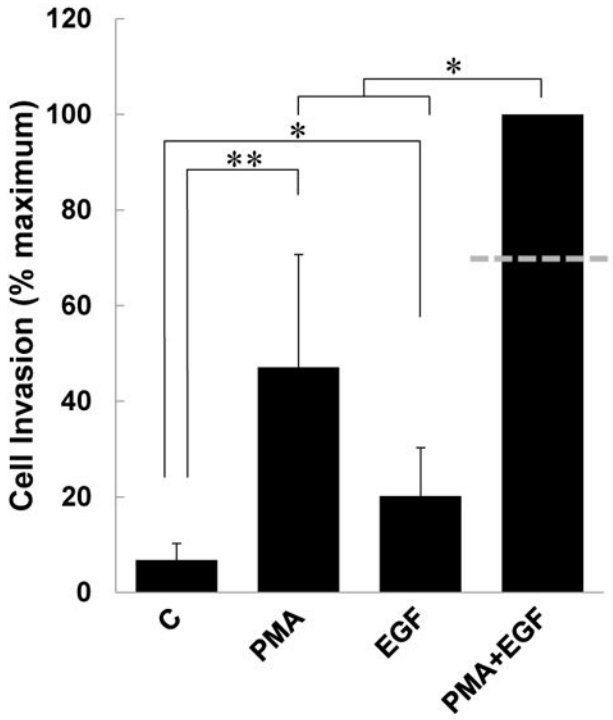
Figure 7. Effects of p38-α knockdown on ACh- and PMA-induced *MMP1* expression

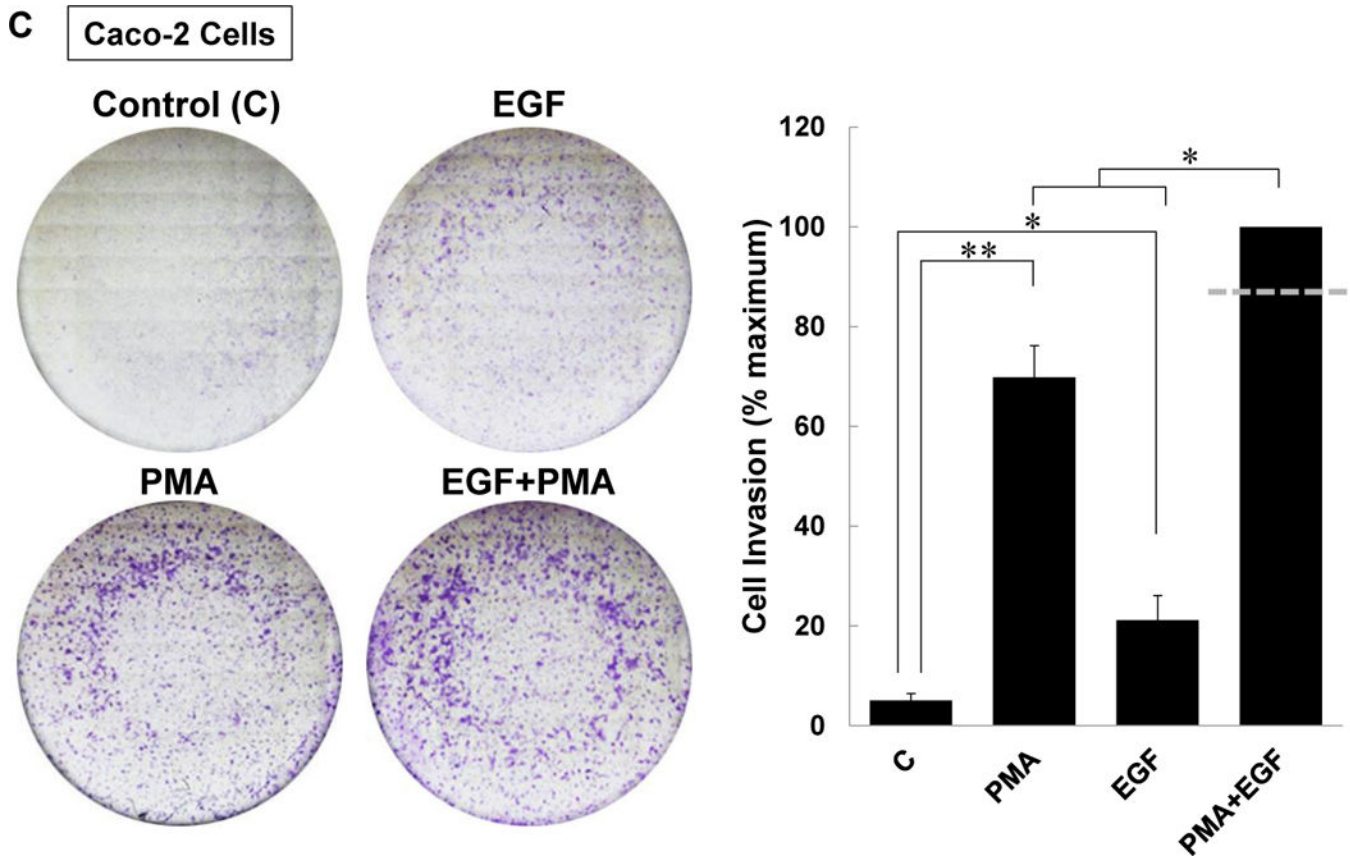
(**A**) HT-29 cells were transfected with Lipofectamine (L), 50 pmoles non-targeting mock siRNA (siR-M), or 50 pmoles siRNA targeting p38-α (siR-p38-α). p38-α, p38-β, total p38, and β-actin expression were measured by immunoblotting with specific antibodies. (**B**) HT-29 cells transfected with Lipofectamine (control), non-targeting mock siRNA, and siRNA targeting p38-α were incubated with saline, 100 μM ACh, or 50 nM PMA for 4 h at 37°C. *MMP1* mRNA levels were measured by qPCR. qPCR data were normalized to GAPDH and are means of four separate experiments. $*P < 0.05$; $***P < 0.001$.











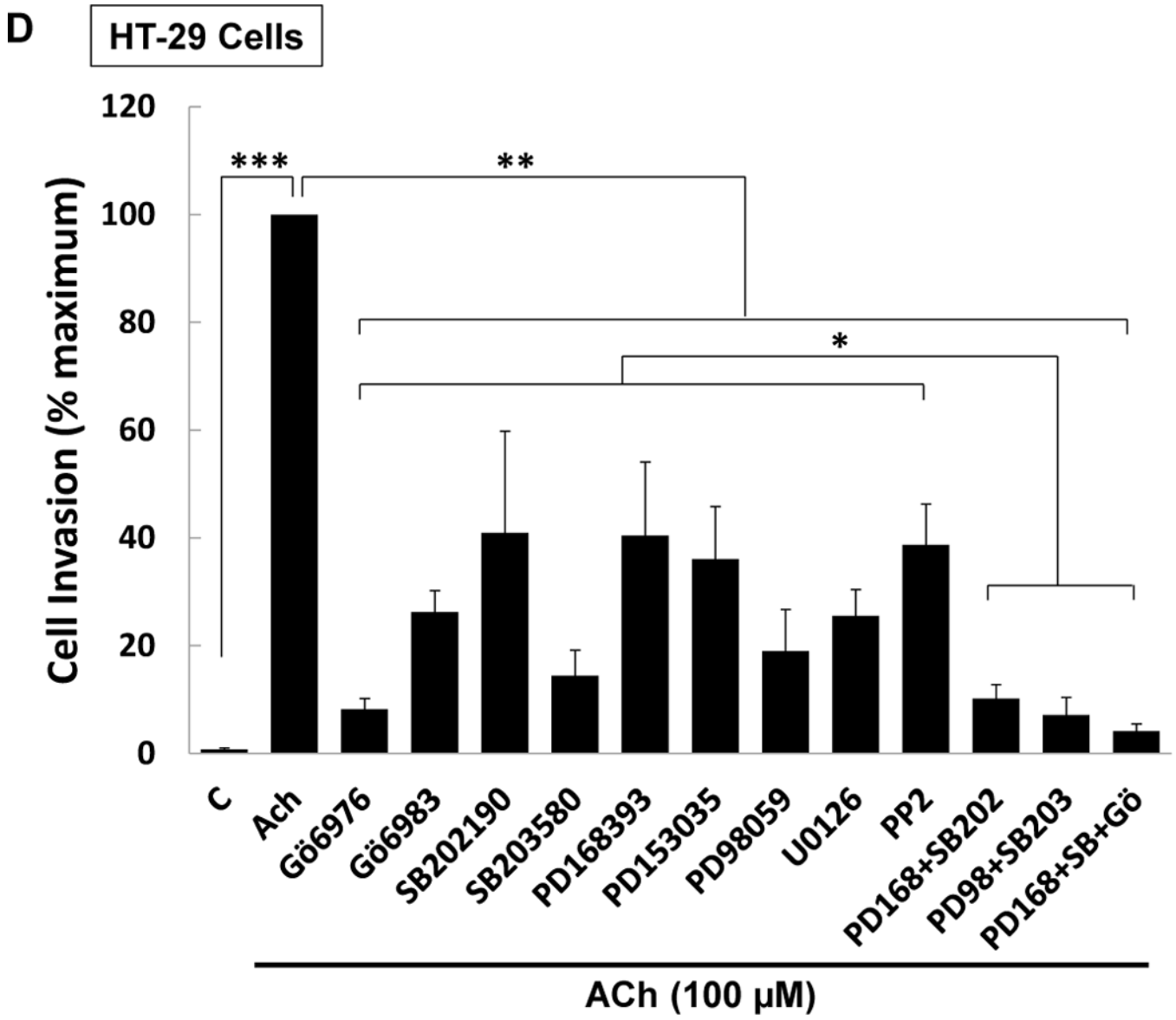


Figure 8. Interactions between PKC and EGFR signaling potentiate colon cancer cell invasion

(**A**) ACh-induced colon cancer cell invasion is blocked by atropine. HT-29 cells were pre-incubated with 5 μM atropine (Atr) for 45 min and then incubated for an additional 48 h with no test agents, or with 100 μM ACh. Atropine alone had no effect but blocked ACh-induced cell invasion. (**B**) Directly activating PKC plus EGFR potentiated colon cancer cell invasion. HT-29 cells were incubated with PMA (50 nM) and EGF (10 ng/ml), alone or in combination, for 48 h. Simultaneous activation of PKC and EGFR significantly potentiates cell invasion; the dashed line represents the calculated additive value for PMA plus EGF ($P < 0.05$ for the observed vs. the calculated addition of PMA plus EGF). (**C**) Caco-2 cells were incubated with PMA (50 nM) and EGF (10 ng/ml), alone or in combination, for 48 h. Simultaneous activation of PKC and EGFR signaling potentiated cell invasion; the dashed line represents the calculated additive value for PMA plus EGF ($P < 0.05$ for the observed vs. the calculated addition of PMA plus EGF). (**D**) ACh-induced colon cancer cell invasion

is blocked by inhibitors of PKC-α/β1 (5 μM Gö6976, 5 μM Gö6983), p38-α/β (10 μM SB202190, 10 μM SB203580), EGFR (5 μM PD168393, 5 μM PD153035), MEK (10 μM PD98059, 10 μM U0126), and Src (10 μM PP2). Pre-incubating cells with a combination of p38-α/β plus EGFR inhibitors or p38-α/β plus MEK inhibitors nearly abolished ACh-induced cell invasion, as did pre-incubation with a combination of EGFR, p38-α/β, and PKC-α/β1 inhibitors. In this set of experiments, cell invasion was measured using BD Biocoat Invasion Chambers with Matrigel inserts. Representative images of crystal violet-stained HT-29 cells that invaded through Matrigel inserts are shown for Fig. 8A–C. Data shown in bar graphs represent mean ± SE of at least three separate experiments. C, untreated control; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

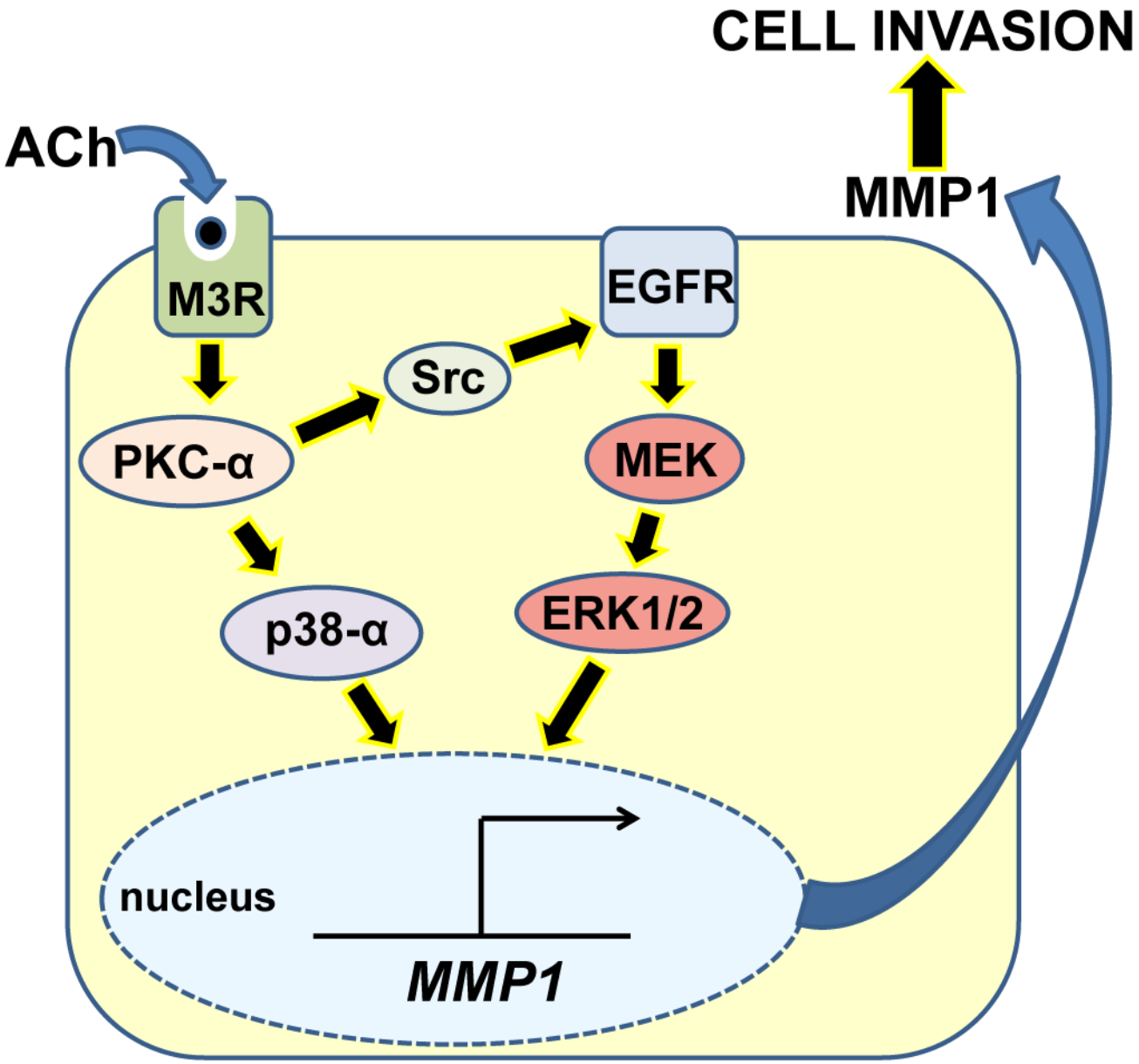


Figure 9. Potentiating interactions between post-M3R signaling pathways govern *MMP1* gene transcription and colon cancer cell invasion

The interaction of a muscarinic receptor agonist (acetylcholine; ACh) with M3 muscarinic receptors triggers downstream PKC-α activation which, in turn, activates Src and p38-α. Src interaction with EGFR stimulates signaling through the MEK/ERK1/2 pathway. Activated p38-α and ERK1/2 coordinately induce *MMP1* gene transcription and enhance MMP1 protein expression. Besides degrading interstitial collagen and facilitating cell invasion, the resulting augmented release of MMP1 into the colon cancer cell microenvironment may catalyze release of EGFR ligands and further enhance ERFR activation. These interactions between post-M3R signaling pathways potentiate MMP1 expression and release, and

culminate in augmented cancer cell invasion, thereby providing potential rescue mechanisms in the event that only one post-M3R signal transduction pathway is blocked.