Small differences in amplitude, duration, and temporal patterns of change in the concentration of free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) can profoundly affect cell physiology, altering programs of gene expression, cell proliferation, secretory activity, and cell survival. We report a novel mechanism for amplitude modulation of [Ca\(^{2+}\)]\(_{i}\), which involves mitogen-activated protein kinase (MAPK). We show that epidermal growth factor (EGF) potentiates gastrin-(1–17) (G17)-stimulated Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores through a MAPK-dependent pathway. G17 activation of the cholecystokinin gastrin receptor (CCK\(_{2}\)R), a G protein-coupled receptor, stimulates release of Ca\(^{2+}\) from inositol 1,4,5-triphosphate-sensitive Ca\(^{2+}\) stores. Pretreating rat intestinal epithelial cells expressing CCK\(_{2}\)R with EGF increased the level of G17-stimulated Ca\(^{2+}\) release from intracellular stores. The stimulatory effect of EGF on CCK\(_{2}\)R-stimulated Ca\(^{2+}\) release requires activation of the MAPK kinase (MEK)1,2/extracellular signal-regulated kinase (ERK)1,2 pathway. Inhibition of the MEK1,2/ERK1,2 pathway by either serum starvation or treatment with selective MEK1,2 inhibitors PD98059 and U0126 or expression of a dominant-negative mutant form of MEK1 decreased the amplitude of the G17-stimulated Ca\(^{2+}\) release response. Activation of the MEK1,2/ERK1,2 pathway either by pretreating cells with EGF or by expression of constitutively active K-ras (K-rasV12G) or MEK1 (MEK1*) increased the amplitude of G17-stimulated Ca\(^{2+}\) release. Although EGF, MEK1*, and K-rasV12G activated the MEK1,2/ERK1,2 pathway, they did not increase [Ca\(^{2+}\)]\(_{i}\), in the absence of G17. These data demonstrate that the activation state of the MEK1,2/ERK1,2 pathway can modulate the amplitude of the CCK\(_{2}\)R-mediated Ca\(^{2+}\) release response and identify a novel mechanism for cross-talk between EGF receptor- and CCK\(_{2}\)R-regulated signaling pathways.

The gastrointestinal peptide hormone gastrin-(1–17) (G17) plays an essential role in the regulation of digestion by stimulating gastric acid secretion, histamine synthesis and release, and proliferation of the gastric epithelium and endocrine pancreas (1, 2). In cancers of the stomach, pancreas, and colon, G17 promotes tumor cell proliferation, motility, and invasion (3–5). The biological effects of G17 are mediated by the cholecystokinin-2 (CCK\(_{2}\)/G17 receptor (CCK\(_{2}\)R) (previously named CCK-B receptor), a member of the G protein-coupled receptor superfamily. Three splice variants of the CCK\(_{2}\)R have been identified (6–8) that bind the structurally related peptides cholecystokinin (CCK) and G17 with high affinities. An early event following agonist activation of CCK\(_{2}\)R is the phospholipase C\(_{i}\)-mediated elaboration of inositol 1,4,5-triphosphate (6) from membrane phospholipids and the subsequent release of calcium (Ca\(^{2+}\)) from inositol 1,4,5-triphosphate-sensitive intracellular Ca\(^{2+}\) stores.

Calcium is an essential intracellular signal involved in many biological processes including fertilization, secretion, contraction, proliferation, differentiation, and apoptosis (9, 10). Small differences in the amplitude, duration, and/or temporal pattern of change in [Ca\(^{2+}\)]\(_{i}\), can have profound effects on cell physiology, altering programs of gene expression (11, 12), secretory activity (13), cell proliferation, and survival (14–16). Calcium is an essential signaling molecule in G17-stimulated cell proliferation. In Chinese hamster ovary cells expressing recombinant CCK\(_{2}\)R, an agonist-induced increase in mitogen-activated protein kinase (MAPK) activity and \(^{3}H\)thymidine incorporation into DNA requires a slow oscillatory increase in [Ca\(^{2+}\)]\(_{i}\), (17). In the rat pancreatic cancer cell line AR4-2J, a CCK\(_{2}\)R-mediated increase in [Ca\(^{2+}\)]\(_{i}\), is required for formation of the Shc-Grb2-Sos protein complex and subsequent activation of MAPK (18). Defining the molecular mechanisms involved in CCK\(_{2}\)R regulation of [Ca\(^{2+}\)]\(_{i}\), is necessary to understand the proliferative effects of G17 on normal and neoplastic cells.

Like G17, epidermal growth factor (EGF) also stimulates the proliferation of gastric epithelial cells (19, 20), and recently, the G17-related peptide CCK has been shown to synergize with EGF to stimulate DNA synthesis (\(^{3}H\)thymidine incorporation), cyclin-D3 expression, and retinoblastoma protein phosphorylation in cells expressing both CCK\(_{2}\)R and EGF receptors (21). EGF and the related growth factors transforming growth factor-\(\beta\) and amphiregulin bind to a family of receptors known as type I receptor tyrosine kinases. This family is composed of four related receptors: the EGF receptor (EGFR/ErB1/HER1), ErB2 (HER2/neu), ErB3 (HER3), and ErB4 (HER4) (22).
The synergistic effect of CCK and EGF on regulation of cell cycle progression indicates cross-talk between CCKR- and EGF-regulated signaling pathways; however, the point at which their signal transduction pathways converge has not been identified. Because a G17-induced increase in [Ca\(^{2+}\)]_i is one of the initial events in CCKR-regulated signal transduction, the aim of this study was to determine the effect of EGF treatment on CCKR regulation of [Ca\(^{2+}\)]_i. We report that EGF affects this very early step in CCKR-mediated signal transduction by potentiating G17-stimulated Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores. The potentiating effect of EGF is mediated by the MEK1/2/ERK1,2 pathway. This study identifies a novel mechanism by which changes in the basal activation state of the MEK1,2/ERK1,2 pathway regulate the amplitude of the CCKR-mediated Ca\(^{2+}\) release response.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—For most experiments, we used a nontransformed rat intestinal epithelial (RIE) cell line expressing recombinant human CCK-R called RIE/RCK-R. These cells were routinely cultured in DMEM supplemented with 400 μg/ml G418 and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C. Other cell lines used included the rat pancreatic cancer cell line AR4-25, the human prostate cancer cell line PC-3, and the human pancreatic carcinoma cell line BON. AR4-25 cells were cultured in DMEM supplemented with 10% FBS. PC-3 cells were cultured as recommended by the American Type Culture Collection (Manassas, VA). BON cells were cultured as described previously (23).

**Intracellular Ca\(^{2+}\) Imaging**—RIE/RCK-R cells were cultured on 25-mm diameter coverslips in DMEM supplemented with 10% FBS. To load the cells with the Ca\(^{2+}\) indicator dye Fura-2, they were first washed with a physiological medium (KRH) containing NaCl (125 mM), KCl (5 mM), KH\(_2\)PO\(_4\) (1.2 mM), MgSO\(_4\) (1.2 mM), CaCl\(_2\) (2 mM), glucose (6 mM), HEPES (25 mM), pH 7.4, and then they were incubated with 2 μM Fura-2/AM (Molecular Probes, Eugene, OR) for 50 min at room temperature. Single cell changes in [Ca\(^{2+}\)]_i were recorded using a Nikon Diaphot inverted microscope (Garden City, NY) and a CCD camera (Dage-MTI, Inc., Michigan City, IN). Data points were collected every 1–8 s from ~35 cells/coverslip and processed using ImageMaster software. Fluorescent ratios were converted into [Ca\(^{2+}\)]_i using the equation (Ca\(^{2+}\)_i) = K_d ([R - R_blank][R_max - R])/([R - R_blank]), as reported previously (48). Maximal (R_max) and minimal (R_blank) values were determined following the addition of either 5 μM ionomycin or 5 mM EGTA, respectively. Analysis of statistical significance was performed using Student’s unpaired t test. For multiple comparisons, one-way analysis of variance combined with the Tukey’s post hoc test was used (GraphPad Software, San Diego, CA). Values are presented as the mean ± S.E. and are considered significant at p < 0.05.

**Competition Binding**—RIE/RCK-R cells were plated into 24-well plates in DMEM supplemented with 10% FBS. After 2 days, cells were cultured in DMEM without FBS for an additional 24 h. Following a 5-min pretreatment with either EGF (1 ng/ml) or vehicle (H\(_2\)O), the cells were washed with binding buffer (DMEM, 25 mM HEPES, 0.1% bovine serum albumin). After washing, the cells were lysed with 300 μl of 1 M NaOH and transferred to a glass tube. The amount of bound radioactivity was measured in a Cobra II gamma counter (Packard Instrument Company, Downers Grove, IL). Total binding averaged ~6% of the total counts added to the assays. Nonspecific binding was defined as the amount of radiolabeled CCK-8 bound in the presence of 1 μM unlabeled CCK-8.

**Western Blotting**—RIE/RCK-R cells were plated into 12-well plates at a density of ~100,000 cells/well in DMEM supplemented with 10% FBS. After 2 days, cells were incubated with DMEM ± FBS for 24 h, treated as described in the figure legends, washed with ice-cold phosphate-buffered saline, and solubilized in lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EGTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100 at 4 °C. Protein concentrations of the supernatant were determined using the Bio-Rad DC protein assay kit. Protein (10 μg) from each sample was resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with an antibody to the dual phosphorylated forms of ERK1 and ERK2 (Ab-Active® MAPK, Cat. No. V9031, Promega, Madison, WI). Immunoreactive proteins were visualized using the ECL Western blotting detection system (Amersham Biosciences). The total ERK content within the cell extracts was determined using an antibody that recognizes both active and inactive forms of ERK1 and ERK2 (Cat. No. SC-94, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

**Transient Transfection**—RIE/RCK-R cells were grown on coverslips in 6-well plates. After 24 h, cells were transiently co-transfected with 1 μg of one of several mutant expression constructs (constitutively active MEK1 (MEK1+), dominant-negative MEK1 (dnMEK1) (Upstate Biotechnology, Placid, NY), and K-rasV12G (Dr. Aubrey Thompson, University of Texas Medical Branch, Galveston, TX)) and 0.1 μg of plasmid containing the cDNA for green fluorescence protein (pEGFP-C1, Clontech, Palo Alto, CA) using LipofectAMINE Plus reagent (Invitrogen). Twenty-four h post-transfection, the G17-stimulated Ca\(^{2+}\) response was measured in the EGFP-positive cells as described above. Transfection efficiency ranged from 10 to 20% of the cells and was assessed by counting the number of EGFP-positive cells/total cells in a microscopic field using a ×40 objective. Typically, 6–8 EGFP-positive cells were observed/field. Intracellular Ca\(^{2+}\) measurements were recorded from 32 to 48 EGFP-positive cells/experiment.

**RESULTS**

**EGF Potentiates G17-stimulated Increases in [Ca\(^{2+}\)]_i**—The binding of G17 to CCKR induced a rapid and transient increase in [Ca\(^{2+}\)]_i, in RIE/RCK-R cells (Fig. 1A). To assess the effects of EGF on the G17-stimulated increase in [Ca\(^{2+}\)]_i, cells were cultured in medium without serum for 24 h. Under these conditions, the amplitude of the G17-induced Ca\(^{2+}\) response decreased by up to 50% (Fig. 1A). However, pretreating the serum-starved cells with EGF (1 ng/ml) reversed the inhibitory effects of serum starvation and increased the amplitude of the G17-stimulated Ca\(^{2+}\) response in a time-dependent manner (Fig. 1B). Simultaneous addition of EGF and G17 (10 nM) had no effect on [Ca\(^{2+}\)]_i when compared with cells treated with G17 alone; however, pretreating cells for 5, 15, or 30 min with EGF significantly increased the amplitude of the G17-induced Ca\(^{2+}\) response when compared with serum-starved cells (Fig. 1B). Five min of pretreatment with EGF was sufficient to increase the amplitude of the G17-stimulated increase in [Ca\(^{2+}\)]_i, to the level observed in cells continuously cultured in 10% FBS (Fig. 1B).

To assess whether the effect of EGF on G17-stimulated increases in [Ca\(^{2+}\)]_i, was mediated by the EGF receptor, we pretreated cells with the EGF receptor tyrosine kinase inhibitor AG1478. Pretreatment with AG1478 (200 nM) had no effect on G17-stimulated increases in [Ca\(^{2+}\)]_i, when used alone but completely blocked the potentiation effect of EGF on the G17-stimulated Ca\(^{2+}\) response (Fig. 1B). These data indicate that EGF potentiation of the G17-stimulated increases in [Ca\(^{2+}\)]_i, required EGF activation. Because EGF can activate phospholipase-γ, resulting in the production of inositol 1,4,5-triphosphate and the release of Ca\(^{2+}\) from intracellular stores (24), we assessed whether the potentiation effect of EGF on the G17-stimulated Ca\(^{2+}\) response was due to direct regulation of [Ca\(^{2+}\)]_i. Pretreating cells with EGF, up to a concentration of 25 ng/ml, had no effect on [Ca\(^{2+}\)]_i, in RIE/RCK-R cells (Fig. 1C), indicating that its potentiation of the G17-stimulated Ca\(^{2+}\) response was not due to parallel regulation of [Ca\(^{2+}\)]_i.

Pretreatment of RIE/RCK-R cells with EGF enhanced the efficacy of the G17-stimulated Ca\(^{2+}\) response but not the sensitivity. Pretreating serum-starved cells with EGF (1 ng/ml) for 5 min significantly increased the amplitude (efficacy) of the Ca\(^{2+}\) response induced by G17 over a broad range of concentrations from 0.01 to 100 nM (Fig. 2A). However, when the Ca\(^{2+}\) data was normalized to a percentage of maximum response there was no effect of EGF pretreatment on the EC\(_{50}\) value of
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is the mitogen-activated protein kinases. MAPks are an evolutionarily conserved family of serine-threonine-directed kinases. Five subfamilies of MAPks have been identified, which include ERK1 and ERK2, the c-Jun N-terminal kinases, the 38-kDa MAPks, ERK5, and ERK-3s (25). The activities of MAPks are regulated by upstream MAPK kinases, which are dual-specificity kinases that activate MAPks by phosphorylating both the tyrosine and threonine residues present in the consensus sequence (TXY). Six MAPK kinase family members have been identified and are designated MEK1 through MEK5 and ERK-3 kinase. In many cell types, EGFR is coupled to the MEK1/2/ERK1,2 MAPK pathway through the Ras family of small GTP-binding proteins (26).

Serum starvation reduces the activity of the MEK1/2/ERK1,2 pathway; therefore, we hypothesized that this pathway may mediate the stimulatory effects of EGF on G17-stimulated increases in $[Ca^{2+}]_i$. To test this hypothesis, we first assessed the time and dose dependence of EGF treatment on the level of phosphorylated (activated) ERK1 (pERK1) and ERK2 (pERK2) in serum-starved RIE/CCK$_2$R cells. EGF (1 ng/ml) induced a time- and dose-dependent increase in the levels of pERK1 and pERK2 (Fig. 3). A long exposure film revealed an increase over baseline levels of pERK1 and pERK2 as early as 2 min after EGF treatment (Fig. 3A). A shorter exposure of an extended time course showed that the levels of pERK1 and pERK2 further increased at 10 and 20 min and began to decrease at 30 min (Fig. 3B). A dose-response analysis at 5 min showed a dose-dependent increase in the levels of pERK1 and pERK2 (Fig. 3C). When compared with untreated control cultures, an increase in the levels of pERK1 and pERK2 was detected in cells treated with as little as 0.1 ng/ml EGF for 5 min. Maximum levels of pERK1 and pERK2 were observed in cells treated with 5 ng/ml EGF for 5 min. An assessment of the effects of EGF on G17-stimulated increases in $[Ca^{2+}]_i$, revealed a good correspondence between both the time (Fig. 1B) and dose effects of EGF on ERK activation (Fig. 3C) and its potentiation of the G17-stimulated increase in $[Ca^{2+}]_i$, (Fig. 3D).

Together, these data suggest that EGF can increase the amplitude of the G17-stimulated $Ca^{2+}$ response by increasing the activation state of the MEK1/2/ERK1,2 pathway. To further assess the role of the MEK1/2/ERK1,2 pathway in CCK$_2$R regulation of $[Ca^{2+}]_i$, we next determined the effects of modulating the activities of MEK1 and MEK2 on G17-induced increases in $[Ca^{2+}]_i$.

**Inhibition of MEK1 and MEK2 Blocked the Potentiating Effects of Serum and EGF on G17-stimulated $Ca^{2+}$ Release—** Agonist binding to CCK$_R$ induces an increase in $[Ca^{2+}]_i$ that involves both the inositol 1,4,5-trisphosphate-mediated release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores and the influx of extracellular $Ca^{2+}$ across the plasma membrane. The total increase in $[Ca^{2+}]_i$, induced by G17 is the sum of $Ca^{2+}$ from these two sources. Because a G17-stimulated release of $Ca^{2+}$ from intracellular stores is required for $Ca^{2+}$ influx across the plasma membrane, we assessed the effects of MEK1,2 inhibitors on G17-stimulated $Ca^{2+}$ release from internal stores. The amount of $Ca^{2+}$ released from intracellular stores can be determined experimentally by bathing the cells in an extracellular solution without added $Ca^{2+}$ and containing the $Ca^{2+}$-chelating agent EGTA. Under these conditions, the change in $[Ca^{2+}]_i$, induced by G17 stimulation is due to the release of $Ca^{2+}$ from intracellular stores alone. To determine whether the activities of MEK1 and MEK2 were required for G17-stimulated $Ca^{2+}$ release, RIE/CCK$_2$R cells cultured in 10% FBS were pretreated for 5 min with different concentrations of the MEK inhibitor PD98059. Pretreatment of cells with PD98059 caused a dose-dependent decrease in the amplitude of the G17-stimulated...
Ca\textsuperscript{2+} release response (Fig. 4A). A concentration of 1 µM PD98059 reduced the amplitude of the change in [Ca\textsuperscript{2+}] \textsubscript{i} by 28% (from 310 ± 12.1 nM to 221 ± 12.3 nM) (Fig. 4B). Treatment with 10 µM PD98059 resulted in a 93% reduction in [Ca\textsuperscript{2+}] \textsubscript{i}. Western blot analysis showed detectable levels of pERK1 and pERK2 in cells cultured in 10% FBS but not in cells pretreated with 10 µM PD98059 for 5 min (Fig. 4B, inset). A similar dose-dependent inhibition of the G17-stimulated Ca\textsuperscript{2+} release was also observed in cells treated with another inhibitor of MEK1 and MEK2, U0126 (data not shown).

The specificity of the chemical inhibitors for MEK1 was confirmed by transiently transfecting cells with a dominant-negative (kinase-dead) mutant of MEK1. To identify transfected cells, we co-transfected cells with an expression vector containing the cDNA for EGFP. G17-stimulated Ca\textsuperscript{2+} release was measured in EGFP-positive cells. When compared with EGFP-positive cells co-transfected with empty vector, EGFP-positive cells co-transfected with dnMEK1 showed a significant increase in [Ca\textsuperscript{2+}] \textsubscript{i} due to a decrease in the amplitude of G17-induced Ca\textsuperscript{2+} release (Fig. 4C). The peak change in [Ca\textsuperscript{2+}] \textsubscript{i} decreased from 221 ± 24.4 nM (empty vector control) to 38.3 ± 12.2 nM (dnMEK1-transfected) (Fig. 4D). Although both the chemical inhibitors and forced expression of dnMEK1 reduced the peak levels of agonist-stimulated Ca\textsuperscript{2+} release, neither PD98059 treatment nor dnMEK expression affected base-line [Ca\textsuperscript{2+}] \textsubscript{i}, which is ~100 nM. Together, these data
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**Fig. 4.** Effects of MEK inhibition on G17-stimulated \([\text{Ca}^{2+}]_i\) release from intracellular stores. A, dose-dependent effect of the MEK inhibitor PD98059 on G17-stimulated \([\text{Ca}^{2+}]_i\) release. RIE/CCK-R cells cultured in 10% FBS were pretreated for 5 min with different concentrations of PD98059 (1, 5, and 10 μM) and stimulated with G17 (10 nM). Control cells were pretreated with MeSO. For each tracing the data represent the average \([\text{Ca}^{2+}]_i\) ± S.E. (n = 40 cells). The arrow indicates when G17 was added to the cells, and the black bar indicates the length of time the cells were exposed to G17. B, summary data from three separate experiments showing the effect of PD98059 (hatched bars) on G17-stimulated \([\text{Ca}^{2+}]_i\) release. Inset, immunoblot showing the levels of pERK1 and pERK2 before (0) and after 5-min treatment with 10 μM PD98059 (10). C, effects of dominant-negative MEK1 expression on G17-stimulated \([\text{Ca}^{2+}]_i\)_release. D, summary data showing the inhibitory effect of dnMEK1 (hatched bar) expression on G17-stimulated \([\text{Ca}^{2+}]_i\)_release. Data are expressed as the average change (Δ) in \([\text{Ca}^{2+}]_i\) ± S.E. (*, p < 0.05; dnMEK1-transfected versus vector-transfected control (filled bar)). E, Western blot showing that pretreatment with PD98059 blocked EGF-stimulated increases in the levels of pERK1 and pERK2. F, PD98059 pretreatment blocked the effects of EGF on G17-stimulated \([\text{Ca}^{2+}]_i\)_release. †, p < 0.05; serum-starved (open bar) versus PD98059. ‡, p < 0.05; EGF versus EGF + PD98059.

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Figure 4. Effects of MEK inhibition on G17-stimulated \([\text{Ca}^{2+}]_i\) release from intracellular stores. A, dose-dependent effect of the MEK inhibitor PD98059 on G17-stimulated \([\text{Ca}^{2+}]_i\) release. RIE/CCK-R cells cultured in 10% FBS were pretreated for 5 min with different concentrations of PD98059 (1, 5, and 10 μM) and stimulated with G17 (10 nM). Control cells were pretreated with MeSO. For each tracing the data represent the average \([\text{Ca}^{2+}]_i\) ± S.E. (n = 40 cells). The arrow indicates when G17 was added to the cells, and the black bar indicates the length of time the cells were exposed to G17. B, summary data from three separate experiments showing the effect of PD98059 (hatched bars) on G17-stimulated \([\text{Ca}^{2+}]_i\) release. Inset, immunoblot showing the levels of pERK1 and pERK2 before (0) and after 5-min treatment with 10 μM PD98059 (10). C, effects of dominant-negative MEK1 expression on G17-stimulated \([\text{Ca}^{2+}]_i\) release. D, summary data showing the inhibitory effect of dnMEK1 (hatched bar) expression on G17-stimulated \([\text{Ca}^{2+}]_i\) release. Data are expressed as the average change (Δ) in \([\text{Ca}^{2+}]_i\) ± S.E. (*, p < 0.05; dnMEK1-transfected versus vector-transfected control (filled bar)). E, Western blot showing that pretreatment with PD98059 blocked EGF-stimulated increases in the levels of pERK1 and pERK2. F, PD98059 pretreatment blocked the effects of EGF on G17-stimulated \([\text{Ca}^{2+}]_i\) release. †, p < 0.05; serum-starved (open bar) versus PD98059. ‡, p < 0.05; EGF versus EGF + PD98059.

Demonstrate that inhibition of MEK activity is sufficient to reduce the amplitude of the G17-stimulated \([\text{Ca}^{2+}]_i\) release from intracellular stores.

To determine whether the MEK1,2/ERK1,2 pathway mediates the effect of EGF on CCK-R-regulated \([\text{Ca}^{2+}]_i\), we assessed the effects of PD98059 treatment on EGF-induced potentiation of G17-stimulated \([\text{Ca}^{2+}]_i\) release from intracellular stores. First, we determined the effects of PD98059 on the levels of pERK1 and pERK2 in serum-starved RIE/CCK-R cells. A long exposure film showed low levels of pERK1 and pERK2 in serum-starved cells compared with cells cultured in 10% FBS (Fig. 4E, lanes 2 and 1, respectively). Pretreating the cells with PD98059 (10 μM for 5 min) reduced pERK1 and pERK2 to undetectable levels (Fig. 4E, lane 3). Treatment of serum-starved cells with EGF (1 ng/ml for 5 min) stimulated an increase in the levels of pERK1 and pERK2, which was completely blocked by PD98059 (10 μM) (Fig. 4E, lanes 5 and 4, respectively). Analysis of G17-stimulated \([\text{Ca}^{2+}]_i\) responses from cells treated the same way showed a good correspondence between the relative levels of pERK1 and pERK2 and the amplitude of the G17-stimulated \([\text{Ca}^{2+}]_i\) release response. PD98059 treatment decreased both basal and EGF-stimulated pERK levels in serum-starved cells and also reduced basal and EGF-enhanced G17-stimulated \([\text{Ca}^{2+}]_i\) release (Fig. 4F).

**Neither G17-stimulated Activation of the MEK1,2/ERK1,2 Pathway nor EGF Activation of the Phosphatidylinositol 3-Kinase Pathway Is Involved in the Potentiation of CCK-R-mediated \([\text{Ca}^{2+}]_i\) Response—**It is well established that in addition to regulation of \([\text{Ca}^{2+}]_i\), CCK-R is coupled to the MEK1,2/ERK1,2 pathway. We reported previously G17-stimulated increases in ERK activation in RIE/CCK-R cells (27). To determine whether G17 stimulation of the MEK1,2/ERK1,2 pathway plays a role in CCK-R-mediated \([\text{Ca}^{2+}]_i\) release, we compared the time courses of G17-stimulated \([\text{Ca}^{2+}]_i\) release and ERK activation (Fig. 5). We found that the G17-stimulated increase in \([\text{Ca}^{2+}]_i\) release from intracellular stores preceded a detectable increase in the levels of G17-stimulated ERK activation, suggesting that CCK-R-mediated activation of the MEK1,2/ERK1,2 pathway is not responsible for the potentiation of the G17-induced \([\text{Ca}^{2+}]_i\) release response.

EGF can activate other pathways in addition to the MEK1,2/ERK1,2 pathway, including the phosphatidylinositol 3-kinase pathway (22). To assess the possible involvement of the phosphatidylinositol 3-kinase pathway in EGF potentiation of G17-stimulated \([\text{Ca}^{2+}]_i\) release, we pretreated cells cultured in 10% FBS with two commonly used inhibitors of the phosphatidylinositol 3-kinase pathway, wortmannin and LY294002. Unlike the MEK inhibitors, which blocked the potentiation effect of
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**Fig. 5.** Time courses of G17-stimulated $\text{Ca}^{2+}$ release from intracellular stores and increase in pERK1 and pERK2 levels. A, the left axis shows the change in $[\text{Ca}^{2+}]_i$. The right axis shows the level of pERK1.2. B, long exposure of a Western blot showing levels of pERK1 and pERK2 at early time points.

EGF on G17-stimulated $\text{Ca}^{2+}$ release, neither wortmannin (100 nM) nor LY294002 (10 $\mu$M) affected EGF potentiation of G17-stimulated $\text{Ca}^{2+}$ release (Fig. 6). Wortmannin and LY294002 also had no effect on G17-stimulated $\text{Ca}^{2+}$ release in the absence of EGF (Fig. 6). Together, the data support the conclusion that EGF potentiates CCK$_2$R-mediated $\text{Ca}^{2+}$ release from intracellular stores by increasing the activation state of the MEK1,2/ERK1,2 pathway. Furthermore, the inhibitory effect of PD98059 on G17-stimulated $\text{Ca}^{2+}$ release in the absence of EGF suggests that altering the basal activation state of the MEK1,2/ERK1,2 pathway may be sufficient to modulate the efficacy of the G17-stimulated $\text{Ca}^{2+}$ release response.

**Fig. 6.** Effects of phosphatidylinositol 3-kinase inhibitor on G17-stimulated $\text{Ca}^{2+}$ release. The filled bar shows G17-stimulated $\text{Ca}^{2+}$ response from the 10% serum control, and the open bar shows the serum-starved control. The hatched bars show treatment groups.

**Fig. 7.** Effects of expression of constitutively active K-rasV12G and MEK1* on G17-stimulated $\text{Ca}^{2+}$ release. RIE/CCK$_2$R cells were transiently co-transfected with pEGFP (0.1 $\mu$g) and constitutively active K-rasV12G (1 $\mu$g), MEK1* (1 $\mu$g), or an empty control vector (1 $\mu$g). Following a 24-h incubation in medium ± 10% FBS, the G17-stimulated change in $[\text{Ca}^{2+}]_i$, was recorded from EGFP-positive cells. A, comparison of the effects of K-rasV12G expression on G17-stimulated $\text{Ca}^{2+}$ release in cells cultured in DMEM ± 10% serum. Data represent the average change ($\Delta$) in $[\text{Ca}^{2+}]_i$ ± S.E. (*, $p < 0.05$; serum-starved K-rasV12G-transfected (right-hand hatched bar) versus serum-starved vector-transfected control (open bar)). B, comparison of the effects of MEK1* expression on G17-stimulated $\text{Ca}^{2+}$ release in cell cultures in DMEM ± 10% serum. Data represent the average change ($\Delta$) in $[\text{Ca}^{2+}]_i$ ± S.E. (*, $p < 0.05$; serum-starved MEK1*-transfected (right-hand hatched bar) versus serum-starved vector-transfected control (open bar)).

Activation of the MEK1,2/ERK1,2 Pathway Is Sufficient to Potentiate G17-stimulated $\text{Ca}^{2+}$ Release—EGF activates the MEK1,2/ERK1,2 pathway through the small GTP-binding protein Ras. To assess whether a constitutively active Ras could substitute for EGF and reverse the effects of serum starvation on G17-stimulated $\text{Ca}^{2+}$ release, RIE/CCK$_2$R cells were transiently transfected with a GTPase-deficient mutant of K-ras (K-rasV12G). When cells were serum-starved for 24 h to down-regulate the MEK1,2/ERK1,2 pathway, expression of K-rasV12G significantly increased the peak amplitude of the G17-stimulated $\text{Ca}^{2+}$ response when compared with serum-starved cells transfected with the empty control vector (280 ± 13.2 nM (K-rasV12G) versus 119 ± 18.2 nM (vector control)) (Fig. 7A).

Ras protein activates Raf family kinases, which in turn activate MEK1 and MEK2. To determine whether increasing the activity of MEK1 was sufficient to prevent the inhibitory effects of serum starvation on G17-induced $\text{Ca}^{2+}$ release, we transfected cells with a constitutively active mutant of MEK1. When compared with cells transfected with the empty control vector, there was no significant effect of MEK1* expression on the level of G17-induced $\text{Ca}^{2+}$ release when cells were cultured in 10% FBS (Fig. 7B). However, a comparison of serum-starved cells expressing the control vector to serum-starved cells expressing MEK1* showed a significant increase in the amplitude of the G17-stimulated $\text{Ca}^{2+}$ response in cells expressing MEK1* (106 ± 33.0 nM (vector control) versus 237 ± 14.0 nM (MEK1*)) (Fig. 7B), demonstrating that MEK1* expression would be necessary to potentiate G17-stimulated $\text{Ca}^{2+}$ release.
mone-stimulated Ca\(^{2+}\) release. To assess the role of the \(G_{i}\) pathway, we measured calcium release from intracellular stores through a MEK1,2/ERK1,2-dependent pathway. Furthermore, we demonstrate that modulation of the basal activation state of the MEK1,2/ERK1,2 pathway regulates a common component in the receptor-mediated Ca\(^{2+}\) release.

**DISCUSSION**

Simple linear models of cell surface receptor-mediated signal transduction have been replaced by complex signaling networks, involving cross-talk between receptor tyrosine kinase-regulated pathways, such as EGFR- and G protein-coupled receptor (GPCR)-regulated pathways. GPCRs can transactivate receptor tyrosine kinase-regulated signaling pathways through both intra- and extracellular mechanisms (19, 28, 29). The transactivation of receptor tyrosine kinase signaling pathways has helped to explain the cell proliferative effects associated with some GPCR agonists (30–32). The proliferative effects of the GPCR agonist G17 have been implicated in a variety of normal and abnormal biological processes, including maintenance of the gastric mucosa, proliferation of ECL cells, and neoplastic transformation (33). Administration of G17 stimulates the growth of human colon cancer cells in culture (34, 35) and human tumors xenographed into nude mice (35).

Additionally, G17 and its related peptide CCK stimulate the growth of stomach (36, 37) and pancreatic cancers (38). EGF receptors and their ligands are also overexpressed and regulate the growth of the same cancers (39, 40), suggesting the possibility for cross-talk between EGFR- and CCKR-regulated pathways in these cancers. This hypothesis is supported by the recent findings that CCK and EGF synergically stimulate DNA synthesis, cyclin-D3 expression, and retinoblastoma phospho-

was sufficient to reverse the inhibitory effects of serum starvation on G17-stimulated Ca\(^{2+}\) release.

**The MEK1,2/ERK1,2 Pathway Modulates the Ca\(^{2+}\) Release Response to Other GI Peptide Hormones**—In addition to CCKR, other GI peptide hormone receptors, such as the gastrin-releasing peptide receptor, which binds members of the bombesin family of peptides, and the neurotensin receptor, are coupled to the regulation of intracellular Ca\(^{2+}\) through an inositol 1,4,5-triphosphate-dependent pathway. To assess the generality of MEK1,2/ERK1,2 regulation of GI peptide hormone-stimulated Ca\(^{2+}\) release from intracellular stores, we determined the effect of MEK inhibition on hormone-induced Ca\(^{2+}\) release in cell lines expressing endogenous CCKR, gastrin-releasing peptide receptor, or neurotensin receptors. Pretreatment with PD98059 inhibited the G17-stimulated Ca\(^{2+}\) release response in the rat pancreatic cancer cell line AR4-2J, which expresses an endogenous CCKR, in a dose-dependent manner (Fig. 8A). Similarly, PD98059 pretreatment inhibited bombesin-stimulated Ca\(^{2+}\) release in the androgen-insensitive human prostate cancer line PC-3, which expresses an endogenous gastrin-releasing peptide receptor, and the neurotensin receptor-mediated Ca\(^{2+}\) response in the human carcinoma cell line BON (Fig. 8, B and C). Together, these data demonstrate that MEK1 and MEK2 regulate the amplitude of the Ca\(^{2+}\) release signal induced by multiple GI peptide hormone receptors expressed in different cell types and suggest that the MEK1,2/ERK1,2 pathway regulates a common component in the receptor-mediated Ca\(^{2+}\) release.

**FIG. 8.** Effects of MEK inhibition on GI peptide hormone-stimulated Ca\(^{2+}\) release. Shown are dose-dependent effects of PD98059 (A) on G17-stimulated Ca\(^{2+}\) release in the rat pancreatic cancer cell line AR4-2J, (B) on bombesin-stimulated Ca\(^{2+}\) release in the human prostate cancer cell line PC-3, and (C) on neurotensin-stimulated Ca\(^{2+}\) release in the human pancreatic carcinoid cell line BON. All cell lines were assayed in Ca\(^{2+}\)-free KRH medium containing 1 mM EGTA. Prior to the addition of GI hormone, the cells were pretreated for 5 min with either the indicated concentration of PD98059 or MeSO (Control). Each tracing is the average \([\text{Ca}^{2+}]_{i}\) ± S.E. (n = 40 cells). The arrow indicates when peptide was added to the cells, and the black bar indicates the length of time the cells were exposed to peptide.

Ca\(^{2+}\) is an important signal in a wide variety of cellular responses, including cell proliferation, differentiation, increased cell survival, neuronal adaptation, and apoptosis. In a resting cell, \([\text{Ca}^{2+}]_{i}\) is maintained at a relatively low level (approximately 100 nm) by intracellular Ca\(^{2+}\)-binding proteins and by sequestration of Ca\(^{2+}\) in intracellular membrane-bound compartments such as the endoplasmic reticulum and mitochondria. When a cell is activated either by membrane depolarization or through a ligand-activated cell surface receptor such as CCKR, \([\text{Ca}^{2+}]_{i}\) can rapidly and transiently increase to concentrations in excess of 1 \(\mu\)M. The information encoded in the transiently increased \([\text{Ca}^{2+}]_{i}\) is deciphered by Ca\(^{2+}\)-binding proteins that convert the Ca\(^{2+}\) signal into specific biochemical changes involving posttranslational modification of proteins by Ca\(^{2+}\)-activated protein kinases, such as Ca\(^{2+}\)-phospholipid-dependent kinase and Ca\(^{2+}\)/calmodulin-dependent kinases, or the activation of Ca\(^{2+}\)-dependent enzymes, such as some forms
of adenyl cyclase and phospholipases (9, 10). Several studies have documented the dependence of specific gene expression, secretion, and cell proliferation on the amplitude, duration, and spacial pattern of change in [Ca\(^{2+}\)](9, 11, 13, 41). Dolmetsch and co-workers demonstrated the differential activation of the proinflammatory transcriptional regulator nuclear factor-\(\kappa\)B, c-Jun N-terminal kinase, and nuclear factor of activated T-cells in B lymphocytes when the amplitude and duration of change in [Ca\(^{2+}\)] was manipulated with ionomycin and Ca\(^{2+}\) chelator EGTA (11). A continuous sustained increase in [Ca\(^{2+}\)], rather than Ca\(^{2+}\) oscillations, was shown to be required for bombesin-stimulated peptide secretion from the human pancreatic carcinoid cell line BON (13). However, Ca\(^{2+}\) oscillations, but not a sustained increase in [Ca\(^{2+}\)], were required for agonist-induced proliferation of Chinese hamster ovary cells expressing CCK\(_2\)R (17). Together, these findings demonstrate that the spacial and temporal patterns of change in [Ca\(^{2+}\)], are important in defining the specificity of the cellular response to the Ca\(^{2+}\) signal. We show that EGFR transactivation of CCK\(_2\)R-mediated signal transduction alters the character of the G17-stimulated Ca\(^{2+}\) release response. Pretreating RIE/CCK\(_2\)R cells with EGF increased the amplitude of the G17-stimulated Ca\(^{2+}\) release response in serum-starved cells over a range of G17 concentrations without altering the affinity of agonist binding. The potentiation effect of EGF on G17-stimulated Ca\(^{2+}\) release suggests that EGFR may play a role in defining the specificity of the cellular response to G17.

Investigations of the intracellular mechanism by which EGFR potentiates G17-stimulated Ca\(^{2+}\) release identified a central role for the MEK1,2/ERK1,2 pathway. Many factors, both extracellular and intracellular, can affect the activity of the MEK1,2/ERK1,2 pathway. The MEK1,2/ERK1,2 pathway receives input from many extracellular signals, including growth factors, cytokines, peptide hormone, and biogenic amines (42). Under certain pathological conditions, such as cancer, activity of the MEK1,2/ERK1,2 pathway can increase due to activating mutations in the GTP-binding protein Ras. Ras mutations occur at high frequencies in some G17-sensitive cancers including cancers of the pancreas and colon (43). Also, age-dependent changes in the activity of the MEK1,2/ERK1,2 pathway have been documented in tissues of the gastrointestinal tract and associated organs (44, 45). Our data suggest that conditions that alter the basal activation state of the MEK1,2/ERK1,2 pathway will alter the efficacy and perhaps the specificity of the CCK\(_2\)R-regulated signaling pathways.

Finally, we have shown that regulation of Ca\(^{2+}\) release by the MEK1,2/ERK1,2 pathway is not limited to the CCK\(_2\)R signaling pathway but also is involved in regulation of the Ca\(^{2+}\) release response induced by bombesin and neurotensin. Like CCK\(_2\)R, the receptors for bombesin and neurotensin are coupled to Gq and regulate [Ca\(^{2+}\)], through an inositol 1,4,5-triphosphate-dependent mechanism (46, 47). The demonstration that PD98059 can inhibit the agonist-stimulated Ca\(^{2+}\) release response of multiple receptors in different cell types suggests the involvement of a common intracellular mechanism. Future studies will attempt to identify the molecular target(s) of MAPK regulation in the peptide hormone receptor-regulated Ca\(^{2+}\) release pathway.

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