Functional role of extracellular signal-regulated protein kinases in gastric acid secretion

YOSHIKI TAKEUCHI, JUNKO YAMADA, TADATAKA YAMADA, AND ANDREA TODISCO

Departments of Internal Medicine and Physiology, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Takeuchi, Yoshiaki, Junko Yamada, Tadataka Yamada, and Andrea Todisco. Functional role of extracellular signal-regulated protein kinases in gastric acid secretion. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1263–G1272, 1997.—Epidermal growth factor (EGF) has acute inhibitory and chronic stimulatory effects on gastric acid secretion. Because a cascade of intracellular events culminating in the activation of a family of serine-threonine protein kinases called extracellular signal-regulated protein kinases (ERKs) is known to mediate the actions of EGF, we undertook studies to explore the functional role of the ERKs in gastric acid secretion. ERK2 was immunoprecipitated from cell lysates of highly purified (>95%) gastric canine parietal cells, and its activity was quantified using in-gel kinase assays. Of the primary gastric secretagogues, carbachol was the most potent inducer of ERK2 activity. Gastrin and EGF had weaker stimulatory effects, whereas no induction was noted in response to histamine. The effect of carbachol appeared to be independent of Ca2+ signaling. PD-98059, a selective inhibitor of the upstream ERK activator mitogen-activated protein kinase/ERK kinase, dose-dependently inhibited both carbachol- and EGF-stimulated ERK2 activity, with a maximal effect observed between 50 and 100 μM. ERKs activation is required for induction of the early gene c-fos via phosphorylation of the transcription factor Elk-1 which binds to the c-fos serum response element (SRE). Carbachol stimulated a two- to threefold induction of luciferase activity in cultured parietal cells transfected with either a SRE-luciferase reporter plasmid or with a chimeric GAL4-ElkC expression vector and the 5′-GAL-luciferase reporter plasmid. To examine the significance of ERK activation in gastric acid secretion, we tested the effect of PD-98059 on carbachol-stimulated uptake of 14C-labeled aminopyrine (AP). Acute inhibition of the ERKs by PD-98059 led to a small increase in AP uptake and a complete reversal of the acute inhibitory effect of EGF on AP uptake induced by either carbachol or histamine. In contrast, exposure of the cells to PD-98059 for 16 h led to a reversal of the chronic stimulatory effect of EGF on AP uptake induced by carbachol. Our data led us to conclude that carbachol induces a cascade of events in parietal cells that results in ERK activation. Although the acute effect of the ERKs on gastric acid secretion appears to be inhibitory, the activation of transcription factors and of early gene expression could be responsible for its chronic stimulatory effects.

mitogen-activated protein kinase; gastric acid secretion; early response genes; transcriptional regulation; c-fos

GASTRIC ACID SECRETION is a complex physiological process that is regulated by numerous hormones and neurotransmitters. Carbachol, gastrin, and histamine are among the major gastric acid secretagogues, and these agents are known to activate multiple signal transduction pathways upon interaction with their specific parietal cell receptors. Although carbachol and gastrin increase Ca2+ mobilization and protein kinase C activation, histamine is known to induce adenylate cyclase activation and adenosine 3′,5′-cyclic monophosphate (cAMP) generation (12).

In addition to these well-established signal transduction pathways, recent studies have indicated that mammalian parietal cells contain multiple isoforms of a recently discovered family of protein kinases known as mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated protein kinases (ERKs) (7, 26). The ERKs are important elements in a cascade of phosphorylation reactions that is known to involve upstream protein kinases such as Raf and MAPK/ERK kinase (MEK) (8, 11). Activation of the ERKs is known to target numerous cellular proteins, including downstream protein kinases such as 90-kDa S6 kinase (8, 11) and transcription factors such as Elk-1 that regulate the activity of the promoter of the early response gene c-fos through the serum response element (SRE) (22, 36). Thus the ERKs, via activation of immediate early gene function and of downstream protein kinases, appear to play a crucial role in the process of amplification, integration, and transmission of the extracellular signals from the cell surface to the nucleus leading to induction of cellular growth and proliferation and, in some systems, cellular differentiation (8, 11, 17, 23). Our understanding of the function and physiological role of the ERK pathway has been improved significantly by the recent development of PD-98059, a highly specific inhibitor of the ERK activator MEK (1). With the use of this compound, it was possible to establish, for example, that activation of the ERK pathway is necessary for the induction of nitric oxide in cardiac myocytes, whereas it is not needed to mediate insulin stimulation of glucose uptake, lipogenesis, and glycogen synthesis (20, 32).

Epidermal growth factor (EGF) is the prototypic member of a large family of peptide growth factors that have biological actions on the function of many organs. In addition to its well-known growth-promoting properties, EGF has been shown to modulate pituitary hormone production, pancreatic amylase secretion, insulin synthesis, and intestinal electrolyte transport (18). One of the best-characterized signal transduction pathways activated in response to EGF stimulation involves the induction of the ERKs and other signaling molecules such as the early response gene c-fos (11, 17, 23).

In the stomach, EGF affects acid secretion in a divergent fashion. Under acute conditions, EGF has a long-recognized inhibitory effect on acid secretion, whereas prolonged administration of EGF increases both basal and maximal acid secretion in vivo and acid production in isolated parietal cells in vitro (7, 18, 37).
The mechanisms responsible for this phenomenon are currently only partially understood. Some studies have suggested that these effects of EGF could be mediated by the activation of protein tyrosine kinases, since they are fully reversed by the addition of protein tyrosine kinase inhibitors (37). In addition, Chew et al. (7) observed that inhibition of the chronic stimulatory effect of EGF by these agents was associated with a decrease in phosphorylation of a 44-kDa protein identified as an ERK isoform. Taken together, these results suggest that the ERKs are likely to play an important role in the regulation of numerous physiological functions of the gastric parietal cells.

In this study, we sought to investigate the regulation and the functional relevance of the ERKs in the process of gastric acid secretion. Using highly purified canine gastric parietal cells in primary culture, we were able to demonstrate that although the acute effect of the ERKs on gastric acid secretion appears to be inhibitory, the activation of transcription factors and of early gene expression could be responsible for its chronic stimulatory effects.

**MATERIALS AND METHODS**

Plasmids. 5×Gal-Luc (16) was a gift from M. Karin (San Diego, CA). Gal4-ElkC (22) was a gift from R. Treisman (London, UK). SRE-Luc (39) was obtained from J. Pessin (Iowa City, IA). pCMV-μGal was a gift from M. Uhler (Ann Arbor, MI).

Primary parietal cell preparation and culture. For preparation of primary parietal cells, we utilized a modification of the method of Soli (33; see also Refs. 4, 5, 13). The mucosal layer of freshly obtained canine gastric fundus was bluntly separated from the submucosa and rinsed in Hanks’ balanced salt solution containing 0.1% bovine serum albumin (BSA). The cells were then dispersed by sequential exposure to collagenase (0.35 mg/ml) and 1 mM EDTA, and parietal cells were enriched by centrifugal elutriation using a Beckman JE-6B elutriator. Our best preparations contained 70% parietal cells as determined by hematoxylin and eosin and periodic acid-Schiff reagent staining. The parietal cells were further purified by centrifugation through density gradients generated by 50% Percoll (Pharmacia Biotech, Piscataway, NJ) at 30,000 g for 20 min. The cell fraction at density = 1.05 consisted of 95–100% parietal cells. In some experiments, the isolated parietal cells (0.8 × 10⁶ cells/well) were cultured for 16 h according to the method of Chew et al. (6) with some modifications (25). Briefly, the cells were cultured in Ham’s F-12-Dulbecco’s modified Eagle’s medium (1:1) containing 50 μg/ml gentamycin, 50 U/ml penicillin G, and 2% dimethyl sulfoxide (DMSO); Sigma, St. Louis, MO) on 12-well culture dishes (Coster, MA) coated with 150 μl of H2O-diluted (1:5) growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA). For our studies, the parietal cells were incubated with carbachol (1–100 μM; Sigma), histamine (100 μM; Sigma), gastrin (10 nM; Bachem, Torrance, CA), EGF (0.1–100 nM; Becton Dickinson), and ionomycin (1 μM; Calbiochem, La Jolla, CA) for various time periods. In some experiments, either PD-98059 (10–100 μM; a gift from Dr. Alan Saltiel, Parke Davis Pharmaceutical Research, Ann Arbor, MI) or 1,2-bis(2-aminoxyethoxy)hexane-N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM; 10 μM; Calbiochem) was added before the addition of the secretagogues. BAPTA-AM, ionomycin, and PD-98059 were dissolved in DMSO. All other test substances were dissolved in culture medium. Control experiments with untreated cells were performed by incubating the cells in either vehicle (0.1% DMSO) or incubation buffer without the test substances. ERK2 induction and aminopyrine uptake in parietal cells obtained from the enriched elutriated fractions were identical to those detected in cells further purified by Percoll density gradients.

Transfection of primary cultured parietal cells. Before transfection, the cells were washed once with 1 ml Opti-MEM I serum-reduced media (GIBCO, Gaithersburg, MD) and fed with 400 μl Opti-MEM I medium supplemented with 2% DMSO. The cells were transfected with 5 μg of the luciferase reporter plasmids and, where indicated, with 0.5 μg of the expression vectors. Transfections were carried out using Lipofectin (GIBCO, Grand Island, NY) as previously described (25). The day after transfection, the medium was removed and the cells were fed with serum-free media for 24 h, then incubated for 5 h with the test substances. At the end of the incubation period, the cells were washed twice with cold calcium-free phosphate-buffered saline. Then, 100 μl cell lysis buffer (25 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.8, 2 mM EDTA, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, and 1% Triton X-100) were added and incubated at room temperature for 15 min. The cells were then scraped and transferred to Eppendorf tubes. After quick centrifugation to pellet large debris, the supernatant was transferred to a new tube. An aliquot of cell lysate (20 μl) was mixed with 100 μl luciferase assay reagent (20 mM tricine, 1.07 mM (MgCO3)2Mg(OH)2·5H2O, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP, final pH 7.8), and luminescent intensity was measured for 10 s utilizing a Lumat LB9501 luminometer (Berthold, Germany). Luciferase activity was expressed as relative light units and normalized for β-galactosidase activity. β-Galactosidase activity was measured by the luminescent light derived from 10 µl of each sample incubated in 100 µl Lumi-Gal 530 (Lumin, Southfield, MI) and used to correct the luciferase assay data for transfection efficiency.

Immunoprecipitations and in-gel ERK2 assay. Immunoprecipitations and in-gel ERK2 assays were performed according to previously described techniques (30, 35) with minor modifications. The parietal cells were lysed in 500 μl of lysis buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl2, 1 mM Na2VO4, 10 mM NaF, 10 mM Na3P2O7·10H2O, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (AEBSF; ICN Biomedicals, Aurora, OH), 1 μg/ml leupeptin, and 1 μg/ml aprotinin), transferred into Microfuge tubes, and spun at 16,000 g for 20 min at 4°C. Equal amounts of protein from each treatment group (1,000 μg) were incubated with an anti-ERK2 specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mixed on a rotating platform for 3 h at 4°C. Protein concentrations were measured by the Bradford method (3). Control experiments were conducted by mixing aliquots of the samples with identical volumes of nonimmune sera. Aliquots of protein A-Sepharose (50 μl) (Pharmacia Biotech) were then added, and the solutions were mixed for one additional hour. After centrifugation, the pellets were washed four times with lysis buffer. The samples were resuspended in 20 μl of electrophoresis buffer [for 10 ml: 1 ml glycerol, 0.5 ml 2-mercaptoethanol, 3 ml of 10% sodium dodecyl sulfate (SDS), 1.25 ml of 1 M Tris buffer, 2 ml of 0.1% bromphenol blue, and 0.6 g urea], boiled for 5 min, and applied to a 10% SDS-polyacrylamide gel containing 0.5 mg/ml myelin basic protein (Sigma). After electrophoresis, the gel was washed with two changes of 20% 2-propanol in 50 mM Tris (pH 8.0) for 1 h and then with two

G1264 ROLE OF THE MAPK PATHWAY ON GASTRIC ACID SECRETION
changes of 50 mM Tris (pH 8.0) containing 5 mM 2-mercaptoethanol for 1 h. The enzyme was denatured by incubating the gel with two changes of 6 M guanidine HCl for 1 h and then renatured with five changes of 50 mM Tris (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol for 1 h. The kinase reaction was performed in conditions inhibitory to cyclic nucleotide-dependent protein kinase and Ca2+-dependent protein kinases, by incubating the gel at 25°C for 1 h with 40 mM HEPES (pH 8.0) containing 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM MgCl2, 2 mM cAMP-dependent protein kinase inhibitor peptide (Sigma), 40 µM ATP, and 2.5 µCi/mI [γ-32P]ATP (6,000 Ci/mmol) (Amersham Life Science, Arlington Heights, IL). After incubation, the gel was washed with a 5% (wt/vol) trichloroacetic acid solution containing 1% (wt/vol) sodium pyrophosphate, dried, and subjected to autoradiography.

Western blots. Equal amounts of immunoprecipitated ERK2 from each treatment group were loaded on a 10% SDS-polyacrylamide gel and run at 20 A for 8 h. The gel was transferred on an Immobilon-P transfer membrane (Millipore, Bedford, MA) using a TE-42 Transphor Electro-Transfer Unit (Hoeffer Scientific Instruments, San Francisco, CA) in 25 mM Tris, 150 mM glycine, and 20% methanol. After transfer, the membrane was blocked in 100 ml TBST (20 mM Tris, 0.15 M NaCl, and 0.3% Tween) and 5% BSA for 2 h and incubated for 20 min at 37°C in TBST and 1% BSA containing a specific antiphosphotyrosine antibody directly conjugated to horseradish peroxidase (RC20, Transduction Laboratories, Lexington, KY) (1:2,500). At the end of the incubation period, the membrane was washed in TBST for 15 min at room temperature and then exposed to the Amersham enhanced chemiluminescence detection system (Amersham Life Science) according to the manufacturer’s instructions.

Aminopyrine uptake. The accumulation of [14C]aminopyrine (Amersham Life Science) was used as an indicator of acid production by parietal cells. A 1-ml aliquot of acutely isolated parietal cells (2 × 106) suspended in Earle’s balanced salt solution (EBSS) was incubated with 0.1 µCi [14C]aminopyrine and the various substances to be tested. In some experiments, the cells were resuspended in Ca2+-free EBSS supplemented with 1 mM EGTA. After a 20-min incubation at 37°C, the cells were centrifuged and the radioactivity of the pellet was measured by 10.2 ± 0.3 on June 9, 2017 http://ajpgi.physiology.org/ Downloaded from

RESULTS

The ERKs are known to be rapidly induced in response to a wide variety of stimuli. Accordingly, we examined the effect of gastric acid secretagogues on ERK activation using isolated gastric parietal cells. For these experiments, we immunoprecipitated the ERKs using an antibody that preferentially recognized ERK2 from parietal cell lysates, and we quantitated its activity using in-gel kinase assays. As shown in Fig. 1, of the primary gastric acid secretagogues, carbachol (100 µM) was the most potent inducer of ERK2 activity (26.5 ± 7.3-fold induction over control, n = 15). Gastrin (10 nM) and EGF (10 nM) had weaker stimulatory effects (3.4 ± 1.1-fold induction over control, n = 8, and 13.0 ± 2.9-fold induction over control, n = 11, in the presence of gastrin and EGF, respectively), whereas no induction was noted in response to histamine (100 µM) (1.0 ± 0.2-fold induction over control, n = 7). The effect of carbachol was dose (1–100 µM) and time dependent, with a maximal stimulatory effect detected after 5 min of incubation (data not shown). Similarly, EGF (0.1–100 nM) time- and dose-dependently induced ERK2 activity with a maximal stimulatory effect detected after 5 min of incubation. No difference in ERK2 induction was detected between 10 and 100 nM EGF (data not shown).

Activation of the ERKs requires phosphorylation on both tyrosine and threonine residues by the dual specificity protein kinase MEK (8, 11). Accordingly, we first tested the effect of the recently described, highly specific MEK inhibitor PD-98059 on EGF-stimulated ERK2 activity. For these experiments, the parietal cells were pretreated for 15 min with either different doses of PD-98059 or vehicle (0.1% DMSO) before the addition of 10 nM EGF. PD-98059 did not affect parietal cell viability measured by trypan blue exclusion at any of the doses tested (data not shown). As shown in Fig. 2, PD-98059 (10–50 µM) dose-dependently inhibited EGF-stimulated ERK2 activity with a complete inhibitory effect observed at the dose of 50 µM. We then tested the effect of PD-98059 on carbachol-stimulated ERK2 activity. For these experiments, the parietal cells were pretreated for 15 min with either vehicle (0.1% DMSO) or different doses of PD-98059 before the addition of 100 µM carbachol. As shown in Fig. 3, PD-98059 (10–100 µM) dose-dependently inhibited, although not completely, carbachol-stimulated ERK2 activity, with a maximal effect observed between 50 and 100 µM (44.9 ± 8.5 and 34.7 ± 8.2% of carbachol-stimulated ERK2 activity in the presence of 50 and 100 µM PD-98059, respectively, n = 3). Neither PD-98059 nor vehicle (0.1% DMSO) had any effect on ERK2 activity (Fig. 4).

In a similar fashion, 50 µM PD-98059 inhibited carbachol-stimulated ERK2 phosphorylation as measured by Western blot analysis using an antiphosphotyrosine antibody, confirming the involvement of MEK in ERK2 activation and phosphorylation (Fig. 5).

Because Ca2+ is an important mediator of carbachol actions on gastric parietal cells (12, 13), we undertook studies to examine the role of both intra- and extracellular Ca2+ on carbachol induction of ERK2 activity. As shown in Fig. 6, both chelation of intracellular Ca2+ by preincubation of the cells for 10 min with either vehicle (0.1% DMSO) or with the cell-permeable Ca2+-chelator BAPTA-AM (10 µM) and deprivation of extracellular Ca2+ by incubation of the cells in Ca2+-free EBSS containing 1 mM EGTA failed to affect the stimulatory
action of carbachol on ERK2 activity. Similarly, no induction was observed in the presence of 1 µM ionomycin, an agent known to induce Ca\(^{2+}\) influx into cells. Thus carbachol appears to activate ERK2 in parietal cells via Ca\(^{2+}\)-independent pathways. In contrast, incubation of the parietal cells in either Ca\(^{2+}\)-free medium or in the presence of BAPTA-AM completely inhibited 100 µM carbachol-stimulated aminopyrine uptake (8.1 ± 1.0- vs. 1.5 ± 0.4-fold induction over control in the presence of either vehicle (0.1% DMSO) or BAPTA-AM and 7.4 ± 1.7- vs. 1.3 ± 0.1-fold induction over control in the absence and presence of Ca\(^{2+}\)-free medium, respectively, n = 3). These results confirm the notion that Ca\(^{2+}\) signaling is of crucial importance in the stimulatory action of carbachol on gastric acid secretion but not on ERK2 activation (Fig. 7).

One of the best-characterized pathways leading to induction of the early response gene c-fos involves ERK2 activation and phosphorylation of Elk-1, a transcription factor known to bind to the c-fos SRE (36). We therefore sought to investigate the effects of carbachol on c-fos gene transcription regulated by the SRE. For these experiments, we transfected Percoll-purified cultured parietal cells with luciferase reporter plasmids containing the c-fos SRE upstream of the thymidine kinase (TK) gene minimal promoter and the firefly luciferase reporter gene together with the pCMV-βGal expression vector. As depicted in Fig. 8, 10 µM carbachol stimulated a twofold increase in luciferase activity, and this induction was completely inhibited by 50 µM PD-98059 (2.19 ± 0.11- vs. 0.97 ± 0.12-fold induction over control in the absence and presence of 50 µM PD-98059, respectively, n = 4). In contrast, parietal cells transfected with a plasmid containing the TK gene minimal promoter but devoid of the SRE exhibited no induction in response to carbachol (data not shown). To examine if carbachol induction of the c-fos SRE was mediated by stimulation of Elk-1 transcriptional activity, we used a yeast hybrid system involving cotransfection of the parietal cells with the Gal4-ElkC and the pCMV-βGal expression vectors and the 5×Gal luciferase reporter plasmids. In this system, Gal4-ElkC transactivates and stimulates luciferase activity only if the carboxy terminus of Elk-1 is phosphorylated by the ERKs (22). As shown in Fig. 9, carbachol (10 µM) stimulated a threefold increase in luciferase activity, and this effect was blocked by 50 µM PD-98059 (3.53 ± 0.54- vs. 1.42 ± 0.24-fold induction over control in the absence and presence of 50 µM PD-98059, respectively, n = 6). Cotransfection of the cells with the expression

Fig. 1. Effect of gastric acid secretagogues on extracellular signal-regulated protein kinase 2 (ERK2) activity. ERK2 in lysates from parietal cells stimulated with histamine (100 µM), carbachol (100 µM), gastrin (10 nM), and epidermal growth factor (EGF; 10 nM) was immunoprecipitated, and its activity was measured by in-gel kinase assays. A: representative assays obtained with a single parietal cell preparation. B: results obtained from densitometric analysis of blots from at least 7 parietal cell preparations. Data are expressed as fold induction over control and are means ± SE. OD, optical density.
vector lacking Gal4-ElkC coding sequences and the 5×Gal luciferase reporter plasmid gave only background luciferase activity (data not shown). Taken together, these data indicate that carbachol activates a cascade of phosphorylation reactions that targets MEK and the ERKs, leading to induction of early gene transcription via phosphorylation and transcriptional activation of Elk-1.

We then examined the functional significance of the ERK pathway in the acid secretory function of the parietal cells. For these experiments, we tested the effects of PD-98059 on carbachol-stimulated uptake of [14C]aminopyrine. As depicted in Fig. 10, addition of 50 µM PD-98059 led to a small but reproducible and significant increase in aminopyrine uptake induced by 100 µM carbachol [9.4 ± 1.3- vs. 10.7 ± 1.6-fold induction over control in the presence of either vehicle (0.1% DMSO) or PD-98059 (10–50 µM)] was immunoprecipitated, and its activity was measured by in-gel kinase assays (A). A linear transformation of densitometric analysis of autoradiograms is depicted (B). Data are expressed as percentage of 10 nM EGF-stimulated ERK2 activity in absence of PD-98059. Identical results were obtained in 1 other separate experiment.

We then examined the functional significance of the ERK pathway in the acid secretory function of the parietal cells. For these experiments, we tested the effects of PD-98059 on carbachol-stimulated uptake of [14C]aminopyrine. As depicted in Fig. 10, addition of 50 µM PD-98059 led to a small but reproducible and significant increase in aminopyrine uptake induced by 100 µM carbachol [9.4 ± 1.3- vs. 10.7 ± 1.6-fold induction over control in the presence of either vehicle (0.1% DMSO) or PD-98059 (10–50 µM)] was immunoprecipitated, and its activity was measured by in-gel kinase assays (A). A linear transformation of densitometric analysis of autoradiograms is depicted (B). Data are expressed as percentage of 10 nM EGF-stimulated ERK2 activity in absence of PD-98059. Identical results were obtained in 1 other separate experiment.

Thus acute activation of the ERKs may not be important for stimulation of gastric acid secretion by carbachol but may, in fact, have a modest inhibitory effect. Because EGF has been shown to have acute inhibitory and chronic stimulatory effects on gastric acid secretion (21–23) and the ERKs are known to mediate some of the cellular effects of EGF, we conducted studies to examine whether PD-98059 influenced EGF inhibition of parietal cell activity stimulated by either carbachol

Fig. 2. Effect of PD-98059 on EGF-stimulated ERK2 activity. ERK2 in lysates from parietal cells stimulated with EGF (10 nM) in presence of either vehicle (0.1% DMSO) or PD-98059 (10–50 µM) was immunoprecipitated, and its activity was measured by in-gel kinase assays (A). A linear transformation of densitometric analysis of autoradiograms is depicted (B). Data are expressed as percentage of 10 nM EGF-stimulated ERK2 activity in absence of PD-98059. Identical results were obtained in 1 other separate experiment.

Fig. 3. Effect of PD-98059 on carbachol-stimulated ERK2 activity. ERK2 in lysates from parietal cells stimulated with carbachol (100 µM) in presence of either vehicle (0.1% DMSO) or PD-98059 (10–100 µM) was immunoprecipitated, and its activity was measured by in-gel kinase assays. A: representative assays obtained with a single parietal cell preparation. B: results obtained from densitometric analysis of blots from 3 parietal cell preparations. Data are expressed as percentage of 100 µM carbachol-stimulated ERK2 activity in absence of PD-98059 and are means ± SE. *P < 0.05.

Fig. 4. Effect of PD-98059 and DMSO on ERK2 activity. ERK2 in lysates from parietal cells treated with carbachol (100 µM) and either DMSO (0.1%) (A) or PD-98059 (50 µM) (B) was immunoprecipitated, and its activity was measured by in-gel kinase assays. These data represent results from a single parietal cell preparation.
or histamine. For these studies, the parietal cells were pretreated for 15 min with either vehicle (0.1% DMSO) or PD-98059 (50 µM) before the addition of EGF (10 nM). After 30 min of incubation, the cells were stimulated with carbachol (100 µM) or histamine (100 µM) for an additional 20 min. Under these conditions, EGF inhibited both carbachol- and histamine-stimulated aminopyrine uptake according to previously published observations (30), and PD-98059 completely reversed this effect [10.02 ± 1.89- vs. 8.08 ± 1.52- and 11.28 ± 1.97-fold induction over control in the presence of carbachol, carbachol after preincubation with EGF, and carbachol after preincubation with EGF in combination with PD-98059, respectively, n = 6; and 2.57 ± 0.4- vs. 1.91 ± 0.23- and 6.23 ± 0.93-fold induction over control in the presence of histamine, histamine after preincubation with EGF, or histamine after preincubation with EGF in combination with PD-98059, respectively, n = 6]. PD-98059 (50 µM) had no effect on basal aminopyrine uptake (1.03 ± 0.13-fold induction over control, n = 8; Fig. 11). Similarly, addition of 0.1% DMSO, EGF, or EGF in combination with PD-98059 had no effect on basal aminopyrine uptake (data not shown). These data confirm the notion that rapid activation of the ERKs results in inhibition of gastric acid secretion stimulated by different classes of gastric acid secretagogues. For these experiments, the parietal cells were cultured in 10 nM EGF in the presence of either vehicle (0.1% DMSO) or PD-98059 (50 µM) for 16 h and then stimulated with carbachol (100 µM) for 30 min. As shown in Fig. 12, EGF enhancement of carbachol-stimulated aminopyrine uptake was completely blocked by pretreatment of the cells with 50 µM PD-98059 (4.16 ± 1- vs. 5.06 ± 1- and 3.25 ± 0.79-fold induction over control in the presence of carbachol, carbachol after preincubation with EGF, or carbachol after preincubation with EGF in combination with PD-98059, respectively, n = 5). Exposure of the parietal cells to PD-98059 (50 µM) alone for 16 h had no effect on basal aminopyrine uptake (0.81 ± 0.23-fold induction over control, n = 5). These data suggest that prolonged activation of the ERKs could be responsible for stimulatory effects on gastric acid production.

DISCUSSION

The ERKs or MAPKs are key elements in a cascade of phosphorylation reactions that is triggered by the interaction of hormones, growth factors, and neurotransmitters with their specific cellular receptors (8, 11). Numerous studies have shown that these enzymes are responsible for the regulation of a wide variety of physiological functions. Activation of the ERKs is in fact necessary for both the induction of cellular proliferation and for the expression of highly differentiated cellular phenotypes (8, 11, 23). Recent studies have shown that seven transmembrane receptors, known to interact with heterotrimeric GTP-binding proteins to transduce extracellular signals to downstream effector molecules (14, 27), are able to activate Ras, Raf, MEK, ERKs, and early response genes in a fashion similar to that employed by receptor tyrosine kinases (2, 9, 10, 15, 28, 29, 30, 31, 35). These findings suggest that common signaling pathways are shared by a diverse array of extracellular messengers that are likely to act in concert at the level of the cell surface to control the expression of diverse physiological functions.

The gastric parietal cell is a highly differentiated cell that secretes gastric acid in response to a broad range of physiological stimuli. Carbachol, gastrin, and histamine are among the major gastric acid secretagogues.
These agents are known to interact with specific seven-transmembrane receptors present on the surface of the parietal cell to stimulate gastric acid secretion. Recent studies have indicated that mammalian parietal cells have receptors for EGF and that this agent regulates gastric acid secretion in a complex manner. Under acute conditions, EGF has a long-recognized inhibitory effect on acid secretion, whereas prolonged administration of EGF increases both basal and maximal acid secretion in vivo and in vitro (7, 18, 37). The mechanisms responsible for this phenomenon are currently only partially understood. Some studies have suggested that these effects of EGF could be mediated by the activation of protein tyrosine kinases since they are fully reversed by the addition of protein tyrosine kinase inhibitors (37). In our study, we have examined the physiological regulation of the ERKs in response to carbachol, gastrin, histamine, and EGF and found that of these agents, carbachol was the most potent inducer. Accordingly, we opted to focus our efforts primarily on the understanding of the intracellular pathways that target the ERKs in response to carbachol and to analyze the functional relevance of this phenomenon.

For these experiments, we took advantage of the recent discovery of PD-98059, a highly selective MEK inhibitor. This compound is known to prevent the activation of MEK1 and to a lesser degree of MEK2 in response to a wide variety of agonists (1). The extent of inhibition, however, seems to depend on how potently MEK or c-Raf is activated by any particular agonist. In fact, in Swiss 3T3 cells, 50 µM PD-98059 almost completely prevents the activation of ERK2 in response to insulin (the weakest activator of MEK in these cells), whereas it inhibits the strong stimulatory effect of platelet-derived growth factor, serum, 12-O-tetradecanoylphorbol 13-acetate, and bombesin by only 60–80% (1, 31). In our study, we demonstrated that PD-98059 dose dependently inhibited both carbachol- and EGF-stimulated ERK2 induction, with a maximal effect detected between 50 and 100 µM. In agreement with previous observations, we found that PD-98059 led to a partial inhibition of carbachol-stimulated ERK2 activity even

![Fig. 7. Effect of intra- and extracellular Ca²⁺ on carbachol (C)-stimulated [¹⁴C]aminopyrine uptake. Canine gastric parietal cells were incubated with 100 µM carbachol, in combination with either vehicle (0.1% DMSO) or BAPTA-AM (10 µM) (A). Identical results were obtained when cells were treated with 100 µM carbachol in presence of Ca²⁺-free medium, as shown in B. Data are expressed as fold induction over control and are means ± SE (n = 3).](image)

![Fig. 8. Effect of PD-98059 (PD) on carbachol (C)-stimulated serum response element (SRE) transcriptional activity. Canine gastric parietal cells were transfected with plasmids SRE-Luc and pCMV-βGal and treated with 10 µM carbachol alone or in combination with 50 µM PD-98059. Data are expressed as fold induction over control and are means ± SE (n = 4). RLU, relative light units. *P < 0.05.](image)

![Fig. 9. Effect of carbachol (C) and PD-98059 (PD) on Elk-1 transcriptional activity. Canine gastric parietal cells were transfected with Gal4-ElkC expression vector, 5×Gal luciferase reporter plasmid, and pCMV-βGal vector and treated with 10 µM carbachol alone or in combination with 50 µM PD-98059. Data are expressed as fold induction over control and are means ± SE (n = 6). *P < 0.01.](image)
in the presence of lower doses of carbachol (10 µM) (data not shown), whereas it completely inhibited ERK2 activity stimulated by EGF, a weaker inducer of the ERKs in the parietal cells.

Numerous studies have examined the role of Ca\(^{2+}\) signaling in activation of the ERKs. Although in some systems, such as cardiac myocytes or PC12 cells, induction of the ERKs requires Ca\(^{2+}\) influx or Ca\(^{2+}\) release from intracellular stores (21), in pancreatic acinar cells this process appears to occur via Ca\(^{2+}\)-independent pathways (15). In agreement with these findings, we observed that activation of the ERKs in the canine parietal cells is not affected by changes in either intracellular or extracellular Ca\(^{2+}\), although this pathway is known to play a crucial role in the process of gastric acid secretion in these cells. Thus carbachol activates the ERKs in gastric canine parietal cells through MEK-dependent, Ca\(^{2+}\)-independent mechanisms.

In previous studies we have shown that carbachol stimulates the expression of the early response gene c-fos in isolated gastric canine parietal cells (34). Accordingly, we examined whether carbachol induction of the ERKs would result in c-fos induction. Transcription of the c-fos gene is under the control of numerous DNA regulatory elements. The SRE, in particular, is known to be the target of growth factor-activated signal transduction pathways (36). Transcriptional activation of the SRE is under the control of numerous nuclear proteins that are known to assemble on the gene forming specific DNA-protein complexes. Some of these transcription factors are thought to be bound constitutively to the DNA and to be phosphorylated rapidly and activated in response to cellular stimulation by growth factors. Elk-1 and SAP-1 are members of a well-characterized family of transcription factors that are known to bind to the SRE and to interact with the serum response factor to form a ternary complex that plays an important role in the activation of c-fos transcription (36). In response to a wide variety of extracellular signals, these nuclear proteins are rapidly phosphorylated and transcriptionally activated by the ERKs, resulting in stimulation of c-fos gene expression (24, 36). In contrast to these findings, ternary complex formation by a third member of this family, SAP-2, seems to be weak and unaffected by serum stimulation (36). In our study, we have shown that in
response to carbachol, the ERKs are rapidly activated and that this effect translates into induction of c-fos expression via phosphorylation and transcriptional activation of Elk-1. Thus carbachol initiates a program of cellular activation that targets the ERKs and c-fos and that is likely, in the end, to induce the transcription of numerous other yet unidentified genes that might play a crucial role in a broad array of physiological functions of the gastric parietal cell. Further studies will be required to elucidate the role of SAP-1 and other nuclear proteins in the regulation of SRE transcriptional activation in these cells.

We then examined the functional role of the ERKs on gastric acid secretion. In the presence of 50 μM PD-98059, we observed a small, albeit very reproducible, increase in aminopyrine uptake, suggesting that acute activation of the ERKs does not play any role in stimulation of gastric acid production but that, on the contrary, it might have a modest inhibitory function, possibly through the phosphorylation of yet unidentified cellular substrates. Because the ERKs are known to mediate some of the cellular effects of EGF, we also examined whether PD-98059 influenced EGF inhibition of parietal cell activity stimulated by either carbachol or histamine, agents known to stimulate gastric acid secretion via different signal transduction pathways. In these experiments, we observed that this compound completely reversed the acute inhibitory effect of EGF on aminopyrine uptake stimulated by carbachol, whereas it potentiated the induction observed in response to histamine. Similar results were reported by Tsunoda et al. (37), who observed a potentiation of histamine-stimulated aminopyrine uptake in isolated rabbit parietal cells in the presence of EGF and the protein tyrosine kinase inhibitor genistein. These data indicate that activation of both protein tyrosine kinases and ERKs leads to the induction of an inhibitory pathway that regulates gastric acid secretion in response to different classes of gastric acid secretagogues. In addition, the potentiation observed in the presence of histamine, but not carbachol, in combination with either PD-98059 or genistein indicates that induction of the MAPK pathway exerts a specific inhibitory effect on still undefined components of histamine signaling in the gastric parietal cells. It is possible that inhibition of the MAPK pathway could alter the coupling of the histamine receptor to Gs, thus regulating receptor function and coupling to downstream effectors (37). Alternatively, MAPK could activate one or multiple phosphatases that could in turn negatively regulate histamine stimulation of gastric acid secretion. However, the exact mechanisms responsible for this phenomenon are still unknown, and additional studies will be necessary to further dissect this interesting observation.

Recent reports have shown that the acute inhibitory effect of EGF on gastric acid secretion could be mediated by a protein kinase C (PKC)-dependent pathway (38). In this report, we did not examine the specific role of PKC on gastric acid production and on ERK activation. However, because PKC has been shown to activate c-Raf directly (19), it seems reasonable to speculate that the acute inhibitory effect of EGF on aminopyrine uptake could be mediated by a signaling cascade involving activation of PKC, c-Raf, and MEK and leading in the end to induction of the ERKs. Further studies will be needed to explore this hypothesis.

In contrast to what was observed in the acute studies, PD-98059 significantly inhibited the chronic stimulatory effect of EGF on carbachol-stimulated aminopyrine uptake, confirming the notion that prolonged activation of the ERKs results in induction of gastric acid secretion. This effect could be attributed to the ability of the ERKs to induce Elk-1 transcriptional activation and c-fos gene expression. In fact, because we previously reported that both EGF and carbachol are able to stimulate the expression of the H+-K+-adenosinetriphosphatase gene in isolated canine gastric parietal cells (5, 18), it is possible that this gene contains specific DNA regulatory elements that could receive input from signaling pathways involving the ERKs and c-fos.

Our data led us to conclude that carbachol induces a cascade of events in parietal cells that results in ERK activation. Although the acute effect of the ERKs on gastric acid secretion appears to be inhibitory, the activation of transcription factors and of early gene expression could be responsible for its chronic stimulatory effects.

We thank Lara Post for assistance with parietal cell isolation. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants RO1-DK-33500 and R01-DK-34306 and funds from the University of Michigan Gastrointestinal Peptide Research Center (NIDDK Grant P30-DK-34933). A. Todisco is a recipient of an American Gastroenterological Association Industry Research Scholar Award and a Clinical Investigator Award from the National Institutes of Health (NIDDK Grant K08-DK-02336).

Address for reprint requests: A. Todisco, 6520 MSRB I, Ann Arbor, MI 48109-0662.

Received 8 January 1997; accepted in final form 19 August 1997.

REFERENCES


